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α-Ketoamide Phe–Pro Isostere as a New Core Structure for the Inhibition of HIV Protease

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Abstract—Studies on the inhibition of HIV-1 protease utilizing a core isostere with replacement of the scissle bond for an α-amino-ketone have resulted in the development of an α-keto-amide isosteric replacement of the Phe–Pro scissle amide bond. The simple dipeptide isostere was shown to be a promising new core structure for the development of the enzyme inhibitors. The K_i of this core structure was determined to be 6 μM, compared to 230 μM and >50 μM for the corresponding phosphinic acid and hydroxyethylamine isosteres.

Introduction

Human immunodeficiency virus (HIV) is a pathogenic retrovirus and the causative agent of acquired immunodeficiency syndrome (AIDS) and its related diseases.[1,2] HIV selectively depletes the CD4+ T-helper cells which results in a profound and fatal impairment of the host immune system.[3–5] Since HIV attacks the components of the immune system that are critical for raising defense against its infection, the present approach towards AIDS therapy focuses on a combination of direct antiviral therapy and immunological enhancement.[5–11] Of several possible new targets for HIV inhibition, the HIV-1 protease[12] is of particular interest as the enzyme is responsible for the release of protease (autoproteolysis), reverse transcriptase, integrase, and other proteins from the gag–pol fusion proteins.[13–15] Recently, it has been demonstrated that mutation of the catalytic residue of the HIV-1 protease results in loss of proteolytic activity as well as the production of noninfectious virons.[16] Moreover, protease inhibitors have exhibited promising antiviral effects.[17,18]

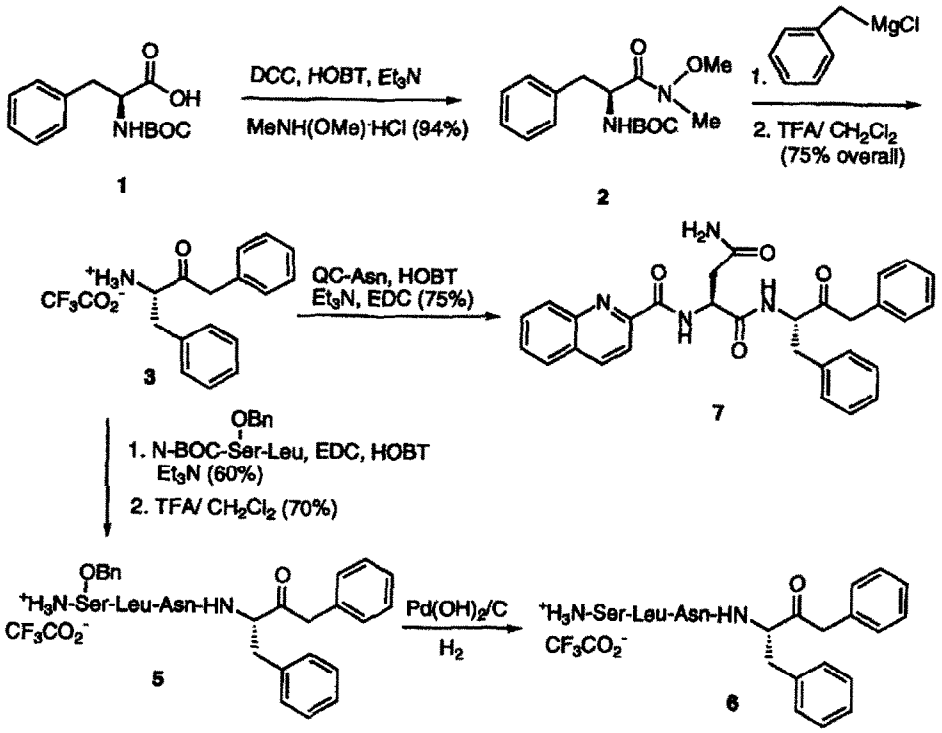
The HIV-1 protease is composed of 99 amino acid residues[19,20] and functions as a homodimer,[21] demonstrating activity mechanistically similar to aspartyl proteases such as pepsin and renin.[22] Recently, another protease of 116 amino residues from a feline immunodeficiency virus, FIV, has been isolated and characterized,[23] and shown to be similar to the HIV protease, i.e. it is also an aspartyl protease and active as a dimer. This new protease appears to be a useful model system for developing effective inhibitors that will become lead compounds for future therapies in the treatment of AIDS.

Although HIV and FIV proteases can cleave a number of specific peptide bonds,[24] both enzymes exhibit high specificity towards the phenylalanine–proline and tyrosine–proline sequences of P_1–P_1' found in gag and pol gene products. Nineteen retroviruses have been documented to display such specificity.[25] The cleavage of this bond is unique for this protease, as cellular proteases are not known to efficiently hydrolyze peptide bonds involving the proline nitrogen.[26] Therefore, synthesis of inhibitors that contain an isosteric unit to resemble the phenylalanine/tyrosine–proline dipeptide has been actively pursued in many laboratories.[27] This approach has proven effective towards the design of potent inhibitors of other aspartic proteinases, in particular renin.[28] Inhibitors that are transition-state mimetics that have proven effective include hydroxyethylene isosteres,[22,29] phosphinic acid,[30] reduced amide,[31] statine types[32] and hydroxyethylamine mimetics.[33]

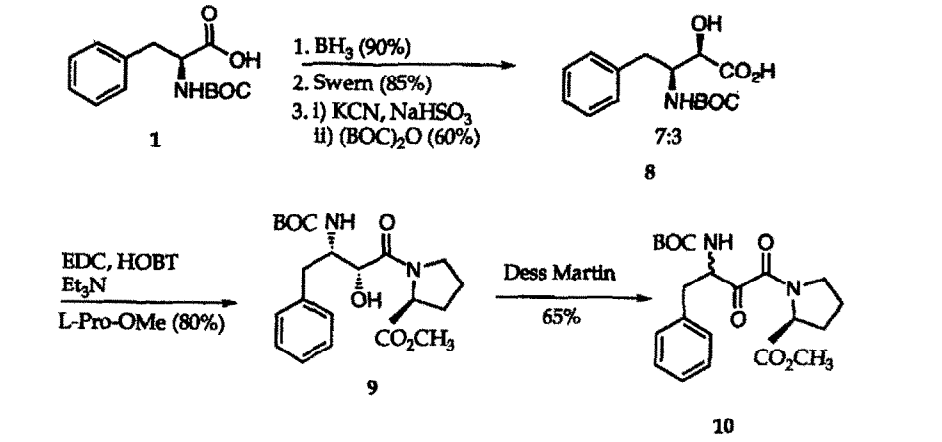
In contrast to inhibitors possessing traditional transition-state analogues, a novel class of inhibitors that capitalizes on the unique C_2 symmetry of the homodimeric enzyme has been reported.[34] Although these inhibitors afford excellent binding, they do not fully utilize the active site hydrogen bonding interactions. The symmetry displayed by the homodimeric enzyme is not translated to the hydrogen bonding network. This limiting factor makes any further improvements in C_2 inhibitor design extremely difficult. However, the results from such C_2 symmetric inhibitors indicate that an aryl moiety could be a suitable replacement for proline at P_1' while maintaining the crucial hydrogen bonding network. Substituents other than proline at P_1' have been successfully used in HIV protease inhibitor design.[26] In order to develop new HIV protease inhibitors, we describe here our efforts directed toward the preparation and inhibitory evaluation of isosteric structures composed of simple ketoamines and ketoamides as core structures.

Results and Discussion

We have begun to investigate the feasibility of utilizing α-amino-ketone inhibitors that contain phenylalanine at P_1 and benzyl moiety at P_1' as inhibitors for HIV and FIV protease (Scheme I). Our initial IC_{50} of 0.55 mM for **3** is promising since the structure does not possess any of the very important additional complementarities that are necessary for maximal binding. The free amino group for the core structure **3** has been extended to afford a new series of potential inhibitors, **5**, **6** and **7**, as illustrated in Scheme I. The initial kinetic results indicate that indeed the potency of the core isosteric unit can be increased. For inhibitor **7**, incorporating the quinolinic-Asn side chain, an IC_{50} of 0.1 mM was observed. However, when the side chain Bz-Ser-Leu-Asn was incorporated, a modest IC_{50} of 45 μM was observed. Since the side chain of Ser-Leu-Asn is found in the P_4, P_3 and P_2 positions of the natural substrate, its incorporation in the inhibitor structure should increase binding affinity. The modest increase of inhibition may be due to the presence of the unnecessary benzyl group in the Ser residue. Deprotection of **5** gave compound **6**, which was still a relatively weak inhibitor with K_i >20 μM.



Scheme I.



Scheme II.

We next prepared an α-keto-amide peptide linkage of Phe–Pro as an isosteric replacement (Scheme II). Interestingly, compound **10** which contains a 1:1 mixture of diastereomers (due to the acidic proton next to the carbonyl group) inhibited the HIV-1 protease with a K_i of 6 μM, approximately 45-fold better than the corresponding phosphinic isostere[30a] (Table 1). This result is very promising since this inhibitor is first a mixture of diastereomers which are spontaneously interconverted and second may be further optimized with incorporation of additional complementary structures to maximize potency. Although α-keto-amide isosteres have been used in the design of inhibitors for metalloproteins,[35–37] this is the first time they have been used in the inhibition of an aspartyl protease. Perhaps they form a *gem*-diol adduct in the active site to resemble the tetrahedral intermediate of the enzymatic reaction. The corresponding hydroxyethylamine isostere **9** was shown to be a much weaker inhibitor (IC_{50} >> 50 μM).

Table 1. Inhibitory activity of synthetic compounds

Compound	IC_{50}
1	0.55 mM
5	45 μM
7	100 μM
10	7 μM, K_i = 6 μM

In summary, this work indicates that the α-keto-amide isostere is a promising new core structure for the design of HIV protease inhibitors, and the principle may be applicable to the development of inhibitors for other aspartyl proteases. Work is in progress to incorporate additional complementary moieties to the keto-amide and determine the mode of their action.

Experimental Section

Melting points were taken on a Uni-Melt Capillary Melting Point Apparatus, and are uncorrected. Infrared spectra were recorded on a Perkin–Elmer 1600 Series FT-IR spectrophotometer. Solids were run either on KBr disks or solution cell using CCl_4 as the solvent. Oils were treated as neat films or in solution cell using CCl_4 as the solvent. ^1H NMR spectra were obtained at 300 MHz and 500 MHz and ^{13}C NMR spectra were obtained at 125 MHz using Bruker instruments. All chemical shifts are reported in δ units (ppm) relative to tetramethylsilane (assigned to 0.0 ppm). Spectra were recorded in $CDCl_3$, D_2O or DMSO-d_6. Thin-layer chromatography (TLC) was performed on silica plates (0.25 mm, Merck) using the following detection methods: UV, visualization under an ultraviolet lamp: I_2 on silica; PMA stain heated on a hot plate. Optical rotations were measured with Perkin–Elmer 241 polarimeter in 1 cm pathlength cell. Flash chromatography was performed with silica gel (230–400 mesh, Merck). The following solvents were used: chloroform (C), ethyl acetate (EA), hexane (H) and methanol (MeOH).

Peptide fragments described herein were synthesized using traditional peptide coupling methodologies [EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide HCl)], HOBt (1-hydroxybenzotriazole) and DMAP (*N*, *N*-dimethyl-4-aminopyridine). Ester were hydrolyzed either by base (NaOH for methyl esters) or acid [TFA (trifluoroacetic acid) for *t*-butyl esters].

*3(S)-1,4-Diphenyl-2-oxo-3-amino-*N*-Boc-butane (2a)*

To a stirred solution of *N*-Boc-L-phenylalanine-*N*-methoxy-*N*-methylamide (**2**) (5.0 g, 14.5 mmol) in anhydrous THF (50 mL) under N_2 at 0 °C was added 2.0 M benzyl magnesium chloride in THF (21.7 mL, 43.5 mmol). The mixture was gradually warmed to room temperature and stirred for an additional 3 h. The reaction mixture was then poured onto 1 N HCl (25 mL). The organic layer was separated and the aqueous layer was extracted with ether (3 × 35 mL). The combined organic layers were dried ($MgSO_4$) and concentrated to give a crude product. Purification of the crude material by flash chromatography (EA:H, 1:4) afforded **2a** as a white solid (4.8 g, 98 %). R_f 0.3 (EA:H, 1:4); mp 86–87 °C; $[\alpha]^{25}_D$ +31.22 ° (c 2.21, CH_2Cl_2); IR 3485, 2978, 1709, 1704, 1490, 1363, 1250 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.14 (s, 9H), 2.9–3.15 (m, 2H), 3.65 (q, 2H, *J* = 11.6 Hz), 4.61 (d, 1H, *J* = 6.9 Hz), 5.1 (bs, 1H), 7.0–7.2 (m, 10H) ppm; ^{13}C NMR ($CDCl_3$) 28.3, 37.8, 47.8, 59.5, 79.9, 127.0, 127.1, 128.8, 129.2, 129.2, 129.6, 133.1, 135.2, 155.1, 206.5 ppm. HRMS: 472.0880, calcd for $C_{21}H_{25}NO_3 + Cs^+$: 472.0889.

3(S)-1,4-Diphenyl-2-oxo-3-amino-butane HCl (3)

To a solution of 3(*S*)-1,4-diphenyl-2-oxo-3-amino-*N*-Boc-butane (**2a**) (1.4 g, 4.12 mmol) in ether (10 mL) was added a saturated solution of HCl(g)/ether (20 mL). After 3 h the precipitate was filtered to afford a crude white solid. Recrystallization (MeOH/ether) gave **3** as a white solid (0.96 g, 85 %). ^1H NMR (300 MHz, CD_3OD) δ 2.96 (dd, 1H, *J* = 8.2 Hz), 3.24 (dd, 1H, *J* = 6.2, 14.3 Hz), 3.73 (q, 2H, *J* = 16.9 Hz), 4.41 (dd, 1H, *J* = 2.0, 8.2 Hz) ppm. HRMS: 240.1388 calcd for $C_{16}H_{18}NO + H^+$: 240.1388.

Compound 4

To a solution of **3** (0.032 g, 0.12 mmol) in CH_2Cl_2 (10 mL) was added *O*-benzyl-*N*-Boc-L-Ser-Leu-Asn (0.075 g, 0.15 mmol), EDC (0.032 g, 0.17 mmol), HOBt (0.045 g, 0.33 mmol) and DMAP (cat.). After 24 h the reaction mixture was washed with sat. $NaHCO_3$ (2 × 5 mL), 1 N HCl (2 × 5 mL), sat. NaCl (2 × 5 mL), dried ($MgSO_4$) and concentrated to give a crude solid. Recrystallization from MeOH/ether afforded compound **4** as a white solid (0.05 g, 58 % yield). ^1H NMR (300 MHz, DMSO-d_6) δ 0.72–0.85 (m, 6H), 1.35 (s, 9H), 1.50–1.72 (m, 1H), 2.30–2.45 (m, 1H), 2.80–2.90 (m, 1H), 3.05 (dd, 1H, *J* = 5.1, 13.9 Hz), 3.5–3.63 (m, 2H), 3.69 (d, 1H), *J* = 17.0 Hz), 3.86 (d, 1H, *J* = 17.3 Hz), 4.20–4.30 (m, 1H), 4.30–4.45 (m, 1H), 4.46

(s, 2H), 4.48–4.51 (m, 1H), 6.80–7.42 (m, 15H) ppm. HRMS: 876.2955 calcd for $C_{41}N_{53}N_5O_8 + CS^+$: 876.2948. Anal. calcd for $C_{41}H_{53}N_5O_8$: C, 60.20 %, H, 5.615 %, N, 7.18 %; S, 9.41 %. Found C, 65.99 %; H, 7.22 %; N, 9.61 %.

Compound 5

To a solution of **4** (0.12 g, 0.16 mmol) in CH_2Cl_2 was added a solution of 305 TFA/CH_2Cl_2. After 24 h the mixture was concentrated to afford a crude white solid which after recrystallization (MeOH/ether) afforded the title compound **5** as a white solid (0.09 g, 75 %). ^{1}H NMR (300 MHz, CD_3OD) δ 0.71–0.85 (m, 6H), 1.40–1.60 (m, 3H), 2.40–2.62 (m, 2H), 2.70–2.91 (m, 1H), 2.91–3.01 (m, 1H), 3.50–3.58 (m, 1H), 3.60–3.72 (m, 2H), 3.75–3.82 (m, 1H), 3.88–4.00 (m, 1H), 4.20–4.30 (m, 1H), 4.44–4.61 (m, 2H), 6.93–7.45 (m, 15H) ppm. HRMS: 644.3448 calcd for $C_{36}H_{46}N_5O_6 + H^+$: 644.3448.

Compound 6

A solution of **5** (80 mg, 0.11 mmol) in glacial acetic acid (10 mL) containing $Pd(OH)_2$/C (cat.) was placed under a H_2 atmosphere at 50 psi. After 12 h the solution was filtered and the crude solid was recrystallized from MeOH/ether to afford the title compound (15 mg, 22 %). ^{1}H NMR (300 MHz, CD_3OD) δ 0.82 (t, 6H, J = 6.6 Hz), 1.39 (t, 2H, J = 7.2 Hz), 1.50–1.60 (m, 1H), 2.36 (dd, 1H, J = 8.1, 15.6 Hz), 2.44–2.50 (m, 1H), 2.38 (dd, 1H, J = 4.5, 13.9 Hz), 3.05 (dd, 1H, J = 4.9, 13.9 Hz), 3.69 (d, 1H, J = 17.3 Hz), 3.85 (dd, 1H, J = 17.2 Hz), 4.28–4.32 (m, 1H), 4.35–4.42 (m, 1H), 4.48–4.53 (m, 1H), 6.80–7.35 (m, 10H) ppm. HRMS: 686.1961 calcd for $C_{31}H_{43}N_5O_8 + CS^+$: 686.1955.

Compound 7

To a solution of **3** (0.17 g, 0.62 mmol) in DMF (5 mL) was added HOBt (0.17, 1.2 mmol), DMAP (cat.), EDC (0.12 g, 0.62 mmol), Et_3N (0.06 g, 0.09 mL, 0.62 mmol) and quinolinic-Asn (0.15 g, 0.52 mmol). After 12 h the reaction mixture was taken up in EA (50 mL). The organic layer was dried ($MgSO_4$) and concentrated to give a crude brown solid. Recrystallization from MeOH/ether gave a white solid (0.12 g, 43 %). ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.62 (dd, 1H, J = 4.9, 15.3 Hz), 2.75 (dd, 1H, J = 6.9, 15.5 Hz), 2.84 (dd, 1H, J = 14.3, 23.4 Hz), 3.06 (dd, 1H, J = 4.6, 13.7 Hz), 3.76 (d, 1H, J = 17.0 Hz), 3.91 (d, 1H, J = 17.0 Hz), 4.51 (q, 1H, J = 7.7 Hz), 4.82 (q, 1H, J = 6.8 Hz), 6.95–7.32 (m, 11H), 7.48 (s, 1H), 7.73 (t, 1H, J = 7.1 Hz), 7.89 (t, 1H, J = 8.3 Hz), 8.13 (q, 4H, J = 8.7 Hz), 8.58 (t, 2H, J = 6.7 Hz) ppm. HRMS: 641.1166 calcd for $C_{30}H_{28}NO_4 + Cs^+$: 641.1165.

Compound 9

To a solution of compound **8** (7:3 diastereomeric mixture) (0.21 g, 0.71 mmol), as previously prepared,[37] in CH_2Cl_2 (10 mL) was added L-proline methyl ester HCl (0.18 g, 1.1 mmol), EDC (0.16 g, 0.85 mmol), HOBt (0.21 g, 1.56 mmol) and DMAP (cat.). After 24 h the reaction mixture was washed with sat. $NaHCO_3$ (2 × 2 mL), 1 N HCl (2 × 2 mL) and sat. NaCl (1 × 1 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated to give a crude solid. Purification by flash chromatography (EA:H, 1:4) gave the white solid compound **9** as a single (2*R*,3*S*) isomer (0.23 g, 80 %). R_f = 0.20 (EA:H,1:4); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.34 (s, 9H), 1.90–2.11 (m, 4H), 2.88–2.91 (m, 2H), 3.11–3.20 (m, 1H), 3.36 (q, 1H, J = 7.6 Hz), 3.65 (s, 3H), 3.89 (d, 1H, J = 5.1 Hz), 4.08 (d, 1H, J = 5.7 Hz), 4.15 (q, 1H, J = 9.7 Hz), 4.39 (d, 1H, J = 7.4 Hz), 4.89 (d, 1H, J = 9.9 Hz), 7.10–7.35 (m, 5H) ppm. HRMS: 539.1169 calcd for $C_{21}H_{30}N_2O_6 + Cs^+$: 539.1158.

Compound 10

To a solution of compound **9** (0.062 g, 0.15 mmol) in anhydrous CH_2Cl_2 under N_2 at room temperature was added the Dess–Martin reagent (0.078 g, 0.18 mmol). After 12 h the reaction was quenched with sat. $NaHCO_3$ (4 mL) and sat. $Na_2S_2O_3$ (4 mL). Ether (40 mL) was added to the reaction and then stirred for 10 min. The reaction mixture was washed with sat. NaCl (2 × 10 mL), dried ($MgSO_4$), filtered and concentrated to give a crude oil. Purification by flash chromatography (EA:H, 1:3) afforded compound **10** as a 1:0.8 L/O diastereomeric mixture of an oil (0.030 g, 65 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.35; (s, 9H), 1.91–2.01 (m, 3H), 2.15–2.23 (m, 1H), 3.09–3.41 (m, 1H), 3.51–3.70 (m, 2H), 3.70 (m, 2H), 3.70 (q, 3H, J = 6.3, 7.8 Hz), 4.40–4.49 (m, 1H), 4.72–4.85 (m, 1H), 4.93–5.00 (m, 1H), 5.10–5.23 (m, 1H), 7.05–7.35 (m, 5H) ppm. HRMS: 537.1002 calcd for $C_{21}H_{28}N_2O_6 + Cs^+$: 537.1002.

Preparation, assay and inhibition analysis of the HIV protease

Plasmid pLAC-PRO 6.5 which contains the *lac*Z-protease fusion construct under the control of the *rac* promoter (composed of the *E. coli* ribosomal RNA promoter fused to *lac* operator) was prepared[38] and transformed to *E. coli* JM103. To induce *lacZ-pro* synthesis, overnight culture of JM103 harboring pLAC-PRO 6.5 (PRO 6–5) was diluted 50 fold with LB broth containing 300 μg/mL ampicillin. Cells were grown at 37 °C for 1 h to A_{600} = 0.2–0.3, lactose (0.4 % final concentration) was then added to induce the synthesis of *lac*Z-protease fusion protein. After overnight growth, cells were harvested by centrifugation in a Sorvall GSA rotor at 6,000 rpm for 7 min. Cell paste from 250 mL culture was suspended in 10 mL lysis buffer (50 mM Tris, 2 mM EDTA, 2 mM DTT, 100 mM NaCl, pH 7.5) containing 200 μg/mL lysozyme and stood at 4 °C overnight. The cell suspension was subsequently sonicated over a salted ice bath 10 times with 10–15 pulses each at the maximal power output of the sonifier (Branson 450). Care was taken during sonication to keep the temperature of cell suspension below 10 °C. The sonicates were then centrifuged at 6,000 rpm in a GSA rotor for 10 min. The majority of the *lacZ-pro* fusion proteins were found in the pellet. The insoluble *lacZ-pro* proteins were solubilized and unfolded in an aqueous solution containing 8 M urea, and 50 mM dithiothreitol (DTT) or 1 % 2-mercaptoethanol

at concentrations of 0.2 mg/mL to 0.4 mg/mL. To refold the *lacZ-pro* proteins, the protein suspension were diluted with 12 volumes of 10 mM Tris at pH 6.0 and incubated at room temperature overnight (final protein concentration at 15 μg/mL to 30 μg/mL). Under these conditions, the *lacZ-pro* proteins underwent auto-proteolysis to yield the mature 10 kDa protease.

The renatured *lac*Z-protease fusion protein mixture was prepared as described above and freeze-dried in a lyophilizer overnight, resuspended in H_2O, and dialyzed against 10 mM Tris buffer at pH 8.0 overnight to remove urea. Under these conditions the mature HIV protease forms an insoluble precipitate and can be readily purified by low speed centrifugation at 2,000 *g* for 20 min. The precipitated protease was redissolved in 8M urea, 10 mM Tris, pH 8.0, 1 mM DTT and passed through a DEAE-Sephacel column or a Pharmacia monoQ column in the same buffer. The flow through fraction was collected and dialyzed against 10 mM Tris, pH 8.0, 1 mM DTT, and concentrated by lyophilization. Both SDS gel electrophoresis and immunoblot analysis and amino acid analysis showed homogeneity of the protease. This procedure routinely gave 2–3 mg of the mature protease per gram of *E. coli* cells.

The synthetic HIV-protease containing a thioester linkage available from CalBiochem (San Diego) can also be used. The enzyme activity was assayed with the fluorogenic substrate Ac-Thr-Ile-Nle-Phe(*P*-NO_2)-Gln-Arg-NH_2 (from CalBiochem, San Diego) according to the procedure described in the literature.[39] Inhibition analysis was performed in the presence of the inhibitor and expressed with IC_{50} (the concentration of inhibitor that causes 50 % inhibition of the enzyme activity).

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