Orthogonal Ligation of Unprotected Peptide Segments through Pseudoproline Formation for the Synthesis of HIV-1 Protease Analogs†,‡

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Abstract: We describe the total synthesis of three active HIV-1 protease analogs by an orthogonal ligation through thiaproline formation of two large, unprotected peptide segments. The central element of this strategy in achieving orthogonality of peptide bond formation is through proximity effect, and the key reaction is a side chain thiol initiated aldehyde capture to overcome the entropic problem of coupling between two large molecular weight peptide segments. The capture step also leads to specific entropic activation of the acyl segment because the respective termini are brought to close proximity, resulting in a spontaneous acyl rearrangement to form the amide bond. A general method using a thioester for introducing an aldehyde moiety to the C-terminus of an unprotected peptide segment was also developed. Three active analogs of HIV-1 protease were obtained in excellent yield by ligating two segments of 38 and 61 residues. Two analogs contained a thioaldehyde residue at position 39, and the third contained a 38–39 non-peptide backbone. Efficient ligation at pH 4 was attained at peptide segment concentrations as low as 50 µM, a concentration which is not feasible with conventional convergent methods using protected peptide segments.

† Abbreviations: Abu, L-α-amino-butyric acid; AcOH, acetic acid; Boc, tert-butoxycarbonyl; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HIV-1 PR, human immunodeficiency virus-1 protease; HOSu, N-hydroxy-succinimide; MALDIMS, matrix-assisted laser desorption mass spectrometry; RP HPLC, reversed-phase high-performance liquid chromatography; SPro, thioaldehyde; TFA, trifluoroacetic acid; Thz, thiazolidine.

‡ Abbreviation: UPS, Unpublished results.

Introduction

Conventional approaches for segment condensation in peptide synthesis focus on an α-amine-directed amide bond formation with an enthalpically activated α-carboxylic group.1,2 These approaches require a full or partial protection scheme for other functionalities to prevent random acylation, and suffer the limitations of solubility and purification difficulties attendant to protected peptide segments.1 Moreover, the intrinsic entropic barrier imposed by the high molecular weights of large protected peptide segments reduces the efficiency of intermolecular peptide bond formation.

Considerable efforts to overcome these limitations have resulted in the development of various approaches using minimally protected or totally unprotected peptide segments.2-6 Those employing the principle of entropy-driven intramolecular acyl transfer for the formation of peptide bonds hold particular promise.4,5 Kemp et al.4 utilized a capture step that brings together the respective C- and N-terminals of the two unprotected peptides through a tricyclic template to facilitate the subsequent amide bond formation through intramolecular acyl rearrangement.


for this purpose in the synthesis of three analogs of the aspartic protease of the human immunodeficiency virus-1, HIV-1 PR. The syntheses of these active HIV-1 PR analogs provide a stringent test for this entropy-driven orthogonal ligation strategy and demonstrate the suitability of a thioalnine as a replacement for Pro residue in protein synthesis.

Results

Overall Synthetic Scheme. HIV-1 PR, a member of the aspartic protease family, is a homodimer in which each of the 99-residue monomers contributes one of the two aspartic acid residues to constitute the active site of the enzyme. Total chemical synthesis of this protease was first achieved by stepwise solid phase peptide synthesis. In addition, several analogs of HIV-1 PR have also been prepared by chemoselective ligation via formation of thioester bonds as peptide bond replacement.

Preparation of Acyl Segment Bearing a Glycol Aldehyde Ester. A general method for the preparation of the acyl segment with a glycol aldehyde ester was developed using an n + 1 strategy by adding a single amino acid derivative, a leucinyglycyl aldehyde dimethyl acetal (Leu-oxycacetalddehyde dimethyl acetal), onto the (1–37)-segment containing a C^\text{40}-thioester, taking the synthesis of [SPro^{90},Abu^{67,95}]HIV-1 PR as example.

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- The aldehyde introduction step consists of coupling of the thioalnine, 1a, activated by silver ion with an \(\alpha\)-amino(acyloxy(amido))acetaldehyde masked as methyl acetal in large excess (\(\geq 100\)-fold) and blocking the acetal by aqueous TFA to yield the desired (1–38)-acyl segment 1e. Ring formation between 1e and 1d upon aldehyde capture of the 1,2-amino thiol of the N-Cys of 1d gave the thiazoline product, 1f, which underwent a spontaneous OH-N-acetyl transfer to yield the final product with a thioalnine (SPro) at position 39. If AA = amino acid residue; X = O or NH. If X = O, [SPro^{90},Abu^{67,95}]PR and [Ala^{90},SPro^{90},Abu^{67,95}]PR were obtained with AA = Leu or Ala, respectively. If X = NH, the synthetic scheme ended at stage 1e and [Leu-NHCH_{2}-Thz^{39} \_^{67,95}]PR was obtained with AA = Leu. Amino acid sequence of HIV-1 protease: H^{PQITLWQRPL\_VTIRIGGQLKEALLDTGADDTYLEEMNL\_P\_KWPKMI\_GGGGFKIVQYDQP\_VEICHGKAITVGLPGTVPNI\_\_NTR\_LETTIGC\_TLP\_\_OH. Amino acid residues are represented by the standard single letter code. The ligation site was at Pro^{90}. The underlined sequence represents the flap region; the highly conserved active site sequence is in italics. The two Cys^{67,95} residues were replaced with L-\(\alpha\)-amino-n-butyric acid (Abu) in our synthetic analogs.

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The synthesis of the (1–37)-segment thioalnine was achieved by the stepwise solid phase synthesis using Boc chemistry on a Boc-Asn-SCH_{2}CH_{2}CO-Gly-OCH_{2}-Pam resin using the method developed by Aimoto et al. &c. HF cleavage of the protected resin-bound (1–37)-thioalnine segment yielded the desired unprotected peptide in excellent yield (Figure 1A). Activation of thioalnine by Ag^{+} in the presence of a large excess of the masked Leu^{15}-oxycacetalddehyde efficiently yielded the (1–38)-

Figure 1. C^38 RP HPLC elution profile of the crude products of HIV-1 PR(1–37)-S(CH_{2})_{2}CO-Gly-OH and [Cys^{90},Abu^{67,95}]HIV-1 PR(39–99) after HF cleavage and dialysis. (A) HIV-1 PR(1–37)-S(CH_{2})_{2}CO-Gly-OH; (B) [Cys^{90},Abu^{67,95}]HIV-1 PR(39–99). HPLC linear gradient: 40–80% buffer B at 1%/min in buffer A.
driven by the silver ion mediated activation which distinguished this feasible because it involves formation of a peptide bond N the probability of undesired random acylation. In addition, groups and by an overwhelming excess of the small amino acid

37)-S(CH$_2$)$_2$CO-Gly-OH (peak 1); (B) purified HIV-1 PR(1

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to give HIV-1 PR(1-38)-OCH$_2$CHO (peak 3) in 10 min at 0 °C; (D) ligation of HIV-1 PR(1-38)-OCH$_2$CHO (Peak 3) with [Cys$_{39}$,Abu$_{67,95}$]HIV-1 PR (39-99) (peak 4), r = O; (E) after 5 h; desired ligation product (peak 5) and peak 6 is the disulfide dimer of peak 4; (F) purified product, the N-acyl product was indistinguishable in HPLC with the O-acyl product. HPLC was run with a linear gradient of 40–80% buffer B at 1%/min in buffer A.

acyl segment, 1b (Figure 2B). No side reactions with other amines and nucleophiles on the (1–37)-acyl segment itself were detectable because of their relatively low molar ratio when compared to the large excess of the small Leu$_{38}$ component. A concern on the stability of the (1–37)-thioester was the presence of Asn at the C-terminus which might be susceptible to aspartimide formation due to the amide side chain participation during the activation by Ag$^+$. However, no aspartimide formation was found in model experiments with Boc-Asn-S(CH$_2$)$_2$COOH. The likelihood of aspartimide formation during the reaction of (1–37)-thioester was further diminished by the large excess of the small amino component.

The masked amino acid glycol aldehyde ester was introduced enzymatically in previously published methods. However, the chemical method via Ag$^+$ described here is more versatile because it overcomes the limitation imposed by the substrate specificity of an enzymatic reaction. Blake and Yamashiro as well as Aimoto et al. have used the silver ion activated thiocarboxylic acid and thioester for the condensation of partially protected peptide segments.$^2$ The use of totally unprotected fragments has not been attempted because of the concern of random acylation on other amine nucleophiles present in the naked amine and acyl components. Our n + 1 coupling makes this feasible because it involves formation of a peptide bond between a large unprotected peptide and a small functionalized amino acid. The selectivity and efficiency of this coupling was driven by the silver ion mediated activation which distinguished the CO$_2$-thioester from other side chain unprotected carboxylic groups and by an overflowing excess of the small amino acid derivative of the masked glycol aldehyde which largely reduced the probability of undesired random acylation. In addition, N-hydroxysuccinimide was also used in large excess as an additive to minimize the possible danger of racemization. In combination with the orthogonal ligation, the n + 1 method of Ag$^+$-mediated thioester activation also provided a convenient approach for the systematic preparation of protein analogs, including those containing small organic functionalities. For example, we have introduced a Boc-protected hydrazide onto the C-terminus of the acyl (1–37)-segment by reaction with Boc-NHNN$_2$ in large excess (unpublished data). Such a derivative was then be used for conjugation, after removal of Boc, with a second segment bearing an N-terminal aldehyde function to form an HIV-1 PR analog containing an unusual backbone (unpublished data).

Acetal was chosen for aldehyde protection because it is easily removed. Treatment of the acetal, 1b, by TFA at low temperature (0 °C) quickly (10 min) demasked the aldehyde, 1c (Figure 2C). The TFA treatment gave side reactions in HIV-1 PR segments as revealed by HPLC when longer reaction time and/or higher temperature was applied (data not shown).

Similarly, two other analogs were also prepared by the n + 1 method. Ala$_{38}$ analog was prepared using the masked Ala-thiocarboxylic acid and the Leu$_{58}$-amidomethyl-Thz$_{59}$ analog by the masked Leu-amidoacetaldehyde.

Ligation of the Amine and Acyl Segments. Ligation between the unmasked acyl segment (1–38) containing a glycol aldehyde, 1c, and [Cys$_{39}$]amide segment 1d gave the HIV-1 PR thiazolidine product 1e, in this case, [SPro$_{39}$,Abu$_{67,95}$]HIV-1 PR. This ring formation was efficient and was 80% completed within 5 h (Figure 2E) even when the concentrations of both segments were low (0.05–0.2 mM). Such high reactivity provided orthogonality of our ligation scheme as well as the driving force to overcome the entropy barrier of the conventional intermolecular reaction between large peptide molecules. This efficiency could be attributed to the high reactivity of an aldehyde with 1,2-amino thiol and possibly to the complementarity between the two structurally folded unprotected peptides of the HIV-1 PR. Such complementarity is usually not possible for protected peptide segments and points to the unique advantage of using unprotected peptide segments as building blocks in our strategy. This ring forming reaction has also found its application in the preparation of large peptide dendrimers with high density.$^{10}$

Unlike the conventional method, our strategy generates an active product because the O,N-acyl transfer reaction also occurs at acidic condition and is the slow step in our scheme.$^3$ This greatly simplifies the synthetic scheme by eliminating the harsh deprotection and the necessity of the refolding steps. In our synthesis, autoproteolytic cleavages$^{11}$ of synthetic HIV proteases and degradation of synthetic segments occurred almost immediately. To prevent enzymatic cleavages, an aspartic protease inhibitor, acetylpepsatin was added to the ligation solution. The product obtained after HPLC purification was further incubated in 6 M guanidine hydrochloride (pH 5.5)/glycerol solution for 3–4 days to assure the completion of the O,N-acyl transfer. Because of the large size of HIV-1 protease, the rearranged N-acyl product was indistinguishable from the unarranged O-acyl product by HPLC. However, studies on small model compounds and peptides have determined that the O,N-acyl transfer reaction occurred at an optimal pH of around 5 with a t$_{1/2}$ of about 20 h.$^{5}$ Furthermore, the formation of the amide bond was confirmed by its stability under saponification


Figure 2. C$_{18}$ RP HPLC monitoring of the progress of the synthesis of [SPro$_{39}$,Abu$_{67,95}$]PR: (A) purified thioester segment, HIV-1 PR(1–37)-S(CH$_2$)$_2$CO-Gly-OH (peak 1); (B) purified HIV-1 PR(1–38)-OCH$_2$-CH(OCH$_3$)$_2$-CO-Gly-OH activated by silver nitrate with H-Leu$_{38}$-OCH$_2$CH(OCH$_3$)$_2$ (large excess) in DMSO; (C) demasking of the acetal by TFA (5% H$_2$O) to give HIV-1 PR(1–38)-OCH$_2$CHO (peak 3) in 10 min at 0 °C; (D) ligation of HIV-1 PR(1–38)-OCH$_2$CHO (Peak 3) with [Cys$_{39}$,Abu$_{67,95}$]HIV-1 PR (39–99) (peak 4), r = O; (E) after 5 h; desired ligation product (peak 5) and peak 6 is the disulfide dimer of peak 4; (F) purified product, the N-acyl product was indistinguishable in HPLC with the O-acyl product. HPLC was run with a linear gradient of 40–80% buffer B at 1%/min in buffer A.
conditions, since the O-acyl intermediate was found susceptible to hydrolysis by 0.05–0.1 N NaOH solution in 5–10 min while the N-acyl product was resistant to the same treatment. Because of the unactivated feature of the α-carboxyl group, this O,N-acyl transfer proceeded smoothly without the side reactions commonly seen in conventional peptide synthesis methods. All three analogs were prepared similarly except for Leu38-amidomethyl-Thz39, which did not involve an acyl rearrangement. All three protease analogs were analyzed by MALDIMS, which gave the expected molecular weight. For example, the [SPro39,Abu67,95]-HIV-1 PR gave a mass unit of 10 805 that agreed closely with the calculated mean unit of 10 803 (Figure 3).

**Enzymatic Activity of the Synthetic HIV-1 PR Analogs.**

Three different peptide substrates were used to determine the activity of these synthetic protease analogs. As shown in HPLC analysis (Figure 4), these HIV-1 PR analogs were active in catalyzing the hydrolysis of these substrates. Detailed kinetic studies (Table 1) with one of these substrates, the Gag p24/p15 synthetic peptide (substrate IV, Figure 4C), showed that all synthetic protease analogs exhibited binding affinity ($K_a$) similar to that of the native enzyme, but showed different catalytic activity ($V_{max}$): ~100% with [SPro39,Abu67,95]-HIV-1 PR, ~70% with Ala38 and ~30% with the Leu38-amidomethyl-Thz39 analog. The catalytic activity ($V_{max}$ or converted to $k_{cat}$) obtained for the native enzyme as well as for our synthetic analogs in this study are, however, lower than those determined by continuous spectrophotometry in the literature. This may be due to different assay methods and conditions. For example, the assay temperature (22 °C) in our study was lower than that (37 °C) used by others. Another reason may be the method for determining the enzyme concentration. Instead of using active-site titration for the folded enzyme solution, we measured the UV absorption of the denatured solution and assumed that all the material would be refolded to the active form when diluted into the assay buffer, which usually leads to overestimation of the actual active concentration. Nevertheless, we used identical assay procedures and conditions for all enzyme preparations including the native HIV-1 PR to provide a consistent basis for comparison.

It is not surprising that [SPro39,Abu67,95]-HIV-1 PR retained full activity when compared to the native enzyme, because the peptide backbone structures of the wild-type protease and this analog are identical and the (hydroxymethyl)thioproline is structurally a close approximation of the Pro residue. The epimeric hydroxymethyl group is on the side chain Pro ring and would not be expected to change the overall conformation of a proline-containing peptide chain, since Pro often occurs at the reverse turn and is solvent exposed. In the Ala38 analog, the change of the isobutyl side chain to a methyl may result in loss of hydrophobic interaction. This loss leads to an increase in the activation energy for the catalytic hydrolysis and accounts for the decrease in catalytic activity. In the case of Leu38-amidomethyl-Thz39 analog, there are three atoms inserted between Leu38 =C=O and Pro39 >NH, and this nonpeptide backbone may cause conformational distortion. Surprisingly, this analog still exhibited about 30% activity and comparable binding affinity toward substrate IV as the native enzyme. The relatively flexible structure of the broad loop8 apparently can tolerate this structural modification.

These results show that protease analogs with a pseudoproline structure in the sequence are able to fold, dimerize correctly into their active conformation, and retain nearly full catalytic activity. It is also interesting to note that our HIV-1 protease analogs already possess partial enzymatic activity at their O-acyl ester stage as concluded from results obtained from the Leu38-NHCH2-Thz39 analog. However, the O,N-acyl transfer was necessary to render these analogs fully active.

**Discussion**

A central element in achieving orthogonality in peptide bond formation between unprotected segments is the exploitation of proximity effect. Thus, our strategy focuses on side chain nucleophile capture for the initial bond formation, an important contributing factor to achieve orthogonality which leads to the subsequent entropy-driven amide bond formation by intramolecular acyl transfer. Interestingly, such a transfer reaction is naturally observed in the posttranslational protein splicing13 in which the ultimate excision of an intervening sequence involves

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an N,O(S)-acyl transfer between the amide and ester linkages of Ser, Thr, or Cys residue. Similar N,O-acyl interconversions are observed in peptides with Gly-Ser/Thr sequences in strongly acidic and mildly basic conditions.14 Thus, the ester formation of an acyl component with the side chains of Ser, Thr, or Cys in the amine component leads to amide bond formation through the proximity-driven O(S),N-acyl transfer. This principle was first utilized for peptide synthesis by Kemp et al.4 and recently adopted by us5 and Dawson et al.6

At present, there are three strategies that utilize the proximity-driven principle besides those using conformation-assisted couplings.15 First, Kemp used a tricyclic aromatic template containing an acyl ester thiol to capture an activated cysteinyl side chain through a disulfide exchange to place both the acyl component with the side chain of Ser, Thr, or Cys in the amine segment containing an aldehyde group.5 The third strategy uses either thioester or disulfide exchange without template to facilitate the proximity-driven S,N-acyl transfer. Dawson et al. have developed a thioester exchange between an acyl thioester and a cysteinyl segment,6 while we have developed similar schemes through a phosphine-assisted reaction as well as thioesterification with a β-bromoalanine peptide segment.16 We have also developed a facile disulfide exchange strategy through an acyl thiocarboxylic acid and an activated unsymmetrical disulfide of the N-Cys amino segment.17 A common theme in these strategies is the involvement of the N-Cys side chain to form a covalent bond between the acyl and the side chain of the amine segment and then an entropy-driven amide bond formation to produce a cysteine residue at the ligation site. Our strategy produces a proline-like backbone that may have the advantage of being more accessible because of the high frequency of proline residues occurring at reverse turns in protein sequences. Furthermore, by varying the aldehyde moiety, other nongenetically coded amino acids could be generated. The thiazolidines are also reversible to give cysteinyl bonds. These chemical approaches, together with the recently developed enzymatic method,18 represent important advances in the total synthesis of proteins.

With large unprotected peptide segments and protein domains readily accessible either by solid phase synthesis or recombinant techniques, a chemical ligation method to form an amide backbone between these high molecular weight building blocks would greatly expand our ability to synthesize and modify proteins with noncoded amino acids as well as to design artificial proteins with unusual architectures.19,20 The syntheses of HIV-1 protease analogs clearly demonstrate the ease and efficiency of our chemical ligation approach. Moreover, our method demonstrates that a pseudoproline structure can be used to replace a proline residue in the sequence of a protein without altering its biological activity. Most importantly, our strategy employs the principle of orthogonality, utilizing the side chain participation in the initial formation of a covalent complex and the subsequent intramolecular acyl transfer to overcome the problems of conventional segment condensation method. In the future, it should open new avenues for other chemical approaches in forming peptide bonds between very large peptide segments.

### Experimental Section

**General.** Analytical C18 HPLC was run on a Shimadzu system with a Vydac column (0.46 cm × 25 cm, C18 reversed phase) at a flow rate of 1.5 mL/min with a linear gradient of buffer B (60% acetonitrile in H2O/0.04% TFA) in buffer A (5% acetonitrile in H2O/0.045% TFA), with UV detection at 225 nm. Mass spectra were obtained with the Matrix-assisted laser desorption mass spectrometry (MALDIMS) with

| Table 1. Kinetic Parameters of the Synthetic HIV-1 PR Analogos on the Hydrolysis of Substrate IV in Comparison with Recombinant HIV-1 PR |
|-----------------|-----------------|-----------------|-----------------|
| Protease        | 38-39 linkage   | K<sub>m</sub> (μM) | V<sub>max</sub> (μmol/min/mg) | V<sub>max</sub>/K<sub>m</sub> (μmol/min/mg·M) |
| HIV-1 PR        | -Leu<sup>38</sup>-N<sup>ε</sup>-H<sub>4</sub> NH-<sup>ω</sup>Ala<sup>39</sup>-Val<sup>40</sup>-H<sub>4</sub> NH-Val<sup>41</sup>-HIV-1 PR | 10.1 | 3.43 | 3.40×10<sup>5</sup> |
| [S-Pro<sup>39</sup>], Abu<sup>57-58</sup>-HIV-1 PR | -Leu<sup>38</sup>-N<sup>ε</sup>-H<sub>4</sub> NH-<sup>ω</sup>Ala<sup>39</sup>-Val<sup>40</sup>-H<sub>4</sub> NH-Val<sup>41</sup>-HIV-1 PR | 11.9 | 3.96 | 3.33×10<sup>5</sup> |
| [Ala<sup>38</sup>, S-Pro<sup>39</sup>], Abu<sup>57-58</sup>-HIV-1 PR | -Ala<sup>38</sup>-N<sup>ε</sup>-H<sub>4</sub> NH-<sup>ω</sup>Val<sup>39</sup>-Val<sup>40</sup>-HIV-1 PR | 8.2 | 2.26 | 2.76×10<sup>5</sup> |
| [Leu-NHCH<sub>2</sub>-Thr<sup>38-39</sup>, Abu<sup>47-58</sup>]-HIV-1 PR | -Leu<sup>38</sup>-N<sup>ε</sup>-H<sub>4</sub> NH-<sup>ω</sup>Thr<sup>39</sup>-Thr<sup>40</sup>-HIV-1 PR | 11.4 | 1.14 | 1.00×10<sup>5</sup> |

Kinetic studies were performed at 22 °C under steady state conditions and substrate hydrolysis was analyzed by RP-HPLC. See Experimental Section for details. Enzyme concentrations were measured by UV spectrophotometry on denatured enzyme preparations in 5 mM guanidine hydrochloride.

an accuracy of ±0.05%. The calculated mass units were given as average values of the isotopic composition.

Recombinant HIV-1 PR was purchased in its highest grade (affinity-purified, crystallizable grade) from BACHEM Bioscience Inc. (Bubendorf, Switzerland). This material gave a \( V_{\text{max}} \approx 19.7 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1} \) toward substrate IV at 37 °C. Acetylpeptatin was also from the same company. All HIV-1 PR substrates were purchased from BACHEM (Torrance, CA).

Synthesis of HIV-1 PR(1–37)-S(CH₂)₃-CO-Gly-OH (1a) and [Cys³⁸,Abu⁶⁷⁹]HIV-1 PR (39–99) (1d). Both the thioester segment, 1a, and amine segment, 1d, were prepared by solid phase synthesis using the conventional Boc-aryl type protection strategy and the BOP coupling protocol. 1a was synthesized on an Asn²²-S(CH₂)₃-CO-Gly-OCH₃-Pam resin which was obtained through coupling of Boc-Asn-S(CH₂)₃-COOG to H-Gly-OCH₃-Pam resin as reported. 1d was synthesized on a Phe⁹⁹-OCH₃-Pam resin. The final cleavage was performed using the low–high HF cleavage procedure. After HF cleavage, the crude products were extracted with 50% AcOH/H₂O, dialyzed against decreasing concentrations of AcOH to 10%, and then purified by preparative HPLC with a Vydac C₄ column. The purified products gave a single peak in analytical RP HPLC (Figure 2A,D, peaks 1 and 4 respectively): MS [M⁺] 4281.8 (calcd), 4282.0 (found); [M+1] 4292.7 (calcd), 4305.0 (found).

Preparation of α-amino(acetyl)- and Amidooacetaldyde Dimethyl Acetal. Aldehyde Introduction. α-amino(acetyl)acetaldyde dimethyl acetal was prepared by esterification of the Z-protected α-amino acid cesium salt with bromoacetaldelyde dimethyl acetal in DMF followed by catalytic hydrogenolysis as reported previously. α-Amino(acylamido)acetaldyde was prepared by coupling of Z-amino acid to aminooacetaldelyde dimethyl acetal followed by catalytic hydrogenolysis. The thioester segment, 1a, was dissolved in DMSO (5–10 mM) containing 0.5–1.0 M of the amino acid derivative, α-aminoacycloxy(amido)acetaldelyde dimethyl acetal, HOSt (0.5–1.0 M), and dimethyl sulfoxide (∼50 mM). AgNO₃ pre-dissolved in DMSO (2 equiv to the thioester segment) was added. In general, the reaction was completed in 20–60 min with >80% yield as determined by analytical HPLC: MS [M + H⁺] : HIV-1 PR(1–38)-OCH₂CH(OH)₂, m/z 4337.9 (calcd), 4338.0 (found); [Ala³⁸,Pro⁶⁷⁹]HIV-1 PR(1–38)-OCH₂CH(OH)₂, m/z 4298.6 (calcd), 4296 (found); HIV-1 PR (1–38)-NHCH₂CH(OH)₂, m/z 4336.9 (calcd), 4338.0 (found).

The acetal deprotection was performed using 95% trifluoroacetic acid in H₂O at 0 °C for 5–10 min to give the desired glycol aldehyde acyl segment, 1c, as shown in Figure 2C for the synthesis of [SPro⁶⁶,Abu²⁶⁶]HIV-1 PR. The reaction mixture was immediately diluted with 10-fold 30% acetonitrile/H₂O and applied to an HPLC column for purification. The collected fractions were concentrated by a centrifuge vacuum and used immediately for the next step.

Ring Formation and Acyl Transfer. The amino segment, [Cys³⁸⁹,Abu⁶⁷⁹]HIV-1 PR (39–99), 1d, was dissolved in a minimal amount of 60% acetonitrile/H₂O (0.1% TFA) and mixed with the acyl segment containing a glycol aldehyde ester, 1c. The solution was diluted with H₂O (0.1% TFA) to the desired concentrations of the two segments (∼50 μM for 1c and ∼100 μM for 1d). Acetylpeptatin (0.5 mM) and EDTA (0.5 mM) were then added. The pH was adjusted by addition of solid sodium acetate to 3.0–4.0 (by pH paper). The thiazolidine ring product, 1e, was 60–80% formed as determined by HPLC in 5–10 h based on the acyl segment of glycol aldehyde ester (the amino segment was used in 2–3 equiv). The ring formation would be faster at higher pH as stated in previous studies, but in the case of HIV-1 protease, pH >4 led to precipitation of both segments (especially the amino segment). Some precipitation (mostly of the ligation product) was indeed observed during the reaction which was dissolved by guanidine hydrochloride before HPLC purification. The ligation product was isolated by HPLC, lyophilized, and redissolved in the denaturing condition of 6 M guanidine hydrochloride (pH 5.5)/40% glycerol (v/v) solution to complete the α,N-aclyl transfer reaction. The protein concentration was determined by UV spectroscopy in its 6 M guanidine hydrochloride solution prior to the addition of glycerol using a \( C_{\text{G280}} = 25\,000\, M^{-1}\mathrm{cm}^{-1} \). This concentration then served as basis for determination of enzymatic activities and for calculation of kinetic parameters such as \( V_{\text{max}} \), assuming that 100% of the material would refold and remained active in enzymatic assays. The guanidine/glycerol solution served as a storage buffer for the protease and to preserve enzymatic activity. A >90% yield of the rearranged N-acyl product, 1f, was obtained after incubating for 3–4 days in the above buffer. These enzyme solutions were stored at 70 °C. The enzyme sample was active immediately when diluted into the assay buffer as shown by the hydrolysis of the synthetic peptides derived from the HIV Gag protein sequences (Figure 4): MS [M + H⁺]: [SPro³⁶,Abu²⁶⁶]HIV-1 PR (monomer), m/z 10803 (calcd), 10805 (found); [Ala³⁸,Pro²⁶⁶]HIV-1 PR, m/z 10761 (calcd), 10764 (found); [Leu-NHCH₂-Thz³⁸,Abu²⁶⁶]HIV-1 PR, m/z 10802 (calcd), 10806 (found).

HPLC Analysis of the Enzymatic Hydrolysis of HIV-1 PR Substrates by the Synthetic Protease Analogs. Three different synthetic substrates derived from the Gag p17/p24 and p24/p15 cleavage sites were used for qualitative HPLC assays: H-Val-Ser-Gln-Tyr-Pro-Ile-Val-OH (substrate VIII) from the natural p17/p24 cleavage site, H-Lys-Ala-Arg-Val-Nle-Phe(p-NO₂)Glu-Ala-Nle-NH₂ (substrate IV) and 2-aminobenzyol-Thr-Ile-Nle-Phe(p-NO₂)Glu-Nle-NH₂β-D derived from the two p24/p15 cleavage sites. Synthetic protease analogs efficiently catalyzed the hydrolysis of these substrates in a weakly acidic buffer. Typically, for HPLC assay, an aliquot of protease analog solution in 6 M guanidine hydrochloride/40% glycerol was directly added to an appropriate assay buffer with a substrate for an indicated time period at 22 °C and stopped with 20% TFA/H₂O before applying to HPLC column (Figure 4). The hydrolitic products were collected from analytic HPLC and confirmed by MS analysis.

Kinetic Studies. Substrate IV, derived from the first cleavage site of the Gag p24/p15, was used for all kinetic studies due to its excellent solubility in aqueous buffer. Assay buffer was 50 mM sodium acetate, pH 4.7, containing 5 mM DTT and 2 mM EDTA, with an ionic strength equal to 1 M NaCl and with glycerol added to give a 10% (v/v). DTT and EDTA were added to prevent the oxidation of the free Cys residues in the recombiant HIV-1 protease. This buffer was used for comparison in all kinetic studies. The enzyme sample was activated by simple dilution into the assay buffer. No further refolding/dialysis procedure was employed. The enzyme concentrations typically used for kinetic assays were 0.02–0.05 μM, and the substrates were between 5 and 60 μM. The reaction proceeded for 3–6 min during which less than 25% of the substrate was hydrolyzed and was stopped with 20% TFA in H₂O. Initial rates were calculated on the basis of the UV absorption (area of HPLC peaks) at 225 nm of the substrate and hydrolyzed products. The ratio of UV absorption of the substrate to the hydrolyzed C-terminal half, H-Phe(p-N₂O)Glu-Ala-Nle-NH₂ was determined to be 1:0.68, calibrated in the presence of internal standards. Typically, five to six data points were used for each experiment. Kinetic parameters were obtained by fitting data into the Michaelis–Menten equation using the Lineweaver–Burk plotting method. All kinetic studies were performed at 22 °C.

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