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A Quick Diversity-Oriented Amide-Forming Reaction to Optimize P-Subsite Residues of HIV Protease Inhibitors

Ashraf Brik,[1] Ying-Chuan Lin,[2] John Elder,[2] and Chi-Huey Wong[1,3]
[1]Department of Chemistry and
The Skaggs Institute for Chemical Biology
[2]Department of Molecular Biology
The Scripps Research Institute
10550 North Torrey Pines Road
La Jolla, California 9203

Summary

We report a new simple method that allows rapid preparation in solution of a library of compounds for in situ high-throughput screening to identify new inhibitors of HIV-1 protease. The method is based on the amide-forming reaction of a C_2-symmetrical diamino diol core with various carboxylic acids, followed by a direct assay of the inhibition activity without product isolation. Sixty-two compounds were made and screened in less than 1 hr. The utility of this method is demonstrated by the identification of new P3-P3′ residues that convert a transition state analog core from a poor binding molecule (1, $K_i > 2$ μM) to a potent inhibitor (AB1, $K_i = 2$ nM) against the wild-type, and the inhibition activities against resistant mutants are better than those of two existing drugs. This method reduces the time required for synthesis and testing of a large number of characterized inhibitors and should find useful applications in other enzyme systems.

Introduction

Most of the drug discovery processes start with efforts to identify lead compounds, either by screening chemical libraries or natural products. Lead optimization is then pursued through analog synthesis. In most cases, the basic structure unit of the lead compound is kept constant, while other parts of the molecule are modified to generate a library. High-throughput screening is often used in both lead identification and lead optimization. A key to the success of this discovery process is efficiency: formation of the expected product must be efficient, with high product purity and high yields. Ideally, the diversification process should be short, if possible a one-step reaction, and requires no further purification before screening.

Solid-phase and solution-phase syntheses have been used widely and successfully in lead identification and lead optimization in drug discovery. The processes often depend on preparation of individual compound or mixture, which are separated from the reaction reagents before biological assays. As a result, the chemistry is often slower than the screening process. Though mixtures have been used for screening, they are often generated through multistep time-consuming processes, and the by-products are often either difficult to separate or interfere with the assay. There are a few reactions (e.g., oxime formation, cycloaddtition reaction, and amide bond formation) known to be rapid, chemoselective, highly quantitative, free of protecting group, and compatible with aqueous solution. Of such reactions, the products, in principle, may be used in situ for screening without concentration and purification. This strategy has not been used effectively in drug discovery, however. To illustrate the effectiveness of this approach, we have selected the human immunodeficiency virus protease (HIV-1 PR) as our target, using a diversity-oriented amide-forming reaction coupled with in situ screening.

Since the early days of the discovery of HIV-1 PR, this enzyme has been selected as an important target for the inhibition of viral replication. The enormous effort over the past two decades to develop effective molecules that inhibit the HIV-1 PR has resulted in the discovery of drugs that have dramatically improved the quality of life and survival of patients infected with HIV-1. To date, there are six different HIV-1 PR inhibitors (PI) that are commercially available (Figure 1) [1–3]. Unfortunately, many drug-resistant and cross-resistant mutant HIV-1 PRs have been identified, rendering AIDS with no definitive cure [4, 5]. Thus, the development of new protease inhibitors which are efficacious against both the wild-type and drug-resistant HIV-1 PR and less prone to development of resistant strains of HIV is urgently needed.

Most of the P2-P2′ and P3-P3′ residues in the structure of FDA-approved HIV-1 protease inhibitors were attached to the cores through an amide bond (Figure 1), making the amide bond-forming reaction of great interest if one wishes to prepare and rapidly screen inhibitors with new P-P′ residues (Figure 2). A vast amount of information is available regarding this chemistry, both in solution and on solid support [6]. Several activation reagents in different solvents have been found to give a quantitative yield in very short reaction time. For example, analogs of phosphonium salts, in particular *N*-[(1-*H*-benzotriazole-1-y) (dimethylamino) methylene]-N-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU) and *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*] pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU), have been found to be very efficient and convenient reagents to activate carboxylic acids for coupling [6].

Herein, we report a useful method that allows rapid preparation of a library of inhibitors for in situ high-throughput screening to identify new HIV-1 PR inhibitors. Once a new inhibitor is identified, it will be synthesized followed by more careful characterization using traditional methods. The new method is based on our observation that the reaction conditions for attaching various P-P′ residues using the amide-forming reaction between the C_2-symmetrical diamino diol core and different carboxylic acids do not interfere with the HIV-1 PR inhibitor assay. The reaction is fast (30 min) and gives the products in quantitative yields. The utility of

[3]Correspondence: wong@scripps.edu

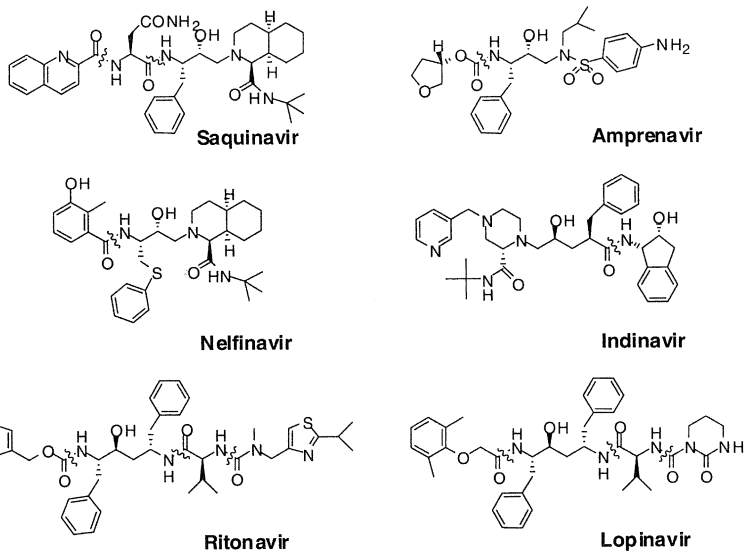


Figure 1. FDA-Approved HIV-1 Protease Inhibitors, Showing the Site of the Attachment of Different P2-P2′ and P3-P3′

our method is demonstrated by the identification of new P-P′ residues that convert the core from a poor binding molecule ($K_i > 2$ μM) to a potent inhibitor ($K_i = 2$ nM), and the inhibition activities against resistant mutants are better than those of two existing drugs.

Results and Discussion

All commercially available drugs for HIV-1 PR and some of the inhibitors currently being tested clinically were developed based on the transition-state mimic concept, in which nonhydrolyzable hydroxyethylene or hydroxyethylamine was used as the basic core for the synthesis of inhibitors. Most commonly, various P-P′ residues were attached to the basic cores to form the active molecules. It was found that subtle changes of the structure of these residues could lead to new inhibitors with unique binding interaction that are effective against drug-resistant strains. For example, changing the tetrahydrofuran ring to *bis*–tetrahydrofyranyl urethane in Amprenavir has furnished an extremely potent inhibitor, UIC-94003 (TMC-126), that is effective against a wide spectrum of HIV strains [7]. Although solid-phase synthesis has been used successfully to diversify several cores using multistep reactions [8–10], finding a method that would allow easier, faster diversification and screening would be highly valuable.

Our previous effort toward the development of protease inhibitors efficacious against both HIV and FIV was focused on the systematic analysis of S3-S3′ subsite

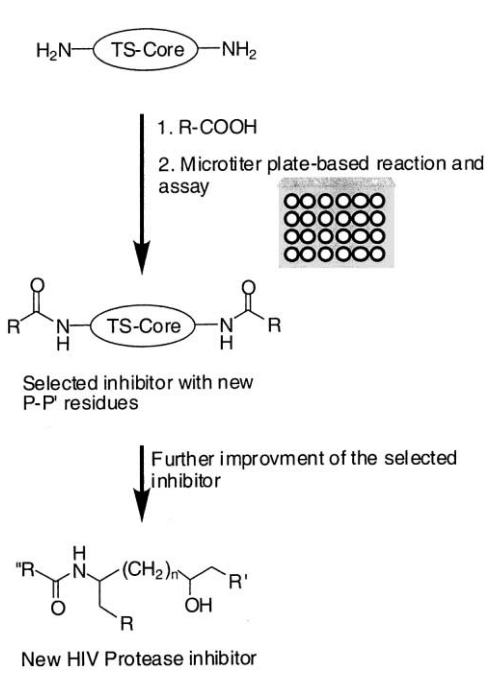


Figure 2. Principle of the Amide Bond-Forming Reaction to Generate a Library of HIV PR Inhibitors, Coupled with High-Throughput Screening

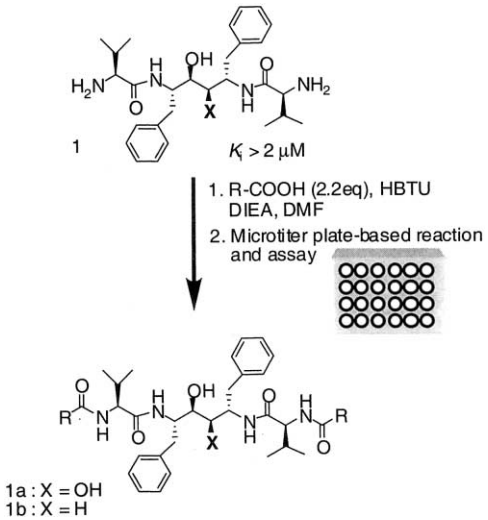


Figure 3. In Situ Preparation and Screening of Different Inhibitors with Various P3-P3′ Residues

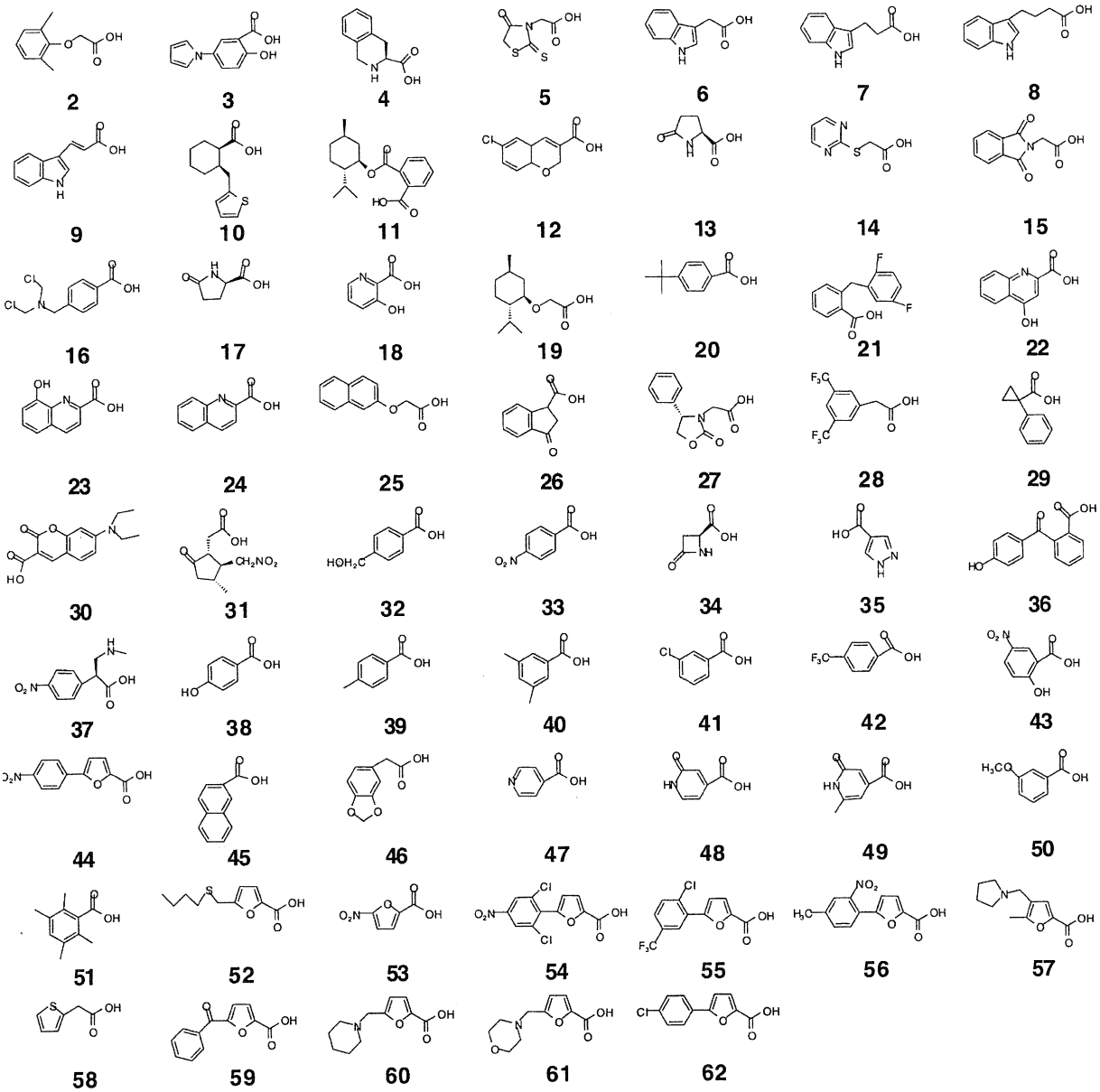
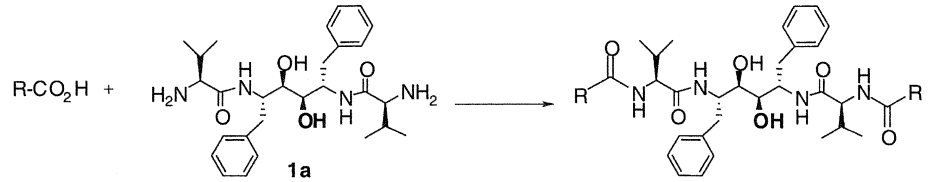


Figure 4. Structure of the 61 Acids that Were Tested as Potential P3-P3′ Residues

specificities of the enzymes using a series of C_2-symmetric inhibitors containing (1S, 2R, 3R, 4S)-1,4-diamino-1,4-dibenzyl-2,3-butan-diol **1a** as a P1-P1′ core and Val as P2-P2′ residues [11–14]. Several inhibitors were synthesized and tested, including a variety of amino acids with different N^{α}-protecting group at the P3-P3′ positions. Although various inhibitors were found to have high potency against HIV-1 PR in vitro, the evaluations of other residues at these positions is a tedious process and is restricted by the time required for the syntheses and purification of the new compounds for testing.

To overcome the above limitations, the C_2-symmetrical diaminodiol **1a** and diamino alcohol **1b** cores were reacted with various acids in solution, and the products were screened for inhibition activity without any purification (Figure 3). This idea was first tested on our previous inhibitor TL-3 [13, 14] (Figure 5). The core **1a** was reacted with Cbz-Ala (2.2 eq) in the presence of HBTU or HATU (2.2 eq) and *N*, *N*-Disopropylethyl amine (DIEA) (4.4 eq) in DMF or DMSO. The reaction product was diluted with buffer and directly screened for its inhibition activity to give very similar results in terms of IC_{50} ($\sim$5 nM) as compared to the pure compound. Indeed, we have found that up to 200 μM concentration of HBTU/HATU and DIEA does not interfere with the activity of the HIV-1 PR. Further investigation of this reaction with various acids revealed that all of reactions went to completion

Table 1. Percent Inhibition of HIV-1 Protease Activity at 100 nM of Product Generated by the Amide-Forming Reaction and In Situ Screening



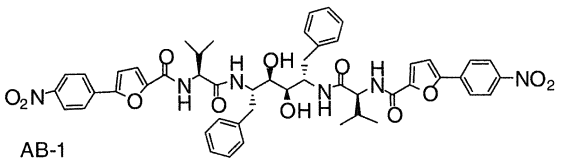
R-CO_2H	HIV-1 protease inhibition (%) at 100 nM	R-CO_2H	HIV-1 protease inhibition (%) at 100 nM
2	50	33	35
3	60	34	95
4	22	35	96
5	21	36	34
6	85	37	29
7	98	38	36
8	87	39	32
9	30	40	45
10	25	41	21
11	14	42	22
12	25	43	27
13	25	44	99
14	75	45	88
15	60	46	70
16	30	47	56
17	40	48	50
18	32	49	34
19	40	50	23
20	45	51	12
21	20	52	86
22	85	53	90
23	50	54	76
24	95	55	67
25	93	56	98
26	30	57	54
27	22	58	86
28	35	59	98
29	29	60	74
30	30	61	66
32	26	62	99

Table 2. IC_{50} Values for Selected Acids at P3-P3′ Positions

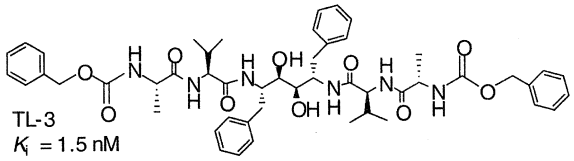
Entry	R-COOH	IC_{50} (nM)
1	6	**17**
2	7	**10**
3	8	**14**
4	22	**19**
5	23	**>50**
6	24	**10**
7	25	**10**
8	44	**6 ± 0.5**
9	53	**20**
10	54	**>50**
11	55	**>50**
12	56	**10**
13	59	**10**
14	60	**>50**
15	61	**>50**
16	62	**10**

Assays were performed directly from reaction mixture after reactions went to completion. For entry 8, IC_{50} was determined for the pure compound. For each IC_{50} determination, assays were performed in triplicate at five inhibitor concentrations.

after 30 min, as monitored by thin-layer chromatography (TLC) and electrospray ionization mass spectrometry (ESI-MS). In addition, the measured IC_{50} for the crude products were nearly identical to the purified compounds.



AB-1
K_i = 2 nM



TL-3
K_i = 1.5 nM

Figure 5. Structure of AB-1 and TL-3

The above results have encouraged us to prepare a library of inhibitors by modifying the core structure **1a** with different P3-P3′ residues. Initially, 50 different acids (**2–51**, Figure 4) were analyzed in the same manner as described. The acids were chosen based on availability, cost, and diversity. Each carboxylic acid was added to a 200 μl well of a 96 microtiter plate containing HBTU and DIEA in DMF followed by addition of core **1a**, and the mixture was kept at room temperature for 30 min. The reaction mixture in each well was diluted to a final concentration of 100 nM, based on complete conversion of the starting material to product, and screened for HIV-1 PR inhibition (Table 1). Wells that inhibited more than 50% of the HIV-1 PR activity were diluted to 10 nM and screened again. We were able to carry out the whole process of activation, coupling, and in situ screening in less than 1 hr.

Several amide products from the acids attached to core **1a** ($K_i > 2$ μM) were found to be very potent inhibitors (Table 2). Some of the acids that formed strong inhibitors have been previously used before (e.g., **24**) as the P3 residue [1]. However, new residues at the P3-P3′ positions also have emerged using our method, most notably acid **44** [5-(4-nitrophenyl)-2-furic acid] (Figure 4), which furnished the potent inhibitor AB1 with $IC_{50} <$ 10 nM for the crude compound (Figure 5). Further synthesis and characterization of this inhibitor on a large scale allowed the determination of the IC_{50} and K_i of the pure compound to be 6 nM and 2 nM, respectively. We also tested compound AB1 against some of the resistant protease mutants, and the IC_{50} values against G48V and V82F were found to be 23 nM and 16 nM, respectively, compared to 110 nM and 30 nM with the existing drug saquniavir and 25 nM and 35 nM with nelfinavir.

With this approach, it is also possible to design a library for structure activity relationship (SAR) studies. For example, once we determined **44** to be a good P3-P3′ residue, we screened a series of acids that are structurally related to **44** (**52–62**, Figure 4). Analysis of the inhibition data shows that separation of the substitution by methylene group from the furan ring causes a loss of activity (Entry 14 and 15, Table 2). Perhaps conjugation of the two aromatic rings is important. In addition, we have found that the number and the position of the substitutions on the phenyl ring are important for the inhibitor potency (Entry 9, 10, and 11, Table 2). Changing the nitro substitution on the *para*-position to the chloro derivative seems to have a small effect on the inhibitor activity [Entry 16, Table 2]. We also have found that reacting the core **1b** instead of **1a** with various acids (**7**, **14**, **44**, **53**, and Cbz-Ala, Figure 4) have provided inhibitors that exhibited 1- to 2-fold decrease in IC_{50}. These results are in agreement with the previous studies using compounds obtained via traditional solution-phase and solid-phase syntheses [10, 15].

In summary, we have applied the amide-forming reaction in solution to generate libraries for in situ identification of new P3-P3′ residues. Sixty-two acids were activated, coupled to the amine core, and screened directly for their inhibition activity in less than 1 hr. In principle, using other amine core or acid intermediates (e.g., in the case of Indinavir [16]), one can rapidly generate libraries of amides from libraries of various acids or amines for in situ screening to optimize the other P-P′ residues.

Significance

We have developed a simple method to rapidly prepare a library of potential inhibitors in solution which are used directly without purification in a high-throughput screening to identify new P3-P3′ residues for the HIV-PR. The method relies on the efficient, flexible, and mild amide-forming reaction, and new inhibitors better than existing drugs against resistant mutants have been rapidly identified within an hour. Although we have demonstrated the usefulness of this method to screen for new P3-P3′ residues in HIV protease inhibitors, it is also possible to use this strategy to identify other new residues in HIV PR inhibitors or other enzyme inhibitors.

Experimental Procedures

General

Analytical TLC was performed on precoated plates (Merck, silica gel 60F-254). Silica gel used for flash column chromatography was Mallinckrodt Type 60 (230–400 mesh). Reagents of the highest purity were purchased from the Aldrich, Sigma, Acros, Novabiochem, or Bachem.

General Procedure for Coupling Reactions and In Situ Screening

Twenty microliters of each carboxylic acid (from a 100 mM stock solution in DMF, 0.0022 mmol) was added to a 200 μl well of a 96 microtiter plate that contained 20 μl of HBTU (from 100 mM stock solution in anhydrous DMF, 1.1 eq), DIEA (0.0044 mmol). To each reaction mixture was added 10 μl of the diaminodiol core **1** (from a stock solution of 100 mM, 0.001 mmol in anhydrous DMF). All the reactions were mixed once all the reagents were added and kept at room temperature. The reactions went to completion in 30 min, based on the disappearance of the free amine monitored by TLC (10:1,$CHCl_3$: MeOH, Rf = 0.28) and analysis of the crude reaction mixture by ESI-MS. The reaction mixture in each well was diluted to a final concentration of 100 nM into second 200 μl wells of 96 microtiter plate using 0.1 M Mes buffer containing 0.2 M NaCl, 1 mM DTT (200 μl final volume). To each well was added enzyme (30 μg/ml) and substrate (5 mM), and the wells were directly assayed for HIV PR inhibition. Wells that inhibited more than 50% of the HIV-1 PR activity were diluted to 10 nM and screened again.

Synthesis of AB1

Diaminodiol core **1a** was prepared as previously reported [14]. To a solution of a free amine **1a** (10 mg, 0.02 mmol) and 5-(4-nitrophenyl)-2-furic acid (10 mg, 0.04 mmol) in 3 ml dry DMF was added HBTU (16 mg, 0.04 mmol) followed by DIEA (10 μl, 0.08 mmol) at 20°C under Ar atmosphere. The reaction mixture was stirred for 30 min and then quenched by addition of brine and extracted with EtOAc. The organic layer was washed with 1 N HCl, saturated aqueous $NaHCO_3$, and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by flash chromatography ($CHCl_3$: MeOH) to give the desired product in 90% yield (16 mg). $[\alpha]^{25}_D = +13.0$ (C = 1, in DMSO) ^{1}H NMR (400 MHZ, DMSO-d_6) δ 0.75 (d, 6.7 Hz, 6H), 0.83 (d, 6.5 Hz, 6H), 2.0 (m, 2H), 2.55 (m, 2H), 2.73 (m, 2H), 3.16 (d, 5.2 Hz, 2H), 4.20 (t, 8.8 Hz 2H), 4.30 (m, 2H), 4.80 (bs, 2H), 6.9 (m, 2H), 7.0 (t, 7.63 Hz, 4H) 7.15 (d, 11 Hz, 4H), 7.36 (d, 3.5 Hz, 2H), 7.45 (d, 3.5 Hz, 2H), 7.55 (d, 9.3 Hz, 2H), 8.16 (d, 9.12 Hz, 4H), 8.23 (d, 8.8 Hz, 2H), 8.33 (d, 8.8 Hz, 4H); ^{13}C NMR (400 MHZ, DMSO-d_6) δ 170.1, 157.0, 152.2, 148.4, 146.7, 138.9, 135.1, 129.1, 127.6, 125.5, 125.1, 124.3, 116.3, 111.6, 73.4, 58.6, 50.9, 29.5, 19.4, 18.7; ESI-MS, calc MH+ for ($C_{50}H_{52}N_6O_{12}$) 929.4, found 929.6.

HIV-1 PR Expression and Purification

A recombinant plasmid bearing a portion of the Pol gene of the BH10 clone of HIV was used for amplification of sequence encoding PR. The 5′ primer was constructed so as to insert an initiator methionine as part of the coding sequence for an NdeI site eight amino acids before the beginning of PR. This primer also encoded a nucleotide change to mutate Gln-7 to Lys, to block a major site of autoproteolysis and thus increase stability of the enzyme. The 3 primer was designed to insert a stop codon immediately after residue 99 of PR and a HindIII site 3′ of the stop codon to facilitate directional cloning. The PCR product was then cut with NdeI and HindIII and inserted into the pET 21a+ vector (Novagen) for protein expression.

The recombinant plasmid was transformed into the BL21(DE3)-pLysS strain of *E. coli*. The washed inclusion body pellet was then solubilized in 200 ml 20 mM Tris-HCl (pH 8) (1 mM DTT, 5 mM EDTA, 8 M urea) with stirring at 4°C for 1 hr. Insoluble material was removed by centrifugation at 8000 × g for 30 min. The supernatant from this centrifugation was treated batchwise by the addition of 20 mg DE 52 anion-exchange resin, and the mixture was stirred at 4°C for 1 hr. After centrifugation, PR was found in the supernatant. The resin was washed once with 50 ml of resuspension buffer, and the wash and supernatant fractions were combined.

The supernatant/wash fraction was then passed over Resource Q anion exchange resin equilibrated in resuspension buffer by using a Pharmacia FPLC apparatus. The fraction that failed to bind to the column was concentrated by using 5 K cut-off UltraFree centrifugal concentrators (Millipore). The retentate was then dialyzed overnight against deionized water, which caused precipitation of PR. The pellet was recovered by centrifugation at 3000 × g for 20 min, then resuspended in 20 mM sodium acetate (pH 5.3) (1 mM DTT, 5 M GuHCl) to a concentration of 1 mg/ml (determined by Lowry assay of the pellet suspended in a known volume of water before final pelleting and solubilization in sodium acetate/DTT/GuHCl buffer). Matrix-assisted laser desorption ionization analysis indicated a mass of 10,792, which is within 1 mass unit of the predicted mass for the properly processed PR. Activity was monitored by using a fluorogenic substrate, as detailed below. Aliquots were stored at 70°C for subsequent use.

Biological Assays

Kinetic determinations for HIV were performed at 37°C at pH 5.6 by using a F-2000 fluorescence spectrophotometer (Hitachi) and Packard-fluorescence spectrophotometer (Fusion-Universal Microplate Analyzer) for microtiter plate assay. For HIV-1 PR, the K_m and V_{max} values for the fluorogenic peptide substrate 2-aminobenzoyl (Abz)-Thr-Ile-Nle∼Phe- (*p*-NO2)-Gln-Arg-NH2 were determined by measuring the initial rate of hydrolysis at different substrate concentrations (2.5, 5.0, 10, 25, 50, and 100 μM), by monitoring the change in fluorescence at an excitation wavelength of 325 nm and an emission wavelength of 420 nm and fitting the obtained data to the Michaelis-Menten equation, using the GRAFIT program (version 3.0, Erithacus Software, UK). Assays were run in 0.1 M Mes buffer containing 0.2 M NaCl, 1 mM DTT (200 μl final volume). The enzyme concentration (30 μg/ml) that gave ideal progress curve was used for assays. Fifty percent inhibitory concentration (IC_{50}) values were determined in triplicate or duplicate with various inhibitor concentrations (for AB1: 0.5, 2.0, 4.0, 8.0, 12, and 16 nM) using the GRAFIT program. K_i of AB1 inhibitor were derived from IC_{50} using the formula for competitive inhibitor $K_i = IC_{50}/(1 + [S]/K_m)$.

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