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Articles

Inhibitors of the Protease from Human Immunodeficiency Virus: Synthesis, Enzyme Inhibition, and Antiviral Activity of a Series of Compounds Containing the Dihydroxyethylene Transition-State Isostere

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A number of potential HIV protease inhibitory peptides that contain the dihydroxyethylene isostere were prepared and evaluated for their enzyme binding affinity and antiviral activity in cell cultures. From the template of a previously reported active peptide A, modifications at the N- and C-terminal groups were assessed for potential maintenance of good inhibitory activity of the resulting peptides. Among the active peptides found, peptide XVIII exhibited potent enzyme inhibitory activity. Interestingly, the previously reported, effective 1(*S*)-amino-2(*R*)-hydroxyindan C-terminal group for the preparation of very active HIV protease inhibitory peptides could not be applied to the template of peptide XVIII. Molecular modeling of peptide XVIII was studied using the X-ray crystal structure of peptide A as a starting point in order to study the likely conformation of peptide XVIII in the active-site cleft. Relative binding conformations of peptide A and XVIII were obtained, although the reason for poor binding affinity for a number of congeneric peptides in this report was not straightforwardly apparent. More importantly, however, peptide XVIII was found to exhibit more effective antiviral activity in the HIV-1/PBMC assay than the reference peptide A which was previously reported to be approximately equal in efficacy to the reverse transcriptase inhibitor AZT in this assay.

Introduction

The rapid spread of the acquired immunodeficiency syndrome (AIDS) epidemic and the difficulties encountered in the development of an efficacious vaccine have stimulated a world-wide quest for therapeutic agents to arrest the replication of the causative virus in AIDS, human immunodeficiency virus (HIV). The formidable task is to develop drugs that can interrupt the life-cycle of HIV without harming the infected host. At present, the most widely used anti-AIDS drug is the nucleoside, reverse transcriptase (RT) inhibitor, 3′-azido-2′,3′-dideoxythymidine (AZT).[1-3] AZT and other such nucleoside derivatives have shown beneficial effects in AIDS patients, but their prolonged use is often compromised by side effects, such as bone marrow suppression.[4] Moreover, it is generally the case that AZT-resistant strains of the virus can overcome the effectiveness of the drug.[5] A number of other non-nucleoside RT inhibitors are also currently undergoing clinical evaluations.[6] There is much interest currently in combination antiviral therapy with drugs directed toward different targets in the viral life cycle.[7]

One promising possibility to interrupt the viral life cycle is the use of inhibitors of the virally encoded protease responsible for viral maturation, possibly alone, or in combination with RT inhibitors. The HIV protease represents another unique aspect of HIV biochemistry, in

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Table I. Inhibitory Activity against HIV-1 Protease[a]

peptide	structure	K_i (nM)
A	Noa—His—Chaψ[CH(OH)CH(OH)]Val-Ile-Amp	<1
B		22
I		36
II		16

[a] Noa = 1-naphthoxyacetyl; Chaψ[CH(OH)CH(OH)]Val = 5(*S*)-amino-6-cyclohexyl-3(*R*),4(*R*)-dihydroxy-2(*R*)-isopropylhexanoyl; Amp = 2-(aminomethyl)pyridine; Ambi = 2-(aminomethyl)benzimidazole; Ahi = 1(*S*)-amino-2(*R*)-hydroxyindan.

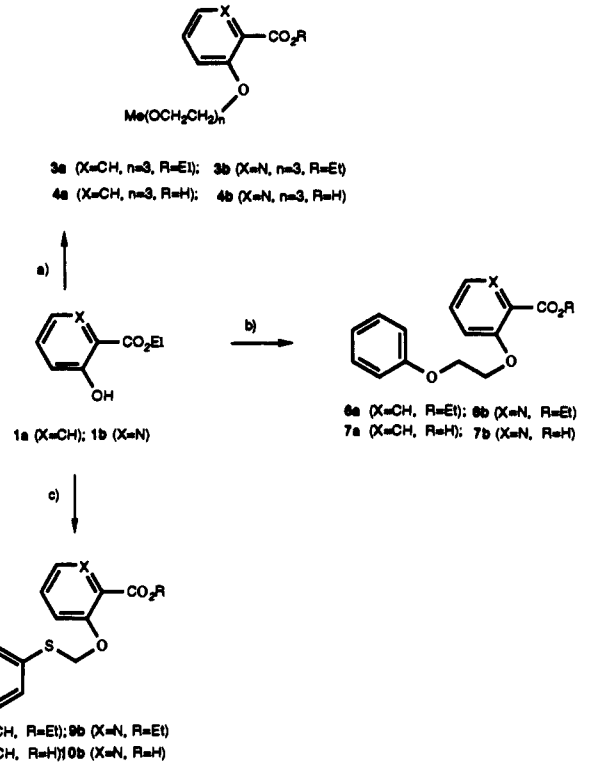
addition to reverse transcription, in that this enzyme is indispensable for generation of an infectious particle.[8,9] HIV protease processes the viral *gag* and *gag/pol* polyproteins in the final stages of viral maturation. It cleaves the p55 *gag* precursor into the four structural proteins forming the core of the virion, p17, p24, p8, and p7. It also processes the p160 *gag/pol* precursor to liberate the structural elements just mentioned, and the enzymes of the virus, i.e., the protease itself, the reverse transcriptase, and the endonuclease or integrase. The HIV protease has been characterized extensively,[10-14] and its three-dimensional structure has been determined both alone and in complexation with a variety of inhibitors.[15-20] It has been well established that when the protease is catalytically defective,[8,9] or if it is inhibited,[21-24] viral maturation in HIV-infected cell culture is blocked, and consequently, infection is arrested.

The most potent inhibitors of the HIV protease reported thus far are pseudopeptidic compounds containing transition-state inserts in place of the residues occupying the P_1 and P_1' positions of the substrate peptides.[25,26] These inserts[27] include reduced peptide bond (methyleneamino, $[CH_2NH]$), hydroxyethylene ($CH(OH)CH_2$), hydroxyethylamine ($CH(OH)CH_2N$), and dihydroxyethylene (CH(OH)CH(OH)) moieties.[28-34] In a previous report,[35] we described the structure–activity study of a series of compounds represented by peptide A (see Table I) with potent inhibitory activity against HIV protease and with desirable antiviral activity in cell culture assays. We noted that a few smaller peptides, such as peptide B, could possess reasonable inhibitory activity, and the antiviral activity is associated with adequate lipophilicity. In this report, we have continued our study of modifications of this template at both N- and C-termini to identify compounds with reduction in size and, more importantly, with increased antiviral activity in vitro. Peptide XVIII emerged from this structure–activity study as a compound with improved antiviral activity over the previous peptide A, which was comparable in antiviral activity to AZT in the HIV-1_{IIIB}/PBMC assay. We have also extended the recently determined X-ray crystal structure of peptide A/HIV-1 protease complex[36] to the modeling of the possible conformation of the peptide XVIII within the active-site cavity of the protease in order to gain some insight in the interaction of the ligand to the enzyme.

Chemistry

Syntheses of the *N*-Terminal Acids. As shown in Scheme I, ethyl salicylate (**1a**) was alkylated with 2-(2-(2-methoxyethoxy)ethoxy)ethyl bromide (**2**) in dimethylformamide in the presence of potassium carbonate to give ethyl 2-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)benzoate (**3a**). The ethyl ester was then hydrolyzed in methanolic sodium hydroxide to yield 2-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)benzoic acid (**4a**). In an analogous manner, ethyl 3-hydroxypicolinate (**1b**) was alkylated with 2-(2-(2-methoxyethoxy)ethoxy)ethyl bromide (**2**) in dimethylformamide in the presence of potassium carbonate to give ethyl 3-(2-(2-(2-methoxyethoxy)ethoxy)-ethoxy)picolinate (**3b**). The ethyl ester was then hydrolyzed in methanolic sodium hydroxide to yield 3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)picolinic acid (**4b**). 3-(2-(2-Methoxyethoxy)ethoxy)picolinic acid could also be prepared in an analogous fashion.

Scheme I. Syntheses of the N-Terminal Acids 4, 7, and 10[a]

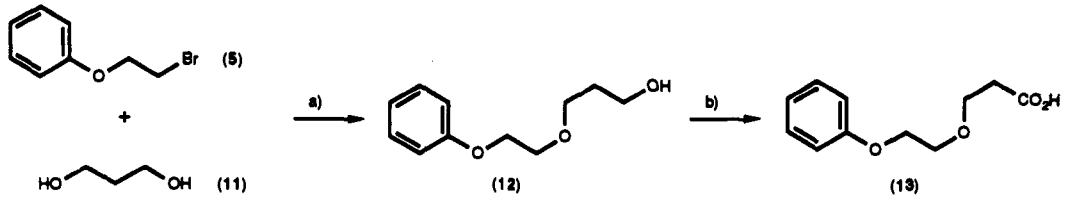


[a] (a) $Me(OCH_2CH_2)_3Br$ (**2**), K_2CO_3, DMF; (b) $C_6H_5OCH_2CH_2Br$ (**5**), K_2CO_3, DMF; (c) $C_6H_5SCH_2Cl$ (**8**), K_2CO_3, DMF.

Ethyl salicylate (**1a**) was alkylated with β-bromophenetole (**5**) in dimethylformamide in the presence of potassium carbonate to give ethyl 2-(2-phenoxyethoxy)benzoate (**6a**). The ethyl ester was then hydrolyzed in methanolic sodium hydroxide to yield 2-(2-phenoxyethoxy)benzoic acid (**7a**). In an analogous manner, ethyl 3-hydroxypicolinate (**1b**) was alkylated with β-bromophenetole (**5**) in dimethylformamide in the presence of potassium carbonate to give ethyl 3-(2-phenoxyethoxy)picolinate (**6b**). The ethyl ester was then hydrolyzed in methanolic potassium hydroxide to yield 3-(2-phenoxyethoxy)picolinic acid (**7b**).

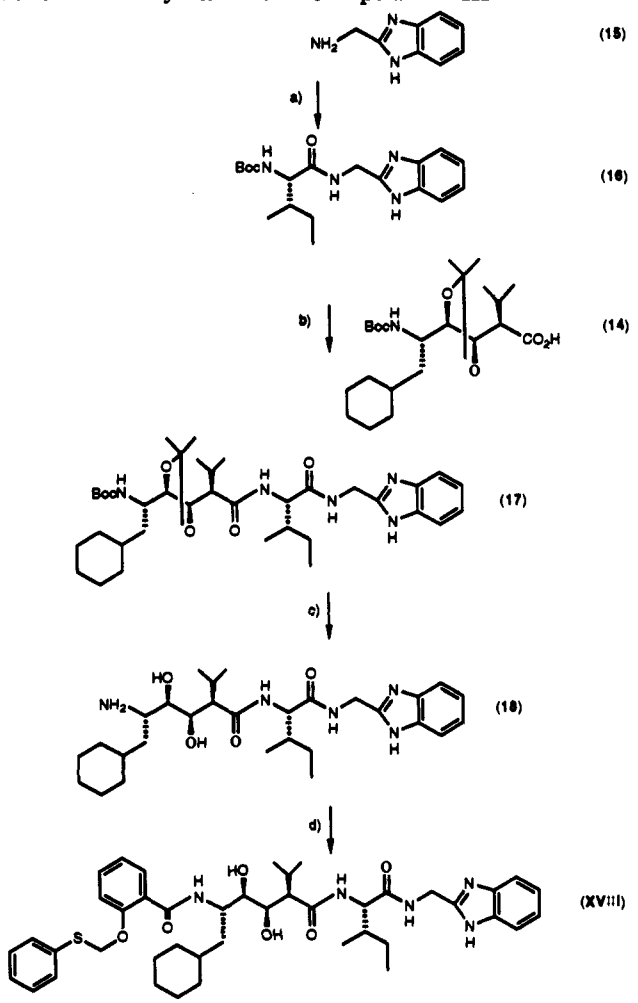
Ethyl salicylate (**1a**) was alkylated with chloromethyl phenyl sulfide (**8**) in dimethylformamide in the presence of potassium carbonate to give ethyl 2-(thiophenoxymethoxy)benzoate (**9a**). The ethyl ester was then hydrolyzed in methanolic potassium hydroxide to yield 2-(thiophenoxymethoxy)benzoic acid (**10a**). In an analogous manner, ethyl 3-hydroxypicolinate (**1b**) was alkylated with chloromethyl phenyl sulfide (**8**) in dimethylformamide in the presence of potassium carbonate to give ethyl 3-(thiophenoxymethoxy)picolinate (**9b**). The ethyl ester

Scheme II. Synthesis of the N-Terminal Acid 13[a]



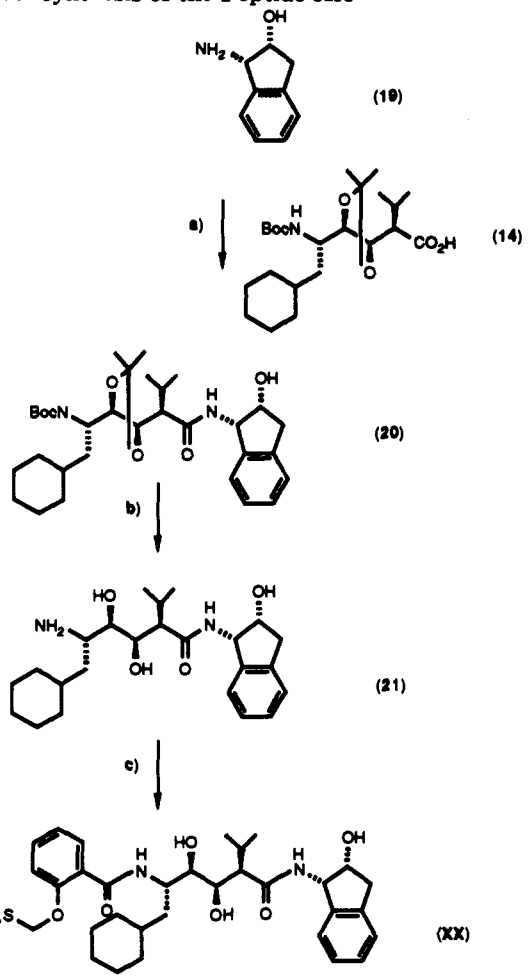
[a] (a) NaH, THF; (b) $RuCl_3$, H_5IO_6, $CH_3CN/CCl_4/H_2O$.

Scheme III. Synthesis of the Peptide XVIII[a]



[a] (a) Boc-Ile-OH, $(EtO)_2P(O)CN$, *i*-Pr_2NEt, CH_2Cl_2; (b) TFA, CH_2Cl_2; acid 14, $(EtO)_2P(O)CN$, *i*-Pr_2NEt, CH_2Cl_2; (c) HCl, CH_3OH, $HSCH_2CH_2SH$; (d) acid **10a**, $(EtO)_2P(O)CN$, *i*-Pr_2NEt, CH_2Cl_2.

Scheme IV. Synthesis of the Peptide XX[a]



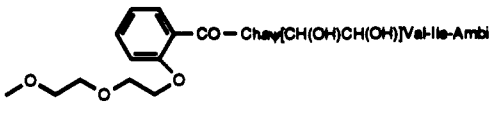
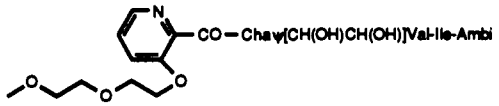
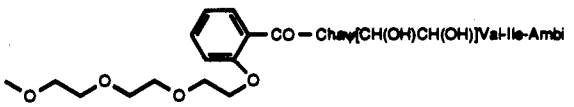
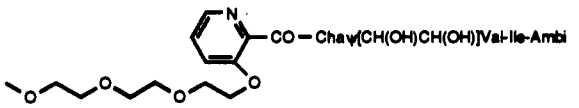
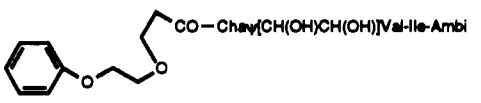
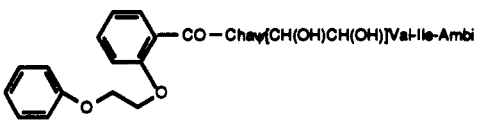
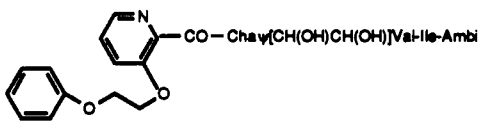
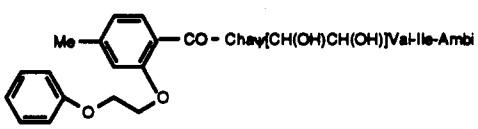
[a] (a) Acid 14, $(EtO)_2P(O)CN$, *i*-Pr_2NEt, CH_2Cl_2; (b) HCl, CH_3OH, $HSCH_2CH_2SH$; (c) acid **10a**, $(EtO)_2P(O)CN$, *i*-Pr_2NEt, CH_2Cl_2.

was then hydrolyzed in methanolic sodium hydroxide to yield 3-(thiophenoxymethoxy)picolinic acid (**10b**).

As shown in Scheme II, 1,3-propanediol (11) was treated with sodium hydride in tetrahydrofuran and followed by β-bromophenetole (**5**) to give 3-(2-phenoxyethoxy)-1-propanol (**12**). This alcohol was oxidized with periodic acid in the presence of ruthenium trichloride[37] in acetonitrile, carbon tetrachloride, and water to give 3-(2-phenoxyethoxy)propanoic acid (**13**).

Synthesis of the Representative Peptide XVIII. As shown in Scheme III, 2-(aminomethyl)benzimidazole (**15**) was condensed with *N*-(*tert*-butyloxycarbonyl)-L-isoleucine using diethyl phosphorocyanidate[38] to give 2-[[[*N*-(*tert*-butyloxycarbonyl)-L-isoleucyl]amino]methyl]benzimidazole (**16**). The *N*-*tert*-butyloxycarbonyl group was removed by treatment with trifluoroacetic acid, and the resulting amine 2-[(L-isoleucylamino)methyl]benzimidazole was condensed with 2(*R*)-[5(*R*)-[1(*S*)-[(*tert*-butyloxycarbonyl)amino]-2-cyclohexylethyl]-2,2-dimethyl-4(*R*)-dioxolanyl]-3-methylbutanoic acid (**14**)[35] using diethyl phosphorocyanidate[38] to give 2-[[[*N*-[2(*R*)-[5(*R*)-[1(*S*)-[(*tert*-butyloxycarbonyl)amino]-2-cyclohexylethyl]-2,2-dimethyl-4(*R*)-dioxolanyl]-3-methylbutanoyl]-L-isoleucyl]-amino]methyl]benzimidazole (**17**). Treatment with acidic methanol removed acid-labile groups to give the amine 2-[[[*N*-[5(*S*)-amino-6-cyclohexyl-3(*R*),4(*R*)-dihydroxy-2(*R*)-isopropylhexanoyl]-L-isoleucyl]amino]methyl]benzimidazole (**18**). Condensation of this amine with 2-(thiophenoxymethoxy)benzoic acid (**10a**) using diethyl phosphorocyanidate[38] gave the desired peptide [[[*N*-[5(*S*)-[[2-(thiophenoxymethoxy)benzoyl]amino]-6-cyclohexyl-3(*R*),4(*R*)-dihydroxy-2(*R*)-isopropylhexanoyl]-L-isoleucyl]-amino]methyl]benzimidazole (XVIII). Other congeneric peptides (peptide I, peptides III–X, and peptide XIX) were prepared from the amine 2-[[[*N*-(5-(*S*)-amino-6-cyclohexyl-3(*R*),4(*R*)-dihydroxy-2(*R*)-isopropylhexanoyl)-L-isoleucyl]amino]methyl]benzimidazole (**18**) by conden-

Table II. Inhibitory Activity against HIV-1 Protease and Antiviral Activity against Vaccinia Virus in CV-1 Monkey Cells[a]

peptide	structure	K_i (nM)	% CV-1 inhibition at 1 μM
III		5	56
IV		100	0
V		10	65
VI		100	0
VII		4	65
VIII		8	86
IX		>200	0
X		48	48

[a] Chaψ[CH(OH)CH(OH)]Val = 5(*S*)-amino-6-cyclohexyl-3(*R*),4(*R*)-dihydroxy-2(*R*)-isopropylhexanoyl. Ambi = 2-(aminomethyl)benzimidazole.

sation to various appropriate acids using diethyl phosphorocyanidate.[38]

Synthesis of the Representative Peptide XX. As shown in Scheme IV, 1(*S*)-amino-2(*R*)-hydroxyindan (**19**)[39] was condensed with 2(*R*)-[5(*R*)-[1(*S*)-[(*tert*-butyloxycarbonyl)amino]-2-cyclohexylethyl]-2,2-dimethyl-4(*R*)-dioxolanyl]-3-methylbutanoic acid (**14**)[35] using diethyl phosphorocyanidate[38] to give 1(*S*)-[[2(*R*)-[5(*R*)-[1(*S*)-[(*tert*-butyloxycarbonyl)amino]-2-cyclohexylethyl]-2,2-dimethyl-4(*R*)-dioxolanyl]-3-methylbutanoyl]amino]-2(*R*)-hydroxyindan (**20**). Treatment with acidic methanol removed acid-labile groups to give the amine 1(*S*)-[[5(*S*)-amino-6-cyclohexyl-3(*R*),4(*R*)-dihydroxy-2(*R*)-isopropylhexanoyl]amino]-2(*R*)-hydroxyindan (**21**). Condensation of this amine with 2-(thiophenoxymethoxy)benzoic acid (**10a**) using diethyl phosphorocyanidate[38] gave the desired peptide 1(*S*)-[5(*S*)-[[2-(thiophenoxymethoxy)benzoyl]-amino]-6-cyclohexyl-3(*R*),4(*R*)-dihydroxy-2(*R*)-isopropylhexanoyl]amino]-2(*R*)-hydroxyindan (XX). Other congeneric peptides (peptide II, peptides XI–XVII, and peptide XXI) were prepared from the amine 1(*S*)-[[5(*S*)-amino-6-cyclohexyl-3(*R*),4(*R*)-dihydroxy-2(*R*)-isopropylhexanoyl]amino]-2(*R*)-hydroxyindan (**21**) by condensation to various appropriate acids using diethyl cyanophosphorocyanidate.[38]

Results and Discussion

Starting with peptide B in Table I, we evaluated a series of peptides with C-terminal modifications of the L-isoleucyl-2-(aminomethyl)pyridine to either L-isoleucyl-2-(aminomethyl)benzimidazole[40] or 1(*S*)-amino-2(*R*)-hydroxyindan.[39] For the N-terminal modifications, we chose the benzoyl group as our starting point so that a variety of substitutions on the phenyl ring can be evaluated. The two reference compounds, peptides I and II, showed reasonable enzyme inhibitory activity with K_i values of 36

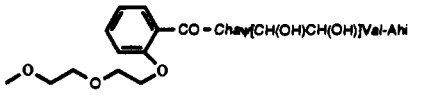
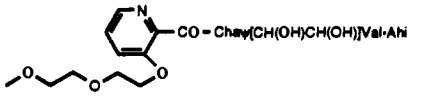
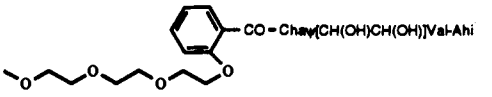
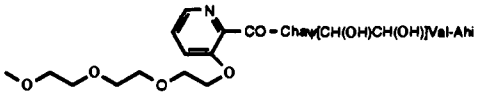
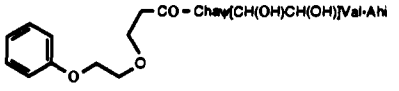
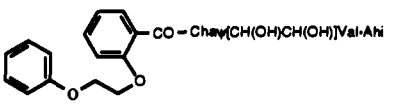
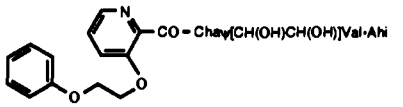
and 16 nM, respectively. Substitutions at the ortho position of the benzoyl group were based on the observation of the crystal structure of peptide A/HIV-1 protease complex that these substitutions may follow the N-terminal backbone.

Table II displays the HIV-1 protease inhibitory activity of congeneric peptides III–X, which contain the 2-[(L-isoleucylamino)methyl]benzimidazole C-terminus[40] and the corresponding antiviral activity (against a vaccinia virus construct in the monkey CV-1 cell). We have studied the processing of the HIV p55 and the maturation of the HIV-like particles in recombinant vaccinia virus (vVK-1)-infected CV-1 cells previously.[21] The vaccinia virus was engineered to express the HIV *gag-pol* genes and resulted in the synthesis and processing of *gag-pol* precursors. The initial prominent polypeptides synthesized are p55, p46, and p41, which are further processed to the mature viral proteins p17 and p24. The HIV-like particles are assembled and undergo a maturation process that closely mimics that occurring in the natural HIV-1 infection. We analyzed the vVK-1 infected CV-1 cell lysates for the processing of p55 to p24 by densitometric analyses of protein immunoblots. We have shown that HIV protease inhibitory compounds that are effective in this assay have arrested infectious HIV-1 maturation and block viral infectivity. We have found that antiviral activity in cell culture as defined by this CV-1 cell assay is associated with compounds that exhibit significantly lipophilicity.[24,35] Peptides III and V, with the 2-(2-methoxyethoxy)ethoxy group and the 2-(2-(2-methoxyethoxy)ethoxy)ethoxy group respectively, are effective inhibitors (with K_i values of 5 and 10 nM, respectively). This is also reflected in the resulting antiviral activity with 56% and 65% inhibition at 1 μM concentration for peptides III and V, respectively. Translocating the phenyl ring of the benzoyl group to the very end of the N-terminus as in peptide VII, which incorporates the 3-(2-phenoxyethoxy)propanoyl group, also resulted in an equally effective inhibitor, with a K_i value of 4 nM and antiviral activity with 65% inhibition at 1 μM concentration. With two phenyl rings at the N-terminus as in peptide VIII, which contains the 2-(2-phenoxyethoxy)benzoyl group, the HIV-1 protease inhibitory activity is maintained with a K_i value of 8 nM; however, the antiviral activity was improved and resulted in 86% inhibition at 1 μM concentration. Addition of a methyl group as in peptide X, with the 4-methyl-2-(2-phenoxyethoxy)benzoyl N-terminus, resulted in a less effective compound with a K_i value of 48 nM and antiviral activity with 48% inhibition at 1 μM concentration.

Congeneric peptides that contain picolinic acid in place of benzoic acid at the N-terminus were found to be uniformly much less effective inhibitors. Peptides IV, VI, and IX all exhibited poor HIV-1 protease inhibitory activity with K_i values of 100 nM or worse. They all show, accordingly, poor antiviral activity with no inhibition at 1 μM concentration.

Table III displays the HIV-1 protease inhibitory activity of congeneric peptides XI–XVII, which contain the 1(*S*)-amino-2(*R*)-hydroxyindan C-terminus.[39] Peptides XI, XIII, XV, and XVI all show very poor HIV-1 protease inhibitory activity, with K_i values of 200 nM or worse, when compared to the active inhibitory peptides III, V, VII, and VIII, respectively. This is in sharp contrast with previous findings that the 1(*S*)-amino-2(*R*)-hydroxyindan C-terminus is a very effective group for the preparation of potent inhibitors.[39] This C-terminal group is not applicable for use in the present peptidic template. Not surprisingly, the corresponding picolinic acid-containing analogues (peptides XII, XIV, and XVII) were also found to be very poor inhibitors.

Table III. Inhibitory Activity against HIV-1 Protease[a]

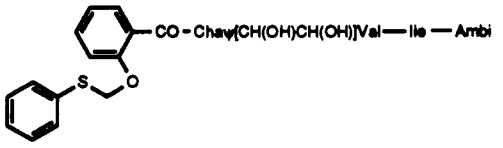
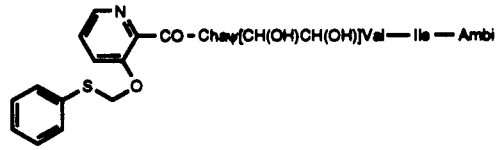
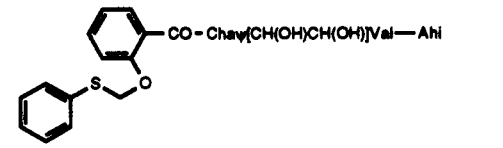
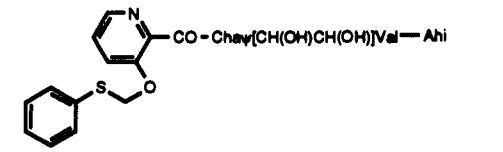
peptide	structure	K_i (nM)
XI		200
XII		>200
XIII		>200
XIV		>200
XV		>200
XVI		>200
XVII		>200

[a] Chaψ[CH(OH)CH(OH)]Val = 5(*S*)-amino-6-cyclohexyl-3(*R*),4(*R*)-dihydroxy-2(*R*)-isopropylhexanoyl. Ahi = 1(*S*)-amino-2(*R*)-hydroxyindan.

Table IV displays the HIV-1 protease inhibitory activity and the antiviral activity (against a vaccinia virus construct in the monkey CV-1 cell) of congeneric peptides XVIII–XXI, which contain the 2-(thiophenoxymethoxy)benzoyl and the 3-(thiophenoxymethoxy)picoloyl C-terminus. Peptide XVIII is an effective inhibitor (as compared to peptide VIII) with a K_i value of 5 nM and possesses good antiviral activity with 86% inhibition at 1 μM concentration. The corresponding picoloyl congeneric peptide XIX is a much poorer inhibitor with a K_i value of 140 nM and also showed poor antiviral activity, with no inhibition at 1 μM concentration. The 1(*S*)-amino-2(*R*)-hydroxyindan C-terminal-containing congeneric peptide XX is also a much poorer inhibitor, with a K_i value of 200 nM. The congeneric peptide XXI, containing the picoloyl N-terminus and the 1(*S*)-amino-2(*R*)-hydroxyindan C-terminus, not surprisingly, has very poor inhibitory activity with a K_i value of 600 nM.

Compounds with reasonably good antiviral activity against the vaccinia virus construct in the monkey CV-1 cell (peptides III, V, VII, VIII, and XVIII) were further evaluated in human peripheral blood mononuclear cells acutely infected with HIV-1. In this assay, the level of HIV-1 replication was assessed 3 and 4 days later after infection as follows: (1) an enzyme-linked immunosorbent

Table IV. Inhibitory Activity against HIV-1 Protease and Antiviral Activity against Vaccinia Virus in CV-1 Monkey Cells[a]

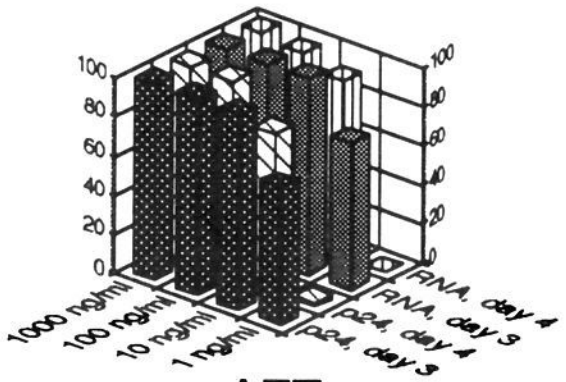
peptide	structure	K_i (nM)	% CV-1 inhibition at 1 μM
XVIII		5	86
XIX		140	0
XX		200	0
XXI		600	0

[a] Chaψ[CH(OH)CH(OH)]Val = 5(*S*)-amino-6-cyclohexyl-3(*R*),4(*R*)-dihydroxy-2(*R*)-isopropylhexanoyl. Ambi = 2-(aminomethyl)benzimidazole; Ahi = 1(*S*)-amino-2(*R*)-hydroxyindan.

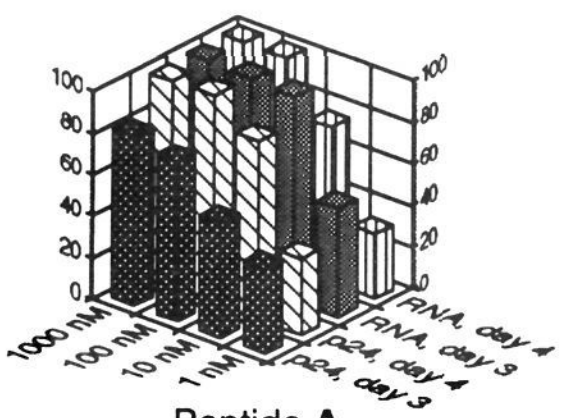
(ELISA) quantified the amount of p24 in culture supernatants; (2) the levels of HIV-1 RNA in culture supernatants were determined by nucleic acid hybridization with HIV-1 specific probes. Results of the dose responses on inhibition of p24 production and RNA synthesis at day 3 and day 4 are shown in Figure 1. The results for the nucleoside RT inhibitor AZT and the HIV protease inhibitory peptide A are also shown for comparison. In this antiviral assay, the HIV protease inhibitor A showed comparable efficacy as AZT, with IC_{50} value between 1 and 10 nM. Peptide III also showed IC_{50} value between 1 and 10 nM. For peptides V and VII, the IC_{50} values are closer to 10 nM, as judged by the inhibition of p24 production. Surprisingly, peptide VIII exhibited poor antiviral activity in this assay with an IC_{50} value between 0.1 and 1 μM. Very interestingly, however, peptide XVIII showed extremely good antiviral activity in this assay with an IC_{50} value of less than 1 nM.

Molecular Modeling of Peptide XVIII/HIV-1 Protease Complex. Molecular model of peptide XVIII was constructed from the recently reported X-ray crystallographic structure of peptide A/HIV-1 protease complex.[36] The inhibitor conformation (molecule in black in Figure 2) shows a roughly C2 symmetric orientation of inhibitor sidechains with respect to the axis of symmetry of the enzyme. The P_2 and P_1' carbonyl oxygen atoms are in position to hydrogen bond to the enzyme through a buried water molecule. The naphthoxyacetyl N-terminus and the 2-(aminomethyl)pyridine C-terminus extend out of the active-site cleft and appear to make some hydrophobic interaction with the enzyme surface. The two hydroxyl groups of the dihydroxyethylene isostere insert interact with the catalytic aspartic acid residues in an asymmetric fashion, in which one hydroxyl group can hydrogen bond to both carboxylic acids while the other hydroxyl group forms a hydrogen bond with only one carboxylic acid. Using the Mosaic modeling system,[41] peptide A in the crystal structure conformation[36] was subjected to energy minimization using the BatchMin[42] implementation of the AMBER force field with PRCG (Polak-Ribiere conjugate gradient) minimizer of BatchMin. Minimization was considered complete when the root mean square gradient of <0.1 kcal/Å was obtained. Figure 2 shows the overlay of the resulting energy-minimized conformation of peptide A (molecule in medium gray in Figure 2) with the original crystal structure conformation[36] of peptide A (molecule in black in Figure 2). The minimized structure closely matched the starting X-ray crystal structure conformation. The noticeable difference is in the orientations of the N-terminal naphthoxyacetyl groups which, as mentioned earlier, extend out of the active site cleft.

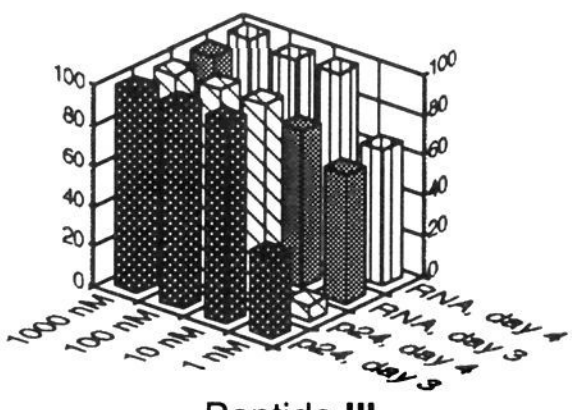
Using this minimized structure of peptide A (molecule in medium gray in Figure 3) as the starting point, the N-terminal (naphthoxyacetyl)-L-histidyl group in peptide A was replaced with the 2-(thiophenoxymethoxy)benzoyl group, and the C-terminal 2-(aminomethyl)pyridine group was replaced with the 2-(aminomethyl)benzimidazole group to generate the starting structure of peptide XVIII. The resulting structure was subjected to energy minimization using the BatchMin[42] implementation of the AMBER force field with PRCG minimizer. Figure 3 shows the overlay of the resulting energy-minimized conformation of peptide XVIII (molecule in black in Figure 3) with the energy-minimized structure of peptide A (molecule in medium gray in Figure 3). The C-terminal 2-(aminomethyl)benzimidazole in peptide XVIII occupies a position which is similar to that of the 2-(aminomethyl)pyridine in peptide A. The P_2 and P_1' carbonyl oxygen atoms of peptide XVIII maintain positions to hydrogen bond to the enzyme through a buried water molecule. Although the conformational constraint of the benzoyl group in peptide XVIII restricts the positioning of the N-terminus,



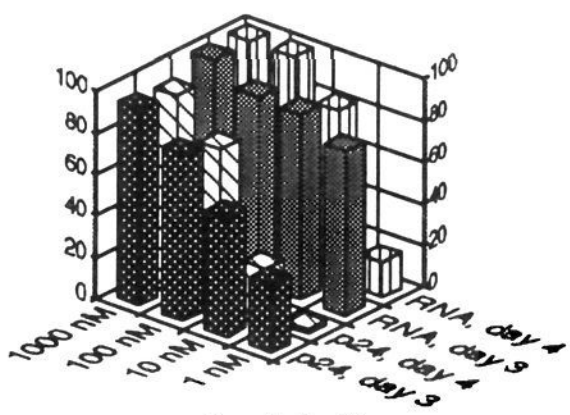
AZT



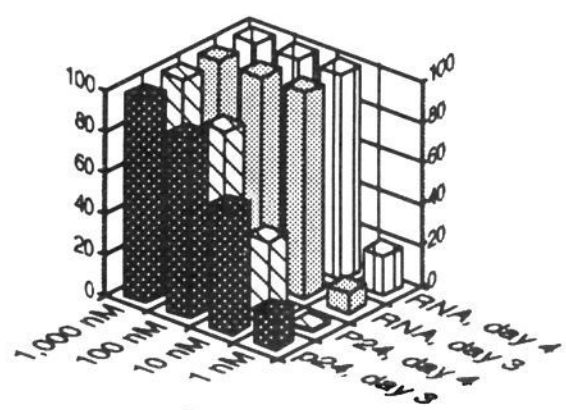
Peptide **A**



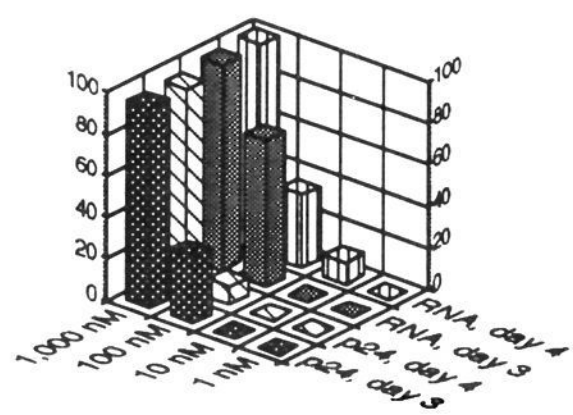
Peptide **III**



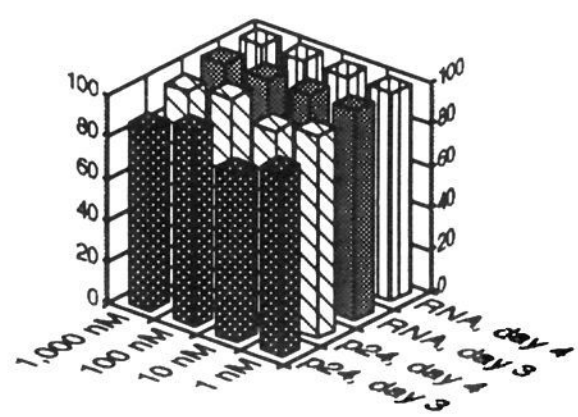
Peptide **V**



Peptide **VII**



Peptide **VIII**



Peptide **XVIII**

Figure 1. Inhibition of p24 production and RNA synthesis in human peripheral blood mononuclear cells actively infected with HIV-1.

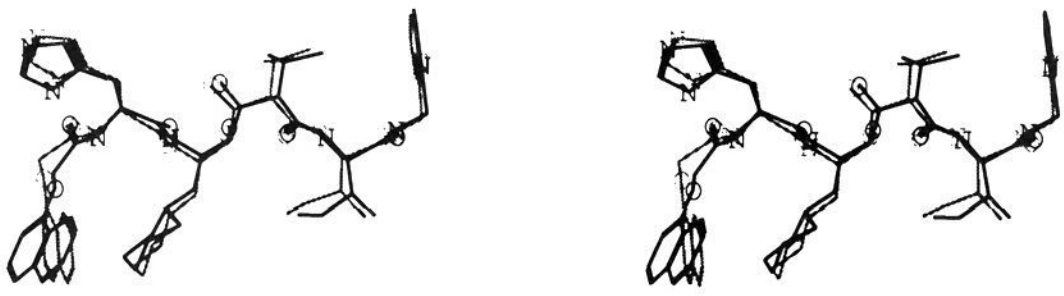


Figure 2. Overlay of crystal structure (in black) and energy-minized (in medium gray) conformations of peptide A in HIV-1 protease active site (in light gray).

the 2-thiophenoxymethoxy group is able to occupy a similar position as the naphthoxyacetyl group in peptide A. We are unable to provide an explanation from the molecular modeling study, however, why the 1(*S*)-amino-2(*R*)-hydroxyindan-containing peptides show much poorer binding affinity to the enzyme. For the picolinic acid-containing analogues, the pyridine ring is positioned in a highly lipophilic pocket, and it is suggested that the more polar nitrogen-containing ring in this binding pocket is less favorable than the corresponding benzene ring.

Summary

A previously reported HIV protease inhibitor (peptide A) exhibited high binding affinity to the enzyme and was also shown to possess active antiviral efficacy in cell cultures. We have investigated series of peptides that contain the dihydroxyethylene isostere insert with emphasis on the reduction in size of the N-terminal groups. The structure–activity relationship on the binding affinity of these compounds to HIV-1 protease suggested that high binding affinity to the enzyme can be maintained with a

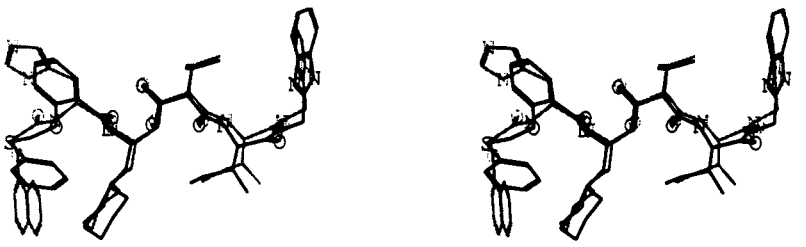


Figure 3. Overlay of energy-minized conformations of peptide A (in medium gray) and peptide XVIII (in black) in HIV-1 protease active site (in light gray).

number of peptides (I, II, III, V, VII, VIII, and XVIII). A molecular modeling study of compound XVIII based upon the crystal structure of compound A/HIV-1 protease complex was conducted and suggested a possible conformation of this compound in the active site. It is interesting to note from the structure–activity findings that nitrogen-containing analogues (picolinic acid in place of benzoic acid) showed much reduced binding affinity to the enzyme. Also surprisingly, the previously proven effective 1(*S*)-amino-2(*R*)-hydroxyindan C-terminus could not be applied to compounds with these N-terminal groups, and the resulting compounds showed very poor inhibitory activity. A number of these active inhibitors were shown to possess antiviral activity in cell culture. In the HIV-1/PBMC assay, peptide A was previously shown to have efficacy similar to AZT, with an IC_{50} value of 1–10 nM. Peptide XVIII could be shown to possess increased antiviral activity, with an IC_{50} value of <1 nM in this assay. This level of activity is desirable for HIV protease inhibitors as potential therapeutic agents for the treatment of HIV infection.

Experimental Section

Chemistry. Mass spectra, infrared spectra, and combustion analyses were obtained by the Physical and Analytical Chemistry Department of Upjohn Laboratories. ^{1}H NMR spectra were recorded at 300 MHz with a Bruker Model AM-300 spectrometer. Chemical shifts were reported as δ units relative to tetramethylsilane as internal standard. Thin-layer chromatography was conducted with Analtech 0.25-mm glass plates precoated with silica gel GF. Chromatography used E. Merck silica gel 60 (70–230 mesh for column chromatography and 230–400 mesh for flash chromatography). All solvents for chromatography were reagent grade.

Reagents were from commercial sources and used without further purification unless otherwise noted. Dichloromethane and dimethylformamide were dried over 4-Å molecular sieves. Diisopropylethylamine was distilled from calcium hydride. Diethyl phosphorocyanidate was distilled before use. Tetrahydrofuran was distilled under argon from sodium metal in the presence of benzophenone.

Ethyl 2-(2-(2-(2-Methoxyethoxy)ethoxy)ethoxy)benzoate (3a). To a solution of 1.47 mL (10 mmol) of ethyl salicylate in 5 mL of dimethylformamide were added 2.27 g (10 mmol) of 2-(2-(2-methoxyethoxy)ethoxy)ethyl bromide (**2**) and 1.52 g (11 mmol) of powdered K_2CO_3. The mixture was heated at 100 °C overnight and then partitioned between diluted aqueous NaOH and 1:1 ether–hexane. The organic phase was washed with 1 N aqueous NaOH and then saturated aqueous NaCl. It was dried ($MgSO_4$) and then concentrated to an oil. Flash chromatography of the residue with 40–70% ethyl acetate in dichloromethane afforded 1.36 g (44%) of ethyl 2-(2-(2-(2-methoxyethoxy)ethoxy)-ethoxy)benzoate (**3a**): ^{1}H NMR ($CDCl_3$) δ 1.36 (t, J = 7.1 Hz, 3 H), 3.35 (s, 3 H), 3.5 (m, 2 H), 3.65 (m, 4 H), 3.75 (m, 2 H), 3.9 (m, 2 H), 4.2 (m, 2 H), 4.33 (q, J = 7.1 Hz, 2 H), 6.9 (m, 2 H), 7.4 (m, 1 H), 7.76 (dd, J = 7.8, 1.8 Hz, 1 H); FAB MS m/z 313 $(M + H)^+$; IR ($CHCl_3$) 2877, 1726, 1705, 1491, 1449, 1305, 1253, 1135, 1107, 1097, 1082 cm^{-1}. Anal. ($C_{16}H_{24}O_6$) C, H.

2-(2-(2-(2-Methoxyethoxy)ethoxy)ethoxy)benzoic Acid (4a). A solution of 0.63 g (2.0 mmol) of ethyl 2-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)benzoate (**3a**) in 3 mL of 1 M aqueous NaOH and 20 mL of methanol was refluxed overnight. Methanol was distilled off, and the residual clear aqueous solution was then washed with diethyl ether. The aqueous phase was acidified and then extracted with four portions of dichloromethane. The combined organic phase was dried ($MgSO_4$) and then concentrated under reduced pressure to give 596 mg of 2-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)benzoic acid (**4a**) as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 3.37 (s, 3 H), 3.35 (m, 2 H), 3.6–3.8 (m, 6 H), 3.9 (m, 2 H), 4.4 (m, 2 H), 7.04 (d, J = 8.4 Hz, 1 H), 7.14 (t, J = 8.4 Hz, 1 H), 7.52 (m, 1 H), 8.15 (dd, J = 7.8, 1.8 Hz, 1 H); IR ($CHCl_3$) 3263, 2919, 2879, 1730, 1604, 1456, 1352, 1243, 1127, 1105, 1041, 758 cm^{-1}; FAB HRMS m/z 285.1342 $(M + H)^+$ (calcd for $C_{14}H_{21}O_6$ 285.1338).

Ethyl 3-(2-(2-(2-Methoxyethoxy)ethoxy)ethoxy)picolinate (3b). A suspension of 836 mg (5.00 mmol) of ethyl 3-hydroxypicolinate (**1b**), 1.14 g (5.00 mmol) of 2-(2-(2-methoxyethoxy)-ethoxy)ethyl bromide (**2**), and 760 mg (5.5 mmol) of powdered K_2CO_3 in 2 mL of dimethylformamide was heated at 60 °C overnight and then partitioned between ethyl acetate and cold water. The organic phase was dried ($MgSO_4$) and then concentrated to give a yellow liquid, which was chromatographed on silica gel with 3–5% methanol in ethyl acetate to provide 586 mg (37%) of ethyl 3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)picolinate (**3b**) as a pale yellow liquid: ^{1}H NMR ($CDCl_3$) δ 1.39 (t, J = 7.1 Hz, 3 H), 3.34 (s, 3 H), 3.51 (m, 2 H), 3.62 (m, 4 H), 3.72 (m, 2 H), 3.87 (m, 2 H), 4.20 (m, 2 H), 4.41 (q, J = 7.1 Hz, 2 H), 7.4 (m, 2 H), 8.25 (m, 1 H); EI MS m/z 313 $(M + H)^+$; IR ($CHCl_3$) 2932, 1732, 1445, 1305, 1198, 1140, 1102 cm^{-1}. Anal. ($C_{15}H_{23}NO_6$) C, H, N.

3-(2-(2-(2-Methoxyethoxy)ethoxy)ethoxy)picolinic Acid (4b). A solution of 521 mg (1.66 mmol) of ethyl 3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)picolinate (**3b**) in 3.3 mL of 1 M aqueous KOH and 8 mL of methanol was stirred overnight. Methanol was removed and the remaining aqueous phase diluted with water and then washed with diethyl ether. The aqueous phase was acidified with 3.3 mL of 1 N aqueous HCl and then extracted with dichloromethane in a continuous extractor overnight. The dichloromethane extract was dried ($MgSO_4$) and then concentrated under reduced pressure to give 379 mg (80%) of 3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)picolinic acid (**4b**) as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 3.35 (s, 3 H), 3.5 (m, 2 H), 3.6 (m, 2 H), 3.75 (m, 2 H), 4.3 (m, 2 H), 7.5 (br, 2 H), 8.2 (br, 1 H); FAB HRMS m/z 286.1296 $(M + H)^+$ (calcd for $C_{13}H_{19}NO_6$ 286.1291); IR (neat) 2881, 1727, 1448, 1288, 1256, 1202, 1108 cm^{-1}.

Ethyl 2-(2-Phenoxyethoxy)benzoate (6a). A mixture of 2.66 g (13.24 mmol) of β-bromophenetole, 2.0 g (12.03 mmol) of ethyl salicylate (**1a**), and 1.83 g (13.24 mmol) of potassium carbonate in 25 mL of dimethylformamide was allowed to stir at room temperature for 4 days. The reaction mixture was concentrated, and the residue was then partitioned between dichloromethane and saturated aqueous NaCl. The organic layer was dried over Na_2SO_4 and then concentrated. The resulting residue was chromatographed on silica gel with 10% ethyl acetate in hexane to give 3.03 g (88%) of ethyl 2-(2-phenoxyethoxy)benzoate (**6a**) as a crystalline product: mp 82–84 °C; ^{1}H NMR ($CDCl_3$) δ 1.31 (t, J = 7.2 Hz, 3 H), 4.32 (m, 4 H), 6.94–7.05 (m, 5 H), 7.29 (m, 2 H), 7.46 (dt, J = 7.5, 1.8 Hz, 1 H), 7.79 (dd, J = 7.5, 1.7 Hz, 1 H); IR ($CHCl_3$) 1716, 1599, 1492, 1444, 1245, 1232, 1086, 767, 760 cm^{-1}. Anal. ($C_{17}H_{18}O_4$) C, H.

2-(2-Phenoxyethoxy)benzoic Acid (7a). To 0.93 g (3.25 mmol) of ethyl 2-(2-phenoxyethoxy)benzoate (**6a**) in 50 mL of methanol and 1 mL of dichloromethane was added 4.87 mL (4.87 mmol) of 1 N aqueous NaOH. After being stirred at room temperature for 90 min, the reaction mixture was heated at reflux for 12 h. It was allowed to cool and then concentrated. The resulting residue was partitioned between dichloromethane and aqueous HCl. The organic layer was dried (Na_2SO_4) and then concentrated. The crude material was crystallized from dichloromethane/hexane to give 0.82 g (98%) of 2-(2-phenoxyethoxy)-benzoic acid (**7a**): mp 80–81 °C; ^{1}H NMR ($CDCl_3$) δ 4.41 (m, 2 H), 4.60 (m, 2 H), 7.0 (m, 3 H), 7.11–7.21 (m, 2 H), 7.28–7.35 (m, 2 H), 7.59 (dt, J = 7.4, 1.8 Hz, 1 H), 8.22 (dd, J = 7.8, 1.8 Hz, 1 H), 11.0 (bs, 1 H); IR (mineral oil) 1686, 1601, 1478, 1317, 1248, 1240, 1166, 762, 750 cm^{-1}. Anal. ($C_{15}H_{14}O_4$) C, H.

Ethyl 3-(2-Phenoxyethoxy)picolinate (6b). A mixture of 836 mg (5.00 mmol) of ethyl 3-hydroxypicolinate (**1b**), 760 mg (5.5 mmol) of powdered K_2CO_3, and 1.06 g (5.3 mmol) of β-bromophenetole in 2.5 mL of dry dimethylformamide was heated at 80 °C for 4 h and then partitioned between ethyl acetate and water. The organic phase was dried ($MgSO_4$) and then concentrated under reduced pressure to give a crude liquid, which was flash chromatographed on silica gel with 70% ethyl acetate in hexane to provide 1.02 g (71%) of ethyl 3-(2-phenoxyethoxy)-picolinate (**6b**): ^{1}H NMR ($CDCl_3$) δ 1.36 (t, J = 7.1 Hz, 3 H), 4.4 (m, 6 H), 6.9 (m, 3 H), 7.3 (m, 2 H), 7.4 (m, 2 H), 8.31 (dd, J = 4.3, 1.5 Hz, 1 H); EI MS m/z 287 (M + H)$^+$; IR ($CHCl_3$) 2952, 2926, 1710, 1441, 1306, 1287, 1249, 1244, 1233, 1207, 1101 cm^{-1}. Anal. ($C_{16}H_{17}NO_4$) C, H, N.

3-(2-Phenoxyethoxy)picolinic Acid (7b). A solution of 575 mg (2.0 mmol) of ethyl 3-(2-phenoxyethoxy)picolinate (**6b**), 10 mL of methanol, and 4.0 mL of 1 N aqueous KOH was refluxed overnight. Methanol was distilled off, and the residual clear aqueous solution was then washed with diethyl ether. The aqueous phase was acidified and then extracted with four portions of dichloromethane. The combined organic phase was dried ($MgSO_4$) and then concentrated under reduced pressure to give 3-(2-phenoxyethoxy)picolinic acid (**7b**) in 95% yield (494 mg): ^{1}H NMR ($CDCl_3$) δ 4.39 (m, 2 H), 4.49 (m, 2 H), 6.9 (m, 3 H), 7.2 (m, 2 H), 7.5 (m, 2 H), 8.30 (br d, J = 5 Hz, 1 H); EI MS m/z 259 (M + H)$^+$; IR ($CHCl_3$) 2954, 2925, 2854, 1451, 1312, 1288, 1247, 1202, 1154, 1129 cm^{-1}. Anal. ($C_{14}H_{13}NO_4$) C, H, N.

Ethyl 2-(Thiophenoxymethoxy)benzoate (9a). A 132-mg (3.31-mmol) portion of sodium hydride (60% in oil) was washed twice with hexane and then suspended in 2 mL of hexamethylphosphoramide. To this stirred suspension was added 0.5 g (3 mmol) of ethyl salicylate (**1a**). After 10 min, 0.525 g (3.31 mmol) of chloromethyl phenyl sulfide was added. The resulting reaction mixture was allowed to stir for 4 h and then partitioned between ethyl acetate and saturated aqueous NaCl. The organic layer was dried (Na_2SO_4) and then concentrated. The resulting residue was chromatographed on silica gel with 10% ethyl acetate in hexane to give 0.48 g (55%) of ethyl 2-(thiophenoxymethoxy)-benzoate (**9a**) as a clear liquid: ^{1}H NMR ($CDCl_3$) δ 1.34 (t, J = 7.2 Hz, 3 H), 4.33 (q, J = 7.2 Hz, 2 H), 5.56 (s, 2 H), 7.02–7.10 (m, 2 H), 7.25–7.33 (m, 2 H), 7.46 (dt, J = 7.4, 1.8 Hz, 1 H), 7.53–7.57 (m, 2 H), 7.81 (dd, J = 7.7, 1.8 Hz, 1 H). Anal. ($C_{16}H_{16}O_3S$) C, H.

2-(Thiophenoxymethoxy)benzoic Acid (10a). To a solution of 0.29 g (1 mmol) of ethyl 2-(thiophenoxymethoxy)benzoate (**9a**) in 20 mL of methanol was added 1.5 mL (1.5 mmol) of 1 N aqueous NaOH. The reaction mixture was allowed to stir at room temperature overnight, after which an additional 1.5 mL (1.5 mmol) of 1 N aqueous NaOH was added. The reaction mixture was allowed to stir for an additional 30 h and then concentrated. The residue was partitioned between diethyl ether and aqueous HCl. The organic layer was dried ($MgSO_4$) and then concentrated to give 0.26 g (100%) of 2-(thiophenoxymethoxy)benzoic acid (**10a**) as a crystalline solid. Recrystallization from diethyl ether/dichloromethane/hexane gave colorless crystals: mp 70–70.5 °C; ^{1}H NMR ($CDCl_3$) δ 5.63 (s, 2 H), 7.06 (d, J = 8.3 Hz, 1 H), 7.19 (t, J = 7.7 Hz, 1 H), 7.35 (m, 3 H), 7.48 (m, 2 H), 7.56 (m, 1 H), 8.20 (d, J = 7.8 Hz, 1 H); IR (mineral oil) 1677, 1483, 1440, 1306, 1298, 1250, 1156, 760, 690 cm^{-1}. Anal. ($C_{14}H_{12}O_3S$) (C, H).

Ethyl 3-(Thiophenoxymethoxy)picolinate (9b). A mixture of 0.18 g (1.175 mmol) of methyl 3-hydroxypicolinate, 0.17 mL (1.29 mmol) of chloromethyl phenyl sulfide, and 0.178 g (1.29 mmol) of potassium carbonate in 10 mL of dimethylformamide was allowed to stir at room temperature for 20 h. The reaction mixture was concentrated, and the resulting residue was then partitioned between dichloromethane and 1 N aqueous NaOH. The organic layer was dried (Na_2SO_4) and then concentrated. The resulting residue was chromatographed on silica gel with 2% methanol in dichloromethane to give 0.122 g (38%) of ethyl 3-(thiophenoxymethoxy)picolinate (**9b**): ^{1}H NMR ($CDCl_3$) δ 3.94 (s, 3 H), 5.58 (s, 2 H), 7.3 (m, 3 H), 7.42 (m, 2 H), 7.52 (m, 2 H), 8.36 (t, J = 2.7 Hz, 1 H); IR (neat) 1736, 1450, 1429, 1304, 1227, 1204, 1099, 985 cm^{-1}.

3-(Thiophenoxymethoxy)picolinic Acid (10b). To 0.122 g (0.443 mmol) of ethyl 3-(thiophenoxymethoxy)picolinate (**9b**) in 5 mL of methanol was added 0.7 mL (0.7 mmol) of 1 N aqueous KOH. After being stirred at room temperature for 5 h, the reaction mixture was concentrated. The residue was partitioned between dichloromethane and aqueous HCl. The organic layer was dried (Na_2SO_4) and then concentrated. The crude product was crystallized from dichloromethane/hexane to give 0.08 g (69%) of 3-(thiophenoxymethoxy)picolinic acid (**10b**): mp 126–128 °C; ^{1}H NMR ($CDCl_3$) δ 5.71 (s, 2 H), 7.32 (m, 3 H), 7.55 (m, 4 H), 8.31 (bs, 1 H); IR (mineral oil) 1701, 1581, 1441, 1313, 1278, 1202, 1122, 986, 740, 654 cm^{-1}. Anal. ($C_{13}H_{11}NO_3S$) C, H, N.

3-(2-Phenoxyethoxy)-1-propanol (12). A 480-mg (12-mmol) portion of 60% NaH in mineral oil, under argon, was washed three times with pentane, followed by removal of supernatant. It was then slurried in 10 mL of dry tetrahydrofuran and cooled to 0 °C. To this stirred suspension was added dropwise 1.4 mL (19 mmol) of 1,3-propanediol. The ice bath was removed, and after 10 min at room temperature, 1.4 mL (10 mmol) of β-bromophenetole was slowly added. The resulting heterogeneous mixture was stirred overnight and then refluxed for 8 h. It was allowed to cool and then partitioned between diethyl ether and pH 7 aqueous phosphate buffer. The organic phase was washed with saturated aqueous NaCl and then dried ($MgSO_4$). Removal of the solvent under reduced pressure, followed by flash chromatography of the residue on silica gel with 70% ethyl acetate in hexane, afforded 758 mg (38%) of 3-(2-phenoxyethoxy)-1-propanol (**12**) as a colorless liquid: ^{1}H NMR ($CDCl_3$) δ 1.8 (m, 2 H), 2.5 (br, 1 H), 3.7–3.8 (m, 6 H), 4.1 (m, 2 H), 6.9 (m, 3 H), 7.3 (m, 2 H); EI MS m/z 196 (M + H)$^+$; IR ($CHCl_3$) 3410, 2929, 2874, 1599, 1587, 1498, 1247, 1125, 1060, 755, 692 cm^{-1}. Anal. ($C_{11}H_{16}O_3$) C, H.

3-(2-Phenoxyethoxy)propanoic Acid (13). To a solution of 702 mg (3.58 mmol) of 3-(2-phenoxyethoxy)-1-propanol (**12**) in 7 mL of acetonitrile and 7 mL of carbon tetrachloride was sequentially added 10 mL of water, 3.26 g (14 mmol) of periodic acid, and 25 mg of $RuCl_3$ hydrate. The resulting heterogeneous mixture was stirred vigorously (initially becoming quite warm) for 3 h and then partitioned between water and dichloromethane. The aqueous phase was extracted with three additional portions of dichloromethane, and the combined extract was dried ($MgSO_4$) and then concentrated to a yellow oil. This material was taken up in ether, and the resulting solution was extracted with dilute aqueous NaOH. The aqueous phase was then acidified and then extracted with four portions of dichloromethane. The combined organic phase was dried ($MgSO_4$) and then concentrated under reduced pressure to afford 434 mg (58%) of 3-(2-phenoxyethoxy)-propanoic acid (**13**): ^{1}H NMR ($CDCl_3$) δ 2.67 (t, J = 6.3 Hz, 2 H), 3.8 (m, 4 H), 4.1 (m, 2 H), 6.9 (m, 3 H), 7.2 (m, 2 H); IR

($CHCl_3$) 3090, 3063, 3042, 3017, 2927, 2881, 1734, 1715, 1599, 1587, 1498, 1247, 1126, 756, 692 cm^{-1}; EI MS m/z 210 $(M + H)^+$.

2-[[[*N*-(*tert*-Butyloxycarbonyl)-L-isoleucyl]amino]methyl]benzimidazole (16). To an ice-cooled, stirred suspension of 440 mg (2.0 mmol) of 2-(aminomethyl)benzimidazole (15) dihydrochloride and 529 mg (2.2 mmol) of *N*-(*tert*-butyloxycarbonyl)-L-isoleucine hemihydrate in 10 mL of dichloromethane was added 1.1 mL (6.3 mmol) of diisopropylethylamine, giving a clear solution, followed by 0.34 mL (2.2 mmol) of diethyl phosphorocyanidate. The solution was stirred and allowed to warm to room temperature overnight. The mixture was concentrated and flash chromatographed on silica gel with 3–5% methanol in ethyl acetate to provide 618 mg (86%) of 2-[[[*N*-(*tert*-butyloxycarbonyl)-L-isoleucyl]amino]methyl]benzimidazole (16) as a yellow solid: ^1H NMR ($CDCl_3$) δ 0.8 (m, 6 H), 1.42 (s, 9 H), 1.8 (m, 1 H), 4.00 (t, J = 7.5 Hz, 1 H), 4.6 (dd, J = 15, 6.2 Hz, 1 H), 4.7 (dd, J = 15, 6.2 Hz, 1 H), 5.4 (br d, J = 8.5 Hz, 1 H), 7.2 (m, 3 H), 7.6 (br), 8.1 (br, 1 H); FAB HRMS m/z 361.2259 $(M + H)^+$ (calcd for $C_{19}H_{29}N_4O_3$ 361.2240).

2-[[[*N*-[2(*R*)-[5(*R*)-[1(*S*)-[(*tert*-Butyloxycarbonyl)amino]-2-cyclohexylethyl]-2,2-dimethyl-4(*R*)-dioxolanyl]-3-methylbutanoyl]-L-isoleucyl]amino]methyl]benzimidazole (17). A solution of 309 mg (0.587 mmol) of 2-[[[*N*-(*tert*-butyloxycarbonyl)-L-isoleucyl]amino]methyl]benzimidazole (16) in 2 mL of 1:1 dichloromethane–trifluoroacetic acid was allowed to stir for 1 h. It was diluted with additional dichloromethane and then added to a stirred solution of aqueous $NaHCO_3$. The resulting mixture was extracted with dichloromethane in a continuous extractor overnight. The dichloromethane extract was dried (Na_2SO_4) and then concentrated under reduced pressure to afford 256 mg of 2-[(L-isoleucylamino)methyl]benzimidazole as a pale yellow solid: ^1H NMR ($CDCl_3$) δ 0.83 (t, J = 7.3 Hz, 3 H), 0.90 (d, J = 6.9 Hz, 3 H), 1.1 (m, 1 H), 1.3 (m, 2 H), 2.0 (m, 1 H), 3.24 (d, J = 4.1 Hz, 1 H), 4.63 (dd, J = 15.2, 5.7 Hz, 1 H), 4.76 (dd, J = 15.2, 6.4 Hz, 1 H), 7.2 (m, 2 H), 7.5 (br, 2 H), 8.57 (t, J = 6.1 Hz, 1 H); FAB HRMS m/z 261.1708 $(M + H)^+$ (calcd for $C_{14}H_{21}N_4O$ 261.1715).

To an ice-cooled, stirred solution of 47 mg (0.18 mmol) of the above 2-[(L-isoleucylamino)methyl]benzimidazole and 64 mg (0.15 mmol) of 2(*R*)-[5(*R*)-[1(*S*)-[(*tert*-butyloxycarbonyl)amino]-2-cyclohexylethyl]-2,2-dimethyl-4(*R*)-dioxolanyl]-3-methylbutanoic acid (14) in 1 mL of dichloromethane was added 31 μL (0.18 mmol) of diisopropylethylamine, followed by 28 μL (0.18 mmol) of diethyl phosphorocyanidate. The resulting solution was stirred overnight and allowed to warm gradually to room temperature. The mixture was concentrated and the resulting residue flash chromatographed on silica gel with 2–4% methanol in ethyl acetate to provide 81.1 mg (81%) of 2-[[[*N*-[2(*R*)-[5(*R*)-[1(*S*)-[(*tert*-butyloxycarbonyl)amino]-2-cyclohexylethyl]-2,2-dimethyl-4(*R*)-dioxolanyl]-3-methylbutanoyl]-L-isoleucyl]amino]methyl]benzimidazole (17) as a white solid: ^1H NMR ($CDCl_3$) δ 0.7–2.0 (m), 1.42 (s), 2.47 (m, 1 H), 3.91 (m, 1 H), 4.56 (dd, J = 16, 4.9 Hz, 1 H), 4.72 (d, J = 10 Hz, 1 H), 4.91 (dd, J = 16, 7.2 Hz, 1 H), 7.2 (m, 3 H), 8.1 (br, 1 H); FAB HRMS m/z 670.4542 $(M + H)^+$ (calcd for $C_{37}H_{60}N_5O_6$ 670.4543).

2-[[[*N*-(5(*S*)-Amino-6-cyclohexyl-3(*R*),4(*R*)-dihydroxy-2(*R*)-isopropylhexanoyl)-L-isoleucyl]amino]methyl]benzimidazole (18). To 1 mL of stirred methanol in an ice bath was slowly added 71 μL (1 mmol) of acetyl chloride. After 5 min, 81 mg (0.121 mmol) of 2-[[[*N*-[2(*R*)-[5(*R*)-[1(*S*)-[(*tert*-butyloxycarbonyl)amino]-2-cyclohexylethyl]-2,2-dimethyl-4(*R*)-dioxolanyl]-3-methylbutanoyl]-L-isoleucyl]amino]methyl]benzimidazole (17) was dissolved in this acidic solution, and then 100 μL (1.2 mmol) of ethanedithiol was added. The resulting solution was allowed to warm to room temperature, and the reaction was monitored by TLC. After 7 h, excess solid $NaHCO_3$ was slowly added and the resulting mixture stirred overnight. It was concentrated and then triturated with dichloromethane, and the slurry was filtered through Celite. The dichloromethane extract was concentrated, and chromatography of the residue on silica gel with 3–6% methanol (saturated with ammonia) in dichloromethane gave 50.2 mg (78%) of 2-[[[*N*-(5(*S*)-amino-6-cyclohexyl-3(*R*),4(*R*)-dihydroxy-2(*R*)-isopropylhexanoyl)-L-isoleucyl]amino]methyl]benzimidazole (18): ^1H NMR ($CDCl_3$) δ 0.6–1.9 (m), 2.1 (m, 1 H), 2.72 (t, J = 7.3 Hz, 1 H), 3.0 (m, 1 H), 3.6 (br, 1 H), 4.14 (d, J = 8.3 Hz, 1 H), 4.5 (m, 2 H), 4.8 (m, 1 H), 7.2 (m, 3 H), 8.4 (br, 1 H); FAB HRMS m/z 530.3713 $(M + H)^+$ (calcd for $C_{29}H_{48}N_5O_4$ 530.3706).

2-[[[*N*-[5(*S*)-[[2-(Thiophenoxymethoxy)benzoyl]amino]-6-cyclohexyl-3(*R*),4(*R*)-dihydroxy-2(*R*)-isopropylhexanoyl]-L-isoleucyl]amino]methyl]benzimidazole (XVIII). To an ice-cooled, stirred solution of 26.5 mg (50 μmol) of 2-[[[*N*-(5(*S*)-amino-6-cyclohexyl-3(*R*),4(*R*)-dihydroxy-2(*R*)-isopropylhexanoyl)-L-isoleucyl]amino]methyl]benzimidazole (18) and 16 mg (60 μmol) of 2-(thiophenoxymethoxy)benzoic acid (10a) in 0.5 mL of dichloromethane was added 11 μL (60 μmol) of diisopropylethylamine, followed by 10 μL (60 μmol) of diethyl phosphorocyanidate. The resulting solution was stirred overnight and allowed to warm gradually to room temperature. The mixture was concentrated and the resulting residue chromatographed on silica gel with 2–4% methanol (saturated with ammonia) in dichloromethane to provide 27.7 mg (72%) of 2-[[[*N*-[5(*S*)-[2-(thiophenoxymethoxy)benzoyl]amino]-6-cyclohexyl-3(*R*),4(*R*)-dihydroxy-2(*R*)-isopropylhexanoyl]-L-isoleucyl]amino]methyl]benzimidazole (XVIII): ^1H NMR ($CDCl_3$) δ 0.63 (t, J = 7.3 Hz, 3 H), 0.7–1.1 (m), 1.2–1.9 (m), 2.5 (m, 2 H), 3.79 (br, 1 H), 3.95 (br, 1 H), 4.3 (m, 3 H), 4.67 (dd, J = 16, 6.7 Hz, 1 H), 5.26 (dd, J = 32, 11 Hz, 2 H), 6.81 (d, J = 8.3 Hz, 1 H), 7.04 (t, J = 7.5 Hz, 1 H), 7.1–7.4 (m, 10 H), 7.82 (d, J = 8.5 Hz, 1 H), 8.04 (dd, J = 7.8, 1.8 Hz, 1 H), 8.22 (br, 1 H); FAB HRMS m/z 772.4156 $(M + H)^+$ (calcd for $C_{43}H_{57}N_5O_6S$ 772.4108).

1(*S*)-[[2(*R*)-[5(*R*)-[1(*S*)-[(*tert*-Butyloxycarbonyl)amino]-2-cyclohexylethyl]-2,2-dimethyl-4(*R*)-dioxolanyl]-3-methylbutanoyl]amino]-2(*R*)-hydroxyindan (20). To a stirred solution of 143 mg (0.33 mmol) of 2(*R*)-[5(*R*)-[1(*S*)-[(*tert*-butyloxycarbonyl)amino]-2-cyclohexylethyl]-2,2-dimethyl-4(*R*)-dioxolanyl]-3-methylbutanoic acid (14) and 50 mg (0.34 mmol) of 1(*S*)-amino-2(*R*)-hydroxyindan (19) in 1.7 mL of dichloromethane was added 64 μL (0.37 mmol) of diisopropylethylamine. The solution was cooled to 0 °C, and then 62 μL (0.40 mmol) of diethyl phosphorocyanidate was added. After allowing to warm to room temperature and stirring overnight, the reaction mixture was concentrated under reduced pressure and the residue was flash chromatographed on silica gel with 25–35% ethyl acetate in hexane to afford 156 mg (0.28 mmol, 83%) of 1(*S*)-[2(*R*)-[5(*R*)-[1(*S*)-[(*tert*-butyloxycarbonyl)amino]-2-cyclohexylethyl]-2,2-dimethyl-4(*R*)-dioxolanyl]-3-methylbutanoyl]amino]-2(*R*)-hydroxyindan (20): ^1H NMR ($CDCl_3$) δ 7.25 (m, 4 H), 6.40 (d, J = 8.4 Hz, 1 H), 5.43 (2 d, J = 8.1, 8.3 Hz, 1 H), 4.86 (d, J = 10 Hz, 1 H), 4.6 (m, 2 H), 4.25 (m, 2 H), 3.94 (dd, J = 9.5, 7.5 Hz, 1 H), 3.78 (m, 1 H), 3.2–2.95 (m, 2 H), 2.52 (dd, J = 9.5, 6.6 Hz, 1 H), 2.15 (m, 1 H), 2.0–0.8 (m, 18 H), 1.42 (s, 9 H), 1.14 (d, 6.9 Hz, 3 H), 1.05 (d, J = 6.8 Hz, 3 H); FAB HRMS m/z 559.3762 $(M + H)^+$ (calcd for $C_{32}H_{51}N_2O_6$ 559.3747).

1(*S*)-[[5(*S*)-Amino-6-cyclohexyl-3(*R*),4(*R*)-dihydroxy-2(*R*)-isopropylhexanoyl]amino]-2(*R*)-hydroxyindan (21). To 1.0 mL of methanol at 0 °C was added 80 μL (1.1 mmol) of acetyl chloride. After 5 min, the acidic methanolic solution was added to 155 mg (0.28 mmol) of 1(*S*)-[[2(*R*)-[5(*R*)-[1(*S*)-[(*tert*-butyloxycarbonyl)amino]-2-cyclohexylethyl]-2,2-dimethyl-4(*R*)-dioxolanyl]-3-methylbutanoyl]amino]-2(*R*)-hydroxyindan (20), and then 0.12 mL (1.4 mmol) of ethanedithiol was added. The resulting solution was allowed to warm to room temperature. After 8 h, excess solid sodium bicarbonate was slowly added, and the resulting suspension was left to stir overnight. The resulting solid was triturated with chloroform and filtered through Celite with additional chloroform washings. The organic filtrate was concentrated under reduced pressure, and the resulting residue was chromatographed on silica gel with 3–6% methanol (saturated with ammonia) in dichloromethane to yield 98 mg (0.23 mmol, 84%) of 1(*S*)-[[5(*S*)-amino-6-cyclohexyl-3(*R*),4(*R*)-dihydroxy-2(*R*)-isopropylhexanoyl]amino]-2(*R*)-hydroxyindan (21): ^1H NMR ($CDCl_3$) δ 7.33 (m, 1 H), 7.22 (m, 3 H), 7.05 (d, J = 8.4 Hz, 1 H), 5.36 (dd, J = 8.4, 5.0 Hz, 1 H), 4.49 (m, 1 H), 4.00 (d, J = 7.4 Hz, 1 H), 3.6 (m, 1 H), 3.15–2.85 (m, 3 H), 2.38 (t, J = 8 Hz, 1 H), 2.1 (m, 1 H), 1.7 (m, 5 H), 1.6–0.8 (m, 10 H), 1.03 (d, J = 6.6 Hz, 3 H), 0.97 (d, J = 6.7 Hz, 3 H); FAB HRMS m/z 419.2894 $(M + H)^+$ (calcd for $C_{24}H_{39}N_2O_4$ 419.2910).

1(*S*)-[[5(*S*)-[[2-(Thiophenoxymethoxy)benzoyl]amino]-6-cyclohexyl-3(*R*),4(*R*)-dihydroxy-2(*R*)-isopropylhexanoyl]amino]-2(*R*)-hydroxyindan (XX). To a stirred solution of 21 mg (0.050 mmol) of 1(*S*)-[(5(*S*)-amino-6-cyclohexyl-3(*R*),4(*R*)-

dihydroxy-2(*R*)-isopropylhexanoyl)amino]-2(*R*)-hydroxyindan (21) and 16 mg (0.060 mmol) of 2-(thiophenoxymethoxy)benzoic acid (**10a**) in 0.5 mL of dichloromethane was cooled to 0 °C and treated with 11 μL (0.060 mmol) of diisopropylethylamine followed by 10 μL (0.060 mmol) of diethyl phosphorocyanidate. The resulting solution was allowed to warm to room temperature. After being stirred overnight, the reaction mixture was concentrated under reduced pressure. The residue was flash chromatographed on silica gel with 50–90% ethyl acetate in dichloromethane to yield 25 mg (0.038 mmol, 77%) of 1(*S*)-[[5(*S*)-[[2-(thiophenoxymethoxy)benzoyl]amino]-6-cyclohexyl-3(*R*),4(*R*)-dihydroxy-2(*R*)-isopropylhexanoyl]amino]-2(*R*)-hydroxyindan (XX): ^{1}H NMR ($CDCl_3$) δ 8.01 (dd, *J* = 7.8, 1.8 Hz, 1 H), 7.87 (d, *J* = 8.8 Hz, 1 H), 7.5–6.9 (m, 12 H), 6.72 (d, *J* = 8.3 Hz, 1 H), 5.56 (m, 2 H), 5.33 (m, 1 H), 4.56 (m, 1 H), 4.35 (m, 1 H), 4.0–3.7 (m, 4 H), 3.15–2.75 (m, 2 H), 2.48 (m, 1 H), 2.18 (m, 1 H), 2.0–0.8 (m, 14 H), 1.05 (d, *J* = 6.7 Hz, 3 H), 1.02 (d, *J* = 6.7 Hz, 3 H); FAB HRMS *m/z* 661.3317 $(M+H)^+$ (calcd for $C_{38}H_{48}N_2O_6S$ 661.3311).

The following are FAB HRMS data for peptides I–XXI:

peptides	formula	calcd	found
I	$C_{36}H_{52}N_5O_5$	634.3968	634.3944
II	$C_{31}H_{43}N_2O_5$	523.3172	523.3196
III	$C_{41}H_{61}N_5O_8$	752.4598	752.4613
IV	$C_{40}H_{60}N_6O_8$	753.4551	753.4566
V	$C_{43}H_{65}N_5O_9$	796.4860	796.4871
VI	$C_{42}H_{64}N_6O_9$	797.4813	797.4835
VII	$C_{40}H_{59}N_5O_7$	722.4492	722.4510
VIII	$C_{44}H_{59}N_5O_7$	770.4492	770.4512
IX	$C_{43}H_{58}N_6O_7$	771.4445	771.4450
X	$C_{45}H_{61}N_5O_7$	784.4649	784.4661
XI	$C_{36}H_{52}N_2O_8$	641.3802	641.3803
XII	$C_{35}H_{51}N_3O_8$	642.3754	642.3766
XIII	$C_{38}H_{56}N_2O_9$	685.4064	685.4106
XIV	$C_{37}H_{55}N_3O_9$	686.4016	686.4002
XV	$C_{35}H_{50}N_2O_7$	611.3696	611.3718
XVI	$C_{39}H_{50}N_2O_7$	659.3696	659.3724
XVII	$C_{38}H_{49}N_3O_7$	660.3649	660.3652
XVIII	$C_{43}H_{57}N_5O_6S$	772.4108	772.4156
XIX	$C_{42}N_{56}N_6O_6S$	773.4060	773.4079
XX	$C_{38}H_{48}N_2O_6S$	661.3311	661.3317
XXI	$C_{37}H_{47}N_3O_6S$	662.3264	662.3245

Biology. HIV Protease Inhibition Assay. Recombinant HIV-1 protease was recovered and refolded from *Escherichia coli* inclusion bodies as described previously. The enzyme was assayed against synthetic peptide H-Val-Ser-Gln-Asn-Tyr-Pro-Ile-Val-OH (GSP) as described previously. The HIV protease activity was measured at 30 °C in 100 mM sodium acetate, pH 5.5, containing 10% glycerol, 5% ethylene glycol, and 0.1% Nonidet P-40 in a total volume of 50 μL. In experiments at pH 7, 100 mM MOPS was substituted for sodium acetate. The reaction was stopped after 30 min by the addition of 75 μL of 1% trifluoroacetic acid, and samples were subjected to HPLC analysis. For inhibitory study, the peptides were added to the incubation mixture before the addition of the HIV-1 protease. In these cases, five to seven inhibitor concentrations were examined with GSP at 2.5 mM concentration. Data were analyzed by Dixon plots.

Inhibition of p55 Processing in vVk-1 Infected CV-1 Cells. CV-1 cells were seeded at 2 × 10^5 cells per well in 24-well Costar dishes and infected 4-6 h later with vVK-1 at 5 plaque-forming units (PFU) per cell. Each peptide was dissolved in Dulbecco's Modified Eagles medium (DMEM) containing 2.5% fetal bovine serum and was added to duplicate wells at the indicated final concentration 2 h after virus addition. After 24 h, the culture medium was removed, the monolayer was washed with 1 mL of PBS, and the cells were lysed by the addition of 0.1 mL of loading buffer (62.5 mM Tris, pH 6.8, 2.3% SDS, 5% β-mercaptoethanol, and 10% glycerol). The cell lysates were collected individually and placed in boiling water for 3 min, and then 0.025 mL of each sample was subjected to electrophoresis on 12% SDS-polyacrylamide gels. The proteins were electroblotted onto nitrocellulose and analyzed by protein immunoblotting. The primary antibodies were sheep antibody to p24 (International Enzyme, Inc., Fallbrook, CA), and the secondary antibody was alkaline-phosphatase-conjugated rabbit antibody to sheep immunoglobulin G (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The levels of immunoreactive proteins were quantified by densitometry (Bio-Rad, Model 260) with the accompanying 1-D Analyst Software. Inhibition refers to the mean percent decrease in p24 levels determined from the duplicate drug-treated samples compared to the nondrug-treated controls. In general, the percent inhibition did not vary more than 10% in the duplicates. The inhibition of p24 levels by treatment of cultures with 1 μM of peptide A was also determined in each experiment.

Inhibition of p24 Production and RNA Synthesis in HIV-1 (D34 Strain) Infected PBMC (DIAGEN, Institut für molekularbiologische Diagonostik, GmbH, Germany). In this assay, 1 × 10^6 PBMC were infected with an inoculum containing 1 ng p24 of HIV-1 (D34 strain). Test compounds were added, and the level of HIV was assessed 3 and 4 days later; nondrug and AZT-treated infected cultures were also included in parallel as appropriate controls in each assay. On each of the days 2, 3, and 4 postinfection, one of the microtiter plates is prepared for determination of viral replication. The viral core protein p24 is detected in the supernatant of the lymphocyte cultures, and the viral RNA is determined within the cells. Accordingly, 0.15 mL of supernatant is removed from each well and transferred to a microtiter plate containing 0.05 mL per well of SDS (sodium dodecyl sulfate, 0.08%). These plates are stored frozen. A 0.05-mL sample of stop solution (1% SDS, 20 mM sodium acetate, pH 5.0, and 0.2 mL/mL of heparine) is added to the cells remaining in each well. The plates are stored frozen.

(1) Determination of the Concentration of Viral Core Protein p24. The concentration of p24 synthesized by the HIV infected cells is determined by means of a "Sandwich ELISA". A standard preparation of recombinant p24 (Micro Gene Sys, USA) is used for calibration of the ELISA.

(2) Determination of the Concentration of Viral RNA. HIV-RNA synthesized in the infected cells is determined by means of the nucleic acid hybridization technique. Cellular RNA is prepared from the infected cells and analyzed by dot blot hybridization. The hybridization solution contains the ^{32}P-labeled DNA probe with an activity of 2 × 10^6 cpm/mL (specific activity of the probe > 10^8 cpm/μg). The probe comprises a 5.5 kilobase DNA fragment of the HIV isolate D31 clone. This fragment covering the *gag/pol* region of the virus is labeled with ^{32}P α-d CTP by oligonucleotide labeling. Plus-strand RNA transcripts derived from the *gag/pol* region of the viral isolate D31 are used as external standard for the hybridization. These "run-off" transcripts are generated by means of the T7 polymerase reaction from negatively polarized HIV-DNA under T7-promoter control. The hybridized probe is detected by autoradiography, and the processed autoradiograms are evaluated densitometrically.

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