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HIV-1 Protease Inhibitors Containing an *N*-Hydroxyamino Acid Core Structure

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Abstract—Two series of peptidomimetics containing an *N*-hydroxyamino acid core structure were prepared by mixed solution solid-phase synthesis and tested for inhibitory activity against the human immunodeficiency virus (HIV-1) protease (Pr) and the virus in cell culture. In general, N-hydroxy Gly containing pseudopeptides displayed modest HIV Pr inhibition ($IC_{50} \geq 930$ nM). In the *N*-hydroxy Phe derivatives, Fmoc-Phe-ψ[CO–N(OH)]-Phe-Pro-NHtBu was the best inhibitor of the series ($IC_{50} = 144$ nM) showing satisfactory inhibition of HIV replication in cell culture ($ED_{50} = 98$ nM) and remarkable stability against cell culture and plasma enzymes.

Introduction

Human immunodeficiency virus (HIV) has been identified as the probable causative agent of AIDS. The different therapeutic strategies for intervention of this disease have been reviewed recently.[1] A critical role in the viral replication is played by the HIV protease (Pr), an enzyme responsible for the processing of the polyproteins to structural proteins and enzymes essential for the viral maturation and infectivity.[2] This essential role makes it a promising target for chemotherapy of AIDS.[3–7] Since the initial characterisation of this enzyme, rapid progress has been achieved toward the development of potent and selective inhibitors.[8–10] The C_2-symmetry of HIV-1 Pr, which functions as a dimer with each subunit contributing an amino acid triad Asp-Thr-Gly to the active site, stimulated the design of symmetry-based inhibitors. On the basis of the hydroxyethylene core as a tetrahedral transition-state replacement for the peptide substrate, various potent and selective C_2-symmetric inhibitors have been designed.[3,8,11,12] Recently, following the Kempf strategy[10] and the aminodiol inhibitors discovery,[13] we disclosed a class of HIV Pr inhibitors which incorporate a novel C_2 symmetrical or pseudosymmetrical diaminohydroxyalkane core structure.[14–17] Some of these pseudotripeptides (lead compounds (LCs)) (Fig. 1, $n = 1$ or 2) show a significant activity against the isolated enzyme ($IC_{50} = 160$–250 nM) and satisfactory inhibition of HIV replication in cell culture ($ED_{50} = 110$–290 nM)[16] but relatively low enzymatic stability. Subsequently, dynamics simulations[18] indicated a binding model suggesting that LC compounds contain a hydroxylic group capable of hydrogen binding to one of the catalytic aspartate (Asp 25^I/Asp 25) residues of the enzyme and P1/P2 hydrophobic groups for placement in the S1/S2 subsites.

Following the indications of this binding model we designed and prepared a new series of HIV-1 Pr inhibitors containing an *N*-hydroxy amino acid as core structure (Fig. 1 and Table 1).

Since the *N*-hydroxy amide function is a strong proton donor[19] its incorporation into pseudopeptides **1**–**12** would result in more potent inhibitors that would occupy, at least in part, the subsites of the enzyme and which are capable of forming strong H bonds to the catalytic aspartates (Fig. 2). Furthermore, the *N*-hydroxy amide motif can increase the stability to enzymatic degradation in comparison with the cognate amide function.[20] Conceptually, pseudopeptides **1**–**12** represent hybrid structures between LCs and potent KNI inhibitors reported by Kiso et al.[21]

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Results and Discussion

Chemistry

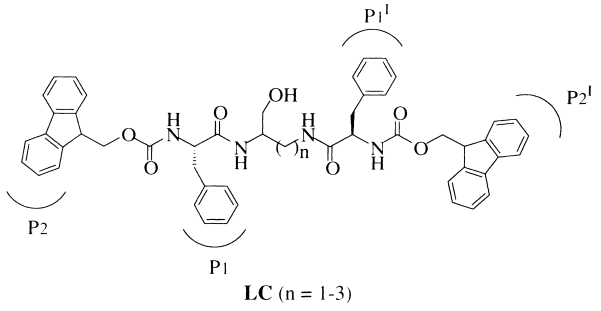
A large number of methods for the synthesis of *N*-hydroxy amino acids, which are building blocks for the incorporation of the *N*-hydroxy amide group into a peptide backbone, have been reported.[22] The insertion of the ψ[CO-N(OH)] modification into peptide sequences has been carried out in solution and more recently also in solid phase.[23–27]

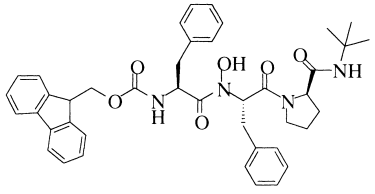
N-Hydroxy pseudopeptides **1**–**12** were obtained trough a convenient mixed solution/solid phase synthetic approach (Scheme 1). *N*-Benzyloxyglycine and *N*-benzyloxy-L or D-phenylalanine were prepared by reaction of benzyloxyamine with the appropriate α-bromo acid.[28] The following synthetic steps were performed by solid phase:[27] each *N*-benzyloxyamino acid (5-fold excess) was coupled with the appropriate amino acid derivatized Wang-resin[29] in the presence of *N*,*N*-diisopropylcarbodiimide (DIPCDI)/1-hydroxybenzotriazole (HOBt). The successive N-hydroxy acylation with the 3-hydroxy-2-methylbenzoic acid (Hmba) or *N*α-benzyloxycarbonylphenylalanine (Z-Phe-OH) (8-fold excess for 24 h) was performed in the presence of 2-(1H-9-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (HATU)/diisopropylethylamine (DIPEA). After trifluoroacetic acid (TFA) cleavage from the solid support, each protected pseudotripeptide was coupled with *tert*-butylamine via 1-ethyl-3-(3[I]-dimethylaminopropyl)carbodiimide (WSC)/HOBt. O-Benzyl hydrogenation gave target compounds **6**, **9** and **12**, whereas benzyl and Z deprotection, followed by the appropriate N-terminal acylation yielded the target pseudopeptides **1**–**5**, **7**, **8**, **10** and **11**.

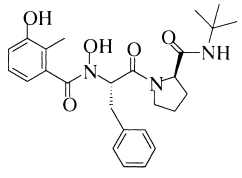
Crude **1**–**12** were purified by RP-HPLC and their structure verification was achieved by ^{1}H NMR and Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass-spectrometry.

Anti HIV-1 activity

Compounds **1**–**12** were tested for their inhibition of purified recombinant HIV-Pr using the peptide substrate His-Lys-Ala-Arg-Val-Leu-Phe(*p*-NO_2)-Glu-Ala-







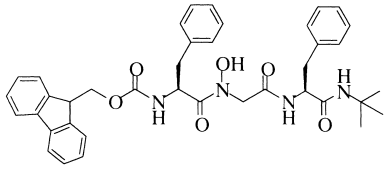


Figure 1. Chemical structures of LC and more representative *N*-hydroxyamide inhibitors.

Table 1. Inhibitory potencies of *N*-hydroxy derivatives

No.	Compound	IC_{50} (nM)[a]	ED_{50} (nM)[a]
1	Boc-Phe-ψ[CO–N(OH)]-Phe-Pro-NHtBu	730	452
2	Z-Phe-ψ[CO–N(OH)]-Phe-Pro-NHtBu	480	371
3	Fmoc-Phe-ψ[CO–N(OH)]-Phe-Pro-NHtBu	144	98
4	Fmoc-Phe-ψ[CO–N(OH)]-D-Phe-Pro-NHtBu	2520	ND[b]
5	Hmba-Phe-ψ[CO–N(OH)]-Phe-Pro-NHtBu	890	630
6	Hmba-ψ[CO–N(OH)]-Phe-Pro-NHtBu	1020	710
7	Fmoc-Phe-ψ[CO–N(OH)]-Gly-Pro-NHtBu	1330	1250
8	Hmba-Phe-ψ[CO–N(OH)]-Gly-Pro-NHtBu	2070	ND
9	Hmba-ψ[CO–N(OH)]-Gly-Pro-NHtBu	2800	ND
10	Fmoc-Phe-ψ[CO–N(OH)]-Gly-Phe-NHtBu	930	450
11	Hmba-Phe-ψ[CO–N(OH)]-Gly-Phe-NHtBu	2200	ND
12	Hmba-ψ[CO–N(OH)]-Gly-Phe-NHtBu	3400	ND
	LC ($n=2$)	160	110

[a]Values are the average of at least two determinations ($n=2$) unless otherwise noted.
[b]Not determined.

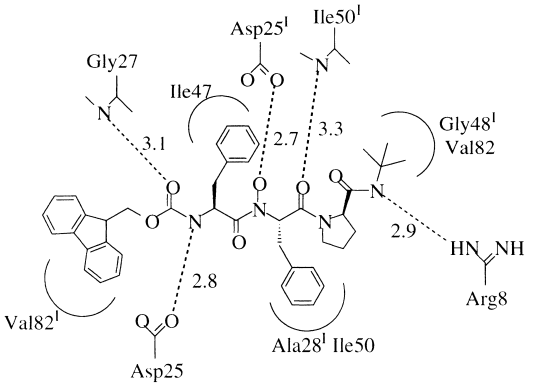
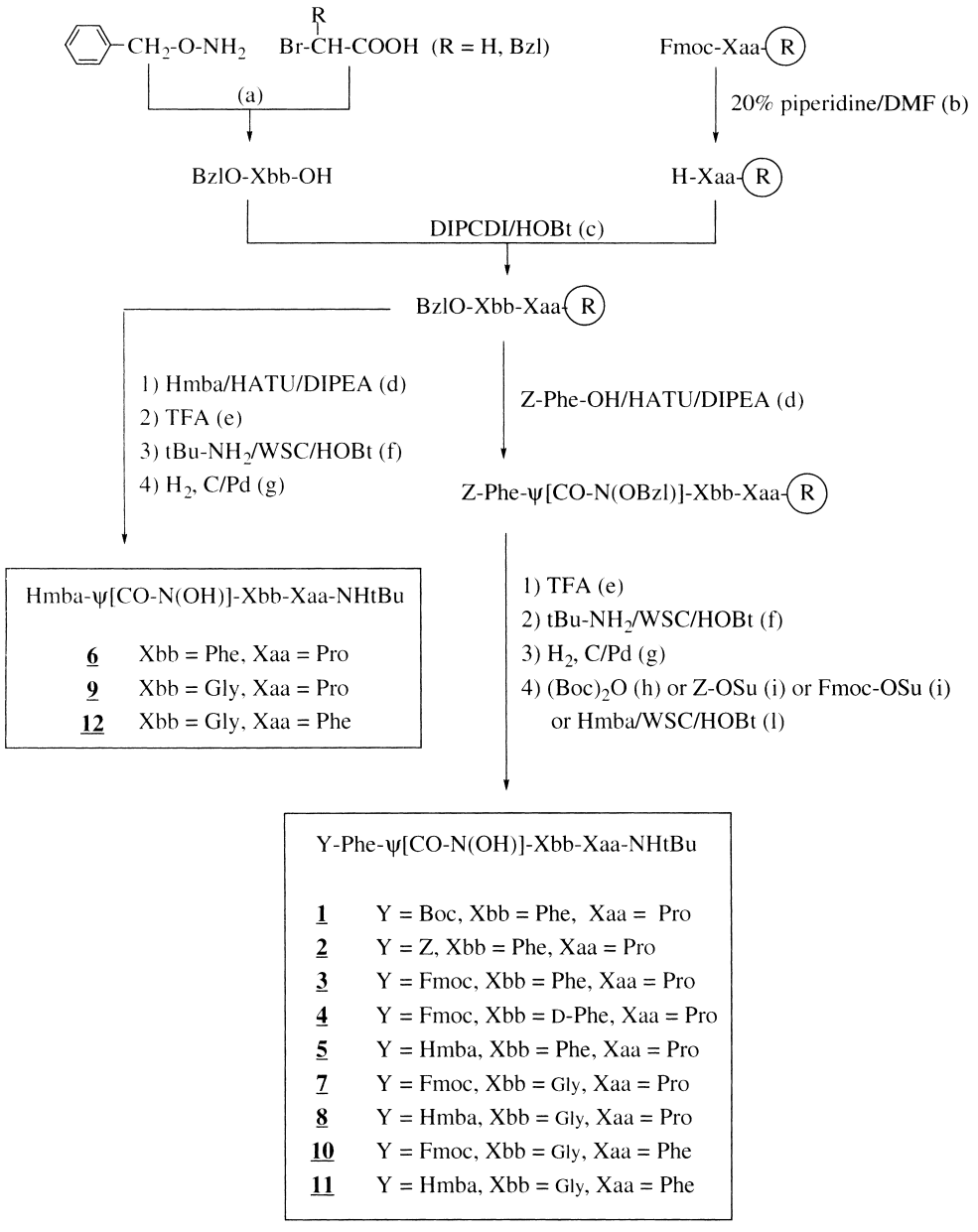


Figure 2. Schematic drawing showing the expected interactions between the HIV-1 protease active site and the inhibitor **3**. Hydrogen bonds are shown as dashed lines with distances in Å. Data were obtained by means of binding model previously suggested by LC analogues dynamic simulations.[18]

Nle-Ser (Bachem Bioscience) as previously described.[14,30,31] The Leu-Phe(*p*-NO_2) amide bond of the substrate is cleaved by enzyme, and substrate and products were separated by reversed-phase HPLC, absorbance being measured at 220 nm. Inhibitory activities are expressed as IC_{50} (concentration of compound which inhibits 50% cleavage of substrate) and are reported in Table 1.

Analysis of the results from the HIV-1 Pr tests showed weak inhibition for the major part of the compounds (IC_{50} values $>0.7\,\mu M$). Only *N*-hydroxy phenylalanine containing pseudotripeptides **2** and **3** exhibited significant activities, comparable to LCs. In detail, *N*-hydroxy-Phe derivatives are more active than the corresponding *N*-hydroxy-Gly containing pseudopeptides (**3** versus **7**, **5** versus **8**) suggesting that the latter core unit can allow less effective interactions between the inhibitors and protease. Comparison of Boc (**1**), Z (**2**), Fmoc (**3**) and Hmba (**5**) functionalities at P2 shows that a bulky tricyclic system is preferred over a single aromatic ring or a branched aliphatic group, confirming the trend previously observed for LCs.[14,16] This preference seems less evident in *N*-hydroxy-Gly compounds (**7** versus **8** and **10** versus **11**). Stereochemical inversion of P_1^1 benzyl group leads to **4** which is 20-fold less potent than its isomeric counterpart **3**. This trend indicates that for this series of inhibitors the presence of a benzyl in the *S* configuration at $P1^1$ may be a critical requirement for the activity. Interestingly, the deletion of Phe residue in pseudotripeptides **5**, **8** and **11** leads to the corresponding pseudodipeptides **6**, **9** and **12** which show activities comparable to the parent inhibitors. These results indicated that in the shorter analogues the Hmba can replace the P1 benzyl of Phe and can fit in the S1 subsite of the enzyme.



Scheme 1. Synthesis of the *N*-hydroxy pseudopeptides.

Seven *N*-hydroxy inhibitors were also tested for their ability to inhibit the replication of the HIV-1 virus in cell culture. Interestingly, the measured ED_{50} values (Table 1) are quite close to the IC_{50} values for enzyme inhibition, as had been noted previously for LCs.[16]

Then, in spite of their modest ability to inhibit the enzyme, **1**–**3** and **10** displayed satisfactory inhibition of HIV replication in cell culture (ED_{50} in the 98–450 nM range), a result that may indicate good cell membrane penetration properties by this class of compounds.

Enzymatic stability

In order to evaluate the stability of these inhibitors against enzyme-mediated hydrolysis, the representative compounds **1**–**3** and **10** were incubated at 37 °C in cell culture medium (RPMI) and in human plasma.[32] The time course of inhibitor degradation was followed by HPLC analysis at varying periods of incubation.[33] As expected, tested pseudopeptides display very high stability in cell culture medium and in human plasma showing no degradation up to 360 min of enzymatic incubation. All new analogues exhibit higher enzymatic resistance compared to the LC series ($T_{1/2} < 90$ min).

Conclusion

Previous efforts in this laboratory resulted in the synthesis of a series of HIV-1 protease inhibitors containing an hydroxyalkyl gem-diamino core structure as exemplified by LCs. In spite of their satisfactory inhibition of HIV replication in cell culture, LCs are quite sensitive to enzymatic degradation. This drawback promoted us to design, through computational studies, LC analogues with improved stability against plasma enzymes while maintaining the lipophilic character of the parent molecules. By the incorporation of an *N*-hydroxy amide motif in the core structure and by modulation of P_1^I/P_2^I of LC, we have been successful in designing the pseudotripeptide **3** (Fig. 2) which has comparable potency against HIV-1 protease and the virus in cell culture but increased metabolic stability.

Experimental

General

Amino acids, amino acid derivatives, resins and chemicals were purchased from Bachem, Novabiochem or Fluka (Switzerland).

Crude pseudopeptides were purified by preparative reversed-phase HPLC using a Water Delta Prep 4000 system with a Waters PrepLC 40 mm Assembly column C_{18} (30×4 cm, 300 A, 15 μm spherical particle size column). The column was perfused at a flow rate of 50 mL/min with a mobile phase containing solvent A (10%, v/v, acetonitrile in 0.1% TFA), and a linear gradient from 0 to 50% of solvent B (60%, v/v, acetonitrile in 0.1% TFA) in 25 min was adopted for the elution of compounds. HPLC analysis was performed by a Beckman System Gold with a Beckman ultrasphere ODS column (5 μm; 4.6×250 mm). Analytical determination and capacity factor (K′) of the peptides were determined using HPLC conditions in the above solvent system (solvents A and B) programmed at flow rates of 1 mL/min using the following linear gradients: (a) from 0% to 100% B in 25 min and (b) from 10 to 70% B in 25 min. All pseudopeptides showed less than 1% impurities when monitored at 220 and 254 nm.

Molecular weights of compounds were determined by a MALDI-TOF analysis using a Hewlett Packard G2025A LD-TOF system mass spectrometer and α-cyano-4-hydroxycinnamic acid as a matrix. The values are expressed as MH^+. TLC was performed in precoated plates of silica gel F254 (Merck, Darmstadt, Germany) using the following solvent systems: (c) AcOEt/*n*-hexane (1:1, v/v), (d) CH_2Cl_2/methanol (9.5:0.5, v/v), (e) CH_2CL_2/methanol (9:1, v/v), f) CH_2CL_2/methanol/toluene (17:2:1, v/v/v). Ninhydrin (1%) or chlorine iodine spray reagents were employed to detect the peptides. Melting points were determined by a Kofler apparatus and are uncorrected. Optical rotations were determined by a Perkin–Elmer 141 polarimeter with a 10-cm water-jacketed cell. ^{1}H NMR spectroscopy was obtained on a Bruker AC 200 spectrometer.

Chemistry

General procedures

***N*-Benzyloxyamino acids (a).** N-Benzyloxy-glycine and *N*-benzyloxy-L or D-phenylalanine were obtained starting to the corresponding α-bromo acids[34] using a slightly modified Kolasa method.[28] To a solution of *N*-benzyloxyamine (5 mmol) in anhydrous DMF (10 mL) the appropriate α-bromo acid (5 mmol) was added. The reaction mixture was allowed to stand at 50 °C for 24 h and after evaporation the residue was poured into 10% sodium carbonate (20 mL). The unreacted benzyloxyamine was extracted with ether (2×10 mL), the aqueous solution was acidified (PH = 3) and the resulting *N*-benzyloxyamino acid was separated by filtration, washed with water and recrystallized from ethanol–water (45–65%). Physicochemical and spectroscopic characteristics of title compounds were in agreement with the data previously reported.[28]

Coupling of *N*-benzyloxyamino acids with the derivatized resin (b, c). Each Fmoc-Xaa-Wang resin (1 g, 0.62 mmol) was treated with 20% piperidine in DMF (15 mL) for 10 min. The resulting H-Xaa-resin was filtrated, washed with DMF (3×20 mL), suspended in DMF (15 mL) and reacted with the suitable *N*-benzyloxyamino acid (1 mmol) in presence of DIPDCI (1 mmol) and HOBt (1 mmol). The heterogeneous mixture was gently stirred for 12 h at room temperature, filtered, washed consecutively with DMF (3×20 mL) and methanol (2×20 mL) to obtain pseudodipeptides BzlO-Xbb-Xaa-resin.

Acylation whit HATU/DIPEA (d). Z-Phe-OH or Hmba (2.2 mmol) were preactivated with HATU (1.8 mmol) and DIPEA (1.8 mol) in DMF (6 mL) for 4 min and added to the suspension of BzlO-Xbb-Xaa-resin (0.4 g) in DMF (4 mL). The mixture was shaken at room temperature overnight and the resulting pseudotripeptide-resin was filtered and whased with DMF (3×10 mL) and methanol (2×10 mL).

Cleavage from the resin (e). Each protected pseudopeptide was cleaved from the solid support by treatment with TFA (7 mL) for 1 h at room temperature. The resin was removed by filtration and washed with TFA (2×1 mL), the filtrate and washing were combined, evaporated under vacuum at 25 °C and the residue triturated with ethyl ether (10 mL). The resulting crude pseudotripeptides were collected by centrifugation and used for the subsequent steps reported in the Scheme 1.

Coupling with *tert*-butylamine (f). To a solution of the appropriate pseudopeptide (0.2 mmol) in DMF (2 mL) were added HOBt (0.2 mmol), WSC (0.2 mmol) and *tert*-butylamine (0.3 mmol). The reaction mixture was stirred at 0 °C for 1 h, 18 h at room temperature and then diluted with ethyl acetate (20 mL), washed successively with 0.1 M HCl, 5% $NaHCO_3$ and brine. The organic solution was dried ($MgSO_4$), filtered and evaporated. The residue was triturated with ethyl ether and the resulting crude amides (71–76%) were used in the successive steps without any further purification.

Hydrogenation deprotection (g). Z and Bzl protecting groups were removed by treating the protected pseudopeptides in methanol (5 mL) with hydrogen (120 min) in the presence of 10% Pd/C. The reaction mixture was filtered, the solvent was evaporated and the resulting target products **6**, **9** and **12** were purified by preparative HPLC. The other crude intermediates were employed in the following steps (Scheme 1).

Introduction of Boc group (h). To a stirred solution of the appropriate pseudotripeptide (0.18 mmol) in DMF (2 mL) was added di-*tert*-butyl dicarbonate (2 mmol). The reaction mixture was stirred overnight at room temperature and evaporated: the resulting target product **1** was purified by preparative HPLC.

Introduction of Z or Fmoc (i). To a solution of H-Phe-ψ[CO–N(OH)]-Xbb-Xaa-NHtBu (0.18 mmol) in DMF (2 mL) was added Z-Osu or Fmoc-Osu (0.18 mmol) at 0 °C. The mixture was stirred for 1 h at 0 °C and 15 h at room temperature and then evaporated. The resulting target compounds **2**, **3**, **4**, **7** and **10** were purified by preparative HPLC.

Introduction of Hmba with WSC/HOBt (l). Suitable pseudotripeptides (0.18 mmol) were added to a solution of Hmba (0.2 mmol), HOBt (0.2 mmol) and WSC (0.2 mmol) in DMF (2 mL) at 0 °C. Each reaction mixture was stirred at 0 °C for 1 h, 18 h at room temperature and then evaporated. The resulting target **5**, **8** and **11** were purified by HPLC.

Boc-Phe-ψ[CO–N(OH)]-Phe-Pro-NHtBu (1). $HPLC_{K'}$ 5.22 (a), 6.03 (b); mp 177–179 °C; $[\alpha]_D^{21}$ −23.9 (*c* 1.0, MeOH); ^1H NMR ($CDCl_3$) 1.34 (br, 18H); 1.80–1.91 (m, 4H); 3.08 (m, 4H); 3.44 (m, 2H); 4.56–4.80 (m, 3H); 5.21 (s, 1H); 6.85–7.03 (m, 10H); 7.73 (s, 1H); 8.49 (sbr, 1H). MS (M + H) 580.7 (calcd 580.7).

Z-Phe-ψ[CO–N(OH)]-Phe-Pro-NHtBu (2). $HPLC_{K'}$ 5.48 (a), 6.78 (b); mp 169–172 °C; $[\alpha]_D^{21}$ −21.4 (*c* 1.0, MeOH); ^1H NMR ($CDCl_3$) 1.31 (s, 9H); 1.76–1.85 (m, 4H); 2.97 (m, 4H); 3.30 (m, 2H); 4.42–4.79 (m, 5H); 5.18 (s, 1H); 6.79–7.11 (m, 15H); 7.67 (s, 1H); 8.52 (sbr, 1H). MS (M + H) 614.7 (calcd 614.7).

Fmoc-Phe-ψ[CO–N(OH)]-Phe-Pro-NHtBu (3). $HPLC_{K'}$ 6.71 (a), 8.14 (b); mp 142–144 °C; $[\alpha]_D^{21}$ −15.3 (*c* 1.0, MeOH); ^1H NMR ($CDCl_3$) 1.28 (s, 9H); 1.93–1.99 (m, 4H); 3.13 (m, 4H); 3.38 (m, 2H); 4.40–4.72 (m, 5H); 5.25 (s, 1H); 7.02–7.89 (m, 20H); 8.40 (sbr, 1H). MS (M + H) 702.8 (calcd 702.8).

Fmoc-Phe-ψ[CO–N(OH)]-D-Phe-Pro-NHtBu (4). $HPLC_{K'}$ 6.82 (a), 8.30 (b); mp 139–142 °C; $[\alpha]_D^{21}$ −6.3 (*c* 1.0, MeOH); ^1H NMR ($CDCl_3$) 1.30 (s, 9H); 1.88–2.00 (m, 4H); 3.18 (m, 4H); 3.40 (m, 2H); 4.44–4.70 (m, 5H); 5.32 (s, 1H); 6.99–7.93 (m, 20H); 8.38 (sbr, 1H). MS (M + H) 702.8 (calcd 702.8).

Hmba-Phe-ψ[CO–N(OH)]-Phe-Pro-NHtBu (5). $HPLC_{K'}$ 4.51 (a), 4.92 (b); mp 190–193 °C; $[\alpha]_D^{21}$ −27.1 (*c* 1.0, MeOH); ^1H NMR ($CDCl_3$) 1.23 (s, 9H); 1.78–1.85 (m, 4H); 2.37 (s, 3H); 3.12 (m, 4H); 3.35 (m, 2H); 4.60–4.79 (m, 3H); 5.23 (s, 1H); 6.85–7.43 (m, 15H); 8.91 (sbr, 1H). MS (M + H) 614.7 (calcd 614.7).

Hmba-ψ[CO–N(OH)]-Phe-Pro-NHtBu (6). $HPLC_{K'}$ 3.70 (a), 4.04 (b); mp 159–161 °C; $[\alpha]_D^{21}$ −35.3 (*c* 1.0, MeOH); ^1H NMR ($CDCl_3$) 1.25 (s, 9H); 1.71–1.86 (m, 4H); 2.41 (s, 3H); 3.08 (m, 2H); 3.40 (m, 2H); 4.66–4.77 (m, 2H); 5.23 (s, 1H); 6.92–7.36 (m, 9H); 8.98 (sbr, 1H). MS (M + H) 467.6 (calcd 467.6).

Fmoc-Phe-ψ[CO–N(OH)]-Gly-Pro-NHtBu (7). $HPLC_{K'}$ 4.60 (a), 5.53 (b); mp 188–190 °C; $[\alpha]_D^{21}$ −18.2 (*c* 1.0, MeOH); ^1H NMR ($CDCl_3$) 1.34 (s, 9H); 1.98–2.13 (m, 4H); 3.09 (dd, $J = 16.5$ and 6.8 Hz, 2H); 3.40 (m, 2H); 4.05–4.77 (m, 6H); 5.11 (s, 1H); 7.12–7.84 (m, 15H); 8.40 (sbr, 1H). MS (M + H) 612.7 (calcd 612.7).

Hmba-Phe-ψ[CO–N(OH)]-Gly-Pro-NHtBu (8). $HPLC_{K'}$ 3.35 (a), 5.02 (b); mp 193–196 °C; $[\alpha]_D^{21}$ −24.6 (*c* 1.0, MeOH); ^1H NMR ($CDCl_3$) 1.31 (s, 9H); 1.97–2.17 (m, 4H); 2.39 (s, 3H); 3.07 (dd, $J = 16{,}7$ and 6.9 Hz, 2H); 3.43 (m, 2H); 4.13–4.91 (m, 4H); 5.23 (s, 1H); 6.97–7.38 (m, 10H); 8.91 (sbr, 1H). MS (M + H) 524.6 (calcd 524.6).

Hmba-ψ[CO–N(OH)]-Gly-Pro-NHtBu (9). $HPLC_{K'}$ 2.90 (a), 3.27 (b); mp 140–143 °C; $[\alpha]_D^{21}$ −26.7 (*c* 1.0, MeOH); ^1H NMR ($CDCl_3$) 1.34 (s, 9H); 1.90–2.02 (m, 4H); 2.34 (s, 3H); 3.58 (m, 2H); 4.08 (s, 2H); 4.88 (m, 1H) 5.31 (s, 1H); 6.87–7.38 (m, 4H); 8.98 (sbr, 1H). MS (M + H) 377.4 (calcd 377.4).

Fmoc-Phe-ψ[CO–N(OH)]-Gly-Phe-NHtBu (10). $HPLC_{K'}$ 5.55 (a), 7.03 (b); mp 151–154 °C; $[\alpha]_D^{21}$ −13.9 (*c* 1.0, MeOH); ^1H NMR ($CDCl_3$) 1.25 (s, 9H); 3.13–3.22 (m, 4H); 4.05 (s, 2H); 4.68–4.94 (m, 4H); 5.15 (s, 1H); 7.08–7.90 (m, 21H); 8.39 (sbr, 1H). MS (M + H) 662.8 (calcd 662.8).

Hmba-Phe-ψ[CO–N(OH)]-Gly-Phe-NHtBu (11). $HPLC_{K'}$ 4.04 (a), 4.55 (b); mp 173–176 °C; $[\alpha]_D^{21}$ −19.5 (*c* 1.0, MeOH); ^1H NMR ($CDCl_3$) 1.35 (s, 9H); 2.41 (s, 3H); 3.08–3.19 (m, 4H); 4.11 (s, 2H); 4.83–4.91 (m, 2H); 5.23 (s, 1H); 6.88–7.50 (m, 16H); 8.79 (sbr, 1H). MS (M + H) 574.7 (calcd 574.7).

Hmba-ψ[CO–N(OH)]-Gly-Phe-NHtBu (12). $HPLC_{K'}$ 3.14 (a), 3.66 (b); mp 185–187 °C; $[\alpha]_D^{21}$ −25.3 (*c* 1.0, MeOH); ^1H NMR ($CDCl_3$) 1.30 (s, 9H); 2.35 (s, 3H); 3.12 (dd, $J = 16.6$ and 6.8 Hz, 2H); 4.07 (s, 2H); 4.85 (m, 1H); 5.23 (s, 1H); 6.93–7.48 (m, 10H); 8.69 (sbr, 1H). MS (M + H) 427.5 (calcd 427.5).

Metabolic stability assay

The kinetics of new inhibitors degradation were studied in culture medium (RPMI) and human plasma. 0.1 mL of a solution of each compound (10 mg/mL in acetonitrile/H_2O 1:1) was added to 1 mL of RPMI containing 20% fetal calf serum. Alternatively, test compound were incubated with plasma (0.6 mL) in a total volume of 1.5 mL of 10 mM Tris–HCl buffer, pH 7.5. Incubation were performed at 37 °C for different times: up to 360 min in the case of human plasma and up to 4 days in the case of RPMI containing 20% FCS. The incubation was terminated by addition of ethanol (0.2 mL), the mixture poured at 21 °C and after centrifugation (5000 rpm for 10 min), aliquots (20 μL) of the clear supernatant were injected into RP-HPLC column. HPLC was performed as described above (see Experimental procedures, general).

Test for the inhibition of HIV-1 protease

For determination of IC_{50} values, affinity-purified HIV-1 protease (Bachem Bioscience), 1.1 nM final concentration, was added to a solution (100 μL final volume) containing inhibitor, 4 mM peptide substrate (His-Lys-Ala-Arg-Val-Leu-*p*-nitro-Phe-Glu-Ala-Nle-Ser, Bachem Bioscience), and 1.0% dimethyl sulfoxide in assay buffer: 1.0 mM dithiothreitol, 0.1% glycerol, 80 mM sodium acetate, 160 mM sodium chloride, 1.0 mM EDTA, all at pH 4.7. The solution was mixed and incubated for 25 min at 37 °C and reaction quenched by the addition of trifluoroacetic acid, 2% final concentration. The Leu-Phe(*p*-NO_2) bond of the substrate was cleaved by the enzyme. The cleavage products and substrate were separated by reversed-phase HPLC. Absorbance was measured at 220 nm, peak areas were determined, and percent conversion to product was calculated using relative peak areas. The data were plotted as percent control (the ratio of percent conversion in the presence and absence of inhibitor) versus inhibitor concentration and fit with the equation $Y = 100/1 + (X/IC_{50})^A$, where IC_{50} is the inhibitor concentration at 50% inhibition and A is the slope of the inhibition curve.

Cell culture activity against HIV-1 IIIB

HIV-1 IIIB was obtained from HIV-1 IIIB chronically infected Molt-4 cells as a supernatant fluid. The 50% tissue culture infection dose (TC ID_{50}) was determined by an endpoint titration procedure.[35] CEM cells (5000/mL) were exposed to HIV-1 IIIB fluid at a multiplicity of infection (m.o.i.) 0.001 TC ID_{50} (mL). Aliquots (0.2 mL) of cells were placed in 96 well microtitre plates with 2 mL of the appropriate concentrations of inhibitors dissolved in DMSO. After incubation for 6 days in RPMI-1640 medium containing 10% fetal calf serum, the p24 antigen of HIV in the supernatant was determined by an ELISA assay kit (RETRO-TEK, Cellular Products Inc., Buffalo, USA). The ED_{50} (50% dose) values were calculated as the dose of the inhibitor that resulted in a 50% reduction in p24 levels as compared to those in control wells.

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