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Synthesis and Activity of *N*-Benzyl Pseudopeptides HIV Protease Inhibitors

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Abstract—A series of *N*-benzyl pseudopeptides was designed, synthesized and tested as HIV-1 protease inhibitors. The ability of the new compounds containing *N*-benzyl hydroxyalkylamino acid core structure to inhibit HIV replication in cell culture is comparable to their capacity to inhibit the isolated enzyme, a result compatible with good pharmacokinetic properties of these derivatives. The pseudotripeptide Fmoc-Leu-N(Bzl)Hse-Met-NH-*t*Bu was the best inhibitor of the series ($IC_{50} = 170$ nM) showing promising inhibition of viral replication ($ED_{50} = 52$ nM). All new compounds exhibit high enzymatic resistance and stability against cell cultures and plasma enzymes.

Introduction

The pandemic spread of human immunodeficiency virus (HIV), the etiologic agent of AIDS, has promoted an unequalled scientific effort to understand and control this disease. The resultant understanding of HIV-1 life cycle have defined many different targets for potential drug intervention. The virally encoded homodimeric aspartyl protease (HIV Pr) is currently one of the more promising therapeutic targets for the treatment of AIDS due to its critical role in the virus maturation and replication.[1] The HIV-1 Pr has an homodimeric C-2 symmetric structure and each monomer contributes one catalytic aspartic residue and flexible flap, which is able to bind the substrates and inhibitors.[2–4] In addition, a characteristic bound water molecule forms an hydrogen bonding network between the flaps and bond substrates creating a tetrahedral transition-state intermediate. Many different classes of HIV-1 protease inhibitors have been developed, showing excellent antiviral profiles.[5–8] On the other hand there is the necessity to introduce new potent inhibitors that show good pharmacokinetics and activity against mutant strains of HIV. On the basis of these features and following the Kempf strategy,[9–11] we designed different series of pseudopeptide inhibitors which incorporate amide bond modified and hydroxyalkane residue on the core structure.[12–18] Some previous compounds containing an hydroxyalkyl gem-diamino or *N*-hydroxy amide function (Fig. 1) showed significant activity against the isolated enzyme ($IC_{50} = 140$–160 nM), satisfactory inhibition of HIV replication in cell culture ($ED_{50} = 98$–110 nM) and good metabolic stability.

In this paper, we describe a new series of HIV-1 inhibitors, which contain a *N*-benzyl hydroxyalkylamino acid in the core structure. The introduction of benzyl group on the N near to hydroxy function was predicted to have the potential to make supplementary favourable interactions at P1 position (Fig. 1).[19]

Compounds **1**–**6** contain *N*-benzyl serine, while derivatives **7**–**12** have homoserine (Hse) in the core structure, to modulate distance and flexibility of the hydroxyalkyl side chain. Bulky protected phenylalanine or leucine were placed at P1/P2 in pseudotripeptides **3**–**6** and **9**–**12** to increase the number of van der Waals interactions with the enzyme. Methionine *t*-butylamide was selected in analogy with other sulfur containing residues at $P2^{I}$ position in potent HIV protease inhibitors.[20,21] Pseudodipeptides **1**, **2** and **7**, **8** were designed to ameliorate pharmacokinetic properties. In effect, for all analogues, the *N*-benzyl amide function can increase metabolic stability in comparison with the cognate amide bond.

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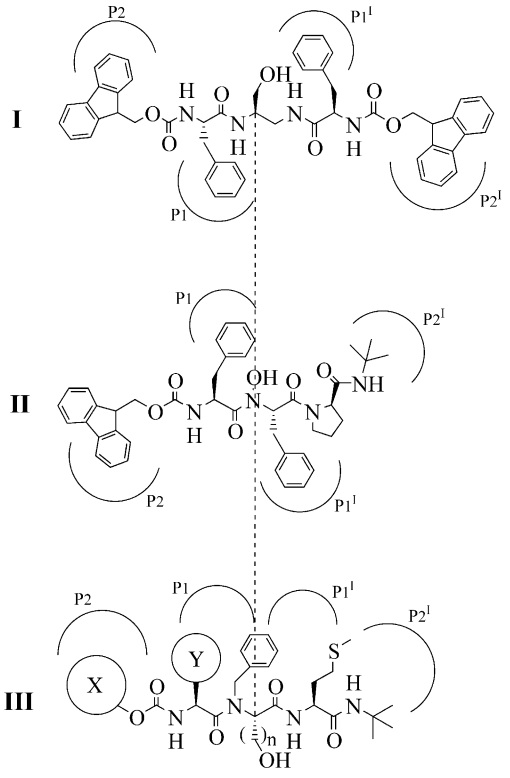


Figure 1. More representative diamino-hydroxyalkane derivative (I), *N*-hydroxyamide inhibitor (II) and general structure of *N*-benzyl pseudotripeptides (III).

Results and Discussion

Chemistry

Synthesis of *N*-benzyl pseudopeptides is outlined in Schemes 1 and 2.

Analogues **1**–**6** containing *N*-benzyl serine were prepared by initial coupling of Fmoc-protected serine *t*-butyl ether with methionine *t*-butylamide (Scheme 1). Resulting dipeptide, after Fmoc removal, was benzylated by reductive alkylation and resulting mixture of mono- and di-*N*-benzylated products was separated by column chromatography.[22] Pseudodipeptides **1** and **2** were obtained by treatment with trifluoroacetic acid (TFA). The key intermediate protected *N*-benzyl dipeptide was acylated by *t*-butyloxycarbonyl-phenylalanine or -leucine via mixed anhydride. *t*-Butyl deprotection, followed by coupling of the Fmoc-OSu or Z-OSu, gave the desired *N*-benzyl pseudotripeptides **3**–**6**.

Compounds **7**–**12** containing *N*-benzyl homoserine core structure were prepared by the same synthetic strategy (Scheme 2). The difficulty in incorporating homoserine into synthetic peptides stem from the well known tendency of γ-hydroxyamino acids and their derivatives to form γ-lactone.[23–25] To avoid this drawback we used an approach where the key of the synthesis was the preparation of *N*-benzylated aspartyl dipeptides and the subsequent conversion in the corresponding Hse-products or intermediates by chemoselective reduction via mixed anhydrides.[26–28]

Crude **1**–**12** were purified by RP-HPLC and their structure verification was achieved by ^{1}H NMR and mass-spectrometry (Table 1).

Antiviral activity

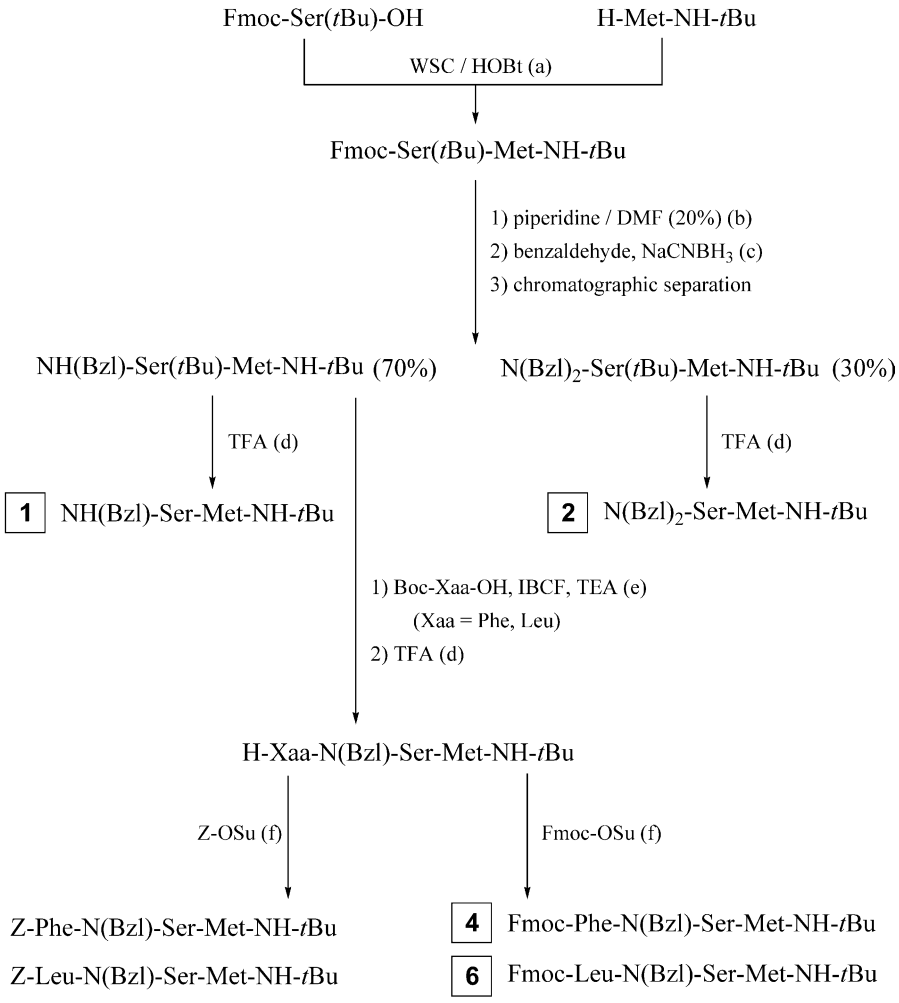
IC_{50} and ED_{50} values, respectively against HIV-1 protease and for inhibition of viral replication in cell culture for pseudopeptides **1**–**12** are shown in Table 2.

IC_{50} values of the inibitory potencies against purified recombinant HIV-Pr were determined using synthetic peptide H-His-Lys-Ala-Arg-Val-Leu-Phe(pNO_2)-Glu-Ala-Nle-Ser-OH (Bachem Bioscience) as a substrate. The C-terminal pentapeptide cleaved product was measured at 220 nm by reversed-phase HPLC analysis at different times of incubation.[29] The HIV Pr inhibition results show a moderate activity for mono- and di-benzylated pseudopeptides **1**, **2**, **7** and **8**, indicating that the reduction of molecular size decreases the number of interactions between enzyme and inhibitor especially at P2 site.

In pseudotripeptides, bulky tricyclic system (Fmoc) is preferred in P2 position confirming the trend previously observed.[14–16] In P1, branched aliphatic side chain (Leu) is slightly better as compared aromatic ring (Phe)

Table 1. Analytical data and physicochemical properties of *N*-benzyl pseudopeptides

No.	Compd	HPLC		Mp (°C)	$[\alpha]_D^{20}$ (C = 1, MeOH)	MS (M + H$^+$)
		K^I (A)	K^I (B)			
1	NH(Bzl)Ser-Met-NH-*t*Bu	5.92	4.83	93–95	−33.4	382.6
2	N(Bzl)$_2$Ser-Met-NH-*t*Bu	7.25	6.41	87–89	−31.8	472.7
3	Z-Phe-N(Bzl)Ser-Met-NH-*t*Bu	10.45	9.28	132–136	−20.7	662.5
4	Fmoc-Phe-N(Bzl)Ser-Met-NH-*t*Bu	11.70	10.34	124–127	−16.3	751.8
5	Z-Leu-N(Bzl)Ser-Met-NH-*t*Bu	9.42	8.51	143–145	−12.1	628.9
6	Fmoc-Leu-N(Bzl)Ser-Met-NH-*t*Bu	10.63	9.14	138–141	−9.5	716.7
7	NH(Bzl)Hse-Met-NH-*t*Bu	6.25	5.52	88–91	−30.3	396.6
8	N(Bzl)$_2$Hse-Met-NH-*t*Bu	7.21	6.43	82–84	−27.1	486.6
9	Z-Phe-N(Bzl)Hse-Met-NH-*t*Bu	10.55	9.11	117–120	−18.5	676.6
10	Fmoc-Phe-N(Bzl)Hse-Met-NH-*t*Bu	12.41	10.76	109–113	−15.4	765.7
11	Z-Leu-N(Bzl)Hse-Met-NH-*t*Bu	9.73	8.39	140–142	−17.7	643.1
12	Fmoc-Leu-N(Bzl)Hse-Met-NH-*t*Bu	11.62	10.22	129–133	−11.2	730.8



Scheme 1. Synthesis of *N*-benzyl pseudopeptides **1**–**6**.

suggesting in this case a non-favourable conformational perturbation due at presence of the adjacent *N*-benzyl moiety.

The higher length of the hydroxyalkyl chain in *N*-benzyl homoserine core residue (**7**–**12**) over *N*-benzyl serine (**1**–**6**), promote easier hydrogen bond formation with catalytic aspartic acid.

Table 2. Inhibitory potencies and metabolic degradation of *N*-benzyl pseudopeptides

No.	IC_{50} (nM)[a]	ED_{50} (nM)[a]	Half-life (min)	
			Culture medium	Human plasma
1	3700	ND[b]	>360	>360
2	2220	ND[b]	>360	>360
3	1170	ND[b]	>360	270
4	789	543	>360	230
5	1010	ND[b]	>360	>360
6	370	310	>360	310
7	2643	ND[b]	>360	>360
8	1730	ND[b]	>360	>360
9	620	245	>360	325
10	297	118	>360	>360
11	365	295	>360	342
12	170	52	>360	>360

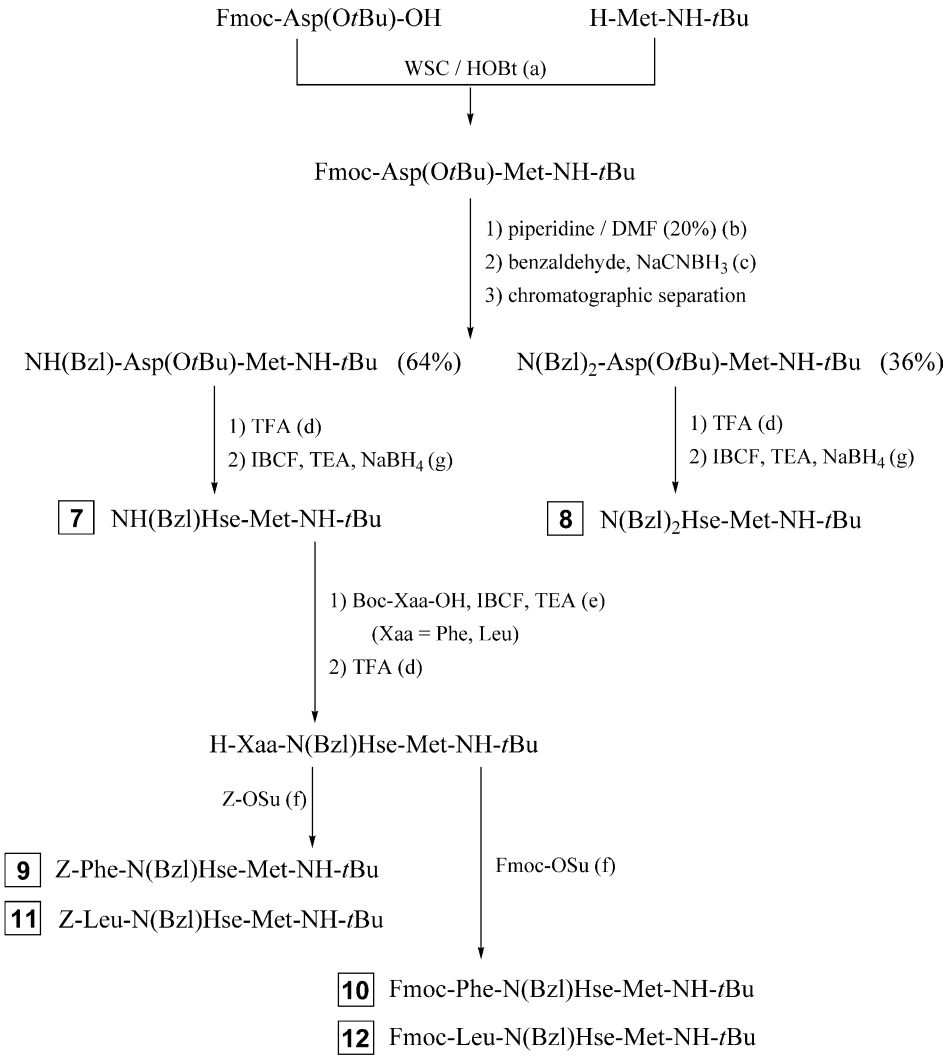
[a]Values are the average of at least two determinations ($n=2$) unless otherwise noted.
[b]Not determined.

In general, methionine *t*-butylamide is well tolerant in $P1^I$ and $P2^I$ sites and the presence of a supplementary benzyl group in P1 catalytic position seems to generate additional van der Waals interactions; this is a favourable condition to decrease drug resistance through enzyme mutation (Fig. 2).

Better isolate enzyme *N*-benzyl inhibitors were also tested for their capacity to inhibit viral replication in the HIV-1 strain IIIB in CEM cells.[30] All compounds displayed satisfactory activity quite close to the IC_{50} values for HIV-1 Pr inhibition. In particular *N*-benzyl pseudotripeptide **12** ($ED_{50}=52$ nM) shows good potency confirming its favourable pharmacokinetic properties, especially an essential cell membrane penetration.

Metabolic stability

The stability against enzymatic hydrolysis of inhibitors **1**–**12** was evaluated in cell culture medium (RPMI) and in human plasma, after incubation at 37 °C. The time course of pseudopeptide degradation was followed by RP-HPLC analysis at varying periods of incubation.[31,32]



Scheme 2. Synthesis of the *N*-benzyl analogues **7**–**12**.

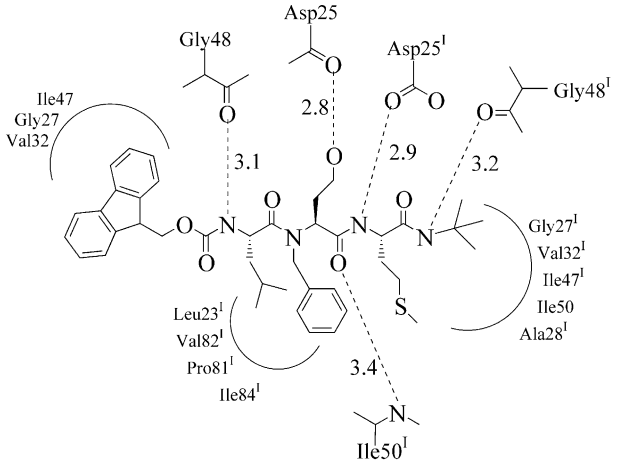


Figure 2. Schematic drawing showing the expected interactions between *N*-benzyl pseudotripeptide **12** and the HIV-1 protease active site. Hydrogen bonds are shown as dashed lines with distances in Å. Data were obtained by means of binding model previously suggested by diamino-hydroxyalkane derivatives (I) dynamic simulations.

All compounds display very high stability in cell culture medium, in human plasma only pseudotripeptides **3**, **4**, **6**, **9** and **11** exhibit an half-life less to 360 min (Table 2). Experiments carried out using human plasma showed essentially identical trend of inhibitor degradation, with a very slowly formation of a single catabolite. The degradation products were isolated by RP-HPLC and after mass spectrometry analysis were found to be compatible with the N-terminal pseudodipeptides.

The data of the present study demonstrate, that the new inhibitors were very weakly cleaved of the amide bond between *N*-benzyl core residue and methionine *t*-butylamide.

Conclusion

On the basis of previous efforts in our laboratories which resulted in the development of HIV-1 protease inhibitors, we designed, synthesized and tested new pseudopeptides containing *N*-benzyl hydroxyalkyl residue in

core structure. Our results show that the *N*-alkylation of central amide bond confer elevated metabolic stability and maintain satisfactory antiviral activity. The *N*-benzyl homoserine pseudotripeptide **12** is endowed with a good inhibition against isolated enzyme and HIV replication in culture cell.

In summary, our work in this field produced a pseudopeptide model which can permit structural changes in order to ameliorate viral activity and pharmacokinetic properties.

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 Hypersil BDS C18 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 weight 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. ^1H NMR spectroscopy was obtained on a Bruker AC 200 spectrometer.

Chemistry

General procedures. Coupling with WSC/HOBt (a). To a solution of the carboxy component (1 mmol) in DMF (10 mL) were added the amino component (1 mmol), NMM (1 mmol), HOBt (1.1 mmol) and WSC (1.1 mmol) in this order at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and 18 h at room temperature; then the solution was diluted with EtOAc (100 mL) and washed consecutively with HCl 0.1 N, brine, $NaHCO_3$ and brine. The organic phase was dried ($MgSO_4$), filtered and evaporated to dryness. The residue was treated with Et_2O and resulting solid, separated by centrifugation.

Fmoc removal (b). The Fmoc group was cleaved with a solution of 20% piperidine in DMF for 25 min. After evaporation the residue was triturated with Et_2O, centrifugated and resulting solid was collected and dried.

***N*-Benzylation (c).** To a solution of dipeptide H-Ser(*t*Bu)-Met-NH-*t*Bu or H-Asp(O*t*Bu)-Met-NH-*t*Bu (2 mmol) in acetonitrile were added benzaldehyde (2 mmol) and $NaCNBH_3$ (3 mmol). The reaction mixture was stirred for 15 min and acetic acid was added dropwise until pH 7; after 45 min, the solution was evaporated under reduced pressure. The residue was diluted with EtOAc (50 mL) and washed with water; the organic phase was dried ($MgSO_4$), filtered and evaporated. The resulting mixture of mono and di *N*-benzylated dipeptides was separated by column chromatography.

TFA deprotection (d). *t*-Butyl protections were removed by treating intermediates with aqueous 90% TFA (1:10, w/v) for 30–40 min. After evaporation, the residue was worked up as described in (b).

Coupling via mixed anhydride (e). To a stirred solution of Boc-amino acid (1 mmol) in DMF (3 mL), triethylamine (TEA, 1 equiv) was added; the mixture was cooled to −10 °C, treated with isobutylchloroformate (IBCF, 1 equiv), and allowed to react for 5 min. A precooled solution of the amino component trifluoroacetate (1.1 mmol) in DMF (3 mL) was added to the mixture, followed by TEA (1.1 equiv). The reaction mixture was stirred for 1 h at −10 °C and 3 h at room temperature and then evaporated. After evaporation the residue was worked up as described in (a).

Introduction of Z or Fmoc (f). To a solution of *N*-benzyl pseudotripeptide (0.5 mmol) in DMF (5 mL) was added Z-OSu or Fmoc-OSu (0.5 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 **3–6** and **9–12** were purified by preparative HPLC.

Reduction of β-carboxylic side chain (g). To a stirred solution of mono or di *N*-benzyl aspartyl dipeptide (1 mmol) in THF (5 mL) at −15 °C, *N*-methilmorpholine

(NMM, 1 mmol) was added, followed by isobutylchloroformate (IBCF, 1 mmol). After 20 min, the precipitate was removed by filtration and the filtrate was added dropwise to a cold (−10 °C) solution of $NaBH_4$ (3 mmol) in EtOH (3 mL). The solution was stirred for 10 min at 0 °C and for additional 15 min at room temperature and then evaporated. The residue was worked up as described in (a).

NH(Bzl)-Ser-Met-NH-*t*Bu (1). ^{1}H NMR ($CDCl_3$) 1.42 (s, 9H), 2.15–2.23 (m, 5H), 2.87 (m, 2H), 3.65 (d, 1H), 3.91–4.02 (m, 4H), 4.53 (m, 1H), 4.90 (sbr, 1H), 6.15 (sbr, 1H), 6.32 (sbr, 1H), 7.21 (m, 5H).

N(Bzl)$_2$-Ser-Met-NH-*t*Bu (2). ^{1}H NMR ($CDCl_3$) 1.40 (s, 9H), 2.11–2.21 (m, 5H), 2.62 (m, 2H), 3.60 (d, 1H), 3.81–4.05 (m, 6H), 4.86 (sbr, 1H), 6.11 (sbr, 1H), 6.35 (sbr, 1H), 7.15–7.34 (m, 10H).

Z-Phe-N(Bzl)-Ser-Met-NH-*t*Bu (3). ^{1}H NMR ($CDCl_3$) 1.32 (s, 9H), 2.04 (s, 3H), 2.27–2.42 (m, 4H), 3.40–3.58 (m, 3H), 4.03 (m, 2H), 4.53–4.86 (m, 5H), 5.34 (S, 2H), 5.86 (sbr, 1H), 6.11 (sbr, 1H), 7.05–7.19 (m, 15H), 7.45 (sbr, 1H).

Fmoc-Phe-N(Bzl)-Ser-Met-NH-*t*Bu (4). ^{1}H NMR ($CDCl_3$) 1.27 (s, 9H), 1.94 (s, 3H), 2.11–2.28 (m, 4H), 3.40–3.56 (m, 3H), 3.99 (m, 2H), 4.45–4.66 (m, 5H), 5.32 (S, 2H), 5.77 (sbr, 1H), 6.01 (sbr, 1H), 7.02–7.25 (m, 19H), 7.37 (sbr, 1H).

Z-Leu-N(Bzl)-Ser-Met-NH-*t*Bu (5). ^{1}H NMR ($CDCl_3$) 1.03 (d, 6H), 1.32 (s, 9H), 1.74–1.82 (m, 3H), 2.09 (s, 3H), 2.24–2.36 (m, 4H), 3.36 (m, 1H), 3.95 (s, 1H), 4.05 (m, 2H), 4.48–4.79 (m, 4H), 5.30 (s, 2H), 5.76 (sbr, 1H), 6.05 (sbr, 1H), 7.15–7.27 (m, 10H), 7.40 (sbr, 1H).

Fmoc-Leu-N(Bzl)-Ser-Met-NH-*t*Bu (6). ^{1}H NMR ($CDCl_3$) 1.09 (d, 6H), 1.37 (s, 9H), 1.70–1.85 (m, 3H), 2.15 (s, 3H), 2.31–2.44 (m, 4H), 3.29 (m, 1H), 3.80 (s, 1H), 4.10 (m, 2H), 4.42–4.73 (m, 4H), 5.22 (s, 2H), 5.59 (sbr, 1H), 5.98 (sbr, 1H), 7.02–7.26 (m, 14H), 7.51 (sbr, 1H).

NH(Bzl)-Hse-Met-NH-*t*Bu (7). ^{1}H NMR ($CDCl_3$) 1.38 (s, 9H), 2.13–2.28 (m, 7H), 2.80 (m, 2H), 3.55 (d, 1H), 3.90–4.09 (m, 4H), 4.65 (m, 1H), 4.87 (sbr, 1H), 6.11 (sbr, 1H), 6.29 (sbr, 1H), 7.13 (m, 5H).

N(Bzl)$_2$-Hse-Met-NH-*t*Bu (8). ^{1}H NMR ($CDCl_3$) 1.44 (s, 9H), 2.24–2.32 (m, 7H), 2.58 (m, 2H), 3.51 (d, 1H), 3.77–3.94 (m, 6H), 4.81 (sbr, 1H), 5.97 (sbr, 1H), 6.19 (sbr, 1H), 7.03–7.25 (m, 10H).

Z-Phe-N(Bzl)-Hse-Met-NH-*t*Bu (9). ^{1}H NMR ($CDCl_3$) 1.30 (s, 9H), 1.99 (s, 3H), 2.17–2.31 (m, 6H), 3.45–3.59 (m, 3H), 4.21 (m, 2H), 4.63–4.79 (m, 5H), 5.32 (S, 2H), 5.80 (sbr, 1H), 6.22 (sbr, 1H), 7.13–7.30 (m, 15H), 7.50 (sbr, 1H).

Fmoc-Phe-N(Bzl)-Hse-Met-NH-*t*Bu (10). ^{1}H NMR ($CDCl_3$) 1.39 (s, 9H), 2.03 (s, 3H), 2.20–2.35 (m, 6H), 3.33–3.45 (m, 3H), 3.97 (m, 2H), 4.31–4.47 (m, 5H), 5.19 (S, 2H), 5.70 (sbr, 1H), 5.98 (sbr, 1H), 7.23–7.47 (m, 20H).

Z-Leu-N(Bzl)-Hse-Met-NH-*t*Bu (11). ^{1}H NMR ($CDCl_3$) 1.07 (d, 6H), 1.39 (s, 9H), 1.79–1.85 (m, 3H), 2.19 (s, 3H), 2.33–2.45 (m, 6H), 3.39 (m, 1H), 4.03 (s, 1H), 4.15 (m, 2H), 4.45–4.70 (m, 4H), 5.25 (s, 2H), 5.67 (sbr, 1H), 6.03 (sbr, 1H), 7.08–7.29 (m, 10H), 7.44 (sbr, 1H).

Fmoc-Leu-N(Bzl)-Hse-Met-NH-*t*Bu (12). ^{1}H NMR ($CDCl_3$) 1.09 (d, 6H), 1.28 (s, 9H), 1.60–1.74 (m, 3H), 2.06 (s, 3H), 2.25–2.38 (m, 6H), 3.21 (m, 1H), 3.82 (s, 1H), 3.96 (m, 2H), 4.35–4.70 (m, 4H), 5.28 (s, 2H), 5.66 (sbr, 1H), 6.04 (sbr, 1H), 7.14–7.44 (m, 15H).

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 compounds 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 was performed at 37 °C for different time: up to 360 min in the case of human plasma and up to 2 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 RP-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 fitted 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 end point 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.

Acknowledgements

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