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ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 874–880
Bioorganic & Medicinal Chemistry

Design, synthesis and structure–activity study of shorter hexa peptide analogues as HIV-1 protease inhibitors

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Received 20 April 2007; revised 5 October 2007; accepted 10 October 2007
Available online 22 October 2007

Abstract—Inhibition of HIV-1 protease enzyme can render the Human Immunodeficiency Virus (HIV-1) non-infectious in vitro. Previous studies have shown that several shorter peptides were discovered as HIV-1 protease inhibitors. In this context, a series of shorter synthetic hexapeptides, Leu-Leu-Glu-Tyr-Val-Xaa (Xaa = Phe, Met, Tyr and Trp), were designed. The synthesized hexa peptides were screened for their HIV-1 protease inhibition. These peptides showed moderately good HIV-1 protease inhibition when compared to acetyl pepstatin.

1. Introduction

The pandemic spread of Human Immunodeficiency Virus (HIV), the etiologic agent of Acquired Immunodeficiency Syndrome (AIDS),[1] has promoted an unprecedented scientific and clinical effort to understand and combat this lethal disease. According to the United Nations AIDS organization,[2] by the end of 2007, there would be 75 million people living with HIV/AIDS globally and an estimated 30 million may die since the beginning of the epidemic. The identification of the HIV retrovirus and the accumulated knowledge about the role of different elements in the viral life cycle made it possible to identify numerous intervention points in the HIV-1 viral life cycle that could be exploited in the development of drugs for AIDS therapy.[3]

Very detailed crystallographic analysis combined with extensive biochemical characterization and site-specific mutagenesis studies made on the HIV-1 protease made it perhaps the best characterized enzyme to date.[4] The virally encoded homodimeric aspartyl protease (HIV-1 protease) is responsible for the processing of the *gag* (Pr 55^{gag}) and *gag/pol* (Pr $160^{gag\text{-}pol}$) gene products that allow for the organization of the core structural proteins (Matrix P17, Capsid P24, and Nucleocapsid P9) and release of viral enzymes (Reverse Transcriptase, RnaseH, Integrase, and Protease). It has been shown that budding immature viral particle that contains catalytically inactive protease cannot undergo maturation into an infective form.[5] The necessity of this enzyme in the virus life cycle makes it a promising target for the design of inhibitors against HIV-1 protease enzyme.

Several methodologies have been applied for drug discovery of HIV-1 protease inhibitors. In these methodologies, the structure of enzyme substrates as a starting point for drug discovery (substrate-based approach), biological screening approach developed to test hundreds/thousands of compounds, and X-ray crystallography data of HIV-1 protease-inhibitor complexes (biostructural approach or ''rational drug design'') were the prominent ones. Mainly the peptidomimetics drug discovery was achieved with the substrate-based approach. Systematic modification of the substrate-based peptides using rational design techniques led to peptidomimetic compounds lacking much of the peptide backbone while retaining the essential functionalities for HIV-1 protease active site inhibition.[6] Among them the marketed drugs Invirase (Saquinavir mesylate, Hoffmann-LaRoche), Norvir (Ritonavir, Abott Laboratories), Crixivan (Indinavir sulfate, Merck) and Viracept (Nelfinavir mesylate, Agouron Pharmaceuticals), as well as Amprenavir (VX-478, Glaxo Wellcome), are currently in clinical trials. These compounds present subnanomolar activities versus the enzyme[7] and demonstrate high selectivity for HIV-1 protease over other

Keywords: Human Immunodeficiency Virus; HIV-1 protease; Hexa peptides; Oyester peptides; Structural analogues; Design and synthesis.
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doi:10.1016/j.bmc.2007.10.052

aspartyl proteases. Non-peptide HIV-1 protease inhibitors, irreversible inhibitors, dimerization inhibitors, and metal based inhibitors were the other class of HIV-1 protease inhibitors.

Another major class of HIV-1 protease inhibitors is the peptide inhibitors. The rapid identification of HIV-1 protease inhibitors through the synthesis and screening of defined tripeptide and acetylated tetrapeptide amides was reported by Owens et al.[8] The inhibition of HIV-1 protease by short peptides derived from the terminal segment of the protease was reported.[9] At the same time the inhibition of HIV-1 protease by its own interface peptides was also reported.[10] An evaluation of the inhibition of the HIV-1 protease by its C- and N-terminal peptides at 100 μM concentration was also reported.[11] Noever[12] had reported the HIV-1 protease inhibitory activity of the naturally occurring common protease inhibitors. The fluorinated peptides incorporating a 4-fluoroproline residue as potential inhibitor of HIV-1 protease was reported by Tran et al.[13] Louis et al.[14] reported the hydrophilic peptides derived from the transframe region of *Gag-Pol* as potent HIV-1 protease inhibitors. Recently it has been shown that the HIV-1 auxiliary protein Vif, and especially the N-terminal half of Vif, inhibits the HIV-1 protease.[15] Based on this fact various Vif derived peptides were synthesized chemically and screened for their HIV-1 protease inhibitory activity. The in vitro HIV-1 protease inhibition by the modified peptides, *N*-glyoxylyl peptides,[16] and boronated peptides[17] has opened up a new vista in understanding the HIV-1 protease enzyme inhibition.

Several inhibitors of HIV-1 protease are currently approved and some are under clinical trials. However, the therapeutic effect of protease inhibitors is limited by the rapid development of inhibitor resistant variants of the protease.

The peptides inhibiting HIV-1 protease were isolated from the hydrolysate of Oyster (Crassostrea Gigas) proteins prepared with thermolysin.[18] The amino acid sequences of peptides were determined as Leu-Leu-Glu-Tyr-Ser-Ile and Leu-Leu-Glu-Tyr-Ser-leu. These sequences exist in some proteins of various major viruses or human cytomegalovirus. Chemically synthesized Leu-Leu-Glu-Tyr-Ser-Ile and Leu-Leu-Glu-Tyr-Ser-Leu showed IC_{50} values of 20 and 15 nM, respectively, as competitive inhibitors of HIV-1 protease with K_i values of 13 and 10 nM, respectively. These peptides were approximately 100-fold more potent as an HIV-1 protease inhibitor than acetyl pepstatin (IVal-Val-Val-Sta-Ala-Sta), a characteristic inhibitor of aspartic protease. The structure–activity studies on these peptides suggest that the *C*- and N-terminal hydrophobic amino acids or the length of the peptides is important for their inhibitory activity. Sarubbi et al.[19] have reported the isolation and structural characterization of an HIV-1 protease inhibitor from the fermentation broth of a *Streptomyces* strain, namely α-microbial alkaline protease inhibitor (α-MAPI). α-MAPI is a tetrapeptide derivative with a C-terminal Phenylalanine in the aldehyde form. This function is essential for the inhibition of HIV-1 protease. The studies suggest that aspartic proteases may be inhibited by peptide aldehydes acting in their tetrahedral hydrated forms as transition state analogues. Valine and Phenylalanine, the two C-terminus amino acid residues of α-MAPI, were among the best suited to occupy S_2 and S_1 sub sites of the HIV-1 protease enzyme, respectively. The studies based on substrate analogues show that hydrophobic/aromatic moieties were preferable in the S_1 sub site of the enzyme, with Phe, Tyr, and Met as best residues.

In the above context we designed a hexapeptide series. The hexapeptide series contains Leu-Leu-Glu-Tyr-Val as the main pentapeptide sequence to which hydrophobic amino acids such as Phe, Met, Tyr, and Trp were attached at the C-terminal end to obtain a set of hexapeptides. These peptides were structurally characterized and analyzed for HIV-1 protease inhibitory activity.

2. Results and discussion

2.1. Design of peptides

The various literature reports on the synthesis and HIV-1 protease inhibitory activity of smaller peptides made us to design and evaluate some small peptides as HIV-1 protease inhibitors. The hexapeptides Leu-Leu-Glu-Tyr-Ser-Ile and Leu-Leu-Glu-Tyr-Ser-leu, isolated from the hydrolysate of Oyster (Crassostrea Gigas), were taken as the platform for the design of the hexapeptide series. Valine and Phenylalanine, the two C-terminus amino acid residues of α-MAPI, were among the best suited to occupy S_2 and S_1 sub sites of the HIV-1 PR enzyme, respectively.[19] The studies based on substrate analogues show that hydrophobic/aromatic moieties are preferable in the S_1 sub site of the enzyme, with Phe, Tyr, and Met as best residues. In this study, we wanted to study the structure–activity of the above hexapeptides. In this context we designed the hexapeptide Leu-Leu-Glu-Tyr-Val-Phe by replacing the Ser-Ile C-terminal end with the more hydrophobic amino acids Val-Phe. As the literature reports say that the hydrophobic amino acids play a prominent role in the HIV-1 protease inhibitors, we designed a hexapeptide series Leu-Leu-Glu-Tyr-Val-(Xaa)-OH by replacing the Phe with Met, Tyr, and Trp.

2.2. HIV-1 protease inhibition studies

The HIV-1 protease inhibitory activity of the synthesized peptides was carried out at a concentration of 100 μg/ml of each of the peptides (Table 3). As the initial screening, we carried the assay at only one concentration of the peptides. The hexapeptide series displayed a very good inhibition of up to 64.92%. The overall order of HIV-1 PR inhibition was found to be Leu-Leu-Glu-Tyr-Val-Trp-OH > Leu-Leu-Glu-Tyr-Val-Tyr-OH > Leu-Leu-Glu-Tyr-Val-Phe-OH > Leu-Leu-Glu-Tyr-Val-Met-OH.

The data shown above clearly show that some of the above peptides may serve as promising lead compounds for a novel type of HIV-1 protease inhibitors. Careful

examination of the sequence of these peptides shows that there is no similarity to any of the natural HIV-1 protease cleavage sites. Furthermore, the fact that the above peptides were not cleaved by HIV-1 protease strongly indicates that they do not serve as HIV-1 protease substrates, but they are the inhibitors. The mechanism in which these peptides inhibit HIV-1 protease is not clear. It is possible that, being non-substrate molecules, they do not function in the active site of the enzyme, but inhibit its action by some allostearic mechanism.

2.3. Structure–activity relationship studies

The peptides related to the Oyster derived hexapeptides were synthesized to study the structure–activity relationship. The results showed that the hexapeptide series exhibited very good HIV-1 protease inhibitory activity compared to acetyl pepstatin. The results revealed that the substitution of the Phe of the C-terminal, with more hydrophobic amino acid residues like Trp and Tyr, resulted in increase in the HIV-1 protease inhibiting activity.

3. Conclusions

Since the outbreak of the AIDS epidemic, tremendous efforts have been directed toward the development of antiretroviral therapies that target HIV-1 in particular (HIV-1). Although there are six HIV-1 protease inhibitors and commercially available reverse transcriptase inhibitors, their effectiveness is hampered by the emergence of drug resistance and cross-resistance mutants, rendering AIDS with no definite cure. In general, the HIV-1 protease inhibitors used today are all substrate-based. Here we presented a novel type of HIV-1 protease inhibitors, non-substrate based, which can serve as lead compounds for the development of a novel type of HIV-1 protease inhibitors. This new approach may be an answer to the acute problem of drug resistance caused by active site mutations, a problem which prevents the long-term usage of HIV-1 protease inhibitors as anti HIV-1 drugs. Inhibitors which are not substrate-based and will not function in the active site should not be affected by the active site mutations. These inhibitors may be active against protease-mutant HIV-1 strains. The potential peptides described above may become promising drug candidates and warrant further development of peptides and peptidomimetics as drug candidates against AIDS.

4. Materials and methods

4.1. General

All the amino acids used were of L-configuration unless otherwise specified. All *tert*-butyloxycarbonyl (Boc) amino acids, amino acid derivatives, 1-hydroxybenzotrizole (HOBt), and trifluoroacetic acid (TFA) were purchased from Advanced Chem. Tech. (Louisville, Kentucky, USA). Isobutylchloroformate (IBCF), *N*-methyl morpholine (NMM), and EDCI were purchased from Sigma Chemicals (St. Louis, USA). Recombinantly produced HIV-1 protease enzyme and Acetyl pepstatin were purchased from Sigma Aldrich (India) Ltd. HIV-1 Substrate III was purchased from Severn Biotech Ltd (UK). All solvents and reagents were of analytical grade or were purified according to the standard procedure recommended for peptide synthesis. Silica gel (60–120 mesh) for column chromatography was purchased from Merck India Ltd (India).

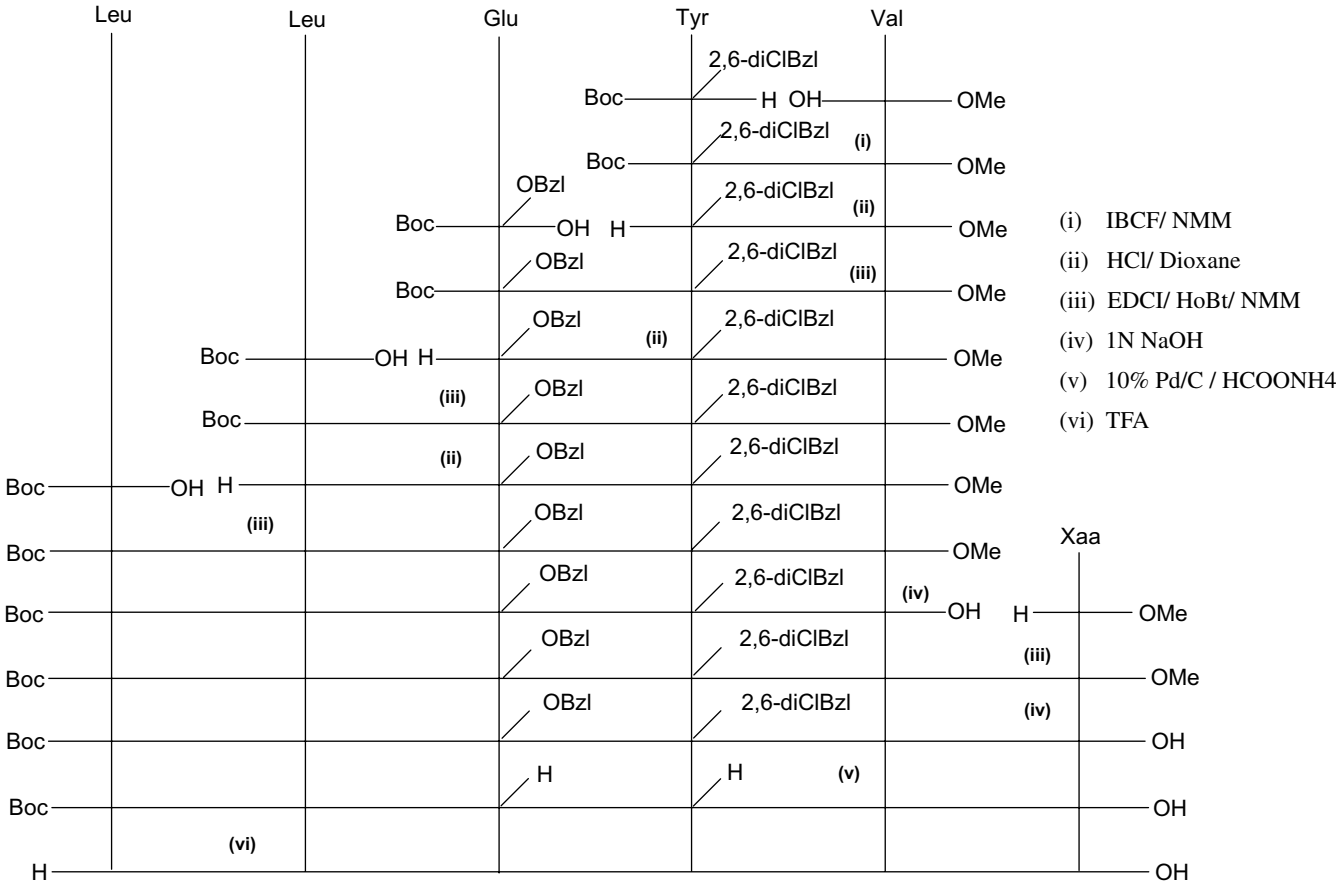
The thin layer chromatography (TLC) was carried out on silica gel plates obtained from Whatman Inc., with the following solvent systems:

1:	$CHCl_3$:CH_3OH:CH_3COOH	(95:05:3)
2:	$CHCl_3$:CH_3OH:CH_3COOH	(90:10:3)
3:	$CHCl_3$:CH_3OH:CH_3COOH	(85:15:3)

The compounds on TLC plates were detected by UV light, by ninhydrin or chlorine/toluidine spray. The purity of the peptides was determined by HPLC analysis by using Thermo Electron with RP C18 column (5CN Cosmogel 215–4.6 mm) and UV detector—UV-VISL7400. Peptide sample (25 μl) in water was injected for analysis. The analysis was carried out using appropriate 0–100% water/acetonitrile linear gradients in the presence of 0.1% TFA (flow rate 1.0 ml/min). The melting points of peptides were determined with Thomas Hoover melting point apparatus and were uncorrected. The optical rotation was measured using Perkin-Elmer 243 digital polarimeter. Elemental analysis was carried out on VARIO EL III CHNOS Elementar. The compounds were dried over P_2O_5 under reduced pressure for 24 h prior to the preparation of samples for all the analyses. ^1H NMR was recorded on AMX-400 MHz spectrometer.

4.2. Peptide synthesis

Synthesis of peptides was achieved by classical solution phase method[20] using an efficient and well established Boc-chemistry instead of more expensive Fmoc-chemistry. The hexapeptide series Leu-Leu-Glu-Tyr-Val-(Xaa)-OH was synthesized by the stepwise solution phase approach as shown in Scheme 1. The Boc group was chosen for temporary N^{α}-protection and its removal was achieved with 4 N HCl in dioxan or trifluoroaceticacid (TFA). The C-terminal carboxyl group was protected by methyl ester, and its removal was effected by saponification with 1 N NaOH. The side chain active hydrogen of the Serine and Glutamic acid was protected by benzyl group. The active hydrogen of OH group on the aromatic side chain of Tyrosine was protected by 2, 6, dichloro benzyl group. The side chain protecting group was deprotected by the catalytic transfer hydrogenolysis by using 10% Pd/C and $HCOONH_4$. All the coupling reactions were achieved with the standard coupling agents isobutylchloroformate (IBCF) and EDCI. The protected peptides were purified by column chromatography over silica gel and characterized by physical and analytical techniques.



Scheme 1. Scheme of synthesis for the hexapeptide analogues.

4.2.1. Boc-Tyr(2,6-diClBzl)-Val-OMe. To Boc-Tyr(2,6-diClBzl)-OH (5.23 g, 12 mmol) dissolved in acetonitrile (53 mL) and cooled to 0 °C was added NMM (1.21 mL, 12 mmol). The solution was cooled to −15 ± 1 °C and IBCF (1.63 mL, 12 mmol) was added under stirring while maintaining the temperature at −15 °C. The reaction mixture was stirred for an additional 10 min and a pre-cooled solution of HCl·H-Val-OMe (2.01 g, 12 mmol) and NMM (1.21 mL, 12 mmol) in DMF (21 mL) was added slowly. After 20 min, the pH of the solution was adjusted to 8 by the addition of NMM and the reaction mixture stirred overnight at room temperature. Acetonitrile was removed under reduced pressure and the residual DMF solution was poured into about 100 mL ice-cold 90% saturated $KHCO_3$ solution and stirred for 30 min. The peptide precipitated was extracted into $CHCl_3$. The organic layer was washed with water, 10% citric acid, water, 5% $NaHCO_3$ solution, water and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure, the obtained peptide was triturated with dry ether, petroleum ether and dried under vacuum. The yields and physical constants are given in Table 1.

4.2.2. Boc-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-OMe. Boc-Tyr(2,6-diClBzl)-Val-OMe (3.64 g, 6.76 mmol) was deblocked with 4 N HCl/dioxane (36.4 mL) for 1.5 h. Excess HCl and dioxane were removed under reduced pressure, triturated with ether, filtered, washed with ether and dried (yield, 100%). The HCl·H-Tyr(2,6-diClBzl)-Val-OMe in DMF (37 mL) was neutralized with NMM (0.64 mL, 6.42 mmol) and coupled to Boc-Glu(OBzl)-OH (2.16 g, 6.42 mmol) in acetonitrile (22 mL) and NMM (0.64 mL, 6.42 mmol) using IBCF (0.87 mL, 6.42 mmol) and worked up the same as Boc-Tyr(2,6-diClBzl)-Val-OMe to obtain Boc-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-OMe. The sample was recrystallized from ether/petroleum ether. The yields and physical constants are given in Table 1.

4.2.3. Boc-Leu-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-OMe. Boc-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-OMe (2.94 g, 3.8 mmol) was deblocked with 4 N HCl/dioxane (29.4 mL) for 1.5 h. Excess HCl and dioxane were removed under reduced pressure, triturated with ether, filtered, washed with ether, and dried (yield, 100%). The HCl·H-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-OMe in DMF (30 mL) was neutralized with NMM (0.38 mL, 3.8 mmol) and coupled to Boc-Leu-OH. H_2O (0.94 g, 3.8 mmol) in acetonitrile (10 mL) and NMM (0.38 mL, 3.8 mmol) using EDCI (0.72 g, 3.8 mmol), HOBt (0.51 g, 3.8 mmol) and worked up the same as Boc-Tyr(2,6-diClBzl)-Val-OMe to obtain Boc-Leu-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-OMe. The sample was recrystallized from ether/petroleum ether. The yields and physical constants are given in Table 1.

4.2.4. Boc-Leu-Leu-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-OMe. Boc-Leu-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-OMe (2.54 g, 2.86 mmol) was deblocked with 4 N HCl/dioxane

Table 1. Physical and analytical data of protected hexapeptides

Peptide	Yield (%)	Mp (°C)	R_f^1	R_f^2	R_f^3	$[\alpha]_D^{25}$ (*C* 1) MeOH	Molecular formula	Elemental analysis found (calculated)		
								% C	% H	% N
Boc-Tyr(2,6-diClBzl)-Val-OMe	87	48–49	0.61	0.73	0.72	−82	$C_{27}H_{34}N_2O_6Cl_2$	57.51 (57.54)	6.00 (6.03)	4.94 (4.97)
Boc-Glu(OBzl)-Tyr (2,6-diClBzl)-Val-OMe	90	60	0.93	0.94	0.96	−56	$C_{37}H_{47}N_3O_9Cl_2$	59.20 (59.27)	6.24 (6.27)	5.56 (5.60)
Boc-Leu-Glu(OBzl)-Tyr (2,6-diClBzl)-Val-OMe	86	49	0.83	0.92	0.94	−64	$C_{43}H_{58}N_4O_9Cl_2$	58.94 (58.97)	6.60 (6.62)	6.38 (6.40)
Boc-Leu-Leu-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-OMe	79	49–50	0.82	0.91	0.90	−63	$C_{51}H_{69}N_5O_{11}Cl_2$	61.30 (61.32)	6.89 (6.91)	7.00 (7.01)
Boc-Leu- Leu-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-Phe-OMe	97	58–59	0.90	0.95	0.98	−73	$C_{60}H_{78}N_6O_{12}Cl_2$	62.85 (62.88)	6.81 (6891)	7.32 (7.33)
Boc-Leu-Leu-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-Met-OMe	80	48–49	0.84	0.94	0.95	−44	$C_{56}H_{78}N_6O_{12}SCl_2$	59.50 (59.52)	6.89 (6.90)	7.40 (7.44)
Boc-Leu-Leu-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-Tyr-OMe	92	56	0.92	0.94	0.95	−67	$C_{60}H_{75}N_6O_{13}Cl_2$	62.10 (62.17)	6.42 (6.47)	7.20 (7.25)
Boc-Leu-Leu-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-Trp-OMe	89	57–58	0.86	0.92	0.95	−63	$C_{62}H_{78}N_7O_{12}Cl_2$	62.84 (62.89)	6.54 (6.59)	8.20 (8.24)

R_f^1: R_f value in solvent 1.
R_f^2: R_f value in solvent 2.
R_f^3: R_f value in solvent 3.

(26 mL) for 1.5 h. Excess HCl and dioxane were removed under reduced pressure, triturated with ether, filtered, washed with ether, and dried (yield, 100%). The HCl·H-Leu-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-OMe in DMF (30 mL) was neutralized with NMM (0.28 mL, 2.86 mmol) and coupled to Boc-Leu-OH. H_2O (0.71 g, 2.86 mmol) in acetonitrile (10 mL) and NMM (0.28 mL, 2.86 mmol) using EDCI (0.54 g, 2.86 mmol), HOBt (0.51 g, 2.86 mmol) and worked up the same as Boc-Tyr(2,6-diClBzl)-Val-OMe to obtain Boc-Leu-Leu-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-OMe. The sample was recrystallized from ether/petroleum ether. The yields and physical constants are given in Table 1.

4.2.5. Boc-Leu-Leu-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-(Xaa)-OMe (Xaa = Phe, Met, Tyr, or Trp). Boc-Leu-Leu-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-OMe (2.55 g, 2.5 mmol) was taken in 26 mL of methanol and saponified with 1 N NaOH (5.0 mL, 5.00 mmol) to remove the methyl ester group. The reaction was stirred up to the completion of the reaction as monitored by TLC, and the MeOH was evaporated, dissolved the product in 25 mL water, washed with $CHCl_3$, then neutralized with cold 1 N HCl (5.0 mL). The precipitate was taken into $CHCl_3$ layer, washed with saturated NaCl, and dried over sodium sulfate anhydrous. The Boc-Leu-Leu-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-OH was neutralized with NMM (0.05 mL, 0.49 mmol) and coupled with individual amino acids HCl. ^{2}HN·Xaa-OMe (0.49 mmol) in DMF (10 mL/1 g of peptide) and NMM (0.05 mL, 0.49 mmol) using EDCI (0.09 g, 0.49 mmol), HOBt (0.06 g, 0.49 mmol) and worked up the same as Boc-Tyr(2,6-diClBzl)-Val-OMe to obtain Boc-Leu-Leu-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-(Xaa)-OMe. The samples were purified over silica gel using chloroform–methanol as elutant. The yields and physical constants are given in Table 1. The ^{1}H NMR spectral data of protected peptides are provided in Table 2.

4.2.6. Leu-Leu-Glu-Tyr-Val-(Xaa)-OH (Xaa = Phe, Met, Tyr, or Trp). The protected peptides were hydrogenolyzed separately using ammonium formate (2 equivalents) and 10% Pd/C (1 equivalent) in methanol (10 mL/1 g) for 2 h at room temperature. The catalyst was filtered and washed with methanol. The combined washings and filtrate were evaporated in vacuo and the residue taken into $CHCl_3$, washed with water, and dried over Na_2SO_4. The solvent was removed under reduced pressure and triturated with ether, filtered, washed with ether, and dried. The resulting peptides were saponified with 1 N NaOH as described in above steps to remove the methyl protecting group. The resulting peptides were treated individually with TFA (10 mL/1 g for 40 min) for final Boc deprotection. The solvent was removed under reduced pressure, triturated with ether to obtain TFA salt of Leu-Leu-Glu-Tyr-Val-(Xaa)-OH. The free peptides were purified by gel filtration using Sephadex G-10 and checked the purity by HPLC, using a linear gradient of 0–100% acetonitrile/0.1% trifluoroacetic acid.

4.3. HIV-1 protease inhibition studies

This assay followed the method previously described by Min et al.[21] Recombinant HIV-1 protease enzyme was diluted with buffer composed of 50 mM sodium acetate at pH 5.0, 1 mM ethylenediamine disodium (EDTA·2-Na), and 2 mM 2-mercaptoethanol and glycerol in the ratio of 75:25. The HIV-1 protease Substrate III, His-Lys-Ala-Arg-Val-Leu-(pNO_2-Phe)-Glu-Ala-Nle-Ser-NH_2, was diluted with a buffer solution of 50 mM sodium acetate (pH 5.0). To a reaction mixture containing 2 μl 50 mM buffer solution (pH 5.0) and 2 μl of substrate solution (1 mg/ml), 2 μl of the samples prepared in water at 100 μg/ml concentration and 4 μl of HIV-1 protease enzyme solution in buffer (0.025 mg/ml) were added. The reaction mixture (10 μl) was incubated at 37 °C for 1 h. A control reaction was performed under the same condition, without the addition of the sample solu-

Table 2. ^{1}H NMR data of protected hexapeptides in $CDCl_3$

Peptide	Components	Chemical shift (δ, ppm)
Boc-Leu-Leu-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-Phe-OMe[a]	Boc[1]	1.40 (s, 9H, $(CH_3)_3$)
	Leu[2]	4.50 (d, 1H, $^{\alpha}$CH), 2.25 (m, 1H, $^{\beta}$CH), 1.0 (m, 3H, $^{\beta 1}$CH), 0.90 (m, 3H, $^{\gamma}$CH), 7.65 (s, 1H, NH)
	Leu[3]	4.10 (d, 1H, $^{\alpha}$CH), 1.9 (m, 1H, $^{\beta}$CH), 1.0 (m, 3H, $^{\beta 1}$CH), 1.22(m, 3H, $^{\gamma}$CH), 7.6 (s, 1H, NH)
	Glu(OBzl)[4]	3.0 (d, 1H, $^{\alpha}$CH), 1.6 (m, 2H, $^{\beta}$CH), 1.58 (t, 2H, $^{\gamma}$CH), 5.1–5.3(m, 2H, CH), 7.2–7.3(m, 5H, Ar–H), 8.0 (s, 1H, NH)
	Tyr(2,6-diClBzl)[5]	3.7 (d, 1H, $^{\alpha}$CH), 2.35 (m, 2H, $^{\beta}$CH), 5.2 (t, 2H, CH), 7.0–7.2(m, 3H, Ar–H), 8.0 (s, 1H, NH)
	Val[6]	4.60 (d, 1H, $^{\alpha}$CH), 3.0 (m, 2H, $^{\beta}$CH), 3.0 (m, 3H, $^{\beta 1}$CH), 1.4 (m, 3H, $^{\gamma}$CH), 8.0 (s, 1H, NH)
	Phe	4.9 (d, 1H, $^{\alpha}$CH), 3.2 (m, 2H, $^{\beta}$CH), 7.4(m, 5H, Ar–H), 8.0 (s, 1H, NH)
	OMe[7]	3.65 (s, 3H, CH_3)
Boc-Leu-Leu-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-Met-OMe[b]	Met	4.5 (m, 1H, $^{\alpha}$CH), 2.65(m, 2H, $^{\beta}$CH), 2.2 (m, 2H, $^{\gamma}$CH), 1.6 (s, 6H, CH_3), 8.45 (s, 1H, NH)
Boc-Leu-Leu-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-Tyr-OMe[c]	Tyr	4.6 (d, 1H, $^{\alpha}$CH), 2.85 (m, 2H, $^{\beta}$CH), 6.8–7.0(m, 5H, Ar–H), 8.35(s, 1H, NH)
Boc-Leu-Leu-Glu(OBzl)-Tyr(2,6-diClBzl)-Val-Trp-OMe[d]	Trp	5.2 (d, 1H, $^{\alpha}$CH), 3.0 (m, 2H, $^{\beta}$CH), 8.25 (s, 1H, NH), 7.2–7.35(m, 5H, Ar–H), 6.85(m, 1H, indole–H), 8.85 (s, 1H, ring NH),

The chemical shift values for the residues Boc[1], Leu[2], Leu[3], Glu(OBzl)[4], Tyr(2,6-diClBzl)[5], Val[6], and OMe[7]of hexapeptides b, c, and d were almost same as obtained for the hexapeptide a.

Table 3. HIV-1 protease inhibition study of the synthesized peptides at 100 μg/ml

Peptide	% of inhibition
Leu-Leu-Glu-Tyr-Val-Phe-OH	52.3 ± 4.3
Leu-Leu-Glu-Tyr-Val-Met-OH	47.2 ± 7.7
Leu-Leu-Glu-Tyr-Val-Tyr-OH	58.6 ± 2.5
Leu-Leu-Glu-Tyr-Val-Trp-OH	64.92 ± 4.7
Acetyl pepstatin (positive control)	98.47 ± 0.27

The results are means ± SD ($n = 3$).

tion. The reaction was stopped by heating the reaction mixture at 90 °C for 1 min. Then 20 μl of double distilled water was added and an aliquot of 10 μl was analyzed by HPLC using RP-18 column. 10 μl of the reaction mixture was injected to RP-18 column and gradiently eluted with acetonitrile (15–40%) and 0.1% trifluoro acetic acid (TFA) in water, at a flow rate of 1 ml/min. The elution profile was monitored at 280 nm. The retention times of the substrate and pNO_2-Phe bearing hydrolysate were recorded at 10.32 and 8.97 min, respectively. The inhibitory activity of HIV-1 protease was calculated as: % inhibition = $(A_{Control} - A_{Sample}) \times 100/A_{Control}$, where A is the relative area of the product hydrolysate. Acetyl pepstatin, which showed 50% inhibitory activity at a concentration of 29 μg/ml, was used as a positive control of inhibition.

Acknowledgments

The authors are grateful to UGC-SAP Phase-I, DST-FIST programmes, New Delhi, India, for financial assistance. The spectral data obtained by the instrumentation facility provided by the above funding agencies are greatly acknowledged. S.N.N.B. is grateful to CSIR, New Delhi, India, for the award of S.R.F.

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