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Inhibition of HIV-1 Protease by Novel Dipeptide Isosteres Containing 2-Isoxazoline or α-Hydroxy Ketomethylene

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Abstract—Human immunodeficiency virus type 1 (HIV-1) protease is essential for the replication of the virus and it is therefore an attractive target for antiviral drugs of HIV-1. Several dipeptide isosteres containing 2-isoxazoline or α-hydroxy ketomethylene have been synthesized and their inhibitory effects on the HIV-1 protease examined. The enzymatically active HIV-1 protease was purified to homogeniety from *E. coli* transformed with a recombinant plasmid (pMAL-pro) containing the entire gene encoding the protease. The purified protease had the substrate specificity with Km value of 9.8 μM when an undecapeptide His-Lys-Ala-Arg-Val-Leu-(p-nitro)Phe-Glu-Ala-Nle-Ser-amide was used as a substrate, and the products from the substrate after specific cleavage by HIV-1 protease were analyzed by HPLC. The synthetic compounds containing dipeptide isosteres showed specific inhibitory effects while a dipeptide isostere containing an isoxazoline ring inhibited the HIV-1 protease competitively with Ki value of 500 μM. Even if the inhibition effects of HIV-1 protease were not very high, these novel dipeptide isosteres can be used as key structural moieties for developing specific inhibitors of HIV-1 protease.

Keywords □ Human Immunodeficiency Virus type 1 (HIV-1), HIV-1 protease, dipeptide isostere, 2-isoxazoline, α-hydroxy ketomethylene.

AIDS epidemic is caused by the human immunodeficiency virus (HIV). The HIV is a member of the retrovirus family, *Retroviridae*. The viral genome possesses *gag*, *pol*, and *env* (Ratner *et al.*, 1985). The *gag* region is initially translated into a 55 kDa fusion protein, $pr55^{gag}$, that is subsequently cleaved into four structural proteins of the virion core (p17, p24, p9, p6) (Veronese *et al.*, 1986). The *gag-pol* region is expressed as a fusion product ($pr160^{gag\text{-}pol}$) by a translational frameshift between the overlapping *gag* and *pol* reading frames (Jacks *et al.*, 1985; Jacks *et al.*, 1988). This $pr160^{gag\text{-}pol}$ is posttranslationally cleaved to yield mature *gag* proteins, protease, reverse transcriptase, RNase H and endonuclease (Steimer *et al.*, 1986; Veronese *et al.*, 1986). The HIV protease is responsible for the cleavages of gag precursor and gag-pol fusion precursor so that the inhibition of the enzyme blocks maturation of the virion core and the enzymes. Therefore, the HIV-1 protease has been considered as a good target for the inhibition of the key viral replication process.

The HIV-1 protease, an aspartyl protease, consists of two identical 99-aa subunits which have a twofold (C_2) axis of symmetry (Lapatto *et al.*, 1989; Navia *et al.*, 1989; Wlodawer *et al.*, 1989). Each subunit of the enzyme contributes to half of the active site with a catalytic aspartic acid in the sequence Asp-Thr/Ser-Gly, which is conserved among all aspartyl proteases (Miller *et al.*, 1989; Navia *et al.*, 1989; Pearl *et al.*, 1987; Wlodawer *et al.*, 1989).

Various specific inhibitors of HIV-1 protease have been developed. Among them, transition-state analog inhibitors such as reduced amide (Dreyer *et al.*, 1989; Moore *et al.*, 1989; Urban *et al.*, 1992), hydroxyethylene and hydroxyethylamine isosteres (Dreyer *et al.*, 1989; Jaskolski *et al.*, 1991; Kempf *et al.*, 1990; Rich *et al.*, 1990; Roberts *et al.*, 1990; Thaisrivongs *et al.*, 1991; Tomasselli *et al.*, 1990; Vacca *et al.*, 1991), α-difluoroketone (Dreyer *et al.*, 1989), phosphinate (Grobelny *et al.*, 1990), and phenylnorstatine (Raju *et al.*, 1991) have been tested for their inhibitory effect on HIV-1 pro-

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tease most extensively. Some of them block the protease function and inhibit HIV-1 replication *in vitro* very efficiently. Recently, such inhibitors face the problem that the genetic mutations of HIV-1 produce variants resistant to the protease inhibitors during the *in vitro* culture (El-Farrash *et al.*, 1994; Ho *et al.*, 1994; Otto *et al.*, 1994). Therefore, new types of HIV-1 protease inhibitors are inevitable for the development of successful drugs.

As a new approach, we synthesized the transition-state analogs mimicking the Phe-Pro cleavage site by using 2-isoxazoline ring or α-hydroxy ketomethylene structure (Fig. 3) (Kim *et al.*, 1992; Kim *et al.*, 1993). The synthetic compounds containing these structures were tested for the inhibition effect on the HIV-1 protease. Here, we report the specific inhibitory effect of new drugs on HIV-1 protease by novel dipeptide isosteres which can be used as key structural moieties for the development of new types of inhibitors.

Materials and Methods

Materials

Isopropyl β-D-thiogalactopyranoside (IPTG), Dimethyl Sulfoxide (DMSO), Acetic acid, Nonidet P-40, and Sodium acetate were obtained from Sigma Chemical.. pMAL-cRI was obtained from New England BioLab. Acetonitrile and Methanol were the ChromAR HPLC grade and obtained from Malinckrodt. Trifluoroacetic acid (TFA) was obtained from Pierce. The substrate, His-Lys-Ala-Arg-Val-Leu-(p-nitro)Phe-Glu-Ala-Nle-Ser-amide was purchased from Bachem.. The columns and resins, DEAE cellulose, SE cellulose, Superose 12 gel filtration column, and Mono S HPLC column were obtained from Pharmacia. Dithiothreitol (DTT) was purchased from Promega.

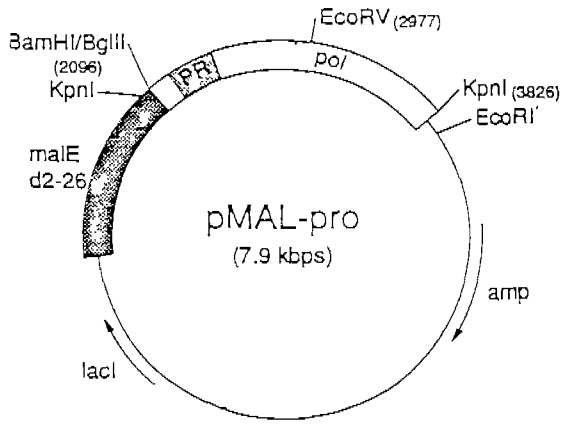


Fig. 1. The recombinant construct expressing HIV-1 protease. The numbers in parenthesis represent the nucleotide positions in HIV-1 proviral DNA sequence and PR represents the protease gene.

Expression and Purification of HIV-1 protease

The entire gene coding for HIV-1 protease was subcloned into an expression vector, pMAL-cRI, resulting in pMAL-pro (Fig. 1). The translational fusion was created between reading frames of both HIV-1 pro/pol gene and maltose binding protein gene on the expression vector. The cloned gene was expressed in *E. coli* XL1-Blue containing pMAL-pro by inducing with 1 mM IPTG for 1 hr. The expressed protease was purified to homogeniety by series of chromatographies using DEAE, SE cellulose, Superose 12 gel filtration, and Mono S HPLC columns as described previously (Kim *et al.*, 1994).

HIV-1 protease assay

HIV-1 protease was assayed by the method described previously (Danley, *et al.*, 1989) with minor modification; the substrate was an undecapeptide, His-Lys-Ala-Arg-Val-Leu-(p-nitro)Phe-Glu-Ala-Nle-Ser-amide (Bachem), mimicking the cleavage site in the natural

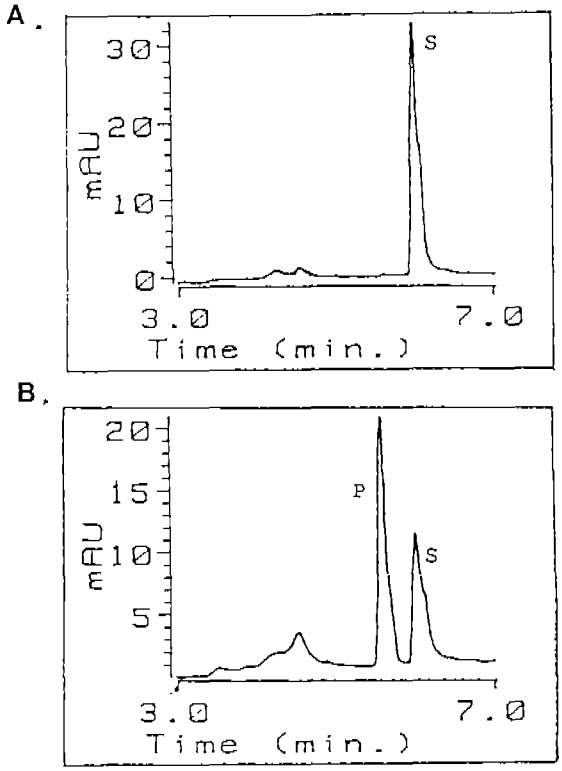


Fig. 2. The chromatograms illustating the hydrolysis of substrate peptide by HIV-1 protease. S and P represent substrate and product peaks, respectively.
(A) Substrate peak before the reaction.
(B) Product and substrate peaks after 4 min incubation with the purified protease.

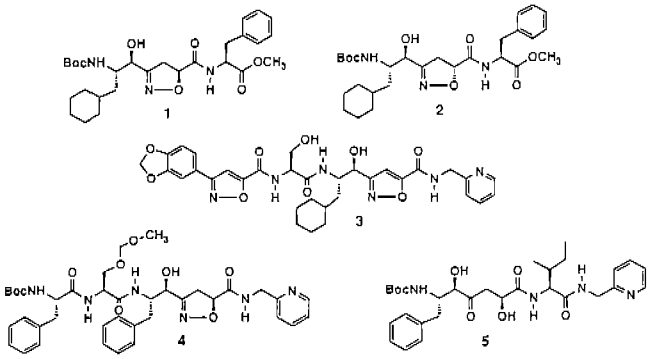
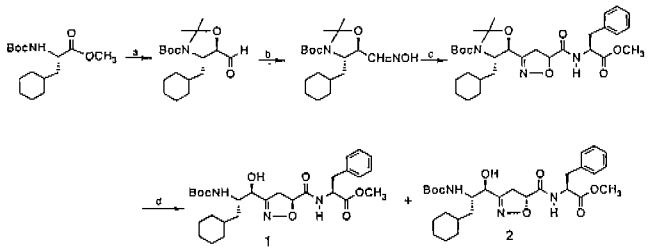


Fig. 3. Designed HIV-1 Protease Inhibitors. 1, JC5401; 2, JC 5402; 3, JC6790; 4, JC6981; 5, JC7241.

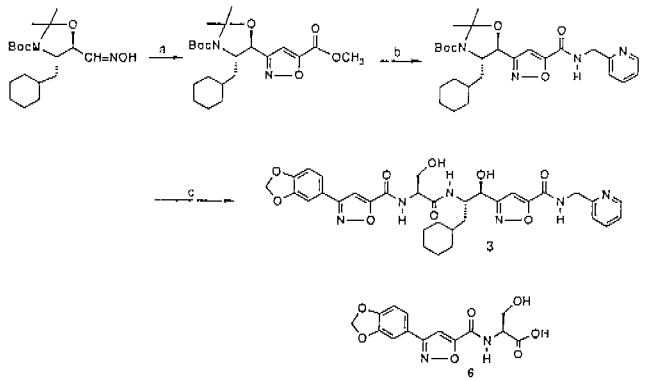


Scheme 1. a: 1) DIBAL, Toluene. 2) Vinylmagnesium bromide, THF. 3) 2-Methoxypropene, ppTs. 4) O_3, Zn-AcOH. b: $NH_2OH.HCl$, pyridine, MeOH, 79%. c: N-acryloyl Phe-OMe, NaOCl, EtOAc, 70%. d: 1) 4M HCl 2) $(Boc)_2O$, Et_3N, MeOH, 77%.

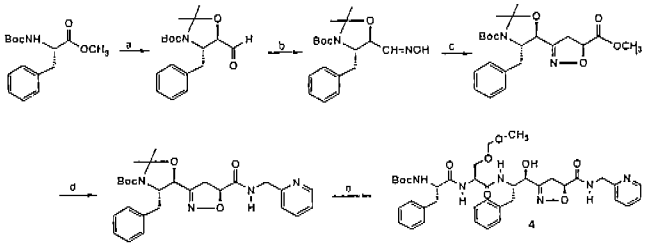
HIV-gag polyprotein in which Ala is replaced by (p-nitro)Phe and Met by Nle (Nashed *et al.*, 1989; Phylip *et al.*, 1990; Richards *et al.*. 1990). The cleavage site of the substrate is located between Leu and (p-nitro) Phe. The reaction mixture of a typical assay contained 4 μl of substrate stock (1.25 $\mu g/\mu l$ in H_2O), 5 μl protease sample, and 15 μl of a reaction buffer (0.1 M sodium acetate, 1 mM DTT, 0.25% NP-40, pH 5.5). Following incubation at 37℃, 210 μl of 10% CH_3COOH was added to stop the reaction. After centrifugation for 15 min at 10,000×g, the supernatant was analyzed by HPLC (Hewlett-Packard HP1090) on a Vydac C_{18} column (4.6 mm ID×15 cm) with a linear gradient of acetonitrile (44%/5 min) containing 0.05% trifluoroacetic acid (w/v) and a flow rate of 1.5 ml/min. Peaks from products and substrate were monitored by measuring absorbance at 280 nm in order to detect the compound containing p-nitro phenyl group (Fig. 2).

Synthesis of inhibitors and inhibition Assays

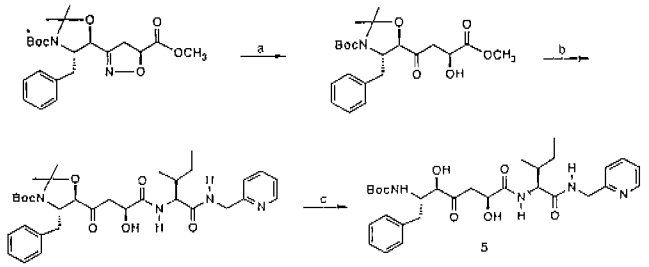
The four compounds, JC5401, JC5402, JC6790, and JC6981, containing an isoxazoline ring structure and the one compound, JC7241, containing an α-hydroxy ketomethylene dipeptide isostere were synthesized according to the schemes 1~4 as described in Fig. 3



Scheme 2. a: Methyl propiolate, NaOCL, EtOAc, 65%. b: 1) 1N NaOH. 2) 2-(Aminomethyl) pyridine, EDC. HOBt, NMM, CH_2Cl_2, 47%. c: 1) 4M HCl. 2) (**6**), EDC, HOBt, Et_3N, CH_2Cl_2, 40%.



Scheme 3. a: 1) DIBAL, Toluene. 2) Vinylmagnesium bromide, THF. 3) 2-Methoxypropene, ppTs. 4) O_3, Zn-AcOH. b: $NH_2OH.HCl$, pyridine, MeOH, 82%. c: Methyl acrylate, NaOCl, EtOAc, 84%. d: 1) 1N NaOH, 2) 2-(Aminomethyl) pyridine, EDC, HOBt, NMM, CH_2Cl_2, 62%. e) 1) 4M HCl. 2) Boc-Phe-(Mom)Ser-OH, EDC, HOBt, Et_3N, CH_2Cl_2, 42%.



Scheme 4. a: H_2, Ra-Ni, McOH/H_2O (5 : 1), $B(OH_3)$, 68%. b: 1) 1H NaOH, 2) He-Amp, EDC, HOBt, NMM, CH_2Cl_2, 79%. c: 1) 4M HCl, 2) $(Boc)_2O$, Et_3N, MeOH, 46%.

(Kim *et al.*, 1992; Kim *et al.*, 1993; Curran *et al.*, 1983). With the purified protease, the Km value was measured in the range of the substrate concentration from 5.17 μM to 77.5 μM. In case of 77.5 μM, the reaction mixture contained 15 μl of the reaction buffer, 2 μl (2.5 μg) of substrate, 1.2 μl of dimethyl sulfoxide, 4.8 μl of distilled water, and 1 μl (50 ng) of enzyme. Since there was a limit in detecting the peptide peaks by HPLC, the amount of substrate could not be lower

than 2.5 μg. With the fixed substrate amount (2.5 μg), the volumes of other components were raised to change the substrate concentration. The synthetic compound JC5402, shown in Fig. 3, was tested for the inhibitory effect on HIV-1 protease. The Ki value of JC 5402 was measured in the same way as described above using the compound dissolved in dimethyl sulfoxide. The IC_{50} values for the compounds, JC5401, JC 6790, JC6981, and JC7241, were measured in 30 μl of the reaction buffer, 4 μl of substrate (2.5 μg), 9.6 μl of distilled water, 2 μl of enzyme, and 2.4 μl of various concentrations of the putative inhibitory compounds.

Results and Discussion

The designed synthetic inhibitors were found to have similar IC_{50} values as shown in Table I. The inhibitory effects of these synthetic compounds containing either an isoxazoline ring or α-hydroxy ketomethylene were not high enough to be compared with highly efficient inhibitors which have been developed so far (Blundell *et al.*, 1990; Huff *et al.*, 1991; Kempf *et al.*, 1990; Roberts *et al.*, 1990; Tucker *et al.*, 1992). Because we wanted to develop unique and novel inhibitors containing dipeptide isosteres with either an isoxazoline ring or α-hydroxy ketomethylene structure, the inhibitory effects of two different dipeptide isostere analogs were compared. Starting with the simple lead compound of a synthetic analog equivalent to the peptide consisting of three amino acids, peptide analogs of various lengths which retain the dipeptide isostere structure were synthesized. The extra moieties other than the dipeptide isostere in the designed inhibitors have the structures which were previously reported in the structures of inhibitors showing a very efficient inhibition of HIV-1 protease (Blundell *et al.*, 1990; Huff *et al.*, 1991). Interestingly, our results showed that similar inhibitory effects were exhibited regardless of the size of the overall length of synthetic inhibitors and the structures of extra moieties introduced in the dipeptide isostere skeleton, suggesting that the region of the dipeptide isostere is important in the inhibitory action of the designed inhibitors (Table I).

Table I. IC_{50} values of the synthetic compounds

Compounds	IC_{50} (mM)
JC5401	1.3
JC6790	1.6
JC6981	1.1
JC7241	1.0

In order to characterize the nature of inhibitory action of the dipeptide isostere analogs, a synthetic compound JC5402 was subjected to a detailed analysis by the method of enzyme kinetics. As shown in Fig. 4, the Lineweaver-Burk plot suggested that the inhibition effect of JC5402 on the HIV-1 protease was competitive to Ki value of 500 μM when the undecapeptide, His-Lys-Ala-Arg-Val-Leu-(p-nitro)Phe-Glu-Ala-Nle-Ser-amide, was used as substrate. This result supports the idea that the major recognition at the active site was made in the region of the dipeptide isostere leading to similar inhibitions of the different structural synthetic inhibitors containing the dipeptide isostere structure.

In the design of the inhibitors, the proline residue in the recognition sequence of the substrates was replaced by an isoxazoline ring structure or α-hydroxy ketomethylene which is the open form of the isoxazoline ring. Either the isoxazoline ring or its open structural form of α-hydroxy ketomethylene showed similar inhibitory effect on HIV-1 protease activity (Table I). The isoxazoline ring structure which is a isostere with a conformationally restricted one gives rigidity to the overall structure in the compounds so that these inhibitors lost the flexibility. On the other hand, the structure of α-hydroxy ketomethylene analogs has more flexibility in the overall structure in the compounds, despite the intramolecular hydrogen bonding between carbonyl oxygen and hydroxyl group. Therefore, the rigidity in the dipeptide isosteres containing the isoxazoline moiety does not seem to be critical.

Despite active development of specific protease inhi-

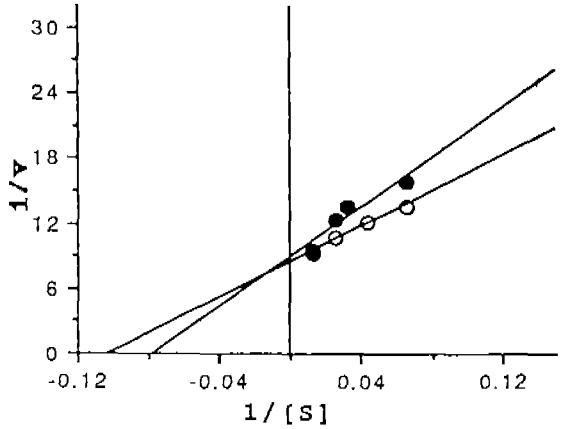


Fig. 4. The Lineweaver-Burk plot showing the inhibition effect of JC5402 on the HIV-1 protease. 1/v versus 1/[S] plot in the absence (○) and presence (●) of the inhibitor JC5402 (155 μM). The measured Km is 9.8 μM and Ki is calculated to be 500 μM. The units of x-axis and y-axis are μM^{-1} and μmol^{-1} min ml, respectively.

bitors as potential drugs to prevent AIDS, the unusual high rate of genetic mutation of HIV-1 protends that many different variants resistant to the protease inhibitors can emerge by mutating the protease gene spontaneously so that HIV-1 may escape the attack of the inhibitors (El-Farrash *et al.*, 1994; Ho *et al.*, 1994, Otto *et al.*, 1993). Many such examples have been reported in which the mutations of HIV-1 protease were made in the amino acid codons at the residue 82, 84, or 8. These HIV-1 mutants appeared from the *in vitro* culture when the virus was treated with specific inhibitors with prolonged period. Hence, for the future development of protease inhibitors preventing the HIV-1 caused diseases, the emergence of new variants resistant to the current anti-HIV drugs must be considered and the determination of potential resistance to various protease inhibitors is imperative.

Therefore we need different types of inhibitors for efficient and selective prevention of many variants of HIV-1. The dipeptide isosteres containing either isoxazoline or α-hydroxy ketomethylene can be added to the variety of other isosteres as a lead structure for the future design of specific inhibitor of HIV-1 protease. Currently, Cbz-asparagine group which is frequently included in the structures of many HIV-1 protease inhibitors has been added to the N-terminus of the dipeptide isostere and is being tested. The addition of planar peptidomimetic group to give the overall β-sheet structure is also being tried. Since the bulky group in C-terminal did not show any significant effect (Table I), these groups may be omitted or substituted by smaller groups in the future development of specific inhibitors.

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