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Design, Synthesis, and Characterization of Dipeptide Isostere Containing *cis*-Epoxide for the Irreversible Inactivation of HIV Protease.

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Abstract: A novel isostere was designed for the inactivation of HIV protease on the basis of mechanistic and structural information. Key factors of the inactivator are the *cis*-epoxide for the nucleophilic addition of the active site aspartic residue and two amide carbonyls at P2 and P1' for the formation of the hydrogen-bondings with an essential structural water molecule.

Peptidomimetic transition state analogues or structure-based designed inhibitors have been widely used for the development of HIV protease inhibitors with nanomolar or subnanomolar K_I value.[1] Those reversible inhibitors would be functionally irreversible if they bound to HIV protease so tight that their dissociation rates were so small.[2] However, the reported apparent dissociation rate constants of 5 inhibitors including quinoline-2-carbonyl-Asn-Phe-Ψ[(1R)-CH(OH)CH_2N]Pro-O-tBu were bigger than 0.1 sec^{-1}.[3] Fast dissociation rates indicate that the inhibitory effect could be maintained only by enough concentration at the drug target site. Therefore, irreversible inactivator would be more desirable in the aspect of the long time *in vivo* inhibitory efficacy which reversible inhibitors could not offer. Hereby, we report the efforts on the design of powerful irreversible inactivator on the basis of structural information as well as mechanistical information. Designed compounds inactivated HIV-1 protease successfully with time-dependent irreversible inhibition pattern, and selected compounds inactivated HIV protease with second order rate constants (k_{ina}/K_I) of 10^7 - 10^8 $M^{-1}min^{-1}$.

The development stratagy was divided into two parts: the design of dipeptide isostere for active site directed inactivation and the full structure optimization for the improvement of binding affinity toward HIV protease. The typical reaction of aspartic protease undergoes via the concerted general acid and base mechanism of the two catalytic aspartyl residues, only one of which is protonated.[4] Kinetic isotope and isotope exchange studies suggested the catalytic mechanism of HIV protease similar to other aspartic protease.[5] While protonated aspartic residue donates a proton to carbonyl at scissile peptide amides, deprotonated aspartic residue abstracts a proton from a water, which attacks the amide carbonyl carbon to make a *gem*-diol transition state intermediate in a concerted manner. The protonated and deprotonated aspartic residues assist the bond breakage of *gem*-diol intermediate by the general base and acid mechanism again. The concerted mechanism suggests that the inactivation of HIV protease is possible by the introduction of a functional group which is labile toward the direct nucleophilic addition of unprotonated aspartic residue coupled with the aid of protonated one. Indeed, it was reported that HIV protease was inactivated by 1,2-epoxy-3-(p-nitrophenoxy)propane.[6] Studies on the pH profile of the inactivation proposed that the nucleophilic attack on the epoxide by aspartic residue was responsible for the inactivation.[6] These results suggest that epoxide could be a good pharmacophore for the inactivation of HIV protease. Even though the reaction mechanism was not clear and the potency was weak,

cerulenine, an epoxide-containing inhibitor of fatty acid biosynthesis, and an epoxide-containing tripeptide analogue inactivated HIV protease with time-dependent irreversible pattern.[7,8]

Another key concept in designing inactivator came from the published X-ray crystal structure of HIV protease-inhibitor complexes. Two amide carbonyls of P2 and P1' are bridged by two hydrogen-bondings with a water molecule, whose oxygen atom is also hydrogen bonded with backbone amide hydrogens of Ile50 and Ile50' at so-called flap region.[9] We regarded these hydrogen bondings with a tetracoordinated water as the crucial factor for catalysis and inhibition by stabilizing the transition state intermediate and analogues, respectively. Therefore, a proper isostere should contain the epoxide for irreversible inactivation and two well-fitting carbonyls which are able to make a water bridge by two hydrogen-bondings (Fig. 1).

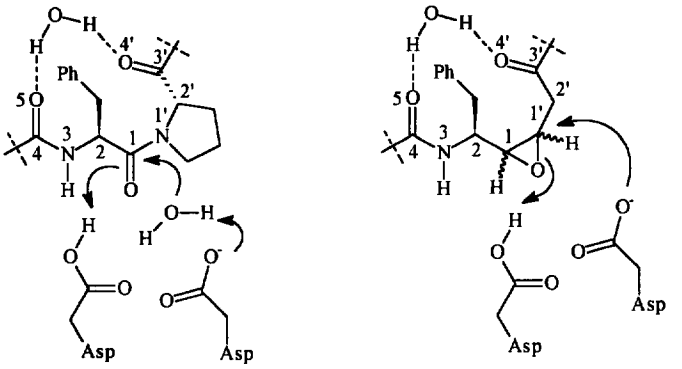


Figure 1: Two dimensional model for the inactivation of HIV protease (right) based on the substrate hydrolysis mechanism (left). Fundamental features are the alkylation at the active site aspartic residue by the nucleophilic attack, and the hydrogen-bondings with an essential structural water molecule.

Two possible structures, one with *trans*-epoxide and the other with *cis*-epoxide, were our starting-point on the basis of the above hypothesis and the substrate structure. The structures of designed dipeptide isostere were rather rigid,[10] which enabled us to fit their minimum energy structures into the published 3-dimensional protease-inhibitor complex structures without major conformational change. The minimum energy structure was obtained using SYBYL program, and docking experiments were carried out using FRODO program. This intuitive modeling suggested that proper positioning of two carbonyls for the tetracoordinated water bridge made either (R,R) for *trans*- or (R,S) for *cis*-epoxide preferable to the other two isomers in the sense of steric interference (Fig. 2). In addition, those epoxides were positioned close enough to aspartic residues for chemical reaction. In case of *trans*-epoxide, the formation of the water bridge located epoxide oxygen at ideal distance for hydrogen-bondings with the oxygens of two aspartyl residues. However, the epoxide oxygen was facing the carboxyl oxygens of two aspartyl residues. In this situation, only the stepwise reaction, epoxide-opening before covalent bond formation, is possible rather than the concerted reaction. On the other hand, in case of *cis*-epoxide, the basic aspartyl residue could be positioned to serve as a nucleophile, and the protonated residue to donate a proton to the leaving group epoxide oxygen at the opposite side for the concerted reaction. According to the proposed mechanism, a water molecule activated by aspartyl carboxylate hydrolyzes the peptide bond.[5] The ring opening of the epoxide to diol would be expected based on this mechanism. However, our model suggested that the proper positioning of two carbonyls for a water bridge would not allow enough space between epoxide ring and two aspartyl residues to accomodate a water molecule.

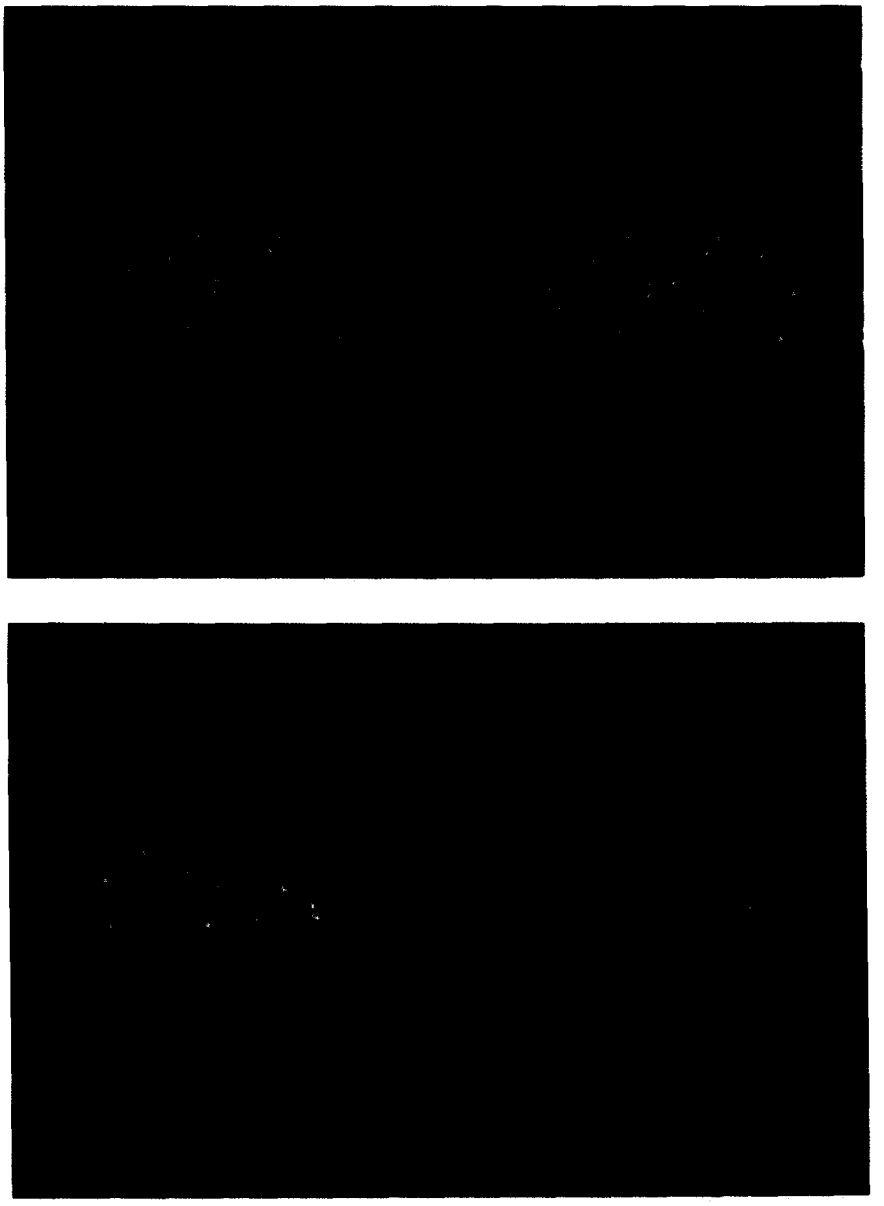
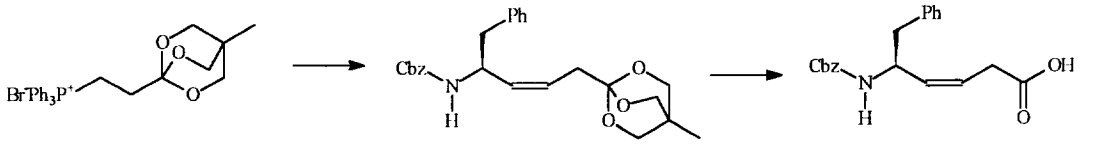


Figure 2. Stereoviews of the binding model of the designed isostere to HIV protease. Tetracoordination of an essential water molecule with isoleucine 50, 50', and P2, P1' carbonyls and location of benzyl group at S1 pocket render *cis*-epoxide with (R,S)-configuration (up) and *trans*-epoxide with (R,R)-configuration (down) close enough for the nucleophilic attack of aspartic residue without steric interferance.

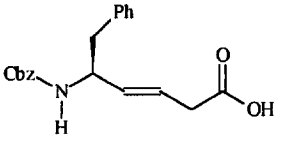
Analogue containing *trans*- and *cis*-isosteres were synthesized by the following route.



2 **3** **1**

Three carbon phosphonium salt **2** was prepared in three steps according to the modified methods of Corey and Raju, and Keinan *et al.*[11] The Wittig reaction of phosphonium salt **2** with cbz-L-phenylalanal using potassium bis(trimethylsilyl)amide in THF at -78°C to room temperature gave **3** in 91% yield without racemization (Z:E ≥ 99:1). Efficient hydrolysis of ortho ester **3** without double bond migration was achieved by acidic hydrolysis with aq. HCl in t-butanol under reflux condition.

trans-Olefinicacid **4** was synthesized according to the method of Johnson.[12]



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Compounds **5** and **6** in Table 1 were synthesized from olefinicacid **1** and **4** via amide coupling reaction with corresponding methyl ester of amino acids using EDC/HOBT followed by epoxidation reaction with mCPBA. The epoxidation was stereoselective toward the desired configuration ((R,S):(S,R) = 4-10:1for *cis*-epoxide and (R,R):(S,S) = 4-10:1 for *trans*-epoxide).[13] These diastereomers were easily separated by column chromatography. Compound **8** was prepared by the cbz-deprotection using Pd/C followed by amide coupling reaction with 2-quinolinecarbonyl-asparagine.

As shown in Table 1, compound **5** containing *cis*-epoxide inhibited protease strongly with time-dependent irreversible pattern (K_I of 1.32 μM and k_{ina} of 1.48 min^{-1}). In spite of time-dependent irreversible pattern, compound **6** in which *cis*-epoxide of compound **5** was replaced by *trans*-epoxide showed much poorer activity (K_I of 200 μM and k_{ina} of 0.20 min^{-1}). Methylene group between epoxide and the carbonyl group corresponding to the α-carbon of P1' site seems to be essential for the proper geometry of a water bridge (Fig. 1 and Fig.2). As shown in compound **7**, the absence of the methylene group reduced the inhibition activity drastically. Therefore, dipeptide isostere containing *cis*-epoxide, PheΨ[(R,S)-*cis*-epoxide]Gly, was chosen as our mother isostere. Then, various natural amino acids, and functionalized N- and C-terminal protecting groups were introduced at the sites corresponding to the binding pockets of HIV protease. In the round of optimization, compound **8** and **9** gave the best inactivation with k_{ina} values of 0.20-0.23 min^{-1} and K_I values of nanomolar range. The extensive dialysis after inactivation of enzyme could not revive the enzyme activity. The time-dependent inactivation of the protease by 50 nM compound **8** was partially blocked in the presence of 500 nM N-acetylpepstatine, a competitive inhibitor with K_I of 20 nM, as evidenced by 90% decreased k_{obs}. These results showed that compound **8** was the active-site directed inactivator. In addition, active site titration using 0.1-1 molar equivalent inhibitor over the concentration of protease fitted data to 1:1 stoichiometric binding of inhibitors to enzyme (Fig. 5), which indicated that in partitioning only aspartic carboxylate attacked the epoxide, not activated water.

Table 1. Enzyme inhibition constants of various compounds. Kinetic constants were obtained as previously described.[14]

compound	structure	$K_I(\mu M)$	$k_{ina}(min^{-1})$
5	Cbz-PheΨ[(R,S)-*cis*-epoxide]Gly-Val	1.32	1.48
6	Cbz-PheΨ[(R,R)-*trans*-epoxide]Gly-Val	200	0.20
7	Cbz-PheΨ[(R,R)-*cis*-epoxide-C(=O)]Val	>10000	0.02
8	Qc-Asn-PheΨ[(R,S)-*cis*-epoxide]Gly-Ile	0.018	0.22
9	Qc-Asn-PheΨ[(R,S)-*cis*-epoxide]Gly-NH-CH(isopropyl)$_2$‡	~0.001	0.20

‡ Qc = 2-quinolinecarbonyl

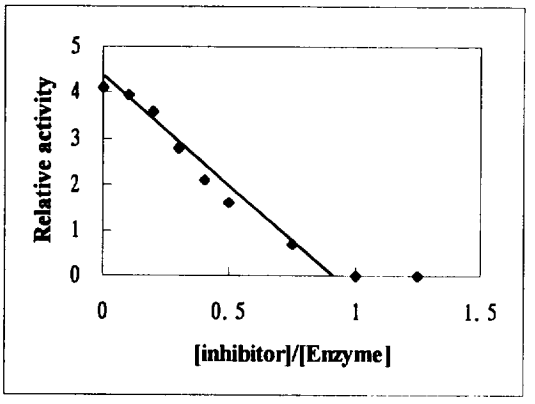

Figure 5. Stoichiometry of inactivation of HIV protease by compound **8**. 0.1-1.0 Molar equivalent inhibitor over 100 nM enzyme were incubated for 30 minutes under the mentioned buffer condition. The remaining activities were measured and plotted against the initial concentration ratio of the inhibitor over the enzyme. The experimental data points were fitted to v_i/v_o(relative activity) = 1 - [compound **8**]/[HIV protease]

Chymotrypsin, digestive serine protease, papain, nonselective cystein protease, and other aspartic protease such as human renin and human cathepsin D were tested as controls. Chymotrypsin, papain, and human renin were not inactivated by 10 μM compound **9**.[15] Human cathepsin D was inhibited by compound **9** reversibly, and IC_{50} value was 12.5 μM.[15] In conclusion, we have shown that compounds containing isostere, PheΨ[(R,S)-*cis*-epoxide]Gly, inactivated HIV protease with high selectivity.

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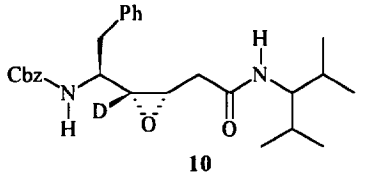
10. Solid state deuterium nuclear magnetic resonance experiments on the compound V showed a pake pattern with full width and long T1, which were major characteristics of slow motion of molecules. Therefore, the local motion of the compound **10** is slow and its structure is rigid.



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15. Chymotrypsin, papain, and their substrates were purchased from Sigma Chemical Co. Human renin inhibition was studied by radioimmunoassay of released angiotensin I using human blood as renin source and RENIN-RIABEAD kit (Abbott Diagnostics). For human cathepsin D inhibition, cleavage reaction between (p-NO_2)-Phe and Phe of Boc-Phe-Ala-Ala-(p-NO_2)-Phe-Phe-Val-Leu-4-(hydroxymethyl)pyridine ester (BACHEM Chemical Co.) by human cathepsin D was followed at pH 3.4 using HPLC according to the method of Marossy, K.; Rich, D. H. *Anal. Biochem.* **1983**, *130* 158. At pH 3.4, compound **9** was stable after 3 days' incubation under the used buffer condition.

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