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RATIONAL DESIGN OF IRREVERSIBLE, *PSEUDO*-C2-SYMMETRIC HIV-1 PROTEASE INHIBITORS

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Abstract. *Pseudo*-C_2-symmetric irreversible inhibitors of HIV-1 protease containing *cis*-epoxide were designed, synthesized, and kinetically characterized. Introduction of a Gly residue into P_1' of the inactivators yielded strong time-dependent irreversible ones with k_{ina}/K_i ranging from 1.5×10^8 to 3.4×10^5 $M^{-1}min^{-1}$.

The human immunodeficiency virus encodes a protease which is responsible for the processing of polyprotein products of the *gag* and *pol* genes into their mature forms.[1] Noninfectious viral particles with immature morphology and dramatically reduced reverse transcriptase activity are formed when the retroviral protease is mutated.[2] Also, cell culture studies using inhibitors of HIV protease have established that this enzyme is essential for viral replication and infectivity.[3] Therefore, chemical inhibition of this critical viral enzyme is regarded as a promising approach for the treatment of AIDS and related diseases.[4]

The enzyme is an aspartyl protease comprising a highly conserved domain Asp-Thr-Gly at the active site and functions as a C_2-symmetric homodimer.[5,6] As the native enzyme can adopt a conformation in which a two-fold symmetry axis extends through the active site, it might also be desirable to include the C_2 symmetry through the center of the inhibitors. As was suggested by Kempf and his coworkers,[7] symmetric inhibitors are expected to exhibit high specificity for HIV protease over other aspartic proteases whose substrate binding sites are less symmetric. In addition, as symmetric inhibitors are less peptide-like than the peptide-based inhibitors using the natural substrate sequences in conjunction with the transition state analogue concept, the stability of symmetric inhibitors *in vivo* would be higher than that of non-symmetric ones.

With this in mind, another important factor was taken into account for the design of inhibitors; that is, irreversibility. Although many transition state analogues have been developed with success so far,[8] their inherent reversible characters gave us a question about their long term efficacy *in vivo*. Meek and his coworkers demonstrated that 3-(4-nitro)phenoxy-1,2-epoxypropane (**EPNP**) irreversibly inactivated HIV-1 protease with a maximal inactivation rate, k_{ina} of 0.004 min^{-1} and K_i of 11 mM.[9] They incorporated irreversible feature of **EPNP** into tripeptide analogues to obtain irreversible tripeptidyl inhibitors with k_{ina} of 0.31 min^{-1} and K_i of 20 μM, of which activities were still not good enough.[10] In an effort to develop epoxide-containing irreversible inhibitors with high potency, we utilized the concept of symmetry for the design of novel inhibitor structures.

The designed compound **3** was prepared from L-phenylalanine as shown in Scheme 1.
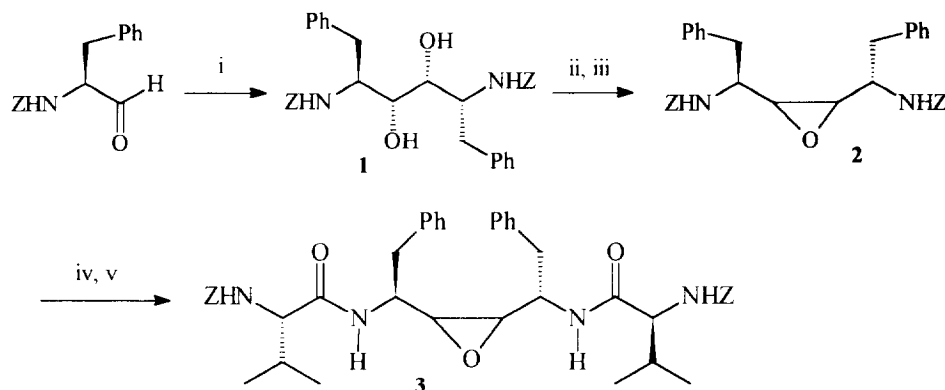
Scheme 1. Reagents i) 1.1 eq. VCl$_3$(THF)$_3$, 0.6eq. Zn; ii) MsCl, pyridine; iii) K$_2$CO$_3$, methanol;
iv) H$_2$, Pd/C, methanol; v) Z-L-valine, EDC, HOBT, DMF.

Homocoupling of cbz-L-phenylalanal was performed by Pederson's method[11] to give 1 in high yields. The key intermediate 2 was obtained from 1 by mesylation followed by cyclization. Coupling of the deprotected diamine with cbz-L-valine afforded the target compound 3.[12]

Compound 3 worked as a reversible inhibitor on the enzyme with K$_i$ of 75nM, which was 300-fold less potent than Abbott's C$_2$-symmetric compound 14, which means that active site aspartates did not attack epoxide ring. In addition, the nucleophilic attack on the epoxide ring by the catalytic water molecule did not occur, which can be inferred from the following observations: If the catalytic water molecule attacks the epoxide ring, the resulting compound would be diol compound 14. In that case, 3 should be converted to 14 after long period of time and observed K$_i$ value of 3 should be same as that of 14. After preincubation of 3 with HIV-1 protease, however, new species such as 14 has not been detected and K$_i$ value of 3 was 300-fold higher than that of 14. The lower potency of 3 compared to 14 could be interpreted as the rigidity of the epoxide ring. In compound 14, the dihedral flexibility involving rotations about the inner three bonds enabled the hydroxyl groups to displace the catalytic water molecule and make hydrogen bonds with the two active site aspartates. Compared with the diol isostere, the epoxide ring in compound 3 was so rigid that the overall conformation of 3 should be totally different from the active conformation of 14, which suggested that P$_1$', P$_2$' and P$_3$' in 3 would be in a different orientation from that of 14. Also, the absence of hydrogen bonding of the epoxide ring with the two aspartyl groups in contrast to the hydroxyl groups in 14 could be another reason for the lower potency of 3 compared to 14.

To overcome this problem, the phenylalanine at P$_1$' was replaced for glycine for two reasons: First, computer modeling based on the X-ray structure of enzyme-14 complex suggested that the removal of P$_1$' benzyl side chain make 3 in the arrangement that the epoxide ring could make a direct contact with the two active site aspartates without interference. If that is the case, new designed compound would exhibit irreversible character. Second, the greater importance of the P region than the P' region was previously observed for the binding of renin inhibitors[13] and a similar observation was noted for the asymmetric peptidomimetic inhibitor complexes of HIV protease.[14]

A representative compound 8 was prepared from so-called en-acid[15] 4 as shown in Scheme 2. EDC coupling of en-acid 4 with ammonium chloride afforded carboxamide in good yields, which was subjected to Hofmann rearrangement[16] with [bis(trifluoroacetoxy)iodo]benzene to give so-called en-amine 5 as a TFA salt.

Cbz protection followed by epoxidation with mCPBA gave the key intermediate 7 as predominantly one isomer. The stereochemistry of 7 was assigned in accordance with literature precedent.[17] Coupling of the deprotected diamine with cbz-L-valine by the EDC/HOBT method afforded the target compound 8.
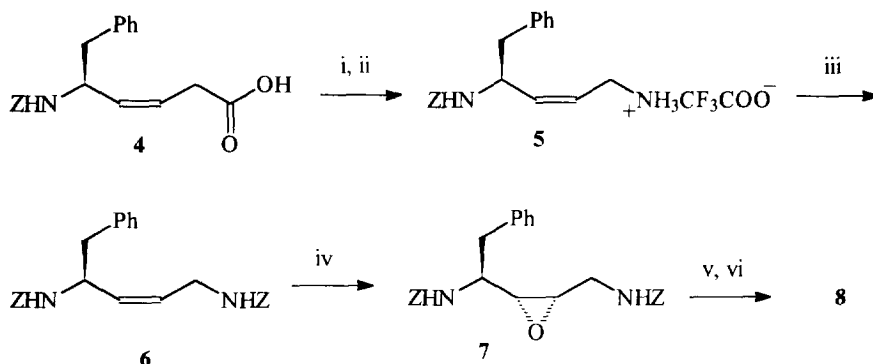
Scheme 2. Reagents: i) NH$_4$Cl, EDC, HOBT, DMF; ii) PhI(OCOCF$_3$)$_2$, CH$_3$CN; iii) ZCl, Et$_3$N; iv) mCPBA, CH$_2$Cl$_2$; v) H$_2$, Pd/C, MeOH; vi) Z-L-valine, EDC, HOBT, DMF.

As was expected, the resulting compound 8 displayed rapid, time-dependent inactivation of HIV-1 protease following pre-incubation of various concentrations of 8 with the enzyme at different time intervals. The calculated bimolecular rate constant for the formation of HIV-1 protease-8 complex value of k_{ina}/K_i was 1.5×10^8 M^{-1}min^{-1} compared with 0.36 M^{-1}min^{-1} for EPNP and 1.56×10^4 M^{-1}min^{-1} for tripeptidyl inhibitor developed by Grant et al.[10] The enzyme activity was not recovered after exhaustive dialysis of the above inactivated enzyme, which indicated that the inactivation was irreversible. In addition, the active site titration studies using 0.1-1 molar equivalent of 8 over the concentration of the protease indicated 1:1 stoichiometric binding ratio of inhibitors to the protease. Therefore, inactivation of HIV-1 protease by 8 involves enzyme-catalyzed alkylation of the unprotonated active site aspartyl residue, in analogy to the inactivation of simian immunodeficiency virus by EPNP.[19] Also, the covalent bond formation could be inferred from the X-ray crystallographic results[20] of the HIV-1 proteinase with another epoxide-containing irreversible inhibitor of our lab.

The compounds in Table 1 were prepared by the similar method to Scheme 2. It is interesting to note that the introduction of pyridylmethyl carbamate or pyridylmethyl urea group between P$_3$ and P$_2$, and P$_2$' and P$_3$' in compound 9, 10, 11 and 12 resulted in more than 100-fold decreases in the inhibitor potency in contrast to the Abbott's diol analogues.[21] This can be explained by the conformational change again. If the active conformation of 8 was different from that of 14 due to the rigid epoxide, the trends in the inhibitory potencies of 8 to 12 should be different from those of Abbott's diol analogues. Also, if the active conformation of 8 was similar to that of 14, same was the case due to the following reason: as was reported by Hosur,[22] compound 15 has the stacked arrangement of the P$_3$/P$_3$' pyridyl groups with Arg8/108 guanidinium side chain of the enzyme, which is partially stabilized by an induced dipole electrostatic interaction. This interaction is stabilized further by the orthogonal interaction of P$_1$/P$_1$' phenyl ring with P$_3$/P$_3$' pyridyl ring. The absence of phenyl ring at P$_1$' in compound 9 to 12 forbade them to have the same arrangement as 15. Therefore, they should exhibit different

trends in the inhibitory potencies from the Abbott's diol analogues. Another interesting point is that phenylalanine (compound **8**) was better than cyclohexylalanine (compound **13**) for P_1 site.

The 50% inhibition constant (IC_{50}) of **8** was 30 nM against HIV-1 in H9 and Sup T1 cell lines as assessed by syncytium formation and reverse transcriptase assay. In the assay system used here, the IC_{50} for AZT and Ro-31-8959 were 50 nM and 20 nM, respectively. The 50% cytotoxic value (CT_{50}) of **8** to the host cell lines was higher than $100\mu M$, indicating a high therapeutic index.

Thus, the design of **8** was successful in that it produced an inhibitor with high affinity and irreversibility. Studies of partitioning ratio and exhaustive dialysis suggested that the inactivation of HIV-1 protease by **8** should be due to covalent modification of an active-site residue. Studies are in progress to assess the potential of this novel compound as a chemotherapeutic agent for the treatment of AIDS.

Table 1. Structure and Potency of HIV Protease Inhibitors.[a]

Compound	X	Y	K_i (nM)	k_{ina}/K_i ($M^{-1}min^{-1}$)
8	O	phenyl	0.1	1.5×10^8
9	O	2-pyridyl	11	2.3×10^7
10	O	3-pyridyl	70	1.4×10^6
11	O	4-pyridyl	300	3.4×10^5
12	NCH_3	2-pyridyl	16	2.2×10^7
13[b]	O	phenyl	4	4.0×10^7
14	Z-Val-PheΨ[CH(OH)CH(OH)]Phe-Val-Z			$K_i < 1$ nM
15	2-pyridyl-CH_2-N(Me)CO-Val-Phe			
	Ψ[CH(OH)CH(OH)]Phe-Val-OCN(Me)-CH_2-2-pyridyl			$K_i < 1$ nM

[a] Z, benzyloxycarbonyl.
[b] P_1 site was cyclohexylalanine.

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18. Determination of Inhibition Constant for the Irreversible Inhibition. Crystallizable grade HIV-1 protease and substrate III (H-His-Lys-Ala-Arg-Val-Leu-(p-nitro)-Phe-Glu-Ala-Nle-Ser-NH₂) were purchased from BACHEM Chemical Co. (Bubendorf, Switzerland). To a buffered solution (A) comprising 50 mM sodium acetate, pH 5.5, 1 mM dithiothreitiol (DTT), 1 mM EDTA, 0.75 M (NH₄)₂SO₄ 0.2 M NaCl, and 0.1% NP-40 (NONIGET P-40; Sigma Chemical Co., U.S.A.), was added various concentrations of inhibitor. Inhibition reaction was started with the addition of 2.6 nM of HIV-1 protease to the preincubation solution. Each 10 μL of the reaction solution was taken at a given time interval and added 80 μL of assay solution containing 100 μM of reaction substrate III (K$_M$=20μM) in the buffer solution (A) to detect the residual activity. The substrate III consisting 11 amino acids was cleaved into two oligopeptides by the breakage of amide bond between (p-nitro)-Phe and Leu upon the attack of HIV-1 protease. The reaction rate was determined by the HPLC separation of the substrate and the product and then the relative amount of the product was determined using absorbance of (p-nitro)-Phe at 280 nm. HPLC were performed under a gradient condition on a reverse phase Nova-Pak ODS C18 column. The mobile phase was a water (solvent A) and 80% acetonitrile (solvent B) system containing 0.1% trifluoroacetic acid. The gradient of solvent B was increased from 10% to 100% for 20 min with retention of 55% solvent B from 10 to 15

min, and the flow rate was 1.5 mL/min. The amount of reduction in enzyme activity according to the elapsed time were measured and the natural logarithmic (ln) of the measured amounts were graphed against time to obtain a linear graph, and k_{obs} was calculated from the slope of the graph. The inhibition constant was calculated according to the following equation:

$$\frac{1}{k_{obs}} = \frac{1}{k_{ina}} + \frac{K_i}{k_{ina}} \cdot \frac{1}{[I]}$$

The above equation was applicable to an experiment carried out under the condition where the concentration of inhibitor was far higher than that of enzyme (Steady State Condition). In case that the concentration of inhibitor and enzyme were not high enough due to the superior inhibition effect of the inhibitor, the mechanism equation of

$$E + I \; \underset{}{\overset{K_i}{\rightleftharpoons}} \; EI \; \overset{k_{ina}}{\longrightarrow} \; EI$$

(where in E means the free enzyme concentration; I means the free inhibitor concentration; EI means a Michaelis-Menten complex; and EI' means a complex having covalent bond between the enzyme and the inhibitor) was used to calculate the relative concentration of active enzyme, i.e. $[E]/([E]+[EI]+[EI'])$ in every given time. The inhibition constants K_i and k_{ina} and the second rate constant k_{ina}/K_i were obtained by inputting the value of $[E]/([E]+[EI]+[EI'])$ into KINSIM/FITSIM program (Williams and Morrison; Zimmerle and Frieden). This method was applicable because the binding ratio of inhibitor to enzyme was 1:1.

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