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Impact of Stereochemistry on Ligand Binding: X-ray Crystallographic Analysis of an Epoxide-Based HIV Protease Inhibitor

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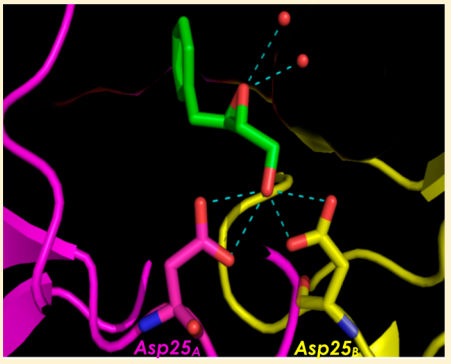
Supporting Information

ABSTRACT: A new pseudopeptide epoxide inhibitor, designed for irreversible binding to HIV protease (HIV-PR), has been synthesized and characterized in solution and in the solid state. However, the crystal structure of the complex obtained by inhibitor−enzyme cocrystallization revealed that a minor isomer, with inverted configuration of the epoxide carbons, has been selected by HIV-PR during crystallization. The structural characterization of the well-ordered pseudopeptide, inserted in the catalytic channel with its epoxide group intact, provides deeper insights into inhibitor binding and HIV-PR stereoselectivity, which aids development of future epoxide-based HIV inhibitors.

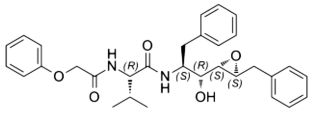


KEYWORDS: *Epoxide inhibitor, HIV protease, irreversible inhibition, stereochemistry, structure-based drug design*

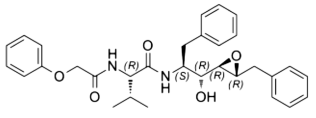
In the treatment of AIDS patients, the so-called highly active antiretroviral therapy (HAART) is based primarily on a cocktail of inhibitors of the reverse transcriptase and aspartic protease of HIV (HIV-PR). HAART has proven to dramatically improve the quality of life of AIDS patients even for critically HIV-infected subjects.[1] However, viral strains that have mutated enzymes less sensitive to the inhibitors may survive the treatment. Viral drug resistance, resulting from mutations of the genes that code for the target enzymes, limits the effectiveness of HAART in an increasing number of patients. Ten first and second generation reversible HIV protease inhibitors (PI) have been approved by the FDA to date.[2,3] All these drugs do not covalently bind to the protease and may undergo loss of activity toward resistant PR mutants in different ways.[3−6]

Irreversible inhibitors could represent a valuable tool in the development of new generation drugs capable of overcoming resistance, as covalent binding may be less sensitive to mutations that decrease the enzyme's affinity for the inhibitor. The catalytic Asp25 residues of HIV-PR must be conserved in all functional mutants[7] and thus are logical targets for irreversible inhibitors. However, irreversible binding in HIV-PR inhibition represents a relatively new chemical expedient. Irreversible inhibition of HIV-PR by EPNP [1,2-epoxy-3-(*p*-nitrophenoxy)propane], a specific inhibitor of aspartyl residues,[8,9] has been reported,[10] and covalent binding has been confirmed by mass spectrometry.[11] Other epoxide inhibitors have been synthesized showing milli- to nanomolar inhibition.[12−15] The main problems in targeting the inhibitor to the Asp25 residues are the low nucleophilicity of aspartate that needs a strongly electrophilic alkylating counterpart in the ligand molecule[16] and the geometrical issues related to the topology of the active site.

The three-dimensional structures of wild-type HIV-PR and several drug-resistant mutants bound to various inhibitors have been obtained by X-ray crystallography.[17,18] Structural information about protease−inhibitor complexes is of paramount importance to study specific HIV-PR/inhibitor interactions and to define the binding efficiency and mechanism of selective drugs. Structure-based drug design may then lead to new, improved inhibitors with increased potency[19] or to the discovery of new lead compounds.[20] Recently, we have used high-resolution X-ray crystallography as a powerful tool to identify the most potent HIV-PR inhibitor in an epimeric mixture.[21] Here, we report the synthesis and structural characterization of two new epimeric inhibitors (**1a** and **1b**) containing an epoxyalcohol moiety within a Phe-Phe pseudodipeptide unit.



1a



1b

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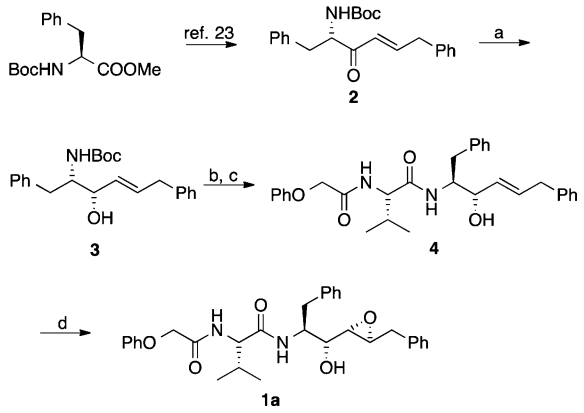
On the basis of our previous work on *trans*-epoxide inhibitors,[16] compound **1a** was designed to target the S_1-S_3' subsites of the enzyme's catalytic site. The inhibitor's P1−P1′ core mimics a Phe-Phe dipeptide; the *R* configuration of the OH group has been found optimal in similar compounds,[22] and the stereochemistry of the epoxide reflects synthetic accessibility (*vide infra*). Proper placement of the molecule in the active site should allow nucleophilic attack on the epoxide by one of the catalytically active aspartyl residues, resulting in ring opening and covalent binding of the inhibitor. Compound **1a** was synthesized from the known enone **2**, derived from phenylalanine,[23] as shown in Scheme 1.

Scheme 1. Synthesis of Inhibitor 1a[a]



[a]Reagents and conditions: (a) L-selectride, THF, −78 °C, diastereoselectivity 9:1; 77% after chromatographic separation; (b) TFA, dichloromethane, 25 °C; (c) POA-Val-OSU, NEt_3, dichloromethane, 96%; (d) mCPBA, dichloromethane, 25 °C, 62%, d.e. > 90%.

L-Selectride reduction[24] of **2** gave a 9:1 mixture of *syn* aminoalcohol **3** and its anti isomer, from which pure **3** was readily obtained by column chromatography. Boc-deprotection and coupling with the *N*-succinimidoyl ester of phenoxyacetylvaline (POA-Val-OSU)[16] was then followed by syn epoxidation[25] of the allylic alcohol **4** giving inhibitor **1a**, in which the phenoxyacetyl group and valine side chain are directed at the enzyme's S3′ and S2′ subsites, respectively. No trace of the (*R*,*R*)-epoxide **1b**, resulting from anti epoxidation of the allylic alcohol, was detected at this stage (see Supporting Information), in agreement with previous findings in the epoxidation of structurally related compounds, and the structure of **1a** has been confirmed by X-ray crystallography (Figure 1A).

In a standard fluorimetric assay, epoxide **1a** inhibited wild-type HIV-PR with a 670 nM IC_{50} (see Supporting Information). However, when **1a** was incubated with the protease, no evidence was found for time-dependent, irreversible inhibition, nor could any covalently modified protease be detected by electrospray ionization mass spectrometry (ESI-MS). Thus, to obtain information on the mode of binding of **1a** to the protease and on the mechanism of inhibition, we solved the crystal structure of the epoxide inhibitor bound to HIV-PR.

Orthorhombic single crystals of the protease−inhibitor complex diffract at 1.45 Å (see Supporting Information), and the electron density maps show a single, ordered inhibitor molecule in the active site of HIV-PR (Figure 1B). As expected from the inhibition data, the crystallographic analysis reveals the intact epoxide ring in the complex with the protease. However, much to our surprise, the configuration of the epoxide carbons in

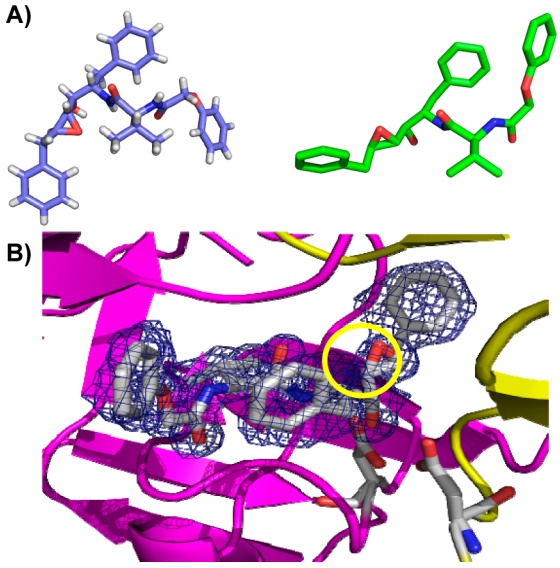


Figure 1. (A) Comparison between the crystal structures of pure **1a** (left) and **1b** as found in the complex with HIV-PR. (B) HIV-PR/**1b** complex electron density $2F_o - F_c$ (1.0 sigma level) in the active site. The epoxide ring is highlighted in the yellow circle.

this complex is *R*,*R*, as in **1b**, and not *S*,*S*, as in **1a** (Figure 1). There is no reasonable mechanism by which **1a** can be converted into **1b**; we must thus conclude that a small amount of **1b** was present in the sample, resulting from anti epoxidation of the alkene **4** (Scheme 1) and was selected by the protease.

Even if the stereochemistry of the HIV-PR bound inhibitor is not as expected, the analysis of this structure, the first for an epoxide-based inhibitor, allows for a better understanding of the binding mode and offers suggestions for improving the inhibitor's design.

Figure 1B shows that, despite the short length of the inhibitor, which can fill asymmetrically only four of the six subsites present in the catalytic channel (corresponding to the recognition of the six amino acid residues of natural substrates), the inhibitor is correctly located with the epoxide group very close to the catalytic center. The analysis of the crystal structure further shows the flap water[26] bridging contacts between the inhibitor's core and the flap tips (Ile50 residues) within the S_1 and S_1' subsites (Figure 2A).

The inhibitor leaves two empty subsites (S_2 and S_3) filled with water molecules (Figure 2B). The hydroxy group present in the inhibitor's core is hydrogen bonded to the catalytic Asp25 residues, a common feature in HIV-PR complexes,[26] contributing to successfully placing the inhibitor in the active site, with the epoxide ring close to the catalytic center. Figure 3 summarizes the hydrogen bond pattern and hydrophobic interactions in the HIV-PR/**1b** complex. Figure 2B clearly shows the water-filled S_2 and S_3 subsites and suggests that hydrophobic substituents on the aromatic ring adjacent to the epoxide might displace the water molecules and improve the inhibitor's binding affinity by making favorable contacts with the protein.

As to the selection of the *R*,*R* **1b** epoxide in the crystals, which appears to be preferred with respect to the main product *S*,*S* **1a**, an overlay of the crystallographic structure of the HIV-PR/**1b** complex and of a docked model of the HIV-PR/**1a** complex is reported in Figure 4.

It can be seen that the epoxide oxygen is flipped down due to the reversed configuration of the oxirane carbons; moreover, the

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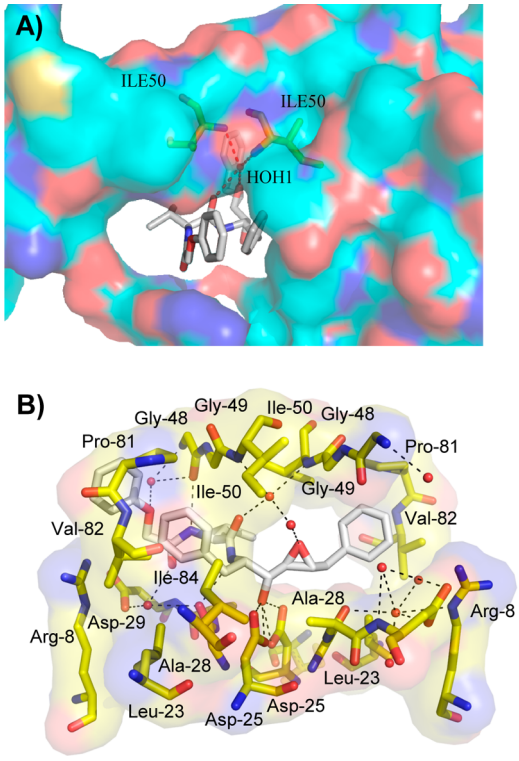

Figure 2. (A) Flap water (red sphere) in the S_1–S_1' pockets of the catalytic site. (B) Water molecules (red spheres) filling S_2–S_3 pockets of the catalytic site.

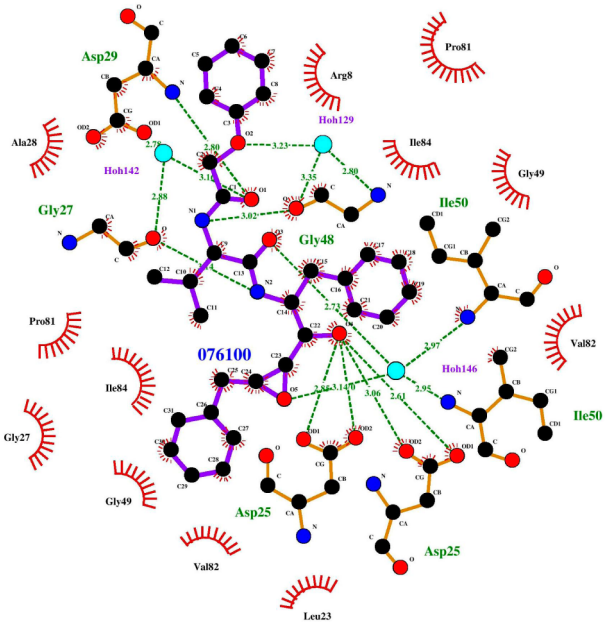

Figure 3. LIGPLOT of hydrophobic and polar contacts between **1b** and amino acid residues in HIV-PR.

benzyl group linked to the epoxide is unable to fit properly inside the S1 subsite. This leads to the loss of several hydrophobic contacts found in the HIV-PR/**1b** complex and supports the hypothesis that **1b** might have been selected thanks to a higher affinity. The same phenomenon was earlier observed in competitive crystallization experiments where the more potent inhibitor occupies the active site of HIV-PR in the crystal, even

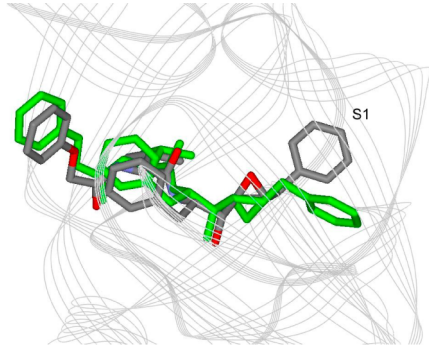

Figure 4. Overlay of the experimental structure of the HIV-PR/**1b** complex and of the docked model for the HIV-PR/**1a** complex (in green).

when added at very low ratio with respect to the other competitive inhibitor.[20]

Epoxides usually show high reactivity toward nucleophiles, leading to ring opening. The intact epoxide ring observed in the X-ray structure of HIV-PR/**1b** was therefore surprising.

The catalytic aspartic 25(B) carboxylate is involved in hydrogen bonding with the hydroxyl group of the inhibitor and is actually rather far from the epoxide carbon atoms. Conversely, the carboxyl group of the other aspartic acid 25(A) is in very close contact with one of the carbons of the oxirane (Figure 5).

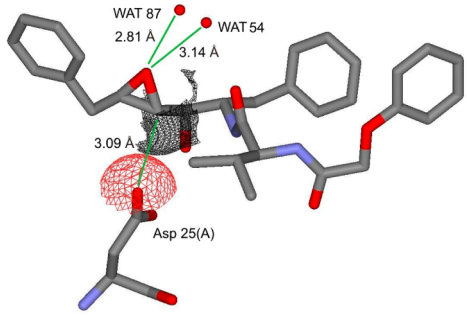

Figure 5. Contact between Asp 25(A) and the epoxide ring. The van der Waals surfaces of the carboxyl oxygen and of the oxirane carbon are shown in red and dark gray, respectively.

The distance between the two atoms is only 3.09 Å, comparable to the sum of their van der Waals radii; the carboxylate oxygen atom is well aligned with the epoxide ring, and the angle with its carbon–carbon bond is 95°. This value is close to the optimal trajectory calculated for the ring opening of an epoxide by oxygen nucleophiles and in particular for the calculated path of epoxide opening by Asp25 in HIV protease models.[27,28]

The epoxide ring opening by HIV protease has been computationally studied by Mavri, who has suggested that the deptrotonated aspartate should attack the oxirane upon proton transfer to the leaving oxygen by the other, protonated aspartic residue.[29] This proton transfer is clearly not possible in our case, as the epoxide oxygen is placed upside and far from the aspartyl side chains. However, two water molecules are found at hydrogen bond distance from the oxygen (Figure 3) and could provide protons to assist the epoxide opening. The reason for the lack of reactivity remains therefore puzzling. This evidence could

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suggest that Asp25(A) is actually protonated in the catalytic site and not available as a nucleophile.

In conclusion, the reported structural characterization of **1a** and HIV-PR/**1b** complex clearly indicated that the configuration of the epoxide carbons plays a crucial role in the binding affinity of the inhibitor and in the alignment of the epoxide group within the active site of HIV-PR.

■ ASSOCIATED CONTENT

Supporting Information

Experimental data for the synthesis and characterization of inhibitor **1a**. Assay protocol for inhibition of HIV-PR. Protocols for expression and purification of HIV-PR, crystallization of HIV-PR/**1b** complex and pure **1a**, and structure determination. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

Coordinates for the HIV-PR/**1b** crystal structure have been deposited in Protein Data Bank (PDB code: 3TOF). CCDC 843044 contains the supplementary crystallographic data for this letter. These data can be obtained free of charge from the (CCDC) Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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