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# INACTIVATION OF HIV-1 PROTEASE BY A TRIPEPTIDYL EPOXIDE

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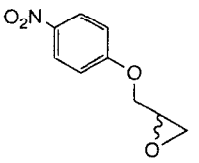
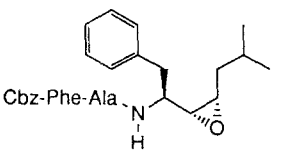
**Abstract:** *(2S,3R,4S)-N-[N-(N-benzyloxycarbonyl)-L-phenylalanyl]-L-alanyl-1-phenyl-2-amino-3,4-epoxy-6-methylheptane, a tripeptidyl epoxide analogue of peptide substrates of the retroviral protease of the human immunodeficiency virus-1, is a potent, active-site directed, irreversible inactivator of this enzyme.*

The initial translation products of retroviral genes are polyproteins, which are in turn processed to active enzymes and structural proteins by the action of a virally-encoded protease. These retroviral proteases belong to the family of aspartic proteases. The retroviral protease of the human immunodeficiency virus-1 (HIV-1) is a homodimer composed of two 11-kDa monomers,[1] and its active site, formed at the interface of the monomers, consists of two aspartyl residues which are highly conserved among the aspartic proteases [2] As these catalytic groups appear to be in opposite states of protonation, the role of these residues in the chemical mechanism of HIV-1 protease is perhaps best described by non-covalent, general acid-general base catalysis in which a single enzyme-bound, tetrahedral adduct of the scissile peptide and the lytic water is involved.[3] However, as first reported by Tang and co-workers, the unprotonated aspartyl residue in the active site of porcine pepsin can also display a nucleophilic role upon treatment with epoxides.[4,5] Hartsuck and Tang demonstrated that 3-(4-nitro)phenoxy-1,2-epoxypropane (EPNP, **1**) irreversibly inactivates the aspartic protease porcine pepsin due to the enzyme-catalyzed ring opening of the epoxide concomitant with alkylation of the β-carboxylic group of an active site aspartyl residue.[6] We have demonstrated that EPNP also inactivates the aspartic protease of the human immunodeficiency virus-1 with a maximal inactivation rate, $k_{inact}$, of 0 004 $min^{-1}$ and a half-maximal inactivation concentration, $K_{inact}$, of 11 mM [1] The pH dependence of this inactivation is consistent with a similar alkylation of the active site aspartyl residue which acts as a general base in the chemical mechanism.[1,3] In an effort to develop epoxide-containing compounds which would prove to be more specific and potent inactivators of HIV-1 protease, we have incorporated some of the salient structural features of EPNP into tripeptide analogues, which contained residues typically found in substrates.[6] As we describe below, one of these tripeptidyl epoxide analogues, (2S,3R,4S)-N-[N-(N-

benzyloxycarbonyl)-L-phenylalanyl]-L-alanyl-1-phenyl-2-amino-3,4-epoxy-6-methylheptane (**2**) is a particularly potent irreversible inactivator of HIV-1 protease.



**1**




**2**

The design of the tripeptidyl epoxide compound **2** was based in part on the specificity of HIV-1 protease for oligopeptide substrates[1,6] and peptide analogue inhibitors.[7] Typically, good oligopeptide substrates consist of hexapeptides which span the P3-P3'[8] positions and contain numerous hydrophobic residues, particularly at the P1 and P1' positions.[1,6] Potent tripeptide analogue inhibitors, spanning the P1-P2' positions have also been identified, in which the P1 residue is phenylalanine.[9] Therefore, we reasoned that compound **2** should provide a highly efficient and potent time-dependent inactivator of HIV-1 protease We thought that occupation of the S3-S1' substrate-binding subsites of the protease by the P3-P1' groups of compound **2** would more optimally position the epoxy group, which replaces the scissile P1-P1' peptide bond, between the catalytic aspartyl residues, Asp-25 and Asp-125, such that enzyme-catalyzed alkylation of the unprotonated aspartyl residue would occur more rapidly than with EPNP.

Indeed, compound **2** displayed rapid, time-dependent loss of HIV-1 protease activity following pre-incubation of micromolar concentrations of **2** with the enzyme at 37° C, pH 6.0 This inactivation was irreversible, since HIV-1 protease activity could not be recovered following exhaustive dialysis of a sample of compound **2**-inactivated enzyme. Analysis of the pseudo-first order rates of inactivation ($k_{obs}$), obtained as described in Experimental Procedures, with respect to concentrations of **2** was done by double-reciprocal analysis,[10] that is by plotting $1/k_{obs}$ vs. 1/[**2**]. That a linear double reciprocal plot of these data (not shown) was obtained indicates that the inactivation of HIV-1 protease by **2** is saturable. From the slopes and intercepts of this plot one obtains values for the apparent maximal rate of inactivation, $k_{inact}$ = 0 31 $min^{-1}$ and the concentration of **2** which results in half-maximal inactivation, $K_{inact}$ = 20 μM. For time-dependent enzyme inactivators, these values are analogous to $V_{max}/E_t$ and $K_m$, respectively, which range from 14-1700 $min^{-1}$ and 1-10 mM, respectively, for typical oligopeptide substrates of HIV-1 protease.[6b] The calculated bimolecular rate constant for formation of the HIV-1 protease-**2** complex value of $k_{inact}/K_{inact}$ was 260 $M^{-1}s^{-1}$, which may be compared with $k_{cat}/K_m$ values of 1,000-15,000 $mM^{-1}s^{-1}$ for substrates [6b]

The tripeptidyl epoxide **2** is a considerably more potent inactivator of HIV-1 protease than is EPNP ($K_{inact}$ = 11 mM, $k_{inact}$ = 0 004 $min^{-1}$, the value of $k_{inact}/K_{inact}$ for **2** is 41,000-fold higher than that of EPNP Since EPNP

may be thought of as a phenylalanine analogue with the *p*-nitrophenoxy group probably occupying the S1 site of the protease, then the greatly enhanced potency of **2** likely results from its binding to the S3, S2, S1, and possibly the S4 and S1' subsites of the enzyme.

Inactivation of HIV-1 protease by **2** probably involves enzyme-catalyzed alkylation of the unprotonated active site aspartyl residue, in analogy to the findings of Tang and co-workers with EPNP inactivation of pepsin. We found that the time-dependent inactivation of the protease by 10 μM **2** was partially blocked in the presence of 1 μM of the competitive inhibitor pepstatin ($K_i$ = 1 μM) as evidenced by a nearly 50% diminution in $k_{obs}$. This result suggests that inactivation of HIV-1 protease by **2** is due to covalent modification of an active-site residue, analogous to the mode of action of EPNP. We previously demonstrated that the isoelectric point (pI) of HIV-1 protease was 8.6 under non-denaturing conditions.[11] Alkylation of a single aspartyl residue in the 22-kDa homodimer of HIV-1 protease should the increase pI by about 0.5 units. As shown in Figure 1, upon treatment of 0.07 nmol of HIV-1 protease homodimer with 0-1.3 molar equivalents of **2**, the isoelectric point shifted from 8.6 to 9.1, in accord with neutralization of a single aspartyl residue. This isoelectric focusing experiment also indicates that approximately 0.9 moles of **2** per mole of protease homodimer is necessary to completely effect this shift in pI. These results suggest that the stoichiometry of inactivation of HIV-1 protease by **2** per mole of active site is 1:1.

We further evaluated the stoichiometry of inactivation of HIV-1 protease by measuring the loss of enzymatic activity upon mixing of 0.1 nmol of HIV-1 protease homodimers (active site concentrations were titrated using a tight-binding inhibitor) with 0-1.1 molar equivalents of **2** (Figure 1). Fitting of the data to $v_i/v_0$ (relative activity) = $1 - [I_{total}]/[E_{total}]$ resulted in an x-intercept of 0.84. Again, the stoichiometry of inactivation of HIV-1 protease by **2** is approximately 1 mol inactivator per mole of homodimer. The present results suggest that the potent and irreversible inactivation of HIV-1 protease results from specific alkylation of a single active site aspartyl residue.

## Experimental Procedures.

*Synthesis of (2S,3R,4S)-N-[N-(N-benzyloxycarbonyl)-L-phenylalanyl]-L-alanyl-1-phenyl-2-amino-3,4-epoxy-6-methylheptane (2).* Compound **2** was prepared as previously described.[12] Briefly, Boc-phenylalaninal was prepared from Boc-Phe-OMe by reduction with DIBAL. The aldehyde was then converted into *cis*-(N-Boc)-1-benzyl-5-methylhex-2-en-amine by Wittig reaction with the ylide of 3-methylbutyl triphenylphosphonium bromide. After deprotection, the olefin was successively coupled with Boc-alanine and Cbz-phenylalanine to yield the tri-peptide olefin. Oxidation with *m*-chloroperbenzoic acid afforded the product epoxide as predominantly one isomer. The stereochemistry of **2** has been provisionally assigned in accordance with literature precedent.[12]

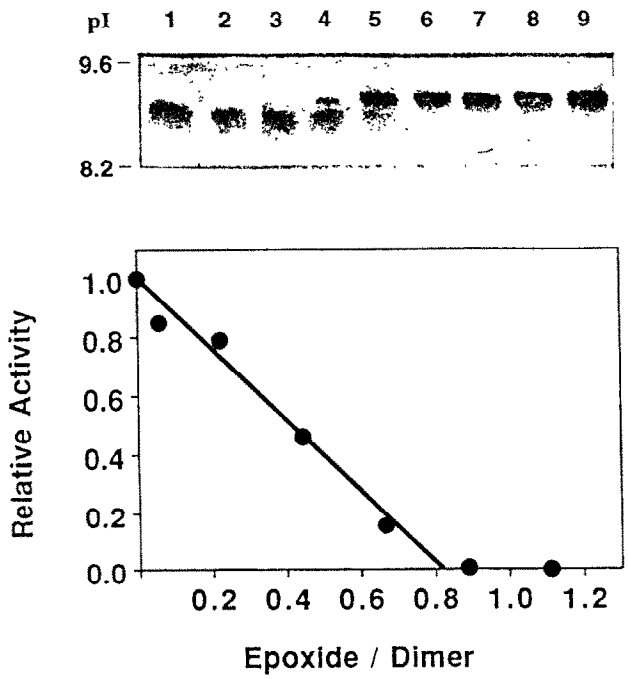



**Figure 1.** *upper panel:* Non-denaturing isoelectric focusing of HIV-1 protease pre-incubated for 24 hrs. at room temperature with various concentrations of **2**. Proteins were visualized by silver stain. Each sample contained 1.6 μg (0.07 nmol) HIV-1 protease, MENDT buffer, 10% dimethyl sulfoxide, and (lanes 1-9) contained concentrations of **2** which were, respectively, 0, 0.05, 0.11, 0.22, 0.44, 0.66, 0.88, 1.10 and 1.32 molar equivalents of the homodimeric protease. *lower panel:* Stoichiometry of inactivation of HIV-1 protease by **2**. The remaining HIV-1 protease activity following pre-incubation with stoichiometric concentrations of **2**, identical to conditions of the isoelectric focusing study, were plotted vs. the ratio of [**2**]·[HIV-1 protease]. The line drawn through the experimental data points was obtained by fitting of the data to $v_i/v_0$ (relative activity) = 1-[**2**]/[HIV-1 protease].

*Enzyme and Chemicals.* HIV-1 protease was expressed in *E. coli* and purified to apparent homogeneity from bacterial lysates as described.[11] The concentration of active sites in preparations of the purified enzyme was determined by titration with a tight-binding inhibitor as described.[11] The preparation and characterization of the oligopeptide substrate Ac-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-$NH_2$ has been previously described.[1,6]

*Analysis of Inactivation of HIV-1 Protease by 2.* HIV-1 protease (≤400 nM) was pre-incubated with variable concentrations of **2** in 50 mM 2-(N-morpholino)ethanesulfonic acid (Mes; pH 6.0), 1 mM dithiothreitol, 1 mM EDTA, 0.2 M NaCl, 0.1% Triton X-100 (MENDT buffer), 10% dimethyl sulfoxide (37° C). At variable time intervals, aliquots

were withdrawn and diluted ten-fold into reaction mixtures containing MENDT buffer and 1 mM Ac-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-$NH_2$ at 37° C After 20 min, remaining enzyme activity ($v_i/v_0$) was determined by the HPLC-based peptidolysis assay.[6b] Kinetic parameters of time-dependent inactivation were determined by fitting values of $v_i/v_0$ to. ln ($v_i/v_0$) = $-k_{obs}t$ in which $v_i$ and $v_0$ are the enzymatic activities remaining after pre-incubation in the presence and absence of **2**, respectively, $k_{obs}$ is the observed pseudo-first order inactivation rate constant, and t is the pre-incubation time in minutes. A half-maximal rate of inactivation concentration, $K_{inact}$, and the apparent maximal rate of inactivation, $k_{inact}$, were then obtained by replotting values of $1/k_{obs}$ vs. 1/[**2**] as described.[1,11] Non-denaturing isoelectric focusing (pH 3 5-9.5) and the subsequent visualization of complexes of HIV-1 protease and **2** was performed as described.[11]

**References.**


1. Meek, T.D.; Dayton, B.D.; Metcalf, B.W.; Dreyer, G.B.; Strickler, J.E.; Gorniak, J.G.; Rosenberg, M.; Moore, M.L.; Magaard, V.W.; Debouck, C. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 1841.

2. Wlodawer, A.; Miller, M.; Jaskolski, M.;Sathyanarayana, B.K.; Baldwin, E.; Weber, I.T.; Selk, L M.; Clawson, L.; Schneider, J.; Kent, S.B.H. *Science* **1989**, *245*, 616.

3. Hyland, L.J.; Tomaszek, T.A., Jr.; Meek, T.D. *Biochemistry* **1991**, *30*, 8454.

4. (a) Tang, J. *J. Biol. Chem.* **1971**, *246*, 4510. (b) Chen, K.C.S.; Tang, J. *J. Biol. Chem.* **1972**, *247*, 2566.

5. Hartsuck, J.A.; Tang, J. *J. Biol. Chem.* **1972**, *247*, 2575.

6. (a) Moore, M.L.; Bryan, W.M.; Fakhoury, S.A.; Magaard, V.W.; Huffman, W.F.; Dayton, B.D.; Meek, T.D.; Hyland, L.; Dreyer, G.B.; Metcalf, B.W ; Strickler, J.E.; Gorniak, J.; Debouck, C. *Biochem. Biophys. Res. Comm.* **1989**, *159*, 450. (b) Hyland, L.J.; Tomaszek, T.A., Jr.; Roberts, G.D.; Carr, S.A.; Magard, V.W.; Bryan, H.L.; Fakhoury, S.A.; Moore, M.L.; Culp, J.S.; DesJarlais, R.L.; Meek, T.D. *Biochemistry* **1991**, *30*, 8441.

7. Dreyer, G.B.; Metcalf, B.;W.; Tomaszek, T.A., Jr.; Carr, T.J.; Chandler, A.C., III; Hyland, L.; Fakhoury, S.A.; Magaard, V.W.; Moore, M.L.; Strickler, J.E.; Debouck, C.; Meek, T. D. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 9752.

8. Schechter, I.; Berger, A. *Biochem. Biophys. Res. Comm.* **1967**, *27*, 157.

9. Dreyer, G.B.; Lambert, D M.; Meek, T.D.; Carr, T.J.; Tomaszek, T.A., Jr.; Fernandez, A.V.; Bartus, H.; Cacciavillani, E.; Hassell, A.; Minnich, M.; Petteway, S.R., Jr.; Metcalf, B.W., Lewis, M. *Biochemistry,* **1992**, *in press.*

10. Kitz, R.; Wilson, I.B. *J. Biol. Chem.* **1962**, *237*, 3245.

11. Grant, S.K.; Deckman, I.C.; Minnich, M.D.; Culp, J.; Franklin, S.; Dreyer, G.B.; Tomaszek, T.A ,Jr.; Debouck, C.; Meek, T. D. *Biochemistry* **1991**, *30*, 8424.

12. Moore, M.L.; Fakhoury, S.A.; Bryan, W.M.; Bryan, H.G.; Tomaszek, T.A., Jr.; Grant, S.K.; Meek, T.D.; Huffman, W.F. in *Peptides, Chemistry and Biology, Proceedings of the 12th American Peptide Symposium,* Smith, J.A.; Rivier, J.E.; (Eds.); ASCOM, Leiden, **1992**, 781.