Irreversible Inhibition of the HIV-1 Protease: Targeting Alkylating Agents to the Catalytic Aspartate Groups

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Received December 4, 1995⊗

Abstract: Irreversible inhibition of the HIV-1 protease by agents that specifically alkylate its catalytic aspartate residues is a potentially useful approach for circumventing the evolution of HIV strains that are resistant to protease inhibitors. Five haloperidol- and two FMOC-based epoxides of differing reactivities have been synthesized and tested as irreversible inhibitors of the HIV-1 protease (HIV-1 PR). Of these, two trisubstituted epoxides, a *cis*-1,2-disubstituted epoxide, a 1,1-disubstituted epoxide, and a monosubstituted epoxide function as irreversible inhibitors, but two *trans*-1,2-disubstituted epoxides do not. The most effective of the epoxides (**6**) inactivates HIV-1 PR with $K_{inact} = 65\ \mu M$ and $V_{inact} = 0.009\ min^{-1}$. 1,2-Epoxy-3-(*p*-nitrophenoxy)propane (EPNP), a nonspecific inactivating agent for aspartyl proteases, has been used to validate a protocol for establishing the stoichiometry and site of protein alkylation. Mass spectrometric analysis of the inactivated enzyme shows that one molecule of either EPNP or the cyclic 1,2-disubstituted epoxide **6** is covalently bound per HIV-1 PR dimer. Mass spectrometric sequencing of labeled proteolytic peptides shows that both inhibitors are covalently bound to a catalytic aspartate residue. The covalent binding of three α,β-unsaturated ketone derivatives of haloperidol has been similarly examined. Analysis of HIV-1 PR inactivated by these agents establishes that they bind covalently to the two cysteines and the N-terminal amino group but not detectably to the catalytic aspartate residues. The results indicate that aspartate-targeted inactivation of HIV-1 PR depends on (a) matching the reactivity of the alkylating functionality to that of the aspartates, preferably by exploiting the two-aspartate catalytic motif of the protease to activate the alkylating agent, and (b) appropriate positioning of the alkylating functionality within the active site. These requirements are readily met by a monosubstituted, 1,1-disubstituted, or cyclic *cis*-1,2-disubstituted epoxide but not by *trans*-1,2-disubstituted epoxides or α,β-unsaturated ketones.

The AIDS epidemic has triggered a massive effort to develop therapeutic strategies to halt the progress of the disease. The focus of these efforts has been the human immunodeficiency virus (HIV), the etiologic agent for the syndrome.[1] As for all retroviruses, the RNA of HIV is transcribed into DNA by a virus-encoded reverse transcriptase and is incorporated into the host genome by a viral integrase. Subsequent expression of the virus by the host cell produces polyprotein precursors that are processed by an HIV-encoded protease into functional capsid proteins and enzymes.[2] The maturation of the precursor proteins occurs at the plasma membrane during particle release, ultimately resulting in infectious viral particles.[3]

HIV infections are currently treated primarily with inhibitors (e.g., AZT) of the viral reverse transcriptase. The toxicity of these reverse transcriptase inhibitors and the rapid development of resistant strains have led to a search for alternative approaches to the treatment of AIDS. This search is currently concentrated on the development of inhibitors of the HIV protease,[4,5] a relatively small protein for which many crystal structures are available.[6] The HIV protease is a member of the aspartyl protease family[7] but differs from most members of the family in that it exists as a dimer of two identical 99-amino acid chains rather than as a monomeric polypeptide.[8,9] Each monomer provides one of the two active-site aspartates required for the catalytic action of the dimer. Many competitive inhibitors of this protease are now available,[10−13] several of which are in clinical trials, but there is growing evidence for the rapid rise of strains that encode mutant proteases resistant to reversible protease inhibitors.[14,15]

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⊗ Abstract published in *Advance ACS Abstracts,* June 1, 1996.

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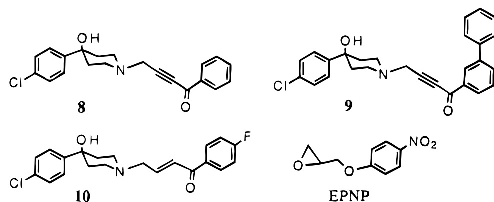

Figure 1. Structures of the α,β-unsaturated ketone derivatives of haloperidol and EPNP.

One strategy for minimizing the development of resistance is to develop irreversible rather than reversible inhibitors of the HIV protease. Irreversible inhibitors are less sensitive to mutations that decrease reversible binding affinity because even a low degree of active site occupancy can lead in time to complete inactivation of the protein. The catalytic aspartate residues are the ideal target for such irreversible inhibitors because their mutation results in complete loss of catalytic activity. This is clearly shown by the early demonstration that a D25N mutation of HIV-1 PR (PR = protease) results in complete loss of protease activity and hence viral maturation.[16] Irreversible inhibition of HIV-1 PR was first achieved with 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP),[17] a small epoxide molecule that is a general irreversible inhibitor of aspartyl proteases.[18] EPNP (Figure 1) is a nonspecific and relatively weak irreversible inhibitor of both HIV-1 PR ($K_{inact} = 9.9$ mM, $V_{inact} = 0.060$ min^{-1}) and the HIV-2 protease (HIV-2 PR; $K_{inact} = 6.7$ mM, $V_{inact} = 0.048$ min^{-1}).[19] An analysis of the binding of EPNP to HIV-2 PR[19] and a crystal structure of the adduct of EPNP with the simian immunodeficiency virus (SIV) protease[20] indicate that EPNP is bound to a catalytic aspartate residue in both of these proteins. A tripeptide with a *cis*-1,2-disubstituted epoxide functionality has also been reported to inactivate HIV-1 PR with $K_{inact} = 20$ μM and $V_{inact} = 0.31$ min^{-1}.[21]

Our search for irreversible inhibitors of the HIV proteases has concentrated on derivatives of haloperidol, a lead structure identified as a reversible inhibitor through computer-assisted inhibitor design methods.[22,23] We have shown in earlier work that haloperidol analogues with a 1,1-disubstituted epoxide or an α,β-unsaturated carbonyl group as the reactive center (Figure 1) irreversibly inhibit HIV-1 PR and HIV-2 PR.[19,23] The generally higher activity of these compounds as inactivators of HIV-1 PR than HIV-2 PR suggests that they bind covalently to several amino acids, but the actual nature of the irreversible inhibition was not established. As part of an effort to identify the key features required to target irreversible inhibitors to the active site aspartyl residues of the HIV proteases, we have (a) synthesized seven additional epoxides with a range of chemical reactivities and evaluated their activities as irreversible inhibitors of HIV-1 PR and (b) undertaken mass spectrometric analyses of HIV-1 PR inactivated by five different agents to define the stoichiometry of the inactivation process and identify the amino acid(s) alkylated by each agent. The sensitivity and ability to provide unambiguous answers make mass spectrometry the method of choice for this purpose.[24] The results provide important information for the development of irreversible inhibitors that specifically target the catalytic aspartates rather than amino acids that can be mutated with the concomitant development of viral strains resistant to the inhibitors.

Results

Inhibitor Design and Synthesis. Seven new epoxide derivatives have been synthesized. Four of the epoxides were constructed using the haloperidol framework for delivery of the reactive group to the HIV-1 PR active site. We have shown in earlier work that a wide range of haloperidol derivatives inhibit both HIV-1 PR and HIV-2 PR.[22,25] Two crystal structures have been reported which show that a single haloperidol derivative can bind in two very different orientations within the active site of the protein.[26] The other three epoxides were incorporated into frameworks based on a new class of FMOC derivatives identified as reversible inhibitors of the HIV proteases (unpublished results). A crystal structure of the lead compound for this series demonstrates that the FMOC agents are also bound within the active site of the protein (unpublished result). The parent haloperidol and FMOC compounds are modest reversible inhibitors of the proteases, with IC_{50} values in the high nanomolar to low micromolar range. These frameworks were chosen for the work described here in preference to frameworks with tighter reversible binding affinities because they better model the affinities that might be expected in inhibitor-resistant variants of the HIV protease.

Of the four epoxides based on the haloperidol framework, two (**1**, **2**) are *trans*-1,2-disubstituted epoxides, one (**3**) is a trisubstituted epoxide, and one (**4**) is a monosubstituted epoxide. Of the three additional compounds based on the FMOC motif one (**5**) is a trisubstituted epoxide, one (**6**) a cyclic *cis*-1,2-disubstituted epoxide, and one (**7**) a 1,1-disubstituted epoxide. In general, the chemical reactivities of epoxides toward S_N2-type reactions decrease in the order monosubstituted > 1,1-disubstituted > 1,2-*cis*-disubstituted > 1,2-*trans*-disubstituted due to steric factors. However, for steric reasons, 1,2-disubstituted epoxides in which the substituents are part of a six-membered ring are less reactive than their acyclic 1,2-disubstituted counterparts. Within each steric reactivity class, the reactivities of the epoxides are further modulated by electronic factors, epoxides with conjugating or electron-releasing functionalities being more reactive than epoxides with simple alkyl substituents.

***trans*-Epoxides.** Direct epoxidation of unsaturated haloperidol analogues is difficult because the reaction also results in oxidation of the piperidine ring nitrogen. The two *trans*-epoxides were therefore synthesized by prefabricating a fragment bearing the epoxide moiety and attaching it to 4-(*p*-chlorophen-

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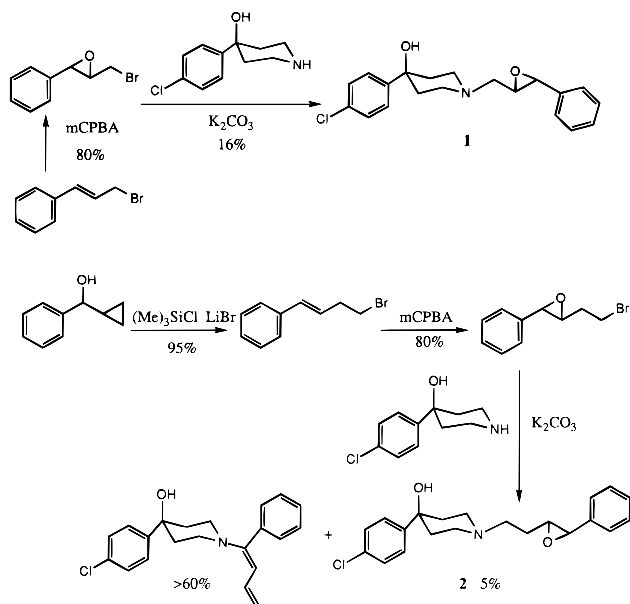
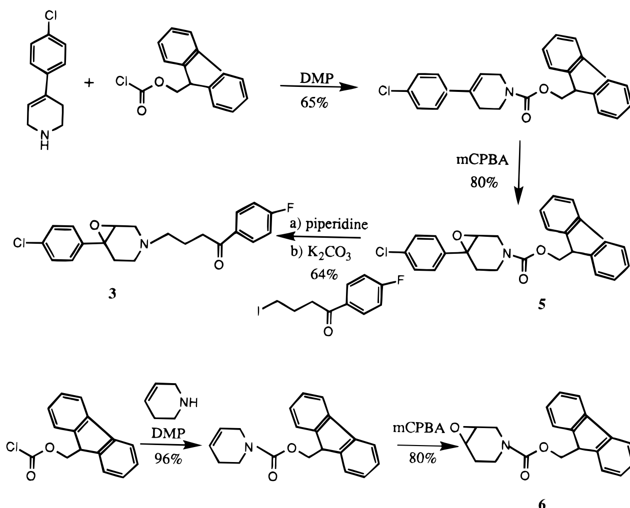
Scheme 1. Synthesis of Epoxides **1** and **2**



Scheme 2. Synthesis of Epoxides **3**, **5**, and **6**



yl)-4-hydroxypiperidine (Scheme 1). Thus, oxidation of cinammyl bromide provided the corresponding bromo epoxide (80% yield) which condensed with the piperidine fragment under conditions mild enough to prevent decomposition of the epoxide to give **1** in 16% isolated yield. In an analogous reaction sequence, epoxidation of the double bond of 4-bromo-1-phenyl-1-butene generated from α-cyclopropylbenzyl alcohol and trimethylsilyl bromide (Scheme 2), followed by reaction with the piperidine fragment, produced **2** in low yield. The major product (60%) is formed by addition of the piperidine to the benzylic terminus of the epoxide followed by base-catalyzed elimination of water and HBr to give a diene product (Scheme 1).

***cis*-1,2-Disubstituted and Trisubstituted Epoxides.** Dehydration of 4-(*p*-chlorophenyl)-4-hydroxypiperidine yielded 4-(*p*-chlorophenyl)-1,2,5,6-tetrahydropyridine which was converted to its FMOC derivative (Scheme 2). Epoxidation of the dihydropyridine double bond with mCPBA produced epoxide **5** in 65% yield. Deprotection of **5** with piperidine in DMF followed by K_2CO_3-catalyzed reaction of the deprotected nitrogen with 4-iodo-4′-fluorobutyrophenone afforded a one-pot synthesis of epoxide **3** (64% yield). 4-Iodo-4′-fluorobutyrophenone was employed because 4-chloro-4′-fluorobutyrophenone was not sufficiently reactive. The iodo derivative was generated *in situ* by reaction of cyclopropyl 4-fluorophenyl ketone with trimethylsilyl iodide. Epoxide **6** was obtained in 80% yield by mCPBA epoxidation of the FMOC derivative of 1,2,5,6-tetrahydropyridine (Scheme 2).

Scheme 3. Synthesis of Epoxides **4** and **7**

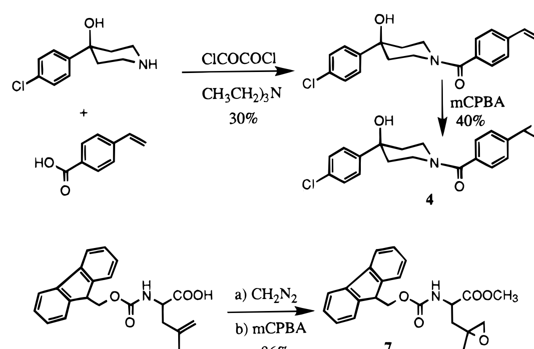


Table 1. Inactivation of HIV-1 PR by Epoxides Synthesized in This Study

inhibitor	[I] (μM)	$t_{1/2}$ min	K_{inact} (μM)	V_{inact} (min^{-1})
3	200	700	ND[a]	ND
4	1000	400	1800	0.001
5	200	600	ND	ND
6	100	110	65	0.009
7	120	800	140	0.0003

[a] ND = not done.

Monosubstituted and 1,1-Disubstituted Epoxides. The monosubstituted epoxide **4** was prepared by oxalyl chloride-mediated condensation of 4-(*p*-chlorophenyl)-4-hydroxypiperidine with 4-vinylbenzoic acid (Scheme 3). The vinyl group was then oxidized to the epoxide with mCPBA (40% yield). Commercially available FMOC-4,5-dehydro-Leu-OH was converted to the corresponding methyl ester with diazomethane. Epoxidation of the resulting ester with mCPBA provided **7** as a mixture of two stereoisomeric products (83% yield).

Enzyme Inactivation. The *trans*-1,2-disubstituted epoxides **1** and **2** do not detectably inactivate HIV-1 PR, but surprisingly, the trisubstituted epoxides **3** and **5** do slowly inactivate the enzyme in a time-dependent fashion. The $t_{1/2}$ values for inactivation of HIV-1 PR by these two agents are 700 and 600 min, respectively (Table 1). These values are comparable to those for the terminally unsubstituted epoxides **4** and **7**. The FMOC-derived *cis*-epoxide **6** inactivates the enzyme approximately 4−8 times more rapidly than the trisubstituted epoxides (Table 1). Solubility limitations restrict the concentrations of these agents that can be achieved, but approximate K_{inact} and V_{inact} values have been obtained for compounds **4**, **6**, and **7** (Table 1). These values confirm that compound **6** is the most effective inhibitor within this series of epoxides.

The kinetic values for the inactivation of HIV-1 PR by EPNP and conjugated ketones **8**−**10** were determined in an earlier study and are as follows: EPNP, K_{inact} = 9900 μM, V_{inact} = 0.06 min^{-1}; **8**, K_{inact} = 62 μM, V_{inact} = 0.380 min^{-1}; **9**, K_{inact} = 10.7 μM, V_{inact} = 0.057 min^{-1}; **10**, K_{inact} = 57 μM, V_{inact} = 0.232 min^{-1}.[19] These results show that the K_{inact} value for **6**, the best epoxide, is comparable to those for **8** and **10** but somewhat worse than that for **9**. On the other hand, the V_{inact} value for **6** is smaller than those for all three unsaturated ketones.

Inactivation by EPNP. EPNP is a nonspecific irreversible inhibitor of classic aspartyl proteases that binds to the active-site aspartates.[18] However, it is not known whether the inactivation of HIV-1 PR, as found for the HIV-2 and SIV proteases,[19,20] involves covalent binding to the catalytic aspartates. A detailed analysis of the HIV-1/EPNP adduct has therefore been carried out both to resolve this ambiguity and to validate with a well-understood agent the methodology for the characterization of protease/inhibitor complexes.

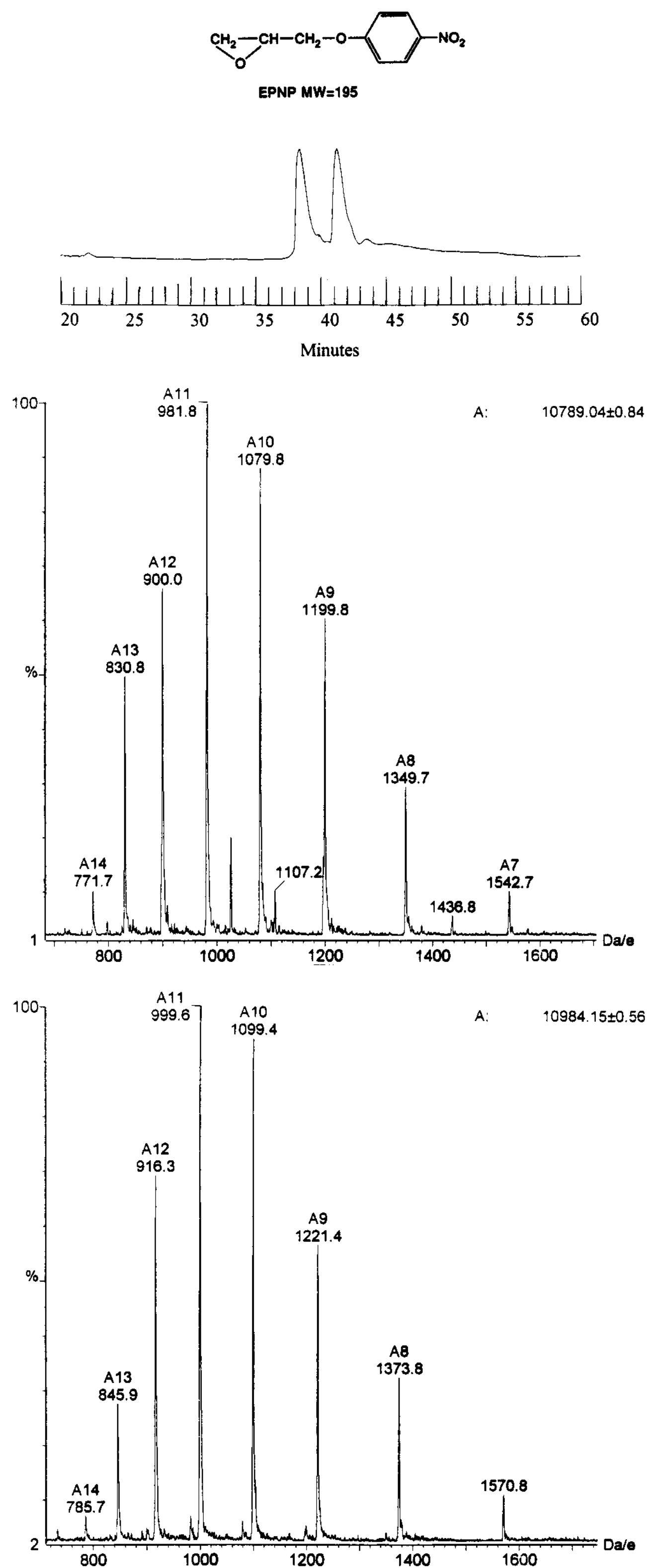


Figure 2. (a, top) HPLC profile of the HIV-1/EPNP complex. Two major peaks are observed by HPLC UV detection. The masses of these peaks have been determined by ESIMS, as shown in panels b and c. (b, middle) ESIMS spectrum of the first HPLC peak shown in panel a. A molecular mass of 10 789 Da is obtained. (c, bottom) ESIMS spectrum of the second peak in panel a. A molecular mass of 10 984 Da is obtained.

HPLC analysis of HIV-1 PR after it is fully inactivated by EPNP yields two well-resolved peaks (Figure 2a). The mass spectrometrically determined molecular mass of the first HPLC peak is 10 789 Da (Figure 2b), which corresponds to the mass of the intact protease monomer (theoretical MW 10 790). The molecular mass of the second peak, 10 984 Da (Figure 2c), is 195 Da higher than that of the intact protease monomer, a difference that corresponds exactly to the molecular weight of EPNP. This product clearly corresponds to an adduct of one molecule of EPNP with a subunit of HIV-1 PR. The presence of the two peaks in approximately equal amounts suggests that covalent attachment of EPNP to one subunit of the protease homodimer inactivates the protein and prevents modification of the second subunit. The stoichiometry of inactivation of HIV-1 PR by EPNP is thus one inhibitor molecule per protease dimer. In accord with this conclusion, HPLC analysis of partially inactivated enzyme shows that both peaks are still present but the peak that corresponds to the unmodified subunit is larger than that for the modified subunit and the ratio of these two peaks approaches a value of 1 as the enzyme approaches complete inactivation.

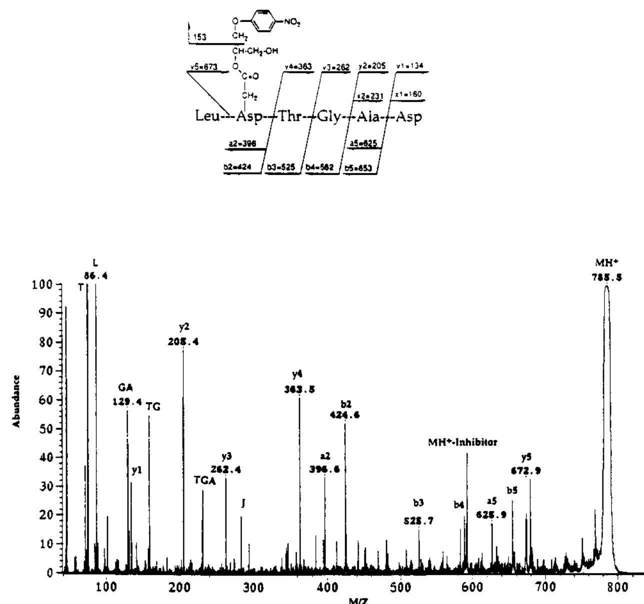


Figure 3. CID spectrum of the EPNP-modified peptide isolated after digestion of the modified protease. Peak assignments are based on the nomenclature of Roepstorff[42] and Biemann.[43] The interpretation, shown on the panel above the spectrum, identifies Asp-25 as the site of covalent modification.

In order to determine the site to which EPNP is covalently attached, the inactivated protease was digested with trypsin or pepsin. The resulting peptides were separated by HPLC, and their molecular masses were determined by HPLC/ESMS. Pepsin, which is not as specific as trypsin, generated peptides of appropriate size for further analysis. Identification of the modified peptides was achieved both by comparing the HPLC profile obtained from the modified protease with that from the unmodified enzyme and by using mass spectrometric peptide mapping. Figure 3 shows the high-energy CID spectrum of an EPNP-modified peptide generated by digestion of the modified protease. This spectrum establishes the sequence of the peptide and identifies the site of the modification. As shown in the upper panel, the fragmentation pattern of this peptide unambiguously establishes its sequence as Leu-Asp-Thr-Gly-Ala-Asp, with EPNP bound to the second residue. The y series of ions at 134, 205, 262, 363, and 673 and the b series of ions at 424, 525, 582, and 653 define the sequence and the site of modification. In addition, the ion at m/z 283, labeled as J, represents the immonium ion of the modified aspartic acid. As this sequence encompasses amino acids 24−29 of HIV-1 PR, the site of the modification is the catalytic residue Asp-25.

Inactivation by Compound 6. HPLC of HIV-1 PR after complete inactivation by compound **6** demonstrates the presence of two peaks of equal size (Figure 4a). The first peak has the

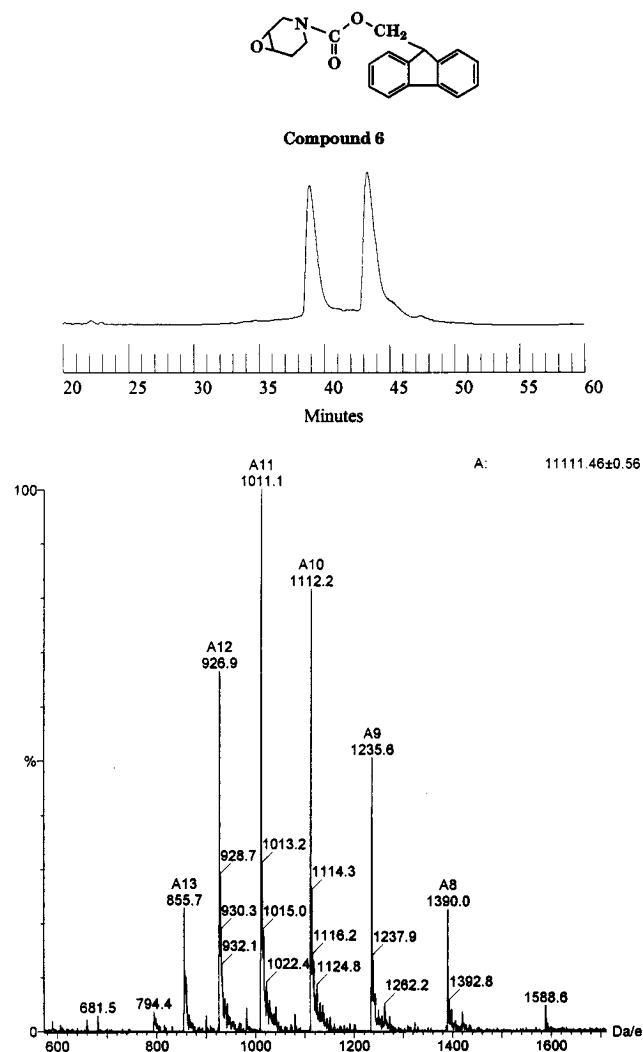


Figure 4. (a, top) HPLC profile of the product of the reaction of HIV-1 with compound **6**. Mass spectrometry identifies the first of the two peaks as the intact protease monomer. (b, bottom) ESIMS spectrum of the second peak in Figure 5a, which has a molecular mass of 11 111 Da.

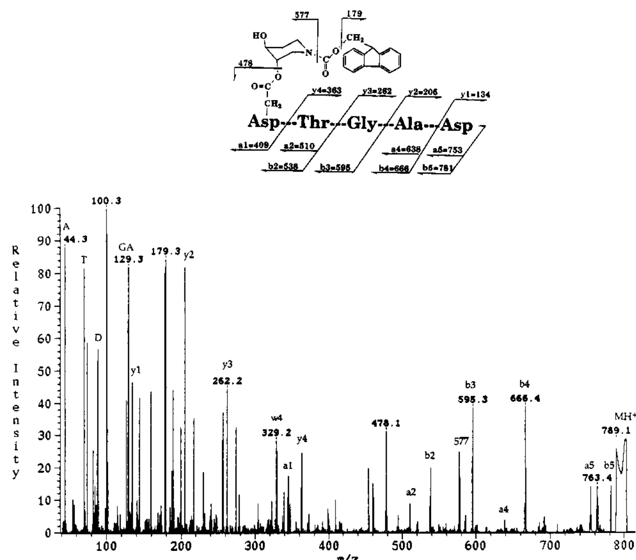


Figure 5. CID spectrum of a labeled peptide from the HIV-1 protease inactivated by **6**. The peptide sequence is Asp-Thr-Gly-Ala-Asp, with the inhibitor attached to the first Asp (Asp-25).

same retention as the intact protease monomer, and mass spectrometric analysis showed that it is indeed the intact protease. The second peak shows a molecular mass of 11 111 Da (Figure 4b), which is 321 Da higher than that of the intact protease monomer. The difference in mass corresponds exactly to the molecular weight of **6**. Covalent binding of one inhibitor molecule to only one of the two subunits of the fully inactivated protease dimer again establishes a stoichiometry of one inhibitor per protease dimer. Cleavage of the intact and modified protease with pepsin and separation of the resulting peptides by HPLC reveal differences in the two HPLC profiles. The peaks unique to the modified protease were analyzed by MS and sequenced by tandem MS (Figure 5). As shown by the CID spectrum of the modified peptide, its sequence is Asp-Thr-Gly-Ala-Asp, with **6** attached to the first aspartic acid residue. This sequence, which corresponds to residues 25−29 of HIV-1 PR, establishes that the inhibitor is attached to Asp-25. As with EPNP, analysis of the partially inactivated protease shows that the ratio of the modified and unmodified subunits is lower than 1 but approaches that value as the enzyme becomes fully inactivated. This confirms that modification of Asp-25 is responsible for the inactivation.

Inactivation by Compound 8. HPLC analysis of the fully inactivated protease after reaction with **8** reveals the presence of three peaks of different sizes (Figure 6a). Mass spectrometry established that the first peak has a molecular mass of 11 144 Da (Figure 6b), 354 Da higher than that of the intact protease monomer. As this corresponds to the molecular weight (MW 353) of compound **8**, it appears that one molecule of **8** is attached to the protease in the most rapidly eluting adduct. The second peak has a molecular mass of 11 498 Da (Figure 6c), 708 Da higher than that of the intact protease. This corresponds to the covalent attachment of two molecules of **8** to the protease monomer. The third peak exhibits a molecular mass of 11 854 Da (Figure 6d). The difference from the mass of the intact protease monomer is 1064 Da, which corresponds to covalent binding of three molecules of **8**. In contrast to the reaction with EPNP, no intact protease is detected.

The three HPLC fractions were digested by pepsin, and the resulting peptides were separated by HPLC and analyzed by HPLC/ESMS. One modified peptide of *m/z* 1349.6 was obtained from the first fraction, and its sequence, as determined by CID analysis (Figure 7a), is Thr-Gln-Ile-Gly-Cys-Thr-Leu-Asn-Phe. A molecule of inhibitor **8** is bound to the cysteine residue. This sequence, which corresponds to amino acids 91−99 of the protease, identifies Cys-95 as the site of the modification. Analysis of the second HPLC fraction provided, in addition to the *m/z* 1349.6 peptide, a new modified peptide of *m/z* 1252.6. The sequence of this second peptide was established by CID analysis as Ile-Cys-His-Lys-Ala-Ile-Gly-Thr, with the inhibitor bound to the cysteine (Figure 7b). As this sequence matches that of residues 66−74 of HIV-1 PR, the site of the alkylation is Cys-67. Analysis of the third HPLC fraction provided, in addition to the two modified peptides already discussed, a modified peptide of *m/z* 923.5. CID analysis identifies this peptide as a Pro-Gln-Ile-Thr-Leu with a molecule of the inhibitor bound to the proline. This sequence is that of the N-terminus of the protease, and the site of alkylation is the N-terminal proline amino function.

The extent of modification could be deduced from the size of the HPLC peaks. Cys-95 was modified completely since it was present in all three peaks. Cys-67 was present in both the second and third peaks, which represent about 70% of the total material recovered. Therefore, roughly 2/3 of Cys-67 was modified. The third HPLC peak represented less than 10% of the total area, so the N-terminus of the protease was only modified by **8** to a limited extent. No modification of Asp-25 was detected.

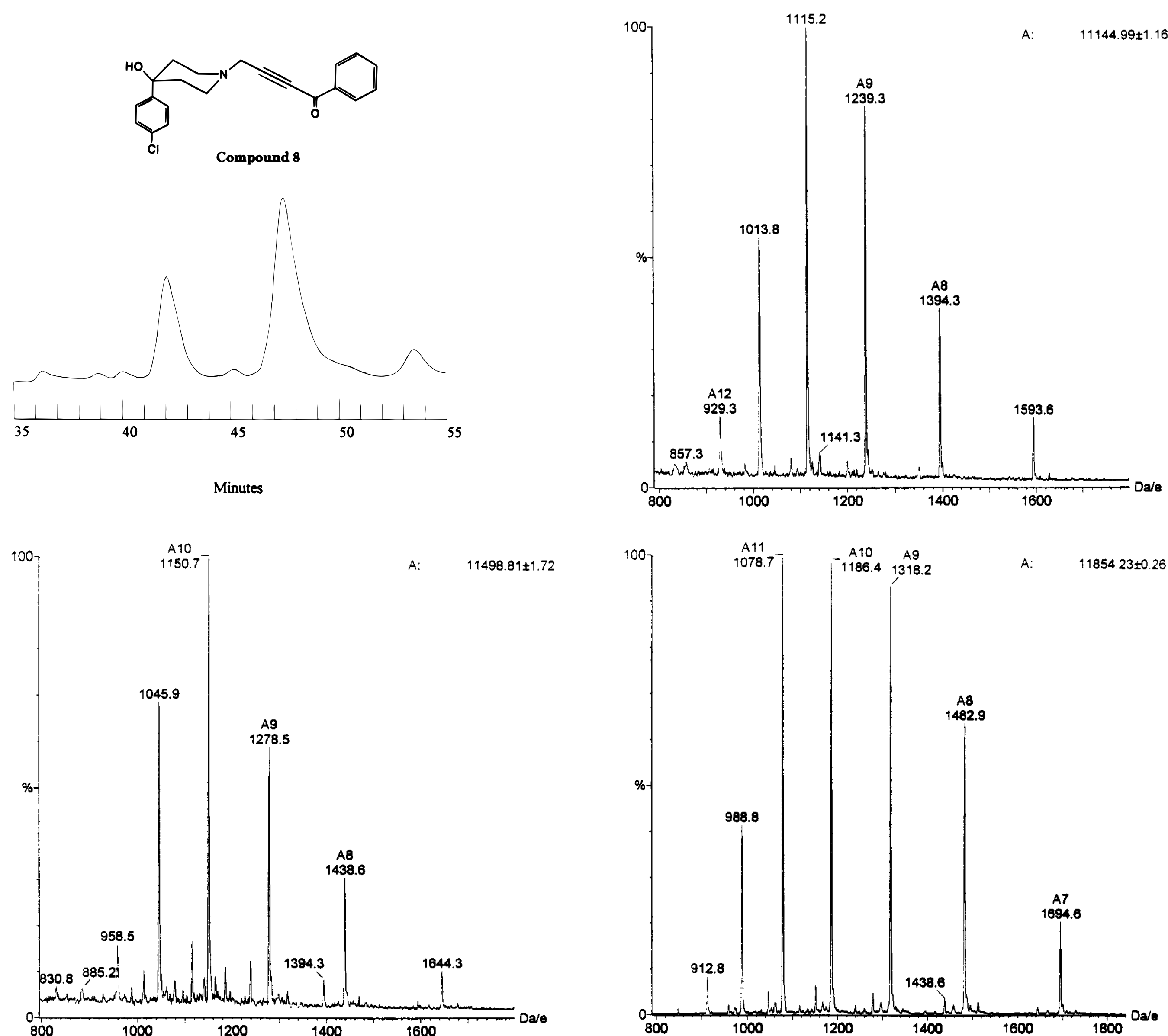


Figure 6. (a, top left) HPLC profile of the product of reaction of HIV-1 with compound **8**. The molecular masses of the three major peaks, determined by ESIMS, are shown in panels b−d. (b, top right) ESIMS spectrum of the first peak in panel a, which has a molecular mass of 11 144 Da. (c, bottom left) ESIMS spectrum of the second peak in panel a, which has a molecular mass of 11 498 Da. (d, bottom right) ESIMS spectrum of the third peak in panel a, which has a molecular mass of 11 854 Da.

Inactivation by Compounds 9 and 10. HPLC purification of the complex after inactivation by compound **9** showed one major HPLC peak with an ESIMS molecular mass of 11 651 Da. The difference of 861 Da between this mass and that of the protease monomer corresponds to the mass of two molecules of the inhibitor. Trypsin digestion followed by HPLC provided two modified peptides, one with a mass spectrometric MH^+ of m/z 1958.9 and the other with a mass spectrometric MH^+ of m/z 1765.8. Mass mapping results indicate that these two masses correspond to the peptides Gln-58 to Lys-70 and Asn-88 to Phe-99, each with one covalently bound inhibitor moiety. MS/MS experiments were carried out to identify the modification sites. The CID spectrum of the precursor ion at m/z 1958.9 confirmed the sequence Gln-Tyr-Asp-Gln-Ile-Pro-Val-Glu-Ile-Cys-Gly-His-Lys, with the inhibitor attached to Cys-67. The b-series ions revealed the sequence, whereas several fragment peaks defined the modification site. Similarly, the CID spectrum of the precursor ion at m/z 1765.8 showed its sequence to be Asn-Leu-Leu-Thr-Gln-Ile-Gly-Cys-Thr-Leu-Asn-Phe, with compound **8** bound to Cys-95.

HPLC analysis of the protease after inactivation by compound **10** provided one major product peak whose molecular mass (11 540 Da) was 750 Da higher than that of the intact monomer, a difference that corresponds to the mass of two inhibitor molecules. Tryptic digestion and MS analysis yielded two modified peptides with the sequences Gln-58 to Lys-70 and Asn-88 to Phe-99, each covalently attached to one inhibitor molecule. CID analysis of these peptides identified the sites of modification of these peptides as Cys-67 and Cys-95, respectively.

Discussion

A shadow has been cast on the promise of the potent inhibitors of the HIV proteases now available by the finding that HIV becomes resistant to the inhibitors due to the evolution of strains encoding protease variants that are resistant to the inhibitors.[14,15] In light of this development, we are investigating approaches to irreversible inhibition of the enzyme because irreversible inhibition is less susceptible to the development of resistance. Mutation-dependent decreases in the affinity of the enzyme for inhibitors are less critical for irreversible than reversible agents because the degree of reversible active site

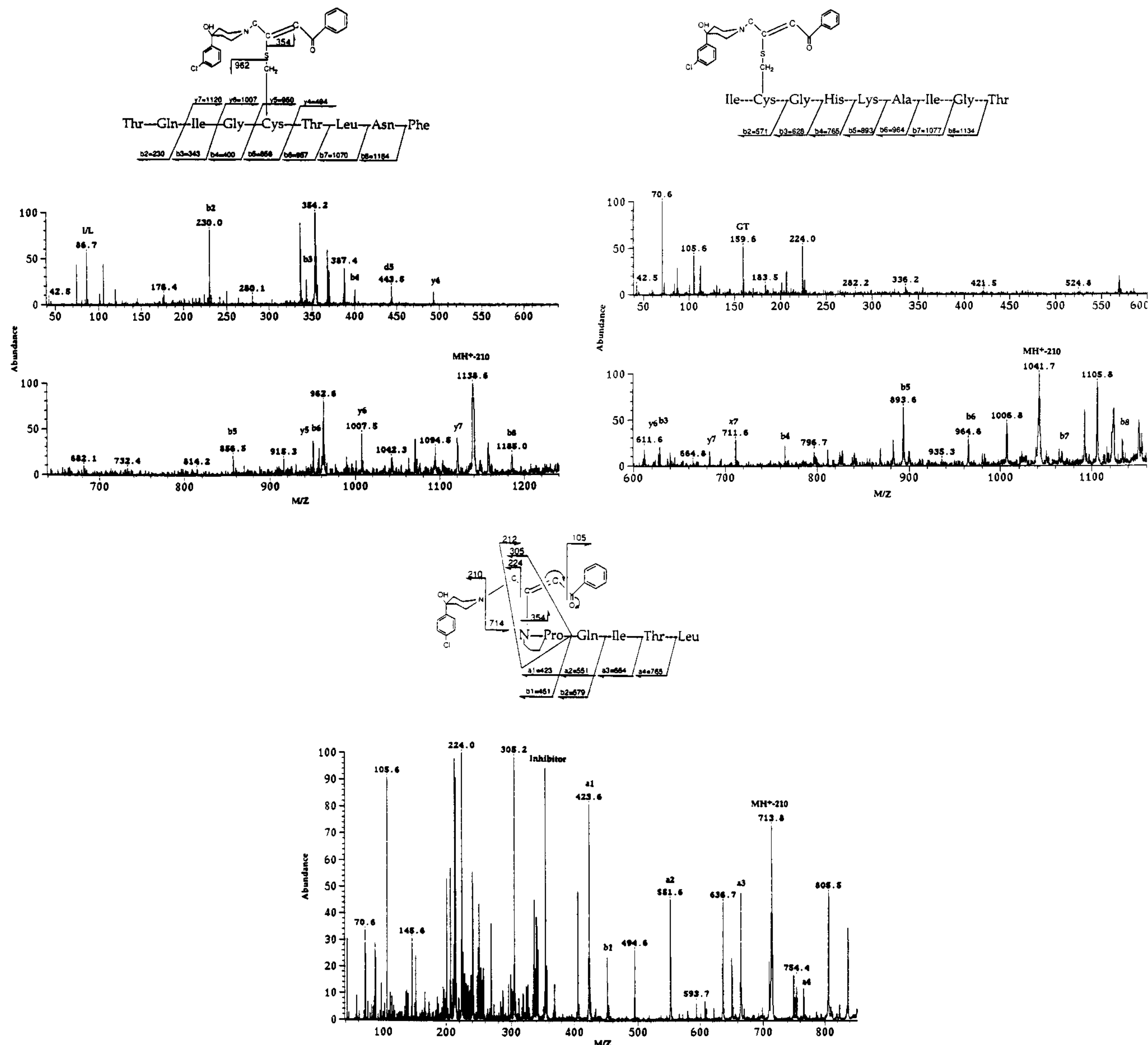


Figure 7. (a, top left) CID spectrum of a modified peptide isolated from the peptic digest of the first peak in Figure 6a. This peptide covers amino acid residues 91−99 of HIV-1 PR and includes a modified Cys-95. (b, top right) CID spectrum of a modified peptide isolated from the peptic digest of the second peak in Figure 6a. The peptide corresponds to amino acids 66−74 of the protease with covalent modification of Cys-67. (c, bottom) CID spectrum of a modified peptide isolated from the third peak in Figure 6a. This peptide corresponds to the N-terminus of the protease with the inhibitor bound to the N-terminus.

occupancy need not be high and need not be continuous to achieve full inactivation of the enzyme. This requires, of course, that the protein residues targeted by irreversible inhibitors not be replaced through mutations by unreactive residues. For this reason, the most desirable amino acids for reaction with irreversible inhibitors are the catalytic aspartate groups of HIV-1 PR because mutations of these residues suppress catalytic activity. It is therefore important to define the parameters that determine whether an irreversible inhibitor reacts with the catalytic aspartates as opposed to other residues of the HIV proteases.

Irreversible inhibitors are constructed by incorporating a reactive functionality, or a latent version of such a functionality, into the framework of a ligand for the enzyme. The bioavailability problems associated with peptide substrates have led us to concentrate on non-peptide inhibitor frameworks. In this study we have synthesized seven new potentially irreversible inhibitors, four based on a haloperidol framework (**1**−**4**) and three FMOC derivatives (**5**−**7**). An epoxide group has been incorporated into each of the seven structures as the reactive locus for potential covalent attachment to HIV-1 PR. However, the intrinsic reactivity of the epoxide group toward S_N2-type addition reactions differs in the seven agents. The least reactive are the trisubstituted epoxide moieties of **3** and **5**, followed by the *trans*-1,2-disubstituted epoxide moieties of **1** and **2**. The most reactive are the 1,1-disubstituted epoxide **7** and, particularly, the monosubstituted epoxide **4**. The cyclic *cis*-1,2-disubstituted epoxide group of **6** is expected to be of intermediate intrinsic reactivity. Studies of the activities of the seven structures show that **1** and **2** have no detectable activity as irreversible inhibitors of HIV-1 PR, either because the epoxide is too unreactive or because it is incorrectly positioned when the compound is bound in the active site. The other five compounds are slow but irreversible HIV-1 PR inhibitors (Table 1). Of the five active compounds, the cyclic *cis*-1,2-disubstituted epoxide **6** is the most effective. The other four compounds, including the trisubstituted epoxides **3** and **5** and the terminally unsubstituted epoxides **4** and **7**, exhibit weaker activities as irreversible inhibitors.

In order to characterize the inactivation process, we have used

mass spectrometric approaches to define the stoichiometry of the reaction and to identify the amino acids that are modified in the process. The major advantages of mass spectrometry for this purpose are its high efficiency, sensitivity, and ability to directly characterize protein modifications. The protocol used in these studies was validated by studying the inactivation of HIV-1 PR by EPNP, a nonselective irreversible inhibitor of aspartyl proteases.[18,20,27] The same protocol was then used to characterize the inactivation of HIV-1 PR by **6**, the most effective inhibitor of the series prepared in this study. For both EPNP and **6**, the data clearly establish that irreversible inactivation involves modification of only one of the two subunits in the homodimeric enzyme by covalent attachment of one molecule of the inhibitor. Mass spectrometric sequencing establishes, furthermore, that both EPNP and **6** bind exclusively to Asp-25, the catalytic aspartate group.

We reported previously that haloperidol derivatives **8**−**10** with an α,β-unsaturated ketone in the flexible side chain are irreversible inhibitors of HIV-1 PR.[19,23] It was not possible at that time, however, to define the nature of the inactivation reaction. We have therefore subjected the protease inactivated by these agents to the same mass spectrometric analyses used to investigate inactivation of the enzyme by the epoxides. The surprising finding from these studies is that inactivation of HIV-PR by the α,β-unsaturated ketones **8**−**10** involves (a) modification of both subunits of the protease dimer rather than only one subunit, (b) binding of more than one molecule of the inhibitor to the protease monomer, and (c) modification of the cysteine residues (Cys-67 and Cys-95) and to a small extent the N-terminal amino group rather than the catalytic aspartate group. As shown by analysis of the enzyme inactivated by **8**, Cys-95 is completely alkylated and Cys-67 is alkylated in most of the subunits of the inactive enzyme. Alkylation of the N-terminal proline amino group occurs in only a minor fraction of the protein. Cys-95 is within the substrate binding channel of the protease, and modification of this residue would be expected to interfere with substrate binding. It is possible that alkylation of this residue alone accounts for the loss of catalytic activity. Cys-67 and the terminal amino group are surface residues, and their alkylation could interfere with dimerization to give the catalytically active homodimer. It is possible, however, that alkylation of Cys-67 does not contribute significantly to the observed loss of catalytic activity because only the alkylation of Cys-95 correlates with complete loss of catalytic activity. No evidence was found for covalent modification of the catalytic aspartate group by compounds **8**−**10**. However, the earlier work with these compounds established that they inactivate HIV-2 PR as well as HIV-1 PR, although the inactivation of HIV-2 PR is much less effective.[19,23] Thus, for the acetylenic ketone **8** $V_{inact} = 380 \times 10^{-3}$ min^{-1} for HIV-1 PR and 7.6×10^{-3} min^{-1} for HIV-2 PR. Inactivation of HIV-2 PR, which has no cysteines, must involve alkylation of other residues. It is not known whether the low rate of inactivation of HIV-2 PR by **8**−**10** results from reaction with the catalytic aspartate, but if it does the differential rates of inactivation of the HIV-1 and HIV-2 enzymes suggest, as found here, that very little if any aspartate alkylation is likely to occur with HIV-1 PR.

One key factor established by the present studies as an important determinant of the mechanism of inactivation of HIV-1 PR by agents that alkylate protein residues is the intrinsic reactivity of the alkylating functionality. This reactivity must not be too high, as that favors reaction with the cysteine residues, or too low, as that prevents reaction with the protein. The catalytic activity of HIV-2 PR in which both cysteines have been replaced by unreactive amino acids provides a clear indication that irreversible inhibitors that target the cysteines of HIV-1 PR may be subject to the development of resistance due to mutation of the cysteines to unreactive amino acids.[19] The *trans*-1,2-disubstituted epoxide functions in **1** and **2** appear to be of too low a reactivity to alkylate either the aspartates or the cysteines. EPNP and the cyclic *cis*-1,2-disubstituted epoxide **6**, on the other hand, appear to be insufficiently reactive to alkylate the cysteines but readily alkylate one, and only one, active site aspartate residue. These results support a mechanism in which the epoxide reactivity is enhanced by hydrogen bonding to the protonated aspartate in the active site with concomitant addition of the second aspartate, which is ionized, to the epoxide ring (Figure 8). The difference in the ability of *cis*- and *trans*-1,2-disubstituted epoxides to inactivate the enzyme presumably is due to the higher steric hindrance to backside addition of the carboxyl group to the *trans*-disubstituted epoxide. This steric factor, however, only accounts for part of the reactivity difference because two trisubstituted epoxides also slowly inactivate the enzyme. The unsaturated ketones **8**−**10**, in contrast, are too reactive toward sulfhydryl groups and primarily inactivate HIV-1 PR by reacting with the cysteine residues. The orientation of the inhibitor when reversibly bound in the active site is likely to contribute to the specificity of the inactivation reaction, although substrate orientation is unlikely to be of much importance in the reactions of a compound as small as EPNP. Furthermore, crystallographic evidence that a haloperidol derivative is readily bound in at least two almost orthogonal orientations in the active site of HIV-1 PR suggests that there is sufficient freedom of movement for these compounds within the active site to allow the catalytic aspartate residues to interact with most regions of the inhibitors.[26]

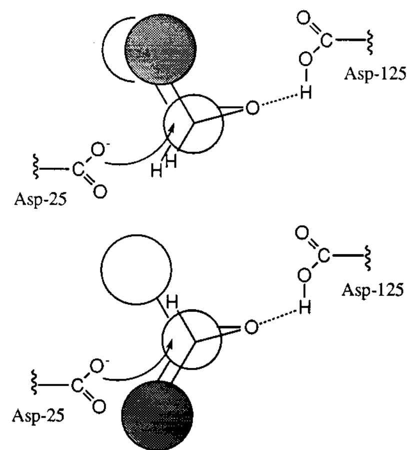


Figure 8. Proposed mechanism for hydrogen-bonding-assisted alkylation of the catalytic aspartate group by 1,2-disubstituted epoxides. The steric basis for the higher reactivity of *cis*- relative to *trans*-disubstituted epoxides is the steric effect shown schematically in the figure, in which the alkyl substituents are represented by spheres.

A model for the alkylation specificity found here for HIV-1 PR is provided by the recent finding that the hydrolysis of epoxides by epoxide hydrolase is a two-step process, the first step of which is addition of an aspartate to the epoxide to give an ester adduct.[28−31] In the second step, the ester is hydrolyzed to the diol metabolite with concomitant regeneration of the enzyme catalyst. Earlier data on the hydrolysis of epoxides

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showed that *cis*-disubstituted epoxides are hydrolyzed more readily than *trans*-disubstituted epoxides by the membrane-bound enzyme. For example, the rates for hydrolysis of the epoxides from *cis*- and *trans*-stilbene by the microsomal epoxide hydrolase differ by a factor of 785.[32] In view of the recently elucidated mechanism of epoxide hydrolysis, the differences in rate for the processing of *cis*- and *trans*-disubstituted epoxides provide a model for the selectivity observed in this study of HIV-1 PR.

The present results indicate that efforts to construct clinically useful inactivating agents for HIV-1 PR should incorporate a functionality with the reactivity of a cyclic *cis*-1,2-disubstituted epoxide, preferably one which, like the epoxide, is activated toward nucleophilic addition by specific hydrogen bonding with one of the catalytic aspartate groups. Moieties with the reactivity of a conjugated ketone are intrinsically too reactive to target the aspartate groups and are unsuitable, and those with the reactivity of a *trans*-1,2-disubstituted epoxide are too unreactive and are likely to be ineffective.

Experimental Section

General Procedures. 1,2-Epoxy-3-(*p*-nitrophenoxy)propane (EPNP) was supplied by Sigma (St. Louis, MO). The syntheses of the haloperidol derivatives **8**−**10** were reported elsewhere.[23] Tetrahydrofuran and ether were dried over sodium/benzophenone and distilled under argon immediately prior to use. HIV-1 protease was expressed and purified as described previously.[19] The Q7K HIV-1 mutant was used instead of the wild type enzyme since it is more resistant to autoproteolysis.[27]

Melting points were determined with a Thomas capillary melting point apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were obtained at 300 MHz on a GE QE-300 instrument. Infrared spectra were recorded on a Nicolet 5DX FT-IR instrument. Mass spectra were obtained at the University of California, San Francisco, Mass Spectrometry Facility. Elemental analyses were performed by the Microanalytical Laboratory, University of California, Berkeley.

1-Phenyl-1,2-epoxy-3-bromopropane. A solution of 80% *m*-chloroperbenzoic acid (mCPBA) (8.5 mmol) in 10 mL of CH_2Cl_2 was added to a solution of cinnamyl bromide (571 mg, 2.9 mmol) in 30 mL of CH_2Cl_2. To the resulting solution was added 20 mL of 0.5 N $NaHCO_3$, and the heterogeneous mixture was stirred at ∼25 °C for 18 h. A few drops of methyl ethyl sulfide were then added to destroy the excess peroxide. The resulting mixture was washed with sodium bicarbonate several times. Evaporation of the solvent provides a crude residue (787 mg): ^1H NMR ($CDCl_3$) 3.31 (m, 1H, C**H**CH_2Br), 3.50−3.52 (dd, 2H, C**H$_2$**Br), 3.82 (d, 1H, J = 2 Hz, C**H**OCHCH_2Br), and 7.3−7.5 (m, 5H, aromatic) ppm; MS *m/z* (%) 215 (MH$^+$, 6), 213, 171 (18). The analytical data are consistent with literature data.[33]

4-Hydroxy-4-(*p*-chlorophenyl)-*N*-(2,3-epoxy-3-phenylpropyl)piperidine (1). K_2CO_3 (386 mg, 2.7 mmol) and 4-(*p*-chlorophenyl)-4-hydroxypiperidine (485 mg, 2.3 mmol) were added to a solution of the crude 1-phenyl-1,2-epoxy-3-bromopropane (700 mg, 12.3 mmol) in DMF, and the mixture was refluxed for 1.5 h. The suspension was then filtered, and the precipitate was washed with ethyl acetate. Evaporation of the solvent from the filtrate followed by flash chromatography on silica gel with ethyl acetate as the eluant provided 128 mg of purified material (16% yield): IR (neat) 3383 (br), 2950, 2821, 1678, 1635, 1604, 1493, 1462, 1370, 1098, 1048, 1011, 906, and 820 cm^{-1}; ^1H NMR ($CDCl_3$) 7.3−7.5 (m, 9H, aromatic), 3.66 (dd, 1 H, J = 2 Hz, CHOC**H**Ph), 3.21 (m, 1 H, C**H**OCHPh), 3.01 (br d, 1 H, J = 11.25 Hz, C**H**$_{eq}$ HN), 2.87 (dd, 2H, J = 3.8 Hz, J = 13 Hz, C**H$_{eq}$**-NCH**H**), 2.62 [m, 3H, (C**H$_{ax}$**)$_2$NCH**H**], 2.15 [m, 2H, (C**H$_{ax}$**)$_2$COH], and 1.77 [m, 2H, (C**H$_{eq}$**)$_2$COH] ppm; ^{13}C NMR ($CDCl_3$) 146.82, 137.01, 132.81, 128.50, 128.42, 126.08, 125.65 (aromatic), 70.75 (**C**OH), 60.7, 60.5 (**C**HO**C**H), 56.9 (N**C**H$_2$), 49.7, 49.9 [piperidine (**C**H$_2$)$_2$N], and 38.3 [piperidine (**C**H$_2$CH$_2$)$_2$N] ppm; MS (LRCI) *m/z* (%) 344 (MH$^+$, 100), 224 (20), 212 (25); HRMS for $C_{20}H_{22}ClNO_2$, calcd 343.1339, found 343.1337.

***trans*-4-Phenyl-1-bromo-3-butene.** Chlorotrimethylsilane (1.24 g, 11.5 mmol) was slowly added at 0 °C to a suspension of lithium bromide (1.1 g, 12.6 mmol) in a solution of α-cyclopropylbenzyl alcohol (1.56 g, 10.5 mmol) in CH_2Cl_2.[34] The mixture was then stirred at room temperature for 3 h before it was poured into water. The organic phase was separated, washed with water and brine, and dried over anhydrous Na_2SO_4. Solvent evaporation yielded a crude product that appears as a single spot on TLC. The crude product was filtered through silica gel to remove polar impurities, yielding 1.41 g (63%) or the desired product: ^1H NMR ($CDCl_3$) 7.20−7.4 (m, 5H, aromatic), 6.4−6.5 (d, J = 15 Hz, 1H, ArC**H**), 6.13−6.23 (dt, J = 15 Hz, J = 7 Hz, 1H, ArCHC**H**), 3.46 (t, J = 7 Hz, 2H, CH_2Br), and 2.77 (AB q, J = 7 Hz, C**H$_2$**CH$_2$Br) ppm; MS (LREI) *m/z* (%) 212, 210 (M$^+$, 28, 30), 131 (M$^+$ − Br, 100), 117 (95), 91 (48). The analytical data are consistent with literature data.[35]

***trans*-4-Phenyl-3,4-epoxy-1-bromobutane.** A solution of *trans*-4-phenyl-1-bromo-3-butene (811 mg, 3.8 mmol) and 800 mg (3.8 mmol) of mCPBA in a biphasic mixture of CH_2Cl_2 and 0.5 N $NaHCO_3$ was stirred for 18 h at ∼25 °C. After workup as described for 1-phenyl-1,2-epoxy-3-bromopropane, 770 mg of product was isolated (90% yield): IR (neat) 3037, 2987, 2358, 1771, 1728, 1505, 1468, 1437, 1277 cm^{-1}; ^1H NMR ($CDCl_3$) 7.2−7.4 (m, 5 H, arom H), 3.72 (d, 1 H, J = 1.8 Hz, ArC**H**O), 3.54 (m, 2 H, C**H$_2$** Br), 3.10 (td, J = 1.8 Hz, J = 5.3 Hz, 1 H, ArCHOC**H**), and 2.24 (m, 2 H, C**H$_2$**CH$_2$Br) ppm; MS (HREI) *m/z* (%) 228 (M$^+$, 25), 226, 147 (M$^+$ − Br, 12), 133 (28), 119 (30), 105 (26), 90 (100), 77(30); HRMS for $C_{10}H_{11}BrO$, calcd 227.9975, found 227.9973.

4-(*p*-Chlorophenyl)-4-hydroxy-*N*-(3,4-epoxy-4-phenylbutyl)piperidine (2). A mixture of 702 mg (3.1 mmol) of 4-(*p*-chlorophenyl)-4-hydroxypiperidine, 675 mg (3.2 mmol) of *trans*-4-phenyl-3,4-epoxy-1-bromobutane, and 441 mg (3.2 mmol) of K_2CO_3 in DMF was refluxed for 2.5 h. The suspension was then filtered and the solvent removed from the filtrate. The residue was redissolved in ether, and the solution was washed with water and brine and then dried over anhydrous Na_2SO_4. Evaporation of solvent provided a residue that was subjected to flash chromatography on silica gel with ethyl acetate as the solvent. The desired product was isolated in low yield (62.7 mg, 5%): ^1H NMR ($CDCl_3$) 7.28−7.5 (m, 9 H, aromatic), 4.27 (t, 2 H, J = 5.9 Hz, NC**H$_2$**-CH$_2$), 4.03 [br, 2 H, (C**H$_{eq}$**)$_2$N], 3.64 (d, 1 H, J = 2 Hz, OC**H**Ph), 3.26 (br, 2 H, (C**H$_{ax}$**)$_2$], 3.05 (br t, 1 H, J = 5.7 Hz, J = 2 Hz, C**H**OCHPh), 2.02 (m, 2 H, NCH$_2$C**H$_2$**), 1.9 [td, 2 H, J = 13.4 Hz, J = 4.55 Hz, (C**H$_{ax}$**)$_2$CH$_2$N], and 1.6−1.7 [d, 2 H, J = 13.4 Hz, (C**H$_{eq}$**)$_2$CH$_2$N] ppm; ^{13}C NMR ($CDCl_3$) 155.1, 146.44, 137.17, 132.97, 128.16, 128.43, 125.93, 125.51, 71.03, 62.15, 60.20, 58.33, 39.91, 37.85, and 32.12 ppm; MS (LSIMS) *m/z* (%) 358 (MH$^+$, 100); (LREI) 357 (M$^+$, 5), 339 (M$^+$ − 18, 10), 238 (M$^+$ − 119, 20), 176 (100), 146 (65). HRMS for $C_{21}H_{24}ClNO_2$, calcd 357.1496, found 357.1507.

4-(*p*-Chlorophenyl)-1,2,5,6-tetrahydropyridine. A mixture of 4-(*p*-chlorophenyl)-4-hydroxypiperidine (1 g, 4.7 mmol), acetic acid (12 mL), and hydrochloric acid (6 mL) was refluxed for 2 h. After pouring the reaction mixture into an ice−water mixture, solid NaOH was added to make the solution basic. The white precipitate that formed was collected and washed extensively with water. After drying, 794 mg of white flakes was isolated (85% yield): mp 124−126 °C; ^1H NMR ($CDCl_3$) 7.29 (m, 4H, aromatic), 6.12 (br s, 1 H, C**H**CH$_2$NH), 3.28 (d, 2 H, J = 2.5 Hz, CHC**H$_2$**NH), 3.10 (dd, 2 H, J = 5.6 Hz, CH$_2$C**H$_2$**-NH), and 2.42 (br s, 2 H, C**H$_2$**CH$_2$NH) ppm. NMR data were identical to those of the free base of a commercial sample.

***N*-FMOC-4-(*p*-Chlorophenyl)-1,2,5,6-tetrahydropyridine.** A solution of 4-(*p*-chlorophenyl)-1,2,5,6-tetrahydropyridine (456 mg, 2.4 mmol) and 4-(dimethylamino)pyridine (2.4 mmol) in $CHCl_3$ was cooled to 0 °C. To this solution was slowly added a $CHCl_3$ solution of 9-fluorenylmethyl chloroformate (619 mg, 2.4 mmol). The reaction mixture was stirred at 0 °C for 30 min and then at ∼25 °C for 2 h before it was sequentially washed with 1 N HCl, water, and brine. Evaporation of the solvent after drying over sodium sulfate gave 650 mg of a light yellow solid (65% yield) that was recrystallized from $CHCl_3$/MeOH: mp 118−120 °C: IR ($CHCl_3$) 3024, 1703, 1493, 1456,

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1431, 1295, 1215, 1116 cm^{-1}; ^{1}H NMR (CDCl$_3$) 7.25−7.42 (m, 8 H, aromatic), 7.6 (d, 2 H, J = 7.3 Hz, aromatic), 7.77 (d, 2 H, J = 7.3 Hz, aromatic), 6.03 (br s, 1 H, NCH$_2$C**H**), 4.47 (d, 2 H, J = 6.78 Hz, FMOC-C**H$_2$**), 4.27 (t, 1 H, J = 6.5 Hz, FMOC-C**H**), 4.13 (br s, 2 H, NC**H$_2$**CH), 3.68 (br s, 2 H, NC**H$_2$**CH$_2$), and 2.49 (br s, 2 H, NCH$_2$C**H$_2$**) ppm; ^{13}C NMR (CDCl$_3$) 174.9, 144.4, 141.3, 138.9, 133.09, 128.54, 127.66, 127.01, 126.18, 124.94, 119.96, 67.3, 47.36, 43.72, 42.67, 40.47, and 27.13 ppm; MS (LSIMS) m/z (%) 416 (MH$^+$, 100), 238 (78); HRMS for $C_{26}H_{22}ClNO_2$, calcd 415.1339, found 415.1329.

***N*-FMOC-4-(*p*-chlorophenyl)-3,4-epoxypiperidine (5).** To a solution of *N*-FMOC-4-(*p*-chlorophenyl)-1,2,5,6-tetrahydropyridine (200 mg, 0.48 mmol) in CH$_2$Cl$_2$ was added a solution of 80% mCPBA (126 mg, 0.7 mmol) in CH$_2$Cl$_2$, and the reaction mixture was stirred for 18 h. A few drops of methyl ethyl sulfide were added to destroy excess peroxide. The solution was then washed sequentially with 5% NaHCO$_3$, water, and brine. Drying over Na$_2$SO$_4$ and evaporation of the solvent provided a crude solid that was purified by flash chromatography on silica gel (hexane/ethylacetate, 6:4). The desired product (163.7 mg, 79% yield) was thus obtained: IR (CHCl$_3$) 3024, 1697, 1456, 1431, 1339, 1246, 1221, 1123, 1098, 1018 cm^{-1}; ^{1}H NMR (CDCl$_3$) 7.77−7.30 (m, 12 H, aromatic), 4.47 (d, 2 H, J = 6.5 Hz, OC**H$_2$**CH), 4.26 (t, 1 H, J = 6.4 Hz, OCH$_2$C**H**), 3.8−4.2 (br m, 2 H, C**H$_2$**NC**H$_2$**), 3.6−3.8 (br d, 1 H, NCH$_2$C**H**OC), 3.1−3.3 (br m, 2 H, C**H$_2$**NC**H$_2$**), and 2.0−2.5 (br m, 2 H, NCH$_2$C**H$_2$**) ppm; ^{13}C NMR (CDCl$_3$) 143.94, 141.33, 133.84, 128.64, 127.67, 127.03, 126.66, 124.83, 119.94, 67.36, 58.52, and 47.53 ppm; MS (LSIMS) m/z (%) 432 (MH$^+$, 90), 391 (54); HRMS for $C_{12}H_{11}NClO_3$, calcd 252.0427, found 252.0418. Anal. Calcd for $C_{26}H_{22}ClNO_3$: C, 73.3; H, 5.13; N, 3.24. Found: C, 73.78; H, 5.37; N, 3.24.

1-(4′-Fluorophenyl)-4-iodobutane. To a solution of 850 mg (5.18 mmol) of *p*-fluorophenyl cyclopropyl ketone in CHCl$_3$ were added 4.6 g (31 mmol) of NaI and a catalytic amount of tetrabutylammonium iodide followed by 1.6 g (15.5 mmol) of (CH$_3$)$_3$SiCl.[36] The solution was stirred for 18 h and was then poured into a solution of NaHCO$_3$. The excess iodide was removed by the addition of a saturated solution of sodium thiosulfate. The organic layer was separated and washed with water and brine. Evaporation of the solvent after drying over anhydrous Na$_2$SO$_4$ yielded a crude product that was dissolved in hexane/ethyl acetate (9:1) and filtered through a short column of silica gel. Evaporation of the solvent yields a dark brown liquid, 1.41 g (94% yield), that is best stored at 0 °C because it decomposes at room temperature: ^{1}H NMR (CDCl$_3$) 8.00 (m, 2 H, aromatic), 7.14 (m, 2 H, aromatic), 3.32 (t, 2 H, J = 6.7 Hz, CH$_2$I), 3.11 (m, 2 H, CH$_2$CO), and 2.25 (m, 2 H, C**H$_2$**CH$_2$I) ppm; ^{13}C NMR (CDCl$_3$) 196.56, 130.41, 130.29, 115.56, 115.27, 38.57, 27.25 and 6.58 ppm.

***N*-(4-*p*-Fluorophenyl-4-oxobutyl)-4-(*p*-chlorophenyl)-3,4-epoxypiperidine (3).** To a solution of 138 mg (0.32 mmol) of *N*-FMOC-4-(*p*-chlorophenyl)-3,4-epoxypiperidine in 1.5 mL of DMF was added 0.1 mL of piperidine. The solution was stirred for 0.5 h at ∼25 °C before the solvent was removed on a rotary evaporator. The solid residue was redissolved in 10 mL of DMF before 44 mg (0.32 mmol) of K$_2$CO$_3$ and 93.4 mg (0.32 mmol) of 1-(4′-fluorophenyl)-4-iodobutane were sequentially added. The reaction mixture was gently refluxed for 0.5 h. The solvent was then evaporated, and the residue was redissolved in CHCl$_3$ and washed with 5% NaHCO$_3$, water, and brine. The solution was dried over anhydrous Na$_2$SO$_4$. Filtration and evaporation of solvent gave a dark brown residue that, after flash chromatography on silica gel with ethyl acetate, provided 76.8 mg (64% yield) of the desired product: IR (neat) 3018, 2950, 1684, 1604, 1221, 1160, and 1098 cm^{-1}; ^{1}H NMR (CDCl$_3$) 7.0−8.1 (m, 8 H, aromatic), 3.18 (m, 1 H, epoxide), 3.12 (m, 1 H, CHCH$_{ax}$H$_{eq}$N), 2.98 (t, 2 H, NC**H$_2$**CH$_2$CH$_2$CO), 2.55 (d, 1 H, J = 11.2 Hz, CHCH$_{ax}$H$_{eq}$N), 2.3−2.55 (m, 5 H, C**H**HC**HH**NCH$_2$CH$_2$C**H$_2$**CO), 2.08 (m, 1 H, CH**H**CH$_2$N), and 1.95 (m, 2 H, NCH$_2$C**H$_2$**CH$_2$CO) ppm; ^{13}C NMR (CDCl$_3$) 133.42, 130.57, 130.68, 128.46, 126.85, 115.76, 115.46, 60.54, 58.56, 56.98, 52.15, 46.52, 36.06, 29.07, and 21.56 ppm; MS (HREI) m/z (%) 373 (5), 356 (12), 235 (100), and 222 (52); HRMS for $C_{21}H_{21}ClFNO_2$, calcd 373.1235, found 373.1245.

4-(*p*-Chlorophenyl)-4-hydroxy-*N*-(4-ethenylbenzoyl)piperidine. To a CH$_2$Cl$_2$ solution of 4-ethenylbenzoic acid (340 mg, 2.3 mmol) was added 2.3 mmol of oxalyl chloride at 0 °C followed by a few drops of DMF to initiate the reaction. After 30 min a solution of 4-(*p*-chlorophenyl)-4-hydroxypiperidine (338 mg, 1.6 mmol) in CH$_2$Cl$_2$ was added, and the reaction mixture was stirred at ∼25 °C for 1.5 h. The solution was sequentially washed with 5% NaHCO$_3$, water, and brine before it was dried and the solvent removed. Purification of the crude residue by flash chromatography on silica gel (hexane/ethyl acetate, 6:4) provided 172 mg of product (30% yield): mp 138−140 °C; ^{1}H NMR (CDCl$_3$) 7.2−7.5 (m, 8 H, aromatic), 6.6−6.7 (dd, 1 H, J = 10.9 Hz, J = 17.6 Hz, vinyl H), 5.79 (d, 1 H, J = 17.6 Hz, vinyl CHC**H$_{cis}$**CH$_{trans}$), 5.32 (d, 1 H, J = 10.9 Hz, vinyl CHCH$_{cis}$C**H$_{trans}$**), 4.6 (br, 1 H, O**H**), 3.2−3.7 [br m, 4 H, (C**H$_2$**)$_2$N)], and 1.8−2.8 [br m, 4 H, (C**H$_2$**CH$_2$)$_2$N] ppm; ^{13}C NMR (CDCl$_3$) 38.17, 38.95, 43.80, 71.39, 115.30, 125.96, 126.23, 127.23, 128.63, 135.10, 136.04, 138.98, 146.08, and 170.19 ppm; MS (HREI) m/z (%) 341 (15), 323 (15), 131 (100); HRMS for $C_{20}H_{20}ClNO_2$, calcd 341.1183, found 341.1179.

4-(*p*-Chlorophenyl)-4-hydroxy-*N*-[4-(1,2-epoxyethyl)benzoyl]piperidine (4). 4-(*p*-Chlorophenyl)-4-hydroxy-*N*-(4-ethenylbenzoyl)-piperidine (102 mg, 0.29 mmol) was epoxidized as described above. Flash chromatography provided 41.5 mg (40%) of the product: mp 90 °C; ^{1}H NMR (CDCl$_3$) 7.2−7.45 (m, 8 H, aromatic), 4.64 (br s, 1 H, CH**H**N), 3.88 (br t, 1 H, PhC**H**OCH$_{cis}$H$_{trans}$), 3.2−3.7 (br m, 3 H, piperidine H), 3.17 (t, 1 H, J = 4.6 Hz, PhCHOC**H$_{cis}$**H$_{trans}$), 2.7 (dd, 1 H, J = 2.3 Hz, J = 5.3 Hz, PhCHOH$_{cis}$**H$_{trans}$**), and 1.6−2.2 (br m, 4 H, piperidine H) ppm; ^{13}C NMR (CDCl$_3$) 169.97, 146.07, 139.36, 135.77, 133.21, 128.58, 127.16, 125.94, 125.6, 71.27, 51.95, 51.26, 43.81, 38.87, 38.29, and 37.72 ppm.

***N*-FMOC-1,2,5,6-tetrahydropyridine.** To a solution of 455 mg (5.4 mmol) of 1,2,5,6-tetrahydropyridine in CH$_2$Cl$_2$ was added 668 mg (5.4 mmol) of 4-(dimethylamino)pyridine, and the resulting solution was cooled to 0 °C. A solution of 1.41 g (5.4 mmol) of 9-fluorenylmethyl chloroformate in CH$_2$Cl$_2$ was slowly added, after which the cooling bath was removed and the mixture was stirred for 2 h. The solution was washed with 1 N hydrochloric acid, water, and brine. Evaporation of solvent after drying over anhydrous Na$_2$SO$_4$ leaves a residue that upon recrystallization from CHCl$_3$/CH$_3$OH gave 1.3 g (77% yield) of fine yellow needles: mp 94 °C; IR (CHCl$_3$) 3024, 1696, 1455, 1431, 1283, 1239, 1215, 1116, and 1029 cm^{-1}; ^{1}H NMR (CDCl$_3$) 7.31−7.75 (m, 8 H, aromatic), 5.6−5.8 (br d, 2 H, **H**CC**H**), 4.41 (m, 2 H, OCH$_2$), 4.24 (m, 1 H, OCH$_2$C**H**), 3.96 (br s, 2 H, NC**H$_2$**CH), 3.56 (br s, 2 H, NC**H$_2$**CH$_2$), and 2.15 (br s, 2 H, NCH$_2$C**H$_2$**) ppm; ^{13}C NMR (CDCl$_3$) 174.69, 144.10, 141.31, 127.61, 126.99, 124.98, 119.93, 67.29, 47.37, 43.44, 40.57, 33.68, and 24.94 ppm; MS (LSIMS) m/z 306 (MH$^+$, 100). Anal. Calcd for $C_{20}H_{19}NO_2$: C, 78.66; H, 6.27; N, 4.59. Found: C, 78.48; H, 6.35; N, 4.51.

***N*-FMOC-3,4-epoxypiperidine (6).** To a solution of 300 mg (0.98 mmol) of *N*-FMOC-1,2,5,6-tetrahydropyridine in CH$_2$Cl$_2$ was added a solution of 700 mg (3.2 mmol) of mCPBA in CH$_2$Cl$_2$ and 10 mL of 0.5 M NaHCO$_3$. The reaction mixture was stirred for 18 h at ∼25 °C. Excess peroxide was destroyed by the addition of a few drops of methyl ethyl sulfide (starch-iodide paper test). The organic phase was washed sequentially with aqueous NaHCO$_3$, water, and brine and was then dried over anhydrous Na$_2$SO$_4$. Solvent removal and purification by flash chromatography on silica gel with hexane/ethyl acetate (6:4) gave 280 mg (89% yield) of crystalline material: mp 110 °C; IR (CHCl$_3$) 3018, 1703 1481, 1450, 1431, 1339, 1289, 1252, 1215, 1110, 1055, 962, and 752 cm^{-1}; ^{1}H NMR (CDCl$_3$) 7.2−7.8 (m, 8 H, aromatic), 4.41 (m, 2 H, OCH$_2$), 4.24 (m, 1 H, OCH$_2$C**H**), 3.70−4.00 (m, 2 H, NC**H**HC**H**O), 3.18−33.6 (m, 4 H, C**H$_2$**NCH**H**CHOC**H**), and 1.8−2.2 (m, NCH$_2$C**H$_2$**) ppm; ^{13}C NMR (CDCl$_3$) 155.28, 143.92, 141.27, 127.61, 126.98, 124.87, 119.90, 67.31, 50.44, 47.28, 42.39, 37.30, and 24.10 ppm; MS (LSIMS) m/z (%) 322 (MH$^+$, 35), 179 (99), 178 (100). Anal. Calcd for $C_{20}H_{19}NO_3$: C, 74.10; H, 5.96; N, 4.29. Found: C, 74.10; H, 5.98; N, 4.29.

FMOC-4,5-epoxy-Leu Methyl Ester (7). A methanol solution of FMOC-4,5-dehydro-Leu-OH (100 mg, 0.28 mmol) was treated with an excess of diazomethane in ether. Evaporation of the solvent gave a quantitative yield of the ester: ^{1}H NMR (CDCl$_3$) 7.25−7.7 (m, 8 H, aromatic), 5.27 (d, 1 H, J = 7.8 Hz, NH), 4.7−4.8 (d, 2 H, J = 30 Hz, vinyl H), 4.52 (m, 1 H, NHC**H**COOH), 4.38 (d, 2 H, J = 7.2 Hz, CHC**H$_2$**O), 4.22 (m, 1 H, J = 7.2 Hz, C**H**CH$_2$O), 3.74 (S, 3 H, OCH$_3$), 2.55 (dd, 1 H, J = 5.2 Hz, J = 13.2 Hz, CHC**H**HC), 2.40 (dd, 1 H, J = 8.3 Hz, J = 13.2 Hz CHCH**H**C), and 1.75 (s, 3 H, −CH$_3$) ppm; MS (HREI) m/z (%) 365.2 (M$^+$, 12), 178.1 (100). The crude FMOC-4,5-

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dehydro-Leu methyl ester (100 mg, 0.27 mmol) was subjected to epoxidation as described above. Purification by flash chromatography yielded 85.4 mg (83%) of the epoxide: ^{1}H NMR ($CDCl_3$) 7.32−7.8 (m, 8H, aromatic), 5.5 (dd, 1 H, NH), 4.42 (m, 1 H, NHC**H**$COOCH_3$), 4.3 (m, 2 H, CHC**H$_2$**O), 4.15 (m, 1 H, C**H**CH_2O), 3.68 (d, 3 H, OCH_3), 22.52 (S, 2 H, epoxide HH), 2.05−2.2 (2 dd, 1 H, $J = 5$ Hz, $J = 14$ Hz, CHC**H**HC), 1.72−1.85 (m, 1 H, CHCH**H**C), and 1.3 (s, 3 H, $-CH_3$) ppm; MS (HREI) m/z (%) 381 (M^+, 8), 178 (100); HRMS for $C_{22}H_{23}NO_5$, calcd 381.1576, found 381.1568.

HIV Protease Expression and Purification. Recombinant HIV-1 PR was expressed in *Escherichia coli* strain X90 using the pT2Cb3 PR vector.[10] The enzyme was purified to homogeneity as previously described.[20] Concentrations of active HIV-1 PR were determined by active site titration using the peptidomimetic inhibitor U-85548 (a gift of Dr. A. Tomasselli, Upjohn), Val-Ser-Gln-Asn-Leu-ψ-[CH(OH)CH_2]-Val-Ile-Val.[5]

Inactivation of HIV Proteases. HIV-1 PR (concentration ∼50 μg/mL) was inhibited by EPNP by preincubation at ∼25 °C in a buffer containing 250 mM sodium acetate buffer (pH 5.5), 0.5 M NaCl, 1 mM EDTA, 0.5 mM DTT, and 10% DMSO in the presence of 5 mM EPNP. For quantitation of the irreversible inactivation of HIV proteases by haloperidol and FMOC derivatives, HIV-1 Q7K PR (15 μg/mL final concentration) was preincubated at 25 °C in 50 mM HEPES, pH 8.0, containing 1 M NaCl, 1 mM EDTA, 0.5 mM DTT, and 5% DMSO in the presence of 10 μM to 1 mM of each inhibitor. Baseline measurements were carried out using 5% DMSO in the absence of inhibitors. At various times, aliquots were removed and assayed for activity. The HIV protease was assayed against the fluorescent substrate ABZ-TInLF(NO_2)QR-NH_2. The enzyme was assayed at pH 5.5 in 50 mM acetate buffer containing 1 M NaCl, 1 mM EDTA, and 1 mM DTT as previously described.[37] For calculation of the rates of inactivation, kinetic data were fit to a pseudo-first-order equation according to the formula $\ln(v_i/v_o) = -k_{obs}t$. From a plot of inactivation rates (k_{obs}) *versus* the square of the inhibitor concentrations, k_{inact}, the inhibitor concentration resulting in half-maximal inactivation, and V_{inact}, the maximum inactivation rate, were calculated. In preparing the proteins for digestion and mass spectrometric work, the loss of activity was monitored by a UV assay using the peptide Arg-Val-nLeu-Phe-(p-NO_2)-Glu-Ala-nLeu-Ser-NH_2 (Bioserv, San Diego, CA). Aliquots of the protease/inhibitor complex were removed, and the protease activity was assessed by measuring the change in UV absorbance at 300 nm, the characteristic absorbance maximum of the substrate.

HPLC Separation of the Inhibited Proteases. The inhibition products were isolated by reversed phase HPLC on a C-4 column (4.6 × 250 mm, Vydac) using a 75-min gradient from 30% to 55% solvent B (acetonitrile containing 0.08% trifluoroacetic acid) against solvent A (water containing 0.1% trifluoroacetic acid). The flow rate was 1 mL/min, and the products were monitored by a UV/vis detector. Fractions were manually collected and lyophilized for further characterization.

Protein Molecular Weight Determination. The HPLC-purified fractions were analyzed by electrospray ionization mass spectrometry[38] on a VG Platform single quadrupole mass spectrometer equipped with an electrospray ion source. The samples were dissolved in an aqueous solvent (40% acetonitrile and 1% acetic acid), and 5 μL aliquots (approximately 10 pmol/μL) were injected into the mass spectrometer. The solvent system for batch mode electrospray contained 10% 2-propanol/40% acetonitrile in H_2O and 50 pmol/μL gramicidin. The flow rate was 5 μL/min, and the mass range was set at 400−2000 with a sweep rate of 7 s/scan. Approximately 10 scans were accumulated into one spectrum for a better signal to noise ratio. The average molecular weight[39] of each species was calculated on the basis of the multiple charge nature of electrospray ionization.

Enzymatic Digestion. The HPLC fractions containing the modified protease were subjected to enzymatic digestion to determine the site of modification of the inhibitor. For tryptic digestion, the samples were dissolved in 20 μL of 8 M urea and then diluted by adding 80 μL of 100 mM ammonium bicarbonate, pH 8.3, to a final concentration of 80 mM NH_4HCO_3 and 1.6 M urea. Enzymes were then added to a concentration of ∼4% of the sample. The digestion was carried out at 37 °C for 4 h but in some cases was continued overnight. For digestion by pepsin, the samples were first dissolved in 70% formic acid, and then 20 volumes of 1 mM HCl containing pepsin was added to the sample. The ratio of protein to enzyme was about 50 to 1. The digestion was carried out at room temperature for 0.5−2 h and stopped by lyophilization or by injection onto a C-18 reversed phase HPLC column.

Peptide Molecular Weight Measurement. Molecular weights of the enzymatic peptides were determined by LC/ESIMS. These experiments were performed on the VG Platform mass spectrometer equipped with an ABI Model 140B HPLC system, using a microbore C-18 reversed phase HPLC column (1 × 100 mm, ABI). A 50-min linear gradient from 2% to 52% solvent B was run at a flow rate of 50 μL/min. Solvent A was 0.1% formic acid in H_2O, and solvent B was 0.05% formic acid in 10% 2-propanol/90% ethanol. The mass spectrometer was set to a m/z range of 350−2100 with a sweep speed of 5 s/scan. Subsequent isolation of the modified peptides was carried out using an analytical C-18 HPLC column (Vydac 4.6 × 250 mm) employing conditions as described above.

Tandem Mass Spectrometry. Selected peptides were subjected to high-energy CID analysis on a Kratos Concept IIHH four-sector tandem mass spectrometer. Samples were introduced into the instrument through a flow probe with a flow rate of 3 μL/min. The delivery solvent contained 5% acetonitrile, 2% thioglycerol, and 0.1% trifluoroacetic acid in H_2O. The precursor ion was subjected to CID process in the collision cell at a helium gas pressure which resulted in an attenuation of its abundance to 30% of the original value. The resulting CID spectra were recorded on a rapid scanning multichannel array detector.[40,41]

Acknowledgment. Financial support was provided by National Institutes of Health Grant GM39522, NIH NCRR Biomedical Research Technology Program Grant RR01614, National Institute of Environmental Health Sciences Grant ES04705, and National Science Foundation Grant DIR 8700766. F.M. was a recipient of an AmFAR Fellowship, award number 70361-14-RF. We thank Fisons/VG for the loan of the platform electrospray mass spectrometer.

JA954069W

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