Inhibition of Human Immunodeficiency Virus-1 Protease by a C_2-Symmetric Phosphinate. Synthesis and Crystallographic Analysis‡

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ABSTRACT: The human immunodeficiency virus type 1 (HIV-1) protease is a potential target of acquired immune deficiency syndrome (AIDS) therapy. A highly potent, perfectly symmetrical phosphinate inhibitor of this enzyme, SB204144, has been synthesized. It is a competitive inhibitor of HIV-1 protease, with an apparent inhibition constant of 2.8 nM at pH 6.0. The three-dimensional structure of SB204144 bound to the enzyme has been determined at 2.3-Å resolution by X-ray diffraction techniques and refined to a crystallographic discrepancy factor, R ($= \sum||F_o| - |F_c||/\sum|F_o|$), of 0.178. The inhibitor is held in the enzyme active site by a set of hydrophobic and hydrophilic interactions, including an interaction between Arg8 and the center of the terminal benzene rings of the inhibitor. The phosphinate establishes a novel interaction with the two catalytic aspartates; each oxygen of the central phosphinic acid moiety interacts with a single oxygen of one aspartic acid, establishing a very short (2.2–2.4 Å) oxygen–oxygen contact. As with the structures of penicillopepsin bound to phosphinate and phosphonate inhibitors [Fraser, M. E., Strynadka, N. C., Bartlett, P. A., Hanson, J. E., & James, M. N. (1992) *Biochemistry 31*, 5201–14], we interpret this short distance and the stereochemical environment of each pair of oxygens in terms of a hydrogen bond that has a symmetric single-well potential energy curve with the proton located midway between the two atoms. Under identical assay conditions, SB204144 binds approximately 2 orders of magnitude more tightly than the monohydroxy analog A74704 [Erickson, J., Neidhart, D. J., VanDrie, J., Kempf, D. J., Wang, X. C., Norbeck, D. W., Plattner, J. J., Rittenhouse, J. W., Turon, M., Wideburg, N., Kohlbrenner, W. E., Simmer, R., Helfrich, R., Paul, D., & Knigge, M. (1990) *Science 249*, 527–33], apparently as a consequence of the stronger hydrogen bonds between the phosphinate oxygens and the catalytic aspartates. Implications for the catalytic mechanism of the novel mode of binding of the phosphinate group are discussed.

The recognition of acquired immune deficiency syndrome (AIDS) as a growing health threat of global proportions has led to extensive research on novel strategies to combat its etiological agent, the human immunodeficiency virus (HIV; Mitsuya et al., 1991). One such strategy is inhibition of the virus-encoded protease required for processing the viral *gag* and *gag–pol* polyproteins into enzymes and structural proteins necessary for the formation of infectious virions (Kohl et al., 1988; Meek et al., 1989). The protease is a 99 amino acid residue protein that functions as a homodimer. It was classified as a member of the aspartyl protease family on the basis of its active site sequence similarity (Toh et al., 1985; Power et al., 1986) and structural analogy to the well-characterized, monomeric enzymes renin, pepsin, rhizopuspepsin, endothiapepsin, and penicillopepsin (Pearl & Taylor, 1987). It is predominantly composed of β-strands; its active site is formed at the interface of the dimer and contains two aspartyl residues, one contributed by each subunit (Lapatto et al., 1989; Navia et al., 1989; Wlodawer et al., 1989). HIV protease undergoes considerable conformational changes upon complexation with inhibitors, particularly in the two "flaps" (flexible β-hairpin structures) which move by as much as 7 Å to tightly embrace the ligands (Miller et al., 1989; Erickson et al., 1990; Fitzgerald et al., 1990; Swain et al., 1990; Bone et al., 1991; Jaskolski et al., 1991; Dreyer et al., 1992, 1993; Murthy et al., 1992; Thompson et al., 1992; Tomaszek et al., 1992).

The catalytic mechanism of aspartyl proteases has been extensively studied by kinetic methods, affinity labeling studies, and X-ray crystallographic techniques [for reviews, see Fruton (1976, 1987), Kostka (1985), Polgar (1987), and Davies (1990)]. These studies are consistent with a general acid–general base mechanism of the two aspartyl residues, with a central water molecule bound between the carboxyl groups of these two residues as the nucleophile (Suguna et al., 1987a). The results of recent kinetic studies of HIV-1 protease are in accord with this general acid–general base mechanism (Hyland et al., 1991a,b).

One goal of our research program is to design inhibitors of the protease of HIV type 1 (the most common strain of this virus in the United States and Europe) as potential AIDS therapeutics. Much of this work has been guided by the crystallographic structure determination of selected inhibitors bound to the enzyme (Dreyer et al., 1992, 1993; Murthy et al., 1992; Tomaszek et al., 1992). Generally these inhibitors are peptide analogs designed to mimic a high-energy intermediate involved in peptidolysis (Dreyer et al., 1989; Moore et al., 1989; Tomasselli et al., 1990; Rich et al., 1990). Various nonhydrolyzable moieties, including the amino acid statine,

‡ The refined coordinates for the complex have been deposited in the Protein Data Bank under file name 1HOS.

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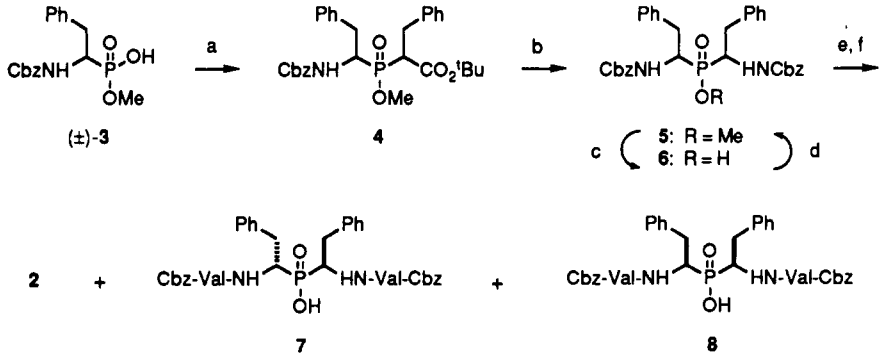
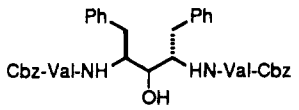
Scheme I[a]

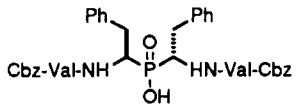


[a] (a) $SOCl_2$, CH_2Cl_2, 22 °C; $Ph(CH_2)_2CO_2tBu$, LDA, THF, −78 → −10 °C (67%). (b) TFA, 25 °C; $(PhO)_2PON_3$, BnOH, Et_3N, toluene, 90 °C, 15 h (28%). (c) NaOH, 2:1 MeOH/H_2O; HCl; HPLC ((±)-**6**, 29%; *meso*-**6**, 60%). (d) CH_2N_2, Et_2O, MeOH ((±)-**5**, 60%; *meso*-**5**, 46%). (e) H_2 (1 atm), Pd/C, MeOH, HCl; Cbz-Val, *i*-BuOCOCl, *N*-methylmorpholine, THF, −30 → 20 °C (From (±)-**5**: **2-OMe**, 25%; **7-OMe**, 24%. From *meso*-**5**: **8-OMe**, 50%). (f) Me_3SiBr, CH_2Cl_2, 25 °C (100%).



1

(A74704)



2

(SB204144)

FIGURE 1: HIV-1 protease inhibitors: **1**, pseudosymmetric monoalcohol; **2**, symmetric phosphinate.

secondary amines, hydroxyethylenes, hydroxyethylamines, and ketones, have been incorporated in these peptides to replace the scissile peptide bond [for reviews, see Greenlee (1990) and Abdel-Meguid (1993)]. An interesting variation on this theme is the recently described pseudosymmetric inhibitor A74704 (Figure 1) designed to complement the C_2 symmetry of HIV protease (Erickson et al., 1990). This inhibitor, however, is not perfectly C_2-symmetric owing to the nature of the carbon atom of the central hydroxymethylene moiety. Here we describe the synthesis of the highly potent, perfectly symmetric phosphinic acid analog SB204144 (Figure 1) and its three-dimensional structure bound to the enzyme. Analysis of the structure of this complex and its comparison with others provide an understanding of the interactions that impart high affinity to this inhibitor.

EXPERIMENTAL PROCEDURES

Synthesis of the Phosphinate Inhibitor SB204144 (Scheme I)

General Synthetic Procedures. THF[1] was distilled from sodium/benzophenone ketyl. Methylene chloride and DMF were anhydrous grade (Aldrich). Flash chromatography was carried out with Kieselgel 60 silica (E. Merck). TLC was performed on 0.25-mm silica gel F-254 plates (E. Merck). Analytical and preparative HPLC were performed in the normal mode with Dynamax silica (5 μm, Rainin) or in the reversed-phase mode with Ultrasphere ODS (Beckman). ^1H NMR spectra were recorded at 250 MHz on a Bruker AM 250 spectrometer. Electrospray ionization mass spectra (MS-(ESI)) were obtained with a Sciex API-III triple quadrupole mass spectrometer.

Methyl [2-Phenyl-1-[(carbobenzyloxy)amino]ethyl][2-phenyl-1-(tert-butylcarboxy)ethyl]phosphinate (**4**). Thionyl chloride (2.72 mL, 37.3 mmol) was added to a solution of methyl 2-phenyl-1-[(carbobenzyloxy)amino]ethylphosphonate (**3**) (11.8 g, 33.9 mmol) in CH_2Cl_2 (80 mL) under Ar. After 2 h the solution was concentrated, and residual HCl was removed by addition and reevaporation of CH_2Cl_2 and then THF (100 mL each) to provide the gummy phosphonyl chloride. Neat *tert*-butyl hydrocinnamate (21.0 g, 0.102 mol) was added over 10 min to a −78 °C solution of LDA prepared from *n*-butyllithium (67.5 mL, 1.51 M in hexane, 0.102 mol) and diisopropylamine (14.3 mL, 0.102 mol) in THF (200 mL) under Ar. After 15 min, a solution of the phosphonyl chloride in 125 mL of THF was added over 5 min, and the stirring mixture was then allowed to warm to ca. −10 °C over 30 min. Excess 10% HCl was added, the mixture was extracted with ether, and the organic layer was concentrated. Flash chromatography of the residue (400 g of silica; successive elution with 2 L each of 4:1, 1:1, and 1:2 hexanes/ethyl acetate) provided compound **4** (12.2 g, 67%) as a mixture of isomers (colorless glass): ^1H NMR ($CDCl_3$) δ 7.25 (15H, m), 5.00 (2H, m), 4.62 (1H, m), 3.89–3.70 (3H, m), 3.35–3.10 (4H, m), 2.95 (1H, m).

Methyl Bis[2-phenyl-1-[(carbobenzyloxy)amino]ethyl]-phosphinate (**5**). Compound **4** (0.88 g, 1.64 mmol) was dissolved in 5 mL of neat TFA. After 1 h, the solution was concentrated under vacuum and then reconcentrated successively from solutions of methanol, CH_2Cl_2, and benzene. The resulting foam was dissolved in toluene (9 mL) and refluxed with triethylamine (0.456 mL, 3.28 mmol) and diphenylphosphoryl azide (0.707 mL, 3.28 mmol) under Ar for 3 h. Benzyl alcohol (0.68 mL, 6.6 mmol) was added, and the mixture was heated for 12 h, cooled, and extracted with 10% HCl, 5% $NaHCO_3$, and water. The organic layer was concentrated, and the residue was purified by flash chromatography on 75 g of silica (gradient, 1:1 to 2:1 ethyl acetate/hexanes) to provide **5** (0.26 g, 27%) as a mixture of isomers.

(±)- *and meso-Bis[2-phenyl-1-[(carbobenzyloxy)amino]-ethyl]phosphinic acid* (**6**). Sodium hydroxide (5 mmol) was

[1] Abbreviations: ^1H NMR, proton nuclear magnetic resonance; Cbz, carbobenzyloxy; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; LDA, lithium diisopropylamide; mp, melting point; MS(ESI), electrospray ionization mass spectrometry; rms, root mean square; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin-layer chromatography.

added to a stirred solution of the isomers (**5**) (1.15 g, 1.96 mmol) in 2:1 methanol/water (25 mL). After 36 h the mixture was acidified with 10% HCl and extracted with CH_2Cl_2. The organic layer was concentrated, and the residue was dissolved in 10 mL of methanol. Crystals of pure *meso*-**6** (0.30 g, 26%) precipitated within 12 h. The supernatant was concentrated, redissolved in 30 mL of 7:2:1:0.01 methanol/water/DMF/TFA, and purified in three portions by HPLC (41 mm × 25 cm Ultrasphere ODS) with the same solvent mixture. The first isomer to elute was (±)-**6** (0.330 g, 29%), followed by additional *meso*-**6** (0.20 g, 18%). For (±)-**6**: mp 164–166 °C; ^{1}H NMR ($CDCl_3/CD_3OD$) δ 7.25–7.00 (20H, m), 4.89–4.46 (4H, m), 4.13 (2H, m), 3.12 (2H, dm), 2.70 (2H, m); MS(ESI) m/z 573 $[M + H]^+$. For *meso*-**6**: mp 211–212 °C; ^{1}H NMR ($CDCl_3/CD_3OD$) δ 7.10–6.97 (20H, m), 4.80 (4H, dd), 4.12 (2H, m), 3.11 (2H, dm), 2.55 (2H, m); MS(ESI) m/z 573 $[M + H]^+$. Analytical HPLC (Ultrasphere ODS, 4.6 mm × 25 cm; binary isocratic solvent system A (30%), 0.2% TFA/water and B (70%), 10% DMF/methanol; 1 mL/min; detection at 254 nM) for (±)-**6**, t_R = 13 min, and for *meso*-**6**, t_R = 16 min.

(±)-*Methyl Bis*[*2-phenyl-1*-[(*carbobenzyloxy*)*amino*]-*ethyl*]*phosphinate* ((±)-**5**). A suspension of (±)-**6** (117 mg, 0.204 mmol) in 5 mL of methanol was stirred at 0 °C, and excess ethereal diazomethane was added. After 1.5 h acetic acid (1 mL) was added. Concentration and flash chromatography of the residue (2:1 ethyl acetate/hexanes, then 25:1 chloroform/methanol) provided the title compound (70 mg, 59%) as a single diastereoisomer (white solid): TLC (25:1 $CHCl_3$/methanol) R_f 0.45; ^{1}H NMR ($CDCl_3$) δ 7.25 (20H, m), 5.92 (1H, d), 5.32 (1H, d), 5.02 (2H, dm), 4.90 (2H, dm), 4.53 (1H, m), 4.43 (1H, m), 3.67 (3H, d, J = 9.9 Hz), 3.24 (2H, m), 2.96 (2H, m).

meso-(R_P,S_P)-*Methyl Bis*[*2-phenyl-1*-[(*carbobenzyloxy*)-*amino*]*ethyl*]*phosphinate* (*meso*-(R_P,S_P)-**5**). By the procedure described for (±)-**5**, *meso*-**6** (114 mg, 0.200 mmol) yielded a 60:40 mixture of diastereoisomers *meso*-(R_P,S_P)-**5** (54 mg, 46%; white solid): TLC (25:1 $CHCl_3$/methanol) R_f 0.45, 0.50; ^{1}H NMR ($CDCl_3$) δ 7.28–7.10 (20H, m), 6.41 (3/5 × 1H, d), 5.52 (2/5 × 1H, d), 5.12–4.94 (4H, m), 4.53 (2H, m), 3.83 (3/5 × 3H, d, J = 10.3 Hz), 3.57 (2/5 × 3H, d, J = 9.6 Hz), 3.34 (2H, m), 2.82 (2H, m).

(*1R*)- *and* (*1S*)-*Methyl Bis*[*2-phenyl-1*-[[(*carbobenzyl-oxy*)*valyl*]*amino*]*ethyl*]*phosphinate* (**2-OMe** *and* **7-OMe**). Compound (±)-**5** (70 mg, 0.12 mmol) was stirred in methanol (5 mL) with 3 N HCl (0.1 mL) and 10% Pd/C (80 mg) under H_2 (1 atm) for 4 h. Filtration and concentration provided the unblocked diamine dihydrochloride (±)-**5a** (39 mg, 84%). Isobutyl chloroformate (39 μL, 0.30 mmol) was added to a stirred solution of Cbz-(L)-valine (75 mg, 0.30 mmol) and *N*-methylmorpholine (38 μL, 0.35 mmol) in 2 mL of THF at –40 °C under Ar. After 10 min a solution of dihydrochloride (±)-**5a** in 1.5 mL of THF and 0.2 mL of DMF was added along with more *N*-methylmorpholine (38 μL), and the cold bath was removed. After 11 h, extractive workup followed by preparative HPLC (silica, 1 in. × 25 cm, 2% methanol in $CHCl_3$, 20 mL/min) provided **2-OMe** (23 mg, 24%), which eluted first (t_R = 14 min), followed by **7-OMe** (22 mg, 23%) (t_R = 17 min). For **2-OMe**: mp 224–225 °C; ^{1}H NMR ($CDCl_3$) δ 7.35–7.09 (22H, m), 5.15–4.75 (6H, m), 4.03 (1H, m), 3.95 (1H, br), 3.76 (3H, br d, J = 9.7 Hz), 3.30 (2H, m), 3.30–2.88 (2H, m), 2.19–1.75 (2H, m), 0.73 (6H, m), 0.63–0.38 (6H, m). For **7-OMe**: mp 195–196 °C; ^{1}H NMR ($CDCl_3$) δ 7.38–7.24 (20H, m), 5.23–4.93 (7H, m), 4.76 (1H, m), 4.23 (1H, dd), 3.77 (1H, m), 3.63 (3H, d, J = 10.3 Hz), 3.42 (2H, br d), 2.96–2.68 (2H, m), 1.68 (2H, m), 0.75 (6H, m), 0.54 (3H, d, J = 6.8 Hz), 0.49 (3H, d, J = 6.6 Hz).

(*1R,1'S,R*$_P$,*S*$_P$)-*Methyl* [*2-Phenyl-1*-[[(*carbobenzyloxy*)-*valyl*]*amino*]*ethyl*][*2'-phenyl-1'*-[[(*carbobenzyloxy*)*valyl*]-*amino*]*ethyl*]*phosphinate* (**8-OMe**). By a procedure analogous to that described for **2-OMe** and **7-OMe**, phosphinic acid *meso*-**6** (54 mg) provided **8-OMe** (37 mg, 50% overall) as a ca. 3:5 mixture of two diastereoisomers at phosphorus: ^{1}H NMR ($CDCl_3$) δ 7.32–7.11 (20H, m), 5.43 (2H, m), 5.14–4.69 (6H, m), 4.24–3.95 (2H, m), 3.89 (3/5 × 3H, d, J = 10.4 Hz), 3.66 (2/5 × 3H, d, J = 9.6 Hz), 3.36 (2H, m), 3.00–2.62 (2H, m), 2.18–1.68 (2H, m), 0.84–0.50 (12H, m).

Deprotection of **2-OMe**, **7-OMe**, *and* **8-OMe**. Trimethylsilyl bromide (10 μL, 76 μmol) was added to a solution of methyl phosphinate **2-OMe** (or **7-OMe** or **8-OMe**) (20 mg, 25 μmol) in 1 mL of CH_2Cl_2. After 7 h, methanol (1 mL) was added, the solution was concentrated, and the residue was triturated with ether to afford the deprotected phosphinic acid **2** (or **7** or **8**, respectively) (20 mg). (1*R*)-Bis[2-phenyl-1-[[(carbobenzyloxy)valyl]amino]ethyl]phosphinic acid (**2**): mp 236–238 °C; ^{1}H NMR ($CD_3OD/CDCl_3$) δ 7.20 (10H, m), 7.18 (10H, m), 5.03 (4H, m), 4.54 (2H, m), 3.86 (2H, d, J = 6.4 Hz), 3.24 (2H, dt), 3.01 (2H, m), 1.92 (2H, m), 0.77 (12H, t); MS(ESI) m/z 771 $(M + H)^+$, 637. (1*S*)-Bis[2-phenyl-1-[[(carbobenzyloxy)valyl]amino]ethyl]-phosphinic acid (**7**): mp 213–215 °C; ^{1}H NMR ($CD_3OD/CDCl_3$) δ 7.33 (10H, m), 7.21 (10H, m), 5.07 (4H, dd), 4.80 (2H, m), 3.96 (2H, d, J = 6.7 Hz), 3.37 (2H, dm), 2.89 (2H, m), 1.67 (2H, m), 0.59 (12H, dd); MS(ESI) m/z 771 $(M + H)^+$, 637, 538. (1*R*,1'*S*)-[2-Phenyl-1-[[(carbobenzyloxy)-valyl]amino]ethyl][2'-phenyl-1'-[[(carbobenzyloxy)valyl]amino]ethyl]phosphinic acid (**8**): ^{1}H NMR (CD_3OD) δ 7.30–7.06 (20H, m), 5.13 (1H, d, J = 12.4 Hz), 4.99 (1H, d, J = 11.5 Hz), 4.79 (1H, d, J = 11.5 Hz), 4.71 (1H, d, J = 12.4 Hz), 4.62 (1H, br m), 4.28 (1H, m), 3.88 (2H, dd), 3.43 (2H, m), 2.86 (1H, m), 2.67 (1H, m), 1.70 (2H, m), 0.75 (3H, d, J = 6.8 Hz), 0.61 (6H, dd), 0.41 (3H, d, J = 6.6 Hz); MS-(ESI) m/z 771 $(M + H)^+$, 637.

Enzymes and Substrates

Recombinant HIV-1 protease, derived from the BH10 clone of HIV-1 (Ratner et al., 1985), was obtained by expression in *Escherichia coli* (Debouck et al., 1987) and was purified to apparent homogeneity as previously described (Grant et al., 1991). The purified protease was stored in 50 mM sodium acetate (pH 5.0), 0.35 M NaCl, 1 mM EDTA, 1 mM DTT, and 20–40% glycerol at –40 °C. The concentration of the enzyme was determined by the chromatographic method of Strickler et al. (1989), and the concentration of enzymatic active sites was determined by titration with a tight-binding inhibitor (Grant et al., 1991). Porcine pepsin A was obtained as a lyophilized powder from Boehringer Mannheim and was used without further purification. The substrates Ac-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 (Moore et al., 1989) and Phe-Gly-His-[(4-nitro)-Phe]-Phe-Ala-Phe-OCH_3 were obtained at >95% purity from Bachem Biosceince, Inc.

Enzyme Inhibition Assays

For recombinant HIV-1 protease, inhibition constants were determined at 37 °C in a buffer composed of 50 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.0), 1 mM EDTA, 200 mM NaCl, 1 mM DTT, 0.1% (v/v) Triton X-100 (MENDT buffer), and 10% (v/v) DMSO in reaction mixtures containing 1–10 mM concentrations of the substrate Ac-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 and 20 nM HIV-1 protease.

Table I: Statistics of Data Measurement and Processing

space group	$P6_1$
lattice constants	$a = 63.2$ Å
	$b = 63.2$ Å
	$c = 83.4$ Å
	$\alpha = 90.0°$
	$\beta = 90.0°$
	$\gamma = 120.0°$
molecules/asymmetric unit	1 protein dimer + 1 inhibitor
resolution limit (Å)	2.3
no. of measured reflections	41 859
no. of unique reflections	8213
data completeness	
∞–2.3 Å	97%
2.4–2.3 Å	79%
R_{sym}[a]	0.073

[a] $R_{sym} = \sum\sum|I_{i(h)} - \bar{I}_{(h)}|/\sum I_{i(h)}$, where $I_{i(h)}$ are the intensities of multiple measurements of reflection h and $\bar{I}_{(h)}$ is their mean.

Table II: Refinement Statistics from PROLSQ

R factor[a]	
6.0–2.3 Å	0.178
2.5–2.3 Å	0.224
resolution range	6.0–2.3
no. of reflections	7014
no. of protein atoms	1520
no. of inhibitor atoms	110
no. of solvent molecules	27
rms deviations from ideal values	
covalent bond distances (Å)	0.016
angle distances (Å)	0.038
planar 1–4 distances (Å)	0.051
planar group (Å)	0.008
chiral centers (Å^3)	0.181
nonbonded contact distances (Å)	
(a) single torsion	0.24
(b) multiple torsion	0.29
(c) possible hydrogen bonds	0.27
planar peptide group torsion angles (deg)	1.4

[a] R factor = $\sum||F_o| - |F_c||/\sum|F_o|$, where $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively.

Enzymatic activity was quantified by HPLC as previously described (Dreyer et al., 1989; Meek et al., 1989; Moore et al., 1989). Apparent inhibition constants were initially determined by Dixon analysis assuming competitive inhibition. Linear competitive inhibition was verified from double-reciprocal plots of initial velocity versus substrate concentration at several fixed concentrations of inhibitor (Lineweaver–Burk analysis). The apparent inhibition constant of SB204144 with HIV-1 protease was obtained by previously described methods for tight-binding inhibitors (Dreyer et al., 1992). Inhibition of porcine pepsin A was performed at 37 °C by spectrophotometric assay (Medzihradzky et al., 1970) of reaction mixtures containing 40 mM sodium formate (pH 4.0), 0.2 M NaCl, 2 nM pepsin, 1.6% DMSO, 33–125 μM Phe-Gly-His-[(4-nitro)-Phe]-Phe-Ala-Phe-OCH_3, and 0–30 nM SB204144. Linear competitive inhibition and the inhibition constant were determined from a Lineweaver–Burk plot.

Crystallography

The complex of SB204144 with HIV-1 protease was crystallized as described previously (Miller et al., 1989). Typically, cocrystals were grown by the method of vapor diffusion in hanging drops (Mcpherson, 1976) by using 18–26% saturated ammonium sulfate buffered at pH 5.0 with 200 mM acetate as the precipitant. The symmetry of the diffraction was consistent with that of the hexagonal space group $P6_122$ or that of $P6_1$ with strong noncrystallographic 22 symmetry resulting from the 2-fold symmetry of the protein. We chose the latter space group to be consistent with the previously reported crystal structure of A74704 complexed to HIV-1 protease (Erickson et al., 1990). The unit cell dimensions were $a = b = 63.2$ Å and $c = 83.4$ Å, with the asymmetric unit containing one complete copy of the complex (a protein dimer plus an inhibitor). X-ray diffraction data were measured from a single crystal using a Siemens two-dimensional, position-sensitive detector. The detector was mounted on a Siemens rotating anode X-ray generator operated at 45 kV and 96 mA, equipped with a 300-μm focusing cup and producing graphite-monochromated Cu Kα radiation. Diffraction images were recorded, reduced, and visualized using FRAMBO, XENGEN (Howard et al., 1987), and XPREP computer programs, respectively. Approximately 42 000 reflections were measured in 2 days to give 8213 unique reflections to 2.3-Å resolution, representing 97% of the data. Details of data measurement and processing statistics are summarized in Table I.

Crystallographic refinement was carried out using the restrained least-squares program PROLSQ (Hendrickson, 1985). Minor modifications to the export version (Smith et al., 1988) of this program allowed for its use on a Silicon Graphics IRIS 4D/380 and the ability to run multiple cycles of refinement without manual intervention. The starting model used in refinement consisted of the protein portion of the 2.8-Å structure of A74704 complexed to HIV-1 protease (Erickson et al., 1990). A few cycles of rigid-body least-squares refinement using data to 3.0-Å resolution for reflections greater than $3\sigma(F_o)$ were carried out to obtain the optimal position and orientation of the starting model. This was followed by iterative cycles of refinement and model building. Fourier maps with coefficients $|F_o| - |F_c|$ and $2|F_o| - |F_c|$ were computed and displayed on an Evans and Sutherland PS300 graphics system using FRODO (Jones, 1985). Maps calculated using refined phases, obtained from data greater than $2.0\sigma(F_o)$ in the resolution range of 8.0 to 2.5 Å, showed clear electron density for the inhibitor. A model of SB204144 derive from the structure of A74704 (Erickson et al., 1990) was positioned in the electron density, and the complex was further refined. Special bond and angle distances were incorporated in the program PROTIN (Hendrickson, 1985) to account for the geometry of the inhibitor. To account for the 2-fold disorder observed in the $P6_1$ crystal lattice resulting from the symmetric nature of the protein (Dreyer et al., 1992, 1993; Murthy et al., 1992), a second inhibitor molecule was introduced and the protein and the two inhibitors (each at half-occupancy) were simultaneously refined. The final model contained 27 water molecules. This was followed by refinement using XPLOR (Brunger et al., 1987) for comparison. The results from both PROLSQ and XPLOR refinement were very similar. Statistics from the crystallographic refinement of HIV-1 protease complexed with inhibitor are detailed in Table II. The refined set of coordinates has been deposited in the Protein Data Bank (Bernstein et al., 1977).

RESULTS AND DISCUSSION

Inhibitor Synthesis. The synthesis of the diaminophosphinate unit employs Curtius rearrangement (Shiori et al., 1972) of the acyl azide derived from α-carboxy phosphinate **4** (Scheme I). Compound **4**, a mixture of eight stereoisomers, leads to a mixture of four diaminophosphinates **5**, i.e., two *meso* isomers (*meso*-(R_P)-**5** and *meso*-(S_P)-**5**) and a racemic pair of C_2-symmetric isomers (±)-**5** nonstereogenic at phosphorus. Partial resolution of the isomers of **5** is achieved by saponification to the phosphinic acids **6**, chromatographic

Table III: Inhibition of HIV-1 Protease[a]

inhibitor	apparent K_i (nM)
2 (SB204144)	2.8 ± 0.3[b]
7	1760 ± 340[c]
8	960 ± 70[c]
1 (A74704)	230 ± 14[c,d]

[a] Assays were conducted at 37 °C, pH 6.0 (0.2 M NaCl), as previously described (Dreyer et al., 1989; Moore et al., 1989). [b] Determined from plots of remaining enzymatic initial rate as a function of inhibitor (0–50 nM) in the presence of 1 mM substrate and 20 nM HIV-1 protease, as previously described for tight-binding inhibitors (Grant et al., 1991; Dreyer et al., 1992). [c] Determined by Dixon analysis. [d] Previously reported (Dreyer et al., 1993).

separation into the two fractions (±)-**6** and *meso*-**6**, and reesterification. This sequence also allows partial configurational assignment of the isomers, since esterification of *meso*-**6** yields the two *meso*-**5** diastereomers, while (±)-**6** yields the racemate (±)-**5**. Coupling the phosphinates **5** to Cbz-valine, separation, and deprotection affords each of the three peptides **2** (SB204144), **7**, and **8** in stereochemically pure form. The synthesis allows unequivocal assignment of the phosphinate **8**, but does not distinguish the absolute configurations of the two C_2-symmetric phosphinates **2** and **7**. The latter two assignments follow from the crystallographic structure of HIV-1 protease complexed with compound **2**. An alternative synthesis of a diastereomeric mixture of **2**, **7**, and **8** has been reported recently (Peyman et al., 1992).

Inhibition of HIV-1 Protease. Phosphinate **2** (SB204144) is a potent competitive inhibitor of HIV-1 protease, with an apparent inhibition constant of 2.8 nM at pH 6.0 (Table III). Clearly, inhibition is stereospecific: the diastereomeric phosphinates **7** and **8** are at least 2–3 orders of magnitude weaker (the residual activity of compounds **7** and **8** could reflect incomplete separation). Thus, as predicted from modeling studies and by analogy with natural amino acids within substrates (e.g., L-phenylalanine), inhibitory potency resides in the isomer possessing the *R* configuration at the carbons joined to phosphorus. Compound **2** is also considerably more potent than the similar alcohol **1** under identical assay conditions (Dreyer et al., 1993).

Despite its low K_i value, compound **2** is very weakly antiviral in Molt4 T-cells acutely infected with HIV-1 (D. M. Lambert, personal communication), most likely due to poor cell penetration (Meek et al., 1990). The methyl phosphinate derivative of **2** is not an HIV-1 protease inhibitor (K_i > 1 mM), nor is it antiviral. However, this may be a surmountable limitation for this class of compounds. For example, the low cell permeability and low potency of a phosphinic acid inhibitor of HMG-CoA reductase were overcome (in cell culture) by the preparation of appropriate ester pro-drugs (Dreyer et al., 1991).

As previously shown for alcohol **1** (Dreyer et al., 1993), phosphinic acid **2** is also a potent competitive inhibitor of porcine pepsin with K_i = 14 nM at pH 4.0. Pepsin is a prototypical monomeric, asymmetric mammalian aspartic protease. Inhibition of pepsin by compound **2** reveals the evolutionary and mechanistic kinship between the monomeric and retroviral aspartic proteases and points out that symmetry within inhibitors is not sufficient to ensure selectivity for retroviral proteases (Dreyer et al., 1993).

The potent inhibition of HIV-1 protease by compound **2** at pH 6.0 is somewhat surprising. In keeping with the accepted mechanism for aspartic proteolysis, pH studies have shown that uncharged competitive inhibitors bind to a form of HIV-1 protease in which the active site pair of catalytic aspartate residues carries one proton and a negative charge, with pK_a values of pK_1 = 3.1–3.3 and pK_2 = 4.9–5.3 (Hyland et al., 1991b). Similarly, one would expect the ionizable phosphinic acid to bind most favorably in its neutral form, preserving the active site −1 charge. Inhibition of aspartic proteases including HIV-1 protease (Dreyer et al., 1989; Grobelny et al., 1990), pepsins (Bartlett & Kezer, 1984; Bartlett et al., 1990; Fraser et al., 1992), and renin (Allen et al., 1989) by phosphinate analogs of pepstatin and hydroxyethylene isosteres is usually relatively weak near pH 6.0 and increases at lower pH values, consistent with preferential binding of the neutral phosphinic acids (which typically exhibit pK_a values in the range 3.0–3.5; Crofts & Kosolapoff, 1953; Grobelny et al., 1989). Nevertheless, phosphinate **2**, for which pK_a = 3.1 (measured spectrophotometrically by titration), is highly effective in a pH range where it is extensively ionized, suggesting either that the intrinsic K_i value of the neutral form must be very low (picomolar) or that the ionized form is a potent inhibitor. An analysis of the pH dependence of K_i failed to definitively resolve this question; this is due in part to the similarity of the pK_a values of the phosphinate inhibitor (pK_a = 3.1) and the active site aspartyl residue (pK_a = 3.1–3.3) that must be protonated for an inhibitor to bind to HIV-1 protease. However, the crystallographic results described below suggest a protonated phosphinate in the enzyme complex.

Crystallographic Refinement of the Structure. The statistics listed in Table II are consistent with a well-refined structure within the limits of precision and resolution of the measured data. The error in the atomic coordinates is estimated to be 0.25 Å by the method of Luzzati (1952), which is known to overestimate errors (Chambers & Stroud, 1979). In previous studies of HIV-1 protease complexed with asymmetric inhibitors (Dreyer et al., 1992; Murthy et al., 1992), and with a C_2-symmetric inhibitor (Dreyer et al., 1993), we observed two distinct orientations of the inhibitor related to each other by the pseudo-2-fold axis of the enzyme dimer, with each orientation present at half-occupancy in the crystal lattice. Although SB204144 is a perfectly symmetric inhibitor, it was clear, after refinement was completed with only one orientation of the inhibitor in the active site, that the two halves of the inhibitor had different conformations. Similar results were reported previously for the HIV-1 protease–A74704 complex (Erickson et al., 1990). As a result, we introduced a second copy of the inhibitor in the active site related to the first by a 2-fold rotation. Further refinement of the structure with both orientations of the inhibitor, each at half-occupancy, resulted in a slight improvement of the *R* factor and a better fit to the electron density. Figure 2 shows that both orientations of the inhibitor fit the electron density well. The density of the inhibitor is almost symmetric except for that of the Cbz phenyl rings, for which the density is weak.

Binding of the Inhibitor. The inhibitor is present in the active site of the enzyme in an extended conformation. It is held by a set of hydrophobic and hydrogen-bonding interactions, many of which are conserved in similar structures (Abdel-Meguid, 1993; Wlodawer & Erickson, 1993). Table IV shows the distances of potential hydrogen bonds between the inhibitor and residues of the active site cleft. Every heteroatom of the inhibitor is hydrogen-bonded to an atom of the protein, either directly or indirectly through a water molecule (Figure 3). Each end of the inhibitor is held to the enzyme by a novel interaction in which one of the terminal nitrogens of Arg8 points toward the center of the benzene ring of the Cbz group (Figure 3), as it is 2.15 Å above the center of the ring. Similar interactions have been reported

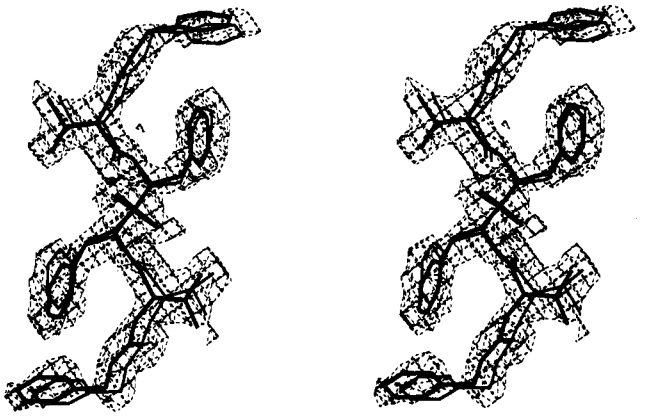


FIGURE 2: Stereoview showing the fit of both orientations of SB204144 (thick and thin lines) to the final $2|F_o|-|F_c|$ electron density map (dashed lines), where F_o and F_c are the observed and calculated structure factor amplitudes, respectively.

in the structure of the phosphotyrosine recognition domain SH2 of v-*src* complexed with a tyrosine-phosphorylated peptide (Waksman et al., 1992) but not in any of the HIV-1 protease structures.

The tetrahedral phosphinate is positioned symmetrically with respect to the Asp25,Asp25′ pair of carboxyl groups. The oxygen of the central water molecule (observed in all structures of HIV-1 protease–inhibitor complexes) and the O–P–O atoms of the inhibitor lie in a common plane, and the water and phosphorus atoms are collinear with the C_2-symmetry axis of the complex (Figure 4). The O–P–O plane intersects the least-squares plane of the two aspartate carboxyl groups along the symmetry axis, forming an angle of approximately 23°. Each oxygen of the phosphinate interacts with a single aspartic acid, establishing one short (2.2–2.4 Å) and one long (3.1–3.2 Å) oxygen–oxygen contact (Table IV). From the geometric placement of the six oxygen atoms of the aspartate side chains and the phosphinate, it seems clear where hydrogen bonds occur (Figure 3). A hydrogen bond to the phosphinate from $O^{\delta 2}$ of either Asp25 or Asp25′ is unlikely due to the acute C^{γ}–$O^{\delta 2}$–O^{P} angles. On the other hand, short hydrogen bonds between the phosphinate and $O^{\delta 1}$ of each aspartate seem a certainty. As a result, we can tentatively associate two protons and a net −1 charge with the complex, consistent with binding of the neutral phosphinate with the monoprotonated form of the enzyme. Direct determination of proton locations would, however, require additional experiments, such as neutron diffraction.

Contacts between phosphorus acids and carboxyl groups as short as 2.2–2.4 Å have also been observed in the structures of thermolysin, carboxypeptidase A, and penicillopepsin

Table IV: Distances of Potential Hydrogen Bonds between Inhibitor and HIV-1 Protease

contact atoms		distance (Å)		
		SB204144		
inhibitor[a]	protease	orientation 1	orientation 2	A74704[c]
Cbz_1 OA	O water2	2.8	3.1	
Cbz_1 O	N Asp29	3.1	3.6	3.1
Val_1 N	O Gly48	2.9	2.5	2.8
Val_1 O	O water1	2.8	2.4	2.7
Phe_1 N	O Gly27	3.0	3.1	3.1
PO_1 (OH)[b]	$O^{\delta 1}$ Asp25	2.4	2.3	2.8
	$O^{\delta 2}$ Asp25	3.1	3.2	2.8
PO_2 (OH)[b]	$O^{\delta 1}$ Asp25′	2.2	2.2	3.2
	$O^{\delta 2}$ Asp25′	3.2	3.0	2.9
Phe_2 N	O Gly27′	3.7	3.1	3.7
Val_2 O	O water1	2.5	2.6	2.7
Val_2 N	O Gly48′	3.0	3.0	3.0
Cbz2 O	N Asp29′	3.5	3.3	2.9
Cbz_2 OA	O water3	3.2	2.7	

[a] Since SB204144 and A74704 are symmetric inhibitors, the designations 1 and 2 for the two halves of the inhibitor are arbitrary. [b] SB204144 differs from A74704 only at the central group: a phosphinate versus a hydroxymethylene (see Figure 1). [c] Atomic coordinates of HIV-1 protease–A74704 were obtained from Dr. John W. Erickson.

complexed with phosphorus-containing inhibitors (Holden et al., 1987; Kim & Lipscomb, 1991; Fraser et al., 1992). Such short distances between oxygen atoms, observed in small-molecule structures, are indicative of strong hydrogen bonds (Jeffrey & Maluszynska, 1982). These short O···H···O bonds ("low-barrier" hydrogen bonds) can be represented by a symmetrical single-well potential energy curve with the proton located midway between the two oxygen atoms (Kreevoy & Liang, 1980; Cleland, 1992).

Additional evidence for the existence of low-barrier hydrogen bonds comes from solvent kinetic isotope effect data on HIV-1 protease (Hyland et al., 1991b). As discussed by Cleland (1992), proton transfers between oxygen atoms (of similar pK_a values) involved in low-barrier hydrogen bonds are characterized by anomalously low fractionation factors and can yield low, normal, or even inverse solvent isotope effects. For a variety of peptide substrates, the solvent isotope effect on the kinetic parameter V/K was consistently ≤ 1.0, indicating that the enzyme-catalyzed prototropic reactions leading to the putative tetrahedral adduct of H_2O and peptide may be characterized by unusually small intrinsic isotope effects ($^{D}k \leq 1$; Hyland et al., 1991b). Similarly, solvent isotope effects of unity or less have been observed on V/K for the aspartyl proteases porcine pepsin (Rebholz & Northrup, 1991) and renin (Green et al., 1990). It is likely then that these proton transfers in the aspartyl proteases also occur

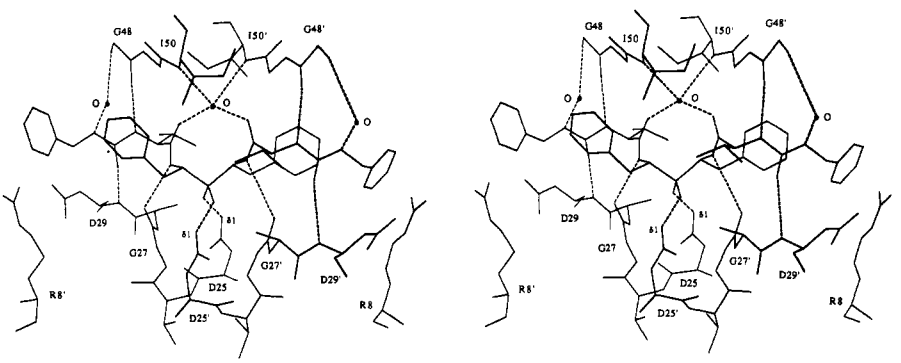


FIGURE 3: Stereoview of the structure of SB204144 bound in the active site of HIV-1 protease. Active site amino acid residues forming potential hydrogen bonds with the inhibitor are labeled by their one-letter codes. Potential hydrogen bonds are indicated by dotted lines, white black circles designate water molecules.

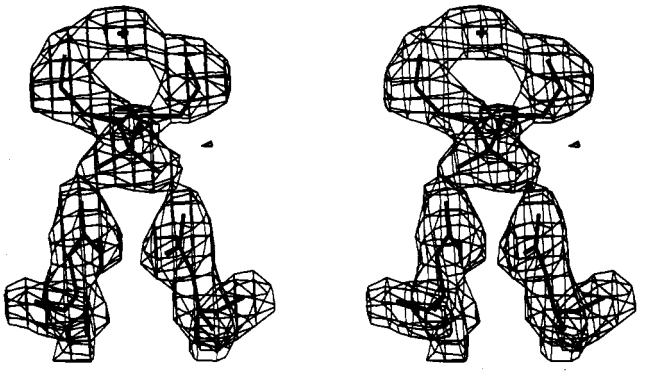


FIGURE 4: Stereoview of the fit of the central portion of SB204144, the central water molecule, and the two catalytic aspartates to the final $2|F_o|-|F_c|$ electron density map, where F_o and F_c are the observed and calculated structure factor amplitudes, respectively. Each of the phosphinate oxygen atoms interacts symmetrically with the outer oxygen atom of a catalytic aspartate.

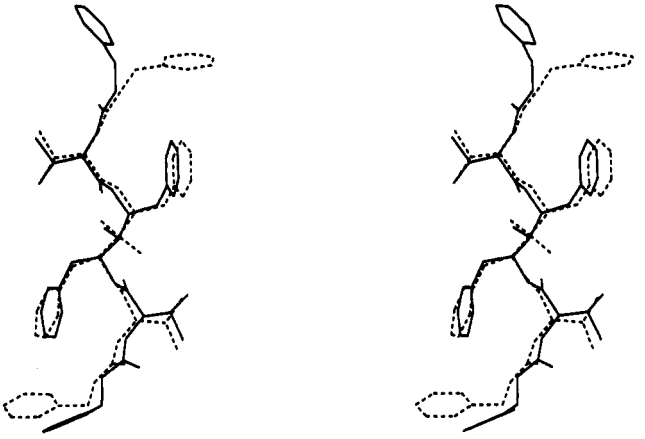


FIGURE 5: Stereoview of the overlay of the active site conformations of SB204144 (one orientation; dotted lines) and A74704 (solid lines). This overlay was achieved by superposition of the protein backbone atoms of the two complexes.

through low-barrier hydrogen bonds, similar to those implicated for adenosine deaminase (Cleland, 1992).

Protein Structure. The structure of the protein is nearly identical to that of previously reported HIV-1 protease–inhibitor complexes. Superposition of backbone atoms from our protease dimer structure with those from the structures containing inhibitors A74704 (Erickson et al., 1990), MVT101 (Miller et al., 1989), and L700,417 (Bone et al., 1991) gives rms deviations of 0.3, 0.6, and 0.6 Å, respectively. Superposition of the backbone atoms of the two protease subunits of this phosphinate structure gives an rms deviation of 0.3 Å, while superposition of all atoms, including side-chain atoms, gives an rms deviation of 1.0 Å, indicating conservation of the symmetry of the homodimer.

Comparison with A74704. The structures of SB204144 and A74704 differ chemically only at the central group: a phosphinate versus a hydroxymethylene (Figure 1). Figure 5 shows the superposition of the two inhibitors in the active site, achieved by superposing all backbone atoms of the two proteins. The two inhibitor structures do not differ significantly except at their ends. However, since the electron density for the Cbz groups in both structures was weak, this difference could be an artifact. Most of the polar interactions are also conserved for the two structures (Table IV). The only meaningful differences between the two inhibitors, and consequently the probable cause of their 80-fold difference in binding affinity, occur around the central atom. The single hydroxyl of A74704 is almost equidistant (2.8–3.2 Å) from all four oxygens of the two catalytic aspartic acids (Erickson et al., 1990) and probably forms two hydrogen bonds similar to those described for a pepstatin analog bound to penicillopepsin (James & Sielecki, 1985). In contrast, each oxygen of the phosphinate interacts with a single aspartic acid, apparently forming one short hydrogen bond and one long van der Waals contact. This requires changes in the orientation of the side chains of the two catalytic aspartic acid residues and small differences in the torsion angles around the central atom of the inhibitors. Since A74704 and SB204144 appear to make the same number of hydrogen bonds with the protein, the enhanced binding affinity of the phosphinate is likely due to its unusually strong, short hydrogen bonds.

Implications for the Catalytic Mechanism. The structure of the HIV-1 protease complex with SB204144 clearly shows that the two phosphinate oxygens bind symmetrically to the outer ($O^{\delta 1}$) oxygens of the catalytic aspartates (Figures 3 and 4; Table IV). This binding mode differs from that of several recently reported structures of phosphinate- and phosphonate-containing (Fraser et al., 1992) and α,α-difluoro ketone-containing (James et al., 1992; Parris et al., 1992; Veerapandian et al., 1992) renin inhibitors bound to fungal pepsins. In the difluoro ketone structures, the carbonyl of the difluoro ketone is hydrated as a geminal diol. One hydroxyl of the hydrate is centered between the two catalytic aspartate carboxyl groups, making one hydrogen bond with the internal oxygen atom of one aspartate and another bond with the external oxygen atom of the other aspartate, while the other hydroxyl interacts with the external oxygen of a single aspartate. The phosphinate and phosphonate renin inhibitors show a binding mode very similar to that of the difluoro ketones, with the two phosphorus oxygens interacting with the aspartates in the same asymmetric manner (Fraser et al., 1992). By comparing the structures of the penicillopepsin-bound difluoro ketone- and phosphinate-containing inhibitors, these authors concluded that the phosphinate provides the more accurate representation of the enzymatic transition state.

In the catalytic mechanism of aspartyl proteases put forth by Suguna et al. (1987a), the water molecule bound symmetrically between the aspartates in unliganded aspartyl protease structures (Suguna et al., 1987b; Wlodawer et al., 1989) is proposed to act as a nucleophile that attacks the substrate carbonyl. The complex of the resulting hydrated peptide intermediate is suggested to resemble the complexes of the phosphorus- and difluoro ketone-containing inhibitors (Fraser et al., 1992; James et al., 1992; Parris et al., 1992; Veerapandian et al., 1992). Kinetic data for HIV-1 protease peptide cleavage are consistent with this mechanism (Hyland et al., 1991a,b; Ido et al., 1991). In addition, Hyland et al. (1991a) reported that HIV-1 protease can catalyze the incorporation of ^{18}O from $H_2{}^{18}O$ into the scissile bond carbonyl of substrates, even in the absence of reverse peptidolysis. This latter observation suggests that at some point the two oxygens of the hydrated amide are chemically equivalent, indicating that peptidolysis by HIV-1 protease proceeds via a pseudosymmetric transition state (or intermediate). A pseudosymmetric reaction intermediate of peptide and water, perhaps with its oxygen atoms symmetrically and intimately disposed between the $O^{\delta 1}$ oxygen atoms of the corresponding aspartyl residues, may be well represented by the protease-bound structure of SB204144. The protonation of either hydroxyl group of this reaction intermediate would be more facile from the outer ($O^{\delta 1}$) oxygen atoms of the catalytic aspartates rather than the inner ($O^{\delta 2}$) oxygen atoms, because a proton on one

of these $O^{\delta 2}$ oxygen atoms is at a greater distance (>3 Å) from the hydroxyl group and is likely to form a strong hydrogen bond with the corresponding ($O^{\delta 2}$) oxygen atom on the opposite aspartyl group.

These differences in the binding modes point out some of the difficulties in attempting to determine mechanisms from static structures. These difficulties are compounded by the lack of definite knowledge about proton positions. All of the aspartyl protease inhibitors discussed here are imperfect mimics of a transition state or intermediate for peptide hydrolysis. The difluoro ketone- and phosphorus-containing substrate analogs complexed to fungal pepsins lack residues beyond P_1' [nomenclature of Schechter and Berger (1967)] and, hence, may not be representative of typical substrates. The correct positioning of the tetrahedral hydrate of the inhibitor may rely on hydrogen-bonding interactions between the inhibitor and the enzyme beyond the P_1' site. SB204144 is not a substrate analog, but is in effect a symmetric composite of the N-terminal portion of a substrate and may well exhibit a conformation around the tetrahedral center that differs from that of a reaction intermediate.

While it is premature to predicate a revision of current mechanisms for aspartyl proteolysis on the structural results with SB204144, the energy difference between symmetrically and asymmetrically positioned intermediates could be sufficiently small that both are accessible during the course of the reaction. It is also possible that the C_2-symmetric HIV-1 protease binds the intermediate slightly differently than do monomeric aspartyl proteases. Structural data on complexes of SB204144 with the pepsins and phosphinate-containing renin inhibitors with HIV-1 protease might provide additional insight into any such differences. Also, it would be interesting to determine whether a peptide with two N-termini (e.g., a symmetric carbonate) is a substrate for either type of protease.

Regardless of the relationship of these inhibitors to the nature of the transition state, clearly the transition-state analog paradigm (Pauling, 1948) continues to be a valuable route to potent inhibitors. Putative mimics of the transition state can retain their binding effectiveness in symmetric peptide analogs and, presumably, in structures further removed from peptides, thus furnishing a molecular tool kit for inhibitor design.

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