Synthesis and Crystallographic Analysis of Two Rhizopuspepsin Inhibitor Complexes†

Kevin D. Parris,‡ Dennis J. Hoover,§ David B. Damon,§ and David R. Davies*,‡

Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, and Department of Medicinal Chemistry, Central Research Division, Pfizer Inc, Groton, Connecticut 06340

Received February 19, 1992; Revised Manuscript Received May 28, 1992

ABSTRACT: The crystal structures of rhizopuspepsin complexed with two oligopeptide inhibitors have been determined. CP-69,799, an azahomostatine dipeptide isostere, had previously been associated with a displacement of the C-terminal subdomain of endothiapepsin [Sali, A., Veerapandian, B., Cooper, J. B., Foundling, S. I., Hoover, D. J., & Blundell, T. L. (1989) *EMBO J. 8*, 2179–2188]. Here, we report the measurement of two data sets, one from crystals soaked in the inhibitor and the other from protein crystallized in the presence of excess inhibitor. In neither case is there any significant movement of the C-terminal subdomain of the rhizopuspepsin. The data suggest that the energy associated with any conformational change is small and is overcome by the crystal packing forces. The second inhibitor, a hydrated difluorostatone, was examined in a search for transition-state analogs that could cast further light on the mechanism of action [Suguna, K., Padlan, E. A., Smith, C. W., Carlson, W. D., & Davies, D. R. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7009–7013]. The *gem*-diol provides a set of contact distances with the enzyme that mimic the interactions with the tetrahedral intermediate of the substrate during catalysis. These data provide support for the suggestion that the polarization of the keto group of the peptide substrate is enhanced by a hydrogen bond from the OD1 of Asp 35 (Suguna et al., 1987).

The aspartic proteinases form a group of enzymes characterized by having two aspartic acids in the catalytic apparatus, extended subsite specificity for substrate, and inhibition by inhibitors such as pepstatin [Kostka, 1985; James & Sielecki, 1987; reviewed by Davies (1990)]. They include the single-chain enzymes (pepsin, renin, chymosin, and the fungal enzymes), as well as the homodimeric retroviral enzymes of which the HIV-1 protease (Navia et al., 1989; Wlodawer et al., 1989; Lapatto et al., 1989) and the Rous sarcoma virus protease (Miller et al., 1989) are examples.

The three-dimensional structures of many of these enzymes have been studied [see James and Sielecki (1987) and Davies (1990) for reviews]. The mechanism of action has also been the subject of considerable investigation, and there have been many mechanistic proposals. No evidence has been observed for a covalent intermediate, and recent mechanisms have invoked general acid–base catalysis (James & Sielecki, 1985; Pearl, 1985; Pearl & Blundell, 1985; Suguna et al., 1987b; Polgar, 1987), although there is still no direct evidence for the location of the nucleophilic water molecule.

On the basis of the extended subsite specificity (Fruton, 1976; Hofmann et al., 1988) of residues in positions P_3 and P_2' on k_{cat} [notation of Schechter and Berger (1967)] and proton inventory measurements (Cunningham et al., 1990), proposals have been made that binding in these positions allows a conformational change in the complex to occur that facilitates peptide bond cleavage (Fruton, 1976; Pearl, 1985). While conformational changes in the binding of pepstatin and analogs to pepsin have been observed by spectroscopic methods (Kunimoto et al., 1974; Schmidt et al., 1982), these changes have been attributed to the reorientation of certain aromatic side chains on binding. Until recently, however, there has been no evidence of conformational change pertinent to the general strain component of catalysis found among many inhibitor complexes of aspartic proteinases. Sali et al. (1989) observed a conformational change that accompanied the binding of the inhibitor CP-69,799 (Figure 1) to endothiapepsin and postulated that this event might relate to catalysis. Also, a recent revision of the pepsin structure and its comparison with other aspartic proteinases has led to the conclusion that there is a flexible portion of these enzymes that includes part of the C-terminal domain (Abad-Zapatero et al., 1990; Sielecki et al., 1990). Additionally, Abad-Zapatero et al. (1991) have observed conformational changes in porcine pepsin upon inhibitor binding. We wished to investigate the possibility of similar conformational changes occurring in rhizopuspepsin and to this end have examined the crystal structure of its complex with the same inhibitor, CP-69,799, employed by Sali and co-workers.

A second inhibitor complex reported herein is that of rhizopuspepsin with CP-82,218 (Figure 1), a renin inhibitor bearing the difluorostatone scissile bond moiety (Gelb et al., 1985; Thaisrivongs et al., 1985, 1986; Fearon et al., 1987) which readily hydrates to the *gem*-diol form and as such resembles the probable tetrahedral intermediate in proteolytic cleavage of the amide bond, both because of the identically placed *gem*-diol functionality and also for the second tetrahedral atom (CF_2 carbon) in the place of tetrahedral nitrogen in this

† The coordinates of the complexes have been deposited in the Brookhaven Protein Data Bank. APR7 (RCP & CP-69,799 soak), APR8 (RCP & CP-69,799 cocrystal), and APR9 (RCP & CP-82,218) have been suggested for the code names.

* Author to whom correspondence should be addressed.

‡ Laboratory of Molecular Biology, NIDDK, National Institutes of Health.

§ Department of Medicinal Chemistry, Central Research Division, Pfizer Inc.

0006-2960/92/0431-8125$03.00/0

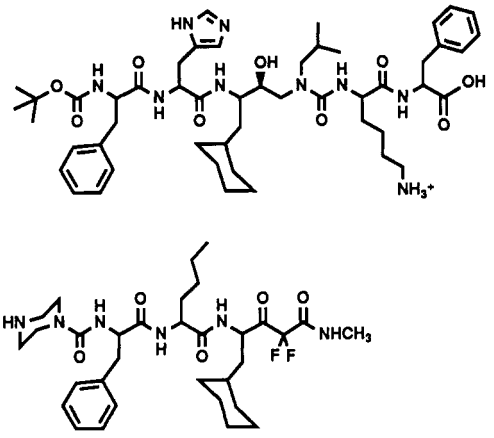


FIGURE 1: Chemical structures of the inhibitors CP-69,799 and CP-82,218 (nonhydrated form).

species. Much interest in the binding of tetrahedral species to aspartic proteinases was stimulated by NMR evidence (Rich et al., 1982; Holladay, 1985) that less potent ketone analogs of pepstatin were bound in the higher-energy hydrate form by porcine pepsin, both forming from and reverting to ketone by highly stereoselective, enzyme-catalyzed processes. Many proposals (James & Sielecki, 1985; Pearl, 1985; Pearl & Blundell, 1985; Suguna et al., 1987b; Polgar, 1987; Jaskolski et al., 1991) for the formation and breakdown of the tetrahedral proteolysis intermediate have been made without the benefit of crystallographic data on such a complex. Blundell and co-workers (Hoover et al., 1991; Veerapandian et al., 1992) and James et al. (1992) have studied the structure of hydrated difluorostatone inhibitor complexes to endothiapepsin and penicillopepsin, respectively, and from these data postulated a proteolysis sequence generally consistent with the Suguna proposal (Suguna et al., 1987b; Parris et al., 1991) and sharing the alternating roles of the catalytic aspartates in proton transfers and charge distribution as proposed by Polgar (1987). The CP-82,218 rhizopuspepsin complex discussed herein is one of the first carbonyl hydrate–aspartic proteinase structures to be determined and is thus important in providing new data in whose light these proposals may be examined.

MATERIALS AND METHODS

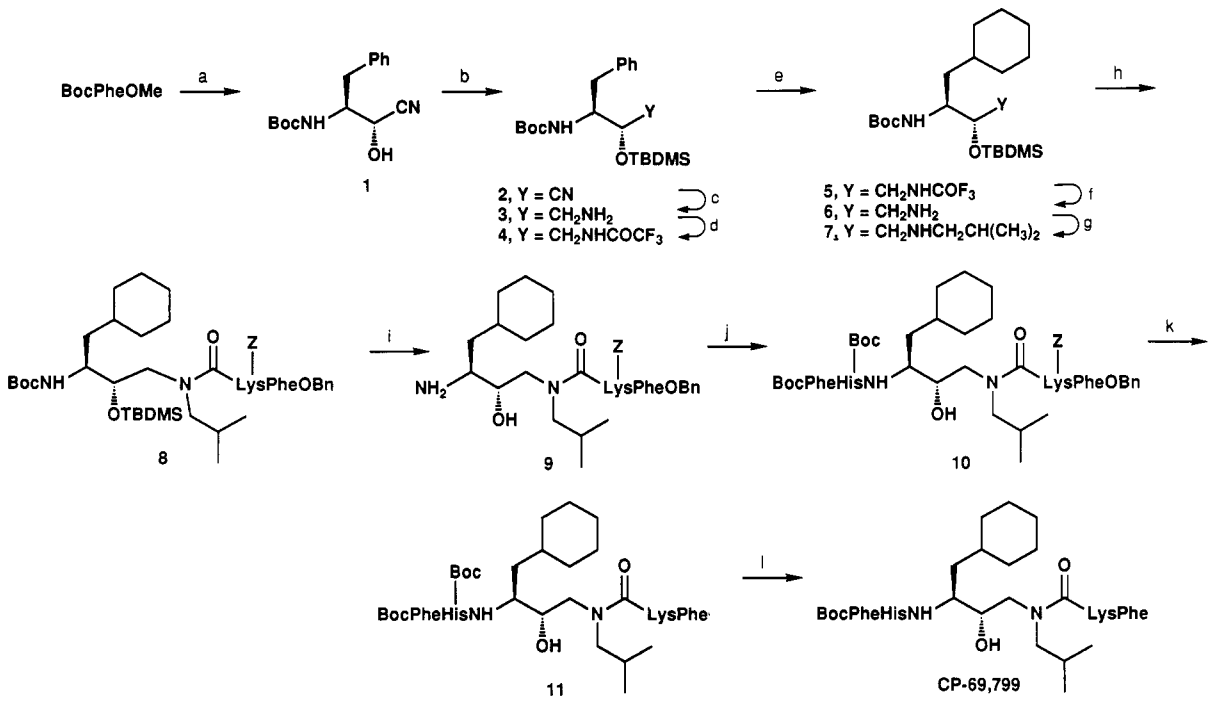
Synthesis of the Inhibitors

General Methods. Melting points were taken on a Buchi 510 apparatus and are uncorrected. Proton and carbon NMR spectra were recorded on a Varian XL-300, Bruker AM-300, or Bruker AM-500 spectrometer at 25 °C. Fluorine NMR spectra were obtained on a Bruker AC-300 spectrometer equipped with a quad probe. Chemical shifts are expressed in parts per million downfield from trimethylsilane (proton, carbon) or from fluorotrichloromethane (fluorine, shifts upfield of $CFCl_3$ expressed as negative). Liquid secondary ion mass spectra (LSIMS) were obtained on a Kratos Concept-1S high-resolution spectrometer using cesium ion bombardment on samples dissolved in a 1:5 mixture of dithioerythritol and dithiothreitol. Reported data are sums of 3–20 scans calibrated against cesium iodide. FAB-MS spectra were obtained on a Kratos MS-80RFA spectrometer operating in the FAB mode on samples dissolved in a thioglycerol matrix. TLC analyses were performed using E. Merck Kieselgel 60 F_{254} silica plates visualized [after elution with the indicated solvent(s)] by staining with 15% ethanolic phosphomolybdic acid and heating on a hot plate. HPLC was performed at 1.5 mL/min with 214-nm detection on a 250 × 4.6 mm Du Pont Zorbax C-8 column eluted isocratically by a two-pump/mixer system supplying the indicated mixture (v/v) of acetonitrile and aqueous pH 2.1 (H_3PO_4) 0.1 M KH_2PO_4, respectively. The terms "concentrated in vacuo" and "coevaporated" refer to removal of solvent at water aspirator pressure on a rotary evaporator with a bath temperature of less than 40 °C. Organic solutions were dried over magnesium sulfate. Woelm silica (32–63 μM) was used for column chromatography. Zinc dust (Aldrich) was activated by stirring with 1 N HCl, washed successively with water, ethanol, acetone, and ether, and dried in vacuo at 56 °C. Anhydrous tetrahydrofuran (THF) was purchased from Aldrich and distilled under nitrogen from sodium benzophenone ketyl. Other rigorously dried solvents (specified below as "dry") were obtained by distillation from calcium hydride at an appropriate pressure. Microanalyses were performed by the Analytical Department of Pfizer Central Research.

Synthesis of the Inhibitor CP-69,799 (Scheme I)

Preparation of 3(S),2(R)-3-[N-(tert-Butyloxycarbonyl)-amino]-2-hydroxy-4-phenylbutyronitrile (**1**). This procedure employs DIBAL reduction conditions previously applied to Boc-leucine (Rich et al., 1978) and Boc-cyclohexylalanine (Boger et al., 1985) methyl esters. A solution of *N*-(*tert*-butyloxycarbonyl)-L-phenylalanine methyl ester (Boger et al., 1985; 250 g, 0.90 mol) in toluene (3.0 L) was treated with diisobutylaluminum hydride in hexane (Aldrich; 2.2 L of 1 M), the latter added over 30 min with stirring and cooling such that the temperature of the mixture was maintained below −70 °C. After being stirred for an additional 15 min, the mixture was treated with absolute methanol at −78 °C (250 mL, dropwise at first until the vigorous effervescence subsided), followed 20 min later by 3.0 L of 50% Rochelle salts solution. Diethyl ether (2 L) was added and the mixture warmed to 25 °C. The aqueous layer after separation was washed with ether (2 × 1.5 L). The combined organic layers were washed with brine, dried (Na_2SO_4), and concentrated, and the crude aldehyde was dissolved in dimethoxyethane (1.5 L). This solution was cooled in an ice bath and treated slowly with a 0 °C solution of $NaHSO_3$ (105 g, 1.0 mol) in H_2O (1.5 L) at such a rate that the temperature did not exceed 10 °C. After being stirred for 16 h while the temperature rose to 25 °C, the solution was concentrated to a volume of 1.0 L, mixed with ethyl acetate (3.5 L), treated at 21 °C with potassium cyanide (65 g, 1.0 mol) in water (300 mL), and stirred at 25 °C for 16 h. The layers were separated, and the aqueous layer was extracted with ethyl acetate. These extracts were washed with brine, dried, and concentrated to an oil which was dissolved in ether (375 mL). Hexanes (1 L) were added to this solution. Crystallization began in several minutes, and the chilled mass was filtered and washed with 1:3 ether–hexane (800 mL). The dried material (122 g, 49%) was a 5:1 mixture of the desired 2(*R*),3(*S*) and the 2(*S*),3(*S*) diastereomers, respectively, as determined by RP-HPLC as described below. Two recrystallizations of this material in the same manner afforded 60.9 g (25% overall) of the title substance. Less than 1% of the 2(*S*),3(*S*) diastereomer was present by HPLC. This analysis was best effected in 40:60 acetonitrile–pH 2.1 buffer as described in the General Methods section above, with analyte injected in the mobile phase immediately after dissolution. The retention times for the 2(*R*),3(*S*) and 2(*S*),3(*S*) diastereomers were 8.0 and 7.7 min, respectively. No isomerization was observed in a few minutes in this acidic mobile phase; in contrast, the substance on standing in acetonitrile–H_2O or acetonitrile–pH 7 buffer isomerized within minutes to an approximately 1:1 mixture

Scheme I: Synthesis of CP-69,799[a]



[a] Conditions: (a) DIBAH, −78 °C, $NaHSO_3$, KCN, recrystallization, 25% overall; (b) TBDMS-Cl, imidazole, DMF; (c) H_2, 5% Rh/C, EtOH–NH_3; (d) $(CF_3CO)_2O$, *i*-Pr_2NEt; (e) H_2, 10% Rh/C, MeOH; (f) $NaBH_4$, EtOH; (g) $(CH_3)_2CHCHO$, NaCNBH, MeOH–HOAc; (h) HCl·Lys(Z)PheOBn (**13**), carbonyldiimidazole, imidazole, Et_3N; (i) HCl–dioxane; HF–pyridine; (j) BocPheHis(Boc) (**14**), DEC, HBT; (k) H_2, 10% Pd/C, MeOH–HOAc; (l) K_2CO_3, MeOH–H_2O; RP-HPLC.

with the 2(*S*),3(*S*) diastereomer. The title substance showed the following: mp 115–116 °C; $[\alpha]^{22}_D$ −58.1° (*c* = 1.14, $CHCl_3$); ^1H NMR ($CDCl_3$, 250 mHz) δ 1.42 (s, 9 H), 2.95 (dd, 1 H), 3.13 (dd, 1 H), 3.86 (m, 1 H), 4.55 (m, 1 H), 4.88 (br, 1 H), 5.00 (br, 1 H), 7.2–7.4 (m, 5 H); ^{13}C NMR (75 mHz, $CDCl_3$) δ 156.4, 136.5, 129.1, 128.8, 127.7, 118.6, 81.1, 63.4, 55.4, 35.7, 28.2. Anal. Calcd for $C_{15}H_{20}N_2O_3$: C, 65.20; H, 7.29; N, 10.14. Found: C, 65.25; H, 7.39; N, 10.14.

Preparation of 2(R),3(S)-3-[N-(tert-Butyloxycarbonyl)-amino]-2-(tert-butyldimethylsilyloxy)-4-phenylbutyronitrile (**2**). A solution of **1** (15.2 g, 55.0 mmol) and imidazole (9.35 g, 137 mmol, 2.5 equiv) in anhydrous dimethylformamide (Aldrich; 125 mL) was cooled to 0 °C, treated with *tert*-butyldimethylchlorosilane (11.6 g, 77.0 mmol, 1.2 equiv), and brought to 25 °C. After 3.5 h, the solution was concentrated and the residue dissolved in ethyl acetate (300 mL). The mixture was washed twice with small portions of aqueous lithium chloride, aqueous 1 N HCl (2 × 100 mL), and brine, dried, and concentrated. The residue was chromatographed on 600 g of silica eluted with 1:25 ethyl acetate–hexanes (2 L), 1:15 ethyl acetate–hexanes and finally 1:7 ethyl acetate–hexanes, giving the title silyl ether as a colorless syrup (21.5 g, 100%): TLC R_f 0.55 in 1:3 ethyl acetate–hexanes; $[\alpha]^{22}_D$ −32.1° (*c* = 1.09, $CHCl_3$); ^1H NMR ($CDCl_3$, 250 mHz) δ 0.17 (s, 3 H), 0.22 (s, 3 H), 0.95 (s, 9 H), 1.37 (s, 9 H), 2.74 (dd, 1 H, *J* = 9.4, 14.2 Hz), 3.13 (dd, 1 H, *J* = 5.4, 14.2 Hz), 4.04 (m, 1 H), 4.63 (d, 1 H, *J* = 3.3 Hz), 4.70 (d, 1 H, *J* = 8.1 Hz), 7.15–7.35 (m, 5 H). HRLSIMS: calculated for $C_{21}H_{34}N_2O_3Si$ + H, 391.2418; found, 391.2429.

Preparation of 3(S),2(S)-3-[N-(tert-Butyloxycarbonyl)-amino]-2-(tert-butyldimethylsilyloxy)-4-phenyl-1-butylamine (**3**). A solution of **2** (15.0 g, 38.4 mmol) in absolute ethanol (200 mL) was cooled in an ice bath while anhydrous ammonia (2 g) was introduced. Rhodium on carbon (5%, Aldrich; 2 g) was added, and the mixture was shaken under 50 psi of hydrogen pressure for 18 h. Filtration through Celite and concentration gave the substance as a colorless oil (15.09 g, 99%): TLC R_f 0.19 in 18:2:1 chloroform–ethanol–acetic acid; ^1H NMR ($CDCl_3$, 250 mHz) δ 0.08 (s, 3 H), 0.10 (s, 3 H), 0.93 (s, 9 H), 1.34 (s, 9 H), 1.48 (br, 2 H, exchanges with D_2O), 2.6–2.75 (m, 2 H), 2.77 (m, 2 H), 3.60 (m, 1 H), 4.09 (m, 1 H), 4.66 (d, 1 H, *J* = 10 Hz), 7.1–7.35 (m, 5 H).

Preparation of N-[3(S),2(S)-3-[N-(tert-Butyloxycarbonyl)amino]-2-(tert-butyldimethylsilyloxy)-4-phenylbut-1-yl]-trifluoroacetamide (**4**). A solution of **3** (9.75 g, 24.7 mmol) and diisopropylethylamine (6.45 mL, 37.1 mmol, 1.5 equiv) in dichloromethane (50 mL) was treated dropwise at 0 °C with trifluoroacetic anhydride (5.2 mL, 36.6 mmol, 1.48 equiv). After 15 min the mixture was diluted with dichloromethane (100 mL), washed with water (2 × 50 mL), ice-cold 1 N HCl (3 × 50 mL), and water (3 × 50 mL), dried, and concentrated to give a pale yellow oil: TLC R_f 0.18 (1:6 ethyl acetate–hexanes; ^1H NMR ($CDCl_3$, 250 mHz) δ 0.14 (s, 3 H), 0.18 (s, 3 H), 0.96 (s, 9 H), 1.36 (s, 9 H), 2.7–2.9 (m, 3 H), 3.8 (m, 2 H), 3.97 (m, 1 H), 4.6 (d, 1 H, *J* = 10 Hz), 7.1–7.4 (m, 5 H); IR ($CHCl_3$) 3450 (m), 1750 (m), 1730 (s), 1710 (s) cm^{-1}. HRLSIMS: calculated for $C_{23}H_{37}N_2O_4SiF_3$ + H, 491.2554; found, 491.2538.

Preparation of N-[3(S),2(S)-3-[N-(tert-Butyloxycarbonyl)amino]-2-(tert-butyldimethylsilyloxy)-4-cyclohexylbut-1-yl]trifluoroacetamide (**5**). A solution of **4** (10.6 g, 21.5 mmol) in methanol (150 mL) was shaken with 10% rhodium on carbon (1.0 g; Engelhard Corp.) at 25 °C and 50 psi of hydrogen pressure for 24 h. The mixture was filtered through Celite and concentrated, giving a colorless foam which was chromatographed on 400 g of silica in 1:20 ethyl acetate–hexanes, giving the title substance as a colorless oil (8.5 g, 79%): TLC R_f 0.4 in 1:6 ethyl acetate–hexanes; ^1H NMR ($CDCl_3$, 250 mHz, partial) δ 0.9 (s overlapping m, 11 H total),

1.1–1.4 (m, ca. 5 H), 1.47 (s, 9 H), 1.6–1.8 (m, ca. 5 H), 2.76 (m, 1 H), 3.6–3.85 (m, 3 H), 4.49 (d, 1 H, J = 10 Hz), 8.0 (br, 1 H).

Preparation of 3(S),2(S)-3-[N-(tert-Butyloxycarbonyl)-amino]-2-(tert-butyldimethylsilyloxy)-4-cyclohexyl-1-butylamine (**6**). A solution of **5** (6.98 g, 14.1 mmol) in absolute ethanol (60 mL) was treated at 0 °C with sodium borohydride (2.13 g, 56.4 mmol). The mixture was stirred at 25 °C for 2 h, stored at 0 °C for 12 h, and concentrated, and the residue was dissolved in ether (200 mL), and the resulting solution was washed with water (3 × 25 mL). The aqueous layers were extracted once with ether, and the combined organic layers were dried and concentrated, giving the product as a colorless oil (6.0 g): TLC R_f 0.31 (18:2:1 chloroform–ethanol–acetic acid); ^{1}H NMR (300 mHz, $CDCl_3$) δ 0.04 (s, 3 H), 0.05 (s, 3 H), 0.87 and 0.88 (s, 9 H total, ca. 6:1, respectively, for apparent rotamers), 1.0–1.4 (s, ca. 7 H), 1.41 and 1.43 (s, 9 H total, ca. 1:6, respectively), 1.49 (br, 2 H, exchanges with D_2O), 1.6–1.8 (m, ca. 7 H), 2.62 (m, 2 H), 3.52 (m, 1 H), 3.89 (m, 1 H), 4.4 and 4.48 (d, 1 H total, ca. 1:6, respectively, J = 10 Hz for major). HRLSIMS: calculated for $C_{21}H_{44}N_2O_3Si$, 401.3200; found, 401.3171.

Preparation of N-Isobutyl-3(S),2(S)-3-[N-(tert-butyloxycarbonyl)amino]-2-(tert-butyldimethylsilyloxy)-4-cyclohexyl-1-butylamine (**7**). A solution of **6** (4.78 g, 11.9 mmol) in absolute methanol (50 mL) was treated at 20 °C with acetic acid (3.5 mL), 3-Å, 600-mesh molecular seives (2 g), sodium cyanoborohydride (1.18 g, 18.8 mmol, 1.58 equiv), and finally dropwise isobutyraldehyde (1.71 mL, 18.8 mmol). After 20 min the mixture was filtered through Celite and concentrated, the residue was dissolved in ethyl acetate, and the resulting solution was washed with aqueous $NaHCO_3$, dried, and concentrated, giving the title amine as a colorless oil (5.70 g, 103%), TLC R_f 0.5 in 18:2:1 chloroform–ethanol–acetic acid, which was used without further purification. The analytical sample was prepared by chromatography on silica in an ethanol–dichloromethane gradient (1–8% ethanol, 0.5% NH_4OH): ^{1}H NMR (300 mHz, $CDCl_3$) δ 0.06 (s, 3 H), 0.07 (s, 3 H), 0.87 and 0.88 (s, 9 H total, ca. 6:1, respectively), 0.90 (d, 3 H, J = 6.7 Hz), 0.91 (d, 3 H, J = 6.7 Hz), 1.0–1.4 (m, ca. 7 H), 1.41 and 1.43 (s, 9 H total, ca. 6:1, respectively), 1.5–1.9 (m, ca. 7 H), 2.35 (dd, 1 H, J = 7, 11.5 Hz), 2.4–2.65 (m, 3 H), 3.7 (m, 2 H), 4.52 and 4.60 (d, 1 H total, ca 1:6, respectively, J = 4.6 Hz). HRLSIMS: calculated for $C_{25}H_{52}N_2O_3Si$ + H, 457.3826; found, 452.3840.

Preparation of N^{α}-[N-Isobutyl-N-3(S),2(S)-3-[N-(tert-butyloxycarbonyl)amino]-2-(tert-butyldimethylsilyloxy)-4-cyclohexylbut-1-yl]carbamoyl]-N^{ϵ}-(benzyloxycarbonyl)-L-lysyl-L-phenylalanine Benzyl Ester (**8**). A solution of N^{ϵ}-(benzyloxycarbonyl)-Lys-Phe benzyl ester hydrochloride (**13**, 1.32 g, 2.39 mmol, 1.3 equiv) and triethylamine (0.33 mL, 2.4 mmol) in dichloromethane (7 mL) was added dropwise at 25 °C to a solution of *N*-isobutyl-3(*S*),2(*S*)-3-[*N*-(*tert*-butyloxycarbonyl)amino]-2-(*tert*-butyldimethylsilyloxy)-4-cyclohexyl-1-butylamine (840 mg, 1.84 mmol), imidazole (162 mg, 2.39 mmol, 1.3 equiv), and carbonyldiimidazole (387 mg, 2.39 mmol) in dichloromethane (3.5 mL). The mixture was stirred for 18 h and diluted with dichloromethane, and the resulting solution was washed with 1 N HCl and water, dried, concentrated, and chromatographed on 100 g of silica eluted with 1:2 ethyl acetate–hexanes, giving the substance (1.32 g, 71%) as a colorless foam: TLC R_f 0.38 in 1:2 ethyl acetate–hexanes; ^{1}H NMR (300 mHz, $CDCl_3$, partial, for major rotamer) δ 0.07 (s, 3 H), 0.10 (s, 3 H), 0.84 (d, 6 H), 0.88 (s, 9 H), 1.1–1.4 (m, ca. 8 H), 1.44 (s, 9 H), 1.6–1.9 (m, ca. 10 H), 2.85–3.15 (m, ca. 9 H), 3.30 (dd, 1 H), 3.68 (m, 1 H), 3.90 (m, 1 H), 4.25 (m, 1 H), 4.68 (d, 1 H), 4.84 (q, 1 H, J = 7.8 Hz), 4.95–5.20 (m, 5 H), 6.42 (br, 1 H), 7.01 (m, 2 H), 7.15–7.40 (m, ca. 15 Hz). HRLSIMS: calculated for $C_{56}H_{85}N_5O_9Si$ + H, 1000.6915; found, 1000.6277.

Preparation of N^{α}-[N-Isobutyl-N-[3(S),2(S)-3-amino-2-hydroxy-4-cyclohexylbut-1-yl]carbamoyl]-N^{ϵ}-(benzyloxycarbonyl)-L-lysyl-L-phenylalanine Benzyl Ester (**9**). Compound **8** (1.25 g, 1.26 mmol) was dissolved in 12 mL of 4 N HCl–dioxane at 25 °C. After 2.5 h the solution was concentrated and the residue dried in vacuo. This solid was dissolved in dry acetonitrile (6 mL) and treated dropwise at 0 °C with 1.0 mL of HF–pyridine (Aldrich). After 3 h the mixture was poured into a chilled mixture of ethyl acetate and 60 mL of 2 N NaOH. The organic layer was separated, dried (Na_2SO_4), and concentrated, giving the title substance (840 mg, 100%).

Preparation of N^{α}-[N-Isobutyl-N-[3(S),2(S)-3-[[[N-(tert-butyloxycarbonyl)-L-phenylalanyl]-N^{τ}-(tert-butyloxycarbonyl)-L-histidyl]amino]-2-hydroxy-4-cyclohexylbut-1-yl]-carbamoyl]-N^{ϵ}-(benzyloxycarbonyl)-L-lysyl-L-phenylalanine Benzyl Ester (**10**). Dicyclohexylcarbodiimide (262 mg, 1.275 mmol) was added to a 0 °C solution of **9** (800 mg, 1.02 mmol), [*N*-(*tert*-butyloxycarbonyl)-L-phenylalanyl]-N^{τ},N^{α}-di(*tert*-butyloxycarbonyl)-L-histidine (**14**, 618 mg, 1.275 mmol), and *N*-hydroxybenzotriazole hydrate (275 mg, 1.8 mmol) in dichloromethane (3 mL). The mixture was stirred at 0 °C for 4 h and at 25 °C for 18 h and filtered, and the solids were washed with dichloromethane. The filtrate was concentrated, the residue suspended in ethyl acetate, and the mixture filtered. The filtrate was washed twice with 1 N NaOH and water, dried, and concentrated, and the residue was chromatographed on silica eluting with a gradient of ethanol in dichloromethane, giving the title substance as a colorless foam (567 mg, 44%): ^{1}H NMR (250 mHz, $CDCl_3$, partial) δ 0.82 (d, 3 H), 0.88 (d, 3 H), 1.38 (s, 9 H), 1.60 (s, 9 H), 1.9 (m, ca. 2 H), 2.8–3.3 (overlapping m, ca. 8 H), 3.4 (m, 1 H), 3.58 (m, 1 H), 3.93 (m, 1 H), 4.03 (m, 1 H), 4.21 (m, 1 H), 4.28 (m, 1–2 H), 4.55 (m, 1 H), 4.87 (m,1 H), 5.06 (m, 4–5 H), 5.41 (m, 1 H), 6.7 (m, 1 H), 7.0–7.4 (m, ca. 15 H), 7.93 (s, 1 H), 8.62 (br, 1 H). HRLSIMS: calculated for $C_{70}H_{95}N_9O_{13}$ + H, 1270.7128; found, 1270.7202.

Preparation of N^{α}-[N-Isobutyl-N-[3(S),2(S)-3-[[[N-(tert-butyloxycarbonyl)-L-phenylalanyl]-N^{τ}-(tert-butyloxycarbonyl)-L-histidyl]amino]-2-hydroxy-4-cyclohexylbut-1-yl]-carbamoyl]-L-lysyl-L-phenylalanine Acetate (**11**). A solution of **10** (550 mg, 0.433 mmol) in methanol (15 mL) and acetic acid (5 mL) was shaken with 10% Pd/C (100 mg) at 25 °C and 40 psi of hydrogen pressure for 2.5 h. The mixture was filtered through Celite, concentrated, coevaporated twice with added toluene and twice with ether, suspended in ether, and filtered. The filtered solid was dried in vacuo (428 mg, 90%): ^{1}H NMR (250 mHz, $CDCl_3$, partial) δ 0.75 (d, 6 H, J = 7 Hz), 1.0–1.2 (m, ca. 6 H), 1.27 (s, 9 H), 1.54 (s, 9 H), 1.87 (s, ca. 3 H), 4.1 (m, ca. 4 H), 4.57 (m, 2 H), 6.25 (m, 1 H), 7.1–7.3 (m, ca. 10 H), 7.57 (d, 1 H), 7.65 (d, 1 H), 8.09 (s, 1 H), 8.38 (d, 1 H). HRLSIMS: calculated for $C_{55}H_{83}N_9O_{11}$ + H, 1046.6290; found, 1046.6287.

Preparation of CP-69,799 [*N^{α}-[N-Isobutyl-N-[3(S),2(S)-3-[[[N-(tert-butyloxycarbonyl)-L-phenylalanyl]-L-histidyl]-amino]-2-hydroxy-4-cyclohexylbut-1-yl]carbamoyl]-L-lysyl-L-phenylalanine Diacetate*]. A solution of **11** (391 mg, 0.354 mmol) in methanol (5 mL) was treated sequentially at 25 °C with 121 mg (0.88 mmol, 2.5 equiv) of potassium carbonate and water (1 mL). After 30 min aqueous HCl was added to

bring the pH to 5.5, and the mixture was concentrated. The residue was dissolved in methanol (5 mL), and water (ca. 15 mL) was added dropwise until precipitation appeared complete. The insoluble oil was collected by centrifugation and washed twice with 1:4 methanol–water. The residue was dried in vacuo and coevaporated several times with added ether to give a solid (200 mg) which was purified by RP-HPLC in the following system: 10 × 250 mm Zorbax C-8 column, 48/52 (v/v) acetonitrile/pH 4.3 0.2 M NH_4OAc/HOAc buffer, 6.3 mL/min, 254-nm detection. Injections were 5 mg each in 0.1 mL mobile phase. The pure fractions were concentrated to dryness, giving 12 mg of pure CP-69,799 per 25 mg of the crude solid injected: ^1H NMR (250 mHz, DMSO-d_6, partial) δ 0.75 (d, 6 H), 1.31 (s, 9 H), 3.84 (m, 1 H), 4.11 (m, 2–3 H), 4.52 (m, 1 H), 6.28 (m, 1 H), 6.8 (s, 1 H), 7.1–7.4 (m, ca. 12 H), 7.45 (s, 1 H), 7.7 (d, 1 H, J = 8 Hz), 8.36 (d, 1 H, J = 8 Hz). LSIMS: calculated for $C_{50}H_{75}N_9O_9$ + H, 946.5766; found, 946.5795.

Preparation of N^{α}-[N-(tert-Butyloxycarbonyl)-L-phenylalanyl]-N^{τ}-(tert-butyloxycarbonyl)-L-histidine (**14**). A slurry of L-histidine methyl ester dihydrochloride (36.5 g, 150 mmol) in dichloromethane (1 L) was treated sequentially at 5 °C with triethylamine (52 mL, 373 mmol), *N*-hydroxybenzotriazole hydrate (30.6 g, 201 mmol), *N*-(*tert*-butyloxycarbonyl)-L-phenylalanine (40.0 g, 150 mmol), and dicylohexylcarbodiimide (30.8 g, 150 mmol), and the mixture was stirred at 0 °C for 4 h and at 20 °C for 90 h. The mixture was filtered, the filtered solid was washed with dichloromethane, the combined filtrates were concentrated, and the residue was suspended in ethyl acetate. After 10 min the mixture was filtered and the filtrate washed with 1 N NaOH (3 × 150 mL) and brine, dried, and concentrated, giving 45.9 g of the crude dipeptide ester. A portion of this crude solid (40.0 g, 96.2 mmol) was dissolved in a mixture of methanol (600 mL) and water (200 mL) and treated at 0 °C with anhydrous potassium carbonate (40 g, 290 mmol). The mixture was stirred for 2.5 h at 20 °C and for 1.5 h at 28 °C, cooled to 10 °C, and treated with 12 N HCl until at pH 4.2. This solution was concentrated to about 250 mL, diluted with water (70 mL) and dioxane (660 mL), and treated at 0 °C with 6 N NaOH until at pH 13.5. Di-*tert*-butyl dicarbonate (29 mL, 125 mmol, 1.3 equiv) was added, the mixture was warmed to 20 °C over 30 min (during which time the pH fell to 9.5), and an additional 10 mL of di-*tert*-butyl dicarbonate (0.45 equiv) was added. After another 1 h, no starting material was present as judged by RP-HPLC, and the mixture was concentrated to remove dioxane, water (300 mL) was added, and the resulting solution was washed twice with ether. The aqueous layer was stirred with ethyl acetate (500 mL), and the pH was adjusted at 10 °C to 1.2 with 12 N HCl. The aqueous layer was separated and washed twice with ethyl acetate, and the combined organic layers were washed with water, dried, concentrated, and coevaporated with added ether, giving the product (44 g, 60% overall) as a colorless foam: HPLC 3.23 min (60/40, 94% of the total integration to 8 min). That no appreciable Boc-L-Phe-D-His(Boc) was present in this sample was determined by HPLC analysis of the material after treatment with NaOH in methanol [a mixture of Boc-L-Phe-L-His and Boc-L-Phe-D-His, prepared by treatment of Boc-L-Phe-L-His methyl ester with LiOH in methanol, was readily separable by RP-HPLC (33/67)]: ^1H NMR ($CDCl_3$, 250 mHz) δ 1.39 (s, 9 H), 1.59 (s, 9 H), 3.0–3.15 (m, 3 H), 3.21 (dd, 1 H), 4.35 (m, 1 H), 4.60 (m, 1 H), 5.03 (d, 1 H), 6.84 (br, 1 H), 7.1–7.35 (m, ca. 6 H), 8.08 (d, 1 H, J = 1.2 Hz). HRLSIMS: calculated for $C_{25}H_{34}N_4O_6$ + H, 503.2507; found, 503.2554.

Preparation of N^{α}-(tert-Butyloxycarbonyl)-N^{ϵ}-(Carbobenzyloxy)-L-lysyl-L-phenylalanine Benzyl Ester (**12**). Dicyclohexylcarbodiimide (14.3 g, 69.4 mmol) was added to a 0 °C solution of N^{α}-(*tert*-butyloxycarbonyl)-N^{ϵ}-(carbobenzyloxy)-L-lysine (26.4 g, 69.4 mmol), L-phenylalanine benzyl ester *p*-toluenesulfonic acid salt (29.7 g, 69.4 mmol), triethylamine (12.5 mL, 90.2 mmol), and *N*-hydroxybenzotriazole hydrate (16.0 g, 104 mmol) in dichloromethane (160 mL), and the mixture was stirred for 16 h, during which time the temperature rose to 20 °C. The mixture was filtered, the filtrate evaporated, the residue suspended in ethyl acetate (600 mL), this suspension filtered, and the filtrate evaporated. The residue was recrystallized twice from 100 mL of 1:1 toluene–hexanes, giving a colorless solid: TLC R_f 0.3 in 2:1 ethyl acetate–hexanes; mp 102.5–105 °C; ^1H NMR ($CDCl_3$, 300 mHz) δ 1.30 (m, 2 H), 1.40 (s, 9 H), 1.45–1.6 (m, ca. 2 H), 1.80 (m, ca. 3 H), 3.08 (m, 2 H), 3.14 (m, 2 H), 4.03 (m, 1 H), 4.85 (m, 1 H), 4.89 (d, 1 H, J = 5.8 Hz), 5.07 (m, 2 H), 5.10 (m, 2 H), 6.44 (d, 1 H, J = 7.9 Hz), 6.98 (dd, 2 H), 7.2–7.4 (m, ca. 15 H). Anal. Calcd for $C_{35}H_{43}N_3O_7$: C, 68.05; H, 7.02; N, 6.80. Found: C, 68.32; H, 6.97; N, 6.82.

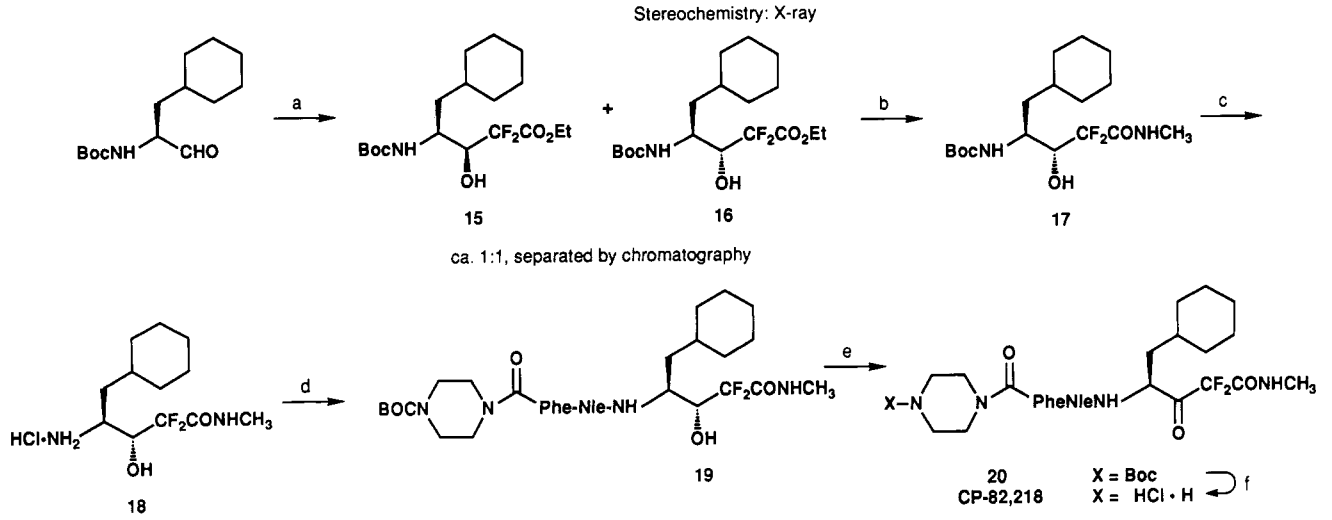
Preparation of N^{ϵ}-(Carbobenzyloxy)-L-lysyl-L-phenylalanine Benzyl Ester Hydrochloride (**13**). A solution of anhydrous HCl in dioxane (4 N, 125 mL) was added in one portion of **12** (32.4 g, 52.5 mmol) at 0 °C. The mixture was stirred at 25 °C for 45 min and concentrated, and the residue was coevaporated several times with added ether and dried, giving a colorless solid which was recrystallized by dissolution in boiling dichloromethane (55 mL) and dilution of the resulting solution with ether (165 mL) to give after filtration at 0 °C and washing with the same solvent mixture 26.4 g of an off-white solid: ^1H NMR (DMSO-d_6, 300 mHz) δ 1.31 (m, 4 H), 1.70 (m, 2 H), 2.93 (m, 2 H), 3.00 (dd, 1 H), 3.08 (dd, 1 H, J = 6.2, 14 Hz), 3.76 (m, 1 H), 4.58 (m, 1 H), 5.01 (m, 2 H), 5.06 (d, 1 H, J = 12.6 Hz), 5.09 (d, 1 H, J = 12.6 Hz), 7.2–7.4 (m, 15 H), 8.24 (br, 1 H), 9.09 (d, 1 H, J = 7.2 Hz). Anal. Calcd for $C_{30}H_{36}N_3O_5Cl$: C, 65.03; H, 6.55; N, 7.58. Found: C, 64.83; H, 6.56; N, 7.53.

Synthesis of the Inhibitor CP-82,218 (Scheme II)

Preparation and Enantiomeric Purity Determination of 2(S)-[(tert-Butyloxycarbonyl)amino]-3-cyclohexylpropanal. This aldehyde was prepared and purified by silica chromatography as has been described (Boger et al., 1985). Assessment of its enantiomeric purity was conveniently made by chiral stationary phase (CSP) HPLC of the derivative prepared by sequential one-flask ethanol–$NaBH_4$ treatment of a 50-mg sample, evaporation, HCl–dioxane deprotection, and 1-naphthoyl chloride acylation (dioxane–water, pH 11). CSP-HPLC of the extracted *N*-(1-naphthoyl)-3-cyclohexyl-L-alaninol on a Pirkle type 1-A ionic (3,5-dinitrobenzoyl)-phenylglycine column (Pirkle et al., 1981; this column is available commercially from the Regis Chemical Co., Morton Grove, IL 60053) in 15:85 2-propanol–hexane at 2 mL/min showed separation of enantiomers (verified with racemate): T_{ret} 10.9 and 12.7 min (ca. 97:3 ratio, respectively, corresponding to 94% enantiomeric purity). The enantiomeric purity of material in both early and late column fractions was also 94%.

Preparation of Ethyl 4(S)-[tert-(Butyloxycarbonyl)-amino]-5-cyclohexyl-2,2-difluoro-3(S)- and 3(R)-hydroxypentanoates (**15** *and* **16**). The following procedure is a modification of Ishihara's method (Ishihara et al., 1984) for the condensation of chlorodifluoromethyl ketones with alde-

Scheme II: Synthesis of CP-82,218[a]



[a] Conditions: (a) $BrCF_2CO_2Et$, Zn–$TiCl_4$, THF, 0–25 °C; (b) $MeNH_2$, EtOH, 25 °C; (c) HCl–dioxane; (d) Boc-piperazino-CO-Phe-Nle (**26**), DEC, HBT, $MeCl_2$; (e) $COCl_2$, DMSO, $MeCl_2$; *i*-Pr_2NEt; (f) HCl–dioxane.

hydes. Ethyl chlorodifluoroacetate was unreactive under these conditions. On a preparative scale the procedure reproducibly gave the difluorocyclohexylstatine isomers in essentially enantiomerically pure form, albeit in modest yield (35–50% combined, ca. 1:1 ratio), and as such proved a useful alternative to the independently reported Reformatsky-like methods (Hallinan & Fried, 1984; Thaisrivongs, 1985, 1986): Titanium tetrachloride (99.995%, Aldrich; 4.47 mL, 0.041 mol, 0.4 mol equiv) was added dropwise by syringe at 10–20 °C to a stirred suspension of dried zinc (activated by washing successively with dilute HCl, water, ethanol, and ether, 20.0 g, 0.31 mol) in THF (85 mL). The resulting dark brown suspension was stirred at 25 °C for 15 min, cooled to 5 °C, and treated with a mixture of purified 2(*S*)-[(*tert*-butyloxycarbonyl)amino]-3-cyclohexylpropanal (26.0 g, 0.102 mol, 1.0 equiv) and ethyl bromodifluoroacetate (SCM Speciality Chemicals, 20.5 g, 0.102 mol, 1.0 equiv) in 40 mL of THF over 15 min with cooling so that the reaction temperature remained below 5 °C. The resulting mixture was stirred at 25 °C for 15 min, cooled to 10 °C, and treated with a further 2.23 mL (0.2 equiv) of $TiCl_4$. The mixture was warmed to 25 °C and poured into a stirred mixture of ether (1200 mL), ice (500 g), and sodium bicarbonate (60 g). The layers were separated, the aqueous layer were extracted with ether, and the combined organic layers were dried and concentrated, giving 32.9 g (85%) of an orange oil which was chromatographed on 1 kg of silica packed and loaded in 12% ethyl acetate–hexane, eluting with 7 L each of 12%, 14%, and 16% ethyl acetate–hexane. Mixed fractions were combined, concentrated, and chromatographed a second time in the same manner. A total of 2.9 g (8%) of the mixture of isomers remained unseparated. The less polar isomer, ethyl 4(*S*)-[(*tert*-butyloxycarbonyl)-amino]-5-cyclohexyl-2,2-difluoro-3(*R*)-hydroxypentanoate (**16**, 9.02 g, 23%, TLC R_f 0.43 in 1:2 ethyl acetate–hexanes), was obtained as a waxy solid $[\alpha]^{20}_D$ –20.6° (*c* = 1.24, $CHCl_3$) [lit. (Thaisrivongs et al., 1986) –19° (*c* = 0.5, $CHCl_3$)]. Recrystallization from 1:4 ethyl acetate–hexanes (60% recovery) gave material having $[\alpha]^{25}_D$ –19.4° (*c* = 1.23, $CHCl_3$) and mp 114.5–117 °C [lit. (Thaisrivongs et al., 1986) 113.0–116.1 °C]. The stereochemistry was unambiguously defined by X-ray analysis of a crystal grown from 4:1:1 ether–chloroform–methanol. X-ray crystal data were collected on a Nicolet R3m/u diffractometer: λ = 1.5418 Å, θ–2θ scan mode, 5° < 2θ < 100°. The substance showed the following: $C_{18}H_{31}NO_5F_2$, 0.18 mm × 0.19 mm × 0.22 mm, $P2_12_12_1$, *Z* = 4, *a* = 10.608 (4) Å, *b* = 11.202 (4) Å, *c* = 17.828 (5) Å, *V* = 2119 (1) Å^3, and ρ_{calcd} = 1.19 g/cm^3. A total of 1277 unique reflections were measured; 1028 were considered observed [$I > 3.0\sigma(I)$] and were used in the subsequent structure analysis, yielding *R* = 0.053 and GOF = 1.39. Positional and thermal parameters are included as supplementary material. The more polar isomer, ethyl 4(*S*)-[(*tert*-butyloxycarbonyl)amino]-5-cyclohexyl-2,2-difluoro-3(*S*)-hydroxypentanoate (**15**, 8.52 g, 22%, TLC R_f 0.38 in 1:2 ethyl acetate–hexanes), was obtained as a colorless syrup. This material could be recrystallized from hexanes (75% recovery) giving material having $[\alpha]^{25}_D$ –39.6° (*c* = 1.01, $CHCl_3$) and mp 73–74 °C [lit. (Thaisrivongs et al., 1986) $[\alpha]^{25}_D$ –40° (*c* = 0.5, $CHCl_3$) and mp 72.8–74.5 °C].

Preparation of 4(S)-[(tert-Butyloxycarbonyl)amino]-5-cyclohexyl-2,2-difluoro-3(R)-hydroxypentanoic Acid N-Methylamide (**17**). Excess anhydrous methylamine was introduced into an ice-cooled solution of **16** (1.66 g) in ethanol (10 mL). The mixture was stirred at 25 °C for 2.5 h and concentrated, and the residue was chromatographed on 60 g of silica packed and loaded in 1:5 ethyl acetate–hexanes, eluting with this mixture (1.5 L) followed by 7:3 ethyl acetate–hexanes. Solvent removal left 1.39 g (87%) of the title substance as a colorless powder: TLC R_f 0.29 in 1:1 ethyl acetate–hexanes. Alternatively, in another experiment, recrystallization of the crude material from 1:5 isopropyl ether–hexanes afforded the title substance in 73% yield: mp 71–76 °C, ^{1}H NMR (300 mHz, $CDCl_3$) δ 0.6–1.4 (m, ca. 6 H), 1.43 (s, 9 H), 1.6–1.9 (m, ca. 8 H), 2.90 (d, 3 H, *J* = 5 Hz), 3.94 (m, 2 H), 4.21 (br, 1 H), 4.78 (d 1 H); ^{19}F NMR (282 mHz, $CDCl_3$) δ –115.9 (dd, 1 F, A of AB, *J* = 12, 261 Hz), –118.8 (dd, 1 F, B of AB, *J* = 11, 261 Hz). Anal. Calcd for $C_{17}H_{30}N_2O_4F_2$: C, 56.03; H, 8.30; N, 7.69. Found: C, 55.81; H, 8.33; N, 7.60.

Preparation of 4(S)-Amino-5-cyclohexyl-2,2-difluoro-3(R)-hydroxypentanoic Acid N-Methylamide Hydrochloride (**18**). A 5 °C a 4 N solution of HCl in dioxane was added in one portion to 1.39 g of **17**, and the resulting mixture was stirred at 25 °C for 40 min. Evaporation and drying gave a colorless powder (1.3 g): TLC R_f 0.05 in 18:2:1 chloroform–ethanol–acetic acid. A sample was recrystallized from 2:1 isopropyl ether–isopropyl alcohol: mp 125–135 °C; ^{1}H NMR (D_2O, 300 mHz) δ 0.0.8–1.8 (m, 13 H), 2.80 (s, 3 H), 3.71

(m, 1 H), 4.34 (ddd, 1 H); ^{19}F NMR (282.4 mHz, D_2O) δ −113.7 (dd, 1 F, A of AB, J = 10, 262 Hz), −120.2 (dd, 1 F, B of AB, J = 16, 262 Hz). Anal. Calcd for $C_{12}H_{22}N_2O_2F_2$·HCl·$^1/_2H_2O$: C, 46.52; H, 7.81; N, 9.04. Found: C, 46.36; H, 7.77; N, 8.96.

Preparation of 4(S)-[[[N-[[4-(tert-Butyloxycarbonyl)piperazino]carbonyl]-L-phenylalanyl]-L-norleucyl]amino]-5-cyclohexyl-2,2-difluoro-3(R)-hydroxypentanoic Acid N-Methylamide (**19**). A stirred mixture of **18** (0.301 g, 1.00 mmol), triethylamine (0.180 mL, 1.3 mmol, 1.3 equiv), **26** (0.491 g, 1.0 mmol, 1.0 equiv), and *N*-hydroxybenzotriazole hydrate (0.230 g, 1.5 mmol, 1.5 equiv) was treated at 0 °C with dicyclohexylcarbodiimide (0.206 g, 1.0 mmol, 1.0 equiv), and the mixture was stirred in an ice bath which was allowed to warm to 20 °C overnight. The mixture was filtered and concentrated, and the residue was stirred with 100 mL of ethyl acetate. The suspension was filtered, and the filtrate was washed twice with 1 N NaOH and brine, dried, and concentrated to give 0.76 g of an off-white foam which was chromatographed on silica (45 g) packed in 1:200 ethanol–dichloromethane. The column was eluted with 800-mL portions of 1:200, 1:100, 1:50, and 1:25 ethanol–dichloromethane, giving the title substance as a colorless foam after evaporation and drying (0.573 g, 78%): TLC R_f 0.58 in 18:2:1 chloroform–ethanol–acetic acid; HPLC (80:20 MeCN–H_2O) 2.84 min (99% of integration to 10 min); ^{1}H NMR ($CDCl_3$, 300 mHz, partial) δ 0.91 (t, 3 H, Nle CH_3), 1.45 (s, 9 H, Boc), 2.89 (d, 3 H, NCH_3), 2.94 (dd, 1 H), 6.35 (d, 1 H, J = 6.5 Hz), 7.2–7.5 (m, 6–7 H); ^{19}F NMR (282 mHz, $CDCl_3$) δ −111.1 (d, 1 F, A of AB, J = 255 Hz), −119.2 (dd, 1 F, B of AB, J = ca. 13 Hz); MS (FAB, thioglycerol) m/e (rel intensity) 737 (MH^+, 60), 378 (50), 304 (68), 265 (100).

Preparation of 4(S)-[[[N-[[4-(tert-Butyloxycarbonyl)piperazino]carbonyl]-L-phenylalanyl]-L-norleucyl]amino]-5-cyclohexyl-2,2-difluoro-3-oxopentanoic Acid N-Methylamide (**20**). A stirred solution of dry dimethyl sulfoxide (0.064 mL, 0.089 mmol, 2.2 equiv) in dry dichloromethane (0.8 mL) was treated at −60 °C with oxalyl chloride (0.43 mL, 0.49 mmol, 1.2 equiv), followed at −60 °C by a solution of **19** (0.300 g, 0.407 mmol, 1.0 equiv) in 0.75 mL of dry dichloromethane (added by cannula under nitrogen pressure with an additional 0.25 mL of dichloromethane used to rinse the donor vessel), and the mixture was stirred for 45 min at −30 °C and cooled again to −60 °C. Dry diisopropylethylamine (0.35 mL, 2.04 mmol, 5 equiv) was added, the mixture was warmed to 25 °C and diluted with dichloromethane (50 mL), and the solution was washed with three 2-mL portions of saturated aqueous $NaHCO_3$ and brine, dried (Na_2SO_4), and concentrated, giving 0.35 g of a colorless foam which was chromatographed on 15 g of silica packed in 3:1 ethyl acetate–hexanes and eluted with 500 mL of this solvent. Evaporation of the appropriate fractions and drying gave the title product as a colorless foam (0.245 g, 82%): TLC R_f 0.33 (ethyl acetate, containing none of the more polar starting material); ^{1}H NMR (300 mHz, $CDCl_3$, partial) δ 0.88 (t, 3 H, Nle CH_3), 1.46 (s, 9 H, Boc CH_3), 2.91 (d, 3 H, NCH_3); ^{19}F NMR (282 mHz, ca. 0.002 M in $CDCl_3$) major compound at δ −111.3 (A of AB, 1 F, J = 270 Hz), −112.5 (B of AB, 1 F, J = 270 Hz), minor singlets (<10% of total integration) at δ −112.13, −112.17, and −112.5. ^{13}C NMR (75.4 mHz, ca. 0.2 M in $CDCl_3$) showed clearly a single (>85%) substance, in the keto form: δ (partial) 195.2 (t), 172.5, 172.1, ca. 160.0 (t), 157.2, 154.5, 136.7, 129.2, 128.6, 127.0, ca. 108.4 (t, CF_2), 80.2, 56.1, 53.3, 52.9, 43.5, 38.0, 36.9, 34.0, 33.7, 31.8, 31.6, 28.3, 27.5, 26.4, 26.2, 26.1, 25.8, 22.3, 13.9; FAB-MS (thioglycerol) m/e (rel intensity) 735 (MH^+, 40), 304 (88), 217 (100).

Preparation of CP-82,218 [4(S)-[[[N-(Piperazinocarbonyl)-L-phenylalanyl]-L-norleucyl]amino]-5-cyclohexyl-2,2-difluoro-3-oxopentanoic Acid N-Methylamide Hydrochloride]. Compound **20** (0.157 g, 0.214 mmol) was dissolved in 4 N HCl–dioxane (2.0 mL), and the resulting solution was stirred at 25 °C for 45 min and concentrated. The resulting solid was coevaporated with ether and dried, giving 145 mg (100%) of CP-82,218 as a colorless solid: TLC R_f 0.18 in 18:2:1 in chloroform–ethanol–acetic acid (the spotted plate was exposed to ammonia vapor prior to development); ^{1}H NMR (300 mHz, D_2O, partial) δ 0.83 (t, 3 H, J = ca. 6 Hz), 1.0–1.8 (overlapping m, ca. 16 H), 2.78 (s, 3 H, NCH_3), 2.89 (dd, 1 H, J = 10.3, 13.6 Hz), 3.07 (m, ca. 4–5 H), 3.18 (dd, 1 H, J = 4.5, 14.5 Hz), 3.51 (m, ca. 14 H), 4.24 (m, 2 H), 4.52 (dd, 1 H, J = 5, 10 Hz), 7.30 (m, ca. 5 H); ^{19}F NMR (282.3 mHz, ca. 0.01 M in D_2O) major resonances at δ −116.8 (d, A of AB, 1 F, J = 260 Hz, −119.3 (d, B of AB, 1 F, J = 260 Hz), minor resonances (ca. 15% of total integration) at δ −117.3 (d, A′ of A′B′, 1 F, J = 255 Hz), −119.0 (d, B′ of A′B′, 1 F, J = 255 Hz), −118.4 (apparently center of A‴B‴ pattern). LSIMS: calculated for $C_{32}H_{48}F_2N_6O_5$ + H, 635.3732; found, 635.3710.

Preparation of tert-Butyl 4-Benzylpiperazine-1-carboxylate (**21**). Di-*tert*-butyl dicarbonate (18 mL, 80 mmol, 1.4 equiv) was added at 0 °C to a pH 12 (with aqueous NaOH) solution of 1-benzylpiperazine (10.0 g, 57 mmol) in 100 mL of dioxane and 50 mL of water, and the pH was maintained (with aqueous NaOH) at 9.5–10.5. After 10 min, an additionl 4 mL of di-*tert*-butyl dicarbonate was added, after 10 min the mixture was concentrated to remove dioxane and extracted with ethyl acetate, and the extracts were washed with brine and dried. Chromatography on silica eluting with an ethyl acetate–hexanes gradient provided 14.2 g (90%) of the substance as a colorless solid: TLC R_f 0.43 in 2:1 ethyl acetate–hexanes; ^{1}H NMR (300 mHz, $CDCl_3$) δ 1.46 (s, 9 H), 2.40 (m, 4 H), 3.44 (m, 4 H), 3.53 (s, 2 H), 7.2–7.35 (m, 5 H).

Preparation of the Acetic Acid Salt of tert-Butyl Piperazine-1-carboxylate (**22**). A solution of **21** (7.7 g, 27.9 mmol) in 70 mL of methanol and 7 mL of acetic acid was shaken with 10% Pd/C (2.0 g) for 2.5 h at 25 °C and 45 psi of hydrogen pressure. The mixture was filtered through Celite, the filtrate concentrated, and the residue coevaporated several times with added toluene. The resulting oily solid was dissolved in ether (75 mL) at reflux and allowed to cool. The resulting crystals were collected by filtration, washed with ether at 0 °C, and dried, giving 6.29 g (92%) of the title substance as colorless needles. A recrystallized sample (ether) had the following: mp 105–106 °C; ^{1}H NMR ($CDCl_3$, 250 mHz) δ 1.43 (s, 9 H), 1.95 (s, 3 H), 2.90 (m, 4 H), 3.49 (m, 4 H), 8.25 (br, 2 H). Anal. Calcd for $C_9H_{18}N_2O_2$·$C_2H_4O_2$: C, 53.64; H, 9.00; N, 11.37. Found: C, 53.56; H, 8.99; N, 11.38.

Preparation of N-[[4-(tert-Butyloxycarbonyl)piperazino]carbonyl]-L-phenylalanine Benzyl Ester (**23**). 2(*S*)-Isocyanato-3-phenylpropionic acid benzyl ester (3.65 g, 13.0 mmol, 1.0 equiv; Lombardino & Gerber, 1964) was added at 25 °C to a solution of **22** (3.2 g, 13.0 mmol) in dichloromethane (30 mL). After 15 min the mixture was concentrated, and the residue was chromatographed on 400 g of silica packed in 3:7 ethyl acetate–hexanes and eluted with 6 L of this solvent followed by 2 L of 2:3 ethyl acetate–hexanes, giving after solvent removal 3.85 g (63%) of an oily solid: TLC R_f 0.24 in 1:1 ethyl acetate–hexanes; ^{1}H NMR (300 mHz, $CDCl_3$) δ 1.45 (s, 9 H), 3.10 (m, 2 H), 3.30 (m, 4 H), 3.38 (m, 4 H),

4.83 (m, 2 H), 5.10 (A of AB, 1 H, J = 12 Hz), 5.18 (B of AB, 1 H, J = 12 Hz), 6.98 (m, 2 H), 7.15–7.45 (m, 8 H). HRLSIMS: calculated for $C_{26}H_{33}N_3O_5$ + H, 468.2500; found, 468.2515.

Preparation of N-[[4-(tert-Butyloxycarbonyl)piperazino]carbonyl]-L-phenylalanine (**24**). A solution of **23** (3.84 g, 8.21 mmol) in 30 mL of 1:10 acetic acid–methanol was shaken with 0.5 g of 10% Pd/C at 50 psi of hydrogen pressure for 1.5 h at 25 °C. The mixture was filtered through Celite and concentrated, giving after coevaporation with added toluene and ether a colorless foam (2.74 g, 88%): TLC R_f 0.48 in 18:2:1 chloroform–ethanol–acetic acid; ^{1}H NMR (300 mHz, $CDCl_3$) δ 1.45 (s, 9 H), 3.10 (dd, 1 H), 3.25 (m, 5 H), 3.36 (m, 4 H), 4.4 (br, 1 H), 4.61 (m, 1 H), 4.98 (m, 1 H), 7.15–7.30 (m, 5 H). LSIMS: calculated for $C_{19}H_{27}N_3O_5$ + H, 378.2030; found, 378.2026.

Preparation of N-[N-[[4-(tert-Butyloxycarbonyl)piperazino]carbonyl]-L-phenylalanyl]norleucine Benzyl Ester (**25**). A mixture of norleucine benzyl ester hydrochloride (1.00 g, 3.88 mmol), triethylamine (0.70 mL, 5.04 mmol, 1.3 equiv), **24** (1.46 g, 3.88 mmol), and *N*-hydroxybenzotriazole hydrate (0.89 g, 5.82 mmol, 1.5 equiv) in dichloromethane (8 mL) was treated at 0 °C with dicyclohexylcarbodiimide (0.80 g, 3.88 mmol, 1.0 equiv). After being stirred for 65 h (the ice bath was allowed to warm to 25 °C), the mixture was filtered and diluted with dichloromethane, and the resulting solution was washed twice with 2 N NaOH and brine, dried, and concentrated to give a yellow foam which was chromatographed on 100 g of silica packed in 1:200 ethanol–dichloromethane and eluted with 500 mL of this solvent followed by 1-L portions of 1:100 and 1:50 ethanol–dichloromethane. Concentration of the appropriate fractions gave the title substance as a colorless foam (1.65 g, 73%): TLC R_f 0.68 in 18:2:1 chloroform–ethanol–acetic acid; ^{1}H NMR (300 mHz, $CDCl_3$) δ 0.80 (t, 3 H, J = 7 Hz), 1.1–1.25 (m, 5 H), 1.45 (s, 9 H), 1.55–1.80 (m, 4–5 H), 3.00 (dd, A of AB, 1 H, J = 7.3, 13.7 Hz), 3.10 (dd, B of AB, 1 H, J = 7.3, 13.7 Hz), 3.30 (m, 4 H), 3.35 (m, 4 H), 4.49 (q, 1 H), 4.56 (q, 1 H), 5.06 (d, 1 H, J = 7 Hz), 5.13 (m, 2 H), 6.34 (d, 1 H, J = 7.7 Hz), 7.15–7.3 (m, 5 H), 7.35 (m 5 H). HRLSIMS: calculated for $C_{32}H_{44}N_4O_6$ + H, 581.3341; found, 581.3354.

Preparation of N-[N-[[4-(tert-Butyloxycarbonyl)piperazino]carbonyl]-L-phenylalanyl]norleucine (**26**). A solution of **25** (1.64 g, 2.82 mmol) in 20 mL 1:10 methanol–acetic acid was shaken with 10% Pd/C (0.30 g) at 50 psi of hydrogen pressure at 25 °C for 40 min. The mixture was filtered through Celite and the cake washed well with methanol. The filtrates were concentrated, and the residue was coevaporated with added toluene and ether and dried, giving the product as a colorless foam (1.40 g, 100%): TLC R_f 0.54 in 18:2:1 chloroform–ethanol–acetic acid; ^{1}H NMR (300 mHz, $CDCl_3$) δ 0.86 (t, 3 H, J = 7 Hz), 1.2–1.4 (m, 4–5 H), 1.46 (s, 9 H), 1.67 (m, 2–3 H), 1.85 (m, 2 H), 3.07 (center of AB, 2 H), 3.25–3.4 (m, ca. 8 H), 4.44 (q, 1 H), 4.67 (q, 1 H, J = 7.4 Hz), 5.32 (d, 1 H), 6.85 (d, 1 H), 7.15–7.35 (m, 5–6 H). HRLSIMS: calculated for $C_{25}H_{38}N_4O_6$ + H, 491.2871; found, 491.2876. Anal. Calcd for $C_{25}H_{38}N_4O_6 \cdot {}^1/_2H_2O$: C, 60.09; H, 7.67; N, 11.21. Found: C, 60.46; H, 7.52; N, 10.99.

Crystallography

Crystals of the native enzyme were grown as described (Suguna et al., 1987a). These crystals belong to the space group $P2_12_12_1$ with a = 60.31 Å, b = 60.60 Å, and c = 106.97 Å. The inhibitor–enzyme complexes were prepared either by soaking a native crystal in a solution containing the inhibitor or by cocrystallization. For CP-69,799, the complex was prepared both by soaking and by cocrystallization. The soaked complex was prepared by soaking a native crystal in a solution containing 50 μL of the mother liquor (1.5 mg of protein dissolved in 220 μL of the crystallization buffer: 20 mM calcium acetate, 50 mM cacodylic acid, pH 6.0) and the inhibitor solution. This inhibitor solution was prepared by dissolving 0.17 mg of CP-69,799 in 21.5 μL of methanol. The resulting crystal of the inhibited enzyme had cell dimensions with a = 60.52 Å, b = 60.66 Å, and c = 107.44 Å. Cocrystals of CP-69,799 and rhizopuspepsin were obtained by dissolving 0.31 mg of inhibitor in 5 μL of methanol and adding it to a solution containing 3.01 mg of rhizopuspepsin dissolved in 220 μL of the buffer described above. The resulting solution was filtered through a 0.2-μ filter (Schleicher & Schuell) and divided into four sitting drops. After 17 months, a crystal was harvested and analyzed. The resulting crystal of the inhibited enzyme had cell dimensions with a = 60.44 Å, b = 60.63 Å, and c = 107.27 Å.

Similarly, for CP-82,218, the complex was prepared by soaking a native crystal in a solution containing mother liquor and the inhibitor solution (0.04 mg of CP-82,218 hydrochloride dissolved in 20 μL of water). The resulting enzyme–inhibitor crystal had the following cell dimensions: a = 60.48 Å, b = 60.71 Å, and c = 106.93 Å. The data collection and refinement parameters for each inhibitor complex are given in Table I.

The intensity data for each inhibitor–protease complex were collected using a Siemens multiwire area detector mounted on a Rigaku RU-200 rotating anode X-ray generator. The 3-kW beam used in the data collection was generated using a 0.2 × 2 mm focal cup, passed through a graphite monochromator, and collimated using a 0.25-mm collimator.

For the CP-69,799 soaked crystal, 124 221 observations of 25 352 reflections between 6.5 to 2.0 Å were collected using a rectangular parallelepiped crystal (1 mm × 0.5 mm × 0.2 mm). After Lorentz, polarization, and absorption corrections (Howard et al., 1985) were applied to the data, the R_{sym} ($\sum|\bar{I} - I|/\sum|I|$) for the 25 352 unique reflections was calculated to be 0.075. This represents 95% of the theoretically available data in the measured range. An electron density map, calculated using $|F_{complex}| - |F_{native}|$ as amplitudes and the phases of the native enzyme (Suguna et al., 1987a), clearly showed five of the six residues of the inhibitor. Initially, only density for the aromatic ring of the phenylalanine at the carboxyl terminus was observed, but upon further refinement, density for the main-chain atoms was also observed for this residue. A model was built and interactively fitted to this density using FRODO (Jones, 1978; Pflugrath et al., 1984) on an Evans and Sutherland PS390 graphics system.

The structure has been refined using the restrained least-squares program PROLSQ developed by Hendrickson and Konnert (Hendrickson & Konnert, 1981; Hendrickson, 1985), which was modified (Finzel, 1987) to make use of Agarwal's (Agarwal, 1978) fast-Fourier-transform (FFT) algorithm for structure factor calculations. It was also modified to correct for any short contacts between symmetry-related molecules (Sheriff, 1987). The starting model for the refinement consisted of the refined native enzyme at 1.8 Å (Suguna et al., 1987a) and the model of the inhibitor fit to the electron density in the cleft. After preliminary phase information was obtained, waters from the native structure were included. Only those waters that had not been displaced by the inhibitor and possessed occupancy of at least 0.7 as well as a B-factor less than 50 Å^2 were included. Special bond distances and angles were incorporated into the program PROTIN (Hendrickson,

Table I: Summary of Data Collection and Geometrical Parameters for the Rhizopuspepsin CP-82,218 and CP-69,799 Inhibitor Complexes[a]

	CP-82,218	CP-69,799 (soak)	CP-69,799 (cocrystal)
cell parameters ($P2_12_12_1$)			
a, Å	60.48	60.55	60.44
b, Å	60.71	60.62	60.63
c, Å	106.93	107.44	107.27
resolution range, Å	10–1.9	6.5–2.0	15–1.9
R_{sym}, %	7.5	7.5	8.2
R value, %	16.6	17.1	16.5
by shell (no. of reflections)			
6.5–2.98		5.53 (6893)	
2.98–2.36		12.49 (7888)	
2.36–2.00		21.38 (9162)	
10–3.5	5.26 (5228)		5.27 (5294)
3.5–2.75	8.75 (5207)		8.51 (5293)
2.75–2.41	15.97 (5072)		12.92 (5271)
2.41–2.19	18.77 (4965)		15.45 (5151)
2.19–2.03	20.24 (4769)		18.81 (4964)
2.03–1.91	20.79 (3318)		25.99 (3682)
rms deviation from ideality for			
distances, Å			
bond	0.011 (0.02)	0.012 (0.02)	0.009 (0.02)
angle	0.033 (0.04)	0.038 (0.04)	0.030 (0.04)
planar 1–4	0.036 (0.05)	0.041 (0.05)	0.030 (0.05)
planar groups, Å	0.009 (0.02)	0.009 (0.02)	0.008 (0.02)
nonbonded distances, Å			
single torsion	0.18 (0.5)	0.18 (0.5)	0.17 (0.5)
multiple torsion	0.19 (0.5)	0.21 (0.5)	0.18 (0.5)
possible H-bonds	0.24 (0.5)	0.23 (0.5)	0.19 (0.5)
torsion angles, deg			
planar	1.7 (3.0)	1.4 (2.0)	1.7 (3.0)
staggered	13.4 (15.0)	14.5 (12.0)	13.4 (15.0)
orthonormal	25.9 (20.0)	29.7 (20.0)	28.9 (20.0)
chiral volume, Å^3	0.14 (0.15)	0.16 (0.15)	0.11 (0.15)
thermal restraints, Å^2			
main-chain bond	0.62 (1.0)	0.69 (1.0)	0.59 (1.0)
main-chain angle	1.02 (1.5)	1.14 (1.5)	1.06 (1.5)
side-chain bond	0.67 (1.0)	0.64 (1.0)	0.60 (1.0)
side-chain angle	1.09 (1.5)	1.04 (1.5)	1.04 (1.5)

[a] The target σ values used in refinement (PROFFT; Hendrickson, 1985; Finzel, 1987; Agarwal, 1978; Sheriff, 1987) are given in parentheses. R_{sym} is defined as the unweighted absolute value R-factor on intensity ($\Sigma|\bar{I} - I|/\Sigma|I|$). The crystallographic R value is defined as $\Sigma|F_o - F_c|/\Sigma|F_o|$.

1985) to account for the geometry at the scissile bond. The crystallographic R-value after 42 cycles of refinement is 17.1%. The final model (non-hydrogen atoms only) contains the 2403 atoms of the enzyme, the 68 atoms of the inhibitor, and 353 waters. Inhibitor CP-69,799 contains the azahomostatine scissile bond surrogate which is isosteric with a dipeptide. For the purposes of refinement, this residue was modeled as two residues. The first residue was defined as cyclohexylalanine (Cha) and the second as a pseudo-leucine residue (Ial).

For the cocrystallized CP-69,799–rhizopuspepsin complex, reflections in the 15–1.9-Å range were measured using a rectangular parallelepiped crystal (1.1 mm × 0.5 mm × 0.25 mm). A total of 140 689 observations were measured and corrected for Lorentz polarization as well as absorption (Howard et al., 1985) prior to merging, yielding 29 655 unique reflections ($R_{sym} = 0.082$) representing 93% of the theoretically available data. An electron density map calculated using ($|F_{complex}| - F_{native}|$) as amplitudes and the refined phases of the native enzyme (Suguna et al., 1987a) exhibited continuous density in the active site cleft. This density defined the conformation of all residues in inhibitor CP-69,799. The structure has been refined using the methodology described above for the CP-69,799 soak experiment, and after 28 cycles of refinement the R-value is 16.5%. The final model contains the 2403 non-hydrogen atoms of the enzyme, the 68 non-hydrogen atoms of the inhibitor, and 293 waters.

Similarly, for the CP-82,218–rhizopuspepsin complex, reflections in the 10.0–1.9-Å range were measured using a rectangular parallelepiped crystal (1 mm × 0.5 mm × 0.2 mm). A total of 169 608 observations were measured and corrected for Lorentz, polarization, and absorption (Howard et al., 1985) prior to merging, yielding 32 949 unique reflections ($R_{sym} = 0.075$) representing 90% of the theoretically available data. An electron density map calculated using ($|F_{complex}| - |F_{native}|$) as amplitudes and the refined phases of the native enzyme (Suguna et al., 1987a) exhibited continuous density in the active site cleft. This density defined the conformation of all residues in inhibitor CP-82,218. The structure has been refined using the methodology described above for the CP-69,799 soak experiment, and after 26 cycles of refinement the R-value is 16.6%. The final model contains the 2403 atoms (non-hydrogen) of the enzyme, the 46 atoms (non-hydrogen) of the inhibitor, and 306 waters.

RESULTS

The final difference ($2|F_o| - |F_c|$) electron density maps for the inhibitor region of the refined complexes are shown in Figure 2. For CP-69,799 (Figures 2A,B), all six residues were clearly defined. Similarly, the three residues of the shorter inhibitor CP-82,218 (Figure 2C) are well-defined. Both CP-82,218 and CP-69,799 bind in an extended conformation which is consistent with other rhizopuspepsin-inhibited structures (Figure 3) and closely resembles the conformations observed in penicillopepsin (James & Sielecki, 1983) and endothiapepsin (Sali et al., 1989). These inhibitors are restrained in this conformation by a series of hydrogen bonds between the main-chain nitrogen and oxygen atoms of the inhibitors to atoms of the enzyme (Table II).

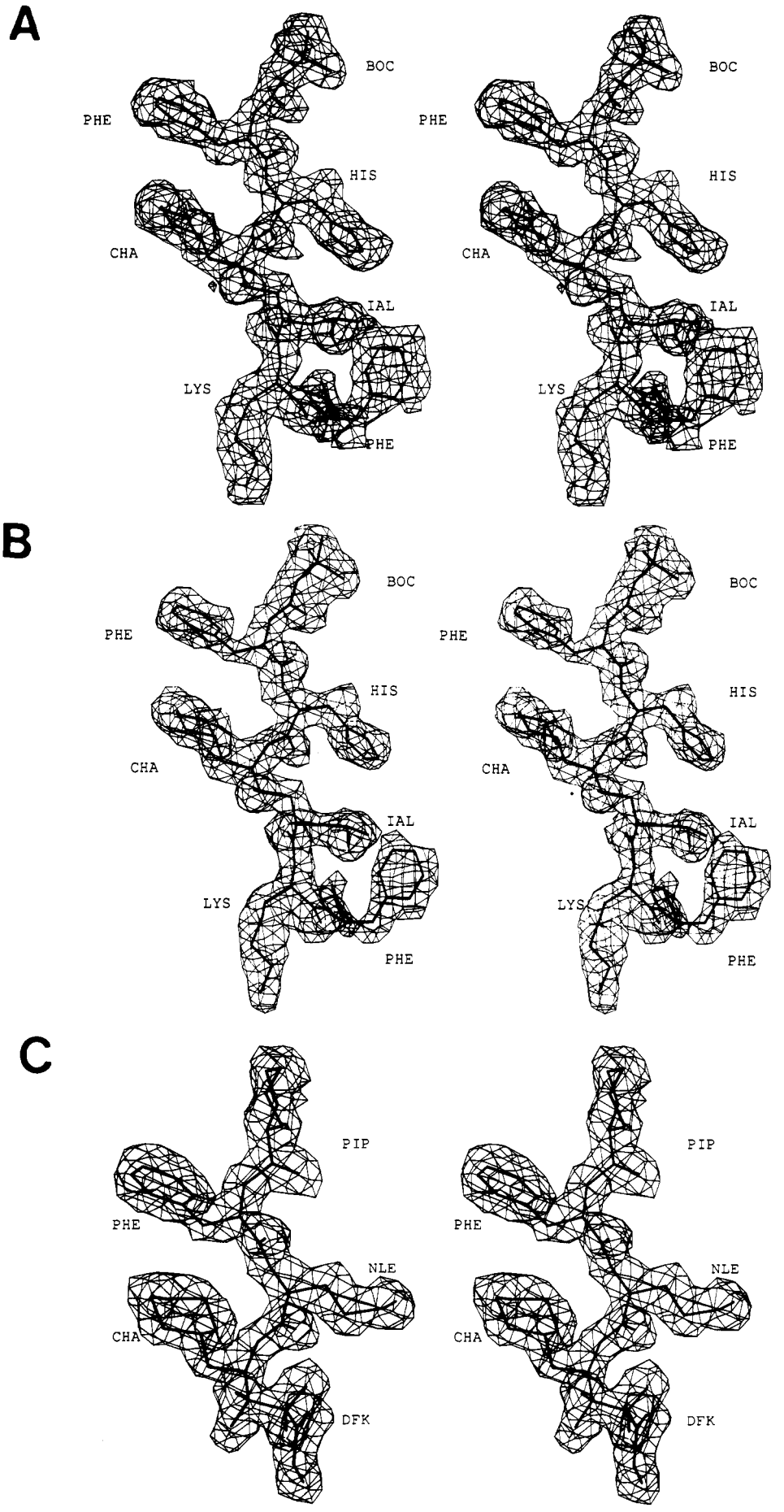


FIGURE 2: Final $2|F_o| - |F_c|$ electron density maps for inhibitors (A) CP-69,799 (soak), (B) CP-69,799 (cocrystal), and (C) CP-82,218 with final models superimposed. Note that there is electron density for all residues of each inhibitor.

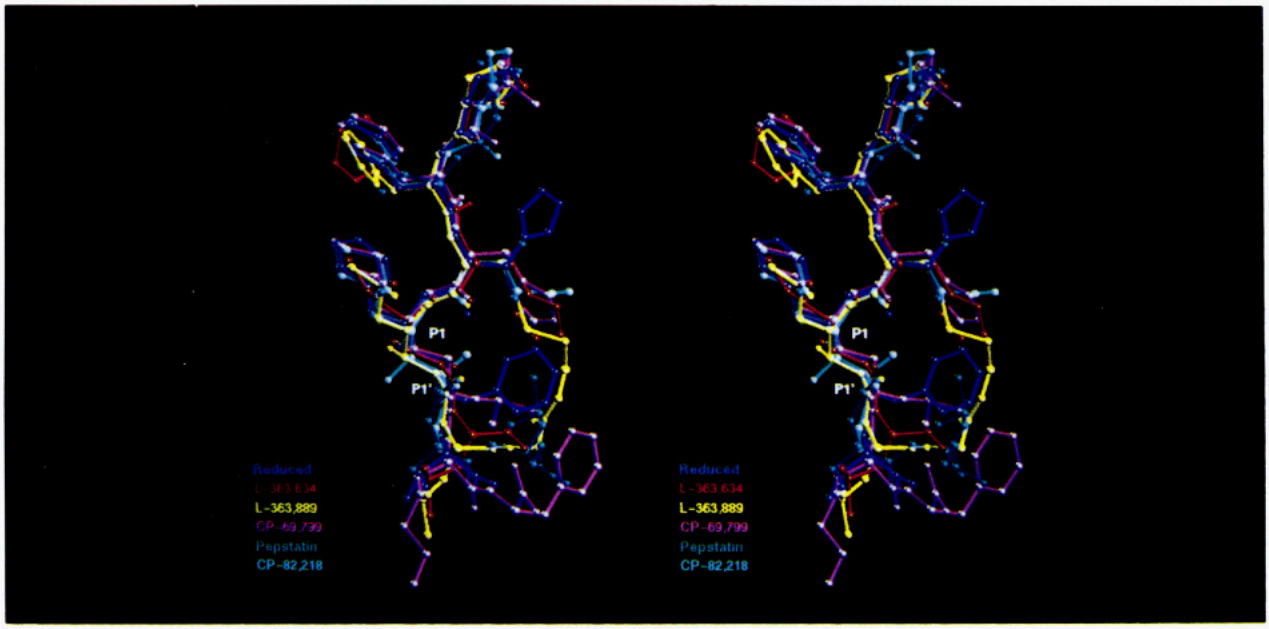


FIGURE 3: Superposition of rhizopuspepsin-bound inhibitors CP-82,218, CP-69,799, pepstatin, reduced peptide inhibitor, L-363,634, and L-363,889.

Table II: Hydrogen Bonds between the Inhibitors and Rhizopuspepsin[a]

inhibitor		protein		hydrogen bond distance, Å		
residue no.	atom	residue	atom	CP-69,799 (soak)	CP-69,799 (cocrystal)	CP-82,218
P_3	N	T222	OG1	2.95	2.84	3.04
	O	T222	N	3.06	2.89	3.00
	OT2	T222	OG1		3.38	
P_2	N	D79	OD1	3.22	3.21	3.07
	O	D79	N	3.08	2.07	3.16
	ND1	T221	OG1	2.78	2.89	
	O	G78	N			3.27
P_1	N	G220	O	2.98	2.99	3.25
	O2[b]	D35	OD1	3.28	3.24	
	O2	D35	OD2	2.53	2.61	2.79
	O2	D218	OD1	2.87	2.92	2.86
	O2	D218	OD2	2.70	2.74	2.58
	O1	D35	OD1			2.57
	O1	D35	OD2			3.24
	O1	G37	O			3.34
	O3	G78	N			2.76
P_1'	O	G78	N	2.98	2.88	
P_2'	N	G37	O	3.03	2.95	
	NZ	I130	O	2.77	2.74	
P_3'	OT	S76	O	2.99	2.92	

[a] Interatomic distances of less than 3.4 Å between electronegative atoms were regarded as potential hydrogen bonds. [b] Oxygens O1 and O2 are part of the dihydroxy moiety of residue Cha. O3 is the carbonyl oxygen of the C-terminal methylamino group.

In the CP-69,799–rhizopuspepsin complex (Figure 4A, both soaked and cocrystallized forms), the backbone of the phenylalanine residue in the P_3 position forms hydrogen bonds with both OG1 and N of Thr222. The next residue, histidine, makes hydrogen bonds to the "flap" (residues 77–83) through OD1 and N of Asp79 as well as to the main body of the enzyme through OG1 of Thr221. The residue at position P_1 (Cha) possesses only one contact with the enzyme through O of Gly220. The statine-like hydroxyl oxygen of the Cha residue at P_1 occupies the position of Wat507 found in the native structure which has been assigned the role of the nucleophile in the catalytic mechanism (Suguna et al., 1987b; Parris et al., 1991; Veerapandian et al., 1992; Hoover et al., 1991; James et al., 1992). This hydroxyl oxygen is located in such a position as to form contacts with all four oxygens of the two aspartic acids at the catalytic center. Three of these contacts are within hydrogen-bonding distance while the fourth can be best classified as a van der Waals interaction. The next residue, lysine, is hydrogen bonded to the enzyme at O of Gly37 as well as O of Ile130. The last residue, phenylalanine, has no hydrogen-bonded contacts although it does have a van der Waals contact with Trp294. Additional van der Waals contacts between the inhibitor and enzyme are given in Table III.

In the CP-82,218–rhizopuspepsin complex (Figure 4B), the N and O of the inhibitor N-terminal residue, phenylalanine, are also hydrogen bonded to the enzyme through OG1 and N of Thr222, respectively. As in the CP-69,799 complexes, the inhibitor makes hydrogen-bonded contacts with the flap through the N and O of the second residue, norleucine, to OD1 and N of Asp79 and from the P_1 NH to Gly220 carbonyl oxygen. An additional hydrogen bond to the flap is made

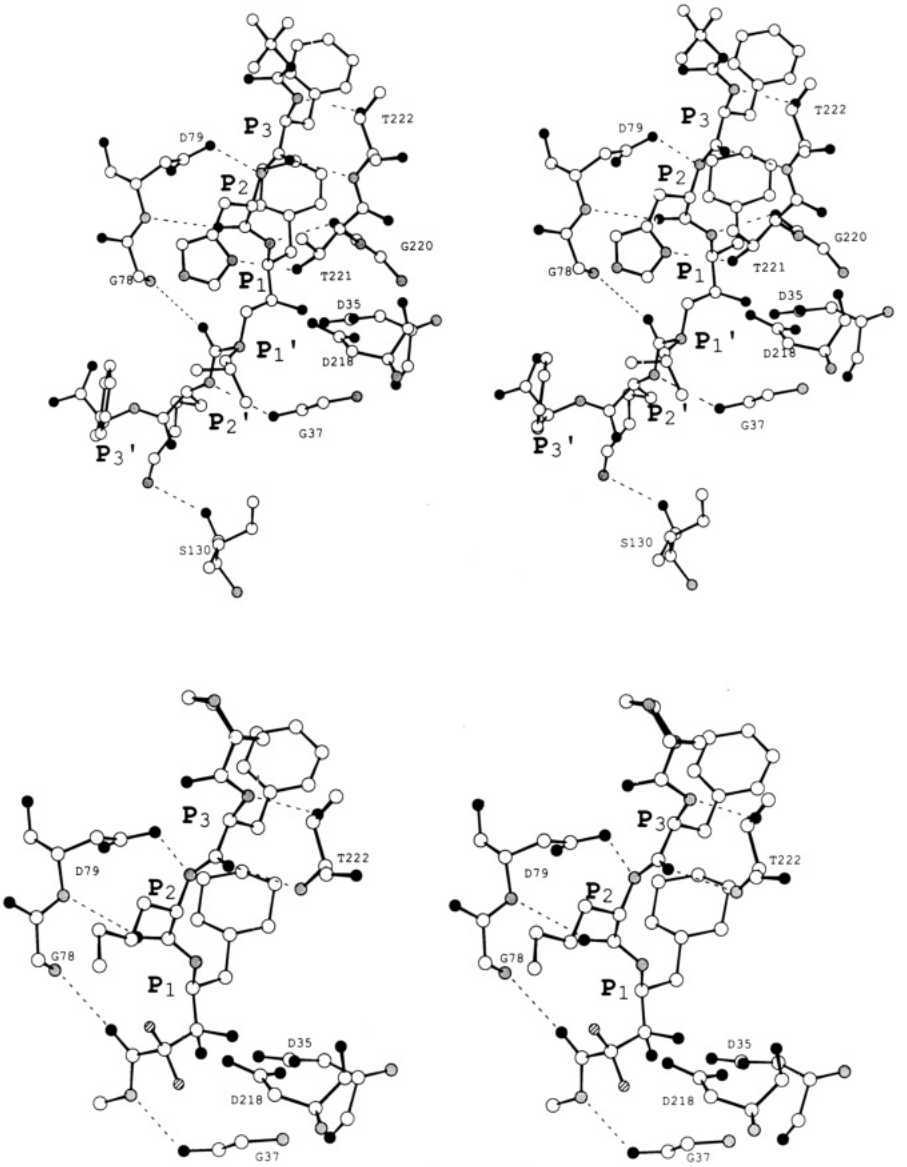


FIGURE 4: Stereoview of inhibitors (A, top) CP-69,799 (soak and cocrystal are identical) and (B, bottom) CP-82,218 showing nearest contacts with the protein. Residues D78 and G79 are part of the flap. Contacts short enough to be hydrogen bonds are shown dotted except for contacts between the hydroxyl group(s) of P_1 and the oxygens of D35 and D218, which for clarity are not drawn. Oxygen atoms are shown are black circles while carbon, nitrogen, and fluorine are shown as open circles, stippled circles, and striped circles, respectively.

between the amide O of the inhibitor carboxyl terminus and N of Gly78. The NH of the truncated P_1' (methylamino) residue is hydrogen bonded to the Gly37 carbonyl. As is clear from examination of the electron density (Figure 2B), the inhibitor is bound in the hydrated form. The oxygens of the *gem*-diol part of the C-terminal residue are positioned between the catalytic aspartic acids in the active site such that one, O1, is within hydrogen-bonding distance of OD1 of Asp35. The oxygen of the second hydroxyl, O2, is located at the position that Wat507 occupies in the native structure (Suguna et al., 1987a) and where the statine-like hydroxyl oxygen in numerous other inhibitors such as CP-69,799 also binds. This O2 is within hydrogen-bonding distance of OD2 of Asp35, OD1 of Asp218, and OD2 of Asp218. Additional contacts between this inhibitor and the enzyme are given in Tables II and III.

In both complexes described above, the flap is closed down upon the inhibitor. This movement of the flap, which has been observed in other inhibitor complexes with rhizopuspepsin (Suguna et al., 1987b, 1992) as well as with endothiapepsin (Cooper et al., 1987) and penicillopepsin (James et al., 1985), enhances the binding of the inhibitor by providing hydrogen bonds and van der Waal interactions between the inhibitor and the flap. These interactions result in a reduction of the mobility of the flap as measured by thermal factors. In the native structure the mean *B*-factor for the flap region is 34.2 Å^2 while in the CP-69,799 complexes the mean *B*-factors are 14.9 and 15.9 Å^2 for the soaked and cocrystallized structures, respectively, and in the CP-82,218 complex the mean *B*-factor is 9.0 Å^2. It has been proposed that enzymes catalyze reactions by excluding water from the reacting molecules, thus producing a "gas-phase" environment for the substrate and the enzyme surface (Dewar & Dieter, 1988; Warshel et al., 1989). This is supported by a calculation of solvent accessibility for CP-69,799 and CP-82,218 in both the bound and unbound states. Examination of Table IV shows that there is a dramatic change in solvent accessibility for the inhibitors upon complexation.

Whereas the analysis of CP-69,799 complexes to endothiapepsin (Sali et al., 1989) revealed a significant rotation of the C-terminal domain relative to the N-terminal domain in the

Table III: van der Waals Contacts between the Inhibitors and Rhizopuspepsin[a]

	CP-82,218		CP-69,799 (soak)		CP-69,799 (cocrystal)	
P_4	Pip401	Thr222 (4, 3.7) Leu223 (3, 4.0) Phe278 (5, 3.5)	Boc401	Thr222 (1, 3.6) Leu223 (1, 3.8) Phe278 (2, 3.7)	Boc401	Thr222 (3, 3.7) Leu223 (1, 3.9) Phe278 (3, 3.5)
P_3	Phe401	Ile15 (2, 3.5) Glu16 (3, 3.6) Asp79 (1, 3.5) Thr221 (1, 3.4) Thr222 (2, 3.0)	Phe401	Ile15 (2, 3.6) Glu16 (5, 3.7) Thr221 (1, 3.5)	Phe401	Ile15 (2, 3.6) Glu16 (2, 3.4) Thr221 (5, 3.6) Thr222 (1, 3.7)
P_2	Nle402	Tyr77 (1, 3.7) Gly78 (1, 3.6) Asp79 (3, 3.8) Thr221 (2, 3.4) Ile225 (2, 3.9) Trp294 (5, 3.5) Ile298 (2, 3.4)	His402	Gly78 (2, 3.8) Asp79 (3, 3.5) Gly220 (1, 3.7) Thr221 (4, 3.6) Trp294 (4, 3.7) Ile298 (3, 3.7)	His402	Gly78 (3, 3.8) Asp79 (3, 3.6) Thr221 (1, 3.6) Trp294 (4, 3.6) Ile298 (3, 3.5)
P_1	Cha403	Asp33 (2, 3.3) Asp35 (6, 3.5) Gly37 (1, 3.4) Ser38 (1, 3.6) Tyr77 (7, 3.9) Asp79 (2, 3.7) Ser81 (1, 3.6) Phe114 (2, 3.6) Leu122 (1, 3.9) Asp218 (2, 3.3) Gly220 (2, 3.6)	Cha403	Asp33 (2, 3.1) Asp35 (3, 3.5) Tyr77 (3, 4.0) Ser81 (1, 3.5) Phe114 (4, 3.8) Leu122 (2, 4.0) Asp218 (1, 3.2) Gly220 (3, 3.6)	Cha403	Asp33 (2, 3.1) Asp35 (3, 3.5) Tyr77 (2, 4.0) Asp79 (1, 3.6) Ser81 (1, 3.6) Phe114 (3, 3.8) Asp218 (1, 3.2) Gly220 (3, 3.5)
P_1'	Dfk403	Gly37 (1, 3.2) Tyr77 (4, 3.4) Asp218 (1, 3.0)	Ial404	Gly37 (1, 3.6) Tyr77 (3, 3.4) Trp194 (4, 3.8) Ile216 (2, 3.4) Ile298 (1, 3.5)	Ial404	Gly37 (1, 3.6) Tyr77 (3, 3.5) Trp194 (5, 3.7) Ile216 (2, 3.2)
P_2'			Lys405	Gly37 (1, 3.7) Ser38 (1, 3.8) Ile75 (4, 3.9) Ile130 (1, 3.5) Thr132 (1, 4.0) Trp194 (2, 3.5)	Lys405	Gly37 (1, 3.7) Ser38 (1, 3.7) Ile75 (1, 4.1) Ile130 (2, 3.5) Trp194 (2, 3.4)
P_3'			Phe406	Trp194 (3, 3.8) Trp294 (4, 3.5)	Phe406	Gly78 (1, 4.0) Trp194 (3, 3.9) Trp294 (6, 3.6)

[a] Atoms separated by less than 4.1 Å were regarded as being in van der Waals contact. In parentheses following each VDW contact are two values (X, Y). X is the number of contacts between the inhibitor residue and a given residue of the enzyme while Y is the average distance of the X contacts.

Table IV: Inhibitor Solvent Accessibility Changes upon Binding to Rhizopuspepsin[a]

	residue						
	P_3 Phe	P_2 His	P_2 Nle	P_1 Cha	P_1' Ial	P_2' Lys	P_3' Phe
CP-69,799 (isolated)							
main-chain atoms	49.87	27.40		30.63	36.91	23.93	31.80
side-chain atoms	180.30	79.72		94.93	53.72	90.10	109.96
total atoms	230.16	107.12		125.56	90.63	114.03	141.76
CP-69,799 (bound)							
main-chain atoms	32.31	0.00		0.00	0.00	6.19	32.20
side-chain atoms	119.05	23.28		14.88	0.00	36.19	62.00
total atoms	151.36	23.28		14.88	0.00	42.38	94.20
CP-82,218 (isolated)							
main-chain atoms	42.04		22.42	41.51			
side-chain atoms	184.99		71.88	191.56			
total atoms	227.03		94.30	233.07			
CP-82,218 (bound)							
main-chain atoms	24.76		0.00	5.13			
side-chain atoms	119.81		29.44	60.20			
total atoms	145.57		29.44	65.33			

[a] Surface areas are given in square angstroms. This solvent accessibility was assessed using the program MS of Connolly (1983) and programs developed by Sheriff et al. (1985). A radius of 1.7 Å was assumed for the solvent probe; standard van der Waals radii (Case & Karplus, 1979) were used. Abbreviations: Cha = cyclohexylalanine; Nle = norleucine; Ial = isoazaleucine.

complex relative to the native structure, equivalent movement is not observed in either soaked or cocrystallized CP-69,799–rhizopuspepsin complexes. Thus, the soaked complex shows less than a 0.2° rotation/0.1-Å displacement, and the cocrystallized complex also shows less than a 0.2° rotation/0.1-Å displacement, relative to the native structures. Although many solvent molecules (waters 507, 525, 570, 577, 663, 671, 678, 694, 721, 760, 789, 817, 827, 858, and 885; calcium 400) have been displaced in binding the inhibitor (Figure 5), only very small conformational changes occur in the enzyme. The

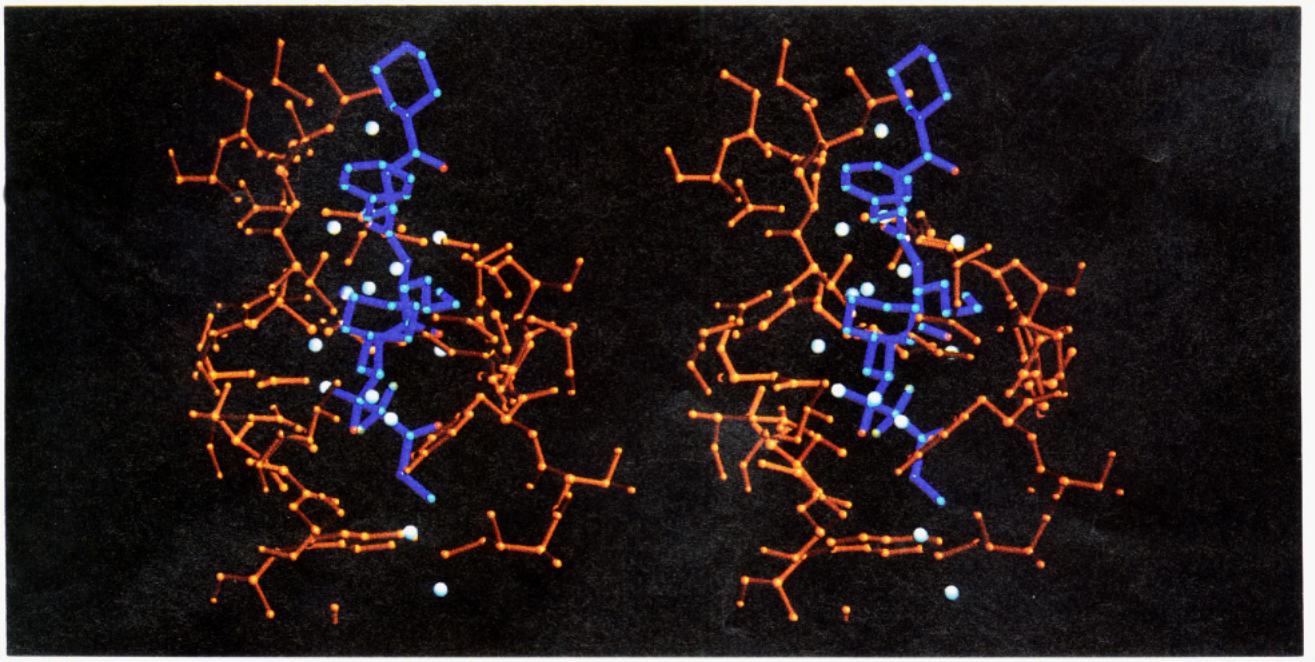


FIGURE 5: Stereoview of the active site of rhizopuspepsin. The enzyme is shown in brown while CP-82,218 is shown in blue. The solvent molecules which are displaced are shown as white spheres.

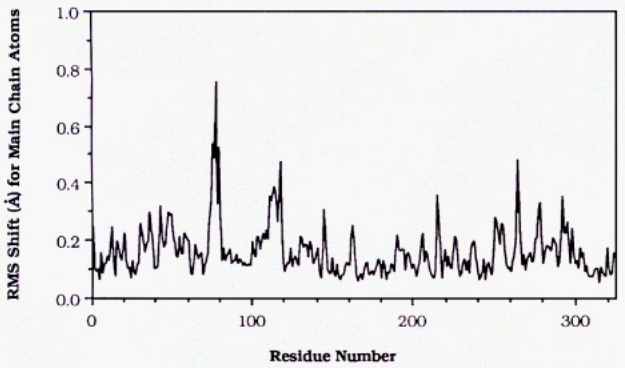


FIGURE 6: Graph showing the rms shift for the main-chain atoms of rhizopuspepsin upon binding inhibitor CP-69,799.

largest change that occurs is the movement in the flap region (Figure 6).

Since CP-82,218 bears essentially no residues C-terminal to the scissile bond region, some solvent remains present in the binding cleft that would be displaced when a typical longer substrate is bound. The final model for the CP-82,218–rhizopuspepsin complex has water molecules in the cleft (570 and 885) that are also observed in the native enzyme but not in previous rhizopuspepsin complexes such as that with the reduced inhibitor. Additional water molecules (863, 864, 869, 886, and 934) that are not observed in the native structure are also present in the C-terminal side of the substrate cleft in this complex.

Another movement that is characteristic of inhibitor binding in rhizopuspepsin structures is the rearrangement of the side chain of Asn119, which is about 5 Å removed from the binding pocket. In the native structure, this residue is hydrogen bonded to Wat698, while in the inhibited structures, Wat698 has been displaced. This leads to a rearrangement of the Asn119 side chain in order that it may adopt a hydrogen-bonding interaction with Glu16. This rearrangement is observed in the CP-82,218 complex and in the cocrystallized CP-69,799 complex, but in the CP-69,799 complex derived from soaking a native crystal, this residue is disordered. Refinement of the position of the Asn119 side chain in the CP-69,799 soaked complex indicates that its conformation is evenly divided between that observed in the native structure and that seen in other inhibited rhizopuspepsin structures, despite the presence of the inhibitor with unit occupancy.

DISCUSSION

The aspartic proteinases differ from most other proteinases by their ability to bind to oligopeptide inhibitors and substrates that contain up to eight or more amino acid residues. Many structural studies have shown that these peptides bind within the groove that lies between the N- and C-terminal domains (Foundling et al., 1987; Suguna et al., 1987b; James et al., 1982). Fruton (1976) and more recently Hofmann et al. (1988) have demonstrated that the addition of a residue at P_3 or at P_2' to tetra- or pentapeptide inhibitors will in either case substantially increase k_{cat} without greatly changing K_m. They concluded that the additional interactions produced a conformational change in either the protein or the substrate (or both) that would favor catalysis. Conformational changes in the substrate could result in distortion of the peptide bond from its planar conformation, lowering the energy barrier for formation of the tetrahedral intermediate and protonation of the nitrogen atom (Pearl, 1985). Until recently, there was little direct evidence for a conformational change within the protein as a result of inhibitor binding except for the well-documented closure of the flap over the substrate. However, it has recently been reported (Sali et al., 1989, 1992) that binding of CP-69,799 to endothiapepsin is accompanied by a displacement and rotation of the N-terminal domain relative to the C-terminal domain. The movement was significant but not large, corresponding to a rotation of about 4° and a translation of 0.3 Å. Flexibility of this region of other aspartic proteinases also became evident when the refined pepsin structures at 2.3-Å resolution (Abad-Zapatero et al., 1990) and at 1.8-Å resolution (Sielecki et al., 1990) were compared to the fungal proteases and a similar displacement of a flexible C-terminal subdomain was observed.

In contrast, rhizopuspepsin undergoes no observable rigid body movement of the flexible domain region, relative to the native enzyme, upon binding of the inhibitor CP-69,799

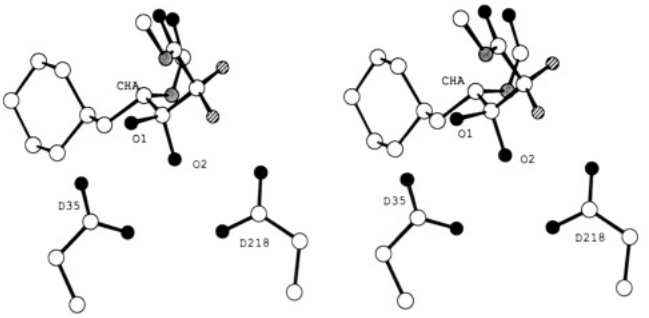


FIGURE 7: Active site as observed in the crystal structure of rhizopuspepsin complexed with CP-82,218.

whether by soaking or cocrystallization. When the CP-69,799 inhibitor was soaked into the crystal, no cracking of the crystal was observed. Also the inhibitor is present in unit occupancy. Both of these observations imply that a lack of conformational change does not result in excessive strain in the molecule, and since the crystal packing forces are generally believed to be weak, the energy associated with any conformational change must be small. The observation that the cocrystallized and soaked complexes are essentially identical supports this conclusion.

Endothiapepsin (Sali et al., 1989) was crystallized in the presence of this inhibitor and resulted in a crystal form different from that of the native enzyme (Blundell et al., 1990). Possible factors contributing to the conformational changes observed are inhibitor binding, different crystal packing forces in the new crystal form, or the differences in crystallization conditions for obtaining the native and complexed crystals. Subsequently, an endothiapepsin complex with inhibitor H-261 has been reported (Veerapandian et al., 1990). This complex is also nonisomorphous with the native enzyme, in that a domain rotation (4°) is again present. Furthermore, the unit cell parameters in this structure are virtually identical to those of the CP-69,799 complex. In the same paper, it is reported that the crystal structure of H-261 and endothiapepsin has been determined in a form isomorphous with native endothiapepsin, where only a 0.6° domain rotation is observed. These observations suggest that here too subdomain rotations can be influenced by the crystal packing forces.

A sequential proteolytic mechanism for aspartic proteinases has been proposed (Suguna et al., 1987b) in which the central water molecule, made nucleophilic by the partial transfer of a proton to Asp218 carboxylate, attacks the carbonyl carbon. It was also proposed that hydrogen bonding with the Asp35 outer oxygen (OD1) would facilitate this process. After formation of this tetrahedral intermediate, in a second step the proton on Asp218 is transferred to the amide nitrogen, resulting in cleavage of the peptide bond. This proposal was made on the basis of crystallographic data on an inhibitor bearing the presumably protonated reduced-bond dipeptide isostere ($-CH_2-NH-$), whose conformation and position relative to the enzyme were used to place a hypothetical substrate's scissile bond relative to the catalytic apparatus. As noted therein, this was a very approximate model, as the reduced isostere lacked both hydroxyl groups of the tetrahedral intermediates, whose interactions with and geometry relative to the catalytic aspartates, if known, could further refine the discussion of the catalytic events.

These interactions are now clearly observed in the rhizopuspepsin complex with the hydrated difluorostatone inhibitor CP-82,218 (Figure 7). The hydrate hydroxyls are positioned such that one (O1) is within hydrogen-bonding distance of only one of the four aspartyl oxygens (2.6 Å from the outer

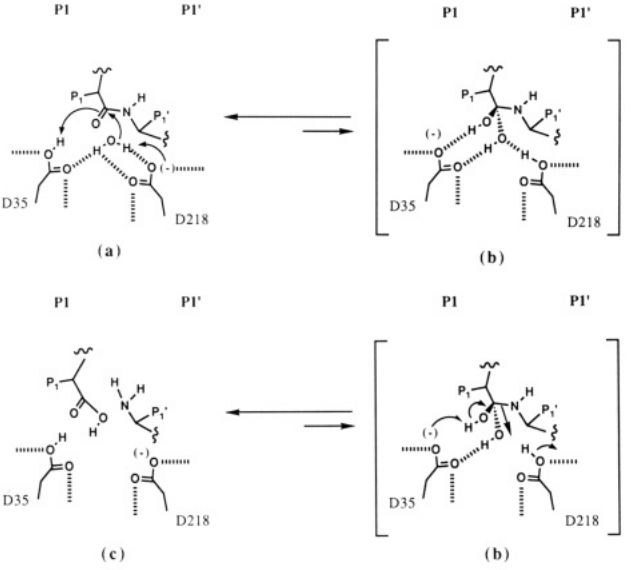
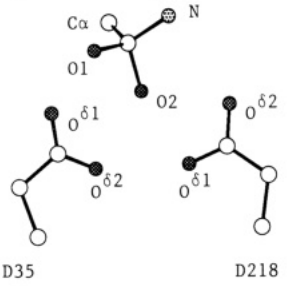


FIGURE 8: Proposed catalytic mechanism for rhizopuspepsin [see also Suguna et al. (1987b) and Parris et al. (1992)].

oxygen of Asp35) and the second (O2), as in pepstatin, occupies the place of Wat507 in the native enzyme. This places the second oxygen in similar proximity to the inner oxygens of Asp35 (2.8 Å) and Asp218 (2.6 Å) (see Table V). These distances support hydrogen bonding in this complex as represented for the stabilized tetrahedral intermediate in Figure 8b. The hydrate hydroxyls are directed toward the two Asp35 carboxylate oxygens, and Asp218 directs a proton from its outer oxygen in a hydrogen bond to the centrally located oxygen (O2) of the hydrate. This arrangement is supported by the observed inter-oxygen distances (Table V) which are consistent with strong hydrogen bonds and by favorable geometries for these proposed hydrogen bonds. The alternative hydrogen-bonding arrangement in which the O2 hydroxyl bonds to OD1 (inner oxygen) of Asp218 (2.9 Å, C–O2–OD1 angle of 161°) rather than with the inner oxygen of Asp35 (2.8 Å, C–O2–OD1 angle of 110°) is less favored. The hydrogen-bonding arrangement of Figure 8b has also been proposed for complexes of similar hydrated difluorostatones with endothiapepsin (Veerapandian et al., 1992; Hoover et al., 1991) and for penicillopepsin (James et al., 1992). The rhizopuspepsin and endothiapepsin difluorostatone hydrate complexes have been compared and are virtually identical with respect to the placement of equivalent atoms in the active site and around the catalytic apparatus; the six oxygen atoms in the *gem*-diol and catalytic aspartate side chains superimpose with a rms deviation of 0.1 Å.

These data consequently support the mechanistic sequence outlined in Figure 8 that contains the essential tenets of the original proposal (Suguna et al., 1987b), as modified in Parris et al. (1991), to take account of the proton transfer from Asp35 OD1. Thus, Asp35 polarizes the carbonyl bond of the substrate in the same way as the oxyanion hole stabilizes the negative charge in the tetrahedral intermediate in the action of serine proteases (Robertus et al., 1972). Simultaneously with carbonyl protonation, nucleophilic attack by Wat507 occurs with transfer of the proton to Asp218. Proteolysis then occurs only after protonation of the nitrogen atom in the tetrahedral intermediate. The distances between the carbon of the CF_2 group, which would be in the approximate position of this nitrogen atom, and the outer aspartyl oxygens are 3.5 Å (Asp218) and 4.8 Å (Asp35). Thus the former, in any mechanism where the positions of atoms in this "model" are

Table V: Distances from Atoms of the Scissile Bond Surrogate to the Oxygens of the Catalytic Aspartic Acids[a]



	native	CP-69,799 (soak)	CP-69,799 (cocrystal)	CP-82,218	reduced	pepstatin	inhibitor 1	inhibitor 2
Oδ1(D35)–N		4.94	4.98	4.88	4.52	4.57	4.73	4.60
Oδ2(D35)–N		4.88	4.99	4.78	4.44	4.84	4.79	4.73
Oδ1(D218)–N		4.75	4.81	4.76	3.78	4.78	4.65	4.60
Oδ2(D218)–N		3.48	3.51	3.52	2.82	3.42	3.15	3.70
Oδ1(D35)–O2	3.37	3.28	3.24	3.68		3.39	3.21	3.01
Oδ2(D35)–O2	2.81	2.53	2.61	2.79		2.79	2.57	2.55
Oδ1(D218)–O2	2.83	2.87	2.92	2.86		2.91	2.90	3.01
Oδ2(D218)–O2	2.92	2.70	2.74	2.58		2.49	2.58	3.21
Oδ1(D35)–C		3.60	3.60	3.55	3.27	3.42	3.46	3.30
Oδ2(D35)–C		3.64	3.72	3.53	3.39	3.72	3.68	3.68
Oδ1(D218)–C		4.26	4.30	4.23	3.57	4.29	4.28	4.37
Oδ2(D218)–C		3.62	3.65	3.66	3.45	3.50	3.45	4.09
Oδ1(D35)–O1				2.57				
Oδ2(D35)–O1				3.24				
Oδ1(D218)–O1				4.75				
Oδ2(D218)–O1				4.61				

[a] The figure at the top of the table shows the atom numbering. For the reduced peptide inhibitor, see Suguna et al. (1987b), and for pepstatin, inhibitor 1, and inhibitor 2, see Suguna et al. (1992).

seriously considered, must be the only enzymatic source of this proton. In the reduced inhibitor (Suguna et al., 1987b), where the CF_2 group is replaced by an NH_2^+, the N to OD2 of Asp218 is 2.8 Å, which corresponds precisely to hydrogen bond formation. The distances observed in the reduced inhibitor may be artificially short, however, since there is a methylene group replacing the *gem*-diol and the nitrogen may be shifted toward the aspartyls to optimize charged interactions. Nevertheless, it is still apparent that proton transfer to the nitrogen, if mediated by the aspartyls rather than by bulk solvent, must occur from OD2 to Asp218. An examination of the stereochemistry of the nucleophilic attack leads to the conclusion, by following the stereoelectronic principles of Deslongchamps (1975) as used by Bizzozero and Dutler (1981) for the serine proteases and by James and Sielecki (1985) for an early mechanism for the aspartic proteinases, that inversion of this nitrogen must occur upon protonation of the nitrogen.

In summary, the mechanism as proposed here for aspartic proteinases is in almost all respects consistent with that proposed earlier (Suguna et al., 1987b; Parris et al., 1991) and equivalent to those recently proposed (Hoover et al., 1991; Veerapandian et al., 1992; James et al., 1992), relying on sequential proton and charge transfers mediated by equally important aspartates of the catalytic diad as proposed by Polgar (1987). The proposed mechanism of action shares considerable resemblance to that of other proteases (Takahashi et al., 1988, 1989; Matthews, 1988).

ACKNOWLEDGMENT

We are grateful to Jon Bordner and Debbie DeCosta (Department of Medicinal Chemistry, Pfizer) for the molecular structure of **16**. We thank Jeffrey P. Kiplinger and Philip Reiche (Department of Medicinal Chemistry, Pfizer) and Daniel T. Pentek (Yale University Chemical Instrumentation Center) for mass spectra and John W. Greene (Department of Analytical Chemistry, Pfizer) for the microanalytical determinations.

SUPPLEMENTARY MATERIAL AVAILABLE

Two figures giving X-ray crystallographic data, experimental detail of single crystal X-ray analysis, and five tables showing positional and thermal X-ray parameters for ethyl 4(*S*)-[(*tert*-butyloxycarbonyl)amino]-5-cyclohexyl-2,2-difluoro-3(*R*)-hydroxypentanoate (**16**) (9 pages). Ordering information is given on any current masthead page.

REFERENCES

Abad-Zapatero, C., Rydel, T. J., & Erickson, J. (1990) *Proteins: Struct., Funct., Genet. 8*, 62–81.

Abad-Zapatero, C., Rydel, T. J., Neidhart, D., Luly, J., & Erickson, J. (1991) in *Structure and Function of the Aspartic Proteases: Genetics, Structure and Mechanism* (Dunn, B. M., Ed.) Advances in Experimental Medicine and Biology, Vol. 306, pp 9–21, Plenum Press, New York.

Agarwal, R. C. (1978) *Acta Crystallogr., Sect. A 34*, 791–809.

Bizzozero, S. A., & Dutler, H. (1981) *Bioorg. Chem. 10*, 46–62.

Blundell, T. L., Jenkins, J. A., Sewell, B. T., Pearl, L. H., Cooper, J., Tickle, I. J., Veerapandian, B., & Wood, S. P. (1990) *J. Mol. Biol. 211*, 919–941.

Boger, J., Payne, L. S., Perlow, D. S., Lohr, N., Poe, M., Blaine, E. H., Ulm, E. H., Schorn, T. W., LaMont, B. I., Lin, T., Kawai, M., Rich, D. H., & Veber, D. F. (1985) *J. Med. Chem. 28*, 1779–1790.

Case, D. A., & Karplus, M. (1979) *J. Mol. Biol. 132*, 343–368.

Connolly, M. L. (1983) *J. Appl. Crystallogr. 16*, 548–558.

Cooper, J., Foundling, S. I., Hemmings, A., Blundell, T. L., Jones, D. M., Hallett, A., & Szelke, M. (1987) *Eur. J. Biochem. 169*, 215–221.

Cunningham, A., Hofmann, M. I., & Hofmann, T. (1990) *FEBS Lett. 276*, 119–122.

Davies, D. R. (1990) *Annu. Rev. Biophys. Biophys. Chem. 19*, 189–215.

Deslongchamps, P. (1975) *Tetrahedron 31*, 2463–2490.

Dewar, M. S., & Dieter, K. M. (1988) *Biochemistry 27*, 3302–3308.

Fearon, K., Spaltenstein, A., Hopkins, P. B., & Gelb, M. H. (1987) *J. Med. Chem. 30*, 1617–1622.

Finzel, B. C. (1987) *J. Appl. Crystallogr. 20*, 53–55.

Foundling, S. I., Cooper, J., Watson, F. E., Cleasby, A., Pearl, L. H., Sibanda, B. L., Hemmings, A., Wood, S. P., Blundell, T. L., Valler, M. J., Norey, C. G., Kay, J., Boger, J., Dunn, B. M., Leckie, B. J., Jones, D. M., Atrash, B., Hallett, A., & Szelke, M. (1987) *Nature 327*, 349–352.

Fruton, J. S. (1976) *Adv. Enzymol. 44*, 1–36.

Gelb, M. H., Svaren, J. P., & Abeles, R. H. (1985) *Biochemistry 24*, 1813–1817.

Hallinan, E. A., & Fried, J. (1984) *Tetrahedron Lett. 25*, 2301.

Hendrickson, W. A. (1985) in *Methods in Enzymology* (Wyckoff, H. W., Hirs, C. W. H., & Timasheff, S. N., Eds.) Vol. 115, pp 252–270, Academic Press, New York.

Hendrickson, W. A., & Konnert, J. H. (1981) in *Biomolecular Structure, Conformation, Function, and Evolution* (Srinivasan, R., Ed.) pp 43–57, Pergamon, Oxford.

Hofmann, T., Allen, B., Bendiner, M., Blum, M., & Cunningham, A. (1988) *Biochemistry 27*, 1140–1146.

Holladay, M. W., Salituro, F. G., Schmidt, P. G., & Rich, D. H. (1985) *Biochem. Soc. Trans. 13*, 1046–1048.

Hoover, D. J., Veerapandian, B., Cooper, J. B., Damon, D. B., Dominy, B. W., Rosati, R. L., & Blundell, T. L. (1991) in *Structure and Function of the Aspartic Proteases: Genetics, Structure and Mechanism* (Dunn, B. M., Ed.) Advances in Experimental Medicine and Biology, Vol. 306, pp 269–273, Plenum Press, New York.

Howard, A. J., Nielsen, C., & Xuong, Ng. H. (1985) in *Methods in Enzymology* (Wyckoff, H. W., Hirs, C. W. H., & Timasheff, S. N., Eds.) Vol. 115, pp 452–472, Academic Press, New York.

Ishihara, T., Yamanka, T., & Ando, T. (1984) *Chem. Lett.*, 1165–1168.

James, M. N. G., & Sielecki, A. R. (1983) *J. Mol. Biol. 163*, 299–361.

James, M. N. G., & Sielecki, A. R. (1985) *Biochemistry 24*, 3701–3713.

James, M. N. G., & Sielecki, A. R. (1987) in *Biological Macromolecules and Assemblies* (Jurnak, F., & McPherson, A., Eds.) Vol. 3, pp 413–482, Wiley, New York.

James, M. N. G., Sielecki, A., Salituro, F., Rich, D. H., & Hofmann, T. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6137–6141.

James, M. N. G., Sielecki, A. R., Hayakawa, K., & Gelb, M. H. (1992) *Biochemistry 31*, 3872–3886.

Jaskolski, M., Tomasselli, A. G., Sawyer, T. K., Staples, D. G., Heinrikson, R. L., Schneider, J., Kent, B. H., & Wlodawar, A. (1991) *Biochemistry 30*, 1600–1609.

Jones, T. A. (1978) *J. Appl. Crystallogr. 11*, 268–272.

Kostka, V. (1985) *Aspartic Proteinases and their Inhibitors*, Walter de Gruyter, Berlin and New York.

Kunimoto, S., Aoyagi, T., Nishizawa, R., Komai, T., Takeuchi, T., & Umezawa, H. (1974) *J. Antibiot. 27*, 413–419.

Lapatto, R., Blundell, T., Hemmings, A., Overington, J., Wilderspin, A., Wood, S., Merson, J. R., Whittle, P. J., Danley, D. E., Geoghegan, K. F., Hawrylik, S. J., Lee, S. E., Scheld, K. G., & Hobart, P. M. (1989) *Nature 342*, 299–302.

Lombardino, J. G., & Gerber, C. F. (1964) *J. Med. Chem. 7*, 97–101.

Matthews, B. W. (1988) *Acc. Chem. Res. 21*, 333–340.

Miller, M., Jaskolski, M., Mohana, R., Leis, J., & Wlodawar, A. (1989) *Nature 337*, 576–579.

Navia, M. A., Fitzgerald, P. M. D., McKeever, B. M., Leu, C.-T., Heimbach, J. C., Herber, W. K., Sigal, I. S., Darke, P. L., & Springer, J. P. (1989) *Nature 337*, 615–620.

Parris, K. D., Hoover, D. J., & Davies, D. R. (1991) in *Structure and Function of the Aspartic Proteases: Genetics, Structure and Mechanism* (Dunn, B. M., Ed.) Advances in Experimental Medicine and Biology, Vol. 306, pp 217–231, Plenum Press, New York.

Pearl, L. (1985) in *Aspartic Proteinases and their Inhibitors* (Kostka, V., Ed.) pp 189–195, Walter de Gruyter, Berlin and New York.

Pearl, L. H., & Blundell, T. L. (1985) *FEBS Lett. 174*, 96–101.

Pflugrath, J. W., Saper, M. A., & Quiocho, F. A. (1984) in *Methods and Applications in Crystallographic Computing* (Hall, S., & Ashida, T., Eds.) pp 404–407, Clarendon, Oxford.

Pirkle, W. H., Finn, J. M., Schreiner, J. L., & Hamper, B. C. (1981) *J. Am. Chem. Soc. 103*, 3964–3966.

Polgar, L. (1987) *FEBS Lett. 219*, 1–4.

Rich, D. H., Sun, E. T., & Boparai, A. S. (1978) *J. Org. Chem. 43*, 3624–3626.

Rich, D. H., Bernatowicz, M. S., & Schmidt, P. G. (1982) *J. Am. Chem. Soc. 104*, 3535–3536.

Robertus, J. D., Kraut, J., Alden, R. A., & Birktoft, J. J. (1982) *Biochemistry 11*, 4293–4303.

Sali, A., Veerapandian, B., Cooper, J. B., Foundling, S. I., Hoover, D. J., & Blundell, T. L. (1989) *EMBO J. 8*, 2179–88.

Sali, A., Veerapandian, B., Cooper, J. B., Moss, D. S., Hofmann, T., & Blundell, T. L. (1992) *Proteins: Struct., Funct., Genet. 12*, 158–170.

Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157–162.

Schmidt, P. G., Bernatowicz, M. S., & Rich, D. H. (1982) *Biochemistry 21*, 6710–6716.

Sheriff, S. (1987) *J. Appl. Crystallogr. 20*, 55–57.

Sheriff, S., Hendrickson, W. A., Stenkamp, R. E., Sieker, L. C., & Jensen, L. H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1104–1107.

Sielecki, A. R., Fedorov, A. A., Boodhoo, A., Andreeva, N. S., & James, M. N. G. (1990) *J. Mol. Biol. 214*, 143–170.

Suguna, K., Bott, R. R., Padlan, E. A., Subramanian, E., Sheriff, S., Cohen, G. H., & Davies, D. R. (1987a) *J. Mol. Biol. 196*, 877–900.

Suguna, K., Padlan, E. A., Smith, C. W., Carlson, W. D., & Davies, D. R. (1987b) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7009–7013.

Suguna, K., Padlan, E. A., Bott, R. R., Boger, J., Parris, K. D., & Davies, D. R. (1992) *Proteins: Struct., Funct., Genet. 13*, 195–205.

Takahashi, L. H., Radhakrishnan, R., Rosenfield, R. E., Meyer, E. F., Jr., Trainor, D. A., & Stein, M. (1988) *J. Mol. Biol. 201*, 423–428.

Takahashi, L. H., Radhakrishnan, R., Rosenfield, R. E., Meyer, E. F., Jr., & Trainor, D. A. (1989) *J. Am. Chem. Soc. 111*, 3368–3374.

Thaisrivongs, S., Pals, D. T., Kati, W. M., Turner, S. R., & Thomasco, L. M. (1985) *J. Med. Chem. 28*, 1553–1555.

Thaisrivongs, S., Pals, D. T., Kati, W. M., Turner, S. R., Thomasco, L. M., & Watt, W. (1986) *J. Med. Chem. 29*, 2080–2087.

Veerapandian, B., Cooper, J. B., Sali, A., & Blundell, T. L. (1990) *J. Mol. Biol. 216*, 1017–1029.

Veerapandian, B., Cooper, J. B., Sali, A., Blundell, T. L., Rosati, R. L., Dominy, B. W., Damon, D. B., & Hoover, D. J. (1992) *Protein Sci. 1*, 322–328.

Warshel, A., Aqvist, J., & Creighton, S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5820–5824.

Wlodawer, A., Miller, M., Jaskolski, M., Sathyanarayana, B. K., Baldwin, E., Weber, I. T., Selk, L. M., Clawson, L., Schneider, J., & Kent, S. B. (1989) *Science 245*, 616–621.