

variants compared to other FDA approved inhibitors. A protein–ligand X-ray structure of 26-bound HIV-1 protease was determined at 1.45 Å resolution. The inhibitor appeared to make extensive interactions throughout the active site. Of particular interest, the 3-(*R*)-hydroxyl of the Cp-THF ligand formed a new water-mediated hydrogen bond interaction with the backbone amide NH of Gly48, and the Cp-THF ether oxygen formed a strong hydrogen bond with backbone amide NH of Asp29. The extensive interactions with the protein backbone may be responsible for inhibitor 26's impressive antiviral activity and drug resistance profile. The design of inhibitors targeting the protein backbone has led us to develop inhibitors characterized by high potency against both wild-type and multidrug-resistant HIV-1 strains. Further design and optimization of inhibitors utilizing this molecular insight are in progress.

EXPERIMENTAL SECTION

General. All anhydrous solvents were obtained according to the following procedures. Diethyl ether and tetrahydrofuran (THF) were distilled from sodium/benzophenone under argon. Toluene, methanol, acetonitrile, and dichloromethane were distilled from calcium hydride, and benzene was distilled from sodium. Other solvents were used without purification. All moisture-sensitive reactions were carried out in flame-dried flasks under argon atmosphere. Reactions were monitored by thin layer chromatography (TLC) using Silicycle 60A-F254 silica gel precoated plates. Flash column chromatography was performed using Silicycle 230–400 mesh silica gel. Yields refer to chromatographically and spectroscopically pure compounds. Optical rotations were recorded on a Perkin-Elmer 341 polarimeter. ^1H NMR and ^{13}C NMR spectra were recorded on a Varian Inova-300 (300 and 75 MHz, respectively), Bruker Avance ARX-400 (400 and 100 MHz), and Bruker Avance ARX-500 (500 and 125 MHz). High and low resolution mass spectra were carried out by the Mass Spectroscopy Center at Purdue University, IN. The purity of all test compounds was determined by HRMS and HPLC analyses in the different solvent systems. All test compounds showed $\geq 95\%$ purity.

(3a*S*,5*R*,6a*R*)-5-Hydroxytetrahydro-2*H*-cyclopenta[*b*]furan-3(3a*H*)-one (7). A solution of ketone 6 (23 mg, 0.09 mmol) in CH_3CN (0.5 mL) was cooled to 0 °C under argon. Pyridine (50 μL) was added followed by dropwise addition of HF–pyridine (0.18 mL). The solution was stirred at 0 °C for 4 h. The reaction was quenched by addition of saturated aqueous NaHCO_3 solution followed by solid NaHCO_3 . The aqueous phase was extracted multiple times with EtOAc, and the combined organic layer was dried over Na_2SO_4 . Following careful evaporation of the solvent, the residue was purified by column chromatography on silica gel using hexanes/EtOAc (1:1 and then 1:2) as the eluent to yield the desired ketone 7 (12 mg, 94%) as a colorless oil. TLC, R_f = 0.32 (hexanes/EtOAc = 1:2); $[\alpha]_D^{20}$ +96.1 (c 0.86, CHCl_3); ^1H NMR (CDCl_3 , 400 MHz) δ 5.04 (t, J = 6.6 Hz, 1H), 4.38 (t, J = 4.0 Hz, 1H), 4.20 (d, J = 17.0 Hz, 1H), 3.97 (d, J = 17.0 Hz, 1H), 2.85 (t, J = 8.4 Hz, 1H), 2.21 (d, J = 13.9 Hz, 1H), 2.15 (d, J = 15.2 Hz, 1H), 2.01 (ddd, J = 3.9, 10.5, 14.1 Hz, 1H), 1.95 (ddd, J = 4.5, 6.0, 15.1 Hz, 1H), 1.90 (br s, 1H); ^{13}C NMR (CDCl_3 , 100 MHz) δ 217.7, 84.0, 72.9, 70.8, 48.8, 43.4, 40.7.

(3*S*,3a*S*,5*R*,6a*R*)-3-Methoxyhexahydro-2*H*-cyclopenta[*b*]furan-5-ol (8). Ketone 6 (35.6 mg, 0.14 mmol) was dissolved in EtOH (1 mL) under argon and cooled to –25 °C. To the solution was added NaBH_4 (10 mg, 0.26 mmol) in one portion, and the resulting mixture was stirred at this temperature for 20 min. Saturated aqueous NH_4Cl solution was added and the volume of solvent reduced under vacuum. Additional water was added, and the aqueous phase was extracted several times with EtOAc. The combined organic layer was washed with brine, dried (Na_2SO_4), and evaporated in vacuo. Purification of the crude alcohol by column chromatography on silica gel using

hexanes/EtOAc (5:1) as the eluent provided the corresponding alcohol (32.2 mg, 90%) as a colorless oil. TLC, R_f = 0.22 (hexanes/EtOAc = 5:1); $[\alpha]_D^{20}$ +21.5 (c 1.05, CHCl_3); ^1H NMR (CDCl_3 , 300 MHz) δ 4.55 (br s, 1H), 4.42 (t, J = 7.0 Hz, 1H), 4.34 (t, J = 4.5 Hz, 1H), 4.11 (m, 1H), 3.87 (d, J = 9.9 Hz, 1H), 3.48 (dd, J = 3.6, 9.9 Hz, 1H), 2.89 (dt, J = 7.8, 10.2 Hz, 1H), 2.13 (dd, J = 2.4, 14.7 Hz, 1H), 1.99 (dd, J = 2.4, 14.7 Hz, 1H), 1.78–1.65 (m, 2H), 0.89 (s, 9H), 0.11 (s, 3H), 0.10 (s, 3H); ^{13}C NMR (CDCl_3 , 75 MHz) δ 85.3, 77.3, 74.9, 72.9, 48.1, 41.8, 34.8, 25.7, 18.0, –4.7, –5.2. HRMS-Cl (m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{13}\text{H}_{27}\text{O}_3\text{Si}$ 259.1729, found 259.1731. To a solution of the above alcohol (27 mg, 0.1 mmol) in CH_3CN (1 mL) was added MeI (0.3 mL, excess) followed by Ag_2O (50 mg, 0.2 mmol). The mixture was gently refluxed for 12 h under argon, and then additional MeI (0.3 mL) and Ag_2O (50 mg) were added. Reflux was continued for an additional 12 h until all SM disappeared. The resulting mixture was diluted in Et_2O and filtered on Celite. After evaporation of the solvent, the residue was purified by column chromatography on silica gel using hexanes/EtOAc (8:1) as the eluent to yield the corresponding 3-(*S*)-methoxy intermediate as a clear oil (23 mg, 85%). TLC, R_f = 0.38 (hexanes/EtOAc = 5:1); $[\alpha]_D^{20}$ +42.6 (c 1.02, CHCl_3); ^1H NMR (CDCl_3 , 300 MHz) δ 4.41 (dt, J = 5.1, 7.2 Hz, 1H), 4.12 (m, 1H), 3.93 (m, 1H), 3.87–3.70 (m, 2H), 3.32 (s, 3H), 2.61 (m, 1H), 2.18 (m, 1H), 1.90–1.75 (m, 2H), 1.63 (m, 1H), 0.88 (s, 9H), 0.05 (s, 6H); ^{13}C NMR (CDCl_3 , 75 MHz) δ 82.5, 81.1, 73.5, 69.0, 57.8, 42.6, 33.5, 25.8, 18.1, –4.8. To a solution of the above intermediate (16 mg, 0.059 mmol) in THF (1 mL) was added TBAF (1 M solution in THF, 0.1 mmol, 100 μL). The solution was stirred at room temperature for 2 h. The solvent was evaporated and the residue purified by flash column chromatography on silica gel using hexanes/EtOAc (1:1 and then 1:2.5) to furnish 8 as a colorless oil (8 mg, 86%). TLC, R_f = 0.32 (CHCl_3 /3% EtOH); ^1H NMR (CDCl_3 , 500 MHz) δ 4.41 (t, J = 4.4 Hz, 1H), 4.21 (dt, J = 5.1, 10.1 Hz, 1H), 4.08 (d, J = 10.3 Hz, 1H), 3.85 (dd, J = 3.0, 7.3 Hz, 1H), 3.83 (d, J = 10.3 Hz, 1H), 3.42 (s, 3H), 2.94–2.86 (m, 1H), 2.13 (d, J = 14.5 Hz, 1H), 2.08 (dd, J = 2.4, 15.1 Hz, 1H), 1.81 (ddd, J = 5.3, 10.6, 14.5 Hz, 1H), 1.74 (ddd, J = 5.3, 6.0, 15.0 Hz, 1H); ^{13}C NMR (CDCl_3 , 125 MHz) δ 86.0, 82.1, 73.6, 71.6, 57.4, 47.2, 42.0, 34.0.

Methyl 2-((1*R*,4*S*)-4-((*tert*-Butyldimethylsilyloxy)cyclopent-2-en-1-yloxy)acetate (9). To an ice-cold suspension of NaH (60% suspension in oil, 360 mg, 9 mmol) in dry THF (5 mL) under argon was slowly added a solution of 2-bromoacetic acid (520 mg, 3.75 mmol) in dry THF (3 mL + 3 mL rinse). The resulting mixture was stirred at room temperature for 30 min and then cooled back to 0 °C. Desymmetrized *meso*-cyclopentenediol 5 (642 mg, 3 mmol) in dry DMF (10 mL) was slowly added to this solution, and the mixture was allowed to warm to room temperature and was stirred for 24 h. A pH 4 phosphate buffer solution was added at 0 °C, and the aqueous phase was extracted with EtOAc ($\times 4$). The combined organic phase was dried (Na_2SO_4), filtered, and evaporated in vacuo to give the corresponding crude carboxylic acid along with residual DMF. The acid was diluted with additional DMF (10 mL). The solution was cooled to 0 °C, and NaHCO_3 (630 g, 7.5 mmol) was added at once followed by MeI (14 mmol, 1.98 g, 871 μL). After the mixture was stirred at room temperature for 12 h, saturated aqueous NH_4Cl solution was added (10 mL), then water (10 mL), and the aqueous phase was successively extracted with hexanes/ Et_2O , 1:1 ($\times 3$). The combined organic phase was dried (Na_2SO_4), filtered, and evaporated. The residue was purified by flash chromatography on silica gel using hexanes/EtOAc (20:1 and then 15:1) to give methyl ester 9 as a colorless oil (654 mg, 76%, two steps). TLC, R_f = 0.45 (hexanes/EtOAc = 3:1); ^1H NMR (CDCl_3 , 300 MHz) δ 5.92 (s, 2H), 4.63 (dd, J = 4.8, 7.0 Hz, 1H), 4.53 (dd, J = 5.1, 7.2 Hz, 1H), 4.10 (s, 2H), 3.74 (s, 3H), 2.64 (dt, J = 7.2, 13.8 Hz, 1H), 1.60 (dt, J = 4.9, 13.7 Hz, 1H), 0.87 (s, 9H), 0.065 (s, 3H), 0.06 (s, 3H); ^{13}C NMR (CDCl_3 , 75 MHz) δ 171.2, 138.4, 131.9, 82.4, 74.6, 64.7, 51.8, 40.5, 25.8, 18.0, –4.7.

(3*R*,3a*R*,5*R*,6a*R*)-5-((*tert*-Butyldimethylsilyloxy)hexahydro-2*H*-cyclopenta[*b*]furan-3-ol (10). A solution of methyl ester 9 (87.2 mg,

0.304 mmol) in dry CH_2Cl_2 (8 mL) was cooled to -78°C under argon. DIBAL-H (1 M solution in hexanes, 0.40 mL) was added slowly, and the reaction mixture was stirred for 1 h at this temperature. The reaction was quenched by addition of MeOH (100 μL), and the mixture was warmed to room temperature. A pH 7 phosphate buffer (0.5 M solution, 2 mL) was added. The aqueous phase was extracted with CH_2Cl_2 ($\times 3$) and the combined organic phase washed with brine, dried (Na_2SO_4), filtered, and evaporated in vacuo. The crude aldehyde solution was passed through a short plug of silica gel, deactivated with 1% Et_3N using hexanes/EtOAc (5:1) as the eluent. After evaporation, the crude product was directly submitted to the next step. The residue was dissolved in dry benzene (degassed, 2 mL) under argon and the solution transferred into a sealable tube. ($n\text{-Bu}_3\text{Sn}$) $_2\text{O}$ (22.5 μL , 26.3 mg, 44 μmol), Ph_2SiH_2 (42 μL , 41.6 mg, 0.22 mmol), EtOH (45 μL), and AIBN (10 mg) were successively added. The sealed tube was placed with stirring in an oil bath at 80°C for 6 h. After cooling to room temperature, the reaction mixture was diluted with Et_2O (2 mL) and aqueous 0.5 M HCl solution (2 mL) was added. After the mixture was stirred at room temperature for 15 min, the aqueous phase was extracted with Et_2O ($\times 3$). The combined organic phase was washed with saturated aqueous NaHCO_3 solution, brine, dried (MgSO_4), filtered, and evaporated. The residue was purified by flash chromatography on silica gel using hexanes/EtOAc (6:1 to 2:1) to afford the (*R*)-alcohol **10** (50 mg, 64%, over two steps) as a white solid; 5 mg of the other 3-(*S*)-diastereoisomer was also isolated, and only traces of 1,2 reduction product was observed. TLC, $R_f = 0.46$ (hexanes/EtOAc = 3:1); $[\alpha]_D^{20} +22.3$ (*c* 0.67, CHCl_3); $^1\text{H NMR}$ (CDCl_3 , 300 MHz) δ 4.69 (dt, $J = 3.8, 7.2$ Hz, 1H), 4.19–4.02 (m, 3H), 3.72 (d, $J = 9.8$ Hz, 1H), 2.48 (q, $J = 7.5$ Hz, 1H), 2.13–1.96 (m, 2H), 1.70–1.55 (m, 1H), 1.47–1.25 (m, 1H), 0.86 (s, 9H), 0.03 (s, 6H); $^{13}\text{C NMR}$ (CDCl_3 , 75 MHz) δ 82.2, 78.3, 73.1, 72.7, 50.9, 42.4, 39.0, 25.8, 17.5, $-4.8, -4.9$.

(3*R*,3*aS*,5*R*,6*aR*)-5-Hydroxyhexahydro-2*H*-cyclopenta[*b*]furan-3-yl Acetate (11). Alcohol **10** (45 mg, 0.175 mmol) was diluted in CH_2Cl_2 (5 mL), and the solution was cooled to 0°C under argon. Et_3N (0.525 mmol, 53 mg, 75 μL) and DMAP (1 crystal) were added followed by acetic anhydride (25 μL , 0.23 mmol). The mixture was warmed to room temperature and stirred overnight. The solution was evaporated to dryness, and purification of the residue by flash column chromatography on silica gel using hexanes/EtOAc (20:1) furnished the corresponding acetate derivative (38 mg, 73%). TLC, $R_f = 0.24$ (hexanes/EtOAc = 10:1); $^1\text{H NMR}$ (CDCl_3 , 300 MHz) δ 5.02 (d, $J = 3.8$ Hz, 1H), 4.69 (dt, $J = 3.2, 7.2$ Hz, 1H), 4.22 (dd, $J = 4.0, 10.4$ Hz, 1H), 4.11 (m, 1H), 3.80 (d, $J = 10.4$ Hz, 1H), 2.56 (m, 1H), 2.09–1.97 (m, 2H), 2.04 (s, 3H), 1.71 (m, 1H), 1.68–1.54 (m, 1H), 0.86 (s, 9H), 0.03 (s, 6H); $^{13}\text{C NMR}$ (CDCl_3 , 75 MHz) δ 170.8, 82.9, 81.2, 72.8, 70.9, 48.5, 42.7, 39.2, 25.8, 21.2, 18.0, -4.9 . The acetate (28 mg, 0.093 mmol) was diluted in THF (1.5 mL). TBAF (1 M solution of THF, 200 μL , 0.2 mmol) was added at 0°C , and the mixture was stirred for 2.5 h while warming to 23°C . The solvent was evaporated and the residue purified by flash column chromatography on silica gel using hexanes/EtOAc (3:1 to 1:1) to give pure alcohol **11** as a colorless oil (17.5 mg, quant). TLC, $R_f = 0.18$ (EtOAc, 100%); $^1\text{H NMR}$ (CDCl_3 , 300 MHz) δ 5.11 (m, 1H), 4.70–4.62 (m, 1H), 4.32 (dd, $J = 5.0, 10.4$ Hz, 1H), 4.28–4.20 (m, 1H), 3.71 (dd, $J = 3.2, 10.4$ Hz, 1H), 2.65–2.56 (m, 1H), 2.30–2.13 (m, 2H), 2.06 (s, 3H), 1.99–1.88 (m, 1H), 1.77–1.68 (m, 1H); $^{13}\text{C NMR}$ (CDCl_3 , 75 MHz) δ 170.8, 84.8, 81.1, 73.4, 71.9, 48.9, 41.9, 39.5, 21.1.

(3*R*,3*aS*,5*R*,6*aR*)-3-Methoxyhexahydro-2*H*-cyclopenta[*b*]furan-5-ol (12). A solution of alcohol **10** (30 mg, 0.116 mmol) in dry DMF (1 mL) was cooled to 0°C under argon, and NaH (60% suspension in oil, 15 mg, 0.348 mmol) was added in one portion. The mixture was warmed to room temperature, stirred for 30 min, and then cooled to 0°C . MeI (22 μL , 50 mg, 0.35 mmol) was added to the solution, and the mixture was warmed to 23°C and stirred for 3.5 h. Saturated aqueous NH_4Cl solution was added, and the aqueous phase was extracted with Et_2O /hexanes (1:1). The combined organic phase

was washed with brine, dried (Mg_2SO_4), filtered, and evaporated in vacuo. The residue was purified by flash chromatography on silica gel using hexanes/EtOAc (10:1 and then 8:1) to afford the corresponding TBS-protected methoxy compound (28.6 mg, 86%) as a clear oil. TLC, $R_f = 0.61$ (hexanes/EtOAc = 3:1); $[\alpha]_D^{20} +20.6$ (*c* 1.52, CHCl_3); $^1\text{H NMR}$ (CDCl_3 , 400 MHz) δ 4.63 (dt, $J = 3.6, 7.2$ Hz, 1H), 4.11 (m, 1H), 4.05 (dd, $J = 4.1, 9.9$ Hz, 1H), 3.81 (dd, $J = 1.3, 9.9$ Hz, 1H), 3.74 (m, 1H), 3.31 (s, 3H), 2.54 (td, $J = 7.7, 8.3$ Hz, 1H), 2.10–1.96 (m, 2H), 1.70–1.60 (m, 1H), 1.47 (m, 1H), 0.87 (s, 9H), 0.04 (6H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz) δ 87.7, 82.5, 72.9, 70.3, 56.6, 47.4, 42.5, 39.5, 25.8, 18.0, $-4.8, -4.9$. To an ice-cold solution of the above methoxy intermediate (27 mg, 0.1 mmol) in THF (2 mL) under argon was added TBAF (1 M solution in THF, 0.2 mL, 0.2 mmol). The mixture was stirred for 1.5 h. The solvent was evaporated, and the crude residue was purified by flash column chromatography on silica gel using hexanes/EtOAc (1:1) as the eluent to furnish alcohol **12** as a clear oil (13 mg, 82%). TLC, $R_f = 0.17$ (hexanes/EtOAc = 1:2); $^1\text{H NMR}$ (CDCl_3 , 300 MHz) δ 4.63 (t, $J = 5.5$ Hz, 1H), 4.26 (m, 1H), 4.20 (dd, $J = 5.3, 9.7$ Hz, 1H), 3.89 (dt, $J = 2.0, 4.8$ Hz, 1H), 3.65 (dd, $J = 4.4, 9.7$ Hz, 1H), 3.34 (s, 3H), 2.64–2.54 (m, 1H), 2.40 (br s, 1H), 2.16 (ddd, $J = 5.5, 10.6, 14.3$ Hz, 1H), 2.02 (dd, $J = 1.7, 14.7$ Hz, 1H), 1.87 (dt, $J = 5.1, 14.7$ Hz, 1H), 1.77–1.61 (m, 1H); $^{13}\text{C NMR}$ (CDCl_3 , 75 MHz) δ 88.4, 85.1, 74.0, 71.9, 57.0, 48.4, 41.8, 40.2.

(3*S*,3*aR*,5*R*,6*aR*)-3-Methylhexahydro-2*H*-cyclopenta[*b*]furan-5-ol (14). To a solution of olefin **13** (29.7 mg, 0.12 mmol) in toluene (3 mL) was added Wilkinson's catalyst, $\text{RhCl}(\text{PPh}_3)_3$ (18 μmol , 17 mg). The resulting solution was then placed under a hydrogen atmosphere and stirred for 3 h. After dilution with additional toluene, the solution was filtered on a Celite pad, and the pad was rinsed with toluene. Evaporation of the solvent and purification of the residue on silica gel using hexanes/EtOAc (100:1 and then 50:1) as the eluent furnished the corresponding 3-(*S*)-methyl compound (29 mg, 97%). TLC, $R_f = 0.28$ (hexanes/EtOAc = 20:1); $[\alpha]_D^{20} +22.7$ (*c* 1.01, CHCl_3); $^1\text{H NMR}$ (CDCl_3 , 400 MHz) δ 4.43 (m, 1H), 4.03 (m, 1H), 3.81 (dd, $J = 7.4, 8.0$ Hz, 1H), 3.42 (dd, $J = 8.0, 10.7$ Hz, 1H), 2.43 (m, 1H), 2.32–2.18 (m, 2H), 1.72 (m, 1H), 1.51 (ddd, $J = 4.8, 8.6, 13.5$ Hz, 1H), 1.44 (m, 1H), 0.95 (d, $J = 6.8$ Hz, 3H), 0.88 (s, 9H), 0.05 (s, 6H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz) δ 82.6, 73.2, 71.6, 44.5, 43.2, 36.2, 34.7, 25.9, 18.2, 11.3, $-4.7, -4.8$. HRMS-Cl (m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{14}\text{H}_{29}\text{O}_2\text{Si}$ 257.1937, found 257.1936. A solution of methyl-based ligand (28 mg, 0.1 mmol) in THF (2 mL) was treated with TBAF (1 M solution in THF, 150 μL) and stirred at room temperature for 2.5 h. The reaction mixture was diluted with Et_2O and filtered on a short silica pad. The solvent containing the alcohol was carefully reduced, and essentially pure alcohol **14** was directly used in the next step without purification ($>99\%$ pure). TLC, $R_f = 0.26$ (hexanes/EtOAc = 1:1); $^1\text{H NMR}$ (CDCl_3 , 400 MHz) δ 4.46 (dt, $J = 2.7, 5.5$ Hz, 1H), 4.20 (m, 1H), 3.88 (t, $J = 7.8$ Hz, 1H), 3.48 (t, $J = 9.2$ Hz, 1H), 2.59–2.40 (m, 2H), 2.22 (d, $J = 6.9$ Hz, 1H), 2.08 (dt, $J = 5.9, 14.1$ Hz, 1H), 1.87 (ddd, $J = 6.0, 9.5, 13.7$ Hz, 1H), 1.83 (m, 1H), 1.68–1.54 (m, 1H), 1.01 (d, $J = 6.8$ Hz, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz) δ 85.3, 73.8, 72.9, 46.3, 42.4, 36.5, 34.5, 12.7.

(3*R*,3*aR*,6*aR*)-3-Methyl-3,3*a*,6,6*a*-tetrahydro-2*H*-cyclopenta[*b*]furan-2-one (16). To a solution of lithium diisopropylamide, prepared by adding *n*-BuLi (2.5 M solution in hexanes, 1.36 mL, 3.39 mmol) to diisopropylamine (477 μL , 3.39 mmol) in THF (15 mL) at 0°C , was added a precooled solution of known (3*aS*,6*aR*)-3,3*a*,6,6*a*-tetrahydro-2*H*-cyclopenta[*b*]furan-2-one (+)-**15** (350 mg, 2.82 mmol) in THF (5 mL + 3 mL rinse) at -78°C , dropwise. The reaction mixture was stirred at this temperature for 30 min. Then methyl iodide (352 μL , 5.65 mmol) was added dropwise and the reaction mixture was stirred at -78°C for 6 h. The reaction mixture was quenched with 2 M aqueous HCl. The aqueous phase was extracted with Et_2O ($\times 3$). The combined organic layer was dried (Na_2SO_4), filtered, and concentrated under vacuum. The residue was purified by column chromatography on silica gel using hexanes/ Et_2O (5:1)

to yield lactone **16** (369 mg, 95%) as a brown oil. TLC, R_f = 0.54 (hexanes/EtOAc = 2:1); $[\alpha]_D^{20}$ +54.2 (c 1.0, CHCl_3); $^1\text{H NMR}$ (CDCl_3 , 400 MHz) δ 5.74 (m, 1H), 5.59 (m, 1H), 5.12 (t, J = 5.6 Hz, 1H), 3.13 (dd, J = 3.8, 1.8 Hz, 1H), 2.65 (m, 2H), 2.52 (q, J = 7.6 Hz, 1H), 1.33 (d, J = 7.6 Hz, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz) δ 180.0, 130.9, 129.4, 81.3, 53.8, 39.9, 39.2, 17.4.

(3R,3aR,5R,6aR)-5-Hydroxy-3-methylhexahydro-2H-cyclopenta[b]furan-2-one (17). To an ice-cold yellow solution of $\text{Hg}(\text{OAc})_2$ (1.45 g, 4.57 mmol) in THF/ H_2O (2.5:1 ratio, 23 mL) was added perchloric acid (~0.7 mL) until the solution became colorless. A solution of lactone **16** (350 mg, 2.54 mmol) in THF (7 mL) was then added at 0 °C, and the mixture was stirred for 1 h. Additional $\text{Hg}(\text{OAc})_2$ (646 mg, 2.03 mmol) similarly pretreated with perchloric acid in THF/ H_2O (2.5:1, 10 mL) was added, and stirring was continued for 2 h at 0 °C. The pH of the mixture was then adjusted to ~10 by addition of a 1 M aqueous NaOH solution. Stirring was then continued for 1 h at room temperature. The solution was cooled to 0 °C, and NaBH_4 (145 mg, 3.81 mmol) was added in small portions. After 1 h, the reaction mixture was acidified to pH 2 with concentrated HCl, and stirring was continued for 1 h. The reaction solution was saturated with NaCl and the aqueous phase extracted several times with EtOAc. The combined organic phase was dried (Na_2SO_4), filtered, and concentrated under vacuum. The residue was purified by column chromatography on silica gel using 2% MeOH in CH_2Cl_2 to yield alcohol **17** (252 mg, 64%) as a colorless oil. TLC, R_f = 0.31 ($\text{CHCl}_3/\text{MeOH}$ = 9/1); $[\alpha]_D^{20}$ +53.5 (c 1.0, CHCl_3); $^1\text{H NMR}$ (CDCl_3 , 400 MHz) δ 4.98 (dt, J = 8.0, 14.9 Hz, 1H), 4.55 (m, 1H), 2.69 (qd, J = 3.9, 7.6 Hz, 1H), 2.58 (m, 1H), 2.55 (m, 1H), 2.09 (d, J = 15.0 Hz, 1H), 1.94 (m, 2H), 1.83 (m, 1H), 1.26 (d, J = 7.6 Hz, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz) δ 181.0, 83.8, 74.6, 51.2, 45.5, 43.9, 41.3, 18.4.

(3R,3aR,5R,6aR)-3-Methylhexahydro-2H-cyclopenta[b]furan-5-ol (18). To a solution of lactone **17** (205 mg, 1.31 mmol) in CH_2Cl_2 (9 mL) was added DIBAL-H (1 M solution in CH_2Cl_2 , 1.45 mL, 1.45 mmol) dropwise at -78 °C. The mixture was stirred for 3 h and then quenched with saturated Rochelle's salt solution. After the mixture was stirred overnight at room temperature, the phases were separated and the aqueous phase was extracted with EtOAc ($\times 3$). The combined organic phase was dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using 2% MeOH in CH_2Cl_2 to yield the corresponding lactol (150 mg, 72%). To a solution of this lactol (143 mg, 0.9 mmol) in CH_2Cl_2 (9 mL) at -78 °C was added TiCl_4 (100 μL , 0.9 mmol) dropwise, and the mixture was stirred for 20 min. Et_3SiH (289 μL , 1.81 mmol) was then added, and the solution was stirred for an additional 1 h. The reaction was quenched with saturated NaHCO_3 solution once completion was reached as observed by TLC. The aqueous phase was extracted with Et_2O , dried (Na_2SO_4), filtered, and concentrated under reduced pressure at 25 °C. The residue was purified by column chromatography on silica gel using 2% MeOH in CH_2Cl_2 to yield alcohol **18** (122 mg, 95%) as a colorless oil. TLC, R_f = 0.38 ($\text{CHCl}_3/\text{MeOH}$ = 9/1); $[\alpha]_D^{20}$ +12.6 (c 1.0, CHCl_3); $^1\text{H NMR}$ (CDCl_3 , 400 MHz) δ 4.47 (t, J = 5.7 Hz, 1H), 4.22 (m, 1H), 4.06 (dd, J = 6.5, 8.7 Hz, 1H), 3.18 (t, J = 8.4 Hz, 1H), 2.60 (d, J = 7.2 Hz, 1H), 2.27–2.18 (m, 1H), 2.18–2.12 (m, 1H), 2.00 (dd, J = 1.5, 16.2 Hz, 1H), 1.98–1.91 (m, 1H), 1.80 (dt, J = 5.2, 14.6 Hz, 1H), 1.69 (ddd, J = 2.3, 5.4, 13.9 Hz, 1H), 1.03 (d, J = 6.7 Hz, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz) δ 85.4, 75.1, 74.7, 50.3, 43.2, 41.6, 41.5, 17.6.

Synthesis of Activated Mixed Carbonates. (3aS,5R,6aR)-3-Oxohexahydro-2H-cyclopenta[b]furan-5-yl (4-nitrophenyl)carbonate (19a). To a solution of ketone **7** (20 mg, 0.14 mmol) in CH_2Cl_2 (1 mL) was added pyridine (30 μL). The solution was cooled to 0 °C under argon, and 4-nitrophenyl chloroformate (42 mg, 0.21 mmol) was added at once. A white suspension formed. The solution was stirred at this temperature and slowly warmed to room temperature until all starting material was consumed. The reaction mixture was evaporated to

dryness and the residue purified by column chromatography on silica gel using hexanes/EtOAc (3:1 and then 2:1) as eluent to furnish pure mixed carbonate **19a** (39 mg, 92%) as a white solid. TLC, R_f = 0.25 (hexanes/EtOAc = 2:1); $^1\text{H NMR}$ (CDCl_3 , 400 MHz) δ 8.27 (d, J = 9.2 Hz, 2H), 7.34 (d, J = 9.2 Hz, 2H), 5.23 (t, J = 4.2 Hz, 1H), 5.13 (t, J = 6.9 Hz, 1H), 4.19 (d, J = 17.1 Hz, 1H), 4.06 (d, J = 17.1 Hz, 1H), 2.99 (dd, J = 8.7, 8.8 Hz, 1H), 2.54–2.41 (m, 2H), 2.22–2.08 (m, 2H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz) δ 216.8, 155.3, 151.5, 145.4, 125.3, 121.9, 82.4, 81.0, 70.6, 48.5, 41.2, 37.1.

(3S,3aS,5R,6aR)-3-Methoxyhexahydro-2H-cyclopenta[b]furan-5-yl (4-nitrophenyl)carbonate (19b). The title compound was obtained from **8** in 82% yield as described for **7** after purification by column chromatography on silica gel using hexanes/EtOAc (3:1 to 2:1) as the eluent (white solid). TLC, R_f = 0.25 (hexanes/EtOAc = 2:1); $^1\text{H NMR}$ (CDCl_3 , 300 MHz) δ 8.27 (d, J = 9.2 Hz, 2H), 7.38 (d, J = 9.2 Hz, 2H), 5.15 (tt, J = 4.5, 6.2 Hz, 1H), 4.56 (dt, J = 2.8, 6.1 Hz, 1H), 4.04 (dt, J = 6.3, 7.5 Hz, 1H), 3.87 (AB, dd, J = 5.2, 9.1 Hz, 1H), 3.86 (AB, dd, J = 6.6, 9.1 Hz, 1H), 3.34 (s, 3H), 2.83 (m, 1H), 2.38–2.02 (m, 4H); $^{13}\text{C NMR}$ (CDCl_3 , 75 MHz) δ 155.6, 152.1, 145.2, 125.2, 121.8, 83.7, 81.4, 77.2, 70.3, 57.9, 44.2, 39.6, 30.2.

(3R,3aS,5R,6aR)-5-((4-Nitrophenoxy)carbonyloxy)hexahydro-2H-cyclopenta[b]furan-3-yl Acetate (19c). The title compound was obtained from **11** in 96% yield as described for **7** after purification by column chromatography on silica gel using hexanes/EtOAc (6:1 and then 4:1) as the eluent (white solid). TLC, R_f = 0.26 (hexanes/EtOAc = 3:1); $^1\text{H NMR}$ (CDCl_3 , 300 MHz) δ 8.27 (d, J = 9.1 Hz, 2H), 7.37 (d, J = 9.1 Hz, 2H), 5.20–5.12 (m, 1H), 5.12–5.08 (m, 1H), 4.76 (dt, J = 2.0, 6.0 Hz, 1H), 4.27 (dd, J = 4.6, 10.4 Hz, 1H), 3.80 (dd, J = 2.5, 10.4 Hz, 1H), 2.79–2.70 (m, 1H), 2.37 (ddd, J = 6.0, 10.2, 15.0 Hz, 1H), 2.29–2.19 (m, 1H), 2.15 (dt, J = 5.6, 15.6 Hz, 1H), 2.07 (s, 3H), 2.02–1.92 (m, 1H); $^{13}\text{C NMR}$ (CDCl_3 , 75 MHz) δ 170.7, 155.4, 151.9, 145.3, 125.3, 121.8, 83.2, 81.0, 80.5, 71.7, 48.8, 39.6, 35.9, 21.1.

(3R,3aS,5R,6aR)-3-Methoxyhexahydro-2H-cyclopenta[b]furan-5-yl (4-Nitrophenyl)carbonate (19d). The title was obtained from **12** in 93% yield as described for **7** after purification by column chromatography on silica gel using hexanes/EtOAc (6:1 to 4:1) as the eluent (white solid). TLC, R_f = 0.40 (hexanes/EtOAc = 3:1); $^1\text{H NMR}$ (CDCl_3 , 400 MHz) δ 8.27 (d, J = 9.2 Hz, 2H), 7.37 (d, J = 9.2 Hz, 2H), 5.19–5.12 (m, 1H), 4.74–4.67 (m, 1H), 4.13 (dd, J = 5.4, 5.5 Hz, 1H), 3.84–3.78 (m, 2H), 3.35 (s, 3H), 2.75–2.68 (m, 1H), 2.36 (ddd, J = 6.1, 10.0, 14.8 Hz, 1H), 2.25–2.11 (m, 2H), 1.82 (dddd, J = 1.2, 3.8, 5.5, 14.7 Hz, 1H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz) δ 155.5, 151.9, 145.3, 125.3, 121.8, 87.5, 83.0, 81.3, 71.3, 56.9, 48.2, 39.4, 36.2.

(3S,3aR,5R,6aR)-3-Methylhexahydro-2H-cyclopenta[b]furan-5-yl (4-Nitrophenyl)carbonate (19e). The title compound was obtained from **14** in 83% yield as described for **7** after purification by column chromatography on silica gel using hexanes/EtOAc (10:1 and then 7:1) as the eluent (white solid). TLC, R_f = 0.25 (hexanes/EtOAc = 3:1); $^1\text{H NMR}$ (CDCl_3 , 300 MHz) δ 8.27 (d, J = 9.0 Hz, 2H), 7.37 (d, J = 9.0 Hz, 2H), 5.09 (m, 1H), 4.57 (dt, J = 2.8, 6.2 Hz, 1H), 3.93 (t, J = 8.1 Hz, 1H), 3.51 (dd, J = 8.1, 10.4, 1H), 2.65 (m, 1H), 2.46 (m, 1H), 2.38 (m, 1H), 2.11 (m, 1H), 2.02 (ddd, J = 2.7, 5.5, 14.8 Hz, 1H), 1.81 (m, 1H), 1.02 (d, J = 6.9 Hz, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 75 MHz) δ 155.5, 152.0, 145.3, 125.3, 121.8, 83.4, 80.8, 72.3, 45.8, 39.7, 36.5, 31.0, 11.8.

(3R,3aR,5R,6aR)-3-Methylhexahydro-2H-cyclopenta[b]furan-5-yl (4-Nitrophenyl)carbonate (19f). The title compound was obtained from **18** in 50% yield as described for **7** following column chromatography on silica gel using hexanes/EtOAc (10:1 and then 7:1) as the eluent (white solid). TLC, R_f = 0.29 (hexanes/EtOAc = 3:1); $[\alpha]_D^{20}$ +40.6 (c 1.0, CHCl_3); $^1\text{H NMR}$ (CDCl_3 , 400 MHz) δ 8.26 (d, J = 9.3 Hz, 2H), 7.39 (d, J = 9.3 Hz, 2H), 5.19–5.12 (m, 1H), 4.57 (dt, J = 2.0, 6.8 Hz, 1H), 4.07 (dd, J = 6.3, 8.6 Hz, 1H), 3.31 (dd, J = 7.1, 8.6 Hz, 1H), 2.35–2.25 (m, 1H), 2.23–2.17 (m, 2H), 2.17–2.07 (m, 2H), 1.88 (ddd, J = 3.8, 5.3, 14.5 Hz, 1H), 1.05 (d, J = 6.8 Hz, 3H); $^{13}\text{C NMR}$

(CDCl₃, 100 MHz) δ 155.6, 151.9, 145.2, 125.2, 121.8, 83.3, 82.2, 74.9, 50.4, 41.8, 39.5, 37.3, 17.5.

General Method for the Synthesis of HIV-Protease Inhibitors. (3aS,5R,6aR)-3-Oxohexahydro-2H-cyclopenta[b]furan-5-yl [(2S,3R)-3-Hydroxy-4-(*N*-isobutyl-4-methoxyphenylsulfonamido)-1-phenylbutan-2-yl]carbamate (21). The *N*-Boc-protected isostere 20a (82 mg, 0.16 mmol) was dissolved in a 2:1 mixture CH₂Cl₂/TFA and stirred at room temperature for 2 h. The solution was then evaporated and dried in vacuo. The residue was redissolved in CH₃CN (1 mL) and the solution cooled down to 0 °C under argon. Et₃N (100 μ L) was added followed by a solution of activated carbonate 19a (40 mg, 0.13 mmol) in THF (1 mL). The solution was stirred for 36 h and the solution evaporated in vacuo. The residue was purified by column chromatography on silica gel using hexanes/EtOAc (3:1 to 1.5:1) as the eluent to furnish inhibitor 21 (46 mg, 61%) as a white solid. TLC, R_f = 0.23 (hexanes/EtOAc = 1:1); ¹H NMR (CDCl₃, 500 MHz) δ 7.70 (d, J = 8.8 Hz, 2H), 7.34–7.28 (m, 2H), 7.25–7.19 (m, 3H), 6.98 (d, J = 8.8 Hz, 2H), 5.03 (t, J = 6.9 Hz, 1H), 4.99 (m, 1H), 4.71 (d, J = 8.7 Hz, 1H), 3.97 (s, 2H), 3.87 (s, 3H), 3.89–3.85 (m, 1H), 3.83–3.70 (m, 2H), 3.09 (dd, J = 8.0, 15.2 Hz, 1H), 3.00 (dd, J = 3.0, 15.1 Hz, 1H), 2.99–2.89 (m, 3H), 2.89–2.82 (m, 1H), 2.79 (dd, J = 6.8, 13.4 Hz, 1H), 2.24–2.12 (m, 2H), 2.06–1.94 (m, 2H), 1.81 (m, 1H), 0.90 (d, J = 6.4 Hz, 3H), 0.86 (d, J = 6.4 Hz, 3H); ¹³C NMR (CDCl₃, 125 MHz) δ 216.2, 163.0, 155.4, 137.5, 129.8, 129.6, 129.5, 128.5, 126.6, 114.3, 82.9, 76.3, 72.2, 70.6, 58.8, 55.6, 55.1, 53.7, 48.7, 41.2, 37.3, 35.1, 27.3, 20.1, 19.9. HRMS-ESI (m/z): [M + Na]⁺ calcd for C₂₉H₃₈N₂O₈SNa 597.2247, found 597.2251.

(3S,3aR,5R,6aR)-3-Hydroxyhexahydro-2H-cyclopenta[b]furan-5-yl [(2S,3R)-3-Hydroxy-4-(*N*-isobutyl-4-methoxyphenylsulfonamido)-1-phenylbutan-2-yl]carbamate (22). A solution of keto inhibitor 21 (10 mg, 17 μ mol) in EtOH was cooled to –25 °C, and NaBH₄ (6 mg) was added at once. The solution was stirred for 30 min. Then saturated aqueous NH₄Cl solution was added. The aqueous phase was extracted with EtOAc (\times 4). The combined organic layer was dried, filtered, and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel using hexanes/EtOAc (2:1, 1:1, then 1:2) as the eluent to afford the desired inhibitor 22 (8 mg, 80%) as a white solid. TLC, R_f = 0.39 (hexanes/EtOAc = 1:5); ¹H NMR (CDCl₃, 500 MHz) δ 7.70 (d, J = 8.8 Hz, 2H), 7.32–7.19 (m, 5H), 6.97 (d, J = 8.8 Hz, 2H), 5.07 (m, 1H), 4.89 (d, J = 7.2 Hz, 1H), 4.38 (t, J = 6.8 Hz, 1H), 4.21 (m, 1H), 3.87 (s, 3H), 3.88–3.74 (m, 4H), 3.54 (dd, J = 3.2, 9.6 Hz, 1H), 3.10 (dd, J = 7.8, 15.0 Hz, 1H), 3.05–2.82 (m, 5H), 2.78 (dd, J = 6.8, 13.4 Hz, 1H), 2.42 (br s, 1H), 2.18–2.04 (m, 2H), 1.94–1.76 (m, 3H), 0.90 (d, J = 6.6 Hz, 3H), 0.86 (d, J = 6.6 Hz, 3H); ¹³C NMR (CDCl₃, 125 MHz) δ 163.0, 155.3, 137.6, 129.9, 129.6, 129.5, 128.6, 126.5, 114.3, 84.9, 78.1, 76.3, 73.0, 72.2, 58.7, 55.6, 55.3, 53.7, 47.3, 39.1, 35.4, 31.3, 27.2, 20.1, 19.9; LRMS-ESI (m/z): [M + Na]⁺ 599.3.

(3S,3aR,5R,6aR)-3-Hydroxyhexahydro-2H-cyclopenta[b]furan-5-yl [(2S,3R)-4-(4-Amino-*N*-isobutylphenylsulfonamido)-3-hydroxy-1-phenylbutan-2-yl]carbamate (23). The ketone intermediate was first synthesized from the coupling of 19a with isostere 20b as described for 21 after stirring 48 h at room temperature in a CH₂Cl₂/THF (1:1) mixture. Purification of the crude compound by column chromatography on silica gel using chloroform with 1–3% EtOH as the eluent afforded the corresponding ketone inhibitor (60%, white solid). TLC, R_f = 0.18 (chloroform/3% EtOH); ¹H NMR (CDCl₃, 500 MHz) δ 7.54 (d, J = 8.6 Hz, 2H), 7.34–7.28 (m, 2H), 7.25–7.18 (m, 3H), 6.68 (d, J = 8.6 Hz, 2H), 5.02 (t, J = 6.9 Hz, 1H), 4.99 (m, 1H), 4.68 (d, J = 8.8 Hz, 1H), 4.14 (br s, 2H), 3.98 (s, 2H), 3.91 (m, 1H), 3.84–3.72 (m, 2H), 3.08 (dd, J = 8.4, 15.1 Hz, 1H), 3.00–2.83 (m, 5H), 2.76 (dd, J = 8.7, 13.4 Hz, 1H), 2.22–2.15 (m, 2H), 2.05–1.97 (m, 2H), 1.80 (m, 1H), 0.90 (d, J = 6.6 Hz, 3H), 0.86 (d, J = 6.6 Hz, 3H); ¹³C NMR (CDCl₃, 125 MHz) δ 216.9, 155.4, 150.6, 137.5,

129.6, 129.5, 128.5, 126.5, 126.3, 114.1, 82.9, 76.3, 72.2, 70.6, 58.9, 55.0, 53.8, 48.7, 41.2, 37.3, 35.2, 27.3, 20.2, 19.9. HRMS-ESI (m/z): [M + Na]⁺ calcd for C₂₈H₃₇N₃O₇SNa 582.2250, found 582.2246. The title compound was obtained in 82% yield by reduction of the above ketone intermediate as described for 22, followed by column chromatography on silica gel using hexanes/EtOAc (1:2 to 1:5) as the eluent. Off-white solid. TLC, R_f = 0.12 (hexanes/EtOAc = 1:2); ¹H NMR (CDCl₃, 500 MHz) δ 7.54 (d, J = 8.8 Hz, 2H), 7.34–7.18 (m, 5H), 6.68 (d, J = 8.8 Hz, 2H), 5.07 (m, 1H), 4.87 (d, J = 7.2 Hz, 1H), 4.38 (t, J = 6.8 Hz, 1H), 4.24–4.19 (m, 1H), 4.14 (s, 2H), 3.86 (br s, 1H), 3.85–3.75 (m, 3H), 3.55 (dd, J = 3.6, 9.6 Hz, 1H), 3.10 (dd, J = 8.2, 15.0 Hz, 1H), 3.04–2.79 (m, 5H), 2.76 (dd, J = 6.8, 13.2 Hz, 1H), 2.37 (d, J = 11.6 Hz, 1H), 2.10 (t, J = 14.4 Hz, 2H), 1.94–1.75 (m, 3H), 0.90 (d, J = 6.8 Hz, 3H), 0.86 (d, J = 6.4 Hz, 3H); ¹³C NMR (CDCl₃, 125 MHz) δ 155.3, 150.6, 137.6, 129.6, 129.5, 128.5, 126.5, 126.4, 114.1, 84.9, 78.0, 70.3, 73.0, 72.2, 58.8, 55.2, 53.7, 47.3, 39.1, 35.5, 31.3, 27.3, 20.2, 19.9. HRMS-ESI (m/z): [M + Na]⁺ calcd for C₂₈H₃₉N₃O₇SNa 584.2406, found 584.2410.

(3S,3aR,5R,6aR)-3-Hydroxyhexahydro-2H-cyclopenta[b]furan-5-yl [(2S,3R)-3-Hydroxy-4-[4-(hydroxymethyl)-*N*-isobutylphenylsulfonamido]-1-phenylbutan-2-yl]carbamate (24). The ketone intermediate was first synthesized from the coupling of 19a with isostere 20c as described for 21. Purification of the crude compound by column chromatography on silica gel using hexanes/EtOAc (2:1 to 1:2) as the eluent afforded the corresponding ketone inhibitor in 49% yield as a white solid. TLC, R_f = 0.38 (hexanes/EtOAc = 1:3); ¹H NMR (CDCl₃, 500 MHz) δ 7.76 (d, J = 8.2 Hz, 2H), 7.51 (d, J = 8.2 Hz, 2H), 7.34–7.28 (m, 2H), 7.25–7.20 (m, 3H), 5.02 (t, J = 6.9 Hz, 1H), 4.96 (m, 1H), 4.79 (s, 2H), 4.67 (d, J = 8.4 Hz, 1H), 3.98–3.93 (m, 2H), 3.84–3.75 (m, 2H), 3.75–3.67 (m, 1H), 3.10 (dd, J = 8.2, 15.1 Hz, 1H), 3.07–2.80 (m, 5H), 2.83 (dd, J = 6.9, 13.5 Hz, 1H), 2.23–2.15 (m, 2H), 2.04–1.96 (m, 2H), 1.88–1.76 (m, 1H), 0.90 (dd, J = 6.6 Hz, 3H), 0.87 (d, J = 6.6 Hz, 3H). The title compound was obtained in 83% yield by reduction of the above ketone intermediate as described for 22, followed by column chromatography on silica gel using hexanes/EtOAc (1:1 to 1:3 and then 100% EtOAc) as the eluent. White solid. TLC, R_f = 0.23 (hexanes/EtOAc = 1:3); ¹H NMR (CDCl₃, 400 MHz) δ 7.76 (d, J = 8.2 Hz, 2H), 7.51 (d, J = 8.2 Hz, 2H), 7.34–7.27 (m, 2H), 7.24–7.20 (m, 3H), 5.06 (m, 1H), 4.82 (d, J = 7.8 Hz, 1H), 4.76 (s, 2H), 4.38 (t, J = 7.0 Hz, 1H), 4.21 (m, 1H), 3.85–3.68 (m, 4H), 3.55 (dd, J = 3.5, 9.8 Hz, 1H), 3.11 (dd, J = 8.3, 15.1 Hz, 1H), 3.05–2.79 (m, 6H), 2.38 (d, J = 8.3 Hz, 1H), 2.15–1.99 (m, 2H), 1.94–1.80 (m, 3H), 0.91 (d, J = 6.6 Hz, 3H), 0.87 (d, J = 6.6 Hz, 3H). HRMS-ESI (m/z): [M + Na]⁺ calcd for C₂₉H₄₀N₂O₈SNa 599.2403, found 599.2401.

(3S,3aS,5R,6aR)-3-Methoxyhexahydro-2H-cyclopenta[b]furan-5-yl [(2S,3R)-3-Hydroxy-4-(*N*-isobutyl-4-methoxyphenylsulfonamido)-1-phenylbutan-2-yl]carbamate (25). The title compound was obtained in 73% yield from 19b and 20a as described for 21 following column chromatography on silica gel using hexanes/EtOAc (1:1) as the eluent. White solid. TLC, R_f = 0.21 (hexanes/EtOAc = 1:1); ¹H NMR (CDCl₃, 500 MHz) δ 7.70 (d, J = 8.9 Hz, 2H), 7.33–7.19 (m, 5H), 6.97 (d, J = 8.9 Hz, 2H), 4.97–4.90 (m, 1H), 4.78 (d, J = 7.5 Hz, 1H), 4.47–4.42 (m, 1H), 3.99 (q, J = 6.9 Hz, 1H), 3.87 (s, 3H), 3.88–3.82 (m, 2H), 3.81–3.76 (m, 2H), 3.70 (dd, J = 6.9, 9.0 Hz, 1H), 3.30 (s, 3H), 3.14–3.06 (dd, J = 7.8, 14.9 Hz, 1H), 3.04–2.88 (m, 4H), 2.78 (dd, J = 6.7, 13.4 Hz, 1H), 2.70 (m, 1H), 2.14 (m, 1H), 1.93 (dd, J = 6.3, 8.6 Hz, 2H), 1.86–1.76 (m, 2H), 0.90 (d, J = 6.6 Hz, 3H), 0.86 (d, J = 6.6 Hz, 3H); ¹³C NMR (CDCl₃, 125 MHz) δ 163.0, 156.3, 137.5, 129.9, 129.6, 129.5, 128.5, 126.5, 114.3, 83.5, 81.3, 76.3, 72.3, 70.0, 58.7, 57.8, 55.6, 54.9, 53.7, 43.8, 39.5, 35.5, 30.4, 27.2, 20.1, 19.9. HRMS-ESI (m/z): [M + H]⁺ calcd for C₃₀H₄₃N₂O₈S 591.2740, found 591.2742.

(3R,3aR,5R,6aR)-3-Hydroxyhexahydro-2H-cyclopenta[b]furan-5-yl [(2S,3R)-3-Hydroxy-4-(*N*-isobutyl-4-methoxyphenylsulfonamido)-1-phenylbutan-2-yl]carbamate (26). The

acetyl intermediate was first synthesized in 84% yield by coupling of **19c** with **20a** as described for **21** followed by purification by column chromatography on silica gel using hexanes/EtOAc (3:1 to 1:1) as the eluent. White solid. TLC, $R_f = 0.23$ (hexanes/EtOAc = 1:2); $^1\text{H NMR}$ (CDCl_3 , 300 MHz) δ 7.71 (d, $J = 8.9$ Hz, 2H), 7.34–7.19 (m, 5H), 6.97 (d, $J = 8.9$ Hz, 2H), 4.91 (m, 1H), 4.88 (m, 1H), 4.75 (d, $J = 8.2$ Hz, 1H), 4.67 (m, 1H), 3.98 (dd, $J = 4.1$, 10.4 Hz, 1H), 3.87 (s, 3H), 3.85–3.77 (m, 3H), 3.73 (dd, $J = 1.5$, 10.4 Hz, 1H), 3.19–2.92 (m, 4H), 2.90–2.72 (m, 2H), 2.67–2.55 (m, 1H), 2.23–2.09 (m, 1H), 2.06 (s, 3H), 2.04–1.76 (m, 3H), 1.50–1.43 (m, 1H), 0.91 (d, $J = 6.6$ Hz, 3H), 0.87 (d, $J = 6.6$ Hz, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 75 MHz) δ 170.6, 163.0, 156.0, 137.6, 129.8, 129.5, 128.5, 126.6, 114.3, 83.5, 80.6, 76.1, 72.6, 71.6, 58.8, 55.6, 54.9, 53.7, 48.5, 39.6, 36.2, 35.7, 27.2, 21.1, 20.1, 19.9. The acetate intermediate (18 mg, 0.029 mmol) was diluted in MeOH (1.5 mL) at 0 °C. K_2CO_3 (5 mg, 0.04 mmol) was added, and the solution was stirred for 6 h. Saturated aqueous NH_4Cl solution (1 mL) was added, and the solvent was reduced under vacuum. The aqueous phase was diluted and extracted with EtOAc ($\times 4$). The combined organic phase was dried (MgSO_4), filtered, and evaporated. The residue was purified by column chromatography on silica gel using (chloroform)/(0.5–3% EtOH) as the eluent to provide inhibitor **26** (15.9 mg, 94%) as a white solid. TLC, $R_f = 0.26$ (hexanes/EtOAc = 1:5); $^1\text{H NMR}$ (CDCl_3 , 500 MHz) δ 7.71 (d, $J = 8.9$ Hz, 2H), 7.33–7.25 (m, 1H), 7.24–7.19 (m, 3H), 6.98 (d, $J = 8.9$ Hz, 2H), 4.86 (m, 1H), 4.81 (d, $J = 8.3$ Hz, 1H), 4.69 (t, $J = 5.4$ Hz, 1H), 4.01 (m, 1H), 3.91–3.76 (m, 4H), 3.87 (s, 3H), 3.64 (dd, $J = 2.0$, 9.7 Hz, 1H), 3.12 (dd, $J = 8.2$, 15.1 Hz, 1H), 3.11–3.05 (m, 1H), 3.03 (dd, $J = 2.7$, 15.2 Hz, 1H), 2.95 (dd, $J = 8.3$, 13.4 Hz, 1H), 2.85–2.75 (m, 2H), 2.52 (m, 1H), 2.12 (ddd, $J = 6.1$, 10.0, 14.7 Hz, 1H), 1.99 (dt, $J = 6.1$, 15.0 Hz, 1H), 1.93–1.79 (m, 3H), 1.35 (m, 1H), 0.91 (d, $J = 6.6$ Hz, 3H), 0.87 (d, $J = 6.6$ Hz, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 125 MHz) δ 163.0, 156.0, 137.8, 129.8, 129.5, 128.4, 126.4, 114.3, 83.1, 78.3, 76.3, 73.8, 72.7, 58.8, 55.6, 54.8, 53.7, 51.3, 39.5, 36.1, 35.8, 27.2, 20.1, 19.9. HRMS-ESI (m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{29}\text{H}_{41}\text{N}_2\text{O}_8\text{S}$ 577.2584, found 577.2572.

(3R,3aR,5R,6aR)-3-Hydroxyhexahydro-2H-cyclopenta[b]furan-5-yl [(2S,3R)-4-(4-Amino-N-isobutylphenylsulfonamido)-3-hydroxy-1-phenylbutan-2-yl]carbamate (27). The acetyl intermediate was first synthesized in 63% yield by coupling of **19c** with **20b** as described for **21** followed by purification by column chromatography on silica gel using (CHCl_3)/(0.25–1.5% EtOH) as the eluent. White solid. TLC, $R_f = 0.44$ (hexanes/EtOAc = 1:3); $^1\text{H NMR}$ (CDCl_3 , 500 MHz) δ 7.54 (d, $J = 8.7$ Hz, 2H), 7.32–7.27 (m, 2H), 7.25–7.19 (m, 3H), 6.67 (d, $J = 8.7$ Hz, 2H), 4.94 (m, 1H), 4.90–4.85 (m, 1H), 4.75 (d, $J = 8.7$ Hz, 1H), 4.66 (t, $J = 5.4$ Hz, 1H), 4.16 (br s, 2H), 3.99 (dd, $J = 4.1$, 10.4 Hz, 1H), 3.88–3.77 (m, 3H), 3.74 (d, $J = 10.3$ Hz, 1H), 3.11 (dd, $J = 8.5$, 15.1 Hz, 1H), 3.05 (dd, $J = 4.0$, 14.1 Hz, 1H), 2.98 (dd, $J = 1.5$, 15.2 Hz, 1H), 2.93 (dd, $J = 8.4$, 13.2 Hz, 1H), 2.84 (dd, $J = 8.8$, 13.9 Hz, 1H), 2.77 (dd, $J = 6.7$, 13.3 Hz, 1H), 2.64–2.56 (m, 1H), 2.20–2.12 (m, 1H), 2.07 (s, 3H), 2.00 (dt, $J = 6.1$, 15.2 Hz, 1H), 1.95–1.88 (m, 1H), 1.81 (m, 1H), 1.51–1.44 (m, 1H), 0.91 (d, $J = 6.6$ Hz, 3H), 0.87 (d, $J = 6.6$ Hz, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 125 MHz) δ 170.6, 155.9, 150.6, 137.7, 129.5, 128.5, 126.5, 126.2, 114.1, 83.5, 80.6, 76.0, 72.6, 71.5, 58.9, 54.9, 53.8, 48.5, 39.6, 36.2, 35.7, 27.3, 21.1, 20.2, 19.9. The title compound was obtained from the above acetate intermediate in 88% yield as described for **26** following purification by column chromatography on silica gel using a gradient, 1–5% EtOH in CHCl_3 , as the eluent. White solid. TLC, $R_f = 0.4$ (CHCl_3 /10% EtOH); $^1\text{H NMR}$ (CDCl_3 , 500 MHz) δ 7.55 (d, $J = 8.7$ Hz, 2H), 7.31–7.26 (m, 2H), 7.24–7.19 (m, 3H), 6.67 (d, $J = 8.7$ Hz, 2H), 4.89–4.83 (m, 1H), 4.79 (d, $J = 8.9$ Hz, 1H), 4.69 (t, $J = 5.4$ Hz, 1H), 4.16 (br s, 2H), 4.01 (m, 1H), 3.88 (dd, $J = 3.7$, 9.9 Hz, 1H), 3.87–3.76 (m, 3H), 3.65 (dd, $J = 1.4$, 9.8 Hz, 1H), 3.14–3.04 (m, 2H), 2.99 (dd, $J = 2.9$, 15.1 Hz, 1H), 2.92 (dd, $J = 8.3$, 13.3 Hz, 1H), 2.81 (dd, $J = 9.1$, 14.0 Hz, 1H), 2.78 (dd, $J = 6.8$, 13.3 Hz, 1H), 2.54–2.47 (m, 1H), 2.11 (ddd, $J = 6.2$, 10.2, 14.6 Hz,

1H), 2.00 (dt, $J = 6.1$, 15.1 Hz, 1H), 1.93–1.85 (m, 1H), 1.85–1.76 (m, 2H), 1.35 (m, 1H), 0.91 (d, $J = 6.6$ Hz, 3H), 0.87 (d, $J = 6.6$ Hz, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 125 MHz) δ 156.0, 150.7, 137.8, 129.5, 128.4, 126.4, 126.2, 114.1, 83.1, 78.3, 76.2, 73.7, 72.7, 58.9, 54.8, 53.8, 51.3, 39.5, 36.0, 35.8, 27.3, 20.2, 19.9. HRMS-ESI (m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{28}\text{H}_{39}\text{N}_3\text{O}_7\text{S}$ 584.2406, found 584.2398.

(3R,3aS,5R,6aR)-3-Methoxyhexahydro-2H-cyclopenta[b]furan-5-yl [(2S,3R)-3-Hydroxy-4-(N-isobutyl-4-methoxyphenylsulfonamido)-1-phenylbutan-2-yl]carbamate (28). The title compound was obtained in 57% yield from **19d** and **20a** as described for **21** following purification by column chromatography on silica gel using hexanes/EtOAc (2:1 to 1:1) as the eluent. White solid. TLC, $R_f = 0.34$ (hexanes/EtOAc = 1:2); $^1\text{H NMR}$ (CDCl_3 , 500 MHz) δ 7.71 (d, $J = 8.8$ Hz, 2H), 7.33–7.25 (m, 2H), 7.26–7.20 (m, 3H), 6.98 (d, $J = 8.8$ Hz, 2H), 4.89 (m, 1H), 4.78 (d, $J = 8.3$ Hz, 1H), 4.61 (t, $J = 5.7$ Hz, 1H), 3.93–3.85 (m, 1H), 3.88 (s, 3H), 3.85–3.79 (m, 2H), 3.73 (d, $J = 2.7$, 9.8 Hz, 1H), 3.60 (m, 1H), 3.31 (s, 3H), 3.13 (dd, $J = 8.3$, 15.2 Hz, 1H), 3.10–3.00 (m, 2H), 2.95 (dd, $J = 8.4$, 13.4 Hz, 1H), 2.88–2.77 (m, 1H), 2.80 (dd, $J = 7.0$, 13.3 Hz, 1H), 2.62–2.53 (m, 1H), 2.19–2.09 (m, 1H), 1.99 (dt, $J = 6.0$, 15.1 Hz, 1H), 1.92 (m, 1H), 1.90–1.78 (m, 2H), 1.47–1.38 (m, 1H), 0.92 (d, $J = 6.6$ Hz, 3H), 0.88 (d, $J = 6.6$ Hz, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 125 MHz) δ 163.0, 156.1, 137.7, 129.9, 129.5, 128.5, 126.5, 114.3, 87.6, 83.3, 76.5, 72.7, 71.1, 58.8, 56.7, 55.6, 54.9, 53.7, 48.0, 39.5, 36.6, 35.8, 27.2, 20.1, 19.9. HRMS-ESI (m/z): $[\text{M} + \text{Na}]^+$ calcd for $\text{C}_{30}\text{H}_{42}\text{N}_2\text{O}_8\text{SNa}$ 613.2560, found 613.2555.

(3S,3aR,5R,6aR)-3-Methylhexahydro-2H-cyclopenta[b]furan-5-yl [(2S,3R)-3-Hydroxy-4-(N-isobutyl-4-methoxyphenylsulfonamido)-1-phenylbutan-2-yl]carbamate (29). The title compound was obtained from **19e** and **20a** in 78% yield as described for **21** following purification by column chromatography on silica gel using hexanes/EtOAc (3:1 to 2:1) as the eluent. White solid. TLC, $R_f = 0.32$ (hexanes/EtOAc = 1:1); $^1\text{H NMR}$ (CDCl_3 , 300 MHz) δ 7.71 (d, $J = 8.9$ Hz, 2H), 7.34–7.19 (m, 5H), 6.98 (d, $J = 8.9$ Hz, 2H), 4.84 (m, 1H), 4.75 (d, $J = 8.2$ Hz, 1H), 4.49–4.40 (m, 1H), 3.87 (s, 3H), 3.86–3.76 (m, 4H), 3.32 (dd, $J = 8.3$, 10.5 Hz, 1H), 3.11 (dd, $J = 7.8$, 15.1 Hz, 1H), 3.06–2.84 (m, 4H), 2.79 (dd, $J = 6.7$, 13.4 Hz, 1H), 2.54–2.45 (m, 1H), 2.40–2.31 (m, 1H), 2.18 (dt, $J = 6.5$, 14.8 Hz, 1H), 1.90–1.77 (m, 2H), 1.74–1.66 (m, 1H), 1.50–1.40 (m, 1H), 0.91 (d, $J = 6.8$ Hz, 3H), 0.90 (d, $J = 6.6$ Hz, 3H), 0.86 (d, $J = 6.6$ Hz, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 75 MHz) δ 162.7, 156.3, 137.6, 129.8, 129.5, 128.5, 126.5, 114.3, 83.5, 76.0, 72.6, 72.2, 58.7, 55.6, 54.9, 53.7, 45.6, 39.8, 36.4, 35.6, 31.3, 27.2, 20.1, 19.8, 11.8. LRMS-ESI (m/z): $[\text{M} + \text{Na}]^+$ 597.3, $[\text{M} + \text{H}]^+$ 575.1.

(3R,3aR,5R,6aR)-3-Methylhexahydro-2H-cyclopenta[b]furan-5-yl [(2S,3R)-3-Hydroxy-4-(N-isobutyl-4-methoxyphenylsulfonamido)-1-phenylbutan-2-yl]carbamate (30). The title compound was obtained from **19f** and **20a** in 96% yield as described for **21** following purification by column chromatography on silica gel using hexanes/EtOAc (5:1) as the eluent. White solid. TLC, $R_f = 0.50$ (hexanes/EtOAc = 1:1); $[\alpha]_D^{20} +24.5$ (c 1.0, CHCl_3); $^1\text{H NMR}$ (CDCl_3 , 400 MHz) δ 7.70 (d, $J = 8.8$ Hz, 2H), 7.30–7.16 (m, 5H), 6.96 (d, $J = 8.8$ Hz, 2H), 4.88 (m, 1H), 4.80 (d, $J = 7.1$ Hz, 1H), 4.46 (m, 1H), 3.91 (dd, $J = 6.1$, 8.5 Hz, 1H), 3.86 (s, 3H), 3.80 (m, 3H), 3.22 (dd, $J = 7.5$, 14.8 Hz, 1H), 3.15–2.99 (m, 3H), 2.94 (dd, $J = 8.2$, 13.4 Hz, 1H), 2.87–2.75 (m, 2H), 2.14 (m, 1H), 2.00–1.77 (m, 5H), 1.50 (d, $J = 12.3$ Hz, 1H), 0.98 (d, $J = 6.6$ Hz, 3H), 0.90 (d, $J = 6.6$ Hz, 3H), 0.85 (d, $J = 6.6$ Hz, 3H); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz) δ 162.9, 156.2, 137.7, 129.8, 129.4, 128.4, 126.4, 114.3, 83.7, 77.2, 74.7, 72.5, 58.7, 55.6, 54.8, 53.7, 50.2, 41.8, 39.4, 37.7, 35.7, 27.2, 20.1, 19.8, 17.6. LRMS-ESI (m/z): $[\text{M} + \text{Na}]^+$ 597.1, $[\text{M} + \text{H}]^+$ 575.3.

(3S,3aR,5R,6aR)-3-(Dimethylamino)hexahydro-2H-cyclopenta[b]furan-5-yl [(2S,3R)-3-Hydroxy-4-(N-isobutyl-4-methoxyphenylsulfonamido)-1-phenylbutan-2-yl]carbamate (31). To a solution of AcOH (~ 15 μL) in dichloromethane (1 mL), a slow

stream of Me₂NH gas was bubbled briefly at 0 °C for 5 min. After the flask was flushed with argon, a solution of ketone inhibitor **21** (13 mg, 0.02 mmol) in dichloroethane (0.5 mL) was added at 0 °C, and after 15 min, NaBH(OAc)₃ (10 mg, 0.05 mmol) was added. The resulting solution was warmed to room temperature. After 24 h, another 10 mg portion of NaBH(OAc)₃ was added and the solution stirred for 48 h. The reaction was quenched by addition of saturated aqueous NaHCO₃ solution, adjusting the pH to 10 with 1 M NaOH solution. The aqueous phase was extracted multiple times with EtOAc. The combined organic phase was dried (Na₂SO₄), filtered, and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel using ethanol (0.5–2%) in CHCl₃ to furnish the corresponding dimethylamine inhibitor **31** (11.3 mg, 82%) as a white solid. TLC, R_f = 0.35 (CHCl₃/8% EtOH); ¹H NMR (CDCl₃, 500 MHz) δ 7.70 (d, J = 8.9 Hz, 2H), 7.32–7.26 (m, 2H), 7.25–7.19 (m, 3H), 6.97 (d, J = 8.9 Hz, 2H), 4.92 (m, 1H), 4.86 (m, 1H), 4.55 (m, 1H), 3.90–3.85 (m, 1H), 3.87 (s, 3H), 3.83–3.75 (m, 2H), 3.70–3.63 (m, 1H), 3.30–3.20 (m, 1H), 3.10 (dd, J = 3.14, 3.06 Hz, 1H), 3.04–2.88 (m, 4H), 2.79 (dd, J = 6.9, 13.4 Hz, 1H), 2.64–2.55 (m, 1H), 2.23–2.18 (m, 1H), 2.24 (br s, 6H), 2.18–2.10 (m, 1H), 2.06–1.97 (m, 1H), 1.88–1.75 (m, 2H), 0.90 (d, J = 6.6 Hz, 3H), 0.85 (d, J = 6.6 Hz, 3H); ¹³C NMR (CDCl₃, 125 MHz) δ 163.0, 156.4, 137.6, 130.0, 129.6, 129.5, 128.5, 126.5, 114.3, 83.8, 77.2, 76.0, 72.3, 69.4, 58.7, 55.6, 55.0, 53.7, 45.4, 45.3, 40.1, 35.5, 31.4, 27.2, 20.1, 19.9. LRMS-ESI (m/z): [M + H]⁺ 604.3

Determination of X-ray Structure of HIV-1 Protease Inhibitor Complex. The optimized HIV-1 protease was expressed and purified as previously described.²³ The protease–inhibitor complex was crystallized by the hanging drop vapor diffusion method with well solutions of 1.2 M ammonium chloride and 0.1 M sodium acetate buffer (pH 4.8). Diffraction data were collected on a single crystal cooled to 90 K at SER-CAT BM beamline 22, Advanced Photon Source, Argonne National Laboratory (Chicago, IL, U.S.), with an X-ray wavelength of 1.0 Å and processed by HKL-2000²⁴ with R_{merge} of 7.2%. The PR structure was used in molecular replacement by PHASER^{25,26} in the CCP4i suite^{27,28} and refined to 1.45 Å resolution using SHELXL-97^{29,30} and COOT³¹ for manual modification. PRODRG-2³² was used to construct the inhibitor and the restraints for refinement. Alternative conformations were modeled, anisotropic atomic displacement parameters (B factors) were applied for all atoms including solvent molecules, and hydrogen atoms were added in the final round of refinement. The final refined solvent structure comprised two Cl[−] ions and 142 water molecules. The crystallographic statistics are listed in Table 3 in the Supporting Information. The coordinates and structure factors of the PR with **26** complex have been deposited in Protein Data Bank³³ with code 3ST5.

■ ASSOCIATED CONTENT

S Supporting Information. HPLC, LRMS, and HRMS data of inhibitors **21**–**31**; crystallographic data collection and refinement statistics for inhibitor **26**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Accession Codes

[†]The PDB accession code for **26**-bound HIV-1 protease X-ray structure is 3ST5.

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■ ABBREVIATIONS USED

bis-THF, bis-tetrahydrofuran; Cp-THF, hexahydrocyclopentafuran; PI, protease inhibitor; APV, amprenavir; DRV, darunavir; SQV, saquinavir; IDV, indinavir; TBS, *tert*-butyldimethylsilyl; DIBAL, diisobutylaluminum hydride

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HIV-1**

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Loss of Protease Dimerization Inhibition Activity of Darunavir Is Associated with the Acquisition of Resistance to Darunavir by HIV-1[∇]

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Dimerization of HIV protease is essential for the acquisition of protease's proteolytic activity. We previously identified a group of HIV protease dimerization inhibitors, including darunavir (DRV). In the present work, we examine whether loss of DRV's protease dimerization inhibition activity is associated with HIV development of DRV resistance. Single amino acid substitutions, including I3A, L5A, R8A/Q, L24A, T26A, D29N, R87K, T96A, L97A, and F99A, disrupted protease dimerization, as examined using an intermolecular fluorescence resonance energy transfer (FRET)-based HIV expression assay. All recombinant HIV_{NL4-3}-based clones with such a protease dimerization-disrupting substitution failed to replicate. A highly DRV-resistant *in vitro*-selected HIV variant and clinical HIV strains isolated from AIDS patients failing to respond to DRV-containing antiviral regimens typically had the V32I, L33F, I54M, and I84V substitutions in common in protease. None of up to 3 of the 4 substitutions affected DRV's protease dimerization inhibition, which was significantly compromised by the four combined substitutions. Recombinant infectious clones containing up to 3 of the 4 substitutions remained sensitive to DRV, while a clonal HIV variant with all 4 substitutions proved highly resistant to DRV with a 205-fold 50% effective concentration (EC₅₀) difference compared to HIV_{NL4-3}. The present data suggest that the loss of DRV activity to inhibit protease dimerization represents a novel mechanism contributing to HIV resistance to DRV. The finding that 4 substitutions in PR are required for significant loss of DRV's protease dimerization inhibition should at least partially explain the reason DRV has a high genetic barrier against HIV's acquisition of DRV resistance.

Currently available combination therapy or highly active antiretroviral therapy (HAART) for human immunodeficiency virus type 1 (HIV) infection and AIDS has been shown to potently suppress the replication of HIV and extend the life expectancy of HIV-infected individuals (32, 34). Recent analyses have revealed that life expectancy in HIV-infected patients treated with HAART has significantly increased, that mortality rates for HIV-infected persons have recently become close to that of general population, and that the appearance of the current first-line antiretroviral therapy with boosted protease inhibitor (PI)-based regimens has made the development of HIV resistance relatively less likely (2, 7, 18, 39). However, the ability to provide effective long-term antiretroviral therapy for HIV infection remains a complex issue since many of those who initially achieved favorable viral suppression to undetectable levels still suffer treatment failure (12, 18, 29).

Dimerization of HIV protease (PR) subunits is an essential

process for the acquisition of proteolytic activity of HIV PR, which plays a critical role in the maturation and replication of the virus (28, 40). Thus, inhibition of PR dimerization by chemical reagents is likely to abolish proteolytic activity and intervene in HIV replication. We have recently developed an intermolecular fluorescence resonance energy transfer (FRET)-based HIV-expression assay that employs cyan fluorescent protein (CFP)- and yellow fluorescent protein (YFP)-tagged HIV PR monomers to detect and quantify PR dimerization (26). Using this assay, we identified a group of nonpeptidyl small molecule inhibitors of HIV PR dimerization. These inhibitors, including darunavir (DRV) and tipranavir (TPV) as well as a series of potent experimental antiretroviral agents such as TMC126 (41), blocked PR dimerization at concentrations of as low as 0.01 μ M and potently blocked HIV replication *in vitro* (26).

DRV contains a structure-based and designed privileged nonpeptidic P2 ligand, 3(R),3a(S),6a(R)-*bis*-tetrahydrofuranylurethane (*bis*-THF) (14, 15, 27), which potently inhibits the enzymatic activity and dimerization of HIV PR (26) and has a high-level genetic barrier against HIV development of resistance to DRV (9, 10). Nevertheless, we have witnessed that HIV acquires significant levels of resistance against DRV among HIV-infected individuals who have received long-term combi-

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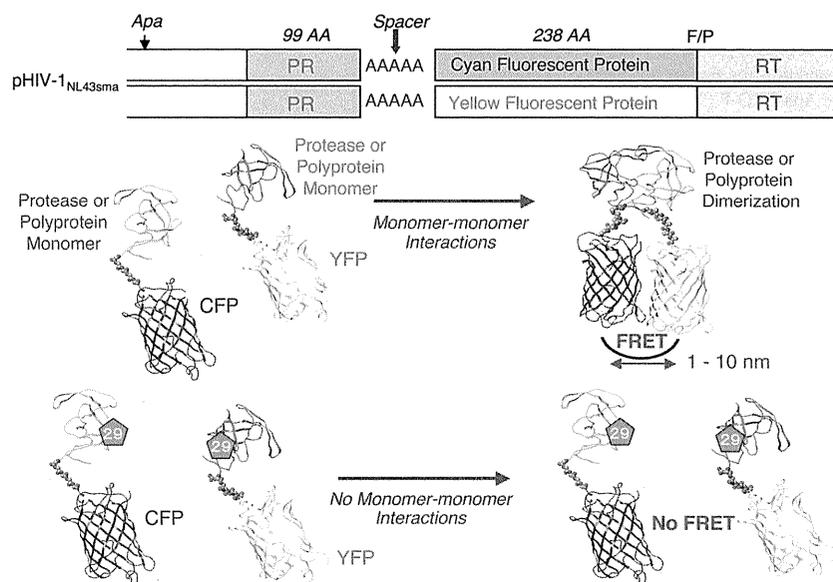


FIG. 1. FRET-based HIV expression system. Plasmids encoding full-length molecular infectious HIV (HIV_{NL4-3}) clones that produce CFP- or YFP-tagged PR were prepared using the PCR-mediated recombination method as described in Materials and Methods. A linker consisting of five alanines was inserted between PR and the fluorescent protein. A phenylalanine-proline site (F/P) that HIV PR cleaves was introduced between the fluorescent protein and RT. Shown are structural representations of PR monomers and dimer in association with the linker atoms and fluorescent proteins. FRET occurs when the two fluorescent proteins become 1 to 10 nm apart. If an agent that is capable of inhibiting the dimerization of PR monomer subunits is present when the CFP- and YFP-tagged PR monomers are produced within the cell upon cotransfection, no FRET occurs. If certain amino acid substitutions (AA) such as D29N (shown below) are introduced, PR subunits do not get dimerized and no FRET occurs.

nation chemotherapy (33, 38). Indeed, a variety of amino acid substitutions that are potentially related to HIV resistance to DRV have been reported (9, 24, 33, 38). Thus, the elucidation of the mechanism of the development of HIV drug resistance represents an urgent subject in the research area of HIV-1 infection/AIDS and therapy.

MATERIALS AND METHODS

Cells, viruses, and antiviral agents. MT-4 cells were grown in RPMI 1640-based culture medium, and 293T and COS7 cells were propagated in Dulbecco's modified Eagle's medium. These media were supplemented with 10% fetal calf serum (FCS; PAA Laboratories GmbH, Linz, Austria) plus 50 U of penicillin and 50 μ g of kanamycin per ml. The following HIV strains were used for the determination of 50% effective concentrations (EC_{50} s) against DRV and to construct plasmids for use in the FRET-based HIV expression assay, including HIV_{NL4-3} and HIV_{8MIX}^{P51} . Three recombinant clinical HIV isolates ($r_{CL}HIV_{F16}$, $r_{CL}HIV_{T45}$, and $r_{CL}HIV_{T48}$) used in this study were produced using recombinant HIV_{NL4-3} -based infectious molecular clones generated by ligating patient-derived amplicons encompassing approximately 200 nucleotides of 3' Gag (beginning at the unique ApaI restriction site), the entire protease, and the first 72 nucleotides of reverse transcriptase (RT) using the expression vector pNLFPB (a generous gift from Tomozumi Imamichi of the National Institute of Infectious Diseases and Allergy). The four clinical HIV isolates examined in the present study were chosen from 32 isolates that had been obtained from multi-PI-treated patients whose protease genotype contained prototypical patterns of PI resistance. The median duration of continuous PI treatment was 7.5 years (range, 6 to 10 years). The median number of PIs received (excluding the use of ritonavir for pharmacokinetic boosting) was 5 (range, 4 to 8). According to the PhenoSense assay, two isolates ($r_{CL}HIV_{T45}$ and $r_{CL}HIV_{F16}$) had high-level resistance to all PIs, including >90-fold decreased susceptibility to DRV and >8-fold decreased susceptibility to TPV, and one isolate ($r_{CL}HIV_{T48}$) had intermediate resistance to DRV and TPV and high-level resistance to the remaining PIs (see Table 2).

DRV was synthesized by A. K. Ghosh as described previously (16, 27). GRL-0216 (37), GRL-98065 (1), and TMC-126 (41) were synthesized in a convergent manner by coupling an optically active P2 ligand and an (*R*)-hydroxyethylamino

sulfonamide isostere (17). TPV was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health.

Generation of FRET-based HIV expression system. Cyan fluorescent protein (CFP)- and yellow fluorescent protein (YFP)-tagged HIV PR constructs were generated using BD Creator DNA cloning kits (BD Biosciences, San Jose, CA). The basic concepts of the intermolecular FRET-based HIV expression assay (FRET-HIV assay) are illustrated in Fig. 1. In brief, XhoI and HindIII fragments from the pCR-XL-TOPO vector containing the HIV PR-encoding gene excised from $pHIV_{NL4-3}$ were inserted into pDNR-1r, the donor vector, which had been digested with XhoI and HindIII. In the transfer of the PR gene from the donor vector into pLP-CFP/YFP-C1 (acceptor vector), the Cre-loxP site-specific recombination method was used according to the manufacturer's instructions. Using Cre-recombinase with the loxP site, the PR gene from pDNR-1r was inserted into pLP-CFP-C1 or pLP-YFP-C1 through Cre-mediated recombination (19), generating a plasmid expressing CFP-tagged wild-type PR (PR_{WT}) and one expressing YFP-tagged PR_{WT} , with which HIV PR was successfully expressed as a fusion protein with CFP and YFP tagged at the C terminus, respectively. Western blot assay using anti-green fluorescent protein-specific rabbit polyclonal antibodies revealed that PR was correctly tagged with CFP or YFP (26).

For the generation of full-length molecular infectious clones containing CFP- or YFP-tagged PR, the PCR-mediated recombination (PMR) method was used (11). To this end, we amplified an upstream proviral DNA fragment containing an ApaI site and HIV PR (excised from $pHIV_{NL4-3}$) with primer pair Apa-PRO-F (5'-TTG CAG GGC CCC TAG GAA AAA GG-3') plus PR-5Ala-R (5'-GGC TGC TGC GGC AGC AAA ATT TAA AGT GCA GCC AAT CT-3'), a middle proviral DNA fragment containing CFP (excised from pCFP-C1) or YFP (excised from pYFP-C1) (Clontech, Mountain View, CA) with primer pair CFPYFP-5Ala-F (5'-GCT GCC GCA GCA GCC GTG AGC AAG GGC GAG GAG CTG-3') plus CFPYFP-FP-R (5'-ACT AAT GGG AAA CTT GTA CAG CTC GTC CAT GCC G-3'), and a downstream proviral DNA fragment containing the 5'-DNA fragment of RT and an SmaI site from $pHIV_{NLSma}$ (13, 25), which had been created to have an SmaI site by changing two nucleotides (2590 and 2593) of $pHIV_{NL4-3}$, with primer pair FRT-F (5'-TTT CCC ATT AGT CCT ATT GAG ACT GTA-3') plus NL4-3-RT263-R (5'-CCA GAA ATC TTG AGT TCT CTT ATT-3'). A linker consisting of five alanines was inserted between the PR and fluorescent protein. The phenylalanine-proline site that HIV PR cleaves

was also introduced between the fluorescent protein and RT. Thus, the three DNA fragments obtained were subsequently joined by using the PMR reaction performed under the standard condition for ExTaq polymerase (Takara Bio, Inc., Otsu, Japan) with 10 pmol of Apa-PRO-F (5'-TTG CAG GGC CCC TAG GAA AAA GG-3') and NL4-3-RT263-R (5'-CCA GAA ATC TTG AGT TCT CTT ATT-3') and the three DNA fragments (100 ng each) in a 20- μ l reaction solution. Thermal cycling was carried out at 94°C for 3 min, followed by 35 cycles of 94°C for 50 s, 53°C for 50 s, and 72°C for 2 min, and finally 72°C for 15 min. The amplified PCR products were cloned into pCR-XL-TOPO vector according to the manufacturer's instructions (Gateway cloning system; Invitrogen). PCR products were generated with pCR-XL-TOPO vector as templates, followed by digestion by both *Apal* and *SmaI*, and the *Apal*-*SmaI* fragment was introduced into pHIV_{NLSma} (13), generating pHIV-PR_{WT}^{CFP} and pHIV-PR_{WT}^{YFP}, respectively.

Analysis of inter- and intramolecular interactions of PR subunits. Analysis of inter- and intramolecular interactions of PR subunits was conducted by examining the crystal structure of DRV with HIV PR (Protein Data Bank identification no. [PDB ID no.] 2IEN). Hydrogens were added and minimized using the OPLS2005 force field with constraints on heavy atom positions. The calculation was performed using MacroModel 9.1 from Schrödinger, LLC. Hydrogen bonds were assigned when the following distance and angle cutoff was satisfied: 3.0 Å for H-A distance, with a D-H-A angle of >90° and an H-A-B angle of >60°, where H is the hydrogen, A is the acceptor, D is the donor, and B is a neighbor atom bonded to the acceptor. The representative distance between the termini of two monomers was determined by analyzing the PR-DRV crystal structure (PDB ID no. 2IEN). The distance between the α carbons at the N termini and C termini is around 0.5 nm, whereas the distance between the α carbons of the N termini ends of two monomers is around 1.8 nm.

FRET procedure. COS7 cells plated on an EZ view cover glass bottom culture plate (Iwaki, Tokyo) were transfected with the indicated plasmid constructs, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions in the presence of various concentrations of each compound, cultured for 72 h, and analyzed under a Fluoview FV500 confocal laser scanning microscope (Olympus Optical Corp., Tokyo) at room temperature. When the effect of each compound was analyzed by FRET, test compounds were added to the culture medium simultaneously with plasmid transfection. The results of FRET were determined by quenching of CFP (donor) fluorescence and an increase in YFP (acceptor) fluorescence (sensitized emission), because part of the energy of CFP is transferred to YFP instead of being emitted. This phenomenon can be measured by bleaching YFP, which should result in an increase in CFP fluorescence. This technique, also known as acceptor photobleaching, is a well-established method of determining the occurrence of FRET (5, 6, 35, 36). Dequenching of the donor CFP by selective photobleaching of the acceptor YFP was performed by first obtaining YFP and CFP images at the same focal plane, followed by illuminating the same image for 3 min at a wavelength of 488 nm with a laser power set at the maximum intensity to bleach YFP and then recapturing the same CFP and YFP images. The changes in the CFP and YFP fluorescence intensity in the images of selected regions were examined and quantified using the Olympus FV500 Image software system (Olympus Optical Corp.). Background values were obtained from the regions where no cells were present and were subtracted from the values for the cells examined in all calculations. For each chimeric protein, the data were obtained from at least three independent experiments. Digitized image data obtained from the experiment were prepared for presentation using Photoshop 6.0 (Adobe Systems, Mountain View, CA). Ratios of intensities of CFP fluorescence after photobleaching to CFP fluorescence prior to photobleaching (CFP^{A/B} ratios) were determined. It is well established that CFP^{A/B} ratios of >1.0 indicate that association of CFP- and YFP-tagged proteins occurred and were interpreted to indicate that the dimerization of PR subunits occurred. CFP^{A/B} ratios of <1 indicated that the association of the two subunits did not occur and were interpreted to indicate that PR dimerization was inhibited. The difference in the CFP^{A/B} ratios determined in the presence or absence of test drugs was evaluated using the nonparametric Mann-Whitney U statistic test.

Replication kinetics of various NL-PR_{mutant}^{YFP} strains. MT-4 cells (10⁵) were exposed to each infectious HIV-PR^{YFP} clone (100 ng of p24 Gag protein/ml) for 6 h, washed twice with phosphate-buffered saline (PBS), and cultured in 7 ml of complete medium with some modification as described previously (3, 13). Culture supernatants (50 μ l) were harvested every other day, and virus replication was monitored by the amounts of p24 Gag produced in the culture supernatants.

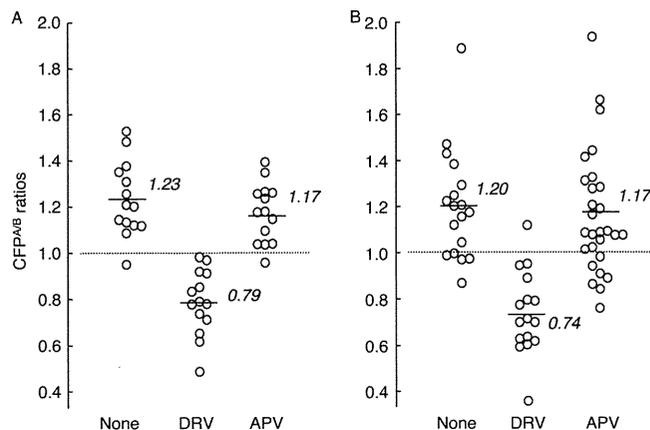


FIG. 2. DRV blocks the dimerization of both pHIV-PR_{WT}-encoded PR and pPR_{WT}-encoded PR. (A) COS7 cells were cotransfected with pHIV-PR_{WT}^{CFP} plus pHIV-PR_{WT}^{YFP} in the absence or presence of 1 μ M DRV or APV. On day 3 after transfection, CFP^{A/B} ratios were determined using an FV500 confocal laser microscope. When the average value of CFP^{A/B} ratios was greater than 1.0, it was judged that the dimerization of PR occurred, whereas when it was less than 1.0, it was judged that the dimerization did not occur. (B) COS7 cells were cotransfected with a pair of wild-type PR-expressing plasmids (pPR_{WT}^{CFP} plus pPR_{WT}^{YFP}) in the absence or presence of 1 μ M DRV or APV, and CFP^{A/B} ratios were determined as described above. Note that DRV inhibited the dimerization of PR when it was expressed as HIV virions and virion-free PR. The results of statistical evaluation of the changes in the CFP^{A/B} ratios, determined in the presence or absence of DRV or APV, using the nonparametric Mann-Whitney U test, are as follows. (A) For the CFP^{A/B} ratios in the absence of drug (CFP^{A/B}_{No Drug}) versus the CFP^{A/B} ratios in the presence of 1.0 μ M DRV (CFP^{A/B}_{1.0 DRV}), $P = 0.00001$, and for CFP^{A/B}_{No Drug} versus CFP^{A/B}_{1.0 APV}, $P = 0.42$. (B) For CFP^{A/B}_{No Drug} versus CFP^{A/B}_{1.0 DRV}, $P = 0.000003$, and for CFP^{A/B}_{No Drug} versus CFP^{A/B}_{1.0 APV}, $P = 0.60$.

RESULTS

DRV inhibits the dimerization of HIV PR expressed as a single protein. The basic concepts of the intermolecular FRET-based HIV-expression assay (FRET-HIV expression assay) to assess PR dimerization are illustrated in Fig. 1 (26). Using the FRET-based HIV expression assay, we previously identified a group of PR dimerization inhibitors (PDIs), including DRV and TPV, although other conventional PR inhibitors (PIs), such as amprenavir (APV), failed to block dimerization (Fig. 2A) (26). In the FRET-based HIV expression assay, YFP- or CFP-tagged PR should be primarily expressed as a part of Pr160^{gag-pol} polyprotein, and it was assumed that DRV blocks the dimerization of the PR subunit within the polyprotein. Thus, it remained to be determined whether DRV also blocks the dimerization of PR in the form of a single PR molecule. We, therefore, generated a pair of plasmids encoding wild-type HIV_{NL4-3} PR tagged with YFP and CFP in the 3' terminus (pPR_{WT}^{YFP} and pPR_{WT}^{CFP}, respectively), transfected COS7 cells with the pair, and determined whether DRV blocked the dimerization of PR_{WT}^{YFP} and PR_{WT}^{CFP}. As shown in Fig. 2B, the average value of CFP^{A/B} ratios obtained in the absence of drug was 1.20 ± 0.24 , which indicated that the dimerization between PR_{WT}^{YFP} and PR_{WT}^{CFP} occurred. The average value of the ratios determined in the presence of 1 μ M DRV was 0.74 ± 0.18 ($P = 0.000003$), signifying that DRV clearly blocked the

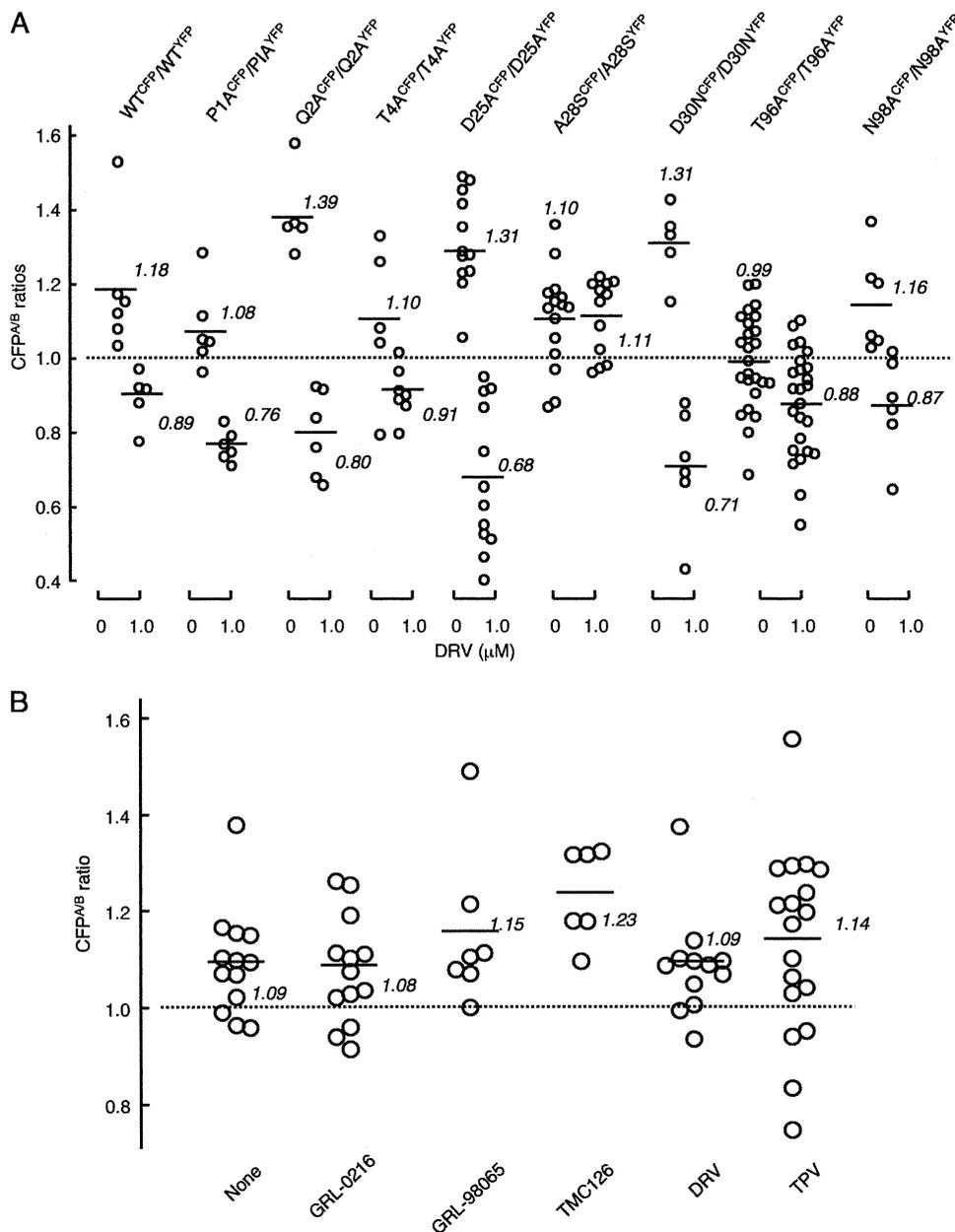


FIG. 3. Dimerization profiles of single PR mutants in the presence of DRV. (A) COS-7 cells were cotransfected with pHIV-PR_{WT}^{CFP} plus pHIV-PR_{WT}^{YFP} (shown as WT^{CFP}/WT^{YFP}) or mutated pairs such as pHIV-PR_{P1A}^{CFP} plus pHIV-PR_{P1A}^{YFP} (shown as P1A^{CFP}/P1A^{YFP}) in the absence or presence of 1 μ M DRV. On day 3 after transfection, CFP^{A/B} ratios were determined. (B) COS7 cells were cotransfected with plasmid pair pHIV-PR_{A28S}^{CFP} and pHIV-PR_{A28S}^{YFP} in the absence or presence of an agent (1 μ M GRL-0216, DRV, GRL-98065, TPV, or TMC126), and CFP^{A/B} ratios were determined as described above. (A) The statistical evaluation of all the changes in the CFP^{A/B} ratios determined in the presence or absence of DRV using the nonparametric Mann-Whitney U test, gave *P* values ranging 0.000037 to 0.044, except for the *P* value for the pair A28S^{CFP} and A28S^{YFP}, which was 0.57. (B) The differences between the CFP^{A/B} ratios in the absence of drug (CFP^{A/B}_{No Drug}) and the CFP^{A/B} ratios in the presence of 1.0 μ M DRV (CFP^{A/B}_{1.0 DRV}) were statistically insignificant, indicating that all of the agents examined failed to block the dimerization of A28S^{CFP}/A28S^{YFP}.

dimerization of PR_{WT}^{YFP} and PR_{WT}^{CFP}, while the value with APV was 1.17 ± 0.27 (*P* = 0.60), indicating that APV failed to block the dimerization, in line with our previous data (26). These results strongly suggest that DRV blocks dimerization of the PR monomer subunit in the form of Pr160^{gag-pol} polyprotein as well as in the form of a single molecule.

Dimerization profiles of single PR mutants in the presence of DRV. Certain amino acids in the termini and active site

interfaces, both of which are critical for the dimerization of PR monomer subunits (28, 40), do not significantly affect the dimerization process of PR. Such amino acids include Pro-1, Gln-2, Thr-4, Asp-25, Ala-28, Asp-30, Thr-96, and Asn-98 (26). It is assumed that DRV blocks PR dimerization by binding to a certain structural domain or domains within or in the proximity of either or both of the two interfaces (4, 22, 23). We, therefore, examined whether amino acid substitutions at posi-

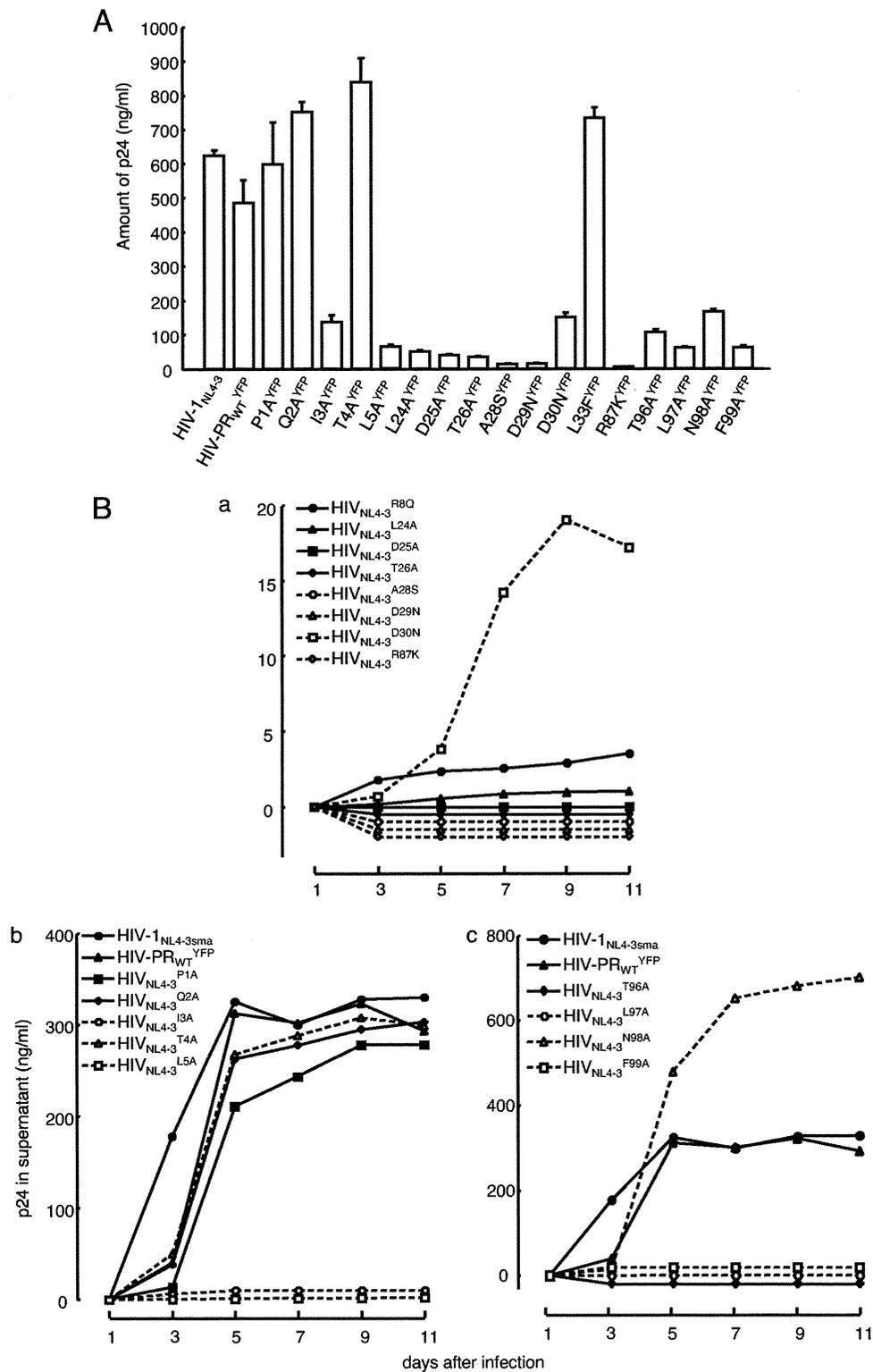


FIG. 4. Replication kinetics of HIV-PR^{YFP} with wild-type or mutated PR. (A) 293T cells were transfected with pHIV-PR_{WT}^{YFP} or mutated pHIV-PR^{YFP} (if pHIV-PR_{P1A}^{YFP} was used, it is shown as P1A^{YFP}), and the amounts of p24 Gag in the culture supernatants were determined 48 h after transfection. (B) MT-4 cells (10⁵) were exposed to the harvested supernatant of each infectious HIV-PR^{YFP} clone shown in panel A (100 ng of p24 Gag protein/ml) for 6 h, washed twice with phosphate-buffered saline (PBS), and further cultured in 7 ml of complete medium. Culture supernatants (50 μl) were harvested every other day, and virus replication was monitored by the amounts of p24 Gag produced in the culture supernatants. Replication kinetics of various HIV-PR^{YFP} mutants are shown over 11 days. In subpanels a, b, and c, the replication kinetics of infectious clones carrying mutations in the active site, N terminus, and C terminus, respectively, are shown. Note that recombinant HIV clones, whose replication rates were relatively poor, are illustrated in subpanel a. The experiments that generated data in subpanels a and b were performed on the same occasion. Thus, two controls (HIV-1_{NL4-3sma} and HIV-PR_{WT}^{YFP}) in subpanel b serve as controls in subpanel a as well.

tions 1, 3, 5, 25, 28, 30, 96, and 98, which allow PR to dimerize, affected the PR dimerization disruption by DRV. We reasoned that if any of the amino acid substitutions at these positions would affect PR dimerization inhibition by DRV, such amino acids could possibly be associated with the binding of DRV to the PR subunit. However, 1 μ M DRV effectively blocked the dimerization of all of the mutated PR species, except that of the species with the A28S substitution (Fig. 3A). These data suggest that all amino acid residues examined except A28S were not associated with the binding of DRV to the PR monomer subunit.

We have previously shown that, in addition to DRV and TPV, the three compounds GRL-0216 (37), GRL-98065 (1), and TMC126 (41) effectively blocked PR dimerization in the FRET-based HIV expression assay (26). Since the structures of these five compounds differ from each other, it was thought that the binding profiles of each compound also differed. We, therefore, examined if the four compounds other than DRV disrupted the dimerization of the A28S-carrying PR subunit. As shown in Fig. 3B, all four compounds failed to block protease dimerization, suggesting that Ala-28 is likely involved directly or indirectly in the binding of all four compounds to the PR monomer subunit.

Replication kinetics of HIV variants with failed PR dimerization. The failure of PR dimerization should completely block or significantly compromise the replication of HIV. In order to confirm that the observed dimerization failure elicited by a single amino acid substitution (26) causes replication failure of HIV, we generated a panel of HIV variants carrying YFP-tagged PR with a single amino acid substitution and examined the replicative capability of each variant. When the amount of p24 antigen produced into culture medium following transfection of COS7 cells with each plasmid was quantified, an HIV variant containing I3A, L5A, L24A, D25A, T26A, A28S, D29N, R87K, T96A, L97A, or F99A produced no or a significantly small amount of p24 (Fig. 4A). Among these variants, we have previously shown that I3A, L5A, T26A, D29N, R87K, T96A, L97A, and F99A disrupted PR dimerization (26). Figure 5 shows that R8A ($P = 0.000099$), R8Q ($P = 0.000084$), and L24A ($P = 0.0000014$) also disrupted PR dimerization, as examined in the FRET-based HIV expression assay (Fig. 5). When fresh MT-4 cells were exposed to each cell-free culture supernatant of the transfected COS7 cells, as described above, no further replication was seen over the 11-day period of the culture (Fig. 4B). The HIV variant carrying A28S also failed to replicate; however, this failure was explained by an observation that protease with A28S has almost no enzymatic activity, as reported by Hong et al. (20, 21). In contrast, recombinant HIV clones containing either of P1A, Q2A, T4A, D30N, or N98A continued to replicate (Fig. 4B).

DRV resistance profiles of PR species carrying DRV-resistance-associated amino acid substitutions. Using the standardized protocol for selection of drug-resistant HIV variants, we along with others have experienced difficulty in selecting DRV-resistant HIV variants *in vitro* (8, 24). The emergence of DRV-resistant HIV variants was substantially slower than that of variants resistant to other FDA-approved PIs when a single HIV strain was employed as a starting viral strain (8, 24). In this respect, we have recently succeeded in selecting a highly DRV-resistant HIV variant by using a mixture of 8 highly multi-PI-resistant, DRV-susceptible clinical HIV strains (HIV_{8MIX}

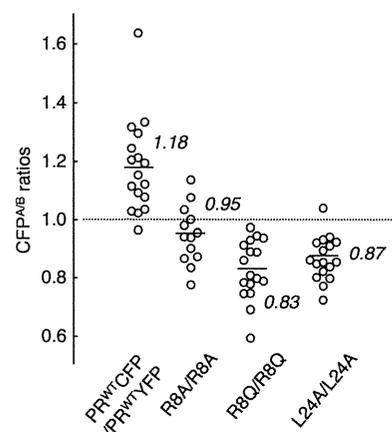


FIG. 5. Dimerization inhibition profiles of selected HIV-1 PR mutants. COS7 cells were cotransfected with a pair of HIV-PR^{CFP} and HIV-PR^{YFP} strains either wild type or carrying single amino acid (AA) substitutions, such as the R8A, R8Q, or L24A, in the absence of drug. The CFP^{A/B} ratios were determined at the conclusion of the 3-day period of culture. The differences between the CFP^{A/B} ratios of the WT and the CFP^{A/B} ratios of the mutant had P values of 0.000099 for R8A, 0.000084 for R8Q, and 0.0000014 for L24A.

strains HIV_A, HIV_B, HIV_C, HIV_G, HIV_{TM}, HIV_{MM}, HIV_{JSL}, and HIV_{SS}), which were originally isolated from patients with AIDS, who had failed then-existing anti-HIV regimens after receiving 9 to 11 anti-HIV drugs over the previous 32 to 83 months in the late 1990s and contained 9 to 14 amino acid substitutions in the PR-encoding region (42). By passage 39 in the selection with DRV, HIV_{8MIX} (HIV_{8MIX}^{P39}) became highly resistant to DRV, with an EC₅₀ ~333-fold greater than that against HIV_{NL4-3}. HIV_{8MIX} at passage 39 (HIV_{8MIX}^{P39}) was capable of replicating in the presence of 1 μ M DRV with a replication fitness comparable to that of HIV_{NL4-3} (24). HIV_{8MIX} at passage 51 (HIV_{8MIX}^{P51}), which was also capable of replicating in the presence of 5 μ M DRV, was found to contain the following 14 mutations: L10I, I15V, K20R, L24I, V32I, L33F, M36I, M46L, I54M, L63P, K70Q, V82I, I84V, and L89M (24). As illustrated in Table 1, when HIV_{8MIX}^{P51} was propagated in the presence of 0.1 and 1.0 μ M DRV in CD4⁺ MT-4 cells, the virus replicated comparably to HIV_{NL4-3} during the 9-day period of culture, while HIV_{NL4-3} completely failed to replicate in the presence of 0.1 or 1.0 μ M DRV, as examined according to the amounts of Gag protein produced in the culture supernatant, indicating that HIV_{8MIX}^{P51} had acquired a high-level resistance against DRV, while it maintained its robust replication fitness.

We, therefore, asked if the dimerization of the PR of HIV_{8MIX}^{P51} was blocked by DRV, exploiting the FRET-based HIV expression system by using a pair of newly generated plasmids encoding a full-length molecular infectious HIV clone containing CFP- or YFP-tagged PR with all 14 amino acid substitutions. As shown in Fig. 6, DRV significantly blocked the dimerization of the wild-type PR of HIV_{NL4-3} at concentrations of 0.1 and 1 μ M. However, DRV failed to block the dimerization of the PR of HIV_{8MIX}^{P51} at 0.1 μ M ($P = 0.42$). These data suggested that all amino acid substitutions present in the PR of HIV_{8MIX}^{P51} or subsets of them were associated with the HIV_{8MIX}^{P51} strain's acquisition of DRV resistance.

TABLE 1. HIV DRV-resistant strain HIV_{8MIX}^{P51} is capable of replicating in the presence of DRV^a

Virus	DRV (μM)	Replication (ng/ml) at day postexposure:				
		1	3	5	7	9
HIV _{NL4-3} (WT)	0	0	33 ± 9.9	955 ± 9.9	993 ± 57	1152 ± 127
	0.1	0	0	0	0	0
	1.0	0	0	0	0	0
HIV _{8MIX} ^{P51}	0	0	701 ± 45	734 ± 68	771 ± 19	877 ± 88
	0.1	1.5 ± 2.1	590 ± 103	682 ± 199	729 ± 3	909 ± 178
	1.0	0.5 ± 0.7	270 ± 10	886 ± 117	936 ± 18	1,201 ± 170

^a CD4⁺ MT-4 cells were exposed to HIV_{NL4-3} or HIV_{8MIX}^{P51} (a highly DRV-resistant HIV variant derived from the mixture of 8 highly-PI-resistant clinical HIV isolates exposed to increasing concentrations of DRV up to 1 μM) (24), cultured in the absence or presence of 0.1 or 1.0 μM DRV. Viral replication was monitored by the amounts of p24 Gag protein (ng/ml) produced in the culture supernatant.

Effects of V32I, L33F, I54M/L, and/or I84V substitutions on HIV susceptibility to DRV and PR dimerization inhibition by DRV. When we examined the sequence of the PR-encoding gene in HIV_{8MIX}^{P51} and three clinical HIV variants isolated from individuals with AIDS who did not respond to DRV-containing antiviral regimens (Table 2) (33), four amino acid substitutions (V32I, L33F, I54M, and I84V) were found to be mostly in common and thought to be relatively unique in such DRV-resistant HIV variants. The locations of the four amino acid substitutions are illustrated in Fig. 7A. This notion was further confirmed when we examined reports by others (9, 24,

33, 38) regarding the sequence of the PR-encoding region of DRV-resistant variants, as illustrated in Table 2. We consequently examined whether the notion described above was plausible by incorporating one of the four amino acid substitutions or subsets of them.

When we introduced each of the four substitutions into the wild-type strain, HIV_{NL4-3}, there was no increase observed in the EC₅₀s of DRV against such infectious recombinant clones,

TABLE 2. Amino acid substitutions associated with DRV resistance

DRV resistance-associated amino acid substitutions identified ^a	Variant ^b	EC ₅₀ , μM (fold change) ^c
L10I, I15V, K20R, L24I, V32I, L33F, M36I, M46L, I54 M, L63P, K70Q, V82I, I84V, L89 M	HIV _{8MIX} ^{P51}	>1 (>333)
L10F, V11I, I13V, L19Q, K20 M, V32I, L33F, E35A, M36I, M46I, I47V, I54 M, R57K, I62V, L63P, I64V, G73T, T74A, I84V, L89V, L90 M	r _{CL} HIV _{F16}	0.30 (97)
L10F, V11I, T12P, I13V, I15V, L19P, K20T, V32I, L33F, E35G, M36I, I54V, I62V, L63P, K70T, A71I, G73S, P79A, I84V, L89V, L90 M	r _{CL} HIV _{T45}	0.33 (105)
L10I, I13V, I15V, L19V, L24I, V32I, L33F, K43E, M46L, I54L, D60E, L63P, A71V, I72V, V82A, I84V, V11I, V32I, L33F, I47V, I50V, I54L/M, G73S, L76V, I84V, L89V, V32I, I50V, I54L, I54 M, L76V, V82F	r _{CL} HIV _{T48}	0.17 (54)

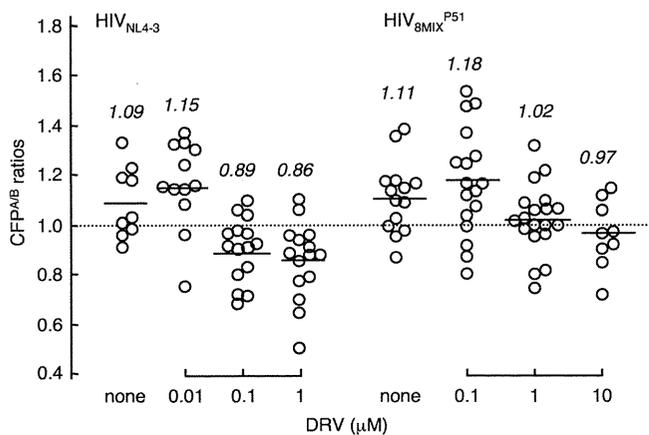


FIG. 6. DRV fails to inhibit the dimerization of the protease of a highly DRV-resistant HIV_{8MIX}^{P51} variant. COS7 cells were transfected with a pair of plasmids encoding a full-length molecular infectious HIV-1 clone (HIV_{8MIX}^{P51}) containing CFP- or YFP-tagged PR with 14 amino acid substitutions (L10I, I15V, K20R, L24I, V32I, L33F, M36I, M46L, I54M, L63P, K70Q, V82I, I84V, and L89M) in the presence or absence of 0.1, 1, or 10 μM DRV. On day 3 after transfection, CFP^{A/B} ratios were determined as described in the legend to Fig. 2. HIV_{NL4-3} served as a reference. Note that 0.1 and 1 μM DRV failed to block the dimerization of the protease of HIV_{8MIX}^{P51}, while the same concentration of DRV blocked protease dimerization in HIV_{NL4-3}. The differences between the CFP^{A/B} ratios in the absence of drug (CFP^{A/B}_{No Drug}) and the CFP^{A/B} ratios in the presence of 0.01 μM DRV (CFP^{A/B}_{0.01 DRV}), between the CFP^{A/B} ratios in the presence of 0.01 μM DRV (CFP^{A/B}_{0.01 DRV}) and 0.1 μM DRV (CFP^{A/B}_{0.1 DRV}), and between the CFP^{A/B} ratios in the presence of 0.1 μM DRV (CFP^{A/B}_{0.1 DRV}) and 1.0 μM DRV (CFP^{A/B}_{1.0 DRV}) had *P* values of 0.32, 0.0025, and 0.34 for HIV_{NL4-3}, respectively. The differences between the CFP^{A/B}_{No Drug} and the CFP^{A/B}_{0.1 DRV}, between the CFP^{A/B}_{0.1 DRV} and CFP^{A/B}_{1.0 DRV}, and between the CFP^{A/B}_{1.0 DRV} and the CFP^{A/B}_{10.0 DRV} had *P* values of 0.42, 0.022, and 0.26, respectively, for HIV_{8MIX}^{P51}.

^a Amino acid substitutions identified in the protease-encoding region of HIV are shown. The amino acid substitutions shown in the second row from the bottom were reported by De Meyer et al. (9) and Mitsuya et al. (33). Those in the bottom row were reported by Van Marck et al. (38) and were reported to have the greatest impact on HIV-1 resistance to DRV. Those for HIV_{8MIX}^{P51} were reported by Koh et al. (24).

^b Three infectious clones (r_{CL}HIV_{F16}, r_{CL}HIV_{T45}, and r_{CL}HIV_{T48}) were derived from clinical strains isolated from patients who failed to respond to DRV-containing regimens.

^c Shown are EC₅₀s of DRV for each infectious clone. Values in parentheses represent fold changes of EC₅₀s of DRV compared to EC₅₀s against a wild-type clinical strain, HIV-1_{ERS104pre}. All assays were conducted in triplicate, and the mean values are shown. The EC₅₀s of ritonavir and lopinavir against HIV_{8MIX}^{P51}, r_{CL}HIV_{F16}, r_{CL}HIV_{T45}, and r_{CL}HIV_{T48} were all >1 μM.

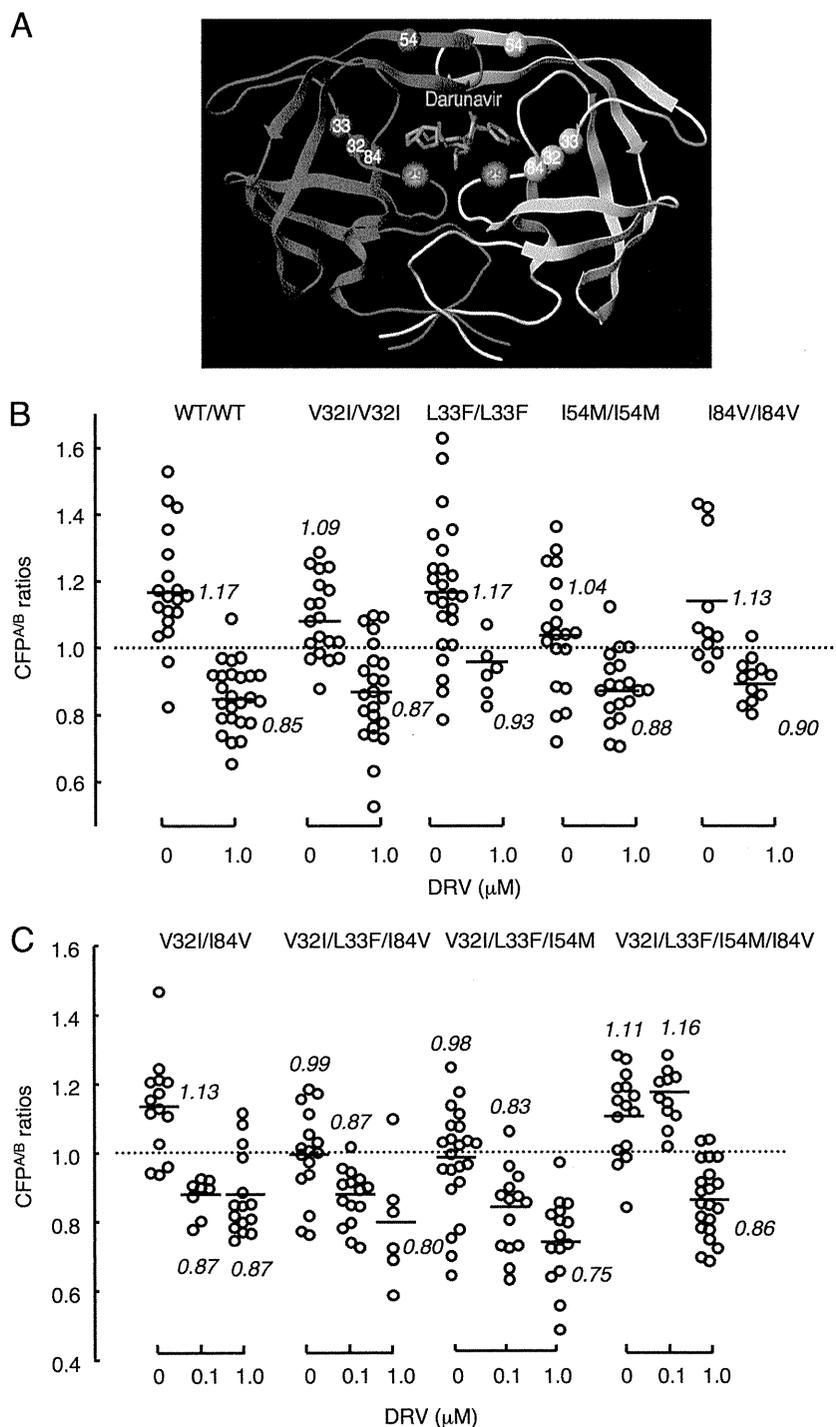


FIG. 7. Amino acid changes conferring DRV resistance on HIV. (A) Locations of amino acid substitutions V32I, L33A/F, I54M, and I84V associated with HIV's DRV resistance. The location of Asp29 (D29), which is known to be an essential amino acid for dimerization, is also shown. (B) Profiles of DRV's dimerization inhibition of PR carrying a single amino acid substitution. COS7 cells were cotransfected with a pair of HIV-PR^{CFP} and HIV-PR^{YFP} variants carrying wild-type PR or a single amino acid substitution such as V32I, L33F, I54M, or I84V, each of which was found to be associated with the development of HIV resistance to DRV, in the presence of 1 μ M DRV, further cultured, and the CFP^{A/B} ratios were determined. Note that none of the amino acid substitutions introduced blocked the dimerization of PR. The statistical evaluation of all the changes in the CFP^{A/B} ratios determined in the presence or absence of DRV, conducted using the nonparametric Mann-Whitney U test, showed *P* values ranging from 0.00000034 ($3.4E-07$) to 0.0026. (C) Profiles of DRV's dimerization inhibition of PR carrying combined amino acid substitutions. COS7 cells were cotransfected with a pair of HIV-PR^{CFP} and HIV-PR^{YFP} variants carrying combined amino acid substitutions such as V32I and I84V, V32I, L33F, and I84V, V32I, L33F, and I54M, or V32I, L33F, I54M, and I84V. The COS7 cells were further cultured in the continuous presence of 0, 0.1, and 1 μ M DRV, and the CFP^{A/B} ratios were determined at the conclusion of the 3-day period of culture. The differences between the CFP^{A/B} ratios in the absence of drug (CFP^{A/B}_{No Drug}) and the CFP^{A/B} ratios in the presence of 0.1 μ M DRV (CFP^{A/B}_{0.1 DRV}) and between the CFP^{A/B} ratios in the presence of 0.1 μ M DRV (CFP^{A/B}_{0.1 DRV}) and 1.0 μ M DRV (CFP^{A/B}_{1.0 DRV}) had *P* values of 0.0015 and 0.42 for V32I and I84V, 0.0047 and 0.15 for V32I, L33F, and I84V, 0.033 and 0.07 for V32I, L33F, and I54M, and 0.3 and 0.0000073 for V32I, L33F, I54M, and I84V, respectively.

TABLE 3. The four amino acid substitutions V32I, L33F, I54M, and I84V confer on HIV_{NL4-3} variants high-level resistance to DRV^a

Wild-type strain or recombinant HIV variant	Amino acid substitution(s) in PR	Mean EC ₅₀ ± SD (μM)	Fold resistance
HIV-1 _{NL4-3}	None (wild type)	0.0031 ± 0.0002	1
HIV-1 _{NL4-3} ^{V32I}	V32I	0.0022 ± 0.00006	0.07
HIV-1 _{NL4-3} ^{L33F}	L33F	0.0028 ± 0.0008	0.9
HIV-1 _{NL4-3} ^{I54M}	I54M	0.0026 ± 0.0001	0.8
HIV-1 _{NL4-3} ^{I84V}	I84V	0.0035 ± 0.0001	1
HIV-1 _{NL4-3} ^{V32I/I54V}	V32I, I54M	0.0017 ± 0.0002	0.5
HIV-1 _{NL4-3} ^{V32I/I84V}	V32I, I84V	0.00028 ± 0.00008	0.09
HIV-1 _{NL4-3} ^{V32I/L33F/I54V}	V32I, L33F, I54M	0.0019 ± 0.0006	0.6
HIV-1 _{NL4-3} ^{V32I/L33F/I84V}	V32I, L33F, I84V	0.0030 ± 0.0004	0.9
HIV-1 _{NL4-3} ^{V32I/L33F/I54M/V82A}	V32I, L33F, I54M, V82A		
HIV-1 _{NL4-3} ^{V32I/L33F/I54M/V82I}	V32I, L33F, I54M, V82I	0.034 ± 0.018	11
HIV-1 _{NL4-3} ^{V32I/L33F/I54V/I84V}	V32I, L33F, I54M, I84V	0.64 ± 0.02	205

^a The data shown represent mean values derived from the results of three independent experiments conducted in triplicate. The EC₅₀s were determined by employing MT-4 cells exposed to each infectious recombinant HIV-1 clone (50 TCID₅₀) and cultured in the presence of various concentrations of DRV, using the inhibition of p24 Gag protein production by DRV by 50% as an endpoint.

^b Note that there are no EC₅₀ or fold resistance values for HIV-1_{NL4-3}^{V32I/L33F/I54M/V82A} because this variant was replication incompetent.

with resistance ranging from 0.07- to 1.0-fold, as shown in Table 3. Introduction of the combinations of two or three amino acid substitutions did not increase the EC₅₀s of DRV against such clones either, with resistance ranging from 0.09- to 0.9-fold. However, when we introduced all four amino acid substitutions (V32I, L33F, I54M, and I84V), into HIV_{NL4-3}, generating HIV_{NL4-3}^{V32I/L33F/I54M/I84V}, the EC₅₀ of DRV against HIV_{NL4-3}^{V32I/L33F/I54M/I84V} was as high as 0.64 ± 0.02 μM with resistance of 205-fold (Table 3). These data suggested that the four amino acid substitutions are associated with the high-level resistance to DRV seen in HIV_{8MIX}^{P51}.

We next examined the effects of one of the four amino acid substitutions or subsets of them on PR dimerization inhibition by DRV using the FRET-based HIV expression assay. Figure 7B shows that each single mutation of the four mutations allowed PR to undergo dimerization, and 1.0 μM DRV effectively blocked the dimerization (*P* values ranging from 3.4E-07 to 0.0026). We next determined the effects of various combinations of the four amino acid mutations on dimerization inhibition by DRV (Fig. 7C). Two mutations such as V32I and I84V still allowed PR to undergo dimerization, and DRV effectively blocked the dimerization at 0.1 and 1 μM. When combinations of three amino acid substitutions such as V32I, L33F, and I84V and V32I, L33F, and I54M were introduced, the mean CFP^{A/B} ratios were close to 1.0, the threshold for indication of the occurrence of dimerization, with 0.99 and 0.98, respectively, in the absence of DRV, suggesting that possibly such groups of three substitutions somewhat compromised PR dimerization, although DRV still significantly blocked the dimerization at 0.1 and 1 μM, giving mean ratios ranging from 0.75 to 0.87. PR dimerization still occurred with all four substitutions, giving a mean CFP^{A/B} ratio of 1.11; however, 0.1 μM DRV clearly failed the dimerization, giving a ratio of 1.16 (*P* = 0.3), while 1.0 μM DRV blocked dimerization (*P* = 0.00000073) (Fig. 7C).

V82I, not V82A, contributes to the loss of DRV's activity to inhibit PR dimerization. Since V82A and V82I were seen in r_{CL}HIV_{T48} and HIV_{8MIX}^{P51}, respectively (Table 2), we examined whether V82A and V82I had effects on the loss of DRV's protease dimerization inhibition activity. Figure 8 shows that with V82A alone, PR dimerization occurred and 1 μM DRV

effectively blocked the dimerization. When V82A was combined with the three amino acid substitutions V32I, L33F, and I54M, PR dimerization still occurred, which DRV effectively blocked at both 0.1 and 1 μM, suggesting that V82A substitution plays no significant role in conferring on HIV reduced sensitivity to the PR dimerization inhibition by DRV. We also examined the effects of the V82I substitution, which was identified in HIV_{8MIX}^{P51}, with or without the three amino acid substitutions, on inhibition of PR dimerization by DRV. Interestingly, the four combined amino acid substitutions V32I, L33F, I54M, and V82I abrogated DRV's PR dimerization inhibition activity at

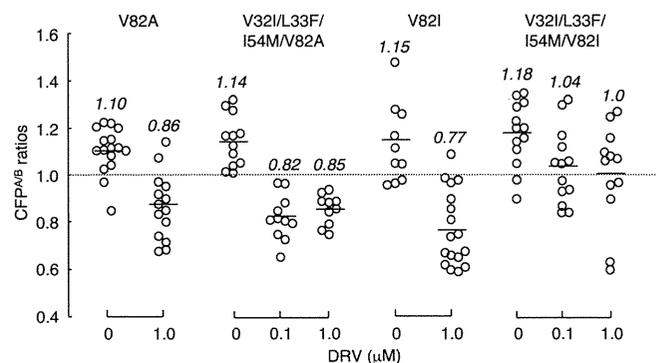


FIG. 8. Effects of V82A and V82I substitutions on DRV's activity to inhibit PR dimerization. COS7 cells were cotransfected with the pHIV-PR^{CFP} and pHIV-PR^{YFP} pair of plasmids carrying a single V82A or V82I substitution or four combined mutations (V32I, L33F, and I54M plus V82A or V82I). The COS7 cells were further cultured in the continuous presence of 0.1 and 1 μM DRV, and the CFP^{A/B} ratios were determined at the conclusion of the 3-day period of culture. Note that combined with other three substitutions V32I, L33F, and I54M, V82A did not have a significant effect on DRV's dimerization inhibition activity, while V82I compromised the dimerization inhibition of DRV at 0.1 and 1.0 μM. The differences between the CFP^{A/B} ratios in the absence of drug (CFP^{A/B}_{No DRV}) and the CFP^{A/B} ratios in the presence of 1.0 μM DRV (CFP^{A/B}_{1.0 DRV}) had *P* values of 0.00017 for V82A and 0.0027 for V82I. The differences between the CFP^{A/B} ratios in the absence of drug (CFP^{A/B}_{No DRV}) and the CFP^{A/B} ratios in the presence of 0.1 μM DRV (CFP^{A/B}_{0.1 DRV}) and between the CFP^{A/B} ratios in the presence of 0.1 μM DRV (CFP^{A/B}_{0.1 DRV}) and 1.0 μM DRV (CFP^{A/B}_{1.0 DRV}) were 0.000055 and 0.38 for V32I, L33F, I54M, and V82A and 0.026 and 0.91 for V32I, L33F, I54M, and V82I, respectively.

both 0.1 and 1.0 μM (Fig. 8), strongly suggesting that V82I with other three amino acid substitutions contributes to the loss of DRV's activity to inhibit PR dimerization.

DISCUSSION

In the FRET-based HIV expression assay we previously reported, YFP- or CFP-containing PR should be initially expressed as a part of Pr160^{gag-pol} polyprotein, and it was assumed that PDIs block the dimerization of PR monomer subunit within the polyprotein. In the present study, we examined whether one of the PDIs, DRV, blocked the dimerization of HIV PR expressed as a single protease molecule. As expected, DRV effectively blocked the dimerization (Fig. 2B), indicating that PDIs can block the dimerization of the PR monomers in the form of polyprotein as well as after autolysis (30, 31). The dimerization of two identical PR monomers is required for the acquisition of PR catalytic activity (28, 40), and the failure of PR dimerization should result in the loss of viral replication or compromised viral replication.

In our previous study (26) and in the present study, we demonstrated that a single amino acid substitution, such as I3A, L5A, R8Q, L24A, T26A, D29N, R87K, T96A, L97A, or F99A, effectively disrupts PR dimerization, as examined in the FRET-based HIV expression assay. In the present work, we constructed plasmids containing HIV_{NL4-3}-based recombinant clones with one of those amino acid substitutions and examined if such clones replicated in MT-4 cells. To this end, we attempted to propagate such recombinant clones in MT-4 cells using the supernatants obtained through transfection of 293T cells; however, all clones failed to replicate (Fig. 4B). In contrast, similarly generated recombinant clones containing a single amino acid substitution such as P1A, Q2A, T4A, D30N, and N98A (26), none of which disrupted PR dimerization, as examined with the FRET-based HIV expression assay (26), continued to replicate in MT-4 cells (Fig. 4B). These data further confirmed the validity of the results obtained in the FRET-based HIV expression assay.

It is of note that HIV-1 protease containing A28S has virtually no enzymatic activity, as previously published by Hong et al. (20), and as expected, recombinant HIV-1_{NL4-3} with A28S (HIV_{NL4-3}^{A28S}) was totally replication incompetent, as shown in Fig. 4B. The D25A substitution is also known to render HIV-1 protease virtually enzymatically inactive; however, that substitution does not disrupt the dimerization of D25A protease as we previously published (26). Yet, 1 μM DRV clearly blocks the dimerization of D25A protease (Fig. 3A), suggesting that D25 is not significantly associated with the putative DRV binding site in monomer subunit. These observations have led us to conclude that A28 is likely involved directly or indirectly in binding of DRV to the protease monomer subunit.

In recent clinical studies, a set of mutations, including V32I, L33F, I47V, I54L, and L89V has been identified in HIV strains isolated from patients failing DRV-containing regimens and has been associated with a diminished virological response to a regimen containing DRV boosted with ritonavir (DRV/r) (9, 33). More recently, Van Marck et al. reported that a set of amino acid substitutions (V32I, I50V, I54L/M, L76V, and V82F) in the PR-encoding region of HIV might be associated with the failure of HIV-1 infected individuals receiving DRV-

containing antiviral regimens (38). We have recently selected a highly DRV-resistant HIV variant (HIV_{8MIX}^{P51}) by using a mixture of eight highly multi-PI-resistant (but DRV-sensitive) clinical HIV strains, isolated from those who had received various PIs and failed to respond to PI (not DRV)-containing regimens, by propagating the mixture in the presence of increasing concentrations of DRV in phytohemagglutinin-activated peripheral blood mononuclear cells (PHA-PBMCs) followed by MT-4 cells. HIV_{8MIX}^{P51} proved to have an EC₅₀ of DRV \sim 333-fold greater than that against a wild-type HIV strain. HIV_{8MIX}^{P51} was highly resistant to amprenavir, indinavir, nelfinavir, ritonavir, lopinavir, and atazanavir (all EC₅₀s of >1 μM) and moderately resistant to saquinavir and TPV (EC₅₀s 33- and 18-fold greater, respectively) and replicated as rapidly as the wild-type HIV_{NL4-3} strain in the presence of 1 μM DRV (Table 1). The amino acid substitutions identified in HIV_{8MIX}^{P51} were L10I, I15V, K20R, L24I, V32I, L33F, M36I, M46L, I54M, L63P, K70Q, V82I, I84V, and L89M (24). When we determined the amino acid sequences of three highly DRV-resistant clinical HIV strains, _{rCL}HIV_{F16}, _{rCL}HIV_{T45}, and _{rCL}HIV_{T48}, which had EC₅₀s of DRV of 0.30 μM (97-fold), 0.33 μM (105-fold), and 0.33 μM (105-fold), respectively, we recognized that all three clinical isolates contained V32I, L33F, I54V, and I84V in common.

Thus, we hypothesized that PR dimerization inhibition by DRV might contribute to the antiviral activity of DRV and that the loss of PR dimerization inhibition by DRV might be associated with the decreased antiviral activity of DRV. When we introduced single, double, or triple amino acid substitutions of the four substitutions into pHIV-PR_{WT}^{CFP} and pHIV-PR_{WT}^{YFP} and cotransfected COS7 cells with such two plasmids, the dimerization of HIV-PR_{WT}^{CFP} and HIV-PR_{WT}^{YFP} clearly occurred in the absence of DRV, giving CFP^{A/B} ratios ranging from 1.04 to 1.17 (Fig. 7B). DRV at 1 μM effectively blocked the dimerization of the PR containing a single one of the four amino acid substitutions (Fig. 7B), giving average CFP^{A/B} ratios ranging from 0.88 to 0.93. DRV at 0.1 and 1.0 μM also blocked dimerization when two amino acid substitutions (V32I and I84V) and three substitutions (V32I, L33F, and I84V or V32I, L33F, and I54M) were introduced. However, when all four combined substitutions were introduced, DRV at 0.1 μM failed to block the dimerization (Fig. 7C), suggesting that the combination of the four substitutions compromised the activity of DRV to block the dimerization, presumably through altering the conformation of the monomer's putative binding site for DRV.

Considering that (i) the four amino acid substitutions in PR V32I, L33F, I54M, and I84V, were identified in the highly DRV-resistant HIV variant (HIV_{8MIX}^{P51}) and various DRV-resistant clinical HIV strains, such as _{rCL}HIV_{F16}, _{rCL}HIV_{T45}, and _{rCL}HIV_{T48}, isolated from individuals failing DRV-containing regimens (9, 33); that (ii) multi-PI-resistant, but DRV-sensitive HIV variants (24) do not contain the combination of the four substitutions; and (iii) that with the four substitutions combined, the activity of DRV to block PR dimerization is compromised, it is strongly suggested that the loss of PR dimerization activity is associated with the acquisition of resistance of HIV against DRV. More critically, the fact that the emergence of the four combined mutations is often seen in individuals failing DRV-containing regimens strongly suggests that

the protein dimerization inhibition activity of DRV is in operation for the drug to exert its anti-HIV activity in a clinical setting. Our finding that four combined amino acid substitutions are required for the loss of PR dimerization inhibition activity of DRV should explain at least in part the reason why DRV has a high level of genetic barrier to HIV acquisition of DRV resistance. It is noteworthy that the crystallographic data of HIV PR monomer complexed with DRV are as yet unavailable; however, if the results of such structural analysis are obtained, it should help our understanding of the dynamics of the dimerization process of HIV PR, the mechanisms of the activity of DRV and other PDIs to block the dimerization, and the mechanisms of the emergence of HIV resistance against PDIs.

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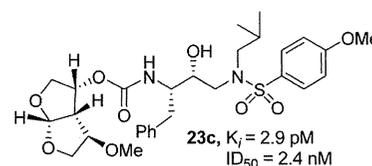
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Design, Synthesis, and X-ray Structure of Substituted Bis-tetrahydrofuran (Bis-THF)-Derived Potent HIV-1 Protease Inhibitors

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Supporting Information

ABSTRACT: We investigated substituted bis-THF-derived HIV-1 protease inhibitors in order to enhance ligand-binding site interactions in the HIV-1 protease active site. In this context, we have carried out convenient syntheses of optically active bis-THF and C4-substituted bis-THF ligands using a [2,3]-sigmatropic rearrangement as the key step. The synthesis provided convenient access to a number of substituted bis-THF derivatives. Incorporation of these ligands led to a series of potent HIV-1 protease inhibitors. Inhibitor 23c turned out to be the most potent ($K_i = 2.9$ pM; $IC_{50} = 2.4$ nM) among the inhibitors. An X-ray structure of 23c-bound HIV-1 protease showed extensive interactions of the inhibitor with the protease active site, including a unique water-mediated hydrogen bond to the Gly-48 amide NH in the S2 site.



KEYWORDS: HIV-1 protease inhibitors, Darunavir, bis-THF, drug-resistant, design, synthesis, X-ray structure

In our continuing studies aimed at the design and synthesis of novel HIV-1 protease inhibitors to combat drug resistance, we have developed a variety of exceedingly potent inhibitors.¹ One of these inhibitors, darunavir (**1**, $K_i = 16$ pM, antiviral $IC_{50} = 4.1$ nM, Figure 1) was first approved in June 2006 for the treatment of HIV/AIDS patients harboring drug-resistant HIV-1 variants.² Later, darunavir received an expanded approval for the treatment of all therapy-naïve HIV/AIDS patients, including pediatric patients.³ Darunavir incorporates a stereochemically defined (3*R*,3*aS*,6*aR*)-bis-tetrahydrofuran (bis-THF) urethane as the P2 ligand in a (*R*)-hydroxyethyl sulfonamide isostere.⁴ Structural studies of darunavir documented that the bis-THF ligand is involved in a network of hydrogen bonding interactions with the protein-backbone of the HIV-1 protease, as formulated in our "backbone binding" concept.⁵ A number of other clinical protease inhibitors (**2**, Breacanavir; **3**, GS-8374) have also incorporated this bis-THF ligand and demonstrated marked antiviral potency against a panel of multidrug-resistant HIV-1 variants.^{6,7}

In our continuing studies toward maximizing ligand-binding site interactions, based upon the X-ray structure of **1**-bound HIV-1 protease, we further speculated that the incorporation of an alkoxy substituent at the C4-position of bis-THF could lead to new interactions with the backbone NH of Gly-48. We therefore required a stereoselective synthesis that would provide access to C4-substituted bis-THF derivatives for further optimization. The bis-THF ligand contains three contiguous stereogenic centers. A

number of syntheses of the bis-THF ligand have been reported, including several optically active routes from our research group.^{8–12} Quaedflieg and co-workers reported a chiral synthesis that involved a conjugate addition of nitromethane.¹³ Other syntheses have been attempted with the use of various catalysts to obtain the desired product.¹⁴ Interestingly, many of these syntheses require late-stage resolution to obtain the enantiomerically pure ligand. Furthermore, the reported syntheses are not convenient for the preparation of C4-substituted derivatives. In view of this shortcoming, we investigated a new optically active synthesis utilizing an efficient [2,3]-sigmatropic rearrangement as the key step and an inexpensive chiral precursor as the starting material. The synthetic route provided convenient access to optically active bis-THF and functionalized bis-THF ligands. We incorporated these ligands in the hydroxyethylsulfonamide isostere, and the resulting HIV-1 protease inhibitors were evaluated in enzyme inhibitory and antiviral assays.

The synthesis of the bis-THF ligand started with known compound **4**.¹¹ It was prepared in multigram quantities by Wittig olefination of (*S*)-glyceraldehyde acetonide with (ethoxycarbonylmethylene) triphenylphosphorane, as described previously.¹¹ The requisite (*S*)-glyceraldehyde can be obtained from ascorbic

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