Supporting Information

Synthesis and Biological Activity of Potent HIV-1 Protease Inhibitors Based on Phe-Pro Dihydroxyethylene Isosters.

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Synthesis and characterization of the Inhibitors.

Inhibitor 17. Method A: 26 mg (12%) from AcNH-Val-Ile-COOH (39 mg, 0.14 mmol) and **3a** (49 mg, 0.14 mmol) first, followed by deprotection and reaction with AcNH-Ser-Leu-Asn-COOH (60 mg, 0.14 mmol). ES-MS *m/z* 861 $[MH]^+$.

Inhibitor 18a. Method B: 30 mg (40%) from AcNH-Trp-Val-OH (165 mg, 0.48 mmol) and **3a** (30 mg, 0.12 mmol). ES-MS *m/z* 906 $[MH]^+$.

Inhibitor 18b. Method C: 146 mg (49%) from dipeptide N-Ac-TrpVal-COOH (224 mg, 0.65 mmol) and Boc-deprotected diol **3b** (118.5 mg, 0.32 mmol). ES-MS *m/z* 832 $[MH]^+$.

Inhibitor 19. Method A: white solid; 128 mg (58%) from N-Ac-TrpVal-OH (125 mg, 0.28 mmol), DMPOA (50 mg, 0.28 mmol) and diol **3a** (98 mg, 0.28 mmol). ES-MS *m/z* 832.3 $[MH]^+$.

Inhibitor 20. Method B: 20 mg (39%) from phenoxyacetic acid (32 mg, 0.2 mmol) and **3a** (26 mg, 0.1 mmol). ^{1}H NMR ($CDCl_3$) δ: 1.85 (m, 3H), 2.15 (m, 1H), 2.74 (dd, 1H, *J* = 9, 14 Hz), 3.15 (m, 2H), 3.28 (m, 1H), 3.45 (m, 2H), 3.83 (d, 1H, *J* = 6 Hz), 4.14 (m, 2H), 4.30 (d, 1H, *J* = 15 Hz), 4.41 (d, 1H, *J* = 15 Hz), 4.67 (d, 1H, *J* = 15 Hz), 4.72 (d, 1H, *J* = 15 Hz), 4.89 (d, 1H, *J* = 4.5 Hz), 6.90–7.35 (m, 16H) ppm.^{13}C NMR ($CDCl_3$) δ: 23.0, 26.6, 37.0, 45.9, 52.1, 58.9, 65.9, 66.9, 70.0, 70.8, 114.5, 114.7, 121.2, 121.8, 126.3, 128.4, 129.3, 129.6, 129.8, 139.0, 157.6, 158.3, 169.1, 169.2 ppm. ES-MS *m/z* 519 $[MH]^+$.

Inhibitor 21. Method B: 35 mg (40%) from 2-methyl-phenoxyacetic acid (62 mg, 0.36 mmol), and Boc-deprotected diol **3a** (55.2 mg, 0.15 mmol). ES-MS *m/z* 547 $[MH]^+$.

Inhibitor 22. Method A: 53 mg form phenoxyacetic acid (23 mg, 0.14 mmol) and **3a** (49 mg, 0.14 mmol) followed by deprotection and reaction with DMPOA (25 mg, 0.14 mmol). ^{1}H NMR ($CDCl_3$) δ: 1.91 (m, 3H), 2.06 (s, 6H), 2.27 (m, 1H), 2.86 (dd, 1H, *J* = 8.8, 14.3 Hz), 3.15 (dd, 1H, *J* = 5.1, 9.1 Hz), 3.20 (dd, 1H, *J* = 6.9, 9.1 Hz), 3.32 (dd, 1H, *J* = 8.8, 14.3 Hz), 3.48 (m, 2H), 3.93 (d, 1H, *J* = 6.2 Hz), 4.09 (d, 1H, *J* = 15 Hz), 4.13 (d, 1H, *J* = 15 Hz), 4.36 (m, 2H), 4.63 (d, 1H, *J* = 15 Hz), 4.67 (d, 1H, *J* = 15 Hz), 4.83 (d, 1H, *J* = 4.7 Hz), 6.72 (d, 1H, *J* = 9.1 Hz), 6.97 (m, 7H), 7.25 (m, 7H) ppm.^{13}C NMR ($CDCl_3$) δ: 15.9, 16.1, 23.6, 26.9, 37.1, 46.7, 51.0, 59.0, 67.5, 69.9, 70.1, 70.9, 121.8, 124.8, 126.6, 128.6, 129.1, 129.1, 129.3, 129.4, 129.6, 129.7, 130.3, 137.7, 153.9, 157.6, 169.5, 169.8 ppm. ES-MS *m/z* 547 $[MH]^+$.

Inhibitor 23a. Method B: white solid; 64 mg (60%) from DMPOA (67.2 mg, 0.37 mmol) and Boc-deprotected diol **3a** (46.7 mg, 0.18 mmol). ^{1}H-NMR ($CDCl_3$) δ: 1.92 (m, 3H), 2.17 (s, 6H), 2.23 (m, 1H), 2.27 (s, 6H), 3.03 (dd, 1H, *J* = 8.8, 14.3 Hz), 3.25 (dd, 1H, *J* = 5.1, 9.1 Hz), 3.37 (dd, 1H, *J* = 6.9, 9.1 Hz), 3.40 (m, 3H), 3.50 (m, 1H), 3.93 (d, 1H *J* = 6.2 Hz), 4.10 (d, 1H, *J* = 15.4 Hz), 4.12 (d, 1H, *J* = 15.4 Hz), 4.20-4.39 (m, 6H), 4.54 (d, 1H, *J* = 4.7 Hz), 6.97 (m, 6H), 7.25 (m, 7H) ppm. ^{13}C-NMR ($CDCl_3$) δ: 16.3, 16.4, 23.7, 27.1, 38.8, 46.6, 52.6, 60.8, 68.6, 70.4, 70.5, 73.2, 124.6, 126.6, 128.5, 129.1, 129.6, 130.6, 130.7, 138.1, 154.4, 155.5, 169.1, 169.6 ppm. ES-MS *m/z* 575 $[MH]^+$.

Inhibitor 23b. Method D: white solid; 41 mg (46%) from DMPOA (65.3 mg, 0.36 mmol) and Boc-deprotected diol **3b** (55.2 mg, 0.15 mmol). ES-MS *m/z* 590 $[MH]^+$.

Inhibitor 24. Method B: 37 mg (55%) from 4-acetylphenoxyacetic acid (42 mg, 0.2 mmol) and **3a** (26 mg, 0.1 mmol). ^{1}H NMR ($CDCl_3$) δ: 1.93 (m, 3H), 2.25 (m, 1H), 2.54 (m, 7H), 3.01 (dd, 1H), 3.04 (dd, 1H), 3.20 (m, 1H), 3.24 (m, 1H), 3.45 (m, 2H), 4.03 (m, 1H), 4.28 (m, 2H), 4.43 (m, 2H), 4.64 (m, 2H), 6.72 (d, 1H), 6.85 (d, 2H), 6.91 (d, 2H), 7.15-7.25 (m, 5H), 7.90 (d, 2H), 7.93 (d, 2H) ppm.^{13}C NMR ($CDCl_3$) δ: 23.7, 26.5, 26.6, 27.0, 36.7, 46.7, 51.6, 59.6, 66.8, 67.0, 69.8, 70.1,

114.3, 114.4, 126.9, 128.8, 129.6, 130.7, 130.9, 131.2, 131.5, 137.1, 160.6, 161.6, 168.5, 168.6, 196.6, 196.8 ppm. ES-MS *m/z* 603 [MH]+.

Inhibitor 25. Method B: 64 mg (60%) from DMPOA (67 mg, 0.37 mmol) and **3c** (46 mg, 0.18 mmol). 1H NMR ($CDCl_3$) δ: 1.92 (m, 3H), 2.17 (s, 6H), 2.23 (m, 1H), 2.27 (s, 6H), 3.03 (dd, 1H, *J* = 8.8, 14.3 Hz), 3.25 (dd, 1H, *J* = 5.1, 9.1 Hz), 3.37 (dd, 1H, *J* = 6.9, 9.1 Hz), 3.40 (m, 3H), 3.50 (m, 1H), 3.93 (d, 1H, *J* = 6.2 Hz), 4.09 (d, 1H, *J* = 15 Hz), 4.13 (d, 1H, *J* = 15 Hz), 4.20-4.39 (m, 6H), 4.54 (d, 1H, *J* = 4.7 Hz), 6.97 (m, 6H), 7.25 (m, 7H) ppm. 13C-NMR ($CDCl_3$) δ: 16.3, 16.4, 23.7, 27.1, 38.8, 46.6, 52.6, 60.8, 68.6, 70.4, 70.5, 73.2, 124.6, 126.6, 128.5, 129.1, 129.6, 130.6, 130.7, 138.1, 154.4, 155.5, 169.1, 169.6 ppm. ES-MS *m/z* 575 [MH]+.

Inhibitor 26. Method B: colourless oil; 56.1 mg (67%) from DMPOA (57.4 mg, 0.32 mmol) and Boc-deprotected **5** (32 mg, 0.16 mmol). 1H-NMR ($CDCl_3$) δ: 1.94-1.98 (m, 2H), 2.24 (m, 7H), 2.36 (m, 1H), 3.25 (m, 1H), 3.43-3.49 (m, 2H), 4.35-4.45 (m, 3H), 4.93 (d, 1H, *J* = 4.4 Hz), 6.95-7.01 (m, 3H) ppm. ^{13}C-NMR ($CDCl_3$) δ: 16.4, 23.7, 27.2, 46.6, 59.3, 70.4, 70.9, 124.6, 129.1, 130.7, 155.6, 169.4 ppm. ES-MS *m/z* 525 [MH]+.

Recombinant HIV-Pr Inhibition Assay.

Solution A: 10 μl of a substrate stock solution in DMSO (10 mg/ml, 10.6 mM) were diluted in 1.99 ml 100 mM MES buffer, pH 5.5, containing 400 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mg/ml BSA, to a final concentration of 53mM.

Solution B: 10 μL of a protease stock solution (0.4 mg/ml) in 10 mM sodium phosphate buffer, pH = 6.5, containing 1 mM EDTA, 10% glycerol, 0.05% mercaptoethanol, 50 mM NaCl, were diluted 100 times with the MES dilution buffer, pH 5.5, to a final concentration of 0.004 mg/ml.

Assay: 114 μL of solution A, 11 μL of solution B and 75 μL of the MES dilution buffer were pre-incubated in a cuvette, at 25 °C. After 1 min, 2 μL of the inhibitor in DMSO or MES were added and the fluorence was recorded at 325 nm excitation and 420 nm emission. Final concentrations in the assay were 1.2 nM protease, 30 μM substrate and 0.5 nM – 5 mM inhibitor. IC_{50} were obtained by measuring the relative residual enzyme activity (ratio of the increase of fluorescence velocities before and after the addition of inhibitor) and by fitting the residual activity vs. inhibitor concentration semilog plots to a tetraparametric logistic function (Sigma plot 2001, SPSS Inc.). The experiments were run in triplicate: results are in Table S1.

Table S1. Average IC_{50} and standard errors for inhibitors **17-26**.[a]

Inhibitor	IC_{50} (nM)	Std. Error (nM)
17	0.6	0.1
18a	<0.6	--
18b	1.8	0.2
19	75	9
20	614	80
21	655	85
22	85	9
23a	9.6	2.1
23b	175	19
24	116000	21000
25	3300	415
26	290000	46000

[a]Atazanavir (IC_{50} = 20 pM) was used in this assay as reference inhibitor for titration of the active enzyme.

Cytotoxicity and anti-viral activity assays.

A stock solution of the inhibitor in ethanol (**18a**: 10 mM; **23a**: 100mM) was diluted in cell growth medium, without fetal serum added, to the concentrations required for the assays. All the assay solutions were freshly prepared. CC_{50} and MNC were detected by MTT–uptake assay Antiviral activity was studied only on MT-2 cells by micro titer infection assay exploring the protection of cells from the cytopathic effect of HIV measured by MTT test. All the compounds were prepared in 2x or 10x dilutions (one dilution/column of plate). The plates were incubated for 72-96 hours at 37 °C and 5% CO_2; MTT test was performed as described (Mossman, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65***,** 55-63) and absorbance of viable cells was measured colorimetrically at 540 nm (A_{540}). For all experiments, the mean value of each column was calculated. The CC_{50} of the test compound was defined as the concentration reducing the absorbance (A_{540}) of uninfected, inhibitor treated cells to 50% compared to the controls. Experiments under conditions of acute infection were performed in 96-well microplates with 6–8 parallels per experiment; each experiment was run in triplicate discarding replicates with standard deviations greater than ±10%. Cell controls (MT-2 cells with medium only) and viral controls (virus infected MT-2 cells) were run with every experiment. For anti-virus assays, HIV (undiluted or diluted to obtain multiplicity of infection approximately 0.1) was added to each well except the cell controls. Virus was allowed to attack the cells for an hour at 37 °C/5% CO_2. The mean values of experimental and control rows were compared and the percent of protected cells (cell survival) was plotted against the concentration of the inhibitor. The cell survival (% of cell protection) was calculated according to the following formula:

$$\% \text{ protection} = 100*(A_{540} - A_{540} \text{ Control HIV})/(A_{540} \text{ Cell Control} - A_{540} \text{ Control HIV})$$

where, A_{540} is the mean value of A_{540} of HIV-infected cells treated with a given concentration of the inhibitor ; A_{540} Control HIV is the mean value of A_{540} of inhibitor-untreated HIV-infected cells, and A_{540} Cell Control is the mean value of A_{540} of uninfected and untreated cells. ABC (Abacavir - well known NRTI) was used as reference in the assays (Harrigan, P. R.; Stone, C.; Griffin, P.; Najera, I.; Bloor, S.; Kemp, S.; Tisdale, M.; Larder, B. Resistance Profile of the Human Immunodeficiency Virus Type 1 Reverse Transcriptase Inhibitor Abacavir (1592U89) after Monotherapy and Combination Therapy. *J. Infect. Dis.* **2000**, *181*, 912–920).

Native protease inhibition assay

(Hinkov, A.; Atanasov, V.; Raleva, S.; Argirova, R. Modified Rapid Screening Method for Evaluation of HIV-1 Protease Inhibitors. *C. R. Acad. Bul. Sci.* **2010**, *63*, 1455-1462)**.**

A suspension of concentrated viral stock (50x) from chronically infected H9/HTLV IIIB cell supernatants was used as the source of native HIV-1 protease. The lysis of viral particles and release of the active protease was performed with a disrupting buffer containing 2.5% Triton X-100 in phosphate buffer. The tissue culture fluid containing the virus was concentrated by ultracentrifugation (Biofuge Stratos, Heraeus) for 1 h, at 4 ºC and 35.000 rev./min. The pellet was resuspended in disrupting buffer to obtain the required 50x concentration. For the test, 20 μL of a freshly prepared solution of HIV protease substrate III (1 μg/mL, 760 μM; Bachem, Switzerland) in DMSO were combined with 20 μL enzyme (stock HIV-20) taken from a solution containing 25 μL disrupting (lysis) buffer + 100 μl HIV-20, incubated 40 min at 37 °C prior to experiment and diluted with 1 mL phosphate buffer (20 mM, pH 6.0). The inhibitors were added to the substrate solution before the addition of enzyme, to final concentrations ranging from 0.1 pM to 1 μM. The HIV-1 protease activity was measured using direct spectrophotometric reading of the substrate utilization at 300nm.