Multivalent Tryptophan- and Tyrosine-Containing [60] Fullerene Hexa-Adducts as Dual HIV and Enterovirus A71 Entry Inhibitors

Marta Ruiz-Santaquiteria, Beatriz M. Illescas,* Rana Abdelnabi, Arnaud Boonen, Alberto Mills, Olaia Martí-Marí, Sam Noppen, Johan Neyts, Dominique Schols, Federico Gago, Ana San-Félix,* María-José Camarasa,* and Nazario Martín*
1. CHEMISTRY

1.1. General Chemistry procedures

All reagents and starting materials were purchased from Sigma-Aldrich and were used directly without previous purification steps. All solvents, including HPLC-grade and some dry ones, were purchased from ScharLab, Fisher, Sigma-Aldrich and Acros Organics. H2O was purified with a Progard TS2 Merck equipment with a Vent Filter MPK01. When dry solvents were employed, reactions were performed under Ar atmosphere. Column chromatography was performed with kiesegel 60, 230-240 mesh, Merck or Scharlau 60, 230-240 mesh, utilizing the medium pressure technique, or by Sephadex LH20 or G25 (GE Healthcare, Barcelona, Spain) gel filtration. TLC was performed on silica gel DC-Alufolien, kiesegel 60 F254 (Merck) with a thickness of 0.2 mm and with detection by UV light (λ = 254 nm) charring with potassium permanganate, phosphomolybdic acid, anisaldehide or cerium sulfate as development reagents.

The NMR instruments were Bruker Avance-300, Bruker AMX-500 and Bruker AMX-700 spectrometers. NMR chemical shifts are reported in ppm (d units) using the residual proton solvent peaks as internal standards (1H NMR experiments), or the characteristic resonances of the solvent nuclei (13C NMR experiments). Coupling constants (J) are reported in Hz and all experiments were performed at 298 K. Spectral assignments were made by routine one- and two-dimensional NMR experiments (1H, 13C, COSY, HSQC and DEPT) where appropriate. Deuterated solvents for NMR are indicated in each case. IR spectra (ν in cm⁻¹) were measured on a Bruker Tensor 27 instrument equipped with an ATR device, and using KBr for water-soluble compounds.
or dissolved in the proper solvent when possible. Copper analysis was carried out using a Varian ICP-MS.

1.2. Synthetic procedures and characterization of the compounds

Compounds 1\(^1\), 2\(^2\), 12\(^3\) and 15\(^4\) have been synthesized following previously reported procedures.

**Compound 3**

DBU (0.67 mL, 4.45 mmol) was added dropwise, under a nitrogen atmosphere, to a solution of C\(_{60}\) (0.16 g, 0.22 mmol), bismalonate derivative 2\(^2\) (0.99 g, 2.23 mmol) and CBr\(_4\) (7.41 g, 22.34 mmol) in anhydrous toluene (250 mL). The reaction mixture was stirred at room temperature for 3 days and the progress of the reaction was monitored by TLC. The residue was washed with a saturated solution of Na\(_2\)S\(_2\)O\(_3\) sat. (2 x 35 mL), HCl 1 N (2 x 40 mL), H\(_2\)O (2 x 40 mL) and brine (2 x 40 mL). The organic layer was dried over anhydrous MgSO\(_4\), filtered and evaporated. The residue was purified by column chromatography, using DCM to give 0.52 g (70%) of 3 as a red solid. \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\): 4.19-4.14 (m, 24H, 12CH\(_2\), COOC\(_2\)H\(_5\)), 2.29-2.12 (m, 24H, 12CH\(_2\), C\(_6\)H\(_5\)COOtBu), 1.90-1.27 (m, 180H, 36CH\(_2\), 36 CH\(_3\), C\(_6\)H\(_5\)CH\(_2\)CH\(_2\), COOC(CH\(_3\))\(_3\)); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\): 172.9, 163.8, 145.9, 141.1, 80.1, 69.1, 66.8, 45.4, 35.4, 28.3, 25.4, 24.7; IR \(\nu\) (cm\(^{-1}\)): 2972, 2867, 1721, 1363, 1215, 754, 532; HRMS (MALDI): m/z calcd for C\(_{198}\)H\(_{228}\)O\(_{48}\): 3373.5395 [M]+; found: 3373.5264.

**Compound 4**

Compound 3 (0.33 g, 98.19 mmol) was dissolved in a mixture of TFA/CH\(_2\)Cl\(_2\) 1:5 (15 mL) and the solution was stirred at room temperature overnight. Then, volatiles were evaporated to dryness and the residue was dissolved in methanol and treated with ethyl acetate to give 0.26 g of dodecaacid 4 in quantitative yield as a red solid. \(^1\)H NMR (300 MHz, DMSO-d\(_6\)) \(\delta\): 11.99 (s, 12H, COOH), 4.48-4.09 (m, 24H, 12CH\(_2\), COO(CH\(_3\))\(_2\)); 2.25-2.07 (m, 24H, 12CH\(_2\), CH\(_2\)COOH), 1.78-1.08 (m, 72H, CH\(_2\)CH\(_2\)CH\(_2\)); \(^{13}\)C NMR (75 MHz, DMSO-d\(_6\)) \(\delta\): 174.3, 162.8, 145.0, 140.8, 67.0, 33.5, 27.7, 25.0, 24.1, 21.1; IR \(\nu\) (cm\(^{-1}\)): 3397, 1647, 1289, 994, 821, 759, 612; HRMS (MALDI): m/z calcd for C\(_{150}\)H\(_{132}\)NaO\(_{48}\): 2723.7780 [M+Na]+; found: 2723.7745.

**General procedure for the synthesis of C\(_{60}\) Trp hexaadducts 5-7**

To a solution containing fullerene dodecaacid 4 in anhydrous DMF, HATU (1.2 eq for each carboxylic acid group), the corresponding amino acid (1.2 eq. for each carboxylic acid group) and diisopropylethylamine (DIPEA) (2.5 eq. for each carboxylic acid group) were added. The reaction mixture was stirred at 30°C during 2 days and then evaporated to dryness. The residue was purified by size-exclusion chromatography (Sephadex® LH-20) using dichloromethane/methanol (1:1) as eluent to give the corresponding fullerene derivative.

**Compound 5**

According to the general procedure, a solution of dodecaacid 4 (0.07 g, 0.03 mmol), HATU (0.14 g, 0.37 mmol), H-Trp-OBn·HCl (0.12 g, 0.37 mmol) and DIPEA (0.14 mL, 0.78 mmol) in anhydrous...
DMF (10 mL) gave 0.15 g (30%) of 5 as a red solid. $^1$H NMR (300 MHz, CDCl$_3$) δ: 8.86-8.56 (m, 12H, NH$_\text{indole}$), 7.49-7.06 (m, 108H, 48CH$_\text{indole}$, 60CH$_\text{Ar}$), 6.85-6.75 (m, 12H, CH$_\text{indole}$), 6.50-6.23 (m, 12H, CONH), 5.20-4.90 (m, 36H, CH$_2$-Bn, NHC$_2$HCO), 4.50-4.00 (m, 24H, COOC$_2$H$_2$), 3.35-3.20 (m, 24H, CH$_2$, CHC$_2$H$_2$), 2.30-1.95 (m, 24H, 12CH$_2$, C$_2$H$_2$CONH), 1.70-1.00 (m, 72H, 36CH$_2$, C$_2$H$_2$C$_2$H$_2$). $^{13}$C NMR (75 MHz, CDCl$_3$) δ: 173.0, 172.1, 163.9, 145.7, 141.4, 136.3, 135.3, 128.6, 128.4, 128.3, 122.1, 119.5, 118.4, 111.6, 109.5, 69.4, 67.2, 62.1, 50.7, 46.1, 36.0, 29.8, 27.6, 25.3, 24.9 ppm; IR ν (cm$^{-1}$): 3380, 3267, 2927, 1737, 1648, 1223, 741, 528; MS (MALDI) m/z: 6041.5 [M+Na]$^+$. 

**Compound 6**

According to the general procedure, a solution of dodecaacid 4 (0.13 g, 0.05 mmol), HATU (0.25 g, 0.67 mmol) and DIPEA (0.24 mL, 1.39 mmol) in anhydrous DMF (20 mL) gave 52 mg (22%) of 6 as a red solid. $^1$H NMR (500 MHz, CDCl$_3$) δ: 9.11-8.57 (m, 12H, NH$_\text{indole}$), 7.48 (t, $J$ = 8.1 Hz, 12H, CH$_\text{indole}$), 7.36-7.25 (m, 12H, CH$_\text{indole}$), 7.19-7.01 (m, 24H, CH$_\text{indole}$), 7.00-6.91 (m, 12H, CH$_\text{indole}$), 6.51-6.04 (m, 12H, CONH), 4.91 (s, 12H, NHC$_2$HCO), 4.27-4.00 (m, 24H, 12CH$_2$, COOC$_2$H$_2$), 3.73-3.60 (m, 36H, 12CH$_3$, COOC$_3$H$_3$), 3.37-3.17 (m, 24H, CH$_2$, CHC$_2$H$_2$), 2.20-1.95 (m, 24H, 12CH$_2$, C$_2$H$_2$CONH), 1.78-1.02 (m, 72H, 36CH$_2$, C$_2$H$_2$C$_2$H$_2$). $^{13}$C NMR (126 MHz, CDCl$_3$) δ: 172.8, 164.0, 162.5, 145.8, 141.4, 136.4, 127.7, 123.2, 122.2, 119.6, 118.5, 111.6, 109.8, 69.4, 67.3, 52.9, 52.5, 46.1, 36.1, 29.8, 27.7, 24.9; IR ν (cm$^{-1}$): 3390, 3310, 2948, 1734, 1654, 1221, 741, 526; MS (MALDI) m/z: 5127.9 [M+Na]$^+$. 

**Compound 7**

According to the general procedure, a solution of dodecaacid 4 (0.14 g, 0.05 mmol), HATU (0.29 g, 0.76 mmol) and DIPEA (0.27 mL, 1.58 mmol) in anhydrous DMF (20 mL) gave 40 mg (14%) of 7 as a red solid. $^1$H NMR (500 MHz, CDCl$_3$) δ: 7.43-7.27 (m, 60H, CH$_\text{Ar}$), 7.01 (d, $J$ = 8.2 Hz, 24H, CH$_\text{Ar}$), 6.87 (d, $J$ = 6.9 Hz, 24H, CH$_\text{Ar}$), 6.54-6.16 (m, 12H, CONH), 5.00 (s, 24H, OCH$_2$Ph), 4.87-4.67 (m, 12H, NHC$_2$HCO), 4.44-4.06 (m, 24H, COOC$_2$H$_2$), 3.75-3.59 (m, 36H, 12CH$_3$, COOC$_3$H$_3$), 3.12-2.88 (m, 24H, 12CH$_2$, C$_2$H$_2$CONH), 1.78-1.02 (m, 24H, 12CH$_2$, C$_2$H$_2$C$_2$H$_2$). $^{13}$C NMR (126 MHz, CDCl$_3$) δ: 172.8, 164.0, 162.5, 145.8, 141.4, 136.4, 127.7, 123.2, 122.2, 119.6, 118.5, 111.6, 109.8, 69.4, 67.3, 52.9, 52.5, 46.1, 36.1, 29.8, 27.7, 24.9; IR ν (cm$^{-1}$): 3390, 3310, 2948, 1734, 1654, 1221, 741, 526; MS (MALDI) m/z: 5127.9 [M+Na]$^+$. 

**Compound 8**

A solution of 7 (0.04 g, 0.01 mmol) in THF/methanol (1:1, 4 mL) containing 30 wt% of Pd/C (10%) was hydrogenated at 2.85 atm (42 psi) at room temperature overnight. The Pd/C was filtered through Celite®, washed with methanol and the solvent was removed under reduced pressure to afford 30 mg (quantitative yield) of the crude deprotected derivative 8 as a red solid. $^1$H NMR (300 MHz, CD$_2$OD) δ: 7.00 (d, $J$ = 7.9 Hz, 24H, CH$_\text{Ar}$), 6.69 (d, $J$ = 8.1 Hz, 24H, CH$_\text{Ar}$), 4.67-4.57 (m, 12H, NHC$_2$HCO), 4.34-4.15 (m, 24H, 12CH$_2$, COOC$_2$H$_2$), 3.65 (s, 36H, 12CH$_3$, COOC$_3$H$_3$), 3.10-2.75 (m, 24H, 12CH$_2$, C$_2$H$_2$CONH), 2.22-2.06 (m, 24H, 12CH$_2$, C$_2$H$_2$CONH). $^{13}$C NMR (75 MHz, CD$_2$OD) δ: 175.7, 173.8, 164.9, 157.4, 146.8, 142.7, 131.3, 128.8, 116.3, 70.7, 61.9, 55.4, 52.7, 47.7, 37.8, 36.5, 29.3, 26.4, 26.3.
Compound 9

To a solution containing the methyl ester derivative 6 (0.04 g, 0.01 mmol) in THF/H$_2$O (1:2, 3 mL) at 0 °C, LiOH·H$_2$O (0.01 g, 0.20 mmol) was added, and the mixture was stirred at room temperature for 5 h. Then, 1N hydrochloric acid aqueous solution was added to reach pH = 2 and volatiles were evaporated to dryness. The residue was dissolved in DMSO and purified after centrifugation with AcOEt to obtain 33 mg of 9 (81%) as a red solid. $^1$H NMR (500 MHz, DMSO-$d_6$) δ: 12.46 (brs, 12H, COOH), 10.80 (s, 12H, NH$_{indole}$), 8.05 (d, J = 3.0 Hz, 12H, CONH), 7.52 (d, J = 7.1 Hz, 12H, CH$_{indole}$), 7.31 (d, J = 7.0 Hz, 12H, CH$_{indole}$), 7.11 (s, 12H, CH$_{indole}$), 7.02 (d, J = 6.9 Hz, 12H, CH$_{indole}$), 6.96 (d, J = 5.5 Hz, 12H, CH$_{indole}$), 4.49 (s, 12H, NHCHCO), 4.32-4.05 (m, 24H, 12CH$_2$, COOCH$_2$), 3.20-2.95 (m, 24H, CH$_2$, CHCH$_2$), 2.21-1.93 (m, 24H, 12CH$_2$, CH$_2$CONH), 1.66-1.03 (m, 72H, 36CH$_2$, CH$_2$CONH); $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ: 173.6, 172.1, 162.8, 145.0, 140.7, 136.1, 127.2, 123.5, 120.9, 118.3, 118.2, 111.3, 110.0, 68.7, 60.6, 52.9, 45.6, 34.9, 27.6, 27.1, 24.7, 24.0; IR ν (cm$^{-1}$): 3370, 2920, 2860, 1730, 1260, 1220, 741, 527.

Compound 10

To a solution containing the methyl ester derivative 8 (0.02 g, 4.6·10$^{-3}$ mmol) in THF/H$_2$O (1:2, 3 mL) at 0 °C, LiOH·H$_2$O (0.01 g, 0.23 mmol) was added and the mixture was stirred at room temperature for 5 h. Then, 1N hydrochloric acid aqueous solution was added to reach pH = 2 and volatiles were evaporated to dryness. The residue was dissolved in DMSO and purified after centrifugation with AcOEt to obtain 18 mg, (84%) of 10 as a red solid. $^1$H NMR (500 MHz, DMSO-$d_6$) δ: 12.48 (brs, 12H, COOH), 9.18 (brs, 12H, PhOH), 8.02 (s, 12H, CONH), 6.99 (d, J = 4.8 Hz, 24H, CH$_x$), 6.64 (d, J = 5.6 Hz, 24H, CH$_x$), 4.34 (s, 12H, NHCHCO), 4.28-4.17 (m, 24H, 12CH$_2$, COOCH$_2$), 2.94-2.67 (m, 24H, 12CH$_2$, CHCH$_2$), 2.09-1.97 (m, 24H, 12CH$_2$, CH$_2$CONH) 1.65-1.11 (m, 72H, 36CH$_2$, CH$_2$CH$_2$CH$_2$); $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ: 173.3, 172.0, 162.8, 155.9, 144.7, 141.1, 130.0, 127.7, 114.9, 68.6, 60.6, 53.7, 46.8, 36.1, 34.8, 27.6, 24.7, 24.0.

Synthesis of the azide precursors 13 and 14

Scheme S1
Benzyl 2-[2-(2-azidoethoxy)ethoxy]acetamido-3-(1H-indol-3-yl)propanoate (13)

To a solution containing 12₃ (0.21 g, 1.13 mmol) in anhydrous DMF (20 mL), HATU (0.52 g, 1.36 mmol), H-Trp-OBn·HCl (0.45 g, 1.36 mmol) and DIPEA (0.49 mL, 2.83 mmol) were added. The reaction mixture was stirred at 30 °C during 2 days and then evaporated to dryness. The residue was purified by silica gel-column chromatography (CH₂Cl₂/MeOH: 9.5:0.5) to give 0.22 g (43%) of 13 as a yellow oil. ¹H NMR (300 MHz, CDCl₃) δ: 8.71 (s, 1H, NHindole), 8.00 (NHCO), 7.54 (d, J = 7.8 Hz, 1H, CHAr), 7.36-7.04 (m, 8H, CHAr), 6.84 (s, 1H, CHAr), 5.09 (s, 2H, Ph-CH₂-COO), 5.05-4.96 (m, 2H, CH-COO), 3.96 (s, 2H, OCH₂CONH), 3.52-3.38 (m, 6H, 3CH₂), 3.34 (d, J = 5.8 Hz, 2H, CH₂-CHCOO), 3.26-3.19 (m, 2H, CH₂). ¹³C NMR (75 MHz, CDCl₃) δ: 171.5, 169.8, 136.2, 135.3, 128.6, 128.4, 127.6, 123.1, 122.0, 119.5, 118.6, 111.3, 109.6, 70.9, 70.5, 70.1, 69.9, 67.2, 52.5, 50.5, 27.7.

Methyl (2-(2-(2-azidoethoxy)ethoxy)acetyl)-L-tryptophanate (14)

To a solution containing 12₃ (0.14 g, 0.72 mmol) in anhydrous DMF (20 mL), HATU (0.33 g, 0.87 mmol), H-Trp-OMe·HCl (0.22 g, 0.87 mmol) and DIPEA (0.31 mL, 1.81 mmol) were added. The reaction mixture was stirred at 30°C for 2 days and then evaporated to dryness. The residue was purified by silica gel-column chromatography in dichloromethane to give 0.09 g (32%) of 14 as a yellow oil. ¹H NMR (300 MHz, CDCl₃) δ: 8.38 (s, 1H, NHindole), 7.55 (d, J = 7.8 Hz, 1H, CHAr), 7.24-7.05 (m, 3H, 2CHAr, CONH), 7.02 (d, J = 2.1 Hz, 1H, CHAr), 4.97 (dt, J = 8.2, 5.8 Hz, 1H, HNC=CO), 3.98 (s, 2H, OC₂H₃CONH), 3.69 (s, 3H, COOC₂H₃), 3.61-3.42 (m, 6H, C₂H₂O(C₂H₅)₂OC₂H₅), 3.34 (d, J = 5.7 Hz, 2H, CH₂CH₂), 3.30-3.19 (m, 2H, OCH₂C₂H₅). ¹³C NMR (75 MHz, CDCl₃) δ: 172.1, 169.9, 136.2, 127.5, 123.1, 122.0, 119.4, 118.4, 111.4, 118.6, 111.3, 110.6, 70.9, 70.4, 70.1, 69.9, 52.4, 50.5, 38.7, 27.7; IR ν (cm⁻¹): 3390, 3322, 2922, 2102, 1739, 1660, 1104, 741, 557; HRMS (ESI): m/z calcd for C₁₈H₂₄N₅O₅: 390.1777 [M+H]+; found: 390.1764.

General procedure for CuAAC reaction to obtain compounds 16 and 17.

A round vessel that contains the corresponding asymmetric hexakis-adduct of [60]fullerene 15 (1 eq.), azide 13 or 14 (10 eq.), CuBr·S(CH₃)₂ (5 eq.) and sodium ascorbate (15 eq.) in DMSO (2 mL), in the presence of a metallic copper wire, was deoxygenated under vigorous stirring for three minutes and maintained at room temperature for 48 h. After that, Quadrasil MP was added and the mixture was stirred for 15 minutes and filtered. The reaction crude was purified by size-exclusion chromatography (Sephadex® LH-20) using dichloromethane/methanol (1:1) as eluent.

Compound 16

Alkyne-functionalized hexakis-adduct 15₄ (0.04 g, 0.02 mmol) was dissolved in DMSO (2 mL) and azide 13 (0.14 g, 0.29 mmol) was added under an argon atmosphere. Then CuBr·S(CH₃)₂ (0.02 g, 0.10 mmol), sodium ascorbate (0.06 g, 0.19 mmol) and a piece of Cu metal wire were added in this order. The mixture was vigorously stirred over a period of 72 h. A metal scavenger resin, quadrasil® MP, was added and the mixture was stirred for 15 min and filtered. The resulting solution was purified by size-exclusion chromatography (Sephadex® LH-20) using dichloromethane/methanol (1:1) as eluent to give 0.12 g of 16 (84%) as a red solid. ¹H NMR (300 MHz, CDCl₃) δ: 9.91-8.80 (m, 12H, NHindole), 7.56-7.35 (m, 12H, CHAr), 7.42-6.94 (m, 120H, CHAr), 7.36-7.04 (m, 8H, CHAr), 6.84 (s, 1H, CHAr), 5.09 (s, 2H, Ph-CH₂-COO), 5.05-4.96 (m, 2H, CH-COO), 3.96 (s, 2H, OCH₂CONH), 3.52-3.38 (m, 6H, 3CH₂), 3.34 (d, J = 5.8 Hz, 2H, CH₂-CHCOO), 3.26-3.19 (m, 2H, CH₂).
96CHAr, 12CH, 12NHamide), 6.82 (s, 12H, 12CHAr), 5.28-5.02 (m, 24H), 5.02-4.85 (m, 12H), 4.34-
4.04 (m, 40H), 3.97-3.78 (m, 24H), 3.56-3.15 (m, 102H), 2.90-2.50 (m, 26H), 2.14-1.80 (m, 24H);
13C NMR (75 MHz, CDCl3) δ: 171.6, 169.8, 163.8, 146.3, 145.9, 141.3, 136.4, 135.3, 128.7, 128.5,
128.5, 127.7, 123.4, 123.3, 122.5, 122.4, 122.1, 119.5, 118.6, 111.6, 109.3, 71.0, 70.5, 70.1, 69.5,
69.3, 67.4, 53.6, 52.5, 50.0, 45.7, 29.8, 28.1, 27.6; ICP-MS (weight percentage): 0.01% Cu (100 ppm).

Compound 17
Alkyne-functionalized hexakis-adduct 15 (0.04 g, 0.02 mmol) was dissolved in DMSO (2 mL) and
azide 14 (0.11 g, 0.29 mmol) was added under argon atmosphere. Then CuBr·SMe2 (0.02 g, 0.10
mmol), sodium ascorbate (0.06 g, 0.19 mmol) and a piece of Cu metal wire were added in this
order. The mixture was vigorously stirred over a period of 72 h. A metal scavenger resin, quadrasil® MP, was added and the mixture was stirred for 15 min and filtered. The resulting
solution was purified by size-exclusion chromatography (Sephadex® LH-20) using
dichloromethane/methanol (1:1) as eluent to give 0.10 g of 17 (80%) as a red solid. 1H NMR (700
MHz, DMSO-d6) δ: 11.34-10.70 (m, 12H, NHindole), 7.90-7.66 (m, 24H, CONH, CHtriazole), 7.45 (s,
12H, CHindole), 7.33 (s, 12H, CHindole), 7.12 (s, 12H, CHindole), 7.04 (s, 12H, CHindole), 6.96 (s, 12H,
CHindole), 4.61 (s, 12H, CONHC2H), 4.52-4.17 (m, 48H, 24CH2, COOC2H2, NC2H2), 3.88-3.77 (m,
24H, 12CH2, OC2H2CO), 3.75-3.65 (m, 24H, 12CH2, NCH2C2H2O), 3.64-3.54 (m, 36H, 12CH3,
OCH3), 3.50-3.30 (m, 48H, 24CH2, OC2H2C2H2O), 3.24-3.09 (m, 24H, 12CH2, CHC2H2), 2.72-2.55
(m, 24H, 12CH2, OCH2CH2C2H2), 2.06-1.77 (m, 24H, 12CH2, OCH2C2H2CH2). 13C NMR (176 MHz,
DMSO-d6) δ: 172.0, 169.3, 162.8, 145.5, 145.1, 140.8, 136.1, 127.1, 123.8, 122.2, 121.0, 118.4,
118.0, 111.5, 109.1, 70.0, 69.6, 69.2, 68.7, 66.6, 60.1, 52.4, 52.0, 49.1, 45.6, 32.3, 27.7, 26.8; IR ν (cm-1): 3607, 3557, 3510, 3481, 3399, 3276, 3255, 2922, 2855, 1741, 1666, 1524, 1439, 1344,
1256, 1217, 1111, 1060, 1011, 744; MS (MALDI) m/z: 6799.0 [M]+; ICP-MS (weight percentage): 0.01% Cu (100 ppm).

Compound 18
To a solution of 17 (0.02 g, 3.6·10-3 mmol) in THF (1 mL) at 0 ºC, a solution of LiOH·H2O (3.6 mg,
0.09 mmol) in water (2 mL) was added, and the mixture was stirred at room temperature for 4 h.
Then, 1N hydrochloric acid aqueous solution was added to reach pH = 2, and volatiles were
evaporated to dryness. The residue was dissolved in DMSO and purified after centrifugation with
AcOEt to give 20 mg of 18 (82%) as a red solid. 1H NMR (700 MHz, DMSO-d6) δ: 12.81 (brs, 12H,
COOH), 10.86 (s, 12 H, NHindole), 7.91-7.65 (m, 24H, CONH), 7.61 (d, J = 7.2 Hz, 12H, CHtriazole),
7.50 (d, J = 7.3 Hz, 12H, CHindole), 7.31 (d, J = 7.2 Hz, 12H, CHindole), 7.11 (s, 12 H, CHindole), 7.04
(s, 12H, CHindole), 6.95 (s, 12H, CHindole), 4.56 (q, J = 7.1 Hz, 12H, NHCHO), 4.44-4.28 (m, 48H,
24CH2, COOC2H2, NCH2), 3.85-3.78 (m, 24H, 12CH2, OCH2CO), 3.67 (s, 24H, 12CH2, NCH2CH2O), 3.61-3.34 (m, 48H, 24CH2, OCH2CH2O), 3.24-3.10 (m, 24H, 12CH2, CHCH2), 2.61
(s, 24H, 12CH2, OCH2CH2CH2), 1.91 (s, 24H, 12CH2, OCH2CH2CH2). 13C NMR (176 MHz, DMSO-
d6) δ: 173.1, 171.7, 169.1, 145.5, 145.1, 140.7, 136.1, 127.3, 123.7, 122.3, 121.0, 118.5, 118.2,
111.4, 109.5, 70.0, 69.7, 69.5, 69.2, 68.7, 52.3, 49.1, 45.5, 27.7, 26.8, 21.3; IR ν (cm-1): 3399, 3255,
2922, 1741, 1666, 1217, 1111, 744. MS (MALDI) m/z: 6799.0 [M]+; ICP-MS (weight percentage): 0.01% Cu (100 ppm).
1.3. $^1$H NMR and $^{13}$C NMR of selected compounds

Compound 9
Compound 10
Compound 18
2. BIOLOGICAL ASSAYS

2.1. Antiviral Activity against HIV

The MT-4 cells used for the anti-HIV assays was a kind gift from Dr. L. Montagnier (formerly at the Pasteur Institute, Paris, France) and cultured in RPMI-1640 medium (Invitrogen, Merelbeke, Belgium) supplemented with 10% Fetal Calf Serum (FCS) (Hyclone, Perbio Science, Aalst, Belgium) and 1% L-glutamine (Invitrogen).

The HIV-1 strain NL4-3 was obtained from the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH) and cultured in MT-4 cells. The virus stock was stored at -80°C.

The compounds were evaluated for their inhibitory activity against HIV-1 (NL4.3) and HIV-2 (ROD) infection in MT-4 cell cultures as have been described in detail earlier. Briefly, MT-4 cells (50 µL, 1 x 10^6 cells/mL) were pre-incubated for 30 min at 37°C with the test compounds (100 µL) in a 96-well plate. Next, the cell-line adapted HIV strains (NL4.3 and ROD) were added according to the TCID_{50} (50% Tissue Culture Infectious Dose) of the viral stock. After 5 days, cytopathic effect (CPE) was scored microscopically and the anti-HIV-1 activity (50% effective concentration, EC_{50}) was scored microscopically and the anti-HIV-1 activity (50% effective concentration, EC_{50}) of each compound were calculated using a colorimetric method based in the in situ reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) according to the manufacturer’s instructions (Promega, Leiden, The Netherlands). Assays are performed by adding a small amount of a solution that contains the tetrazolium compound MTS and an electron coupling reagent (phenazine ethosulfate; PES), directly to culture wells, incubating for 1–4 hours and then recording absorbance at 490 nm (A_{490}) with a 96-well plate reader. The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture. Cytotoxicity in MT-4 cells was measured after 5 days using the MTS/PES method. Data are the mean ± S.D. of at least 3 independent experiments.
2.2. Surface plasmon resonance (SPR) analysis (HIV)

SPR technology was used to determine the binding of 18 and Pradimicin A (PRM-A) (as positive control) to gp120, which was immobilized on a CM5 chip in a Biacore T200 instrument (GE Healthcare, Uppsala, Sweden). The gp120 immobilization was performed using a standard amine coupling procedure. Briefly, the surface was activated using a 7-minute injection of EDC/NHS 1:1. Following this, recombinant gp120 (ImmunoDx, Woburn, Massachusetts), diluted in pH 4 acetate immobilization buffer to 10 µg/ml, was injected over the surface for 420 seconds resulting in a response of ± 1400 RU. Afterwards, the surface was deactivated by injecting 1.0 M ethanolamine-HCl pH 8.5 for 7 minutes. Finally, the surface was regenerated with 12-second injections of NaOH 50 mM and HCl pH 1.5 at 30 µl/min to remove any unbound ligand. All injections were performed with an injection speed of 5 µl/min excluding the surface regeneration step (30 µl/min). Interaction studies were performed at 25 °C in HBS-P+ (10 mM HEPES, 150 mM NaCl, 0.05% surfactant P20; pH 7.4) supplemented with 5% DMSO. For PRM-A, 1mM of CaCl₂ was added to the running buffer. All compounds were diluted in 2-fold dilution steps with 18 ranging from 6.25 to 0.1 µM and PRM-A ranging from 50 to 3.125 µM. The analytes were injected, using multiple cycle kinetics, for 2 minutes at a flow rate of 30 µl/min, and the dissociation was measured for 4 minutes. A 12-second injection of 50 mM NaOH was used to regenerate the surface. Several buffer blanks were included for double referencing. Apparent binding kinetics (K_D, k_a, k_d) were derived after fitting the experimental data to the 1:1 Langmuir binding model in the Biacore T200 Evaluation Software 3.1. A solvent correction was applied to compensate for the DMSO bulk effects. The experiments were performed in quadruplicates.

2.3. Antiviral Activity against EV71

2.3.1. Cells and viruses

Human rhabdomyosarcoma (RD) cells were cultured in DMEM supplemented with 10 % heated-inactivated fetal bovine serum (FBS). EV71 BrCr was a kind gift from Prof. F. van Kuppeveld (University of Utrecht, The Netherlands), and EV71 B2-11316-86 from the National Institute for Public Health and the Environment (RIVM, The Netherlands). Both viruses were propagated on RD cells in 2 % FBS-DMEM.

2.3.2. Anti-EV71 assay laboratory strain

EV71 BrCr laboratory adapted strain and the clinical isolate representative of B genogroup (B2-11316-86) were used at a low multiplicity of infection (MOI = 0.1) in a standardized antiviral assay. Briefly, rhabdosarcoma (RD) cells (2 x 10⁴ cells/well) were seeded in a 96 well-plate. The day after, a serial dilution of the compounds and the virus inoculum were added to the cells. The assay plates were incubated at 37°C, 5% CO₂ with virus inoculum and compounds until full virus–induced cell death was observed in the untreated, infected controls (3 days post-infection). Subsequently, the antiviral effect was quantified using a colorimetric readout with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine methosulfate (MTS/PMS method) and the concentration of compound at which 50% inhibition of virus-induced cell death was observed (EC50), was calculated from the antiviral dose-response curves. A similar assay setup was used to determine the adverse effect of the compound on uninfected, treated cells for calculation of the CC50 (concentration of compound that reduces overall cell health with 50% as determined by the MTS/PMS method). Selectivity index (SI) was
calculated as the ratio of CC<sub>50</sub> to EC<sub>50</sub>. Data are the mean ± S.D. of at least 3 independent experiments.

All viruses were obtained and used as approved according to the rules of Belgian equivalent of IRB (Departement Leefmilieu, Natuur en Energie, protocol SBB 219 2011/0011, and the Biosafety Committee at the KU Leuven).

3. COMPUTER-ASSISTED MOLECULAR MODELING

In silico model building of ligands and HIV-1 Env glycoproteins

The molecular graphics program PyMOL<sup>7</sup> was employed in the construction of the initial molecular models, trajectory visualization, and 3D figure preparation.<sup>18</sup> was model-built using as a template the x-ray crystal structure of the [60]fullerene dodecaacid derivative<sup>8</sup> deposited in the Cambridge Structural Database<sup>9</sup> with CSD identifier ADOVUN. Geometry optimization and derivation of atom-centered charges for suitably capped spacers and the terminal Trp residues were carried out with the default AM1-BCC option<sup>10</sup> implemented in the sqm program<sup>11</sup> that is distributed with AmberTools20 (http://ambermd.org/AmberTools.php). The gaff<sup>12</sup> and ff14SB<sup>13</sup> force field parameters in AMBER18 (http://ambermd.org) were used for ligands and proteins, respectively.

The structure of the HIV-1 envelope protein (Env, UniProt code Q2N0S6), consisting of a trimer of fully cleaved gp120 and gp41 subunits, was taken from the PG16-Env complex deposited in the Protein Data Bank with accession code 6ULC.<sup>14</sup> Disulfide bonds were explicitly defined for the following cysteine pairs: 54-74, 119-205, 126-196, 131-157, 218-247, 228-239, 296-331, 378-445, 385-418, and 598-604. Replacing the standard ASN name for asparagine with the N-linked NLN residue in AMBER allowed attachment (i) of Man<sub>3</sub>GlcNAc<sub>2</sub> at glycosylation positions 133, 156, 160 and 197, and Man<sub>1</sub>GlcNAc<sub>2</sub> at 262, 295, 301, 332 and 386 in gp120; and (ii) GlcNAc<sub>2</sub> at 611, 625 and 637 in gp41. This can be considered a representative (and minimal) consensus glycosylation pattern<sup>14, 15</sup> because of the existence of an “evolving glycanshield”<sup>16</sup> and the high diversity among HIV-1 strains and isolates regarding glycosylation positions, composition, extent and processing (“trimming”).<sup>17</sup> The Carbohydrate and Glycoprotein Builders publicly available at the GLYCAM-Web server (https://dev.glycam.org/) were used for generation of free Man<sub>9</sub>GlcNAc<sub>2</sub>-OMe and interactive protein glycosylation, respectively. The AMBER-compatible GLYCAM06 force field<sup>18</sup> was used for these oligosaccharide moieties.

For completion, the membrane-proximal external region (MPER) and the transmembrane (TM) segments of gp41 (residues 664–683) were taken from PDB entry 6E8W<sup>19</sup> and embedded in a cholesterol-rich lipid bilayer using the CHARMM-GUI Membrane Builder (http://www.charmmgui.org/?doc=input/membrane.bilayer) pipeline.<sup>20</sup> The outer layer consisted of 54 sphingomyelin (PSM, 18:1/16:0), 26 dipalmitoylphosphatidylcholine (DPPC, 16:0/16:0), and 90 cholesterol molecules; the inner layer was composed of 25 dipalmitoylphosphoserine (DPPS, 16:0/16:0) and 112 cholesterol molecules, interspersed with 44 units of the plasmalogens or ether lipid DPPE-E (16:0/16:0), in accord with data from the HIV lipidome.<sup>21</sup> The AMBER lipid17 and lipid17_ext force fields<sup>22, 23</sup> were used for lipids already in the database; consistent parameters and point charges were derived for PSM and DPPE-E fragments using antechamber in AMBER18. The central positioning of the TM segment with respect to the membrane normal was unbiasedly performed by the PPM utility implemented in the Orientations of Proteins in Membranes (OPM) database (http://opm.phar.umich.edu).<sup>24</sup> A 10-Å thick layer of TIP3P water (~21,400 molecules in all) was then placed along the Z axis on both sides of the membrane to solvate the protein ends and the
lipid head groups. Finally, the bulk ion concentration was set at 0.15 M with potassium and chloride ions.

**Molecular dynamics simulations**

For conformational sampling of the full dendrimer, 18 was immersed in a truncated octahedral box of 21,523 TIP3P water molecules that also contained 50 sodium and 38 chloride ions in locations corresponding to minima and maxima of the calculated molecular electrostatic potential. The same procedure was then used for one molecule of 18 in the presence of two molecules of Man9GlcNAc2-OMe, in which case there were 40 Na⁺, 28 Cl⁻ and 16,230 TIP3P water molecules in the truncated octahedron subjected to periodic boundary conditions. System coordinates were first relaxed by performing 25,000 steps of steepest descent followed by 100,000 steps of conjugate gradient energy minimization. The resulting configurations were thereafter used as input for molecular dynamics (MD) simulations at 300 K and 1 atm using the AMBER18 implementation of the pmemd.cuda engine,²⁵ essentially as described earlier for AL-385.²⁶ In both cases, a weak harmonic restraint of 1 kcal mol⁻¹Å⁻² was initially imposed on the Trp Cα atoms to promote water and counterion equilibration. Trajectory snapshots over 300 ns of unrestrained MD simulations were saved every 0.5 ns for further analysis and movie preparation.

The glycosylated gp120:gp41 trimer complex, with each protein fragment conveniently "capped" by acetyl (ACE) and N-methyl amide (NME) groups at N- and C-termini, respectively, was immersed in a cubic box of 90,960 TIP3P water molecules and 13 chloride ions. The simulations were run for 225 ns essentially as described before for VP1 of EV71 but in the absence of any positional restraints.²⁶

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