TRYPTOPHAN DENDRIMERS THAT INHIBIT HIV REPLICATION, PREVENT VIRUS ENTRY AND BIND TO THE HIV ENVELOPE GLYCOPROTEINS GP120 AND GP41

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Abstract

Dendrimers containing from 9 to 18 tryptophan residues at the periphery have been efficiently synthesized and tested against HIV replication. These compounds inhibit an early step of the replicative cycle of HIV, presumably virus entry into its target cell. Our data suggest that HIV inhibition can be achieved by the preferred interaction of the compounds herein described with glycoproteins gp120 and gp41 of the HIV envelope preventing interaction between HIV and the (co)receptors present on the host cells. The results obtained so far indicate that 9 tryptophan residues on the periphery are sufficient for efficient gp120/gp41 binding and anti-HIV activity.

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1. Introduction

AIDS still remains one of the leading infectious diseases for which there is no curative treatment and no vaccine [1]. Current antiretroviral therapy against the human immunodeficiency virus (HIV), the etiological agent of AIDS, consists of 26 clinically approved drugs, primarily focused on the viral enzymes protease and reverse transcriptase, which are administered in combination in the so-called Highly Active AntiRetroviral Therapy (HAART) [2]. HAART modalities have been improved and extend the lives of patients infected with HIV. However, despite this major therapeutic success, the combination regimen is associated with long-term side-effects and the emergence of drug-resistant HIV strains [3]. Therefore, there is a need for the development of improved drugs targeting events in the HIV-1 replication cycle other than those catalyzed by protease and reverse transcriptase enzymes [4]. In this respect, HIV inhibitors able to block viral fusion/entry are particularly attractive [5]. To date, only two drugs among this category have been approved by the FDA, enfuvirtide (T20) [6], a 36-amino acid peptide that binds viral glycoprotein gp41, and maraviroc [7], a small molecule CCR5-antagonist. Therefore entry/fusion inhibitors represent a promising and still unexploited area of research.

Relative to the other classes of antiretroviral drugs (i.e. reverse transcriptase, protease and integrase inhibitors), which act once the cells have been already infected by HIV, fusion/entry inhibitors have the advantage of blocking *de novo* cell infection, preventing the uptake of the virus by the uninfected CD4-positive cells and therefore the spread of infection [8]. In addition, because their mechanism of action is different from the existing antiretroviral drugs, entry/fusion inhibitors would show full efficacy against viruses resistant to the existing anti-HIV inhibitors. Moreover, acting outside the cellular environment represents an additional advantage of this class of anti-retroviral drugs.

The entry of HIV into its target cells is a multistep time-ordered process involving multiple sequential interactions between the viral envelope glycoproteins (gp120 and gp41) and host cell surface proteins (CD4, CCR5 or CXCR4). It may be divided into three distinct steps: (i) attachment of the viral gp120 to the CD4 T cell receptor, (ii) binding of the gp120 to the host cell

co-receptors (CCR5 or CXCR4) and (iii) fusion of the viral and cellular membranes [5d]. All these three steps have been considered for drug design of HIV-1 entry inhibitors [9]. However, as it was proposed by Teixeira *et al* [9a], it might be preferable to develop anti-HIV drugs targeting viral proteins (gp120 and gp41) rather than host cell molecules (CD4, CCR5 and CXCR4), because binding these host cellular molecules might interfere with their normal functions, causing toxic or adverse effects. Despite this, reports about entry inhibitors with HIV activity associated to gp120 and gp41 binding are still scarce [10].

We have previously reported [11] that triazine derivatives containing 6 Tryptophan (Trp) amino acids, exemplified by compound **I** (Figure 1), inhibit HIV infection at an early stage of the replicative cycle, presumably by interaction with the viral glycoproteins gp 120 and gp41. We have now undertaken the synthesis and anti-HIV evaluation of a series of Trp dendrimers easily constructed by combining a core structure of 3, 4, 5 or 6 carboxylic acids with an aminotriester as a branching unit that facilitates the incorporation of up to 18 Trp on the periphery.

Fig. 1

2. Results and discussion

2.1. Chemical results

Firstly, dendrimers with 9 tryptophans on the periphery were obtained. For the synthesis of these compounds the commercially available aminotriester **1** (Behera's amine) [12] was used as spacer arm and tri- carboxylic acids, **a-d**, with different conformational flexibility, as scaffolds (Scheme 1). Carboxylic acid **a**, the same used in the previously described triazine derivative **I**, was obtained following literature procedure [13], while carboxylic acids **b-d** were commercially available. Reaction of these carboxylic acids **a-d** with aminotriester **1**[12] in the presence of HATU/DIPEA and subsequent deprotection of the *tert*-butyl protected intermediates **2a-d**, afforded compounds **3a-d** (nude acids), containing nine carboxylic acids on the periphery (Scheme 1). These compounds were easily isolated in almost quantitative yield by precipitation with ethyl acetate-methanol.

Scheme 1

Coupling of nonaacids **3a** and **3d** [14] with H-Trp-OMe·HCl in the presence of HATU and DIPEA gave compounds **4a** (36%) and **4d** (82%), while reaction of nonaacids, **3b** [15] and **3c**, with H-Trp-OBn·HCl afforded **4b** (94%) and **4c** (86%) (Scheme 2). Methyl ester deprotection of **4a** and **4d**, under basic conditions (LiOH/H₂O), followed by acidification at pH = 2 and purification of the crude product by reverse phase chromatography afforded compounds **5a** and **5d** in 43% and 49% yields respectively (Scheme 2). On the other hand, **4b** and **4c** were debenzylated by catalytic hydrogenation (H₂, 10% Pd-C) to give **5b** and **5c** in 20 and 25% yields respectively (Scheme 2).

Scheme 2

Synthesis of compounds containing 12, 15 and 18 Trps on the periphery was performed using a strategy similar to those described earlier for compounds with 9 tryptophans. In this case, functionalized compounds (**e-g**), with 4, 5 and 6 carboxylic acids as attachment points, were used as central scaffolds. Reaction of these compounds with aminotriester **1** [12] in the presence of HATU and DIPEA afforded the *tert*-butyl derivatives **2e-g**. Subsequent acidic deprotection and precipitation using ethyl acetate/methanol gave carboxylic acids **3e-g** in 80-88 % yields (Scheme 3).

Scheme 3

Reaction of carboxylic acid **3e-g** with H-Trp-OBn-HCl in the presence of HATU and DIPEA gave compound **4e** (61%) that was subsequently debenzylated by catalytic hydrogenation (H_2 , 10% Pd-C) to give **5e** in 38% yield (Scheme 4). Alternatively, reaction of **3f** and **3g** with H-Trp-OMe-HCl in the presence of HATU and DIPEA gave compounds **4f** and **4g**. Methyl ester deprotection as described above (LiOH/ H_2O) and purification of the crude material by reverse phase chromatography gave the desired compounds **5f** and **5g** in 24% and 78% yields, respectively (Scheme 4).

Scheme 4

Finally, nitrodendron **8** with 9 tryptophans on the periphery, was prepared by reaction of dendron **6** [16] with H-Trp-OMe-HCl in the presence of HATU/DIPEA, followed by methyl ester deprotection (LiOH. H_2O) and purification of the crude product by reverse phase chromatography (Scheme 5).

Scheme 5

The characterization of these compounds was performed by 1H and ^{13}C NMR spectroscopies and HRMS.

2.2 Biological results

2.2.1. Anti-HIV activity of the test compounds

The new tryptophan derivatives have been evaluated for their inhibitory effects against HIV replication in cell culture. Table 1 summarizes the results of this evaluation. The antiviral data of the best compound (**I**) of the triazine series is also reported as a reference. Dextran sulfate-5000 (DS-5000), pradimicin A and tenofovir acted as relevant controls. DS-5000 is a negatively charged HIV adsorption inhibitor [17], pradimicin A represents a gp120 carbohydrate-binding entry inhibitor [18] and tenofovir is an HIV RT inhibitor [19]. These compounds showed activities that were in the same range as previously reported [17-19].

None of the compounds **3a**, **3c** and **3e** (nude acids) with free carboxylic acids on the periphery proved inhibitory against HIV (EC_{50} : > 250 μ M). Alternatively compounds **5a–g** and **8**, with Trps on the periphery, provided significant anti-HIV activity, showing a concentration-dependent inhibition of the virus infection. Some of them showed a markedly improved (~ 3- to 7-fold) antiviral potency (EC_{50} : lower μ M range) than the previously described triazine derivative **I** (EC_{50} : 16 μ M). Among these compounds, compounds **5b** and **5f** were the most active (EC_{50} : 2.6 and 2.2 μ M respectively). Compound **5c** showed an EC_{50} of 4.5 μ M, and proved the least toxic compound in this series (CC_{50} : > 250 μ M), resulting in a selectivity index of >55. The compounds tested against HIV-2 (**5e–g**) also were active at subtoxic concentrations, but the inhibitory potential proved inferior to HIV-1 inhibition.

Compound **5b**, containing a rigid central scaffold, and **5c** and **5d**, containing more flexible central scaffolds, showed similar anti-HIV-1 activity (EC_{50} = 2.6, 4.5 and 3.2 μ M respectively). Very comparable data were also obtained for HIV-1 (NL4.3) infection of MT-4 cell cultures (EC_{50} for **5b**, **5c**, and **5d**: 1.3, 2 and 2.5 μ M, respectively). From these data it can be concluded that the flexibility of the central core does not play a significant role on activity.

Interestingly, compounds **5e–g**, bearing 12, 15 and 18 Trps on the periphery were at least equal (**5f**) or less (**5e**, **5g**) active than compounds with only 9 Trps (**5b–d**) showing that the concomitant presence of 9 tryptophan residues on the periphery seems to be sufficient for pronounced anti-HIV activity.

Dendron **8**, with 9 Trps on the periphery did not show improved activity compared with dendrimers **5a–d**, that also contained 9 Trps on the periphery.

Table 1.

Compound	EC_{50}^a (μ M) HIV-1	EC_{50}^a (μ M) HIV-2	CC_{50}^b (μ M)
3a	>250	>250	>250
3c	>250	>250	>250
3e	>250	>250	>250
5a	6.2 ± 2.5	- ^c	>100
5b	2.6 ± 2.6	- ^c	53
5c	4.5 ± 4.9	- ^c	>250
5d	3.2 ± 1.8	- ^c	59
5e	11.3 ± 2.8	≥ 30	>30
5f	2.2 ± 2.1	10	>100

5g	8.0 ± 2.1	30	>100
8	9.9 ± 1.6	44 ± 2.8	>100
I	16 ± 0.71	22 ± 2.8	>250
DS-5000	0.07 ± 0.02	0.03 ± 0.01	>20
Pradimicin A	3.3 ± 1.2	5.9 ± 3.7	>100
Tenofovir	3.9 ± 0.9	3.7 ± 1.2	>100

Data are the mean $\pm$ S.D. of at least 2 to 3 independent experiments

^a 50% Effective concentration, or the concentration required to inhibit HIV-induced cytopathicity by 50%

^b 50% Cytostatic concentration, or the concentration required to inhibit CEM cell proliferation by 50%

^c Not tested

2.2.2. Time-of-addition experiments

In order to reveal which particular stage of the HIV replicative cycle was inhibited by the compounds, time-of-addition (TOA) experiments [20,21] were performed with the selected active compounds **5b** and **5f**. In this experiment, the virus-associated p24 antigen (p24 is the major structural protein of the virion), quantified by a commercially available ELISA assay (Enzyme-linked immunosorbent assay), was used to examine HIV replication. At different time-points after virus infection, the test compounds were added to the virus-infected cell cultures. If a compound inhibits virus infection at an early stage in the virus replicative cycle, addition of such compound cannot be too much delayed in the virus-infected cultures without losing its antiviral activity. Instead, if a compound acts at a late stage in the virus replicative cycle, addition of such compound can be much longer delayed in the virus-infected cell cultures before losing its antiviral efficacy. Different well-characterized anti-HIV drugs were therefore used as reference drugs. As inhibitors of the entry/fusion process the carbohydrate-binding pradimicin S (PRM-S), the CXCR4 antagonist bicyclam AMD3100 and the adsorption inhibitor DS8,000 were used. AZT was used as reverse transcriptase inhibitor. The protease inhibitor ritonavir was also included in the study to reflect an agent that acts late in the viral replication cycle.

As it was shown in Figure 2 the curves for compounds **5b** and **5f** nicely overlap with those of the established entry inhibitors. Thus, this experiment clearly shows that **5b** and **5f** inhibit early step(s) of the replicative cycle of HIV, and more specifically they inhibit the entry of the virus into its target cells. This target may be not so unlikely since the investigated structures have a rather high molecular weight, and thus, it might be most likely, although not proven, that they are not efficiently taken-up by the exposed infected cells. The rather steep dose-response inhibition curves of **5b** and **5f** obviously resulted in the full inhibition of HIV replication (i.e. entry) when continuously present at the start of the virus infection (zero time point) at 10 μ M.

Fig. 2.

2.2.3. SPR experiments

The compounds found inhibitory against HIV-1 in cell culture presumably inhibit HIV entry into its target cells as based on the target profiling found in time-of-addition experiments. It would, therefore, be of particular interest to investigate potential binding of the compounds to the virus-encoded gp120/gp41 envelope glycoproteins. Using surface plasmon resonance (SPR) technology, recombinant HIV-1 III_B gp120 and HIV-1 HxB2 gp41 (produced in Chinese hamster ovary cells) were bound to a sensor chip as monomers and the interaction of the compounds with the viral envelope glycoproteins were determined at one fixed concentration (50 μ M). The two dendrimers that contained the highest number of tryptophans on the periphery, **5f** (15 Trp) and **5g** (18 Trp), showed the highest binding signal to both gp120 and gp41 (Fig. 3). The highest binding efficiency was then followed by compounds **5a-d**. Compound **5c**, containing the cyclohexyl core, was slightly better than **5a,b,d**. Compound **5e** and **8** showed clearly an inferior binding amplitude to gp120 and gp41 than the other compounds (data not shown).

Fig. 3.

Detailed SPR-directed affinities were then measured for **5f** that proved to be the most efficient binding derivative to gp120/gp41. For this purpose compound concentrations ranging between 0 and 40 μ M were used. The apparent K_D value for **5f** binding to gp120 (Figure 1 Supplementary Material and Table 2) was in the low micromolar range (K_D : 6.7 μ M), being 2 times more pronounced than those found for the triazine prototype **I** (K_D : 14 μ M), showing that the affinity of **5f** to gp120 is 2-fold more pronounced than that observed for **I**. The apparent K_D value for **5f** binding to gp41 was 18 μ M, that means that the interaction of **5f** with gp41 was 3-fold less pronounced than with gp120.

Table 2.

Complex	K_D (μ M)
5f-gp120	6.7
5f-gp41	18
I-gp120	14

The polyanionic nature of **5f** may rise the question whether the observed affinity for HIV-1 gp120 is due to the binding to the positively charged V3 loop of gp120 or whether the compound binds to other areas on gp120 such as the glycans present on this glycoprotein [22]. To solve this question, the association and dissociation of **5f** to the gp120-bound sensorchip was also

studied in the presence of the mannose trimer mann(α -1,3-1,6)₃, that represents a mannose oligomer composition that is also present in gp120 glycans. As shown in Figure 4 this mannose trimer was not at all able to affect the interaction of compound **5f** with gp120, neither with gp41. This finding suggests that compound **5f** may interact with gp120 and gp41 in a manner that is different from mannose α 1,3/ α 1,6 oligomer binding.

Fig. 4.

3. Conclusions

Dendrimers with more than six tryptophan residues (i.e. 9, 12, 15 and 18) on the periphery have been readily prepared by combining a core structure of 3, 4, 5 or 6 carboxylic acids with an amino triester as branching unit. These compounds have been evaluated as anti-HIV agents in cell culture. Several of them showed a markedly improved ($\sim$ 3- to 7-fold) antiviral potency (EC_{50} : lower μ M range) than the previously described triazine derivative **I** (EC_{50} : 16 μ M), with six tryptophan residues on the periphery. Selectivity indices were often > 10 - to > 55 -fold.

The results obtained so far indicate that the presence of Trp (at least at a total of 9 residues on the periphery) is crucial for the antiviral activity since the compounds without this moiety (nude acids) were inactive against HIV. The triazine moiety present in **I** is not crucial for activity. This moiety can be substituted by other simplified spacer arms. The rigidity of the central scaffold does not appear to be important for antiviral activity.

Time-of-addition (TOA) experiments carried out with two representative members of this compound family, i.e. **5b** and **5f**, clearly show that these compounds inhibit early steps of the replicative cycle of HIV, presumably virus entry into its target cells. Surface plasmon resonance (SPR) studies revealed that all compounds showed binding to HIV-1 gp120 and gp41.

Compound **5f**, containing 15 tryptophans on the periphery, showed the most significant anti-HIV-1 activity (EC_{50} : 2.2 μ M) and pronounced interaction with gp120/gp41.

4. Materials and methods

4.1. Synthesis

4.1.1. General Procedure for the synthesis of dendrimers **3a-g**

To a solution containing the corresponding central scaffold **a-g** in DMF (20 mL), aminotriester **1** [12] (1.5 eq for each carboxylic acid group), HATU (1.5 eq for each carboxylic acid group) and DIPEA (4.5 eq for each carboxylic acid group) were added. The reaction mixture was stirred at room temperature during 2 days and then evaporated to dryness. The residue was

dissolved in dichloromethane (50 mL) and washed successively with aqueous solutions of citric acid (10%) (3×20 mL), saturated NaHCO_3 (3×20 mL), and brine (3×20 mL). The organic phase was dried over anhydrous Na_2SO_4 , filtered, and evaporated to dryness. The residue, without further purification, was subsequently dissolved in formic acid and the solution was stirred at 40°C for 48 h. After cooling, volatiles were evaporated to dryness and the residue was dissolved in methanol and treated with ethyl acetate to give the corresponding dendrimer **3a-g** with 9, 12, 15 or 18 carboxylic acids on the periphery as a pale yellow solid.

4.1.2. Dendrimer nonaacid (**3a**)

According to the general procedure a solution of 1,3,5-triethyl-2,4,6-tris(carboxymethyl)benzene **a** [13] (400 mg, 1.2 mmol), aminotriester **1** [12] (2.24 g, 5.4 mmol), HATU (2.03 g, 5.4 mmol) and DIPEA (2.9 mL, 16.2 mmol) in DMF (20 mL) gave 1.28 g (99%) of **3a** as a pale yellow solid; m.p. $170\text{--}172^\circ\text{C}$. ^1H NMR (300 MHz, DMSO-d_6) δ : 0.98 (m, 9H, CH_3CH_2), 1.60 (m, 9H, CH_2), 1.75 (m, 9H, CH_2), 2.19 (m, 9H, CH_2), 2.25 (m, 9H, CH_2), 2.52 (m, 6H, CH_2CH_3), 3.60 (m, 6H, CH_2CO), 7.65 (bs, 3H, NH). HRMS (ESI) m/z : Calcd. for $\text{C}_{48}\text{H}_{69}\text{N}_3\text{O}_{21}$ 1023.4424. Found 1023.4460.

4.1.3. Dendrimer nonaacid (**3c**)

According to the general procedure a solution of *cis,cis*-1,3,5-cyclohexanetricarboxylic acid **c** (200 mg, 0.93 mmol), aminotriester **1** [12] (1.69 g, 4.19 mmol), HATU (1.58 g, 4.19 mmol) and DIPEA (2.26 mL, 12.6 mmol) in DMF (20 mL) afforded 784 mg (94%) of **3c** as a pale yellow solid; m.p. $247\text{--}249^\circ\text{C}$. ^1H NMR (400 MHz, DMSO-d_6) δ : 1.78 (m, 21H, CH_2 , CH), 2.05 (m, 24H, CH_2 , CH), 7.09 (s, 3H, NH), 11.96 (bs, 9H, COOH). ^{13}C NMR (125 MHz, CD_3OD) δ : 29.44 (CH_2), 30.40 (CH_2), 31.98 (CH_2), 32.74 (CH_2), 57.44 (C), 175.36 (C=O), 175.77 (C=O). HRMS (ESI) m/z : Calcd. for $\text{C}_{39}\text{H}_{57}\text{N}_3\text{O}_{21}$ 903.3485. Found 903.3489.

4.1.4. Dendrimer dodecaacid (**3e**)

According to the general procedure a solution of ethylenediaminetetraacetic acid (EDTA) compound **e** (100 mg, 0.34 mmol), aminotriester **1** [12] (2.04 g, 2.04 mmol), HATU (954 mg, 2.04 mmol) and DIPEA (2.13 mL, 6.12 mmol) in DMF (20 mL) afforded 340 mg (82%) of **3e** as a pale yellow solid; m.p. $173\text{--}175^\circ\text{C}$. ^1H NMR (500 MHz, CD_3OD) δ : 2.64 (m, 24H, CH_2), 2.91 (m, 24H, CH_2), 3.42 (s, 4H, CH_2), 3.88 (m, 8H, CH_2NH), 8.47 (m, 4H, NH). ^{13}C NMR (125 MHz, CD_3OD) δ : 29.62 (CH_2), 30.51 (CH_2), 53.84 (C), 57.85 (CH_2), 59.77 (CH_2), 171.17 (C=O), 175.88 (C=O). HRMS (ESI) m/z : Calc. for $\text{C}_{50}\text{H}_{76}\text{N}_6\text{O}_{28}$ 1209.1588. Found 1209.1584.

4.1.5. Dendrimer pentadecaacid (**3f**)

According to the general procedure a solution of penta(carboxymethyl)diethylenetriamine (DTPA) **f** (400 mg, 1.02 mmol), aminotriester **1** [12] (3.20 g, 7.6 mmol), HATU (2.94 g, 7.6 mmol) and DIPEA (4.1 mL, 22.95 mmol) in DMF (20 mL) afforded 1.10 g (80%) of **3f** as a pale yellow solid; m.p. $184\text{--}186^\circ\text{C}$. ^1H NMR (400 MHz, DMSO-d_6) δ : 1.63 (t, $J = 7.1$ Hz, 30H, CH_2), 2.14 (m, 8H, CH_2), 2.19 (t, $J = 7.5$ Hz, 30H, CH_2), 3.03 (s, 10H, NCH_2). HRMS (ESI) m/z : Calcd. for $\text{C}_{64}\text{H}_{98}\text{N}_8\text{O}_{35}$ 1538.6135. Found 1538.6216.

4.1.6. Dendrimer octadecaacid (**3g**)

According to the general procedure a solution of hexa(carboxymethyl)triethylenetetramine **g** (500 mg, 1.01 mmol), aminotriester **1** [12] (3.73 g, 9.09 mmol), HATU (3.46 g, 9.09 mmol) and DIPEA (4.76 mL, 27.27 mmol) in DMF (20 mL) afforded 1.55 g (88%) of **3g** as a pale yellow solid; m.p.: 183-185 °C. ¹H NMR (300 MHz, DMSO-d₆) δ: 1.83 (m, 36H, CH₂), 2.11 (m, 36H, CH₂), 2.26 (m, 12H, CH₂), 3.08 (bs, 12H, NCH₂). HRMS (ESI) *m/z*: Calcd. for C₇₈H₁₂₀N₁₀O₄₂ 1868.7562. Found 1868.7542.

4.1.7. General Procedure for the synthesis of OBn or OMe protected Trp dendrimers (**4 a-g**)

To a mixture of the corresponding poly acid **3a-g** and H-(L)-Trp-OMe.HCl or H-(L)-Trp-OBzl.HCl (1.5 eq for each carboxylic acid group) in DMF (20 mL), HATU (1.5 eq for each carboxylic acid group) and DIPEA (4.5 eq for each carboxylic acid group) were added. The reaction was stirred at room temperature during 3 days (for compounds with 9 or 12 Trp moieties) or 4 days (for compounds with 15 or 18 Trp moieties) and then evaporated to dryness to afford a residue that was dissolved in dichloromethane (30 mL) and washed successively with aqueous solutions of citric acid (10%) (3 × 20 mL), saturated NaHCO₃ (3 × 20 mL), and brine (3 × 20 mL). The organic layer was dried over anhydrous Na₂SO₄, filtered and evaporated. The residue was purified on a Biotage HPFC (High Performance Flash Chromatography) purification system on reverse phase using water/acetonitrile (100:0 to 0:100) as eluent.

4.1.8. Dendrimer (**4a**)

According to the general procedure a mixture containing **3a** (250 mg, 0.24 mmol), H-(L)-Trp-OMe.HCl (825 mg, 3.24 mmol), HATU (1.23 g, 3.24 mmol) and DIPEA (1.74 mL, 9.72 mmol) in DMF (20 mL) gave 245 mg (36%) of **4a** as an amorphous yellow solid. ¹H-RMN (300 MHz, CDCl₃) δ: 1.25 (m, 9H, CH₃CH₂), 1.80 (m, 18H, CH₂), 2.31 (m, 24H, CH₂, CH₃CH₂), 3.09 (m, 9H, β-CH₂Trp), 3.26 (m, 9H, β-CH₂Trp), 3.62 (s, 27H, OCH₃), 3.79 (m, 6H, CH₂CO), 4.74 (m, 9H, α-CHTrp), 6.99-7.47 (m, 45H, Ar), 7.98 (s, 3H, NH), 9.04 (m, 9H, NH). HRMS (ESI⁺) *m/z*: Calcd. for C₁₅₆H₁₇₇N₂₁O₃₀ 2824.2970. Found 2824.3040.

4.1.9. Dendrimer (**4b**)

According to the general procedure a mixture containing **3b** (250 mg, 0.24 mmol), H-(L)-Trp-OBzl.HCl (1.07 g, 3.24 mmol), HATU (1.23 g, 3.24 mmol) and DIPEA (1.74 mL, 9.72 mmol) in DMF (20 mL) gave 880 mg (94%) of **4b** as an amorphous yellow solid. ¹H NMR (300 MHz, CDCl₃) δ: 2.40 (m, 18H, CH₂), 2.67 (m, 18H, CH₂), 3.29 (dd, *J* = 14.9 Hz, *J* = 7.8 Hz, 9H, β-CH₂Trp), 3.44 (m, 9H, β-CH₂Trp), 4.41 (td, *J* = 7.7 Hz, *J* = 4.4 Hz, 9H, α-CHTrp), 5.13 (dd, *J* = 14.1 Hz, *J* = 2.3 Hz, 18H, CH₂Ar), 7.03-7.47 (m, 90H, Ar), 8.73 (m, 6H, Ar, NH). HRMS (ESI⁺) *m/z*: Calcd. for C₂₀₁H₁₉₅N₂₁O₃₀ 3382.4379. Found 3382.4370.

4.1.10. Dendrimer (**4c**)

According to the general procedure a mixture containing **3c** (450 mg, 0.5 mmol), H-(L)-Trp-OBzl.HCl (2.01 g, 6.21 mmol), HATU (2.36 g, 6.21 mmol) and DIPEA (3.57 mL, 20.25 mmol) in DMF (20 mL), afforded 1.46 g (86%) of **4c** as an amorphous yellow solid. ¹H NMR (300 MHz, CDCl₃) δ: 1.93-2.44 (m, 45H, CH₂, CH), 3.19 (m, 18H, β-CH₂Trp), 4.80 (s, 9H, α-CHTrp), 5.03 (m, 18H, CH₂Ar), 6.85-7.27 (m, 90H, Ar), 9.04 (m, 9H, NH). HRMS (ESI⁺) *m/z*: Calcd. for C₂₀₁H₂₀₁N₂₁O₃₀ 3388.4848. Found 3388.4859.

4.1.11. Dendrimer (**4d**)

According to the general procedure a mixture containing **3d** (400 mg, 0.46 mmol), H-(L)-Trp-OMe.HCl (1.58 g, 6.21 mmol), HATU (2.36 g, 6.21 mmol) and DIPEA (3.25 mL, 18.63 mmol) in DMF (20 mL) afforded 1.00 g (82%) of **4d** as an amorphous yellow solid. ^1H NMR (300 MHz, CDCl_3) δ : 1.50 (m, 18H, CH_2), 1.78 (m, 18H, CH_2), 3.06 (m, 9H, $\beta\text{-CH}_2\text{Trp}$), 3.25 (m, 15H, $\beta\text{-CH}_2\text{Trp}$, CH_2NH), 3.62 (s, 27H, OCH_3), 4.80 (m, 9H, $\alpha\text{-CHTrp}$), 6.82-7.75 (m, 90H, Ar), 9.82 (bs, 12H, NH). ^{13}C NMR (75 MHz, CDCl_3) δ : 27.53 (CH_3), 30.22 (CH_2), 52.71 (CH_2), 53.28 (CH_2), 53.82 (C), 58.07 (CH), 109.96 (C), 111.84 (CH), 118.49 (CH), 119.55 (CH), 122.15 (CH), 123.89 (CH), 127.43 (C), 136.56 (C), 170.82 (C=O), 173.21 (C=O), 174.37 (C=O). HRMS (ESI^+) m/z : Calcd. for $\text{C}_{144}\text{H}_{162}\text{N}_{22}\text{O}_{30}$ 2679.1827. Found 2679.1739.

4.1.12. Dendrimer (**4e**)

According to the general procedure a mixture containing **3e** (150 mg, 0.12 mmol), H-(L)-Trp-OBzl.HCl (714 mg, 1.92 mmol), HATU (821 mg, 1.92 mmol) and DIPEA (1.13 mL, 6.48 mmol) in DMF (20 mL), afforded 345 mg (61%) of **4e** as an amorphous yellow solid. ^1H NMR (300 MHz, CDCl_3) δ : 2.46 (m, 24H, CH_2), 2.76 (m, 28H, CH_2), 3.31-3.38 (m, 20H, $\beta\text{-CH}_2\text{Trp}$, CH_2NH), 3.50 (dd, 12H, $J = 14.9$ Hz, $J = 3.5$ Hz, $\beta\text{-CH}_2\text{Trp}$), 4.44 (m, 12H, $\alpha\text{-CHTrp}$), 5.19 (d, 24H, CH_2Ar), 5.76 (d, $J = 4.25$ Hz, 4H), 7.07-7.50 (m, 120H, H-2^bTrp, Ar), 8.02 (s, 12H, NH), 8.75 (s, 4H, NH). HRMS (ESI^+) m/z : Calcd. for $\text{C}_{266}\text{H}_{268}\text{N}_{30}\text{O}_{40}$ 4521.9859. Found 4521.9927.

4.1.13. Dendrimer (**4f**)

According to the general procedure a mixture containing **3f** (500 mg, 0.32 mmol), H-(L)-Trp-OMe.HCl (1.83 g, 7.2 mmol), HATU (2.79 g, 7.2 mmol) and DIPEA (3.96 mL, 21.6 mmol) in DMF (20 mL) afforded 771 mg (60%) of **4f** as an amorphous yellow solid. ^1H NMR (400 MHz, CDCl_3) δ : 1.43 (m, 30H, CH_2), 1.94 (m, 30H, CH_2), 2.18 (m, 8H, CH_2), 3.28 (m, 30H, $\beta\text{-CH}_2\text{Trp}$), 3.49 (s, 10H, NCH_2), 3.67 (m, 45H, OCH_3), 4.88 (m, 15H, $\alpha\text{-CHTrp}$), 6.95-7.49 (m, 75H, H-2^bTrp, Ar), 8.77 (m, 15H, NH). HRMS (ESI^+) m/z : Calcd. for $\text{C}_{244}\text{H}_{278}\text{N}_{38}\text{O}_{50}$ 4540.0379. Found 4540.0424.

4.1.14. Dendrimer (**4g**)

According to the general procedure a mixture containing **3g** (550 mg, 0.29 mmol), H-(L)-Trp-OMe.HCl (1.99 g, 7.83 mmol), HATU (2.9 g, 7.83 mmol) and DIPEA (4.1 mL, 23.49 mmol) in DMF (20 mL) afforded 561 mg (35%) of **4g** as an amorphous yellow solid. ^1H NMR (400 MHz, CDCl_3) δ : 1.52 (m, 36H, CH_2), 1.94 (m, 36H, CH_2), 2.20 (m, 12H, CH_2), 3.30 (m, 36H, $\beta\text{-CH}_2\text{Trp}$), 3.45 (s, 12H, NCH_2), 3.62 (m, 54H, OCH_3), 4.86 (m, 18H, $\alpha\text{-CHTrp}$), 6.95-7.45 (m, 90H, H-2^bTrp, Ar), 8.50 (m, 18H, NH). HRMS (ESI^+) m/z : Calcd. for $\text{C}_{294}\text{H}_{336}\text{N}_{46}\text{O}_{60}$ 5470.4655. Found 5470.4648.

4.1.15. General Procedure for the deprotection of the methyl esters **4a**, **d**, **f**, **g**

To a solution containing the corresponding methyl ester derivative (1.0 mmol) in THF (10 mL), a solution of LiOH·H₂O (2 equiv. for each methyl ester group) in water (10 mL) was added, and the mixture was stirred at room temperature during 3 days (for compounds with 9 or 12 tryptophan residues) or 5 days (for compounds with 15 or 18 tryptophan residues). Then, formic acid was added to reach pH = 2, and volatiles were evaporated to dryness. The residue was purified on a Biotage HPFC system (High Performance Flash Chromatography) on reverse phase using water/acetonitrile (100:0 to 0:100) as eluent.

4.1.16. Dendrimer (**5a**)

Following the general procedure for the deprotection of methyl esters, compound **4a** (245 mg, 0.09 mmol) gave 100 mg (43%) of **5a** as an amorphous white solid. ¹H NMR (500 MHz, DMSO-*d*₆) δ : 0.91 (m, 9H, CH₃CH₂), 1.67 (m, 24H, CH₂), 1.97 (m, 18H, CH₂), 2.47 (m, 6H, CH₃CH₂), 3.12 (m, 9H, β -CH₂Trp), 3.29 (m, 9H, β -CH₂Trp), 3.51 (s, 6H, CH₂CO), 4.70 (m, 9H, α -CHTrp), 6.96 (m, 9H, Ar), 7.03 (m, 9H, Ar), 7.08 (s, 9H, Ar), 7.28 (d, *J* = 7.9 Hz, 9H, Ar), 7.55 (d, *J* = 7.9 Hz, 9H, Ar), 8.17 (s, 9H, NH), 10.29 (bs, 9H, NH-1stTrp). ¹³C NMR (125 MHz, DMSO-*d*₆) δ : 14.18 (CH₃), 22.49 (CH₂), 23.59 (CH₂), 27.57 (CH₂), 29.93 (CH₂), 30.59 (CH₂), 47.24 (C), 54.10 (C), 110.10 (C), 111.45 (CH), 118.35 (CH), 118.81 (CH), 121.41 (CH), 123.69 (CH), 127.63 (C), 136.89 (C), 174.19 (C=O). HPLC [gradient: A:B, 10-100% of A in 10 min]: 8.20 min. HRMS (ESI) *m/z*: Calcd. for C₁₄₇H₁₅₉N₂₁O₃₀ 2698.1562. Found 2698.1543.

4.1.17. Dendrimer (**5d**)

Following the general procedure for the deprotection of methyl esters, compound **4d** (1.00 g, 0.34 mmol) gave 46.7 mg (49%) of **5d** as an amorphous white solid. ¹H NMR (500 MHz, DMSO-*d*₆) δ : 1.72 (m, 18H, CH₂), 2.01 (m, 18H, CH₂), 2.97 (m, 9H, β -CH₂Trp), 3.11 (m, 9H, β -CH₂Trp), 3.51 (s, 6H, CH₂NH), 4.42 (m, 9H, α -CHTrp), 6.91 (m, 9H, Ar), 6.98 (m, 9H, Ar), 7.10 (s, 9H, Ar), 7.27 (m, 9H, Ar), 7.50 (m, 9H, Ar), 8.15 (s, 9H, NH), 10.78 (bs, 9H, NH-1stTrp). ¹³C NMR (125 MHz, DMSO-*d*₆) δ : 14.40 (CH₂), 29.45 (CH₂), 31.37 (CH₂), 53.72 (C), 63.21 (CH₂), 69.79 (CH), 111.73 (CH), 118.66 (CH), 121.18 (CH), 123.98 (CH), 127.77 (C), 136.44 (C), 158.36 (C=O), 172.90 (C=O), 174.57 (C=O). HPLC [gradient: A:B, 10-100% of A in 10 min]: 8.17 min. HRMS (ESI) *m/z*: Calcd. for C₁₃₅H₁₄₄N₂₂O₃₀ 2553.0419. Found 2553.0437.

4.1.18. Dendrimer (**5f**)

Following the general method for deprotection of methyl esters, compound **4f** (1.56 g, 0.34 mmol) gave 352 mg (24%) of **5f** as an amorphous white solid. ¹H NMR (500 MHz, DMSO-*d*₆) δ : 1.75 (m, 30H, CH₂), 2.05 (m, 38H, CH₂), 2.99 (m, 25H, β -CH₂Trp, CH₂NH), 3.13 (m, 15H, β -CH₂Trp), 4.49 (q, *J* = 7.7 Hz and *J* = 7.1 Hz, 15H, α -CHTrp), 6.90 (t, *J* = 7.6 Hz, 15H, Ar), 6.97 (t, *J* = 7.6 Hz, 15H, Ar), 7.09 (s, 15H, Ar), 7.26 (d, *J* = 7.9 Hz, 15H, Ar), 7.48 (d, *J* = 7.9 Hz, 15H, Ar), 8.09 (s, 15H, NH), 10.72 (bs, 15H, NH-1stTrp), 12.55 (bs, 15H, COOH). ¹³C NMR (125 MHz, DMSO-*d*₆) δ : 27.64 (CH₂), 29.22 (CH₂), 30.53 (CH₂), 53.76 (CH₂), 110.00 (CH), 111.61 (CH), 118.58 (CH), 118.67 (CH), 121.34 (CH), 124.14 (CH), 127.74 (C), 136.73 (C), 172.76 (C=O), 176.56 (C=O). HPLC [gradient: A:B, 10-100% of A in 10 min]: 8.83 min. HRMS (ESI) *m/z*: Calcd. for C₂₂₉H₂₄₈N₃₈O₅₀ 4329.8032. Found 4329.8070.

4.1.19. Dendrimer (**5g**)

Following the general method for deprotection of methyl esters, compound **4g** (66 mg, 0.012 mmol) gave 49 mg (78%) of **5g** as an amorphous white solid. ¹H NMR (500 MHz, DMSO-*d*₆) δ : 1.50-1.97 (m, 84H, CH₂), 2.96-3.23 (m, 48H, β -CH₂Trp, NCH₂), 4.42 (m, 18H, α -CHTrp), 6.87-7.47 (m, 90H, Ar, H-2ndTrp), 7.91 (s, 18H, NH), 10.86 (s, 18H, NH-1stTrp). ¹³C NMR (125 MHz, DMSO-*d*₆) δ : 24.67 (CH₂), 31.88 (CH₂), 31.97 (CH₂), 52.43 (C), 55.89 (C), 78.86 (CH₂), 80.80 (CH₂), 103.03 (C), 114.94 (C), 118.88 (CH), 122.09 (CH), 127.66 (C), 137.36 (C), 160.59 (C=O), 173.21 (C=O), 220.92 (C=O). HPLC [gradient: A:B, 10-100% of A in 10 min]: 9.17 min. HRMS (ESI) *m/z*: Calcd. for C₂₇₆H₃₀₀N₄₆O₆₀ 5218.1838. Found 5218.1873.

4.1.20. General Procedure for the deprotection of the benzyl esters **4b**, **c**, **e**

A solution of the corresponding OBn protected derivative (1.0 mmol) in THF/methanol (1:1; 20 mL) containing Pd (10% on C; 30 wt.-%) was hydrogenated at 2.85 atm (42 psi) at 30 °C overnight. The Pd/C was filtered through Whatman[®] filter paper 42, washed with methanol and the solvent was removed under reduced pressure to afford the crude deprotected derivative

which was then purified on a Biotage HPFC system (High Performance Flash Chromatography) on reverse phase using water/acetonitrile (100:0 to 0:100) as eluent.

4.1.21. Dendrimer (**5b**)

Following the general method for deprotection of benzyl esters, compound **4b** (880 mg, 0.26 mmol) gave 134 mg (20%) of **5b** as a white amorphous solid. ¹H NMR (400 MHz, CD₃OD) δ : 1.33 (m, 18H, CH₂), 2.08 (m, 18H, CH₂), 3.13 (m, 9H, β -CH₂Trp), 3.32 (m, 9H, β -CH₂Trp), 4.53 (m, 9H, α -CHTrp), 6.88 (m, 9H, Ar), 6.99 (m, 9H, Ar), 7.15 (m, 9H, Ar), 7.32 (d, J = 8.0 Hz, 9H, Ar), 7.49 (m, 9H, Ar), 8.35 (bs, 3H, NH). ¹³C NMR (100 MHz, CD₃OD) δ : 13.83 (CH₂), 29.54 (CH₂), 29.66 (CH₂), 54.78 (CH), 104.97 (C), 111.57 (CH), 118.57 (CH), 118.86 (CH), 121.41 (CH), 121.44 (CH), 123.68 (CH), 127.98 (CH), 136.71 (C), 186.66 (C=O), 199.59 (C=O). HPLC [gradient: A:B, 10-100% of A in 10 min]: 8.05 min. HRMS (ESI) m/z : Calcd. for C₁₃₈H₁₄₁N₂₁O₃₀ 2572.0153. Found 2572.0182.

4.1.22. Dendrimer (**5c**)

Following the general method for deprotection of benzyl esters, compound **4c** (1.46 g, 0.43 mmol) gave 265 mg (25%) of **5c** as an amorphous white solid. ¹H NMR (400 MHz, CD₃OD) δ : 1.45 (m, 3H, CH₂), 1.82 (m, 18H, CH₂), 2.06 (m, 18H, CH₂), 2.10 (m, 3H, CH₂), 3.13 (m, 9H, β -CH₂Trp), 3.25 (m, 9H, β -CH₂Trp), 4.09 (m, 3H, CH), 4.61 (m, 9H, α -CHTrp), 6.99 (t, J = 7.8 Hz, 9H, Ar), 7.05 (t, J = 7.3 Hz, 9H, Ar), 7.15 (s, 9H, Ar), 7.32 (d, J = 8.1 Hz, 9H, Ar), 7.57 (d, J = 8.0 Hz, 9H, Ar). ¹³C NMR (100 MHz, CD₃OD) δ : 16.74 (CH₂), 29.87 (CH₂), 32.32 (CH₂), 43.95 (CH₂), 45.01 (CH), 54.09 (C), 56.22 (CH), 104.95 (C), 111.24 (CH), 118.44 (CH), 118.82 (CH), 121.32 (CH), 123.59 (CH), 127.67 (C), 136.83 (C), 176.58 (C=O). HPLC [gradient: A:B, 10-100% of A in 10 min]: 8.07 min. HRMS (ESI) m/z : Calcd. for C₁₃₈H₁₄₇N₂₁O₃₀ 2578.0623. Found 2578.0663.

4.1.23. Dendrimer (**5e**)

Following the general method for deprotection of benzyl esters, compound **4e** (345 mg, 0.076 mmol) gave 162 mg (38%) of **5e** as a white solid; m.p.: 285-287 °C. ¹H NMR (500 MHz, DMSO-d₆) δ : 1.79 (m, 24H, CH₂), 2.03 (m, 24H, CH₂), 2.62 (m, 4H, CH₂), 2.98 (m, 12H, β -CH₂Trp), 3.13 (m, 12H, β -CH₂Trp), 3.65 (m, 8H, CH₂NH), 4.47 (m, 12H, α -CHTrp), 6.90 (t, J = 7.3 Hz, 12H, Ar), 6.98 (t, J = 7.4 Hz, 12H, Ar), 7.10 (s, 12H, Ar), 7.20 (m, 12H, Ar), 7.48 (m, 12H, Ar), 8.12 (s, 12H, NH), 10.73 (bs, 12H, NH-1^HTrp). ¹³C NMR (125 MHz, DMSO-d₆) δ : 27.64 (CH₂), 29.84 (CH₂), 30.53 (CH₂), 53.76 (CH₂), 110.34 (C), 111.77 (CH), 118.63 (CH), 118.79 (CH), 121.27 (CH), 124.04 (CH), 127.66 (C), 136.45 (C), 172.86 (C=O), 174.12 (C=O). HPLC [gradient: A:B, 10-100% of A in 10 min]: 8.99 min. HRMS (ESI) m/z : Calcd. for C₁₈₂H₁₉₆N₃₀O₄₀ 3441.4225. Found 3441.423.

4.1.24. Dendron nitro-nonaTrp(OMe) (**7**)

A solution of dendron **6** [16] (500 mg, 0.52 mmol) and H-(L)-Trp-OMe.HCl (2.08 g, 7.3 mmol) in DMF (20 mL) was treated with HATU (2.62 g, 7.3 mmol) and DIPEA (3.7 mL, 21.8 mmol). The mixture was stirred at room temperature during 3 days and then evaporated to dryness to afford a residue that was dissolved in dichloromethane (30 mL) and washed successively with aqueous solutions of citric acid (10%) (3 $\times$ 20 mL), saturated NaHCO₃ (3 $\times$ 20 mL), and brine (3 $\times$ 20 mL). The organic layer was dried over anhydrous Na₂SO₄, filtered and evaporated. The residue was purified on a Biotage HPFC system (High Performance Flash Chromatography) on reverse phase using water/acetonitrile (100:0 to 0:100) as eluent to obtain 1.3 g (91%) of dendron **7** as a yellow solid; m.p. 210-212 °C. ¹H NMR (300 MHz, DMSO-d₆) δ : 1.76 (m, 12H, CH₂), 2.06 (m, 36H, CH₂), 3.07 (m, 18H, β -CH₂Trp), 3.53 (s, 27H, OCH₃), 4.49 (m, 9H, α -CHTrp), 6.96 (t, J = 7.4 Hz, 9H, Ar), 7.04 (t, J = 7.4 Hz, 9H, Ar), 7.13 (m, 9H, Ar), 7.32 (d, J = 7.6 Hz, 9H, Ar), 7.47 (d, J = 7.7 Hz, 9H, Ar), 8.27 (d, J = 7.3 Hz, 9H, NH), 10.82 (m, 9H,

NH-1^hTrp). HPLC [gradient: A:B, 10-100% of A in 10 min]: 8.51 min. HRMS (ESI⁺) *m/z*: Cald. for C₁₄₈H₁₆₈N₂₂O₃₂ 2765.2195. Found 2765.2200.

4.1.25. Dendron nitro-nonaTrp(OH) (**8**)

To a solution containing the methyl ester derivative dendron **7** (200 mg, 0.07 mmol) in THF (10 ml), a solution of LiOH·H₂O (52.86 mg, 1.26 mmol) in water (10 ml) was added, and the mixture was stirred at room temperature during 3 days. Then formic acid was added to reach pH = 2, and volatiles were evaporated to dryness. The residue was purified on a Biotage HPFC system (High Performance Flash Chromatography) on reverse phase using water/acetonitrile (100:0 to 0:100) as eluent to afford 80 mg (42%) of **8** as a white solid; m.p. 219-221 °C. ¹H NMR (500 MHz, DMSO-d₆) δ: 1.72 (m, 12H, CH₂), 2.00 (m, 36H, CH₂), 2.97 (m, 9H, β-CH₂Trp), 3.13 (m, 9H, β-CH₂Trp), 4.43 (m, 9H, α-CHTrp), 6.94 (t, *J* = 7.5 Hz, 9H, Ar), 7.01 (t, *J* = 7.6 Hz, 9H, Ar), 7.10 (m, 9H, Ar), 7.29 (d, *J* = 7.8 Hz, 9H, Ar), 7.50 (d, *J* = 7.8 Hz, 9H, Ar), 8.08 (m, 9H, NH), 10.80 (s, 9H, COOH). ¹³C NMR (125 MHz, DMSO-d₆) δ: 27.65 (CH₂), 29.78 (CH₂), 30.65 (CH₂), 53.60 (C), 57.01 (CH), 110.40 (C), 111.80 (CH), 118.59 (CH), 118.79 (CH), 121.33 (CH), 124.01 (CH), 127.64 (C), 136.49 (C), 170.52 (C=O), 172.59 (C=O), 174.06 (C=O), 204.53 (C=O). HPLC [gradient: A:B, 10-100% of A in 10 min]: 7.96 min. HRMS (ESI) *m/z*: Cald. for C₁₃₉H₁₅₀N₂₂O₃₂ 2639.0787. Found 2639.0764.

4.2. General biological methods

For testing all the compounds were dissolved in dimethyl sulfoxide, stored at -20°C, protected from light and further diluted in culture medium prior to use.

4.2.1. Anti-HIV Activity Assays in CEM and MT-4 cell cultures

Inhibition of HIV-1(III_B)- and HIV-2(ROD)-induced cytopathicity in CEM cell cultures was measured in 200 µl-microtiter 96-well plates containing ~ 3 × 10⁵ CEM cells/ml, infected with 100 CCID₅₀ of HIV per milliliter and containing appropriate dilutions of the test compounds. After 4-5 days of incubation at 37°C in a CO₂-controlled humidified atmosphere, CEM giant (syncytium) cell formation was examined microscopically. The EC₅₀ (50% effective concentration) was defined as the compound concentration required to inhibit HIV-induced giant cell formation by 50%. Cytostatic activity was examined by counting the number of the CEM cells that were exposed to the different compound concentrations for 3 days by the use of a Coulter Particle Counter ZI (Harpender Herz, Analis, Ghent, Belgium).

Some of the compounds were also evaluated for their inhibitory activity against HIV-1 infection in MT-4 cell cultures. Briefly, MT-4 cells (1 × 10⁶ cells/mL) were pre-incubated for 30 min at 37°C with the test compounds in a 200 µl-96-well plate. Next, NL4.3 virus was added at 100 CCID₅₀ of the viral stock. The cytopathic effect was scored microscopically 5 days post-infection, and the 50% effective concentration (EC₅₀) values were determined using the MTS/phenazine ethosulfate method.

4.2.2. Time-of-addition (TOA) experiments

Time-of-addition experiments were adapted from previously reported methods [20,21]. Briefly, MT-4 cells were infected with HIV-1(III_B) at an m.o.i. of 0.5. Following a 1 hour adsorption period, cells were washed, distributed in a 96-well tray at 100,000 cells per well and incubated at 37°C. Test compounds were added at different times (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 24, and 25h) after infection. Product added at time point 0h, was present during the start of infection and was washed away after 1 hour. After washing, fresh product was added to maintain viral suppression throughout the rest of the experiment.

HIV-1 production was determined at 31h postinfection via a p24 enzyme-linked immunosorbent assay (Perkin Elmer, Brussels, Belgium). AMD3100 was used at 625 nM, dextran sulfate at 12.5 µM, AZT at 3.8 µM, ritonavir at 554 nM, compounds **17b** and **17f** at 10 µM.

4.2.3. Surface Plasmon Resonance Experiments for evaluation of the binding of the compounds to gp120 and gp41

Recombinant gp120 protein from the HIV-1 IIIB strain (ImmunoDiagnostics Inc., Woburn, MA) (produced in cell cultures of chinese hamster ovary cell cultures) and recombinant gp41 HIV-1(HxB2) (Acris Antibodies GmbH, Herford, Germany) were covalently immobilized on the carboxymethylated dextran matrix of a CM7 sensor chip in 10 mM sodium acetate, pH 4.0, using standard amine coupling chemistry up to a final density of respectively 11,241 RUs and 3,836 RUs. All interaction studies were performed at 25 °C on a Biacore T200 instrument (GE Healthcare, Uppsala, Sweden). The compounds were diluted in HBS-P (10 mM HEPES, 150 mM NaCl and 0,05% surfactant P20; pH 7.4) supplemented with 5% dimethyl sulfoxide (DMSO, Merck) and 10 mM Ca^{2+} at a concentration of 50 μM . Samples were injected for 2 minutes at a flow rate of 30 $\mu\text{L}/\text{min}$ followed by a dissociation phase of 2 minutes. The sensor chip surface was regenerated with an injection of 50 mM NaOH. A reference flow cell was used as a control for non-specific binding and refractive index changes. Several buffer blanks were used for double referencing. A DMSO concentration series was included to eliminate the contribution of DMSO to the measured response.

Binding affinities (K_D) were calculated for **5f** using a CM5 sensor chip at which 1450 RUs gp120 and 600 RUs gp41 were immobilized. For this purpose compound concentrations ranging between 0 and 40 μM were used and the K_D value was derived from steady state analysis in the Biacore T200 Evaluation Software 2.0. Following formula was used to calculate the K_D value of **5f**: $R_{\text{eq}} = C \cdot R_{\text{max}} / (K_D + C)$, where R_{eq} = equilibrium binding level; R_{max} = maximum binding level; K_D = equilibrium dissociation constant; C = concentration of analyte.

Supporting information

General chemistry and biological procedures as well as copies of representative ^1H and ^{13}C NMR spectra are included.

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LIST OF CAPTIONS

Fig. 1. Illustration of the structure of the synthesized compounds

Scheme 1. Synthesis of compounds **2a-d** and **3a-d**. *Reagents and conditions:* (i) HATU/DIPEA (ii) HOOCH, 40°C, 48 h

Scheme 2. Synthesis of compounds **4a-d** and **5a-d**. *Reagents and conditions:* (i) H-(L)-Trp-OMe.HCl or H-(L)-Trp-OBn.HCl HATU/DIPEA (ii) a) LiOH.H₂O (for **4a** and **4d**) b) H₂, Pd/C (for **4b** and **4c**), 40°C, 48 h.

Scheme 3. Synthesis of compounds **2e-g** and **3e-g**. *Reagents and conditions:* (i) HATU/DIPEA (ii) HOOCH, 40°C, 48 h

Scheme 4. Synthesis of compounds **4e-g** and **5e-g**. *Reagents and conditions:* (i) H-(L)-Trp-OMe.HCl or H-(L)-Trp-OBn.HCl HATU/DIPEA (ii) a) H₂, Pd/C (for **4e**), b) LiOH.H₂O (for **4f** and **4g**)

Scheme 5. Synthesis of compounds **7-9**. *Reagents and conditions:* (i) H-(L)-Trp-OMe.HCl, HATU/DIPEA (ii) a) LiOH.H₂O

Fig. 2. Time of drug-addition experiment in HIV-1-infected MT-4 cell cultures

Fig. 3. Binding amplitudes of test compounds against HIV-1 gp120/gp41

Fig. 4. Effect of α 1-3/ α 1-6-mannose trimer on the binding of **5f** against HIV-1 gp120/gp41

Table 1. Anti-HIV activity of the test compounds

Table 2. Apparent K_D value for **5f**-gp120 and **5f**-gp41