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Targeting HIV Entry through Interaction with Envelope Glycoprotein 120 (gp120): Synthesis and Antiviral Evaluation of 1,3,5-Triazines with Aromatic Amino Acids§

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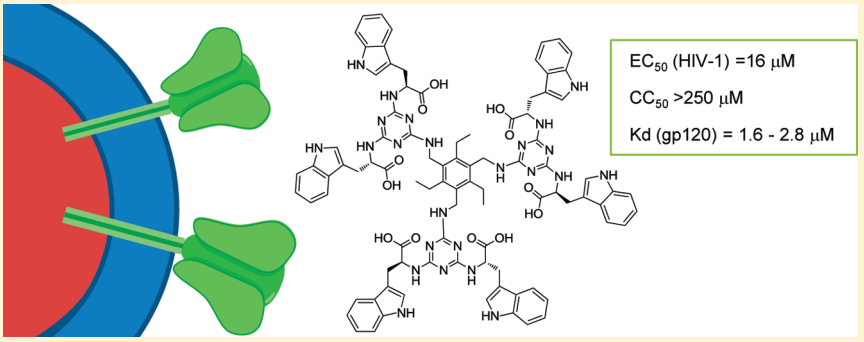
Supporting Information

ABSTRACT:



On the basis of the interesting inhibitory properties that lectins show against HIV-replication through their interaction with glycoprotein 120 (gp120), we here describe the design, synthesis, and anti-HIV evaluation of three series of 1,3,5-triazine derivatives (monomers, dimers, and trimers) functionalized with aromatic amino acids meant to mimic interactions that lectins establish with gp120. While monomers were inactive against HIV replication, dimers showed limited anti-HIV activity that is, however, considerably more significant in the trimers series, with EC_{50} values in the lower μM range. These findings most likely reflect the requirement of multivalency of the 1,3,5-triazine derivatives to display anti-HIV activity, as lectins do. The pronounced anti-HIV activity ($EC_{50} \sim 20\ \mu M$) is accompanied by the absence of toxicity in CEM T-cell line ($CC_{50} > 250\ \mu M$). Moreover, SPR experiments revealed that the prototype trimers with a central core of 2,4,6-triethylbenzene and six L-Trp or six L-Tyr residues at the periphery were efficient binders of CXCR4- and CCR5-tropic HIV-1 gp120 (estimated K_D: lower micromolar range). The collected data support the interest of this novel family of anti-HIV agents and qualify them as potential novel microbicide lead compounds.

INTRODUCTION

Despite the enormous efforts devoted during the last 25 years to human immunodeficiency virus (HIV) drug development and the current availability of anti-HIV agents, there is a continuous need to develop new strategies and/or active principles to act against this pandemic pathogen.[1,2] Some of the reasons to continue the search of novel therapeutic agents against HIV are: (1) an effective vaccine is still far from being available, (2) the current drug regimens need to be better tolerated and/or should reach a wider target population, and (3) the increasing emergence of resistance to existing drugs demand the search for new agents that do not afford cross-resistance or are not affected by such resistance mutations.[3]

Recently, a conceptually new strategy for anti-HIV therapy has been proposed targeting the glycans of the glycoprotein gp120 of the viral envelope.[4,5] This strategy is based on experimental results obtained with carbohydrate-binding proteins that interact with HIV-1 gp120. Glycans of the viral envelope play a crucial role in viral transmission and act as a shield blocking the recognition of the underlying immunogenic epitopes by the immune system of the host.[5,6] It is well-known that lectins (i.e., carbohydrate-binding proteins) from different origin are able to inhibit HIV infection and HIV transmission by blocking virus-to-cell and cell-to-cell contact.[7,8] It is thought that these lectins exert their anti-HIV activity in the early steps of the replicative cycle by binding to the carbohydrates present on gp120 and compromising the required conformational changes in gp120/gp41 for optimal interaction with the (co)-receptors and fusion of the virus envelope with the target cell membrane.[4] Prolonged exposure to lectins, as with any other antiviral drug, results in the emergence of drug resistance. However, pronounced phenotypic

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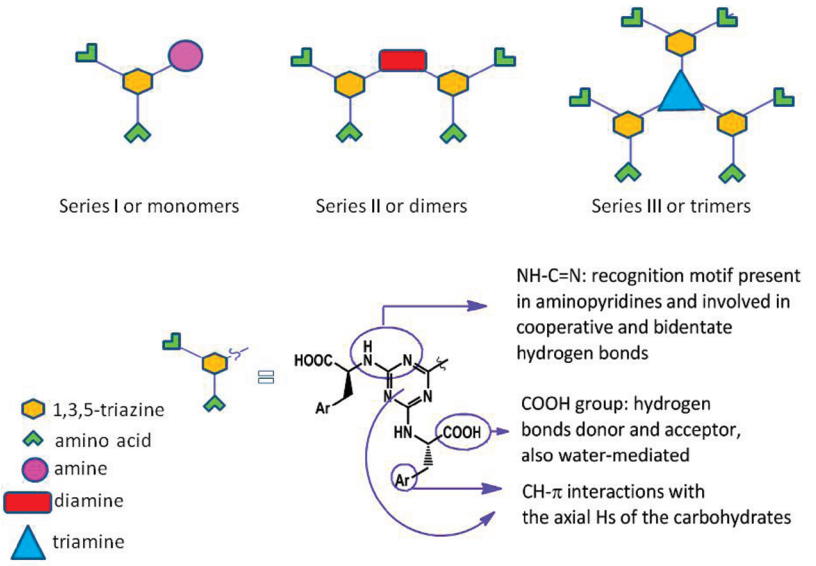


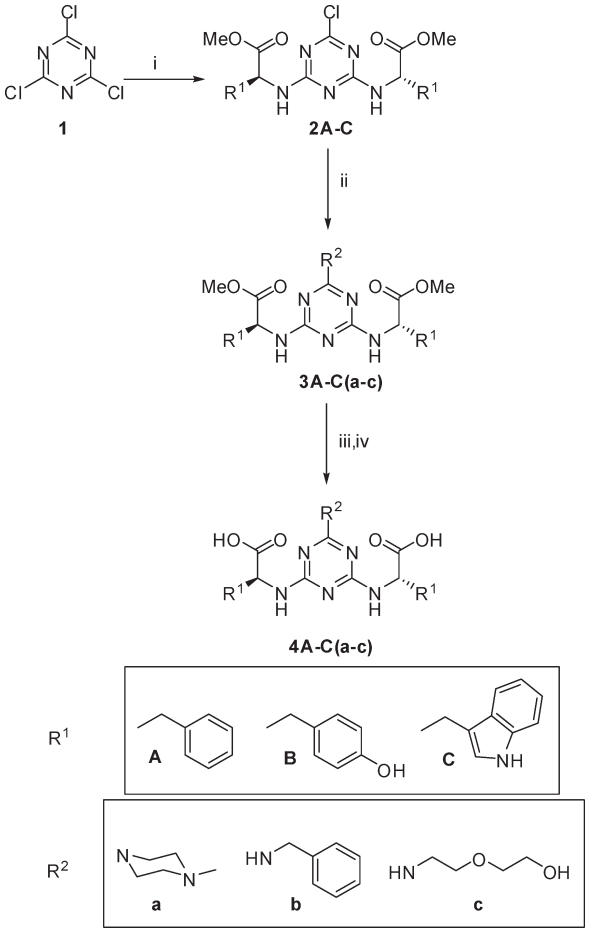
Figure 1. Schematic representation of the proposed structures.

resistance to lectins only appears when several mutations are accumulating in gp120.[5,8] Moreover, these mutations predominantly affect the *N*-glycosylation sites of gp120 and up to 16 different sites have been found deleted under lectin pressure.[9−16] As a consequence, mutant viruses contain a much less glycosylated envelope. On the basis of data obtained in monkeys challenged with mutated simian immunodeficiency virus (SIV) that has two *N*-glycosylation sites deleted in the envelope,[17] it has been found that a humoral response to the previously hidden epitopes on the envelope of such a virus occurred. Therefore, lectins may have a dual mechanism of action: (1) directly by binding to the glycans of the HIV envelope and thus blocking viral entry, and (2) indirectly by favoring deletions in the envelope glycan shield, triggering the immune system to recognize previously hidden immunogenic epitopes.[4,5]

Lectins suffer from a number of drawbacks that may compromise their development as therapeutic agents,[7] including their high molecular weight and peptidic nature. Therefore it would be desirable to find small-sized nonpeptidic molecules that behave in a similar way as lectins do in their interaction with the viral gp120. Very recent data with two nonpeptidic small-size antibiotics, Pradimicin-A (PRM-A) and its more soluble analogue Pradimicin-S, provide evidence that this search can be fruitful.[13,15]

On the basis of these promising data, our objective has been to design, synthesize, and evaluate against HIV novel compounds able to mimic the interactions that lectins establish with the glycans of gp120 so that eventually we could design a synthetic affordable molecule that interacts with gp120 in a similar way as the lectins do. For this purpose, we have considered three series of compounds that are schematically represented in Figure 1. The three compound series have a common motif: a 1,3,5-triazine ring functionalized with two aromatic amino acids through each of their amino groups. The number of amino acid residues goes from 2 in the first series (series I), also designated as monomers, to 4 in the second series designated as dimers (series II), and amounts up to 6 amino acids in the third series of trimers (series III). The selection of this structural motif is based on their richness in recognition elements commonly found in natural lectins or lectin mimetics. Thus, the NH-C═N motif is present in aminopyridines

Scheme 1. Synthesis of Monomers[a]



[a] Reagents and conditions: (i) H-Phe(OMe).HCl, THF, DIPEA, 25 °C, 24 h; (ii) *N*-methylpiperazine (a), benzylamine (b), or 2-(2-aminoethoxy)ethanol (c), 1,4-dioxane, DIPEA, 60−100 °C, 24 h or 100 °C (MW), 1−2 h; (iii) $LiOH \cdot H_2O$, THF:H_2O (1:1), rt, 16 h; (iv) acidification with HCOOH and purification with Amberlite IRA400 ($HCOO^-$ form); when R^2═c neutralization with HCl and purification through reverse phase chromatography.

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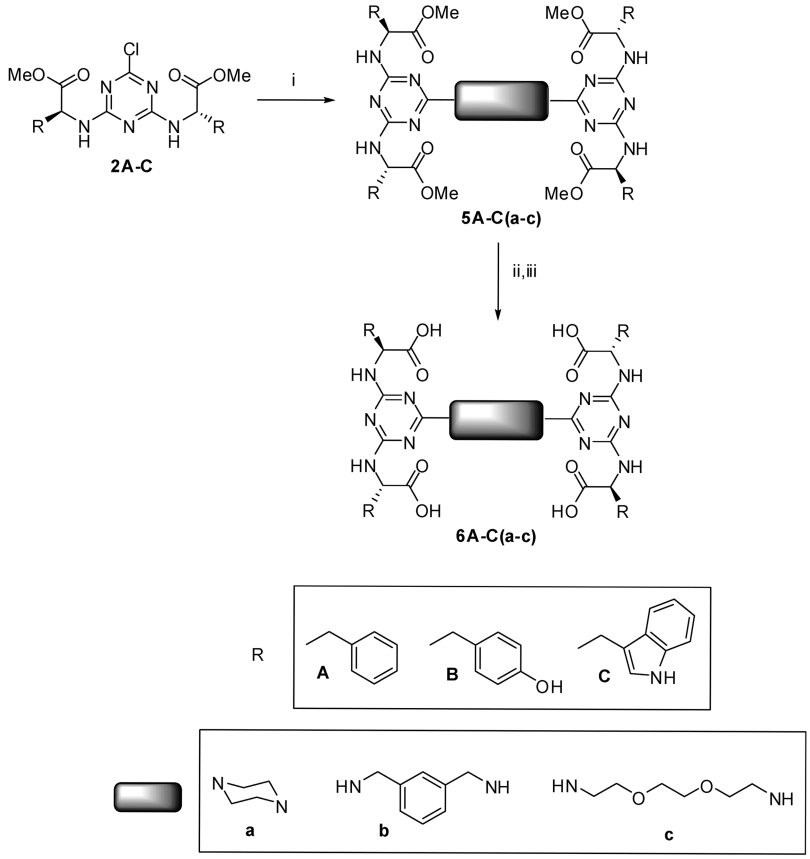
Scheme 2. Synthesis of the Dimers 6A−C(a−c)[a]



[a] Reagents and conditions: (i) piperazine (a), *m*-xylylenediamine (b), or 2,2′-(ethylenedioxy) diethylamine (c), 1,4-dioxane, DIPEA, 100 °C, 48 h or 100−120 °C (MW), 2−3 h; (iii) LiOH·H_2O, THF:H_2O (1:1), rt, 16 h; (iv) acidification with HCOOH and purification with Amberlite IRA400 ($HCOO^-$ form).

and aminopyrimidines, extensively studied for their ability to recognize carbohydrates, and proposed to participate in hydrogen bonding with the OHs of the sugars.[18−20] Carboxylates of the amino acids are good candidates for hydrogen bonding with the sugars and, importantly, are crucial for the water solubility of the proposed structures. It should be kept in mind that the interaction between the synthesized compounds and the glycans of the viral envelope would take place in a highly solvated environment at the surface of the virus. Last, but not least, the presence of the aromatic side chains of the amino acids may participate in CH−π interactions between the aromatic clouds of the side chains and the CH of the sugars, a type of interaction that is being given a predominant role and that is frequently observed in X-ray complexes of lectins with carbohydrates.[21−24] Thus, the proposed structures offer a wide variety of potential interactions with the carbohydrates of gp120.

The carbohydrate binding site in lectins is usually a shallow depression at the surface of the lectins. Therefore, the interactions between lectins and carbohydrates are quite labile and occur in solvent-exposed recognition sites.[21,25] However, nature tries to compensate this lability by the establishment of concomitant multiple interactions, a concept that has been designated as multivalency.[21,25] Therefore, in the proposed series of compounds, going from monomers to dimers and further to trimers, we expected to increase the binding of the compounds to the carbohydrates of gp120 due to an increase of the number of recognition elements.

RESULTS AND DISCUSSION

Chemistry. The proposed structures have been synthesized in a three-step strategy: (1) reaction of cyanuric chloride with the free amino groups of aminoacids to obtain monochlorotriazines, (2) nucleophilic substitution reactions with mono-, di-, or triamines to obtain monomers, dimers, or trimers, respectively, and finally, (3) saponification of the methyl esters of the amino acids to afford the desired free acids. This strategy fulfills our demands to have access to the proposed structures following a robust and highly versatile pathway so that the compounds are easily accessible and can be subjected to further modifications.

Thus, reaction of cyanuric chloride (**1**) with 2 equiv of H-L-Phe-OMe, H-L-Tyr-OMe, or H-L-Trp-OMe in THF and in the presence of DIPEA at rt for 24 h afforded the monochlorotriazines **2A−C** in high yields (72−82%) (Scheme 1). Nucleophilic substitution reactions in monochlorotriazines require heating either under standard thermal conditions or assisted by microwave.[26,27] Either of the two procedures has been used for the reaction of the three chlorotriazines **2A−C** with three different amines (*N*-methylpiperazine (**a**), benzylamine (**b**), or 2-(2-aminoethoxy)ethanol (**c**)) in dioxane to afford nine trisubstituted compounds **3A−C(a−c)** in good yields (57−88%) (Scheme 1). Finally, saponification of the methyl esters with LiOH·H_2O overnight followed by acidification and a "catch and release" purification protocol by anchoring to an anion exchange

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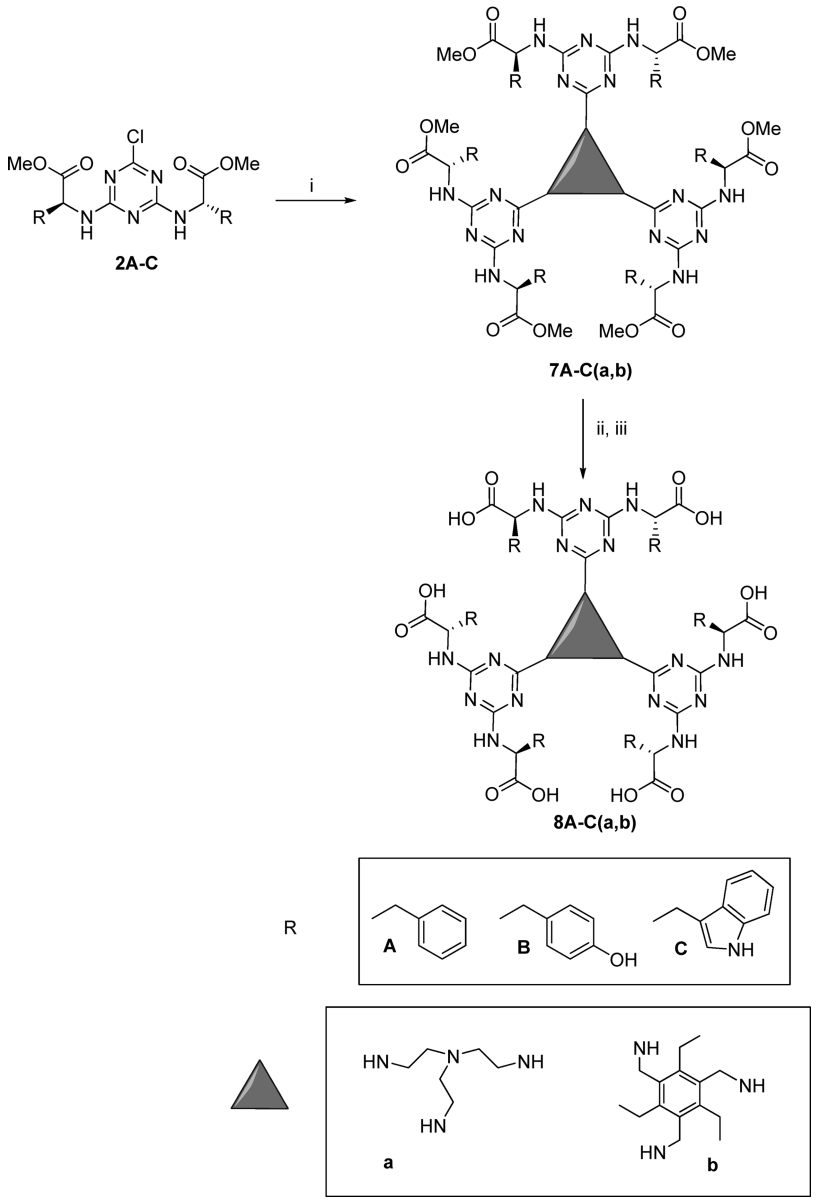
Scheme 3. Synthesis of the Trimers 8A−C(a,b)[a]



[a] Reagents and conditions: (i) tris(2-aminoethyl)amine (a) or 1,3,5-tris(aminoethyl)-2,4,6-triethylbenzene (b), 1,4-dioxane, DIPEA, 100−120 °C (MW), 3 h; (iii) LiOH·H_2O, THF:H_2O (1:1), rt, 16 h; (iv) acidification with HCOOH and purification with Dowex 1 × 2 ($HCOO^-$ form).

resin ($HCOO^-$) and elution with dioxane:HCOOH[28] afforded the free acids **4A−C(a−c)** in good to excellent yields. It should be mentioned that those compounds with a 2-(2-aminoethoxy)-ethanol substituent **4A−C(c)** could not be purified under these conditions due to partial incorporation of a formyl moiety at the terminal hydroxyl group. Instead, these compounds were purified by reverse phase flash chromatography.

The monochlorotriazines **2A−C** were used for the synthesis of the dimers **5A−C(a−c)** by reaction with symmetrical diamines as depicted in Scheme 2. The diamines employed were piperazine (**a**), 1,3-xylylenediamine (**b**), or 2,2′-(ethylenedioxy)diethylamine (**c**). Also, in this case, either standard heating or microwave-assisted conditions can be employed. The yields under both procedures are similar, but the reaction time is clearly shortened from 48 h at 100 °C under standard thermal conditions to only 2 h when the reaction was microwave-irradiated. Thus, the use of the microwave significantly reduced the reaction time and therefore had a significant impact in the speed-up of the synthesis. In all cases, the optimal number of diamine equivalents to favor dimer formation was established as 0.6 equiv independently of the heating procedure and/or the nature of the diamine. Thus nine dimers **5A−C(a−c)** were obtained in yields varying between 58% and 83%. Saponification of the four methyl esters in each dimer with LiOH·H_2O followed by purification with a formate anion exchange resin afforded the dimers **6A−C(a−c)** (Scheme 2).

Following a similar approach, the monochlorotriazines **2A−C** reacted with the commercial tris(2-aminoethyl)amine (**a**) or the synthesized 1,3,5-tris(aminoethyl)-2,4,6-triethylbenzene[29] (**b**) (Scheme 3) to afford the trimers **7A−C(a,b)** in moderate yields (Scheme 3). In this case, only microwave-assisted conditions

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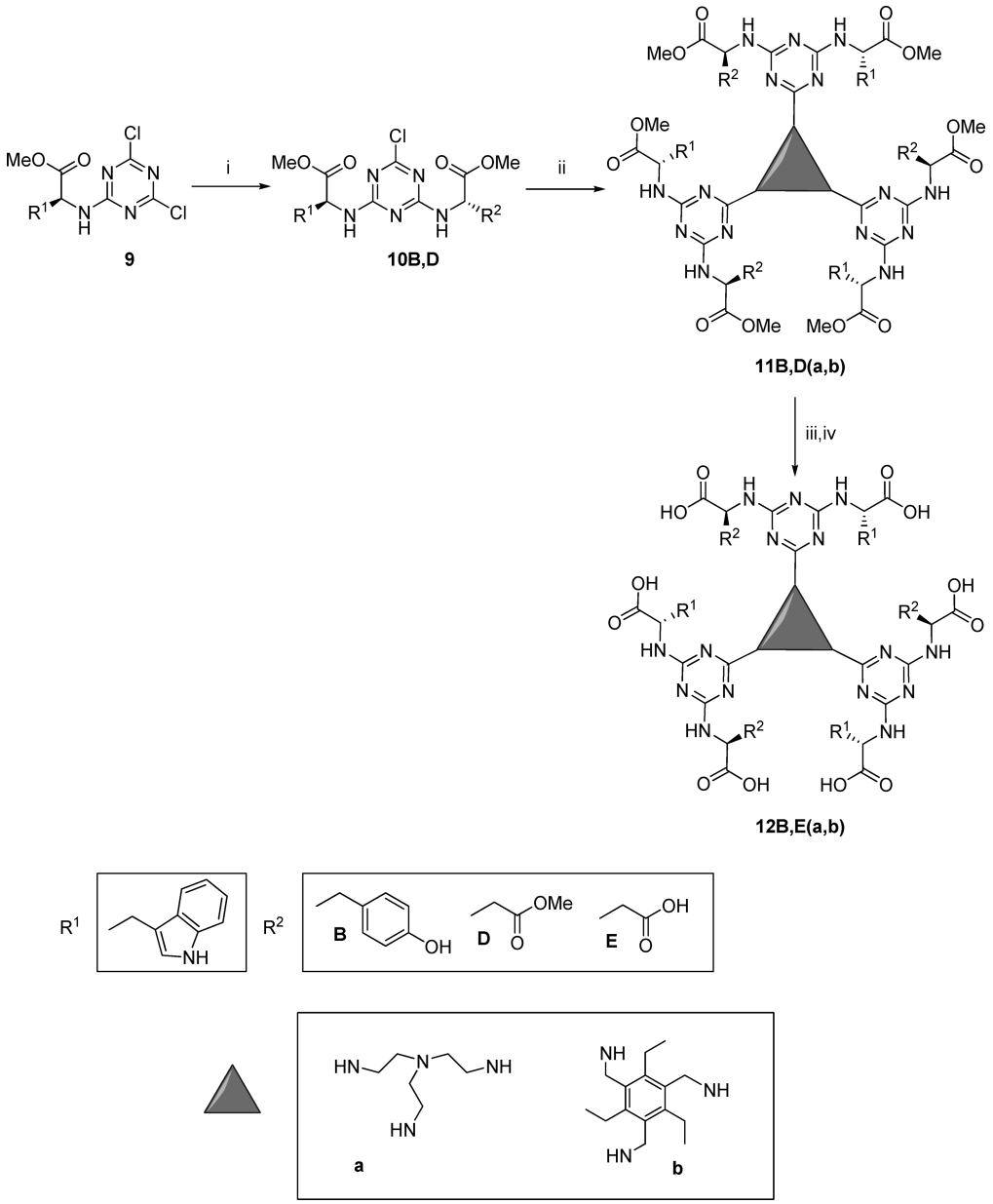
Scheme 4. Synthesis of Trimers 12B,E(a,b)[a]



[a] Reagents and conditions: (i) H-Tyr(OMe)·HCl or H-Asp(OMe)-OMe·HCl, THF, DIPEA, 25 °C, 16 h; (ii) tris(2-aminoethyl)amine (a) or 1,3,5-tris(aminoethyl)-2,4,6-triethylbenzene (b), 1,4-dioxane, DIPEA, 100−120 °C (MW), 3 h; (iii) LiOH·H_2O, THF:H_2O (1:1), rt, 16 h; (iv) acidification with HCOOH and purification with Dowex 1 × 2 ($HCOO^-$ form).

were employed and the best results were obtained with 0.3 equiv of the triamines at 100−120 °C for 3 h. Treatment with LiOH·H_2O overnight for the saponification of the six methyl esters and purification with a formate anion exchange resin afforded the target compounds **8A**−**C**(**a**,**b**) in good to high yields.

As will be later discussed in the Antiviral Evaluation section, some trimers, in particular compounds with tryptophans both with a central scaffold of tris(2-aminoethyl)amine and with a skeleton of 1,3,5-tris(aminoethyl)-2,4,6-triethylbenzene (compounds **8Ca** and **8Cb**, respectively), afforded significant anti-HIV activity in cell culture. Also, the tyrosine derivative (**8Bb**) with a central scaffold of triethylbenzene was similarly active. Thus our next series of modifications were performed based on these findings.

Taking into account the anti-HIV activity of **8Bb** and **8Cb**, both with a central core of triethylbenzene and differing in the presence of Tyr (**8Bb**) or Trp (**8Cb**) at the periphery, we considered of interest the synthesis of a similar analogue alternating Trp and Tyr residues. Thus, reaction of cyanuric chloride (**1**) with 1 equiv of H-L-Trp-OMe, in this case at low temperature, led to the monosubstituted compound **9** in 84% yield (Scheme 4). Then, reaction of **9** with H-L-Tyr-OMe at room temperature afforded the mixed chlorotriazine **10B** in 81% yield. Treatment of **9** with the triamine of triethylbenzene under microwave irradiation allowed the synthesis of the mixed trimer **11Bb** in 38% yield (Scheme 4). Deprotection of the methyl esters afforded compound **12Bb**, which may be considered as a hybrid of compounds **8Bb** and **8Cb**.

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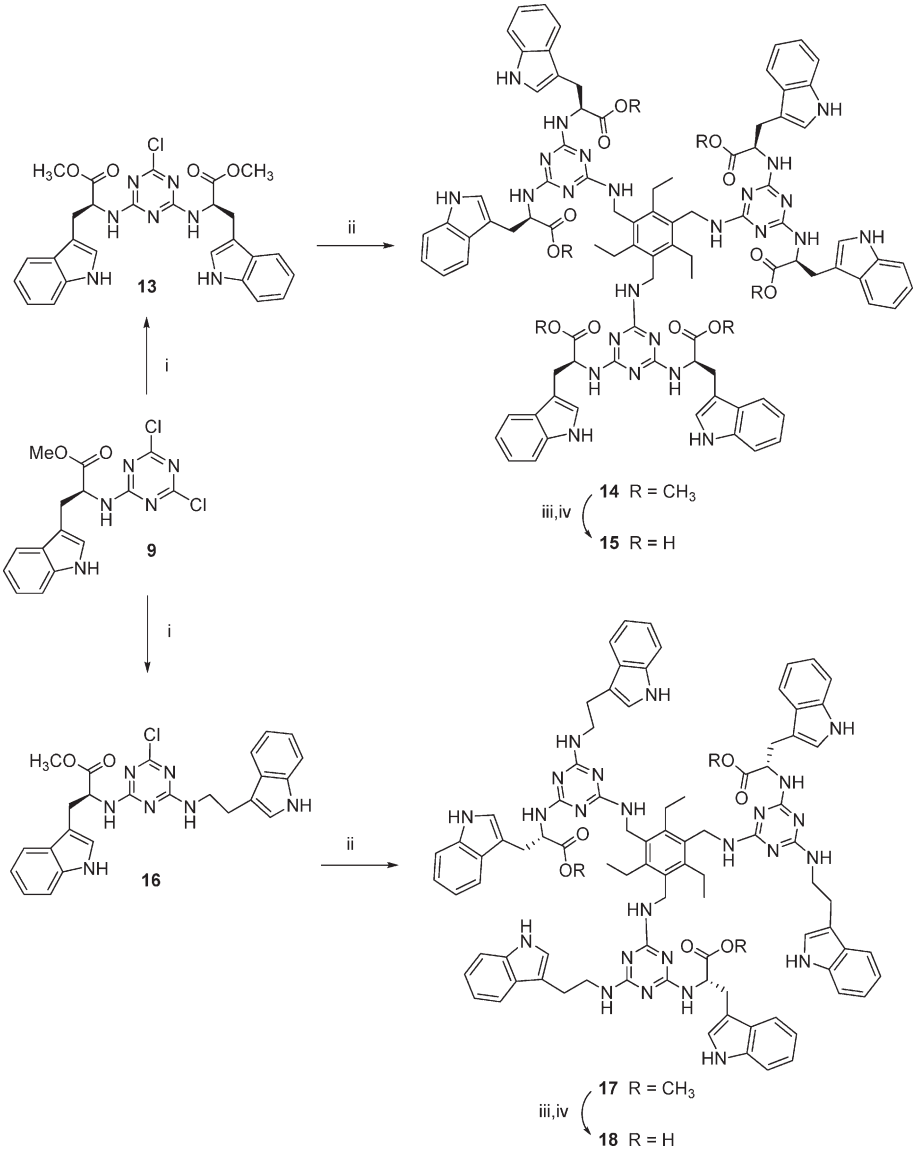
Scheme 5. Synthesis of Trimers 15 and 18[a]



[a] Reagents and conditions: (i) H-D-Trp(OMe).HCl or triptamine, THF, DIPEA, 25 °C, 16 h; (ii) 1,3,5-tris(aminoethyl)-2,4,6-triethylbenzene, 1,4-dioxane, DIPEA, 100−120 °C (MW), 3 h; (iii) LiOH·H_2O, THF:H_2O (1:1), rt, 16 h; (iv) acidification with HCOOH and purification with Dowex 1 × 2 ($HCOO^-$ form).

With the idea to increase the number of carboxylates that could potentially participate in hydrogen bonding interactions, new trimers with Trp and Asp residues in the periphery were envisioned. Reaction of **9** with H-L-Asp(OMe)-OMe in THF at rt afforded the mixed chlorotriazine **10D** in 62% yield (Scheme 4). Treatment of this triazine with the two triamines (tris(2-aminoethyl)amine (**a**) or 1,3,5-tris(aminoethyl)-2,4,6-triethylbenzene (**b**)) in dioxane and DIPEA under microwave conditions yielded the mixed trimers **11Da** and **11Db**, respectively. Treatment of both compounds with LiOH·H_2O saponified the α-methyl esters of the Trp and Asp and the esters of the side chain of the Asp residues. After purification with an anion exchange resin, the acids **12Ea** and **12Eb** were obtained in 96% and 91% yield, respectively (Scheme 4).

Compound **9** was used as starting material for the synthesis of two additional analogues of the Trp derivative **8Cb**. We could take advantage of the chiral nature of the amino acids to synthesize an analogue of **8Cb** where half of the Trp units were substituted by the non-natural D-Trp. In this way, we kept the same recognition elements but we varied their relative spatial disposition. Thus, reaction of **9** with H-D-Trp-OMe in THF at rt afforded the chlorotriazine **13** in 90% yield (Scheme 5). Reaction of **13** with the triamine of triethylbenzene in dioxane, in the presence of DIPEA, in the microwave at 120 °C, afforded after 3 h the trimer **14** that was transformed into the acid **15**.

Finally, we addressed the synthesis of a trimer alternating L-Trp and triptamine, a "decarboxylated" Trp analogue. In this case, reaction of **9** with triptamine at rt afforded the monochlorotriazine **16** in 59% yield (Scheme 5). Reaction of **16** with 1,3,5-tris(aminoethyl)-2,4,6-triethylbenzene followed by saponification of the methyl ester residues afforded the trimer **18**.

Antiviral Evaluation. The synthesized compounds were evaluated for their inhibitory activity against HIV-1(III_B) and

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Table 1. Anti HIV-1(III_B) and anti-HIV-2(ROD) Activity and Cytostatic Properties of the Test Compounds in Human T-Lymphocyte (CEM) Cell Cultures

	$EC_{50}(\mu M)$[a]		
compd	HIV-1(III_B)	HIV-2(ROD)	CC_{50} (μM) [b]
6Aa	106 ± 8.2	121 ± 36	>250
6Ab	>10 [c]	>10 [c]	>10 [c]
6Ac	≥250	≥250	>250
6Ba	≥250	≥250	>250
6Bb	148 ± 28	80 ± 72	>250
6Bc	>250	>250	>250
6Ca	65 ± 9.9	63 ± 13	>100
6Cb	56 ± 24	81 ± 6.4	>250
6Cc	112 ± 0.0	120 ± 9.3	>250
8Aa	65 ± 21	216 ± 49	>250
8Ba	≥250	≥250	>250
8Bb	22 ± 0.0	22 ± 2.8	>250
8Ca	20 ± 3	65 ± 21	>250
8Cb	16 ± 0.71	22 ± 2.8	>250
12Bb	26 ± 4.2	24 ± 0.0	>250
12Ea	216 ± 49	>250	>250
12Eb	18 ± 0.71	33 ± 2.1	>250
15	19 ± 2.8	22 ± 2.8	>250
18	>10[c]	>10[c]	>10[c]
PRM-A[d]	5.2	5.9	>50
PRM-S[d]	8.9	9.6	>250

[a] 50% effective concentration or compound concentration required to protect 50% of the cells against the cytopathic effect of the virus. [b] 50% cytostatic concentration or compound concentration required to inhibit CEM cell proliferation by 50%. All data are mean values ± standard deviation for at least three independent experiments. [c] Compound precipitation was detected at higher compound concentration. [d] Data taken from Balzarini et al.[15]

HIV-2(ROD) replication in CEM T-cell cultures. The monomers **4A**−**C**(**a**−**c**) were devoid of anti-HIV activity at the highest concentration tested (250 μM). The data obtained from the evaluation of the dimers and trimers are shown in Table 1. PRM-A and PRM-S are included as reference compounds. Among the dimers containing Phe, only compound **6Aa** with piperazine as the central core showed marginal but measurable anti-HIV activity (EC_{50} 106−121 μM). Concerning the dimers containing Tyr, the *m*-xylylenediamine derivative **6Bb** was the only one that afforded measurable antiviral activity. However, among the Trp-containing dimers (**6Ca**, **6Cb**, **6Cc**), all showed anti-HIV activity at EC_{50} values between 56 and 120 μM. With the exception of compound **6Ab**, which could only be tested at 10 μM due to a limited solubility, none of these compounds were cytotoxic at the highest concentration tested (250 μM). Thus, although the antiviral activity of these compounds could be considered as rather moderate based on the EC_{50} values, it can be stated that the observed activity reflects a specific antiviral effect because there was no inhibitory effect of the compounds on the proliferation rates of the cell cultures and there were no morphologically visible changes observed at compound concentrations as high as 250 μM.

Interestingly, when the trimers are considered, the antiviral effect is clearly further improved. Among those compounds with tris(2-aminoethyl)amine as the central core, the most active compounds showed EC_{50} values against HIV-1(III_B) of ∼20 μM. The most interesting results were obtained with the trimers containing a triethylbenzene as the central unit. Thus, compounds **8Bb** and **8Cb** showed EC_{50} values against HIV-1(IIIB) of 22 ± 0 and 16 ± 0.71 μM, respectively. The mixed trimer **12Bb**, alternating Tyr and Trp residues at the periphery, also showed comparable activity (EC_{50} = 24−26 μM). Other mixed trimers in the triethylbenzene series with Trp/Asp alternation (**12Eb**) or L-Trp/D-Trp alternation (**15**) were similarly active in cell culture against HIV as the parent Trp-containing trimer compound **8Cb**. Compound **18**, an **8Cb** analogue where half of the Trp residues have been replaced by tryptamines, could not be tested at a concentration higher than 10 μM, but at this concentration no significant antiviral effect was detected.

Compounds **8Bb** and **8Cb** were also tested against the HIV-1/Ba-L, a CCR5-tropic HIV-1 strain, in PBMC. A similar anti-HIV-1 activity for these compounds (EC_{50} ∼ 20 μM) was found as observed for the CXCR4-tropic HIV-1(III_B) strain, pointing to an efficient suppression of both CXCR4- and CCR5-tropic virus strains in cell culture.

It should be mentioned that, in general, EC_{50} values against HIV-2 were often slightly higher than those against HIV-1, but it is not clear whether this difference is physiologically relevant. The antibiotics PRM-A and PRM-S, reference compounds, are only 2- to 5-fold more active than the most active compound in the series **8Cb**.

Many lectins have shown to be mitogenic, and this compromises their use as potential anti-HIV agents. The **8Bb** and **8Cb** derivatives have also been evaluated for their potential to be mitogenic and for their capacity to induce cellular (i.e., CD25, CD69, and HLA-DR) activation markers in freshly isolated PBMC. Interestingly, none of them proved mitogenic or were able to affect expression of CD25, CD69, or HLA-DR. At 50 μM, there were no signs of cytotoxicity in the PBMC cultures.

Interestingly, the methyl esters of all the compounds included in Table 1 (i.e., **7A**−**C**(**a**,**b**); **11B**−**D**(**a**,**b**); ...), which acted as their synthetic precursors, were devoid of antiviral activity. These findings point to the crucial importance of the free carboxylic acids for the anti-HIV activity and support our design strategy. Also, sodium salt preparations of several of the compounds showed similar activities as the free acid compounds (data not shown).

In summary, in both the dimer and trimer series, but not the monomer series, antivirally active compounds were found. Trp derivatives were endowed with the most significant antiviral activities. Moreover, trimers afforded better EC_{50} values than dimers, and this fact may possibly be ascribed to the requirement of a certain degree of multivalency to show antiviral activity. In this respect, it should be noticed that the putative gp120 target present in the HIV-1 envelope or on virus-infected cells is trimeric[30] and thus the trimeric triazine derivatives may interact with several gp120 molecules of the gp120 trimers at the same time.

Not only the nature of the amino acids and their number have an impact on the antiviral activity, but also the central core seems to be quite a determinant for their eventual activity. In general, derivatives of triethylbenzene afforded the most potent inhibitors. This finding may be ascribed to the known property of trisubstituted triethylbenzene derivatives to sterically predispose the substituents in such a way that the final architecture is more

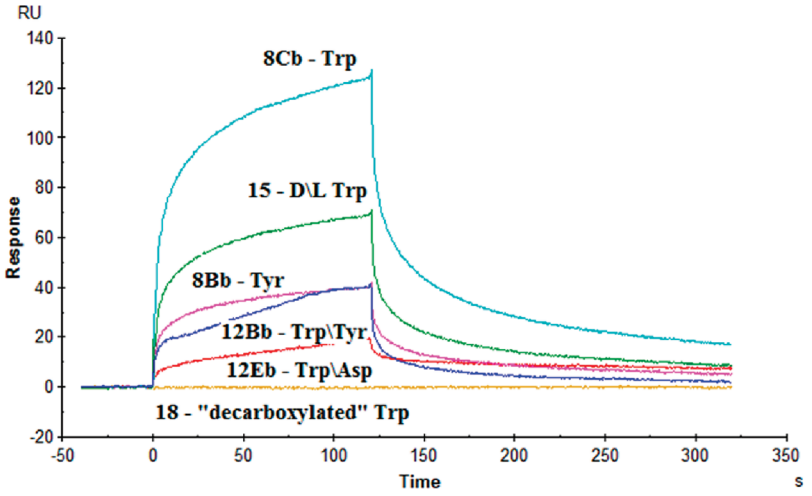

Figure 2. SPR analysis of the binding of compounds **8Bb** (magenta), **8Cb** (cyan), **12Bb** (blue), **12Eb** (red), **15** (green), and **18** (gold) to gp120 (III_B) injected over the surface at a fixed concentration of 12.5 μM. The biosensor chip density was 10400 RUs .

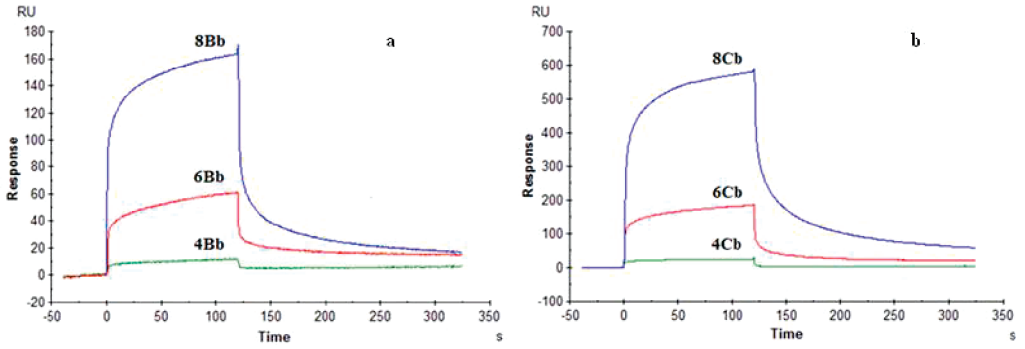

Figure 3. SPR analysis of the binding mode of monomers, dimers and trimers of Try and Trp to HD gp120 chip at a fixed concentration of 50 μM. (a) Tyr derivatives: trimer **8Bb** (blue), dimer **6Bb** (red), and monomer **4bB** (green). (b) Trp compounds: trimer **8Cb** (blue), dimer **6Cb** (red), and monomer **4Cb** (green).

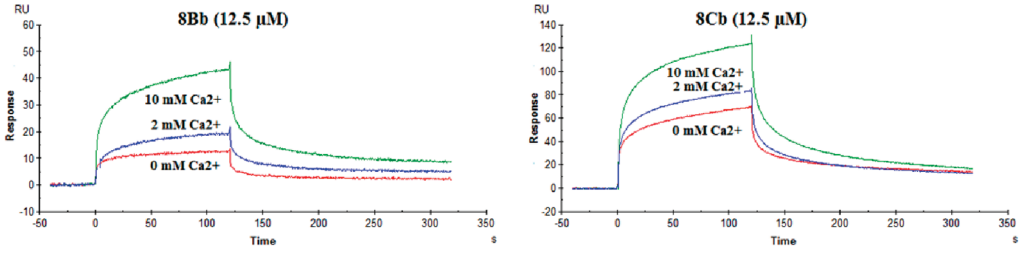

Figure 4. SPR analysis of the influence of different Ca^{2+} concentrations on the binding of the Tyr trimer **8Bb** (on the left) and the Trp trimer **8Cb** (on the right) to HD gp120. The compounds were both at a fixed concentration of 12.5 μM. The concentrations of Ca^{2+} tested were: 10 mM (green), 2 mM (blue), and 0 mM (red).

preorganized.[31] Thus it may be postulated that the whole architecture of the molecules determined by the central core as well as the nature of the different amino acids at the periphery of the molecules are important for the eventual antiviral activity.

Several types of anionic compounds have been previously identified for their inhibitory activity against virus infections. They include the polyvalent phosphothiolated 8mer G-quartet oligonucleotides (i.e., ISIS 5320),[32] antisense oligonucleotides (i.e., ISIS 2922, fomivirsen),[33−35] polyanionic sulfated carbohydrate polymers (i.e., dextran sulfate, cellulose sulfate),[36−38] and sulfonated polymers (i.e., PRO 2000).[37,39,40] Several of them failed during clinical trials due to toxic side effects and/or lack of proven antiviral activity, whereas some of them were approved to be used for antiviral treatment. Fomivirsen has indeed shown to provide effective treatment for peripheral HCMV retinitis in patients with AIDS and in those with relapsed HCMV retinitis unresponsive to conventional therapy.[35] Thus, although the eventual clinical success of such compounds seems to be rather unpredictable, some polyanions may be useful for clinical use.

Surface Plasmon Resonance (SPR) Studies. The most interesting compounds based on the HIV-infected cell culture assays were subjected to SPR analysis to determine their capacity

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Table 2. Kinetic Analysis of 8Bb and 8Cb versus HIV-1 gp120 Using SPR Technology

		low density HIV-1 (III_B) gp120			low density HIV-1 (ADA) gp120			high density gp120		
	conc range (μM)	K_D (μM)	k_{on} (1/Ms)	k_{off} (1/s)	K_D (μM)	k_{on} (1/Ms)	k_{off} (1/s)	K_D (μM)	k_{on} (1/Ms)	k_{off} (1/s)
8Bb	1.6−12.5	1.97 ± 0.64	$(2.31 \pm 0.17) \times 10^3$	$(4.01 \pm 1.77) \times 10^{-3}$	1.71 ± 0.63	$(2.09 \pm 0.71) \times 10^3$	$(3.36 \pm 0.10) \times 10^{-3}$	2.61 ± 0.37	$(2.48 \pm 0.01) \times 10^3$	$(6.44 \pm 0.84) \times 10^{-3}$
8Cb	1.6−12.5	1.61 ± 0.94	$(2.92 \pm 0.08) \times 10^3$	$(4.73 \pm 2.87) \times 10^{-3}$	2.51 ± 0.04	$(2.33 \pm 0.07) \times 10^3$	$(5.83 \pm 0.09) \times 10^{-3}$	2.82 ± 0.02	$(2.58 \pm 0.17) \times 10^3$	$(7.26 \pm 0.44) \times 10^{-3}$

to interact with the viral envelope glycoprotein gp120. In particular, we have focused on the most active compounds in cell culture (i.e., **8Cb** and **8Bb**) and their closest structural analogues. HIV-1 (IIIb) gp120 was covalently immobilized on the sensor chip at high density (HD) (10400 RU) and low density (LD) (792 RU).

In a first series of experiments, the most active anti-HIV compounds were investigated for their binding to HIV-1 gp120 at 12.5 μM, using the HD gp120 chip (10400 RU). There were striking differences in terms of binding amplitude to gp120 (Figure 2). The Trp derivative **8Cb** showed the highest binding amplitude followed by the L-Trp/D-Trp derivative **15** and the Tyr and Trp/Tyr derivatives **8Bb** and **12Bb**. Whereas the Trp/Asp derivative **12Eb** showed a rather poor binding interaction with gp120, the "partially decarboxylated" Trp derivative **18** containing three carboxylic acid units less than **8Cb** was devoid of any measurable binding to gp120 (Figure 2). For further more in-depth studies, we mainly focused on the Trp derivative **8Cb** and the Tyr derivative **8Bb**.

We first wanted to compare the trimers **8Cb** and **8Bb** with their corresponding antivirally poorly active dimers **6Cb** and **6Bb** and inactive monomers (**4Cb** and **4Bb**). The compounds were injected at a relatively high concentration (50 μM) to the HD gp120 chip to enable detection of a potential low binding affinity of the test compounds. Whereas the trimers **8Cb** and **8Bb** showed the highest binding amplitudes to gp120, the dimers **6Bb** and **6Cb** showed a much lower amplitude of binding , whereas the binding of the monomers **4Bb** and **4Cb** to HIV-1 gp120 could barely be detected (Figure 3). These striking differences in binding to gp120 correlate well with the observed antiviral activity of the test compounds (EC_{50} trimer < dimer ≪ monomer (inactive)).

A number of natural lectins such as mannose-binding lectin (MBL),[24] dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN),[23] but also the nonpeptidic Pradimicin antibiotics,[13,15] belong to the class of C-type lectins that are Ca^{2+}-dependent to exert their carbohydrate-binding properties, whereas other lectins such as a broad variety of plant lectins are Ca^{2+}-independent.[7] It was therefore of particular interest to investigate the effect of the presence of Ca^{2+} on the binding of the Trp and Tyr trimers to gp120 (Figure 4). In the presence of 10 mM Ca^{2+}, both **8Bb** and **8Cb** trimers efficiently bound gp120, and this binding was slightly reduced in the absence of Ca^{2+} (at only a 2- to 4-fold lower amplitude). Therefore, it could be concluded that the trimers were only partially dependent on the presence of Ca^{2+} and could still markedly bind to gp120 in the complete absence of Ca^{2+}.

Next, detailed kinetics were performed for the Trp and Tyr trimers **8Cb** and **8Bb**. For this purpose, kinetics of **8Bb** and **8Cb** were performed on both a high-IIIB gp120 density chip (X4) (10400 RU) and the low-III_B gp120 density chip (X4) (792 RU) (see Figure S1 in the Supporting Information). The binding of **8Cb** and **8Bb** to gp120 clearly showed biphasic binding signals (as similarly observed for PRM-A or PRM-S). When examined at compound concentrations ranging between 1.6 and 12.5 μM, the apparent K_D values for both **8Bb** and **8Cb** were in the lower micromolar range irrespective of the density of the gp120 chip (K_D: 1.61−2.82 μM) (Table 2).

The polyanionic nature of our compounds may rise the question whether the observed affinity to HIV-1 gp120 is due to the binding to the glycans of gp120 or whether the compounds bind to other areas on gp120 such as the positively charged V3 loop of

gp120. Because the antiviral activity of polyanionic compounds is usually higher against CXCR4-tropic (X4) than CCR5-tropic (R5) virus,[41] we examined the affinity of **8Cb** and **8Bb** against HIV-1 X4-derived gp120 (III_B) and HIV-1 R5-derived gp120 (ADA) by SPR technology. The data obtained indicate that both molecules showed equal binding affinities and kinetics against both types of gp120s (Table 2 and Figure S2 in the Supporting Information). Thus, the triazine derivatives are able to efficiently interact with both X4- and R5-derived envelope gp120.

CONCLUSIONS

We have efficiently synthesized three series of triazine derivatives functionalized with aromatic amino acids that have been designated as monomers, dimers, and trimers starting from disubstituted chlorotriazines. The synthesis of dimers and trimers is considerably sped up through the aid of microwave-assisted synthesis, reducing the reaction times from days to hours.

The antiviral evaluation indicated that the total number of amino acids at the periphery of the molecules is crucial for the anti-HIV activity. Thus, monomers were antivirally inactive ($EC_{50} > 250\ \mu M$) and dimers showed a limited anti-HIV activity (higher micromolar range) while trimers afforded the most potent antiviral compounds active in the lower micromolar range. The prototype compounds **8Cb** and **8Bb** were shown to be efficient binders of gp120 (both X4 and R5 strains) according to SPR, with a K_D estimated value in the lower micromolar range. It was of interest to demonstrate a strong correlation for the mono-, di-, and trimers between their antiviral activity observed in cell culture and their affinity to gp120 as recorded by SPR.

As for every new family of compounds, a number of questions arise related to the possible mechanism(s) of action. On the basis of the antiviral activity data with X4 and R5 virus strains, further corroborated by the SPR experiments, it seems that this class of compounds inhibits both X4- and R5 virus strains at comparable efficacy. This implicates that the target of interaction on both types of gp120 must be rather conserved (i.e., *N*-glycans, but not excluding other interaction points with gp120 as well). Indeed, the exact locations of the binding of the most active compounds to the HIV-1 envelope gp120 are currently unknown. It is also unclear whether binding with one single gp120 molecule is sufficient to eventually afford antiviral activity or whether binding (cross-linking) between different gp120 molecules within the gp120 trimer or even between two gp120 trimers need to occur as a prerequisite for antiviral activity.

Although the antiviral activity of the test compounds may still seem modest, it should be emphasized that the observed activity is already significant for this new family of compounds and should be considered as potential novel anti-HIV lead candidates that need to be further explored for their potential usefulness as antiviral agents. Also, because these type of compounds are directly binding to their target (HIV gp120) and do not need to be taken up by the target cells, nor metabolized to an active species, low micromolar activity may afford significant disruption of the viral infection, as exemplified by the pradimicin antibiotics that are also active in the lower micromolar range.[13]

Thus, these results support the interest of this new family of compounds to interact with gp120 and show anti-HIV activity both by inhibiting cell-free virus infection and cell-to-cell virus transmission. In this respect, this family of compounds may qualify as potential novel HIV microbicide lead compounds to be further examined.

EXPERIMENTAL SECTION

Chemistry Procedures. The structures and purity of each target compound were confirmed by TLC, ^{1}H NMR, HPLC/MS, HRMS, and/or elemental analysis. Analytical TLC was performed on silica gel 60 F_{254} (Merck) precoated plates (0.2 mm). Spots were detected under UV light (254 nm) and/or charring with ninhydrin. The monitoring of the reactions was also performed by HPLC/MS through a HPLC-Waters 12695 connected to a Waters Micromass ZQ spectrometer. Separations on silica gel were performed by preparative centrifugal circular thin-layer chromatography (CCTLC) on a Chromatotron (Kiesegel 60 PF_{254} gipshaltig (Merck)), with layer thickness of 1 and 2 mm and flow rate of 4 or 8 mL/min, respectively. Column chromatography was performed with silica gel 60 (230−400 mesh) (Merck). Flash chromatography was performed in a Biotage Horizon instrument.

Melting points were obtained using a Mettler Toledo MP70 melting point system and are uncorrected. The elemental analysis was performed with a Heraeus CHN-O-RAPID instrument. The purity of compounds **3A**−**C**(**a**−**c**), **4A**−**C**(**a**−**c**), **5A**−**c**(**a**−**c**), **7A**−**C**(**a**,**b**), **11B**,**D**(**a**,**b**), **14**, and **17** was determined by elemental analysis, and the elemental compositions were within ±0.4% of the calculated values. The purity of compounds **6A**−**C**(**a**−**c**), **8A**−**C**(**a**,**b**), **12B**,**E**(**a**,**b**), **15**, and **18** was determined by HPLC and was >95%. HPLC spectra were recorded on a Waters 2690 instrument using a diode array detector (230−400 nm) and an analytical Sunfire C18 column 3.5 μm (4.6 mm × 50 mm). Solvents used were acetonitrile (0.04%TFA) for bottle A and H_2O (0.05%TFA) for bottle B, and the flow rate was 1 mL/min. Gradients used were as follows: gradient A, from 10% to 100% of bottle A in 5 min; gradient B, from 10% to 70% of A in 10 min. Electrospray mass spectra were measured on a quadrupole mass spectrometer equipped with an electrospray source (Hewlett-Packard, LC/MS HP 1100). ^{1}H NMR spectra were recorded on a Varian INNOVA 300 operating at 299 MHz and on a Varian 500 operating at 500 MHz.

Microwave reactions were performed using the Biotage Initiator 2.0 single-mode cavity instrument from Biotage (Uppsala). Experiments were carried out in sealed microwave process vials utilizing the standard absorbance level (400 W maximum power). The temperature was measured with an IR sensor on the outside of the reaction vessel.

The synthesis of the different series of compounds has been performed applying the here described general procedures. A few key compounds are described in this Experimental Section. Full details and analytical and spectroscopic data for all the compounds are included in the Supporting Information.

*General Procedure for the Synthesis of Disubstituted 1,3,5-Triazines (**2A**−**C**).* A mixture containing cyanuric chloride (1.0 mmol), the corresponding amino acid methyl ester (2.4 mmol), and DIPEA (6.0 mmol) in THF (12 mL) was stirred at room temperature overnight. Then, volatiles were removed to dryness in vacuo and the residue was dissolved in ethyl acetate (20 mL) and washed with a saturated NH_4Cl solution (10 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered, and evaporated. The residue was then purified as indicated for each compound.

*Compound **2A**.* According to the general procedure for the synthesis of disubstituted triazines, reaction of cyanuric chloride (150 mg, 0.81 mmol), H-Phe(OMe)·HCl (420 mg, 1.95 mmol), and DIPEA (2.0 mL, 11.71 mmol) in THF (20 mL) afforded a residue that was purified by column chromatography (hexane/ethyl acetate 1:1) to yield 308 mg (82%) of **2A** as a white solid; mp 108−110 °C. MS (ES, positive mode): m/z 470 $(M + H)^+$ with a Cl isotopic pattern. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 2.90−3.15 (m, 4H, β-CH_2Phe), 3.61 (s, 6H, CH_3O), 4.83−4.97 (m, 2H, α-CHPhe), 5.81 (bs, 2H, NH), 6.90−7.27 (m, 10H, Ar).

Compounds **2B** and **2C** were similarly prepared (see Supporting Information)

General Procedure for the Synthesis of the Triazine Monomers ***3A*****−****C****(****a****−****c****).** *Procedure A.* A solution containing the disubstituted triazine **2A**−**C** (1.0 mmol), the corresponding amine (3.0 mmol), and DIPEA (3.0 mmol) in dioxane (10 mL) was stirred at 60−100 °C for 24 h in a sealed tube. After cooling to room temperature, volatiles were evaporated and the residue was dissolved in ethyl acetate (20 mL) and washed with a NH_4Cl saturated solution (10 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered, and evaporated. The residue was then purified as indicated for each compound.

Procedure B. A microwave vial was loaded with disubstituted triazine **2A**−**C** (1.0 mmol), the corresponding amine (3.0 mmol), DIPEA (3.0 mmol), and 1,4-dioxane (10 mL). The reaction vessel was sealed and heated in a microwave reactor at 100 °C for 1−2 h. After cooling, volatiles were evaporated to dryness and the residue was dissolved in ethyl acetate (20 mL) and washed with a NH_4Cl saturated solution (10 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered, and evaporated. The residue was then purified as indicated for each compound.

Compound ***3Aa****.* According to procedure A for the synthesis of triazine monomers, *N*-methylpiperazine (87 μL, 0.81 mmol) and DIPEA (139 μL, 0.81 mmol) were added to a solution of **2A** (160 mg, 0.27 mmol) in dioxane (5 mL), and the mixture was stirred at 60 °C for 24 h in a sealed tube, affording a residue that was purified by column chromatography (dichloromethane/methanol, 10:1) to yield 114 mg (80%) of a colorless oil identified as **3Aa**. MS (ES, positive mode): *m*/*z* 534 $(M + H)^+$, ^1H NMR ($CDCl_3$, 300 MHz) δ: 2.24 (s, 3H, CH_3N), 2.32−2.35 (m, 4H, CH_2N), 3.12 (m, 4H, β-CH_2Phe), 3.68 (s, 6H, CH_3O), 3.77 (m, 4H, CH_2N), 4.70−4.89 (m, 2H, α-CHPhe), 5.15 (bs, 1H, NH), 5.35 (bs, 1H, NH), 7.02−7.22 (m, 10H, Ar). Anal. Calcd for $C_{28}H_{35}N_7O_4$: C, 63.02; H, 6.61; N, 18.37. Found: C, 62.97; H, 6.63; N, 18.09.

Compound ***3Ab****.* According to procedure B for the synthesis of triazine monomers, a microwave vial was charged with **2A** (300 mg, 0.61 mmol), benzylamine (200 μL, 1.85 mmol), and DIPEA (320 μL, 1.85 mmol) in dioxane (5 mL), and the mixture was heated at 100 °C for 2 h. The residue was purified by flash chromatography (hexane/ethyl acetate 2:1); yield 214 mg (65%); mp 54−56 °C. MS (ES, positive mode): *m*/*z* 541 $(M + H)^+$. ^1H NMR (DMSO-d_6, 300 MHz) δ: 2.80−3.18 (m, 4H, β-CH_2Phe), 3.62 (bs, 6H, CH_3O), 4.31−4.73 (m, 4H, CH_2N, α-CHPhe), 6.71−7.35 (m, 18H, NH, Ar). Anal. Calcd for $C_{30}H_{32}N_6O_4$: C, 66.65; H, 5.97; N, 15.55. Found: C, 66.47; H, 6.33; N, 15.69.

The synthesis, analytical, and spectroscopic data of all the other triazine monomers **3A**−**C**(**a**−**c**) is fully described in the Supporting Information.

General Procedure for the Deprotection of the Methyl Esters. To a solution containing the methyl ester derivative (1.0 mmol) in THF (10 mL), a solution of $LiOH·H_2O$ (2 equiv for each methyl ester group) in water (10 mL) was added, and the mixture was stirred at room temperature for 8 h. Then formic acid was added to reach pH = 2, and volatiles were evaporated to dryness. The residue was dissolved in 2 mL of dioxane/water (9:1) and was gently stirred with the resin Amberlite IRA400 ($HCOO^-$) for monomers and dimers, or Dowex 1 × 2 ($HCOO^-$) for trimers through orbital stirring overnight. Then, the resin was washed with 4 volumes of dioxane/water (9:1). Cleavage of the product from the resin was performed by treatment with formic acid/water (9:1) for 1 h with orbital stirring, followed by washing with 4 volumes of formic acid/water (9:1). The filtrate was evaporated to dryness in vacuo and lyophilized.

Compound ***4Ca****.* According to the general procedure for the deprotection of methyl esters, compound **3Ca** (100 mg, 0.16 mmol) in THF (1 mL) was treated with $LiOH·H_2O$ (28 mg, 0.64 mmol) in water (1 mL). The residue was purified with Amberlite IRA400 ($HCOO^-$) resin to yield 65 mg (43%) of **4Ca** as a white solid; mp > 250 °C, decompose. MS (ES, positive mode): *m*/*z* 584 $(M + H)^+$. ^1H NMR (DMSO-d_6, 300 MHz) δ: 2.18 (s, 3H, CH_3N), 2.34 (m, 4H, CH_2N), 3.08−3.18 (m, 4H, β-CH_2Trp), 3.71 (m, 4H, CH_2N), 4.30−4.75 (m, 2H, α-CHTrp), 6.49 (bs, 1H, NH), 6.75 (bs, 1H, NH), 6.89−7.59 (m, 10H, H-2^i Trp, Ar), 10.81 (s, 2H, NH-1^i Trp), 12.30 (bs, 2H, COOH). Anal. Calcd for $C_{30}H_{33}N_9O_4$: C, 61.74; H, 5.70; N, 21.60. Found: C, 61.42; H, 5.86; N, 21.73.

The synthesis, analytical, and spectroscopic data of all the other triazine monomers **4A**−**C**(**a**−**c**) is fully described in the Supporting Information.

General Procedure for the Synthesis of the Triazine Dimers ***5A*****−****C****-(****a****−****c****).** *Procedure A.* A mixture containing a disubstituted triazine **2A**−**C** (1.0 mmol), the corresponding diamine (0.6 mmol), and DIPEA (3.0 mmol) in dioxane (10 mL) was stirred at 100 °C for 48 h in a sealed tube. After cooling to room temperature, volatiles were evaporated to dryness in vacuo, and the residue was dissolved in ethyl acetate (20 mL) and treated with a saturated solution of NH_4Cl (10 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered, and evaporated. The residue was then purified as indicated for each compound.

Procedure B. A microwave vial was charged with the disubstituted triazine **2A**−**C** (1.0 mmol), the corresponding diamine (0.6 mmol), DIPEA (3.0 mmol), and dioxane (10 mL). The reaction vessel was sealed and heated in a microwave reactor at 100−120 °C for 2−3 h. After cooling to room temperature, the volatiles were evaporated to dryness in vacuo, and the residue was dissolved in ethyl acetate (20 mL) and treated with a saturated solution of NH_4Cl (10 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered, and evaporated. The residue was purified as indicated for each compound.

Compound ***5Aa****.* According to procedure A for the synthesis of triazine dimers, reaction of **2A** (128 mg, 0.25 mmol) with piperazine (70 mg, 0.15 mmol) in the presence of DIPEA (131 μL, 0.75 mmol) in dioxane (8 mL) afforded a residue that was purified by column chromatography (hexane/ethyl acetate, 2:1); yield 197 mg (83%); mp 138−139 °C. MS (ES, positive mode): *m*/*z* 477 $(M + 2H)^{2+}$, 953 $(M + H)^+$. ^1H NMR ($CDCl_3$, 300 MHz) δ: 3.05 (m, 8H, β-CH_2Phe), 3.60 (m, 20H, CH_3O, CH_2N), 4.76 (m, 4H, α-CHPhe), 5.60−5.95 (bs, 4H, NH), 7.02−7.21 (m, 20H, Ar). Anal. Calcd for $C_{50}H_{56}N_{12}O_8$: C, 63.01; H, 5.92; N, 17.64. Found: C, 62.74; H, 6.28; N, 17.66.

Compound ***5Ab****.* Following procedure B for the synthesis of triazine dimers, a microwave vial was charged with **2A** (386 mg, 0.80 mmol), 1,3-xylylenediamine (66 μL, 0.48 mmol), DIPEA (360 μL, 2.04 mmol), and dioxane (10 mL), and the mixture was irradiated at 100 °C for 2 h, affording a residue that was purified by flash chromatography (hexane/ethyl acetate, 1:1 to 1:2); yield 179 mg (58%); mp 102−104 °C. MS (ES, positive mode): *m*/*z* 502 $(M + 2H)^{2+}$, 1003 $(M + H)^+$. ^1H NMR ($CDCl_3$, 300 MHz) δ: 3.20 (m, 8H, β-CH_2Phe), 3.33−3.64 (m, 12H, CH_3O), 4.52−4.59 (m, 8H, α-CHPhe, CH_2N), 5.30−6.34 (m, 2H, NH), 7.02−7.48 (m, 24H, NH, Ar). Anal. Calcd for $C_{54}H_{58}N_{12}O_8$: C, 64.66; H, 5.83; N, 16.76. Found: C, 64.79; H, 5.68; N, 16.98.

The synthesis, analytical and spectroscopic data of all the other triazine dimers **5A**−**C**(**a**−**c**) is fully described in the Supporting Information.

Compound ***6Aa****.* Following the general procedure for the deprotection of methyl esters, compound **5Aa** (100 mg, 0.10 mmol) in THF (1 mL) was treated with $LiOH·H_2O$ (36 mg, 0.84 mmol) in water (1 mL). The residue was purified with Amberlite IRA400 ($HCOO^-$) resin to yield 42 mg (47%) of **6Aa** as a white solid; mp > 165 °C, decompose; .MS (ES, positive mode): *m*/*z* 449 $(M + 2H)^{2+}$, 897 $(M + H)^+$. ^1H NMR (DMSO-d_6, 300 MHz) δ: 3.19 (m, 8H, β-CH_2Phe), 3.46 (m, 8H, CH_2N), 4.40 (m, 4H, α-CHPhe), 6.68−6.93 (m, 4H, NH), 7.12−7.21 (m, 20H, Ar), 12.30 (s, 4H, COOH). HRMS (ES, negative mode) Calcd for $C_{46}H_{48}N_{12}O_8$: 896.3718. Found: 896.3710. HPLC: (system A) 6.5 min (98%); (system B) 3.7 min (99%).

Compound ***6Ab****.* Following the general procedure for the deprotection of methyl esters, compound **5Ab** (120 mg, 0.12 mmol) in THF (1.5 mL) was treated with $LiOH·H_2O$ (41 mg, 0.95 mmol) in water (1.5 mL). The residue was purified with Amberlite IRA400 ($HCOO^-$)

resin to yield 83 mg (73%) of **6Ab** as a white solid; mp > 215 °C, decompose. MS (ES, positive mode): m/z 474 $(M + 2H)^{2+}$, 947 $(M + H)^{+}$. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.95−3.17 (m, 8H, β-CH_2Phe), 4.22−4.58 (m, 8H, α-CHPhe, CH_2N), 6.03−6.56 (m, 2H, NH), 7.09−7.35 (m, 28H, NH, Ar), 12.61 (s, 4H, COOH). HRMS (ES, negative mode) Calcd for $C_{50}H_{50}N_{12}O_8$: 946.3875. Found: 946.3883. HPLC: (System A) 6.6 min (99%); (system B) 3.9 min (99%).

The synthesis, analytical and spectroscopic data of all the other triazine dimers **6A**−**C**(**a**−**c**) is fully described in the Supporting Information.

*General Procedure for the Synthesis of the Triazine Trimers **7A**−**C**(**a**,**b**), **11B**,**D**(**a**,**b**), **14** and **17***. A microwave vial was loaded with a disubstituted triazine (1.0 mmol), the corresponding triamine (0.3 mmol), DIPEA (3.0 mmol), and dioxane (10 mL). The reaction vessel was sealed and heated in a microwave reactor at 120 °C for 3 h. After cooling to room temperature, the volatiles were evaporated to dryness in vacuo, and the residue was dissolved in ethyl acetate (20 mL) and treated with a saturated solution of NH_4Cl (10 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered, and evaporated. The residue was then purified as indicated for each compound.

*Compound **7Ca***. Following the general procedure for the synthesis of triazine trimers, a mixture containing **2C** (400 mg, 0.73 mmol), tris(2-aminoethyl)amine (36 μL, 0.22 mmol), DIPEA (380 μL, 2.16 mmol), and dioxane (5 mL) was stirred in a microwave reactor, affording a residue that was purified by column chromatography (dichloromethane/methanol, 25:1 to 10:1); yield 187 mg (30%); mp 109−110 °C. MS (ES, positive mode): m/z 841 $(M + 2H)^{2+}$. ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 2.52 (m, 6H, CH_2N), 3.09−3.34 (m, 18H, β-CH_2Trp, CH_2N), 4.37−4.71 (m, 6H, α-CHTrp), 6.39−6.66 (m, 6H, NH), 6.92−7.45 (m, 33H, Ar, NH, H-2^i Trp), 10.85 (bs, 6H, NH-1^i Trp). Anal. Calcd for $C_{87}H_{93}N_{25}O_{12}$: C, 62.17; H, 5.58; N, 20.83; Found: C, 62.42; H, 5.76; N, 20.46.

*Compound **7Cb***. Following the general procedure for the synthesis of triazine trimers, a mixture containing **2C** (200 mg, 0.36 mmol), 1,3,5-tris(aminomethyl)-2,4,6-triethylbenzene[29] (29 mg, 0.12 mmol), DIPEA (190 μL, 1.12 mmol), and dioxane (4 mL) was stirred in a microwave reactor, affording a residue that was purified by column chromatography (dichloromethane/methanol, 20:1 to 10:1); yield 70 mg (33%); mp 183−185 °C. MS (ES, positive mode): m/z 892 $(M + 2H)^{2+}$. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 1.00 (m, 9H, CH_2CH_3), 2.56 (m, 6H, CH_2CH_3), 3.12 (m, 12H, β-CH_2Trp), 3.50 (s, 18H, CH_3O), 4.26 (s, 6H, CH_2N), 4.41−4.78 (m, 6H, α-CHTrp), 5.85−6.68 (m, 6H, NH), 6.85−7.58 (m, 33H, NH, H-2^{i}Trp, Ar), 10.83 (s, 6H, NH-1^i Trp). Anal. Calcd for ($C_{96}H_{102}N_{24}O_{12}$): C, 64.63; H, 5.76; N, 18.84; Found: C, 64.52; H, 6.01; N, 18.61.

The synthesis, analytical and spectroscopic data of all the other triazine trimers **7A**−**C**(**a**,**b**), **11B**,**D**(**a**,**b**), **14** and **17** is fully described in the Supporting Information.

*Compound **8Ca***. According to the general procedure for the methyl esters deprotection, treatment of **7Ca** (80 mg, 0.05 mmol) and LiOH·H_2O (24 mg, 0.57 mmol) in THF (1 mL) and water (1 mL) afforded a residue that was purified with Dowex 1 × 2 ($HCOO^-$) resin; yield 56 mg (78%); mp > 230 °C, decompose. MS (ES, positive mode): m/z 799 $(M + 2H)^{2+}$. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.56 (m, 6H, CH_2N), 3.02−3.30 (m, 18H, β-CH_2Trp, CH_2N), 4.61 (m, 6H, α-CHTrp), 5.99−6.70 (m, 6H, NH), 6.90−7.58 (m, 33H, NH, H-2^i Trp, Ar), 10.84 (bs, 6H, NH-1^i Trp), 12.31 (bs, 6H, COOH). HRMS (ES, negative mode) Calcd for $C_{81}H_{81}N_{25}O_{12}$: 1595.6497. Found: 1595.6527 HPLC: (system A) 6.8 min (98%); (system B) 3.7 min (98%).

*Compound **8Cb***. According to the general procedure for the methyl esters deprotection, treatment of **7Cb** (67 mg, 0.04 mmol) and LiOH·H_2O (19 mg, 0.45 mmol) in THF (1 mL) and water (1 mL) afforded a residue that was purified with Dowex 1 × 2 ($HCOO^-$) resin; yield 47 mg (74%); mp > 230 °C, decompose. MS (ES, positive mode): m/z 850 $(M + 2H)^{2+}$,1700 $(M + H)^{+}$. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 0.98 (m, 9H, CH_2CH_3), 2.71 (m, 6H, CH_2CH_3), 3.18 (m, 12H, β-CH_2Trp), 4.36−4.79 (m, 12H, CH_2N, α-CHTrp), 5.89−6.38 (m, 6H, NH), 6.86−7.93 (m, 33H, NH, H-2^i Trp, Ar), 10.81−10.89 (m, 6H, NH-1^i Trp). HRMS (ES, negative mode): Calcd for $C_{90}H_{90}N_{24}O_{12}$: 1698.7170. Found: 1698.7158. HPLC: (system A) 7.4 min (98%); (system B) 3.8 min (99%).

The synthesis and spectroscopic data of all the other triazine trimers **8A**−**C**(**a**,**b**), **12B**,**E**(**a**,**b**), **15** and **18** is fully described in the Supporting Information.

Antiviral Activity. The methodology of the anti-HIV assays was as follows: human T-lymphocyte CEM cells (~3 × 10^5 cells/ml) were infected with 100 $CCID_{50}$ of HIV-1(III_B) or HIV-2(ROD)/ml and seeded in 200 μL wells of a microtiter plate containing appropriate dilutions of the test compounds. After 4 days of incubation at 37 °C, HIV-induced CEM giant cell formation was examined microscopically. The 50% effective concentration (EC_{50}) was defined as the compound concentration required to inhibit virus-induced cytopathicity by 50%. The 50% cytostatic concentration (CC_{50}) was defined as the compound concentration required to inhibit CEM cell proliferation by 50% as counted by a Coulter counter. PBMC from healthy donors were stimulated with PHA at 2 μg/mL (Sigma, Bornem, Belgium) for 3 days at 37 °C. The PHA-stimulated blasts were then seeded at 0.5 × 10^6 cells per well into a 48-well plate containing various concentrations of compound in cell culture medium containing 10% FCS and IL-2 (25 U/mL; R&D Systems Europe, Abingdon, United Kingdom). The virus stocks were added at a final dose of 250 pg p24 Ag. Cell supernatant was collected at day 10 and HIV-1 core Ag in the culture supernatant was analyzed by a specific p24 Ag ELISA kit (Perkin-Elmer, Zaventem, Belgium). Mock-infected cell cultures exposed to the different drug concentrations were examined for cell viability by trypan blue staining.

Effect of Test Compounds against the Expression of Cellular Activation Markers. Expression of cellular activation markers was measured after 3 days incubation of PBMC with varying concentrations of **8Bb** and **8Cb** at 37 °C. Briefly, after washing with PBS containing 2% FBS, cells were incubated with FITC-conjugated anti-CD4 mAb in combination with PE-conjugated anti-CD25, anti-CD69, or anti-HLA-DR mAbs for 30 min at 4 °C. For aspecific background staining, cells were stained in parallel with Simultest Control IgG γ1/γ2a (BD Biosciences, Erembodegem, Belgium). Finally, the cells were washed, fixed with 1% formaldehyde solution, and analyzed with a FACSCalibur, and data were acquired and analyzed with CellQuest software.

Surface Plasmon Resonance (SPR) Analysis. The recombinant monomeric gp120 protein from HIV-1(III_B) (ImmunoDiagnostics Inc., Woburn, MA) was covalently immobilized on the carboxymethylated dextran matrix of a CM5 sensor chip using standard amine coupling chemistry.[42] The sensor surface was activated upon a 7-min injection of a 1:1 ratio of 0.4 M *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide and 0.1 M *N*-hydroxysuccinimide. Gp120 was coupled randomly to the surface in 10 mM sodium acetate, pH 4.0. Remaining activated groups were blocked upon a 7 min injection of 1.0 M ethanolamine, pH 8.5. A reference flow cell was used as a control for nonspecific binding and refractive index changes. All interaction studies were performed at 25 °C on a Biacore T100 instrument (GE Healthcare, Uppsala, Sweden). The compounds were serially (2-fold) diluted in HBS-P (10 mM HEPES, 150 mM NaCl, and 0.05% surfactant P20; pH 7.4) supplemented with 5% DMSO and 10 mM Ca^{2+}. Samples were injected for 2 min at a flow rate of 30 μL/min, and the dissociation was followed for 5 min. One duplicate sample and several buffer blanks were used as a positive control and for double referencing, respectively. The CM5 sensor chip surfaces were regenerated with 1 injection of 50 mM NaOH. All studied interactions resulted in specific binding signals. The

shape of the association and dissociation phases reveals that the curves are not following 1:1 Langmuir binding kinetics.

A panel of test compounds were evaluated at 12.5 or 50 μM for binding to immobilized gp120 (10400 RU ~ 86.7 fmol gp120). The interaction between **8Bb** and **8Bc** and HIV-1(III_B) gp120 were studied in detail. Gp120 was covalently immobilized at low (792 RU ~ 6.6 fmol gp120) and at high (10400 RU) density. Serial 2-fold analyte dilutions (concentration range between 1.6 and 12.5 μM) were injected over the gp120-coated surfaces of the sensor chip. The experimental data were fit using the 1:1 binding model (Biacore T100 evaluation software 2.0.2) to determine the binding kinetics. The affinity and kinetic values are apparent values as the injected concentrations of the evaluated compounds did result in biphasic binding signals.

To determine whether the interaction between gp120 and the compounds was Ca^{2+}-dependent, **8Bb** and **8Cb** were diluted in HBS-P buffer supplemented with 5% DMSO and also supplemented with either 2 or 10 mM Ca^{2+}. The compounds were also diluted in HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.05% surfactant P20; pH 7.4) supplemented with only 5% DMSO to mimic the situation without calcium. Then 12.5 μM of each sample was injected on immobilized gp120 (10400 RU).

In a last set of experiments, the III_B X4 gp120 and the ADA R5 gp120 proteins (ImmunoDiagnostics) were covalently immobilized on the carboxymethyl dextran matrix of a CM5 sensor chip using standard amine coupling chemistry. Identical experimental conditions as described for the high- and low-density III_B gp120 experiments were used to determine the kinetic rate constants of **8Bb** and **8Cb** versus III_B gp120 and ADA gp120.

■ ASSOCIATED CONTENT

Supporting Information. Synthesis and spectroscopic data of all the synthesized compounds. SPR analyses are included. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ DEDICATION

[§]Dedicated to Prof. José-Luis García-Ruano on the occasion of his 65th anniversary.

■ ABBREVIATIONS USED

DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin; gp120, glycoprotein 120; HD, high density; HIV, human immunodeficiency virus; LD, low density; MBL, mannose binding lectin; PBMC, peripheral blood mononuclear cells; PRM-A, Pradimicin-A; PRM-S, Pradimicin-S; SIV, simian immunodeficiency virus; SPR, surface plasmon resonance

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