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Inhibition of DC-SIGN-Mediated HIV Infection by a Linear Trimannoside Mimic in a Tetravalent Presentation

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ABSTRACT HIV infection is pandemic in humans and is responsible for millions of deaths every year. The discovery of new cellular targets that can be used to prevent the infection process represents a new opportunity for developing more effective antiviral drugs. In this context, dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN), a lectin expressed at the surface of immature dendritic cells and involved in the initial stages of HIV infection, is a promising therapeutic target. Herein we show the ability of a new tetravalent dendron containing four copies of a linear trimannoside mimic to inhibit the *trans* HIV infection process of CD4+ T lymphocytes at low micromolar range. This compound presents a high solubility in physiological media, a neglectable cytotoxicity, and a long-lasting effect and is based on carbohydrate-mimic units. Notably, the HIV antiviral activity is independent of viral tropism (X4 or R5). The formulation of this compound as a gel could allow its use as topical microbicide.

Human immunodeficiency virus (HIV) remains one of the main health problems of the 21st century. Almost three decades have elapsed since the discovery of this virus, and despite the variety of the available therapeutic arsenal, over 3 million people die of AIDS every year. It is evident that more efficient drugs and strategies are needed. Unfortunately, the hope to achieve a successful vaccine against HIV in the near future remains elusive (*1*). New strategies have been envisaged and evaluated, and HIV-entry inhibitors appear one of the most promising alternatives (*2*). Many efforts have been devoted to the search for novel antagonists with the ability to target different HIV receptors and coreceptors, such as CD4, CCR5, or CXCR4, at the cell surface. In this context, a potential therapeutic target was identified in dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) a molecule involved in the early stages of HIV infection (*3*). DC-SIGN is a tetrameric calcium-dependent (C-type) lectin, expressed by dendritic cells (DC), which specifically recognizes highly glycosylated structures displayed at the surface of several pathogens such as viruses, bacteria, yeasts, and parasites (*4–7*). Recognition by DC-SIGN plays a key role in the infection process of some of these pathogens, most notably HIV, and it is considered an interesting new target for the design of anti-infective agents (*8–12*).

DC-SIGN+ immature DC, located in vaginal, cervical, and rectal mucosae, are among the first cell types to en-

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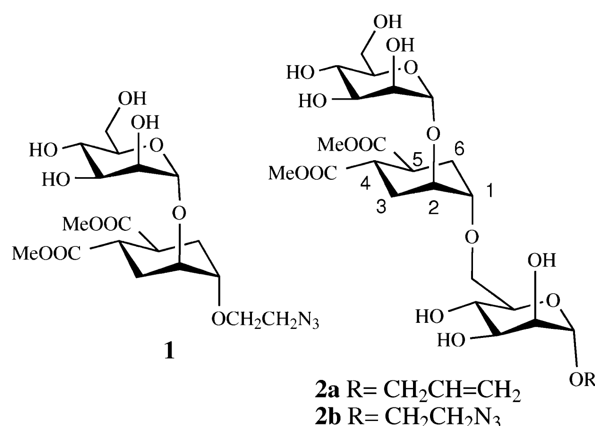


Figure 1. Mimics of linear mannose disaccharide 1 **and trisaccharide** 2.

counter HIV during sexual transmission. DC-SIGN expressed by DC at mucosal tissues captures HIV at low titer by binding the envelope glycoprotein gp120. DC-SIGN acts as an attachment factor rather than an entry receptor, binding and concentrating HIV on the cell surface. Two different pathways are involved in the transmission of DC-SIGN-bound HIV to T lymphocytes (*13, 14*). The first, responsible for short-term HIV transfer (24 h after HIV exposure), involves virion internalization into intracellular compartments where the virus is protected from degradation and retains a high infective capacity during DC migration to lymphoid tissues (*15*). The second, involved in long-term HIV transfer (72 h after exposure), follows DC infection in *cis* by transfer of DC-SIGN-bound virus to canonical HIV entry receptors, CD4 and CCR5, resulting in infected DC with a continuous production of virus for the T cells (*14, 16*). In both cases, upon arrival at lymphoid tissues, DC efficiently transmit HIV to CD4+ T lymphocytes, a process called infection in *trans*. Hence, blocking the interaction between DC-SIGN and HIV could be an efficient strategy to prevent HIV transmission and infection of the host.

DC-SIGN efficiency in HIV capture is associated with two main characteristics of this molecule. First, the carbohydrate binding domains (CRD) of DC-SIGN spike up (320 Å) from the cell surface and are the first to encounter the virus (*17*). Second, the protein is organized as a tetramer containing 4 CRDs and is distributed as clustered patches at the cellular surface (*18, 19*). Altogether, these features lead to attachment platforms of high avidity for virus particles, allowing multipoint attachment with the numerous glycans exposed by the viral gp120 glycoprotein. Thus, the choice of DC-SIGN as a therapeutic target implies a double challenge: first, the development of a ligand unit featuring a good affinity for the DC-SIGN CRD, and second, its functionalization on a backbone allowing multiple ligand presentation to compete against this natural multivalent-based interaction.

The main carbohydrate ligand recognized by the carbohydrate recognition domain of DC-SIGN is the high mannose glycan, $(Man)_9(GlcNAc)_2$, also known as Man_9, a branched oligosaccharide found in multiple copies on several pathogen glycoproteins (gp120, GP1, *etc.*). Therefore, structural analogues of Man_9 terminal di- or trisaccharides, able to compete with binding of gp120 to DC-SIGN, could be suitable for the development of new anti-infective drugs. Indeed, recent data (*20*) have shown that linear fragments of Man_9 presented in high density arrays bind with high affinity to DC-SIGN and crystal structures of DC-SIGN complexes with linear oligomannosides have been solved (*21*). We have reported that the pseudo-dimannoside **1** (Figure 1), mimicking the three-dimensional structure and conformational behavior of the natural disaccharide Manα1-2Man, binds to DC-SIGN and exhibits moderate anti-infective action in an Ebola virus infection model (*8*). Additional results indicate that 2-C-substituted branched D-mannose analogues bind to DC-SIGN with affinity significantly greater than that of mannose (*10*). Non-carbohydrate inhibitors with IC_{50} values in the low micromolar range were identified by Kiessling and Borrok *via* high-throughput screening of ~36,000 compounds from commercial libraries (*9*). Multivalent oligomannosides blocking DC-SIGN were described by Wong *et al.* (*22*) Finally, Penadés *et al.* have just reported that gold nanoparticles displaying different multivalent linear and branched mannosyl oligosaccharides behave as potent inhibitors of HIV *trans* infection processes at the cellular level (*23*). We have previously demonstrated that commercially available hyperbranched dendritic Boltorn polymers are good platforms for sugar multivalent presentation (*24, 25*). Using a biosensor with a SPR detection method, we proved that this mannosylated glycodendritic polymer binds to DC-SIGN and inhibits interaction to gp120-coated SPR chips (*26*). Also, using an infection model based on pseudo-typed viral particles of Ebola virus, we have found that at least the third generation of Boltorn polymer bearing 32 copies of man-

nose at the surface was able to inhibit with an IC_{50} of 0.3 μM the *cis* infection of Jurkat cells *via* the interaction with DC-SIGN (*25*).

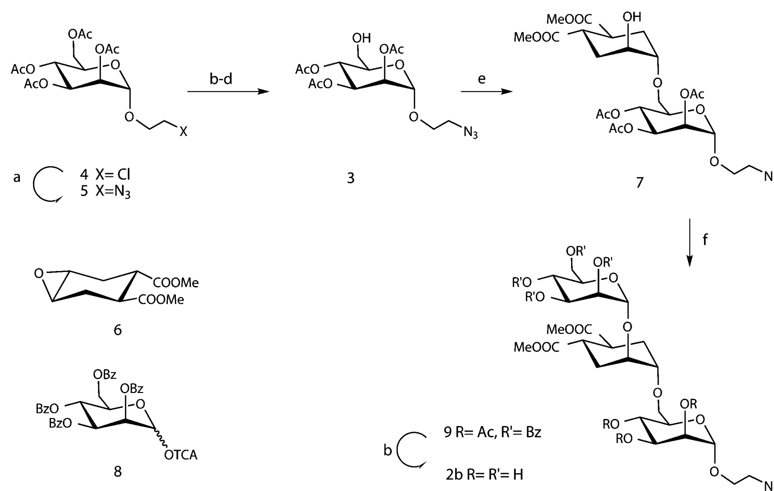
The previous data from our groups convinced us to explore the possibility of combining dendritic platforms with glycomimetic compounds to obtain novel DC-SIGN antagonists endowed with strong binding affinities and able to block this lectin and to inhibit the HIV infection process. The pseudo-trisaccharide **2a** (R = allyl) was recently designed in one of our laboratories as a mimic of the linear mannotrioside Manα1-2 Manα1-6 Manα by replacing the central mannose unit with a carbocyclic diol (Figure 1) (*27*). The results described here show that a tetravalent construct presenting four copies of the pseudo-trimannoside **2** on a Boltorn-type dendron inhibits DC-SIGN-mediated HIV *trans* infection of CD4+ T lymphocytes at low micromolar concentration. Infection inhibition data of this functionalized dendron were also compared to the activity of the monovalent ligand **2b** and of the corresponding tetravalent construct presenting four copies of mannose as the active component.

RESULTS AND DISCUSSION

Synthesis of Monovalent Units. The synthesis of **1** (Figure 1) was previously described (*8*). The synthesis of **2b** (Figure 1) was based on the reported synthesis of **2a** (*27*), with appropriate modifications. Briefly, (2-azidoethyl)-2,3,4-tri-*O*-acetyl-α-D-mannopyranoside **3** was prepared from (2-chloroethyl)-2,3,4,6,-tetra-*O*-acetyl-α-D-mannopyranoside **4** (*28*) *via* the known azide **5** (*27, 29*) by standard steps (Scheme 1). Opening of the epoxide **6** (*30*) with **3** was promoted by $Cu(OTf)_2$ to afford the pseudo-1,6-dimannoside **7** in 53% yield. This was mannosylated in 85% yield using the tetra-*O*-benzoyl trichloroacetimidate **8** (*31*) as donor and TMSOTf (20%) as the promoter in methylene chloride at −20 °C. Zemplen deprotection of the pseudo-trisaccharide **9** afforded **2b** quantitatively.

Evaluation of Monovalent Glycomimetics by Surface Plasmon Resonance (SPR). Surface plasmon resonance (SPR) was used to compare the DC-SIGN recognition properties of the two monovalent ligands **1** and **2b** (Figure 1). As previously described (*11*), because of the natural low affinity of monovalent compounds, evaluation of their relative affinity cannot be obtained in a direct interaction mode and can only be accessed through a competition assay that we recently reported (*11*). For this assay, we used a CM4 SPR chip functionalized with Man-BSA containing 15 glycosylation sites displaying the Manα1-3[Manα1-6]Man trisaccharide. The DC-SIGN extracellular domain (ECD) exhibited good affinity (in the micromolar range) for this surface. Inhibition studies were then performed using DC-SIGN ECD, at a fixed concentration, injected alone or in the presence of an increasing amount of the ligands. As a reference, a competition experiment has been performed with mannose. Sensograms are included in the Supporting Information (Supplementary SPR Data).

Scheme 1. Synthesis of linear trimannoside mimic 2b



a.NaN_3 DMF b. MeONa/MeOH. c. TBDMSCl Pyridine, CH_2Cl_2 followed by Ac_2O. d. TBAF-AcOH in THF. e. 6, CH_2Cl_2, 50% $Cu(OTf)_2$. f. 8, TMSOTf, -20°C

The efficiency of inhibition as a function of a compound's concentration is directly related to the ligand affinity toward DC-SIGN ECD (Figure 2). An IC_{50} of 2.5 mM was estimated for mannose. Using this assay, an IC_{50} of 1.8 mM has been obtained previously for mannose (*11*), and a K_I of 7.7 mM has been also reported using a solid-phase competition assay (*6*). Indeed, within experimental deviations, the IC_{50} is in the range of previously reported experiments, thus validating the test. From Figure 2, the IC_{50} values of **1** and **2b** were determined to be 1.0 mM and 125 μM, respectively. While the interaction properties of the pseudo-disaccharide **1** with DC-SIGN have already been demonstrated by NMR (*8*), it is shown here for the first time that its affinity is better than that of mannose, thus confirming a direct contribution of the additional carbocyclic-diol in the binding to DC-SIGN CRD. In compound **2b**, the addition

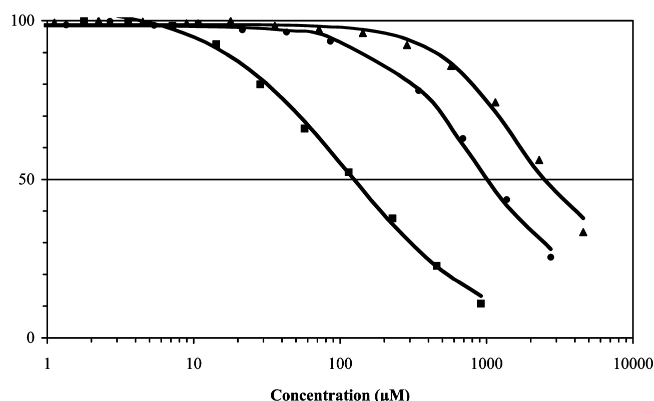


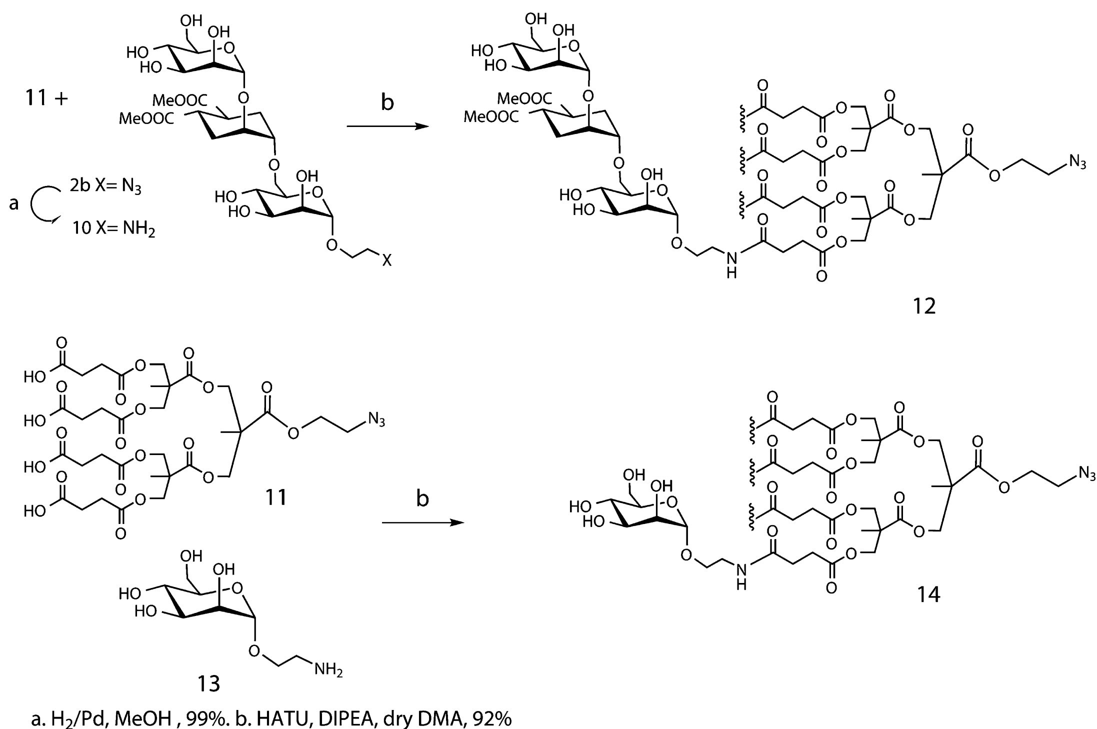
Figure 2. Comparison of inhibitory properties of different compounds on DC-SIGN/BSA-mannotriose interaction. DC-SIGN ECD at 20 μM is incubated with mannose (▲), 1 **(●), or** 2b **(■) and injected on a BSA-mannotriose functionalized surface (1208 RU immobilized). IC_{50} values are 2515 μM for mannose, 1005 μM for compound** 1**, and 125 μM for compound** 2b**.**

of a second mannose unit mimicking an α1-6 link and resulting in a linear trimannoside mimic improves the affinity by 1 order of magnitude relative to mannose.

Both mimics discussed here represent interesting ligands to pursue with multivalent versions. Considering the highest affinity of the trimannoside mimic **2b**, the latter has been selected for further synthetic effort toward multivalent presentation versions for biological testing.

Synthesis of Multivalent Compounds. The functionalized monovalent pseudo-trimannoside **2b** (Figure 1, R = $CH_2CH_2N_3$) was used for the synthesis of the corresponding tetravalent dendron. Hydrogenolytic reduction of azide **2b** with Pd(C) catalyst afforded amine **10** in 99% yield, which was coupled to dendron **11** (*32*) using HATU (*33, 34*) as the condensing agent. LH-20 Sephadex chromatography of the reaction crude afforded the tetrafunctionalized dendron **12** (92%), which was fully characterized by NMR and MS analysis (Scheme 2). The tetravalent mannosylated analogue **14** was prepared using the same approach from mannosyl derivative **13** and the same dendritic core **11**. Coupling conditions and purification steps were as described for glycodendron **12**.

Scheme 2. Synthesis of dendrons 12 **and** 14



a. H_2/Pd, MeOH , 99%. b. HATU, DIPEA, dry DMA, 92%

Infection Studies. Synthesized compounds were tested for the ability to inhibit HIV transmission in an *in vitro trans* infection assay. B-THP-1/DC-SIGN cells are derived from B-THP-1 human B cell line by transfection with DC-SIGN expression vector in order to express high levels of the DC-SIGN receptor. This cell line supports efficient DC-SIGN-mediated HIV transmission and is a widely used model system to mimic HIV capture and transmission to T lymphocytes by dendritic cells (*3*). In a first series of experiments, B-THP-1/DC-SIGN cells preincubated for 30 min in the presence or in the absence of the DC-SIGN inhibitors were subsequently exposed to HIV (the R5 tropic laboratory-adapted strain HIV-1 BaL) in the continued presence of inhibitors. Mannan is known to inhibit DC-SIGN-mediated viral infection (*4, 35, 36*) and was used as positive control (0.25 mg mL^{-1}). Non-transfected B-THP-1 cells were used as a negative control and, as expected, did not transmit infection (Figure 3, panel a). After washing, the B-THP-1/DC-SIGN cells were co-cultured with activated CD4+ T lymphocytes from healthy volunteer donors. Viral infection of CD4+ T lymphocytes was assessed by measuring the concentration of the HIV core protein p24 in the co-culture supernatants by ELISA. p24, immunologically distinct from the protein of most other retroviruses, is a major structural core component of HIV-1and is estimated to be present at 2000−4000 molecules in each virion. The measurement of p24 levels is therefore a commonly exploited method to verify the successful infection by the virus. Each point was obtained in triplicate using CD4+ T lymphocytes from at least three different healthy donors. Results showed that the tetravalent compound **12** at 50 μM reduced the *trans* infection of CD4+ T lymphocytes by over 90%; moreover, at 100 μM and at 250 μM inhibition was almost complete. Although the uncertainties deriving from the involvement of three different donors are very high in these experiments, the data suggest that higher concentrations of the monovalent compound **2b** (1 mM and 0.5 mM) are necessary to obtain a comparable inhibitory effect (Figure 3, panel a).

In a second set of experiments the B-THP-1/DC-SIGN cells were first incubated with the inhibitors and then washed with buffer *prior* to exposure to HIV (BaL). In this case (Figure 3, panel b) the tetravalent system **12** displayed highly efficient anti-infective properties with >94% inhibition at 100 μM. On the contrary, the monovalent compound **2b**, even when used at 5 mM concentration (data not shown), did not show anti-infective activity. Although alternative explanations cannot be discarded (see Discussion), these data suggest that the tetravalent system possesses significant affinity to DC-SIGN, which allows it to deploy its antiviral activity and to prevent the interaction between HIV gp120 and DC-SIGN even after a washing cycle. On the contrary, the affinity of **2b** is not sufficient for the DC-SIGN/**2b** com-

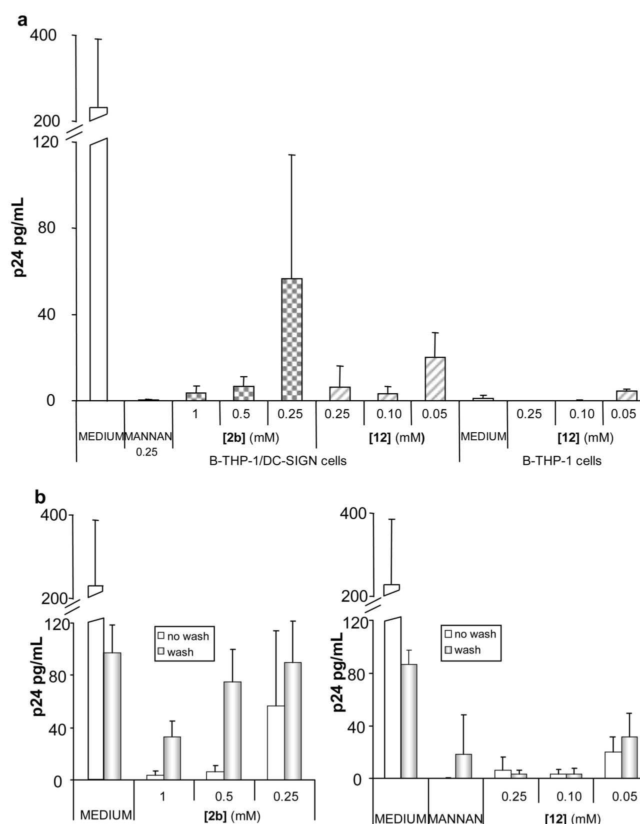


Figure 3. Inhibition of HIV transmission after treatment with 2b **and** 12**. Data obtained in triplicate, from 3 different healthy donors. Values are mean ± SD. a) After 30 min of preincubation with** 2b **and** 12**, B-THP-1/DC-SIGN cells or B-THP-1 cells were incubated for 3 h with HIV-1 BaL in the presence of the indicated concentrations of the DC-SIGN inhibitors, then washed and co-cultured with CD4+ T lymphocytes for 3 days. Viral infection was assessed by measuring the concentration of p24 in the co-culture supernatants. Mannan (0.25 mg mL^{-1}) was used as positive control. Non transfected B-THP-1 cells do not trasmit infection. b) Effect of cell washing after treatment with the inhibitors. B-THP-1/DC-SIGN cells were washed after 30‘ min of incubation with the inhibitor, before exposure to HIV-1 BaL. Results obtained with or without intermediate wash step are compared. Different concentrations of** 2b **and** 12 **were assayed. Mannan (0.25 mg mL^{-1}) was included as a control.**

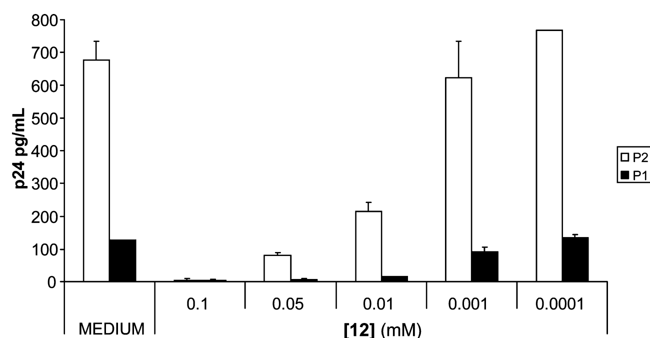


Figure 4. Dose−response results for 12. B-THP1/DC-SIGN cells were incubated for 3 h with HIV-1 BaL in the presence of different concentrations of 12. After washing, B-THP1/DC-SIGN cells were co-cultured for 3 days with CD4+ lymphocytes of individuals genetically less (P1) or more (P2) susceptible to HIV infection. Viral infection was assessed by measuring the concentration of p24 in the supernatant. Each donor was tested in duplicate. Values are mean ± SD.

plex to survive the washing step, and hence viral transmission can occur when the cells are challenged with BaL. A quantitative assessment of the relative potency of **12** and **2b** was obtained in dose–response single-donor experiments that will be described below. The protocol in which inhibitors were not removed by washing was applied in the following *trans* infection experiments, unless otherwise specified.

The properties of **12** were further analyzed. Comparison with dendron **14**, the corresponding tetravalent system presenting four mannose units (Scheme 2) was performed. Results showed that dendron **12** displayed a stronger inhibitory activity at both concentrations tested (100 and 10 μM). Notably, at 100 μM **12** abrogated almost totally HIV BaL transmission to CD4+ T lymphocytes, whereas the inhibition provided by **14** was only partial (approximately 65%) (Supplementary Figure 1). The results obtained are consistent with a 1 order of magnitude difference in affinity between **12** and **14**, which corresponds to the affinity difference estimated by SPR for the corresponding monovalent binding elements, the linear trimannoside mimic **2b** in **12** and mannose in **14**.

Dose–response curves were obtained for **12** using CD4+ T lymphocytes from healthy donors characterized as being endowed with the genetic markers associated with reduced susceptibility to HIV infection (P1) or lack thereof (augmented susceptibility to infection, P2). B-THP-1/DC-SIGN cells were challenged with HIV BaL in the presence of increasing concentrations of **12**. After washing and co-culture with the CD4+ T lymphocytes, viral infection was assessed using analysis of p24 concentration in the supernatants. At 100 μM the inhibition of infection was complete for both donors and an IC_{50} of 5 μM could be estimated (Figure 4). Dose–response data were also obtained for **2b** using CD4+ T lymphocytes from a susceptible donor and allowed estimation of an IC_{50} of 80 μM for the monovalent ligand (Supplementary Figure 2).

Dendritic cells are permissive toward infection supported both by X4- and R5-tropic strains. Thus, we next verified the capability of **12** to block transmission of both IIIB (X4 tropic) and 89.6 (dual tropic, R5/X4) strains of HIV. Results showed that transmission of virus to CD4+ T lymphocytes was almost completely prevented at 250 and 100 μM, and about 90% at 50 μM, when B-THP-1/DC-SIGN cells were challenged with either viral strains (Figure 5, panels a and b). In subsequent analyses the primary HIV isolates V6 (R5 tropic) and V17 (X4 tropic) were utilized; also in this case results indicated an inhibition of HIV infection with both types of virus >99% at all concentration tested (Figure 5, panels c and d).

To evaluate the duration of the inhibitory properties of **12**, B-THP-1/DC-SIGN cells were treated for either 30 min or 2 h with **12** at 250 μM. The compound was subsequently removed, and cells were exposed to HIV-1 BaL either immediately thereafter or after 6 or 12 h. Subsequently B-THP-1/DC-SIGN cells were co-cultured with CD4+ T lymphocytes as described above. Virus transmission to CD4+ T lymphocytes was almost completely abrogated at 0 and 6 h post compound removal; notably, infection was still reduced by over 80% after 12 h. Two hour pretreatment did not appear to prolong inhibitory effect of **12** compared to 30 min pretreatment (Figure 6).

Evaluation of Toxicity of Compounds. 7-Aminoactinomicin D (7-AAD) labeling of the cells after the incubation period with **12** showed that the anti-infective properties of the inhibitors are not an epiphenomenon due to cell death. The data obtained for **12** indicate that the percentage of 7-AAD positive cells (apoptotic cells) was below 1% and did not change significantly in the absence of the compound or in its presence up to 250 μM (Supplementary Figure 3).

Effect of Compounds on DC-SIGN Expression. Compounds **2b** and **12** could exert their effect secondarily to the suppression of DC-SIGN expression. To examine this

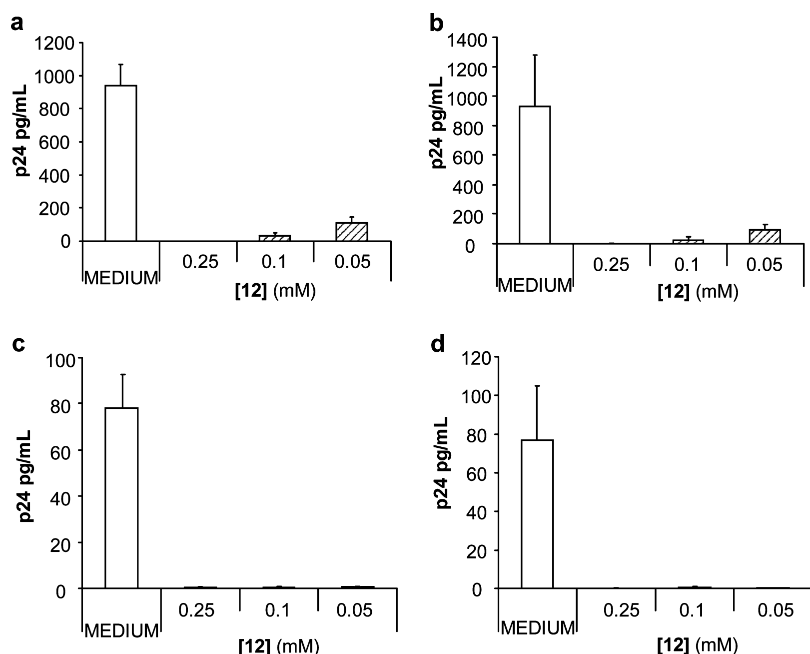


Figure 5. Inhibition of *trans* infection mediated by laboratory-adapted HIV strain (IIIB and 86.9) or by primary viral isolates (V6 and V17). B-THP1/DC-SIGN cells were incubated for 3 h with (a) HIV-1 strains IIIB, (b) 89.6 HIV, (c) V6 (primary isolate; R5- tropic), and (d) V17 (primary isolate; X4-tropic) in the presence of indicated concentration of 12. Virus transmission was determined by measuring the concentration of p24 in the supernatants. Experiments were performed on 3 healthy donors. Values are mean ± SD.

with anti-DC-SIGN antibody, and both percentage of DC-SIGN positive cells and mean fluorescence intensity (MFI) were evaluated after 30 min or 3.5 h of incubation with different concentrations of **2b** and **12** or in the absence of inhibitor. Results showed that incubation of cells with the compounds did not modify percentages of DC-SIGN-positive cells (data not shown). A reduction of MFI was observed after treatment of the cells with **12** (Supplementary Figure 4). The effect is dose-dependent: ~20% reduction of fluorescence intensity was observed for a 50 μM concentration of **12**, up to a 52% reduction for a 250 μM concentration of **12**. The monovalent ligand **2b** was tested at higher concentrations (Supplementary Figure 4). A change in MFI was observed only after 3.5 h of incubation and for ligand concentrations ≥0.5 mM. The effects observed may be at least partially due to increased internalization of DC-SIGN receptors after binding of the dendron. However, the concentration required to exert a noticeable effect in the flow cytometry studies seems somewhat higher than the infection inhibition concentration.

possibility, DC-SIGN expression was studied by flow cytometry experiments. B-THP1/DC-SIGN cells were stained

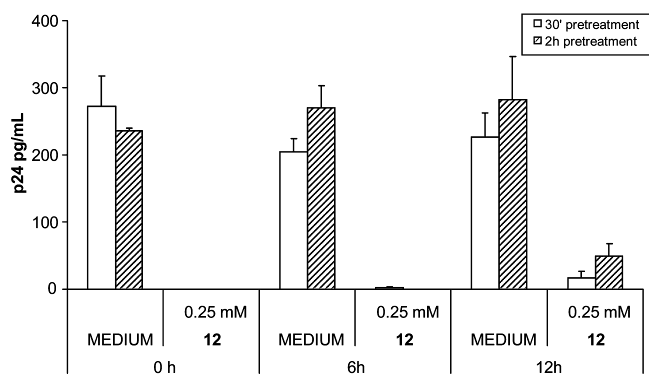


Figure 6. Persistence of inhibitory effect of 12 after compound removal. B-THP-1/DC-SIGN cells were incubated with 12 (0.25 mM) for 30 min or 2 h. After extensive washing, cells were pulsed with HIV-1 BaL following 0, 6, or 12 h. Levels of infection (determined by p24 concentration in co-culture supernatants) 3 days post infection were shown. Experiment was performed on two donors. Each condition was tested in duplicate. Values are mean ± SE.

Discussion. DC-SIGN recognizes highly mannosylated glycoproteins at the surface of a broad variety of pathogens. At least for some of these agents, including human immunodeficiency virus (HIV) (*3*), Dengue virus (*37*), Ebola virus (*38*), hepatitis C virus (*39*), SARS (*40*), and *Mycobacterium tuberculosis* (*41*), this interaction appears to be an important part of the infection process, and as a consequence, the initial stages of the infec-

tion could be prevented by inhibiting DC-SIGN mediated pathogen recognition.

Therapies based on antimicrobial drugs that kill pathogens apply a strong selective pressure to the microbial population, which in turns favors the emergence of resistant strains: hence the never-ending search for new, more powerful agents capable of fighting such strains. For HIV, this is particularly exacerbated by the rapid rate of mutations of the viral population. The mechanism by which DC-SIGN antagonists work aims at interfering with the initial steps in the infection process without killing the virus. Preventing the adhesion of HIV to its specific dendritic cell receptor, the lectin DC-SIGN, can block the infection while applying minimal selective pressure to the pathogen, which is simply made non-harmful.

Current antiviral therapies are based on drugs directed at different targets of the virus or of the microbial infection cycle. Highly active antiretroviral therapy (HAART) markedly reduces morbidity and mortality of HIV-infected individuals but is not able to eradicate HIV infection, and lifelong treatment is required. Moreover, people living in developing countries often do not have access to this therapy (*42*). Several efforts have been made to develop a vaccine against HIV, but vaccine candidates have so far met with limited success (*43, 44*).

It is the general consensus that the development of efficient, low-cost microbicides and entry inhibitor molecules that can be applied topically to prevent sexually transmitted HIV infections should be given high priority (*45*). Mannan is known to inhibit DC-SIGN-mediated viral uptake, but it cannot be used *in vivo* because it is highly toxic and mitogenic, which limits its therapeutic applications *in vivo* (*46, 47*). The polyvalent ligand **12** described herein inhibits viral uptake by DC-SIGN-expressing cells, thus avoiding viral dissemination. Application of this new tetravalent pseudo-trimannoside dendron as topical formulations may lead to topical anti-infective agents with a protective action against HIV.

Some topical anti-infective agents against HIV have been recently disclosed, all of which attempt to exploit inhibitory pathways independent of the DC-SIGN receptor. New microbicides were developed and studied in cell, tissue, and animal models. Glycerol monolaurate (*48*) (GML) blocks CCR5+ cell-attracting chemokines and pro-inflammatory cytokines induced by SIV, thus inhibiting the recruitment of SIV target cells expressing the CCR5 receptor. Therefore GML action is dependent on viral tropism, acting only on the R5 tropic strain, as opposed to the molecules described here. Griffithsin (GRFT) (*49*), like cyanovirin (CNV) (*50*), acts by binding glycoproteins of the HIV envelope and is capable of blocking cell-to-cell HIV transmission. So far, all clinical trials involving microbicides designed to prevent mucosal transmission of HIV infection have failed (*51*).

A handful of compounds have been developed so far in an effort to target DC-SIGN-mediated viral transmission (Supplementary Tables 2 and 3 collect the available affinity information). Among them, some potent, highly mannosylated polyvalent compounds have been described. The Wong group has reported oligomannose dendrons that display complex oligomannoses in high density and inhibit binding of gp120 to recombinant dimeric DC-SIGN with IC_{50} in the nanomolar range (*22*). These systems are excellent models of DC-SIGN inhibition, but their practical application as therapeutic agents seems unlikely, given the complexity of the oligosaccharides used. More remarkably, Penadés, Alcami, and their groups have reported that gold nanoparticles displaying various linear and branched mannosyl oligosaccharides (Manno-GNPs) are potent inhibitors of DC-SIGN-mediated HIV *trans* infection of human activated peripheral blood mononuclear cells (*23*). However, *in vivo* use of GNPs raises some concerns, mostly because of the potential toxicity produced by gold accumulation.

The development of glycomimetic drugs appears as a new, expanding, and promising approach to circumvent chemical drawbacks associated with the use of carbohydrates for therapeutic purpose (*12*). The pseudo-glycosylated dendron disclosed here is the first example of a low-valency compound based on a sugar mimic that, used in low micromolar concentrations, can completely block DC-SIGN-mediated *trans* infection of CD4+ T lymphocytes. The anti-infective action is exerted upstream of cytokine involvement and therefore is independent of viral tropism, as shown by inhibition of a series of laboratory-adapted strains and primary isolates with different tropism.

The distance spanned by two ligand units of **12** at full extension of the dendron arm is 28–30 Å at best, too short to allow simultaneous binding to two CRDs of the same DC-SIGN tetramer (from the recently published tetrameric model of DC-SIGN, derived from small-angle X-ray scattering studies, the distance between two adja-

cent Ca^{2+} sites is close to 40 Å) (*17*). Depending on protein density on the cell surface, simultaneous binding to two CRDs on different tetramers could occur. Most probably, however, the ligand presentation on dendrons allows achievement of a high local concentration of recognition elements. With dendrimers this is obtained without increasing the viscosity of the system and thus is fully compatible with use in physiological fluids.

The time-course studies (Figure 6) and the experiments shown in Figure 3, panel b show that the antiviral effect of **12** persists for hours even after the cells have been washed. The mechanism of this inhibition after removal could be based on the persistency of the multivalent ligand on the receptor binding site (slow off-rate of the tetravalent compound from the protein). However, the dose-dependent reduction in MFI observed in the flow cytometry studies (Supplementary Figure 4) suggests that exposure to **12** may also alter somewhat significantly the observed cell surface concentration of DC-SIGN, possibly by induced endocytosis. Depletion of receptor membrane concentration is an interesting feature of **12** and could at least partially account for its antiviral activity. Complex mechanisms can be involved, and for this reason further studies are in progress to quantitatively understand the correlation between anti-infectivity and modification of cell-surface receptor concentration in this system, as well as to establish the underlying mechanisms.

The structural simplicity of ligand **12**, which displays only four copies of a simple linear mannose trisaccharide mimic on a Boltorn-type dendron, allows efficient synthesis, amenable to large-scale production. The compound is highly soluble in physiological media and shows negligible cytotoxicity. Its chemical stability in aqueous buffer was evaluated at pH 5 and 7.4 using MALDI-MS and ^{1}H NMR spectroscopy. At pH 5, which is relevant for topical vaginal application, **12** was found to be stable for at least 1 week. In pH 7.4 aqueous buffer the stability is reduced to 6–12 h (*52*). Although stability to mannosidase has not been tested yet for **2** or **12**, the parent pseudo-disaccharide **1** was found to resist mannosidase hydrolysis (*30*).

The anti-infective activity of **12** appears to depend both on the good affinity for DC-SIGN of its pseudo-trimannoside binding unit and on the tetravalent presentation on the dendron scaffold. Indeed, the infection studies show that neither the monovalent pseudo-trisaccharide **2b** nor the tetravalent mannosylated dendron **14** perform as well as **12** as inhibitors of viral transmission. Thus, the structure of this lead can be improved by increasing the affinity of the monovalent binding unit, but it can also be tuned on the controlled level of presentation depending on the dendrimer/dendron used. Furthermore, functionalization of the dendron focal point in **12** allows further structural manipulations (*i.e.*, introduction of a lypophilic tail), which will help to modulate the pharmacokinetics of the molecule without affecting the affinity for DC-SIGN. Further developments along these lines are currently actively pursued in our laboratories.

METHODS

Surface Plasmon Resonance. DC-SIGN ECD protein (residues 66–404) has been overexpressed and purified as described previously (*17*). All experiments were performed on a BIAcore 3000 using functionalized CM4 chips and the corresponding reagents from BIAcore. Two flow cells were activated as previously described (*53*). Flow cell one was then blocked with 50 μL of 1 M ethanolamine and served as the control surface. The second one was treated with BSA-Manα1-3[Manα1-6]Man (BSA-Mannotriose, Dextra) (60 μg mL^{-1}) in 10 mM acetate buffer, pH 4. Remaining activated groups were blocked with 50 μL of 1 M ethanolamine. The final density immobilized on the surface of the second flow cell was 1200 RU. The BSA-Mannotriose used to functionalize the CM4 chip harbors 15 glycosylation sites according to manufacturer. The affinity of the various sugars and mimics was then estimated through a DC-SIGN ECD binding inhibition assay. The ECD of DC-SIGN was injected onto the BSA-Mannotriose surface, at a 20 μM alone or in presence of an increasing concentration of the sugar derivatives. Injection were performed at 20 μL/min using 25 mM Tris-HCl, pH 8, 150 mM NaCl, 4 mM $CaCl_2$, and 0.005% of P20 surfactant as running buffer. The original sensograms are reported as Supporting Information.

Infection Studies. *Cell Culture and Virus.* Human B cell lines B-THP1 and B-THP1/DC-SIGN (contributed by Drs Li Wu and Vinet N.Keval Ramani) were grown in RPMI 1640 (PBI International) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin (P/S), and L-glutamine (L-Gln) (all from EuroClone).

The following laboratory-adapted HIV-1 strains were used in the experiments: the R5 tropic HIV-1 BaL (contributed by Drs. S. Gartner, M. Popovic, and R. Gallo), the R4 tropic HIV-1 IIIB (contributed by Drs. M. Popovic and R. Gallo), and the dual tropic strain HIV-1 89.6 (contributed by Dr. R Collman). All strains were grown to high titer in peripheral blood mononuclear cells (PBMC) from healthy donors. Primary isolates HIV-1 V6 (CCR5 tropic) and V17 (CXCR4 tropic) were kindly provided by Dr. M. Andreoni (University of Rome Tor Vergata, Italy).

CD4+ T Lymphocytes Preparation. Peripheral blood was collected from healthy donors and PBMC were isolated by centrifugation on a Ficoll discontinuous density gradient (Lympholyte-H,

Cederlane Laboratories). CD4+ T lymphocytes were separated from PBMC by direct magnetic labeling using the CD4+ microbeads (Miltenyi Biotech) according to manufacturer's protocol.

CD4+ T cells (at density of 2×10^6 cells mL^{-1}) were activated by culturing them in complete RPMI (RPMI 160 with 20% FBS, P/S, and L-Gln), in presence of IL2 (15 ng mL^{-1}; R&D Systems) and PHA (7.5 $\mu g\ mL^{-1}$; Sigma Aldrich) for two days.

Inhibition of HIV Infection in "trans". Inhibitors were diluted to desired concentration into culture medium (complete RPMI).

B-THP1/DC-SIGN cells or B-THP1 cells (10^6 cells mL^{-1}) were preincubated with different concentration of compounds **2b**, **12**, **14**, and mannan (Sigma) or culture medium alone (negative control) for 30 min prior to exposure to HIV-1 BaL, IIIB, 89.6, V6, or V17 (final concentration of all HIV-1 strains = 0.08 ng mL^{-1} of p24) in the continued presence of the inhibitors for 3 h at 37 °C and 5% CO_2.

After extensive washing, to remove unbound virus and inhibitors, B-THP1/DC-SIGN cells were co-cultured with activated CD4+ T cells in complete RPMI with IL2 (15 ng mL^{-1}) at 37 °C and 5% CO_2 for 3 days. Cellular concentration of both B-THP1/DC-SIGN cells and CD4+ T lymphocytes was 2×10^6 cells mL^{-1}, and the ratio of B-THP1/DC-SIGN to CD4+ T lymphocytes was 1:4.

For some experiments B-THP-1/DC-SIGN cells were treated 30 min or 2 h with the inhibitor or medium alone and washed with PBS to eliminate unbound compounds. After wash B-THP-1/DC-SIGN cells were put in culture medium and then were exposed for 3 h to HIV BaL (0.08 ng mL^{-1} of p24) immediately or after 6 or 12 h. After incubation with the virus the cells were the co-cultured with CD4+ T lymphocytes, as previously described.

ELISA. Co-culture supernatants were collected at day 3, and p24 concentration, as a measure of HIV infection, was assayed using the Alliance HIV-1 p24 Antigen kit (Perkin-Elmer) following manufacturer's instruction.

Flow Cytometry. DC-SIGN expression in the cell line B-THP1/DC-SIGN was checked by staining with the PE-labeled anti-human DC-SIGN monoclonal antibody (clone AZND1, Beckman Coulter). To evaluate the toxicity of **12** B-THP-1/DC-SIGN cells were incubated with different concentrations of **12** for 3 h and 30 min. The apoptosis was monitored evaluating the number of dead cells according to forward and side scatters of flow cytometry analysis and the staining with DNA incorporating the dye 7-aminoactinomicin D (7-AAD, Beckman Coulter). All flow cytometric analyses were performed using a CYTOMICS FC-500 flow cytometer interfaced with CXP 21 software (Beckman Coulter).

Synthesis. General information is reported in Supporting Information. The following compounds are known and have been previously described: **4** (*28*), **5** (*28, 29*), **6** (*30*), **8** (*8*), and **13** (*29*). Full synthetic procedures, compound characterization for **2b**, **10**, and **14**, and numbering used in spectral assignment and isotopic distribution of dendron **12** are reported as Supporting Information.

Tetravalent Dendron of the α(1,2)α(1,6)Pseudomannotriose **12**. To a solution of the scaffold dendron **11** (*32*) (4.5 mg, 5.39 μmol, 1 equiv) in 100 μL of dry *N,N*-dimethylacetamide (DMA) under nitrogen atmosphere were added HATU (17 mg, 43.36 μmol, 8 equiv) and diisopropylethylamine (DIPEA) (15 μL, 86.7 μmol, 16 equiv). After 15 min a solution of **10** (26 mg, 43.36 μmol, 8 equiv) in dry DMA (170 μL) was added. The reaction was stirred at RT for 3 days. MALDI mass analysis of a reaction aliquot showed completion of the reaction. The reaction mixture was diluted in methanol and charged directly onto a LH-20 Sephadex column equilibrated in methanol. Slow elution led to the purification of product **12**, which was isolated in good yield (15.6 mg, 92% yield). $[\alpha]^{20}_D = +55.2$ (*c* 0.7, CH_3OH). ^1H NMR (400 MHz, D_2O, ppm): δ 5.00 (s, 4H, $H_{1M'}$ assigned on the basis of interglycosidic NOE signal in ROESY experiment between $H_{1M'}$ and D_1 and D_{3eq}), 4.84 (s, 4H, H_{1M}), 4.33 (bs, 6H, $OCH_2CH_2N_3$, CH_2O a), 4.24 (bs, 8H, CH_2O b), 4.03 (bs, 1H, D_2), 3.97 (bs, 4H, $H_{2M'}$), 3.92 (bs, 4H, H_{2M}), 3.89–3.80 (m, 12H, $H_{3M'}$, $H_{6M'B}$, H_{6MB}), 3.80–3.53 (m, 38H, D_1, $H_{6M'A}$, H_{6MA}, H_{3M}, H_{4M}, $H_{4M'}$, H_7, H_{5M}, $H_{5M'}$, CH_2N_3), 3.69 (s, 24H, $COOCH_3$), 3.41 (bs, 8H, H_8), 3.46–3.38 (m, 8H, D_4, D_5), 2.72–2.62 (m, 8H, CH_2COO), 2.60–2.49 (m, 8H, CH_2CONH), 2.24–2.06 (m, 8H, D_{6eq}, D_{3eq}), 1.92–1.72 (m, 8H, D_{6ax}, D_{3ax}), 1.32 (s, 3H, CH_3 a′), 1.25 (s, 6H, CH_3 b′). ^{13}C NMR (100 MHz, D_2O, ppm): δ 181.9 (COO); 178.2, 177.9 ($\underline{C}OOCH_3$); 174.7, 174.66 ($COOCH_2$ b, CONH); 174.3 ($COOCH_2$ a); 100.5 ($C1_M$); 99.2 ($C1_{M'}$); 75.1 ($C1_D$); 71.5 ($C2_D$); 74.0, 72.5, 71.2, 71.1 ($C3_M$, $C3_{M'}$, $C4_M$, $C4_{M'}$); 71.1 ($C2_{M'}$); 70.6 ($C2_M$); 68.5 ($C6_M$); 67.5, 67.4 ($C5_M$, $C5_{M'}$); 66.8 (C7, OCH_2 a, OCH_2 b), 66.5 ($OCH_2CH_2N_3$), 61.6 ($C6_{M'}$), 53.2 (CH_3O); 50.0 (CH_2N_3); 47.3, 47.0 (Cquat a″, b″); 39.6 ($C4_D$, $C5_D$); 39.5 (C8); 30.6 (CH_2CONH); 29.8 (CH_2COO); 27.6, 27.3 ($C6_D$, $C3_D$), 17.5 (CH_3 a′, b′). MS (MALDI) calcd for $[C_{129}H_{201}N_7O_{82}Na]^+$ = 3184.97, found = 3184.60. ESI-HRMS calcd for $[C_{129}H_{201}N_7O_{82}]$ monoisotopic peak = 3160.17735, found = 3160.18380. Calculated and found isotopic distribution are reported in Supporting Information (Supplementary Figure 5 and Table 1)

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Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

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