Novel Hyperbranched Glycomimetics Recognized by the Human Mannose Receptor: Quinic or Shikimic Acid Derivatives as Mannose Bioisosteres

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The mannose receptor mediates the internalization of a wide range of molecules or microorganisms in a pattern recognition manner. Therefore, it represents an attractive entry for specific drug, gene, or antigen delivery to macrophages and dendritic cells. In an attempt to design novel effective synthetic mannose receptor ligands, quinic and shikimic acid were selected as putative mannose mimics on the basis of X-ray crystallographic data from the related rat mannose-binding lectin. As the mannose receptor preferentially binds to molecules displaying several sugar residues, fluorescein-labeled cluster quinic and shikimic acid derivatives with valencies of two to eight were synthesized. Their mannose receptor mediated uptake was assayed on monocyte-derived human dendritic cells by cytofluorimetric analysis. Mannose-receptor specificity was further assessed by competitive inhibition assays with mannan, by confocal microscopy analysis, and by expression of the mannose receptor in transfected Cos-1 cells. Constructs derived from both quinic and shikimic acid were efficiently recognized by the mannose receptor with an optimum affinity for the molecules with a valency of four. As a result, commercially available quinic and shikimic acids appear as stable mannose bioisosteres, which should prove valuable tools for specific cell delivery.

KEYWORDS:

drug targeting · glycomimetics · mannose receptor · quinic acid · shikimic acid

Introduction

The human mannose receptor is expressed on resident macrophages,[1] on dendritic cells, and on subsets of epithelial and endothelial cells.[2] The mannose receptor is a multidomain membrane-associated receptor which binds selectively to molecules or microorganisms carrying multiple, exposed, terminal sugars such as D-mannose, *N*-acetyl-D-glucosamine, or L-fucose, but not D-galactose. Interaction with the ligands is achieved through cooperative binding with eight carbohydrate recognition domains (CRDs) within the ectodomain of the mannose receptor,[2a, 3] in accordance with the cluster effect.[4] The mannose receptor is capable of mediating internalization of both soluble and particulate carbohydrate structures; it regulates levels of endogenous proteins and contributes to the clearance of potentially harmful glycoproteins and of a wide variety of infectious agents, and so takes part in the innate immunity.[5] The mannose receptor strongly enhances effector immune functions through efficient antigen uptake.[6] The broad pattern recognition displayed by the mannose receptor together with its implication in adaptive immunity has stimulated considerable efforts toward the selective delivery of enzymes,[7] drugs,[8] oligonucleotides or genes,[9] and antigens[10] to cells expressing the mannose receptor, for therapeutic and vaccine strategies. For successful targeting, these entities have been conjugated to numerous glycosides and have given rise to mannose receptor ligands such as manno- or fucosylated neoglycoproteins,[7, 8b, 9b, 10c, 11] mannosylated liposomes,[8b, 9e, 10b] or polymers.[8a, 9a, 9c, 9d, 12] In the search for synthetic, chemically defined, high-affinity ligands for the mannose receptor, Ponpipom et al., Robbins et al., and Biessen et al. synthesized a series of lysine-based cluster mannosides containing 2 – 6 terminal D-mannose groups connected with the backbone by flexible spacers.[13] The latter study showed that recognition of low molecular weight mannosides by the mannose receptor was

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indeed possible, and was consistently enhanced with valencies increasing from 2 up to 6.

Nevertheless, the synthesis of glycopolymers or chemically characterized dendrimeric or cluster mannoside structures is demanding, with challenges stemming from the need to selectively functionalize the carbohydrate anomeric position, prior to bonding. Moreover, carbohydrates might be degraded upon synthesis or in the biological environment. Thus, we have sought to develop a glycomimetic approach which, in addition to improved yields and purities and lowered production costs, would produce compounds with enhanced stability and affinity.

Recent structure – function studies of recombinant truncated forms of the mannose receptor proposed that CRD-4 is the only CRD capable of binding monosaccharides when expressed in isolation.[14] CRD-4 shares sequence homology with the CRD of the rat mannose-binding lectin A (MBL-A), another surface-pattern recognition molecule of innate immunity; residues which participate directly in the binding are identical in both CRDs as revealed by sequence alignments. Moreover, CRD-4 and MBL-A bind to a similar spectrum of monosaccharides and interact with sugars with only slight differences.[3c, 15] As the only available CRD-4 crystallographic structure represents a non-sugar binding form of the domain,[16] it is tempting, in view of the mentioned similarities, to design C-type CRD ligands from data collected with MBL-A. A major contribution to the binding is provided by an extensive network of hydrogen bonds and coordination bonds between two equatorial, vicinal hydroxy groups in the (+)-synclinal configuration of the sugar ligand (that is, the 3- and 4-hydroxy groups for D-mannose), a calcium ion, two asparagines, and two glutamic acid residues of the protein (Figure 1).[17] Smaller contribution is provided by a stacking interaction between a tyrosine residue of the CRD and the C-5 and C-6 atoms of D-mannose (C-2 for *N*-acetyl-D-glucosamine).[15c] Moreover, the mannose receptor tolerates ligands with large substituents at the C-1 or C-6 positions and is not affected by the anomeric configuration of the sugar.[18] These observations reflect well the sugar specificity exhibited by the mannose receptor and, in particular, how it can discriminates between D-mannose and D-galactose since the 3- and 4-hydroxy groups are equatorial in the former and axial in the latter (Scheme 1).

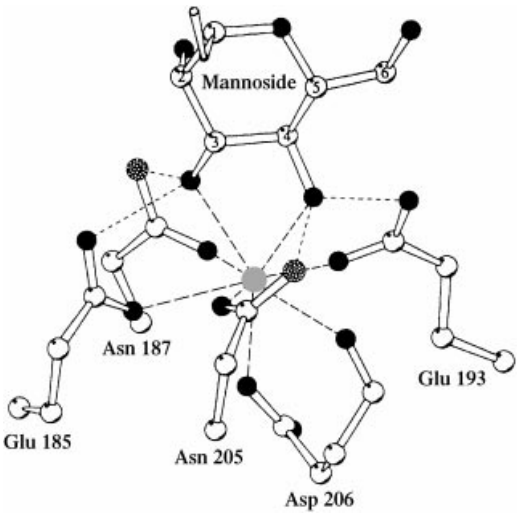
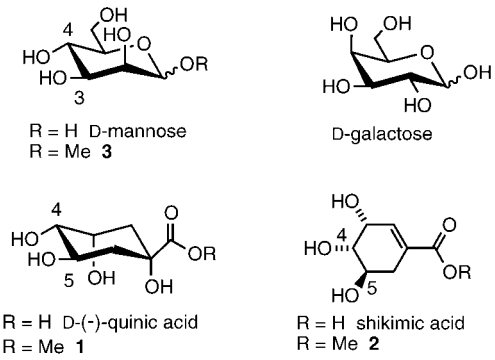


Figure 1. *Mannose binding at the calcium site of MBL-A. Calcium is shown as a grey sphere. White, spotted, and black spheres represent carbon, nitrogen, and oxygen, respectively. Long-dash lines represent calcium coordination bonds. The α-glycosidic bond to the next sugar (at C-1) has been removed for clarity. Oxygen atoms from the side chains of Glu 185, Asn 187, Glu 193, and Asn 205 and the main-chain carbonyl oxygen of Asp 206 form the base of the pentagonal bipyramid coordination set of Ca^{2+}; a carboxylate oxygen of Asp 206 forms one apex of the bipyramid, and the 3- and 4-hydroxy groups of the mannose bisect the other apex. Adapted with permission from ref. [17]. Copyright (1992) Macmillan publishers.*



(ClCH$_2$C=O)-L-Lys-βAla-NH$_2$, monovalent core **4**

(ClCH$_2$C=O)-L-Lys(ClCH$_2$C=O)-L-Lys-βAla-NH$_2$, divalent core **5**

((ClCH$_2$C=O)-L-Lys(ClCH$_2$C=O))$_2$-L-Lys-L-Lys-βAla-NH$_2$, trivalent core **6**

((ClCH$_2$C=O)-L-Lys(ClCH$_2$C=O))$_4$-(L-Lys)$_2$-L-Lys-L-Lys-βAla-NH$_2$, tetravalent core **7**

Scheme 1. *Structures of the monovalent mannose mimics **1** – **3** and the lysine cores **4** – **7** used for the synthesis of the cluster mannose mimics.*

We postulated that the commercially available carbocyclic D-(−)-quinic and -shikimic acids could act as carbohydrate mimics, since they possess a conveniently arranged vicinal diol (at the C-4 and C-5 positions) in (+)-synclinal *trans*-diequatorial or *pseudo*-diequatorial relationships, respectively (Scheme 1). Equally, they might offer a greater stability than mannose since the pyranose ring is replaced by a cyclohexane or cyclohexene. Finally, the presence of a carbocyclic acid group might facilitate functionalization in comparison with mannose, particularly when a solid-phase strategy is utilized.

To investigate the actual mannose mimicry of quinic and shikimic acids, we elected to quantify the mannose receptor mediated uptake of fluorescent-labeled multivalent constructs by monocyte-derived human dendritic cells with flow cytometric analysis. Such an assay, as close as possible to the physiological conditions, will be easily amenable to further evaluation of vaccine formulation. Lysine-based cluster quinic and shikimic acids were thus prepared. Quinic acid has previously been identified from the chiral pool as a valuable precursor of carboxylic isosteres of sugars. However, numerous modifications were necessary to accentuate its mimicry.[19] Furthermore, to our knowledge, sugar mimics have never been combined with the multivalency concept.[20]

The biomimetic activities of the quinic- or shikimic-derived molecules were evaluated with reference to analogue constructs decorated with D-galactonamide, designed as negative controls in order to differentiate the mannose receptor specific internalization from the nonspecific endocytosis of the dendritic cells. The mimics were also compared with cluster mannosides, related to the ligands of Ponpipom et al. and Biessen et al.,[13] together with their corresponding cluster galactosides as negative constructs. Since dendritic cells are known to express numerous lectins or adhesion molecules, uptake specificity was assessed by inhibition experiments with mannan, an established competitor of mannose receptor mediated uptake,[6a] by confocal microscopy analysis and by use of mannose receptor transfected Cos-1 cells.[3c]

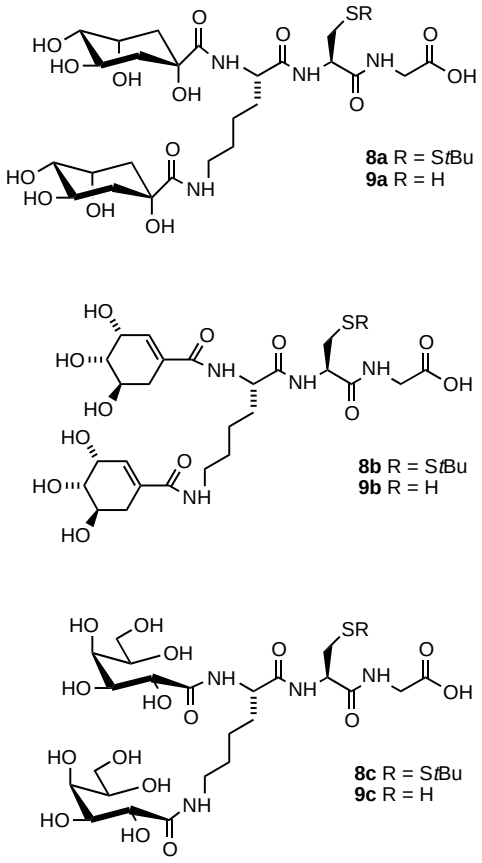
Results

Synthesis of the monovalent or cluster mannose mimics

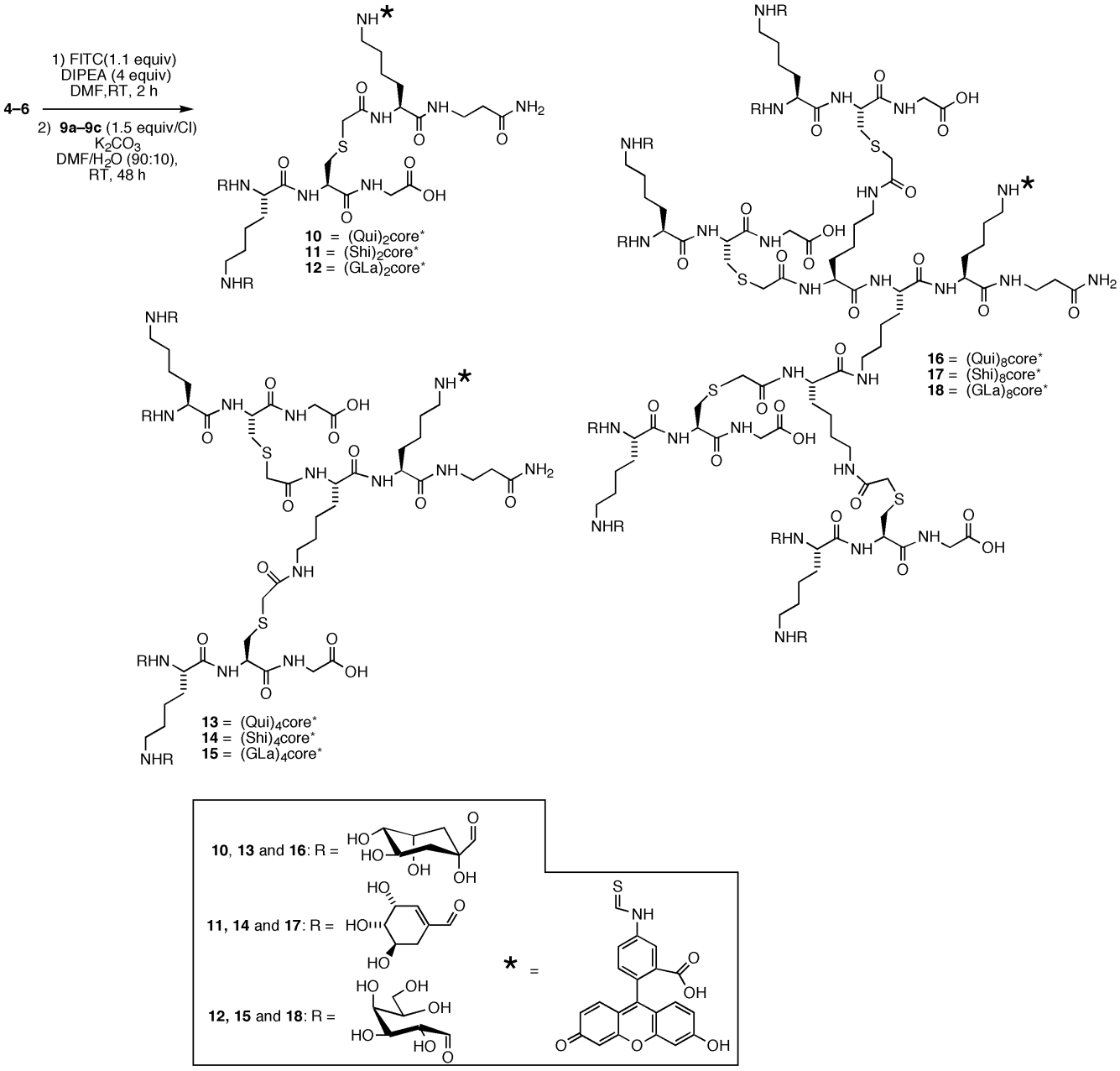
The carboxylic function of the quinic or shikimic acids was masked in a first series of putative monovalent ligands, methyl quinate (**1**)[21] and methyl shikimate (**2**),[22] evaluated as mannose receptor ligands with reference to methyl α-mannopyranoside (**3**; Scheme 1).

We then decided to synthesize dendrimers based on L-lysine trees, in view of their biocompatibility and, in particular, their lack of intrinsic immunogenicity.[23] The use of poly-L-lysine scaffolds has been extended more recently to the preparation of glycodendrimers.[24] However, the reported syntheses have been modified for our purpose, as previously described.[25] Briefly, the ε-amino group of the first lysinyl residue was not incorporated into the scaffold, in order to permit an ulterior linkage with fluorescent labels or peptide antigens. The terminus of each dendrimer arm was derivatized with chloroacetyl groups, to be further reacted with the glycomimetic or the carbohydrate moieties through sulfide ligation. Four levels of complexity, compounds **4** – **7**, were selected in order to display 2 – 8 residues (Scheme 1). For their attachment to the lysine cores, commercially available D-(−)-quinic and -shikimic acids or D-galactonolactone should be functionalized by a thiol group. Quinic and shikimic acids were linked through an amide bond on the α- and ε-positions of a H-L-Lys-L-Cys(S*t*Bu)-Gly-OH tripeptide assembled stepwise on a solid-support Sasrin or Wang resin to provide bivalent glycomimetic moieties, whose *tert*-butylthiocysteinyl radical represents the thiol precursor (Scheme 2).[26]

The coupling constants between protons 3-H/4-H and 4-H/5-H of both quinic acid residues in **8a**, as determined by ^{1}H NMR spectroscopy, were 2.9 and 9.6 Hz, respectively. These values are consistent with axial – equatorial and axial – axial couplings and with a chair-like conformation of the cyclohexanes which has an equatorial amide group. Similarly, the coupling constants between protons 3-H/4-H and 4-H/5-H of both shikimic acid residues in **8b** were 4.6 and 9.2 Hz, respectively. These values are compatible with a pseudoequatorial orientation of hydroxy groups at the C-4 and C-5 positions of the shikimic acid residues, and support the structural analogy made between the D-(−)-quinic and -shikimic acids and D-mannose. The galactonoyl residues were introduced by condensation of D-galactonolactone with the H-L-Lys-L-Cys(S*t*Bu)-Gly-OH tripeptide in MeOH under reflux conditions to form **8c**. Having prepared the different bivalent synthons **8a** – **8c** and the chloroacetyl core building blocks **4** – **7**, we next examined their final assembly into fluorescein-labeled cluster glycomimetics through a straightforward two-step procedure (Scheme 3). The chloroacetyl cores **4** – **6** were treated with FITC, in the presence of DIPEA in degassed DMF. After completion of the reaction (monitored by reverse-phase high-pressure liquid chromatography (RP-HPLC)), the crude mixtures were transferred and mixed with thiols **9a** – **9c** (1.5 equiv per chloroacetyl group to be substituted) dissolved in degassed H_2O. Compounds **9a** – **9c** were obtained from **8a** – **8c** by prior treatment with tri-*n*-butylphosphine in a degassed mixture of *n*PrOH/H_2O (1:1) followed by evaporation to dryness under reduced pressure to remove any *tert*-butylthiol liberated. The pH value of the mixture was further adjusted to 8 – 8.5 by adding solid potassium carbonate and the reaction was monitored by RP-HPLC. After 48 – 72 h, the reactions were essentially complete and constructs **10** – **18** were obtained in 27 – 80% yield following RP-HPLC purification (Scheme 3). Known fluorescent, multivalent lysine-based cluster mannopyranoside and galactopyranoside **19** – **24** were used as reference compounds (Scheme 4).[27]



Scheme 2. *Structures of the* tert*-butyl-protected and the unprotected bivalent building blocks* ***8a*** *–* ***c*** *and* ***9a*** *–* ***c****.*

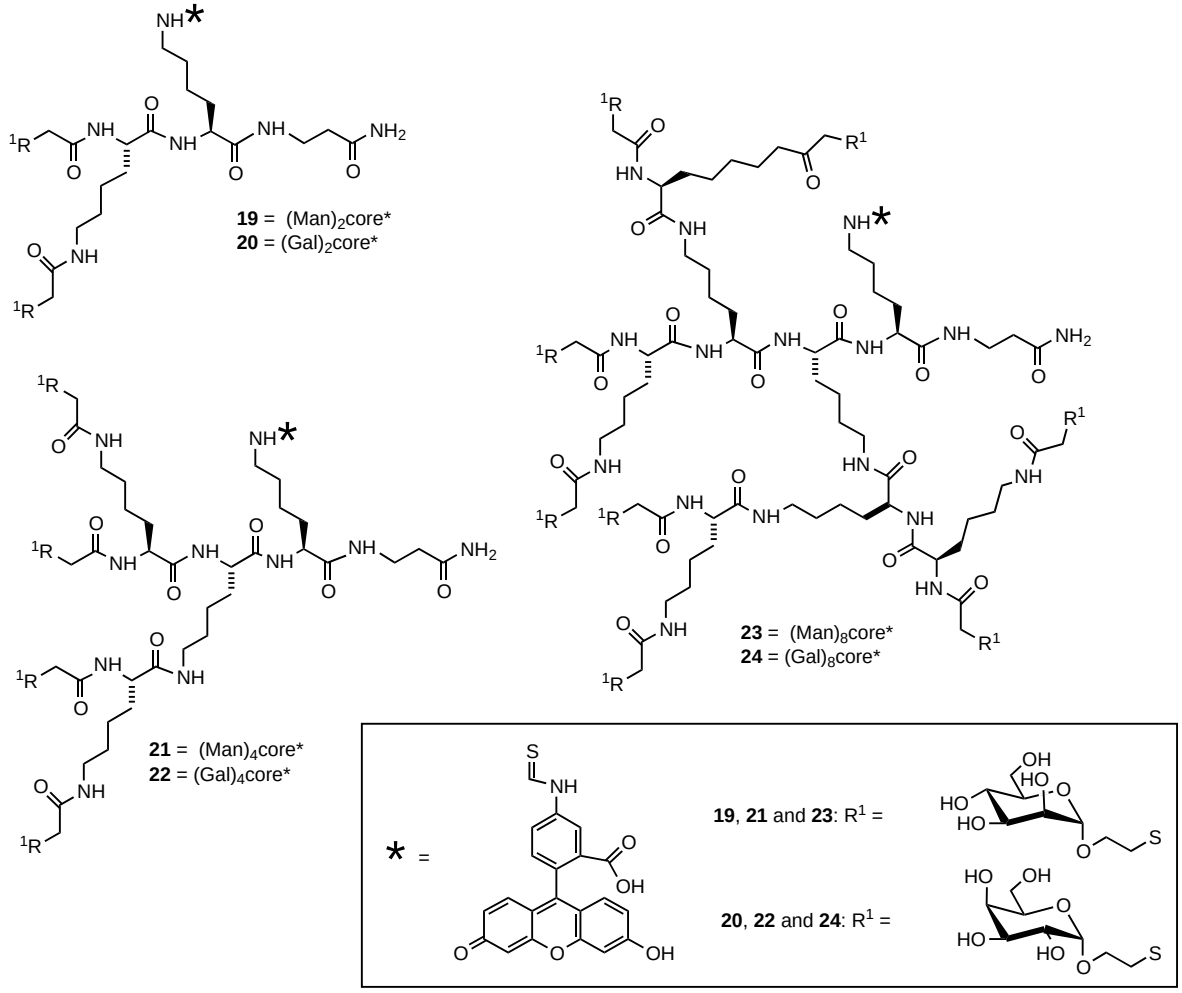


*Scheme 3. Preparation of the di-, tetra-, and octavalent fluorescein-labeled cluster glycomimetics **10** – **18**. FITC = fluorescein isothiocyanate, DIPEA = diisopropylethylamine, DMF = N,N-dimethylformamide, Qui = quinoyl, Shi = shikimoyl, and GLa = galactonoyl.*

Glycomimetic uptake in human dendritic cells

Monomer uptake: Binding of methyl quinate (**1**) and methyl shikimate (**2**) to the mannose receptor was assessed by comparison with methyl α-D-mannopyranoside (**3**) by competitive inhibition experiments with the $(Man)_8$core* (**23**). This molecule was preferred to FITC-mannosylated bovine serum albumin for the test because the uptake of neoglycoconjugates or proteins is known to be enhanced rather than inhibited by monomeric ligands such as mannose.[11c, 13c] Cells were preincubated for 10 min with concentrations of the three monomers ranging from 0 – 250 mmol, then coincubated for 20 min with **23**. As shown in Figure 2 A, the ability of **1** to block uptake of **23** is similar to that of **3**. A contrasted behavior was observed with **2** which favored capture of **23** at these concentrations (data not shown).

Specific uptake of the $(Qui)_8$core* and the $(Shi)_8$core*: Cells were pulsed for 20 min at 37 °C with fluorescein-labeled constructs at concentrations of 1, 5, and 10 μM, then washed three times with cold phosphate-buffered saline (PBS) and fixed for 20 min in paraformaldehyde solution (1 %) before FACScan analysis. The reference compound $(Man)_8$core* (**23**) was used to calibrate the test parameters (concentration, time of incubation); the optimum ratio of mannose receptor dependent uptake versus nonspecific endocytosis was obtained with an incubation time of 20 min as already observed.[3c] $(GLa)_8$core* (**18**) was also



Scheme 4. *Fluorescent multivalent lysine-based cluster mannopyranoside and galactopyranoside compounds which were used for reference.*

incorporated into the test; as expected from the sugar specificity displayed by the mannose receptor,[2a] this compound was poorly internalized by the dendritic cells at any concentration (Figure 2B), and the resulting mean fluorescent intensities were attributed to nonspecific pinocytosis. As can be seen in Figure 2B, the $(Qui)_8$core* and $(Shi)_8$core* compounds (**16** and **17**, respectively) were both internalized by the dendritic cells, with the mean fluorescent intensities obtained being dose dependent in the tested range. The uptake of **16** reached 52–62% of the mean fluorescent intensities attained with the reference **23**, while the uptake of **17** was lower (25–31%) although still significantly higher that the uptake of the negative control compound **18** (<3%).

Mannan inhibition test: Mannan, a bacterial polysaccharide that binds with high affinity to the mannose receptor, was used in a competitive inhibition test to confirm the specificity of the uptake of the ligands. As shown in Figure 2C, mannan inhibits uptake of $(Man)_8$core* (**23**), $(Qui)_8$core* (**16**), and $(Shi)_8$core* (**17**) in a dose-dependant manner. Maximal inhibition was reached at 1000 $\mu g\,mL^{-1}$, with more than 98% inhibition for **16** and **17**.

Effect of the valency of the cluster glycomimetics: We next examined the influence of the size of the cluster glycomimetics on the internalization mediated by the mannose receptor. Specific uptake of bi-, tetra- and octavalent fluorescein-labeled cluster glycomimetics, $(Qui)_n$core* and $(Shi)_n$core* (with $n=2$, 4, and 8), was deduced from endocytosis by testing against the corresponding $(GLa)_n$core* (with $n=2$, 4, or 8) at a 10 μmol ligand concentration. In Figure 3, results are expressed as the percentage of specific uptake of fluorescein-labeled ligands in comparison with nonspecific internalization. The assay was conducted in parallel with $(Man)_n$core* (with $n=2$, 4, or 8) compounds and their corresponding related $(Gal)_n$core* (with $n=2$, 4, or 8) structures as negative constructs.

Nonspecific endocytosis diminished consistently with valencies increasing from 2–8 (data not shown); no difference between specific uptake and endocytosis was observed for the bivalent mannoside construct. Significant specific uptake was observed from the divalent $(Qui)_2$core* (**10**) upwards and from the tetravalent $(Shi)_4$core* (**14**) and $(Man)_4$core* (**21**) upwards (Figure 3). Strikingly, optimal specific uptake was obtained for tetravalent constructs in the glycomimetic series whereas the higher-branched, octavalent structure proved to be superior in the natural ligand series. Nonequivalent interactions with the CRD domains might be expected due to the differences of these

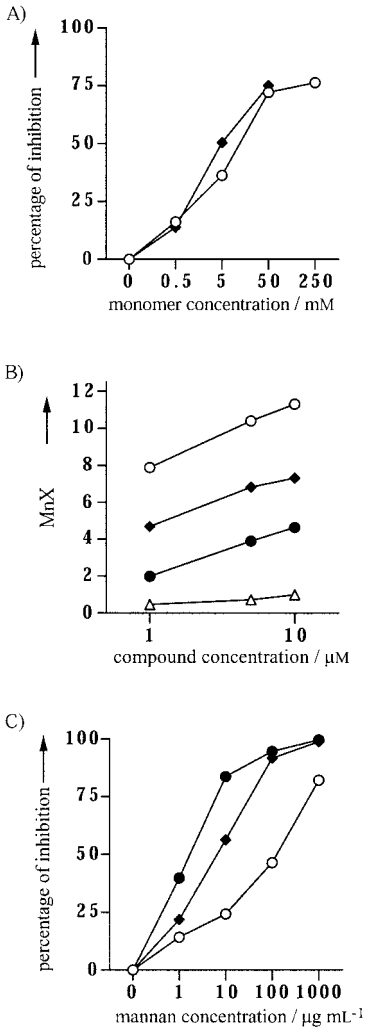


Figure 2. *a) Monomers inhibitory test: Cells were incubated with different concentrations of monomers* ***1*** *(○) or* ***3*** *(◆), and coincubated with FITC-labeled* $(Man)_8$*core* before cytofluorimetric analysis. Results are expressed in percentage of inhibition, compared to the maximal signal obtained in the absence of inhibitors. b) Uptake of FITC-labeled compounds by dendritic cells: Cells were pulsed with fluorescein-labeled constructs. The efficiency of the uptake was evaluated at ligand concentrations of 1, 5, or 10 μM. Cell-associated fluorescence, due to the uptake of fluorescein-labeled compounds, was quantified by mean fluorescence intensity (arbitrary units of fluorescence). c) Mannan inhibitory test: cells were incubated with different concentrations of mannan derived from* Saccharomyces cerevisiae *and coincubated with the glycomimetics before cytofluorimetric analysis. For b) and c):* $(Man)_8$*core* (○),* $(Qui)_8$*core* (◆),* $(Shi)_8$*core* (●), and* $(GLa)_8$*core* (△). The results shown are representative of at least three independent experiments. For further details, see the Expermental Section.*

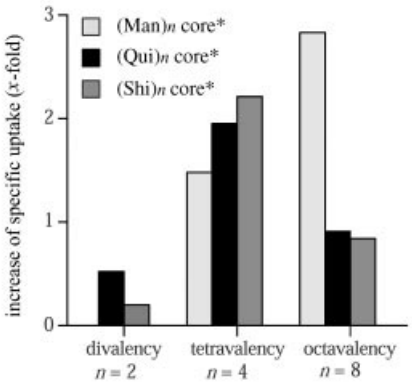


Figure 3. *Effect of valency on mannose receptor specific internalization: Results are expressed as percentage of mannose receptor-specific uptake. Nonspecific uptake due to endocytosis was measured with the corresponding cluster galactosides and subtracted from the mean fluorescence intensity detected.* n *represents the valency of the ligands used: 2, 4, or 8. The results shown are representative of at least three independent experiments. For further details, see the Experimental Section.*

ligands in their shape and nature (see above). Given these results, tetravalent structures were selected for the confocal microscope analysis and the transfection experiments.

Confocal microscopy: Dendritic cells derived from peripheral blood mononuclear cells (PBMC) after 5 days of culture are competent for antigen capture and processing; they express the mannose receptor and use it for efficient capture of a variety of antigens. In order to minimize the contribution of nonspecific fluid-phase macropinocytosis, cells were first incubated with the glycomimetics for 45 min over ice. In these conditions, the binding of the fluorescent probes was mostly dependent upon the interaction with receptors expressed at the cell surface. After several washings, the cells were cultured for 5 min at 37 °C in marker-free medium, to permit active uptake at the receptor-bound compounds.

Cells were then analyzed for the green fluorescence (excitation at 488 nm) of the constructs (Figure 4A, E, and I), the red fluorescence (excitation at 568 nm) of the mannose receptor (Figure 4B, F, and J) and the blue fluorescence (excitation at 647 nm) of the nucleus (Figure 4C, G, and K). The superposition of the three fluorescences is shown in Figure 4D, H, and L. Data for the $(Shi)_4$core* construct (**14**) are not shown. The glycomimetics were visualized by using an anti-fluorescein antibody to amplify the signal.

Internalization of constructs occurs rapidly, since following the 5 min incubation, the $(Man)_4$core* and, to a lesser extent, the glycomimetics were detected intracellularly. Except for the $(GLa)_4$core*, which is marginally internalized (Figure 4E and H), the other three constructs are colocalized with the mannose receptor (Figure 4D and L, data for $(Shi)_4$core* (**14**) are not shown). The constructs seem to accumulate in vesicular compartments under the plasma membrane, suggesting internalization into early endosomes.

Expression of the mannose receptor in Cos-1 cells: Colocalization of $(Qui)_4$core* (**13**) with the mannose receptor, visualized by confocal microscopy, is a strong argument that glycomimetic cluster uptake passes through the mannose receptor. To demonstrate the specificity of glycomimetics to the receptor, we chose to express the human mannose receptor transiently in Cos-1 cells.

As described earlier,[3c] mannose receptor CD8 vector, which contains the cDNA (complimetary DNA) for the human mannose receptor was transfected in the presence of polyethylenimine (PEI). After 48 hours, cells were harvested and mannose receptor expression was checked with the PE-conjugated anti-mannose receptor monoclonal antibody (mAb). The transgene was being

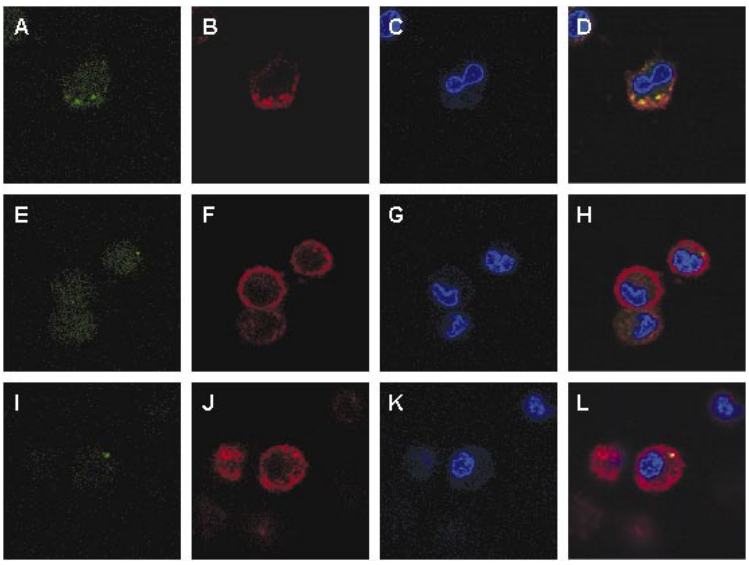


Figure 4. *Confocal study of the cellular distribution of the glycomimetics and the mannose receptor. Cellular localization of the FITC-labeled constructs was amplified with a monoclonal rabbit anti-fluorescein antibody coupled to Alexa 488 (green fluorescence (488 nm): A, E, and I). The mannose receptor was detected by indirect immunochemistry using a monoclonal antibody against human mannose receptor and a goat anti-mouse IgG Alexa 568 conjugated as a secondary antibody (red fluorescence (568 nm): B, F, J). Nuclear DNA was stained with TO-PRO-3 iodide (blue fluorescence (647 nm): C, G, K). Triple exposure shows the overlapping fluorescence of the different constructs with the mannose receptor (D, H, L). Magnification: × 1000. A – D: $(Man)_4$core* (**21**); E – H: $(GLa)_4$core* (**15**); I – L: $(Qui)_4$core* (**13**). Data for the $(Shi)_4$core* construct (**14**) are not shown.*

expressed by 10 – 15 % of the Cos-1 cells. Cells were pulsed for 20 min at 37 °C with FITC-labeled glycomimetics, then washed and fixed in paraformaldehyde solution (1 %) before FACScan analysis. As shown in Figure 5 empty vector transfected Cos-1 cells take up very low quantities of $(Qui)_4$core* (**13**). In mannose receptor transfected Cos-1 cells $(GLa)_4$core* (**15**) internalization is close to the background, as nonspecific pinocytosis is low. The percentage of mannose receptor transfected Cos-1 cells internalizing **13** is 17.2 %, which corresponds to the cells expressing the receptor. Moreover, the uptake of **13** is inhibited by mannan, which indicates the specificity of the uptake by the mannose receptor.

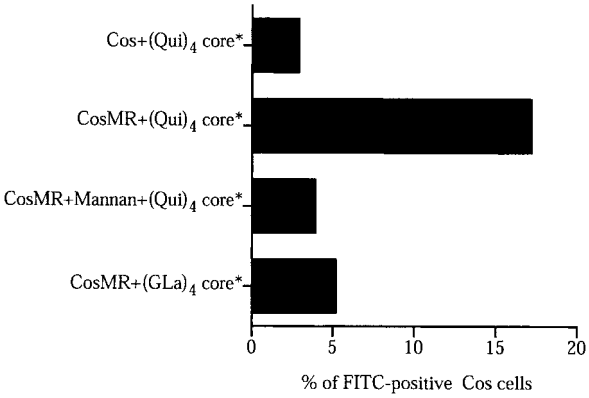


Figure 5. *Glycodendrimers are specifically taken up in mannose receptor expressing Cos-1 cells. Percentages of fluorescein positive cells are expressed. CosMR = cells with the plasmid coding for the mannose receptor; Cos = cells with the empty vector. The results shown are representative of at least three independent experiments. For further details, see the Experimental Section.*

Discussion

In this study, we have investigated the possibility of adapting the concept of carbohydrate mimics to the design and synthesis of ligands of the mannose receptor expressed by the human dendritic cell. With the identification of molecular events critical for binding to the mannose receptor, we have proposed to replace the natural mannoside or fucoside ligands with stable, commercially available, polyhydroxylated carbocyclic derivatives. In this article, we have described the preparation of a set of lysine-based cluster quinic and shikimic acid derivatives with valencies of 2, 4, or 8, fluorescein-labeled to allow evaluation of their uptake by cytofluorimetry. However, dendritic cells are known to take up a large variety of substances by macropinocytosis in addition to mannose receptor specific capture.[6a] We planned to synthesize markers of the nonspecific macropinocytosis to discriminate between the two modes of internalization: D-galactonolactone, a structure derived from D-galactose and known to have a very low affinity for mannose receptor,[2a] was linked to the same lysinyl cores to provide structurally related constructs for negative control. Thus, *N*-chloroacetylated L-lysinyl trees were labeled with FITC at the lysine residue near the C terminus and further reacted through thioether ligation with cysteinyl tripeptides bearing two quinoyl, shikimoyl, or galactonoyl (for negative control) residues. These divalent synthons were independently prepared from commercially available D-(−)-quinic or shikimic acids and D-galactonolactone; these were linked to the α and ε positions of a L-Lys-L-Cys-Gly tripeptide (Scheme 2). To calibrate the assays (concentration, time of pulsation), mannosylated trees, related to the mannose receptor ligands reported by Biessen et al. and Ponpipom et al.[13] were used. Nonspecific endocytosis in dendritic cells was assessed with the corresponding galactosylated trees (Scheme 4), and subtracted as the background. The glycomimetics were then tested in the same conditions, in order to evaluate receptor-specific internalization.

Binding properties of monomeric, methyl quinate (**1**) and methyl shikimate (**2**) were first evaluated in a competitive inhibition experiment with $(Man)_8$core* (**23**), with methyl α-D-mannopyranoside (**3**) as the reference ligand. Compound **1** was as potent as the mannoside equivalent. As shown in Figure 2 A the uptake of **23** was efficiently inhibited with 500 – 1000 times higher concentrations of the monomers, which suggests a strong cluster effect. Compound **2** caused enhancement of the uptake of **23** (data not shown). Such a stimulatory effect has been previously observed in inhibition studies using tissue-type plasminogen activator, ribonuclease B,[13c] or bovine serum albumin (BSA).[11c] This effect is particularly exerted by weak ligands, which suggests that shikimic acid is recognized by

mannose receptor, albeit poorly. Binding discrepancies between shikimic and quinic acids were expected from the less favorable orientation of the 4- and 5-hydroxy groups, which is pseudo-equatorial rather than equatorial in shikimic acid.

To confirm these preliminary results, we next examined the mannose receptor mediated uptake of the octavalent fluorescein-labeled glycomimetics most likely to be potent ligands.[4a, 13c] Significant uptake was detected following 20 min incubation at 37 °C from glycomimetics at a concentration of 1 μM (Figure 2B). By comparison, uptake of the negative control compound, $(Gla)_8$core* (**18**), was very low and close to the background signal due to the auto-fluorescence of dendritic cells. This result suggests a specific capture of the $(Qui)_8$core* (**16**) and $(Shi)_8$core* (**17**) by human dendritic cells. Furthermore, this capture involves mannan-competitive interaction with a surface-expressed receptor (Figure 2C); the mannan doses ($\sim 1\ mg\ mL^{-1}$) required to inhibit the uptake of the constructs were comparable with those used by others in similar competitive inhibition experiments.[6a] Thus, as anticipated, D-(−)-quinic and -shikimic derivatives behave as mannose mimics, and are recognized by receptors, possibly the mannose receptor, expressed at the cell membrane of human dendritic cells.

Influence of the valency of the trees was next studied by comparison with the $(Man)_n$core* ligands ($n = 2$, 4, and 8) (Figure 3). Among bivalent constructs, only $(Qui)_2$core* (**10**) was specifically taken up by dendritic cells. Active capture of $(Shi)_2$core* (**11**) or $(Man)_2$core* (**19**) was not observed, as a result of important endocytic uptake of these small molecules rather than an absence of recognition; nonspecific macropinocytosis diminished steadily with valencies increasing from 2 – 8 (data not shown). Strikingly, specific capture was better for the tetravalent glycomimetics than for the octavalent ones, whereas it was consistently enhanced in the mannosylated series from $(Man)_2$-core* (**19**) to $(Man)_8$core* (**23**). The observed differences might originate from divergences in the fine structure of the mannoside and glycoside trees. Indeed, the nature, size, and shape of such multivalent ligands can deeply influence their interaction with receptors as previously mentioned.[13a, 13c, 28] presently mannoside and glycoside clusters have been elaborated on the same lysinyl cores but these mainly differ in their branching pattern and their overall charge at physiological pH values (neutral or negative). This interesting valency effect needs further study to explore the complex multiinteractions between mannose receptors and ligands.

Since tetravalent clusters were the most effective in our test, they were retained for further investigations. In order to identify the cell membrane receptor involved in the uptake of the D-(−)-quinic and -shikimic cluster derivatives, we attempted to visualize, by confocal microscopy, the early membrane events during internalization. As shown in Figure 4B, F, and J, mannose receptors are distributed close to the cell membrane and in intracellular vesicles as expected.[6a, 29] After 5 min incubation at 37 °C, $(Man)_4$core* (**21**), as well as $(Qui)_4$core* (**13**), and $(Shi)_4$core* (**14**) have been internalized and are fully co-localized with the mannose receptor, in vesicles close to the cell membrane (Figure 4A, I, D, and L, data not shown for **14**). Comparatively, $(GLa)_4$core* (**15**) was only poorly taken up by dendritic cells and does not colocalize with the mannose receptor (Figures 4E and H).

To confirm the mannose receptor mediated internalization of the glycomimetics, which was suggested by the competitive inhibition experiments and the confocal microscopy analysis, we took the benifit of the mannose receptor expressing model in Cos-1 cells, developed by Ezekowitz et al.[3c] Cos-1 cells, which have low endocytic capacity, take up D-(−)-quinic cluster derivatives only when the mannose receptor is expressed. Moreover, this uptake is specifically inhibited by mannan, a well-known mannose receptor ligand (Figure 5). When these results are taken together, it is tempting to identify the target receptor as the mannose receptor of the human dendritic cells.

Conclusion

Synthetic lysine-based clusters of quinic and shikimic acid derivatives have been shown to be effective ligands for the mannose receptor of dendritic cells. In this respect quinic and shikimic acids appear as mannose bioisosteres. In particular, shikimic and quinic acids differ from mannopyranoside by their hydroxy-substitution pattern and, for the former, probably by a more flattened conformation of the ring induced by the unsaturation. This study thus indicates that the mannose receptor can accommodate structures which significantly diverge from previously identified natural ligands.

The herein described glycomimetics have been evaluated in reference with cluster mannosides closely related to the more potent mannose receptor ligands so far designed.[13c] The mimetics do not perform as well as the references but the comparison must be balanced by the obvious discrepancies, notably in term of shape, between the two sets of compounds. Moreover, neither affinity for the mannose receptor or synthesis of the glycomimetics have been optimized in this study. In this way, chemical modification of D-(−)-quinic and -shikimic acids (that is, the preparation of deoxy-analogues) will be helpful to further investigate the intimate ligand – receptor interactions. Their carboxylic function might also facilitate the synthesis of multivalent ligands through conventional peptide solid-phase synthesis. These advantageous properties open the way for the employment of quinic or shikimic acid derivatives for targeting of antigens or toxins to dendritic cells to enhance immunogenicity. In this sense, facilitating the uptake of antigen peptides should allow more effective immune responses, while significantly reducing the amounts of antigens which need to be used.

Experimental Section

Materials: D-Mannose, D-galactose, D-galactonolactone, and shikimic acid were purchased from Acros Organics (Noisy le Grand, France); methyl α-D-mannopyranoside, D(−)-quinic acid, and fluorescein isothiocyanate (FITC) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

Instrumental Analysis: Analytical and semipreparative reverse-phase high-performance liquid chromatography (RP-HPLC), separations were performed as previously described.[19] Solvent system A:

0.05% trifluoroacetic acid (TFA) in water; solvent system B: 0.05% TFA in acetonitrile/water (8:2); solvent system C: 0.05% TFA in acetonitrile/water (6:4); solvent system D: phosphate buffer (50 mmol, pH 6.95); solvent system E: phosphate buffer (50 mmol, pH 6.95)/acetonitrile (1:1). TOF-PDMS spectra were recorded with a Bio-Ion 20 Plasma Desorption Mass Spectrometer (Uppsala, Sweden) and ESI-MS spectra were recorded with a Micromass Quatro II Electrospray Mass Spectrometer. Hyperbranched compounds were verified for homogeneity by analytical capillary zone electrophoresis in a 75 μm × 500 mm fused silica capillary, with a 18 mA current and a 30 kV field in an Applied Biosystems Model 270A-HT system (Foster City, USA). Separations were performed at 45 °C using a 50 mM sodium borate migration buffer (pH 9.2) or a 20 mM citrate buffer (pH 2.47) at 40 °C. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX 300 spectrometer. Chemical shifts are given in ppm and referenced to internal [2,2,3,3-d$_4$]-3-(trimethylsilyl)propionic acid, sodium salt (TMSP).

***N*$^{\alpha}$-(Chloroacetyl)-L-lysyl-β-alanine-amide (4)**: This compound was synthesized according to the reported procedure.[19] Compound **4** (184 mg, 65%) was obtained as a white powder, after RP-HPLC purification (gradient: 100:0 to 90:10 (solutions A to B), 20 min) followed by lyophilization; TOF-PDMS: *m/z*: 292.6 $[M+H]^+$; ^{1}H NMR (D_2O/H_2O (10:90)): $\delta = 8.37$ (d, $^3J(H,H) = 6.8$ Hz, 1H, lysyl α-NH), 8.07 (t, $^3J(H,H) = 5.7$ Hz, 1H, β-alanyl NH), 7.40 (br. s, 1H, NH_2), 4.11 (dt, $^3J(H,H) = 6.8$ and 6.8 Hz, 1H, lysyl α-H), 4.01 (s, 2H, CH_2), 3.31 (dt, $^3J(H,H) = 5.7$ and 6.6 Hz, 2H, β-alanyl β-H), 2.84 – 2.81 (m, 2H, lysyl ε-H), 2.33 (t, $^3J(H,H) = 6.6$ Hz, 2H, β-alanyl α-H), 1.72 – 1.53 (m, 4H, lysyl β- and δ-H), 1.42 – 1.22 (m, 2H, lysyl γ-H); ^{13}C NMR (D_2O/H_2O (10:90)): $\delta = 175.7$, 172.4, and 168.6 (3 × CON), 53.2 (lysyl α-C), 41.0 (CH_2), 38.4 (lysyl ε-C), 34.8 and 33.5 (β-alanyl α-C and β-C), 29.4 (lysyl β-C), 25.3 (lysyl δ-C), 21.0 (lysyl γ-C).

Compounds 5 – 7 and 8a – 8c: Syntheses and full characterization of these compounds have been reported elsewhere.[25]

Synthesis of Fluorescein-Labeled Lysine-Based Cluster Glycomimetics: General Procedure: *n*Bu$_3$P (1.5 equiv per Cl to be substituted) was introduced into a solution containing one of compounds **8a – 8c** (1.5 equiv per Cl to be substituted) in a mixture of degassed *n*PrOH/H_2O (50:50; 1 mL). Each mixture was stirred at room temperature overnight under N_2. The solvent was evaporated under reduced pressure. The residue was dissolved in H_2O (50 μL). FITC (1 equiv) was added to a solution containing one of the L-lysine cores **4 – 6** (1 – 6 μmol, 1 equiv) in *N*,*N*-dimethylformamide (DMF; 300 μL) which also contained diisopropylethylamine (DIPEA; 4 equiv). The mixture was stirred under N_2 in the dark for 2 h. On completion of the reaction, as determined by RP-HPLC, the reaction mixture was transferred to the above aqueous mixture containing the reduced tripeptidyl compounds. The reaction vessel was rinsed with DMF (150 μL). The pH values of the mixtures were adjusted to a value of 8 – 8.5 by addition of solid K_2CO_3. The reaction was stirred under N_2 in the dark at room temperature for 24 h, diluted in H_2O, lyophilized, and purified by RP-HPLC to furnish compounds **10 – 18**.

(Qui)$_2$core* (10): Compound **10** (5.04 mg, 80%), was obtained as an orange powder, after RP-HPLC purification (gradient: 100:0 to 85:15 (solutions A to C), 15 min, then 85:15 to 75:25 (A to C), 30 min, then isocratic) followed by lyophilization; positive ESI-MS: *m/z*: 1300.3 $[M+H]^+$, 650.8 $[M+2H]^{2+}$; ^{1}H NMR (DMSO-d_6): $\delta = 10.2$ (br. s, 1H, NH-fluor), 8.40 – 8.32 (m, 3H, H-fluor, cysteinyl NH, and lysyl α-NH), 8.31 (t, $^3J(H,H) = 5.1$ Hz, 1H, glycyl NH), 8.24 (d, $^3J(H,H) = 7.8$ Hz, lysyl α-NH), 8.10 (t, $^3J(H,H) = 5.2$ Hz, 1H, β-alanyl NH), 7.82 – 7.80 (m, 2H, lysyl α-NH and lysyl ε-NH), 7.80 (d, $^3J(H,H) = 8.3$ Hz, H-fluor), 7.41 (br. s, 1H, N*H*), 7.22 (d, $^3J(H,H) = 8.3$ Hz, H-fluor), 6.90 (br. s, 1H, N*H*), 6.74 – 6.60 (m, 6H, 6H-fluor), 4.60 (m, 1H, cysteinyl α-H), 4.34 – 4.26 (m, 2H, 2 × lysyl α-H), 4.036 (br. s, 2H, 2H-3), 3.67 (d, 2H, 2 × glycyl α-H), 3.60 – 3.52 (m, 2H, 2H-5), 3.49 – 3.38 (m, 2H, 2 × lysyl ε-H), 3.30 – 3.27 (m, 4H, 2 β-alanyl, β-H, and CH_2), 3.25 (dd, 2H, $^3J(H,H) = 3.1$ and 9.7 Hz, 2H-4), 3.11 – 2.98 (m, 2H, 2 × lysyl ε-H), 2.98 – 2.88 (m, 1H, cysteinyl β-H), 2.84 – 2.69 (m, 1H, cysteinyl β-H), 2.29 (t, $^3J(H,H) = 6.3$ Hz, β-alanyl α-H), 1.91 – 1.65 (m, 8H, 4H-2 and 4H-6), 1.72 – 1.52 (m, 4H, 4 × lysyl β-H), 1.22 – 1.08 (m, 8H, 4 × lysyl δ-H and 4 × lysyl γ-H).

(Shi)$_2$core* (11): Compound **11** (2.42 mg, 80%) was obtained as an orange powder, after RP-HPLC purification (gradient: 100:0 to 85:15 (solutions A to C), 15 min, then 85:15 to 70:30 (A/C), 50 min) followed by lyophilization; positive ESI-MS: *m/z*: 1264.4 $[M+H]^+$, 632.8 $[M+2H]^{2+}$; ^{1}H NMR (DMSO-d_6): $\delta = 10.07$ (s, 1H, OH), 9.81 (br. s, 1H, NH-fluor), 8.22 (t, $^3J(H,H) = 5.7$ Hz, 1H, glycyl NH), 8.20 (br. s, 1H, H-fluor), 8.11 (d, $^3J(H,H) = 8.0$ Hz, 2H, cysteinyl NH and lysyl α-NH), 8.01 (t, $^3J(H,H) = 5.6$ Hz, 1H, lysyl ε-NH), 7.99 (t, $J = 5.8$ Hz, β-alanyl NH), 7.77 (t, $^3J(H,H) = 5.6$ Hz, 1H, lysyl ε-NH), 7.75 (d, $^3J(H,H) = 7.6$ Hz, 1H, lysyl α-NH), 7.69 (br. d, $^3J(H,H) = 8.3$ Hz, H-fluor), 7.28 (br. s, 1H, N*H*), 7.12 (d, $^3J(H,H) = 8.3$ Hz, H-fluor), 6.80 (br. s, 1H, N*H*), 6.74 – 6.60 (m, 6H, 6H-fluor), 6.31 and 6.22 (2 × br. s, 2 × shikimoyl H-3), 4.50 – 4.30 (m, 1H, cysteinyl α-H), 4.24 – 4.12 (m, 4H, 2 × lysyl α-H and 2 × H-2), 3.80 – 3.74 (m, 2H, 2 × H-5), 3.71 (d, 2 × glycyl α-H), 3.76 – 3.71 (m, 2H, 2 × H-4) 3.36 – 3.44 (m, 2H, 2 × lysyl ε-H), 3.26 – 3.13 (m, 2H, 2 × β-alanyl β-H), 3.18 (s, 2H, CH_2), 3.12 – 2.94 (m, 2H, 2 × lysyl ε-H), 2.88 (dd, 1H, $^3J(H,H) = 5.3$ and 14.9 Hz, cysteinyl β-H), 2.73 (dd, 1H, $^3J(H,H) = 8.7$ and 14.9 Hz, cysteinyl β-H), 2.60 – 2.39 (m, 2H, 2 × H-6), 2.19 (t, $^3J(H,H) = 7.1$ Hz, 2 × β-alanyl α-H), 1.94 (dt, $^3J(H,H) = 3.6$ and 17.9 Hz, 2 × H-6), 1.72 – 1.57 (m, 4H, 4 × lysyl β-H), 1.62 – 1.42 and 1.41 – 1.30 (m, 4H, 4 × lysyl δ-H), 1.29 – 1.16 (m, 4H, 4 × lysyl γ-H).

(Gla)$_2$core* (12): Compound **12** (2.57 mg, 65%), was obtained as an orange powder, after RP-HPLC purification (gradient: 100:0 to 85:15 (solutions D to E), 15 min, then 85:15 to 80:20 (D to E), 20 min, then isocratic) followed by lyophilization, desalting, and lyophilization; positive ESI-MS: *m/z*: 1308.4 $[M+H]^+$, 654.9 $[M+2H]^{2+}$; ^{1}H NMR (DMSO-d_6): $\delta = 10.40$ (br. s, 1H, NH-fluor), 8.68 (t, $^3J(H,H) = 5.7$ Hz, 1H, glycyl NH), 8.27 (t, $^3J(H,H) = 7.5$ Hz, 1H, lysyl α-NH), 8.08 (t, $^3J(H,H) = 5.4$ Hz, β-alanyl NH), 8.01 (d, $^3J(H,H) = 8.0$ Hz, lysyl α-NH), 7.99 (t, $^3J(H,H) = 5.6$ Hz, 1H, cysteinyl NH), 7.86 (d, $^3J(H,H) = 1.8$ Hz, 1H, H-fluor), 7.79 (dd, $^3J(H,H) = 1.8$ and 8.4 Hz, H-fluor), 7.70 – 7.63 (m, 2H, 2 × lysyl ε-NH), 7.45 (br. s, 1H, N*H*), 6.94 (d, $J = 8.4$ Hz, H-fluor), 6.81 (br. s, 1H, N*H*), 6.73 (dd, $^3J(H,H) = 1.5$ and 9.2 Hz, 2H, 2 × H-fluor), 6.16 (d, $^3J(H,H) = 9.2$ Hz, 2H, 2 × H-fluor), 6.08 (d, $^3J(H,H) = 1.5$ Hz, 2H, 2 × H-fluor), 4.51 – 4.40 (m, 1H, cysteinyl α-H), 4.30 – 4.09 (m, 4H, 2 × lysyl α-H and 2 × H-4), 4.10 – 3.96 (m, 8H, 2 × H-3 and 2 × H-5), 3.73 – 3.30 (m, 10H, 2 × glycyl α-H, 2 × lysyl ε-H, 2 × H-2, 2 × H-5, and 4 × H-6), 3.30 – 3.22 (m, 4H, 2 × β-alanyl β-H and CH_2), 3.13 – 3.00 (m, 2H, 2 × lysyl ε-H), 2.91 (dd, 1H, $^3J(H,H) = 5.2$ and 14.5 Hz, cysteinyl β-H), 2.92 – 2.77 (m, 1H, cysteinyl β-H), 2.23 (t, 2H, $^3J(H,H) = 7.1$ Hz, 2 × β-alanyl α-H), 1.77 – 1.52 (m, 6H, 4 × lysyl β-H and 2 × lysyl δ-H), 1.46 – 1.20 (m, 6H, 2 × lysyl δ-H and 4 × lysyl γ-H).

(Qui)$_4$core* (13): Compound **13** (1.82 mg, 46%) was obtained as an orange powder, after RP-HPLC purification (gradient: 100:0 to 85:15 (solutions A to C), 15 min, then 85:15 to 75:25 (A to C), 40 min) followed by lyophilization; positive ESI-MS: *m/z*: 2124.1 $[M+H]^+$, 1062.3 $[M+2H]^{2+}$, 721.2 $[M+2H+K]^{3+}$.

(Shi)$_4$core* (14): Compound **14** (1.42 mg, 27%) was obtained as an orange powder, after RP-HPLC purification (gradient: 100:0 to 75:25 (solutions A to C), 15 min, then 75:25 to 65:35 (A to C), 40 min) followed by lyophilization; positive ESI-MS: *m/z*: 1045.1 $[M+H+K]^{2+}$, 1025.9 $[M+2H]^{2+}$, 722.6 $[M+3K]^{3+}$.

$(GLa)_4$core* (15): Compound **15** (2.23 mg, 65%) was obtained as an orange powder, after RP-HPLC purification (gradient: 100:0 to 75:25 (solutions D to E), 20 min, then 75:25 to 65:35 (D to E), 25 min), followed by lyophilization, desalting, and lyophilization; positive ESI-MS: calcd: 2139.2, found: 2139.0; *m/z*: 1070.4 $[M+H]^+$, 726.6 $[M+H+K]^{2+}$, 714.9 $[M+2H]^{2+}$.

$(Qui)_8$core* (16): Compound **16** (4.56 mg, 62%) was obtained as an orange powder, after RP-HPLC purification (gradient: 100:0 to 75:25 (solutions A to B), 15 min, then 75:25 to 70:30 (A to B), 30 min) followed by lyophilization; negative ESI-MS: calcd: 3769.1, found: 3769.0; *m/z*: 1254.9 $[M-3H]^{3-}$, 941.2 $[M-4H]^{4-}$.

$(Shi)_8$core* (17): Compound **17** (3.57 mg, 50%) was obtained as an orange powder, after RP-HPLC purification (gradient: 100:0 to 85:15 (solutions A to B), 15 min then 85:15 to 70:30 (A to B), 30 min) followed by lyophilization; negative ESI-MS: calcd: 3640.9, found: 3640; *m/z*: 1214.2 $[M-3H]^{3-}$, 909.2 $[M-4H]^{4-}$.

$(GLa)_8$core* (18): Compound **18** (1.64 mg, 35%) was obtained as an orange powder, after RP-HPLC purification (gradient: 100:0 to 80:20 (solutions D to E), 35 min) followed by lyophilization, desalting, and lyophilization; positive ESI-MS: calcd: 3801.0, found: 3801.0; *m/z*: 1901.2 $[M+2H]^{2+}$, 1280.0 $[M+2H+K]^{3+}$, 1267.7 $[M+3H]^{3+}$.

Culture of Human Dendritic Cells from Peripheral Blood: Dendritic cells were generated from Buffycoats provided by the EFS (Etablissement Français du Sang Nord-Pas-de Calais, Lille, France) according to a recently described protocol.[2a] Briefly, peripheral blood mononuclear cells (PBMC) were obtained by Ficoll Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation and resuspended in cell culture medium consisting of RPMI 1640 (Gibco, Courbevoie, France) supplemented with 5×10^{-5} M β-mercaptoethanol (Merck, Darmstadt, Germany), 2 mM L-glutamine (Merck), 1 mM sodium pyruvate (Gibco), 10% heat-inactivated feotal calf serum (Gibco), and 50 μg mL^{-1} gentamycin (Gibco) at 37 °C in a 5% CO_2 atmosphere. Cells were allowed to adhere to cell culture dishes (100 mm diameter, $8-12 \times 10^7$ cells/plaque) for 3–4 h at 37 °C in an humidified CO_2 incubator. After removal of the nonadherent cells, the adherent cells were resuspended at 1×10^6 cells mL^{-1} and cultured for 6–8 days in medium supplemented with the cytokines GM-CSF (800 U mL^{-1}; Pepro Tech Inc., Rocky Hill, USA) and IL-4 (1000 U mL^{-1}; Pepro Tech Inc.) in 24-well tissue culture plates.

Cell Surface Antigen Detection: After several days of culture, cells were harvested and washed in PBS (Gibco) containing BSA (bovine serum albumin; 0.03 mg mL^{-1}; Sigma Immunochemicals, St. Louis, USA). Cells were then incubated for 30 min on ice with the following monoclonal antibodies (MAb): phycoerythrine (PE) conjugated anti-CD11c or purified nonblocking anti-mannose receptor (clone 19.2; Pharmingen, San Diego, CA, USA). After incubation cells were washed and resuspended in PBS. Mannose receptor expression was assessed with a goat anti-mouse IgG conjugated to Alexa 488 as the secondary antibody (Molecular Probes, Leiden, The Netherlands). The cell-associated fluorescence was further analyzed with the flow cytometer Epics XL-MCL system (Coulter Corporation, Miami, USA).

Glycomimetics Uptake: Cells were harvested after 5–8 days of culture and washed in PBS. They were first preincubated for 10 min at 37 °C, then pulsed for 20 min at 37 °C with FITC-labeled di-, tetra-, and octavalent cluster glycomimetics or glycosides, respectively, washed three times with cold PBS and then fixed for 20 min in paraformaldehyde solution (1%). Cell-associated fluorescence was further analyzed using the FACScan described above.

For the monomer inhibitory test, cells were preincubated for 10 min at 37 °C with different concentrations of monomers: methyl D-(−)-quinate (**1**), methyl shikimate (**2**), or methyl α-D-mannopyranoside (**3**). Cells were then coincubated for 20 min at 37 °C with 10 μM FITC-labeled $(Man)_8$core* (**23**), washed three times with cold PBS, and fixed for 20 min in paraformaldehyde solution (1%) before cytofluorimetric analysis.

For the mannan inhibitory test, cells were first incubated with different concentrations of mannan derived from *Saccharomyces cerevisiae* (Sigma) for 20 min at 37 °C, then coincubated for 20 min at 37 °C with glycomimetics and treated as previously described, before cytofluorimetric analysis.

Confocal Microscopy: To address the intracellular delivery of the glycomimetics and their cellular localization, we used the glycomimetics modified by an FITC group. Monoclonal mouse nonblocking antibody directed against the human mannose receptor (clone 19.2) was purchased from Pharmingen. Monoclonal rabbit anti-fluorescein antibody coupled to Alexa Fluor 488, monoclonal goat anti-mouse IgG conjugated to Alexa Fluor 568, and TO-PRO-3 iodide were purchased from Molecular Probes.

Dendritic cells (0.5×10^5 per condition), harvested after 5 or 6 days of culture, were incubated for 45 min at 4 °C with 5 μM of the different fluoresceinated glycomimetics and 10 μg of purified anti-mannose receptor antibody. The cells were further incubated at 37 °C for 5 minutes. Following incubation, cells were washed twice in PBS and then fixed with PBS containing (4%) paraformaldehyde solution, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, and 4% sucrose for 45 min at 4 °C. Cells were washed in PBS and resuspended in 50 mM NH_4Cl medium to stop the fixation reaction, and, after 10 min at room temperature, cells were washed three times in PBS. Fixed cells were placed on glass coverslips, permeabilized, and blocked for 10 min at room temperature in permeabilizing buffer (PBS containing 1 mg mL^{-1} BSA and 0.05% saponin). Then cells were incubated for 30 min at 37 °C with both the nonblocking antibody, the rabbit anti-fluorescein antibody coupled to Alexa 488 (used at 1:200 dilution in permeabilizing buffer; 5 mg mL^{-1}) and the goat anti-mouse IgG-Alexa 568 conjugate (used at 1:500 dilution in permeabilizing buffer; 1 mg mL^{-1}). Cells were washed twice with permeabilizing buffer and then the nuclear DNA was stained with TO-PRO-3 iodide (0.1 μM) for 30 min at 37 °C.

Slides were mounted using Vectaschield (Vector Laboratories, Compiègne, France). Fluorescence-stained slides were examined under a Leica TCS NT laser-scanning confocal microscope (Leica, Heidelberg, Germany) comprising a krypton/argon laser at 488, 568, and 647 nm excitation wavelength. Simultaneous three channel recording was performed. Frame scanning was performed at 1000 magnification and a single optical section was collected per field.

Mannose Receptor Expression in Cos-1 Cells: Cos-1 cells were transiently transfected as previously described with the plasmid CD8 containing a sequence coding for the wild-type mannose-receptor transcripts, mannose receptor CD8; this was a gift from Dr. R. A. B. Ezekowitz.[3c] Briefly, transfections were performed using polyethylenimine (PEI) reagent EXGEN 500 (Euromedex, Souffelweyersheim, France). 1 μg of mannose receptor CD8 plasmid or the empty vector, as a control, and 5 μl of PEI per well were used for transfection of cells cultured in a 24 well plate. Cells were incubated for 5 hours with the reagents in OptiMEM (Gibco) followed by a change of the culture medium. 48 hours after transfection, the cells were harvested and the mannose receptor expression was checked using the PE-conjugated anti-mannose receptor monoclonal antibody. 10–15% of Cos-1 cells were expressing the transgene. Internalization tests were performed as described earlier. Briefly, cells were pulsed for 20 min at 37 °C with 10 μM of FITC-labeled

glycomimetics, then washed three times with cold PBS and fixed for 20 min in paraformaldehyde solution (1%) before FACScan analysis.

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