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Synthesis and Mannose Receptor-Mediated Uptake of Clustered Glycomimetics by Human Dendritic Cells: Effect of Charge

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Abstract—Effect of charge and shape of multivalent lysine-based cluster glycomimetics on their mannose receptor-mediated uptake by human dendritic cells has been evaluated: The capture is strongly affected by the shape of the ligands. The effect of charge is less pronounced although positive charges on the ligands seem to favor non-specific endocytosis capture.

Among the immune cell populations, dendritic cells appear as the most potent antigen presenting cells, being the most efficient in activating both naive and memory T lymphocytes.[1] But, their low number in peripheral tissues (0.5–2%) limits considerably the efficient delivery of vaccinal antigens. Thus, selective targeting of receptors preferentially expressed on dendritic cells should considerably improve vaccine efficacy. The mannose receptor involved in the uptake of particulate and soluble antigenic products of bacterial origin represents therefore a promising target. The mannose receptor is a monomeric membrane-bound protein displaying eight carbohydrate-recognition domains (CRDs), mainly expressed on dendritic cells and macrophages, being directly involved in the endolysosomic antigen processing and presentation pathway.[2] It was shown that polymannosylation or -fucosylation of antigen peptides induces an improved uptake and presentation by the dendritic cells. This has been demonstrated by using in vitro T cell clones proliferation experiments.[3]

In search of effective mannose receptor ligands, which could be further associated with antigen following minor modifications, we propose to use D-(−)-quinic and shikimic acid derivatives clustered on lysinyl trees: These acids possess two *trans*-di-equatorial (or *pseudo*-di-equatorial, respectively), vicinal hydroxy groups in the (+)-synclinal configuration. As deduced from X-ray crystallographic data,[4] collected with the rat mannose binding lectin which shares similarities with the mannose receptor CRDs, this is the configuration required for interaction with the mannose receptor (Fig. 1).[5]

These commercially available carbocyclic compounds were selected not only for their putative mannose

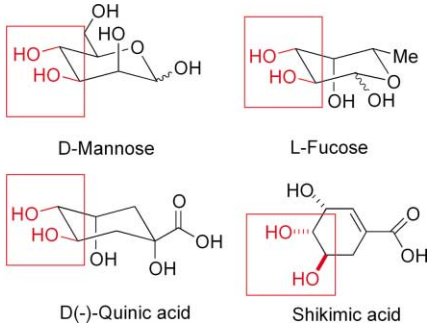

Figure 1. Structures of monomeric carbohydrates or glycomimetics recognized by the mannose receptor: Major contribution to the binding is provided by coordination bonds between two hydroxy groups of the ligand (in the open box), a calcium ion, and four residues of the lectin.

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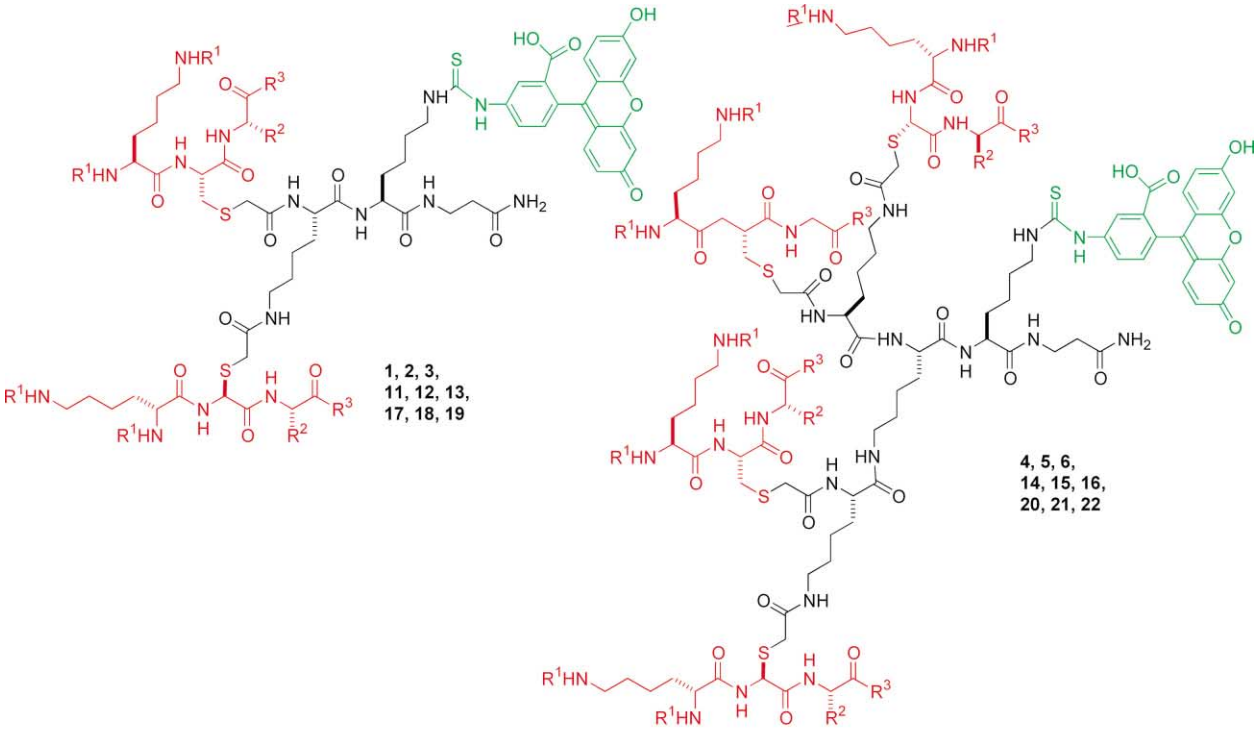
mimicry but also for their chemical stability and their ease of functionalization via their carboxylic acid groups.

Thus, a set of fluorescein-labeled quinic and shikimic acids-containing clusters among with **1**, **2**, **4** and **5** (Fig. 2) were synthesized from L-lysinyl cores[6] and their internalization was assayed on peripheric blood monocyte-derived human dendritic cells by cytofluorimetry analysis. The mannose receptor capture specificity was further assessed by competitive inhibition experiments assays with mannan, by confocal microscopy analysis and by expression of the mannose receptor in transfected Cos-1 cells.[5b]

The mimics were evaluated with reference to mannosylated trees, for example **7** and **9**, closely related to the most potent mannose receptor ligands so far reported,[7] and to analogue constructs, for example, **3**, **6**, **8** and **10** decorated with galactonamide or galactoside as positive or negative controls, respectively (Figs. 2 and 3). The latter were designed in order to discriminate the mannose receptor specific uptake from nonspecific binding to or pinocytosis of dendritic cells.

From this study, it appeared that divalent compounds were poorly internalized and mainly through nonspecific endocytosis. Otherwise, specific uptake became significant from the tetravalent constructs. Internalization of the tetravalent glycomimetics **1** and **2**, was comparable to the corresponding cluster mannoside **7** (Fig. 5a). Surprisingly, the capture was better for the tetravalent trees than for the octavalent ones **4** or **5** whereas, in the mannosylated series, it was consistently enhanced with increasing valency (Figs. 5a and b). The observed divergence may originate from differences in the



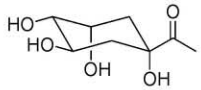
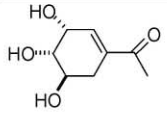
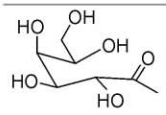
R^1	R^2	R^3	Compds
	**H**	**OH**	1, 4
	H	**NH_2**	11, 14
	$(CH_2)_3NH(C{=}NH)NH_2$	**NH_2**	17, 20
	**H**	**OH**	2, 5
	H	**NH_2**	12, 15
	$(CH_2)_3NH(C{=}NH)NH_2$	**NH_2**	18, 21
	**H**	**OH**	3, 6
	H	**NH_2**	13, 16
	$(CH_2)_3NH(C{=}NH)NH_2$	**NH_2**	19, 22

Figure 2. Hyperbranched cluster glycomimetics.

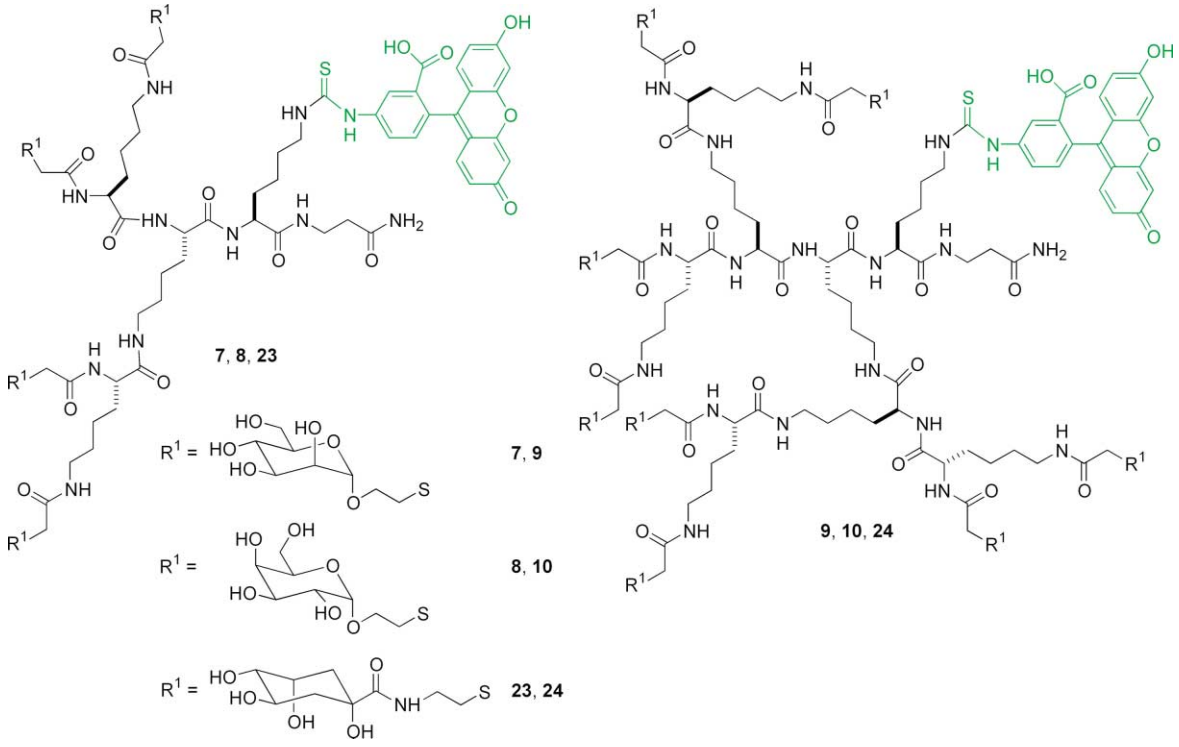


Figure 3. Branched cluster glycosides and glycomimetics.

hydroxy substitution pattern which might confer extra binding capacities to mannose compared with quinic and shikimic acids.[8] It more likely arises from:

(a) the distinct topologies of the ligands: The mannosides were reacted at the extremities of *N*-chloroacetylated-L-lysinyl trees to provide *branched* glycoclusters whereas glycomimetics were first coupled to a cysteine-containing tripeptide, H-L-Lys-L-Cys(S^{t}Bu)-Gly-OH, to give divalent intermediates which, in turn, were linked to the lysinyl cores to provide *hyperbranched* constructs,
(b) the overall charge of the ligands at physiological pH which is neutral or negative: the glycine rest on the tripeptide indeed introduces 2 or 4 negative charges on the tetra- or octavalent cluster glycomimetics, respectively.

To document these assumptions, the synthesis and the biological evaluation of positively charged, neutral as well as branched or hyperbranched cluster glycomimetics **11**–**24** are reported here on.

Synthesis

Neutral and positively charged, branched constructs **11**–**22** were obtained as described for clusters **1**–**6**,[5] yet replacing the glycines by glycine-amides or arginine-amides, respectively. Briefly, shikimic acid was coupled to H-L-Lys-L-Cys(S^{t}Bu)-Gly or H-L-Lys-L-Cys(S^{t}Bu)-Arg (Pmc) 2,2,5,7,8-pentamethylchroma-6-sulfonyl peptidyl-resins **25** and **26**, assembled stepwise on a Rink-amide-AM-PS resin using the Fmoc/*tert*-butyl strategy.[9] Bivalent intermediates **27** and **28** were obtained after release from the resin and RP-HPLC purification in 40 and 26% yields (Scheme 1). To avoid the immediate formation of the bicyclic 1,5-lactone[10] upon activation of the carboxylic acid group, D(−)-quinic acid was reacted to peptidyl-resins **25** and **26** as its per-*O*-acetylated protected form **29**.[11] Compounds **30** and **31** were obtained after acidic release from the resin, methanolic sodium methoxide deacetylation[12] and RP-HPLC purification in 24 and 25% yields.

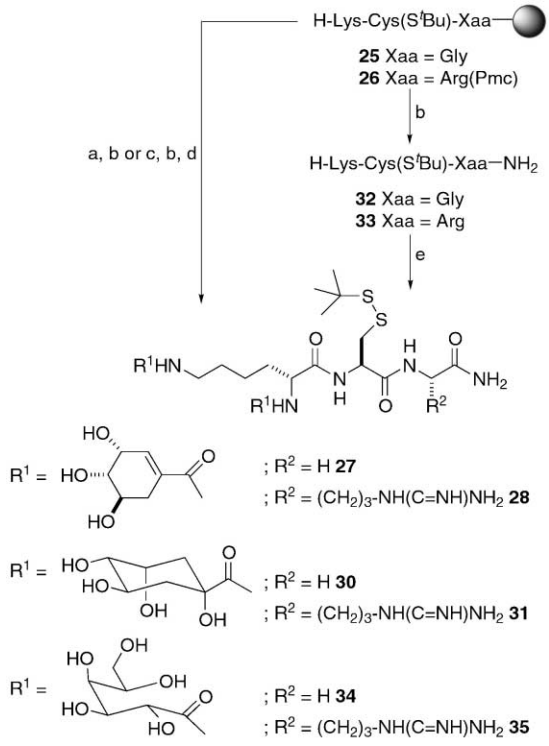
Finally, D-galactonolactone was condensed in refluxing methanol to tri-peptides **32** and **33** to provide negative construct precursors **34** and **35** in 28 and 33% overall yields.

According to our previously reported one-pot two-steps procedure,[13] *N*-chloroacetylated L-lysinyl cores **36** and **37** (Fig. 4) were labeled with fluorescein isothiocyanate and further reacted in a carbonated DMF/H_2O mixture with *n*-Bu_3P-reduced disulfides **27**, **28**, **30**, **31**, **34** and **35** and 2-thioethyl quinoyl-amide to give hyperbranched and branched constructs **11**–**13**, **15**, **17**–**21** in 26–66% purified yields.[14]

Compounds **13**, **14**, **22** and **24** were best prepared using lysinyl cores **36**–**38**, following a reverse one-pot or sequential (for the last two compounds) procedure, that is thioetherification then labeling, in 40, 72, 50 and 22% yields, respectively.[14]

Biological Assays

Having the fluorescein-labeled glycomimetics in hands, we next examined their mannose receptor-mediated uptake using flow cytometry. We chose the model of human monocyte-derived dendritic cells, as they express homogenously, at their immature state of differentiation, large amounts of functionally active mannose receptor.[2c] Specific uptake was calculated by substracting nonspecific pinocytosis or binding, corresponding to the D-galactonolactone-derived constructs. The assay was conducted in parallel with their corresponding mannosylated (positive control) and galactosylated (negative control) structures. Capture specificity was confirmed by competitive inhibition assay using mannan, a bacterial polysaccharide known to bind with high affinity to the mannose receptor as depicted previously.[5b]



Scheme 1. Reagents and conditions: (a) shikimic acid (2.2 equiv), O-(benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-(dimethylamino)methylene-*N*-methylmethanaminium hexafluorophosphate *N*-oxide(HBTU)/1-hydroxy-1*H*-benzatriazole(HOBt)/DIPEA (2.2/2.2/4.4 equiv), DMF, rt, 40 min; (b) TFA/H_2O/*i*-Pr_3SiH (95:2.5:2.5), rt, 1 h; (c) **29** (3 equiv), HBTU/HOBt/DIPEA (3:3:9 equiv), DMF, rt, 40 min, (twice); (d) MeONa, MeOH, rt, 30 min; (e) D-galactonolactone (4+4 equiv), DIPEA (8 equiv), MeOH, reflux, 48+48 h.

(ClCH$_2$C=O)-L-Lys(ClCH$_2$C=O)-L-Lys-βAla-NH$_2$,
divalent core **36**

[(ClCH$_2$C=O)-L-Lys(ClCH$_2$C=O)]$_2$-L-Lys-L-Lys-βAla-NH$_2$,
tetravalent core **37**

{[(ClCH$_2$C=O)-L-Lys(ClCH$_2$C=O)]$_2$-L-Lys}$_2$-L-Lys-L-Lys-βAla-NH$_2$,
octavalent core **38**

Figure 4. Structure of the lysine cores.

Most of the tetravalent compounds, neutral and basic ligands were similarly internalized yet in a slightly higher extent than the acidic ones (Fig. 5a). For the octavalent series, uptake was very high for the basic constructs but mainly mediated by nonspecific endocy-

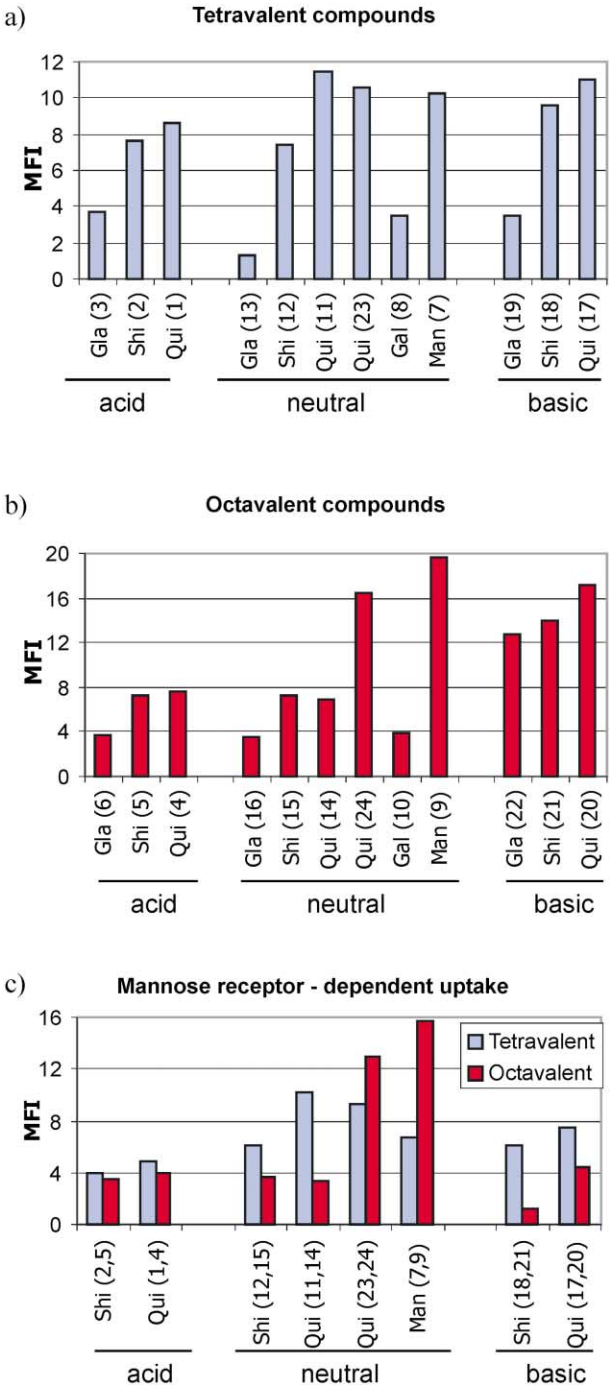


Figure 5. Dendritic cells, obtained as previously described,[5b] were pulsed for 20 min at 37 °C with fluorescein-labeled constructs at concentration of 10 μM, then washed three times with cold PBS and fixed in paraformaldehyde 1% before FACScan analysis: (a) uptake of tetravalent compounds, (b) uptake of octavalent compounds, (c) mannose receptor specific uptake, calculated by substracting for each compound its corresponding negative control. MFI stands for mean fluorescence intensity. Cumulative results representative of at least four independent tests for each compound are shown. Gla, Qui, Shi, Man and Gal corespond to galactonoylated, quinoylated, shikimoylated, mannosylated and galactosylated trees, respectively.

tosis as judged by the level of internalization of the corresponding negative cluster **22** (Fig. 5b). In fact, receptor-dependent uptake appeared close for most of the quinic and shikimic constructs when the non specific endocytosis is substracted (Fig. 5c). These observations probably result from privileged interaction between positively charged ligands and the negatively charged membrane of the dendritic cells. This effect became apparent from the octavalent clusters upwards as they display more charges.

For the tetravalent series, quinic acid-based mimetics were systematically better recognized than the shikimic acid-derived constructs. This result might reflect differences in the structure of quinic and shikimic acids, the latter having a more flattened conformation and thus resembling less to natural mannose receptor sugar ligands.

For the hyperbranched glycomimetic compounds, optimal uptake was observed for the tetravalent ones compared to octavalents, whatever their charges. Such an optimum (tetravalent versus octavalent) was not observed for the mannosylated series, whose uptake was considerably enhanced as valency increases: If the tetravalent glycomimetics uptake was equivalent or higher than that of the tetravalent mannosylated tree **7**, the octavalent glycomimetics did not compare favorably with the octavalent mannosylated tree **9**. However, the observed discrepancies can be attributed to the distinct topologies of the constructs. Indeed, the internalization level and supremacy of octavalent over tetravalent trees were restored when using branched quinoylated clusters **23** and **24** rather than the hyperbranched ones. These trees solely differ from the reference trees **7** and **9** by one bond length and replacement of an acetal by an amide linkage.[15]

In summary, this study confirms the mannose mimicry of quinic and shikimic acid towards mannose receptor expressed on human dendritic cells. These derivatives can lead to the design of constructs as efficient as the most potent synthetic ligands designed so far. Strong modulation of the recognition can be expected by varying the shape of the clusters. On the other hand, introducing positive charges on the ligands, while increasing the overall capture, diminishes the specific uptake, by favoring electrostatic ligand–cell membrane interactions.

Acknowledgements

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8. In such a case, noticeable effect would have probably been detected for the tetravalent series; moreover, methyl α-D-mannopyranoside and methyl quinate have been shown to inhibit dendritic cells' uptake of ligands similarly.
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14. All fluorescein-labeled conjugates were characterized by ES–MS recorded on a Micromass Quatro II Electrospray or by MALDI-TOF-MS recorded on a Finnigan Vision 2000 Mass Spectrometer: Conjugate **11** (47% yield); MALDI-TOF-MS: m/z 2320.0 $(M+H)^+$. Conjugate **12** (43%); ES–MS: Found: 2086; Calcd: $M+K$, 2087.3; m/z 1044.1 $(M+H+K)^{2+}$, 696.4 $(M+2H+K)^{3+}$. Conjugate **13** (40%); MALDI-TOF-MS: m/z 2138.0 $(M+H)^+$. Conjugate **14** (72%); ES–MS: found: 3764; calcd: M, 3765.1; m/z 1883.1 $(M+2H)^{2+}$, 1255.4 $(M+3H)^{3+}$. Conjugate **15** (36%); ES–MS: found: 3620; calcd: M, 3621.0; m/z 1207.7 $(M+3H)^{3+}$, 905.9 $(M+4H)^{4+}$. Conjugate **16** (45%); ES–MS: found: 3834; calcd: $M+K$, 3835.0; m/z 1279.3 $(M+2H+K)^{3+}$, 959.5 $(M+3H+K)^{4+}$. Conjugate **17** (33%); ES–MS: found: 2120; calcd: M, 2121.3; m/z 1061.2 $(M+2H)^{2+}$, 707.9 $(M+3H)^{3+}$. Conjugate **18** (45%); MALDI-TOF-MS: m/z 2248.8 $(M+H)^+$. Conjugate **19** (46%); MALDI-TOF-MS: m/z 2336.9 $(M+H)^+$. Conjugate **20** (26%); ES–MS: found: 4160; calcd: M, 4161.6; m/z 1387.6 $(M+3H)^{3+}$, 1041.0 $(M+4H)^{4+}$. Conjugate **21** (61%); ES–MS: found: 4016; calcd: M, 4017.6; m/z 1339.9 $(M+3H)^{3+}$, 1005.2 $(M+4H)^{4+}$. Conjugate **22** (50%); ES–MS: found: 4193; calcd: M, 4193.6; m/z 1398.1 $(M+3H)^{3+}$, 1048.9 $(M+4H)^{4+}$. Conjugate **23** (66%); MALDI-TOF-MS: m/z 2156.7 $(M+H)^+$. Conjugate **24** (22%); MALDI-TOF-MS: m/z 3834.5 $(M+H)^+$.
15. These results were confirmed on testing a set of linear glycomimetic constructs differing in their spacer arms: Unpublished results.