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Synthesis of glycopeptide dendrimers, dimerization and affinity for Concanavalin A

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ABSTRACT

We described herein the synthesis of second generation glycopeptide dendrimers **G2a–g** presenting variable amino acids placed internally into the multivalent scaffold. The effect of such structural modulation on recognition processes by Concanavalin A (Con A), was then estimated by enhanced-sensitivity Enzyme-Linked Lectin Assay (ELLA). In a complementary study, glycopeptide dendrons of different valencies and including a L-cysteine residue before the dendritic core (**G0SH**, **G1SH** and **G2SH**), were also synthesized and homodimerized. Then, the disulfide-containing glycopeptide dendrimers generated by this convergent approach (**G0$_2$S$_2$**, **G1$_2$S$_2$** and **G2$_2$S$_2$**) were used as Con A inhibitors and assayed by ELLA.

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1. Introduction

Dendrimers are monodisperse tree-like molecules built in a generation-wise manner. These hyperbranched systems have found many applications in homogeneous catalysis, organic synthesis or in biology.[1] Glycodendrimers, such as glycoclusters and glycopolymers, have thus appeared as very useful molecules to probe carbohydrate–lectin interactions.[2,3] Construction of well-defined multivalent scaffolds and their use as molecular probes is then still a challenge.[4]

In this context, some peptide and glycopeptide dendrimers presented an interesting propensity to interact with lectins.[2,5] We showed for example that screening of combinatorial libraries could lead to potent bacterial or plant lectin inhibitors.[2b–g] We also recently completed these studies by underlining that nature of amino acids located close to external carbohydrate moieties of such glycopeptide dendrimers, could tune affinity for Concanavalin A (Con A).[2a] Nevertheless, influence of amino acids incorporated more internally into multivalent scaffolds was not fully explored.

We showed also, in agreement with previously reported data, that valency of these lectin inhibitors was a key feature for recognition processes.[6] In many dendrimers studies, valency could be increased by dimerization of thiol-functionnalized dendrons.[7] For example, subunits derivatized from L-cysteine (Cys) could lead to disulfide-containing dendrimers, displaying hydrolytic activities.[1b,8] However, to the best of our knowledge, few examples took advantage of such a convergent strategy for designing multivalent lectin inhibitors.[7a]

Therefore, and considering our interest in (glyco-)peptide dendrimers, we evaluated herein in a first part, if amino acids placed relatively internally into mannosylated peptide dendrimers could tune affinity for Con A.[1b,2,7c,8,9] This protein was selected as a model receptor since this mannose-binding plant lectin and its derivatives were commercially available and widely used to probe carbohydrate–protein interactions.[6,10] After experiments, we found by enhanced-sensitivity Enzyme-Linked Lectin Assay (ELLA) that modulating internal amino acids composition of presented glycopeptide dendrimers did not improve significantly their inhibition properties. On the basis of these results, mono-, di- and tetravalent glycodendrons including Cys far from carbohydrate moieties were synthesized and then homodimerized to give, respectively, the corresponding di-, tetra- and octavalent glycodendrimers. The inhibition potency of these disulfides was also measured by ELLA. As expected, binding properties increased with valency of glycopeptide dendrimers. Their abilities to inhibit Con A activity were comparable to these of glycopeptide dendrimers obtained previously within a divergent approach. Consequently, these results suggested that inhibition properties of presented glycopeptide dendrimers depended mainly on their valency, on the amino acids located close to carbohydrate units and little on the manner to construct the rest of multivalent scaffolds.

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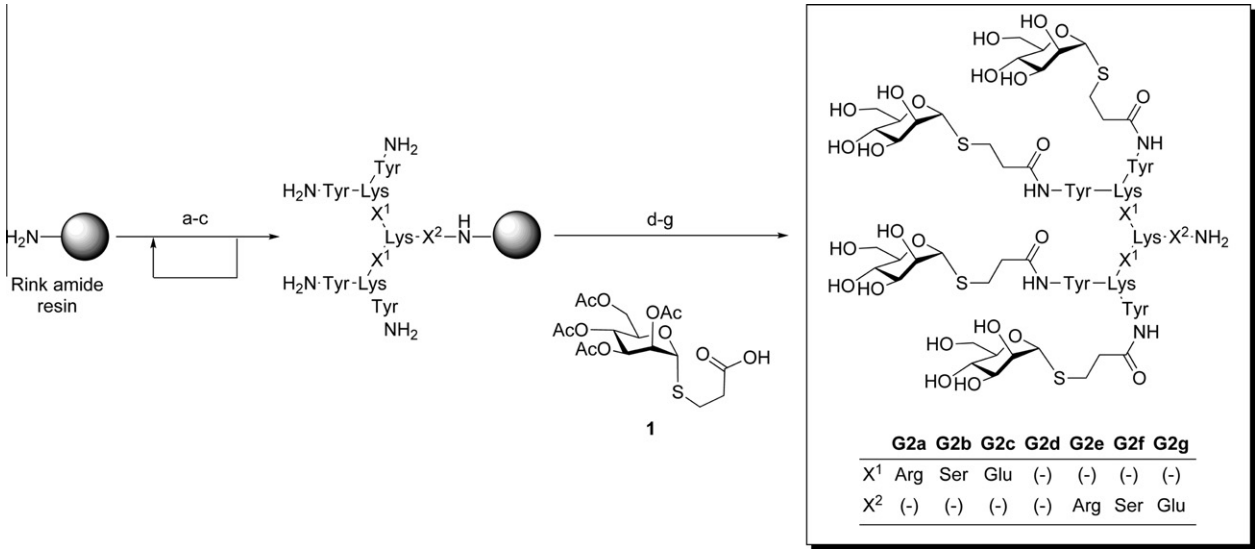


Scheme 1. General synthesis of second generation glycopeptide dendrimers **G2a–g**. Reagents and conditions: (a) protected amino acid [Fmoc–Arg(Pbf)–OH, Fmoc–Ser(tBu)–OH, Fmoc–Glu(OtBu)–OH or Fmoc–Lys(Fmoc)–OH], BOP, DIPEA, NMP, rt, 1–12 h; (b) Ac_2O, CH_2Cl_2, rt, 10 min.; (c) piperidine, DMF, rt, 2× 10 min; (d) **1**, HCTU, DIPEA, NMP, rt, 12 h; (e) NH_3, H_2O, MeOH, rt, 24 h; (f) TFA, TIS, H_2O, rt, 4 h; (g) NH_3, H_2O, MeOH, rt, 24 h.

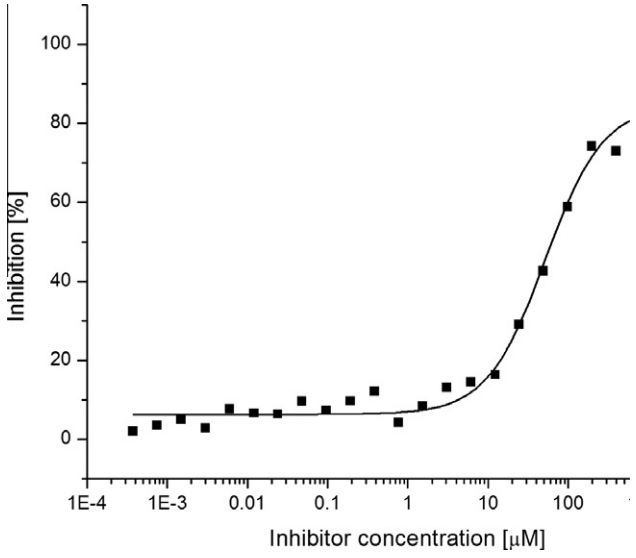


Figure 1. Example of the inhibition curve obtained for the second generation glycopeptide dendrimer **G2d** by enhanced-sensitivity ELLA.

2. Results and discussion

2.1. Synthesis of second generation glycopeptide dendrimers G2a–c and G2e–g

Glycopeptide dendrimers **G2a–c** incorporating variable amino acids (X^1) between branching points were initially synthesized (Scheme 1). L-Arginine (Arg), L-serine (Ser) or L-glutamic acid (Glu) were indeed selected as variable amino acids given their complementary charges at neutral pH. These multivalent glycoconjugates were synthesized by standard Fmoc solid phase peptide synthesis (SPPS).[11] *N,N′*-Bis-Fmoc protected L-lysine (Lys) was then coupled to Rink amide resin using benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophos-phate (BOP) as a condensation agent. After capping of potentially unreacted amines and Fmoc groups removal by treatment with piperidine, protected variable amino acids could be coupled. Terminal Fmoc groups were then cleaved as previously described and all N-termini were acylated once again with *N,N′*-bis-Fmoc protected Lys in order to introduce the last branching units. Protected L-tyrosine (Tyr) moieties were afterwards coupled to the supported tetravalent peptides before being involved in the last piperidine-mediated deprotection step. Finally, all N-termini were glycosylated using 2-(6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HCTU) as a coupling reagent and the known thiomannosidic building block **1**.[12] This latter could be easily obtained in an anomerically pure form from D-mannose (Man), in few steps and with a good overall yield. After on-bead carbohydrates deacetylation reaction, acidolytic cleavage from resin, in-solution removal of the remaining acetyl groups and purification by reversed phase preparative HPLC (RP-HPLC), the target glycopeptide dendrimers **G2a–c** could be isolated with 5–7% yields.[13] The second generation glycopeptide dendrimers **G2e–g**, including no variable amino acid between branching units but one copy of Arg, Ser or Glu before the dendritic core (X^2), were also synthesized by SPPS. After coupling, deprotection, cleavage reactions and purification, **G2e–g** were obtained with 15%, 22% and 18% yields, respectively.

2.2. Affinity of second generation glycopeptide dendrimers G2a–c and G2e–g for Con A

G2a–c were involved in an enhanced-sensitivity ELLA.[2a] As reported, this competitive test initially relies on yeast mannan adsorption on a 96-wells microtitration plate. Inhibitor is then introduced as serial dilutions followed by biotinylated Con A. After washings, the remaining lectin can be covered by a preformed Neutravidin™-biotinylated horseradish peroxidase complex. Finally, addition of a chromogenic enzyme substrate leads quickly to reliable absorptions. This methodology, inspired by original works of Duk et al. gives sigmoidal inhibition response curves from which IC_{50} values can be measured (Fig. 1).[14] These latters are then compared to inhibition properties obtained, in the same conditions (Table 1), with monovalent carbohydrate derivatives such as Man, *p*-nitrophenyl α-D-mannoside (*p*NP α-Man) or methyl α-D-mannoside (Me α-Man). This latter glycoconjugate is indeed used as a monovalent reference in our ELLA. Within this approach, **G2a–c** then displayed IC_{50}'s of 92, 64 and 60 μM corresponding to relative potencies per carbohydrate moiety of 3.6, 5.2 and 5.5, respectively.

Table 1
Inhibition properties of glycopeptide dendrimers **G1**, **G2a–g**, **G3**, **G02S2**, **G12S2** and **G22S2**

Compound	n[a]	IC_{50} (μM)	r.p.[b]	r.p./n[c]
Man[d]	1	10456 (±1953)	0.13 (±0.04)	0.13 (±0.04)
Me α-Man[d]	1	1320 (±57)	1.0	1.0
***p*NP α-Man**[d]	1	644 (±25)	2.1 (±0.2)	2.1 (±0.2)
G2a	4	92 (±42)	14.3 (±8.6)	3.6 (±2.1)
G2b	4	64 (±19)	20.6 (±9.0)	5.2 (±2.3)
G2c	4	60 (±17)	22.0 (±9.3)	5.5 (±2.3)
G2d[d]	4	52 (±11)	25.4 (±6.5)	6.4 (±1.6)
G2e	4	62 (±26)	21.3 (±11.2)	5.3 (±2.8)
G2f	4	55 (±15)	24.0 (±9.1)	6.0 (±2.3)
G2g	4	93 (±30)	14.2 (±6.1)	3.6 (±1.5)
$G0_2S_2$	2	104 (±32)	12.7 (±4.5)	6.4 (±2.3)
$G1_2S_2$	4	52 (±8)	25.4 (±5.0)	6.4 (±1.6)
$G2_2S_2$	8	2.0 (±0.9)	660 (±326)	83 (±41)
G1[d]	2	150 (±34)	8.8 (±2.5)	4.4 (±1.3)
G3[d]	8	2.9 (±1.1)	455 (±203)	57 (±25)

[a] Number of mannosyl residues.
[b] Relative potency compared to Me α-Man.
[c] Relative potency per carbohydrate moiety.
[d] See Ref. 2a.

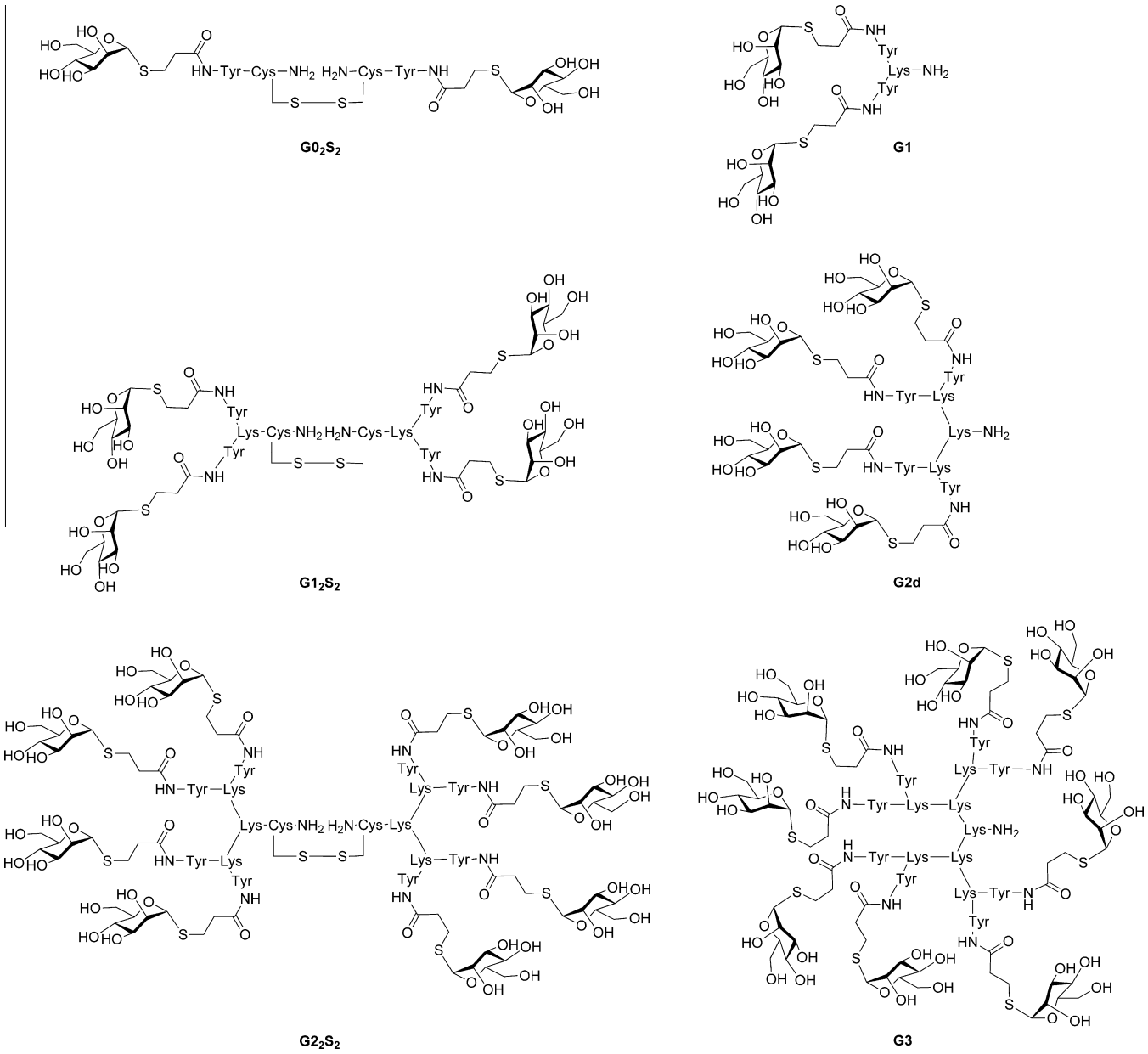
These values highlighted an improvement of affinity toward Con A by cluster effect compared to that obtained with the monovalent reference.[15] In other words, the affinity of the lectin for inhibitor increased with its valency. This result might arise from higher local ligand concentration. However, these IC_{50}'s remained close to that found previously for the glycopeptide dendrimer **G2d**, presenting only four external Tyr residues. Indeed, this latter compound was associated within the same assay, to an IC_{50} value of 52 μM and then to relative potency per carbohydrate of 6.4. Then, introduction of variable amino acids between branching points influenced modestly the inhibition properties of our glycopeptide dendrimers. The inhibition properties of **G2e–g** were also estimated by enhanced-sensitivity ELLA. This assay gave, respectively, IC_{50} values of 62, 55 and 93 μM associated to relative potencies par carbohydrate of 5.3, 6.0 and 3.6. Thus, introduction of variable amino acids before the first branching unit did not affect significantly inhibition properties of glycopeptide dendrimers in agreement with results found with **G2a–c**. Consequently, although introduction close to carbohydrate moieties of amino acids charged at neutral pH seemed to disrupt recognition processes, this behavior was not observed for further variable positions.[2a] These results then contrasted with those previously observed with external amino acids.

2.3. Synthesis of disulfide-based glycopeptide dendrimers $G0_2S_2$, $G1_2S_2$ and $G2_2S_2$

Valency of multiantennary scaffolds is known to be an important structural feature to tune affinity for Con A.[6] This point was also underlined in our previous study involving multivalent inhibitors built owing a divergent approach.[2a] Although convergent strategies, and more particularly these relying on thiol-presenting subunits dimerization, are well known in dendrimers chemistry, disulfide-including glycopeptide dendrimers were rarely involved as lectin inhibitors. Interestingly, in a recent study Lindhorst et al. reported by employing a uropathogenic strain of *Escherichia coli* that Cys-based mannoside glycoclusters were inhibitors of type 1 fimbriae bacterial adhesion.[7a] We then envisioned the synthesis of di-, tetra- and octavalent disulfides **$G0_2S_2$**, **$G1_2S_2$** and **$G2_2S_2$** (Scheme 2) from the corresponding glycodendrons **G0SH**, **G1SH** and **G2SH**, respectively (see Supplementary data). These latters, incorporating a Cys residue before the first branching Lys, could be produced by classical SPPS. Once released from resin, analytical RP-HPLC profiles unfortunately showed the formation of many products. Complexity of such reaction mixtures was however greatly simplified by a subsequent reduction with DTT (1,4-dithio-D,L-threitol), thus allowing a partial purification of expected thiols by preparative RP-HPLC.[11] Then, air-promoted dimerization reactions were conveniently performed. Completion of such an oxidation process was easily monitored by analytical RP-HPLC. After work-up and purification, the target disulfides **$G0_2S_2$**, **$G1_2S_2$** and **$G2_2S_2$** were finally isolated with 16%, 6% and 4% overall yields, respectively. Then, **$G0_2S_2$** and **$G1_2S_2$** were prepared in slightly lower yields than these associated to the synthesis of known counterparts **G1** and **G2d** obtained within a divergent approach.[2a] However, the octavalent derivatives **$G2_2S_2$** and the previously described analog **G3** were isolated with comparable yields.[2a] Although this latter could only be isolated in a 4% yield by decreasing of resin loading, this condition was not required for preparing **$G2_2S_2$**.

2.4. Affinity of disulfide-based glycopeptide dendrimers $G0_2S_2$, $G1_2S_2$ and $G2_2S_2$ for Con A

The ability of **$G0_2S_2$**, **$G1_2S_2$** and **$G2_2S_2$** to inhibit Con A activity was also estimated by enhanced-sensitivity ELLA. The obtained data were additionally confirmed by standard ELLA.[16] **$G0_2S_2$** then presented an IC_{50} value of 104 μM corresponding to a relative potency per mannosyl residue of 6.4. In other words, this divalent disulfide was an inhibitor as potent as the known first generation glycopeptide dendrimer **G1**, synthesized owing to a divergent approach. The dimer **$G1_2S_2$** was also assayed. With an IC_{50} of 52 μM, the inhibitory properties of this tetravalent compound were close to these measured for its counterpart **G2d**. Indeed, a relative potency per carbohydrate of 6.4 was found for both glycopeptide dendrimers. Finally, affinity of **$G2_2S_2$** for Con A was also estimated. The IC_{50} value of 2.0 μM obtained for this octavalent derivative could be associated to a relative potency per carbohydrate of 83. Then, inhibition properties of **$G2_2S_2$** and **G3** were both found in the micromolar range. Considering the uncertainties of ELLA for high affinity ligands, relatively similar inhibition behaviours were herein observed for both glycopeptide dendrimers. Moreover, IC_{50} values obtained by enhanced-sensitivity ELLA for **$G0_2S_2$**, **$G1_2S_2$** and **$G2_2S_2$** were compared to these measured by standard ELLA. Indeed, in this latter method, attachment of a 40 kDa peroxidase to the lectin would prevent aggregation processes and thereby virtual increase of affinity.[16] Given that **$G0_2S_2$**, **$G1_2S_2$** and **$G2_2S_2$** were associated, respectively, to IC_{50} values of 102, 52 and 2.4 μM by standard ELLA, it was then likely that presented disulfides were not involved in cross-linking of



Scheme 2. Structure of glycopeptide dendrimers **G1**, **G2d**, **G3**, **G02S2**, **G12S2** and **G22S2**.

biotinylated Con A within enhanced-sensitivity ELLA. Consequently, affinity for Con A increased as expected with valency. Our results were moreover consistent with pioneer works of Roy et al. in which di- and tetravalent inhibitors were associated to IC_{50} values reaching several dozens of μM, and in which inhibition activity of an octamer was in the μM range.[6e] It could be also deduced from presented results that ability of our glycopeptide dendrimers to inhibit Con A activity was relatively independent on the nature of the employed multivalent scaffold, as long as external amino acids were judiciously chosen.

3. Conclusions

In conclusion, we contributed herein to study the relationship existing between structure of multivalent glycoconjugates and affinity for lectins. Different glycopeptide dendrimers, presenting various amino acids inside or before the dendritic core, were initially synthesized and used as ligands toward Con A. It was then underlined by ELLA that incorporation of internal amino acids did not modify significantly the affinity for this plant lectin. This result then contrasted with these previously obtained with variable amino acids placed externally on the multivalent scaffold.[2a] Additionally, disulfide-containing glycopeptide dendrimers were built by homodimerization of Cys-based glycodendrons. The affinity for Con A of these dimers obtained by a convergent strategy, was also evaluated by ELLA. Comparison of inhibition properties with results obtained for known derivatives synthesized by a divergent approach showed that affinity for Con A depended mainly on valency and few on the way to assemble the multivalent scaffolds.

4. Experimental section

4.1. General methods

All standard chemicals were purchased either from Acros, Aldrich or Fluka (Switzerland). Amino acids derivatives and

coupling reagents were purchased from Seen Chemicals or Novabiochem (Switzerland). Rink amide resin (NovaSyn® TGR, 0.23 mmol g^{-1}) was purchased from Novabiochem (Switzerland). All solvents used were analytical grade. Biotin labelled Concanavalin A (type IV, from *Canavalia ensiformis*) and yeast mannan (from *Saccharomyces cerevisiae*) were purchased from Sigma (Switzerland). Neutravidin™ biotin binding protein and ImmunoPure® biotinylated horseradish peroxydase were purchased from Pierce Biotechnology (Switzerland). C96 Maxisorp Nunc-Immuno plates and nontreated mixing plates (EIA/RIA 96 well plates) were, respectively, purchased from Milian (Switzerland) and Corning Incorporated (USA). Peptide syntheses were performed manually in a glass reactor. Analysis by reversed phase high performance liquid chromatography (RP-HPLC) was performed with a Waters (996 Photodiode array detector) chromatography system using a chromolith performance RP-18e column (4.6 × 100 mM, flow rate 3 mL min^{-1}). Preparative RP-HPLC was performed with HPLC-grade CH_3CN and MilliQ deionized H_2O in a Waters prepak cartridge 500 g (RP-C18 20 nm, 300 Å pore size) installed on a waters Prep LC4000 system from Millipore (flow rate 100 mL min^{-1}). The gradients of eluent and the percentages referring to mixtures of solvents were given as a ratio of volumes. Abbreviation 'eluent A' and 'eluent B' were used to refer, respectively, to H_2O containing 0.1% TFA (trifluoroacetic acid), and CH_3CN/H_2O (3:2) containing 0.1% TFA. Compounds were detected by UV absorption at 214 nm. The structure of all compounds was ascertained by 1D [^1H, ^{13}C, DEPT-135 (Distorsionless Enhancement by Polarization Transfer)] and/or 2D NMR experiments [COSY (^1H–1H Correlation Spectroscopy), HMQC (^1H–13C Heteronuclear Multiple Quantum Correlation) and HMBC (^1H–13C Heteronuclear Multiple Bond Correlation) experiments]. The NMR spectra were recorded on a DRX400 spectrometer (Bruker) at 400 MHz for ^1H and 100 MHz for ^{13}C, or on DRX500 spectrometer (Bruker) at 500 MHz for ^1H and 125 MHz for ^{13}C (Analytical Research and Services, University of Berne). Chemical shifts were given in δ units, measured from the solvent signal. MS spectra were performed by ESI (Electrospray Ionization) either in positive or negative mode, and were recorded on an Applied Biosystems/Sciex Qtrap spectrometer (Analytical Research and Services, University of Berne).

4.2. Chemical synthesis

4.2.1. Synthesis of the peptide backbones

The Rink amide resin was washed and swollen successively with CH_2Cl_2 (2 × 5 mL) and NMP (*N*-methylpyrrolidone, 1 × 5 mL). The coupling reaction was then performed by stirring the resin (ca. 200 mg, 46 μmol) with 3 equivalents (per amine) of N-Fmoc protected amino acid, 3 equiv of BOP [benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophos-phate] and 6 equiv of DIEA (*N*,*N*-diisopropylethylamine) in NMP (5 mL) for 1–12 h. Subsequently, the solid support was washed successively with NMP, MeOH and CH_2Cl_2 (3 × 5 mL of each solvent) and the completion of the condensation reaction was controlled by using the TNBS (2,4,6-trinitrobenzensulfonic acid) test or selectively by using the acetaldehyde–chloranil test when traces of free amines derived from proline had to be detected.[11] The condensation procedure was repeated if the reaction was found to be uncompleted and was also systematically renewed in the case of arginine coupling. Once completed the reaction, the potentially free amines were acetylated by stirring the resin for 10 min with an equivolumic mixture of Ac_2O and CH_2Cl_2 (5 mL), followed by washings as described above. Then, the Fmoc groups were then removed by stirring the resin with a solution (5 mL, 1:4) of piperidine in DMF (*N*,*N*-dimethylformamide) for 10 min. After washings with NMP, MeOH, CH_2Cl_2 (3 × 5 mL of each solvent), this deprotection procedure was repeated and the completion of the reaction was monitored with the previously mentioned tests. Finally, the whole procedure was repeated as many times as necessary to achieve the synthesis of the desired oligopeptide.

4.2.2. Mannosylation reactions

Once realized the assembly of the peptide backbone and the cleavage of the last Fmoc residue, the resin was acylated using 5 equiv per amine of the mannosidic building block **1**, 5 equiv of HCTU [2-(6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] and 10 equiv of DIEA in NMP (5 mL). The reaction was carried out overnight in dark and the resin was then washed with NMP, MeOH and CH_2Cl_2 (3 × 5 mL of each solvent). The completion of the glycosidic capping was checked with the previously mentioned TNBS test. The reaction was systematically restarted and, after washings, the potentially free amines were acetylated as described above.

4.2.3. On-bead *O*-deacetylation

The on-bead deacetylation of carbohydrate moieties was realized by suspending beads in a mixture of MeOH, H_2O and 25% aqueous solution of NH_4OH (4 mL, 8:1:1). After stirring for 24 h and filtration, the beads were washed with NMP, MeOH and CH_2Cl_2 (3 × 5 mL of each solvent).

4.2.4. One-pot side chains deprotection and cleavage from resin

The side-chains deprotection and cleavage from resin were carried out concomitantly by stirring the beads for 4 h in a mixture of TFA, TIS (triisopropylsilane) and H_2O (3 mL, 94:5:1). For cysteine-containing dendrimers, 1% of EDT (1,2-ethandithiol) was added to this cleavage mixture. After filtration and washing of the solid support with TFA (2 × 1 mL), the peptide was precipitated by addition of MTBE (methyl *tert*-butyl ether, 45 mL) and collected by centrifugation (10 min, 4 °C, 4400 rpm). The crude peptide was then resuspended in clean MTBE (50 mL), recentrifugation and finally dried under a gentle stream of N_2.

4.2.5. In-solution *O*-deacetylation

The in-solution removal of residual acetyl groups was achieved by stirring for 24 h the crude glycopeptide in a mixture of MeOH, H_2O and 25% aqueous solution of NH_4OH (4 mL, 8:1:1). Then, the solution was concentrated under reduced pressure, dissolved in eluent A (10 mL) and freeze-dried. The optimal purification conditions were then estimated by analytical RP-HPLC, and the target glycopeptide was finally purified by preparative RP-HPLC.

4.2.6. Second generation glycopeptide dendrimer G2a

The glycopeptide dendrimer **G2a** was synthesized from Rink amide resin (212 mg, 49 μmol). After purification by RP-HPLC, the target TFA salt was isolated as a white amorphous (9 mg, 7%): Preparative RP-HPLC: t_r = 23.6 min (A/B = 100:0→40:60, 40 min); Analytical RP-HPLC: t_r = 8.11 min (A/B = 100:0→40:60, 10 min); ^1H NMR (500 MHz, CD_3CN/D_2O (3:2) + 0.1% [D]-TFA): δ 7.72–7.69 (m, 8H, H-$3'_{Tyr}$, H-$5'_{Tyr}$), 7.41–7.39 (m, 8H, H-$2'_{Tyr}$, H-$6'_{Tyr}$), 5.87 (br s, 4H, H-1_{Man}), 5.14–5.10, 5.08–5.05 (2m, 4H, CHα_{Tyr}), 4.93–4.67 (m, 5H, CHα_{Arg}, CHα_{Lys}), 4.54, 4.52 (2br s, 4H, H-2_{Man}), 4.50–4.40 (m, 8H, H-5_{Man}, H-$6a_{Man}$), 4.35 [dd, ^2J(H-$6a_{Man}$, H-$6b_{Man}$) = 12.2 Hz, ^3J(H-5_{Man}, H-$6b_{Man}$) = 5.8 Hz, 4H, H-$6b_{Man}$], 4.28–4.23 (m, 8H, H-3_{Man}, H-4_{Man}), 3.77–3.57, 3.50–3.35 (2m, 26H, $CH_2\varepsilon_{Lys}$, $CH_2\delta_{Arg}$, $CH_2\alpha_{Lys}$, SCH_2), 3.23–3.12 (m, 8H, SCH_2CH_2), 2.48–2.37, 2.34–2.27, 2.25–2.14, 2.13–2.07, 2.01–1.89, 1.84–1.69 ppm (6m, 26H, $CH_2\beta_{Arg}$, $CH_2\beta_{Lys}$, $CH_2\gamma_{Arg}$, $CH_2\gamma_{Lys}$, $CH_2\delta_{Lys}$); ^{13}C NMR (125 MHz, CD_3CN/D_2O (3:2) + 0.1% [D]-TFA): δ 175.7 [C(=O)NH_2], 173.6, 173.1, 173.0 [C(=O)NH], 156.5 (C-$1'_{Arg}$), 155.0 (C-$1'_{Tyr}$), 130.3 (C-$3'_{Tyr}$, C-$5'_{Tyr}$), 127.9, 127.7 (C-$4'_{Tyr}$), 115.2, 115.2 (C-$2'_{Tyr}$, C-$6'_{Tyr}$), 84.9, 84.6 (C-1_{Man}), 73.1 (C-5_{Man}), 71.7, 71.6 (C-2_{Man}), 71.2 (C-3_{Man}), 66.9 (C-4_{Man}), 60.8 (C-6_{Man}), 55.4, 55.3 (CHα_{Tyr}), 53.6, 53.2, 52.9, 52.7

($CH\alpha_{Arg}$, $CH\alpha_{Lys}$), 40.8 ($CH_2\delta_{Arg}$), 38.8, 38.7 ($CH_2\varepsilon_{Lys}$), 36.5, 35.9, 35.9 ($CH_2\beta_{Tyr}$), 35.9, 35.4 (SCH_2CH_2), 30.7, 30.2, 30.2 ($CH_2\beta_{Arg}$), 30.4 ($CH_2\beta_{Lys}$), 27.9 ($CH_2\delta_{Lys}$), 26.5, 26.3 (SCH_2), 24.5, 24.4, 24.4 ($CH_2\gamma_{Arg}$), 22.3 ppm ($CH_2\gamma_{Lys}$); MS (ESI+) calcd for $C_{102}H_{157}N_{19}O_{37}S_4^{2+}$ $[M+2H]^{2+}$: 1184.0. Found: 1184.6.

4.2.7. Second generation glycopeptide dendrimer G2b

The synthesis of the dendrimer **G2b** was performed on Rink amide resin (226 mg, 52 μmol). After work-up, this molecule was isolated as a colourless solid by RP-HPLC (6 mg, 5%): Preparative RP-HPLC: t_r = 22.7 min (A/B = 100:0→60:40, 40 min); Analytical RP-HPLC: t_r = 7.97 min (A/B = 100:0→60:40, 10 min); ^{1}H NMR (500 MHz, CD_3CN/D_2O (3:2) + 0.1% [D]-TFA): δ 7.70–7.67 (m, 8H, H-$3'_{Tyr}$, H-$5'_{Tyr}$), 7.39–7.37 (m, 8H, H-$2'_{Tyr}$, H-$6'_{Tyr}$), 5.85 (s, 4H, H-1_{Man}), 5.15–5.11, 5.07–5.04 (2m, 4H, $CH\alpha_{Tyr}$), 5.00–4.98, 4.92–4.89 (2m, 2H, $CH\alpha_{Ser}$), 4.87–4.71 (m, 3H, $CH\alpha_{Lys}$), 4.52–4.51 (m, 4H, H-2_{Man}), 4.48–4.36 (m, 12H, H-5_{Man}, H-$6a_{Man}$, $CH_2\beta_{Ser}$), 4.33 [dd, ^2J(H-$6a_{Man}$, H-$6b_{Man}$) = 12.0 Hz, ^3J(H-5_{Man}, H-$6b_{Man}$) = 5.9 Hz, 4H, H-$6b_{Man}$], 4.26–4.21 (m, 8H, H-3_{Man}, H-4_{Man}), 3.81–3.55, 3.51–3.31 (2m, 22H, $CH_2\varepsilon_{Lys}$, $CH_2\beta_{Tyr}$, SCH_2), 3.20–3.09 (m, 8H, SCH_2CH_2), 2.45–2.37, 2.35–2.26, 2.24–2.15, 2.12–2.05, 2.00–1.88, 1.82–1.68 ppm (6m, 18H, $CH_2\beta_{Lys}$, $CH_2\gamma_{Lys}$, $CH_2\delta_{Lys}$); ^{13}C NMR (125 MHz, CD_3CN/D_2O (3:2) + 0.1% [D]-TFA): δ 175.8 [C(=O)NH_2], 175.0, 174.2, 173.5, 173.0, 173.0 [C(=O)NH], 154.9, 154.9 (C-$1'_{Tyr}$), 130.3 (C-$4'_{Tyr}$), 127.9, 127.7 (C-$3'_{Tyr}$, C-$5'_{Tyr}$), 115.2, 115.1 (C-$2'_{Tyr}$, C-$6'_{Tyr}$), 84.8, 84.7 (C-1_{Man}), 73.1 (C-5_{Man}), 71.6 (C-2_{Man}), 71.2 (C-3_{Man}), 66.9 (C-4_{Man}), 66.3 ($ch_2\beta_{Ser}$), 60.8, 60.8 (C-6_{Man}), 55.2, 55.1 ($CH\alpha_{Ser}$, $CH\alpha_{Tyr}$), 53.6, 53.5 ($CH\alpha_{Lys}$), 36.5, 36.4, 36.0 ($CH_2\varepsilon_{Lys}$), 35.5, 35.4, 35.4 ($CH_2\beta_{Tyr}$), 35.5, 35.4, 35.4 (SCH_2CH_2), 30.4, 30.4 ($CH_2\beta_{Lys}$), 27.9, 27.9, 27.8 ($CH_2\delta_{Lys}$), 26.5, 26.5, 26.4 (SCH_2), 22.3 ppm ($CH_2\gamma_{Lys}$); MS (ESI+) calcd for $C_{96}H_{143}N_{13}O_{39}S_4^{2+}$ $[M+2H]^{2+}$: 1114.9. Found: 1115.4.

4.2.8. Second generation glycopeptide dendrimer G2c

The dendrimer **G2c** was obtained from Rink amide resin (203 mg, 47 μmol) and was isolated by RP-HPLC as a white foam (6 mg, 6%): Preparative RP-HPLC: t_r = 22.8 min (A/B = 100:0→40:60, 40 min); Analytical RP-HPLC: t_r = 8.18 min (A/B = 100:0→40:60, 10 min); ^{1}H NMR (500 MHz, CD_3CN/D_2O (3:2) + 0.1% [D]-TFA): δ 7.72–7.69 (m, 8H, H-$3'_{Tyr}$, H-$5'_{Tyr}$), 7.40–7.38 (m, 8H, H-$2'_{Tyr}$, H-$6'_{Tyr}$), 5.86 (br s, 4H, H-1_{Man}), 5.15–5.12, 5.09–5.06 (2m, 4H, $CH\alpha_{Tyr}$), 4.95–4.91, 4.87–4.82 (2m, 2H, $CH\alpha_{Glu}$), 4.87–4.67 (m, 3H, $CH\alpha_{Lys}$), 4.53–4.52 (m, 4H, H-2_{Man}), 4.50–4.45 (m, 4H, H-5_{Man}), 4.44–4.40 (m, 4H, H-$6a_{Man}$), 4.35 [dd, ^2J(H-$6a_{Man}$, H-$6b_{Man}$) = 12.2 Hz, ^3J(H-5_{Man}, H-$6b_{Man}$) = 6.0 Hz, 4H, H-$6b_{Man}$], 4.28–4.23 (m, 8H, H-3_{Man}, H-4_{Man}), 3.80–3.57 (m, 10H, $CH_2\varepsilon_{Lys}$, $CH_2\beta_{Tyr}$), 3.50–3.32 (m, 12H, $CH_2\beta_{Tyr}$, SCH_2), 3.23–3.11 (m, 8H, SCH_2CH_2), 3.05–2.96 (m, 4H, $CH_2\gamma_{Glu}$), 2.74–2.68, 2.60–2.49 (2m, 4H, $CH_2\beta_{Glu}$), 2.44–2.37, 2.35–2.25, 2.22–2.15, 2.13–2.06, 2.01–1.90, 1.82–1.68 ppm (6m, 18H, $CH_2\beta_{Lys}$, $CH_2\gamma_{Lys}$, $CH_2\delta_{Lys}$); ^{13}C NMR (125 MHz, CD_3CN/D_2O (3:2) + 0.1% [D]-TFA): δ 175.9, 175.8, 175.7 [C(=O)NH_2, CO_2H], 173.4, 173.0, 172.9, 172.9, 172.8, 172.3, 172.2, 172.0 [C(=O)NH], 155.0, 155.0, 154.9 (C-$1'_{Tyr}$), 130.3 (C-$3'_{Tyr}$, C-$5'_{Tyr}$), 127.9, 127.7, 127.7 (C-$4'_{Tyr}$), 115.2, 115.2 (C-$2'_{Tyr}$, C-$6'_{Tyr}$), 84.9, 84.6, 84.6 (C-1_{Man}), 73.1, 73.1 (C-5_{Man}), 71.6 (C-2_{Man}), 71.2 (C-3_{Man}), 66.9, 66.9 (C-4_{Man}), 60.8, 60.8 (C-6_{Man}), 55.3, 55.2 ($CH\alpha_{Tyr}$), 53.7, 53.6, 53.3 ($CH\alpha_{Lys}$), 52.8, 52.8 ($CH\alpha_{Glu}$), 38.8, 38.7 ($CH_2\varepsilon_{Lys}$), 36.6, 36.6, 36.0 ($CH_2\beta_{Tyr}$), 35.5, 35.4, 35.3 (SCH_2CH_2), 30.7, 30.5, 30.4 ($CH_2\beta_{Lys}$), 30.0, 29.9 ($CH_2\gamma_{Glu}$), 27.9 ($CH_2\delta_{Lys}$), 26.5, 26.3 (SCH_2), 26.3, 26.0 ($CH_2\beta_{Glu}$), 22.4, 22.3, 22.3 ppm ($CH_2\gamma_{Lys}$); MS (ESI–) calcd for $C_{100}H_{143}N_{13}O_{41}S_4^{2-}$ $[M-2H]^{2-}$: 1154.9. Found: 1155.6.

4.2.9. Second generation glycopeptide dendrimer G2e

The glycopeptide dendrimer **G2e** was obtained from Rink amide resin (232 mg, 53 μmol) and was isolated as a colourless TFA salt (19 mg, 15%) by RP-HPLC purification: Preparative RP-HPLC: t_r = 28.4 min (A/B = 100:0→50:50, 50 min); Analytical RP-HPLC: t_r = 9.13 min (A/B = 100:0→50:50, 10 min); ^{1}H NMR (400 MHz, CD_3CN/D_2O (3:2) + 0.1% [D]-TFA): δ 7.72–7.70 (m, 8H, H-$3'_{Tyr}$, H-$5'_{Tyr}$), 7.41–7.38 (m, 8H, H-$2'_{Tyr}$, H-$6'_{Tyr}$), 5.87 (br s, 4H, H-1_{Man}), 5.17–5.13, 5.09–5.05 (2m, 4H, $CH\alpha_{Tyr}$), 4.88–4.72 (m, 4H, $CH\alpha_{Arg}$, $CH\alpha_{Lys}$), 4.56–4.52 (m, 4H, H-2_{Man}), 4.51–4.40 (m, 8H, H-5_{Man}, H-$6a_{Man}$), 4.35 [dd, ^2J(H-$6a_{Man}$, H-$6b_{Man}$) = 12.0 Hz, ^3J(H-5_{Man}, H-$6b_{Man}$) = 5.8 Hz, 4H, H-$6b_{Man}$], 4.29–4.23 (m, 8H, H-3_{Man}, H-4_{Man}), 3.77–3.57, 3.51–3.31 (2m, 24H, $CH_2\beta_{Tyr}$, $CH_2\delta_{Arg}$, $CH_2\varepsilon_{Lys}$, SCH_2), 3.23–3.11 (m, 8H, SCH_2CH_2), 2.47–2.37, 2.37–2.24, 2.24–2.13, 2.13–2.03, 2.00–1.89, 1.83–1.67 ppm (6m, 22H, $CH_2\beta_{Arg}$, $CH_2\beta_{Lys}$, $CH_2\gamma_{Arg}$, $CH_2\gamma_{Lys}$, $CH_2\delta_{Lys}$); ^{13}C NMR (100 MHz, CD_3CN/D_2O (3:2) + 0.1% [D]-TFA): δ =175.7 [C(=O)NH_2], 173.3, 173.2, 172.9, 172.8, 172.7, 172.5, 172.4, 172.2 [C(=O)NH], 155.0, 155.0, 154.9 (C-$1'_{Arg}$, C-$1'_{Tyr}$), 130.3 (C-$3'_{Tyr}$, C-$5'_{Tyr}$), 127.9, 127.7 (C-$4'_{Tyr}$), 115.2, 115.2 (C-$2'_{Tyr}$, C-$6'_{Tyr}$), 84.9, 84.7, 84.6 (C-1_{Man}), 73.1 (C-5_{Man}), 71.7 (C-2_{Man}), 71.2 (C-3_{Man}), 67.0 (C-4_{Man}), 60.8 (C-6_{Man}), 55.2, 55.1, 55.0 ($CH\alpha_{Tyr}$), 53.5, 53.2 ($CH\alpha_{Arg}$, $CH\alpha_{Lys}$), 38.9, 38.7, 38.7 ($CH_2\varepsilon_{Lys}$), 36.6, 36.1, 36.0 ($CH_2\beta_{Tyr}$), 35.5, 35.4 (SCH_2CH_2), 30.8, 30.7, 30.7, 30.6, 30.6, 30.6 ($CH_2\beta_{Arg}$, $CH_2\beta_{Lys}$), 28.0, 27.9 ($CH_2\delta_{Lys}$), 26.6, 26.5, 26.4 (SCH_2), 22.5, 22.3, 22.3 ppm ($CH_2\gamma_{Lys}$); MS (ESI–) calcd for $C_{96}H_{149}N_{15}O_{40}S_4^{2-}$ $[M+2H_2O+2OH]^{2-}$: 1139.9. Found: 1140.8.[17]

4.2.10. Second generation glycopeptide dendrimer G2f

The glycodendrimer **G2f** was obtained from Rink amide resin (218 mg, 50 μmol) and isolated as a colourless solid (24 mg, 22%) by RP-HPLC: Preparative RP-HPLC: t_r = 9.02 min (A/B = 100:0→50:50, 50 min); Analytical RP-HPLC: t_r = 9.02 min (A/B = 100:0→50:50, 10 min); ^{1}H NMR (500 MHz, CD_3CN/D_2O (3:2) + 0.1% [D]-TFA): δ 7.69-7.63 (m, 8H, H-$3'_{Tyr}$, H-$5'_{Tyr}$), 7.39–7.32 (m, 8H, H-$2'_{Tyr}$, H-$6'_{Tyr}$), 5.84–5.80 (m, 4H, H-1_{Man}), 5.14–5.10, 5.06–5.03 (2m, 4H, $CH\alpha_{Tyr}$), 4.97–4.95 (m, 1H, $CH\alpha_{Ser}$), 4.86–4.70 (m, 3H, $CH\alpha_{Lys}$), 4.51–4.50 (m, 4H, H-2_{Man}), 4.49–4.36 (m, 10H, H-5_{Man}, H-$6a_{Man}$, CH2βSer), 4.32 [dd, ^2J(H-$6a_{Man}$, H-$6b_{Man}$) = 12.2 Hz, ^3J(H-5_{Man}, H-$6b_{Man}$) = 5.8 Hz, 4H, H-$6b_{Man}$], 4.25–4.19 (m, 8H, H-3_{Man}, H-4_{Man}), 3.75–3.54 (m, 10H, $CH_2\varepsilon_{Lys}$, $CH_2\beta_{Tyr}$), 3.47–3.30 (m, 12H, $CH_2\beta_{Tyr}$, SCH_2), 3.19–3.08 (m, 8H, SCH_2CH_2), 2.45–2.37, 2.34–2.23, 2.19–2.09, 2.09–2.01, 1.97–1.85, 1.79–1.65 ppm (6m, 18H, $CH_2\beta_{Lys}$, $CH_2\gamma_{Lys}$, $CH_2\delta_{Lys}$); ^{13}C NMR (125 MHz, CD_3CN/D_2O (3:2) + 0.1% [D]-TFA): δ 173.4, 173.4, 173.2, 173.1, 172.9, 172.9, 172.7, 172.5, 172.4, 172.1 [C(=O)NH_2, C(=O)NH], 154.9, 154.9 (C-$1'_{Tyr}$), 130.3 (C-$3'_{Tyr}$, C-$5'_{Tyr}$), 127.9, 127.7 (C-$4'_{Tyr}$), 115.2, 115.2 (C-$2'_{Tyr}$, C-$6'_{Tyr}$), 84.8, 84.7, 84.6 (C-1_{Man}), 73.1 (C-5_{Man}), 71.6 (C-2_{Man}), 71.2 (C-3_{Man}), 66.9 (C-4_{Man}), 61.2 (CH2βSer),60.8 (C-6_{Man}), 55.1, 55.1, 55.0 ($CH\alpha_{Ser}$, $CH\alpha_{Tyr}$), 53.7, 53.5, 53.4 ($CH\alpha_{Lys}$), 38.6, 38.6 ($CH_2\varepsilon_{Lys}$), 36.5, 36.0, 36.0 ($CH_2\beta_{Tyr}$), 35.5, 35.4 (SCH_2CH_2), 30.7, 30.5, 30.3 ($CH_2\beta_{Lys}$), 27.9 ($CH_2\delta_{Lys}$), 26.5, 26.4, 26.3 (SCH_2), 22.4, 22.3 ppm ($CH_2\gamma_{Lys}$); MS (ESI–) calcd for $C_{93}H_{134}N_{12}O_{37}S_4^{2-}$ $[M-2H]^{2-}$: 1069.4. Found: 1069.8.

4.2.11. Second generation glycopeptide dendrimer G2g

The glycopeptide dendrimer **G2g** was synthesized from Rink amide resin (216 mg, 50 μmol) and isolated by RP-HPLC as a colourless foam (19 mg, 18%): Preparative RP-HPLC: t_r = 28.0 min (A/B = 100:0→50:50, 50 min); Analytical RP-HPLC: t_r = 9.16 min (A/B = 100:0→50:50, 10 min); ^{1}H NMR (400 MHz, CD_3CN/D_2O (3:2) + 0.1% [D]-TFA): δ 7.69–7.66 (m, 8H, H-$3'_{Tyr}$, H-$5'_{Tyr}$), 7.38–7.34 (m, 8H, H-$2'_{Tyr}$, H-$6'_{Tyr}$), 5.83 (br s, 4H, H-1_{Man}), 5.16–5.08, 5.05–5.02 (2m, 4H, $CH\alpha_{Tyr}$), 4.91–4.87 [dd, ^3J($CH\alpha_{Glu}$,$CH_2\beta a_{Glu}$) = 9.6 Hz, ^3J($CH\alpha_{Glu}$,$CH_2\beta b_{Glu}$) = 4.7 Hz, 1H, $CH\alpha_{Glu}$], 4.83–4.69 (m, 3H, $CH\alpha_{Lys}$), 4.50–4.49 (m, 4H, H-2_{Man}), 4.47–4.36 (m, 8H, H-5_{Man}, H-$6a_{Man}$), 4.31 [dd, ^2J(H-$6a_{Man}$, H-$6b_{Man}$) = 12.2 Hz, ^3J(H-5_{Man}, H-$6b_{Man}$) = 5.8 Hz, 4H, H-$6b_{Man}$], 4.25–4.19 (m, 8H, H-3_{Man}, H-4_{Man}), 3.75–3.53 (m, 10H, $CH_2\varepsilon_{Lys}$, $CH_2\beta_{Tyr}$), 3.47–3.28 (m, 12H,

$CH_2\beta_{Tyr}$, SCH_2), 3.19–3.09 (m, 8H, SCH_2CH_2), 3.03–2.99 (m, 2H, $CH_2\gamma_{Glu}$), 2.74–2.66, 2.55–2.47 (2m, 2H, $CH_2\beta_{Glu}$), 2.44–2.33, 2.33–2.21, 2.21–2.09, 2.09–1.98, 1.98–1.83, 1.79–1.63 ppm (6m, 18H, $CH_2\beta_{Lys}$, $CH_2\gamma_{Lys}$, $CH_2\delta_{Lys}$); ^{13}C NMR (100 MHz, CD_3CN/D_2O (3:2) + 0.1% [D]-TFA): δ 175.8 (CO_2H), 174.9 [C(=O)NH_2],173.4, 173.2, 173.2, 173.1, 172.9, 172.9, 172.8, 172.5, 172.3, 172.1 [C(=O)NH], 154.9, 152.9 (C-$1'_{Tyr}$), 130.3 (C-$3'_{Tyr}$, C-$5'_{Tyr}$), 127.9, 127.7 (C-$4'_{Tyr}$), 115.1 (C-$2'_{Tyr}$, C-$6'_{Tyr}$), 84.8, 84.7, 84.6 (C-1_{Man}), 73.1 (C-5_{Man}), 71.7 (C-2_{Man}), 71.2 (C-3_{Man}), 66.9 (C-4_{Man}), 60.8 (C-6_{Man}), 55.1, 55.0 ($CH\alpha_{Tyr}$), 53.7, 53.4 ($CH\alpha_{Lys}$), 52.3 ($CH\alpha_{Glu}$), 38.7, 38.6 ($CH_2\varepsilon_{Lys}$), 36.5, 36.0, 35.9 ($CH_2\beta_{Tyr}$), 35.5, 35.4, 35.3 (SCH_2CH_2), 30.7, 30.5, 30.3 ($CH_2\beta_{Lys}$), 29.9 ($CH_2\gamma_{Glu}$), 27.9, 27.9 ($CH_2\delta_{Lys}$), 26.5, 26.4 (SCH_2), 26.3 ($CH_2\beta_{Glu}$), 22.4, 22.3 ppm ($CH_2\gamma_{Lys}$); MS (ESI−) calcd for $C_{95}H_{136}N_{12}O_{38}S_4^{2-}$ $[M-2H]^{2-}$: 1090.4. Found: 1090.8.

4.2.12. Synthesis of cysteine-containing glycopeptides

After peptide assembly, glycosylation reaction, on-bead deacetylation and cleavage from the solid support as described above, the crude material was solubilised in 25 mL of a 50 mM aqueous solution of $(NH_4)_2CO_3$. DTT (1,4-dithio-D,L-threitol) was added (ca. 71 mg, 460 μmol) and the mixture was stirred under argon for 2 h. The solution was freeze-dried, the residue thus obtained dissolved in eluent A (20 mL) and subjected to an other freeze-drying step. The composition of the reaction mixture was evaluated by analytical RP-HPLC and partial purification of intermediates **G0SH**, **G1SH** and **G2SH** was achieved by preparative RP-HPLC. The fractions containing mercaptans were then pooled and freeze-dried.

4.2.13. Monovalent glycopeptide G0SH

The intermediate **G0SH** was synthesized from Rink amide resin (410 mg, 94 μmol) and partially purified by RP-HPLC: Preparative RP-HPLC: t_r = 8.4 min (A/B = 90:10→40:60, 50 min); Analytical RP-HPLC: t_r = 3.34 min (A/B = 90:10→40:60, 10 min); MS (ESI+) calcd for $C_{21}H_{32}N_3O_9S_2^+$ $[M+H]^+$: 534.2. Found: 534.2.

4.2.14. Divalent glycopeptide G1SH

The synthesis of the intermediate **G1SH** was performed from Rink amide resin (403 mg, 93 μmol) and partially purified by RP-HPLC: Preparative RP-HPLC: t_r = 25.4 min (A/B = 100:0→40:60, 60 min); Analytical RP-HPLC: t_r = 7.36 min (A/B = 100:0→40:60, 10 min); MS (ESI+) calcd for $C_{45}H_{67}N_6O_{18}S_3^+$ $[M+H]^+$: 1075.4. Found: 1075.6.

4.2.15. Tetravalent glycopeptide G2SH

The intermediate thiol **G2SH** was synthesized on Rink amide resin (411 mg, 94 μmol) and partially purified by RP-HPLC: Preparative RP-HPLC: t_r = 22.0 min (A/B = 90:0→40:60, 50 min); Analytical RP-HPLC: t_r = 7.56 min (A/B = 90:0→40:60, 10 min); MS (ESI+) calcd for $C_{93}H_{138}N_{12}O_{36}S_5^{2+}$ $[M+2H]^{2+}$: 1079.4. Found: 1080.2.

4.2.16. Homodimerization of cysteine-containing glycopeptides

The intermediate thiol was solubilised in a mixture MeOH, H_2O and 25% aqueous solution of NH_4OH (4 mL, 8:1:1) and the reaction medium subjected to an air-bubbling for 48 h. After concentration under reduced pressure, acidification by adding a small amount of eluent A (ca. 10 mL) and freeze-drying, the optimal purification conditions were estimated by analytical RP-HPLC. Finally, the desired disulfide was isolated by preparative RP-HPLC.

4.2.17. Divalent disulfide $G0_2S_2$

The thiol **G0SH** was then dimerized to give the disulfide **$G0_2S_2$** which was isolated by RP-HPLC as a colourless foam (8 mg, 16%, overall): Preparative RP-HPLC: t_r = 22.9 min (A/B = 100:0→50:50, 50 min); Analytical RP-HPLC: t_r = 7.36 min (A/B = 100:0→50:50, 10 min); ^{1}H NMR (500 MHz, CD_3CN/D_2O (3:2) + 0.1% [D]-TFA): δ 7.76–7.71 (m, 4H, H-$3'_{Tyr}$, H-$5'_{Tyr}$), 7.44–7.40 (m, 4H, H-$2'_{Tyr}$, H-$6'_{Tyr}$), 5.89–5.85 (m, 2H, H-1_{Man}), 5.25–5.15 (m, 4H, CHαCys, $CH\alpha_{Tyr}$), 4.57–4.36 (m, 8H, H-2_{Man}, H-5_{Man}, H-$6a_{Man}$, H-$6b_{Man}$), 4.29–4.24 (m, 4H, H-3_{Man}, H-4_{Man}), 3.84–3.78 (m, 2H, CH2βCys), 3.70–3.64 (m, 2H, $CH_2\beta_{Tyr}$), 3.61–3.47 (m, 4H, CH2βCys, $CH_2\beta_{Tyr}$), 3.46–3.36 (m, 4H, SCH_2), 3.20–3.16 ppm (m, 4H, SCH_2CH_2); ^{13}C NMR (125 MHz, CD_3CN/D_2O (3:2) + 0.1% [D]-TFA): δ 173.1,173.1, 172.3, 172.3 [C(=O)NH_2, C(=O)NH], 154.7, 154.7 (C-$1'_{Tyr}$), 130.1, 130.1 (C-$3'_{Tyr}$, C-$5'_{Tyr}$), 127.5, 127.5 (C-$4'_{Tyr}$), 115.0, 115.0 (C-$2'_{Tyr}$, C-$6'_{Tyr}$), 84.6, 84.6 (C-1_{Man}), 72.9, 72.8 (C-5_{Man}), 71.4, 71.4 (C-2_{Man}), 71.0, 71.0 (C-3_{Man}), 66.7, 66.7 (C-4_{Man}), 60.6, 60.6 (C-6_{Man}), 55.0, 54.9 ($CH\alpha_{Tyr}$), 51.9, 51.9 (CHαCys), 38.7, 38.7 (CH2βCys), 35.8, 35.8 ($CH_2\beta_{Tyr}$), 35.2, 35.2 (SCH_2CH_2), 26.2, 26.2 ppm (SCH_2); MS (ESI−) calcd for $C_{42}H_{59}N_6O_{16}S_4^-$ $[M-H]^-$: 1063.3. Found: 1063.0.

4.2.18. Tetravalent disulfide $G1_2S_2$

After dimerization of the thiol **G1SH** and purification by RP-HPLC, the disulfide **$G1_2S_2$** was obtained as a colourless amorphous solid (6 mg, 6%, overall): Preparative RP-HPLC: t_r = 28.8 min (A/B = 100:0→50:50, 50 min); Analytical RP-HPLC: t_r = 8.62 min (A/B = 100:0→50:50, 10 min); ^{1}H NMR (500 MHz, CD_3CN/D_2O (3:2) + 0.1% [D]-TFA): δ 7.74–7.66 (m, 8H, H-$3'_{Tyr}$, H-$5'_{Tyr}$), 7.43–7.36 (m, 8H, H-$2'_{Tyr}$, H-$6'_{Tyr}$), 5.88–5.85 (m, 4H, H-1_{Man}), 5.25–5.20 (m, 2H, CHαCys), 5.18–5.12, 5.12–5.05 (2m, 4H, $CH\alpha_{Tyr}$), 4.82–4.79 (m, 2H, $CH\alpha_{Lys}$), 4.56–4.32 (m, 16H, H-2_{Man}, H-5_{Man}, H-$6a_{Man}$, H-$6b_{Man}$), 4.29–4.23 (m, 4H, H-3_{Man}, H-4_{Man}), 3.89–3.82 (m, 2H, CH2βCys), 3.76–3.54 (m, 10H, CH2βCys, $CH_2\beta_{Tyr}$, $CH_2\varepsilon_{Lys}$), 3.52–3.32 (m, 12H, $CH_2\beta_{Tyr}$, SCH_2), 3.22–3,11 (m, 8H, SCH_2CH_2), 2.40–2.29, 2.28–2.15, 2.03–1.88, 1.84–1.68 ppm (4m, 12H, $CH_2\beta_{Lys}$, $CH_2\gamma_{Lys}$, $CH_2\delta_{Lys}$); ^{13}C NMR [125 MHz, CD_3CN/D_2O, 3:2 (v:v) + 0.1% TFA-*d*]: δ 173.5, 172.9 [C(=O)NH_2, C(=O)NH], 154.8, 154.8 (C-$1'_{Tyr}$), 130.2 (C-$3'_{Tyr}$, C-$5'_{Tyr}$), 127.9, 127.6 (C-$4'_{Tyr}$), 115.1, 115.1 (C-$2'_{Tyr}$, C-$6'_{Tyr}$), 84.8, 84.5 (C-1_{Man}), 73.0 (C-5_{Man}), 71.6 (C-2_{Man}), 71.1 (C-3_{Man}), 66.9 (C-4_{Man}), 60.7 (C-6_{Man}), 55.1 ($CH\alpha_{Tyr}$), 53.7 ($CH\alpha_{Lys}$), 52.0 ($CH\alpha_{Cys}$), 38.6, 38.6 (CH2βCys, $CH_2\varepsilon_{Lys}$), 36.5 ($CH_2\beta_{Tyr}$), 35.4, 35.3 (SCH_2CH_2), 30.3, 30.3 ($CH_2\beta_{Lys}$), 27.8, 27.8 ($CH_2\delta_{Lys}$), 26.5, 26.3 (SCH_2), 22.3, 22.2 ppm ($CH_2\gamma_{Lys}$); MS (ESI−) calcd for $C_{90}H_{128}N_{12}O_{36}S_6^{2-}$ $[M-2H]^{2-}$: 1072.3. Found: 1073.0.

4.2.19. Octavalent disulfide $G2_2S_2$

The synthesis of **$G2_2S_2$** could then achieved from the mercaptan **G2SH** and the target disulfide was obtained, after RP-HPLC purification, as a colourless foam (9 mg, 4%, overall): Preparative RP-HPLC: t_r = 22.6 min (A/B = 100:0→40:60, 60 min); Analytical RP-HPLC: t_r = 8.39 min (A/B = 100:0→40:60, 10 min); ^{1}H NMR (500 MHz, CD_3CN/D_2O (3:2) + 0.1% [D]-TFA): δ 7.73–7.70 (m, 16H, H-$3'_{Tyr}$, H-$5'_{Tyr}$), 7.42–7.39 (m, 16H, H-$2'_{Tyr}$, H-$6'_{Tyr}$), 5.89–5.87 (br s, 8H, H-1_{Man}), 5.36–5.33, 5.29–5.26 (2m, 2H, $CH\alpha_{Cys}$), 5.20–5.16, 5.11–5.07 (m, 8H, $CH\alpha_{Tyr}$), 4.90–4.70 (m, 6H, $CH\alpha_{Lys}$), 4.56–4.54 (m, 8H, H-2_{Man}), 4.52–4.41 (m, 16H, H-5_{Man}, H-$6a_{Man}$), 4.37 [dd, ^2J(H-$6a_{Man}$, H-$6b_{Man}$) = 12.4 Hz, ^3J(H-5_{Man}, H-$6b_{Man}$) = 5.9 Hz, 8H, H-$6b_{Man}$], 4.29–4.26 (m, 16H, H-3_{Man}, H-4_{Man}), 3.89–3.84 (m, 2H, $CH_2\beta_{Cys}$), 3.77–3.58 (m, 22H, $CH_2\beta_{Cys}$, $CH_2\beta_{Tyr}$, $CH_2\varepsilon_{Lys}$), 3.51–3.35 (m, 24H, $CH_2\beta_{Tyr}$, SCH_2), 3.19–3.15 (m, 24H, SCH_2CH_2), 2.46–2.39, 2.39–2.26, 2.26–2.13, 2.13–2.00, 2.00–1.90, 1.82–1.68 ppm (6m, 36H, $CH_2\beta_{Lys}$, $CH_2\gamma_{Lys}$, $CH_2\delta_{Lys}$); ^{13}C NMR (125 MHz, CD_3CN/D_2O (3:2) + 0.1% [D]-TFA): δ 173.4, 172.6, 172.0 [C(=O)NH_2, C(=O)NH], 154.9 (C-$1'_{Tyr}$), 130.5 (C-$3'_{Tyr}$, C-$5'_{Tyr}$), 127.4 (C-$4'_{Tyr}$), 116.1 (C-$2'_{Tyr}$, C-$6'_{Tyr}$), 85.8 (C-1_{Man}), 74.0 (C-5_{Man}), 72.6 (C-2_{Man}), 72.2 (C-3_{Man}), 67.8 (C-4_{Man}), 61.8 (C-6_{Man}), 55.9 ($CH\alpha_{Tyr}$), 54.3 ($CH\alpha_{Lys}$), 53.0 ($CH\alpha_{Cys}$), 40.1 ($CH2\beta_{Cys}$), 39.7 ($CH_2\varepsilon_{Lys}$), 37.6 ($CH_2\beta_{Tyr}$), 36.4 (SCH_2CH_2), 31.5 ($CH_2\beta_{Lys}$), 29.6 ($CH_2\delta_{Lys}$), 27.3 (SCH_2), 23.4 ppm ($CH_2\gamma_{Lys}$); MS (ESI−) calcd for $C_{186}H_{266}N_{24}O_{72}S_{10}^{4-}$ $[M-4H]^{4-}$: 1078.0. Found: 1076.9.

4.3. Enzyme-linked lectin assays (ELLA)

4.3.1. Preparation of buffers used for the inhibition measurements

PBS (phosphate saline buffer, 10 mM, pH 7.3): To a solution of NaCl (8.77 g, 150 mmol) in deionized water (500 mL) were added successively NaH_2PO_4, H_2O (0.26 g, 1.9 mmol) and Na_2HPO_4, $7H_2O$ (2.17 g, 8.1 mmol). If necessary, the pH could be adjusted to 7.3 by adding few drops of 1 M aqueous solutions of NaOH or HCl. The volume was then completed to 1 L with deionized water. PBST (phosphate saline-Tween 20 buffer, 6.4 mM, pH 7.2): NaCl (9.00 g, 154 mmol) was dissolved in deionized water (500 mL). NaH_2PO_4, H_2O (0.32 g, 2.3 mmol), Na_2HPO_4, $7H_2O$ (1.09 g, 4.1 mmol) and Tween 20 (500 μL) were then added. The pH value could also be corrected as mentioned above if necessary. Finally, the volume was adjusted to 1 L by addition of deionized water. Tris [tris(hydroxymethyl)aminomethane, 50 mM, pH 7.6]: Tris base (6.05 g, 50 mmol) was dissolved in deionized water (800 mL). The pH was then adjusted to 7.6 by adding successively concentrated and molar aqueous solution of HCl. The volume was finally completed to 1 L with deionized water. Citrate–phosphate buffer (200 mM, pH 4.0): Citric acid (7.70 g, 40 mmol) was dissolved in PBS (100 mL). The pH was then adjusted to 4.0 by adding the minimal amount of aqueous solutions of NaOH (5.0 M and 1.0 M solutions were successively used). Finally, the volume was completed to 200 mL with PBS.

4.3.2. Enhanced-sensitivity ELLA

A solution of yeast mannan (10 μg mL^{-1}) in PBS was prepared by diluting ten times a stock solution. $CaCl_2$ and $MnCl_2$ were then added to reach the final concentrations of 1 μmol mL^{-1} for each salt. Maxisorp microtitration plates were coated overnight with this diluted yeast mannan solution and the plates were washed three times with PBST (250 μL/well). The wells were then blocked for 1 h at 37 °C with PBSA [PBS containing 10 mg mL^{-1} of BSA (bovine serum albumin), 100 μL/well] and were washed as described above. Separately, serial two-fold dilutions of inhibitors (60 μL/well) were performed in nontreated mixing plates. Stock solutions of monosaccharides (1–2 M) and of glycopeptide derivatives (1–10 mM) in PBS were then used. These wells were completed with a solution of biotin-conjugated Con A (60 μL/well of a 5 μg mL^{-1} solution in PBS) and the plates were incubated 1 h at 37 °C. These solutions were transferred into the yeast mannan coated plates, incubated 1 h at 37 °C and washed as mentioned previously. The complex Neutravidin™-biotinylated HRP was then preformed separately: to Tris buffer (9.6 mL) were successively added a solution (1.2 mL) of Neutravidin™ (100 μg mL^{-1} in Tris) and a solution (1.2 mL) of biotin-conjugated HRP (25 μg mL^{-1} in Tris). The mixture was shaken for 30 min at rt and immediately transferred into the yeast mannan-biotinylated Con A coated plates (60 μL/well). After 1 h at 37 °C, these plates were washed with Tris (2 × 250 μL/well) and an ABTS solution (0.25 mg mL^{-1} in citric–phosphate buffer containing 0.015% of a 30% aqueous solution of H_2O_2) was added (50 μL/well). After 5 min at rt, the absorbances (Abs) were measured at 415 nm and the inhibition percentages were calculated as follows: % Inhibition = $100 \times (Abs_{no\ inhibitor} - Abs_{with\ inhibitor})/Abs_{no\ inhibitor}$. The concentration of the inhibitor leading to 50% of the maximal activity was reported as IC_{50}. Each test was done at least as a triplicate and the relative potency of all glycoconjugates was calculated from the IC_{50} of methyl α-D-mannoside (Me α-Man) measured in the same conditions and on the same plate. The inhibitory properties of each neoglycoconjuagte were virtually made independent of the experiment series by correcting the IC_{50} values obtained for the reference to a common experimental value (the value of 1320 μM was chosen for the IC_{50} associated to methyl α-D-mannoside among all the obtained measurements since this experimental data was the closest to the average value). The relative potencies (r.p. and r.p./n) were then calculated as follows: r.p. = $IC_{50\text{-Me α-Man}}/IC_{50\text{-inhibitor}}$ and r.p./n = $IC_{50\text{-Me α-Man}}/(IC_{50\text{-inhibitor}} \times n)$. The corresponding errors were then Δr.p. = $(IC_{50\text{-Me α-Man}}/IC_{50\text{-inhibitor}}) \times (\Delta IC_{50\text{-Me α-Man}}/IC_{50\text{-Me α-Man}} + \Delta IC_{50\text{-Inhibitor}}/IC_{50\text{-Inhibitor}})$ and Δ(r.p./n) = $(IC_{50\text{-Me α-Man}}/IC_{50\text{-inhibitor}}) \times (\Delta IC_{50\text{-Me α-Man}}/IC_{50\text{-Me α-Man}} + \Delta IC_{50\text{-Inhibitor}}/IC_{50\text{-Inhibitor}})/n$. If the glycopeptide dendrimers were not completely soluble in pure PBS, DMSO (from 10% to 30%) could be added without significant modification of the IC_{50} values (data not shown).

4.3.3. Standard ELLA

Maxisorp microtitration plates were treated as described above. Serial two-fold dilutions of the inhibitors were performed as previously mentioned and completed with a solution of HRP-conjugated Con A in PBS (60 μL/well of a 5 μg mL^{-1} solution). After preincubation for 1 h at 37 °C, the mixtures were transferred into Maxisorp microtitration plates and incubated. These plates were washed with three times with PBST (250 μL/well) and the ABTS solution was added (50 μL/well). After 20 min at rt, a molar solution of sulphuric acid was added (50 μL/well) and the absorbances were measured at 415 nm. The inhibition percentages, relative potencies and uncertainties were then calculated as described previously.

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Supplementary data

Supplementary data (characterization data, including ELLAs, NMR, MS spectra and HPLC profiles are provided for **G2a–c**, **G2e–g**, **G02S2**, **G12S2** and **G22S2**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.03.047.

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