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Scaffold Optimisation of Tetravalent Antagonists of the Mannose Binding Lectin

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Abstract: Antagonists of mannose binding lectin (MBL) have shown a protective role against brain reperfusion damage after acute ischemic stroke. Here we describe the design and streamlined synthesis of glycomimetic MBL antagonists based on a new tetravalent dendron scaffold. The dendron was developed by optimisation of a known polyester structure previously demonstrated to be very efficient for ligand presentation to MBL. Replacement of a labile succinyl ester bond with a more robust amide functionality, use of a longer and more hydrophilic linker, fast modular synthesis and orthogonal functionalisation at the focal point are the main features of the new scaffold. The glycoconjugate constructs become stable to silica gel chromatography and to water solutions at physiological pH, while preserving water solubility and activity in an SPR assay against the murine MBL-C isoform. Higher-order constructs were easily assembled, as demonstrated by the synthesis of a 16-valent dendrimer, which leads to two orders of magnitude increase in activity over the tetravalent version against MBL-C.

Complex glycans on cell surfaces often function as recognition signals for specific sugar-binding proteins known as lectins. The C-type lectins are the largest lectin family found in animals and are known to be involved in numerous physiological and pathological processes, mostly in the immune system. Its members are characterised by a carbohydrate recognition domain (CRD) that requires a calcium ion to properly bind to sugars. As most other lectins, C-lectins are oligomeric structures that compensate low affinity of individual CRDs with avidity enhancements sustained by multiple interactions with polyglycosylated targets. Many efforts have been devoted to antagonising these interactions using multivalent glycosylated constructs, varying from nanoparticles, to polymers, dendrimers and other multivalent platforms.[1]

The serum mannose binding lectin (MBL) is a C-type lectin that recognizes sugars in a Ca^{2+}-dependent manner. Binding of serum MBL to specific carbohydrate patterns[2] on the surface of pathogens is an essential part of the innate immune response.[3] MBL may also target "altered self" or damage-associated molecular patterns, such as dying or damaged cells[4] and has been shown to have a crucial role in the pathophysiology of brain ischemia/reperfusion injury.[5]

Over the past few years we have reported on two tetravalent pseudo-mannosylated dendrons Polyman1 (**1**) and Polyman2 (**2**; Figure 1) that bind with good affinity to serum MBL[6] as well as to a second immune system C-lectin, the dendritic cell receptor DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin).[7] The two dendrons are characterised by a common tetravalent polyester scaffold built from 2,2-bishydroxymethyl-propionic acid units **3** and bear four copies of either a pseudo-dimannoside (in **1**) or a pseudo-trimannoside (in **2**) ligand (Figure 1).

Both pseudo-saccharides were designed and synthesised to mimic the terminal moieties of the natural mannose glycan $(Man)_9(GlcNAc)_2$, also known as Man_9, that is a major binding determinant of both lectins. In particular, **2** has found application as antagonist of DC-SIGN-mediated viral infections[7,8] and in the protection from brain reperfusion damage triggered by MBL after acute ischemic stroke.[6]

Despite these promising results, **1** and **2** suffer from chemical instability that arise from the unhindered succinyl ester bond (highlighted in red, Figure 1), which is labile to nucleophiles both under basic and mild acidic catalysis. This instability prevents chromatographic analysis and purification on silica (direct and reverse phase), hinders the scale up of the synthetic process and hampers a full pharmacokinetic characterisation of the molecules, all fundamental requirements for a potential drug. Additionally, 30% hydrolytic degradation of **2** was measured (by NMR spectroscopy) after 6 h in water solution at physiological pH (7.4, PBS buffer),[7] which is a clear drawback for many in vivo applications. Not surprisingly, MS analysis of hydrolysis mixtures revealed that the main decomposition occurred by hydrolysis of the succinate bond, while the other,

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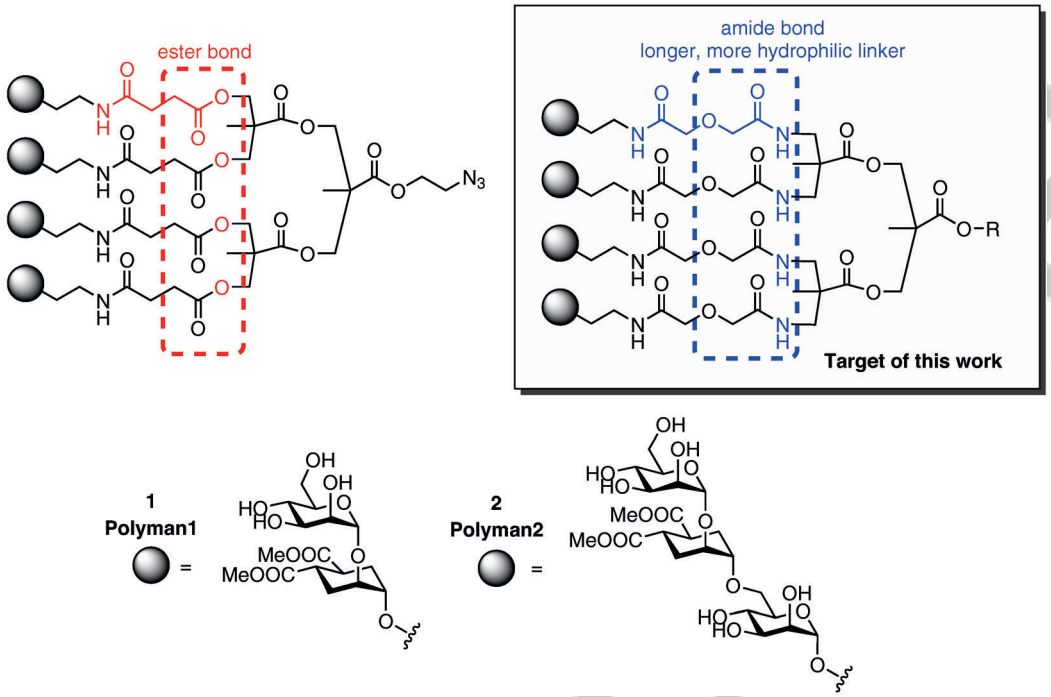


Figure 1. Tetravalent polyester dendrons Polyman1 (**1**) and Polyman2 (**2**) bearing, respectively, four copies of a pseudo-disaccharide and a pseudo-trisaccharide. The labile succinyl ester bond is highlighted in the red box. The target of this work replaces the succinyl ester with an amide bond (blue box).

more hindered esters remained untouched. For all these reasons, an optimisation of the dendron structure by replacement of the succinyl ester bond was sought after.

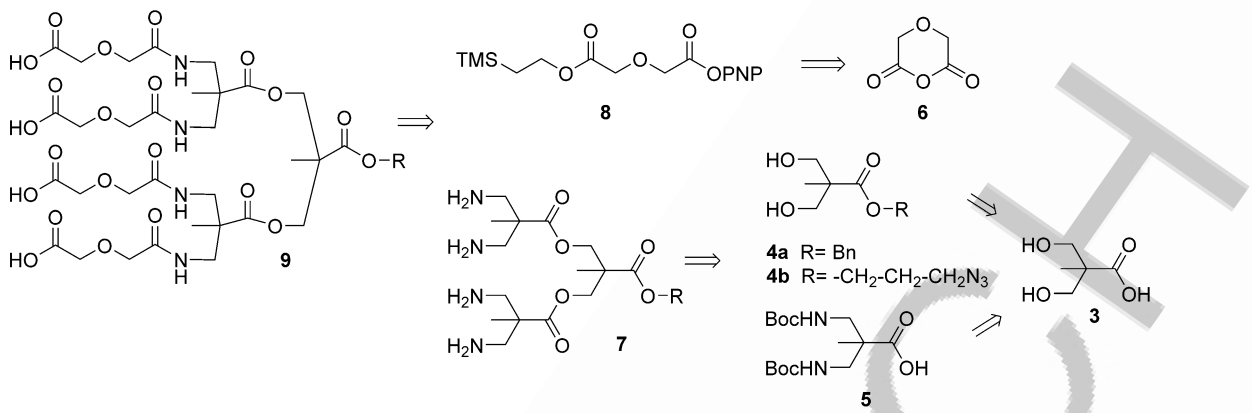
Here we report on the synthesis of new tetravalent analogues of **1** and **2**, which have been stabilised against hydrolysis by appropriate modifications of the original scaffold. We show that the new dendrons remain water soluble while displaying improved stability and comparable activity as MBL antagonists. Focal point functionalisation also allowed the easy synthesis of higher order constructs with MBL affinity improved by 2-orders of magnitude (valency corrected).

In order to achieve a significant stabilisation of **1** and **2** while introducing minimal changes to the original structure, we sought to replace the labile succinyl ester linker at the dendron periphery with a more robust amide moiety (Figure 1). This can be accomplished by connecting a 2,2-bis(aminomethyl)propionic acid building block (**5**) to the 2,2-bis(hydroxymethyl)propionic ester focal unit **4** to obtain tetraamine **7** (Scheme 1). The synthesis of the stabilised dendron **9** can thus be completed by reaction with a capping unit (**8**) derived from diglycolic anhydride (**6**, Scheme 1). This 5-atom linker was preferred over succinate to minimize intramolecular cyclisation of the amide group over the activated carboxylic acid during the final functionalisation of **9**. This side-reaction is well-known to occur for aspartic acid derivatives that can form 5-member ring imides upon activation.[9] Moreover, the linker derived from **6** contains an ether bond that is expected to bestow a more hydrophilic character to the final dendron. The ester group at the dendron focal point can also be used to arm the molecule with a terminal azide moiety, as in **4b**, in order to include an anchoring point for further functionalisation and to enable the synthesis of higher valency compounds.

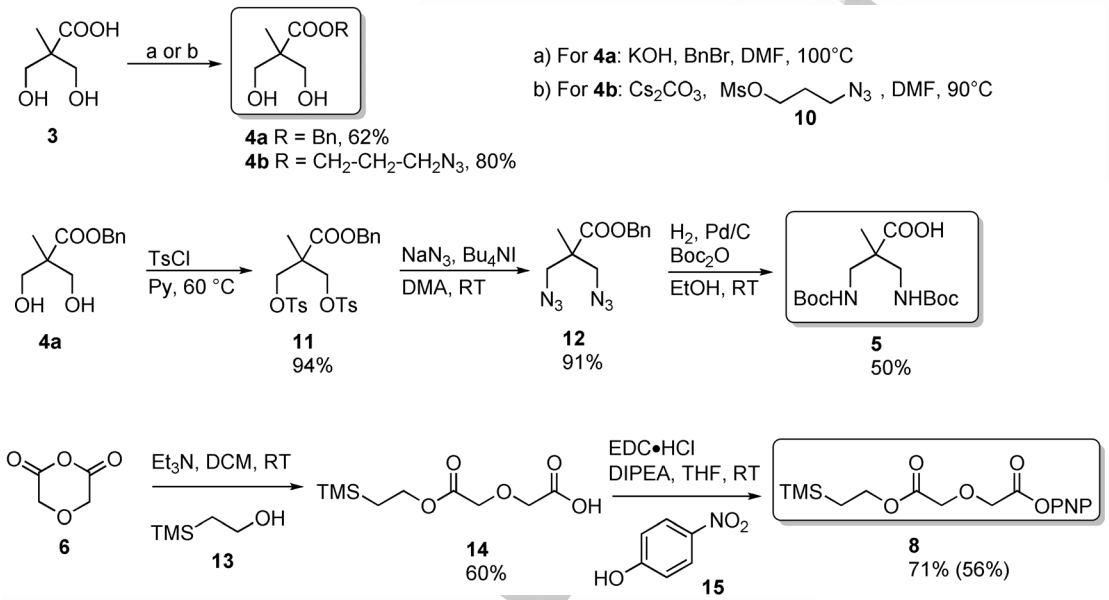
The synthesis we have designed strives to minimize protection/deprotection steps[8] and involves the three building blocks **4**, **5** and **8**. The focal unit **4** is obtained from 2,2-bis(hydroxymethyl)propionic acid **3**; the benzyl ester **4a** (R = Bn) is also an intermediate in the synthesis of **5**. The synthesis of the building blocks is depicted in Scheme 2. The 2,2-bis(hydroxymethyl)propionic acid **3** was transformed in the corresponding benzyl ester **4a** using KOH and benzyl bromide (62% yield). Similarly, **4b** was obtained in 80% yield with Cs_2CO_3 and the mesylate of 3-azidopropanol **10**.[10] For the synthesis of **5**, **4a** was treated with TsCl and pyridine to get **11** (94%), which was subjected to a double nucleophilic substitution with sodium azide affording intermediate **12** in 85% yields over the two steps.

The Boc-protected amino acid **5** was finally obtained through a one-pot catalytic hydrogenation of **12** in the presence of di-*tert*-butyl dicarbonate. This reaction sequentially affords reduction of the azido groups to amines, protection of the amines as Boc carbamates and removal of the benzyl ester in 50% overall yield, after chromatographic purification.

Finally, the last building block **8** was synthesised by reaction of diglycolic anhydride **6** with trimethylsilylethanol **13**, followed by EDC-mediated esterification of **14** with *p*-nitrophenol **15**. The activated ester **8** can be used in the next step as a crude (71% yield, determined by ^{1}H NMR spectroscopy), although flash chromatography purification was performed for characterisation, affording pure **8** as a waxy white solid (56% yield).



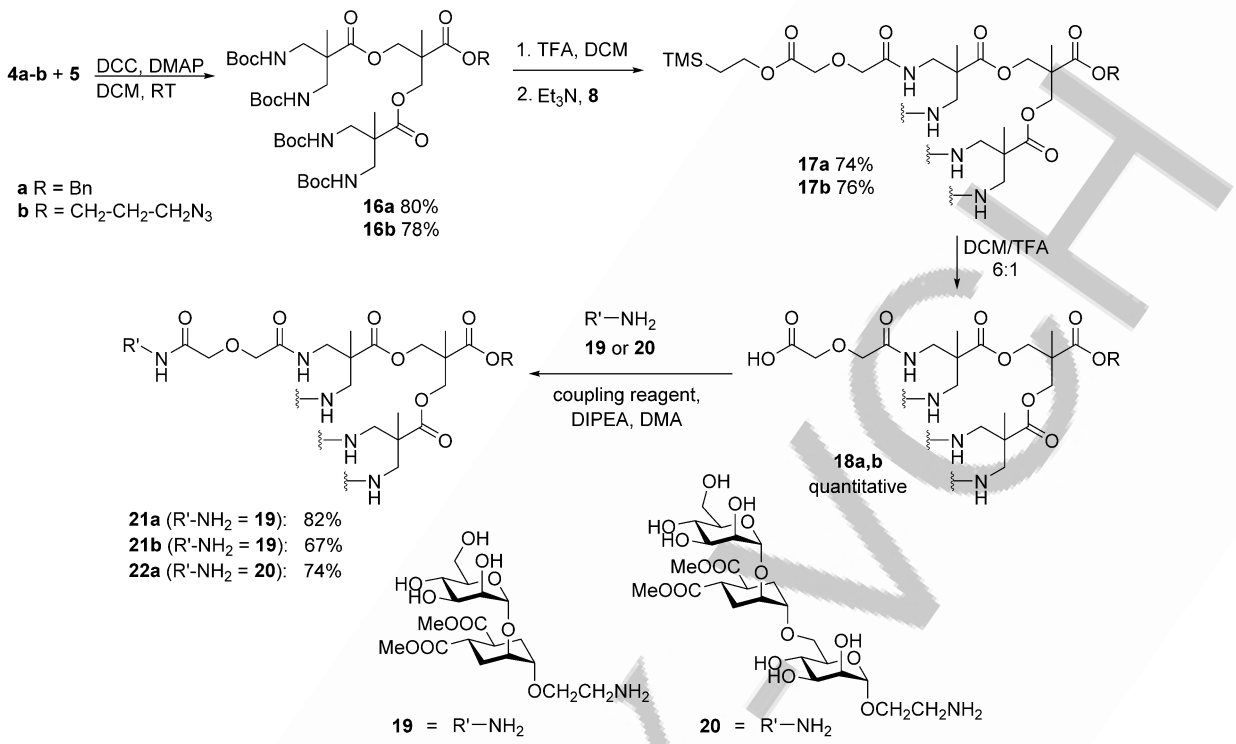
Scheme 1. Retrosynthetic analysis of the stabilised tetravalent scaffold **9**.



Scheme 2. Synthesis of the three building blocks **4a**,**b**, **5** and **8**.

The initial studies for the synthesis of the tetravalent constructs (Scheme 3) were performed using benzyl ester **4a** as the focal point unit. The tetravalent construct **16a** was obtained in 80% yield through a double condensation between **4a** and two equivalents of **5** using DCC (dicyclohexylcarbodiimide) as the coupling agent (Scheme 3). Deprotection of **16a** (TFA, CH_2Cl_2) afforded tetraamine **7** that was not isolated but treated with an excess of the activated ester **8**, leading to the protected tetravalent dendron **17a** in 74% yield. Direct reaction of **7** with diglycolic anhydride **6** was also attempted, but abandoned for the difficulty of isolating and purifying the resulting tetra-acid **18a** from the reaction mixture. The corresponding trimethylsilylethyl ester **17a**, on the contrary, is a very convenient precursor, which can be stored without degradation for months and treated with TFA to deliver **18a** when needed. An analytical sample of **18a** was purified by reverse phase chromatography (Biotage SNAP C18, gradient elution: from 100% H_2O to 100% MeOH) but the acid was generally used as a crude in the next synthetic step. Finally, the glycodendrons **21a** and **22a** were synthesised in good yields (82 and 74%, respectively) by condensation of **18a** and either the pseudo-disaccharide **19**[11] (HATU, DMA, 30 °C, 12 h) or the pseudo-trisaccharide **20**[7] (HATU/HOAT, DMA, 37 °C, 12 h). Notably, under these mild conditions, no nucleophilic attack of the amide group over the activated carboxylic acid was observed by MALDI analysis (HCCA, MeOH) of the crude reaction mixtures. On the contrary, imide side-products were clearly detected when the reaction was performed at 70 °C. A similar sequence starting from the azide-armed ester **4b** afforded the tetravalent ester **17b** (Scheme 3), from which the tetravalent glycodendron **21b** was obtained in good yields (HATU, DMA, 35 °C, 12 h, 67%).

The resulting glycodendrons **21a-b** and **22a** were isolated from their crude reaction mixtures by size-exclusion chromatography (Sephadex LH-20) and, as opposed to **1** and **2**, were successfully purified by a further direct-phase flash chromatog-

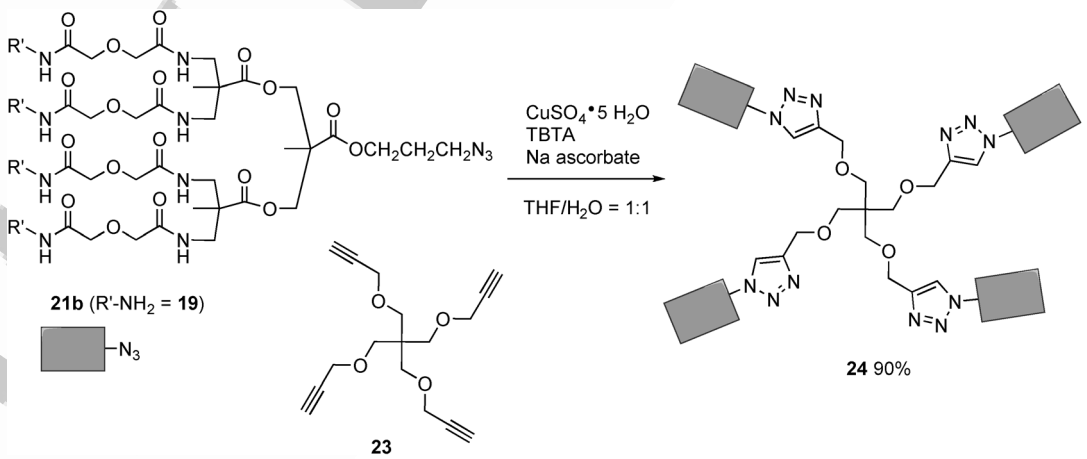


Scheme 3. Synthesis of dendrons **21a,b** and **22a**.

raphy on silica gel. Additionally, **21a** and **21b** were shown to be stable to reverse phase HPLC purification conditions (H_2O/CH_3CN in the presence of 0.1% of formic acid), and no hydrolysis of **22a** was detected (^{1}H NMR spectroscopy) in water solution at physiological pH (PBS buffer, pH 7.4) when monitoring for up to four days. All glycodendrons were fully characterised by NMR, ESI and MALDI (HCCA) mass spectroscopy.

Encouraged by the increased stability of the novel teatravalent constructs, we employed the azide-armed dendron **21b** to generate a higher valency construct by Cu catalysed cycloaddition reaction with the tetravalent propargyl ether pentaeritritol-derived core **23**[12] (Scheme 4). This reaction proceeded successfully affording in high yield (90%) the hexadecavalent glycodendrimer **24**, which could be isolated by size-exclusion chromatography (Sephadex LH-20 column). An analytical sample was further purified by reverse phase HPLC and fully characterised by NMR and ESI mass spectroscopy.

In order to compare the activity of the new constructs with that of Polyman2 (**2**), their inhibition potency against MBL was assayed. MBL is found in mammalian sera as oligomers of trimers[2] and is a paradigm of multivalent interactions. The lectin normally has a protective role, as a complement activator in the innate immune system and a scavenger of pathogens and damaged cells. However, this role can be reversed in some



Scheme 4. Synthesis of the 16-valent dendrimer **24**.

acute inflammatory conditions, for example upon reperfusion after an ischemic event. Animal model studies have indicated that the MBL-mediated pathway increases the reperfusion injury, possibly by recognising and attacking the damaged tissue.[4] Indeed, MBL antagonists have been shown to reduce the lesion extent.[4–6]

The inhibition potency of the new dendrons towards mouse MBL-C (mMBL-C) was assessed by a recently established assay[13] performed by surface plasmon resonance (SPR) directly on murine plasma, which contains the two murine MBL isoforms A and C. In this assay the dendrons were incubated for 30 min at 25 °C at different concentrations in diluted mouse plasma and the solutions were flowed onto a SPR sensor chip on which mannosylated bovine serum albumin (Man-BSA) had been previously immobilised. The amount of mMBL-C bound to immobilised mannose residues was revealed flowing anti-MBL-C antibody, the binding of which resulted in a clear SPR signal (Supporting Information Figure S1). The preincubation of plasma with the dendrons resulted in a concentration-dependent decrease of the binding of the anti-MBL-C antibody (Supporting Information Figure S1 and Figure 2■■*ok?*), that is, a decrease of binding of mMBL-C to mannose residues. From these inhibition curves IC_{50} values were determined (Table 1). The data clearly show that the inhibitory activities of the novel tetravalent constructs **21 a** and **22 a** are comparable to that of the original lead **2** (Table 1). Almost two orders of magnitude were gained with dendrimer **24**, which corresponds to a relative inhibitory potency (rIP) of 19, relative to **21 a**. The activity of mannose was not measured in this test, but, for comparison, the dissociation constant of the MBL–mannose complex was estimated by various techniques to fall in the millimolar range.[14]

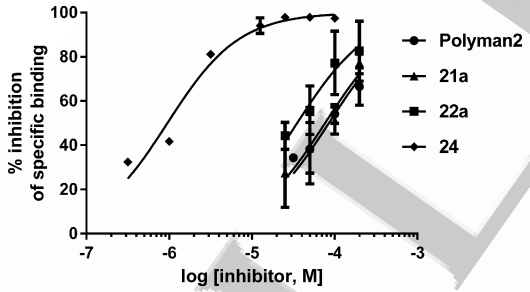


Figure 2. Inhibition of mMBL-C binding to Man-BSA immobilised on a SPR sensor. Compounds were preincubated at different concentrations with diluted murine plasma (containing the mMBL-C isoform). These solutions were then injected over a sensor chip where Man-BSA had been immobilised. The amount of mMBL-C bound on the chip was revealed by flowing anti-MBL-C antibodies. Dendrons inhibited in a concentration-dependent manner the binding of mMBL-C to mannose residues. Each curve was obtained in 2 or 3 independent experiments; the points represent the mean ± SD (SD shown for $n = 3$).

In conclusion, we have succeeded in stabilising the structure of tetravalent glycodendron antagonists of MBL, while preserving their biological activity against the target lectin. The novel dendron design replaces a labile succinyl ester bond with a more robust amide functionality, incorporating a longer and more hydrophilic linker. As a result, the construct becomes stable to silica gel chromatography and to water solutions at physiological pH. The remaining ester linkages, bearing a quaternary carbon in the α position, are clearly stabilised against hydrolytic cleavage under physiological conditions. They may also be partially stabilised against esterases,[15] a proposition that we are now in the position to test experimentally. The streamlined synthesis of the dendron and the possibility of orthogonal functionalisation at the focal point are very attractive features of this system, as demonstrated by the easy synthesis of the 16-valent construct **24**. This dendrimer afforded activity improvements against MBL-C by two-orders of magnitude relative to tetravalent **21**. It is tempting to speculate that this strong effect depends on the ability of **24** to bridge two binding sites in the MBL trimer, that are separated by 5 nm.[16] Perhaps more importantly, this feature should allow the engineering of higher valency constructs with controlled ligand density and spacing, which are important structural elements for pattern-recognising receptors, such as MBL.[14] Finally, the stabilised dendron scaffold **18**, which is easily accessible in a few steps of a highly modular synthesis, can find numerous additional applications as multivalent support of biologically active payloads.

Table 1. Inhibition of mMBL-C binding to Man-BSA immobilised on a SPR sensor (IC_{50} values).[a]

Compounds	**2**	**21 a**	**22 a**	**24**
IC_{50} [μM][b]	84 (61–118)	77 (57–102)	35 (25–50)	1.0 (0.7–1.3)

[a] Inhibitory effects were evaluated on the murine isoform mMBL-C in mouse plasma. Values were obtained by fitting of the inhibition curves shown in Figure 2, using the "one-site competition" equation. [b] 95% confidence.

Acknowledgements

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Keywords: glycodendrimers · glycomimetics · mannose binding lectin · multivalency · surface plasmon resonance

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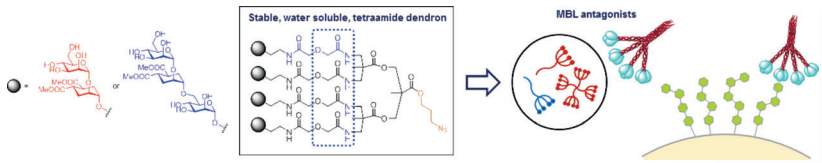

Novel dendrons for multipresentation: Water soluble, tetravalent glycodendrons that perform as powerful mannose binding lectin (MBL) antagonists are described. The stable scaffold adopted (see figure) is a versatile tool for nested-layer multipresentation which enabled the preparation of higher valency antagonists ■■ok■■. All the constructs were tested as MBL-C antagonist by an SPR assay and showed binding with IC_{50} values in the low micromolar range.

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