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Divergent and convergent synthesis of GalNAc-conjugated dendrimers using dual orthogonal ligations

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Abstract

The synthesis of glycodendrimers remains a challenging task. In this paper we propose a protocol based on both oxime ligation (OL) to combine cyclopeptide repeating units as the dendritic core and the copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) to conjugate peripheral α and β propargylated GalNAc. By contrast with the oxime-based iterative protocol reported in our group, our current strategy can be used in both divergent and convergent routes with similar efficiency and the resulting hexadecavalent glycodendrimers can be easily characterized compared to oxime-linked analogues. A series of glyconjugates displaying four or sixteen copies of both α and β GalNAc have been prepared and their ability to inhibit the adhesion of the Soybean agglutinin (SBA) lectin to polymeric-GalNAc immobilized onto microtiter plates have been evaluated. As it was anticipated, the higher inhibitory effect (IC$_{50}$ = 0.46 µM) was measured with the structure displaying αGalNAc with the higher valency (compound 13), which demonstrates that the binding properties of these glycoconjugates are strongly dependent on the orientation and the distribution of the GalNAc units.

Graphical abstract
Introduction

The chemistry of multivalent glycosystems has made impressive progress over the past decade. Various structures such as glycodendrimers, glycocalixarenes, or glycocyclodextrines are now commonly used for biomedical applications and represent relevant tools for studying carbohydrate-protein interactions. In this broad research field, our group has developed glyoclusters and glycodendrimers which have been assembled at the surface of conformationally stable cyclopeptide scaffolds using either identical or different sugar head groups - with valency of four, sixteen and sixty - and with other biomolecular entities in a well-defined spatial orientation. Some of these structures have revealed sub-nanomolar affinities towards vegetal and bacterial lectins as well as potent immunoactivation effect against cancer cells in murine models. However, the construction of glycodendrimers remains a difficult and challenging task. To this aim, we have developed a divergent strategy that allows the controlled assembly of cyclopeptide and carbohydrate building blocks in a repetitive fashion and with excellent yields. In this approach, properly functionalized cyclopeptides (i.e. with aminooxy and oxo-aldehyde functions) can be self-condensed using an iterative oxime ligation (OL) protocol to provide dendritic framework to be functionalized with aminooxy carbohydrates (Fig. 1A). While stable in vivo, the main drawback of the resulting compounds is the fragility of the peripheral oxime-linked carbohydrates during analysis by mass spectrometry which strongly hampers the complete structural characterization. In addition, the utilization of the alternative convergent approach which is more reliable to construct glycodendrimers with lower risk of formation of partially glycosylated intermediates was found unsuccessful in our hands due to side reactions (Fig. 1B). Herein, we report first a versatile synthetic strategy that can be used either in a divergent (Fig. 1C) or a convergent (Fig. 1D) protocol to build hexadecavalent glycocyclopeptide dendrimers. To demonstrate the feasibility of the proposed synthetic route, we have selected two orthogonal ligation methods that are the copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) strategy to attached sugar moieties while the
dendritic core was built using OL, similarly to the “onion peel” strategy previously described by the group of R. Roy.\textsuperscript{23}

Fig. 1 General strategy for the divergent (A) and the convergent (B) assembly by OL; for the divergent (C) and the convergent (D) assembly by CuAAC/OL, similarly to the “onion peel” method. Trz: triazole; Ox: oxime ether.

Results and Discussion
We focused the present study on N-acetylgalactosamine (GalNAc) which represents key building block for the synthesis of antitumoral vaccines,\textsuperscript{24,25} uridine diphosphate (UDP) mimics\textsuperscript{26} or antifreeze glycoproteins (AFGP).\textsuperscript{27} In addition, when GalNAc is presented in a suitable multivalent fashion, the resulting structures have shown interesting properties towards specific carbohydrate-binding proteins.\textsuperscript{28-30} In the course of our activities in this field, we have designed here molecules displaying both $\alpha$ and $\beta$ GalNAc that have been synthesized using propargylated compounds 1a and 1b (Fig. 2). It was indeed largely demonstrated that besides valency, the orientation of the sugar unit within the scaffold is a structural parameter that strongly impacts the binding affinity for these proteins.\textsuperscript{1,16}

![Fig. 2 Structure of compounds 1a and 1b.]

**Synthesis of prop-2-ynl 2-acetamido-2-deoxy-$\alpha$-D-galactopyranoside**

A few groups recently proposed different synthetic strategies for the preparation of $O$-propargyl GalNAc 1a and 1b. In 2009, Fairbanks and co-workers\textsuperscript{26} described the preparation of the $\alpha$ anomer 1a by a Fisher-type glycosylation. The unprotected GalNAc was treated with sulphuric acid in presence of silica and equimolar amount of propargyl alcohol. The $\alpha/\beta$ mixture (3:2) was obtained in 54\% yield after recrystallization. In the same year, the Brimble group\textsuperscript{31} described another protocol to obtain the acetylated compound 1a, but later the authors confirmed that only the furanose form was synthesized.\textsuperscript{32} To avoid this problem, the 1,3,4,6-tetra-$O$-acetyl-2-azido-2-deoxy-D-galactose has been glycosylated with propargyl alcohol in the presence of BF$_3$OEt$_2$ as promoter. After the reduction/acetylation of the azido group using Zn and Ac$_2$O/pyridine, both $\alpha$ and $\beta$ anomers 1a and 1b have been separated by silica gel chromatography. In 2010, Sewald and co-workers\textsuperscript{27} also described the synthesis of 1a starting from the azidochloride precursor using a
Koenigs-Knorr reaction as the key step. The azido group was reduced and subsequently acetylated with AcSH/pyridine and the fully deprotected compound 1a was obtained using Zemplén conditions after separation of α and β anomers.

As a synthetic alternative, we have used here the fluoride donor 2 that was glycosylated with propargyl alcohol in CH₂Cl₂ using boron trifluoroethoxide (BF₃·OEt₂) as promoter (Scheme 1). An inseparable mixture of α and β anomers 3 was obtained in 90% yield (α/β, 6:4). The azido group was next converted into -NHAc group using triphenylphosphine (PPh₃) in presence of acetic anhydride.

![Scheme 1. Synthesis of compounds 1a and 1b.](image)

The separation of α and β anomers was performed by silica gel chromatography to obtain the alpha anomer 4a in 45% (36% for the β anomer 4b). Compound 4a has been further crystallized as single crystals in a mixture of dichloromethane and pentane. Both the coupling constant measured by ¹H NMR (J_H1-H2 = 4.0 Hz) and the RX diffraction analysis (Fig. 3) have confirmed the stereochemistry of the anomeric carbon of 4a, which is in good agreement with the literature data. Both anomers were finally deacetylated using the Zemplén condition to afford the corresponding prop-2-ynl 2-acetamido-2-deoxy-α/β-D-galactopyranosides (1a-b) in 23% and 12% over yield, respectively.
**Fig. 3** X-ray structure of compound 4a (Crystallization: CH₂Cl₂/pentane; Formula: C₁₇H₂₃N₁O₉; Unit Cell Parameters: a = 9.1230(18); b = 14.399(3); c = 15.260(3); P2₁2₁2₁). Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 1423098. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: +44(0)-1223-336033 or e-mail: deposit@ccdc.cam.ac.uk].

**Synthesis of hexadecavalent glycodendrimers**

We first followed a divergent route to prepare glycodendrimers following the onion peel method. To do this, we introduced an aminooxyacetyl linker using N-Boc-O-(carboxymethyl)hydroxylamine succinimide ester 6 in presence of DIPEA to the scaffold 5 (Scheme 2). Further removal of the Boc protecting group gave the appropriate template 8 in 91% yield calculated over two steps.
Scheme 2 Synthesis of compound 8 and glyoclusters 9-10.

This compound was next conjugated to the cyclopeptide-containing aldehyde 11 by oxime ligation in water containing trifluoroacetic acid (0.1% TFA in H$_2$O) at 37°C (Scheme 3). After 4 h, analytical HPLC indicated the quantitative conversion of 11 to 12. The crude mixture finally was purified by semi-preparative HPLC to remove the excess of 8 and provided scaffold 12 with an excellent yield (85%) and purity.

Scheme 3 Synthesis of compound 12.
The efficiency of the CuAAC reaction is closely dependent on the experimental conditions.\(^{37}\) In a previous study, we observed that tetravalent glyoclusters can be obtained in good yields using a catalytic amount of copper micropowder in a mixture of isopropanol and sodium acetate buffer.\(^{38}\) Because copper-mediated generation of oxygen species can lead to the formation of aggregates in these conditions, we have decided to follow another procedure reported recently.\(^{39}\) We have first tested this procedure for the preparation of more simple compounds, i.e. tetravalent glyoclusters 9-10 (Scheme 2). Compounds 1a and 1b have been reacted with 5 in the presence of CuSO\(_4\), THPTA (3(tris(3-hydroxypropyltriazolylmethyl)amine) and sodium ascorbate in a mixture of DMF and phosphate buffer. Complete reaction has been observed in 2 h for both compounds and the pure glyoclusters 9 and 10 have been isolated after purification by preparative HPLC in 84% and 83% respectively. Due to the efficiency of this protocol, we thus decided to follow the same procedure from 12 (Scheme 4).

Scheme 4 Divergent route to synthesize glycodendrimers 13-14.
Despite steric hindrance generated during the molecular assembly of these glycodendrimers, the HPLC profile of the crude mixture indicated once again the formation of a single compound in both cases and no trace of partially glycosylated structures (Figure 3). After HPLC purification, compounds 13 and 14 have been recovered in 70% and 69% yields, respectively.

![RP-HPLC profile of crude mixture (λ = 214 nm) for compound 13. Analysis was carried out at 1.0 mL/min using a linear A–B gradient (buffer A: 0.09% CF$_3$CO$_2$H in water; buffer B: 0.09% CF$_3$CO$_2$H in 90% acetonitrile) in 20 min.](image)

These compounds have been first characterized by NMR spectroscopy. $^1$H NMR has shown characteristic signal for the triazole protons at 8.04 - 7.75 ppm, the oxime protons at 7.77 - 7.74 ppm and the anomic protons at 4.96 - 4.97 ppm (α anomer) and 4.93 - 4.90 ppm (β anomer) with the expected integration values (i.e. 16, 4 and 16, respectively). By contrast with the previous synthetic approach based on OL, mass spectrometry has provided the expected spectra without requiring specific sample preparation. Moreover no peak corresponding to fragmentation during the analysis has been observed, thus confirming the monodispersity of the glycodendrimers.

We next evaluated whether the convergent approach can be used to prepare the same series of glycodendrimers. For this purpose, the compound 7 was first conjugated with the propargylated
compound 1a under the conditions described previously (Scheme 5). After semi-preparative HPLC, the aminooxy group was deprotected with TFA and the resulting tetravalent structure 15 was coupled to 11 by OL. As it was expected, the glycodendrimer 13 was finally obtained in 90% yield after purification, thus confirming that the convergent approach can be followed successfully by using both oxime and CuAAC conjugations.

Scheme 5 Synthesis of glycodendrimer 13.

**Biological evaluation**

Several groups have studied the recognition of GalNAc by specific lectins. For example, Bertozzi et al. developed microarrays in which glycans are presented on linear polymer backbones mimicking the spatial arrangements of native mucins. By modulating the molecular composition and surface density of these mucin mimetics, they have shown how parameters such as GalNAc valency and interligand spacing affect their recognition by several GalNAc-specific lectins. In addition, if other studies have demonstrated the influence of glycan density on lectin binding, the recognition mechanism is still not fully addressed. Dam and co-workers reported isothermal titration microcalorimetry (ITC) and hemagglutination inhibition measurements for the binding of the Soybean agglutinin (SBA) lectin to modified forms of porcine submaxillary mucin (PSM), which possesses GalNAc residues. This SBA lectin is a tetramer, in which the GalNAc binding domains are located at the apexes of quadrangle spaced by 5 and 7 nm. Its high affinity \(K_d = 0.2 \text{ nM}\) for a modified form of PSM \(\approx 2300\) GalNAc residues) indicates that increasing the numbers of GalNAc
epitopes leads to higher affinities. Further investigations suggested that a lectin “bind and jump” from carbohydrate to carbohydrate epitope along the mucin peptide backbone before complete dissociation from the mucin.\textsuperscript{41,42} In our study, we have selected this lectin to evaluate how parameters such as GalNAc valency and anomeric configuration of GalNAc can affect the recognition process. To do this, we have performed competitive enzyme-linked lectin assays (ELLA) using GalNAc-polymer coated to microtiter plates with GalNAc as the monovalent reference, tetravalent glyoclusters 9 and 10 and the hexadecavalent glycodendrimers 13 and 14 (Fig. 4). IC\textsubscript{50} values which correspond to the concentration of glycoconjugates required to prevent 50\% of the binding are reported in Table 1.

![Fig. 4 Inhibition curves for the binding of SBA-HRP to GalNAc-polymer by (A) GalNAc monomer (■), 9 (○) or 13 (△); (B) GalNAc monomer (■), 10 (○) or 14 (△).](image)

<p>| Table 1. Inhibition of the adhesion of SBA lectin to a GalNAc-coated plates determined by ELLA. |
|---------------------------------|------|------------------|-------|-------------------|</p>
<table>
<thead>
<tr>
<th>Compound</th>
<th>n\textsuperscript{[a]}</th>
<th>IC\textsubscript{50} (µM)\textsuperscript{[b]}</th>
<th>rp \textsuperscript{[c]}</th>
<th>rp/n\textsuperscript{[d]}</th>
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</thead>
</table>
The results showed that all glyoclusters and glycodendrimers, in both anomer series are able to prevent SBA lectin adhesion at micromolar concentrations. In the beta series, an improvement of the lectin binding was obtained when the valency increases, since tetravalent 10 and hexadecavalent 14 derivatives exhibited IC$_{50}$ of 27.3 µM and 8.6 µM, respectively, while an IC$_{50}$ of 154.8 µM was measured for GalNAc. Although, when reported to the number of sugars (rp/n), the value only reaches 1, which denotes that inhibition is only due to a simple concentration effect instead of multivalency. More interestingly, stronger inhibition effects were obtained in the alpha series. Indeed, if significant improvement was obtained with tetravalent glyocluster 9 (IC$_{50}$ = 4.9 µM, rp = 31), the higher inhibitory effect was measured with the hexadecavalent structure 13 since an IC$_{50}$ of 0.46 µM corresponding to a 330-fold improvement compared to the GalNAc monomer. When reported to the number of GalNAc, the inhibition enhancement achieved 20, suggesting that the glycodendrimer 13 display alpha GalNAc residues in more favourable orientation to ensure multivalent interaction with the SBA binding sites. While moderate effects have been observed, all these results demonstrate that the binding properties of these glycoconjugates are strongly dependent on structural parameters that are the orientation and the distribution of the GalNAc units.

Conclusions
We have reported an “onion peel” strategy based on OL to combine cyclopeptide repeating units as the dendritic core and CuAAC to conjugate peripheral α and β propargylated GalNAc. By contrast with our previous oxime-based protocol, this strategy offers the advantage to allow both divergent and convergent routes with similar efficiency. In addition, the characterization by mass spectrometry of the resulting glycodendrimers is significantly easier than for the oxime-linked analogues. The capacity of the resulting glycodendrimers 13 and 14 to prevent the binding of the SBA lectin to polymeric-GalNAc has been studied by ELLA. As it was anticipated, the structure displaying αGalNAc (13) inhibit the interaction with higher efficacy (IC₅₀ = 0.46 µM, 330-fold improvement compared to GalNAc), which suggests that the binding properties of these glycoconjugates are strongly dependent on the orientation and the distribution of the GalNAc units. Due to both facility and versatility, this strategy will be used in our laboratory to synthesize large glycodendrimers¹³ as inhibitors against bacterial lectins.

**Experimental section**

**General procedures**

All chemical reagents were purchased from Aldrich (Saint Quentin Fallavier, France) or Acros (Noisy-Le-Grand, France) and were used without further purification. All protected amino acids and Fmoc-Gly-Sasrin®resin was obtained from Advanced ChemTech Europe (Brussels, Belgium), BachemBiochimie SARL (Voisins-Les-Bretonneux, France) and France Biochem S.A. (Meudon, France). For peptides and glycopeptides, analytical RP-HPLC was performed on Waters system equipped with a Waters 600 controller and a Waters 2487 Dual Absorbance Detector. Analysis was carried out at 1.0 mL/min (EC 125/3 nucleosil 300-5 C₁₈) with UV monitoring at 214 nm and 250 nm using a linear A–B gradient (buffer A: 0.09% CF₃CO₂H in water; buffer B: 0.09% CF₃CO₂H in 90% acetonitrile). Preparative HPLC was performed on Gilson GX 281 equipped with a fraction collector or on Waters equipment consisting of a Waters 600 controller and a Waters 2487 Dual Absorbance Detector. Purifications were carried out at 22.0 mL/min (VP 250/21 nucleosil 100-7
C\textsubscript{18}) with UV monitoring at 214 nm and 250 nm using a linear A–B gradient. For carbohydrate, progress of reactions was monitored by thin layer chromatography using silica gel 60 F254 precoated plates (Merck). Spots were visualised by UV light and by charring with 10% H\textsubscript{2}SO\textsubscript{4} in EtOH for protected derivatives or 1% ninhydrine in EtOH for hydroxylamine derivatives. Silica gel 60 (0.063-0.2 mm or 70-230 mesh, Merck) was used for column chromatography. \textsuperscript{1}H and \textsuperscript{13}C NMR spectra were recorded on BrukerAvance 400 MHz or BrukerAvance III 500 MHz spectrometers and chemical shifts (\(\delta\)) were reported in parts per million (ppm). Spectra were referenced to the residual proton solvent peaks relative to the signal of CDCl\textsubscript{3} (\(\delta\) 7.27 and 77.23 ppm for \textsuperscript{1}H and \textsuperscript{13}C) and D\textsubscript{2}O (4.79 ppm for \textsuperscript{1}H), assignments were done by GCOSY and GHMQC experiments. The anomeric configuration was established from \(J_{1,2}\) coupling constant. Standard abbreviations s, d, t, dd, br s, m refer to singlet, doublet, triplet, doublet of doublet, broad singlet, multiplet. ESI mass spectra of peptides and glycopeptides were measured on an Esquire 3000 spectrometer from Bruker or on an Acquity UPLC/MS system from Waters equipped with a SQ Detector 2. MALDI-TOF were performed on a AutoFlex I Bruker after sample pre-treatment in an OligoR3 microcolumn (Applied Biosystems, USA) using 2,5-dihydroxybenzoic acid matrix. HRMS analyses were performed on a Waters Xevo\textsuperscript{®} G2-S QTof.

\textbf{3-O-(2'-Deoxy-2'-azido-3',4',6'-tri-O-acetyl-\(\alpha/\beta\)-D-galactopyranosyl)propyne 3}

To a stirred solution of glycosyl fluoride \(2^{33}\) (533 mg, 1.6 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (10 mL) at 0°C propargyl alcohol (369 \(\mu\)L, 6.4 mmol) and BF\textsubscript{3}.Et\textsubscript{2}O (471 \(\mu\)L, 3.2mmol) were slowly added. After 2h the reaction mixture was diluted with water (25 mL) and extracted with CH\textsubscript{2}Cl\textsubscript{2} (3x 25 mL). The combined organic layers were washed with aqueous NaHCO\textsubscript{3} (2x25 mL), dried over MgSO\textsubscript{4}, filtered and the solvent removed \textit{in vacuo}. The resulting residue was purified by flash column chromatography (Et\textsubscript{2}O:cyclohexane, 1:1) to afford 3 (531 mg, 90%) as a mixture of anomers (\(\alpha/\beta, 3/2\) ).
3-O-(2'-Deoxy-2'-acetamido-3',4',6'-tri-O-acetyl-αβ-D-galactopyranosyl) propyne 4a-4b

Triphenylphosphine (472 mg, 1.8 mmol) and acetic anhydride (440 µL, 4.31 mmol) were added to a solution of 3 (531 mg, 1.44 mmol) in CH₂Cl₂ (5 mL). After 16 h stirring at room temperature, the reaction mixture was diluted with water (25 mL) and extracted with CH₂Cl₂ (4x 25 mL). The combined organic layers were washed with aqueous NaHCO₃ (2x 25 mL), dried over MgSO₄, filtered and the solvent removed in vacuo. The resulting residue was purified by flash chromatography (AcOEt:CH₂Cl₂, 4:1) to afford 4a (248 mg, 45%) and 4b (200 mg, 36%), both as a white solid.

For 4a: Rᵢ = 0.39 (AcOEt:CH₂Cl₂, 4:1); [α]D²⁵ = -21 (c, 1.0 in CHCl₃); ¹H NMR (CDCl₃, 400 MHz) δ = 5.60 (1H, d, J = 9.8 Hz, NHAc), 5.38 (1H, app dd, J = 1.2, 4.0 Hz, H-4), 5.17 (1H, dd, J = 12.0, 4.0 Hz, H-3), 5.06 (1H, d, J = 4.0 Hz, H-1), 4.62 (1H, ddd, J = 12.0, 9.8, 4.0 Hz, H-2), 4.26 (2H, dd, J = 5.4, 2.4 Hz, OCH₂-), 4.19 (1H, m, H-5), 4.10 (2H, dd, J = 6.4, 1.8 Hz, H-6a,b), 2.47 (1H, t, J = 2.4 Hz, -C≡CH), 2.14 (3H, s, OAc), 2.04 (3H, s, OAc), 1.99 (3H, s, OAc), 1.97 (3H, s, OAc); ¹³C NMR (CDCl₃, 100 MHz) δ 171.02-170.21 (C=O), 96.82 (C-1), 78.40 (C-3'), 75.5 (C≡C), 68.37 (C-3), 67.40(C-4), 67.44(C-5), 61.85 (C-6), 61.54 (OCH₂), 47.7 (C-2), 23.41 (CH₃), 20.94 (CH₃), 20.83 (CH₃); HRMS (ESI⁺-TOF) m/z: calcd for C₁₇H₂₃NO₉Na [M+Na]⁺: 408.1270, found: 408.1264 (error = -1.5 ppm).

For 4b: Rᵢ = 0.36 (AcOEt:CH₂Cl₂, 4:1); [α]D²⁵ = +83 (c, 1.0 in CHCl₃); ¹H NMR (CDCl₃, 400 MHz) δ = 5.58 (1H, d, J = 8.8 Hz, NHAc), 5.36 (1H, br d, J = 3.4 Hz, H-4), 5.32 (1H, dd, J = 11.0, 3.4 Hz, H-3), 4.88 (1H, d, J = 8.0 Hz, H-1), 4.38 (2H, br d, J = 2.4 Hz, -CH₂), 4.14 (2H, m, H-6a,b), 4.03 (1H, td, J = 11.0, 8.0 Hz, H-2), 3.94 (1H, t, J = 6.6 Hz, H-4), 2.46 (1H, t, J = 2.4 Hz, -C≡CH), 2.14 (3H, s, OAc), 2.04 (3H, s, OAc), 1.99 (3H, s, OAc), 1.96 (3H, s, OAc); ¹³C NMR (CDCl₃, 100 MHz) δ 170.63-170.39 (C=O), 98.75 (Cβ-1), 78.71 (C-3), 75.39 (-C≡CH), 70.94 (-C≡CH), 70.04(C-4), 66.83(C-5), 61.54 (C-6), 56.03 (OCH₂), 52.29 (C-2), 23.61 (CH₃), 20.85 (CH₃), 20.83 (CH₃); HRMS (ESI⁺-TOF) m/z: calcd for C₁₇H₂₃NO₉ [M+Na]⁺: 408.1270, found: 408.1280 (error = +2.4 ppm), m/z: calcd for C₁₇H₂₄NO₉ [M+H]⁺: 386.1451, found: 386.1458 (error = +1.8 ppm).
3-O-(2'-Deoxy-2'-acetamido-α-D-galactopyranosyl) propyne 1a

NaOMe (63 µL, 0.06 mmol) was added to a solution of 4a (222 mg, 0.58 mmol) in MeOH (10 mL) at room temperature. After 4 h, the reaction mixture was neutralized with Dowex 50W-X8 (H⁺) resin, filtered and concentrated in vacuo to afford 1a (143 mg, 96%) as a white solid. Rf = 0.39 (CH₂Cl₂:MeOH, 4:1); [α]D²⁵ = +230 (c, 1.0 in MeOH); ¹H NMR (D₂O, 400 MHz) δ = 5.12 (1H, d, J = 4.0 Hz, H-1), 4.41-4.36 (2H, m, H-2, H-3), 4.27 (1H, dd, J = 12.0, 3.8 Hz, H-4), 4.06-4.03 (2H, m, H-5, H-6a), 3.95 (1H, dd, J = 12.0, 4.0 Hz, H-6b), 3.82-3.80 (2H, m, -OCH₂), 2.94 (1H, br s, -C≡CH), 2.10 (3H, s, NHAc); ¹³C NMR (D₂O, 100 MHz) δ = 174.7 (C=O), 96.1 (C-1), 71.42 (C-4), 68.51 (C-5), 67.64 (C-3), 61.17 (C-6), 55.15 (OCH₂), 49.6 (-C≡CH), 21.9 (CH₃); HRMS (ESI⁺-TOF) m/z: calcd for C₁₁H₁₇NO₆Na [M+Na]⁺: 282.0954, found: 282.0962 (error = +2.8 ppm).

3-O-(2'-Deoxy-2'-acetamido-β-D-galactopyranosyl) propyne 1b

β Anomer was obtained following the conditions described for 1a (49 mg, 95%). Rf = 0.35 (CH₂Cl₂:MeOH, 4:1); [α]D²⁵ = -38 (c, 1.0 in H₂O); ¹H NMR (D₂O, 400 MHz) δ = 4.71 (1H, d, J = 8.0 Hz, H-1), 4.38 (2H, d, J = 2.2 Hz, OCH₂-), 3.90 (1H, t, J = 3.4 Hz, H-3), 3.86 (1H, dd, J = 8.6, 3.4 Hz, H-2), 3.73 (3H, m, H-6, H-5), 3.65 (1H, dd, J = 7.6, 3.4 Hz, H-4), 2.96 (1H, t, J = 4.0 Hz, -C≡CH), 2.10 (3H, s, NHOAc); ¹³C NMR (D₂O, 100 MHz) δ = 175.02 (C=O), 99.73 (C-1), 79.01 (C-2), 75.78 (-C≡CH), 75.29 (C-3), 70.58 (C-4), 67.49 (C-5), 60.98 (C-6), 56.65 (OCH₂), 51.98 (-C≡CH), 22.4 (CH₃); HRMS (ESI⁺-TOF) m/z: calcd for C₁₁H₁₇NO₆Na [M+Na]⁺: 282.0954, found: 282.0956 (error = +0.7 ppm).

General procedure for solid-phase peptide synthesis

Assembly of protected linear peptide was performed manually or automatically (Syro II, Biotage) by employing solid-phase peptide synthesis (SPPS) protocol using the Fmoc/tBu strategy and the Fmoc-Gly-SasrinTM resin (loading of 0.7 mmol/g). Coupling reactions were performed using,
relative to the resin loading, 1.5-2 eq. of N-Fmoc-protected amino acid in situ activated with PyBOP (1.5-2 eq.) and DIPEA (3-4 eq.) in DMF (10 mL/g resin) for 30 min. Coupling reaction was checked by TNBS test using a solution of 1% trinitrobenzenesulfonic acid in DMF. N-Fmoc protecting groups were removed by treatment with piperidine/DMF (1:4, 10 mL/g resin) for 10 min. The process was repeated three times and the resin was further washed five times with DMF (10 mL/g resin) for 1 min. The peptide was released from the resin using cleavage solution of TFA: CH₂Cl₂ (1:99) and linear protected peptide was obtained as a white solid powder after precipitation, triturating and washing with diethyl ether and was used without further purification.

**General procedure for CuAAC ligation**

A solution of CuSO₄ (1 eq./azide) and THPTA (5 eq./azide) in PBS buffer (pH 7.4, 100 mM) was added to a mixture of cyclopeptide and propargyl glycoside (1.5 eq./azide) in DMF at room temperature. To this reaction mixture was added a solution of sodium ascorbate (7 eq./azide) in PBS buffer (10 mM). All solutions were previously degassed under argon. The reaction was stirred at room temperature under argon, after 2h analytical HPLC indicated complete reaction coupling. Chelex™ resin was added to remove excess copper and the reaction mixture was purified by RP-HPLC to afford pure compound as a white powder.

**Compound 5**

The linear peptide A (0.44 mmol) was synthesized following the general procedure for solid phase peptide synthesis, then dissolved in DMF (0.5 mM) and the pH was adjusted to 9 by addition of DIPEA. PyBOP (1.2 eq.) was added and the solution stirred at room temperature for 1h. Solvent was removed under reduced pressure and precipitation in diethyl ether afforded Boc-protected cyclic peptide B as white solid. After cleavage of Boc protecting group, using a solution of TFA/CH₂Cl₂ (10 mL, 3:2, v/v) for 1h, the crude reaction mixture was purified by RP-HPLC affording 5 (455 mg, 92 %) as a white powder after lyophilisation. RP-HPLC: Rₜ = 12.2 min (C₁₈, λ
Compound 8

To a solution of cyclopeptide 5 (11.7 mg, 10.4 µmol) and N-Boc-O-(carboxymethyl)hydroxylamine succinimide ester\(^{35}\) (4.5 mg, 15.6 µmol) in DMF (4 mL), DIPEA was added and the pH adjusted at 8. After 2h stirring at room temperature analytical HPLC indicated formation of compound 7 and the reaction mixture was evaporated in vacuo. The crude product was then subjected to deprotection of Boc protecting group by using a solution of TFA/CH\(_2\)Cl\(_2\) (20 mL, 3:2 v/v) at room temperature. The crude mixture was purified (acetonitrile/water with 0.1% TFA with gradient 5-60% B in 25 min) and lyophilized to give 8 (11.4 mg, 91%) as a white powder. \(R_t = 12.1\) min (C\(_{18}\), \(\lambda = 214\) nm, gradient 5-100% B in 20 min); HRMS (ESI\(^+\)-TOF) \(m/z\): calcd for C\(_{47}\)H\(_{77}\)N\(_{23}\)O\(_{10}\)Na [M+Na]: 1146.6121, found: 1146.6146 (error = +2.2 ppm).

Compound 12

A solution of 8 (27.1 mg, 22.7 µmol) and 11\(^{36}\) (4.7 mg, 3.8 µmol) in a mixture water/acetonitrile (50:50) with 0.1% TFA (10 mL) was incubated at 37°C for 45 min. The crude reaction mixture was purified by semi-preparative HPLC (acetonitrile/water with 0.1% TFA with gradient 5-60% B in 25 min) and lyophilized to give 12 (19.2 mg, 85%) as a flocculent white powder. RP-HPLC \(R_t = 17.0\) min; (C\(_{18}\), \(\lambda = 214\) nm 5-100% B in 20 min); MALDI-TOF \(m/z\): calcd for C\(_{251}\)H\(_{398}\)N\(_{111}\)O\(_{62}\) [M+H]: 5962.6, found: 5962.7

Compound 9

A mixture of CuSO\(_4\) (8.9 mg, 0.03 mmol) and THPTA (77.3 mg, 0.18 mmol) in PBS buffer (0.5 mL, pH 7.4, 10 mM) was added to a solution of 5 (10 mg, 8.9 µmol) and 1\(\alpha\) (13.8 mg, 53.2 µmol)
in DMF (1 mL) at room temperature under argon atmosphere. To this reaction mixture was added a solution of sodium ascorbate (49.4 mg, 0.25 mmol) in PBS buffer (0.5 mL, pH 7.4, 10 mM) and the reaction was stirred for 2 h. The crude reaction mixture was purified by semi-preparative HPLC (acetonitrile/water with 0.1% TFA with gradient 5-60% B in 25 min) to afford 9 (16.2 mg, 84%) as a white powder after lyophilisation. RP-HPLC R_t = 11.5 min (C_{18}, \lambda = 214 nm, gradient 5-100% B in 20 min); HRMS (ESI^+-TOF) m/z: calcd for C_{91}H_{146}N_{27}O_{34} [M+H]^+: 2161.0525, found : 2161.0474 (error = -2.3 ppm).

**Compound 10**

The synthesis was performed using 5 (10 mg, 8.8 \mu mol) and 1b (13.8 mg, 53.2 \mu mol) by following the procedure described for 9. Compound 10 (15.8, 83%) was obtained as a white powder after purification (semi-preparative HPLC, acetonitrile/water with 0.1% TFA with gradient 5-60% B in 25 min) and lyophilisation. RP-HPLC R_t = 11.0 min (C_{18}, \lambda = 214 nm, gradient 5-100% B in 20 min); HRMS (ESI^+-TOF) m/z: calcd for C_{91}H_{146}N_{27}O_{34} [M+H]^+: 2161.0525, found : 2161.0471 (error = -2.5 ppm).

**Compound 13**

The synthesis was performed using 12 (3.5 mg, 0.59 \mu mol) and 1a (3.6 mg, 13.9 \mu mol) by following the procedure described for 9. Compound 13 (4.2 mg, 70%) was obtained as white powder after purification (semi-preparative HPLC, acetonitrile/water with 0.1% TFA with gradient 5-60% B in 25 min) and lyophilisation. RP-HPLC R_t = 11.9 min (C_{18}, \lambda = 214 nm, gradient 5-100% B in 20 min); MALDI-TOF m/z: C_{427}H_{670}N_{127}O_{158} [M+H]^+: 10110.6, found: 10106.1; ESI^-MS m/z: calcd for C_{427}H_{670}N_{127}O_{158} [M+H]^+: 10110.6, found: 10111.2

**Compound 14**
The synthesis was performed using 12 (4.5 mg, 0.75 µmol) and 1b (4.8 mg, 18.5 µmol) by following the procedure described for 9. Compound 14 (5.2 mg, 69%) was obtained as white powder after purification (semi-preparative HPLC, acetonitrile/water with 0.1% TFA with gradient 5-60% B in 25 min) and lyophilisation. RP-HPLC R_t = 11.5 min (C_{18}, λ = 214 nm, gradient 5-100% B in 20 min); MALDI-TOF m/z: calcd for C_{427}H_{670}N_{127}O_{158} [M+H]^+: 10110.6, found: 10106.1; ESI^+-MS m/z: calcd for C_{427}H_{670}N_{127}O_{158} [M+H]^+: 10110.6, found: 10110.6

**Compound 15**

Compound 15 (13.2 mg, 77% over two steps) was obtained using 7 (10 mg, 7.7 µmol) and 1a (12 mg, 46.3 µmol) by following the procedure described for 9. The resulting Boc-protected glycosylated scaffold (15.6 mg, 6.7 µmol) RP-HPLC R_t = 11.1 min (C_{18}, λ = 214 nm, gradient 5-100% B in 20 min); HRMS (ESI^+-TOF) m/z: calcd for C_{98}H_{156}N_{28}O_{38}Na [M+Na]^+: 2356.1033; found 2356.0977 (error = -2.4 ppm) was treated with 60% TFA in CH_2Cl_2 (10 mL) at room temperature for 1 h. Solvents were removed under reduced pressure and the crude reaction mixture was purified by semi-preparative HPLC (acetonitrile/water with 0.1% TFA with gradient 5-60% B in 25 min) to afford the expected product as a white powder after lyophilisation. RP-HPLC R_t = 10.4 min; (C_{18}, λ = 214 nm 5-100% B in 20 min); HRMS (ESI^+-TOF) m/z: calcd for C_{93}H_{148}N_{28}O_{36}Na [M+Na]^+: 2256.0509; Found 2256.0588 (error = +3.5 ppm).

**Compound 13 (Convergent pathway)**

A solution of 15 (10.7 mg, 4.8 µmol) and 11 (1.0 mg, 0.8 µmol) in 0.1% aq. TFA (10 mL) was incubated at 37°C for 45 min. The crude reaction mixture was purified by semi-preparative HPLC (acetonitrile/water with 0.1% TFA with gradient 5-60% B in 25 min) and lyophilized to afford 13 (7.3 mg, 90%) as a white powder.

**ELLA (enzyme-linked lectin assay) experiments**
ELLA experiments were conducted using 96-well microtiter Nunc-Immuno plates (Maxi-Sorp) coated with 100 μL of polymeric sugar (PAA-α-N-acetyl-D-galactosamine, 5 μg.mL⁻¹; Lectinity Holding, Inc., Moscow) diluted in carbonate buffer, pH 9.6 for 1 h at 37°C. Excess of PAA-sugar was removed, then wells were blocked with BSA in PBS (3% w/v, 100 μL.well⁻¹) at 37°C for 1 h. The Soybean agglutinin (SBA) lectin conjugated HRP (0.1 μg.mL⁻¹) was mixed with various concentrations of inhibitors for 1 h at 37°C. Then the mixture was added to the PAA-sugar-coated microwells and incubated for 1 h at 37°C. The wells were washed with T-PBS (3x100 μL.well⁻¹) then the colour was developed using 100 μL per well of 0.05 M phosphate/citrate buffer containing O-phenylenediamine dihydrochloride (OPD, 0.4 mg.mL⁻¹) and urea hydrogen peroxide (0.4 mg.mL⁻¹, Sigma-Aldrich). The reaction was stopped after 10 min by the addition of 50 μL of 30% H₂SO₄. The absorbance was read at 490 nm using a microtiter plate reader (SPECTRAmax, model PLUS384, Molecular Devices). Percentage inhibition was calculated as follows: inhibition (％)=(A_max-A)/A_max)*100, where A_max is the absorbance of the SBA lectin without inhibitor and A is the absorbance of the SBA lectin with inhibitor. Percent of inhibition was plotted against log [inhibitor].

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Notes and references


