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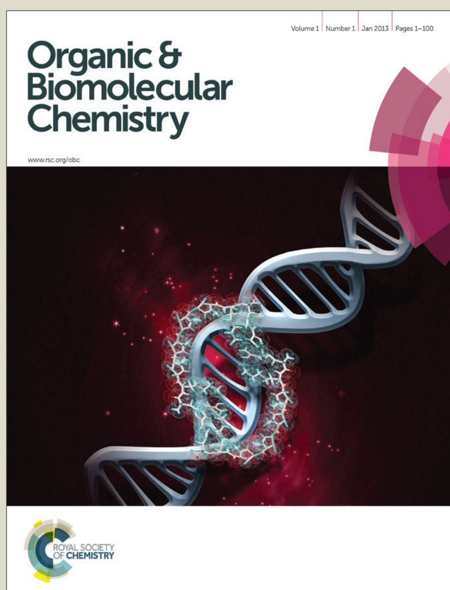

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ARTICLE

Multivalent Presentation of Carbohydrates by 3_{14}-Helical Peptide Templates: Synthesis, Conformational Analysis by CD Spectroscopy and Saccharide Recognition

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Well defined 3_{14}-helical tetravalent β-galactopeptide site specific functionalised template (SSFT) **1** was prepared containing D-galactose units, with free anomeric carbons as aldehyde tags, that was explored by ligation with different aminoxy sugars (α-/β-D-glucose, α/β-D-galactose, α-D-mannose and β-D-lactose) to get 3_{14}-helical carbohydrate functionalised multivalent glycoconjugates **2**-**7**. Preliminary recognition studies of tetramannosyl glycoconjugate **4** with a specific lectin (Concanvalin A) using fluorescence anisotropy showed an increase in binding affinity and the multivalency effect was found to be increased by 6.5 times per glycan.

Introduction

Intercellular interactions involving multivalent carbohydrate motifs are associated with a various biological processes such as cell adhesion, fertilisation, antigen/antibody interactions, cancer metastasis and infection of bacteria or viruses.[1] The major difficulty in understanding the mechanism of carbohydrate recognition is the large-scale preparation and isolation of complex oligosaccharides or glycoproteins.[2] In this direction, design of well organised motifs in which sugar units are attached to a rigid peptidomimetic template, with defined distance, is highly desirable.[3] Conceptually, carbohydrates are ligated to helical structures with rigid and defined conformation derived from natural or unnatural peptides and peptiods.[4] Amongst these, the β-peptide derived peptidomimetic foldamers which are known to adopt 12/14-helical stable secondary structures (even with short peptide sequences) are used as templates on which carbohydrate units are tied up to get a defined secondary motif.[5,6,7,8] Gallaher and co-workers used 12 helical β-peptide backbones (which requires five amino acids per turn) derived from D-galactosylated-pyrrolidine-β-amino acids to synthesise trivalent-*galacto*-foldamers.[5] On the contrary, the 3_{14}-helical secondary structures in β-peptides require only three amino acids per turn and every third side chain (*i* and *i*+3) is oriented on the same side of the helix at 5 Å intervals.[9] This concept proved to be highly beneficial for base pair recognition of entropically preorganised β-peptide nucleic acids, leading to high duplex stabilities.[10] In spite of this, the β-glycopeptide scaffolds that are capable of adopting defined secondary structures and carrying multiple carbohydrate epitopes with defined distances on one side of the 14-helix have received limited attention and to the best of our knowledge only two reports are known so far. Arvidsson and co-workers incorporated one D-galactosamine unit as a sugar amino acid on a β-peptide 3_{14}-helical topology which was found to bind with a lectin.[6] In another report, Taillefumier and co-workers linked a D-mannose sugar unit to the β-peptide amino acid side chain by using an azide-alkyne cycloaddition reaction in order to study multivalent interactions.[7] Our recent finding suggested that, incorporation of four sugar β-amino acids (derived from D-glucose, D-xylose and D-galactose) in β-peptide templates allow to adopt well defined 3_{14}-helical conformation. Among these functionalised β-glycopeptides, the trivalent β-galactopeptide with free anomeric carbon (potential aldehyde functionality) showed highest 3_{14}-helical propensity.[11] In continuation of our interest in this area,[10-12] we explored newly synthesised tetravalent site specific *galacto*-functionalised template (SSFT) **1**, as an aldehyde tag,[13] that was ligated with six different aminooxy sugars via oxime bonds to afford tetravalent *O*-linked glyconjugates **2**-**7** (Fig. 1). All glycoconjugates showed 3_{14}-helical conformation by organising a sugar unit on one face of the peptide helix. The lectin binding study of tetravalent α-D-*manno*-glycoconjugate **4** showed an increase in specific binding with concanavalin A showing a multivalent effect of 6.5 fold increased binding affinity per glycan.

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†Electronic Supplementary Information (ESI) available: Copies of ^{1}H and ^{13}C NMR spectrum for new compound and HPLC traces of purified glycoconjugates **1**-**7**.
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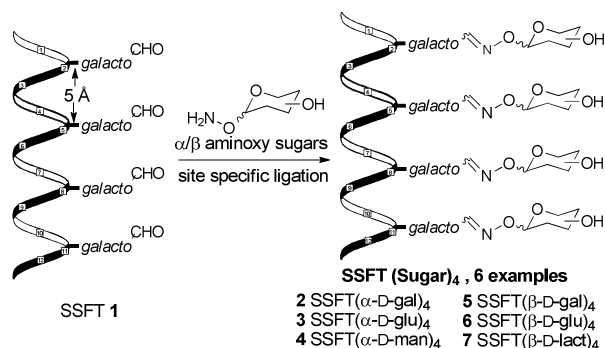


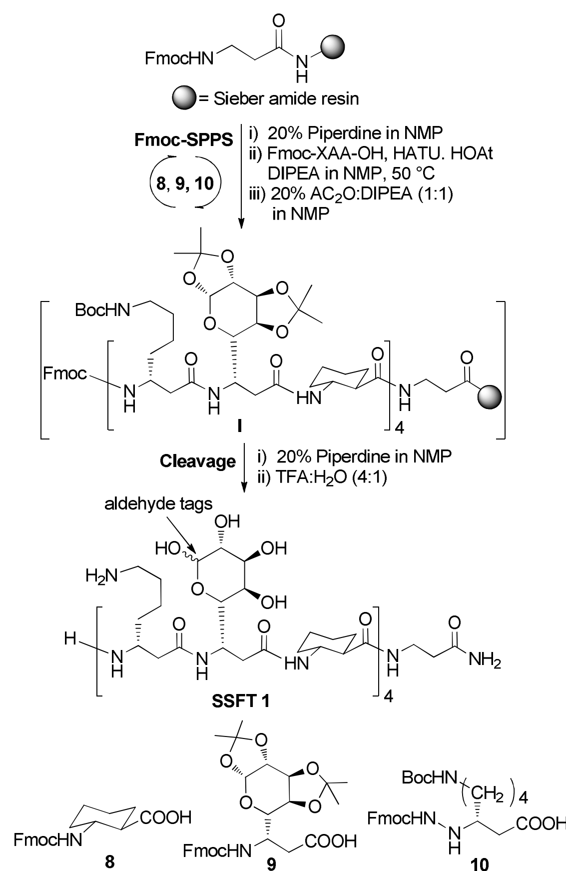
Fig. 1: Right-handed SSFT **1**-**9**.

Results and discussion

Preparation of the sugar functionalised β-peptide was provided by SPPS on a Sieber amide resin preloaded with Fmoc-β-Ala-OH (Scheme 1). Deprotection of *N*-Fmoc using 20% piperdine in NMP was followed by coupling with Fmoc-ACHC-OH **8**[14] using HATU, HOAt, DIPEA, NMP, at 50 °C. Capping of unreacted amine was achieved using 20% Ac_2O/DIPEA 1:1 in NMP. Further coupling steps with Fmoc-L-*glycero*-galactose-OH **9**[11] and with Fmoc-β-HLys(Z)-OH **10**[15] were achieved using an analogous protocol as described for the first coupling. Subsequently, the sequence was repeated one by one to get the required resin bound tetravalent galactopeptide **I**. Deprotection of the Fmoc group followed by removal of acetonide groups, Boc-cleavage and deliberation of the peptide from solid support using TFA/water (4:1) provided tetravalent β-galactopeptide as a site specific functionalised template (SSFT) **1**.

The structural integrity of β-peptide **1** was ensured by high resolution ESI mass spectrometry. In order to ensure the helical conformation, CD spectra of **1** were measured in triethylammonium acetate buffer (pH 7) at various temperatures. A positive Cotton effect at 215 nm indicates the 3_{14}-helix conformation[8,9,16] by organising four D-galactose units on one face of the helix (Fig. 2) with the availability of aldehyde tags, in the form of hemiacetals, for further functionalization.

The required aminooxy sugars **11**-**14**, for oxime bond ligation, were prepared by the protocol of Wang et al. (Scheme 2).[17] Thus, treatment of β-D-galactopyranosepenta-acetate with *N*-hydroxysuccinimide and TMSOTf gave 2,3,4,6-tetra-*O*-acetyl-*O*-α-D-galactopyranosyl-*N*-oxysuccinimide predominantly as an α-anomer in 61% yield. Subsequent reaction with an excess of hydrazine in methanol gave α-D-galactopyranosyl-oxyamine **11a** as a white solid in 92% yield. Alternatively, treatment of β-D-galactopyranosepenta-acetate with borontrifluoride etherate and *N*-hydroxysuccinimide provided 2,3,4,6-tetra-*O*-acetyl-*O*-β-D-galactopyranosyl-*N*-oxysuccinimide as an exclusive β-anomer in 87% yield, that on hydrazine treatment gave β-D-galactopyranosyl-oxyamine **11b** as a white solid in 86% yield. The same method was applied to β-D-glucopyranosepenta-acetate and β-D-mannopyranosepenta-acetate to get **12a**, **12b**,[18] and **13**.[19] The β-D-Lactopyranosyl-oxyamine **14** was prepared following literature procedure.[20]



Scheme 1: Synthesis of SSFT **1**.

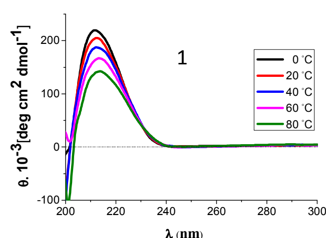


Fig. 2: CD spectra of SSFT **1** (20 µM) in triethylammonium acetate buffer (5mM, pH 7) at various temperatures.

In the final step, site specific oxime bond formations were carried out between aldehyde scaffold **1** and aminooxy sugars **11**-**14**. Thus, individual treatment of **1** with 10 equivalents of aminooxy sugars **11**-**14** in 0.1 M AcONa aqueous buffer (pH 4.1) at room temperature afforded glycoconjugates **2**-**7**, respectively (Scheme 3). The progress of reaction was analysed by reverse-phase HPLC monitoring at 214

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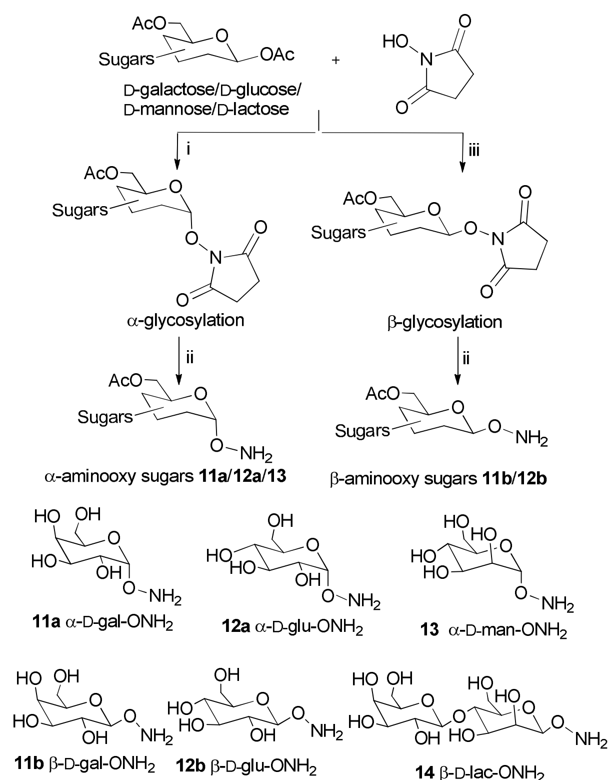
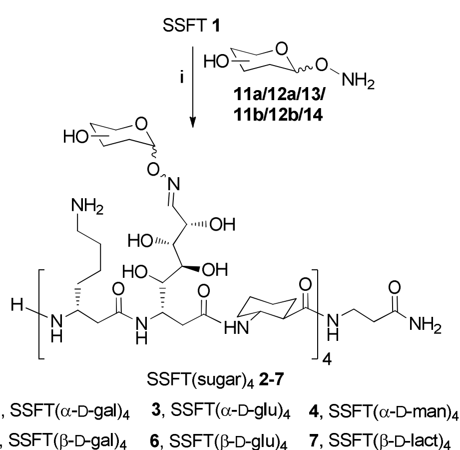
nm. The glycoconjugates **2**-**7** were purified by semi preparative HPLC to get **2**-**7** in ~ 90% yield. The structural integrity of β-peptides **2**-**7** was ensured by the high resolution ESI mass spectrometry.



Scheme 2: Synthesis of aminooxy sugars **11a-b**, **12a-b**, **13** and **14**; *Reagents and conditions*; i,TMSOTf, ACN; ii, N_2H_4, MeOH; iii, BF_3-Et_2O, DCM.



Scheme 3: Synthesis of tetravalent *O*-linked glyconjugates **2**-**7**; *Reaction conditions*; i, 0.1 M AcONa buffer, pH 4.1, 24h (90-95%).

The CD spectra of tetravalent glyconjugates **2**-**7** (Fig. 3) showed a strong positive Cotton effect at 215 nm indicating a right-handed 3_{14}-helix conformation.[9,16] Even at elevated temperature (80 °C), the helical propensity drops only about 20-40%, depending on the kind or sugar units. The observed CD spectra of glyconjugates **2**-**7** were compared with precursor (SSFT) **1**. It was noted that the SSFT **1** adopts a higher 3_{14}-helix propensity as compared to glyconjugates **2**-**5** and **7**. This could be due to the hemiacetal form of the galactosesugar units in **1** which is in equilibrium with the open chain aldehyde form- thus releasing the strain due to cyclic hemiacetal structure. It was found that the attachment of aminoxy sugar with the galactoaldose tag forming an oxime bond is sterically not demanding. This fact is evident from the propensity of the 3_{14}-helix in glycoconjugates **2**-**7**, which is affected by a little extent as compared to SSFT **1**. Similarly, the glycopeptide containing β-lactose also demonstrated 3_{14}-helix conformation indicating that the disaccharide group, which is more sterically demanding, has a small effect on the β-peptide conformation. Amongst the tetravalent glyconjugates the SSFT (β-glu)$_4$ **6** showed higher helical content.

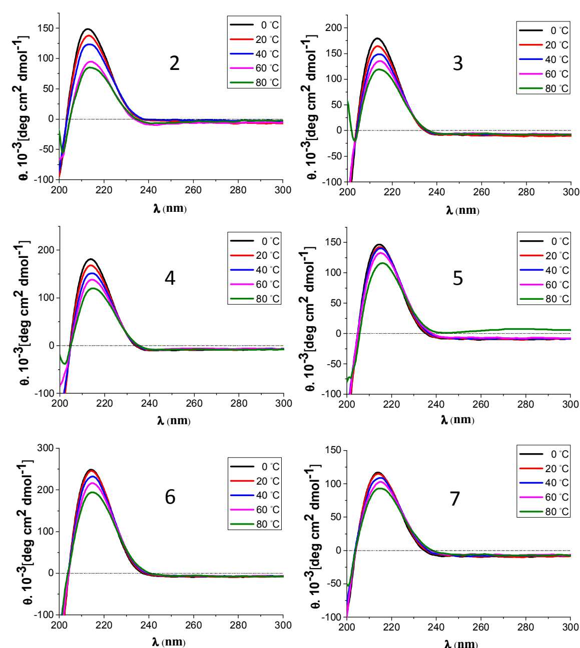


Fig. 3: The CD spectra of β-glycopeptides **2**-**7** (20 μM) in triethylammonium acetate buffer (5mM, pH 7) at various temperatures.

Saccharide Recognition Studies

The affinity of the multivalent α-D-mannoglycoconjugate **4** ligand with a specific mannose binding protein (lectin) was examined using the fluorescence anisotropy method. The basis of this technique is the finding a changes in the anisotropy of fluorescence (r) of a fluorophore attached to a sugar ligand.

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When a lectin binds to the fluorophore labelled carbohydrate ligand, there is an enhancement in the rotational correlation time of the labelled fluorophore and vice-versa. This method is sensitive and can detect even weak binding characteristics of carbohydrate-protein interactions.[21] In view of this, we decided to determine the binding affinity of tetramannosylglycoconjugate SSFT (α-man)$_4$ **4** with concanavalin A (Con A)- an α-D-mannose specific tetrameric plant lectin.

The required fluoromannosyl derivative (Fl-*manno*) **15** (labelled ligand) was synthesised in four steps as reported (Fig. 4).[13c] The fluoromannosyl derivative **15** was assayed for binding by measuring the anisotropy of fluorescence as a function of Con A concentration. The binding property of mannose fluorescent derivative **15** with the lectin (Con-A) is shown in the titration curve (Fig.5) providing free energies of binding Fl-gal **15** with Con-A.[22]

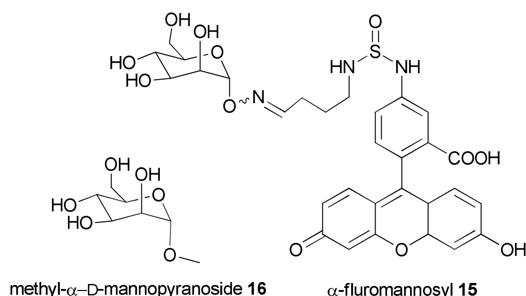

Fig. 4: Fluro-α-D-mannosylpyranoside **15** and methyl α-D-mannopyranoside **16**.

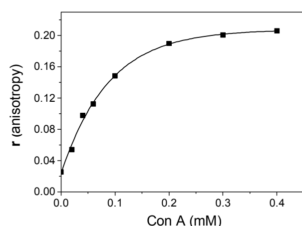

Fig. 5: Fluorescence anisotropy titration of labelled ligand **15** against Con-A.

Subsequently, competition experiments were performed using commercially available methyl α-D-mannopyranoside **16** and SSFT (α-man)$_4$ **4**. The unlabelled ligand concentration was then gradually increased until further addition of unlabelled ligand failed to notably affect the anisotropy measurement. Inhibitor solutions (12.0 mM for **16** and 4.0 mM for **4**) were added in varying concentrations to labelled fluoromannosyl compound **15** bound to Con A and the decrease in r was measured (Fig.6) and inhibition profiles thus obtained are shown in Fig.7. The compound presenting site-specific functionalised mannose cluster **4** replaced the entire fluorescent mannose probe **15** from Con A. This experiment suggests a complete inhibition of the monovalent binding process at about 0.2 mM concentration of **4**. However, in sharp contrast a 30-times higher concentrated solution of α-methyl mannopyranoside **16** was not able to displace the entire labelled fluoromannosyl compound **15** from the complex. The IC_{50} value for monomannosyl compound **16** was found to be 1.065 mM which is 26-fold less (0.041 mM) than for tetramannosyl compound **4**. Thus, the binding enhancement towards Con A was found for the multivalent compound **4** compared to the monovalent mannose **16** and the multivalent effect was found to be 6.5 fold per glycan.

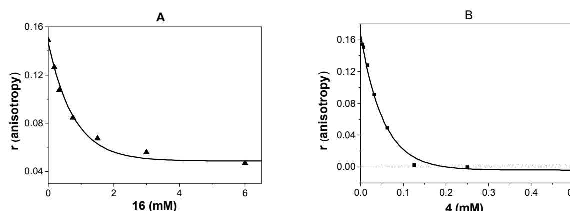

Fig.6: Fluorescence anisotropy competition experiment: To a solution containing fluorescent α-D-manno-pyranoside **15** and Con-A, (A) increasing amounts of nonfluorescent ligand **16** and (B) increasing amounts of nonfluorescent ligand **4**.

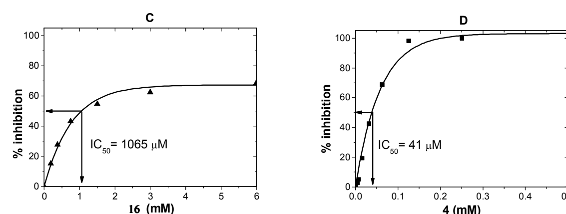

Fig.7: Fluorescence anisotropy competition experiments: Inhibition of binding Con A/fluorescent ligand **15**, (C) versus concentration of **16** and (D) versus **4** as an inhibitor.

Conclusions

To summarise, we have synthesised a tetrameric glycoconjugate template SSFT **1**, having free anomeric carbon as an aldehyde tag that was coupled with a variety of six aminooxy sugars (α/β-D-galactose, α-/β-D-glucose, α-D-mannose and β-D-lactose) to get multivalent glyconjugates **2**-**7**. All β-peptide glycoconjugates showed 3_{14}-helical conformation as indicated by CD spectroscopy. The important aspect of **2**-**7** is the availability of carbohydrate epitopes (α/β-D-galactose, α-/β-D-glucose, α-D-mannose and β-D-lactose) at equidistance to the same side of peptide helix. The presence of a hydrophilic linker containing free hydroxyl groups, assisted additional interactions in the design of water-soluble peptides. The binding enhancement towards Con A was found for the tetravalent mannopeptide **4** compared to the monovalent methyl α-D-mannopyranoside **16** and thus a multivalent effect was found to be 6.5 fold per glycan. It is interesting to note that this

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type of sugar arrangement on one face of peptide helix is present in antifreeze glycoproteins which are essential for antifreeze activity.[23] Therefore, this kind of sugar organisation could be used as a potential tool to investigate multivalent effects in cell targeting/cell-surface mimetics.[24]

Experimental

General procedure: Fmoc-Sieberamide resin for solid phase peptide synthesis was purchased from Novabiochem (Merck) and stored at 4 °C. Optical rotations were measured on a Perkin–Elmer 241 polarimeter. Melting points were obtained with a Bibby-SMP10 melting point apparatus and are uncorrected. IR spectra were recorded on a Digilab Excalibur FTIR spectrometer. ^{1}H and ^{13}C NMR spectra were recorded at 298 K in appropriate deuterated solvents using BrukerAvance 300/500 MHz spectrometers. Mass spectra (ESI-MS) were recorded on a Finnigan LCQ mass spectrometer. High-resolution mass spectra (HRMS-ESI) were obtained with the Bruker Apex-Q IV FT-ICR-MS instrument using TOF (Time-of-flight) analyser. For HPLC analysis HPLC grade CH_3CN and ultra pure H_2O (Millipore, Bedford, UK) were used. HPLC analysis and purification of the oligomers were performed on a Pharmacia Äkta Basic system (GE Healthcare, London, UK) with a pump type P-900, variable wavelength detector UV-900 using C18 MN_Nucleodur 100_5_C-18 (250 × 4.6 mm, 5 μm) analytical HPLC column. All HPLC runs were performed by using a linear gradient of A (0.1% aq. TFA) and B (80% aq. CH_3CN and 0.1% TFA). Flow rates were taken as 1 mL min^{-1} for the analytical HPLC. For HPLC, glycoconjugates were dissolved in Milli Q H_2O. All sample solutions were filtered prior to injection. UV detection was conducted at 215 nm. CD spectra were recorded on a JASCO J-810 spectrometer equipped with a JASCO ETC-505S/PTC-423S temperature controller. All CD measurements were carried out in 5 mM triethylammoniumacetate buffer (pH 7.0) with a quartz cell of 1 cm path length. Spectra represent the average of 6 scans after baseline correction. Concanavalin A and methyl α-D-mannopyranoside **16** were purchased from Sigma Aldrich and used without further purification.

General procedure for solid-phase synthesis of β-glycopeptide: Peptide synthesis was done by manual solid-phase peptide synthesis in a 10 ml BD syringe, using mild acid sensitive Fmoc-Sieber Amide resin with a loading capacity of 0.42 mmol g^{-1}. For peptide syntheses the resin preloaded with H-β-HGly-OH (238.0 mg resin, 100 μmolhomoglycine) was used. Double coupling of the amino acids at 50 °C was needed for peptide bond formation. Initial coupling was done using excess of 5 equivalents amino acid (500.0 μmol), activated by *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N′*,*N′*-tetramethyluroniumhexafluorophosphate (HATU; 171.1 mg, 450.0 μmol, 4.5 equiv), 1-hydroxy-7-azabenzotriazole (HOAt; 680.0 mg, 500.0 μmol, 5 equiv) and *N*,*N*diisopropylethylamine (DIPEA; 243.8 μL, 1.40 mmol, 14 equiv) in NMP (4 mL); however, the second coupling was performed with 3 equivalents of amino acid (300.0 μmol) and activation with HATU (102.6 mg, 270 μmol, 2.7 equiv), HOAt (40.8 mg, 300.0 μmol, 3 equiv), and DIPEA (156.76 μL, 900.0 μmol, 9 equiv) in NMP (3 mL). The loaded resin was allowed to swell for 2 h in dichloromethane (5 mL). The following procedure was carried out for each coupling step: 1) Fmoc-deprotection twice for 10 min with 20% piperidine in NMP (6 ml); 2) Washing with NMP (4 × 6 mL), then with dichloromethane (4 × 6 mL) and then again with NMP (4 × 6 mL); 3) Double coupling steps, each 1.5 h gently moving at 50 °C; 4) Washing with NMP (3 × 6 mL), dichloromethane (3 × 6 mL), and NMP (3 × 6 mL); 5) Capping twice for 3 min with NMP/Ac_2O/DIEA (8:1:1, 4 mL). After the final coupling cycle, *N*-terminal Fmoc group was deprotected and resin washed with NMP (4 × 5 ml), DCM (4 × 5 ml), NMP (4 × 5 ml), MeOH (4 × 5 ml), eventually with DCM (4 × 5 ml) and dried overnight in vacuum.

H-[β-HLys-β-(*S*)Hal(gal)-ACHC]$_4$-HGly-NH$_2$ (1): The deprotection of acetonide groups of D-galactose, Boc-group of β-HLys and cleavage of peptide from the solid support were done in one step using TFA/water (4:1). The cleavage reactions were carried out for 1 h shaking at ambient temperature. The solution was filtered and TFA was removed under N_2 stream; the resulting solutions were directly lyophilized. The crude peptide was dissolved in water/acetonitrile and purified by HPLC. (Nucleodur column, MN-C18, 250 × 4.6 mm, 5 μm, gradient 0-50% B2): t_R = 26.4 min. ESI-MS: m/z = 509.54 $[M+4H]^{4+}$, 679.05 $[M+3H]^{3+}$, 1018.08 $[M+2H]^{2+}$, 2034.14 $[M+H]^{+}$. HRMS (ESI): $C_{91}H_{160}N_{18}O_{33}$ m/z = 509.5430 $[M+4H]^{4+}$, calcd 509.5429, 679.0549 $[M+3H]^{3+}$, calcd 679.0548, 1018.0787 $[M+2H]^{2+}$, calcd 1018.0786, 2034.1447 $[M+H]^{+}$, calcd 2034.1468.

General experimental procedure for α-glycosylation: To a stirred solution of 1,2,3,4,6-penta-*O*-acetyl-α-D-galactopyranose, 1,2,3,4,6-penta-*O*-acetyl-α-D-glucopyranose or 1,2,3,4,6-penta-*O*-acetyl-α-D-mannopyranosein anhydrous CH_3CN (0.3M) *N*-hydroxysuccinimide (3 equiv), 4Å MS (50 weight %) and TMSOTf (5 equiv) were added at 0 °C. The resulting mixture was stirred and warmed up from 0 °C to room temperature within 18h. The reaction mixture filtered through celite, the organic phase was washed with saturated $NaHCO_3$, H_2O, brine and dried over anhydrous Na_2SO_4. Solvent evaporation followed, chromatographic purification using EtOAc/pentane afforded 2,3,4,6-tetra-*O*-acetyl-*O*-α-D-galactopyranosyl-*N*-oxysuccinimide (61%), 2,3,4,6-tetra-*O*-acetyl-*O*-α-D-glucopyranosyl-*N*-oxysuccinimide (69%) or 2,3,4,6-tetra-*O*-acetyl-*O*-α-D-mannopyranosyl-*N*-oxysuccinimide (74%), respectively. 2,3,4,6-Tetra-*O*-acetyl-*O*-α-D-mannopyranosyl-*N*-oxysuccinimide: white solid; R_f = 0.55 (EtOAc/pentane 7:3); mp 130 °C; $[\alpha]D^{22}$ = + 105.2 (c, 0.59, methanol); IR (neat, ν, cm^{-1}): 1753, 1741, 1717, 1700, 1424; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.95 (s, 3H, CH_3), 2.02 (s, 3H, CH_3), 2.04 (s, 3H, CH_3), 2.12 (s, 3H, CH_3), 2.70 (s, 4H, 2 × CH_2), 4.09 (dd, *J* = 12.6, 2.4 Hz, 1H, H-6a),), 4.18 (dd, *J* = 12.6, 4.2 Hz, 1H, H-6b), 4.70-4.80 (m, 1H, H-5), 5.24-5.34 (m, 3H, H-1, H-2, H-4), 5.54-5.58 (m, 1H, H-3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 20.4, 20.5, 20.6 (strong), 25.3 (strong), 61.7, 64.9, 67.0, 68.6, 70.8, 101.4, 169.3, 169.5, 169.6, 170.3 (strong), 170.4; MS (ESI) m/z : 444.1 $[M-H]^{+}$, 468.1 $[M+Na]^{+}$; HR-MS (ESI) m/z : calcd. $C_{18}H_{23}NO_{12}$ $[M+Na]^{+}$: 468.1112, found: 468.1119.

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General experimental procedure for β-glycosylation: A solution of BF_3-Et_2O (5 equiv) in anhydrous dichloromethane (2 M) was added to an ice-cooled suspension of 1,2,3,4,6-penta-*O*-acetyl-α-D-galactopyranose or 1,2,3,4,6-penta-*O*-acetyl-α-D-glucopyranose, *N*-hydroxysuccinimide (3 equiv) and 4Å MS (50 weight %) in anhydrous dichloromethane (0.3 M). The resulting mixture was stirred at room temperature for 18 h, filtered through celite and the organic phase was washed with saturated $NaHCO_3$, H_2O and dried over anhydrous Na_2SO_4. Chromatographic purification using EtOAc/pentane (1:1) and solvent evaporation gave 2,3,4,6-tetra-*O*-acetyl-*O*-β-D-galactopyranosyl-*N*-oxysuccinimide (87%) or 2,3,4,6-tetra-*O*-acetyl-*O*-β-D-glucopyranosyl-*N*-oxysuccinimide (78%), respectively.

General experimental procedure for α/β-aminooxy sugars: To a suspension of various 2,3,4,6-tetra-*O*-acetyl-*O*-D-glycopyranosyl-*N*-oxysuccinimide derivatives in dry methanol (0.1 M) hydrazine (0.7 M) was added and the resulting mixture was stirred at room temperature for 18h. The solvent was evaporated, the residue was dissolved in MeOH/CH_2Cl_2 (1:2) and filtered. The filtrate was again concentrated and recrystallised in MeOH/CH_2Cl_2. The precipitate was collected through gravity filtration and dried under vacuum to give aminooxy sugars **11-13**, respectively.

General procedure for synthesis of tetravalent glycoconjugates 2-7: To a solution of SSFT$(CHO)_4$ **1** (2 mg; 0.98 μmol) in sodium acetate buffer 0.1M (400 μL; pH 4.1) the respective aminooxy sugar **11**-**14** (2.5 equivalents/aldehyde function) was added at room temperature. The reaction mixture was stirred at ambient temperature for 24 h and disappearance of the starting material was determined by reverse-phase HPLC. Purification by reverse-phase HPLC (gradient 0–50% B2 in 30 minutes; detection: λ=214 and 250nm) followed by lyophilisation gave glycoconjugates **2**-**7** as white powders with 90-95% yield.

SSFT (*α*-D-gal)$_4$ (2): HPLC (Nucleodur column, MN-C18, 250 × 4.6 mm, 5 μm, gradient 0-50% B2): t_R = 25.5 min. ESI-MS: m/z = 686.61 $[M+4H]^{4+}$, 915.14 $[M+3H]^{3+}$, 1372.21 $[M+2H]^{2+}$. HRMS (ESI): $C_{115}H_{204}N_{22}O_{53}$ m/z = 915.1406 $[M+3H]^{3+}$, calcd 915.1398, 1372.2079 $[M+2H]^{2+}$, calcd 1372.2060.

SSFT (*α*-D-glu)$_4$ (3): HPLC (Nucleodur column, MN-C18, 250 × 4.6 mm, 5 μm, gradient 0-50% B2): t_R = 25.7 min. ESI-MS: m/z = 686.61 $[M+4H]^{4+}$, 915.14 $[M+3H]^{3+}$, 1372.21 $[M+2H]^{2+}$. HRMS (ESI): $C_{115}H_{204}N_{22}O_{53}$ m/z = 686.6079 $[M+4H]^{4+}$, calcd 686.6066, 915.1393 $[M+3H]^{3+}$, calcd 915.1398, 1372.2082 $[M+2H]^{2+}$, calcd 1372.2060.

SSFT (*α*-D-man)$_4$ (4): HPLC (Nucleodur column, MN-C18, 250 × 4.6 mm, 5 μm, gradient 0-50% B2): t_R = 25.7 min. ESI-MS: m/z = 686.61 $[M+4H]^{4+}$, 915.14 $[M+3H]^{3+}$, 1372.21 $[M+2H]^{2+}$. HRMS (ESI): $C_{115}H_{204}N_{22}O_{53}$ m/z = 915.1435 $[M+3H]^{3+}$, calcd 915.1398, 1372.2099 $[M+2H]^{2+}$, calcd 1372.2060.

SSFT (β-D-gal)$_4$ (5): HPLC (Nucleodur column, MN-C18, 250 × 4.6 mm, 5 μm, gradient 0-50% B2): t_R = 25.6 min. ESI-MS: m/z = 686.61 $[M+4H]^{4+}$, 915.14 $[M+3H]^{3+}$, 1372.21 $[M+2H]^{2+}$. HRMS (ESI): $C_{115}H_{204}N_{22}O_{53}$ m/z = 686.6083 $[M+4H]^{4+}$, calcd 686.6066, 915.1429 $[M+3H]^{3+}$, calcd 915.1398, 1372.2073 $[M+2H]^{2+}$, calcd 1372.2060.

SSFT (β-D-glu)$_4$ (6): HPLC (Nucleodur column, MN-C18, 250 × 4.6 mm, 5 μm, gradient 0-50% B2): t_R = 25.8 min. ESI-MS: m/z = 686.61 $[M+4H]^{4+}$, 915.14 $[M+3H]^{3+}$, 1372.21 $[M+2H]^{2+}$. HRMS (ESI): $C_{115}H_{204}N_{22}O_{53}$ m/z = 686.6083 $[M+4H]^{4+}$, calcd 686.6066, 915.1406 $[M+3H]^{3+}$, calcd 915.1398, 1372.2072 $[M+2H]^{2+}$, calcd 1372.2060.

SSFT (β-D-lac)4 (7): HPLC (Nucleodur column, MN-C18, 250 × 4.6 mm, 5 μm, gradient 0-50% B2): t_R = 25.4 min. ESI-MS: m/z = 848.66 $[M+4H]^{4+}$, 1131.21 $[M+3H]^{3+}$, 1696.81 $[M+2H]^{2+}$, 3390.61 $[M+H]^{+}$. HRMS (ESI): $C_{139}H_{244}N_{22}O_{73}$ m/z = 848.6587 $[M+4H]^{4+}$, calcd 848.6595, 1131.2087 $[M+3H]^{3+}$, calcd 1131.2102.

General procedure for fluorescence anisotropy: Fluorescence anisotropy measurements were done at 23 °C with a Perkin-Elmer Fluorescence Spectrometer with Data Manager LS501 nm at a final volume of 0.6 mL in a quartz cell of 1 cm path length under magnetic stirring. For fluorescence anisotropy measurements, fluorescein-labelled mannose **15** was excited at 590 nm and the emission measured at 610 nm. First the G factor was determined according to $G = I_{HV}/I_{HH}$. Subsequently, the anisotropy (r) was determined according to $r = (I_{VV} – G \times I_{VH})/(I_{VV} + 2 \times G \times I_{VH})$, where I is the fluorescence intensity and the first and second subscript letter indicate the polarisations of the excitation light and emission mirrors, respectively. Fluorescence anisotropy reports local conformational flexibility of the labelled residue and is a measure of mobility. The anisotropy changes upon complex formation or dissociation. Con A was dissolved in 0.1 M HEPES buffer, pH 7.5, containing 0.9 M NaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$, which were added to the solution containing 200 nM ligand (Fl-man **15**) in 0.1 M HEPES buffer, pH 7.5, containing 1 mM $MnCl_2$, 1 mM $CaCl_2$, 0.9 M NaCl and varying the lectin (Con A) concentration from 1 to 400 μM. The binding energy was determined by using the BIOEQS curve fitting programme. Competition titration experiments were performed using 150 μM concanavalin A in 0.1 M HEPES buffer (pH 7.5) containing 1 mM $MnCl_2$, 1 mM $CaCl_2$, 0.9 M NaCl and 160 nM fluorescent α-D-manno-pyranoside **15**.

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Notes and references

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