Green glycosylation using ionic liquid to prepare alkyl glycosides for studying carbohydrate–protein interactions by SPR

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Several simple glycosides of d-glucose (Glc) and N-acetyl-d-galactosamine (GalNAc) were prepared in a single step glycosylation reaction using unprotected and unactivated sugar donors. The resulting GalNAc glycoside, containing a bifunctional linker, was used to immobilize this glycoconjugate to a self assembled monolayer on a gold biosensor chip. Surface plasmon resonance (SPR) experiments demonstrated that this immobilized glycoconjugate bound to GalNAc specific lectin, Viscum album agglutinin.

Introduction

Glycoconjugates including proteoglycans, glycoproteins and glycolipids are involved in many different biological events, such as molecular recognition for cell–cell interaction. Owing to their ubiquitous presence at the cell membrane surface, carbohydrates are located in an environment containing many proteins, such as growth factors, cytokines, receptors, enzymes, and viruses and bacterial proteins. The numerous biological roles of carbohydrates are attributed to their interactions with these proteins, called lectins. Membrane surface carbohydrates, called glycoconjugates, modulate lectin activity at the cell-extracellular interface. Many studies have been directed towards understanding the recognition of these cell surface glycoconjugates by lectins from different sources. The most widely used techniques (i.e., enzyme linked immnosorbent assays, microarrays, atomic force microscopy and surface plasmon resonance (SPR)) for the study of such molecular interactions require the immobilization of either the lectin or the glycoconjugate.

A number of strategies have been developed to mimic the presentation of glycoconjugates on the membrane surface. In addition, many different methodologies have been investigated for the synthesis of glycoconjugates. Protecting groups for hydroxyl moieties in carbohydrates (acyl, ethers, etc.) and activation methods for the anomic position (halides, thioglycosides, trichloroacetimidates, etc.) are required in traditional synthetic chemistry to obtain new glycoconjugates. A major problem in the synthesis of glycoconjugates is the need for multiple protection, deprotection and activation steps. The crucial importance of glycoconjugates in the study of glycobiology cries out for alternative, simpler methods for their synthesis. Enzymatic synthesis of carbohydrates can be used to avoid many protection, deprotection and activation steps and the products are often obtained with high regio- and stereoselectivity. However, enzymes are often less efficient for the functionalization of glycoconjugates with linker chains required their immobilization to study their interaction with lectins.

Glycosylation using unprotected and unactivated donors is often preferable as it can reduce the number of steps, enhance reactivity, allow a different stereochemistry, and increase prospects for further process modifications. The chemical synthesis of unprotected carbohydrates possesses a number of challenges, including their poor solubility in most conventional solvents. It is important to investigate new solvent systems that dissolve carbohydrates and support glycosylation reactions of unprotected sugars.

Recently, room temperature ionic liquids (RTILs) have received attention as solvents for a wide range of chemical processes. RTILs show exceptional solvent properties including thermal stability, low volatility and an ability to dissolve both polar and non-polar compounds. RTILs also have been widely researched as possible “green” replacements for organic solvents because they have very low vapour pressures and may be used to replace volatile organic solvents and may be easier to efficiently reuse than organic solvents. Carbohydrate chemistry is not an exception to this trend. In the past few years ionic liquids have been used in both protection and glycosylation reactions, resulting in new methodologies and enhanced yield.

We report a convenient method for the modification of unprotected and unactivated carbohydrates, of biological interest, with a bifunctional linker. The use of RTIL solvent allowed us to introduce a linker chain into carbohydrates in a single, simple step. The resulting glycoconjugates were immobilized
on a carboxylated functionalized matrix and used in lectin interaction studies performed by SPR.

Results and discussion

Synthesis of functionalized carbohydrates

In this study, we present the functionalization of non-activated carbohydrates in a single step to give a glycoconjugate having a free amino group. Thereby, it is possible to obtain various kinds of functionalized glycoconjugates without the need of protection, activation and deprotection steps required for the chemical synthesis chemistry of carbohydrates. For this purpose, we selected three substrates, D-glucose (Glc), N-acetyl-D-galactose (GalNAc) and lactose as model carbohydrates for study.

Glycosylation reactions were carried out in the presence of the RTIL, 1-ethyl-3-methylimidazolium benzoate [emIm][ba], and Amberlite IR-120 [H+] providing the acid milieu necessary to promote glycosylation. The application of [emIm][ba] RTIL was previously demonstrated in the protection and functionalization of carbohydrate.

This RTIL was also previously used for the glycosylation of bulky acceptors, including monosaccharides and benzyl alcohol. These initial successes prompted us to examine if the same one-step glycosylation reaction could be similarly carried out using linear alkyl chain acceptors. For these purposes we initially tested the reactivity of an α,β-anomeric mixture of Glc monosaccharide donor with the allyl alcohol as acceptor (Table 1).

The reaction, carried out following the conditions described in the Experimental section, led to formation of the desired compound in 35% yield (Scheme 1, Table 2). Some stereoselectivity was observed in this glycosylation reaction, giving primarily the α-glycoside (α:β, 77:23), as previously described for similar processes.

One of the most remarkable features of [emIm][ba] is its high viscosity at room temperature. The viscous nature of this solvent posed a problem that was overcome by increasing the reaction temperature to 50 °C. Under the conditions, allyl alcohol afforded the complete conversion of Glc to the allyl glycoside 1. However, the viscosity of this RTIL solvent posed additional problems in product purification. While a nearly quantitative yield was observed by TLC, the recovered yield of purified glucoside product was only 35%. Despite a yield of only 35%, the requirement of only one step makes this approach promising for glycosylation reactions, as protection, activation and deprotection steps would certainly decrease more greatly the yield of isolated product.

Allyl alcohol was selected as we expected it would be an excellent glycosidation acceptor due to its very small size when compared to the benzylamine, benzylmercaptan, 4-aminobenzyl alcohol or 2-(2¢-aminoethoxy)ethanol acceptors (Table 2). This was demonstrated by the case when 4-aminobenzyl alcohol was used as acceptor; no reaction was observed, probably due to the poor solubility of the linker chain in the reaction medium. In addition, benzylamine, benzylmercaptan, and 2-(2¢-aminoethoxy)ethanol acceptors afforded a mixture of products by TLC, possibly including the α,β-glycosides, the signals of which correspond to the anomeric protons identified by 1H-NMR. Further isolation failed to afford the desired product due to the high viscosity of the RTIL at room temperature (Table 2). [emIm][ba] RTILs are relatively viscous RTILs with a viscosity of 425 cP at 25 °C, making both stirring and product recovery difficult.

Next, a bifunctional linker chain was tested as a glycosylation acceptor in [emIm][ba], the tert-butoxycarbonyl (tBoc) amino protected 2-(2¢-aminoethoxy)ethanol. The advantage of this linker is that once bound to the carbohydrate through the glycosylation of the hydroxyl group, selective removal of tBoc exposes an amino group that could be used for a covalent immobilization of the resulting glycoconjugate onto surfaces. Compound 7 was obtained as a mixture of α- and β-isomers.

Table 1 Functionalization of carbohydrates with different linker chains*

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>Compound</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc</td>
<td>Allyl alcohol</td>
<td>1</td>
<td>35%</td>
</tr>
<tr>
<td></td>
<td>Benzyamine</td>
<td>2</td>
<td>n.p.</td>
</tr>
<tr>
<td></td>
<td>Benzymercaptan</td>
<td>3</td>
<td>n.p.</td>
</tr>
<tr>
<td></td>
<td>4-Aminobenzyl alcohol</td>
<td>4</td>
<td>n.r.</td>
</tr>
<tr>
<td></td>
<td>2-(2¢-Aminoethoxy)ethanol</td>
<td>5</td>
<td>n.p.</td>
</tr>
<tr>
<td></td>
<td>tBoc-2-(2¢-Aminoethoxy)ethanol (6)</td>
<td>7</td>
<td>12%</td>
</tr>
<tr>
<td>Lac</td>
<td>Allyl alcohol</td>
<td>8</td>
<td>n.p.</td>
</tr>
<tr>
<td>GalNAc</td>
<td>Allyl alcohol</td>
<td>9</td>
<td>35%</td>
</tr>
<tr>
<td></td>
<td>tBoc-2-(2¢-Aminoethoxy)ethanol (6)</td>
<td>10</td>
<td>7%</td>
</tr>
</tbody>
</table>

*a.n.p.: no product isolated. n.r.: no reaction observed.

Table 2 Glycosylation of Glc with different linker chains*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Product</th>
<th>t</th>
<th>Yield</th>
<th>α:β</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1.png" alt="Image" /></td>
<td>17 h</td>
<td>35%</td>
<td>77:23</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2.png" alt="Image" /></td>
<td>6 h</td>
<td>n.p.</td>
<td>17:83</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3.png" alt="Image" /></td>
<td>6 h</td>
<td>n.p.</td>
<td>59:41</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4.png" alt="Image" /></td>
<td>7 d</td>
<td>n.r.</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5.png" alt="Image" /></td>
<td>6 h</td>
<td>n.p.</td>
<td>50:50</td>
</tr>
<tr>
<td>7</td>
<td><img src="image6.png" alt="Image" /></td>
<td>24 h</td>
<td>12%</td>
<td>40:60</td>
</tr>
</tbody>
</table>

*a.n.p.: no product isolated. n.r.: no reaction observed.
by 1HN M R. From which we were unable to detect the desired glycoconjugate lactose as a disaccharide donor afforded a mixture of products and lactose using allyl alcohol as acceptor (Table 1). Using was next extended to other glycosyl donors, including GalNAc.

Interestingly, glycosylation by GalNAc exclusively afforded the galactosylglycoside (Table 4). The high polarity of the solvent may explain the low reactivity towards this non-polar acceptor and also may have complicated the isolation of pure products. Again, the α-selectivity was also described for the glycosylation of Man, Glc and GalNAc with benzyl alcohol by other authors.

The bifunctional linker, tBoc amino protected 2-(2-aminoethoxy) ethanol was also examined as a glycosylation acceptor in [emIm][ba] using both Glc and GalNAc donors. The conversion was very low in both cases, 12% for Glc and 7% for GalNAc (Table 3); however, the Glc initial donor completely disappeared in 17 h, while the GalNAc reaction was terminated because no further evolution of product was observed after 28 h. On the other hand, the α-stereoselectivity observed using both Glc and GalNAc donors was surprising, as neighboring group participation of the 2-acetamido group typically affords the β-product using such donors. However, the α-selectivity was also described for the glycosylation of Man, Glc and GalNAc with benzyl alcohol by other authors.

The investigation of the glycosylation reaction in [emIm][ba] was next extended to other glycosyl donors, including GalNAc and lactose using allyl alcohol as acceptor (Table 1). Using lactose as a disaccharide donor afforded a mixture of products from which we were unable to detect the desired glycoconjugate by 1H NMR.

Using GalNAc as donor and carrying out reactions under identical experimental conditions as described for Glc, afforded the desired glycoside product in 35% yield (Table 3). Interestingly, the conversion Glc and GalNAc afforded identical 35% isolated yields (Table 3); however, the Glc initial donor completely disappeared in 17 h, while the GalNAc reaction was terminated because no further evolution of product was observed after 28 h. On the other hand, the α-stereoselectivity observed using both Glc and GalNAc donors was surprising, as neighboring group participation of the 2-acetamido group typically affords the β-product using such donors. However, the α-selectivity was also described for the glycosylation of Man, Glc and GalNAc with benzyl alcohol by other authors.

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Glycosylation of monosaccharides with 2-(2-aminoethoxy) ethanol protected with different protecting groups

<table>
<thead>
<tr>
<th>Product</th>
<th>Donor</th>
<th>Linker chain</th>
<th>Yield</th>
<th>Percentage of α-derivative</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Glc</td>
<td>HOCH₂CH₂OCH₂CH₂NH-tBoc</td>
<td>12%</td>
<td>43%</td>
</tr>
<tr>
<td>10</td>
<td>GalNAc</td>
<td></td>
<td>7%</td>
<td>99%</td>
</tr>
</tbody>
</table>

Functionality of a biosensor chip

The basic SPR sensor chip contains a gold surface to which a ligand is immobilized. Commercially available biosensor chips, such as the CM5 chip, typically contain a functionalized matrix on the gold surface. Thus, when immobilizing low-molecular-weight ligands onto these types of surfaces, the greater the distance from the gold surface the lower the relative response (measured as “response units”, RU), or sensitivity when a binding partner, in the solution phase, interacts with immobilized ligand. The problem of low sensitivity has been reported by others when immobilizing small molecules on a commercial CM5 biosensor chips (Biacore).

Compound 11 was immobilized directly on a gold chip through a self-assembled monolayer (SAM) prepared using compound 14 (Fig. 1).

Fig. 1 Preparation of the chip with mixed chains and immobilization of functionalized glycoconjugate 14.

This thiol-reactive heterobifunctional linker 14 was synthesized as described in the Experimental section with good yield (Scheme 2). Tetra(ethylene glycol) reacted with 1,8-dibromo-octane to afford compound 12 in 40% yield. Compound 12 was then oxidized with Jones reagent to afford 13 in good yield (70%). And finally, reaction with thiourea followed by base catalyzed hydrolysis afforded thiol group and carboxyl containing compound 14 in 60% yield. The resulting monolayer was very homogeneous judging from the similar RU values obtained for both flowcells. The linker chains forming the SAM contained a free carboxylic group for functionalization. The SAM was formed quickly and was quite stable due to the avidity of the thiol group for gold. Several washings with 50% aqueous MeOH were required to eliminate non-covalently bound linker.
The GalNAc glycoconjugate I1 was immobilized to the SAM on the biosensor chip using standard EDC/NHS activation (Fig. 1). After several injections of glycoconjugate I1, the response increased 600 RU and after the standard blocking of the remaining activated binding sites with ethanolamine, the increase of the response decreased to 374 RU. These results compare favorably to those obtained for the immobilization of the same concentration of glycoconjugate I1 on the carboxy dextran matrix of a CM5 chip, where no increase in RU was observed. Thus, the self-assembled monolayer considerably improves the sensitivity of SPR detection using small molecule ligands.

Interaction studies with lectin

The final step of this study was to determine the utility of SAM containing glycoconjugates to study a carbohydrate–protein interaction. VAA, a potent toxin and biohazard, was selected as a model lectin to examine the bioactivity of this surface. VAA recognizes and selectively binds to GalNAc residues. Different concentrations of VAA were injected over the GalNAc containing biochip and sensorgrams were obtained and analyzed (Fig. 2). The affinity parameters obtained were $K_a: 6.72 \times 10^6$ M$^{-1}$; $K_D: 1.49 \times 10^{-7}$ M, clearly demonstrating binding in the micromolar range (Fig. 2). SPR, using the CM5 surface, showed no observable interaction.

Experimental

The RTIL [emIm][ba] was synthesized according to the reported procedure and all chemicals were purchased from Fisher or Sigma–Aldrich. $^1$H-NMR and $^13$C-NMR experiments were performed in a Varian 500 MHz (COSY or $^1$H–$^13$C heteronuclear experiments were also carried out as required for making assignments). Chemical shifts ($\delta$) are indicated in parts per million (ppm). LRMS was carried out with an Agilent 1100 series LC/MSD trap. TLC was performed on Kieselgel plates 60 F$_254$ (Merck). Product detection on TLC plates was carried out under a UV-lamp or with 5% H$_2$SO$_4$ in CH$_3$OH and heating. Column chromatography used silica gel 230–400 mesh (Natland International Corporation). Sensor chips and solutions used for the SPR assays were purchased from Biacore. SPR experiments were carried out with a BIACore-3000 and the sensorgrams were analyzed with BIAEvaluation software version 4.1, 2003 (Biacore).

The Viscum album agglutinin (VAA) lectin was purified from mistletoe extracts of dried leaves by affinity chromatography on lactosylated Sepharose 4B obtained by divinyl sulfone activation and ligand coupling as crucial step and assays to ascertain purity and activity were run by one- and two-dimensional gel electrophoresis and gel filtration or haemagglutination and solid-phase/cell binding.

General method for glycosylation reactions in RTIL

Carbohydrate was dissolved in [emIm][ba] in the presence of Amberlite IR-120 (H$^+$) and a excess of the linker (Scheme 1 and Table 1). The reaction mixture was stirred at 50 °C under argon atmosphere and monitored by TLC.

When reaction was complete, or no further conversion of substrates into products was observed, the Amberlite was removed by filtration and washed with CH$_3$OH. Solvent was removed and product purified by column chromatography (CH$_3$Cl–CH$_3$OH in the appropriate ratio depending on the polarity of compounds). If necessary, traces of RTIL could be removed from the product using a Dowex MR-3 mixed bed ion-exchange resin. All fractions containing carbohydrate were analyzed by $^1$H-NMR.

Synthesis of 1-O-allyl-glucopyranoside (1)

Allyl alcohol (322 µL, 4.1 mmol) was added to a mixture of Amberlite IR-120 (H$^+$) (500 mg) and Glc (50 mg, 0.28 mmol) [emIm][ba] (300 mg, 1.3 mmol) under the conditions described above. Compound 1 was purified by column chromatography eluting with CH$_3$Cl–CH$_3$OH gradient (9:1 to 3:1) affording 21 mg of product in 35% yield (Table 1).

$^1$H-NMR (500 MHz, D$_2$O): Allyl: 5.97 (m, 1H, H-2), 5.36 (dd, 1H, $J = 17.0$, $J = 1.8$ Hz, H-3a), 5.26 (dd, 1H, $J = 10.6$, $J = 0.7$ Hz, H-3b), 4.23 (dd, 1H, $J = 13.0$, $J = 5.7$ Hz, H-1a), 4.07 (dd, 1H, $J = 13.0$, $J = 6.2$ Hz, H-1b). Glc: 4.96 (d, 1H, $J = 3.8$ Hz, H-1o), 3.85 (dt, 1H, $J = 12.1$, $J = 1.9$ Hz, H-6a), 3.75 (dd, 1H, $J = 11.6$, $J = 5.3$ Hz, H-6b), 3.69 (m, 1H, H-5), 3.68 (t, 1H, $J = 10.2$ Hz, H-3), 3.55 (dd, 1H, $J = 9.8$, $J = 3.9$ Hz, H-2), 3.40 (t, 1H, $J = 9.3$ Hz, H-4). $^1$C-NMR (125 MHz, D$_2$O): 133.69, 118.34, 97.47, 73.26, 71.99, 71.38, 69.81, 68.61, 60.66.
Synthesis of 1-[(2′-aminoethoxy)ethyl]-2-acetamido-2-deoxy-galactopyranoside (9)

Allyl alcohol (300 μL, 5.0 mmol) was added to a mixture of Amberlite IR-120 (H⁺) (50 mg) and Glc (10 mg, 0.023 mmol) in [emIm][ba] (30 mg, 0.13 mmol) and reaction conditions as described in the general procedure. Purification by column chromatography CH₂Cl₂–CH₃OH (5:1) afforded compound 9 (1 mg, 12% yield) (Table 1).

1H-NMR (500 MHz, D₂O): 5.16 (d, 1H, J = 3.9, H-1α), 4.57 (d, 1H, J = 7.9, H-1β), 3.84–3.16 (14H, H-2 to H-6, and sugar), 1.44 (s, 9H, –(CH₃)₃). 13C-NMR (125 MHz, D₂O): 156.08, 107.78, 81.76, 79.38, 77.78, 75.83, 73.16, 70.15, 70.09, 65.30, 64.87, 40.54, 27.92. MS: [M + Na] calc: 390.2, found: 389.1. [M + Cl] calc: 402.7, found: 403.5.

Synthesis of 1-(O-allyl-2-acetamido-2-deoxy-galactopyranoside (10)

Tetrahydrofuran (1 mL, 5 mmol) and GalNAc (25 mg, 0.11 mmol) was added to a mixture of Amberlite IR-120 (H⁺) (150 mg) and of GalNAc (25 mg, 0.11 mmol) in [emIm][ba] (150 mg, 0.65 mmol) under identical purification and reaction conditions as described in the general procedure. Purification by column chromatography CH₂Cl₂–CH₃OH (5:1) afforded compound 10 (3 mg, 7%) (Table 1).

1H-NMR (500 MHz, D₂O): linker chain: 3.61–3.59 (4H, H-1′ and H-2′), 3.53 (t, 2H, J = 5.5 Hz, H-1″), 3.20 (t, 2H, J = 5.2 Hz, H-2″), 1.27 (s, 9H, –(CH₃)₃). GalNAc: 4.96 (d, 1H, J = 2.1 Hz, H-1α), 4.11 (dd, 1H, J = 2.3, J = 4.4 Hz, H-2), 4.07 (dd, 1H, J = 4.5, J = 6.6 Hz, H-4), 3.96 (dd, 1H, J = 3.4, J = 6.6 Hz, H-3), 3.79 (m, 2H, H-5 and H-6a), 3.64 (dd, 1H, J = 3.7, J = 6.6 Hz, H-6b), 1.96 (s, 3H, –CH₃). 13C-NMR (125 MHz, D₂O): 174.99, 156.08, 101.78, 80.22, 79.01, 72.83, 71.16, 70.02, 69.91, 64.07, 63.85, 41.00, 27.82, 23.20. MS: [M + Na] calc: 431.2, found: 430.8. [M + Cl] calc: 443.4, found: 444.5.

Synthesis of 2′-(2′-aminoethoxy)ethyl-2-acetamido-2-deoxy-galactopyranoside (11)

Trifluoroacetic acid (100 μL) was added to 1 mg of compound 10 and reaction was stirred for 1 min. The mixture was dried under vacuum and compound 11 was analyzed by NMR without purification.

1H-NMR (500 MHz, D₂O): 5.10 (d, 1H, J = 3.6, H-1α), 4.51 (d, 1H, J = 8.3, H-1β), 4.04–3.51 (H-2 to H-6, and H-2′), 3.51 (t, 2H, J = 5.5 Hz, H-1″), 3.09 (t, 2H, J = 5.5 Hz, H-2″), 1.92 (s, 3H, –CH₃). 13C-NMR (125 MHz, D₂O): 173.54, 100.35, 81.22, 74.55, 73.71, 71.39, 70.04, 65.11, 64.54, 60.01, 40.89, 23.21. ESI-MS: [M+ Na] calc: 331.2, found: 331.0.

Synthesis of 20-bromo-3,6,9,12-tetraoxatrieicosan-1-ol (12)

NaH (160 mg, 6.5 mmol) was added to a solution of tetaethylenglycol (1 g, 5 mmol) in anhydrous THF and the reaction mixture was stirred under argon atmosphere at 0 °C. After 3 h the reaction mixture, under argon atmosphere at 0 °C, was added over a period of 4 h to a solution of 1,8-dibromo-octane (1.4 g, 5 mmol). The NaBr formed was removed by filtration and the solvent was evaporated. Purification of final product was carried out by column chromatography (CH₂Cl₂–CH₃OH 20:1) affording 12 in 40% yield.

1H-NMR (300 MHz, CDCl₃): 3.62 (m, 16H, H-1 to H-8), 3.43 (t, 2H, J = 6.56 Hz, H-9), 3.38 (t, 2H, J = 7.03 Hz, H-19), 1.83 (q, 2H, J = 7.2 Hz, H-18), 1.55 (q, 2H, J = 6.90 Hz, H-10), 1.38 (m, 2H, H-11), 1.25 (m, 12H, H-12 and H-17). 13C-NMR (125 MHz, CDCl₃): 72.63 (C-9), 71.52 (C-7), 70.61 (C-3, C-4, C-5, C-6), 70.28 (C-8), 70.05 (C-2), 61.50 (C-1), 34.00 (C-19), 32.89 (C-18), 29.56 (C-10, C-12, C-13, C-14, C-15), 28.72 (C-11), 28.18 (C-16), 26.13 (C-17). Analysis calculated for C₃₇H₇₅BrO₆: C: 49.87%; H: 8.63%; Found: C: 49.88%; H: 8.65%. ESI-MS: [M + Na] Calcd: 407.2. Found: 407.3.
Synthesis of 20-bromo-3,6,9,12-tetraoxatrieicosan-1-oic acid (13)
A mixture of 12 (650 mg, 1.7 mmol) in acetonitrile (50 mL) was stirred at room temperature and Jones reagent (2.4 mmol in CrO₃) was added dropwise over 15 min. The reaction was stirred for an additional 30 min followed by the addition of few drops of 2-propanol. Saturated NaCl solution (20 mL) was added to the mixture and stirred for an additional 30 min followed by removal of the acetonitrile. The aqueous mixture was extracted with CH₂Cl₂ (3 × 30 mL) and the solvent was removed by rotary evaporation. Purification of final product was carried out by column chromatography (CH₂Cl₂–CH₃OH 10:1) yielding 13 in 70%.

¹H-NMR (300 MHz, CDCl₃): 6.71 (s, 1H, COOH), 4.13 (s, 2H, H-2), 3.56 (m, 12H, H-3 and H-8), 3.39 (t, 2H, J = 6.90 Hz, H-9), 3.34 (t, 2H, J = 6.85 Hz, H-14), 1.81 (q, 2H, J = 7.16 Hz, H-15), 1.52 (m, 2H, H-10), 1.34 (m, 2H, H-11), 1.28 (m, 6H, H-12, H-13, H-14). ¹³C-NMR (125 MHz, CDCl₃): 173.03 (C-1), 72.07 (C-9), 71.97 (C-8), 70.84 (C-7), 70.74 (C-6), 70.65 (C-5), 70.45 (C-4), 70.34 (C-3), 69.11 (C-2), 34.44 (C-16), 33.10 (C-15), 29.70 (C-10), 29.55 (C-12), 29.00 (C-11), 28.41 (C-13), 26.24 (C-14). Analysis calculated for C₁₆H₃₁BrO₆: C: 48.12%; H: 29.70; Br: 15.46%; O: 16.64%. Found: C: 48.02%; H: 29.62%. ESI-MS: [M + Na] Calcd: 421.2. 

Immobilization of glycoconjugate 11 on the Au chip (Biacore).
For the immobilization of GalNAc glycoconjugate (11), the carboxylated groups of the self-assembled monolayer were activated by the injection of the amino coupling kit (Biacore): EDC/NHS, 35 μL, at a flow rate of 5 μL min⁻¹, at 25 °C, and HBS-P as running buffer. At the same conditions of flow and temperature, a solution of 11 (2 mM, 150 μL) in HBS-P was injected to get the maximum immobilization (605 RU), followed by the injection of ethanolamine (1 M, 35 μL) to block the possible remaining active sites and another injection of MeOH (5 μL). On the second flow cell, the activation and blocking standard procedures were carried out as described above, and it was left as a negative control of the interaction.

Immobilization of glycoconjugate 11 on the CM5 chip. The immobilization of the functionalized GalNAc glycoconjugate (11) was carried out on commercially available CM5 chips (Biacore). The carboxylated dextran matrix was activated by the standard procedure (Biacore), as described above. A solution of glycoconjugate 11 (150 μL, 2 mM) in the running buffer (HBS-P) was injected at a flow rate of 5 μL min⁻¹ at 25 °C followed by the injection of ethanolamine (1 M, 35 μL) to block the remaining active sites. The same activation and blocking procedures were repeated in a different flow cell and used as negative control for the interaction studies.

Interaction with Viscum album lectin. The interaction experiments were carried at a constant flow of 5 μL min⁻¹ at 25 °C over GalNAc glycoconjugate 11 immobilized on the Au chip functionalized with the thiol chains as described above. For that purpose, 15 μL of different solutions of the VAA in the running buffer (HBS-P) were injected: 2.2, 5.4, 8.9, 17.9 and 35.7 μM. Sensorgrams obtained for the interaction of VAA with 11, were analyzed and kinetic parameters of the interaction were determined with BIAEvaluation software (Biacore).

Conclusions
Different glycoconjugates were prepared in the presence of ionic liquids in order to carry out carbohydrate–protein interaction studies.

The direct functionalization of carbohydrates (Glc, GalNAc, lactose) with different linker chains (in the presence of ionic liquids) was carried out. Despite the poor yields obtained, the decrease in the number of steps made it a green alternative to the traditional carbohydrate chemistry.

An amino functionalized GalNAc was directly synthesized in the presence of ionic liquids and further immobilized on an alkanethiol-coated surface. The interaction of immobilized GalNAc derivative with a carbohydrate binding protein was studied, and kinetic parameters of this procedure were determined.

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References