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.[1–3] 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.[3,4] The most widely used techniques (*i.e.*, enzyme linked immunosorbent 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.[5]

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 moieities in carbohydrates (acyl, ethers, *etc.*) and activation methods for the anomeric position (halides, thioglycosides, trichloroacetimidates, *etc.*) are required in traditional synthetic chemistry to obtain new glycoconjugates.[6] 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.[7–9] 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.[10,11] 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 glycosidation reactions of unprotected sugars.

Recently, room temperature ionic liquids (RTILs) have received attention as solvents for a wide range of chemical processes.[12] 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.[13,14] 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.[15–21]

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

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Table 1 Functionalization of carbohydrates with different linker chains[a]

Donor	Acceptor	Compound	Yield
Glc	Allyl alcohol	**1**	35%
	Benzylamine	**2**	n.p.
	Benzylmercaptan	**3**	n.p.
	4-Aminobenzyl alcohol	**4**	n.r.
	2-(2′-Aminoethoxy)ethanol	**5**	n.p.
	*t*Boc-2-(2′-Aminoethoxy)ethanol (**6**)	**7**	12%
Lac	Allyl alcohol	**8**	n.p.
GalNAc	Allyl alcohol	**9**	35%
	*t*Boc-2-(2′-Aminoethoxy)ethanol (**6**)	**10**	7%

[a] n.p.: no product isolated. n.r.: no reaction observed.

on a carboxylated functionalized matrix and used in lectin interaction studies performed by SPR.

Results and discussion

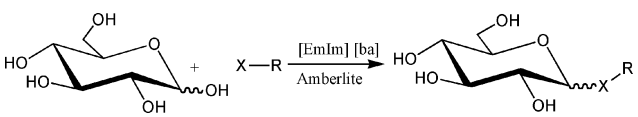
Synthesis of functionalized carbohydrates

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.[21]

This RTIL was also previously used for the glycosylation of bulky acceptors, including monosaccharides and benzyl alcohol.[15] 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).

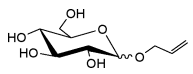
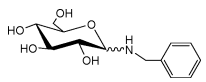
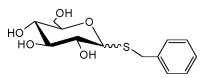
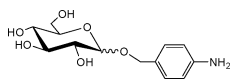
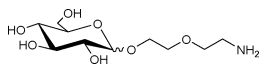
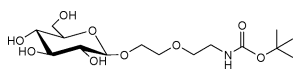
The reaction, carried out following the conditions described in the Experimental section, led to formation of the desired compound in 35% yield (Scheme 1, Table 2). Some stereoselectivity was observed in this glycosylation reaction, giving primarily the α-glycoside (α:β, 77:23), as previously described for similar processes.[15,22]



Scheme 1 General glycosylation reaction of Glc with various acceptors.

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.

Table 2 Glycosylation of Glc with different linker chains[a]

Compound	Product	*t*	Yield	α:β
1		17 h	35%	77:23
2		6 h	n.p.	17:83
3		6 h	n.p.	59:41
4		7 d	n.r.	—
5		6 h	n.p.	50:50
7		24 h	12%	40:60

[a] n.p.: no product isolated. n.r.: no reaction observed.

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 ^{1}H-NMR. Further isolation failed to afford the desired product due to the high viscosity of the RTIL at room temperature (Table 2). [emIm][ba] RTLs are relatively viscous RTLs with a viscosity of 425 cP at 25 °C, making both stirring and product recovery difficult.[15]

Next, a bifunctional linker chain was tested as a glycosylation acceptor in [emIm][ba], the *tert*-butoxycarbonyl (*t*Boc) 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 *t*Boc 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

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Table 3 Glycosylation of non-modified monosaccharides with allyl alcohol as linker chains in the presence of [emIm][ba]

Compound	Donor	Linker chain	Yield	Percentage of α-derivative
1	Glc	$HOCH_2CH{=}CH_2$	35%	77%
9	GalNAc		35%	80%

(40:60) in 12% yield (Table 2). The α and β configurations were inferred from the chemical shifts and coupling constant of the anomeric proton singnal.

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 ^{1}H 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.[15,22]

The bifunctional linker, *t*Boc 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 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, compared to traditional glycosylation procedures, requiring several intermediate steps, the low overall yields associated with direct glycosylation in RTIL was considered acceptable. Interestingly, glycosylation by GalNAc exclusively afforded the α-glycoside (Table 4).

Deprotection of *t*-Boc with trifluoroacetic acid (TFA) quantitatively afforded the glycoconjugate **11**. This reaction, in the absence of water and at room temperature was complete within 3 min, and deprotected compound **11** decomposed to a complex mixture unless residual TFA was quickly removed and the sample was stored cold (< 4 °C). Thus, compound **11** was best immediately immobilized on the surface of the biosensor chip for carbohydrate–protein interaction studies.

Table 4 Glycosylation of monosaccharides with 2-(2′-aminoethoxy)-ethanol protected with different protecting groups

Product	Donor	Linker chain	Yield	Percentage of α-derivative
7	Glc	$HOCH_2CH_2OCH_2CH_2NH$-*t*Boc	12%	43%
10	GalNAc		7%	99%

Functionalization 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[23] 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).

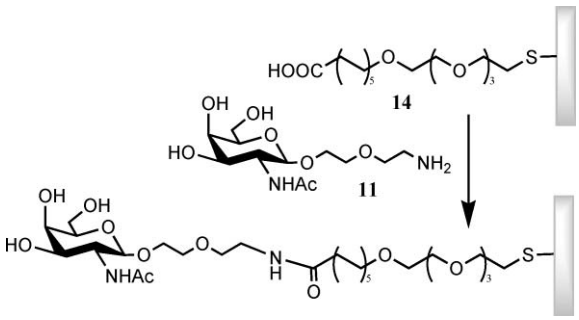
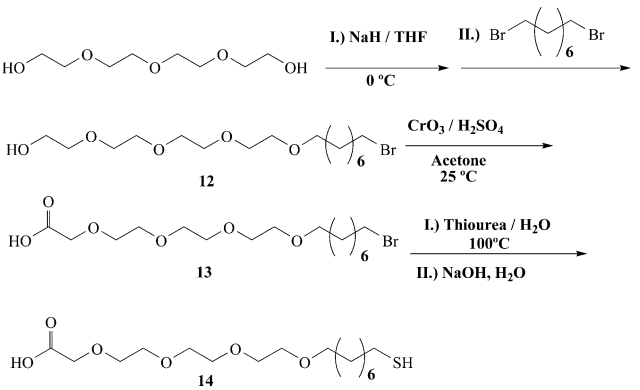


Fig. 1 Preparation of the chip with mixed chains and immobilization of functionalized glycoconjugate **14**.

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-dibromooctane 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.



Scheme 2 Synthesis of compound **14**.

The GalNAc glycoconjugate **11** was immobilized to the SAM on the biosensor chip using standard EDC/NHS activation (Fig. 1). After several injections of glycoconjugate **11**, 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 **11** 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: 6.72×10^4 M^{-1}; K_D: 1.49×10^{-5} M, clearly demonstrating binding in the micromolar range (Fig. 2). SPR, using the CM5 surface, showed no observable interaction.

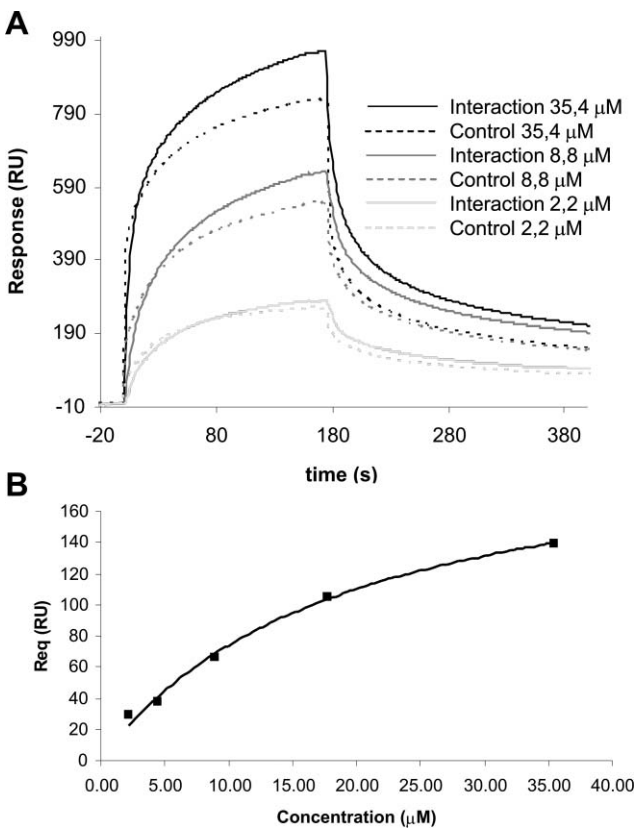


Fig. 2 A. Sensorgrams of the glycoconjugate–lectin interaction compared to the control sensorgrams. B. Steady-state affinity study of the interaction between B subunit from *Viscum album* lectin with glycoconjugate **11**, immobilized on an Au chip.

Experimental

The RTIL [emIm][ba] was synthesized according to the reported procedure[15] 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 (δ) 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_2SO_4 in CH_3OH 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.[24,25]

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_3OH. Solvent was removed and product purified by column chromatography (CH_2Cl_2–CH_3OH 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^+) (300 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_2Cl_2–CH_3OH gradient (9:1 to 3:1) affording 21 mg of product in 35% yield (Table 1).

^{1}H-NMR (500 MHz, D_2O): 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-1α), 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). ^{13}C-NMR (125 MHz, D_2O): 133.69, 118.34, 97.47, 73.26, 71.99, 71.38, 69.81, 68.61, 60.66.

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MS: [M + Na] calcd: 243.1, found: 242.7. [M+ Cl] calcd: 255.6, found: 256.9.

Synthesis of *t*-Boc-2-(2′-aminoethoxy)ethanol (6)

2-(2′Aminoethoxy)ethanol (10 5 μL, 0.95 mmol) was dissolved in 15 mL of anhydrous THF and cooled by stirring in an ice-water bath, NEt_3 (13 μL) was added and the reaction mixture was stirred under argon atmosphere at 0 °C. $(t\text{-Boc})_2O$ (311 mg, 1.42 mg) was slowly added to the reaction mixture at 0 °C, and then allowed to warm to room temperature with stirring. When reaction was complete, aqueous $NaHCO_3$ was added and this mixture was extracted with ethyl acetate (3 × 50 mL). The organic phase was dried with anhydrous $MgSO_4$, filtered and the solvent was removed by rotary evaporation. The final product was purified by column chromatography (hexanes–EtOAc gradient 1:1 to 1:9) affording compound **6** (158 mg, 81%).

^{1}H-NMR (500 MHz, $CDCl_3$): 5.15 (s, 1H, –NH–), 3.66 (t, 2H, J = 4.8 Hz, H-2′), 3.53 (t, 2H, J = 4.8 Hz, H-1′), 3.50 (t, 2H, J = 5.5 Hz, H-2), 3.23 (t, 2H, J = 5.5 Hz, H-1), 2.73 (s, –OH), 1.44 (s, 9H,–$(CH_3)_3$). ^{13}C-NMR (125 MHz, $CDCl_3$): 156.10, 79.35, 72.16, 70.26, 61.62, 40.27. ESI-MS: [M + Na] calcd: 228.1, found: 228.0.

Synthesis of *t*-Boc-2-(2′-aminoethoxy)ethyl-glucopyranoside (7)

t-Boc-2-(2′aminoethoxy)ethanol (58 mg, 0.28 mmol) was added to a mixture of Amberlite IR-120 (H^+) (30 mg) and Glc(5 mg, 0.023 mmol) in [emIm][ba] (30 mg, 0.13 mmol) under identical purification and reaction conditions as described in the general procedure. Column chromatography was performed in CH_2Cl_2–CH_3OH (50:1 to 4:1) gradient, affording compound **7** (1 mg, 12% yield) (Table 1).

^{1}H-NMR (500 MHz, D_2O): 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,–$(CH_3)_3$). ^{13}C-NMR (125 MHz, D_2O): 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] calcd: 390.2, found: 389.1. [M + Cl] calcd: 402.7, found: 403.5.

Synthesis of 1-*O*-allyl-2-acetamido-2-deoxy-galactopyranoside (9)

Allyl alcohol (300 μL, 5.0 mmol) was added to a mixture of Amberlite IR-120 (H^+) (60 mg) and GalNAc (10 mg, 0.05 mmol) in [emIm][ba] (60 mg, 0.26 mmol) under identical purification and reaction conditions as described in the general procedure. The solvent was removed with a high-vacuum pump to afford compound **9** (4 mg, 35%) (Table 1).

^{1}H-NMR (500 MHz, D_2O): Allyl: 5.90 (m, 1H, H-2), 5.28 (dd, 1H, J = 17.2, J = 1.5 Hz, H-3a), 5.23 (dd, 1H, J = 10.6, J = 1.1 Hz, H-3b), 4.13 (1H, H-1a), 3.98 (dd, 1H, J = 13.0, J = 6.1 Hz, H-1b). GalNAc: 4.89 (d, 1H, J = 3.8 Hz, H-1α), 4.10 (dd, 1H, J = 10.9 J = 3.8 Hz, H-2), 3.94–3.90 (m, 2H, H-6), 3.86 (dd, J = 10.9 J = 3.2 Hz, H-3), 3.72–3.64 (m, 2H, H-4, H-5), 1.90 (s, 3H, –CH_3). ^{13}C-NMR (125 MHz, D_2O): 175.04, 134.10, 122.20, 118.34, 96.66, 71.41, 68.89, 68.11, 61.65, 50.33, 22.34. MS: [M + Na] calcd: 284.1, found: 283.7. [M + Cl] calcd: 296.6, found: 296.9.

Synthesis of *t*-Boc-2′-(2″-aminoethoxy)ethyl-2-acetamido-2-deoxy-galactopyranoside (10)

t-Boc-2-(2′aminoethoxy)ethanol (232 mg, 1.13 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 CH_2Cl_2–CH_3OH (5:1) afforded compound **10** (3 mg, 7%) (Table 1).

^{1}H-NMR (500 MHz, D_2O): 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, –$(CH_3)_3$). 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, –CH_3). ^{13}C-NMR (125 MHz, D_2O): 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] calcd: 431.2 found: 430.8. [M + Cl] calcd: 443.5 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.

^{1}H-NMR (500 MHz, D_2O): 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, H-1′ 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, –CH_3). ^{13}C-NMR (125 MHz, D_2O): 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] calcd: 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 tetraethyleneglycol (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-dibromooctane (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 (CH_2Cl_2–CH_3OH 20:1) affording **12** in 40% yield.

^{1}H-NMR(300 MHz, $CDCl_3$): 3.62 (m, 16H, H-1 a 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). ^{13}C-NMR (125 MHz, $CDCl_3$): 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 $C_{16}H_{33}BrO_5$: 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 acetone (50 mL) was stirred at room temperature and Jones reagent (2.4 mmol in CrO_3) 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 acetone. The aqueous mixture was extracted with CH_2Cl_2 (3 × 30 mL) and the solvent was removed by rotary evaporation. Purification of final product was carried out by column chromatography (CH_2Cl_2–CH_3OH 10:1) yielding **13** in 70%.

^{1}H-NMR (300 MHz, $CDCl_3$): 6.71 (s, 1H, COOH), 4.13 (s, 2H, H-2), 3.56 (m, 12H, H-3 a 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). ^{13}C-NMR (125 MHz, $CDCl_3$): 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 $C_{16}H_{31}BrO_6$: C: 48.12%; H: 7.82%. Found: C: 48.20%; H: 7.81%. ESI-MS: [M + Na] Calcd: 421.1. Found: 421.0.

Synthesis of 20-mercapto-3,6,9,12-tetraoxatrieicosan-1-oic acid (14)

A solution of **13** (230 mg, 0.5 mmol) and thiourea (120 mg, 1.5 mmol) in 30 mL of water was heated under reflux for 6 h. After the addition of NaOH (600 mg, 15 mmol), the heating was resumed for an additional period of 6 h. After the mixture was cooled at room temperature, the pH was adjusted to 1 with concentrated HCl. The resulting solution was extracted with CH_2Cl_2 (3 × 20 mL). The combined extracts were dried on $MgSO_4$ and evaporated to dryness, leaving an oil, which was purified by column chromatography (CH_2Cl_2–CH_3OH 5:2.) with a 60% yield.

^{1}H-NMR (300 MHz, $CDCl_3$): 8.23 (s, 1H, COOH), 4.0 (s, 2H, H-2), 3.56 (m, 12H, H-3 and H-8), 3.38 (t, 2H, J = 6.93 Hz, H-9), 2.58 (t,2H, J = 7.35 Hz, H-19), 1.58 (m, 4H, H-10, H-18), 1.20 (m, 14H, H-11, H-12, H-13, H-14, H-15, H-16, H-17). ^{13}C-NMR (125 MHz, $CDCl_3$): 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 $C_{16}H_{32}O_6S$: C: 54.52%; H: 9.15%; S: 9.10%. Found: C: 54.53%; H: 9.17%; S: 9.09%. ESI-MS: [M + Na] Calcd: 375.2. Found: 375.0.

Immobilization of glycoconjugates on the SPR chip

Immobilization of thiol chains on the gold surface. For the immobilization of the thiol linker functionalized with a carboxyl group, an Au chip (Biacore) was required. Flow was set at 2 μL min^{-1} and temperature at 25 °C using HBS-P (0.01 M HEPES, 0.15 M NaCl, pH= 7.4) as running buffer. A solution of the linker **14** was injected into two different flow cells until 721 and 1228 RU (relative response unit) were obtained.

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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