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To cite this version:

Arnaud Masselin, Antoine Petrelli, Maxime Donzel, Sylvie Armand, Sylvain Cottaz, et al.. Unprecedented Affinity Labeling of Carbohydrate-Binding Proteins with s -Triazinyl Glycosides. Bioconjugate Chemistry, 2019, 30 (9), pp.2332-2339. 10.1021/acs.bioconjchem.9b00432 . hal-02316484

HAL Id: hal-02316484

https://hal.science/hal-02316484

Submitted on 19 Oct 2022

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Unprecedented affinity-labelling of carbohydrate-binding proteins with *s*-triazinyl glycosides

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Abstract

Carbohydrate- protein interactions trigger a wide range of biological signalling pathways, the mainstays of physiological and pathological processes. However, there are an incredible number of carbohydrate-binding proteins (CBPs) that remain to be identified and characterised. This study reports for the first time the covalent labelling of CBPs by triazinyl glycosides, a new and promising class of affinity-based glycoprobes. Mono- and bis-clickable triazinyl glycosides were efficiently synthesised from unprotected oligosaccharides (chitinpentaose and 2'-fucosyl-lactose) in a single step. These molecules allow the specific covalent labelling of chitin-oligosaccharide-binding proteins (wheat germ agglutinin WGA and *Bc* ChiA1 D202A, an inactivated chitinase) and fucosyl-binding lectin (UEA-I), respectively.

Introduction

Protein–carbohydrate interactions are at the heart of many biological processes in all living organisms.[1] A small set of monosaccharides make up a fantastic glycocode that triggers physiological or pathological events through interactive binding between complex oligosaccharides and specific carbohydrate-binding proteins (CBPs), also called lectins, or

cell surface receptors. *N*-acetyl glucosamine (GlcNAc) is certainly the most ubiquitous monosaccharide. In addition to being a major building block of cell-surface glycoconjugates and glycoproteins in mammals, GlcNAc is also the monomeric unit of chitin, the second most abundant polysaccharide after cellulose. Chitin (β-(1–4)-poly-*N*-acetyl-D-glucosamine) is a structural polysaccharide in microbial and fungal cell walls, in animal conjunctive tissues and in arthropod exoskeletons.[2] Its oligomers, namely chitin oligosaccharides (COs), exhibit various biological properties often related to their degree of polymerisation (DP). For instance, COs have been reported to display anti-cancer and anti-inflammatory activities.[3] Oral administration of a CO mixture to a colon-26-bearing mouse model suppressed tumour growth through the induction of apoptosis and stimulation of the immune system.[4] COs (DP 4-6) also exhibit a protective effect in mice against opportunistic pathogens *Candida albicans*, *Pseudomonas aeruginosa* and *Listeria monocytogenes*.[5,6] The most thoroughly investigated activities of COs are, by far, those participating in plant defence and plant growth.[7] Long-chain COs (DP 6-8) are potent inducers of the immune response in rice and wheat, among others.[8] In contrast, short-chain COs (DP 4-5) induce oscillations of the calcium concentration in the cell nucleus, which is a hallmark of early arbuscular mycorrhizal and root-nodule symbiosis signalling in various legumes (*Pisum sativum*, *Medicago truncatula*, *Lotus japonicus*) and rice.[9,10]

Understanding how these closely related molecules can trigger such different and sometimes contradictory plant responses is a major challenge in agronomy, and requires isolation and characterisation of the related biological receptors. More generally, identifying CBPs – not only in plants but also in microbes and animals – is a major goal for the determination of the biological roles of carbohydrates.

Efficient labelling and isolation of CBPs requires the synthesis of affinity glycoprobes. The structure of these probes must be close to that of the original ligand, but should also allow the

introduction by bio-orthogonal reactions of chemical reporters, such as a fluorescent group or an isotope, and of an affinity tag for purification. To date, most glycoprobes reported in the literature are photoactivatable crosslinking compounds derived from mono- and disaccharides.[11,12] The synthesis of oligosaccharide probes is much less well studied. Among the few examples, gangliosides and Lewis X saccharidic portions have been successfully functionalised with AffiLight CHO,[13] an aminoxy-biotinyl-phenyldiazirine reagent but, to the best of our knowledge, no biological study with these molecules has been reported to date. Thus far, few plant receptors have been labelled with photoactivatable COs. CEBiP, a chitin elicitor receptor, was identified in rice cells by photoaffinity labelling with a radiolabelled azidophenyl-chitinoctaose.[14,15] More recently, a lipochitin oligosaccharide (LCO)-binding protein, LYR3 was identified in *Medicago truncatula* (a lucerne) by combining photoaffinity-labelling experiments with proteomics and transcriptomics.[16] Affinity probes, lacking a photo-crosslinking group, have also been developed in recent years. For example, ligand-directed chemistry (LDC) involves a probe that incorporates an electrophilic reactive group such as an arylsulfonyl or an acyl pyridinium.[17] Protein labelling occurs spontaneously by nucleophilic attack of an amino-acid residue in close proximity to the binding site on the activated ligand. Dimethylaminopyridine-tethered carbohydrates efficiently and specifically label concanavalin A, wheat germ agglutinin and congerin II.[18] Noteworthily, most of the synthetic procedures reported for the preparation of ligand-directed or photoactivatable oligosaccharidic glycoprobes generally suffer from low efficiency. There is thus a need for new and practical methods to synthesise affinity or photoaffinity-based glycoprobes and these new methods should be applicable to any type of carbohydrate, including COs whose chemical modification is particularly complex.[19,20]

Here, we report the preparation and biochemical evaluation of new class of affinity glycoprobes for the specific labelling of CBPs consisting in *s*-triazinyl glycosides.

Results and discussion

The first synthesis of *s*-triazinyl glycosides was reported as water-soluble analogues of the anti-tumour drug hexamethylmelamine.[21] Later, Shoda *et al.* reported the rapid preparation of dimethoxy-triazinyl (DMT) glycosides from unprotected carbohydrates and their use as novel donors in enzymatic reactions.[22,23] In particular, α-DMT-GlcNAc allows the synthesis of the terminal disaccharide of gastric mucin.[24] In addition to being readily installed at the anomeric position of free carbohydrates, the *s*-triazine moiety can carry three different substituents, thereby providing for the unique possibility of designing multifunctional compounds. In the present study, we designed and assessed novel triazinyl glycosides from oligosaccharides for the labelling of CBPs and more specifically for CO-binding proteins. We first prepared a chitinpentaose (CO-V) probe containing an azidoaryl photo-crosslinking moiety and a propargyl group for the subsequent introduction via copper(I)-catalysed alkyne-azide cycloaddition (CuAAC) of a fluorescent tag and assessed it against two model CO-binding proteins: wheat germ agglutinin (WGA) and an hydrolytically inactive mutant of *Bacillus circulans* WL-12 chitinase A1.[25] Unexpectedly, covalent protein labelling occurred under photoactivation and in the dark. To confirm this unique property of triazinyl glycosides to specifically label CBPs, new probes lacking a photoactive group, but containing two bio-orthogonal clickable groups (propargyl for CuAAC and furan for Diels-Alder conjugation on maleimide derivatives) were synthesised from CO-V and 2'-fucosyl-lactose and assessed against CO- and fucosyl-binding proteins, respectively (Figure 1).

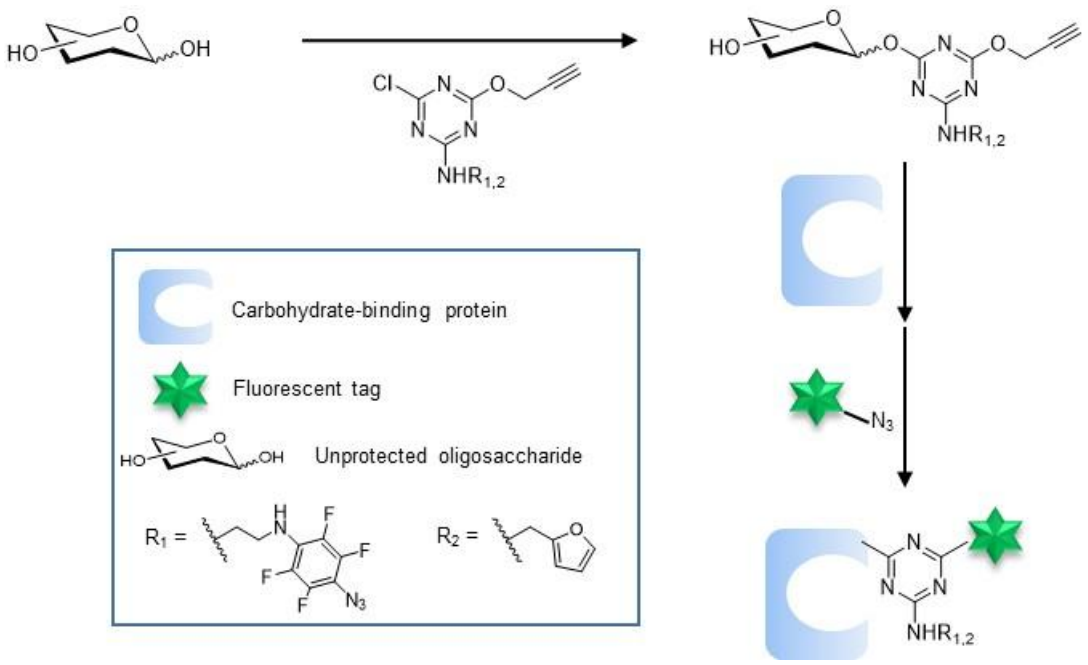
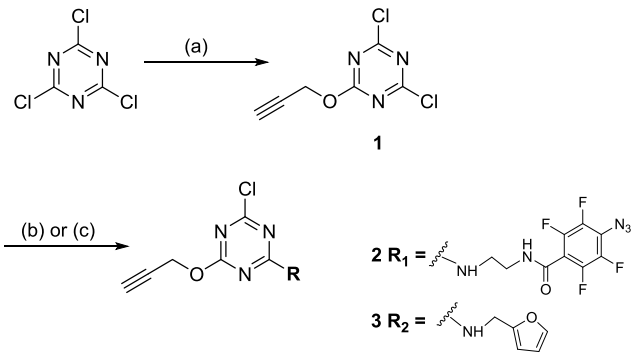


Figure 1. General approach for the synthesis of new *s*-triazinyl-based affinity glycoprobes from unprotected oligosaccharides and their use for the specific labelling of carbohydrate-binding proteins

Synthesis of multifunctional s-triazine scaffolds

s-Triazine scaffolds were prepared from commercially available cyanuric chloride (Scheme 1). A literature survey of cyanuric chloride reactivity confirmed that controlled nucleophilic substitution of each chloride can be carried out by taking advantage of the decrease in reactivity with the number of substituents. It also appeared that 2,4-dialkoxy-6-chloro-*s*-triazines are less stable than their 2,6-diamino counterparts but that the displacement of the chlorine atom therefore requires mild reaction conditions.[26,27] Preliminary experiments conducted on 2,4-dialkoxy-6-*s*-triazines confirmed the poor stability of the substituents. We have therefore decided to synthesise a 2-alkoxy-4-amino-6-chloro-*s*-triazine, which would combine both sufficient stability and reactivity with the anomeric hydroxyl of a carbohydrate. First, propargyloxy-*s*-triazine **1** was prepared with 80% yield as previously reported[28] Briefly,

the reaction was carried out in tetrahydrofuran (THF) at room temperature with one equivalent of propargyl alcohol and *N*,*N*-diisopropylethylamine (DIPEA). Among common photoreactive crosslinkers, azido-fluoro-aryls show high reactivity, low non-specific labelling and are sterically less cumbersome than benzophenone derivatives.[29] *N*-(2-aminoethyl)-4-azido-tetrafluorobenzamide (NH_2-ATFB)[30] was prepared and reacted with **1** under similar reaction conditions except for the temperature (0°C) to produce di-substituted *s*-triazine **2** with 50% yield. Amino-substituted *s*-triazines often exist as mixtures of rotamers in conformational equilibrium generating multiple peaks on NMR spectra.[31,32] As expected, most of the ^{1}H and ^{13}C NMR signals of **2** were split, making assignment difficult. Nevertheless, the unambiguous characterisation of **2** was achieved by mass spectrometry and NMR spectroscopy (see Supporting Information). The synthesis of propargyl, furyl-*s*-triazine **3** was carried out by reacting **1** with furfurylamine and DIPEA in THF. After purification, **3** was isolated with 89% yield and characterised by MS and NMR with the same difficulties met for **2**.

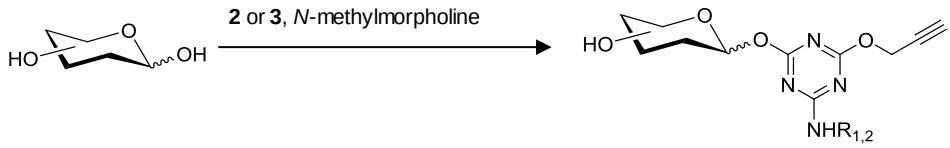


Scheme 1. Synthesis of di-substituted *s*-triazines containing one or two bio-orthogonal clickable groups. Reagents and conditions: (a) Propargyl alcohol (1 equiv.), DIPEA (1 equiv.) THF, 80% yield, (b) NH_2-ATFB (1 equiv.), DIPEA (1 equiv.), THF, 50% yield; (c) Furfurylamine (1 equiv.), DIPEA (1 equiv.), THF, 89% yield.

Synthesis of triazinyl glycosides

The anomeric functionalisation of CO-V was then evaluated directly with *s*-triazines **2** and **3** in a one-pot procedure (Figure 2). In the original syntheses,[22] triazinyl glycosides were obtained by reacting carbohydrates with a morpholinium salt of *s*-triazine (DMTMM) in the presence of 2,6-lutidine. We tested the one-pot activation of chloro-*s*-triazines and the subsequent reaction with sugar. Thus, **2** and **3** were dissolved in THF and added to an aqueous solution of CO-V containing four equivalents of *N*-methylmorpholine. We observed the formation of triazinyl glycosides under these conditions and, after purification on a C18 reverse-phase solid-phase extraction cartridge, compounds **4** and **5** were isolated with the same yields of 46%. Forming the morpholinium salts first and then performing the carbohydrate functionalisation in the presence of 2,6-lutidine did not improve the yield of the reaction, nor did using four equivalents of reagents **2** or **3**. The lower reactivity of COs relative to GlcNAc often requires a large excess of reactant to reach high conversion rates.[33] However, due to the poor solubility of **2** and **3** in water and for atom saving reasons, no further improvements were attempted. Considering the ease of purification of the triazinyl CO-V conjugates and the recovery of about 30% of the remaining unreacted CO-V, an overall yield close to 50% appeared satisfactory. MS and NMR analyses confirmed the expected structures of **4** and **5**. In particular, the anomeric proton next to the triazine moiety was shifted downfield to 6.3 ppm and appeared as two doublets (rotamers) with a coupling constant of 3 Hz, as previously reported for similar compounds.[24] To evaluate the versatility of the method and to assess the specificity of **4** and **5** toward CO-binding proteins, a fucosylated glycoprobe was also synthesised. When conducted on 2'-fucosyl lactose[34] with two equivalents of **3**, the reaction led to the formation of the expected glycoside **6** together with a di-substituted triazinyl derivative. Modifications at O1 and O2 of the glucosyl unit were reported by Shoda

et al. under similar conditions.[23] To avoid the formation of di-substituted products, the reactions were carried out with one equivalent of **2** or **3** and the triazinyl glycosides **6** and **7** were isolated with yields of 30% and 35%, respectively. MS and NMR (see Supporting Information) confirmed their structure. In particular, the expected β-configuration of the glycosides was confirmed with a coupling constant of 8 Hz of the anomeric signal at 5.82 ppm.



Sugar	NMM (equiv.)	*s*-Triazine (equiv.)	Product	Yield (α/β)
CO-V	4	**2** (2)	**4**	45% (α only)
CO-V	4	**3** (2)	**5**	45% (α only)
FucLac	2	**2** (1)	**6**	30% (β only)
FucLac	2	**3** (1)	**7**	35% (β only)

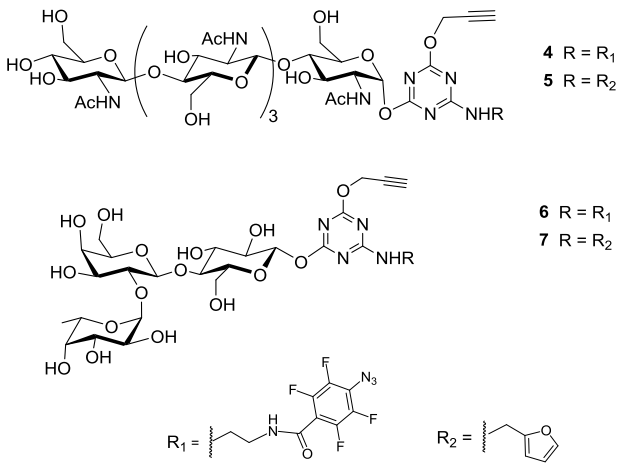


Figure 2. Direct synthesis of triazinyl-oligosaccharide glycoprobes

Protein labelling with triazinyl glycoprobes

The biochemical evaluation of the CO glycoprobes **4** and **5** was carried out with two GlcNAc-binding proteins. The first one is WGA which is a well-characterised plant lectin whose binding affinity for CO-III to V is in the low micromolar range.[35] The second one is BcChiA1 D202A, a hydrolytically inactive mutant of the Bacillus circulans WL-12 chitinase A1 (BcChiA1); it belongs to the GH-18 family that also displays low micromolar affinity for CO-V.[36] The BcChiA1 D202A mutant is devoid of hydrolytic activity but can still carry out

transglycosylation, and is used for the synthesis of long chitin oligosaccharides from oxazoline-activated donors.[25]

After checking that the triazinyl glycosides were stable in phosphate buffer pH 7 for 48 h using ^{1}H NMR monitoring (see Supporting Information), binding experiments were carried out with **4** and WGA in phosphate buffer. A CuAAC reaction with azido-fluorescein (FITC-N_3)[37] was then conducted to visualise the crosslinked ligand-protein after SDS-PAGE (Figure 3A-B).

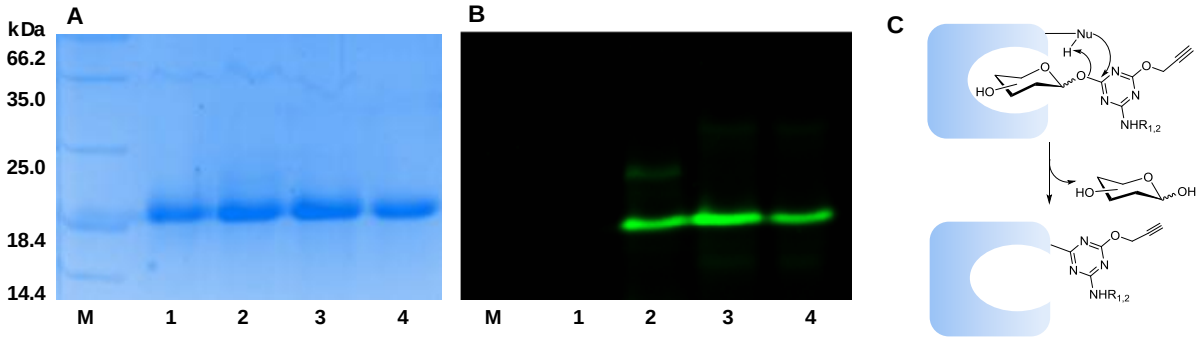


Figure 3. Crosslink of glycoprobes **4** and **5** to WGA. Glycoprobes **4** and **5** (5 μM) were incubated with WGA (0.1 mg/mL) in PBS buffer (60 mM, pH 7.2, 150 mM NaCl, 0.1 mM $MnCl_2$, 0.1 mM $CaCl_2$) and then labelled with FITC-N_3. After SDS-PAGE, WGA was detected by Coomassie blue staining (A) and by fluorescence detection (B): Lane M) Molecular weight marker; Lane 1) WGA; Lane 2) WGA, **4**, hυ 254 nm; Lane 3) WGA, **4,** dark; Lane 4) WGA, **5**; (C) Proposed mechanism for protein labelling in the absence of UV irradiation.

Without the glycoprobe, a polypeptide corresponding to the expected molecular mass of WGA (18 kDa) was detected by coomassie blue staining and no fluorescence was detected (Figure 3, Lane 1).[38] After photoactivation of **4** at 254 nm and tagging with FITC-N_3 using CuAAC, a single fluorescent signal at the same molecular mass than the WGA was observed

(Figure 3A-B, Lane 2). The fluorescence of WGA under denaturing conditions attested to the covalent binding of the glycoprobe to the protein. Unexpectedly, a similar result was observed when WGA was incubated with **4** in the dark before fluorescein tagging (Figure 3B, Lane 3). To confirm the ability of the triazinyl glycoside to label the CBP without photoactivation, WGA was incubated with **5** and subsequently tagged with FITC-N_3. Once again, Coomassie blue detected WGA at its expected molecular mass (18 kDa) and fluorescence detection confirmed the covalent binding of the propargyloxy-triazine moiety (Figure 3B, Lane 4). When conducted in presence of an excess of CO-V, the labelling of WGA by **5** was significantly decreased, indicating that the interaction between the triazinyl glycoside and the lectin takes place at its binding site (see Supporting Information). To explain the mode of action of **5**, we propose that a nucleophilic amino acid of the protein reacts with triazine, thereby causing the substitution of the carbohydrate moiety (Figure 3C). Apart from quaternary amines, which are good leaving groups, primary and secondary amino substituents of *s*-triazines, are quite stable. The substitution of the furyl part is thus unlikely to occur. In contrast, alkoxy substituents are prone to substitution and this property has already been used for the slow release of drugs from cellulose triazinyl ethers.[39] The substitution of both the propargyloxy group and the oligosaccharide part can take place, however only the second can generate a fluorescent protein after CuAAC with FITC-N_3. To confirm this hypothesis, the lability of the alkoxy substituents of *s*-triazine was then evaluated in phosphate buffer in the presence of a large excess of butylamine (100 equiv.) as a model nucleophile. Analysis of the crude reaction mixture by mass spectrometry confirmed the substitution of the oligosaccharide part of the *s*-triazinyl probes (see Supporting Information). The presence of products whose propargyl moiety has been substituted were also detected, albeit in lower amounts (see Supporting Information). These data seem to suggest that the oligosaccharide

moiety is a better leaving group than the propargyloxy group although this result requires further investigation to be confirmed.

To confirm the potential of **5** as a new specific probe for CO-binding proteins, we then performed labelling experiments with another CO-binding protein, the chitinase *Bc* ChiA1 D202A. *Bc* ChiA1 D202A has a molecular mass of 74 kDa and shares only 20% of sequence identity with WGA. Furthermore, their binding sites have different topologies as shown by their X-ray 3D structures.[40,41] The low homology between these two proteins is appropriate to assess the versatility of our new trazinyl glycosides as new CBP probes. Again, incubation of *Bc* ChiA1 D202A and **5** without any UV irradiation followed by CuAAC with FITC-N_3 resulted in protein labelling. The detection of *Bc* ChiA1 D202A was at the expected molecular mass (74 kDa) after SDS-PAGE, Coomassie blue staining and fluorescence detection (Figure 4A-B, Lane 1). Although the two proteins have no similarity apart from their affinity for chitin oligosaccharides, both were labelled with **5**, constituting strong evidence for affinity labelling.

To assess the specificity of the new probe, competitive labelling assays were carried out with the two above-mentioned CO-binding proteins and *Ulex europaeus* lectin UEA-I, a glycoprotein of 28 kDa which exhibits affinity for oligosaccharides containing α-L-fucosyl residues[42] but not for chitin oligosaccharides. An equimass solution of WGA and UEA was incubated with **5** for 1 h prior to conjugating FITC-N_3 using CuAAC. The reaction mixture was then analysed by SDS PAGE. The gel revealed by Coomassie blue staining displayed the bands corresponding to WGA and UEA-I at 18 kDA and 28 kDa, respectively. Detection using fluorescence showed that only the WGA was labelled by fluorescein (Figure 4, lane 2). As expected, the CO-glycoprobe **5**, which has no affinity for UEA-I, reacted exclusively with the CO-binding protein. The same result was observed when **5** was incubated with a mixture of *Bc* ChiA1 D202A and UEA-I. Specific labelling occurred exclusively on the chitinase

(Figure 4, lane 4). To further attest to the versatility of the newly synthesised probes and to confirm that the absence UEA-I labelling by **5** was not due to an inactive form of the fucose-binding lectin, the labelling of UEA-I by **7** was assessed in presence of WGA. When **7** was incubated in the presence of WGA and UEA-I, only UEA-I was fluorescently tagged (Figure 4, lane 3). Taken together, these data confirm that triazinyl glycosides hold promise as a new family of affinity-based probes for the labelling of CBPs.

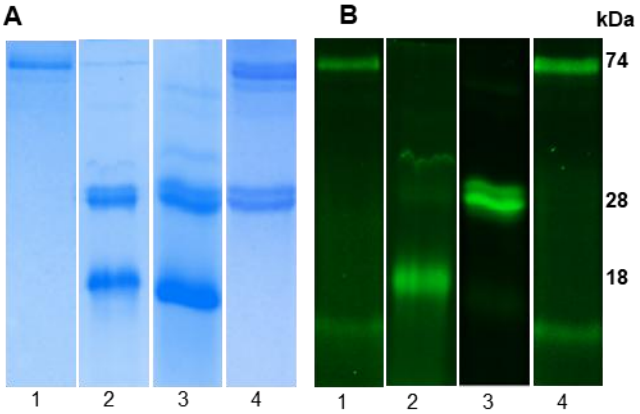


Figure 4. Specificity of glycoprobes **5** and **7** crosslink to GlcNAc and fucosyl binding proteins respectively. Glycoprobes **5** or **7** (5 μM) were incubated with *Bc* ChiA1 D202A, WGA or UEA-I (0.1 mg/mL) in PBS buffer (60 mM, pH 7.2, 150 mM NaCl, 0.1 mM $MnCl_2$, 0.1 mM $CaCl_2$) and then labelled with FITC-N_3. After SDS-PAGE, proteins were detected by Coomassie blue staining (A) and by fluorescence detection (B): Lane 1) *Bc* ChiA1 D202A, **5**; Lane 2) WGA and UEA-I, **5**; Lane 3) UEA-I and WGA, **7**; Lane 4) *Bc* ChiA1 D202A and UEA-I, **5**.

Conclusion. In this study, we designed and assessed novel affinity glycoprobes consisting of clickable *s*-triazinyl glycosides. These probes are conveniently synthesised from unprotected oligosaccharides and 2-chloro-2,5-di-substituted-*s*-triazine in one step in aqueous media.

Chitinpentaose and 2'-fucosyl-lactose probes containing two bio-orthogonal clickable groups (alkyne and furyl) on the triazinyl moiety were isolated with 30-40% yield after simple purification on a reversed-phase C18 cartridge. Biochemical assays conducted with two model lectins (WGA, UEA-I) and an inactivated chitinase (*Bc* ChiA1 D202A) demonstrated the ability of the chitinpentaose probe to specifically crosslink to two unrelated CO-binding proteins and of the 2'-fucosyl-lactose probe to specifically crosslink to a fucosyl binding protein. Protein labelling was shown to occur by substitution of the oligosaccharide part of the triazine scaffold. The covalent protein-triazine conjugate is stable enough to allow its detection after the CuAAC reaction with a fluorescent tag. Further work is now necessary to quantify the protein labelling and to determine which amino acid(s) are involved in the reaction with the triazinyl moiety. In particular, it will be interesting to determine if the labelling is conditioned by the presence of a specific amino acid in close proximity to the protein binding site. So far, only the alkyne function of the probes has been used to introduce a fluorescent tag. With the aim of discovering new carbohydrate receptors, the furyl group should allow the introduction of a biotin or another molecule allowing their isolation. These versatile new probes are of particular interest for identification of plant receptors for short-chain COs and will be thus evaluated on plant extracts.

Material and methods

General. Chitinpentaose was prepared by *N*-acetylation of a commercially available mixture of chitosan oligosaccharides.[43] Recombinant *Bc* ChiA1 D202A was expressed in *E. coli.*[25] 2,4-Dichloro-6-prop-2-ynyloxy-[1,3,5]-triazine **1,**[28] *N*-(2-aminoethyl)-4-azido-tetrafluorobenzamide (ATFB- NH_2)[30] and FITC-N_3[37] were synthesized as reported in the literature. Other chemicals were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier) or TCI (Europe) and used directly without further purification.

NMR spectra were recorded in $CDCl_3$, D_2O or CD_3OD, with a liquid Brücker Avance III 400MHz spectrometer (^1H: 400 MHz, ^{13}C: 100 MHz, ^{19}F: 376 MHz) or a liquid Brücker Avance III 500 MHz spectrometer (^{13}C: 125 MHz). The solvent residual peaks of D_2O, CD_3OD, and $CDCl_3$ were used as internal standards, at 4.79 ppm (298 K), 3.31 ppm (298 K), and 7.26 ppm (298 K), respectively. Chemical shifts δ are reported in ppm relative to the solvent residual peak. Coupling constants *J* are expressed in Hz. Multiplicity is designated by: s (singulet), d (doublet), t (triplet), m (multiplet). Rotamers are indicated by a *.

Reactions were monitored by TLC (Merck Silica gel 60 F254). Detection of COs was achieved under UV (254 nm) then by dipping into a diphenylamine-aniline-phosphoric acid staining solution (4 g diphenylamine, 4 ml of aniline, 20 ml of 85% H_3PO_4 in H_2O, 200 ml of acetone) followed by heating with a heatgun. Detection of FucLac oligosaccharides was achieved under UV (254 nm) and by dipping into a 30% ammonium bisulfate water solution followed by heating. Detection of non-carbohydrate molecules was achieved under UV (254 nm) and staining with a KMnO4 solution (1.5 g $KMnO_4$, 10 g K_2CO_3, 1.25 mL 10% NaOH in 200 mL water) followed by heating. Purifications were performed by manual flash chromatography (wet-packed silica, Merck 0.04-0.063 mm) or by automated flash chromatography on a Grace Reveleris instrument using prepacked silica columns.

Mass analyses (ESI and APCI) were recorded with an ESQUIRE 3000+ (Brücker Daltonics). MALDI-TOF spectra were recorded with a Maldi-ToF-Tof Speed (Brücker Daltonics) and HRMS spectra were recorded with a ESI/QTOF –Xevo G2-S QTof-Waters.

Photochemical reactions were performed with a RPR-100 Rayonet photochemical reactor.

SDS-polyacrylamide gel electrophoresis was performed in 15% gel according to Laemmli's procedure.[44] The gels were analyzed with UV sample tray on a Biorad Gel Doc™ EZ System and staining with brilliant blue R-250.

Synthesis

2-Chloro-4-(*N*-(2-aminoethyl)-4-azido-tetrafluorobenzamide)-6-(prop-2-ynyloxy)-1,3,5-triazine (2). *N*-(2-aminoethyl)-4-azido-tetrafluorobenzamide (50 mg, 0.18 mmol) was added at 0 °C to a stirred solution of **1** (37 mg, 0.18 mmol)[28] and DIPEA (31 µL, 0.18 mmol) in THF (1 mL). The reaction mixture was stirred and allowed to warm up to room temperature over 3h. The solution was diluted with CH_2Cl_2 (3 mL) and washed with H_2O (3 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated. After purification by flash chromatography on silica gel eluting with a gradient of EtOAc in Petroleum Ether (10% to 20%), **2** was isolated with 50% yield (40 mg). R_f 0.7 (Petroleum ether/ EtOAc 6:1); ^{1}H NMR (400 MHz, CD_3OD) δ 5.03 and 4.98 (2d*, *J* = 2.4 Hz, 2H, OC*H*$_2$C≡CH), 3.67-3.61 (m, 4H, NCH_2CH_2N) 2.99-2.97 (m, 1H, OCH_2C≡C*H*); ^{1}H-decoupled ^{13}C NMR (126 MHz, CD_3OD) δ 170.8, 170.0, 169.9, 169.4, 167.2, 159.0, 143.5 (d, *J* = 250 Hz, *C*F), 140.4 (d, *J* = 252 Hz, *C*F), 121.9-121.7, 111.8-111.5, 77.0, 75.5, 55.0, 39.9-39.8, 38.9-38.7; ^{19}F-decoupled ^{13}C NMR (126 MHz, CD_3OD) δ 171.2, 170.6, 170.4, 169.3, 167.7, 159.5, 144.1, 141.0, 122.3, 112.2, 77.8-77.1 (m), 75.1-75.0 (m), 56.9-54.1 (m), 41.5-38.1 (m); ^{19}F (376 MHz, CD_3OD) δ ppm -140 (m, 2F), -150 (m, 2F); HRMS (ESI) *m/z* calcd for $C_{15}H_9ClN_8O_2F_4$: 445.0551 $[M+H]^+$; found: 445.0555.

*Rotamers.

2-Chloro-4-(furan-2-ylmethylamino)-6-(prop-2-ynyloxy)-1,3,5-triazine (3). Furfurylamine (433 µL, 4.9 mmol) was added at 0 °C to a stirred solution of **1** (1g, 4.9 mmol)[28] and DIPEA (854 µL, 4.9 mmol) in THF (15 mL). The reaction mixture was stirred and allowed to warm up to room temperature over 1h. The solution was diluted with CH_2Cl_2 (70 mL) and washed with H_2O (30 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated. After purification by flash chromatography on silica gel eluting with a gradient of EtOAc in Petroleum Ether (10% to 30%), **3** was isolated with 89% yield (1.15 g). R_f 0.4 (Petroleum ether/EtOAc 6:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37-7.36 (m, 1H, OCH^{furyl}), 6.51 (s, 0.6H,

NH), 6.34-6.27 (m, 2H, CH^{furyl}), 6.19 (s, 0.4H, NH), 5.00 and 4.94 ($2d^*$, J = 2.4 Hz, 2H, $OCH_2C{\equiv}CH$), 4.67-4.65 (m, 2H, CH_2NH), 2.51 and 2.49 ($2t^*$, J = 2.4 Hz, 1H, $OCH_2C{\equiv}CH$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.4, 170.5, 170.2, 169.8, 166.7, 166.6, 150.0, 149.9, 142.4, 142.3, 110.4, 110.3, 107.9, 107.7, 77.0, 76.9, 75.5, 75.4, 55.4, 55.2, 38.1, 38.0; HRMS (ESI) *m/z* calcd for $C_{11}H_9N_4O_2Cl$: 265.0502 $[M+H]^+$; found: 265.0492.

* Rotamers.

CO-V glycoprobe (4). In a plastic microtube, *N*-methylmorpholine (8.4 µL, 77.4 µmol) was added to stirred solution of CO-V (20 mg, 19 µmol) in deionized water (1 mL). A solution **2** (17 mg, 38 µmol) in THF (100 µL) was then added and the reaction mixture was stirred overnight. The solution was poured into acetone (10 mL) and the precipitate was recovered by centrifugation (4°C, 7000 rpm, 20 min). This crude solid was purified by reversed phase chromatography (1g C18 SPE cartridge) eluting with a gradient of MeOH (0 to 100%) in water. After freeze-drying, **4** was isolated with 46% yield (13 mg). R_f 0.1 (H_2O/ACN 2:8) ^1H NMR (400 MHz, D_2O) δ 6.44 and 6.34 ($2d^*$, J = 3.2 Hz, 1H, $H\text{-}1^{GlcNAc(I)}$), 4.93 (m, 2H, $OCH_2C{\equiv}CH$), 4.56-4.54 (m, 4H, $H\text{-}1^{GlcNAc(II\text{-}V)}$), 3.90-3.43 (m, 34H, $H\text{-}2,3,4,5,6^{GlcNAc(I\text{-}V)}$, NCH_2CH_2N), 2.03-1.89 (m, 15H, $COCH_3$); ^{13}C NMR (101 MHz, D_2O) δ 174.6, 174.5, 169.5, 168.0, 101.4, 101.2, 101.1, 79.1, 78.9, 79.8, 78.4, 78.5, 75.9, 74.5, 74.4, 73.4, 72.5, 72.4, 72.1, 72.0, 69.7, 67.0, 68.4, 60.5, 59.9, 59.4, 55.5, 55.1, 55.0, 54.1, 52.6, 52.4, 39.5, 22.1, 22.0; ^{19}F NMR (376 MHz, D_2O) δ -143.7-143.9 (m, 2F), -151.1/-151.2 (m, 2F); HRMS (ESI) *m/z* calcd for $C_{55}H_{75}N_{13}O_{28}F_4$: 1464.4678 $[M+Na]^+$; found: 1464.4645.

*Rotamers.

CO-V glycoprobe (5). In a plastic microtube, *N*-methylmorpholine (8.4 µL, 77.4 µmol) was added to stirred solution of CO-V (20 mg, 19 µmol) in water (1 mL). A solution of **3** (10.2 mg, 38 µmol) in THF (100 µL) was then added and the reaction mixture was stirred

overnight. The solution was poured into acetone (10 mL) and the precipitate was recovered by centrifugation (4°C, 7000 rpm, 20 min). This crude solid was purified by reversed phase chromatography (1g C18 SPE cartridge) eluting with a gradient of MeOH (0 to 100%) in water. After freeze-drying, **5** was isolated with 45% yield (11.5 mg). R_f 0.3 (H_2O/ACN 2:8) ^{1}H NMR (400 MHz, D_2O) δ 7.47 (m, 1H, OCH^{furyl}), 6.55-6.34 (m, 3H, CH^{furyl}, H-$1^{GlcNAc(I)}$), 5.09 and 4.81 (2s*, 2H, OC*H*$_2$C≡CH), 4.61-4.57 (m, 6H, H-$1^{GlcNAc(II-V)}$, CH_2NH), 4.13-3.45 (m, 30H, H-2,3,4,5,$6^{GlcNAc(I-V)}$), 2.09-1.96 (m, 15H, $COCH_3$); ^{13}C NMR (101 MHz, D_2O) δ 174.6, 160.4, 151.3, 142.5, 110.5, 107.4, 101.4, 101.2, 79.1, 78.9, 78.6, 75.9, 74.5, 73.4, 72.1, 69.7, 69.0, 60.5, 59.9, 55.6, 55.0, 52.5, 37.5, 34.3, 21.1, 21.7; HRMS (ESI) *m/z* calcd for $C_{51}H_{75}N_9O_{28}$: 1284.4592 $[M+Na]^+$; found: 1284.4619.

*Rotamers.

FucLac glycoprobe (6) In a plastic microtube, *N*-methylmorpholine (13 µL, 123 µmol) was added to stirred solution of 2'-fucosyllactose (30 mg, 61 µmol) in water (1 mL). A solution of **2** (27 mg, 61 µmol) in THF (100 µL) was then added and the reaction mixture was stirred overnight. The solution was poured into acetone (10 mL) and the precipitate was recovered by centrifugation (4°C, 7000 rpm, 20 min). This crude solid was purified by reversed phase chromatography (1g C18 SPE cartridge) eluting with a gradient of MeOH (0 to 100%) in water. After freeze-drying, **6** was isolated with 30% yield (15 mg). R_f 0.2 (ACN/H_2O, 8:2); ^{1}H NMR (400 MHz, D_2O) δ 5.85 and 5.81 (2d*, *J* = 8.0 Hz, 1H, H-1^{Glc}), 5.32 (m, 1H, H-1^{Fuc}), 5.00 (m, 2H, OC*H*$_2$C≡CH), 4.57 (d, *J* = 7.6 Hz, 1H, H-1^{Gal}), 4.24 (m, 1H, H-5^{Fuc}), 3.93-3.61 (m, 19H, NC*H*$_2$C*H*$_2$N, H-2,3,4^{Fuc}, H-2,3,4,5,6^{Gal}, H-2,3,4,5,6^{Glc}), 2.27 and 2.23 (2s*, OCH_2C≡C*H*), 1.25 (m, 3H, CH_3^{Fuc}); ^{13}C NMR (101 MHz, D_2O) δ 170.9, 170.8, 170.5, 170.4, 168.1, 167.9, 160.7, 144.7, 142.2, 141.5, 139.9, 100.2, 99.5, 99.4, 96.6, 96.4, 77.6, 76.6, 76.4, 5.9, 75.6, 75.2, 75.1, 74.0, 73.6, 73.5, 71.8, 71.7, 69.6, 69.0, 68.2, 67.0, 66.9, 61.0, 59.6, 55.2,

39.6, 15.3; ^{19}F NMR (376 MHz, D_2O) δ -143.4-143.59 (m, 2F), -151.1/-151.2 (m, 2F); HRMS (ESI) *m/z* calcd for $C_{33}H_{40}N_8O_{17}F_4$: 897.2526 $[M+H]^+$; found: 897.2505.

*Rotamers.

FucLac glycoprobe (7) In a plastic microtube, *N*-methylmorpholine (22.5 µL, 0. µmol) was added to stirred solution of 2'-fucosyllactose (50 mg, 0.1 mmol) in water (1 mL). A solution of **3** (30 mg, 0.1 mmol) in THF (100 µL) was then added and the reaction mixture was stirred overnight. The solution was poured into acetone (10 mL) and the precipitate was recovered by centrifugation (4°C, 7000 rpm, 20 min). This crude solid was purified by reversed phase chromatography (1g C18 SPE cartridge) eluting with a gradient of MeOH (0 to 100%) in water. After freeze-drying, **7** was isolated with 30% yield (15 mg). R_f 0.2 (ACN/H_2O, 8:2); ^{1}H NMR (400 MHz, D_2O) 7.45 (m, 1H, OCH^{furyl}), 6.41-6.31 (m, 2H, CH^{furyl}), 5.89 and 5.81 ($2d^*$, *J* = 8.0 Hz, 1H, H-1^{Glc}), 5.32-5.30 (m, 1H, H-1^{Fuc}), 5.06 and 5.02 (2d*, *J* = 2.8 Hz, 2H, $OCH_2C{\equiv}CH$), 4.65-4.48 (m, 3H, *H*-1^{Gal}, CH_2NH), 4.24-4.18 (m, 1H, H-5^{Fuc}), 3.99-3.59 (m, 14H, H-2,3,4^{Fuc}, H-2,3,4,5,6^{Gal}, H-2,3,4,5,6^{Glc}), 2.97 and 2.79 (2t*, 1H, *J* = 2.8 Hz, $OCH_2C{\equiv}CH$), 1.23 (2d*, 3H, *J* = 6.5 Hz, CH_3^{Fuc}); ^{13}C NMR (101 MHz, D_2O) δ 171.0, 170.8, 170.6, 170.3, 167.4, 167.3, 151.4, 151.2, 142.6, 142.5, 142.4, 110.6, 110.5, 110.4, 107.4, 102.8, 100.2, 100.1, 99.5, 99.4, 96.5, 96.4, 76.9, 76.3, 76.0, 75.9, 75.3, 75.0, 74.2, 74.0, 73.5, 72.9, 72.5, 71.8, 69.6, 69.0, 66.9, 61.1, 59.7, 59.6, 55.4, 55.2, 37.5, 15.3; HRMS (ESI) *m/z* calcd for $C_{29}H_{40}N_4O_{17}$: 739.2293 $[M+Na]^+$; found: 739.2286.

*Rotamers.

Protein labelling with photoactivatable probes. In a plastic microtube (1.5 mL), protein (WGA, *Bc*Chi A1 D202A or UEA-I, 0.1 mg/mL) and photoactivatable probe **4** or **6** (5 µM) in PBS (60 mM, pH 7.2, NaCl 150 mM, $CaCl_2$ 0.1 mM, $MnCl_2$ 0.1mM, 200 µL) were incubated in a heat block at 22 °C in the dark during one hour. The mixture was then irradiated by UV-

radiation in a RPR-100 Rayonet photochemical reactor for 10 min. Grafting of fluorescein was achieved by addition of freshly prepared aqueous solutions of sodium ascorbate (1 mM final), $CuSO_4$ (1 mM final) and FITC-N_3 (5 µM final). After two hours at 25°C in dark, proteins were purified on Sephadex G-25 in PD-10 desalting columns and freeze dried. Proteins were taken back in 100 µL of water and 10 µL of this solution was analysed by SDS-PAGE. Protein visualization was performed by fluorescence and then by staining with Coomassie Brillant Blue dye.

Labelling of protein mixtures was achieved following the same protocol with the same amount of each protein (0.1 mg/mL).

Protein labelling of with probes (5 or 7). In a plastic microtube (1.5 mL), protein (WGA, *Bc*Chi A1 D202A or UEA-I, 0.1 mg/mL) and probe **5** or **7** (5 µM) in PBS (60 mM, pH 7.2, NaCl 150 mM, $CaCl_2$ 0.1 mM, $MnCl_2$ 0.1mM, 200 µL) were incubated at 22 °C in dark during one hour. Grafting of fluorescein was directly achieved by addition of freshly prepared aqueous solutions of sodium ascorbate (1 mM final), $CuSO_4$ (1 mM final) and FITC-N_3 (5 µM final). After two hours at 25°C in dark, proteins were purified on Sephadex G-25 in PD-10 desalting column and freeze dried. Proteins were taken back in 100 µL of water and 10 µL of this solution was analysed by SDS-PAGE. Protein visualization was performed by fluorescence and then by staining with Coomassie Brillant Blue dye.

Labelling of protein mixtures was achieved following the same protocol with the same amount of each protein (0.1 mg/mL).

Acknowledgments

A.M. thanks the “Fondation pour le développement de la Chimie des substances Naturelles et ses applications” for a PhD Grant. We acknowledge the financial support of ANR NiceCrops (ANR-14-CE18-0008-01), Labex ARCANE and CBH-EUR-GS (ANR-17-EURE-0003), Glyco@Alps (ANR-15-IDEX-02) and PolyNat Carnot Institut (ANR-16-CARN-0025-01). ICMG (FR 2607) mass spectrometry and NMR platforms are also acknowledged for their technical support.

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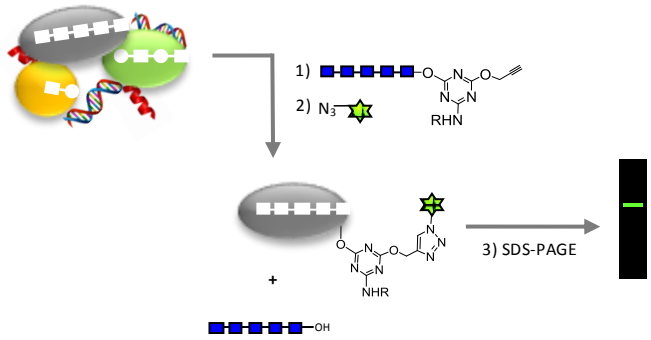
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