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**The Myrosinase-Glucosinolate system to generate neoglycoproteins:
a case study targeting mannose binding lectins.**

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Highlights :

- Enzymatically induced isothiocyanate formation through a Lossen rearrangement
- A natural myrosinase-glucosinolate/ enzyme-substrate system used for bioconjugation
- A one-pot strategy for the preparation of neoglycoproteins without isothiocyanate handling.

Abstract

A convenient strategy for a 'one-pot' synthesis of neoglycoproteins (NGP) was developed using the myrosinase-glucosinolate couple, a natural enzyme-substrate system. This enzymatic reaction allowed us to generate an isothiocyanate *in situ* which then reacted with the lysine residues of bovine serum albumin protein (BSA) to produce multivalent neoglycoproteins. Using two models, glucomoringin which is a natural glucosinolate bearing a L-rhamnose unit, and an artificial glucosinolate specifically designed for mannose type lectins, an average of up to 17.8 and 28.7 carbohydrate residues could be respectively grafted onto the BSA protein. This process is comparable to commercial approaches using BSA-Man_c without the disadvantage of handling harmful chemical reagents. Lectin binding screening (GLYcoPROFILE®) showed that among all NGPs synthesized, BSA-Man **16** gave similar and in some cases better affinities in comparison with commercial BSA-Man_c towards various mannose-specific lectins.

Keywords: myrosinase, glucosinolate, neoglycoprotein, isothiocyanate, lectin

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Conflict of interest

Giuliano Cutolo, Blanka Didak, Josip Tomas, Marie Schuler, Pierre Lafite, Reine Nehmé, and Arnaud Tatibouët declare that they have no conflict of interest. Benoit Roubinet and Ludovic Landemarre are employees of GLYcoDiag company.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors

Introduction

Glycoproteins are involved in diverse recognition processes (cell adhesion, trafficking, differentiation, migration and signalling). The complexity of the glycan structures decorating the surface of proteins makes the formation of well-defined glycoproteins a chemical challenge. Synthetic glycoproteins, also called neoglycoproteins (NGP) could serve as invaluable tools to better understand the biological properties of specific carbohydrates structures. More specifically, neoglycoproteins have an important role in targeting lectins interactions, taking advantage of the avidity effect through multivalent systems. [1] Indeed, numerous chemical methods have been developed to conjugate mono or oligosaccharides to the side chains of amino acids of a peptide or a protein scaffold to form these compounds. Construction using Solid Phase Peptide Synthesis (SPPS) has revealed to be long and tedious [2]. More straightforward approaches have been explored for the functionalization of selected proteins such as HSA (Human Serum Albumin), BSA (Bovine Serum Albumin) or OVA (Ovalbumin) [3] with better modularity for various applications and studies. New assembly methods have also been developed, such as the use of squaric acid derivatives through sequential nucleophilic additions [4]. This same chemistry was also applied to human galectin-3 inhibitors by linking chitooligomers to BSA [5]. *N*-hydroxysuccinimide (NHS) activated esters have been used to prepare fluorescent neoglycoproteins based on OVA and to develop gold nanoparticles as sensors of plant lectins [6]. The NHS coupling methodology was also applied to BSA to vary the density of antigens in order to pick up the full spectrum of antibodies in a mixture [7]. Incorporation of α -Gal epitope using the reactivity of thiols on maleimides functionalized BSA led to neoglycoproteins as tools to develop vaccines against Cutaneous leishmaniasis [8,9]. Copper Azide Alkyne Cycloaddition (CuAAC) coupling was recently applied to an alkyne modified BSA

to incorporate mannose-like structures resulting from an Amadori rearrangement [10]. Despite the development of newer synthetic methodology, more “classical approaches” are still worthwhile : reductive amination to incorporate linkers, but also grafting onto BSA or HSA with isothiocyanates [11,12,13]. The isothiocyanate strategy is the historical approach and remains a convenient technique for reaction with the free amino function of amino acids, thus creating a stable thiourea [14]. This strategy has been largely employed to obtain highly functionalized and complex NGPs [15,16]. However, some disadvantages can be highlighted: the tedious synthesis of an isothiocyanate on highly water-soluble saccharides, the instability of this function which affects their synthesis, isolation and storage, and above all the inherent toxicity of such a strong electrophile. Indeed, strong and toxic reagents such as thiophosgene, carbon disulfide, or thiocarbonyldiimidazole are the main reagents used to prepare isothiocyanates.

In this context, we wished to develop an enzymatic alternative and a simplified methodology to form an isothiocyanate function *in situ* that could be used for bioconjugation and applied to the preparation of NGPs. This method would avoid the main drawbacks of organic solvent handling, toxicity and stability of the ITC together with reduced effects on biological substrates. With this aim in mind, we set up a process to induce the formation of the isothiocyanate by taking advantage of the enzymatic activity of myrosinase. Myrosinase is a *S*-glucosidase able to hydrolyze glucosinolates (GLs) into glucose and a transient *O*-sulfated thiohydroximate species, which decomposes in water to sulfate and isothiocyanate [17]. GLs are secondary metabolites and the main components of the chemical defense of the plants of the Brassicales order against insects [18]. Thus, myrosinase is able to form toxic and reactive reagents (isothiocyanates) from stable and non-toxic substrates, namely glucosinolates (GLs) (Figure 1).

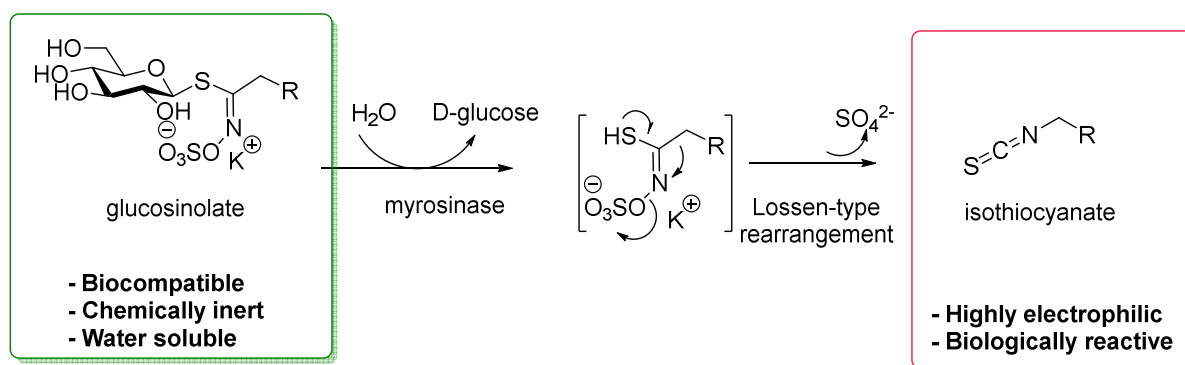


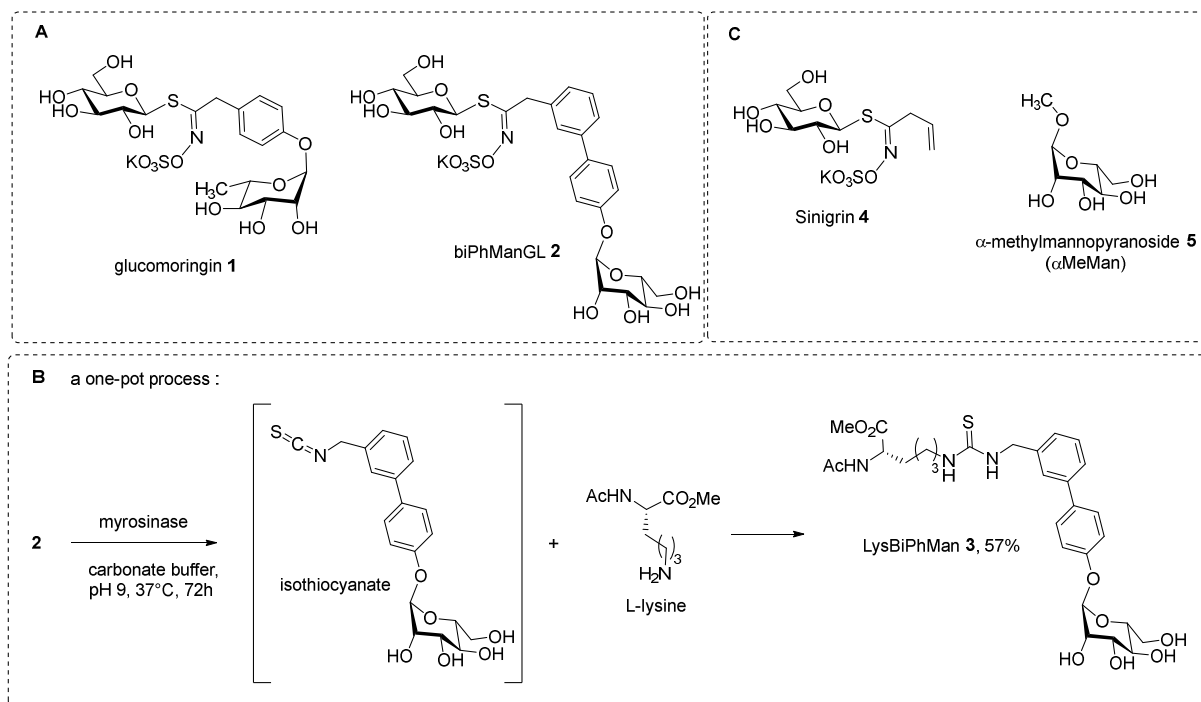
Figure 1: hydrolysis of a glucosinolate by myrosinase and formation of an isothiocyanate.

Myrosinase from *Sinapis alba* is able to hydrolyse not only natural GLs, but also artificial ones [19]. We have recently shown that the myrosinase-glucosinolate system (MG system) can be used as an enzymatic tool in bioconjugation [20,21]. Indeed, we have demonstrated the ability of myrosinase to produce isothiocyanates from constructs dedicated to target FimH while maintaining lectin recognition [20]. More recently, we have developed a process to incorporate fluorescent moieties or a biotin group that can be used to label BSA and furthermore be applied for fluorescent labelling in cells, thus demonstrating the compatibility of the MG system with living cells [21]. Recently the synthesis and the myrosinase degradation of a BODIPY fluorescent GL using CuAAC coupling was also reported [22].

In the present study, we demonstrate that the MG system can be applied to develop novel platforms for multivalency via the formation of NGPs. This sets up a premise for a general protocol using artificial and/or natural GLs with myrosinase for the *in situ* generation of an isothiocyanate and its direct conjugation with a protein.

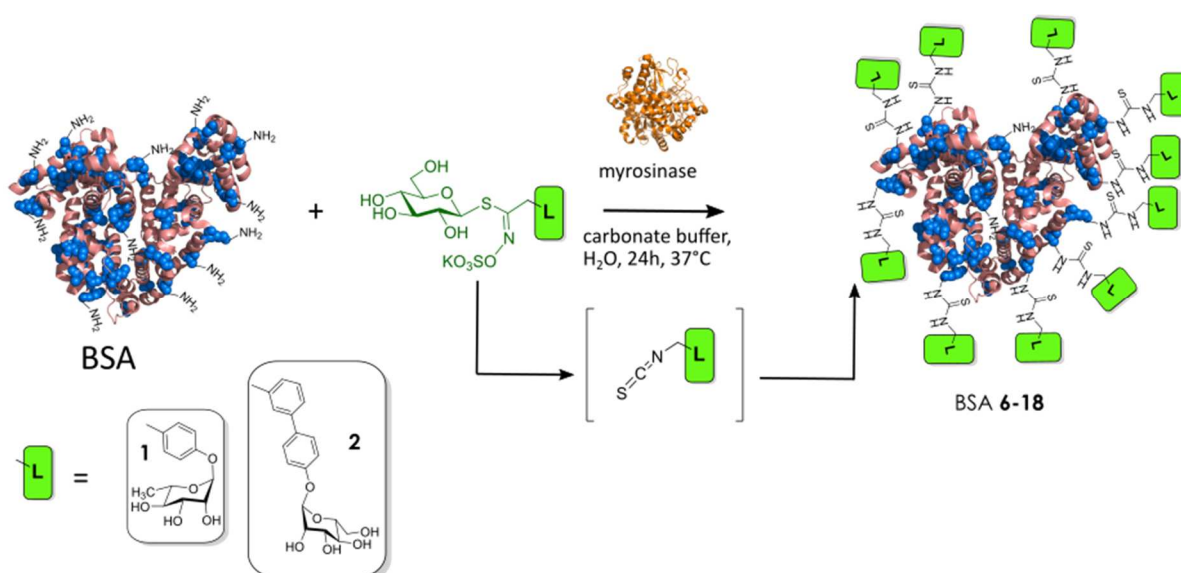
Results and Discussion

To prepare our neoglycoproteins, two different GLs were chosen: the natural Glucomoringin (GMG) **1**, and the synthetic biphenyl mannoside GL **2** (Scheme 1). GMG **1** bears a α -L-rhamnopyranoside functionalized aglycon that can be extracted from seeds of *Moringa Oleifera* [23]. Unnatural GL **2** was recently synthesized and studied in our group [20] and showed efficient binding with mannose specific lectins such as *E.coli* bacterial lectins FimH, BC2L-A and plant lectin ConA. The action of myrosinase on GL **2** led to the formation of an isothiocyanate that could be trapped by simple thiols. We first synthesized thiourea **3** as a monovalent model of the coupling of lysine residues with isothiocyanates. As L-lysines are the primary amine residues on proteins we used a protected L-lysine aminoacid (Scheme 1-B). The coupling reaction was performed applying the previously developed protocol using myrosinase hydrolysis and *in situ* trapping of the resulting isothiocyanate. To our surprise, no coupling could be detected at pH 7.4. Taking into consideration the high pKa of the primary amine, we changed the conditions to pH 9 where the amine is partially deprotonated and the myrosinase is still active [24]. Under these conditions, the enzyme proved to be stable enough and the L-lysine derived model thiourea **3** was obtained in 57% yield.



Scheme 1: A) natural glucomoringin **1** and the mannoside biphenylmethylglucosinolate (BiPhManGL) **2** used to functionalise BSA. B) myrosinase hydrolysis of GL **2** to produce the reference biphenylmannoside thiourea (BiPhManLys) **3** as a monoligand. C) Sinigrin **4** and α -methylmannopyranoside **5** structures.

Our next step was to develop a simple and efficient one-pot protocol. A typical reaction was set up by mixing GL **1** or **2**, myrosinase and BSA in a basic buffered solution. The MG system triggers the *in situ* formation of the isothiocyanate which can then react with the L-lysine residues of BSA (Scheme 2).



Scheme 2 : BSA functionalization in a one pot reaction using the MG tandem to produce *in situ* the isothiocyanate.

Based on our previous studies regarding myrosinase activity with artificial GLs, we explored the conditions necessary for the multivalent functionalization of BSA with the biphenyl mannose GL **2**[21]. We developed a one-pot procedure by mixing the enzyme with fluorescent GLs (5 equiv. relative to BSA) and BSA. Under those conditions, the bioconjugation of the fluorescent tags to BSA was obtained with a satisfactory yield. The average number of glycans per BSA was determined by comparative MALDI-TOF analysis of native BSA for two fluorescent tags and gave a ratio of 3.4 and 2.1 equiv. of fluorophore on the protein. We felt it was necessary to enhance the multivalent bioconjugation onto BSA, to achieve this goal, different parameters were studied: the excess of GL, as well as the optimum pH and the concentrations of BSA and myrosinase (Table 1).

| Entry | GL | Reaction volume | BSA (mg/mL) | pH | GL (equiv.) | U _E / μ mol ^a | NGP | Average number of glycans/BSA ^b |
|-------|----|-----------------|-------------|-----|-------------|---|------------|--|
| 1 | 2 | 1 mL | 1 | 7.4 | 160 | 0.5 | BSA-Man 6 | 11.2 |
| 2 | 2 | 1 mL | 1 | 8 | 160 | 0.5 | BSA-Man 7 | 17.2 |
| 3 | 2 | 1 mL | 1 | 9 | 160 | 0.5 | BSA-Man 8 | 18.7 |
| 4 | 2 | 1 mL | 5 | 9 | 160 | 0.5 | BSA-Man 9 | 10.8 |
| 5 | 2 | 1 mL | 1 | 9 | 80 | 0.5 | BSA-Man 10 | 20.4 |
| 6 | 2 | 1 mL | 2 | 9 | 80 | 0.5 | BSA-Man 11 | 19.7 |
| 7 | 2 | 0.5 mL | 1 | 9 | 120 | 0.5 | BSA-Man 12 | 25.2 |
| 8 | 2 | 0.5 mL | 1 | 9.5 | 120 | 0.5 | BSA-Man 13 | 23.2 |
| 9 | 2 | 0.5 mL | 1 | 9.5 | 80 | 0.5 | BSA-Man 14 | 27.5 |
| 10 | 2 | 0.5 mL | 1 | 9 | 80 | 0.25 | BSA-Man 15 | 28.5 |
| 11 | 2 | 4 mL | 1 | 9 | 80 | 0.25 | BSA-Man 16 | 28.7 |
| 12 | 1 | 0.5 mL | 1 | 9 | 80 | 0.25 | BSA-Rha 17 | 17.8 |
| 13 | 1 | 4 mL | 1 | 9 | 80 | 0.25 | BSA-Rha 18 | 16.8 |

Table 1: optimization of the BSA multivalent functionalization; ^a Unit of myrosinase per μ mol of GL; ^b Average number of glycans/BSA measured by MALDI-TOF analysis.

From Table 1, it can be seen that the choice of the pH is critical to reach the maximum number of glycans/BSA. As previously described, bioconjugation to BSA can be performed at pH 7.4, with a large excess of of glucosinolate per BSA (160 equivalents) which corresponds

to a ratio of 2.7 equivalents of GL **2** per amine residue [25]. MALDI-TOF detection (entry 1) indicated a number of glycans/BSA close to 11. Considering that the lysine residues need to be amines and not protonated in their ammonium form for an optimum ligation, the pH of the buffer was enhanced to 8 (entry 2) then 9 (entry 3), which increased the ratio of glycans/BSA to 18.7. Increasing the concentration of BSA to 5mg/mL sharply reduced the ligation efficiency (entry 4), thus suggesting that a concentrated solution of protein hampers the ligation process. Indeed, at a concentration of 10 mg/mL of BSA no conjugation was detected (results not shown in Table 1) . In this case, the GL **2** was mostly recovered from the reaction mixture. The amount of glucosinolate was also considered and reduced to 80 equivalents (2.5 equivalents of GL **2** per amine residue, entries 5 and 6). At 1 or 2 mg/mL of BSA, the results were comparable, with an average of glycans/BSA of respectively 20.4 and 19.7 detected by MALDI-TOF. Using an intermediate amount of 120 equivalents of GL **2** (entry 7), the number of mannose reached 25.2 units per BSA. A nearly identical number of ligations was observed with a slight increase of the pH to 9.5 (entry 8) but a clear increase to 27.5 ligations was obtained with 80 equivalents of GL (entry 9). We also reduced the amount of myrosinase to 0.25 U at pH 9 and it proved as efficient (entry 10), giving the optimum number of mannoside units of 28.5 per BSA. These conditions could be scaled up to 4 mg of BSA (entry 11) with 28.7 ligations observed. When transposing these optimized conditions to GMG **1** (entry 12), a lower number of rhamnose units/BSA was observed but the reaction was still effective and allowed the grafting of 17.8 rhamnoses to BSA. Again, this protocol was scaled up from 0.5 mg to 4 mg of BSA with a similar number of mannosides per BSA of 16.8 .

From these results, it can be concluded that the optimum conditions of ligation (entry 10) can be transposed to different starting glucosinolates (GMG **1** and GL **2**) and at different

reaction scales. Variation of the number of glycans/BSA observed depends on both the glucosinolate related myrosinase activity and on the chemical reactivity/stability of the resulting isothiocyanate.

Purification of the NGPs was also an important issue to address, in order to ensure an easy separation of the myrosinase from the desired NGP, as this enzyme has a molecular weight of 61 190 Da which is very close to that of BSA (66 463 Da). MALDI analysis after 24h of several crude samples showed no presence of a protein with a molecular weight close to that of myrosinase, thus suggesting a degradation of the enzyme in the reaction mixture. A blank test where only myrosinase was incubated at pH 9, 37°C for 24h showed that the protein decomposed in smaller peptides with molecular weights ranging from 15 to 18 kDa. These fragments are easily detected by MALDI-TOF analysis and can also be separated from the newly functionalized protein by a size exclusion chromatography.

We have also investigated the immobilization of myrosinase on a classical and compatible support, cyanogen bromide (CNBr) activated sepharose beads, in order to simplify the purification protocol using easy centrifugation or filtration of the reaction medium. Thus, the activated sepharose gel was used to support myrosinase using standard protocol. With this immobilization protocol, it was possible to quantitatively fix 3.36 mg of myrosinase on 1 mL of sepharose gel. Having this material in hand, we first determined the activity of the supported myrosinase. Using capillary electrophoresis, a method previously developed in our lab [26], it was possible to determine the hydrolysis rate of the immobilized myrosinase using sinigrin **4** as a natural and standard substrate (Figure 2).

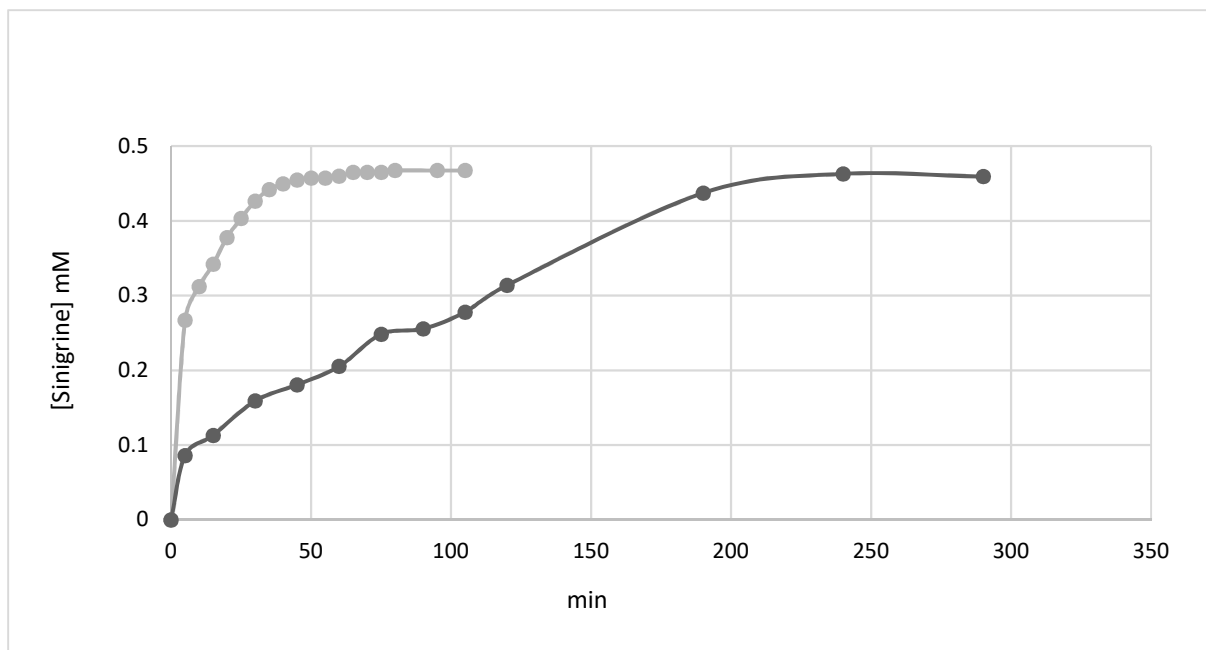


Figure 2: Capillary electrophoresis detection of sulfate ion from Sinigrin (**4**) hydrolysis with free (light grey) and supported myrosinase (dark grey).

Hydrolysis of a 0.5 mM solution of the natural GL sinigrin **4** (Scheme 1, Figure 2) was performed using both myrosinase (light grey curve) and the sepharose-supported myrosinase (dark grey curve). During this experiment, the amount of liberated sulfate ion was quantified using $C^{4}D$ detection. Satisfyingly, both types of myrosinase were able to quantitatively hydrolyse GL **4** in the solution (0.5 mM) demonstrating that the immobilization of myrosinase did not severely alter its hydrolytic activity. The concentration of sulfate ion was determined to be close to 0.5 mM after nearly 50 min of reaction time with the free myrosinase while completion of the reaction was obtained in 200 min for the immobilized one. The difference between both curves clearly shows that the supported enzyme activity was reduced by 4 times compared to free myrosinase. This result is in agreement with the general slower kinetics of immobilized enzymes and could be attributed

to the partially inactivated enzyme together with a slow diffusion of the substrate in the polymer matrix [27].

We then explored the ability of the sepharose supported myrosinase to promote the multivalent conjugation of GL **2** to BSA. We again explored different pH conditions, the concentration of BSA and the amount of both glucosinolate and myrosinase.

| Entry | GL | Reaction volume | BSA (mg/mL) | pH | GL (equiv.) | U _E / μ mol ^a | NG | Number of ligations/BSA ^b |
|-------|----------|-----------------|-------------|-----|-------------|---|----------------------|--------------------------------------|
| 1 | 2 | 1 mL | 1 | 7.4 | 160 | 0.5 | BSA-Man 19 | 8.1 |
| 2 | 2 | 0.5 mL | 1 | 9 | 120 | 0.5 | BSA-Man 20 | 8.8 |
| 3 | 2 | 0.5 mL | 1 | 9 | 80 | 0.5 | BSA-Man 21 | 11.6 |
| 4 | 2 | 0.5 mL | 1 | 9 | 120 | 0.25 | BSA-Man 22 | 15.3 |
| 5 | 2 | 0.5 mL | 1 | 9 | 80 | 0.25 | BSA-Man 23 | 13.9 |

Table 2: reaction conditions using sepharose supported-myrosinase. ^aUnits of native myrosinase per μ mol of GL, based on the amount of myrosinase fixed on sepharose;^b average number of ligations/BSA measured using MALDI-TOF analysis.

Overall, the observed number of ligations was significantly lower with the sepharose-supported myrosinase, and the difference observed was pH dependent. At pH 7 the number of ligations was similar for both enzymes (BSA-Man **6**, 11.2 for free myrosinase and BSA-Man **19**, 8.8 for the sepharose supported myrosinase), while at pH 9 a higher gap was observed,

respectively 25.8 (BSA-Man **15**) and 13.9 (BSA-Man **23**). The number of conjugations was thus reduced by more than two-fold. Also, from a practical point of view, it was not possible to increase the amount of sepharose-myrosinase in the reaction mixture. With up to 0.5 units of enzyme per μmol of GL, the required amount of sepharose gel was too voluminous and compromised the homogeneity of the reaction solution (entry 1). To avoid this issue, 0.25 unit of enzyme per μmol of GL was used and ligation amounts were improved (entries 4 and 5). The reduced amount of ligations per BSA could be rationalized by the decreased performance of the sepharose-supported myrosinase material, together with a phenomenon of adsorption of BSA on the sepharose beads thus decreasing the accessibility of the GL **2** for myrosinase as well as the availability of the isothiocyanate and BSA-protein for the conjugation[29]. In spite of these drawbacks, it was possible to obtain a functionalized neoglycoprotein without the need of size exclusion chromatography.

The lectin binding profile of the synthesized neoglycoproteins was then explored using the GLYcoPROFILE method, a technique developed to evaluate and determine the glycobiological interactions between lectins and glycans [30]. This assay is based on a competition between a fluorescent tracer and the tested sample for the recognition of a lectin immobilized in microplate wells [31]. Five mannose specific lectins were used: Jack bean lectin ConA (*Canavalia ensiformis*), two recombinant bacterial lectins BC2L-A (*Burkholderia cenocepacia*) and FimH (*Escherichia coli*), and two recombinant extracellular domains (ECD) of human lectins Langerin and DC-SIGN. All samples were compared with references for mannose specific lectins, α -methylmannopyranoside ($\alpha\text{MeMan } \mathbf{5}$) and a BSA- αMan_c used as a tracer (BSA- αMan_c was prepared by reacting BSA with 4-isothiocyanatophenyl α -D-mannopyranosides yielding a neoglycoprotein with 25

glycans/BSA). Furthermore, both GL **2** and thiourea **3** were used as monovalent references for comparison. The results are reported in Table 3.

Comparison of the monovalent ligands, GL **2** and thiourea **3** against α MeMan showed a stronger binding affinity (Table 3) regarding all five lectins except for BC2L-A. The best affinity was observed for FimH lectin as expected. Indeed, ligand **2** was designed to interact more specifically with FimH [20] and the observed IC_{50} is submicromolar (0.39 μ M). The thiourea LysbiPhMan **3** exhibited a similar IC_{50} of 1.45 μ M which shows that the L-lysine conjugate form of GL **2** retains similar interaction with FimH. Thus, we can assume that the biphenylmannopyranoside moiety is mainly responsible for the interaction. In comparison with α MeMan **5**, the most impressive effect was observed with the DC-SIGN lectin for which the interaction showed a 200-fold enhancement for the monovalent ligand **3**.

The multivalent effect of the prepared NGPs was evaluated by looking at the Relative Inhibitory Potency (RIP (**5**)) against α MeMan **5**, and against the L-lysine conjugated monovalent ligand **3** (RIP (**3**)). For ConA lectin, an important multivalency effect was indeed observed with all the BSA-Man, reaching a thousand-fold with BSA-Man **16** while the RIP value for BSA-Man_C was more similar to the one of BSA-Man **22** which displayed a lower number of ligands.

Very interesting results were obtained regarding FimH (Table 3). Glucosinolate **2**, the conjugate lysin ligand **3**, tracer BSA-Man_C and BSA-Man **22** have similar interactions on FimH with 0.39 μ M, 1.45 μ M, 0.27 μ M and 0.56 μ M IC_{50} values respectively. BSA-Rha **17**, which is not supposed to inhibit FimH, showed a good affinity with an unexpected IC_{50} of 0.76 μ M. BSA-Man **16** stayed as the NGP with the strongest avidity with an IC_{50} of 4.57 nM. The RIP related to α -methylmannopyranoside showed strong values for the monovalent ligands **2**

and **3** (respectively 148.7 and 40) but with only weak multivalency effects. In these cases, the RIP related to monovalent ligand **3** gave significant information, showing multivalency is somehow detrimental to the interactions with reduced affinity. Only the BSA-Man **16** proved efficient in terms of relative inhibitory potency with a ten-fold enhancement .

For BC2L-A, the IC_{50} for the monovalent ligand **2** (15.7 μ M) and **3** (44.3 μ M) were higher compared to α MeMan (7.45 μ M). The RIP for α MeMan with BSA-Man **22** was insignificant (0.6) whereas a multivalency effect was observed with a higher ligand level: BSA-Man_C (RIP = 12.1) and BSA-Man **16** (RIP = 17). Those two NGPs showed the strongest enhancement of avidity regarding ligand **3** (RIP (**3**)= 96.5 and 68.15 respectively), while a low value was observed with BSA-Man **22** (RIP (**3**) = 3.4). With these neoglycoproteins the IC_{50} reached the nM range (BSA-Man **16**, IC_{50} = 16 nM). Thus, it is clear that a high number of ligands is required for a good inhibition of BC2L-A . Surprisingly, the negative control with BSA-Rha **17** showed an efficient IC_{50} but with no multivalency effect.

The two human lectins, Langerin and DC-Sign belong to the C-type Lectins and share the same primary binding sites with specificity for oligomannosides. They are important lectins involved in pathogen recognition and immunity development [32]. Interesting results with Langerin ECD were observed. Indeed, all of the tested compounds were more efficient than α MeMan especially with the monovalent ligand **3** with an improved IC_{50} of 0.215 mM, which gives a 150-fold affinity enhancement for the monovalent structure. The multivalency effect was also pronounced. The IC_{50} of the BSA-Man_C and BSA-Man **16** were in the nanomolar range with 21 nM and 2.1 nM respectively. These results are associated with a very impressive RIP (**5**) and RIP (**3**) with an enhancement of the avidity by respectively half a million and 3567-fold.

When tested with DC-SIGN ECD, all compounds were good inhibitors and gave lower IC_{50} than α MeMan **5**. The two monovalent ligands **2** and **3** showed significant inhibition with IC_{50} values of 19.5 μ M and 33.2 μ M. The mannose conjugated BSA-Man_C and BSA-Man **16** were the most potent ligands with IC_{50} in the nanomolar range (respectively 22 nM and 5.2 nM) but the RIP values were less important than with Langerin (60.4 and 222.5 respectively). Still, the BSA-Man **16** proved much more efficient than the BSA-Man_C.

The results observed for the monovalent ligands **2** and **3** with the different lectins reflect the design of these biphenylmannosides targeting FimH and its recognition site, and more specifically the clamp π -stacking effect of the two tyrosine residues. Nonetheless, they both proved to show interesting inhibition against BC2L-A and DC-SIGN ECD with IC_{50} values in the 15-45 μ M range. Again, considering the interaction of the two molecules, of the five lectins, the glucosinolate **2** is the most potent inhibitor whereas with the L-Lysine conjugate **3**, the interaction is slightly reduced. The ligand **3** only showed a better affinity for Langerin ECD lectin.

When considering the overall interaction of the BSA conjugated mannoside and rhamnoside, the pattern of recognition is clearly in favour of the mannoside moiety (Table 3, entries 4-6) compared to the reference BSA-Rha **17** (Table 3, entry 7). Furthermore, when comparing inhibition with BSA conjugated mannosides (entries 4-6), a strongly improved efficiency with the biphenylmannoside conjugated protein BSA-Man **16** was clearly seen. Indeed, even if the BSA-Man_C possesses a reduced number of conjugated mannoside per BSA, the BSA-Man **16** showed improved inhibition by 15.8 fold for ConA, 121 fold for FimH, 1.6 fold for BC2L-A, 10 fold for Langerin and 4.2 fold for DC-SIGN. The strongest enhancement with FimH could almost certainly be attributed to the selective design of the biphenyl moiety [33]. A possible

explanation of this improved efficiency could be correlated with the structure of the biphenyl system. Its rigidity and length (close to 10 Å) compared to phenylisothiocyanate (5 Å) allows a better separation of the mannopyranoside moiety from the surface of the protein and improves interaction with lectins. In addition to these results the interaction with myrosinase (entry 8) was also studied. Myrosinase proved to have a strong interaction with all 5 lectins with IC₅₀ values in the μM range, similar to BSA-Rha **18**. Myrosinase was degraded during the conjugation process and removed during purification of the NGP via size exclusion chromatography, using Sephadex G25. [30]

| Entries | Compound↓ | Lectins → | ConA | | | FimH | | | BC2L-A | | | Langerin ECD | | | DC-SIGN ECD | | |
|---------|----------------------------|--------------|-------------------|-----------------------|---------|---------|-----------------------|---------|-----------------|-----------------------|---------|-------------------|-----------------------|---------|-------------------|-----------------------|---------|
| | | | ligands number | IC ₅₀ (μM) | RIP (5) | RIP (3) | IC ₅₀ (μM) | RIP (5) | RIP (3) | IC ₅₀ (μM) | RIP (5) | RIP (3) | IC ₅₀ (μM) | RIP (5) | RIP (3) | IC ₅₀ (μM) | RIP (5) |
| 1 | <i>αMeMan 5</i> | 1.00 | 923.0 ±20 | 1.0 | | 58.0 | 1.0 | | 7.85 ±0.8 | 1.0 | | 32327.0 ±500.0 | 1.00 | | 6610.0 ±360 | 1.0 | |
| 2 | <i>biPhManGL 2</i> | 1.00 | 126.0 ±5.0 | 7.3 | | 0.39 | 148.7 | | 15.66 ±2.0 | 0.5 | | 312.0 ±4.0 | 103.6 | | <10.0 | ND | |
| 3 | <i>LysBiPhMan 3</i> | 1.00 | 209.0 ±6.0 | 4.42 | 1.0 | 1.45 | 40.0 | 1.0 | 44.32 ±5.0 | 0.2 | 1.0 | 214.6 ±6.0 | 150.23 | 1.0 | 33.2 ±5.0 | 199.1 | 1.0 |
| 4 | <i>BSA-Man 22</i> | 15.30 | 0.252 ±0.03 | 241.3 | 54.6 | 0.27 | 14.0 | 0.3 | 0.84 ±0.01 | 0.6 | 3.4 | 1.365 ±0.5 | 1540.96 | 10.26 | 0.64 ±0.06 | 675.0 | 3.4 |
| 5 | <i>BSA-Man 16</i> | 28.70 | 0.0076 ±0.0006 | 4231.6 | 958.2 | 0.0046 | 439.3 | 11.0 | 0.016 ±0.007 | 17.1 | 96.5 | 0.0021 ±0.0005 | 535921.7 | 3567.3 | 0.0052 0.001 | 44291.1 | 222.5 |
| 6 | <i>BSA-Man_c</i> | 25.00 | 0.12 ±0.02 | 300.16 | 67.97 | 0.56 | 4.14 | 0.1 | 0.026 ±0.005 | 12.1 | 68.15 | 0.0210 ±0.005 | 61523.81 | 409.5 | 0.02169 ±0.005 | 12018.2 | 60.4 |
| 7 | <i>BSA-Rha 17</i> | 17.80 | 3.97 ±0.6 | 13.1 | 3.0 | 0.76 | 4.3 | 0.1 | 2.11 ±0.3 | 0.2 | 1.2 | >1.25 | ND | ND | 0.625 ±0.01 | 322.9 | 1.6 |
| 8 | <i>Myrosinase</i> | - | 5.343 ±0.4 | ND | ND | ND | | | 1.517 ±0.2 | ND | ND | 9.845 ±0.5 | ND | ND | 0.312 ±0.07 | ND | ND |

Table 3: The ligand potencies, IC₅₀ (μM) were determined for αMeMan **5**, the monovalent ligands **2** and **3** and the synthesized neoglycoproteins BSA-Man **16**, BSA-Rha **18** and BSA-Man **22**, as well as for commercial myrosinase. The RIP values were determined from the IC₅₀ of the αMeMan **5** but also calculated against the monovalent ligand **3** (RIP(**3**)), and corrected with valency (the number of conjugated mannosyl or rhamnosyl units) on BSA. The valency-calculated values take the number of conjugated mannosyl units into account. RIP (**5**) = IC₅₀ (α-MeMan **5**)/[IC₅₀ (NGPs)] · 1/n with n = 15 for **22**, 29 for **16** and 18 for **18**; ND: not determined; ECD: extracellular domain

Conclusion

A convenient strategy for a 'one-pot' synthesis of NGPs was developed based on an enzymatically triggered system. In comparison to previously published methods used to prepare neoglycoproteins, the myrosinase-glucosinolate system allows to generate an isothiocyanate *in situ* thus avoiding stability and solubility problems that might occur when handling reactively labelled molecules. It also avoids the use of organic solvents commonly used for bioconjugation such as DMF or DMSO. The *in situ* generated isothiocyanates were simultaneously reacted and linked to BSA. Optimisation of ligation conditions led to the optimum amount of ligands on one BSA with up to 29 biphenylmannosides on the surface of the protein, which is comparable to the commercial BSA-Man_c. The lectin profile (GLYcoPROFILE) of the newly synthesised NGP showed that BSA-Man **16** produced in this way showed the same pattern of recognition as the commercial BSA-Man_c commonly used as a tracer with improved efficiency. This phenomenon could be attributed to the biphenyl moiety, which could allow a better availability of the mannopyranoside motif for recognition. In general, the avidity of BSA-Man **16** increased by 2 to 5 orders of magnitude in comparison with the monovalent mannose ligand. Although FimH is known for its strong affinity toward mannose, the highest avidity was observed for Langerin ECD (RIP in 10⁵ range) in comparison with αMeMan5.

The protocol developed was successfully applied to synthesize a NGP functionalised with rhamnopyranoside moieties, using glucomoringin **1**, a natural glucosinolate, as the substrate. It produced BSA-Rha **17** with up to 17 rhamnopyranoside moieties on the surface of the protein.

This simple and reliable *in situ* enzymatic method, inspired by the natural glucosinolate-myrosinase system, could be used as an alternative to other bioconjugation protocols.

Materials and methods:

Materials: flash silica column chromatography was performed on a Reveleris® flash chromatography system. The reactions were monitored by thin layer chromatography (TLC) on silica gel 60F254 precoated aluminium plates. Compounds were visualised under UV light and by charring with a 10% H₂SO₄ ethanolic solution or a solution of potassium permanganate. Optical rotation was measured at 20°C using a Jasco P-2000 polarimeter with a path length of 1 dm, values are given in deg.dm⁻¹ g⁻¹ mL⁻¹ with concentrations reported in g.100 mL⁻¹. ¹H NMR and ¹³C NMR were recorded with Bruker Avance II 250 or an Avance III HD Nanobay spectrometers. Assignments were based on DEPT 135 sequence, homo- and heteronuclear 2D correlations. Chemical shifts were reported in parts per million (ppm). Coupling constants (*J*) are reported and expressed in Hertz (Hz), splitting patterns are designated as b (broad), s (singlet), d (doublet), dd (doublet of doublet), q (quartet), ddd (doublet of doublet of doublet), m (multiplet). High-resolution mass spectra (HRMS) were performed on a Maxis Bruker 4G by the “Federation de Recherche” ICOA/CBM (FR2708) platform in the electrospray ionisation (ESI) mode. Thioglucosidase from *Sinapis alba* (white mustard) seed (myrosinase, EC 3.2.1.147, 25U, ≥100 units. g⁻¹) was purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). Stock solution was prepared using phosphate buffer pH = 7.4 (C = 0.1 M). Bovine serum albumine (BSA) was purchased from Seqens in vitro diagnostics (France, Ref: 1035-70)

Synthesis of compound 3

methyl (2R)-2-acetamido-6-[[[4'- α -D-mannopyranosyloxy-[1,1'-biphenyl]-3-yl]methyl]carbamoithioyl]amino}hexanoate **3**

Myrosinase solution 10 U/mL (67 μ L) was added to a solution of glucosinolate **2** (30 mg, 0.042 mmol, 1 equiv.) and *N*-acetyl-L-lysine methyl ester hydrochloride (10.2 mg, 0.042 mmol, 1 equiv.) in potassium carbonate buffer (0.1M, pH = 9.2 mL) then the reaction mixture was stirred with a thermomixer at 37°C and followed by using TLC. The solvent was evaporated under reduced pressure, and the crude was purified using reverse phase column chromatography (water/MeOH: 100/0 to 0/100) to afford the pure product (15 mg, 0.024 mmol, 57%). $[\alpha]_D^{20} = +7.5$ (c 0.4, MeOH). $^1\text{H NMR}$ (400 MHz, CD_3OD) δ (ppm) = 7.56-7.53 (m, 3H, Ar-H), 7.46 (d, 1H, $J = 7.9$ Hz, Ar-H), 7.37 (t, 1H, $J = 7.8$ Hz, Ar-H), 7.25 (d, 1H, $J = 7.8$ Hz, Ar-H), 7.20-7.17 (m, 2H, Ar-H), 5.53 (d, 1H, $J_{1,2} = 1.9$ Hz, H-1), 4.75 (m, 2H, ArCH_2N), 4.33 (dd, 1H, $J = 5.1$ Hz, $J = 9.0$ Hz, H-a), 4.04 (dd, 1H, $J_{1,2} = 1.9$ Hz, $J_{2,3} = 3.4$ Hz, H-2), 3.93 (dd, 1H, $J_{3,2} = 3.4$ Hz, $J_{3,4} = 9.4$ Hz, H-3), 3.80-3.70 (m, 3H, H-4, H-6), 3.69 (s, 3H, OCH_3) 3.63 (ddd, 1H, $J_{4,5} = 9.7$ Hz, $J_{5,6a} = 2.8$ Hz, $J_{5,6b} = 4.8$ Hz, H-5), 3.52-3.40 (bs, 2H, H-e), 1.96 (s, 3H, CH_3CON), 1.84-1.75 (m, 1H, H-b_A) 1.72-1.64 (m, 1H, H-b_B), 1.58 (q, 2H, $J = 7.3$ Hz, H-d) 1.45-1.32 (m, 2H, H-c). $^{13}\text{C NMR}$ (100 MHz, CD_3OD) δ (ppm) = 174.3 (C=O), 173.5 (C=O), 157.4 ($\text{C}_{\text{Ar-O}}$), 142.1 (C_{Ar}), 140.5 (C_{Ar}), 136.4 (C_{Ar}), 130.0 (C-H_{Ar}), 129.1 (C-H_{Ar}), 127.0 (C-H_{Ar}), 126.7 (C-H_{Ar}), 126.5 (C-H_{Ar}), 118.1 (C-H_{Ar}), 100.1 (C-1), 75.3 (C-5), 72.4 (C-3), 71.9 (C-2), 68.3 (C-4), 62.6 (C-6), 53.8 (C-a), 52.7 (OCH_3), 48.7 (ArCH_2N), 44.9 (C-e), 32.1 (C-b), 29.7 (C-d), 24.0 (C-c), 22.39 (CH_3). HRMS (ESI⁺): m/z calculated for $\text{C}_{29}\text{H}_{39}\text{CaN}_3\text{O}_9\text{S}$ ($[\text{M}+\text{Ca}]^{2+}$): 322.6011, found 322.6015.

Capillary Electrophoresis Analysis

The procedure follows protocols earlier developed in our laboratory using an HP^{3D} capillary electrophoresis (CE) apparatus (Agilent, Waldbronn, Germany).[19] Agilent software 3D-CE

Chemstation (rev B.04.02) was used to pilot the CE. The HP^{3D}CE was equipped with an on-capillary TraceDec capacitively coupled contactless conductivity detector (C⁴D) (Innovative Sensor Technologies GmbH, Strasshof, Austria). The detection signal was acquired with the Tracemon software (Istech, version 0.07a). CE analyses were performed in an uncoated fused-silica capillary of 35 cm total length and 50 µm i.d., purchased from Polymicro Technologies (Phoenix, AZ, USA).[19] Substrates are used at large excess relative to the enzyme (about 100 times). For this reason, “free or immobilized” myrosinase was used at 0.05 U.mL⁻¹ for all assays. The myrosinase activity was determined by following the hydrolysis of the glucosinolate substrate, the SO₄²⁻ produced was detected by the C⁴D and quantified using external standard calibration. The volume of the reaction mixture was set down to 7 µL and the reaction was performed in a micro-vial of the CE instrument autosampler.

Neoglycoproteins synthesis using the MG system:

Buffers and solutions: pH 7 to 8: phosphate buffer (KH₂PO₄/K₂HPO₄), *c* = 33 mM.; pH 9 to 10: carbonate buffer (KHCO₃/K₂CO₃), *c* = 33 mM. For the reaction followed by LC-MS the phosphate buffer pH 7 was replaced by AcONH₄, 33 mM. All buffers, myrosinase and protein (BSA) solutions were prepared using purified water with a Milli-Q system (purified to 18.2 MΩ cm).

General procedure: a 10 U_E/mL Myrosinase was added to a solution of the GL and BSA in the appropriate buffer. The solution was shaken with a thermomixer during 24h at 37 °C. After this period, the solution was frozen at -78°C and stored at -18°C ready for analysis and purifications.

The obtained neoglycoproteins were purified by gel filtration using Sephadex G25 (GE Healthcare, ref: GE17-0032-01) before biological testing.

Optimized conditions (Table 1, entry 10): 15 μL of a Myrosinase solution (10 $\text{U}_\text{E}/\text{mL}$) were added to a solution of GL 2 (0.42 mg, 0.6 μmol , 80 equiv.) and BSA (0.5 mg) in 485 μL of carbonate buffer.

Myrosinase sepharose-supported preparation: myrosinase was coupled to CNBr-activated 4B Sepharose from GE Healthcare following the supplier recommended procedure.

Optimised conditions with sepharose-supported myrosinase (Table 2, entry 4) : 94 μL of a settled sepharose-myrosinase solution were added to a solution of GL (0.63 mg, 0.9 μmol , 120 equiv.) and BSA (0.5 mg) in 405 μL of carbonate buffer.

Lectin Interaction

Kinetic Studies: Lectin array interaction analysis was performed by using customized 96-wells plates functionalized with plant or human recombinant lectins (LEctPROFILE® plates from GLYcoDiag (Orléans, France))[34] [30]. The interaction profiles of each compound were determined through an indirect method based on the inhibition by the compound of the interaction between a specific lectin-glycan couple (neoglycoprotein BSA-Man). Briefly, a mix of neoglycoprotein (fixed concentration) and the corresponding compounds (range of concentrations) prepared in PBS supplemented with 1 mM CaCl_2 and 0.5 mM MgCl_2 was deposited in each well (50 μL each) in triplicates and incubated two hours at room temperature. After washing with PBS buffer, the conjugate streptavidine-DTAF was added (50 μL) and incubated 30 min more. The plate was washed again with PBS. Finally, 100 μL of PBS was added for the readout of fluorescent plate performed with a fluorescence reader ($\lambda_{\text{ex}} = 485 \text{ nm}$, $\lambda_{\text{em}} = 530 \text{ nm}$, Fluostar OPTIMA, BMG LABTECH, France). The signal intensity is

inversely correlated with the capacity of the compound to be recognized by the lectin and expressed as inhibition percentage with comparison with the corresponding tracer alone (neoglycoprotein BSA-Man). The RIP value was determined from the IC_{50} of the α MeMan **5** but also calculated against the monovalent ligand **3** (RIP(**3**)), corrected with the valencies (the number of conjugated mannosyl or rhamnosyl units) on BSA. The valency-calculated values take the number of conjugated mannosyl units into account. $RIP = IC_{50} (\alpha\text{-MeMan } \mathbf{5}) / [IC_{50} (\text{NGPs})]$

Abbreviations

BC2L-A :*Burkholderia cenocepacia* lectin - A

BSA : Bovine Serum Albumine

C⁴D : capacitively coupled contactless conductivity detector

CNBr: cyanogen bromide

ConA : Concanavalin A, *Canavalia ensiformis* lectin

CuAAC : Copper Azide Alkyne Cycloaddition

DC-SIGN : Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin

ECD : extracellular domain

FimH : *Escherichia coli* type 1 fimbrial lectin

GLs : Glucosinolates

GMG :Glucomoringin

HAS : Human Serum Albumin

MALDI-TOF : Matrix-assisted laser desorption ionization time-of-flight

NGP : Neoglycoprotein

NHS : *N*-hydroxysuccinimide

OVA: Ovalbumine

PBS : Phosphate buffered saline

SPPS : Solid Phase Peptide Synthesis

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Graphical Abstract :

