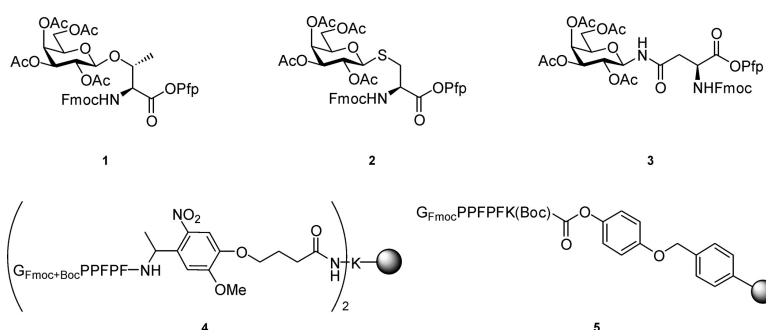Article

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Affinity Determination of *Ricinus communis* Agglutinin Ligands Identified from Combinatorial *O*- and *S*-,*N*-Glycopeptide Libraries

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Two combinatorial glycopeptide libraries were synthesized on solid support via the "split-and-mix" method combined with the ladder synthesis strategy. The *O*-glycopeptide library contained Gal(β1-O)Thr, whereas the *S*-,*N*-glycopeptide library contained both Gal(β1-S)Cys and Gal(β1-N)Asn. In this model study, the two libraries were screened against the fluorescently labeled lectin *Ricinus communis* agglutinin (RCA$_{120}$). The screening results showed that both *O*- and *S*- or *S*-,*N*-glycopeptides were recognized by the lectin with similar amino acid recognition patterns. Surface plasmon resonance interaction studies demonstrated that both the selected *S*- or *S*-,*N*-glycopeptide hits and the *O*-glycopeptides bound to the lectin with a similar affinity. Structure **19**, containing two glycosylated cysteine residues, bound to the receptor with the highest affinity ($K_A = 3.81 \times 10^4$ M^{-1}), which is comparable to *N*-acetyllactosamine. Competition assays, in which some selected glycopeptides and methyl β-D-galactopyranoside competed for the binding site of immobilized RCA$_{120}$, showed that the glycopeptide−lectin interaction was carbohydrate-specific. Incubation of the *O*- and *S*-,*N*-glycopeptides with β-galactosidase demonstrated the complete stability of *S*-,*N*-glycopeptides toward enzymatic degradation, whereas *O*-glycopeptides were not completely stable.

Introduction

Interactions between membrane-bound glycoconjugate glycans and carbohydrate-binding proteins are important in mediating intercellular recognition processes. Typical examples of these processes are cell−cell recognition; cell growth regulation; cancer cell metastasis; and viral, bacterial, and parasitical infections.[1,2] Fundamental interaction studies to understand at a molecular level, and possibly to intervene with these processes, are hampered by the low availability of complex carbohydrates. Over the years, it has been shown that not the complete glycan structure is involved in the interaction with its receptor.[3,4] In most cases, only the residues at the nonreducing end of the glycans that are in close contact with the external environment are important for the interaction. This allows the use of simplified structures, such as a carbohydrate epitope attached to a scaffold, as mimics of complex glycans in interaction studies.[2,5−7]

Previously, simple *O*-glycopeptides have been successfully used as mimics of complex glycans.[8,9] These glycopeptides can be generated in a library format via a combinatorial approach. The most frequently implemented method for the generation of "one-bead-one-compound" (glyco)peptide libraries is the split-and-mix method.[10,11] This method, combined with the ladder synthesis strategy,[12,13] offers facile synthesis and characterization of thousands of potential ligands that can be used in interaction studies. *O*-Glycopeptide libraries generated via this strategy have been screened with lectins,[12,14] and effective mimics were identified. Some of these mimics were shown to have higher affinities toward their receptor, as compared to their natural carbohydrate ligands.

The in vivo stability of compounds is of great importance in the development of new therapeutics. In the field of oligosaccharide synthesis, it has been shown that replacing an O-glycosidic linkage with an S-glycosidic linkage greatly enhances the stability of the product toward acidic conditions and glycan-degrading enzymes.[15−17] In an analogous way, it can be expected that the stability of glycopeptides can be increased by replacing the carbohydrate−peptide O-glycosidic linkage with an S- or N-glycosidic linkage. To our knowledge, the preparation and screening of an *S*-glycopeptide library has not been described before. The synthesis and screening of an *N*-glycopeptide library was reported recently,[18] however, with the glycosylated amino acid placed in one fixed position.

In this article, the preparation and evaluation of an *O*-glycopeptide library containing Gal(β1-O)Thr introduced via building block **1**[19] and an *S*-,*N*-glycopeptide library containing Gal(β1-S)Cys and Gal(β1-N)Asn introduced via building blocks **2** and **3**,[20] respectively, is presented (Figure 1). Both libraries were screened with the model lectin *Ricinus communis* agglutinin. The strength and specificity of the interaction of some of the lead glycopeptides from both

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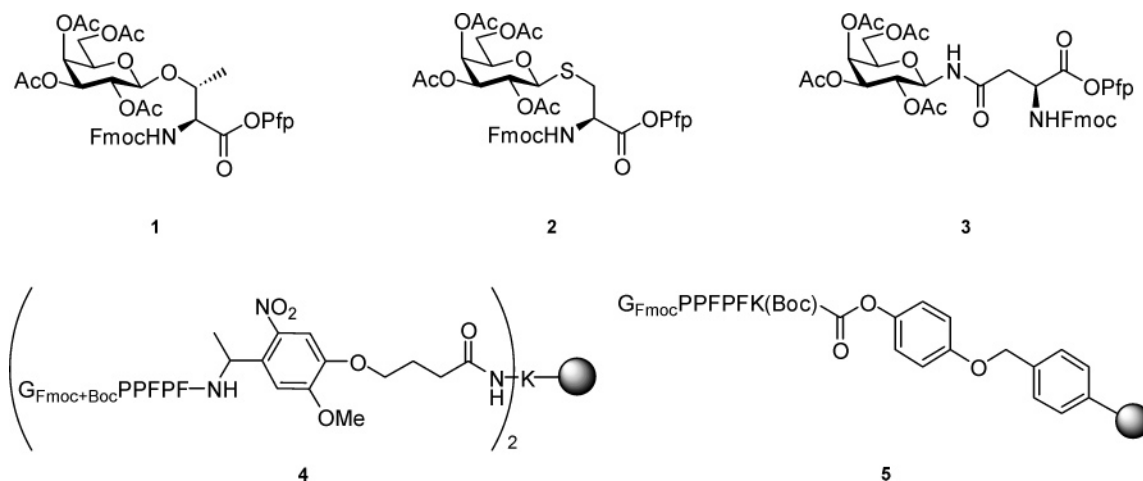
Figure 1. Glycosylated amino acid building blocks **1** (for library A) and **2** and **3** (for library B). The hexaglycopeptide libraries were generated on lysine-functionalized PEGA$_{1900}$ resin, prefunctionalized with general library construct **4**, containing a photolabile linker and an ionization mass peptide (IMP) spacer. Lead glycopeptides were resynthesized on Wang resin prefunctionalized with general solid-phase construct **5** containing an acid-labile Rink linker and an IMP spacer.

libraries with RCA$_{120}$ was determined by SPR. In addition, some glycopeptides were incubated with β-galactosidase to assess the enzymatic stability of both the *O*- and the *S*-,*N*-glycopeptides. The value of *S*- or *S*-,*N*-glycopeptides in comparison with *O*-glycopeptides in molecular recognition studies will be discussed.

Results and Discussion

The General Library Construct. Two "one-bead-one-compound" glycopeptide libraries were generated on general library construct **4** (Figure 1) using the split-and-mix method and chemically encoded using the "ladder synthesis strategy".[10] General library construct **4** was prepared by the attachment of an IMP spacer (GPPFPF) to lysine-functionalized PEGA$_{1900}$ resin via a photolabile linker. All couplings were performed using TBTU/NEM activation. Lysine was introduced to double the loading of the resin by using both amine functions of the amino acid for subsequent couplings. The introduced photolabile linker allowed direct, solid-phase biological assays and facilitated the cleavage of the active glycopeptides from the solid support under relatively mild conditions. As shown before, this linker is stable in the acidic and alkaline conditions that are used for the deprotection of amino acid side chains and carbohydrate residues, respectively.[21] The IMP spacer was included to facilitate the MALDI-TOF MS analysis of the glycopeptide fragments generated during the ladder synthesis. It increases the mass of the glycopeptide fragments in the library beyond 600 Da, out of the region containing the matrix adduct peaks. In addition, the IMP spacer serves to improve MALDI-TOF MS sensitivity and to ensure a reasonably uniform response from the various intermediate structures.[12,14]

Combinatorial Glycopeptide Library Synthesis. Two random hexaglycopeptide libraries were generated in a custom-made, 20-well, multiple-column peptide synthesizer.[22] Library A (1 g, ~420 000 beads) contained the *O*-glycosylated amino acid building block **1**,[19] whereas library B (0.8 g, ~330 000 beads) contained both the *S*- and *N*-glycosylated amino acid building blocks **2** and **3**[20] (Figure 1). In both libraries, all natural amino acids except Cys, Leu,

Lys, Asp, and Glu were included. Furthermore, the non-natural amino acid Cha was incorporated. In library A, glycosylated amino acid building block **1** was duplicated to achieve a carbohydrate preponderance equal to library B.

To allow unambiguous identification of the sequence of each individual library member, the ladder synthesis strategy was applied to generate a series of capped intermediates on each bead during the synthesis of the library.[13] Subsequent MALDI-TOF MS analysis of the peptide ladder formed by these terminated intermediates allows facile sequence elucidation. To obtain capped intermediates, a small portion of the growing peptide chain was terminated in each reaction step by using Boc-protected amino acids that are stable under the basic deprotection conditions used in an Fmoc-based synthesis protocol. Therefore, Fmoc- and Boc-protected amino acids were incorporated in a 9:1 ratio using TBTU/NEM activation. Simple pentafluorophenyl-derivatized carboxylic acids that differ in mass from all (glycosylated) amino acid building blocks were used as capping agents for the glycosylated amino acid building blocks (nonanoic acid-OPfp for **1** and **2**, pentadecanoic acid-OPfp for **3**). To ensure reproducible and detectable capping, the relative reactivities of the glycosylated amino acid building blocks and their complementary capping agents toward the solid phase were determined as reported previously.[12] During library synthesis, the glycosylated amino acids and their complementary capping agents were incorporated in the established ratios using Dhbt-OH activation.

After the last amino acid coupling, all Boc and amino acid side-chain protecting groups were removed under acidic conditions, and the carbohydrate protecting groups were cleaved under alkaline conditions. The efficiency of the library syntheses was evaluated by MALDI-TOF MS analysis of 25 randomly selected members of each library; all spectra clearly showed the ladder of terminated intermediates, allowing correct sequence determination and complete structure elucidation. In Figure 2, as a typical example, the MALDI-TOF mass spectrum of T(Gal)IIQChaY is depicted. In some spectra, monosaccharide elimination was observed by the presence of a peak of 162 Da less than the product
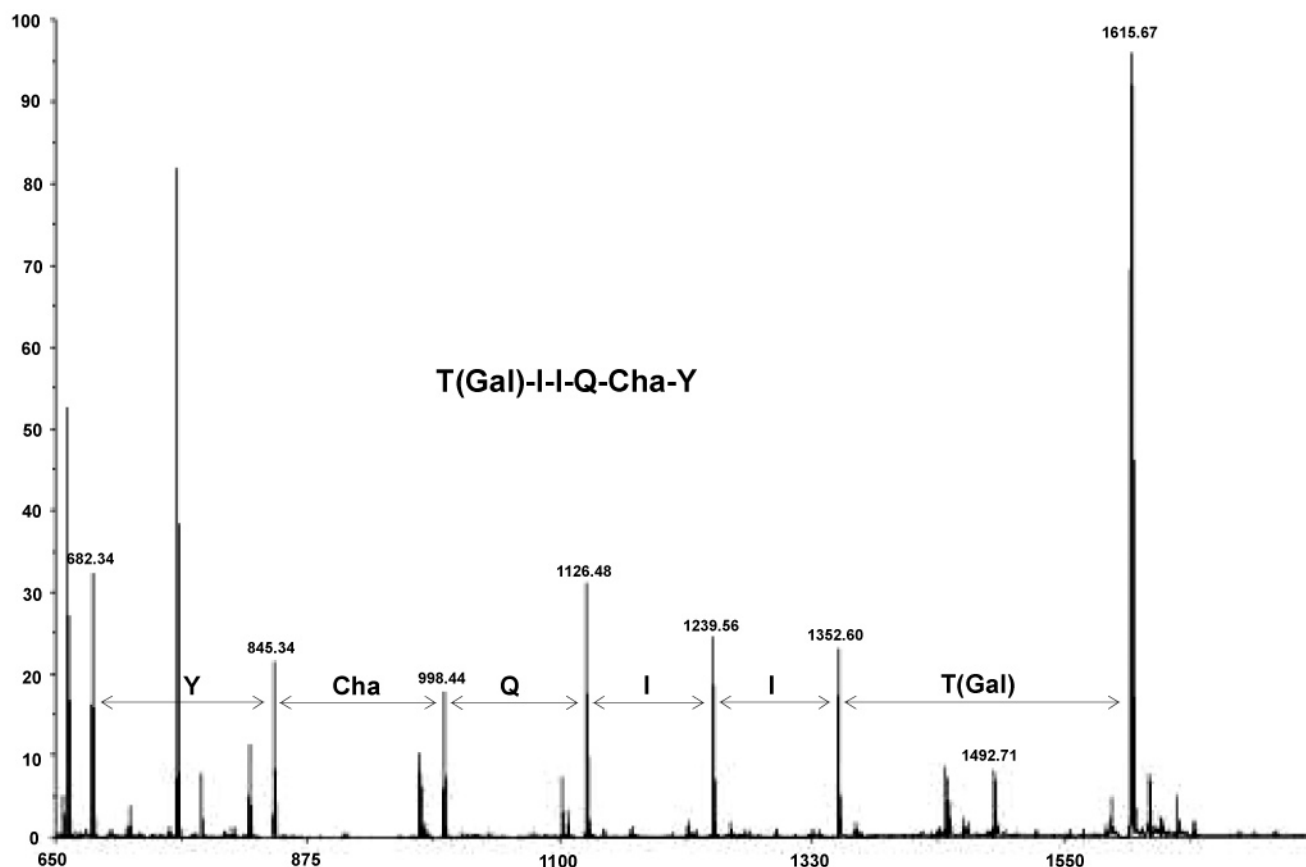
Figure 2. Example of a MALDI-TOF mass spectrum of a bead from library A. Each marked peak in the spectrum represents the mass of a terminated intermediate. The base peak in the spectrum corresponds to the IMP spacer (m/z 682.3, $[M + Na]^+$). The mass difference between peaks 1492.7 and 1352.6 corresponds to the mass of the capping agent for glycosylated amino acid building block **1**.

peak. Most likely, this elimination occurs under MALDI-TOF MS conditions, because an increase of the laser power led to an increase in the extent of monosaccharide loss.

Solid-Phase Screening of Libraries A and B with *R. communis* agglutinin. To identify active (glyco)peptides, both libraries were screened with fluorescently labeled *R. communis* agglutinin. RCA_{120} was chosen as a model lectin because of its ability to recognize a broad range of ligands that contain terminal galactose. RCA_{120} is a 120-kDa tetramer, consisting of two As-sB dimers that are noncovalently linked to each other; the B-chain contains the lectin domain.

Portions of libraries A and B (50 mg, ~21 000 beads) were incubated with Alexa Fluor 488-labeled RCA_{120} for either 2 h or overnight and analyzed by fluorescence microscopy. After each screening, ~2−3% of the beads were fluorescent, and 25 of the most fluorescent beads were manually selected and analyzed by MALDI-TOF MS. High-quality mass spectra were obtained for all selected hits, and from the ladder of peptide fragments and carboxylic acid labels, their complete sequence could be determined (See Supporting Information for the sequences of all selected hits). For both libraries, only glycopeptides were detected as active compounds, with up to five glycosylated amino acid building blocks in one sequence. In general, small hydrophobic amino acids (Val, Ala, Ile, Gly) were often found next to the glycosylated amino acids. After the 2-h screening of both libraries, aromatic amino acids (Phe, Tyr, and predominantly

His) were often found next to the glycosylated amino acid building blocks; however, this trend was less obvious after the overnight screenings. Strikingly, all hits from library B contained at least one glycosylated cysteine. Glycosylated asparagine, on the other hand, did not occur by itself, but rather, was only found in combination with glycosylated cysteine. This might suggest that the lectin shows a preference for binding to the S-glycosylated amino acid.

Affinity Determination by Surface Plasmon Resonance. To verify the results from the library screening, a series of representative active glycopeptides was resynthesized (Tables 1 and 2). The selection was based on the presence of aromatic or small hydrophobic amino acids next to the glycosylated amino acid according to the observed trends as described above. The affinity of the resynthesized glycopeptides for the lectin was measured by SPR on a Biacore instrument. The glycopeptides were prepared on Wang resin containing general solid-phase construct **5** (Figure 1). The acid-labile Rink linker allows the release of the glycopeptide from the resin under acidic conditions with concomitant deprotection of the amino acid side chains. The reported exact masses (Tables 1 and 2), which were obtained with internal calibration, confirmed the identity of the HPLC-purified compounds. The incorporation of Lys(Boc) into the IMP spacer allows the use of the glycopeptides in other applications, such as their immobilization on a surface via the side chain of Lys, after acetylation of the N terminus of the glycopeptide.

Table 1. Exact Masses and Affinity Constants for the Selected *O*-Glycopeptides

	sequence[a]	yield (%)	mass expected (M + H)	observed	K_A $(10^4$ $M^{-1})$
6	T(Gal)T(Gal)T(Gal)AT(Gal)T(Gal)	21	2176.972	2176.977	1.5 ± 0.12
7	AT(Gal)VT(Gal)TG	9	1643.805	1643.800	2.24 ± 0.19
8	PT(Gal)THT(Gal)Q	16	1778.848	1778.830	1.96 ± 0.15
9	GT(Gal)TATS	16	1509.729	1509.727	0.264 ± 0.06
10	GAMTPT(Gal)	20	1469.716	1469.722	1.55 ± 0.09
11	ChaGT(Gal)QYT(Gal)	19	1735.798	1735.786	1.85 ± 0.24
12	TT(Gal)MSTT(Gal)	15	1719.800	1719.891	0.55 ± 0.001
13	PGSYT(Gal)T(Gal)	19	1816.889	1816.880	1.07 ± 0.04
14	HT(Gal)SFNT(Gal)	20	1800.833	1800.827	2.31 ± 0.13

[a] All structures were synthesized on IMP-K.

Table 2. Exact Masses and Affinity Constants for the Selected *S*- or *S*-,*N*-Glycopeptides

	sequence[a]	yield (%)	mass expected (M + H)	observed	K_A $(10^4$ $M^{-1})$
15	C(Gal)GYGIN(Gal)	23	1720.778	1720.778	0.479 ± 0.01
15b	N(Gal)GYGIN(Gal)	24	1731.726	1731.775	0.13 ± 0.004
16	TIYANC(Gal)	40	1616.767	1616.763	0.529 ± 0.06
17	PHC(Gal)HYC(Gal)	15	1854.790	1854.803	1.55 ± 0.03
18	VC(Gal)N(Gal)MVP	19	1756.817	1756.800	2.43 ± 0.27
19	AC(Gal)YPYC(Gal)	22	1813.770	1813.765	3.81 ± 0.04
20	ChaN(Gal)C(Gal)MMP	15	1842.836	1842.836	0.61 ± 0.07
21	ChaHC(Gal)C(Gal)GG	17	1723.771	1723.738	2.05 ± 0.27
22	AC(Gal)VHSN(Gal)	18	1724.774	1724.767	1.62 ± 0.14
23	STMTIC(Gal)	18	1587.743	1587.741	0.229 ± 0.01

[a] All structures were synthesized on IMP-K.

To obtain binding data of the various glycopeptides in solution toward immobilized RCA$_{120}$, the lectin was immobilized on two flow cells of a CM5 chip at 4000 RU. To create a suitable reference surface, the lectin on one flow cell was denatured with guanidine hydrochloride (6 M, pH 1) and 0.5% SDS. Binding data of different glycopeptide concentrations (500−3.9 μM) were collected at a flow rate of 5 μL/min. In Figure 3a, a typical binding response with increasing concentrations of glycopeptide **7** is shown. Affinity constants were obtained from nonlinear fits of the data in plots of R_{eq} (response at equilibrium) versus the glycopeptide concentration (Figure 3b).

Previously, the binding constants of various small ligands toward RCA$_{120}$ were determined by ITC; the values ranged from 2.2×10^3 M^{-1} for galactose to 4.84×10^4 M^{-1} for *N*-acetyllactosamine.[23] As is evident from Tables 1 and 2, the affinity constants obtained for the glycopeptides are similar, or 1 order of magnitude higher than the value reported for galactose, which may indicate a favorable interaction of the peptide scaffold with the receptor. On average, the affinity constants of the *S*- or *S*-,*N*-glycopeptides are similar to those measured for the *O*-glycopeptides, indicating that the S-,N-glycosidic linkages did not disturb the biological interaction or influence the binding strength. Structure **19**, containing two glycosylated cysteine residues, bound to the receptor with the highest affinity, $K_A = 3.81 \times 10^4$ M^{-1}, which is comparable to *N*-acetyllactosamine. Comparison of the lead compounds obtained after the 2-h (**6**−**10**, Table 1; **15**−**19**, Table 2) and the overnight screenings (**11**−**14**, Table 1; **20**−**23**, Table 2) did not indicate significant differences in their affinity for RCA$_{120}$.

Since the N-glycosylated amino acid was found only in combination with the S-glycosylated amino acid, structure
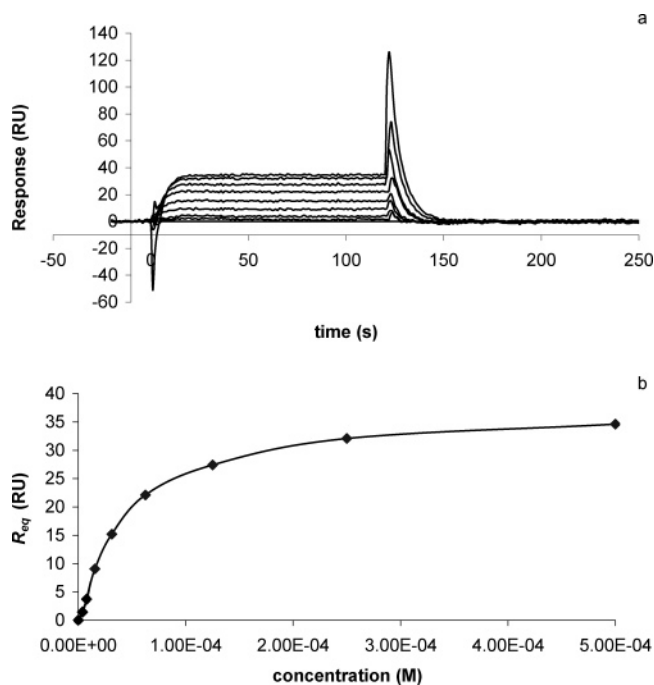
Figure 3. (a) Binding of glycopeptide **7** to immobilized RCA$_{120}$ at eight different concentrations between 500 (top) and 3.9 μM (bottom) obtained by 2-fold dilution. (b) Plot of the equilibrium response versus the glycopeptide concentration. The experimental data were fitted using the steady-state model to obtain the value for K_A.

15b, an analogue of structure **15**, was prepared to verify whether a glycopeptide containing only the N-glycosylated amino acid building block was also recognized by the lectin. SPR interaction studies showed that structure **15b** had a 4-fold lower affinity for RCA$_{120}$ than structure **15** (Table
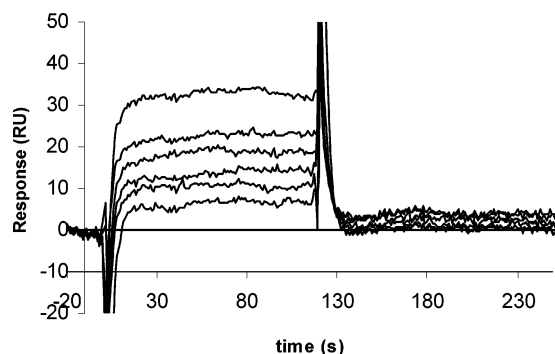
Figure 4. Competition of glycopeptide **15** (250 μM) and methyl β-D-galactopyranoside for the binding site of immobilized RCA$_{120}$. Inhibitor concentrations ranged from 0 (top) to 10 mM (bottom) and were obtained by 2-fold dilution from a 10 mM stock solution in Tris−HCl buffer pH 7.7.

2), indicating that a glycopeptide containing only the N-glycosylated amino acid building block can still be recognized by the lectin, although with a lower affinity than the original *S-*,*N*-glycopeptide that was identified during the library screening.

To confirm the carbohydrate specificity of the interaction, some selected glycopeptides and methyl β-D-galactopyranoside were allowed to compete for the binding site of immobilized RCA$_{120}$ in a competition assay. The selected glycopeptides **13**, **15**, **15b**, and **16** (Tables 1 and 2) contained either one of the three different glycosylated amino acid building blocks or a combination of the glycosylated amino acid building blocks **2** and **3**; therefore, they were considered to be representative of the total series of lead glycopeptides. In Figure 4, a typical binding response (glycopeptide **15**) in the presence of increasing amounts of methyl β-D-galactopyranoside is shown. The results of the competition assays showed that the binding of all glycopeptides containing the different glycosylated amino acid building blocks to RCA$_{120}$ was inhibited by methyl β-D-galactopyranoside. This proves that the lectin binds to *O-* and *S-* or *S-*,*N*-glycopeptides in a carbohydrate-specific way.

Investigation of the Enzymatic Stability of *O-*, and *S-*,*N*-Glycopeptides. The stability of *O-* and *S-*,*N*-glycopeptides toward enzymatic degradation was verified by treatment of glycopeptides **11** and **18** (Tables 1 and 2) with β-galactosidase. Glycopeptide **11** contained the O-glycosylated amino acid building block, and structure **18** contained both the S- and N-glycosylated amino acid building blocks. Lactose was used as a positive control to confirm the activity of the enzyme. All substrates were incubated overnight at 37 °C in the presence and absence of β-galactosidase. GC/MS analysis of the lactose references indicated efficient cleavage of the glycosidic linkage in the presence of the enzyme (data not shown). The glycopeptide mixtures were analyzed by MALDI-TOF MS. In the reference spectrum of **11** without β-galactosidase (Figure 5a), a small peak at $m/z = 1655.4$ is observed, indicating carbohydrate loss under MALDI-TOF MS conditions ($\Delta m/z = 162.1$). This peak is significantly increased in the spectrum of β-galactosidase-treated **11** (Figure 5b), indicating that *O*-glycopeptides are not completely stable toward β-galactosidase, although they are far less susceptible toward enzymatic degradation than lactose.

A possible explanation for this could be that these *O*-glycopeptides are structurally very different from the natural substrates of this enzyme. Furthermore, steric hindrance around the glycosidic linkage may reduce its accessibility for the enzyme. As can be seen from Figure 5c,d, both the reference spectrum in the absence of enzyme and the MALDI-TOF mass spectrum of β-galactosidase-treated **18** show a small peak at m/z 1595.8, which corresponds to traces of carbohydrate loss. Since the extent of carbohydrate loss is similar in both cases, it probably occurs under MALDI-TOF MS conditions; therefore, it is concluded that *S-*,*N*-glycopeptides are completely stable toward β-galactosidase.

Conclusions

In summary, we can conclude that *S-* or *S-*,*N*-glycopeptides mimic carbohydrates with the same efficiency as *O*-glycopeptides. They bound to the lectin in a carbohydrate-specific way, and the measured affinity constants were similar to those measured for the *O*-glycopeptides. Structure **19**, containing two glycosylated cysteine residues, had the highest affinity for the receptor, $K_A = 3.81 \times 10^4 \text{ M}^{-1}$, which is similar to the value reported for *N*-acetyllactosamine. In addition, the *S-*,*N*-glycopeptides were shown to be stable toward β-galactosidase treatment. *O*-glycopeptides, on the other hand, were not completely stable under these conditions.

Our finding that the affinity constants of the *O-* and *S-* or *S-*,*N*-glycopeptide mimics for RCA$_{120}$ are similar to those measured for a disaccharide ligand is in agreement with very recent findings on interactions between GlcNAc-containing glycopeptides and WGA.[18] In contrast, the affinity constants of glycopeptide mimics for Siglec-1, having a characteristic specificity for both the type of sialic acid and the linkage to the penultimate sugar,[14] were higher than mono- to trisaccharides but lower than complex oligosaccharides. In view of the aforementioned, it is interesting to explore whether screening the *O-*, and *S-*,*N*-glycopeptide libraries with a lectin that is more specific than RCA$_{120}$ will result in the detection of high-affinity ligands. This work is currently under investigation with some galectins.

Experimental Section

Materials and General Methods. PEGA$_{1900}$ resin (0.2 mmol/g, 300−500 μm) and Wang resin (0.68 mmol/g, 200−400 mesh, prefunctionalized with a Rink linker) were obtained from NovaBiochem (Läufelfingen, Switzerland). Suitably protected N^α-Fmoc and N^α-Boc amino acids were purchased from Bachem (Bubendorf, Switzerland). RCA$_{120}$ and BSA were obtained from Sigma (Zwijndrecht, The Netherlands). The Alexa Fluor 488 labeling kit was purchased from Molecular Probes (Leiden, The Netherlands). All solvents were of HPLC grade and were used without further purification.

Preparative HPLC was performed on a Knauer HPLC system using a reversed-phase Polaris C18-A column (250 × 4.6 mm) with UV detection at 214 nm. Eluent A (0.1% TFA in 5% aqueous acetonitrile) and eluent B (0.08% TFA in 90% aqueous acetonitrile) were mixed using a linear gradient starting from 90% A to 60% A with a slope of 1.5%/min and a flow rate of 1 mL/min.
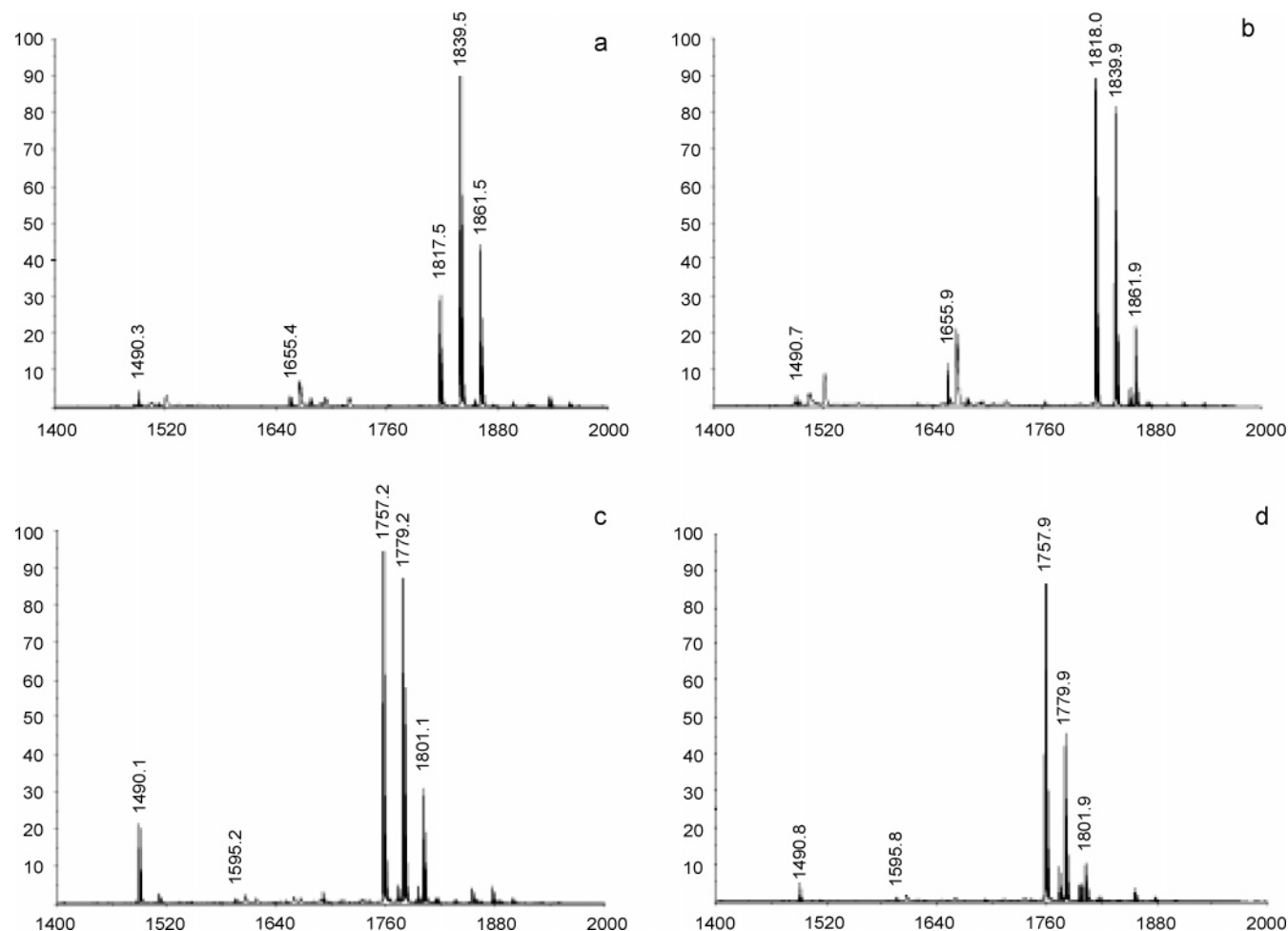
Figure 5. (a) Reference MALDI-TOF mass spectrum of glycopeptide **11**. (b) MALDI-TOF mass spectrum of β-galactosidase-treated **11**. (c) Reference MALDI-TOF mass spectrum of glycopeptide **18**. (d) MALDI-TOF mass spectrum of β-galactosidase-treated **18**.

MALDI-TOF mass spectra were recorded using a Voyager-DE Pro (Applied Biosystems) instrument in the reflector mode at a resolution of 5000 fwhm. Beads containing glycopeptides were placed on a stainless steel target and irradiated with UV light (254 nm, 30 min). The glycopeptides were extracted on the target from the beads using 0.2 μL of a 50% aqueous acetonitrile solution, and 0.2 μL of α-CHC (10 mg/mL) in 50% aqueous acetonitrile was added as a matrix. Exact masses of soluble lead glycopeptides were measured by using α-CHC as a matrix, and a mixture of peptides (Peptide calibration Mix4 (Proteomix) 500−3500 Da, LaserBio Labs, France) was added as the internal standard.

General Methods for Solid-Phase Synthesis. All solid-phase washings were performed for 2 min, and an excess of solvent was used for all washing and deprotection protocols. All resins were washed with DMF (6×) and CH$_2$Cl$_2$ (4×) and lyophilized for at least 24 h prior to use. All manipulations (synthesis and screening) of the glycopeptides that are linked to the resin via a photolabile linker were carried out in subdued light (protected from UV radiation). Solid-phase (glyco)peptide couplings were performed in DMF overnight, and reaction progress was monitored using the Kaiser test.[24] After each coupling reaction, the resin was washed with DMF (10×). The Fmoc-protecting groups were removed by treatment with 20% piperidine in DMF (2 + 18 min),

followed by washing the resin with DMF (10×). De-*O*-acetylation of the monosaccharides was achieved by treating the resin with hydrazine monohydrate (55 μL) in MeOH (1 mL) for 6 h, followed by washing with MeOH (3×), CH$_2$-Cl$_2$ (2×), MeOH (3×), and H$_2$O (5×).

Ladder Synthesis of Libraries A and B. General library construct **4** was prepared in a syringe fitted with a polypropylene filter. Fmoc-K(Fmoc)-OH (2 equiv) and the photolabile linker (4-[4-(1-(Fmoc-amino)ethyl)-2-methoxy-5-nitrophenoxy)]butanoic acid (4 equiv) were sequentially coupled to PEGA$_{1900}$ resin (1.8 g, ~750 000 beads). Fmoc-amino acids were used to successively introduce the first five amino acids (4 equiv; 3.9 equiv of TBTU and 6 equiv of NEM) of the IMP spacer (GPPFPF). The last amino acid of the IMP-spacer, Gly, was coupled as a mixture (9:1 ratio, 4 equiv) of Fmoc and Boc amino acids to generate a base peak in the MALDI-TOF MS spectra of the glycopeptides.

General library construct **4** (0.4 mmol/g loading; library A, 1.0 g, ~420 000 beads; library B, 0.8 g, ~330 000 beads) was equally distributed over the wells of a 20-well, multiple-column peptide synthesizer[22] (2.0-mL capacity). To each well, one of the following amino acids was coupled as a 9:1 mixture of Fmoc and Boc amino acids after 5 min of preactivation (4 equiv; 3.9 equiv of TBTU and 6 equiv of NEM): Val, Met, Arg, His(Boc), Ser(*t*Bu), Cha, Ile, Pro, Asn, Ala, Thr(*t*Bu), Gly, Gln, Trp, Phe (2×) and Tyr(*t*Bu)

(2×). The glycosylated Fmoc-amino acids and their complementary capping agents were coupled to the two remaining wells in the appropriate ratios to achieve 10% capping (4 equiv of **1**,[19] 0.17 equiv of nonanoic acid-OPfp; 4 equiv of **2**,[20] 0.7 equiv of nonanoic acid-OPfp; 4 equiv of **3**,[20] 0.7 equiv of pentadecanoic acid-OPfp). All (glycosylated) amino acids and capping agents were coupled directly after activation with Dhbt-OH (1 equiv). After each coupling, the resin was pooled, mixed, and distributed again over all wells prior to Fmoc removal. The washing protocol (general methods) followed each coupling and deprotection step. After six coupling steps, the Fmoc-protecting groups were removed, and the resin was washed with DMF (10×) and CH_2Cl_2 (5×). Subsequently, the resin was treated with a mixture of TFA/EDT/thioanisole/H_2O/CH_2Cl_2 (82.5:2.5:5:5:5 v/v, 2 min + 2.5 h) to remove the Boc and amino acid side-chain protecting groups. The resin was then washed with 90% HOAc (4 × 5 min), 5% DIPEA/DMF (2 × 2 min), DMF (4×), CH_2Cl_2 (10×), and MeOH (5×).[12] After de-O-acetylation of the monosaccharides (general methods), the libraries were lyophilized, protected from light, and stored at −20 °C. Taking into account that in each step, 10% of the incorporated amino acids were glycosylated, ∼45% of all library members should be glycopeptides.

Solid-Phase Library Screening. Prior to library screening, RCA_{120} was labeled with the Alexa Fluor 488 fluorescent dye according to the manufacturer's protocol, with the exception that the excess of dye was removed by centrifugal filtration using a 30K MWCO filter (Nalgene) and repeated washings with a 10 mM PBS buffer, pH 7.4, containing 2.7 mM KCl and 137 mM NaCl (10×). The library screening was carried out at room temperature. The beads (50 mg, ∼21 000 beads) were swollen in PBS buffer, and the resin was blocked with a 1% BSA in PBS buffer solution (1 mL, 30 min) to minimize nonspecific binding. The beads were then incubated with fluorescently labeled RCA_{120} (80 μg/mL) in PBS buffer containing 1% BSA (312 μL) for either 2 h or overnight. The solution was removed by careful suction, and the resin was washed with PBS buffer (2×) and water (1×). Small portions of beads were transferred to a glass plate, swollen in water, and inspected under a fluorescence microscope. The most fluorescent beads were manually collected and analyzed by MALDI-TOF MS.

Synthesis of Soluble Lead Glycopeptides. General solid-phase construct **5** was prepared in a syringe fitted with a polypropylene filter. Fmoc-K(Boc)-OH (3 equiv) was activated with MSNT (3 equiv) and MeIm (2.5 equiv) and coupled to Wang resin (1.9 g) prefunctionalized with a Rink linker. The reaction was repeated to ensure complete reaction of all hydroxyl functions of the linker, and subsequently, the Fmoc protecting group was removed. Then the IMP spacer, GPPFPF, was synthesized as described previously (2 equiv of Fmoc-Aa-OH; 1.9 equiv TBTU; 3 equiv NEM).

Soluble lead glycopeptides were synthesized on construct **5** (15 mg each) using 2 equiv of Fmoc-Aa-OH, and 3 equiv of the glycosylated amino acid building blocks **1**, **2**, and **3**, using TBTU/NEM (1.9 and 4 equiv) and Dhbt-OH (1 equiv), respectively. After the last coupling step, the glycopeptides were de-O-acetylated as described in the general methods.

In a single step, using a mixture of TFA/H_2O/TIS (95:2.5:2.5; 4 × 30 min), the amino acid side chains were deprotected, and the glycopeptides were released from the resin. The cleaved glycopeptides were extracted from the resin with 10% aqueous acetonitrile (4×), then concentrated and purified by reversed-phase HPLC. After purification, the glycopeptides were lyophilized and analyzed by high-resolution MALDI-TOF MS using a mixture of peptides as the internal reference.

Surface Plasmon Resonance. SPR measurements were carried out on a BIAcore 2000 instrument using a CM5 sensor chip. A 10 mM HEPES buffer, pH 7.5, augmented with 150 mM NaCl, 0.005% p20, and 3 mM EDTA, was used during the immobilization of the ligand. A 10 mM Tris−HCl buffer, pH 7.7, augmented with 150 mM NaCl, 2 mM $CaCl_2$, and 2 mM $MgCl_2$, was used as the running buffer in the binding and competition experiments. All buffers were filtered and degassed before use. The data collected were deconvoluted using BIAevaluation 4.1 software. All experiments (surface preparation, affinity determination, and competition assays) were carried out at a flow rate of 5 μL/min.

The carboxymethylated dextran layer in flow cell 2 was activated by injecting a 1:1 mixture (35 μL) of 0.05 M NHS and 0.2 M EDC, and RCA_{120} was immobilized (20 μL, 50 μg/mL in 10 mM NaOAc buffer, pH 5.0) to a level of 4000 RU. The remaining *N*-hydroxysuccinimide esters were blocked by the injection of a 1.0 M ethanolamine hydrochloride solution (35 μL, pH 8.5). A reference surface was created in flow cell 1 by applying the same activation and immobilization procedure, followed by denaturation of the lectin with 6 M guanidine hydrochloride (2 × 20 μL, pH 1.0) and 0.5% SDS (2 × 20 μL).

The competition assays and the experiments for measuring the affinity of lead glycopeptides toward immobilized RCA_{120} were carried out in duplicate, and the average of the two results is reported. For measuring the affinity of the glycopeptides, varying concentrations of the glycopeptides (500−3.6 μM, obtained by 2-fold dilution) in Tris−HCl buffer (pH 7.7) were injected, allowing 10 min for dissociation. The surfaces were not regenerated because the glycopeptides dissociated completely. The data were double-reference-subtracted, and K_A was determined from nonlinear fitting of the plot of the response at equilibrium (R_{eq}) versus the glycopeptide concentration using the average model with steady state affinity in BIAevaluation 4.1.

For the competition experiments, glycopeptides **13**, **15**, **15b**, and **16** (250 μM) were mixed with different concentrations of methyl β-D-galactopyranoside and injected over flow cells 1 and 2 for 2 min, allowing 5 min for dissociation. The inhibitor concentrations ranged from 10 to 0.625 mM, obtained by 2-fold dilution from a 10 mM stock solution in Tris−HCl buffer.

***β*-Galactosidase Digestion of Glycopeptides.** Lactose and glycopeptides **11** and **18** (5.6 nmol each) in Tris−HCl buffer (10 mM, pH 7.7) were kept overnight at 37 °C in the presence and absence of β-galactosidase (50 mU per 20 μL of digest; *Escherichia coli*, Sigma). The samples were diluted with H_2O (300 μL) and filtered through a 5-kDa-cutoff

centrifugal filter (Millipore), and the filter was washed with H_2O (2 × 300 μL). The digested lactose mixture was lyophilized, trimethylsilylated with a mixture of pyridine/hexamethyldisilazane/TMSCl (5:1:1 v/v, 20 min), and analyzed by GC/MS. The glycopeptide mixtures were analyzed by MALDI-TOF MS.

Abbreviations. Boc, *tert*-butyloxycarbonyl; BSA, bovine serum albumin; *t*Bu, *tert*-butyl; Cha, cyclohexylalanine; α-CHC, α-cyano-4-hydroxycinnamic acid; Dhbt-OH, 3,4-dihydro-3-hydroxy-4-oxobenzotriazine; DIPEA, *N,N*-diisopropylethylamine; EDC, *N*-ethyl-*N′*-(dimethylaminopropyl)-carbodiimide; EDT, ethanedithiol; EDTA, ethylenediaminetetraacetic acid; Fmoc, 9-fluorenylmethoxycarbonyl; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid; HOAc, acetic acid; IMP, ionization-mass-peptide; MeIm, 1-methylimidazole; MSNT, 1-(mesitylene-2-sulfonyl)-3-nitro-1*H*-1,2,4-triazole; NEM, *N*-ethyl morpholine; NHS, *N*-hydroxysuccinimide; p20, polyoxyethylenesorbitan; PBS, phosphate-buffered saline; PEGA, poly(ethylene glycol)/dimethylacrylamide copolymer; Pfp, pentafluorophenyl; RCA$_{120}$, *R. communis* agglutinin; SDS, sodium dodecyl sulfate; SPR, surface plasmon resonance; TBTU, *O*-(1*H*-benzotriazoyl-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TIS, triisopropylsilane; TMSCl, trimethylsilyl chloride; Tris, tris(hydroxymethyl)-aminomethane; WGA, wheat germ agglutinin.

Supporting Information Available. All identified hits from the screening of libraries A and B. This material is available free of charge via the Internet at http://pubs.acs.org.

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