Oligosaccharide Mimetics Obtained by Novel, Rapid Screening of Carboxylic Acid Encoded Glycopeptide Libraries

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Abstract: Glycopeptides that mimic the action of oligosaccharides have been rapidly identified through the implementation of combinatorial library methodology combined with a novel, easy, screening and analysis method. A glycopeptide library containing three different glycosyl amino building blocks, Fmoc-Asn(β-Ac$_3$-GlcNAc)-OPfp (**5**), Fmoc-Thr(α-Ac$_4$Man)-OPfp (**6**), and Fmoc-Thr[α-Ac$_4$Man(1→3)α-2-*O*-Bz-4,6-Ac$_2$Man]-OPfp (**7**), was synthesized by the portion-mixing method on PEGA solid support. The library was designed to facilitate rapid and unambiguous analysis of the active glycopeptides detected during the high throughput-screening step. Consequently, the library was synthesized using the ladder synthesis approach and linked to the solid support via a photolabile linker. The glycosyl amino acids were labeled with carboxylic acid tags to allow unambiguous identification of the glycan moiety. Photolytic release of active glycopeptide from the resin was induced by irradiation of the bead with the MALDI-TOF-MS laser, and analysis of the resulting spectrum presenting the ladder of glycopeptide fragments yielded the sequence of the active glycopeptide. Glycopeptide ligands were identified for the C-type lectin from *Lathyrus odoratus* by screening the fluorescent-labeled protein in a solid-phase binding assay of the PEGA resin-bound glycopeptide library. Of the several glycopeptide ligands detected, most contained Man or GlcNAc, glycans that display specificity for the lectin in hemagglutination assays. The most active glycopeptides detected from the library screening were T(α-D-Man)ALKPTHV, LHGGFT(α-D-Man)HV, T(α-D-Man)EHKGSKV, GT(α-D-Man)FPGLAV, and T(α-D-Man)LFKGFHV.

Introduction

It is a well-established fact that glycoconjugates on cell surfaces play a vital role in a wide variety of biological phenomena including immune response, intercellular recognition, cellular adhesion, intracellular targeting, cell growth regulation, cancer cell metastasis, and inflammation.[1,2] A plethora of viral, bacterial, mycoplasmal, and parasitic infections are also mediated by the tight associative interaction of glycoconjugates with a protein receptor. As a consequence, there has been an impetus to develop carbohydrate-based therapeutics that ameliorate such pathological conditions either by direct intervention at the cell-surface binding level or by inhibition of the appropriate carbohydrate processing enzyme or transport mechanism.

The glycosylation of proteins confers to them some proteolytic resistance and has a pronounced influence on their conformational stability. The mechanisms of the majority of the above-mentioned interactions are not yet well-elucidated. It is therefore of great interest to understand at the molecular level, the nature of protein−carbohydrate interactions. To facilitate these studies, it is necessary to have ready access to both protein receptors and carbohydrate ligands alike. In Nature, the majority of oligosaccharides involved in ligand−receptor interactions are complex glycans, which are not readily accessible. Often, only a few residues at the nonreducing end of a complex glycan are necessary for tight interaction to the receptor. Therefore, the use of simplified synthetic molecules

that can be rapidly generated and that can mimic the natural ligand can give important information about the nature and topology of the ligand−receptor interaction.[3] Furthermore, such compounds may serve as leads in the development of drugs. For example, glycopeptides that mimic SLe^x and other complex carbohydrates have been identified by rational design[4,5] or from the screening of glycopeptide libraries obtained by parallel synthesis.[6,7] When screened for activity as inhibitors of selectin binding, SLe^x mimics show increased binding compared to the original ligand, probably due to favorable interaction of the peptide scaffold with the receptor.

Combinatorial synthesis of libraries has developed into a useful method for the rapid identification of lead compounds for drug discovery. While there are several methods for the generation of libraries, the portion-mixing or split-and-mix method[8,9] has been implemented most, giving rise to "one-bead-one-compound" libraries.[10] One major liability of this method is identifying the structure of the minute amounts of compound

(1) Varki, A. *Glycobiology* **1993**, *3*, 97−130.
(2) Dwek, R. *Chem. Rev.* **1996**, *96*, 683−720.

(3) Meldal, M.; Christiansen-Brams, I.; Christensen, M.; Mouritsen, S.; Bock, K. In *Complex Carbohydrates in Drug Research*; Bock, K., Clausen, H., Eds.; Munksgaard: Copenhagen, 1994; pp 153−164.
(4) Liu, A.; Dillon, K.; Campbell, R.; Cox, D. C.; Huryn, D. M. *Tetrahedron Lett.* **1996**, 3785−3788.
(5) Marron, T.; Woltering, T.; Weitz-Schmidt, G.; Wong, C.-H. *Tetrahedron Lett.* **1996**, *37*, 9037−9040.
(6) Christensen, M.; Meldal, M.; Bock, K.; Cordes, H.; Mouritsen, S.; Elsner, H. *J. Chem. Soc., Perkin Trans. 1* **1994**, 1299−1310.
(7) Franzyk, H.; Meldal, M.; Bock, K. *J. Chem. Soc., Perkin Trans. 1* **1995**, 2883−2898.
(8) Furka, A.; Sebestyen, F.; Dibo, G. *Abstr. 10th Int. Symp. Med. Chem., Budapest* **1988**, *9*, 288
(9) Furka, A.; Sebestyen, F.; Asgedom, M.; Dibo, G. *Int. J. Peptide Protein Res.* **1991**, *37*, 487−493.

attached to a single bead. With peptide libraries containing only natural amino acids, the sequence of active compounds has conventionally been achieved by Edman degradation sequence analysis, whereas the structures of DNA libraries can be amplified by PCR methods and deduced by nucleotide sequencing. In the case of libraries containing small organic molecules or modified amino acids, there are currently few reliable and direct methods of analysis, and many of these require considerable quantities of compound.

The number of methods for analyzing non-peptide libraries are ever increasing and fall into two categories: direct methods usually based on mass spectrometry and NMR spectroscopy or indirect methods employing encoding, chemical,[11−14] chemoluminescent,[15] or otherwise.[16−19] Many of the methods of chemical encoding are restricted by the additional synthetic effort required and the need to design orthogonal reaction conditions required for the two sets of syntheses. Nonchemical encoding has been reported and includes the use of laser-etched barcodes[17] or the encryption of radio frequency transmitters embedded in the resin beads.[16] Currently, the limitation of the indirect methods seems to be the number of compounds which can be encoded, and although elegant, they are all technologically difficult to perform. For example, the commercial version of the microchip resin requires a volume of approximately 1 mL for each library member. More direct methods, largely based on mass spectrometry, include an isotopic mass encoding strategy[18] and electrospray ionization Fourier transform mass spectrometry.[20] The screening and identification processes have been combined into one by coupling capillary zone electrophoresis of the assay solution with electrospray mass spectroscopy.[19]

Despite the increasing numbers of analytical methods, rapid and unambiguous analysis of modified peptide-like library components on resin beads remains challenging. *In the current work, we present a novel method of rapidly screening and identifying resin-bound active compounds from one-bead-one-compound glycopeptide libraries*. The method, *encoded ladder synthesis*, combines the principles of chemical encoding and ladder synthesis, a method in which a small portion of the growing oligomer chain is capped in each synthetic step.[21] The "ladder" of terminated oligomer fragments is subsequently evaluated in one step by MALDI-TOF mass spectrometry directly indicating the structure and sequence of the oligomer. In the seminal work describing the principle of ladder synthesis,
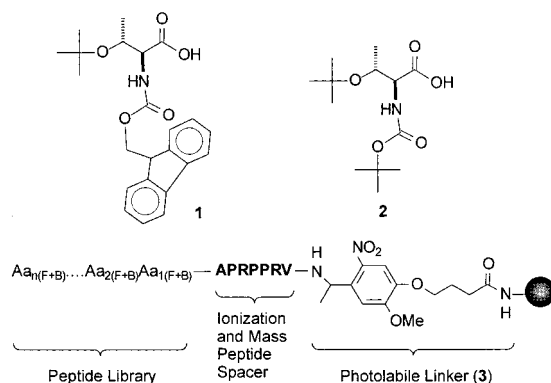

Figure 1. The construct of peptide and peptide libraries containing an IMP spacer and the photolabile linker **3**. A 9:1 mixture of orthogonally protected amino acids (e.g., Fmoc (**1**) and Boc (**2**)) were used in the ladder synthesis of the glycopeptide libraries.

the same compound was used as the capping agent for all of the amino acid building blocks.[21] The use of a single capping agent poses a potential problem because neither the reactants nor the various nucleophiles in a library have the same reactivity and nonuniform capping results. Therefore, our initial attempts to reproduce the ladder synthesis strategy as described were unsuccessful. We then reasoned that a simple solution was to utilize different, but closely related, compounds as the capping agent for each of the building blocks used in the library synthesis. Consequently, in the Fmoc-based synthesis of a peptide, the related capping agent was the Boc-protected counterpart of the Fmoc amino acid building block (Figure 1).

To identify the structure and to distinguish glycans of identical mass, the glycosyl amino acid building blocks (Figure 2) incorporated in the library, were encoded with simple carboxylic acid labels (Figure 3). While the method is illustrated here for glycosyl amino acids, it could be used for any nonnatural or modified amino acid building block. To facilitate on-bead identification of the sequence of active glycopeptides by mass spectroscopy, a photolabile linker was used to attach the glycopeptides to solid support. *Thus, by utilizing an* in situ *encoded capping strategy combined with rapid, photolytic release of glycopeptide fragments, we were able to identify glycopeptide ligands (oligosaccharide mimetics) for the* Lathyrus odoratus *lectin from a 300 000 member glycopeptide library*. The lectin is a C-type lectin from the *Leguminosae* family and exhibits a Man/Glc specificity similar to that of Concanavalin A, favin and pea lectins and other members of the *Lathyrus* genus.[22,23]

Results and Discussion

Construction of the Glycopeptide Library. Linker. To increase the speed of glycopeptide library analysis, direct on-bead assays, and facile cleavage of the active peptides from the solid support, photolabile amide linker **3** was used. This linker is stable to both acidic and basic conditions used for the deprotection of amino acid side chains and carbohydrate residues, respectively, but is more easily cleaved than other photolabile linkers (Figure 1).[24] However, our efforts to effect rapid, complete cleavage of the glycopeptide under the conditions described were futile. Yields were variable, and in some instances, the glycan moiety was cleaved from the glycopeptide

(10) Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. *Nature (London)* **1991**, *354*, 82−84.

(11) Balkenhohl, F.; von dem Bussche-Hunnefeld, C.; Lansky, A.; Zechel, C. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 2288−2337.

(12) Liang, R.; Yan, Y.; Loebach, J.; Ge, M.; Uozumi, Y.; Sekanina, K.; Horan, N.; Gildersleeve, J.; Thompson, C.; Smith, A.; Biswas, K.; Still, C.; Kahne, D. *Science* **1996**, *274*, 1520−1522.

(13) Ohlmeyer, M.; Swanson, R.; Dillard, L.; Reader, J.; Asouline, G.; Kobayashi, R.; Wigler, M.; Still, C. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 10922−10926.

(14) Nestler, H. P.; Bartlett, P. A.; Still, W. C. *J. Org. Chem.* **1994**, *59*, 4723−4724.

(15) Egner, B.; Rana, S.; Smith, H.; Bouloc, N.; Frey, J.; Brocklesby, W.; Bradley, M. *Chem. Commun.* **1997**, 735−736.

(16) Nicolaou, K. C.; Xiao, X.-Y.; Parandoosh, Z.; Senyei, A.; Nova, M. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 2289−2291.

(17) Xiao, X.-Y.; Zhao, C.; Potash, H.; Nova, M. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 780−782.

(18) Geysen, M.; Wagner, C.; Bodnar, W.; Markworth, C.; Parke, G.; Schoenen, F.; Wagner, D.; Kinder, D. *Chem. Biol.* **1996**, *3*, 679−688.

(19) Chu, Y.-H.; Dunayevskiy, Y.; Kirby, D.; Vouros, P.; Karger, B. *J. Am. Chem. Soc.* **1996**, *118*, 7827−7835.

(20) Winger, B.; Campana, J. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1811−1813.

(21) Youngquist, S. R.; Fuentes, G.; Lacey, M.; Keough, T. *J. Am. Chem. Soc.* **1995**, *117*, 3900−3906.

(22) Kolberg, J.; Michaelsen, T.; Sletten, K. *FEBS Lett.* **1980**, *117*, 281−283.

(23) Sakakibara, M.; Noguchi, H.; Makino, S. *Agric. Biol. Chem.* **1979**, *43*, 1647−1658.

(24) Holmes, C.; Jones, D. *J. Org. Chem.* **1995**, *60*, 2318−2319.
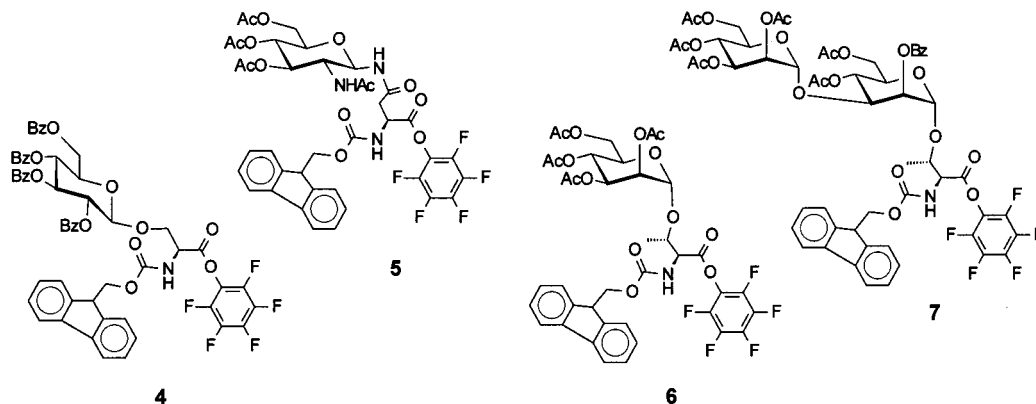
Figure 2. Glycosyl amino acid building blocks used in glycopeptide and glycopeptide library synthesis. The building blocks were prepared by glycosylation of Fmoc amino acid OPfp esters.
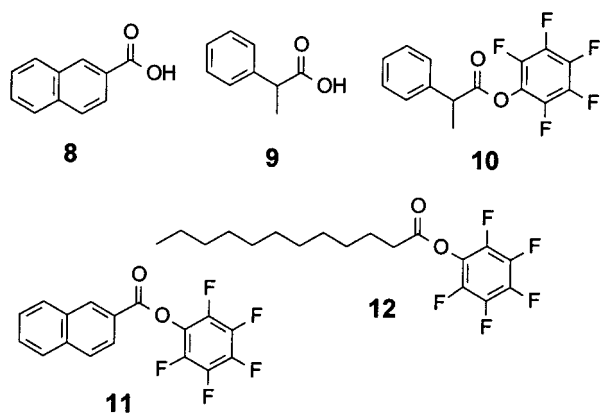

Figure 3. Carboxylic acids and OPfp esters used as labels for encoding of glycopeptides and glycopeptide libraries.

under the basic conditions that developed during photolysis. In a recent article, the cleavage of peptides linked to the solid support by an α-methyl-phenacyl linker by direct radiation of the beads by the MALDI N_2 laser was reported.[25] Although the N_2 laser irradiates at 337 nm and the optimum wavelength given for the cleavage of the *o*-nitrobenzyl linker is 365 nm,[24] it was possible to facilitate on-bead cleavage of the peptide from the resin using the MALDI N_2 laser. A 20-min irradiation with a Hg lamp prior to analysis is also effective.

Ionization/Mass Peptide Spacer (IMP Spacer). To analyze the peptide fragments generated during the ladder synthesis by MALDI, it was necessary to incorporate an ionization/mass peptide spacer (IMP) into the library construct (Figure 1).

The spacer of choice, **APRPPRV**, had a mass greater than 600 which placed the mass of the peptide fragments out of the region containing the matrix adduct peaks. Arginine was incorporated into the IMP spacer, to improve MALDI sensitivity and to ensure a reasonably uniform response from the various fragments. The preponderance of prolines in the spacer was employed to increase both the mass and the protease stability. The prolines are expected to show little specific interactions with the carbohydrate receptor being analyzed, and this premise was supported by our results.

Ladder Synthesis of a Pentapeptide of Known Sequence. For the ladder synthesis methodology to succeed, it is important that accurately measured quantities of the capping reagent and building block are added simultaneously and that complete coupling and capping occurs at each step. In work by Younguist and co-workers,[21] a single compound, Ac-Ala-OH (10%), was

(25) Fitzgerald, M.; Harris, K.; Shevlin, C.; Siuzdak, G. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 979–982.

used as the capping agent. Because the reactivities of the building block and capping agent were not matched, a 10-fold excess of reagent was used drive the reaction to completion. However, it is unclear how this would improve the control of capping. A particular problem arises from the presence in the library of a large number of nucleophiles with different reactivities.

Matching reactivities of capping agent and building block is possible by using species with identical side chains but with orthogonal N^α-urethane amino protecting groups (Figure 1). Consequently, we used Boc-protected amino acids as the temporary capping agent in an Fmoc-based peptide synthesis. The converse approach is also possible. To test the success of this novel method, a pentapeptide VSAFT attached to the resin via the IMP spacer, **PPPRM**, and the photolabile linker **3** was synthesized using TBTU/NEM activation. A methionine residue was incorporated into this IMP spacer to allow alternative CNBr promoted cleavage of the peptide from the resin to compare with release of the peptide by irradiation with the MALDI N_2 laser. A mixture of N^α-Fmoc (**1**) and N^α-Boc (**2**) protected amino acids (9:1 ratio; 2 equiv total) was added during the coupling step. The N-terminus was acetylated prior to acidic cleavage of the N^α-Boc and other side-chain protecting groups. Analysis was achieved by grinding a few beads, adding matrix, and irradiating the resulting slurry with the MALDI laser. The correct sequence of the peptide was obtained (Figure 9, Supporting Information).

Encoded Ladder Synthesis of a Glycopeptide of Known Sequence. To test the efficacy of the carboxylic acid encoding procedure for glycopeptide synthesis, a hexapeptide, Ac-A-S(Glc)AVS(Glc)F, attached to PEG–sarcosine resin via the IMP spacer, **PRPPRV**, and the photolabile linker **3** was synthesized. Because MALDI analysis of the ladder sequence of the nonglycosylated test peptide cleaved with CNBr from a single bead showed weak signal intensities, it was decided to use a higher capacity resin and an additional arginine in the IMP spacer in order to improve signals from peptide fragments. The nonglycosylated amino acid was coupled as described for the nonglycosylated test peptide. A preblocking strategy was initially employed, in which the free amino groups were reacted with 0.1 equiv of the acid using TBTU/NEM activation. The remaining groups were then reacted with the glycosyl amino acid building block Fmoc-S(Bz$_4$Glc)-OPfp **4** (2 equiv). The first S(Glc) was labeled with phenylpropionic acid, **9**, and the second with naphthoic acid, **8**. MALDI-TOF-MS indicated that the encoding was successful, and the two S(Glc) residues were readily identified. The preblocking strategy works well, provided that all of the reacting amino groups have similar reactivities.

However, in a laddered synthesis of a library, where different peptide amino groups have considerably different reactivities, a preblocking strategy will not be effective. Consequently, the acid labels were synthesized as OPfp esters to achieve comparable reactivity with the activated glycosyl amino acids used routinely in our laboratory.

Synthesis of Glycosyl Amino Acid Building Blocks and Acid Label Esters. Glycosyl amino acids. The glycosyl amino acid building blocks **4**,[26] **5**,[27] **6**,[28] and **7**[7] were synthesized as described.

Carboxylic Acid Labels. The carboxylic acid labels (Figure 3) were chosen such that they would differ in mass from all of the amino acids or other building blocks used. These acids were converted to OPfp either by activation with DCC[29] or by reaction with pentafluorophenyl trifluoroacetate (TFA-OPfp).[30] The latter method was superior in that better yields (90% vs 40−75%) were obtained, the reaction times were short (5 min), and the products, if solid, could be obtained pure by precipitation from the crude reaction mixture.

Relative Reactivity Assessment of Label and Glycosyl Amino Acid by MALDI-TOF-MS. The relative reactivities of the acid labels were determined using two resin-bound nucleophiles, IMP spacers **VPRPPRV** and **APRPPRV**. The valine reactant, **VPRPPRV**, was selected because its amino group is less reactive than that of other amino acids and should clearly indicate any differences in reactivity between two reactants. The less hindered amino nucleophile, **APRPPRV**, was also used in the reactivity tests. Fmoc-Thr(α-Ac$_4$Man)-OPfp (**6**) was used as the representative glycosylated building block; however, for comparison, selected labels were also reacted with **5** and **7**. An equimolar mixture of the label and glycosylated building block was coupled to the resin-bound nucleophiles. After deprotection, the intensities of the product mass peaks in the MALDI spectrum were measured, and the relative reactivity was correlated with peak intensity. It must be noted that this is not a quantitative assessment of reactivity. However, since both products have identical IMP spacers, their ionization and flight properties are similar. Therefore, the relative product signal intensities are a measure of the relative reactivities. Signal intensities are thought to be as accurate as peak areas and were used in these calculations.[31]

The relative reactivities of selected labels and building blocks are presented in Table 1. Generally, there is a larger difference in reactivity rates between the label and the building block with hindered nucleophiles. In addition, the relative reactivity of label **10** compared to that of glycosyl amino acids **5** and **6** (entries 1 and 2), is very similar, suggesting that the reactivities of carbohydrate building blocks and a particular label toward a particular nucleophile are essentially the same. The relative reactivities obtained from studies with **VPRPPRV** were used in the synthesis of the library.

To ensure that the relative reactivities calculated were correct, a capping procedure was carried out on **VPRPPRV**, adjusting the quantity of label to yield 10% capping based on the relative

Table 1. Relative Reactivities of Selected Glycosyl Amino Acid Building Blocks and Carboxylic Acid Ester Labels towards Two Resin-Bound Amino Nucleophiles

entry	glycosyl amino acid (GA)	acid label	relative reactivity of label:GA[a]	relative reactivity of label:GA[b]
1	**5**	**10**	1:3	1:1
2	**6**	**10**	1:2.5	
3	**6**	**11**	3:1	
4	**6**	**12**	9.5:1	5:1
5	**7**	**11**		1.5:1

[a] A 1:1 mixture of building block and label was coupled to VPRPPRV−photolinker-resin. [b] A 1:1 mixture of building block and label were coupled to APRPPRV−photolinker-resin.

reactivities determined. The results (not shown) indicated that the relative reactivities are generally valid and useful both for hindered and less hindered nucleophiles.

Synthesis of Glycopeptide Library by Encoded Ladder Synthesis. A random heptaglycopeptide library was synthesized on 250 mg of resin consisting of approximately 300 000 beads giving rise to roughly 300 000 members. The IMP spacer, **APRPPRV** was the initial nucleophile. Three glycosylated amino acid building blocks (Figure 2) and their acid labels (Figure 3) were incorporated into the library as follows: Fmoc-Asn(β-Ac$_3$GlcNAc)-OPfp (**5**) and label **10** (label mass difference, 132.2); Fmoc-Thr[β-Ac$_4$Man(1→3)α-2-O-Bz-4,6-Ac$_2$-Man]-OPfp (**7**) and label **11** (label mass difference, 154.2); and Fmoc-Thr(α-Ac$_4$Man)-OPfp (**6**) and label **12** (label mass difference, 182.3). Five amino acids, Arg, Cys, Gln, Ile, and Met, were excluded from the library to allow for the incorporation of the glycosylated building blocks. To use all of the columns of the custom-made 20-column synthesizer,[32] **5** and **6** were coupled in two columns during the synthesis. The nonglycosylated amino acids were incorporated using Fmoc/TBTU/NEM methodology, and the glycosylated building blocks and their labels were coupled using the pentafluorophenyl ester strategy. The Boc-protected counterpart (10%) of each amino acid was utilized as the capping agent. The library was synthesized using a high loading PEGA resin (0.46 mequiv/g) equipped with a photolabile linker, and the progress of the coupling reactions was monitored using the Kaiser test.[33]

Results from the mass analysis of some library members indicated that encoded ladder synthesis was very efficient. The labels were clearly detected and easily distinguished from amino acids of relatively close mass difference (e.g., lauric acid (182) vs Trp (186)). The glycosylated amino acids were thus easily identified. In addition to elucidating the sequence of library members, the mass analysis was useful in identifying some problems that occurred during the synthesis of the library. Often, a peak 97 mu higher than the mass of the parent compound, corresponding to a TFA adduct formed during deprotection of the amino acid side chains, was present. This product, however, can easily be reverted to the free amino group by treatment with piperidine. The dehydration of Asn was sometimes observed by the presence of a peak 18 mu less that the mass of the fragment peak. Finally, carbohydrate elimination was occasionally observed. It is most likely that the loss of the carbohydrate occurred under MALDI-TOF-MS conditions and not during deacylation since increasing laser power led to an increase in the extent of carbohydrate loss. Changing from CHC matrix to DHB matrix or using a high-performance MALDI-TOF-MS instrument reduced the extent of carbohydrate loss.

(26) Reimer, K.; Meldal, M.; Bock, K. *J. Chem. Soc. Perkin Trans. 1* **1993**, 925−932.

(27) Meinjohanns, E.; Meldal, M.; Bock, K. *Tetrahedron Lett.* **1995**, *36*, 9205−9208.

(28) Andrews, D. M.; Seale, P. W. In *Peptides 1992*; Schneider, C. H., Eberle, A. N., Eds.; ESCOM: Leiden, 1992; p 355.

(29) Atherton, E.; Sheppard, R. C. In *Solid-Phase Peptide Synthesis: A practical approach*; IRL Press at Oxford University Press: Oxford, 1989; pp 76−79.

(30) Breslav, M.; Kalejs, U.; Pupikina, S.; Doviborov, N. *J. Chem. Res. (S)* **1992**, 272−273.

(31) Krone, J. R.; Nelson, R. W.; Dogruel, D.; Williams, P.; Granzow, R. *Anal. Biochem.* **1997**, *244*, 124−132.

(32) Meldal, M.; Holm, C. B.; Bojesen, G.; Jacobsen, H.; Holm, A. *Int. J. Pept. Protein Res.* **1993**, *42*, 250−260.

(33) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595−598.

13316   *J. Am. Chem. Soc., Vol. 120, No. 51, 1998*
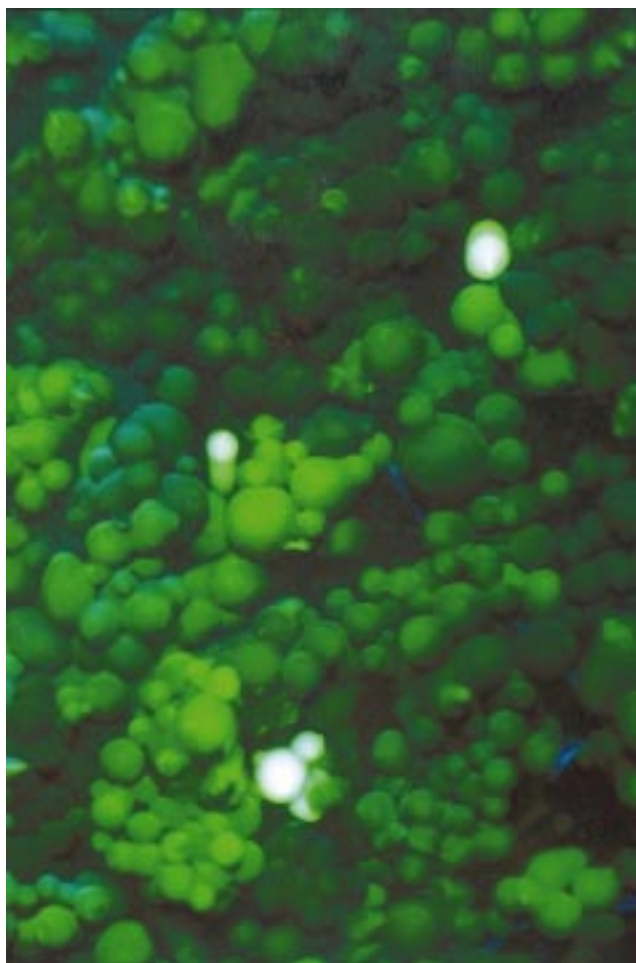
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Figure 4. A selection of library beads after incubation with fluorescent labeled *Lathyrus odoratus* lectin. Active beads varied in fluorescence intensity. The beads with the most intense fluorescence were collected manually and the compounds analyzed by MALDI-TOF-MS.

Figure 5. The MALDI-TOF-MS spectrum of a nonglycosylated active peptide **30** showing the peptide ladder sequence.

Figure 6. The MALDI-TOF-MS spectrum of the monoglycosylated active peptide **23** showing the peptide ladder sequence and carboxylic acid label, lauric acid (Lau), which indicates that the glycosylated residue is Thr(α-D-Man) (Tm) and is at the N terminus. The peak at 1936.1 represents the peptide−TFA adduct.

Library Screening. Solid-Phase Assay of *Lathyrus odoratus* Lectin and MALDI-TOF-MS Analysis. Portions of the library were incubated with FITC-labeled sweet pea lectin, *L. odoratus* and compared to the nonfluorescent background. Between 0.1 and 0.05% of the library beads showed varying degrees of fluorescence (Figure 4). Bright beads (approximately 80) were transferred to the MALDI-TOF-MS target, and spectra were recorded. High quality spectra showing the ladder of peptide fragments and acid labels were obtained from single beads. Often, good spectra were obtained from a single laser pulse. Noticeably absent from these spectra were the peaks due to sodium and potassium adducts often observed when recording spectra of compounds that had been cleaved from the resin prior to mass analysis. MALDI-TOF-MS spectra of selected peptides are shown in Figures 5−7. Both nonglycosylated and glycosylated peptides were detected as active compounds (Table 2). The glycopeptides contained either Man or GlcNAc or both. Interestingly, no active compounds contained the Man disaccharide, suggesting that the lectin's sugar binding site may only accommodate a monosaccharide. In most cases, the specificity for Man arose from N-terminal positioning of the glycan, whereas the GlcNAc specificity was observed primarily in the C-terminal to the middle position of the glycan. While there was no clear sequence consensus for the nonglycosylated peptides, they all contained a preponderance of polar or hydroxyl groups that could form hydrogen bonds to the carbohydrate binding site of the lectin.
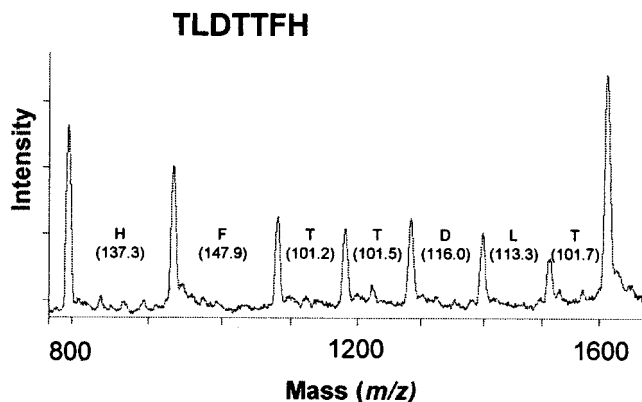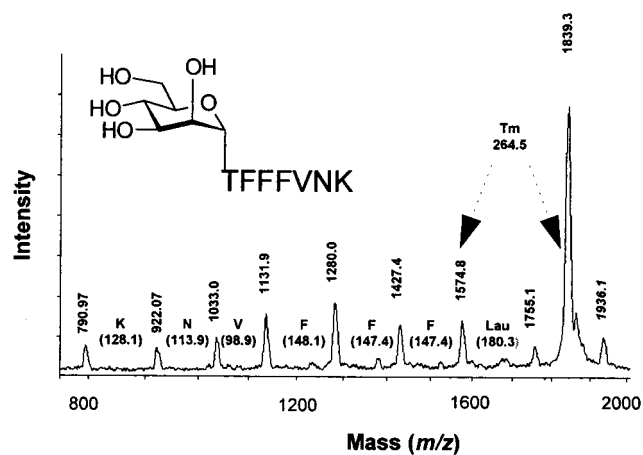
Test of Lead Glycopeptides. To verify the results from the library, representative active peptides were synthesized and treated with the fluorescent-labeled lectin while still attached to the solid phase. The results are summarized in Figure 8 and are compared to the binding of the fluorescent-labeled lectin to resin bound α-D-Man (**32**) and β-D-GlcNAc (**33**). The most active compounds were glycopeptides containing only Man (peptides **22**, **23**, **24**, **25**, **26**, and **27**) displaying up to a 25-fold increase in fluorescence compared to lectin binding to resin-bound Man. Peptides containing both glycans or only β-D-GlcNAc exhibited moderate fluorescence, a 2−4-fold increase over resin-bound Man. Of the nonglycosylated peptides, **29** and **31** demonstrated lower fluorescence intensity compared to GlcNAc or Man, whereas **30** showed a brightness equivalent to peptides **14** and **19** and almost 2-fold greater than that of Man. Interestingly, peptides **15** and **16** exhibited very low fluorescence intensity. Substituting a manosylated amino acid for a glycosylated one resulted in a doubling of the fluorescence, whereas replacement of a GlcNAc-Asn with an Asn resulted in a reduction of fluorescence intensity (Figure 8, peptides **27** and **28**; **13** and **14**).

Most of the tightest binding glycopeptides (peptides **22**, **23**, **24**, **25**, **26**, and **27**) contain α-D-Man at the N terminus and hydrophobic residues (F, L, A, and H) in the sequence. These results are consistent with the Man binding specificity of the lectin. In hemagglutination assays using chicken erythrocytes, *L. odoratus* lectin exhibited a weak (mM) specificity for methyl
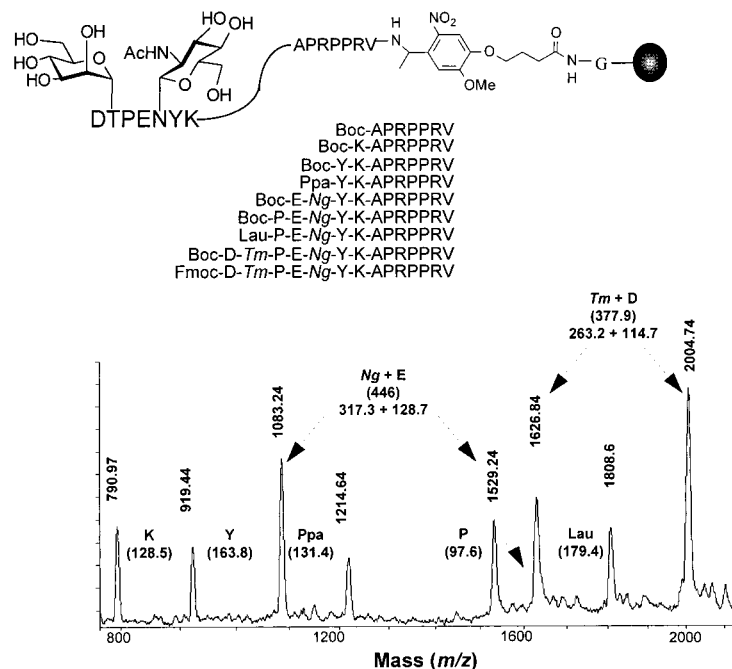
Boc-APRPPRV
Boc-K-APRPPRV
Boc-Y-K-APRPPRV
Ppa-Y-K-APRPPRV
Boc-E-*Ng*-Y-K-APRPPRV
Boc-P-E-*Ng*-Y-K-APRPPRV
Lau-P-E-*Ng*-Y-K-APRPPRV
Boc-D-*Tm*-P-E-*Ng*-Y-K-APRPPRV
Fmoc-D-*Tm*-P-E-*Ng*-Y-K-APRPPRV

Figure 7. The MALDI-TOF spectrum of the diglycosylated active peptide **16** showing the peptide ladder sequence and carboxylic acid labels, lauric acid (Lau), and phenylpropionic acid (Ppa) which correspond to the glycosylated residues Thr(α-D-Man) (**Tm**) and Asn(β-D-GlcNAc) (*Ng*), respectively. Also shown are the partial peptide fragments generated during ladder synthesis.

Table 2. Mass of Resin-Bound Lead Peptides[a]

peptide	sequence	mass expected (M+, MNa+)	mass observed
13	**Tm**FHFVE*Ng*V	1355.6, 1378.6	1356.0, 1378.3
14[b]	**Tm**FHFVENV	1153.3, 1176.3	1154.1, 1176.7
15	**Tm**T*Ng*SLE*Ng*V	1443.6, 1466.6	1469.1
16	D**Tm**PE*Ng*YKV	1328.6, 1351.6	1329.1, 1352.0
17	**Tm**HA*Ng*VPTV	1202.3, 1255.3	1203.9
18	*Ng*TFF**Tm**PTV	1290.4, 1313.4	1315.1
19	PHG*Ng*GTEV	1011.4, 1034.4	1012.0, 1032.4
20	VYYG*Ng*FLV	1175.6, 1198.6	1199.0
21	TWF*Ng*GFSV	1158.5, 1181.5	1180.6
22	LHGGF**Tm**HV	1028.2, 1051.2	1028.9
23	**Tm**FFFVNKV	1161.6, 1184.6	1182.4
24	**Tm**EHKGSKV	1046.2, 1069.2	1047.5
25	**Tm**ALKPTHV	1027.2, 1050.2	1028.4
26	G**Tm**FPGLAV	922.1, 945.1	923.0
27	**Tm**LFKGFHV	1108.6, 1131.6	1130.8
28[b]	**Tm**LFK**Tm**FHV	1315.5, 1338.5	
29	EFPWLSEV	1004.5, 1032.5	1011.6, 1030.9
30	TLDTTFHV	930.5, 953.5	951.1
31	YGEASTTV	825.9, 848.9	848.5

[a] All MALDI spectra obtained using CHC matrix with bradykinin (1060.2 mu) as the reference except for peptides **19**, **22**, **24**, **25**, **27**, and **29** when Substance P (1347.7 mu) was used as the reference. [b] Not obtained from direct library screen.

α-D-Man, methyl α-D-Glc, and GlcNAc.[23,34] In that assay, the lectin bound two times more tightly to methyl α-D-Man than to Man and two times more tightly to Man than to GlcNAc. Results from our solid-phase assay are in agreement with these observations since resin bound α-D-Man exhibits an increase in fluorescence 3-fold greater than that of resin-bound β-D-GlcNAc after incubation with labeled lectin. It is also noteworthy that the glycopeptides contain a phenylalanine residue in close proximity to the glycan-containing residue. It is well-documented that lectins contain a preponderance of aromatic amino acids in the binding site and that these help tighten the binding of a carbohydrate ligand by interacting with the β-face
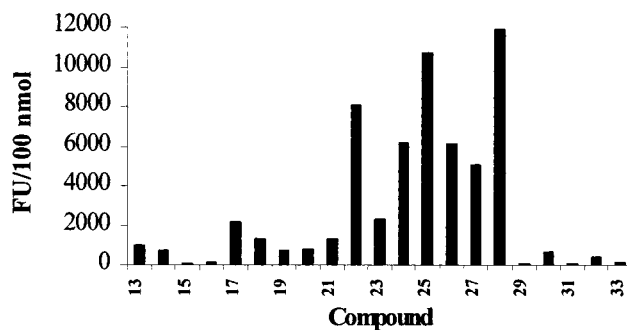
(34) Ticha, M.; Zeineddine, I.; Kocourek, J. *Acta Biol. Med. Germ.* **1980**, *39*, 649−655.

Figure 8. Bar graph showing the binding of fluorescent-labeled *Lathyrus odoratus* lectin to different resin-bound compounds. Fluorescence intensity has been normalized for resin weight and loading. Compounds **32** and **33** represent resin-bound mannose and GlcNAc, respectively.

or nonpolar parts of the saccharide. In this case, it is possible that binding is enhanced by hydrophobic interactions between the phenylalanines of the glycopeptide and complementary surfaces in the lectin active site. In an illustrative example, Concanavalin A was found to bind nine times more tightly to α-4-nitrophenyl D-Man than to methyl α-D-Man.[35]

Influence of Spacer and Ladder Fragments. Peptides **15** and **16** represent interesting deviations because, on the basis of the sequence, they were expected to have a binding activity comparable to peptides **13** and **17**. The reason for their low binding activity is as yet unclear. It was initially thought that the peptide ladder fragments enhance binding of the receptor to a bead during the screening process, leading to a high percentage of false positives. However, when some of the lower binding peptides (**14**, **16**, and **31**) were resynthesized with the ladder and incubated with fluorescent-labeled lectin, the results suggested that the ladder caused little binding enhancement.[36]

(35) Page, D.; Zanini, D.; Roy, R. *Bioorg. Med. Chem.* **1996**, *4*, 1949−1961.

(36) FU/mg of resin-bound compound after incubation with lectin. Peptide, without ladder/with ladder, **14:** 2181/1541**;** **16:** 2655/2822**;** and **31:** 758/1502.

We also investigated the influence of the spacer on binding to the lectin using peptide **23** as an example. Results indicate that the spacer has no significant effect on the binding (FU/mg resin for peptide without spacer, 2889, and FU/mg resin for peptide without spacer, 2950).

The presence of nonglycosylated peptides was initially attributed to normal occurrence of false positives that plagues any screening method. Selected peptides **29** and **31**, showed little binding, half that of GlcNAc. However, **30** demonstrated a 2-fold increase in binding compared to resin-bound Man and a 6-fold increase in binding compared to GlcNAc, suggesting that it is an active ligand and that the binding specificity of oligosaccharides can be mimicked not only with glycopeptides but also with peptides. This phenomenon was previously observed with Concanavalin A.[37] Similarly, several peptides from a peptide phage display library, were shown to cross-react with monoclonal antibodies directed against Streptococcus group A cell wall polysaccharide.[38]

Inhibition of Binding of Lectin to Resin-Bound Compounds. To establish that the glycopeptides obtained from the library screen are indeed mimetics of methyl α-D-Man and GlcNAc, attempts were made to carry out the traditional Landsteiner hapten inhibition assays. Our efforts at inhibition were thwarted since the glycopeptides themselves induce the agglutination of rabbit erythrocytes at certain critical concentrations (data not shown). We then attempted to inhibit the binding of the fluorescent-labeled lectin to selected resin-bound glycopeptides using a modification of a solid-phase inhibition assay.[39] Binding of labeled lectin to glycopeptides **14** and **28** and peptide **30** were inhibited using methyl α-D-Man. In no case was complete inhibition achieved at the concentrations of Man used (25.6−250 mM). At a Man concentration of 31.25 mM, there was 34% inhibition of binding to **14**, 17% inhibition to **28**, and 45% inhibition to peptide **30**.

We also compared the ability of methyl α-D-Man and **28** to inhibit the binding of the lectin to resin-bound Man. As previously, there was no complete inhibition at the concentrations used, and to achieve a 30% inhibition of binding of lectin to resin-bound Man required 62.5 mM methyl α-D-Man or 9.0 mM **28**.

The results from the inhibition studies suggest that the peptide **30** and methyl α-D-Man binds to *L. odoratus* in related binding sites. It is also clear that glycopeptide **19** obtained from the screening binds tighter to the fluorescent-labeled lectin than methyl α-D-Man. As previously noted,[39,40] an extrapolation of results from a solid-phase, heterogeneous assay in which the ligands are presented polyvalently to a solution phase where the ligands can be presented in a homogeneous monovalent fashion is not necessarily a direct one. As with any screening procedure, further characterization and optimization of the leads obtained is necessary. The need for an optimization process, however, by no means negates the merits of the solid-phase screening process which quickly provides a convenient starting point.

Conclusions

A novel method has been developed for rapid and easy analysis of glycopeptide libraries as ligands for carbohydrate-

binding proteins. The protocol is based on the generation of a library of resin-bound glycopeptides. During library synthesis, the capping of a small portion of the growing oligomer chain with derivatives of comparable reactivities produces a ladder of compounds for facile mass spectrometric analysis. The resulting encoded glycopeptide "one-bead-one-ligand" library is then screened in a simple solid-phase fluorescence-binding assay. In the system tested, the labels and the fragments generated do not appear to greatly influence the screening process. The methodology is useful in identifying not only the glycan moiety but also other amino acids and building blocks with identical mass. This method can be extended to most libraries and does not involve tedious or complicated synthetic steps. By implementation of the encoded ladder synthesis strategy, we have identified glycopeptide ligands for the *L. odoratus* lectin. However, as with any screening process, lead optimization and further characterization of the ligand-receptor interaction must be carried out, and the methodology presented in this paper is well-designed to yield good leads for compounds involved in carbohydrate−protein interactions.

Experimental Section

Materials and General Methods. All solvents were used without further purification unless otherwise indicated. DMF was distilled under reduced pressure and stored over activated 4-Å molecular sieves. PEGA 800 resin (0.25 mmol/g, 150−300 μm) was obtained from Polymer Laboratories (Amherst, MA), whereas all other resins used were custom synthesized as previously reported.[41] Suitably protected amino acids (N^α-Fmoc-Aa-OPfp, N^α-Fmoc-Aa-OH, and N^α-Boc-Aa-OH) and Rink amide linker were purchased from Bachem (Bubendorf, Switzerland) and NovaBiochem (Läufelfingen**,** Switzerland). BSA and *L. odoratus* lectin−FITC conjugate were obtained from Sigma (Lot # 86F4038, 3 mol FITC per mol of lectin).

Flash chromatography was performed on silica gel 60, 0.040−0.060-mm mesh (Merck, Darmstadt, Germany). For the purification of OPfp esters, the silica gel was dried by heating at 120 °C for at least 24 h before use. Solid-phase peptide coupling reactions were monitored using the Kaiser test,[33] and solution-phase reactions were monitored by TLC performed on silica gel 60 F_{254} aluminum-backed sheets with detection by UV radiation or charring in 5% sulfuric acid in methanol. Reaction mixtures were dried with anhydrous Na_2SO_4 and then concentrated in vacuo.

^{1}H and ^{13}C chemical shifts are given in ppm and referenced to CDCl$_3$ at 7.26 for ^{1}H NMR and 77.00 for ^{13}C ppm at 300 K. Analytical and semipreparative reversed phase HPLC separations were performed using analytical RCM (8 × 200 mm) and delta PAK (15 μM, 300 Å, 25 × 200 mm) C-18 columns with flow rates of 1 mL/min and 10 mL/min. Detection was at 235 and 280 nm with a photodiode array detector. Solvent system A: 0.1% TFA in water; solvent system B: 0.1% TFA in 10% aqueous acetonitrile. Water was obtained Nanopure at 18.4 Ω. Fluorescence intensity was measured on an HTS 7000 plate reader with excitation at 485 nm and emission at 510 nm.

Synthesis of Carboxylic Acid Ester Labels. Carboxylic acid labels were synthesized according to literature procedure.[29,30] Synthesis and characterization are detailed in Supporting Information.

Synthesis of Resin-Bound β-D-GlcNAc and α-D-Man. Allyl β-D-GlcNAc and allyl α-D-Man were coupled to 3-mercaptopropanoic acid under UV radiation according to literature procedure.[42] The resulting acid was then coupled to resin-bound valine under TBTU activation.

Solid-Phase Synthesis of Peptides and Glycopeptides. All resins were washed with CH$_2$Cl$_2$ (6×) and then dried under vacuum (lyophilizer) for at least 24 h before use. The photolabile linker **3** was synthesized as previously described.[24] All manipulations (synthesis and

(37) Oldenburg, K.; Druaikkannu, L.; Goldstein, I.; Schlutz, P. G.; Gallop, M. A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 5393−5397.

(38) Harris, S.; Craig, L.; Mehroke, J.; Rasheed, M.; Zwick, M.; Kenar, K.; Toone, E. J.; Greenspan, N.; Auzanneau, F.-I.; Marino-Alberas, J.-R.; Pinto, B. M.; Scott, J. K. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, 2454−2459.

(39) Liang, R.; Loebach, J.; Horan, N.; Ge, M.; Thompson, C.; Yan, Y.; Kahne, D. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10554−10559.

(40) Lees, W. J.; Spaltenstein, A.; Kingery-Wood, J. E.; Whitesides, G. M. *J. Med. Chem.* **1994**, *37*, 3419−3433.

(41) (a) Renil, M.; Meldal, M. *Tetrahedron Lett.* **1995**, *36*, 4647−4650. (b) Auzanneau, F.-I.; Meldal, M.; Bock, K. *J. Pept. Sci.* **1995**, *1*, 31−44.

(42) Lee, R. T.; Lee, Y. C. *Carbohydr. Res* **1974**, *37*, 193−201.

screening) of peptides linked to the resin via the photolabile linker were carried out in subdued light (protected from UV radiation).

Deprotection Protocols. After acetylation, the resin was washed with DMF (2×), 5% DIPEA in DMF (2×), DMF (4×), and CH_2Cl_2 (10×) and then dried by suction for 1 h using a water aspirator or by lyophilization overnight. The side-chain protection groups were removed using a cocktail consisting of TFA 82.5%, EDT 2.5%, thioanisole 5%, and H_2O 5% for 2.5 h. The resin was then washed with 90% aqueous acetic acid, (4 × 5 min), 5% DIPEA in DMF (2 × 2 min), DMF (4 × 2 min), CH_2Cl_2 (10 × 2 min), and finally methanol (5 × 2 min) before being dried by suction for 1 h or by lyophilization overnight. Carbohydrate acetyl protecting groups were removed by hydrolysis with hydrazine hydrate (55 μL) in methanol (1 mL) for 6 h followed by washing with methanol (3×), CH_2Cl_2 (3×), methanol (3×), H_2O (3×), toluene (3×), and then diethyl ether (3×). When both benzoyl and acetyl groups were present, the glycopeptide was treated with 0.1 N sodium methoxide (pH 10) for 3 h and then washed with methanol and water. If deprotection was incomplete as determined by MALDI-TOF-MS (particularly in the case of benzoyl groups), the deacylation step was repeated.

Synthesis of Test Peptides for Ladder Synthesis Methodology. Both nonglycosylated and glycosylated test peptides of known sequence were synthesized manually using the syringe technique as described in the Supporting Information.

Acid Label: Glycosyl Amino Acid Relative Reactivity Test. Reactivity test reactions were carried out using resin (2.5 mg, 1.15 μmol) derivatized with photolabile linker and IMP spacer in a disposable syringe (1 mL) fitted with a Teflon filter. To the deprotected peptide was added an equimolar mixture of glycosyl amino acid and carboxylic acid label (2.0 equiv). The reaction was catalyzed by the addition of Dhbt-OH (1 equiv) and proceeded for 2 h. The peptide was completely deprotected and analyzed by MALDI-TOF-MS as described in the Supporting Information.

Encoded Glycopeptide Library Ladder Synthesis. PEGA$_{1900}$ resin (250 mg, 200−300 μm beads, 0.46 mmol/g loading) was used for the peptide libraries. Reactions were run on a 0.12 mmol scale. The peptide was attached to the resin via a photolabile linker and an IMP spacer, **XPRPPRV**, where X is Val or Ala, and was first synthesized using the syringe technology prior to library generation. Randomized positions in the library were generated using the split synthesis approach using a 20-well custom-made (2.0-mL capacity) multiple-column synthesizer. After each coupling, the resin was pooled, mixed, and divided before Fmoc removal with 20% piperidine. The usual washing protocol followed each coupling and deprotection step. For the coupling of nonglycosylated amino acids, a mixture of the Fmoc- and Boc-protected amino acid (90% Fmoc and 10% Boc, 4 equiv) from stock solutions was activated with TBTU/NEM for 6 min and then added to the wells. For the glycosylated amino acids, a mixture of the carboxylic acid capping agent and the glycosyl amino acid in the appropriate ratio to give about 10% capping was added. Coupling times ranged from 4 to 12 h, and reaction completion was checked by the Kaiser test. After the final coupling, the peptide was completely deprotected using scavengers as described above and stored at 4 °C.

Preparation of Lead Glycopeptides. Synthesis. PEGA$_{1900}$ resin (200−300 μm beads, 0.46 mmol/g loading) was used for the synthesis of lead peptides, which were were attached to the resin via a Rink amide linker, and all contained Val at the carboxy terminus. The synthesis was carried out on a 0.018 mmol scale using the Fmoc/OPfp ester methodology and nonglycosylated amino acids (3.0 equiv) and glycosylated amino acid (2.0 equiv). Completion of acylation was monitored using the Kaiser test.

Partial Cleavage. Partial cleavage of the peptides from the resin was effected by treatment with 50% TFA in CH_2Cl_2 (500 μL) for 25 min. The resin was washed with 50% TFA in CH_2Cl_2 (2 × 500 μL) and then with water (2 × 500 μL). The cleaved peptide solution was diluted with water (2 mL), lyophilised, purified by HPLC, and analyzed by MALDI-TOF-MS**.** The resin-bound peptides were washed and carbohydrate-deprotected as usual. Glycopeptides from a few resin beads were cleaved by treatment with 95% TFA for 10 min and the masses analyzed by MALDI-TOF-MS.

Solid-Phase Binding Assays. Glycopeptide Library. Solid-phase

assays were carried out at room temperature (25 °C) in 5-mL syringes that had been cut to a height of 1.5 cm and fitted with a Teflon filter and a stopper. Library beads were washed with 0.01 M PBS buffer pH 7.2 containing 0.05% Tween 20 (PBST) (3 × 5 min), and then the resin was blocked with 1% BSA in PBS buffer solution (600 μL) for 30 min. The beads were then incubated with the fluorescent-labeled *L. odoratus* lectin (0.2 mg/mL) in PBS buffer containing 1% BSA (600 μL) and then incubated for 1.5 h. The lectin solution was sucked away, and the resin was washed with PBST buffer (3 × 5 min), water (3 × 2 min), transferred to a glass plate in small portions (approximately 1000 beads at a time), swollen in water, and visualized using fluorescence microscopy. Bright and medium bright beads were isolated with closed glass capillaries and transferred directly onto the MALDI stainless steel target for ladder analysis. The assay was performed 5 times using different portions of the library.

Lead Glycopeptides. Resin-bound lead peptides were tested using a protocol similar to that for screening of the library. The assays were carried out in duplicate in a custom-made 20-well synthesizer. The beads (3 mg) were blocked with 1% BSA in PBS buffer solution (200 μL) for 30 min and then incubated with the fluorescent-labeled *L. odoratus* lectin (0.15 mg/mL) in PBS buffer containing 1% BSA (100 μL) and incubated for 3 h. The lectin solution was sucked away and the resin washed with PBST buffer (2 × 5 min), water (2 × 2 min). After incubation, the beads were carefully transferred to a 96-well transparent immuno plate (NUNC, Denmark) containing 100 μL of water for reading of the fluorescence by the plate reader. Beads were placed in every other well in order to avoid scattering of luminescence from one sample to the other.

Inhibition Assay. Resin-bound compound (3 mg) was transferred to a micro-Eppendorf tube (250 μL) and blocked with 1% BSA in PBS buffer solution (50 μL) for 30 min and then washed 3 times with PBST. (Washing consisted of centrifugation for 5 min, removal of the supernatant, and then agitation with 100 μL of PBTST.) In another Eppendorf tube, the fluorescent-labeled lectin (0.15 mg/mL) was incubated with inhibitor at 4−5 different concentrations for 2 h at room temperature. The lectin inhibitor solution was added to the washed beads, and then the resulting mixture was incubated for 3 h. The beads were then washed as described above, transferred to a 96-well immunoplate, and the fluorescence measured.

Mass Spectrometry. General Methods. MALDI-TOF mass spectra were recorded in the positive ion mode. The samples, on stainless steel targets, were irradiated with a pulsed nitrogen laser emitting at 337 nm until a suitable spectrum was obtained (1−20 laser pulses) at the lowest power required to facilitate desorption and ionization. Ions were accelerated toward the discrete dynode multiplier detector with an acceleration voltage of 20 kV. The matrix α-cyano-4-hydroxycinnamic acid (CHC) was used to analyze both peptides and glycopeptides. 2,5-Dihydroxybenzoic acid (DHB) was also used for the analysis of glycopeptides. Matrixes were prepared by dissolving 10 mg of compound in 30% aqueous acetonitrile. 2,5-Dihydroxyacetophenone (DHAP) matrix was used to analyze OPfp ester acid labels and was dissolved in dry acetonitrile. Bradykinin (1060.2 mu), substance P (1347.4 mu), or mellitin (2846.5 mu) was used as the standards for internal calibration of the mass. Spectra obtained were smoothed depending on the signal-to-noise ratio.

Sample Preparation. Soluble Peptides and Glycopeptides. One microliter of the analyte was mixed with an equal volume of matrix and 10−25 pmol of the reference peptide on the target and dried at 40 °C.

Carboxylic Acid Labels. Two microliters of the analyte was added to 0.6 μL of matrix on the target and dried at 40 °C. The high analyte-to-matrix ratio is necessary to cause suppression of the matrix peaks and result in analysis of low molecular weight.[43]

Library Components. Fluorescent beads containing active peptides that were isolated from the library and transferred directly to the sample target were first swelled in 1 μL of water and squashed with a thin metal spatula. Matrix was added, the sample dried at 40 °C, and the spectrum recorded. The mass difference between adjacent peptide

(43) St. Hilaire, P. M.; Cipolla, L.; Tedebark, U.; Meldal, M. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 1475−1484.

fragment peaks of the ladder was calculated manually or by using the Lasermat 2000 software version 1.1 mass match option.

Supporting Information Available: Synthesis and characterization of carboxylic acid labels **8−12**, synthesis and characterization of IMP spacers to PEGA and PEG sarcosine resin. Experimental details for peptide and glycopeptide synthesis, acid label reactivity tests, and Figure 9 showing laddered sequence of nonglycosylated peptide of known sequence (7 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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