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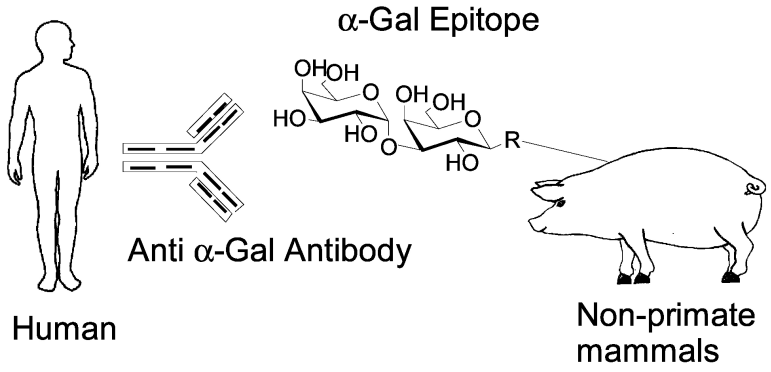

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Identification of α-Galactosyl Epitope Mimetics through Rapid Generation and Screening of C-Linked Glycopeptide Library

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A general methodology has been established for rapid generation and screening of combinatorial glycopeptide library and subsequent mass spectrometric sequencing to identify the mimetics of Galα(1,3)Gal epitopes. Using this approach, several active glycopeptide sequences were recognized and found to inhibit the binding of human natural anti-Gal antibodies with comparable IC_{50}s to synthetic Galα(1,3)Gal oligosaccharides. The most active glycopeptides detected from the library included Gal-Tyr-Trp-Arg-Tyr, Gal-Thr-Trp-Arg-Tyr, and Gal-Arg-Trp-Arg-Tyr. These glycopeptides showed higher affinities to anti-Gal antibodies than known Galα(1,3)Gal peptide mimetics, such as DAHWESWL and SSLRGF. Our results suggest that, by combining a peptide sequence (the "functional" mimic part) with a terminal α-linked galactose moiety (the "structural" mimic part), the resulting glycopeptide could be a very good Galα(1,3)Gal mimetic. Analysis of these active glycopeptides provided first-hand information regarding the binding site of anti-Gal antibodies to facilitate the structurally based design of more potent and stable inhibitors.

Introduction

Carbohydrates are of fundamental importance in a broad range of biological phenomena, including cell communication, recognition, and adhesion.[1,2] Carbohydrate structures also play important roles in the immune response and have been identified as the major antigenic epitopes of many microorganisms and xenoantigens (antigens differing in the various species) and as neoantigens on many human tumors.[3,4] Therefore, carbohydrates are important for many therapeutic approaches. The low natural abundance, however, of many complex carbohydrates dictates that alternative means of supply are usually required to further investigate and exploit their biological activities.[5,6] Chemical synthesis of complex carbohydrates involves many time-consuming and low-yielding steps and is accordingly very expensive. A possible solution to this problem is to mimic the carbohydrate by other compounds that are easier to prepare.

Functional equivalence of chemically dissimilar molecules, such as carbohydrates and proteins sharing common surface topology, has been identified previously as a result of combinatorial technologies as well as naturally occurring phenomena.[5] It has suggested that peptides could be true molecule mimics of carbohydrate determinants. Peptides that mimic carbohydrate structures have significant advantages as vaccines. First, peptide synthesis is more practical than the synthesis of carbohydrates. Second, the immunogenicity of the peptides can be significantly enhanced by polymerization or addition of relatively small carrier molecules that reduce the total amount of antigen required for immunization. Third, peptide mimeotopes can be engineered into DNA plasmids for DNA vaccination to further manipulate both B-cells and T-cells. Until now, many peptide mimics of different carbohydrates have been identified from the screening of phage display peptide libraries.[7−13] The demonstrated ability of a peptide to mimic a carbohydrate determinant indicates that, although mimicry is accomplished using amino acids in place of sugar units, the specificity pattern can be reproduced.

Our group has been working on chemical and enzymatic synthesis of Galα(1,3)Gal epitopes for years. With respect to hyperacute rejection of xenotransplantation, the Galα(1,3)-Gal epitope is the only antigen to which all humans have naturally occurring antibodies, and therefore, it is of importance when animal (such as pig) tissue is transplanted to humans. Studies indicate that the initial step of this rejection is the recognition of human natural anti-Gal antibody to carbohydrate epitopes bearing a Galα1−3Gal*β* terminus (termed α-Gal epitope or α-Gal) on the surface of animal cells.[14−16] Trisaccharides Galα1−3Gal*β*1−4Glc*β*-R (**1**) and Galα1−3Gal*β*1−4GlcNAc*β*-R′ (**2**) and pentasaccharide Galα1−3Gal*β*1−4GlcNAc*β*1−3Gal*β*1−4Glc*β*-R″ (**3**) have been identified as the major α-Gal epitopes, which are abundantly expressed on the cells of most mammals, with the exception of humans, apes, and other Old World primates (Figure 1).[17−23] Conversely, anti-Gal antibodies (known as anti-Gal) are the most abundant human natural antibodies in blood serum, constituting 1−2% of total serum IgG and 3−8% of total serum IgM.[24,25] In attempts to prevent rejection using a neutralization approach, a number of α-Gal oligosaccharides have been tested for the inhibition of anti-Gal binding to the porcine endothelial cells. Results indicate[26]

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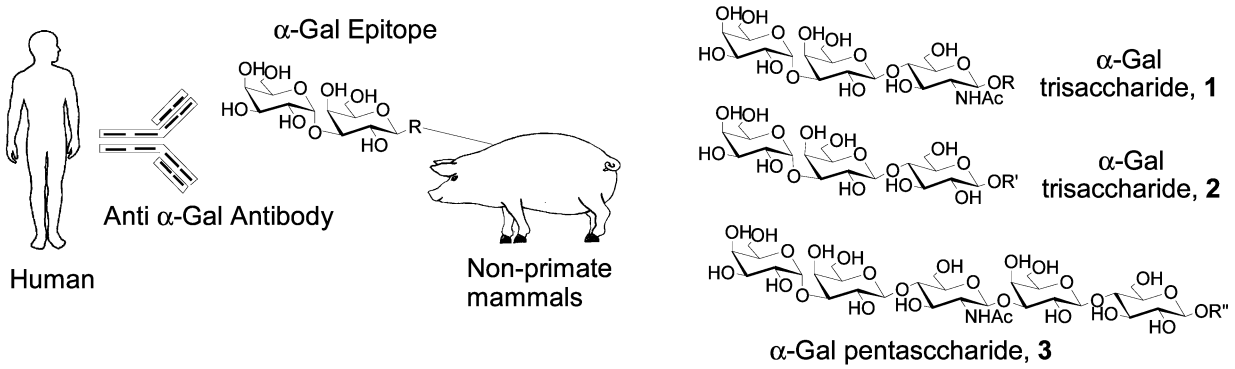


Figure 1. The structures of major α-galactosyl epitopes.

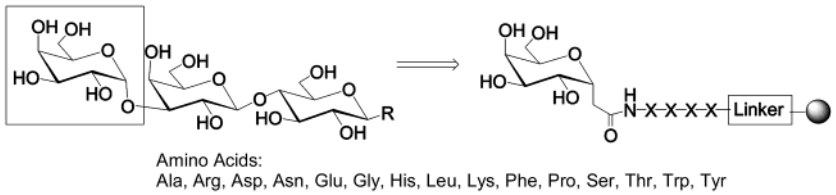


Figure 2. α-Gal glycopeptide mimetic library design.

that it would require a millimolar level of α-Gal trisaccharide Galα1−3Galβ1−4GlcNAc to achieve 90% inhibition of anti-Gal binding to the porcine endothelial cells, which is not sufficient for practical application. Since α-Gal oligosaccharides are difficult to synthesize, there exists a need for compounds capable of mimicking α-Gal eptiopes.

In 1996, McKenzie et al. demonstrated that a synthetic octapeptide, DAHWESWL, isolated from a peptide library using α-galactosyl-specific lectin IB4, mimics the carbohydrate epitope α-Gal.[27−29] In another study, Kooyman et al. reported the identification of a hexapeptide (SSLRGF) that binds to lectin GS-1-B4 (which binds α-Gal epitopes) and blocks the binding of GS-1-B4 to pig endothelial cells.[30] However, the affinities of these peptide mimics for lectin and anti-gal antibodies are much weaker than α-Gal sugars. For example, the affinity of DAHWESWL to lectin IB4 is ∼10-fold weaker than that of Galα(1,3)Gal. The affinity of DAHWESWL to anti-Gal antibodies is 300-fold lower than that of Galα(1,3)Gal.

In an effort to find better mimics of α-Gal epitopes, we designed a glycopeptide library, which combined a "structure-mimic" (i.e., a molecule that is structurally similar to α-Gal epitopes. In this study, it refers to an α-linked Gal moiety) and a functional-mimic (i.e., a molecule which is structurally dissimilar but is functionally similar to α-Gal epitopes; in this study, it refers to a peptide sequence). Previously, glycopeptides that mimic complex carbohydrates have been identified by rational design or from the screening of glycopeptide libraries.[31−35] Some mimics were shown to have increased binding activities compared to original ligands, probably due to the favorable interaction of peptide scaffold with the receptor.

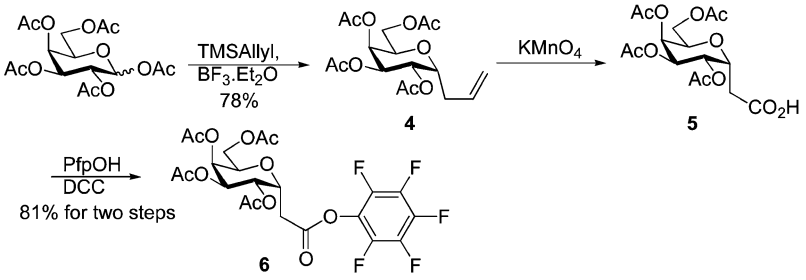
In this report, a general approach for rapid generation and screening of a combinatorial glycopeptide library followed by mass spectrometric sequencing has been established to identify the mimetics of α-Gal epitopes. Using this approach, several active glycopeptide sequences were recognized. Some of them showed improved activities over known peptide mimics and were found to inhibit the binding of human natural anti-Gal antibodies with comparable IC_{50}s to synthetic α-Gal oligosaccharides.

Results and Discussion

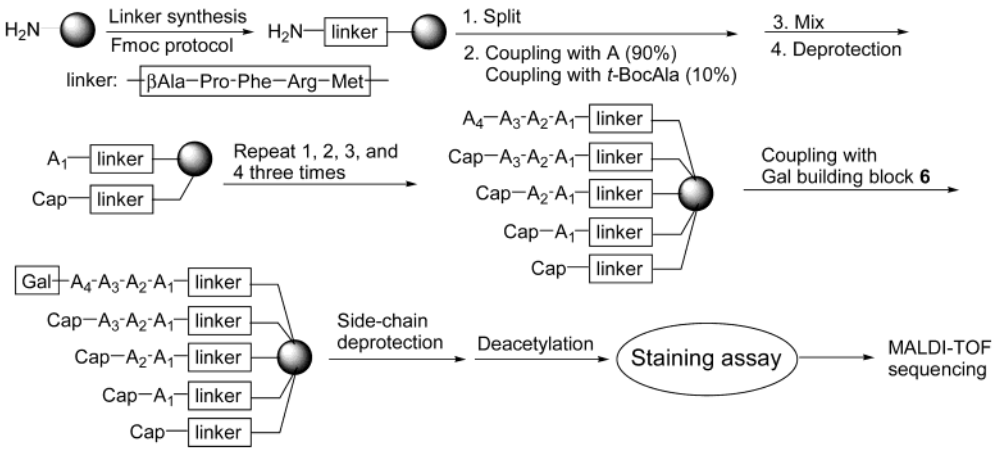
Library Design. One of the most important features of carbohydrate-protein binding is that the recognition process is generally initiated from the nonreducing end of oligosaccharides, and only part of the structure, usually one terminal sugar unit, is deep in the binding pocket in intimate contact with the protein. This specific sugar unit is a so-called "immunodominant sugar" with regard to antibody binding. For α-Gal epitopes, the terminal Galα(1−3) is the key sugar unit that is recognized by anti-Gal antibodies and plays a crucial role in α-Gal/anti-Gal binding. Therefore, our design of a α-Gal mimetic glycopeptide library retained the terminal α-Gal to maintain the binding specificity and substituted the rest of the structure with peptide sequences containing protein binding functionalities to increase the binding affinity (Figure 2). The galactose residue was linked to the peptide through an amide bond in an α configuration. The library has a randomized region of 4 amino acids chosen from 15 amino acids. Theoretically, this library has $15 \times 15 \times 15 \times 15 = 50\,625$ members.

Synthesis of Activated *C*-Galactosyl Building Block. Since there is no general glycosylation method to directly attach a sugar unit to a peptide in solid phase, a sugar building block is needed which could be coupled to a peptide under standard solid-phase conditions. *O*-Glycoside is labile to hydrolysis catalyzed by glycosidase in vivo, whereas *C*-glycoside is much more stable under such conditions. Thus, a *C*-glycoside galactose building block (**6**) was prepared as shown in Scheme 1. 3-(Tetra-*O*-acetyl-α-D-galactopyranosyl)-1-propene (**4**) was obtained from the treatment of galactose peracetate with allyltrimethylsilane.[36] After one-step oxidation by $KMnO_4$, carboxylic acid **5** was then protected with a pentafluorophenyl group, which served as the activating group for solid-phase coupling. The α

Scheme 1



Scheme 2



linkage of compound **6** was confirmed with NMR and X-ray crystal structure analysis.

Construction of C-Linked Glycopeptide Library. The portion-mixing method has been implemented most, giving rise to the "one-bead-one-compound" library.[35,37] In this study, the library was generated using a portion-mixing synthetic approach and chemically encoded using a partial termination (ladder synthesis) method.[35,38] This strategy encodes the peptide on each bead by generating a series of sequence-specific, partially terminated products during the synthesis of the library. The sequence of the full-length peptide is determined by analyzing the peptide ladder formed by these termination products in a mass spectrum. This method has been successfully applied to both peptide and peptidomimetic libraries.[38−41] In our study, during coupling of each position in the randomized region, a small proportion (10%) of capping agent, *N*-*t*-Boc alanine was added to the reaction mixture. Thus, at each step, a small percentage of peptide chains is terminated. At the end of the synthesis, each resin bead contained a full-length peptide as the major product, as well as a series of corresponding terminated products reflecting the peptide sequence. The construction of this library is described in Scheme 2.

TentaGel S NH_2 was used as a solid support for the library synthesis. The nonglycosylated amino acids were incorporated using standard Fmoc chemistry, and the glycosylated building block was coupled using the pentafluorophenyl ester strategy. A pentapeptide linker, β-Ala-Pro-Phe-Arg-Met was first coupled onto the resin to enable efficient release of the peptide and improve the quality of MALDI mass analysis. The methionine at the C terminus ensured the peptide to be cleaved from the solid support by cyanogen digestion,[42,43] whereas β-Ala provided a flexible connection between peptide and linker unit. This peptide linker unit also effectively increased the molecular mass of peptides in the libraries beyond 500 Da, which is an area with heavy chemical noise produced by desorption of UV-absorbing matrix.

Library Screening and Lead Glycopeptides Identification. After completing the full-length glycopeptide synthesis, the side chains of resin-bound glycopeptides were deprotected first with a TFA cocktail containing EDT 2.5%, thioanisole 5%, and water 5%, and then with MeONa to remove the acetyl protecting groups on the sugar residue. After washing and drying, the beads were blocked by BSA (3%) and then incubated with human anti-Gal antibodies (IgG, IgM, and IgA), followed by adding anti-human IgG (or IgM, IgA) alkaline phosphatase conjugate. The staining was effected using a standard mixture of 5-bromo-4-chloro-3′-indolyl phosphate *p*-toluidine salt (BCIP) and nitro-blue tetrazolium chloride (NBT) in Tris−HCl buffer at pH = 9. The color developing was monitored under a microscope.

The most intensely colored beads were selected under a low-magnification microscope and treated with BrCN in dilute HCl to cleave the peptide from the resin at the methionine residue and generate a homoserine lactone at the C terminus.[42,43] The cleaved peptides were then dried and mixed with MS matrix, α-cyano-4-hydroxycinnamic acid, and a small aliquot of the mixture was analyzed by MALDI-TOF spectroscopy. Since each bead carried a unique full-length glycopeptide and four truncation peptide products, MALDI analysis of the cleavage mixture generated a peptide ladder containing five individual peaks. A typical MALDI-TOF spectrum of an active bead is shown in Figure 3. The

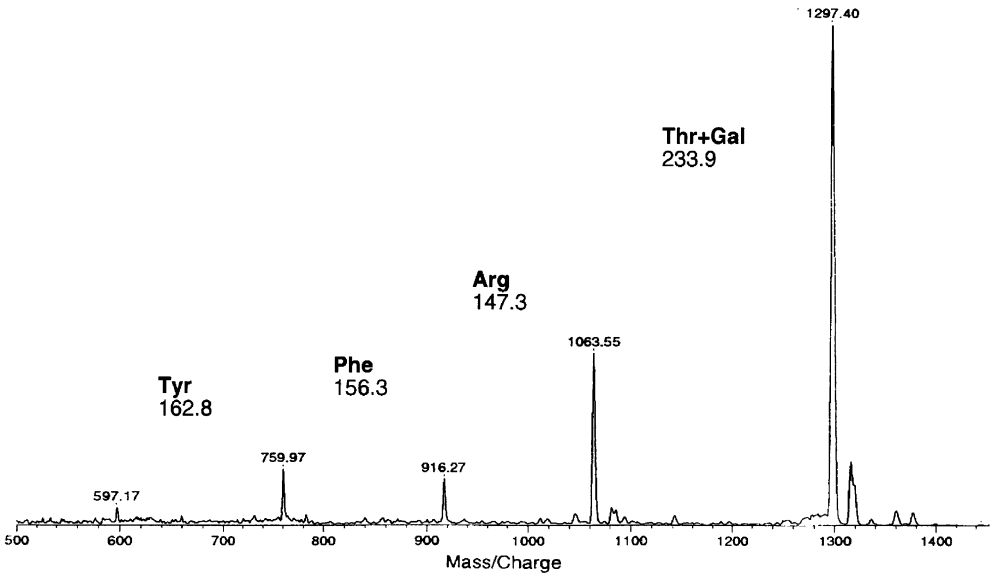


Figure 3. MALDI-TOF spectrum of an active glycopeptide.

full-length glycopeptide gave a peak at *m*/*z* 1297.40. The truncation products produced four peaks at *m*/*z* 1063.55, 916.27, 759.97, and 597.17. The mass difference between the full-length glycopeptide and truncation product with *m*/*z* 1063.55 was 233.9, indicating that the N-terminal residue of the glycopeptide was Thr. Likewise, the rest of the residues were dertermined to be Phe, Arg, and Tyr, respectively, on the basis of the mass differences between adjacent peaks in the ladder. Thus, this analysis yielded the sequence of the active glycopeptide as Gal-Thr-Phe-Arg-Tyr.

A total of 130 beads were analyzed by this method, and 117 (90%) produced high-quality spectra, which allowed unambiguous sequence assignment at all randomized positions. The active glycopeptides' sequences are summarized in Table 1. Taking the results of IgG for example, the possibility of each amino acid appearing in a different position is shown in Scheme 3. For the first position next to terminal Gal, Arg appeared most frequently, 32% of the time. Tyr was the second most frequently appearing residue, with a 13% possibility. In the second position, Trp and Phe were shown to be the most likely amino acid residues. Interestingly, Arg was found in the third position in 46% of the glycopeptide sequences. Tyr and Trp were the most frequently appearing residues in the fourth position with 26 and 22% possibilities, respectively. Overall, the consensus for α-Gal epitope mimics (for IgG) was determined to be Gal-Arg/Tyr-Trp/Phe-Arg-Tyr/Trp.

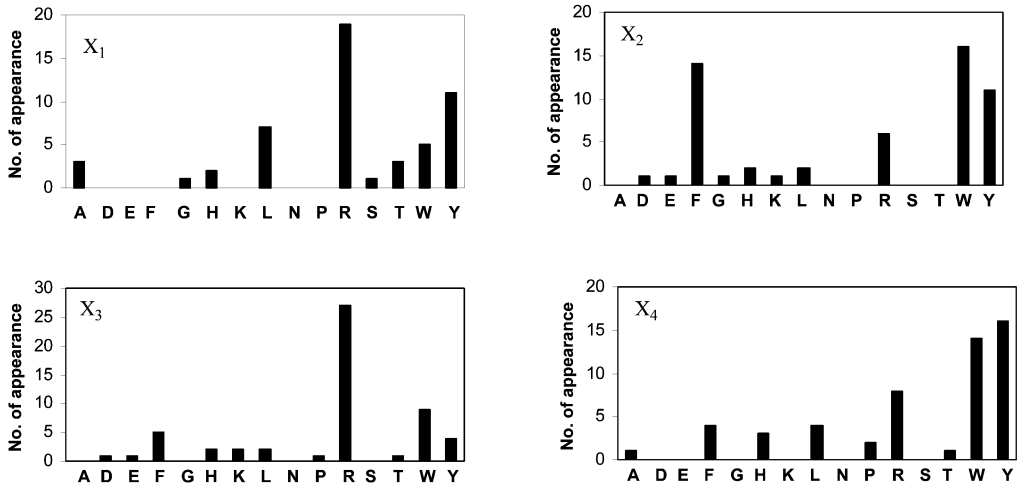
Similar analysis for IgM and IgA indicated that Gal-His-Tyr/Lys/Leu/Arg-Arg-Arg was the active sequence for IgM, and Gal-His/Arg-Arg-Arg/His-Arg/His was the active sequence for IgA. The frequent appearance of an Arg residue in the active sequences strongly suggested the existence of negative charge(s), either from Asp or from Glu, in the binding site of anti-Gal. It is also conceivable that aromatic-containing residues are present in the active site, since Tyr, Trp, and Phe are also heavily populated in the active glycopeptides.

It is interesting to compare the sequence selected here to other peptide sequences known to interact with carbohydrate binding sites.[27] Although there is no apparent motif conserved in the mimicking peptides, there appears to be an abundance of the amino acids tryptophan, tyrosine, and phenylalanine or charged residues, such as arginine. The preference for aromatic groups is not unreasonable, since they resemble sugar moieties in their size, and cyclic shape, particularly in the case of tyrosine. In most cases, the sequence of the binding site of the carbohydrate binding proteins is unknown. Thus, the molecular nature of the mimic interaction is still unclear. The exact nature of these interactions must await the solution of the three-dimensional structure of carbohy-

Table 1. Glycopeptide Sequences Identified by Screening the Library with Anti-Gal IgG, IgM, and IgA

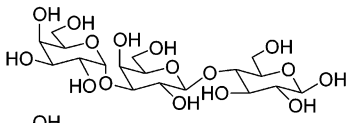
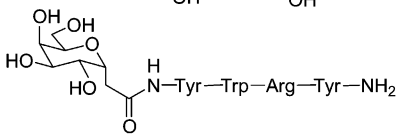
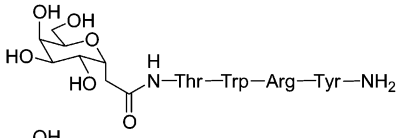
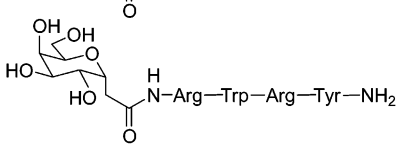
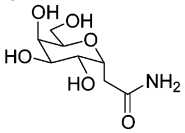
Gal-$X_1X_2X_3X_4$-				
IgG				
YWRY	YRFY	YRWY	SRFY	RKWF
LWRY	TRYR	RGRW	RFRY	RWWH
AWRY	WYRA	SYWR	WDHR	TFRW
TFRY	RFYL	LWWR	RWYF	RLWW
YLRY	RHPF	TWRW	LFRE	YHRL
LFRY	HFRY	RLRY	YFKW	YFRL
YERW	LWRW	RRWY	RWFT	LFRS
AWRW	AYRW	RYPW	YFEW	LFRH
RWYW	RFFP	RYWR	LFRK	YWRP
GRWW	RYRL	RWTY	WYDR	RFLW
HYRH	LWHR	WYLR	WFYY	RWFY
YFKF				
IgM				
HFRR	HLRR	NHYR	HYRW	RRYT
FKLR	EYRR	HLRF	RLRF	HKHR
PYHR	YKLR	HYYR	FWRW	RWKY
TKWK	KRRH	AYHR	RRFW	AERR
LFKR	LGRR	NYYR	HFRW	HPRR
WRHH	WYRY	KWRW	WRRW	FRDR
HKWF	HARY	TKRR	YWRP	FRRN
NLRF	AYHR			
IgA				
LRYR	NWRY	YHRW	RFHR	HNRF
AYRY	RRHK	RHWA	TRRH	HRFW
HYGR	WPRH	TRYH	GKHR	SPHR
YRRH	TRHH	HKHR	GRWF	RGRK
NRRN	YWTR	RRNF	RWYW	

Scheme 3. Substrate Specificity of IgG[a]



[a] Displayed are the amino acids identified at each of the four N-terminal positions (X_1 is the N-terminal residue). Number of appearance on the *y* axis represents the number of selected sequences that contain a particular amino acid at a certain position.

Table 2. Inhibitory Effects of α-Gal and Its Mimetics[a]

Compound	IC_{50} (mM)		
	IgG	IgM	IgA
	0.082	0.44	0.09
	0.94	0.18	1.03
	2.98	0.16	0.98
	0.50	0.23	0.85
	>10	>10	>10
Ac—Tyr—Trp—Arg—Tyr—NH_2	>10	>10	>10
Ac—Arg—Trp—Arg—Tyr—NH_2	>10	>10	>10
Asp-Ala-His-Trp-Glu-Ser-Trp-Leu	5.2	7.4	3.3
Ser-Ser-Leu-Arg-Gly-Phe	2.1	0.4	0.6
Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro	1.3	1.2	0.5

[a] See references 44-47 for the synthesis of α-Gal trisaccharide derivatives. See the Experimental Section for the details of the synthesis of 2-(D-galactosyranosyl)acetamide and peptides.

drate binding proteins with both the carbohydrate and the peptide mimics.

Evaluation of Active Glycopeptides. To verify the results from library screening, a series of representative active glycopeptides were synthesized for iteration, along with the truncated peptides representing the partially terminated glycopeptide sequences in solid-phase library synthesis. A standard ELISA inhibition assay was carried out to study the binding affinity of these glycopeptides or peptides toward anti-Gal IgG, IgA, and IgM. Three known peptide

mimics of α-Gal (i.e. DAHWESWL, SSLRGF, and SAPDTRPAP)[28−30] were also tested in order to compare their activities with glycopeptides. Although several active glycopeptide sequences (obtained from the library screening) did not show significant inhibition even at high concentration (>10 mM) (data not shown), some glycopeptides did exhibit good activities toward anti-Gal. The results obtained from the most active glycopeptides are listed in Table 2. As can be seen, in terms of IgM's binding affinity, glycopeptides Gal-Tyr-Trp-Arg-Tyr, Gal-Thr-Trp-Arg-Tyr, and Gal-Arg-Trp-Arg-Tyr had IC_{50}s's comparable (0.18, 0.16, and 0.23 mM, respectively) to α-Gal trisaccharide (0.44 mM), whereas their IgG and IgA binding affinities were lower than the trisaccharide. It is also important to note that truncated peptides, such as Ac-Tyr-Trp-Arg-Tyr and Ac−Arg-Trp-Arg-Tyr, and terminal Gal-$CONH_2$ did not show any significant binding (IC_{50} > 10 mM) toward all three anti-Gal isotypes. In addition, the mixture of a terminal sugar moiety (i.e., Gal-$CONH_2$) with truncated peptides (such as Ac-Tyr-Trp-Arg-Tyr and Ac−Arg-Trp-Arg-Tyr) did not show any binding activity (IC_{50} > 50 mM). These results suggest that the covalent attachment of Galα sugar moiety and the peptide sequence is necessary for a glycopeptide to be a good mimic of α-Gal. Our results also showed that the activities of some glycopeptides were better than that of the known peptide mimics. For example, the binding affinities to IgG of Galα-Tyr-Trp-Arg-Tyr and Galα-Arg-Trp-Arg-Tyr were about one magnitude higher than that of DAHWESWL.

Conclusion

A general approach for rapid generation and screening of a combinatorial glycopeptide library followed by mass spectrometric sequencing has been established to identify the mimetics of α-Gal epitopes. First-hand information regarding the binding site of anti-Gal was obtained on the basis of the analysis of active glycopeptides. The active sequences of glycopeptide include Galα-Arg/Tyr-Trp/Phe-Arg-Tyr/Trp (for IgG), Galα-His-Tyr/Lys/Leu/Arg-Arg-Arg (for IgM), and Galα-His/Arg-Arg-Arg/His-Arg/His (for IgA). Using this approach, active glycopeptide sequences were recognized and synthesized. Some of them were found to inhibit the binding of human natural anti-Gal antibodies with comparable IC_{50}s's (IgM) to synthetic α-Gal oligosaccharides. Compared with the inhibition activities of known Galα(1,3)Gal peptide mimics (DAHWESWL, SSLRGF, and SAPDTRPAP), these glycopeptides showed better activities. Our results suggest that, by combined a peptide sequence (the "functional" mimic part) with a terminal α-linked galactose moiety (the "structural" mimic part), the resulting glycopeptide could be an efficient Galα(1,3)Gal mimic. Detailed inhibitory studies on these glycopeptides and structure modification are being actively pursued.

Experimental Section

General. ^{1}H and ^{13}C spectra were recorded on 400-MHz Varian VXR400 NMR and 500-MHz Varian Unity spectrometers. Mass spectra were run at the mass spectrometry facility at the University of California, Riverside. Baker silica gel (40-μm) was used for column chromatography, and E. Merck precoated TLC plates for thin-layer chromatography. Size-exclusion chromatography was performed on Biogel P2 resin using distilled water as the eluent. Dialysis was performed using Spectra/Por Molecularporous membrane (16-mm cylinder diameter, molecular weight cutoff 14 kD) against deionized water.

Protected amino acids were purchased from either Advanced ChemTech or NovaBiochem. TentaGel S NH_2 (100−200 mesh, 0.3 mmol/g) resin was purchased from Advanced ChemTech. Rink amide resin (200−400 mesh, 0.48 mmol/g) was purchased from NovaBiochem.

Anti-Gal anti-body was isolated from human sera (Sigma) using an α-Gal affinity column. Anti-human (goat) IgG (Fc specific)-alkaline phosphatase was purchased from Sigma.

Synthesis of *C*-Galactosyl Building Block (6) and 2-(D-Galactosyranosyl)acetamide. 3-(Tetra-*O*-acetyl-α-D-galactopyranosyl)-1-propene (4). Galactose peracetate (3.9 g, 10 mmol) was dissoved in 50 mL of absolute acetonitrile, then allyltrimethylsilane (4.75 mL, 30 mmol) and $BF_3.Et_2O$ (6.4 mL, 50 mmol) were added successively under an atmosphere of argon. After the reaction had been kept at 0 °C for 2 days, the mixture was poured into a saturated solution of sodium bicarbonate. The product **4** was extracted three times with dichloromethane (50 mL each), the organic solution was dried with sodium sulfate, and the solvent was evaporated. Finally, the product **4** (2.9 g, 78%) was isolated by flash chromatography using ethyl acetate/hexane (1:1) for elution. ^{13}C NMR ($CDCl_3$) δ 5.80−5.67 (m, 1H), 5.37 (m, 1H), 5.24−5.15 (m, 2H), 5.11−5.03 (m, 2H), 4.31−4.24 (m, 1H), 4.18−4.10 (m, 1H), 4.09−4.00 (m, 2H), 2.47−2.36 (m, 1H), 2.28−2.20 (m 1H), 2.08 (s, 3H), 2.03 (s, 3H), 2.00 (s, 3H), 1.99 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 170.8, 170.3, 170.2, 170.0, 133.5, 117.9, 71.6, 68.4, 68.1, 67.9, 67.8, 61.7, 31.1, 21.0, 20.9, 20.8. MS (ESI) 395 ($M + Na^+$).

Pentafluorophenyl 2-(2,3,4,5-Tetra-*O*-acetyl-D-galactosyranosyl)acetate (6). A 250-mL round-bottom flask was charged with $KMnO_4$ (1.37 g) and 30 mL of water. The flask was immersed in an ice bath and stirred vigorously. A solution of **4** (1.0 g, 2.7 mmol), 30 mL of toluene, 6 mL of glacial acetic acid, and tetrabutyl hydrogensulfate (0.1 g) was added in one portion. Stirring was continued without any further addition of ice to the bath for ∼4 h. A total of 3.5 g Na_2SO_3 was added to the cooled reaction mixture, followed by the slow addition of a solution of 3.5 mL of concentrated HCl in 3.5 mL of water. Two clear layers resulted. The layers were separated, and the aqueous layer was extracted three times with ethyl acetate. The organic layer was combined and dried over anhydrous sodium sulfate. The drying agent was removed by filtration, and the bulk of the solvent was removed on a rotary evaporator. The residue was further dried under high vacuum and then dissolved in anhydrous ethyl acetate. After the solution was cooled with an ice bath, pentaflurophenol (600 mg, 3.0 mmol) and DCC (1.0 g, 4.9 mmol) were added. After the reaction had been conducted at 0 °C for 5 h, the solid byproduct was removed by filtration. The solution was concentrated. Product **6** (1.2 g, 81% for two steps) was isolated by flash chromatography using ethyl acetate/hexane (1:2) for elution. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.45 (t, J = 6.8 Hz, 1H), 5.38 (dd, J = 4.8, 8.8 Hz, 1H),

5.21 (dd, $J = 3.2$, 9.2 Hz, 1H), 4.82 (dt, $J = 9.2$, 5.2 Hz, 1H), 4.25−4.17 (m, 2H), 4.14 (dd, $J = 4.0$, 9.6 Hz, 1H), 3.07 (dd, $J = 9.2$, 16 Hz, 1H), 2.94 (dd, $J = 4.8$, 15.6 Hz, 1H), 2.13 (s, 3H), 2.09 (s, 3H), 2.05 (s, 3H), 2.01 (s, 3H); ^{13}C (100 MHz; $CDCl_3$) δ 170.6, 170.0, 169.9, 169.6, 166.2, 69.7, 68.8, 67.9, 67.5, 67.0, 61.0, 32.8, 20.7, 20.7, 20.6. MS (ESI) 579 ($M + Na^+$).

2-(2,3,4,5-Tetra-*O*-acetyl-D-galactosyranosyl)acetamide. 2-(2,3,4,5-Tetra-*O*-acetyl-D-galactosyranosyl)acetic acid (0.33 g, 0.85 mmol), which was prepared from the procedure for compound **6**, pyridine (0.067 mL), and Boc_2O (0.26 g, 1.4 equiv) were dissolved in 3 mL of DMF. After adding NH_4HCO_3 (0.087 g, 1.3 equiv), the mixture was stirred at room temperature overnight. Then, 30 mL EtOAc was added into the mixture, and the organic solution was washed using water, 5% H_2SO_4, and brine. The solution was concentrated. Product (0.23 g, 70%) was isolated by flash chromatography. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.20 (br, 1H), 5.65 (br, 1H), 5.42 (t, $J = 3.0$ Hz, 1H), 5.26 (dd, $J = 4.5$, 8.5 Hz, 1H), 5.17 (dd, $J = 3.0$, 8.0 Hz, 1H), 4.68−4.64 (m, 1H), 4.29 (dd, $J = 6.5$, 10.0 Hz, 1H), 4.20−4.15 (m, 2H), 2.62 (dd, $J = 10.0$, 15.5 Hz, 1H), 2.44 (dd, $J = 4.0$, 15.5 Hz, 1H), 2.12 (s, 3H), 2.08 (s, 3H), 2.05 (s, 3H), 2.04, (s, 3H). ^{13}C (125 MHz; $CDCl_3$) δ171.9, 170.5, 170.2, 170.1, 169.8, 69.8, 68.8, 68.2, 67.9, 67.1, 61.4, 34.2, 28.4, 20.95, 20.91, 20.86. MS (ESI) 389.9 ($M + H^+$), 411.9 ($M + Na^+$).

2-(D-Galactosyranosyl)acetamide. To a solution of 2-(2,3,4,5-Tetra-*O*-acetyl-D-galactosyranosyl)acetamide (0.125 g, 0.32 mmol) in anhydrous MeOH (3 mL) was added $NaOCH_3$ portionwise until pH 10. Stirring was continued for 3 h, followed by the addition of Dowex resin (H form) to neutralize the solution. The resin was filtered off and the filtrate was concentrated and dried to afford the desired product as a white solid (95%). ^{1}H NMR (400 MHz, D_2O) δ 4.42 (m, 1H), 3.95 (t, $J = 2.8$ Hz, 1H), 3.92−3.80 (m, 3H), 3.69−3.62 (m, 3H), 2.65 (dd, $J = 10.8$, 15.6 Hz, 1H), 2.50 (dd, $J = 4.4$, 15.6 Hz, 1H). MS (ESI) 244 ($M + Na^+$).

Glycopeptide Library Synthesis. TentaGel S NH_2 (90 μm, 0.3 mmol/g) resin was used as the solid support for constructing the *C*-glycopeptide library, and an Fmoc coupling protocol was applied for the synthesis. The solid-phase reaction was run on a 500-mg (~1.5 million beads) scale. At the beginning of the library synthesis, the five amino acid linker unit, (β-Ala)PPRM, was first attached on the resin. The portioning/mixing procedure was then used to generate the randomized positions in the library. The resin was divided such that there was one reaction vessel for each amino acid to be incorporated at that position in the library. The resin was deprotected with 20% piperidine in DMF for 20 min, followed by washing five times with DMF. Each amino acid was coupled to the resin using a 3-fold excess of the protected amino acid and a coupling reagent combination of TBTU (3 equiv), HOBt (3 equiv), and DIPEA (6 equiv). The reaction was monitored by a Kaiser test. After each coupling, the resin was mixed, deprotected, and redistributed to the reaction vessels. This process was repeated to produce the library. Partial termination was effected by adding 10% *N*-acetyl alanine to the amino acid solutions used for coupling at the randomized positions. Upon completion of the peptide synthesis, the α-Gal moiety was introduced into the sequence by the reaction of glycosylated building block **6** (5 equiv) and HOBt (5 equiv) for 4 h.

Upon the completion of the final coupling reaction, the resin was washed with DMF (3 × 5 mL), CH_2Cl_2 (5 × 5 mL), and methanol (3 × 5 mL) and dried under high vacuum for 10 h. The peptide side-chain deprotection was achieved by the treatment of resin for 3 h using the following mixture (1.5 mL per 100 mg of resin): TFA 90%, thioanisole 5%, EDT 2.5%, and water 2.5%. After side-chain deprotection, the resin was washed with 90% TFA (water 10%, 2 × 1 mL), 10% DIPEA (in DMF, 2 × 3 mL), CH_2Cl_2 (5 × 5 mL), and methanol (3 × 5 mL) and dried under high vacuum for 3 h. Acetyl protecting groups were removed using MeONa treatment (pH adjusted to 10) in methanol for 6 h. Upon the completion of the deprotection, the resin was washed with methanol (3 × 5 mL) and dried by suction.

Library Screening. A 20-mg portion of beads was incubated with 3% BSA in PBS buffer (containing 0.05 mM TWEEN) for 2 h to block nonspecific protein binding sites. The resin was then washed with 1% BSA in PBS buffer and mixed with anti-Gal antibody. After incubating for 1 h, the solution was removed, and the beads were washed with PBS buffer three times. Following the washing, anti-human IgG−alkaline phosphatase was added to the beads, and incubation was allowed to proceed for 1 h. The beads were washed with PBS buffer five times. A standard alkaline phosphatase substrate mixture of 5-bromo-4-chloro-3′-indolyl phosphate *p*-toluidine salt and nitro-blue tetrazolium chloride (1 mg each in 10 mL of PBS buffer) was added to the beads, and the staining reaction was allow to proceed for 30 min and monitored by microscope. The staining was terminated by removing substrate and washing the beads with deionized water. Positive beads were identified by their dark blue color and were removed under a low magnification microscope.

MALDI-TOF Mass Spectrometry. Individual stained beads were placed into 500-μL Eppendorf tubes, and 20 μL of a cyanogen bromide in 0.1 N HCl (20 mg/mL) solution was added. The reaction was allowed to proceed at room temperature in the dark for 15 h. The reaction was then stopped by freezing and lyophilizing to dryness.

Mass spectroscope analysis was carried out at the mass spectrometry facility in Park-Davis Pharmaceutical Research. All MALDI mass spectra were obtained in the positive ion mode on a Vestec VT2000 linear time-of-flight mass spectrometer.

Syntheses of Active Glycopeptides and Other Peptides. Rink amide AM resin was used as the solid support for synthesizing the active glycopeptides. Coupling reactions were run on a 0.1-mmol scale using standard Fmoc protocol and a 3-fold excess of amino acid. Upon completion of the peptide synthesis, the α-Gal moiety was introduced into the sequence by the reaction of glycosylated building block **6** (5 equiv) and HOBt (5 equiv) for 4 h. Then the resin was washed with DMF (3 × 5 mL) and DCM (3 × 5 mL). Acetyl protecting groups on the sugar moiety were removed using MeONa treatment (pH adjusted to 10) in methanol for 6 h. The desired glycopeptide was cleaved from the resin and deprotected using the following mixture in a ratio of 2 mL

per 100 mg of resin: 10 mL of TFA, 0.3 g of phenol, 0.3 mL of TIS, and 0.3 mL of water. The reaction was allowed to proceed for 2 h at room temperature with gentle shaking. The resin was washed with TFA (3 × 2 mL), and the solutions were concentrated. The ether precipitation was applied, and the precipitate was dissolved in water and lyophilized. The glycopeptides were purified by HPLC. Gal-Tyr-Trp-Arg-Tyr-NH_2 ESI-MS [M + H^+] calcd 890.4, found 890.2. Gal-Thr-Trp-Arg-Tyr-NH_2 ESI-MS [M + H^+] calcd 828.4, found 828.2. Gal-Arg-Trp-Arg-Tyr-NH_2 ESI-MS [M + H^+] calcd 883.4, found 883.6.

Truncated peptides and known peptide mimics were prepared by standard Fmoc protocol using Rink amide AM resin (for truncated peptides) or Wang resin (for known peptide mimics).

ELISA Competitive Inhibition Assay. An ELISA was conducted using mouse laminin, a basement membrane glycoprotein containing 50−70 α-Gal epitopes per molecule, as the immobilized antigen. The purified human polyclonal anti-Gal antibodies (32 ng/mL) or human sera (2.5-fold dilute) was first incubated with varying concentrations of α-Gal mimics for 1.5 h at room temperature with gentle shaking. An aliquot (50 μL) of the mixture was then added to each microtiter plate that had been well-precoated with mouse laminin (50 μL/well of 10 μg/mL in 0.1 N Na_2CO_3−$NaHCO_3$ buffer, pH = 9.5). After incubation for 1.5 h at room temperature, unbound antibodies were washed out with PBS-Tween (pH = 7.4, 0.5% Tween, 5 × 200μL). A secondary antibody (1/1000 peroxidase conjugated goat anti-human IgG or IgM or IgA; 50 μL/well) was introduced, and the incubation was allowed to proceed for 1 h at room temperature. After washing with PBS-Tween buffer (5 × 200 μL), standard substrate (3,3′,5,5′-tetramethylbenzidine: H_2O_2, 9:1; 100 μL/well) was added. The enzymatic oxidation reaction produced a blue stain in each well. The staining reaction was stopped by adding 1 N H_2SO_4 (100 μL/well). Readings of optical absorption were taken at 450 nm (BioRad Microplate Reader, model 3550-UV). PBS with the secondary antibody was used as a background control. Purified anti-Gal antibody or human sera with secondary antibody was used as maximum staining (0% inhibition). The % inhibition was calculated using eq 1.

$$(M - S)/(M - B) = \%\ \text{inhibition} \qquad (1)$$

where S is the OD_{450} reading of the sample with different concentrations of α-Gal inhibitors (mouse laminin + α-Gal mimics + purified anti-Gal Ab or human sera + 2nd Ab), B is the OD_{450} value of the background staining (mouse laminin + 2nd Ab), and M is the OD_{450} value of the maximum staining (mouse laminin + purified anti-Gal Ab or human sera + 2nd Ab). The percent inhibition versus the concentration of inhibitors was plotted for each α-Gal compound. IC_{50} was calculated from the curve obtained.

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