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Authors

Wu, Zhigang
Liu, Yunpeng
Ma, Cheng
et al.

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Identification of the binding roles of terminal and internal glycan epitopes using enzymatically synthesized *N*-glycans containing tandem epitopes†

Zhigang Wu[a], **Yunpeng Liu**[a], **Cheng Ma**[a], **Lei Li**[a], **Jing Bai**[b], **Lauren Byrd-Leotis**[c], **Yi Lasanajak**[d], **Yuxi Guo**[a], **Liuqing Wen**[a], **He Zhu**[a], **Jing Song**[a], **Yanhong Li**[e], **David A. Steinhauer**[c], **David F. Smith**[d], **Baohua Zhao**[b], **Xi Chen**[e,‡], **Wanyi Guan**[a,b], and **Peng George Wang**[a]

[a]Department of Chemistry and Center of Diagnostics & Therapeutics, Georgia State University, Atlanta, GA 30303, USA

[b]College of Life Science, Hebei Normal University, Shijiazhuang, Hebei 050024, China

[c]Departments of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322, USA

[d]Department of Biochemistry and Emory Comprehensive Glycomics Core, Emory University School of Medicine, Atlanta, GA 30322, USA

[e]Department of Chemistry, University of California, Davis, CA 95616, USA

Abstract

Glycans play diverse roles in a wide range of biological processes. Research on glycan-binding events is essential for learning their biological and pathological functions. However, the functions of terminal and internal glycan epitopes exhibited during binding with glycan-binding proteins (GBPs) and/or viruses need to be further identified. Therefore, a focused library of 36 biantennary asparagine (Asn)-linked glycans with some presenting tandem glycan epitopes was synthesized *via* a combined Core Isolation/Enzymatic Extension (CIEE) and one-pot multienzyme (OPME) synthetic strategy. These *N*-glycans include those containing a terminal sialyl *N*-acetyllactosamine (LacNAc), sialyl Lewis x (sLe^x) and Siaα2–8-Siaα2–3/6-R structures with *N*-acetylneuraminic acid (Neu5Ac) or *N*-glycolylneuraminic acid (Neu5Gc) sialic acid form, LacNAc, Lewis x (Le^x), α-Gal, and Galα1–3-Le^x; and tandem epitopes including α-Gal, Le^x, Galα1–3-Le^x, LacNAc, and sialyl LacNAc, presented with an internal sialyl LacNAc or 1–2 repeats of an internal LacNAc or Le^x component. They were synthesized in milligram-scale, purified to over 98% purity, and used to prepare a glycan microarray. Binding studies using selected plant lectins, antibodies, and viruses demonstrated, for the first time, that when interpreting the binding between glycans and GBPs/

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Correspondence to: Xi Chen; Wanyi Guan; Peng George Wang.

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viruses, not only the structure of the terminal glycan epitopes, but also the internal epitopes and/or modifications of terminal epitopes needs to be taken into account.

Introduction

In nature, glycans are displayed universally at the surface of living cells[1] and are the mediators of numerous biological events, including, but not limited to protein folding and trafficking, cell signaling and intercellular interactions, and cell–pathogen interactions.[2–4] Aberrant processing of glycans may lead to malfunctions, such as the development of neurological diseases and cancer.[5–7] Thus, interpretation of the structures and functions of glycans will be crucial to better understand carbohydrate-associated biological and pathological processes, and to develop diagnostics and therapeutics. The diverse functions of glycans are directly related to their structures. Most studies, including glycan microarray studies, however, have been focused on the terminal glycan epitopes whose important functions are now well appreciated.[8] For example, the outermost sialyl-Lewis x (sLe^x) sequences [Neu5Acα2–3Galβ1–4(Fucα1–3)GlcNAc-R] on human zona pellucida have been found to mediate human sperm-egg binding and form a zygote.[9,10] The α-Gal (Galα1–3Galβ1–4GlcNAc-R) epitope on the cells of non-primate mammals including pigs binds to anti-Gal antibodies naturally existed in humans, posing barriers of pig-human xenotransplantation.[11,12] The presence of α-Gal as a terminal epitope on *N*-glycan of glycoprotein pharmaceuticals produced in Chinese hamster ovary (CHO) cells has also been shown to induce anaphylaxis reactions.[13] In *N*-glycans isolated from membrane glycoproteins of miniature pig kidneys, Galα1–3Gal and Fucα1–3GlcNAc have been found to be presented in the same LacNAc core, generating Galα1–3-Le^x [Galα1–3Galβ1–4(Fucα1–3)GlcNAc-R] epitope.[14] In many cases, nature presents glycans containing tandem epitopes as exemplified by poly-*N*-acetyllactosamine [poly-LacNAc, $(–3Galβ1–4GlcNAcβ1–)_n$] structures in glycoproteins and glycolipids in mammals[15–19] as well as capsular polysaccharides and lipopolysaccharides in bacteria.[20–22] Poly-LacNAc, composing of LacNAc repeats, exhibits critical roles in cell adhesion, immune response, and carcinomatosis through binding with galectins.[23,24] Poly-LacNAc can also be modified by α2–3-sialylation at the terminal galactose (Gal) residue or α2–6-sialylation at the terminal and internal Gal residue in LacNAc repeats.[25–27] It can also be fucosylated to generate polymeric Lewis x (poly-Le^x) structures.[17,28] Studies on the recognition between sialylated poly-LacNAc and glycan-binding proteins (GBPs) indicate that, the degree of polymerization (DP) of poly-LacNAc with only terminal sialylation modification affects the recognition of glycans by influenza A virus hemagglutinins.[25] When modified by both terminal sialic acid and internal sialic acids (could be viewed as tandem sialylated LacNAc), more divergent binding profiles to different GBPs were reported.[26] Another example is polysialic acid (polySia), which is a crucial glycan epitope with tandem sugar residues. Its DP ranges from 2 to over 8, and polySia with different DPs binds differentially to different GBPs, and mainly plays an irreplaceable role in nervous system.[29,30] In another study, a number of *N*-glycans with biantennary structure clusters tandemly linked together were chemoenzymatically synthesized, and the generated unnatural *N*-glycans showed some unexpected strong binding affinity to some lectins, broadening our knowledge about the binding profile of lectins.[31]

Several recent studies have been focusing on preparation of *N*-glycans with various structures. For instance, Boons[32] and one of our groups[33,34] used chemoenzymatic strategy to synthesize asymmetric *N*-glycans, most of which have only a single glycan epitope in each branch. Using bacterial sialyl-transferases, Paulson synthesized *N*-glycans containing poly-LacNAc structures with only terminal sialylation[25] or with both terminal and internal sialylation.[26] Inspired by the naturally existing tandem glycan epitopes, the unnatural tandem *N*-glycan clusters, and their different binding profiles to GBPs, we describe herein the synthesis of a library of biantennary *N*-glycans with some containing tandem glycan epitopes, including those with a terminal sialyl LacNAc, sLe^x and Siaα2–8Siaα2–3/6-R and with *N*-acetylneuraminic acid (Neu5Ac) or *N*-glycolylneuraminic acid (Neu5Gc) sialic acid form, LacNAc, Le^x, α-Gal, and Galα1–3-Le^x; as well as those containing α-Gal, Le^x, Galα1–3-Le^x, LacNAc, and sialyl LacNAc, which is linked to an internal sialyl LacNAc or 1–2 repeats of an internal LacNAc or Le^x as an internal glycan component.

Since chemical synthesis of target complex glycans from scratch is usually tedious, we have developed a Core Synthesis/Enzymatic Extension (CSEE) strategy, in which the relatively simple core glycans were chemically synthesized and then the outer parts of glycans were extended enzymatically, to prepare a series of complex glycans.[33,35] For some of the core glycans which are readily available from an abundant natural source, purification followed by additional processing such as trimming using exoglycosidases or acid hydrolysis[36–38] is a good alternative. We name the strategy Core Isolation/Enzymatic Extension (CIEE) and use it for the desired biantennary *N*-glycans. To do this, sialylated biantennary *N*-glycan with the attached asparagine (Asn) was obtained from sialylglycopeptide (SGP)[39] by pronase E treatment. Enzymatic desialylation then led to the formation of the core glycan which was enzymatically extended with a diverse array of glycosyltransferases to obtain the target *N*-glycans (Fig. 1). One-pot multi-enzyme (OPME)[40] synthetic strategies were further adopted to make the synthesis more efficient and with lower cost. Each target glycan was synthesized in a milligram-scale and was purified to over 98% purity using a hydrophilic interaction liquid chromatography (HILIC) column on a high-performance liquid chromatography (HPLC) system monitored by UV absorption. The roles of the internal glycan structure in affecting the binding of the glycans to GBPs and viruses were investigated using a glycan microarray format. The internal glycan epitopes such as LacNAc, Le^x, and sialyl LacNAc, as well as sialylation and fucosylation of terminal glycans hindered or enhanced the binding of GBPs or viruses to the *N*-glycans.

Results and discussion

Preparation of core glycan

Sialylglycopeptide (SGP) was obtained from egg yolks as previously described.[41] In order to assist immobilization of glycans for glycan microarray studies, the Asn residue to which the *N*-glycan was attached was retained by treating SGP with pronase E to provide Asn-linked biantennary complex-type glycan terminated with α2–6Neu5Ac (**BA-01**, Scheme 1A). The terminal Neu5Ac was removed by a sialidase BiNanH2 from *Bifidobacterium longum* subsp. infantis ATCC15697[42] to expose the penultimate Gal residue in **BA-02** (Scheme 1A) that can be further extended enzymatically.

Enzymatic extension of *N*-glycans

To decrease the cost and make the enzymatic glycosylation reactions more efficiently, one-pot multienzyme (OPME) strategy was adopted in the *N*-glycan synthesis whenever possible. For example, in all sialic acid transfer reactions, sialic acid, CTP, and *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS),[43] the enzyme responsible for the synthesis of the activated sugar nucleotide form of sialic acid (CMP-sialic acid), were added together with a suitable sialyltransferase, allowing *in situ* generation and consumption of CMP-sialic acid. Also, in sequential extensions with several monosaccharides other than sialic acid (*e.g.* the synthesis of **BA-08** in Scheme 1B), multiple glycosyltransferases were added successively once the previous reaction reached completion, and only the final products were purified. For elongation with sialic acids, glycan products were purified right after each OPME sialylation reaction. *Pasteurella multocida* α2–3-sialyltransferase double mutant E271F/R313Y (PmST1 E271F/R313Y)[44] was used to catalyze the addition of α2–3-sialic acid. Once monosialylation products were observed, adding more enzymes and donors into the same reaction mixtures did not lead to additional sialylation. Disialylation could be achieved, however, by purifying the monosialylated products followed by another round of sialylation reaction which led to sialylation on both branches of the *N*-glycans.

In addition to α2–6Neu5Ac-terminated **BA-01** which was readily obtained by purification of SGP followed by pronase E digestion (Scheme 1A), 3 sialylated *N*-glycans were prepared from **BA-02** with a single sialic acid extension at each branch including **BA-03** with terminal α2–6Neu5Gc, **BA-04** with terminal α2–3Neu5Ac and **BA-05** with terminal α2–3Neu5Gc (Scheme 1B). Le^x (**BA-06**), α-Gal (**BA-07**) and Galα1–3-Le^x (**BA-08**) epitopes were produced by reactions catalyzed by *Helicobacter pylori* α1–3-fucosyltransferase (Hpα1,3FT),[45,46] bovine α1–3-galactosyltransferase (Bα1,3GalT),[47] and Bα1,3GalT followed by Hpα1,3FT, respectively (Scheme 1B).

For sialyl Le^x (sLe^x; **BA-17** and **BA-18**), α1–3-fucosylation was performed after sialylation (Scheme 1B).[33,48]

Enzymatic extension of *N*-glycans with tandem glycan epitopes

To synthesize *N*-glycans with tandem epitopes, glycosyltransferases were added sequentially according to the monosaccharide sequence in the desired glycans. For example, alternating utilization of *Neisseria meningitidis* β1–3-*N*-acetylglucosaminyl-transferase (NmLgtA)[15] and *Neisseria meningitidis* β1–4-galacto-syltransferase (NmLgtB)[49] allowed the formation of glycans with tandem LacNAc sequence (–3Galβ1–4GlcNAcβ1–) (Scheme 2, **BA-24** as di-LacNAc and **BA-25** as tri-LacNAc). These tandem LacNAc glycans were further β1–3-galactosylated at the outermost Gal, forming an α-Gal epitope (**BA-26** and **BA-27**). The LacNAc could also be fucosylated at GlcNAc with α1–3-linkage, generating Le^x epitope. It is worth noting that, Hpα1,3FT[45,46] could only attach α1–3Fuc to GlcNAc residue in the existing LacNAc unit. For instance, in **BA-30**, fucosylation occurred on both LacNAc because Hpα1,3FT was added after both LacNAc were produced, leading to tandem Le^x epitope, while **BA-28** was only internally fucosylated since the non-reducing terminal GlcNAc in GlcNAc–LacNAc was not an acceptable receptor for this fucosylation, resulting in LacNAc-Le^x (LacNAc at the non-reducing end) tandem epitopes. We also observed that

NmLgtA could not extend the Gal residue in Le^x epitope, therefore, during synthesis, the terminal LacNAc needed to be extended by GlcNAc before fucosylated into Le^x. Based on this principle, we have synthesized glycans with di-LacNAc (**BA-24**), tri-LacNAc (**BA-25**), LacNAc-Le^x (VIM-2, CD65, **BA-28**),[50] LacNAc-Le^x-Le^x (**BA-33**), di-Le^x (**BA-30**), tri-Le^x (**BA-34**), α-Gal-LacNAc (**BA-26**), α-Gal-LacNAc-LacNAc (**BA-27**), α-Gal-Le^x (**BA-31**), α-Gal-Le^x-Le^x (**BA-35**), Galα1–3-Le^x-Le^x (**BA-32**) and Galα1–3-Le^x-Le^x-Le^x (**BA-36**). In addition, the LacNAc-Le^x in **BA-28** was further sialylated with α2–3Neu5Ac, generating a sialylated VIM-2 structure (sLacNAc-Le^x, CD65s, **BA-29**).[50]

Another group of glycans were terminally extended with sialic acids, leading to disialic acid (diSia) determinant. The diSia determinant was found abundantly existing in human brain (mostly in gangliosides),[30] but evidence also showed its presence in protein *N*-glycosylation,[51] although the function was unknown. Therefore, we employed *Campylobacter jejuni* 2–3/8-sialyltransferase (CjCstII)[52] to accomplish the diSia modification. CjCstII worked efficiently, and added α2–8-linked Neu5Ac or Neu5Gc to the terminal α2–6/α2–3-linked Neu5Ac or Neu5Gc in sialylated LacNAc, generating a series of glycans with tandem sialic acids (Scheme 3A, **BA-09** to **BA-16**, Table S2†). The di-LacNAc was also modified with sialyltransferases (Scheme 3B), and the sialyltransferases used exhibited different glycosylation profile. The α2–3-sialyltransferase (PmST1 E271F/R313Y) could only add terminal sialic acids (**BA-19** and **BA-20**), while *Photobacterium damselae* α2–6-sialyl-transferase (Pd2,6ST) added both terminal and internal sialic acids (**BA-21** and **BA-22**), different from mammalian α2–6-sia-lyltransferase ST6Gal-1 that only sialylated terminal LacNAc.[32] However, to produce the glycan with both terminal α2–3-Sia and internal α2–6-Sia (**BA-23**), the synthetic route was strict since Pd2,6ST was capable of adding α2–6-Sia to the penultimate Gal to which α2–3-Sia was already linked (Scheme S1,† lower route), resulting in a mixture of glycans with 1–3 α2–6-Sia added. To circumvent this limitation, the internal α2–6-Sia was added to the Gal of the inner LacNAc after the GlcNAc of the outer LacNAc was added, and then the outer LacNAc was completed by adding Gal and further modified by α2–3-Sia (Scheme S1,† upper route).

Since PmST1 E271F/R313Y and CjCstII have glycosidase activity although the α2–3-sialidase activity of wild-type PmST1[53] was reduced 6333-fold by E271F/R313Y mutations,[44] the incubation time for reactions was controlled to be less than 45 min. During sialylation, altered activity of PmST1 E271F/R313Y was observed towards glycans with different structures (Table S1†). Single LacNAc was an acceptor (**BA-02**) for α2–3-sialylation, with a moderate percentage conversion (71%) during reaction within 45 min. If the LacNAc was modified with α1–3Fuc to form Le^x, no α2–3-sialylation was detected, consistent with the previous reports.[33,48] When di-LacNAc was in the acceptor (**BA-24**), within 45 min, the percentage conversion of α2–3-sialylation was 96%, much higher compared to single LacNAc in **BA-02**. When the inner LacNAc in di-LacNAc was converted to Le^x, the α2–3-sialylation efficiency of the outer LacNAc was even higher than di-LacNAc acceptor,

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reaching 99%. This indicates that longer glycans are preferred by PmST1 E271F/R313Y than shorter glycans (**BA-24** *vs.* **BA-02**). Also, fucosylation of terminal LacNAc would hinder sialylation of the same LacNAc (**BA-06**), but fucosylation of internal LacNAc would not impede and would even enhance the sialylation of the terminal LacNAc (**BA-28**).

Glycan microarray of *N*-glycans

To explore the roles of internal and terminal epitopes of these synthetic glycans in glycan-binding events, glycan microarrays prepared from the library of *N*-glycans synthesized were screened with three classes of glycan-binding reagents, including plant lectins, antibodies, and viruses.

Plant lectins—Eight subarrays were interrogated with lectins exhibiting defined binding specificities including MAL-I (specific to Siaα2–3Gal-linkage),[58] SNA (specific to Siaα2–6Gal-linkage),[59] ECL (binds terminal LacNAc),[60] GSL-I-B_4 (binds Galα1–3Gal-R),[61] and WGA (binds GlcNAc residue and Neu5Ac residue;[62,63] four subarrays for four concentrations). The lectins were biotinylated and detected with Cy5-labeled-strepta-vidin. The results are shown as histograms in Fig. 2, and the average relative fluorescence units (RFUs) and coefficient of variation (%CV) are provided in Dataset S1.

Glycans with terminal sLacNAc (Siaα2–3Galβ1–4GlcNAc) were bound by MAL-I,[58] which is specific to Siaα2–3Gal-linkage. MAL-I did not distinguish between Neu5Ac and Neu5Gc. Even if the terminal sialic acid in sLacNAc was extended with another sialic acid in the C8 position (**BA-13** to **BA-16**), the glycans were still bound by MAL-I, as reported previously.[54] However, its binding was abolished if the sLacNAc was fucosylated to form sLe^x (**BA-17** and **BA-18**), demonstrating the interruption of binding caused by Fuc. With regard to internal epitope, when terminal sLacNAc was linked with internal LacNAc (**BA-19** and **BA-20**), stronger binding was observed in comparison with glycans in which sLacNAc was directly linked with Man in core pentasaccharide of *N*-glycans (**BA-04** and **BA-05**). Nevertheless, if the internal LacNAc was further modified by α2–6-sialylation (as in **BA-23**) or α1–3-fucosylation (as in **BA-29**), the enhancement in binding for MAL-I was compromised.

Glycans terminating in s6LacNAc (Siaα2–6Galβ1–4GlcNAc) were bound by SNA.[59] The data for binding were consistent with the given structures in the analysis. SNA also did not distinguish between Neu5Ac and Neu5Gc. However, unlike MAL-I, SNA did not bind to the s6LacNAc determinant if α2–8-sialylation occurred to the terminal s6LacNAc, which illustrates the interruption of binding by sialylation of terminal epitope. The internal s6LacNAc does not contribute to binding with SNA, as no detectable binding was observed for **BA-23**. The comparable binding affinity for glycans with single or double s6LacNAc moieties (**BA-01** *vs.* **BA-21**, **BA-03** *vs.* **BA-22**) further indicates the neglectable roles the internal s6LacNAc plays in binding with SNA.

All tested glycans possessing a terminal LacNAc, including glycans **BA-02**, **BA-24**, **BA-25**, **BA-28** and **BA-33**, were bound by ECL.[60] Any modification to the terminal LacNAc epitope (sLacNAc, s6LacNAc, Le^x or α-Gal as illustrated by **BA-04**, **BA-01**, **BA-06** or **BA-07**, respectively) hampered the binding of ECL, demonstrating the strict glycan structural

requirement for ECL binding. With respect to the roles of internal epitopes, internal LacNAc determinant is not attributable to ECL binding (as in **BA-19**, **BA-20**, **BA-26** and **BA-27**). Nor does internal LacNAc or Le^x determinant exhibit distinguishable effect on ECL binding, being exemplified by similar binding between the following groups of glycans, *i.e.* **BA-02** *vs.* **BA-24** *vs.* **BA-25** for internal LacNAc, and **BA-24** *vs.* **BA-28**, and **BA-25** *vs.* **BA-33** for internal Le^x.

Glycans with Galα1–3Gal-R structures were bound by GSL-I-B_4,[61] which is specific to α-Gal determinant. Contrast to the binding profile of MAL-I, fucosylation of the GlcNAc residue in terminal α-Gal epitope had no hindrance for the binding to GSL-I-B_4 (**BA-07** *vs.* **BA-08**), or it could even augment the binding (**BA-32** > **BA-31**, **BA-36** > **BA-35**). Internal LacNAc moiety did not pose apparent influence on the binding (**BA-07** *vs.* **BA-26** *vs.* **BA-27**), whereas internal Le^x epitope generally decreased the binding (**BA-35** < **BA-31** < **BA-07**, **BA-36** < **BA-32**), with the exception that no decrease was detected from **BA-08** to **BA-32**.

Another set of interesting observations is the binding patterns of WGA to these glycans. WGA is known to be specific to GlcNAc residue, including internal GlcNAc, and Neu5Ac residue.[62,63] According to our results, WGA only recognizes α2–3Sia epitope rather than α2–6Sia, and is more sensitive to Neu5Ac compared to Neu5Gc. In addition, as the concentration of WGA increased from 0.5 to 10 μg mL^{-1}, the number of bound glycans increased. At the lowest concentration we tested (0.5 μg mL^{-1}), glycans with terminal sLacNAc (regardless of fucosylation, **BA-4**, **BA-17**, **BA-19**, **BA-23** and **BA-29**), as well as tri-LacNAc (**BA-25**) and α-Gal-LacNAc-LacNAc (containing tri-LacNAc in total, **BA-27**) were bound by WGA. As the concentration of WGA increased to 2 μg mL^{-1}, besides the aforementioned glycans, single/double LacNAc (**BA-02** and **BA-07**) and α-Gal epitope containing single/double LacNAc (**BA-24** and **BA-26**) were also bound by WGA. At the concentration of 5 μg mL^{-1}, WGA exhibited broader binding to glycans. Terminal Neu5Gcα2–3Galβ1–4GlcNAc (**BA-05** and **BA-20**) was bound at this concentration. The Neu5Ac/Gcα2–3Galβ1–4GlcNAc epitopes were still bound by WGA even when they were further modified by α2–8-Neu5Ac (**BA-13** and **BA-15**). LacNAc(-Le^x)-Le^x (**BA-28** and **BA-31**) and α-Gal(-Le^x)-Le^x (**BA-33** and **BA-35**) with non-fucosylated terminal epitopes were bound by WGA, while terminal single Le^x (**BA-06**) and Galα1–3-Le^x (**BA-08**) only displayed weak affinity to WGA. When the WGA concentration reached 10 μg mL^{-1}, Le^x–Le^x (**BA-30**) and Galα1–3-Le^x-Le^x (**BA-32**) can be additionally bound. Collectively, fucosylation of terminal sLacNAc, LacNAc or α-Gal epitope reduced to some extent the binding to WGA (**BA-17** < **BA-04** at 0.5 μg mL^{-1}, BA-06 < BA-02, and BA-08 < BA-07 at 5 μg mL^{-1}, BA-30 < BA-28, **BA-32** < **BA-31** at 10 μg mL^{-1}). While internal LacNAc improved the binding for both sialosides and asialosides to WGA (**BA-19** > **BA-04** at 0.5 μg mL^{-1}), an effect that may be caused by WGA's binding with internal GlcNAc,[62] α2–6-sialylation or fucosylation of internal LacNAc decreased the binding (**BA-23** < BA-19 and BA-29 < BA-19 at 0.5 μg mL^{-1}, BA-28 < BA-24, BA-31 < BA-26, BA-33 < BA-25, BA-35 < BA-27 at 5 μg mL^{-1}).

Antibodies—Anti-CD15 and anti-CD15s antibodies were selected to perform the binding study (Fig. 3, Dataset S1).

Anti-CD15 antibody is an antibody that binds to Le^x epitope. In our results, this antibody exhibited strong binding to the glycans containing di-Le^x (**BA-30**) and tri-Le^x (**BA-34**), but failed to bind glycans with only internal Le^x epitopes (**BA-28** and **BA-33**), indicating that only the terminal Le^x determinant participates in the binding. No binding was observed with single terminal Le^x directly connected to the mannose in core pentasaccharide in *N*-glycans (**BA-06**), which is consistent with the glycan binding data published by Consortium for Functional Glycomics (CFG, http://www.functionalglycomics.org), highlighting the negative effect of internal core pentasaccharide on the binding. In addition, the terminally modified Le^x, *i.e.* by sialylation as in **BA-17** and **BA-18** or by α-galactosylation as in **BA-08**, **BA-32** and **BA-36**, were not binding ligand for this antibody.

Anti-CD15s antibody binds to sLe^x epitope. As consistent with the data reported by CFG, sLe^x directly linked to the mannose in core pentasaccharide (**BA-17** and **BA-18**) also failed to bind anti-CD15s antibody. However, **BA-21**, which has tandem s6LacNAc structure, unexpectedly showed considerable binding to this antibody, implying that glycan with this structure may mimic the sLe^x that leads to the binding of anti-CD15s antibody, which means that binding of anti-CD15s antibody for sLe^x epitope is not too strict.

Viruses—The binding specificity of three strains of influenza A virus, representing influenza viruses of swine, avian and human origin, were examined (Fig. 4, Dataset S1).

A/sw/Minnesota/02749/2009 (H1N1) isolated from swine has a known binding specificity towards Neu5Acα2–6Gal-linked glycans.[63] As expected, **BA-01** and **BA-21**, both with terminal Neu5Acα2–6Galβ1–4GlcNAc (s6LacNAc) epitope, presented strong binding, and the binding to **BA-21** appeared higher than **BA-01**. Although the internal s6LacNAc could not directly bind the swine virus (no binding to **BA-23**, which only has internal s6LacNAc), its presence still resulted in the higher binding of **BA-21** compared to **BA-01**, clarifying that internal s6LacNAc facilitated the binding of terminal s6LacNAc. No binding was observed if the terminal of Neu5Acα2–6Gal-linked glycans was further modified with α2–8-linked sialic acids (**BA-09** and **BA-10**), consistent with the previous report.[64] Nor did Neu5Gcα2–6Gal-linked glycans (**BA-03** and **BA-22**) show any binding.

The avian virus strain A/Ruddy Turnstone/DE/650625/2002, isolated from the shorebird, Ruddy Turnstone, was previously reported to bind Neu5Acα2–3Gal-linked glycans and Neu5Acα2–6Gal-linked glycans.[64] In our array, internal LacNAc improved the binding of virus (**BA-20** > **BA-05**). Internal s6LacNAc significantly enhanced the binding between α2–6-sialosides and virus (**BA-21** > **BA-01**), but only slightly increased the binding of α2–3-sialosides (**BA-23** > **BA-04**). Sialylation and fucosylation of adjacent internal LacNAc did not alter the binding (**BA-19** *vs.* **BA-23**, **BA-19** *vs.* **BA-29**). With α2–8-sialylation of terminal sLacNAc (**BA-13** and **BA-14** *vs.* **BA-04**) or s6LacNAc (**BA-09** and **BA-10** *vs.* **BA-01**), binding with the virus was no longer detectable. Unlike lectins, fucosylation of terminal sLacNAc has enhanced binding affinity (**BA-17** > **BA-04**, **BA-18** > **BA-05**). The virus also showed strong binding to some Neu5Gcα2–3Gal-linked glycans (**BA-18**,

fucosylated Neu5Gcα2–3LacNAc, and **BA-20**, Neu5Gcα2–3LacNAcβ1–3LacNAc). However, the failure of **BA-05** (Neu5Gcα2–3LacNAc) to bind this virus may suggest that fucosylation of terminal epitope and internal LacNAc are favorable factors for the binding.

The third virus, A/Brisbane/59/2007 (H1N1), is a human vaccine strain, and can bind both Neu5Acα2–3Gal-linked glycans and Neu5Acα2–6Gal-linked glycans.[64] It showed similar binding profile to that of the Ruddy Turnstone virus we tested. The difference existed in that no binding to Neu5Gcα2–3Gal-linked glycans (**BA-18** and **BA-20**) but binding to Neu5Gcα2–6Gal-linked glycan (**BA-22**) were detected with Brisbane virus. This could be attributed to the α2–6-sialoside preference for human virus *versus* α2–3-sialoside preference for avian virus. The binding patterns of this virus demonstrate the enhancement of binding by internal s6LacNAc epitope (**BA-21** > **BA-01**, **BA-22** > **BA-03**).

Taken together, some general observations can be made that the influence caused by internal epitope or the modification of terminal epitope on the binding characteristics of plant lectins, antibodies, or viruses are obvious. In some cases, the internal epitope totally blocked the binding. For instance, direct connection with core pentasaccharide in *N*-glycans completely abolished the binding of Le^x and sLe^x with the corresponding antibodies. The binding between glycans and specific GBPs was eliminated by fucosylation of terminal epitope, as for MAL-I and ECL. α2–8-Sialylation of terminal α2–3/6-sialylated LacNAc also abolished the binding, as was observed for the three viruses. Although α2–8-sialylation did not influence binding between α2–3-sialylated LacNAc and MAL-I, it blocked the binding between α2–6-sialylated LacNAc and SNA. In some other cases, the internal epitope or modification of terminal epitope reversed the binding profile from no binding to binding, or even strong binding. For example, internal LacNAc converted the non-ligand **BA-05** into a ligand **BA-20** for Ruddy Turnstone virus, whereas fucosylation of terminal LacNAc in **BA-05** generated a good ligand **BA-18** for the same virus. Internal s6LacNAc, in the similar fashion, altered the non-bound **BA-03** into a bound ligand **BA-22** for Brisbane virus.

Other than the abovementioned binding switch, increase or decrease of binding caused by internal epitope or modification of terminal epitopes was also common in our assay. The divergent effects led by the same epitope or modification appeared dependent on the inherent binding activity of the binding partners. Fucosylation of terminal epitope enhanced viral binding to α2–3-sialosides, yet reduced binding of WGA. Internal LacNAc usually strengthens the binding, as for MAL-I, WGA, avian and human viruses, but does not affect the binding with ECL and GSL-I-B_4. When internal LacNAc was fucosylated, the binding was generally hampered, as for MAL-I, GSL-I-B_4, WGA, avian and human viruses, with the exception of no influence for ECL binding. Sialylation of internal LacNAc, on the other hand, exhibited three different effects –no effect on SNA binding, decreased MAL-I and WGA binding, yet improved binding with all three viruses.

These results suggest that a re-examination of the binding patterns that were assigned to the known GBPs and viruses and their application in glycan-binding investigation is needed. For example, quite a few plant lectins are commonly used to define the structures of isolated glycans.[55–57,64] With our results, the expected binding could not be observed because the

internal epitope or modification of terminal epitope caused binding decrease to an undetectable level, or unexpected binding could be observed by the enhancing effect.

Conclusions

By using the “Core Isolation/Enzymatic Extension (CIEE)” strategy combined with one-pot multienzyme (OPME) synthesis, a series of *N*-glycans, which include those containing a terminal sialyl LacNAc, sLe^x and Siaα2–8-Siaα2–3/6-R structures with Neu5Ac or Neu5Gc sialic acid form, LacNAc, Le^x, α-Gal, and Galα1–3-Le^x; and tandem epitopes including α-Gal, Le^x, Galα1–3-Le^x, LacNAc, and sialyl LacNAc, presented with an internal sialyl LacNAc or 1–2 repeats of an internal LacNAc or Le^x component, were prepared in milligram-scale and over 98% purity by HPLC-HILIC purification monitored by UV absorption. One-pot synthesis has been proven as an efficient strategy for complex *N*-glycan preparation. All glycosyltransferases and sugar nucleotide synthesizing enzymes used in this study were over-expressed by *E. coli* and were robust in catalyzing the synthesis of the 36 *N*-glycans including those with tandem epitopes, providing us a methodology to produce more complex structurally defined *N*-glycans. Interrogation of microarrays displaying such glycans revealed the participation of the internal epitopes in the binding with GBPs and viruses. In conclusion, when interpreting the binding between glycans and GBPs/viruses, not only the structure of the terminal glycan epitopes, but also the internal epitopes and/or modifications of terminal epitopes, especially tandem glycan epitopes need to be taken into account.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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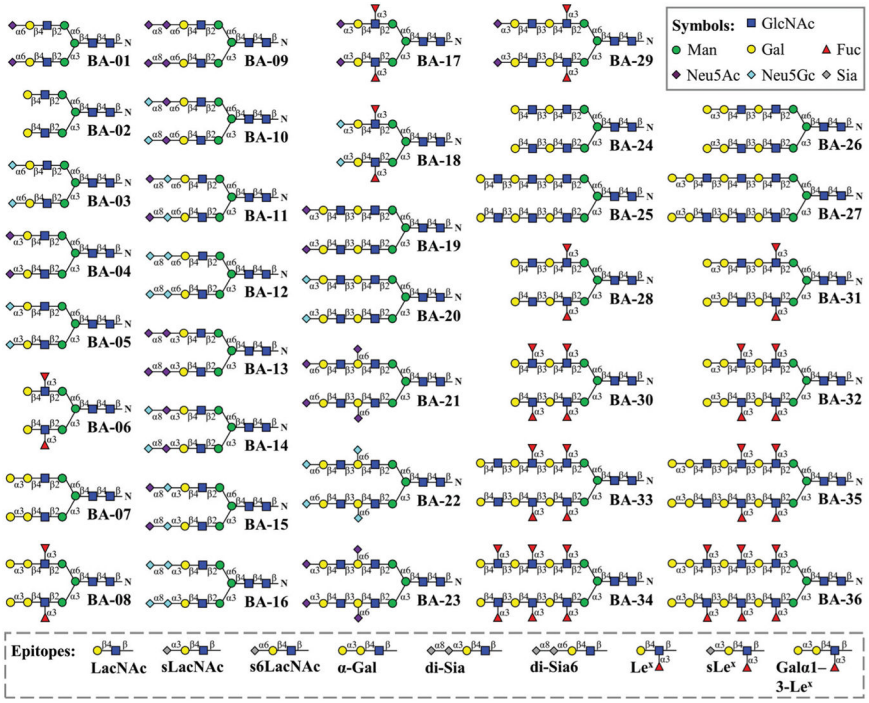


Fig. 1.

Biantennary *N*-glycans synthesized in this study. Glycan epitopes are shown in the dashed box.

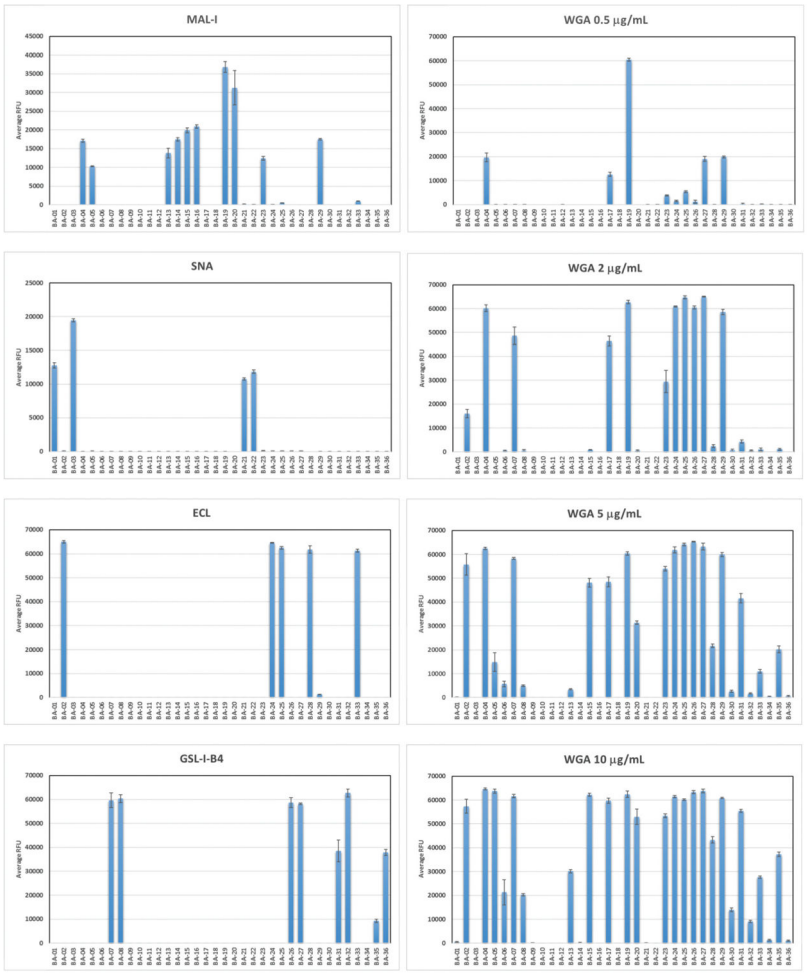


Fig. 2.

Binding profile of plant lectins with defined binding specificities. Five lectins were inspected including *Maackia amurensis* lectin I (MAL-I), *Sambucus nigra* lectin (SNA), *Erythrina cristagalli* lectin (ECL), B subunit of *Griffonia simplicifolia* lectin I (GSL-I-B_4), and wheat germ agglutinin (WGA).

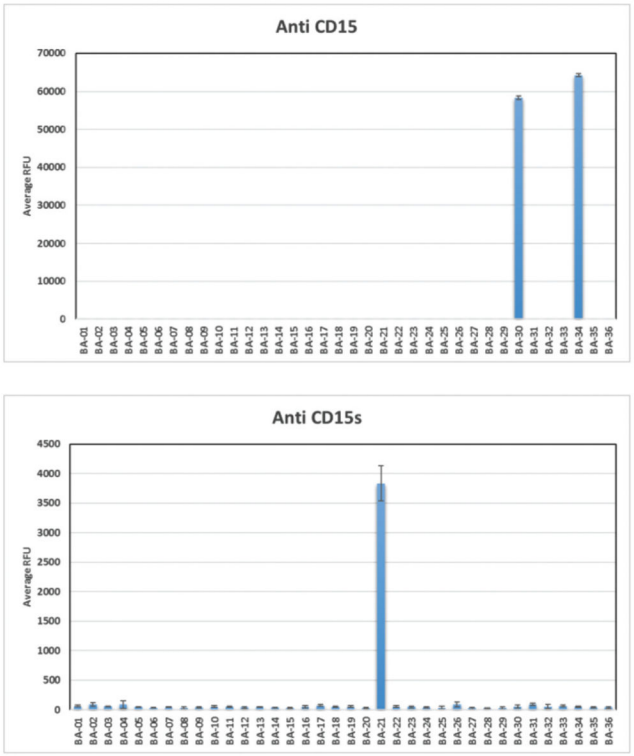


Fig. 3.

Binding profile of antibodies. Two antibodies were tested, including anti-CD15 antibody and anti-CD15s antibody.

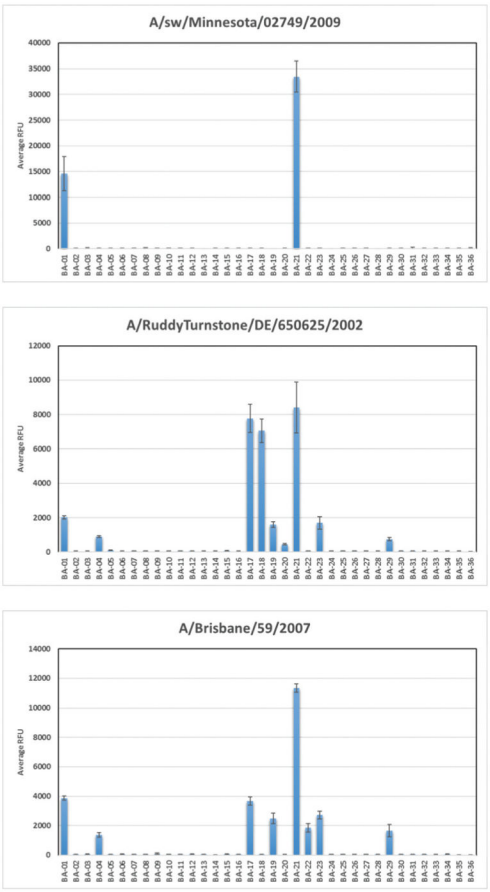
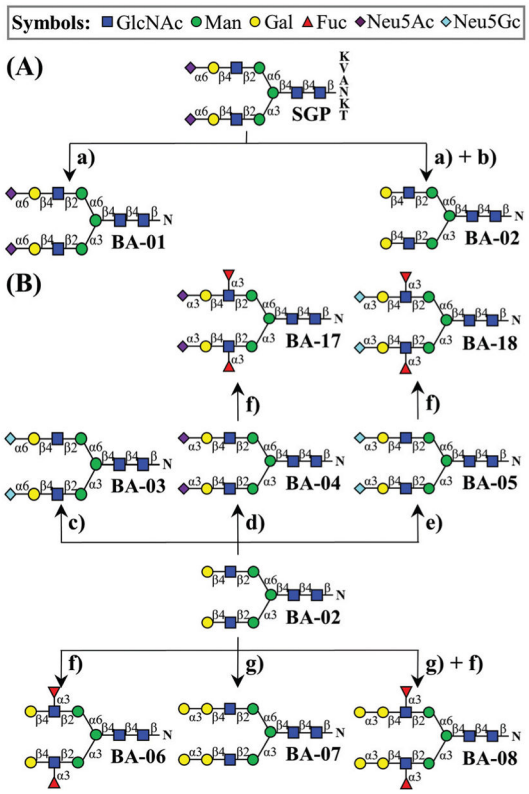


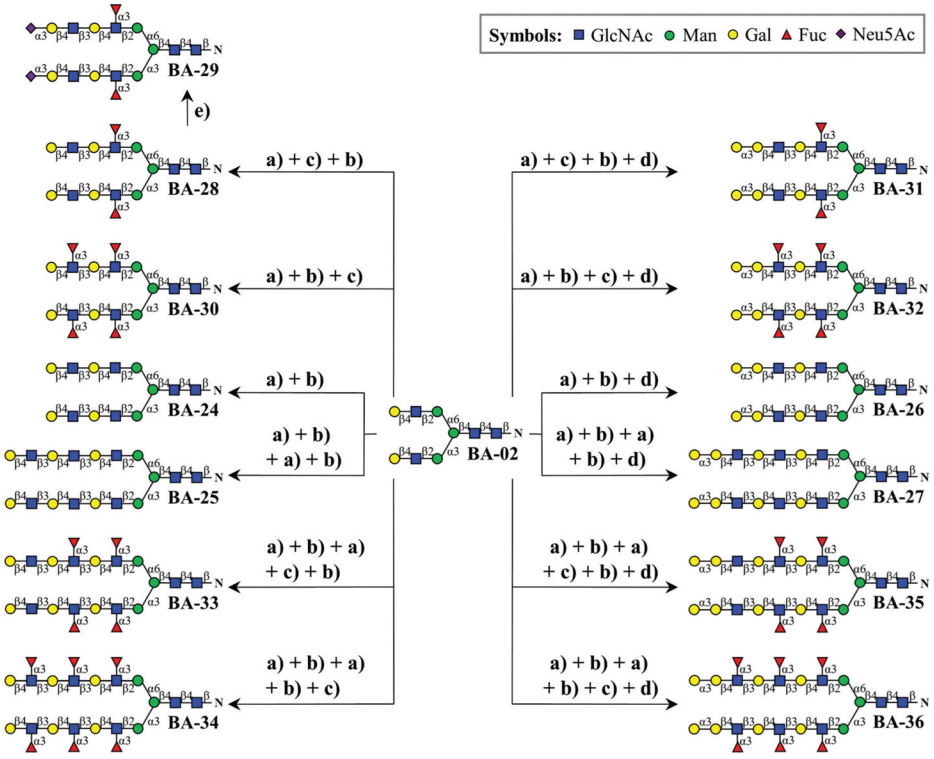
Fig. 4.

Binding profile of influenza A viruses. Three viruses were examined, including subtypes from swine (A/sw/Minnesota/02749/2009), avian (A/Ruddy Turnstone/DE/650625/2002) and human vaccine (A/Brisbane/59/2007) origin.



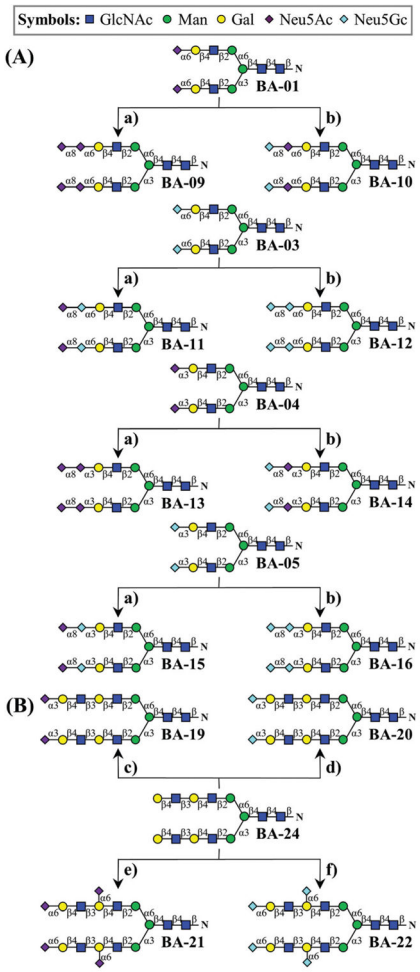
Scheme 1.

Preparation of disialylated *N*-glycan **BA-01**, the core *N*-glycan (**BA-02**) and one-pot enzymatic synthetic schemes for producing *N*-glycans (**BA-03** to **BA-08**, **BA-17** and **BA-18**) with only terminal glycan epitopes. Reagents and conditions: (a) pronase E; (b) sialidase BiNanH2; (c) Neu5Gc, CTP, Mg^{2+}, *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS) and *Photobacterium damselae* α2–6-sialyltransferase (Pd2,6ST); (d) Neu5Ac, CTP, Mg^{2+}, NmCSS and *Pasteurella multocida* α2–3-sialyltransferase double mutant E271F/R313Y (PmST1 E271F/R313Y); (e) Neu5Gc, CTP, Mg^{2+}, NmCSS and PmST1 E271F/R313Y; (f) GDP-Fuc, Mn^{2+} and *Helicobacter pylori* α1–3-fucosyltransferase (Hpα1,3FT); (g) UDP-Gal, Mn^{2+} and bovine α1–3-galactosyltransferase (Bα1,3GalT). The plus sign in the reaction conditions denotes that the subsequent reactions were performed without purification of the product of the previous reaction.



Scheme 2.

One-pot synthetic scheme for *N*-glycans with tandem sugar epitopes (**BA-24** to **BA-36**). Reagents and conditions: (a) UDP-GlcNAc, Mn^{2+} and NmLgtA; (b) UDP-Gal, Mn^{2+} and NmLgtB; (c) GDP-Fuc, Mn^{2+} and Hpα1,3FT; (d) UDP-Gal, Mn^{2+} and Bα1,3GalT; (e) Neu5Ac, CTP, Mg^{2+}, NmCSS and PmST1 E271F/R313Y. The plus sign denotes the subsequent reactions were performed without purification of the product of the previous reaction.



Scheme 3.

One-pot synthetic scheme for *N*-glycans with sugar epitopes with sialic acids (**BA-09** to **BA-16**, **BA-19** to **BA-22**). Reagents and conditions: (a) Neu5Ac, CTP, Mg^{2+}, NmCSS and *Campylobacter jejuni* α2–3/8-sialyltransferase CstII (CjCstII); (b) Neu5Gc, CTP, Mg^{2+}, NmCSS and CjCstII; (c) Neu5Ac, CTP, Mg^{2+}, NmCSS and PmST1 E271F/R313Y; (d) Neu5Gc, CTP, Mg^{2+}, NmCSS and PmST1 E271F/R313Y; (e) Neu5Ac, CTP, Mg^{2+}, NmCSS and Pd2,6ST; (f) Neu5Gc, CTP, Mg^{2+}, NmCSS and Pd2,6ST.