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Efficient Convergent Synthesis of Bi-, Tri-, and Tetraantennary Complex Type *N*-Glycans and Their HIV-1 Antigenicity

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Abstract

The structural diversity of glycoproteins often comes from posttranslational glycosylation with heterogeneous *N*-glycans. Understanding the complexity of glycans related to various biochemical processes demands a well-defined synthetic sugar library. We report herein a unified convergent strategy for the rapid production of bi-, tri-, and tetra-antennary complex type *N*-glycans with and without terminal *N*-acetylneuraminic acid residues connected via the α-2,6 or α-2,3 linkages. Moreover, using sialyltransferases to install sialic acid can minimize synthetic steps through the use of shared intermediates to simplify the complicated procedures associated with conventional sialic acid chemistry. Furthermore, these synthetic complex oligosaccharides were compiled to create a glycan array for the profiling of HIV-1 broadly neutralizing antibodies PG9 and PG16 that were isolated from HIV infected donors. From the study of antibody PG16, we identified potential natural and unnatural glycan ligands, which may facilitate the design of carbohydrate-based immunogens and fasten the HIV vaccine development.

Introduction

The process of *N*-linked glycosylation of HIV-1 envelope gp120 and gp41 glycoproteins results in diverse carbohydrate structures, masking the underlying glycoprotein domains and thus forming a "glycan shield" that can evade immune recognition.[1] The outer envelope of glycoprotein gp120 contains 24 glycosylation sites. Among those, 13 are linked to complex type *N*-glycans, and 11 are occupied by *N*-glycans of the high-mannose or hybrid type, which in fact account for half of the mass of gp120.[2] The biosynthesis of *N*-glycans begins in endoplasmic reticulum (ER), where the *N*-linked glycan precursor, $Glc_3Man_9GlcNAc_2$, is transferred co-translationally to the nascent gp160 polypeptide.[3] The terminal glucose moieties are then trimmed by ER resident glucosidase I and II, freeing gp160 from calnexin/calreticulin to exit ER. As the gp160 transported from ER to Golgi, the glycan moieties get trimmed further by mannosidase to form $Man_5GlcNAc_2$, a substrate for various Golgi resident cellular enzymes.[4] N-acetyl glucosamine transferase (GnT) catalyses the transfers of GlcNAc to the D1 arm of $Man_5GlcNAc_2$ substrate, converting it to a hybrid type glycan. This hybrid glycoform is then a substrate for modification into complex glycans, in which the D2 and D3 arm mannose is cleaved, allowing addition of GlcNAc moieties by a series of GnT family enzymes to form multiantennary mature oligosaccharides (Figure 1a). The occurrences of bi-, tri-, and tetra-antennary structures with various degrees of sialylations on gp120 surface were characterized by mass spectrometric analysis.[2, 5]

A series of broadly neutralizing antibodies (bNAbs) that recognize a cluster of oligomannose type glycans in the V3 loop of gp120 is recently described.[6] This epitope has fueled considerable interests in exploiting oligomannose clusters as possible carbohydrate-based vaccines. However, most of the oligomannose-based glycoconjugate vaccines so far are unable

to achieve exact mimicry of the carbohydrate epitope on cell surface and, therefore, failed to elicit effective neutralizing antibodies.[7] In contrast to previously identified oligomannose targeting bNAbs, Mouquet et al. reported the first example of complex type *N*-glycans binding bNAb PGT121,[8] which emphasized the role of complex type *N*-glycans in eliciting neutralizing antibodies.[6] Thereafter, two studies demonstrated the dependence of sialylated complex and hybrid type *N*-glycans at a secondary binding site in addition to $Man_5GlcNAc_2$ at the primary binding site recognized by two somatically related bNAbs, PG9 and PG16.[9] However, the binding preference of PG9 and PG16 to isolated carbohydrates in the absence of peptide backbone has not been attempted, so there is no binding study for glycans only. As a part of our interests in the development of carbohydrate-based HIV-1 vaccines, we developed a glycan microarray of synthetic *N*-linked oligosaccharides found on gp120 for use to investigate the glycan binding specificities of newly discovered HIV-1 bNAbs. Our preliminary results showed that without multiantennary glycans, we could not achieve a complete profiling since we know the existence and abundance of the multiantennary glycans on gp120 surface. We established that access to bi-, tri-, and tetra-antennary glycans would allow validation of the glycan processing in the biosynthesis pathway to analyze the impact of various glycoforms on antibody recognition.

Much of the complexity associated with multiantennary oligosaccharide synthesis was addressed by Danishefsky,[10] Unverzagt,[11] Huang[12] and Reichardt[12] in developing synthesis strategies and methods. Not surprisingly, due to the difficulty of sialic acid chemistry, only a few examples of fully sialylated tri- and tetra-antennary *N*-glycans have been reported.[14] Consequently, building a comprehensive complex type *N*-glycan-based sugar library for the study of HIV glycosylation and related immune response would be a major challenge. Thus, an

efficient strategy that generates diverse complex oligosaccharides would be advantageous. Recently, Boons and coworkers developed a general strategy to chemoenzymatically synthesize a library of unsymmetrical multi-antennary *N*-glycans.[13] Despite the advancement in synthetic methodologies, the complete access to all possible HIV-gp120 related *N*-linked oligosaccharide structures is still not avaliable to date. In this study, we described the development of a convergent strategy that utilized oligosaccharyl fluoride as a versatile donor to cleanly conjugate highly branched carbohydrate building blocks in a highly stereo- and regioselective manner to prepare unnatural and naturally occurring versions of multiantennary glycans. Then, sialyltransferases were used to perform terminal sialylation that permitted access to both α-2,6 and α-2,3-linked glycans of desired purity to avoid complications in chemical synthesis. With a larger and more complete sugar library, we conducted a glycan microarray-based profiling of PG9 and PG16 to understand the binding specificity of these antibodies and the information was used for the design of carbohydrate-based vaccines.

Results and Discussion

Bi-, tri- and tetra-antennary complex glycans are composed of a central trisaccharide core and equipped with two, three, and four antennae, respectively, with varying degree of sialylation (Figure 1b). The analysis of strategic bond disconnection provides a guiding principle of total chemical synthesis that tetra-antennary complex glycans (structure **VII)** could be assembled by conjugation of three building units in two glycosylation events, followed by one enzymatic sialylation (Figure 1c). Our general synthetic strategy envisioned the double regio- and stereoselective glycosylations at the 3-*O* and 6-*O* positions of the core trisaccharide with strategically protected tri-/pentasaccharide donors. The resulting galactose terminated systems would be further elaborated by sialyl transferases mediated enzymatic extension to recruit diverse complex structures to our library. At the reducing end, the amino pentyl linker, which was aimed at preserving the native glycan structure, could serve as a handle for covalent immobilization on NHS-coated glass slides for microarray analysis and also for conjugation to a carrier protein.

Based on previous reports, the most challenging phase of complex oligosaccharide synthesis involves the installation of highly branched tri- or pentasaccharide antennae at the sterically hindered sites in the central core. Danishefsky and coworkers recently accomplished the total synthesis of fully sialylated tri-antennary *N*-glycan and elaborated the intricacy of its assembly.[14-15] Dealing with the branched architecture, our strategy is to first gain access to antennae **7**, **8**, and **9** (Scheme 1). The synthesis of key intermediate **3**, which was commenced with NIS/TMSOTf[16] catalyzed coupling of glucosamine **1** and properly functionalized galactosyl thioglycoside donor[17] **2** (See Supporting Information), required the complete β-selectivity. Condensation of disaccharide donor **3** with mannosyl acceptors **4**, **5**, and **6** under the promotion

of NIS and TMSOTf provided pentasaccharides **7**, **8**, and trisaccharide **9**, respectively, in excellent yield. It is noteworthy that the disaccharide **3**, without further manipulation, was used directly as a donor for a single or double glycosylation to facilitate antennae assembly in just two glycosylation events from appropriate monosaccharide building blocks. Anticipating upcoming difficulties associated with incorporation of antennae to the core, the D1/D2 arm building blocks **7**, **8**, and **9** were transformed into anomeric trichloroacetimidates and fluorides. The trichloroacetimidate donors **7a**, **8a**, and **9a** were prepared by selective cleavage of anomeric allyl or *p*-methoxyphenyl (PMP) ethers and subsequent reaction with trichloroacetonitrile in the presence of DBU. Similarly, the synthesis of glycosyl fluorides **7b**, **8b**, and **9b** was approached through the exposure of anomeric -OH to DAST[18]. With two kinds of glycosyl donors in hand, we next sought to evaluate the compatibility of these branched oligosaccharide donors for regio- and stereoselective glycosylation.

The key intermediate **10**, a chitobiose trisaccharide precursor, was synthesized on multigram scales through a sequence of coupling of the reducing end di-glucosamine acceptor with a gluco-type donor. Next, the β-Mannoside structure was accessed via 2-OTf mediated SN_2 inversion of *β*-glucoside (See Supporting Information). With all required elements in place, the assembly of skeleton was initiated by 3-O glycosylation of the core trisaccharide. We initially used Schmidt's[19] trichloroacetimidate donor, which was extensively utilized by Unverzagt[12] in the synthesis of multiantennary glycans. Installation of the D1 arm trisaccharide **9a** via glycosidation at the 3-O position of core **10** in the presence of $BF_3{\cdot}Et_2O$ afforded desired hexasaccharide **11** in 60% yield. However, using fluoride donor **9b** in Cp_2HfCl_2/AgOTf [20,21] promoted glycosylation, we observed a slight improvement in yield (Scheme 2). Then, *p*-toluene sulfonic acid mediated reductive benzyledene ring opening of **11** afforded diol **12,** which served

as a common precursor in the construction of bi- and tri-antennary glycans. Then, diol **12** was glycosidated with donor **9a** in the presence of $BF_3{\cdot}Et_2O$ or TMSOTf to afford nonasaccharide **13** in low yield (Table 1). Likewise, glycosidation with **7a** under similar conditions led to donor decomposition. We suspected that trichloroacetimidates may experience the steric hindrance at the attachment point and the low stability of donor in acidic conditions, resulting in the formation of side products from the donor. On the other hand, engagement of fluorides **7b** and **9b** in the reaction pathway to supply the corresponding bi- and tri-antennary glycans **13** and **15**, respectively, proceeded very efficiently without forming any major side products. The stereochemistry of all the glycosidic linkages was confirmed by NMR analysis (see Supporting Information).

After establishing the effective use of fluoride donor, we proceeded to obtain highly branched tetra-antennary glycans. The preparative routes for undeca- and tri-decasaccharide derivatives are via regioselective 3-*O* glycosylation of **10** with **8b** catalyzed by Cp_2HfCl_2/AgOTf[22] to furnish the desired octasaccharide **17** in 68% yield (Scheme 3). Using another trichloroacetimidate donor **8a** and catalyzed by $BF_3{\cdot}Et_2O$, the desired octasaccharide **17** was obtained in 25% with minor acceptor recovery (Table 1). Compound **17** was undergone reductive ring opening in the presence of *p*-toluene sulfonic acid to afford diol **19**. To exploit the higher reactivity of primary -OH, compound **19** was condensed with the **D2** arm **9b** and pentasaccharide **7b** in the presence of Cp_2HfCl_2/AgOTf. The tri-antennary **20** and tetra-antennary **22** glycans were obtained in satisfactory yields (Scheme 3). Our results showed that glycosylation with fluorides (**7b**, **8b,** and **9b**) under Cp_2HfCl_2/AgOTf mediated condition proceeded very efficiently with short reaction time and less side products (Table 1). Throughout the above-mentioned protocols, we utilized a versatile glycosyl fluoride donor and exclusive

glycosylation condition (Cp_2HfCl_2/AgOTf) to prepare multiple target products, which effectively reduce the synthetic steps.

With compounds **13**, **15**, **17**, **20**, and **22** in hand, we next proceeded to the stage of global deprotection. The process commenced with removal of phthalimide protection by using *n*-butyl alcohol and ethylene diamine at 90 °C. This mixture was then exposed to N-acetylation of the free amine followed by deacetylation using sodium methoxide. Finally, the compounds were subjected to hydrogenolysis to supply **14, 16**, **18**, **21**, and **23**, respectively. Throughout the global deprotection process, and three rounds of column purifications, the products were, however, obtained in relatively low yields. Structure studies are done by extensive NMR analysis (See Supporting Information II for spectra). Motivated by $Man_4GlcNAc_2$ (D1 arm of $Man_9GlcNAc_2$) recognition by HIV-1 bNAb 2G12,[23] we also prepared the unnatural form **18** for comparative study.

The chemical installation of sialic acid-containing antennae to assemble complex oligosaccharides is particularly challenging due to the lack of participating group to achieve the desired stereochemistry and the complexity of tedious multistep protection and de-protection processes[24]. However, the enzymatic glycosylation with glycosyl transferases is a single step process with high regio- and stereo-specificity in excellent yield. As a result, the enzymatic method of sialylation,[20,21] which was well demonstrated previously[25] especially through the synthesis of sLe^x, was implemented in our strategy with regeneration of sugar nucleotide.[26] In general, bacterial enzymes are more stable and easier to express in *Escherichia coli*, and bacterial glycosyltransferases have broader acceptor-substrate specificity than mammalian-derived enzymes[27]. Yamamoto and coworkers have reported that α-2,3-sialyltransferase from Vibrio sp. bacterium JT-FAJ-16[28] and α-2,6-sialyltransferase from photobacterium sp. JT-ISH-224[29] have

very broad acceptor specificity, excellent productivity, high specific activity and no intrinsic sialidase activity. The unique properties of these sialyltranferases provide a powerful tool to construct the library of α-2,3/2,6 sialosides. Therefore, the galactose terminated acceptor substrates **14**, **16**, **18**, **21**, and **23** were subjected to sialylation by using CMP-NeuAc as donor in the presence of α-2,6 and α-2,3 sialyltransferases (see Supporting Information I) to furnish the desired sialosides **24**, **25**, **26**, **27**, **28**, **29**, **30**, **31**, **32**, and **33**, respectively, in respectable yields (Scheme 4). Overall, our chemoenzymatic synthesis offers an efficient and convenient way to access both α-2,6 and α-2,3 linked glycans of highly branched structures.

Profiling glycan specificities of HIV-1 bNAbs PG9 and PG16. A series of broadly neutralizing antibodies (bNAbs) that recognize a cluster of oligomannose type glycans in the V3 loop of gp120 have been described.[6] This glycan recognition phenomenon was well documented by Burton and Wilson through their study of bNAbs 2G12 and PGTs 127/128, which recognize oligomannose clusters in the V3 loop of gp120.[6,9,30] Previous structural and biochemical studies showed the necessity of both the carbohydrate and peptide domains in forming an epitope of PG9,[9a,31] PG16,[9b] and PGT128,[30b] in contrast to 2G12, which apparently does not interact with the gp120 peptide backbone. However, past work has largely relied on recombinant gp120, supplied as mixtures of glycoforms that might not represent the actual glycosylation pattern on native envelope. The glycan heterogeneity complicates the precise correlation between glycan structure and immunoactivity. In the present study, we attempted to reveal the glycan specificity of PG9 and PG16 by using the glycan array platform.

In order to examine the glycan binding specificity of antibodies PG9 and PG16 using carbohydrate microarray, the synthetic multiantennary *N*-glycan ligands were printed on N-

hydroxy succinimide-activated glass slides through covalent immobilization. The slides were spotted by taking an aliquot from stock solution of individual glycans (100 μm) (Fig. 2). Each sample was printed with 10 replicates horizontally to form an array of 15 x 10 spots in each sub array, and the slide image was obtained from a fluorescence scan after incubation with DyLight649-conjugated donkey anti-Human IgG antibody. Our microarray analysis resulted in almost no detectable binding for PG9, probably due to (1) very weak binding affinity towards protein/peptide free glycans; (2) the requirement of closely spaced $Man_5GlcNAc_2$ (N160) and complex type glycan (N156/163) as PG9 epitopes; and (3) the heterogeneous distribution of NHS groups on glass slides resulting in uneven and low density glycan array. However, using the glycan array on aluminum-oxide coated glass slides, we were able to identify the ligands recognized by PG9 and the results will be published separately. On the other hand, PG16 showed detectable binding to only α-2,6-linked sialic acid terminated complex oligosaccharides, implying significant structural specificity. Moreover, we observed that PG16 exhibited high affinity binding to the tri-antennary glycans **26**, **30**, and the tetra-antennary glycan **32** in addition to the bi-antennary glycan **28**, a major complex type glycan observed on gp120, probably the increase in number of antennae would offer additional binding sites for PG16.

Determination of dissociation constants ($K_{D.surf}$). To determine the dissociation constants of α-2,6-sialylated complex glycans **24**, **26**, **30**, and **32**, that are bound on array surface to interact with PG16, we used various concentrations (1.25-0.039 μM) of PG16 and printed sugars (100-12.5 μM) to obtain binding curves, which were analyzed by the Langmuir isotherm to generate surface dissociation constants ($K_{D, surf.}$).[32] To calculate the K_D for glycan **32**, we plotted PG16 concentrations against the fluorescence intensities at various printing concentrations (Figure 4). The dissociation constants (K_D) and relative binding affinity of PG16

for additional carbohydrate structures were calculated using the same method (Table 2, Figure S5, and Table S2 SI). Our data revealed that the tetra-antennary **32** (K_D = 0.1 μM) and tri-antennary **30** (K_D = 0.093 μM) exhibit highest binding affinity. Interestingly, the unnatural glycan **28** (K_D = 0.117 μM)**,** a truncated version of tetra-antennary **32**, showed at least three-fold higher affinity compared to its structural surrogate, bi-antennary glycans **24** (K_D = 0.327 μM). However, it should be pointed out that the relative binding affinity of PG16 towards bi-antennary complex type glycan **24** (K_D = 0.327 μM) was found to be significantly higher than the glycan affinity (K_D = 1.19 mM) reported by Pancera et. al.[9b] by using STD NMR, probably due to multivalent interactions between PG16 and glycan **24** printed on array. Careful structural observation of **28** revealed that deletion of one mannose residue of naturally occurring bi-antennary complex type structure might impose restriction on the flexibility of antennae. Such restricted orientation may project two antennae at appropriate distance necessary for strong PG16 binding. Our detailed survey of structures and binding constants (K_D) suggest that glycan **28** could be considered as an important unnatural glycan ligand of HIV-1 bNAb PG16 in relation to $Man_4GlcNAc_2$ (an important unnatural ligand of HIV-1 bNAb 2G12). Further studies are needed to understand the exact mode of binding of **28** to PG16 in comparison with the natural form **24**. Theoretically, immunization with vaccine constructs bearing different copies of unnatural glycan **28** would induce antibodies that may not target the host glycoproteins on their surfaces. In principle, glycan **28** is structurally simpler and its synthesis could be achieved in fewer steps than other glycans in the multiantennary series. For these reasons, we select glycan **28** as a potential candidate for the development of carbohydrate-based HIV-1 vaccine and immune response studies.

Conjugation of carbohydrate antigen to carrier protein. Carbohydrate immunogenicity can be enhanced by their covalent attachment to antigenic carrier proteins such as tetanus toxoid (TT), diphtheria toxoid (DT), keyhole lympet hamocyanin (KLH), etc. Our group has recently demonstrated that for Globo H (GH) based cancer vaccine, GH-DT adjuvanted with glycolipid C34 showed the highest enhancement of anti-GH IgG.[33] Thus, we explored DT as a potential carrier protein for our carbohydrate-based HIV-1 vaccine candidate. We adopted the thiol-maleimide coupling chemistry to increase the carbohydrate epitope ratio on carrier protein to reduce the amount of precious glycosyl thiol. Thus, glycan **28** with amino pentyl spacer at the reducing end was modified with 3,3'-dithiobis (sulfosuccinimidyl- propionate) (DTSSP) in phosphate buffer (pH 7.2). Then, the disulfide bond was reduced by dithiothreitol (DTT) to afford the desired Michael donor **34** after P2-Gel column purification. Surface modification of DT with thio-active maleimide functional group was performed by reaction with an excess of N-(- ε -maleimidocaproyloxy) sulfosuccinimide ester (Sulfo-EMCS) in phosphate buffer (pH 7.2) for 2 h (Scheme 5). The loading of maleimide linkers on DT was confirmed by MALDI-TOF mass analysis. Finally, maleimide derivatized DT was incubated with 5 and 20 equivalents of thiolated decasaccharide **34** to afford glycoconjugates **36** with epitope ratio of four and **37** with epitope ratio of eleven respectively. The unreacted maleimide functional groups on DT were deactivated by excess amount of thioethanol (supporting information).

To assess the affinity of PG16-glycoconjugate antigens, we used synthetic glycoconjugates as a coating antigen to detect PG16 IgG antibody. The synthetic constructs were immobilized on microtiter plate and titrated against serial PG16 dilutions. We found that DT modified with carbohydrates could detect PG16 in a clear dose response (Figure 5). We also observed some dose dependent non-specific binding of PG16 to carrier protein DT. A glycoconjugate construct

37 with high carbohydrate content showed enhancement in sensitivity of the detection in comparison with **36**, perhaps due to multivalent interactions between PG16 and the glycans on carrier protein. It should be noted that the relative binding affinity of our synthetic glycoconjugates is still much lower than the affinity of PG16 to gp120. Taken together, the present study provides the synthetic glycoconjugate constructs in an attempt to mimic the putative epitope of PG16 for HIV-1 vaccine design.

Conclusion

We have successfully developed a highly efficient strategy based on the use of oligosaccharyl fluorides for the convergent installation of branched galactose terminated antennae to achieve high regio- and stereoselectvity with excellent yields. Combining with sialyltransferases mediated sialylation, this method allows easy access to α-2,6 and α-2,3-sialylated glycans of bi-, tri- and tetra-antennary complex type. With the glycans found on gp120 available from this synthetic approach, a glycan array was thus prepared on glass slides, and the binding specificity of PG16 towards a panel of complex *N*-glycans in the carbohydrate microarray was used to define the glycan composition required for strong antibody binding. Furthermore, the potential candidate **28** was conjugated to the carrier protein DT for antibody binding studies. The results confirmed that the glycoconjugates indeed bind to PG16.This study also revealed the problem of glycan array on glass slides, which led to the development of glycan array on aluminum oxide-coated glass slides for use to identify mixed ligands in PG9-glycan interaction, and the result will be reported separately.

Supporting Information.

Materials, supplementary schemes, synthetic methods, spectroscopic and analytical data for new compounds, and experimental protocols.

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Figure Legends

Figure 1. (a) Golgi dependent glycan biosynthesis pathway ; (b) Representation of bi-, tri- and tetra-antennary *N*-glycan structures selected for synthesis; (c) Overall synthetic strategy. D1/D2 building blocks could be installed in two glycosylation steps, followed by enzymatic sialylation.

Figure 2. Pictorial representation of multiantennary complex type *N*-glycan structures printed on NHS-activated glass slide through amide bond formation.

Figure 3. (a) Slide obtained from fluorescent scan after secondary antibody incubation; (b) Binding of PG16 to a panel of glycans in glycan microarray represented in bar chart.

Figure 4. Binding curves for glycan 32 printed on microarray surface at various concentrations (100, 50, 25, and 12.5 μM). The curves were obtained by using DyLight649-conjugated donkey anti-Human IgG secondary antibody. Error bars indicate the SEM of K_D values obtained from two independent experiments while each experiment was performed in triplicates and average of fluorescent intensity was taken.

Figure 5. ELISA binding of PG16 to glycoconjugate constructs in serial antibody dilutions.

Scheme 1. Reagents and Conditions : (a) NIS, TMSOTf, 4 Å MS, CH_2Cl_2, -50°C, **3**: 82%, **7**: 85%, **8**: 76%, **9**: 72%; (b) (1) CAN, ACN:toluene:H_2O (4 : 2 : 1), 1 h, (2) DBU, CCl_3CN, CH_2Cl_2, 0 °C, 3h, **7a**: 75%, **8a**: 70%, over 2 steps; (c) (1) CAN, ACN : toluene : H_2O (4 : 2 : 1), 1 h, (2) DAST, CH_2Cl_2, -10°C, 1 h, **7b**: 72%, **8b**: 69%, over 2 steps; (d) (1) $PdCl_2$, CH_2Cl_2 : MeOH (1/1), 3 h, (2) DBU, CCl_3CN, CH_2Cl_2, 0 °C, 85% over 2 steps; (e) (1) $PdCl_2$, CH_2Cl_2 : MeOH (1/1), 3 h, (2) DAST, CH_2Cl_2, -10 °C, 1 h , 76% over 2 steps. NIS : N-iodosuccinimide; TMSOTf : trimethylsilyl trifluoromethanesulfonate; CAN : cerium ammonium nitrate; DBU : 1,8-

Diazabicyclo[5.4.0]undec-7-ene; CCl_3CN : trichloroacetonitrile; DAST : diethylaminosulfur trifluoride.

Scheme 2. Reagents and Conditions : (a) **9b**, AgOTf, Cp_2HfCl_2, 4 Å MS, toluene, -40 °C, 2 h, 71%; (b) *p*-TSA, acetonotrile, rt, 3 h, 70%; (c) **9b**, AgOTf, Cp_2HfCl_2, 4 Å MS, toluene, -40 °C, 3 h, 69%; (d) **7b**, AgOTf, Cp_2HfCl_2, 4 Å MS, toluene, -40 °C, 2 h, 51%; (e) (1) $NH_2CH_2CH_2NH_2$, *n*BuOH, 90 ^oC, overnight; (2) Ac_2O, pyridine, overnight; (3) NaOMe, MeOH, overnight; (4) $Pd(OH)_2$, MeOH : H_2O : HCOOH (5 : 3 : 2), H_2, **14**: 60%; **16**: 39%. Cp_2HfCl_2 : Bis (cyclopentadienyl) hafnium Dichloride, AgOTf : silver trifluromethanesulfonate.

Scheme 3. Reagents and Conditions : (a) **8b**, AgOTf, Cp_2HfCl_2, 4 Å MS, toluene, -50 °C to -30 °C, 2 h, 68%; (b) *p*-TSA, acetonotrile, rt, 8 h, 72%; (c) **9b**, AgOTf, Cp_2HfCl_2, 4 Å MS, toluene, -40 °C, 3 h, 64%; (d) **7b**, AgOTf, Cp_2HfCl_2, 4 Å MS, toluene, -50 °C to -30 °C, 2 h, 73%; (e) (1) $NH_2CH_2CH_2NH_2$, *n*-BuOH, 90 ^oC, overnight; (2) Ac_2O, pyridine, overnight; (3) NaOMe, MeOH, overnight; (4) $Pd(OH)_2$, MeOH : H_2O : HCOOH (5 : 3 : 2), H_2, **18**: 51%; **21**: 37%; **23**: 27%.

Scheme 4. Reagents and Conditions : (a) CMP-Neu5Ac, α-2,6/2,3- sialyltransferase, alkaline phosphatase, 2 d for α-2,6 and 8 d for α-2,3, **24**: 72%; **25**: 75%; **26**: 66%; **27**: 67%; **28**: 55%; **29**: 70%; **30**: 64%; **31**: 66%; **32**: 60%; **33**: 70%.

Scheme 5. Reagents and Conditions : (a) (1) DTSSP, PBS, pH 7.4, rt, overnight; (2) DTT, PBS, pH 7.4, 40°C, 2 h ; (b) Sulfo-EMCS, PBS, pH 8.0, rt, 2 h ; (c) (1) PBS, pH 7.2, rt, 2 h; (2) thioethanol, PBS, pH 7.2, rt, overnight.

Figure 1.

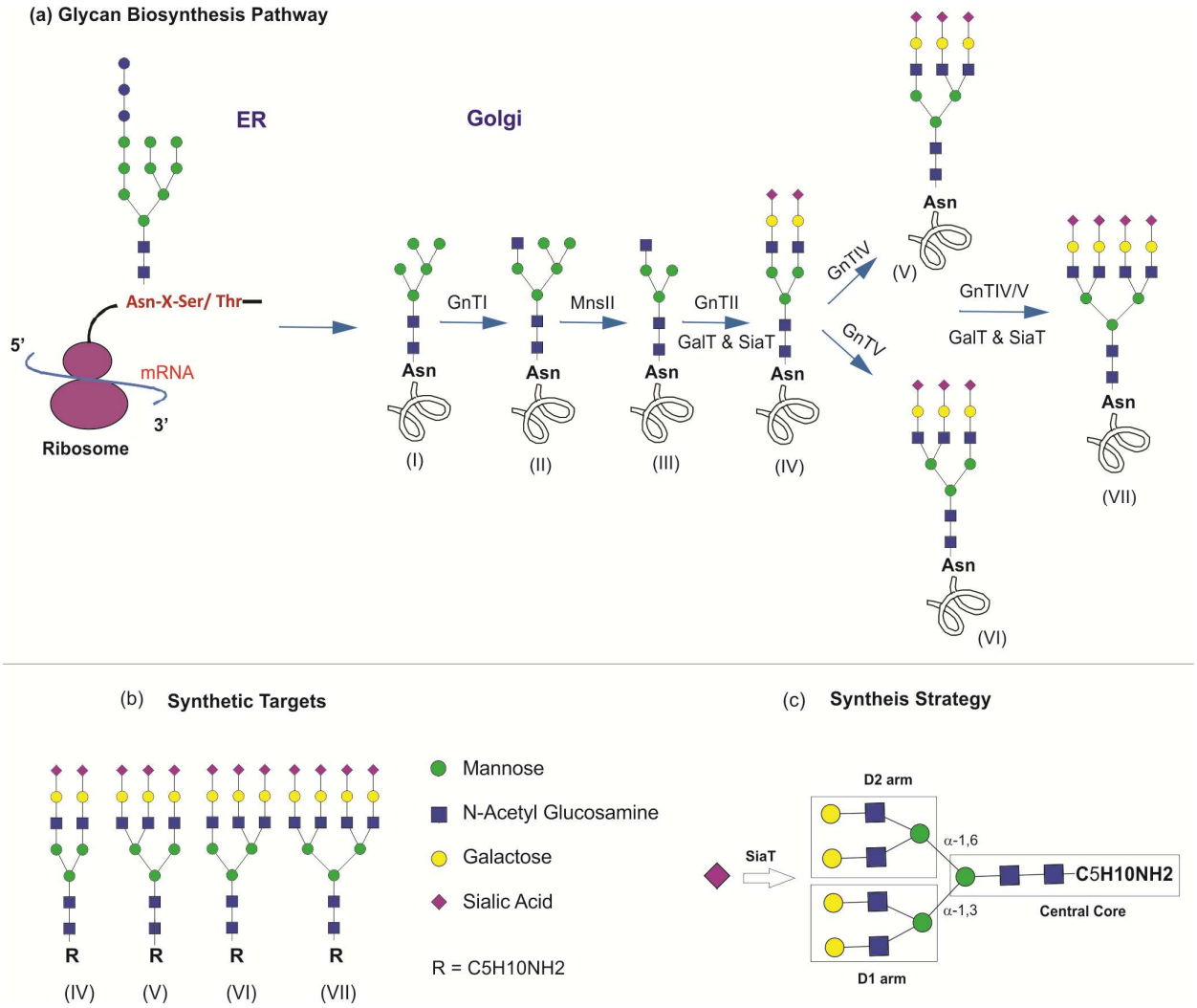

(a) Glycan Biosynthesis Pathway
ER
Golgi
Asn-X-Ser/ Thr
5'
mRNA
3'
Ribosome
GnTI
MnsII
GnTII
GalT & SiaT
GnTIV
GnTV
GnTIV/V
GalT & SiaT
Asn
(I)
(II)
(III)
(IV)
(V)
(VI)
(VII)
(b) Synthetic Targets
R
(IV)
(V)
(VI)
(VII)
Mannose
N-Acetyl Glucosamine
Galactose
Sialic Acid
R = C5H10NH2
(c) Syntheis Strategy
SiaT
D2 arm
D1 arm
α-1,6
α-1,3
C5H10NH2
Central Core

Figure 2.

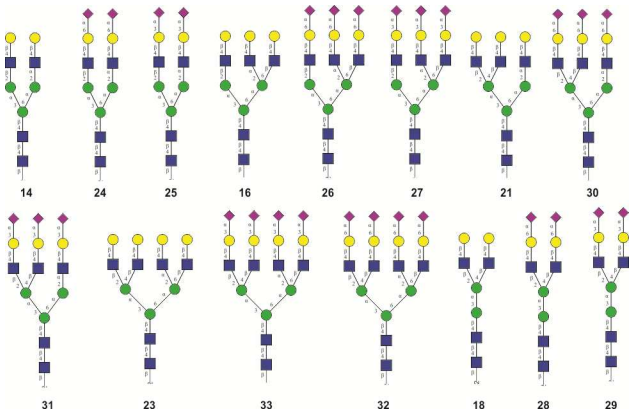


Figure 3.

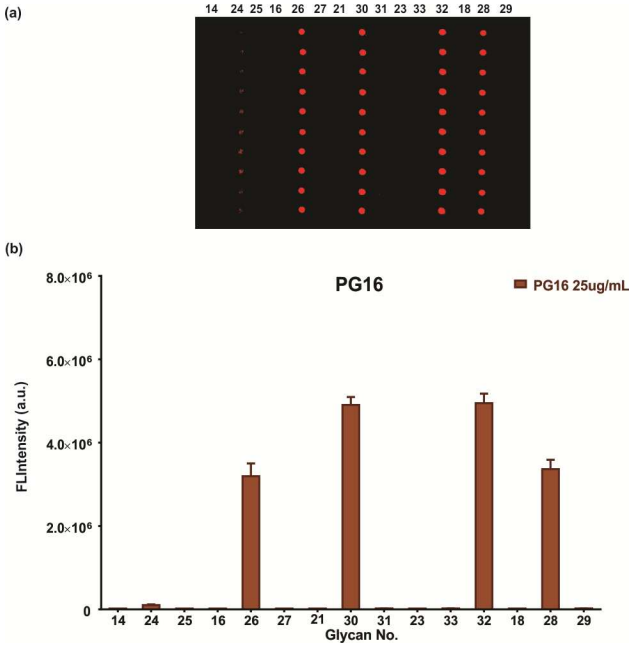

(a)
14 24 25 16 26 27 21 30 31 23 33 32 18 28 29
(b)
PG16
PG16 25ug/mL
FLintensity (a.u.)
8.0×10⁶
6.0×10⁶
4.0×10⁶
2.0×10⁶
0
14 24 25 16 26 27 21 30 31 23 33 32 18 28 29
Glycan No.

Figure 4.

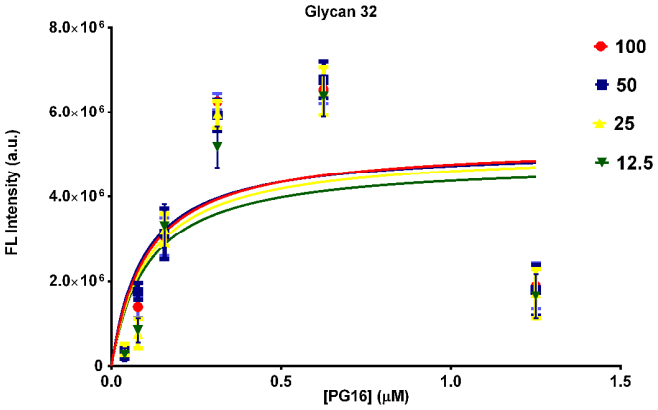
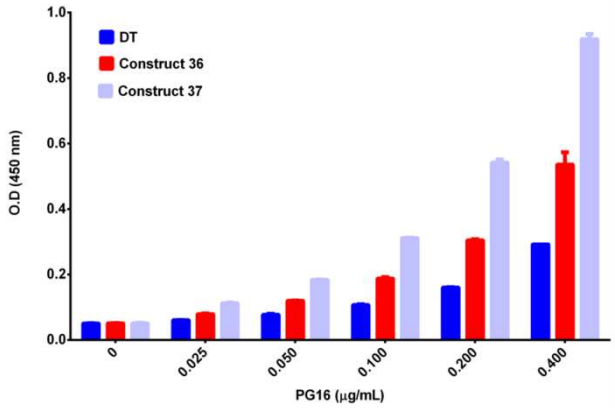
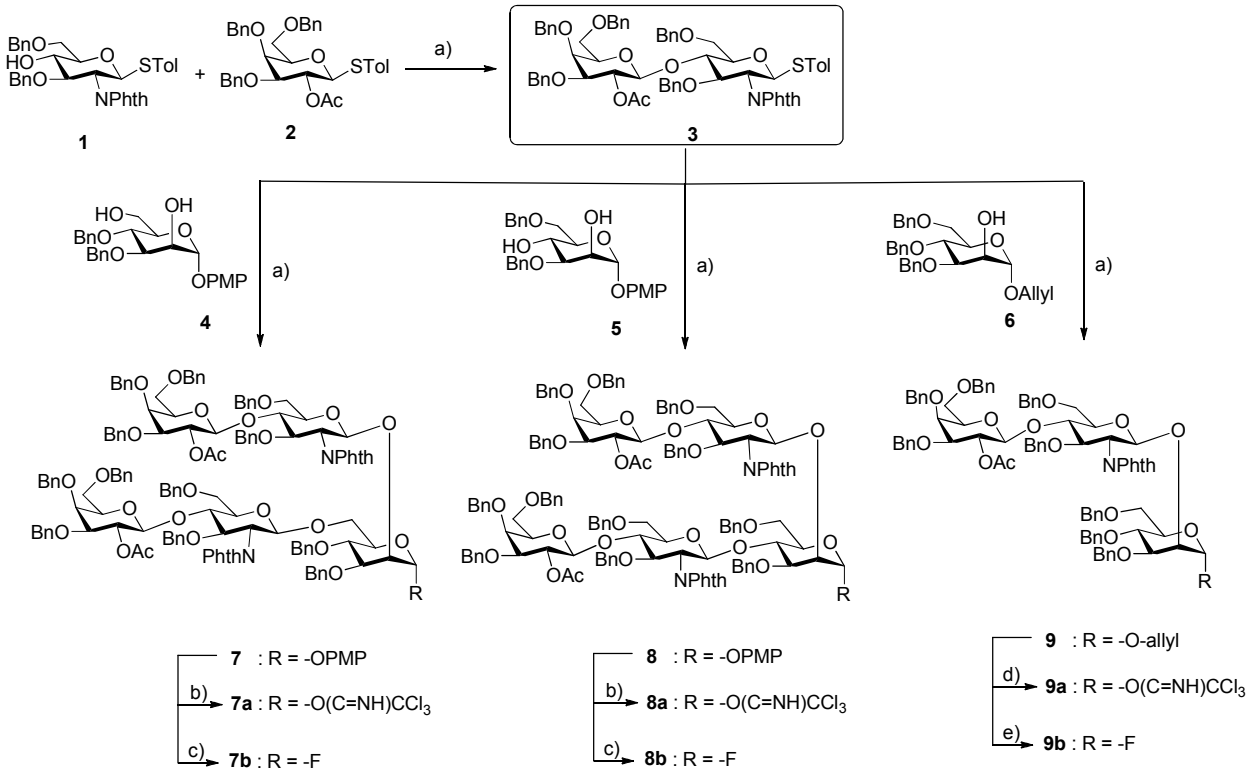


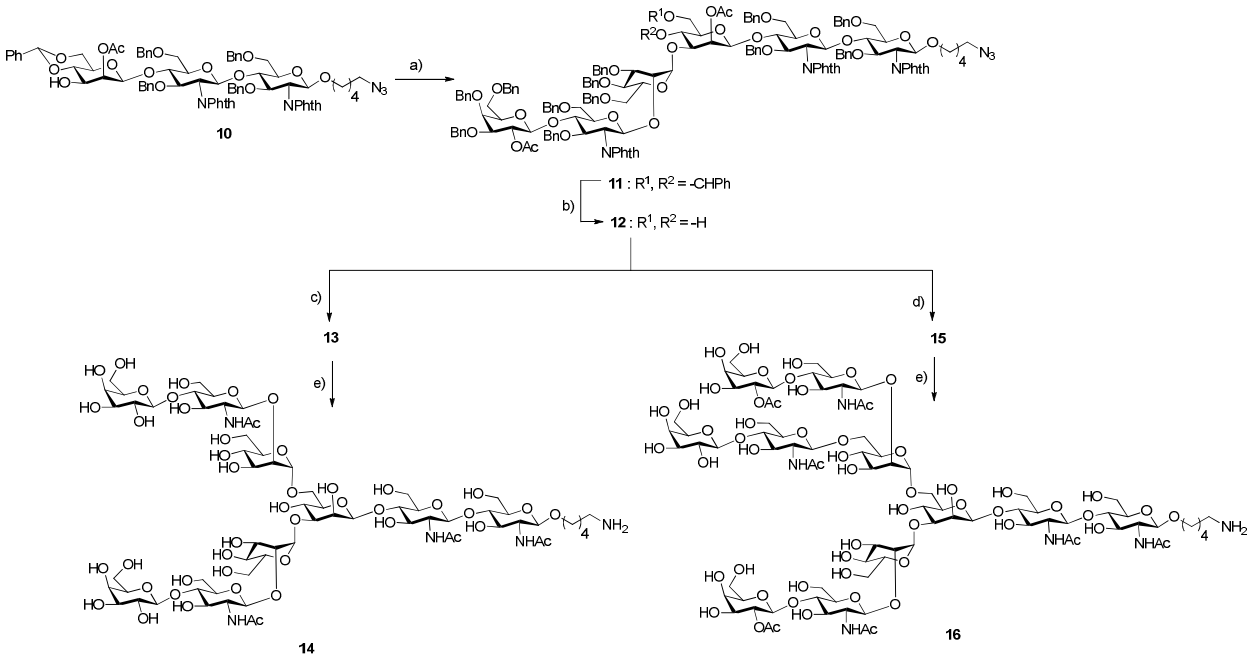
Figure 5.



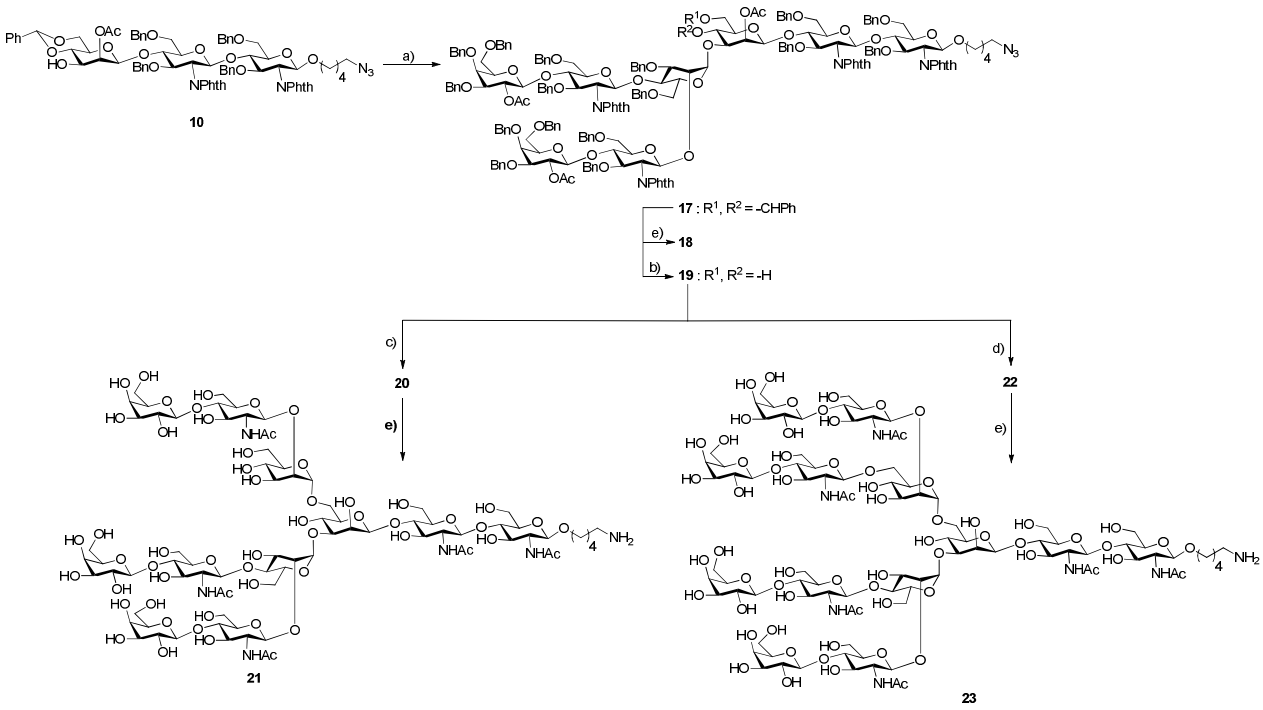
Scheme 1. Synthesis of D1/D2 arm tri- and pentasaccharides.



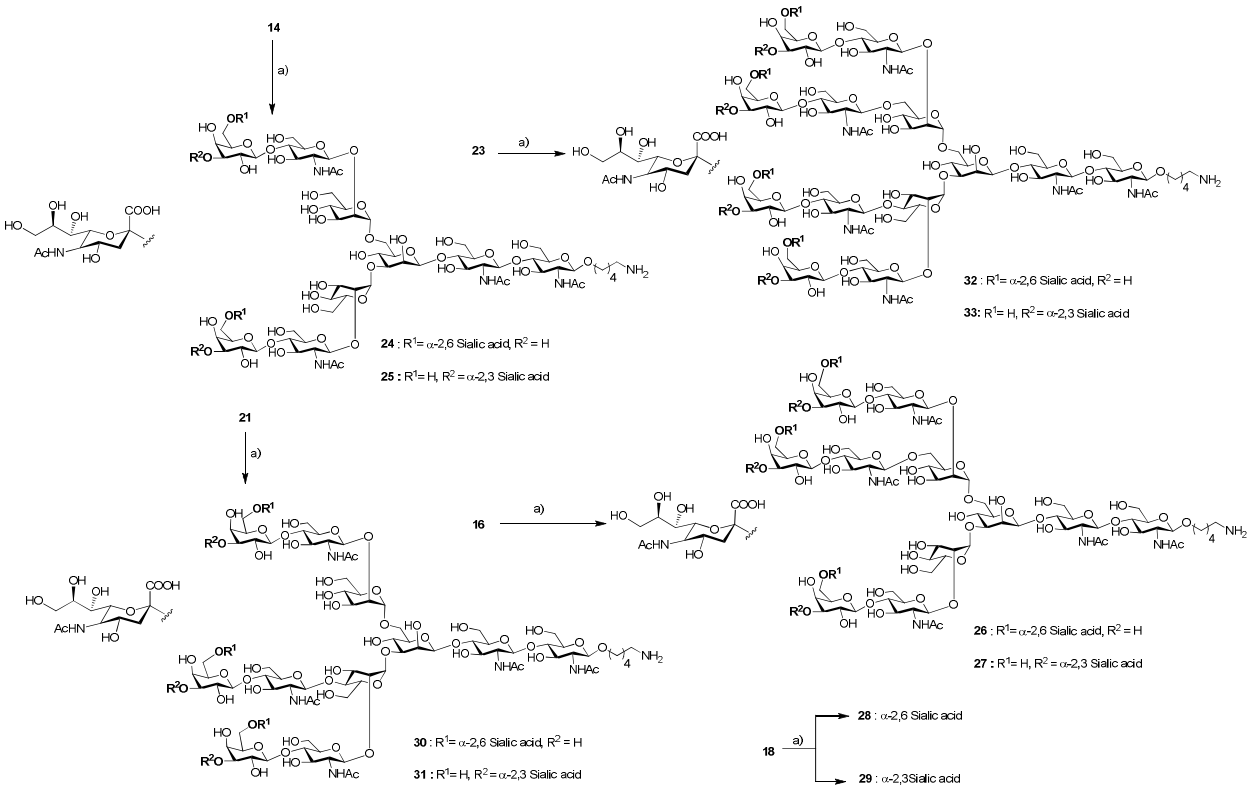
Scheme 2. Synthesis of bi- and tri-antennary glycans 14 and 16.



Scheme 3. Synthesis Tri- and tetraantennary glycans 21 and 23.



Scheme 4. Enzymatic extension to get fully sialylated multiantennary *N*-glycans.



Scheme 5. Conjugation of glycan 28 to carrier protein for immunization study.

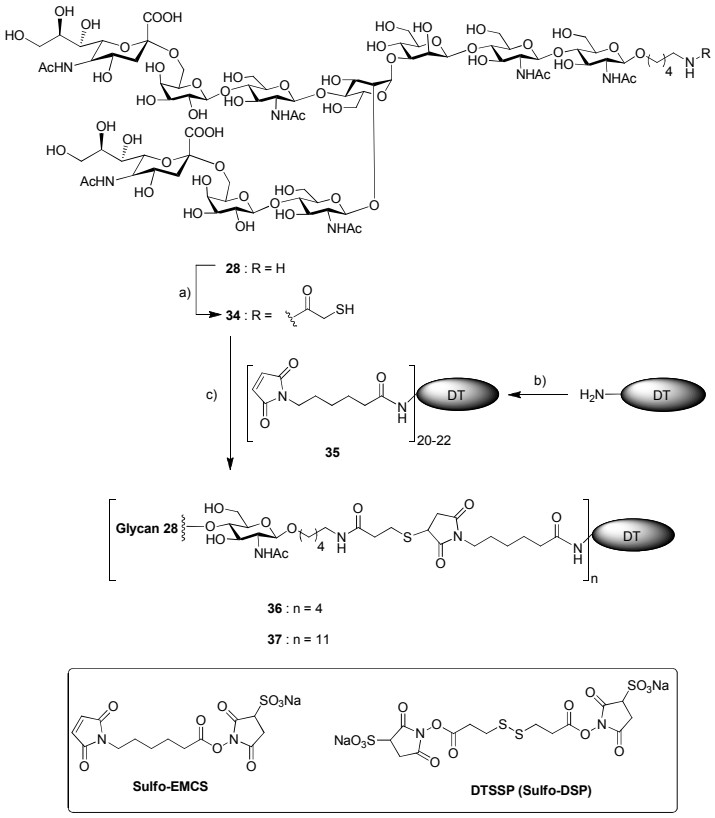


Table 1. Coupling of trichloroacetimidate and fluoride donors at 3-*O* and 6-*O* positions of core trisaccharide.

Entry	Acceptor	Donor	Promoter	Product	Yield (%)
1	10	9a	$BF_3{\cdot}Et_2O$	11	60
		9b	Cp_2HfCl_2/AgOTf		71
2	12	9a	TMSOTf	13	32[a]
		9b	Cp_2HfCl_2/AgOTf		69
3	12	7a	$BF_3{\cdot}Et_2O$/TMSOTf	15	ND
		7b	Cp_2HfCl_2/AgOTf		51[a]
4	10	8a	$BF_3{\cdot}Et_2O$	17	25
		8b	Cp_2HfCl_2/AgOTf		68
5	19	9a	$BF_3{\cdot}Et_2O$	20	16[a]
		9b	Cp_2HfCl_2/AgOTf		64
6	19	7a	$BF_3{\cdot}Et_2O$/TMSOTf	22	ND
		7b	Cp_2HfCl_2/AgOTf		73

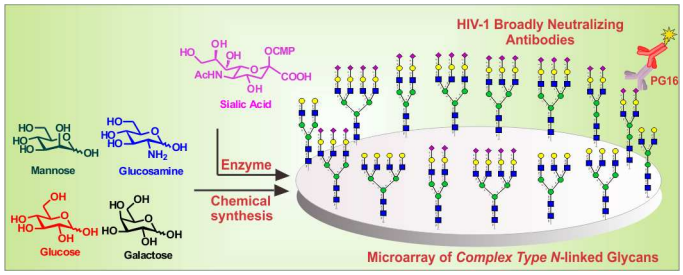
ND : none detected

[a]Acceptor Recovery : 21% (Entry 2), 40% (Entry 3), 31% (Entry 5).

Table 2. $K_{D,surf}$ (μM) values of antibody PG16 and sialylated complex type glycans.

Glycan no.	$K_{D,surf}$ (μM) ± SD (μM)
24	0.327 ± 0.087
26	0.120 ± 0.003
28	0.117 ± 0.003
30	0.093 ± 0.019
32	0.1 ± 0.006

TOC


HIV-1 Broadly Neutralizing Antibodies
PG16
Sialic Acid
Mannose
Glucosamine
Glucose
Galactose
Enzyme
Chemical synthesis
Microarray of Complex Type N-linked Glycans