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An Efficient Approach to the Discovery of Potent Inhibitors against Glycosyltransferases

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We describe a standardized approach for searching potent and selective inhibitors of glycosyltransferases by high throughput quantitative MALDI-TOFMS-based screening of focused compound libraries constructed by 1,3-dipolar cycloaddition of the desired azidosugar nucleotides with various alkynes. An aminooxy-functionalized reagent with a stable isotope was conjugated with oligosaccharides to afford glycopeptides as acceptor substrates with improved ion sensitivity. Enhanced ionization potency of new substrates allowed for MALDI-TOFMS-based facile and quantitative analysis of enzymatic glycosylation in the presence of glycosyl donor substrates. A non-natural synthetic sugar nucleotide was identified to be the first highly specific inhibitor for rat recombinant α2,3-(*N*)-sialyltransferase (α2,3ST, IC_{50} = 8.2 μM), while this compound was proved to become a favorable substrate for rat recombinant α2,6-(*N*)-sialyltransferase (α2,6ST, K_m = 125 μM). Versatility of this strategy was demonstrated by identification of two selective inhibitors for human recombinant α1,3-fucosyltransferase V (α1,3-FucT, K_i = 293 nM) and α1,6-fucosyltransferase VIII (α1,6-FucT, K_i = 13.8 μM).

Introduction

Posttranslational modification with glycans is a series of dynamic and complicated biosynthetic processes to control essential functions of glycoconjugates such as glycoproteins, glycosphingolipids, and proteoglycans.[1] It is obvious that glycan structures (glycoforms) of cell surface glycoconjugates have pivotal functions in various cellular recognition systems involving cell differentiation, development, inflammation, immune response, tumor metastasis, bacterial/viral infections, and many other intercellular communication and signal transductions.[2a−e] However, glycan expression and its microheterogeneity in common glycoproteins cannot be predicted because protein glycosylation is not template-driven and is subject to multiple sequential and competitive enzymatic pathways.[3a−c] Complex glycoforms of glycoproteins are synthesized through multistep glycosylation reactions catalyzed by a variety of glycosyltransferases (GTs[a]) sharing several sugar nucleotides in collaboration with many glycoside hydrolases (GHs) to define further synthetic pathways of two important subgroups, namely hybrid- and complex-type glycans.[4a,b] Therefore, a tremendous number of enzymes are considered to participate in highly complicated synthetic pathways of naturally occurring glycans and share their substrates with both sugar nucleotides and acceptor oligosaccharide structures during biosynthesis and metabolic degradation. In other words, it is extremely difficult to identify every enzyme responsible for synthesizing biologically relevant glycoforms even though a large-scale glycomics would reveal whole human glycoforms and profiles in their structural alterations during cellular differentiation, proliferation, carcinogenesis, malignant alteration, and metastasis.[5a−d] It is therefore evident that insight into precise substrate specificity and mechanisms in catalytic action by major enzymes might become a key to elicit functional roles of individual enzyme and the hierarchical relationship of crucial GTs/GHs in the course of biosynthetic pathway of human normal and disease-relevant glycan structures.

Specific and strong inhibitors of GTs appear to become potential tools for the investigation of structure−function relationship of human glycomes and the regulation mechanism of glycan biosynthesis as well as the discovery research of new class therapeutic reagents. Actually, extensive efforts have been paid to the development of such inhibitors for various GTs.[6a−j] However, there is no efficient and standardized strategy for searching promising inhibitors due to the complex and dynamic mechanism in the GT-catalyzed glycosylation reactions that involve many components such as some sugar nucleotides as donor substrates, multiform acceptor substrates, and a metal ion. Three dimensional (3D) structural information of enzyme at atomic resolution obtained by crystallographic or NMR analysis may be utilized for conclusive identification of the active site amino acid residues, often noted as conserved key amino acid residues in the catalytic pocket. Considering the success of some antivirus therapeutic drugs to inhibit the activity of influenza viral neuraminidases,[7a,b] it is believed that structural biology-based molecular design had often accelerated rational molecular design

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[a]Abbreviation: GTs, glycosyltransferases; GHs, glycoside hydrolases; MALDI-TOFMS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; βGal-T1, β1,4-galactosyltransferase; α2,3ST, α2,3-(*N*)-sialyltransferase; α2,6ST, α2,6-(*N*)-sialyltransferase; α1,3FucT, α1,3-fucosyltransferase; α1,6FucT, α1,6-fucosyltransferase; UDP-Gal, uridine-5′-diphospho-α-D-galactopyranose; CMP-Neu5Ac (CMP-NANA), cytidine-5′-monophospho-5-acetamido-3,5-dideoxy-β-D-*glycero*-α-D-*galacto*-non-2-ulosonic acid; GDP-Fuc, guanosine-5′-diphospho-β-L-fucopyranose; ATP, adenosine-5′-triphosphate; CTP, cytidine-5′-triphosphate; TBTA, tris-(benzyltriazolylmethyl)amine; SPPS, solid phase peptide synthesis; DEAE, diethylaminoethyl-sepharose; TMS, tetramethylsilane; TSP, trimethylsilylpropionate sodium salt.

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of potential compounds leading toward practical therapeutic reagents. We should consider that general 3D structural information is still not enough for understanding dynamic reaction mechanisms in unique GTs' catalytic action,[8a−h] although it is likely that 3D structures of some known GTs[3c] may be helpful to design some "*static inhibitors*" for the target GT. In fact, most human protein kinases are known to interconvert between at least two structural conformations, that is, active and inactive, and the phosphorylation of key amino acid residues of kinases can shift the balance between these dynamic states.[9a,b] These two states are characterized by conformational changes in flexible activation-loops bearing such as DFG (Asp-Phe-Gly) motif that borders or blocks the conserved ATP-binding pockets of active kinase conformations. Thus, a growing number of kinase inhibitors that bind *selectively* to inactive conformation, namely type II inhibitors, have been developed. The merit of this type of inhibitors is evident because the inhibitors that bind selectively to inactive conformation will not compete with cellular ATP. The rational design of the type II inhibitors has been therefore a promising strategy toward the practical kinase inhibitors,[10] whereas most of type I inhibitors that preferentially bind with active conformation must face significant competition by cellular ATP as a result of their similar binding affinity to both conformations.

In our previous study,[6i] we succeeded in the first rational molecular design of mechanism-based specific inhibitors for human β1,4-galactosyltransferase (βGal-T1), a member of the most important and ubiquitous superfamily of GTs in eukaryotes, transfer galactose from uridine-5′-diphosphogalactose (UDP-Gal) to various acceptor substrates bearing terminal GlcNAc residue(s). Our attention was then focused on the fact that βGal-T1 undergoes critical conformational changes upon UDP-Gal binding from an open (inactive) conformation to a closed (active) conformation conducted by the flexible loop region near the active site.[8c] Upon donor substrate binding, tryptophan 310 (Trp310) involved in the small loop plays an important role for the conformational changes in the long flexible loop by moving toward the catalytic pocket to interact with the complex of donor substrate and Mn^{2+} ion. This step seems to be essential for generating new binding pocket designated for the acceptor substrates. Although it seemed that this dynamic conformational change at around the catalytic site makes rational molecular design of a favorable inhibitor for βGal-T1 difficult, we hypothesized that compounds freezing molecular motion of this crucial Trp310 residue in the small loop should interrupt the access of the long flexible loop to the catalytic pocket. Using a designated suicide substrate to probe Trp310 residue based on the irreversible reaction mechanism,[6i,8c] we revealed that the distance between Trp310 and UDP-Gal binding pocket of βGal-T1 is estimated to be approximately 15 Å in the open conformation. Fortunately, this approach allowed us to develop the first class specific inhibitor for human βGal-T1 (K_i = 1.86 μM).[6i] Recently, Wagner et al. reported a profound insight into a unique mechanism of a new, base-modified UDP-Gal derivative that blocks the closure of a flexible loop in the active site of a human blood group GalT and acts, toward five different galactosyltransferases, as an inhibitor, with K_i values in the low micromolar to nanomolar range.[8h] It should be, however, emphasized that the above approach may be available only when the precise 3D structural formats of the target GTs, in which the flexible loop structures can be traced, had been already uncovered. At present, it seems likely that only limited number of human GTs may become targets of such mechanism-based specific inhibitors.[3c] Thus, advent of an efficient and high throughput approach for searching potential inhibitors of human GTs is now strongly required. In the present communication, we report a standardized protocol for the development of potent and selective inhibitors against GTs by MS-based rapid and quantitative screening of the focused compound libraries constructed by click chemistry[11a−e] between azidosugar nucleotides and various alkynes.

Results and Discussion

A. General Concept. Recently, we have developed mass spectrometry-based quantitative assay for the characterization of human protein kinases and their inhibitors.[12] It was demonstrated that synthetic peptide substrates of clinically important human kinases, in which *C*-terminus is conjugated with tryptophanylarginine (WR) and *N*-terminus is capped by acetylation with $(CH_3CO)_2O$ or $(CD_3CO)_2O$, allow for rapid and highly sensitive MALDI-TOFMS-based kinetic analyses of recombinant human kinases. It was revealed that concurrent labeling of designated peptides with stable isotope and ion sensitivity-enhancement reagents greatly facilitate multiple-kinase assay and inhibitor profiling using human lung cancer K562 cells in the presence of known inhibitors in a quantitative manner.[12] The advantage of this high throughput protocol is evident because doubly probed peptide sets will accelerate the investigation of the relationship between the expression levels of cellular kinases and inhibitor sensitivity for any specific kinases without any radioactive isotope labeling and prerequisite tedious chromatographic separation.

Our attention has been directed toward the versatility of this strategy in a wide range of biological and medical/pharmaceutical sciences. Protein glycosylation by GTs is an alternative important biosynthetic process for posttranslational modifications to phosphorylation by protein kinases. Glycosylation patterns of mammalian cells, namely "glycotypes" affecting cellular adhesion, seem to be strongly dependent on a variety of cellular stages because whole cellular glycomes change significantly during cell differentiation, proliferation, and malignant alteration.[2,13] Judging from the importance of identifying disease-related GTs and the specific inhibitors, it is not surprising that advent of a standard protocol for rapid and real activity-based characterization of GTs has long been needed. It is clear that such high throughput screening system should accelerate discovery research of potent inhibitors against GTs of interest. We hypothesized that tagging sugars with WR and stable isotope concurrently at suited positions will readily enhance ion-sensitivity of various glycosyl acceptor substrates[14a,b] and allow for rapid and quantitative characterization of GTs reactions by MALDI-TOFMS-based protocol as outlined in Figure 1A,B. When a focused compound library made by feasible click reactions[11a−e] between a designated azidosugar nucleotide and various alkynes is available (Figure 1C), high throughput screening for searching selective inhibitors of GTs can be established by using *an internal standard substrate* labeled with stable isotope moiety (Gal-$GlcNAc_3$-WR-OCD_3). For instance, the effect of an inhibitor on α2,3/2,6-sialyltransferases-catalyzed glycosylation (sialylation) can be determined quantitatively in the presence of CMP-Neu5Ac and an acceptor substrate (Gal-$GlcNAc_3$-WR-OCH_3) by comparing signal intensities at m/z 1511.223 and m/z 1514.244 due to the expected products bearing OCH_3 or OCD_3 group in the absence of inhibitor as indicated in Figure 1B.

B. Focused Compound Library Derived from Azidosugar Nucleotides. Three azidosugar nucleotides were selected

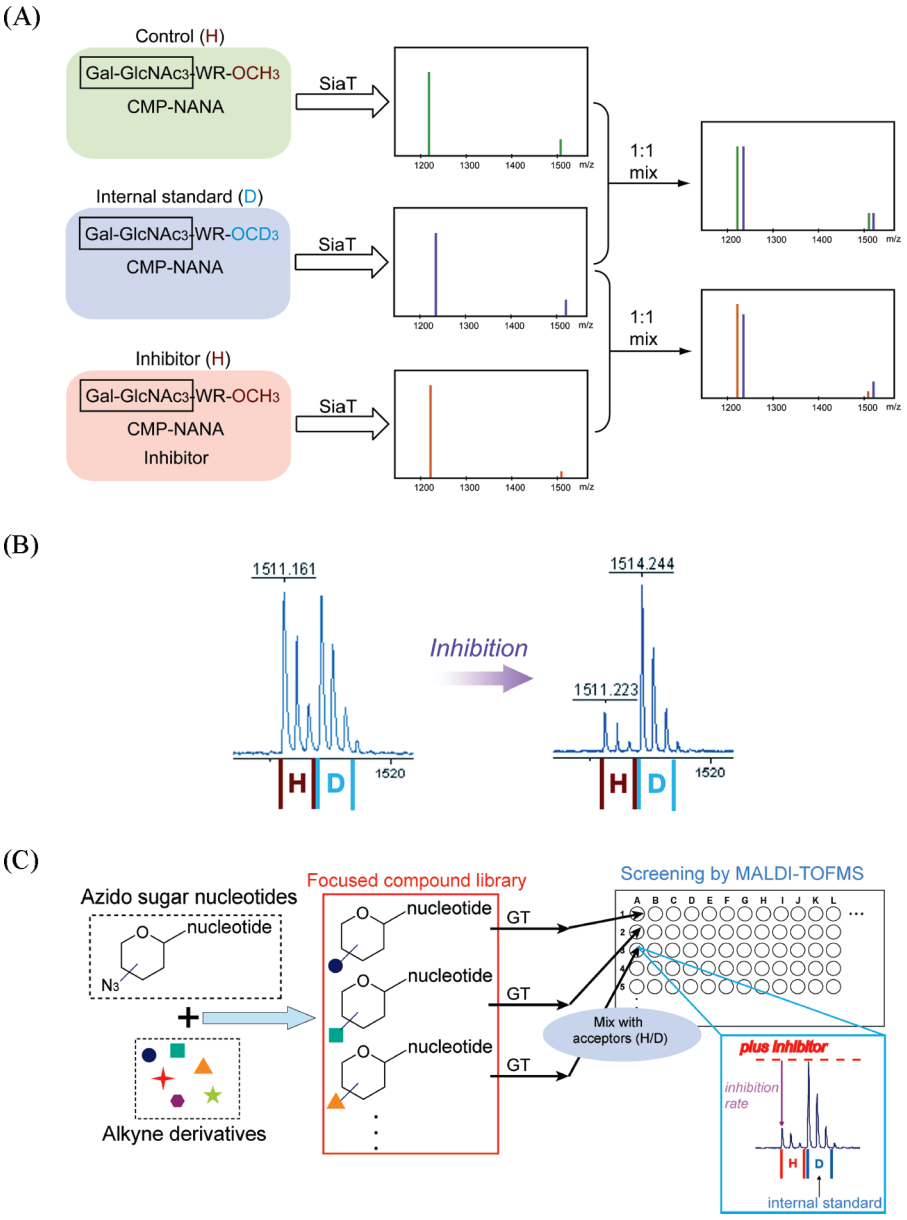


Figure 1. Concept of mass spectrometry-based rapid and quantitative assay of glycosyltransferase reactions by means of doubly probed glycopeptides. (A) General protocol illustrating for direct monitoring of the sialylation of doubly tagged acceptor Galβ1,4(GlcNAcβ1,4)$_3$ by α2,3/2,6-sialyltransferases in the presence of CMP-Neu5Ac (CMP-NANA). (B) Inhibitory effects of tested compounds determined by spiking an internal standard substrate tagged with stable isotope (OCD_3). The signals at m/z 1511.2 and m/z 1514.2 are due to the molecular weights of expected products bearing OCH_3 or OCD_3. (C) High throughput and quantitative screening of large numbers of compounds using a focused compound library constructed by click reactions between azidosugar nucleotides and commercially available alkynes.

tentatively as key intermediates for the construction of focused compound library related to CMP-Neu5Ac and GDP-Fuc analogues (Figure 2) because growing importance of the functions of various human sialyltransferases and fucosyltransferases in disease-relevant glycans synthesis prompted us to challenge the discovery of specific inhibitors showing the highest level of affinity with these enzymes. Our attention was then paid to the facts that modifications at C-9 and C-5 positions of CMP-Neu5Ac and C-6 position of GDP-Fuc should not reduce significantly the original affinity with their binding pockets of GTs for sugar nucleotides.[15] It is expected that divergent derivatization at these carbon atoms of **1**−**3** with various alkynes might be greatly beneficial for the discovery research of potential candidates exhibiting mechanism-based inhibitory effect through the interaction with unknown key amino acid(s) locating on the flexible loop near the active site. It is likely that this approach leads to new competitive inhibitors having additional function to freeze molecular motion of flexible loop involving nucleophilic amino acid residue(s) as we have succeeded rationally in the synthesis of mechanism-based inhibitors against human recombinant βGal-T1.[6i]

Schemes 1 and 2 indicate the synthetic route of **1** and **2** from sialic acid on the basis of combined chemical and enzymatic reactions. **1** was prepared readily from a known 9-azido sialic acid derivative **4**[16] by treatment with CMP-sialic acid synthetase[17] in the presence of CTP (cytidine-5′-triphosphate) in 70% yield. In case for the synthesis of **2**, 5-azidoacetamide sialic acid derivative **8**[18] prepared using 5-azidoacetamido-3,5-dideoxy-D-*glycero*-β-D-*galacto*-non-2-thiophenyl-1-methyl ester (**6**) was subjected to

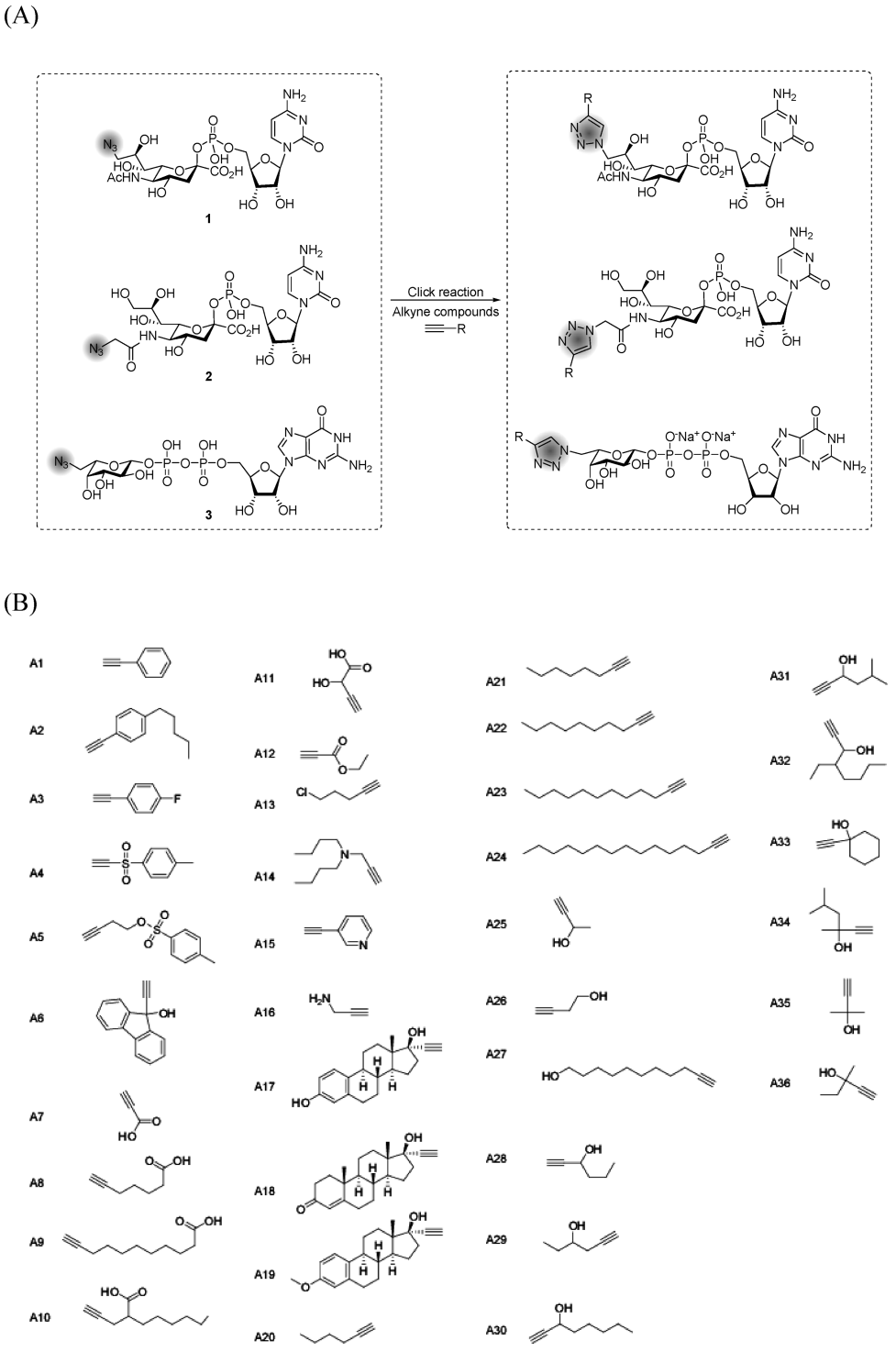
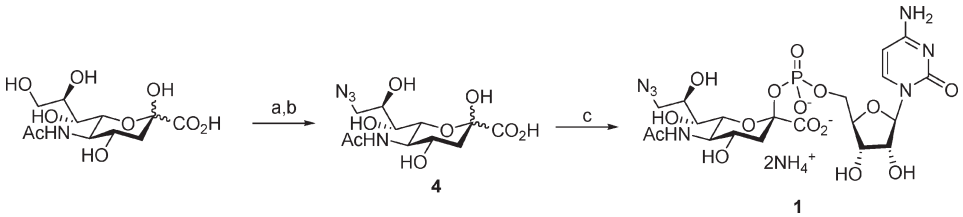


Figure 2. Azidosugar nucleotides used in this study and general scheme for the click reaction (A), notably 1,3-dipolar cycloaddition in a mild aqueous solution, between these azidosugar nucleotides and commercially available alkynes (B) allowed for the construction of focused compound library.

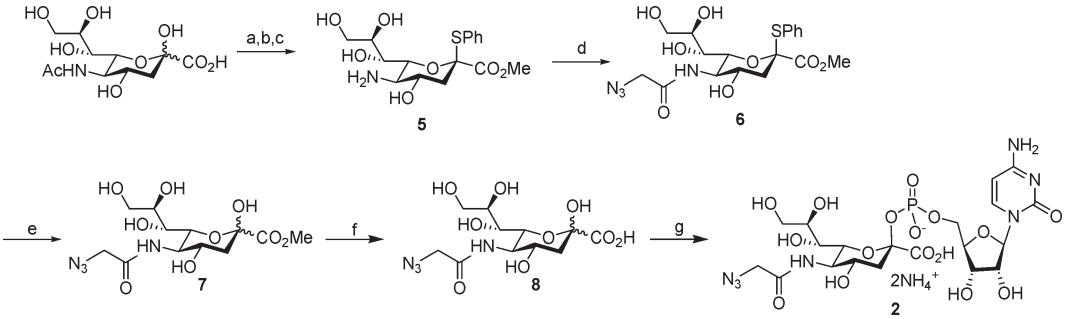
the enzymatic reaction with CTP under a similar condition with that of the synthesis of **1** to afford **2** in 50% yield. It is clear that use of CMP-sialic acid synthetase greatly facilitates synthetic procedure of various CMP-Neu5Ac analogues and the tolerance observed in the reactions with 5-azidoacetamide and 9-azido sialic acid derivatives[17] should encourage us to expand this method to the synthesis of much more complicated sialic acid-related compounds. **3** was synthesized through a key intermediate, 6-azide-1,2,3,4-tetra-*O*-benzoyl-6-deoxy-β-D-galactopyranose derived from D-galactose as a starting material, according to the procedure described in our previous report.[19] Azidosugar nucleotides were employed for 1,3-dipolar cycloaddition reaction with commercially available 36 kinds of alkynes containing typical aromatic, aliphatic, hydrophilic, carboxyl, amino groups, or steroid scaffolds under a mild aqueous solution according to the condition reported previously.[11] As seen from the plausible yields of the click reactions determined by ESI-MS (Supporting Information), it seems that yields in the reaction

Scheme 1. Synthetic Route of Azidosugar Nucleotide **1** from Sialic Acid[a]



[a] Reagents and conditions: (a) (i) TFA, MeOH, rt. 12 h, (ii) TsCl, pyridine, rt, 12 h, 56%; (b) NaN_3, acetone/H_2O, reflux, 10 h, 75%; (c) CMP−sialic acid synthetase, CTP, pH 9.0, 5 h, 70%.

Scheme 2. Synthetic Route of **2** from Sialic Acid[a]



[a] Reagents and conditions: (a) (i) Dowex 50W X8 (H^+), MeOH, rt, 1 d, (ii) Ac_2O, pyridine, rt, 3 d, 90%; (b) PhSH, BF_3, Et_2O, CH_2Cl_2, rt, 2 d, 89%; (c) CH_3SO_3H, MeOH, 60 °C, 2 d, 51%; (d) (i) $N_3CH_2CO_2Na$, DPPA, Et_3N, DMF, rt, 12 h, (ii) NaOMe, MeOH, rt, 1.5 h, 69%; (e) NBS, acetone/H_2O, 0 °C to rt, 1 h, 75%; (f) 1 N NaOH aq, 1 h, qy; (g) (i) CMP−sialic acid synthetase, CTP, pH 9.0, 37 °C, 2 h, (ii) calf intestine alkaline phosphatase (Takara Bio), pH 9.0, 37 °C, 2 h, 50%.

between **1**−**3** and alkynes depend significantly on the reactivity of an azide functional group substituted at C5, C6, or C9 positions of sugar residues, and steric hindrance effect during the 1,3-dipolar cycloaddition reaction with various alkynes. Therefore, we tested the effects of click reagents such as $CuSO_4$, TBTA, sodium ascorbate, and DMSO in order to set an appropriate criteria in the high throughput screening of the inhibitory effects by these click products.

C. Double Probing of Glycosyl Acceptor Substrates. Carbohydrates generally need to be modified chemically by some sensitive labeling groups such as photosensitive/fluorescent groups or radioactive isotopes when they are used as substrates for monitoring the reactions catalyzed by GTs and GHs. Mass spectrometry is an attractive analytical method because changes of the mass (molecular weight) from substrate to product can be directly detected during enzymatic reactions without special probing. Because of the poor potentials of the ionization and ion suppression effect, however, it is usually difficult to detect ions elaborated from free (unmodified) carbohydrates in the presence of many other biological substances such as peptides, nucleotides, lipids, and other metabolites. In the course of our studies on the comprehensive glycomics based on glycoblotting technology,[13,14b,20a,b] it was demonstrated that carbohydrates can be predominantly detected by tagging the reducing end with some ion-sensitivity enhancement reagents such as aminooxy-tryptophanylarginine (ao-WR)[14a,b] or some similar peptides having hydrophobic and basic amino acid residues. As illustrated in Figure 1, we considered that novel glycosyl acceptors having such improved ion-sensitivity would greatly facilitate MS-based protocol for monitoring glycosylation by GTs, trimming by GHs, and inhibitory effects by large numbers of synthetic and naturally occurring compounds. Figure 3 shows the chemical structures of glycosyl acceptor substrates used in this study. In the present study, **9** and **10** were prepared from chitotriose as a starting material by conjugating with aminooxy-WR-OCH_3/OCD_3, **13** or **14**,[14a,b] because the terminal *N*-acetyllactosamine unit generated by galactosylation with βGal-T1 should become a highly convenient acceptor substrate for a lot of major GTs such as α2,3/2,6-sialyltransferases, β1,3-*N*-acetylglucosaminyltransferase, and α1,3-fucosyltransferases known as crucial human glycosyltransferases. In addition, we employed a commercially available glycoamino acid carrying a typical biantennary *N*-glycan core[21] as a starting material that can be converted into **11** and **12**, doubly probed functional glycopeptides, by solid phase glycopeptide synthesis and selective modification at *C*-terminal lysine residue with stable isotope moiety (Supporting Information).

D. MS-Based High Throughput Screening of Glycosyltransferases Inhibitors. Focused compound library derived from **1**−**3** and **A1**−**A36** were subjected to high throughput screening of inhibitory effects on sugar transferring activities by using four glycosyltransferases in the presence of **9**−**12**, designated acceptor substrates, according to the general protocol illustrated in Figure 1. Advantage of MS-based protocol used in this study is that crude click products can be employed directly for the first screening to survey potent candidates exhibiting significant inhibition when other components such as alkynes, $CuSO_4$, tris-(benzyltriazolylmethyl)amine (TBTA), and sodium ascorbate have no or little influence to this assay system (Supporting Information).[11] As anticipated, **9**−**12** exhibiting enhanced ion sensitivity allowed for rapid and large-scale screening to identify some potential compounds from crude click products without any purification, notably focused compound library derived from 36 alkynes and three azidosugar nucleotides (Figure 2). It was demonstrated that analysis of inhibitory effect by using large number of compounds can be performed quantitatively by focusing the ion intensities of a pair of expected glycosylated products at *m*/*z* 1511.3 and *m*/*z* 1514.3 for α2,3/α2,6-STs, at *m*/*z* 1366.4 and *m*/*z* 1369.4 for α1,3-FucT, and at *m*/*z* 2386.9 and *m*/*z* 2389.2 for α1,6-FucT (Figure 4).

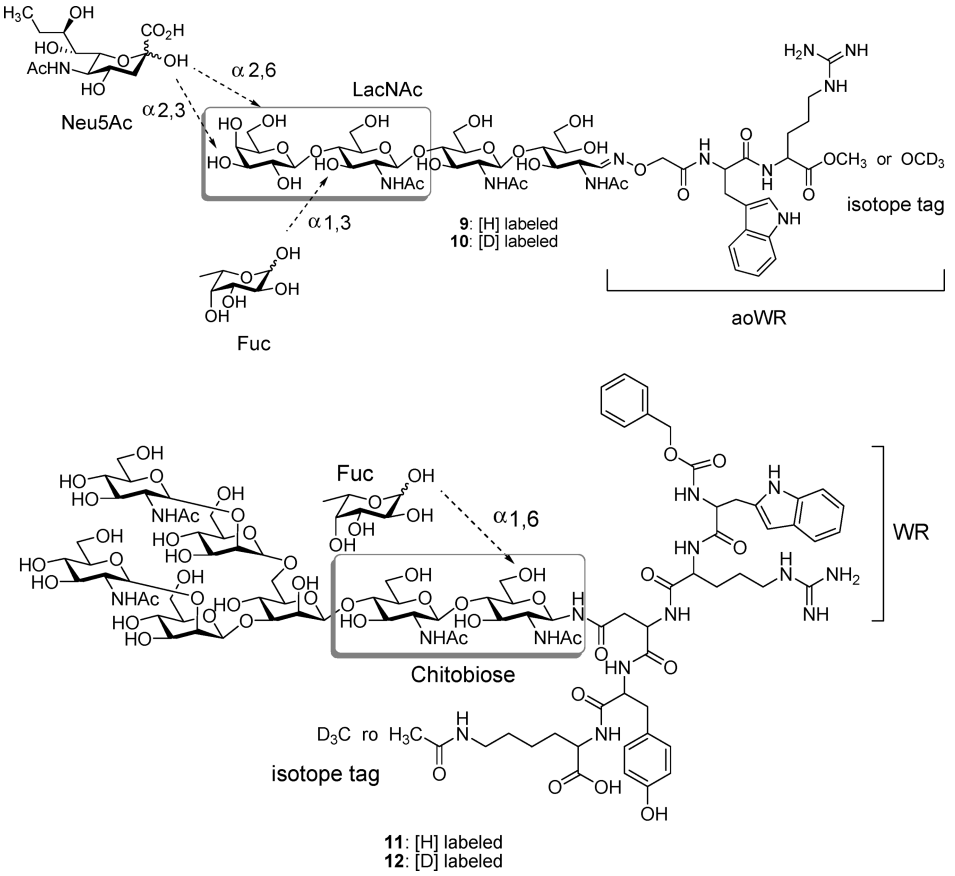


Figure 3. Glycosyl acceptor substrate sets designed for the characterization of recombinant rat α2,3-ST/ST3Gal III and α2,6-ST/ST6Gal I and recombinant human α1,3-FucT/FUT V and α1,6-FucT/FUT VIII. WR represents dipeptide tryptophanyl-arginine moiety as an ion-sensitivity enhancement reagent.

As summarized in Figure 5, the results of MALDI-TOFMS-based high throughput screening suggest that 17 compounds [3 compounds for α2,3-ST (Figure 5A,B), four compounds for α1,3-FucT (Figure 5E), and 10 compounds for α1,6-FucT (Figure 5F)] among 108 click entries showed potent inhibitory effects judged by a tentative criteria (relative inhibition rate $>50\%$). Most derivatives from **1** did not exhibit significant inhibition against α2,3/α2,6-STs, and they seemed to become donor substrates of both enzymes because signals corresponding to the expected products were detected during the inhibition assay even in the presence of native CMP-Neu5Ac. In addition, we could not detect any significant inhibitory effect on recombinant α2,6-ST by derivatives from **2**, indicating that modifications both at C-5 and C-9 positions of sialic acid do not influence dynamic mechanism in the glycosylation catalyzed by this enzyme. On the contrary, it was also revealed that modification at C-5 position of CMP-Neu5Ac has strong impact to the catalytic action by α2,3-ST when bulky steroid analogues such as **A17** and **A19** were subjected to the click reaction with **2** (Figure 5B). However, the click product between compound **2** and a similar steroidal alkyne **A18** had little inhibitory effect, indicating that the terminal aromatic moiety of **A17** and **A19** appears to be crucial in addition to the steroidal skeleton for this activity rather than α,β-unsaturated ketone structure of **A18**. These results suggest that 3D structure of the donor binding site and dynamic mechanism including function of a flexible loop of mammalian α2,6-STs might be quite different from those of α2,3-STs, although both α2,3/α2,6-STs share a natural CMP-Neu5Ac as a redundant donor substrate. As shown in Figure 5E, it was revealed that activity of α1,3-FucT is inhibited significantly by four click products derived from **3** with **A6**, **A17**, **A33**, or **A35** having some bulky alcohols. No steroidal compound showed significant inhibition against α1,6-FucT, while simple aromatic and aliphatic 10 alkynes, **A1**, **A2**, **A3**, **A4**, **A6**, **A8**, **A9**, **A21**, **A22**, and **A28** provided **3** with potent inhibitory effects (Figure 5F). Interestingly, any compounds derived from **3** did not become substrate for both α1,3- and α1,6-FucTs in contrast to the results observed in cases for α2,3-ST/α2,6-ST and compounds derived from **1** or **2**.

To evaluate potent inhibitory effects more precisely, we concluded that c **15** (derived from **2** and **A17**), **16** (derived from **3** and **A17**), and **17** (derived from **3** and **A2**) among 17 hit derivatives should be synthesized by simply scale-up in the click reactions and employed for further in vitro characterization (Figure 6). It was demonstrated that **15** is highly promising inhibitor against α2,3-ST (ST3Gal III) ($IC_{50} = 8.2\ \mu M$) as shown in Figure 7A. Surprisingly, this inhibitor was proved to be a good donor substrate for α2,6-ST (ST6Gal I) ($K_m = 125\ \mu M$) from the LB plot indicated in Figure 7B. These results clearly show that **15** is the first highly selective inhibitor for mammalian α2,3-ST. It should also be noted that the binding mode of α2,6-ST with CMP-Neu5Ac appears to be different from that of α2,3-ST, in which chemical substitution at C-5 and C-9 positions of CMP-Neu5Ac did not interfere sugar transfer reaction toward bulky glycosyl acceptor substrates. This may suggest that major parts of sialic acid moiety are exposed to outer side from the CMP-Neu5Ac bound in the catalytic pocket of α2,6-ST, although cytidine and negative charges locating around the anomeric carbon seem to be crucial for the interaction with α2,6-ST.[22] In contrast, modification at C-5 position of Neu5Ac

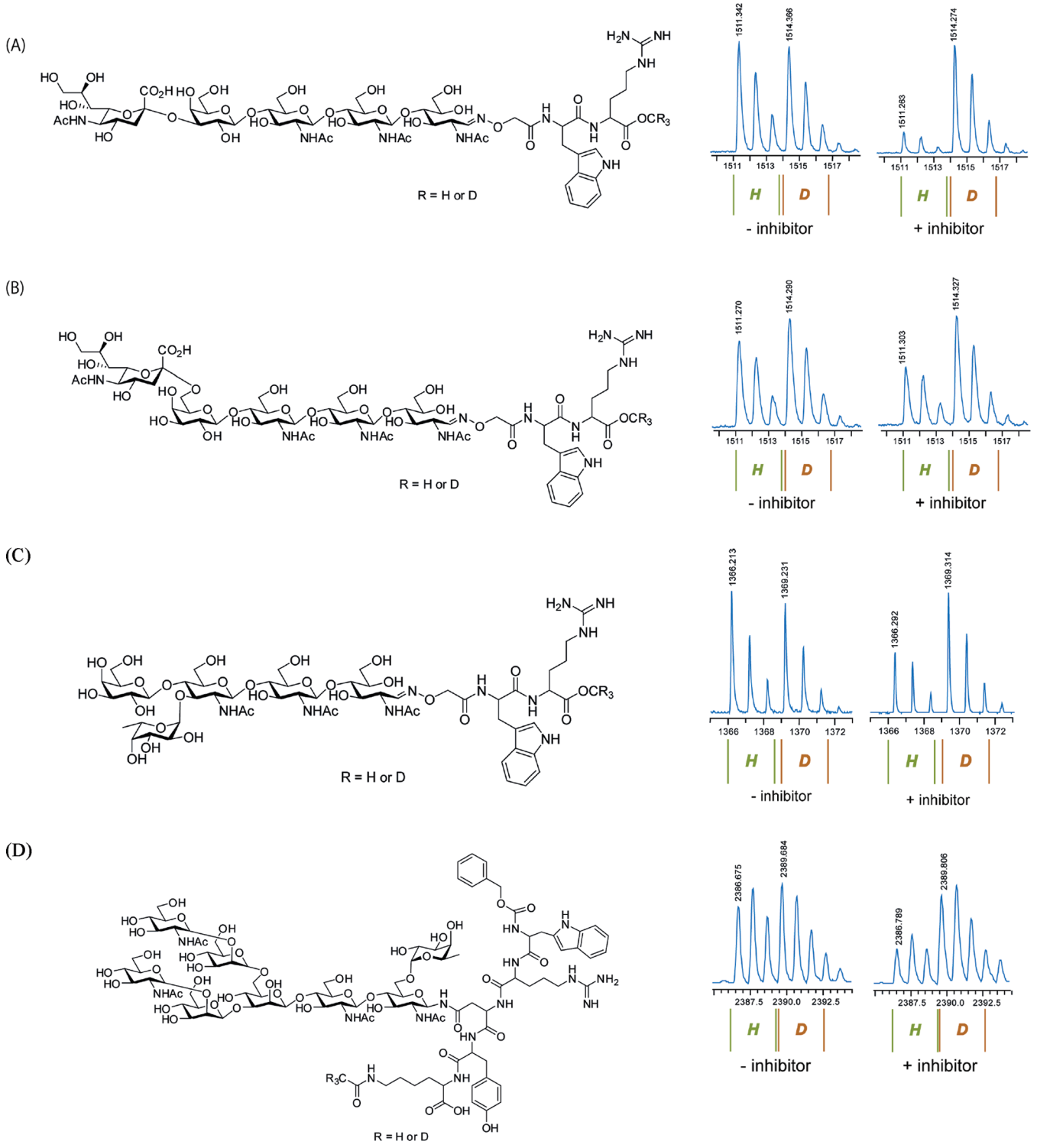


Figure 4. MALDI-TOFMS-based rapid and highly efficient quantification of inhibitory effects by focused compound library. Ion intensities of a pair of expected glycosylated products should be determined at *m*/*z* 1511.3 and *m*/*z* 1514.3 for α2,3/α2,6-STs (A) and (B), at *m*/*z* 1366.4 and *m*/*z* 1369.4 for α1,3-FucT (C), and at *m*/*z* 2386.9 and *m*/*z* 2389.2 for α1,6-FucT (D), respectively.

greatly affected the activity of α2,3-ST, indicating that this class of enzymes recognize sialic acid residue more precisely than α2,6-STs through the interaction with C-5 acetamide group as well as above essential motifs of CMP-Neu5Ac. Considering that many inhibitors inspired from transition-state analogues during glycosylation catalyzed by α2,3-/α2,6-STs showed much higher inhibitory effects on α2,6-STs rather than α2,3-STs,[22] we may conclude that there is no discrepancy between the present results and these previous pioneering reports. It was also documented that most bisubstrate analogous inhibitors having designated pseudo α2,6- or α2,3-glycoside linkage exhibited potent but a similar level inhibition toward both enzymes.[6,22] In addition, it was also reported that CMP shows almost similar level of inhibitory effects on rat α2,3-ST (K_i = 90 μM)[23a,b] and α2,6-ST (65 μM),[23b] respectively. Therefore, our present data clearly suggest that modification at C-5 position of Neu5Ac

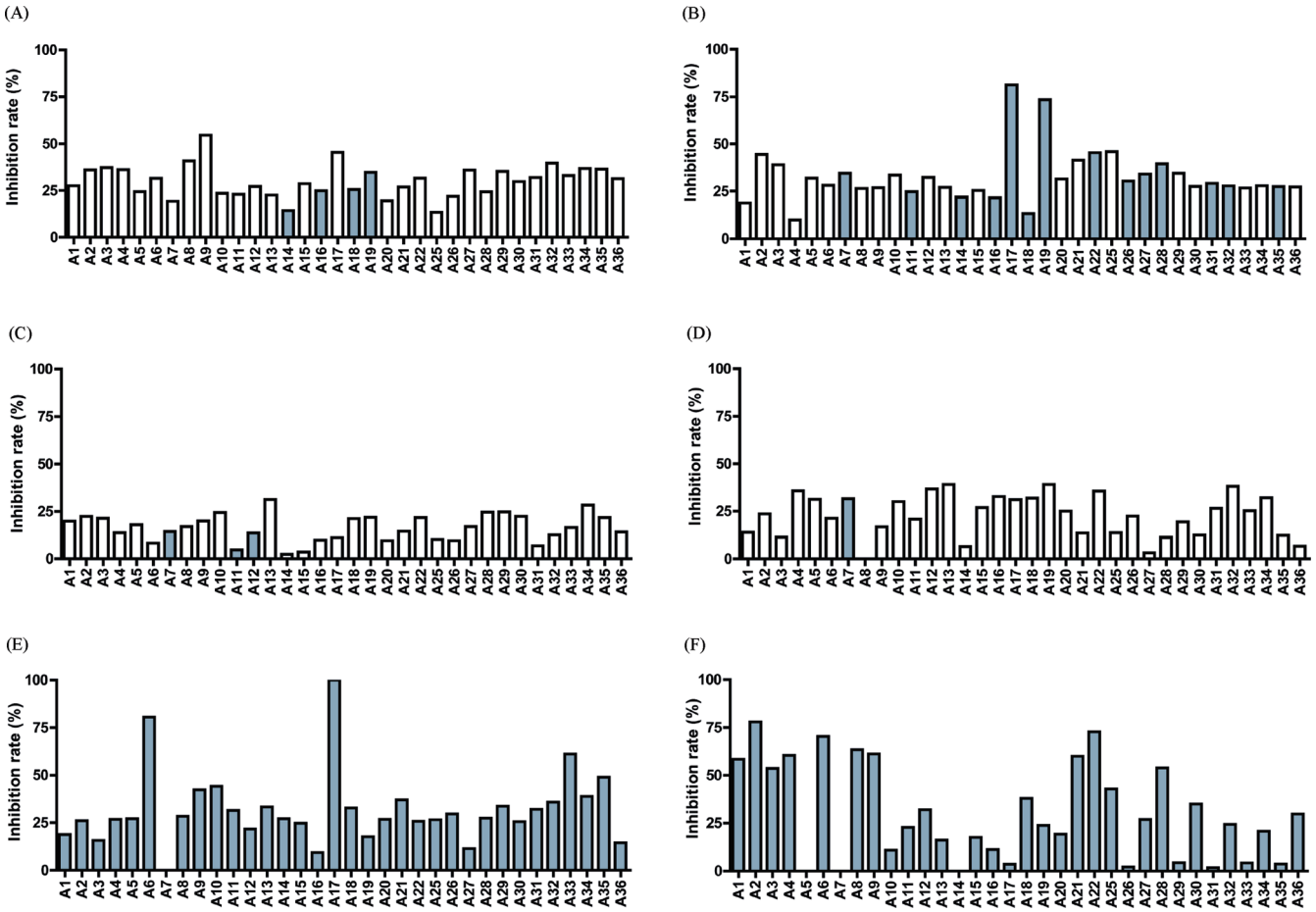


Figure 5. High throughput screening of inhibition by 108 click trials products from **1**−**3** and 36 kinds of commercially available alkynes. (A) and (B) show the effects of compounds derived from **1** and **2** on α2,3-ST, (C) and (D) exhibit the effects of compounds derived from **1** and **2** on α2,6-STs, and (E) and (F) represent the effects of compounds derived from **3** on α1,3-Fuc and α1,6-Fuc, respectively. In (A−F), the results represented by gray-colored panel mean the compounds were not consumed as donor substrate, while others (white panel) became substrate during inhibition assay.

without loss of an original amide linkage should allow for ideal molecular design and further optimization study of highly selective inhibitors against various α2,3-STs.

Inhibitory effects by **16** and **17** were also estimated as K_i = 293 nM (vs α1,3-FucT) and K_i = 13.8 μM (vs α1,6-FucT), respectively (Supporting Information). As anticipated from the scores in the high throughput screening by means of click derivatives from **3** (Figure 5E,F), both compounds were proved to be highly potential and selective inhibitors for two important classes of human FucTs. Difference in the structural features between **16** and **17** derived from the same azidosugar nucleotide suggests that the strong inhibition by these compounds against α1,3-FucT and α1,6-FucT must be conducted in a different manner, although the formation of triazene ring at C-6 position of L-fucose residue seems to be required to acquire the minimal inhibitory activity.[6f] It is also clear from the results shown in parts E and F of Figure 5 that all click products derived from **3** were not consumed as donor substrate by both α1,3/α1,6-FucTs. Given the fact that a similar derivative from **3**, 6-deoxy-6-*N*-(2-naphthalene-2-yl-acetamide)-β-L-galactopyranos-1-yl-guanosine 5′-diphosphate disodium salt,[19] becomes an excellent substrate for α1,3/α1,6-FucTs, difference in the anchor moiety between GDP-Fuc and hydrophobic elements, namely amide and triazene ring, markedly influence the dynamic interaction with these enzymes. Because GDP was proven to inhibit both human α1,3-FucT (K_i = 16 μM and IC_{50} = 50 μM)[24a,b] and

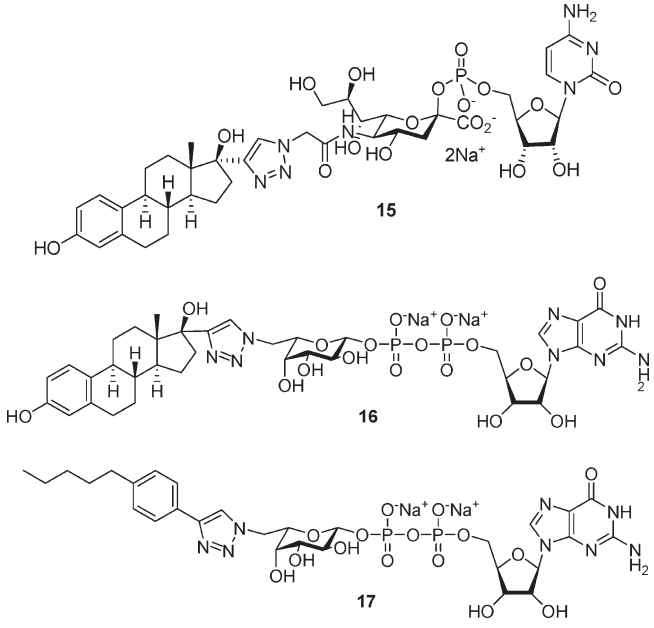


Figure 6. Selected compounds **15**−**17** showing the highest inhibitory effects against α2,3-ST, α1,3-FucT, and α1,6-FucT among 108 click trials using azidosugar nucleotides **1**−**3** and 36 kinds of alkynes. Synthetic procedures and characterizations of these compounds (see, Experimental Section and Supporting Information).

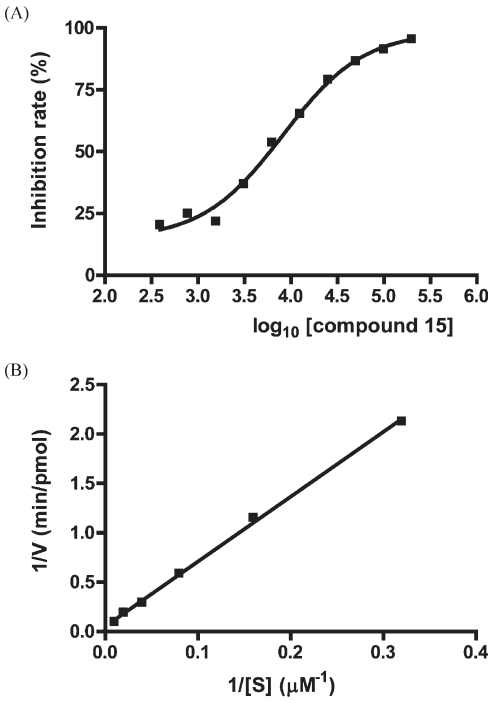


Figure 7. **15** is a heterobifunctional molecule. (A) Relationship between concentration of **15** (0.39−200 μM) and relative inhibitory effect to estimate IC_{50} against α2,3-ST (0.5 mU) in the presence of CMP-Neu5Ac (200 μM), and **9** (1 mM). (B) LB plot of the reaction catalyzed by α2,6-ST (0.5 mU) in the presence of **15** (12.5−200 μM) and **9** (1 mM).

human α1,6-FucT (K_i = 36 μM)[24c] in a similar manner, it is clear that enzyme-selective inhibitors become nice tools for investigating functions and mechanism of individual glycosyltransferases. However, it is thought that considerable efforts should be paid toward practically potent drug candidates through further molecular design and optimization for controlling properties such as stability, organ/tissue targeting, and cellular permeability.

Conclusion

An efficient and versatile approach for searching potent inhibitors against glycosyltransferases was established on the basis of high throughput mass spectrometry. Double probing of common glycosyl acceptor substrates with an ion-sensitivity enhancement reagent and stable isotope tag allowed for highly sensitive and quantitative screening of large number of compounds prepared by click reactions.[11] Advantage of the present MS-based protocol is evident because direct monitoring molecular mass of interest would greatly facilitate data annotation/analysis when the method makes highly sensitive and quantitative analysis of the target ion possible. Actually, we have demonstrated that MALDI-TOFMS-based screening is feasible for the assay using heterogeneous cell lysate in cases for profiling whole kinases activity of human cancer K562 cells.[12] Therefore, it is our belief that the present strategy would greatly facilitate lead discovery research toward new generation drug candidates controlling posttranslational modifications such as protein phosphorylation, glycosylation, and other important modification processes by means of cell-based experiments as well as in vitro common assay systems.

Experimental Section

General Methods and Materials. Recombinant rat α2,3-(*N*)-sialyltransferase (α2,3-ST, one unit of this enzyme is defined as the amount of enzyme that transfer 1.0 μmol of sialic acid from CMP-Neu5Ac to *N*-acetyllactosamine per min at 37 °C, pH 7.4), α 2,6-(*N*)-sialyltransferase (α2,6-ST, one unit of this enzyme is defined as the amount of enzyme that transfer 1.0 μmol of sialic acid from CMP-Neu5Ac to *N*-acetyllactosamine per min at 37 °C, pH 6.0), and recombinant human α1,3-fucosyltransferase V (α1,3-FucT/FUT V, one unit of this enzyme is defined as the amount of enzyme that will transfer 1.0 μmol of fucose from GDP-Fuc to *N*-acetyllactosamine per min at 37 °C, pH 7.5) were purchased from Calbiochem (San Diego, CA). Recombinant human α1,6-fucosyltransferase VIII (α1,6-FucT/FUT VIII, one unit of this enzyme is defined as the amount of enzyme that will transfer 1.0 μmol of fucose from GDP-Fuc to asparagine-linked *N*-acetyl-β-D-glucosamine of the chitobiose moiety per min at 37 °C, pH 7.5) and recombinant human β1,4-galactosyltransferase (β1,4-GalT/βGal-T I, one unit of this enzyme is defined as the amount of enzyme that will transfer 1.0 μmol of galactose from UDP-gal to D-glucose per min at 30 °C, pH 8.4 in the presence of α-lactoalbumin) were purchased from Toyobo Co., Ltd. Recombinant influenza CMP sialic acid synthetase (CSS, one unit of this enzyme is defined as the amount of enzyme that transfer 1.0 μmol of CMP from CTP to sialic acid per min at 37 °C, pH 8.0) was provided from Yamasa Co (Japan). Recombinant calf intestine alkaline phosphatase (one unit of this enzyme is defined as the amount of enzyme that decomposes 1.0 μmol of *p*-nitrophenyl phosphate per min at 37 °C, pH 9.8) was purchased from Takara Bio.

6-Azide-6-deoxy-β-L-galactopyranos-1-yl-guanosine 5′-diphosphate disodium salt (**3**) was synthesized from D-galactose as a starting material according to the procedure reported previously by our group.[19] Acetylene compounds (alkynes represented as **A1**−**A36**) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Solvents and other reagents for chemical synthesis were purchased from Aldrich Chemical Co. (Milwaukee, WI), Calbiochem (San Diago, CA), Seikagaku Co. Ltd. (Tokyo, Japan), Tokyo Chemical Industry (Tokyo, Japan), and Wako Pure Chemical Industries Ltd. (Tokyo, Japan) and used without further purification. Proton and carbon NMR was recorded with Bruker Lambda 600 MHz (Bruker BioSpin Corp., Germany). Chemical shifts are given in ppm and referenced to internal TMS (δ_H 0.00 in $CDCl_3$), $CHCl_3$ (δ_H 7.26 in $CDCl_3$), TSP (δ_H 0.00 in D_2O), or $CDCl_3$ (δ_C 77.00). Assignments in ^{1}H NMR were made by first-order analysis of the spectra and were verified by H−H COSY and HMQC experiments. Elemental analyses were performed with MT-6 CHN CORDER (Yanako, Japan). TLC was performed on Merck precoated plates (20 cm × 20 cm; layer thickness, 0.25 mm; Silica Gel $60F_{254}$); spots were visualized by spraying a solution of 90:5:5 (v/v/v) MeOH-*p*-anisaldehyde-concentrated sulfuric acid and heating at 180 °C for ca. $^1/_2$ min, a solution of 95: 5 (v/v) MeOH-concentrated sulfuric acid and heating at 180 °C for ca. $^1/_2$ min, and by UV light (256 or 365 nm) when applicable. All new compounds were examined by HRMS and/or HPLC, and the purity was proven to be more than 95%. Column chromatography was performed on Silica Gel 60 (spherical type, particle size 40−50 μm; Wako Pure Chemical Co. Ltd.), Iatrobeads (6RS-8060, Jatron Laboratories, Inc.), Sephadex G-15, Sephadex G-10 (GE Healthcare Bio-Sciences Corp., NJ), or Wako-gel C-18 (Wako Pure Chemical Co. Ltd.) with the solvent systems specified, and the ratio of solvent systems was gived in v/v. High performance liquid chromatography (HPLC) was conducted by HITACHI L-7100 HPLC system equipped with a Inertsil-ODS column (4.6 mm ×250 mm, GL Sicence Inc.) and HITACHI L-7420 UV−vis detector.

Samples for MALDI-TOFMS were desalted and concentrated using 10-μL C_{18} ZipTips (Millipore) according to the manufacturer's instructions. Typically, samples were dissolved in 1 mL of 90% (v/v) acetonitrile containing trifluoroacetic acid and mixed with the same volume of a saturated solution of 2,5-dihydroxubenzoic acid (DHB) in 33% acetonitrile containing 0.1% trifluoroacetic acid. The above mixtures (1 μL) were applied to a polished stainless steel target MALDI plate and air-dried before analysis in the mass spectrometer. All measurements were performed using an

Ultraflex TOF/TOF mass spectrometer equipped with a reflector and controlled by the Flexcontrol 1.2 software package (Bruker Daltonics GmbsH, Bremen, Germany). Ions generated by a pulsed UV laser beam (nitrogen laser, λ = 337 nm) were accelerated to a kinetic energy of 23.5 kV. External calibration of MALDI-mass spectra was carried out using singly charged monoisotopic peaks of a mixture of human angiotensin II (m/z 1046.542), bombesin (m/z 1619.823), ACTH (m/z 2465.199), and somatostain 28 (m/z 3147.472). The mixture of these peptides was measured on the central spot of a 3 × 3 square with external calibration. To achieve mass accuracy better than 60 ppm, internal calibration was carried out by doping the matrix solution with a mixture of the calibration peptides. Calibration of these mass spectra was performed automatically utilizing a customized macro command of the XMASS 5.1.2 NT software package. The macro command was used for the calibration of the monoisotopic singly charged peaks of the abovementioned peptides.

Synthesis. Cytidine-5′-monophospho-5-acetamido-9-azido-3,5,9-trideoxy-β-D-*glycero*-α-D-*galacto*-non-2-ulosonic acid (1). To the solution of **4**[16] (50 mg, 150 μmol) in buffer (6.0 mL, 100 mM Tris-HCl, pH 8.8, 20 mM $MgCl_2$) was added CTP ($3Na^+$) (79 mg, 165 μmol) and CMP-sialic acid synthetase (70 μL of 7 U/100 μL, *Campylobacter influenzae*, Yamasa Co.) and incubated at 37 °C for 5 h. During the reaction, 1 N NaOH was added to keep pH of the reaction mixture. The reaction mixture was filtered and concentrated, and the residue was purified by reverse phase column chromatography (WakoGel C-18) and ion exchange chromatography (Diethylaminoethyl-sepharose (DEAE): 0.05−0.1 M $NH_4^+HCO_3^-$) to give **1** in 53% yield (52 mg, 80 μmol). NMR data was identical with that of previous paper.[17] ^{1}H NMR (600 MHz, D_2O, 27 °C): δ 1.66 (dt, 1H, $J_{3ax,4}$ 13.2 Hz, $J_{3ax,3eq}$ 13.2 Hz, H-3ax), 2.07 (s, 3H, Ac), 2.50 (dd, 1H, $J_{3eq,4}$ 4.7 Hz, $J_{3ax,3eq}$ 13.2 Hz, H-3 equiv), 3.48 (d, 1H, $J_{7,8}$ 9.6 Hz, H-7), 3.50−3.53 (m, 1H, H-9), 3.64−3.66 (m, 1H, H-9′), 4.09 (t, 1H, $J_{5,6}$ 10.4 Hz, $J_{4,5}$ 10.4 Hz, H-5), 4.19−4.23 (m, 2H, H-4, H-8), 4.28 (d, 1H, $J_{5,6}$ 10.5 Hz, H-6), 4.25−4.35 (m, 5H, H-2 of ribose, H-3 of ribose, H-4 of ribose, H-5 of ribose, H-5′ of ribose), 5.99 (d, 1H, $J_{1,2}$ 4.2 Hz, H-1 of ribose), 6.21 (d, 1H, $J_{5,6}$ 7.7 Hz, H-5 of cytosine), 8.07 (d, 1H, $J_{5,6}$ 7.7 Hz, H-6 of cytosine).

5-Azidoacetamido-3,5-dideoxy-D-*glycero*-β-D-*galacto*-non-2-thiophenyl-1-methyl Ester (6). To a solution of methyl 2-azidoacetate (470 mg, 4.08 mmol) in 95% EtOH (1 mL) was added 1 N NaOH aq (4.08 mL, 4.08 mmol) and stirred at room temperature for 3 h. Then the solution was added to **5**[25] (1.59 g, 3.89 mmol) in 95% EtOH (10 mL). After stirring at rt for 7 h, the solution was concentrated and coevaporated with DMF three times. The residue was dissolved in DMF (15 mL), and the solution was added to Et_3N (1.14 mL, 8.18 mmol) and diphenylphosphorylazide (880 μL, 4.08 mmol). After stirring at room temperature for 12 h, the reaction mixture was added to MeOH (58 mL) and NaOMe (28% MeOH solution, 725 μL, 3.76 mmol). After 1.5 h, the reaction mixture was neutralized with Dowex 50WX8 (H^+) and filtered and concentrated in vacuo. The residue was purified on a column of silica gel ($CHCl_3$/MeOH = 20/1) to give compound **6** in 69% yield (1.22 g, 2.67 mmol) as a syrup. R_f = 0.6 ($CHCl_3$/MeOH = 3/1). ^{1}H NMR δ (600 MHz, CD_3OD) 2.00 (t, 1H, $J_{3ax,4}$ 12.0 Hz, H-3ax), 2.70 (dd, 1H, $J_{3ax,3eq}$ 13.6 Hz, $J_{3eq,4}$ 4.68 Hz H-3 equiv), 3.57 (d, 1H, $J_{7,8}$ 9.12 Hz, H-7), 3.68 (q, 1H, $J_{8,9}$ 5.4 Hz, $J_{9,9'}$ 11.3 Hz, H-9), 3.77−3.80 (m, 1H, H-8), 3.83 (dd, 1H, $J_{8,9}$ 2.82 Hz, $J_{9,9'}$ 11.3 Hz, H-9′), 3.93−4.01 (m, 3H, H-5, N_3-CH_2-CO), 4.17 (td, 1H, $J_{3eq,4}$ 4.62 Hz, $J_{4,5}$ 10.7 Hz, $J_{3ax,4}$ 10.8 Hz, H-4), 4.60 (d, 1H, $J_{5,6}$ 10.6 Hz, H-6), 7.33−7.39 (m, 3H, aromatic), 7.59 (d, 2H, aromatic). ^{13}C NMR δ (CD_3OD) 42.27 (C-3), 53.10−53.14 (methyl ester and methylene of azideacetamide), 54.24 (C-5), 65.21 (C-9), 68.09 (C-4), 70.72 (C-7), 71.46 (C-8), 73.27 (C-6), 91.59 (C-2), 130.02−137.41 (thiophenyl), 170.82 (C-1), 171.43 (C-10). HRMS (FAB) Anal. calcd for $C_{18}H_{25}N_4O_8S$ $[M + H]^+$ 457.1393; found 457.1386.

5-Azidoacetamido-3,5-dideoxy-D-*glycero*-β-D-*galacto*-non-2-ulosonic-1-methyl Ester (7). To a solution of **6** (50 mg, 0.11 mmol) in acetone (9.1 mL) and water (0.9 mL) was added NBS (78 mg, 0.44 mmol) at 0 °C. After stirred at 0 °C for 1 h, the reaction mixture was filtered on a silica gel plaque ($CHCl_3$/MeOH = 3/1) and concentrated. The residue was purified on a column of silica gel ($CHCl_3$/MeOH = 10/1 to 5/1) to yield compound **7** (30 mg, 75%). NMR data was identical with published data.[18] ^{1}H NMR (600 MHz, CD_3OD, 27 °C): δ 1.88 (t, 1H, $J_{3ax,4}$ 13.2 Hz, $J_{3ax,3eq}$ 13.2 Hz, H-3ax), 2.22 (dd, 1H, $J_{3ax,3eq}$ 12.9 Hz, $J_{3eq,4}$ 4.8 Hz H-3 equiv), 3.49 (d, 1H, $J_{7,8}$ 9.0 Hz, H-7), 3.62 (q, 1H, $J_{8,9}$ 5.4 Hz, $J_{9,9'}$ 13.0 Hz, H-9), 3.68−3.71 (m, 1H, H-8), 3.78 (s, 3H, Me), 3.79−3.81 (m, 1H, H-9′), 3.86−3.97 (m, 3H, H-5, N_3-CH_2-CO), 4.05−4.10 (m, 2H, H-4, H-6).

5-Azidoacetamido-3,5-dideoxy-D-*glycero*-α,β-D-*galacto*-non-2-ulosonic Acid (8).[18] To a solution of **7** (52 mg, 114 μmol) in H_2O (1.0 mL) was added 1 N NaOH aq (125 μL, 125 μmol). After stirred at rt for 1.5 h, the solution was neutralized with Dowex 50WX8 (H^+). After filtration, the solution was concentrated in vacuo to yield compound **8** (50 mg, qy). NMR data was identical with that of previously published.[18] ^{1}H NMR (600 MHz, D_2O, 27 °C): δ 1.91 (t, 1H, $J_{3ax,4}$ 12.0 Hz, $J_{3ax,3eq}$ 12.0 Hz, H-3ax), 2.31 (dd, 1H, $J_{3ax,3eq}$ 12.9 Hz, $J_{3eq,4}$ 5.4 Hz H-3 equiv), 3.58 (d, 1H, $J_{7,8}$ 9.0 Hz, H-7), 3.67 (q, 1H, $J_{8,9}$ 6.6 Hz, $J_{9,9'}$ 12.0 Hz, H-9), 3.80−3.82 (m, 1H, H-8), 3.90 (dd, 1H, $J_{8,9}$ 2.4 Hz, $J_{9,9'}$ 11.7 Hz, H-9′), 4.05 (t, H-5, $J_{5,6}$ 10.2 Hz, $J_{4,5}$ 10.2 Hz), 4.13−4.15 (m, 4H, N_3-CH_2-CO, H-4, H-6).

Cytidine-5′-monophospho-5-azidoacetamide-3,5-dideoxy-β-D-*glycero*-α-D-*galacto*-non-2-ulosonic Acid (2). To the solution of **8** (10 mg, 29 μmol) in buffer (2 mL, 100 mM Tris-HCl, pH 9.0, 20 mM $MgCl_2$) was added CTP ($3Na^+$) (13.6 mg, 26 μmol) and CMP-sialic acid synthetase (14.3 μL of 7 U/100 μL) and incubated at 37 °C for 2 h. The reaction mixture was added buffer (3 mL, 900 mM Tris-HCl, pH 9.0) and alkaline phosphatase (50 μL of 1 U/μL, calf intestine, Takara Bio). After incubation at 37 °C for 2 h, the reaction mixture was filtered through a cotton plaque. The solution was applied to a column of Sephadex G15 and eluted with H_2O. Fractions containing the product were lyophilized. The residue was dissolved in water and purified by ion exchange chromatography (DEAE:H_2O to 0.1 M $NH_4^+HCO_3^-$) to give compound **2** in 51% yield (9.9 mg, 15 μmol). NMR data was in good agreement with that previously reported.[18] ^{1}H NMR (600 MHz, D_2O, 27 °C): δ 1.72 (dt, 1H, $J_{3ax,4}$ 12.3 Hz, $J_{3ax,3eq}$ 12.3 Hz H-3ax), 2.56 (dd, 1H, $J_{3eq,4}$ 4.2, $J_{3ax,3eq}$ 13.2 Hz, H-3 equiv), 3.50 (d, 1H, $J_{7,8}$ 9.6 Hz, H-7), 3.68 (q, 1H, $J_{8,9b}$ 6.6, $J_{9,9'}$ 12.0 Hz, H-9), 3.94 (d, 1H, $J_{9,9'}$ 11.1 Hz, H-9′), 4.00 (m, 1H, H-8), 4.08(t, 1H, $J_{5,6}$ 10.8 Hz, $J_{4,5}$ 10.8 Hz, H-5), 4.15−4.21 (m, 3H, H-4, N_3-CH_2-CO), 4.27−4.33 (m, 4H, H-6, H-4 of ribose, H-5 of ribose, H-5′ of ribose), 4.36 (t, 1H, $J_{1,2}$ 4.2 Hz, $J_{2,3}$ 4.2 Hz H-2 of ribose), 4.40 (t, 1H, $J_{2,3}$ 4.2 Hz, $J_{3,4}$ 4.2 Hz, H-3 of ribose), 6.05 (d, 1H, $J_{1,2}$ 4.8 Hz, H-1 of ribose), 6.19 (d, 1H, $J_{5,6}$ 7.2 Hz, H-5 of cytosine), 8.04 (d, 1H, $J_{5,6}$ 7.2 Hz, H-6 of cytosine).

Cytidine-5′-monophospho-5-(2-(4-((8*R*,9*S*,13*S*,14*S*,17*S*)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6*H*-cyclopenta[*a*]phenanthren-17-yl)-1*H*-1,2,3- triazol-1-yl)acetamido)-3,5-dideoxy-β-D-*glycero*-α-D-*galacto*-non-2-ulosonic Acid (15). The solution containing compound **2** (2.0 mM), **A17** (2.0 mM), 1 mM $CuSO_4$, 10 mM sodium ascorbate, 1 mM TBTA, 40% *t*-BuOH, 10% DMSO, and 20 mM Tris-HCl (pH 7.6) in total volume 4.0 mL was incubated for 6 h at rt. The mixture was concentrated by speedvac (25 °C, 6 h) and the residue was dissolved in water, and the solution was filtered by membrane filter. The solution was then purified with reversed-phase HPLC to give compound **15** (2.69 mg, 39%). ^{1}H NMR δ (600 MHz, D_2O), 1.06 (s, 3H), 1.27−1.53 (m, 4H), 1.63−1.71 (m, 2H), 1.72 (td, 1H, H-3ax), 1.86 (br, 1H), 1,95−2.00 (br, 2H), 2.16−2.20 (m, 2H), 2.47−2.52 (m, 1H), 2.56 (dd, 1H, H-3 equiv), 2.80 (s, 2H), 3.70 (q, 1H, H-9b), 3.94 (d, 1H, H-9a), 4.00 (m, 1H, H-8), 4.06 (t, 1H, H-5), 4.21(td, 1H, H-4), 4.29(m, 4H, H-6, H-4 of ribose, H-5a of ribose, H-5b of ribose), 4.34 (t, 1H, H-2 of ribose), 4.39 (br, 1H, H-3 of ribose), 5.38 (s, 2H, methylene at acetamide), 6.03 (d, 1H, H-1 of ribose), 6.17 (d, 1H, H-5 of cytosine), 6.65 (s, 1H, aromatic), 6.69 (d, 1H, aromatic),

7.16 (d, 1H, aromatic), 8.00 (s, 1H, triazole), 8.02 (s, 1H, H-6 of cytosine). ^{13}C NMR δ (125 MHz, D_2O) 14.44, 23.67, 26.52, 27.57, 29.73, 33.30, 37.49, 39.84, 41.79, 43.55, 47.65, 48.72, 52.93, 63.68, 65.57, 67.42, 69.63, 70.05, 70.35, 72.25, 75.02, 83.23, 83.61, 89.75, 97.31, 125.79, 127.40, 139.49, 142.33, 154.05, 158.53, 166.92, 169.00, 174.94. HRMS (ESI) Anal. calcd for $C_{40}H_{53}N_7O_{18}P$ $[M - H]^-$ 950.31847; found 950.31749.

6-Deoxy-6-(2-(4-((8*R*,9*S*,13*S*,14*S*,17*S*)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6*H*-cyclopenta-[*a*]phenanthren-17-yl)-1*H*-1,2,3-triazol-1-yl))-*β*-L-galactopyranos-1-yl-guanosine 5′-Diphosphate Disodium Salt (16). Azidosugar nucleotide **3**[25b] (8.5 mg, 12.6 μmol) was dissolved in 2 mL of H_2O, 1 mL of DMSO, and 1 mL MeOH. The solution was added alkyne **A17** (1.1 equiv, 3.7 mg), $CuSO_4$ (0.5 equiv, 1.0 mg), sodium ascorbate (5.0 equiv, 12.5 mg), and TBTA (0.16 equiv, 1.1 mg) and incubated for 1 day at rt. The mixture was subjected to the purification by reverse phase chromatography (Wako gel C18, eluted with water) to remove $CuSO_4$ and TBTA, and the fractions containing product were concentrated. The residue was finally purified by HPLC equipped Inertsil ODS-3 column (4.6 mm × 250 mm, GL Science Inc.) and eluted by 3% acetonitrile/water containing 20 mM ammonium bicarbonate. The residue was evaporated to afford white amorphous powdery **16** (5.0 mg, 40%). ^{1}H NMR δ (D_2O) 1.49 (3H, $-CH_3$), 3.68 (2H, H-2″, H-3″), 3.88 (1H, H-4″), 4.11 (1H, H-5″), 4.17 (2H, H-5′, H-6α″), 4.31 (1H, H-5′), 4.49 (1H, H-4′), 4.67−4.76 (4H, H-2′, H-3′, H-6′, H-6β″), 4.95 (1H, H-1″), 5.87 (1H, H-1′), 6.47, 6.53, 6.90 (each 1H, aromatic), 8.07 (1H, triazole), 8.25 (1H, H-8). ^{13}C NMR δ (D_2O) 65.0 (C-6), 68.8 (C-4″), 70.1 (C-4′), 71.1 (C-3″), 72.1 (C-2″), 73.6 (C-6′), 73.9 (C-5″), 83.7 (C-5′), 87.7 (C-1′), 98.6 (C-1″), 112.6, 115.0, 126.5 (aromatic). HRMS (ESI): calcd for $C_{36}H_{46}O_{17}N_8P_2Na$ $[M - Na]^-$ 947.2359; found 947.2326.

6-Deoxy-6-(1-methyl-4-(4-pentylphenyl)-1*H*-1,2,3-triazol-1-yl)-*β*-L-galactopyranos-1-yl-guanosine 5′-Diphosphate Disodium Salt (17). Azidosugar nucleotide **3**[19] (3.3 mg, 4.9 mmol) was dissolved in 1:1 (v/v) of THF−water (1 mL). The solution was added **A2** (1.1 equiv, 1.0 mg), $CuSO_4$ (0.4 mg), TBTA (2.5 mg), and sodium ascorbate (4.9 mg) and incubated at rt for 5 h. The solution was subjected to the chromatography on Wako-gel C18 and eluted by water. The fractions were concentrated. The residue was chromatographed on Sephadex G-10 and eluted water. The fractions were collected and lyophilized to afford white amorphous powdery **17** (2.5 mg, 61%). ^{1}H NMR δ (D_2O) 0.87 (3H, $-CH_3$), 1.21−1.31 (4H, CH_2 × 2), 1.49−1.56 (2H, CH_2), 2.41−2.55 (2H, CH_2), 3.73 (2H, H-2″, H-3″), 4.03 (2H, H-4″, H-5′), 4.19 (1H, H-6α″), 4.27 (1H, H-5″), 4.32 (1H, H-6β″), 4.57 (1H, H-4′), 4.67 (1H, H-6α′), 4.93 (1H, H-1″), 5.75 (1H, H-1′), 7.21, 7.71 (each 2H, aromatic), 7.92 (1H, triazole), 8.50 (1H, H-8). ^{13}C NMR δ (D_2O) 74.8 (C-1″), 89.296 (C-1′), 124.81 (triazole). HRMS (ESI): calcd for $C_{29}H_{39}N_8Na_2O_{15}P_2$ $[M + H]^+$ 847.1806; found 847.1801.

Preparation of Glycosyl Acceptor Substrates. Compound s **9** and **10** were synthesized from chitotriose as a starting material as follows: Chitotriose (6.2 mg, 0.01 mmol) was dissolved in 1 mL of 100 mM acetate buffer (pH 4.0). The solution was divided into 10 microfuge tubes by 100 μL. Aminooxy-WR-OCH_3/OCD_3(**13**)/(**14**) (200 μL of 20 mM) and acetonitrile (700 μL) were added to each tube. The mixture was reacted at 90 °C without closing the cap to concentrate the solvent. After confirming that starting material was completely converted into the target product by monitoring MALDI-TOFMS, the residues were collected in one microfuge tube by 500 μL of water. To the solution was added 250 mM Hepes buffer (500 μL, pH 7.2, containing 20 mM $MnCl_2$), UDP-Gal (2.0 equiv, 12.2 mg), and Gal-T I (20 mU), and the mixture was incubated at 37 °C. When the enzymatic galactosylation of the chitotriose derivatives was completed, the reaction was stopped by heat shock (90 °C, 5 min). The mixture was purified by Sephadex G-10 (eluted with 0.1% aq AcOH) and concentrated. The syrup was dissolved in 1 mL of 0.1% aq AcOH to adjust the concentration to 10 mM. The standard stock solutions of pure acceptor substrates **9** and **10** were stored at −20 °C as mother solution, respectively, HRMS (ESI): **9**, calcd for $C_{50}H_{79}O_{25}N_{10}$ $[M + H]^+$ 1219.5212, found 1219.5218; **10**, calcd for $C_{50}H_{76}D_3O_{25}N_{10}$ $[M + H]^+$ 1222.5401, found 1222.5406 (see also Supporting Information for the calibration curve to indicate the linearity between mixed rate and rate of MS intensity).

Compound s **11** and **12** were customized products prepared by Sigma Genosys (Ishikari, Japan) using common solid phase peptide synthesis (SPPS) on Fmoc 4-hydroxymethylphenoxyacetyl (HMPA)-PEGA resin with Fmoc-Asn(heptasaccharide)-OH,[21] Z-Trp-OH, general Fmoc amino acids, and coupling reagents (benzotriazol-1-yl-*N*-tetramethyl-uronium tetrafluoroborate (TBTU)/1-hydroxy-1*H*-benzotriazole (HOBt) or *N*,*N*′-diisopropyl carbodiimide (DIC)/HOBt). In the final step after releasing the *N*-glycopeptides from resin by treating with TFA, normal or deuterated acetyl group was incorporated at ε-amino group of *C*-terminus lysine residue by using $(CH_3CO)_2O$ or $(CD_3CO)_2O$. HRMS (ESI): **11**, calcd for $C_{96}H_{143}O_{46}N_{15}$ $[M + 2H]^{2+}$ 1120.9656, found 1120.9633; **12**, calcd for $C_{96}H_{140}D_3O_{46}N_{15}$ $[M + 2H]^{2+}$ 1122.4750, found 1122.4732 (see also Supporting Information for demonstrating purity of these compounds and the calibration curve to indicate the linearity between mixed rate and rate of MS intensity).

Acceptors **10** and **12** (deuterium-labeled compounds) were subjected to the preparation of internal standards to be employed in the following inhibition assays by using corresponding glycosyltransferases and sugar nucleotides (see Figure 4).

Standard Conditions for Click Reactions Tested in High Throughput Screening. Condition for **1** and **2**: Each click reaction was carried out in 1: 4: 5 (v/v/v) DMSO-*t*-BuOH-water (50 μL), in the presence of alkynes (**A1**−**A36**, 2 mM), **1** or **2** (2 mM), $CuSO_4$ (1 mM), tris-(benzyltriazolylmethyl)amine (TBTA, 1 mM), sodium ascorbate (10 mM), and Tris-HCl (20 mM, pH 7.6). The mixture was kept at room temperature for 6 h and then concentrated by speedvac (25 °C, 3 h). To the residue was added 2 mM EDTA ($2Na^+$) to a total volume 100 μL. The solutions were stored at −20 °C as stock solutions. The yields of all click reactions were summarized in Supporting Information.

Condition for **3**: 4: 6 (v/v) DMSO−water (25 μL), in the presence of alkynes (**A1**−**A36**, 2 mM), **3** (2 mM), $CuSO_4$ (1 mM), tris-(benzyltriazolylmethyl)amine (TBTA, 1 mM), and sodium ascorbate (5 mM). The mixture was kept at room temperature, and the reactions were monitored by ESI-MS. After 4 h, 25 μL of water was added to adjust the concentration to 1 mM. The solutions were stored at −20 °C as stock solutions. The yields of all click reactions and the effects of free alkynes and reagents on the glycosylation by α1,3/α1,6-FucTs were summarized in the Supporting Information.

General Procedure for Monitoring Glycosyltransferases Reactions by MALDI-TOFMS. The sample was dropped on the polished steel sample plate and dried by blower. The laser was irradiated to the edge of sample crystal because the WR-labeled compounds were concentrated by edge of crystal. The quantitative analysis was carried out by means of the ratio of mass intensity due to H-labeled product and D-labeled internal standard as indicated in Figure 4. The yield of the glycosylation in the presence of each click product was calculated from the peak intensities corresponding to product and internal standard as shown in eq 1.

$$\text{yield}(\%) = P/\text{In} \times 100 \quad (1)$$

Where P is peak intensity due to the product and In represents the peak intensity due to the internal standard. The data processing and calculation of inhibition constant were performed by Microsoft Excel and Graphpad Prism.

Conditions for High Throughput Inhibition Assay of Click Products. α2,3/α2,6-STs: To the mixture containing 200 μM CMP-Neu5Ac, 200 μM click product (**A1**−**A36**, calculated from the concentration of azidosugar nucleotide and alkyne), 1 mM **9**, 12 mM sodium cacodylate buffer (pH 7.4), 0.15 mg/mL BSA,

and 0.03% Triton CF-54 was added 0.5 mU α2,3- or α2,6-ST (total volume was adjusted to 25 μL). The reaction mixture was incubated at 37 °C for 30 min. After incubation, 100 μL of CH_3CN was added to stop the reaction, and the total volume was adjusted to 250 μL by addition of 125 μL of water. To the 20 μL of DHB solution (10 mg/mL DHB in 30% CH_3CN) was added 2 μL of each reaction mixture and deuterium labeled internal standard, and then 0.5 μL of mixed solution was mounted on MALDI-TOFMS target plate.

α1,3/α1,6-FucTs: To the mixture of GDP-Fuc (200 μM), **9** (1 mM), 30 μM click product (**A1**−**A36**, calculated from the concentration of azidosugar nucleotide and alkyne) in 50 mM Hepes buffer (20 μL, pH 7.2) was added α1,3Fuc-T (1 mU). The mixture was incubated at 37 °C for 20 min. In the case of α1,6-Fuc-T, the reaction was carried out in 50 mM sodium cacodylate buffer (20 μL, pH 7.5) in the presence of GDP-Fuc (50 μM), **11** (100 μM), click product (**A1**−**A36**, 50 μM), and α1,6-Fuc-T (80 μU). All reactions were stopped by addition of CH_3CN and employed for MALDI-TOFMS assay by the same procedure described above.

Kinetic Analysis. IC_{50} of compound **15** againstα2,3-ST: The inhibitory effect of compound **15** on α2,3-ST was evaluated and determined as IC_{50} value by using 11 different concentrations of **15** from 0.39 to 200 μM. The solution containing 200 μM CMP-Neu5Ac, 1 mM acceptor substrate **9**, 40 mM sodium cacodylate buffer (pH 7.4), 0.5 mg/mL BSA, 0.1% Triton CF-54, and α2,3-ST (0.5 mU) in a total volume 25 μL was incubated at 25 °C for 15 min. After incubation, 100 μL of CH_3CN was added to stop the reaction, and 125 μL of water was added to the mixture to be a total volume 250 μL. To the 20 μL of DHB solution (10 mg/mL DHB in 30% CH_3CN) was added 2 μL of each reaction mixture and deuterium labeled internal standard, and then 0.5 μL of mixed solution was mounted on MALDI-TOFMS target plate.

K_m of compound **15** to α2,6-ST: The kinetic parameter of K_m value of compound **15** for α2,6-ST was determined at six different concentrations in a range of 12.5−200 μM. The reaction solution containing compound **7**, 1 mM **9**, 40 mM sodium cacodylate buffer (pH 7.4), 0.5 mg/mL BSA, 0.1% Triton CF-54, and α2,6-ST (0.5 mU) in a total volume 25 μL was incubated at 37 °C for 20 min. After incubation, 100 μL of CH_3CN was added to stop the reaction, and the mixture was added by 125 μL of water to be a total volume 250 μL. To the 20 μL of DHB solution (10 mg/mL DHB in 30% CH_3CN) was added 2 μL of each reaction mixture and deuterium labeled internal standard, and then 0.5 μL of mixed solution was mounted on MALDI-TOFMS target plate.

K_i of compound **16** against α1,3-FucT: The assays were carried out in 50 mM Hepes buffer (20 μL, pH 7.2) in the presence of GDP-Fuc (50−125 μM), **11** (1 mM), **16** (0.25−2 μM), and α1,3-FucT (0.1 mU). The mixtures were incubated at 37 °C for 60 min, and the inhibitory effect by compound **16** was evaluated by general MALDI-TOFMS protocol described above (Supporting Information).

K_i of compound **17** against α1,6-FucT: The assays were carried out in 50 mM sodium cacodylate buffer (20 μL, pH 7.5) in the presence of GDP-Fuc (5−50 μM), **9** (100 μM), **17** (0.5−50 μM), and α1,6-FucT (80 μU). The mixtures were incubated at 37 °C for 60 min, and the inhibitory effect by compound **17** was evaluated by general MALDI-TOFMS protocol described above (Supporting Information).

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Supporting Information Available: HPLC profiles of compounds **11** and **12**, standard curves for the quantitation of products, characterization of click reactions, and kinetic analysis data. This material is available free of charge via the Internet at http://pubs.acs.org.

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