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Interaction assay of oligosaccharide with lectins using glycosylasparagine

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Abstract—Glycosyl amino acids having natural glycan were useful for the interaction assay of oligosaccharides. A glycochip containing the whole structure of an oligosaccharide was easily prepared by the immobilization of the glycosyl amino acid. Furthermore, fluorescence probes were introduced into the glycosyl amino acid while maintaining the whole structure of oligosaccharide. By using these labeled oligosaccharides, fluorescence polarization (FP) and fluorescence correlation spectroscopy (FCS) analyzed the carbohydrate–lectin interaction in a solution assay system.

Glycoconjugates, that is, glycopeptides and glycolipids, play an important role in biological processes, such as cell recognition, cell adhesion, immunogenic recognition, etc.[1] Proteins almost have an oligosaccharide on their side-chain, and the carbohydrate moiety is a post-translational product. The study of carbohydrates (*glycomics*) is very interesting, and important as a post-genome and postproteome. The immobilization or a fluorescence modification of oligosaccharides has been investigated in order to understand the biological interaction between various species and oligosaccharides.[2] Especially, a glycochip is expected to be diagnostic tool for the investigation of new lectins. Some immobilizations of carbohydrates have been reported, however, the structure of a glycan was relatively simple (from mono- to tetrasaccharide) because the synthesis of an oligosaccharide is not easy compared with an oligopeptide and oligonucleotide.[3] Although the immobilization of a natural type *N*-glycan was demonstrated, the immobilization by a reductive amination produced the ring-opening form of the reduced terminal of the oligosaccharide and the complete structure of the carbohydrate was lost.[4,5] Fluorescence modification of an oligosaccharide needs an enormous excess amount of fluorescence reagent, and the ring-opening of the reduced terminal is also produced under this condition.[6,7]

Various kinds of glycosylasparagines are obtained by the peptidase digestion of glycopeptides from hen eggs,[8] and these are used for the transglycosylation by *endo*-β-*N*-acetylglucosaminidases as a glycosyl donor.[9] Glycosylasparagine has an amino group and a carboxyl group in its molecular structure. These functional groups allow the oligosaccharide to be easily modified or introduced to various substrates. We demonstrated that glycosylasparagine having a natural *N*-glycan was introduced to a cyclodextrin,[10] a calixarene derivative[11] and peptides.[12] We postulated that using a glucosyl amino acid produces immobilization or fluorescence modification by maintaining the whole structure of the oligosaccharide.

In this report, we describe the straightforward approach to apply a glycosyl amino acid to an interaction assay of oligosaccharides. We prepared the glycochip by immobilization of the glycosyl amino acid and demonstrated the carbohydrate–lectin interaction on the glycochip. Furthermore, some fluorescence probes were able to be easily introduced to the glycosyl amino acid, and using these labeled oligosaccharides, carbohydrate–lectin interactions in the solution assay system were performed by fluorescence polarization (FP)[5] and fluorescence correlation spectroscopy (FCS).[13]

Keywords: Glycochip; Oligosaccharide; Glycosyl amino acid; Fluoresence polarization; Fluoresence correlation spectroscopy.
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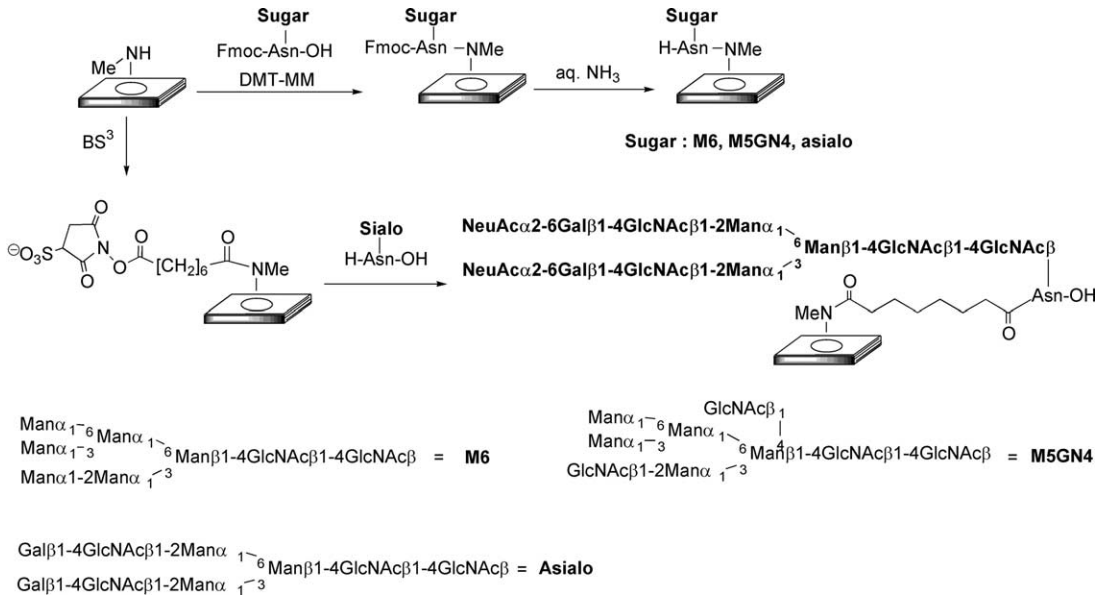


Scheme 1. Preparation of glycochip using glycosylasparagines.[20]

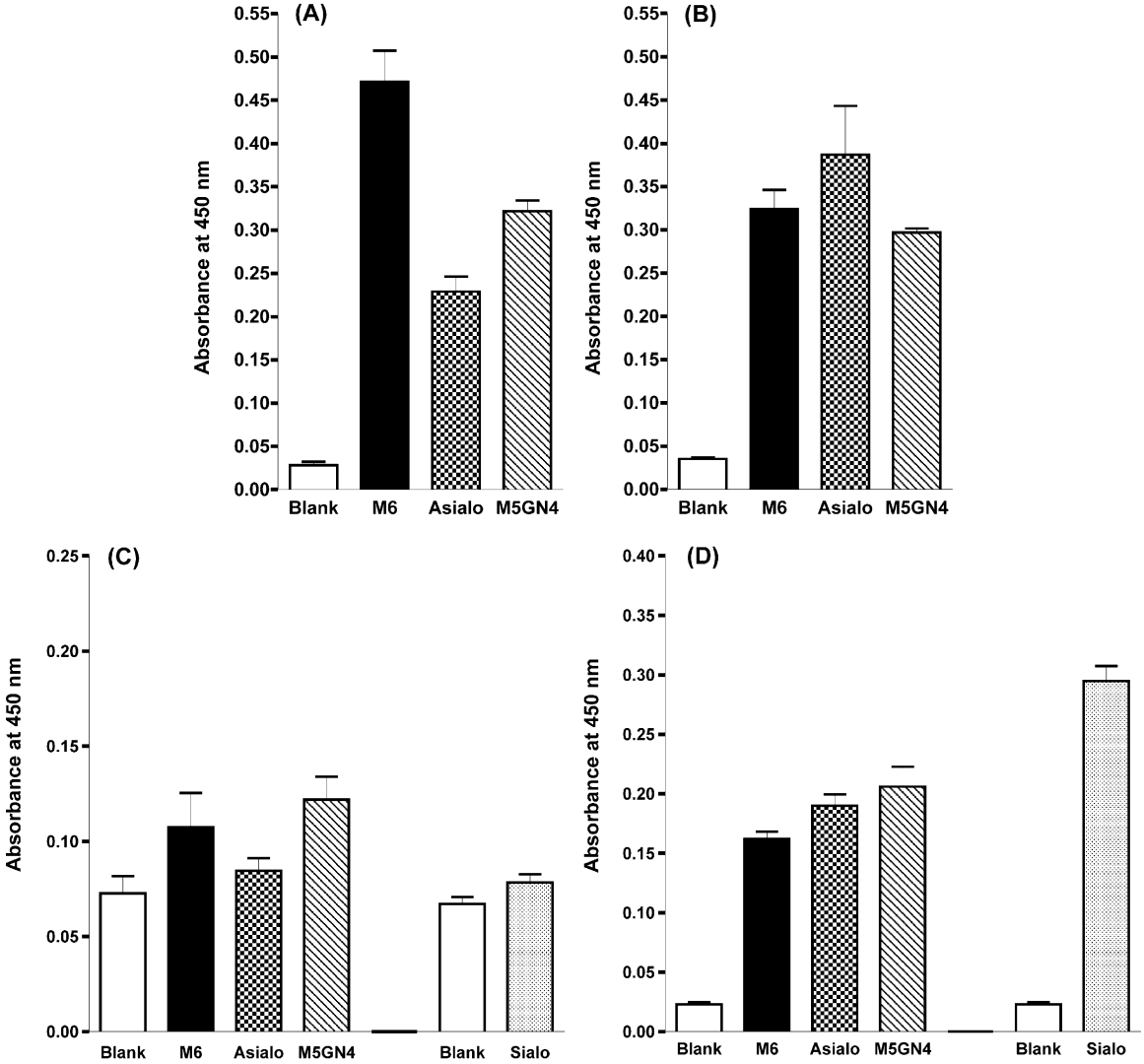


Figure 1. Binding assay of HRP-lectins to glycochip: (A) interaction of ConA with M6, M5GN4 and Asialo chip. (B) interaction of RCA with M6, M5GN4 and Asialo chip. (C) interaction of DSA with M6, M5GN4, Asialo and Sialo chip. (D) interaction of WGA with M6, M5GN4, Asialo and Sialo chip.

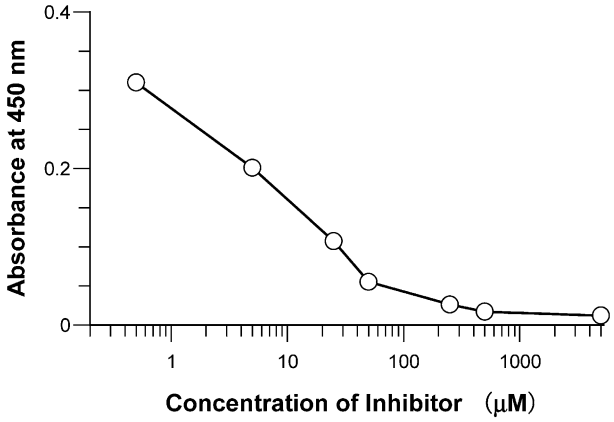


Figure 2. Inhibition assay of binding of HRP-ConA to M6 chip. Inhibitor was α-methyl mannoside.

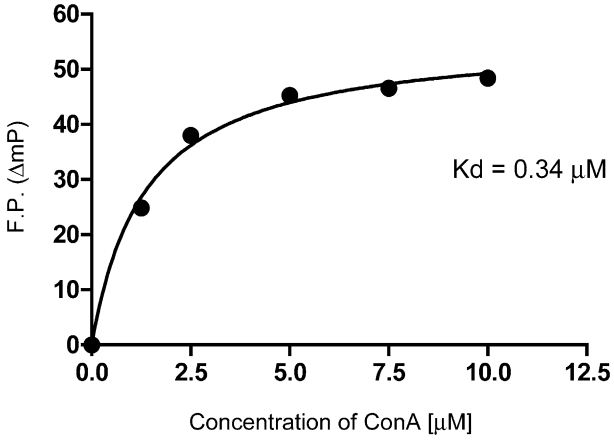


Figure 3. Binding of Dns-Asn(M6)-OH by ConA based on fluorescence polarization assay. The concentration of Dns-Asn(M6)-OH was 2 μM.

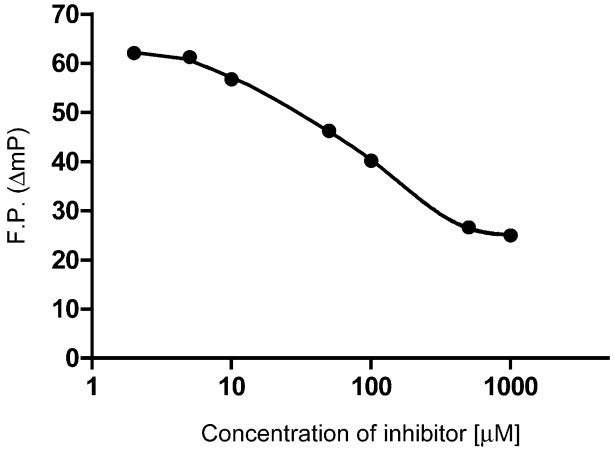


Figure 4. Inhibition of HRP-ConA binding to Dns-Asn(M6)-OH based on fluorescence polarization assay. The concentration of Dns-Asn(M6)-OH was 2 μM, and the inhibitor was α-methyl mannoside.

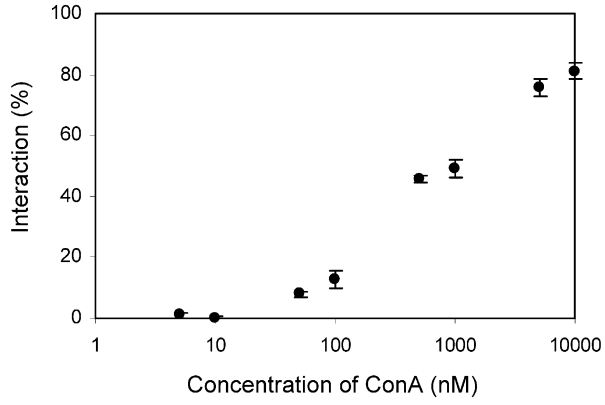


Figure 5. Binding of TMR-Asn(M6)-OH by ConA studied based on fluorescence correlation spectroscopy. The concentration of TMR-Asn(M6)-OH was 5 nM.

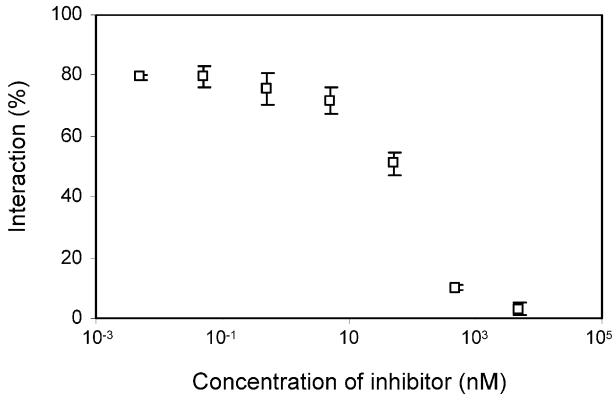


Figure 6. Inhibition of ConA binding to TMR-Asn(M6)-OH by fluorescence polarization assay. The concentration of TMR-Asn(M6)-OH was 5 nM, and the inhibitor was α-methyl mannoside.

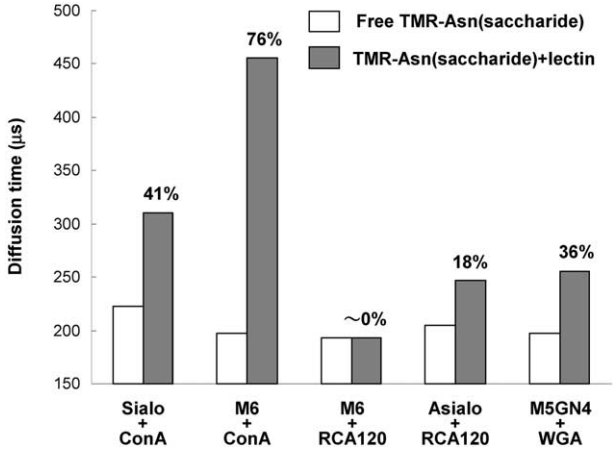
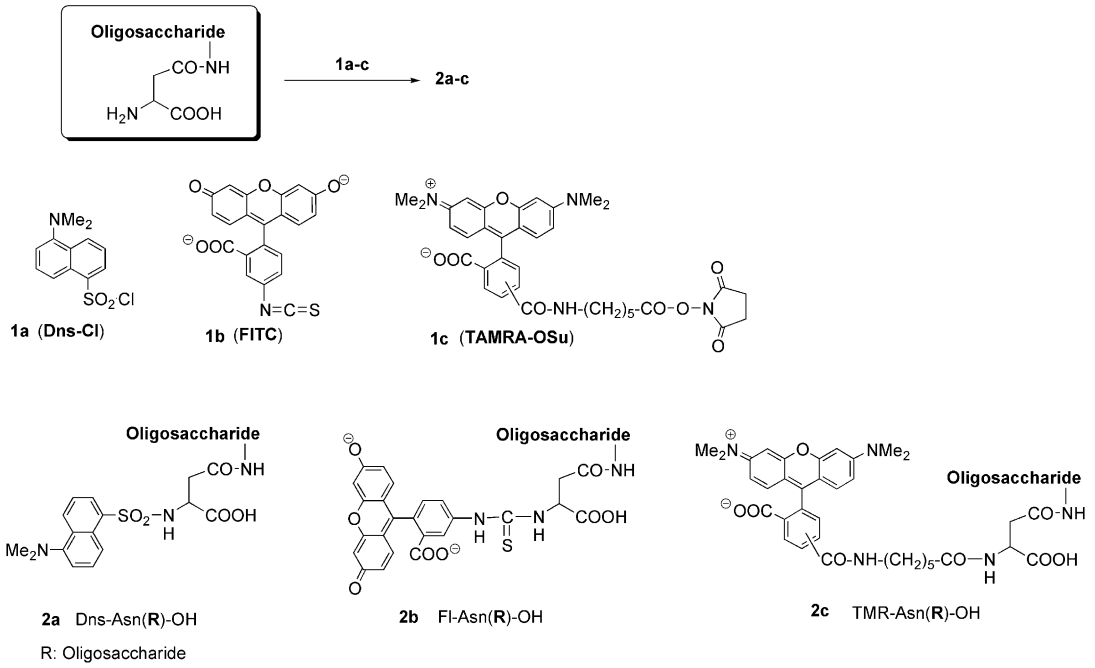


Figure 7. Interaction of TMR-labeled glycosylasparagines with lectins measured by fluorescence correlation spectroscopy. The concentration of TMR-Asn(M6)-OH was 5 nM, and ConA was 5 μM.

Initially, various kinds of Fmoc-glycosylasparagines were prepared. The glycosylasparagine having the disialo complex-type, H-Asn[(NeuAc-Gal-GlcNAc-Man)$_2$-Man-(GlcAc)$_2$]-OH [*H-Asn(Sialo)-OH*], and the glycosylasparagine having the asialo complex-type, H-Asn[(Gal-GlcNAc-Man)$_2$-Man-(GlcNAc)$_2$]-OH [*H-Asn(Asialo)-OH*] derived from egg yolk, were prepared as previously described.[14] Various kinds of high-mannose type asparagines and hybrid-type asparagines were obtained from ovalbumin.[15] The ovalbumin was first treated with α-chymotrypsin or trypsin, and then with actinase E.[16] To the amino group of the obtained glycosylaspar-



Scheme 2. Fluorescence modification of glycosylasparagines.

agines, the 9-fluorenylmethyloxycarbonyl (Fmoc) group was introduced.[10,12] The resulting Fmoc-glycosylasparagines were separated into 6 peaks by an ODS column.[17]

Fmoc-Asn(Asialo)-OH, a high-mannose type Fmoc-Asn($Man_6GlcNAc_2$)-OH [*Fmoc-Asn(M6)-OH*], and the bisect-type, Fmoc-Asn($Man_5GlcNAc_4$)-OH [*Fmoc-Asn(M5GN4)-OH*], were immobilized in a 96-well plate, on which the amino group is modified[18] using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM).[19] H-Asn(Sialo)-OH was immobilized using bis(sulfosuccinimidyl)suberate (BS^3) through amino group because the carboxyl group contained not only the asparagine moiety but also the sialic acid moiety in H-Asn(Sialo)-OH (Scheme 1).

The binding assay of horseradish peroxydase (HRP)-labeled lectins, ConA, RCA120, DSA, WGA, to the glycochip and subsequent inhibition of a binding event of M6-ConA were performed by enzyme-linked immunosorbent assay (ELISA) (Figs. 1 and 2).[21,22] From these results, ConA, RCA120, DSA and WGA showed distinctly different binding specificities toward different oligosaccharides. This result showed that the immobilizations of the oligosaccharides occurred and the prepared glycochip worked as an interaction assay tool.

Furthermore, the carbohydrate-lectin interactions in the solution assay system were carried out using glycosylasparagines by FP and FCS. Fluorescence groups, that is, the dansyl (Dns) group, fluorescein (Fl) group, and tetramethylrhodamine (TMR) group, were introduced to the glycosylasparagines (Scheme 2).[23] The interaction of Dns-Asn(M6)-OH with ConA was analyzed using FP. This result showed significant binding of ConA with Dns-Asn(M6)-OH. A nonlinear regression analysis using a one-site model gave a K_d (dissociation constant) for ConA of 0.34 μM (Fig. 3). The FCS assay using TMR-Asn(M6)-OH also gave a K_d for ConA of 0.56 μM (Fig. 5). These K_d values are consistent with these determined by other methods.[10,24] The inhibition assay using α-methyl mannoside as an inhibitor was also demonstrated by FP (Fig. 4) and FCS (Fig. 6).

TMR-labeled glycosylasparagine (M6, sialo, asialo, M5GN4) and lectine (ConA, RCA120, WGA) were mixed. After a 10-min incubation at room temperature, its diffusion time was measured and provided the interaction ratio. As shown in Fig. 7, for example, the interaction of ConA with M6 was stronger (76%) than Sialo (41%), and the M6-RCA120 interaction was not observed (∼0%). From this result, the screening of the interaction between the lentins and fluorescence-labeled glycosylasparagine was able to be easily carried out by FCS.

In conclusion, glycosyl amino acid is useful for the interaction assay of oligosaccharides. Immobilization of the glycosyl amino acid allowed the preparation of a glycochip that maintained the whole structure of the oligosaccharide. The interaction on the glycochip was detected by ELISA. Use of the glycosyl amino acid made fluorescence modification of the oligosaccharide facile. The fluorescence-labeled glycosyl amino acid is very eminent for the interaction assay in solution (FP, FCS).

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assay. We thank Prof. Kazuaki Kakehi and Mr. Mitsuhiro Kinoshita (Kinki University) for their helpful advice and calculation of the Kd value in the FP method. MF20TM (OLYMPUS CORPORATION) was used in the FCS, and we thank Dr. Noriko Kato, Dr. Naoaki Okamoto and Mr. Masayoshi Kusano (OLYMPUS CORPORATION) for operation of the MF20. Plate CHAMELEON™ (HIDEX) was used in the FP. We thank Prof. Munetaka Kunishima (Kobe Gakuin University) for providing DMT–MM (Kunishima reagent).

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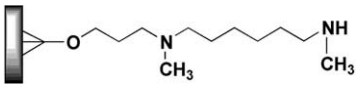
16. In a previous method, ovalbumin was treated 4 or 5 times by actinase E. In present method, one treatment of α-chymotrypsin (or trypsin) and once with actinase E gave the glycosylasparagine from ovalbumin.
17. Preparative condition. Column: Inertsil ODS-3 (20×750 mm, GL Sciences Inc., Tokyo, Japan). Elution: 26–28%B/0–40 min, 28%B/40–120 min (A: 0.1% TFA/H_2O, B: 0.1% TFA/MeCN). Flow rate: 10.0 mL min^{-1}.
18. 96-well plate is Nunc CovaLink™ NH Module (spacer length = 2 nm).



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20. Immobilization of glycosylasparagine using Fmoc-Asn (saccharide)OH and DMT-MM: To a solution of Fmoc-glycosylasparagine (60 nmol) in water (55 μL) is added 0.1 M aqueous DMT–MM (5 μL) and 5% (w/v) aqueous $NaHCO_3$ (1 μL). This mixture was added to one well of amino modified 96-well plate. After incubation for 2 h at room temperature, the solution was removed and the well was washed three times with PBS (10 mM, pH 7.4). A 28% (w/v) aqueous NH_3 solution containing 0.05% (w/v) Tween 20 (60 μL) was added to the well and allowed to stand for 1 h at room temperature. After washing three times with 0.05% (w/v) Tween 20/PBS, the immobilized glycosylasparagines were obtained. Immobilization of H-Asn(Sialo)-OH using BS^3: 2% (w/v) aqueous BS^3 (100 μL) was added to the amino-well, and incubated for 30 min at room temperature. After washing three times with 0.05% (w/v) Tween 20/PBS, 1 mM H-Asn(Sialo)-OH/PBS (60 μL) was added and allowed to stand for 2 h at room temperature. The well was washed three times with 0.05% (w/v) Tween 20/PBS. 1 M 2-aminoethanol/PBS (60 μL) was added to the well, and incubated for 1 h at room temperature. Washing three times with 0.05% (w/v) Tween 20/PBS gave the glycowell immobilized sialo complex-type oligosaccharide.
21. ELISA condition: The glycosylated well was treated with 3% (w/v) BSA/PBS (420 μL) for 18 h at room temperature, and then the well was washed three times with PBS. The HRP–lectin solution (100 μL) was added to each well, and incubated for 45 min at room temperature. The wells were washed three times with 0.05% (w/v) Tween 20/PBS and developed with 100 μL of TMB One Solution (Promega Co., Madison, WI). A 80 μL aliquot of the mixture was transferred to another blank well and the color development was stopped by the addition of 80 μL of 1M HCl. The absorbance of each well was then read at 450 nm using a microplate reader.

22. ConA has specificity with α-D-Man, α- D-Glc and N-glycans (biantennary complex-type, high-mannose type, hybrid type). RCA 120 has specificity with terminal Gal. DSA has with chitin oligosaccharide and N-acetyllactosamine, tri- and tetraantennary complex-type oligosaccharide. WGA has with D-GlcNAc and hybrid type oligosaccharide, sialyl oligosaccharide.
23. To the solution of Glycosyl amino acid and 3 equivalents of *N,N*-diisopropylethylamine in DMF was added 1.5–2.0 equivalents of fluorescence reagent **1a**–**c**, and stirred at room temperature for 18 h. HPLC purification of the mixture gave **2a**–**c** in 64–97%.
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