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A Combined Strategy for Glycan Profiling: a Model Study with Pyridylaminated Oligosaccharides

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Structural glycomics plays a fundamental role in glycoscience and glycotechnology. In this paper, a novel strategy for the structural characterization of glycans is described, in which MS^2 analysis involving a LIFT-TOF/TOF procedure is combined with frontal affinity chromatography (FAC). As model compounds, 20 neutral pyridylaminated (PA) oligosaccharides were chosen, which included four groups of structural isomers differing in sequence, linkage, position, or branching features. By depicting significant diagnostic ions on MS^2, most of the analyzed oligosaccharides were successfully differentiated, while two pairs of linkage isomers, *i.e.*, L*N*T/L*N*nT, and L*N*H/L*N*nH were not. For subsequent analysis by FAC, 14 lectins showing significant affinity to either L*N*T (type 1) or L*N*nT (type 2) were screened, and a galectin from the marine sponge *Geodia cydonium* (GC1) and a plant seed lectin from *Ricinus communis* (RCA-I) were used for determination of type 1 and 2 chains, respectively. With these specific probes, both of the isomeric pairs were unambiguously differentiated. Furthermore, a pair of triantennary, asparagine-linked oligosaccharide isomers could also be successfully differentiated. Thus, the combination of MS^2 and FAC is a practical alternative for the structural characterization of complex glycans.

Key words: frontal affinity chromatography, glycan mass-fingerprinting, lectin, LIFT, MALDI-TOF-MS.

Abbreviations: DHB, dihydroxybenzoic acid; FAC, frontal affinity chromatography; GC1, *Geodea cydoinum* galectin-1; IDI, intensity-defined diagnostic ion; MALDI, matrix-assisted laser desorption ionization; MCI, multiply cleaved ion; MS, mass spectrometry; PA, pyridylaminated; PSD, post-source decay; RCA-I, *Ricinus communis* agglutinin-I; SDI, structure-defined diagnostic ion; TOF, time-of-flight.

Structural glycomics plays an essential role in elucidation of the biological functions of diverse glycans (*1–6*). In particular, recent progress in mass spectrometry (MS) is remarkable in enabling us to perform a repeated process of ion selection and fragmentation, called MS^n. The method proved to be effective for structural analysis of even complex glycans including isomers (*7–16*). From a practical viewpoint, however, the use of advanced MS machines for glycan analysis still has some basic and technical problems, *e.g.*, machine availability (*i.e.*, economy), reproducibility of fragment signals, machine dependency, technical skills, consensus regarding experimental protocols and MS databases. In such a situation, a simple but stable method is much preferred. In this context, a LIFT-TOF/TOF procedure recently developed for matrix-assisted laser-desorption ionization time-of-flight MS (MALDI-TOF-MS) (*17*) is promising as an advanced, but extremely simple technique for performing post-source decay (PSD) analysis, comparable to MS^2 (*18–20*). However, the discrimination of isomeric oligosaccharides with the MS^2 strategy is not always assured. Therefore, the method must be supplemented with an alternative separation principle. For this, 2-D/3-D mapping of pyridylaminated (PA) oligosaccharides by means of liquid chromatography (LC) has been developed and widely utilized (*21*, *22*). However, LC mapping generally takes a long time for each analysis, and thus high-throughput and systematic analysis is difficult although LC separation is necessary for the purification of glycans. More importantly, it does not provide direct answers to questions about epitope structures, which are often associated with biological functions. Rather, it is practical for obtaining the most essential information (*i.e.*, major characteristics of individual glycan structures) required of the context of functional glycomics.

Here, we propose a novel strategy for glycan analysis, in which LIFT-TOF/TOF MS analysis and frontal affinity chromatography (FAC) are combined. Since investigation of the principle of FAC by Kasai and Ishii (*23*), it has now been acknowledged as an established technique for determining bio-molecular interactions in terms of dissociation constants (K_ds) (*24*, *25*) as well as for high-throughput ligand screening (*26*). More recently, its accuracy and throughput were further improved by introducing an automated injecting system (*1*, *27*). An apparent advantage of using lectins for structural analysis is the provision of the most relevant epitope structures, which are often associated with biological functions, while neither MS nor LC mapping can achieve this in a direct manner. To establish a novel strategy for glycan analysis, however, evaluation must be performed with a relatively small set of simple

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glycans, of which the structures are defined with closely related structures (*i.e.*, isomers).

In a model experiment, all 20 neutral oligosaccharides examined, including 13 isomeric pairs, were successfully differentiated, with the two different principles coupled. An extended study involving more complex, asparagine-linked oligosaccharides has supported the validity of the proposed strategy. Thus, the combination of MS^2 and FAC is a promising alternative for structural analysis of glycans.

MATERIALS AND METHODS

Pyridylaminated (PA) Oligosaccharides—Standard neutral pyridylaminated (PA) oligosaccharides (Fig. 1), originating from glycolipids (**01**–**17**) and glycoproteins (**21**, **22**), were purchased from Takara Bio (10 pmol/ml). Other PA-glycans (GN_3, GN_4 and LN_3), derived from polysaccharides, were prepared by using non-labeled purified saccharides essentially as described previously (*21*). The obtained PA-oligosaccharides were further purified until >95% purity was attained by means of reversed-phase chromatography according to the established procedure (*22*).

MS Measurements—MS and LIFT-TOF/TOF spectra were acquired with an Ultraflex^{TM} TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) under the control of Flexcontrol 2.0 software. Ionization was accomplished with a 337 nm beam from a nitrogen laser. Ions of MS spectra were accelerated at 25 kV. The external calibration of MALDI mass spectra was performed using the singly charged monoisotopic peaks of Peptide Calibration Standards (Bruker Daltonics), *i.e.*, a mixture of angiotensin II (*m/z* 1,046.542), angiotensin I (*m/z* 1,296.685), substance P (*m/z* 1,347.735), bombesin (*m/z* 1,619.822), ACTH clip1-17 (*m/z* 2,093.083), ACTH clip18-39 (*m/z*

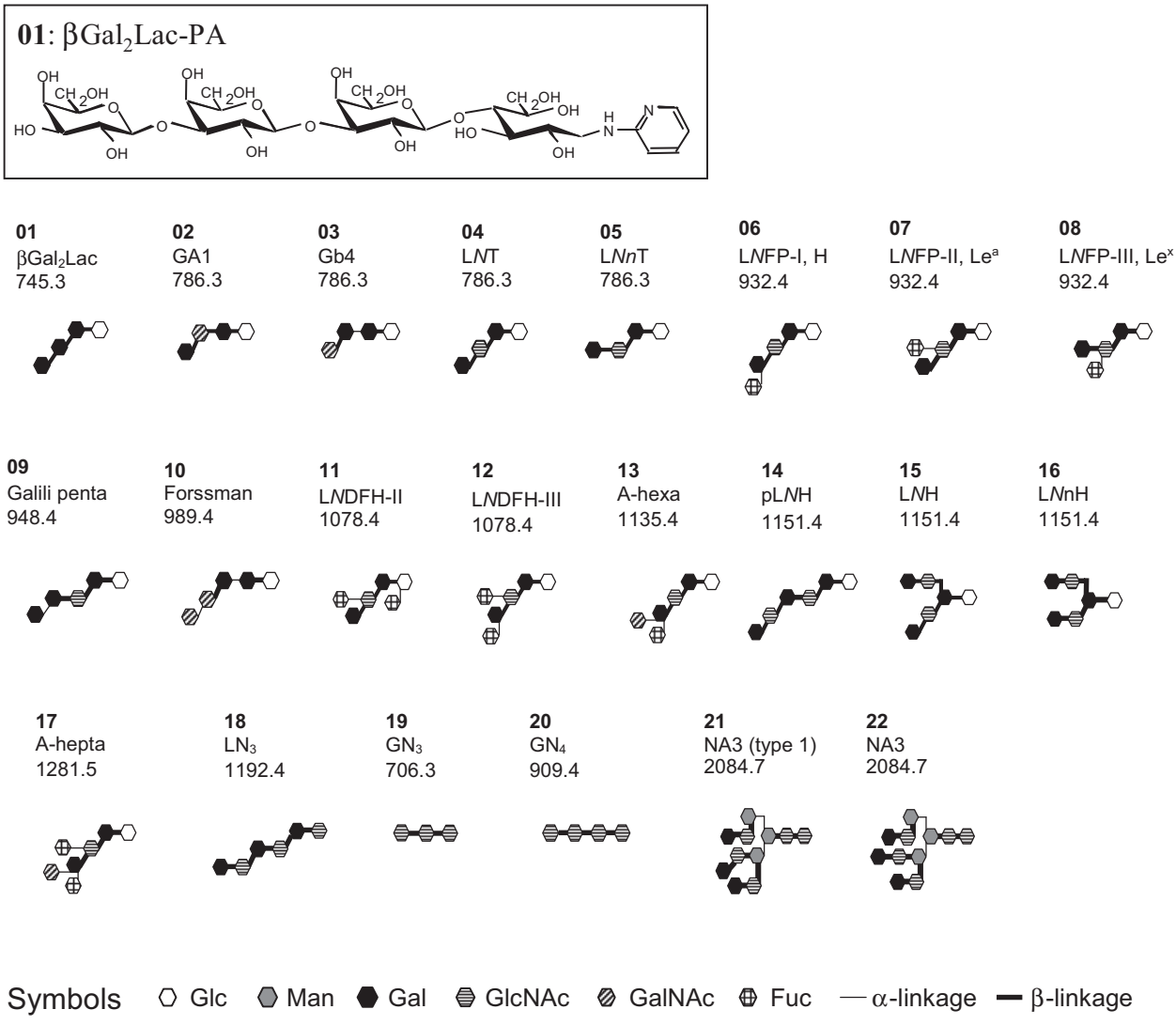


Fig. 1. **A list of the PA-oligosaccharides analyzed in this work.** Identification numbers (in bold type), trivial names and theoretical molecular weights are shown. Symbols and anomeric linkages used to represent glycan structures are shown at the bottom. For the sake of simplicity, the reducing terminal PA group is omitted. For chemical structures, see the case of PA-oligosaccharide **01** in the inset. Note that reducing terminal hexose (Glc in this case) takes an open-ring structure as a result of fluorescence labeling reaction with 2 aminopyridine.

2,465.198), and somatostatin (*m/z* 3,147.471). In the LIFT-TOF/TOF mode, precursor ions were accelerated to 8 kV and selected in a timed ion gate. In the LIFT-cell the fragments were further accelerated to 19 kV. The reflector potential was 29 kV.

Samples were prepared by a standard dried-droplet method on MALDI targets of Ground steel™ or Anchorchip™ for application of 5 pmol or 1 pmol of PA-sugars, respectively, using 2,5-dihydroxybenzoic acid (DHB) as a matrix. LIFT-TOF/TOF spectra were obtained by targeting singly charged [M + H] + ions derived from three different positions on the same MALDI plate. In this work, relative intensity (%) was defined as the fragment intensity relative to the highest intensity (*i.e.*, base peak ion) observed in each LIFT-TOF/TOF spectrum. The thus calculated relative intensities in three experiments for each oligosaccharide were averaged and SD values were determined. Correlation coefficients between MS^2 profiles of glycans were obtained as described previously (*28*).

Frontal Affinity Chromatography—Frontal affinity chromatography was performed using an automated machine (FAC-1) with miniature columns (capsule type) as previously described (*27*). Briefly, 0.5 ml of a PA-oligosaccharide solution in Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 130 mM NaCl) at the concentration of 5 nM was applied to a lectins column at a flow rate of 0.125 ml/min. Elution of the PA-sugars was monitored as fluorescence (excitation and emission wavelengths, 310 and 380 nm, respectively), and the elution point was determined as described originally by Arata *et al.* (*29*). All the chromatographic procedures were performed at 25°C. Lectin agarose for BPA, ECA and RCA-I was purchased from Seikagaku Corporation, Ltd. (Tokyo, Japan). Other lectin columns were prepared by coupling lectin proteins to NHS-activated Sepharose (Amersham, NJ, USA) as described previously (*24*). *Geodia cydonium* galectins (GC1 and GC2) and rat galectin-2 (rGal2) were generous gifts from W.E.G. Muller (University of Mainz) and M. Futai (Osaka University), respectively. GST fusions to the human galectin-4 N-terminal domain (GST-hG4N), galectin-7 (GST-hG7), galectin-8 N-terminal domain (GST-hG8N), galectin-9 N-terminal (GST-hG9N), C-terminal domain (GST-hG9C) were generous gifts from N. Nishi and M. Hirashima (Kagawa University). Chicken galectins, C14 and C16, and human galectin-3 (hGal3) were described previously (*24*).

RESULTS AND DISCUSSION

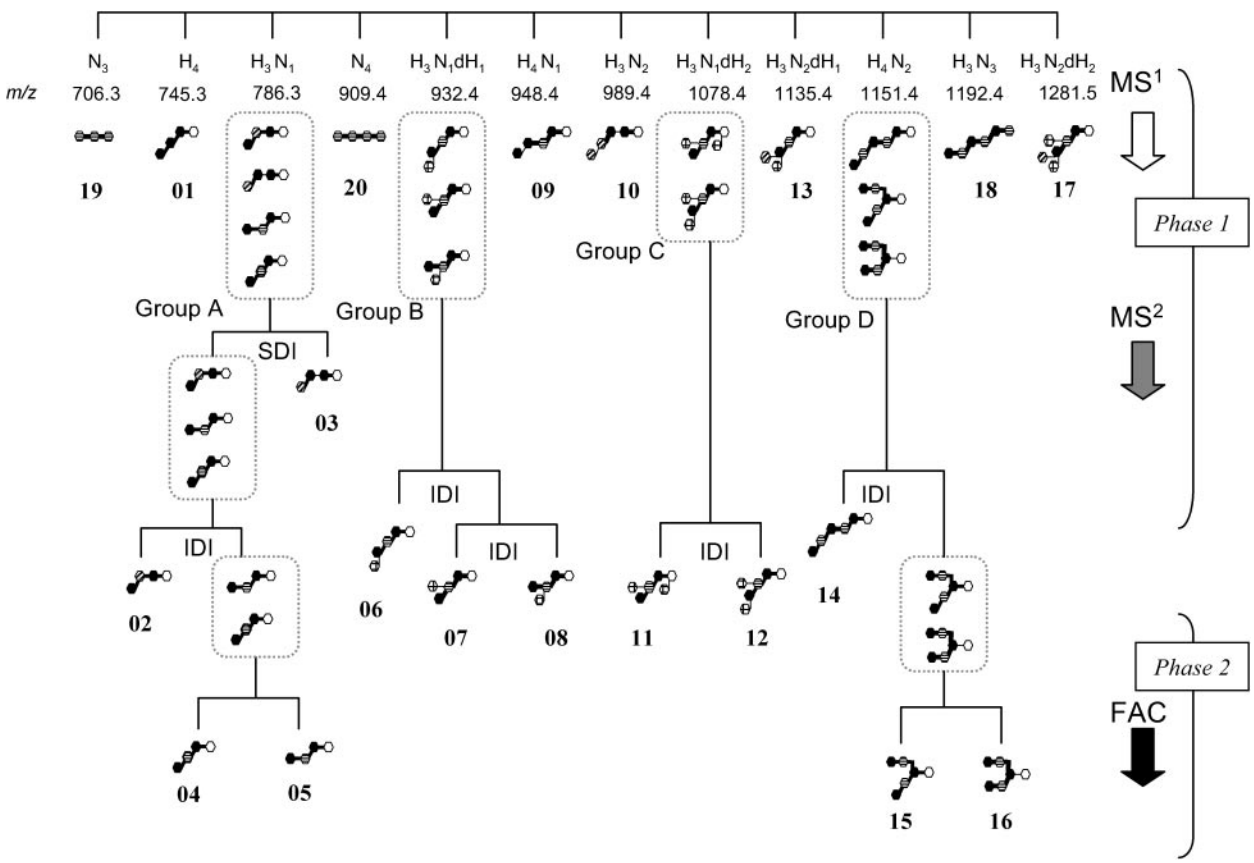
Overall Strategy—The proposed strategy consists of two phases: *phase I* comprises so called "glycan mass-fingerprinting" by means of LIFT-TOF/TOF MS^2, while *phase II* is achieved by quantitative lectin affinity chromatography, *i.e.*, FAC. *Phase I* further comprises MS^1 and MS^2 steps: in the MS^1 step, an accurate molecular mass is obtained to estimate the "sugar composition" for each oligosaccharide, although diastereomers (*e.g.*, Glc/Man/Gal and GlcNAc/GalNAc) cannot be distinguished from one another. In the subsequent LIFT-TOF/TOF MS^2 step, fragmentation patterns are compared. In *phase II*, FAC is used for further characterization in a particular context to differentiate isomeric glycans, which is not possible in *phase I*. The results and overall strategy are summarized in Scheme 1.

LIFT-TOF/TOF Analysis of 20 PA Oligosaccharides—All of the 20 PA-oligosaccharides were observed in a high proportion (>50% total ion) as an H^+ adduct on MS^1, when 2,5-dihydroxybenzoic acid (DHB) was used as the matrix (spectra not shown). In contrast, targeting of Na^+ adducts resulted in unexpected fragmentation, which accompanies counter ion exchange, as reported for previous experiments involving Ultraflex (*30*). Therefore, these H^+ adducts were targeted as precursor ions in the subsequent MS^2 step. As a result of LIFT-TOF/TOF measurements targeting H^+ adducts as parent ions, most of the PA-oligosaccharides were differentiated from one another in terms of either fragment type or intensity, *i.e.*, diagnostic fragment ions (Table 1: data for **18**–**20** not shown).

From a theoretical viewpoint, diagnostic fragment ions can be categorized into two types: "structure-defined" diagnostic ions (SDIs), and "intensity-defined" diagnostic ions (IDIs). The former are fragment ions theoretically derived from only one of the isomeric pairs, while the latter are fragment ions derived from both of the isomers, but differ in their intensities. Compared with SDIs, however, intensities of IDIs are more likely to vary depending on the experimental parameters, types of machines, adducts, fragmentation methods, *etc*. Therefore, IDIs should be evaluated more rigorously than SDIs. In this work, fragment ions showing >5% relative intensity were taken to be "significant" SDIs, whereas those of >20% relative intensity for either of the isomers and showing a >1.5-fold difference between the two were considered as "significant" IDIs [*Note*: relative intensity (%) in this work was defined as the fragment intensity relative to the base peak ion in each LIFT-TOF/TOF spectrum].

As a general feature of labeled glycans, B/Y-type fragmentation was extensively observed (*7*, *8*, *26*), whereas C/Z-type and A/X-type fragmentation was rare (nomenclature according to Domon and Costello, *31*). This simple feature is preferable for solid judgment of diagnostic fragment ions. In-source decay fragment ions were also insignificant. In the following paragraphs, the fragmentation patterns of four groups (A–D) of structural isomers are described (Fig. 2, original data from Table 1).

Among the group A tetrasaccharides, GA1 (Galβ1-3GalNAcβ1-4Galβ1-4Glc-PA, **02**) and Gb4 (GalNAcβ1-3Galα1-4Galβ1-4Glc-PA, **03**) are sequence isomers, while L*N*T (Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA, **04**) and L*N*nT (Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA, **05**) are linkage isomers. On comparison of their MS^2 spectra (not shown), a distinguishing Y-ion corresponding to Gal-Gal-Glc-PA (*m/z*, 583; relative intensity, 67%) was observed that is an SDI specific for Gb4 (Fig. 2a). In other words, at this stage, the possibility of either GA1, L*N*T or L*N*nT is excluded. Among these three, GA1 was characterized by its much increased (by ~40%) Y-ion corresponding to lactosyl-PA (*m/z*, 421), and hence could be clearly differentiated from the others (L*N*T/L*N*nT) by this IDI. However, these linkage isomers, L*N*T/L*N*nT, gave almost the same fragment patterns, and thus could not be fully differentiated. In terms of correlation coefficient, they (**04/05**) showed relatively high similarity (0.97) in comparison with other combinations (0.71–0.91). None of the potential IDIs (*e.g.*, GlcNAc-Gal-Glc-PA; *m/z*, 624) fulfilled the requirement (*i.e.*, >20%) defined above because of the too-low intensity (Table 1).



Scheme 1. **Summary of differentiation of 20 PA-oligosaccharides by the combination of LIFT-TOF/TOF MS (phase 1) and FAC (phase 2).** On MS^1, the compositions of elementary saccharides were deduced (shown at the top); N (*N*-acetylhexosamine), H (hexose), and dH (deoxyhexose). On MS^2, the structural isomers boxed in dashed lines were differentiated by depicting either structure-defined (SDIs) or intensity-defined diagnostic fragment ions (IDIs). For the differentiation of two pairs of linkage isomers, **04/z05** and **15/16**, FAC was performed using GC1 and RCA-I to probe type 1 and type 2 chains, respectively.

Among the group B isomers (*m*/*z*, 932.4), L*N*FP-I (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA, **06**) and L*N*FP-II [Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc-PA, **07**], -III [Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc-PA, **08**] are positional isomers as regards fucosylation, while L*N*FP-II and L*N*FP-III are linkage isomers. On MS^2, a Y-ion corresponding to GlcNAc-Gal-Glc-PA (*m*/*z*, 624) was a useful IDI for L*N*FP-I (45%; Fig. 2b; spectra not shown). Apparently, generation of this fragment is disadvantageous for the others (L*N*FP-II and III), because they require simultaneous multiple cleavages for their generation (hereafter, designated as multiply cleaved fragment ions, MCI), while only a single site cleavage is necessary for L*N*FP-I. With this MCI, L*N*FP-I could be clearly differentiated from the others. For the differentiation of L*N*FP-II and III, a Y-ion, Gal-GlcNAc-Gal-Glc-PA (*m*/*z*, 786), was found to be a useful IDI. The correlation coefficients among them are in the range of 0.83–0.89.

Group C isomers (*m*/*z*, 1078.4), *i.e.*, L*N*DFH-II [Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc-PA, **11**] and L*N*DFH-III [Le^b, Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc-PA, **12**], contain two fucose residues. They are positional isomers as regards the "second" fucose residue, while the first one (Fucα1-4) is common. A significant difference in the relative intensity of a Y-ion, Gal-Glc-PA (*m*/*z*, 421), was observed, which is an MCI for L*N*DFH-II (Fig. 2c; spectra not shown). A Y-ion, Gal-GlcNAc-Gal-Glc-PA (*m*/*z*, 770), is also an MCI for L*N*DFH-II. A monodefucosylated Y-ion (*m*/*z*, 932) was detected 2.5-times more abundantly in L*N*DFH-II than L*N*DFH-III, and thus was also a useful IDI. As a result, these two positional isomers could be easily differentiated with a considerably low correlation coefficient (0.51).

Among group D isomers (*m*/*z*, 1,151.4), *p*L*N*H (**14**) is a linear oligosaccharide, while L*N*H (**15**) and L*N*nH (**16**) are branched, linkage isomers as regards the β1-3 branch. As expected, only *p*L*N*H generated a B-ion, Gal-GlcNAc-Gal-GlcNAc (*m*/*z*, 732, 14%), as an SDI, although not in great abundance (Fig. 2d; spectra of *p*L*N*H and L*N*H are shown in Fig. 3). On the other hand, two Y-ions common to **14**, **15**, and **16**, *i.e.*, Gal-Glc-PA (*m*/*z*, 421) and GlcNAc-Gal-Glc-PA (*m*/*z*, 624), were generated 7–14-times more abundantly from *p*L*N*H. This results in relatively low correlation coefficients between linear and branched isomers (0.46–0.53). Apparently, this unevenness is again due to the difficulty in production of MCIs from L*N*H and L*N*nH. On the other hand, differentiation between branched saccharides (L*N*H and L*N*nH) was found to be very difficult (correlation coefficient, 0.99), as far as the LIFT-TOF/TOF procedure was used.

In summary, 11 of 13 isomeric pairs were differentiated by depicting either useful SDIs or IDIs (Scheme 1). The

Table 1. Summary of LIFT/TOF/TOF analyses. The fragment ions observed with the LIFT-TOF/TOF procedure for a set of model PA-oligosaccharides are shown with relative intensities.[a]

Isomer group	01	Group A				Group B			09	10	Group C		13	Group D			17
Sugar ID		02	03	04	05	06	07	08			11	12		14	15	16	
Precursor, *m/z*	745.3	786.3	786.3	786.3	786.3	932.4	932.4	932.4	948.3	989.4	1,078.4	1,078.4	1,135.4	1,151.4	1,151.4	1,151.4	1,281.5
Fragment, *m/z*																	
91	23.2																
145	25.5																
163	30.9																
168				14.8	2.9	3.3	6.7	10.0	19.2		7.2	9.4		9.1	14.4	15.0	4.0
186		12.3	3.9	3.4	5.6	4.5	4.6	2.7	7.4	2.7	3.9	5.2	5.7	11.3	5.1	3.1	6.1
204		29.4	15.0	12.3	24.7	27.1	32.0	23.6	29.3	18.0	29.2	51.2	39.2	55.0	19.3	8.4	47.6
259	100.0	81.9	75.8	100.0	100.0	100.0	98.2	100.0	100.0	38.4	66.6	100.0	100.0	94.3	77.0	66.4	100.0
271													7.3				
350				2.3			3.8				2.5	7.0	3.7				12.8
366		23.2		23.8	19.1	13.0	10.6	18.5	9.0		6.6	9.9	11.9	65.0	43.6	40.4	13.3
405	2.9					9.0	3.2	14.2			68.6	14.1	6.1	3.4			8.1
407										9.6							
421	90.0	100.0	100.0	68.3	61.1	61.8	100.0	89.0	94.7	65.3	37.6	92.6	53.7	100.0	14.4	11.6	90.1
512						9.5	6.2	4.8			4.4	6.0	3.2				3.8
528									24.2								
567						7.0	2.2	10.6			67.8	14.3					9.2
569													4.2				
571				2.1										4.7			
583	23.1	2.6	67.2				2.3			100.0							
589	6.8																
624		23.3	12.8	6.9	18.5	44.5	2.9	2.5	18.7			2.3	95.1	47.7	6.7	3.4	5.4
637		4.2	3.9	4.9	3.9												
715													5.5				3.4
732														14.0			
754														4.5			
770							16.4				3.8	24.4	4.6				61.5
786						46.0	10.9	70.5	5.2	75.6	2.7		4.6	40.4	100.0	100.0	
827										4.4							
916											5.2						
932											100.0	40.1	20.4				5.7
989													35.3	11.2	9.6	3.8	
1,079																	19.9
1,136																	46.5

[a]Relative intensity is defined as the signal intensity relative to that of a fragment showing the highest intensity in each spectrum, and is expressed in %.

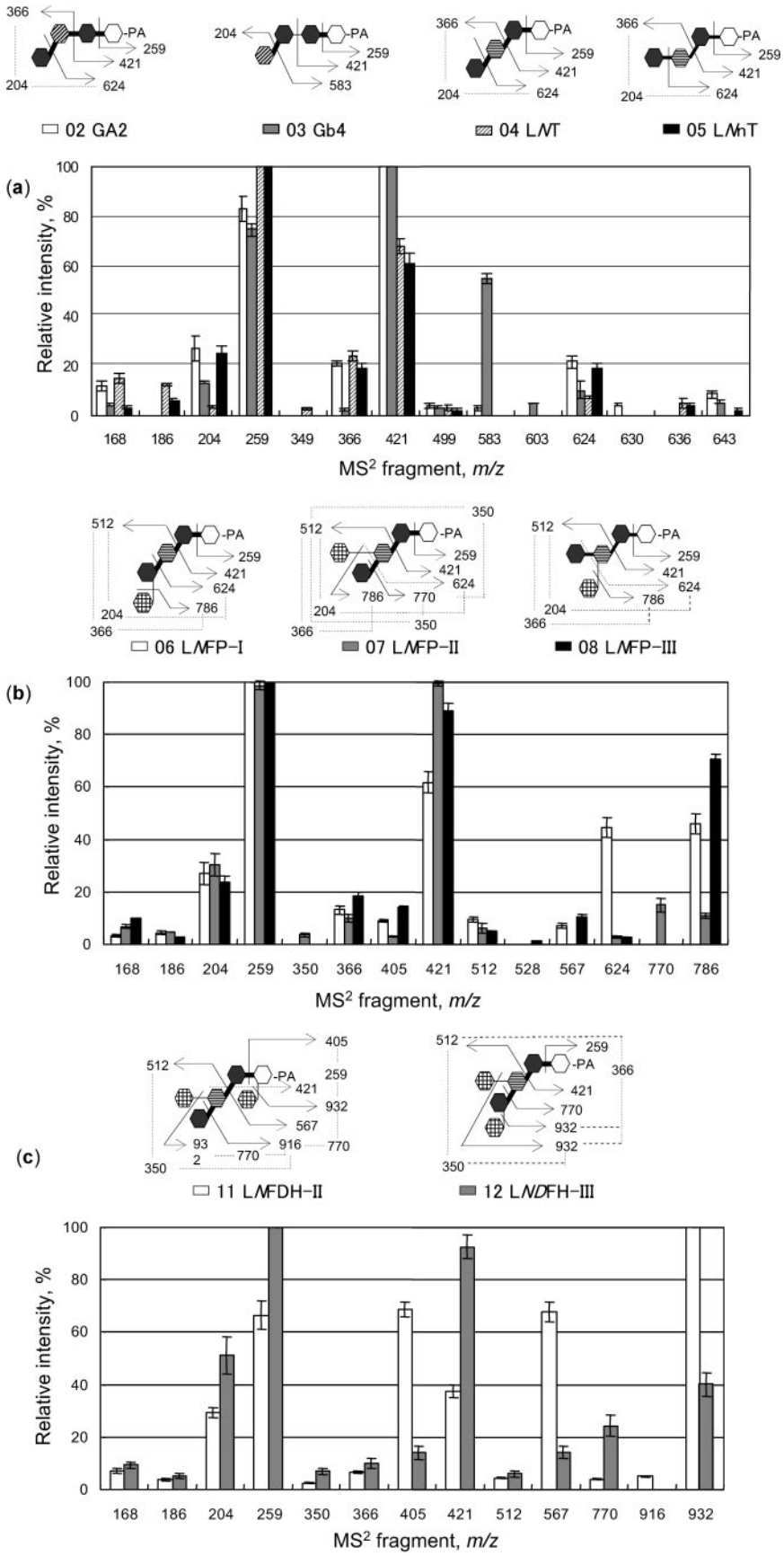


Fig. 2. **Continued.**

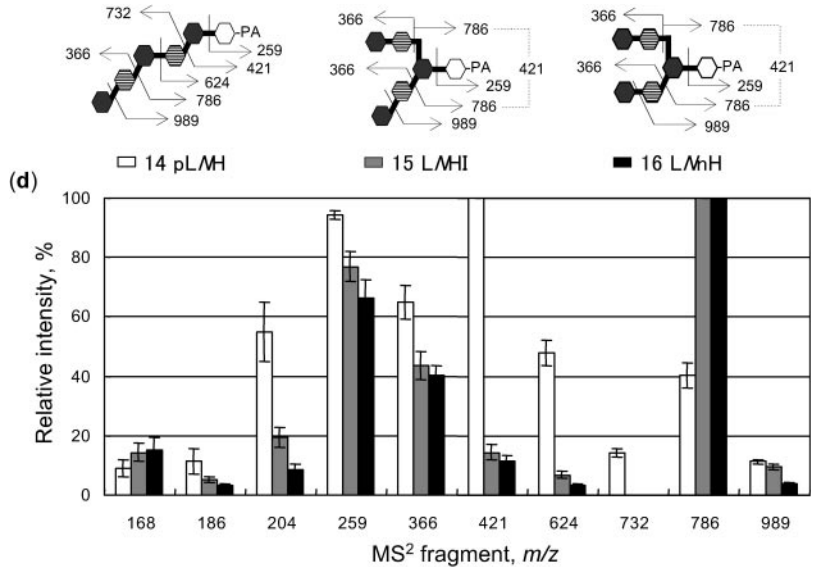


Fig. 2. **Differentiation of structural isomers by MS^2 profiling.** Structural isomers comprise four groups with different *m*/*z* values; *i.e.*, group A (GA2, Gb4, L*N*T and L*N*nT), group B (L*N*FP-I, II and III), group C (L*N*DFH-II and III), and group D (*p*L*N*H. L*N*H and L*N*nH).

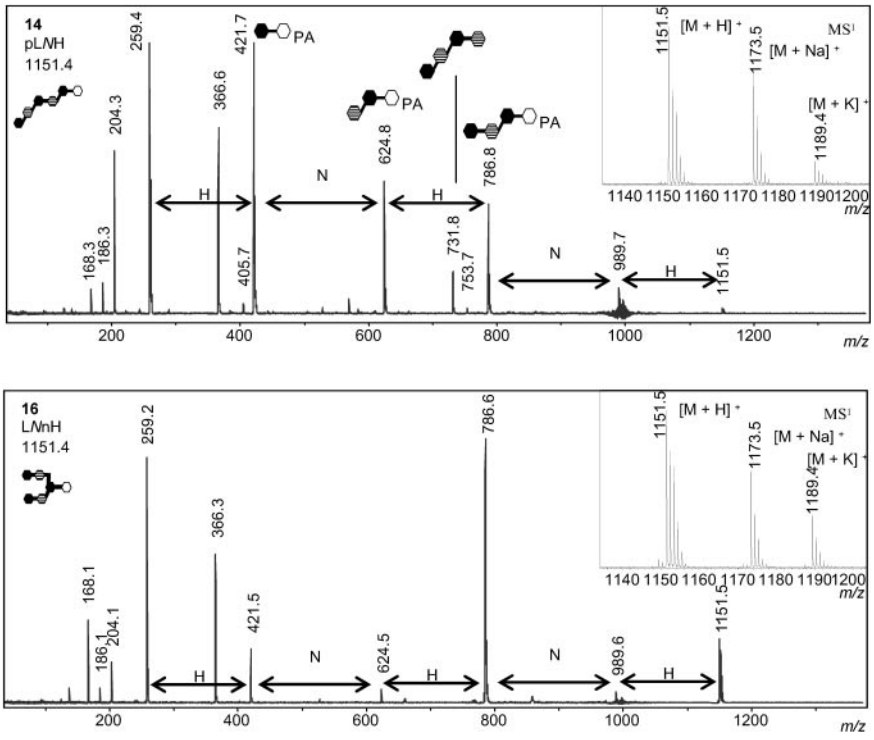


Fig. 3. **Typical LIFT-TOF/TOF spectra of structural isomers, pL*N*H (14) and L*N*nH (16).** The spectra include useful fragment ions, structure-defined diagnostic ions (SDIs) and intensity-defined diagnostic fragment ions (IDIs), for which the assumed fragment structures are shown with *m*/*z* values. The fragment with *m*/*z* = 731 corresponds to an SDI, and the others (*m*/*z* = 786, 624 and 421) to IDIs.

result was well correlated with statistical scoring in terms of correlation coefficient. Notably, both of the remaining unsuccessful cases, *i.e.*, those with a correlation coefficient close to 1.0, were linkage isomers, *i.e.*, L*N*T/L*N*nT (0.97) and L*N*H/L*N*nH (0.99).

Further Characterization of Linkage Isomers by FAC—For further characterization, the second analytical method, FAC, was used. To discriminate type 1 (Galβ1-3GlcNAc)/type 2 (Galβ1-4GlcNAc) chains, we searched for useful lectins by comprehensive lectin-oligosaccharide interaction analysis (*1*, *25*). Among 14 lectins, which showed significant affinity to either L*N*T (type 1) or L*N*nT (type 2) (Fig. 4), galectins from the marine sponge *Geodia cydonium* (GC1 and GC2; *22*) and a plant seed lectin from *Ricinus communis* (RCA-I; *32*) were found to be most relevant for differentiating type 1/type 2 chains: the former bound exclusively to type 1 (Galβ1-3GlcNAc), while the latter much preferred type 2 (Galβ1-4GlcNAc). Thus, in the present analysis, GC1 was used as a type 1-specific probe. As expected, L*N*T and L*N*H containing a type 1 chain were strongly recognized by GC1, whereas L*N*nT and L*N*nH, which lacked a type 1 chain, were scarcely recognized (Fig. 4). Instead, both of the latter glycans were more strongly recognized by RCA-I in a compensatory manner. Thus, GC1 and RCA-I are the best combination for characterizing type 1 and type 2 structures, respectively. For the sake of simplicity, affinity constants (K_a) obtained by FAC are expressed as relative affinities (for

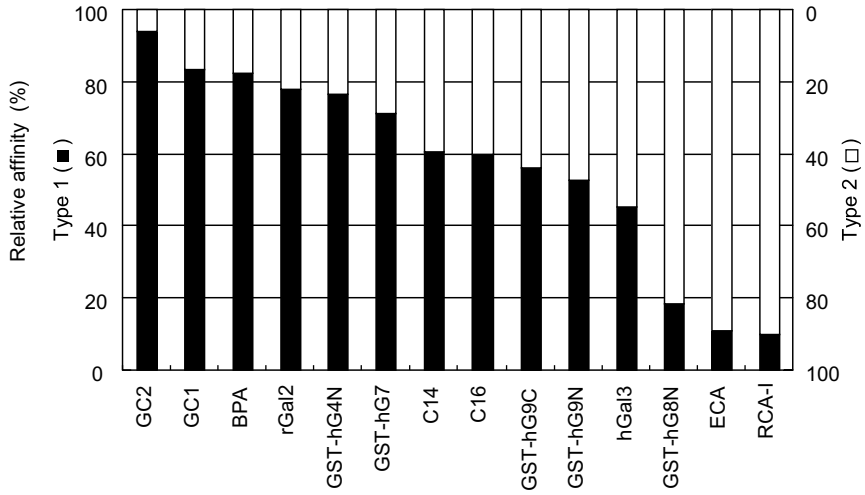


Fig. 4. **Screening probe lectins to discriminate type 1 and 2 chains.** Here, the results of FAC of 14 lectins in terms of relative affinities to L*N*T (**04**) and L*N*nT (**05**) are shown as representative oligosaccharides for type 1 and 2 chains, respectively. Abbreviations: RCA-I, *Ricinus communis* agglutinin; ECA, *Erythrina cristagalli* agglutinin; GST-hG8N, GST (glutathione-*S*-transferase) fusion to human galectin-8 N-terminal domain; hGal3, human galectin-3; GST-hG9N, GST fusion to human galectin-9 N-terminal domain; GST-hG9C, GST fusion to human galectin-9 C-terminal domain; C16, chicken 16K galectin; C14, chicken 14K galectin; GST-hG7, GST fusion to human galectin-7; GST-hG4N, GST fusion to human galectin-4 N-terminal domain (data obtained in this work); rGal2, rat galectin-2; BPA, *Bauhinia purpurea* agglutinin; GC1, *Geodia cydonium* galectin isolectin-1; and GC2, *Geodia cydonium* galectin isolectin-2. The relative affinity of type 1 (solid squares) and type 2 (open squares) was calculated with the following equation; [type 1/(type 1 + type 2)] and [type 2/(type 1 + type 2)], respectively.

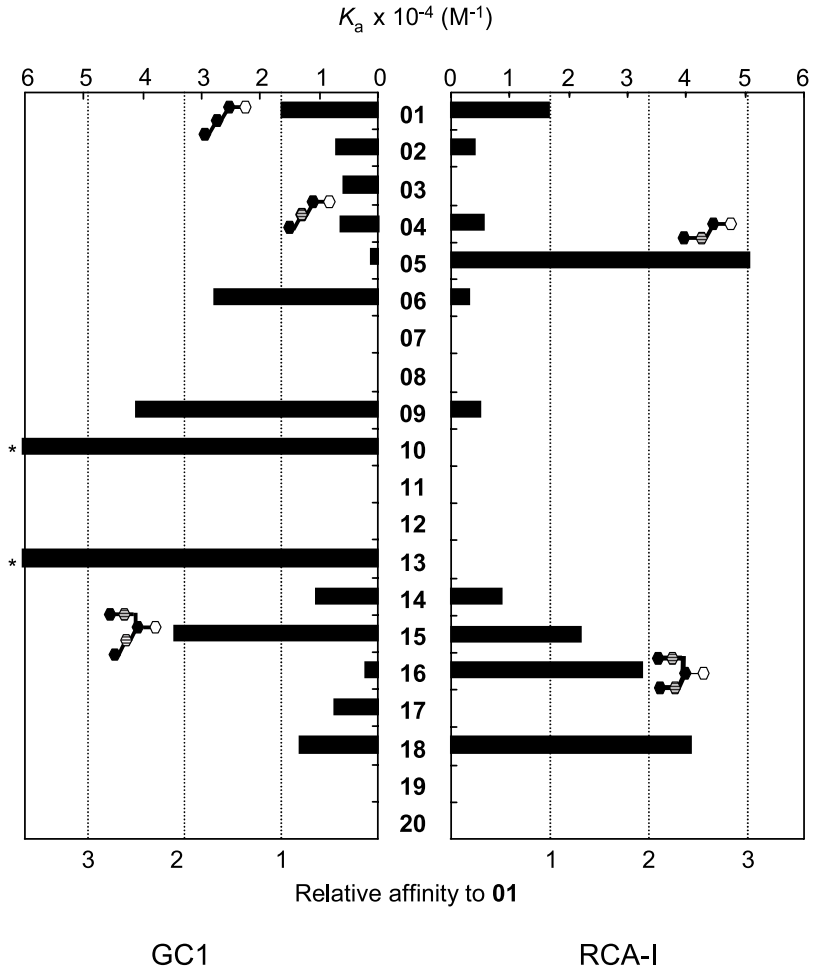


Fig. 5. **The second step of profiling by FAC.** The affinities to two probe lectins, GC1 and RCA-I, of 20 PA-oligosaccharides are shown in terms of both affinity constant (K_a, M^{-1}; the top scale) and relative affinity to oligosaccharide **01** (the bottom scale), respectively.

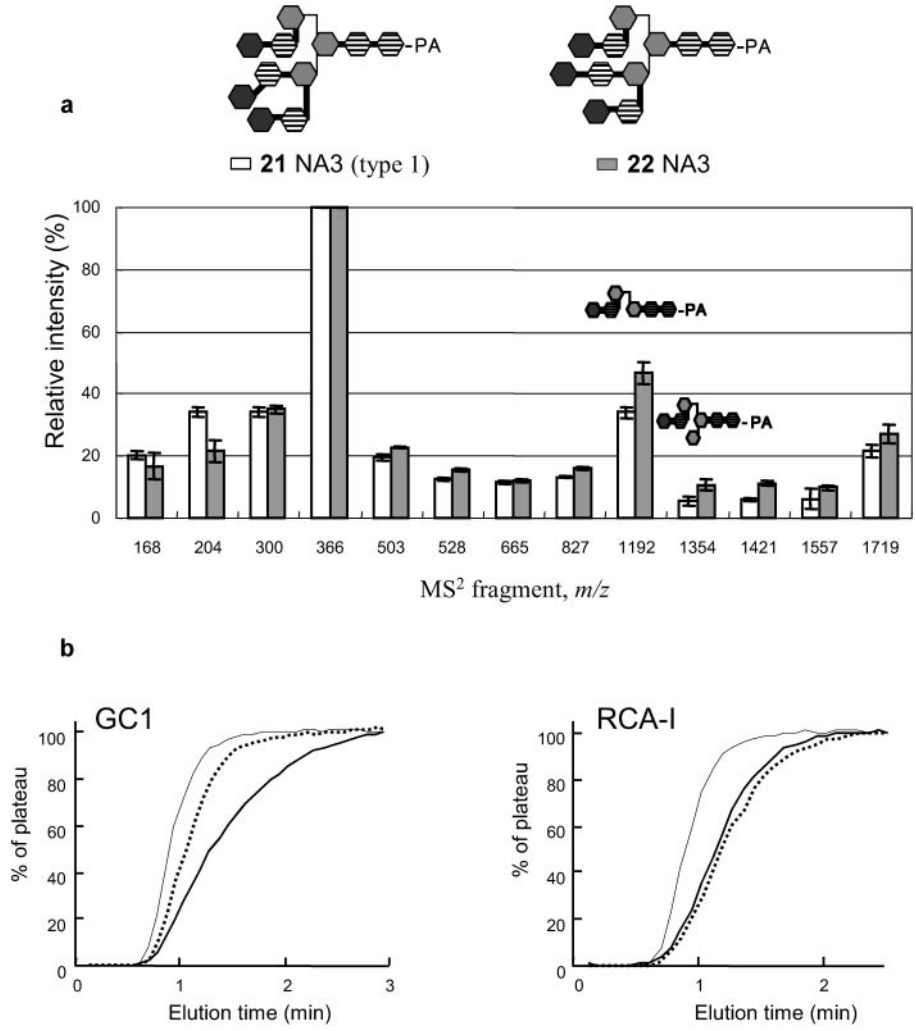


Fig. 6. **Differentiation of isomeric triantennary *N*-glycans (21, 22) by FAC.** (a) Comparison of relative intensities of observed fragment ions in their LIFT-TOF/TOF spectra. (b) Chromatograms obtained for PA-oligosaccharides **21** and **22** are shown by thick solid and broken lines, respectively, whereas those for a negative control PA-sugar (in this case, trimannosyl core M3 structure) are shown by a thin line. The calculated retardation on the GC1 column is 59 (**21**) and 16 μl (**22**), respectively, whereas that on the RCA-I column is 31 (**21**) and 39 μl **(22)**, respectively, in terms of V-V_0 (for experimental details, see Ref. *27*).

normalization, βGal_2Lac (**01**) was taken as a standard for both GC1 and RCA-I) in symmetrical horizontal bar graphs in Fig. 5.

Application of the Strategy to Linkage Isomers of Complex-Type N-Glycans—Recently reinforced FAC has proved to afford highly reproducible data in rapid (5 min for each analysis), simple (isocratic elution), and sensitive (1 pmol for each analysis) manners (*27*). As shown above, FAC provides further structural information, which results in complementation of the defect of LIFT-TOF/TOF MS^2 analysis. To ensure wide applicability, however, the proposed strategy must be evaluated by using more complex oligosaccharides. On this basis, two isomeric triantennary, asparagine-linked oligosaccharides (**21** and **22**; Fig. 1) were chosen: one is a complex-type, triantennary *N*-glycan designated as NA3 (**22**), and the other is one containing a single type 1 chain (**21**). As expected, both oligosaccharides showed closely similar spectra on MS^2 with a correlation coefficient of 0.99 (spectra not shown). In fact, two fragment ions having *m/z* values of 1,192 and 1,421 common to these oligosaccharides showed slightly different relative intensities (Fig. 6a). However, none of them fulfilled the criteria defined in this work (*i.e.*, the 1,192 fragment did not show a >1.5-fold difference, and neither of the 1,421 fragments derived from the two oligosaccharides exceeded >20% relative abundance). Therefore, it was not possible to depict significant IDIs in a conclusive manner as far as the present MS^2 analysis was concerned.

However, these isomers were easily differentiated by employing FAC with the above two lectins as specific probes: the calculated retardation in terms of V-V_0 from the GC1 column is 59 μl (**21**) and 16 μl (**22**), respectively. Thus, GC1 recognized **21** containing a type 1 chain 3.7-times more strongly than **22** lacking one (Fig. 6b). On the other hand, RCA-I recognized **22** (V-V_0, 39 μl) 1.4-times more strongly than **21** (V-V_0, 31 μl). Therefore, in this case, FAC proved to be effective for the discrimination of type 1/type 2 chains, and thus compensated for the defect of the LIFT-TOF/TOF performance.

Although the demonstrated examples are limited, active usage of FAC should be promising as a supplementary means to MS. In the present study, we emphasize that FAC worked well for the discrimination of linkage isomers with the use of GC1 and RCA-I. However, there are many other types of isomers in complex glycans, which cannot be discriminated by simple MS methodology. For this, many useful lectins are available: these include *Aleuria aurantia*

lectin (AAL; *33*) and *Aspergillus oryzae* lectin (AOL; *34*) for detecting the presence of core α1-6Fuc, *Phaseolus vulgaris* leukoagglutinating (PHA-L; *35*) and erythroagglutinating lectins (PHA-E; *36*) for profiling β1-6 linked GlcNAc and bisecting GlcNAc, respectively, and *Bandeiraea simplicifolia* isolectin II (GSL-II; *37*), *Boletopsis leucomelas* lectin (BLL; *38*), and *Agaricus bisporus* agglutinin (ABA; *39*) for characterizing the branching features of *N*-glycans (*40*, *41*). Galectin-1 binds more preferentially to highly-branched *N*-glycans than the repeated structures of *N*-acetyllactosamine, while galectin-3 binds to the latter with much enhanced affinity (*24*). Thus, FAC should contribute to structural analysis of glycans by providing useful information on the glycan epitope (*i.e.*, glycotope) structures of even unknown glycans, which are often associated with biological recognition events. This cannot be easily achieved by any other principle. To our knowledge, there has been no approach combining MS^n and quantitative lectin affinity analysis. In this study, we used for MS^2 profiling using an MALDI-LIFT-TOF/TOF MS machine (Ultraflex). Basically, however, the proposed strategy should be widely applicable to other MS^2 procedures; e.g., electrospray ionization for ionization, and collision-induced dissociation for fragmentation purposes. Special emphasis is made on depicting the essential features of fragmentation in terms of SDIs, IDIs and MCIs.

In this context, we have recently developed an advanced machine for FAC (designated as FAC-T) for systematic glycan analysis, which is equipped with four miniature columns and specialized software, in collaboration with Shimadzu Co., Ltd. The results of glycan analysis using this renewed glycan profiler will be described elsewhere.

CONCLUSION

In this paper, we focused on the differentiation of linkage isomers of relatively small PA-oligosaccharides by means of the combination of MS^2 and FAC. Even though this is a rather simple, model experiment, it is a necessary step toward the development of a universal protocol for glycan analysis that can be applied to diverse glycans including unknown ones. Special emphasis has been made that lectins can be utilized as identification tools for glycan structures, when FAC is employed with established information of lectin-carbohydrate interactions. Although LC separation and MS analysis are required as ever prior to analysis by FAC, the combination of these analytical methods, of which the principles are basically different, proved to be effective to some degree. For this extension, a more systematic study involving >100 lectins and >100 glycans is in progress with the aid of bio-informatics. The results and a related public database will be described elsewhere.

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