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High-Yielding and Controlled Dissociation of Glycosides Producing B- and C-Ion Species under Collision-Induced Dissociation MS/MS Conditions and Use in Structural Determination

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Collision-induced dissociation (CID) in mass spectrometry is a powerful technique with which to understand gas-phase chemical reactions. A mass spectrometer is used to carry out the reaction, isolation, and analysis. On the other hand, structural analysis of glycan structures is of extreme importance in the analysis of biomolecules, such as glycoproteins and glycolipids. In the analysis of glycan structures based on CID, certain ion species, including B-/Y-, C-/Z-, and A-/X-ions, are produced. Among these ions, we are interested in C-ion species that carry a glycosyl oxygen atom at the anomeric center and that possibly provide information regarding anomeric configuration. A method for generating C-ion species when necessary is thus considered to be important; however, none is currently available. In this study, synthetic glycosides carrying a series of aglycons were analyzed with the aim of identifying suitable glycosides with which to produce C-ions to be used in the structural determination of oligosaccharides. The results showed a 4-aminobutyl group was an excellent candidate. Furthermore, the use of C-ion species obtained in this manner in the structural characterization of a ganglioside, GM3, is described. The type of glycoside is believed to be valuable not only in structural analysis but also in biological investigation, because of the existing amino functionality that has been proven to be useful by enabling the generation of conjugates with other molecules and materials.

The structural characteristics of oligosaccharides are quite different from other biopolymers such as nucleic acids and peptides. Diversity is generated from sequential combination in the latter two types of polymers, whereas anomers, linkage position, branching, and sequence are factors in oligosaccharide structure. Furthermore, the molecular diversity of oligosaccharides is described using the term glycoform. This class of molecule thus has potential to form an enormous number of structures.[1] Despite the difficulties encountered in structural determination, it has been reported that glycosylation as a posttranslational modification (PTM) of protein affects the secretion of glycoprotein.[2,3] The involvement of PTM in cellular polarity has also been reported.[4,5] It is widely recognized that changes in the composition of glycosphingolipids are associated with development and differentiation.[6] Considering the need to analyze minute amounts of glycoconjugates obtained from biological samples as well as the existence of glycoforms, it is believed that the use of mass spectrometry-based analytical methods in the analysis of glycoconjugates is a logical consequence.[7−9] Structural analysis based on collision-induced dissociation (CID) is a very powerful technique in this regard.[10,11] It is known that there are spectral differences among isomeric compounds. In most cases, there are some differences in signal intensities, while in other cases, different fragment ions can be found. These phenomena are now used in a more systematic manner to identify oligosaccharides by spectral matching.[11−17] In structural elucidation, descriptive ion

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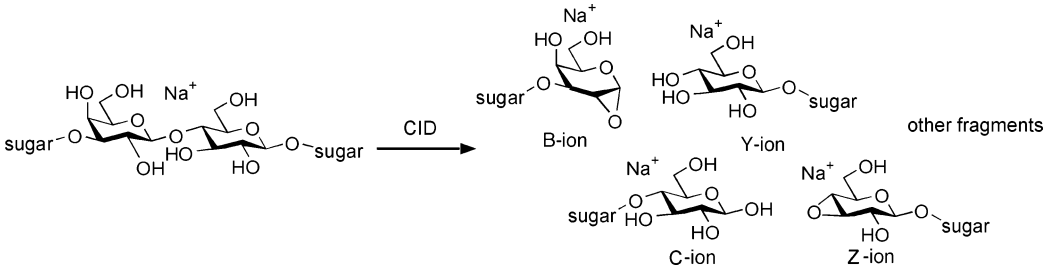
Scheme 1. Precursor Ion of a Glycan Producing a Series of Fragment Ions under CID Conditions



species, such as B-/Y-, C-/Z-, and A-/X-ions, are analyzed (Scheme 1).[18] The elucidation of a totally unknown structure that is not present in a database is an important problem that still remains after introduction of this efficient methodology. In such a case, an approach based on principal component analysis is considered to be effective; however, the source of information to be analyzed has been far from adequate and consists of limited structural isomers only.[19,20] For this reason, a combinatorial oligosaccharide library should be used as a source of information.[21−26] A library consisting of isomeric compounds with logical structural arrangements should provide indispensable information regarding structural characteristics. We have previously reported that important information could be obtained from library compounds based on energy-resolved mass spectrometry (ERMS).[27,28] In order to obtain fragment ions possessing useful information, we investigated glycosides carrying various aglycons and determined that some of the aglycons specifically provide B- and C-ions. Although B-ions are produced from various glycosides, no report has described the production of C-ions despite the fact that the ions are often obtained as a fragment under CID conditions. We report herein our findings regarding high-yielding and selective dissociation of sodiated glycosides; that is, 4-bromobutyl and 4-(*n*-octyloxy)phenyl glycosides are useful for producing B-ions and 4-aminobutyl glycoside produces C-ions in good yield.

MATERIALS AND METHODS

Materials. *General Method for Chemistry.* Dried solvents were used for all chemical reactions. Solutions were evaporated under reduced pressure at a bath temperature not exceeding 50 °C. Analytical thin-layer chromatography was performed on Merck Art. 5715, Kieselgel 60 F_{254}, 0.25-mm-thickness plates. Visualization was accomplished with UV light and phosphomolybdic acid or sulfuric acid solution followed by heating. Column chromatography was performed with Merck Art. 7734 silica gel 60, 70−230 mesh. Optical rotations were measured in a 1.0-dm tube with a Horiba SEPA-200 polarimeter. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded with an Avance 500 spectrometer (Bruker Biospin Inc.) in deuterated solvents using tetramethylsilane as an internal standard.

Synthesis of Compounds **1**α/β*−*__8__α/β. The detailed procedures and structural characterizations for the synthesis of compounds **1**α/β−**8**α/β are described in the Supporting Information. For structures, see Chart 1. A typical procedure is described for the precursor of compound **5** (**12**) as follows (Scheme 2). A phenylthio glycoside of lactose (**9**) was treated with NaH and BnBr in *N*,*N*-dimethylformamide (DMF) to give perbenzylated lactoside (**10**), which was then dissolved in dichloroethane (DCE) glycosylated with benzyloxycarbony (Cbz) protected 4-aminobutanol in the presence of *N*-iodosuccinimide (NIS), and a catalytic amount of trifluoromethanesulfonic acid (TfOH) to afford the glycosylated compound **11** in moderate yield. Compound **11** was hydrogenated, using $Pd(OH)_2$ on charcoal in the presence of di-*tert*-butyldicarbonate (Boc_2O), to provide a mixture of compounds (**12**). The Cbz group was first removed to give an amino functionality, which was then protected in situ with a Boc group. Compounds **12**α and **12**β were finally separated using HPLC.

Derivatization of GM3. A ganglioside, GM3 (>98%, bovine brain), was purchased from HyTest Ltd. (Turku, Finland). GM3 was esterified using MeI (extra pure; Nacalai Tesque, Inc., Kyoto, Japan) in dimethyl sulfoxide (extra pure, Merck, Darmstadt, Germany).

Solvents for Mass Analysis. For analyses using a mass spectrometer, HPLC grade solvents were used; MeOH and $CHCl_3$ (Wako Pure Chemical Industries, Ltd, Osaka, Japan). Deuterated methanol-d_4 (NMR Grade) was used for the D−H exchange experiments.

Instrumentation and Data Collection. Samples of disaccharides were analyzed using a quadrupole ion trap mass spectrometer coupled with an electrospray interface (Bruker Esquire 3000 plus, Bruker Daltonics GmbH, Bremen, Germany). Samples dissolved in MeOH (0.01−0.1 μmol/mL) were introduced into the ion source via infusion (flow rate, 120 μL/h). The parameters for the analysis were as follows: (1) "dry temperature", 250 °C; (2) nebulizer gas (N_2), 10 psi; (3) dry gas (N_2), 4.0 l/min; (4) "Smart frag.", off; (5) scan range, m/z 50−750; (6) compound stability,

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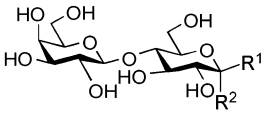
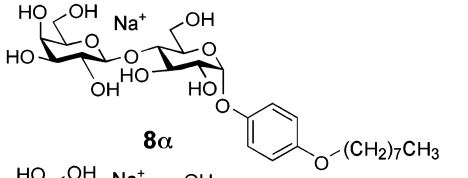
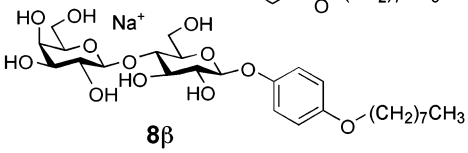
Chart 1



compound	R^1	R^2	n	X	adduct
1α	H		8	H	Na^+
2α			4	Br	
3α			2		
4α			3		
5α			4	NH_2	
6α			5		
7α			6		
1β			8	H	
2β			4	Br	
3β			2		
4β		H	3		
5β			4	NH_2	
6β			5		
7β			6		
5β			4		H^+

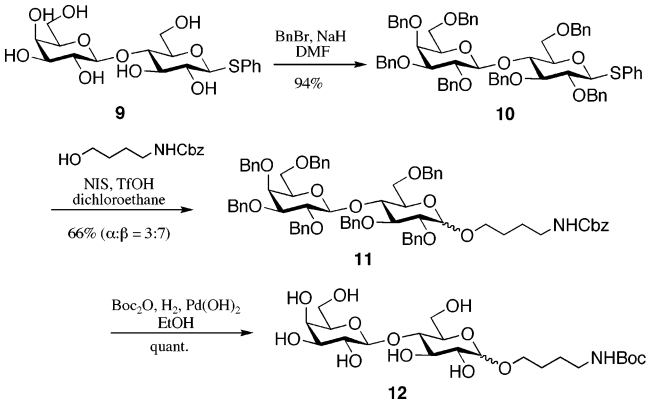


8α



8β

Scheme 2. Synthesis of Compound 12



9 **10** **11** **12**

300%; (7) ICC target, 5000; (8) maximum acquisition time, 200 ms; (9) average, 10 spectra; and (10) "cutoff", 27.6% of the corresponding precursor ion.

In our MS^n experiments, the end cap rf amplitude was raised by 0.02-V increments until the precursor ion could no longer be detected (plateau at less than 0.9% of total ion current). Only end cap rf amplitude was controlled during the CID experiments. The He pressure was 4.86×10^{-6} mbar, and the CID time was 40 ms. Averages of $m - 4$ spectra were used for CID experiments ($m = 14$: where m is the number of spectra obtained during the experiment); the first and the last two data sets, which are associated with a transient period to steady state, in an rf amplitude step were not used in order to avoid any inaccuracy.

Isotopic peaks with $[I^i + 1]$ and $[I^i + 2]$, where I^n indicates a fragment ion, were included in the calculations. (See also Data Handling.) For the isolation of a product ion, $m/z \pm 2$ ($w = 2$) were isolated and subjected to the CID experiments to include isotopes. Standard MS/MS spectra are the extracts of these ERMS at a designated amplitude.

Data Handling. In order to obtain graphs of the ERMS, the following equations were used. When an ion "I^P" produces a series of product ions, I^1, I^2, I^3,... I^i, the relative ion currents for individual ions were defined by the equation,

$$^{\mathrm{rel}}C = \frac{C^{\mathrm{I}^i}}{C^{\mathrm{I}^P}} \times 100 \quad (1)$$

$$^{\mathrm{rel}}C = \frac{C^{\mathrm{I}^i}}{C^{\mathrm{I}^P} + \sum_{i=1}^{n} C^{\mathrm{I}^i}} \times 100 \quad (2)$$

where $^{\mathrm{rel}}C$ indicates the ion current of a given ion among observed ions in percentage, C^{I^i} is the observed ion current in focus, and C^{I^P} is the ion current of a precursor ion. The calculations were performed using a program we developed with Excel (Excel 2000 (Microsoft Co.)), which was based on DSUM function and programmed to choose a range of isotopes (w) to be taken into consideration ($w = 2$ in the experiments).

RESULTS AND DISCUSSION

As explained above, B- and C-ion species are often observed under CID conditions during structural investigation of oligosaccharides. For this reason, if these ion species of specific sequences can be made readily available, they can be used as references for comparisons in the structural determination. We have been attempting to identify suitable aglycons to be used in structural elucidation, providing such glycon-oriented ion species when needed. ERMS is a very powerful tool for the structural analysis of isomeric compounds such as oligosaccharides and is considered to be a suitable method with which to examine the details of the dissociation profile under CID conditions.[29−40] With

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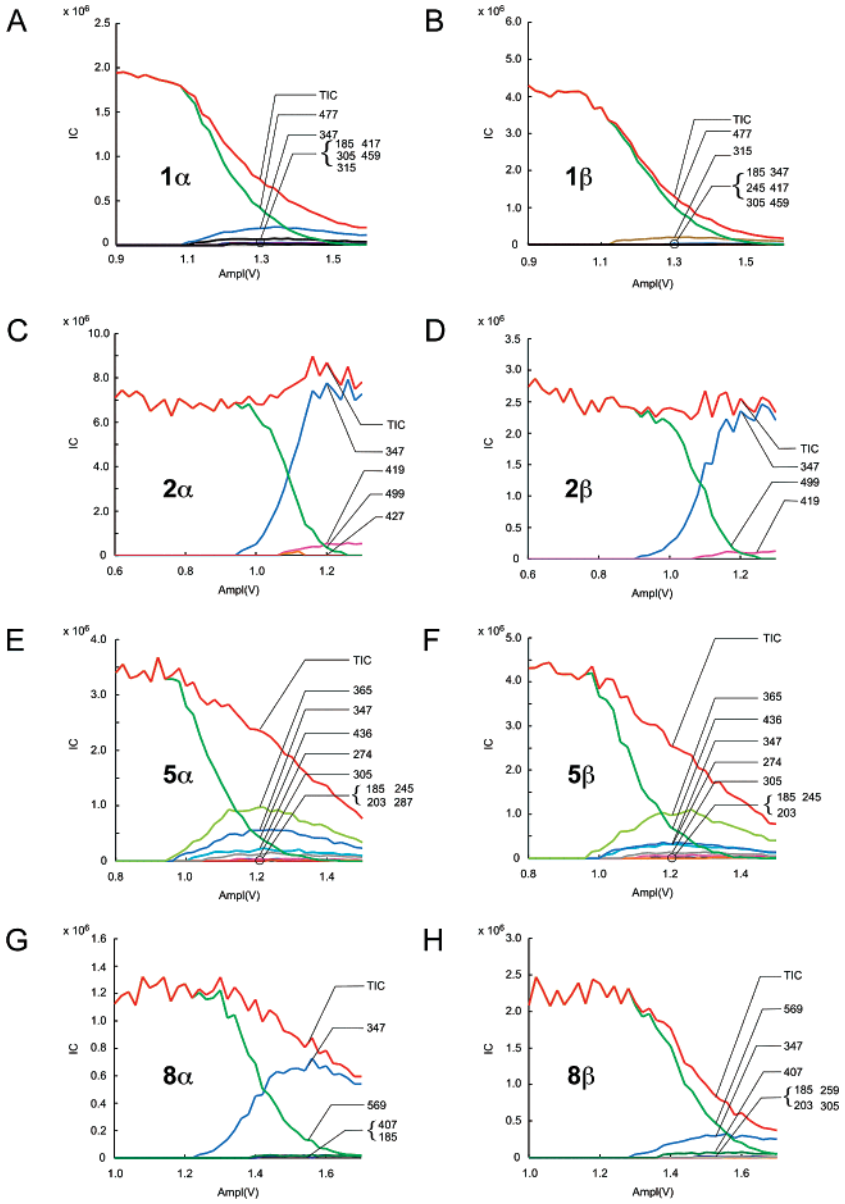

Figure 1. Representative energy-resolved mass spectra for $[M + Na]^+$. Representative fragment ions: red, TIC; green, precursor ion; blue, B_2-ion (*m/z* 347); beige, Y_1-ion; pink, butenyl compound; light green, C_2-ion (*m/z* 365).

this aim in mind, we prepared a series of compounds (**1**α/β−**8**α/β). (Details of syntheses are provided in Supporting Information (SI) section 1).

Analysis of Energy-Resolved Mass Spectrum of a Series of Glycosides. We conducted CID experiments using these compounds to determine suitable glycosides that produce B- and C-ion species using a disaccharide (lactose) structure as an example. Sodiated precursor ions ($[M + Na]^+$) were collided with He gas in an ion trap mass spectrometer to produce a series of product ions with various end-cap electrode amplitudes. Representative ERMS thus obtained are shown in Figure 1, and the fragmentation pathways during the CID process are depicted in Scheme 3. An *n*-octyl α-lactoside (**1**α) produced B_1-ion (*m/z* 347) as a major fragment ion followed by Y_1-ion (*m/z* 315) in about one-third of the B_2-ions, whereas its counterpart, β-glycoside (**1**β) produced Y_1-ion as a major fragment (Figure 1A,B). This type of difference in fragmentation depending on anomeric configuration is often observed and is useful in discriminating anomers.[1,27,28,39,41] Here, we have focused on the overall ionic profiles where the total ion current (TIC) gradually decreased to 10.3 and 4.0% for octyl α- and β-lactosides, respectively, over a range of 0.7 V during the CID experiments (This amplitude range was determined based on the observation that all compounds used in the experiments dissociated.). This drastic decrease in "ion quantity" will most likely be problematic in future glycan analysis using an oligosaccharide library as a structural information source. Therefore, the ion recovery needs to be improved. Furthermore, B_2- and C_2-ions in these cases will be used for further MS/MS analysis to provide more detailed information regarding the structures of these ions. These data obtained at the MS^n stage of CID experiments are

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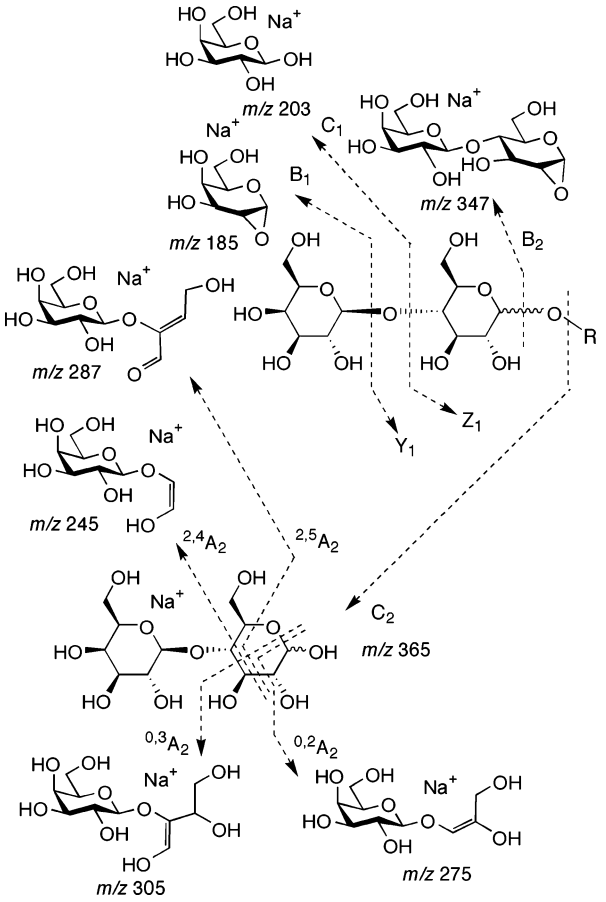
Scheme 3. Generalized Fragmentation Pathways of the CID Process of Lactosyl Glycosides



useful in structural analysis based on "spectral matching" between a fragment ion generated from a glycan and one from a library.

Dissociation of 4-Bromobutyl Lactoside. We examined the CID of Na^+ adducts of 1-bromobutyl α- and β-lactosides (**2**α and **2**β). The ERMS are presented in Figure 1C,D. As is clear from the spectra, the "ion yields" for both anomers were improved, which was surprisingly found to be quasi-quantitative over a 0.7-V range. It was found that B_2-ion is generated preferentially in the dissociation of bromobutyl glycosides. It is thought that lower activation energy was required for the cleavage of a Br−C bond and is responsible for the high-yield reaction. We believe that the difference in dissociation energy for Br−C bonds and coordination of Na^+ on multiple oxygen atoms is particularly important because desired ion species can be generated before Na^+ dissociates leaving a neutral molecule. Also, involvement of a cyclic intermediate was suggested in this preferable dissociation because a sodiated lactosyl bromide (*m/z* 427) was observed as well in an α-glycoside (Scheme 4A). The presence of a sodiated butenyl glycoside (*m/z* 419) observed in both anomers suggested the involvement of three competing paths, B-Ion generation (path a), bromination (path b), and an elimination (path c), in the dissociation. Regardless of the transition states and stereochemistry of the products, it was shown that the bromobutyl glycoside might be useful in structural analysis by providing B-ion.

Dissociation of 4-Aminobutyl Lactoside. We further investigated the dissociation of glycosides based on a similar mechanism proceeding via a possible five-membered transition-state. The next examples are 4-aminobutyl glycosides (**5**α and **5**β) obtained from the Boc-protected precursor at MS^2, where we expected a long-distance hydrogen abstraction reaction might take place through a cyclic transition state (Scheme 4 B, path a). (See SI, section 2.1 for the generation of the ion from the precursor ion **5**.) In this reaction, we anticipated that a pyrollidine would be released when a C_2-ion species is generated. The ERMS indicated that the C_2-ion (sodiated lactose equivalent) was indeed a main product in both anomers (Figure 1E,F). The ion yields obtained for aminobutyl α- and β-glycosides were 22.6 and 18.1%, respectively, over a 0.7-V range. Although the ion yields were not comparable to those of bromobutyl glycosides over this range of rf amplitudes, MS/MS analysis is usually carried out at an rf amplitude where the desired ion species is produced in the greatest amount, which is ~1.2 V in each anomer in this case. The ion yields at 1.2 V were 69.9 and 59.3%. A series of fragment ions was obtained where C_2-ion was the most abundant.

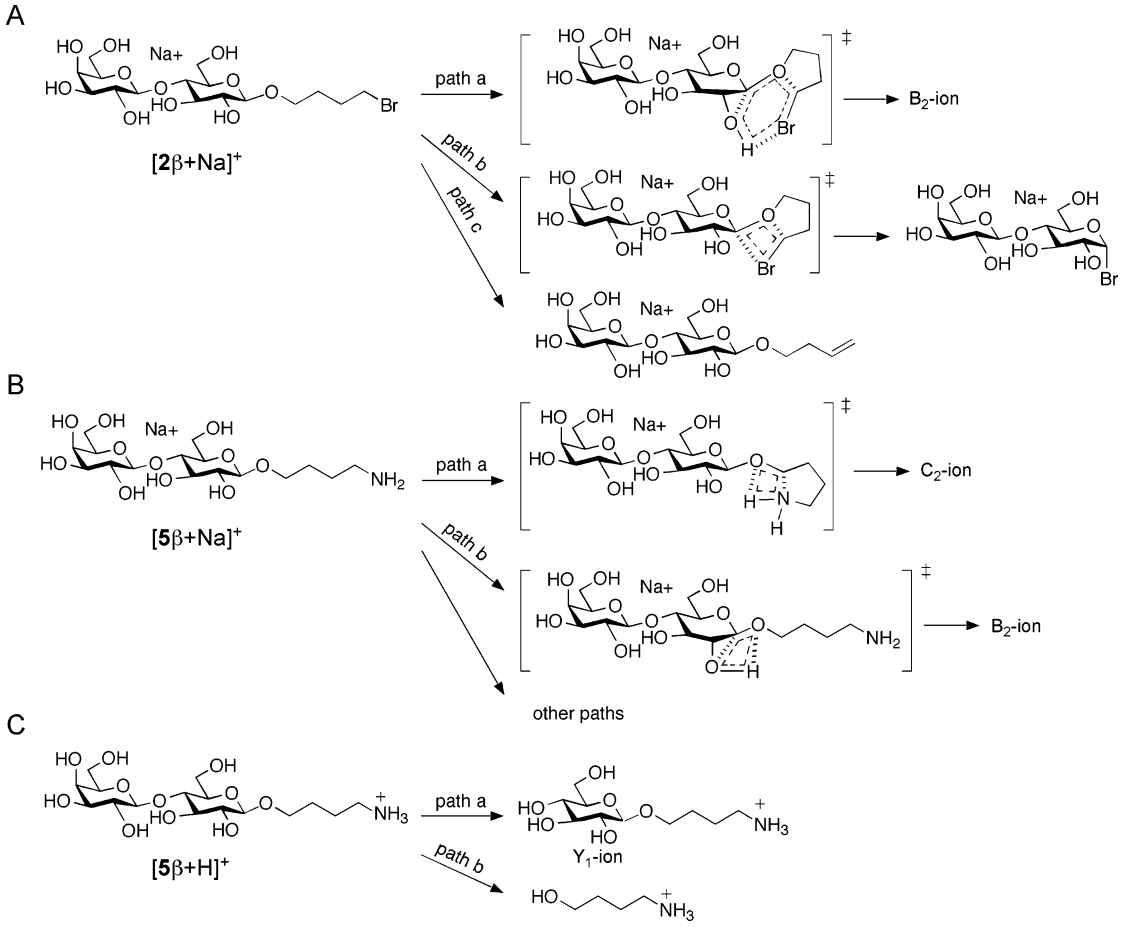
Table 1

	generated ion species, %							
	B_2-ion (*m/z* 347)		Y_1-ion (Gal-cleavage)		$^{0,3}A_2$-ion (*m/z* 305)		C_2-ion (*m/z* 365)	
compd	α	β	α	β	A	β	α	β
1	9.4	0.6	3.5	4.2	0.4	0.7	0.0[a]	0.0[a]
2	99.5	79.2	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]
3	16.2	3.6	11.0	11.2	0.4	2.1	0.0[a]	0.0[a]
4	16.4	7.5	11.4	10.9	0.5	2.4	0.0[a]	0.2
5	15.8	7.8	5.9	6.9	3.8	3.0	26.7	23.3
6	21.0	3.7	9.4	10.7	1.0[a]	1.5	4.3	3.5
7	17.9	2.8	8.6	12.0	0.0[a]	1.0	0.4	0.2
8	50.0	12.4	1.6	2.8	0.0[a]	0.0[a]	0.0[a]	0.0[a]

[a] Not observed. Yields were calculated at an amplitude range of 0.1 V where the graphs of a series of ions were maximum.

Possible Dissociation Mechanism. In order to ascertain the mechanism of this dissociation reaction, the effect of chain length on the dissociation reaction was evaluated based on ERMS. It was found that the generation profiles of C_2-ions from sodiated compounds **3**−**7** were similar to those in the radical cyclization reactions (Table 1). Furthermore, the ratio of the C_2-ion yields from α- and β-lactosides is 1.1, which indicates that dissociation of the aglycon was independent of the stereochemistry at C-1. This is in contrast to the fact that the energy requirement for the α- and β-glycosides differs[27,28,39] and supports the reaction mechanism shown in Scheme 4B. Since no radical species were observed, it is believed that the reaction proceeded in a concerted fashion or the hydrogen abstraction process was completed within an experimental period (40 ms). We looked for a pyrrolidine, possibly produced in the reaction, since it would represent strong evidence; however, one was not observed. However, a CID experiment using a deuterated compound did produce a deuterated C_2-ion, indicating that deuterium was transferred from an amino group and strongly supporting our model (SI; section 2.2). In the dissociation, a considerable amount of A-series ions was observed. A-Ion species are usually generated from hemiacetals; therefore, we believe that the observed A-ions were produced *via* C_2-ion (Scheme 3). Regardless of the actual mechanism, it should be noted that this is the first report that has described obtaining C-ions based on a mechanism-based dissociation reaction.

Scheme 4. Anticipated Reaction Mechanism of 4-Substituted Butyl Lactosides.



Further analysis of ERMS showed that the profiles of B_2-ion generation are similar among *n*-octyl (**1**), 4-aminobutyl (**5**), and octyloxyphenyl glycosides (**8**), with the exception of 4-bromobutyl glycoside (**2**) (Figure 2). The general tendency that α-glycosides in each set required less activation energy (provided by rf amplitude) is in agreement with our previous results, which indicated a dependency on anomeric configuration. The profile of B_2-ion generation for **2** (Figure 2B) therefore suggests that the mechanism of the bromobutyl group occurred independently of the anomeric configurations (Scheme 4A). The generation profile for C_2-ion in **5** (Figure 2D) is similar to that of **2**, which indicates again that the dissociation reaction to produce the ion species is independent of anomeric configurations and supports our proposed mechanism (Scheme 4B).

One of the possible reasons that neither a furan nor a pyrrolidine was detected in these reactions was that the reactions were not promoted by the Na^+, as is usually the case. In this model, Na^+ is coordinated most likely at multiple oxygen atoms between Gal and Glc, but the dissociation took place at a location remote from the site around the aglycon. We conducted more extensive synthetic and CID experiments and confirmed the formation of pyrrolidine (Data will be reported elsewhere.). Contrary to the dissociations of sodiated compounds, no C_2-ion was observed in the dissociation of protonated **5***β* (Scheme 4C).

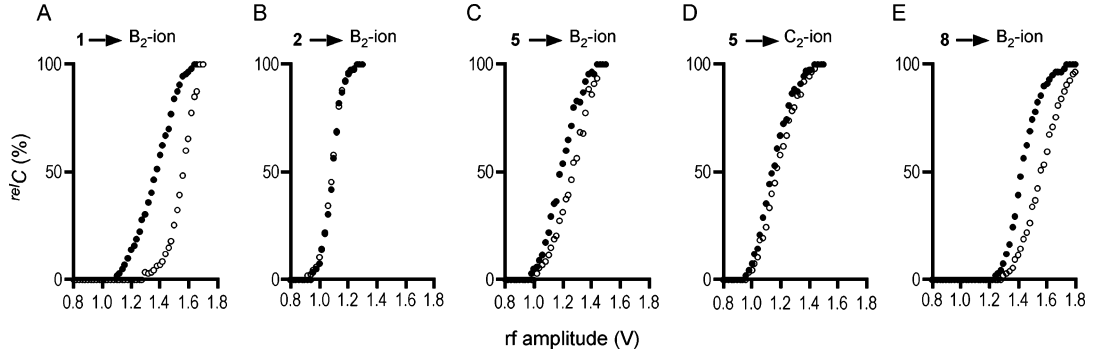


Figure 2. ERMS of sodiated compounds **1**, **2**, **5**, and **8**. Ratios of individual product ions (*m/z* 347 and 365) over respective precursors were calculated to enable discussion of generation profiles. Closed circle, α-glycoside; open circle, *β*-glycoside. The relative ion current (^{rel}C) was calculated based on eq 1.

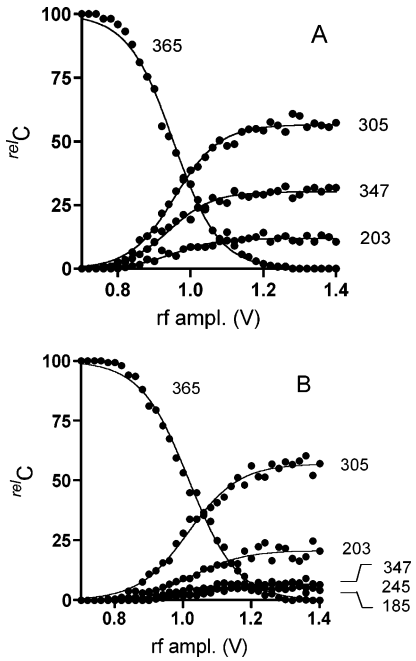


Figure 3. ERMS of C-ion species from [**5**α + Na]$^+$ and [**5**β + Na]$^+$. (A, B) ERMS spectra obtained for C_2-ions from 4-aminobutyl α- and β-lactosides, respectively. The relative ion current (^{rel}C) was calculated based on eq 2.

Both Y-ions that originated with a reducing terminus were observed, although no nonreducing end associated ion (B- and C-ions) was observed. (SI; section 2.3.1) This suggests an amino group is protonated, and yet glycosidic bond cleavages took place. The above two reactions can be explained by a charge remote process. This might be related to our and other observations that α-glycosides are labile under CID conditions, which is the opposite of commonly found protonation-associated solution chemistry.[27,28,39,41] Similar phenomena can be found for a pair of pyridylaminated oligosaccharides as well (SI; section 2.3.2). Although other mechanisms including cation-induced dissociation may exist, the possibility of a charge-independent dissociation mechanism should be considered.

With regard to bromobutyl glycosides, their high potential value for producing B-type ion species in good ion yield is going to be useful in the future development of MS-based analytical methods for glycan structures. It was also found that a phenyl glycoside (**8**), especially α-glycoside (**8**α), produced B-ions in a good yield (50.0%) (Table 1) and that it could be an alternative to a bromobutyl glycoside when a synthesis does not permit its use (Figure 1G,H).

C-Ions from a Pair of Anomers. We examined a series of ERMS spectra obtained for α- and β-aminoalkyl glycosides of

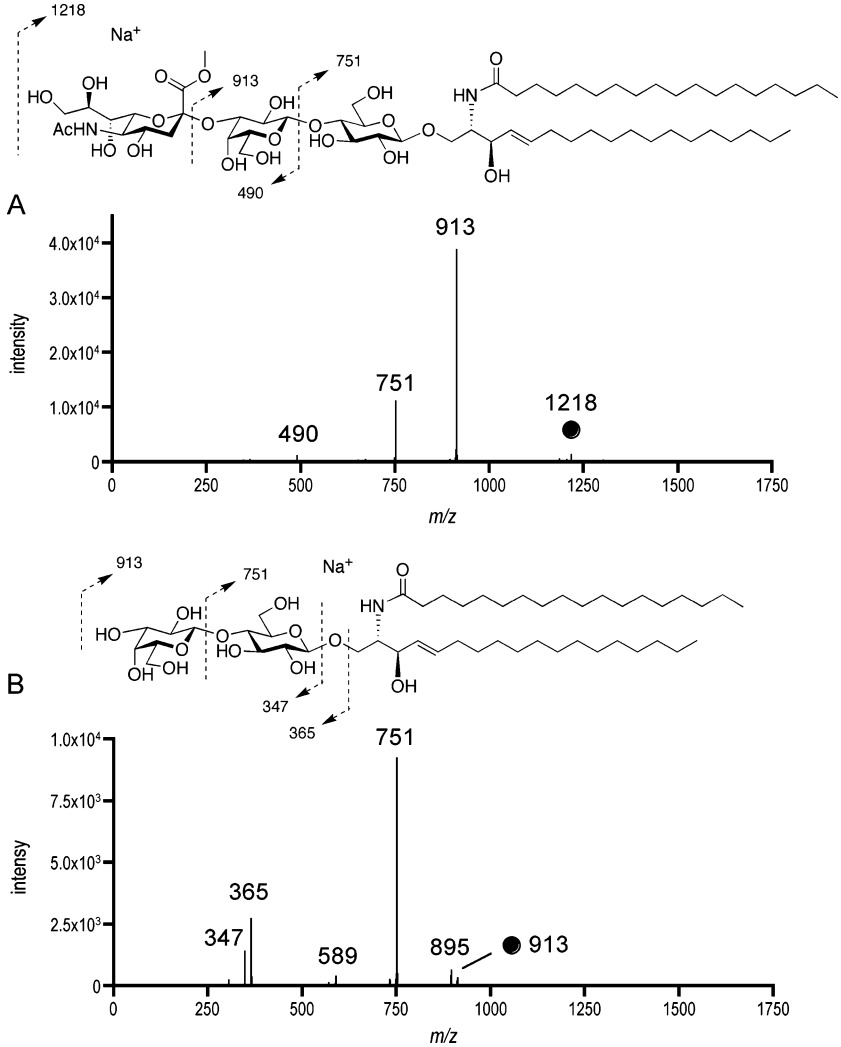


Figure 4. MS/MS analyses of a ganglioside GM3. (A) MS/MS analysis of sodiated GM3 methyl ester; (B) MS/MS of Y_2-ion (sodiated lactosyl ceramide).

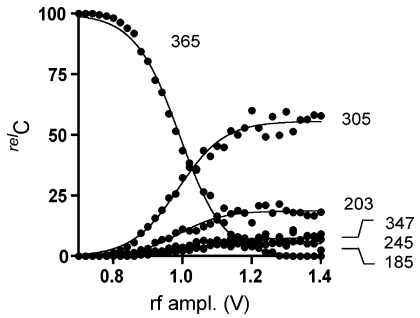


Figure 5. ERMS of C_2-ion obtained after MS^3 of GM3. The relative ion current (^{rel}C) was calculated based on eq 2.

lactose focusing on the generation of C_2-ions and found that the 4-aminobutyl glycosides are excellent candidates for this particular purpose. We now discuss the stereochemistry of thus obtained C_2-ion species, the chemical structures of which resemble sodiated α- and β-hemiacetals, and in this case lactose. The individual C_2-ions were further examined under CID conditions (ERMS), and the analyses resulted in completely different spectra. (Figure 3A,B) According to the fragmentation process of the respective precursors and the fragment ions generated (see fragments presented in Scheme 3), we are convinced that both C-ions are lactoses, although with different anomeric configurations. This indicates that anomers of hemiacetals have distinct behaviors and are distinguishable from each other, and furthermore, this suggests the C-ion species can be used in the structural determination of fragments, including their anomeric configurations. In order to utilize this finding, we decided to perform a CID analysis of a ganglioside, GM3, which contains a β-lactosyl glycoside in its structure. We conducted a CID experiment of a sodiated ion of a methyl ester of GM3 (m/z 1218)[42] from bovine brain that produced Y_2-ion (m/z 913), the structure of which resembled a sodiated lactosyl ceramide, as the most abundant ion. (Figure 4A) We used a methyl ester of GM3 because it is known that the glycosidic bond of esterified sialic acid becomes more stable under CID conditions, which would enable the production of various ion species. Among the fragment ions generated, the Y_2-ion was isolated and further analyzed under CID conditions. Figure 4B shows that the C_2-ion (m/z 365) was produced while the abundant ion was the Y_1-ion (m/z 751). C_2-Ion obtained in this manner was then analyzed by means of ERMS, as shown in Figure 5. Comparison of the ERMS and ones obtained from α- and β-aminobutyl glycosides of lactose (Figure 3) resulted in confirmation that the C_2-ion derived from the ganglioside GM3 had indeed a β-glycosidic linkage between glucose and the ceramides. This suggests that C-ion species are useful in the structural determination of fragments obtained at MS^n and also indicates the importance of an aglycon that generates C-ions.

(42) Handa, S.; Nakamura, K. *J. Biochem.* **1984**, *95*, 1323−1329.

CONCLUSION

We succeeded in identifying very useful aglycons that provide a series of fragment ions that are useful in MS/MS-based structural analysis. It has been impossible until now to obtain C-ion species when needed. A 4-aminobutyl glycoside was introduced for the first time to overcome this problem. The use of a specific type of aglycon is advantageous because of their adaptability in solid-phase extraction, when they are suitably protected, and their potential to couple with other materials using the amino group after deprotection. Thus, it is hoped that the use of the type of glycoside will not be limited to analyses but will also be very useful when constructing conjugates and arrays for functional investigation. The importance of C-ion species in the structural determination of naturally occurring glycoconjugates was also presented by confirming the β-lacotsyl linkage in the GM3.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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