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# Ion-trap mass spectrometry unveils the presence of isomeric oligosaccharides in an analyte: stage-discriminated correlation of energy-resolved mass spectrometry

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ABSTRACT

Mass spectrometry, especially tandem mass spectrometry, has been widely used in the field of analytical sciences for handling biological and chemical samples. The technique resolves molecular and fragment ions based on the mass to charge ratio. Energy-resolved mass spectrometry (ERMS) further provides an activation energy-related factor in the dissociation reaction. Therefore, it is a very powerful technique that can discriminate isomeric compounds. Despite the power of ERMS, useful information cannot be obtained when an analyte contains structural isomers. Carbohydrates carry multiple chiral centers, thus oligomers of monosaccharides can form a vast number of structural isomers. We decided to use such species in our endeavors to establish a method of identifying the 'purity' of an analyte solely based on mass spectrometry. In the present paper, we describe a stage-discriminated spectral correlation of ERMS, which not only enables identification of the presence of contaminants in an analyte, but also provides information regarding the 'purity' of fragment ions.



## 1. Introduction

Glycans are one of the major components of a variety of biological substances that include glycoproteins, proteoglycans, and glycolipids. The important roles of carbohydrate moieties in a wide range of biological functions, such as cell proliferation, cell differentiation, and cell–cell recognition, are well recognized.[1] Structural analysis is equally important for understanding such functions at the molecular level. The diversity of oligosaccharides consists not only of sequential isomers but also of regioisomers, stereoisomers, as well as different ring-sizes and branching patterns, which makes structural analysis of these compounds a particularly difficult task. Thus, the development of new analytical methods capable of identifying miniscule amounts of glycoconjugates is of extreme importance. The advantages of mass spectrometry (MS) are its sensitivity and low sample consumption. Methods based on collision-induced dissociation (CID) are very proficient for obtaining the detailed structure of a given sample by analyzing a fragment ion produced from a precursor ion.[2,3] Most current research efforts on the use of MS techniques in the field of carbohydrate science are being directed at establishing methods for determining glycan structures.[4–12]

In addition to the important issue of using the analytical method to identify isomeric oligosaccharides, questions regarding the purity of the analyte under investigation are often asked.[3] This is particularly important when the possibility of structural isomers in an analyte exists. The isolation of individual compounds is quite difficult because the structures of oligosaccharides are very complex due to the presence of stereoisomers at the anomeric center (anomers), linkage isomers, and branching patterns. Although sufficiently purified compounds can be analyzed by state-of-the-art MS-based methods, it is not possible to draw any useful information from a mixture. This issue was addressed where the ratio of individual compounds in an unresolved fraction can be estimated using a standard curve obtained for the specific isomeric oligosaccharides.[9,13–15] While this method is powerful, to use such an approach it is necessary to prepare first a set of compounds in the mixture. Thus, there is no known method allowing the investigation of the purity of a totally unknown analyte, and investigations on developing such methods are important.

Energy-resolved mass spectrometry (ERMS) provides useful information concerning the energy dependence of the dissociation reaction under CID conditions, and has been shown to be useful not

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only in the identification of ions generated from structurally isomeric molecules but also in the analysis of noncovalently associated complexes.[16–25] Furthermore, the method has been used in the analysis of intramolecular hydrogen-bonding and entropy effects in protonated peptides.[26] (Fig. 1A) In our efforts to identify the structural characteristics of oligomeric carbohydrates, we have found that ions with the same ionic structure result in the same fragmentation pattern, including not only the *m*/*z* value but also the various descriptive characters obtained by ERMS.[27,28] This finding indicated that isomeric oligosaccharides containing different chemical bond(s) can be distinguished by ERMS. Thus, the ions of diastereomers, even though they have the same *m*/*z* values, in general, give rise to different fragmentation patterns.

Based on the fact that ERMS provides the energy dependence of an analyte, we have strived to develop a method that relies on ERMS to judge the purity of a given sample. A method of this type would not require the use of separation technique such as high-pressure liquid chromatography (HPLC) or capillary electrophoresis (CE). In this report, we present our results with regard to examining the 'purity' of unknown oligosaccharide samples with ERMS.

## 2. Materials and methods

### 2.1. Samples

Pairs of anomers and positional isomers from both natural and synthetic compounds were used in the experiments. A detailed list of compounds is provided in Table 1. Compounds synthesized in house have been reported elsewhere.[29–34] The total number of samples and fragment ions used in this statistical investigation was 227. Of these, 27 analytes showed inconsistent numbers of fragment ions at $MS^n$ and $MS^{n+1}$, indicating that these analytes can easily be identified as a mixture of isomeric compounds (see Section 2.2.3). The data were found to be unreliable for 13 analytes (the method for determining the reliability is shown below in Section 2.2.3). The remaining analytes were used in the statistical analysis. There were 49 pure compounds, 44 mixtures of compounds, 23 hemiacetals, 17 A-ions, 17 B-ions, 18 C-ions, and 17 Y-ions (total: 185). The purity of the 'pure' compounds was usually over 99% for ions having the same *m*/*z*. Mixtures were prepared from pure compounds where the ratio varied from 5:95 to 95:5. For the purity or the ratios of mixtures for individual analytes, see Table 1. The annotation of fragment ions was conducted according to previous suggestions.[35]

### 2.2. Mass spectrometric analysis

All experiments were performed using sodiated molecules and fragments, because such species were suitable in the ERMS analysis. Our initial investigation indicated that interpretation of protonated and deprotonated ion species was more difficult than that of sodiated species.

#### 2.2.1. Instrumentation and data collection

Samples were analyzed using a quadrupole ion-trap mass spectrometer (QIT-MS) 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, 300%; (7) ICC target, 5000; (8) maximum acquisition time,

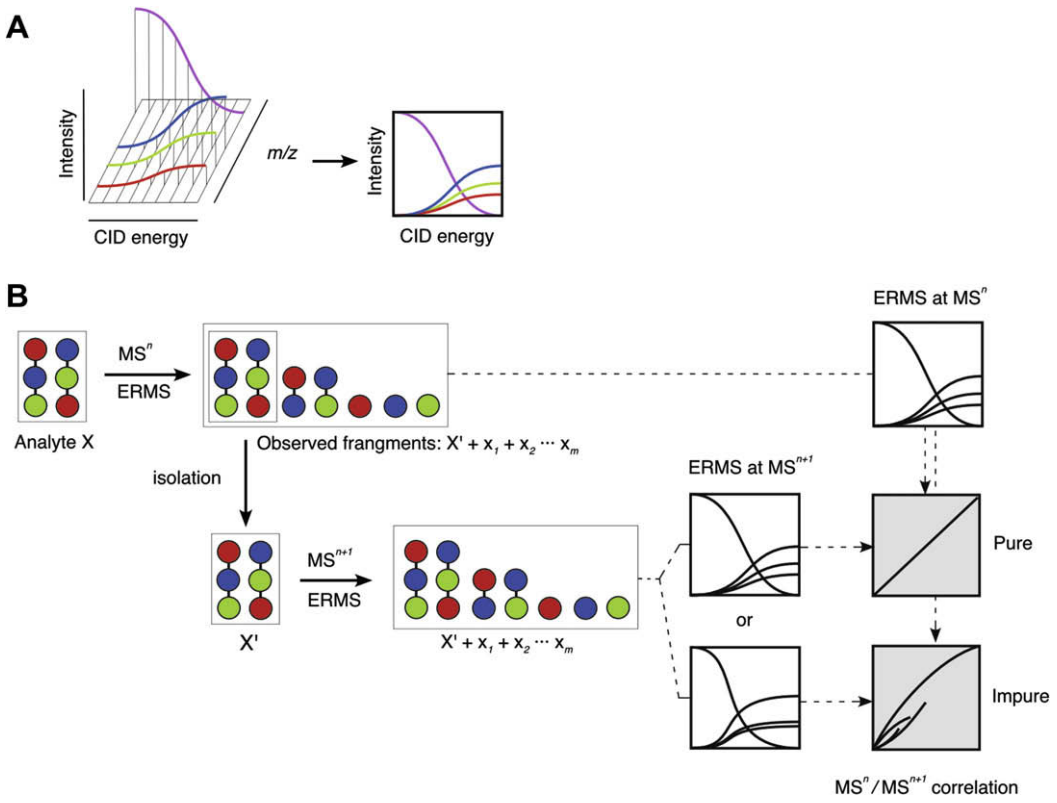



**Figure 1.** Concept of stage-discriminated correlation (SDC) of ERMS. (A) Energy-resolved mass spectrometry. (B) ERMS data sets obtained for the analyte X at $MS^n$ and $MS^{n+1}$ are compared. When these spectra are identical, which is the case when the analyte was pure or indistinguishable, an excellent correlation would be obtained. A poor correlation indicates the analyte was impure.

**Table 1**
Reaction paths, stages of MS/MS and other information for samples

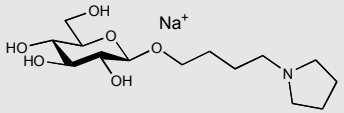
| Sample name | MS/MS sequence and $m/z$ | Stages ($n$) versus ($n+1$) compared $n$ | Maker, etc. | Species | Assignment | Purity[a] (%) |
|---|---|---|---|---|---|---|
| β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 730→670 | 3 | Dextra Laboratories | A | $^{0,2}A_4$ | >95 |
| β-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc | 876→816 | 3 | Dextra Laboratories | A | $^{0,2}A_3$ | >95 |
| β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 730→670 | 3 | Dextra Laboratories | A | $^{0,2}A_4$ | >95 |
| β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 1095→1035 | 3 | Dextra Laboratories | A | $^{0,2}A_6$ | >95 |
| β-Gal-(1→4)-GlcNAc | 406→305 | 3 | Sigma–Aldrich | A | $^{0,2}A_2$ | >95 |
| α-Man*p*-(1→6)-[α-Man*p*-(1→3)-]-α-Man*p*-(1→6)-[α-Man*p*-(1→3)-]-Man | 851→599 | 3 | Dextra Laboratories | A | $^{0,3}A_3$ | >95 |
| β-Glc*p*-(1→4)-β-Glc*p*-(1→4)-Glc | 527→467 | 3 | Seikagaku Corporation | A | $^{0,2}A_3$ | >95 |
| β-Gal*p*-(1→4)-β-Glc | 365→305 | 3 | Nacalai Tesque | A | $^{0,2}A_2$ | Not provided |
| β-Glc*p*NAc-(1→2)-αMan*p*-(1→3)-[αMan*p*-(1→6)-]-β-Man*p*-(1→4)-β-Glc*p*NAc-(1→4)-GlcNAc | 1136→1035 | 3 | Dextra Laboratories | A | $^{0,2}A_5$ | >95 |
| α-Glc*p*-(1→4)-α-Glc*p*-(1→4)-Glc | 527→467 | 3 | Wako Pure Chemical Industries | A | $^{0,2}A_3$ | >97 |
| α-Fuc*p*-(1→2)-β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 876→816 | 3 | Dextra Laboratories | A | $^{0,2}A_5$ | >95 |
| β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 730→610 | 3 | Dextra Laboratories | A | $^{2,4}A_4$ | >95 |
| β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 730→610 | 3 | Dextra Laboratories | A | $^{2,4}A_4$ | >95 |
| α-Man*p*-(1→3)-Man | 365→275 | 3 | Sigma–Aldrich | A | $^{1,4}A_2$, $^{0,3}A_2$ | >95 |
| β-Glc*p*-(1→4)-β-Glc*p*-(1→4)-Glc | 527→407 | 3 | Seikagaku Corporation | A | $^{2,4}A_3$ | >99 |
| β-Gal*p*-(1→6)-GlcNAc | 406→305 | 3 | Dextra Laboratories | A | $^{0,2}A_2$ | >95 |
| β-Glc*p*NAc-(1→6)-Gal | 406→346 | 3 | Sigma–Aldrich | A | $^{0,2}A_2$ | >98 |
| β-Gal*p*-(1→4)-β-Glc*p*NAc-OPNP | 527→388 | 3 | Toronto Research Chemicals | B | $B_2$ | 98 |
| β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→3)-β-Gal-(1→4)-Glc-PA | 550 | 3 | Takara Bio | B | $B_3$ | >95 |
| β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal-(1→4)-Glc-PA | 550 | 3 | Takara Bio | B | $B_3$ | >95 |
| α-Fuc*p*-(1→6)-α-Gal*p*-(1→3)-α-Glc*p*-*O*-octyl | 623→493 | 3 | Synthesized in house | B | $B_3$ | >99 |
| β-Fuc*p*-(1→6)-α-Gal*p*-(1→3)-α-Glc*p*-*O*-octyl | 623→493 | 3 | Synthesized in house | B | $B_3$ | >99 |
| β-Fuc*p*-(1→6)-β-Gal*p*-(1→3)-α-Glc*p*-*O*-octyl | 623→493 | 3 | Synthesized in house | B | $B_3$ | >99 |
| β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 730→550 | 3 | Dextra Laboratories | B | $B_3$ | >95 |
| α-Fuc*p*-(1→2)-β-Gal*p*-OPNP | 470→331 | 3 | Toronto Research Chemicals | B | $B_2$ | 98 |
| β-GlcpNAc-(1→3)-β-Gal*p*-OMe | 420→388 | 3 | Sigma–Aldrich | B | $B_2$ | 98.5 |
| β-Gal*p*-(1→3)-α-Gal*p*NAc-OPNP | 388 | 3 | CALBIOCHEM | B | $B_2$ | >99 |
| β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 1095.4→753 | 3 | Dextra Laboratories | B | $B_4$, $Y_5/C_5(-H_2O)$ | >95 |
| β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 730→550 | 3 | Dextra Laboratories | B | $B_3$, $Y_3-H_2O$ | >95 |
| β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 730→388 | 3 | Dextra Laboratories | B | $B_2$, $Y_3/B_3(-H_2O)$ | >95 |
| β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 730→388 | 3 | Dextra Laboratories | B | $B_2$, $Y_3/B_3$ | >95 |
| β-Gal*p*-(1→3)-β-Gal*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc | 730→388 | 3 | Dextra Laboratories | B | $B_2$, $Y_3/B_3$ | >99 |
| β-Glc*p*NAc-(1→2)-αMan*p*-(1→3)-[αMan*p*-(1→6)-]-βMan*p*-(1→4)-β-Glc*p*NAc-(1→4)-GlcNAc | 1136→712 | 3 | Dextra Laboratories | B | $B_2$, $Y_4/B_4$ | >95 |
| β-Gal*p*-(1→3)-β-Gal*p*NAc-(1→4)-β-Gal-(1→4)-Glc-PA | 808→388 | 3 | Takara Bio | B | $B_2$, $B_3/Y_3$ | >95 |
| β-Gal*p*-(1→4)-β-Glc*p*NAc-O$(CH_2)_4$NHBoc | 577→477→406 | 4 | Synthesized in house | C | $C_2$ | >99 |
| βLactosyl-O$(CH_2)_4$NHBoc | 536→436→365 | 4 | Synthesized in house | C | $C_2$ | >99 |
| β-Gal*p*-(1→3)-[α-Fuc*p*-(1→4)-]-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 876→406 | 3 | CALBIOCHEM | C | $C_2/Y_3\beta$ | >95 |
| β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 730→406 | 3 | Dextra Laboratories | C | $C_2$ | >95 |
| β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 568 | 3 | Dextra Laboratories | C | $C_3$, $Y_3$ | >95 |
| β-Gal*p*-(1→3)-α-Glc*p*NAc-O$(CH_2)_4$NHBoc | 477→406 | 4 | Synthesized in house | C | $C_2$ | >99 |
| β-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc | 876→406 | 3 | CALBIOCHEM | C | $C_2/Y_3\beta\beta$ | >95 |
| α-Fuc*p*-(1→2)-β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 876→406 | 3 | Dextra Laboratories | C | $C_3/Y$ | >95 |
| β-Gal*p*-(1→3)-β-Glc*p*NAc-O$(CH_2)_4$NHBoc | 477→406 | 4 | Synthesized in house | C | $C_2$ | >99 |
| β-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 954→406 | 3 | Takara Bio | C | $C_2/Y_3\beta$ | >95 |
| β-Gal*p*-(1→3)-β-Gal*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc | 730→568 | 3 | Dextra Laboratories | C | $C_3$, $Y_3$ | >99 |
| β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 406 | 3 | Dextra Laboratories | C | $C_2$ | >95 |
| β-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 954→808→406 | 4 | Takara Bio | C | $C_2/Y_3\beta$ | >95 |
| β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc-PA | 406 | 3 | Takara Bio | C | $C_2$ | >95 |
| α-Lactosyl-O$(CH_2)_4$NHBoc | 365 | 4 | Synthesized in house | C | $C_2$ | >99 |
| β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal-*p*-(1→4)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 1095→933 | 3 | Dextra Laboratories | C | $C_5$, $Y_5$ | >95 |
| β-Gal*p*-(1→3)-[α-Fuc*p*-(1→4)-]-β-Glc*p*NAc-(1→3)-β-Gal-(1→4)-Glc-PA | 954→406 | 3 | Takara Bio | C | $C_2/Y_3\beta$ | >95 |
| α-Fuc*p*-(1→2)-β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 876→552 | 3 | Dextra Laboratories | C | $C_3$ | >95 |

| | | | | | | |
|---|---|---|---|---|---|---|
| β-Gal*p*-(1→4)-GlcNAc | 406 | 2 | Sigma–Aldrich | Free | | >95 |
| β-Gal*p*-(1→3)-GlcNAc | 406 | 2 | Dextra Laboratories | Free | | >95 |
| β-Glc*p*NAc-(1→2)-αMan*p*-(1→3)-[αMan*p*-(1→6)-]-β-Man*p*-(1→4)-β-Glc*p*NAc-(1→4)-GlcNAc | 1136 | 2 | Dextra Laboratories | Free | | >95 |
| α-Glc*p*-(1→4)-α-Glc*p*-(1→4)-Glc | 527 | 2 | Wako Pure Chemical Industries | Free | | >97 |
| β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 730 | 2 | Dextra Laboratories | Free | | >95 |
| β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 730 | 2 | Dextra Laboratories | Free | | >95 |
| β-Gal*p*NAc-(1→3)-Gal | 406 | 2 | Dextra Laboratories | Free | | >95 |
| β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 730 | 2 | Dextra Laboratories | Free | | >95 |
| β-Gal*p*-(1→3)-Gal*p*NAc | 406 | 2 | Sigma–Aldrich | Free | | >99 |
| β-Gal*p*-(1→4)-Glc | 365 | 2 | Nacalai Tesque | Free | | Unknown |
| α-Man*p*-(1→6)-[αMan*p*-(1→3)-]-Man | 527 | 2 | Dextra Laboratories | Free | | >95 |
| α-Gal*p*NAc-(1→3)-Gal | 406 | 2 | Dextra Laboratories | Free | | >95 |
| α-Gal*p*-(1→3)-Gal | 365 | 2 | Sigma–Aldrich | Free | | 95 |
| α-Man*p*-(1→3)-Man | 365 | 2 | Dextra Laboratories | Free | | 95 |
| α-Man*p*-(1→6)-[α-Man*p*-(1→3)-]-Man | 365 | 3 | Dextra Laboratories | Free/$Y_1$α, $Y_1$β/free | | >95 |
| β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc | 730→365 | 3 | Dextra Laboratories | Free/$Y_2$/free | | >95 |
| GD1a methyl ester | 969→1610 | 3 | Seikagaku Corp. modified in house | Free/$Y_4$α, $Y_3$β | | >98 |
| 3Sulfo-Lewis x | 608 | 2 | Dextra Laboratories | Free | | >95 |
| 3Sulfo-Lewis x | 654 | 2 | Dextra Laboratories | Free | | >95 |
| 3Sulfo-Lewis a | 654 | 2 | Dextra Laboratories | Free | | >95 |
| α-Gal*p*-(1→3)-α/β-Gal*p*-OMe | 379 | 2 | Sigma–Aldrich | Mixt | 95:5 | |
| β-Gal*p*-(1→3)-β-Gal*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc*p*-PA + β-Gal*p*NAc-(1→3)-α-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 808 | 2 | Takara Bio | Mixt | 90:10 | |
| α/β-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 605 | 2 | Dextra Laboratories + Sigma–Aldrich | Mixt | 90:10 | |
| α-Fuc*p*-(1→2)-β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→6)-[α-Glc*p*NAc-(1→4)-β-Gal*p*-(1→3)-]-GalNAc-ol + α-Glc*p*NAc-(1→4)-β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→6)-[α-Fuc*p*-(1→4)-β-Gal*p*-(1→3)-]-GalNAc-ol | 1122 | 2 | Kanto Chemical | Mixt | 10:90 | |
| α/β-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 605 | 2 | Dextra Laboratories + Sigma–Aldrich | Mixt | 30:70 | |
| β-Gal*p*-(1→3)-β-Gal*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc*p*-PA + β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc-PA | 808 | 2 | Takara Bio + Dextra Laboratories modified in house | Mixt | 30:70 | |
| β-Gal*p*-(1→3)-β-Gal*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc*p*-PA + β-Gal*p*NAc-(1→3)-α-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 808 | 2 | Takara Bio | Mixt | 10:90 | |
| α-Fuc*p*-(1→2)-β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→6)-[α-Glc*p*NAc-(1→4)-β-Gal*p*-(1→3)-]-GalNAc-ol + α-Glc*p*NAc-(1→4)-β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→6)-[α-Fuc*p*-(1→4)-β-Gal*p*-(1→3)-]-GalNAc-ol | 1122 | 2 | Kanto Chemical | Mixt | 90:10 | |
| β-Gal*p*-(1→3)-β-Gal*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc*p*-PA + β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc-PA | 808 | 2 | Takara Bio + Dextra Laboratories modified in house | Mixt | 70:30 | |
| GD1a,b methyl ester | 1916 | 2 | Seikagaku Corporation modified in house | Mixt | 50:50 | |
| α/β-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 605 | 2 | Dextra Laboratories + Sigma–Aldrich | Mixt | 95:5 | |
| α/β-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 605 | 2 | Dextra Laboratories + Sigma–Aldrich | Mixt | 50:50 | |
| α/β-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 605 | 2 | Dextra Laboratories + Sigma–Aldrich | Mixt | 70:30 | |
| β-Gal*p*-(1→3)-β-Gal*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc*p*-PA + β-Gal*p*NAc-(1→3)-α-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 808 | 2 | Takara Bio | Mixt | 30:70 | |
| α-Gal*p*-(1→3)-α/β-Gal*p*-OMe | 379 | 2 | CALBIOCHEM | Mixt | 90:10 | |
| α-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-*O*-$(CH_2)_5$COOMe + β-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-*O*-$(CH_2)_5$COOMe | 688 | 2 | Synthesized in house | Mixt | 20:80 | |

(*continued on next page*)

**Table 1** (*continued*)

| Sample name | MS/MS sequence and $m/z$ | Stages ($n$) versus ($n+1$) compared $n$ | Maker, etc. | Species | Assignment | Purity[a] (%) |
|---|---|---|---|---|---|---|
| α/β-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 605 | 2 | Dextra Laboratories + Sigma–Aldrich | Mixt | 50:50 | |
| β-Gal*p*-(1→3)-β-Gal*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc*p*-PA + β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc-PA | 808 | 2 | Takara Bio | Mixt | 70:30 | |
| α-Fuc*p*-(1→2)-β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→6)-[α-Glc*p*NAc-(1→4)-β-Gal*p*-(1→3)-]-GalNAc-ol + α-Glc*p*NAc-(1→4)-β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→6)-[α-Fuc*p*-(1→4)-β-Gal*p*-(1→3)-]-GalNAc-ol | 1122 | 2 | Kanto Chemical | Mixt | 50:50 | |
| GD1a,b methyl esters | 1916 | | Seikagaku Corp. modified in house | Mixt | 90:10 | |
| β-Gal*p*-(1→3)-β-Gal*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc*p*-PA + β-Gal*p*NAc-(1→3)-α-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 808 | 2 | Takara Bio | Mixt | 70:30 | |
| α-Fuc*p*-(1→2)-β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→6)-[α-Glc*p*NAc-(1→4)-β-Gal*p*-(1→3)-]-GalNAc-ol + α-Glc*p*NAc-(1→4)-β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→6)-[α-Fuc*p*-(1→4)-β-Gal*p*-(1→3)-]-GalNAc-ol | 1122 | 2 | Kanto Chemical | Mixt | 70:30 | |
| α-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-*O*-$(CH_2)_5$COOMe + β-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-*O*-$(CH_2)_5$COOMe | 688 | 2 | Synthesized in house | Mixt | 80:20 | |
| α-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-*O*-$(CH_2)_5$COOMe + β-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-*O*-$(CH_2)_5$COOMe | 688 | 2 | Synthesized in house | Mixt | 5:95 | |
| α/β-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 605 | 2 | Dextra Laboratories + Sigma Aldrich | Mixt | 5:95 | |
| α/β-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 605 | 2 | Dextra Laboratories + Sigma–Aldrich | Mixt | 10:90 | |
| β-Gal*p*-(1→3)-β-Gal*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc-ol + β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc-ol | 1122 | 2 | Dextra Laboratories | Mixt | 50:50 | |
| α/β-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc-ol | 529 | 2 | Dextra Laboratories + Sigma–Aldrich | Mixt | 50:50 | |
| α-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-*O*-$(CH_2)_5$COOMe + β-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-*O*-$(CH_2)_5$COOMe | 688 | 2 | Synthesized in house | Mixt | 95:5 | |
| 3Sulfo-Lewis a | 654 | 2 | Dextra Laboratories | Mixt | Mixt | |
| α/β-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc-ol | 529 | 2 | Dextra Laboratories + Sigma–Aldrich | Mixt | 70:30 | |
| α-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-*O*-$(CH_2)_5$COOMe + β-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-*O*-$(CH_2)_5$COOMe | 688 | 2 | Synthesized in house | Mixt | 95:5 | |
| GD1a,b methyl ester | 1916 | | Seikagaku Corporation modified in house | Mixt | 5:95 | |
| α-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-*O*-$(CH_2)_5$COOMe + β-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-*O*-$(CH_2)_5$COOMe | 688 | 2 | Synthesized in house | Mixt | 80:20 | |
| α-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-*O*-$(CH_2)_5$COOMe + β-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-*O*-$(CH_2)_5$COOMe | 688 | 2 | Synthesized in house | Mixt | 20:80 | |
| α-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-*O*-$(CH_2)_5$COOMe + β-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-*O*-$(CH_2)_5$COOMe | 688 | 2 | Synthesized in house | Mixt | 5:95 | |
| α-Gal*p*-(1→3)-α/β-Gal*p*-OMe | 379 | 2 | CALBIOCHEM | Mixt | 5:95 | |
| α-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-*O*-$(CH_2)_5$COOMe + β-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-*O*-$(CH_2)_5$COOMe | 688 | 2 | Synthesized in house | Mixt | 50:50 | |
| α-Gal*p*-(1→3)-α/β-Gal*p*-OMe | 379 | 2 | CALBIOCHEM | Mixt | 70:30 | |
| α-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc*p*NAc-PA + β-Gal*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 646 | 2 | Dextra Laboratories + Takara Bio | Mixt | 50:50 | |
| α-Gal*p*-(1→3)-α/β-Gal*p*-OMe | 379 | 2 | CALBIOCHEM | Mixt | 50:50 | |
| α-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc*p*NAc-PA + β-Gal*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 646 | 2 | Dextra Laboratories + Takara Bio | Mixt | 50:50 | |
| α-Gal*p*-(1→3)-α/β-Gal*p*-OMe | 379 | 2 | CALBIOCHEM | Mixt | 30:70 | |
| α/β-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 605 | 3 | Dextra Laboratories | Mixt | 50:50 | |

| | | | | | |
|---|---|---|---|---|---|
| α-Gal*p*-(1→4)-β-Gal*p*-(1→4)-GlcNAc-PA | 646 | 2 | Dextra Laboratories | Pure | >95 |
| β-Gal*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 646 | 2 | Takara Bio | Pure | >95 |
| α-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 605 | 2 | Dextra Laboratories | Pure | >95 |
| α-Fuc*p*-(1→6)-α-Gal*p*-(1→6)-βMan*p*-*O*-octyl | 623 | 2 | Synthesized in house | Pure | >99 |
| β-Gal*p*-(1→3)-β-Gal*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc-ol | 732 | 2 | Dextra Laboratories | Pure | >95 |
| β-Gal*p*-(1→3)-β-Gal*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 808 | 2 | Takara Bio | Pure | >95 |
| α-Fuc*p*-(1→6)-β-Gal*p*-(1→3)-α-Glc*p*NAc-*O*-octyl | 664 | 2 | Synthesized in house | Pure | >99 |
| β-Gal*p*NAc-(1→3)-α-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 808 | 2 | Takara Bio | Pure | >95 |
| β-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 954 | 2 | Takara Bio | Pure | >95 |
|  | 328 | 2 | Synthesized in house | Pure | >99 |
| β-Cyclodextrin | 1157 | 2 | Wako Pure Chemical Industries | Pure | >97% |
| β-Fuc*p*-(1→6)-α-Gal*p*-(1→6)-α-Glc*p*-*O*-octyl | 623 | 2 | Synthesized in house | Pure | >99 |
| β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc-PA | 808 | 2 | Dextra Laboratories modified in house | Pure | >99 |
| α-Fuc*p*-(1→6)-β-Gal*p*-(1→6)-α-Glc*p*NAc-*O*-octyl | 664 | 2 | Synthesized in house | Pure | >99 |
| β-Gal*p*-(1→3)-β-Gal*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 808 | 2 | Takara Bio | Pure | >95 |
| α-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-*O*-$(CH_2)_5$COOMe | 688 | 2 | Synthesized in house | Pure | >99 |
| β-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 605 | 2 | Seikagaku Corporation | Pure | >99 |
| α-Glc*p*NAc-(1→4)-β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→6)-[α-Fuc*p*-(1→2)-β-Gal*p*-(1→3)-]-GalNAc-ol | 1122 | 2 | Kanto Chemical | Pure | >95 |
| α-Fuc*p*-(1→6)-α-Gal*p*-(1→3)-β-Glc*p*NAc-*O*-octyl | 664 | 2 | Synthesized in house | Pure | >99 |
| Lewis y-β-O$(CH_2)_5$COOMe | 826 | 2 | Carbohydrate synthesis | Pure | ca. 95 |
| β-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-*O*-$(CH_2)_5$COOH | 666 | 2 | Synthesized in house | Pure | >99 |
| α-Lactosyl-*O*-$(CH_2)_4$NHBoc | 536 | 2 | Synthesized in house | Pure | >99 |
| α-Gal*p*-(1→3)-α-Gal*p*-OMe | 379 | 2 | CALBIOCHEM | Pure | >95 |
| GD1a methyl ester | 1916 | 2 | Seikagaku Corporation modified in house | Pure | >98 |
| GD1a methyl esters | 1916 | 2 | Seikagaku Corporation modified in house | Pure | >98 |
| β-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-O$(CH_2)_5$COOMe | 688 | 2 | Synthesized in house | Pure | >99 |
| Lewis b-β-O$(CH_2)_5$COOMe | 826 | 2 | Dextra Laboratories | Pure | ca. 95 |
| β-Fuc*p*-(1→6)-α-Gal*p*-(1→3)-α-Glc*p*-*O*-octyl | 623 | 2 | Synthesized in house | Pure | >99 |
| 3′Sulfate Lewis x-β-OMe | 668 | 2 | Toronto Research Chemicals | Pure | 98 |
| α-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-O$(CH_2)_5$COOH | 666 | 2 | Synthesized in house | Pure | >99 |
| Cellopentaose-PA | 929 | 2 | Seikagaku Corporation | Pure | >95 |
| α-Gal*p*-(1→3)-β-Gal*p*-OMe | 379 | 2 | CALBIOCHEM | Pure | >95 |
| α-Fuc*p*-(1→2)-β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→6)-[α-Glc*p*NAc-(1→4)-β-Gal*p*-(1→3)-]-GalNAc-ol | 1122 | 2 | Kanto Chemical | Pure | >95 |
| β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc-PA | 808 | 2 | Takara Bio | Pure | >95 |
| β-Fuc*p*-(1→6)-β-Gal*p*-(1→3)-α-Glc*p*-*O*-octyl | 623 | 2 | Synthesized in house | Pure | >99 |
| α-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc-ol | 529 | 2 | Dextra Laboratories | Pure | >95 |
| 4′Sulfate Lewis x-β-OMe | 668 | 2 | Toronto Research Chemicals | Pure | 98 |
| α-Fuc*p*-(1→6)-α-Gal-(1→6)-α-Glc *p*NAc-*O*-octyl | 664 | 2 | Synthesized in house | Pure | >99 |
| β-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc-ol | 529 | 2 | Seikagaku Corporation | Pure | >95 |
| β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc-PA | 808 | 3 | Takara Bio | Pure | >95 |
| Maltopentaose-PA | 929 | 2 | Hayashibara Co., Ltd | Pure | 98.5 |
| β-Gal*p*-(1→4)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc-ol | 732 | 2 | Dextra Laboratories | Pure | >95 |
| β-Gal*p*-(1→3)-β-Gal*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 808 | 2 | Takara Bio | Pure | >95 |
| α-Fuc*p*-(1→6)-β-Gal*p*-(1→6)-αMan*p*-*O*-octyl | 623 | 2 | Synthesized in house | Pure | >99 |
| GM3 methyl ester | 1245 | | Hytest Ltd. Modified in house | Pure | >98 |
| α-Cyclodextrin | 995 | 2 | Tokyo Chemical Industry | Pure | >98 |
| β-Fuc*p*-(1→6)-α-Gal-(1→6)-β-Glc*p*-*O*-octyl | 623 | 2 | Synthesized in house | Pure | >99 |

(*continued on next page*)

**Table 1** (*continued*)

| Sample name | MS/MS sequence and $m/z$ | Stages ($n$) versus ($n+1$) compared $n$ | Maker, etc. | Species | Assignment | Purity[a] (%) |
|---|---|---|---|---|---|---|
| Lactosyl ceramide | 982 | | Merck KGaA | Pure | | >98 |
| β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc-PA | 808 | 2 | Takara Bio | Pure | | >95 |
| β-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 954→808 | 3 | Takara Bio | Y | $Y_3\beta$ | >95 |
| α-Fuc*p*-(1→6)-α-Gal*p*-(1→3)-β-Glc*p*NAc-*O*-octyl | 664→518 | 3 | Synthesized in house | Y | $Y_2$ | >99 |
| α-Fuc*p*-(1→6)-α-Gal-(1→3)-β-Glc*p*-*O*-octyl | 623→477 | 3 | Synthesized in house | Y | $Y_2$ | >99 |
| β-Glc*p*-(1→4)-β-Glc*p*-(1→4)-β -Glc*p*-(1→4)-β-Glc*p*-(1→4)-Glc-PA | 929→767 | 3 | Seikagaku Corporation | Y | $Y_4$ | >95 |
| α-Fuc*p*-(1→6)-β-Gal*p*-(1→6)-β-Glc*p*NAc-*O*-octyl | 664→518 | 3 | Synthesized in house | Y | $Y_2$ | >99 |
| α-Gal*p*-(1→3)-[α-Fuc*p*-(1→2)-]-β-Gal*p*-O$(CH_2)_8$COOMe | 681→535 | 3 | Carbohydrate synthesis | Y | $Y_1\beta$ | >95 |
| α-Gal*p*NAc-(1→3)-[α-Fuc*p*-(1→2)-]-β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc-PA | 1157→1011 | 3 | Takara Bio | Y | $Y_4\beta$ | >95 |
| β-Gal*p*NAc-(1→3)-[α-Fuc*p*-(1→2)-]-β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-Glc-PA | 1157→1011 | 3 | Takara Bio | Y | $Y_4\beta$ | >95 |
| β-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-(1→4)-β-Gal-(1→4)-Glc-PA | 954→443 | 3 | Takara Bio | Y | $Y_2$ | >95 |
| α-Gal*p*NAc-(1→3)-[α-Fuc*p*-(1→2)-]-β-Gal*p*-O$(CH_2)_8$COOMe | 722→576 | 3 | Carbohydrate synthesis | Y | $Y_1\beta$ | >95 |
| α-Fuc*p*-(1→6)-β-Gal*p*-(1→3)-α-Glc*p*-*O*-octyl | 623→477 | 3 | Synthesized in house | Y | $Y_2$ | >99 |
| β-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)-]-β-Glc*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc-PA | 954→808 | 3 | Takara Bio | Y | $Y_3\beta$ | >95 |
| β-Glc*p*-(1→4)-β-Glc*p*-(1→4)-β-Glc*p*-(1→4)-β-Glc*p*-(1→4)-Glc-PA | 929→605 | 3 | Seikagaku Corporation | Y | $Y_3$ | >95 |
| α-Fuc*p*-(1→6)-β-Gal*p*-(1→3)-β-Glc*p*NAc-*O*-octyl | 664→518 | 3 | Synthesized in house | Y | $Y_2$ | >99 |
| α-Fuc*p*-(1→6)-α-Gal*p*-(1→3)-α-Glc*p*-*O*-octyl | 623→477 | 3 | Synthesized in house | Y | $Y_2$ | >99 |
| α-Fuc*p*-(1→6)-β-Gal*p*-(1→3)-α-Glc*p*NAc-*O*-octyl | 664→518 | 3 | Synthesized in house | Y | $Y_2$ | >99 |
| β-Glc*p*NAc-(1→6)-[β-Gal*p*-(1→3)-]-α-Gal*p*NAc-*O*-$C_6H_4$O-octyl | 813→610 | 3 | Synthesized in house | Y | Yβ | >99 |

[a] The purity of samples that were confirmed by HPLC and/or NMR independent of the MS analysis.




**Figure 2.** Flow chart of analysis of compounds.

200 ms; (9) average, 10 spectra; and (10) 'cut-off', 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 the end cap rf amplitude was controlled during the CID experiments. The He pressure was $4.86 \times 10^{-6}$ mbar and the CID time was 40 ms. Enough cooling time was given between CID experiments at $MS^n$ and $MS^{n+1}$ to allow excited ions to loose

internal energy through collision cooling. 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 Section 2.2.2). For the isolation of a product ion, $m/z \pm 1$ ($w = 2$) were isolated and subjected to the CID experiments to include isotopes.

### 2.2.2. Data handling

The following equation was used in order to obtain graphs of the ERMS. 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,

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

where $^{rel}C$ 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 2000 (Microsoft Co.), which is based on the DSUM function and programed to choose a range of isotopes ($w$) to be taken into consideration ($w = 1$; excluding isotopes in the experiments).

The ERMS plot was first generated using all the data to estimate maximum response (plateau) values. The ions not reaching 5% of total ions were excluded and the remaining signals were treated as the total ion intensity, and once again an ERMS plot was generated. The thus obtained ERMS data were used for the following analysis to judge whether they were considered to be pure or impure.

### 2.2.3. Flow chart of judgment procedure

The flow chart of the judgment procedure is shown in Figure 2. Data that contained any number of fragments observed at either $MS^n$ or $MS^{n+1}$ showing a coefficient of determination ($r^2$) smaller than 0.8 were not used for further analysis. This was because the series of signals was weak due to a sensitivity problem associated with the nature of analyte, and thus contained considerably large experimental errors. Data consisting of individual data sets for the fragment ions having a good $r^2$ ($\geqslant 0.8$) only were used for the following analysis. Two criteria needed to be fulfilled in the judgment process: (1) the number of fragment ions obtained at $MS^n$ and $MS^{n+1}$ independently should be the same and (2) the $m/z$ values of the observed ion species in both $MS^n$ and $MS^{n+1}$ stages should be the same. Data that could be judged were subjected to the following statistical analysis, which used PRISM 4 software (GRAPHPAD Software, Inc.). Some statistical parameters were shown in Table 2.

## 2.3. Demonstration of 'stage-discriminated correlation' of HPLC-unresolved analyte

A 7:3 mixture of α-Gal*p*-(1→3)-β-Gal*p*-(1→4)-GlcNAc-PA and β-Gal*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc-PA was prepared and used in the experiment. Suppliers are listed in a Table 1.

### 2.3.1. HPLC conditions

Solvent A: 200 mM acetic acid–triethylamine (pH 7.3)/acetonitrile = 20:80. Solvent B: 200 mM acetic acid–triethylamine (pH 7.3)/acetonitrile = 50:50. Column: PALPAK TypeN 240 mm (l) × 4.6 mm (i.d.) (Takara BIOMEDICAL). Column temp: 40 °C. Flow rate: 1.0 mL/min. Gradient: 0→100% (0→50 min). Detector: RF-10AXL (Ex: 300 nm; Em: 400 nm), Shimadzu Corp.

## 3. Results and discussion

### 3.1. Concept of stage-discriminated correlation (SDC) of ERMS

The general use of the MS/MS technique with an ion-trap mass spectrometer is to obtain further structural information of a fragment ion ($X_i$) generated from a precursor ion (X) observed at an $MS^n$ stage. This can be done at several stages using ion-trap MS equipment enabling detailed analysis of an ionized molecule. Let us consider isolating and trapping the remaining precursor ion (X′) after $MS^n$ of an ion X, and analyze its fragmentation process at $MS^{n+1}$ as it was carried out at the stage of $MS^n$ (Fig. 1B). When an analyte is pure, the first ion (X) introduced into the ion trap is considered to be pure under the given low energy collision conditions and the second precursor ion (X′) obtained after $MS^n$ is considered to be also pure. Therefore, if $MS^{n+1}$ analysis of X′ is further carried out, the ion will undergo exactly the same fragmentation process as that occurring for ion X. The cooling process, as it is applied for the isolation of the ion X, has to be applied after $MS^n$ to secure initial states of X and X′ same. Thus, the MS/MS spectra obtained at $MS^n$ and $MS^{n+1}$ should, in theory, be identical. The concept is feasible based on the fact that indistinguishable ERMS spectra can be obtained for an ion regardless of the stage of the CID.[27,28] Structurally isomeric ion species that resulted in a distinguishable ERMS were also reported.[27,28,36,37]

If an analyte (X) is not pure, the precursor ion (X′) for the next stage of MS/MS is most probably impure because the dissociation constants of individual components producing fragments may be different and such ion species shall undergo independent fragmentation processes according to the individual dissociation pathways and kinetics. Thus, in an ordinary case, a precursor ion consisting of a mixture of ions with isomeric structures (X′) may result in a different composition of fragment ions compared to those obtained for an original precursor (X). The CID profiles obtained at $MS^n$ and $MS^{n+1}$ for each 'precursor ion' are therefore anticipated to become different when a sample is not pure.

**Table 2**
Statistical data

| | All | Pure | Mixture | Hemiacetal | A-ions | B-ions | C-ions | Y-ions |
|---|---|---|---|---|---|---|---|---|
| Number of data | 185 | 49 | 44 | 23 | 17 | 17 | 18 | 17 |
| Minimum | 0.392 | 0.4194 | 0.658 | 0.507 | 0.7485 | 1.03 | 0.585 | 0.392 |
| 25% Percentile | 0.8798 | 0.6287 | 1.187 | 1.375 | 1.141 | 1.44 | 0.8335 | 0.8407 |
| Median | 1.358 | 0.8448 | 1.666 | 2.185 | 2.109 | 2.635 | 1.065 | 1.109 |
| 75% Percentile | 2.296 | 1.265 | 2.838 | 6.354 | 5.798 | 11.45 | 1.398 | 1.588 |
| Maximum | 28.69 | 3.887 | 19.06 | 19.88 | 22.05 | 28.69 | 2.15 | 2.304 |
| Mean | 2.911 | 1.008 | 3.180 | 4.923 | 4.758 | 6.678 | 1.156 | 1.222 |
| Std. deviation | 4.527 | 0.5939 | 3.999 | 6.280 | 6.144 | 7.546 | 0.4289 | 0.4958 |
| Std. error | 0.3328 | 0.08484 | 0.6028 | 1.309 | 1.49 | 1.83 | 0.1011 | 0.1203 |
| Lower 95% CI of mean | 2.254 | 0.8377 | 1.964 | 2.207 | 1.599 | 2.798 | 0.9423 | 0.9674 |
| Upper 95% CI of mean | 3.568 | 1.170 | 4.395 | 7.639 | 7.918 | 10.56 | 1.369 | 1.477 |

In the SDC, ERMS spectra were compared at the stages of $MS^n$ and $MS^{n+1}$. The spectrum at $MS^{n+1}$ was obtained using isolated precursor ions at $MS^n$ when ca. 50% remained (consumed). If the ERMS spectra obtained at $MS^n$ and $MS^{n+1}$ are identical, we expect a perfect correlation can be observed between these data sets. Thus, it is clear that the sum of distances from a hypothetical curve ($y = x$) describes the differences of a set of spectra. To examine the difference between data for a set of spectra obtained at $MS^n$ and $MS^{n+1}$, we define the distance $d$ to be,

$$d = \frac{1}{m}\sum_{m=1}^{m}\frac{(a_i^n - a_i^{n+1})^2}{2} \quad (2)$$

where $a_i$ is the intensity of a fragment $i$, $n$ indicates the stage of CID experiments, and $m$ is the total number of observed ions. Therefore, the $d$ value indicates departure from the theoretical value ($d = 0$). Note that all data obtained for ERMS, except for the data sets of fragment ions with lesser intensities as described in Section 2.2.2, were used as $a_i$.

### 3.2. Analysis of 'pure' compounds and 'mixtures' based on SDC

To examine the theory, we selected three categories of compounds to be used in a statistical analysis, namely 'pure' compounds, 'mixtures', and hemiacetals. It was necessary to examine the data by statistics to determine if the SDC can be used for the judgment. As shown in Figure 3A, the mean of $d$ for the 'pure' compounds was small compared to that of the 'mixtures'. Also, the 95% confidence interval of the mean for the former is considerably narrower than that of mixture. The large value of 95% confidence interval for the mixture is due to unpredictable nature of a pair of data sets ($MS^n$ vs $MS^{n+1}$) obtained from a sample consisting of structural isomers. These indicate that the $d$ value is useful in distinguishing mixtures of isometric compounds. Analytes having a larger $d$ indicate that they are mixtures of diastereomers. According to this method commercial hemiacetals were judged as mixtures. This observation is consistent with our previous findings that each anomer of a pair of hemiacetals can be distinguished by ERMS.[33] Importantly, no information regarding the structure of an analyte is required prior to examination. This is a significant advantage compared to the existing methods where standard compounds have to be used as references. Another advantage is that the method does not require any isolation technique that is usually necessary for this type of investigation. Therefore, SDC may become an analytical gateway in various fields.

### 3.3. Analysis of ion species generated under CID conditions

Having a basis for judging the presence of contaminants in an analyte in hand, we became interested in the 'purity' of fragment ions produced under CID conditions (Fig. 3B). Fragment ions have been used in structural elucidation by performing further MS/MS and by comparing the spectrum with one obtained from 'a standard ion' obtained from a known compound.[9,13–15] Our question is whether a fragment used in such comparisons is pure enough to be used for the structural investigation.

Various ion species, that is, A-, B-, C-, and Y-ions, were therefore examined by SDC, which suggested that A- and B-ion species were generally a mixture of isomeric ions and C- and Y-ion species might be treated as 'pure' (a borderline of $d$ value might be set to be 1.5.). The reason that A- and B-ions are believed to be mixtures can be explained by a reaction mechanism of the dissociation to produce individual ions. For example, possible mechanisms to produce B- and C-ions are shown (Fig. 3C). As is clear from the mechanism, two components can be formed depending on the reaction path for B-ion species; however, either path provides a single Y-ion. The dissociation mechanism can be confirmed by an experiment using compounds, all exchangeable protons of which are replaced with deuterium atoms.[38] The behavior of other ion species can be interpreted by an analogy of this dissociation. It should be noted that we confirmed that the Z-ion, which is the counter part of the C-ion, was a mixture based on an experiment using deuterated compounds. Note that the sites of coordination of $Na^+$ may vary and this creates 'pseu-

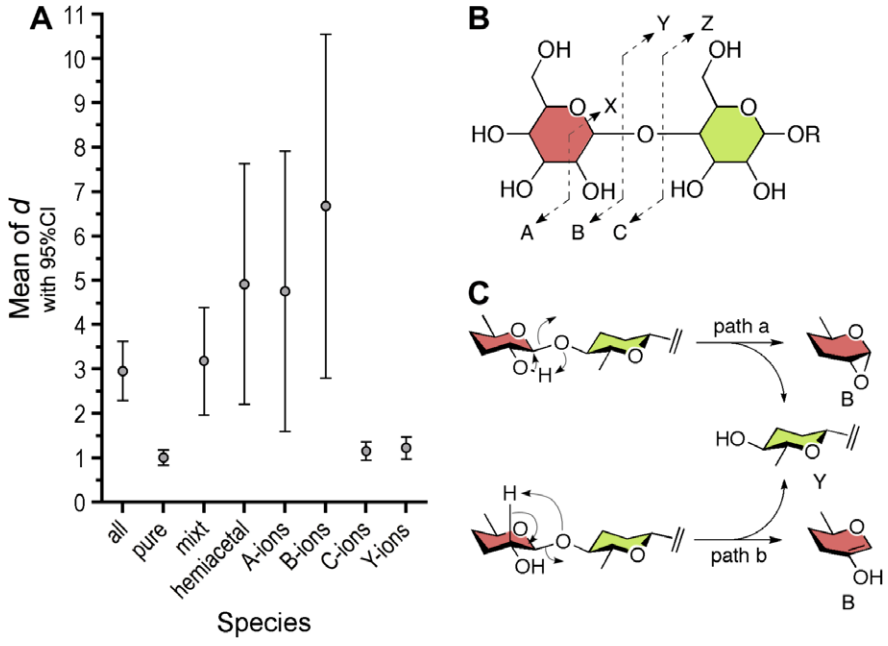



**Figure 3.** Statistical analysis of a variety of compounds and fragment ion species. (A) The differences between ERMS data obtained at $MS^n$ and $MS^{n+1}$ can be described by the $d$ as defined by Eq. 2. The mean and 95% confidence intervals of the mean are shown. (B) Fragmentation patterns of a glycan and the assignments. (C) The dissociation mechanism for the B/Y rapture producing a mixture consisting of a pair of structural isomers (B-ion species) and a Y-ion. The $Na^+$ adducts were analyzed in this investigation but $Na^+$ was omitted in the scheme for clarity.

do-isomers', but our experiments indicate that other factors related to isomeric structures are considerably larger and the difference in localization of $Na^+$ cannot be distinguished. Also, isomeric structures according to the different network of hydrogen bonds and conformations may exist. However, these were not observed in the current investigation.

### 3.4. SDC-based purity inspection of a mixture of sequence isomers before and after HPLC separation

In order to demonstrate the potential of SDC, we set up an experiment where SDC analyses were made before and after separation (HPLC) of isomeric compounds (Fig. 4). Compounds used in the experiments were α-Gal*p*-(1→3)-β-Gal*p*-(1→4)-GlcNAc-PA carrying the P1 antigenic disaccharide from *Escherichia coli*[39] and β-Gal*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc-PA associated with *Pseudomonas cepacia*[40] and *Moraxella catarrhalis*[41] infections. These compounds share the same element composition and thus have the same *m*/*z* values. The SDC of ERMS of a mixture of these compounds (Fig. 4A, condition A) resulted in a *d* value that was to be judged as a mixture (*d* 7.95) (Fig. 4B), whereas the isolated compounds resulted in smaller *d* values (Fig. 4C and D). In a real world analytical process, we are frequently confronted with defining whether any given sample is pure or contaminated. Mass spectrometric analysis determines the presence of contaminants with different *m*/*z* values, while the SDC further provided information concerning the presence of structural isomers (but not isomers with a single chiral center) without HPLC separation. Therefore, it is believed that the method will speed up the analytical process dramatically.

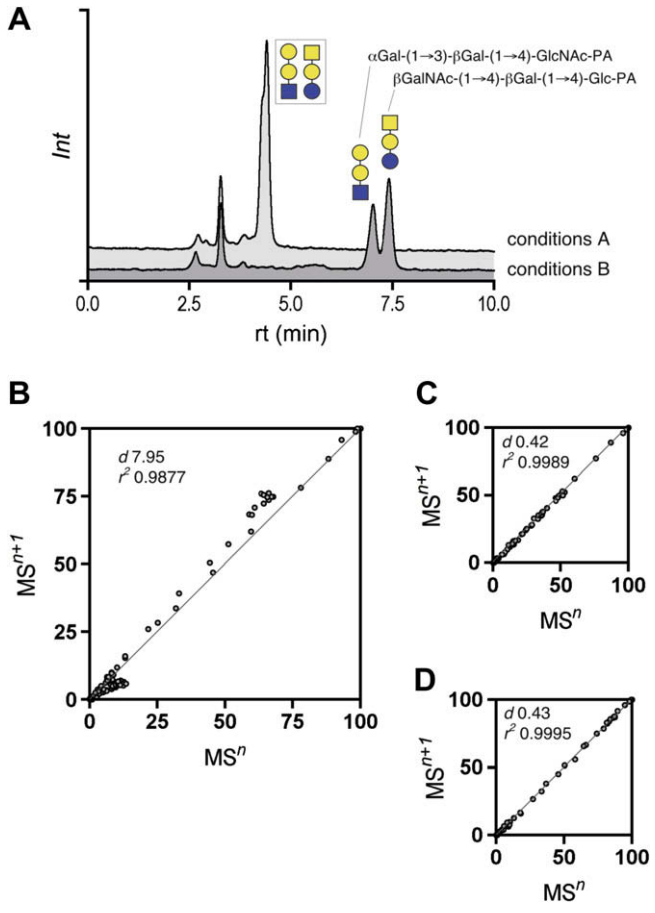



**Figure 4.** A simulation of analysis of an unknown analyte. (A) HPLC profiles of an analyte where the components were not resolved (conditions A) and were well separated (conditions B) (for details, see material and methods section). (B) SDC plot for an analyte without resolution. C. SDC plot for a peak of α-Gal*p*-(1 →3)-β-Gal*p*-(1→4)-GlcNAc-PA. (D) SDC plot for a peak of β-Gal*p*NAc-(1→4)-β-Gal*p*-(1 →4)-Glc-PA.

### 3.5. Relationship between *d* value and the ratio of contents

The value *d* is affected by the ratio and the physicochemical properties of contents in an analyte. When we assume individual compounds have same ionizability during the ionization process, electrospray ionization (ESI) in this case, the *d* values obtained for mixtures of different ratios may follow a simple bell-shaped profile with zero skewness, where the values are proportional to the difference in activation energy of each compound. In reality, the ionizability of all compounds are different, therefore, the shape of a curve become asymmetric (skewness ≠ 0).

We present such an example using β-Gal*p*-(1→3)-α- and -β-Gal*p*-OMe. Using the *d* values that had been used to judge the purity, a curve describing the ionization profile of a mixture of these compounds could be obtained (Fig. 5). This experimentally shows β-Gal*p*-(1→3)-β-Gal*p*-OMe has higher ionizability compared to β-Gal*p*-(1→3)-α-Gal*p*-OMe, and it is also the first result of introducing a method of evaluating ionization potency of isomeric compounds under ESI process.

### 3.6. Further considerations

In this article, a method for checking the purity of a carbohydrate sample was introduced based on a stage-discriminated correlation (SDC) of ERMS of 'a precursor ion' at different stages of CID ($MS^n$ vs $MS^{n+1}$). The method, however, should not be limited to carbohydrate analysis, but also can be applied to other compounds carrying diastereomeric and sequentially isomeric structures. A problem associated with the current SDC method is that it cannot be applied reliably to detect a minor contamination that is less than 10% due to the differences in ionization potency as it was clearly shown in Figure 5. The different ionizabilities are always a problem when analyzing a mixture consisting of various compounds with different characteristics. This is the case that it is difficult to detect glycopeptides in a proteinase digest that also contains unmodified peptides.

One of the ways to overcome this problem would be to use a dilute solution for spraying the sample where ideally only one molecule occupies a droplet of solvent. As the solvent evaporates, an ion is left. Each droplet contains an independent molecule, but these molecules are isolated and thus individual ions are formed independent of the ionizability of molecules. The next problem

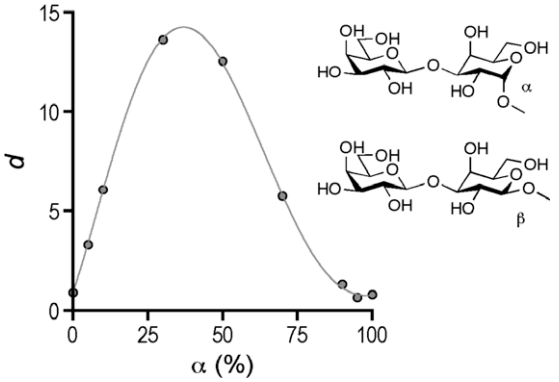



**Figure 5.** Standard curve for a mixture of β-Gal*p*-(1→3)-α- and -β-Gal*p*-OMe. Mixtures (9:1, 7:3, 5:5, 3:7, and 1:9) of individual components were prepared and the *d* values were obtained from an Eq. 2 after SDC analysis.

would be the amount of ions formed. Thus, a future mass spectrometer needs to have an extra 'well' to concentrate thin ionic gas. In this manner, a difficulty in the current report, which is associated with the ionization tendencies of individual species, might be overcome. Regardless of the problem, SDC-ERMS does not require any chromatographic purification methods. Therefore, this technique may become a first choice of analysis of potential mixtures of structural isomers.

## 4. Conclusion

The stage-discriminated correlation of ERMS provides a new way to assess even an analyte whose contents are totally unknown. The obvious advantage is that no separation protocol except for MS is necessary. The stage-discriminated correlation may thus speed up the analysis stages on occasions such as the analysis of synthetic pharmaceuticals and other compounds and structural determination of natural products including glycoconjugates. Furthermore, it was shown for the first time that C- and Y-ion species obtained under CID conditions can be treated as pure, and are suitable for further structural analysis, especially in spectral matching.[8–10] On the other hand, it is recommended that precautions be taken when using B- and A-ion species in such comparisons. It was also shown that the method can be used to observe differences in the ionization potencies of isomeric compounds.

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