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# Collisional Activation Dissociation Mass Spectrometry Studies of Oligosaccharides Conjugated with $Na^+$-Encapsulated Dibenzo-18-Crown-6 Ether

**Jungeun Bae[#], Hwangbo Song[#], Bongjin Moon*, and Han Bin Oh***

*Department of Chemistry, Sogang University, Seoul 04107, Korea*

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**Abstract :** To determine the influence of the cationization agent on the collision activated dissociation (CAD) fragmentation behavior of oligosaccharides, the CAD spectra of the singly protonated, sodiated oligosaccharides and singly sodiated and dibenzo-18-crown-6 ether conjugated oligosaccharides were carefully compared. Each of these three different species showed quite different fragmentation spectra. The comparison of singly protonated and sodiated oligosaccharide CAD spectra revealed that different cationization agents affected the cationization agent adduction sites as well as the fragmentation sites within the oligosaccharides. When the mobility of $Na^+$ was limited by the dibenzo-18-crown-6 ether encapsulation agent, the examined linear oligosaccharides showed fragmentation patterns quite different from the unmodified ones. For the dibenzo-18-crown-6 ether conjugated oligosaccharides, the *charge-remote* fragmentation pathways were more likely to be activated than the *charge-directed* pathways. This work demonstrates that dibenzo-18-crown-6 ether conjugation can potentially provide a route to selectively activate the *charge-remote* fragmentation pathways, albeit to a limited extent, in tandem mass spectrometry studies.

**Keywords :** collisional activation dissociation, mass spectrometry, crown ether, oligosaccharides, sodium

## Introduction

Glycosylation is one of the most common posttranslational modifications. In the framework of glycosylated proteins, glycans play an important role in a variety of biological processes, such as protein conformation, cell signaling, cell adhesion, and cell recognition.[1] Furthermore, disease states are often reflected in the glycosylated states of many proteins.[2] For these reasons, extensive effort has been made to reveal the structures and functions of glycans. In the mass spectrometry field, much work has been conducted to develop universal and effective mass spectrometry methods to elucidate glycan structures.[3-9] However, glycan analysis is more challenging than protein analysis since glycans present an enormous structural diversity that is derived from variability in interglycosidic linkage and branching.[10]

In the mass spectrometric analysis of glycans, monosaccharide composition, sequence of monosaccharide residues, interglycosidic linkage, branching, and anomeric states must be characterized.[10] To acquire this information, a variety of tandem mass spectrometry methods are being used in glycan analysis: collision activation dissociation (CAD), infrared multiphoton dissociation (IRMPD), ultraviolet photodissociation (UV-PD), and electron-aided fragmentation methods.[8,9,11] Among those, CAD is the most widely used method. In the CAD of protonated glycans, glycosidic bond cleavage is a prevalent process, which reveals the sequence of glycans through the laddering approach. However, interglycosidic linkage and branching information cannot be deduced from only glycosidic bond cleavage products. Cross-ring cleavage products can provide such information, but these products are rarely observed in the CAD of protonated glycans. Instead, alkali-metal adducted glycan ions are known to produce cross-ring cleavage products upon CAD.[12-16] The alkali-metal has been proposed to interact with polar groups of oligosaccharide species and to catalyze local fragmentations by lowering the threshold energy for decomposition.[16] Furthermore, alkali-metal adducted oligosaccharide ions do not undergo the rearrangement of monosaccharide units in the collisional activation process, whereas protonated oligosaccharide ions were reported to often experience rearrangement of composing sugar units.[9] In this regard, the



#Both authors contributed equally to this work.
*Reprint requests to Han Bin Oh and Bongjin Moon
E-mail: hanbinoh@sogang.ac.kr, bjmoon@sogang.ac.kr

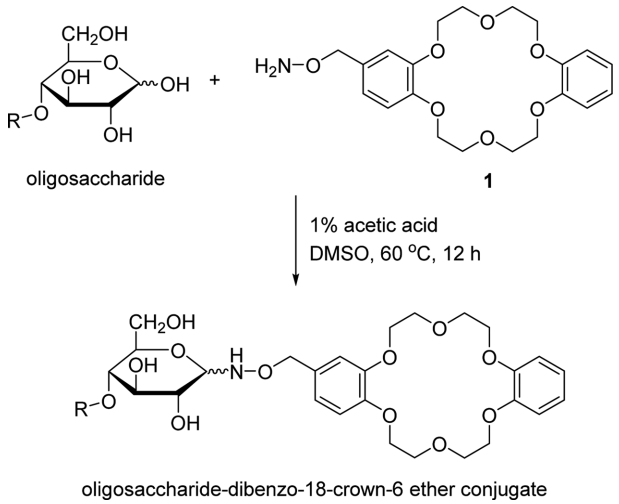
CAD of protonated and sodiated oligosaccharides can be complementary to each other, revealing more structural information.

In the fragmentation of oligosaccharides, it was suggested that glycosidic bond cleavage involves a charging agent, either $H^+$ or $M^+$ (particularly, $Na^+$), known as *charge-directed* fragmentation. On the other hand, cross-ring cleavage occurs through the *charge-remote* fragmentation pathways.[13,17-19] However, the effect of an alkali metal ion, *e.g.*, $Na^+$, which is localized and immobilized at a certain position of the oligosaccharide species, has not been investigated. The immobilization of $Na^+$ can be achieved by covalently incorporating a dibenzo-18-crown-6 ether that is known to form stable complexes with $Na^+$ (see **Scheme 1**).[20] In the present study, singly protonated, sodiated oligosaccharides and singly sodiated, dibenzo-18-crown-6 ether conjugated oligosaccharides were subjected to CAD and carefully compared. The comparison of these CAD spectra is expected to show the effects of $Na^+$ adduction on the fragmentation behavior of oligosaccharide ions and also to shed a light on how the immobilization of $Na^+$ influences the sodiated oligosaccharide ion fragmentation patterns.

## Experimentals

### Materials

Maltose, maltotetraose, and maltoheptaose, dimethyl sulfoxide (DMSO), and sodium hydroxide were purchased from Sigma (St. Louis, MO, USA). Acetic acid, acetonitrile, and water were obtained from Burdick and Jackson (Ulsan, Korea). A dibenzo-18-crown-6 ether alkoxyamine tag reagent (see **Scheme 1**) was synthesized in 6 steps starting from 3,4-dihydroxybenzaldehyde (See Supporting Information).




**Scheme 1.** A reaction scheme for oligosaccharide conjugation with the dibenzo-18-crown-6 ether alkoxyamine reagent.

### Oligosaccharide conjugation with a dibenzo-18-crown-6 ether alkoxyamine tag reagent

For glycan derivatization, the oligosaccharide (0.3 μmol) and dibenzo-18-crown-6 ether alkoxyamine tag reagent (355 μg, 0.876 μmol) were dissolved in DMSO (25 μL) with 1% acetic acid in a clean Eppendorf tube (Scheme 1). The reaction mixture was incubated at 60°C for 12 h. The resulting solution of conjugated oligosaccharide was diluted to 20 μM with a solution of water/methanol (20:80 v/v) and subjected to characterization by mass spectrometry.

### Mass spectrometry

Electrospray ionization (ESI) mass spectrometry and tandem mass spectrometry experiments were carried out in positive ion mode on a linear quadrupole ion-trap mass spectrometer (LTQ-XL, Thermo Scientific, San Jose, CA, USA). Protonated oligosaccharide ions were generated by directly infusing the above mentioned sample solution using a built-in syringe pump at 20 μL/min. For the generation of sodiated oligosaccharide ions, a few aliquots of 0.1 mM NaOH solution were added into the oligosaccharide solution. The following mass spectrometer operation parameters were used: nebulizer gas, 10 (arbitrary unit) $N_2$; ESI source voltage, +5.0 kV; capillary voltage, +35 V; capillary temperature, 275°C; tube lens voltage, +50 V.

## Results and Discussion

Figure 1 shows the comparison of two CAD mass spectra of singly protonated and sodiated disaccharide (maltose) ions. These spectra clearly indicate that the fragmentation patterns are quite different, consistent with previous literature reports.[16,18,19] For the protonated maltose ions, a peak originating from OH loss is dominant, followed by glycosidic bond cleavage products such as $B_1$ and $Y_1$. It is also notable that cross-ring cleavage products, such as $^{3,5}X_2$ and $^{2,5}X_2$, are observed in significant amounts (Here, the glycan fragments are annotated according to the nomenclature of Domon and Costello).[21] In comparison, the CAD of singly sodiated maltose ions produced $Y_1$ and $^{0,2}A_2$ as major products, and the relative abundance of $B_1$ is much lower than these two peaks. This noticeable difference suggests that the different cationization agents, *i.e.*, $H^+$ and $Na^+$, strongly influence the location of a cation adduction and the fragmentation sites within the disaccharide maltose structures.[13-16,18,19]

The location and immobilization of $Na^+$ may affect the fragmentation patterns of sodiated oligosaccharides. To examine how the location and immobilization of $Na^+$ influences the disaccharide fragmentation behavior, a dibenzo-18-crown-6 ether, which acts as a $Na^+$ immobilizer by forming strong complexes with $Na^+$, is conjugated at the maltose reducing end (see Figure 2). In this structure, it can

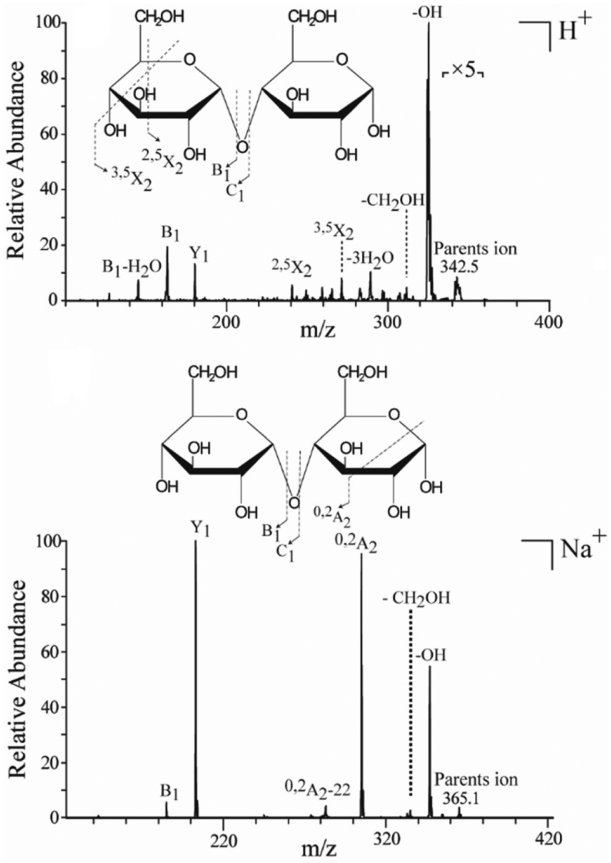



**Figure 1.** CAD spectra of singly (a) protonated and (b) sodiated maltose.

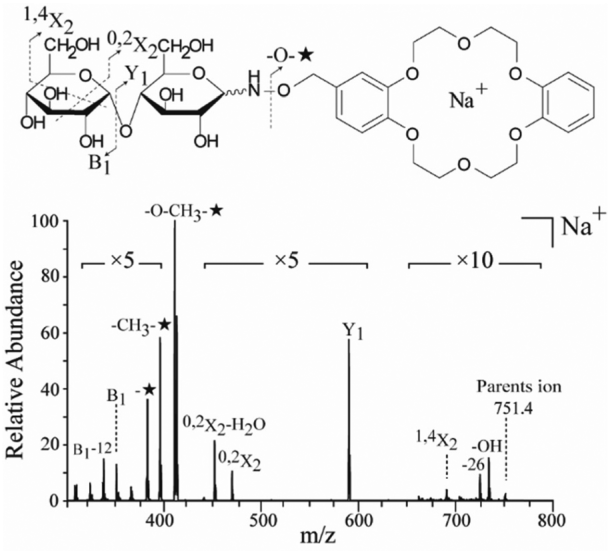



**Figure 2**. Above: the structure of maltose conjugated with dibenzo-18-crown-6 ether. Below: a CAD spectrum of the sodiated conjugated disaccharide, wherein $Na^+$ is envisaged to be encapsulated within the crown ether.

be envisaged that $Na^+$ is trapped within the crown ether. Therefore, for the sodiated dibenzo-18-crown-6 ether conjugated maltose, it can be conceived that if any fragmentation would occur within the disaccharide, it would do so through the so-called *charge-remote* fragmentation pathway.

The sodiated, conjugated maltose produced a fragmentation pattern quite different from that of the unmodified maltose counterpart. For example, bond cleavage between the N-O bond appeared as the most abundant cleavage for the conjugated maltose ion, which is denoted as –O-$CH_3$-★ in Figure 2. Of course, this bond cleavage product could not be observed at all for the unmodified maltose ions, due to the absence of the conjugated group linkage. The cross-ring cleavage product $^{0,2}A_2$, which is observed in high abundance for the unmodified maltose ion, did not appear

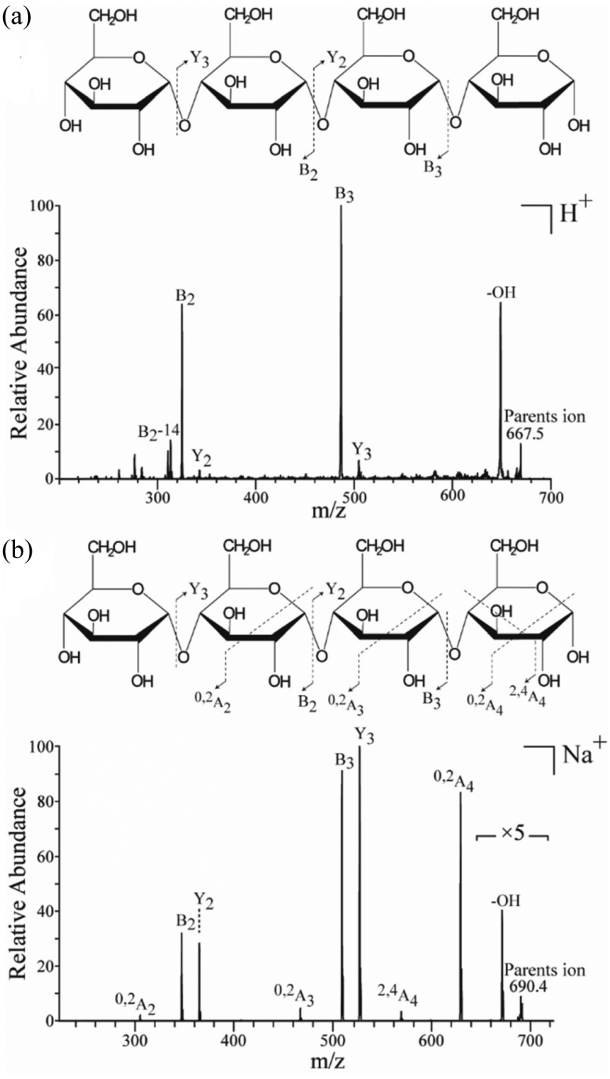



**Figure 3.** CAD spectra of singly (a) protonated and (b) sodiated maltotetraose.

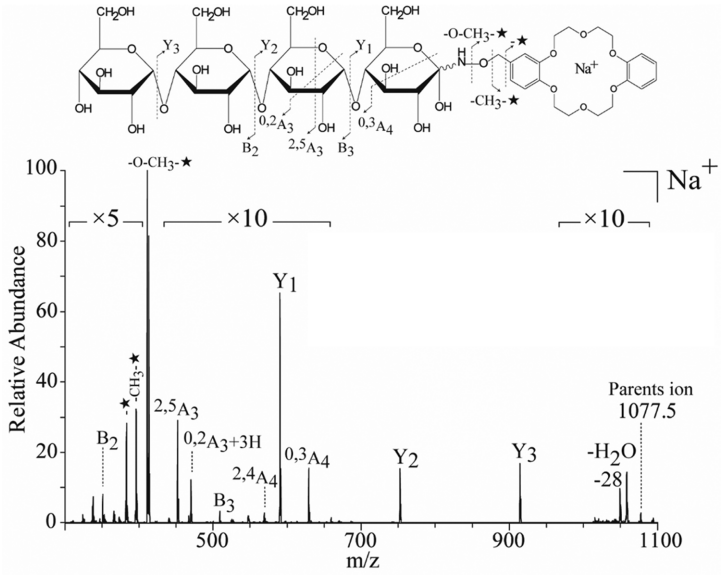



**Figure 4.** The structure of dibenzo-18-crown-6 conjugated, singly sodiated maltotetraose and its CAD spectrum.

at all for the maltose ions conjugated with the dibenzo-18-crown-6 ether. It is also noteworthy that $Na^+$ migrated to the first monosaccharide residue, to a certain extent, exhibiting the product (sodiated) $B_1$, despite our expectation that $Na^+$ was strongly trapped by the dibenzo-18-crown-6 ether.

To further examine the influence of the cationization agent on the CAD spectra of oligosaccharides, a linear tetrasaccharide maltotetraose was also examined. Figure 3 shows the CAD spectra of (a) singly protonated and (b) sodiated maltotetraose. As expected, these two cations show two contrasting MS/MS spectra. For protonated maltotetraose, $B_2$ and $B_3$ fragments are most abundant along with the OH loss peak. $Y_2$ and $Y_3$ fragments are also observed but in low abundances. In contrast, the sodiated tetrasaccharide yielded abundant Y fragments along with abundant B fragments. It is also notable that cross-ring cleavage products, such as $^{0,2}A_2$, $^{0,2}A_3$, $^{2,4}A_4$, and $^{0,2}A_4$ ions (which can be formed only through two bond fragmentations in the sugar ring and thus have higher fragmentation threshold energies), are observed for sodiated maltotetraose, but no such ring cleavage product is observed for protonated maltotetraose. It appears that cross-ring cleavages were promoted by the presence of $Na^+$.[16] The $Na^+$ adduction lowered the threshold energy values of glycosidic bond cleavage and also of cross-ring cleavage. Therefore, the cross-ring cleavage products, which have higher fragmentation thresholds, could be observed for the sodiated maltotetraose. The observation of cross-ring cleavage products for the sodiated maltotetraose is consistent with previous literature reports that the CAD spectra of $Na^+$-adducted glycans often produced more information-rich fragmentation patterns than the protonated glycans.[16,17,22] These cross-ring cleavage products are useful for determining the linkage position between the adjacent monosaccharide residues.

A dibenzo-18-crown-6 ether conjugated, singly sodiated maltotetraose shows a quite different fragmentation pattern from the unmodified sodiated maltotetraose (see Figure 4). Due to the charge ($Na^+$) localization at the reducing end (dibenzo-18-crown-6 ether), Y series fragments are observed in high abundances, and the relative abundances of B series fragments are much lower. On the other hand, the appearance of highly abundant Y series fragments suggests that the glycosidic bond cleavage can also occur through the *charge-remote* fragmentation pathway (note that $Na^+$ is immobilized by the dibenzo-18-crown-6). This is in stark contrast to previous reports that glycosidic bond cleavage occurred via a *charge-directed* fragmentation mechanism.[13,17,19] However, this interpretation should be taken with caution. The observation of (sodiated) B series fragments indicates that even with the dibenzo-18-crown-6 that is known to form a strong complex with $Na^+$, $Na^+$ migrated to one of the tetrasaccharide sugar units during the collisional activation process, although it occurred to a limited extent. Nevertheless, the spectral difference between the unmodified and dibenzo-18-crown-6 conjugated, singly sodiated maltotetraose suggests that the location and immobilization of $Na^+$ may significantly influence the overall fragmentation pattern of maltotetraose.

A tandem mass spectral difference between the singly protonated and sodiated oligosaccharide is much clearer for a linear heptosaccharide, maltoheptaose (see Figure 5). Maltoheptaose is a good linear glycan model that has been studied by many other researchers.[23,24] Singly protonated maltoheptaose shows only B series glycosidic bond cleavage products as main fragments. In contrast, the CAD spectrum of singly sodiated maltoheptaose yields abundant

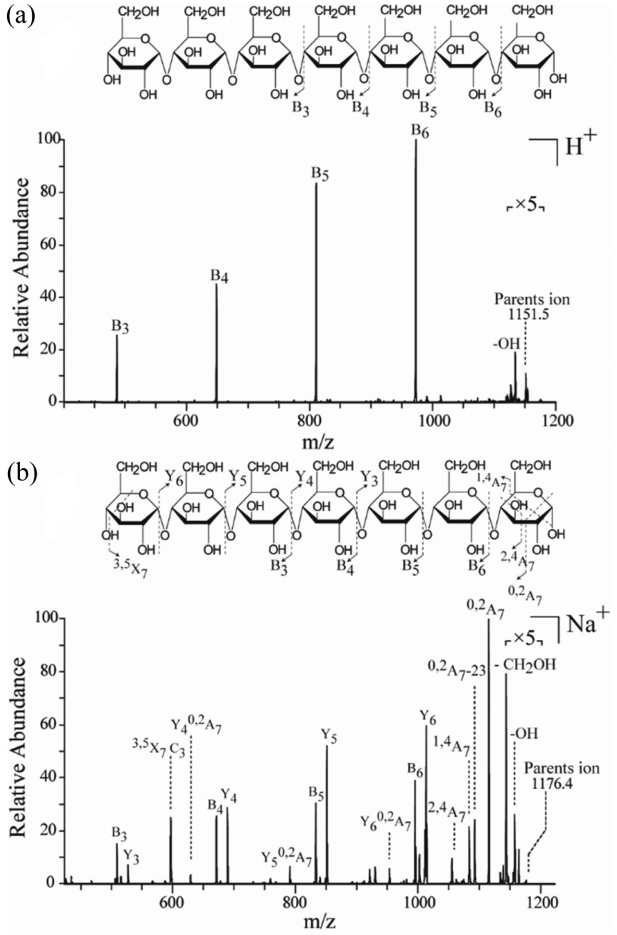



**Figure 5.** CAD spectra of singly (a) protonated and (b) sodiated maltoheptaose.

Y series fragments as well B series fragments. Furthermore, a number of cross-ring cleavage products are also observed in the CAD spectra of singly sodiated maltoheptaose: $^{0,2}A_7$, $^{1,4}A_7$, $^{2,4}A_7$, and $^{3,5}X_7$.

The influence of $Na^+$ location and immobilization on the oligosaccharide fragmentation patterns can be understood by comparing the spectra of singly sodiated unmodified maltoheptaose and the dibenzo-18-crown-6 conjugated maltoheptaose. As shown in Figure 6, the CAD spectrum of the modified maltoheptaose shows a much more complex fragmentation pattern compared with the unmodified maltoheptaose spectrum. Specifically, the C series of glocosidic bond cleavage products, which were often observed in negative-ion CAD experiments,[25] appear in significant abundances. In addition, different cross-ring cleavage products are more substantial: $^{0,1}A_7$, $^{2,4}A_7$, and $^{0,2}X_{2\text{-}5}$. This difference suggests that $Na^+$ immobilization activated different oligosaccharide fragmentation pathways, more likely enhancing the *charge-remote* fragmentation pathways compared with the *charge-directed* pathways.[13,17-19] However, as mentioned earlier, the observation of (sodiated) B and C series fragments implies that $Na^+$ migration to the sugar unit from the dibenzo-18-crown-6 ether occurs albeit to a limited extent. Nevertheless, from these observations, it can be deduced that a cationization agent, either $H^+$ or $Na^+$, and the immobilization of $Na^+$ strongly affect the oligosaccharide fragmentation pathways.

## Conclusions

CAD fragmentation patterns of a number of singly protonated, sodiated, and 18-crown-6 conjugated

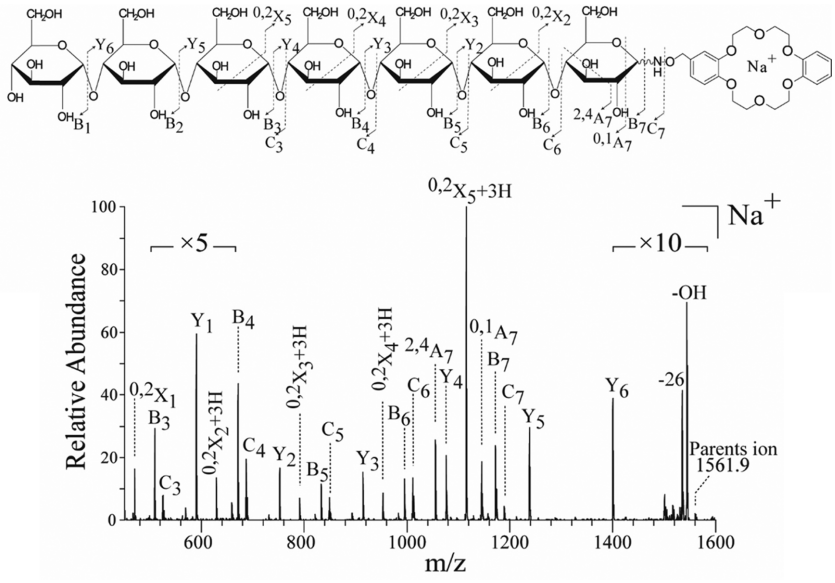



**Figure 6.** The structure of dibenzo-18-crown-6 conjugated, singly sodiated maltoheptaose and its CAD spectrum.

oligosaccharides are examined to understand the influence of the cationization agent and $Na^+$ immobilization on the overall oligosaccharide fragmentation behavior. It is shown that the protonated oligosaccharides tend to induce a much simpler fragmentation pattern, mostly with B series fragments, while $Na^+$ cationized oligosaccharides show Y series fragments along with B series products. Furthermore, $Na^+$ adducted oligosaccharides also yielded more cross-ring cleavage products. Interestingly, dibenzo-18-crown-6 conjugated oligosaccharides, which were expected to limit the $Na^+$ mobility within the oligosaccharide framework, showed fragmentation patterns quite different from singly sodiated but unmodified oligosaccharides. Particularly, for the dibenzo-18-crown-6 conjugated oligosaccharides, the *charge-remote* fragmentation pathways were presumed to be more activated than the *charge-directed* pathways. From these comparative examinations, it can be further confirmed that a cationization agent, either $H^+$ or $Na^+$, strongly influences the overall fragmentation behavior of oligosaccharides.

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