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FOCUS: BIO-ION CHEMISTRY: INTERACTIONS OF BIOLOGICAL IONS WITH IONS, MOLECULES, SURFACES, ELECTRONS, AND LIGHT : RESEARCH ARTICLE

Gas-Phase Intercluster Thiyl-Radical Induced C–H Bond Homolysis Selectively Forms Sugar C2-Radical Cations of Methyl D-Glucopyranoside: Isotopic Labeling Studies and Cleavage Reactions

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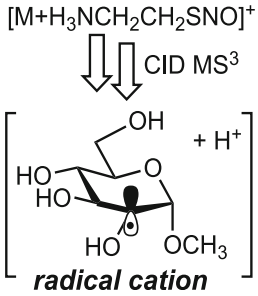

Abstract. A suite of isotopologues of methyl D-glucopyranosides is used in conjunction with multistage mass spectrometry experiments to determine the radical site and cleavage reactions of sugar radical cations formed via a recently developed 'bio-inspired' method. In the first stage of CID (MS^2), collision-induced dissociation (CID) of a protonated noncovalent complex between the sugar and *S*-nitrosocysteamine, $[H_3NCH_2CH_2SNO + M]^+$, unleashes a thiyl radical via bond homolysis to give the noncovalent radical cation, $[H_3NCH_2CH_2S^{\bullet} + M]^+$. CID ($MS^3$) of this radical cation complex results in dissociation of the noncovalent complex to generate the sugar radical cation. Replacement of all exchangeable OH and NH protons with deuterons reveals that the sugar radical cation is formed in a process involving abstraction of a hydrogen atom from a C–H bond of the sugar coupled with proton transfer to the sugar, to form $[M - H^{\bullet} + D^+]$. Investigation of this process using individual C-D labeled sugars reveals that the main site of H/D abstraction is the C2 position, since only the C2-deuterium labeled sugar yields a dominant $[M - D^{\bullet} + H^+]$ product ion. The fragmentation reactions of the distonic sugar radical cation, $[M - H^{\bullet} + H^+]$, were studied by another stage of CID (MS^4). ^{13}C-labeling studies revealed that a series of three related fragment ions each contain the C1–C3 atoms; these arise from cross-ring cleavage reactions of the sugar.

Keywords: Noncovalent complex, *S*-nitrosocysteamine, Carbohydrate, Collision-induced dissociation, Radical chemistry

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Introduction

Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry are routinely used to sequence oligosaccharides by collision-induced dissociation (CID) of their even electron ions [1, 2]. However, relatively few studies have investigated the use of gas-phase radical ion chemistry involving odd electron species to direct the fragmentation of sugars. Early pioneering electron ionization (EI) studies by Finan and other workers highlighted the rich chemistry of sugar radical cations, which included cross-ring cleavage reactions [3–7]. Unfortunately the generation of traditional EI mass spectra is restricted by volatility issues to monosaccharides and small oligosaccharides, which can in some cases be overcome by derivatization strategies [8]. To overcome such limitations, several approaches that exploit the gas-phase chemistry of ions formed using ESI have been developed that unleash radical sites on sugars. These include: the use of electron capture dissociation (ECD) and electron transfer dissociation (ETD), which result in radical-directed

Dedicated to Professor Scott McLuckey on the occasion of his ASMS award and in recognition of his important contributions to gas-phase ion chemistry and service to the American Society of Mass Spectrometry. One of us (R.A.J.O.) fondly remembers sabbatical visits with Scott at Oak Ridge National Laboratories and at Purdue.

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cleavage of oligosaccharides [9–11]; formation of radical ions by electron detachment dissociation (EDD) and the related negative electron transfer dissociation (NETD) method [12–16], or by photo-activated electron detachment [17–20]; the use of CID of a ternary metal complex containing the carbohydrate to form radical anions [21]; and an extension of Beauchamp's free radical initiated peptide sequencing (FRIPS) method [22] to oligosaccharides [23, 24].

Inspired by Nature's use of enzyme radical chemistry to biocatalytically transform carbohydrate-based substrates [25–31], we have previously described an approach that utilizes a charged noncovalent complex between a sugar and a radical to generate a charged sugar radical [32, 33]. The method (Scheme 1) involves: (1) transfer to the gas phase of a charged noncovalent complex containing a sugar and a radical precursor (an *S*-nitrosoamine); (2) formation of the radical cation noncovalent complex by unleashing the radical site from the precursor in preference to dissociation of the noncovalent complex [step (a) of Scheme 1]; and (3) transfer of both the radical and charge sites to the sugar upon dissociation of the radical cation non-covalent complex [step (b) of Scheme 1]. A wide range of noncovalent complexes were found to produce varying amounts of radical cations of mono- and oligosaccharides; however, the sites of the hydrogen atom transfer (HAT) from the sugar and the mechanistic details of the radical fragmentation reactions were not examined. Here we use a series of isotopically labeled methyl D-glucopyranosides (shown in Scheme 2) to identify: (1) the site(s) of hydrogen atom abstraction; and (2) which bonds within the sugar are cleaved upon CID of the radical cation. Based on previous studies that revealed that the best *S*-nitrosoamines to form the highest yield of sugar radical cations are those with a short linker X [33], *S*-nitrosocysteamine was chosen for this work.

Materials and Methods

Materials

Methyl α-D-glucopyranoside, cysteamine, methanol-d_4 (>99.8 atom-% and *tert*-butyl nitrite were purchased from Sigma Aldrich Chemical Co. (Castle Hill, NSW, Australia) and used as received. Deuterium-labeled [(2-^{2}H)-, (3-^{2}H)-, (4-^{2}H)-, (5-^{2}H)-, and (6,6'-$^2H_2$)-] D-glucose (98 atom-%), ^{13}C-labeled [(1-^{13}C)-, (2-^{13}C)-, (3-^{13}C)-, (4-^{13}C)-, (5-^{13}C)-, (6-^{13}C)-] D-glucose (99 atom-%), and doubly labeled (1-^{2}H, 1-^{13}C)-D-glucose (98,99 atom-%) were purchased from Omicron Biochemicals (South Bend, IN, USA).

Preparation of Labeled Methyl D-Glucopyranosides

A solution of isotopically labeled D-glucose (3.0 mg, 0.017 mmol) in dry MeOH (0.5 mL) was treated with 3 M HCl in dry MeOH (0.3 mL) (prepared by addition of the appropriate amount of acetyl chloride to dry methanol), and heated to reflux overnight. The reaction was cooled to room temperature, then was adjusted to neutral pH by the addition of a small quantity of Dowex 1X8 (HO^- form), filtered and the solvent evaporated. Methanol-d_4 was used in place of methanol for the synthesis of the anomeric methyl-labeled isotopologue. Analysis of the products by ^{1}H NMR spectroscopy revealed a typically 4:1 α/β ratio of pyranose anomers.

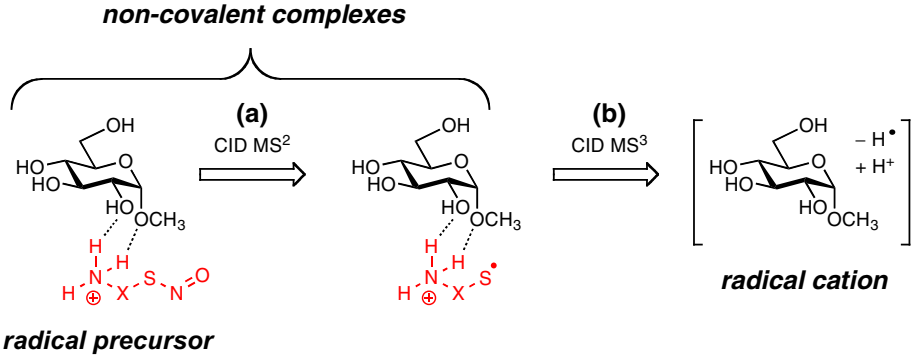
Preparation of S-Nitrosocysteamine

A mixture of *tert*-butyl nitrite (1.5 equivalents) and a 1 mM solution of the cysteamine (in 1:1 methanol:water with 1% acetic acid) was allowed to react for 10 min at room temperature. The reaction mixture was then diluted 100-fold using 50/50 methanol:water with 1% acetic acid.

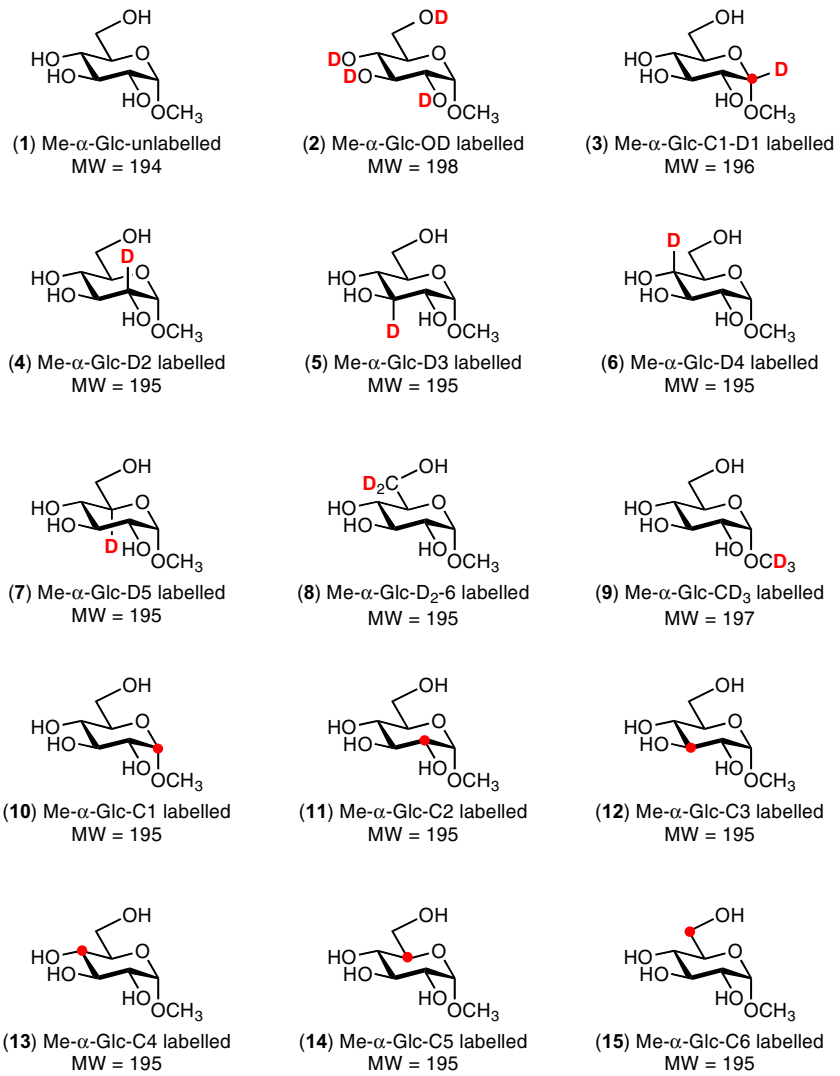
Mass Spectrometry

Solutions containing the sugar and *S*-nitrosocysteamine were made by mixing two equivalents of the sugar with one equivalent of *S*-nitrosocysteamine in 1:1 methanol:water with 1% acetic acid to give a final concentration of 1 μM. The labeled noncovalent complex of **2** was formed in a similar fashion, except that **1** was mixed with *S*-nitrosocysteamine in D_2O and 1% acetic acid-d4 for 15 min, and this solution was then subjected to ESI.

ESI mass spectra were collected using a Thermo Scientific LTQ-FT-ICR hybrid mass spectrometer (Bremen, Germany), consisting of a linear ion trap coupled to a 7T FT-ICR mass spectrometer. Analyte solutions were injected onto the ion trap



Scheme 1. Sequential CID steps used to form radical cations of methyl α-D-glucopyranoside. X = a short linker. For this work, X = CH_2CH_2



Scheme 2. Structures and MWs of methyl α-D-glucopyranoside isotopologues. A red dot denotes the site of a ^{13}C labeled atom

mass spectrometer using a syringe pump with a flow rate of 5.0 μL min^{-1}. The instrument settings were optimized for maximum $[H_3NCH_2CH_2SNO + M]^+$ signal intensity by using the auto-tune routine within the Tune program. The spray needle voltage, nitrogen sheath gas flow rate, and the capillary temperature were maintained at +5 kV, 5 arbitrary units, and 200 °C, respectively. Helium gas was used as the collision gas for CID experiments, and all MS^n experiments were carried out in the linear ion trap by mass selecting the precursor ion and subjecting it to collisional activation. The normalized collision energy (RCE), which determines the amplitude of the rf energy applied to the end-cap electrodes, was set between 16% and 18% (arbitrary units) depending on the precursor ion. The activation Q was set at 0.25, and the activation time was 30 ms.

Results and Discussion

A series of multistage mass spectrometry experiments were carried out on the noncovalent complexes formed between protonated *S*-nitrosocysteamine and various isotopically-labeled methyl D-glucopyranosides. MS/MS of the unlabeled noncovalent complex **1** (Figure 1a) gave rise to a radical cation of the complex (Equation 1, *m/z* 271) as well as protonated *S*-nitrosocysteamine (Equation 2, *m/z* 107) and the radical cation of cysteamine (Equation 3, *m/z* 77). Isolation of the radical cation complex, followed by a second stage of CID (Figure 1b), gave the sugar radical cation at *m/z* 194 (Equation 4), as well as fragment ions at *m/z* 177, 176, and 158. A minor competing loss of the cysteamine radical cation is also observed (Equation 5, *m/z* 77). Hydrogen atom abstraction (Equation 6) and formation of the protonated sugar (Equation 7) are not competitive fragmentation pathways. The fragment ions at *m/z* 176 and 158 are likely to occur from loss of one or two molecules of H_2O from the radical cation, the ion at *m/z* 177 can occur from loss of $HO^{\bullet}$ form the radical cation or from H_2O loss from a completely dissociating $[M + H]^+$ (initially formed via Equation 7). Isolation of the radical cation of the sugar

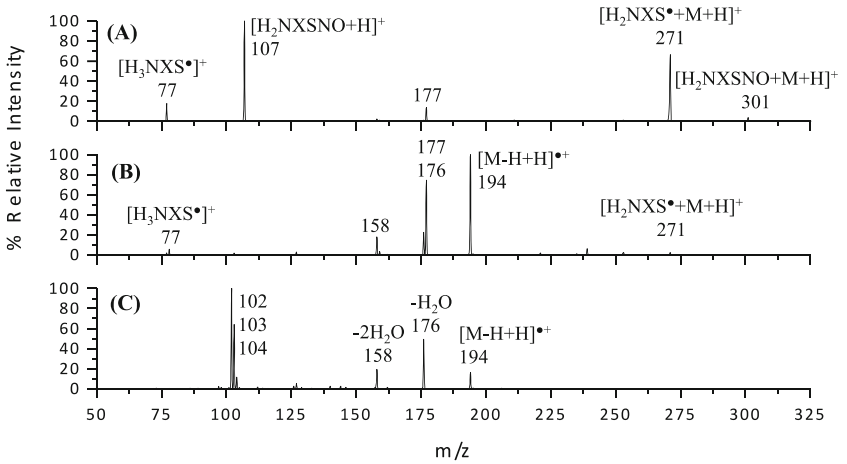


Figure 1. MS^n CID mass spectra of: (**a**) [**1** + $H_3NCH_2CH_2SNO]^+$, MS^2; (**b**) [**1** + $H_3NCH_2CH_2S^•]^+$, MS^3; (**c**) [**1** − $H^•$ + H^+], MS^4. X = CH_2CH_2. The spectra were collected using an LTQ-FT-ICR hybrid mass spectrometer. The RCE value was 18%

followed by another stage of CID (Figure 1c) proceeded through the loss of 1 and 2 water molecules (*m/z* 176 and 158, respectively) as well as by fragmentation of the sugar ring to give the peaks at *m/z* 102, 103, and 104.

$$[H_3NCH_2CH_2SNO + M]^+ \quad (1)$$

$$\rightarrow [H_3NCH_2CH_2S^• + M]^+ + NO^•$$

$$\rightarrow [H_3NCH_2CH_2SNO]^+ + M \quad (2)$$

$$\rightarrow [H_3NCH_2CH_2S^•]^+ + NO^• + M \quad (3)$$

$$[H_3NCH_2CH_2S^• + M]^+ \quad (4)$$

$$\rightarrow [M–H^• + H^+] + H_2NCH_2CH_2SH$$

$$\rightarrow [H_3NCH_2CH_2S^•]^+ + M \quad (5)$$

$$\rightarrow [H_3NCH_2CH_2SH]^+ + [M–H]^• \quad (6)$$

$$\rightarrow [M + H]^+ + H_2NCH_2CH_2S^• \quad (7)$$

Site of Hydrogen Atom Abstraction

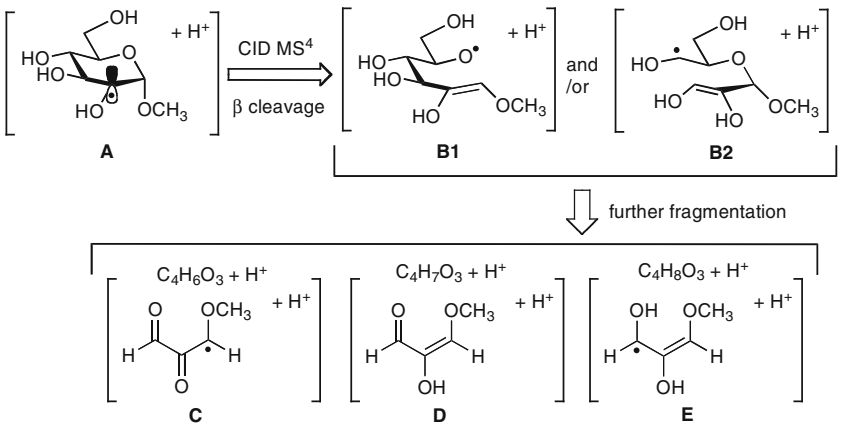
Based on the fact that monosaccharides have lower gas-phase basicities (GBs) than primary amines [compare GB(α-D-glucopyranose) = 188 kcal/mol [34] with GB($EtNH_2$) = 210 kcal/mol [35]] and that sugars readily form hydrogen bonded ammonium ion complexes $[M + NH_4]^+$ (and not $[M + H + NH_3]^+$) [36], we assume that complexes of the type (Scheme 1) play a key role in the formation of the sugar radical cation, which requires both hydrogen atom abstraction from the sugar and proton transfer to the resultant sugar radical. To separate out these two distinct intra-cluster reactions, we performed a deuterium labeling experiment in which all of the labile hydrogens of the noncovalent complex were exchanged for deuterium (Supporting Information, Supplementary Figure S1). In the second stage of CID (MS^3), the peak corresponding to the radical cation of the sugar (*m/z* 194) shifted to *m/z* 199 (Supplementary Figure S1b), confirming that abstraction of a hydrogen from one of the C–H bonds of the sugar coupled with D^+ transfer had occurred. This suggests that the radical is located on a carbon atom and the charge is located on an oxygen atom (i.e., formation of a *distonic* ion [37]). This observation is consistent with the lower bond dissociation energy of the C–H versus O–H bonds in sugars [23], and that the only sites for protonation in sugars are the O atoms [34, 38]. Thus the structure of the radical cation formed in our method is different to the initially formed canonical structures of radical cations of sugars generated via EI/MS [8].

Although the preceding experiment is silent on the site of protonation, by using the regiospecifically deuterium labeled precursors **3**–**9** (note: **3** contains both ^{13}C and ^2H) we reasoned that we could determine the site(s) of H atom abstraction from C–H bond(s). Surprisingly, HAT was found to occur almost exclusively from the C2 position (Supplementary Figure S3), whereas essentially no HAT occurs from the C1, 3, 4, 5, or 6 positions (Supplementary Figures S2, S4–S7). How do these results compare to previous studies? Radical reactions of sugars have been extensively examined in solution, and the regioselectivity of these reactions has been found to be determined by a range of factors, including: (1) the nature/reactivity of the radical species that abstracts a hydrogen atom; (2) relative stabilities of sugar radicals formed by hydrogen atom

abstraction; (3) stereochemical effects; and (4) steric effects of alcohol protecting groups [39, 40]. Owing to the absence of protecting groups, this last effect is not significant in this work. Regarding (1), "hot" hydroxyl radicals, $HO^{\bullet}$, are nonselective [41], whereas the resonance stabilized radical anion $SO_4^{\bullet-}$ produces radicals at the C1, C2, C5, and C6 positions of α-D-glucose [42]. With respects to (2), a previous study utilizing DFT calculations has revealed that the C–H bond dissociation energies (BDEs) of methyl α-D-glucopyranoside fall into a narrow range, with the C1 position having the lowest BDE [20]. While C1 hydrogen atom abstraction often occurs in solution [27, 28, 34–37], other C–H bonds can also be abstracted [39–42]. Abundant H atom abstraction from the C2 position in our experiments are consistent with a previous study that suggests the resultant C-based radical is stabilized by SOMO-σ* interactions between the unpaired electron at C2 and the eclipsing C1–OMe bond [42]. The observed regioselectivity for HAT is likely further controlled by hydrogen bonding within the noncovalent complex.

Origin of the m/z 102, 103, and 104 Fragment Ions in the CID Spectrum of the Radical Cation of Methyl D-Glucopyranoside

The use of the isotopically-labeled sugars allows not only the site of H atom abstraction to be established but allows the suggestion of likely intermediates in the fragmentation reactions (**A** and **B** in Scheme 3) and the assignment of product ion formulae for the fragment ions *m/z* 102 (**C**), *m/z* 103 (**D**), and *m/z* 104 (**E**) formed in the CID spectra of the sugar distonic ion, $[M - H^{\bullet} + H^{+}]$. All CID spectra are given in the Supporting Information, and Scheme 3 provides a summary of how the *m/z* values for these three fragment ions either remain the same or change as a function of the site(s) of the labels in the sugars. The CD_3 sugar, **9** (Scheme 2), gives product ions that are shifted by 3 Da, highlighting that the anomeric methoxy group is maintained in these fragment ions. Use of the ^{13}C labeled sugars **10**–**15** clearly identifies that the product ions each contain the C1, C2, and C3 carbons of the starting sugar. Thus fragment ions in the CID spectra of $[M - H^{\bullet} + H^{+}]$ are all shifted by 1 Da for **10**, **11**, and **12**, but not for **13**, **14**, and **15**. The deuterium labeled sugars **3**–**8** (note: **3** contains both ^{13}C and ^{2}H) provide fragments with *m/z* values consistent with the structures shown in Scheme 3. Finally, more than one bond must be cleaved to form fragment ions **C**–**E** and thus the mechanisms for their formation are likely to be complicated. Ignoring H-atom shifts, it seems likely that the initially formed distonic radical cation **A** undergoes β cleavage [43] of either the C1–O5 and/or the C3–C4 bond to give the ring opened ions **B1** and **B2**. Subsequent fragmentation reactions of **B1** and **B2** ultimately induce cleavage of the C3–C4 and C1–O5 bonds, respectively.



labelling	ion	*m/z* observed	ion	*m/z* observed	ion	*m/z* observed
none	**C1**:	*m/z* 102	**D1**:	*m/z* 103	**E1**:	*m/z* 104
C1-D1	**C3**:	*m/z* 104	**D3**:	*m/z* 105	**E3**:	*m/z* 106
D2	**C4**:	*m/z* 102	**D4**:	*m/z* 103	**E4**:	*m/z* 104
D3	**C5**:	*m/z* 103	**D5**:	*m/z* 104	**E5**:	*m/z* 105
D4	**C6**:	*m/z* 102	**D6**:	*m/z* 103	**E6**:	*m/z* 104
D5	**C7**:	*m/z* 102	**D7**:	*m/z* 103	**E7**:	*m/z* 104
D_2-6	**C8**:	*m/z* 102	**D8**:	*m/z* 103	**E8**:	*m/z* 104
CD_3	**C9**:	*m/z* 105	**D9**:	*m/z* 106	**E9**:	*m/z* 107
C1	**C10**:	*m/z* 103	**D10**:	*m/z* 104	**E10**:	*m/z* 105
C2	**C11**:	*m/z* 103	**D11**:	*m/z* 104	**E11**:	*m/z* 105
C3	**C12**:	*m/z* 103	**D12**:	*m/z* 104	**E12**:	*m/z* 105
C4	**C13**:	*m/z* 102	**D13**:	*m/z* 103	**E13**:	*m/z* 104
C5	**C14**:	*m/z* 102	**D14**:	*m/z* 103	**E14**:	*m/z* 104
C6	**C15**:	*m/z* 102	**D15**:	*m/z* 103	**E15**:	*m/z* 104

Scheme 3. Possible structures of key intermediates, **A** and **B1**, **B2**, and fragment ions, **C**–**E**, formed by CID of the radical cation; *m/z* values of fragment ions are given for the sugar isotopologues used as precursors (see Scheme 2 for structures and Supplementary Figures for MS^4 spectra)

Conclusions

This study emphasizes the power of experimental studies utilizing systematic isotope labeling to unravel fragmentation reactions of natural products [44]. The use of a suite of systematically stable-isotope labeled methyl glucosides has allowed the establishment of: (1) C2 as the site of the H atom abstraction within the noncovalent radical cation complex, $[H_3NCH_2CH_2S^{\bullet} + M]^+$, formed by S–NO bond homolysis (Equation 1); and (2) the bonds that are cleaved to form the fragment ions **C**, **D**, and **E** involve cross-ring cleavage of the sugar to release a fragment ion derived from the sugar C1–C3 atoms. A unique feature of this system, which uses intra-cluster proton and hydrogen transfer reactions facilitated by *N*-nitrosocysteamine to generate the distonic sugar radical is the highly regiospecific HAT from the sugar C2 position, and results in a simple series of fragment ions through one major fragmentation channel.

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