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**Gas-phase intercluster thiyl-radical induced C-H bond
homolysis selectively forms sugar C2-radical cations of methyl D-
glucopyranoside: isotopic labelling studies and cleavage reactions.^ψ**

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^ψ Dedicated to Prof. 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.

ABSTRACT:

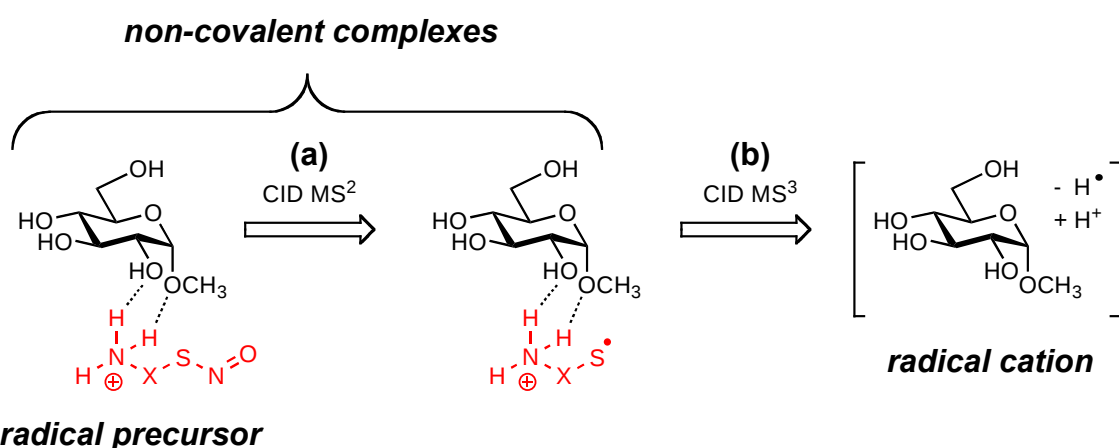
A suite of isotopologues of methyl D-glucopyranosides is used in conjunction with multistage mass spectrometry experiments to determine the radical site and cleavage reactions of sugar radical cations formed via a recently developed 'bio-inspired' method. In the first stage of CID (MS^2), collision-induced dissociation (CID) of a protonated non-covalent complex between the sugar and S-nitrosocysteamine, $[H_3NCH_2CH_2SNO+M]^+$, unleashes a thiyl radical via bond homolysis to give the non-covalent radical cation, $[H_3NCH_2CH_2S^{\bullet}+M]^+$. CID (MS^3) of this radical cation complex results in dissociation of the non-covalent 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 labelled 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 -labelling 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.

Running Title: Fragmentation of sugar radical cations in the gas-phase.

Keywords: non-covalent complex; S-nitrosocysteamine; carbohydrate; collision-induced dissociation; radical chemistry.

1. Introduction:

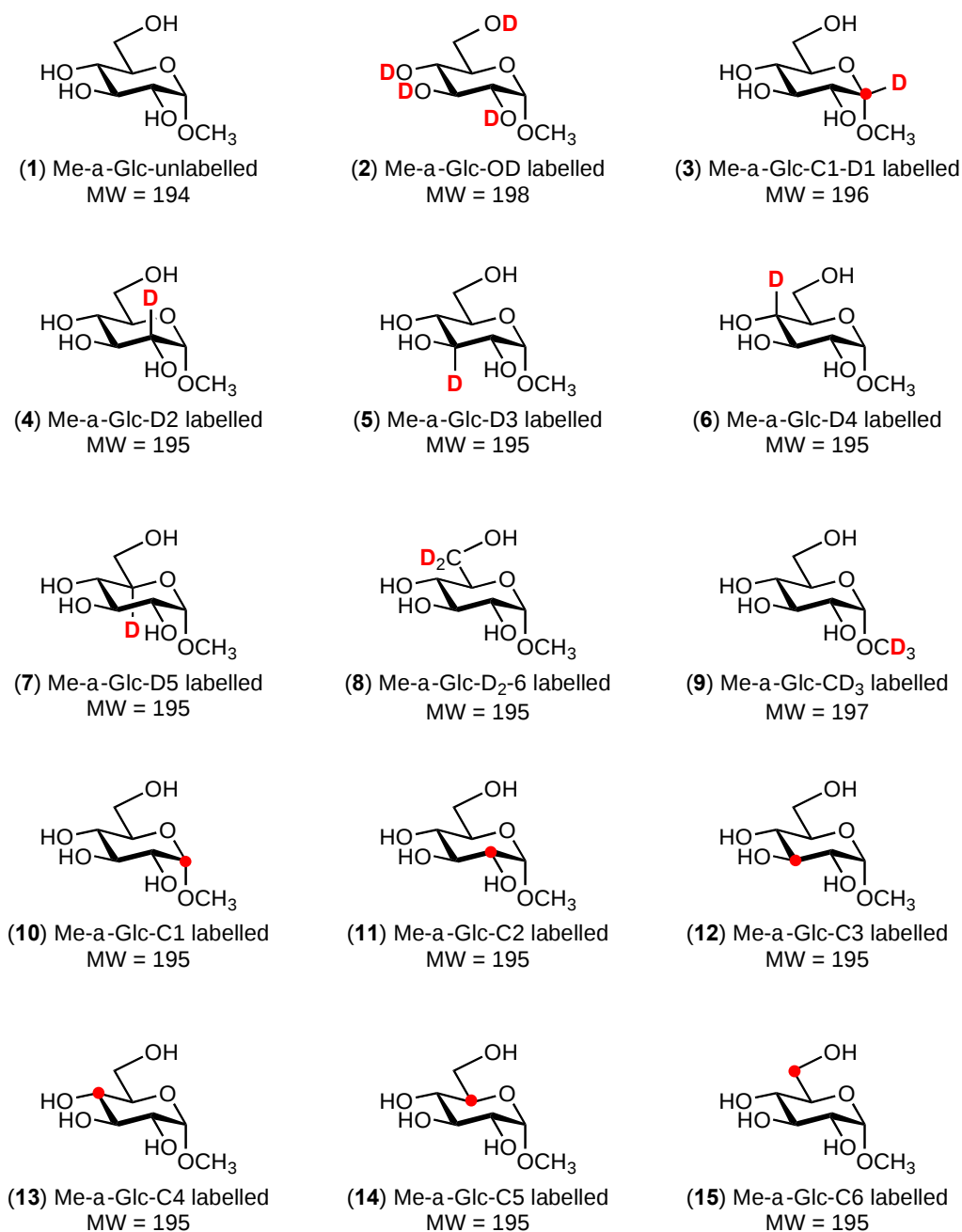
Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry are routinely used to sequence oligosaccharides by collision-induced dissociation (CID) of their even electron ions [1,2]. However, relatively few studies have investigated the use of gas phase radical ion chemistry involving odd electron species to direct the fragmentation of sugars. Early pioneering electron ionization (EI) studies by Finan et al. 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 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 Beachamp's free radical initiated peptide sequencing (FRIPS) method [22] to oligosaccharides [23,24].



Scheme 1. Sequential CID steps used to form radical cations of methyl α -D-glucopyranoside.

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 non-covalent complex between a sugar and a radical to generate a charged

sugar radical [32,33]. The method (Scheme 1) involves: (i) transfer to the gas phase of a charged non-covalent complex containing a sugar and a radical precursor (an *S*-nitrosoamine); (ii) formation of the radical cation non-covalent complex by unleashing the radical site from the precursor in preference to dissociation of the non-covalent complex (step (a) of Scheme 1); and (iii) 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 non-covalent 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 labelled methyl D-glucopyranosides (shown in Scheme 2) to identify: (i) the site(s) of hydrogen atom abstraction; and (ii) 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 [33], *S*-nitrosocysteamine was chosen for this work.



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

2. Materials and Methods

2.1 Materials

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

Preparation of labelled methyl D-glucopyranosides

A solution of isotopically labelled 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-labelled isotopologue. Analysis of the products by 1H 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.

2.2 Mass spectrometry

Solutions containing the sugar and *S*-nitrosocysteamine were made by mixing 2 equivalents of the sugar with 1 equivalent of *S*-nitrosocysteamine in 1:1 methanol:water with 1% acetic acid to give a final concentration of 1 μM . The labelled non-covalent complex of **2** was formed in a similar fashion, except that **1** was mixed with *S*-nitrosocysteamine in D_2O and 1% deuterated acetic acid for fifteen minutes, 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 mass spectrometer using a syringe pump with a flow rate of $5.0 \mu\text{L min}^{-1}$. The instrument settings were optimized for maximum $[\text{H}_3\text{NCH}_2\text{CH}_2\text{SNO}+\text{M}]^+$ 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.

3. Results and discussion

A series of multistage mass spectrometry experiments were carried out on the non-covalent complexes formed between protonated *S*-nitrosocysteamine and various isotopically-labelled methyl D-glucopyranosides. MS/MS of the unlabelled non-covalent complex **1** (Figure 1(a)) gave rise to a radical cation of the complex (eq. 1, m/z 271) as well as protonated *S*-nitrosocysteamine (eq. 2, m/z 107) and the radical cation of cysteamine (eq. 3, m/z 77). Isolation of the cluster, followed by a second stage of CID (Figure 1(b)), gave the sugar radical cation at m/z 194 (eq. 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 (eq. 5, m/z 77). Hydrogen atom abstraction (eq. 6) and formation of the protonated sugar (eq. 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 $\text{HO}^\bullet$ from the radical cation or from H_2O loss from a completely dissociating $[\text{M}+\text{H}]^+$ (initially formed via eq. 7). Isolation of the radical cation of the sugar followed by another stage of CID (Figure 1(c)) 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.

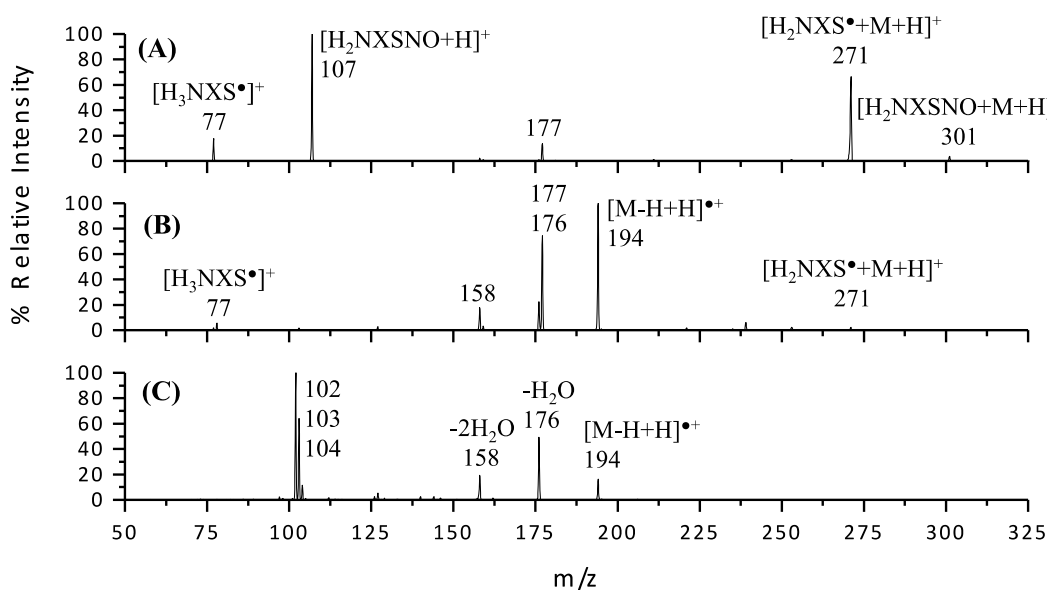
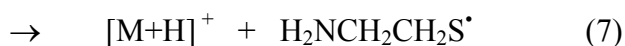
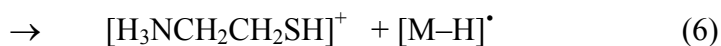
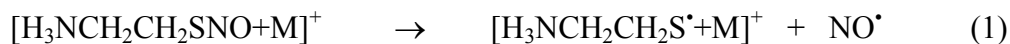


Figure 1: MSⁿ CID mass spectra of: (A) $[\mathbf{1} + \text{H}_3\text{NCH}_2\text{CH}_2\text{SNO}]^+$, MS²; (B) $[\mathbf{1} + \text{H}_3\text{NCH}_2\text{CH}_2\text{S}^\bullet]^+$, MS³; (C) $[\mathbf{1} - \text{H}^\bullet + \text{H}^+]$, MS⁴. X = CH₂CH₂. The spectra were collected using an LTQ-FT-ICR hybrid mass spectrometer. The RCE value was 18%.

3.1 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(EtNH₂) = 210 kcal/mol [35]) and that sugars readily form hydrogen bonded ammonium ion complexes

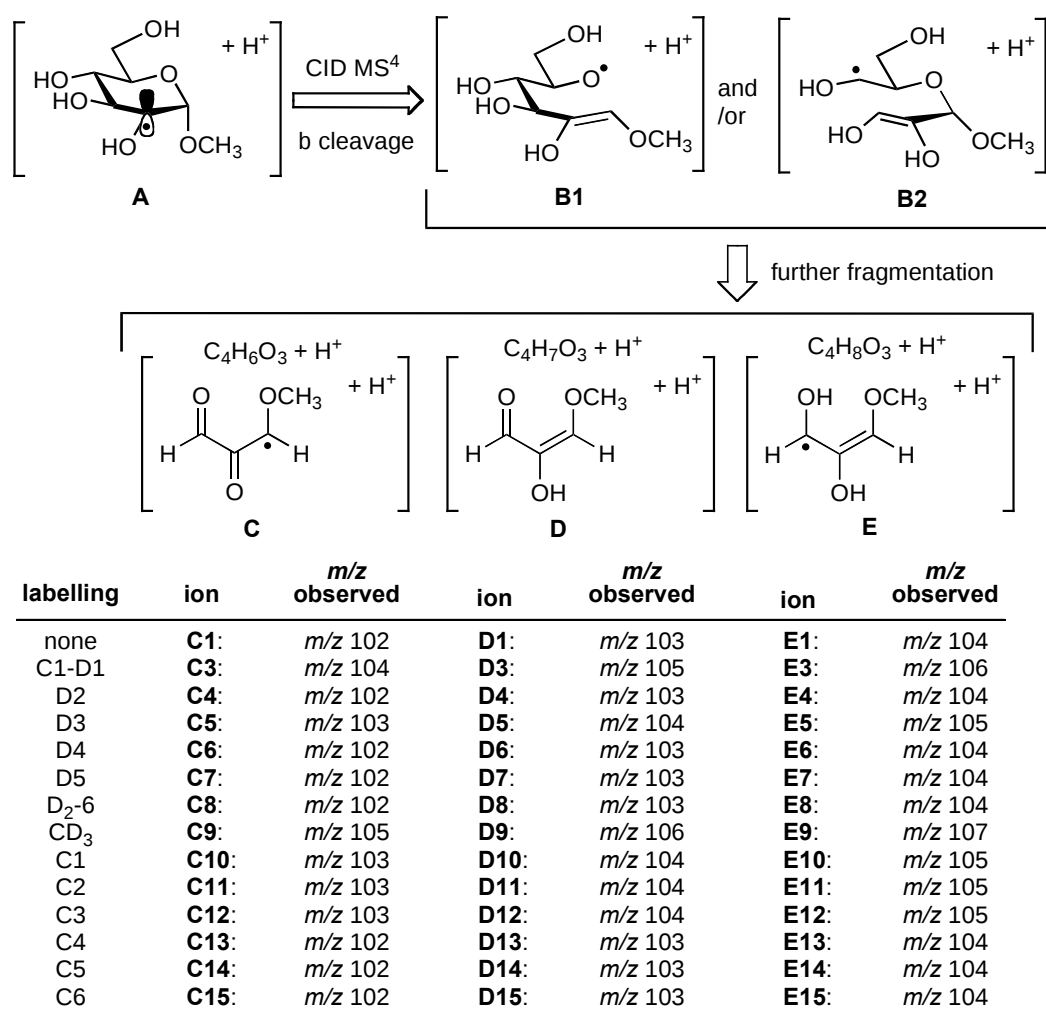
$[M+NH_4]^+$ (and not $[M+H+NH_3]^+$) [36], we assume that complexes of the type (Scheme 1) play a key role in the formation of the sugar radical cation, which requires both hydrogen atom abstraction from the sugar and proton transfer to the resultant sugar radical. To separate out these two distinct intracuster reactions, we performed a deuterium labelling experiment in which all of the labile hydrogens of the non-covalent complex were exchanged for deuterium (Supporting Information 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 (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 labelled 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 (Supporting Information Figure S3), whereas essentially no HAT occurs from the C1, 3, 4, 5 or 6 positions (Supporting Information 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: (i) the nature/reactivity of the radical species that abstracts a hydrogen atom; (ii) relative stabilities of sugar radicals formed by hydrogen atom abstraction; (iii) stereochemical effects; and (iv) 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 (i), “hot” hydroxyl radicals, $HO^\bullet$, are non-selective [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 (ii), 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 non-covalent complex.

3.2 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-labelled 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 CD₃ 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 ¹³C labelled 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 labelled sugars **3–8** (note: **3** contains both ¹³C and ²H) 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.



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 MS⁴ spectra).

4. Conclusions

This study emphasizes the power of experimental studies utilizing systematic isotope labelling to unravel fragmentation reactions of complex natural products [44]. The use of a suite of systematically stable-isotope labelled methyl glucosides has allowed the establishment of: (i) C2 as the site of the H atom abstraction within the non-covalent radical cation complex, $[\text{H}_3\text{NCH}_2\text{CH}_2\text{S}^+\text{M}]^+$, formed by S–NO bond homolysis (eq. 1); and (ii) 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 intracuster 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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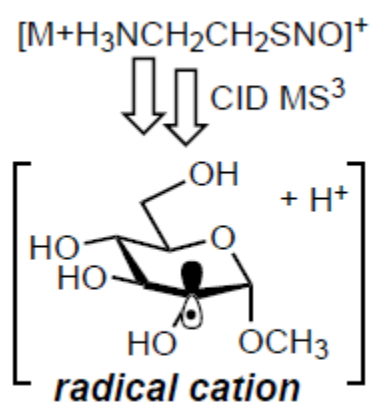
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