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Collision-induced dissociation mass spectra of glucosinolate anions

Jason B. Bialecki,[a] Josef Ruzicka,[a†] Carl S. Weisbecker,[a] Meena Haribal[b] and Athula B. Attygalle[a*]

Collision-induced dissociation (CID) mass spectra of differently substituted glucosinolates were investigated under negative-ion mode. Data obtained from several glucosinolates and their isotopologues (^{34}S and ^{2}H) revealed that many peaks observed are independent of the nature of the substituent group. For example, all investigated glucosinolate anions fragment to produce a product ion observed at *m/z* 195 for the thioglucose anion, which further dissociates via an ion/neutral complex to give two peaks at *m/z* 75 and 119. The other product ions observed at *m/z* 80, 96 and 97 are characteristic for the sulfate moiety. The peaks at *m/z* 259 and 275 have been attributed previously to glucose 1-sulfate anion and 1-thioglucose 2-sulfate anion, respectively. However, based on our tandem mass spectrometric experiments, we propose that the peak at *m/z* 275 represents the glucose 1-thiosulfate anion. In addition to the common peaks, the spectrum of phenyl glucosinolate (β-D-Glucopyranose, 1-thio-, 1-[*N*-(sulfooxy)benzenecarboximidate] shows a substituent-group-specific peak at *m/z* 152 for C_6H_5-C(=NOH)S^-, the CID spectrum of which was indistinguishable from that of the anion of synthetic benzothiohydroxamic acid. Similarly, the *m/z* 201 peak in the spectrum of phenyl glucosinolate was attributed to C_6H_5-C(=S)OSO_2^-. Copyright © 2009 John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article.

Keywords: glucosinolates; deuterium-labeled compounds; isotopologues; negative ions; even-electron ions; collision-induced dissociation; fragmentation

Introduction

Glucosinolates are an important group of natural products isolated predominately from plants of the Brassicaeae family. Enzymatic action on glucosinolates during food preparation, cooking, and chewing releases the characteristic flavors, or off-flavors, associated with many cruciferous vegetables such as cabbage, broccoli, and cauliflower. These off-flavors are attributed primarily to the formation of isothiocyanates, which have been demonstrated to have anti-cancer effects.[1,2] Glucosinolates also play an important role in the ecology of Brassicaceae vegetables because many pest insects take cues from these compounds to locate host plants.[3] Moreover, the biosynthetic pathways of glucosinolates are widely investigated to understand plant genetics and genomics.[4] Consequently, a significant research effort has been devoted to developing chromatographic and spectroscopic procedures for analyzing of glucosinolates found in biological and food samples often as complex mixtures.[5–8]

Chemically, glucosinolates are a group of substituted *cis*-hydroxyimine sulfate β-D-thioglucopyranosides (Fig. 1). Over 120 different glucosinolates have been identified from natural sources.

Several ionization methods have been utilized for generating gaseous ions from glucosinolates for mass spectrometry.[9–12] For earlier studies, fast-atom bombardment (FAB) ionization has been the most informative.[13] For more recent investigations negative-ion electrospray ionization (ESI) has been employed, however, direct ESI has been considered to generate very little diagnostically useful fragment ions because spectra are dominated by the glucosinolate anion derived from the glucosinolate salt.[14]

Several mass spectrometric techniques have been used for qualitative and quantitative determination of glucosinolates. Recently, it has been shown that glucosinolates upon negative-mode ESI produce abundant ions, which fragment under collision-induced dissociation (CID) conditions to form several product ions. One of the dominant fragment ions derived from glucosinolates is that observed at *m/z* 97 for the HSO_4^- anion.[11] Most quantitative determinations of glucosinolates have been performed by LC-MS multiple reaction monitoring (MRM) procedures using the parent glucosinolate anion,[10] and the product ion *m/z* 97,[11] as the key ions. Two recent papers have reported quantitative LC/MS–MS analysis of glucosinolates from plant extracts using this MRM technique.[15,16] The advantages of LC-MS/MS procedure is that no desulfation or derivatization of the glucosinolates are necessary. Although most MRM analyses have used the product ion at *m/z* 97, this ion is common for most organic sulfate compounds. Therefore, the use of more specific product ions would be more beneficial.[17]

* *Correspondence to: Athula B. Attygalle, Department of Chemistry, Chemical Biology and Biomedical Engineering, Center for Mass Spectrometry, Stevens Institute of Technology, Hoboken, NJ 07030, USA. E-mail: athula.attygalle@stevens.edu*

† *Current address: Josef Ruzicka, Scientific Instruments Division, Thermo Fisher Scientific, 265 Davidson Avenue, Suite 101, Somerset, NJ 08873, USA.*

a *Department of Chemistry, Chemical Biology and Biomedical Engineering, Center for Mass Spectrometry, Stevens Institute of Technology, Hoboken, NJ 07030, USA*

b *Boyce Thompson Institute, Tower Road, Ithaca, NY 14853, USA*

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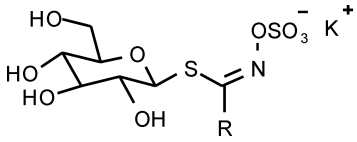


Figure 1. General structure of a glucosinolate.

In order to properly identify more selective product ion(s) suitable for MRM quantification of glucosinolates, a better understanding of their fragmentation pathways is necessary.

Glucosinolates can be categorized into several chemical classes based on the chemistry of the R-substituent.[18] In our quest for understanding intricate rearrangement mechanisms involved in the fragmentation processes of organic anions, we investigated spectra of glucosinolates bearing allylic, aromatic, and sulfur-containing R-substituent (Fig. 2), and we present here our results.

Experimental

Materials

All materials and reagents, including sinigrin (**1**) as the potassium salt, were obtained from Sigma-Aldrich Chemical Company (St Louis, MO) unless otherwise stated. Glucotropaeolin (**5**) and gluconasturtiin (**6**) were purchased as potassium salts from Carl Roth GmbH (Karlsruhe, Germany). Glucoerucin (**7**) and glucoiberin (**8**)were isolated and from Waltham-29 broccoli seeds, and progoitrin was separated from Red Russian kale seeds (Organica Seed, Wilbraham, MA). The synthesis of phenyl-d_5 desulfoglucosinolate (**11**) and benzothiohydroxamic acid (the acid of **21**) are reported below. The synthesis of phenyl glucosinolate (**3**), which is reported below, was based on a previously reported synthesis for phenyl-d_5 glucosinolate (**4**).[19] All synthesized products were detected as one spot when subjected to thin layer chromatographic analysis (precoated 0.25-mm silica plates from Fisher Scientific, Pittsburgh, PA).

NMR spectrometry

All nuclear magnetic resonance (NMR) spectra were recorded on a Varian INOVA 400 spectrometer as $CDCl_3$ or D_2O solutions. Chemical shifts for ^1H NMR are reported in ppm relative to the internal reference TMS or DMSO. All coupling constants (J values) are reported in Hertz (Hz). All ^{13}C NMR spectra are proton decoupled and all chemical shifts are reported relative to the central peak of $CDCl_3$ or DMSO.

Mass spectrometry

The CID mass spectra were recorded on a Micromass Quattro I tandem mass spectrometer equipped with an electrospray ion source. Samples were infused as acetonitrile–water–ammonia (9 : 1 : 0.00001, v/v/v) solutions at a flow rate of 6 µl min^{-1}. The source temperature was held at 80 °C. The capillary voltage was held at 4000 V. The argon gas pressure in the collision cell was adjusted to attenuate precursor transmission by 30–50%. Accurate mass measurements were performed at high resolution (R ≈ 15000) on a Waters-Micromass Q-ToF API-US spectrometer equipped with a nanoelectrospray ion source. Signals were acquired in the W-mode operation. Ion series generated by water clusters charged by the bicarbonate anion were used as reference mass peaks. The source temperature was held at 80 °C. The capillary voltage was held at 3000 V. Pressure of argon gas in the collision cell was held at 5.1 × 10^{-5} Pa.

For deuterium exchange experiments, samples were dissolved in 1 ml of 90 : 10 (v/v) acetonitrile: D_2O (99% isotopic purity, Sigma-Aldrich) and 0.05 ml of 1.0% (v/v) ND_4OD (99 atom % D; Cambridge Isotope Laboratories, Andover, MA) in D_2O.

Extraction

Glucosinolates were extracted from seeds based on a previously described procedure.[20] Seeds (0.1 g) of a specific variety were placed in a closed vial and submerged in boiling water for 15 min to deactivate the enzyme myrosinase. The seeds were then pulverized and extracted three times with boiling water–methanol (19 : 1). After each hot water–methanol extraction, samples were allowed to cool and were centrifuged at 11 000 rpm for 2–3 min. The supernatant layers were separated, combined and passed through a DSC-18 plug (Supelco). The eluent was concentrated under reduced pressure to yield a crude mixture of natural glucosinolates. This concentrated extract was taken up in 0.5 ml of water and centrifuged at 11 000 rpm for 2 min to give the final seed extract as the supernatant.

Chromatography

The preparative high performance liquid chromatography (HPLC) was conducted on an HP1090 (Hewlett–Packard) liquid chromatograph. The method used was based on a previously reported procedure.[21] The final seed extract was injected (50 µl) onto an Aqua C_{18} RP column (150 × 4.60-mm i.d., 3-µm particle size) from Phenomenex (Torrance, CA). The column was eluted at a flow rate of 1 ml min^{-1} with mobile phase A (water with 0.1% HCOOH) to which mobile phase B (acetonitrile with 0.1% HCOOH) was added by a linear gradient (initially 2% B for 5 min, and then increased to 40% at 20 min, to 100% at 25 min), and then kept isocratic for 5 min. The column was maintained at ambient temperature and the peaks were monitored by an ultraviolet (UV) detector set at 235 nm. Progoitrin (**2**) was collected (multiple times) at retention time 3–4 min from the final Red Russian kale seed extract. Glucoiberin (**8**) and glucoeurcin (**7**) were collected (multiple times) at retention times 3–4 min and 16–17 min, respectively, from the final Waltham-29 broccoli seed extract. Solvents were removed under reduced pressure to yield off-white solids, which appeared pure under negative-ion electrospray MS.

Synthetic reactions

*Phenyl-d_5 desulfoglucosinolate (**11**)*

S-2′,3′,4′,6′-Tetra-*O*-acetyl-β-D-glucopyranosyl [2,3,4,5,6-$^2H_5$] phenylthiohydroxamate (0.20 g, 0.36 mmol), which was previously prepared,[19] was dissolved in anhydrous ammonia in methanol (1.0 ml, 7 M, Aldrich) and allowed to stand for 48 h at −10 °C. The solvent was removed under reduced pressure to afford a white, solid residue as the final product (**11**, 0.10 g, 76%). δ_H (400 MHz; $CDCl_3$) δ 2.58 (1H, m), 3.12 (1H, t, J = 9.2 Hz), 3.31 (2H, m), 3.58 (1H, dd, J = 12.4 Hz, J = 5.2 Hz), 3.70 (1H, dd, J = 12.4 Hz, J = 2.4 Hz), 4.20 (1H, d, J = 10.0 Hz), 7.46 (3H, m), 7.59 (2H, t, J = 7.2 Hz); δ_C (400 MHz; $CDCl_3$) 62.4, 70.9, 74.1, 79.9, 82.2, 85.3, 129.8, 131.1, 135.3, 155.2; m/z (negative-ion electrospray) [M − H]$^-$ required for $C_{21}H_{19}D_5NO_9$ 319.1018, found 319.1031.

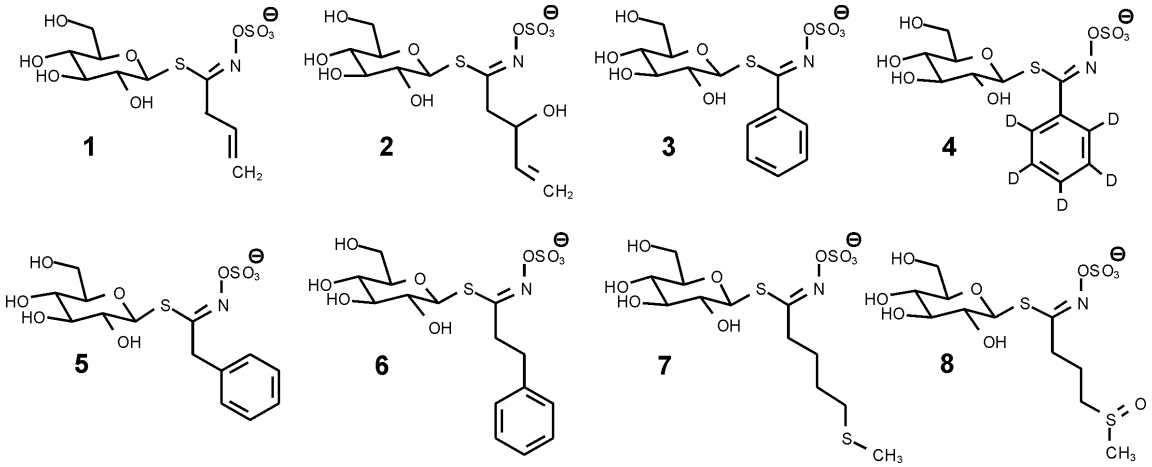


Figure 2. Structures of the anions of the investigated glucosinolate salts. Allyl class: sinigrin (**1**), progoitrin (**2**). Aromatic class: phenyl glucosinolate (**3**), phenyl-d_5 glucosinolate (**4**), glucotropaeolin (**5**), gluconasturtiin (**6**). Sulfur class: glucoerucin (**7**), glucoiberin (**8**).

Benzothiohydroxamic acid (N-hydroxybenzenecarbothioamide, the free acid of anion **21***)*

Magnesium turnings (0.32 g, 13.3 mmol) and a crystal of iodine were heated until purple vapors formed, and a solution of bromobenzene (2.0 g, 12.7 mmol) in Et_2O (8 ml) was added.[22] The mixture was refluxed for 30 min, cooled in an ice bath to 0 °C, and carbon disulfide (1.1 ml, 18.4 mmol) was added. After refluxing for 1 h, solvents were removed, and the dark residue was taken up in KOH (6 ml, 2 M) and filtered over Celite® to give a clear, reddish solution. A solution of hydroxylamine hydrochloride (0.9 g, 13.0 mmol) in water (4 ml) was added to the reddish solution and the mixture was stirred overnight. The aqueous mixture was extracted with Et_2O (3 × 5 ml); the Et_2O layer was separated, dried over $MgSO_4$ and evaporated to yield the crude product which was dissolved in MeOH, filtered hot over charcoal and evaporated to give the final product as a yellowish solid (the acid of anion **21**, 0.20 g, 66%). δ_H (400 MHz; $CDCl_3$) δ 7.40 (2H, t, J = 7.4 Hz), 7.46 (1H, t, J = 7.6 Hz), 7.65 (2H, d, J = 7.2 Hz); δ_C (400 MHz; $CDCl_3$) 126.7, 128.8, 131.2, 135.1; *m/z* (negative-ion electrospray) $[M - H]^-$ required for C_7H_6NSO 152.0176, found 152.0181.

Benzaldehyde oxime

A solution of sodium carbonate (0.28 g, 2.7 mmol in 5 ml of H_2O) was slowly added to a solution of hydroxylamine hydrochloride (0.35 g, 4.9 mmol in 5 ml of H_2O) and benzaldehyde (0.52 g, 4.8 mmol), and the mixture was stirred at RT for 2 h. The reaction mixture was extracted with Et_2O (15 ml); the Et_2O layer was separated, dried over Na_2SO_4 and evaporated to give the product, a colorless oil, as mixture of *E* and *Z* isomers. (0.57 g, 94%). δ_H (400 MHz; $CDCl_3$) 7.36 (3H, m), 7.56 (2H, d, J = 7.4), 8.18 (1H, s), 10.20 (1H, s); δ_C (400 MHz; $CDCl_3$) 127.0, 128.7, 130.1, 131.97, 150.4; *m/z* (EI) 121 (M^+, 100%), 120 (20), 103 (15), 94 (40), 78 (80), 51 (70), 39 (20).

Phenylhydroxamic chloride (N-hydroxybenzenecarboximidoyl chloride)

To a solution of benzaldehyde oximes from the previous step (0.45 g, 3.7 mmol in 5 ml of DCM) and pyridine (0.1 ml) cooled to 0 °C, *N*-chlorosuccinimide (0.49 g, 3.7 mmol) was slowly added.[23] Upon completion, the ice bath was removed and the reaction mixture was stirred for 2 h at RT. The organic layer was separated and washed with H_2O (10 ml), dried over Na_2SO_4 and evaporated to give the product (PhC(Cl)=NOH, 0.53 g, 89%), which was used in the next step without further purification. δ_H (400 MHz; $CDCl_3$) 7.52 (5H, m), 10.5 (1H, s); δ_C (400 MHz; $CDCl_3$) 126.8, 127.6, 130.3, 137.03, 144.2; *m/z* (positive-ion electrospray) 137 ($[M - HCl + NH_4]^+$, 100%), 120 (20, $[M - HCl + H]^+$), 104 (30), 77 (25).

S-2′,3′,4′,6′-Tetra-O-acetyl-β-D-glucopyranosyl phenylthiohydroxamate

A solution of Et_3N (0.2 ml) and Et_2O (1.0 ml) was added to a stirred mixture of 1-thio-β-D-glucose tetraacetate (0.4 g, 1.1 mmol) and phenylhydroxamic chloride (0.21 g, 1.4 mmol) in dry Et_2O (4.0 ml) and DCM (2.0 ml). A precipitate of triethylamine hydrochloride was formed immediately. The reaction mixture was stirred for 2 h at RT, and washed with aq. HCl (10 ml, 1 M) and H_2O (10 ml). The organic layer was separated, dried over Na_2SO_4, and evaporated. The crude product was recrystallized from ethanol to afford the pure white *cis* product (0.34 g, 62%). δ_H (400 MHz; $CDCl_3$) 2.03 (3H, s), 2.04 (3H, s), 2.09 (3H, s), 2.12 (3H, s), 3.04 (1H, s), 3.97 (1H, d, J = 12.4), 4.09 (1H, dd, J = 12.4 Hz, J = 4.8 Hz), 4.45 (1H, d, J = 9.6 Hz), 5.01 (2H, d, J = 8.4 Hz), 5.08 (1H, td, J = 7.2 Hz, J = 2.8 Hz), 7.46 (3H, m), 7.52, (2H, d, J = 7.4 Hz), 8.94 (1H, s); δ_C (400 MHz; $CDCl_3$) 20. 4, 20.5, 20.6, 20.7, 61.7, 67.8, 69.8, 73.71, 75.7, 81.3, 128.5, 129.0, 130.1, 132.3, 169.2, 170.2 and 170.6; *m/z* (positive-ion electrospray) $[M - H]^-$ required for $C_{21}H_{24}NO_{10}S$ 482.1126, found 482.1134.

Pyridinium S-2′,3′,4′,6′-tetra-O-acetyl-β-D-glucopyranosyl phenylthiohydroxamic N-sulfate

A solution of S-2′,3′,4′,6′-tetra-*O*-acetyl-β-D-glucopyranosyl phenylthiohydroxamate (0.60 g, 1.26 mmol in 4.0 ml of DCM) and dry pyridine (5 ml) was cooled to 0 °C and a cold solution of chlorosulfonic acid (1.6 ml, 24.2 mmol in 4.0 ml of dry Et_2O) was slowly added. After the addition, the ice bath was removed and the mixture was stirred overnight at RT. All solvents were removed under reduced pressure at 60 °C. The residue was dissolved in DCM (10 ml) and washed with H_2SO_4 (20 ml, 1 M) and H_2O (10 ml). The organic layer was separated and dried over $MgSO_4$ and evaporated to afford the white product (0.36 g, 44%). δ_H (400 MHz; $CDCl_3$) 1.96, 1.97, 2.06 and 2.10, 3.09 (1H, s), 4.01 (1H, d, J = 11.6 Hz), 4.09 (1H, dd, J = 11.6 Hz, J = 4.8 Hz), 4.58 (1H, d, J = 7.6 Hz), 5.03 (3H,

m), 7.38 (2H, t, $J = 7.2$ Hz), 7.46 (1H, t, $J = 7.2$ Hz), 7.50 (2H, d, $J = 7.6$ Hz), 7.87 (2H, t, $J = 6.4$ Hz), 8.39 (1H, t, $J = 6.4$ Hz), 8.86 (2H, d, $J = 5.2$ Hz); δ_C (400 MHz; $CDCl_3$) 20.4, 20.6, 20.7, 61.5, 67.6, 69.4, 73.6, 75.7, 81.5, 126.9, 128.4, 129.2, 131.2, 142.5, 145.5, 157.0, 169.2, 169.3, 170.0 and 170.6; *m/z* (negative-ion electrospray) $[M - C_6H_6N]^-$ required for $C_{21}H_{24}NO_{13}S_2$ 562.0695, found 562.0704.

Ammonium β-D-glucopyranosyl phenylthiohydroxamic N-sulfate (ammonium salt of phenyl glucosinolate, **3***) (β-D-Glucopyranose, 1-thio-, 1-[N-(sulfooxy)benzenecarboximidate ammonium salt)*

Pyridinium S–2′,3′,4′,6′-tetra-*O*-acetyl-β-D-glucopyranosyl phenylthiohydroxamic *N*-sulfate (0.35 g, 0.55 mmol) was dissolved in anhydrous ammonia in methanol (1.5 ml, 7 M, Aldrich) and allowed to stand for 48 h at −10 °C. The solvent was removed under reduced pressure to afford a crude product. The crude product was dissolved in water (2 ml), mixed with activated charcoal, heated and filtered to give a colorless solution. The solution was evaporated, and the residue was subjected to repeated dissolution in methanol and evaporation. The final residue was dissolved in a minimal amount of methanol (0.1 ml), and triturated with THF (0.5 ml) followed by Et_2O (1.5 ml) to form a white precipitate. The solvent was decanted and the white solid was washed with Et_2O and dried to afford the final product as a slightly off-white amorphous solid (**3**, 0.17 g, 65%). Reverse phase-HPLC with UV detection at 235 nm showed that the purity of the product was >99%. δ_H (400 MHz; D_2O) 2.47 (1H, m), 2.89 (1H, t, $J = 8.6$ Hz), 3.06 (2H, td, $J = 8.4$ Hz, $J = 4.2$ Hz), 3.35 (1H, dd, $J = 12.2$ Hz, $J = 5.6$ Hz), 3.51 (1H, d, $J = 9.6$ Hz), 3.95 (1H, d, $J = 7.6$ Hz), 7.46 (3H, m), 7.53 (2H, d, $J = 7.4$ Hz); δ_C (400 MHz; D_2O) 54.9, 60.4, 69.2, 72.3, 78.2, 81.1, 128.1, 129.0, 129.5, 132.4, 154.9; *m/z* positive-ion electrospray) $[M - NH_4]^-$ required for $C_{13}H_{16}NO_9S_2$ 394.0272, found 394.0275.

Computational methods

All QM calculations were performed using the Gaussian 03 W program package. Computations were performed using the restricted Hartree–Fock method as described in the review article by Mercero *et al.*,[24] using the 6-31G(d) basis set.[25] Structures depicted in the results section were energy minimized to obtain a stationary state. Calculated relative energies include a correction for the zero-point energy of each structure (scaled by a factor of 0.9135).[26]

In addition, structures were also energy minimized using the B3LYP exchange-correlation functional and the 6-311++G(d,p) basis set. Calculated relative energies include a correction for the zero-point energy scaled by a factor of 0.9877.[27] Energies of optimized structures were also calculated using the MP2 method with the 6-311++G(d,p) basis set.

Results and Discussion

Glucosinolates are ideal candidates for negative-ion electrospray ionization mass spectrometry because of the sulfate moiety in their molecular structure. Thus, the anions derived from glucosinolates have been widely used for their qualitative and quantitative determinations. The product ion spectra of all glucosinolate anions[16] are known to show an intense signal at *m/z* 97, representing a bisulfate anion (HSO_4^-). For example, the low-energy CID spectrum of *m/z* 394 of phenyl glucosinolate (**3**), which

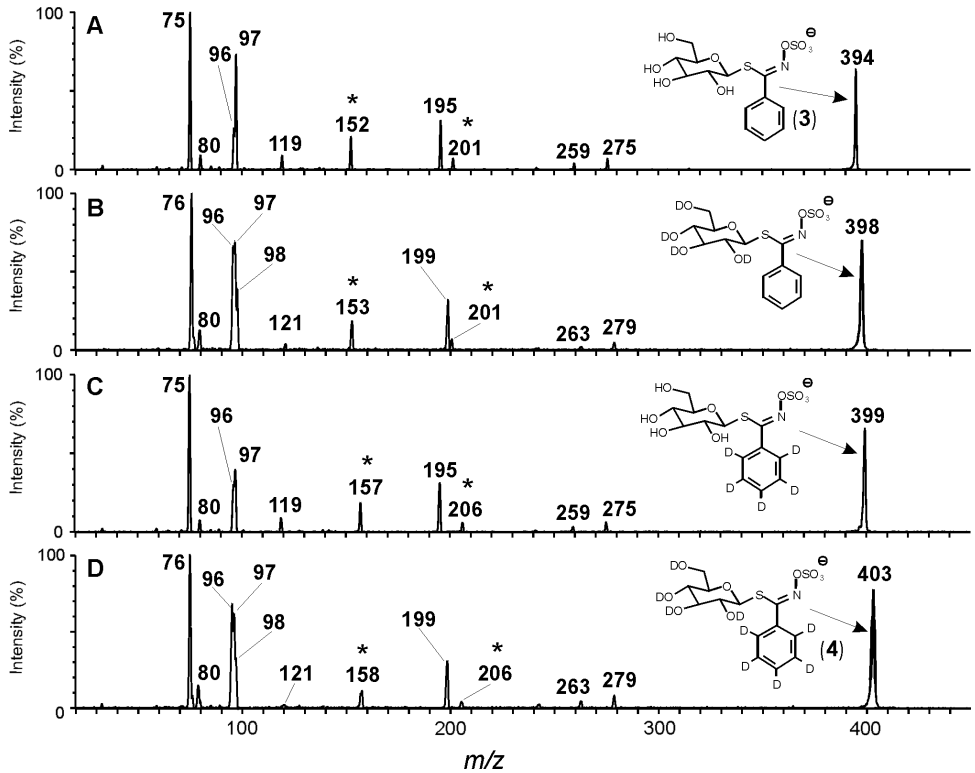


Figure 3. Product ion spectra of ions derived from phenyl glucosinolate (**3**, *m/z* 394) (A), phenyl glucosinolate (**3**) in D_2O (*m/z* 398) (B), phenyl-d_5 glucosinolate (**4**, *m/z* 399) (C) and phenyl-d_5 glucosinolate (**4**) in D_2O (*m/z* 403) (D). Peaks for ions containing the R-substituent are denoted with an '*'. Laboratory-frame collision energy was set at 25 eV.

was synthesized in our laboratory, showed a significant peak at *m/z* 97 (Fig. 3A). Similar results were obtained from several other glucosinolate anions (Fig. 4). However, the *m/z* 97 product ion is not specific for glucosinolates because most organic sulfate anions form this anion via a six-membered, cyclic *syn*-elimination mechanism as depicted in Scheme 1.[28]

However, a six-membered transition state for a similar hydrogen transfer (from R substituent) cannot be attained from glucosinolates because the *N*-sulfated thiohydroximate moiety of glucosinolates bears a (*Z*)-configuration.[29] For some molecules, particularly for those without a hydrogen atom at the C-2 position, an alternative mechanism in which two neutral molecules are eliminated via an eight-membered transition state has been proposed.[28] In fact, this mechanism entails properties of heterolytic fragmentations described by Grob and Schiess.[30] H/D exchange studies carried out in solution with phenyl glucosino-

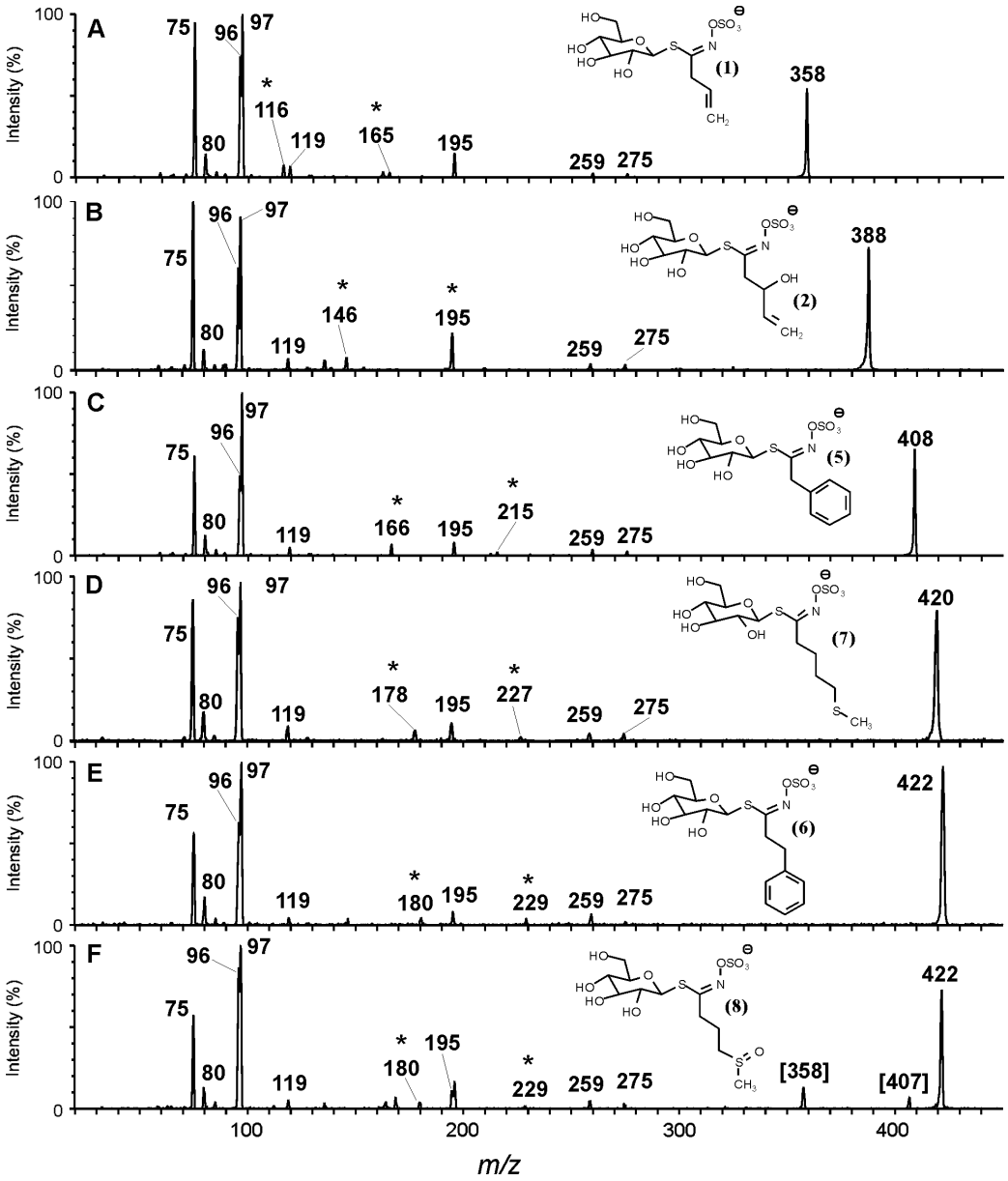
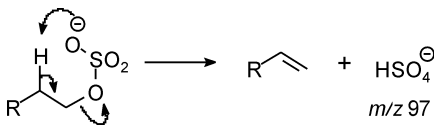


Figure 4. Product ion spectra of ions derived from sinigrin (**1**, *m/z* 358) (A), progoitrin (**2**, *m/z* 388) (B), glucotropaeolin (**5**, *m/z* 408) (C), glucoerucin (**7**, *m/z* 420) (D), gluconasturtiin (**6**, *m/z* 422) (E) and glucoiberin (**8**, *m/z* 422) (F). Note: the *m/z* 195 in spectrum (B) represents two different ions. Peaks for ions containing the R-substituent are denoted with an '*', and the *m/z* values of the ions originating from a dissociation within the substituent are given in brackets. Laboratory-frame collision energy was set at 25 eV.



Scheme 1. Production *m/z* 97 ion from most organic sulfates.[25].

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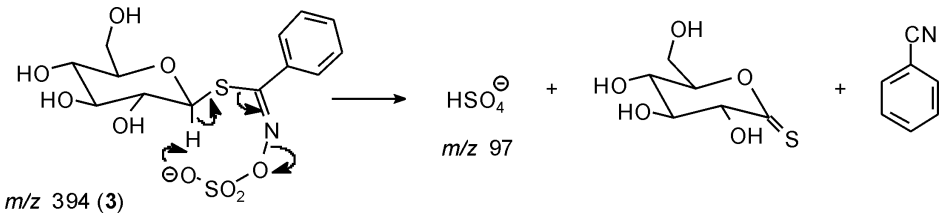


Scheme 2. Formation of *m/z* 97 ion from glucosinolate anions.

Scheme 3. Fragmentation of phenyl glucosinolate anion (**3**, *m/z* 394).

late (**3**) and its deuteriated analog **4** (Fig. 3B, D) confirmed that the major source of the hydrogen atom in the HSO_4^- is a non-exchangeable hydrogen atom from the sugar moiety. The most plausible atom for this transfer is the hydrogen atom attached to the C-1 carbon. Thus, we propose a mechanism similar to the reported eight-member transition mechanism,[28] to rationalize the formation of *m/z* 97 (HSO_4^-) from glucosinolate ions (Scheme 2).

The product ion spectra of all investigated glucosinolates (Figs 3 and 4) also show peaks for two odd-electron fragment ions at *m/z* 80 and *m/z* 96. The formation of an *m/z* 80 ($SO_3^{-\bullet}$) product ion from both *m/z* 97 (HSO_4^-) and 98 (DSO_4^-) ions, by loss of a hydroxyl or deuteroxyl radical, respectively (Fig. 3A, B), confirmed that the bisulfate anion fragments by a homolytic fission of the S–OH(D) bond.[28] Based on the reported fragmentation of organic *N*-sulfate anions,[31] the facile formation of *m/z* 96 ($SO_4^{-\bullet}$) from glucosinolate anions occur through a direct homolytic cleavage of the N–O bond of the *N*-sulfate function (Scheme 3).

The characteristic fragment peak at *m/z* 195, observed in all recorded glucosinolate spectra (Figs 3A, C, 4 and 5), is analogous to that observed under negative-ion FAB conditions for the 1-thiohexose anion (**9**).[5,29] The origin of the *m/z* 195 fragment ion (**9**) from the 1-thiohexose moiety of glucosinolates can be attributed to a charge-mediated multiple bond cleavage mechanism similar to that proposed for phenyl glucosinolate in Scheme 4.

Although a mechanism of eliminating SO_3 and phenylnitrile oxide (**10**) by a stepwise pathway can be considered, a peak for a SO_3 loss was not observed in the spectrum of phenyl-d_5 glucosinolate (Fig. 5A), or any other glucosinolate. On the other hand, the anion of synthetic phenyl-d_5 desulfoglucosinolate, *m/z* 319 (**11**), which was synthesized in our laboratory, dissociated under very low collision energy to produce an intense signal at *m/z* 195 (Fig. 5B). It appears that without the sulfate group to stabilize the negative charge, the desulfo species of *m/z* 319, readily transfers the charge from the oxime oxygen to the thio function and eliminates phenyl-d_5-nitrile oxide (**12**) (Scheme 5).

Moreover, a comparison of the product ion spectrum of *m/z* 399 ion from phenyl-d_5 glucosinolate (**4**) with that from the *m/z* 319 ion from phenyl-d_5 desulfoglucosinolate (**11**) (Fig. 5), established the identity of product ions that still contain the sulfur atom from the sulfate moiety. The product ions observed at 75, 119, 157 and 195 are common to both spectra (Fig. 5). Because phenyl-d_5

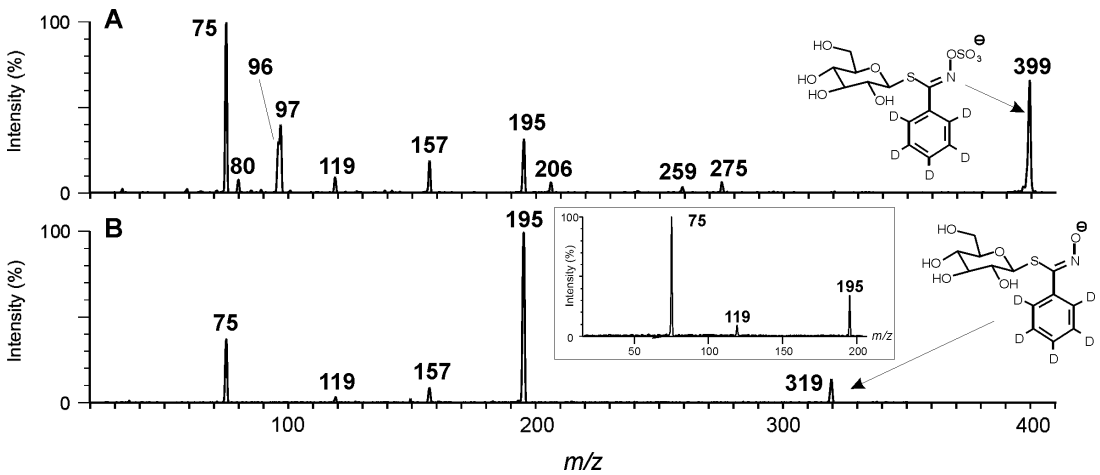
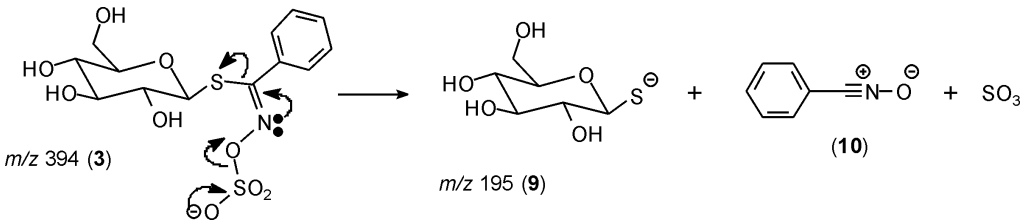
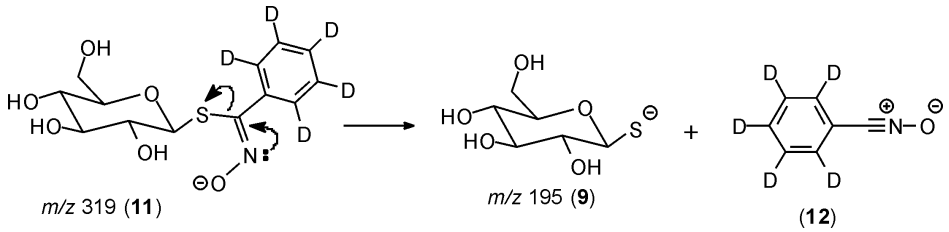


Figure 5. Product ion spectra of *m/z* 399 ion derived from phenyl-d_5 glucosinolate at collision energy setting of 30 eV (A), and the *m/z* 319 ion derived from phenyl-d_5 desulfoglucosinolate at collision energy setting of 15 eV (B). The inset in Figure B shows the product ion spectrum of in-source generated *m/z* 195 ion (cone voltage 25 V). Laboratory-frame collision energy was set at 25 eV.



Scheme 4. Formation of *m/z* 195 ion from phenyl glucosinolate anion.



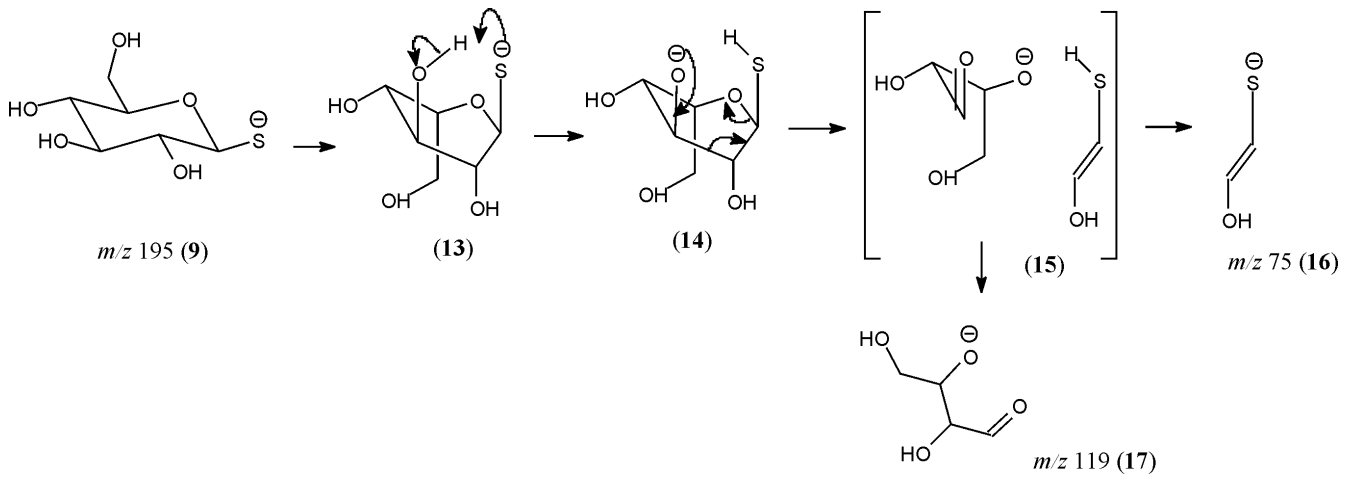
Scheme 5. Fragmentation of *m/z* 319 anion of phenyl-d_5 desulfoglucosinolate (**11**).

desulfoglucosinolate (**11**) contains no sulfate residue, it was clear that these ions do not contain the sulfur atom from the sulfate moiety. In contrast, the product ions observed at *m/z* 80, 96, 97, 206, 259, and 275 all contain the sulfur atom from the sulfate moiety (Fig. 5A).

A tandem mass spectrometric experiment conducted with the thioglucose anion of *m/z* 195 (**9**) indicated its fragmentation to two product ions observed at *m/z* 75 and 119 (see inset in Fig. 5B). For the formation of these two ions, the negative charge on the sulfur atom of thioglucose anion should be transferred to the hydroxy group on position 3 by a proton transfer via a six-membered transition state (Scheme 6). Computations carried out at Hartree–Fock level with a 6-31G(d) basis set, and B3LYP and MP3 levels with a 6-311++G(d,p) set showed not only that the transition state energy barrier for proposed flip from a chair conformation (**9**) is relatively small (about 6 kcal/mol) but also the boat conformation **13** (about −2 kcal/mol) that is formed is relatively more stable than the chair form **9** (0.00 kcal/mol) (Supplementary Fig. S1). Moreover, when the stationary states were verified by a frequency analysis, it confirmed that only the chair and boat conformations bear real vibrational modes, as opposed to the transition state between them which contained one imaginary vibrational mode. Presumably, after the initial transfer of the acidic proton, the carbon–carbon bond between C-2 and C-3 positions elongates to form a transition state which eventually breaks down to form an ion/neutral complex (**15**) between thioglycal and tetrose anion both of which remain closely associated as depicted in Scheme 6.

The formation of such ion/neutral complexes have been noted in several other anion fragmentation mechanisms.[32,33] A simple dissociation of the complex leads to the ion observed at *m/z* 119 (**17**) (Scheme 6). Alternatively the complex can break via a proton transfer[34] to give a peak *m/z* 75 (**16**). This fragmentation pathway is considered to be the more favorable pathway in the product ion spectra of glucosinolates (Figs 3 and 4). The structural identity of *m/z* 119 was established to be a deprotonated tetrose by a comparison of its product ion spectrum with that derived from deprotonated D-(−)-erythrose (*m/z* 119). Both spectra were nearly identical (Fig. S2). Evidently, the formation *m/z* 75 and *m/z* 119 ions appear to be characteristic for all glucosinolate anions.

Alternatively, a ring-opening mechanism similar to that proposed for the fragmentation of deprotonated glucose can be considered.[35] Although calculations at the HF/6-31G(d) level for



Scheme 6. Proposed Fragmentation pathway for thioglucose anion (*m/z* 195).

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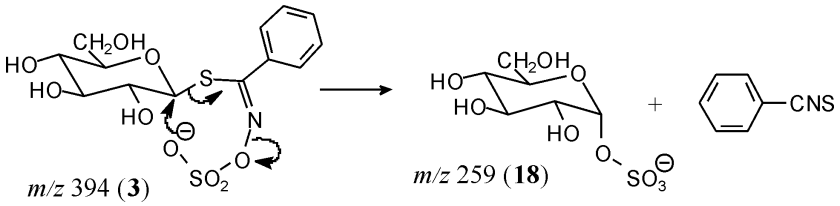
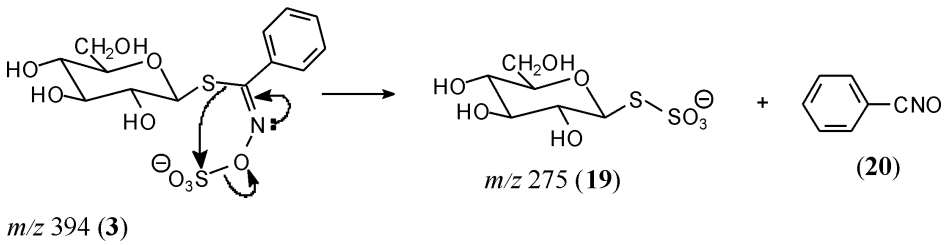


Scheme 7. Formation of *m/z* 259 ion from glucosinolates.



Scheme 8. Proposed rearrangement to form glucose 1-thiosulfate anion (**19**).

the glucose anion identified a ring-opened intermediate structure with a terminal aldehyde group, when the aldehyde oxygen of this intermediate was replaced with a sulfur atom and the structure was reoptimized, the structure failed to remain open anymore. Instead, the thioglucose anion from a ring-opened starting point optimized to a ring-closed ground state. This computational result suggested that ring opening of thioglucose anion is energetically less favorable than the ring opening of glucose anion, and does not lead to an open intermediate structure. Furthermore, the general appearance of the CID spectrum of glucose anion is very different to that obtained from the thioglucose anion (Fig. S3).

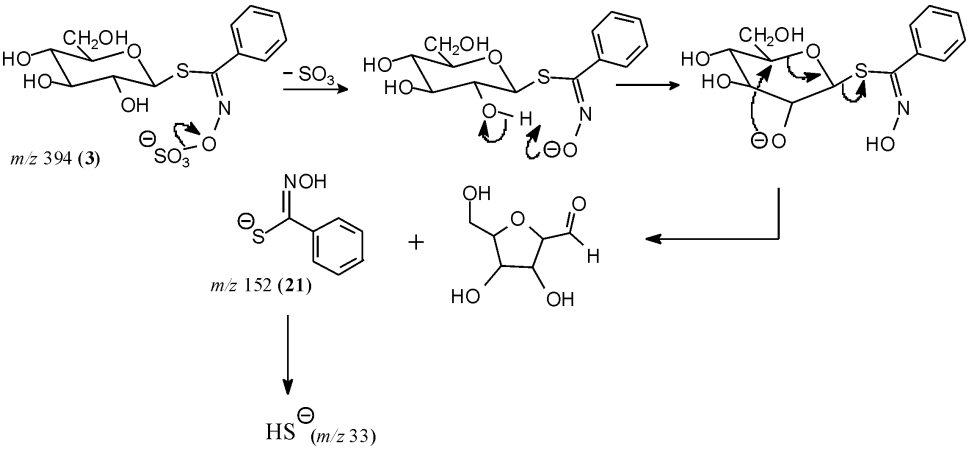
Further support to the proposed mechanism in Scheme 6 was obtained by H/D exchange experiments conducted in solution. When glucosinolates without any exchangeable hydrogens in the R group were dissolved in D_2O and spectra were recorded, the peak for the glucosinolate anion underwent a shift of 4 *m/z* units indicating the exchange of the four hydroxyl hydrogen atoms in the glucose ring with deuterium (Fig. 3A, B). Under these conditions, the peak for the 1-thiohexose product ion, (*m/z* 195), underwent a shift to *m/z* 199. This result confirmed that the four exchangeable hydroxyl hydrogen atoms were still intact in the glucose ring after the fragmentation. Upon fragmentation of the *m/z* 199 ion, the *m/z* 75 peak shifted to *m/z* 76 indicating the presence of one exchangeable hydrogen atom in the thioglycal anion. Similarly, the peak at *m/z* 119 was shifted to *m/z* 121 indicating the presence of two exchangeable hydrogen atoms in the tetrose anion (Fig. 3A, B).

The CID spectra of glucosinolates (Figs 3A, C and 4) also show two minor peaks at *m/z* 259 and 275. Both these ions have been previously reported from negative-ion FAB spectra of glucosinolates.[5,36] Thus, we inferred that the *m/z* 259 fragment ion (Fig. 3) represents the glucose 1-sulfate anion (**18**) which presumably originates by an elimination of phenyl isothiocyanate from the precursor anion (Scheme 7). An open-ring structure has been previously proposed for the *m/z* 259 ion by Rochfort *et al.*[37]

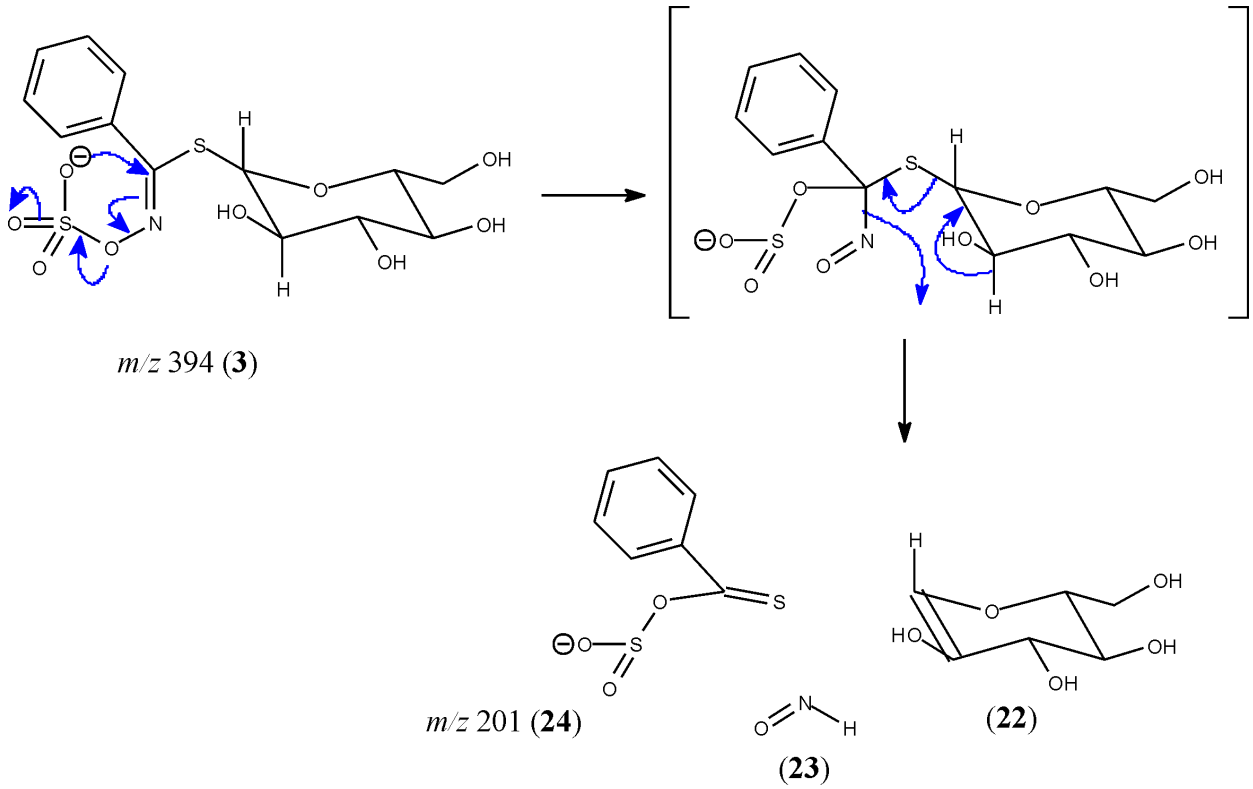
A product ion experiment conducted with the *m/z* 259 ion, revealed that it does not undergo a direct loss of SO_3[31,37] In contrast, the *m/z* 275 ion underwent a direct loss of SO_3, upon CID. Because glucose sulfates are not known to eliminate SO_3, we propose that the *m/z* 394 anion undergoes a fragmentation to eliminate phenyl cyanate (**20**) and produce a glucose 1-thiosulfate anion (**19**). Because the S–S bond in *m/z* 275 would cleave more easily than the S–O bond in *m/z* 259 (**18**), an SO_3 loss can be expected from a thiosulfate but not from a sulfate (a sulfate moiety loses SO_3 only when it is attached directly to a phenyl ring or a double bond,[28,31] or when another acidic group is present in the molecule which can accommodate the negative charge[38]). Thus, the thioglucose 2-sulfate structure, in which the sulfate group is linked directly to the C-2 position on the sugar ring, suggested by Kokkonen *et al.*,[5] Bojen and Larsen,[36] and Fabre *et al.*,[39] for the *m/z* 275 ion appears to be erroneous.

Product ion experiments conducted with samples under H/D exchange conditions (Fig. 2B, D) revealed a shift of *m/z* 259 and 275 peaks to *m/z* 263 and *m/z* 279, respectively. This result indicated that each ion bears four exchangeable hydrogen atoms, and the glucose ring remains intact during the rearrangement as illustrated in Scheme 8.

Two other significant fragment ion peaks observed in glucosinolate product ion spectra are denoted by asterisks in Figs 3 and 4. Unlike the product ions discussed thus far, these ions contain the R-substituent. Even though the *m/z* values of these two fragment ions vary according to the mass of the R-substituent of the glucosinolate, the mechanisms for their formation appear to follow a generalized pathway. For example, the CID spectrum of phenyl glucosinolate (**3**) show two peaks at *m/z* 152 and 201, and that from the phenyl-d_5 glucosinolate (**4**) show the corresponding peaks at *m/z* 157 and 206 (Fig. 3A, C). This observation confirmed that these peaks are attributable to ions bearing the phenyl substituent. Deuterium/hydrogen exchange experiments (Fig. 3B, D) showed that fragment ions *m/z* 152 and 157 bear only one exchangeable hydrogen. An accurate mass determination experiment established the molecular composition of the *m/z* 152 ion to be $C_7H_6NSO^-$ (Supplementary Table S1). Moreover, the product ion spectrum of *m/z* 152 was indistinguishable from that obtained under identical conditions from the anion of benzothiohydroxamic acid (**21**), which was synthesized in our laboratory. Both *m/z* 152 and 157 ions fragmented to give a single intense product ion peak at *m/z* 33 (HS^-), which becomes *m/z* 34 (DS^-) under H/D exchange conditions. The fragmentation occurs by a loss of a phenylnitrile oxide molecule from the *m/z* 152 ion (**21**). Although the structure of the *m/z* 152 product ion (**21**) formed from phenyl glucosinolate (**3**) is confirmed, the exact pathway of its formation is less certain.



Scheme 9. Proposed pathway for the formation of *m/z* 152 ion from phenyl glucosinolate anion.



Scheme 10. Formation of *m/z* 201 ion (**24**) from phenyl glucosinolate anion.

A proposed pathway based on available experimental details is illustrated in Scheme 9.

Another intriguing peak in the spectrum of phenyl glucosinolate (**3**) is that observed at *m/z* 201. Unlike the ions discussed above, H/D exchange experiments revealed that the *m/z* 201 ion (**24**) contains no exchangeable hydrogen atoms (Fig. 3). An accurate mass determination experiment of the *m/z* 201 ion confirmed its molecular formula to be $C_7H_5O_3S_2$ (Table S1). In other words, the precursor *m/z* 394 ion should undergo an unprecedented skeletal rearrangement and then fragment to eliminate the nitrogen atom while retaining both sulfur atoms. To rationalize the observations, we propose that upon collisional activation the *m/z* 394 ion rearranges to a nitroso derivative, which undergoes a charge remote fragmentation via six-membered system to eliminate an anhydroglucose molecule (**22**) and a nitrosyl hydride molecule (**23**) (Scheme 10).

The *m/z* 201 ion (**24**) represents the deprotonated anhydride of thiobenzoic acid and sulfurous acid (Fig. 6). The proposed structure is supported by the results obtained with phenyl-d_5 glucosinolate (**4**), which showed a peak at *m/z* 206 in its product ion spectrum (Fig. 3C). Upon CID, both *m/z* 201 and 206 ions underwent a facile loss of SO_2 confirming the proposed sulfite anhydride structure (Fig. 6). The thiobenzoate anion (*m/z* 137 or 142) formed in this way, loses COS to form the phenyl anion (*m/z* 77 or 82), or loses CO by a rearrangement to give the thiophenoxide anion (*m/z* 109 or 114).

To obtain further support for the proposed structures of the fragment ions of glucosinolates, we recorded and compared

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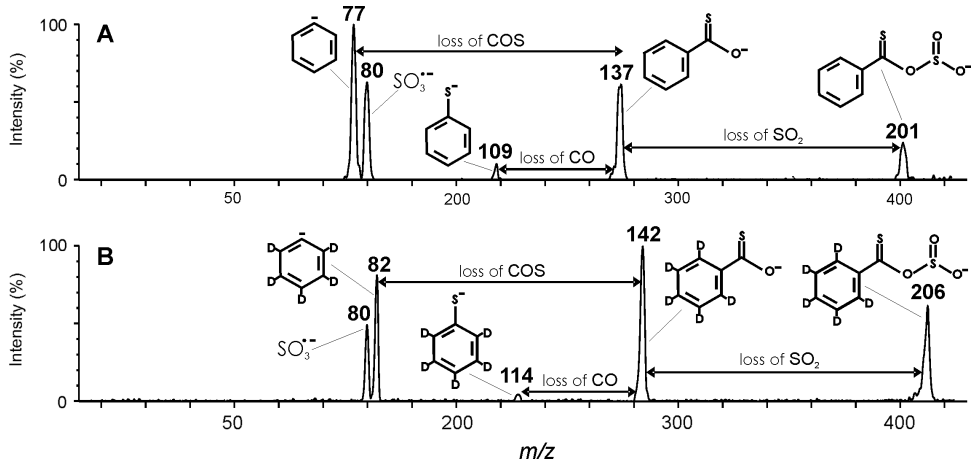

Figure 6. Product ion spectra of in-source generated fragment ions of *m/z* 201 from phenyl glucosinolate (A), and that of *m/z* 206 from phenyl-d_5 glucosinolate (B). (Laboratory-frame collision energy was set at 25 eV, and cone voltage 30 V).

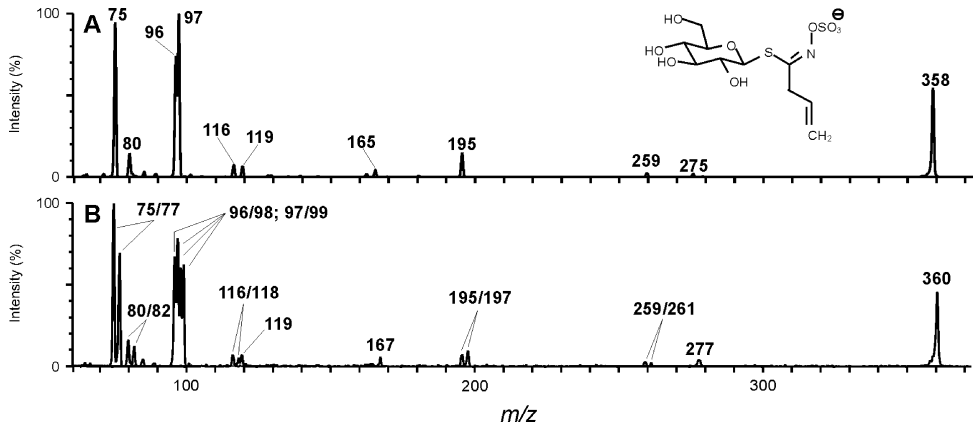

Figure 7. Product ion spectra derived from *m/z* 358 of [$^{32}S_2$]sinigrin (A), and *m/z* 360 of [$^{34}S_1{}^{32}S_1$]sinigrin (B). Laboratory-frame collision energy was set at 25 eV.

the product ion spectra of $^{32}S_2$ and $^{32}S_1{}^{34}S_1$ isotopologues of sinigrin (Fig. 7). In the spectrum recorded from the *m/z* 360 ion of [$^{34}S_1{}^{32}S_1$]sinigrin, peaks for all product ions bearing one sulfur atom appeared as double peaks, whereas those bearing two sulfur atoms showed only one peak at a value two *m/z* units higher than that observed from the $^{32}S_2$ isotopologue. For example, the peak at *m/z* 275 for the 1-phenyl[$^{32}S_2$]thiosulfate anion, increased to *m/z* 277 for the 1-phenyl[$^{32}S_1{}^{34}S_1$]thiosulfate anion indicating the presence of two sulfur atoms. The product ion at *m/z* 259 on the other hand, changed to a double peak at *m/z* 259 and 261 indicating the presence of one sulfur atom. Under this experiment, product ions *m/z* 75, 80, 96, 97 and 195 all behaved in the same fashion as that observed from the *m/z* 259 ions confirming that each of them contain a single sulfur atom. The product ion at *m/z* 119 for the tetrose (**17**) on the other hand remained unchanged in this experiment because it is not a sulfur-containing ion. The product ion $CH_2{=}CH\text{-}CH_2\text{-}C(=NOH)S^-$ ion (*m/z* 116) from sinigrin (**1**), which corresponds to the product ion at *m/z* 152 from phenyl glucosinolate (as shown in Table 1) changed to a double peak at *m/z* 116 and 118 indicating the presence of one sulfur atom. Finally, product ion $CH_2{=}CH\text{-}CH_2\text{-}C(=S)OSO_2^-$ (*m/z* 165) from sinigrin, which corresponds to the product ion *m/z* 201 from phenyl glucosinolate (as shown in Table 1) changed to a single peak at *m/z* 167 indicating the presence of two sulfur atoms.

Gluconasturtiin (**6**) and glucoiberin (**8**), two common glucosinolates in Brassica seeds, are considered isobaric compounds because they have the same nominal mass. A comparison of their product ion spectra revealed that they can be differentiated directly by MS/MS. In addition to the usual fragment ions, the CID spectrum of glucoiberin displays two other significant peaks because of the distinctive chemistry of its R-substituent (peaks denoted by brackets in Fig. 4). Both gluconasturtiin (Fig. 4E) and glucoiberin (Fig. 4F) show essentially the same peaks, with the notable exception of *m/z* 358 and 407 peaks in the spectrum of glucoiberin, which is a unique three-sulfur-atom glucosinolate with a sulfoxide moiety. The two peaks at *m/z* 358 and 407 represent neutral losses of methyl sulfoxide (64 Da) and methyl radical (15 Da), from the *m/z* 422 ion of glucoiberin, respectively.

Conclusions

The fragmentation pathways of glucosinolate anions under CID conditions are dictated by the chemistry of the sulfate, thiohydroxamate, and sugar moieties. Consequently, all glucosinolates generate common ions that are diagnostically useful for the general group. In the low-mass region, besides the peak at *m/z* 97, intense peaks observed at *m/z* 75 and *m/z* 96 are also useful to identify glucosinolates in a complex mixture. Although the *m/z* 97

Table 1. A list of peaks observed in negative-mode CID fragmentation of $[M - X]^-$ ions of some glucosinolates[a]

Glucosinolate	$[M - X]^-$ (*m/z*)	R-substituent and its nominal mass (Da)	Fragment ions common to all glucosinolates (*m/z*)	Fragment ions specific to each R-substituent (*m/z*)	
				R-C(=NOH)S$^-$	R-C(*S*)OSO$_2^-$
Sinigrin (**1**)	358	-$CH_2CH{=}CH_2$ (41)	75, 80, 96, 97, 119, 195, 259, 275	116	165
Progoitrin (**2**)	388	-$CH_2CH(OH)CH{=}CH_2$ (71)	75, 80, 96, 97, 119, 195, 259, 275	146	195
Phenyl glucosinolate (**3**)	394	-C_6H_5 (77)	75, 80, 96, 97, 119, 195, 259, 275	152	201
Phenyl-d_5 glucosinolate (**4**)	399	-C_6D_5 (82)	75, 80, 96, 97, 119, 195, 259, 275	157	206
Glucotropaeolin (**5**)	408	-$CH_2C_6H_5$ (91)	75, 80, 96, 97, 119, 195, 259, 275	166	215
Gluconasturtiin (**6**)	422	-CH_2-CH_2-C_6H_5 (105)	75, 80, 96, 97, 119, 195, 259, 275	180	229
Glucoerucin (**7**)	420	-$(CH_2)_4SCH_3$ (103)	75, 80, 96, 97, 119, 195, 259, 275	178	227
Glucoiberin (**8**)	422	-$(CH_2)_3SOCH_3$ (105)	75, 80, 96, 97, 119, 195, 259, 275	180	229

[a] X = K or NH_4.

ion is widely used for qualitative and quantitative investigations of glucosinolates, the thioglycosyl ion at *m/z* 75, which is specific for thioglucosyl moiety, appears to be a better and more specific choice for MRM determinations.

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Supporting information

Supporting information may be found in the online version of this article.

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