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The synthesis and enzymic hydrolysis of (*E*)-2-[2,3-$^2H_2$]propenyl glucosinolate: Confirmation of the rearrangement of the thiohydroximate moiety

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Abstract

(*E*)-2-[2,3-$^2H_2$]propenyl glucosinolate was synthesised starting from (*E*)-[3,4-$^2H_2$]but-3-en-1-ol, which was produced by reduction of but-3-yn-1-ol with deuterium gas in the presence of Lindlar's catalyst. The synthesis of (*E*)-2-[2,3-$^2H_2$]propenyl glucosinolate was completed *via* the nitro intermediate to form the basic desulphoglucosinolate skeleton. The (*E*)-2-[2,3-$^2H_2$]propenyl glucosinolate was fully characterised and deuterium NMR spectroscopy used to examine the rearrangement of the thiohydroximate to the isothiocyanate and thiocyanate.

Keywords: Glucosinolate; Sinigrin; Thiocyanate; Isothiocyanate; Synthesis; Isotope; Mechanism; Deuterium NMR spectroscopy

1. Introduction

Glucosinolates are a class of sulfur-containing secondary metabolites present in sixteen families of dicotyledonous angiosperms which includes the important edible cruciferous plants (Brassicaceae) (Fahey et al., 2002). Glucosinolates have now been recognised as important dietary constituents of the *Brassica* vegetables due to their anticancer properties (Holst and Williamson, 2004) as well as providing an important constitutive defence mechanism towards pests and diseases in *Brassica* crops (Kliebenstein et al., 2005). The biosynthesis of glucosinolates has been under intense study in recent years with most of the genes functionally characterised using the model plant *Arabidopsis thaliana* (Grubb and Abel, 2006; Halkier and Gershenzon, 2006). Myrosinase (E.C. 3.2.1.147) catalyses the hydrolysis of glucosinolates to give rise to an unstable thiohydroximate-*O*-sulfate (Fig. 1), which can further rearrange to an isothiocyanate (Bones and Rossiter, 1996, 2006).

Alternatively, in the presence of epithiospecifier protein (ESP) and ferrous ions, epithionitriles or nitriles are produced depending on the functionality of the glucosinolate side chain (Bones and Rossiter, 1996, 2006; Foo, 1998; Foo et al., 2000; Lambrix et al., 2001). ESP is unique in as much as it has no activity towards the initial substrate, but only with the unstable thiohydroximate-*O*-sulfate intermediate which is captured by an assumed iron-ESP complex to give a nitrile or epithionitrile. Interestingly, another protein, the epithiospecifier modifier protein, appears to modulate the activity of ESP towards certain classes of glucosinolate (Zhang et al., 2006). Most recently, the thiocyanate forming factor has been successfully cloned from *Lepidium sativum* and intriguingly has in addition ESP-like activity (Burow et al., 2007) and is iron dependent.

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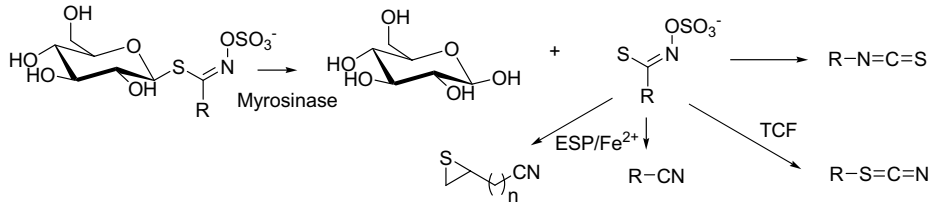


Fig. 1. Generalised enzymic hydrolysis of glucosinolates (TCF = thiocyanate forming factor, ESP = epithiospecifier protein).

As a consequence of recent advances in glucosinolate biology (Halkier and Gershenzon, 2006) there is a need to investigate glucosinolate metabolism to understand the various metabolic pathways in animals and plants. Thus there is a requirement for both radioactive and stable isotope labelled glucosinolates for metabolism studies and as standards for qualitative analysis, for example by mass spectroscopy.

The chemical synthesis of glucosinolates and their analogues (Bourderioux et al., 2005) has received a great deal of attention, while there have been fewer synthetic routes to labelled glucosinolates described. These labelled glucosinolates have included ^{14}C-phenethyl glucosinolate (gluconasturtiin) (Svanem et al., 1997), ^{3}H-butenyl glucosinolate (gluconapin) (Rossiter and James, 1990), ^{3}H-indolyl glucosinolate (glucobrassicin) (Chevolleau et al., 1993) and ^{2}H-phenethyl glucosinolate (Morrison and Botting, 2005). The synthesis of labelled glucosinolates can give high specific activities as compared to feeding plants with radiolabelled amino acids, which can require extensive purification steps (Chen and Halkier, 2000). Glucosinolates are usually synthesised *via* a chloro-oxime which can be generated from nitro groups or oximes and subsequent reaction with *O*-acetylated thioglucose in the presence of a suitable base to give the basic glucosinolate skeleton.

2-Propenyl glucosinolate (sinigrin) has previously been synthesised by Benn and Ettlinger using the nitro route to the chloro-oxime (Benn and Ettlinger, 1965). This synthesis was improved to produce large amounts of 2-propenyl glucosinolate (Abramski and Chmielewski, 1996) using essentially the same route as Benn and Ettlinger (1965). The formation of thiocyanates has been studied in some detail by Luthy and Benn (1977), who used a *Thlaspi arvense* seed flour extract to show that 2-[1-^{14}C]propenyl glucosinolate is converted to the 3-thiocyanato[3-^{14}C] prop-1-ene. Here the aglycone undergoes an putative S_N2' rearrangement to give the thiocyanate (Fig. 2).

We have used the same methodology as Abramski and Chmielewski (1996) to synthesise (*E*)-2-[2,3-$^{2}H_2$]propenyl glucosinolate and show that it is structurally similar to the purified natural compound. By using deuterium

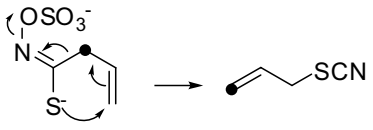


Fig. 2. The S_N2' rearrangement of (NO-sulfate)-but-3-enethiohydroximate to 3-thiocyanatoprop-1-ene.

NMR spectroscopy we were able to show that there was no rearrangement of the isothiocyanate while the thiocyanate underwent the expected rearrangement.

2. Results and discussion

By using the synthetic method described by Abramski and Chmielewski (1996) it was possible to synthesise (*E*)-2-[2,3-$^{2}H_2$]propenyl glucosinolate. A previous route (Rossiter and James, 1990) to tritiated 3-butenyl glucosinolate has been described where pent-4-ynaldoxime was reduced to the tritiated alkene using tritium in the presence of Lindlar's catalyst. However, all attempts to prepare the corresponding but-3-ynaldoxime were unsuccessful and this route could not be used for the preparation of 2-propenyl glucosinolate. The reduction of but-3-yn-1-ol with deuterium gas in the presence of Lindlar's catalyst gave the deuterated alkene in good yield. Further conversion to (*E*)-4-bromo[1,2-$^{2}H_2$]but-1-ene and generation of sodium salt of the (*E*)-4-nitro[1,2-$^{2}H_2$]but-1-ene enabled the construction of a thiohydroximate which was followed by sulfation and deacetylation (Fig. 3). The synthesis of (*E*)-4-nitro[1,2-$^{2}H_2$]but-1-ene was carried out using silver nitrite in water (Ballini et al., 2004) which is reported to give high yields of aliphatic nitro compounds. However, we only obtained a yield of 29% after purification and this method (Ballini et al., 2004) did not seem to offer any advantages in terms of yield over sodium nitrite in dimethyl formamide (Kornblum et al., 1956), at least for this particular compound. The proton and deuterium NMR spectra of (*E*)-2-[2,3-$^{2}H_2$]propenyl glucosinolate show that the regioselectivity of the reduction was not entirely 100%. However, by examining the NMR integration pattern, it was estimated that the proportion of deuterium was 81% based on the deuterium NMR spectroscopy and 85% for the proton NMR spectroscopy. Thus it would appear that some formation and subsequent addition of H–D occurs across the alkyne. This was confirmed by negative ion EMS where a ion of mass of 359.1 corresponded to the monodeuterated 2-propenyl glucosinolate with an intensity of 16.5%. The negative ion EMS of the selected ion at 360.1 corresponding to (*E*)-2-[2,3-$^{2}H_2$]propenyl glucosinolate is shown in Fig. 4. The small amount of hydrogen present at C2 is seen throughout all of the synthetic intermediates and final product in the same proportion. An enzyme assay with partially purified myrosinase gave the expected isothiocyanate with the corresponding abundant ion (EI) of 101 (Fig. 5).

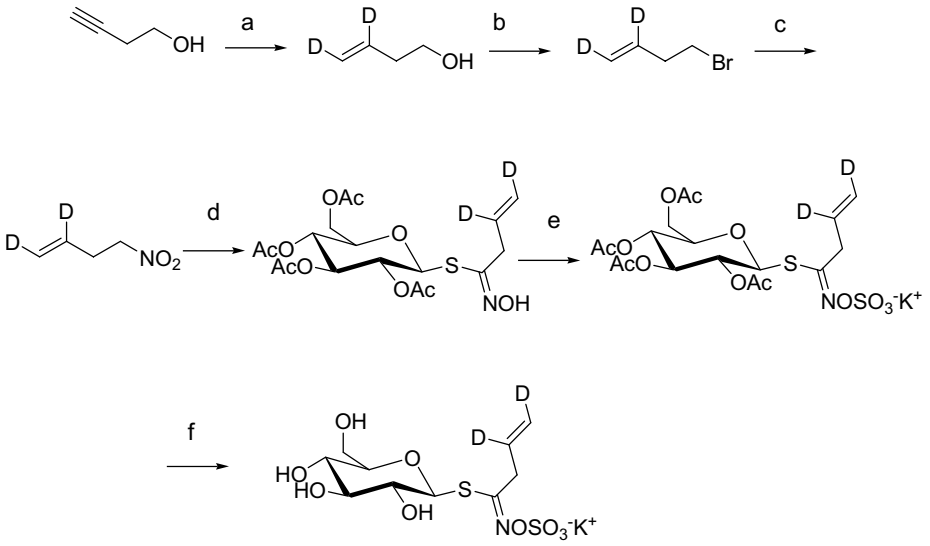


Fig. 3. The synthesis of (*E*)-2-[2,3-$^2H_2$]propenyl glucosinolate; a = D_2/Lindlar's catalyst/quinoline/ethanol; b = PBr_3; c = $NaNO_2$/water; d = sodium methoxide/methanol, HCl/triethylamine/diethyl ether/2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranose; e = Pyr.SO_3/pyridine; f = NH_3/methanol.

Thiocyanates are thought to arise from glucosinolates that can give rise to a stable carbocation (Luthy and Benn, 1977) such as benzyl-, 2-propenyl- and 4-methylthiobutyl-glucosinolates. With specifically deuterium labelled 2-propenyl glucosinolate we were able to re-explore the rearrangement of the thiohydroximate intermediate to the isothiocyanate and thiocyanate. Previous work by Luthy and Benn (Luthy and Benn, 1977) using 2-[1-^{14}C]propenyl glucosinolate had shown (Fig. 2) a change in the labelling pattern indicating that a rearrangement had taken place. This was achieved by synthesising the allyl benzyl sulfone from the enzymatically produced allyl thiocyanate followed by degradation to formaldehyde (isolated as the dimedone derivative) and the benzyl methyl sulfone and subsequent crystallisation and ^{14}C radioactive counting to locate the labelled carbon. However, having available deuterated sinigrin we were able to rapidly confirm Luthy and Benn's (1977) mechanism by carrying out deuterium NMR spectroscopy of the hydrolysis products with defatted and glucosinolate-free *T. arvense* and partially purified *S. alba* myrosinase. The quantitative formation (>90% of total products) of the thiocyanate was confirmed by GC using

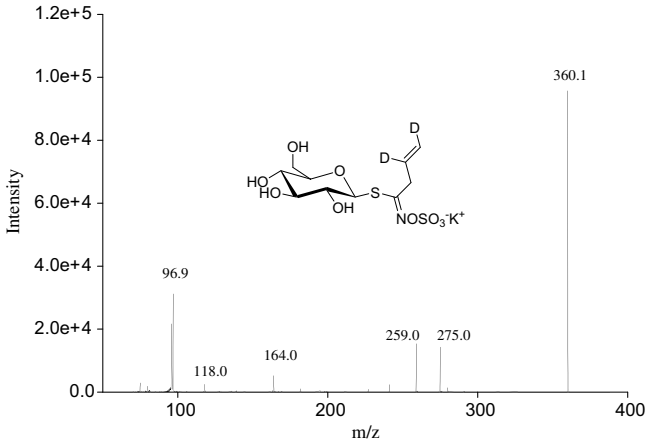


Fig. 4. Negative EPI spectrum of (*E*)-2-[2,3-$^2H_2$]propenyl glucosinolate.

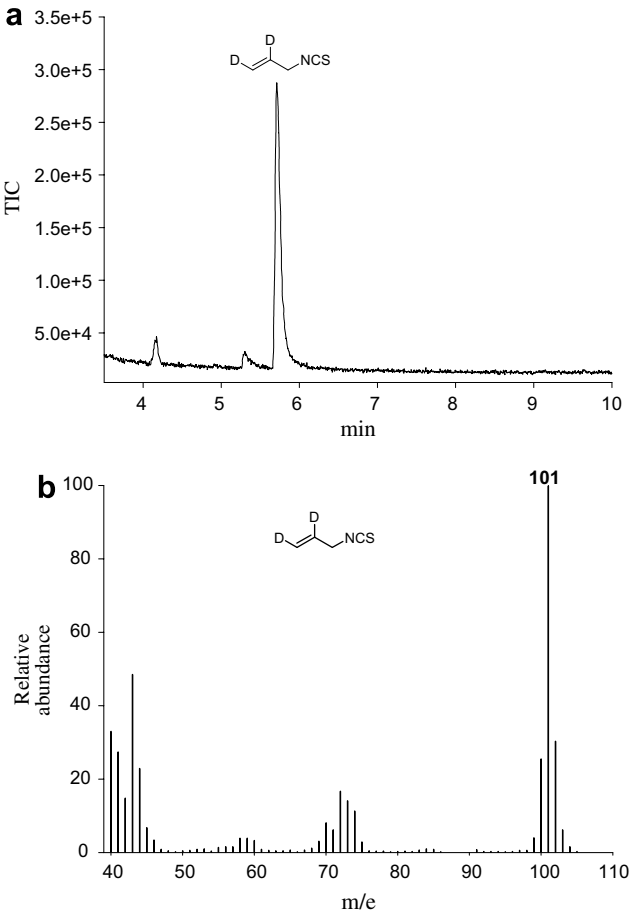
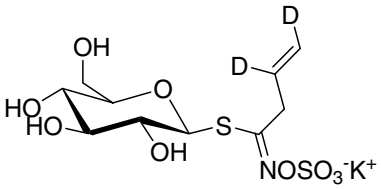
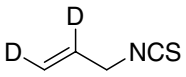
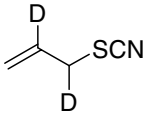


Fig. 5. GC–MS profile of the hydrolysis of (*E*)-2-[2,3-$^2H_2$]propenyl glucosinolate by partially purified *S. alba* myrosinase. A = GC trace, B = mass spectrum (EI) of deuterated 3-isothiocyanatoprop-1-ene.

the extracted seed meal of *T. arvense*. Deuterium NMR spectroscopy was carried out with the deuterated sinigrin (Fig. 6a) and *S. alba* myrosinase. After extraction of the products with dichloromethane it was found that the chemical shifts of the deuterons (Table 1, Fig. 6b) correlated with those of the protons at the C3 and C2 positions of allyl isothiocyanate, showing that no rearrangement had occurred. However, when the experiment was repeated with *T. arvense* seed meal, the pattern of the chemical shifts for the deuterons corresponded to those of the protons at the C1 and C2 positions of allyl thiocyanate, showing that a rearrangement had occurred (Fig. 6c, Table 1). The deuterium NMR spectroscopy experiment with *T. arvense* shows the presence of comparatively low levels of other deuterons which don't correspond to either the thiocyanate or isothiocyanate. It is possible that these represent minor non-enzymic degradation compounds of the thiocyanate. It is also evident that over the time scale of the experiment the [3,3]-sigmatropic rearrangement of the thiocyanate to the isothiocyanate did not occur.

Table 1

Structure	Chemical shift of D	Ratio of deuterium C1:C2:C3
	C3-D, 5.05 C2-D, 5.75	0:1.02:1.0
	C3-D, 5.37 C2-D, 5.86	0:1.0:1.0
	C2-D, 5.93 C1-D, 3.52	1.0:1.21:0

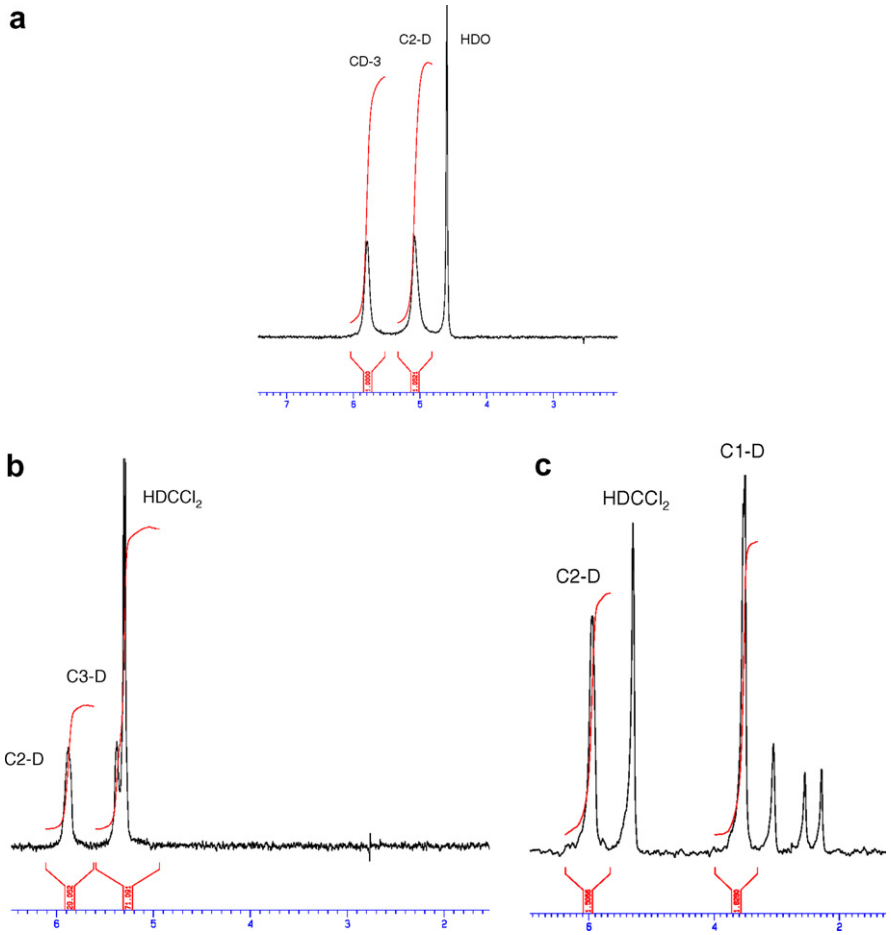


Fig. 6. (a) Deuterium NMR spectrum of (*E*)-2-[2,3-$^2H_2$]propenyl glucosinolate; (b) deuterium NMR spectrum of dichloromethane extract of (*E*)-2-[2,3-$^2H_2$]propenyl glucosinolate incubated with a myrosinase preparation from *S. alba*; (c) deuterium NMR spectrum of dichloromethane extract of (*E*)-2-[2,3-$^2H_2$]propenyl glucosinolate incubated with defatted and deglucosinolated *T. arvense* seed meal.

3. Conclusion

Deuterium NMR spectroscopy proved to be a powerful tool to examine the mechanism of product formation in the degradation of 2-propenyl glucosinolate, and the synthesis of (*E*)-2-[2,3-$^2H_2$]propenyl glucosinolate provides material that can be used for tracer experiments in plant metabolism and as a standard for mass spectroscopy.

4. Methods

4.1. Spectroscopy

Solid samples were run on a JEOL AX505W instrument (a double focussing sector machine) at 700 resolution. Accelerating voltage was 3 kV, beam current 1 μA, scan range 35–800 Da, scan speed 3 s/decade and detector voltage 1.2 kV. The intact glucosinolate was also run on an Applied Biosystems QTrap. The mass spectrum of volatile compounds was obtained on a Hewlett Packard 6890 GC linked to a 5973 MSD mass spectrometer. NMR spectra were recorded either on a Bruker 400 or 360 MHz machine. Chemicals were purchased from Sigma-Aldrich while allyl thiocyanate was synthesised.

4.2. (*E*)-[3,4-$^2H_2$]but-3-en-1-ol

But-3-yn-1-ol (10 g, 0.142 mol) was dissolved in ethanol (50 ml) containing Lindlar's catalyst (650 mg) and quinoline (100 mg). The mixture was then deuterated with deuterium gas (99.8 at.%) until the reaction was complete (monitored by gas chromatography). The mixture was filtered, poured onto water (100 ml) and extracted with diethyl ether (100 ml, ×5). The combined extracts were washed with HCl_{aq} (50 ml, 1 M) and finally with water (50 ml). The solvents were carefully distilled using a 30 cm Vigreux column. The residue was distilled (8 g, 80% yield; b.p. 112–114 °C) to give the (*E*)-[3,4-^{2}H]but-3-en-1-ol. ^{1}H NMR (360 MHz): δ 2.25 (*t*, 2H, DHC=CD CH_2—CH_2, *J* 6.0 Hz), 3.60 (*q*, 2H, DHC=CDCH_2—CH_2, *J* 6.0 Hz), 5.05–5.06 (*m*, 1H, D*H*CH=CD—CH_2CH_2—); *m*/*z* 74.1 (M^+, 8.2%), 58.1 (7.6), 57.1 (3.2), 45.1 (39.2), 44.1 (100), 43.1 (56.8), 31.1 (62.5), 29.1 (16).

4.3. (*E*)-4-Bromo [1,2-$^2H_2$]but-1-ene

(*E*)-[3,4-$^2H_2$]but-3-en-1-ol (9 g, 122 mmol) was added to dry pyridine (2.85 g, 36 mmol) and phosphorous tribromide (13.3 g, 49 mmol) carefully added dropwise to the cooled solution at 0 °C. The mixture was allowed to reach room temperature and carefully distilled to give the product. The distillate was washed with 5% sodium bicarbonate and extracted into diethyl ether (50 ml). The organic layer was separated and the aqueous layer re-extracted with diethyl ether (50 ml). The extracts were combined and carefully distilled to give the product (7.22 g, 44% yield; b.p. 98 °C).

^{1}H NMR (360 MHz): δ 2.55 (*t*, 2H, DHC=CDCH_2—CH_2, *J* 7.0 Hz), 3.35 (*t*, 2H, DHC= $CDCH_2$—CH_2, *J* 7.0 Hz), 5.06–5.04 (*m*, 1H, D*H*CH=CD— CH_2CH_2—); *m*/*z* 138 (M^+, 6.0%), 136 (6.0), 107 (1.3), 105 (0.5), 95 (2.2), 93 (2.7), 79 (2.4), 81 (2.4), 58.1 (67), 57.1 (100), 40.1 (15.5), 28.1 (14).

4.4. (*E*)-4-Nitro [1,2-$^2H_2$]but-1-ene

(*E*)-4-Bromo [1,2-$^2H_2$]but-1-ene (7 g, 51.1 mmol) was added to water containing silver nitrite (31.5 g, 205 mmol) and allowed to react for 2.5 h at 60 °C. The aqueous mixture was extracted with diethyl ether (50 ml, ×2), dried with anhydrous magnesium sulfate, filtered and distilled to remove the ether. The residue was then distilled under vacuum to give the product (1.5 g, 29% yield; b.p. 144 °C, 680 mmHg). ^{1}H NMR (360 MHz): δ 2.55 (*t*, 2H, DHC=CDCH_2—CH_2, *J* 7.0 Hz), 3.35 (*t*, 2H, DHC= $CDCH_2$—CH_2, *J* 7.0 Hz), 5.06–5.04 (*m*, 1H, D*H*CH=CD —CH_2CH_2—); *m*/*z* 103 (M^+, 0.014 %), 57.1 (86.5), 56.1 (100), 46.1 (7.5), 40 (36.9), 30 (39.2).

4.5. 2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranosyl-(*E*)-[3′,4′-$^2H_2$]but-3′-enethiohydroximate

(*E*)-4-Nitro [1,2-$^2H_2$]but-1-ene (1.1 g, 10.7 mmol) was added to anhydrous methanol (3.7 ml), sodium methoxide (21.4 ml, 0.5 M) was added and the mixture left for 10 min. Anhydrous ether (37 ml) was added and the precipitate filtered and washed with anhydrous ether and the salt dried over P_2O_5. The salt (0.42 g, 3.3 mmol) was added to diethyl ether (11 ml) and cooled to −70 °C. $HCl_{(g)}$ in diethyl ether (2 M HCl, 14 ml, 28 mmol) was slowly added over a period of 1 h and left for 30 min. Triethylamine (anhydrous) was added (6.3 ml) in ether (13 ml) not exceeding −40 °C. 2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranose (1.3 g, 35.7 mmol) was added to the mixture and the temperature allowed to reach 15 °C. The mixture was stirred for 30 min and poured onto ice cold sulfuric acid (6 ml sulfuric acid on 77 ml ice water mix), extracted with ethyl acetate (40 ml, ×2) and the combined extracts washed with water (50 ml, ×3). The ethyl acetate extract was dried over anhydrous magnesium sulfate, filtered and the solvent evaporated under reduced pressure. The product was purified by flash silica chromatography by elution with hexane:ethylacetate (60:40) and then hexane:ethylacetate (50:50) to give a white amorphous solid (800 mg, 54% yield; m.p. 165–166 °C [lit 165–166°C (Abramski and Chmielewski, 1996), 164–165 °C (Benn and Ettlinger, 1965) for non-deuterated material]). Elemental analysis (Found: C, 48.29; H, 5.94; N, 3.09). Calculated for $C_{18}H_{23}D_2NO_{10}S$: C, 48.09; H, 6.01; N, 3.11%. ^{1}H NMR (360 MHz): δ 1.94, 1.07, 1.98, 2.02 (4*s*, 12H, 4 × Ac), 3.23–3.34 (*q*, 2H, DHC=CDCH_2—, J_{gem} 16.7, *J* 7.8 Hz), 3.70–3.65 (*ddd*, 1H, *H*-5, *J* 3.3, 5.3 and 10.0 Hz), 4.09–4.11 (*m*, 2H, H-6, H-6′), 4.96–5.06 (*m*, 3H,

H-1,2,4), 5.15–5.21 (*m*, 2H, D*H*C=CDCH$_2$—, *H*-3), 8.10 (*s*, 1H, —NO*H*); *m*/*z* 449 (M^+, 0.82%), 331 (82.30), 271 (21.20), 229 (12.82), 169 (100), 127 (61.48), 109 (89.73), 43 (97.12).

4.6. S-(2,3,4,6-Tetra-O-acetyl-1-thio-β-D-glucopyranosyl)-O-(potassium sulfonato)-(E)-[3′,4′-$^2H_2$]but-3′-enethiohydroximate

2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranosyl-(*E*)-[3′, 4′-$^2H_2$]but-3′-enethiohydroximate (300 mg, 0.67 mmol) was added to freshly prepared pyridine sulfur-trioxide complex (334 mg, 2.1 mmol) in anhydrous pyridine (3.1 ml) and left at room temperature for 48 h. The pyridine solution was poured onto a solution of potassium bicarbonate (0.423 g in 2.5 ml water) and stirred for 20 min. Diethyl ether (18 ml) was added to the pyridine mixture and shaken. The excess of diethyl ether was carefully removed and the precipitated salts were filtered. Water was added to rinse the remaining salts from the flask and used to wash the main precipitate. The precipitate was washed with saturated potassium sulfate (370 μl, ×2) and then ethanol (600 μl). The precipitate was dissolved in boiling ethanol (85%, 4 ml) and immediately filtered to give fine crystals which were dried under vacuum over phosphorous pentaoxide (150 mg, 40% yield; m.p. 188–192 °C [lit 193–194 °C (Abramski and Chmielewski, 1996) for non-deuterated material]). Elemental analysis (Found: C, 38.31; H, 4.50; N, 2.52. Calculated for $C_{18}H_{22}D_2KNO_{13}S_2$: C, 38.09; H, 4.62; N, 2.47%). ^{1}H NMR (360 MHz, $CDCl_3$): *δ* 1.76, 1.80, 1.85 (3*s*, 12H, 4 × Ac), 3.17–3.26 (*m*, 2H, DHC=CDCH_2-), 3.70–3.65 (*ddd*, 1H, *H*-5, *J*2.2, 4.8 and 10.0 Hz), 3.93–3.95 (*m*, 2H, *H*-6, *H*-6′), 4.73–4.85 (*m*, 2H, *H*-2,4), 4.99–5.04 (*m*, 3H, D*H*C=CDCH$_2$—, *H*-1, 3); *m*/*z* 331 (13.20), 169 (49.91), 109 (28.46), 43(100).

4.7. (E)-2-[2,3-$^2H_2$]propenyl glucosinolate

S-(2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranosyl)-*O*-(potassium sulfonato)-(*E*)-[3′,4′-$^2H_2$]but-3′-enethiohydroximate (90 mg, 0.16 mmol) was added to anhydrous methanol (3.6 ml). Ammonia in methanol (2 M, 100 μl) was then added and the reaction mixture was stirred overnight. The reaction mixture was then centrifuged to remove a slight white precipitate and activated charcoal was added (15 mg). After mixing for 10 min, the methanolic solution was centrifuged to remove the charcoal and the solution was carefully decanted. The methanol was then removed under a stream of nitrogen and water was added (1 ml) and then freeze dried. The freeze dried material was then dissolved in water (15 μl) and ethanol (96 %, 1.12 ml) was slowly added and the solution left overnight at 4 °C. The crystalline (*E*)-2-[2,3-$^2H_2$]propenyl glucosinolate was centrifuged, the excess ethanol was removed and the crystals were dried overnight under vacuum over phosphorous pentaoxide to give 55 mg of product (86% yield; m.p. 127.5–129 °C [lit 128–129 °C (Abramski and Chmielewski, 1996) for 2-propenyl glucosinolate]). Elemental analysis (Found: C, 29.81; H, 4.34; N, 3.51. Calculated for $C_{10}H_{14}D_2KNO_9S_2$: C, 30.07; H, 4.54; N, 3.51%). ^{1}H NMR (400 MHz, D_2O): *δ* 3.42–3.55 (*m*, 6H, *H*-2, 3, 4, 5, DHC=CDCH_2—), 3.69 (*dd*, 1H, *H*-6, *J* 5.84 and 12.6 Hz), 3.89 (dd, 1H, *H*-6′, *J* 2.0 and 12.6 Hz), 5.04 (d, 1H, *H*-1, *J* 9.81 Hz), 5.26 (*m*, 1H, D*H*C=CDCH$_2$—); *m*/*z* 147 (7.71), 119 (18.34), 73 (46.46), 42 (100).

A solution of (*E*)-[2,3-$^2H_2$]propenyl glucosinolate (2 μg/ml of water) was infused into an Applied Biosystems QTrap at a rate of 10 μl/min. The sample was analysed in both positive and negative Enhanced Mass Spectrum (EMS) modes. The prominent peak at 360 *m*/*z*, observed in negative EMS, was further analysed in Enhanced Product Ion mode (EPI). The MS conditions (EPI) were: Turbo ion spray source, Temperature 420 °C, gas1 25 psi, gas2 30 psi, Voltage −4500, the declustering potential was set to −20 V and the collision energy to −35 mV. Data were acquired at a scan rate of 1000 amu/s and analysed with Analyst 1.4.1. The molecular ions in the negative EMS gave the following proportion of ions: 359.1 (16.5%), 360.1 (49.7%), 361.1 (24.6%), 362.1 (1.06).

4.8. Enzymic hydrolysis of (E)-2-[2,3-$^2H_2$] propenyl glucosinolate

(*E*)-2-[2,3-$^2H_2$]propenyl glucosinolate (0.1 mg) was dissolved in acetate buffer (0.1 M, pH 5.5) containing ascorbate (0.7 mM) and partially purified (60–90% ammonium sulfate cut) myrosinase extract from *S. alba* and incubated for 2 h at 37 °C. The enzyme assay was extracted with dichloromethane (1 ml) dried with anhydrous magnesium sulfate and the dichloromethane was carefully evaporated to 100 μl under a stream of nitrogen. The extract was analysed on a Hewlett Packard 6890 GC linked to a 5973 MSD. Injections were made onto a HP-5MS 5% phenylmethylsiloxane (30 m × 250 mm) column in the pulsed splitless or split mode using the following temperature programme: inlet temperature 225 °C; initial temperature 40 °C, 5 min; 5 °C/min until 180 °C; 10 °C/min until 280 °C; hold 10 min.

4.9. Deuterium NMR spectroscopy of enzymic hydrolysis products

Seeds of *T. arvense* were ground to a powder and the oil was removed by repeated extraction with hexane. Glucosinolates were removed by extraction with 80% acetone/water at 0 °C and the powder residue was washed with acetone and air dried. The seed powder was assayed for thiocyanate activity by adding 25 mg of seed powder to 200 μl of 0.1 M sodium acetate buffer (pH 5.8), 0.7 mM ascorbate, 2 mg of (*E*)-2-[2,3-$^2H_2$]propenyl glucosinolate and incubating for 3 h at 37 °C. The aqueous mixture was extracted with dichloromethane (1 ml, ×2) and the organic phases were combined and reduced in volume to 500 μl by careful evaporation. The experiment was repeated using partially

purified (60–90% ammonium sulfate cut) myrosinase from *S. alba* and the products were extracted with dichloromethane as before. Spectra were then recorded on a Bruker 400 MHz NMR spectrometer using dichloromethane as solvent.

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