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Synthesis Of Deuterium Labelled Desulfoglucosinolates as Internal Standards for LC-MS Analysis

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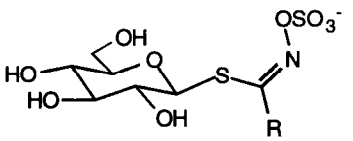
Abstract: The syntheses of three deuterium labelled desulfoglucosinolates are described. These are the phenethyl, 1-methoxyindolyl and 4-methoxyindolyl derivatives. The compounds were prepared as internal standards for use in the quantitative LC-MS analysis of glucosinolates to improve the sensitivity of the analytical procedure.

Keywords: thioglycosides; labelling; plants; insects

Introduction

Glucosinolates are a group of thioglycosides that occur in all members of the *Cruciferae*, including the brassica crops such as cabbage, Brussels sprouts and oilseed rape.[1] Glucosinolates contain a common structure (1) and over one hundred different examples have been isolated and characterised,[1] with a variety of substituents in the side chain R, including allyl (sinigrin), benzyl, 4-hydroxybenzyl and indoyl. Glucosinolates possess a range of important biological activities. Anti-nutritional and potentially toxic effects have been observed following consumption of high concentrations by mammals,[2] whereas reduced risk of cancer has been linked with consumption of brassicas by humans.[3] The anti-carcinogenic effects are thought to arise from isothiocyanates which are glucosinolate breakdown products.[4]



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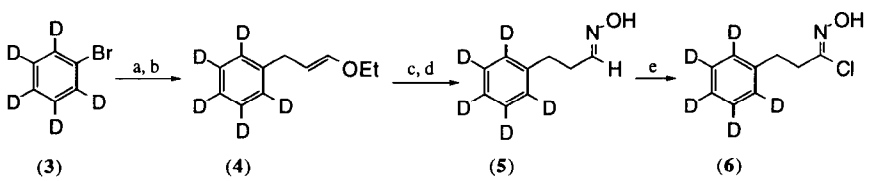
In plants the breakdown products play an important defensive role. They show anti-fungal and anti- bacterial effects and deter attack by non-adapted herbivores.[5] However in some cases glucosinolates play a role in host-plant recognition[5] and leaf surface glucosinolates can act as ovi-position (egg-laying) stimulants for brassica adapted insects, e.g. cabbage and turnip root flies *(Delia radicum* and *Delia floralis).*[6,7] In collaboration with the Scottish Crop Research Institute (SCRI), we are interested in further understanding the molecular interactions involved in the stimulation of ovi-position activity. This research requires a sensitive method for the accurate quantitative analysis of glucosinolates,[2] at the low levels observed on the leaf surface. This would allow the levels to be measured on single leaves of plants at different stages of development and under different amounts of environmental stress, which could then be correlated with the ovi-position activity.

LC-MS techniques for the identification of glucosinolates have been developed[8] but due to variations in ionisation in the source, reproducible quantification requires the use of a suitable internal standard. Ideally the internal standard should have a similar structure to the analyte with the most appropriate being the per-deuterated analogue of the analyte. In the case of glucosinolate analysis by high performance liquid chromatography the most commonly adopted methodologies are based on the separation and quantification of the desulfoglucosinolates normally prepared by enzymatic degradation of the extracted semi-purified glucosinolates. Consequently desulfoglucosinolates were chosen as the most appropriate synthetic targets.

Results and Discussion

In order to examine the feasibility of the analytical procedure it was decided to first prepare a glucosinolate that was readily amenable to labelling with deuterium. As many deuterated benzene derivatives are available, desulfogluconasturtiin (1, R = CH_2CH_2PH) was chosen as a suitable target. The most commonly used synthesis of glucosinolates involves the coupling of an oximyl chloride derivative to tetraacetyl thioglucose (2) followed by sulfation if required, or in our case simple deprotection to afford the desulfoglucosinolate.[9]



(3) (4) (5) (6)

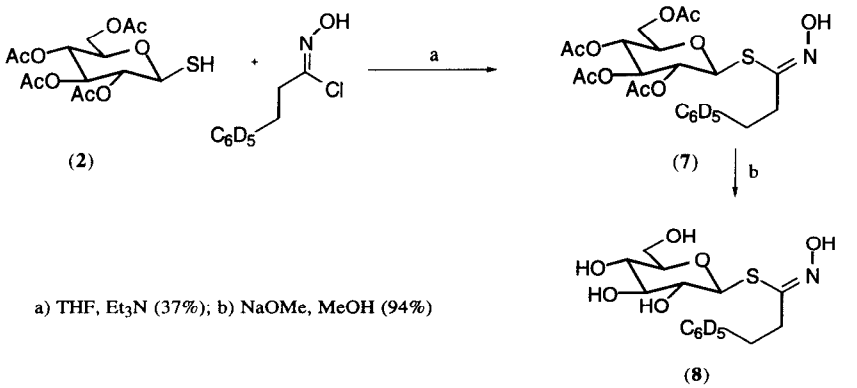
a) Mg, I_2, Et_2O; b) Acrolein diethyl acetal, 5% CuBr, THF (100% over two steps);
c) Acetone-water (4:1), conc. HCl (87%); d) $NH_2OH.HCl$, water, ethanol, $NaOAc.(H_2O)_3$ (49%);
e) $CHCl_3$, pyridine, *N*-chlorosuccinimide (97%)

Scheme 1

The oximyl chloride can either be prepared from an aldehyde or a nitro alkane and the former was found most suitable for gluconasturtiin synthesis (Scheme 1).

The starting material was pentadeuterated bromobenzene (3), commonly used as a solvent for NMR spectroscopy and thus fairly inexpensive. This was converted to the Grignard reagent and then reacted with acrolein diethyl acetal. Conjugate addition took place to give a mixture of E and Z isomers of the enol ether (4), in quantitative yield over the two steps.[10] There was no need to separate the two isomers as the mixture could be directly hydrolysed in aqueous acid to give the aldehyde (5) which was then readily converted to the oxime using hydroxylamine hydrochloride. Finally the oximyl chloride (6) was produced using *N*-chlorosuccinimide and used without purification in the coupling reaction. The NMR data was identical to that of the unlabelled compounds except that it lacked any signal for the aromatic protons and mass spectrometry confirmed the structures.

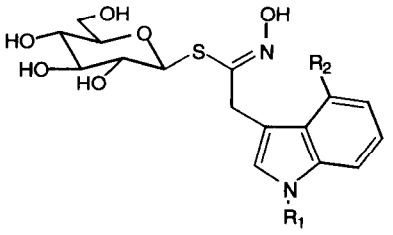
Coupling of the oxime to the tetraacetyl thioglucose was carried out using literature procedures,[9] although it gave the acetylated desulfoglucosinolate (7) in only moderate yield (37%). However, this was then efficiently deprotected in methanol using sodium methoxide to give the final product (8) in 94% yield (Scheme 2). The structure of the product was confirmed by NMR spectroscopy by comparison with previously prepared unlabelled material, and mass spectrometry showed that there was >95% incorporation of deuterium.



Scheme 2

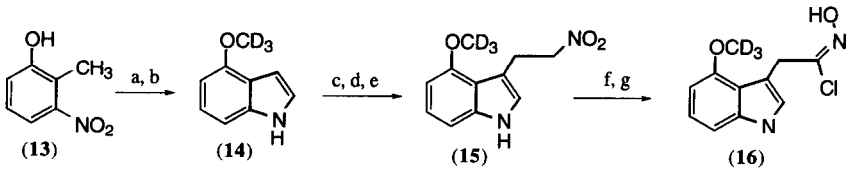
Behavioural studies of the oviposition behaviour of the flies using artificial leaves, and electrophysiological measurements of the responses of isolated tarsal sensilla have shown that the indole glucosinolates are the most active ovi-position stimuli.[6, 11] These are glucobrassicin (9), neoglucobrassicin (10), 4-hydroxyglucobrassicin (11) and 4-methoxyglucobrassicin (12). It was therefore necessary to prepare perdeuterated internal standards of

some of these indole derivatives. The indole glucosinolates are extremely challenging to prepare and were first synthesised by Rollin et al.[12,13] These existing methods were adapted for our purposes. The methoxy derivatives were targeted as these allowed the incorporation of deuterium into the methyl group of the methoxy substituent.



(**9**; R_1 = H, R_2 = H)
(**10**; R_1 = OMe, R_2 = H)
(**11**; R_1 = H, R_2 = OH)
(**12**; R_1 = H, R_2 = OMe)

In the case of the 4-methoxy derivative, the label was incorporated at the very start of the synthesis *via* deuteromethylation of 2-methyl-3-nitrotoluene (13) in quantitative yield (Scheme 3). This was then cyclised to the 4-methoxyindole (14) in 71% yield following reduction of the nitro group with titanium (III) chloride.[15] The side chain at the 3-position was introduced using a sequence of formylation, chain extension with nitromethane, and reduction to give the desired nitroalkane (15). Conversion to the oximyl chloride (16) was achieved by treatment with sodium in methanol followed by purified thionyl chloride.



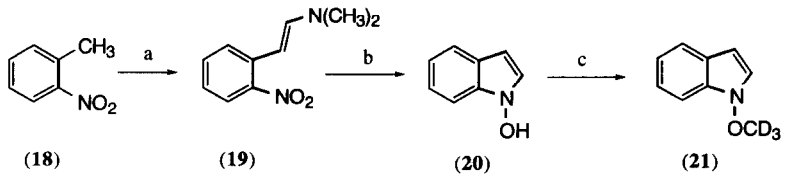
a) DMSO, KOH, CD_3I (87%); b) Pyrrolidine, DMF.DMA, $TiCl_3$ (71%);
c) $POCl_3$, DMF (73%); d) CH_3NO_2, NH_4OAc (89%); e) $NaBH_4$, $CHCl_3$, ^iPrOH, Silica gel (37%);
f) Na, MeOH; g) $SOCl_2$, DME, -40 °C (100% over two steps).

Scheme 3

This material was immediately coupled to tetraacetyl thioglucose as above, to provide the protected desulfoglucosinolate in 17% yield over the three steps. These proved to be the most difficult steps of the reaction and in order to get reasonable yields it was necessary to rigorously dry and purify all of the solvents and starting materials. Examination of the NMR spectra of all the intermediates and comparison with authentic samples of the non-deuterated materials, prepared during optimisation of the synthetic procedures, clearly demonstrated that the

deuterium atoms were retained throughout the synthesis. Finally deprotection gave the labelled desulfo 4-methoxyglucobrassicin (17).

The 1-methoxy derivative was prepared using a very similar procedure, however, the starting material, 1-methoxyindole first had to be synthesised. Thus 2-nitrotoluene (18) was reacted with *N,N*-dimethylformamide dimethyl acetal (DMF.DMA) in DMF to add on a one carbon side chain to give the intermediate (19). Reduction of the nitro group using zinc under acidic conditions was followed by cyclisation to 1-methoxyindole (20), which was then trapped *in situ* with deuteromethyl iodide and pyridine to give the desired starting material (21) in 40% yield over the three steps (Scheme 4).

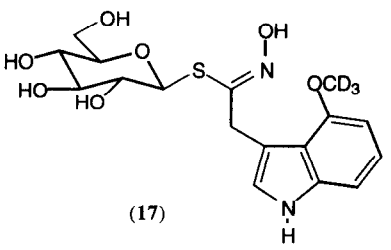


(18) (19) (20) (21)

a) DMF.DMA, DMF; b) Zn, NH_4Cl, Et_2O, H_2O;
c) CD_3I, Pyridine (40% over three steps)

Scheme 4

The remainder of the synthesis was identical to that for the 4-methoxy derivative and produced the desired deuterium labelled desulfo 1-methoxyglucobrassicin (22). This desulfoglucosinolate has not been previously prepared in either labelled or unlabelled form.



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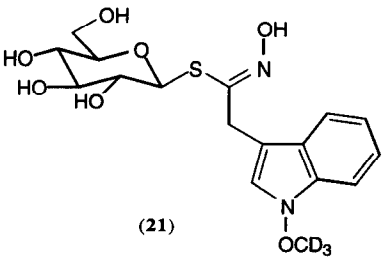

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Preliminary investigations using LC-APCI-MS have commenced utilising the synthesised desulfoglucosinolates and their per-deuterated analogues.[14] The results indicate that linear responses were obtained between normalised peak areas and analyte concentration for all three desulfoglucosinolates. These experiments suggest that using the perdeuterated internal standards combined with single ion monitoring increased the levels of detection a hundred

fold as compared with traditional hplc methods. Further studies are currently being undertaken to evaluate their value using plant derived samples.

With this methodology it is possible to detect the desulfoglucosinolate at the levels found on the surface of a single leaf, rather than having to combine leaves of similar ages from different plants in order to obtain sufficient sample. Subtle changes in the concentration of leaf surface glucosinolate could therefore be monitored to investigate their variation with age of leaf, plant development and environmental stress, opening up new areas of research in this field.

The problem with the methodology described herein is that the deuterium atoms are incorporated into the side chain of the desulfoglucosinolate and so a different synthetic procedure will be required for each compound. Work is now underway in our laboratory to develop a synthesis whereby the deuterium is incorporated into the glucose so that the same sugar fragment can be used for the preparation of any desulfoglucosinolate, regardless of the nature of the side chain, thus making the procedure much more flexible.

Experimental

Materials and Methods

[$^2H_3$]Methyl iodide, 99.5+ atom % D and [$^2H_5$]bromobenzene-d_5, 99.5+ atom % D were obtained from Aldrich. ^1H and ^{13}C NMR spectra were obtained using a Varian 2000 f.t spectrometer (^1H, 300 MHz;^{13}C, 75.42 MHz) and a Varian Gemini f.t spectrometer (^1H 200 MHz; ^{13}C 50.31 MHz). Mass spectra were recorded on an A.E.I MS-902 spectrometer. Optical rotations were measured at room temperature using an Optical Activity Ltd. AA 1000 polarimeter with 20 cm path-length cells. IR spectra were obtained on a Perkin-Elmer 1420 instrument or a Perkin-Elmer 1710 FT-IR spectrometer.

1-Ethoxy-3-[$^2H_5$]phenylprop-1-ene (4)

[$^2H_5$]Bromobenzene (1.0 g, 6.4 mmol) was added to magnesium turnings (1.63 g, 63.25 mmol) and iodine (1 crystal) in dry diethyl ether (10 ml). The reaction was warmed slightly until the iodine colour had disappeared. The remaining dry diethyl ether (20 ml) and [$^2H_5$]bromobenzene (8.0 g, 51.1 mmol) were added dropwise and the reaction stirred for 1 hour. To acrolein diethyl acetal (5.0 g, 5.85 ml, 38.4 mmol) and copper bromide (0.275 g, 2.0 mmol) in dry THF (50 ml) was added the [$^2H_5$]phenyl magnesium bromide, dropwise at room temperature. The reaction was exothermic and turned purple in colour on completion. The course of the reaction

was followed by t.l.c. on silica, ethyl acetate-hexane (1:4). A solution of saturated aqueous ammonium chloride (50 ml) was added before the organic layer was dried ($MgSO_4$) and concentrated at reduced pressure. The crude product was purified by distillation at reduced pressure to give 1-ethoxy-3-[$^2H_5$]phenylprop-1-ene as a clear oil (6.4 g, 100%) as a mixture of *E* and *Z* isomers in a 3:5 ratio; b.p. 100 °C/ 0.8 mm Hg; ν_{max} (thin film)/cm^{-1} 1650, 935 (C=C); δ_H (200 MHz; $CDCl_3$) 1.4 (6H, t, $J_{10,11}$ 7, H-11, *E* and *Z*), 3.4 (2H, dd, $J_{7,8}$ 7, $J_{7,9}$ 2.5, H-7, *E*), 3.7 (2H, dd, $J_{7,8}$ 7, $J_{7,9}$ 2.5, H-7, *Z*), 3.85 (2H, q, $J_{10,11}$ 7, H-10, *E*), 4.0 (2H, q, $J_{10,11}$ 7, H-10, *Z*), 4.75 (1H, m, H-8, *Z*), 5.1 (1H, m, H-8, *E*), 6.2 (1H, 2t, $J_{8,9}$ 7, $J_{7,9}$ 2.5, H-9, *Z*), 6.5 (1H, 2t, $J_{8,9}$ 13, $J_{7,9}$ 2.5, H-9, *E*); δ_C (50.3 MHz; $CDCl_3$) 15.4 (C-11, *E*), 16.0 (C-11, *Z*), 30.9 (C-7, *Z*), 34.7 (C-7, *E*), 65.1 (C-10, *E*), 68.3 (C-10, *Z*), 103.4 (C-8, *Z*), 106.1 (C-8, *E*), 146.0 (C-9, *Z*), 147.8 (C-9, *E*); *m/z* (EI) 167 ([M]$^+$, 87%), 138 (51, [M-C_2H_5]$^+$), 110 (100, [M-C_3H_5O]$^+$), 96 (29, [$C_6{}^2H_5CH_2$]$^+$), 83 (20, [$C_6{}^2H_5H$]$^+$).

[$^2H_5$]Hydrocinnamaldehyde

Two drops of concentrated HCl were added to a mixture of acetone (8 ml), water (2 ml) and 1-ethoxy-3-[$^2H_5$]phenylprop-1-ene (0.25 g, 1.54 mmol). The reaction was brought to reflux for 1 hour and then concentrated under reduced pressure. Purification by distillation at reduced pressure gave [$^2H_5$]-hydrocinnamaldehyde as a clear oil (3.74 g, 87%); b.p. 110 °C/ 0.8 mm Hg; ν_{max} (thin film)/cm^{-1} 1720 (C=O); δ_H (200 MHz; $CDCl_3$) 2.8 (2H, t, *J* 7, PhCH_2), 3.0 (2H, t, *J* 7, CH_2CHO), 9.8 (1H, s, C*H*O); δ_C (50.3 MHz; $CDCl_3$) 28.6 (Ph*C*H$_2$) 45.8 (*C*H$_2$CHO), 141.0 (C-1), 202.1 (*C*HO); *m/z* (EI) 164 ([M]$^+$, 91%), 110 (47, [M-CHO]$^+$), 96 (100, [M-CH_2CHO]$^+$), 83 (45, [M-$CHCH_2CHO$]$^+$).

[$^2H_5$]Hydrocinnamaldehyde oxime (5)

To a solution of sodium acetate trihydrate (5 g), hydroxylamine hydrochloride (3 g, 43 mmol) and water was added [$^2H_5$]hydrocinnamaldehyde (3.27 ml, 22.36 mmol) and the minimum volume of ethanol required to get the aldehyde into solution. The reaction was warmed in a water bath for 30 minutes with vigorous stirring then cooled to 0 °C to allow the product to crystallise from the solution. The product was removed by filtration, washed with ice-cold water and dried in a desiccator. The solid product was recrystallised from toluene to give a white crystalline solid (1.10 g, 49%) which was a mixture of *E* and *Z* isomers; m.p. 85-87 °C; (Found: C, 70.02; H, 7.25; N, 8.98. Calc for $C_9H_6{}^2H_5NO$: C, 70.09; H, 7.19; N, 9.08%); ν_{max} (nujol)/cm^{-1} 3110-3200 (OH); δ_H (300 MHz; $CDCl_3$) 2.5 (2H, t, *J* 7, CH_2CNH, *E*), 2.6 (2H, t, *J* 3.5, CH_2CNH, *Z*), 2.7-2.9 (4H, m, PhCH_2,

E and *Z*), 6.8 (1H, t, *J* 3.5, CN*H*, *Z*), 7.4 (1H, t, *J* 3.5, CN*H*, *E*), 7.7 (1H, br.s., OH); δ_C (75.45 MHz; $CDCl_3$) 27.1 (CH_2CHN, *Z*), 31.8 (CH_2CHN, *E*), 32.3 ($PhCH_2$, *Z*), 33.2 ($PhCH_2$, *E*), 141.2 (C-1, *E*), 141.4 (C-1, *Z*), 151.7 (*C*NH, *E*), 151.8 (*C*NH, *Z*); $^m/_z$ (EI) 154 ([M]$^+$,10%), 121 (13, [M-NH_2OH]$^+$), 109 (37, [M-CH_2NOH]$^+$), 96 (100, [M-CH_2CHNOH]$^+$) 82 (6, [M-CH_2CH_2CHNOH]$^+$).

[$^2H_5$]Hydrocinnamaldehyde oximyl chloride (6)

[$^2H_5$]Hydrocinnamaldehyde oxime (1.03 g, 6.7 mmol) was dissolved with chloroform (15 ml) and dry pyridine (0.27 ml, 3.35 mmol) then *N*-chlorosuccinimide (0.9 g, 6.7 mmol) was added slowly at 0°C. After 30 minutes the reaction mixture was poured onto ice water (20 ml) and extracted with diethyl ether (2 x 40 ml). The organic layers were washed with water (2 x 20 ml), dried ($MgSO_4$) and concentrated under reduced pressure to give a viscous yellow oil (1.23 g, 97%). This material was used in the next reaction without further purification; δ_H (200 MHz; $CDCl_3$) 2.8 (2H, m, $PhCH_2$), 2.9 (2H, m, CH_2CNCl), 9.65 (1H, s., OH); δ_C (50.3 MHz; $CDCl_3$) 33.0 ($PhCH_2$), 38.9 (CH_2CNCl), 144.0 (C-1), 146.0 (C=N).

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-[$^2H_5$]gluconasturtiin thiohydroximate (7)

A solution of [$^2H_5$]hydrocinnamaldehyde oximyl chloride (1.23 g, 6.5 mmol) was added to a solution of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (0.68 g, 4.35 mmol) in dry THF (150 ml) under nitrogen with stirring. Dry triethylamine (5.73 ml, 40.95 mmol) was added and the reaction stirred for 18 hours before being concentrated under reduced pressure. The residue was taken up in diethyl ether (50 ml) and washed with 1 M H_2SO_4 (50 ml). The acid layer was then extracted with ethyl acetate (20 ml). The combined organic extracts were dried ($MgSO_4$) and concentrated under reduced pressure. The resulting solid was purified by column chromatography on silica using 1:1 ethyl acetate-hexane as the eluant to give a white amorphous solid (1.24 g, 37%); m.p. >150 °C decomposes; (Found: C, 52.62; H, 5.42; N, 2.60. Calc for $C_{23}H_{24}{}^2H_5NO_{10}S.0.5H_2O$: C, 52.56; H, 5.0; N, 2.66%); $[\alpha]_D^{20}$ -24.2 (c 1.0 in $CHCl_3$); ν_{max} (nujol)/cm^{-1} 3300 (OH), 1750 (C(O)CH_3); δ_H (200 MHz; $CDCl_3$) 1.9-2.2 (12H, 4 x s, OC(O)CH_3), 2.75-2.9 (2H, m, H-8), 2.9-3.0 (2H, m, H-9), 3.7 (1H, m, H-5), 4.1 (2H, m, H-6), 4.95-5.15 (3H, m, H-1, 2, 4), 5.25 (1H, t, *J* 10, H-3), 8.2 (1H, s, OH); δ_C (50.3 MHz; $CDCl_3$) 21.0-21.3 (4 x $COCH_3$), 33.7 (CH_2), 34.7 (CH_2), 62.8 (C-6), 68.4 (C-4), 70.5 (C-2), 74.1 (C-5), 76.5 (C-3), 80.2 (C-1), 140.9 (C-1'), 153.2 (C-7), 169.8, 169.9, 170.8, 171.2 (4 x C=O); *m/z* (CI) 519 ([MH_2]$^+$, 2.0%), 518 (1.5, [M]$^+$), 517 (3.0, [MH]$^+$), 331 (94, [M-aglycone]$^+$), 271 (59, [M-

$C_{11}H_9{}^2H_5NO_3S]^+$), 169 (73, $[C_8H_9O_4]^+$), 155 (93, $[C_6{}^2H_5(CH_2)_2CHNHOH]^+$), 137 (100, $[C_6{}^2H_5(CH_2)_2NH]^+$).

Desulfo-[$^2H_5$]gluconasturtiin (8)

To 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl-[$^2H_5$]gluconasturtiin thiohydroximate (1.116 g, 2.16 mmol) in methanol was added a catalytic amount of sodium metal under nitrogen. The reaction was stirred for 18 hours before amberlite IR-120 resin was added. Stirring was continued for a further 15 minutes before the amberlite was removed by filtration and the solvent evaporated at reduced pressure to give a white amorphous solid (0.71 g, 94%); m.p. 89-92 °C; (Found: C, 49.55; H, 5.59; N, 3.75. Calc for $C_{15}H_{16}{}^2H_5NO_6S.H_2O$: C, 49.17; H, 5.78; N, 3.82%); $[\alpha]_D{}^{20}$ -52.0 (c 1.0 in CH_3OH); ν_{max} (nujol)/cm^{-1} 3300 (OH); δ_H (200 MHz; $C^2H_3O^2H$) 2.95-3.0 (4H, m, CH_2CH_2), 3.3-3.5 (4H, m, H-2,3,4,5), 3.65 (1H, dd, $J_{5,6a}$ 5, $J_{6a,6b}$ 12.5, H-6a), 3.85 (1H, dd, $J_{5,6b}$ 2.5, $J_{6a,6b}$ 12.5, H-6b), 4.85 (1H, d, $J_{1,2}$ 10, H-1); δ_C (50.3 MHz; $C^2H_3O^2H$) 35.1 (CH_2), 35.8 (CH_2), 62.9 (C-6), 71.4 (C-4), 74.8 (C-2), 79.8 (C-5), 82.4 (C-3), 84.1 (C-1), 142.7 (C-1'), 152.8 (C=N); *m/z* (FAB) 371 ([MNa]$^+$, 21%), 349 (3, [MH]$^+$).

2-[$^2H_3$]Methoxy-6-nitrotoluene

Powdered potassium hydroxide (0.79 g, 14 mmol) was added to DMSO (7 ml, 98.7 mmol) and stirred for 5 minutes. 2-Methyl-3-nitrophenol (0.54 g, 3.5 mmol) and [$^2H_3$]methyl iodide (0.436 ml, 7.0 mmol) were added in quick succession and the reaction stirred for 15 minutes. On completion the solution was poured onto water (70 ml) and extracted with dichloromethane (3 x 70 ml). The combined organic layers were washed with water (5 x 35 ml), dried ($MgSO_4$) and evaporated at reduced pressure. A light yellow powder of 2-[$^2H_3$]methoxy-6-nitrotoluene was obtained (4.423 g, 87%); m.p. 51-53 °C; (Found: C, 56.35; H, 5.10; N, 8.02. Calc for $C_8H_6{}^2H_3NO_3$: C, 56.46; H, 5.33; N, 8.23 %); δ_H (200 MHz; $CDCl_3$) 2.35 (3H, s, CH_3), 7.0 (1H, d, *J* 6, H-6), 7.25 (1H, t, *J* 6, H-5), 7.4 (1H, d, *J* 6, H-4); δ_C (50.3 MHz; $CDCl_3$) 12.0 (CH_3), 114.2 (C-4), 116.2 (C-6), 122.4 (C-2), 127.2 (C-5), 151.8 (C-3), 158.9 (C-1); *m/z* (EI) 170 ([M]$^+$, 85 %), 153 (83, [M-OH]$^+$), 135 (17, [M-$OC^2H_3H]^+$), 105 (20, [M-$C^2H_3HNO_2]^+$), 93 (100, $[C_7H_9]^+$).

4-[$^2H_3$]Methoxyindole (14)

A stirred solution of 2-[$^2H_3$]methoxy-6-nitrotoluene (1.0 g, 6.0 mmol) in DMF (10 ml, 129 mmol) was treated with DMF-dimethyl acetal (0.88 ml, 6.64 mmol) and pyrrolidine (0.58 ml, 7.08 mmol) then heated under nitrogen at 125 °C for 3 hours. The solution was evaporated under reduced pressure, taken up in the minimum volume of acetone and added to a mixture of titanium trichloride (30% wt soln in 2 N HCl, 18.2 ml) and ammonium acetate buffer (53 ml, 4 M). This was shaken for 10 minutes then extracted with diethyl ether (3 x 75 ml), dried ($MgSO_4$) and evaporated under reduced pressure. The resulting dark oil was purified by flash chromatography on silica gel with 40-60 petroleum ether-diethyl ether (2:1) as the eluant. A white crystalline solid was obtained (1.53 g, 57%); m.p. 68-69.5 °C; (Found: M^+, 150.0879. Calc for $C_9H_6{}^2H_3NO$: M^+, 570.0872); ν_{max} (nujol)/cm^{-1} 3300 (NH); δ_H (200 MHz; $CDCl_3$) 6.55 (1H, d, *J* 8.1, H-5), 6.68 (1H, m, H-3), 7.04 (1H, d, *J* 8.1, H-7), 7.10 (1H, t, *J* 4.0, H-2), 7.18 (H, t, *J* 8.1, H-6), 8.15 (1H, s, NH); δ_C (50.3 MHz; $CDCl_3$) 100.0 (C-5), 100.3 (C-3), 105.0 (C-7), 113.0 (C-9), 123.2 (C-2,6), 137.7 (C-8), 153.9 (C-4); *m/z* (EI) 150 ([M]$^+$, 100%), 132 (93, [M-C$^2H_3$]$^+$), 117 (18, [M-OC$^2H_2$H]$^+$), 104 (85, [C_6H_4CO]$^+$).

1-[$^2H_3$]Methoxyindole (21)

2-Nitrotoluene (3.0 g, 21.9 mmol) was brought to reflux with *N,N*-dimethylformamide dimethyl acetal (5.52 g, 6.15 ml, 46.3 mmol) in dry DMF (25 ml) for 18 hours under nitrogen while stirring vigorously. The reaction mixture was concentrated under reduced pressure, dissolved in diethyl ether (150 ml) and added to a solution of ammonium chloride (4.3 g, 80.4 mmol) in distilled water (30 ml) with zinc dust (27 g, 413 mmol). The reaction was mechanically stirred under nitrogen for 8 hours before being filtered through silica gel to remove the zinc, washing through with diethyl ether (50 ml). The filtrate was washed with saturated $NaHCO_3$ (2 x 75 ml) then treated with [$^2H_3$]methyl iodide (16.4 g, 7.2 ml, 116 mmol), 10% NaOH (aq) solution (150 ml) and tri(n-octyl)methyl ammonium chloride (1.0 g, 2.47 mmol). The reaction was stirred for 20 hours at room temperature, washed with brine (2 x 75 ml), dried ($MgSO_4$) then concentrated under reduced pressure to give a dark-red viscous oil. Purification by column chromatography on silica gel using DCM-hexane (3:7) as the eluant gave 1-[$^2H_3$]-methoxyindole as a pale-yellow oil (2.40 g, 44%); ν_{max} (thin film)/cm^{-1} 1710 (N-OC$^2H_3$), 2850 (OC$^2H_3$); δ_H (200 MHz; $CDCl_3$) 6.6 (1H, d, *J* 3.0, H-3), 7.35 (1H, t, *J* 7.7, H-5), 7.45 (1H, t, *J* 7.7, H-6), 7.5 (1H, d, *J* 7.7, H-2), 7.7 (1H, d, *J* 7.7, H-7), 7.9 (1H, d, *J* 7.7, H-4); δ_C (50.3 MHz; $CDCl_3$) 98.7 (C-3), 108.9 (C-7), 120.7 (C-4), 122.0 (C-5), 123.1 (C-2), 123.6 (C-9), 123.8 (C-6), 132.6 (C-8); *m/z* (EI) 150 ([M]$^+$, 100%), 132 (68, [M-C$^2H_3$]$^+$), 116 (81, [M-OC$^2H_3$]$^+$).

4-[$^2H_3$]Methoxyindole-3-carboxaldehyde

Phosphorous oxychloride (0.48 ml, 5.0 mmol) was added dropwise to dry DMF (3 ml) at 0°C. A solution of 4-[$^2H_3$]methoxyindole (0.5 g, 3.4 mmol) in dry DMF (2 ml) was added over a period of 30 mins at 0°C. The reaction mixture was warmed to 45 °C and stirred for 2 hours monitoring the reaction by t.l.c. (silica, ethyl acetate-hexane (1:3)). The reaction was poured onto ice water (8 ml), extracted twice with diethyl ether (2 x 10 ml) and the ethereal extracts discarded. The aqueous layer was then treated with aqueous sodium hydroxide until the solution was basic and extracted with diethyl ether (3 x 20 ml). The organic extracts were washed with brine, dried ($MgSO_4$) and concentrated under reduced pressure to give the crude product as a pale-yellow crystalline solid (1.24 g, 73%); m.p. 158.5-161.0 °C; ν_{max} (nujol)/cm^{-1} 1600 (C(O)H), 3200 (NH); δ_H (200 MHz; $C^2H_3O^2H$) 6.7 (1H, d, *J* 7.5, H-5), 7.0-7.3 (3H, m, H-2,6,7), 8.0 (1H, s, C*H*O); δ_C (50.3 MHz; $(C^2H_3)_2SO$) 102.4 (C-5), 106.0 (C-7), 115.8 (C-9), 118.4 (C-3), 123.8 (C-6), 129.8 (C-2), 138.2 (C-8), 154.1 (C-4), 186.7 (*C*HO); *m/z* (EI) 178 ([M]$^+$, 100%), 160 (31, [M-C^2H_3]$^+$), 149 (15, [M-COH]$^+$).

1-[$^2H_3$]Methoxyindole-3-carboxaldehyde

As described for 4-[$^2H_3$]methoxyindole-3-carboxaldehyde using 1-[$^2H_3$]methoxyindole (2.0 g, 13.3 mmol). The crude product was obtained as a dark-yellow oil (2.35 g, 100%); ν_{max} (thin film)/cm^{-1} 1600 (C(O)H); δ_H (200 MHz; $CDCl_3$) 7.1 (1H, m, H-5), 7.25 (1H, m, H-6), 7.7 (1H, d, *J* 7.5, H-7), 7.75 (1H, s, H-2), 8.1 (1H, d, *J* 7.5, H-4), 9.7 (1H, s, C*H*O); δ_C (50.3 MHz; $CDCl_3$) 109.2 (C-7), 114.3 (C-3), 121.9 (C-9), 122.4 (C-4), 123.8 (C-5), 125.0 (C-6), 132.9 (C-2), 133.1 (C-8), 184.7 (*C*HO); *m/z* (EI) 178 ([M]$^+$, 100%), 143 (10, [M-C^2H_3OH]$^+$), 132 (58, [M-C^2H_3CO]$^+$), 116 (64, [M-OC^2H_3CO]$^+$).

3-(2-Nitrovinyl)-4-[$^2H_3$]methoxyindole

Ammonium acetate (0.125 g, 1.60 mmol) was added to 4-[$^2H_3$]methoxyindole-3-carboxaldehyde (1.0 g, 5.6 mmol) in nitromethane (10 ml) and stirred vigorously while heating under reflux for 2 hours. The reaction was followed by t.l.c. (silica, 1:1, ethyl acetate-hexane) and the product showed as a bright-yellow spot. The resulting solution was concentrated under reduced pressure and the remaining bright-red solid purified by column chromatography on silica gel with ethyl acetate-hexane (1:1) as the eluant. The product was isolated as a bright-yellow solid (1.10 g, 89%); m.p. >105 °C decomposes; ν_{max} (nujol)/cm^{-1} 3200 (NH), 1300 (NO_2), 950 (C=C); δ_H (200 MHz; $C^2H_3O^2H$) 6.7 (1H, d, *J* 7.5, H-5), 7.05 (1H, d, *J* 7.5, H-7), 7.2 (1H, t, *J* 7.5, H-6), 7.85 (1H,

s, H-2), 8.05 (1H, d, *J* 12.5, C*H*NO_2) 8.6 (1H, d, *J* 12.5, C*H*=CH); δ_C (50.3 MHz; (C^2H_3)SO) 102.7 (C-5), 106.3 (C-7), 108.2 (C-3), 115.4 (C-9), 124.6 (C-6), 132.5 (C-2), 135.8 (*C*H=CH and *C*HNO_2), 139.0 (C-8), 153.9 (C-4); *m/z* (EI) 221 ([M]$^+$, 100 %), 174 (56, [M-NO_2H]$^+$), 157 (35, [M-$C^2H_3NO_2$]$^+$).

3-(2-Nitrovinyl)-1-[$^2H_3$]methoxyindole

As described for (21) using 1-[$^2H_3$]methoxyindole-3-carboxaldehyde (2.16 g, 12.1 mmol). The purified product was given as a bright-yellow solid (2.08 g, 78%); m.p. 81-85 °C decomposes; ν_{max} (nujol)/cm^{-1} 1300 (NO_2), 950 (C=C); δ_H (200 MHz; $CDCl_3$) 7.32 (1H, t, *J* 7.5, H-5), 7.40 (1H, t, *J* 7.5, H-6), 7.52 (1H, d, *J* 7.5, H-7), 7.73 (1H, s, H-2), 7.73 (1H, d, *J* 14.0, C*H*NO_2), 7.74 (1H, d, *J* 7.5, H-4), 8.2 (1H, d, *J* 14.0, C<u>H</u>=CH); δ_C (50.3 MHz; $CDCl_3$) 104.4 (C-3), 109.4 (C-7), 120.6 (C-4), 122.4 (C-9), 123.5 (C-5), 125.0 (C-6), 129.6 (C-2), 133.2 (*C*H=CH), 133.4 (*C*HNO_2), 133.5 (C-8); *m/z* (EI) 221 ([M]$^+$, 80%), 178 (100, [M-CHNO]$^+$), 140 (83, [M-C^2H_3OH-NO_2]$^+$), 132 (64, [M-C^2H_3-CCHNO_2]$^+$), 114 (53, [C_8H_4N]$^+$).

3-(2-Nitroethyl)-4-[$^2H_3$]methoxyindole (15)

Chloroform (200 ml), isopropanol (38 ml), silica gel (25.2 g) and 3-(2-nitroethyl)-4-[$^2H_3$]methoxyindole (2.4 g, 10.8 mmol) were stirred together for 15 minutes. Sodium borohydride (2.60 g, 68.7 mmol) was then added portionwise over 1 hour and the reaction followed by t.l.c. (silica, 1:1, ethyl acetate-hexane). Once the reaction was complete, the excess sodium borohydride was destroyed using 2 N HCl and the reaction was filtered, washing through with DCM. The filtrate was washed with brine, dried (Na_2SO_4) and concentrated under reduced pressure to give a dark oil which was purified three times by column chromatography on silica using ethyl acetate-hexane (1:1) as the eluant. The purified product was given as a light-brown coloured solid (0.9 g, 37%); m.p. 85-89 °C; (Found: C, 59.18; H, 5.12; N, 12.52. Calc for $C_{11}H_9{}^2H_3N_2O_3$: C, 59.18; H, 5.42; N, 12.55 %); ν_{max} (nujol)/cm^{-1} 3350 (NH); δ_H (200 MHz; $CDCl_3$) 3.6 (2H, t, *J* 7.5, CH_2), 4.8 (2H, t, *J* 7.5, CH_2NO_2), 6.6 (1H, d, *J* 10, H-5), 6.85 (1H, d, *J* 2.5, H-2), 7.0 (1H, d, *J* 10, H-7), 7.2 (1H, t, *J* 7.5, H-6), 8.15 (1H, s, H-1); δ_C (50.3 MHz; $CDCl_3$) 26.2 (CH_2), 77.7 (CH_2NO_2), 100.1 (C-5), 105.4 (C-7), 110.6 (C-3), 117.2 (C-9), 122.6 (C-2), 123.6 (C-6) 138.6 (C-8), 154.8 (C-4); *m/z* (EI) 223 ([M]$^+$, 97%), 176 (100, [M-NO_2H]$^+$), 159 (57, [M-$C^2H_3NO_2$]$^+$), 130 (43, [M-OC^2H_3-CHNO_2]$^+$).

3-(2-Nitroethyl)-1-[$^2H_3$]methoxyindole

As described for (26) using 3-(2-nitrovinyl)-1-[$^2H_3$]methoxyindole (1.9 g, 8.6 mmol). The product was purified three times using column chromatography on silica gel using ethyl acetate-hexane (1:2) as the eluant. The product was given as a pale-yellow oil (1.242 g, 65%); (Found: C, 59.28; H, 4.84; N, 12.10. Calc for $C_{11}H_9{}^2H_3N_2O_3$: C, 59.18; H, 5.42; N, 12.55 %); δ_H (200 MHz; $CDCl_3$) 3.5 (2H, t, *J* 7.5, CH_2), 4.65 (2H, t, *J* 7.5, CH_2NO_2), 7.2 (1H, s, H-2), 7.25 (1H, t, *J* 7.5, H-5), 7.35 (1H, t, *J* 7.5, H-6), 7.5 (1H, d, *J* 7.5, H-7), 7.65 (1H, d, *J* 7.5, H-4); δ_C (50.3 MHz; $CDCl_3$) 33.9 (CH_2), 76.1 (CH_2NO_2), 106.3 (C-3), 109.2 (C-7), 119.0 (C-4), 120.6 (C-5), 122.2 (C-2), 123.5 (C-6), 123.7 (C-9) 132.9 (C-8); *m/z* (EI) 223 ([M]$^+$, 100%), 176 (73, [M-NO_2H]$^+$), 163 (24, [M-CH_2NO_2]$^+$), 142 (60, [M-C^2H_3OH-NO_2]$^+$), 130 (32, [M-OC^2H_3-CH_2NO_2]$^+$), 115 (70, [M-C^2H_3-$CH_2CH_2NO_2$]$^+$).

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-4-[$^2H_3$]methoxyglucobrassicin thiohydroximate

Sodium metal (0.18 g, 7.8 mmol) was added to dry methanol (15 ml) under nitrogen and treated with 3-(2-nitroethyl)-4-[$^2H_3$]methoxyindole (1.0 g, 4.5 mmol) in dry diethyl ether (10 ml). After 15 minutes the reaction was concentrated at reduced pressure giving the nitronate as a white solid which was dried under a high vacuum for 15 minutes. The nitronate was cooled to -40 °C under nitrogen then treated with dry DME (37 ml) at -40 °C. A solution of purified thionyl chloride (1.86 ml) in dry DME (12.5 ml) at -40 °C was added to the nitronate dropwise to give a burgundy coloured solution. After 30 minutes at -40°C, water (60 ml) was added and the solution extracted with DCM (2 x 100 ml). The organic extracts were dried ($MgSO_4$) and concentrated under reduced pressure to give indol-3-yl acethydroximoyl chloride which was left under a high vacuum for 15 minutes and reacted directly in the next step. Indol-3-yl acethydroximoyl chloride in dry ether (39 ml) and dry DCM (19.5 ml) was treated with a solution of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (2.51 g, 6.9 mmol) and dry triethylamine (2.5 ml, 17.52 mmol) in dry DCM (19.5 ml). The reaction was stirred for 1 hour giving an orange solution. The reaction mixture was acidified with 0.5 M H_2SO_4 (100 ml) then extracted using DCM (2 x 100 ml). The organic extracts were dried ($MgSO_4$) and concentrated under reduced pressure to give a dark, sticky oil. The oil was purified twice by column chromatography on silica gel. Firstly, with 40-60 petroleum ether-ethyl acetate (6:4), secondly, using DCM-methanol (97:3) to give the product as a cream coloured solid (0.5 g, 20%); m.p. 90-96 °C; (Found: C, 51.66; H, 5.12; N, 4.79; [*M* + H]$^+$, 570.1848. Calc for $C_{25}H_{27}{}^2H_3N_2O_{11}S.0.5H_2O$: C, 51.90; H, 5.40; N, 4.84%; [*M* + H]$^+$, 570.1837); $[\alpha]_D^{20}$ -2.6 (c 1.1 in $CHCl_3$); ν_{max} (nujol)/cm^{-1} 1750 ($C(O)CH_3$), 3350 (NH, OH); δ_H (200 MHz; $CDCl_3$) 1.8-2.1 (4 x 3H, 4s,

OC(O)C*H*3), 3.0 (1H, m, H-5), 3.7 (1H, d, J_{gem} 12.5, H-6'), 4.0 (1H, d, J_{gem} 12.5, H-6), 4.25 (2H, s, H-8,8'), 4.95-5.0 (3H, m, H-2,3,4), 5.1 (1H, d, $J_{1,2}$ 10, H-1), 6.5 (1H, d, $J_{5i,6i}$ 8.0, H-5i), 6.9 (1H, s, H-2i), 6.9 (1H, d, *J* 8.0, H-7i), 7.04 (1H, t, *J* 8.0, H-6i), 8.6 (1H, s, N*H*), 10.0 (1H, s, O*H*); δ_C (50.3 MHz; $CDCl_3$) 21.0, 21.1, 21.5 (4 C(O)*C*H3), 30.6 (C-8), 61.0 (C-6), 67.9 (C-4), 70.4 (C-2), 74.4 (C-3), 75.7 (C-5), 80.2 (C-1), 100.2 (C-5i), 105.7 (C-7i), 110.6 (C-3i), 116.7 (C-9i), 122.6 (C-2i), 123.4 (C-6i), 138.3 (C-8i), 153.4 (C-7), 154.7 (C-4i), 169.9, 170.0, 170.8, 171.4 (4 x *C*(O)CH3); *m/z* (CI) 570 ($[MH]^+$, 3%), 569 (2, $[M]^+$), 331 (87, [M-aglycone]$^+$), 271 (24, [M-aglycone-AcOH]$^+$), 190 (68, $[M\text{-}C_{15}H_{23}O_9S]^+$), 169 (41, $[C_8H_9O_4]^+$), 163 (100, $[C_6H_{11}O_5]^+$).

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-1-[$^2H_3$]methoxyglucobrassicin thiohydroximate

As described for (29) using 3-(2-nitroethyl)-1-[$^2H_3$]methoxyindole (0.9 g, 4.0 mmol). The product was given as a cream coloured solid (0.816 g, 40%); m.p. 63-67 °C; (Found: C, 52.85; H, 4.80; N, 4.63; $[M + H]^+$, 570.1824. Calc for $C_{25}H_{27}{}^2H_3N_2O_{11}S$: C, 52.72; H, 5.31; N, 4.92%; $[M + H]^+$, 570.1837); $[\alpha]_D{}^{20}$ +9.0 (c 0.5 in $CHCl_3$); ν_{max} (nujol)/cm^{-1} 1750 (C(O)CH3); δ_H (200 MHz; $CDCl_3$) 1.9-2.1 (4 x 3H, 4s, OC(O)C*H*3), 3.3 (1H, m, H-5), 4.0 (3H, m, H-6,8,8'), 4.1 (1H, m, H-6'), 4.95 (4H, m, H-1,2,3,4), 7.15 (1H, t, *J* 7.5, H-5i), 7.15 (1H, s, H-2i), 7.2 (1H, t, *J* 7.5, H-6i), 7.45 (1H, d, *J* 7.5, H-7i), 7.55 (1H, d, *J* 7.5. H-4i), 9.65 (1H, s, O*H*); δ_C (50.3 MHz; $CDCl_3$) 21.0, 21.2 (4 C(O)*C*H3), 29.5 (C-8), 62.6 (C-6), 68.3 (C-4), 70.6 (C-2), 74.2 (C-3), 76.1 (C-5), 80.1 (C-1), 106.5 (C-3i), 109.2 (C-7i), 119.0 (C-4i), 120.6 (C-5i), 122.0 (C-9i), 122.2 (C-2i), 123.5 (C-6i), 132.9 (C-8i), 151.4 (C-7), 169.7, 169.9, 170.7, 171.2 (4 *C*(O)CH3); *m/z* (CI) 570 ($[MH]^+$, 2%), 569 (1, $[M]^+$), 331 (100, [M-aglycone]$^+$), 271 (30, [M-aglycone-AcOH]$^+$), 169 (52, $[C_8H_9O_4]^+$), 163 (92, $[C_6H_{11}O_5]^+$).

Desulfo-4-[$^2H_3$]methoxyglucobrassicin (17)

To a stirred solution of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-4-[$^2H_3$]methoxyglucobrassicin thiohydroximate (0.15 g, 0.263 mmol) in anhydrous methanol (5 ml) was added a catalytic amount of sodium metal. After stirring for 18 hours at room temperature under nitrogen, amberlite IR-120 resin was added. The solution was stirred for a further 15 minutes before the amberlite was removed by filtration and the solvent evaporated at reduced pressure. The residue was purified by column chromatography on silica gel using DCM-methanol (85:15) to give the product as a cream coloured amorphous solid (0.061 g, 58%); m.p. 94-100 °C;

(Found: C, 49.01; H, 5.81; N, 6.63. Calc for $C_{17}H_{19}{}^2H_3N_2O_7S.H_2O$: C, 48.68; H, 5.77; N, 6.68%); $[\alpha]_D{}^{20}$ -21.6 (c 0.4 in CH_3OH); ν_{max} (nujol)/cm^{-1} 3000-3300 (OH); δ_H (200 MHz; $C^2H_3O^2H$) 3.0 (1H, m, H-5), 3.05 (1H, t, *J* 10, H-3), 3.2 (1H, t, *J* 10, H-2), 3.35 (1H, t, *J* 10, H-4), 3.65 (1H, dd, $J_{5,6'}$ 5, $J_{6,6'}$ 12.5, H-6'), 3.7 (1H, dd, $J_{5,6}$ 2.5, $J_{6,6'}$ 12.5, H-6), 4.1 (1H, d, J_{gem} 17.5, H-8'), 4.5 (1H, d, J_{gem} 17.5, H-8), 4.75 (1H, d, $J_{1,2}$ 10, H-1), 6.5 (1H, d, $J_{5i,6i}$ 7.5, H-5i), 6.9 (1H, s, H-2i), 6.95-7,05 (2H, m, H-6i,7i); δ_C (50.3 MHz; $C^2H_3O^2H$) 31.7 (C-8), 62.5 (C-6), 70.8 (C-4), 74.5 (C-2), 79.8 (C-3), 81.8 (C-5), 83.7 (C-1), 100.4 (C-5i), 106.3 (C-7i), 112.2 (C-3i), 118.0 (C-9i), 122.7 (C-2i), 123.7 (C-6i), 140.0 (C-8i), 155.6 (C-7), 156.2 (C-4i); *m/z* (CI) 402 ($[MH]^+$, 100%), 240 (13, $[Aglycone+H]^+$), 163 (71, $[C_6H_{11}O_5]^+$).

Desulfo-1-[$^2H_3$]methoxyglucobrassicin (21)

As described for (34) using 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-4-[$^2H_3$]methoxyglucobrassicin thiohydroximate (0.5 g, 0.88 mmol). The product was given as a cream coloured amorphous solid (0.18 g, 51%); m.p. 69-71 °C; (Found: C, 49.50; H, 5.77; N, 6.54. Calc for $C_{17}H_{19}{}^2H_3N_2O_7S.0.5H_2O$: C, 49.75; H, 5.65; N, 6.82%); $[\alpha]_D{}^{20}$ -10.2 (c 0.5 in CH_3OH); ν_{max} (nujol)/cm^{-1} 3000-3300 (OH); δ_H (200 MHz; $C^2H_3O^2H$) 3.1-3.45 (4H, m, H-2,3,4,5), 3.65 (1H, dd, $J_{5,6'}$ 5, $J_{6,6'}$ 12.5, H-6'), 3.75 (1H, dd, $J_{5,6}$ 2.5, $J_{6,6'}$ 12.5, H-6), 4.0 (1H, d, J_{gem} 17.5, H-8'), 4.25 (1H, d, J_{gem} 17.5, H-8), 4.75 (1H, d, $J_{1,2}$ 10, H-1), 7.1 (1H, t, *J* 7.5, H-5i), 7.2 (1H, t, *J* 7.5, H-6i), 7.3 (1H, s, H-2i), 7.4 (1H, d, *J* 7.5, H-7i), 7.7 (1H, d, *J* 7.5, H-4i); δ_C (50.3 MHz; $C^2H_3O^2H$) 30.1 (C-8), 62.7 (C-6), 71.2 (C-4), 74.4 (C-2), 79.4 (C-3), 82.2 (C-5), 82.9 (C-1), 108.7 (C-3i), 109.3 (C-7i), 120.3 (C-4i), 120.9 (C-5i), 123.0 (C-9i), 123.7 (C-2i), 124.8 (C-6i), 134.1 (C-8i), 153.7 (C-7); *m/z* (CI) 402 ($[MH]^+$, 100%), 240 (27, $[Aglycone+H]^+$), 163 (12, $[C_6H_{11}O_5]^+$).

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