Biosynthesis of (*R*)-2-Hydroxybut-3-enylglucosinolate (Progoitrin) from [3,4-^{3}H]But-3-enylglucosinolate in *Brassica napus*

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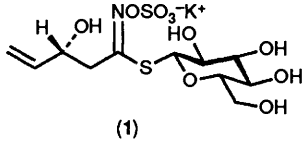
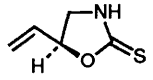
The synthesis of [3,4-^{3}H]but-3-enylglucosinolate is described and its role in (*R*)-2-hydroxybut-3-enylglucosinolate biosynthesis is discussed. This is the first time radio labelled but-3-enylglucosinolate has been synthesized and its incorporation into (*R*)-2-hydroxybut-3-enylglucosinolate demonstrated.

Glucosinolates are a class of naturally occurring thioglucosides found in the botanical family Cruciferae. (*R*)-2-Hydroxybut-3-enylglucosinolate (**1**) is present in the commercially important crop oil seed rape. Its importance lies in its enzyme-mediated degradation to 5-vinyloxazolidine-2-thione (**2**) commonly

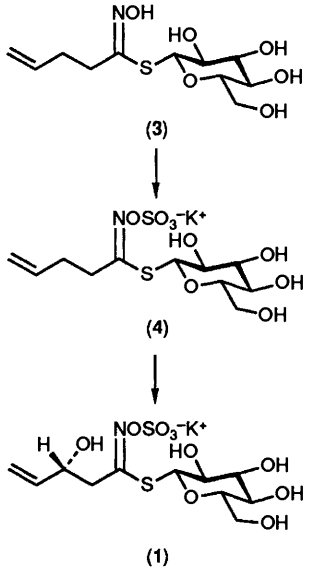


(1)



(2)

known as goitrin. Goitrin is a potent goitrogen and constitutes a serious problem in the economic utilisation of this crop.[1] In order to develop genetic strategies to eliminate compound (**1**) from the crop, more information on the biosynthesis and enzymes that control this pathway is desirable. But-3-enylglucosinolate (**4**) is believed to be the biosynthetic precursor[2] of compound (**1**) (Scheme 1). Recently it has been



Scheme 1. Late-stage biosynthesis of progoitrin.

shown[3] that [3,4-^{3}H]-desulphobut-3-enylglucosinolate (**11**) is quantitatively incorporated into compounds (**1**) and (**4**). The data obtained in these experiments also strongly support the hypothesis that compound (**4**) is the precursor of compound (**1**).

In order to establish compound (**4**) as the precursor of the hydroxy derivative (**1**) it was necessary to carry out a radio labelled synthesis of compound (**4**) with high specific activity. Compound (**4**) has previously been synthesized *via* a reaction involving pent-4-enohydroximinoyl chloride (**9**) and tetra-acetylthioglucose in the presence of base to give the glucosinolate skeleton; in this synthesis compound (**9**) was obtained by treatment of the sodium salt of 5-nitropent-1-ene with lithium chloride and conc. hydrochloric acid. However, this route was not considered suitable for a radiochemical synthesis and the alternative route described in this paper was devised.

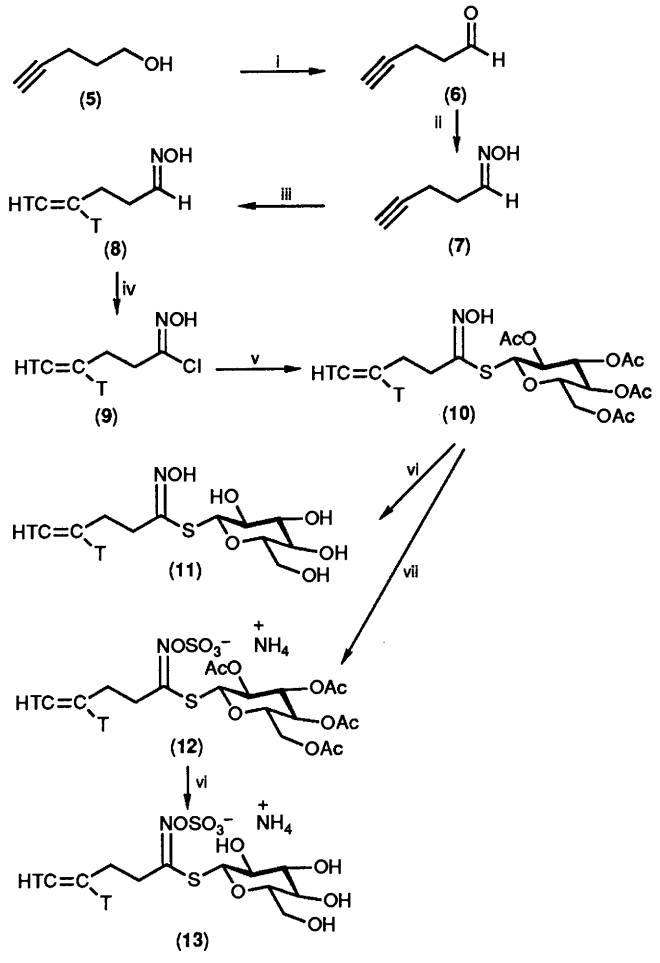
Results and Discussion

Scheme 2 details the radiochemical synthesis of compound (**4**) to give the biosynthetic substrates (**11**) and (**13**). The key to this synthesis is the reduction of pent-4-ynaloxime (**7**) with tritium gas in the presence of Lindlar's catalyst. The oxime (**7**) was prepared from pent-4-ynal in good yield and was fully characterised. Reduction of the triple bond in compound (**7**) with hydrogen in the presence of Lindlar's catalyst showed that some over-reduction, possibly to pentanaloxime, has occurred but could be minimised by using a slight excess of substrate (**7**). Reduction of compound (**7**) with tritium gas to give [4,5-^{3}H]pent-4-enal oxime (**8**) was confirmed by tritium NMR spectroscopy. Tritium NMR spectroscopy also revealed that some over-reduction had occurred (to the extent of 11.7%). The oxime (**8**) was diluted with unlabelled oxime (**8**) and the mixture was treated with *N*-chlorosuccinimide (NCS) to yield the chloro oxime (**9**), which was not characterised but used directly in the construction of the glucosinolate skeleton (**10**). The 1D ^{1}H NMR spectrum of compound (**10**) was complex but the resonances of the butenyl side-chain and the pyranose ring were distinguishable in the 2D COSY NMR spectrum. The tritium NMR spectrum of compound (**10**) (Figure 1) was consistent with the addition of HT across the acetylenic bond, showing resonances in the alkenyl region of the spectrum. The biosynthetic substrate (**11**) was prepared by ammonolysis of the tetra-acetate (**10**) and was purified by preparative TLC (PLC). Preparation of the salt (**12**) was achieved by reaction with pyridine–sulphur trioxide complex. However, because of the small scale of this preparation compound (**12**) was not purified but was used directly in the next stage of the synthesis, the ammonolysis step, to give its deacetylated product (**13**). The labelled glucosinolate (**13**) was purified by ion-exchange chromatography with DEAE Sephadex A-25 to give the ammonium salt. The radiochemical purity of compound (**13**) was shown by desulphoglucosinolate HPLC analysis to be 95%. All compounds synthesized after construction of the glucosinolate

Table. Administration of [3,4-^{3}H]but-3-enylglucosinolate to 4-day-old *Brassica napus* seedlings.

Time (h)	Uptake[a] (μCi)	Glucosinolate (mg)	% Incorporation	Specific activity mCi mmol^{-1}	Dilution
(*R*)-2-Hydroxybut-3-enylglucosinolate					
48	1.06	9.8	3.91	1.95	15 815
72	1.11	12.9	4.75	1.75	19 055
96	1.1	7.7	4.48	2.28	13 812
But-3-enylglucosinolate					
48		0.52	2.31[b]	19.9	1 668
72		0.38	2.27	30.3	1 292
96		0.3	1.54	24.0	1 307

[a] Refers to uptake of compound (**13**). [b] Recovery.



Scheme 2. *Reagents:* i, PCC; ii, $^+NH_3OHCl^-$, Na_2CO_3, water; iii, ^3H/Lindlar's catalyst; iv, NCS; v, 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose; vi, NH_3–MeOH; vii, pyridine–sulphur trioxide complex.

skeleton were authenticated by comparison of spectroscopic and chromatographic data using samples prepared by Kjaer's synthesis.[4] The biosynthetic substrate (**11**) was used in a series of previously described experiments,[3] and compound (**13**) was used in feeding experiments described in this paper.

Compound (**13**) was fed to 4-day-old developing seedlings of *Brassica napus* cv Bienvenu for periods of 48, 72, and 96 h (Table 1). The results show low incorporation (3.91–4.75%) of (**13**) into compound (**1**) over this period. However, the amount of compound (**13**) recovered was low (Table 1) (1.75–2.28%), indicating that some enzymic hydrolysis by endogenous thioglucosidase (E.C. 3.2.3.1) may have occurred on feeding to give, as yet, undetermined products. These results are in contrast to those previously obtained when feeding compound (**11**), the desulpho analogue, to the seedling system. In this case incorporation of compound (**11**) into products (**4**) and (**1**) was virtually quantitative,[3] giving incorporations of 31.4 and 50.4% respectively. This would imply that substrate (**11**) reaches its biosynthetic target site protected from endogenous thioglucosidase activity, since desulphoglucinolates are not substrates for this enzyme. These and previous results[3] indicate that there is little turnover of compound (**1**) in early stages of seedling development.

Both (2*R*) and (2*S*) forms of 2-hydroxybut-3-enylglucosinolate are known to exist at various developmental stages within some plant species,[5] and it therefore became desirable to examine the stereospecificity of the hydroxylation at the prochiral C-2 of substrates (**11**) and (**13**) in our seedling system. The (2*R*) and (2*S*) forms of desulpho-2-hydroxybut-3-enylglucosinolate are diastereoisomeric and separable by reverse-phase HPLC.[6] Previously synthesized compound (**1**), which contained both (2*R*) and (2*S*) forms,[7] was mixed with an extract from a feeding experiment, and HPLC analysis showed that radiolabelled 2-hydroxybut-3-enylglucosinolate eluted entirely as the (2*R*) form with both compounds (**11**)[3] and (**13**) as substrates, thus confirming that stereospecificity had been maintained.

In conclusion, these results confirm the latter stages of the biosynthesis of compound (**1**) as depicted in Scheme 1.

Experimental

IR spectra were recorded on a Pye-Unicam SP 1000 instrument; mass spectra on a Kratos MS25 mass spectrometer operating at 70 eV; ^3H NMR spectra on a Bruker WH-300 instrument operating at 320 MHz, and ^1H NMR spectra on a Bruker WH-250 instrument operating at 250 MHz. Radioactivity (dpm) was measured on a LKB 1211 Rackbeta liquid scintillation counter, and TLC plates were scanned on a Berthold Radio Scanner.

A Packard 428 with heated FID was used for gas chromatography, and a Waters HPLC (gradient) system for glucosinolate analysis. Microanalyses were carried out commercially, and reagents were obtained from the Aldrich Chemical Company. All solvents and reagents were purified before use. M.p.s were measured on a Gallenkamp m.p. apparatus and are uncorrected.

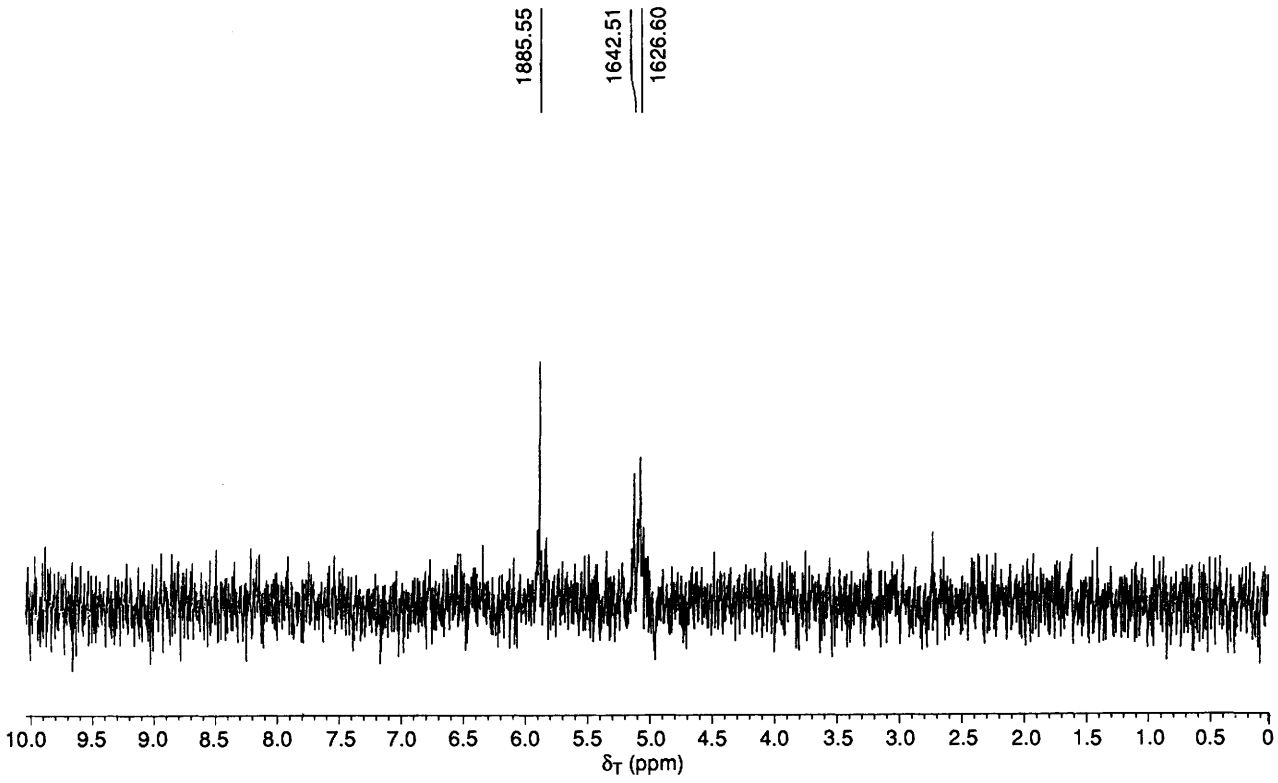


Figure 1. Tritium resonances from compound (**10**).

Pent-4-enal Oxime.—Pent-4-en-1-ol (5 g, 58 mmol) was added to a stirred solution of anhydrous dichloromethane (40 ml) containing pyridinium chlorochromate (PCC) (18.8 g, 87.2 mmol) and sodium acetate (1.42 g, 17.3 mmol) and allowed to react for 2 h. Anhydrous diethyl ether (100 ml) was added to the solution, the mixture was shaken, the organic plane was decanted, and the residue was extracted with further diethyl ether (2 × 50 ml). The combined extracts were filtered through a pad of Florisil, the ether was distilled through a Vigreux column, and the residue was distilled to give a liquid (2 g, 23.8 mmol), b.p. 104 °C (corresponding to that of the aldehyde), which was immediately added to water (4 ml) containing hydroxylamine hydrochloride (2.2 g, 31.7 mmol), and a solution of sodium carbonate (1.57 g, 14.3 mmol) in water (4 ml) was slowly added. After cessation of the reaction the oxime was extracted with diethyl ether (3 × 10 ml), the extract was dried with anhydrous $MgSO_4$ and then filtered, and the ether was removed by distillation. The residue was distilled under reduced pressure to give the title oxime as a liquid (1.41 g, 61% from pent-4-enal), b.p. 68 °C/12 mmHg (lit.,[8] 70 °C/13 mmHg) (Found: C, 60.1; H, 9.3; N, 13.75. Calc. for C_5H_9NO: C, 60.60; H, 9.09; N, 14.1%; ν_{max}(film) 3 280, 2 980, 2 920, 1 642, 1 445, 1 340, 1 300, 995, 920, and 705 cm^{-1}; δ_H(250 MHz; $CDCl_3$) 9.35 (1 H, br s, OH), 7.44 (0.5 H, t, *J* 6 Hz, C*H*=NOH), 6.74 (0.5 H, t, *J* 5 Hz, C*H*=NOH), 5.82 (1 H, m, CH_2=C*H*), 4.99–5.12 (2 H, m, H_2C=CH), and 2.21–2.54 (4 H, m, CH_2CH_2); *m/z* 99 (M^+, 1.1%), 82 (30), 67 (21), 55 (33), 54 (31), 53 (13), 41 (100), and 39 (62).

Pent-4-ynal Oxime (**7**).—Compound (**7**) was prepared using the previous procedure with pent-4-ynol. Pent-4-yn-1-ol (**5**) (5 g, 59 mmol) gave pent-4-ynal (1.4 g, 28%) as a liquid, b.p. 122 °C, which gave the *oxime* (**7**) as a liquid (1.28 g, 75.7% from pent-4-ynal), b.p. 85–90 °C/13 mmHg, which slowly crystallised at 4 °C (Found: C, 60.8; H, 7.4; N, 13.95. C_5H_7NO requires C, 61.84; H, 7.26; N, 14.41%); ν_{max}(Nujol) 3 300, 2 105, 1 670, 1 590, 1 075, 1 040, 920, 825, and 720 cm^{-1}; δ_H(250 MHz; $CDCl_3$) 8.63 (0.5 H, br s, OH), 8.20 (0.5 H, br s, OH), 7.52 (0.5 H, t, *J* 5.4 Hz, C*H*=NOH), 6.86 (0.5 H, t, *J* 5.1 Hz, C*H*=NOH), 2.58 (1 H, ddt, *J* 7.4, 5.7, and 0.7 Hz, CH*H*CH=NOH), 2.44 (3 H, m, CH_2C*H*HCH=NOH), and 2.02 (1 H, t, *J* 2.5 Hz, HC≡C); *m/z* 97 (M^+, 1.9%), 96 (16), 80 (12), 67 (22), 53 (35), 39 (64), 32 (26), and 28 (100) (Found: M^+, 96.0446. Calc. for C_5H_6NO: *M*, 96.0450).

Pent-4-enal Oxime (*by Reduction with Hydrogen*).—Compound (**7**) (200 mg, 2.1 mmol) was added to EtOH (95%; 5 ml) containing Lindlar's catalyst (12 mg) and was hydrogenated for 2 h. During this period the reaction was monitored by GC on a 2 m × 2 mm i.d. glass column packed with 10% diethylene glycol succinate coated on Chromosorb W, operating isothermally at 100 °C with carrier gas (N_2) flow of 30 ml min^{-1}. After 60 min, 75% of the yne compound (**7**) had been converted into pent-4-enal oxime, after which levels began to decrease with the formation of a new product. The new product was not characterized but the ^1H NMR spectrum of the unpurified residue showed resonances consistent with the formation of pentanal oxime.

[4,5-^{3}H]*Pent-4-enal Oxime* (**8**).—Compound (**7**) (12.3 mg, 0.13 mmol) was added to absolute EtOH (300 μl) containing Lindlar's catalyst (6 mg). The vessel was frozen, evacuated, and flushed with helium. Tritium (0.4 ml, 0.1 mmol; 10 Ci $mmol^{-1}$) in hydrogen (2 ml) was passed into the vessel, which was allowed to reach room temperature and the mixture was then stirred. After 2 h the reaction mixture was treated with EtOH and filtered. The EtOH was removed by distillation at reduced pressure and further EtOH (4 × 4 ml) was added to remove any labile tritium. The residue was taken up in anhydrous diethyl ether, the solution was dried over anhydrous $MgSO_4$ and then

filtered, and the ether was distilled off to give the crude oxime (**8**) (10 mg, 14 mCi; 1 mCi mmol^{-1}); δ_T(320 MHz; $CDCl_3$) 5.86, 5.85, 5.75, 5.71, and 5.62 (36.8%, m, H_2C=CT), 5.36 (4.38%), 5.12, 5.11, 5.07, 5.06, 5.02, 5.01, 4.97, and 4.96, (47.1%, m, HTC=CH), and 2.01, 1.98, 1.39, and 1.37 (11.7%).

2,3,4,6-*Tetra*-O-*acetyl*-β-D-*glucopyranosyl*[4,5-^{3}H]*Pent*-4-*enethiohydroximate* (**10**).—The tritiated oxime (**8**) (6.49 mg, 0.67 mmol; 74 mCi) was diluted with unlabelled oxime (63.5 mg, 0.65 mmol) and the mixture was added to anhydrous dimethylformamide (800 μl) containing NCS (95.4 mg, 0.71 mmol) under nitrogen. The reaction was initiated by warming to 50 °C and this temperature was maintained for 15 min. For convenience this reaction was best carried out in a Reacti-Vial. The reaction mixture was poured onto water (3.5 ml) and extracted with diethyl ether (2 × 2.5 ml); the combined extracts were washed with water (3 × 5 ml), dried over anhydrous $MgSO_4$, and filtered.

The ethereal solution was added directly to a stirred solution of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (154 mg, 0.42 mmol) in anhydrous diethyl ether (15 ml) at 0 °C; anhydrous triethylamine (42 mg, 0.42 mmol) in anhydrous diethyl ether (5 ml) was added and the reaction mixture was left at room temperature for 30 min. The ethereal solution was washed successively with 0.5M-H_2SO_4 (2 × 13 ml) and water (2 × 13 ml), dried over anhydrous $MgSO_4$, filtered, and evaporated to dryness to give a solid residue (119 mg). The crude residue was purified by 'flash' chromatography on silica (32–63 mesh; hexane–ethyl acetate, 3:1) to give pure thioester (**10**) [59 mg, 18.1% overall yield from (**9**), 5.3% radiochemical yield from (**9**); specific activity 30.8 mCi mmol^{-1}]; δ_T(320 MHz; $CDCl_3$) 5.89 (s, H_2C=CT), 5.13 (s, HTC=CH), and 5.08 (s, HTC=CH). The following spectroscopic data were obtained from a synthesis carried out with unlabelled material: m.p. 151–153 °C (lit.,[4] 150–152 °C) (Found: C, 49.7; H, 6.0; N, 3.2; S, 7.1. Calc. for $C_{19}H_{27}NO_{10}S$: C, 49.44; H, 5.90; N, 3.04; S, 6.98%); ν_{max}(Nujol) 3 330, 1 750, 1 710, 1 610, 1 075, 1 040, 920, 825, and 720 cm^{-1}; δ_H(250 MHz; $CDCl_3$) 5.86 (1 H, ddt, *J* 17.1, 10.4, and 6.4 Hz, H_2C=C*H*), 5.26 (1 H, ddd, *J* 8.5, 7.5, and 2.5 Hz, pyranose ring-H), 5.12 (1 H, m, *H*HC=CH), 5.05 (4 H, m, H*H*C=CH and 3 × pyranose ring-H), 4.17 (2 H, m, pyranose 6-H), 3.75 (1 H, m, pyranose ring), 2.63 (2 H, m, CH_2-CH_2C=NOH), 2.42 (2 H, q, *J* 6.9 Hz, CH_2CH_2=NOH), and 2.07, 2.05, 2.04, and 2.02 (4 × 3 H, 4 × s, OAc); *m*/*z* 331 (7%), 169 (52), 139 (19), 126 (32), 109 (34), 97 (57), 81 (20), 69 (10), 67 (23), and 43 (100). The ^{1}H NMR spectrum (320 MHz) of labelled (**10**) was identical with that of the unlabelled material.

[3,4-^{3}H]*But*-3-*enyldesulphoglucosinolate* (**11**).—Compound (**10**) (10 mg, 0.0217 mmol; 0.67 mCi) was added to anhydrous MeOH (1 ml) (previously saturated with NH_3 at 0 °C) and the mixture was left overnight, then reduced to dryness by rotary evaporation at reduced pressure and the residue was purified by PLC (silica gel; BuOH–MeOH–water, 8:1:1). The radiochemical purity of compound (**11**) was shown to be >99% on scanning of the TLC plate for tritium. The radiolabelled compound was eluted with 70% MeOH and the eluant was evaporated by rotary evaporation at reduced pressure to give compound (**11**) (~3 mg) (specific activity was taken as 30.8 mCi mmol^{-1} assuming no loss of tritium in this step). The chemical purity of compound (**11**) was checked by TLC using an authentic sample of but-3-enyldesulphoglucosinolate obtained by enzymic desulphonation of but-3-enylglucosinolate.

[3,4-^{3}H]*But*-3-*enylglucosinolate* (**13**).—Compound (**11**) (16.5 mg, 0.036 mmol; 1.1 mCi) was added to a stirred solution of anhydrous pyridine (200 μl) containing freshly prepared pyridine–sulphur trioxide complex (20 mg, 0.14 mmol) under nitrogen. The reaction mixture was left overnight and was then poured onto water (2 ml), a solution of potassium hydrogen carbonate (75 mg) in water (400 μl) was slowly added. The aqueous solution was extracted with diethyl ether (3 × 2 ml) and was then evaporated to dryness by rotary evaporation at reduced pressure <40 °C. The residue was dissolved in anhydrous MeOH (1 ml) (saturated with NH_3 at 0 °C) and the solution was left overnight at room temperature. The reaction mixture was reduced to dryness by rotary evaporation at reduced pressure to give a solid residue, which was applied to a column of DEAE Sephadex A25 (2 g, equilibrated in 0.2M-NH_4HCO_3), which was washed with water and the glucosinolate was eluted with 0.1M-NH_4HCO_3. The fractions containing compound (**13**) were combined and freeze dried to give the title compound (**13**) as the ammonium salt [~5 mg, 33% from (**10**); the specific activity was taken as 30.8 mCi mmol^{-1} assuming no loss of tritium in this step]. The purity of compound (**13**) was examined by desulphoglucosinolate analysis and was found to be >95%; compound (**11**) was found to be present in <0.0334%.

2,3,4,6-*Tetra*-O-*acetyl*-1-*thio*-β-D-*glucopyranose*.—This compound was prepared from α-D-glucose in four steps as previously described.[9]

Feeding Experiments.—Compound (**13**) (1.5 μCi; 30.8 mCi mmol^{-1}) in water (3 ml) was added to 100 four-day-old seedlings of *Brassica napus* cv Bienvenu contained in Petri dishes at 25 °C for periods of 48, 72, and 96 hr, on a day-and-night cycle of 12 h day, 12 h night. All incubations were carried out in duplicate. Seedlings were harvested by washing with water (dpm were measured to enable calculation of precursor uptake), freeze dried, and homogenized in boiling MeOH [80%; 20 ml; benzylglucosinolate (2 mg) as internal standard] for 5 min (repeated × 2). The combined extracts were reduced in volume by evaporation at reduced pressure <40 °C, reconstituted in water (8 ml), and extracted with light petroleum (40–60 °C; 3 × 20 ml). The analysis was carried out using a previously described protocol.[10] Ba/Pb acetate solution (0.1 ml) was added to the extract (2 ml) which was centrifuged (200 g) and the clear supernatant was applied to a column of DEAE Sephadex A25 (1 ml). The columns were washed successively with water (2 × 1 ml) and sodium acetate buffer (0.02M; pH 5; 2 × 1 ml), and sulphatase (75 μl) was added. After incubation overnight at room temperature the desulphoglucosinolates were eluted with water (3 × 0.5 ml) and analysed by reverse-phase HPLC using a Spherisorb S5 ODS2 column. An aliquot (1 ml) of the desulphoglucosinolate solution was reduced in volume (to 200 μl) and a portion (20 μl) was injected onto the HPLC column, and fractions corresponding to compounds (**1**) and (**4**) were collected and counted in Fluoran-HV scintillation fluid (BDH) for dpm. The validity of this method was presented in a previous paper.[3]

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