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Research Article

Synthesis of [2,3,4,5,6-$^2H_5$]phenyl glucosinolate

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Abstract: Starting from commercially available [2,3,4,5,6-$^2H_5$]benzoic acid, [2,3,4,5,6-$^2H_5$]phenyl glucosinolate was synthesized. Under negative-ion electrospray-ionization mass spectrometric conditions, this compound affords a peak at m/z 399. Since this m/z value is not known from the ions derived from natural glucosinolates, the [2,3,4,5,6-$^2H_5$]phenyl glucosinolate reported here is useful as an internal standard for the quantification of glucosinolates by negative-ion mass spectrometry (MS) and liquid chromatography (LC)/MS techniques. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: glucosinolates; phenyl glucosinolate; collision-induced dissociation (CID); internal standard; electrospray-ionization mass spectrometry

Introduction

Convincing evidence demonstrates that the consumption of broccoli and other cruciferous vegetables decreases the risk of cancer. Although cruciferous plants contain many important vitamins and minerals, the anticancer effects have been attributed to the presence of glucosinolates.[1–3] Intact glucosinolates appear to have no biological functions against cancer. However, glucosinolates are known to be metabolized into biologically active substances, such as isothiocyanates and indole-3-carbinol, on contact with the plant enzyme myrosinase.[4] Glucosinolates present in plant vacuoles react with myrosinase when the plant material is crushed or macerated. It is known that the concentration of glucosinolates drastically drops within a few minutes of maceration.[4] Apparently, this phenomenon has evolved as a defense mechanism against herbivore attack.

More than 120 different glucosinolates have been identified. All glucosinolates represent the basic chemical structure shown in Figure 1, and differ from each other by the composition of the aglycon moiety (the R function).[5] Wide interest in glucosinolates demand accurate, and reproducible analytical methods for quantification. Several chromatographic and mass spectrometric methods have been developed for the qualitative and quantitative determination of glucosinolates and their metabolites.[5–11] To develop accurate and reproducible quantification assays for low molecular weight compounds similar to glucosinolates by modern techniques such as liquid chromatography/mass spectrometry (LC/MS^n) procedures, isotopically labeled derivatives are required as internal standards.[11–13] However, preparation of only a few isotopically labeled glucosinolates has been reported. Botting and his coworkers have synthesized glucosinolates with deuterium atoms incorporated into the side chain[13] or the sugar moiety.[14] For LC/MS^n method development and validation, details of low-energy collision-induced dissociation (CID) pathways of the analytes (glucosinolates) must be established. However, fragmentation mechanisms of glucosinolates under negative-ion mass spectrometric conditions have not been fully established.[15,16]

Here, we report the synthesis of [2,3,4,5,6-$^2H_5$]phenyl glucosinolate, a deuterium-labeled analog of an uncommon but naturally occurring[1] glucosinolate. This analog is the first glucosinolate to be prepared bearing a perdeuterated aglycon moiety (R function). Although the compound is simple, its perdeuteration on the aglycon moiety renders it well suited for extensive CID fragmentation studies. Furthermore, this compound is useful as an internal standard for

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LC/MS^n quantification procedures of glucosinolates since the m/z value of the anion of the deuteriated compound is different from those reported from natural glucosinolates.

Results and discussion

The synthesis of [2,3,4,5,6-$^2H_5$]phenyl glucosinolate was accomplished by a seven-step process starting with commercially available [2,3,4,5,6-$^2H_5$]benzoic acid, which was first reduced to [2,3,4,5,6-$^2H_5$]benzyl alcohol (**1**) (Scheme 1). The key step of our synthesis was the nucleophilic addition of the 1-thio-β-D-glucose moiety to the phenyl-d_5 nitrile oxide, which was prepared *in situ* by the treatment of phenyl-d_5-hydroxamic chloride with a base.[17] This reaction produced the thiohydroxamate (**4**) in the desired *cis* configuration.

[2,3,4,5,6-$^2H_5$]Benzoic acid was first reduced to the deuteriated benzyl alcohol (**1**) with lithium aluminum hydride in a 1:1 mixture of diethyl ether and tetrahydrofuran. [2,3,4,5,6-$^2H_5$]Benzyl alcohol was then converted to the labeled benzaldehyde (**2**) by pyridinium chlorochromate (PCC) oxidation. The labeled aldehyde (**2**) was then derivatized to an oxime using hydroxylamine hydrochloride, and then to the hydroxamic chloride (**3**) with *N*-chlorosuccinimide.[18] Basification of an ethereal solution of deuterium-labeled phenylhydroxamic chloride (**3**) and 1-thio-β-D-glucose tetraacetate with triethylamine formed the desired *cis*-isomer of thiohydroxamate (**4**) in moderate yield (60%).[19] Sulfonation of the thiohydroxamate (**4**) with chlorosulfonic acid in the presence of pyridine afforded the pyridinium salt of the tetra-*O*-acetyl glucosinolate anion (**5**) in fair yield (45%).[20] Finally, deacetylation with ammonical methanol[19] provided the desired product [2,3,4,5,6-$^2H_5$]phenyl glucosinolate (**6**) in good yield (66%). A purity check of the final product showed that a final chromatographic purification was unnecessary. The negative-ion electrospray-ionization (ESI) CID mass spectra of synthetic compounds (**5**) and (**6**) are presented in Figure 2.

All synthetic reactions were first carried out using the unlabeled compounds. The ^1H and ^{13}C NMR data of the phenyl-d_5-labeled derivatives were identical to those of the unlabeled compounds, except for the absence of signals for the aromatic protons and carbon atoms adjacent to the aromatic protons. The mass spectrometric peaks for the anions from the phenyl-d_5-labeled compounds were observed 5 Da higher that those for the unlabeled compounds.

Synthesis of the phenyl-d_5 analog of phenyl glucosinolate was chosen for several reasons. To the best of our knowledge, the mass peak of the anion derived from phenyl-d_5 glucosinolate does not overlap with the m/z values known from the anions of natural glucosinolates or their ^{13}C and ^{34}S isotopomers including those of the unlabeled phenyl glucosinolate. Moreover, the chromatographic affinity of the phenyl-d_5 analog differs sufficiently from that of the unlabeled analog to afford a chromatographic separation. The phenyl-d_5 analog of phenyl glucosinolate is useful also for mass spectrometric fragmentation studies. For example, the fragment ions that contain the aromatic moiety can be easily recognized because the R function is perdeuterated. Moreover, the perdeuterated R function is helpful in identifying the origin of the hydrogen atom(s)

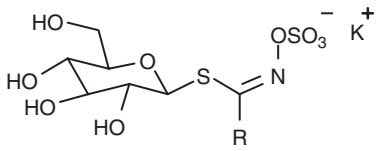
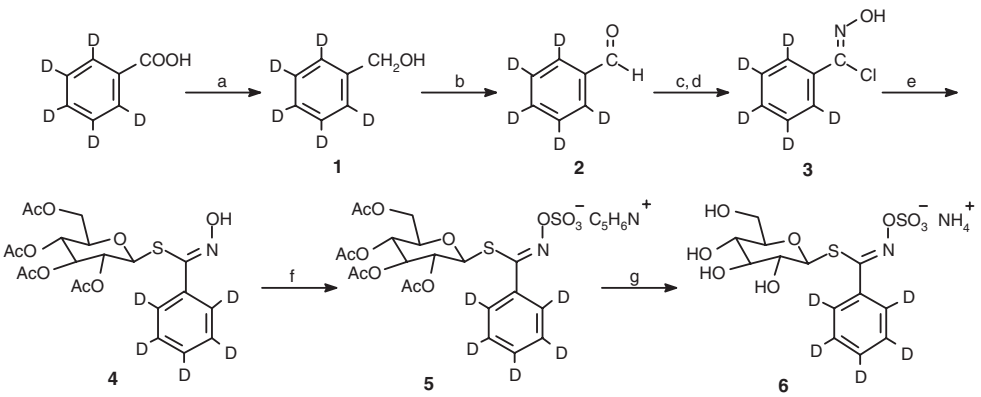


Figure 1 Structure of glucosinolates.



Scheme 1 Synthesis of [2,3,4,5,6-$^2H_5$]phenyl glucosinolate. Reagents and conditions: (a) $LiAlH_4$, Et_2O/THF (1:1) (83%); (b) PCC, DCM (84%); (c) $NH_2OH \cdot HCl$, Na_2CO_3, H_2O (92%); (d) *N*-Chlorosuccinimide, pyridine, DCM (90%); (e) Et_3N, Et_2O, 1-thio-β-D-glucose tetraacetate (60%); (f) $ClSO_3H$, pyridine, DCM (45%); (g) NH_3/MeOH,-10°C (66%).

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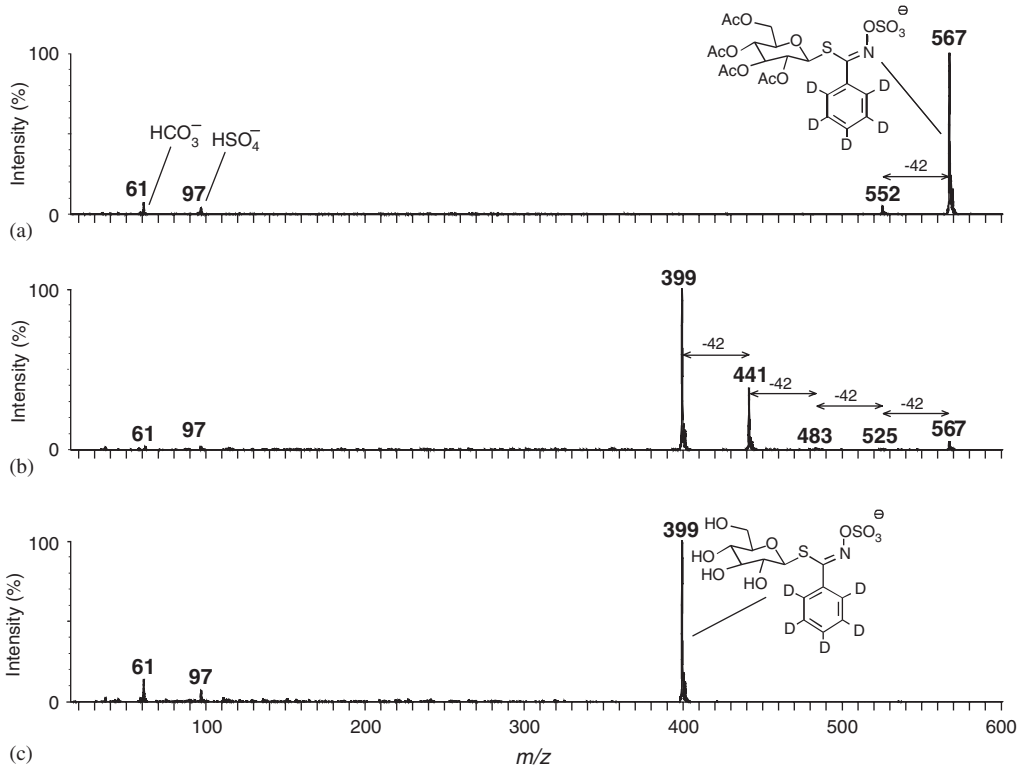

Figure 2 Negative-ion ESI mass spectra recorded from a sample of product **5** (a), and those obtained from the reaction mixture of the hydrolysis of **5** by methanolic ammonia at −10°C after 16 h (b) and 48 h (c). All spectra were recorded from 90:10 acetonitrile/water solutions. Note: Each loss of 42 mass units represents a deacetylation of the 1-thio-β-D-glucose tetraacetate moiety, and *m/z* 61 and 97 are analytical artifacts.

involved in various hydrogen transfer processes that occur under CID conditions.

Currently, compounds **4**, **5** and **6** are being employed in a detailed low-energy CID fragmentation investigation; compound **6** is also being employed as an internal standard for developing MS/MS procedures for rapid quantification of glucosinolates in cruciferous plant materials.

Experimental

General

All materials and reagents were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO). All 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 decoupled and all chemical shifts are reported relative to the central peak of $CDCl_3$ or DMSO.

GC–MS analysis carried out on an HP 5989B quadrupole mass spectrometer linked to a Hewlett Packard 5890 Series II gas chromatograph showed the purity of each sample to be >99%. 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 ($9:1:10^{-5}$) solutions at a flow rate of $6\,\mu L\,min^{-1}$. The source temperature was held at 80°C. The argon gas pressure in the collision cell was adjusted to attenuate precursor ion transmission by 30–50%.

High resolution ($R \approx 15\,000$) and accurate mass spectra were measured 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. Pressure of argon gas in the collision cell was held at 5.1×10^{-5} Pa.

The high-performance liquid chromatography (HPLC) analysis was conducted on an HP1090 (Hewlett-Packard) liquid chromatograph instrument. The method used was based on a previously reported procedure.[21] The final product (0.05 g) was dissolved in water

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(0.5 mL) and 15 μL of the solution was injected 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 ultraviolet (UV) detector was set at 235 nm.

[2,3,4,5,6-$^2H_5$]Benzyl alcohol (**1**)

[2,3,4,5,6-$^2H_5$]Benzoic acid (0.3 g, 2.4 mmol in 1.0 mL of dry THF) was added to lithium aluminum deuteride (0.144 g, 3.4 mmol in 1.0 mL of dry Et_2O). The reaction mixture was stirred at 40°C for 16 h, cooled to 0°C and quenched with water (0.2 mL). The mixture was shaken with 2 M HCl (10 mL) to dissolve the inorganic solids and the aqueous layer was discarded. The organic layer was dried over $MgSO_4$ and evaporated to yield the product as a clear liquid (205 mg, 83%). δ_H (400 MHz; $CDCl_3$) 2.52 (1H, s, O*H*), 4.62 (2H, s, CH_2OH); δ_C (400 MHz; $CDCl_3$) 64.8 (Ph*C*H_2OH), 140.6 (Ar–C_1); *m/z* (EI) 113 ($M^{+\cdot}$, 100%), 112 (40), 96 (20), 84 (90), 83 (85), 81 (40), 68 (10), 54 (40), 42 (15).

[2,3,4,5,6-$^2H_5$]Benzaldehyde (**2**)

A solution of [2,3,4,5,6-$^2H_5$]benzyl alcohol (**1**) (0.6 g, 5.4 mmol) in DCM (5 mL) was added to PCC (2.4 g, 11.2 mmol) in DCM (15 mL). The dark solution obtained was stirred for 1 h at RT. The dark solids were filtered from the reaction mixture and washed with DCM. The filtrate obtained was washed with aq. NaOH (40 mL, 1 M) and HCl (20 mL, 1 M). The organic layer was separated, dried over Na_2SO_4 and evaporated to yield the final product as an oil (0.51 g, 84%). δ_H (400 MHz; $CDCl_3$) 9.92 (1H, s, PhC*H*O); δ_C (400 MHz; $CDCl_3$) 136.8 (Ar–C_1), 179.9 (Ph*C*HO); *m/z* (EI) 111 ($M^{+\cdot}$, 100%), 110 (100), 83 (20), 82 (100), 54 (70), 52 (40), 42 (10), 40 (10).

[2,3,4,5,6-$^2H_5$]Benzaldehyde oxime

A solution of sodium carbonate (0.28 g, 2.6 mmol in 5 mL of H_2O) was slowly added to a solution of hydroxylamine hydrochloride (0.34 g, 4.8 mmol in 5 mL of H_2O) and [2,3,4,5,6-$^2H_5$]benzaldehyde (0.50 g, 4.6 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 as a colorless oil (0.52 g, 92%). δ_H (400 MHz; $CDCl_3$) 7.88 (1H, s, PhC*H*NOH), 10.20 (1H, s, O*H*); δ_C (400 MHz; $CDCl_3$) 132.0 (Ar–C_1), 174.8 (Ph*C*HNOH); *m/z* (EI) 126 ($M^{+\cdot}$, 100%), 124 (15), 109 (15), 83 (90), 82 (80), 71 (40), 52 (40), 42 (10), 40 (10).

[2,3,4,5,6-$^2H_5$]Phenylhydroxamic chloride (**3**)

A solution of [2,3,4,5,6-$^2H_5$]benzaldehyde oxime (0.45 g, 3.6 mmol in 5 mL of DCM) and pyridine (0.1 mL) was cooled to 0°C in an ice bath, and *N*-chlorosuccinimide (0.48 g, 3.6 mmol) was slowly added. 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 (0.49 g, 90%), which was used in the next step without further purification. δ_C (400 MHz; $CDCl_3$) 137.0 (Ar–C_1), 142.5 (*C*=N); *m/z* (+ve ion electrospray) 142 ([M–HCl+NH_4]$^+$, 100%), 125 (10, [M–HCl+H]$^+$), 109 (20), 82 (30).

S-2′,3′,4′,6′-tetra-O-acetyl-β-*D*-glucopyranosyl [2,3,4,5,6-$^2H_5$]phenylthiohydroxamate (**4**)

A solution of triethylamine (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 [2,3,4,5,6-$^2H_5$]phenylhydroxamic chloride (0.22 g, 1.4 mmol) in dry Et_2O (4.0 mL) and DCM (2.0 mL). A precipitate of triethylamine hydrochloride formed immediately. The reaction mixture was stirred for 2 h at RT. The mixture was then washed with aq. HCl (10 mL, 1 M) and H_2O (10 mL), and the organic layer was separated, dried over Na_2SO_4 and evaporated. The crude product was recrystallized from ethanol to afford the pure white product (0.30 g, 60%). δ_H (400 MHz; $CDCl_3$) 2.03, 2.04, 2.09 and 2.12 (12H, 3 × s, OCOCH_3), 3.04 (1H, s, H-4), 3.97 (1H, d, J = 12.4 Hz, H-3), 4.09 (1H, dd, J = 12.4, 4.8 Hz, H-2), 4.45 (1H, d, J = 9.6 Hz, H-1), 5.01 (2H, d, J = 8.4 Hz, H-3), 5.08 (1H, td, J = 7.2, 2.8 Hz, *H*-5), 8.90 (1H, s, PhCSNO*H*); δ_C (400 MHz; $CDCl_3$) 20.5, 20.5, 20.6 and 20.7 (4 × OC(O)*C*H_3), 61.8 (C-6), 67.8 (C-4), 69.8 (C-2), 73.7 (C-3), 75.7 (C-5), 81.4 (C-1), 132.3 (Ar–C_1), 169.2, 169.2, 170.2 and 170.6, (4 × O*C*(O)CH_3); *m/z* (–ve ion electrospray) [M–H]$^-$ $C_{21}H_{19}D_5NSO_{10}$ required 487.1435, found 487.1444.

Pyridinium *S*-2′,3′,4′,6′-tetra-O-acetyl-β-*D*-glucopyranosyl [2,3,4,5,6-$^2H_5$] phenylthiohydroxamic *N*-sulfate (**5**)

A stirred solution of the thiohydroxamate (**4**) (0.60 g, 1.24 mmol in 4.0 mL of DCM) and dry pyridine (5 mL) was cooled to 0°C in an ice bath and a cold solution of

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chlorosulfonic acid (1.6 mL, 24.2 mmol in 4.0 mL of dry Et_2O) was slowly added. After the addition was complete, 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, 45%). δ_H (400 MHz; $CDCl_3$) 1.96, 1.97, 2.06 and 2.11 (12H, 4 × s, OCOC*H*$_3$), 3.08 (1H, s), 4.01 (1H, d, $J = 11.6$ Hz), 4.09 (1H, dd, $J = 11.6$, 4.8 Hz), 4.55 (1H, d, $J = 7.6$ Hz), 5.02 (3H, m), 7.87 (2H, t, $J = 6.4$ Hz, Pyr–C_2), 8.39 (1H, t, $J = 6.4$ Hz, Pyr–C_3), 8.86 (2 H, d, $J = 5.2$ Hz, Pyr–C_1); δ_C (400 MHz; $CDCl_3$) 20.5, 20.6 and 20.7 (4 × OC(O)*C*H$_3$), 61.5 (C-6), 67.6 (C-4), 69.4 (C-2), 73.6 (C-3), 75.6 (C-5), 81.5 (C-1), 126.9 (Pyr–C_2), 131.3 (Ar–C_1), 142.5 (Pyr–C_1), 145.4 (Pyr–C_3), 157.0 (*C*═N), 169.2, 169.3, 170.0 and 170.5 (4 × O*C*(O)CH$_3$); *m/z* (–ve ion electrospray) [M–C_6H_6N]$^-$ $C_{21}H_{19}D_5NS_2O_{13}$ required 567.1022, found 567.1015.

Ammonium *β*-*D*-glucopyranosyl [2,3,4,5,6-$^2H_5$]phenylthiohydroxamic N-sulfate (**6**)

Pyridinium *S*-2′, 3′, 4′, 6′-tetra-*O*-acetyl-*β*-D-glucopyranosyl [2,3,4,5,6-$^2H_5$] phenylthiohydroxamic *N*-sulfate (**5**) (0.35 g, 0.54 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 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 pale yellow amorphous solid (0.15 g, 66%). Reversed-phase (RP) HPLC with UV detection at 235 nm showed that the purity of the product was >99%. δ_H (400 MHz; D_2O) 2.50 (1H, m), 2.91 (1H, t, $J = 8.8$ Hz), 3.05 (2H, td, $J = 8.4$, 4.4 Hz), 3.34 (1H, dd, $J = 12.0$, 5.6 Hz), 3.51 (1H, d, $J = 10.0$ Hz), 3.95 (1H, d, $J = 7.6$ Hz); δ_C (400 MHz; D_2O) 54.9 (C-6), 60.4 (C-4), 69.2 (C-2), 72.2 (C-3), 78.2 (C-5), 81.2 (C-1), 132.2 (Ar–C_1), 155.5 (*C*═N); *m/z* (–ve ion electrospray) [M–NH_4]$^-$ $C_{13}H_{11}D_5NS_2O_9$ required 399.0586 found, 399.0589.

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