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Research Article

The synthesis of [*phenyl*-$^2H_5$]gluconasturtiin and its metabolites for metabolic studies

John J. Morrison and Nigel P. Botting*
School of Chemistry, University of St. Andrews, St. Andrews, Fife KY16 9ST, UK

Summary

The synthesis of a deuterium labelled glucosinolate, [$^2H_5$]gluconasturtiin, is described starting from [$^2H_5$]bromobenzene. The potential metabolites of the glucosinolate, namely the [*phenyl*-$^2H_5$]phenethyl isothiocyanate, nitrile, thiocyanate, amine and the mercapturic acid conjugate of phenethyl isothiocyanate are also described. This series of compounds has been prepared for use in feeding studies to examine the mammalian metabolism of gluconasturtiin and search for new biomarkers of exposure.

Key Words: glucosinolates; isothiocyanates; gluconasturtiin; cancer prevention

Introduction

There is convincing epidemiological evidence that consumption of broccoli and other cruciferous vegetables is associated with a decreased risk of cancer and this association is strongest for cancers of the gastrointestinal and respiratory tracts.[1] The anti-cancer effects have been attributed to the presence of glucosinolates in these vegetables. Glucosinolates (**1**) are a class of naturally occurring thioglucosides which are metabolized by the plant enzyme myrosinase during food preparation, cooking and chewing (Scheme 1).[2,3] The major product of this metabolism is the corresponding isothiocyanate (**2**), formed *via* a Lossen-type rearrangement of the unstable thiohydroximate-*O*-sulphonate aglycone initially produced. In plants glucosinolates and myrosinase are compartmentalized and only interact following tissue damage. In mammals there also appears to be myrosinase activity in intestinal bacteria which may contribute to glucosinolate degradation *in vivo*.[4]

*Correspondence to: Nigel P. Botting, School of Chemistry, University of St. Andrews, St. Andrews, Fife KY16 9ST, UK. E-mail: npb@st-andrews.ac.uk

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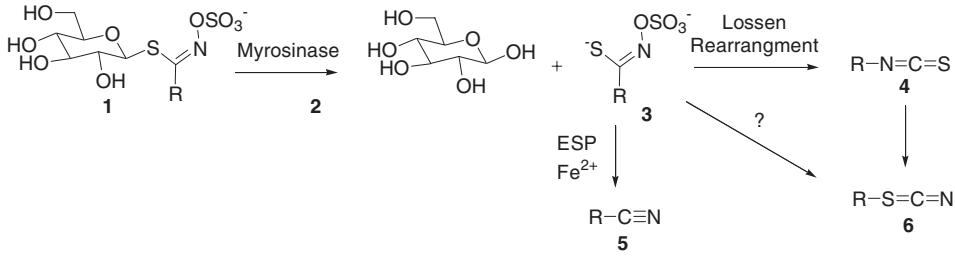


Scheme 1. General metabolism for glucosinolates

The anti-cancer activity appears to reside with the isothiocyanate[5] although the mechanism is still controversial and could be a result of inhibition of the Phase 1 detoxification enzymes,[6] upregulation of the Phase 2 detoxification enzymes[7] or even induction of apoptosis of tumour cells.[8] There are also many unanswered questions regarding the metabolism and bioavailability of glucosinolates and isothiocyanates. It is known that the aglycone can also break down to give nitriles (**5**) and thiocyanates (**6**), depending on the conditions and presence of other factors such as ESP (epithiospecifier protein) and ferrous ions.[9] The biological importance and metabolic fate of some of these metabolites is as yet unknown.

A project is being developed to employ a stable isotopically labelled derivative of gluconasturtiin, which has a phenethyl side chain and is one of the most common glucosinolates, in feeding studies with rats. These studies are aimed at further elucidating glucosinolate metabolism and identifying possible new biomarkers of glucosinolate exposure.

Results and discussion

In previous studies in our laboratory a deuterated desulfogluconasturtiin was prepared as an internal standard for glucosinolate analysis.[10] This was synthesized from [$^2H_5$]bromobenzene, commonly used as a solvent for NMR spectroscopy and fairly inexpensive. It was thus decided to repeat this synthesis but carry it through to the required [*phenyl*-$^2H_5$]gluconasturtiin (**7**). Similarly labelled versions of potential gluconasturtiin metabolites were also needed as internal standards, producing a family of synthetic targets. These were the isothiocyanate (**8**), amine (**9**), thiocyanate (**10**), nitrile (**11**) and the mercapturic acid conjugate of the isothiocyanate (**12**), a known human metabolite (Figure 1).

[$^2H_5$]Bromobenzene (**13**) was thus converted to the Grignard reagent which was reacted with acrolein diethyl acetal (Scheme 2). The conjugate addition took place to give a mixture of *E* and *Z* isomers of the enol ether (**14**), in quantitative yield over the two steps.[10] There was no need to separate the two isomers and the mixture was directly hydrolysed in aqueous acid to give the

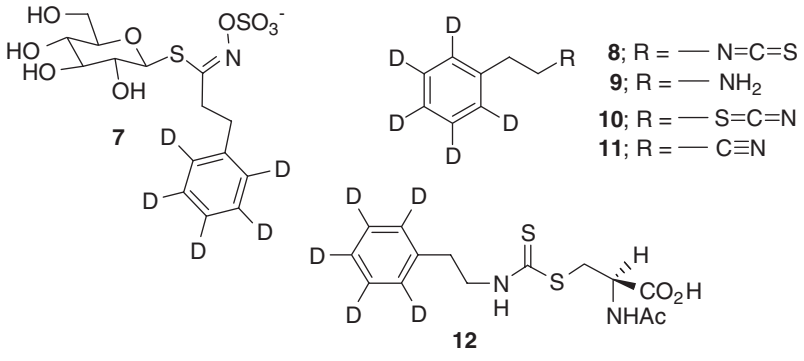
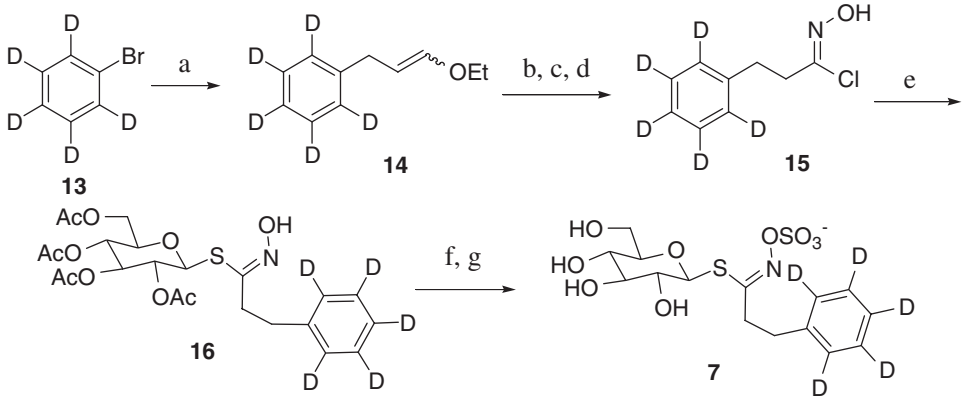
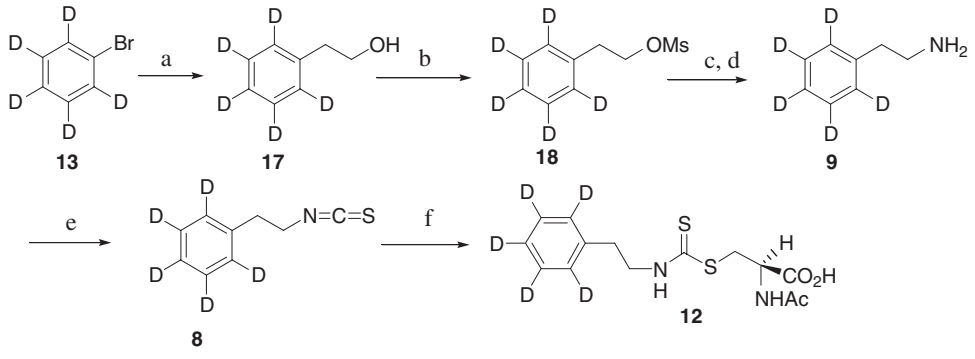


Figure 1. Synthetic targets



Scheme 2. Synthesis of [phenyl-$^2H_5$]Gluconasturtiin. Reagents and conditions: (a) Mg, THF, then 5% CuBr, THF then acrolein diethyl acetal (100%); (b) Acetone/H_2O (1:4), HCl (93%); (c) $NH_2OH.HCl$, NaOAc, EtOH, reflux (88%); (d) *N*-Chlorosuccinimide, pyridine, $CHCl_3$ (82%); (e) Et_3N, THF, tetraacetyl thioglucose (74%); (f) $ClSO_3H$, pyridine, DCM (20%); (g) KOMe, MeOH (99%)

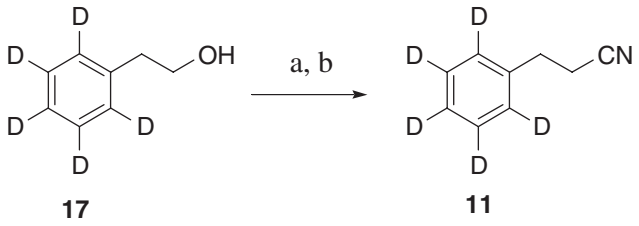
aldehyde, converted to the oxime using hydroxylamine hydrochloride and then chlorinated using *N*-chlorosuccinimide. The oximyl chloride (**15**) was used without purification in the coupling reaction employing modified literature procedures,[11] whereby under basic conditions elimination gives the nitrile oxide which is trapped out by the thiolate from tetraacetyl thioglucose to give the protected desulfoglucosinolate (**16**) in 74% yield. The *Z*-isomer is obtained exclusively due to stereoelectronic effects.[12] For the synthesis of the complete glucosinolate sulfonation was carried out using conditions established in our laboratory employing chlorosulfonic acid in pyridine.[13] The yield at this stage



Scheme 3. Synthesis of gluconasturtiin metabolites. Reagents and conditions: (a) Mg, THF, then ethylene oxide (99%); (b) MsCl, Et_3N, DCM (99%); (c) NaN_3, DMF (95%); (d) $LiAlH_4$, Et_2O (81%); (e) $CSCl_2$, Na_2CO_3, DCM, H_2O (82%); (f) *N*-Acetylcysteine, aqueous buffer (pH 6.6) (82%)

(20%) is the lowest on the sequence, although it reflects difficulties in purification rather than incomplete reaction. Final deprotection was carried out using potassium methoxide in methanol to give the [*phenyl*-$^2H_5$]gluconasturtiin as its potassium salt. The ^1H and ^{13}C NMR data were identical to those of the unlabelled compounds except for the lack of any signal for the aromatic protons and electrospray mass spectrometry confirmed the molecular mass.

In order to obtain the same labelling pattern for each metabolite [$^2H_5$]bromobenzene (**13**) was retained as the starting material. It was thought that the nitrile (**11**) would be accessible *via* a similar conjugate addition reaction to that used to make the aldehyde intermediate but using acrylonitrile instead of the acrolein diethyl acetal. However this turned out to be a very poor reaction and was abandoned. An alternative, more flexible approach involved reaction of the Grignard reagent with ethylene oxide to give the alcohol (**17**) (Scheme 3). This reaction could be carried out on a large scale to provide material for further reactions as **17** can then be modified to prepare all the required metabolites. Mesylation with mesyl chloride and triethylamine gave **18** in 99% yield. Nucleophilic substitution using azide ion as the nucleophile followed by reduction with $LiAlH_4$ then gave the required amine (**8**), in 81% yield. Conversion of the amine to the [*phenyl*-$^2H_5$]phenethyl isothiocyanate (**9**) was achieved in high yield using thiophosgene in a two-phase system of dichloromethane (DCM) and aqueous sodium carbonate solution. Synthesis of the deuterium labelled mercapturic acid conjugate (**12**) was carried out by reaction of the isothiocyanate and *N*-acetylcysteine in an aqueous buffer at pH 6.6 and the product purified by recrystallization.[14]



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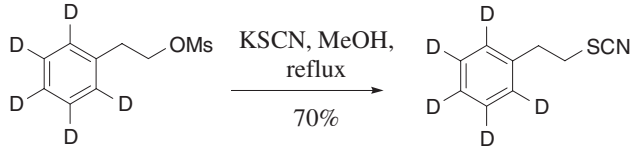
Scheme 4. Synthesis of nitrile. Reagents and conditions: (a) Ph_3P, CBr_4, DCM (36%); (b) KCN, 16-crown-6, MeCN (73%)



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Scheme 5. Synthesis of phenethyl thiocyanate

The nitrile (**11**) was synthesized from the same alcohol (**17**) in two steps *via* conversion to the bromide and nucleophilic substitution using potassium cyanide in acetonitrile (MeCN), with 18-crown–6 to assist solubilization (Scheme 4).

The final metabolite required was the thiocyanate (**10**). This molecule can be prepared using thiocyanate anion as a nucleophile, which is an ambident nucleophile and can react either through sulphur to give the thiocyanate or through nitrogen to give the isothiocyanate. The product distribution has been shown to depend on a number of factors including the solvent employed. It was found that reaction of potassium thiocyanate with the mesylate (**18**) in refluxing acetonitrile gave a 70% yield of the desired thiocyanate (**10**) after purification (Scheme 5). Under these conditions only a trace of the alternative isothiocyanate was produced and this was easily removed on purification.

All the unlabelled compounds have been previously reported in the literature and the spectral data obtained herein were identical apart from the absence of a signal for the aromatic protons in the ^1H NMR spectrum and an increase of 5 mass units in the observed molecular ion. The compounds are now being employed in feeding studies using rats to examine the metabolism and search for novel biomarkers. The experiments involve comparing the products derived from rats fed with unlabelled compounds and rats fed with the equivalent deuterium labelled compounds. LC–MS provides a rapid search

method to look for compounds with a 5 unit mass difference between the two sets of experiments which will then be further investigated. Feeding studies are being carried out using the glucosinolate, the glucosinolate plus added myrosinase, the isothiocyanate and the nitrile. The other compounds are being used as reference standards.

Experimental

General

Melting points were determined in open capillary tubes and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75.46 MHz, respectively, using Bruker Avance 500 and Varian Gemini 2000 spectrometers. The chloroform peaks (7.27 ppm for ^{1}H, 77.00 ppm for ^{13}C) were used as references. The EI and CI mass spectra were obtained on a VG Autospec mass spectrometer, and the ESI and APCI mass spectra on a Micromass LCT mass spectrometer. Diethyl ether and tetrahydrofuran were distilled over sodium and dichloromethane over calcium hydride. DMSO was freshly distilled from calcium hydride.

*2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl-[phenyl-$^{2}H_5$]gluconasturtiin (**16**)*

Chlorosulfonic acid (4.2 ml, 62.6 mmol) was diluted with dry DCM (50 ml) and this mixture was slowly added under argon to a solution of pyridine (62.0 ml, 626 mmol) in DCM (50 ml) and allowed to stir for 40 min. 2,3,4, 6-tetra-*O*-acetyl-β-D-glucopyranosyl-1-[*phenyl*-$^{2}H_5$]phenethyl thiohydroximate (**15**) (2.80 g, 5.4 mmol) was dissolved in DCM (100 ml) and added dropwise and the mixture was stirred for 24 h. Potassium hydrogen carbonate (6.26 g, 62.6 mmol in 100 ml water) was added and the mixture stirred for 30 min prior to evaporation of all the liquids below 40°C and addition of small aliquots of toluene to maintain a pyridine:toluene azeotrope. This provided a yellow gum which was purified by column chromatography on silica using 1:5 methanol:ethyl acetate as the eluant to give a white amorphous solid (0.680 g, 20%); $R_f = 0.61$ (1:4 methanol-ethyl acetate); δ_H (300 MHz; CD_3OD) 1.87 (3H, s, OC(O)CH_3), 2.00–2.10 (9H, 3 × s, OC(O)CH_3), 2.92–3.02 (2H, m, H-8), 3.02–3.18 (2H, m, H-9), 3.90–4.00 (1H, m, H-5), 4.10–4.20 (2H, m, H-6), 4.95–5.15 (2H, m, H-2, 4), 5.25 (2H, t, $J = 10$, H-1, 3); δ_C (75.4 MHz; CD_3OD) 20.7 (4 × OC(O)CH$_3$), 34.4 (CH$_2$), 35.6 (CH$_2$), 63.6 (C-6), 69.7 (C-4), 71.5 (C-2), 75.1 (C-3), 77.0 (C-5), 80.9 (C-1), 140.1 (C-1′), 158.9 (C═N), 172.0,171.3, 171.6 and 172.3 (4 × OC(O)CH$_3$); Mpt 168–170°C decomp. (Lit[15] 198–200°C decomp [from recryst in EtOH]); M.S. (−ve ion electrospray) $C_{23}H_{23}D_5O_{13}NS_2$ required 595.1334 found 595.1326.

*[phenyl-$^2H_5$]Gluconasturtiin (**7**)*

2,3,4,6-Tetra-O-acetyl-*β*-D-glucopyranosyl-[*phenyl*-$^2H_5$]gluconasturtiin (**16**) (0.73 g, 1.2 mmol) was dissolved in dry methanol (10 ml) and potassium metal (about 100 mg) was carefully added under a flow of nitrogen. The mixture was stirred at room temperature and after 4 h TLC showed all the starting material had been consumed. Amberlite IR 120 resin (1 g) was added and the mixture stirred for 1 h then the mixture was concentrated by evaporation and purified by column chromatography on silica using 1:4 methanol:ethyl acetate as the eluant to give a pale yellow amorphous solid (0.53 g, 99%); $R_f = 0.23$; δ_H (300 MHz; D_2O) 2.95–3.18 (4H, m, CH_2CH_2), 3.41–3.58 (4H, m, H-2,3,4,5), 3.65–3.75 (1H, m, H-6a), 3.75–3.90 (1H, m, H-6b), 4.85 (1H, d, $J = 12.0$, H-1); M.S. (−ve ion electrospray) $C_{15}H_{15}D_5O_9NS_2$ required 427.0889 found 427.0903.

*[phenyl-$^2H_5$]Phenethyl alcohol (**17**)*

[$^2H_5$]Bromobenzene (**13**) (20 g, 123 mmol in 50 ml dry THF) was added dropwise under N_2 onto magnesium turnings (3.36 g, 140 mmol in 75 ml dry THF). The reaction was activated with a few iodine crystals. The addition was regulated to maintain reflux and when addition was complete the mixture was heated under reflux for a further 4 h then cooled over ice. Ethylene oxide (9.0 g, 200 mmol) was decanted into ice cold dry THF (100 ml) and slowly added to the cooled Grignard reagent. A slight exotherm was observed on this addition. This procedure was done with due care as ethylene oxide boils at 11°C. The mixture was allowed to warm to room temperature then stirred overnight prior to cooling over ice then hydrolysed by careful addition of HCl (100 ml 10%). The mixture was extracted with EtOAc (2 × 100 ml), then washed with brine, dried over $MgSO_4$ and evaporated to give a mixture found to contain the desired product and 2-bromoethanol which was removed on the evaporator at 85°C to give [*phenyl*-$^2H_5$]phenethyl alcohol (15.6 g, 99%) as a colourless oil. δ_H (300 MHz; $CDCl_3$) 1.62(1H, s, O*H*) 2.90 (2H, t, $J = 6.8$, PhCH_2CH_2), 3.88 (2H, t, $J = 6.8$, CH_2CH_2OH); δ_C (75.4 MHz; $CDCl_3$) 38.7 (Ph*C*H_2CH_2OH), 63.3 (CH_2*C*H_2OH), 138.65 (Ar-C_1); M.S. (C.I.) $C_8H_5D_5O$ required 127.1045 found 127.1048.

*[phenyl-$^2H_5$]Phenethyl methanesulfonate (**18**)*

[*phenyl*-$^2H_5$]Phenethyl alcohol (**17**) (9.53 g, 75 mmol) and methanesulfonyl chloride (7.74 ml, 100 mmol) and dry triethylamine (14 ml, 100 mmol) were stirred in DCM (100 ml) under nitrogen at 0°C for 4 h. The products were washed swiftly with HCl (150 ml, 0.5 M), then saturated $NaHCO_3$ followed by brine. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to give the title compound as a colourless oil (15.4 g, 100%); δ_H (300 MHz;

$CDCl_3$) 2.85 (3H, s, CH_3), 3.08 (2H, t, $J = 7.6$, $PhCH_2CH_2$), 4.44 (2H, t, $J = 7.6$, CH_2CH_2O); δ_C (75.4 MHz; $CDCl_3$) 35.5 ($PhCH_2CH_2CN$), 37.2 (CH_3), 70.3 (CH_2CH_2O), 136.1 (Ar-C_1); M.S. (+ve ion electrospray) $C_9H_7D_5O_3NaS$ required 228.0719 found 228.0729.

[phenyl-$^2H_5$]Phenethyl azide

[*phenyl*-$^2H_5$]Phenethyl methanesulfonate (**18**) (15.46 g, 75 mmol) and sodium azide (4.47 g, 106 mmol) were heated to 70°C overnight in DMF (300 ml) then cooled by adding ice. The mixture was diluted with water (600 ml) and extracted with diethyl ether (3 × 200 ml). The extracts were washed with HCl (250 ml, 0.1 M) and brine and then dried over anhydrous Na_2SO_4 and evaporated to give a pale yellow oil (10.9 g, 95%); δ_H (300 MHz; $CDCl_3$) 2.91 (2H, t, $J = 7.2$, $PhCH_2CH_2$), 3.52 (2H, t, $J = 7.2$, $CH_2CH_2N_3$); δ_C (75.4 MHz; $CDCl_3$) 35.3 ($PhCH_2CH_2$ N_3), 52.5 (CH_2CH_2 N_3), 137.8 (Ar-C_1). The azide was used in the next step without further purification.

*[phenyl-$^2H_5$]Phenethylamine (**9**)*

Lithium aluminium hydride (5.0 g, 132 mmol) was added portionwise to dry diethyl ether (200 ml) and stirred under nitrogen until all effervescence had ceased. [*phenyl*-$^2H_5$]Phenethyl azide (5.0 g, 33 mmol) was dissolved in dry diethyl ether (80 ml) and added dropwise with care to ensure the rate of gas evolution did not increase to the point where excessive frothing would result. Once all the azide had been added the mixture was heated under reflux for 3 h then stirred overnight. The solvent was evaporated off and replaced with DCM (50 ml) and the organic layer filtered through celite washing with copious DCM, then washed with water, then brine, prior to evaporation of the DCM. The residue was purified by column chromatography on silica using 2:1 DCM:MeOH as the eluant. This gave the product as a colourless oil (3.78 g, 91%); δ_H (300 MHz; $CDCl_3$) 1.32 (2H, s, NH_2), 2.77 (2H, t, $J = 6.8$, $PhCH_2CH_2$), 2.99 (2H, t, $J = 6.8$, $CH_2CH_2NH_2$); δ_C (75.4 MHz; $CDCl_3$) 39.9 ($PhCH_2CH_2NH_2$), 43.5 ($CH_2CH_2NH_2$), 139.5 (Ar-C_1); M.S. (C.I.)[$M^+ + H$] $C_8H_7D_5$ required 127.1284 found 127.1286.

*[phenyl-$^2H_5$]Phenethyl isothiocyanate (**8**)*

Thiophosgene (34.5 g, 300 mmol) and sodium carbonate (6.89 g, 65 mmol) were stirred in water (100 ml) at room temperature until dissolved. [*phenyl*-$^2H_5$]Phenethylamine (**9**) was dissolved in 90 ml DCM and the mixture stirred vigorously. CO_2 evolution is quite rapid at first and slowly decreases over 5 h so the mixture was allowed to stir overnight. More DCM was added before the mixture was washed with HCl (100 ml 1 M) and brine prior to the organic layer being dried over anhydrous Na_2SO_4 and evaporated. The residue

was purified by column chromatography on silica using 1:1 petroleum ether:diethyl ether as eluant to give the product as a light yellow oil (4.12 g, 82%); δ_H (300 MHz; $CDCl_3$) 3.00 (2H, t, $J = 7.2$, PhCH_2CH$_2$), 3.76 (2H, t, $J = 7.2$, CH$_2$CH_2NCS); δ_C (75.4 MHz; $CDCl_3$) 36.8 (Ph*C*H$_2$CH$_2$NCS), 46.8 (CH$_2$*C*H$_2$NCS), 128.2 (N*C*S), 137.2 (Ar-C$_1$); (C.I.)[$M^+ + H$] $C_9H_5D_5NS$ required 169.0844 found 169.0847.

*Mercapturic acid conjugate of [phenyl-$^2H_5$]phenethyl isothiocyanate (**12**)*[14]

[*phenyl*-$^2H_5$]Phenethyl isothiocyanate (**8**) (0.672 g, 4 mmol) and *N*-acetyl cystine (1.31 g, 8 mmol) were stirred in aqueous phosphate buffer at pH 6.6. The oily isothiocyanate eventually dissolved and the product was precipitated out by adding 2 M HCl dropwise. The product was extracted into DCM and washed with HCl (100 ml, 0.01 M) then dried over anhydrous Na_2SO_4 and $NaHCO_3$. The DCM was evaporated at reduced pressure and the residue freeze dried from water to give light brown flakes (1.09 g, 82%); δ_H (300 MHz; $CDCl_3$) 1.92 (3H, s, COCH_3), 2.90 (2H, t, $J = 7.2$, PhCH_2CH$_2$), 3.58–3.72 (2H, m, SCH_2CH) 3.78–3.92 (2H, m, PhCH$_2$CH_2NCS), 4.58–4.70 (1H, m, SCH$_2$C*H*), 7.42 and 8.25 (2×1 H, br,s $2 \times$ N*H*) 9.95 (1H, br,s, COO*H*); δ_C (75.4 MHz; $CDCl_3$) 22.8 (CO*C*H$_3$), 33.9 (Ph*C*H$_2$CH$_2$NCS), 34.6 (S*C*H$_2$CH), 48.8 (CH$_2$*C*H$_2$NCS), 53.9 (SCH$_2$*C*H), 137.8 (Ar-C$_1$), 172.0 and 172.7 (2C, *C*OCH$_3$ and *C*OOH), 197.4 (N*C*SS); M.S. (−ve ion electrospray) $C_{14}H_{13}D_5O_3N_2S_2$ required 331.1073 found 331.1076.

[phenyl-$^2H_5$]Phenethyl bromide

[*phenyl*-$^2H_5$]Phenethyl alcohol (**17**) (2.5 g, 19.7 mmol) triphenylphosphine (10.5 g, 40 mmol) and carbon tetrabromide (13.26 g, 40 mmol) were dissolved in dry DCM (100 ml) over ice then allowed to warm to room temperature and stirred overnight. The original yellow solution gave a thick milky white precipitate that did not dissolve on addition of saturated $NaHCO_3$. The mixture was filtered through celite and the celite washed with copious EtOAc. The solvents were evaporated and the residue purified by column chromatography on silica using 1:1 petroleum ether:diethyl ether as eluant to remove the phosphines. The products were further purified by column chromatography on silica using 9:1 petroleum ether:DCM as eluant to give the product as a colourless oil (1.36 g, 36%); δ_H (300 MHz; $CDCl_3$) 3.16 (2H, t, $J = 7.5$, PhCH_2CH$_2$), 3.56 (2H, t, $J = 7.5$, CH$_2$CH_2Br). The bromide was used in the next step without further purification.

*3-[phenyl-$^2H_5$]Phenylpropionitrile (**11**)*

[*phenyl*-$^2H_5$]Phenethyl bromide (0.89 g, 4.7 mmol), 18-crown-6 (1.88 g, 5.2 mmol) and potassium cyanide (0.34 g, 5.2 mmol) were combined in acetonitrile (20 ml)

and heated under reflux for 4 h. The solvent was evaporated off and replaced with DCM (50 ml) and the organic layer washed with water then brine prior to evaporation of the DCM. The residues were purified by bulb to bulb distillation at 240°C at 6 mmHg to give the product as a colourless oil (0.46 g, 73%); δ_H (300 MHz; $CDCl_3$) 2.53 (2H, t, $J = 7.5$, CH_2CH_2CN), 2.88 (2H, t, $J = 7.5$, $PhCH_2CH_2$); δ_C (75.4 MHz; $CDCl_3$) 19.3 (CH_2CH_2CN), 31.4 ($PhCH_2CH_2CN$), 119.1 (CH_2CH_2CN), 137.8 (Ar-C_1); M.S. (C.I.)[$M^+ + H$] $C_9H_4D_5N$ required 136.1049 found 136.1053.

[phenyl-$^2H_5$]Phenethyl thiocyanate

[*phenyl*-$^2H_5$]Phenethyl methanesulfonate (**18**) (2.0 g, 10 mmol) and potassium thiocyanate (1.1 g, 11 mmol) were heated to reflux in acetonitrile (50 ml) overnight. The acetonitrile was evaporated and the residue dissolved in warm diethyl ether and filtered. The diethyl ether was evaporated off and the residues purified by column chromatography on silica using 1:1 petroleum ether:diethyl ether as eluant. This gave the product as a pale yellow oil (1.18 g, 70%); δ_H (300 MHz; $CDCl_3$) 3.10–3.21 (4H, m, $PhCH_2CH_2$); δ_C (75.4 MHz; $CDCl_3$) 35.1 and 35.9 ($PhCH_2CH_2SCN$ and $PhCH_2CH_2SCN$), 112.0 (S*C*N), 138.0 (Ar-C_1); M.S. (C.I.)[$M^+ + H$] $C_9H_5D_5NS$ required 169.0844 found 169.0848.

Acknowledgements

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