Pergamon

PII: S0031-9422(96)00741-8

Phytochemistry, Vol. 44, No. 7, pp. 1251–1255, 1997

0031-9422/97 $17.00+0.00

METABOLISM OF [α-^{14}C]-DESULPHOPHENETHYLGLUCOSINOLATE IN *NASTURTIUM OFFICINALE*

PÅL-JOHAN SVANEM, ATLE M. BONES* and JOHN T. ROSSITER†

Department of Biological Sciences, Wye College, University of London, Wye TN25 5AH, U.K.; *Unigen Centre for Molecular Biology and Department of Botany, MTFS, University of Trondheim, N-7005 Trondheim, Norway

(*Received in revised form* 19 *September* 1996)

Key Word Index—*Nasturtium officinale*; Brassicaceae; water cress; phenethylglucosinolate; biosynthesis; isothiocyanate.

Abstract—[α-^{14}C]-Desulphophenethylglucosinolate was synthesized in six steps from phenethylbromide, the label being introduced via substitution with ^{14}C-cyanide. Feeding experiments with [α-^{14}C]-desulphophenethylglucosinolate into water cress (*Nasturtium officinale*) gave good incorporation into phenethylglucosinolate, and *in vivo* metabolism at six days showed phenethylisothiocyanate and phenethylnitrile to be metabolites and an as yet an undetermined polar metabolite. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Glucosinolates are a diverse group of sulphur containing glycosides present in all cruciferous plants [1]. These secondary compounds co-occur with myrosinases which catalyse their hydrolysis. Glucosinolate degradation initially involves enzymic cleavage of the thioglucoside linkage by myrosinase, yielding D-glucose and an unstable thiohydroximate-*O*-sulphonate that spontaneously rearranges, resulting in the production of sulphate and one or more of a wide range of products. The products are generally a thiocyanate, isothiocyanate or nitrile, depending on factors such as substrate, pH or the availability of ferrous ions. The glucosinolates are derived from protein amino acids and are formed by a common pathway [1]. Glucosinolates can be fed in their desulpho analogue forms to plants. These undergo quantitative conversion into the corresponding glucosinolate [2, 3] and this provides a useful method of following their metabolism. Throughout the life cycle of cruciferous plants the glucosinolate profile can change quite markedly. Thus, for example, in developing seedlings of *Brassica napus* it has been demonstrated in single low varieties that the seedling content of aliphatic glucosinolates diminishes quite markedly [4]. Similar studies have also been carried out [5, 6] on turnip, cauliflower and forage rape. More recently it has been shown that changes in glucosinolate profile in cabbage (*B. oleracea* var. acephala and capitata) can change throughout the period of a day [7]. However, little is known about the *in vivo* metabolism of glucosinolates or their metabolites, although some previous work with *S*-2-hydroxybut-3-enylglucosinolate metabolism in *Crambe abyssinica* seedlings has shown goitrin and the nitrile to be the main metabolites [8]. Using [α-^{14}C]-desulphophenethylglucosinolate we have set out to examine the fate of this compound in water cress (*Nasturtium officinale*). Water cress was chosen, since the main glucosinolate in this plant is phenethylglucosinolate and this provides a convenient model system.

RESULTS AND DISCUSSION

Phenethylglucosinolate has been synthesized previously via direct chlorination of the oxime [9]. [α-^{14}C]-2-Desulpho-phenethylglucosinolate was successfully synthesized in good yield from 2-phenethylbromide (Fig. 1). After incorporation of labelled carbon the nitrile was reduced to the aldehyde with diisobutylaluminium hydride (DIBAL) in good yield. Attempts at reducing the nitrile with lithium triethoxyaluminium hydride were unsuccessful. Preparation of 3-phenyl-1-hydroxyamoylchloride-propane was carried out using a previously described method [10]. Throughout the synthesis, product purity was monitored by TLC using authentic compounds synthesized cold. The intermediate [α-^{14}C]-2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-2-phenethylthiohydroximate was further checked for authenticity using 2D COSY ^{1}H–^{1}H-NMR. The purity of the compound was confirmed by TLC and HPLC methods of analysis.

†Author to whom correspondence should be addressed.

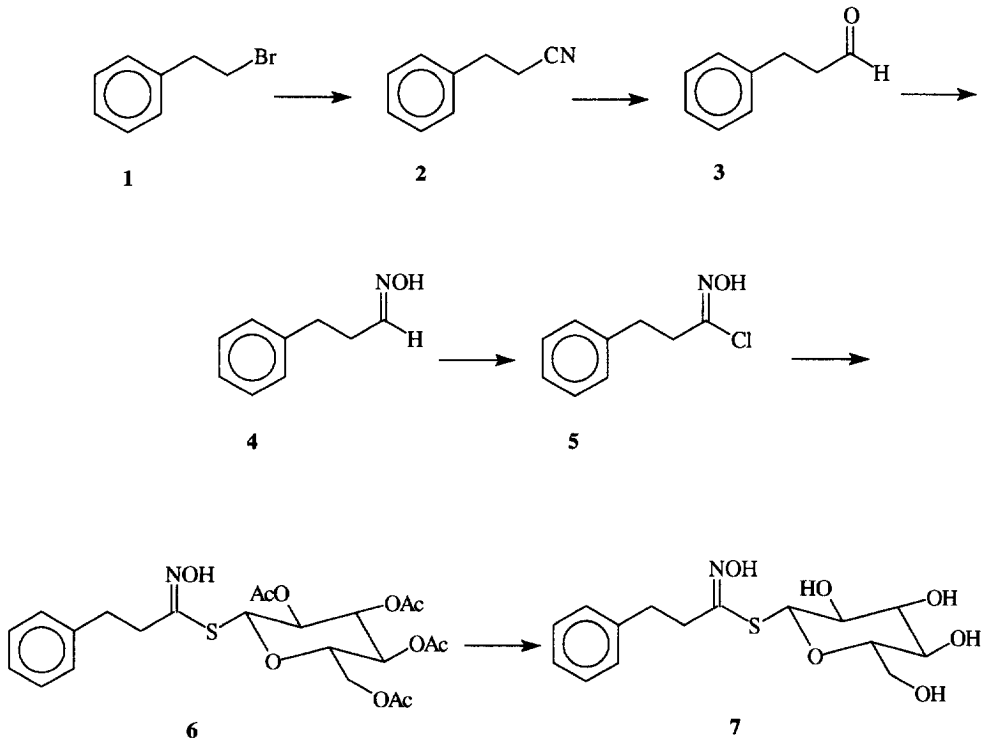

Fig. 1. Synthesis of [α-^{14}C]-2-desulphophenethylglucosinolate.

Uptake of the precursor by water cress was more than 70% after day 1 and virtually 100% by day 3. Incorporation into phenethylglucosinolate was rapid and reached *ca* 42% at day 3 and was maintained in this region for the remaining days (Table 1). These results are consistent with previous precursor feeding work [2] where high incorporation of desulphoglucosinolates into glucosinolates was observed.

In order to examine possible *in vivo* metabolites it was necessary to establish a method for extracting both glucosinolates and their degradation products. Simple extraction of cress material with ice-cold ethyl acetate resulted in a substantial loss of phenethylglucosinolate due to enzymic hydrolysis. The addition of an excess of sinigrin to compete out this hydrolysis was not entirely sufficient to overcome the problem. However, this problem of hydrolysis was overcome by low temperature extraction with ethanol and liquid nitrogen. Each set of analyses was carried out in triplicate, giving a mean of 125.3 μg (SD 8.5) of phenethylglucosinolate g^{-1} (fr. wt) of water cress for the conventional method, while for extraction at low temperature a mean of 130.7 μg (SD 5.8) of phenethylglucosinolate g^{-1} (fr. wt). Thus, by comparison with the existing method, which involves extraction with boiling 80% methanol, it was shown that no significant loss of phenethylglucosinolate occurred. The conventional method of extracting glucosinolates is not suitable for isothiocyanate analysis since the process results in the complete loss of this compound.

In order to determine the *in vivo* metabolites three separate extractions were carried out on cress tissue fed with precursor for six days. The incorporation of precursor into phenethylglucosinolate ranged between 41.3 and 57.2% while the amount of. isothiocyanate isolated accounted for between 5.1 and 11.8% (Table 2). Given the conditions of extraction it is not likely that the isothiocyanate is a product of the grinding

Table 1. Incorporation of [α-^{14}C]-2-desulphophenethylglucosinolate into phenethylglucosinolate in water cress

	Day				
	1	2	3	4	5
Uptake (%)	72	93	100	100	99
kBq in phenethylglucosinolate fraction	1.39	1.18	1.76	1.92	1.63
Incorporation (%)	33.5	28.3	42.2	46.1	39.2
Specific activity (MBq mol^{-1})	0.37	0.27	0.20	0.15	0.33
Dilution	70	96	129	170	79

Table 2. Analysis of metabolites at day 6 in water cress

	Experiment		
	1	2	3
kBq in phenethylglucosinolate fraction (% of total)	2.93 (77.9%)	3.43 (84.1%)	4.08 (78.3%)
kBq in phenethylisothiocyanate fraction (% of total)	0.44 (11.8%)	0.21 (5.1%)	0.53 (10.2%)
kBq in washings from glucosinolate ion exchange column (% of total)*	0.39 (10.8%)	0.44 (10.8%)	0.57 (11.5%)

*Percentage of total of kBq in phenethylglucosinolate, phenethylisothiocyanate and washings (minor metabolites from TLC analysis have not been included).

process as enzymic hydrolysis is unlikely to occur in a liquid nitrogen/ethanol slush ($< -50°$). In our experiments, phenethylisothiocyanate and phenethylnitrile were shown to be metabolites (Fig. 2), although a more polar compound was also found which has not yet been identified and does not correspond to phenylpropanoic acid, which is potentially derived from the nitrile. The efficiency of the method was determined by the addition of cold phenethylisothiocyanate and measurement of its recovery, which was quantitative.

Washings from the DEAE-A25 column used in the analysis of glucosinolates contained between 10.3 and 11.5% of the radioactivity and was most likely to contain unmetabolized [α-^{14}C]-2-desulphophenethylglucosinolate or perhaps polar metabolites. The percentage incorporation into phenethylglucosinolate does not markedly change after day 3, which suggests little turnover of this compound in leaves of cress, and the isothiocyanate isolated represents a potential *in vivo* metabolite together with the nitrile. It has been suggested [11] that natural pathways exist for isothiocyanate degradation, possibly via the formation of an amine. It is known, for example, that benzylisothiocyanate [12] can be broken down to the corresponding amine by *Enterobacter cloacae* to give benzylamine and hydrogen sulphide. However, such a route has yet to be shown in the plant. It has been suggested that isothiocyanates might bind to proteins in the plant [13] although extraction of protein from the tissues of water cress showed only 0.38% of radioactivity accounted for by this potential route. Binding of isothiocyanates to proteins [14] can occur, but only at pHs greater than 7. In our process, tissue was extracted in buffer of pH 5.8, together with an excess of phenethylisothiocyanate, which would effectively compete out any endogenous labelled isothiocyanate, thus precluding any binding in the extraction process. It would seem, therefore, that glucosinolates are hydrolysed *in vivo* by myrosinases to isothiocyanates.

However, it is possible that degradation profiles might alter, depending on the stage of plant development, and switches to nitrile formation may occur. Similarly, plants undergoing abiotic and biotic stress may also have altered glucosinolate metabolism, particularly when considering the number of myrosinase genes and the factors (e.g. ferrous ions) that may alter product profiles [13]. For example, it has recently been shown [15] that sulphur is involved in resistance to *Verticillium dahliae* in *Theobroma cacao* and it is possible that in *Brassica* plants sulphur deposition from glucosinolates may be of importance in disease resistance.

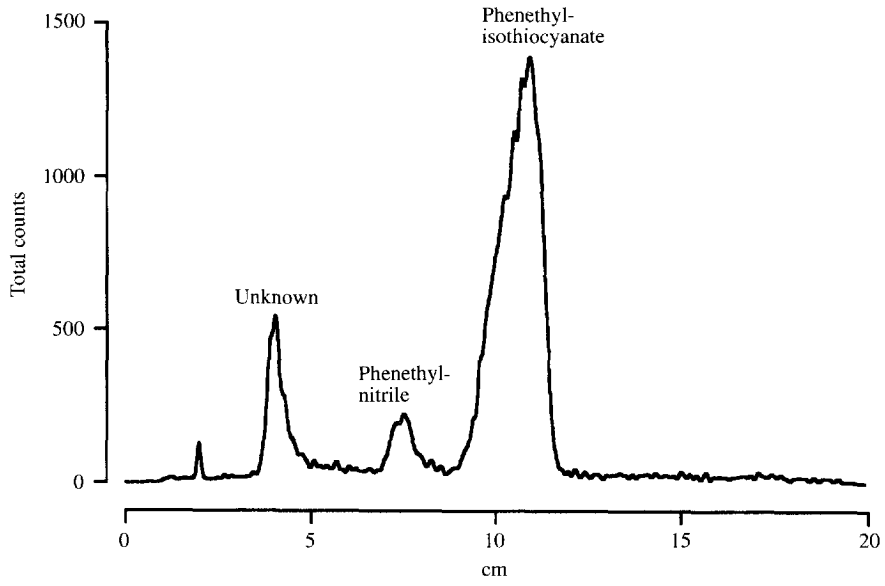

Fig. 2. Radioactive scan of TLC plate. Total counts were accumulated over 100 min.

The actual fate of the isothiocyanate in the developing plant is at this stage unknown although it seems likely that some detoxification pathways will be invoked or that these compounds can be salvaged into primary metabolism. Interestingly, a large proportion of the radiolabel could not be accounted for at day 6, suggesting that metabolism beyond the isothiocyanate/nitrile stage has occurred.

EXPERIMENTAL

Synthesis of [α-^{14}C]-*desulphophenethylglucosinolate*

Preparation of [α-^{14}CN]-3-*phenylpropionitrile* (**2**). [^{14}CN]-KCN (37 MBq, 3.6 mg) was dissolved in H_2O (0.5 ml) and added to cold KCN (73.8 mg, 1.1 mmol). H_2O was removed by azeotropic distillation with EtOH under red. pres. and finally dried *in vacuo* over P_2O_5, giving 66.6 mg material. DMSO (3.5 ml) was added, followed by phenethylbromide (172 mg, 0.93 mmol) in DMSO (0.5 ml), and the reaction mixt. was stirred under Ar at 70° for 2 hr. The cooled mixt. was poured on to H_2O (25 ml) and extracted with Et_2O (3 × 10 ml), and the combined organic layers were washed with 6 M HCl (20 ml) and H_2O (20 ml), dried over $MgSO_4$ and evapd. The residue was distilled under red. pres. (Kuhn-Roth apparatus) to give [^{14}CN]-3-phenylpropionitrile (28.5 MBq, 92.5 mg, 0.70 mmol). The yield was 76%, and the radiochemical yield 77%.

Preparation of [α-^{14}C]-3-*phenylpropanal* (**3**). [^{14}C]-3-Phenylpropionitrile (91.5 mg, 0.70 mmol) was dissolved in hexane (15 ml) and cooled to −63° ($CHCl_3$ and liquid N_2 slush). 1 M DIBAL in hexane (1.4 mmol) was added and the reaction mixt. was stirred under Ar at −63° for 30 min and at room temp. for 5 hr. $EtCO_2H$ (1 ml) was added and stirring continued for 1 hr. The mixt. was poured on to satd NH_4Cl soln (10 ml) and after 20 min 25% H_2SO_4 (5 ml) was added. The phases were sepd and the aq. phase extracted with Et_2O (3 × 10 ml). The combined organic phases were washed with brine (2 × 5 ml) and the solvents evapd. The residue was used directly in the next step without further purification.

Preparation of [α-^{14}C]-3-*phenylpropanaloxime* (**4**). A soln of $H_2NOH.HCl$ (58.4 mg, 0.84 mmol) in H_2O (1 ml) was added to the aldehyde from the previous step. Na_2CO_3 (41.1 mg, 0.39 mmol) dissolved in H_2O (2 ml) was added dropwise and the reaction mixt. stirred for 10 min at room temp. The product was extracted with Et_2O (3 × 2 ml), and the Et_2O soln was dried over $MgSO_4$, filtered and evapd. The residue was distilled under red. pres. (Kuhn-Roth apparatus) to give the oxime (57.4 mg, 0.38 mmol) as a crystalline solid. The yield from the nitrile was 55%.

Preparation of [α-^{14}C]-3-*phenyl*-1-*hydroxyamoyl-chloride-propane* (**5**). [^{14}C]-3-Phenylpropanal oxime (57.4 mg, 0.38 mmol) was dissolved in $CHCl_3$–pyridine (50:1) (0.8 ml). A soln of *N*-chlorosuccinimide (51.4 mg, 0.38 mmol) in $CHCl_3$ (1.5 ml) was added dropwise at 0° and the reaction mixt. was stirred under Ar for 2 hr at room temp. The mixt. was washed with H_2O (3 × 1 ml), dried over $MgSO_4$ and the solvent evapd. The residue was not purified, but used directly in the next step.

Preparation of [α-^{14}C]-2,3,4,6-*tetra*-O-*acetyl*-β-D-*glucopyranosyl*-2-*phenethylthiohydroximate* (**6**). 3-Phenyl-1-hydroxamoylchloride-propane was dissolved in Et_2O–CH_2Cl_2 (3.5 ml, 2:1). 2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranose (85 mg, 0.23 mmol) dissolved in $CHCl_3$ (1.2 ml) and Et_3N (0.7 mmol) were successively added, and the reaction mixt. was stirred under Ar at room temp. for 1 hr. The organic phase was washed with H_2SO_4 (2 M, 5 ml) and H_2O (2 × 5 ml) and dried over $MgSO_4$. The solvent was evapd and the residue purified on a silica gel column (hexane–EtOAc, 1:1), giving **6** (83 mg, 0.17 mmol) as a solid. The yield from the oxime was 42%. The ^{1}H NMR spectrum of the radiolabelled material was identical to that of cold synthesized material. δ_H (400 MHz; $CDCl_3$), 7.25 (5H, *m*, Ph), 5.22 (1H, *m*, pyranose), 5.05 (3H, *m*, pyranose), 4.12 (2H, *m*, pyranose), 3.68 (1H, *m*, pyranose), 2.98 (2H, *m*, $PhCH_2CH_2$), 2.83 (2H, *m*, $PhCH_2$ CH_2), 2.06, 2.03, 2.01, 1.91, (4 × 3 H, 4 × *s*, OAc).

Preparation of desulpho-[α-^{14}C]-2-*phenethylglucosinolate* (**7**). Compound **6** (20 mg, 0.039 mmol) was added to MeOH (10 ml) previously satd with NH_3 at 0°. The reaction mixt. was left overnight and evapd. The residue was purified on a silica gel column (CH_2Cl_2–MeOH, 17:3) to give the desulphoglucosinolate (9 mg, 0.026 mmol) as a glassy solid. The yield was 67% with a spec. act. of 26.6 MBq mmol^{-1}.

Analysis of glucosinolates. Water cress plants were extracted with boiling 80% aq. MeOH (50 ml) for 10 min, benzylglucosinolate (0.25 mg) was added as int. standard and the extraction repeated. The soln was evapd to a small vol. at 35°, reconstituted in H_2O (5 ml) and extracted with Et_2O (2 × 10 ml). The aq. soln was extracted with petrol (2 × 10 ml) and analysed as described in ref. [16]. Ba/Pb acetate soln (0.1 ml) was added to the extract (2 ml), which was centrifuged (2000 g). The supernatant was applied to a DEAE-A25 Sephadex ion exchange column with a bed vol. of *ca* 1 ml. The column was washed with 0.02 M NaOAc buffer (pH 5, 2 × 0.5 ml), and 75 μl sulphatase prepn was added. The column was left overnight, and desulphoglucosinolates were washed off the column with H_2O (3 × 0.5 ml) and analysed by reverse phase HPLC on a Spherisorb S5 ODS2 column.

Extraction of glucosinolates and degradation products. Cress plants were fed labelled precursor (7.1 kBq, 26.6 MBq mmol^{-1}) by placing the stems in a soln of H_2O and precursor (usually no more than 300 μl) in 1.5 ml Eppendorf tubes. Plants were kept at 23° on a 14 hr: 10 hr daylight–dark cycle in a moist chamber. Uptake of precursor soln was rapid (3–5 hr) and the tubes were kept topped up with H_2O. Plants together with 20 μl of phenethylisothiocyanate (as carrier) were placed into a pestle and liquid N_2 added and the plants

ground to a fine powder. The fine frozen powder was kept immersed in liquid N_2 and 25 ml EtOH added and the slush further ground. The freezing slush was immediately filtered *in vacuo* and the plant residue rinsed (×2) with ice-cold EtOH. The residue was immediately transferred to boiling 80% MeOH (50 ml) together with benzyl glucosinolate (0.25 mg) as int. standard for 10 min and the process repeated. The extract was combined with the initial EtOH extract and H_2O was added (100 ml) followed by CH_2Cl_2 (100 ml) and the 2 layers sepd. The aq. phase was re-extracted with a further 100 ml CH_2CH_2. The CH_2CH_2 extract was dried over $MgSO_4$ and evapd to give a residue that was reconstituted in 1 ml of CH_2CH_2. The residue was applied to silica gel plates and developed in hexane–EtOAc (4:1) and scanned on a Bioscan System 200 imaging system. Efficiency of extraction was determined by eluting the phenethylisothiocyanate from the plate together with elution of a known amount of the pure compound run on the same plate. Quantitative estimation was obtained by measuring *A* at 247 nm. The aq. extract was concn by rotary evapn (<35°) to a vol. of 5 ml and analysed for glucosinolates as before.

Protein analysis. Cress plants (3 g of tissue) were ground in NaOAc buffer (10 ml, pH 5.8, 0.1 M) containing 100 μl of phenethylisothiocyanate and centrifuged for 15 min at 15 000 *g*. The supernatant was desalted (M_r cut off 10k) against NaOAc buffer (pH 5.8, 0.01 M) and counted on a Wallac 1211 Rackbeta liquid scintillation counter.

REFERENCES

1. Underhill, E. W., *Secondary Plant Products*, 1971, **10**, 2279.
2. Rossiter, J. T., James, D. C. and Atkins, N., *Phytochemistry*, 1990, **29**, 2509.
3. Rossiter, J. T. and James, D. C., *Journal of the Chemical Society, Perkin Transactions I*, 1990, 1909.
4. MaCregor, D. I., *Canadian Journal of Plant Science*, 1988, **68**, 367.
5. Cole, R. A., *Phytochemistry*, 1978, **17**, 1563.
6. Cole, R. A., *Journal of the Science of Food and Agriculture*, 1980, **31**, 549.
7. Rosa, A. S. E., Heaney, R. K., Rego, F. C. and Fenwick, R., *Journal of the Science of Food and Agriculture*, 1994, **66**, 457.
8. Tookey, H. L. and Wolff, I. A., *Canadian Journal of Biochemistry*, 1970, **48**, 1024.
9. Gil, V. and MacLeod, A. J., *Tetrahedron*, 1979, **36**, 779.
10. Brochard, L., Joseph, B., Viaud, M. C. and Rollin, P., *Synthetic Communications*, 1994, **24**, 1403.
11. Ettlinger, M. G. and Kjaer, A., in *Recent Advances in Phytochemistry*, Vol. 1, eds T. J. Mabry, R. E. Alston and V. C. Runeckles. Appleton-Century Crofts, New York, 1968, p. 89.
12. Tang, C., Bhothipasksa, H and Frank, H., *Applied Microscopy*, 1972, **23**, 1145.
13. Bones, A. M. and Rossiter, J. T., *Physiologia Plantarum*, 1996, **97**, 194.
14. Bjorkman, R., *Phytochemistry*, 1973, **12**, 1585.
15. Cooper, R. M., Resende, M. L. V., Flood, J., Rowan, M. G., Beale, M. H. and Potter, U., *Nature*, 1996, **379**, 159.
16. Spinks, E. A., Sones, K. and Fenwick, G. R., *Fette Siefen Anstrichmittel*, 1986, **86**, 228.