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BIOSYNTHESIS OF 3-METHYLTHIOPROPYLGLUCOSINOLATE AND 3-METHYLSULFINYLPROPYLGLUCOSINOLATE IN WALLFLOWER *CHEIRANTHUS KEWENSIS**

M. D. CHISHOLM

National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan, Canada

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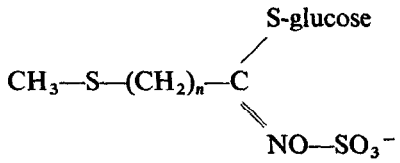
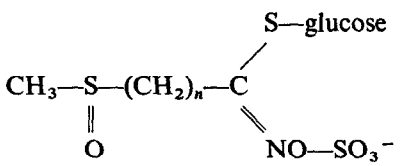
Abstract—Incorporation of DL-methionine-2-^{14}C and DL-homomethionine-2-^{14}C into 3-methylthiopropylglucosinolate and into 3-methylsulfinylpropylglucosinolate have been demonstrated using *Cheiranthus kewensis* The reduction of 3-methylsulfinylpropylglucosinolate to 3-methylthiopropylglucosinolate has also been shown The results support the following sequence of biochemical reactions Methionine → homomethionine → 3-methylthiopropylglucosinolate ⇌ 3-methylsulfinylpropylglucosinolate A method of separating the two glucosinolates on a preparative scale is described

INTRODUCTION

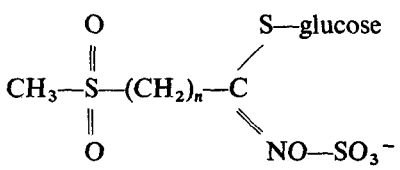
THE LARGE number of known homologues, each differing by a single methylene group, is a striking feature of the naturally occurring glucosinolates. Additionally, the oxidation level of the sulfur in the methylthio group is the only distinguishing feature between the following homologous series:



$n = 3, 4, 5$ and 6



$n = 3, 4, 5, 6, 7, 8, 9$ and 10



$n = 3$ and 4

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The homologues with ω-methylthio side chains are likely to arise biogenetically by chain extension of methionine via acetate [1,2] Subsequent oxidation of the methylthio group would produce the corresponding glucosinolate with ω-methylsulfinyl and ω-methylsulfonyl side chains Alternatively these glucosinolates could arise directly from the amino acid at the corresponding level of oxidation Isolation of the enzymes involved will likely be required before this question can be conclusively resolved

Feeding experiments using wallflower (*Cheiranthus kewensis*), a plant in which both 3-methylthiopropylglucosinolate (IV, Fig. 1) and 3-methylsulfinylpropylglucosinolate (V) occur, have provided useful information toward clarifying the biosynthesis of these glucosinolates [3] This paper presents the experimental data obtained from those experiments

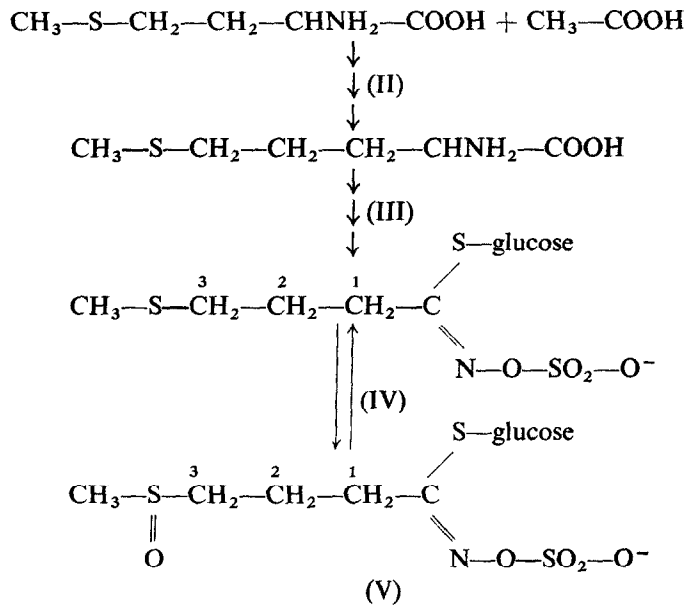

FIG 1 SCHEME PROPOSED FOR THE BIOSYNTHESIS OF 3-METHYLTHIOPROPYLGLUCOSINOLATE AND 3-METHYLSULFINYLPROPYLGLUCOSINOLATE
(II)—L-Methionine, (III)—L-homomethionine, (IV)—3-methylthiopropylglucosinolate, (V)—3-methylsulfinylpropylglucosinolate

RESULTS AND DISCUSSION

Each feeding experiment consisted of administering each of four compounds to four groups of plants (Table 1) The 3-methylthiopropylglucosinolate (IV) was quantitatively determined as the isothiocyanate in a sample of the plant extract by the method of Youngs and Wetter,[4] and the specific radioactivity was determined as described in the Experimental, using these two values, the percent incorporation was calculated

[1] E W UNDERHILL and L R WETTER, in *Biosynthesis of Aromatic Compounds* Proceedings of the 2nd meeting of the Federation of European Biochemical Societies (edited by G BILLEK), Vol 3, p 129, Pergamon Press, Oxford (1966)
[2] M G ETTLINGER and A KJAER, *Sulfur Compounds in Plants, Recent Advances in Phytochemistry*, Vol I, p 59
[3] A KJAER, *Prog Chem Org Nat Prods* **18**, 1505 (1962).
[4] C G YOUNGS and L R WETTER, *J Am Oil Chem Soc* **44**, 551 (1967)

TABLE 1. INCORPORATION OF ^{14}C LABELED COMPOUNDS INTO 3-METHYLTHIOPROPYLGLUCOSINOLATE AND 3-METHYLSULFINYLPROPYLGLUCOSINOLATE

Administered			Isolated				
			3-Methylthiopropylglucosinolate				
Compound	Specific activity mμCi/ m-mole	Total mμCi	m-mole	Specific activity mμCi/ m-mole	Total mμCi	Dilution value‡	% Incorp†
DL-Methionine-2-^{14}C	15,700	1570			not isolated		
DL-Homomethionine-2-^{14}C	16,500	1681	0 0729	347	36 2	47	2 15
3-Methylsulfinylpropyl-[^{14}C=N]glucosinolate	1539	354	0 1044	49 5	3 6	71	1·02
3-Methylsulfinylpropyl-glucosinolate-$^{14}CH_3$	1854	188	0 0666	22 1	1 47	84	0 78

Table continued on following page (p 200)

Pure crystalline potassium 3-methylsulfinylpropylglucosinolate (V) was isolated from another sample of the same plant extract and its specific radioactivity was determined The total quantity in the extract was obtained by assuming the yield of 3-methylsulfinylpropylglucosinolate (V) per g of fresh plant material would be the same as that in a parallel isolation where the isotope dilution method was used (see Experimental)

The results of a typical experiment are presented in Table 1 which shows that DL-methionine-2-^{14}C (II) and DL-homomethionine-2-^{14}C (III) were good precursors of 3-methylsulfinylpropylglucosinolate (V) and that DL-homomethionine-2-^{14}C (III) was a precursor of 3-methylthiopropylglucosinolate (IV) They also show that DL-homomethionine-2-^{14}C (III), 3-methylsulfinylpropyl[^{14}C=N]glucosinolate (V) and 3-methylsulfinylpropylglucosinolate-$^{14}CH_3$ (V) were precursors of 3-methylthiopropylglucosinolate (IV) The average yield of 3-methylsulfinylpropylglucosinolate (V) is 18 times that of 3-methylthiopropylglucosinolate (IV)

The reduction of 3-methylsulfinylpropylglucosinolate (V) to 3-methylthiopropylglucosinolate (IV) is also clearly demonstrated and is a very efficient reaction when the relative pool sizes are considered The dilution values for 3-methylsulfinylpropylglucosinolate-$^{14}CH_3$ (V) and for 3-methylsulfinylpropyl[^{14}C–N]glucosinolate (V) are approximately equal which shows that the basic skeleton of the sulfoxide remains intact during the reduction When homomethionine-2-^{14}C (III) is fed, the 3-methylthiopropylglucosinolate (IV) isolated has a higher specific radioactivity than has the 3-methylsulfinylpropylglucosinolate (V) The dilution values are 47 and 105 respectively which indicates that the thioether is formed first and then oxidized The greater per cent incorporation of homomethionine-2-^{14}C into the sulfoxide is primarily an indication of a larger pool

DL-Homomethionine-2-^{14}C (III) was more efficiently incorporated into 3-methylsulfinylpropylglucosinolate (V) than DL-methionine-2-^{14}C (II) This suggests that the chain lengthening process that has been reported in previous publications[5–7] is operating.

A number of biological systems have the ability to reduce organic sulfoxides to their corresponding thioethers. Reports of the reduction of methionine sulfoxide in cells of

[5] E. W UNDERHILL, *Can J Biochem* **46**, 401 (1968)
[6] M D CHISHOLM and L R WETTER, *Can J Biochem* **42**, 1033 (1964)
[7] M D CHISHOLM and L R WETTER, *Plant Physiol* **42**, 1726 (1967)

TABLE 1—*Continued*

Administered Compound	Isolated 3-Methylsulfinylpropylglucosinolate m-mole*	Specific activity mμCi/ m-mole	Total mμCi	Dilution value‡	% Incorp†
DL-Methionine-2-^{14}C	1 40	92 9	130	169	8 3
DL-Homomethionine-2-^{14}C	1 45	156 4	226	105	13 4
3-Methylsulfinylpropyl-[^{14}C=N]glucosinolate	1 52	232 2	352	15	—
3-Methylsulfinylpropylglucosinolate-$^{14}CH_3$	1 37	136 0	187	13 5	—

* Weight estimated by the isotope dilution method

† $\%^{14}C$ incorporated $= \dfrac{\text{total m}\mu\text{Ci in compound} \times 100}{\text{m}\mu\text{Ci administered}}$

‡ Dilution value $= \dfrac{\text{specific activity of compound fed (m}\mu\text{Ci/m-mole)}}{\text{specific activity of compound isolated (m}\mu\text{Ci/m-mole)}}$

Escherichia coli have been presented by Sourkes and Trano[8]. Stekol[9] has suggested that the rat must reduce methionine sulfoxide to methionine, since rats fed a diet in which the only sulfur amino acid was methionine sulfoxide grow as well as those fed a diet containing methionine. Black *et al*[10] has described a three enzyme system from yeast which catalyzes the specific reduction of one of the four isomers of methionine sulfoxide to the corresponding thioether, and Porque *et al*[11] have isolated a similar system from yeast in which the enzymes function as proton carriers in both sulfoxide and sulfate reduction Finally Doney and Thompson[12] have shown that *S*-methylcysteine sulfoxide and methionine sulfoxide are reduced to their corresponding thioethers in turnip leaves under anaerobic conditions, and that the same sulfoxides are reduced to thioethers under anaerobic or aerobic conditions in bean leaves.

This paper presents experimental data which show that wallflower plants can reduce 3-methylsulfinylpropylglucosinolate (V) to 3-methylthiopropylglucosinolate (IV), thus adding another class of compound to the list of sulfoxides that can be reduced to thioethers *in vivo*

EXPERIMENTAL

Cultivation of plants and administration of labelled compounds The experiments were performed using greenhouse grown plants at the flowering stage of development The radioactive compounds were administered through the cut ends of the stocks as described previously[13] Approximately 100 μmole of radioactive compound was administered for every 75 g of plant material The metabolic period was 24 hr under 100 lx of continuous light

[8] J A SOURKES and W TRANO, *Arch Biochem Biophys* **42**, 321 (1953)
[9] J A STEKOL, in *Symposium on Amino Acid Metabolism*, (edited by W D MCELROY and B GLASS), p 509, The John Hopkins Press, Baltimore (1955)
[10] S BLACK, E M HARTE, B HUDSON and L WORTOFSKY, *J Biol Chem* **235**, 2910 (1960)
[11] P G PORQUE, A BLADESTEN and P RICHARDS, *J Biol Chem* **245**, 2371 (1970)
[12] R C DONEY and J F THOMPSON, *Biochem Biophys Acta* **124**, 39 (1966)
[13] E W UNDERHILL, M D CHISHOLM and L R WETTER, *Can J Biochem Physiol* **40**, 1505 (1962)

Radioactive materials DL-Methionine-2-^{14}C (II) was obtained from Volk Chemical Company DL-Homomethionine-2-^{14}C (III) was obtained by synthesis[14] 3-Methylthiopropyl[^{14}C=N]glucosinolate (IV), 3-methylsulfinylpropyl[^{14}C=N]glucosinolate (V), and 3-methylsulfinylpropylglucosinolate-$^{14}CH_3$ (V) were biosynthesized by wallflower plants and isolated by the methods described below

Extraction and isolation of the two glucosinolates The plants were extracted by grinding with hot absolute methanol in a Waring Blendor After filtering, the fibrous residue was re-extracted with hot 80% methanol (v/v) The methanol in the combined filtrates was removed by evaporation *in vacuo* The solids were removed by filtering the aqueous solution through Celite and a sample was saved for the determination of total glucosinolates The remaining filtrate was passed through a cation exchange column of Amberlite IR 120 (H^+) resin (2 × 14 cm), and the effluent was neutralized to pH 7 0 with 1 N KOH The glucosinolates were isolated by passing the aqueous extract through a column of Amberlite IR 4B (Cl^-) resin (2 × 16 cm) After washing the resin with water (100 ml) the glucosinolates were eluted with 1 l of 0 1 M K_2SO_4 The eluate was evaporated to dryness and the glucosinolates were extracted from the residual inorganic salts with 4 × 50 ml of hot 80% ethanol

Additional purification was obtained by passing the 80% ethanol extract through a column of basic aluminium oxide pH 10 0–10 5 After washing with 80% ethanol (100 ml) the glucosinolates were eluted together with 60% ethanol (200 ml) TLC on Silica Gel G developed with MeCoEt–EtOH–H_2O (45 5 10)[15] showed 3-methylthiopropylglucosinolate (IV) and 3-methylsulfinylpropylglucosinolate (V) to be the major products The potassium salt of 3-methylsulfinylpropylglucosinolate (V) crystallized from 80% ethanol and after 2 recrystallizations from 90% ethanol had a constant specific radioactivity, m p 141–143° Found C, 27 72, H 4 91 Calc for $C_{11}H_{20}O_{10}N_3K$ $1H_2O$ C, 27 58, H,4 63 The NMR spectrum of the compound at 100 M Hz in D_2O using tetramethylsilane as an external reference showed signals at σ 3 22 (singlet, CH_3=O—S—), 2 66 (quartet, J = 7 5 Hz, C-2 protons), 3 41 (triplet, J = 7 5 Hz, C-1 and C-3 protons, see Fig 1 for numbering system) Appropriate signals for the glucose protons were also present The assignments were supported by integration

3-Methylthiopropylglucosinolate (IV) in the mother liquors from the above crystallization was separated from the residual 3-methylsulfinylpropylglucosinolate (V) by elution from a column of basic aluminum oxide (2 × 21 cm, 50 g alumina) with a linear gradient of 500 ml of 80% acetone to 500 ml of 20% acetone[16] The mother liquors were evaporated to dryness and the residue was taken up in 1 ml of water and then 4 ml of acetone was added This solution was pipetted onto the column and washed in with 2 × 2 ml of 80% acetone The linear gradient was started and 10 ml fractions were taken fraction numbers 25–27 contained 3-methylthiopropylglucosinolate (IV), numbers 28–32 3-methylthiopropylglucosinolate (IV) and 3-methylsulfinylpropylglucosinolate (V), and numbers 33–50 3-methylsulfinylpropylglucosinolate (V) Fraction numbers 28–32 were re-run using the procedure just described to obtain additional 3-methylthiopropylglucosinolate (IV) All fractions containing the 3-methylthiopropylglucosinolate (IV) were combined and the solvent was removed *in vacuo* Identification was established by TLC[15] by paper chromatography[17] and by GLC of the 3-methylthiopropyl isothiocyanate as described below The NMR spectrum in D_2O showed signals at σ 2 62 (singlet, CH_3–S–), 3 10 and 3 30 (triplets, J = 7 5 Hz, C-1 and C-3 protons), and at 2 46 (quartet, J = 7 5 Hz, C-2 protons) Attempts to crystallize this glucosinolate were unsuccessful The total 3-methylthiopropylglucosinolate (IV) isolated was estimated by treating a sample with thioglucosidase (E C 3.2 3 1, thioglucoside glucohydrolase) and then determining the glucose release by glucose oxidase 'glucostat' method[18] Another sample of the same material was used to determine its total radioactivity A specific radioactivity was calculated from the two values

GLC analysis of 3-methylthiopropyl isothiocyanate The GLC method of Youngs and Wetter[4] was used to quantitatively assay a 5 per cent sample of the extract A Hewlett Packard gas chromatograph, model 5754 with 5 ft × ⅛ in o d stainless steel column packed with 20% FFAP on acid washed, DMCS-treated, Chromosorb W, 60–70 mesh, helium flow 30 ml/min, hydrogen 20 ml/min; injector and detector temperatures were maintained at 250° At an oven temperature of 210°, an authentic sample of 3-methylthiopropyl isothiocyanate synthesized by the method of Kjaer[17] had a retention time of 0 51 relative to β-phenylethyl isothiocyanate at 1 00 β-Phenylethyl isothiocyanate was used as an internal standard in all the subsequent GLC analyses

Isotope dilution method The GLC method[4] could not be used to measure the total quantity of 3-methylsulfinylpropylglucosinolate because the 3-methylsulfinylpropyl isothiocyanate produced by thioglucosidase hydrolysis of this glucosinolate did not give quantitative yields A small quantity of pure crystalline 3-methylsulfinylpropylglucosinolate of known specific radioactivity was added to each of five nonradioactive extracts from a known weight of plant material The glucosinolate was isolated and purified and its specific activity

[14] A Kjaer and S Wagner, *Acta Chem. Scand* **9**, 721 (1955)
[15] M Matsuo, *J Chromatog* **49**, 323 (1970)
[16] R M Bock and N Ling, *Analyt Chem* **26**, 1543 (1954)
[17] A Kjaer, *Acta Chem Scand* **9**, 1143 (1955)
[18] E W Underhill and L R Wetter, *Plant Physiol* **44**, 584 (1969)

was determined The total amount of glucoside in the original extract was calculated by the dilution method, the weight ranged from 8 0 to 9 5 mg per g of fresh plant material The average for the five isolations was 8 6 mg per g of fresh weight This value was used to calculate the total weight of 3-methylsulfinylpropylglucosinolate (V) in the plants fed In practice only half of this amount of pure crystalline material could be isolated

Isotope analyses Radioactive samples were assayed in a liquid scintillation spectrometer (Nuclear-Chicago Mark I) using a modified Toluene/Triton X-100 liquid scintillant solution[19] in which the PPO and POPOP were replaced by 0 5% (w/v) butyl-PBD (Beckman Instruments, Fullerton, California) Radiochemical purity was secured by radioautography of thin layer and paper chromatograms

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[19] J C TURNER, *Intern J Appl Radiat Isotopes* **19**, 557 (1968)

Key Word Index—Cheiranthus kewensis, Cruciferae, glucosinolate, biosynthesis, 3-methylthiopropylglucosinolate, 3-methylsulfinylpropylglucosinolate