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BIOSYNTHESIS OF ALLYLGLUCOSINOLATE AND 3-METHYLTHIOPROPYLGLUCOSINOLATE IN HORSERADISH, *ARMORACIA LAPATHIFOLIA**

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Abstract—3-Methylthiopropylglucosinolate (V) has been identified as a naturally occurring glucosinolate in *Armoracia lapathifolia* Gilib The biosynthesis of allylglucosinolate (VII) and 3-methylthiopropylglucosinolate (V) have been studied The data show that 4-methylthiobutyrothiohydroximate (III), desulfo-3-methylthiopropylglucosinolate (IV), 3-methylthiopropylglucosinolate (V), and 3-methylsulfinylpropylglucosinolate (VI) are efficient precursors of allylglucosinolate (VII) They also show that 3-methylsulfinylpropylglucosinolate (VI) and homomethionine (I) are precursors of 3-methylthiopropylglucosinolate (V) A biosynthetic scheme is proposed.

INTRODUCTION

GENERALLY the aglycones of the glucosinolates are derived from α-amino acids with the corresponding molecular structure [1,2]

In the biosynthesis of benzylglucosinolate from phenylalanine, Underhill and Wetter[3] demonstrated that phenylacetothiohydroximate and desulfobenzylglucosinolate are intermediates on the pathway, it has previously been shown that phenylacetoaldoxime is on the same pathway [4–6] Matsuo and Underhill[7] have isolated an enzyme from *Tropaeolum majus* L that catalyzes the transfer of glucose from uridine diphosphate glucose to phenylacetothiohydroximate They have also demonstrated that a similar enzyme is present in cell free extracts of other glucosinolate bearing plants including *Armoracia lapathifolia* Gilib [8]

In the biosynthesis of allylglucosinolate (VII, see Fig 1) from homomethionine (I) in *A. lapathifolia*,[9–11] Matsuo[12] demonstrated that 4-methylthiobutyraldoxime (II) is an intermediate on the pathway; he[13] also proposed a complete biosynthetic scheme The

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experimental data in this report show that 4-methylthiobutyrothiohydroximate (III), desulfo-3-methylthiopropylglucosinolate (IV), 3-methylthiopropylglucosinolate (V) and 3-methylsulfinylpropylglucosinolate (VI) are efficient precursors of allylglucosinolate (VII). Methylthiopropylglucosinolate (V) which has not been previously reported in this plant has been identified and its precursors have been established

TABLE 1 COMPARISON OF ^{14}C LABELLED COMPOUNDS AS PRECURSORS OF ALLYLGLUCOSINOLATE

Administered			Allylglucosinolate isolated			
Compound	Total mμCi	Specific activity mμCi/ m-mole	Total mμCi	Specific activity mμCi/ m-mole	Dilution value	% Incorp
DL-Homomethionine-2-^{14}C	3210	162,000	1100 0	799 0	203	34 3
4-methylthiobutyrothiohydroximate-1-^{14}C	644	41,400	323 0	249 5	166	50 2
Desulfo-3-methylthiopropyl[^{14}C=N] glucosinolate	644	41,400	408 0	347 0	199	63 5
3-Methylthiopropyl[^{14}C=N]glucosinolate	88 5	4490	64 4	44 1	102	72 7
3-Methylsulfinylpropyl[^{14}C=N]glucosinolate	73 7	3740	51 5	36 8	101	69 9

Metabolism period—24 hr

$$\%\ ^{14}C \text{ incorp} = \frac{\text{Total m}\mu\text{Ci in compound isolated} \times 100}{\text{m}\mu\text{Ci administered}}.$$

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

RESULTS AND DISCUSSION

The tracer results in Table 1 were obtained from allylglucosinolate (VII) isolated from horseradish plants, after they had metabolized the radioactive compounds for 24 hr. The percent incorporations of each succeeding compound in Table 1 increase and the dilu-

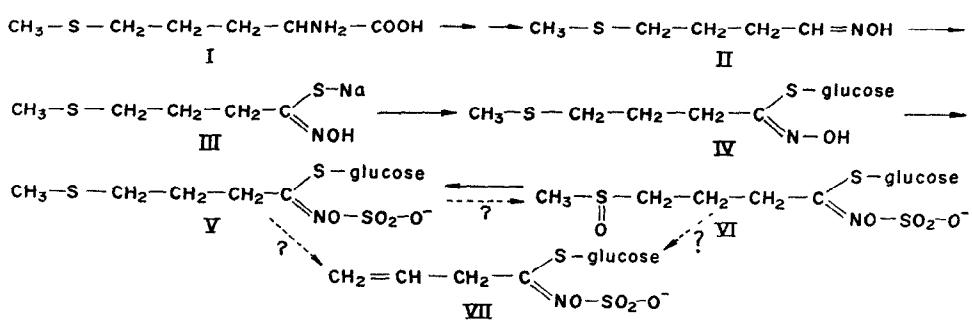

FIG 1 PROPOSED PATHWAY FOR THE BIOSYNTHESIS OF ALLYLGLUCOSINOLATE
(I)—Homomethionine, (II)—4-methylthiobutyraldoxime; (III)—4-methylthiobutyrothiohydroximate, (IV)—desulfo-3-methylthiopropylglucosinolate, (V)—3-methylthiopropylglucosinolate, (VI) 3-methylsulfinylpropylglucosinolate, (VII)—allylglucosinolate

tion values decrease and, therefore, support the biosynthetic scheme proposed for allylglucosinolate (VII) in Fig 1. The incorporation of 3-methylthiopropylglucosinolate (V) and 3-methylsulfinylpropylglucosinolate (VI) are almost identical; consequently it is impossible to decide which of these compounds is the immediate precursor

3-Methylthiopropylglucosinolate (V) has not been reported previously in horseradish plants, so another experiment was performed to study its biosynthesis and to see if data could be obtained that would fix its position in the biosynthetic sequence leading to allylglucosinolate (VII) Homomethionine-2-^{14}C (I) and 3-methylsulfinylpropyl[^{14}C=N]glucosinolate (VI) were administered to two lots of leaves After a 5 hr metabolic period, the plants were extracted and the isothiocyanates of both 3-methylthiopropylglucosinolate (V) and of allylglucosinolate (VII) were isolated by the procedure described. The results are in Table 2. Making no allowance for losses during isolation we have estimated that 100 g of fresh plant material contains approximately 30 μg of 3-methylthiopropylglucosinolate (V). The small amount of this glucosinolate in the plant prevented a quantitative assay Therefore, a per cent incorporation value of the compound administered is not reported, and the dilution value is used as a measure of precursor efficiency

Chisholm and Wetter,[10] and Matsuo and Yamazaki[11,14] working with horseradish leaves demonstrated that allylglycine, the amino acid structurally similar to the aglycone of allylglucosinolate (VII) was not a precursor, nor was 2-amino-5-hydroxyvaleric acid. However, homomethionine (I) was an excellent precursor. On the basis of these findings, we[10,11,13] proposed that one of: 3-methylthiopropylglucosinolate (V), 3-methylsulfinylpropylgluco-

TABLE 2. COMPARISON ^{14}C LABELLED COMPOUNDS AS PRECURSORS OF ALLYLGLUCOSINOLATE AND 3-METHYLTHIOPROPYLGLUCOSINOLATE

	Administered		Isolated					
			Allylglucosinolate*				3-Methylthiopropyl-glucosinolate*	
	Total mμCi	Specific activity mμCi/m-mole	Total mμCi	Specific activity mμCi/m-mole	Dilution value	% Incorp	Specific activity mμCi/m-mole	Dilution value
DL-Homomethionine-2-^{14}C	1262	128,200	417 0	774 0	166	33 0	22,100	5 8
3-Methylsulfinylpropyl[^{14}C=N]-glucosinolate	37	3690	15 5	25 4	145	41 9	180	20 5

* Isolated as the isothiocyanate, metabolic period—5 hr

$$\% \ ^{14}C \text{ incorp} = \frac{\text{Total m}\mu\text{Ci in compound isolated} \times 100}{\text{m}\mu\text{Ci administered}}$$

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

[14] M MATSUO and M YAMAZAKI, *Chem Pharm Bull* **16**, 1034 (1968)

sinolate (VI) or 3-methylsulfonylpropylglucosinolate, by elimination of methanethiol or a comparable compound at the appropriate level of oxidation, would produce the terminal olefin of allylglucosinolate (VII) All of the foregoing glucosinolates occur naturally in other plants

After treating the aqueous horseradish extract with thioglucosidase (E C 3 2 3 1, thioglucoside glucohydrolase) we isolated and purified 3-methylthiopropyl isothiocyanate by GLC We propose that 3-methylthiopropylglucosinolate (V) is present in horseradish on the reasonable assumption that the 3-methylthiopropyl isothiocyanate isolated is the enzymatic degradation production of the glucosinolate

The combined data presented in Tables 1 and 2 support the scheme proposed in Fig 1 for the biosynthesis of allylglucosinolate (VII) The increasing per cent conversion of homomethionine (I), 4-methylthiobutyrothiohydroximate (III) and desulfo-3-methylthiopropylglucosinolate (IV) to allylglucosinolate (VII) are comparable to those observed for the biosynthesis of another glucosinolate in another plant [3] However, the final step in which the methylthio group is eliminated to form the terminal olefin of allylglucosinolate (VII) is not clear The data of Table 1 show that 3-methylthiopropylglucosinolate (V) and 3-methylsulfinylpropylglucosinolate (VI) are converted with equal efficiency to allylglucosinolate (VII) The results presented in Table 2 show that the plant has a very efficient mechanism with which it can reduce the sulfoxide to the thioether We have not shown, however, that 3-methylsulfinylpropylglucosinolate (VI) occurs naturally in this plant, as the GLC method used to obtain the data for 3-methylthiopropyl isothiocyanate retained most of the sulfoxide on the column When an authentic sample of 3-methylsulfinylpropyl isothiocyanate was subjected to GLC (UC-W98 column) an unidentified unsymmetrical peak was obtained From the amount of aglycone injected into the gas chromatograph and the peak area of the eluted material the detector response was disproportionately low We were therefore unable to employ GLC as a means of detecting the aglycone of this glucosinolate

It is clear from the dilution values of Table 2 that homomethionine (I) and 3-methylsulfinylpropylglucosinolate (VI) are converted to allylglucosinolate (VII) with about equal efficiency But when compared as precursors of 3-methylthiopropylglucosinolate (V), homomethionine (I) is a three fold more efficient precursor than is 3-methylsulfinylpropylglucosinolate (VI) One might speculate that a relatively large pool of the 3-methylsulfinylpropylglucosinolate (VI) could be present in the plant which would account for this result, and consequently could be interpreted to mean that the sulfoxide formed from the thioether is the immediate precursor of allylglucosinolate (VII).

EXPERIMENTAL

Cultivation of plants The experiments were performed on mature leaves from 2- to 3-month-old horseradish plants The radioactive compounds were administered to the leaves through the petioles [15] Approximately 15 μmole of radioactive compound was administered for every 100 g of fresh leaf, and the metabolic period was either 5 or 24 hr under continuous light

Identification of 3-methylthiopropylglucosinolate (V) The presence of 3-methylthiopropylglucosinolate (V) in horseradish plants has not been reported, so an experiment was designed to detect and isolate its isothiocyanate Horseradish leaves (100 g) were extracted by grinding with 400 ml of hot absolute methanol in a Waring Blendor and filtered The fibrous residue was re-extracted with 400 ml of hot 80% methanol (v/v) The methanol in the combined filtrates was removed by evaporation *in vacuo*, the residue was dissolved in water and filtered through Celite After an extraction with CH_2Cl_2, the aqueous solution was incubated with thioglucosidase in 0 1 M phosphate buffer (pH 7 0) for 24 hr, and the isothiocyanates were steam distilled After recovery by continuous CH_2Cl_2 extraction, the 3-methylthiopropyl isothiocyanate was

[15] E W. UNDERHILL, M D CHISHOLM and L R WETTER, *Can J Biochem Physiol* **40**, 1505 (1962)

separated from the allyl isothiocyanate by GLC and identified on both UC-W98 and 20% FFAP columns by comparing with an authentic sample which was synthesized by the method of Kjaer [16]

Isolation of products A chlorophyll free aqueous solution, prepared from a hot 80% methanol extract of macerated plants, was used as the starting material in each of the two isolation methods In the first method the total allylglucosinolate (VII) was determined in an aliquot of the extract by a modified method of Wetter [17] From the remainder allylglucosinolate (VII) was recovered by ion exchange on a column of Dowex 2 × 8 (Cl^-) The 0 1 M K_2SO_4 eluate from this column was evaporated to dryness and the glucosinolates were extracted from the residue with hot 80% ethanol Additional purification was obtained by adsorbing the glucosinolates from the 80% ethanol onto a column of basic aluminum oxide and then eluting them with 60% ethanol [18] The product, potassium allylglucosinolate (VII), was recrystallized to a constant specific radioactivity

In the second method allyl isothiocyanate and 3-methylthiopropyl isothiocyanate were released from their glucosinolates by hydrolysis with thioglucosidase. The GLC method of Youngs and Wetter[19] was used to assay a 5% sample of the extract The assay was performed on 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 130° allyl isothiocyanate had a retention time of 0 85 relative to an internal standard of *n*-butyl isothiocyanate at 1 00 at 210° 3-methylthiopropyl isothiocyanate had a retention time of 0 51 relative to an internal standard of β-phenylethyl isothiocyanate at 1 00

From the remaining 95 per cent of the hydrolyzed extract, purified isothiocyanates were prepared for counting A steam distillate was continuously extracted with CH_2Cl_2 After drying over anydrous Na_2SO_4, the CH_2Cl_2 was removed by evaporation Allyl isothiocyanate was separated from 3-methylthiopropyl isothiocyanate using a 6 ft × ⅛ in o d stainless-steel column packed with 10% silicone rubber UC-W98 on Diatoport S, 80–100 mesh (Hewlett Packard), operated at 170°, and equipped with a 10 1 steam splitter Each isothiocyanate was collected by condensation in a glass tube, and the purity of the collected fractions was checked by injecting an aliquot onto the UC-W98 and the FFAP columns The amount collected was assayed by GLC (internal standard method), its radioactivity was determined, and specific activity was calculated

Radioactive compounds DL-Homomethionine-2-^{14}C (I) was synthesized by the method of Kjaer and Wagner [20] 4-Methylthiobutyrothiohydroximate-1-^{14}C (III) (sodium salt) was prepared by the method of Matsuo and Underhill [8] Desulfo-3-methylthiopropyl[^{14}C=N]glucosinolate was prepared by incubating 4-methylthiobutyrothiohydroximate-1-^{14}C (III) 9 5 mg (50 μmole, 41 4 μCi/mM) and uridine diphosphate glucose 32 0 mg (50 μmole) in 1 5 ml of 0 1 M tris-HCl buffer (pH 7 4) containing 0 05 M β-mercaptoethanol for 2 5 hr at 30° with a crude glucosyltransferase [7] After incubation, 3 ml of methanol was added to the reaction mixture and the precipitate was removed by centrifugation The supernatant was concentrated *in vacuo* and the residue was extracted with a small amount of methanol The methanol extract was streaked onto a sheet of chromatographic paper (Whatman No 1) and after developing with *n*-BuOH-HOAc-H_2O (4 1 1 8) a radioactive band corresponding to desulfo-3-methylthiopropylglucosinolate was cut out and extracted with methanol After removal of the methanol the desulfo-3-methylthiopropyl[^{14}C=N]glucosinolate (IV) 5 08 mg (15 5 μmole, 0 644 μCi) was obtained Yield 31 per cent

3-Methylthiopropyl[^{14}C=N]glucosinolate (V) was isolated from an extract of *Cheiranthus kewensis* that had been fed DL-homomethionine-2-^{14}C (I) as described earlier,[18] and 3-methylsulfinylpropyl[^{14}C=N]glucosinolate (VI) was isolated by the same method from another feeding experiment

Isotope analyses Radioactive samples were assayed as described earlier [18]

Acknowledgements—The authors are grateful to Mr J Dyck for ^{14}Canalysis, and to Dr L R Wetter for helpful discussion

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Key Word Index—Armoracia lapathifolia, Cruciferae, glucosinolates, biosynthesis, allylglocosinolate, 3-methylthiopropylglucosinolate.