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Synthesis of Glucosinolate Precursors and Investigations into the Biosynthesis of Phenylalkyl- and Methylthioalkylglucosinolates*

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The alkenyl and aromatic glucosinolates in oilseed rape (*Brassica napus*) are biosynthesized from chain-extended homologues of protein amino acids, including methionine and phenylalanine. Homologues of these two amino acids, homophenylalanine (2-amino-4-phenylbutyric acid) and dihomomethionine (2-amino-6-methylthiohexanoic acid) were synthesized both with and without a 1-^{14}C label. Microsomal preparations from oilseed rape leaves were shown to contain enzyme systems which metabolize these compounds, with loss of $^{14}CO_2$, and produce the aldoxime intermediates possible in the biosynthetic pathway utilizing homophenylalanine. These were characterized by comparison with authenticated synthetic compounds. Potential intermediates on the pathway between homophenylalanine and its corresponding aldoxime, the *N*-hydroxyamino- and the oximino acids, were synthesized and their possible role in the pathway investigated.

Secondary metabolism in plants can yield compounds active in defense against pests and pathogens which may be exploited by plant molecular biology for crop plant protection (Hallahan *et al.*, 1992). Various plants, but particularly those in the family Cruciferae (= Brassicaceae) produce glucosinolates (I). These, and the associated organic isothiocyanate (II) formed by thioglucosidase (myrosinase) enzyme action released on plant damage, act potently against unadapted invading organisms (Vaughan *et al.*, 1976; Fenwick *et al.*, 1983) (Scheme I). To minimize pesticide use on arable crop crucifers, mainly oilseed rape *Brassica napus*, this natural defensive mechanism needs to be enhanced, although for commercial considerations, the seed glucosinolate content should be low (Dawson *et al.*, 1989). Furthermore, organisms adapted to feed on crucifers employ glucosinolates and their catabolites as semiochemical cues in locating and colonizing these plants. Coleopterous pests of *B. napus*, the weevil *Ceutorhynchus assimilis* and the chrysomelid beetle *Psylliodes chrysocephala*, are particularly well adapted to employ the alkenyl homologues, 3-butenyl and 4-pentenyl isothiocyanates (Blight *et al.*, 1989, 1992) as attractants. Thus, any molecular genetic modification to enhance glucosinolate production may need to be directed away from the corresponding alkenyl glucosinolates and toward aromatic compounds (Dawson *et al.*, 1989), bearing in mind that such compounds may act as feeding stimulants for adapted herbivores (Traynier and Truscott, 1991; Renwick *et al.*, 1992). This molecular genetic approach may be facilitated by studies on the crucifer *Arabidopsis thaliana*, which has a relatively smaller and thereby more accessible genome than *Brassica* spp, but which produces a large range of glucosinolates.

To ensure that genetic modification is rationally directed, the biosynthetic pathways to the aromatic and alkenyl glucosinolates must be fully understood. It has been established that glucosinolates are derived from amino acids, or chain-extended amino acids, and that aldoximes form an intermediate on this pathway. The conversion from amino acid to aldoxime occurs in the microsomal fraction of crucifers (Larsen, 1981; Chapple *et al.*, 1990) and, for many glucosinolates, chain extension of normal protein amino acids to the homoamino acid (III) (Haughn *et al.*, 1991) is the first step on the biosynthetic pathway. Earlier proposals for the biosynthesis of glucosinolates from amino acids or the homoamino acids (Larsen, 1981) may be modified by recent advances in understanding the route to the biosynthetically related cyanogenic glycosides (Halkier *et al.*, 1991; Koch *et al.*, 1992), *e.g.* to the aldoxime (IV) (Scheme I). Formation of the aldoxime would then be followed by conversion to the thiohydroximic acid (V) and glycosylation and sulfation to give the glucosinolate (I) (Jain *et al.*, 1989). The alkenylglucosinolates (*e.g.* Ic) are then formed after another step involving the elimination of methanethiol, most likely as an oxidized product, from the ω-methylthioalkylglucosinolates (*e.g.* Ib) (Glover *et al.*, 1988) or the corresponding sulfoxides or sulfones.

In this study, synthesis of putative precursors and feeding studies with the 1-^{14}C-radiolabeled compounds using the microsomal fraction of *B. napus* were made using a representative of the aromatic and the alkenylglucosinolates, homophenylalanine (2-amino-4-phenylbutyric acid) (IIIa) and dihomomethionine (2-amino-6-methylthiohexanoic acid) (IIIb). In addition, other intermediate precursors, *N*-hydroxyhomophenylalanine (2-hydroxylimino-4-phenylbutyric acid) (VIa) and 2-oximino-4-phenylbutyric acid (VIIa) for the aromatic series have been synthesized for examination of their possible role in this pathway. Another potential intermediate found in the cyanogenic glycoside pathway, the nitroalkane (IXa) has been synthesized by oxidation of the aldoxime (IVa) (Ballini *et al.*, 1992), and its presence has been searched for in microsomal preparations known to produce the aldoxime.

EXPERIMENTAL PROCEDURES

NMR spectra were recorded in either deuterochloroform ($CDCl_3$), d_6-acetone or d_6-dimethyl sulfoxide with tetrameth-

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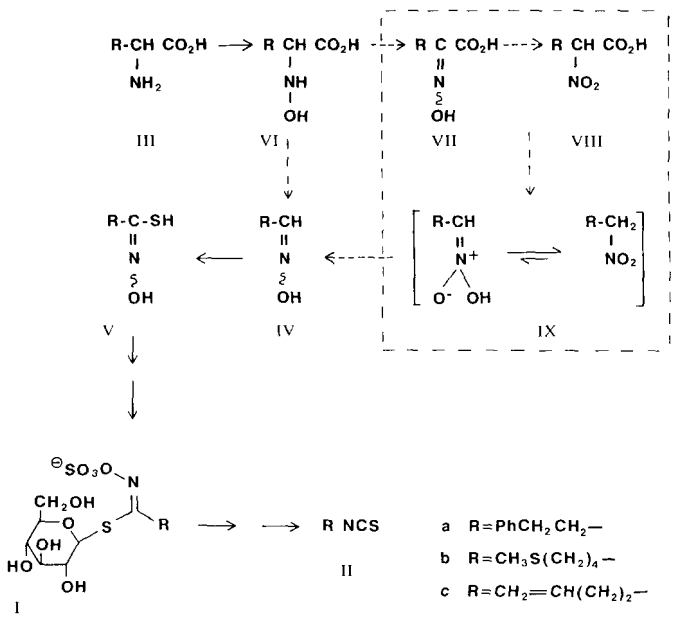

SCHEME I. Proposed glucosinolate biosynthetic pathway.

ylsilane as an internal standard (δ = 0.0 parts/million) on a JEOL GNX 400 spectrometer at 400 MHz for ^{1}H spectra and at 100 MHz for ^{13}C spectra.

Gas Chromatography (GC)[1]

HP5890, 50 m, 0.32 mm inner diameter, 0.25 μm HP-1, temperature programmed on-column injector (3 °C above column temperature), 40 °C for 1 min, 10 °C/min to 250 °C isothermal for 25 min, flame ionization detector.

Gas Chromatography-Mass Spectroscopy (GC-MS)

GC: HP5890, 30 m, 0.32 mm inner diameter, 0.25 μm DB-1 (J. & W. Scientific, Folsom), cold on-column injector, 30 °C for 5 min, 5 °C/min to 250 °C, isothermal.

MS: directly coupled 70–250 (VG Analytical, Manchester) 70 eV, 250 °C. Melting points are uncorrected.

Chemical Synthesis

DL-Homophenylalanine (IIIa)—Potassium cyanide (65 mg, 1.0 mmol), ammonium chloride (51 mg, 1.0 mmol), and ammonia (1.0 ml, specific gravity 0.88) were added to a mixture of hydrocinnamaldehyde (107 mg, 1.0 mmol) in ethanol (20 ml) and water (5 ml) and stirred overnight at room temperature. The mixture was partitioned between diethyl ether (50 ml) and water (30 ml), and the organic layer was extracted with hydrochloric acid (50 ml, 0.1 N). The acid layer was basified with sodium hydroxide solution (50 ml, 0.2 N) and re-extracted with diethyl ether (2 × 100 ml). The ether layers were combined, dried, and concentrated *in vacuo*. The residue was treated with concentrated hydrochloric acid (3 ml) and left at room temperature for 3 days. Addition of water (10 ml) was followed by reflux for 5 h, and then concentration *in vacuo*. The residue was dissolved in sodium hydroxide solution (1.0 ml, 1.0 N) and applied to an ion-exchange resin (Amberlite CG-400, -OH form, 5 g). This was washed with water (100 ml) and then the product eluted with hydrochloric acid (25 ml, 1.0 N). The acid eluate was concentrated to give the product as its hydrochloride (48 mg, 28%).

NMR, in d_6-dimethyl sulfoxide: ^{1}H: δ = 8.46(br s, 3H), 7.22–7.33(m, 5H), 3.90(br s, 2H), 3.70(br s, 1H), 2.75(m, 1H), 2.66(m, 1H), 2.08(br s, 2H). ^{13}C: δ = 170.9, 140.4, 128.6, 128.3, 126.3, 51.6, 31.9, 30.4.

DL-[1-^{14}C]Homophenylalanine (IIIa)—In the same manner as DL-homophenylalanine. Hydrocinnamaldehyde (3.13 mg, 0.023 mmol) in ethanol (580 μl), ammonium chloride solution (120 μl, 10.5 mg/ml), potassium cyanide (800 MBq/mmol, 1.50 mg, 0.023 mmol) (Sigma) in water (200 μl), and ammonia (120 μl) were stirred together for 2 days. Partitioning, extraction of the amino-nitrile into acid, re-extraction after aqueous base treatment, followed by acid hydrolysis (all as above) gave the product. This was purified using an ion-exchange resin (as above) to give a radiochemical yield of 6.0 MBq (32%).

3-Phenylpropanaldoxime (IVa)—Hydroxylamine hydrochloride (7.63 g, 0.11 mol) was added over 0.25 h to a mixture of hydrocinnamaldehyde (13.40 g, 0.10 mol) and sodium bicarbonate (8.5 g, 0.10 mol) in water (100 ml) and ethanol (100 ml). The mixture was refluxed for 2.5 h, and then the ethanol was removed *in vacuo* to precipitate the crude aldoxime. This was filtered and dried (12.1 g, 81%). Recrystallization from diethyl ether/hexane gave a pure mixture of the (*E*)- and (*Z*)-isomers in a 3:2 ratio. Further slow recrystallization (over 3 days) from diethyl ether/hexane gave exclusively the (*Z*)-isomer (melting point 91–3 °C). NMR in d_6-acetone: (*E*)-isomer: ^{1}H: δ = 7.39(t, 1H), 7.16–7.31(m, 5H), 2.79(t, 2H), 2.45(m, 2H). ^{13}C: δ = 150.0, 142.1, 129.2, 129.1, 126.8, 33.5, 32.0. (*Z*)-isomer, ^{1}H: δ = 7.16–7.31(m, 5H), 6.67(t, 1H), 2.81(t, 2H), 2.65(m, 2H). ^{13}C: δ = 150.7, 141.2, 129.2, 129.1, 126.8, 32.7, 27.2. MS, see Fig. 1.

Phenethylmalonic Acid—Potassium *t*-butoxide (11.2 g, 0.10 mol) was added to a solution of diethyl malonate (16.0 g, 0.10 mol) in dry glyme (200 ml). The mixture was stirred at room temperature for 0.5 h, then phenethyl bromide was added, and the mixture refluxed for 1.0 h. After cooling, the solvent was removed *in vacuo*, sodium hydroxide (20 g, 0.5 mol) in ethanol (100 ml), and water (100 ml) was added and the reflux was continued for 2.0 h. The mixture was cooled, the ethanol removed *in vacuo*, and the residue extracted with diethyl ether (100 ml). The aqueous phase was then cooled with ice and acidified to pH 2 with concentrated hydrochloric acid and extracted with ethyl acetate (2 × 100 ml). The combined ethyl acetate extracts were washed with water, saturated brine, dried, and concentrated. The solid residue was recrystallized from benzene (melting point 125–7 °C, 10.8 g, 52%). NMR in d_6-dimethyl sulfoxide: ^{1}H: δ = 12.76(br s, 2H), 7.18–7.31(m, 5H), 3.20(t, 1H), 2.59(t, 2H), 2.01(m, 2H). ^{13}C: δ = 170.6, 140.9, 128.3, 128.2, 125.9, 50.9, 32.6, 30.2.

2-Oximino-4-phenylbutyric acid (VIIa)—Phenethylmalonic acid (5.2 g, 0.025 mol) and *n*-propylnitrite (2.5 g, 0.028 mol) in glyme was cooled in an ice/salt bath to −10 °C and treated with hydrogen chloride gas until the solution became brownish-yellow. The mixture was then left at room temperature for 2 days, concentrated *in vacuo*, and then the residue recrystallized as a single isomer from benzene, 10% ethyl acetate (melting point 150–2 °C, 3.8 g, 80%). NMR in d_6-dimethyl sulfoxide: ^{1}H: δ = 12.70(br s, 1H), 12.20(br s, 1H), 7.14–7.28(m, 5H), 2.71(s, 4H). ^{13}C: δ = 165.4, 151.4, 141.2, 128.7, 128.5, 126.2, 31.5, 26.3.

N-Hydroxyhomophenylalanine (VIa)—2-Oximino-4-phenylbutyric acid (1.93 g, 0.01 mol) in acetic acid (10 ml) and water (10 ml) was treated with sodium cyanoborohydride (1.1 g, 0.02 mol) (Ahmad, 1974) and stirred at room temperature for 16.0 h. Hydrochloric acid (5 ml, 12 N) was added and stirring continued for a further 1.0 h. The mixture was evaporated to dryness, dissolved in water, and again taken to dryness. The residue was dissolved in water and applied to a sulfonic acid resin column (Amberlyst 15, H^+ form, 10 g). This was washed with water (500 ml), and the product was eluted with aqueous ammonia (2% w/v). The ammoniacal eluate was taken to dryness to give the product as its ammonium salt (1.05 g, 55%). NMR in d_6-dimethyl sulfoxide: ^{1}H: δ = 7.14–7.30(m 5H), 4.50(br s, 4H), 3.25(t, 1H), 2.66(m, 2H), 1.80(m, 2H). ^{13}C: δ = 176.1, 142.4, 128.4, 128.3, 125.6, 65.4, 31.9, 31.3.

3-Nitropropylbenzene (IXa)—Trifluoroacetic anhydride (10.60 g, 0.05 mol) in acetonitrile (10 ml) was added to a stirred mixture of urea-hydrogen peroxide complex (5.82 g, 0.06 mol) in acetonitrile (30 ml) dropwise at 0 °C over 0.5 h. This solution was then added dropwise to a stirred mixture of hydrocinnamaldoxime (1.00 g, 6.7 mmol) and disodium hydrogen phosphate (21 g, 0.15 mol) in acetonitrile (50 ml) at 0 °C. After stirring for 2.0 h at room temperature, the solvent was removed *in vacuo*. The residue was treated with saturated sodium bicarbonate solution (100 ml) and extracted with diethyl ether (2 × 100 ml). The ether layers were combined, washed with sodium bisulfite solution (100 ml, 5% (w/v)), and dried. Removal of the solvent *in vacuo* gave the crude product. This was purified by "flash" chromatography (Merck Kieselgel 60, hexane/ethyl acetate, 80:20) to give the pure product as a light brown oil (252 mg, 23%). NMR in $CDCl_3$: ^{1}H: δ = 7.16–7.32(m, 5H), 4.34(t, 1H), 2.71(t, 1H), 2.31(m, 2H). ^{13}C: δ

[1] The abbreviations used are: GC, gas chromatography; MS, mass spectometry.

= 139.5, 128.7, 128.4, 126.6, 74.6, 32.2, 28.8. MS: m/z 165 (M^+, 6%), 147 (33), 135 (62), 131 (30), 117 (25), 107 (17), 104 (20), 91 (100), 65 (23).

5-Methylthiopentanal—A solution of sodium methoxide was formed from sodium (0.77 g, 0.033 mol) and methanol (50 ml). This was treated with gaseous methanethiol (1.8 g, 0.037 mol). To this solution was added 5-bromopentanenitrile ((5-bromovaleronitrile) 5.4 g, 0.033 mol). After 3.0 h diethyl ether (100 ml) and water (200 ml) were added. The aqueous phase was re-extracted with diethyl ether (100 ml) and the combined organic phases washed with water, dried, and concentrated. The residue was distilled (74 °C/0.7 torr, 3.2 g, 75%).

5-Methylthiopentanenitrile (1.7 g, 0.013 mol) in hexane (20 ml) and benzene (10 ml) at 0 °C under nitrogen was treated with diisobutylaluminium hydride (2.5 ml, 2.0 g, 0.014 mol) dropwise over 5 min and stirred at room temperature for 1.0 h. The mixture was poured onto ice and hydrochloric acid (100 ml, 2.0 N) and stirred for 1.0 h. Then diethyl ether (200 ml) was added and the mixture partitioned. The organic phase was washed with water (100 ml), saturated sodium bicarbonate (50 ml), saturated brine (50 ml), dried, filtered, and concentrated. The residue was distilled (60 °C/0.6 torr, 0.4 g, 24%). NMR in $CDCl_3$: ^1H: δ = 9.77 (t, 1H), 2.50(m, 4H), 2.10(s, 3H), 1.73(m, 2H), 1.64(m, 2H). ^{13}C: δ = 201.9, 43.2, 33.6, 28.2, 20.9, 15.3.

DL-Dihomomethionine (IIIb)—5-Methylthiopentanal (1.32 g, 0.01 mol), potassium cyanide (0.65 g, 0.01 mol) ammonium chloride (0.545 g, 0.01 mol) in ethanol was treated with ammonia (5 ml, 0.1 mol) and stirred for 48 h. Diethyl ether (100 ml) and water (100 ml) were added and the mixture partitioned. The organic layer was washed with water (100 ml), then extracted with hydrochloric acid (2 × 25 ml, 0.5 N). The combined aqueous phases were treated with sodium hydroxide solution (2 N, to pH 9) and extracted with diethyl ether (2 × 50 ml). The combined organic phase was washed with water (50 ml), dried, and concentrated *in vacuo* to give the amino-nitrile (0.7 g). The crude amino-nitrile was treated with hydrochloric acid (10 ml, 12 N) and left for 24 h. Water (10 ml) was added and the mixture refluxed for 2.0 h. The mixture was then taken to dryness *in vacuo* and then dissolved in aqueous sodium hydroxide solution (1 ml, 2 N). This was applied to an ion-exchange resin (Amberlite CG-400, —OH form, 10 g), which was washed with water (500 ml), then eluted with hydrochloric acid (50 ml, 0.5 N). The eluate was taken to dryness to leave the product as its hydrochloride (0.45 g, 22%) NMR in d_6-dimethyl sulfoxide: ^1H: δ = 9.15(s, 3H), 4.45(m, 1H), 3.07(t, 2H), 2.65(s, 3H), 2.43(m, 2H), 2.12(m, 4H). ^{13}C: δ = 170.9, 51.8, 32.9, 29.6, 28.2, 23.5, 14.7.

DL-[1-^{14}C]Dihomomethionine (IIIb)—In the same manner as (ix), DL-dihomomethionine. 5-Methylthiopentanal (3.0 mg, 0.023 mmol) in ethanol (300 μl), ammonium chloride solution (120 μl, 10.5 mg/ml), potassium [^{14}C]cyanide (800 MBq/mmol, 1.50 mg, 0.023 mmol) (Sigma) in water (200 μl) and ammonia (120 μl) were stirred for 2 days. Partitioning, extraction of the amino-nitrile into acid, re-extraction after aqueous base treatment, followed by acid hydrolysis (all as above) gave the product. This was purified using an ion-exchange resin (as above) to give a radiochemical yield of 3.52 MBq (19%).

5-Methylthiopentanaldoxime (IVb)—5-Methylthiopentanal (0.33 g, 2.5 mmol), hydroxylamine hydrochloride (0.25 g, 3.5 mmol), and sodium hydrogen carbonate (0.3 g, 3.5 mmol) were stirred in ethanol (10 ml) at room temperature for 4.0 h. The solution was diluted with water (50 ml) and partitioned with diethyl ether (50 ml). The organic phase was washed with water, saturated brine, dried, filtered, and concentrated to leave a mixture (3:2) of (*E*)- and (*Z*)-isomers, unchanged in ratio after recrystallization from diethyl ether/hexane (melting point 46–9 °C, 0.28 g, 77%). Further slow recrystallization from hexane/diethyl ether gave exclusively the (*Z*)-isomer. NMR in $CDCl_3$: (*E*)-isomer: ^1H: δ = 7.42(t, 1H), 2.50(t, 2H), 2.23(m, 2H), 2.09(s, 3H), 1.60(m, 4H). ^{13}C: δ = 151.7, 33.8, 29.0, 28.4, 25.6, 15.5. (*Z*)-isomer: ^1H: δ = 6.74(t, 1H), 2.52(t, 2H), 2.41(m, 2H), 2.09(s, 3H), 1.60(m, 4H). ^{13}C: δ = 152.2, 33.8, 28.7, 25.1, 24.6, 15.5. MS: (*E*)- and (*Z*)-isomers similar. *m/z* 147 (M^+, 1.5%), 129 (31), 82 (30), 61 (100), 55 (25), 48 (21), 47 (15), 45 (17), 41 (19).

Plant Material

Oilseed rape (*B. napus* L., cv Bienvenu) seeds were obtained from Rothamsted farm stocks. Plants were grown in compost in a controlled environment room under the following conditions: 12 h light period (a combination of tungsten and fluorescent lamps, average PAR 350 μE m^{-2} s^{-1}), temperature day/night 17–14 °C, relative humidity day/night 80–90%.

TABLE I

The effect of preincubation of DL-[1-^{14}C]dihomomethionine with amino acid oxidases on $^{14}CO_2$-release catalyzed by rape leaf microsomes

DL-[1-^{14}C]Dihomomethionine (25-μl aliquot) was incubated, in a water bath at 30 °C, for 1 h with either 20 μl of L-amino acid oxidase (L-AAO, Type III, *Crotalus atrox*; 0.5 units/20 μl) or 40 μl of D-amino acid oxidase (D-AAO, Type I, porcine kidney; 0.2 units/40 μl). Bovine catalase (5 μl, 2.5 units/5 μl) was included to remove the hydrogen peroxide generated during the incubation. The incubation was stopped by boiling the substrate-enzyme mixture in a water bath for 5 min. The mixture was cooled on ice and centrifuged in a Biofuge at 4 °C for 10 min at 10,000 revolutions/min. The supernatants (50 μl of L-AAO mix or 70 μl of D-AAO mix) were then used for the normal $^{14}CO_2$ release assays. Activity is expressed as percent of that found with no preincubation treatment.

Preincubation treatment	Activity
	%
None	100
L-AAO	5
D-AAO	101

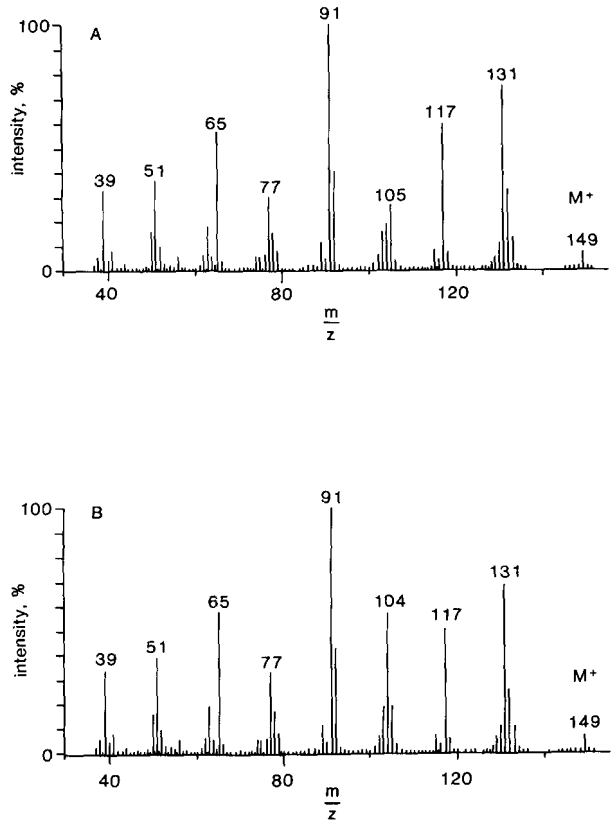

FIG. 1. **MS of authentic 3-phenylpropanaldoxime.** *A*, (*E*)-isomer; *B*, (*Z*)-isomer.

Microsomal Preparation

All operations were done on ice in a cold room (4 °C), and the pestle and mortar and all buffer solutions were prechilled. A mixture of leaves five and six from 48-day old plants were used in all experiments as these gave the highest activity. Twenty g (fresh weight) of leaves were chopped and then homogenized in a pestle and mortar with acid-washed sand (10 g) and homogenization buffer (100 ml, 0.25 M sucrose, 50 mM sodium bisulfite, 10 mM ascorbic acid, 0.1 M

potassium dihydrogen phosphate, adjusted to pH 7.0 with potassium hydroxide). The resulting homogenate was slurried with polyvinylpyrrolidone (10 g) and Amberlite XAD-4 (2g) for 2 min, then filtered through four layers of muslin. The filtrates were combined and centrifuged at 27,000 × *g* for 20 min. The supernatant was centrifuged at 100,000 × *g* for 1 h, and the resulting pellet resuspended in suspension buffer (2 × 500 μl, 20% (v/v) glycerol, 0.1 M potassium dihydrogen phosphate, adjusted to pH 7.5 with potassium hydroxide). The suspension was transferred to Eppendorf tubes and centrifuged in a Biofuge for 10 min. The pooled supernatants were made up to 5 ml with resuspension buffer, and the resulting solution comprised the crude microsome preparation used in all assays.

Enzyme Assays

Metabolism of homophenylalanine or dihomomethionine by the microsome preparations was monitored by following the release of $^{14}CO_2$ from amino acid substrates with the label at C-1. Both substrates were synthesized at a specific activity of 800 MBq mmol^{-1} and were diluted to 222 MBq mmol^{-1}. Glass vials (5 × 2 cm) were used for the assays, with a smaller glass vial placed within containing potassium hydroxide (1 ml, 1 M) plus a wick of Whatman No. 1 filter paper. Assays consisted of microsomes (200 μl) in a final volume of 500 μl. Reactions were started by the addition of substrate (25 μl, 6.8 nmol), and the vials were capped with a Subaseal prior to incubation at 30 °C. The reaction was stopped by addition of hydrochloric acid (30 μl, 40% (v/v)) to the assay mix. After overnight incubation at 30 °C (to allow $^{14}CO_2$ released from the acidified assay mix to be trapped in the potassium hydroxide solution), an aliquot (500 μl) of potassium hydroxide solution was added to "Cocktail T" (5 ml, BDH Chemicals). Radioactivity was determined using a Packard 2500T scintillation counter. All assays were run in triplicate, and compared to control assays containing microsomes, substrate, and buffer.

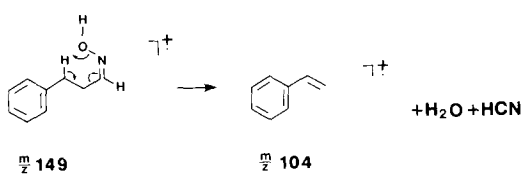

FIG. 2. Proposed MS rearrangement of (*Z*)-3-phenylpropanaldoxime to give intense ion, *m/z* 104.

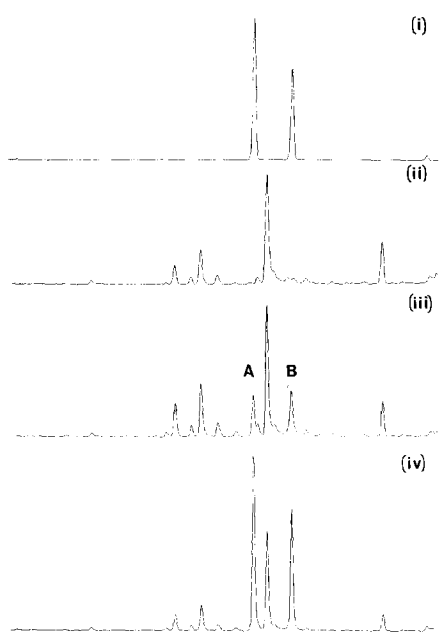

FIG. 3. **GC traces of synthetic and isolated aldoximes.** *i*, synthetic 3-phenylpropanaldoxime. *ii*, microsomes only. *iii*, microsomes incubated with homophenylalanine. *iv*, coinjection of *iii* with synthetic 3-phenylpropanaldoxime.

TABLE II

The effect of potential reaction intermediates and products on $^{14}CO_2$ release from homophenylalanine by rape leaf microsomes

DL-[1-^{14}C]Homophenylalanine was incubated with rape leaf microsomes as described under "Experimental Procedures," and the $^{14}CO_2$ released during incubation at 30 °C for 30 min in the presence of NADPH was collected and counted. Other homophenylalanine derivatives were dissolved in resuspension buffer and added to the reaction before addition of the substrate. Activity is expressed as percent of that found without additions; 100 activity is equivalent to between 250 and 370 pmol of CO_2 released h^{-1} g^{-1} fresh weight.

Addition	Concentration	Activity
	μM	%
None		100
VIa	100	53
VIa	250	32
VIIa	100	48
VIIa	250	19
IVa	100	52
IVa	250	30

The assays for aldoxime determination were run using unlabeled substrate (200 μl, 10 mM), incubated at 30 °C for 0, 30, or 60 min using microsomes (400 μl) in resuspension buffer (200 μl) containing NADPH (200 μl, 12.5 mM). Control samples contained microsomes (400 μl) and resuspension buffer (600 μl). The assay was then diluted with water to 5 ml and extracted with diethyl ether/hexane (5 ml, 50:50). The organic layer was separated, dried over magnesium sulfate, and concentrated to 50 μl. This was then analyzed by GC and GC-MS.

RESULTS AND DISCUSSION

The aldoxime forming enzyme systems involved in cyanogenic glycoside biosynthesis in cassava and sorghum (Halkier and Linberg-Moller, 1991; Koch *et al.*, 1992), and the different enzyme responsible for indolacetaldoxime synthesis in Chinese cabbage (Ludwig-Muller and Hilgenberg, 1988), are all membrane-bound. Here rape leaf microsomes were prepared to search for comparable enzyme systems that might be involved in glucosinolate biosynthesis. A convenient and potentially sensitive assay system was designed, in which $^{14}CO_2$ release from 1-^{14}C-labeled amino acids was monitored. The advantage of this assay is that it does not require complex analysis of the reaction products, and activity can be readily monitored irrespective of any enzymatic or non-enzymatic reactions that might degrade or modify the (presumed) oxime(s) formed. The two amino acids synthesized are the precursors of 2-phenethylglucosinolate Ia, *i.e.* homophenylalanine (IIIa), and the 3-butenylglucosinolates Ic, *i.e.* dihomomethionine (IIIb), both of which have been shown to accumulate in young developing rape leaves (Porter *et al.*, 1991). Microsomes prepared from young expanding leaves did not contain any nonspecific decarboxylases active with either substrate, as there was no significant $^{14}CO_2$ release in the absence of added cofactors. In the presence of NADPH, but not NAD or ATP, $^{14}CO_2$ release from each of the amino acids could readily be detected. The oxidative decarboxylation is clearly NADPH-dependent and therefore cannot involve a decarboxylase, specific or nonspecific. Incubation of microsomes with L-[U-^{14}C]glutamate, L-[U-^{14}C]arginine, or L-[1-^{14}C]phenylalanine did not lead to any detectable $^{14}CO_2$ release, in either the presence or absence of NADPH (data not shown), suggesting that the enzyme system(s) in rape leaves active with homophenylalanine and dihomomethionine are specific for the chain-extended amino acids.

Preincubation of [1-^{14}C]dihomomethionine (IIIb) with L-amino acid oxidase or D-amino acid oxidase (Meister, 1957) and subsequent incubation with rape leaf microsomes showed

loss of activity only with L-amino acid oxidase (Table I). This suggests that the enzyme system(s) involved are specific for L-amino acids as would be expected.

The respective aldoximes, from the two homoamino acids, IVa and IVb, were synthesized and fully characterized so that the identification of any such metabolites could be confirmed unequivocally in the microsome preparations. The synthetic 3-phenylpropanaldoxime (IVa) comprised (*E*)- and (*Z*)-isomers by both GC and NMR. However, slow recrystallization of the isolated mixture gave exclusively one isomer. This was the later eluting peak by GC and was identified as the (*Z*)-isomer by comparison with ^{1}H and ^{13}C NMR data with related oxime ethers (Elliott *et al.*, 1988). In the ^{1}H spectrum, the proton on C-1 in the (*E*)-isomer is shifted 0.7 parts/million downfield compared to the (*Z*)-isomer. The ^{13}C shift of C-2 in the (*E*)-isomer is also downfield, by 4.8 parts/million relative to the (*Z*)-isomer. This assignment also agrees with the MS data (Fig. 1), which show a greater intensity at *m/z* 104 in spectrum B arising from the rearrangement which is favored stereochemically in the (*Z*)-isomer (Fig. 2). Sigma bond fragmentation in the (*E*)-isomer gives the predominant ion *m/z* 105 in the spectrum (A). Both isomers give spectra which include loss of water (M^{+}-H_2O) to give *m/z* 131, with the base peak from the tropylium ion at *m/z* 91.

The synthetic 5-methylthiopentaldoxime (IVb) also comprised (*E*)- and (*Z*)-isomers by GC and NMR in a 3:2 ratio. Isolation of the pure (*Z*)-isomer was possible by slow recrystallization as before. Comparison with the data for the 3-phenylpropanaldoxime and analysis of the ^{1}H and ^{13}C spectra suggests that the elution order by GC is the same as for the 3-phenylpropanaldoxime with the (*E*)-isomer eluting before the (*Z*)-isomer. However, the mass spectra for these two isomers were almost identical.

In addition a number of possible intermediates on the pathway between homophenylalanine and 3-phenylpropanaldoxime were prepared to test their possible involvement in the pathway, including the *N*-hydroxyamino acid (VIa), the 2-oximino acid (VIIa), and 3-nitropropylbenzene (IXa).

The microsomal fraction was incubated with unlabeled homophenylalanine for different times. The extract from this preparation was analyzed by GC and gave two peaks at retention time 14.48 (*A*) and 14.80 (*B*) corresponding to the two aldoxime isomers (Fig. 3, *iii*). GC-MS of the sample showed that the ions *m/z* 131, 117, and 91 had intensity maxima at the same relative positions with a 2:1 ratio in the total ion current and that *m/z* 104 was maximal for the second peak. Background subtraction for the two peaks gave spectra similar to those from the synthetic sample. However, the parent ion was only present for the first, more abundant peak, and the *m/z* 131 ion was less intense, possibly as the ion reaction M^{+}-H_2O was less favored at the reduced source pressure arising from the low concentration of this sample. Nonetheless, the identity of (*E*)- and (*Z*)-3-phenylpropanaldoxime was confirmed by peak enhancement using coinjection GC (Fig. 3, *iv*). The ratio of total ion currents for the two peaks of 2:1 suggests that the (*E*)-aldoxime is formed in greater amounts than the (*Z*)-aldoxime. However, this is not confirmed by the GC integration which shows very little difference between the two peaks. The sample was also searched for the presence of the nitroalkane (IXa), using as the diagnostic ions the parent *m/z* 165, loss of water giving *m/z* 147, and other abundant ions at *m/z* 135, 117, and 91 (base ion), but no trace was found. The nitroalkane is found in the cyanogenic glycoside pathway, but only in significant amounts when the substrate is the *N*-hydroxyamino acid (VIa) (Halkier *et al.*, 1991).

Studies with dihomomethionine (IIIb) did not show generation of the corresponding aldoxime (IVb) by GC. In GC-MS mass fragmentography the base peak *m/z* 61 gave a peak with the correct retention time for the (*E*)-isomer but as no other diagnostic ions peaked, identification of this product (IVb) cannot be confirmed. Addition of the aldoxime (IVb) to the microsomal preparation showed greater than 80% loss of added material over 60 min. Although other possible metabolites, including the sulfoxides and sulfones, were searched using the GC-MS data, none were detected. These data suggest that although the aldoxime may be formed, its detection is unlikely under these experimental conditions. This could be due to oxidation at sulfur, and either subsequent reaction to form a polar compound or elimination to form the alkene, or the aldoxime undergoes further steps on the biosynthetic pathway.

The clear demonstration that homophenylalanine (IIIa) is converted to the aldoxime (IVa) by the *B. napus* preparation is an important development in understanding glucosinolate biosynthesis. The enzymology is elucidated elsewhere.[2] The isolation of the aldoxime (IVa) as a mixture of (*E*)- and (*Z*)-isomers may result from chemical equilibration. The (*E*)-aldoxime would be expected to give the glucosinolate with the accepted (*Z*)-stereochemistry (Ettlinger and Lundeen, 1956; Ettlinger *et al.*, 1961).

Addition of VIa, IVa, or VIIa to the microsomal system inhibited $^{14}CO_2$ release from homophenylalanine (Table II). *N*-Hydroxyamino acids have been reported to be better substrates for aldoxime synthesis than the parent amino acid (Kindl and Underhill, 1968), so it is possible that VIa and VIIa are simply diluting the product of an initial *N*-hydroxylation reaction. This might imply that VIIa is a natural intermediate, but this remains to be clearly demonstrated. Inhibition of the reaction by the product (IVa) is perhaps surprising, but could represent a control mechanism to restrict glucosinolate biosynthesis when sulfur donors for the next step (to V) are limiting. More detailed investigations are under way to discover the precise nature of the enzymic reaction(s), the intermediate metabolites, and the regulation of the pathway.

Further studies will be made on the dihomomethionine (IIIb) system, but the compounds synthesized have been of value in the enzymology studies (Bennett *et al.*, 1993).

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