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BIOSYNTHESIS OF 2-(2′-METHYLTHIO)ETHYLMALATE IN *BRASSICA CARINATA*

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Key Word Index—*Brassica carinata*, Brassicaceae, biosynthesis, 2-(2′-methylthio)ethylmalate, glucosinolates, allylglucosinolate; 2-(2′-methylthio)ethylmalate synthase, enzymology

Abstract—2-(2′-methylthio)Ethylmalate (2-MTEM) is thought to be an intermediate in the biosynthesis of allylglucosinolate from methionine in *Brassica*. When chemically synthesized 2-MTEM and labelled methionine are administered simultaneously to leaves of *B. carinata*, radiolabelled 2-MTEM can be recovered from the tissue but the incorporation of label into allylglucosinolate is unaffected. A non-glucosinolate-producing plant, *Phaseolus vulgaris* does not transform ^{3}H-methionine into ^{3}H-2-MTEM under the same conditions. All efforts to isolate the enzyme responsible for the synthesis of 2-MTEM from 2-keto-4-methylthiobutyrate (KMTB) and acetyl CoA have been unsuccessful.

INTRODUCTION

The biosynthesis of many glucosinolates in *Brassica* and related genera requires the chain extension of amino acid precursors to homologous non-protein amino acids. This has been demonstrated in radiotracer studies of the synthesis of phenethylglucosinolate in *Nasturtium* [1], allylglucosinolate in *Armoracia* [2] and 2-hydroxybut-3-enylglucosinolate in *Brassica* [3]. The chain extension pathway is thought to proceed via 2- and 3-substituted malic acid intermediates such as those in leucine biosynthesis [4] since labelled 2-benzylmalate and 3-benzylmalate are efficient precursors of phenethylglucosinolate in *Nasturtium officinale* [1, 5]. By analogy, allylglucosinolate biosynthesis would require 2-(2′-methylthio) ethylmalate (2-MTEM) to be formed from the condensation of the alpha-keto acid of methionine, 2-keto-4-methylthiobutyrate (KMTB), with acetyl Coenzyme A. 2-MTEM has been synthesized and its formation from radiolabelled precursors has been examined *in vivo* and *in vitro*

RESULTS AND DISCUSSION

Synthesis of 2-MTEM

The synthesis of 2-MTEM was attempted employing conditions described for the synthesis of isopropylmalate [6]; however, this method resulted in the liberation of large quantities of methanethiol from the starting material, methyl 3-methylthiopropionate. To circumvent this problem, the acetonitrile anion was generated using *n*-butyl lithium and was then added to the starting material. The other steps of the synthesis were performed essentially as previously described [6]

Feeding experiments

When L-[*Me*-^{3}H]methionine was administered to leaves of *Brassica carinata* simultaneously with synthetic 2-MTEM, reisolation of the 2-MTEM by two consecutive HPLC purification procedures showed significant incorporation of radiolabel into 2-MTEM (Table 1). Oxidation of the putative ^{3}H-2-MTEM with hydrogen peroxide and re-analysis of the oxidized material by reverse phase HPLC, resulted in the recovery of only one peak of radioactivity, which co-chromatographed with 2-MTEM sulphoxide. The highest incorporation of label into 2-MTEM in the smaller leaf 2 is consistent with the higher rate of glucosinolate biosynthesis reported earlier for immature upper leaves [7].

The observed labelling of 2-MTEM might have resulted from a non-enzymatic methylthio exchange reaction during extraction of the tissue, but reisolation of 2-MTEM from extracts to which ^{3}H-methionine and 2-MTEM were added only at the time of extraction showed no label in the recovered 2-MTEM. The biotransformation of methionine to 2-MTEM in *Brassica* leaves was also found to correlate with their glucosinolate biosynthetic capability since identical uptake experiments

Table 1 Recovery of 2-MTEM from leaves of *B. carinata* administered 3.7×10^5 Bq (10 nmol) L-[*Me*-^{3}H]methionine and synthetic 2-MTEM (1 μmol)

Experiment no	1	2	3
Leaf fresh weight (g)	1.20	0.78	1.37
Uptake time (min)	40	40	100
% Uptake	94	66	100
Total dpm in 2-MTEM ($\times 10^{-4}$)	5.1	7.2	6.6
% Incorporation	0.23	0.46	0.28

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using leaves of *Phaseolus vulgaris*, a plant which does not produce glucosinolates, showed no production of labelled 2-MTEM from ^{3}H-methionine

In an attempt to confirm 2-MTEM as an intermediate in the allylglucosinolate biosynthetic pathway, 10 nmol of both ^{14}C-methionine and ^{3}H-tryptophan, precursors of the major glucosinolates in *Brassica*, were administered simultaneously to leaves of *B carinata* in the presence and absence of 1 μmol 2-MTEM If 2-MTEM were an effective cold trap, it would be expected to diminish the incorporation of radioactivity from methionine into allylglucosinolate, while the incorporation of label from tryptophan into indoleglucosinolates would act as an internal standard of glucosinolate biosynthesis. The inclusion of 2-MTEM in the uptake solution did not disturb the ratio of ^{14}C/^{3}H incorporated into the two glucosinolates (Table 2) While this result could be interpreted as evidence against the proposed chain extension pathway in glucosinolate biosynthesis [1, 5], it is more likely that exogenous 2-MTEM does not have access to the allylglucosinolate biosynthetic pathway and therefore cannot act as a cold trap. Further support for this concept is the observation that the accumulation of label into 2-MTEM in leaves at similar developmental stages (Table 1, experiments 1 and 3) is not time dependent ^{3}H-2-MTEM formed *in vivo* does not appear to freely exchange with the administered compound The radioactivity recovered at the time of extraction may represent the liberation of the small pool of ^{3}H-2-MTEM formed *in vivo* into the excess unlabelled 2-MTEM supplied in the experiment The lack of a substantial endogenous pool of 2-MTEM in the leaf tissue was confirmed by HPLC analyses which indicated no measureable amount of 2-MTEM (detection limit 0 03 μmol 2-MTEM per g fr. wt) in leaf extracts

The production of 2-MTEM *in vivo* indicated that an enzyme which condenses acetyl CoA with KMTB to produce 2-MTEM (2-MTEM synthase) might be detectable *in vitro* For these studies, leaf tissue was extracted in potassium phosphate or Tris buffer at pH 6 8, 7 5, or 8.2, in the presence of various combinations and concentrations of potassium chloride, sodium fluoride, 2-mercaptoethanol, dithioerythritol, polyclar AT, L-methionine, L-valine, L-cysteine, KMTB, 2-ketoisovalerate, 3-[3-cholamidopropyldimethylammonio]-1-propanesulphonate (CHAPS) and phenylmethanesulphonylfluoride (PMSF) High acetyl CoA thioesterase activity was observed in crude extracts, which made these preparations unsuitable for direct assay Gel filtration and anion exchange fractionations of the crude extract were employed to separate potential 2-MTEM synthase activity from the competing thioesterase and other potentially inhibitory factors, but 2-MTEM synthase activity was not detected in any fraction of these preparations Although the related enzyme, 2-isopropylmalate synthase, has been detected in plants [8, 9], conditions reported to be suitable for its assay in extracts of plants [8] as well as yeast [10], and bacteria [11, 12] were ineffective in the search for a 2-MTEM synthase activity in *Brassica carinata* The failure to detect this enzyme activity may be due to the presence (or absence) of regulatory substances in the assay mixture, or to the instability of 2-MTEM synthase under the conditions employed

In summary, while the radiotracer results indicate that an enzyme catalysing the formation of 2-MTEM must be present in *B carinata* leaf extracts, conditions for its study *in vitro* have not yet been established It may be advantageous to first fully characterize the analogous 2-isopropylmalate synthase responsible for leucine biosynthesis as a means of developing appropriate isolation and assay systems for this class of enzymes in plants

Table 2 Labelling of glucosinolates from L-[U-^{14}C]-methionine and L-[5-^{3}H]tryptophan administered to leaves of *B carinata* in the presence and absence of 1 μmol synthetic 2-MTEM

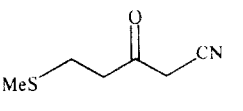
	10^{-4} × dpm in glucosinolates	
Treatment	Allyl	Indolylmethyl
Control	1 9	6 0
+2-MTEM	2 1	5 8

Data shown represent the means of triplicate incubations

EXPERIMENTAL

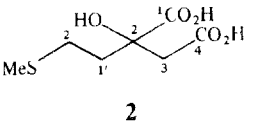
Dry THF was obtained from a Na–benzophenone ketyl ^{1}H and ^{13}C spectra were recorded on a Bruker WH-400 spectrometer, with TMS or DHO as internal standard in the solvents indicated TLC analyses were performed on silica gel K6F (Whatman)

3-Oxo-5-methylthiovaleronitrile (**1**) To a soln of 28 9 ml (72 mmol) *n*-butyl lithium (2 5 M in hexanes, Aldrich) in 20 ml dry THF at −80°, was added 4 15 ml (79 mmol) MeCN dissolved in 20 ml dry THF A dry N_2 atmosphere was maintained at all times and the addition was complete in 10 min The white ppt which formed was kept in suspension with vigorous stirring The mixture was then transferred through a stainless steel needle by positive N_2 pressure to a receiving flask containing 9 ml (72 mmol) methyl 3-methylthiopropionate in 20 ml dry THF Vigorous stirring was maintained at −80° An additional 10 ml of THF was used to rinse the first flask The soln was allowed to warm and a gelatinous ppt began to form 100 ml of 10% HCl was rapidly added with stirring and the products were immediately extracted into Et_2O (3 × 100 ml), washed with H_2O (1 × 100 ml), dried over $MgSO_4$ and concd to yield a yellow oil (12 g) This material was not purified further due to its tendency to polymerize at high concn Yield >90% by TLC TLC (MeOH–$CHCl_3$ 1 49) R_f 0 54, IR ν_{max}^{film} cm^{-1} 2290, 2255 (CN), 1727 (C=O), 1420, 1390, 1355, 1300, 1070, ^{1}H NMR ($CDCl_3$) δ 3 6 (2H, s), 2 92 (2H, *t*, 7 0), 2 76 (2H, *t* 7 0), 2 14 (3H, s), ^{13}C NMR ($CDCl_3$) 196 3 (s), 113 6 (s), 41 7 (*t*), 37 7 (*t*), 27 3 (*t*), 15 6 (*q*), EIMS *m/z* 143 [M]$^+$ (45), 103 (10), 75 (43), 68 (22)



1

2-(2-methylthio)Ethylmalate (**2**) 6 5 ml HCl (conc) was added slowly to a cooled mixt (ice bath) of 5 7 g (40 mmol) **1**, and 5 21 g



2

(80 mmol) KCN Stirring at 5° was maintained for 3 hr after which 100 ml 10% HCl was added The mixt. was extracted into Et_2O (3 × 100 ml), dried over $MgSO_4$ and concd *in vacuo* The crude yellow oil (5 7 g) was added to 2 vols of concd HCl and incubated for 70 hr at room temp The mixture was concd *in vacuo*, 3 vols of 2 M HCl was added and the mixture was refluxed for 3 hr The hydrolysis mixture was extracted into EtOAc (3 × 100 ml) and washed with H_2O (1 × 100 ml) Concn of the organic phase yielded 2 ml of a dark yellow oil which was dissolved in 20 ml H_2O, treated with activated charcoal and filtered through Celite The filtrate was taken to dryness and stored in a vacuum desiccator overnight. Recrystallization from hot EtOAc yielded 730 mg of **2**, (yield from methyl 3-methylthiopropionate, 10%). TLC (EtOH–NH_4OH 30.1) R_f 0 21, IR ν_{max}^{Nujol} cm^{-1} 3510 (O-H), 3200–2700 *br* (COO-H), 1695 *br* (C =O), 1220, ^1H NMR (D_2O) δ2 82 (1H, *d*, 16.3, H-3), 2 50 (1H, *d*, 16 3, H-3), 2 37 (1H, *ddd*, 5 2, 12 7, 12 7, H-2′), 2.19 (1H, *ddd*, 5 2, 12 7, 12 7, H-2′), 1 88–1 73 (2H, *m*, H-1′), 1 83 (3H, *s*, methylthio), ^{13}C NMR (D_2O) 180 1 (*s*), 176 5 (*s*), 77 6 (*s*), 45 8 (*t*), 41 0 (*t*), 29 6 (*t*), 16 8 (*q*), EIMS *m/z*. 208 $[M]^+$ (23), 190 (12), 145 (16), 75 (74), 61 (100), 47 (12), acc mass calculated for $C_7H_{12}O_5S$ 208 04055, found 208 03980 Analysis calculated %C, 40 38, H, 5 81, S, 15 40, found %C, 40 60, H, 5 85, S, 15 73 Mp 121–123°

Radiotracer feeding experiments. Brassica carinata var. 4218 (Agriculture Canada Research Station, Saskatoon, Saskatchewan) were maintained as described in [7] Leaves from 5-to 6-week-old plants were cut from plants while the petiole was submerged in H_2O L-[U-^{14}C]Methionine and L-[5-^3H]tryptophan (each 3 7 × 10^4 Bq, 10 nmol, total vol 200 μl) or L-[Me-^3H]methionine (3 7 × 10^5 Bq, 10 nmol, total vol 100 μl) was administered via the transpiration stream. Where *ca*, 1 μmol 2-MTEM was included in the uptake soln 30–90 min after beginning the feeding, leaves were extracted in MeOH as described in [7]

Extraction and analysis of glucosinolates Isolation of desulphoglucosinolates followed a modified procedure of ref [13] as described in [7], using 0 2 μmol benzylglucosinolate as an internal recovery standard Desulphoglucosinolate analysis was performed on an Alltech 5 μm C-18 column

Reisolation of administered 2-MTEM. Leaf extracts were separated into cationic, anionic and neutral fractions on SP-C-25 and QAE Sephadex as described by Redgewell [14] QAE column HCO_2H eluates were dried *in vacuo* and dissolved in 500 μl H_2O A 50 μl aliquot was fractionated on a Synchropak Q-300 HPLC anion exchange column (detection UV 210) with a NaCl gradient in 20 mM Tris–HCl pH 7.5 (0.5 ml/min, 0% NaCl for 4 min, 1.0 ml/min, gradient to 0 5 M NaCl over 11 min). 2-MTEM-containing fractions were further fractionated on a Serva 5 μm RP-8 column (detection UV 210; 1 ml/min, 0 2 M H_3PO_4 for 5 min; gradient to 20% MeCN over 15 min; composition constant for a further 10 min) Aliquots of the 2-MTEM-containing fractions were extracted with Et_2O (5 × 1 ml), the pooled organic fractions were dried, taken up in 200 μl 10 mM H_2O_2, and incubated overnight at room temp. An aliquot of the mixture was then analysed using the reverse phase system described above 1 min fractions were collected in all cases, and assayed for radioactivity by liquid scintillation counting.

Enzyme assays. B carinata leaf enzyme extracts were incubated with 1 mM ^3H-KMTB (prepared by oxidation of L-[Me-^3H]methionine with L-amino acid oxidase) and 1 mM acetyl CoA (final vol 100 μl) After 60 min, assays were spiked with 0.2 μmol 2-MTEM, transferred to a 100° heating block for 10 min and clarified by centrifugation. Radiolabelled substrate was separated from product on a Synchropak Q-300 HPLC anion exchange column (1 0 ml/min 20 mM Tris–HCl pH 7.5 containing 100 mM NaCl) The 2-MTEM-containing fraction was collected and assayed by liquid scintillation counting

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