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Glucosinolate biosynthesis: demonstration and characterization of the condensing enzyme of the chain elongation cycle in *Eruca sativa*

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Abstract

Glucosinolates are a group of sulfur-rich thioglucoside natural products common in the Brassicaceae and related plant families. The first phase in the formation of many glucosinolates involves the chain extension of the amino acid methionine. Additional methylene groups are inserted into the side chain of methionine by a three-step elongation cycle involving 2-oxo acid intermediates. This investigation demonstrated the first step of this chain elongation cycle in a partially-purified preparation from arugula (*Eruca sativa*). The 2-oxo acid derived from methionine, 4-methylthio-2-oxobutanoic acid, was shown to condense with acetyl-CoA to form 2-(2′-methylthioethyl)malate. The catalyst, designated as a 2-(ω-methylthioalkyl)malate synthase, belongs to a family of enzymes that mediate the condensation of acyl-CoAs with 2-oxo acids, including citrate synthase of the citric acid cycle, and 2-isopropylmalate synthase of leucine biosynthesis. The 2-(ω-methylthioalkyl)malate synthase studied here shares properties with other enzymes of this class, but appears chromatographically distinct and is found only in extracts of plant species producing glucosinolates from chain-elongated methionine derivatives. Although the principal glucosinolates of arugula are formed from methionine that has undergone two rounds of chain elongation to form dihomomethionine, studies with substrates and substrate analogs of different chain lengths showed that the isolated enzyme is responsible only for the condensation step of the first round of elongation.

Keywords: *Eruca sativa*; Brassicaceae; Arugula; Biosynthesis; Glucosinolate; Chain elongation; Methionine; Condensing enzyme; Methylthioalkylmalate synthase

1. Introduction

One of the best known groups of sulfur-containing secondary metabolites in plants are the glucosinolates. Approximately 120 individual glucosinolates have been isolated chiefly from species of the Brassicaceae and allied families (Fahey et al., 2001). Glucosinolates share a core structure containing a β-D-glucopyranose residue linked via a sulfur atom to a (*Z*)-*N*-hydroximinosulfate ester, and are distinguished from each other by a variable R group derived from one of several amino acids (Fig. 1A). They are stored in the vacuoles of plant cells, but following cell damage and exposure to myrosinase, a thioglucosidase found in glucosinolate-containing plants, they are hydrolyzed to form isothiocyanates and other products (Rask et al., 2000). These hydrolysis products have long been of interest for their role in the resistance of plants to herbivores and pathogens (Wittstock et al., 2003), in the flavor of cruciferous vegetables, such as cabbage and broccoli, and in the quality of rape seed oil and seed meal. In the last decade, glucosinolate hydrolysis products, such as sulforaphane (Fig. 1B), have also been identified as the agents responsible for lowering cancer risk in people eating diets rich in cruciferous vegetables (Hecht, 2000).

Glucosinolates originate from a group of amino acids, including alanine, leucine, methionine, phenylalanine and tryptophan. The biosynthetic process can be divided

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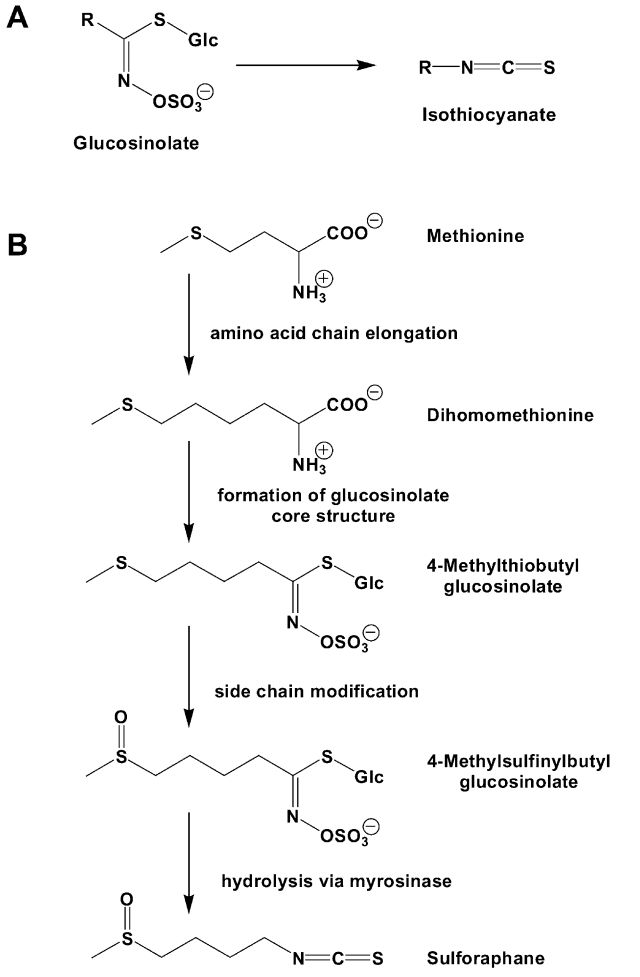


Fig. 1. Glucosinolate structures, biosynthesis and hydrolysis. (A) Basic structures of glucosinolates and isothiocyanates, one of the principal classes of glucosinolate hydrolysis products. (B) Outline of glucosinolate biosynthesis and hydrolysis showing the pathway leading to the isothiocyanate, sulforaphane, a potent anti-carcinogen.

into three stages (Fig. 1B): (1) amino acid chain elongation, in which additional methylene groups are inserted into the side chain, (2) conversion of the amino acid moiety to the glucosinolate core structure, and (3) subsequent, largely oxidative, side chain modifications (Halkier, 1999; Graser et al., 2001). We are currently studying the process of amino acid chain elongation because chain- extension has a significant influence on both the insect resistance and anti-cancer activity of glucosinolates (Morse et al., 1989).

Our knowledge of the chain elongation pathway comes from in vivo studies carried out with radioactive precursors (e.g., Chisholm and Wetter, 1964; Lee and Serif, 1970; Löffelhardt and Kindl, 1975) and stable isotope-labeled tracers (Graser et al., 2000), and from the isolation of several intermediates (Underhill, 1968; Dörnemann et al., 1974; Chapple et al., 1988). These investigations are consistent with a three-step elongation cycle involving amino acids as their 2-oxo acid derivatives, depicted for methionine in Fig. 2. Following deamination of methionine, the resulting 2-oxo acid condenses with acetyl-CoA to form a malate derivative. Subsequent isomerization and oxidation-decarboxylation generate a 2-oxo acid possessing one more methylene group than the original 2-oxo acid. This series of three reactions is homologous to other 2-oxo acid chain-elongation processes, such as that occurring in leucine biosynthesis (converting 2-oxoisovalerate to 2-oxoisocaproate) (Bryan, 1980). The chain-elongated 2-oxo acid resulting from the cycle can be transaminated and enter the next phase of glucosinolate biosynthesis, or it can pass through the cycle again adding another methylene group up to 8 more times (Fig. 2) (Fahey et al., 2001). To date, nothing has been reported on the enzymology of any steps of this process despite several attempts to demonstrate activities in vitro (Chapple et al., 1988; Dörnemann, 1977).

We set out to investigate the first step of the elongation cycle for methionine-derived glucosinolates, since this is the largest group of chain-elongated glucosinolates. In the first step of methionine chain-elongation, the 2-oxo acid produced from methionine, 4-methylthio-2-oxobutanoate (MTOB), condenses with acetyl-CoA to form 2-(2′-methylthioethyl)malate (MTEM) (Fig. 2). The enzymes catalyzing this reaction and the first step of successive rounds of methionine chain elongation can be referred to as 2-(ω-methylthioalkyl)malate synthases, abbreviated as MAMS. Recently, we isolated a gene encoding a MAMS from *Arabidopsis thaliana* based on sequence similarity to genes encoding the first step in the chain elongation cycle in leucine biosynthesis, as well as the results of mapping studies, mutant analyses and heterologous expression (Kroymann et al., 2001). However, no investigation of the enzyme properties was carried out at this time. Isolation of such a MAMS will furnish essential in vitro evidence for the chain-elongation pathway of glucosinolates, and characterization of the activity will provide insight into the mechanisms controlling glucosinolate chain length in plants.

2. Results

2.1. Isolation of MAMS activity

The MAMS activity catalyzing the condensation of acetyl-CoA with 4-methylthio-2-oxobutanoate (MTOB), the first step of methionine elongation in glucosinolate biosynthesis, had already been unsuccessfully sought in extracts of *Brassica carinata* (Chapple et al., 1988). We attempted to find MAMS activity in *Eruca sativa* (arugula, rocket), which synthesizes 4-methylsulfinylbutylglucosinolate. Similarly, we were unable to find MAMS in crude extracts of *E. sativa*, but were more successful when the soluble fraction of an enriched

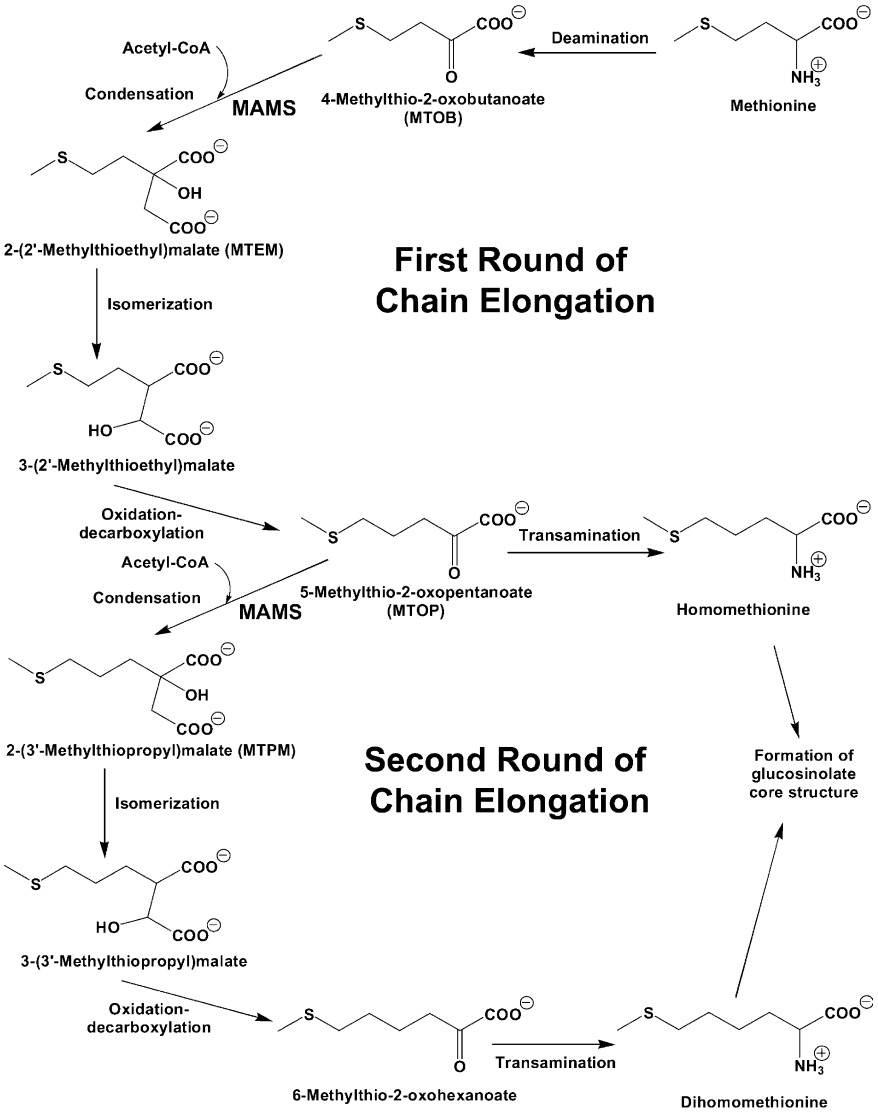


Fig. 2. The pathway of amino acid chain elongation in glucosinolate formation. Depicted are the first two rounds of the elongation of methionine. Up to 9 rounds of methionine elongation are known to occur in plants.

chloroplast preparation was used as the enzyme source. However, the presence of substantial, fluctuating amounts of thioesterase activity made the activity very variable from preparation to preparation. Thus, the enriched chloroplast extract was subjected to gel filtration chromatography and the pool of active fractions assayed in the presence of an acetyl-CoA regenerating system to prevent depletion of this substrate by residual thioesterase activity. To facilitate detection of small amounts of the assay product, 2-(2′-methylthioethyl)-malate (MTEM), a radio-labeled substrate, [1-^{14}C]acetyl-CoA, was employed with analysis by a sensitive HPLC protocol using an ion exclusion column. This procedure resulted in a measured rate of MTEM formation of approximately 2.25 pmol min^{-1} g plant tissue^{-1} (300 pmol min^{-1} g protein^{-1}) from a typical preparation. Under the standard assay conditions described under "Experimental", the activity was linear for over 12 h of incubation as checked by analyses of aliquots removed periodically over the time course. The final preparation could be frozen in liquid nitrogen and stored at −20 °C for up to a month with only a 33% decline in its activity.

2.2. Identification of MTEM product

The identity of the radiolabeled assay product as MTEM was verified by mass spectrometry and co-chromatography with an authentic standard. Fig. 3 compares the radioactivity trace from HPLC chromatography of a typical assay (panel A) to the trace of a control assay lacking only the 2-oxo acid cosubstrate, MTOB (panel B), and to a UV trace of an authentic MTEM standard run under identical conditions (panel C). A new peak with the same retention time as the authentic MTEM standard appeared in the complete

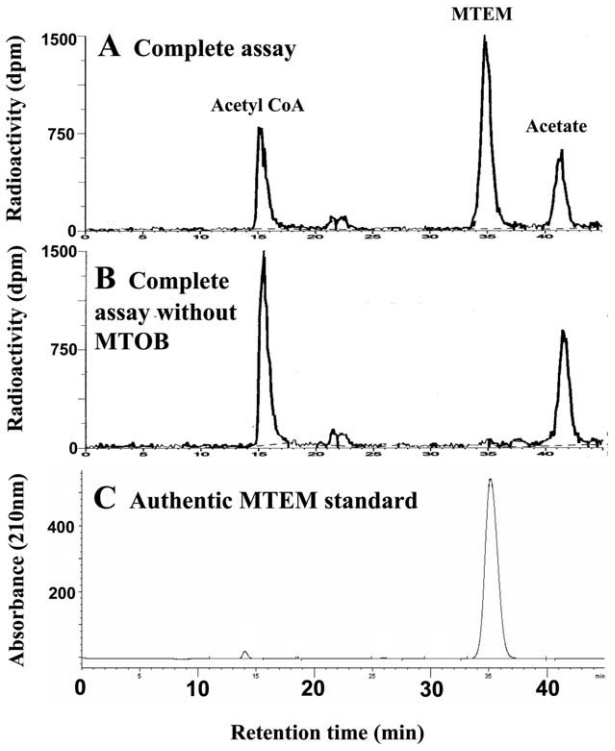


Fig. 3. Demonstration of MAMS activity in *E. sativa* by HPLC analysis of the assay products. (A) Trace of radioactivity monitor for complete assay run under standard conditions with acetyl-CoA and MTOB as substrates, as described under "Experimental". The trace shows peaks for the radiolabeled substrate, [1-^{14}C]acetyl-CoA, the product MTEM, and hydrolyzed acetyl-CoA. (B) Trace of radioactivity monitor for complete assay without co-substrate MTOB. (C) Trace of UV monitor for injection of authentic MTEM standard.

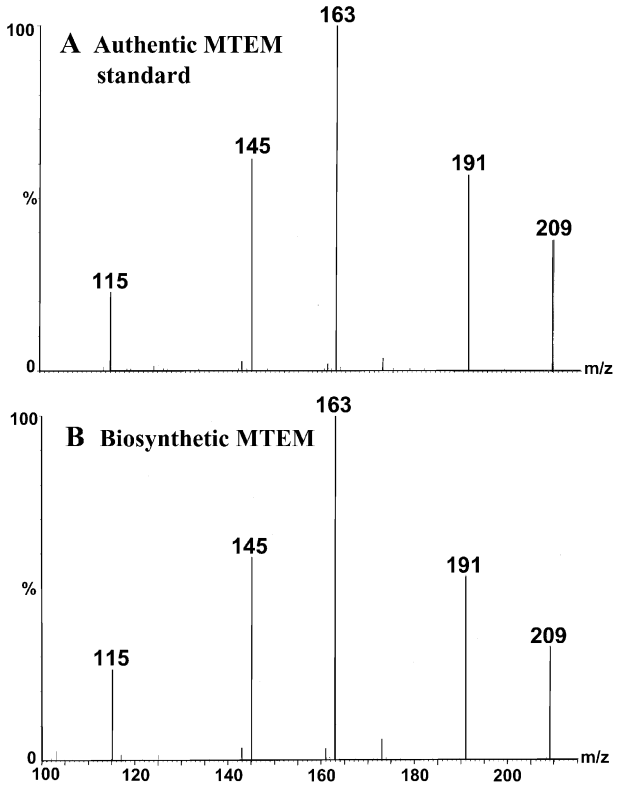


Fig. 4. Identification of MTEM as MAMS enzyme product by mass spectrometry. (A) Spectrum of authentic standard of MTEM synthesized by protocol adapted from Chapple et al. (1988). (B) Spectrum of MTEM produced in MAMS assay carried out under standard conditions. Spectra were obtained by LC-MS using a tandem quadrupole instrument with an electrospray interface. Depicted are MS-MS daughter ion spectra recorded by fragmentation of the protonated parent ion at m/z 209.

assay, but not in the assay without MTOB, nor in assays in which the protein preparation was first boiled. Similar results were observed when the products of the assay were separated on a different chromatographic system using a C18 reverse phase column in the presence of an ion pair reagent (data not shown). Mass spectrometry of the biosynthetic MTEM gave a spectrum nearly identical to that obtained from the authentic MTEM standard (Fig. 4). As an additional proof that the assay produces MTEM, the deuterated substrate, [methyl-$^2H_3$]4-methylthio-2-oxobutanoate, was supplied to the MAMS preparation by administering [methyl-$^2H_3$]methionine in a coupled assay with L-amino acid oxidase. The product was [methyl-$^2H_3$]2-(2′-methylthioethyl)malate which had a mass spectrum and retention time identical to that of an authentic standard (Fig. 5).

2.3. Catalytic properties of MAMS

The MAMS activity in the partially-purified preparation from *E. sativa* had a molecular mass of 84±1.5 kDa based on gel filtration chromatography with molecular mass standards. This can be compared to the predicted masses of the proteins encoded by MAMS genes reported from *A. thaliana*, approximately 48–52 kDa ignoring the predicted chloroplast transit peptide (Kroymann et al., 2001) which should not be present in the mature protein. If the *E. sativa* MAMS protein is similar to those of *A. thaliana*, it has a homo-dimeric architecture. The pH optimum of the enzyme was determined to be 9.0 with half-maximal values at 7.9 and 9.9. The temperature optimum was broad with a peak of activity observed at 32 °C.

A divalent metal ion appeared to be required for MAMS catalysis, since activity was promoted by addition of various metal species and was drastically reduced by addition of 1 mM EDTA (Table 1). Several different divalent ions supported catalysis at 1 mM, including Mn^{2+}, Ca^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+}, and Zn^{2+}. Although Co^{2+}, Fe^{2+} and Zn^{2+} gave more activity at 1 mM, Mn^{2+} was used in the standard assay because it gave more consistent results when working with our partially-purified preparations. The metal ion requirement may be associated with a requirement for ATP, as reported for enzymes catalyzing similar condensation reactions (Masurekar and Demain, 1974; Gray and Bhattacharjee, 1976). The *E. sativa* MAMS assay was

supplied with ATP along with *Saccharomyces cerevisiae* acetyl-CoA synthetase as part of an acetyl-CoA regenerating system, and showed almost no activity without this system. There is some evidence for an independent requirement for ATP, since ATP alone, added in the absence of acetyl-CoA synthetase, supported substantial catalysis. However, confirmation of the need for ATP must await the availability of pure protein because our partially-purified preparation required the ATP-containing acetyl-CoA regenerating system for linear assay conditions. DTT was also required for optimal MAMS activity, and was routinely added to give a concentration of 0.5 mM in the assay buffer. Samples lacking DTT had less than 10% of the activity with 0.5 mM DTT.

MAMS displayed Michaelis—Menten kinetics with both of its substrates when tested at concentrations ranging from 4 μM–8 mM (acetyl-CoA) and 1 μM–7 mM (MTOB). K_m values derived from Lineweaver–Burk plots were 340 μM for acetyl-CoA and 315 μM for MTOB. In comparison, the condensing enzyme of the chain-elongation process in leucine biosynthesis, 2-isopropylmalate synthase (IPMS), had K_m values of 5 μM for acetyl-CoA and 75 μM for its 2-oxo acid substrate, 2-oxoisovalerate, in spinach (Hagelstein and Schultz, 1993).

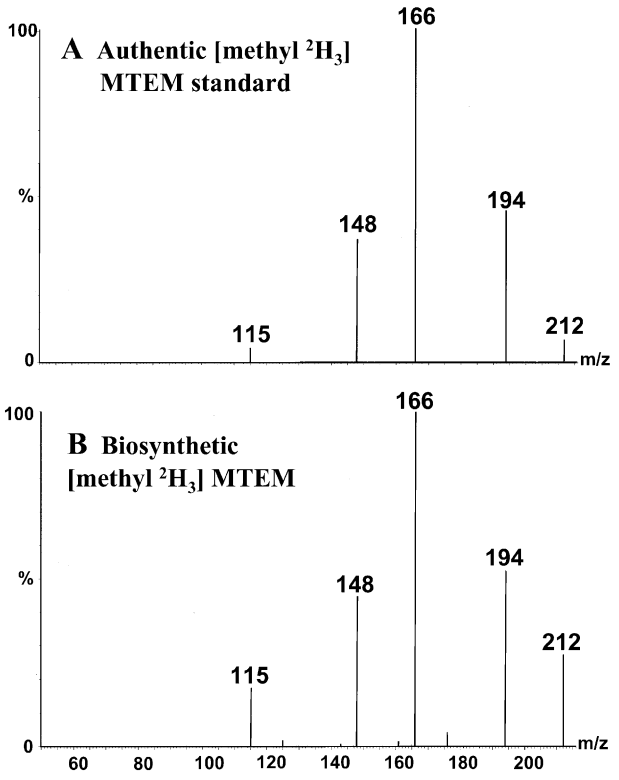


Fig. 5. Confirmation of MTEM as MAMS enzyme product by conversion of [methyl-$^2H_3$]MTOB to [methyl-$^2H_3$]MTEM. (A) Mass spectrum of authentic standard of [methyl-$^2H_3$]MTEM obtained by chemical synthesis. (B) Spectrum of [methyl-$^2H_3$]MTEM produced in MAMS assay from [methyl-$^2H_3$]MTOB. The latter was formed from [methyl-$^2H_3$]methionine in a coupled assay with L-amino acid oxidase.

Table 1
Effect of divalent cations at 1 mM on the activity of *E. sativa* MAMS

Cation	Relative activity (%)[a]
Mn^{2+}	100[b]
Ca^{2+}	83
Mg^{2+}	69
Ni^{2+}	83
Co^{2+}	170
Fe^{2+}	118
Zn^{2+}	130
Cu^{2+}	0
EDTA	0

[a] Activity measured under standard assay conditions as described under "Experimental". $MnCl_2$ was omitted from assay when other divalent cations were tested.

[b] Activity with 1 mM Mn^{2+} was 300 pmol min^{-1} mg $protein^{-1}$

2.4. 2-Oxo acid specificity

To assess the substrate specificity of the *E. sativa* MAMS preparation, assays were performed with a series of 2-oxo acids other than MTOB that serve as substrates for related acetyl-CoA-using condensation enzymes. Of the naturally-occurring 2-oxo acids tested, activity was only observed with oxaloacetate and 2-oxoisovalerate (Table 2), indicating citrate synthase and IPMS activity, respectively. Citrate synthase, an enzyme of the citric acid cycle, is found in the mitochondrial matrix. Its presence here suggests mitochondrial contamination of the chloroplast preparation. The presence of IPMS raises the question of whether MAMS in *E. sativa* is in fact a side activity of IPMS, the condensation enzyme of leucine biosynthesis. Using a fine scale gel filtration column, we were able to separate the peaks of these two activities (Fig. 6), suggesting that they arise from different proteins.

Table 2
Comparison of MAMS activity with MTOB and 2-oxo acid substrates of other condensing enzymes

2-Oxo acid	Relative activity (%)[a]
4-Methylthio-2-oxobutanoate (MTOB)	100[b]
2-Oxoisovalerate	33
Oxaloacetate	264
Glyoxylate	0
Pyruvate	0
β-Phenylpyruvate	0
2-Oxoadipate	0

[a] Activity measured under standard assay conditions as described under "Experimental" with 3 mM of the 2-oxo acid substrates indicated.

[b] Activity with MTOB was 300 pmol min^{-1} mg $protein^{-1}$.

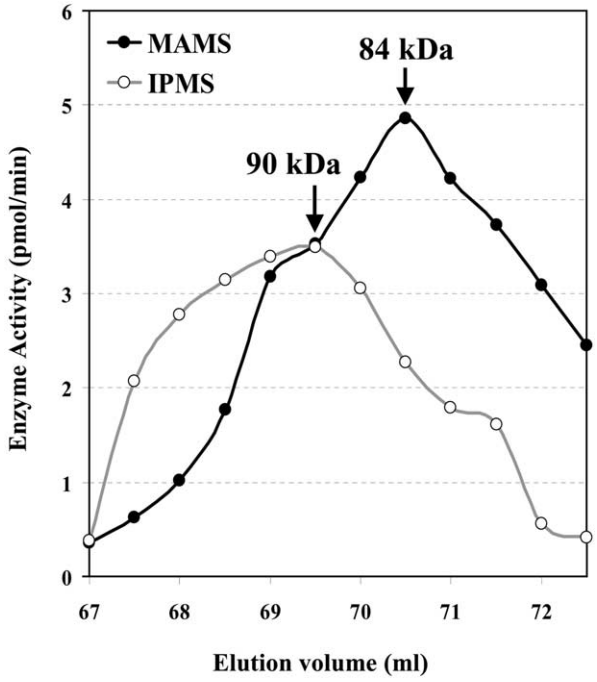


Fig. 6. Separation of MAMS and IPMS from a soluble preparation of lysed chloroplasts by gel filtration chromatography. Fractions (0.5 ml) from a Superdex 200 column (flow rate- 1 ml/min) were divided for simultaneous MAMS and IPMS activity determination. Assays were performed under standard conditions as described under "Experimental" with 3 mM MTOB and 3 mM 2-oxoisovalerate as substrates for MAMS and IPMS assays, respectively. This separation was repeated three times with similar results.

The presence of IPMS activity in a preparation derived from a soluble chloroplast fraction is at variance with the work of Hagelstein and Schulz (1993), who reported spinach IPMS to be confined to the thylakoid membranes. However, in our hands spinach IPMS was also obtained in a soluble, chloroplast-enriched extract with an activity of 4400 pkat/mg chlorophyll compared to only 380 pkat/mg chlorophyll as reported by Hagelstein and Schulz (1993) for spinach thylakoid membranes.

To further explore the specificity of our MAMS activity for methionine chain elongation as a part of glucosinolate biosynthesis, a range of other plant species was surveyed for both MAMS and IPMS activities using a similar extraction procedure (Table 3). While IPMS activity was found in all five plants surveyed, MAMS was restricted solely to the three species that synthesize chain-elongated glucosinolates derived from methionine (Fahey et al., 2001). A fourth species, *Tropaeolum majus*, also produces glucosinolates, but does not make any chain-elongated substances and did not possess any detectable MAMS activity.

2.5. Catalysis of the second round of chain elongation

Since *E. sativa* accumulates principally glucosinolates derived from dihomomethionine, the product of two rounds of chain elongation, we sought to determine if our MAMS preparation could catalyze the condensation reactions of both rounds. The condensation of the second round is the reaction of 5-methylthio-2-oxopentanoate (MTOP) with acetyl-CoA to form 2-(3′-methylthiopropyl)malate (MTPM) (Fig. 2). Since MTOP is not commercially available, this substrate was generated in a coupled assay using homomethionine and L-amino acid oxidase. As a control, MTOB, the substrate of the first condensation reaction, was generated from methionine in a similar coupled assay. While the MAMS coupled assay with methionine and amino acid oxidase produced MTEM at nearly half the rate obtained when MTOB was provided directly, no MTPM was found when the coupled assay was done with homomethionine (Table 4).

To confirm the absence of any activity for the second round reaction, 2-oxo acid substrate analogs were tested in which the sulfur atom is replaced by an additional methylene group. The MAMS preparation accepted 2-oxohexanoate (Table 4), the non-sulfur analog of MTOB. However, the longer non-S analogs, 2-oxoheptanoate, an analog of MTOP, and 2-oxooctanoate, an analog of 5-methylthio-2-oxohexanoate (MTOH), were not accepted as substrates. Also, neither MTOH itself was accepted, nor was 4-ethylthio-2-oxobutanoate, an elongated substrate formed from ethionine by amino

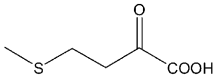
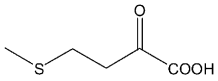
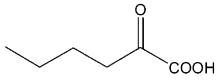
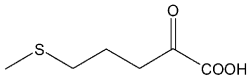
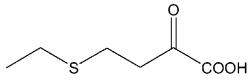
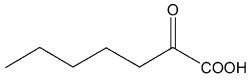
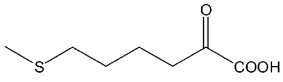
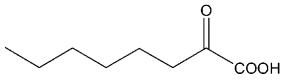
Table 3
Correlation of MAMS and IPMS activities with glucosinolate formation in various plant species

Species	Aliphatic glucosinolates[a]	Aromatic or indole glucosinolates[a]	MAMS[b]	IPMS[b]
Eruca sativa (Brassicaceae)	+	+	+	+
Diplotaxis tenuifolia (Brassicaceae)	+	+	+	+
Arabidopsis thaliana (Brassicaceae)	+	+	+	+
Tropaeolum majus (Tropaeolaceae)	–	+	–	+
Spinacia oleracea (Chenopodiaceae)	–	–	–	+

[a] Glucosinolate occurrence is taken from literature references (Fahey et al., 2001; Reichelt et al., 2002).

[b] Protein extracts from each species were prepared, purified and assayed as described under "Experimental" using MTOB for MAMS activity and 2-oxoisovalerate for IPMS measurements. Activity was deemed present if product (MTEM or 2-isopropylmalate) was seen in HPLC radiotrace at a level at least five times background.

Table 4
Comparison of MAMS activity with MTOB and other 2-oxo acid substrates and substrate analogs of varying chain lengths

Substrate		Relative activity (%)[a]	
		MAMS fraction	Crude chloroplast supernatant
MTOB (4-methylthio-2-oxobutanoate)		100[b]	100[b]
MTOB formed from methionine[c]		76	100
2-Oxohexanoate		64	–[d]
MTOP (5-methylthio-2-oxopentanoate) formed from homomethionine[c]		0	26
ETOB (4-ethylthio-2-oxobutanoate) formed from ethionine[c]		0	6
2-Oxoheptanoate		0	–[d]
MTOH (6-methylthio-2-oxopentanoate) formed from dihomomethionine[c]		0	0
2-Oxooctanoate		0	–[d]

[a] Activity measured under standard assay conditions as described under "Experimental" with 3 mM of the substrate indicated.
[b] Activity with MTOB was 300 pmol min^{-1} mg $protein^{-1}$ for the MAMS fraction and 130 pmol min^{-1} mg $protein^{-1}$ for the crude chloroplast supernatant.
[c] 2-Oxo acid substrate was formed from the amino acid indicated in a coupled assay with L-amino acid oxidase.
[d] Not measured.

acid oxidase in a coupled reaction. Thus, our *E. sativa* MAMS could only catalyze the condensation reaction of the first round of chain elongation. But, when the crude, chloroplast-enriched, soluble fraction was examined prior to gel filtration, both first-round and second-round reactions were observed, implying that these two activities are associated with separate proteins.

2.6. CoA ester specificity

Given the role of malonyl-CoA as an acyl donor in other elongation reactions, the partially purified MAMS preparation was also tested with this substrate. However, the incorporation of radioactivity from [2-^{14}C]malonyl-CoA into product was less than 10% of that with [1-^{14}C]acetyl-CoA. Moreover, the radio-HPLC chromatogram of the products of the assay with malonyl-CoA showed a peak of acetate (Fig. 7), suggesting that malonyl-CoA was only incorporated into MAMS products after first being converted to acetyl-CoA.

2.7. Effectors

Since IPMS has been shown to be strongly inhibited by leucine in many organisms (Webster and Gross, 1965; Kohlaw et al., 1969; Ulm et al., 1972; Wiegel and Schlegel, 1977; Hagelstein and Schultz, 1993), it was of interest to determine whether MAMS was inhibited by leucine or by the end products of chain elongation. Neither leucine, homomethionine nor dihomomethionine had a significant effect on *E. sativa* MAMS activity when preincubated with the enzyme at concentrations of 20 μM–2 mM at pH 9.0.

3. Discussion

The biosynthesis of many glucosinolates begins with the chain elongation of amino acids, such as methionine, phenylalanine and leucine. Although the chain elongation pathway had been elucidated by in vivo tracer experiments (summarized in Graser et al., 2000)

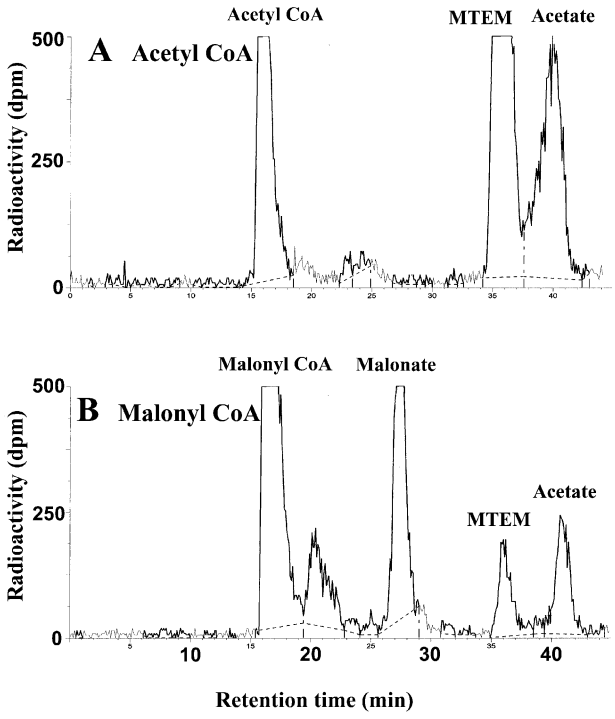


Fig. 7. Comparison of MAMS activity with acetyl-CoA and malonyl-CoA as substrates. Depicted are traces of the radioactivity monitor for HPLC injections of MAMS assays carried out under standard conditions with [1-^{14}C]acetyl-CoA and [2-^{14}C]malonyl-CoA as substrates.

and the isolation of a few intermediates (Chapple et al., 1988; Dörnemann et al., 1974; Underhill, 1968), until now none of the enzymatic conversions had been demonstrated in vitro. Here we were able to show the action of the first step in the chain elongation cycle of methionine, the condensation of acetyl-CoA with 4-methylthio-2-oxobutanoate (MTOB), the deamination product of methionine, to form 2-(2′-methylthioethyl)-malate (MTEM). This 2-(ω-methylthioalkyl)malate synthase (MAMS) activity had been sought unsuccessfully in earlier work, but we were able to demonstrate it using a partially-purified, chloroplast-enriched extract and a sensitive, radiolabeled assay incubated in the presence of an acetyl-CoA regenerating system to prevent the losses of substrate due to thioesterase activity. The results provide important confirmation of the proposed three-step, 2-oxo acid-mediated chain elongation cycle in glucosinolate biosynthesis, and are consistent with all of the previous in vivo feeding studies.

MAMS belongs to a group of enzymes that catalyze the condensation of acetyl-CoA and other acyl-CoA species with 2-oxo acids. Among the better known members of this group are citrate synthase of the citric acid cycle (substrates: acetyl-CoA and oxaloacetate) and 2-isopropylmalate synthase (IPMS) of the leucine biosynthesis pathway (substrates: acetyl-CoA and 2-oxoisovalerate). Other condensing enzymes of plant secondary metabolism include those that elongate 2-oxo acids involved in sucrose ester formation in glandular hairs of the Solanaceae (Kruomova and Wagner, 2003). The properties of *E. sativa* MAMS are broadly similar to those of other condensing enzymes. Nearly all members of this class share an alkaline pH optimum and a catalytic requirement for a divalent metal ion and ATP (Hagelstein and Schultz, 1993; Gray and Bhattacharjee, 1976; Masurekar and Demain, 1974; Zheng et al., 1997). However, despite its similar properties, MAMS from *E. sativa* was not able to use any of the substrates of the other enzymes of the group. Our partially-purified preparation did show some IPMS activity, but this could be separated from MAMS by further chromatography, so the two activities are distinct. In *A. thaliana,* the genome appears to possess separate genes encoding IPMS and MAMS (Kroymann et al., 2001). Further evidence for the distinctiveness of these two activities comes from a survey of several additional plant species. IPMS activity was found in all species examined, while MAMS was only found in those that synthesize methionine-derived, chain-elongated glucosinolates.

In *E. sativa*, the major glucosinolates require two rounds of chain elongation. However, the *E. sativa* MAMS performed only the condensation reaction of the first round of elongation. A second MAMS activity capable of catalyzing the condensation reaction of the second round appeared to be present in the crude chloroplast extract of *E. sativa*. The participation of separate MAMS activities in each of the first two rounds of chain elongation is consistent with a recent genetic study of glucosinolate formation in *Brassica oleracea* (Li et al., 2001). Here it was shown that separate, independently-segregating genes regulate the formation of glucosinolates derived from homomethionine (requiring one round of elongation) and dihomomethionine (requiring two rounds of elongation). Additional biochemical and molecular genetic investigations are now underway with the MAMS enzymes of *A. thaliana* to address the specificity of these catalysts for substrates of differing chain length in more detail. Since glucosinolates of different chain lengths often give rise to hydrolysis products with very different flavor properties, anti-cancer activities and toxicities to herbivores, an understanding of what regulates the formation of glucosinolates of different chain lengths will have many important applications in human health and agriculture.

4. Experimental

4.1. Plant material

Seeds of arugula (*Eruca sativa*) and spinach (*Spinacea oleracea*) were purchased from Saatzucht GmbH,

Germany. *Diplotaxis tenuifolia* seeds were obtained from Exhibition Seeds, UK, *Tropaeolum majus* seeds were purchased from Julius Wagner GmbH, Germany, and *Arabidopsis thaliana* seeds were from Lehle seeds, USA. Plants were germinated and grown for 3 weeks in ordinary potting soil mixed with vermiculite (3:1) in a controlled environment chamber under a diurnal cycle of 16 h light at 22 °C and 8 h dark at 18 °C. Illumination was with high-pressure sodium lamps (100% light intensity = 880 μmol m^{-2} s^{-1}) according to the following program for the 16 h light period: 5 h with 50% illumination, 6 h with 75% illumination, 5 h with 50% illumination. Plants were watered daily and fertilized three times a week with 15:11:13 (N:P:K) (Osmocote, Scotts Europe). Prior to chloroplast isolation, plants were kept in the dark for 3 days.

4.2. Chemicals

The substrate [1-^{14}C]acetyl-CoA (2.18 GBq/mmol) was purchased from Amersham Biosciences, and [2-^{14}C]malonyl-CoA (1.48-2.22 GBq/mmol) was purchased from NEN Life Science. The sources of the 2-oxo acids were MTOB: Sigma, 2-oxohexanoate: Aldrich and 2-oxooctanoate: Fluka, while L-[methyl-$^{2}H_3$]-methionine was obtained from Isotec. The enzyme product MTEM (Chapple et al., 1988), and homomethionine and dihomomethionine (Dawson et al., 1993) were synthesized as previously described. All other chemicals were obtained from Sigma-Aldrich unless otherwise noted.

4.3. Synthesis of 2-oxoheptanoic acid

Diethyloxalate (1.125 mol) and ethyl hexanoate (250 mmol) were added to a solution of 275 mmol sodium ethanolate in 125 ml absolute ethanol. After 0.5 h of stirring at room temperature, the ethanol and diethyloxalate were removed under reduced pressure. Then, 15 ml acetic acid and 20 ml water were added to the residue, and the solution extracted with ether (3×100 ml). The combined organic layers were washed with water (3×20 ml), saturated sodium bicarbonate (2×20 ml), water (30 ml) and brine (30 ml), and then dried with sodium sulfate. Evaporation of the solvent under reduced pressure gave 36.8 g of diethyl 2-butyl-3-oxosuccinate as a colorless oil (60% yield). Of this product, 50 mmol were refluxed with 25 ml concentrated HCl and 50 ml water for 12 h. The reaction mixture was then cooled to room temperature and 50 ml ether were added. The aqueous layer was extracted with ether (3×50 ml), and the combined organic layers dried with sodium sulfate. After removal of the solvent under reduced pressure, the residual oil was distilled (110 °C, 4 mbar) to give 7.21 g of 2-oxoheptanoic acid (66% yield) verified by mass spectrometry and NMR.

4.4. Synthesis of [methyl-$^{2}H_3$]MTEM

A mixture of 3-mercaptopropionic acid methyl ester (4.8 g, 40 mmol) and potassium carbonate (6.91 g, 50 mmol) was refluxed in 50 ml acetone for 20 min. Then, [$^{2}H_3$]iodomethane (5.86 g, 40 mmol) was added and the mixture was refluxed for 3 h. Next, 10 ml of water were added and the mixture was extracted with ethyl acetate (3×10 ml). The combined organic extracts were dried over magnesium sulfate, and the solvent was removed under reduced pressure. Distillation yielded [methylthio-$^{2}H_3$]-3-(methylthio)propionic acid methyl ester as a colorless oil (4.94 g, 90%). $Bp._{7\ mbar}$: 75–77 °C. ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.56 (2H, *m*-CH_2–CO–), 2.72 (2H, *m*, –S–CH_2–), 3.64 (3H, *s*, –OCH_3). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 14.8 (*sept.*–CD_3), 28.9 (*t*, –S–CH_2–), 34.2 (*t*, –CH_2–CO–), 51.8 (*q*, –O–CH_3), 172.1 (*s*, –*C*O–). EIMS *m*/*z* (rel. int.): 137 $[M]^+$ (100), 106 (42), 78 (59), 64 (70).

[Methylthio-$^{2}H_3$]-3-(methylthio)propionic acid methyl ester was converted to [methyl-$^{2}H_3$]MTEM in analogy with a protocol previously reported (Chapple et al., 1988) to yield 870 mg (29%) of colorless needles. Mp.: 123–125 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.98 (1H, *ddd*, *J* = 14.1, 11.1, 5.3 Hz, –CH_2–COH–), 2.07 (1H, *ddd*, *J* = 14.1, 11.0, 5.5 Hz, –CH_2-COH–), 2.42 (1H, *ddd*, *J* = 13.1, 11.1, 5.5 Hz, –S–CH_2–), 2.63 (1H, *ddd*, *J* = 13.1, 11.0, 5.3 Hz, –S–CH_2–), 2.73 (1H, *d*, *J* = 16.3 Hz, –CH_2–CO_2H), 3.04 (1H, *d*, *J* = 16.3 Hz, –CH_2–CO_2H). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 15.2 (*sept.*–CD_3), 29.5 (*t*, –CH_2–COH–), 41.1 (*t*, –S–CH_2–), 46.0 (*t*, –CH_2–CO_2H), 77.7 (*s*, –*C*–OH) 176.7 (*s*, –CH_2–CO_2H), 180.3 (*s*, –CO_2H). EIMS *m*/*z* (rel. int.): 211 $[M]^+$ (72), 148 (32), 134 (36), 116 (42), 106 (24), 88 (24), 78 (90), 64 (100); HR-MS: calc. for $C_7H_9D_3O_5S_2$ 211,0591, found 211.0584; IR (KBr) cm^{-1}: 3524 (O–H), 3200–2700 *br* (COO–H), 2130 (C–D), 1704 versus (C–OOH), 1430, 1395, 1339, 1264, 1232, 1170, 1133, 1001, 907, 806, 780, 653.

4.5. Isolation and partial purification of MAMS from E. sativa

Chloroplasts were isolated by a protocol modified from a published procedure (Hagelstein and Schultz, 1993). Briefly, mature leaves were ground in a buffer of 50 mM Mes, pH 6.1, containing 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM KH_2PO_4, 2 mM $NaNO_3$ and 5 mM DTT. After centrifugation at 6000*g*, the pellet containing intact chloroplasts was resuspended in a washing buffer identical to the grinding buffer except that the pH was 7.6 and no DTT was present. After a second centrifugation, the purified chloroplasts were suspended in a small volume of lysis buffer of 50 mM Tris, pH 7.6, containing 10% (v/v) glycerol, 1% (w/v) BSA, 1% (w/v) polyvinylpyrrolidone

40, 1% (w/v) polyvinylpolypyrrolidone, 1% (w/v) Amberlite XAD-4 resin, 2 mM $MgCl_2$, 1 mM NaCl, 1 mM ascorbate, 1 mM DTT, leupeptin (5 μg/ml), antipain (5 μg/ml) and 1 mM each of the following additional protease inhibitors: benzamide, benzamidine, phenylmethylsulfonylfluoride and γ-aminocaproate. The suspension was sonicated twice with a Sonoplus HD2070 sonicator (Bandelin, Berlin) using a microprobe at 60% full power for 5 min to burst the chloroplasts. The resulting extract was centrifuged at 48,000*g* for 20 min, the supernatant filtered through a 0.2 μ filter, and the filtered supernatant subjected to gel filtration chromatography on a Superdex 200 column (Amersham Biosciences). The column was equilibrated with 50 mM Tris, pH 8.0, containing 1 mM NaCl, 1 mM $MgCl_2$ and 1 mM DTT, and separation performed under isocratic conditions at a flow rate of 1 ml/min. Active fractions were pooled and concentrated 10-fold on a 30 kDa Filtron membrane (Pall Gelman Sciences) and the DTT concentration adjusted to 0.5 mM for storage and assay. These fractions were used in all characterization experiments except when otherwise noted.

MAMS preparations from all additional species studied, except *T. majus* and *A. thaliana*, were prepared in the same manner. Due to the difficulty in obtaining chloroplasts from *T. majus* and *A. thaliana*, crude extracts were prepared instead. For *T. majus*, 5 g of young leaves were homogenized in 25 ml of chloroplast lysis buffer with a Polytron. The resulting suspension was filtered through 8 layers of cheesecloth and 1 layer of nylon before centrifugation at 48,000 *g* for 20 min. The supernatant was then subjected to Superdex 200 chromatography as described above. For *A. thaliana*, 5 g young leaves were frozen in liquid nitrogen, ground in a mortar, and the powder resuspended in 10 ml 50 mM Tris buffer, pH 8.0, containing 10% glycerol, 10 mM $Na_2S_2O_5$, 2 mM $MgCl_2$, 1 mM ascorbate, 1% PVP-40, 1% PVPP, 1% XAD, 1 mM benzamidine, 1 mM PMSF, 1 mM γ-aminocaproate and 1 mM DTT. After filtration through cheesecloth, ammonium sulfate was added to 30% saturation, and the solution stirred for 30 min. The precipitated protein was removed by centrifugation at 26,000 *g* and 90,000 *g*, successively, and the supernatant loaded onto a Phenylsepharose (Amersham Biosciences) column equilibrated with 50 mM Tris buffer, pH 8.0, containing 1 mM $MgCl_2$ and 1 mM DTT. After washing with 60 ml buffer, a linear gradient was run from 1 to 0 M ammonium sulfate in this buffer at 3 ml/min, and fractions eluting between 850–500 mM ammonium sulfate collected and combined. Parallel experiments with *E. sativa* extracts prepared in the same manner indicated that MAMS activity was readily detectable with these alternate procedures at levels similar to those obtained using the protocol described in the previous paragraph.

4.6. Assay of MAMS activity

Standard assays were in a 250 μl volume in a 100 mM Ampso buffer, pH 9.0, containing 50 mM KCl, 1 mM NaCl, 4 mM $MnCl_2$, and 0.5 mM DTT. Substrates added were 3 mM MTOB and 1 mM [1-^{14}C]acetyl-CoA at a specific activity of 14.8 MBq/mmol. To prevent excessive losses of acetyl-CoA to thioesterase, an acetyl-CoA regenerating system was also added, consisting of 0.25 U *Saccharomyces cerevisieae* acetyl-CoA synthetase (Roche) and 20 mM ATP. For coupled assays in which an amino acid was added for *in situ* conversion to a 2-oxo acid, 3 mM methionine, homomethionine or ethionine were included along with 50 mU L-amino acid oxidase from *Crotalus adamanteus*. The assay was initiated by addition of MAMS extract, and assays were allowed to incubate up to 12 h at 32 °C. Reactions were stopped by mixing briskly with 750 μl absolute ethanol, and the resulting precipitate removed by centrifugation at 16,000 *g*. The supernatant was concentrated under vacuum to 50 μl for HPLC analysis. Assay products were separated isocratically on an ion exclusion column (Nucleogel Ion-300 OA, Macherey-Nagel) at 60 °C with 0.005 N H_2SO_4 (0.25 ml/min) as the mobile phase. Detection was by UV absorption (210 nm) followed by radioactivity monitoring (Radiomatic C505TR Flow Scintillation Analyzer, Canberra-Packard). The liquid flow cell was 0.5 ml, and the scintillation fluid (Ultima-Flo AP, Packard) was provided at a 4:1 ratio to the column eluent. The counting efficiency for ^{14}C in this solvent system was 60%. Approximate retention times were: acetyl-CoA—16.5 min, MTOB—28 min, MTEM—36.5 min, acetate—41 min and MTPM—43.5 min. All assays were repeated at least twice, except for kinetic determinations where substrates were tested at concentrations from 0.004–8.0 mM (acetyl-CoA) and 0.001–7.0 mM (MTOB) in 3 independent experiments. The values given are means, with variance representing less than 15% of the mean value. When varying acetyl-CoA concentration, 15 mM MTOB was used. When varying MTOB concentration, 10 mM acetyl-CoA was used.

4.7. Identification of MAMS assay products

Co-chromatography of biosynthetic MTEM with an authentic standard was carried out on two different HPLC columns: the ion exclusion column as described above and on a C18 column eluted with 50 mM potassium phosphate, pH 7.0, containing the ion-pairing reagent, *tert*-butylammonium hydrogen sulfate (5 mM), with a 0–30% CH_3CN gradient. Under the latter conditions, approximate retention times were: acetate-solvent front, MTEM—14.5 min, MTOB—15.5 min and acetyl-CoA—22.3 min.

Identification of MTEM, [methyl-$^2H_3$]MTEM and MTPM was performed by LC-MS using a Quattro II

tandem quadrupole mass spectrometer (Micro-Mass) equipped with an electrospray interface (capillary—2.5 kV, sample cone—12 V, desolvation temp—375 °C). HPLC employed the ion exclusion column described above using 0.003% (w/v, aqueous) CF_3COOH with a flow rate of 0.2 ml/min. MS-MS daughter ion spectra were recorded by fragmentation of the protonated parent ion, $[M+H]^+$, at m/z 209 (MTEM), m/z 212 ([methyl-$^2H_3$] MTEM) or m/z 223 (MTPM). Argon was used as the collision gas at 1.4×10^{-3} mbar, and a collision energy of 7 eV was employed to achieve fragmentation. The scanned mass range was m/z 50 to m/z 230 and the scan time 1 s. The citrate synthase reaction product, oxaloacetate, and the IPMS reaction product, 2-isopropylmalate, were identified by mass spectrometry and co-chromatography with authentic standards on the ion exclusion column.

4.8. MAMS molecular mass determination

Gel filtration chromatography was performed on a Superdex 200 column equilibrated as described above. Elution was carried out with the same buffer at a flow rate of 1 ml/min, and fractions of 0.5 ml were collected. The column was calibrated with a mixture of standard proteins: beta-amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; bovine serum albumin, 66 kDa, carbonic anhydrase, 29 kDa; cytochrome c, 12.4 kDa. These were measured by their absorbance at 280 nm in a prior run under identical conditions. The mass reported for MAMS is the mean of three independent experiments.

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