The Biosynthesis of 4-Methylsulfinyl-3-butenylglucosinolate (Glucoraphenin) in *Matthiola* Species

Evidence for Homomethionine and 2-Amino-6-methylthiocaproic Acid as Intermediates

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[^{35}S] L-Methionine and [methyl-^{14}C] L-methionine were fed to shoots of *Matthiola annua* and *Matthiola sinuata*. The label from both precursors was incorporated to a high degree into 4-methylsulfinyl-3-butenylglucosinolate, the main mustard oil glucoside of *Matthiola* species. Two homologues of methionine, homomethionine and 2-amino-6-methylthiocaproic acid, were also labelled in these experiments. 2-Amino-6-methylthiocaproic acid, deriving from methionine *via* homomethionine by two successive C_1-chain elongation steps, was shown to be a naturally occurring compound in higher plants.

Introduction

4-Methylsulfinyl-3-butenylglucosinolate (**4**) is an already long known mustard oil glucoside present in seeds of radish. The structure of its "aglycone", **4-methylsulfinyl-3-butenyl isothiocyanate** (**5**), derivable from the glucosinolate by the enzymatic action of myrosinase, was elucidated 1948 by Schmid and Karrer [1]. More recent investigations by Gmelin and Kjaer [2] revealed this glucosinolate, also known as glucoraphenin, as the major glucosinolate of *Matthiola* species.

Besides this compound also other mustard oil glucosides with a C_4-, C_5- or C_6-skeleton are found in *Matthiola* and related genera. Plants synthesizing a number of homologues, differing by one or more methylene groups, should provide a suitable model system for the investigation of chain elongation mechanisms and their regulation. Such a lengthening mechanism was hypothetically proposed by Wetter and coworkers [3, 4] for the biosynthesis of allylglucosinolate in analogy to the biosynthesis of leucine from valine [5, 6] (Scheme 1).

In the case of the analogous elongation of phenylalanine to 2-amino-4-phenylbutyric acid this mechanism including substituted malic acids (Scheme 1) could be verified by *in vivo* experiments [7, 8]. Less is known about the pathway leading to the aliphatic glucosinolates originating from methionine. In agreement with the general scheme the conversion of [2-^{14}C]methionine and [2-^{14}C]-homomethionine into 3-methylthiopropylglucosinolate and the incorporation of 2-amino-6-methylthiocaproic acid into 3-hydroxy-4-butenylglucosinolate could be demonstrated using *Cheiranthus kewensis* [10, 11] and *Brassica napobrassica* [12], respectively.

In addition to studies which established homomethionine as precursor in the biosynthesis of sinigrin [3] and methylthiopropylglucosinolate [11] we intended to demonstrate a two-fold chain elongation of methionine (**1**) leading to homomethionine (**2**) and further to 2-amino-6-methylthiocaproic acid (**3**). These amino acids are shown to be intermediates in the biosynthesis of glucoraphenin (**4**).

Materials and Methods

Plant material

M. annua R. Br. and *M. sinuata* R. Br. were grown in a greenhouse. 4–6 leaves of plants in the flowering stage were cut off from the stems (4–7 g fresh weight) and immersed into 0.2 ml of a solution containing the radioactive tracer. After 4 hours this solution was completely absorbed. Distilled

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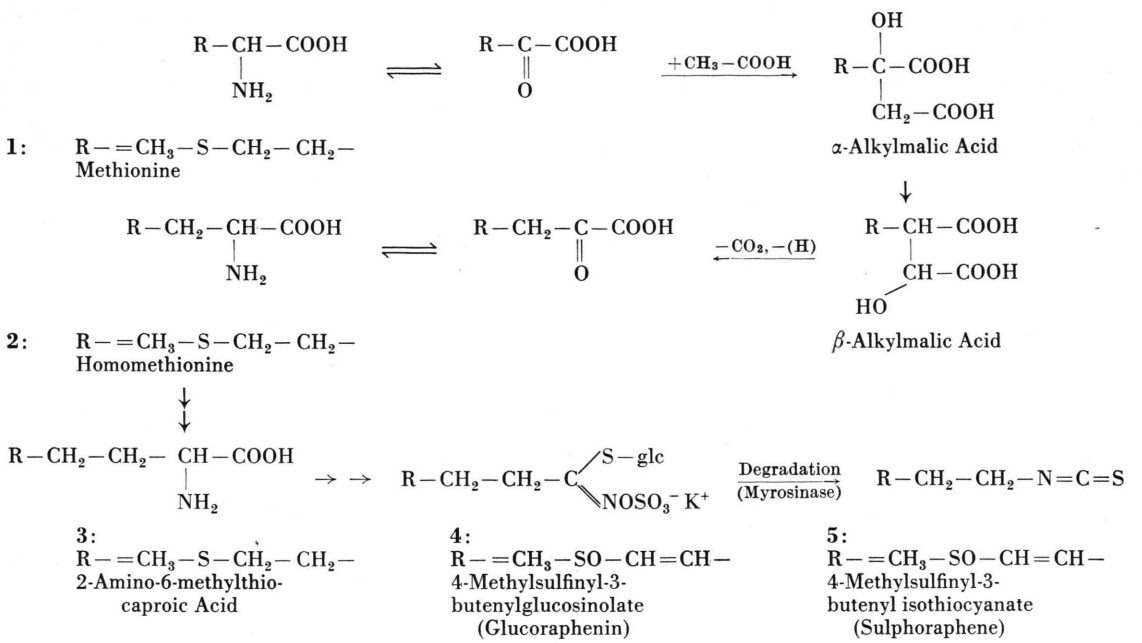

Scheme 1.

water was then added and the leaves were allowed to metabolize for the period of time indicated. These application experiments were performed in a controlled environment cabinet at 18 °C, 80% humidity and under continous light.

Labelled compounds

[^{35}S]L-Methionine (50 mCi/mmol), [methyl-^{14}C]-L-methionine (60 mCi/mmol), sodium [^{35}S]sulphate (50 mCi/mmol), [1-^{14}C]acetate (40 mCi/mmol) and [2-^{14}C]acetate (40 mCi/mmol) were purchased from the Radiochemical Centre, Amersham. For the preparation of [methyl-^{14}C]L-homomethionine [12 μCi/mmol) plants of *M. annua* where fed with [methyl-^{14}C]L-methionine. Isolation and purification of the biosynthetically formed amino acid are described later.

Non-labelled compounds

DL-Methionine sulfoxide, S-methylmethionine, DL-methionine were obtained from Sigma Chem. Comp.

DL-Homomethionine was synthesized by alkylation of dimethyl formamidomalonate [13] and photochemical addition of methyl mercaptan. The product was purified by repeated recrystallization from water/ethanol.

DL-2-Amino-6-methylthiocaproic acid was prepared according to Lee and Serif [10] starting with diethyl acetylamidomalonate and 1-chloro-4-(methylthio)-butane. 4-Methylsulfinyl-3-butenylglucosinolate was isolated from seeds of *Raphanus sativus* and *M. annua*, respectively, as follows: The seeds were defatted by mixing 20 g portions with 50 ml benzene/chloroform (2:1, v/v). Glucosinolates could be extracted then with a hot mixture of ethanol/water (7:3, v/v). The combined ethanolic extracts were concentrated *in vacuo* and purified by treatment with cation exchanger (Dowex 50 × 4, 200 – 400 mesh, K^+-form) and successive centrifugation. 30 ml of an aqueous solution containing approximately 6 g of glucosinolates and carbohydrates were layered onto the top of a 15 × 150 cm column filled with cellulose powder (Macherey and Nagel, MN 300) which was equilibrated with acetone/water (85:15, v/v). The compounds were eluted using a gradient of water in acetone reaching from 85:15 to 65:35. Fractions of 400 ml were collected and tested by PC using n-butanol/acetic acid/water (4:1:3, v/v/v, upper phase) (= system A) and acetone/water (85:15, v/v) (= system B) as solvent systems. The spots corresponding to glucoraphenin were visualized by a triplicate procedure: $AgNO_3$/NaOH for the carbohydrate moiety [14], KOH/sodium nitroprusside for the thioglucoside moiety [15] and a sensitive test for the sulfoxide group using a KJ/starch solution [16]. The fractions containing the maximum of glucoraphenin were combined and concentrated. Glucoraphenin showed a $R_{glc} = 0.45$ in

system A and a $R_{glc} = 0.87$ in system B. An aliquot of glucoraphenin was further purified by preparative PC (on MN 218[14]) and then used as a reference and as a carrier for the tracer experiments. Another part of the solution containing 4-methylsulfinyl-3-butenylglucosinolate was incubated for three hours with a partially purified myrosinase from seeds of *Sinapis alba* at 35 °C. Prior to the incubation, the pH was adjusted to 6.5 and ascorbic acid was added. The product of the enzymatic reaction was repeatedly extracted with chloroform and then converted into the thiourea derivative by reacting with saturated methanolic ammonia at 20 °C. Purification by TLC on Silica Gel HF_{254} developed with chloroform/methanol (85:15, v/v) showed a major band at $R_f = 0.50$, which was also visualized with Grote's reagent. After elution of this band with methanol, concentration and recrystallization from methanol/diethyl ether white crystalls with a m.p. 86 – 89 °C were obtained.

MS: *m/e* 192 (M^+), 177 ($-CH_3$), 176 ($-NH_2$), 129 ($-CH_3-SO$), 103 ($CH_3-SO-CH=CH-CH_2$). These data are in good agreement with those recently published for R-1-(methylsulfinyl-3-butenyl)-thiourea by Hansen and Kjaer[17].

Isolation and identification of products after feeding experiments

Leaves were immersed in 200 ml of boiling 80% ethanol containing DL-homomethionine, DL-2-amino-6-methylthiocaproic acid, DL-methionine sulfoxide, DL-S-methylmethionine (2 mg of each) and 4-methylsulfinyl-3-butenylglucosinolate as carriers. The plant material was cut into small pieces and extracted repeatedly with 80% ethanol. After removal of ethanol the extracts were concentrated *in vacuo*. The sticky residue was diluted with water and extracted several times with chloroform. The aqueous layer containing the amino acids and glucosinolates was concentrated and placed on a 1.5 × 20 cm column of cation exchanger (Dowex 50 × 4, 200 mesh, H^+-form) carefully conditioned with water. After passing through 200 ml of water the effluent was concentrated, neutralized with diluted potassium hydroxide and subjected to PC in solvent system A. The intensive peak ($R_{glc} = 0.45$) that accounted for about 80% of the radioactivity present in the anion + neutral fraction coincided with the position of glucoraphenin. This zone was eluted from the paper and rechromatographed in solvent system B.

Again the peak matched the glucosinolate test at $R_{glc} = 0.87$. The radioactive glucosinolate was eluted and an aliquot was treated with myrosinase and converted into the thiourea as described. Upon analysis of the thiourea developed with chloroform/methanol (85:15, v/v) yielded one major radioactive peak which coincided with 4-methylsulfinyl-3-butenylthiourea. A minor peak at $R_f = 0.40$ could account for a cyclization product[17].

The amino acid fraction was eluted from the cation exchanger column described above with 1.4 N NH_4OH, concentrated and separated by PC in solvent system A. The R_f-values were: S-methylmethionine 0.24, methionine sulfoxide 0.36, methionine 0.56, homomethionine 0.62, 2-amino-6-methylthiocaproic acid 0.71. The major peaks of radioactivity could be attributed to S-methylmethionine and methionine sulfoxide. These zones as well as areas containing methionine and its two homologues were cut off, the paper eluted and the substances rechromatographed in the solvent *n*-butanol/ammonia/ethanol/benzene (5:3:2:1, v/v/v/v) (= system C). The radioactive methonine sulfoxide ($R_f = 0.28$) was eluted and reduced to methionine by heating in 10^{-2} M mercaptoethanol at 120 °C for 15 min[18]. The radioactivity was retained in the methionine zone ($R_f = 0.40$ after chromatography in solvent system C). The incorporation of tracer into S-methylmethionine ($R_f = 0.20$, in system C), was finally proved by preparative GLC as will be described later for homomethionine and 2-amino-6-methylthiocaproic acid. These two homologues (R_f = 0.50, 0.62) were separated from methionine by GLC as follows: A solution of compounds was concentrated and then evaporated to dryness under a stream of nitrogen at 60 °C. Silylation was performed by heating the residue in a sealed vial with 200 µl of N-methyl-N-trimethylsilyltrifluoracetamide (MSTFA) for 30 min at 120 °C. A stainless steel column (2 m × 1/8″) with 10% OV-17 on Varaport 30 (80 – 100 mesh) was used for the separation of the TMS-amino acids. The temperature program started at 160 °C and rised at 4 °C/min. The relative retention times (RRT) were: S-methylmethionine 1.10 (only for comparison since already separated by PC), methionine 1.00, homomethionine 1.34, 2-amino-6-methylthiocaproic acid 1.66. Fractions were collected by means of glass trapping tubes connected to the heated exit tip. The bent lower end of a tube was immersed in a scintillation vial containing 10 ml of a scintillation fluid (*e. g.* 5% butylphenylbisphenyloxadiazole in toluene). After rinsing the trapping tube with methanol the radioactivity was determined in the scintillation counter. Distinct maxima of radioactivity corresponded to the mass peaks of the GLC of the TMS-amino acids. In order to confirm the radiochemical purity the fractions from GLC were collected in water containing carrier and either subjected to PC in solvent system A and C or evaporated to dryness and silylated again. GLC of the

single homologues showed that there was no contamination arising from tailing of methionine or homomethionine in 2-amino-6-methylthiocaproic acid.

Determination of radioactivity

Radioactive samples were counted in a liquid scintillation spectrometer equipped with a dpm-automatic (Packard Tri-Carb model 3380 + 544). Paper chromatograms were scanned with a Packard Radiogram Scanner, model 7201 (4π double flow chambers) with an efficiency of 25%. Radioactivity on TLC-plates was detected using a thin layer scanner (Berthold-Friesecke II) with a 2π proportional chamber (12% efficiency).

Results

Precursors of mustard oil glucosides in two Matthiola species

Incorporation of radioactivity from various labelled precursors into-4-methylsulfinyl-3-butenylglucosinolate, was investigated using shoots of two *Matthiola* species, *M. annua* and *M. sinuata*. Another labelled product was tentatively identified as 4-methylthio-3-butenylglucosinolate, known already as a minor constituent of seeds and leaves of *Matthiola* species [2].

Investigations were started with the application of [^{35}S]sulphate to plants of different stages of development. It became evident that mustard oil glucosides were not formed in etiolated leaves, whereas older green plants of *Matthiola* species produced – compared to other Cruciferae – remarkably high amounts of a glucosinolate which was also found as the main mustard oil glucoside in *Raphanus sativus*.

Table I presents the results obtained with *M. annua* when precursors were allowed to metabolize in leaves of 2 months old plants during 24 hours. The methyl ether group as well as the sulphur of methionine were incorporated at a high rate. The fact that the sulphur of methionine can also be the precursor of the thioglucose moiety of a mustard oil glucoside may account for higher efficiency of [^{35}S]-labelled methionine compared to [^{14}C]-labelled methionine. An even better conversion into the glucoraphenin could be demonstrated using homomethionine.

Competition experiments carried out by feeding [^{35}S]L-methionine together with 2-amino-6-methylthiocaproic acid (Exp. No. 4) showed a significant decrease in incorporation in comparison to the conversion rate in Exp. No. 3. This does not only indicate a function of the C_6-acid as intermediate but is also in agreement with the assumption made before that [^{35}S] is only partly incorporated into the thioglucose moiety of the glucosinolate.

Two control experiments with differently labelled acetate are reconcilable with the hypothesis (*cf.* Scheme 1) which requires the loss of C-1 of acetate during elongation and thiohydroxamic acid formation.

Using [methyl-^{14}C]L-methionine or [^{35}S]L-methionine, respectively, as precursors at least 80% of the total radioactivity found in the fraction representing the anions and water soluble neutral compounds was contributed by glucoraphenin. A small peak found at lower migration values (R_f = 0.33, in system A) most likely accounts for 4-methylthio-3-butenylglucosinolate: A glucosinolate with a similar behavior and the same R_f-value in system A and B could be obtained by reduction of 4-methylsulfinyl-3-butenylglucosinolate. Treatment

Table I. Incorporation of label from various precursors into 4-methylsulfinyl-3-butenylglucosinolate in leaves of *M. annua*.

Exp. No.	Compound fed	Activity [μCi]	Spec. Act. [μCi/μmol]	4-Methylsulfinyl-3-butenyl-glucosinolate formed [% Incorporation]
1	[methyl-^{14}C]L-Methionine	50	58	8.9
2	[methyl-^{14}C]L-Methionine	50	56	9.2
3	[^{35}S]L-Methionine	42	282	17.9
4	[^{35}S]L-Methionine + 1.1 mg DL-2-Amino-6-methylthiocaproic acid	42	282	10.6
5	[methyl-^{14}C]L-Homomethionine	0.017	0.012	19.4
6	[1-^{14}C]Acetate	50	10	0.1
7	[2-^{14}C]Acetate	50	14	5.8

Table II. Formation of [^{35}S]4-methylsulfinyl-3-butenylglucosinolate in leaves of *M. sinuata*.

Exp. No.	Compound fed	Activity [μCi]	Spec. Act. [μCi/μmol]	Metabolic Period [hours]	4-Methylsulfinyl-3-butenylglucosinolate [% Incorporation]
8	[^{35}S] L-Methionine	54	282	40	12.1
9	[^{35}S] L-Methionine	54	282	110	11.0

Table III. Formation of homomethionine and 2-amino-6-methylthiocaproic acid in leaves of *M. annua*.

Exp. No.	Compound fed	Activity [μCi]	Spec. Act. [μCi/μmol]	Metabolic Period [hours]	Activity incorporated [nCi] Homomethionine	Activity incorporated [nCi] 2-Amino-6-methylthiocaproic acid
1	[methyl-^{14}C] L-Methionine	50	58	24	290	19
2	[methyl-^{14}C] L-Methionine	50	56	24	200	16
3	[^{35}S] L-Methionine	42	282	24	85	11
4	[^{35}S] L-Methionine +1.1 mg 2-Amino-6-methylthiocaproic acid	42	282	24	11	1.4
8	[^{35}S] L-Methionine	100	300	144	280	25
5	[methyl-^{14}C] L-Homomethionine	0.017	0.012	24	4 *	0.4

* Recovered.

of labelled glucoraphenin with either a hot solution of sodium dithionite or with mercaptoethanol (10%) at 120 °C, conditions that lead to a reduction of sulphoxides, yielded one major product which behaved identical to our compound isolated.

Investigations with *M. sinuata* led to similar results as found using *M. annua*. This shows that also in this species glucoraphenin represents the major glucosinolate which derives from methionine. In this case, we tested the influence of prolonged metabolic periods on the formation of glucoraphenin. The results (Table II) were comparable with those outlined for *M. annua* in Table I. The rate of incorporation was not altered significantly upon a 2.5-fold increase of reaction time.

The role of homomethionine and 2-amino-6-methylthiocaproic acid

Homomethionine is formed from methionine (Table III) with a yield of about 0.5%; it is also subjected to a considerable turnover as shown in Exp. No. 5 where only 1/4 of the radioactive homomethionine fed could be recovered after a period of 24 hours. Also 2-amino-6-methylthiocaproic acid was found in labelled form when radioactive L-methionine was fed. But compared to a low incorporation of L-methionine (0.04% in Exp. No. 1), a significantly higher conversion into this C_6-compound (more than 2%) was demonstrated with homomethionine as precursor (Exp. No. 5).

Table IV. Conversion of L-methionine into homomethionine and 2-amino-6-methylthiocaproic acid in *M. sinuata*.

Exp. No.	Compound fed	Activity [μCi]	Spec. Act. [μCi/μmol]	Metabolic Period [hours]	Activity incorporated [nCi] Homomethionine	Activity incorporated [nCi] 2-Amino-6-methylthiocaproic acid
8	[^{35}S] L-Methionine	54	282	40	48	8
9	[^{35}S] L-Methionine	54	282	110	28	6

In a trapping experiment with labelled L-methionine and inactive 2-amino-6-methylthiocaproic acid (Exp. No. 4) both the formation of homomethionine and of 2-amino-6-methylthiocaproic acid were considerably decreased.

Homomethionine and 2-amino-6-methylthiocaproic acid could also be demonstrated as intermediates in experiments carried out with *M. sinuata* (Table IV).

Other products of L-*methionine metabolism*

The major peaks of radioactivity on paper chromatograms of the amino acid fraction after feeding [^{35}S]L-methionine or [methyl-^{14}C]L-methionine to *M. annua* and *M. sinuata* were S-methylmethionine and methionine sulphoxide. The incorporation rates found are 0.6 – 2.2% for S-methylmethionine and 1.0 – 3.6% for methionine sulphoxide. The highest values were obtained in the competition experiment using inactive 2-amino-6-methylthiocaproic acid. Prolonging of the metabolic period decreased the percentage of radioactivity incorporated into these amino acids.

To exclude the possibility that methionine sulphoxide could have been formed artefactually on paper chromatograms developed with the acidic solvent system A we used in one case only the alkaline mixture C. However, the same results were obtained.

Discussion

A sequential conversion of homologous amino acids with a C_4-, C_5- and C_6-carbon skeleton, respectively, could be established as initial part of the pathway leading to the C_5-mustard oil glucoside glucoraphenin (*cf.* Scheme 1). The results are in agreement with the chain lengthening mechanism postulated by Underhill and Wetter [19] for the biosynthesis of glucosinolates deriving from methionine. Noteworthy is the high efficiency (9%) with which [methyl-^{14}C]L-methionine is incorporated into glucoraphenin compared to a 2.4% conversion of [2-^{14}C]L-methionine into 3-butenylglucosinolate [20], where a 2-fold chain elongation of methionine is involved as well.

[methyl-^{14}C]L-Homomethionine was a precursor of glucoraphenin about twice as effective as [methyl-^{14}C]L-methionine. Only one chain elongation is necessary to convert it into the direct amino acid precursor of the glucosinolate. As expected, the incorporation rate is in good agreement with those reported for sinigrin using [2-^{14}C]L-methionine as precursor.

Both homologous intermediary amino acids, homomethionine and 2-amino-6-methylthiocaproic acid, were isolated from the amino acid fractions in labelled form. Thus, a 2-fold chain elongation of methionine was shown to occur *in vivo* and 2-amino-6-methylthiocaproic acid was established not only as a precursor of the glucosinolate but also as an intermediate in its biosynthesis.

The co-application of inactive 2-amino-6-methylthiocaproic acid in a competition experiment resulted not only in a reduced labelling of glucoraphenin but also in a pronounced decrease in the amounts of radioactive homomethionine and 2-amino-6-methylthiocaproic acid. Control mechanisms, *e. g.* a feedback inhibition of the chain elongation step, seem to be involved. Decreased radioactivity in these compounds was balanced by a higher incorporation into ethanol-insoluble leaf material and into S-methylmethionine and methionine sulphoxide.

Long time experiments gave less radioactivity in all amino acids, accompanied by an increase of labelling of ethanol-insoluble material, whereas the incorporation into 4-methylsulphinyl-3-butenylglucosinolate remained constant. Thus, a rapid turnover of the mustard oil glucoside in the plant is not very likely.

Surprisingly, far more radioactivity was found in homomethionine than in 2-amino-6-methylthiocaproic acid when L-methionine was fed. As a glucosinolate directly derived from homomethionine has not been reported for *Matthiola* species and no indications for the occurrence of such a glucoside could be found in our mustard oil glucoside fractions, a direct unbranched pathway homomethionine → 2-amino-6-methylthiocaproic acid → glucoraphenin appears to be most likely. Then our results would point towards different pool sizes of these two amino acids.

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