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γ-GLUTAMYL PEPTIDES IN THE BIOSYNTHESIS OF *S*-ALK(EN)YL-L-CYSTEINE SULPHOXIDES (FLAVOUR PRECURSORS) IN *ALLIUM*

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Key Word Index—*Allium cepa*; *A. sativum*; *A. siculum*; Alliaceae; onion; garlic; biosynthesis; *S*-alk(en)yl-L-cysteine sulphoxides; γ-glutamyl peptides.

Abstract—Pulse-chase experiments with $^{35}SO_4^{2-}$ fed for 10 min to leaves of *Allium cepa* (onion), *A. sativum* (garlic) and *A. siculum* showed that label appeared in γ-glutamyl peptides within 15 min, reached a maximum amount at 1 hr and had decreased by 6 hr. Label was not detected in free *S*-alk(en)yl-L-cysteine sulphoxides (flavour precursors) until 6 hr after pulse feeding, and then increased steadily for at least 24 hr and for onions until day 3. It is concluded that glutathione, and γ-glutamyl peptides are intermediates in the biosynthetic pathway to flavour precursors in *Allium* spp.

INTRODUCTION

Allium spp. characteristically contain a high concentration of non-protein sulphur amino acids (between 1 and 5% dry wt). One class of these secondary metabolites, the *S*-alk(en)yl-L-cysteine sulphoxides (flavour precursors) gives rise to the characteristic aroma and flavour of *Allium* spp. In the intact cell the sulphoxides are located in the cytoplasm and the hydrolytic enzyme alliinase in the vacuole [1]. Disruption of the cell results in hydrolysis of the sulphoxides to volatile sulphides.

Four sulphoxides are found to occur in *Allium* spp.: (+)-*S*-methyl-L-cysteine sulphoxide (methyl CSO), (+)-*S*-propyl-L-cysteine sulphoxide (propyl CSO), *trans*-(+)-*S*-(1-propenyl)-L-cysteine sulphoxide (propenyl CSO) and (+)-*S*-(2-propenyl)-L-cysteine sulphoxide, more commonly called allyl cysteine sulphoxide (allyl CSO). All *Allium* spp. contain methyl CSO. Propyl CSO and propenyl CSO are also present in onions and allyl CSO and propyl CSO in garlic. The ornamental *A. siculum* contains mainly methyl CSO, with minimal amounts of propyl CSO [2].

The quantitative and qualitative differences in flavour precursor content result in the different flavours of many of the *Allium* spp., particularly the presence or absence of propenyl CSO which is responsible for the lachrymatory effect of onions and allyl CSO which produces the characteristic taste of garlic.

Allium spp. also contain other non-volatile sulphur compounds, the γ-glutamyl peptides, which are not acted upon by alliinase and do not give rise to flavour. In *Allium* spp. 18 γ-glutamyl peptides of sulphur compounds have been identified by Virtanen and co-workers in Finland and Suzuki and co workers in Japan [3]. The significance of the γ-glutamyl peptides in the metabolism of the plant has been unclear. They have generally been considered to function as reserves of nitrogen and sulphur [3].

Early biosynthetic studies by Suzuki *et al.* on garlic [4–6] and by Granroth on onion [7] showed that the uptake of labelled sulphur compounds resulted in many labelled γ-glutamyl peptides, as well as flavour precursors. They also found that radioactivity from uniformly labelled valine was incorporated into propenyl CSO (in onion) and allyl CSO (in garlic). However, propyl and methyl cysteine sulphoxides were thought to be formed from a thioalkyl conjugation with serine. These early studies give rise to two important questions: (i) what is the biosynthetic relationship between peptides and free flavour precursors? Are the γ-glutamyl peptides intermediates to or metabolites of the flavour precursors. (ii) Do flavour precursors which are structurally homologous (i.e. with a C_3 alkyl chain) share a common pathway?

In order to answer these questions we have used pulse chase experiments with labelled sulphate $^{35}SO_4^{2-}$ to investigate the sequence of appearance of sulphur compounds and thus to determine the biosynthetic relationship between free flavour precursors and γ-glutamyl peptides. The results are reported below.

RESULTS

Pulse labelling of S *compounds in onion seedlings*

Radioactivity was found in the γ-glutamyl peptides (1.0 and 2.0 M HOAc fractions eluted from Dowex-1 ion-exchange columns) within 15 min of uptake of the $^{35}SO_4^{2-}$ pulse (Fig. 1). One hr after feeding, *ca* 35% of the total label in the leaf was present in γ-glutamyl peptides. Radioactivity in these fractions declined sharply by 24 hr and gradually thereafter to a level of 5% (2.0 M HOAc) and 2% (1.0 M HOAc) of the total label by day 7.

Radioactivity in the free flavour precursors (electrophoresed 0.1 M HOAc fraction) increased slowly and steadily up to 6 hr after feeding when it reached 7.8% of the total radioactivity (Fig. 1). Label increased to a maximum of 18.7% by day 3. Thus the pulse of labelled

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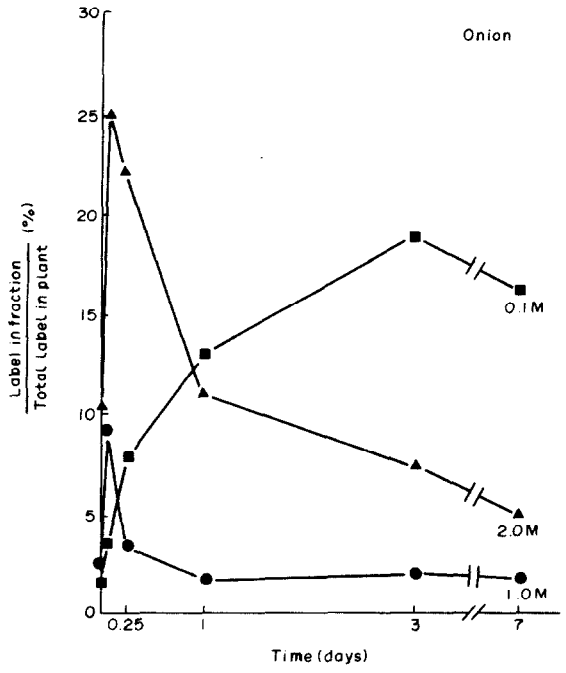

Fig. 1. Changes with time in incorporation of 10 min pulse of $^{35}SO_4^{2-}$ into *S* compounds of onion seedlings. Compounds separated on Dowex-1 ion-exchange column: 0.1 M HOAc fraction (■–■) was electrophoresed to remove cysteine and methionine and contains alk(en)yl cysteine sulphoxides; 1.0 M (●–■) and 2.0 M HOAc (Δ—Δ) fractions contain γ-glutamyl peptides of increasing acidity.

sulphur was incorporated into γ-glutamyl peptides before free flavour precursors.

The S compounds comprising each of the HOAc fractions were separated by TLC and radioactivity determined (Table 1). Glutathione and γ-glutamyl cysteine (overlap on TLC silica gel) were labelled most rapidly and strongly reaching a maximum of 248 × 1000 cpm/g fr. wt by 1 hr. By day 1 the amount of label had fallen to a low level of 23 × 1000 cpm/g fr wt which was maintained for the duration of the experiment. Radioactivity was detectable after 15 min. in compounds identified as methyl gluthathione, γ-glutamyl methyl cysteine, *S*-2-carboxypropyl glutatione and γ-glutamyl propenyl CSO, it reached a maximum after 1 hr, and had dispersed by 24 hr. A similar pattern was observed in unidentified compounds A and B (1.0 M HOAc) and E and H (2.0 M HOAc). Unidentified compound *J* (2.0 M HOAc) was very highly labelled at 1 hr (155 × 1000 cpm/g fr wt) and declined to very low levels by day 7 (similar to glutathione and γ-glutamyl cysteine).

In contrast to the peptides, the amount of label in the free sulphoxides was minimal until 6 hr after uptake of $^{35}SO_4^{2-}$ (Table 1). Over the 7 day duration of the experiment the amount of label increased (from the 6 hr levels) only slightly for methyl CSO (× 1.2), five-fold for propyl CSO and eight-fold for propenyl CSO.

Calculations of specific activities for each of the sulphoxides showed that after three days propenyl CSO was the most strongly labelled (859 × 1000 cpm/mg); three times more than propyl CSO and 10 × more than methyl CSO (Table 2). The three sulphoxides showed different trends in sp. act. Propyl CSO increased steadily in specific activity during seven days, whereas propenyl CSO dropped by *ca* 40% between day 3 and 7 and methyl CSO peaked after 24 hr, suggesting metabolism of these two compounds.

Pulse labelling of S *compounds in garlic and A. siculum*

In leaves of garlic, radioactivity was detectable in γ-glutamyl peptides (1.0 and 2.0 M HOAc fractions) after 1 hr (5%), reached a maximum at 6 hr (15%) and declined by 1 day (2%). Label in free sulphoxides (0.1 M HOAc) reached 1% by 24 hr (Fig. 2).

The pattern of labelling of garlic S compounds on TLC

Table 1. Changes with time in the amount of ^{35}S (cpm/g fr wt) in γ-glutamyl peptides and alk(en)yl cysteine sulphoxides of onion seedlings fed with a 10 min. pulse of $^{35}SO_4^{2-}$

Compound	Ion exchange fraction (HOAc) (M)	^{35}S (cpm × 10^3/g fr wt)					
		15 min	1 hr	6 hr	24 hr	3 day	7 day
Methyl CSO*	0.1	0	0	19	22	20	23
Propenyl CSO	0.1	0	0	37	121	280	319
Propyl CSO	0.1	0	0	11	36	83	60
γ-Glupropenyl CSO†	1.0	16	20	1	0	0	0
γ-Glumethyl cysteine	1.0	tr	13	1	0	0	0
Methyl glutathione	1.0	6	41	15	0	0	0
A	1.0	6	54	17	0	0	0
B	1.0	6	10	5	0	0	0
Glutathione } γ-Glucysteine }	2.0	89	248	100	23	25	24
E	2.0	0	71	13	7	0	0
S-2-Carboxypropyl glutathione	2.0	tr	63	57	7	0	0
H	2.0	34	116	19	9	0	0
J	2.0	41	155	56	21	14	4

*CSO Cysteine sulphoxide.
†γ-Glu, γ-Glutamyl.

Table 2. Changes with time in the specific activity (cpm/mg compound) of flavour precursors of onion, fed with a 10 min pulse of $^{35}SO_4^{2-}$

Flavour precursor	Sp. activity (cpmx $\times 10^3$/mg)					
	15 min	1 hr	6 hr	24 hr	3 day	7 day
Methyl CSO*	0	0	87	130	81	75
Propenyl CSO	0	0	171	557	859	495
Propyl CSO	0	0	40	130	236	404

*CSO, Cysteine sulphoxide.

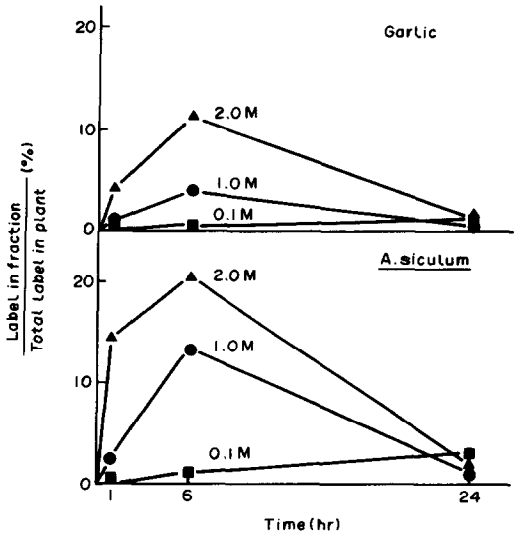

Fig. 2. Changes with time in incorporation of 10 min pulse of $^{35}SO_4^{2-}$ into *S* compounds of garlic and *A. siculum* leaves. Compounds separated on Dowex-1 ion-exchange column: 0.1 M HOAc fraction (■—■) was electrophoresed to remove cysteine and methionine and contains alk(en)yl cysteine sulphoxides; 1.0 M (●—●) and 2.0 M HOAc (▲—▲) fractions contain γ-glutamyl peptides of increasing acidity.

plates was very similar to that in onion since γ-glutamyl derivatives of allyl cysteine and allyl CSO are not resolved from propenyl analogues by silica gel TLC. However, total cpm was lower in garlic, probably because the leaves used were older, fully expanded and of lower metabolic activity.

Allium siculum contains methyl CSO as the major flavour precursor with minor amounts of propyl CSO. Feeding $^{35}SO_4^{2-}$ to *A. siculum* means that the S compounds on the pathway to methyl CSO are highlighted. As with onions, radioactivity appeared in the γ-glutamyl peptides (1.0 and 2.0 M HOAc) within 1 hr, reached a maximum of 33% by 6 hr and declined to 3% by 24 hr. Radioactivity in the free flavour precursors (0.1 M HOAc) was negligible at 1 hr, and increased gradually to 3% by 24 hr (Fig. 2).

A more detailed TLC analysis of the labelled sulphur compounds in the HOAc fractions showed that label was detectable in glutathione, γ-glutamyl cysteine, methyl glutathione and γ-glutamyl methyl cysteine after 1 hr, reached a maximum at 6 hr and declined by 24 hr (Table 3). Eight unidentified sulphur compounds showed a pattern of early labelling which had dropped off by 24 hr. However, neither sulphur label, nor amino acid was detectable at R_f's corresponding to those of γ-glutamyl propenyl CSO and S-2-carboxypropyl glutathione. The amount of radioactive label detectable in methyl CSO increased 12-fold from 1 to 24 hr (Table 3).

DISCUSSION

The results above for onion, garlic and *A. siculum* showed that the pulse of labelled sulphur was first incorporated into γ-glutamyl peptides from which it was rapidly lost. It was later incorporated into free flavour precursors where it was maintained before slowly decaying. The results indicate that γ-glutamyl peptides are intermediates on the pathway to flavour precursors rather than metabolites of flavour precursors.

Any biosynthetic pathway from sulphate to flavour precursors needs to account for: (i) known pathways of sulphate assimilation and reduction, and the glutathione cycle; (ii) the pulse-labelling of γ-glutamyl peptides before free sulphoxides; (iii) the pulse-labelling patterns in *A. siculum* and the similar labelling of peptides in garlic and onion.

The biosynthetic scheme outlined in Fig. 3 meets these criteria. Sulphate is reduced and assimilated into cysteine and thence into the glutathione cycle [8]. γ-Glutamyl cysteine and glutathione are the first peptides labelled and are proposed as the starting compounds for the pathway to all sulphoxides. Methylation of glutathione produces the tripeptide methyl glutathione; when the glycine moiety is cleaved the result is γ-glutamyl methyl cysteine. Oxidation of the sulphur produces γ-glutamyl methyl cysteine sulphoxide. The final step is the hydrolysis of the γ-glutamyl residue to produce methyl CSO. Labelled methyl glutathione and γ-glutamyl methyl cysteine were identified in each of the *Alliums* within 1 hr of feeding $^{35}SO_4^{2-}$. γ-Glutamyl methyl cysteine may also be formed directly from methylation of γ-glutamyl cysteine. Label in the former compound decayed faster than label in methyl glutathione, suggesting that label to γ-glutamyl methyl cysteine may be supplied by an alternative source, such as γ-glutamyl cysteine.

Granroth [7] had shown in onions and Suzuki *et al.* in garlic [4–6] that ^{14}C-labelled valine gave rise to the intermediate methacrylic acid which could react with glutathione to produce S-2-carboxypropyl glutathione (2CPGTH). Labelled 2CPGTH was detectable in both onion and garlic within 1 hr but not in *A. siculum*. We suggest that 2CPGTH gives rise to the remaining 3 flavour precursors, allyl CSO, propenyl CSO and propyl CSO via (i) cleavage of the glycine moiety to produce γ-glutamyl S-2-carboxypropyl cysteine, (ii) decarboxy-

Table 3. Changes with time in the amount of ^{35}S (cpm/g fr wt) in γ-glutamyl peptides and alk(en)yl cysteine sulphoxides of *A. siculum* leaves fed with a 10 min pulse of $^{35}SO_4^{2-}$

Compound	Ion exchange fraction (HOAC) (M)	^{35}S(cpm × 10^3/g fr wt) 1 hr	6 hr	24 hr
Methyl CSO*	0.1	8	31	102
†γ-Glumethyl cysteine	1.0	4	7	0
Methyl glutathione	1.0	trace	14	0
A	1.0	31	16	0
B	1.0	0	49	0
C	1.0	0	55	0
Glutathione) γ-Glucysteine)	2.0	32	37	5
F	2.0	60	77	8
G	2.0	17	30	6
H	2.0	15	10	0
I	2.0	1	10	0
K	2.0	53	68	6

*CSO, Cysteine sulphoxide.
†γ-Glu, γ-Glutamyl.

lation of the alkyl chain to produce either allyl or propenyl residues, (iii) reduction of the allyl residue to propyl (as would happen in garlic) or of the propenyl residue to propyl (as would happen in onion), (iv) oxidation of the γ-glutamyl alk(en)yl cysteine to the sulphoxides, (v) hydrolysis of the γ-glutamyl residue to produce free allyl, propyl or propenyl cysteine sulphoxide.

γ-Glutamyl-*S*-2-carboxypropyl cysteine may also be formed directly from the reaction of γ-glutamyl cysteine with methacrylic acid. Evidence for this alternative route is provided by the fact that label moved more quickly through γ-glutamyl propenyl CSO than it did through the upstream compound 2CPGTH.

All of the intermediates in the proposed pathway with the exception of γ-glutamyl allyl cysteine sulphoxide and methyl glutathione have been previously isolated from either onion, garlic or chives [3]. In an earlier paper, we could not detect methyl glutathione above levels of 0.005 mg/g fr wt and thus the compound was used as an internal standard for recovery of γ-glutamyl peptides from 1.0 M HOAc fractions [9]. Evidence in this paper suggests that methyl glutathione is a highly labelled intermediate but present in very small amounts.

Although the pulse of *S* moved quickly through the γ-glutamyl peptides it was accumulated in the free sulphoxides during the first 3 days of the experiment. The specific activity of ^{35}S in propenyl CSO fell by half between days 3 and 7, providing evidence for the metabolism of this compound. However, the enzymes involved in the metabolism of sulphoxides remain unknown.

Sugii *et al.* [5] and Granroth [7] have suggested that methyl CSO is formed by either methylation of cysteine or thioalkylation of serine and that propyl CSO is formed by thioalkylation of serine. Although this does not appear to be the main route to these sulphoxides one cannot rule out the possibility that it may be a route under particular circumstances (e.g. if methionine is the sole sulphur source for the plant).

In the biosynthetic pathway outlined in Fig. 3, the enzyme γ-glutamyl transpeptidase has an important function in hydrolysing the γ-glutamyl moiety to produce the free flavour precursors. The pH optimum of the enzyme in the hydrolytic mode is 6.5 [10]. When the enzyme is not active one would expect the peptides to accumulate. This is found in stored bulb material where there is no transpeptidase activity but high levels of peptides. During sprouting the enzyme is synthesized, the final step in the pathway is activated and peptides are metabolised to free flavour precursors [11]. γ-Glutamyl transpeptidase is also very active in germinating *Allium* seeds, and peptides in the seed are metabolized to free flavour precursors during germination [12, 13].

Glutathione is a key metabolite in the proposed pathway to the free sulphoxides. It has been suggested that photosynthetic tissues carry out net synthesis of glutathione for export to the rest of the plant and that chloroplasts may be the site of this synthesis [8, 14]. Roots although capable of producing some glutathione are thought to be unable to synthesize sufficient organic *S* for their own requirements [15]. In intact onion plants, green leaves function as the major site of synthesis of flavour precursors [16]. However, in both garlic and onion plants, bulb tissue and roots have been shown to be capable of flavour precursor synthesis [5–7]. The cellular localization of glutathione and the biosynthetic pathway of flavour precursors in onions are currently being investigated in this laboratory.

Three of the onion γ-glutamyl peptides and five of the γ-glutamyl peptides in *A. siculum* are unidentified. Work is continuing on identifying these putative intermediates, and isolating the enzymes involved in their metabolism.

EXPERIMENTAL

Material. Onion (*A. cepa* cv Pukekohe Long Keeper) seeds, garlic (*A. sativum*) cloves and *A. siculum* bulbs were raised in a growth chamber at 75% relative humidity, with day temperatures 21 ± 1.5° for 12 hr and an abrupt change to 14 ± 2.0° for the 12 hr night. Irradiance was 200 ± 30 μmol/m^2/sec. Plants were watered with half strength New North Carolina nutrient soln [17]. Fully expanded leaves of garlic and *A. siculum* and the first true leaf of onion seedlings were used for the feeding experiments. $^{35}SO_4^{2-}$ was purchased from the Radiochemical Centre, Amersham, U.K.

Feeding methods. Labelled $Na_2^{35}SO_4$ (1078 Ci/mmol = 2.04 mCi/ml) was fed to cut leaves of each species for 10 min (*ca* 20 μCi/g fr. wt). Leaves were rinsed and maintained on nutrient soln in the growth chamber for 15 min, 1 hr, 6 hr, 24 hr, 3 days, 7 days, for onions and 1 hr, 6 hr, 24 hr for garlic and *A. siculum*.

Isolation of products. Leaves (*ca* 1.5 g fr. wt) were extracted twice in MeOH–$CHCl_3$–H_2O (12:5:3) and once in 80% EOH at −20°. The extractants were combined and phases separated by the addition of $CHCl_3$ and H_2O. The aq phase was separated by Dowex-1 ion-exchange chromatography. Flavour precursors were eluted with 0.1 M HOAc and further purified on cellulose plates by electrophoresis to remove other amino acids (particularly cysteine and methionine) and organic acids. γ-Glutamyl peptides were eluted in 1.0 M HOAc and 2.0 M HOAc and separated by TLC on silica gel in solvent I (MeCOEt–pyridine–H_2O–glacial HOAc 8:15:15:2) and then in solvent II (*n*PrOH–H_2O–propyl acetate–HOAc–pyridine 120:60:20:4:1) in the same direction. Compounds were visualized with ninhydrin. Flavour precursors in the electrophoresed 0.1 M HOAc were separated on cellulose plates run in

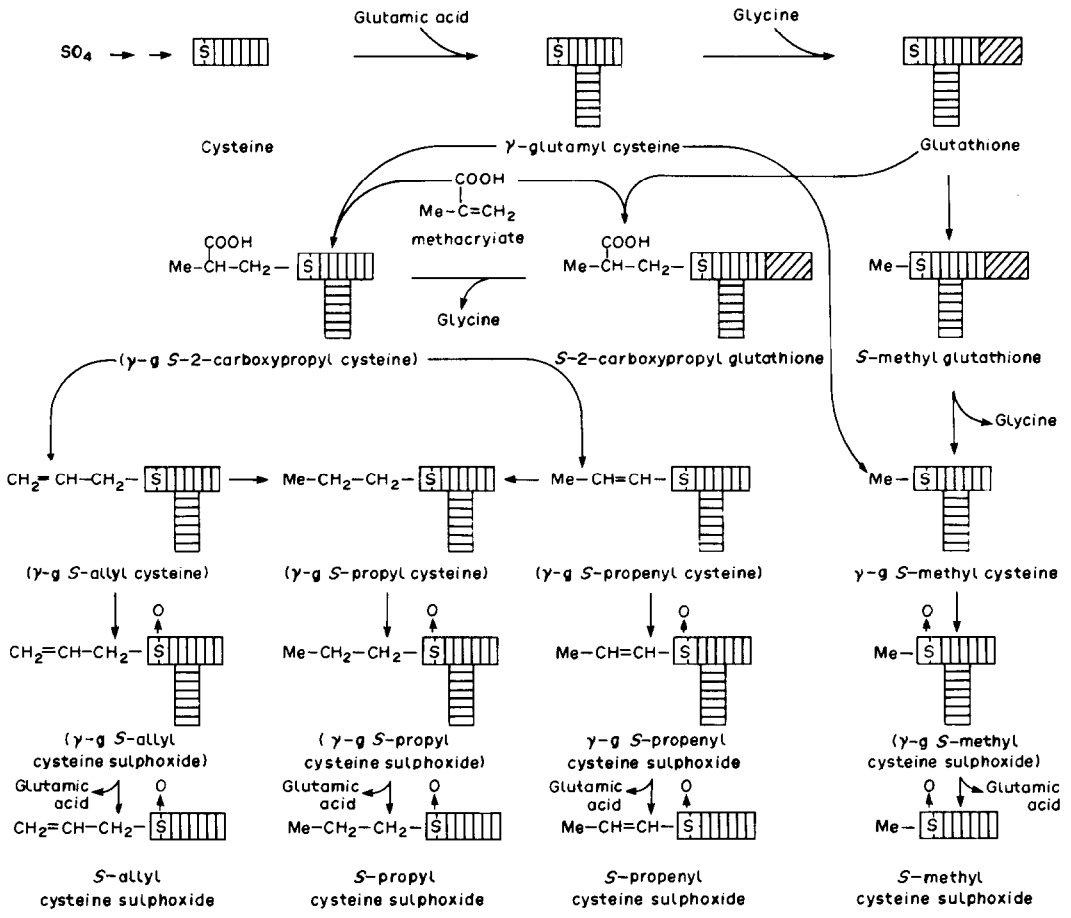

Fig. 3. Proposed pathway of alk(en)yl cysteine sulphoxide biosynthesis.

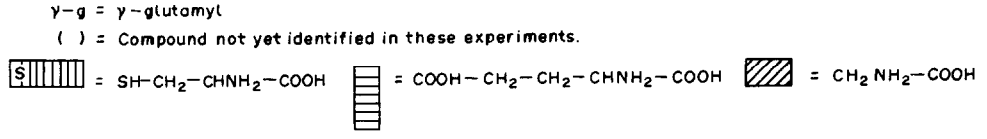

Fig. 3.

BUOH–HOAc–H_2O (12:3:5). They were visualized with fluorescamine.

Quantitation was by scanning densitometry. Recoveries were computed by the addition of known amounts of *S*-butyl cysteine sulphoxide (for 0.1 M fraction), methyl glutathione (1.0 M HOAc) and γ-glutamyl glutamic acid (2.0M HOAc) to each sample. Analytical details are described in refs [9, 18].

Radiochemical methods. Portions of tissue residue, $CHCl_3$ fraction, 0.1–2.0 M HOAc fractions and electrophoresed 0.1 M HOAc fraction were added to a Triton X-100 scintillant [19]. ^{35}S was radioassayed by liquid scintillation counting using a ^{14}C window and ext. standard technique [20]. To reduce quenching $CHCl_3$ was evapd before adding scintillant and pigments were bleached [21]. Any residual quenching was corrected for with a chemical and optical quench curve. Counts from each extract were summed to give total counts/g fr wt for each sample.

Labelled compounds, separated on TLC plates, as described above were measured in the following way. TLC plates were dipped in a commercial fluor, dried and exposed to Kodak X-Omat AR film at −70° for 24 hr [22]. The X-ray film was developed and image intensity was determined by a scanning densitometer and calibrated against images of known radioactivity.

Identification of compounds labelled with ^{35}S. Free flavour precursors and several of the γ-glutamyl peptides have been either purchased (glutathione, methyl glutathione) or synthesized (methyl CSO, allyl CSO, propenyl CSO, propyl CSO, γ-glutamyl methyl cysteine, γ-glutamyl cysteine) or isolated from *Allium tissue* (γ-glutamyl propenyl CSO, *S*-2-carboxypropyl glutathione). Their identity has been confirmed by GC-MS and NMR [Lane, G. A., Shaw, M. L. and Lancaster, J. E. unpublished work]. Extracts were co-run on silica gel and cellulose TLC with γ-glutamyl peptide and flavour precursor standards; some of the labelled compounds have yet to be identified. The pattern of elution of compounds on Dowex 1 and R_f on TLC of the ^{35}S labelled compounds is shown in Table 4.

Table 4. Elution on ion-exchange (Dowex 1) and R_f on silica gel and cellulose TLC for ^{35}S labelled intermediates in the biosynthetic pathway to flavour precursors in garlic, onion and *A. siculum*

	Ion exchange fraction (HOAc (M)	Silica gel‡ (R_f)	Cellulose§ (R_f)
Methyl CSO*	0.1	—	0.30
Propenyl CSO	0.1	—	0.47
Allyl CSO	0.1	—	0.47
Propyl CSO	0.1	—	0.49
+γ-Glupropenyl CSO	1.0	0.32	0.33
γ-Glumethyl cysteine	1.0	0.37	0.39
Methyl glutathione	1.0	0.43	0.42
A	1.0	0.49	0.48
B	1.0	0.51	—
C	1.0	0.57	0.56
Glutathione	2.0	{0.17}	0.17
γ-Glucysteine	2.0	—	0.18
E	2.0	0.24	0.33
F	2.0	0.25	0.36
S-2-Carboxypropyl glutathione	2.0	0.38	0.42
G	2.0	0.42	0.49
H	2.0	0.45	0.55
I	2.0	0.48	0.65
J	2.0	0.53	0.64
K	2.0	0.56	0.68

*CSO Cysteine sulphoxide

γ-Glu, γ-Glutamyl

‡Solvent system I MeCOEt–pyridine–H_2O–glacial HOAc (8:15:15:2). II *n*PrOH–H_2O–propylacetate–HOAc–pyridine (120:60:20:4:1).

§Solvent BuOH–HOAc–H_2O (12:3:5).

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