ChemBioChem

Research Article
doi.org/10.1002/cbic.202300056

Chemistry Europe
European Chemical Societies Publishing

www.chembiochem.org

VIP *Very Important Paper*

Isotope-Guided Metabolomics Reveals Divergent Incorporation of Valine into Different Flavor Precursor Classes in Chives

Ricarda Freke[+],[a] Björn Heinemann[+],[b] Samuel Edward Hakim,[c, d] Claus-Peter Witte,[e] Marco Herde,*[e] Tatjana M. Hildebrandt,*[b] and Jakob Franke*[c, d]

Plants of the genus *Allium* such as chives, onions or garlic produce *S*-alk(en)yl cysteine sulfoxides as flavor precursors. Two major representatives are *S*-propenyl cysteine sulfoxide (isoalliin) and *S*-propyl cysteine sulfoxide (propiin), which only differ by a double bond in the C_3 side chain. The propenyl group of isoalliin is derived from the amino acid valine, but the source of the propyl group of propiin remains unclear. Here, we present an untargeted metabolomics approach in seedlings of chives (*Allium schoenoprasum*) to track mass features containing sulfur and/or ^{13}C from labeling experiments with valine-$^{13}C_5$ guided by their isotope signatures. Our data show that propiin and related propyl-bearing metabolites incorporate carbon derived from valine-$^{13}C_5$, but to a much lesser extent than isoalliin and related propenyl compounds. Our findings provide new insights into the biosynthetic pathways of flavor precursors in *Allium* species and open new avenues for future untargeted labeling experiments.

Introduction

As the major stable flavor precursors of onions, garlic, leek, chives and related species of the genus *Allium*, *S*-alk(en)yl cysteine sulfoxides are prime examples of plant natural products with almost daily relevance to humans. The four most common representatives are *S*-allyl cysteine sulfoxide (alliin, **1**), *S*-*trans*-prop-1-enyl cysteine sulfoxide (isoalliin, **2**), *S*-propyl cysteine sulfoxide (propiin, **3**) and *S*-methyl cysteine sulfoxide (methiin, **4**; Figure 1).[1,2] *Allium* plants containing alliin (**1**) typically produce no or only trace amounts of isoalliin (**2**) and vice versa.[3] In addition, *S*-ethyl cysteine sulfoxide (ethiin, **5**) and *S*-butyl cysteine sulfoxide (butiin, **6**) occur in few *Allium* species as minor components.[2] The molecular mechanisms by which these compounds are activated by the enzyme alliinase to produce volatile sulfur flavor compounds that exert various biological effects are already well understood, thanks to decades of vigorous investigation.[2,4–6] In contrast, how these flavor precursors are produced in *Allium* plants is only partially

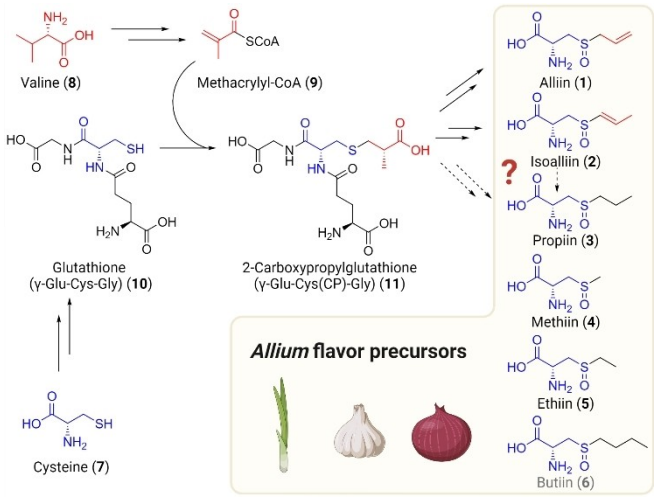

Figure 1. Biosynthesis of the flavor precursor *S*-alk(en)yl cysteine sulfoxides **1**–**6** in *Allium* plants. The C_3 side chains of alliin (**1**) and isoalliin (**2**) are derived from valine (**8**), but the link to the saturated analog propiin (**3**) is not yet clear. The conversion of valine (**8**) to the C_3 side chains of alliin (**1**) and isoalliin (**2**) probably involves 2-carboxypropyl key intermediates such as 2-carboxypropylglutathione (**11**).

[a] *R. Freke[+]*
Institute of Plant Genetics, Leibniz University Hannover
Herrenhäuser Str. 2, 30419 Hannover (Germany)
[b] *Dr. B. Heinemann,[+] Prof. Dr. T. M. Hildebrandt*
Institute for Plant Sciences
Cluster of Excellence on Plant Sciences (CEPLAS)
University of Cologne
Zülpicher Str. 47 B, 50674 Cologne (Germany)
E-mail: t.hildebrandt@uni-koeln.de
[c] *S. E. Hakim, Prof. Dr. J. Franke*
Institute of Botany, Leibniz University Hannover
Herrenhäuser Str. 2, 30419 Hannover (Germany)
E-mail: jakob.franke@botanik.uni-hannover.de
[d] *S. E. Hakim, Prof. Dr. J. Franke*
Centre of Biomolecular Drug Research (BMWZ)
Leibniz University Hannover
Schneiderberg 38, 30167 Hannover (Germany)
[e] *Prof. Dr. C.-P. Witte, Dr. M. Herde*
Institute of Molecular Nutrition and Biochemistry of Plants
Leibniz University Hannover
Herrenhäuser Str. 2, 30419 Hannover (Germany)
E-mail: mherde@pflern.uni-hannover.de
[+] *These authors contributed equally to this work.*

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbic.202300056

This article is part of the Special Collection ChemBioTalents2022. Please see our homepage for more articles in the collection.

understood. Based on labeling experiments with radioactively labeled amino acids, it was established that all of these compounds are derived from the sulfur-containing amino acid cysteine (**7**) as the source of sulfur and part of their carbon backbone (Figure 1).[2] The allyl/propenyl side chains of alliin (**1**) and isoalliin (**2**) were traced back to the amino acid valine (**8**).[2,7] Modified glutathione-like and glutamyl peptides were identified as putative key intermediates in the pathway.[8–10] The transformation of valine to the C_3 side chain likely involves addition of the valine catabolism intermediate methacrylyl-CoA (**9**)[11] to glutathione (**10**) to yield *S*-(2-carboxypropyl) glutathione (γ-Glu–Cys(CP)–Gly, **11**); this intermediate is then hypothesized to undergo an unusual oxidative decarboxylation towards alliin (**1**) or isoalliin (**2**).[8,12–14] In contrast, the biosynthetic origin of the propyl side chain of propiin (**3**) remains unclear.[2,7] In light of the structural similarity to alliin (**1**) and isoalliin (**2**), it has been proposed that the propyl side chain of propiin (**3**) is obtained by reduction of allyl or propenyl side chains of alliin (**1**), isoalliin (**2**) or their precursors, and hence is ultimately also derived from valine (**8**). However, the enzymatic hydrogenation of C=C double bonds with limited activation is biosynthetically unusual. Also, the existence of the other saturated *S*-alkyl cysteine sulfoxides methiin (**4**), ethiin (**5**) and butiin (**6**), whose origin from γ-Glu–Cys(CP)–Gly (**11**) is improbable, raises the question if there is a different biosynthetic route to propiin (**3**) and other *S*-alkyl cysteine sulfoxides.

In recent years, advances in mass spectrometry and metabolomics have revolutionized the way how complex metabolite mixtures and metabolic pathways can be investigated.[15–19] Previous studies used high resolution metabolomics to identify sulfur-containing metabolites in *Allium* plants;[20,21] universal ^{13}C-labeling was employed to determine the elemental composition of unknown ions.[22] Similar strategies were also applied to other plants to identify metabolites containing sulfur or nitrogen in an untargeted manner.[23,24] However, so far no selective stable isotope labeling experiments combined with untargeted metabolomics were conducted to shed light on *S*-alk(en)yl cysteine sulfoxide biosynthesis in *Allium* plants. Here, we developed an untargeted metabolomics workflow to investigate *S*-alk(en)yl cysteine sulfoxide biosynthesis in seedlings of chives (*Allium schoenoprasum*). We demonstrate how untargeted isotopolog detection enables the reliable detection of mass features that natively contain sulfur, incorporate ^{13}C carbon labels from fed valine-$^{13}C_5$, or a combination of both in a quick and unbiased manner. With this approach, we prove that the propyl side chains of propiin (**3**) and four other propyl-bearing metabolites are indeed derived from valine, but surprisingly the incorporation of labeled carbons was much weaker compared to isoalliin (**2**) and the four other corresponding propenyl analogs. As such, our findings shed new light on the biosynthetic relationship of the flavor precursors isoalliin (**2**) and propiin (**3**) in *Allium* species, and our untargeted labeling method sets the basis for future pathway elucidation.

Jakob Franke has been intrigued by natural products since his studies of biochemistry and chemistry in Munich (Germany). After his PhD in the group of Prof. Christian Hertweck (Jena, Germany) on bacterial metabolites, he switched to plant natural product research during his postdoctoral research in the group of Prof. Sarah O'Connor (John Innes Centre, Norwich, UK). The research of his group at Leibniz University Hannover (Germany) is now focused on understanding the biosynthetic pathways of plant natural products and harnessing this biochemical knowledge for metabolic engineering to improve biotechnological access to plant specialized metabolites.

Results and Discussion

To develop a workflow for untargeted investigation of *S*-alk(en)yl cysteine sulfoxide biosynthesis in *Allium* species, we first set out to establish an in vitro culture system that permits facile feeding of stable isotope-labeled precursors via the medium. Previous studies were based on excised garlic roots or full onion plants,[13,14,25] but these approaches were not considered as ideal for labeling studies due to the relatively large biomass involved, requiring unnecessarily large quantities of labeled substrates. Instead, we first attempted to employ a callus culture of onions as our model system. Although we succeeded in the generation of an onion callus culture, no alliin (**1**) or isoalliin (**2**) could be detected in extracted calli. This finding is in agreement with observations by others,[26–29] and excluded onion callus cultures as a suitable model system, despite their ready availability. We therefore turned our attention to seedlings obtained by in vitro germination. Due to the good availability of seeds, we selected chives (*A. schoenoprasum*) as our model system. Gratifyingly, chive seeds germinated readily in liquid as well as solid Murashige & Skoog (MS) medium. Further experiments were performed in liquid MS medium, as it allowed easier handling and substrate feeding. Chive seeds started to germinate at around 3 days post inoculation (dpi), and seedlings were afterwards grown for up to 28 days (31 dpi). The presence of isoalliin (**2**) as the major cysteine sulfoxide between 10 dpi and 24 dpi was confirmed by LC-MS (Figure S1 in the Supporting Information). As the seedlings started to outgrow the liquid medium after 18–19 dpi, it was decided to harvest further samples at 17 dpi. Taken together, this suggested that chive seedlings are indeed a suitable model system to investigate *S*-alk(en)yl cysteine sulfoxide biosynthesis.

Next, we wanted to establish an untargeted metabolomics approach that can automatically identify sulfur-containing metabolites based on the presence of ^{34}S isotopologs (Figure 2). This is made possible by state-of-the-art mass spectrometers that provide sufficiently high spectral resolution to allow distinguishing $^{13}C_2$ and ^{34}S isotopolog peaks.[19] Measurements with an Orbitrap mass analyzer at a spectral resolution of 140 000 provided baseline resolution of ^{34}S and $^{13}C_2$ peaks up to *m/z* 396, corresponding to one of the largest known metabo-

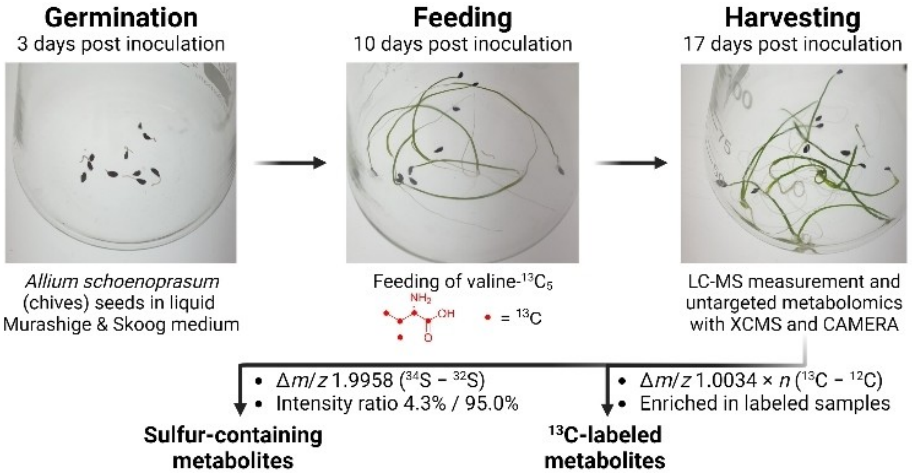

Figure 2. Experimental workflow for stable isotope labeling and metabolomics of chive (*A. schoenoprasum*) seedlings. Samples were analyzed by LC-MS-based untargeted metabolomics using XCMS[30] and CAMERA[31] to identify features containing sulfur and/or incorporating carbon from ^{13}C-labeled precursors (here: valine-$^{13}C_5$) based on their isotope signatures.

lites of the pathway (Figure S2). To ensure that also the chromatographic separation was suitable for analysis of highly polar metabolites, we used a C_{18} column with polar endcapping. Indeed, with this column and an optimized chromatographic method starting at 100% water, we achieved good separation of the major, highly polar sulfoxides isoalliin (**2**), propiin (**3**) and methiin (**4**; Figure 3). We then developed a custom R script based on previous work by Nett *et al.*[32] to automatically identify sulfur-containing mass features based on the following criteria: 1) Co-occurrence of a coeluting feature shifted by $\Delta m/z$ 1.9958, corresponding to an ^{34}S instead of ^{32}S isotope signature. 2) An intensity ratio of both mass features close to the natural isotope abundance of 95.0%/4.3% (or multiples of this value in the case of multiple sulfur atoms). Mass features were identified using the XCMS package,[30] followed by deconvolution with CAMERA[31] to reduce redundancy from adducts and undesired isotope peaks. Then, a pairwise comparison of all mass features following the strategy by Nett *et al.*[32] was performed to extract features exhibiting the desired mass shift and intensity ratio. After individual optimization of parameters and thresholds for XCMS and isotope filtering, we detected 162 sulfur-containing features (Figure 3).

Next, we wanted to combine our metabolomics method with selective stable isotope labeling to shed light on the biosynthetic origin of propiin (**3**) in a global and untargeted manner. From past experiments with radioactively labeled valine it is known that the C_3 side chain of alliin (**1**) and isoalliin (**2**) is most likely derived from this amino acid via the intermediate γ-Glu–Cys(CP)–Gly (**11**) and/or (2-carboxypropyl)cysteine (Cys(CP)) (**12**),[2,10,14] but it is not yet clear if there is also a link to propiin (**3**). We administered 1 mM valine-$^{13}C_5$ to the culture medium after 10 dpi (i.e., 1.2 mg per 10 mL culture). Labeled cultures as well as unlabeled control cultures were harvested at 17 dpi and analyzed by LC-MS (Figure 2). We adjusted our R script to not only search for sulfur-containing metabolites, but likewise to detect co-eluting mass features with a mass shift of multiples of $\Delta m/z$ 1.0034, corresponding to the mass difference between ^{13}C and ^{12}C. This resulted in 366 features suggestive of isotope labeling events, either because heavy features appeared in labeled samples that were absent in unlabeled samples, or because heavy features were at least tenfold enriched in labeled samples compared to unlabeled samples. To link mass features from our metabolomics study to metabolites, we identified putative sum formulas and searched for potentially matching metabolites from previous studies on *Allium* metabolites and in Reaxys. Wherever possible, metabolites were verified by comparison of retention times and MS/MS spectra to authentic reference compounds (Table S1). To provide structural support for metabolites without available reference compounds, we harnessed parallel reaction monitoring (PRM)[33] based on an inclusion list of about 60 prioritized ions to obtain MS/MS spectra of key metabolites. This enabled us to successfully obtain reliable MS/MS spectra even for low abundance ions and labeled isotopologs from crude *Allium* extracts (Figures S3–S25). In total, we could confidently identify 23 metabolites (Figure 3 and Table S2). These included the following groups of metabolites: (Group I) Cysteine sulfoxides isoalliin (**2**), propiin (**3**) and methiin (**4**) as well as the deoxy precursors deoxyisoalliin (Cys(Propenyl) (**13**) and deoxypropiin (Cys(Propyl)) (**14**); (group II) glutamyl peptides[9] γ-Glu–Cys(Propenyl) (**15**), γ-Glu–Cys(Propyl) (**16**), γ-Glu–Cys(Propenyl) sulfoxide (**17**) and γ-Glu–Cys(Me) (**18**); (group III) 2-carboxypropyl intermediates γ-Glu–Cys(CP)–Gly (**11**),[14] Cys(CP) (**12**)[14] and Cys(CP)–Gly (**19**);[20] (group IV) pathway-related disulfides[20] Cys(SPropenyl) (**20**), Cys(SPropyl) (**21**), γ-Glu–Cys(SPropenyl)-Gly (**22**) and γ-Glu–Cys(SPropyl)–Gly (**23**); (group V) labeled primary metabolites linked to valine metabolism, that is, valine (**8**), leucine (**24**), pantothenic acid (**25**) and pantetheine (**26**); (group VI) unlabeled sulfur metabolites glutathione (**10**), methionine (**27**) and methylthioadenosine (**28**). Methiin (**4**) was found as a sulfur-containing metabolite, but did not show any carbon enrichment from labeling.

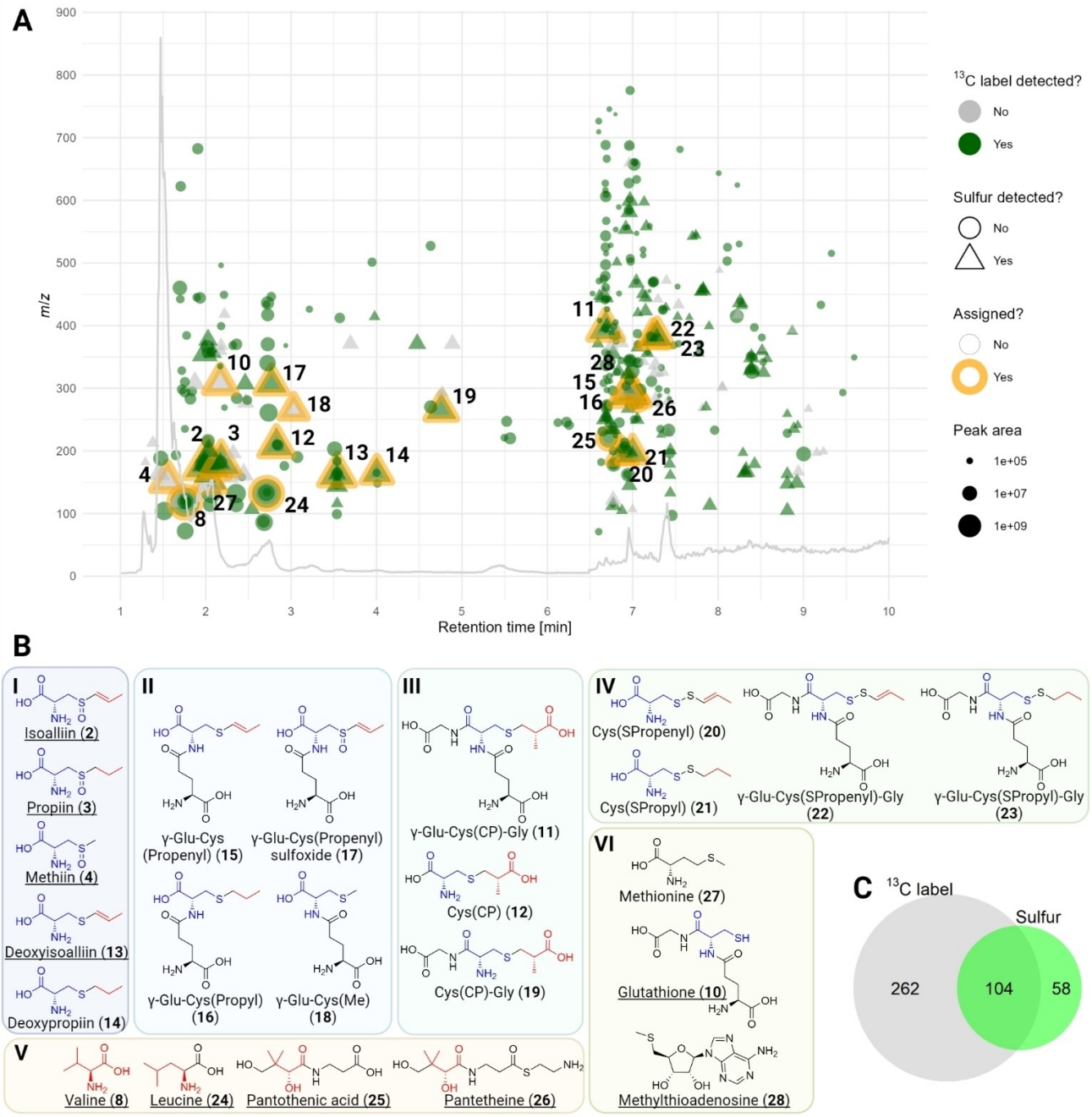

Figure 3. Identification of 23 metabolites from *A. schoenoprasum* by isotope-guided untargeted metabolomics. A) Cloud plot of mass features that natively contain sulfur (triangle vs. circle) and/or incorporate ^{13}C from valine-$^{13}C_5$ (green vs. gray). Compounds assigned to metabolites based on comparison to authentic reference compounds or analysis of MS/MS spectra are highlighted in gold. The background shows a representative base peak chromatogram. B) Structures of identified metabolites grouped by structural similarities or properties. Group I: Cysteine sulfoxides and deoxy precursors; group II: glutamyl peptides; group III: 2-carboxypropyl (CP) intermediates; group IV: disulfides; group V: labeled primary metabolites related to valine metabolism; group VI: unlabeled sulfur metabolites. Underlined metabolites were identified by using reference compounds; all other metabolites were supported by MS/MS data. C) Venn diagram showing numbers of detected features containing sulfur and/or ^{13}C labeling.

Importantly, the top hits from our labeling dataset included the expected +3 labeling event for isoalliin (**2**), but also +3 labeling of propiin (**3**) (Figure 3). This finding supports the previous proposal that propiin (**3**) follows the biosynthetic route of isoalliin (**2**).[7] In addition, it shows that *S*-alk(en)yl cysteine sulfoxides are actively produced in chive seedlings de novo and not just obtained from seeds.

To get further insights into the connection between propenyl- and propyl-containing cysteine metabolites, we next analyzed the incorporation of ^{13}C labels into the different metabolites identified by us in more detail (Figure 4). Isotopo-

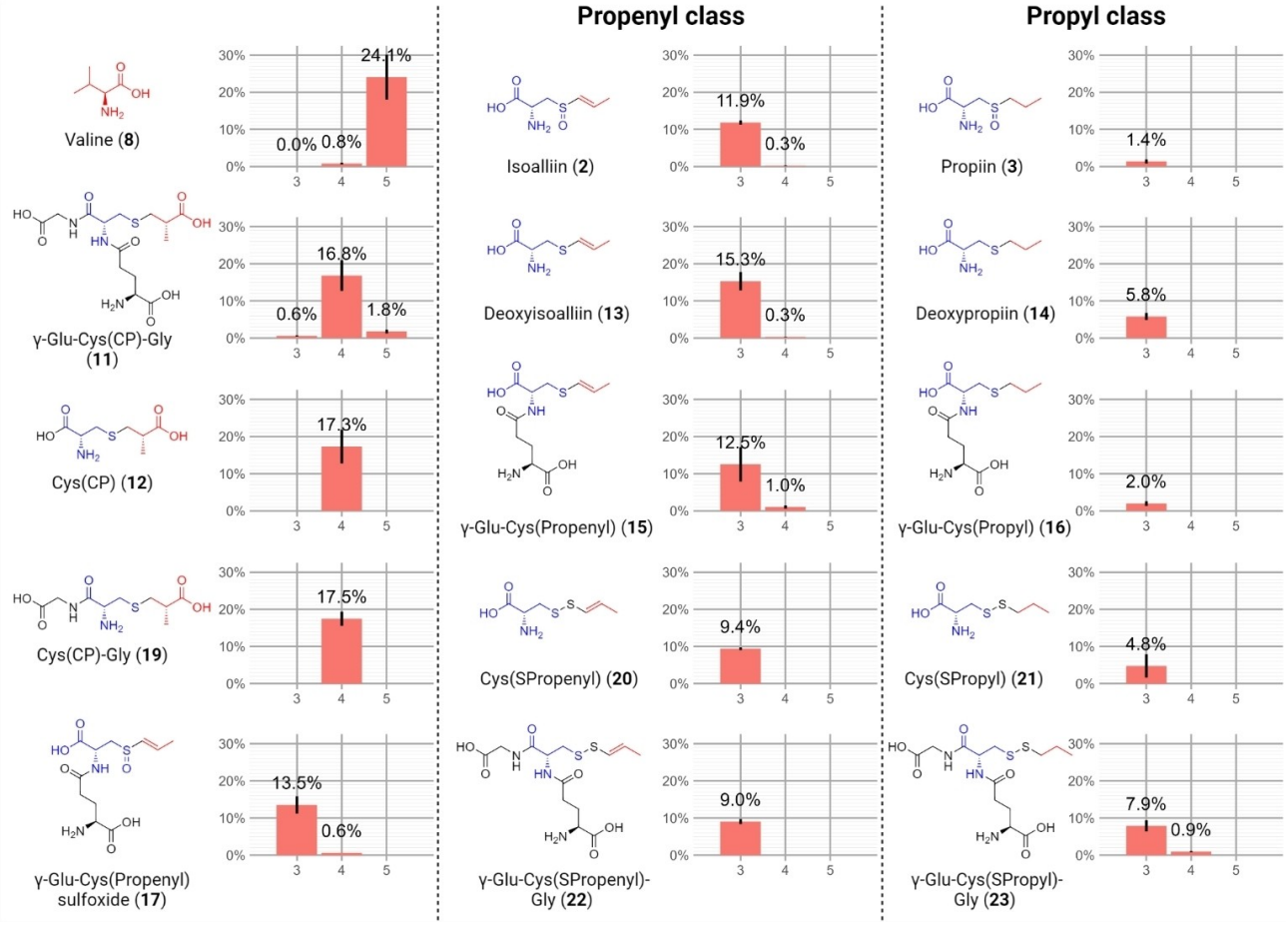

Figure 4. Isotopolog analysis of labeled metabolites reveals different degrees of labeling between propenyl-containing metabolites **2**/**13**/**15**/**20**/**22** and their propyl-bearing analogs **3**/**14**/**16**/**21**/**23**. The bar plots show peak areas of +3/+4/+5 isotopologs (1.0034×*n*) relative to the main ^{12}C isotopolog peak from three replicate samples. Isotopologs not shown were not detected. Error bars are ± standard deviation ($n = 3$).

log peaks were picked and integrated with XCMS to determine and plot the isotope patterns in R. The robustness of this approach was verified by inspection of extracted ion chromatograms (Figures S26–S30) and manual integration. The highest isotopolog enrichment was found – not surprisingly – for valine itself with 24.1% at +5. The three metabolites containing 2-carboxypropyl (CP) groups, namely γ-Glu–Cys(CP)–Gly (**11**), Cys(CP) (**12**) and Cys(CP)–Gly (**19**), showed +4 labeling events at comparable intensities of 16.8%, 17.3% and 17.5%, respectively. In addition, we compared five pairs of analogous propenyl vs. propyl metabolites: 1) Isoalliin (**2**) vs. propiin (**3**); 2) deoxyisoalliin (**13**) vs. deoxypropiin (**14**); 3) γ-Glu–Cys(Propenyl) (**15**) vs. γ-Glu–Cys(Propyl) (**16**); 4) Cys(SPropenyl) (**20**) vs. Cys(SPropyl) (**21**); 5) γ-Glu–Cys(SPropenyl)–Gly (**22**) vs. γ-Glu–Cys(SPropyl)–Gly (**23**). Strikingly, for most of these pairs the incorporation of +3 labels was drastically lower for propyl compounds than for propenyl metabolites, particularly for isoalliin (**2**; 11.9%) vs. propiin (**3**; 1.4%) and the glutamyl conjugates γ-Glu–Cys(Propenyl) (12.5%) vs. γ-Glu–Cys(Propyl) (2.0%). For the immediate biosynthetic precursors deoxyisoalliin (**13**, 15.3%) and deoxypropiin (**14**, 5.8%), this effect was less pronounced, but still noticeable. Only for the disulfide metabolite pairs **20**/**21** and **22**/**23** were the differences small or negligible (9.4 vs. 4.8% and 9.0 vs. 7.9%, respectively). These labeling results indicate that – even though there was clear incorporation of valine-$^{13}C_5$ into propiin (**3**) and related propyl metabolites – the relationship between isoalliin (**2**) and propiin (**3**) is more complex than previously recognized.

Two main explanations are conceivable regarding the differences in labeling efficiencies of propenyl and propyl metabolites. First, the propenyl and propyl classes might underlie different flux dynamics. This could occur if genes specific for either propenyl or propyl metabolites are differentially expressed, or if an enzymatic step required for propyl metabolites but not for propenyl metabolites is a rate-limiting step. For example, a putative reductase capable of reducing the propenyl side chain to propyl could represent a bottleneck in propiin (**3**) biosynthesis and limit the flux of valine to this section of the pathway. This would lead to apparently lower labeling rates in comparison to the background of unlabeled metabolites accumulated before the feeding period. Second, methacrylyl-CoA (**9**) derived from valine (**8**) might not be the only or direct carbon source for propiin (**3**). In plants, valine (**8**)

is eventually degraded to acetyl-CoA following a route involving multiple C_3 (and C_4) intermediates which overlap with other metabolic pathways.[11] It is possible that not methacrylyl-CoA (**9**) but a different intermediate with more links to other metabolic pathways is the ultimate propyl carbon source for propiin (**3**) and hence the flux of ^{13}C label towards propyl metabolites is more strongly diluted. Future labeling experiments will be needed to clarify the exact biosynthetic relationship between the metabolite classes represented by isoalliin (**2**) and propiin (**3**), which will be strongly facilitated by the untargeted metabolomics method presented here.

Previously described labeling setups for *Allium* plants were based on excised roots, intact onion bulbs or full plants.[12,22] In our in vitro germination system, labeled compounds are easily administered by addition to the liquid medium, and only small quantities in the range of 1 mg of labeled compound are required due to the small size of chive seedlings and the sensitivity of modern mass analyzers. Thereby, this approach not only avoids the risks of working with radioactive substrates employed previously,[8,10,14] but also provides an unbiased labeling picture at the full metabolome level. Compared to previous labeling studies of this biochemical pathway,[12,14] our analysis method also does not require any tedious purification of labeled products, but directly operates on crude extracts. The use of parallel reaction monitoring (PRM) enables MS/MS measurements of low abundance ions including isotopologs, which are very helpful for assigning mass data to metabolites. Our workflow is therefore easy to perform experimentally, accounts for minor components which could be overlooked with traditional methods, and even provides direct structural information thanks to MS and MS/MS spectra.

Conclusion

In summary, we have developed a facile metabolomics system to probe the biosynthesis of *S*-alk(en)yl cysteine sulfoxides in chive seedlings by isotope-guided untargeted metabolomics. We show that the propyl chains of propiin (**3**) and related metabolites incorporate carbon atoms from valine (**8**), but to a much lower extent than their corresponding propenyl analogs. These new insights into the relationship of propiin (**3**) and isoalliin (**2**) together with our metabolomics method are an important basis for further unraveling the biosynthesis of flavor precursors of everyday relevance for human nutrition and health in *Allium* plants.

Experimental Section

Plant material and in vitro germination: Seeds of *A. schoenoprasum* "Staro" were obtained from Little Plants (Germany). About 30–50 seeds were sterilized in a microcentrifuge tube with 1 mL ethanol by shaking for 5 min. Afterwards, the liquid was removed. Then, 1 mL of a 6% NaOCl solution (*v*/*v*) was added and the mixture incubated for 5 min with shaking. The liquid was removed again. Finally, the seeds were washed 5× with 1 mL sterile ddH_2O. The seeds were kept in the fridge for 2 days to break their dormancy before further use.

Seeds were germinated on liquid half-strength Murashige & Skoog medium, prepared from 10 g/L saccharose and 2.2 g/L MS medium (Sigma Aldrich, Germany) at pH 5.7–5.8. Ten seeds were placed per 10 mL culture medium in a 100 mL Erlenmeyer flask. The cultures were incubated by shaking at 70 rpm in a phytochamber under long-day conditions (16/8 h day/night cycle, temperature 22 °C, 70% humidity, light intensity 120 $\mu mol\,m^{-2}\,s^{-1}$).

Stable isotope labeling experiments: L-valine-$^{13}C_5$ (99 atom% ^{13}C, 97% purity) was obtained from Sigma-Aldrich. A stock solution of 100 mM valine-$^{13}C_5$ in ddH_2O was prepared and sterilized by filtration through a 0.2 μm syringe filter. Then, 100 μL of this 100 mM stock solution was added per 10 mL culture to achieve a final concentration of 1 mM valine-$^{13}C_5$. The feeding was performed 10 days post inoculation (ca. 7 days post germination). Cultures were incubated for further 7 days after feeding and harvested on day 17 (ca. 14 days post germination) for LC-MS analysis.

Sample preparation for LC-MS: *A. schoenoprasum* seedlings were collected in a sieve and washed twice with ddH_2O. Then, the black seed coats were manually removed. The seedlings were blotted dry on tissue paper, flash frozen in liquid nitrogen and dried for 24 h by freeze-drying on a Christ Alpha 1–2 LD+ (Germany). The dried material was pulverized in a Retsch MM 400 bead mill (Germany) for 60 s at 23.0 Hz with a Ø 7 mm steal bead. The powder was stored at −20 °C until LC-MS measurements. 3 mg of powdered tissue was mixed with 100 μL methanol, centrifuged at 14000 *g* for 5 min and the supernatant transferred to a glass vial for LC-MS measurement.

LC-MS analysis and data access: For the chromatography a Vanquish LC (Thermo Fisher) was used in combination with a Polar C_{18} column (Kinetex 2.6 μm, 150×4.6 mm, Phenomenex) and a matching security guard ultra cartridge (Polar C_{18}, 4.6 mm, Phenomenex). Chromatographic parameters were: flowrate, 1 $mL\,min^{-1}$; column temperature, 35 °C; injection volume, 5 μL; mobile phase A, water with 0.1% formic acid; mobile phase B, acetonitrile with 0.1% formic acid. Gradient: 0 min, 100% A; until 4 min, 100% A; 4 to 8 min, 100% to 5% A; 8 to 12 min, 5% A; 12 to 12.5 min, 5% to 100% A; until 15 min, 100% A.

The metabolites were analyzed with an Orbitrap Q Exactive Plus mass spectrometer (Thermo Fisher) at a resolution of 140000 in full MS mode. Samples were measured in positive polarity mode. Automatic gain control (AGC) target and maximum injection time were set to 3×10^6 and 100 ms, respectively. The scan range was 60–900 *m/z*. The heated electrospray-ionization (ESI) source was operated at 0 eV collision-induced dissociation (CID), sheath gas flow 36, auxiliary gas flow 10, sweep gas flow 1, spray voltage 3.5 kV, capillary temperature 250 °C, S-lens RF level 50.0, and aux gas heater 220 °C. Samples were first measured in full MS mode. Subsequently, *m/z* values corresponding to major chromatographic features with an isotopic fine structure indicating the presence of the element sulfur or incorporation of ^{13}C from valine-$^{13}C_5$ were used for an inclusion list guiding the selection of precursors for MS/MS experiments. Parallel reaction monitoring (PRM) was employed for MS/MS measurements with an isolation window of 1.0 *m/z*, a normalized collision energy of 35 V, an AGC target of 2×10^5 and a maximum injection time of 100 ms. All source parameters were identical to those described for the full MS experiment.

Metabolomics data have been deposited in the EMBL-EBI MetaboLights database (DOI: 10.1093/nar/gkz1019, PMID:31691833) with the identifier MTBLS6921. The complete dataset can be accessed here https://www.ebi.ac.uk/metabolights/MTBLS6921.

Preliminary low-resolution LC-MS analyses were performed on an Agilent Infinity II 1260 system consisting of a G7167A autosampler, G7116A column thermostat, G7111B quaternary pump, G7110B make-up pump, G7115A diode array detector, G1364F fraction collector, and G6125B single quadrupole mass spectrometer equipped with an ESI source (positive mode, 4000 V, 12 L min^{-1} drying gas, 350 °C gas temperature) using the same chromatographic method and conditions as described above.

Isotopolog detection by untargeted metabolomics in R: RAW files from Orbitrap measurements were converted into mzXML format using MSConvert 3.0.21105-a3d3afac6,[34] employing peak picking using the vendor algorithm and filtering for MS level 1. Then, mass features matching the mass differences of $^{34}S/^{32}S$ or $^{13}C/^{12}C$ were identified using a custom R script with XCMS[30,35,36] based on scripts by Nett et al.[32] and Baumeister et al.[15] Features were dereplicated using the CAMERA package.[31] Our script is available on GitHub: https://github.com/Franke-Lab/Allium-metabolomics.

Acknowledgements

We thank Alena Zinecker for initial work on this project. We like to acknowledge the support by the Deutsche Forschungsgemeinschaft (HE 5949/4-1 to M.H.), and grant no. INST 187/741-1 FUGG for support of the Orbitrap mass analyzer. J.F. gratefully acknowledges financial support by the SMART BIOTECS alliance between the Technische Universität Braunschweig and the Leibniz Universität Hannover, supported by the Ministry of Science and Culture (MWK) of Lower Saxony. Research in T.M.H.'s lab is funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence Strategy – EXC-2048/1 – project ID 390686111. In addition, this work was supported by the LUH compute cluster, which is funded by the Leibniz Universität Hannover, the MWK, and the German Research Association (DFG). Figures 1–4 were created with BioRender.com. Open Access funding enabled and organized by Projekt DEAL.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: *Allium* · isoalliin · isotope labeling · propiin · *S*-alk(en)yl cysteine sulfoxide

[1] N. Yoshimoto, K. Saito, *Sulfur Metabolism in Higher Plants: Fundamental, Environmental and Agricultural Aspects* (Eds.: L. J. De Kok, M. J. Hawkesford, S. H. Haneklaus, E. Schnug), Springer International Publishing **2017**, pp. 49–60.
[2] N. Yoshimoto, K. Saito, *J. Exp. Bot.* **2019**, *70*, 4123–4137.
[3] R. Kubec, M. Svobodová, J. Velíšek, *J. Agric. Food Chem.* **2000**, *48*, 428–433.
[4] J. Borlinghaus, F. Albrecht, M. C. H. Gruhlke, I. D. Nwachukwu, A. J. Slusarenko, *Molecules* **2014**, *19*, 12591–12618.
[5] J. Borlinghaus, J. Foerster (née Reiter), U. Kappler, H. Antelmann, U. Noll, M. C. H. Gruhlke, A. J. Slusarenko, *Molecules* **2021**, *26*, 1505.
[6] M. G. Jones, *The Chemistry and Biology of Volatiles*, Wiley **2010**, pp. 203–229.
[7] M. G. Jones, J. Hughes, A. Tregova, J. Milne, A. B. Tomsett, H. A. Collin, *J. Exp. Bot.* **2004**, *55*, 1903–1918.
[8] J. E. Lancaster, M. L. Shaw, *Phytochemistry* **1989**, *28*, 455–460.
[9] T. Suzuki, M. Sugii, T. Kakimoto, *Chem. Pharm. Bull.* **1961**, *9*, 77–78.
[10] B. Granroth, *Biosynthesis and Decomposition of Cysteine Derivatives in Onion and Other Allium Species*, Suomalainen Tiedeakatemia **1970**.
[11] T. M. Hildebrandt, A. Nunes Nesi, W. L. Araújo, H.-P. Braun, *Mol. Plant Pathol.* **2015**, *8*, 1563–1579.
[12] R. J. Parry, G. R. Sood, *J. Am. Chem. Soc.* **1989**, *111*, 4514–4515.
[13] R. J. Parry, F. L. Lii, *J. Am. Chem. Soc.* **1991**, *113*, 4704–4706.
[14] T. Suzuki, M. Sugii, T. Kakimoto, *Chem. Pharm. Bull.* **1962**, *10*, 328–331.
[15] T. U. H. Baumeister, N. Ueberschaar, W. Schmidt-Heck, J. F. Mohr, M. Deicke, T. Wichard, R. Guthke, G. Pohnert, *Metabolomics* **2018**, *14*, 41.
[16] A. T. Aron, E. C. Gentry, K. L. McPhail, L.-F. Nothias, M. Nothias-Esposito, A. Bouslimani, D. Petras, J. M. Gauglitz, N. Sikora, F. Vargas, J. J. J. van der Hooft, M. Ernst, K. B. Kang, C. M. Aceves, A. M. Caraballo-Rodríguez, I. Koester, K. C. Weldon, S. Bertrand, C. Roullier, K. Sun, R. M. Tehan, C. A. B. P, M. H. Christian, M. Gutiérrez, A. M. Ulloa, J. A. T. Mora, R. Mojica-Flores, J. Lakey-Beitia, V. Vásquez-Chaves, Y. Zhang, A. I. Calderón, N. Tayler, R. A. Keyzers, F. Tugizimana, N. Ndlovu, A. A. Aksenov, A. K. Jarmusch, R. Schmid, A. W. Truman, N. Bandeira, M. Wang, P. C. Dorrestein, *Nat. Protoc.* **2020**, *15*, 1954–1991.
[17] M. A. Beniddir, K. Bin Kang, G. Genta-Jouve, F. Huber, S. Rogers, J. J. J. van der Hooft, *Nat. Prod. Rep.* **2021**, *38*, 1967–1993.
[18] R. Nakabayashi, K. Saito, *Curr. Opin. Plant Biol.* **2020**, *55*, 84–92.
[19] R. Nakabayashi, K. Saito, *Curr. Opin. Biotechnol.* **2017**, *43*, 8–16.
[20] C. Böttcher, A. Krähmer, M. Stürtz, S. Widder, H. Schulz, *Metabolomics* **2017**, *13*, 35.
[21] R. Nakabayashi, Y. Sawada, M. Aoyagi, Y. Yamada, M. Y. Hirai, T. Sakurai, T. Kamoi, D. D. Rowan, K. Saito, *J. Nutr.* **2016**, *146*, 397S–402S.
[22] R. Nakabayashi, Y. Sawada, Y. Yamada, M. Suzuki, M. Y. Hirai, T. Sakurai, K. Saito, *Anal. Chem.* **2013**, *85*, 1310–1315.
[23] R. Nakabayashi, T. Mori, N. Takeda, K. Toyooka, H. Sudo, H. Tsugawa, K. Saito, *Anal. Chem.* **2020**, *92*, 5670–5675.
[24] R. Nakabayashi, Y. Yamada, T. Nishizawa, T. Mori, T. Asano, M. Kuwabara, K. Saito, *J. Agric. Food Chem.* **2021**, *69*, 8571–8577.
[25] S. J. Edwards, G. Britton, H. A. Collin, *Primary and Secondary Metabolism of Plants and Cell Cultures III: Proceedings of the Workshop Held in Leiden, The Netherlands, 4–7 April 1993* (Eds.: J. Schripsema, R. Verpoorte), Springer, Dordrecht **1995**, pp. 181–188.
[26] N. Yoshimoto, T. Asano, A. Kisanuki, C. Kanno, M. Asanuma, M. Yamazaki, I. Fujii, K. Saito, *J. Nat. Med.* **2022**, *76*, 803–810.
[27] C. Selby, I. J. Galpin, H. A. Collin, *New Phytol.* **1979**, *83*, 351–359.
[28] J. Hughes, A. Tregova, A. B. Tomsett, M. G. Jones, R. Cosstick, H. A. Collin, *Phytochemistry* **2005**, *66*, 187–194.
[29] C. Ohsumi, T. Hayashi, K. Sano, *Phytochemistry* **1993**, *33*, 107–111.
[30] C. A. Smith, E. J. Want, G. O'Maille, R. Abagyan, G. Siuzdak, *Anal. Chem.* **2006**, *78*, 779–787.
[31] C. Kuhl, R. Tautenhahn, C. Böttcher, T. R. Larson, S. Neumann, *Anal. Chem.* **2012**, *84*, 283–289.
[32] R. S. Nett, X. Guan, K. Smith, A. M. Faust, E. S. Sattely, C. R. Fischer, *AIChE J.* **2018**, *64*, 4319–4330.
[33] A. C. Peterson, J. D. Russell, D. J. Bailey, M. S. Westphall, J. J. Coon, *Mol. Cell. Proteomics* **2012**, *11*, 1475–1488.
[34] D. Kessner, M. Chambers, R. Burke, D. Agus, P. Mallick, *Bioinformatics* **2008**, *24*, 2534–2536.
[35] R. Tautenhahn, C. Böttcher, S. Neumann, *BMC Bioinf.* **2008**, *9*, 504.
[36] H. P. Benton, E. J. Want, T. M. D. Ebbels, *Bioinformatics* **2010**, *26*, 2488–2489.

Manuscript received: January 25, 2023
Revised manuscript received: February 27, 2023
Accepted manuscript online: February 28, 2023
Version of record online: April 17, 2023