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Allium Discoloration: Precursors Involved in Onion Pinking and Garlic Greening

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Precursors involved in the formation of pink and green-blue pigments generated during onion and garlic processing, respectively, have been studied. It has been confirmed that the formations of both pigments are of very similar natures, with (*E*)-*S*-(1-propenyl)cysteine sulfoxide (isoalliin) serving as the primary precursor. Upon disruption of the tissue, isoalliin and other *S*-alk(en)ylcysteine sulfoxides are enzymatically cleaved, yielding 1-propenyl-containing thiosulfinates [$CH_3CH{=}CHS(O)SR$; R = methyl, allyl, propyl, 1-propenyl] among others. The latter compounds have been shown to subsequently react with amino acids to produce the pigments. Whereas the propyl, 1-propenyl, and methyl derivatives form pink, pink-red, and magenta compounds, those containing the allyl group give rise to blue products after reacting with glycine at pH 5.0. The role of other thiosulfinates [RS(O)SR′] (R, R′ = methyl, allyl, propyl) and (*Z*)-thiopropanal *S*-oxide (the onion lachrymatory principle) in the formation of the pigments is also discussed.

KEYWORDS: ***Allium*; onion; leek; garlic; pinking; reddening; greening; discoloration; thiosulfinate; isoalliin**

INTRODUCTION

During the processing of garlic (*Allium sativum* L.), onion (*Allium cepa* L.), and leek (*Allium porrum* L.) intensely colored pigments are often formed. In the case of garlic, green, blue-green, or blue compounds are generated, whereas onion and leek homogenates may turn pink or red within several hours (*1*−*9*). Although no definitive evidence has been obtained thus far, it is believed that the pinking of onion and leek and the greening of garlic are of the same nature.

Despite having been studied for almost 50 years, this phenomenon is still very poorly understood. It has been shown that the discoloration is a multistep process. The first step is considered to be an enzyme-mediated degradation of an amino acid precursor, leading to formation of an ether-soluble colorless compound. The latter compound, called the "color developer", subsequently reacts with various amino acids and carbonyl compounds, yielding the pigments. Shannon et al. (*8*, *9*) proposed that the primary amino acid precursor involved in the pinking of onion is (*E*)-*S*-(1-propenyl)cysteine sulfoxide (isoalliin, **1**). Isoalliin is the major free amino acid occurring in onion, and it is also present as a minor *S*-substituted cysteine sulfoxide derivative in garlic. The key role of isoalliin in the discoloration of garlic was later confirmed by Lukes (*5*). Although Bandyopadhyay and Tewari (*10*) tentatively identified the major color-developing compound to be (*Z*)-thiopropanal *S*-oxide (**5**), the lachrymatory factor (LF) of onion, this finding has never been confirmed. Furthermore, the structures of the pigments remain unknown.

Described herein are our investigations into the identification of the precursors involved in the discoloration of onion, leek, and garlic during processing. Our attention was mainly focused on the roles of isoalliin and its analogues, together with their primary decomposition products, that is, (*Z*)-thiopropanal *S*-oxide and thiosulfinates.

MATERIALS AND METHODS

Chemicals. Glycine, potassium *tert*-butoxide, formaldehyde (36%), acetaldehyde, propionaldehyde, and acrolein were obtained from Fluka. Dipropyl disulfide, dimethyl disulfide, diallyl disulfide, propanethiol, sulfuryl chloride, 3-chloroperoxybenzoic acid (*m*-CPBA), propyl bromide, allyl bromide, propargyl bromide, methyl thiocyanate, lithium, sodium, lithium aluminum hydride, potassium thiocyanate, potassium cyanide, tri-*n*-butylamine, and methanesulfonyl chloride were purchased from Aldrich. Solvent grade methanol, acetonitrile, dichloromethane, tetrahydrofuran, petroleum ether (40−60 °C), and dimethyl sulfoxide were purchased from Merck. Allyl methyl disulfide, allyl propyl disulfide, and methyl propyl disulfide were obtained from Oxford Chemicals (Hartlepool, U.K.). All other chemicals used were of analytical grade and of the highest available purity. These were purchased from Lachema (Brno, Czech Republic). Distilled and deionized water was used throughout this study.

Plant Material. Garlic (China) and white onion (France) were purchased in a local store in Prague in March 2003.

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Synthesis of Reference Compounds. *S*-Alk(en)yl-L-cysteines were synthesized by alk(en)ylation of L-cysteine with the appropriate alk(en)yl halides according to the procedure of Stoll and Seebeck (*11*). (*E,Z*)-*S*-(1-Propenyl)-L-cysteine was synthesized by base-catalyzed isomerization of *S*-allyl-L-cysteine with potassium *tert*-butoxide according to the procedure of Carson and Boggs (*12*). (R_S/S_S)-Diastereomers of *S*-alk(en)yl-L-cysteine sulfoxides (**1**−**4**) were prepared by oxidation of the corresponding *S*-alk(en)yl-L-cysteines with hydrogen peroxide following the procedure of Yu et al. (*13*).

(*E*)-1-Propenyl-containing disulfides [allyl (*E*)-1-propenyl, methyl (*E*)-1-propenyl, and (*E*)-1-propenyl propyl disulfides] were prepared as described by Wijers et al. (*14*) and Block et al. (*15*) using the appropriate alk(en)yl thiocyanates. Propyl and allyl thiocyanates were synthesized following the methods of Reeves et al. (*16*) and Slater (*17*), respectively. (*E,E*)-Bis(1-propenyl) disulfide was synthesized as reported by Block et al. (*15*). Thiosulfinates (**6**−**23**) were prepared by oxidation of the corresponding disulfides with *m*-CPBA (1 equiv) in CH_2Cl_2 (−20 °C, 30 min) and purified by preparative C-8 HPLC (CH_3CN/H_2O). *S*-(*E,Z*)-(1-Propenyl) (*E*)-1-propenethiosulfinates (**24**, **25**) were synthesized from the corresponding disulfide by oxidation with *m*-CPBA (CH_2Cl_2, −60 °C, 30 min). The solution was then filtered, the solvent was quickly stripped off under a stream of nitrogen, and the product was used immediately without further purification. (*Z*)-Thiopropanal *S*-oxide (**5**) was obtained from propanesulfinyl chloride according to the method of Block et al. (*18*). Propanesulfinyl chloride was synthesized from dipropyl disulfide and sulfuryl chloride (*19*).

The identity and purity of the reference compounds were checked by ^1H and ^{13}C NMR, IR, GC/MS, TLC, and HPLC.

Isolation of Alliinase. The procedure described by Thomas and Parkin was followed for the isolation of both onion and garlic alliinase (*20*). The purity and specific activity of the obtained preparations were not examined in detail.

Model Pigment Isolation. Garlic (540 g) was peeled and homogenized with 200 mL of distilled H_2O using a blender, and the slurry was filtered through cheesecloth. The filtrate obtained (390 mL) was mixed with 400 mL of acetone and allowed to stand for 15 min. The precipitate that formed was filtered off, and the acetone was removed by evaporation under reduced pressure. Glycine (3.8 g) was added to the solution, and the pH was adjusted to 5.5 using 1 M NH_4OH. After incubation at 40 °C for 18 h, the dark blue-green solution was cooled to room temperature and filtered, and the filtrate was loaded onto a C-18 SPE cartridge (5 mL, Analtech, Newark, NJ). The cartridge was washed first with 50 mL of distilled water and then with 50 mL of acetone. The pigment was eluted with 100 mL of acidified methanol (CH_3OH/HCl, 97/3, v/v), and the resulting solution was evaporated to dryness. The remaining residue (27 mg) was then subjected to ICP/MS analysis. An analogous procedure was followed for the isolation of the red pigment from white onion.

Model Experiments. Aliquots (1 mL) of stock solutions of *S*-alk(en)ylcysteine sulfoxides (5 mg/mL) and glycine (15 mg/mL) in 0.1 M KH_2PO_4 buffer (pH 5.5) were placed in 13-mL glass vials. A portion of 0.5 mL of an alliinase solution (40 mg/100 mL) was added, the total volume was adjusted to 5 mL with the buffer, and the vials were capped, shaken, and immersed in a water bath (40 °C) overnight. Alternatively, 20 μL of a carbonyl compound (formaldehyde, acetaldehyde, propionaldehyde, or acrolein) or 1 mmol of a metal sulfate ($FeSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, or $ZnSO_4 \cdot 7H_2O$) was added before incubation. When the role of thiosulfinates and the LF was studied, 2 μL of a thiosulfinate or (*Z*)-thiopropanal *S*-oxide was mixed with 3 mL of a glycine solution (10 mg/mL 0.1 M KH_2PO_4, pH 5.0) in a 10-mL glass vial. The vials were capped, briefly sonicated (1 min), and kept overnight at 45 °C. After cooling to room temperature, the solutions were filtered (0.45 μm) and their UV−vis spectra were recorded. In the case of the LF, amounts of up to 100 μL were also used (at 40−95 °C). In all cases, at least three parallel experiments were conducted.

Instrumentation. HPLC separations were performed on a Dynamax SD-200 binary pump system (Varian, Walnut Creek, CA), employing a Varian PDA 330 detector and a preparative C-8 column (Rainin Dynamax-100 Å, 250 × 21.4 mm, 8 μm). An Agilent 6890N chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a 5973 Agilent MS detector and an HP-INNOWax fused silica capillary column (30 m × 0.25 mm i.d.; film thickness of 25 μm; Agilent Technologies) was used for GC-MS analyses. ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini 300 HC spectrometer, IR spectra were obtained using a Perkin-Elmer RX I FTIR spectrometer, and UV spectra were measured on a Shimadzu UV-1601PC spectrophotometer (Kyoto, Japan). An ICP/MS Elan 6000 spectrometer (Perkin-Elmer, Wellesley, MA) with a concentric nebulizer and a cyclonic spray chamber was used for ICP/MS analyses. TLC was performed on precoated Aldrich plastic plates (silica gel polyester) with petroleum ether/diethyl ether/glacial acetic acid (60:40:1, v/v/v) as the mobile phase.

Table 1. Color Formation in Model Solutions Composed of *S*-Alk(en)ylcysteine Sulfoxides, Glycine, and Onion Alliinase (pH 5.5)

isoalliin	alliin	methiin	propiin	glycine	alliinase	resulting color
+					+	pink
+				+	+	magenta
+	+			+	+	dark blue
+		+		+	+	magenta
+			+	+	+	magenta
	+			+	+	none
		+		+	+	none
			+	+	+	none
				+	+	none
+	+	+	+	+		none
	+	+	+	+	+	none

RESULTS AND DISCUSSION

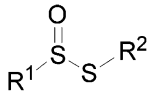
Because the observed discoloration seems to occur only in members of the *Allium* genus, the role of the most typical compounds of these plants, *S*-alk(en)ylcysteine sulfoxides, was investigated in the first stage of the study. The four major derivatives occurring in the *S*-alk(en)ylcysteine sulfoxide pool of alliaceous plants, that is, *S*-1-propenyl-, *S*-methyl-, *S*-propyl-, and *S*-allylcysteine sulfoxides (isoalliin, methiin, propiin, and alliin, respectively) (**1**−**4**), were prepared by synthesis. Crude preparations of alliinase (EC 4.4.1.4), an enzyme that cleaves these amino acids upon disruption of the tissue, were obtained from both onion and garlic. Various combinations of the *S*-alk(en)ylcysteine sulfoxides, glycine, and onion alliinase were mixed and incubated overnight at 40 °C, and the resulting change in color was evaluated. As can be seen in **Table 1**, a pigment was formed in only those systems containing both isoalliin and alliinase. Although the addition of glycine significantly enhanced the intensity of the color formed, its presence was not essential for color development (perhaps isoalliin and the other cysteine derivatives were incorporated into the pigments instead of glycine). Furthermore, no significant difference in the resulting color was observed between pigments obtained by employing either onion or garlic alliinase (not shown).

Bandyopadhyay and Tawari (*10*) reported (*Z*)-thiopropanal *S*-oxide (LF, **5**) to be the major color-developing compound in onion. We were puzzled by this finding, given that the pigments observed in both onion and garlic are presumed to form via similar mechanisms. If this is the case, then the LF would not be expected to be the color-developing compound, because it is absent in garlic. Indeed, the formation of the LF in onion was recently shown to be catalyzed by a novel enzyme, LF-synthase, which is not present in garlic (*21*). We have repeated the experiments reported by Bandyopadhyay and Tewari (*10, 22*) and, in contrast to their results, no pigment was formed in model systems containing (*Z*)-thiopropanal *S*-oxide and glycine under any of the conditions studied (temperatures 40−95 °C,

Table 2. Color Formation in Model Solutions Consisting of Thiosulfinates and Glycine (pH 5.0)

	thiosulfinate						
	9/10	**14/15**	**19/20**	**21**	**22**	**23**	**24/25**
λ_{max}[a]	pink (548 nm)	faint pink (538 nm)	light blue (580 nm)	magenta (548 nm)	pink (538 nm)	dark blue (582 nm)	pink-red (536 nm)

[a] Absorption maximum in the region of 380–650 nm



R^1, R^2 = —CH_3 (Me)
—$CH_2CH_2CH_3$ (Pr)
—$CH_2CH{=}CH_2$ (All)
—$CH{=}CHCH_3$ (Pe)

R^1	R^2 = Me	Pr	All	(*E*)-Pe	(*Z*)-Pe
Me	**6**	**7**	**8**	**9**	**10**
Pr	**11**	**12**	**13**	**14**	**15**
All	**16**	**17**	**18**	**19**	**20**
(*E*)-Pe	**21**	**22**	**23**	**24**	**25**

Figure 1. Structures of synthesized thiosulfinates.

various concentrations). Interestingly, Bandyopadhyay and Tewari (*10*) reported the color developer as having a TLC R_f value of 0.33 (petroleum ether/diethyl ether/acetic acid, 60:40:1, v/v/v). In our hands, however, a fully characterized sample of (*Z*)-thiopropanal *S*-oxide, when analyzed by TLC using the same mobile phase, showed an R_f value of 0.62. This reproducible result implies that the compound observed in their study (*10*) was not the presumed LF.

Thus, our attention was drawn to another class of compounds arising from enzymatic degradation of isoalliin and analogous compounds, the thiosulfinates (**Figure 1**). All of the major *Allium*-occurring thiosulfinates [RS(O)SR′; R, R′ = CH_3—, $CH_3CH_2CH_2$—, $CH_2{=}CHCH_2$—, and $CH_3CH{=}CH$—] (**6**−**23**) were synthesized and purified by preparative C-8 HPLC. Due to their instability (*23*), no attempt was made to isolate (*E*)- and (*Z*)-*S*-(1-propenyl) (*E*)-1-propenethiosulfinates (**24** and **25**) in a pure form. The NMR, IR, and UV data of the thiosulfinates synthesized were in good agreement with those published previously (*24*, *25*) except for some discrepancies in the assignment of the methine ^{13}C signals in **9**/**10**, **14**/**15**, and **19**/**20**. The spectral data (NMR and IR) can be found in the Supporting Information.

Thiosulfinates (**6**−**25**) were allowed to react with glycine at 45 °C to test their ability to form the pigments. Indeed, pink, pink-red, magenta, and blue compounds were produced overnight in several mixtures (**Table 2**). As can be seen, only the 1-propenyl-containing thiosulfinates gave rise to colored products. Whereas the onion and leek-occurring thiosulfinates (**9**/**10**, **14**/**15**, **21**, **22**, and **24**/**25**) yielded pink, pink-red, or magenta compounds, the solutions containing the garlic-related derivatives (**19**/**20** and **23**) turned blue at pH 5.0. Although both regiomers, namely, (*E*,*Z*)-$CH_3CH{=}CHSS(O)R$ (**9**/**10**, **14**/**15**, **19**/**20**) and (*E*)-$RSS(O)CH{=}CHCH_3$ (**21**−**23**), are capable of forming the pigments, the latter ones seem to be severalfold more potent as judged by the intensity of the observed color. For instance, the absorbance ratio of A_{548}(**21**)/A_{548}(**9**/**10**) was found to be 4.7. Even higher ratios were observed for the pigments formed from the propyl (5.3 at 538 nm) and allyl (6.2 at 582 nm) derivatives. However, it remains to be determined whether the pigments produced from both regiomers are identical.

The key role of 1-propenyl-containing thiosulfinates in onion discoloration was first advanced by Shannon et al. (*9*). Surprisingly, this suggestion has never been tested until now, perhaps because of the relatively elaborate synthesis and instability of these compounds. The only paper describing the possible role of thiosulfinates in *Allium* discoloration was published by Lee and Parkin (*26*), who studied variations in coloration as a function of *total* tissue thiosulfinate and pH. They observed a positive correlation between thiosulfinate concentration and pigment formation. However, their results were quite incon-

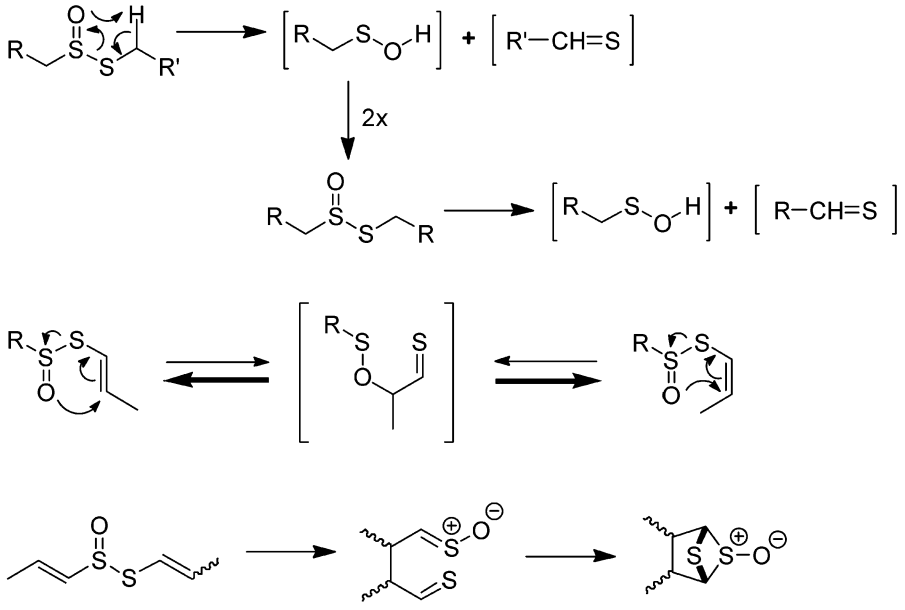


Figure 2. Formation of various thioaldehydes from thiosulfinates.

Table 3. Content of Metals in Crude Onion and Garlic Model Pigments As Determined by ICP/MS

	metal content (mg·kg^{-1})												
	Al	Cr	Mn	Fe	Co	Ni	Cu	Zn	Mo	Ag	Cd	Tl	Pb
onion	35.6	0.0	6.6	4.4	0.14	14.4	136.7	234.5	11.6	0.20	4.1	8.4	5.1
garlic	218.2	27.9	11.9	848.2	0.11	19.5	27.4	77.5	0.29	2.4	0.18	0.03	6.2

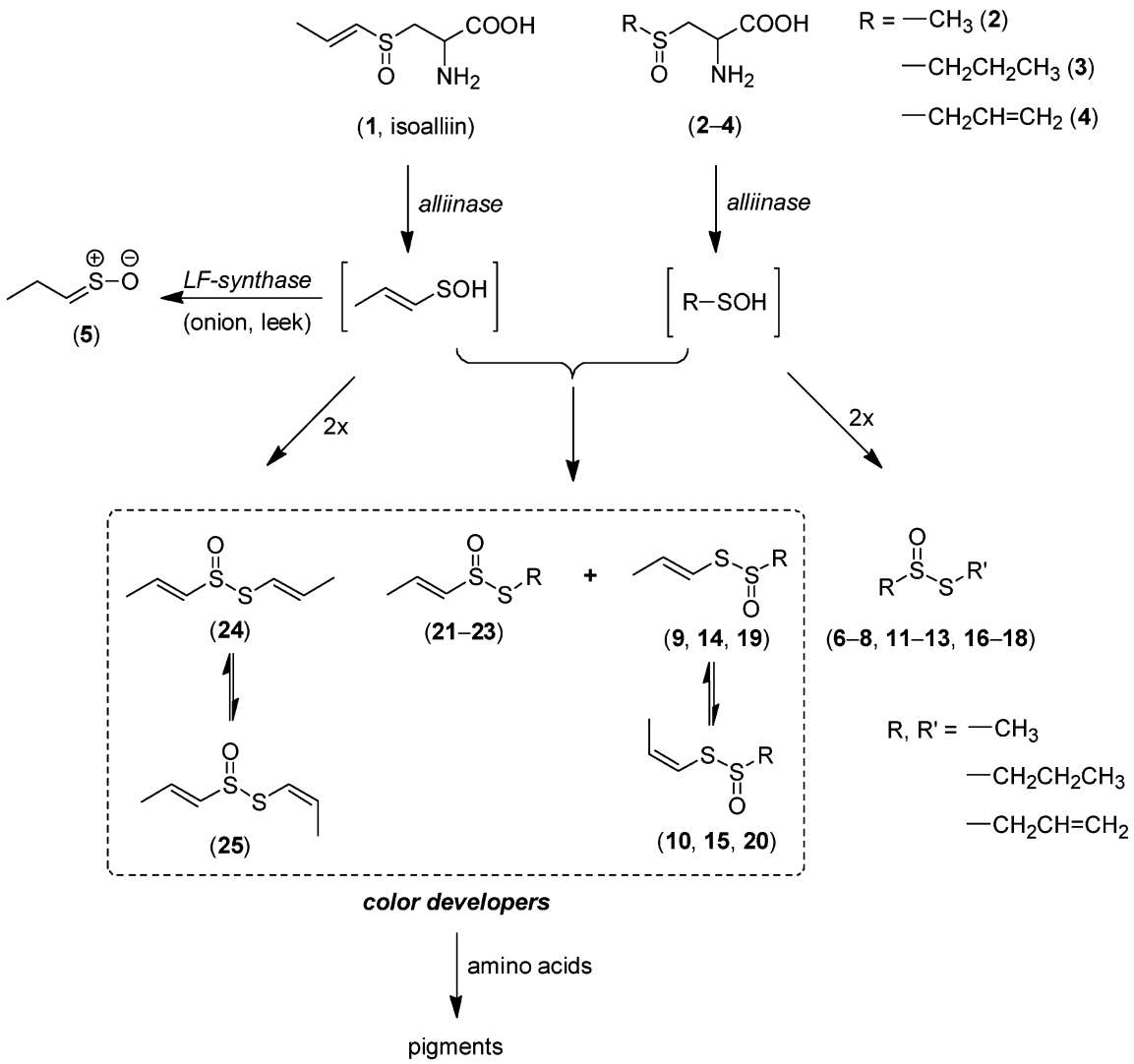


Figure 3. Formation of pigments in *Allium* species.

clusive, because color formation was monitored as a function of total thiosulfinate concentration and not individual thiosulfinate compounds.

Joslyn and Peterson (*3*) were the first to report on the significant influence of carbonyl compounds on the rate of pigment formation. Their findings were later confirmed by Yamaguchi et al. (*6*) and Shannon et al. (*8*), who demonstrated that some carbonyl compounds were apparently involved in the discoloration process. We have made very similar observations. Addition of a carbonyl compound (e.g., formaldehyde, acetaldehyde, propionaldehyde, and acrolein) to model solutions had a significant effect on the intensity of the resulting color. Whereas the model solutions (consisting of isoalliin, glycine, and onion alliinase) with added formaldehyde, acetaldehyde, or propionaldehyde were pink, those containing acrolein turned dark blue. The rate of pigment formation in the carbonyl compound-enriched samples was somewhat higher than in those with no aldehyde added. However, as we have demonstrated, the pigments are also formed in solutions consisting only of 1-propenyl-containing thiosulfinates and glycine. This may indicate that some carbonyl or even thiocarbonyl compounds formed via decomposition of thiosulfinates are intermediates in pigment formation. Several very reactive thiocarbonyl species can be generated from 1-propenyl-containing thiosulfinates (*23*) (**Figure 2**), and it is conceivable that some of them may be involved in pigment formation. If that is the case, then it is possible that thiosulfinates devoid of the 1-propenyl moiety (**6**−**8**, **11**−**13**, **16**−**18**) may still contribute to pigment formation by serving as alternative sources of thiocarbonyl compounds. The apparent involvement of both amino and (thio)carbonyl compounds is reminiscent of Maillard-type reaction chemistry.

The propensity of sulfur toward metal binding to produce highly colored complexes prompted us to consider whether the presence of ubiquitous trace metals might be involved in pigment formation. To study the role of metals in discoloration, model pigments were prepared by mixing glycine with juice

from onion and garlic, respectively, and analyzed by ICP/MS. The analyses showed that neither pigment contained considerable quantities of metals (**Table 3**). On the basis of the low percentage of total metals found (0.05 and 0.12% in the onion and garlic pigments, respectively), we conclude that they most likely represent only impurities and that the pigments are not metal complexes. Nevertheless, the influence of the presence of the most abundant metals (i.e., Fe^{2+}, Cu^{2+}, and Zn^{2+}) was studied in more detail. Sulfates of the selected metals were added to model solutions (consisting of **9**/**10**/**21** and glycine), and the mixtures were incubated at 45 °C. No significant differences in the intensity or rate of color formation were observed between the added metal-containing solutions and those without added metal salts. On the basis of these findings, we conclude that metals probably do not significantly influence the formation of the pigments. Some effect of metals on the resulting color cannot, however, be completely ruled out.

Our findings indicate that the sequence of reactions leading to pigment formation includes (1) enzymatic cleavage, upon tissue disruption, of isoalliin and other *S*-alk(en)ylcysteine sulfoxides, ultimately yielding 1-propenyl-containing thiosulfinates, among others; and (2) reaction of the thiosulfinates with amino acids to produce the pigments (**Figure 3**). The major color-developing compounds formed in onion and leek homogenates are **9**/**10**, **14**/**15**, **21**, **22**, **24**, and **25**. Their relative proportions would be expected to vary significantly from batch to batch, depending mostly on the relative ratios of the corresponding amino acid precursors (**1**−**3**) present in the tissue. Because **9**/**10** and **21** (and to a very limited extent **24** and **25**) are also present as minor constituents in garlic homogenates (*23*−*25*), a small amount of the red pigment may be formed in garlic. In fact, some red compounds are perhaps generated even from **19**/**20** and **23**, as indicated by the shoulder at 538 nm in the UV−vis spectra at pH 5.0. However, the presence of the red pigment in garlic may be masked by the much more abundant green-blue compounds formed from **19**/**20** and **23**, which are the key color-developing compounds occurring in crushed garlic. Of additional relevance is the study reported by Lawson et al. (*25*), who observed a severalfold increase in the content of 1-propenyl-containing thiosulfinates (**9**/**10**, **19**/**20**, **21**, and **23**) upon storage of garlic bulbs at 4 °C. This interesting finding may explain the observation reported by Lukes (*5*) that garlic stored for 2−4 weeks at 3 °C was much more susceptible to greening than that stored at room temperature.

Apparently, discoloration can occur upon tissue disruption of any *Allium* species that contains at least traces of isoalliin. In many cases, however, pigment formation would be expected to be masked by chlorophyll (e.g., in chive, scallion, or Chinese chive) or other natural pigments (e.g., anthocyanins in red varieties of onion). On the other hand, some cysteine derivatives (e.g., *S*-ethyl- and *S*-butylcysteine sulfoxides) that occur in onion, leek, and garlic in only trace amounts (*27*−*29*) may contribute significantly to the discoloration of other members of the *Allium* genus. This is, for instance, the case of *Allium siculum*, which contains both isoalliin and *S*-butylcysteine sulfoxide as major cysteine sulfoxide derivatives (*30*).

Further research aimed at elucidating the structures of the pigments and determining the reaction pathways leading to their formation is ongoing. Preliminary HPLC results indicate that the discoloration process is very complex, yielding several colored products even when only one pair of thiosulfinate regiomers and a single amino acid are allowed to react. Apparently, the natural pigment is a mixture of structurally related compounds whose differences are correlated with the structures of the specific amino acid(s) and (thio)carbonyl compound(s) that serve as the reactants.

ABBREVIATIONS USED

A, absorbance; DMSO, dimethyl sulfoxide; ICP, inductively coupled plasma; LF, lachrymatory factor [(*Z*)-thiopropanal *S*-oxide]; *m*-CPBA, 3-chloroperoxybenzoic acid; PDA, photodiode array.

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Supporting Information Available: Spectral data (NMR and IR) of thiosulfinates. This material is available free of charge via the Internet at http://pubs.acs.org.

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