

Allium Discoloration: Color Compounds Formed during Pinking of Onion and Leek

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S Supporting Information

ABSTRACT: Structures and formation pathways of compounds responsible for pink discoloration of onion and leek were studied. A procedure was developed for the isolation and purification of the color compounds from various model systems and their identification by HPLC-DAD-MS/MS. In total, structures of 15 major color compounds were tentatively determined. It was found that the pigment is a complex mixture of highly conjugated species composed of two *N*-substituted 3,4-dimethylpyrrole-derived rings linked by either a methine or a propenylidene bridge. These two-ring units are further modified by various C₁- and C₃-side chains. Experiments with isotope-labeled thiosulfates revealed that the methine bridge and C₁-side chains originate from the methyl group of methiin, whereas the C₃ units are derived from the propenyl group of isoalliin.

KEYWORDS: discoloration, plant pigment, pinking, onion, leek, Allium, thiosulfinate, isoalliin, dipyrromethane, pyrrole

INTRODUCTION

The formation of intensely colored compounds is often observed during the processing of many genus *Allium* species, including garlic (*Allium sativum* L.), onion (*Allium cepa* L.), leek (*Allium porrum* L.), or giant onion (*Allium giganteum* Regel).^{1–6} In the case of garlic, a vividly blue-green pigment is generated, whereas onion and leek homogenates may turn pink or red (Figure 1). The formation of these pigments significantly lowers the organoleptic quality of processed alliacious vegetables and causes great economic losses to producers.

Whereas the discoloration of garlic has already been studied quite thoroughly,^{1,7–17} the pinking of onion and leek has received considerably less attention.^{2–4,18–22} It is mainly due to much slower formation and paler intensity of the pink color compared with the vividly blue-green discoloration of garlic.

The pinking of onion was once believed to be caused by the formation of a betanine type pigment² or by the presence of some microorganisms.³ Surprisingly, only a single study has thus far been specifically dedicated to the pinking of leek.⁵ The authors (erroneously) concluded that this discoloration was a result of enzymatic oxidation of phenolic substances to quinones, which in turn reacted with amino acids.

Despite the great visual difference in the colors formed, the discoloration of both garlic and onion/leek are of a closely related nature. In both cases, it is a very complex multistep process, consisting of enzymatic and nonenzymatic stages. (*E*)-*S*-(1-Propenyl)cysteine sulfoxide (isoalliin, **1**), together with other *S*-alk(en)ylcysteine sulfoxides (e.g., methiin, **2**), is enzymatically cleaved upon disruption of the tissue, yielding a great number of various sulfur compounds. Among them, 1-propenyl-containing thiosulfates and cepathiolanes (often referred to as “color developers”) have been shown to subsequently react with amino acids to give *N*-substituted 3,4-dimethylpyrroles (called “color precursors”).^{8,10–12,22} These colorless pyrroles further react with various (thio)carbonyl species to produce the color compounds. It was reported that this process can also proceed without the catalytic action of alliinase on isoalliin. In that case, however, the formation of the pink color is considerably slower and takes several weeks.²³

Although the primary precursor (isoalliin, **1**) and general mechanism of the discoloration of onion and leek have already been identified, structures of the color compounds and exact pathways of their formation are still poorly understood. A

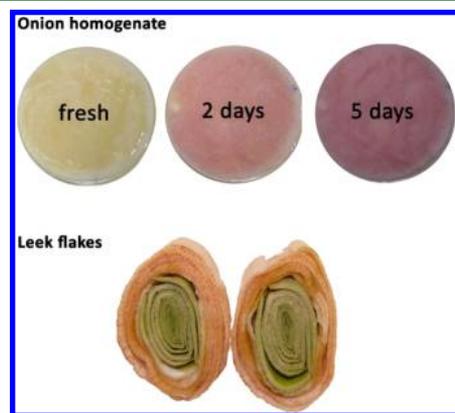


Figure 1. Discoloration of homogenized onion and leek flakes stored at 23 °C for 5 days.

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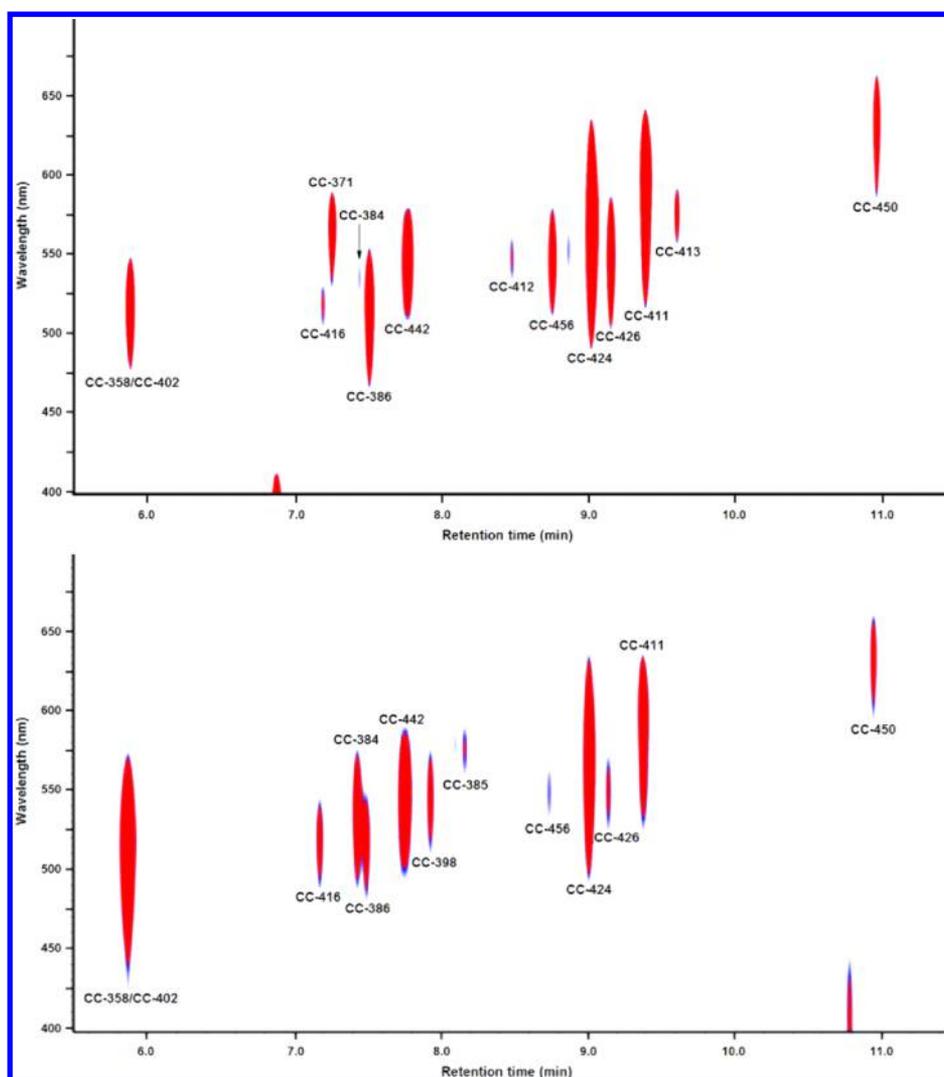


Figure 2. 2D-HPLC chromatograms of color compounds formed after mixing extracts of homogenized onion (above) and leek (below) with alanine.

better knowledge of the structure and formation of these compounds may help in the prevention of this undesirable process. Thus, this work was aimed to identify color compounds formed during pinking of onion/leek homogenates. Due to the overwhelming complexity of reactions taking place in real systems, structures and formation pathways of the color compounds were studied in model mixtures including the utilization of several isotopically labeled precursors.

MATERIALS AND METHODS

General Methods. UHPLC analyses were performed using an UltiMate 3000 RS UHPLC system (Thermo Scientific, Waltham, MA, USA) equipped with an Acquity UPLC HSS T3 column (100 × 2.1 mm, 1.8 μm, Waters, Milford, MA, USA) maintained at 45 °C. The mobile phase consisted of 10 mM formic acid in Milli-Q water (solvent A) and methanol (solvent B). The following gradient was used: A/B 80:20 (0 min) (flow rate = 0.4 mL min⁻¹) and 0:100 (15 min) (flow rate = 0.6 mL min⁻¹). UV-vis data were collected by using a diode array detector (UltiMate 3000, Thermo Scientific). ESI HRMS data were obtained by an AB SCIEX TripleTOF 5600 (AB SCIEX, Framingham, MA, USA), provided with a DuoSpray ion source. The needle voltage in (+)ESI was +5.0 kV and that in (-)ESI, -4.5 kV. Other ion source parameters were identical for both polarities: curtain gas, 35 psi; nebulizer and drying gas, 60 psi; turbo gas temperature, 600 °C; declustering potential, 60 V. The mass spectrometer was

operated using an information-dependent acquisition (IDA) method to collect full scan MS and MS/MS information simultaneously. The method consisted of a survey scan (m/z 100–800, 100 ms accumulation time) and a product ion scan (m/z 50–800, 40 ms accumulation time). Product ion spectra were collected automatically for the 10 most intense ions in the survey scan throughout the chromatographic run. Spectra were collected using a collision energy of 45 V with a collision energy spread of ±15 V. The mass resolving power over the acquired mass range was always $R > 30000$ fwhm. Collected data were processed with PeakView software 1.1.1.2 (AB SCIEX).

The isolation of compound CC-371 was achieved by using a Dynamax SD-210 binary pump HPLC system (Varian, Palo Alto, CA, USA), employing a diode array detector (Varian 335) and a preparative Ascentis C-8 column (250 × 21.2 mm, 5 μm; Supelco, St. Louis, MO, USA).

GC analyses were conducted on a Varian 3800 chromatograph (Varian), equipped with a Varian 4000 MS detector and an HP-SMS fused silica capillary column (30 m × 0.25 mm i.d.; film thickness = 0.25 μm; Agilent Technologies, Santa Clara, CA, USA). Mass spectra were obtained by EI ionization at 70 eV over the range of 30–350 mass units. NMR spectra were recorded on a Bruker AVANCE III 700 MHz spectrometer equipped with a cryoprobe (Bruker, Billerica, MA, USA). ¹H and ¹³C chemical shifts were referenced to the signals of residual MeOH (δ 3.31 and 49.0, respectively).

Chemicals and Plant Material. 1-Propenyl bromide, 1,2-dibromoethane, magnesium, (¹³C)methyl iodide (99% ¹³C), propane-

thiol, D,L-(3-D₃)alanine (99% D), D,L-(¹⁵N)alanine (99% ¹⁵N), D,L-(2-D)alanine (98% D), D,L-(1-¹³C)alanine (99% ¹³C), formic acid, Milli-Q water, and methanol for LC-MS were obtained from Sigma-Aldrich (St. Louis, MO, USA). 3-Chloroperoxybenzoic acid, dansyl chloride, methyl thiocyanate, potassium thiocyanate, sodium, lithium, lithium aluminum deuteride (98% D), propargyl bromide, and tetrahydrofuran were obtained from Acros Organics (Geel, Belgium). Other chemicals were supplied by Lach-Ner (Neratovice, Czech Republic). Onion and leek were purchased in a local store.

Synthesis of Thiosulfates. Alk(en)yl 1-propenyl disulfides [alk(en)yl = CH₃–, ¹³CH₃–, or CH₃CH=CH–] were synthesized by following the methods reported in our previous study.⁸ ¹³CH₃SCN was obtained by the reaction of potassium thiocyanate with ¹³CH₃I. (1,2-D₂)1-Propenyl propyl sulfide was prepared according to the method of Block et al.²⁴ by using LiAlD₄ and used for the preparation of CH₃CD=CDSSCH₃. Thiosulfates [3a/b, (D₂)3a/b, and (¹³CH₃)3a/b] were obtained by oxidation of the corresponding disulfides with 3-chloroperoxybenzoic acid and used without further purification.⁸ Representative MS data of the synthesized disulfides and detailed description of experimental procedures can be found in the Supporting Information.

Extraction of Thiosulfates and Related Compounds from Plant Material. Peeled onions (500 g) were homogenized with 500 mL of H₂O by a kitchen blender and allowed to stand for 30 min at room temperature. The slurry was filtered through a layer of cotton wool, and the filtrate was extracted by diethyl ether (3 × 300 mL). The combined ether portions were dried over MgSO₄, and the solvent was removed at reduced pressure (<30 °C). The viscous residue obtained was stored at –28 °C before use. Extracts of homogenized white parts of leek were prepared analogously.

Model Experiments. Thiosulfates 3a/b (2.4 g in 24 mL of CH₃CN) were mixed with 500 mL of L-alanine solution (25 mM in 0.5 M KH₂PO₄, pH 5.5) and the mixture was continuously stirred at 40 °C for 18 h. Experiments with isotopically labeled compounds or extracts from onion and leek were conducted analogously.

Pigment Purification. Model solutions were cooled to room temperature, filtered (if needed), and loaded onto a C-18 SPE column (60 mL, 10 g, Supelco). The column was washed successively with 50 mL of the following solvents: H₂O, acetone, pentane, CH₂Cl₂, diethyl ether, and CH₃CN. These fractions were discarded. Finally, the color compounds were eluted with 50 mL of CH₃OH. The solvent was removed at reduced pressure (<35 °C) and the residue dissolved in 1 mL of CH₃OH/H₂O (1:9, v/v). The solution was loaded onto a C-8 SPE column (3 mL, 500 mg, Supelco), and the column was washed successively with 3 mL of H₂O, 25 mL of CH₃CN, and 3 mL of CH₃OH. The fraction eluted by CH₃OH was analyzed by HPLC. Color compounds obtained from isotopically labeled precursors were purified analogously using appropriately smaller SPE columns and amounts of solvents.

Isolation of CC-371. The compound was isolated from selected model mixtures by preparative C-8 HPLC using the following gradient: 10 mM HCOOH (solvent A) and methanol (solvent B), A/B 65:35 (0–5 min), 25:75 (10–12 min), 10:90 (17 min), and 65:35 (22 min) (flow rate = 16.8 mL min⁻¹). The collected fractions (t_R 11.9 min) were combined, and methanol was carefully removed at reduced pressure (<30 °C). The aqueous portion was loaded onto a C-8 SPE cartridge (500 mg), the cartridge was washed with H₂O (5 mL), and the compound was eluted by methanol and carefully dried at reduced pressure.

Determination of S-Substituted Cysteine Sulfoxides. Relative proportions of isoalliin (1), methiin (2), and propiin in studied onion and leek samples were determined by HPLC after derivatization with dansyl chloride.²⁵

RESULTS AND DISCUSSION

Pigment Formation and Isolation. Thiosulfates and related sulfur compounds present in freshly homogenized onion were extracted and mixed with aqueous solution of alanine. During 18 h at 40 °C, the solution turned intensely red.

The color compounds formed were isolated by SPE and analyzed by HPLC-DAD-MS/MS. As shown in Figure 2, the sample consisted of numerous compounds exhibiting absorption maxima in the range of 517–634 nm (pink, red, magenta, and blue shades; see the picture of collected fractions in the Supporting Information).

Analogous experiments were also conducted with homogenized leek. It was found that the pattern of color compounds formed was very similar to that observed in the onion homogenates, differing only in relative proportions of the individual components (Figure 2).

As determined by HPLC, the onion and leek used in our experiments contained isoalliin (1) and methiin (2) in the ratios of 79:21 and 84:16, respectively, but no propiin (S-propylcysteine sulfoxide). Thus, the major color-forming thiosulfates present in the extracts were S-(1-propenyl) methanethiosulfinate [CH₃S(O)SCH=CHCH₃, 3a] and S-methyl 1-propenethiosulfinate [CH₃SS(O)CH=CHCH₃, 3b]. These thiosulfates were prepared by synthesis and allowed to react with alanine under the same conditions as the extracts of homogenized onion/leek. Indeed, all color compounds formed from the extracts were also generated in the model mixture consisting of synthetic thiosulfates 3a/b and alanine (although in different relative proportions, see the Supporting Information). These results are consistent with our previous conclusions that 1-propenyl-containing thiosulfates are precursors of the color compounds responsible for discoloration of onion and leek.^{8,12}

Structure Elucidation. As documented in Figure 2, the pigment is a very complex mixture of difficult-to-separate compounds. Despite a great effort to isolate some of the major components by preparative HPLC, only compound CC-371 was obtained in a sufficient quantity and purity for NMR measurements.

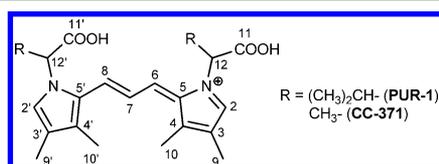
The ¹H NMR spectrum of CC-371 contained seven signals, revealing the presence of three different CH₃– groups (δ 1.84, 2.14, and 2.46), one –CH– moiety (δ 5.42), and three –CH= hydrogens (δ 7.41, 7.82, and 8.00). The ¹³C NMR spectrum showed the presence of 11 different carbon atoms, attributable to 4 sp³- and 6 sp²-hybridized carbons together with a carboxylic group. These NMR data were found to be very similar to those reported by Imai et al.¹⁰ for valine-derived compound PUR-1 formed during garlic greening (Table 1; Figure 3). This assumption was fully confirmed by additional 2D NMR experiments, including COSY, HSQC, and HMBC (see the Supporting Information). The UV–vis maximum of CC-371 (λ_{max} 568 nm) was also very similar to that of PUR-1 (λ_{max} 570 nm). Further evidence that CC-371 is the alanine analogue of PUR-1 was obtained from ESI-HRMS data, which revealed the elemental composition of C₂₁H₂₇N₂O₄ (Table 2). The MS/MS fragmentation pattern and isotopic distribution were also in perfect agreement with the structure proposed for CC-371 (see the Supporting Information). This assumption was subsequently verified by experiments with isotope-labeled precursors, as will be described in the following sections.

Due to chromatographic behavior, UV–vis spectra, and identical precursors similar to those of CC-371, it seemed reasonable to assume that the other color compounds shown in Figure 2 were also 3,4-dimethylpyrrole-based species. Their elemental composition was determined from ESI-HRMS data obtained in both negative and positive ion modes. As summarized in Table 1, their relative molecular weights ranged between 358 and 456 Da, and elemental composition

Table 1. ^1H and ^{13}C NMR Data of Compounds CC-371 and PUR-1

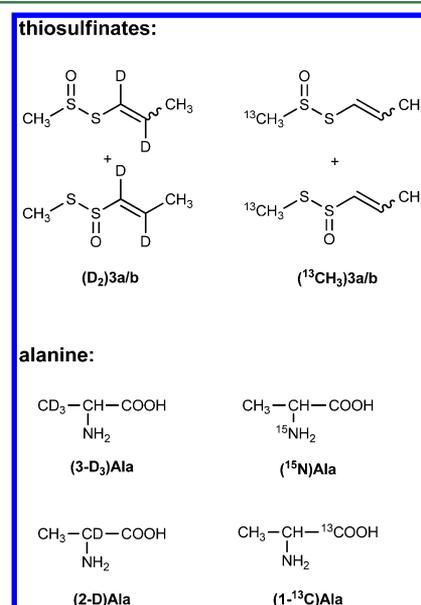
atom ^a	chemical shifts (ppm), signal intensity, multiplicity, coupling constants (Hz)			
	CC-371		PUR-1	
	^1H	^{13}C	^1H	^{13}C
2/2'	7.82 (2H, s)	140.9	7.75 (s)	140.9
3/3'		127.9		128.0
4/4'		140.2		138.8
5/5'		135.0		135.4
6/8	8.00 (2H, d, 13.2)	146.4	8.07 (d, 13.5)	146.4
7	7.41 (1H, t, 13.2)	119.4	7.39 (dd, 13.5)	119.6
9/9'	2.14 (6H, s)	9.8	2.02 (s)	9.8
10/10'	2.46 (6H, s)	13.4	2.36 (s)	13.5
11/11'		173.4		173.6
12/12'	5.42 (2H, q, 7.1)	56.1		
13/13'	1.84 (6H, d, 7.1)	18.2		

^aAtom numbers correspond to Figure 3.

**Figure 3.** Structures of CC-371 and PUR-1.

corresponded to two general formulas of either $\text{C}_x\text{H}_y\text{N}_2\text{O}_4$ or $\text{C}_x\text{H}_y\text{N}_2\text{O}_5$ ($x = 20-27$, $y = 27-37$). Structures of the compounds were proposed on the basis of evaluation of their MS/MS fragmentation and isotopic patterns. The interpretation of MS data and structure assignment of individual fragments was greatly facilitated by the use of several isotope-labeled precursors, including four alanine isotopologues [(3- D_3)Ala, (^{15}N)Ala, (2- D)Ala, and (^{13}C)Ala] and two

selectively labeled thiosulfinates ($^{13}\text{C}_3$)3a/b and (D_2)3a/b (Figure 4).

**Figure 4.** Isotopically labeled precursors used in our model experiments.

Detailed inspection of the MS/MS spectra revealed that the color compounds could be divided into two structurally distinct groups. The first one included compounds having spectra containing a dominant fragment of 160 Da attributable to $[\text{C}_{11}\text{H}_{14}\text{N}]^+$. Their fragmentation patterns were analogous to that of CC-371 (see the Supporting Information). It was obvious that these compounds are closely structurally related to CC-371, differing only in the presence of various side chain alk(en)yl moieties (CH_3- in CC-385, $\text{CH}_3\text{CH}=\text{CH}-$ in CC-411, and $\text{CH}_3\text{CH}_2\text{CH}_2-$ in CC-413). On the other hand, the

Table 2. UV-Vis and ESI-HRMS Data of Color Compounds Formed by the Reaction of Onion/Leek Ether Extracts with Alanine

compound ^a	λ_{max}^b (nm)	elemental composition	RDB ^c	(+)ESI			(-)ESI		
				$[\text{M} + \text{H}]^+$ calcd	$[\text{M} + \text{H}]^+$ found	deviation (ppm)	$[\text{M} - \text{H}]^-$ calcd	$[\text{M} - \text{H}]^-$ found	deviation (ppm)
CC-358	518	$\text{C}_{20}\text{H}_{26}\text{N}_2\text{O}_4$	9	359.1965	359.1972	1.9	357.1820	357.1820	0
CC-402	518	$\text{C}_{22}\text{H}_{30}\text{N}_2\text{O}_5$	9	403.2227	403.2227	0	401.2082	401.2083	0.3
CC-416	519	$\text{C}_{23}\text{H}_{32}\text{N}_2\text{O}_5$	9	417.2384	417.2383	0.3	415.2238	415.2238	0
CC-371 ^d	568	$\text{C}_{21}\text{H}_{27}\text{N}_2\text{O}_4$	10	371.1965	371.1970	2.1	369.1820	369.1820	0
CC-384	537	$\text{C}_{22}\text{H}_{28}\text{N}_2\text{O}_4$	10	385.2122	385.2129	1.9	383.1976	383.1979	0.7
CC-386	517	$\text{C}_{22}\text{H}_{30}\text{N}_2\text{O}_4$	9	387.2278	387.2277	0.3	385.2133	385.2137	1.1
CC-442	548	$\text{C}_{25}\text{H}_{34}\text{N}_2\text{O}_5$	10	443.2540	443.2542	0.4	441.2395	441.2396	0.2
CC-398	544	$\text{C}_{23}\text{H}_{30}\text{N}_2\text{O}_4$	10	399.2278	399.2283	1.2	397.2133	397.2133	0
CC-385 ^d	576	$\text{C}_{22}\text{H}_{29}\text{N}_2\text{O}_4$	10	385.2122	385.2119	0.7	383.1976	383.1972	1.1
CC-456	548	$\text{C}_{26}\text{H}_{36}\text{N}_2\text{O}_5$	10	457.2697	457.2706	2.0	455.2551	455.2546	1.2
CC-424	570	$\text{C}_{25}\text{H}_{32}\text{N}_2\text{O}_4$	11	425.2435	425.2435	0	423.2289	423.2290	0.2
CC-426	548	$\text{C}_{25}\text{H}_{34}\text{N}_2\text{O}_4$	10	427.2591	427.2587	0.9	425.2446	425.2444	0.4
CC-411 ^d	600	$\text{C}_{24}\text{H}_{31}\text{N}_2\text{O}_4$	11	411.2278	411.2272	1.5	409.2133	409.2133	0
CC-413 ^d	576	$\text{C}_{24}\text{H}_{33}\text{N}_2\text{O}_4$	10	413.2435	413.2443	2.0	411.2288	411.2288	0
CC-450	634	$\text{C}_{27}\text{H}_{34}\text{N}_2\text{O}_4$	12	451.2591	451.2594	0.5	449.2446	449.2441	1.1

^aThe names follow the format CC-number, where the CC stands for color compound and the number corresponds to the molecular weight. ^bData from DAD (in $\text{H}_2\text{O}/\text{CH}_3\text{OH}/\text{HCOOH}$). ^cRDB, the number of rings and double bonds present. ^d(+)ESI and (-)ESI values correspond to ions $[\text{M}]^+$ and $[\text{M} - 2\text{H}]^-$, respectively.

Table 3. Mass Increase Observed in the Color Compounds Formed from Isotope-Labeled Precursors (in Da)^a

compound	elemental composition	isotope-labeled precursor used						structural subunits present
		(3-D ₃)Ala	(¹⁵ N)Ala	(2-D)Ala	(1- ¹³ C)Ala	(¹³ CH ₃)3a/b	(D ₂)3a/b	
CC-358	C ₂₀ H ₂₆ N ₂ O ₄	+6	+2	+2	+2	+2	0	2C ₉ NO ₂ ^b + 2C ₁
CC-402	C ₂₂ H ₃₀ N ₂ O ₅	+6	+2	+2	+2	+1	+2	2C ₉ NO ₂ + C ₃ + C ₁ + OH
CC-416	C ₂₃ H ₃₂ N ₂ O ₅	+6	+2	+2	+2	+1	+2	2C ₉ NO ₂ + C ₃ + C ₁ + OCH ₃
CC-371	C ₂₁ H ₂₇ N ₂ O ₄	+6	+2	+2	+2	0	+1-2 ^c	2C ₉ NO ₂ + C ₃
CC-384	C ₂₂ H ₂₈ N ₂ O ₄	+6	+2	+2	+2	+1	+2	2C ₉ NO ₂ + C ₃ + C ₁
CC-386	C ₂₂ H ₃₀ N ₂ O ₄	+6	+2	+2	+2	+1	+2	2C ₉ NO ₂ + C ₃ + C ₁
CC-442	C ₂₅ H ₃₄ N ₂ O ₅	+6	+2	+2	+2	+1	+4	2C ₉ NO ₂ + 2C ₃ + C ₁ + OH
CC-398	C ₂₃ H ₃₀ N ₂ O ₄	+6	+2	+2	+2	+2	+2	2C ₉ NO ₂ + C ₃ + 2C ₁
CC-385	C ₂₂ H ₂₉ N ₂ O ₄	+6	+2	+2	+2	+1	+1-2 ^c	2C ₉ NO ₂ + C ₃ + C ₁
CC-456	C ₂₆ H ₃₆ N ₂ O ₅	+6	+2	+2	+2	+1	+4	2C ₉ NO ₂ + 2C ₃ + C ₁ + OCH ₃
CC-424	C ₂₅ H ₃₂ N ₂ O ₄	+6	+2	+2	+2	+1	+4	2C ₉ NO ₂ + 2C ₃ + C ₁
CC-426	C ₂₅ H ₃₄ N ₂ O ₄	+6	+2	+2	+2	+1	+4	2C ₉ NO ₂ + 2C ₃ + C ₁
CC-411	C ₂₄ H ₃₁ N ₂ O ₄	+6	+2	+2	+2	0	+3-4 ^c	2C ₉ NO ₂ + 2C ₃
CC-413	C ₂₄ H ₃₃ N ₂ O ₄	+6	+2	+2	+2	0	+3-4 ^c	2C ₉ NO ₂ + 2C ₃
CC-450	C ₂₇ H ₃₄ N ₂ O ₄	+6	+2	+2	+2	0	+5-6 ^c	2C ₉ NO ₂ + 3C ₃

^aExact masses determined by (+)ESI-HRMS are given in the Supporting Information. ^bC₉NO₂ represents a subunit with *N*-(1-carboxyethyl)-3,4-dimethylpyrrole backbone. ^cIsotopologues with either one or two deuterium atoms in the C₃-bridge were detected.

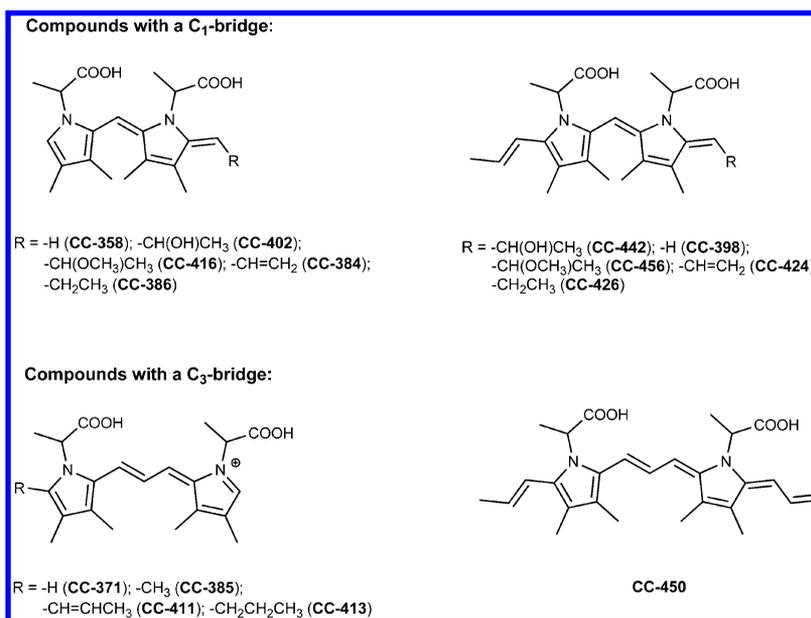


Figure 5. Tentative structures of compounds responsible for pinking of onion and leek.

fragment of 160 Da was absent in spectra of compounds belonging to the second group. As described in the following sections, these compounds were deduced to be composed of two 3,4-dimethylpyrrole-derived moieties linked via a one-carbon unit.

When isotopically labeled alanine was used in our model mixtures, molecular weights of all compounds changed by the same increment. In the case of (3-D₃)Ala, the relative molecular weights increased by 6 Da. On the other hand, an increase of 2 Da was observed when (¹⁵N)Ala, (2-D)Ala, or (1-¹³C)Ala was used (Table 3, for exact masses see the Supporting Information). These results unambiguously showed that two molecules of alanine were incorporated into each color compound without any structural changes (except for the loss of the -NH₂ hydrogens). Thus, two alanine molecules contributed 6 carbon, 10 hydrogen, 2 nitrogen, and 4 oxygen atoms to the overall elemental composition of each color compound, whereas the remaining atoms originated from the 1-

propenyl and methyl groups of 3a/b as demonstrated by experiments with isotopically labeled thiosulfates (D₂)3a/b and (¹³CH₃)3a/b (Table 3).

Experiments with (¹³CH₃)3a/b showed that molecular weights of all C₁-bridged compounds increased by 1–2 Da (Table 3), with the ¹³C label always occurring in the C₁-bridge between the two rings. In compounds CC-358 and CC-398, a second ¹³C label was detected, which could be attributed to the presence of a C₁-side chain derived from the methyl group of (¹³CH₃)3a/b (see the MS spectra including fragment assignments in the Supporting Information). On the contrary, no ¹³C label was detected in the C₃-bridged compounds (except for the methyl side group of CC-385). These compounds were thus composed of two molecules of alanine and C₃-units derived from the 1-propenyl group of isoalliin (1).

The number of C₃-units involved in the formation of the color compounds was deduced from the experiments with CH₃CD=CD-labeled thiosulfates (D₂)3a/b. As shown in

were rather evenly distributed at all three positions of the propenylidene unit. In most cases, isotopologues with only one deuterium atom in the C₃-bridge were also detected. On the other hand, the side C₃-chains of all compounds (if present) always contained the two deuterium atoms at positions 1 and 2. No deuterium labels were detected in the pyrrole units of compounds with an unsubstituted α -position (i.e., CC-358, CC-402, CC-416, CC-384, CC-386, CC-371, CC-385, CC-411, and CC-413). However, the α -hydrogens of pyrroles are known to readily exchange under acidic conditions.²⁶ It is therefore not clear whether the deuterium atoms were replaced by hydrogens during the formation of the compounds or the exchange occurred during their isolation/analysis.

In general, the collected MS data including fragmentation and isotopic patterns were found to be in excellent agreement with the proposed structures. MS spectra of compounds obtained by the use of isotopically labeled precursors were also fully consistent with the structures shown in Figure 5 (see the Supporting Information). Differences between observed and calculated $[M + H]^+$ or $[M]^+$ values typically varied in the range of 0–2.1 ppm with a mean of 1.0 ppm. In all cases, structures could be assigned to fragments representing at least 96% of the total fragment intensity within the mass tolerance of 10 ppm.

Absorption maxima of the color compounds were also in a very good correlation with the proposed structures. Structurally related C₁-bridged compounds with one double bond in their side chain (i.e., CC-358, CC-386, CC-402, and CC-416) exhibited λ_{\max} of 517–519 nm (Table 2). The presence of additional propenyl group in their counterparts (i.e., CC-398, CC-426, CC-442, and CC-456) caused a shift of the maximum to 544–548 nm. On the other hand, the C₃-bridged compounds exhibited significantly higher λ_{\max} values (568–634 nm) that also closely correlated with their degree of unsaturation. In general, the C₁-bridged compounds seem to be responsible for pink or red tones, whereas the C₃-bridged species contribute to the violet or blue shades of homogenized onion/leek.

Although the proposed structures are based on rather strong experimental evidence, they should be considered as tentative until confirmed by NMR. It should also be noted that the structures differ from those reported previously by other authors. As depicted in Figure 6, Lee et al.²¹ tentatively identified a color compound (Ala-p) generated upon mixing alanine with ether extract of homogenized onion. Given the fact that the formation of Ala-p would involve modification of the amino acid backbone, we consider this pathway as very unlikely on the basis of our experiments with isotopically labeled alanine. We rather suspect that Ala-p (M_r 358 Da) could be misidentified with isomeric CC-358. On the other hand, Kato et al.²² reported tentative identification of a compound named “pink pigment α ” in a model mixture consisting of isoalliin (1), alliinase, valine, and formaldehyde (Figure 6). On the basis of MS data, they deduced the compound to be composed of two 3,4-dimethylpyrrole moieties bridged by a vinylene group presumably derived from two formaldehyde molecules. However, our experiments with (¹³CH₃)₃a/b did not show that such C₂-bridged compounds are formed. We thus suspect that “pink pigment α ” could in fact be the valine analogue of CC-358.

Possible Formation Pathways. Imai et al.¹⁰ showed that *N*-substituted 3,4-dimethylpyrroles (e.g., 6) are key intermediates formed during discoloration of garlic. These colorless pyrroles apparently play a pivotal role also in the formation of

the color compounds in onion and leek homogenates (Figure 7). They are probably generated by the reaction of amino acids with 1-propenyl-containing thiosulfonates (e.g., 3a/b)^{8,12} or cepathiolanes (5).²² These pyrroles subsequently react with various one- and three-carbon (thio)aldehydes derived from thiosulfonates. Another source of aldehydes is likely to be the lachrymatory sulfine, thiopropanal sulfoxide (11), which is known to readily decompose to propanal (12).²⁷ On the other hand, the role of other carbonyl compounds naturally occurring in onion/leek seems to be rather marginal. For example, only traces of color compounds containing C₂-side chains (presumably derived from acetaldehyde) were detected (not shown).

We propose that the compounds with the methine bridge are generated by the well-known reaction of 6 with (thio)formaldehyde to form colorless bis[*N*-(1-carboxyethyl)-3,4-dimethylpyrrol-2-yl]methane (10).²⁸ In accordance with this proposal, the valine analogue of 10 (named CC α) was recently found by Kato et al.²² in model systems consisting of valine, isoalliin, alliinase, and formaldehyde. Our experiments with (¹³CH₃)₃a/b showed that (thio)formaldehyde is derived from CH₃SS(O)R thiosulfonates according to the mechanism proposed by Block et al.²⁷ The color compounds are subsequently generated by further modifications of 10. For example, 10 can react with another molecule of (thio)formaldehyde to yield pink CC-358 (via hydroxymethylation and subsequent dehydration) or analogously with a C₃-carbonyl compound (probably propanal) to give magenta CC-386. 2-[(Methylthio)oxy]propanethial (7), the reactive intermediate formed during the (*E/Z*)-interconversion of thiosulfonate 3a,^{24,27} is probably the source of the 2-hydroxypropenylene unit present in CC-402 and CC-442. Compounds CC-384 and CC-424 are likely to be generated by dehydration of CC-402 and CC-442, respectively. On the other hand, the two compounds containing the methoxy moiety in their side chains (CC-416 and CC-456) are probably only artifacts generated by the reaction of acidified methanol with CC-402 and CC-442, respectively, during isolation. In accordance with this assumption, no ¹³C-label was present in the CH₃O– moiety when (¹³CH₃)₃a/b was used.

The C₃-bridged compounds (CC-385, CC-411, and CC-413) are probably formed by analogous modifications of CC-371 as shown in Figure 7. However, the exact mechanism leading to the formation of the parent compound (CC-371) remains unclear, although Block²⁷ proposed its formation via Diels–Alder reaction of thioacrolein with 6.

Although compounds containing more than two pyrrole subunits were not found at significant levels in our model mixtures, increased formation of tri- or tetrameric compounds in advanced stages of discoloration can be expected.

Our experiments also showed that a single amino acid could give rise to more than 15 color compounds. In the presence of two amino acids, the number will be considerably higher due to the formation of “mixed” color compounds containing different *N*-bound residues in the two pyrrole-based moieties. It is thus likely that the pigment formed in homogenized onion and leek may consist of several hundreds of compounds.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b04564.

(+)ESI-HRMS spectra of the color compounds (including fragment assignments), EI-MS spectra of isotopically labeled disulfides, detailed procedures for synthesis of isotopically labeled compounds, NMR spectra of CC-371, a picture of color fractions collected by preparative HPLC, 2D-HPLC chromatogram of color compounds formed after mixing 3a/b with alanine, IUPAC names of the color compounds (ZIP)

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

CC, color compound; COSY, correlation spectroscopy; DAD, diode array detector; EI, electron impact; ESI, electrospray ionization; GC-MS, gas chromatography–mass spectrometry; IDA, information-dependent acquisition; HMBC, heteronuclear multiple-bond correlation; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; HSQC, heteronuclear single-quantum correlation; LFS, lachrymatory factor synthase; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance; PTFE, polytetrafluoroethylene; subg, subgenus; PUR, reddish-purple pigment (adopted from ref 10); RDB, (the number of) rings and double bonds; UV–vis, ultraviolet–visible; 2D, two-dimensional

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