JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Identification of Two Novel Pigment Precursors and a Reddish-Purple Pigment Involved in the Blue-Green Discoloration of Onion and Garlic

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By using a model reaction system representing blue-green discoloration that occurs when purees of onion (*Allium cepa* L.) and garlic (*Allium sativum* L.) are mixed, we isolated two pigment precursors (PPs) and a reddish-purple pigment (PUR-1) and determined their chemical structures. PPs were isolated from a heat-treated solution containing color developer (CD) and either L-valine or L-alanine, and their structures were determined as 2-(3,4-dimethylpyrrolyl)-3-methylbutanoic acid (PP−Val), and 2-(3,4-dimethyl-1H-pyrrolyl) propanoic acid (PP−Ala), respectively. Next, PUR-1 was isolated from a heat-treated solution containing PP−Val and allicin, and its structure was determined as (1*E*)-1-(1-((1*S*)-1-carboxy-2-methylpropyl)-3,4-dimethyl-1H-pyrrol-2-yl)-prop-1-enylene-3-(1-((1*S*)-1-carboxy-2-methylpropyl)-3,4-dimethyl-1H-pyrrol-2-ylidenium). The structure of PUR-1 suggested that PP molecules containing a 3,4-dimethyl pyrrole ring had been cross-linked by an allyl group of allicin to form conjugated pigments. While PUR-1 is a dipyrrole compound exhibiting a reddish-purple color, a color shift toward blue to green can be expected as the cross-linking reaction continues to form, for example, tri- or tetrapyrrole compounds.

KEYWORDS: Allium; garlic; onion; greening; pinking; discoloration; structure; mechanism.

INTRODUCTION

Blue pigment formation that occurs in a mixed puree of onion and garlic is the same phenomenon known as "greening" of garlic. "Pinking" of onion also occurs thorough essentially the same reaction mechanism. In our companion paper (*1*), we presented a model reaction system capable of producing blue pigments from only chemically defined components.

The model reaction system of our study comprises four steps, namely: (i) formation of *S*-1-propenyl 1-propenethiosulfinate termed color developer (CD) by the catalytic action of alliinase (EC 4.4.1.4) on *trans*-(+)-*S*-(1-propenyl)-L-cysteine sulfoxide (1-PeCSO); (ii) formation of colorless, ether-insoluble pigment precursor (PP) from the CD and amino acids; (iii) formation of *S*-2-propenyl 2-propenethiosulfinate (allicin) by the catalytic action of alliinase on *S*-allyl-L-cysteine sulfoxide (2-PeCSO); and (iv) formation of blue pigments from PP and allicin. These reaction steps are in agreement with those predicted by earlier investigators (*2*−*7*).

In this paper, we report isolations and structure elucidations of two PPs and a reddish-purple pigment named PUR-1, and discuss possible reaction mechanisms leading to pigment formation at a molecular level.

MATERIALS AND METHODS

Materials. All chemicals used were purchased from Wako Pure Chemical (Osaka, Japan) or Kanto Chemical (Tokyo, Japan) unless otherwise noted. All solvents used were chromatography grade. Acidic water was prepared by adjusting the pH of distilled water to 3.3 with trifluoroacetic acid (TFA). Yellow onion and garlic were purchased locally. Juices of onion and garlic, purified garlic alliinase, 1-PeCSO, 2-PeCSO, CD, and allicin were prepared as in our companion paper (*1*).

Apparatus. A JNM-A 500 spectrometer equipped with a pulsed field gradient (PFG) unit (JEOL, Tokyo, Japan), operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C, was used for NMR experiments. Coupling constants are expressed in Hz, and chemical shifts of PP−Val and PP−Ala are expressed in δ (parts per million), referring to the solvent peaks δ H 2.49 and δ C 39.5 for DMSO-d_6. Chemical shifts of PUR-1 are expressed relative to TMS (tetramethylsilane) as the internal standard. 1H, 13C, 1H-1H COSY, NOESY, PFG-HMQC, and PFG-HMBC NMR experiments were carried out by using the conventional pulse sequences as described in standard textbooks. High-resolution fast atom bombardment (HRFAB) mass spectra were recorded in positive mode on a VG ZAB-HF (Micromass, Manchester, U.K.) by using glycerol as the matrix and Xe for atom bombardment. High-resolution electron impact (HREI) mass spectra were obtained by using a JMS-SX102A (JEOL) or DX-303 (JEOL). Samples were introduced by a direct probe inlet system. The ionization voltage was 70 eV. UV−visible spectra (200−700 nm) in methanol were obtained with a UV-3100 PC (Shimadzu, Kyoto, Japan). Infrared spectra (400−4000 cm^{-1}, KBr method) were obtained with a JIR-7000 (JEOL). The final purification of two pigment precursors and a pigment was performed by medium-pressure liquid chromatography (MPLC) with a YFLC system (Yamazen, Osaka, Japan).

Preparation of a Pigment Precursor (PP−Val) from CD and L-Valine. 1-PeCSO (2.0 g) was dissolved in 0.1 M acetic acid buffer (pH 5.6, 200 mL) and treated with purified alliinase (27 000 units) at

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10.1021/jf0519818 CCC: $33.50
Published on Web 01/12/2006

Table 1. Physicochemical Properties of PP–Val (**1**), PP–Ala (**2**), and PUR-1 (**3**)

	1	2	3
appearance	colorless needles	colorless needles	purple powder
MP (°C)	70–72	38–40	161–164
molecular formula	$C_{11}H_{17}NO_2$	$C_9H_{13}NO_2$	$C_{25}H_{35}N_2O_4$
HREI-MS (*m/z*) found	195.1265 (M^+)	167.0947 (M^+)	
calcd	195.1260	167.0946	
HRFAB-MS (*m/z*) found			427.2589 (M+)
calcd			427.2581
UV λ_{max}^{MeOH} nm (ϵ)	230 (5200)	228 (6300)	570 (94 000)
UV λ_{max}^{MeOH} + HCl nm (ϵ)	230 (5900)	228 (6300)	570 (148 000)
UV λ_{max}^{MeOH} + NaOH nm (ϵ) max	220 (4900)	222 (5600)	300 (20 000)
IR ν_{max} (KBr) cm^{-1}	3000, 2960, 1720	3050, 2990, 1700	3430, 2970, 1680
$[\alpha]_D^{24}$ (c 0.2, CH_3OH)	–6.5°	+22°	

Table 2. ^1H and ^{13}C NMR Assignments for PP–Val (1), PP–Ala (**2**), and PUR-1 (**3**)

	PP–Val		PP–Ala		PUR-1	
	1[a]		2[a]		3[b]	
atom	δ_C mult	δ_H (mult, *J*)	δ_C mult	δ_H (mult, *J*)	δ_C mult	δ_H (mult, *J*)
2	118.1 (d)	6.47 (s)	117.5 (d)	6.45 (s)	140.9 (d)	7.75 (s)
3	115.7 (s)		116.3 (s)		128.0 (s)	
3-Me	10.1 (q)	1.87 (s)	10.0 (q)	1.87 (s)	9.8 (q)	2.02 (s)
4	115.7 (s)		116.3 (s)		138.8 (s)	
4-Me	10.1 (q)	1.87 (s)	10.0 (q)	1.87 (s)	13.5 (q)	2.36 (s)
5	118.1 (d)	6.47 (s)	117.5 (d)	6.45 (s)	135.4 (s)	
6	69.2 (d)	4.00 (d, 10.0)	55.7 (d)	4.64 (q, 7.5)	68.5 (d)	4.57 (d, 10.0)
7	172.2 (s)		172.9 (s)		173.6 (s)	
7-OH		12.83 (s)				
8	31.2 (d)	2.20 (dqq, 10.0, 6.5, 6.5)	18.0 (q)	1.48 (d, 7.5)	32.9 (d)	2.41 (dqq, 10.0, 6.5, 6.5)
9	18.5 (q)	0.65 (d, 6.5)			19.1 (q)	0.76 (d, 6.5)
10	19.4 (q)	0.90 (d, 6.5)			20.0 (q)	1.06 (d, 6.5)
11					146.4 (d)	8.07 (d, 13.5)
12					119.6 (d)	7.39 (dd, 13.5, 13.5)
2′					140.9 (d)	7.75 (s)
3′					128.0 (s)	
3′-Me					9.8 (q)	2.02 (s)
4′					138.8 (s)	
4′-Me					13.5 (q)	2.36 (s)
5′					135.4 (s)	
6′					68.5 (d)	4.57 (d, 10.0)
7′					173.6 (s)	
8′					32.9 (d)	2.41 (dqq, 10.0, 6.5, 6.5)
9′					19.1 (q)	0.76 (d, 6.5)
10′					20.0 (q)	1.06 (d, 6.5)
11′					146.4 (q)	8.07 (d, 13.5)

[a] In DMSO-d_6. [b] In MeOH-d_4.

37 °C for 3 min. CD was extracted with ether (200 mL × 3) and concentrated in vacuo. To convert CD to PP−Val, 0.1 M acetic acid buffer (pH 5.6, 200 mL) and L-valine (9.34 g) were added to CD extract and heated in boiling water for 15 min. The reaction products were extracted with ether (200 mL × 3), concentrated in vacuo, redissolved in methanol/acidic water (7:3 v/v), and subjected to MPLC with a reverse-phase LiChroprep RP-18 column (310 mm × 25 mm i.d. 40−60 μm, Merck, Darmstadt, Germany) eluted with methanol/acidic water (7:3 v/v) at 8.5 mL/min. The eluent was monitored at 230 nm. Fractions that formed red color when heated for 2 min at 60 °C with formaldehyde, or for 10 min in boiling water with allicin, were collected as active fractions. Active fractions eluted around 42 min were evaporated to dryness in vacuo to give 186.7 mg of PP−Val.

Preparation of a Pigment Precursor (PP−Ala) from CD and L-Alanine. PP−Ala was prepared and purified by the same procedure as described above with minor modifications. PP−Ala was prepared from CD (757.2 mg), purified alliinase (10 000 units), and L-alanine (2.69 g). The reaction products were extracted with ether, concentrated in vacuo, redissolved in methanol/acidic water (6:4 v/v), and subjected to MPLC with a reverse-phase LiChroprep RP-18 column (310 mm × 25 mm i.d. 40−60 μm) eluted with methanol/acidic water (6:4 v/v) at 8.5 mL/min. The eluent was monitored at 230 nm and active fractions eluted around 26 min were evaporated to dryness in vacuo to give 20.3 mg of PP−Ala.

Preparation of a Pigment (PUR-1) from PP−Val and Allicin. PP−Val (18.7 mg) was dissolved in a 0.1 M acetic acid buffer (pH 5.6, 162 mL) and mixed with an equal volume of the 0.1 M acetic acid buffer containing allicin (0.6 mM). The mixture of PP−Val and allicin was heated for 25 min in boiling water and cooled to room temperature. Insoluble pigment was removed by filtration, and the filtrate was washed with ether (320 mL × 2). The water layer was collected, concentrated in vacuo, applied to a Sep-pak C18 Cartridge (Waters, Milford, MA), and eluted with methanol/acidic water (7:3 v/v). The eluate was subjected to MPLC with a reverse-phase LiChroprep RP-18 column (310 mm × 25 mm i.d. 40−60 μm) eluted with methanol/acidic water (7:3 v/v) at 8.5 mL/min. Eluent was monitored at 570 nm, and fractions corresponding to the largest peak around 42 min were pooled and evaporated to dryness in vacuo to give 3.2 mg of PUR-1.

RESULTS AND DISCUSSION

Structure Elucidations. The physicochemical properties of PP−Val, PP−Ala, and PUR-1 prepared as described in the

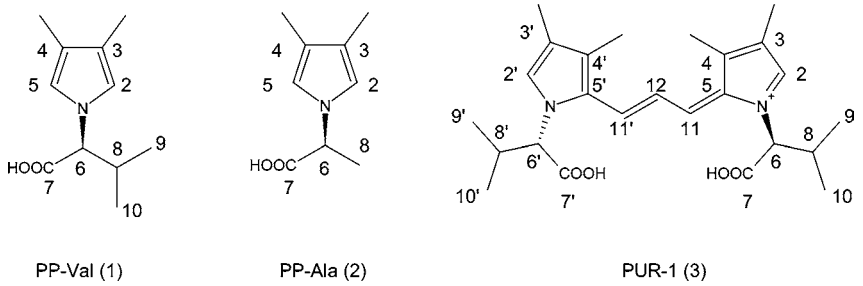

Figure 1. Structures of PP–Val, PP–Ala, and PUR-1.

material and methods section are summarized in **Table 1**, and their ^{1}H- and ^{13}C NMR spectral data in **Table 2**.

Because of the close resemblance of their UV and NMR spectra, it was anticipated that PP−Val and PP−Ala would have similar molecular structures. We, therefore, elucidated the structure of PP−Val first, and making use of the result, determined the structure of PP−Ala, and finally, the structure of PUR-1. Their structures are summarized in **Figure 1**.

Structure of PP−Val (1). The molecular formula of PP−Val was deduced to be $C_{11}H_{17}NO_2$ on the basis of HREI-MS and ^{13}C NMR spectral data. The IR absorption bands at 3000 cm^{-1} and at 1720 cm^{-1} indicated the presence of a hydroxyl group and a carbonyl residue, respectively. The ^{1}H NMR spectrum of PP−Val showed an exchangeable broad signal at 12.83 ppm (H-7-OH), ascribable to a hydroxyl proton. COSY spectrum demonstrated that the signal at 2.20 ppm (dqq, H-8) was coupled to the signals at 0.65 ppm (d, H-9), 0.90 ppm (d, H-10), and 4.00 ppm (d, H-6). Thus, 2-methylpropyl unit was established. The ^{13}C NMR spectrum of PP−Val showed three overlapping signals at 118.1 ppm (d, C-2, C-5), 115.7 ppm (s, C-3, C-4), and 10.1 ppm (q, C-3-Me, C-4-Me), which suggested existence of a symmetric unit. The gross structure of PP−Val was established by PFG-HMBC experiment. The α-methine proton at 4.00 ppm (H-6) showed cross-peaks with a carbonyl carbon at 172.2 ppm (C-7) and the methine signals at 118.1 ppm (C-2, C-5), whereas the methyl proton at 1.87 ppm (H-3-Me, H-4-Me) had cross-peaks with the methine signals at 118.1 ppm (C-2, C-5) and a quaternary signal at 115.7 ppm (C-3, C-4)-(**Figure 2-1**). Thus, the whole structure of PP−Val was determined to be 2-(3,4-dimethylpyrrolyl)-3-methylbutanoic acid (**Figure 1-1**). The structure indicated that 2-amino-3-methylbutanoic acid unit was derived from L-valine, and hence, the configuration at C-6 was assumed to be *S*.

Structure of PP−Ala (2). The molecular formula for PP−Ala was established as $C_9H_{13}NO_2$ by HREI-MS and ^{13}C NMR spectra. The IR absorption bands at 3050 cm^{-1} and at 1700 cm^{-1} suggested the presence of a hydroxyl group and a carbonyl group, respectively. Comparison of ^{1}H NMR spectrum of PP−Ala with that of PP−Val revealed that two methyl proton signals at 0.65 ppm (H-9), 0.90 ppm (H-10), and one methine proton signal at 2.20 ppm (H-8) found in PP−Val were replaced by a doublet methyl signal at −1.48 ppm (H-8), which was coupled to the signal at 4.64 ppm (H-6) in PP−Ala. Thus, the structure of PP−Ala was deduced as 2-(3,4-dimethylpyrrolyl) propanoic acid (**Figure 1-2**) and was confirmed by PFG-HMBC experiment (**Figure 2-2**).

From the structures of PP−Val and PP−Ala, it can be seen that the side-chain of an amino acid incorporated into the PP molecule determines the substituent group at C-6. Although glycine had been used most frequently in the "pinking" or "greening" studies (*2*, *3*, *5*, *6*), and gave strong pigment formation in our model reaction system as well, isolation of PP derived from CD and glycine (PP-Gly) was not successful.

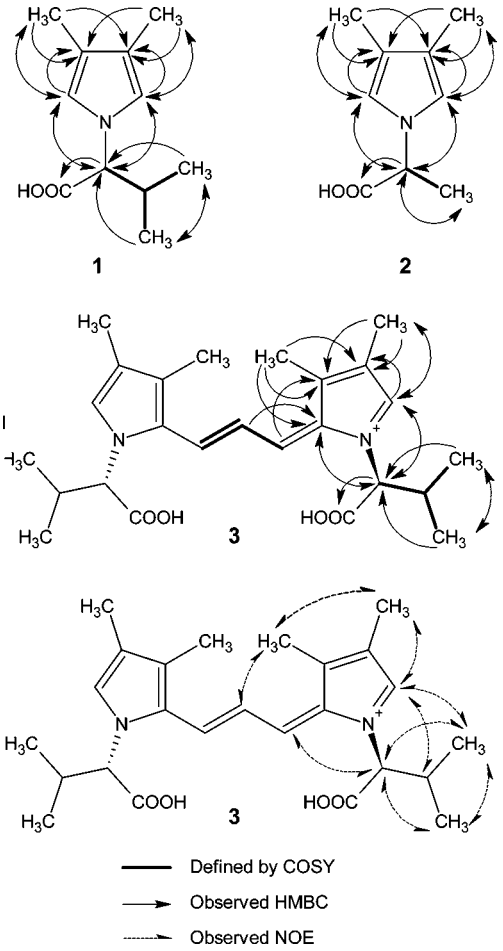

Figure 2. COSY, PFG-HMBC, and NOE analyses of PPs and PUR-1.

Judging from chromatograms of the reaction mixture (data not shown), we postulated that PP-Gly was less stable than PP−Val or PP−Ala and that the substituent group at C-6 had an influential role on the stability of PPs.

Structure of PUR-1 (3). The molecular formula for PUR-1 was established as $C_{25}H_{35}N_2O_4$ by HRFAB-MS and ^{13}C NMR spectra. The peak areas of all the signals except for the proton signal at 7.39 ppm (dd, J = 13.5, 13.5 Hz, H-12) in the ^{1}H NMR spectrum suggested that they were overlapping signals. This result, along with the PFG-HMQC spectra (data not shown), indicated that PUR-1 had a symmetric structure with C-12 at the center. COSY spectrum demonstrated that the methine signal at 7.39 ppm (dd, H-12) was coupled to 8.07 ppm (d, H-11, H-11′), and the methine signal at 2.41 ppm (dqq, H-8, H-8′) was coupled to 0.76 ppm (d, H-9, H-9′), 1.06 ppm (d, H-10, H-10′), and 4.57 ppm (d, H-6, H-6′). Thus, a −CH═CH−CH═ unit and two 2-methylpropyl units were established. The ^{1}H NMR spectrum also exhibited two overlapping singlet methyl signals at 2.02 ppm (H-3-Me, H-3′-Me), 2.36 ppm (H-

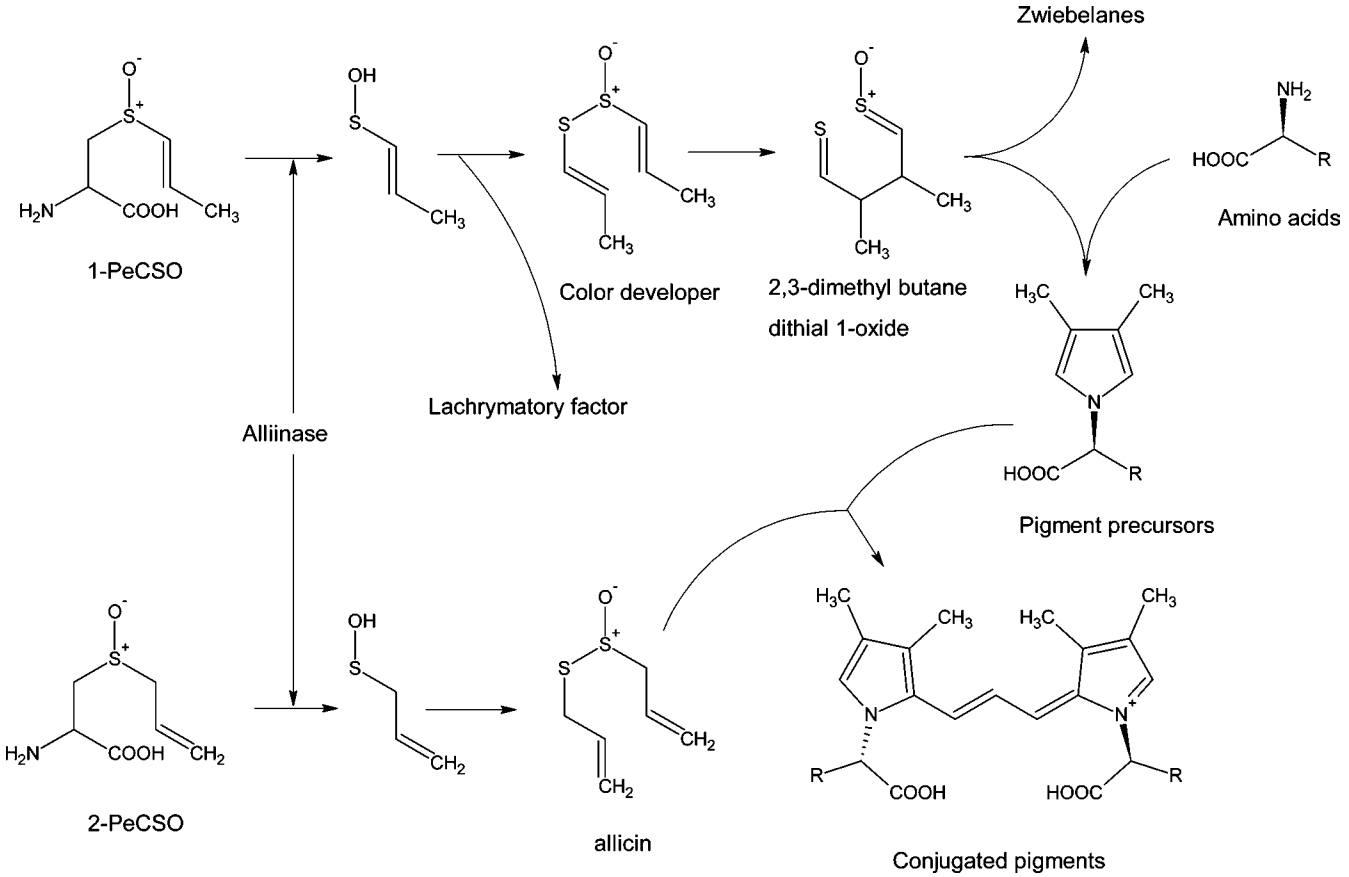

Figure 3. Proposed reaction mechanism leading to pigment formation in the model reaction system comprising 1-PECSO, 2-PECSO, purified alliinase, and amino acids.

4-Me, H-4′-Me), and an overlapping singlet methane signal at 7.75 ppm (H-2, H-2′). The ^{13}C NMR spectrum showed three overlapping quaternary carbon signals at 138.8 ppm (C-4, C-4′), 135.4 ppm (C-5, C-5′), 128.0 ppm (C-3, C-3′), and an overlapping carbonyl carbon signal at 173.6 ppm (C-7, C-7′). Connectivities of the revealed partial structures and signals were elucidated by the PFG-HMBC spectrum as shown in **Figure 2-3** (middle). The α-methine proton at 4.57 ppm (H-6, H-6′) showed cross-peaks with the carbonyl carbon signal at 173.6 ppm (C-7, C-7′), the methine signal at 140.9 ppm (C-2, C-2′), and quaternary carbon signal at 135.4 ppm (C-5, C-5′), whereas the olefinic methine proton at 7.39 ppm (H-12) had cross-peaks with the signal at 135.4 ppm (C-5, C-5′). The geometrical configurations were determined to be 11*E* and 11′*E* from their relevant proton coupling constants ($J_{11-12} = 13.5$, $J_{11'-12} = 13.5$ Hz), and these configurations were supported by NOESY correlations (**Figure 2-3** (bottom)). The configuration at C-6 and C-6′ was assumed to be *S*, as with PP−Val. Thus, the structure of PUR-1 was established as (1*E*)-1-(1-((1*S*)-1-carboxy-2-methylpropyl)-3,4-dimethyl-1H-pyrrol-2-yl)-prop-1-enylene-3-(1-((1*S*)-1-carboxy-2-methylpropyl)-3,4-dimethyl-1H-pyrrol-2-ylidenium), as shown in **Figure 1-3**.

Joslyn et al. (*8*) reported that the pigment responsible for pinking (reddening) of onion contained nitrogen, but not sulfur, and considered it as a good example of a then-uncharacterized class of pigments termed "nitrogenous anthocyanines". The structure of PUR-1 revealed in our study is in agreement with their findings.

Hayase et al. (*9*) identified the structure of a blue pigment formed in a D-xylose−glycine reaction system and named it as Blue-1. PUR-1 and Blue-1 share a common feature in that they both have a pyrrolylium group.

Proposed Reaction Mechanisms. The structures of PP−Val and PP−Ala suggested that the 3,4-dimethylpyrrole unit formed though a reaction between CD and amine residue of an amino acid. In our companion paper (*1*), we postulated that CD was *S*-1-propenyl 1-propenethiosulfinate on the basis of the fact that it was derived from 1-PeCSO by the action of alliinase, as well as on the observed positive correlation between the intensity of the *N*-ethylmaleimide color reaction and the amount of blue pigment formation. Block et al. (*10*) proposed that *S*-1-propenyl 1-propenethiosulfinate would rearrange itself rapidly to a more stable heterocyclic zwiebelanes via 2,3-dimethylbutanedithial-*S*-oxide. Further, Sircar et al. (*11*) reported that diketones, such as hexane-2,5-dione, would form pyrroles when heated with amino acids. Because of its structural similarity, we assumed that the dithial 1-oxide moiety had reactivity similar to that of diketone and that the 2,3-dimethylbutanedithial 1-oxide reacted with amino acids to form PPs.

When heated, an aqueous solution of PP−Val and allicin turned reddish purple first, then to blue to dark blue, and finally, formed green-colored precipitate. Because isolation of individual pigments became increasingly difficult as the number of pigmented molecular species increased with heating time, we isolated only PUR-1, which formed almost exclusively during the initial stage of heating. Its structure suggested that PUR-1, a dipyrrole compound, was a product of two molecules of PP−Val connected together by the allyl moiety of allicin and that tri-, tetra-, and polypyrrole compounds were expected to form as polymerization progressed with heating. As phycocyanobilin, a blue food colorant derived from *Spirulina* sp., has a tetrapyrrole structure (*12*), we conclude that the blue to green pigments that form from the reaction between PPs and allicin also have

tetra- or polypyrrole structures. The proposed reaction scheme of the color formation is summarized in **Figure 3**.

ABBREVIATIONS USED

1-PeCSO, *trans*-(+)-*S*-(1-propenyl)-L-cysteine sulfoxide; 2-PeCSO, *S*-allyl-L-cysteine sulfoxide or *S*-2-propenyl-L-cysteine sulfoxide; CD, color developer; PP, pigment precursor; HRFAB, high-resolution fast atom bombardment; HREI, high-resolution electron impact; COSY, correlated spectroscopy; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; PFG, pulsed field gradient; HMQC, heteronuclear multiple-quantum coherence; HMBC, heteronuclear multiple-bond coherence; MPLC, medium-pressure liquid chromatography; PUR-1, reddish-purple pigment

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Received for review August 12, 2005. Revised manuscript received December 5, 2005. Accepted December 10, 2005.

JF0519818