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# Characterization of yellow pigments formed on reaction of 2-(1*H*-pyrrolyl)carboxylic acids with pyruvic acid in garlic greening model systems

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ABSTRACT

Two new pyrrole derivatives 2-(1*H*-pyrrol-1-yl)succinic acid (P-Asp) and 2-(1*H*-pyrrol-1-yl)pentanedioic acid (P-Glu) were synthesized to study their effect on garlic greening, the structures of which are similar to that of a previously proposed pigment precursor for garlic greening. The puree of freshly harvested garlic bulbs turned green after being soaked in solutions of the two compounds. Also, it was found that yellow pigments can be produced by reacting the two model compounds with pyruvic acid at room temperature. Four major new yellow pigments from these two model systems were formed. Two of them named AUP-1 and AUP-2 produced from model system II consisting of P-Asp and pyruvic acid have the same molecular weight 417.1294 $[M+H]^+$ but having different retention times in HPLC, suggesting that there are isomers with each other. Likewise, other two isomer compounds GUP-1 and GUP-2 from model system I containing P-Glu and pyruvic acid have molecular weight as 445.1586 $[M+H]^+$. The molecular formula for AUP-1 and AUP-2 was $C_{20}H_{20}N_2O_8$ while the molecular formula for GUP-1 and GUP-2 was $C_{22}H_{24}N_2O_8$. All these results confirmed previous proposal that pyrrole derivatives acted as PP (pigment precursor) for *Allium* discoloration.



## 1. Introduction

As food, spice, and traditional medicine, garlic has been widely planted and used in the world for more than 4000 years. It is processed in various forms, such as powder, granules, puree, minced paste, and oleoresin. During processing, green pigments are often formed, which limit commercial utilization and reduce economic value (Adams & Brown, 2007; Kim, Cho, & Kim, 1999; Lukes, 1986). On the contrary, the green color is desirable and required for preparation of the traditional homemade Chinese "Laba" garlic product (Bai, Chen, Wang, Liao, Zhao, Hu, 2005). The discoloration phenomenon and mechanism of the green color formation have been subjects of intense study, but little information is available for structure for final pigment(s). As early as 1958, it was reported that the pigment responsible for pinking of onion contained nitrogen, but not sulfur, and considered it as a good example of a then-uncharacterized class of pigments termed "nitrogenous anthocyanines" (Joslyn & Peterson, 1958). Lee et al. tried to identify the structure of the green pigment in garlic homogenate, but they were not successful as judged from the reported MS and $^{13}C$ NMR spectra (Lee, Cho, Kim, & Lee, 2007). In 2006, Imai et al. reported that they obtained a reddish-purple pigment in the model reaction system comprising *S*-(1-propenyl)-L-cysteine sulfoxide (1-PeCSO), *S*-allyl-L-cysteine sulfoxide (2-PeCSO), purified alliinase and amino acids, which was named PUR-1 (Imai, Akita, Tomotake, & Sawada, 2006a, 2006b). Recently, Jedelská et al. found that the pigment responsible for pinking of an exotic species *Allium giganteum* and its allies contained nitrogen and sulfur, and it was identified as 3, 3′ –dithio– 2,2′ dipyrrole (Jedelská, Vogt, Reinscheid, & Keusgen, 2008).

To date, the mechanism on garlic greening is not completely understood, it has been established that the discoloration is a multistep process including enzymatic and non-enzymatic reactions (Imai et al., 2006a, 2006b; Kubec, Hrbáčová, Musah, & Velíše, 2004; Kubec & Velíšek, 2007) and that garlic greening occurs with garlic containing enough isoalliin which is usually accumulated by low-temperature storage (0–10 °C) (Adams & Brown, 2007; Bai et al., 2005; Kim et al., 1999; Lukes, 1986). Such storage can increase the amount of *S*-(1-propenyl)-L-cysteine sulfoxide (1-PeCSO) which is necessary for the development of garlic greening (Lukes, 1986). It was known that garlic greening is similar to onion reddening (Kubec et al., 2004), and it comprises four steps. The formation of di(1-propenyl) thiosulfinate (Imai et al., 2006a, 2006b) or 1-propenyl containing thiosulfinate under the action of alliinase on its substrates (Kubec & Velíšek, 2007) represents step 1. The

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second step corresponds to the formation of pigment precursor(s) (PP) by reactions between thiosulfinates and amino acids. Since 2-(3,4-dimethylpyrrolyl)-3-methylbutanoic acid (PP-Val) reacts with di(2-propenyl) thiosulfinate to produce pigment(s), this compound and its derivatives were considered to be the pigment precursor(s) (Imai et al., 2006a, 2006b). Support for this idea comes from recent observation that model compounds of PP-Val, 2-(1*H*-pyrrol-1-yl)carboxylic acids with a hydrophobic side chain facilitate garlic puree prepared from newly harvested garlic to turn green (Wang, Nanding, Han, Chen, & Zhao, 2008). Moreover, these model compounds react with pyruvic acid, a product from the action of alliinase on either 1-PeCSO or 2-PeCSO to generate yellow pigment(s). The formed yellow pigment(s) was believed to mix with blue pigment(s) to result in the formation of green color responsible for garlic greening (Bai et al., 2005; Kubec & Velíšek, 2007; Wang et al., 2008). Our recent study showed that the side chain has an important effect on the garlic greening (Wang et al., 2008).

In the present study, two new compounds, 2-(1*H*-pyrrol-1-yl)carboxylic acids with a hydrophilic side chain were prepared and characterized to obtain insights into the mechanism of garlic greening. It was found that they can facilitate garlic greening in garlic puree prepared from freshly harvested garlic. Furthermore, these two model compounds reacted with pyruvic acid to form yellow pigments. The formed two pigments from the above system were identified by high-resolution mass spectroscopy.

## 2. Materials and methods

### 2.1. Chemicals

L-Glutamic acid and L-aspartic acid were purchased from Biodee Biotechnology Co., Ltd. (Beijing China). Hydrochloric acid, acetic acid, sodium acetate anhydrous, ethyl acetate, sodium sulfate anhydrous, and potassium hydroxide were purchased from Beijing Chemistry Co. (Beijing, China). Pyruvic acid was obtained from Sinopharm Chemical Reagent Co. (Beijing, China). Formic acid ($\geqslant$99%), methanol (HPLC-grade) and 2,5-dimethoxytetrathydrofuran were purchased from J&K Chemical Ltd. (Beijing, China). All solvent/chemicals except methanol used were of analytical grade or purer.

### 2.2. Plant materials

Freshly harvested (May 2008) garlic bulbs were obtained from a local market at China Agriculture University, stored at room temperature (28 °C) for less than 3 days, and used immediately for following experiments.

### 2.3. Synthesis of P-Glu and P-Asp

(*S*)-2-(1*H*-pyrrol-1-yl)pentanedioic acid (P-Glu) and (*S*)-2-(1*H*-pyrrol-1-yl)succinic acid (P-Asp) were synthesized by reacting corresponding amino acids with 2,5-dimethoxytetrathydrofuran as recently described (Wang et al., 2008). The identity and purity of the synthesized compounds were determined by $^{1}$H NMR and $^{13}$C NMR which were performed on a dpx-300 MHz spectrometer (Brucker Co.). DMSO-$d_6$ was used as an NMR solvent with tetramethylsilane (TMS) as an internal standard.

### 2.4. Effect of P-Glu and P-Asp on garlic greening of garlic purees from newly harvested garlic

After the garlic bulbs had been cracked, cloves (45 g) were peeled and rinsed with distilled water. Garlic was homogenized in a blender. Resulting materials were equally divided into three parts and each part was 15 g. Two parts were immersed in 25 mL of P-Glu and P-Asp (40 mM), respectively, for up to 6 days at room temperature (28 °C). As a control sample, the remaining part was soaked in 25 mL pH 2.0 water (which was prepared by adjusting pH of dd$H_2O$ pH to 2.0 with 10 mM HCl) instead of P-Glu or P-Asp under the same experimental conditions. After 1-day storage, resulting solutions (1 mL) were filtered with qualitative filter paper and placed into a cuvette for UV–vis spectral measurement. After UV–vis spectral measurement, the solutions were recombined with the supernatant and the residual for further incubation. The same procedure was repeated every day at the same time as above described until the 6th day. UV–vis spectra were recorded with a Cary 50 UV–vis spectrophotometer (Varian Co.). All experiments were performed in triplicate.

### 2.5. Reactions of P-Glu or P-Asp with pyruvic acid

Both pyruvic acid (40 mM) and P-Glu (40 mM) in de-ionized water were mixed thoroughly with a volumetric ratio of 1–1 followed by standing at room temperature (28 °C) for 3 days under dark conditions, which corresponds to reaction system I. A reaction of P-Asp with pyruvic acid was carried out with the same procedure used for P-Glu, representing reaction system II. The two chemicals have good solubility in water. After 3 days of incubation, resulting solutions were filtrated with syringe filter units (0.22 μm) and placed into a cuvette for spectral measurement.

### 2.6. HPLC-MS analysis

HPLC-MS/MS analysis was performed by an Alliance 2695 Separations Module (Waters, Milford, MA, USA) coupled to a Micromass Quattro Micro triple-quadrupole mass spectrometer (Micromass, Manchester, UK) with MassLynx software. After 3 days of incubation, resulting solution (20 μL) from the above model systems was filtrated with syringe filter units (0.22 μm) followed injection into a reversed MP-$C_{18}$ column (4.6 × 250 mm, 5 μm, Venusil, Agela, USA) maintained at 30 °C. The elution mode was isocratic using a mixture of 19% methanol and 81% water containing 0.2% formic acid as mobile phase at a flow rate of 0.4 mL/min. All chemicals were measured at 430 nm and detected using electrospray ionization in the positive ion mode. The optimized MS instrument parameters obtained by the tuning were as follows: capillary voltage, 2.5 kV; cone voltage, 10 V; source temperature, 110 °C; desolvation temperature, 400 °C; desolvation gas ($N_2$) flow, 700 L h$^{-1}$; cone gas ($N_2$) flow, 50 L h$^{-1}$.

High-resolution MS analysis was performed by a quadrupole ion-trap time-of-flight mass spectrometer (LCMS-IT-TOF, Shimadzu, Kyoto, Japan). The following method parameters were used for sample analysis: mass range, *m/z* 220–600 in MS and *m/z* 50–600 in MS$^n$ mode; ion source temperature, 200 °C; heated capillary temperature: 200 °C; ESI voltage, +4.5 kV; ESI nebulisation gas ($N_2$) flow, 1.5 L/min; detector voltage, 1.7 kV; and ion accumulation time, 10–50 ms. Positive ion mode was used. Argon gas was used as cooling gas and collision gas at a pressure of 0.15 MPa, respectively.

LCMS solution Ver. 3.41 and the Formula Predictor software were used to verify component identification (Shimadzu Corp., Kyoto, Japan). Predicting a candidate list based on MS and MS$^n$ data takes into account a number of variables, including isotopic profile analysis, mass accuracy and mass resolution of the experimentally derived pseudo-molecular peak and related fragment ion data.

### 2.7. Purification of yellow pigment from model reaction systems

After incubation for 3 days, 1 mL of reaction solution from system I was filtrated with syringe filter units (0.22 μm), and subjected to HPLC with a MP $C_{18}$ column (21.5 × 250 mm, 10 μm,