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Cepaic acid, a novel yellow xanthylium pigment from the dried outer scales of the yellow onion *Allium cepa*

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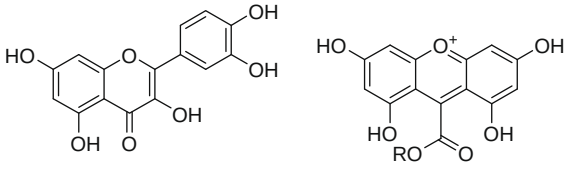
ABSTRACT

Cepaic acid was isolated as a novel xanthylium yellow pigment from the dried outer scales of the yellow onion *Allium cepa* Linne. Its structure was elucidated on the basis of ESI-MS and 2D NMR spectroscopy as a 9-carboxy-1,3,6,8-tetrahydroxyxanthylium, which suggests that cepaic acid and other yellow pigments in the dried outer skin of onion were formed by the nucleophilic reaction of phloroglucinol derived from quercetin, a flavonol in onion scales, by autoxidation to glyoxylic acid. To our knowledge, this is the first report of such a pigment in yellow onion.

The yellow pigment from the dried outer scales of the common yellow onion *Allium cepa* Linne has a warm and quiet tone. The pigment is hydrophilic, stable against heat and light exposure, and has an excellent dyeing ability against proteins. Therefore, the extract of the dried outer scales of the yellow onion *A. cepa* has been used as a common natural dyestuff, and the partially purified pigment has been permitted as a natural food color for food additives. Surprisingly, the chemical structure of the yellow pigment from onion has remained unknown, although various anthocyanin derivatives, such as the 3-O-glucoside of cyanidin, have been identified as red pigments of the red onion *A. cepa*.[1–3] It has been believed that quercetin (**1**, Fig. 1) and its glucosides (3-O-glucoside and 3,4′-O-diglucoside), the main flavonols in the scales of *A. cepa*, are responsible for the yellow color in the dried outer scales of onion. However, **1** and its glucosides are very pale yellow and the absorbance maximum of these (370 nm) does not fit well with that of the yellow pigment from the dried outer scales of onion which is located around 450 nm. In fact, the fresh scales of onion are absolutely white, regardless of how much glycosides of **1** they contain. The browning of the outer scales goes from the top to the bottom of the onion bulb when onions are dried during storage. Therefore, it is assumed that the yellow pigments in the dried outer scales are generated from constituents in the scales by oxidative reaction. In fact, various oxidized and degraded products of **1** such as 3,4-dihydroxybenzoic acid (protocatechuic acid) have been found from the dried outer scales which showed antioxidative and antifungal activities.[4]

As part of the study on natural food additives, we investigated the extract of the dried outer scales of yellow onion *A. cepa* by LC/ESI-MS. In this work the isolation and structural elucidation of a new xanthylium pigment, cepaic acid (**2**), are reported. A mechanism of its formation in the dried outer scales of the yellow onion is also proposed.

The dried outer scales of a yellow onion (20.56 g) were manually cut into small pieces and homogenized in 10% ethanol (1.0 L) with a blender for 1 h. The debris was then removed by filtration and centrifugation (2000 rpm, 15 min), and the obtained extract was concentrated in vacuo. A small volume of the extract was injected in the LC/PDA/ESI-MS system.[5] The PDA chromatogram of the extract recorded at 250 nm showed the appearance of various compounds, as previously reported.[4] The presence of 3,4-dihydroxybenzoic acid, 4′-O-glucoside of **1,** and aglycon **1** was confirmed by using standards. When the chromatogram was detected at 450 nm, an intense peak (**2**) was detected in 6.5 min



1 **2**: R = H **3**: R = Me

Figure 1. Structures of quercetin (**1**), cepaic acid (**2**), and cepaic acid methyl ester (**3**).

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followed by a huge unresolved broad hump over a wide range of the chromatogram (7.5–27.5 min). The UV–visible spectrum of **2** on the PDA showed an absorption maximum at 430 nm. The ESI-MS analysis in the positive ion mode of **2** gave an intense peak at *m/z* 289 and the analysis in the negative ion mode showed a peak at *m/z* 287 and a fragment peak at *m/z* 243 that suggested the presence of a carboxylic acid group in a molecule of **2**. For the isolation of **2**, the whole extract was first partitioned between *n*-BuOH and H_2O, which resulted in the yellow pigment staying in the aqueous layer. Subsequently, the aqueous layer was acidified to pH 1.0 with concd HCl and again partitioned with fresh *n*-butanol. Then the yellow pigment was entirely extracted in the *n*-butanol layer, which was subsequently concentrated in vacuo. The brown residue obtained was subjected to a flash ODS column, followed by elution with 0%, 10%, 50%, and 100% MeOH in 0.05% TFA. The light yellow 10% MeOH fraction was concentrated and purified by reversed-phase HPLC to yield **2** as an orange solid (5.13 mg).[6]

The ^{1}H NMR spectrum (500 MHz) of **2** in DMSO-d_6–TFA (9:1) gave only two signals at δ_H 6.46 and 6.63 ppm (Table 1). The appearance of these signals as two doublets with small coupling constants J = 2.3 Hz indicated that both were *meta* aromatic protons. The ^{13}C NMR analysis of **2** exhibited a spectrum including eight signals (Table 1). The ^{1}H-^{13}C HMQC experiment of **2** assigned two carbon signals at δ_c 96.2 and 101.1 ppm to aromatic methine carbons corresponding to the protons at δ_H 6.63 and 6.46 ppm, respectively (Table 1). The ^{1}H–^{13}C HMBC experiment of **2** showed the correlations from the proton at δ_H 6.63 to two oxygenated aromatic carbons at δ_c 162.3 and δ_c 172.8 ppm (Fig. 3). Analogously, the correlations from the proton at δ_H 6.46 to carbons at δ_c 158.8 ppm and δ_c 172.8 ppm were observed. Furthermore, the correlations from both the protons to a carbon signal at δ_c 105.6 ppm were also observed. These HMBC correlations indicated the presence of a probable phloroglucinol (benzene-1,3,5-triol) moiety in **2**. In this stage, two carbon signals at δ_c 151.5 and 166.6 ppm remained unassigned.

In order to obtain more structural information, methylation of **2** was carried out. Although the addition of trimethylsilyldiazomethane to **2** in MeOH resulted in various products, the heating (110 °C for 2 h) of **2** in HCl–MeOH (6%) yielded a single product (**3**). The ESI-MS spectra of **3** showed ion peaks at *m/z* 303 and *m/z* 301 in the positive and negative-ion mode, respectively, indicating the introduction of a methyl group in **3**. In the ^{1}H NMR spectrum of **3,** the appearance of a new methyl signal at δ_H 3.88 was confirmed in addition to two doublet signals at δ_H 6.63 and 6.46 ppm (Table 1). The HMBC correlation from the methyl signal to the unassigned carbon signal at δ_c 165.5 of **3** demonstrated that the carbon signal at δ_c 165.5 was a carboxy carbon and **3** was a methyl ester derivative of **2** (Fig. 3). Furthermore, in the ^{1}H NMR spectrum of **3**, the integral ratio of the methyl signal to the proton signal at δ_H 6.63 or 6.46 ppm was 3:2, not 3:1. This implied that two phloroglucinol moieties symmetrically existed in a molecule

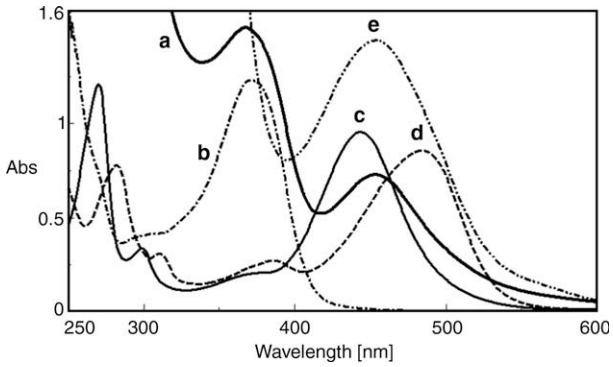

Figure 2. UV–visible spectra of (a) the 10% ethanol extract of the dried outer scales of onion, (b) **1** in 10% ethanol, (c) **2** in 1 N HCl, (d) **2** in 1 N HCl + 3 N NaOH, and (e) the reaction mixture of phloroglucinol and glyoxylic acid.

of **3** and they were bridged by a carboxyvinyl structure that consisted of the last unassigned carbon at δ_c 151.5 and the carboxylic acid group. The molecular weight of **2** estimated by ESI-MS analyses was 18 mass units smaller than the phloroglucinol dimer structure linked by the carboxyvinyl structure. Therefore, the dehydration between two phloroglucinol moieties was required to give a tricyclic 1,3,6,8-tetrahydroxyxanthylium structure. The chemical shifts of **2** were in good accordance with the shifts of the 1,3,6,8-tetrahydroxyxanthylium structure reported previously.[7] The UV–visible spectrum of **2** in the 1 N HCl solution showed absorption maxima at 430 and 269 nm. The addition of base (NaOH) shifted the absorption maxima bathochromically to 484 and 284 nm (Fig. 2), as reported previously about the 1,3,6,8-tetrahydroxyxanthylium structure.[7,8] Finally, the molecular formulas of **2** and **3** were confirmed by HRESI-MS as $C_{14}H_9O_7$ (*m/z* 289.03450 (M^+), δ 0.22 mmu) and $C_{15}H_{11}O_7$ (*m/z* 303.05021 (M^+), δ 0.28 mmu), respectively. Thus, the structure of **2** was elucidated to be a 9-carboxy-1,3,6,8-tetrahydroxyxanthylium and was named cepaic acid as a novel compound. This is the first time that the chemical structure of yellow pigment of the dried outer scales of onion was determined.

Xanthylium is often found in artificial dyes such as fluorescein,

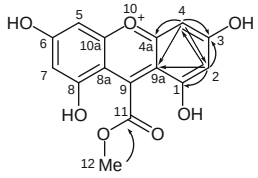

Figure 3. ^{1}H–^{13}C HMBC correlations of **3**.

Table 1
^{1}H and ^{13}C NMR assignments of cepaic acid (**2**) and its methyl ester (**3**) in DMSO-d_6–TFA (9:1)

Position	Cepaic acid (**2**)		Cepaic acid methyl ester (**3**)	
	δ^1H (ppm)	δ^{13}C (ppm)	δ^1H (ppm)	δ^{13}C (ppm)
1, 8 or 4a, 10a		162.3		161.8
2, 7 or 4, 5	6.63 (d, J = 2.3 Hz)	96.2	6.63 (d, J = 2.3 Hz)	96.1
3, 6		172.8		173.0
2, 7 or 4, 5	6.46 (d, J = 2.3 Hz)	101.1	6.46 (d, J = 2.3 Hz)	100.9
1, 8 or 4a, 10a		158.8		158.5
8a, 9a		105.6		105.9
9		151.5		148.9
11		166.6		165.5
12			3.88 (s)	53.6

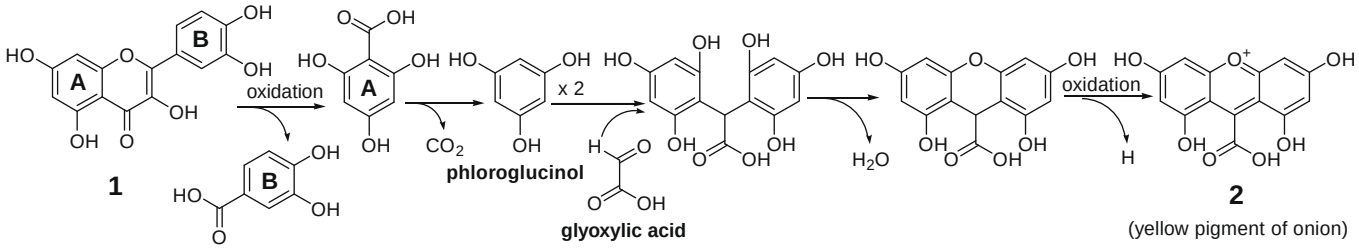

Figure 4. Proposed scheme of the formation from **1** to **2** in the outer scales of onion.

rhodamine, and an artificial food color, erythrosine. It has, however, seldom been reported as a natural dye except as a browning dye generated in grape extract and wine while storing and aging.[8,9] Es-Safi et al. proposed that xanthylium pigments in wine were formed by the first polymerization of (+)-catechin units, which are found in abundance in grape extract, with glyoxylic acid (formylformic acid) generated from tartaric acid by autoxidation,[10] and the subsequent dehydration and oxidation processes.[7,11,12] On the basis of this proposition, it was postulated that **2** was formed by the interaction between two units of phloroglucinol, a strong nucleophile, and glyoxylic acid. To confirm this, glyoxylic acid (25 mM) was added to the solution of phloroglucinol (50 mM in 5 mM phosphate buffer, pH 7.5). After stirring for 48 h at room temperature, the reaction mixture turned from colorless to brown, and had a maximum absorbance at 454 nm (Fig. 2). HPLC analysis of the mixture clearly showed the production of **2** and an unresolved broad hump as well as the crude extract of the dried outer scales of onion. The broad hump observed in the mixture might be caused by xanthylium products formed from higher polymerized phloroglucinol derivatives than **2**, which suggests the possibility that the majority of yellow pigments in the dried outer scales of onion were composed of xanthylium compounds. It was also demonstrated that the oxidation process was indispensable for the yellow pigment formation in the mixture, because the reaction mixture was kept colorless under the reductive condition with the addition of L-ascorbic acid (50 mM). As mentioned above, various oxidized products of **1** have been found in the dried outer scales of onion,[4] and it was also reported that under a mild oxidized condition, **1** was degraded into 3,4-dihydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid, corresponding to the B and A ring of **1**, respectively (Fig. 4). In addition, the latter was easily converted to phloroglucinol by the decarboxylation process.[13] While 3,4-dihydroxybenzoic acid was habitually observed in the extract of the dried outer scales of onion, both 2,4,6-trihydroxybenzoic acid and phloroglucinol were almost absent. These results and facts seem to show that during the storage of onion bulb, phloroglucinol is generated from **1** by autoxidation and decarboxylation and converted soon into yellow xanthylium pigments as **2** by the nucleophilic addition of phloroglucinol to aldehyde groups such as glyoxylic acid and the further dehydration and oxidation process (Fig. 4). This proposition fairly explained well the fact that the browning of outer scales started from cutting plane of the top of onion bulb and was completely synchronized with bulb drying. Although the origin of the glyoxylic acid of **2** is unknown, it might be generated from organic acid or phenolic compounds including phloroglucinol by a ring cleavage process under an oxidative condition.[14] An investigation of the reaction products in the mixture of phloroglucinol and glyoxylic acid and a comparison of them with the extract from the dried outer scales of onion are currently in progress.

Acknowledgments

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5. HPLC (Waters) was performed with a COSMOSIL C_{18}-MS-II column (4.6 i.d. × 150 mm, 40 °C, Nacalai Tesque Inc.) with gradient elution from 10% to 100% of acetonitrile containing 0.1% formic acid for 30 min (flow rate: 0.5 mL/min). ESI-MS (Waters) was performed with the following ion optics: Capillary 3 kV, cone 30 V, and extractor 5 V. The source block was 120 °C and the desolvation temperature was 350 °C. The cone gas flow was 62 L/h and desolvation gas flow was 500 L/h.
6. HPLC (Shimadzu) was performed with a COSMOSIL C_{18}-MS-II column (20.0 i.d. × 250 mm, 40 °C, Nacalai Tesque Inc.) with gradient elution from 10% to 30% of acetonitrile containing 0.1% formic acid for 10 min (flow rate: 9.0 mL/min).
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