Structure of Acid-Stable Carmine

(Received September 17, 2001)

Naoki Sugimoto*1, Yoko Kawasaki*1, Kyoko Sato*1, Hiromitsu Aoki*2, Takahito Ichii*2, Takatoshi Koda*2, Takeshi Yamazaki*1 and Tamio Maizani*1

(*1National Institute of Health Sciences: 1–18–1, Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan; *2San-Ei Gen F.F.I., Inc.: 1–11, Sanwa-cho, Toyonaka-shi, Osaka 561–8588, Japan; †Corresponding author)

Acid-stable carmine has recently been distributed in the U.S. market because of its good acid stability, but it is not permitted in Japan. We analyzed and determined the structure of the major pigment in acid-stable carmine, in order to establish an analytical method for it. Carminic acid was transformed into a different type of pigment, named acid-stable carmine, through amination when heated in ammonia solution. The features of the structure were clarified using a model compound, purpurin, in which the orientation of hydroxyl groups on the A ring of the anthraquinone skeleton is the same as that of carminic acid. By spectroscopic means and the synthesis of acid-stable carmine and purpurin derivatives, the structure of the major pigment in acid-stable carmine was established as 4-aminocarminic acid, a novel compound.

Key words: food additive; acid-stable carmine; carminic acid; 4-aminocarminic acid; purpurin; 1-amino-2,4-dihydroxyanthraquinone

Introduction

Cochineal extract, the extract from Coccus cacti LINNE. (Dactylopius coccus COSTA), is well-known as a natural red colorant1). The List of Existing Food Additives in Japan2) describes cochineal extract as “a substance composed mainly of carminic acid3) 4) obtained from cochineal insects”.

Carmines5), namely the aluminum lake of carminic acid and its salt, and the derivatives of carminic acid obtained by any chemical procedure are not permitted for use as food additives in Japan. However, carmines are used in foods in foreign countries. In addition, acid-stable carmine, which is synthesized from carminic acid by chemical modification, has recently been distributed in the U.S. market. While the color of carminic acid changes from orange to red depending on the pH of the solution, the color of acid-stable carmine is always purple-red. The acid stability of acid-stable carmine is better than that of anthocyanins, which are generally used for coloring acid foods red. These features of acid-stable carmine favor its use in red colorations for foods.

It is likely that acid-stable carmine, not permitted in Japan, is present in imported foods, but there is no information on the structure of the major pigment of acid-stable carmine or a detection method for this illegal food colorant in processed foods. It is supposed that acid-stable carmine is prepared by the method described in the U.S. patent6), since the features of acid-stable carmine are identical with the description in the patent. However, the patent does not describe the structure of the major pigment. Moreover, there is no report on the determination of its structure.

In this paper, in order to establish an analytical method for acid-stable carmine, the structure of the major pigment was determined by the synthesis of a model compound and by spectroscopic means.

Materials and Methods

Materials

A sample of acid-stable carmine was obtained in the U.S. market. Carminic acid was purchased from Wako Pure Chemical Industries Co., Ltd. and Merck Co., Ltd., and purpurin was purchased from Tokyo Kasei Kogyo Co., Ltd. They were used as standards for NMR and HPLC analyses, and as starting materials for synthesis. Dimethyl sulfate ((CH3)2SO4), 25% ammonia solution (NH4OH), and potassium carbonate (K2CO3) were obtained from Wako Pure Chemical Industries Co., Ltd.

HPLC conditions

Analytical HPLC was performed using an HP-1100 series (Hewlett Packard Co., Ltd.) equipped with a photodiode array (PDA) detector under the following two conditions. Condition A: column, YMC-ODS-L80 (4.6 mm i.d. × 250 mm) (YMC Co., Ltd.); mobile phase, 2% acetic acid:acetonitrile = 4:1; flow rate, 0.6 mL/min; detection, 460 nm; column temperature, 40°C. Condition B: column, YMC-ODS-L80 (4.6 mm i.d. × 250 mm); mobile phase, 2% acetic acid:acetonitrile = 3:2; flow rate, 0.6 mL/min; detection, 460 nm; column tempera-
ture, 40°C. LC/MS analysis was performed with an API-electrospray LC/MS system (Hewlett Packard Co., Ltd.) (mass spectrometer, HP-5989B MS engine; interface, HP-59987A API-electrospray LC/MS interface; HPLC, HP-1090 liquid chromatograph). Preparative HPLC was performed using an HPLC system LC-6A (Shimadzu Co., Ltd.) under the following conditions: column, YMC-ODS-L80 (20 mm i.d.×250 mm); mobile phase, 2% acetic acid–acetoni-trile=4:1; flow rate, 7.0 mL/min; detection, 460 nm; column temperature, 40°C.

Spectroscopic analysis

1H- and 13C-NMR spectra were recorded on a JEOL JNM-alpha (500 MHz) in dimethyl sulfoxide-d6 (DMSO-d6) as the solvent. Spectra were referenced internally to tetramethylsilane (TMS) in 1H-NMR and to the solvent in 13C-NMR. Assignments of the proton and carbon signals of all isolated compounds were confirmed by pulse field gradient (PGF) heteronuclear multiple quantum coherence (HMQC), PGF heteronuclear multiple bond connectivity (HMBC), and nuclear Overhauser effect (NOE) experiments. High-resolution fast atom bombardment mass spectra (HR-FAB-MS) were recorded on a JEOL JEMS-700 with p-nitrobenzyl alcohol as a matrix and [M-H]− and [M+H]+ peaks are indicated as m/z. UV/Vis spectra were recorded using a Shimadzu UV-240 and given by λmax in nm (log ε). Melt- ing points were determined using a Yanako MP-3 and are uncorrected.

Isolation of the major pigment (1) from acid-stable carmine

Acid-stable carmine (10 mL) obtained in the U.S. market was evaporated and the solvent was removed. The purple residue was fractionated repeatedly by preparative HPLC, and the major pigment (1, 20 mg) was isolated. 1: purple-red powder, mp >300°C, UV (H2O): 555 (3.65), 527 (3.64), 288 (4.08). HR-FAB-MS (neg.): [M–H]− m/z 490.1033 (calcld. for C22H20O13) 490.0986. The 1H- and 13C-NMR chemical shifts of 1 are shown in Table 1.

Synthesis of the major pigment (1) from carminic acid

Acid-stable carmine was synthesized in accordance with the U.S. patent. A mixture of carminic acid (0.3 g), citric acid (0.36 g), 25% ammonia solution (1 mL), and water (0.36 mL) in a sealed tube was heated at 115–120°C for 70 min. The reaction mixture was evaporated and the solvent was removed. The residue was purified by preparative HPLC to afford compound 1 (30 mg). The spectral data of compound 1 were identical with those of the major pigment isolated from acid-stable carmine obtained in the U.S. market.

Reaction of purpurin in ammonia solution

A mixture of purpurin (0.5 g) and 25% ammonia solution (2.5 mL) in methanol (50 mL) was refluxed for 2 hours. After evaporation of the solvent, compound 2 (490 mg) was obtained. 2: purple powder, UV (methanol): 549 (3.78), 517 (3.83), 284 (3.92). HR-FAB-MS (neg.): [M−H]− m/z 254.0405 (calcld. for C14H8NO4 254.0453). The 1H- and 13C-NMR chemical shifts of 2 are shown in Table 2.

Methylation of 2

A mixture of 2 (200 mg), K2CO3 (1.2 g), and (CH3)3SO4 (1 mL) in acetone (10 mL) was refluxed for 4 hours. The reaction mixture was added to 2 mol/L hydrochloric acid (50 mL), and the product was extracted with a sufficient amount of ethyl acetate. The solvent was removed in vacuo, then the purple residue was chromatographed on an ODS column (Fuji Silica Chemical Co., Ltd, Chromatorex ODS, mesh 200–350) using methanol–water=5:2 to give the methylated compound 3 (70 mg). 3: green paste, UV (methanol): 544 (3.14), 389 (3.07), 328 (3.16). HR-FAB-MS (pos.): [M+H]+ m/z 298.1097 (calcld. for C17H16NO4 298.1079). The 1H- and 13C-NMR chemical shifts of 3 are shown in Table 2.

Results and Discussion

HPLC analysis of the major pigment in acid-stable carmine

To clarify the difference between carminic acid and the major pigment (1) isolated from acid-stable carmine obtained in the U.S. market, analytical HPLC (condition A) with a PDA detector was performed. The PDA spectra and chromatograms at 460 nm of the two are shown in Fig. 1. The pigment (1) showed a different retention time and PDA spectrum from carminic acid. In the LC/MS, a deprotonated molecule [M−H]− of major pigment (1) was observed at m/z 490, whereas that of carminic acid was at m/z 491. In addition, we synthesized the pigment, according to the preparation method presented in the U.S. patent (see Materials and Methods). The reaction mixture gave only one peak on HPLC, and the retention time, the PDA spectrum, and the LC/MS spectrum of the peak were identical to those of the major pigment (1) isolated from acid-stable carmine obtained in the U.S. market. The results indicate that acid-stable carmine obtained in the U.S. market was prepared according to the U.S. patent.

Spectroscopic analyses of the major pigment (1)

The major pigment (1) is a purple-red powder, mp >300°C (recrystallized from methanol and water). In negative-mode HR-FAB-MS of 1, a deprotonated molecule [M−H]− at m/z 490.1033 was observed. The ion peak indicates that the molecular formula can be represented as C22H20O13, corresponding to carminic acid (C17H16NO4) minus one oxygen and plus one nitrogen and plus one hydrogen. In the 1H-NMR spectrum, it showed a methyl group at δ 3.08 (3H, s), C-glucosyl at δ 3.13–4.60 (7H), one hydroxyl proton at δ 8.06, and an olefinic proton at δ 7.38 as a singlet. In the 13C-NMR spectrum, it had 22 carbon signals and appeared similar to carminic acid except for the signals derived from the anthraquinone skeleton. The C-glucosyl, methyl, and carboxyl signals were observed at δ 60.6–80.5, δ 20.9, and δ 171.0, respectively. Among the other 14 carbons,
the one at $\delta$ 110.8, which correlated with $\delta$ 7.38 in the HMQC spectrum, was attributable to $=C-H$. In addition, the carbons of 1, except two carbons at $\delta$ 104.0 and 145.9 were correlated with at least one proton in the HMBC spectrum. The observed HMBC correlations are shown in Scheme 1, and the $^1$H- and $^{13}$C-NMR chemical shifts for carminic acid and 1 are summarized in Table 1.

Fariña et al.\textsuperscript{7,8} reported in detail the reactions of anthraquinones with ammonia solution. They revealed that anthraquinone derivatives with hydroxyl groups at the peri position, are converted to 9- or 10-monoiminoanthraquinone derivatives. Following from these reports, the 9-monoiminoanthraquinone structure 1a was assumed firstly, but the signal of the 9 position of 1 was observed at $\delta$ 175.5, whereas an imino carbon is generally observed at $\delta$ ca. 160 in $^{13}$C-NMR (Table 1, Scheme 1). The 4- aminoanthraquinone structure 1b (in which one hydroxyl group was replaced by an amino group) was thus considered, and the observed NMR chemical shifts on 1 were considered to be more readily assignable to 1b than to 1a (Table 1, Scheme 1). However, there is no rigorous proof that the structure of 1 is 1b. In order to clarify the position of replacement of $=O$ with $=NH$ or of $=OH$ with $=NH_2$, various attempts at derivatization, such as methylation and acetylation, were made, but were fruitless, because 1 has many functional groups.

**Synthesis of 2 from a model compound, purpurin**

It was assumed that the structure of 1 was either 1a or 1b. Since such conversions seemed to be reasonable for an anthraquinone derivative whose orientation of hydroxyl groups is the same as in carminic acid, we carried out the reaction with a model compound, purpurin (1,3,4-trihydroxyanthraquinone: for convenience, the numbering is that of carminic acid), which has the same orientation of hydroxyl groups on the A ring of the anthraquinone skeleton as carminic acid. Purpurin gave 2 quantitatively when heated in ammonia solution. The chromatograms of analytical HPLC (condition B) with a PDA detector and the PDA spectra of purpurin and 2 are shown in Fig. 2. Only one peak of 2 was observed after the reaction, and the retention time was different from that of purpurin. The
PDA spectrum of 2 was very similar to that of 1, which showed the two $\lambda_{\text{max}}$ at about 550 nm. This result suggested that the orientation of the functional groups (C=O, –OH, and –NH) of 2 was identical to that of 1.

In negative-mode HR-FAB-MS of 2, a deprotonated molecule [M–H]$^-$ at $m/z$ 254.0405 was observed. The ion peak indicates that the molecular formula can be represented as $C_{14}H_{8}NO_4$, corresponding to purpurin ($C_{14}H_{8}O_5$) minus one oxygen plus one nitrogen and plus one hydrogen. The $^1$H-NMR spectrum of 2, showed four aromatic protons on the B ring at $\delta$ 7.81, 7.82, 8.19, 8.23, and a singlet aromatic proton on the A ring at $\delta$ 6.42.
and a broad singlet hydroxyl proton at δ 15.02. The 13C-NMR spectrum showed 14 carbons, which appeared at δ 104.9–181.0. The five signals at δ 105.3, 132.5, 133.7, 125.4, and 126.2, which correlated with δ 6.42, 7.81, 8.21, and 8.23, respectively, in the HMQC spectrum, were attributable to the anthraquinone skeleton. The NOE enhancements and HMBC correlations of 2 and 3 are demonstrated in Scheme 2, and the NMR chemical shifts of purpurin, 2, and 3 are summarized in Table 2. In particular, the NOE enhancements of 3 observed between the aromatic proton at δ 7.13 and the two O-methyl groups at δ 3.98, 4.05, and between the O-methyl group at δ 4.05 and N-methyl group at δ 3.08, enabled the assignments of the positions of the two O-methyl groups and N-methyl group. Thus, the structure of 2 is confirmed as 4-amino-1,3-dihydroxyanthraquinone.

**Conclusion**

A major pigment (1) was isolated from acid-stable carmine. The acid-stable carmine had been prepared by the method in the U.S. patent, as we actually synthesized 1 from carminic acid by using the preparation procedure in the patent. Carminic acid is transformed into 1, when heated in ammonia solution. Since such a transformation seemed reasonable for any anthraquinone derivative, in which the orientation of the hydroxyl groups on the A ring of the anthraquinone skeleton is the same as that of carminic acid, we carried out the reaction with a model compound, purpurin, in order to clarify the structure of 1. The structure of the product

---

**Table 2.** 1H- and 13C-NMR Chemical Shifts (δ, ppm) of Purpurin, 2, and 3 in DMSO-d6

<table>
<thead>
<tr>
<th>No.</th>
<th>Purpurin</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1H</td>
<td>13C</td>
<td>1H</td>
<td>13C</td>
</tr>
<tr>
<td>1</td>
<td>13.27 (1H, br s, 1-OH)</td>
<td>160.4</td>
<td>15.02 (1H, br s, 1-OH)</td>
</tr>
<tr>
<td>2</td>
<td>6.55 (1H, s, 2-H)</td>
<td>109.6</td>
<td>6.42 (1H, s, 2-H)</td>
</tr>
<tr>
<td>3</td>
<td>11.56 (1H, br s, 3-OH)</td>
<td>157.0</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>13.04 (1H, br s, 4-OH)</td>
<td>149.3</td>
<td>—</td>
</tr>
<tr>
<td>4a</td>
<td>112.2</td>
<td>106.0</td>
<td>113.4</td>
</tr>
<tr>
<td>4b</td>
<td>132.3</td>
<td>134.4</td>
<td>133.2</td>
</tr>
<tr>
<td>5</td>
<td>8.11 (1H, d, J=8 Hz, 5-H)</td>
<td>126.3</td>
<td>8.23 (1H, d, J=8 Hz, 5-H)</td>
</tr>
<tr>
<td>6</td>
<td>7.84 (1H, t, J=8 Hz, 6-H)</td>
<td>134.0</td>
<td>7.82 (1H, t, J=8 Hz, 6-H)</td>
</tr>
<tr>
<td>7</td>
<td>7.86 (1H, t, J=8 Hz, 7-H)</td>
<td>134.8</td>
<td>7.81 (1H, t, J=8 Hz, 7-H)</td>
</tr>
<tr>
<td>8</td>
<td>8.06 (1H, d, J=8 Hz, 8-H)</td>
<td>126.1</td>
<td>8.19 (1H, d, J=8 Hz, 8-H)</td>
</tr>
<tr>
<td>8a</td>
<td>183.1</td>
<td>179.3</td>
<td>179.6</td>
</tr>
<tr>
<td>8b</td>
<td>186.5</td>
<td>181.0</td>
<td>184.5</td>
</tr>
<tr>
<td>9</td>
<td>105.0</td>
<td>104.9</td>
<td>113.4</td>
</tr>
<tr>
<td>10</td>
<td>3.98 (3H, s)</td>
<td>57.0</td>
<td></td>
</tr>
<tr>
<td>1-OCH3</td>
<td>4.05 (3H, s)</td>
<td>59.9</td>
<td></td>
</tr>
<tr>
<td>3-OCH3</td>
<td>3.08 (3H, s)</td>
<td>34.7</td>
<td>5.19 (br s, NH)</td>
</tr>
</tbody>
</table>
formed from purpurin was confirmed to be 4-amino-1,3-dihydroxyanthraquinone (IUPAC name: 1-amino-2,4-dihydroxyanthraquinone) \( (2) \). On the basis of spectral data and the results of the synthesis of \( 2 \) from a model compound, purpurin, the structure of the major pigment \( (1) \) in acid-stable carmine was concluded to be 4-aminocarminic acid \( (1b) \).

References