Original Paper

Preparation of a Ammonia-Treated Lac Dye and Structure Elucidation of Its Main Component

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Lac dye and cochineal extract contain laccaic acids and carminic acid as the main pigments, respectively. Both laccaic acids and carminic acid are anthraquinone derivatives. 4-Aminocarminic acid (acid-stable carmine), an illegal colorant, has been detected in several processed foods. 4-Aminocarminic acid is obtained by heating cochineal extract (carminic acid) in ammonia solution. We attempted to prepare ammonia-treated lac dye and to identify the structures of the main pigment components. Ammonia-treated lac dye showed acid stability similar to that of 4-aminocarminic acid. The structures of the main pigments in ammonia-treated lac dye were analyzed using LC/MS. One of the main pigments was isolated and identified as 4-aminolaccaic acid C using various NMR techniques, including 2D-INADEQUATE. These results indicated that ammonia-treatment of lac dye results in the generation of 4-aminolaccaic acids.

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Introduction

Natural food colorants are used worldwide to stain various processed foods and to make the products more attractive to consumers. Lac dye, the extract from *Laccifer* spp., and cochineal extract, the extract from *Coccus cacti* Linnaeus, are well-known as natural red food colorants. The List of Existing Food Additives in Japan^{*1} describes lac dye as “a substance composed mainly of a mixture of laccaic acids A (1), B (2), C (3) and E (4),”^{1), 2)} and cochineal extract as “a substance mainly composed of carminic acid (5)”³⁾. Both laccaic acids and carminic acid (5) are anthraquinone derivatives (Fig. 1).

Carmine is the aluminum lake of carminic acid (5) and its salt, and is authorized for use in the USA^{*2} and the European Union^{*3}, but not in Japan. The name “car-

mine” is often used loosely to refer to any cochineal-based product, such as acid-stable carmine, which is prepared from cochineal extract containing carminic acid (5) by treatment with ammonia solution⁴⁾. It is well-known that the colors of carminic acid (5) and laccaic acids change from orange to purple depending on the pH of the solution, but the color of acid-stable carmine remains purple regardless of the pH. However, the use of acid-stable carmine in processed foods is not authorized in any country since it does not comply with specifications^{*1, *2, *3}. Based on structure analysis of ammonia-treated purpurin, which contains hydroxyl groups at the same positions on the A ring of the anthraquinone skeleton as in carminic acid (5), acid-stable carmine was concluded to be 4-aminocarminic acid (5')⁵⁾ (Fig. 1). This report lead to investigations in various countries that revealed the widespread presence of 4-aminocarminic acid (5') in processed foods^{6)–9)}.

4-Aminocarminic acid (5') is easily synthesized by replacing the hydroxyl group (–OH) with an amino group (–NH₂) at the C-4 position of carminic acid (5). This replacement occurs in nature to provide anthraquinone derivatives, including laccaic acids (Lac dye), and the resulting derivatives do not comply with the Japanese specifications for lac dye^{*1}. The Japanese specifications do not cover the presence of ammonia-treated

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*¹ Notification No. 120 (Apr. 16, 1996) List of Existing Food Additives, Ministry of Health and Welfare.

*² 21CFR73.100 (Apr. 1, 2015) Code of Federal Regulations, FDA (U.S. Food and Drug Administration).

*³ CELEX No. 31994 L0036 (Jun. 30, 1994) European Parliament and Council Directive 94/36/EC of 30 June 1994 on Colours for Use in Foodstuffs, European Parliament, Council of the European Union.

Fig. 1. Structures of anthraquinone derivatives in this study

lac dye in processed foods, but illegal colorant may be intentionally added to processed foods.

Herein we prepared ammonia-treated lac dye according to previously reported strategies^{4), 5)} and analyzed the main dye components using LC/MS. Using various NMR techniques, we determined that 4-aminolaccacid acids are the main components of ammonia-treated lac dye.

Materials and Methods

Materials

Lac dye powder was purchased from The Japan Shellac Industries, Ltd. (Osaka, Japan). ¹⁴N- and ¹⁵N-Ammonia solutions (¹⁴NH₄OH and ¹⁵NH₄OH) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and Sigma-Aldrich (St. Louis, MO, USA), respectively.

Preparation of ammonia-treated lac dye for LC/MS analysis

Lac dye (300 mg) was dissolved in 100 mL of 10% ammonia solution, placed in a screw-cap test tube, heated at 120°C for 2 h using an aluminum block heater, and then cooled overnight. The solvent was evaporated in vacuo and the residue was obtained as ammonia-treated lac dye. The color changes of solutions of lac dye and of ammonia-treated lac dye at various pH values were observed by dissolving 300 mg of lac dye or ammonia-treated lac dye in 200 mL of 0.05% sodium carbonate solution, and then diluting each sample solution 100-fold with 0.1% formic acid (for pH 2), 10 mM ammonium formate (for pH 6), and 0.05% sodium carbonate solution (for pH 10). The colors of the solutions were observed under a fluorescent lamp.

The components of lac dye and ammonia-treated lac dye were investigated by diluting each sample solution 10-fold with 20% methanol containing 1% formic acid,

filtering the mixture through a 0.20 µm filter (Millex-LG, Merck KGaA, Darmstadt, Germany), and analyzing the filtrate by LC/MS using an ACQUITY UPLC/SQD system (Waters, Milford, MA, USA). The following LC conditions were used: column, XTerra MS C18 column (2.1 mm i.d. × 150 mm; Waters); solvent A, 1% formic acid; solvent B, methanol containing 1% formic acid; elution, A : B = 80 : 20 for 0–10 min and A : B = 75 : 25 for 10–35 min; flow rate, 0.3 mL/min; detection, 480 nm; column temperature, 40°C. The MS conditions used were: capillary voltage, 3.0 kV; cone voltage, 30 V; source temperature, 120°C; desolvation temperature, 350°C; scan range, ESI neg. *m/z* 200–700.

Purification of laccacid acid C (3) and synthesis of 4-aminolaccacid acid C (3')

Lac dye (2.5 g) was dissolved in 500 mL of 0.05% sodium carbonate solution and filtered through a filter paper. Then, the filtrate was loaded onto an ODS (Chromatorex ODS; Fuji Silysia Chemical Ltd., Aichi, Japan) open column (40 mm i.d. × 400 mm) equilibrated with 30% methanol containing 0.1% formic acid. Crude laccacid acid C (3) was obtained by elution with 200 mL of 30% methanol containing 0.1% formic acid and 200 mL of 40% methanol containing 0.1% formic acid. Twenty fractions (each 20 mL) were subjected to LC/MS as described above. The column fractionation step was repeated twenty times to obtain sufficient material for NMR analysis. The fractions containing laccacid acid C (3) were combined and the solvent was evaporated in vacuo. The residue was dissolved in 300 mL of 0.05% sodium carbonate solution and the solution was subjected to preparative LC/MS (Shimadzu, Kyoto, Japan) as described below to yield about 1,700 mg of laccacid acid C (3). ¹⁴N- and ¹⁵N-4-Aminolaccacid acid C (3' and 3'') were synthesized by heating laccacid acid C (3) in ¹⁴N- or ¹⁵N-ammonia solution, respectively, and then the samples were subjected to preparative LC/MS using the condi-

tions described below to yield about 600 mg of ^{14}N -4-aminolaccaic acid **C** (**3'**) and about 50 mg of ^{15}N -4-aminolaccaic acid **C** (**3''**).

Preparative LS/MS conditions: column, XTerra Prep PR18 column (19 mm i.d. $\times$ 100 mm; Waters); solvent A, 0.1% formic acid; solvent B, methanol containing 0.1% formic acid; elution, A : B = 75 : 25 for 0–10 min (for laccaic acid **C** (**3**)) and A : B = 75 : 25 to 40 : 60 for 0–10 min (for ^{14}N - and ^{15}N -4-aminolaccaic acid **C** (**3'** and **3''**)); flow rate, 8 mL/min; detection, ESI neg. m/z 538 $[\text{M}-\text{H}]^-$ (for laccaic acid **C** (**3**)) and m/z 537 $[\text{M}-\text{H}]^-$ (for ^{14}N -4-aminolaccaic acid **C** (**3'**)).

NMR analysis

NMR spectra of the samples dissolved in dimethyl sulfoxide- d_6 (DMSO- d_6) were recorded on a JEOL JNM-ECA800 spectrometer equipped with an Ultra COOL NMR probe (for ^1H - and ^{13}C -NMR) or HCN probe (for ^{15}N -NMR). Both probe temperatures were set at 50°C. Assignments of the proton and carbon signals of laccaic acid **C** (**3**) and 4-aminolaccaic acid **C** (**3'**) were confirmed by correlation spectroscopy (COSY), ^1H -detected multiple quantum coherence (HMQC), ^1H -detected multi-bond heteronuclear quantum coherence (HMBC) and incredible natural abundance double quantum transfer experiment (INADEQUATE) techniques. ^1H - and ^{13}C -NMR chemical shifts were referenced to the residual solvent (DMSO- d_6) signal at δ_{H} 2.50 and δ_{C} 39.5, respectively. The INADEQUATE conditions were: relaxation delay, 1 s; J constant, 70 Hz; X points, 8,192; Y points, 128; scans, 320,000 (laccaic acid **C** (**3**)) (four days) and 486,400 (4-aminolaccaic acid **C** (**3'**)) (six days). The ^{15}N -NMR conditions were: relaxation delay, 2 s; spectral width, -50 to 650 ppm; X points, 32,768; scans, 880; ^1H decoupling, on; nuclear Overhauser effect (NOE), false.

Results and Discussion

LC/MS analysis of ammonia-treated lac dye

We investigated the color changes of lac dye and ammonia-treated lac dye dissolved in solutions of different pH. The lac dye solutions changed from orange to purple as the pH of the solutions increased, whereas the ammonia-treated lac dye solutions remained purple regardless of the pH (Table 1) (Fig. S1). The results suggested that ammonia-treatment makes the color of lac dye stable to changes in pH, and that under acidic conditions, the pH stability of the color is equivalent to that of acid-stable carmine (4-aminocarmine acid (**5'**)).

In LC/MS analysis, lac dye and ammonia-treated lac

dye each showed five peaks (a–e, lac dye; a'–e', ammonia-treated lac dye) (Fig. 2). The PDA spectra of peak a to peak e showed λ_{max} at about 490 nm, whereas peak a' to peak e' showed two λ_{max} values at approximately 550 nm (Fig. 2). The mass spectra of peak a to peak e and peak a' to peak e' provided deprotonated molecular ions $[\text{M}-\text{H}]^-$, as summarized in Table 2. Peaks a, b, d, and e gave m/z 494, 538, 495, and 536, so these peaks were identified as corresponding to laccaic acids **E** (**4**), **C** (**3**), **B** (**2**), and **A** (**1**), respectively^{11, 10–14}. Peak c was identified as anthraquinonedicarboxylic acid due to its m/z 359, and because $[\text{M}-\text{H}-\text{CO}_2]^-$ and $[\text{M}-\text{H}-2\text{CO}_2]^-$ appeared at m/z 315 and m/z 271, respectively, consistent with the results of ESI-MS/MS analysis by Oka *et al.*¹⁵. On the other hand, the m/z values of peaks a', b', c', d', and e' of ammonia-treated lac dye were each one mass unit lower than those of laccaic acids **E** (**4**) and **C** (**3**), anthraquinonedicarboxylic acid (molecular mass of 360 Da), and laccaic acids **B** (**2**) and **A** (**1**), respectively (Table 2). Based on a previous report on acid-stable carmine (**5'**)⁵, it appears that these one mass unit discrepancies are due to the replacement of -OH with -NH₂. These results suggested that the acid-stability of ammonia-treated lac dye is due to the ammonia-induced replacement of -OH with -NH₂ in laccaic acids **A** (**1**), **B** (**2**), **C** (**3**), and **E** (**4**), and of the anthraquinonedicarboxylic acid derivative.

Structure analysis of laccaic acid **C** (**3**)

Laccaic acids **A** (**1**), **B** (**2**), **C** (**3**), and **E** (**4**), and the anthraquinonedicarboxylic acid derivative, are not commercially available. We confirmed the structures of the laccaic acids and ammonia-treated derivatives by isolating one laccaic acid from lac dye. Laccaic acid **C** (**3**) could be easily separated and purified from the other laccaic acids by means of ODS column fractionation and preparative HPLC. Laccaic acid **C** (**3**) (500 mg) was dissolved in 600 μL of DMSO- d_6 and the NMR spectra were recorded. The ^1H -NMR spectrum of **3** showed four aromatic protons at δ_{H} 6.92, 7.04, 7.13, and 7.56, along with two other protons at δ_{H} 3.11 and 4.12 (Table 3). The ^{13}C -NMR spectrum of **3** showed the presence of 25 carbons (Fig. 3) (Table 3). The HMQC experiment with **3** showed that the six signals at δ_{H} 3.11, 4.12, 6.92, 7.04, 7.13, and 7.56 correlated with δ_{C} 35.36, 54.05, 116.48, 132.83, 130.82, and 115.86, respectively. The HMBC experiment with **3** showed correlations between δ_{H} 6.92, δ_{C} 119.36, and 125.04, between δ_{H} 7.04, δ_{C} 35.36, 122.55, 130.82, and 155.00, between δ_{H} 7.13, δ_{C} 132.83, and 155.00, and between δ_{H} 7.56, δ_{C} 118.32, 121.23, 168.54, 183.12, and 186.75. The remaining 10 carbons were unclear, so we

Table 1. The color of lac dye and ammonia-treated lac dye at different pH values

Lac dye			Ammonia-treated lac dye		
pH 2	pH 6	pH 10	pH 2	pH 6	pH 10
Orange	Red	Purple	Purple	Purple	Purple

The solutions consisted of 0.1% formic acid (pH 2), 10 mM ammonium formate (pH 6), and 0.05% sodium carbonate solution (pH 10).

Fig. 2. LC/MS analysis of lac dye and ammonia-treated lac dye

(A) Lac dye; (B) Ammonia-treated lac dye. HPLC chromatograms were recorded at 480 nm. PDA spectra of peaks a to e and a' to e' are shown below. MS analyses of peaks a to e and a' to e' are summarized in Table 2.

Table 2. The observed m/z of peaks a–e and a'–e' in lac dye and ammonia-treated lac dye, respectively, as determined by LC/MS analysis

Lac dye			Ammonia-treated lac dye		
Peak	m/z	Compound	Peak	m/z	Compound
a	494	Laccaic acid E (4)	a'	493	4-Aminolaccaic acid E (4')
b	538	Laccaic acid C (3)	b'	537	4-Aminolaccaic acid C (3')
c	359	Anthraquinonedicarboxylic acid derivative	c'	358	4-Aminoanthraquinonedicarboxylic acid derivative
d	495	Laccaic acid B (2)	d'	494	4-Aminolaccaic acid B (2')
e	536	Lacciac acid A (1)	e'	535	4-Aminolaccaic acid A (1')

Peaks a–e and a'–e' are shown in Fig. 2.

attempted a 2D-INADEQUATE experiment to elucidate the structure of laccaic acid C (**3**). The 2D-INADEQUATE experiment showed two fragments with contiguous carbon skeletons as follows: δ_c 121.23–140.17–(169.53)–118.32–136.53–(115.86)–(169.65)–186.75–118.83–(104.90)–(183.12)–148.50–153.12–122.55–(158.93)–(104.90)–119.36–(155.00)–(116.48)–132.83–125.04–130.82, and δ_c 54.05–170.76 (Fig. 4). The results of these NMR experiments and LC/MS analyses con-

firmed that the structure is consistent with that of laccaic acid C (**3**) proposed in a previous report study¹³⁾.

Structure analysis of 4-aminolaccaic acid C (3')

Purified laccaic acid C (**3**) (800 mg) was treated with ammonia solution to obtain the derivative (**3'**). LC/MS analysis showed that the retention time, PDA spectrum, and mass spectrum of the derivative (**3'**) matched those of peak b' (Fig. 2). The NMR spectra of the purified de-

Table 3. ^1H - and ^{13}C -NMR chemical shifts of laccaic acid C (**3**) and 4-aminolaccaic acid C (**3'**)

No.	Laccaic acid C (3)		4-Aminolaccaic acid C (3')	
	^1H	^{13}C	^1H	^{13}C
1	—	158.93	—	164.43*
2	—	122.55	—	116.08
3	—	153.12	—	162.84*
4	—	148.50	—	144.11
4a	—	118.83	—	104.76
4b	—	136.53	—	138.33
5	7.56 (1H, s)	115.86	7.57 (1H, s)	113.88
6	—	169.65	—	167.20
7	—	121.23	—	119.99
8	—	140.17	—	138.66
8a	—	118.32	—	119.62
8b	—	104.90	—	102.00
9	—	183.12	—	177.15
10	—	186.75	—	180.00
1'	—	119.36	—	122.59
2'	7.04 (1H, s)	132.83	7.32 (1H, s)	133.78
3'	—	125.04	—	124.83
4'	7.13 (1H, d, $J=5.50$)	130.82	7.05 (1H, d, $J=5.50$)	129.06
5'	6.92 (1H, d, $J=7.34$)	116.48	6.81 (1H, d, $J=7.34$)	118.12
6'	—	155.00	—	155.85
7-COOH	—	168.54	—	169.41
8-COOH	—	169.53	—	170.59**
7'	3.11 (2H, br s)	35.36	2.99 (1H, s)	35.78
			3.11 (1H, s)	
8'	4.12 (1H, s)	54.05	3.83 (1H, s)	54.99
8'-COOH	—	170.76	—	171.07**

Solvent, DMSO- d_6 . br s, broad singlet; d, doublet; s, singlet. Sets of * and ** indicate that assignments may be reversed.

Fig. 3. ^{13}C -NMR spectra of laccaic acid C (**3**) and 4-aminolaccaic acid C (**3'**)

^{13}C – ^{15}N spin coupling of ^{15}N -4-aminolaccaic acid C (**3''**) was observed at δ_{C} 141.44 (15.41 Hz) (Fig. S2).

rivative (**3'**) were similar to those of laccaic acid C (**3**). The ^1H -NMR spectrum of **3'** showed four aromatic protons at δ_{H} 6.81, 7.05, 7.32, and 7.57, along with three other protons at δ_{H} 2.99, 3.11, and 3.83 (Table 3). The ^{13}C -NMR spectrum of **3'** showed the presence of 25 carbons (Fig. 3) (Table 3). An HMQC experiment with **3'** showed that the five signals at δ_{H} 3.83, 6.81, 7.05, 7.32, and 7.57 correlated with δ_{C} 54.99, 118.12, 129.06, 133.78, and 113.88, respectively, and that the signals at δ_{H} 2.99 and 3.11 correlated with δ_{C} 35.78. An HMBC experiment with **3'** showed correlations between δ_{H} 6.81, δ_{C} 122.59, 124.83, and 155.85, between δ_{H} 7.05, δ_{C} 35.78, 122.59, 133.78, and 155.85, between δ_{H} 7.32, δ_{C} 35.78,

116.08, 129.06, and 155.85, and between δ_{H} 7.57, δ_{C} 119.62, 138.33, 167.20, 169.41, 177.15, and 180.00 (Fig. 5). A 2D-INADEQUATE experiment of **3'** showed four fragments with contiguous carbon skeletons as follows: δ_{C} 138.66–119.99–167.20–113.88–138.33–119.62–138.66, δ_{C} 180.00–104.76–(144.11)–102.00–177.15, δ_{C} 116.08–122.59–133.78–124.83, and δ_{C} 129.06–118.12–155.85 (Fig. 5). The remaining 4 carbons (δ_{C} 162.84, 164.43, 170.59, and 171.07) were estimated from the chemical shifts of laccaic acid C (**3**).

Consistent with the NMR spectra of laccaic acid C (**3**), NMR experiments using the synthetic derivative (**3'**) showed the presence of 7 protons linked with carbon and

Fig. 5. 2D-INADEQUATE spectrum of 4-aminolaccaic acid C (3')

In this study, we prepared ammonia-treated lac dye by heating lac dye in ammonia solution. The acid stability of the product under acidic conditions was similar to that of acid-stable carmine (4-aminocarminic acid (5')). LC/MS analysis indicated that ammonia-treated lac dye is mainly composed of 4-aminolaccaic acids A (1'), B (2'), C (3'), and E (4'), generated by the replacement of $-\text{OH}$ with $-\text{NH}_2$. We isolated laccaic acid C (3) from lac dye and heated it in ammonia solution. Structure elucidation based on various NMR techniques confirmed that the synthetic derivative is 4-aminolaccaic acid C (3'). Accordingly, the study established that heating lac dye in ammonia solution imparts acid stability to the compound, and that the acid-stability is due to substitution of $-\text{OH}$ with $-\text{NH}_2$ at the C-4 position of laccaic acids.

Examination of ammonia-treated lac dye in processed foods by HPLC with PDA and MS detection would be aided by the ability to separate and quantify 4-aminolaccaic acids from laccaic acids. Further study would then be required to determine whether or not ammonia-treated lac dye is used in processed foods.

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