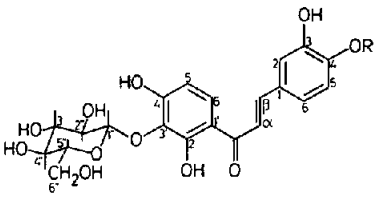

1 R = Me
2 R = H

Isolation. The extract of the dried leaves (1 kg) with aqueous methanol was successively partitioned between H_2O, trichloroethylene and EtOAc The residue obtained after evapn of the EtOAc extract (20 g) was chromatographed over Sephadex® LH-20 with MeOH–H_2O (1 4) and increasing amounts of MeOH, 13 fractions were collected Fraction 8 (3300 mg) was separated by RLCC using the system $CHCl_3$–MeOH–*iso*-PrOH–H_2O (8 7 1 6) in the descending mode and finally purified over LH-20 with *iso*-PrOH as eluent to yield 46 mg of **1**. R_f s **1** on TLC silica gel $60F_{254}$ were 0.16 in $CHCl_3$–MeOH–H_2O (13 7.4, lower phase) 0.56 in EtOAc–MeOH–H_2O (100:17 13) and 0 77 in EtOAc–HCOOH–H_2O (15 3:4).

Mp. (uncorr) 196°, UV λ_{max}^{MeOH} 376, 311 (sh), 260, +$AlCl_3$ 433, 330 (sh), 270, +$AlCl_3$+HCl 433, 323 (sh), 269, 240 (sh); +NaOAc 397, 275 (sh), 262, +NaOAc+H_3BO_3 482 (sh), 447, 429, 398 (sh), 293 (sh), 263, ^{1}H NMR (CD_3OD, solvent int standard, 400 MHz, δ-values, ppm) 7 80 (1H, *d*, 9 Hz, H-6'), 7.74 (1H, *d*, 15 Hz, H-β), 7 56 (1H, *d*, 15 Hz, H-α), 7.22 (1H, *d*, 2 Hz, H-2), 7.18 (1H, *dd*, 8 Hz, 2 Hz, H-6), 6 95 (1H, *d*, 8 Hz, H-5), 6 50 (1H, *d*, 9 Hz, H-5'), 4.80 (1H, *d*, 8 Hz, H-1''), 3 89 (3H, *s*, –OMe), 3.81 (1H, *dd*, 12 Hz, 2 Hz, H-6''), 3 74 (1H, *dd*, 12 Hz, 4 Hz, H-6''), 3.42–3 55 (3H, *m*, H-2'', H-3'', H-4''), 3 28–3 30 (1H, *m*, H-5'')

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CYANIDIN 3-ACETYLRUTINOSIDE IN *EURYA JAPONICA* BERRIES

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Key Word Index—*Eurya japonica*; Theaceae; berries; acylated anthocyanin; acetic acid; cyanidin 3-acetylrutinoside

Abstract—The major anthocyanin in the berries of *Eurya japonica* was identified as cyanidin 3-acetylrutinoside, from chromatographic and spectral methods.

INTRODUCTION

The pigment in the black berries of *Eurya japonica* Thunberg was earlier identified as cyanidin 3-glucoside (Cy 3-G) by Shibata *et al.*[1] In the course of an acylated anthocyanin survey, however, the major pigment from the same plant was observed to have different R_f values on TLC from authentic Cy 3-G. This prompted us to re-examine the anthocyanin. This paper deals with the elucidation of the major anthocyanin in the *Eurya japonica* berries.

RESULTS AND DISCUSSION

Dried black berries of *Eurya japonica* were extracted with aqueous methanol containing formic acid, and the major pigment was purified by standard procedures To avoid the possibility of acetylation occurring during manipulation [2], acetic acid was replaced by formic acid throughout the isolation and purification processes The major pigment **1** gave the chromatographic and spectral data listed in Table 1, compared with those of cyanidin 3-rutinoside (Cy 3-R) and 3-glucoside (Cy 3-G) which also occurred in the berries of *Eurya japonica* as minor pigments When left standing in methanolic hydrochloric acid, pigment **1** decomposed to Cy 3-R, also obtained by alkaline deacylation of **1** (Table 1), which suggested that **1** was Cy 3-R acylated with an aliphatic carboxylic acid [3, 4]. In the IR spectrum for **1**, the presence of an acylating aliphatic acid was clearly indicated by the ester carbonyl absorption at 1720 cm^{-1} which could not be observed in the spectrum of Cy 3-R or Cy 3-G However, TLC detection of the acid after alkaline deacylation failed, due to its volatility Through careful HPLC analysis using acidic elution, acetic acid was detected in the deacylated product of **1** in comparison with the authentic acid (Table 1) Consequently, pigment **1** is an acetylated Cy 3-R

SIMS measurement verified the structure of **1** from the molecular ion (*m/z* 637) corresponding to Cy 3-R monoacetate and the fragment ions, 595 $[M-Ac]^+$ to Cy 3-R, 449 (low intensity) to Cy 3-G, and 287 to cyanidin Moreover, in the ^1H NMR spectrum, the strong singlet signal at δ 1 96 ppm could be assigned to the methyl protons of the acetyl residue, and the signals in the lower magnetic field than δ 6 ppm as typical cyanidin protons [5] In addition, the carbon skeleton of the aglycone moiety of **1** was ascertained by the signal assignments of the ^{13}C NMR spectra (Table 2), in which the signals in the region lower than δ 95 ppm were quite similar to those of Cy 3-R or were on general accord with those of Cy or Cy 3-G reported by Ikuta *et al* [6] By comparison with the results obtained on violanin [7, 8], the stereostructure of each sugar of the rutinose in **1** was also determined by ^{13}C NMR and ^1H NMR measurements The glucose was shown to be in the β-D-pyranose form because the anomeric proton resonated at δ 5 44 ppm with large coupling constant ($J = 7$ 4 Hz) as well as those of H-3″ ($J = 9$ Hz) and H-4″ ($J = 9$ Hz) The other sugar moiety was confirmed to be α-L-rhamnopyranose for it had methyl group at δ 0 9 ppm ($J = 1$ 2 Hz), and a large value at H-3″ ($J = 9$ 7 Hz) The interglycosidic linkage was determined by ^{13}C NMR measurement (Table 2) as rhamnosyl (1→6)-glucose, [9, 10] Moreover, the position of the acetyl group was at the 4-hydroxyl of rhamnose, since, (Table 2) a shift downfield (+2ppm) was observed only at C-4 of the rhamnose moiety in **1** [11] Thus **1** in the berries of *Eurya japonica* is cyanidin 3-*O*-(6″-*O*-(4‴-*O*-acetyl-α-L-rhamnopyranosyl)-β-D-glucopyranoside), a new anthocyanin. Although many acetylated anthocyanidin 3-monoglucosides and 3,5-diglucosides occur in grape skins [12–14], this is the first report of an acetylated anthocyanin in a fruit other than grape

EXPERIMENTAL

Plant materials Black berries of *Eurya japonica* Thunberg were harvested at the campus of Minami-Kyusyu University and dried at 50° overnight The authentic anthocyanins, Cy 3-G and Cy 3-R, were isolated from the seed coats of *Glycine max* [15] and from the flowers of *Antirrhinum majus* [16], respectively

Isolation and purification Dried berries were (*ca* 100 g) extracted with MeOH–HCO_2H–H_2O (10 1 10), and the pigments extract was conc under 40° The concentrate was purified by prep PC (Toyo No 526 filter paper) in the solvents 10% HCO_2H and *n*-BuOH–HCO_2H–H_2O (4 1 2) and TLC (Funakoshi Avicel SF cellulose) in 10% HCO_2H successively followed by Sephadex LH-20 CC with MeOH HCO_2H–H_2O (10 1 10) to give a red powder (*ca* 20 mg), **1**.

HPLC analysis HPLC analysis of **1** and the related pigments were performed with Hitachi L-6200 system, with Hitachi gel no 3056 reversed phase column (C_{18}, 150 mm × 4 mm i d) at 520 nm at 30° using the solvent system of ref [17]

Acyl group analysis Pigment 1 (*ca* 1 mg) was deacylated with

Table 1 Chromatographic and spectral properties of *Eurya japonica* anthocyanin (**1**) and its acylating acid

	R_f × 100 in				Retention time (min)		In 1% HCl–MeOH			
Compound	AHW	BAW	BFW	10% HCO_2H*	R_{t1}	R_{t2}	λ_{max}^{UV}(nm)	λ_{max}^{VIS}(nm)	$\frac{E_{320}}{E^{VIS}}$(%)	$AlCl_3$ shift‡
Eurya japonica anthocyanin (**1**)	49	55	51	48	2 80		282	530	9	+
Cyanidin 3-rutinoside and deacylated anthocyanin of **1**	30	33	30	30	2 04		282	531	14	+
Cyanidin 3-glucoside	15	33	30	15	2 01		282	530	8	+
Acetic acid and acylating acid of **1**						2 52				
Formic acid						1 61				

*TLC was carried on Funakoshi avicel SF microcrystalline cellulose plates using AHW = HOAc–HCl–H_2O (15 3 82), BAW = *n*-BuOH–HOAc–H_2O (6 1 2), BFW = *n*-BuOH–HCO_2H–H_2O (4 1 2)

†HPLC was run with the reversed phase column at 30°, R_{t1} detected at 520 nm using solvent A B = 2 3, solvent A = HCO_2H–H_2O (1.9); solvent B = HCO_2H–H_2O–MeOH (1 4 5), ref [17], R_t2 detected at 210 nm using 15 mM $(NH_4)H_2PO_4$ (pH 2 4)

‡Observed after addition of 5% $AlCl_3$–MeOH to 0 01% HCl–MeOH solution of each pigment

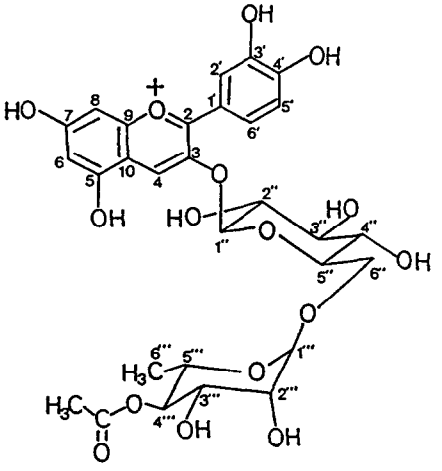
Table 2 ^{13}C NMR spectral data of *Eurya japonica* anthocyanin (**1**) and related compounds in δ ppm from TMS as an internal standard

	Aglycone moiety*					Sugar moiety†			
Carbon	1	Cy 3-R	Cy	Cy3-G	Carbon	1	Cy 3-R	MG	MR
2	161 8	161 8	161.3	164.2	1″	101 6	101 8	104.3	
3	144 1	144 2	146.3	145 6	2″	74 0	73.0	74.2	
4	134 4	134 4	133 9	136 8	3″	76.2[a]	76.3[a]	76 9	
5	157 6	157 6	157 9	159 2	4″	69 5[b]	69 7[b]	70.8	
6	102.4	102 4	103 1	103 3	5″	75 7[a]	76.0[a]	76.9	
7	168 3	168 3	168 8	170 4	6″	66 1	66.3	61 9	
8	94 1	94 1	94 8	95 1	1‴	100.3	100.7		101.9
9	157 5	157 5	156 6	157 6	2‴	70 2[b]	70 3[b]		71.0
10	113 0	113 1	113 4	113 3	3‴	70 4[b]	70.7[b]		71 3
1′	121 5	121.7	121 7	121 2	4‴	74 0	72.0		73 1
2′	119 6	119 6	117 7	118 4	5‴	68.3	68.4		69 4
3′	146 2	146 1	147 2	147 4	6‴	17 3	17 9		17 7
4′	155 8	155 8	155.0	155 8					
5′	117 4	117 5	117 2	117 4					
6′	127 0	127 0	127 1	128 3					
C=O	170 1								

*1, Cy 3-R *Eurya japonica* anthocyanin and cyanidin 3-rutinoside respectively were measured in CF_3CO_2D–DMSO-d_6 (1·9); Cy, Cy 3-G: cyanidin and cyanidin 3-glucoside respectively were measured in DCl–CD_3OD [6]

† MG,MR methyl-β-D-glucopyranoside and methyl-α-L-rhamnopyranoside respectively, were measured in D_2O [9]

[a] [b] Assignments bearing the same superscript in any one spectrum may be reversed



1

0.5 N NaOH–MeOH aq under N_2 for 30 min at room temp., acidified with 2% HCl–MeOH and dried with Na_2SO_4. After removal of salts by centrifugation, the red supernatant was added with excess ether and the pptd pigment was separated for analysis Resulting supernatant was evapd to a small volume and the residue was analysed by the same HPLC system described above at 210 nm with 5 mM $(NH_4)H_2PO_4$ (adjusted to pH 2 4 with phosphoric acid) as an isocratic eluent.

Instrumental analyses SIMS were determined on a Hitachi M-80B mass spectrometer using Xe gas in DMSO with glycerol or benzyl alcohol as a matrix ^1H NMR and ^{13}C NMR spectra were measured in CF_3CO_2D–DMSO-d_6 (1 9) contained TMS as an int standard ^{13}C NMR was operated at 67 94 MHz with a complete proton decoupling mode

Cyanidin 3-O-(6″-O-(4‴-O-*acetyl*-α-L-*rhamnopyranosyl*)-β-D-*glucopyranoside*) (**1**) IR ν_{max}^{KBr} cm^{-1}. 3400, 1720 (acetoxyl C=O), 1630, 1580, 1270, 1040, SIMS *m/z*: 637 $[M+H]^+$ ($C_{29}H_{33}O_{16}$ requires 637 as the flavylium ion), 595 $[M+H-Ac]^+$ (loss of acetyl group), 449 [Cy 3-G]$^+$, 287 [Cy]$^+$; ^1H NMR(270 MHz, CF_3CO_2D–DMSO-d_6 (1 9) δ8 85 (1H, *s*, H-4), 8 26 (1H, *dd*, *J* = 8 8, 2 3 H-6′), 8 02 (1H, *d*, *J* = 2.3 Hz, H-2′),7 06 (1H, *d*, *J* = 8 7 Hz, H-5′), 6 93(1H, *br*, *s*, H-8), 6 75 (1H, *br s*, H-6), 5 44 (1H, *d*, *J* = 7 4 Hz, H-1″), 4 73 (1H, *t*, *J* = 9 7 Hz, H-4‴), 4 60 (1H, *d*, *J* = 1 2 Hz, H-1‴), 3 90 (1H, *br*, *d*, *J* = 10.4, H-6″a), 3 72 ~ 3 50 (5H, *m*, H-5″, 3‴, 2″, 2‴, 5‴), 3 41 (1H, *t*, *J* = 8.8 Hz, H-6″b), 3.27 (1H, *t*, *J* = 9 1 Hz, H-3″) 1 96 (3H, *s*, COMe), 0 90 (1H, *d*, *J* = 6 3 Hz, H-6‴)

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