A Diacylated Anthocyanin from Tibouchina urvilleana Flowers

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A DIACYLATED ANTHOCYANIN FROM *TIBOUCHINA URVILLEANA* FLOWERS

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ABSTRACT.—The structure of the major pigment in the purple flowers of *Tibouchina urvilleana* (Melastomataceae) has been identified as malvidin 3-(*p*-coumarylglucoside)-5-acetylxyloside [**1**], a new anthocyanin, by using chromatographic and various nmr techniques involving nOe, HOHAHA, DQF-COSY, DEPT, HMQC, and HMBC. The pigment was found to exist as an equilibrium mixture of the flavylium and the pseudobase forms in the nmr solvent.

An anthocyanin from the purple flower petals of *Tibouchina urvilleana* (Melastomataceae) was isolated by Harborne (1) and Lowry (2) and identified as malvidin 3-(*p*-coumaroylglucoside)-5-glucoside on the basis of chemical evidence. In the course of surveying such acylated anthocyanins for utilization as natural food colorants, we have reinvestigated the structure of the major anthocyanin isolated from the same plant material and found it to be novel. We describe here its precise determination.

RESULTS AND DISCUSSION

Petals of *T. urvilleana* were extracted with 5% HOAc, and the extract was purified by HP-20 and LH-20 resin columns and preparative ODS-hplc using HOAc solvent

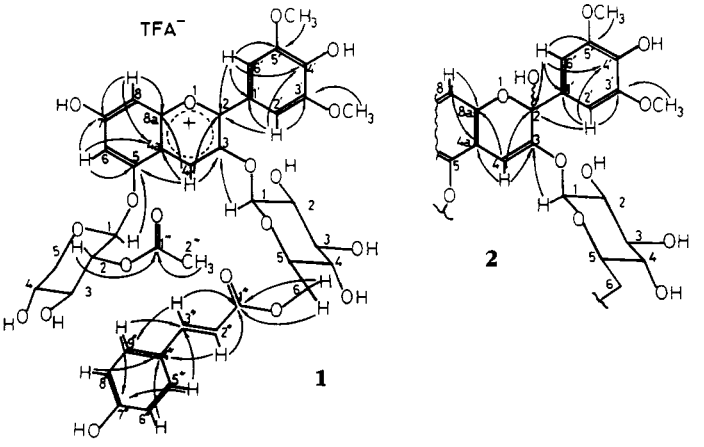

FIGURE 1. Structures of *Tibouchina urvilleana* flower pigment **1** and its pseudobase form **2**. Solid arrows indicate the long range C-H correlatons by the HMBC method.

systems. Among the four pigments TS1–TS4, the major one, TS4 [**1**], was obtained as the trifluoroacetic acid (TFA) salt in the form of a reddish purple amorphous powder.

On acidic hydrolysis, **1** gave malvidin (Mv) as the aglycone and D-glucose (Glc) and D-xylose (Xyl) as sugar components. In the uv-vis spectra of **1**, the presence of the characteristic absorptions at 301 nm suggested that **1** was acylated with aromatic acid(s). This was also confirmed by the conjugated ester carbonyl band at 1686 cm^{-1} in the ir spectrum. The number of binding aromatic acids was estimated to be 1–2 mol in **1** due to the value (75%) of E_{301}/E_{542} (3). On alkaline hydrolysis, the aromatic acid of **1** was identified to be *p*-coumaric acid (pC). Other than pC, the alkaline hydrolysis of **1** gave the deacylated anthocyanin, which was determined to have the di-monoglycosidic substitution pattern by partial acid hydrolysis (4). H_2O_2 oxidation of the deacylated anthocyanin gave Glc, supporting the presence of a 3-glucoside (5).

Molecular weights of the flavylium ions of the deacylated anthocyanin and **1** were determined as 625 and 813, corresponding to $C_{28}H_{33}O_{16}^{+}$ and $C_{39}H_{41}O_{19}^{+}$, respectively, based on fabms data, showing the deacylated anthocyanin to be composed of Mv, Glc, and Xyl, and **1** with additional pC and HOAc. Hence, the chemical structures of the deacylated anthocyanin and **1** were suggested as Mv 3-Glc-5-Xyl and its acylated derivative with pC and HOAc, respectively.

Of the assignable peaks in the ^{1}H-nmr spectra of the deacylated anthocyanin, four proton peaks in the downfield and one peak in upfield regions showed the aglycone moiety to be Mv. The anomeric protons of two sugars in the downfield region with large *J* values (7–8 Hz) showed the sugar moieties to have the β form. The ^{1}H-nmr time-course spectra of **1** in DMSO-d_6 containing CF_3COOD showed the appearance of a new component [**2**] in addition to **1**. The signal intensity of **1** prevailing in the freshly prepared sample was decreasing, and finally the equilibrium of the two components was established. In the ^{1}H- and ^{13}C-nmr spectra, signals assignable to **1** and **2** could be differentiated and analyzed by a combination of difference nOe (6), difference HOHAHA (7,8), DQF-COSY (9), DEPT (10), HMQC (11), and HMBC (12) methods.

In the sugar region, four anomeric protons (H-X1 and H-G1 for **1** and **2**), four methylene protons (H_a-G6 and H_b-G6 for **1** and **2**), and two methine protons (H-X2 for **1** and **2**) appeared as the separated signals in the downfield region (δ 4.2–5.5 ppm),

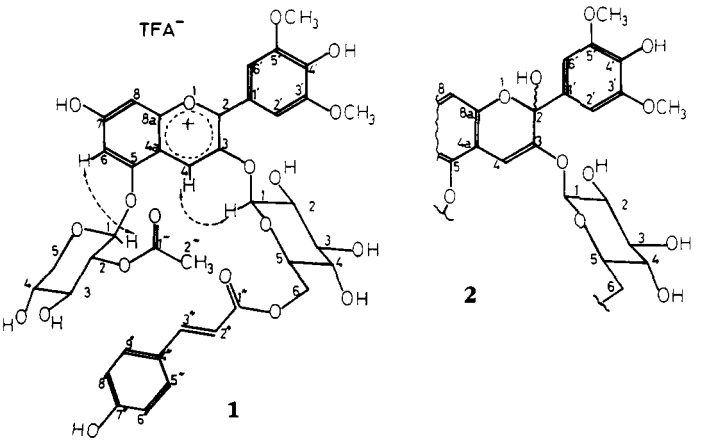

FIGURE 2. Structures of *Tibouchina urvilleana* flower pigment **1** and its pseudobase form **2**. Dotted arrows indicate the negative nOe's.

indicating the sugar parts to be β anomers and the G6-OH and X2-OH to be acylated, while the other sugar protons gave overlapped signals in the upfield region (3.3–3.9 ppm). However, the complete assignment of the complicated proton signals was clarified by the analysis of the HOHAHA and DQF-COSY spectra with the aid of ^{13}C-nmr analysis by HMQC spectra.

When the lowest anomeric proton (H-X1, 5.45 ppm) of **1** was irradiated by using a long pulse mode excitation with the spin locking time of 100 msec in the Haltmann-Hahn experiments (7,8), the magnetization was propagated along with the acyloxy methine (H-X2) and hydroxy methines (H-X3 and -H4) and finally reached the methylene protons (H-X5), in which they were attributed to a series of a scalar coupling network. The assignment and the connectivities of the five carbons having OH groups were determined with HMBC and HMQC spectra based on the above results; therefore, the Xyl moiety was completely clarified. With the exception of H_a-X5, all five other protons exhibited large vicinal couplings (J=7–10 Hz, Table 1) assignable to be the trans diaxial correlations with each other, which suggests a pyranosyl configuration. Therefore, the pentose moiety was determined to be in the 2-acylated-β- xylopyranoside (2-acyl-β-Xyl) form. Similarly, when H-G1 (5.34 ppm) of the glucose moiety was irradiated, the spin energies were transferred by a series of Glc ring protons (H-G2, -G3, -G4, and -G5) and reached to the H-G6. Since, with the exception of the methylene protons (H-G6), these had large vicinal couplings (Table 1), the sugar portion was regarded to be 6-acylated-β-glucopyranoside (6-acyl-β-Glc).

With **2**, analogous results and assignments were obtained in the same manner by irradiation of the appropriate anomeric protons which appeared up-field from those of **1**. Xyl and Glc moieties were confirmed to be 2-acyl-β-Xyl and 6-acyl-β-Glc, respectively.

^{1}H-nmr signals of the Me protons (H-2‴) of **1** and **2** were assigned to the singlet peaks at δ 2.12 and 1.99 ppm, respectively, with consideration of the time-course spectra. The Me and carbonyl carbons of both components could be assigned by the inverse type HMQC and HMBC techniques. The long range correlation between the carbonyl (C-1‴) and H-X2 confirmed that the Xyl moiety was in the 2-acetyl-β-xylopyranoside form in the HMBC spectrum.

The structure of the sugar moiety at the 3 position of **1** and **2** was unambiguously determined to be 6-*p*-coumaryl-β-glucopyranoside by the analysis of ^{1}H- and ^{13}C-nmr spectra based on the HMQC and HMBC methods.

On the aglycone moieties, residual two aromatic methoxyls and five aromatic proton signals of **1** suggested a flavylium skeleton, and those five aromatic methine carbons (C-4, -6, -8, -2′, and -6′) and especially ten quaternary carbons (C-2, -3, -4a, -5, -7, -8a, -1′, -3′, -5′, and -4′) with the long range correlations confirmed that the aromatic moiety was 3,5,7,4′-tetrahydroxy-3′,5′-dimethoxyflavylium cation, malvidin.

To clarify the glycosidic linkages, 1D difference nOe was applied at 500 MHz. Irradiations at H-X1 and H-G1 showed negative nOe's at H-6 and H-4, respectively. Furthermore, long range correlations were observed between H-X1 and H-G1 and C-5 and C-3, respectively. Based on difference nOe and HMBC data, the glycosidic linkages of both **1** and **2** were determined to be 3-Glc-5-Xyl. Thus, the chemical structure of the major anthocyanin **1** is 3-*O*-[6-*O*-(*E*)-*p*-coumaryl-β-D-glucopyranosyl]-5-*O*-(2-*O*-acetyl-β-D-xylopyranosyl)malvidin (Figures 1 and 2).

For **2**, large upfield chemical shift changes were observed for the aromatic proton and carbon signals when compared to those of **1**, while the signals on the sugar moieties were maintained as with those of **1** (Table 1). Especially, a remarkable upfield shift occurred at C-2 (ca. 60 ppm, Table 1), suggesting that the valence state and the configuration at C-2 is converted to the sp^3 of a hemiacetal from the sp^2 of an aromatic carbon as in **1**. Based

TABLE 1. ^{13}C- and ^{1}H-nmr Data of *Tibouchina urvilleana* Flower Pigment **1** and Related Compound **2** in DMSO-d_6–CF_3COOD (10:1).[a]

Position	Compound			
	1		**2**	
	δ_c	δ_H	δ_c	δ_H
Aglycone				
2	162.06 s		102.51 s	
3	145.0 s		141.27 s	
4	130.78 d	8.56 s	101.41 d	6.12 s
4a	111.69 s		102.02 s	
5	154.49 s		153.39 s	
6	103.29 d	7.00 d (1.7)	97.87 d	6.20 d (2)
7	167.46 s		158.52 s	
8	96.88 d	7.29 d (1.7)	98.55 d	6.24 d (2)
8a	155.34 s		150.99 s	
1′	118.20 s		130.0 s	
2′,6′	110.02 d	8.02 s (2H)	103.3 d	6.75 s (2H)
3′,5′	148.51 s		147.76 s	
4′	145.65 s		136.68 s	
OMe	56.64 q	3.97 s (6H)	56.18 q	3.73 s (6H)
p- Coumaryl				
1″	166.9 s		166.92 d	
2″	113.76 d	6.31 d (15.8)	114.17 d	6.49 d (15.6)
3″	145.33 d	7.50 d (15.8)	145.33 d	7.63 d (15.6)
4″	125.20 s		125.39 s	
5″,9″	130.47 d	7.44 d (2H, 8.8)	130.61 d	7.60 d (2H, 9)
6″,8″	115.77 d	6.79 d (2H, 8.8)	115.97 d	6.82 d (2H, 9)
7″	160.07 s		160.07 s	
Acetyl				
1‴	170.71 s		169.71 s	
2‴	21.32 q	2.12 s (3H)	20.91 q	1.99 s (3H)
Xylosyl				
X1	99.74 d	5.45 d (7.3)	100.57 d	4.88 d (7.8)
X2	73.76 d	5.00 dd (9.3, 7.8)	73.66 d	4.79 dd (9.3, 7.8)
X3	73.22 d	3.60 t (ca. 9)	73.55 d	3.46 t (ca. 9)
X4	76.75 d	3.48 m	76.75 d	3.51 m
X5	65.94 t	3.80 dd (11.2, 4,4)	65.94 t	3.86 dd (ca. 8, 4)
		3.55 m		3.30 t (ca. 9)
Glucosyl				
G1	100.67 d	5.34 d (7.8)	97.66 d	5.16 d (8.3)
G2	73.22 d	3.61 t (ca. 8.6)	75.65 d	3.84 t (ca. 9)
G3	76.75 d	3.50 t (9)	72.79 d	3.56 t (9)
G4	69.92 d	3.39 t (9)	70.60 d	3.32 t (ca. 9)
G5	74.20 d	4.24 m	75.31 d	3.74 m
G6	63.53 t	4.53 d (10.8)	63.61 t	4.51 d (11)
		4.26 m		4.25 m

[a]*J* (Hz) in parentheses.

on the above facts, the structure of **2** was deduced to be as a 2-carbinol Mv, the pseudobase of **1** (Figures 1 and 2).

This hydration of **1** to form **2** was suggested to be due to a trace of H_2O in the nmr solvent, causing a nucleophilic attack to the electron-deficient C-2 of **1**; this occurred as well in weak acidic and neutral aqueous solutions of ordinary anthocyanins (13–16). Although the existence of the pseudobases in the anthocyanin equilibrium mixtures was

previously detected by nmr spectroscopic methods (17,18), the complete structural assignment of the pseudobase **2** is reported here for the first time.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Tlc was carried out on microcrystalline cellulose plates (Avicel SF, Funakoshi). The sample spots on chromatograms were detected with uv for aromatic compounds and aniline hydrogenphthalate (AHP) spray reagent for sugars. Hplc was performed on an L-6200 Intelligent pump system (Hitachi). Analytical hplc was run on a Develosil ODS-5 (4.6ϕ×250 mm, Nomura Chemical) column at 35° with a flow rate of 1 ml/min, monitoring at 312 nm for uv-absorbing compounds and 530 nm for anthocyanins. Solvent systems were employed as follows: a linear gradient elution for 50 min from 25% to 100% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1.5% H_3PO_4 in H_2O) (19). Preparative hplc was done on a Develosil ODS-5 (20ϕ×50 mm+20ϕ×250 mm, Nomura Chemical) column with 9 ml/min by isocratic elution using 1:1 mixture 15% HOAc in H_2O and 15% HOAc, 30% MeCN in H_2O (20) at 530 nm. Mp's were measured on a micro mp apparatus MP-J3 (Yamnako). Ir spectra were recorded on 270–30 (Hitachi) spectrophotometer in KBr, uv-vis spectra on MPS-2000 (Shimadzu) spectrophotometer, fabms spectra on JMX DX-300 (JEOL), and ^1H (400 and 500 MHz) and ^{13}C (100.53 and 125.78 MHz) nmr on JNM GX-400 and GX-500 (JEOL) in DMSO-d_6–CF_3COOD (9:1) with internal standard TMS.

PLANT MATERIAL.—The purple flower petals of *T. urvilleana* were collected in November 1990 in the garden of Minami-Kyushu University and dried at 50° overnight. The material was identified by Dr. Kenjiro Toki, Laboratory of Floriculture, College of Horticulture, Minami-Kyushu University, Japan. A voucher specimen is available for inspection at the herbarium of the Minami-Kyushu University.

ISOLATION OF PIGMENT **1**.—The dried petals (20 g) were steeped in 5% HOAc (1 liter×3) overnight and filtered. In the crude pigment filtrate were detected four anthocyanins named tentatively as TS1–TS4 at retention times (contents %) TS1 30.7 (2%), TS2 33.1 (18%), TS3 34.6 (5%), and TS4 [**1**] 36.6 (69%) min, respectively, by hplc analysis. The reddish purple extract was applied on HP-20 (Diaion) resin column (45ϕ×200 mm). The column was washed with 1% HOAc (2 liters) and then eluted with 1% HOAc in 70% EtOH. The pigment eluate was separated by a Sephadex LH-20 (Pharmacia) column (16ϕ×800 mm) using HOAc-EtOH-H_2O (1:2:7) and finally purified by preparative hplc using an HOAc solvent system. The major anthocyanin fractions were evaporated to dryness, dissolved in a small amount of TFA, and precipitated with excess Et_2O to give TFA salts as reddish purple amorphous powders. The predominant anthocyanin **1** was obtained in a yield of about 130 mg.

PREPARATION OF THE DEACYLATED ANTHOCYANIN.—Compound **1** (100 mg) was dissolved in 8 ml of 2 N NaOH, and N_2 was bubbled into the solution and saturated for 5 min. The solution was kept standing for 30 min, acidified with 9 ml of 2 N HCl, and washed five times with 20 ml of Et_2O. The Et_2O washings were used for acylated acid analysis. The aqueous layer was applied on an HP-20 column (40ϕ×140 mm), washed with 1% HOAc, and eluted with 1% HOAc in 70% EtOH; the eluate was evaporated in vacuo to dryness. The residue was powdered with TFA/Et_2O as described above, and 43 mg of deacylated anthocyanin TFA salt was obtained as a dark purple powder.

HYDROLYTIC ANALYSES.—Complete and partial acid hydrolyses, alkaline hydrolysis, and H_2O_2 oxidation (5) were performed as usual.

DEACYLATED ANTHOCYANIN TFA SALT.—Mp>300°; ir ν max (KBr) cm^{-1} 3432 (O-H), 1636 (aromatic C=C), 1606 (aromatic C=C); uv-vis ν max (0.01% HCl-MeOH) nm 537 (not shifted bathochromically with $AlCl_3$) 275, E_{440}/E_{537}=13%; fabms (positive mode in *m*- benzyl alcohol) *m/z* $[M]^+$ 625 ($C_{28}H_{33}O_{16}$); ^1H nmr δ ppm [400 MHz, DMSO-d_6–CF_3COOD (9:1) internal standard TMS] 8.95 (1H, s, H-4), 8.01 (2H, s, H-2′, -6′), 7.27 (1H, s, H-8), 6.98 (1H, s, H-6), 5.39 (1H, d, J=8, H-G1), 5.14 (1H, d, J=7, H-X1), 3.93 (6H, s, 3′- ,5′- OMe).

PIGMENT **1** TFA.—Mp>300°; ir ν max (KBr) cm^{-1} 3408 (O−H), 1736 (C=O), 1686 (C=O), 1636 (aromatic C=C), 1606 (aromatic C=C); uv-vis λ max (0.01% HCl/MeOH) nm 542 (not shifted bathochromically with $AlCl_3$), 301, 281, E_{301}/E_{542}=75%, E_{440}/E_{542}=9%; fabms (positive mode in *m*- benzyl alcohol) *m/z* $[M]^+$ 813 ($C_{39}H_{41}O_{19}$).

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