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ANTHOCYANIDIN MALONYLGLUCOSIDES IN FLOWERS OF *HIBISCUS SYRIACUS*

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Key Word Index--Hibiscus *syriacus;* Malvaceae; anthocyanidin malonylglucosides; ^{1}H NMR; FABMS.

Abstract-The methanolic formic acid extraction of the petals of *Hibiscus* syriacus yielded 3-*O*-malonylglucosides of delphinidin, cyanidin, petunidin, pelargonidin, peonidin and malvidin. The structures were determined by hydrolytic studies and ^{1}H NMR and FABMS examination.

INTRODUCTION

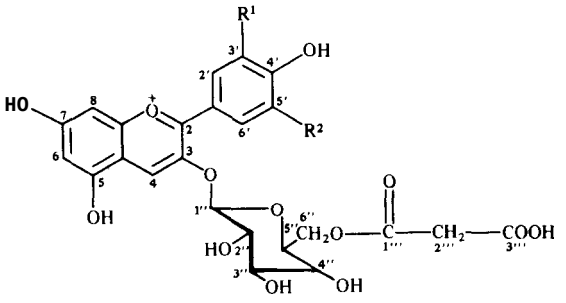
Previous work on the floral anthocyanins of *Hibiscus syriacus* L. (Malvaceae) showed the occurrence of 3-*O*-glucosides of delphinidin, petunidin, cyanidin and malvidin [1]. During the systematic survey of anthocyanins in the species of genus *Hibiscus, we* have however found that *H. syriacus* contains unidentified anthocyanin pigments which are extremely labile in methanolic HCl [2]. Successive large-scale extraction using methanol-formic acid has now resulted in the isolation of six anthocyanidin 3-*O*-malonylglucosides, among which three were found to be new. We report here the isolation and characterization of these compounds.

RESULTS AND DISCUSSION

Preliminary examination of the methanol-formic acid extract by HPLC showed the presence of 12 anthocyanin pigments, of which the first six eluted were identified as 3-0-glucosides of delphinidin (**1**), cyanidin (**2**), petunidin (**3**), pelargonidin (**4**), peonidin (5) and malvidin (6) (Table 1) by comparing their R_fs with those of authentic samples [2].

The remaining six compounds (7-12) were considered to be acylated, since it was reported that, in the reverse-phase HPLC, acylation characteristically increases the R_fs twofold as compared with those of the original anthocyanin glucosides [3, 4]. Moreover, when the anthocyanins of *H. syriacus* were extracted with methanolic HCl, peak areas of **7**–**12** continuously decreased, whereas those of the 3-0-glucosides (**1**–**6**) increased.

Compounds 7-12 were isolated by column and Sep Pak chromatography, PC, TLC and finally by Sephadex LH-20 chromatography. Of these, compound 7 was identified as delphinidin 3-*O*-(6″-*O*-malonyl)glucoside by TLC and HPLC with authentic sample which was donated by Dr K. Toki [5]. Alkaline treatment of 8-12 yielded malonic acid as the acidic component, together with anthocyanins whose R_f values and R_t coincided with the corresponding 3-*O*-glucosides (2-6). Additionally, complete acid hydrolyses of **8**–**12** yielded only glucose as the sugar component. Based on this chemical evidence, 7-12 were assumed to be the malonyl esters of 1-6, respectively. This was further supported by FAB-MS analyses, which exhibited the corresponding $[M]^+$ peak, viz., 535 (**8**), 565 (**9**), 519 (**10**), 549 (**11**) and 579 (**12**) respectively, together with the fragment peak $[M-86]^+$ due to the loss of the malonyl group, viz. 449 (**8**), 479 (**9**), 433 (**10**), 463 (11) and 493 (12) respectively.



	R^1	R^2
7	OH	OH
8	OH	H
9	OMe	OH
10	H	H
11	OMe	H
12	OMe	OMe

The location of the malonyl ester group in **8**–**12** were determined by ^{1}H NMR examinations (Table 2). Each of the compounds **8**–**12** gave a sharp singlet at δ3.38–3.40 assignable to the malonyl methylene group, and two lowfield signals in the regions of δ4.28–4.30 and 4.52-4.56 which were attributable to the protons germinal to the acyl group. The coupling patterns (AB portion of the ABX system) clearly indicated that these signals are due to the C-6 methylene protons of the glucose moiety, thus confirming the location of the malonyl group to be at the

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Table I. R_f values and retention times (R_t) of anthocyanins (1-12)

Pigment	R_f (×100) in*				R_t (min)
	BAW	BuHCl	1% HCl	HOAc–HCl	
Delphinidin 3-*O*-glucoside (1)	26	11	4	17	8.62
Cyanidin 3-*O*-glucoside (2)	38	24	7	26	11.29
Petunidin 3-*O*-glucoside (3)	34	14	4	21	13.48
Pelargonidin 3-0-glucoside (4)	48	36	12	34	14.47
Peonidin 3-*O*-glucoside (5)	46	31	8	32	16.21
Malvidin 3-*O*-glucoside (6)	40	15	6	30	18.53
Delphinidin 3-*O*-malonylglucoside (7)	32	22	5	23	17.15
Cyanidin 3-*O*-malonylglucoside (8)	43	39	15	32	20.73
Petunidin 3-*O*-malonylglucoside (9)	38	29	6	30	22.74
Pelargonidin 3-*O*-malonylglucoside (10)	57	48	16	39	24.14
Peonidin 3-*O*-malonylglucoside (11)	52	42	14	38	25.97
Malvidin 3-*O*-malonylglucoside (12)	51	35	12	37	27.08

*R_f values were measured on microcrystalline cellulose TLC in the solvents of BAW; *n*-BuOH–HOAc–H_2O (4: 1:5), BuHCl; *n*-BuOH–2 M HCl (1: 1), 1% HCl; conc HCl–H_2O (3:97), and HOAc–HCl; H_2O–HOAc–HCl (82: 15:3).

C-6" position. In addition, the large coupling constant (J = 7.6-7.8) of the sugar anomeric proton appearing at δ5.26–5.32 indicated that **8–12** have the /?-configuration. Thus, **8–12** are 3-*O*-(6''-*O*-malonyl)-β-D-glucopyranosides of cyanidin, petunidin, pelargonidin, peonidin and malvidin, respectively. The cyanidin malonate (8) was found to be abundant in the petal eyes of all the cultivars examined so far (about 30%), malvidin (12) in blue petal tips (about 50%), and petunidin (9) and delphinidin (7) in most mauve flowers (about 30% in total), while pelargonidin (10) and peonidin (11) were invariably minor components.

Hibiscus ***syriacus*** has very diverse anthocyanidin compositions compared with those of other ***Hibiscus*** species. A survey of Malaysian ***Hibiscus*** [6] and other preliminary studies [7–13] have shown that only delphinidin and cyanidin glycosides are contained in the ***Hibiscus*** species. Furthermore, acylated anthocyanins were not reported previously. The reason why acylated anthocyanins were not detected in the previous reports of ***H. syriacus*** [1, 2] and related species [6–13] may be explained by the classical methodology which employs methanolic HCI for the extraction and purification. Hence, it is emphasized that more precise reexamination of the anthocyanins of ***Hibiscus*** will be required. Recently, many zwitterionic anthocyanins were discovered [4, 14, 15] and the function of such acylations in living cells were discussed [4,16]. However, the role of such acylated anthocyanins, especially that of mono and di-glucosides, in flower colour production is not fully resolvèd.

EXPERIMENTAL

Plant material. Many horticultural cultivars of ***H. syriacus*** were obtained from the Botanical Garden of Osaka City University, and old cultivars were also collected from Ryukyu islands, Formosa, China and Korea. The fresh petals of several cultivars were collected in the morning and frozen at -40" until analysed. When necessary, petals were separated into the basal blotch 'eye' and the upper 'tip'.

Isolation and *purification.* Petals were extracted with MeOH–HCO_2H (19: 1) at 5° for *ca* 24 hr. The extract was concd and sepd on a polyamide column (3.5 × 45 cm) using MeOH–H_2O–HCO_2H (5 : 13 : 2) into 3-0-glucosides and acylated 3-*O*-glucosides. The earlier eluted 3-*O*-glucosides were identified by the methods of ref. [17] and by cochromatography (TLC and HPLC) with authentic samples. The later fraction containing acylated anthocyanins was concd to ca 3/4 vol. The aq. soln was applied to a C-18 Sep Pak cartridge and eluted with small vol. of MeOH–HCO_2H (19: 1). The eluate was concd under N_2 and purified by prep. PC (57 × 60 cm) with *n*-BuOH–HOAc–H_2O (4: 1:5) and TLC (Avicel SF cellulose) with 10% HCO_2H. Each pigment was further purified by chromatography on a Sephadex LH-20 column (3 × 45 cm) using 10% HCO_2H. The eluate was adsorbed on a C-18 Sep Pak cartridge and eluted with MeOH–HCO_2H. The purity of each sample was checked by HPLC at 280nm absorbance. Since the acylated anthocyanins readily decomposed in soln releasing malonic acid, the solvent was immediately evapd in N_2 at each isolation stage.

HPLC analysis. Anthocyanins were analysed on a Shimpak CLC-ODS reverse-phase column (0.45 x 15 cm) by the slightly modified methods of ref. [3]. The initial solvent system was 10% of HCO_2H–MeOH (1:9) in HCO_2H–H_2O (1:9) (10 min) and then the concn immediately Increased to 22%. After 10 min, the concn gradually increased to 45% (25 min). The flow rate was 1 ml/min and the detector wavelength was 530 nm. R_ts are summarized in Table 1.

Hydrolysis. Deacylation was accomplished by treatment with 0.05 M NaOH under N_2 gas for 5 min. After acidification with 3 M H_2SO_4, the organic acid was extracted with Et_2O. The solvent was evapd under N_2 and the residue examined by cellulose TLC. In all acylated pigments, malonic acid was the only acid detected with Bromocresoi Green in NaOH–EtOH. In EtOH–H_2O–NH_4OH, 16:3:1 R_f: 0.13, *n*-BuOH–HOAc–H_2O, 4: 1: 1 R_f: 0.74, EtOAc–HOAc–H_2O, 3: 1: 1 R_f: 0.82, *n*-BuOH–HOAc–H_2O, 1: 1 : 5 R_f: 0.69. The deacylated anthocyanins were identified by HPLC. After complete acid hydrolysis, the sugar was determined as glucose in all pigments.

Spectral ***analyses.*** FABMS was obtained in a positive mode with glycerol-HCI (glycerol–0.1 M HCI. 1:1) as the matrix.

Table 2. ^{1}H NMR data for malonyl anthocyanins (812)

	8	9	10	11	12
Anthocyanidin moiety					
H	Cyanidin	Petunidin	Pelargonidin	Peonidin	Malvidin
4	8.95 (s)	*8.95 (s)*	*9.02 (s)*	9.00 (s)	*8.98 (s)*
6	6.68 *(d, J = 2* Hz)	*6.68 (d, J = 2* Hz)	*6.69 (d, J = 2* Hz)	6.69 *(d, J =* 2 Hz)	*6.69 (d, J = 2* Hz)
8	6.90 *(d, J =* 2 Hz)	*6.91 (d, J = 2* Hz)	*6.94 (d, J = 2* Hz)	6.95 *(d, J =* 2 Hz)	*6.97 (d, J = 2* Hz)
2'	8.03 *(d, J =* 2 Hz)	*7.79 (d. J = 2* Hz)		8.23 *(d, J =* 2 Hz)	
			8.61 (d, J = 9 Hz)		*8.08 (s)*
6	8.28 *(dd, J = 2, 9* Hz)	*7.98 (d, J = 2* Hz)		8.28 *(dd, J = 2, 9* Hz)	
3'					
			7.06 (d, J = 9 Hz)		
5'	*7.03 (d, J = 9* Hz)			*7.09 (d, J = 9* Hz)	—
H	Glucose moiety				
1"	*5.27 (d, J = 8* Hz)	*5.32 (d, J = 8* Hz)	*5.26 (d, J = 8* Hz)	*5.30 (d, J = 8* Hz)	*5.32 (d, J = 8* Hz)
2"	*3.68 (dd, J = 8, 9* Hz)	*3.68 (dd, J = 8, 9* Hz)	*3.65 (dd, J = 8, 9* Hz)	*3.66 (dd, J = 8, 9* Hz)	*3.66 (dd, J = 8, 9* Hz)
3"	*3.54 (t, J = 9* Hz)	*3.56 (t, J = 9* Hz)	*3.53 (t, J = 9* Hz)	*3.54 (t, J = 9* Hz)	*3.55 (t, J = 9* Hz)
4"	*3.42 (t, J = 9* Hz)	*3.42 (t, J = 9* Hz)	*3.41 (t, J = 9* Hz)	*3.41 (t, J = 9* Hz)	*3.39 (t. J = 9* Hz)
5"	3.81 (m)	*3.82 (m)*	3.80 *(m)*	3.81 *(m)*	3.81 (m)
6"a	4.28 *(dd, J = 7,* 12 Hz)	*4.29* (dd, *J =* 7, 12 Hz)	*4.28 (dd, J = 7, 12* Hz)	4.29 *(dd, J = 7, 12* Hz)	4.30 *(dd, J = 7, 12* Hz)
6"b	*4.56 (dd, J = 2, 12* Hz)	4.53 *(dd, J = 2, 12* Hz)	*4.56* (dd, *J =* 2, 12 Hz)	*4.54 (dd, J = 2, 12* Hz)	*4.52 (dd, J = 2, 12* Hz)
H	Malonyl moiety				
2"'	*3.40 (s)*	*3.39 (s)*	3.38 (s)	*3.38 (s)*	*3.38 (s)*
H	Methoxyl group				
3'-Me		*4.01 (s)*	—	*4.03 (s)*	
					4.01 (s, 2 x Me)
S-Me			—	—	

^{1}H NMR spectra were measured at 270 MHz in CF_3CO_2D–DMSO-d_6 (1: **9)** containing **TMS** as int. standard.

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