



ACYLATED CYANIDIN 3-RUTINOSIDE-5,3'-DIGLUCOSIDE FROM THE PURPLE-RED FLOWER OF *LOBELIA ERINUS*

NORIO SAITO, KENJIRO TOKI,* HARUMITSU KUWANO,† HIDEKAZU MORIYAMA,* ATSUSHI SHIGIHARA† and TOSHIO HONDA†

Chemical Laboratory, Meiji-Gakuin University, Totsuka, Yokohama 224, Japan; *Laboratory of Floriculture, Minami-Kyushu University, Takanabe, Miyazaki 884, Japan; †Institute of Science and Technology, Inc., Shinagawa Tokyo 140, Japan; ‡Institute of Medicinal Chemistry, Hoshi University, Shinagawa, Tokyo 142, Japan

(Received 19 September 1994)

Key Word Index—*Lobelia erinus*; Lobeliaceae; flower colour; acylated anthocyanin; cyanidin 3-rutinoside-3',5'-diglucoside; *p*-coumaric, caffeic, malonic acids; intramolecular stacking structure.

Abstract—A new acylated anthocyanin was isolated from the purple-red flowers of *Lobelia erinus* cv 'Rosamond'. Its structure was elucidated to be cyanidin 3-*O*-(6-*O*-(4-*O*-*trans*-*p*-coumaryl- α -L-rhamnopyranosyl)- β -D-glucopyranoside)-5-*O*-(6-*O*-malonyl- β -D-glucopyranoside)-3'-*O*-(6-*O*-*trans*-caffeyl- β -D-glucopyranoside) by spectroscopic evidence. The three dimensional structure of this pigment was suggested from the observation of difference negative NOE spectrum analysis that cyanidin (chromophore) and caffeic acid (intracopigment) occupy a folding conformation as a stacking structure.

INTRODUCTION

Lobelia erinus is a popular ornamental plant with white, purple-red, purple and purple-blue flowers. Previously two acylated delphinidin glycosides were isolated from the purple-blue flowers of *L. erinus* [1, 2].

In continuing work on the flower colour variation due to acylated anthocyanins with aromatic acids [3-7], we undertook the isolation and elucidation of the anthocyanin in the purple-red flowers of *L. erinus* cv 'Rosamond'. We now report a structural study of this new acylated cyanidin glycoside.

RESULTS AND DISCUSSION

An acylated anthocyanin **1** was extracted from the purple-red flowers of *L. erinus* with 5% acetic acid and purified using the chromatographic techniques according to the previous reports [7, 8]. By HPLC analysis of crude extract, we observed the presence of three major anthocyanins. However, the other two could not be isolated.

The chromatographic and spectral data of **1** are shown in Table 1. On acid hydrolysis, **1** gave cyanidin, D-glucose, L-rhamnose, *p*-coumaric acid, caffeic acid and malonic acid. The alkaline hydrolysis of **1** yielded a deacylanthocyanin (**2**), *p*-coumaric acid, caffeic acid and malonic acid. Compound **2** was identified as cyanidin 3-rutinoside-5,3'-diglucoside from its chromatographic and spectral analyses containing partial acid hydrolysis (Table 1).

The FAB-mass spectrometry of **1** showed a molecular ion peak at 1313 *m/z* (calcd for C₆₀H₆₅O₃₃, 1313.341) and fragment peaks (*m/z* 859, 611, 459), indicating the presence of one molecule of cyanidin, two of glucose, one of rhamnose, caffeic acid, *p*-coumaric acid and malonic acid. The ¹H and ¹³C NMR spectra of **1** were measured on 400 MHz (¹H) and 100.53 MHz (¹³C) FT-NMR in trifluoroacetic acid-*d*₁-DMSO-*d*₆ (1:9) at room temperature.

The ¹H and ¹³C signals of **1** could be analysed by a combination of difference NOE [9, 10], phase sensitive DQF-COSY [11], HMQC [12], HMBC [13] and DEPT [14]. The H-4 signal of cyanidin nucleus was observed at δ 8.78, and also a signal at δ 7.15 was assigned to H-8 by observing a long range spin coupling between H-4 and H-8 in DQF-COSY spectrum. A doublet (*J* = 2 Hz, H-6) at δ 6.95 was spin-coupled with H-8. A doublet (*J* = 2 Hz, H-2') at δ 8.21 was spin-coupled with H-6' (δ 8.57, *dd*, *J* = 2, 8.8 Hz), which was also spin-coupled with H-5' (δ 7.21, *d*, *J* = 8.8 Hz). The aromatic proton signals of *p*-coumaric and caffeic acids were assigned by the analysis of DQF-COSY spectrum analysis as shown in Table 2. Two pairs of olefinic protons in both cinnamic acid units had the large coupling constants (12.7 and 15.6 Hz) between α and β proton signals indicated that all of the double bonds in these cinnamic acid units have a *trans* configuration. The proton signals of sugar part were observed in the region of δ 5.37-0.87, and four anomeric proton signals present in the spectrum indicated the presence of four sugar moieties. To elucidate the structure of sugar portion, difference NOE and DQF-COSY

Table 1. Chromatographic and spectral properties of anthocyanins from the purple-red flowers of *Lobelia erinus*

Anthocyanin*	R_f values ($\times 100$)†				R_t	Spectral data in 0.1% HCl–MeOH			FAB-MS [M] ⁺
	BAW	BuH	1% HCl	HAc–HCl	(min)	λ_{\max} (nm)	$E_{\text{acyl}}/E_{\text{max}}$ (%)	AlCl ₃	
1	47	34	32	65	27.6	295, 318, 528	86	—	1313
2	23	2	69	75	4.4	276, 518	—	—	919

*1, Cyanidin 3-(*p*-coumarylrutinoside)-5-(malonylglucoside)-3'-(caffeoylglucoside).

2, Deacyl pigment of 1; cyanidin 3-rutinoside-5,3'-diglucoside.

†BAW, *n*-BuOH-HOAc-H₂O (4:1:5); BuH, *n*-BuOH-2M HCl (1:1); HAc-HCl, HOAc-HCl-H₂O (15:3:82), analytical conditions: see Experimental.Table 2. NMR spectral data of acylated cyanidin glycoside from the purple-red flowers of *Lobelia erinus* (CF₃CO₂D-DMSO-*d*₆, 1:9 at 25°, standard TMS)

Position	δ_C	δ_H	Position	δ_C	δ_H
Cyanidin			Glucose A		
2	161.8		1	101.9	5.37 <i>d</i> (7.3)
3	145.2		2	73.6	3.63 <i>t</i> (8.3)
4	133.2	8.78 <i>s</i>	3	76.8	3.56 <i>t</i> (8.8)
4a	112.5		4	69.7	3.47 <i>t</i> (8.8)
5	155.4		5	71.1 ^a	3.83 <i>dm</i> (7.8)
6	105.5	6.95 <i>d</i> (1.5)	6	65.9	3.73 <i>dd</i> (3, 9.7)
7	168.4				3.94 <i>dm</i> (9.7)
8	97.1	7.15 <i>d</i> (1.5)	Glucose B		
8a	155.5		1	100.5	5.35 <i>d</i> (7.3)
1'	120.1		2	73.6	3.54 <i>m</i>
2'	116.8	8.21 <i>d</i> (2.0)	3	76.4	3.54 <i>m</i>
3'	146.3		4	76.2 ^a	3.37 <i>tm</i> (8.8)
4'	155.9		5	75.1 ^a	3.97 <i>t</i> (8.8-9.3)
5'	117.8	7.21 <i>d</i> (8.8)	6	64.0	4.29 <i>t</i> (8.3-)
6'	130.6	8.57 <i>dd</i> (2.0, 8.8)			4.67 <i>dm</i> (11)
<i>p</i> -Coumaric acid [I]			Glucose C		
1	126.1		1	101.8	5.16 <i>d</i> (7.8)
2,6	133.3	7.68 <i>d</i> (8.8)	2	73.8	3.59 <i>t</i> (7.3-9)
3,5	115.4	6.79 <i>d</i> (8.8)	3	76.3	3.56 <i>t</i> (7.8-9.7)
4	159.4		4	70.2	3.43 <i>t</i> (7.8)
α	115.9	5.57 <i>d</i> (12.7)	5	75.0	3.83 <i>t</i> (7.8-9)
β	144.2	6.80 <i>d</i> (13.2)	6	64.7	4.27 <i>dd</i> (6.8, 11.7)
C=O	166.1				4.54 <i>d</i> (11.7)
Caffeic acid [II]			Rhamnose		
1	126.0		1	100.6	4.69 <i>s</i>
2	114.6	6.51 <i>d</i> (2.0)	2	71.0	3.74 <i>d</i> (1.5)
3	145.8		3	69.0	3.70 <i>m</i>
4	148.5		4	74.1	4.84 <i>t</i> (9.8)
5	116.3	6.69 <i>d</i> (7.8)	5	66.6	3.66 <i>m</i>
6	121.6	6.49 <i>dd</i> (2, 7.8)	-Me	17.7	0.88 <i>d</i> (6.4)
α	114.8	6.02 <i>d</i> (15.6)			
β	144.9	7.20 <i>d</i> (15.6)			
C=O	166.8				
Malonic acid					
C=O	167.5				
-CH ₂ -	49.0*	3.43-3.48			
C=O	168.6				

^aTentative assigned (these values may be exchangeable with each other).Coupling constants (*J* in Hz) in parentheses.

spectra were measured and all signals corresponding to the protons in the four sugar moieties were assigned as shown in Table 2. In this experiment at room temperature, difference NOE spectra appeared all negative type and the signals of proton of the sugar unit are confirmed by the spin diffusion effect so called negative NOE relief method [9]. The signals of three anomeric protons appeared at $\delta 5.37$ (*d*, $J = 7.3$ Hz, Glc A), $\delta 5.35$ (*d*, $J = 7.3$ Hz, Glc B) and $\delta 5.16$ (*d*, $J = 7.8$ Hz, Glc C), and the vicinal coupling constants of these three glucoses were $J = 6.8$ – 11.7 Hz. Therefore, these glucoses must be β -D-glucopyranose form. The anomeric proton of rhamnose unit was observed at $\delta 4.69$ as a singlet. The ^1H NMR spectrum of vicinal coupling constants ($J_{3,4}$ and $J_{4,5} = 9.8$ Hz) and results of NOE experiments in the rhamnose moiety showed it to have the α -L-pyranorhamnose configuration. By analysis of DQF-COSY spectrum four protons of the methylene in Glc B and C were shifted to low magnetic field (Glc B, $\delta 4.29$ and 4.67 ; Glc C, $\delta 4.27$ and 4.54). Another proton (H-4) of rhamnose was also shifted downfield at $\delta 4.84$. Therefore, the OH-6 of Glc C and Glc B and also the OH-4 of rhamnose were confirmed to be acylated with malonic, *p*-coumaric and/or caffeic acids.

In order to determine the attachments and/or positions of the sugars and acyl units in **1**, difference NOE spectra were measured. By irradiation at H-1 of Glc A, a strong NOE was observed at H-4 of cyanidin. Therefore, Glc A is attached to OH-3 of cyanidin through a glucosidic bond. Similarly Glc C was deduced to be attached at the OH-5 of cyanidin through a glucosidic bond, because of the presence of NOEs between H-6 of cyanidin and H-1 of Glc C. Glc B was determined to be glucosylated at OH-3' of cyanidin, because of the presence of NOEs between H-2' of cyanidin and H-1 of Glc B. By irradiation on the H-1 of rhamnose moiety NOEs were observed at H-6a ($\delta 3.73$) and H-6b ($\delta 3.94$) of Glc A. This linkage of Glc A and rhamnose was determined by direct comparison with rutinose obtained from **1** by H_2O_2 degradation process [15]. To confirm the linkages between sugar and acid units, ^1H and ^{13}C signals of **1** were correlated by the HMQC spectrum as shown in

Table 2, and the ^1H - ^{13}C long range correlations of **1** were observed by the HMBC spectrum. The HMBC spectrum of **1** showed that the three long distance correlations between the carbonyl carbon ($\delta 166.8$) of caffeic acid and H-6 ($\delta 4.29$, 4.67) of Glc B, the carbonyl carbon ($\delta 167.5$) of malonic acid and H-6 ($\delta 4.27$) of Glc C, and the carbonyl carbon ($\delta 166.1$) of *p*-coumaric acid and H-4 ($\delta 4.84$) of rhamnose were confirmed, indicating that *p*-coumaric acid unit is attached to OH-4 of rhamnose, caffeic acid unit is attached to OH-6 of Glc B, and malonic acid unit is bonded to OH-6 of Glc C. Therefore, the structure of **1** is cyanidin 3-*O*-(6-*O*-(4-*O*-(*trans-p*-coumaryl)- α -L-rhamnopyranosyl)- β -D-glucopyranoside)-5-*O*-(6-*O*-malonyl- β -D-glucopyranoside)-3'-*O*-(6-*O*-*trans*-caffeyl- β -D-glucopyranoside), which is a new anthocyanin. Furthermore, careful detail difference NOE measurements of **1** showed several weak long distant NOEs as well as strong NOEs as shown in Table 3, indicating that caffeic acid unit and cyanidin (chromophore) were closely located in three dimensions. These results suggested that cyanidin and caffeic acid occupy a folding conformation as a stacking structure [16–20].

EXPERIMENTAL

Materials. Seeds of *Lobelia erinus* cv 'Rosamond' were obtained from Sakata Seed Co., Ltd and cultivated in the experimental farm of Minami-Kyushu University. Fresh petals were collected in spring and air-dried at 45° .

Extraction and isolation. The dried petals (30 g) were extracted with 5% HOAc at room temp. overnight. The filtered extract was adsorbed on Diaion HP-20 column, washed with 1% HOAc and then eluted with 5% HOAc in 70% MeOH. After concn, the eluate was fractionated over Sephadex LH-20 CC using HOAc–MeOH– H_2O (1:6:12) and HPLC. Prep. HPLC was performed on a Hitachi 6200 system, using a Inertsil ODS-2 (20 Φ \times 250 mm) column and HOAc solvent system. Pigment (ca 20 mg) was obtained as a red-purple powder.

Analysis. Characterization of **1** and **2**, obtained by alkaline deacylation of **1**, was carried out with UV-Vis, FAB-mass, and ^1H and ^{13}C NMR spectrometry, and

Table 3. Observed negative NOEs in *Lobelia anthocyanin 1* by irradiation of each proton

Irradiation (H)*	Observed NOE	
	Strong	Weak
Cy-H4	Glc A-H1, Glc A-H3, Glc A-H5	Caf-H α , Caf-H β , Caf-H2, Caf-H5, Caf-H6, Glc B-H6, Glc C-H1, Glc D-H1
Cy-H2'	Glc B-H1, Glc B-H3, Glc B-H5	Caf-H α , Caf-H β , Caf-H2, Caf-H5, Caf-H6, Glc A-H2
Cy-H6	Glc C-H1, Glc C-H3, Glc C-H5	
Caf-H α	Caf-H2, Caf-H6	
Caf-H2	Caf-H α , Caf-H β	Glc C-H1
Caf-H6	Caf-H α	Glc A-H1
pCou-H2, 6	pCou-H3, 5	pCou-H α , pCou-H β
pCou-H α	pCou-H2, 6	

*Cy, cyanidin; Caf, caffeic acid (II); pCou, *p*-coumaric acid (I); Glc, glucose.

also TLC and HPLC [7, 15]. ^1H and ^{13}C NMR spectra of anthocyanins were measured in 10% TFA-*d*-90% DMSO-*d*₆ by 400 and 100.5 MHz NMR (JNM GX-400 Jeol) with internal standard TMS. The other products from **1**, by acid hydrolysis, alkaline deacylation and H₂O₂ oxidation, were analysed as previously [7, 15]. Analytical HPLC was performed on a Inertsil ODS-2 column (4.6Φ × 250 mm) at 35° with a flow rate of 0.8 ml min⁻¹ monitoring at 520 nm. Solvent systems used were as follows; a linear gradient elution for 40 min from 25 to 85% B (1.5% H₃PO₃, 20% HOAc and 25% MeCN in H₂O) in Solvent A (1.5% H₃PO₄).

REFERENCES

1. Yoshitama, K. (1977) *Phytochemistry* **16**, 1857.
2. Kondo, T., Yamashiki, J., Kawahori, K. and Goto, T. (1989) *Tetrahedron Letters* **30**, 6055.
3. Saito, N., Osawa, Y. and Hayashi, K. (1972) *Bot. Mag. Tokyo* **85**, 105.
4. Saito, N., Abe, K., Honda, T., Timberlake, C. F. and Bridle, P. (1985) *Phytochemistry* **24**, 1583.
5. Lu, T. S., Saito, N., Yokoi, M., Shigihara, A. and Honda, T. (1992) *Phytochemistry* **31**, 289.
6. Saito, N. and Harborne, J. B. (1992) *Phytochemistry* **31**, 3009.
7. Saito, N., Toki, K., Uesato, K., Shigihara, A. and Honda, T. (1994) *Phytochemistry* **37**, 245.
8. Toki, K., Saito, N. and Honda, T. (1991) *Phytochemistry* **30**, 3769.
9. Kondo, T., Kawai, T., Tamura, H. and Goto, T. (1987) *Tetrahedron Letters* **28**, 2273.
10. Lu, T. S., Saito, N., Yokoi, M., Shigihara, A. and Honda, T. (1991) *Phytochemistry* **39**, 2387.
11. Piantini, U., Sorensen, O. W. and Ernst, R. R. (1982) *J. Am. Chem. Soc.* **104**, 6800.
12. Bax, A. and Subramanian, S. (1986) *J. Magn. Reson.* **67**, 565.
13. Bax, A. and Summers, M. F. (1986) *J. Am. Chem. Soc.* **108**, 2093.
14. Doddrell, D. M., Pegg, D. T. and Bendall, M. R. (1982) *J. Magn. Reson.* **48**, 323.
15. Harborne, J. B. (1984) *Phytochemical Methods*, 2nd edn. Chapman & Hall, London.
16. Brouillard, R. (1981) *Phytochemistry* **20**, 143.
17. Goto, T. (1987) *Prog. Chem. Org. Nat. Prod.* **52**, 113.
18. Goto, T. and Kondo, T. (1991) *Angew. Chem. Int. Edn Engl.* **30**, 17.
19. Dangles, O., Saito, N. and Brouillard, R. (1993) *Phytochemistry* **34**, 119.
20. Brouillard, R. and Dangles, O. (1994) *The Flavonoids. Advances in Research since 1986* (Harbone, J. B., ed.), pp. 565–588. Chapman & Hall, London.