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Anthocyanins from flowers of *Lilium* (Liliaceae)

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Abstract

The perianth segments of 10 cultivars including Asiatic and Oriental hybrids and one species of *Lilium*, were investigated by HPLC for their content of anthocyanins. The investigation revealed the presence of one new and one known anthocyanin. The novel anthocyanin, cyanidin 3-*O*-*β*-rutinoside-7-*O*-*β*-glucoside and cyanidin 3-*O*-*β*-rutinoside were both isolated from the red flowers of *Lilium* 'Holean'. Within both Asiatic and Oriental hybrids, cultivars with or without the novel anthocyanin were found, whereas the known anthocyanin was always present in none-white genotypes. The structural determination of the compounds was achieved by 1D and 2D NMR techniques and other spectral evidence.

Keywords: Lilium; Liliaceae; Flower pigments; Anthocyanins; HPLC

1. Introduction

Lilies are popular ornamental plants with red, pink, orange, yellow or white flowers, often with dark red spots. The lilies are grouped in sections. Two major sections are the Asiatic and Oriental hybrids, both interspecific hybrids, including different species. Chromatographic studies of anthocyanins in flowers of *Lilium* showed the presence of two aglycones, cyanidin and petunidin, but attached sugars were not identified (Lawrence, Price, Robinson, & Robinson, 1938). However, the 3-*O*-*β*-rutinosyl-7-*O*-*β*-glucosyl moiety has earlier been identified in another genus as a part of a delphinidin derivative (Brandt, Kondo, Aoki, & Goto, 1993). Anthocyanin content in extracts of freeze-dried perianth segments of selected cultivars and one species of *Lilium* were measured by analytical HPLC. The red colours and spots are due to the anthocyanins, cyanidin 3-*O*-*β*-rutinoside being the major component and the novel compound, cyanidin 3-*O*-*β*-rutinoside-7-*O*-*β*-glucoside, being present in small amounts or non-detectable. Petunidin glycosides were not found.

2. Results and discussion

HPLC chromatograms detected in the visible spectral region of the crude anthocyanin extract from perianth segments, occasionally showed one minor component (**1**) while the major anthocyanin (**2**) was always present (Table 1), with the exception of the white flowered *L. longiflorum*, that did not contain anthocyanins (Table 1). The anthocyanins were isolated by column chromatography on Amberlite XAD-7 with subsequent preparative HPLC.

Compound **1** showed λ_{max} at 280 and 525 nm in 0.1% HCl–MeOH and FAB-MS established $[M]^+$ at *m*/*z* 757, supporting the molecular formula $C_{33}O_{20}H_{41}{}^+$ with fragments corresponding to cyanidin 3-rutinoside (*m*/*z* 595), cyanidin 3-glucoside (*m*/*z* 449) and cyanidin (*m*/*z* 287). The proton signals of cyanidin, the aglycone of both **1** and **2**, were assigned (Table 2) using information regarding coupling constants and chemical shifts. The assignment of the sugar protons was carried out by 1D-HOHAHA spectra and ^{1}H–^{1}H-COSY (Table 2) and the position of the glucosidic linkages determined by NOE difference spectra (Goto & Kondo, 1991). The chemical shifts of the two glucosidic anomeric protons of **1** appeared at δ 5.34 (*d*, J=8.0 Hz, H-1″) and δ 5.22 (*d*, J=7.3 Hz, H-1‴), while the anomeric proton of rhamnosyl (H-1‴) was observed at δ 4.66 (*br s*). By irradiation of H-1‴ of rhamnosyl (δ 4.66) NOE was observed to H-6″ of glucosyl (δ 4.07; 3.75) indicating rutinose. The linkage at 3-OH

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Table 1
Distribution of anthocyanins in flower extracts from cultivars and one species of *Lilium*

Cultivars/species	Approximate concentrations (μM)		Flower colour
	Cy-3-Ru-7-Glu	Cy-3-Ru	
Asiatic hybrids			
L. 'Holean'	20	130	lavender red
L. 'Red Carpet'	5	80	lavender red
L. 'Hollandicum'	5	40	red
L. 'Monte Negro'	bd	80	lavender red
L. 'Montreux'	bd	40	red
L. 'Avignon'	bd	50	reddish orange
L. 'Compas'	bd	10	orange with spots
L. 'Las Vegas'	bd	10	yellow with spots
Oriental hybrids			
L. 'Star Gazer'	5	30	white with red patches
L. 'Le Reve'	bd	10	pink
L. longiflorum	bd	bd	white

Cy-3-Ru-7-Glu: cyanidin 3-*O*-*β*-rutinoside-7-O-*β*-*D*-glucoside and Cy-3-Ru: cyanidin 3-*O*-*β*-rutinoside. Concentrations were calculated from the HPLC chromatograms by using the extinction coefficient of cyanidin 3-glucoside $\log \varepsilon = 4.47$ (Boyles & Wrolstad, 1993).bd: below detection limit.

was determined from the presence of a strong negative NOE at H-4 (δ 8.87) of the cyanidin nucleus by irradiation of the anomeric proton (H-1″) (δ 5.34). Negative NOEs appeared at H-6 (δ 6.87) and H-8 (δ 7.21) by irradiation of (H-1‴″) (δ 5.22). Thus, **1** is cyanidin 3-*O*-*β*-rutinoside-7-*O*-*β*-D-glucoside (Fig. 1).

The FAB-MS spectrum of **2** showed $[M]^+$ at *m*/*z* 595, in good agreement with the mass calculated for $C_{33}O_{20}H_{41}^+$ with same fragmentation as **1** corresponding to cyanidin 3-glucoside and the aglycone. A strong negative NOE was observed at H-4 (δ 8.91) of the nucleus by irradiation of H-1″ of glucosyl (δ 5.27) indicating that this sugar moiety is linked to 3-OH of cyanidin. Furthermore there was a weak negative NOE between H-6 of glucosyl (δ 4.07; 3.59) and H-1‴ of rhamnosyl (δ 4.65) indicating a 1→6 linkage (Fig. 1). The spectral data of **2** was found to be in accordance with data found in literature of cyanidin 3-*O*-*β*-rutinoside (Saito, Yokoi, Ogawa, Kamijo, & Honda, 1988; Nørbæk, Christensen, Bojesen, & Brandt, 1996).

Table 2
^{1}H-NMR spectral data of anthocyanins (**1**, **2**) from *Lilium* (in CD_3OD, containing 10% TFA-*d*)

H no.	Cyanidin (ppm)	H no.	3-Glucoside (H″) (ppm)	Rhamnoside (H‴) (ppm)	7-Glucoside (H⁗) (ppm)
1					
4	8.87 *br s*	1	5.34 *d* (8.0)	4.66 *br s*	5.22 *d* (7.3)
6	6.87 *d* (2.2)	2	3.70 *t* (9.0)	3.78 *m*	3.60 *t* (9.6)
8	7.21 *d* (2.2)	3	3.59 *t* (8.4)	3.61 *dd* (3.6, 10.2)	3.57 *t* (9.0)
2′	8.02 *d* (2.2)	4	3.43 *t* (8.4)	3.30 *t* (9.0)	3.44 *t* (8.4)
5′	6.97 *d* (8.8)	5	3.60 *m*	3.53 *m*	3.66 *m*
6′	8.30 *dd* (3.0, 9.0)	6	3.75 *m*	1.15 *d* (6.6)	3.76 *dd* (4.8, 12.0)
			4.07 *m*		4.00 *m*
2					
4	8.91 *br s*	1	5.27 *d* (8.4)	4.65 *br s*	
6	6.67 *d* (1.8)	2	3.68 *t* (9.0)	3.78 *m*	
8	6.88 *d* (1.8)	3	3.58 *t* (9.0)	3.60 *dd* (3.6, 10.2)	
2′	8.01 *d* (2.4)	4	3.43 *t* (9.6)	3.30 *t* (9.0)	
5′	7.01 *d* (9.0)	5	3.73 *m*	3.53 *m*	
6′	8.24 *dd* (3.0, 9.0)	6	3.59 *dd* (6.6, 11.4)	1.15 *d* (6.0)	
			4.07 *m*		

Coupling constants *J* (in Hz) in parentheses.

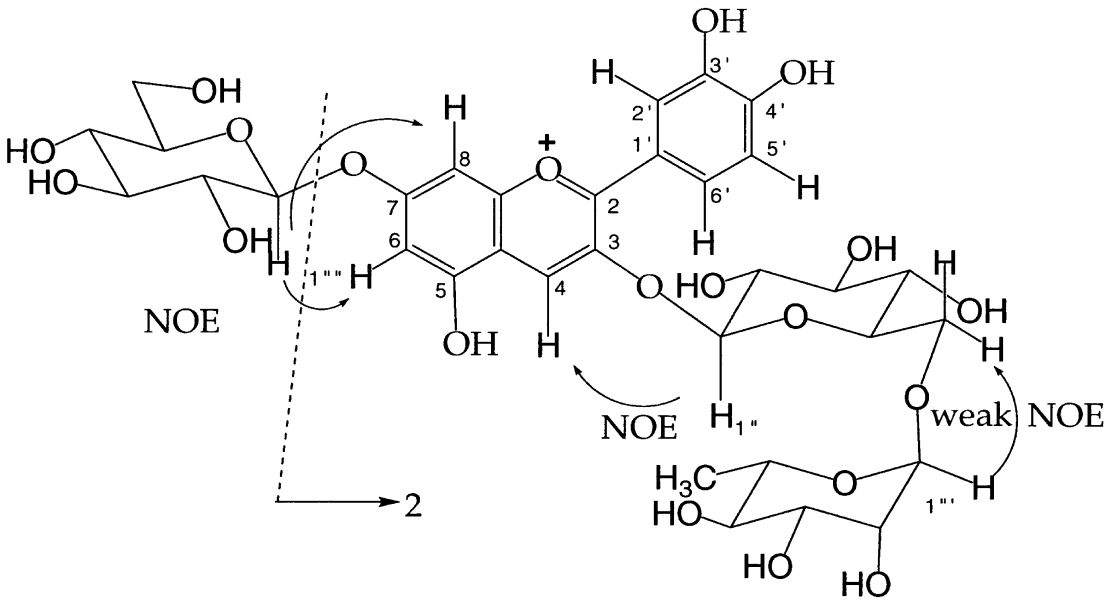

Fig. 1. By irradiation of H-1″ of the glucosyl of **1** in CD_3OD, containing 10% TFA-d, a strong negative NOE was observed at H-4 of the nucleus and strong negative NOEs of H-6 and H-8 of the nucleus appeared by irradiation of H-1‴″ of the glucosyl and H-1‴ of the rhamnosyl. Strong NOE was observed at H-4 of the nucleus of **2** by irradiation of H-1″ of the glucosyl in CD_3OD, containing 10% TFA-d. A negative NOE was also observed between H-6″ of the glucosyl and H-1‴ of the rhamnosyl.

The genotypes investigated include Asiatic and Oriental hybrids as well as one species, *L. longiflorum* clone (Table 1). The cultivars cover the shade of flower colours found in *Lilium* (Table 1). Flowers containing high amounts of anthocyanin have a red hue (Table 1) while some of the flowers have yellow or orange colours due to carotenoids (Mummery & Valadon, 1974; Valadon & Mummery, 1977; Toth & Szabolcs, 1981; Marki-Fischer & Eugster, 1985).

Among the investigated Asiatic hybrids *L.* 'Holean' produces significant amounts of cyanidin 3-*O*-*β*-rutinoside-7-*O*-*β*-D-glucoside. Both anthocyanins were also detected in *L.* 'Hollandicum' and *L.* 'Red Carpet'. *L.* 'Hollandicum' is believed to be a common ancestor to the Asiatic hybrids (Synge, 1980). In other Asiatic cultivars, like *L.* 'Monte Negro', *L.* 'Montreux', *L.* 'Avignon', *L.* 'Compas' and *L.* 'Las Vegas' the new anthocyanin was not present or in amounts below the detection limit. The Oriental *Lilium* 'Star Gazer' also contained cyanidin 3-*O*-*β*-rutinoside-7-*O*-*β*-D-glucoside, whereas in *L.* 'Le Reve' the content was below the detection limit. The ability to produce the novel anthocyanin is thus present in several distantly related species. However, all cultivars may posses the enzymes involved in 7-glycosylation but the enzyme activity in some genotypes may be to low for cyanidin 3-*O*-*β*-rutinoside-7-*O*-*β*-D-glucoside to be detected.

3. Experimental

3.1. Plant material

Cutflowers of eight *Lilium* cultivars and one species were purchased from GASA, Odense (8 Asiatic, 2 Oriental cultivars and *L. longiflorum*. *L.* 'Red Carpet' and *L.* 'Hollandicum' were grown at the Department of Botany, Dendrology and Forest Genetics, The Royal Veterinary and Agricultural University, Copenhagen. The identity was verified by Professor N. Jacobsen, Royal Veterinary and Agricultural University, Copenhagen. The investigated cultivars cover the colour range present in *Lilium*.

3.2. Isolation and quantification of anthocyanins

Freeze-dried perianth segments of *Lilium* 'Holean' (80 g) was extracted with 50% aq. CH_3CN containing 0.5% TFA at room temp. for 1 h. The conc. extract was adsorbed on an Amberlite XAD-7 column, washed with 0.5% TFA aq. soln and then eluted stepwise from 5 to

14% aq. CH_3CN containing 0.5% TFA. The pigments were further purified by prep. ODS-HPLC (20ϕ × 250 mm, Develosil ODS-HG-5, Nomura Chemicals) in the same solvent system; flow rate of 7 ml min^{-1}. From *Lilium* 'Holean' 10 mg of **1** and 40 mg of **2** were stored at −80°C as pure TFA salts.

For analytical HPLC about 1 g of the freeze-dried perianth segments were extracted with 13 ml 50% aq. CH_3CN containing 3.0% TFA and after filtration the extracts were analyzed by ODS-HPLC (4.6ϕ × 250 mm, Develosil ODS-HG-5, Nomura Chemicals) at 40°C, detection on a 3D diode-array detector at 260–530 nm. Two gradient systems were used to verify the presence of the anthocyanins. One was, a linear gradient elution with a flow rate of 1 ml min^{-1} for 30 min using from 0 to 30% aq. CH_3CN containing 0.5% TFA. Retention times for the anthocyanins were 6.1 (**1**) and 14.4 min (**2**), respectively. The second elution profile was as follows: 0 min, 16% B; 3 min, 38% B; 10 min, 44% B; 20 min, 50% B; 25 min, 67% B and 40–50 min, 100% B using solvent A (H_2O–TFA, 99:1) and solvent B (CH_3CN–H_2O–TFA, 60:140:1), with a flowrate of 1.5 ml min^{-1}. The anthocyanins were eluted at 9.5 (**1**) and 19.8 min (**2**), respectively. The limit for detection was 1.2 μM for each anthocyanin.

3.3. Spectral analysis

UV–Vis spectra were measured in MeOH containing 0.1% HCl. FAB-MS spectra were obtained in a positive mode using glycerol (1 drop of HCl aq. was added) as a matrix of the isolated anthocyanins. ^1H NMR and other NMR-spectra were measured in CD_3OD containing 10% TFA-d by 600 MHz (JNM alpha 600, JEOL) with internal standard CD_2HOD (3.326 ppm). 1D HOHAHA and 2D spectra were obtained using a pulse sequence supplied from JEOL.

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