

CYANIDIN 3-*O*-(6''-SUCCINYL- β -GLUCOPYRANOSIDE) AND OTHER ANTHOCYANINS FROM *PHRAGMITES AUSTRALIS*

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(Received 17 November 1997)

Key Word Index—*Phragmites australis*; Gramineae; acylated anthocyanins; succinic acid; cyanidin 3-*O*-(6''-*O*-succinyl- β -glucopyranoside); cyanidin 3-*O*-(6''-*O*-malonyl- β -glucopyranoside); ^{13}C NMR.

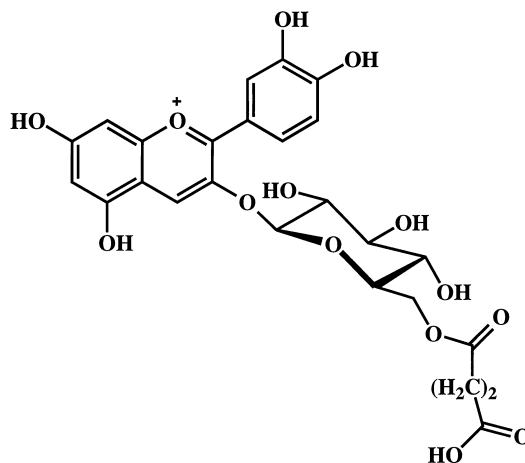
Abstract—Three anthocyanins were isolated from the flowering tops of *Phragmites australis*. Their structures were elucidated by means of homo- and heteronuclear two-dimensional NMR techniques and mass spectrometry to be cyanidin 3-*O*-(6''-*O*-succinyl- β -glucopyranoside) (2%), cyanidin 3-*O*-(6''-*O*-malonyl- β -glucopyranoside) (64%) and cyanidin 3-*O*- β -glucopyranoside (34%). The former pigment, which is a new anthocyanin, represents the first identification of an anthocyanin acylated with succinic acid found outside the dicotyledon genus *Centaurea* (Compositae). © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Recent work has shown that anthocyanins acylated with aliphatic acids have a rather widespread occurrence [1]. Most reports include malonic or acetic acids as the acyl moieties. Similar acylation with oxalic, malic or succinic acid has a very restricted distribution, respectively in the orchid family [2], the genera *Dianthus* (Caryophyllaceae) [3] and *Centaurea* (Compositae) [4]. While continuing our investigation of acylated anthocyanins in plants [5–12], we here report on an anthocyanin acylated with succinic acid in addition to two cyanidin derivatives in flowers of the grass *Phragmites australis*. In an electrophoretic survey of floral tissue this species has previously been proved to be positive with respect to the presence of zwitterionic anthocyanins [13], however, without identification of the anthocyanins.

RESULTS AND DISCUSSION

The HPLC chromatogram of the acidified methanolic extract from flowers of *Phragmites australis* detected in the visible region, showed mainly three anthocyanins, **1** (34%), **2** (64%) and **3** (2%). Pigments **1–3** were purified by partition against ethyl acetate followed by Amberlite XAD-7 column chro-



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matography and finally Sephadex LH-20 gel filtration and preparative HPLC. The pure anthocyanins were checked for homogeneity by analytical HPLC and TLC (Table 1).

The UV-vis spectrum of pigments **1–3**, taken during on-line HPLC showed in all cases visible maxima at 520 nm with A_{440}/A_{520} of 29%, indicating the presence of an anthocyanin 3-glycoside with a cyanidin or peonidin nucleus. In the UV-region of 290–340 nm there was no indication of acylation with an aromatic

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Table 1. Chromatographical and spectral data for the anthocyanins in flowers of *Phragmites australis*, cyanidin 3-*O*- β -glucopyranoside (**1**), cyanidin 3-*O*-(6''-*O*-malonyl- β -glucopyranoside) (**2**) and cyanidin 3-*O*-(6''-*O*-succinyl- β -glucopyranoside) (**3**)

Compound	MS (<i>m/z</i>)	TLC (<i>R_f</i>)		On-Line HPLC Vis.max. (nm)	<i>A</i> ₄₄₀ / <i>A</i> _{max} (%)	<i>R_t</i> (min)
		FHW	BAW			
1	451*	0.30	0.26	520	29.1	14.10
2	535	0.39	0.33	520	29.1	16.54
3	549	0.49	0.69	520	29.1	18.01

* Molecular ion plus deuterium in the aglycone 6- and 8- positions.

acid for **1–3**. Acid hydrolysis of pigments **1–3** produced in all instances cyanidin and glucose.

The assignment of the ¹H and ¹³C NMR shift values (Tables 2, 3) based on one- and two-dimensional NMR experiments identified **1** as cyanidin 3-*O*- β -glucopyranoside [14]. Co-chromatography (HPLC) with authentic anthocyanin and a molecular ion of *m/z* 451 in the mass spectrum confirmed the identity of **1**. After storage of pigment **1** in the NMR solvent (acidified deuterated methanol) the molecular ion in the mass spectrum showed two mass unit higher than the normal value (*m/z* 449) because of deuterium exchange of the protons in the 6- and 8-positions of the aglycone [15].

The relative long HPLC retention of **2**, the similarity between the UV-vis spectra of **1** and **2** (Table 1) and the fact that **2** turned into **1** after alkaline

Table 2. ¹H NMR spectral data for cyanidin 3-glucoside (**1**), cyanidin 3-(6''-malonylglucoside) (**2**) and cyanidin 3-(6''-succinylglucoside) (**3**) in CD₃OD–CF₃COOD (95:5, v/v) at 25°

	1 δ (ppm)	2 δ (ppm)	3 δ (ppm)
Cyanidin			
4	9.06	8.98	9.02
6	6.75	6.75	6.75
8	6.94	6.94	6.94
2'	8.10	8.08	8.11
5'	7.11	7.08	7.10
6'	8.31	8.31	8.34
3- <i>O</i> - β -glucopyranoside			
1''	5.37	5.33	5.37
2''	3.77	3.76	3.77
3''	3.63	3.65	3.64
4''	3.52	3.52	3.51
5''	3.65	3.88	3.89
6''A	4.02	4.66	4.64
6''B	3.79	4.39	4.38
6''-malonyl			
CH ₂		3.44	
6''-succinyl			
CH ₂			4.05
CH ₂			3.51

Table 3. ¹³C NMR spectral data for cyanidin 3-glucoside (**1**), cyanidin 3-(6''-malonylglucoside) (**2**) and cyanidin 3-(6''-succinylglucoside) (**3**) in CD₃OD–CF₃COOD (95:5, v/v) at 25°

	1 δ (ppm)	2 δ (ppm)	3 δ (ppm)	SEFT*
Cyanidin				
2	164.31	164.31	164.62	↑
3	145.62	145.52	145.52	↑
4	136.78	136.78	136.88	↓
5	159.13†	159.13†	159.08†	↑
6	103.50	103.55	103.46	↓
7	170.57	170.57	170.46	↑
8	95.17	95.32	95.27	↓
9	157.73†	157.13†	157.75†	↑
10	113.38	113.27	§	↑
1'	121.21	121.21	121.22	↑
2'	118.47	118.47	118.47	↑
3'	147.42	147.42	147.49	↑
4'	155.77	155.87	155.94	↑
5'	117.46	116.84	117.41	↓
6'	128.27	128.46	128.51	↓
3- <i>O</i> - β -glucopyranoside				
1''	103.76	103.64	103.66	↓
2''	74.76	74.66	74.68	↓
3''	78.10	77.93	77.90	↓
4''	71.08	71.33	71.30	↓
5''	78.75	75.96	75.95	↓
6''	62.39	65.49	65.48	↑
6''-malonyl¶				
1'''		168.70‡		↑
3'''		169.07‡		↑
6''-succinyl¶				
1'''			170.50‡	↓
4'''			168.21‡	↑

* SEFT = coupling modulated spin echo NMR experiment C_q and CH₂, †: CH and CH₃, ↓.

‡‡ Assignments with the same superscript may be reversed.

§ Hidden under solvent peak.

¶ The resonances in the SEFT of the CH₂-units of the acyl groups are absent due to deuterium effects.

hydrolysis, indicated **2** to be a derivative of **1** acylated with an aliphatic acid. The downfield shifts of C-6'' (δ 65.49) and H-6''A/H-6''B (δ 4.66 and 4.39, respec-

tively) revealed the acyl group to be connected to the sugar 6''-position. The proton signal at δ 3.44 integrating for two protons, and the two ^{13}C carbonyl signals at δ 168.70 and 169.07 identified the acyl group as malonic acid. Co-chromatography (HPLC) with authentic pigment and a molecular ion $[\text{M}]^+$ of m/z 535 found in the MS spectrum, confirmed the identity of **2** as cyanidin 3-*O*- β -(6''-malonylglucopyranoside).

The long HPLC retention time of **3**, the high R_f -values in both TLC solvents (BAW and FHW), the similarity between the UV vis spectra of **1** and **3** (Table 1), the similarities between the ^1H and ^{13}C shift values (Tables 2, 3) and the fact that **3** turned into **1** after alkaline hydrolysis, indicated **3** to be a derivative of **1** acylated with an aliphatic acid. The number of carbon atoms of **3** was determined by a coupling modulated spin-echo (SEFT) NMR experiment, which also gave the number of protons attached on each carbon. The spectral region between δ 80 and 60 showed five resonances which together with the anomeric carbon resonance (δ 103.66), the corresponding sugar protons assigned by the DQF-COSY and HSC NMR experiments (Tables, 2, 3), and the anomeric ^1H - ^1H coupling constant of 7.8 Hz were in accordance with a substituted β -D-glucopyranoside [9]. The downfield shifts of C-6'' (δ 65.48), H-6''A (δ 4.64) and H-6''B (δ 4.38) revealed the connection point between the acyl group and the sugar 6'' position. The acyl group was indicated as succinic acid by the 2 CH_2 multiplets at δ 4.05 and 3.51 in the ^1H spectrum and 2 carbonyl signals at δ 170.50 and 168.21 in the ^{13}C SEFT spectrum. Due to rapid exchange of the succinyl CH_2 protons with deuterium, none of the corresponding ^{13}C signals were detected. The mass spectrum of pigment **3** stored in acidified deuterated NMR solvent showed the molecular ion as a cluster of signals at m/z 551, 552, 553, 554, 555, 556, and 557, which was in accordance with a molecular ion with two to seven protons exchanged with deuterium (H-6 and H-8 on the aglycone and up to five protons on the succinyl moiety). The corresponding molecular ion (m/z 451) of cyanidin 3-glucoside (**1**) which was stored in the same deuterated solvent, revealed two exchangeable protons, namely H-6 and H-8 [15]. A molecular ion $[\text{M}^+]$ of m/z 549 measured on a freshly prepared sample of **3** in a non-deuterated solvent confirmed the identity of the novel pigment as cyanidin 3-*O*- β -(6''-succinylglucopyranoside).

The only other reported source for an anthocyanin acylated with succinic acid is some species in the genus *Centaurea* (Compositae), which contain the 3-(6''-succinylglucoside)-5-glucosides of cyanidin and pelargonidin [4]. The former pigment has later been found to be part of the self-assembled supramolecule protocyanin [16]. The identification of both cyanidin 3-*O*-(6''-*O*-succinyl- β -glucopyranoside) and cyanidin 3-*O*-(6''-*O*-malonyl- β -glucopyranoside) in *Phragmites australis* represents the first report of anthocyanins containing different aliphatic acyl moieties from the same species.

EXPERIMENTAL

Extraction and separation

Flowering tops of *Phragmites australis* Trin. ex Steudel [*P. communis* Trin.] were collected in August 1996 in Lake Røyrvannet, Haugesund, Norway, and a voucher specimen has been deposited in BG. The flowering tops were cut into pieces with a pair of scissors and extracted with 1% TFA in MeOH at 5°. The filtered extract was concd under red. pres., purified by partition against ethyl acetate and applied to an Amberlite XAD-7 column [17]. The pigments were separated by Sephadex LH-20 column chromatography using MeOH-H₂O-TFA (99:100:1) as eluent. Pigment **3** was further purified by prep. HPLC on an Econosil C18 column (25 \times 2.2 cm, 10 μm). Two solvents were used for elution: HCO₂H-H₂O (1:9) (A) and HCO₂H-H₂O-MeOH (1:4:5) (B). The elution profile consisted of isocratic elution (90% A, 10% B) in 4 min, linear gradient from 10% B to 100% B during the next 17 min, isocratic elution (100% B) in 12 min followed by linear gradient from 100% B to 10% B over 1 min. The flow rate was 4.0 ml min⁻¹. Acid and alkaline hydrolysis were performed according to standard procedures [18].

Analytical chromatography

TLC was carried out on microcrystalline cellulose (F1440, Schleicher and Schüll) with the solvents BAW (1-butanol-HOAc-H₂O; 4:1:5 v/v, upper phase) and FHW (HCO₂H-conc. HCl-H₂O; 1:1:2 v/v). Analyt. HPLC was performed with an ODS-Hypersil column (20 \times 0.5 cm, 5 μm) using isocratic elution (90% A, 10% B) in 4 min, linear gradient from 10% B to 100% B during the next 17 min, isocratic elution (100% B) in 4 min followed by linear gradient from 100% B to 10% B over 1 min. The flow rate was 1.0 ml min⁻¹, and aliquots of 15 μl were injected.

Spectroscopy

UV-vis absorption spectra were recorded on-line during HPLC analysis using a photodiode array detector (HP 1050, Hewlett-Packard). Spectral measurements were made over the wavelength range 240–600 nm in steps of 2 nm. The relative quantitative data were based on the average values of the absorptions on every second nm between 500 and 540 nm, without taking to account the different molar absorption coefficients of the pigments. The NMR experiments on pigment **3** were obtained at 600.13 MHz and 150.92 MHz for ^1H and ^{13}C respectively, on a Bruker DRX-600 instrument at 25°, and for pigment **1–2** at 400.13 MHz and 100.62 MHz on an AM Bruker 400 MHz instrument. The 1D ^1H and the 2D homonuclear correlation experiment (DQF-COSY) were performed on a 5 mm multinuclear TXI-probe. The heteronuclear spin-echo experiments (SEFT) and the 2D het-

eronuclear shift correlation (HSC) experiments were obtained on a 5 mm BBO probe. The deuteriomethyl ^{13}C signal and the residual ^1H signal of the solvent ($\text{CF}_3\text{CO}_2\text{D}-\text{CD}_3\text{OD}$; 5:95, v/v) were used as secondary references (δ 49.0 and δ 3.4) from TMS, respectively. The mass spectra were obtained on a Quattro II MS/MS (Micromass, U.K.) by flow injection into the electrospray sources. The instrument was operated in the positive ion mode and calibrated by NaI. The mobile phase carrier was a methanol–water (50:50) mixture containing 0.1% formic acid. The carrier was pumped into the source at a flow rate of $100\ \mu\text{l min}^{-1}$. Data acquisition was obtained by scanning from 50–1000 Da in 3 s scans. The samples were dissolved in 3% formic acid (in methanol) prior to analysis.

Acknowledgements—The authors thank Mr Åsmund Larsen (Nycomed) for running the MS spectra. T.F. gratefully acknowledges the Norwegian Research Council for a fellowship.

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