PERGAMON

Phytochemistry 60 (2002) 357–359

PHYTOCHEMISTRY

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Anthocyanins from flowers of *Cichorium intybus*

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Received 14 November 2001; received in revised form 4 February 2002

Abstract

From the blue perianth segments of *Cichorium intybus* we isolated four anthocyanins. The pigments were identified as delphinidin 3,5-*di*-*O*-(6-*O*-malonyl-β-D-glucoside) and delphinidin 3-*O*-(6-*O*-malonyl-β-D-glucoside)-5-*O*-β-D-glucoside and the known compounds were delphinidin 3-*O*-β-D-glucoside-5-*O*-(6-*O*-malonyl-β-D-glucoside) and delphinidin 3,5-*di*-*O*-β-D-glucoside. In addition 3-*O*-*p*-coumaroyl quinic acid has been identified. © 2002 Published by Elsevier Science Ltd.

Keywords: *Cichorium intybus*; Compositae; Flower pigments; Malonated anthocyanins; Quinic acid derivative; FABMS; ^{1}H NMR; DIF NOE

1. Introduction

Chicory, a typical Mediterranean plant indigenous to Europe, Western Asia, Egypt and North America, varies in perianth colour from white, red to blue and the flowering period is from June to September (Fernald, 1950; Clapham et al., 1962). From λ_{max} measured on the flower extract it has been suggested that delphinidin glycosides are present, but the pigments were not further identified (Proctor and Creasy, 1969). Subsequently, from blue perianth segments of *Cichorium intybus*, the presence of delphinidin 3,5-*di*-*O*-malonylglucoside has been suggested by FAB–MS measurement but the positions of sugars and malonic acid moieties has not been clarified (Takeda et al., 1986). From a leaf extract, cyanidin 3-*O*-(6-*O*-malonyl-β-D-glucoside) (Timberlake et al., 1971; Bridle et al., 1984), quercetin 3-*O*-β-D-glucoside (Saleh et al., 1975), several coumarin and cinnamic acid derivatives such as chicoric acid (Scarpati and Oriente, 1958), caffeic acid and chlorogenic acid (Trease, 1969) have been found. However, in the flowers no other compound with UV-absorption besides anthocyanins have yet been studied.

In this paper we report on the identification of four anthocyanins and one quinic acid derivative from blue perianth segments of *C. intybus*.

2. Results and discussion

Blue perianth segments of *C. intybus* were extracted with aqueous acetonitrile containing 0.5% TFA. The HPLC chromatogram showed four peaks of anthocyanin and one peak of UV-absorbing substance. The anthocyanins were isolated by column chromatography on Amberlite XAD-7 with subsequent preparative HPLC. Pigment **1**, **2**, **3** and **4** were obtained as dark red TFA salts.

The HR FAB–MS spectrum of **1** showed a molecular ion at m/z 799.1561, in good agreement with the mass calculated for $C_{33}O_{23}H_{35}^{+}$ to be comprised of delphinidin, and two malonylhexoses and that of **2** gave a molecular ion peak at m/z 713.1566 ($C_{33}O_{20}H_{33}^{+}$) corresponding to monomalonyl dihexosyl delphinidin. Partial hydrolysis of **1** gave the products corresponding to **2**, **3** and **4** (Scheme), and after 60 min hydrolysis the remaining products were **4** and delphinidin. These results strongly suggested that **1** is monomalonated compound of **3** and **2** is an isomer of **3**.

Analysis of the ^{1}H NMR spectrum of **1** agreed well with the MS data of the presence of delphinidin and two malonylated hexosyl residues (Table 1). The assignment of the two hexoses was done by ^{1}H HOHAHA spectra, ^{1}H COSY and ^{1}H TCOSY. All vicinal coupling constants (J) of both sugars were 7.8–9.8 Hz, including two anomeric protons at δ 5.44 (d, $J=7.8$ Hz, glucoside A) and δ 5.14 (d, $J=7.8$ Hz, glucoside B). Therefore, both sugar units were determined to be β-D-glucopyranoside. The 6-methylene protons of glucoside A and B are lowfield

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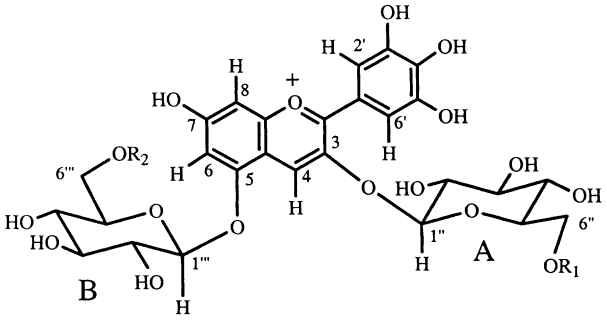
0031-9422/02/$ - see front matter © 2002 Published by Elsevier Science Ltd.
PII: S0031-9422(02)00055-9

Table 1
^{1}H NMR spectral data of two novel acylated anthocyanins from *Cichorium intybus* (in CD_3OD, containing 10% TFA-*d*)

	1	2[a]
Delphinidin		
4	8.56 *s*	8.67 *s*
6	6.71 *d* (1.8)	6.84 *d* (1.8)
8	6.69 *br d*	6.80 *br d*
2′	7.43 *s*	7.57 *s*
6′	7.43 *s*	7.57 *s*
Glucoside A		
1″	5.32 *d* (7.8)	5.25 *d* (7.8)
2″	3.55 *dd* (7.8, 9.0)	3.56 *dd* (7.8, 9.0)
3″	3.43 *t* (9.0)	3.39 *t* (9.0)
4″	3.24 *t* (9.0)	3.24 *t* (9.0)
5″	3.72 *ddd* (2.2,7.7,9.0)	3.71 *ddd* (2.4,7.0,9.0)
6a″	4.24 *dd* (2.2,12.0)	4.25 *dd* (2.4, 12.0)
6b″	4.14 *dd* (7.7, 12.0)	4.15 *dd* (7.0, 12.0)
Glucoside B		
1‴	5.02 *d* (7.8)	4.98 *d* (8.4)
2‴	3.52 *dd* (7.8, 9.0)	3.49 *dd* (8.4, 9.0)
3‴	3.40 *t* (9.0)	3.35 *t* (9.0)
4‴	3.29 *t* (9.0)	3.25 *t* (9.0)
5‴	3.62 *ddd* (1.8, 7.3, 9.0)	3.38 *ddd* (2.2, 6.2, 9.0)
6a‴	4.32 *dd* (1.8, 12.0)	3.75 *dd* (2.2, 12.0)
6b‴	4.16 *dd* (7.3, 12.0)	3.54 *dd* (6.2, 12.0)

Coupling constants *J* (in Hz) in parentheses.
The methylene protons of malonic acid disappeared by displacement of deuterium.

[a] By irradiation of H-1″ of glucoside A at 0 °C in CD_3OD containing 10% TFA-*d*, a strong negative NOE was observed at H-4 of delphinidin. By irradiation of H-1‴ of glucoside B, a strong negative NOE was observed at H-6 of the nucleus. A weak negative NOE was also observed between the same anomeric proton and H-4.



	R_1	R_2
1	malonyl	malonyl
2	malonyl	H
3	H	malonyl
4	H	H

Scheme 1.

shifted by ca. 0.6 ppm more than **4** (Nørbæk and Kondo, 1999). Therefore, the malonyl groups were esterified to the 6-OH of each glucoside (Kondo et al., 1989, 1990). The positions of the glucosidic linkages were determined by ^{1}H NOESY. NOE was observed between H-4 (δ 8.68) and the anomeric proton (H-1″). Also, NOE between H-6 (δ 6.83, *d*, $J = 1.8$ Hz) and H-1‴ (glucoside B) was observed, but no NOE was found between H-8 (δ 6.81, *br d*) and H-1‴. Thus, **1** is delphinidin 3,5-*di*-*O*-(6-*O*-malonyl-β-D-glucoside) (Scheme 1).

Using the same NMR experiments **2** gave the similar NMR data as that of **1** but only showed lowfield shifted methylene protons (H-6a″ at δ 4.37 and H-6b″ at δ 4.27), of the glucoside A. Thus, **2** is delphinidin 3-*O*-(6-*O*-malonyl-β-D-glucoside)-5-*O*-β-D-glucoside (Scheme 1).

The presence of **1** in perianth segments of *C. intybus* and of **2** in flowers of Labiatae have previously been suggested by Takeda et al. (1986) and Saito and Harborne (1992), respectively. Therefore, this is the first time that the glucosidic linkages and the acyl positions have been determined.

Structures of **3** and **4** were established as delphinidin 3-*O*-β-D-glucoside-5-*O*-(6-*O*-malonyl-β-D-glucoside) and delphinidin 3,5-*di*-*O*-β–D-glucoside, respectively (Nørbæk and Kondo, 1999; Nørbæk et al., 1996) by comparison of retention time on HPLC and ^{1}H NMR with authentic samples.

Also 3-*O*-*p*-coumaroylquinic acid (Loponen et al., 1998; Ossipov et al., 1996) was isolated as a major UV-absorbing product in *Cichorium intybus* for the first time.

3. Experimental

3.1. Plant material

The blue perianth segments of *Cichorium intybus* were collected in September from plants growing wild around the area Aarslev, Denmark. The plant material was identified by N. Jacobsen, The Royal Veterinary and Agricultural University, Dept. of Ecology, Botanical Section, Copenhagen, Denmark.

3.2. Spectroscopic analysis

UV-visible spectra were recorded in MeOH containing 0.1% HCl. FAB–MS (JMS-700) spectra were obtained by using glycerol–HCl as a matrix. Furthermore MALDI-TOF (Voyager DE-PRO) were measured by using cyano-4-hydroxycinnamic acid in H_2O:CH_3CN (1:1) as a matrix. ^{1}H NMR was measured in CD_3OD containing 10% TFA-*d* by 600 MHz (JNM alpha 600, Jeol) with internal standard CD_2HOD (3.326 ppm). 2D

spectra were obtained using a pulse sequence supplied from Jeol.

3.3. HPLC analysis

Analytical ODS-HPLC was carried out by using reversed phase column (4.6ϕ×250 mm, Develosil ODS-HG-5, Nomura Chemicals) at 40 °C, detected at λ_{max} 530 nm. A linear gradient from 0 to 30% aq. CH_3CN containing 0.5% TFA during 60 min with a flow rate of 1 ml min^{-1} was used.

3.4. Isolation of anthocyanins

Freeze-dried perianth segments of *Cichorium intybus* (50 g) were extracted with 50% aq. CH_3CN containing 0.5% trifluoroacetic acid (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 7 to 25% aq. CH_3CN containing 0.5% TFA. For further purification, the crude anthocyanins were sepd on prep. ODS-HPLC (20ϕ×250 mm, Develosil ODS-HG-5, Nomura Chemicals) using stepwise elution (8–25% aq. CH_3CN containing 0.5% TFA) at a flow rate of 5 ml min^{-1}. The pigment frs. were conc. to dryness in vacuo and stored at −80 °C as TFA salts; **1** (10 mg), **2** (5 mg), **3** (7 mg), **4** (8 mg).

3.5. Partial acid hydrolysis

Hydrolysis of anthocyanin **1** was carried out in 1 M HCl at 100 °C. At suitable time intervals (2, 5, 10, 20, 40 and 60 min) samples were taken out, cooled in an icebath and analyzed by HPLC.

3.6. Physical data of isolated compounds

1: Rt (min) of 25.6, UV-vis (0.1% HCl–MeOH): 270, 532 nm. **2**: Rt (min) of 19.0, UV-vis 271, 533 nm, Rt (min) of **3**: 21.4, UV-vis of **3**: 271, 535 nm, Rt (min) of **4**: 14.2, UV-vis of **4**: 269, 532 nm.

1: λ_{max} 538 (ε 27800) , 345, 274 nm (in 1% HCl–MeOH).

NMR and MS data for *trans*-3-*p*-coumaroylquinic acid. ^{1}H NMR: δ 7.41 (1H, *d*, J = 16.2 Hz, H-β), δ 7.23 (2H, *d*, J = 8.4 Hz, H-2′, H-6′), δ 6.57 (2H, *d*, J = 8.4 Hz, H-3′, H-5′), δ 6.11 (1H, *d*, J = 16.2 Hz, H-α), δ 5.10 (1H, *m*, H-3), δ 3.96 (1H, *m*, H-5), δ 3.50 (1H, *m*, H-4), δ 2–03–1.81 (4H, *m*, H-2, H-2). MS: 338 $[M]^+$.

Acknowledgements

We thank Niels Poulsen, Danish Institute of Agricultural Sciences, for collecting the plant material of *Cichorium intybus,* Berta Gonçalves, University of Trás-os-Montes e Alto Douro, for helping with isolation of the pigments, and Sin-ichi Oyama, Chemical Instrument Center, Nagoya Univesity, for taking of the MS data.

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