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CYANIDIN 3-MALONYLGLUCOSIDE IN *CICHORIUM INTYBUS*

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Key Word Index—*Cichorium intybus*; Compositae; chicory; FABMS; ^{13}C NMR; ^{1}H NMR; acylated anthocyanin; cyanidin 3-*O*-β-(6-*O*-malonyl)glucoside.

Abstract—The major anthocyanin of red leaves of *Cichorium intybus* has been identified as cyanidin 3-*O*-β-(6-*O*-malonyl)-D-glucopyranoside by fast atom bombardment mass spectrometry and NMR spectroscopy.

We had previously reported the presence of cyanidin 3-glucoside derivatives of unknown acylation pattern in red leaves of *Cichorium intybus* (cv Rossa di Verona) [1]. The power of fast atom bombardment mass spectrometry (FABMS) as a tool in structure elucidation of anthocyanins has recently become apparent, since molecular ions can readily be identified [2]. FABMS of the purified chicory anthocyanin gave its molecular mass as 535, with fragments corresponding to cyanidin 3-glucoside (*m/z* 449) and cyanidin (*m/z* 287). Accurate mass determination for the molecular ion gave *m/z* 535.1084 ± 0.0015, in good agreement with the mass calculated for $C_{24}H_{23}O_{14}$ (*m/z* 535.1088), establishing the composition of the compound. The presence of a malonyl group, suspected from the empirical formula and fragmentation pattern, was confirmed by NMR spectroscopy, which also defined the position of attachment of the malonyl group to the glucose moiety.

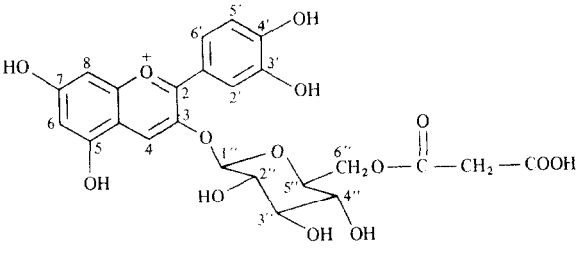
In most of the solvent systems commonly used for ^{1}H NMR of anthocyanins, such as D_2O–DCl [3] and CD_3OD–DCl [4], malonyl CH_2 protons exchange rapidly with exchangeable protons from the solvent and cannot be observed. We have found, however, that in 10% TFA–90% (v/v) DMSO-d_6, acid-catalysed exchange is slow and labile carbon-bonded protons in anthocyanins can be observed without difficulty, while the hydroxyl protons are completely exchanged with the TFA and do not obscure the spectrum; the combined signal of the TFA and the hydroxyl groups occurs at low field and can be removed by selective irradiation.

Under these conditions, the chicory anthocyanin gave a sharp singlet at δ3.42 which can be assigned to the malonyl CH_2. Acylated sugars normally show signals in the region δ4.0–5.0 attributed to the proton(s) geminal to the acyloxy group. The chicory compound gave rise to two such signals, at δ4.51 and 4.17, indicating that the acyl group was attached to C-6″; both these signals showed the characteristic geminal coupling (J = 11 Hz) usually observed for the magnetically non-equivalent C-6″ methylene protons, giving further evidence that the acyl group is attached to C-6″. The anomeric proton (δ5.44) was strongly coupled to H-2″ (J = 6.8 Hz), indicating that the compound is a β-D-glucopyranoside.

Further confirmation of the structure came from ^{13}C NMR spectroscopy. In particular, two additional signals at low field (170.09 and 168.56 ppm), attributable to the carbonyl C of the malonate moiety, were observed, in comparison with the ^{13}C chemical shifts of other anthocyanins [5] and of cyanidin-3-glucoside (Loeffler, R. S. T. and Timberlake, C. F., unpublished results).

The malonyl methylene C was found at 45.1 ppm.‡ Characteristic acylation shifts were observed for the signals assigned to C-5″ and C-6″, further evidence that the point of attachment of the malonyl group is C-6″. The ^{13}C NMR spectrum of the compound resembled that of 6-*O*-malonyl-β-methyl-D-glucopyranoside [6], allowing for the differences in the aglycone. Thus, the ^{1}H NMR and ^{13}C NMR evidence together with the FAB mass spectral data indicated that the structure of the major chicory anthocyanin is cyanidin 3-*O*-β-(6-*O*-malonyl)-D-glucopyranoside (**1**).

Documented instances of malonylated anthocyanins are rare. Malonyl cyanidin 3-glucoside has been reported only twice previously, in *Spirodela polyrrhiza* [7] and in *Mimulus luteus* [8], but its complete structure was not determined. Other malonates have been found in *Brassica oleracea* [9], *Papaver nudicaule* [3] and *Commelina*



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‡In DMSO-d_6 + 0.5% v/v 20% HCl–H_2O. In CD_3OD–DCl, we failed to observe the malonyl CH_2, presumably due to exchange with the solvent to give CD_2, which could not be observed above the noise level owing to its multiplet nature.

communis [10]. Earlier, malonic acid was reported in salvianin [11] and monardein [11, 12], but was not confirmed by Harborne [13]. However, Goto *et al.* [10] believe that monardein contains malonic acid, and FABMS of some complex plant pigments by one of us (R.S., unpublished) indicates the presence of malonic acid, hitherto unsuspected. Thus, malonic acid may not be as rare in anthocyanins as supposed previously. Goto *et al.* [10] have pointed out the facile hydrolysis of malonylated anthocyanin derivatives, and suggested that this is the reason why they have not been found to be common. More careful extraction and purification procedures coupled with the sophisticated analytical techniques now available (FABMS, FT-NMR) should aid future identifications.

The utilization of malonate, a precursor of both flavonoid and fatty acid biosynthesis, as an acylating agent is considered especially interesting in view of the possible controlling or regulating effects of acylating reactions in cell biosynthetic processes [14]. A malonated anthocyanin is significant also in being anionic above pH 3, since it contains a free ionizable carboxyl group [3]. Consequently, cyanidin 3-malonylglucoside should possess distinctive properties, which are currently being investigated.

EXPERIMENTAL

Methods. Coloured leaves of cv Rossa di Verona were extracted with 50% MeOH–H_2O containing 4% HOAc. After removal of MeOH *in vacuo*, the anthocyanins were adsorbed on a column of Techoprep (40–60 μm C18, HPLC Technology). The column was washed well with 4% HOAc and the anthocyanins were eluted with MeOH containing a trace of HOAc and evapd to dryness *in vacuo*.

Anthocyanin and UV (280 nm) composition of samples was monitored throughout by comparing peak areas. After Techoprep treatment, the crude pigment contained 7% cyanidin (Cy) 3-glucoside, formerly band III [1], 82% Cy 3-malonylglucoside, formerly band IV [1], and 6% of an additional component, formerly band V [1]. Previously, Cy 3-glucoside (band III) was the main component. The present use of HOAc rather than HCl in the extracting solvent probably accounts for the greatly increased yield of Cy 3-malonylglucoside. Further purification was by prep. HPLC and separately by PPC (in BAW and 15% HOAc on Whatman 3 MM paper). Samples of Cy 3-malonylglucoside so obtained were of similar purity (92–93%), containing 2–3% Cy 3-glucoside, with little additional UV-absorbing material. Samples recovered from NMR had hydrolysed slightly (5–8% Cy 3-glucoside); some hydrolysis during handling seemed unavoidable. The malonate had a longer HPLC retention time than Cy 3-glucoside (cf. ref. [7]); under our conditions it eluted consistently 2.1 times later.

High performance liquid chromatography. A Spectra-Physics HPLC was operated at 280 nm with simultaneous monitoring at 520 nm using a Pye-Unicam LC-UV variable-wavelength detector. A reversed-phase column (100 × 5 mm) packed with Spherisorb hexyl, 5 μm, was used at 35° and 20 μl samples were injected. Solvent A was 0.6% perchloric acid and solvent B was MeOH. The gradient was 20% B for 15 min, 30% B for 5 min, 40% B for 8 min, followed by 98% B at a flow rate of 1 ml/min. For preparative separation, a Gilson 303 microprocessor controlled gradient generator and pumping system attached to a column (250 × 16 mm) of Spherisorb hexyl 5 μm packing was used. The flow rate was 15 ml/min and the temp. 35°. The eluate was monitored using a Knauer variable-wavelength detector at 520 nm. The gradient, using the same solvent pair as that used for analytical HPLC, was 20% B for 15 min, 30% B for 10 min, 40% B for 6 min, 50% B for 1 min and 98% B for 10 min. In order to remove $HClO_4$, fractions were adsorbed on to Sep-Pak C18 cartridges, washed with 4% HOAc and eluted with methanol.

Mass spectrometry. FABMS were obtained on a MS 9/50TC mass spectrometer (Kratos Analytical Instruments) using a 5–7 kV xenon beam (source pressure 10^{-5} Torr). Samples were taken up in MeOH and a 1–2 μl aliquot transferred to the copper tip of the FAB insertion probe. After solvent evapn, 1–2 μl glycerol and 1 μl 1 M HCOOH were added and the acid was taken to dryness. Spectra were recorded at 8 kV accelerating potential and at 1000 resolution. For accurate mass determinations, the matrix was changed to a mixture of polyethylene glycols 200, 400 and 600 (BDH, Poole, U.K.) in the ratio of 1:2:4 [15] and the resolution was increased to 6600 (150 ppm).

Nuclear magnetic resonance. ^{13}C NMR (50.3 MHz, CD_3OD + 0.5% v/v 20% DCl–D_2O): δ65.46 (C-6″), 71.26 (C-4″), 74.56 (C-2″), 75.87 (C-5″), 77.83 (C-3″), 95.25 (C-1″), 103.46, 113.11, 117.35, 118.20, 121.04, 128.46, 136.45, 145.49, 147.35, 155.84, 157.54, 158.98, 164.04, 168.56 (C=O), 170.09 (C=O) and 170.33, all *s* (broad-band ^1H decoupling); ^{13}C NMR (50.3 MHz, DMSO-d_6 + 0.5% v/v 20% HCl–H_2O): similar, with additional signal at δ45.1; ^1H NMR (199.5 MHz, 10% v/v TFA–90% DMSO-d_6): δ3.42 (2H, *s*, malonyl CH_2), 3.2–4.0 (4H, *m*, H-2″, H-3″, H-4″ and H-5″), 4.17 (1H, *dd*, J = 7.8 Hz, 9.7 Hz, H-6″ A), 4.51 (1H, *d*, J = 11.2 Hz, H-6″ B), 5.44 (1H, *d*, J = 6.8 Hz, H-1″), 6.75 (1H, *d*, J = 2.0 Hz, H-6), 6.93 (1H, *br s*, H-8), 7.07 (1H, *d*, J = 8.3 Hz, H-5′), 8.04 (1H, *d*, J = 2.0 Hz, H-2′), 8.27 (1H, *dd*, J = 8.3 Hz, 2.5 Hz, H-6′) and 8.86 (1H, *s*, H-4).

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