



Covalent anthocyanin–flavonol complexes from flowers of chive, *Allium schoenoprasum*

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Abstract

The structures of eight anthocyanins have been determined in acidified methanolic extract of pale-purple flowers of chive, *Allium schoenoprasum*. Four of them have been identified as the anthocyanin–flavonol complexes (cyanidin 3-*O*- β -glucoside^{AII}) (kaempferol 3-*O*-(2-*O*- β -glucosyl^{FIII}- β -glucoside^{FII})-7-*O*- β -glucosiduronic acid^{FIV}) malonate^{AIII} (AII-6 \rightarrow AIII-1, FIV-2 \rightarrow AIII-3), **1**, (cyanidin 3-*O*-(3-*O*-acetyl- β -glucoside^{AII}) (kaempferol 3-*O*-(2-*O*- β -glucosyl^{FIII}- β -glucoside^{FII})-7-*O*- β -glucosiduronic acid^{FIV}) malonate^{AIII} (AII-6 \rightarrow AIII-1, FIV-2 \rightarrow AIII-3), **2**, and their 7-*O*-(methyl-*O*- β -glucosiduronate^{FIV}) analogous, **3** and **4**. Pigments **1** and **2** are the first final identification of covalent complexes between an anthocyanin and a flavonol, while **3** and **4** are formed during the isolation process. The other four anthocyanins (**5–8**) were found to be the 3-acetylglucoside, 3-glucoside, 3-(6-malonylglucoside) and 3-(3,6-dimalonylglucoside) of cyanidin. The three latter pigments have earlier been identified as the major anthocyanins of the chive stem. The covalent anthocyanin–flavonol complexes show intramolecular association between the anthocyanidin (cyanidin) and flavonol (kaempferol) units, which influence the colour. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Allium schoenoprasum*; Liliaceae; Covalent anthocyanin–flavonol complexes; (Cyanidin 3-glucoside) (kaempferol 3-(2-glucosylglucoside)-7-glucosiduronic acid) malonate; Acetylation; Co-pigmentation; Intramolecular association; Flower colour

1. Introduction

After the report of widespread occurrence in angiosperms of anthocyanins substituted by aliphatic dicarboxylic acids (Harborne and Boardley, 1985), an increase in complete identification of such pigments has been experienced (Strack and Wray, 1994; Harborne and Williams, 1995, 1998). The function(s) of dicarboxylic acid moieties in anthocyanins are, however, at the discussion level. It is clear that dicarboxylic acids, among which malonic acid is the most common type, make a covalent linkage between the anthocyanin

to another moiety through the dicarboxylic acid possible. Nevertheless, very few of such complexes have been identified. The only covalent complex between an anthocyanin and another flavonoid moiety has been isolated from *Eichhornia crassipes* (Pontederiaceae) (Toki et al., 1994). This pigment contains delphinidin 3-gentiobiosyl and the flavone apigenin 7-glucosyl attached to each end of a malonyl unit. The existence of similar complexes has been indicated in orchids (Uphoff, 1982; Strack et al., 1989) and lupins (Takeda et al., 1993). A modified version of a diesterified dicarboxylic acid has recently been reported for an anthocyanin having the same malic acid unit connected to both the 3- and 5-sugar (Bloor, 1998).

Chives, *Allium schoenoprasum* L., is used in salads and culinary cookery. From the stem the following four anthocyanins have been reported: the 3-(3,6-dima-

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lonylglucoside), 3-(6-malonylglucoside), 3-(3-malonylglucoside) and 3-glucoside of cyanidin (Fossen et al., 1996). Chemotaxonically, species belonging to the *Allium* genus seem to be remarkable containing at least some anthocyanins with substitution by either sugar or aliphatic acid in their sugar 3-position (Du et al., 1974; Terahara et al., 1994; Andersen and Fossen, 1995; Fossen et al., 1996; Fossen and Andersen, 1997). The anthocyanin content of *Allium* flowers has not been reported previously.

This paper describes the identification of two novel anthocyanin–flavonol covalent complexes in addition to other anthocyanins from the pale–purple flowers of chives, *Allium schoenoprasum*. A new intramolecular association mechanism is indicated.

2. Results and discussion

The HPLC chromatogram of the weakly acidified methanolic extract of chives detected in the visible spectral region, revealed a complex anthocyanin mixture. On the basis of the UV–VIS spectra recorded on-line during HPLC analysis (Table 1 and Section 3.4), the anthocyanins could be assigned into two groups. Pigments of one of these groups showed visible absorption maxima around 540 nm and extraordinary absorbance around 354 nm, indicating anthocyanin–flavonol complexes. This group constituted above 65% of the total anthocyanin content. The anthocyanins in the extract were purified by partition against ethyl acetate followed by chromatography on a bed of Amberlite XAD-7 material. They were separated on a Sephadex LH-20 column, and individual anthocyanins were isolated by preparative HPLC.

Anthocyanins **5–8** had visible maxima around 520 nm, and their A_{440}/A_{520} ratio of 29% indicated 3-substituted cyanidin or peonidin derivatives (Andersen, 1987). The identity of **5**, **7** and **8** were determined to be cyanidin 3-glucoside, cyanidin 3-(6-malonylglucoside) and cyanidin 3-(3,6-dimalonylglucoside), respectively, by on-line co-chromatography (UV–VIS spectra

and t_R -values) with authentic pigments from other *Allium* species (Fossen et al., 1996). The identity of **5** and **7** were also confirmed by their molecular ions in the electrospray mass spectra (m/z 449 and 535). These three pigments have previously been reported to constitute jointly 96% of the anthocyanins in the stem (Fossen et al., 1996). The identity of **6** was tentatively determined to be cyanidin 3-acetylglucoside by its on-line UV–VIS spectrum and molecular ion at m/z 491.

The downfield region of the ^1H NMR spectrum of pigment **1** showed a singlet at δ 8.72 (H-A4), a 3H AMX system at δ 8.31 (dd , 8.6 Hz, 1.6 Hz, H-A6^I), δ 8.00 (d , 1.6 Hz, H-A2^I) and δ 7.05 (d , 8.6 Hz, H-A5^I) and an AX system at δ 6.87 (s , broad, H-A8) and δ 6.60 (d , 1.8 Hz, H-A6) in accordance with a cyanidin derivative. This region also contained a 4H AA'XX' system at δ 8.03 (d , 8.7 Hz, H-F2^I/6^I) and δ 6.90 (H-F3^I/5^I) and one AX system at δ 6.58 (H-F8) and δ 6.22 (H-F6) corresponding to a kaempferol derivative.

The sugar region in the ^1H NMR spectrum of **1** revealed the presence of four sugar units. To determine the linkages between the building blocks and to assign all the ^1H and ^{13}C resonances (Tables 2 and 3), it was necessary to apply a combination of several 1D and 2D NMR spectroscopic techniques. The seven ^1H resonances (Table 2) revealed in the 1D TOCSY spectrum, the corresponding six ^{13}C resonances (Table 3) assigned from the HSQC spectrum, and the large anomeric coupling constant (7.7 Hz) of three of the sugars, were in accordance with three β -glucopyranosyl moieties. The crosspeak at δ 5.15/145.7 (H-A1^{II}/C-A3) in the HMBC spectrum confirmed that one of these units was connected to the anthocyanidin 3-position. The downfield shifts of H-A6A^{II} and H-A6B^{II} of this sugar indicated 6^{II}-substitution. The crosspeaks at δ 4.69/167.9 (H-A6A^{II}/C-A1^{III}) and δ 4.34/167.9 (H-A6B^{II}/C-A1^{III}) confirmed the A6^{II}-linkage between the anthocyanidin 3-sugar and the malonate moiety. The five ^1H resonances in the 1D TOCSY spectrum of the last sugar unit (Table 2), the corresponding six ^{13}C resonances (Table 3) assigned from the HSQC spectrum, and the large ^1H – ^1H coupling constants showing that

Table 1
Chromatographic and spectral data for cyanidin 3-glucoside (**5**) and the covalent anthocyanin–flavonol complexes (**1–4**)

| Compound | TLC (R_F) | | UV–Vis (nm) ^a | | Absorbance (%) | | On-line ^b UV–Vis (nm) | | HPLC t_R (min) | MS [M^+] |
|----------|---------------|------|--------------------------|-----------|---|------------------------------|----------------------------------|-----------|------------------|--------------|
| | FHW | BAW | An-band I | Fl-band I | $A_{\text{An-band I}}/A_{\text{Fl-band I}}$ | $A_{440}/A_{\text{Vis-max}}$ | An-band I | Fl-band I | | |
| 1 | 0.79 | 0.17 | 537 | 352 | 65 | 22 | 538 | 354 | 10.51 | 1303 |
| 2 | 0.88 | 0.25 | 537 | 352 | 69 | 24 | 540 | 354 | 10.79 | 1345 |
| 3 | 0.79 | 0.17 | 537 | 352 | 63 | 22 | 538 | 354 | 12.00 | 1317 |
| 4 | 0.88 | 0.25 | 537 | 352 | 67 | 23 | 540 | 354 | 12.64 | 1359 |
| 5 | 0.29 | 0.32 | 528 | | | 24 | 522 | | 9.49 | 449 |

^a An = anthocyanin moiety; Fl = flavonol moiety.

^b Recorded on-line during HPLC analysis.

all the sugar ring protons were positioned di-axial to each other, were in accordance with a β -glucuronic acid on a pyranosyl form. The crosspeak at δ 5.07/163.7 (H-F1^{IV}/C-F7) in the HMBC spectrum confirmed that this sugar unit was connected to the kaempferol 7-position. The downfield shift value of H-F2^{IV} (δ 5.00) of this sugar indicated F2^{IV}-substitution. The crosspeak at δ 5.00/167.3 (H-F2^{IV}/C-A3^{III}) confirmed the linkage between the kaempferol 7-sugar and

the malonic acid at the F2^{IV}-position. The crosspeak at δ 5.51/135.3 (H-F1^{II}/C-F3) confirmed that one of the β -glucopyranosyl moieties was connected to the kaempferol 3-position. The downfield shift value of C-F2^{II} (δ 82.34) of this moiety and the crosspeak at δ 4.89/82.3 between C-F2^{II} and the anomeric proton of the last β -glucopyranosyl moiety confirmed the sugar in the kaempferol 3-position to be the disaccharide sophorose. Together with a molecular ion at m/z 1303

Table 2

¹H NMR spectral data for covalent anthocyanin–flavonol complexes in CD₃OD:CF₃COOD (19:1, v/v) at 25°C

| | 1 δ_{H} (ppm) J (Hz) | 2 δ_{H} (ppm) J (Hz) | 3 δ_{H} (ppm) J (Hz) | 4 δ_{H} (ppm) J (Hz) | 5 (Fossen et al., 1996) δ_{H} (ppm) |
|---|---------------------------------------|---------------------------------------|---------------------------------------|---------------------------------------|--|
| cyanidin | | | | | |
| A4 | 8.72 <i>s</i> | 8.71 <i>s</i> | 8.72 <i>s</i> | 8.70 <i>s</i> | 9.03 |
| A6 | 6.60 <i>d</i> , 1.8 | 6.61 <i>d</i> , 1.8 | 6.61 <i>d</i> , 1.8 | 6.61 <i>d</i> , 1.8 | 6.71 |
| A8 | 6.87 <i>s</i> (broad) | 6.90 <i>s</i> (broad) | 6.86 <i>s</i> (broad) | 6.89 <i>s</i> (broad) | 6.90 |
| A2 ^I | 8.00 <i>d</i> , 1.6 | 7.99 <i>d</i> , 1.5 | 8.01 <i>m</i> * | 7.99 <i>d</i> , 1.8 | 8.05 |
| A5 ^I | 7.05 <i>d</i> , 8.6 | 7.07 <i>d</i> , 8.6 | 7.07 <i>d</i> , 8.8 | 7.08 <i>d</i> , 8.7 | 7.04 |
| A6 ^I | 8.31 <i>dd</i> , 8.6, 1.6 | 8.29 <i>dd</i> , 8.6, 1.5 | 8.32 <i>dd</i> , 8.7, 1.8 | 8.29 <i>dd</i> , 8.6, 1.8 | 8.25 |
| A3- <i>O</i> - β -glucopyranosyl | | | | | |
| A1 ^{II} | 5.15 <i>d</i> , 7.7 | 5.31 <i>d</i> , 7.8 | 5.14 <i>d</i> , 7.8 | 5.33 <i>d</i> , 7.7 | 5.37 |
| A2 ^{II} | 3.72 <i>m</i> | 3.77 <i>dd</i> , 7.7, 9.5 | 3.71 <i>m</i> | 3.79 <i>m</i> | 3.76 |
| A3 ^{II} | 3.53 <i>t</i> , 9.5 | 5.10 <i>t</i> , 9.5 | 3.52 <i>t</i> , 9.2 | 5.12 <i>t</i> , 9.3 | 3.67 |
| A4 ^{II} | 3.45 <i>t</i> , 9.5 | 3.58 <i>t</i> , 9.5 | 3.44 <i>t</i> , 9.2 | 3.60 <i>m</i> | 3.56 |
| A5 ^{II} | 3.72 <i>m</i> | 3.91 <i>m</i> | 3.71 <i>m</i> | 3.94 <i>m</i> | 3.67 |
| A6A ^{II} | 4.69 <i>d</i> (broad), 12.0 | 4.74 <i>d</i> (broad), 12.0 | 4.67 <i>d</i> (broad), 12.0 | 4.77 <i>d</i> (broad), 12.0 | 4.02 |
| A6B ^{II} | 4.34 <i>dd</i> , 12.0, 8.2 | 4.43 <i>dd</i> , 11.9, 8.0 | 4.33 <i>dd</i> , 11.9, 8.0 | 4.46 <i>dd</i> , 12.0, 8.0 | 3.83 |
| A3 ^{II} - <i>O</i> -acetyl | | | | | |
| 2 | | 2.27 <i>s</i> | | 2.27 <i>s</i> | |
| A6 ^{II} - <i>O</i> -malonate | | | | | |
| A2 ^{III} | 3.64 <i>s</i> | 3.66 <i>s</i> | 3.63 <i>s</i> | 3.67 <i>s</i> | |
| kaempferol | | | | | |
| F6 | 6.22 <i>s</i> (broad) | 6.19 <i>s</i> (broad) | 6.24 <i>s</i> (broad) | 6.18 <i>s</i> (broad) | |
| F8 | 6.58 <i>s</i> (broad) | 6.56 <i>s</i> (broad) | 6.58 <i>d</i> , 1.8 | 6.54 <i>d</i> , 1.8 | |
| F2 ^I /6 ^I | 8.03 <i>d</i> , 8.7 | 8.09 <i>d</i> , 8.6 | 8.02 <i>d</i> , 8.7 | 8.09 <i>d</i> , 8.8 | |
| F3 ^I /5 ^I | 6.90 <i>d</i> , 8.7 | 6.94 <i>d</i> , 8.6 | 6.90 <i>d</i> , 8.8 | 6.94 <i>d</i> , 8.8 | |
| F3- <i>O</i> - β -glucopyranoside | | | | | |
| F1 ^{II} | 5.51 <i>d</i> , 7.7 | 5.43 <i>d</i> , 7.7 | 5.51 <i>d</i> , 7.7 | 5.44 <i>d</i> , 7.7 | |
| F2 ^{II} | 3.84 <i>m</i> | 3.86 <i>m</i> | 3.85 <i>m</i> | 3.88 <i>dd</i> , 7.9, 9.4 | |
| F3 ^{II} | 3.71 <i>t</i> , 9.0 | 3.70 <i>m</i> | 3.71 <i>t</i> , 9.0 | 3.72 <i>t</i> , 9.0 | |
| F4 ^{II} | 3.41 <i>t</i> , 9.4 | 3.43 <i>m</i> | 3.41 <i>t</i> , 9.7 | 3.47 <i>t</i> , 9.5 | |
| F5 ^{II} | 3.35 <i>m</i> | 3.32 <i>m</i> | 3.35 <i>m</i> | 3.35 <i>m</i> | |
| F6A ^{II} | 3.84 <i>m</i> | 3.82 <i>m</i> | 3.84 <i>m</i> | 3.84 <i>d</i> (broad), 12.0 | |
| F6B ^{II} | 3.56 <i>m</i> | 3.57 <i>m</i> | 3.57 <i>dd</i> , 11.9, 6.4 | 3.60 <i>dd</i> , 12.0, 6.4 | |
| F2 ^{II} - <i>O</i> - β -glucopyranosyl | | | | | |
| F1 ^{III} | 4.89 <i>d</i> , 7.7 | 4.92 <i>d</i> , 7.8 | 4.89 <i>d</i> , 7.7 | 4.94 <i>d</i> , 7.7 | |
| F2 ^{III} | 3.50 <i>m</i> | 3.52 <i>m</i> | 3.50 <i>m</i> | 3.54 <i>m</i> | |
| F3 ^{III} | 3.47 <i>m</i> | 3.54 <i>m</i> | 3.51 <i>m</i> | 3.56 <i>m</i> | |
| F4 ^{III} | 3.47 <i>m</i> | 3.47 <i>m</i> | 3.45 <i>m</i> | 3.50 <i>m</i> | |
| F5 ^{III} | 3.44 <i>m</i> | 3.47 <i>m</i> | 3.42 <i>m</i> | 3.48 <i>m</i> | |
| F6A ^{III} | 3.92 <i>d</i> (broad), 12.1 | 3.95 <i>m</i> | 3.92 <i>dd</i> , 12.0, 1.8 | 3.97 <i>d</i> (broad), 11.9 | |
| F6B ^{III} | 3.81 <i>dd</i> , 12.1, 5.7 | 3.84 <i>m</i> | 3.81 <i>dd</i> , 12.1, 5.0 | 3.86 <i>dd</i> , 11.7, 5.0 | |
| F7- <i>O</i> - β -glucopyranosiduronic acid | | | | | |
| F1 ^{IV} | 5.07 <i>d</i> , 7.7 | 4.92 <i>d</i> , 7.7 | 5.07 <i>d</i> , 8.0 | 4.91 <i>d</i> , 8.0 | |
| F2 ^{IV} | 5.00 <i>dd</i> , 7.7, 9.5 | 5.03 <i>dd</i> , 7.8, 9.5 | 4.98 <i>dd</i> , 8.0, 9.5 | 5.02 <i>dd</i> , 8.0, 9.5 | |
| F3 ^{IV} | 3.74 <i>t</i> , 9.4 | 3.75 <i>t</i> , 9.5 | 3.72 <i>t</i> , 9.5 | 3.74 <i>t</i> , 9.5 | |
| F4 ^{IV} | 3.86 <i>t</i> , 9.4 | 3.87 <i>t</i> , 9.5 | 3.84 <i>t</i> , 9.5 | 3.86 <i>t</i> , 9.4 | |
| F5 ^{IV} | 4.19 <i>d</i> , 9.4 | 4.19 <i>d</i> , 9.7 | 4.22 <i>d</i> , 9.5 | 4.18 <i>d</i> , 9.5 | |
| F6 ^{IV} -OMe | | | 3.96 <i>s</i> | 3.98 <i>s</i> | |

Table 3

¹³C NMR spectral data for covalent anthocyanin–flavonol complexes in CD₃OD:CF₃COOD (19:1, v/v) at 25°C^a

| | 1 | 2 | 3 | 4 | 5 (Fossen et al., 1996) |
|---|----------------------|----------------------|----------------------|----------------------|-------------------------|
| | δ _C (ppm) | δ _C (ppm) | δ _C (ppm) | δ _C (ppm) | δ _C (ppm) |
| cyanidin | | | | | |
| A2 | 164.11 | 164.99 | 164.18 | 163.88 | 163.91 |
| A3 | 145.73 | 145.60 | 145.74 | 145.60 | 145.57 |
| A4 | 135.94 | 135.96 | 135.91 | 135.91 | 136.68 |
| A5 | 158.73 | 158.7 | 158.7 | 158.9 | 159.25 |
| A6 | 103.39 | 103.45 | 103.41 | 103.40 | 103.45 |
| A7 | 169.95 | 170.50 | 169.97 | 170.44 | 170.55 |
| A8 | 95.63 | 95.54 | 95.63 | 95.5 | 95.17 |
| A9 | 157.18 | 157.12 | 157.21 | 157.35 | 157.58 |
| A10 | 112.72 | 112.57 | 112.74 | 112.58 | 113.32 |
| A1 ^I | 121.10 | 121.02 | 121.13 | 121.03 | 121.16 |
| A2 ^I | 118.66 | 118.70 | 118.70 | 118.60 | 118.39 |
| A3 ^I | 147.32 | 147.44 | 147.34 | 147.41 | 147.34 |
| A4 ^I | 156.21 | 156.40 | 156.21 | 156.41 | 155.77 |
| A5 ^I | 117.56 | 117.78 | 117.56 | 117.74 | 117.46 |
| A6 ^I | 129.29 | 129.30 | 129.26 | 129.43 | 128.25 |
| A3- <i>O</i> -β-glucopyranoside | | | | | |
| A1 ^{II} | 104.23 | 103.59 | 104.25 | 103.59 | 103.72 |
| A2 ^{II} | 74.41 | 72.58 | 74.42 | 72.59 | 74.79 |
| A3 ^{II} | 77.94 | 78.56 | 77.84 | 78.54 | 78.14 |
| A4 ^{II} | 71.33 | 69.30 | 71.34 | 69.34 | 71.08 |
| A5 ^{II} | 76.15 | 76.13 | 76.15 | 76.11 | 78.77 |
| A6 ^{II} | 66.11 | 65.55 | 66.08 | 65.57 | 62.36 |
| A3 ^{II} - <i>O</i> -acetyl | | | | | |
| 1 | | 172.65 | | 172.68 | |
| 2 | | 21.13 | | 21.13 | |
| A6 ^{II} - <i>O</i> -malonate | | | | | |
| A1 ^{III} | 167.86 | 167.74 | 167.86 | 167.77 | |
| A2 ^{III} | 41.4 | 41.7 | 41.6 | 41.4 | |
| A3 ^{III} | 167.31 | 167.41 | 167.31 | 167.45 | |
| kaempferol | | | | | |
| F2 | 159.33 | 159.1 | 159.4 | 159.3 | |
| F3 | 135.31 | 135.75 | 135.34 | 135.41 | |
| F4 | 179.56 | 179.52 | 179.47 | 179.52 | |
| F5 | 162.48 | 162.47 | 162.54 | 162.48 | |
| F6 | 100.76 | 100.57 | 100.77 | 100.55 | |
| F7 | 163.69 | 163.63 | 163.68 | 163.64 | |
| F8 | 96.15 | 96.63 | 96.20 | 96.6 | |
| F9 | 157.45 | 157.12 | 157.45 | 157.35 | |
| F10 | 107.89 | 107.89 | 107.93 | 107.88 | |
| F1 ^I | 122.32 | 122.26 | 122.29 | 122.26 | |
| F2 ^I /6 ^I | 132.52 | 132.62 | 132.52 | 132.63 | |
| F3 ^I /5 ^I | 116.27 | 116.40 | 116.30 | 116.42 | |
| F4 ^I | 161.73 | 161.87 | 161.74 | 161.87 | |
| F3- <i>O</i> -β-glucopyranoside | | | | | |
| F1 ^{II} | 100.97 | 101.19 | 100.98 | 101.16 | |
| F2 ^{II} | 82.34 | 82.52 | 82.37 | 82.55 | |
| F3 ^{II} | 77.81 | 77.93 | 77.82 | 77.93 | |
| F4 ^{II} | 71.20 | 71.06 | 71.15 | 71.02 | |
| F5 ^{II} | 78.31 | 78.37 | 78.35 | 78.31 | |
| F6 ^{II} | 62.58 | 62.62 | 62.59 | 62.62 | |
| F2 ^{II} - <i>O</i> -β-glucopyranosyl | | | | | |
| F1 ^{III} | 104.62 | 104.81 | 104.65 | 104.81 | |
| F2 ^{III} | 75.46 | 75.56 | 75.48 | 75.57 | |
| F3 ^{III} | 78.21 | 77.97 | 77.95 | 77.97 | |
| F4 ^{III} | 71.21 | 71.39 | 71.20 | 71.39 | |
| F5 ^{III} | 78.21 | 78.21 | 78.20 | 78.21 | |
| F6 ^{III} | 62.72 | 62.65 | 62.71 | 62.66 | |

Table 3 (continued)

| | 1 δ_C (ppm) | 2 δ_C (ppm) | 3 δ_C (ppm) | 4 δ_C (ppm) | 5 (Fossen et al., 1996) δ_C (ppm) |
|---|-----------------------|-----------------------|-----------------------|-----------------------|---|
| F7- <i>O</i> - β -glucopyranosiduronic acid | | | | | |
| F1 ^{IV} | 100.14 | 100.45 | 100.22 | 100.44 | |
| F2 ^{IV} | 75.03 | 75.04 | 75.04 | 75.02 | |
| F3 ^{IV} | 74.53 | 74.57 | 74.53 | 74.55 | |
| F4 ^{IV} | 72.84 | 72.81 | 72.81 | 72.81 | |
| F5 ^{IV} | 76.72 | 76.78 | 76.74 | 76.78 | |
| F6 ^{IV} | 170.58 | 171.52 | 170.56 | 170.55 | |
| F6 ^{IV} -OMe | | | 53.25 | 53.27 | |

^a Values given with one decimal are determined from the heteronuclear experiments.

in the electrospray MS spectrum, these data confirmed the identity of pigment **1** to be the covalent anthocyanin-flavonol complex (cyanidin 3-*O*- β -glucoside^{AII}) (kaempferol 3-*O*-(2-*O*- β -glucosyl^{FIII}- β -glucoside^{FII})-7-*O*- β -glucosiduronic acid^{FIV}) malonate^{AIII} (AII-6 \rightarrow AIII-1, FIV-2 \rightarrow AIII-3).

The ¹H and ¹³C NMR spectra of pigment **2** contained, in addition to the analogous signals of **1**, a 3H singlet at δ 2.27 and two ¹³C resonances at δ 172.65 and δ 21.13, respectively, which is in accordance to a unit of acetic acid (Tables 2 and 3). The downfield shift of H-A3^{II} (δ 5.10) and the crosspeak at δ 5.10/172.7 (H-A3^{II}/C-1 acetyl) in the HMBC spectrum of **2** verified that the acetyl was connected to the A3^{II}-position. After recording of the molecular ion at m/z 1345, pigment **2** was found to be (cyanidin 3-*O*-(3-*O*-acetyl- β -glucoside^{AII}) (kaempferol 3-*O*-(2-*O*- β -glucosyl^{FIII}- β -glucoside^{FII})-7-*O*- β -glucosiduronic acid^{FIV}) malonate^{AIII} (AII-6 \rightarrow AIII-1, FIV-2 \rightarrow AIII-3).

The ¹H and ¹³C NMR spectra of **3** contained, in addition to the corresponding signals of **1**, a 3H singlet at δ 3.96 and a ¹³C resonance at δ 53.25 (Tables 2 and 3). The crosspeak at δ 3.96/ δ 170.7 (OCH₃/C-F6^{IV}) in the HMBC spectrum of **3** showed that the acid function of the glucuronyl was methylesterified. The molecular ion at m/z 1317 confirmed **3** to be (cyanidin 3-*O*- β -glucoside^{AII}) (kaempferol 3-*O*-(2-*O*- β -glucosyl^{FIII}- β -glucoside^{FII})-7-*O*-(methyl-*O*- β -glucosiduronate^{IV})) malonate^{AIII} (AII-6 \rightarrow AIII-1, FIV-2 \rightarrow AIII-3).

The ¹H and ¹³C NMR spectra of **4** contained, in addition to the analogous signals of **3**, resonances corresponding to one acetic acid moiety (Tables 2 and 3). The downfield shift of H-3^{II} (δ 5.12) and the crosspeak at δ 5.12/172.7 (H-A3^{II}/C-1 acetyl) in the HMBC spectrum of **4** were in accordance with acetylation at a A3^{II}-linkage position. A molecular ion at m/z 1359 confirmed **4** to be (cyanidin 3-*O*-(3-*O*-acetyl- β -glucoside^{AII}) (kaempferol 3-*O*-(2-*O*- β -glucosyl^{FIII}- β -glucoside^{FII})-7-*O*-(methyl-*O*- β -glucosiduronate^{IV})) malonate^{AIII} (AII-6 \rightarrow AIII-1, FIV-2 \rightarrow AIII-3).

Pigments **1** and **2** are the first final identification

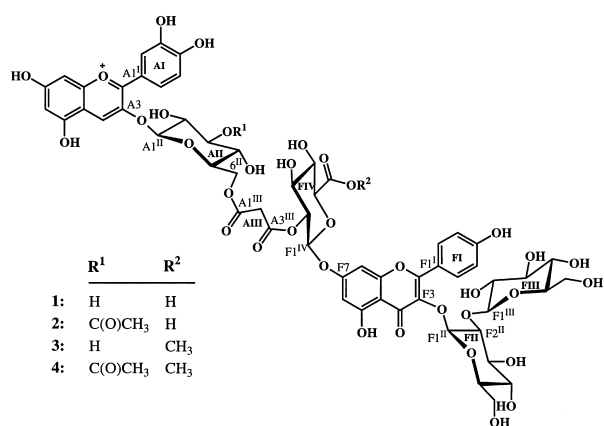
of covalent complexes between an anthocyanin and a flavonol. Uphoff (1982) has suggested that the anthocyanin orchicyanin I, which was isolated from several orchids, contained a co-pigment. Later Strack et al. (1989) gave a hypothetical structure for this pigment, cyanidin oxalyl-3,5-diglucoside-kaempferol 7-glucoside. A covalent complex between an anthocyanin and a flavone isolated from *Eichhornia crassipes* (Pontederiaceae) has recently been fully characterised (Toki et al., 1994). A similar complex has been suggested to occur in lupins (Takeda et al., 1993).

Pigments **3** and **4** are the 7-*O*-(methyl-*O*- β -glucosiduronate^{FIV}) analogous of **1** and **2**, respectively, formed during extraction with acidified methanol. The acid function of the glucuronic acid was considerably methylesterified during extraction with methanol containing as little as 1% trifluoroacetic acid.

Table 1 shows that the R_F-values of **1–4** are higher and lower than the corresponding values of simple anthocyanins like cyanidin 3-glucoside in acidified aqueous and acidified alcoholic TLC solvent systems, respectively. However, in a typical HPLC-RP18 system their retention times are similar to those of ordinary anthocyanins (Table 1).

Co-pigmentation is known to play a crucial role in the pigmentation of fruit and flowers (Brouillard, 1988; Kondo et al., 1998). The visible absorption maxima in spectra of **1–4** showed significant bathochromic shifts compared to analogous values of similar anthocyanins (e.g. **5**) without the flavonol moiety (Table 1). This association between anthocyanin and flavonol units was also supported by the NMR data on **1–4**. Especially ¹H chemical shifts are very sensitive to the magnetic anisotropy of aromatic systems and may thus serve as a probe for the spacial proximity of aromatic rings to specific protons. The chemical shifts of the anthocyanidin H-4 in all the anthocyanin-flavonol complexes (**1–4**) were 0.3 ppm upfield for the same shifts of anthocyanins without connection to a flavonol moiety (Table 2). These spectroscopic data may be interpreted as intramolecular 'π-π' stacking (Nerdal

and Andersen, 1992; Yoshida et al., 1992; Gakh et al., 1998) between the anthocyanidin (cyanidin) and the flavonol (kaempferol). Unusual high field shift of H-4 has previously been used as indicator for stacking between an anthocyanin and an aromatic acyl group like hydroxy-cinnamic acids (e.g. Gläbgen et al., 1992). Intramolecular association involving an anthocyanin and a flavonol has previously not been reported, however, flavonols like rutin has been reported as excellent co-pigments in intermolecular associations (Brouillard and Dangles, 1994). The covalent linkage through the malonic acid in **1–4** seems to allow the molecules enough rotational freedom for intramolecular association. Work is now in progress to investigate this intramolecular association at the molecular level.



3.4. Mass spectral and HPLC-diode array detector data for 6–8

Cyanidin 3-acetylglucoside, **6**: $[M^+]$ 491; Vis-max 520 nm; $A_{440}/A_{\text{Vis-max}}$ 29%; t_R 10.85 min. Cyanidin 3-(6-malonylglucoside), **7**: $[M^+]$ 535; Vis-max 520 nm; $A_{440}/A_{\text{Vis-max}}$ 29%; t_R 11.56 min. Cyanidin 3-(3,6-dimalonylglucoside), **8**: Vis-max 522 nm; $A_{440}/A_{\text{Vis-max}}$ 29%; t_R 13.39 min.

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