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NMR identification of ethyl-linked anthocyanin–flavanol pigments formed in model wine ferments

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Abstract—The ethyl-linked pigments produced by the reaction between either catechin or epicatechin and malvidin 3-O-glucoside with added acetaldehyde have been isolated and characterised by NMR spectroscopy. These pigments are generated in high concentrations in model fermentations containing added malvidin 3-O-glucoside and (*epi*)catechin when inoculated with *Saccharomyces cerevisiae* yeast. This confirms that these pigments are produced during fermentation from metabolically produced acetaldehyde and provides evidence that the formation of these pigments may be a significant contributor to the purple colouration of young red wines.

Condensation reactions between anthocyanins and other phenolic compounds to form more stable polymeric pigments are believed to be responsible for alterations in colour and astringency that occur during the maturation and ageing of red wine.[1] Mechanisms and reaction pathways leading to the formation of various pigments have been described in the literature. In particular, the reaction between anthocyanins and flavan-3-ols is believed to proceed by two main processes: (i) condensation between anthocyanins and flavanols either through the formation of an interflavan bond or through a vinyl-flavanol intermediate yielding yellow-orange pigments,[1–5] or (ii) via a Baeyer-type condensation step between a flavanol and acetaldehyde to produce purple pigments.[6–15] The latter reaction is well known and involves the formation of an ethyl bridge between the electrophilic positions of the anthocyanin and flavanol phloroglucinol rings. The ethyl-linked pigments formed by this process are characterised by an absorbance maximum around 540 nm with a shoulder at 450 nm in the UV–vis spectrum.[11] The acetaldehyde involved in this process may be produced by yeast metabolism during red wine fermentation[16] or by ethanol oxidation.[17]

The acetaldehyde-mediated condensation between malvidin 3-O-glucoside (**1**) and catechin (**2**) (Fig. 1) is known to produce two major pigments that have been proposed to be two diastereomers of a flavanol–anthocyanin dimer linked by an ethyl bridge through their C-8 positions.[6–15] While the structures of acetaldehyde-mediated condensation products with **1** and **2** were elucidated using thiolysis and LC/ESI-MS,[12] no NMR data are available for these specific pigments. However NMR data were reported for very similar ethyl-linked pigments formed between synthetic flavylium ions and **2**,[18] while NMR spectroscopy was used to confirm that cyanidin 3-O-galactoside and (−)-epicatechin (**3**) in rosé cider were linked by a CH_3–CH bridge at the 8-position.[14] NMR spectroscopic analyses of red-wine anthocyanin-derived pigments have only recently appeared in the literature, and we present here the first report of the NMR data for the ethyl-linked pigments formed between **1** and the flavanols **2** and **3**.

As part of our studies on the formation and stability of pigments in red wine, **1** (500 mg) was condensed with 600 mg each of **2** and **3** in pH 2.0 model wine solutions consisting of 20% v/v aqueous acetaldehyde (200 mg) and 0.02 M tartaric acid (250 mL). The reaction solutions were monitored with a conveniently short HPLC method involving chromatography on a Platinum™ 'EPS C18 100 Å, 1.5 μm, 33 × 7 mm Rocket' column and a 2% HCOOH/CH_3CN gradient solvent system flowing at 1.5 mL/min, starting with 10% CH_3CN for 2 min and

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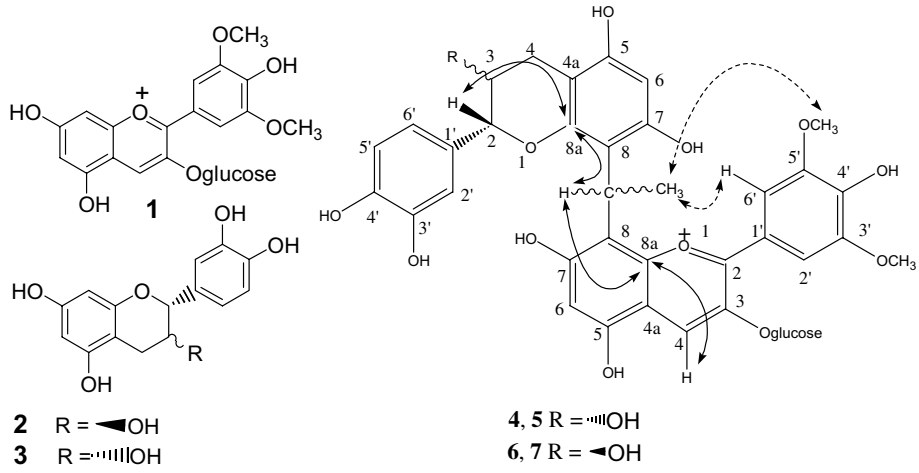

Figure 1. Structures of pigments **1**–**7**; HMBC (◀——▶), ROESY (◀----▶) correlations.

proceeding to 60% (8 min) and then 100% (10–12 min) CH_3CN. After 2 days of reaction in the dark at room temperature, the HPLC chromatograms (520 nm) of the reaction solutions showed the maximum yield of newly formed pigments **4** (6.43 min), **5** (6.54 min), **6** (6.43 min) and **7** (6.70 min) (Fig. 1), the latter two being products between **1** (6.03 min) and **2** (2.87 min), and **4** and **5** arising from **1** and **3** (4.20 min). The on-line UV–visible spectra of each pigment showed a shoulder at around 450 nm and two maxima at 280 nm and around 540 nm, characteristic of ethyl-linked anthocyanin–flavanol pigments.[11]

Concentration of the reaction mixtures on C_{18}Sep-Pak® (10 g) was followed by consecutive preparative column chromatography on Sephadex LH-20 (MeOH, H_2O, TFA; 50, 49.9, 0.1) and sulfoxyethylcellulose. Elution of the pigments adsorbed on the latter resin with increasing concentrations of 0.2 M NaCl in ethanol, followed by concentration on C_{18}Sep-Pak® (10 g) and subsequent lyophilisation afforded the pigments **4** (125 mg), **5** (102 mg), **6** (44 mg) and **7** (79 mg). LC/ESI-MS analyses showed molecular ion peaks at m/z 809 for each pigment, which was typical for ethyl-linked anthocyanin–flavonol dimers.[12]

1D and 2D NMR experiments were performed for each pigment in CD_3OD/HCl (9:1). ^1H and ^{13}C chemical shifts are shown in Tables 1 and 2. The ^1H NMR spectra of **4**,

Table 1. ^1H NMR (600 MHz) assignments: δ (ppm); m; J (Hz)

Position	4	5	6	7
Malvidin				
4	8.70; s	8.85; s	8.67; s	8.77; s
6	6.76; s	6.70; s	6.75; s	6.67; s
2′	7.73; s	7.76; s	7.70; s	7.88; s
6′	7.73; s	7.76; s	7.70; s	7.88; s
OMe	3.83; s	3.90; s	3.83; s	3.90; s
Glucose				
1	5.30; br d	5.47; d; 7.2	5.35; d; 7.8	5.30; d; 7.8
2	3.70; m	3.73; m	3.71; m	3.68; m
3	3.62; m	3.64; m	3.73; m	3.58; m
4	3.60; m	3.62; m	3.59; m	3.48; m
5	3.69; m	3.72; m	3.62; m	3.64; m
6	3.89; m	3.89; m	3.89; m	3.83; m
	3.99; m	3.99; m	4.03; m	4.01; m
Ethyl link				
CH	5.08; br q	5.41; br q	4.97; br q	5.20; br q
CH_3	1.70; d; 7.8	1.70; d; 8.4	1.62; d; 7.8	1.78; d; 7.8
Flavan-3-ol				
2	4.38; br d	4.59; d; 8.4	4.02; br d	4.03; d; 8.4
3	3.87; m	3.84; m	3.64; m	3.72; m
4	2.72; dd; 16.2, 4.0	2.78; dd; 16.8, 4.2	2.84; dd; 16.2, 5.4	2.94; dd; 16.2, 6.0
	2.58; dd; 16.8, 2.4	2.65; dd; 16.8, 2.4	2.34; dd; 16.2, 9.6	2.37; dd; 16.2, 9.6
6	6.17; s	6.20; s	6.17; s	6.11; s
2′	6.02; br d	6.11; br d	5.91; br d	5.96; d; 1.8
5′	6.32; d; 7.8	6.25; d; 7.8	6.34; d; 7.8	6.36; d; 7.8
6′	5.98; br d	5.98; d; 7.8	5.94; br d	5.98; d; 7.8

Table 2. ^{13}C NMR (150 MHz) assignments: δ ^{13}C

Position	4	5	6	7
Malvidin				
2	162.1	161.0	161.9	162.7
3	145.4	145.8	145.5	146.6
4	135.5	135.0	135.3	135.5
4a	114.6	113.7	114.4	114.6
5	155.5	155.8	155.2	157.0
6	103.7	104.4	104.1	103.8
7	168.2	167.7	167.9	168.0
8	113.0	113.0	113.0	113.7
8a	156.7	156.6	156.5	155.8
1′	120.4	120.5	120.5	120.3
2′	110.4	110.7	113.0	111.0
3′	149.5	149.3	149.4	149.9
4′	144.6	145.5	144.5	146.0
5′	149.5	149.3	149.4	149.9
6′	110.4	110.6	110.5	111.0
OMe	57.7	57.4	57.6	57.5
Glucose				
1	103.4	102.8	103.1	103.9
2	75.4	75.4	75.4	75.7
3	78.4	78.3	78.2	78.8
4	71.1	71.0	71.1	71.4
5	78.4	77.9	78.2	79.1
6	62.2	61.6	62.2	62.7
Ethyl link				
CH	27.7	26.9	27.7	28.1
CH_3	20.8	19.2	20.6	20.6
Flavan-3-ol				
2	80.5	81.0	83.2	84.0
3	66.7	66.3	68.7	68.4
4	29.7	29.2	29.6	30.4
4a	100.7	101.2	102.4	103.8
5	156.0	156.6	155.0	155.7
6	97.0	97.1	97.4	96.7
7	155.2	155.6	155.2	155.5
8	110.6	108.7	110.5	108.7
8a	155.6	156.7	155.2	155.5
1′	132.4	131.7	130.0	131.4
2′	115.3	113.8	115.2	115.8
3′	145.4	145.3	145.3	146.7
4′	145.6	145.5	145.3	146.7
5′	116.7	115.4	116.7	117.0
6′	120.4	119.8	119.8	120.4

5, **6** and **7** showed signals attributed to a CH_3–CH group as well as characteristic singlets for the malvidin glucoside H-4, equivalent H-2′,6′ and OCH_3-3′,5′[19] together with typical chemical shifts for the catechin and *epi*-catechin moieties.[18] However there was a conspicuous absence of a second proton signal for the A-ring of malvidin glucoside and the flavanol nucleus, indicating that the ethyl bridge was connected at either the 6- or 8-position of the constituent units. The position of the ethyl linkage was established by HMBC and ROESY experiments. Strong ROESY correlations were observed between the bridge CH_3 and the malvidin glucoside H-2′,6′ and OCH_3-3′,5′, which suggested a spatial proximity facilitated by a link to C-8 of malvidin glucoside rather than a C-6 link. This was confirmed in the HMBC spectra where the methine proton signal (**4**: δ 5.08; **5**: δ 5.41; **6**: δ 4.97; **7**: δ 5.20) showed a strong correlation with the malvidin 8a carbon signal (**4**: δ 156.7; **5**: δ 156.6; **6**: δ 156.5; **7**: δ 155.8). This 8a carbon showed connectivity to H-4 (**4**: δ 8.70; **5**: δ 8.85; **6**: δ 8.67; **7**: δ 8.77). Linkage to C-8 of the flavanols was confirmed by HMBC experiments, which showed correlations between the bridge CH and C-8a, the latter correlating to H-2 (**4**: δ 4.38; **5**: δ 4.59; **6**: δ 4.02; **7**: δ 4.03). The glucose protons were assigned using 2D ^1H COSY experiments.

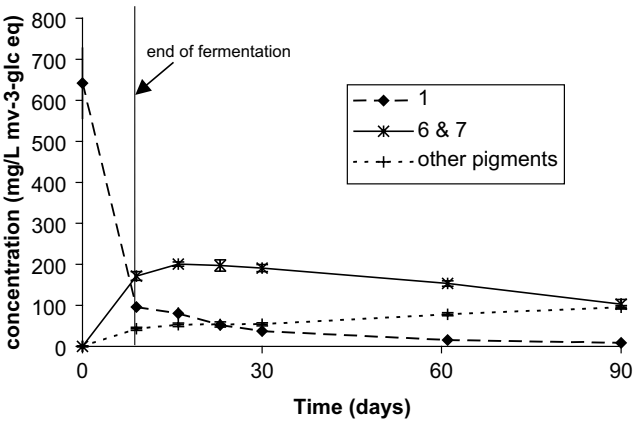

Figure 2. Change in pigment concentrations in model wine ferments with time.

The formation of pigments **6** and **7** (Fig. 2) during fermentation by *Saccharomyces cerevisiae* yeast in a chemically defined grape juice containing **1** and **2** was confirmed by HPLC detection at 520 nm, HPLC on-line spectra and co-chromatography with the synthetic analogues. It is confirmation that these pigments are produced with the involvement of acetaldehyde secreted by the yeast during fermentation and provides evidence that the formation of these pigments contributes to the purple colouration of red wines immediately after fermentation, an observation that has previously been ascribed to the effects of co-pigmentation. In addition to the ethyl-linked pigments other pigments were observed, one of which produced an LCMS m/z 561 signal and was identified as vitisin A.[20,21] The concentration of the various pigments in the ferment over time, as shown in Figure 2, was determined by HPLC and expressed as malvidin-3-glucoside equivalents.

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