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Structures and colour properties of new red wine pigments

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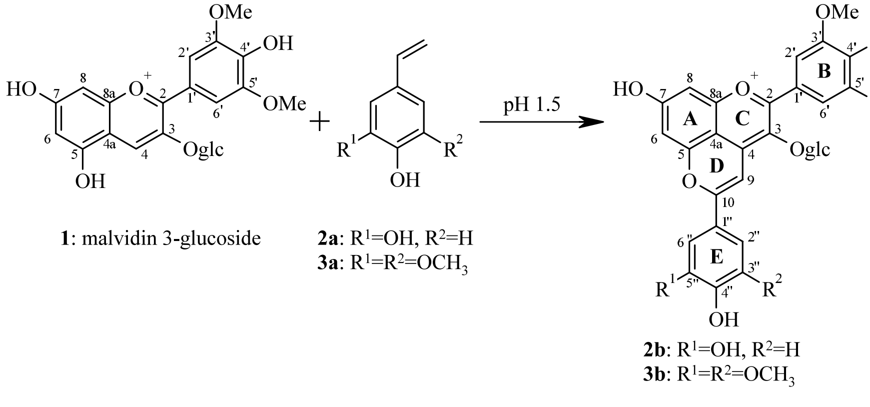
Abstract—Two new red pigments were synthesized by nucleophilic addition of vinylphenols to malvidin 3-glucoside. The structures of the resulting pyranoanthocyanins were confirmed by electrospray-mass spectrometry and NMR spectroscopy (gHMQC, gHMBC and CIGAR experiments). By means of UV–vis spectroscopy the colour properties of the pigments were characterized; it could be demonstrated that the pyranoanthocyanins retained their red colour at pH 3.6 in model wine and were resistant to bisulfite-mediated bleaching. Finally, HPLC–MS analysis confirmed the presence of both anthocyanin-derived pigments in red wine. © 2003 Elsevier Science Ltd. All rights reserved.

The importance of grape-derived anthocyanins to red wine colour is well established. These pigments are quite reactive and their colour properties are significantly modulated by oxidation and aging reactions, bisulfite bleaching and changes in pH.[1] Furthermore, only small amounts of grape anthocyanins that are extracted from skins during fermentation can be detected in aged red wines even though the wine colour is largely maintained.[2] Together with the anthocyanins, two types of pigments contribute to the colour of red wines: Firstly, *pigmented polymers*,[3] a very heterogeneous group of biomacro-molecules, are formed by the condensation of anthocyanins with other grape-derived polyphenols such as tannins. Secondly, *pyranoanthocyanins* result from the nucleophilic addition of vinylphenols or carbonyls (as enols) to anthocyanins followed by subsequent cyclization and oxidation reactions.[4]

The isolation and identification of individual red wine pigments is a tedious process and is hampered by the relatively low concentrations found in complex wine samples, and by the structural diversity of these pigments. For the rapid detection of potential pigments we reported a screening protocol based on nanoelectrospray-tandem mass spectrometry experiments. Applica-



1: malvidin 3-glucoside

2a: R^1=OH, R^2=H
3a: R^1=R^2=OCH_3

2b: R^1=OH, R^2=H
3b: R^1=R^2=OCH_3

Figure 1. Formation of pyranoanthocyanins.

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tion of these screening experiments on grape marc and wine samples from *V. vinifera* cv. Shiraz allowed us to postulate the presence of 14 anthocyanidin-derived pigments including 4-vinylcatechol and 4-vinylsyringol adducts of malvidin that had not been described previously.[5] To establish the colour properties of these previously unknown wine constituents the potential pigments were synthesized. Here we report the outcomes of the structure elucidation by spectroscopic techniques, establish the colour properties and confirm the presence of the new pigments in red wine by HPLC–MS analysis.

Anthocyanidin glucosides, including malvidin 3-glucoside **1**, were isolated from grape marc extracts by multi-layer coil countercurrent chromatography (MLCCC). Further purification of **1** was achieved by reversed-phase column chromatography.[6] The identity of **1** was confirmed by HPLC and HPLC–MS by comparison with an authentic reference compound. 2-Hydroxy-4-vinylphenol **2a** and 2,6-dimethoxy-4-vinylphenol **3a** were synthesized via Wittig reactions from the corresponding silyl protected benzaldehyde derivatives. After removal of the protecting groups, vinylphenols **2a** and **3a** were purified by column chromatography[7] and the identity of **2a** and **3a** was confirmed by NMR spectroscopy and GC–MS analysis.[8] To minimize side reactions during formation of pyranoanthocyanins **2b** and **3b** (Fig. 1) malvidin 3-glucoside **1** was dissolved in 160 ml of acetonitrile and acidified with 40 ml aqueous HCl (pH 1.5), vinylphenols **2a** or **3a** were added and the mixture was kept in the dark under N_2 until HPLC analysis indicated substantial conversion of **1**.[9] Following removal of vinyl phenols **2a** and **3a** by solvent extraction, the aqueous reaction mixture was concentrated and further purified by reversed phase column chromatography.[10]

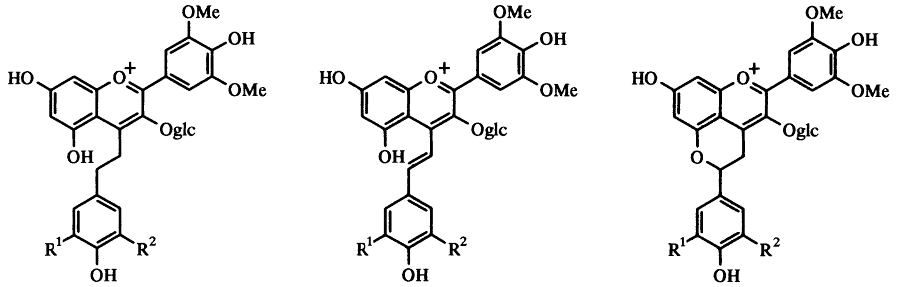
ESI-MS analysis established the molecular mass of **2b** and **3b**, and demonstrated the characteristic neutral loss of glucose from the anthocyanin backbone. The molecular formulas were further corroborated by high resolution MS spectra.[11]

Additional HPLC–MS analysis demonstrated formation of at least three clearly separated intermediates **2c** (*m*/*z* 629; $[M+4H]^+$), **2d** and **2e** (*m*/*z* 627; $[M+2H]^+$). Following isolation these compounds (postulated structures are shown in Fig. 2) could be converted into the target pyranoanthocyanin **2b**.[12] The corresponding intermediates **3d** and **3e** with molecular ions at *m*/*z* 671 ($[M+2H]^+$) could be detected in the reaction mixture together with **3b**. The presence of these intermediates indicated that the formation of pyranoanthocyanins by nucleophilic addition of vinylphenols to **1** involved a complex combination of addition, ring closure and oxidation reactions.[13]

In the ^1H NMR spectra of **2b** and **3b** the absence of the characteristic signal at 9 ppm (H4 in **1**) verified that the purified pigments did not contain any precursor **1** and also proved exclusive addition of the vinylphenols to C4 (Table 1). gHMQC experiments confirmed the identity of the hydrogen and geminal carbon atoms. All other carbon atoms were assigned by comparison with published data[2,3,14] and further substantiated by gHMBC experiments (Table 2). The only remaining signals at 146.38 ppm for **2b** and 147.85 ppm for **3b** were attributed to C4 as described previously.[14] Again, the two-dimensional ^{13}C NMR data clearly supported the proposed 4-linkage as strong crosspeaks identified $^3J_{C,H}$ couplings between H9 and C3 and C4a. The problematic carbon C4, however, did not yield any crosspeak with H9 in various gHMBC and CIGAR NMR experiments.[15]

After completing the structure elucidation, the presence of both pyranoanthocyanin pigments **2b** and **3b** in red wine was unambiguously confirmed by HPLC–MS and HPLC–UV analysis. Furthermore, addition of the reference compounds to a randomly selected 1995 Australian Shiraz wine resulted in co-elution and symmetric peak enhancement.

Comparison of the UV–vis spectra of **1**, **2b** (Fig. 3) and **3b** in model wine revealed a hypsochromic shift of the absorbance maxima to 506 nm for **2b** and 512 nm for **3b**. The resulting visible colour of both pyranoanthocyanins and their CIELab values showed clearly enhanced redness and larger a* values.[16] Compared to **2b**, **3b** had also enhanced violet/blue characters and smaller b* values, an effect that reflected the malvidin-like substitution pattern in the E-ring of **3b**. Most importantly, **2b** and **3b** were almost inert to SO_2-mediated bleaching, while **1** was instantly decolourized.[17]



[M+4H]+
2c: R1=OH, R2=H

[M+2H]+
2d: R1=OH, R2=H
3d: R1=R2=OCH3

[M+2H]+
2e: R1=OH, R2=H
3e: R1=R2=OCH3

Figure 2. Postulated intermediates.

Table 1. ^{1}H NMR data for pigments **2b** and **3b**

	2b CD_3OD+D_2O/DCl (2+1; pH 1.5)		**3b** CD_3OD+D_2O/DCl (2+1; pH 1.5)	
Position	δ ^{1}H (ppm), m, J (Hz) 600 MHz	gHMBC, CIGAR 600 MHz	δ ^{1}H (ppm), m, J (Hz) 300 MHz	CIGAR 600 MHz
9	7.74; s; 1H	C4a,1″,3,10	7.85; s 1H	C4a,1″,10
2′,6′	7.60; s; 2H	C2,2′,6′,1′,4′,3′,5′	7.61; s; 2H	C2,2′,6′,1′,4′,3′,5′
6″	7.57; d (8.4); 1H		7.22; s; 2H$^{\#}$	C10,2″,6″,3″,5″,4″
2″	7.51; s; 1H	C6″,3″,4″,10	7.22; s; 2H$^{\#}$	
6*	7.06; s; 1H	C6,8,4a,5,8a,7	7.06; d (1.8); 1H	
8*	7.03; s; 1H		7.03; d (1.8); 1H	
5″	6.92; d; (8.4); 1H	C1″,6″,3″,4″	–	
3′,5′ OCH_3	3.92; s; 6H	C3′,5′	3.9; s 12H$^{\#\#}$	C3′,5′
3″,5″OCH_3	–	–	3.9; s 12H$^{\#\#}$	
Glc				
1	5.00$^{@}$; d (7.8); 1H		Solvent overlap	
2–6	3.2–3.9		3.2–3.8	

* Assignments interchangeable.

$^{\#}$ Signals overlapped.

$^{@}$ By HMQC in CD_3CN+D_2O/DCl (2+1; pH 1.5).

Table 2. ^{13}C NMR data for pigments **2b** and **3b**

	2b CD_3CN+D_2O/DCl (2+1; pH 1.5)	**3b** CD_3OD+D_2O/DCl (2+1; pH 1.5)
Position	δ ^{13}C (ppm); 75 MHz	
10	168.50	169.00
7	166.77	168.30
2	161.36	162.51
5	153.48**	154.50
8a	152.58**	153.59
4″	152.95**	144.49
3′,5′	148.28	149.37
3″	147.82	150.30$^{\#}$
4	146.38	147.85
4′	141.80	143.52
3	134.36	135.52
6″	123.24	106.83$^{\#\#}$
1″	122.30	121.80
1′	120.36	121.21
5″	117.48	150.30$^{\#}$
2″	115.29	106.83$^{\#\#}$
2′,6′	109.00	110.06
4a	107.96	108.73
6	101.26*	102.21*
8	101.17*	101.93*
9	97.96	98.88
3′,5′-OCH_3	57.29	58.00**
3″,5″-OCH_3	–	57.92**
glc		
1	104.71	106.29
5	77.50	78.98
3	76.80	78.69
2	74.95	76.53
4	70.60	72.04
6	61.94	63.24

*, **: assignments interchangeable.

$^{\#}$, $^{\#\#}$: signals overlapped.

Of equal importance was the hyperchromic effect observed for **2b** and **3b** during pH change from pH 1.5 to pH 3.6 in model wine. Higher extinction coefficients and smaller lightness data (L*) demonstrated that **1** was the more effective pigment at pH 1.5, while the pyranoanthocyanins **2b** and **3b** showed superior colour properties and enhanced extinction coefficients in model wine at pH 3.6.[18] This significantly reduced sensitivity to pH-induced colour loss probably resulted from a combination of various effects. The additional aromatic rings (D and E in **2b** and **3b**) could contribute to the delocalization of the positive charge and thus help to stabilize the coloured flavylium cation. In addition, decolorization of **1** at wine pH has been explained by the formation of colourless hemiketals and ring-opened chalcones. While pyranoanthocyanins **2b** and **3b** are likely to undergo similar reactions at C2 in less acidic media, the presence of the additional aromatic rings D and E should result in highly conjugated and thus still coloured derivatives.

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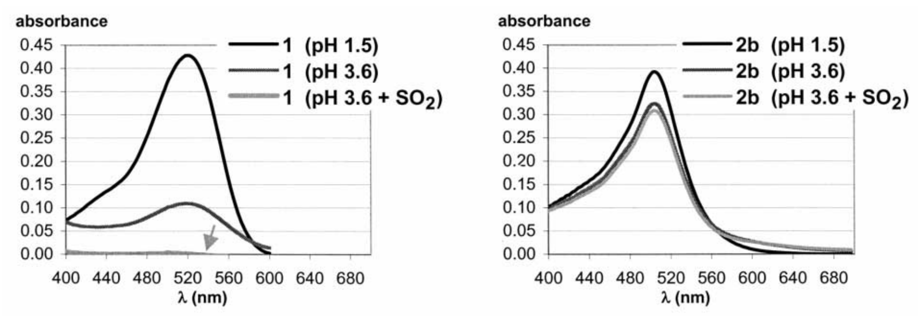

Figure 3. Visible spectra of **1** (7.7 mg/l) and **2b** (19.2 mg/l).

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6. (a) Schwarz, M.; Hillebrand, S.; Habben, S.; Degenhardt, A.; Winterhalter, P. *Biochem. Eng. J.* **2003**, *3705*, 1–11; (b) Column 15×4 cm packed with RP18-silica (ODS), solvents: 2% aqueous formic acid adjusted to pH 1.5 with HCl and increasing amounts of acetonitrile, **1** was obtained at 7–9% acetonitrile with a typical purity >95% by HPLC (peak area% at 520 nm).
7. (a) Cardona, L.; Fernandez, I.; Garcia, B.; Pedro, J. R. *Tetrahedron* **1986**, *42*, 2725–2730; (b) column 15×4 cm packed with silica 60 (230–400 mesh), solvents: 10% methanol, 90% dichloromethane, with a typical purity (by GC–MS) of **2a** and **3a** >90%.
8. DB1701 column (J&W, 30 m×0.25 mm, 0.25 μm film df); **2a**: EI-MS (70 eV) *m*/*z* (%) 51 (4.6), 63 (6.2), 77 (8.5), 79 (7.3), 89 (23.8), 90 (11.4), 108 (6.2), 110 (8.3), 119 (2.5), 135 (9.2), 136 (100, $M^{+\bullet}$); ^{1}H NMR ($(CD_3)_2SO$) δ 8.89 (2H, br s), 6.90 (1H, s), 6.74 (2H, s), 6.57 (1H, dd, $J=17.6$ Hz, $J=10.9$ Hz), 5.52 (1H, d, $J=17.6$ Hz), 5.03 (1H, d, $J=10.9$ Hz); ^{13}C NMR ($(CD_3)_2SO$) δ 147.2, 146.9, 138.4, 130.5, 119.7, 117.2, 114.6, 112.0.
   3a: EI-MS (70 eV) *m*/*z* (%) 51 (6.0), 65 (6.7), 77 (12.7), 91 (11.1), 94 (6.5), 105 (3.6), 122 (12.4), 137 (28.8), 165 (40.4), 180 (100, $M^{+\bullet}$); ^{1}H NMR ($CDCl_3$) δ 6.65 (2H, s), 6.62 (1H, dd, $J=17.5$ Hz, $J=10.8$ Hz), 5.60 (1H, dd, $J=17.5$ Hz, $J=1.0$ Hz), 5.55 (1H, s), 5.15 (1H, dd, $J=10.8$ Hz, $J=1.0$ Hz), 3.91 (6H, s); ^{13}C NMR ($CDCl_3$) δ 147.1, 136.8, 134.8, 129.2, 111.8, 103.0, 56.2.
9. (a) To 0.27 mM of **1** (142.2 mg as hydrochloride) in 200 ml 80% acetonitrile/20% aqueous HCl (pH 1.5), 1.67 mM of **2a** (227.2 mg in 1 ml acetonitrile) was added. After 7 days under N_2 at room temperature the reaction yielded 35% pyranoanthocyanin **2b** together with 35% intermediates **2c**–**e** which could be subsequently converted into **2b**, 12% educt **1** and 5% 'hydrophobic polymers'; (b) To 0.20 mM of **1** (103.6 mg as hydrochloride) in 200 ml 80% acetonitrile/20% aqueous HCl (pH 1.5) a total 1.44 mM of **3a** (260 mg in acetonitrile) was added in four batches at day 0, day 3, day 9 and day 12. After 9d under N_2 at room temperature the reaction yielded 22% pyranoanthocyanin **3b** together with 59% educt **1**. To improve the yield the reaction temperature was raised to 45°C and the mixture was stirred under air exposure for a further 5 days when HPLC analysis demonstrated formation of 61% **3b** together with 7% intermediates (not isolated), 20% educt **1** and 11% 'hydrophobic polymers'; (c) HPLC analysis was performed with a Phenomenex Hydro C18 column (150×2 mm), gradient elution with acetonitrile in aqueous H_3PO_4 (pH 1.5) at a flow rate of 0.2 ml/min and detection of UV spectra by means of a photodiode array detector.
10. Column chromatography as described in Ref. 6b; intermediates **2c**–**e** eluted at 11% acetonitrile, pyranoanthocyanin **2b** at 17% acetonitrile, and pyranoanthocyanin **3b** at 16–20% acetonitrile.
11. 7-hydroxy-2-(4-hydroxy-3,5-dimethoxy-phenyl)-10-(3,4-dihydroxy-phenyl)-pyrano[4,3,2-*de*]chromen-1-ylium-3-O-glucoside **2b**: ESI-MS *m*/*z* 625 $[M]^+$, 463 $[625\text{-glc}]^+$; high resolution MS 625.1541 (calcd. 625.1548); 7-hydroxy-2-(4-hydroxy-3,5-dimethoxy-phenyl)-10-(3,5-dimethoxy-4-hydroxy-phenyl)-pyrano[4,3,2-*de*]chromen-1-ylium-3-O-glucoside **3b**: ESI-MS *m*/*z* 669 $[M]^+$, 507 $[669\text{-glc}]^+$; high resolution MS 669.1816 (calcd. 669.1809).
12. Heating of 72 mg of **2c**–**e** in 20 ml aq. HCl (pH 1.5) under air at 45°C for 16 h yielded 72% **2b** together with 9% **2c**–**e** and 10% 'hydrophobic polymers'.
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15. gHMBC experiments selective for $J_{C,H} = 8$ Hz in $CD_3OD/D_2O/TFA$, $CD_3OD/D_2O/DCl$ and $CD_3CN/D_2O/DCl$; CIGAR experiments selective for $J_{C,H} = 8\text{-}15$ Hz in $CD_3OD/D_2O/DCl$.
16. (a) Solutions of **1** (9.9 mg/l), **2b** (43.6 mg/l) and **3b** (44.4 mg/l) in model wine at pH 3.6 (0.25% w/v potassium bitartrate in 12% v/v aq. ethanol, pH 3.6) and in model wine at pH 1.5 (model wine containing 50 mM/l HCl) were measured at 1 cm optical pathlength in a Varian 300 UV–vis spectrophotometer; (b) CIELab data were calculated from absorbance at 400–700 nm in 1 nm increments using the Varian standard software, D_{65} illuminant and the 10° standard observer. In the CIE 1976 colour space (or CIELab) individual colours are defined by rectangular coordinates L* (as a measure of lightness), a* (as a measure of the red–green component) and b* (as a measure of the yellow–blue component); (c) CIE *Colorimetry*; Central Bureau of the Commission Internationale de l'Eclairage, Publication CIE No. 15.2 (2nd ed.): Vienna, 1986.

	L*	a*	b*
1 pH 1.5	82.5	37.6	-2.2
1 pH 3.6	96.5	5.0	0.2
2b pH 1.5	88.8	22.6	10.4
2b pH 3.6	90.0	19.2	10.1
3b pH 1.5	87.8	27.4	6.5
3b pH 3.6	87.6	25.9	6.9

17. Addition of 1 volume of a freshly prepared solution of 2 g/l $Na_2S_2O_3$ in model wine to pigments in model wine (pH 3.6); bleaching started immediately and was measured 2 h later.
18. Extinction coefficients in model wine:

	ε 506 nm	ε 512 nm	ε 520 nm
1 pH 1.5	-	-	27600
1 pH 3.6	-	-	7100
2b pH 1.5	12200	-	10000
2b pH 3.6	10000	-	8400
3b pH 1.5	-	8300	7800
3b pH 3.6	-	6200	5800