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IDENTIFICATION OF AN ANTHOCYANIN OCCURRING IN SOME RED WINES

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Key Word Index—Red wine; anthocyanins; vitisin A; vitisidin A; NMR; FAB mass spectrometry.

Abstract—An anthocyanin-type pigment, vitisin A, was found in small amounts in some red wines and at trace levels in stored grapes. HPLC and spectrophotometric analysis showed that vitisin A exhibited a unique UV–Vis spectrum. Vitisin A was isolated, purified and identified using FAB mass spectrometry and NMR. The aglycone, vitisidin A, is 68 mass units greater than malvidin, accounted for by an additional C_3O_2 substituent. Further accurate mass determinations and NMR studies of both malvidin 3-glucoside and vitisin A confirmed that vitisin A is based on malvidin 3-glucoside with an additional C_3O_2 between position 4 and the 5-hydroxyl of the molecule. Five different isomers were distinguished by NMR, namely the flavylium cation, quinonoidal base, chalcone form and two carbinol pseudobases. The assignment of one of the protons remains tentative due to the rapid hydrogen/deuterium exchange. The structure was determined to be 3-formyl-4-D-β-glucopyranosyloxy-8-hydroxy-5-(4-hydroxy-3,5-dimethoxy)phenyl-2-oxo-1,6-dioxa-2,3-dihydrophenalene. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Structural analyses of anthocyanins by ^{1}H NMR have only appeared in the literature comparatively recently, owing to the difficulty in obtaining analysable spectra. Goto *et al.* [1] first made it possible to determine unambiguously the structure and stereochemistry of complex anthocyanins; probably, the first anthocyanin identification relying solely on physical techniques (mass spectrometry and NMR) followed several years later [2].

Earlier analyses of red fortified port wine pigments by HPLC revealed a number of small unidentified peaks eluting soon after malvidin 3-glucoside [3]. This author reported some properties of one of these anthocyanins, now named vitisin A, particularly its resistance to colour bleaching by sulphur dioxide—a property, indicative, by analogy with flavylium cation studies [4, 5], of a 4-substituent on the anthocyanin molecule. During our studies on maturation of red table wines, the same anthocyanin was observed, often accompanied by another unidentified anthocyanin; lesser amounts of two other anthocyanins were also observed in fortified red port wines. These three latter pigments, apparently possessing similar properties to vitisin A, will be described elsewhere [6]. The structure of another red wine pigment has been published recently [7]. Herein, we report the isolation and structural determination of the main new anthocyanin, vitisin A, using FAB mass spectrometry and ^{1}H and ^{13}C NMR.

RESULTS AND DISCUSSION

Occurrence

Vitisin A was first encountered in small quantities in fortified red wines [3]. Our laboratory studies also revealed the presence of this novel anthocyanin in maturing red table wines. Recently, we found a small amount of vitisin A in the skins of some deep frozen stored grapes, but it does not occur in the skins of any fresh grapes we have examined (unpublished data).

HPLC analysis

Quantitatively, vitisin A occurred in red wines as <7% of the total anthocyanin concentration. Using our HPLC conditions, vitisin A eluted 3 min later

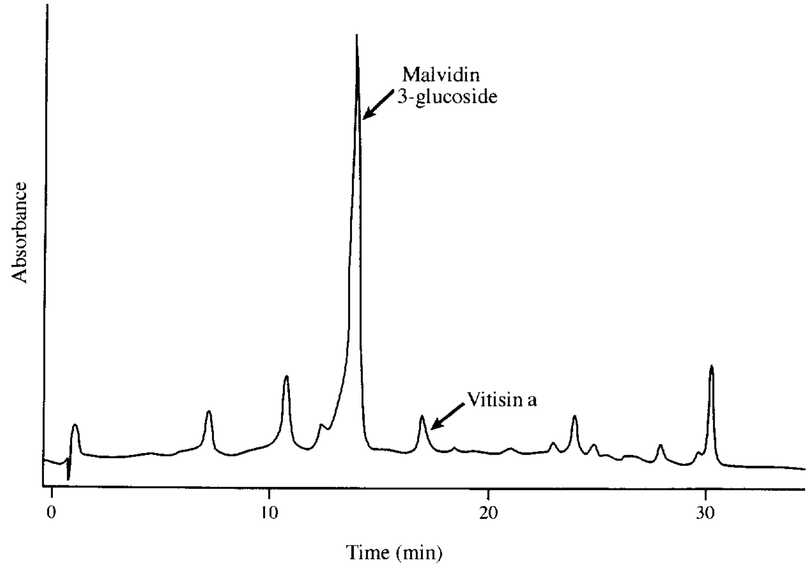

Fig. 1. HPLC chromatogram of red wine recorded at 520 nm, showing malvidin 3-glucoside and vitisin A.

than malvidin 3-glucoside (Fig. 1 and Table 1), indicating that vitisin A is less polar than malvidin 3-glucoside. The UV–Vis spectra of vitisin A and malvidin 3-glucoside recorded in aqueous 0.6% $HClO_4$–methanol (65:35) during HPLC are compared in Fig. 2. The λ_{max} of vitisin A is 15 nm less than that of malvidin 3-glucoside (Table 1); the pronounced peak at 374 nm is an unusual feature in anthocyanins. Similarly, the UV peak at 302 nm has only been observed previously in purpurinidin 3-glucoside—also believed to be 4-substituted [8, 9]. These data showed clearly that vitisin A is an anthocyanin-type pigment not previously described in the literature.

Structural identification

Classical identification methods for unknown anthocyanins rely upon destructive hydrolytic procedures and subsequent identification of the fragments produced. Since only small quantities of purified vitisin A were recovered from moderate volumes of wine, there was insufficient material available for this type of analysis. Hence, the structural characterization of vitisin A is based solely on the physical methods of FAB mass spectrometry and ^{1}H and ^{13}C NMR, which are able to provide data from minimal amounts of sample.

FAB mass spectrometry

Analysis of vitisin A produced a $[M]^+$ of 561 mass units, accompanied by a fragment ion of 399 (162 mass units less, corresponding to the loss of glucose), compared to $[M]^+$ of 493 and an aglycone of 331 for malvidin 3-glucoside. Thus, it is evident that vitisin A is a monoglucoside having an aglycone 68 mass units greater than malvidin. The additional mass can be accounted for by proposing an aglycone of malvidin with an additional C_3O_2 substituent (vitisidin A). To confirm the composition of the extra mass on vitisin A, a further mass spectral study was done to determine the accurate mass of the aglycones of malvidin 3-glucoside and vitisin A. Malvidin gave an accurate mass of 331.0817, which gives the best fit for $C_{17}H_{15}O_7$, with a mass deviation of −0.2 ppm. Vitisidin A gave an accurate mass of 399.0716, which gives the best fit for $C_{20}H_{15}O_9$, with a mass deviation of 0.0 ppm. The difference in accurate mass between malvidin and vitisidin A was 67.9899, corresponding to C_3O_2, with a mass deviation of 1.1 ppm, indicating that this substituent must form an integral part of the structure of vitisin A.

^{1}H *NMR of vitisin A*

The exact configuration of vitisin A was confirmed by detailed ^{1}H and ^{13}C NMR. The flavylium form of

Table 1. Comparison of HLPC elution times and spectral data measured in 0.6% $HClO_4$–methanol (65:35)

Anthocyanin	Elution time (min)	λ_{max} (nm)
Malvidin 3-glucoside	13.8	280, 350, 529
Vitisin A	16.8	238, 302, 374, 514, sh 263–272

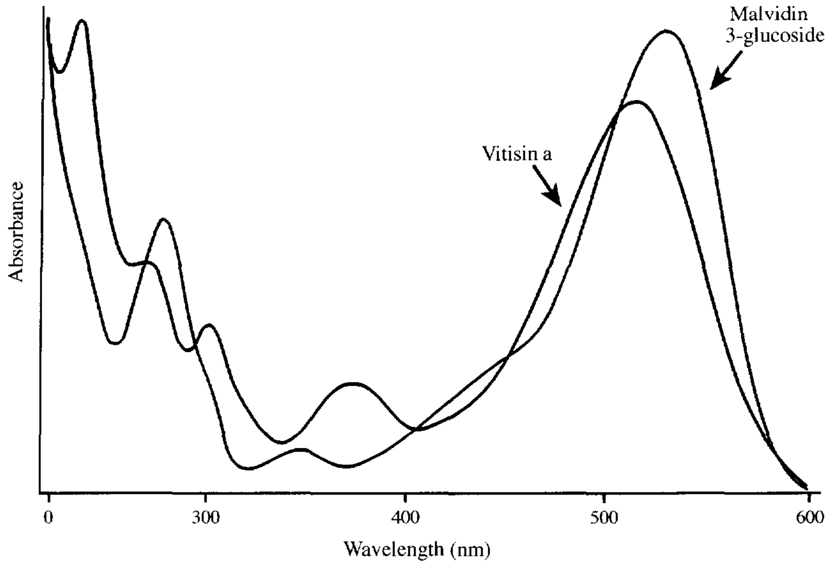

Fig. 2. UV–Vis spectra of vitisin A and malvidin 3-glucoside recorded in aqueous 0.6% $HClO_4$–methanol (65:35), from HPLC diode array detector.

an anthocyanin is stabilized in strong acid, such as hydrochloric acid, and hence NMR determinations of anthocyanins are usually performed in methanol containing hydrochloric acid. However, this solvent system is unsuitable to record different NOE spectra. Thus, in order to record good DIFNOE spectra of anthocyanins, deuterated dimethylsulphoxide (DMSO-d_6)–trifluoroacetic acid (TFA) is a good solvent, although the acidity of the solvent is not strong enough to stabilize the flavylium forms. In particular, malvidin and delphinidin readily produce the chalcone form in weak acid conditions. The solvent DMSO–d_6–TFA (9:1) allowed easy solubilization of the anthocyanins, but caused the flavylium cation of the anthocyanin to assume several isomeric forms. Proton NMR analyses were recorded at several time intervals (0, 2, 5, 7 and 16 days) after dissolving the sample. The spectrum on the first day revealed an equilibrium of five different isomers of an anthocyanin-like structure. Subsequent spectra showed that during the 16 days, the isomers slowly reverted to the chalcone form, presumably the most stable isomer in this solvent. In order to interpret the spectra, the spectrum recorded after 16 days was subtracted from that recorded on the first day, giving a difference spectrum of mainly the flavylium cation and the quinonoidal base. Similarly, by subtracting the spectrum recorded on the first day from that recorded after 16 days, spectra of mainly the chalcone and the two carbinol pseudobases were obtained.

The ^1H signals of the two most stable isomers, the flavylium cation (**I**) and the chalcone form (**Va**/**b**) (Fig. 3), were assigned by two-dimensional spectral analysis difference spectra, using HOHAHA and DQF-COSY. These data are presented in Table 2. Of the assignable peaks in the ^1H NMR spectrum of the aglycone, the proton peaks confirm the aglycone moiety to be malvidin [10]. Thus, these ^1H NMR spectra confirm the presence of H-6 and H-8 in the flavylium A ring, H-2′6′ and two methoxyl groups in the B ring for both malvidin 3-glucoside and vitisin A [3]. However, the low-field H-4 singlet characteristic for flavylium cations and anthocyanins [11] (e.g. 9.0 ppm for malvidin 3-glucoside [12]), is missing. Instead there is a more upfield singlet at 8.018 ppm for vitisin A, assigned to H-11. The flavylium H-11 is a new signal, and is differentiated from the H-4 signal of malvidin 3-glucoside by its chemical shift as well as its J_{CH} correlations (see Fig. 4). Evidence for ring opening of the C ring as well as the D ring to form the chalcone form (**Va** or **Vb**) is provided by the single signal for H-6 and H-8. The proton signals for the quinonoidal base and the two stereoisomers of the carbinol pseudobases were tentatively assigned for the aglycone, but the signals for their glucosides could not be assigned (Table 2). There is probably a strong intramolecular bond between the hydroxyl at C-11 and the carbonyl group in compounds I–V; hence, the structures are likely to be *Z*-forms.

The proton signals of the sugar moiety of the flavylium cation (**I**) appeared in the region of 3.093–3.052 ppm, in addition to the signal for the anomeric proton at 4.766 ppm (*d*, 7.8 Hz). The proton signals for the chalcone (**Va**/**b**) appeared between 2.936 and 3.690 ppm, in addition to the anomeric proton signal at 4.314 ppm (*d*, 7.8 Hz). The glucose signals were assigned by HOHAHA and DQF-COSY. Since all the observed vicinal coupling constants of the glucose were between 7.8 and 12 Hz, the glucose unit is β-D-glucopyranose. There is an upfield shift of the anom-

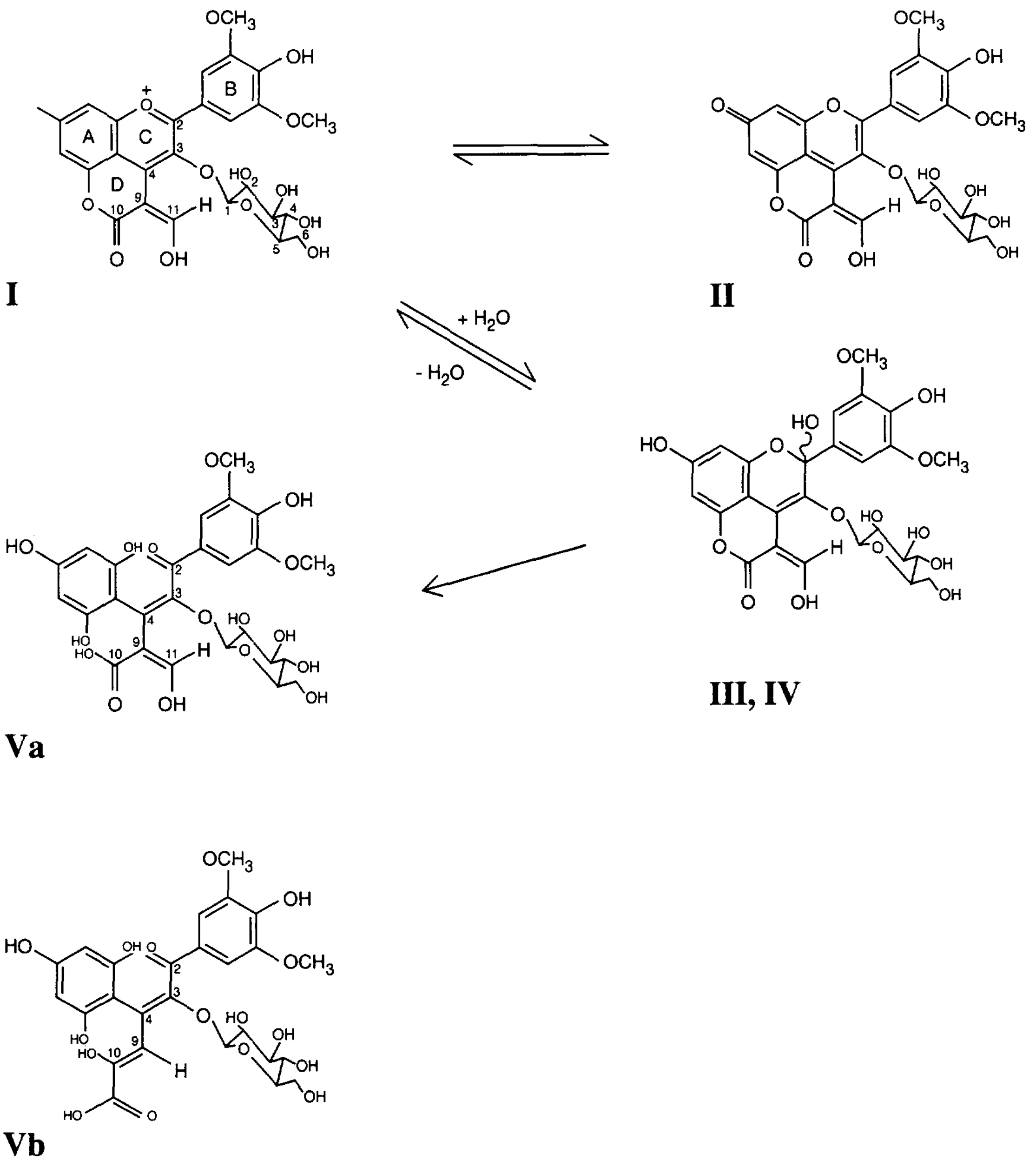

Fig. 3. Structures of vitisin A: flavylium cation (**I**), quinonoidal base (**II**), carbinol pseudobases (**III** and **IV**) and chalcone (**Va** and **Vb**).

eric proton (4.766) compared to the signal for malvidin 3-glucoside (5.36) [12]. This can be explained by the close proximity of H-11 to the anomeric proton of the sugar, as observed by the NOE experiments (Fig. 4); thus, the H-11 is forced out of plane with the B ring, resulting in a more upfield signal.

Based on the information from the ^{1}H NMR spectra the five isomers of vitisin A are shown in Fig. 3. Vitisin A is based on malvidin 3-glucoside with a 4-substitution of $C_3H_2O_2$ by linkage with the oxygen from the 5-hydroxyl.

^{13}C *NMR of vitisin A*

The ^{13}C NMR spectra recorded on the first day showed mainly the flavylium cation (**I**) while after 22 days the chalcone (**Va**/**b**) was dominant, as was also observed in the ^{1}H NMR analysis. Hence, the spectra recorded on the first day and after 22 days were used to interpret the data for the flavylium cation and the chalcone form, respectively. The signals of the ^{13}C NMR spectra of both forms were assigned using two different two-dimensional techniques, HMBC and HMQC, which relate ^{1}H and ^{13}C NMR data (Table

Table 2. ^{1}H NMR spectral data for vitisin A isolated from young red wines of *Vitis vinifera* grapes, analysed in DMSO-d_6 (0.5 ml) with TFA (0.055 ml)

H	Flavylium cation (I) δ^1H	J	Quinonoidal base (II) δ^1H	J	Carbinol (III) δ^1H	J	Carbinol (IV) δ^1H	J	Chalcone form (V) δ^1H	J
Aglycone										
6	7.267	*d*, 2.0	7.171	*d*, 2.0	6.141	*d*, 2.0	6.026	*d*, 2.0	5.761	*s*
8	7.497	*d*, 2.0	7.416	*d*, 2.0	6.286	*d*, 2.0	6.157	*d*, 2.0	5.761	*s*
2′,6′	7.872	*s*	7.900	*s*	6.754	*s*	6.781	*s*	7.053	*s*
3′,5′-OMe	3.955	*s*	3.937	*s*	3.740	*s*	—		3.712	*s*
11	8.018	*s*	7.926	*s*	6.843	*s*	6.948	*s*	7.318	*s*
Glucose										
G-1	4.766	*d*, 7.8	—		—		—		4.314	*d*, 7.8
G-2	3.478	*dd*, 8.8/7.8	—		—		—		2.936	*dd*, 8.8/8.3
G-3	3.256	*t*, 8.3–8.8	—		—		—		3.156	*t*, 8.3–8.8
G-4	3.136	*dd*, 9.8/8.3	—		—		—		3.076	*t*, 7.8–9.8
G-5	3.093	*ddd*, 10.9/5.5/2.0	—		—		—		3.106	*ddd*, 9/5.5/2.0
G-6a	3.521	*dd*, 11.7/1.5–2.0	—		—		—		3.690	*dd*, 11.0/2.0
G-6b	3.293	*dd*, 11.7/5.4	—		—		—		3.456	*dd*, 11.9/4.5–5.4

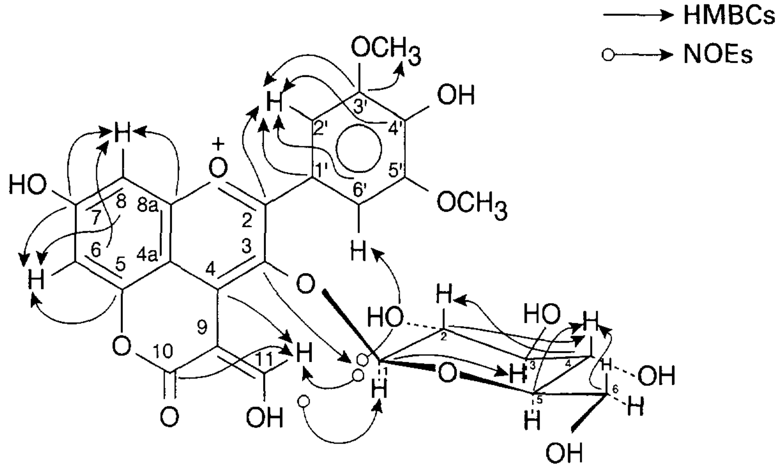

Fig. 4. Structure of vitisin A flavylium cation (AH$^+$) showing the long range C–H correlations using the HMBC method and the NOE effects of Hs in close vicinity.

3). In Fig. 4 the correlations for the flavylium cation are shown. The HMBC for the flavylium cation and HMQC and HMBC data for the chalcone were obtained 2, 16 and 21 days, respectively, after preparation of the sample solution. These data were compared with those obtained for the *Tibouchina* anthocyanin [10], which was identified as malvidin 3-(*p*-coumaroyl glucoside)-5-acetylxyloside; this comparison also confirmed that the aglycone of vitisin A had the malvidin structure.

Using these techniques the most probable structures of the flavylium and chalcone forms were deduced, although some of the correlations between the signals were only tentative. The chemical shifts on the flavylium cation were difficult to determine because the flavylium structure transformed rapidly into the chalcone form in the solvent used. For the flavylium cation (Fig. 3, I), C-2 could be assigned to 163.42. The assignments of carbon signals of the 3, 4, 9, 10 and 11 positions were estimated using HMBC correlations with the anomeric proton (G1-H) and H-11. Using the three correlations that appeared (Table 3) the signal at 134.89 correlating with G1-H was assigned to C-3, while the signals at 109.56 and 154.20 correlating with H-11 were assigned to C-4 and C-10, respectively. The residual signal at 160.02 was attributed to C-11, and

Table 3. ^{13}C NMR data for vitisin A isolated from young red wines of *Vitis vinifera* grapes, analysed in DMSO-d_6 (0.5 ml) with TFA (0.055 ml)

Flavylium form (**I**)			Chalcone form (**V**)			
Assign	$\delta^{13}C$	HMBC*	Assign	$\delta^{13}C$	HMQC†	HMBC‡
Aglycone						
2	163.42	H-2′6′	2	183.14	—	H-2′6′,H-G1
3	134.89	H-G1	3	149.12	—	H-9,H-G1
4	109.56	H-11	4	124.57	—	H-9
4a	109.56	H-6,H-8	4a	98.48	—	H-6,8
5	152.62	H-6	5	156.26	—	H-6,8
6	100.77	H-8	6	94.32	H-6,8	H-6,8
7	167.84	H-6,H-8	7	159.54	—	H-6,8
8	101.03	H-6	8	94.32	H-6,8	H-6,8
8a	152.71	H-8	8a	156.26	—	H-6,8
1′	118.42	H-2′,6′	1′	126.92	—	H-2′,6′
2′,6′	109.41	H-2′,6′	2′,6′	107.27	H-2′,6′	H-2′,6′
3′,5′	148.12	H-3′,5′, OMe	3′,5′	147.32	—	H-2′,6′, OMe
4′	143.70	H-2′,6′	4′	140.72	—	H-2′,6′
3′,5′-OMe	56.54	(OMe)	3′,5′-OMe	55.84	OMe	—
4-Substituent						
9	108.85 or 103.41		9	122.21	H-9	—
10	154.20	H-11	10	144.43	—	H-9
11	160.02	—	11	163.54	—	—
3-Glucoside						
G1	104.51	H-G3	G1	97.10	H-G1	H-G2
G2	76.21	H-G4	G2	74.97	H-G2	—
G3	74.24	H-G4	G3	76.92	H-G3	H-G1,H-G4
G4	69.82	H-G2	G4	70.48	H-G4	H-G3
G5	77.86	H-G4	G5	76.96	H-G5	H-G1,-G4,-G6
G6	60.98	H-G4	G6	61.42	H-G6a	—

* 2D NMR spectra after 2 days.
† 2D NMR spectra after 16 days.
‡ 2D NMR spectra after 21 days.

finally 108.85 or 103.41 could be ascribed to C-10. The flavylium methoxyl signal could only be assigned by residual $^{1}J_{CH}$-satellite peaks on the HMBC spectrum.

The assignment of the signals to the chalcone form was even more difficult. Complete unambiguous assignment of the ^{13}C NMR signals of the chalcone was not possible, due to the rapid hydrogen/deuterium exchange at the C-9 position. This rapid exchange at the C-9 position could be explained by keto–enol tautomerism. The chalcone form (**Va**) would be predicted by hydration of the flavylium cation. However, the chemical shifts and the interpretation using the correlations do not fit this structure. The chemical shift at 122.21 attributable to C-9 indicated an olefinic methin carbon, and hence this signal could not be confined to the proposed chalcone (**Va**) structure. For H-9 the long-range correlations appeared at 124.57, 144.43 and 149.12 (Table 3). The slightly weak correlation between the anomeric proton (G1-H) and the carbon signal 149.12 was observed; hence, this carbon signal could be attributed to C-3, which has the glycosidic link and corresponds to the quaternary olefin oxy-carbon. Another quaternary olefin-oxy carbon (144.43) and the quaternary olefin carbon (124.57) could be ascribed to C-10 and C-4, respectively. Considering these assignments, structure **Vb** is proposed and the residual carboxylic carbon of C-11 is assigned to 163.54.

Formation

The formation mechanism of this new compound remains to be explained; its absence in fresh grapes and the presence of small quantities in stored red wines indicates a chemical formation. Since vitisin A is based on the malvidin 3-glucoside structure, the most dominant anthocyanin in the grapes and wines used for our studies [12, 13], it is likely that vitisin A is formed from malvidin 3-glucoside reacting with a naturally occurring small molecule, such as an acid or aldehyde. One possible mechanism is an addition of malonic acid, whereby the negatively charged C-2 of malonic acid attacks the 4-position of malvidin 3-glucoside. This addition would result in a loss of OH^-, and subsequent ring closure via the 5-*C*-hydroxyl and the carboxyl group of the malonic acid, accompanied by a loss of water. In principle, such a mechanism could be enzymically induced, but since formation of vitisin A was observed in frozen grapes and maturing wines, a more plausible explanation may involve another mechanism.

The unusual structure of vitisin A fits well with some of the observed chemical properties, such as its resistance to bleaching by sulphur dioxide; thus, structural features of this compound may indicate, in general terms, the molecular features required to overcome the well known shortcomings of anthocyanins as food colours. Further data on the properties of vitisin A will be presented elsewhere [6]. The formation of this compound remains unsolved; the absence in fresh fruit and the presence of small quantities in stored red wines indicates a chemical formation. Since the structure of vitisin A is based on that of the major grape anthocyanin malvidin 3-glucoside, it is likely that vitisin A is formed from malvidin 3-glucoside by reaction with a naturally occurring small molecule, such as an acid or aldehyde.

EXPERIMENTAL

HPLC of wine. Wines were analysed on a Hewlett-Packard 1090 M Series II chromatograph with auto injector (25 μl). A Hypersil ODS column, 2.1 mm × 100 mm (5 μm), was used at 40°, with diode array detection at 280, 320 and 520 nm, and linear gradient elution (0.3 ml min^{-1}) with solvent A (0.6% $HClO_4$) and solvent B (MeOH) from 20 to 53%B over 33 min.

Source of vitisin A. Vitisin A occurs in red fortified port wines and many red table wines; the source used for confirmatory ^1H and ^{13}C NMR studies was a table wine produced by normal fermentation during the 1992 vintage in Portugal, from *Vitis vinifera* (var. Roriz) grapes.

Isolation of vitisin A. Red wine (*ca* 1.5 years old) was concd by rotary evapn at 40° to approx. half its vol., applied to a column of Sephadex LH-20 in 3% HCO_2H. Frontal elution was followed by elution with aq. 3% HCO_2H. The early eluate contained mainly vitisin A, later becoming diluted with other anthocyanins. Crude vitisin A was retained on a ODS Sep-Pak cartridge, washed with aq. 3% HCO_2H, eluted with 3% HCO_2H in MeOH and dried *in vacuo*. Vitisin A was obtained in highly purified form by semi-prep. HPLC on a Spherisorb ODS column (8 × 100 mm) at 40° and detection at 520 nm. Linear gradient elution (2 ml min^{-1}) was used with solvent A (1% TFA) and solvent B (MeOH) from 20 to 46% B over 26 min. The vitisin A peak was collected, rotary evapd to small vol. and dried *in vacuo*; its purity was tested by HPLC (as for wine).

FAB-MS analysis. A small sample of anthocyanin was dissolved in 1 M HCl, placed on a clean copper tipped probe, dried *in vacuo* and mixed with glycerol on the probe before insertion into the FAB source. Spectra were obtained using a resolution of 1000, with the gun producing a 5–7 kV beam of Xe atoms.

NMR analysis. ^1H NMR (400 and 500 MHz) and ^{13}C NMR (100.53 and 125.78 MHz) spectra of vitisin A at 10^{-3} M concn, were measured in DMSO-d_6 (0.5 ml) with TFA (0.055 ml) and TMS as int. standard, on JNM GX-400 and GX-500 (Jeol). ^1H NMR analyses were recorded after 0, 2, 5, 7 and 16 days. 5 isomers were observed and their ^1H signals were assigned using NOE and 2D spectral analysis using HOHAHA and DQF-COSY. ^{13}C NMR spectra were recorded after 1 and 22 days. ^{13}C NMR signals of 2 isomers were assigned using two different 2D techniques which relate ^1H and ^{13}C NMR data: HMBC and HMQC [14–16].

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