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ARTICLE

Pyranoanthocyanin Dimers: A New Family of Turquoise Blue Anthocyanin-Derived Pigments Found in Port Wine

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In the present work, several compounds bearing similar spectroscopic features were found to occur in aged Port wines and respective sediments (lees). The data obtained revealed two new families of compounds with unique spectroscopic characteristics, displaying a wavelength of the maximum absorption at high wavelength in the visible spectrum at ~730 and ~680 nm. The structure of these pigments was elucidated by liquid chromatography/diode array detector−mass spectrometry (LC/DAD−MS) and nuclear magnetic resonance (NMR), and their formation pathway in wines was established. Their structure is constituted by two pyranoanthocyanin moieties linked together through a methyne bridge. This new family of compounds displays an attractive and rare turquoise blue color at acidic conditions and has never been reported in the literature.

KEYWORDS: Pyranoanthocyanins; wine; lees; unusual spectroscopic features

INTRODUCTION

Red wine is a very complex matrix because of the extraction of a wide variety of compounds from red grape skins. Among red wines, Port wine has a higher complexity, owed to the addition of wine spirit (to stop the fermentation), which confers them great potential for the formation of new compounds (*1*, *2*). Anthocyanins are the main pigments present in grapes, being responsible for the red−violet color observed in young red wines. During wine aging and maturation, anthocyanins participate in several reactions leading to the formation of new pigments. The transformation mechanisms involved in the formation of new pigments were first described as condensation reactions of anthocyanins with flavanols mediated or not by acetaldehyde (*3*−*10*). However, over the past decade, new anthocyanin-derived pigments have been detected and identified, namely, pyranoanthocyanins that are known to be formed from the reaction of anthocyanins with small molecules, such as acetaldehyde (*11*), acetoacetic acid (*12*), pyruvic acid (*13*), vinylphenol (*14*), vinylguaiacol (*15*), vinylcatechol (*16*), and vinylcatechin (*17*). Pyranoanthocyanins are thought to contribute to the orange hues observed during wine maturation. Among the different pyranoanthocyanins present in red wines, carboxypyranoanthocyanins are the main pigments detected by high-performance liquid chromatography (HPLC) after only 1 year of aging (*18*). A few years ago, a new family of anthocyanin-derived compounds (portisins) had been detected and isolated and was found to display unusual spectroscopic features, presenting a bluish color in acidic conditions (*19*). Nuclear magnetic resonance (NMR), liquid chromatography/diode array detector−mass spectrometry (LC/DAD−MS), and studies performed in model solutions revealed that these latter pigments were formed from the reaction of carboxypyranoanthocyanins with flavanols mediated by acetaldehyde (*19*, *20*). Similar compounds were also detected and identified, with the flavanol moiety replaced by a phenolic molecule in their structure (catechol, phenol, syringol, or guaiacol) (*21*). The detection and identification of portisins point to new chemical pathways that can occur in red wines and belong to the second generation of compounds, where the main precursors involved are no longer anthocyanins but anthocyanin-derived pigments.

The detection and identification of new pigments in wines help to better understand the color changes observed during maturation. The present work deals with the detection and identification of two new families of compounds in a 9-year-old Port wine and respective sediments (lees).

MATERIALS AND METHODS

Reagents. 4-Vinylphenol was purchased from Sigma-Aldrich (Madrid, Spain). TSK Toyopearl gel HW-40(S) was purchased from Tosoh (Tokyo, Japan). 8-Vinylcatechin, vinylcatechol, carboxypyranomalvidin-3-glucoside, and methylpyranomalvidin-3-glucoside were synthesized and purified as previously described (*12*, *22*, *23*).

Wine and Lees Samples. The studied wine was a commercial red Port wine (vintage style from the year 1998) aged in a bottle for 9 years [pH 3.7, 20% alcohol (v/v), 6.3 g/L total acidity, and 22 mg/L total SO_2]. The lees were obtained from the bottom of the bottle by filtration.

Wine and Lees Fractionation. The compounds present in lees were extracted with a solution of acidulated methanol (100 mL) by stirring for 2 h. After that, the mixture was filtered and the methanol was evaporated in a rotator evaporator at room temperature.

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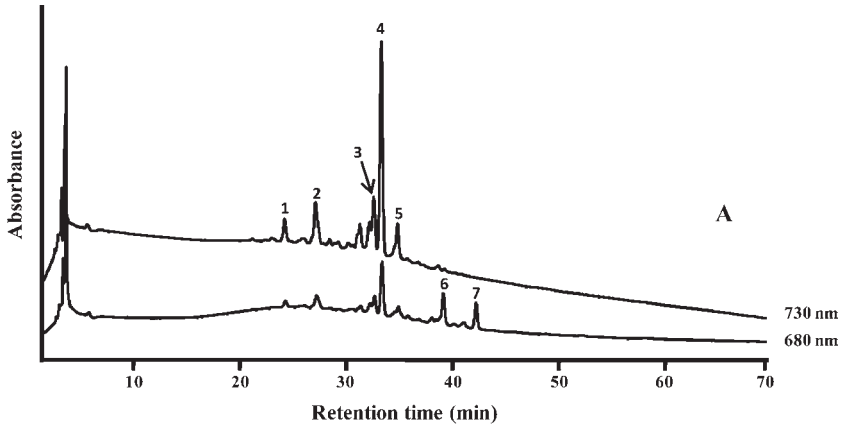

Figure 1. HPLC−DAD chromatograms of the fraction of the lees extract eluted from TSK Toyopearl gel with 60% (v/v) aqueous methanol, recorded at 730 and 680 nm.

The lees extract and the Port wine filtered were fractionated by TSK Toyopearl HW-40(S) column chromatography using different aqueous solutions with increasing percentages of methanol up to 80% (v/v). The fractions (20, 40, 60, and 80% methanol) obtained from wines and lees were analyzed by HPLC−DAD−MS.

Reaction of Carboxypyranomalvidin-3-glucoside with Other Wine Phenolics. Five solutions of carboxypyranomalvidin-3-glucoside (4 mM) were incubated in an aqueous solution of ethanol 20% (v/v) at pH 4.0 with malvidin-3-glucoside (4 mM), vinylphenol (4 mM), 8-vinylcatechin (4 mM), vinylcatechol (4 mM), and vinylpyranomalvidin-3-glucoside-catechin (4 mM). All of the reaction mixtures were left to react at 30 °C. The formation of the new compounds was monitored by HPLC−DAD.

Reaction of Methylpyranomalvidin-3-glucoside with Carboxypyranomalvidin-3-glucoside and Purification of the New Formed Compound. Methylpyranomalvidin-3-glucoside (1.4 μM) and carboxypyranomalvidin-3-glucoside (2 μM) were incubated in 40 mL of an aqueous solution of 20% ethanol (v/v) at pH 4.0. The reaction mixture was left to react at 30 °C. The formation of new compounds was monitored by HPLC−DAD. A similar reaction was performed between the coumaroyl derivative of methypyranomalvidin-3-glucoside (1.4 mM) and carboxypyranomalvidin-3-glucoside (2 mM) in 5 mL of an aqueous solution of ethanol 20% (v/v). After the maximum formation of the new compound (14 days) was reached, the reaction mixtures were pre-purified by TSK Toyopearl HW-40(S) gel column chromatography, with the major compound formed being eluted with a 50% (v/v) methanol aqueous solution. After that, each compound was isolated by semi-preparative HPLC using the conditions described above. The structures of the compounds were characterized by LC/DAD/electrospray ionization (ESI)−MS and NMR.

HPLC Analysis. The fractions and reaction mixture were analyzed with Knauer K-1001 HPLC on a 250 × 4.6 mm inner diameter reversed-phase C18 column (Merck, Darmstadt); detection was carried out at 676 and 730 nm using a Knauer K-2800 DAD. The solvents were A, H_2O/HCOOH (9:1), and B, HCOOH/H_2O/CH_3CN (0.5:19.5:80). The gradient consisted of 20−85% B for 70 min at a flow rate of 1.0 mL/min. The column was washed with 100% B for 20 min and then stabilized with the initial conditions for another 20 min.

LC−MS Analysis. All of the fractions and the reaction mixture were analyzed by LC−MS to detect the presence of new polyphenolic compounds. A Finnigan Surveyor series liquid chromatograph, equipped with a 150 × 4.6 mm inner diameter, 5 μm LicroCART reversed-phase C18 column thermostatted at 25 °C, was used. The mass detection was carried out by a Finnigan LCQ DECA XP MAX (Finnigan Corp., San Jose, CA) mass detector with an atmospheric pressure ionization (API) source of ionization and an ESI interface. Solvents were A, H_2O/TFA (99.9:0.1), and B, HCOOH/H_2O/CH_3CN (0.5:19.5:80). The HPLC gradient used was the same reported above for the HPLC analysis. The capillary voltage was 4 V, and the capillary temperature was 300 °C. Spectra were recorded in positive-ion mode between m/z 300 and 1500. The mass spectrometer was programmed to do a series of three scans: a full mass, a zoom scan of the most intense ion in the first scan, and a MS−MS of the most intense ion using a relative collision energy of 30 and 60 V.

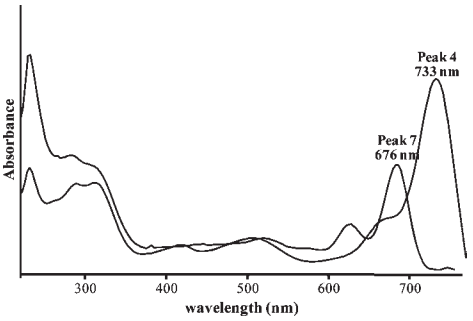

Figure 2. UV−vis spectra of compounds **4** and **7** recorded from the HPLC−DAD.

NMR Analysis. ^{1}H NMR (500.13 MHz) and ^{13}C NMR (125.77 MHz) spectra of compound **8** was measured in dimethylsulfoxide (DMSO)/trifluoroacetic acid (TFA) (9:1) on a Bruker-Avance 500 spectrometer at 293 K with tetramethylsilane (TMS) as the internal standard. ^{1}H chemical shifts were assigned using 1D and 2D ^{1}H NMR (*g*COSY), while ^{13}C resonances were assigned using 2D NMR techniques (*g*HMBC and *g*HSQC) (*24*, *25*). The delay for the long-range C/H coupling constant was optimized to 7 Hz.

RESULTS AND DISCUSSION

Detection of New Polyphenolic Compounds in Port Wine and Respective Lees. The presence of new polyphenolic compounds was assessed in a 9-year-old red Port wine and the respective lees. The wine fractions obtained from TSK Toyopearl gel column chromatography revealed compounds already described in the literature, such as carboxypyranoanthocyanins (*13*, *26*−*29*), portisins (*17*), and other pyranoanthocyanins (data not shown) (*6*, *30*, *31*). However, in the fraction eluted with 60% (v/v) methanol, several pigments were detected with spectroscopic features never observed before in red wines or lees (**Figure 1**). Most of the pigments detected in this fraction displayed a UV−vis spectrum with a λ_{max} around 730 and 680 nm (**Figure 2**), which probably correspond to two new families of compounds. These λ_{max} values are quite bathochromically shifted compared to that of other pigments present in wines (anthocyanins, around 525 nm; carboxypyranoanthocyanins, 510 nm; and portisins, 570 nm). The molecular weight and mass spectra of those new pigments are shown in **Table 1**. Compounds **6** and **7** are present in wines only in trace amounts in comparison to compounds **1**−**5**. For all compounds, the fragmentation patterns are consistent

Table 1. Molecular Ion and Respective Fragments MS^2 and MS^3 Obtained by LC−ESI−MS of Several Compounds Detected in Wine Lees (L) and Port Wine (W) (with λ_{max} Recorded from the LC−DAD)

peak (compound)	λ_{max} (nm)	m/z [M^+]	m/z (MS^2)	fragment	m/z (MS^3)	fragment	source
1	730	1205	1043	glucose	735	coumaroylglucose	W + L
2	730	1205	1043	glucose	735	coumaroylglucose	W + L
3	727	1337	1029	coumaroylglucose	721	coumaroylglucose	W + L
4	733	1351	1043	coumaroylglucose	735	coumaroylglucose	W + L
5	727	1321	1013	coumaroylglucose	705	coumaroylglucose	W + L
6	676	1191	1029	glucose	721	coumaroylglucose	W[a] + L
7	676	1337	1029	coumaroylglucose	721	coumaroylglucose	W[a] + L

[a] Trace amounts.

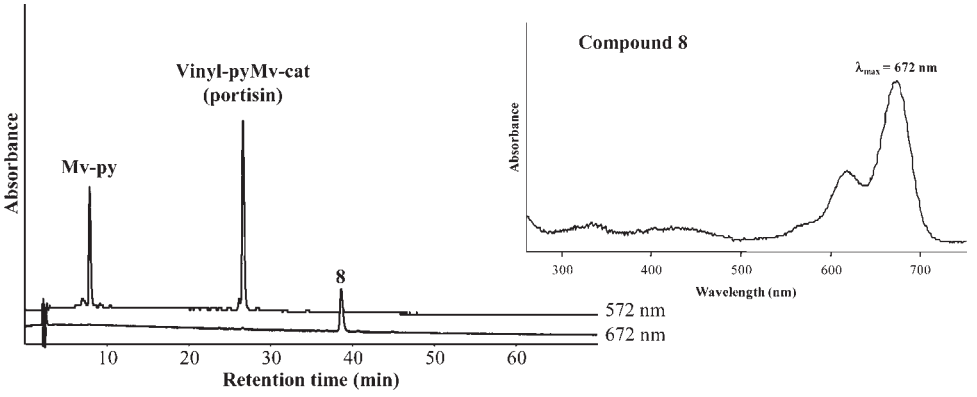

Figure 3. HPLC−DAD of the reaction of mv-3-glc-py with 8-vinylcatechin after 2 days and UV−vis spectrum recorded from the LC−DAD detector.

with the loss of two sugar moieties, acylated or not with coumaric acid. These data point that the structure of the new pigments detected includes a double anthocyanin-derived arrangement. The LC−MS data point to some similarity between the structures of compounds **1**−**5**, differing by 16 and 30 amu from each other, which probably correspond to different hydroxylation and methoxylation patterns. These structural differences are particularly visible in the MS^3 spectrum, where the sugar moieties are released. This could mean that this family of pigments results from the different anthocyanins present in wine, with a different pattern of substitution of ring B, and differing from the presence or absence of acylated sugars. Additionally, as shown in **Table 1**, compounds **3** and **7** have the same ion mass and same fragmentation pattern but they displayed different spectroscopic features ($\lambda_{max} \sim 727$ and 676 nm, respectively). Therefore, these compounds are likely to have some structural differences and belong to different families of pigments.

The isolation of the referred compounds from wines and lees is very difficult because of the complexity of the compounds present in these matrixes. Therefore, the amounts of compounds obtained were very low, which made their structural identification by NMR difficult. Thus, the synthesis of these new polyphenolic compounds was attempted in model solutions to better understand their formation in wines and to obtain sufficient amounts for NMR structural characterization.

Tentative Synthesis of the New Detected Compounds in Model Solutions. Because it was assumed that these newly formed pigments are likely to bear a double anthocyanin-derived arrangement, several reactions were undertaken in the wine model solution involving anthocyanins, different classes of pyranoanthocyanins [carboxypyranoanthocyanins, methylpyranoanthocyanins and vinylpyranoanthocyanins (portisins)], and other compounds present in wines, such as vinylphenol, vinylcatechol, and vinylcatechin. Compounds displaying similar HPLC−MS−DAD spectroscopic features than some of the new pigments were detected from different reactions involving necessarily carboxypyranomalvidin-3-glucoside and pyranoanthocyanins (portisins and methylpyranoanthocyanins) or the vinyl compounds referred to above.

However, a single chromatographic peak **8** with a λ_{max} at 672 nm and with an ion mass at m/z 1045 was observed after 2 days of reaction between carboxypyranomalvidin-3-glucoside with vinylphenol, vinylcatechol, or vinylcatechin (**Figure 3**).

The UV−vis spectrum and mass fragmentation pattern of this newly formed pigment **8** is consistent with those of two of the new compounds detected in Port wine lees (pigments **6** and **7**). Thus, the compound obtained in model solutions and these ones detected in Port wine and respective lees are likely to have a related structure. Unfortunally, the reaction yields were very low, and the formation of this new compound was found to be very limited because the major pigments formed from the reaction of carboxypyranomalvidin-3-glucoside with vinylphenolics were the respective portisins already described in the literature (*19*−*21*, *28*) (**Figure 3**).

However, in the reaction of carboxypyranomalvidin-3-glucoside with methylpyranomalvidin-3-glucoside, pigment **8** started to appear in less than 1 day and attained the maximum formation after 14 days in a much higher quantity (~12 mg; yield of 20%).

Compound **6** was obtained from the reaction of the coumaroyl derivative of methylpyranomalvidin-3-glucoside with carboxypyranomalvidin-3-glucoside with a λ_{max} at 676 nm. The molecular weight of this later compound has more 146 amu than compound **8**, corresponding to the presence of a coumaroyl group. The LC−MS data of this compound showed an ion mass at m/z 1191 and the same two fragments at m/z 1029 and 721 than compound **6** detected in Port wine and lees. Furthermore, the HPLC retention time and the visible spectra are in agreement with the results obtained for pigment **6**. After purification by Toyopearl gel column chromatography and isolation by semi-preparative HPLC, compounds **6** and **8** were characterized by NMR spectroscopy.

NMR Spectroscopy. The structural characterization of compounds **6** and **8** was performed by NMR analysis in DMSO/TFA (9:1). The assignment of the proton and carbon chemical shifts (**Table 2**) of compound **8** led to the proposed structures shown in **Figure 4**. The double pyranoanthocyanin moieties are linked through a methyne bridge. Because this structure has a symmetry plan that passes through the methyne carbon-11, only five signals in the proton spectrum (excluding the sugar moieties) were expected. Indeed, for compound **8**, protons 2′,6′ and 3′,5′-$(OMe)_2$ of B rings appeared as singlets at 7.55 and 3.87 ppm, respectively. The chemical equivalence was also observed for protons 9 of both D rings that were assigned at 6.78 ppm. Protons 8 and 6 of A rings also appeared as a singlet at the same chemical shift at 6.91 ppm. The fifth signal situated at 5.89 ppm is consistent with the chemical shift of the proton of the methyne bridge. The assignment of the carbon resonances was possible using 2D NMR techniques (*g*HSQC and *g*HMBC). Carbons C-2′,6′, C-9, C-6, C-8, C-11, and OCH_3 were assigned through their direct correlation $^1H-^{13}C$ in the heteronuclear single-quantum coherence (HSQC) spectrum at 107.3, 99.5, 99.5, 95.3 and 55.7 ppm, respectively. All of the glucose carbons were attributed through their direct $^1H-^{13}C$ correlation with the glucose protons. Carbons C-2, C-3, C-1′, and C-4′ were attributed through their long-range correlation with protons H-2′,6′. Carbons C-3′,5′ were assigned at 147.3 ppm from the correlation with protons H-2′,6 and OCH_3 in the heteronuclear multiple-bond correlation (HMBC) spectrum. The correlation of protons H-8 and H-6 in the HMBC spectrum led to the assignment of carbons C-4a, C-5, C-7, and C-8a. Carbons C-10 from D rings were attributed to 160.1 ppm by their long-range correlation with the methyne proton H-11.

Table 2. ^1H and ^{13}C Chemical Shifts of the New Compound **8**, Determined in DMSO/TFA (9:1)[a]

position	δ ^1H (ppm); *J* (Hz)	δ ^{13}C	HSQC	HMBC
		Pyranomalvidin Moieties		
2C		155.3		H-2′,6′
3C		131.9		H-2′,6′
4C		na		
4aC		103.3		H-6,8
5A		150.0		H-6,8
6A	6.91; s	98.8	H-6	
7A		163.7		H-6,8
8A	6.91; s	98.8	H-8	
8aA		150.0		H-6,8
9D	6.78	99.5	H-9	
10D		160.1		H-11
1′B		119.5		H-2′,6′
2′,6′B	7.55; s	107.3	H-2′,6′	
3′,5′B		147.3		H-2′,6′; OCH_3
4′B		153.2		H-2′,6′
3′,5′-OMe	3.89; s	55.7	OCH_3	
		Methyne Linkage		
11	5.89; brs	95.3	=CH−	
		Glucose Moiety		
1″	4.76; d, 7.6	103.0	H-1″	
2″	3.44; ∗	74.1	H-2″	
3″	3.29; t, 8.5	76.2	H-3″	
4″	3.15; ∗	69.8	H-4″	
5″	3.11; ∗	77.6	H-5″	
6a″	3.35; ∗	60.7	H-6″a, 6″b	
6b″	3.57; ∗	60.7	H-6″a, 6″b	

[a] s, singlet; brs, broad singlet; d, doublet; t, triplet; ∗, unresolved; na, not assigned.

Because of the small amount of material obtained for compound **6**, its NMR data only allowed for a partial structural characterization. Although all protons were assigned, only the carbons attached to the protons of the molecule were assigned by HSQC (**Table 3**).

Similar to the previously described compound **8**, an overall symmetry was observed. Indeed, the chemical shifts of protons 2′B and 6′B, protons 3′,5′-$(OMe)_2$, and protons 9D appeared as only one singlet at 7.48, 3.87, and 6.88 ppm, respectively. Also, protons 8 and 6 of rings A appeared as a singlet at the same chemical shift at 6.85 ppm. The chemical shift of the proton of the methyne bridge was assigned at 5.83 ppm.

The major difference between this compound **6** and compound **8** regards the sugar moieties. The region of the signals of the sugar moieties of compound **6** is much more complex, displaying a duplication of signals. These data suggest that the two sugar moieties are not equivalent, as confirmed by the detection of two anomeric protons at 4.71 and 4.66 ppm. One of the sugar moieties is acylated with a coumaroyl group, which yielded more signals attributed to the coumaroyl protons; protons 2‴,6‴ and 3‴, 5‴ were attributed to two doublets (J = 8.3 Hz) at 7.00 and

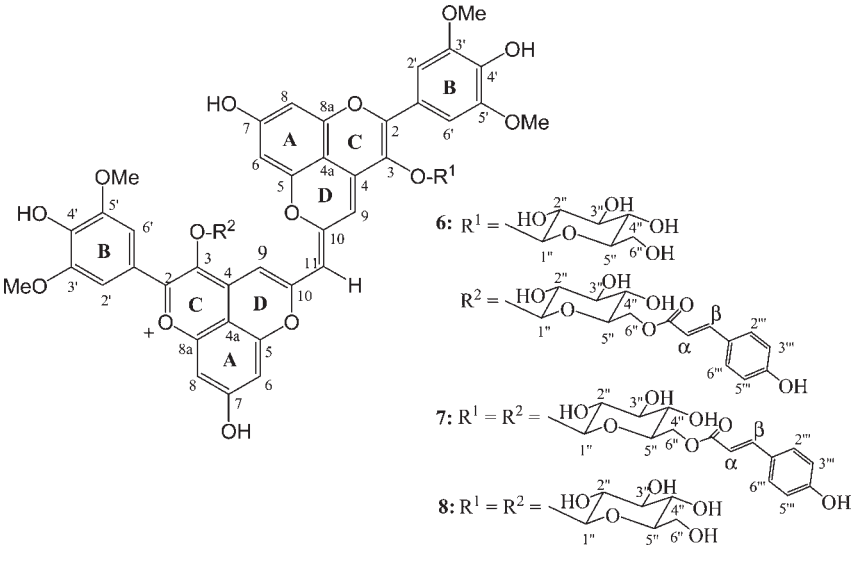

Figure 4. General structure of the new pyranoanthocyanin dimers **6**, **7**, and **8** detected in wine, lees, and model solutions.

Table 3. ^{1}H and ^{13}C Chemical Shifts of the New Compound **6**, Determined in DMSO/TFA (9:1)[a]

position	δ ^{1}H (ppm); J (Hz)	δ ^{13}C
	Pyranomalvidin Moieties	
6A	6.85	99.0
8A	6.85	99.0
9D	6.88	99.2
2′,6′B	7.48	107.6
3′,5′-OMe	3.87; s	56.2
	Methyne Linkage	
11	5.83	96.1
	Glucose Moiety	
1″	4.71	102.9
	Coumaroylglucose Moiety	
1‴	4.66	102.9
CH=CH$_\alpha$CO$_2$R	5.95	113.7
CH$_\beta$=CHCO$_2$R	7.25	144.9
2‴,6‴	7.00; d, 8.3	127.7
3‴,5‴	6.66; d, 8.3	115.4

[a] s, singlet; d, doublet.

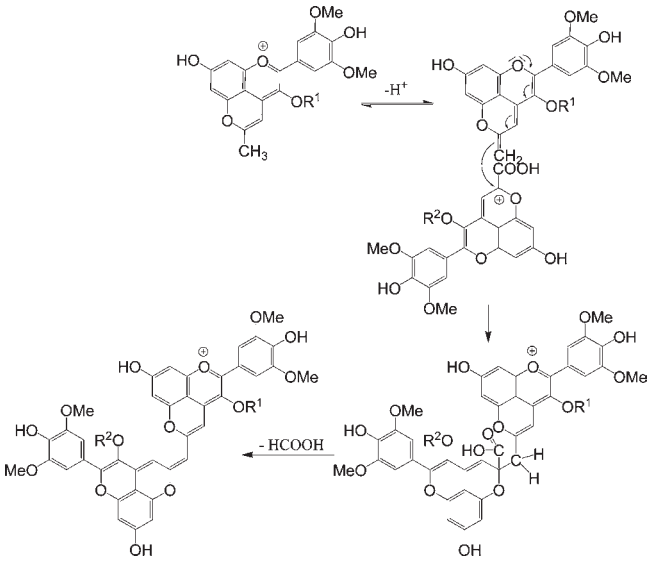

Figure 5. Proposed chemical pathway for the formation of compounds **6**, **7**, and **8** in red wine and model solutions.

6.66 ppm, respectively. Protons α and β were situated at 5.95 and 7.25 ppm.

Mechanism of Formation. Figure 5 shows a proposal for the formation mechanism of compounds **6**, **7**, and **8**. At wine pH (~3.5), deprotonation of the methyl group of the methylpyranoanthocyanin may occur, giving rise to the formation of a methylene group at carbon C-10. Therefore, these new pigments may result from the nucleophilic attack of the double bond of this methylene group to the electrophilic carbon C-10 of the carboxypyranoanthocyanin molecule. The last step should involve the loss of a formic acid molecule, leading to the formation of a structure with two pyranoanthocyanin moieties linked through a methyne group.

Attending to the yield obtained (~20%), this mechanism probably explains the great part of those compounds formed in Port wines. The mechanism involving the vinyl-phenolics to give these compounds seems to be more complex, difficult to explain, and hence, still unknown.

Figure 6. Color of pyranoanthocyanin dimers in ethanol aqueous solution at pH 2.0.

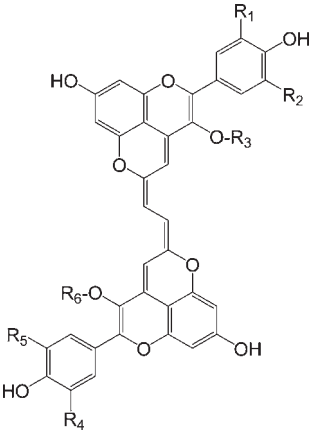

Figure 7. Hypothetic general structure for compounds **1**−**5** detected in wine and lees. R_1, R_2, R_4, and R_5 = H, OH, or OMe. R_3 and R_6 = glucose or coumaroylglucose.

In conclusion, two new families of anthocyanin-derived pigments were detected in a 9-year-old red Port wine and the respective lees, displaying unusual spectroscopic features. One group of these newly formed pigments (compounds **6** and **7**) displayed a λ_{max} at 676 nm in the UV−vis spectrum and was detected in higher levels in wine lees probably because of their lower solubility in 20% aqueous ethanol, characteristic of Port wine. In fact, during the synthesis in a 20% (v/v) ethanol aqueous Port wine model solution, the formation of a sediment corresponding to that compound was observed. The structure of these compounds was found to correspond to a double pyranoanthocyanin arrangement linked by a methyne bridge. These pigments may arise from the reaction of carboxypyranoanthocyanins with vinylphenolics and mainly with other pyranoanthocyanins occurring in the wine, such as methylpyranoanthocyanins. Carboxypyranoanthocyanins had already been found to be the major precursors for the formation of new wine pigments, such as portisins (*16*), after only 1 year of aging. Therefore, it is not surprisingly that they were found to be once again at the origin of some of these new pigments. At acidic and neutral pH, these compounds revealed a very attractive unusual turquoise blue color, appearing as blue sky pigments in acidic conditions (**Figure 6**).

Another group of these newly formed pigments was also detected in both Port wine and lees (compounds **1**−**5**). The LC−MS data of these compounds also suggested that they are likely to be characterized by a double anthocyanin-derived arrangement, as previously discussed. The difference between both families of compounds seems to be an unsaturated carbon involved in the conjugation system, which would also explain the higher λ_{max}. However, to fit the mass data, this is only possible if the corresponding compound has no charge (**Figure 7**). A comparison of the MS spectra of the two families indicates that they probably have the same substitution pattern on the ring B

corresponding to malvidin. The differences observed could correspond to the presence of an unsaturated carbon group in the structure and their charge. Their structures and the mechanism of formation in wines remain to be clarified.

Because most of these pigments were found to occur in wine lees, probably because of their low solubility, their contribution to the overall color of red wine is thought to be negligible. Nevertheless, unravelling their formation mechanism allows us to establish new chemical pathways involving different wine pigments and, by this way, contributing indirectly to the color evolution of red wine. After portisins, pyranoanthocyanin dimers constitute a second group found in wines belonging to the second generation of anthocyanin-derived pigments in which grape anthocyanins are no longer involved directly in their formation.

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