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ARTICLE

Oxovitisins: A New Class of Neutral Pyranone-anthocyanin Derivatives in Red Wines

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A new class of stable yellowish pigments with similar unique spectral features, displaying only a pronounced broad band around 370 nm in the UV−vis spectrum, was detected in an aged Port wine fraction obtained by a combination of chromatography on TSK Toyopearl HW-40(s) and Polyamide resins. These compounds were identified by liquid chromatography-diode array detector/electrospray ionization mass spectrometry (LC-DAD/ESI/MS) and shown to be direct oxidative derivatives of carboxy-pyranoanthocyanins (vitisins A) by synthesis experiments performed in a wine model solution. Their structures were fully characterized by MS and NMR spectroscopy (^{1}H, gCOSY, gHSQC, and gHMBC) and found to correspond to α-pyranone-anthocyanins (lactone or pyran-2-one-anthocyanins). Their formation involves first the nucleophilic attack of water into the positively charged C-10 position of vitisins, followed by decarboxylation, oxidation, and dehydration steps, yielding a new and neutral pyranone structure. The occurrence of these novel pigments in aged wines points to a new pathway involving anthocyanin secondary products (vitisins A) as precursors of new pigments in subsequent stages of wine aging that may contribute to its color evolution.

KEYWORDS: Carboxy-pyranoanthocyanin; oxidation; pyranone-anthocyanins; oxovitisins; vitisin, NMR; red wine aging

INTRODUCTION

Anthocyanins are the main pigments that contribute to the color of young red wines. During maturation and aging, the wine color is progressively shifted from the initial red-purple of young red wines toward more orange-like hues of aged old wines. These changes are generally attributed to several different chemical reactions (oxidation, reduction, polymerization, and complexation) in which anthocyanins participate, being by this way the precursors of new pigments that become responsible for the color change and the longevity of wines.

These newly formed pigments were initially thought to arise mainly from the condensation reactions between anthocyanins and flavanols directly or mediated by aldehydes (*1*−*7*) and dimerization of anthocyanins (*8*, *9*). Nevertheless, over the last two decades, new classes of anthocyanin-derived pigments, described as pyranoanthocyanins, were found to occur in red wines. This includes compounds formed by the reaction of anthocyanins with small molecules such as acetaldehyde (*10*), pyruvic acid (*11*, *12*), acetoacetic acid (*13*), vinyl-phenol (*14*), vinyl-guaiacol (*15*), and vinyl-catechol (*16*). One of the most important pyranoanthocyanin groups is carboxy-pyranoanthocyanins (type A vitisins), which result from the oxidative cycloaddition of pyruvic acid with anthocyanins. In Port red wine, this kind of pigment is the main pigment detected by HPLC after one year of wine aging (*12*, *17*).

Further studies on aged Port wines over recent years have revealed the presence of another family of purple/blue pigments, named portisins, which arise from reactions between carboxy-pyranoanthocyanins and flavanol in the presence of acetaldehyde (*18*, *19*) or with hydroxycinnamic acids (*20*). The detection and identification of these portisins point to new chemical reactions that possibly occur in aging red wines, in which the major precursors involved are no longer anthocyanins but anthocyanin-derived pigments such as carboxy-pyranoanthocyanins. More recently, a new class of pyranoanthocyanin dimers that present an outstanding, rare turquoise color was identified in an aged Port wine, and were shown to be formed from the reaction of a carboxy-pyaranoanthocyanin with a methyl-pyranoanthocyanin (*21*).

In the present work, a new series of yellowish pigments exhibiting similar spectral properties has been discovered, with a structure that has not been identified or postulated before. Structural elucidation indicated that they were yielded from the direct oxidation reaction of carboxy-pyranoanthocyanins, thus corresponding to a further step in the formation of new types of structures involved in the color changes of red wines.

MATERIALS AND METHODS

Reagents. TSK Toyopearl gel HW-40(S) was purchased from Tosoh (Tokyo, Japan); polyamide gel was from SINOPEC (60−80 mesh;

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Hunan, China); and pyruvic acid (d = 1.267; 97%) was purchased from Sigma-Aldrich (Madrid, Spain). All solvents were of HPLC grade.

Wine Samples. A 7-year-old Port red wine (pH 3.5, 18% alcohol (v/v), total acidity = 6.4 g/L, total SO_2 = 20 mg/L), was made from the typical grape varieties (*Vitis vinifera*) from Douro Demarcated Region (northern Portugal) throughout the traditional Port wine-making process.

Wine Fractionation. The aged Port wine sample was fractionated by a combination of two types of chromatographic techniques on TSK Toyopearl gel HW-40(s) and polyamide (60−80 mesh, SINOPEC, Hunan, China) gel, according to the procedures previously described (*22*). Different fractions were obtained by elution with increasing percentages of methanol aqueous solutions. First, 0.75 L of the old wine sample was applied onto a Toyopearl column and eluted with 20% aqueous methanol to remove the original anthocyanins and their pyruvic acid adducts (vitisins A). When practically no more colored compounds were eluted from the column, the other anthocyanin derivative fractions were recovered by elution with 50% acidified aqueous methanol. After evaporation under vacuum to remove the solvents, the above fraction was fractionated on a second column of polyamide gel. After progressive elution with water with an increasing percentage of methanol up to 30% aqueous methanol, the solvent was changed to 70% (v/v) methanol and different fractions were collected and analyzed by HPLC/DAD and LC/ESI-MS in the positive ion mode.

HPLC/DAD Conditions. A Knauer K-1001 HPLC with a 250 × 4.6 mm i.d. reversed-phase C18 column (Merck, Darmstadt, Germany) was used to analyze the wine fractions. The detection was carried out at 375 nm using a Knauer K-2800 diode array detector. The solvents were A, H_2O/HCOOH (9:1), and B, HCOOH/H_2O/CH_3CN (1:1:8). The gradient consisted of 15−35% B over 70 min, 35−80% B over 5 min, and then isocratic for 10 min at a flow rate of 1.0 mL/min. The column was then stabilized with the initial conditions for another 10 min.

LC-MS Analysis. For the LC-MS analysis was used a *Finnigan Surveyor* series liquid chromatograph, equipped with a 150 × 4.6 mm i.d., 5 μm LicroCART reversed-phase C18 column thermostatted at 35 °C. The mass detection was carried out by a Finnigan LCQ DECA XP MAX (Finnigan Corp., San José, CA, USA) mass detector with an API (Atmospheric Pressure Ionization) source of ionization and an ESI (ElectroSpray Ionization) interface. The HPLC gradient used was the same as that reported above for the HPLC analysis. Solvents were A, H_2O/HCOOH (99:1), and B, HCOOH/H_2O/CH_3CN (0.5:19.5:80). The capillary voltage and temperature were 4 V and 190 °C, respectively. Spectra were recorded in positive ion mode between m/z 120 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 relative collision energies of 30 and 60 V.

Malvidin-3-*O*-glucoside and its Pyruvic Acid Adduct (Vitisin A). Malvidin-3-glucoside was isolated and purified from grape skin anthocyanin extract according to the procedure described previously (*23*). The carboxy-pyranomalvidin-3-glucoside was achieved through the reaction of malvidin-3-glucoside with pyruvic acid in water at pH 2.6 and at 35 °C with a molar ratio pyruvic acid/malvidin-3-glucoside of 50:1 over 5 days according to the method of Oliveira et al. (*23*). The resulting major compound vitisin A was then purified by TSK Toyopearl gel HW-40(s) column chromatography with 20% aqueous methanol. The isolated pigment was then concentrated under vacuum and lyophilized.

Formation of the Pyranone-malvidin-3-glucoside Derivative (Oxovitisin A). The purified carboxy-pyranomalvidin-3-glucoside (20 mg) was dissolved in 20 mL of 20% aqueous ethanol solution (pH 3.8) in a screw-cap vial, sealed, and stored at 50 °C. Every three days, it was enriched with air oxygen through energetic shaking (oxidative medium). After 25 days of incubation, the solution was analyzed by HPLC-DAD using the conditions described above. The new pigment detected at 373 nm was then purified by TSK Toyopearl gel HW-40(s) column chromatography. The fraction containing the derived pigment eluted with 40% aqueous methanol was collected and then submitted to semipreparative HPLC using the above indicated reversed-phase C18 column using an injection volume of 500 μL and the same gradient program. The isolated compound was concentrated under vacuum and then submitted to further purification with distilled methanol, which consisted of a final elution on silica gel 100 C18 reversed phase using a vacuum filtration system. After concentration under vacuum and lyophilization, the amount of purified product was ca. 5 mg and was structurally characterized by NMR and mass spectrometry.

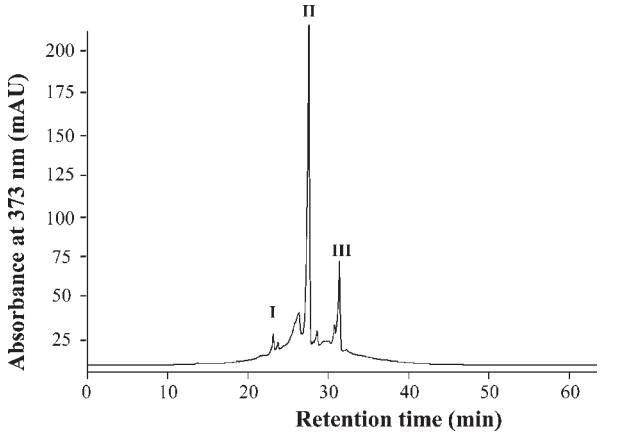

Figure 1. HPLC profile recorded at 370 nm of the light yellowish fraction from the aged Port wine after Toyopearl and polyamide purification, showing three peaks **I**, **II**, and **III** that correspond to the new pigments.

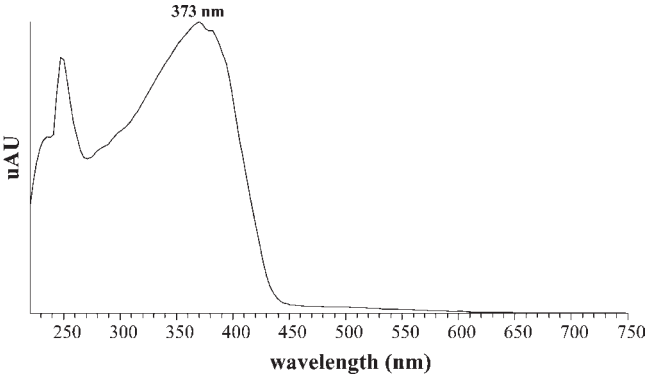

Figure 2. UV−visible spectra of peaks **I**, **II**, and **III** as recorded with the HPLC diode array detector.

NMR Analysis. ^{1}H NMR (500.13 MHz) and ^{13}C NMR (125.77 MHz) spectra were measured in CD_3OD/TFA (98:2) and $CDCl_3$ on a Bruker-Advance 500 spectrometer at 303 K with TMS as the internal standard. ^{1}H chemical shifts were assigned using 1D and 2D ^{1}H NMR (*g*COSY and NOESY). ^{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 and Structural Identification of Pyranone-anthocyanin Pigments. HPLC analysis of an aged Port red wine extract obtained after the fractionation on Toyopearl gel HW-40(s) revealed unusual components showing an absorbance maximum around 370 nm, along with a complex mixture of polyphenols including pyranoanthocyanin-flavanols as previously identified (*22*). The extract was further applied on a polyamide column for fractionation. Progressive elution with water having an increasing percentage of methanol (up to 70% aqueous methanol) yielded a light yellowish fraction which was then analyzed by HPLC (**Figure 1**). The UV−vis spectra, obtained by means of the diode array detector coupled with the chromatographic system, showed a pronounced broad band at 373 nm (**Figure 2**). This spectrum was different from all of those anthocyanin-derived flavylium pigments previously characterized (*4*, *11*, *13*, *26*−*29*). The fractionation of these compounds by polyamide chromatography, originally described for the separation of flavonoids that strongly adsorb and are eluted with over 50%, up to 70%, of

Table 1. Ion Peaks of Pyranoanthocyanins (Vitisins B), Carboxy-pyranoanthocyanins (Vitisins A), and the New Derivatives Detected in Aged Wine Fraction Analyzed by LC/M[a]S

				vitisin B	vitisin A			new derivatives	
anthocyanin skeleton	R_1	R_2	R_3	$[M]^+$	$[M]^+$	pigment no.	T_R (min)	$[M + H^+]$	aglycon
Pt-3-gluc	OCH_3	OH	Gluc	503	547	**I**	21.98	519	357
Mv-3-gluc	OCH_3	OCH_3	Gluc	517	561	**II**	28.85	533	371
Mv-3-acetylgluc	OCH_3	OCH_3	Acetyl-Gluc	559	603	**III**	34.67	575	371

[a] Pt = petunidin; Mv = malvidin; gluc = glucoside.

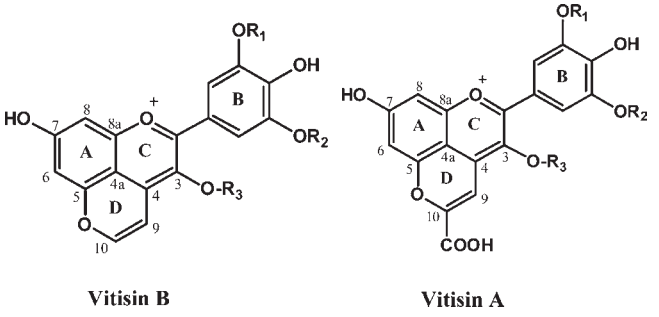

Figure 3. General structure of two pyranoanthocyanins: vitisin B and vitisin A.

methanol (*30*, *31*), and also the UV−vis spectral similarities between these new compounds and flavonoids indicated that they could possibly have a flavone-like skeleton. However, an absorption band around 370 nm is also the characteristic of 4-substituted anthocyanins (pyranoanthocyanins) (*11*, *32*). Therefore, the unique spectra of the new compounds with a pronounced broad band at 373 nm suggested that they could possibly have the pyranoanthocyanin-like skeleton as well.

Structural analysis of the above fraction was also performed by LC-MS. Pigments **I**, **II**, and **III** showed ion peaks at *m*/*z* 519, 533, and 575, respectively. The *Mr* related to each *m*/*z* value could be ascribed to that of the quasi molecular ions $[M + H]^+$ of one pryanoanthocyanin (vitisin B) plus an additional 15 a.m.u. or of one carboxy-pyranoanthocyanin (vitisin A) with less than 29 amu (**Table 1** and **Figure 3**). MS^2 and MS^3 spectra showed characteristic fragmentations as presented in the mass spectrum of malvidin-based derivative **II** (**Figure 4**). The loss of 162 amu was ascribed to the cleavage of the glucosyl moiety. The aglycone mass of each derivative is 15 amu larger than that of its pyranoanthocyanin (vitisin B), which can be due to an extra oxo-group replacing the hydrogen in the pyran ring D of vitisin B giving rise to a pyranone ring (**Figure 5**). The spectral features also suggest that these compounds might be both a neutral 4-substituted anthocyanin (pyranoanthocyanins) and have a flavone-like skeleton.

Moreover, the appearance of a fragment at *m*/*z* 343 in the MS^3 spectrum could then correspond to the further loss of the 10-carbonyl (C═O) group (equal to 28 a.m.u.) of the new derivatives. **Figure 5** resumes the fragmentation pattern of the new pigment **II** structure. The other two quasi molecular ions $[M + H]^+$ at *m*/*z* 519 (compound **I**) and *m*/*z* 575 (compound **III**) and the fragmentation pattern are consistent with the pyranone-petunidin-3-glucoside and the pyranone-malvidin-3-acetylglucoside, respectively. Therefore, the resulting new pyranone structure was probably formed by a pyran ring oxidation reaction to give a neutral and unsaturated lactone (pyran-2-one). This assumption was checked by oxidative incubation of carboxy-pyranomalvidin-3-glucoside (vitisin A) in 20% (v/v) aqueous ethanol at high temperature (50 °C) using the same procedure as that described in the Materials and Methods section. The formation of the

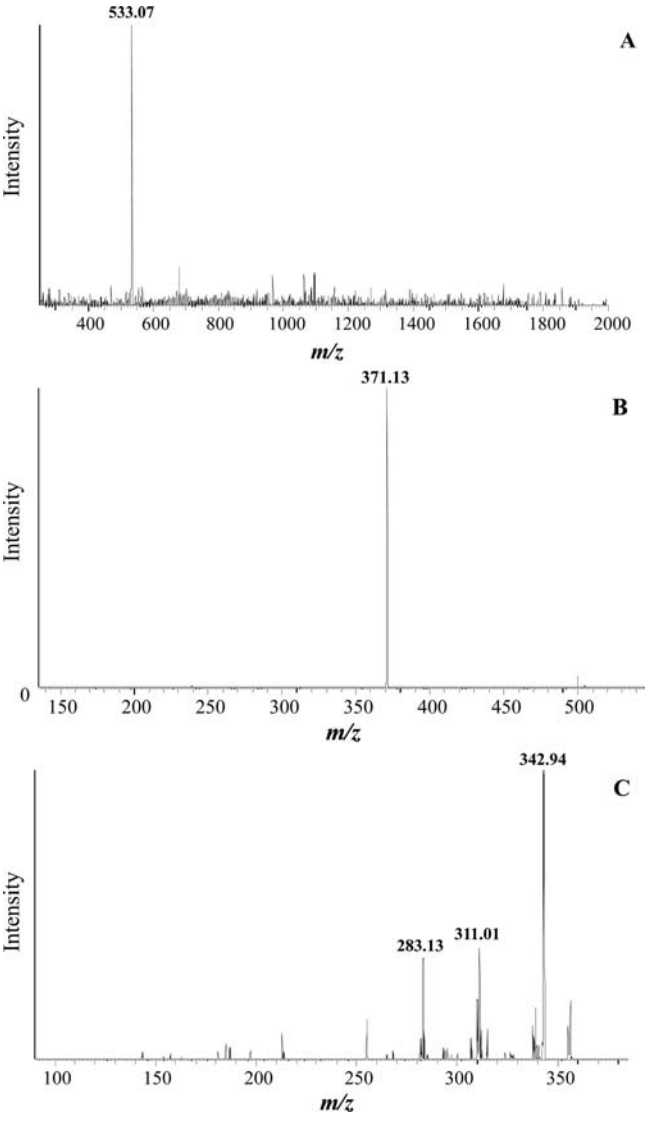

Figure 4. LC/ESI-MS analysis performed in the positive-ion mode of the new derivative peak **II**: **A**, full mass spectrum (quasi molecular ion); **B**, MS^2 spectrum, and **C**, MS^3 spectrum of the major ion fragment in the last spectrum.

corresponding pyranone-anthocyanin derivative (Pigment **II**) was confirmed both by UV−visible and mass spectrometry, following the HPLC separation.

Upon increasing the incubation time, we observed that the amount of vitisin A decreased along with the appearance of a new chromatographic peak. The HPLC-DAD analysis (**Figure 6**) of the solution after 25 days of oxidation reaction revealed the presence of vitisin A (a), together with a new significant peak (b) with the same retention time, UV−vis, and mass spectroscopic properties as the major new pigment **II** of the aged wine fraction. These results strongly suggest that the proposed new

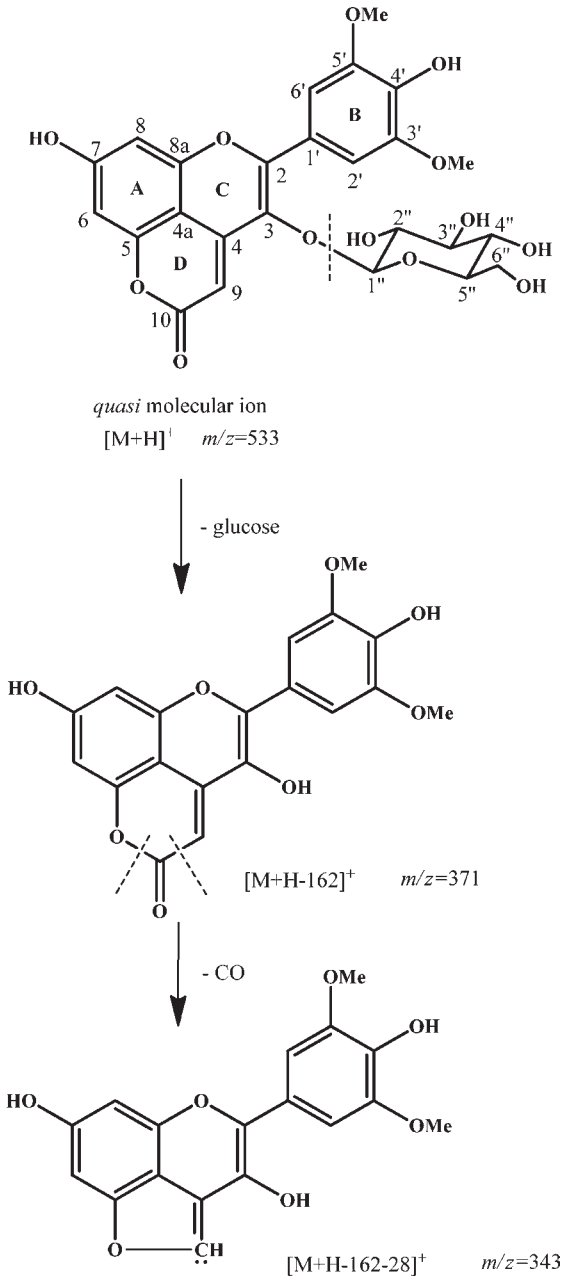

Figure 5. Structure of pyranone-malvidin-3-glucoside and fragmentation pattern obtained from the LC/ESI-MS analysis.

pyranone-anthocyanin derivatives found in aged wine fractions could be formed in wine by oxidative transformation of vitisins. Thereafter, this newly formed pyranone-anthocyanin A was purified by a series of column chromatography, and its structure was further confirmed by mono- and bidimensional NMR spectroscopy (**Table 2**).

Protons H-6 and H-8 of the ring A were attributed to two doublets (J = 1.8 Hz) at 6.59 and 6.51 ppm, respectively. Protons H-2′, 6′, and those of the methoxyl groups at ring B were situated at 7.39 and 3.94 ppm, respectively. Proton H-9 was assigned at 6.01 ppm. Concerning the glucose moiety, the anomeric proton was attributed to a doublet (J = 7.7 Hz) at 4.71 ppm. The coupling constant observed for the anomeric proton suggests a β configuration for the glucose molecule. The proton H-2″ was assigned to a double doublet (J = 7.7 and 9.5 Hz) at 3.49 ppm. Protons H-3″ and H-4″ were attributed to two triplets (J = 9.5 Hz) at 3.36 and 3.22 ppm, respectively. Proton H-5″ was assigned to a multiplet at 3.13 ppm. The proton H-6″a was attributed to a broad doublet (J = 11.2 Hz) at 3.68 ppm. Proton H-6″b was situated at 3.40 ppm.

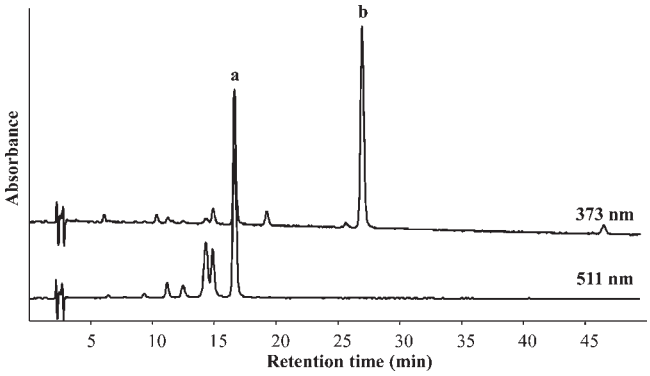

Figure 6. HPLC/DAD chromatograms recorded at 373 and 511 nm of the hydroalcoholic solution containing carboxy-pyranomalvidin-3-glucoside (vitisin A) after 25 days of oxidative incubation: (a), carboxy-pyranomalvidin-3-glucoside; (b), the corresponding pyranone-malvidin-3-glucoside (oxovitisin A).

Table 2. ^{1}H and ^{13}C Chemical Shifts and HMBC and HSQC Correlations of Oxovitisin **A** (Pigment II, Pyranone-malvidin-3-glucoside) Determined in CD_3OD/TFA (98:2)[a]

position	δ^{1}H (ppm); J (Hz)	δ^{13}C	HMBC	HSQC
	Pyranone-malvidin moiety			
2	—	153.6	H-2′, 6′	
3	—	132.5	H-9, 1″	
4	—	n.a.		
4a	—	103.0	H-6, H-8	
5	—	155.6	H-6	
6	6.59; d, 1.8	98.7	H-8	H-6
7	—	164.0	H-6, H-8	
8	6.51; d, 1.8	99.4	H-6	H-8
8a	—	152.3	H-8	
9	6.01; s	92.3		H-9
10	—	165.7	H-9	
1′	—	122.6	H-2′, 6′	
2′, 6′	7.39; s	108.4		H-2′, 6′
3′, 5′	—	148.9	OMe, H-2′, 6′	
4′	—	139.8	OMe, H-2′, 6′	
3′, 5′ − OMe	3.94; s	56.0		OMe
	Glucose Moiety			
1″	4.71; d, 7.7	103.9		H-1″
2″	3.49; dd, 9.5/7.7	76.1		H-2″
3″	3.36; t, 9.5	76.7		H-3″
4″	3.22; t, 9.5	71.6		H-4″
5″	3.13; m	78.0		H-5″
6a″	3.68; bd, 11.2	63.2		H-6a″
6b″	3.40; *	63.2		H-6b″

[a] Key: s, singlet; d, doublet; dd, double doublet; bd, broad doublet; t, triplet; m, multiplet; *, unresolved; n.a., not assigned.

The carbon resonances were attributed using two-dimensional techniques (HSQC and HMBC). Carbons C-6, C-8, C-9, C-2′,6′, and OCH_3 were assigned to the signals at 98.7, 99.4, 92.3, 108.4, and 56.0 ppm, respectively, through their direct ^{1}H−^{13}C correlation in the HSQC spectrum. Carbons C-7 and C-4a were assigned to the signals at 164.0 and 103.0 ppm, respectively, by their correlations in the HMBC spectrum with protons H-6 and H-8. Carbon C-5 and C-8a were attributed to the signals at 155.6 and 152.3 ppm, respectively, by their long distance correlation with

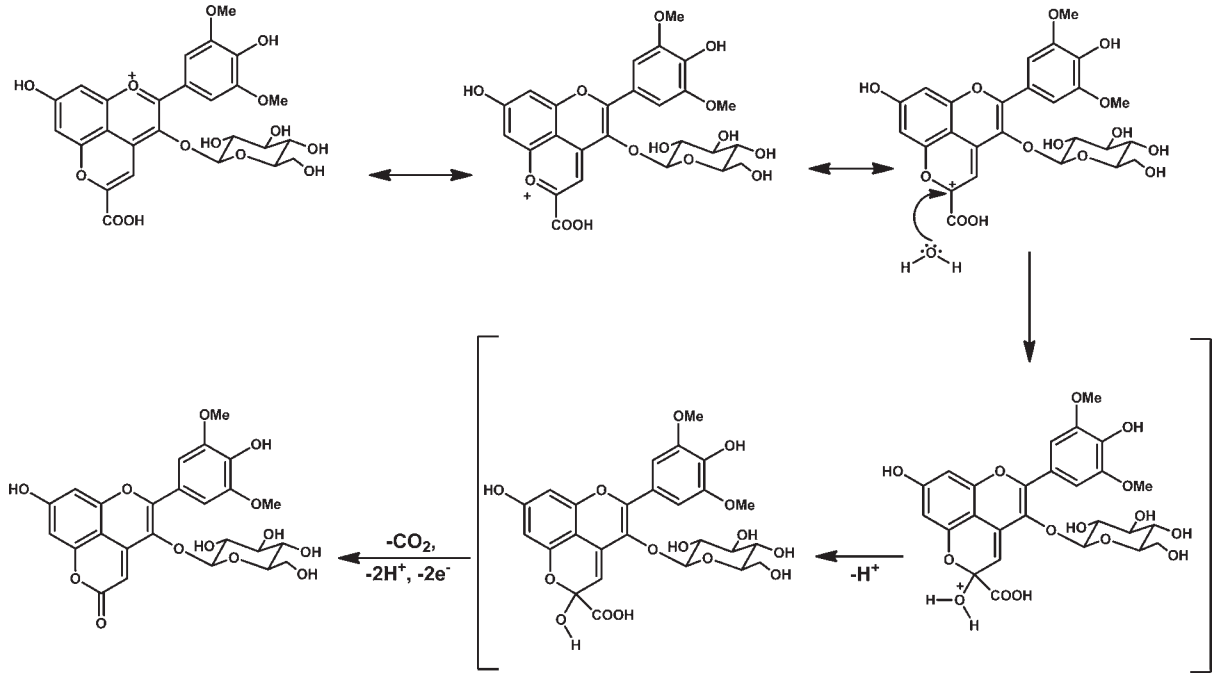

Figure 7. Proposed mechanism for the oxidative formation of pyranone-anthocyanins (oxovitisins).

proton H-6 and H-8, respectively. Carbons C-2, C-1′, C-3′,5′, and C-4′ were attributed to the signals at 153.6, 122.6, 148.9, and 139.8 ppm, respectively, by their long distance correlation with protons H-2′,6′. Carbon C-3 was assigned to the signal at 132.5 ppm through its correlations in the HMBC spectrum with protons H-9 and the anomeric proton H-1″. Carbon C-10 was attributed to the signals at 165.7 ppm by its long distance correlation with proton H-9. The higher chemical shift observed for this carbon, compared with that of other pyranoanthocyanins described in the literature (*11*−*13*, *33*), is due to the presence of an oxo-group attached to this carbon. With respect to the carbons of the glucose molecule, they were assigned by their $^{1}H-^{13}C$ direct correlation in the HSQC spectrum.

Mechanism of Formation. The electrophilic character of carboxy-pyranoanthocyanin pigments was already evidenced in some reactions occurring in wines and leading to the formation of portisins, pyranoanthocyanin dimers, etc (*18*−*21*). Thus, the carboxy-pyranoanthocyanin pigments can undergo hydration at their positively charged carbon C-10 (*34*). On the basis of these data, we propose that the pyranone-anthocyanin A may arise from the nucleophilic attack of water to the electrophilic C-10 of the carboxy-pyranoanthocyanin, leading to hemiacetal formation (**Figure 7**). Decarboxylation of this intermediate under mild conditions and further oxidation of the hydroxyl group of the hemiacetal to the pyran-2-one result in the formation of the final product, a stabilized neutral pyranone-anthocyanin derivative. The final steps of the proposed mechanism for the oxidative generation of an oxo group present at the carbon C-10 are in agreement with the fact that a pyrylium cation with a hydroxyl anion substituent in the α-position is not the zwitterionic aromatic compound but a neutral unsaturated lactone or α-pyrone, to which class belong the coumarins (*35*). Opposite to anthocyanins, it has been recently shown (*33*) that vitisins B are not in equilibrium with the hemiacetal form resulting from the nucleophilic attack by water. These results show that the nucleophilic attack may occur very slowly and that this should be the first step for the irreversible change of carbonium vitisins to the formation of the neutral pyranone-anthocyanins. Therefore, the resulting new pyranone structure (named herein as oxovitisins) was formed by the oxidation reaction of the pyran ring of vitisins with the addition of a hydroxyl group to give a neutral unsaturated lactone (pyran-2-one).

Because of the pure chemical nature of the novel pathway in without the need for enzymatic support, the oxidative formation of oxovitisins could take place during years of storage. Studies on the stability and evolution of vitisin A pyranoanthocyanins in Port wines during aging (*36*) had shown much higher losses (70%) in wines stored in oak barrels (oxidative conditions) after two years, compared with that of in-bottle aging (9−18%) during the same period of time. The single oxidative transformation of vitisins to form the respective oxovitisins through hydration reactions under wine conditions could be an important pathway contributing to the color changes in wine from the red color of original anthocyanins to the yellow color of oxovitisins through the orange vitisins. However, this work introduces a new family of compounds that involves carboxy-pyranoanthocyanin compounds as precursors in the subsequent stages of wine color evolution. These compounds, together with flavonols and flavones, could represent an important class of compounds that contribute to the yellow hues observed in aged red wines. Nevertheless, further studies are still required in order to elucidate the factors that govern the formation of different pigment families in wine aging and to assess their actual contribution to the color changes of red wines.

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