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Reaction between Hydroxycinnamic Acids and Anthocyanin−Pyruvic Acid Adducts Yielding New Portisins

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Three new anthocyanin-derived pigments were found to occur in a 2-year-old Port red wine. Their structures were elucidated through LC/DAD-MS and NMR analysis and were found to correspond to a pyranoanthocyanin moiety linked to substituted cinnamyl substituents. The structures of these compounds are very similar to the one already reported for portisins, with a phenolic moiety replacing the catechin moiety. The newly formed anthocyanin-derived compounds display a bathochromic shift of the λ_{max} (∼540 nm) when compared with their anthocyanin−pyruvic acid adduct precursor (λ_{max} = 511 nm), which may be due to the extended conjugation of the π electrons in the structures of those pigments. Studies performed in model solutions helped to clarify the formation mechanism of these pigments that can result from the nucleophilic attack of the olefinic double bond of a hydroxycinnamic acid to the eletrophilic C-10 position of the anthocyanin−pyruvic acid adduct, followed by the loss of a formic acid molecule and decarboxylation. The chromatic characterization of these malvidin-3-glucoside-derived compounds revealed a higher resistance to discoloration against the nucleophilic attack by water and bisulfite when compared to malvidin-3-glucoside that is almost converted into its colorless hemiacetal form. However, the resistance to discoloration of these new pigments is not as high as the one reported for catechin-derived portisins. This could be explained by the presence of a smaller group (hydroxycinnamyl group), which does not protect so efficiently the chromophore against nucleophilic attack at the C-2 position. The occurrence of these pigments in red wine highlights new chemical pathways involving anthocyanin−pyruvic acid derivatives as precursors for the formation of new pigments in subsequent stages of wine aging that may contribute to its color evolution.

KEYWORDS: Portisins; hydroxycinnamic acids; wine; model solutions; anthocyanin; pyruvic acid

INTRODUCTION

Anthocyanins are the most abundant pigments in young red wines, being responsible for their red/purple color. However, in solution, the color displayed by anthocyanins is dependent on the pH of the medium (*1*, *2*). At the wine pH (pH ∼3.5) anthocyanins are likely to be present mainly in their colorless hemiacetal form. Nevertheless, the flavylium cation structure of anthocyanins is stabilized through copigmentation mechanisms, which protect the molecule chromophore against nucleophilic attack by water. These mechanisms may include molecular association between anthocyanins and other molecules of anthocyanins (self-association) or complexation with other molecules, usually noncolored, referred to as copigments (*3*−*5*). During maturation and aging, the wine color changes from red to red/orange due to several chemical reactions (oxidation, reduction, and polymerization) in which anthocyanins participate, being in this way the precursors of new pigments. **Figure 1** summarizes the different families of anthocyanin-derived pigments that have been detected by HPLC in red wines. The transformations involved in the formation of anthocyanin-derived pigments were first thought to arise mainly from the dimerization between anthocyanins (*6*) and condensation of anthocyanins and flavanols in the presence or absence of acetaldehyde (*7*−*14*). However, over the past years new families of pigments, namely, pyranoanthocyanins, have been identified and are known to result from the reaction between anthocyanins and small molecules such as acetaldehyde (*15*), acetoacetic acid (*16*), pyruvic acid (*17*), vinylphenol (*18*), vinylguaiacol (*19*), and vinylcatechol (*20*). This kind of pigment is supposed to contribute to the orange hues of red wines observed during maturation. In Port red wine, the anthocyanin−pyruvic acid adducts (formed from the reaction of anthocyanins with pyruvic

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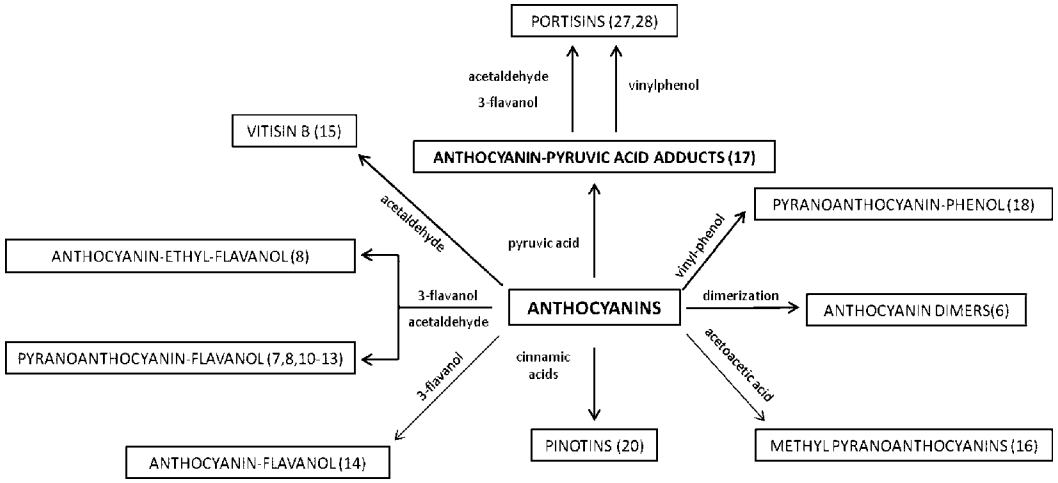

Figure 1. Summary of the main classes of anthocyanin-derived pigments already reported in red wines by HPLC.

acid) were the main pigments detected by HPLC after 1 year of wine aging (*21*).

Hydroxycinnamic acids are phenolic compounds very abundant in grape juice and wine, and the three most common ones are *p*-coumaric acid, caffeic acid, and ferulic acid. In grape berries hydroxycinnamic acids exist as esters of tartaric acid and are named *p*-coutaric acid, caftaric acid, and fertaric acid, respectively. These compounds are present mainly in the pulp of fruits and thus found in grape juices and wines. Caftaric acid is by far the predominant hydroxycinnamic acid in grapes and wines followed by the *p*-coutaric acid and in smaller amounts the fertaric acid (*22*). These naturally occurring esters are susceptible to hydrolysis by the cinnamyl esterase enzyme (*23*), and this occurs in the aqueous acidic solution of wine, releasing the free hydroxycinnamic acids, which are readily detected in a few weeks old wine (*24*). The levels of hydroxycinnamic acids observed in wines are usually 130 mg/L for whites and 60 mg/L for red ones (*25*). With respect to red wines, hydroxycinnamic acids may contribute to their color evolution because they can participate as copigments through hydrophobic stacking interactions with anthocyanin chromophores. Haslam (*26*) has also referred to copigmentation as the first step that led to the formation of a covalent bond between the pigment (anthocyanin) and the copigment (*8*). In fact, it has already been shown that hydroxycinnamic acids can react directly with anthocyanins, giving rise to new pigments with different color hues (*20*).

More recently, a new class of anthocyanin-derived pigments with unusual color properties, presenting a blue color in acidic conditions, were found to occur in a 2-year-old Port wine and were named portisins (*27*). Their structural characterization and studies performed in model solutions revealed that these pigments arise from the reaction between the anthocyanin−pyruvic acid adducts and flavanols in the presence of acetaldehyde (*27*, *28*). The detection and identification of portisins point to new chemical reactions that possibly occur in red wines, in which the major precursors involved are no longer anthocyanins but anthocyanin-derived pigments. The present work deals with the detection in Port wine and characterization of new portisin-type pigments arising from the reaction between anthocyanin−pyruvic acid adducts and hydroxycinnamic acids.

MATERIALS AND METHODS

Reagents. TSK Toyopearl gel HW-40(S) was purchased from Tosoh (Tokyo, Japan); caffeic acid (99%) was purchased from Sigma-Aldrich (Madrid, Spain); sinapic acid (≥97%), ferulic acid (≥98%), and *p*-coumaric acid (≥98%) were purchased from Fluka (Madrid, Spain).

Wine Fractionation. The studied wine was a 2-year-old red Port wine [pH 3.6, 20% alcohol (v/v), total acidity = 6.5 g/L, total SO_2 = 20 mg/L], made from Touriga Nacional and Touriga Francesa varieties (*Vitis vinifera*) grown in the Douro Demarcated Region (northern Portugal). Several fractions were obtained from the Port wine sample using two types of resins, TSK Toyopearl gel HW-40(S) and polyamide (60−80 mesh, SINOPEC, Hunan, China), and different percentages of methanol aqueous solutions. The wine fractionation was performed according to the method of He et al. (*29*). The fractions obtained were analyzed by LC/DAD-MS in positive ion mode.

Synthesis and Purification of Malvidin-3-glucoside−Pyruvic Acid Adduct. Grape skin anthocyanins were extracted with an aqueous solution of ethanol (1:1) acidified with HCl, for 1 day at room temperature. The anthocyanin extract was purified by TSK Toyopearl gel column (250 × 16 mm i.d.) chromatography, the malvidin-3-glucoside fraction being recovered with a solution of 20% (v/v) aqueous ethanol.

The malvidin-3-glucoside−pyruvic acid adduct was synthesized through reaction of the previous purified malvidin-3-glucoside with pyruvic acid and later purified according to the method of Oliveira et al. (*30*).

Reaction of Malvidin-3-glucoside−Pyruvic Acid Adduct with Several Hydroxycinnamic Acids (Caffeic, Sinapic, Ferulic, and *p*-Coumaric Acids) and Isolation of the Newly Formed Pigments. Four aqueous solutions of ethanol (30%, v/v) with a 700 μM concentration of malvidin-3-glucoside−pyruvic acid adduct were prepared. To each solution was added caffeic acid, sinapic acid, ferulic acid, or *p*-coumaric acid to obtain a molar ratio of hydroxycinnamic acid/malvidin-3-glucoside−pyruvic acid adduct of 50:1. The pH of the solutions was adjusted to 1.5 with HCl, and the solutions were left to react at 35 °C in the dark. The formation of new pigments was monitored by HPLC-DAD. After the maximum formation of the pigments had been reached, the reaction was stopped, the solvent was removed, and the major new pigments were purified by Toyopearl gel column (250 × 16 mm i.d.). The elution was carried out with aqueous methanol with increasing percentage of methanol up to 60% (v/v). The 50% (v/v) aqueous methanol fraction, containing the major compounds, was then submitted to isolation of the individual pigments by semi-preparative HPLC. The structure of each compound obtained was elucidated by LC/DAD-MS and NMR.

HPLC Analysis. All of the extracts were analyzed using a Knauer K-1001 HPLC on a 250 × 4.6 mm i.d. reversed-phase C18 column (Merck, Darmstadt, Germany); detection was carried out at 528 and 540 nm using a Knauer K-2800 diode array detector. The solvents were (A) H_2O/HCOOH (9:1) and (B) $CH_3COOH/H_2O/CH_3CN$ (0.5:19.5:80). The gradient consisted of 20−85% B for 70 min at a flow rate of 1.0

Table 1. ^{1}H Chemical Shifts of Vinylpyranomalvidin-3-*O*-glucoside–Catechol (Pigment I), Determined in CD_3OD/TFA (98:2)[a]

position	δ ^{1}H; *J* (Hz)	δ ^{13}C	HMBC	HSQC
pyranomalvidin moiety				
2		160.8	H-2′, 6′	
3		132.8	H-1″	
4		na		
4a		106.8	H-6, 8	
5		152.6	H-6	
6	7.14; bs	99.4		H-6A
7		166.7	H-6, 8	
8	7.30; bs	101.1		H-8A
8a		133.2	H-8	
9	8.08; s	na		
10		167.2	H_α, H_β	
1′		119.2	H-2′, 6′	
2′,6′	7.65; s	107.7		H-2′B, 6′B
3′,5′		147.5	CH_3, H-2′, 6′	
4′		141.5	H-2′, 6′	
3′,5′-OMe	3.98; s	55.6		CH_3
vinylcatechol group				
H_α	7.06; d, 15.7	115.1		H_α
H_β	7.87; d, 15.7	143.9		H_β
1‴		126.7	H_α, H-6‴	
2‴	7.22; s	126.9		H-2‴
3‴		145.8	H-2‴, 5‴	
4‴		149.8	H-2‴, 5‴, 6‴	
5‴	6.85; d, 8.1	114.4		H-5‴
6‴	7.16; d, 8.1	99.6	H_β, H-5‴	
glucose moiety				
1″	4.75; d, 7.7	103.1		H-1″
2″	3.64;*	73.9		H-2″
3″	3.37;*	76.3		H-3″
4″	3.16;*	77.0		H-4″
5″	3.24;*	69.8		H-5″
6a″	3.71;*	61.3		H-6a″, 6b″
6b″	3.42;*	61.3		H-6a″, 6b″

[a] *, unresolved; bs, broad singlet; s, singlet; d, doublet; na, not assigned.

Table 2. ^{1}H Chemical Shifts of Vinylpyranomalvidin-3-*O*-glucoside–Syringol (Pigment II), Determined in CD_3OD/TFA (98:2)[a]

position	δ ^{1}H; *J* (Hz)	δ ^{13}C	HMBC	HSQC
pyranomalvidin moiety				
2		168.9.0	H-2′, 6′	
3		134.4	H-1″	
4		na		
4a		109.3	H-6, 8	
5		148.9	H-6	
6	6.45; bs	105.5		H-6
7		169.5	H-6, 8	
8	7.68; bs	105.6		H-8
8a		143.3	H-8	
9	8.09; s	109.5	H_α	
10		169.3	H_α, H_β	
1′		115.3	H-2′, 6′	
2′,6′	6.89; s	105.5		H-2′, 6′
3′,5′		149.9	CH_3, H-2′, 6′	
4′		139.4	H-2′, 6′	
3′,5′-OMe	3.88; s	56.7		CH_3
vinylsyringol group				
H_α	6.38; d, 15.9	114.9		H_α
H_β	7.59; d, 15.9	145.8		H_β
1‴		127.2	H_α, H_β, H-2‴, 6‴	
2‴,6‴	6.89; s	105.5		H-2‴, 6‴
3‴,5‴		147.1	H-2‴, 6‴	
4‴		139.4	H-2‴, 6‴	
3‴,5‴-OMe	3.88; s	56.7		CH_3
glucose moiety				
1″	4.80; d, 7.7	104.0		H-1″
2″	3.65; t, 7.7	74.8		H-2″
3″	3.41; *	77.0		H-3″
4″	3.27; *	70.2		H-4″
5″	3.21; *	77.6		H-5″
6a″	3.72; *	61.5		H-6a″, 6b″
6b″	3.43; *	61.5		H-6a″, 6b″

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

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. A Finnigan Surveyor series liquid chromatograph, equipped with a 150 × 4.6 mm i.d., 5 μm LicroCART reversed-phase C18 column thermostated 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 electrospray ionization (ESI) interface. Solvents were (A) H_2O/TFA (99.9:0.1) and (B) $CH_3COOH/H_2O/CH_3CN$ (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 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 relative collision energies of 30 and 60.

NMR. ^{1}H NMR (500.13 MHz) and ^{13}C NMR (125.77 MHz) spectra were measured in CD_3OD/TFA (98:2) on a Bruker-Avance 500 spectrometer at 298 K with TMS as internal standard. ^{1}H chemical shifts were assigned using 1D and 2D ^{1}H NMR (*g*COSY and NOESY), whereas ^{13}C resonances were assigned using 2D NMR techniques (*g*HMBC and *g*HSQC) (*31*, *32*). The delay for the long-range C/H coupling constant was optimized to 7 Hz. NMR data of pigments I, II, and III are summarized in **Tables 1**, **2**, and **3**, respectively. The data obtained for pigment IV (vinylpyranomalvidin-3-*O*-glucoside−phenol) are already described in the literature (*33*).

RESULTS AND DISCUSSION

Pigment Detection in Port Wine. Different classes of anthocyanin-derived pigments occurring in red wine such as pyranoanthocyanins have been described over the past years, and others are still being researched. Port wine has always been found to be a good source for searching for new polyphenolic compounds due to its physical−chemical characteristics (*21*). Wines are a very complex matrix, which makes it difficult to screen for new pigments. Therefore, wine samples must be fractionated to simplify the further interpretation by liquid chromatography coupled to mass spectrometry (LC/DAD-MS). For this work, a previously developed procedure was used for the fractionation of wine samples using two types of resins: TSK Toyopearl HW-40(S) and polyamide (60−80 mesh) gel (*29*). As a result, several fractions were obtained and analyzed by LC/DAD-MS. The aim was to detect chromatographic peaks that display a λ_{max} in the visible range different from those of genuine anthocyanins or other known pigments. Special emphasis was given to compounds displaying a higher λ_{max} than that of anthocyanins. Several chromatographic peaks with these features were detected in some wine fractions and were assigned to different known portisins (*34*). Among these compounds, in the fraction T50P50 [obtained first from the Toyopearl gel with a 50% (v/v) methanol aqueous solution and then subfractionated in a polyamide resin and recovered with a methanol aqueous solution 50% (v/v)] a chromatographic peak was detected with a $\lambda_{max} \sim 540$ nm that revealed a $[M]^+$ molecular ion at *m*/*z* 635 with a fragment ion $[M - 162]^+$ at *m*/*z* 473, possibly corresponding to the loss of the glucosyl moiety. This molecular ion was attributed to the portisin already described in the literature, vinylpyranomalvidin-3-*O*-glucoside−phenol (*33*).

The same fraction revealed two other $[M]^+$ molecular ions at *m*/*z* 797 and 783. These peaks have a major fragment ion $[M - 308]^+$ at *m*/*z* 489 and 475, respectively, possibly correspond-

Table 3. ^{1}H Chemical Shifts of Vinylpyranomalvidin-3-*O*-glucoside–Guaiacol (Pigment III), Determined in CD_3OD/TFA (98:2)[a]

position	δ ^{1}H; *J* (Hz)	δ ^{13}C	HMBC	HSQC
pyranomalvidin moiety				
2		162.7	H-2′, 6′	
3		na		
4		na		
4a		108.8	H-6, 8	
5		153.6	H-6	
6	7.14; bs	100.8		H-6
7		167.9	H-6, 8	
8	7.32; bs	102.6		H-8
8a		134.4	H-8	
9	8.08; s	na		
10		169.4	H_α, H_β	
1′		120.9	H-2′, 6′	
2′,6′	7.67; s	109.4		H-2′, 6′
3′,5′		149.5	CH_3, H-2′, 6′	
4′		143.3	H-2′, 6′	
3′,5′-OMe	3.99; s	56.8		CH_3
vinylguaiacol group				
H_α	6.35; d, 15.8	115.7		H_α
H_β	7.60; d, 15.8	146.5		H_β
1‴		127.5	H_α, H_β, 2‴, 5‴, 6‴	
2‴	7.17; dd, 8.1/1.3	111.2		H-2‴
3‴		150.0	H-2‴, 5‴	
4‴		146.5	H-2‴, 5‴, 6‴	
5‴	6.81; d, 8.1	116.5		H-5‴
6‴	7.07; dd, 8.1/1.3	124.0		H-6‴
3‴-OMe	3.96; s	56.2		CH_3
glucose moiety				
1″	4.74; d, 7.8	104.7		H-1″
2″	3.65; *	71.2		H-2″
3″	3.36; *	77.9		H-3″
4″	3.23; *	71.4		H-4″
5″	3.16; *	78.8		H-5″
6a″	3.70; *	62.5		H-6a″, 6b″
6b″	3.41; *	62.5		H-6a″, 6b″

[a] *, unresolved; bs, broad singlet; s, singlet; d, doublet; dd, double doublets; na, not assigned.

ing to the loss of a coumaroylglucosyl moiety. The similarity of the UV−vis spectrum and the fragmentation pattern between these two compounds with the previously identified vinylpyranomalvidin-3-*O*-glucoside−phenol suggested that they might have a closer structure bearing a different phenolic moiety, such as catechol. The molecular ions at *m*/*z* 797 and 783 concur with the structures of vinylpyranomalvidin-3-*O*-coumaroylglucoside−catechol and vinylpyranopetunidin-3-*O*-coumaroylglucoside−catechol, respectively.

The compound already reported in the literature, vinylpyranomalvidin-3-*O*-glucoside−phenol, had been found to arise from the reaction between malvidin-3-glucoside−pyruvic acid adduct and vinylphenol (*33*). The latter precursor could possibly be formed from the decarboxylation of *p*-coumaric acid (*35*). By similarity, the other two pigments could arise from the reaction of malvidin-3-coumaroylglucoside and petunidin-3-coumaroylglucoside pyruvic acid adducts with vinylcatechol, formed from decarboxylation of caffeic acid.

Pigment Formation in Model Solution. To clarify the structures of these pigments, their formation was thus attempted in model solutions through the reaction between anthocyanin−pyruvic acid adducts and hydroxycinnamic acids such as caffeic, sinapic, ferulic, or *p*-coumaric acid, which can all be present in Port wines (*18*, *20*, *35*, *36*). A malvidin-3-glucoside−pyruvic acid adduct extract was left to react with the hydroxycinnamic acids in aqueous ethanol solutions, and the formation of new pigments was monitored by HPLC/DAD. After 5 days, it was possible to observe the appearance of new peaks in the HPLC chromatogram (**Figure 2**). These compounds revealed a λ_{max} (~540 nm) bathochromically shifted when compared with that observed for the malvidin−pyruvic acid adduct precursor (λ_{max} = 511 nm). The retention time and the λ_{max} are similar to those of the pigments detected in the wine fraction. After 14 days, the maximum formation of the pigments was reached, and the

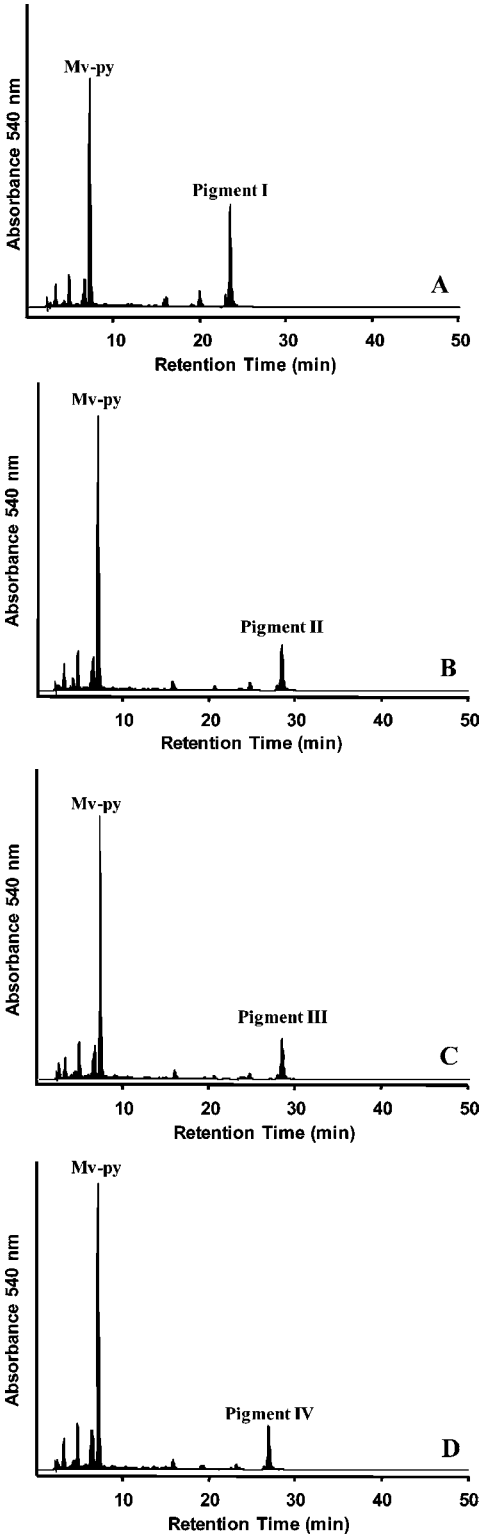

Figure 2. Chromatograms recorded from the HPLC-DAD at 540 nm of the reaction of a malvidin-3-glucoside–pyruvic acid adduct (mv-3-glc-py) extract with different hydroxycinnamic acids after 14 days of reaction: (**A**) caffeic acid (pigment I); (**B**) sinapic acid (pigment II); (**C**) ferulic acid (pigment III); (**D**) *p*-coumaric acid (pigment IV).

Table 4. Portisins Identified in Port Wine Fractions and Model Solutions by LC/DAD-MS

m/z (M^+)	peak identity	*m/z*	fragment
651*	vinylpyranomalvidin-3-*O*-glucoside–catechol	489	vinylpyranomalvidin–catechol
695*	vinylpyranomalvidin-3-*O*-glucoside–syringol	533	vinylpyranomalvidin–syringol
665*	vinylpyranomalvidin-3-*O*-glucoside-–guaiacol	503	vinylpyranomalvidin–guaiacol
635[26]**	vinylpyranomalvidin-3-*O*-glucoside–phenol	473	vinylpyranomalvidin–phenol
783***	vinylpyranopetunidin-3-*O*-coumaroylglucoside–catechol	475	vinylpyranopetunidin–catechol
797***	vinylpyranomalvidin-3-*O*-coumaroylglucoside–catechol	489	vinylpyranomalvidin–catechol

[a] *, model solutions; **, model solution and Port wine; ***, Port wine.

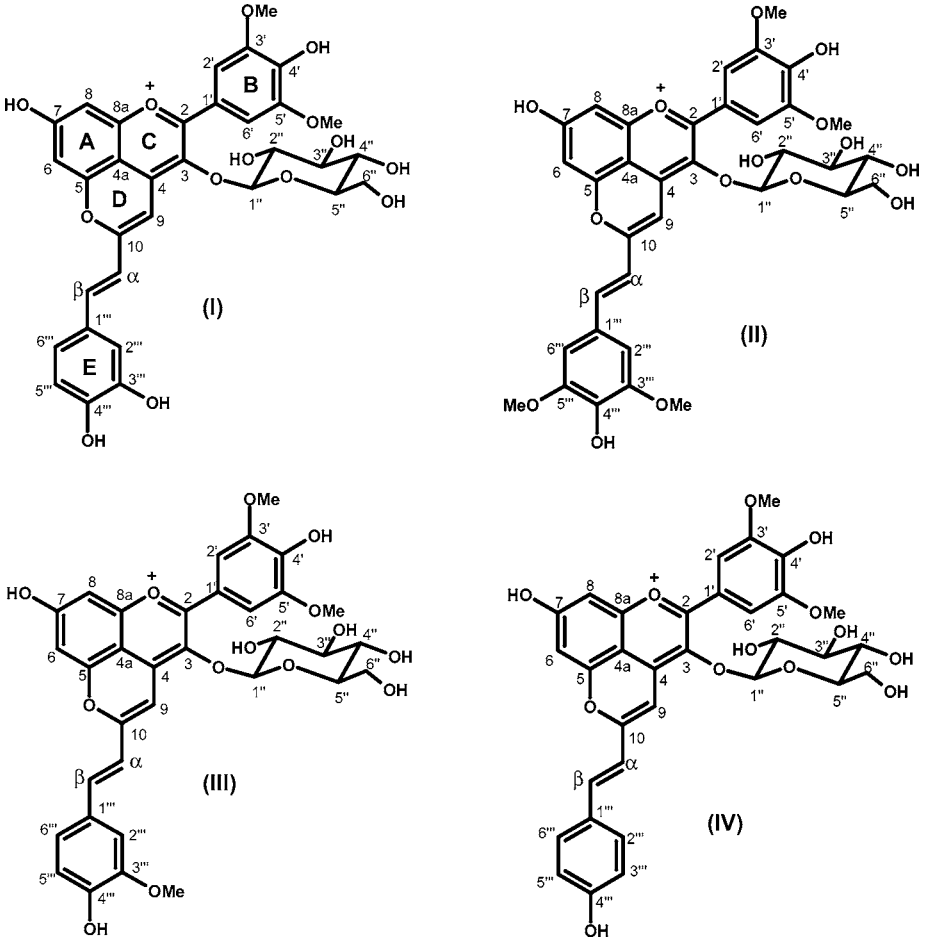

Figure 3. Structures of the synthesized pigments: vinylpyranomalvidin-3-*O*-glucoside–catechol (I); vinylpyranomalvidin-3-*O*-glucoside–syringol (II); vinylpyranomalvidin-3-*O*-glucoside–guaiacol (III); vinylpyranomalvidin-3-*O*-glucoside–phenol (IV).

major compounds were purified for further structural characterization. The purification was performed by TSK Toyopearl gel HW-40(S) column chromatography, and the main pigments were isolated by semipreparative HPLC. Their structures were elucidated by LC/DAD-MS analysis (**Table 4**) and by 1D and 2D NMR analysis (**Tables 1−3**). The structural characterization of the pigments revealed a structure in which a pyranoanthocyanin moiety is linked to a hydroxycinnamyl substituent such as vinylcatechol (pigment I), vinylsyringol (pigment II), vinylguaiacol (pigment III), or vinylphenol (pigment IV) (**Figure 3**).

The formation mechanism of these pigments can involve a nucleophilic attack of the olefinic double bond of the hydroxycinnamyl derivative to the C-10 position of the malvidin-3-glucoside−pyruvic acid adduct that presents eletrophilic characteristics, followed by the loss of a formic acid molecule and a decarboxylation (**Figure 4**).

The formation kinetics of these compounds are different, and this may be explained by the different substitution patterns of ring E. The reaction kinetics of the di- and trisubstituted acids (pigments I−III) with malvidin-3-glucoside−pyruvic acid adduct seem to be slightly enhanced when compared to the monosubstituted one (pigment IV). This feature has already been described in the literature and is in agreement with the fact that electron-donating substituents such as hydroxyl and methoxyl groups stabilize electron-deficient transition states (*36*).

Pigment Chromatic Features. Following this, some chromatic features of these compounds were also studied, namely, their color resistance toward discoloration by the nucleophilic attack by water (color stability at different pH values) and by bisulfite anion. In acid aqueous buffer conditions (pH 1.0) the four pigments (I−IV) obtained present a red/violet color. At these pH conditions, the λ_{max} of the pigments in the visible region is located at 537, 540, 537, and 533 nm, respectively.

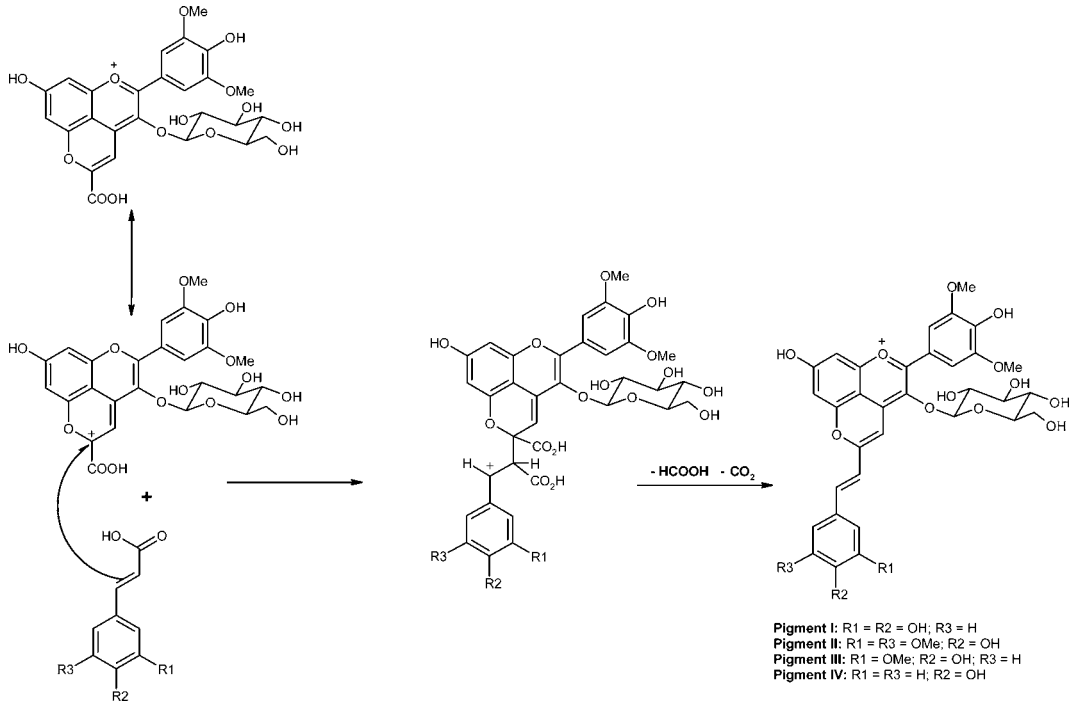

Figure 4. Proposed mechanism for the synthesis of the hydroxycinnamyl-derived portisins.

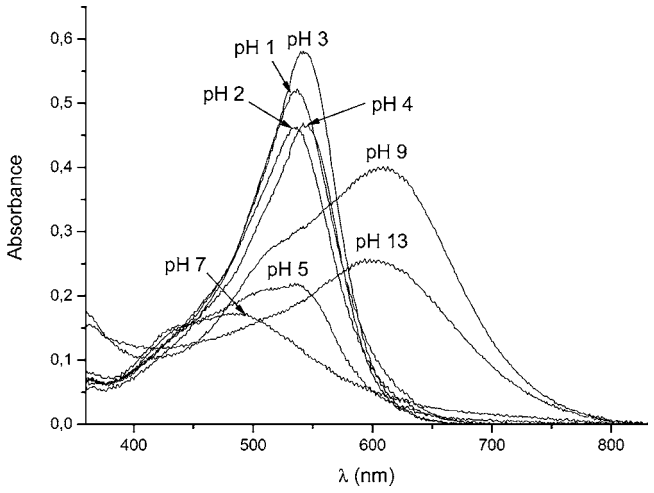

Figure 5. UV–vis spectra of pigment I in model solution at different pH values (1.0–11.0).

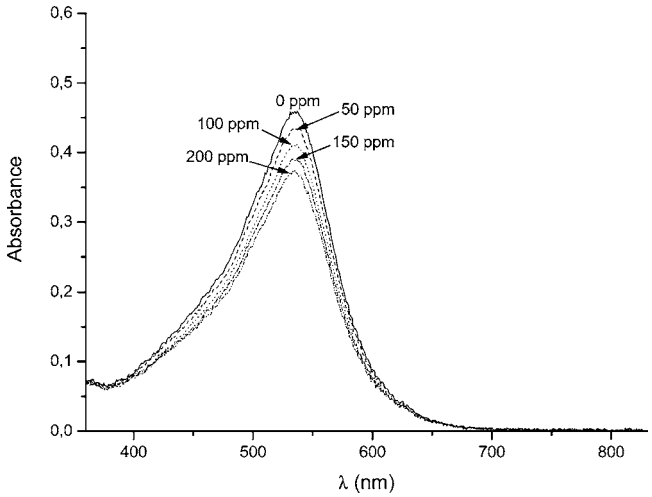

Figure 6. UV–vis spectra of pigment I at pH 2.0 with increasing amounts of bisulfite (0–200 ppm).

These values are bathochromically shifted to the one observed for the malvidin-3-glucoside−pyruvic acid adduct precursor (511 nm) in similar conditions (data not shown). The substitution pattern (hydroxylation and/or methoxylation) of ring E affected slightly the λ_{max} of these pigments. The resistance to discoloration against the nucleophilic attack by water or bisulfite anion of these four pigments has been revealed to be similar. Thus, the results obtained for pigment I will be used as an example. The color displayed by pigment I was shown to change only slightly with increasing pH of the aqueous solutions (pH 1.0−4.0) (**Figure 5**). At the same pH conditions, malvidin-3-glucoside is almost converted into its colorless hemiacetal form (*37*). The higher resistance of the compounds described herein to discoloration can be explained as for the portisins previously described in the literature (*37*), by a greater protection of the chromophore moiety against the nucleophilic attack by water to give the respective hemiacetal form. However, the resistance to discoloration of these new pigments is not as high as that reported for vinylpyranoanthocyanin−catechin pigments (*37*).

This minor resistance may be explained by the presence of a smaller group (hydroxycinnamyl group), which does not protect so efficiently the chromophore against the nucleophilic attack of water at the C-2 position. The bathochromic shift observed in the λ_{max} for higher pH values (9.0 and 11.0) probably corresponds to the equilibrium displacement toward the formation of the quinoidal forms of the pigments. The hydroxycinnamyl-derived pigments also revealed a great resistance to discoloration against the nucleophilic attack by bisulfite (**Figure 6**), especially when compared to the malvidin-3-glucoside solution that become almost uncolored after the addition of high concentrations of SO_2 (200 ppm) (*37*). This feature has previously been shown to occur with the vinylpyranoanthocyanin−catechin pigments (*37*). According to Bérké et al., the nucleophilic addition of anionic bisulfite, in the anthocyanin structure, can occur at the positively charged C-4 position. The lower steric hindrance in C-4 makes this position more available to nucleophilic attack by bisulfite (*38*). Like vinylpyranoanthocyanin−catechin pigments, these new pigments have a substitute

group in C-4 (ring D), which prevents nucleophilic attack of bisulfite in that position.

Anthocyanin−pyruvic acid adducts and hydroxycinnamic acids, the precursors of the newly formed pigments reported herein, are present in appreciable amounts in red wines (*12*, *15*, *17*, *21*−*26*). In fact, pigments derived from hydroxycinnamic acids have already been identified in wines (*18*, *20*). The characterization of these pigments highlights new chemical pathways involving anthocyanin−pyruvic acid derivatives as precursors for the formation of new pigments in subsequent stages of wine aging that may contribute to its color evolution.

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LITERATURE CITED

(1) Brouillard, R. The *in vivo* expression of anthocyanins colour in plants. *Phytochemistry* **1983**, *22*, 1311−1323.

(2) Von Elbe, J. H.; Schwartz, S. J. Colorants. In *Food Chemistry*, 3rd ed.; Fennema, O. R., Ed.; Dekker: New York, 1996; pp 651−723.

(3) Brouillard, R.; Dangles, O. Anthocyanin molecular interactions: the first step in the formation of new pigments during wine aging? *Food Chem.* **1994**, *51*, 365−371.

(4) Goto, T.; Kondo, T. Structure and molecular stacking of anthocyanins−flower color variation. *Angew. Chem. Int.* **1991**, *30*, 17−33.

(5) Mistry, T. V.; Cai, Y.; Lilley, T. H.; Haslam, E. Polyphenol interactions. Part 5. Anthocyanin copigmentation. *J. Chem. Soc., Perkin Trans. 2* **1991**, 1287−1296.

(6) Atanasova, V.; Fulcrand, H.; Cheynier, V.; Moutounet, M. Effect of oxygenation on polyphenol changes occurring in the course of wine-making. *Anal. Chim. Acta* **2002**, *458*, 15−27.

(7) Somers, T. C. The polymeric nature of wine pigments. *Phytochemistry* **1971**, *10*, 2175−2186.

(8) Liao, H.; Cai, Y.; Haslam, E. Polyphenols interactions. Anthocyanins: Copigmentation and colour changes in red wines. *J. Agric. Food Chem.* **1992**, *59*, 299−305.

(9) Rivas-Gonzalo, J. C.; Bravo-Haro, S.; Santos-Buelga, C. Detection of compounds formed through the reaction of malvidin 3-monoglucoside and catechin in the presence acetaldehyde. *J. Agric. Food Chem.* **1995**, *43*, 1444−1449.

(10) Timberlake, C. F.; Bridle, P. Interactions between anthocyanins, phenolic compounds and acetaldehyde and their significance in red wines. *Am. J. Enol. Vitic.* **1976**, *27*, 97−105.

(11) Vivar-Quintana, A. M.; Santos-Buelga, C.; Francia-Aricha, E.; Rivas-Gonzalo, J. C. Formation of anthocyanin-derived pigments in experimental red wines. *Food Sci. Technol. Int.* **1999**, *5*, 347.

(12) Mateus, N.; Pascual-Teresa, S.; Rivas-Gonzalo, J. C.; Santos-Buelga, C.; de Freitas, V. Structural diversity of anthocyanin-derived pigments in port wines. *Food Chem.* **2002**, *76*, 335−342.

(13) Francia-Aricha, E. M.; Guerra, M. T.; Rivas-Gonzalo, J. C.; Santos-Buelga, C. New anthocyanin pigments formed after condensation with flavanols. *J. Agric. Food Chem.* **1997**, *45*, 2262−2266.

(14) Salas, E.; Atanasova, V.; Poncet-Legrand, C.; Meudec, E.; Mazauric, J. P.; Cheynier, V. Demonstration of the occurrence of flavanol−anthocyanin adducts in wine and in model solutions. *Anal. Chim. Acta* **2004**, *513*, 325−332.

(15) *15*) Bakker, J.; Timberlake, C. F. Isolation, identification, and characterization of new color-stable anthocyanins occurring in some red wines. *J. Agric. Food Chem.* **1997**, *45*, 35−43.

(16) He, J.; Santos-Buelga, C.; Silva, A. M. S.; Mateus, N.; de Freitas, V. Isolation and structural characterization of new anthocyanin-derived pigments in aged red wines. *J. Agric. Food Chem.* **2006**, *54*, 9598−9603.

(17) Fulcrand, H.; Benabdeljalil, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. A new class of wine pigments generated by reaction between pyruvic acid and grape anthocyanins. *Phytochemistry* **1998**, *47*, 1401−1407.

(18) Fulcrand, H.; Cameira dos Santos, P. J.; Sarni-Manchado, P.; Cheynier, V.; Favre-Bonvin, J. Structure of new anthocyanin-derived wine pigments. *J. Chem. Soc., Perkin Trans. 1* **1996**, 35−739.

(19) Hayasaka, Y.; Asenstorfer, R. E. Screening for potential pigments derived from anthocyanins in red wine using nanoelectrospray tandem mass spectrometry. *J. Agric. Food Chem.* **2002**, *50*, 756−761.

(20) Schwarz, M.; Jerz, G.; Winterhalter, P. Isolation and structure of pinotin A, a new anthocyanin derivative from Pinotage wine. *Vitis* **2003**, *42*, 105−106.

(21) Mateus, N.; de Freitas, V. A. P. Evolution and stability of anthocyanin-derived pigments during Port wine aging. *J. Agric. Food Chem.* **2001**, *49*, 5217−5222.

(22) Ong, B. Y.; Nagel, C. W. Hydroxycinnamic acid−tartaric acid ester content in mature grapes and during the maturation of white Riesling grapes. *Am. J. Enol. Vitic.* **1978**, *29*, 277−281.

(23) Somers, T. C.; Verette, E.; Pocock, K. F. Hydroxycinnamates esters of *V. vinifera*: changes during white vinification and effects of exogenous enzyme hydrolysis. *J. Sci. Food Agric.* **1987**, *40*, 67−78.

(24) Singleton, V. L.; Zaya, J.; Trousdale, E. Compositional changes in ripening grapes: caftaric and coutaric acids. *Vitis* **1986**, *25*, 107−117.

(25) Waterhouse, A. L. Wine phenolics. *Ann. N. Y. Acad. Sci.* **2002**, *957*, 21−36.

(26) Haslam, E. *Plant Polyphenols, Vegetable Tannins Revisited*; Cambridge University Press: Cambridge, U.K., 1989.

(27) Mateus, N.; Silva, A. M. S.; Rivas-Gonzalo, J. C.; Santos-Buelga, C.; Freitas, V. A new class of blue anthocyanin-derived pigments isolated from red wines. *J. Agric. Food Chem.* **2003**, *51*, 1919−1923.

(28) Mateus, N.; Oliveira, J.; Santos-Buelga, C.; Silva, A. M. S.; de Freitas, V. A. P. NMR structure characterization of a new vinylpyranoanthocyanin−catechin pigment. *Tetrahedron Lett.* **2004**, *45*, 3455−3457.

(29) He, J.; Santos-Buelga, C.; Mateus, N.; de Freitas, V. Isolation and quantification of oligomeric pyranoanthocyanin−flavanol pigments from red wines by combination of column chromatographic techniques. *J. Chromatogr., A* **2006**, *1134*, 215−225.

(30) Oliveira, J.; Fernandes, V.; Miranda, C.; Santos-Buelga, C.; Silva, A.; de Freitas, V.; Mateus, N. Color properties of four cyanidin−pyruvic acid adducts. *J. Agric. Food Chem.* **2006**, *54*, 6894−6903.

(31) Bax, A.; Subramanian, S. Sensitivity enhanced two-dimensional heteronuclear shift correlation NMR spectroscopy. *J. Magn. Res.* **1986**, *67*, 565−569.

(32) Bax, A.; Summers, M. F. ^{1}H and ^{13}C assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. *J. Am. Chem. Soc.* **1986**, *108*, 2093−2094.

(33) Mateus, N.; Oliveira, J.; Pissarra, J.; González-Paramás, A. M.; Rivas-Gonzalo, J. C.; Santos-Buelga, C.; Silva, A. M. S.; de Freitas, V. A new vinylpyranoanthocyanin pigment occurring in aged red wine. *Food Chem.* **2006**, *97*, 689−695.

(34) Mateus, N.; Oliveira, J.; González-Paramás, A. M.; Santos-Buelga, C.; de Freitas, V. Screening of portisins (vinylpyranoanthocyanin pigments) in port wine by LC/DAD-MS. *Food Sci. Technol. Int.* **2005**, *11*, 353−358.

(35) Chatonnet, P.; Dubourdieu, D.; Boidron, J. N.; Lavigne, V. Synthesis of volatile phenols by *Saccharomyces cerevisiae* in wines. *J. Sci. Food Agric.* **1993**, *62*, 191−202.

(36) Schwarz, M.; Wabnitz, T. C.; Winterhalter, P. Pathway leading to the formation of anthocyanin−vinylphenol adducts and related pigments in red wines. *J. Agric. Food Chem.* **2003**, *51*, 3682−3687.

(37) Oliveira, J.; Santos-Buelga, C.; Silva, A. M. S.; de Freitas, V.; Mateus, N. Chromatic and structural features of blue anthocyanin-derived pigments present in Port wine. *Anal. Chim. Acta* **2006**, *563*, 2−9.

(38) Berké, B.; Chèze, C.; Vercauteren, J.; Deffieux, G. Bisulfite addition to anthocyanins: revisited structures of colourless adducts. *Tetrahedron Lett.* **1998**, *39*, 5771−5774.

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