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Structural Characterization of New Malvidin 3-Glucoside−Catechin Aryl/Alkyl-Linked Pigments

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The condensation reaction between malvidin 3-glucoside and catechin mediated by isobutyraldehyde, benzaldehyde, and isovaleraldehyde was conducted in model solutions at two pH values (1.5 and 3.2). The formation of new alkyl/aryl-linked adducts corresponding to the structures malvidin 3-glucoside−isobutylcatechin, malvidin 3-glucoside-benzylcatechin, and malvidin 3-glucoside−3-methylbutylcatechin was respectively observed from each aldehyde. The structural characterization of these new structures has been elucidated by 1D and 2D NMR, mass spectrometry, and UV−vis techniques. These new adducts showed the same λ_{max} in the visible region at 540 nm, which is bathochromically shifted 15 nm when compared with the original anthocyanin (λ_{max} = 525 nm).

KEYWORDS: Pigment; anthocyanin; catechin; isobutyraldehyde; benzaldehyde; isovaleraldehyde

INTRODUCTION

The visual appreciation of a red wine is strongly related to its quality. During aging, the wine color undergoes some important changes from an initial purple-red to a more reddish brown hue, mainly due to progressive structural changes of anthocyanins. The reaction of these pigments with tannins, directly or mediated by other molecules such as acetaldehyde, is supposed to play a crucial role in color evolution, leading to the formation of new pigments with different chromatic features (*1*, *2*).

The contribution of acetaldehyde to the wine color evolution has been long recognized (*3*−*6*). Its involvement in anthocyanin/flavanol associations leading to the formation of ethyl-linked adducts has been extensively studied in model solutions (*7*−*12*) and pigments resulting from this reaction recently detected in wines (*13*−*16*). Furthermore, acetaldehyde can also intervene in the formation of other anthocyanin-derived pigments such as pyranoanthocyanins (*16*−*21*) and vinylpyranoanthocyanins (portisins) recently described in Port wines (*22*).

The diversity and levels of aldehydes in Port red wine are much higher compared to those in common table red wines, because the wine spirit used to stop the fermentation in Port winemaking is rich in aldehydes and alcohols susceptible to oxidation during aging, leading to the formation of new aldehydes (*23*, *24*). Thus, apart from acetaldehyde, other minor aldehydes such as propionaldehyde, isovaleraldehyde, isobutyraldehyde, and benzaldehyde contribute to the aldehyde composition in wine spirits. The total aldehyde content present in the commercial wine spirits used in the Port wine industry could ranges between 30 and 250 mg/L (data not shown).

Minor aldehydes are supposed to interact with both anthocyanins and flavanols similarly to the mechanism described for acetaldehyde in the literature. Recent studies have shown that these aldehydes induce some chromatic changes in wine model solutions due to the formation of adducts between anthocyanins and flavanols linked by alkyl/aryl bridges as detected by LC-MS (*25*, *26*).

The aim of the present work was to structurally characterize the newly formed pigments resulting from the interaction between malvidin 3-glucoside and (+)-catechin in the presence of isobutyraldehyde, benzaldehyde, and isovaleraldehyde.

MATERIALS AND METHODS

Standards. (+)-Catechin and isobutyraldehyde were purchased from Sigma Chemical Co. (St. Louis, MO), isovaleraldehyde was purchased from Fluka Chemika (Buchs, Switzerland), and benzaldehyde was obtained from Merck (Darmstadt, Germany).

Isolation of Malvidin 3-Glucoside. Anthocyanins were extracted from red grape skins (*Vitis vinifera*) with 40% aqueous ethanol. Grape skin extract was applied onto a 300 × 250 mm TSK Toyopearl gel HW-40(S) (Tosoh) column and eluted with water acidified with 2% HCl at a flow rate of 0.8 mL/min in order to yield nonacylated

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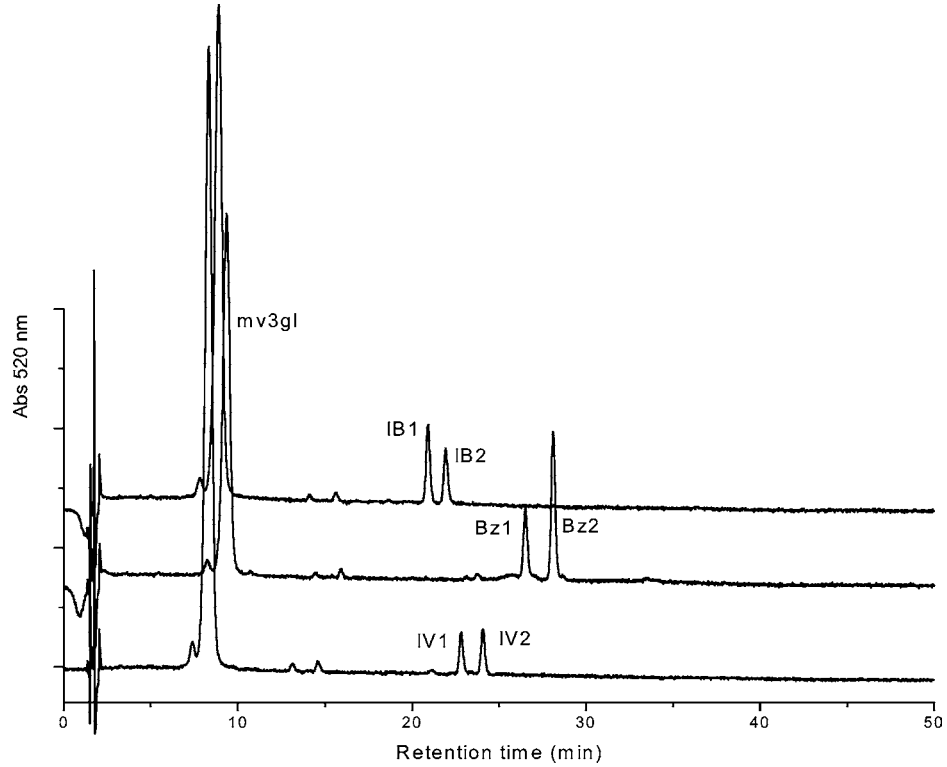

Figure 1. HPLC chromatograms recorded at 520 nm of model solutions (pH 3.2, 35 °C) containing malvidin 3-glucoside, catechin, and different aldehydes: isobutyraldehyde (IB1 + IB2), benzaldehyde (Bz1 + Bz2), and isovaleraldehyde (IV1 + IV2), after 29 days of reaction.

anthocyanins. The isolation of malvidin 3-glucoside was performed by semipreparative HPLC using a 250 × 4.6 mm i.d. reversed-phase C18 column as reported elsewhere (*25*).

Preparation of Anthocyanin-Derived Pigments. Solutions (200 mL) composed of malvidin 3-glucoside (2 mM)/catechin/aldehyde (molar ratio of 1:4:10) were prepared in 12% ethanol, pH 1.5, for each aldehyde (isobutyraldehyde, isovaleraldehyde, and benzaldehyde). These solutions were kept at 35 °C and protected from light to obtain the maximum amount of adducts from the respective aldehyde. The appearance of newly formed pigments was followed by HPLC-DAD. The kinetics studies were performed following similar experimental conditions in two sets at pH 1.5 and 3.2.

HPLC Analysis. Samples were analyzed by HPLC-DAD using a 250 × 4.6 mm i.d. reversed-phase C18 column at 25 °C. Solvents were (A) water/formic acid (95:5) and (B) acetonitrile. The elution gradient was performed using a K-1001 Knauer pump from 10 to 35% B for 50 min at a flow rate of 1.5 mL/min. Detection was carried out at 520 nm with a K-2800 UV−vis Knauer detector. The molar ratio for each pair of isomeric pigments obtained from every aldehyde was estimated on the basis of the respective peak area in the corresponding HPLC chromatogram.

Isolation of Anthocyanin-Derived Pigments. The solvent was evaporated using a rotary evaporator at 30 °C to a volume of ∼5 mL. The solution was applied on a 300 × 25 mm TSK Toyopearl gel HW-40(S) column (Tosoh), and the pigments were eluted with water/ethanol (7:3) at a flow rate of 0.8 mL/min. The major anthocyanin-derived pigments were collected and submitted to semipreparative HPLC using a 250 × 4.6 mm i.d. reversed-phase C18 column for the isolation of compounds. Solvents were (A) water/formic acid (90:10) and (B) water/formic acid/acetonitrile (6:1:3), with the following gradient: 20−80% B over 30 min; 80−100% B over 5 min; isocratic 100% B over 10 min; and 100−20% B over 5 min; at a flow rate of 1.0 mL/min. The isolated pigments were submitted to a final purification by TSK Toyopearl gel HW-40(S) column chromatography eluted with distilled methanol acidified with 2% HCl. The solvent was partially evaporated in a rotary evaporator at 30 °C, and the sample was freeze-dried and stored at −18 °C until use.

LC-MS Analysis. A Hewlett-Packard 1100 series liquid chromatograph, equipped with a 150 × 4.6 mm, 5 μm AQUA reversed-phase C18 columm (Phenomenex, Torrance, CA) thermostated at 35 °C was used. Solvents were (A) aqueous 0.1% trifluoroacetic acid and (B) acetonitrile, using the gradient reported by Pissarra et al. (*25*). The capillary voltage was 3 V and the capillary temperature 190 °C. 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 an MS-MS of the most intense ion using a relative collision energy of 30.

NMR Analysis. ^{1}H NMR (500.13 MHz) and ^{13}C NMR (125.77 MHz) spectra were acquired in CD_3OD/TFA (98:2) on a Bruker-DRX500 spectrometer at 298 K with TMS as internal standard. ^{1}H chemical shifts were assigned using 1D and 2D ^{1}H NMR (gCOSY), whereas ^{13}C resonances were assigned using 2D NMR (gHMBC, gHSQC, and gNOESY) techniques (*27*−*31*). The delay for the long-range C/H coupling constant was optimized to 7 Hz.

RESULTS AND DISCUSSION

Identification of Newly Formed Pigments. The reaction between malvidin 3-glucoside and catechin mediated by different aldehydes (isobutyraldehyde, benzaldehyde, and isovaleraldehyde) led to the formation for each aldehyde of two major red pigments named IB1 + IB2, Bz1 + Bz2, and IV1 + IV2, respectively. For each reaction, the compounds were numbered 1 or 2 according to their order of elution in the respective HPLC chromatogram (**Figure 1**). MS analysis of these new pigments showed molecular ions consistent with the structure of malvidin 3-glucoside−8-isobutyl-8-catechin (*m*/*z* $[M]^+$ at 837) for IB1 and IB2, malvidin 3-glucoside−8-benzyl-8-catechin (*m*/*z* $[M]^+$ at 871) for Bz1 and Bz2, and malvidin 3-glucoside−8−3methylbutyl-8-catechin (*m*/*z* $[M]^+$ at 851) for IV1 and IV2 (**Figure 2**), by analogy with the ethyl-linked adducts obtained after acetaldehyde-induced reaction (*12*, *32*, *33*). The fact that two pigments with identical molecular ions are formed in each case is explained by the existence of two diasterisomers, which are supposed to differ in the stereochemistry of the asymmetric carbon (C9) of the interflavanolic linkage, as demonstrated by

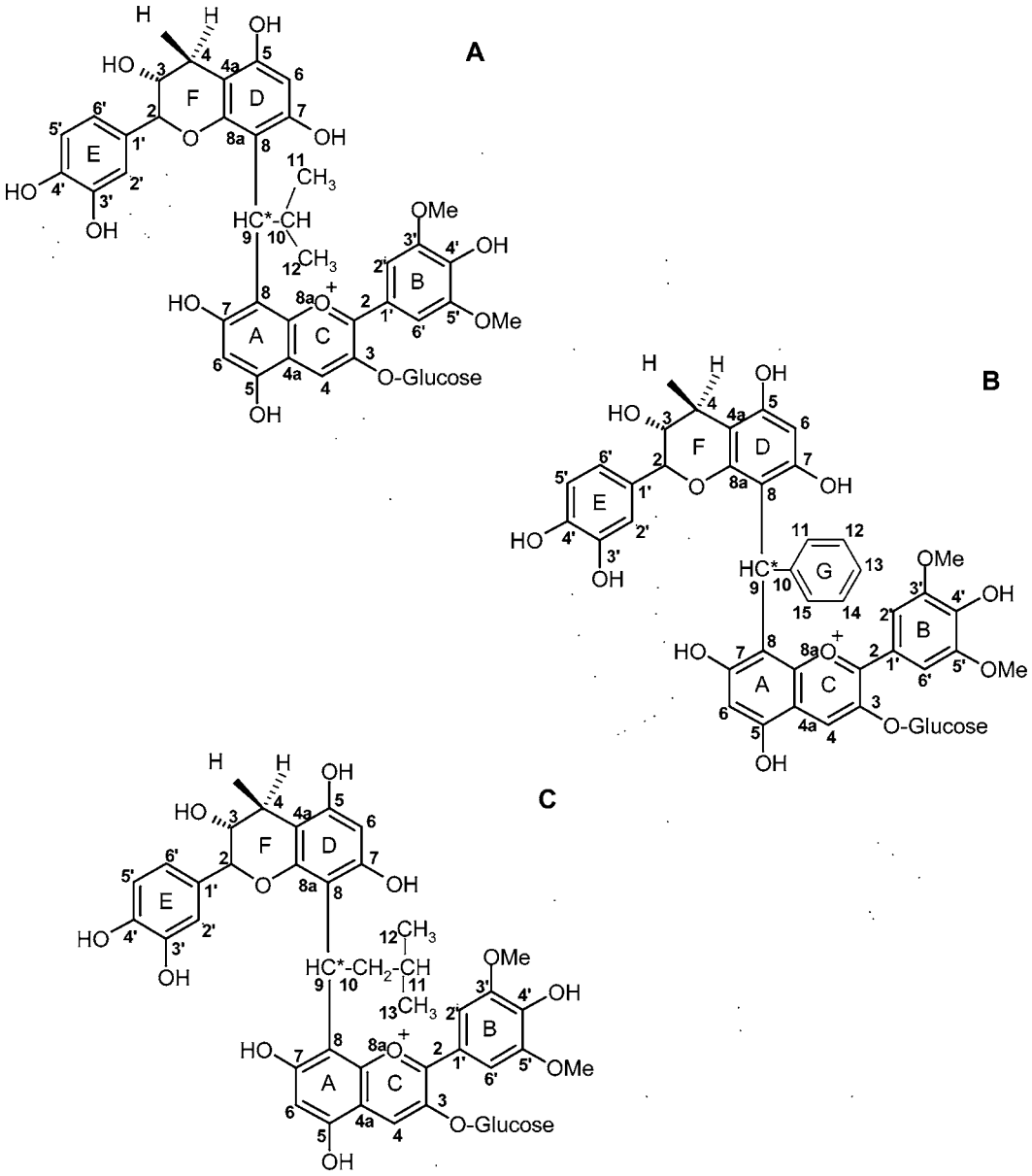

Figure 2. Structures proposed for pigments IB1 + IB2 (**A**), Bz1 + Bz2 (**B**), and IV1 + IV2 (**C**).

Escribano-Bailon et al. (*10*). MS^2 fragmentation of these newly formed pigments showed a typical pattern, with a loss of the catechin moiety ($[M - 290]^+$, fragments at *m*/*z* 547, 581, and 561, for pigments IB1 + IB2, Bz1 + Bz2, and IV1 + IV2, respectively) and a loss of the glucose moiety ($[M - 162]^+$, fragments at *m*/*z* 675, 709, and 689, for pigments IB1 + IB2, Bz1 + Bz2, and IV1 + IV2, respectively).

The ^1H and ^{13}C NMR chemical shifts of the isolated compounds in CD_3OD/TFA (98:2) are indicated in **Tables 1**−**4**. The ^1H chemical shifts were assigned using 1D and 2D NMR techniques (COSY and NOESY), and the assignment of the carbon resonances was made using 2D techniques (HSQC and HMBC). The HMBC spectra were optimized to visualize long-distance coupling $^1H-^{13}C$ in $^2J_{C,H}$ and $^3J_{C,H}$ in order to assign most of the carbons, especially the ones of the alkyl/aryl linkage.

Anthocyanidin Moiety. All of the protons of the anthocyanidin moiety were easily assigned by comparison with those of anthocyanin−ethylcatechin adducts reported in the literature (*10*, *33*). The ^1H spectrum showed the presence of protons 2′,6′B integrating two protons as a singlet between 7.5 and 8.0 ppm for all structures. The two methoxyl groups of ring B were also attributed to singlets around 3.8 ppm for all structures. Protons H-4C and H-6A were attributed to singlets around 8.9 and 6.6 ppm, respectively.

The carbons assigned through HSQC and HMBC correlations are presented in **Tables 1**−**4**, and all structures showed close resemblance in their chemical shift. The assignments of carbon C-4C around 134 ppm, carbon C-6A around 104 ppm, carbons C-2′,6′B around 110 ppm, and the methoxyl carbons around 57 ppm were obtained by HSQC. The long-range $^1H-^{13}C$ connectivities were obtained in the HMBC spectrum, allowing the assignment of the remaining carbons of the anthocyanidin moiety.

Flavanol Moiety. The protons H-4αF and H-4βF of the flavanol moiety were assigned through the characteristic AMX spin system of the flavanol pyran ring observed in the COSY spectrum. Thus, protons H-4αF and H-4βF correspond, respectively, to the double doublets at 2.50 and 2.97 ppm (Bz1) and at 2.45 and 3.06 ppm (Bz2). For the other compounds, the resonances of these protons appear at a similar chemical shift (δ) for both isomers at 2.42 and 2.83 ppm (IB1 + IB2) and at 2.45 and 2.88 ppm (IV1 + IV2), respectively. The proton H-2F

Table 1. ^{1}H and ^{13}C NMR Data and HMBC and HSQC Correlations of Pigments IB1 and IB2, Determined in CD_3OD/TFA (98:2)[a]

Pigments IB1 and IB2: Malvidin 3-Glucoside–Isobutylcatechin				
position	δ^1H; *J* (Hz)	δ ^{13}C	HMBC	HSQC
Anthocyanidin Moiety				
2C		161.9	H-4C; H-2′,6′B	
3C		146.2	H-4C	
4C′	8.91; s	134.2		H-4C
4C″	8.87; s			
4aA		113.1	H-6A	
5A		156.6	H-6A; H-4C	
6A′	6.62; s	104.7		H-6A
6A″	6.63; s			
7A		167.1	H-6A	
8A		111.1	H-6A; H-9	
8aA		154.1	H-4C; H-9	
1′B		110.1	H-2′,6′B	
2′,6′B	8.00; s	110.5		H-2′,6′B
3′,5′B		149.2	OMe; H-2′,6′B	
4′B		146.2	H-2′,6′B	
OMe	3.85; s	57.0		OCH_3
Flavanol Moiety				
1′E		131.8	H-6′E; H-2F	
2′E	5.89; s	113.5		H-2′E
2F′	4.58; d, 7.4	82.8		H-2F
2F″	4.40; d, 7.4	83.9		
3′E		146.2	H-5′E; H-2′E	
3F′	3.85–3.91; m*	68.9		H-3F
3F″	3.56–3.60; m*			
4aD		102.3	H-4F	
4′E		146.2	H-6′E; H-5′E; H-2′E	
4αF	2.42; dd*	28.2		H-4F
4βF	2.83; dd, 5.0/15.1			
5D		na		
5′E	5.84; d, 8.0	118.9		H-5′E
6D	6.07 br s	116.9		
6′E	6.22; d, 8.0	116.2		H-6′E
7D		na		
8D		100.8	H-4F	
8aD		155.4	H-4F; H-9	
Sugar Moiety				
Gl-1	5.37*	103.1		H-Gl-1
Gl-2	3.80*	75.0		H-Gl-2
Gl-3	3.61*	78.5		H-Gl-3
Gl-4	3.47*	70.9		H-Gl-4
Gl-5	3.68*	78.5		H-Gl-5
Gl-6a	3.93*	62.0		H-Gl-6a
Gl-6b	3.73*	62.0		H-Gl-6b
Isobutyl Bridge				
9′ CH	5.08; d, 12.0	40.5		H-9
9″ CH	4.99; d, 11.5	40.5		H-9
10 CH	2.96–3.00 m	30.7		H-10
11′ CH_3	1.07; d, 6.3	22.8		H-11
11″ CH_3	1.05; d, 6.7			
12′ CH_3	0.80; d, 6.4	22.8		H-12
12″ CH_3	0.74; d, 6.2			

[a] Key: ′ and ″, isomers; *, unresolved; s, singlet; m, multiplet; d, doublet; dd, double doublets; br, broad; na, not attributed.

Table 2. ^{1}H and ^{13}C NMR Data and HMBC and HSQC Correlations of Pigment Bz1, Determined in CD_3OD/TFA (98:2)[a]

Pigment Bz1: Malvidin 3-Glucoside–Benzylcatechin				
position	δ^1H; *J* (Hz)	δ ^{13}C	HMBC	HSQC
Anthocyanidin Moiety				
2C		162.5	H-4C; H-2′,6′B	
3C		145.7	H-4C	
4C	8.87; s	134.2		H-4C
4aA		113.2	H-6A	
5A		156.7	H-6A; H-4C	
6A	6.72; s	103.6		H-6A
7A		167.7	H-6A; H-9	
8A		110.2	H-6A	
8aA		153.9	H-4C; H-9	
1′B		110.5	H-2′,6′B	
2′,6′B	7.64; s	110.8		H-2′,6′B
3′,5′B		148.2	OMe; H-2′,6′B	
4′B		145.7	H-2′,6′B	
OMe	3.85; s	56.9		OCH_3
Flavanol Moiety				
1′E		131.5	H-6′E	
2′E	5.9–6.1*	114.9		H-2′E
2F	4.3; d, 9.4	83.7		H-2F
3′E		146.0	H-5′E; H-2′E	
3F	3.63–3.66; m*	67.8		H-3F
4aD		102.2	H-4F	
4′E		146.0	H-6′E; H-5′E; H-2′E	
4αF	2.50; dd, 9.6/16.2	30.1		H-4F
4βF	2.97; dd, 7.1/16.2			
5D		na		
5′E	6.03; d*	119.9		H-5′E
6D	*	na		
6′E	6.28; d, 8.00	117.2		H-6′E
7D		na		
8D		107.2	H-9	
8aD		155.9	H-4F; H-9	
Sugar Moiety				
Gl-1	5.48*	103.0		H-Gl-1
Gl-2	3.71*	75.1		H-Gl-2
Gl-3	3.58*	78.2		H-Gl-3
Gl-4	3.47*	71.4		H-Gl-4
Gl-5	3.67*	78.3		H-Gl-5
Gl-6a	4.00*	62.3		H-Gl-6a
Gl-6b	3.80*	62.3		
Benzyl Bridge				
9CH	6.92, s	36.6		H-9
10G		131.8	H-12,14G	
11,15G	8.00; d, 8.0	130.7		H-11,15G
12,14G	7.44; t, 8.0	129.5		H-12,14G
13G	7.57; t, 8.0	134.0		H-13G

[a] Key: *, unresolved; s, singlet; m, multiplet; d, doublet; dd, double doublets; t, triplet; na, not attributed.

of the flavanol pyran ring was assigned at ~4.0 ppm, and the proton H-3F was assigned as a multiplet at ~3.5−3.9 ppm. The relative 2,3-stereochemistry is concluded to be trans as proton H-2F appears as a large doublet ($J_{2-3} = 7-9$ Hz) corresponding to (+)-catechin, instead of a broad singlet or a doublet with a small coupling constant (0−3 Hz) characteristic of a (−)-epicatechin unit (*34*).

Interflavanolic Linkage. The assignment of proton H-4C (anthocyanidin moiety) proves that the alkyl/aryl group is not linked to this position, and so the alkyl/aryl linkage is assumed to be situated between ring A of anthocyanidin moiety and ring D of the catechin moiety. The position of the alkyl/aryl linkage was elucidated by NOESY experiments. Indeed, for all pigments, spatial couplings were observed between protons of ring B (H-2′6′B and OMe groups) and protons H-9. This indicates a spatial proximity between the B ring of the anthocyanidin and the alkyl/aryl bridge, which is possible only in the case of a C8 linkage rather than a C6 linkage. In the isomer Bz1, the NOESY spectrum showed other correlations between all of the protons of the benzyl group with the same protons of ring B of anthocyanidin moiety.

The NMR data do not clarify the position of the alkyl/aryl linkage to ring D of the catechin moiety. Thus, a ^{1}H−^{13}C correlation was found in the HMBC spectrum between protons H-9 (interflavonoid linkage) and a signal around 154−155 ppm, which could be assigned to the resonance of carbons either at C-8aD (C8 linkage) or at C-5D (C6 linkage). The unequivocal assignment of these carbon resonances could be elucidated through an HMBC correlation between proton H-2F and carbon

Table 3. ^{1}H and ^{13}C NMR Data and HMBC and HSQC Correlations of Pigment Bz2, Determined in CD_3OD/TFA (98:2)[a]

Pigment Bz2: Malvidin 3-Glucoside–Benzylcatechin

position	δ^1H; *J* (Hz)	δ ^{13}C	HMBC	HSQC
		Anthocyanidin Moiety		
2C		162.0	H-4C; H-2′,6′B	
3C		145.5	H-4C	
4C	8.78; s	134.2		H-4C
4aA		113.2	H-6A	
5A		156.7	H-6A; H-4C	
6A	6.75; s	103.6		H-6A
7A		167.7	H-6A	
8A		110.2	H-6A; H-9	
8aA		155.0	H-4C; H-9	
1′B		110.5	H-2′,6′B	
2′,6′B	7.49; s	110.8		H-2′,6′B
3′,5′B		148.2	OMe; H-2′,6′B	
4′B		145.5	H-2′,6′B	
OMe	3.83; s	56.9		OCH_3
		Flavanol Moiety		
1′E		131.5	H-6′E	
2′E	5.9–6.1*	114.9		H-2′E
2F	4.05; d, 9.4	83.7		H-2F
3′E		146.0	H-5′E; H-2′E	
3F	3.85–3.90; m	69.0		H-3F
4aD		102.2	H-4F	
4′E		146.0	H-6′E; H-5′E; H-2′E	
4αF	2.45; dd, 9.5/16.2	30.1		H-4F
4βF	3.06; dd, 5.9/16.2			
5D		na		
5′E	5.94; d, 8.3	119.4		H-5′E
6D		na		
6′E	6.30; d, 8.01	117.2		H-6′E
7D		na		
8D		106.8	H-9	
8aD		155.9	H-4F; H-9	
		Sugar Moiety		
Gl-1	5.48*	103.0		H-Gl-1
Gl-2	3.67*	75.1		H-Gl-2
Gl-3	3.58*	78.2		H-Gl-3
Gl-4	3.45*	71.4		H-Gl-4
Gl-5	3.67*	78.5		H-Gl-5
Gl-6a	3.97*	62.3		H-Gl-6a
Gl-6b	3.80*	62.3		H-Gl-6b
		Benzyl Bridge		
9CH	6.68; s	36.1		H-9
10G		*		
11,15G	6.99–7.21*	*		
12,14G	6.99–7.21*	*		
13G	6.99–7.21*	*		

[a] Key: *, unresolved; s, singlet; m, multiplet; d, doublet; dd, double doublets; na, not attributed.

Table 4. ^{1}H and ^{13}C NMR Data and HMBC and HSQC Correlations of Pigments IV1 and IV2, Determined in CD_3OD/TFA (98:2)[a]

Pigments IV1 and IV2: Malvidin 3-Glucoside–3-Methylbutylcatechin

position	δ^1H; *J* (Hz)	δ ^{13}C	HMBC	HSQC
		Anthocyanidin Moiety		
2C		162.5	H-4C; H-2′,6′B	
3C		146.0	H-4C; H-2′,6′B	
4C′	8.78; s	134.6		H-4C
4C″	8.85; s			
4aA		112.1	H-6A	
5A		156.6	H-6A; H-4C	
6A	6.65; s	104.6		H-6A
7A		167.8	H-6A	
8A		113.5	H-6A	
8aA		154.1	H-4C	
1′B		120.1	H-2′,6′B	
2′,6′B′	7.93; s	111.5		H-2′,6′B
2′,6′B″	7.97; s			
3′,5′B		149.3	OMe; H-2′,6′B	
4′B		146.0	H-2′,6′B	
OMe′	3.91; s	57.5		OCH_3
OMe″	3.99; s			
		Flavanol Moiety		
1′E		na		
2′E	5.94; s	114.4		H-2′E
2F	4.51; d, 7.51	82.9		H-2F
3′E		146.0	H-5′E; H-2′E	
3F	3.63–3.66; m	67.8		H-3F
4aD		102.0	H-4F	
4′E		146.0	H-6′E; H-5′E	
4αF	2.45; dd, *	29.0		H-4F
4βF	2.88; dd, 4.8/16.2			
5D		na		
5′E	5.92; d, 8.85	119.4		H-5′E
6D		na		
6′E	6.26; d, 8.02	116.3		H-6′E
7D		na		
8D		na		
8aD		na		
		Sugar Moiety		
Gl-1	5.39; d, 7.6	102.9		H-Gl-1
Gl-2	3.72*	75.1		H-Gl-2
Gl-3	3.60*	78.5		H-Gl-3
Gl-4	3.52*	70.9		H-Gl-4
Gl-5	3.68*	78.2		H-Gl-5
Gl-6a	4.08*	62.4		H-Gl-6a
Gl-6b	3.87*	62.4		H-Gl-6b
		3-Methylbutyl Bridge		
9CH	5.42; s	30.7		CH
10CH_2	2.0–2.1; m*	27.0		CH_2
11CH	1.6–1.9*	*		
12CH_3	0.9–1.6*	*		
13CH_3	0.9–1.6*	*		

[a] Key: *, unresolved; s, singlet; m, multiplet; d, doublet; dd, double doublets; na, not attributed.

C-8aD (154−155 ppm), which is hardly detected in these kinds of compounds. These data unequivocally support the proposed structure. This is in agreement with the proposed mechanism; the interflavonoid linkage is more likely attached to carbon C-8 of the flavanol moiety that presents a higher negative charge than at C-6 (*35*).

For the structures IB1 + IB2, the resonance of proton H-9 appears as a doublet integrating one proton around 5.0 ppm, whereas the proton H-10 appears as a multiplet at 2.96−3.00 ppm integrating one proton for both structures. The two methyl groups act as nonequivalent groups and resonate as doublets integrating three protons at 1.07 and 0.80 ppm for IB1 and at 1.05 and 0.74 ppm for IB2.

For the structures Bz1 + Bz2, the proton H-9 appears as a singlet at 6.92 and 6.68 ppm for Bz1 and Bz2, respectively, whereas the benzyl group seems to have different behaviors between the two diasteroisomers. For the structure Bz1, the protons H-11,15G were assigned as equivalent protons, and their resonance appears as a doublet at 8.00 ppm (J = 8.0 Hz). The protons H-12,14G were also assigned as equivalent protons, and their signal appears as a triplet at 7.44 ppm (J = 8.0 Hz), whereas the resonance of proton H-13G appears as a triplet at 7.57 ppm. All of the carbons in the structure Bz1 were easily attributed through correlations found in the HSQC and HMBC spectra.

For the other diasteroisomer (Bz2) the NMR data shows all of the protons of the benzyl group (H-11G to H-15G) acting as nonequivalents and resonating individually, probably due to restricted rotation of the benzyl group (ring G) by the C9−C10 linkage. These protons as well as their corresponding carbons could not be individually attributed due to the complex region

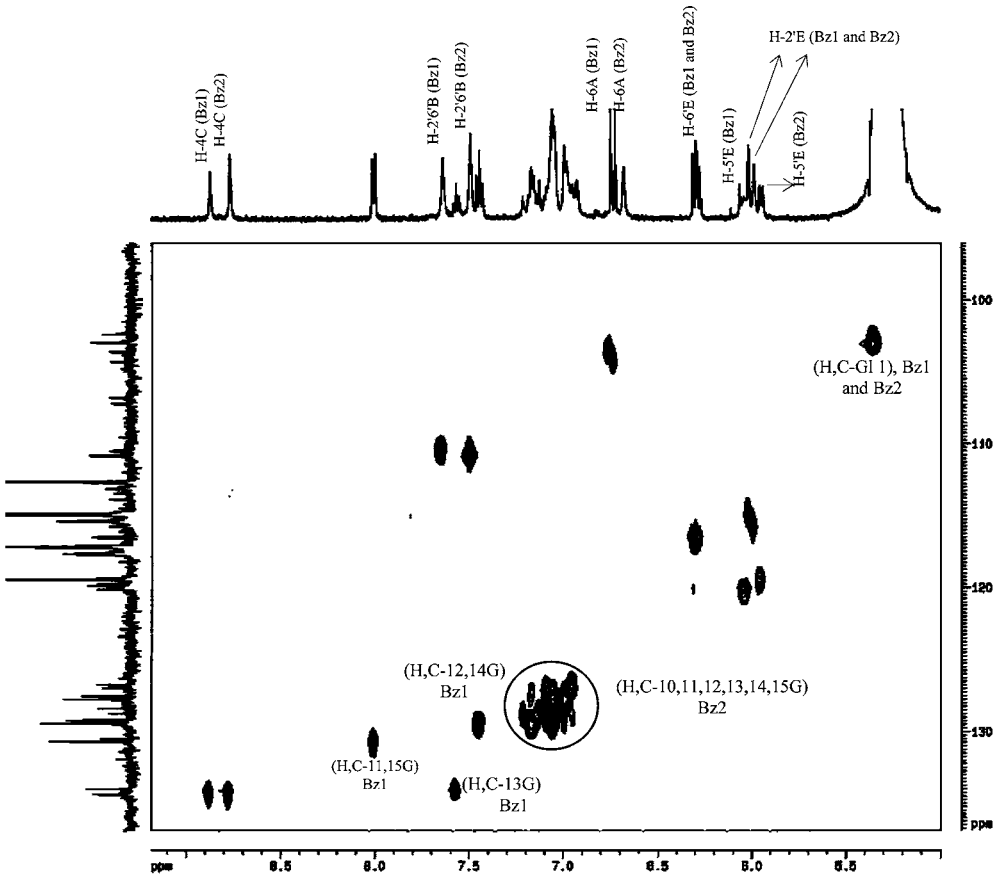

Figure 3. HSQC spectrum evidencing the benzyl linkage of both pigments Bz1 (with the protons H-11,15G and H-12,14G resonates as equivalent protons respectively) and Bz2 (all protons resonate individually).

present in both COSY (from 6.99 to 7.21 ppm) and HSQC spectra (**Figure 3**).

For the structures IV1 + IV2, the proton H-9 was easily assigned at 5.42 ppm for both isomers IV1 and IV2. The other protons present in this alkyl linkage could not be exactly assigned mainly due the complexity of the spectrum at higher magnetic field. Thus, and from some correlations found in the COSY spectrum, the methylenic protons were assigned as a multiplet (2.0−2.1 ppm), the proton H-11 was assigned between 1.6 and 1.9 ppm, and the protons in the two CH_3 groups are nonequivalent protons and were assigned at different regions of the spectrum between 0.9 and 1.6 ppm. The assignment of the respective carbons could not be achieved, except for the carbons C-9 and C-10, which were assigned at 30.7 and 27.0 ppm, respectively.

Pigment Formation. The UV−vis spectra of these new pigments (IB1, IB2, Bz1, Bz2, IV1, and IV2) are quite similar and revealed a significant bathochromic shift of the λ_{max} to 540 nm, into a more red-purple color, compared to the red color of the original anthocyanin at λ_{max} 525 nm (**Figure 4**). Their UV−vis spectra do not seem to be influenced by the substituent group at C9 (isobutyl, benzyl, or 3-methylbutyl) as all of them show the same λ_{max}.

The formation of these pigments is more favored at pH 1.5 than at pH 3.2 (data not shown). The higher reactivity at lower pH values was already reported (*12*, *36*). According to the formation mechanism proposed in the literature, the reaction involves first the protonation of the aldehyde with the formation of the respective carbocation (R−HC^+OH), followed by addition to a nucleophilic position of the phloroglucinol ring of the flavanol (A) to form the two diastereoisomers [catechin−HC*(OH)−R] named herein as 1 and 2. A dehydration of the resulting protonated adduct occurs, yielding a new carbocation (catechin−HC^+−R), which suffers a nucleophilic attack from the anthocyanin [catechin−HC*(R)−Mv3gl]. Insufficient acidic media could prevent the protonation of the aldehyde and, consequently, it reacts with catechin to a lesser extent.

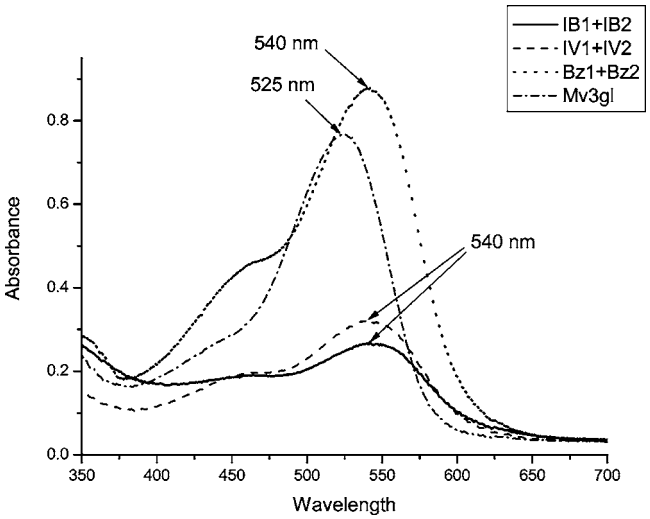

Figure 4. UV–vis spectra of original anthocyanidin (malvidin 3-glucoside) and new colored compounds showing their λ_{max}. Each spectrum was determined with a pigment concentration of 0.08 mM in a citrate–phosphate buffer solution at pH 2.2.

The speed and rate of pigment formation, followed by HPLC, showed differences depending on the aldehyde. In the assays carried out at pH 3.2, maximum concentration of IV1 + IV2 (i.e., malvidin 3-glucoside−3methylbutylcatechin) was obtained

at 14 days, whereas pigments IB1 + IB2 (i.e., malvidin 3-glucoside−isobutylcatechin) and Bz1 + Bz2 (i.e., malvidin 3-glucoside−benzylcatechin) reached their maximum formation at about 30 and 50 days, respectively. Similar behavior was observed at pH 1.5, at which the maximum level of pigments IV1 + IV2 was obtained in only 3 days or 5 days in the case of IB1 + IB2 and 11 days for Bz1 + Bz2. Because the charge stabilization of the carbocations found at the different stages of the proposed mechanism (R−HC^+OH and catechin−HC^+−R) is probably not significantly different between them, steric hindrance should be the major factor influencing the condensation between catechin and malvidin 3-glucoside mediated by the different aldehydes. Thus, the slower rate of formation of pigments Bz1 + Bz2 could be explained by the existence of an opposite inductive effect (−I) and a lower mesomeric effect of the phenyl substituent group (R) compared to the substituent groups (R) resulting from isobutyraldehyde and isovaleraldehyde, which have similar inductive (+I) and mesomeric (+M) effects between them (*37*). On the other hand, the ratios of formation estimated for each pair of pigments derived from every aldehyde were similar (IB1/IB2 = Bz2/Bz1 = IV1/IV2 = 0.8), indicating that the stereoselectivity observed in the adduct formation seems not to be dependent on the aldehyde involved.

Conclusion. The present work shows that aldehydes commonly present in wine spirits, such as isobutyraldehyde, benzaldehyde, and isovaleraldehyde, may contribute to the formation of new pigments showing an anthocyanin−alkyl/aryl−flavanol structure. This pigment family, in which the alkyl/aryl bridge results from different aldehydes, could have an important role in the initial stages of Port wine aging. Thus, especially for the Port wine industry, proper knowledge of the aldehyde composition of the commercial wine spirit added could be an important factor for the definition of the wine color. Besides the aldehyde profile of the spirits, their contents and the pH value of the wine could favor the formation of pigments with different chromatic features.

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