JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization of a Colorless Anthocyanin−Flavan-3-ol Dimer Containing Both Carbon−Carbon and Ether Interflavanoid Linkages by NMR and Mass Spectrometry

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Direct addition of anthocyanins and flavan-3-ols was investigated in a model system by incubating malvidin 3-glucoside and (−)-epicatechin in ethanol. Analysis of reaction products by high-performance liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC/ESI-MS) before and after thiolysis showed the formation of colorless dimers detected at *m*/*z* 781 in the negative ion mode, with retention times and spectroscopic characteristics identical to those of compounds detected in wine, which contain one malvidin 3-glucoside unit and one flavanol unit. On the basis of their resistance to thiolysis, these compounds were postulated to be bicyclic dimers linked with both carbon−carbon and ether bonds as observed in the case of A type proanthocyanidins. The major dimer analyzed by NMR experiments was identified as malvidin 3-glucoside(C2−O−C7,C4−C8)epicatechin, confirming this hypothesis. A similar assay was performed with (+)-catechin instead of (−)-epicatechin, and the formation of bicyclic dimers was also observed.

KEYWORDS: Anthocyanins; malvidin 3-glucoside; flavanols; adducts; A type linkages; thiolysis; LC/ESI-MS; MS-MS; NMR

INTRODUCTION

In the course of red wine making and aging, modifications affect organoleptic properties such as astringency (*1*) and color hue, passing from purple red in young wines to reddish-brown in older wines (*2*). These changes have been attributed to the formation of various reaction products, particularly including new, more stable, polymeric pigments proceeding from reactions between anthocyanins (A) and tannins (T) (*3*−*4*). Two main processes are considered to account for the formation of these polymeric pigments: direct tannin−anthocyanin reactions (*4*) and reactions involving acetaldehyde (*5*). Two direct reaction pathways between anthocyanins and tannins have been postulated, leading to tannin−anthocyanin (T−A) and to anthocyanin−tannin (A−T) adducts. In fact, both types of compounds can react either as a nucleophile or as an electrophilic species.

In the first reaction, the electrophilic species is a carbocation resulting from cleavage of a proanthocyanidin interflavanic bond (*6*), which reacts with the C-8 or C-6 position of an anthocyanin in its nucleophilic hydrated form. The resulting flavanol−hemiketal adduct (T−AOH) can be converted into the corresponding flavylium form (T−A^+). Such pigments were detected in wine by liquid chromatography/mass spectrometry (LC/MS), and the position of the anthocyanin as the terminal unit was confirmed by thiolysis (*7*). The other reaction starts with a nucleophilic attack by the flavan unit (C-8 or C-6) at the electrophilic C-4 of the anthocyanin flavylium form (A^+) to yield an A−T adduct. In previous work (*7*), analysis of a wine extract showed the presence of colorless compounds, which were tentatively identified as A−T dimers in the flavene form in earlier studies (*8*−*9*). These flavene adducts have been reported to oxidize to the corresponding flavylium, which in turn proceeds to the yellow xanthylium ion (*4*), but no evidence of such species was found in our wine sample. However, resistance of the A−T dimers to thiolytic cleavage (*7*) led us to consider an alternative structure, consisting of a bicyclic A−T structure linked by both carbon−carbon and ether−interflavanoid bonds (i.e., A type linkage) analogous to the malvidin 3,5-diglucoside−catechin dimer obtained by Bishop and Nagel (*10*).

The purpose of this work was to determine the structure of the colorless A−T adducts. Their purification from wine was impossible due to their very small concentration. Their production was therefore achieved by incubation of malvidin 3-glucoside with (−)-epicatechin in ethanolic solution. The colorless products thus obtained were analyzed by LC/ESI-MS, and the major one was isolated and analyzed by NMR spectroscopy.

MATERIALS AND METHODS

Materials. Malvidin 3-glucoside was isolated from a grape anthocyanin extract obtained from skins of *Vitis vinifera* var. Syrah as described by Sarni et al. (*11*). Briefly, the crude extract was purified by chromatography on Polyclar SB 100 (BDH Laboratory Supplies,

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10.1021/jf021227b CCC: $25.00
Published on Web 05/02/2003

Poole, England). After sugars were removed by washing with water/hydrochloric acid (99:1, v/v), malvidin 3-glucoside was eluted with methanol/water/hydrochloric acid (70:29:1, v/v/v). Anthocyanin purity was controlled by high-performance liquid chromatography coupled to diode array detection and mass spectrometry (HPLC-DAD/MS). (−)-Epicatechin and (+)-catechin were purchased from Sigma (St. Louis, MO).

Preparation of Solutions. The reaction mixture used for the production of the condensation product (A−T dimer) consisted of an equimolar solution of malvidin 3-glucoside (2.2 g/L, 4 mM) and (−)-epicatechin (1.2 g/L, 4 mM) in 30 mL of ethanol. The mixture was purged with argon and maintained at 35 °C in a tube sealed with a Teflon septum. Samples (0.1 mL) were taken periodically by puncturing the septum and were diluted 10-fold in water/formic acid (95:5, v/v) before analysis. The reaction products were monitored by HPLC-DAD/MS. After 16 days, the amount of A−T dimer was maximum and the reaction was stopped and stored at −80 °C until purification. For comparison, a similar solution was prepared with (+)-catechin instead of (−)-epicatechin in 1 mL of ethanol and after it was incubated for 19 days, it was stored at −80 °C until its analysis.

Purification of A−T Dimers. To separate malvidin 3-glucoside from the reaction products, a first purification step consisting of liquid chromatography on the semipreparative scale was carried out. The apparatus was a Gilson system including a 305 master and a 306 slave pump, an 811 dynamic mixer, a 7161 Rheodyne valve injector, an 875 UV−visible detector Jasco (Tokyo, Japan) set at 280 nm, and an HP3396A integrator. The column was a 250 mm × 41.4 mm i.d., 5 μm reversed-phase Microsorb C18 (Rainin, Woburn, MA). Elution conditions were as follows: 10 mL/min flow rate at room temperature; solvent A, methanol/water/acetic acid (50:49:1, v/v/v); solvent B, methanol/water/acetic acid (90:9:1, v/v/v); elution from 15 to 41% B in 11 min followed by washing and reequilibrating the column. The fractions collected (from 5 to 7 min) were pooled, concentrated under vacuum, and loaded onto preconditioned Environmental model Sep Pak tC18 cartridges (Waters, Milford, MA). Elution was started with 40 mL of water. After the samples were dried under an air stream, flavanol monomers were eluted with 70 mL of diethyl ether. After they were dried, the A−T dimers were recovered together with 70 mL of ethyl acetate and this fraction was taken to dryness by rotary evaporation and dissolved in water/acetic acid (99:1, v/v). The A−T dimers were isolated from the ethyl acetate fraction by HPLC on a semipreparative scale. The latter separation was performed using the same equipment as described above but under the following conditions: column, reversed-phase 125 mm × 25 mm i.d., 5 μm Lichrospher 100 RP-18 (Merck, Darmstadt, Germany); 12 mL/min flow rate at room temperature; solvent A, water/acetic acid (99:1, v/v); solvent B, methanol/water/acetic acid (80:19:1, v/v/v); elution from 15 to 50% B in 15 min; isocratic for 3 min with 50% B and from 50 to 90% B in 3 min followed by washing and reequilibrating the column. Fractions containing the AT dimers were collected, concentrated to dryness under vacuum, and lyophilized.

Thiolysis Conditions. The samples taken from the reaction mixture or the purified A−T dimer previously dissolved in methanol were treated in a glass vial with an equal volume of a 5% solution of toluene-α-thiol in methanol containing HCl (0.2 M). After the vials were sealed, the mixture was shaken and heated at 90 °C for 2 min. The thiolyzed solutions were then analyzed by LC/MS.

HPLC-DAD/Electrospray Ionization (ESI)-MS. Mass detection and fragmentation experiments were performed on a ThermoFinnigan LCQ Advantage (San Jose, CA) mass spectrometer equipped with an ESI source and an ion trap mass analyzer, which was controlled by the LCQ navigator software. The mass spectrometer was operated in the negative and positive ion modes under the following conditions: source voltage, 4.5 kV; capillary voltage, 23.5 V; capillary temperature, 250 °C; collision energy for fragmentation, 40% for MS^2 and 35% for MS^3. During the chromatographic run, mass spectra of the eluate were recorded from *m*/*z* 100 to *m*/*z* 2000. Chromatographic separation was achieved using the following conditions: the HPLC apparatus was a Waters 2690 including an autosampler, a Waters 996 photodiode array detector, and a Millennium 32 chromatography manager software. The column was a 250 mm × 2 mm i.d., 5 μm reversed-phase Lichrospher 100-RP18 (Merck) protected with a guard column of the same material. Elution conditions were as follows: 0.250 mL/min flow rate at 30 °C; solvent A, water/formic acid (95:5, v/v); solvent B, acetonitrile/water/formic acid (80:15:5, v/v/v); isocratic conditions with 2% B for 7 min, linear gradients from 2 to 20% B in 15 min, from 20 to 30% B in 8 min, from 30 to 40% B in 10 min, and from 40 to 50% in 5 min, followed by washing and reequilibrating the column.

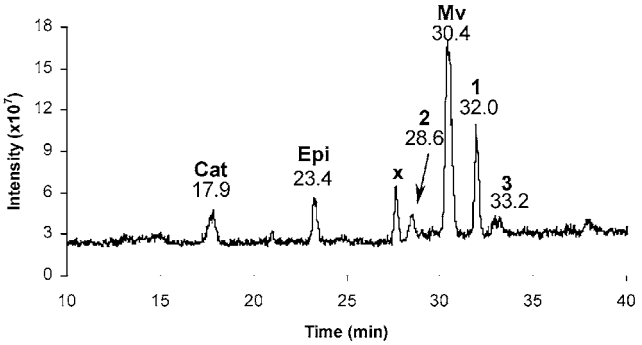

Figure 1. HPLC trace recorded at 280 nm of the malvidin 3-glucoside and epicatechin solution after 16 days of reaction. Cat, (+)-catechin; Epi, (−)-epicatechin; Mv, malvidin 3-glucoside; ×, unidentified compound. Peaks 1–3 refer to A–T dimers.

NMR Spectroscopy. NMR experiments were performed on a Varian UNITY INOVA 500 MHz spectrometer (Varian NMR instruments, Palo Alto, CA) operating at 500 MHz for ^{1}H and 125.7 MHz for ^{13}C, equipped with a 3 mm indirect detection probe. One-dimensional (1D) ^{1}H, two-dimensional (2D) (^{1}H) COSY, and 2D (^{1}H−^{13}C) HSQC and HMBC spectra of sample dissolved in DMSO-d_6 were recorded at 298 K. Chemical shifts are given in ppm, and coupling constant *J* values are given in Hz. The solvent signals of DMSO were used as an internal reference (^{1}H signal at 2.5 ppm and ^{13}C signal at 39.5 ppm).

RESULTS AND DISCUSSION

The reaction mixture of (−)-epicatechin and malvidin 3-glucoside was monitored at intervals from the beginning of incubation by LC-DAD/ESI-MS. After only 3 days, analysis showed the presence of a new compound (**1**) (λ_{max} = 276 nm) detected at *m*/*z* 781 in the negative ion mode, which corresponds to the signal expected for the A−T colorless adduct formed by direct condensation between malvidin 3-glucoside and (−)-epicatechin. A second (**2**) and a third (**3**) colorless derivative with the same mass and the same λ_{max} were also detected after 10 and 13 days of incubation, respectively (**Figure 1**). The retention times, UV−vis spectra, and mass data of these dimers are the same as those of compounds found in a two year old Cabernet sauvignon wine (*7*), indicating that they are probably identical. The progressive formation of other compounds was also observed in the solution, such as catechin (resulting from the epimerization of the epicatechin) and an unidentified product (×), which has an absorbance maximum between 295 and 330 nm and a mass signal at *m*/*z* 507. After 16 days of reaction, the amount of the major colorless product was maximum and the reaction was stopped by freezing at −80 °C. The yield of this reaction was estimated to be 11% in relation of the initial quantity of malvidin 3-glucoside. The signal at *m*/*z* 781 may correspond either to the malvidin 3-glucoside flavene−epicatechin dimer as postulated by Santos-Buelga et al. (*9*) or to a derivative with an A type linkage (a structure analogous to that of A type proanthocyanidins) similar to that identified by Bishop and Nagel (*10*). According to the literature, the flavene adduct can proceed to the corresponding flavylium (*4*) and xanthylium (*2*−*5*) salts. However, no evidence of such species was found in the solution. A previous study carried out in our laboratory

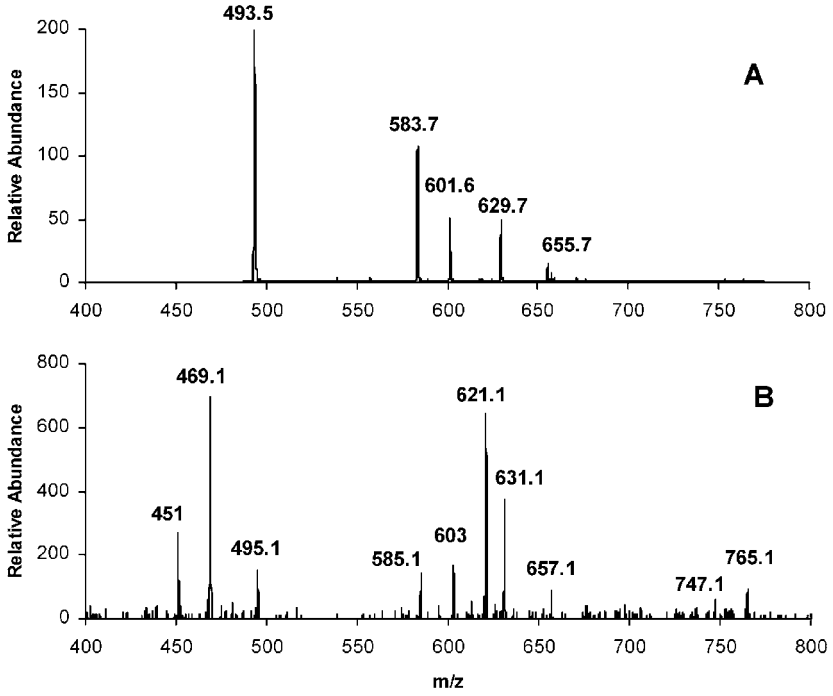

Figure 2. MS^2 analysis of the A type malvidin 3-glucoside–(–)-epicatechin molecular ion in the negative (**A**) and the positive (**B**) ion modes.

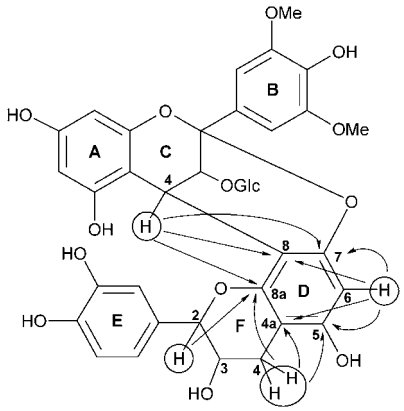

Figure 3. Postulated main fragmentation pathways of the A type A–T dimer in the negative ion mode.

(*12*) showed that thiolysis, a reaction used to characterize tannins (*13*), failed to cleave A type proanthocyanidins. In contrast, a dimer containing malvidin 3-glucoside in a flavene form is expected to directly release a cationic anthocyanin by thiolysis. Thiolysis therefore seems to be an appropriate method to discriminate both types of structure, and a thiolysis reaction was performed on the reaction mixture. LC/MS analysis showed that the three compounds detected at *m*/*z* 781 remained unchanged after thiolysis, allowing a flavene−epicatechin structure to be ruled out.

The A type dimers were analyzed by HPLC/MS in the negative ion mode, using fragmentation conditions, so that the compounds were detected in their deprotonated forms $[M - H]^-$. It can be noted that the three A−T dimers (**1**−**3**) detected in the model solution gave comparable results, but only mass analysis of the major dimer is presented here.

As expected, the major A−T A type species was detected at *m*/*z* 781 and MS^2 of the molecular ion yielded several fragments as shown in **Figure 2A**. The corresponding main fragmentation pathways are shown in **Figure 3**. The attributions of these fragments were as follows: the fragment at *m*/*z* 629 results from the retro Diels−Alder decomposition (RDA) (*14*) of the flavanol

Table 1. ^{1}H NMR (500 MHz) and ^{13}C NMR (125.7 MHz) Spectral Data for Dimeric Compound (DMSO-d_6, 298 K)[a]

ring	proton	δ (ppm)	carbon	δ (ppm)
C			2	114.7
	3	4.62 (d, $J = 3.4$)	3	69.3
	4	4.79 (d, $J = 3.4$)	4	23.9
F	2	4.99 (bs)	2	78.4
	3	4.08 (bs)	3	65.2
	4α	2.44 (dd, $J = 16.0, 4.8$)	4	27.7
	4β	2.72 (dd, $J = 16.0, 3.9$)		
A	6	5.87 (d, $J = 1.9$)	6	96.7
	8	5.81 (d, $J = 1.9$)	8	94.1
			4a	105.0
			8a	153.0
			5	155.3
			7	157.5
D	6	6.09 (s)	6	95.3
			8	102.7
			4a	101.1
			8a	152.0
			5	154.7
			7	151.0
B			1′	129.4
	2′, 6′	6.95 (s)	2′, 6′	105.4
			3′, 5′	147.4
			4′	136.2
	OCH_3	3.79	OCH_3	56.1
E			1′	130.5
	2′	6.98 (bs)	2′	114.7
			3′, 4′	144.9
	5′	6.75 (d, $J = 8.5$)	5′	115.4
	6′	6.98 (d, $J = 8.5$)	6′	118.2
glucose	1	4.19 (d, $J = 7.8$)	1	99.2
	2	2.78 (m)	2	73.9
	3	3.04 (m)	3	76.8
	4	2.93 (m)	4	70.0
	5	2.95 (m)	5	77.3
	6a	3.30 (dd, $J = 11.6, 5.1$)	6	61.3
	6b	3.55 (d, $J = 11.6$)		

[a] bs, broad singlet; m, multiplet. For resolved multiplets, couplings are given in Hz. Chemical shifts are given relative to the internal DMSO residual proton signal at 2.5 ppm and carbon signal at 39.5 ppm.

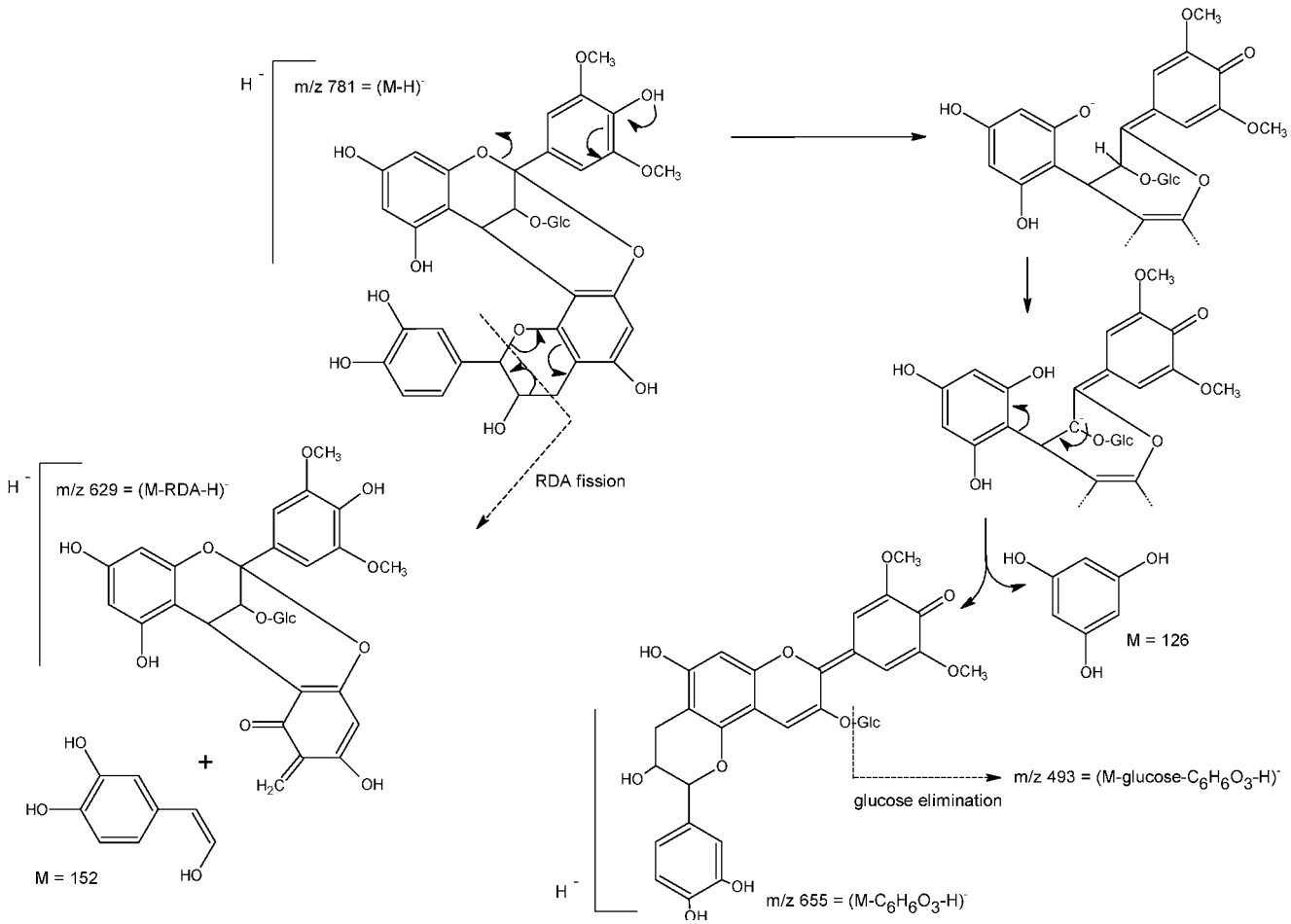

Figure 4. Main HMBC correlations allowing linkage positions to be established.

unit of the A−T dimer ($[M - H - 152]^-$). This result allowed the presence of the flavanol unit in the lower position in the initial A−T dimer to be confirmed. In addition, two ions were detected at *m*/*z* 655 and 493, corresponding to the loss of 126 u from the A−T dimer $[M - H - 126]^-$ and its aglycone $[M - H -$ glucose $- 126]^-$, respectively. This mass was attributed to a $C_6H_6O_3$ residue (**Figure 3**) as reported by Miketova et al. (*15*) and Friedrich et al. (*16*). In addition, values of the subsequent water elimination were detected at *m*/*z* 601 and 583 and could be interpreted as follows: $[M -$ glucose $- H_2O - H]^-$ and $[M -$ glucose $- 2(H_2O) - H]^-$. Experiments were also performed in the positive ion mode and presented the same fragmentation patterns with additional masses (**Figure 2B**): two ion peaks detected at *m*/*z* 621 and 469, which correspond to the loss of the glucose moiety $[M + H -$ glucose$]^+$ and to the RDA fission of the aglycone $[M + H -$ glucose $- 152]^+$, respectively. The fragments at *m*/*z* 765, 747, and 451 were attributed to the water elimination from the parent and fragment ions ($[M - H_2O + H]^+$, $[M - 2(H_2O) + H]^+$, and $[M -$ RDA $-$ glucose $- H_2O + H]^+$, respectively). The fragment ion of *m*/*z* 469 obtained in the positive ion mode was then further fragmented (MS^3) to *m*/*z* 343 and 451, which corresponded to the elimination of $C_6H_6O_3$ fragment (126 u) and of a water molecule. In the same way, MS^3 of the fragment at *m*/*z* 495 ($[M + H -$ glucose $- 126]^+$) yielded a major ion peak at *m*/*z* 343 resulting from its RDA fission. These data are consistent with the malvidin 3-glucoside−(−)-epicatechin A type structure and are comparable with the fragmentation patterns obtained for the dimeric A type procyanidin A2.

To check the A type structural hypothesis, the major dimer was purified and analyzed by NMR spectroscopy. Both 1D ^1H and 2D homonuclear ^1H and heteronuclear $^1H-^{13}C$ NMR experiments were performed, allowing assignment of all proton and carbon signals of the molecule (**Table 1**). The 1D ^1H spectrum showed the presence of only one type of dimeric compound and allowed both malvidin monoglucoside and epicatechin moieties to be identified. An ABC spin system in the aromatic region could be unambiguously attributed to the epicatechin B ring. The heterocyclic ring protons of this flavan-3-ol unit were easily recognized with the help of their typical chemical shifts and coupling patterns. H4α and H4β gave signals as two doubled doublets at 2.44 and 2.72 ppm, whereas H3 and H2 signals appeared at 4.08 and 4.99 ppm, respectively, as broad singlets, which are characteristic of 2−3 cis relative stereochemistry. The presence of a singlet at 6.95 ppm with a relative intensity of two as well as a signal of six methoxy protons at 3.79 ppm was characteristic of a symmetrical malvidin B ring. Two mutually coupled doublets at 4.62 and 4.79 ppm were attributed to H3 and H4 protons of the heterocycle of the malvidin moiety, ruling out the structural hypothesis of the anthocyanin in a flavene form. Moreover, the absence of H2 and of one H4 protons of the C ring of the malvidin indicated a double linkage between the two units implying both 2C and 4C positions of the malvidin heterocycle. One of the linkage positions on the epicatechin unit was either C6 or C8 of the A ring since only one A ring proton signal of the epicatechin unit appearing as a singlet at 6.09 ppm could be observed. The

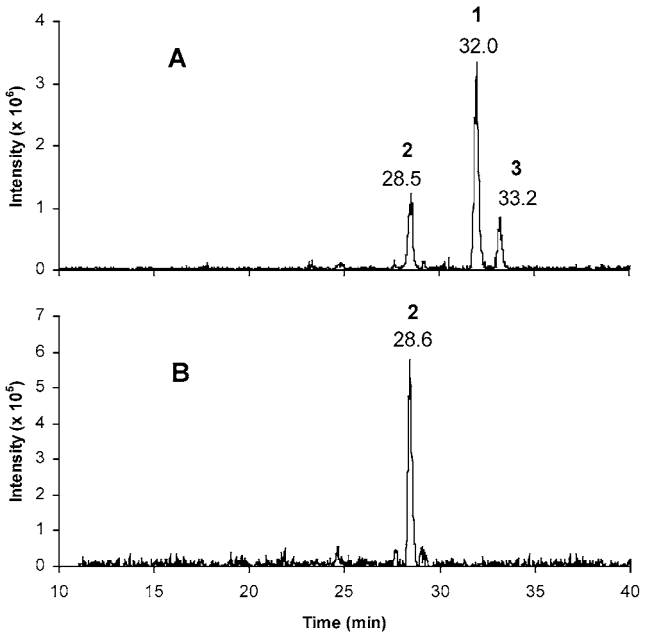

Figure 5. Ion current extracted from the total ion current of the solution of (**A**) malvidin 3-glucoside–(–)-epicatechin and (**B**) malvidin 3-glucoside–(+)-catechin at *m*/*z* 783 in the positive ion mode.

determination of lower linkage positions was achieved with HMBC long-range correlations (**Figure 4**).

Once protonated carbons were assigned with a HSQC spectrum, long-range correlations allowed quaternary carbons to be attributed. A carbon at 152 ppm, which showed correlations with H2F and both H4F, could readily be attributed to C8aD. The residual proton of the D ring at 6.09 ppm gave correlations with four quaternary carbons at 154.7, 151.0, 101.1, and 102.7 ppm, whereas no correlation was observed for the carbon 8aD (152.0 ppm). The four quaternary carbons were attributed through their correlation with other protons as shown in **Figure 4**. These results allowed the residual proton to be readily assigned to H6D. Consequently, one linkage implied the C8D position of the epicatechin unit. Furthermore, the chemical shift of the linked carbon 4C at 23.9 ppm allowed this bond to be established as a carbon−carbon linkage. Consequently, the second bond was a C2−O−C7 ether linkage since it is the only possible ether linkage position (*17*).

In their study, Bishop and Nagel (*10*) specified that they failed to obtain the bicyclic condensation product with malvidin 3-glucoside and (+)-catechin, suggesting that glycosylation of malvidin may affect its stereospecificity and its activity as an electrophile. To check whether formation of such a compound was possible, a reaction mixture was prepared with malvidin 3-glucoside and (+)-catechin instead of (−)-epicatechin in ethanol and was incubated for 19 days. LC/MS monitoring of this solution over this period showed the presence of an ion peak at *m*/*z* 783 in the positive ion mode with an absorbance maximum around 276 nm, which eluted at the same retention time as dimer **2** (**Figure 5**). The MS^2 and MS^3 analyses of the molecular ion yielded identical fragmentation patterns as for the A−T A type dimers previously identified, indicating that this dimer was also an A type compound. The solution was then submitted to the thiolysis conditions. The analysis showed that this compound also resisted to thiolysis. All of these results show that malvidin 3-glucoside and (+)-catechin condensed in an A−T dimer containing an A type interflavanoid bond.

On the other hand, because the malvidin−catechin A type dimer exhibited similar retention time as the dimer **2** obtained during incubation of malvidin 3-glucoside and epicatechin, this compound is postulated to be a malvidin 3-glucoside−(+)-catechin A type dimer. Concerning the third dimer, no result allowed conclusions to be drawn about its structure; however, it may be a C4−C6, C2−O−C7, or C2−O−C5 malvidin 3-glucoside−flavanol.

The results obtained in this study showed that malvidin 3-glucoside can directly react with (−)-epicatechin as well as with (+)-catechin to give colorless dimers. Mass spectrometric and NMR data confirmed that these compounds were A type A−T dimers linked by both carbon−carbon and ether bonds, the flavanol unit being in the lower position. In addition, the retention times and spectral characteristics of the compounds detected in the model solution are the same as those obtained earlier in a wine fraction, confirming the occurrence in wine of A type A−T dimers. Moreover, thiolysis combined with LC/MS fragmentation appeared to be an efficient method to detect such compounds. Concerning their formation mechanism, the literature suggested that they are probably formed from the intermediate flavene compound by intramolecular addition of the flavanol hydroxyl to the C2,C3 double bond (*10*, *18*).

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Received for review December 18, 2002. Revised manuscript received March 24, 2003. Accepted March 24, 2003. The work presented here was funded by the European Union (FAIR CT-950653 and QLRT-1999-00505).

JF021227B