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Synthesis and Structural Characterization of Oaklin−Catechins

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ABSTRACT: Condensation reactions of procyanidin dimer B4 with two representative oak wood cinnamic aldehydes (coniferaldehyde and sinapaldehyde) were conducted in winelike model solutions. Coniferaldehyde led to the formation of guaiacylcatechin−pyrylium−catechin (GCP−catechin, 737 m/z), whereas sinapaldehyde led to the formation of syringylcatechin−pyrylium−catechin (SCP−catechin, 767 m/z). The former was also structurally characterized by 1D and 2D NMR, allowing an elucidation of the formation mechanism of these oaklin−catechin adducts and demonstrating the importance of procyanidins in the formation of colored compounds through the reaction with cinnamic aldehydes extracted from oaks during storage.

KEYWORDS: *red wine, aging, oak barrels, procyanidin B4, aldehydes, oaklin, NMR, mass spectrometry*

INTRODUCTION

Wine storage in oak barrels during the first years of aging is a common procedure in wine industry and the extraction of volatile and nonvolatile compounds from the wood influences important characteristics in matured wine, namely aroma, taste, and color.[1,2] The extraction of these compounds depends on the period of contact between wine and wood and on the chemical composition of the wood, which is affected by the species and origin of the trees, the seasoning of the staves, the age of the barrel and most important by the heat treatment or toasting of barrels.[3−5] In this procedure, macromolecular components like lignins and polysaccharides of the wood are degraded into smaller compounds such as several aldehydes[6] (e.g., furfural, hydroxymethylfurfural, hydroxybenzaldehyde, vanillin, coniferaldehyde, sinapaldehyde) which are of particular interest because they are aroma compounds and they can also interact with some wine compounds like anthocyanins and catechins, hence contributing to color changes.[7−11]

Indeed, one of these reactions most studied is the aldehyde-mediated association of anthocyanins and catechins through a Bayer acid-catalyzed condensation, yielding new pigments with different chromatic properties than the anthocyanin precursor.[8,12−18] More recently, cinnamic aldehydes have been shown to react with catechin, yielding a new class of brick-red catechin−pyrilium pigments (3-deoxyanthocyanidin derivatives) named oaklins.[10] The formation of these compounds was confirmed in winelike model solutions containing oak wood extract, and an oaklin-derived pigment was already found in a commercial table red wine.[19] Analogous compounds may be formed through the reaction of oak-derived aldehydes with proanthocyanidins. The latter are extracted from grapes to wine during winemaking and contribute to the astringency of red wines.[20−22]

The present work deals with the formation of new oaklin pigments (see Figure 4, structure **IX**) resulting from a direct reaction of a procyanidin dimer B4 ((+)-catechin-(4−8)-(−)-epicatechin) (**III**) with two cinnamic aldehydes (**I**), coniferaldehyde and sinapaldehyde. The newly formed compounds described herein for the first time point out the importance of procyanidins and cinnamic aldehydes in the formation of colored compounds during storage.

MATERIALS AND METHODS

Samples. Coniferaldehyde and sinapaldehyde were purchased from Sigma-Aldrich. Procyanidin B4 was obtained by hemisynthesis following the procedure described in the literature.[23]

Study of the Reaction between Procyanidin B4 and Cinnamic Aldehydes. Procyanidin B4 (1.7 mM, 2 mg) was incubated with coniferaldehyde (2.1 mM) and sinapaldehyde (2.1 mM) separately in 2 mL of 12% (v/v) hydroalcoholic solutions at pH 3.5 with a molar ratio of 1:1.2 (phloroglucinol:cinnamic aldehyde). These model solutions were kept at a temperature of 35 °C and protected from light. The formation of new compounds was followed over time by HPLC−DAD using a reversed phase C-18 (Merck) column (250 × 4.6 mm i.d., particle size 5 μm), at 25 °C. Solvents were (A) water/formic acid (95:5) and (B) acetonitrile. The elution gradient was performed using a L-2130 Merck pump from 10 to 35% B for 55 min at a flow rate of 1.5 mL min^{-1}.

LC−MS Conditions. Mass spectrometry analysis was performed using a Finnigan SurVeyor series liquid chromatograph, equipped with an API source, using an electrospray ionization (ESI) probe. Solvents were (A) aqueous 0.1% acetic acid and (B) acetonitrile. The elution conditions were as follows: flow rate, 0.5 mL min^{-1}; oven temperature, 35 °C; elution began with linear gradient from 5 to 30% B in 40 min, from 30 to 40% in 10 min and from 40 to 100% in 5 min, followed by washing and re-equilibration of the column. The capillary voltage was 11 V, and the capillary temperature was 200 °C. Spectra were recorded in positive ion mode between m/z 100 and 1200. The mass spectrometer was programmed to do a series of three scans: a full mass spectrum, a MS^2 spectrum of the most intense ion, and a MS^3 spectrum of the most intense ion in the second scan, using a relative collision energy of 45 V.

Synthesis and Purification of the Guaiacylcatechin−Pyrylium−Catechin (GCP−Catechin) Adduct. Procyanidin B4 (1.7 mM, 80 mg) was incubated with coniferaldehyde (8.3 mM) in 80 mL of a 12% (v/v) hydroalcoholic solution at pH 1 with a molar ratio of 1:4.8

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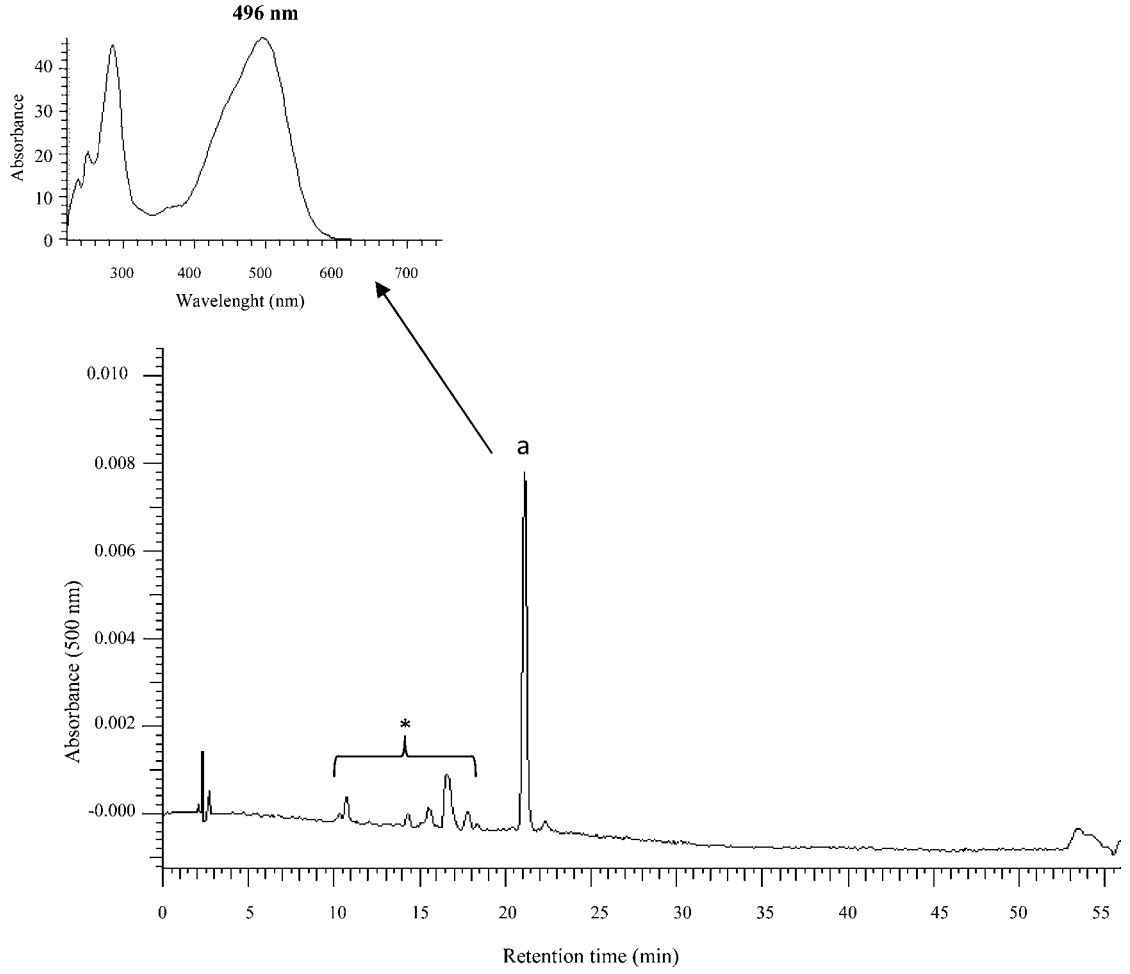

Figure 1. HPLC chromatogram recorded at 500 nm of the model solution containing B4 and coniferaldehyde, after 5 days of reaction at pH 3.5 and 35 °C. UV/vis spectrum of GCP−catechin (*, unidentified peaks; a, GCP−catechin).

(phloroglucinol:coniferaldehyde). Before the synthesis, several experiments at different pH values and molar ratios were tested in order to improve the yield of the reaction to a maximum of 9%. The model solution was kept at a temperature of 35 °C and protected from light, and the formation of new compounds was followed by HPLC−DAD. When the reaction was completed, the sample was applied on a silica gel C-18 reversed phase SPE cartridge in order to remove inorganic salts and other impurities and the pigments were eluted with methanol acidulated with 2% HCl. Methanol was evaporated in a rotary evaporator at 38 °C, and the sample was freeze-dried and stored at −18 °C until use.

The sample was further applied into a 5 cm diameter medium-porosity sintered glass funnel with TSK Toyopearl gel HW-40(S) (Tosoh), connected to standard vacuum filtration glassware and gradually eluted with increasing percentages of acidified methanol (F1, 30%; F2, 40%; F3, 50%; F4, 60%; and F5, 80%). The criterion used for changing the percentages of methanol was the decrease in color intensity of the solution eluted from the column. The solvent of each fraction was partially evaporated in a rotary evaporator at 38 °C, and the samples were freeze-dried and stored at −18 °C until use.

Semipreparative HPLC. Semipreparative HPLC was performed in order to further isolate and purify the oaklin−catechin aducts, eluted in fraction F2 from Toyopearl gel. This fraction was injected into a reversed phase C-18 (Merck) column (250 × 4.6 mm i.d., particle size 5 μm) at room temperature (volume injected was 1 mL). Solvents were (A) water/formic acid (95:5) and (B) acetonitrile. The elution gradient was performed using a L-2130 Elite LaChrom pump from 10 to 35% B for 65 min at a flow rate of 1.5 mL min^{-1} and detection was carried out at 500 nm using a L-2420 Elite LaChrom detector.

The sample collected from semipreparative HPLC was further chromatographed on a Toyopearl HW40(S) column (30 cm × 1 cm) at a flow rate of 1 mL min^{-1}, with an injection volume of 2.0 mL. The fractions obtained were gradually eluted with increasing percentages of acidified methanol from 30% to 100%. The solvent of each fraction was partially evaporated in a rotary evaporator at 38 °C, and the samples were freeze-dried and stored at −18 °C until use.

NMR Measurements. In the NMR characterization of GCP−catechin adduct, ^{1}H NMR (500.13 MHz) and ^{13}C NMR (125.77 MHz) spectra were measured in DMSO:TFA (90:10) and on a Bruker Avance 500 spectrometer at 25 °C with TMS as internal standard. ^{1}H assignments were made with the aid of 2D gCOSY (^{1}H−^{1}H), whereas ^{13}C assignments were made on the basis of 2D gHSQC (^{1}H−^{13}C) and gHMBC experiments.

RESULTS AND DISCUSSION

The reaction between cinnamic aldehydes and procyanidin B4 at pH 3.5 led to the formation of new compounds that showed maximum absorption in the visible range at λ_{max} 496 nm and at λ_{max} 506 nm from the reaction with coniferaldehyde and sinapaldehyde, respectively, indicating an orange/red color (Figure 1).

The mass spectra and the respective MS^2 and MS^3 fragmentations of the new compounds formed in the reaction with coniferaldehyde ([M]$^+$ at m/z 737) and sinapaldehyde ([M]$^+$ at m/z 767) are presented in Figure 2 and the respective fragmentation pattern in Figure 3.

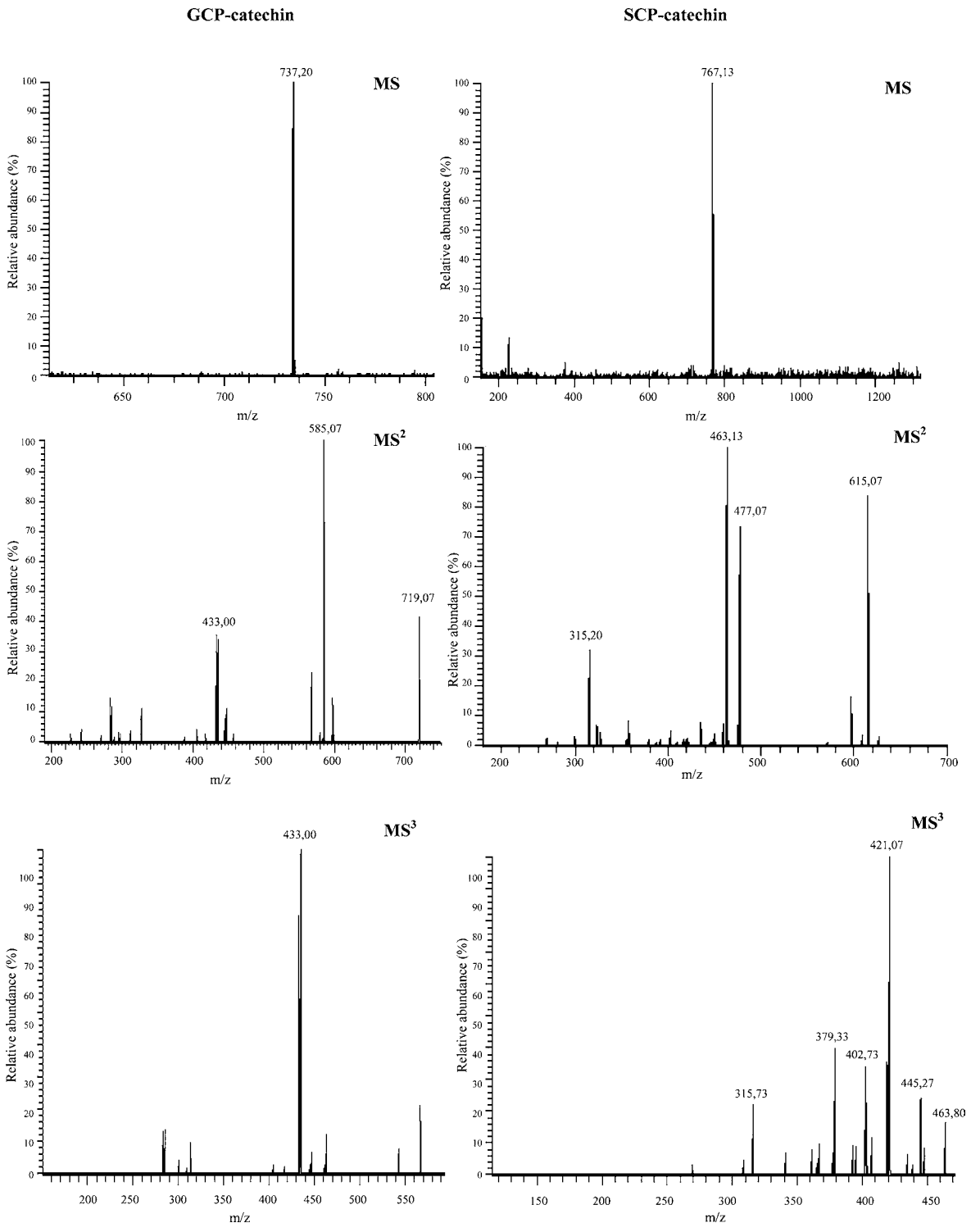

Figure 2. Mass spectra and respective MS^2 (of the molecular ion) and MS^3 (of the main fragment in MS^2) fragmentations for GCP−catechin and SCP−catechin adducts.

The molecular ions of these new compounds correspond to the sum of the molecular weights of procyanidin B4 (578 g mol^{-1}) and the cinnamic aldehydes (coniferaldehyde and sinapaldehyde, 178 and 208, respectively) and the loss of a water molecule. Coniferaldehyde led to the formation of guaiacylcatechin−pyrylium−catechin adduct (GCP−catechin, 737 m/z), whereas sinapaldehyde led to the formation of syringylcatechin−pyrylium−catechin adduct (SCP−catechin, 767 m/z). Analyzing in more detail the MS fragmentation (the products of the reaction of procyanidin B4 with coniferaldehyde) of GCP−catechin, the MS^2 spectrum shows a major fragment at m/z 585 ($[M - 152]^+$), resulting from a retro-Diels−Alder fragmentation (RDA), a fragment at m/z 719 corresponding to the loss of a water molecule ($[M - 18]^+$), and two fragments corresponding to another RDA fragmentation ($[M - 152 - 152]^+$ at m/z 433) and more two hydrogens ($[M - 152 - 152 + 2H])^+$ at m/z 435). Further MS^3 fragmentation of the ion at m/z 585 yielded two fragments at m/z 433 and 435. The other pigment

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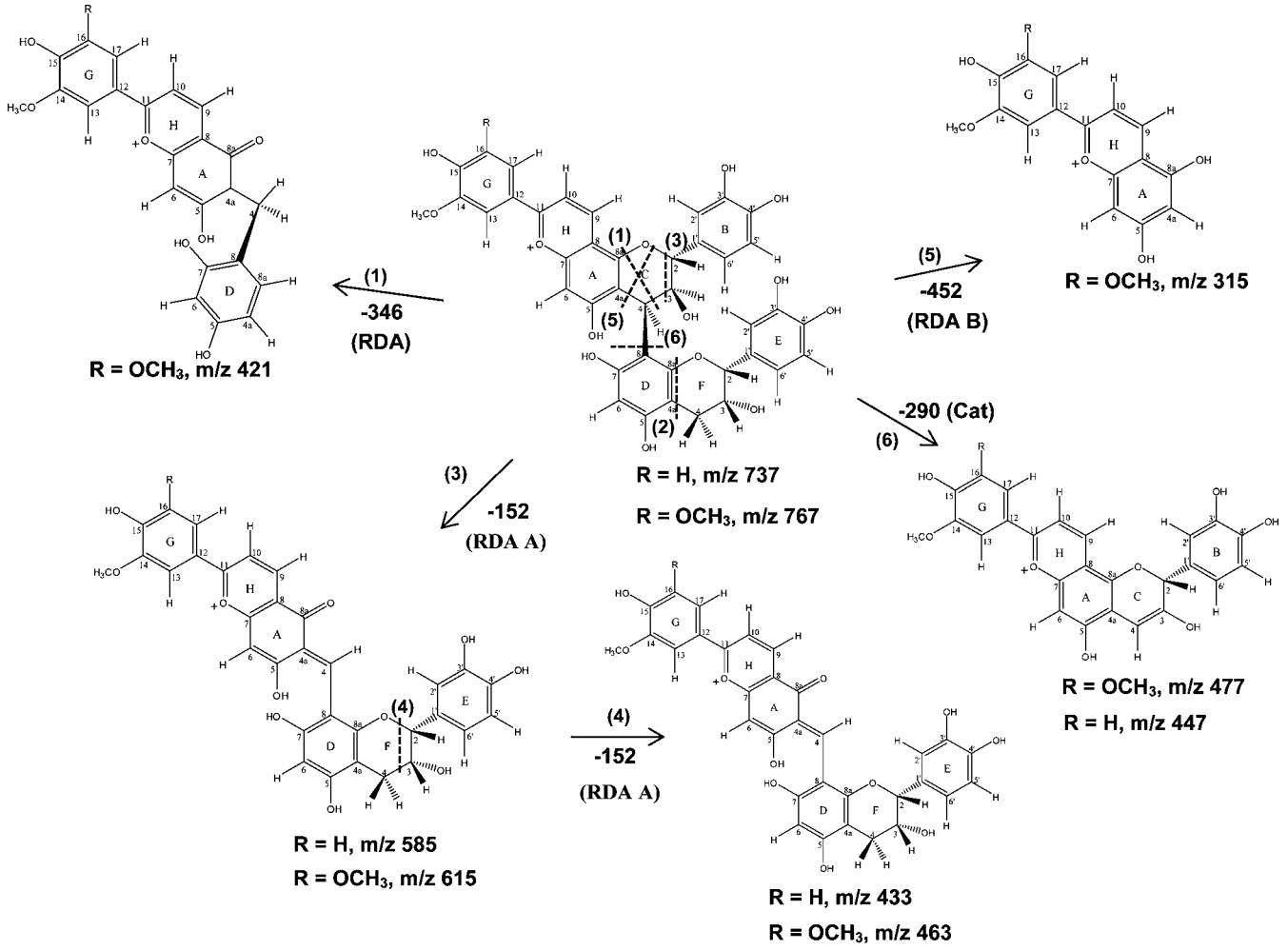

Figure 3. Fragmentation pattern of GCP−catechin (R = H) and SCP−catechin (R = OCH_3) adducts in the positive ion mode.

formed in the reaction of procyanidin B4 with sinapaldehyde followed a similar fragmentation scheme.

Synthesis and Structural Characterization of GCP−Catechin Adduct. The synthesis reaction was carried out at pH 1.0 and the sample collected from semipreparative HPLC. In these conditions, three pigments were formed and coeluted in HPLC at 21 min and were only detected after further purification by Toyopearl gel chromatography. These pigments were analyzed separately by LC−MS and correspond to two compounds with the same molecular weight (GCP−catechin isomers) and another one with a molecular weight corresponding to a GCP−catechin−catechin adduct ($[M]^+$ at m/z 1025). GCP−catechin isomers should correspond to positional isomers, as there are no chiral centers in their structure.

The GCP−catechin−catechin structure was not detected in the reaction at pH 3.5. This structure should only be formed at lower pH because procyanidin B4 may be decomposed through its interflavonoid linkage, yielding catechin monomers in solution that may react by acid-catalyzed condensations with a molecule of procyanidin dimer B4 forming procyanidin dimer−catechin adducts. Moreover, a GCP monomer structure was also detected in the mass spectra ($[M]^+$ at m/z 449) in the reaction at pH 1.0, as it is expected to be formed due to the presence of catechin monomers in solution that may react with the cinnamic aldehydes, like the mechanism described in the literature for the formation of these pigments.[10] However, only the major GCP−catechin isomer was obtained in enough quantity to be characterized by NMR.

The ^{1}H chemical shifts were assigned using 1D and 2D NMR techniques (gCOSY), and the assignment of the carbon resonances was made using 2D techniques (gHSQC and gHMBC techniques) (Table 1).

The new vinylic protons 9H and 10H revealed a clear correlation in the COSY spectrum and were attributed to the two doublets located at δ 9.00 and 8.30 ppm, respectively. The assignment of the carbon resonances was possible using two-dimensional NMR techniques (HSQC and HMBC). Carbons 9H and 10H were assigned at δ 146.9 and 111.3 ppm through HSQC correlation with the respective protons.

Concerning the procyanidin B4 moiety, the proton 4C was assigned to two different signals, δ 3.74 and 3.84 ppm, H-$4C_1$ and H-$4C_2$, respectively, through the characteristic ABMX spin system of the pyran ring C observed in the COSY spectrum. Proton 3C was also assigned to two different signals, δ 3.52 and 3.50 ppm, H-$3C_1$ and H-$3C_2$, respectively, from its correlations with proton 4C. These two signals may be attributed to two rotamers resulting from a rotation throughout the interflavonoid C-4C−C-8D linkage characterized by different C3−C4−C8−C2 dihedral angles.[24] Only protons from pyranic rings F and C show different chemical shifts for the two rotamers, probably because their chemical environment is the most affected by the C-4C−C-8D linkage rotation.

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Table 1. ^{1}H and ^{13}C NMR Data and HMBC and HSQC Correlations of Isomer 1 of GCP−Catechin, Determined in DMSO/TFA (90:10)[a]

position	δ ^{1}H (ppm); J (Hz)	δ ^{13}C (ppm)	HMBC	HSQC
		Ring G		
12G		120.3	H-16G	
13G	7.92; s	112.4		H-13G
14G		149.0	H-16G, H-13G, OMe	
15G		156.0	H-13G, H-16G	
16G	7.09*	117.5		H-16G
17G	8.08; d	126.0		H-17G
OMe	3.96; s	56.2		OCH3
		Ring H		
9H	9.00; d	146.9		H-9H
10H	8.30; d	111.3		H-10H
11H		170.2	H-10H, H-13G	
		Rings A, B, C		
2C	na	na		H-2C
$3C_1$	3.52*	60.4		H-$3C_1$
$3C_2$	3.50*			H-$3C_2$
$4C_1$	3.74*	65.5		H-$4C_1$
$4C_2$	3.84*			H-$4C_2$
4aA		110.5	H-6A	
5A		168.8	H-6A	
6A	7.17; s	95.4		H-6A
7A		156.9	H-6A	
8A		111.5	H-6A, H-10H	
8aA		153.7	H-9H	
1′B		129.6	H-2′B	
2′B	6.76*	118.0		H-2′B
3′B		145.3	H-2′B	
4′B		145.5	H-6′B	
5′B	6.77*	115.4		H-5′B
6′B	6.81*	118.4		H-6′B
		Rings D, E, F		
$2F_1$	5.20*	80.2		H-$2F_1$
$2F_2$	5.30*			H-$2F_2$
$3F_1$	4.85*	66.3		H-3αF
$3F_2$	4.26*			H-3βF
4$\alpha$$F_1$	2.86*	40.0		H-4$\alpha$$F_1$
4$\beta$$F_1$	2.92*			H-4$\beta$$F_1$
4$\alpha$$F_2$	3.44*			H-4$\alpha$$F_2$
4$\beta$$F_2$	3.49*			H-4$\beta$$F_2$
4aF		na		
5D		145.0	H-6D	
6D	7.02; s	115.5		H-6D
7D		145.0	H-6D	
8D		na		
8aD		na		
1′E		128.4	H-2F, H-6′E	
2′E	6.88*	115.0		H-2′G
3′E		144.8	H-2′E	
4′E		145.0	H-6′E	
5′E	6.88*	118.5		H-5′G
6′E	6.74*	115.4		H-6′G

[a]na, not attributed; s, singlet; d, doublet; brs, broad singlet; *, unresolved.

Proton H-4αF was assigned to two different signals, δ 2.86 and 2.91 ppm, H-4$\alpha$$F_1$ and H-4$\alpha$$F_2$, respectively, through the characteristic ABMX spin system of the pyran ring C observed in the COSY spectrum. The same occurs to proton H-4βF with two different signals, δ 3.44 and 3.49 ppm, H-4$\beta$$F_1$ and H-4$\beta$$F_2$, respectively. Proton H-3F was also assigned to two different signals, δ 3.52 and 3.52 ppm, H-3F_1 and H-3F_2, respectively, from its correlations with protons H-4αF (weak) and H-4βF (strong). H-3F also correlates with H-2F, which also shows two different signals, δ 5.20 and 5.30 ppm, H-2F_1 and H-2F_2, respectively. The only proton detected on ring D (H-6D) was assigned to the singlet at δ 7.02 ppm.

Carbons C-4aA and C-7A were determined from their long-range $^{1}H-^{13}C$ correlation with H-6A. The quaternary carbons C-5A and C-8aA were assigned at δ 168.8 and 153.7 ppm from their long-distance correlations with protons H-6A and H-9H, respectively, observed in the HMBC spectrum.

These correlations as well as the lack of a long-distance correlation between carbon C-8aA and the singlet at 7.17 ppm (H-6A) identify unambiguously the position of the pyrylium ring H linkages onto carbons C-7A and C-8A, as they could not be observed if ring H was formed between the hydroxyl group at carbon C-5A and the carbon C-6A.

The chemical shifts of the remaining protons and carbons identified in Table 1 were easily established by HSQC and HMBC techniques.

Formation Mechanism. The hypothetic mechanism of formation of these new pigments from the reactions between procyanidin B4 and cinnamic aldehydes is represented in Figure 4. By analogy with the mechanism described in the literature for the formation of monomeric oaklins, the reaction starts with the protonation of the cinnamic aldehyde **I** in acidic medium, forming a carbocation in the carbonyl carbon **II**, followed by a nucleophilic attack of ring A of procyanidin B4 **III**, leading to structure **IV**. The A ring attack may occur from position C6 or preferentially from position C8. Indeed, the negative formal charge in ring A is expected to be higher at carbon 8, as it is well-documented for flavylium compounds in the reaction leading to the formation of catechin−alkyl/aryl−anthocyanin adducts.[18,25] The putative presence of compound **IV** was evidenced in the mass spectra by the presence of a protonated molecular ion ($[M + 1]^+$) at m/z 757. The dehydration of the resulting protonated adduct **V** yields a new carbocation, **VI**, which undergoes a rearrangement leading to carbocation **VII**. This carbocation suffers an intramolecular nucleophilic attack by the hydroxyl group at carbon 7 of ring A, leading to structure **VIII**. The putative presence of compound **VIII** was evidenced in the mass spectra by the presence of a protonated molecular ion ($[M + 1]^+$) at m/z 739. The final oxidation yields the structure **IX**, which has the pyrylium ring H associated with the aromatic ring A and constitutes a chromophore group. The extended conjugation of the π electrons are at the origin of their maximum absorption around 500 nm. The GCP−catechin position isomer formed at pH 1.0 in lower amount may result from an initial nucleophilick attack from position C6 to the aldehyde, resulting in the formation of a new pyranic ring involving positions 5 and 6 of ring A.

Procyanidin B4 showed the ability to react directly with cinnamic aldehydes, namely, coniferaldehyde and sinapaldehyde, to give orange pigments (oaklins). Oaklins monomers formed from the reaction between catechin and cinnamic aldehydes have already been detected in wine model solutions (11-guaiacylcatechinpyrylium) and wine.[19] Therefore, attending to the relatively high amount of procyanidin dimers in real wines, it is also expected that oaklin−catechin adducts may also occur in wine and even play a role in some color

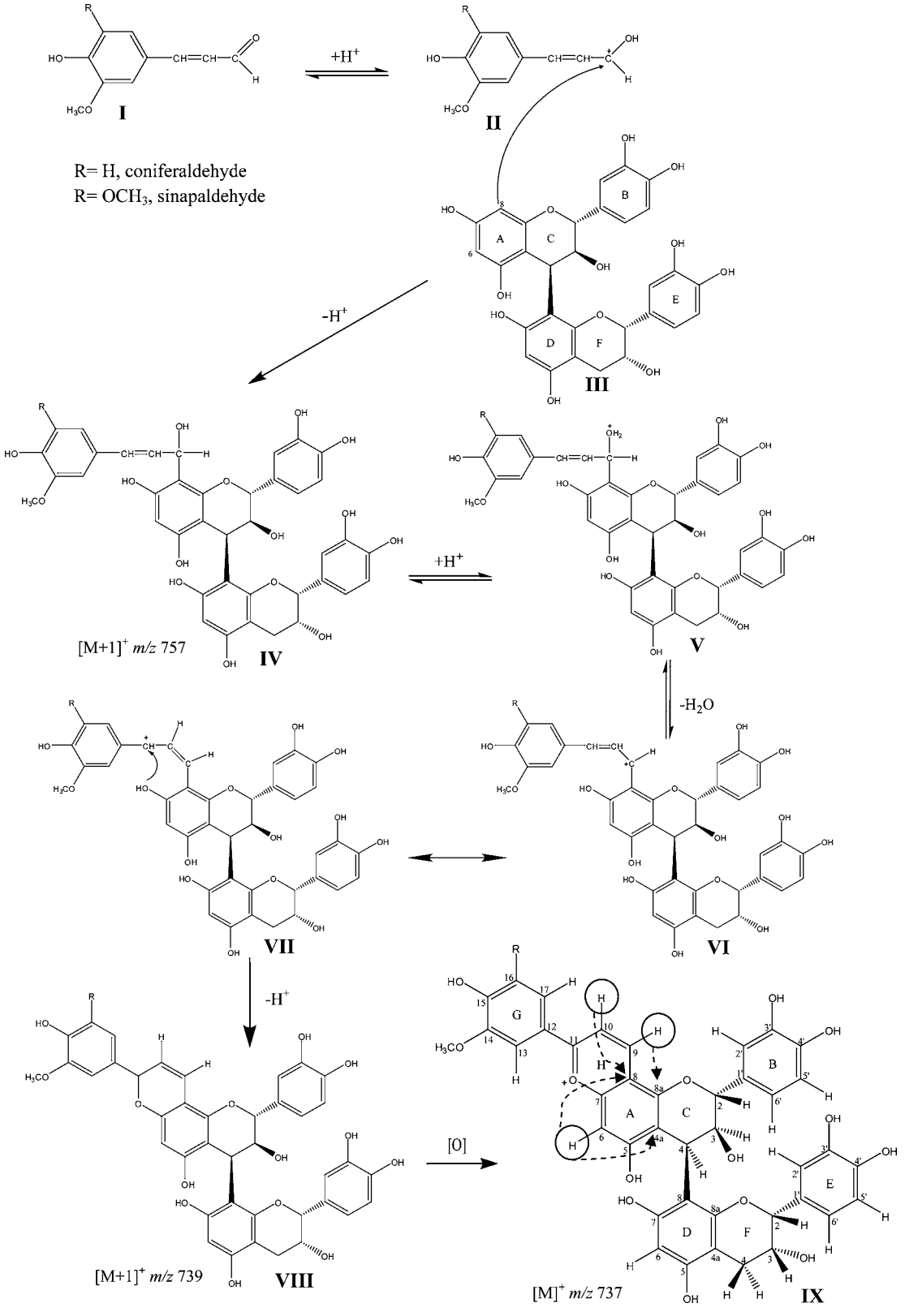

Figure 4. Hypothetical mechanism for the formation of oaklin−catechins **IX** obtained from the reaction between procyanidin B4 **III** and cinnamic aldehydes **I**.

changes observed during the aging process. Nonetheless, further studies are still required to unequivocally detect the formation of oaklin−catechins in wine and to understand their overall contribution to wine properties, such as color and taste.

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