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Identification and Sensory Evaluation of Dehydro- and Deoxy-ellagitannins Formed upon Toasting of Oak Wood (*Quercus alba* L.)

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Traditionally, spirits such as whiskey are matured in toasted wood barrels to improve the sensory quality of the final beverage. In order to gain first insight into the puzzling road map of thermal ellagitannin transformation chemistry and provide evidence for the changes in sensory active nonvolatiles in oak wood during toasting, the purified oak ellagitannins castalagin and vescalagin, their corresponding dimers roburin A and roburin D, and 33-carboxy-33-deoxyvescalagin were thermally treated in model experiments. Besides mouth-coating and golden-brown colored melanoidin-type polymers, individual major reaction products were produced as transient intermediates which were identified for the first time by means of LC-MS/MS and 1D/2D-NMR spectroscopy. Depending strongly on the stereochemistry, castalagin is oxidized to the previously unreported dehydrocastalagin, whereas its diastereomer vescalagin, differing only in the stereochemistry at carbon C-1, is most surprisingly converted into deoxyvescalagin. Comparative model experiments with 33-carboxy-33-deoxyvescalagin revealed castalagin, vescalagin, dehydrocastalagin, and deoxyvescalagin as typical reaction products, thus indicating decarboxylation as a key step in the thermal degradation of that ellagitannin. Similar to the ellagitannin monomers, LC-MS/MS analyses gave strong evidence that the corresponding dimer roburin A, containing the vescalagin configuration at C-1, was converted into the deoxyroburin A, whereas roburin D, exhibiting the castalagin configuration at C-1, was oxidized to give the dehydroroburin D. Human sensory experiments revealed that the ellagitannin derivatives imparted an astringent mouth-coating sensation with threshold concentrations ranging from 1.1 to 126.0 μmol/L, depending strongly on their chemical structure.

KEYWORDS: Ellagitannins; castalagin; vescalagin; roburin A; roburin D; taste; astringency; oak wood; whiskey

INTRODUCTION

Over centuries, spirits such as whiskey have been matured in toasted wood barrels for several years to give the beverage a final improvement in color, aroma, taste, and mouth fullness, the later of which is perceived as body, viscosity, or complexity in the oral cavity. For this maturation procedure American white oak (*Quercus alba* L.) and European oak (*Quercus robur* L.) have emerged as the wood of choice but, in particular, the toasting of the oak wood is commonly accepted as one of the most important technological steps in barrel manufacturing. During the toasting process, the inner surface of the oak wood barrels is burned with an open fire, generating a thin layer of charcoal and inducing severe changes in the wood chemical composition (*1*). During the last decades, multiple studies have been focused on the thermal generation of odor-active volatiles from nonvolatile oak-derived precursors; for example, toasting was demonstrated to liberate the key odorant vanillin upon lignin decomposition (*2*, *3*), and the so-called whiskey lactone, recently confirmed as a key aroma compound of whiskey (*4*), was found to be released from its progenitors 3-methyl-4-(3′,4′-dihydroxy-5′-methoxybenzoyl) octanoic acid (*5*) and 3-methyl-4-(1-*O*-β-D-glucopyranosyl) octanoic acid (*6*), respectively. In comparison, the information available on sensory active nonvolatiles formed from ellagitannin precursors in oak wood is rather scarce.

Nonvolatile oak wood ellagitannin monomers such as castalagin (**1**) (**Figure 1**) and vescalagin (**2**), the recently reported 33-carboxy-33-deoxyvescalagin (**3**), and ellagitannin dimers such as roburin D (**4**) and roburin A (**5**) are well characterized in their chemical structure (*7*−*9*), and their sensory attributes were recently evaluated (*9*). Depending on the degree of thermal treatment, such macromolecular ellagitannins are well accepted to be degraded to give castalin (**6**), vescalin (**7**), and ellagic

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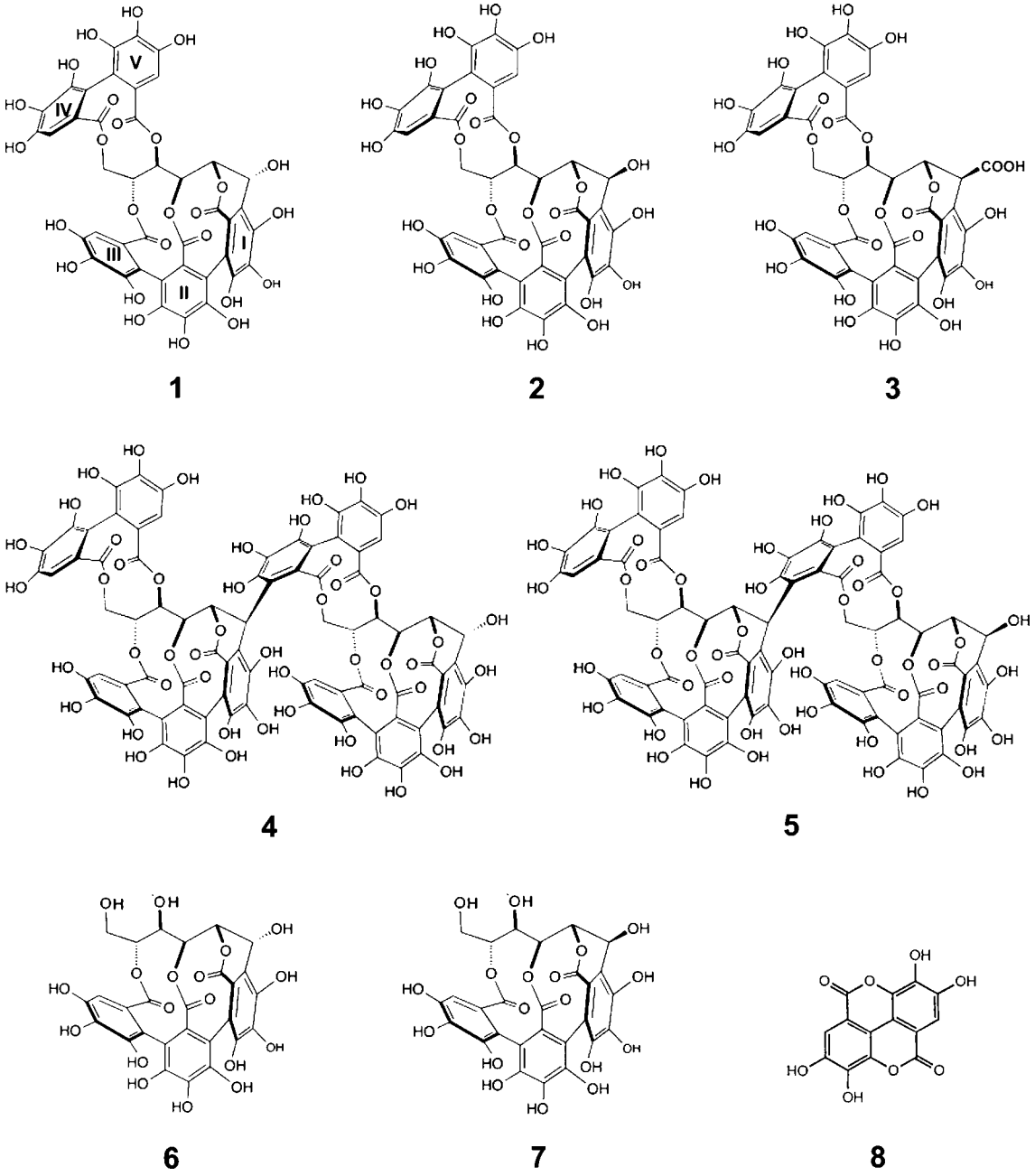

Figure 1. Chemical structures of the ellagitannin monomers castalagin (**1**), vescalagin (**2**), and 33-carboxy-33-deoxyvescalagin (**3**), the ellagitannin dimers roburin D (**4**) and roburin A (**5**), as well as chemical structures of castalin (**6**), vescalin (**7**), and ellagic acid (**8**) released from castalagin (**1**) and vescalagin (**2**) upon thermohydrolysis.

acid (**8**) besides colored melanoidin-type macromolecules, thus explaining the small amounts of native ellagitannins in spirits matured in toasted oak wood (*10*−*12*). Although such colored macromolecules are believed to impart mouth fullness as well as the golden-brown color to spirits such as whiskey, neither the chemical structures and sensory properties of the thermal ellagitannin metabolites nor the reaction mechanisms underlying the ellagitannin degradation are known to date (*13*).

Therefore, the purpose of the present study was to isolate the main nonvolatile reaction products formed upon thermal treatment of purified ellagitannins, to determine their chemical structure using LC/MS and 1D/2D-NMR spectroscopy, and to investigate the sensory activity of these thermally generated ellagitannin metabolites by means of human sensory experiments.

MATERIALS AND METHODS

Chemicals and Materials. Chips from a seasoned and 2 years air-dried oak wood (*Q. robur* L. and *Q. alba* L.) were obtained from the cooperaging industry (USA); formic acid, hydrochloric acid, and sodium hydroxide were from Grüssing (Filsum, Germany); solvents were of HPLC grade (Merck, Darmstadt, Germany); deuterated solvents were from Euriso-top (Saarbrücken, Germany). Deionized water used for chromatography was purified by means of a Milli-Q Gradient A10 system (Millipore, Billerica, USA). For sensory analyses, bottled water (Evian, low mineralization: 405 mg/L) was adjusted to pH 4.5 with trace amounts of formic acid prior to use. The oak wood ellagitannins castalagin (**1**), vescalagin (**2**), 33-carboxy-33-deoxyvescalagin (**3**), roburin D (**4**), and roburin A (**5**) were isolated from *Q. robur* and *Q. alba* L. closely following the procedure reported recently (*9*).

Preparation of Castalin (6) and Vescalin (7). Castalagin and vescalagin (each 100 mg), respectively, were maintained for 24 h at 60 °C in an aqueous hydrochloric acid solution (200 mL; 1 mol/L in

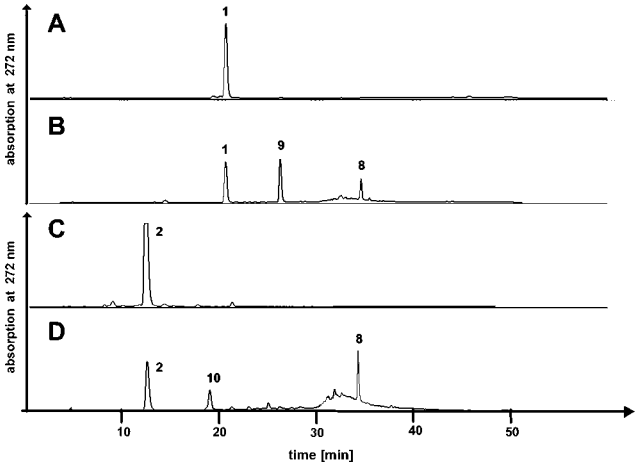

Figure 2. RP-HPLC chromatogram of native castalagin (**A**) and vescalagin (**C**) and after thermal treatment (**B**, **D**) for 60 min at 180 °C.

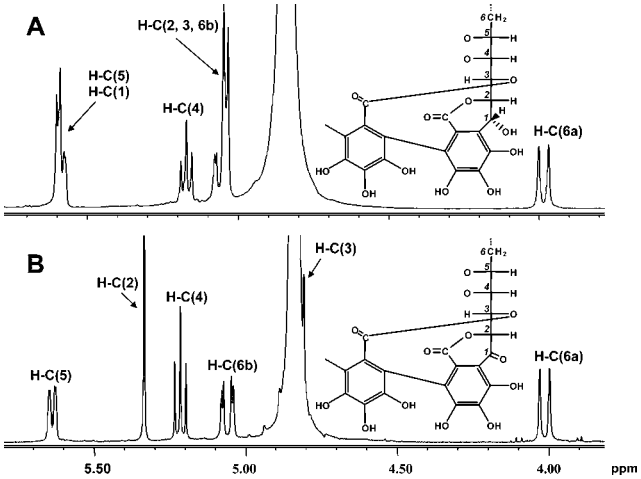

Figure 3. Excerpt of ^{1}H NMR spectrum (400 MHz, methanol-d_4) of castalagin (**A**) and dehydrocastalagin (**B**).

water), then adjusted to pH 6.0 with sodium hydroxide (1 mol/L), freeze-dried, and, finally, isolated by means of preparative RP-HPLC. Castalin and vescalin (yield: ~20% each) were obtained as white amorphous powders in a purity of more than 98% as measured by LC-MS.

Castalin, ***6*** *(**Figure 1**).* UV/vis (water) λ_{max} = 229 nm. LC/TOF-MS: $C_{27}H_{20}O_{18}$. LC/MS (ESI$^-$): *m/z* 315 (100, $[M - 2H]^{2-}$, 631 (87, $[M - H]^-$). ^{1}H NMR (400 MHz; D_2O): δ 3.80 [dd, 1H, J = 3; 12 Hz, H−C(6)], 3.88 [d, 1H, J = 3; 12 Hz, H−C(6)], 3.96 [t, 1H, J = 7 Hz, H−C(4)], 4.88 [d, 1H, J = 7 Hz, H−C(3)], 5.02 [q, 1H, J = 3, 7 Hz, H−C(5)], 5.11 [d, 1H, J = 3.0 Hz, H−C(2)], 5.55 [s, 1H, H−C(1)], 6.78 [s, 1H, H−C(2′, III)]. ^{13}C NMR (100 MHz; D_2O): δ 60.9 [C(6)], 65.8 [C(1)], 67.5 [C(4)], 68.7 [C(3)], 72.3 [C(2)], 74.5 [C(5)], 108.6 [C(2′, III)], 112.0 [C(6′, I)], 113.0 [C(6′, III)], 113.2 [C(6′, II)], 115.5 [C(2′, II)], 116.5 [C(2′, I)], 120.7 [C(1′, I)], 124.8 [C(1′, III)], 126.7 [C(1′, II)], 134.3 [C(4′, II)], 135.7 [C(4′, III)], 137.9 [C(4′, I)], 143.3 [C(5′, III)], 143.6 [C(5′, II)], 143.7 [C(5′, I)], 145.0 [C(3′, III)], 145.0 [C(3′, I)], 145.6 [C(3′, II)], 166.5 [C(7′, I)], 166.9 [C(6′, II)], 168.4 [C(7′, III)].

Vescalin, ***7*** *(**Figure 1**).* UV/vis (water) λ_{max} = 229 nm. LC/TOF-MS: $C_{27}H_{20}O_{18}$. LC/MS (ESI$^-$): *m/z* 315 (100, $[M - 2H]^{2-}$, 631 (75, $[M - H]^-$). ^{1}H NMR (400 MHz; D_2O): δ 3.82 [dd, 1H, J = 3; 12.5 Hz, H−C(6)], 3.88 [d, 1H, J = 3; 12.5 Hz, H−C(6)], 3.95 [t, 1H, J = 7 Hz, H−C(4)], 4.46 [d, 1H, J = 7 Hz, H−C(3)], 4.78 [s, 1H, H−C(1)], 5.04 [q, 1H, J = 3; 7.0 Hz, H−C(5)], 5.32 [s, 1H, H−C(2)], 6.79 [s, 1H, H−C(2′, III)]. ^{13}C NMR (100 MHz; D_2O): δ 60.8 [C(6)], 64.0 [C(1)], 67.6 [C(4)], 70.7 [C(3)], 74.2 [C(5)], 76.0 [C(2)], 108.5 [C(2′, III)], 112.3 [C(6′, I)], 113.0 [C(6′, III)], 113.9 [C(6′, II)], 115.2 [C(2′, II)], 116.8 [C(2′, I)], 123.5 [C(1′, I)], 124.8 [C(1′, III)], 126.8 [C(1′, II)], 134.4 [C(4′, II)], 135.7 [C(4′, III)], 137.8 [C(4′, I)], 143.6 [C(5′, III)], 143.6 [C(5′, II)], 143.8 [C(5′, I)], 144.2 [C(3′, I)], 145.0 [C(3′, III)], 147.4 [C(3′, II)], 166.7 [C(7′, I)], 166.8 [C(7′, II)], 168.3 [C(7′, III)].

Thermal Treatment of Ellagitannins. The purified ellagitannins **1**−**7** (50 mg each) were separately dry-heated in a lab oven for 15, 30, 60, 90, 120, and 240 min at 180 °C. After cooling, the reaction mixtures were taken up in water (5 mL), and an aliquot (200 μL) was analyzed by means of RP-HPLC/DAD (**Figure 2**). The thermally treated samples of castalagin and vescalagin were then separated by means of preparative RP-HPLC and, after purification by rechromatography, the main reaction products dehydrocastalagin (**Figure 3B**) and deoxyvescalagin, respectively, were analyzed by means of LC/TOF-MS, LC-MS/MS, and 1D/2D-NMR experiments. The reaction products generated upon thermal treatment of castalin, vescalin, roburin A, roburin D, and 33-carboxy-33-deoxyvescalagin, respectively, were analyzed by means of LC/TOF-MS and LC-MS/MS.

Dehydrocastalagin, ***9*** *(**Figure 4**).* UV/vis (water) λ_{max} = 229 nm. LC/TOF-MS: $C_{41}H_{24}O_{26}$. LC/MS (ESI$^-$): *m/z* 465 (100, $[M - 2H]^{2-}$, 931 (80, $[M - H]^-$). ^{1}H NMR (400 MHz; CD_3OD): δ 4.02 [d, 1H, J = 12.5 Hz, H−C(6)], 5.06 [d, 1H, J = 12.9 Hz, H−C(6)], 4.81 [d, 1H, J = 7 Hz, H−C(3)], 5.21 [t, 1H, J = 7 Hz, H−C(4)], 5.33 [d, 1H, J = 1 Hz, H−C(2)], 5.64 [d, 1H, J = 7.3 Hz, H−C(5)], 6.59 [s, 1H, H−C(2′, V)], 6.77 [s, 1H, H−C(2′, IV)], 6.83 [s, 1H, H−C(2′, III)]. ^{13}C NMR (100 MHz; CD_3OD): δ 64.5 [C(6)], 68.5 [C(4)], 69.7 [C(3)], 70.6 [C(5)], 77.7 [C(2)], 106.7 [C(2′, V)], 107.0 [C(2′, IV)], 108.0 [C(2′, III)], 111.5 [C(2′, I)], 112.1 [C(6′, I)], 113.4 [C(6′, II)], 113.9 [C(6′, III)], 114.3 [C(6′, V)], 115.2 [C(6′, IV)], 115.4 [C(2′, II)], 120.5 [C(1′, I)], 123.0 [C(1′, III)], 123.4 [C(1′, IV)], 125.4 [C(1′, V)], 126.3 [C(1′, II)], 134.4 [C(4′, II)], 135.8 [C(4′, V)], 136.4 [C(4′, III)], 136.7 [C(4′, IV)], 137.7 [C(4′, I)], 143.3−145.7 [10C, C(3′, I−V), C(5′, I−V)], 162.6 [C(7′, I)], 164.4 [C(7′, II)], 165.9 [C(7′, IV)], 166.5 [C(7′, III)], 168.9 [C(7′, V)], 190.1 [C(1)].

Deoxyvescalagin, ***10*** *(**Figure 4**).* UV/vis (water) λ_{max} = 229 nm. LC/TOF-MS: $C_{41}H_{26}O_{25}$. LC/MS (ESI$^-$): *m/z* 458 (100, $[M - 2H]^{2-}$, 917 (67, $[M - H]^-$). ^{1}H NMR (400 MHz; D_2O): δ 2.98 [d, 1H, J = 15.0 Hz, H−C(1)], 3.40 [d, 1H, J = 15.0 Hz, H−C(1)], 4.16 [d, 1H, J = 12.5 Hz, H−C(6)], 4.89 [d, 1H, J = 12.9 Hz, H−C(6)], 4.79 [d, 1H, J = 7 Hz, H−C(3)], 5.11 [t, 1H, J = 7 Hz, H−C(4)], 5.41 [d, 1H, J = 1 Hz, H−C(2)], 5.54 [d, 1H, J = 7.3 Hz, H−C(5)], 6.70 [s, 1H, H−C(2′, V)], 6.77 [s, 1H, H−C(2′, IV)], 6.93 [s, 1H, H−C(2′, III)]. ^{13}C NMR (100 MHz; D_2O): δ 28.2 [C(1)], 65.5 [C(6)], 68.8 [C(4)], 70.4 [C(3)], 71.0 [C(5)], 72.0 [C(2)], 107.1 [C(2′, V)], 108.8 [C(2′, IV)], 109.1 [C(2′, III)], 111.3 [C(2′, I)], 112.1 [C(6′, I)], 113.4 [C(6′, II)], 113.5 [C(6′, V)], 113.7 [C(6′, III)], 115.2 [C(6′, IV)], 115.4 [C(2′, II)], 120.5 [C(1′, I)], 123.0 [C(1′, III)], 123.4 [C(1′, IV)], 125.4 [C(1′, V)], 126.3 [C(1′, II)], 134.4 [C(4′, II)], 135.0 [C(4′, V)], 136.4 [C(4′, III)], 136.6 [C(4′, IV)], 137.6 [C(4′, I)], 143.3−146.7 [10C, C(3′, I−V), C(5′, I−V)], 165.7 [C(7′, II)], 166.3 [C(7′, I)], 167.2 [C(7′, IV)], 167.6 [C(7′, III)], 170.2 [C(7′, V)].

Dehydrocastalin, ***11*** *(**Figure 4**).* UV/vis (water) λ_{max} = 229 nm. LC/TOF-MS: $C_{27}H_{18}O_{18}$. LC/MS (ESI$^-$): *m/z* 314 (100, $[M - 2H]^{2-}$, 629 (87, $[M - H]^-$).

Deoxyvescalin, ***12*** *(**Figure 4**).* UV/vis (water) λ_{max} = 229 nm. LC/TOF-MS: $C_{27}H_{20}O_{17}$. LC/MS (ESI$^-$): *m/z* 307 (100, $[M - 2H]^{2-}$, 615 (66, $[M - H]^-$).

Dehydroroburin D, ***13*** *(**Figure 4**).* UV/vis (water) λ_{max} = 229 nm. LC/TOF-MS: $C_{82}H_{48}O_{51}$. LC/MS (ESI$^-$): *m/z* 615 (100, $[M - 3H]^{3-}$, 923 (82, $[M - 2H]^{2-}$).

Deoxyroburin A, ***14*** *(**Figure 4**).* UV/vis (water) λ_{max} = 229 nm. LC/TOF-MS: $C_{82}H_{50}O_{50}$. LC/MS (ESI$^-$): *m/z* 610 (100, $[M - 3H]^{3-}$, 916 (75, $[M - 2H]^{2-}$).

Sensory Analyses. Twelve assessors (five male, seven female), who had given informed consent to participate in the sensory tests of the present investigation and had no history of known taste disorders, were trained in sensory experiments at regular intervals for at least 2 years as described earlier (*9*, *14*−*16*) and were, therefore, familiar with the techniques applied. Sensory analyses were performed in a sensory panel room at 22−25 °C in three independent sessions. Prior to sensory analysis, the fractions or compounds isolated were suspended in water, and, after removing the volatiles in high vacuum (<5 mPa), were freeze-

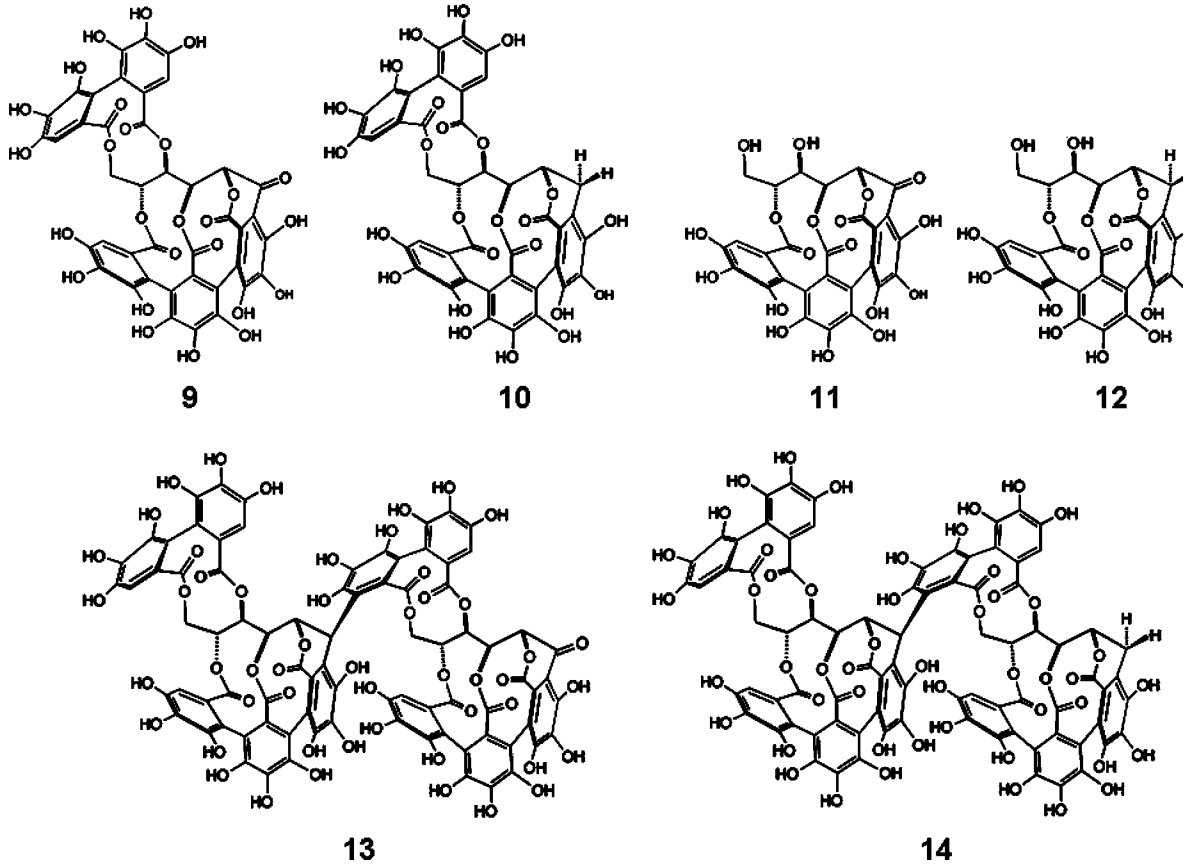

Figure 4. Chemical structures of the thermal ellagitannin transformation products dehydrocastalagin (**9**), deoxyvescalagin (**10**), dehydrocastalin (**11**), deoxyvescalin (**12**), dehydroroburin D (**13**), and deoxyroburin A (**14**).

dried twice. GC-MS and ion chromatographic analysis revealed that food fractions treated by that procedure are essentially free of the solvents and buffer compounds used.

To overcome carry-over effects of astringent compounds, oral recognition threshold concentrations of astringent compounds were determined in bottled water (pH 4.5) by means of the half-tongue test as reported in detail in previous publications (*9*, *14*, *16*). The threshold value of the sensory group was approximated by averaging the threshold values of the individuals in three independent sessions. Values between individuals and separate sessions differed not more than plus or minus one dilution step; that is, a threshold value of 1.1 μmol/L for castalagin represents a range of 0.55−2.2 μmol/L.

Thermal Treatment of Oak Wood. Oak wood chips were heated in a lab oven for 15, 30, 60, 90, 120, and 240 min at 180 °C. After cooling, the wood was extracted three times with methanol/water (70/30, v/v), freed from solvent, and freeze-dried. An aliquot (10 mg) of the extract was dissolved in solvent mixture (10 mL) and was analyzed by means of HPLC. Quantitation of ellagitannins was performed by means of a five-point external calibration.

High-Performance Liquid Chromatography (HPLC). Liquid chromatography was performed on a HPLC apparatus (Jasco, Groβ-Umstadt, Germany) consisting of two pumps (PU 2086/2087), a gradient mixer (1000 μL), a Rheodyne injector with a 200 μL loop, and a MD 2010plus diode array detector (Jasco, Germany) monitoring the effluent in a wavelength range between 220 and 500 nm. Analytical HPLC was done on a 250 × 10 mm i.d., 5 μm, RP-18, ODS-Hypersil column (ThermoHypersil, Kleinostheim, Germany) equipped with a guard column of the same type. Using an aqueous formic acid solution (0.3% in water) as solvent A and acetonitrile as solvent B, chromatography was started at a flow rate of 3 mL/min with 5% solvent B for 10 min, then increasing solvent B to 15% within 15 min, then to 60% within another 15 min, and, finally, maintaining for 3 min at 60% solvent B. Preparative HPLC was performed using a 2.0 mL sample loop and a 250 × 21.2 mm i.d., 5 μm, RP-18, ODS-Hypersil column (ThermoHypersil, Kleinostheim, Germany) equipped with a guard column of the same type. Using an aqueous formic acid solution (0.3% in water) as solvent A and acetonitrile as solvent B, chromatography was started at a flow rate of 18 mL/min with 0% solvent B for 5 min, increasing solvent B to 5% within 15 min, then to 15% within 15 min, then to 60% within 10 min, and, finally, maintaining for 3 min at 60% solvent B.

Liquid Chromatography/Time-of-Flight Mass Spectrometry (LC/TOF-MS). High-resolution mass spectra of the compounds were measured on a Bruker Micro-TOF (Bruker Daltronics, Bremen, Germany) mass spectrometer and referenced on sodium formate. The deviation of the measured from the calculated molecular mass was less than 1.5 ppm.

Liquid Chromatography/Mass Spectrometry (LC/MS). Electrospray ionization (ESI) spectra were acquired on a API 4000 Q-Trap LC/MS/MS system (AB Sciex Instruments, Darmstadt, Germany) with an Agilent 1100 HPLC system operating at a flow rate of 200 μL/min with direct loop injection of the sample (2−20 μL). The spray voltage was set at −4500 V in ESI^- mode and at 5500 V in ESI^+ mode. Nitrogen served as curtain gas (20 psi); the declustering potential was set at −10 to −40 V in ESI^- mode and at 30 V in ESI^+ mode. The mass spectrometer was operated in the full scan mode monitoring positive and negative ions. For analysis of the thermal reaction products of 33-carboxy-33-deoxyvescalagin, the selected ion monitoring (SIM) mode was used in negative mode with *m*/*z* 917, 931, and 933 as target ions. Fragmentation of $[M - H]^-$ and $[M + H]^+$ pseudomolecular ions into specific product ions was induced by collision with nitrogen (4×10^{-5} Torr) and a collision energy of −40 V.

Nuclear Magnetic Resonance Spectroscopy (NMR). The ^1H, ^{13}C, COSY, HMQC, and HMBC spectroscopic experiments were performed on a DPX 400 NMR spectrometer from Bruker (Rheinstetten, Germany). Samples were dissolved in D_2O and CD_3OD, respectively, and placed into NMR tubes (Schott Professional 178 × 5 mm) prior to measurement. Spectrum analysis was done with NMR Software Mestre-C.

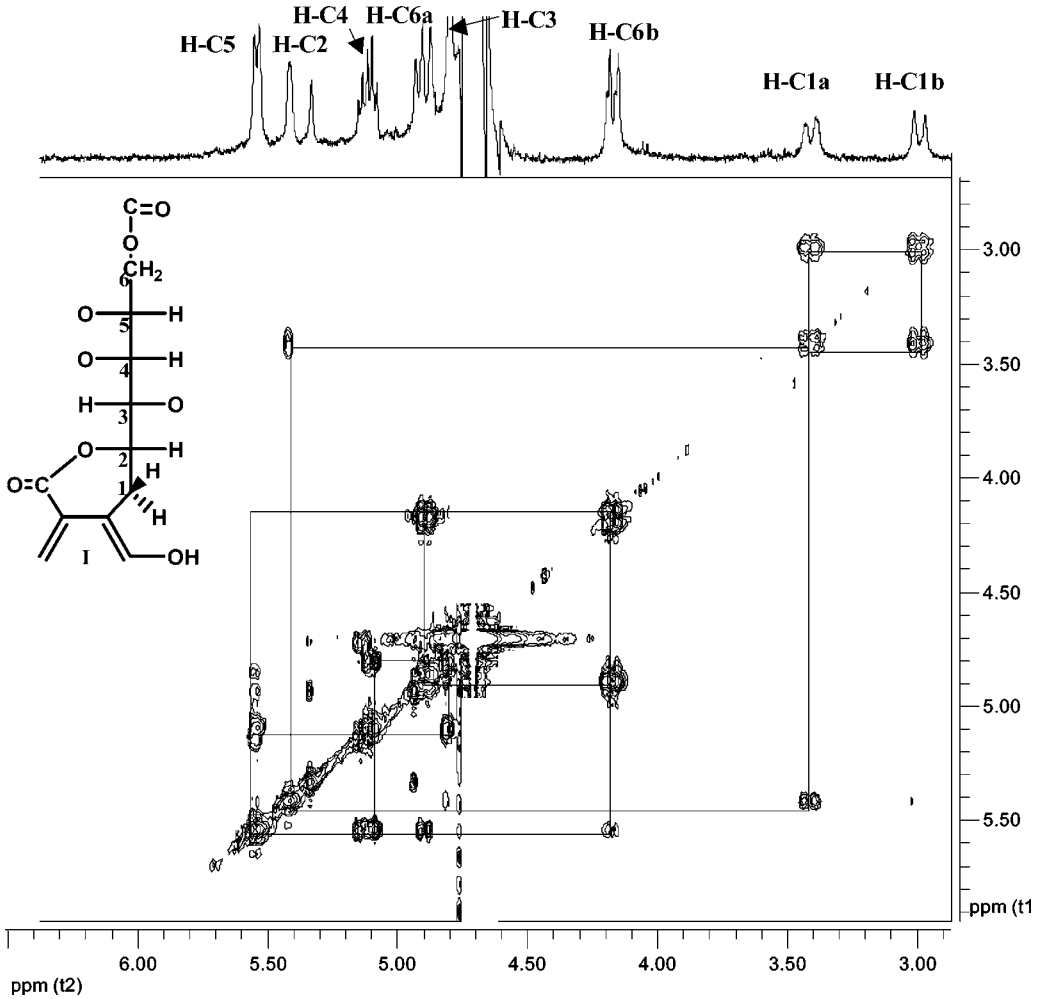

Figure 5. Excerpt of the COSY spectrum of the main reaction product (**10**) generated upon thermal treatment of vescalagin.

RESULTS AND DISCUSSION

Aimed at investigating the chemical transformations of ellagitannins upon wood toasting, the purified oak wood ellagitannins castalagin (**1**), vescalagin (**2**), 33-carboxy-33-deoxyvescalagin (**3**), roburin D (**4**), roburin A (**5**), as well as the thermohydrolysis products castalin (**6**) and vescalin (**7**) were individually dry-heated in a lab oven at 180 °C, and the reaction products obtained were analyzed by HPLC-DAD and LC-MS.

Thermal Degradation of Monomeric Ellagitannins. In a first set of experiments, the ellagitannin monomers castalagin and vescalagin were thermally treated for 60 min at 180 °C in a lab oven. After the reaction mixture was taken up in water, the product profiles of both reaction mixtures were monitored by means of HPLC coupled to a diode array detector. Thermal treatment of castalagin (**1**) (**Figure 2A**) generated one main reaction product (**9**) (**Figure 2B**) besides ellagic acid coeluting after 35 min with a small "hump" of polymeric material. In comparison, vescalagin (**2**) (**Figure 2C**) degraded to give the reaction product **10** and a rather large amount of polymer materials coeluting with ellagic acid (**Figure 2D**).

After isolation and purification of reaction product **9** by means of preparative RP-HPLC, a pale yellow colored amorphous powder was obtained, thus indicating a bathochromic shift of the absorption maximum of **9** when compared to the ellagitannin **1**. LC-MS analysis in the ESI^- mode revealed a pseudomolecular ion with *m*/*z* 931 for compound **9**. This indicated a molecular weight of 932 Da for **9**, and a loss of 2 amu compared to castalagin (**1**) most likely corresponding to the oxidation of either a 1,2-dihydroxybenzene moiety of a galloyl residue to an *ortho*-quinone system or the hydroxyl group HO−C(1) to a carbonyl function.

To unequivocally identify the chemical structure of reaction product **9**, 1D- and 2D-NMR experiments were performed with **9** as well as with the educt **1** in CD_3OD. Compared to castalagin (**1**), showing seven proton signals corresponding to the glucose skeleton (**Figure 3A**), compound **9** revealed six proton signals only (**Figure 3B**). Signal assignment by means of a COSY experiment demonstrated that proton H−C(1) of the hexose backbone of **1** was absent in the reaction product **9**. Starting from the diastereotropic protons H−C(6a) and H−C(6b) showing a coupling constant of 13 Hz, protons H−C(5) and H−C(4) showed similar chemical shifts at 5.33 and 5.21 ppm as found for castalagin (**1**). In contrast to the corresponding protons in **1**, H−C(2) and H−C(3) were significantly different in **9**. In addition, H−C(2) showed homonuclear coupling only with H−C(3) and not with H−C(1) as observed for castalagin. As all the other resonance signals were close to those observed for **1**, the hydroxyl group at C(1) was supposed to be oxidized to a carbonyl function in **9**. This was further strengthened by the data obtained from a ^{13}C NMR spectrum differing from that of castalagin by an additional carbonyl signal resonating at 190.1 ppm. Heteronuclear correlation spectroscopy (HMBC) revealed a long-range coupling of H−C(2) and H−C(3) with this carbonyl atom, thus confirming the suggested structure of a keto function at C(1) of the glucose core (**Figure 3**). Taking all the spectroscopic data into account, the structure of the reaction

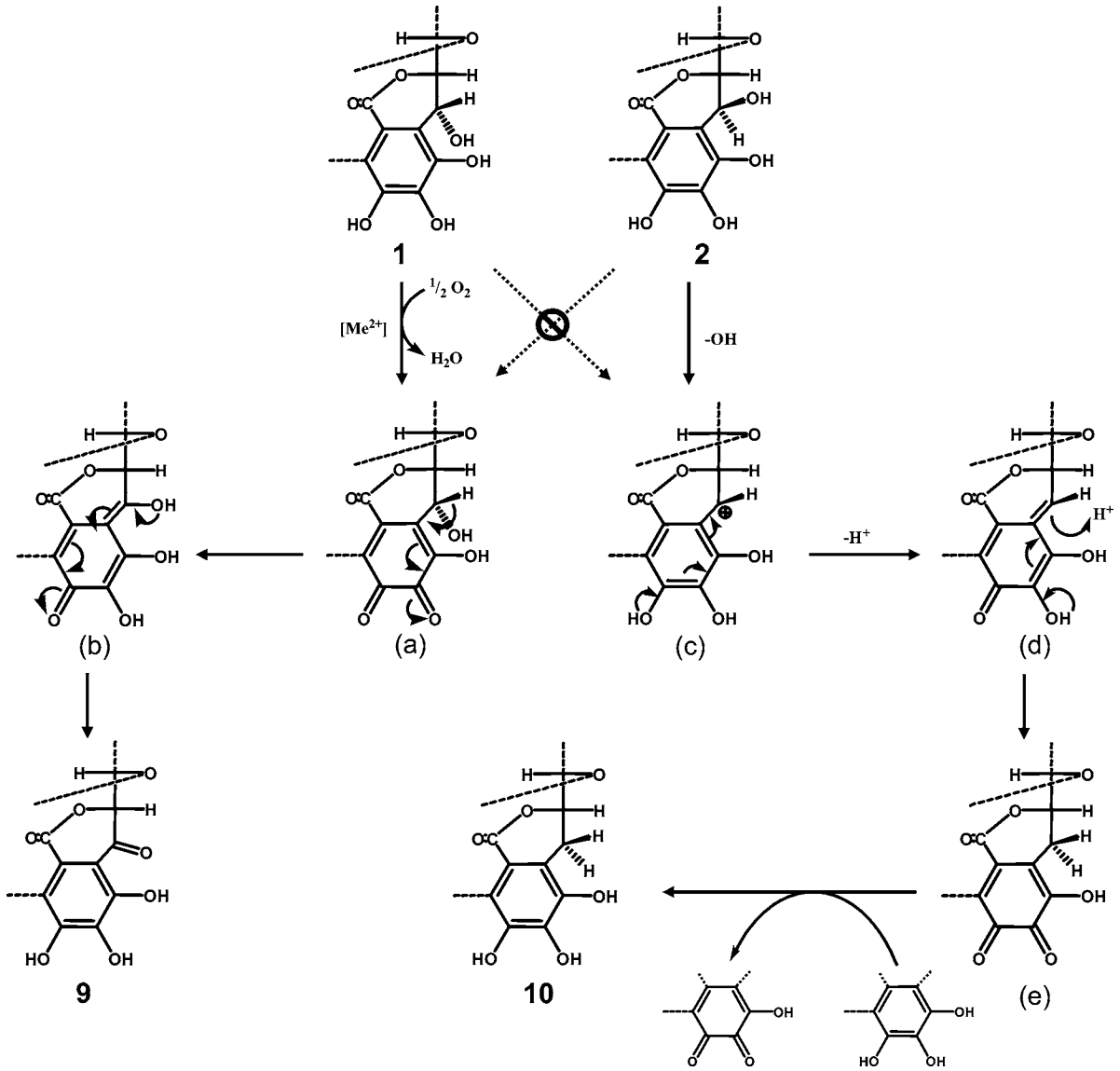

Figure 6. Postulated sequence of reactions leading to the formation of dehydrocastalagin (**9**) and deoxyvescalagin (**10**) upon thermal treatment of castalagin (**1**) and vescalagin (**2**), respectively.

product generated upon thermal treatment of castalagin (**1**) was identified as its previously unreported oxidation product **9** (**Figure 4**) named dehydrocastalagin.

As the oxidation of C(1) in castalagin is associated with the loss of the chirality at C(1), thermal treatment of the stereoisomer vescalagin was expected to give the same thermal reaction product. However, as outlined in **Figure 2D**, the reaction product (**10**) formed from vescalagin did not show the same retention time as dehydrocastalagin (**9**) (**Figure 2B**), thus indicating that vescalagin is thermally degraded via another reaction pathway as compared to castalagin. In order to investigate the influence of the stereochemistry at C(1) of the ellagitannin monomers on the structure of the thermal reaction products formed, compound **10** was isolated and purified from thermally treated vescalagin by means of preparative RP-HPLC. LC-MS (ESI^-) analysis of **10** revealed a pseudo molecular ion with *m*/*z* 917, thus differing from that of vescalagin (**2**) by a loss of 16 amu and corroborating with the lack of a hydroxyl group in **10**.

To clarify the chemical structure of compound **10**, 1D- and 2D-NMR experiments were performed in D_2O. In comparison to vescalagin (**2**), the ^1H NMR spectrum of **10** displayed eight instead of seven proton signals resonating between 2.98 and 5.55 ppm and representing the protons of the hexose chain in the molecule. In addition, H−C(2) was somewhat low-field shifted and H−C(3) high-field shifted whereas the chemical shifts of the hexose protons H−C(4) to H−C(6) were nearly unaffected. The noticeable difference in the ^1H NMR data of compounds **2** and **10** were the two high-field doublets resonating at 2.98 and 3.40 ppm which were not detectable for vescalagin. In the COSY spectrum (**Figure 5**), both protons showed geminal coupling with a coupling constant of 15 Hz. Finally, heteronuclear multiple quantum correlation (HMQC) experiments revealed that both of these protons H−C(1a) and H−C(1b) were bound to the carbon atom resonating at 28.2 ppm in the ^{13}C dimension. In addition, long-range couplings, observed by means of an HMBC experiment, revealed connectivities between H−C(2) and C(1) as well as between H−C(3) and C(1) and further confirmed the presence of a methylene group at position 1 of the hexose core in **10**. Taking all these data into account, the structure of compound **10**, thermally generated from vescalagin (**2**), was identified as the previously unreported deoxyvescalagin (**Figure 4**).

On the basis of the data obtained, it has to be concluded that the stereochemistry at C(1) of the hexose skeleton in the ellagitannins is controlling their degradation pathways. While

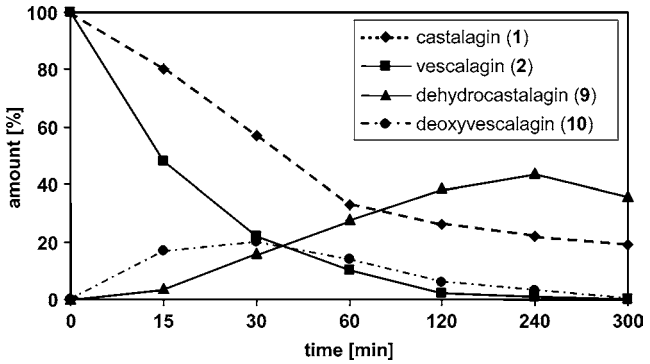

Figure 7. Influence of the heating time on the model degradation of castalagin (**1**) and vescalagin (**2**), respectively, and the time course of dehydrocastalagin (**9**) and deoxyvescalagin (**10**) generated.

thermal treatment of castalagin (**1**) was shown to induce its oxidation to dehydrocastalagin (**9**), vescalagin (**2**) was found to be reduced to deoxyvescalagin (**10**). Possible reaction mechanisms explaining the formation of **9** and **10** from their precursor ellagitannins are proposed in **Figure 6**. Oxidation of the galloyl ring I of castalagin by air oxygen leads to the corresponding *ortho*-quinone (a) and, upon enolization, to the *p*-hydroxymethylidene-cyclohexadienone (b) which, upon rearomatization, gives rise to dehydrocastalagin (**9**). In contrast, vescalagin is generating the intermediary carbocation (c) as recently reported to occur also upon ethanolysis of vescalagin (*17*). Stabilization of the carbocation by elimination of a proton leads to the *p*-methylidene-cyclohexadienone (d) which, upon enolization, gives the *ortho*-quinone (e). Finally, redox reaction of (e) with another ellagitannin molecule generates deoxyvescalagin (**10**).

Beside the formation of dehydrocastalagin (**9**) and deoxyvescalagin (**10**), a "hump" of unresolved, golden-brown colored components was detected in the HPLC chromatogram between 30 and 38 min, coeluting with ellagic acid as confirmed by comparison of spectroscopic (LC/MS, UV/vis) and chromatographic data with those obtained for the reference compound. Because castalagin and vescalagin are well-known to release ellagic acid (**8**) as well as the ellagitannin subunits castalin (**6**) and vescalin (**7**) (*7*) and these compounds were found to be generated upon thermally treatment of castalagin and vescalagin (data not shown), we were interested in investigating the thermal degradation products generated from castalin and vescalin. To achieve this, first, castalin and vescalin were preparatively isolated by HPLC from an acidic hydrolysate of purified castalagin and vescalagin. After freeze-drying, the highly pure castalin and vescalin were thermally treated and the reaction products formed were analyzed by means of LC-MS. Besides the high amount of polymers formed, the reaction products formed from castalin and vescalin showed molecular weights of 630 and 616 Da, respectively, which is 2 or 16 amu below their corresponding educts. In consequence, there is strong evidence that castalin and vescalin are converted into the corresponding dehydro (**11**) and deoxy derivatives (**12**) as already found for the ellagitannins castalagin and vescalagin (**Figure 4**).

In order to gain first insights into the time course of ellagitannin degradation, castalagin and vescalagin were thermally treated at 180 °C for up to 300 min and the remaining concentrations of the educts as well as the amounts of the redox products **9** and **10** formed were quantified by means of HPLC-DAD. As outlined in **Figure 7**, 32% of the starting amount of castalagin was left after 60 min of thermal treatment, whereas

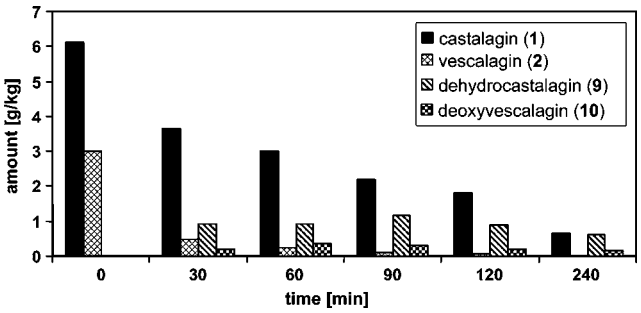

Figure 8. Influence of oak wood toasting on the degradation of castalagin (**1**) and vescalagin (**2**) and the generation of dehydrocastalagin (**9**) and deoxyvescalagin (**10**), respectively.

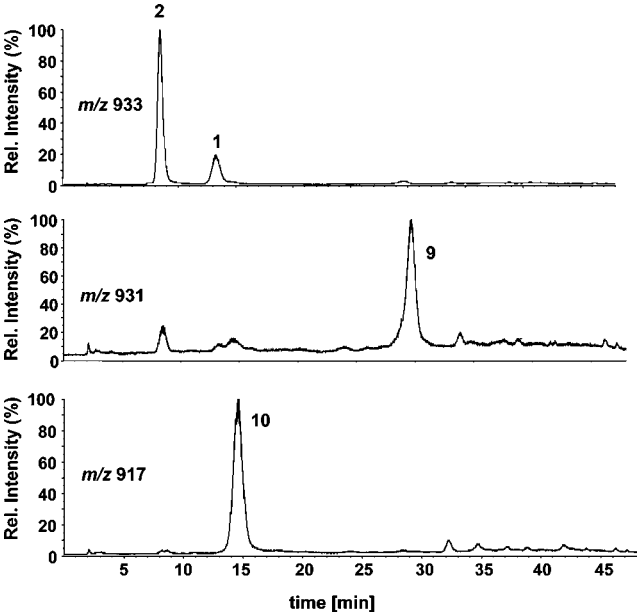

Figure 9. LC-MS analysis of a sample of 33-carboxy-33-deoxyvescalagin (**3**) thermally treated for 60 min at 180 °C. Using the selected ion monitoring (SIM) mode, the target ions *m*/*z* 917, 931, and 933 were recorded.

only 8% of the initial amount of vescalagin was detectable after the same reaction time. After 120 min, vescalagin was almost completely absent, whereas 26% of the initial amount of castalagin was still detectable, thus demonstrating the higher thermal stability of castalagin when compared to vescalagin. In parallel to the degradation of the ellagitannins, both the dehydrocastalagin (**9**) and deoxyvescalagin (**10**) were generated but strongly differed in their time courses (**Figure 7**). Whereas deoxyvescalagin was rapidly formed from the reactive vescalagin and went through a maximum after only 30 min, the dehydrocastalagin started to be generated after an induction period of about 15 min and reached maximum concentration levels after 240 min. Although deoxyvescalagin was rapidly formed from vescalagin, its concentration decreased again rapidly. The low stability of deoxyvescalagin (**10**) indicated that this compound is more a transient reaction intermediate rather than a stable end product, the degradation of which was accompanied with the generation of polymeric materials as outlined in **Figure 2**. Taking all these findings into consideration, the C(1)-stereochemistry of the ellagitannin monomers is driving their reactivity as well as the product formation.

To verify the formation of compounds **9** and **10** upon thermal treatment of wood, oak wood chips were toasted at 180 °C for up to 240 min, and **9** and **10** as well as their precursors castalagin

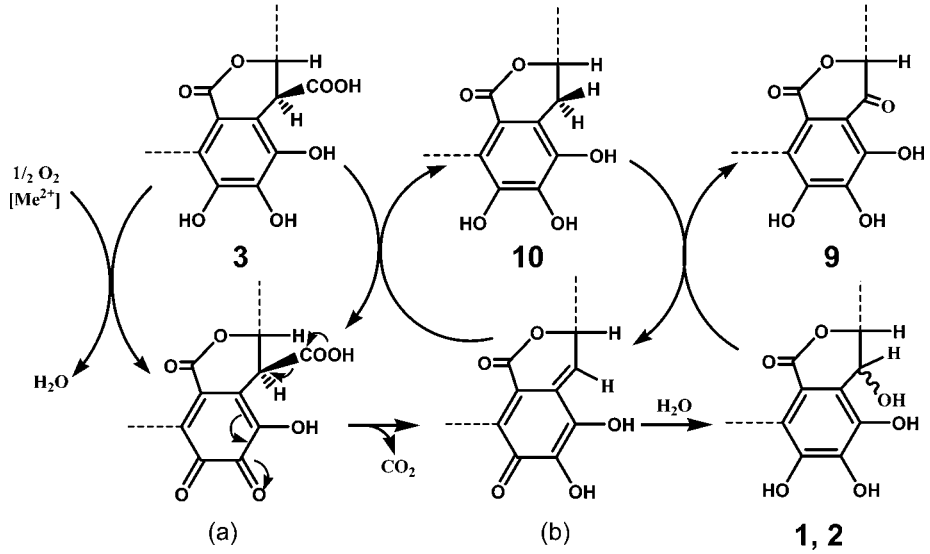

Figure 10. Proposed reaction sequence leading to the formation of castalagin (**1**), vescalagin (**2**), dehydrocastalagin (**9**), and deoxyvescalagin (**10**) upon thermal treatment of 33-carboxy-33-deoxyvescalagin (**3**).

and vescalagin were quantified in methanol/water extracts prepared from the toasted wood material. As shown in **Figure 8**, castalagin and vescalagin were present in amounts of 6.1 and 3.0 g/kg in oak wood prior to toasting. Thermal treatment induced a significant degradation of these ellagitannins depending on their stereochemistry; for example, after 60 min about 50% of the starting amount of castalagin was still present whereas more than 90% of the vescalagin was already degraded, confirming again the higher reactivity of the later ellagitannin. Independent from the heating time, both dehydrocastalagin and deoxyvescalagin were produced (**Figure 8**). As already found in the heating experiments with individual ellagitannins (**Figure 7**), dehydrocastalagin was formed in higher concentrations compared to the unstable deoxyvescalagin. Whereas the formation of dehydrocastalagin (**9**) reached a maximum at 90 min, the deoxyvesclagain (**10**) was detectable in maximum concentrations after only 60 min.

In order to study the influence of C(1)-carboxylation on thermal stability of ellagitannin monomers, 33-carboxy-33-desoxyvescalagin (**3**), recently identified in *Q. alba* and *Q. robur* (*9*), was thermally treated, and the reaction products formed were analyzed by HPLC-DAD and HPLC-MS. The HPLC chromatogram exhibited a complex pattern of reaction products with four main reaction products showing the same retention times found for vescalagin (**2**), deoxyvescalagin (**10**), castalagin (**1**), and dehydrocastalagin (**9**) (data not shown). To confirm the identify of these compounds, the thermally treated 33-carboxy-33-desoxyvescalagin was analyzed by LC-MS (ESI^-) operating in the SIM mode with *m*/*z* 933 as target ion for vescalagin and castalagin, *m*/*z* 917 for deoxyvescalagin, and *m*/*z* 931 for dehydrocastalagin. As shown in **Figure 9**, two compounds were found with the pseudo molecular ion *m*/*z* 933 matching that of castalagin (**1**) and vescalagin (**2**), and the compounds detected with *m*/*z* 931 and 917 were identical with dehydrocastalagin (**9**) and deoxyvescalagin (**10**), respectively. Therefore, it can be concluded that upon thermal treatment, 33-carboxy-33-deoxyvescalagin is degraded to give castalagin, vescalagin, dehydrocastalagin, and deoxyvescalagin.

In **Figure 10**, we propose a hypothetical reaction route showing the formation of **1**, **2**, **9**, and **10** from 33-carboxy-33-deoxyvescalagin (**3**). Oxidation of the galloyl ring I of **3** gives an σ-quinone exhibiting a vinylogous β-keto acid structure (a) which upon decarboxylation gives the intermediary *p*-methylidene-cyclohexadienone (b) already discussed as a key intermediate in the degradation of vescalagin (**Figure 6**). This intermediate might either be reduced to give the deoxyvescalagin (**10**) or might add a molecule of water to give castalagin (**1**) and vescalagin (**2**), respectively. Under participation of another molecule of deoxyvescalagin (**10**), castalagin can be oxidized to dehydrocastalagin (**9**) as shown in more detail in **Figure 6**.

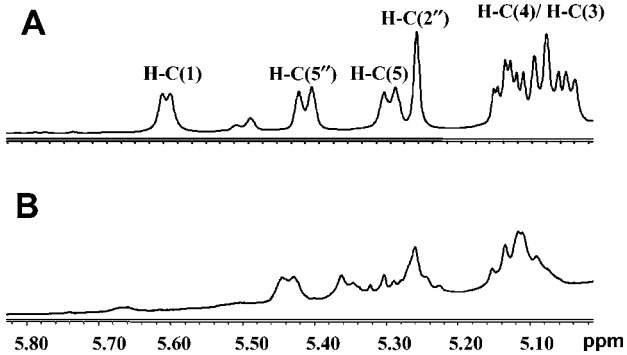

Figure 11. Excerpt of ^{1}H NMR spectra (400 MHz, methanol-d_4) of roburin D (**4**) and dehydroroburin D (**13**).

Thermal Degradation of Ellagitannins Dimers. In order to answer the question as to how ellagitannin dimers degrade upon toasting processes, roburin D (**4**) and roburin A (**5**) were thermally treated for 60 min at 180 °C. LC-MS analysis of the reaction mixtures revealed that the molecular weight of the roburin A degradation product was 16 amu below that of the educt, whereas the reaction product generated from roburin D showed a molecular mass which was 2 amu below that of the corresponding ellagitannin. Although the sample amounts were not enough to obtain a complete NMR signal assignment, the ^{1}H NMR spectra of both reaction products gave evidence for the presence of dehydroroburin D (**13**) and deoxyroburin A (**14**) (**Figure 4**). For example, the ^{1}H NMR spectrum of the degradation product of roburin D reflected the loss of the H−C(1) as given in **Figure 11**, thus indicating the oxidation of the hydroxyl group to a carbonyl. In contrast, the degradation product of roburin A showed doublets at 2.98 and 3.40 ppm, thus indicating the presence of the methylen group at C1 position

Table 1. Taste Threshold Concentrations[a] of Ellagitannins and Degradation Products

compound	recognition threshold concentration in μmol/L	recognition threshold concentration in mg/L
castalagin (**1**)	1.1	1.0
vescalagin (**2**)	1.1	1.0
castalin (**6**)	126.0	79.0
vescalin (**7**)	126.0	79.0
ellagic acid (**8**)	6.6	2.0
dehydrocastalagin (**9**)	4.4	4.1
deoxyvescalagin (**10**)	3.4	3.1
polymeric fraction[b]	–	13.8

[a] Taste threshold concentrations were determined by means of the half-mouth test in bottled water (pH 4.5). [b] The polymeric fraction was isolated from thermally treated vescalagin (60 min, 180 °C) by means of preparative HPLC.

in deoxyroburin A. In consequence, it can be suggested that the ellagitannin dimers roburin A and roburin D follow the same type of redox reaction chemistry as shown above for the corresponding monomers vescalagin and castalagin.

Sensory Evaluation of Ellagitannin Derivatives. In order to evaluate the taste quality and taste activity of the ellagitannin degradation products, first, the purity of the native ellagitannin monomers castalagin (**1**) and vescalagin (**2**) and the redox reaction products dehydrocastalagin (**9**) and deoxyvescalagin (**10**) was checked by LC-MS as well as ^{1}H NMR spectroscopy prior to sensory analysis. In addition, the polymeric fraction isolated from vescalagin, the cleavage product ellagic acid (**8**), as well as the thermohydrolysis products castalin (**6**) and vescalin (**7**), prepared by acidic hydrolysis of castalagin and vescalagin, were included in the experiments. The human taste threshold concentrations of these compounds were determined in water (pH 4.5) using the half-mouth test described above (**Table 1**). The oral sensation imparted by these ellagitannins was described as mouth-coating and astringent, detectable at relatively low threshold concentrations spanning from 1.1 to 126.0 μmol/L, strongly depending on the ellagitannin structure.

The lowest threshold of 1.1 μmol/L was found for the astringent sensation of castalagin (**1**) and vescalagin (**2**), whereas their thermal degradation products dehydrocastalagin (**9**), deoxyvescalagin (**10**), and ellagic acid (**8**) exhibited the same oral sensation at somewhat higher threshold levels of 4.4, 3.4, and 6.6 μmol/L (in water), respectively. Interestingly, the golden-brown colored polymeric fraction isolated from thermally treated vescalagin imparted complexity and mouth fullness as well as an astringent sensation at a threshold concentration of 13.8 mg/L which is 14-fold above the threshold concentration of the native vescalagin. These findings demonstrate that the oak wood toasting process is converting the highly astringent vescalagin and castalagin into less astringent degradation products. The role of these redox products as well as the golden-brown polymeric fraction formed from castalagin and vescalagin in the balanced gustatory profile of oak-matured spirits is currently under investigation.

To investigate the contribution of the individual subunits on the astringency of ellagitannins, the taste thresholds of vescalagin and castalagin were compared with those of castalin, vescalin, and ellagic acid. It is interesting to note that the threshold of castalin and vescalin, representing the lower part of the ellagitannins, shows a rather high taste threshold of 126 μmol/L, whereas the upper part represented as the ellagic acid exhibited a significantly lower threshold concentration of 6 μmol/L.

In summary, individual ellagitannin degradation products could be identified as transient intermediates of thermal transformation of ellagitannins into brown colored and mouth-coating melanoidin-type polymers. These studies offer first insights into the puzzling road map of thermal ellagitannin transformation chemistry and give some preliminary evidence for the changes in sensory active nonvolatiles in oak wood during toasting.

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