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Pathway Leading to the Formation of Anthocyanin−Vinylphenol Adducts and Related Pigments in Red Wines

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On the basis of observations from *Vitis vinifera* cv. Pinotage wines and experiments performed in model wine medium, a new chemical pathway responsible for the formation of anthocyanin−vinylphenol adducts in red wines is described. Until now, these pigments have been considered to be reaction products of anthocyanins and vinylphenols, the latter being generated during fermentation by enzymatic decarboxylation of the respective cinnamic acids. The mechanism of the novel pathway, involving intact hydroxycinnamic acid and anthocyanin, is explained. Only cinnamic acids with electron-donating substituents on the aromatic ring, such as coumaric acid, ferulic acid, caffeic acid, and sinapic acid, undergo this conversion, as they stabilize an intermediately formed carbenium ion. Decarboxylation and oxidation of the pyran moieties are the final steps in the generation of the corresponding 4-vinylphenol, 4-vinylguaiacol, 4-vinylcatechol, and 4-vinylsyringol adducts of anthocyanins in red wine.

KEYWORDS: Anthocyanins; red wine; *Vitis vinifera*; malvidin 3-glucoside; vinylphenols; aging products; pinotin A; hydroxycinnamic acids

INTRODUCTION

The most abundant red wine pigments are the 3-*O*-glucosides of malvidin and peonidin, as well as their 6″-acetylated and coumaroylated derivatives. The 3-*O*-glucoconjugates of petunidin, delphinidin, and cyanidin are also widespread. During wine aging, these pigments polymerize to a large extent and form a heterogeneous and not well characterized group of compounds, which is thought to be of major importance for the color of aged wines (*1*). Anthocyanins and colorless flavanols such as catechin and epicatechin form oligomeric pigments (*2*−*4*)—possibly the starting point in the genesis of polymers. Multiple other reactions take place during the aging of red wines, and several colored low molecular reaction products have been isolated and characterized in the past decade. Some of the aged pigments bear an additional pyran ring between C-4 and the hydroxyl group in position 5 of the aglycon moiety. Among these, the vitisin-type pigments originate from the reaction of malvidin 3-glucoside and acylated derivatives with pyruvic acid (*5*, *6*). Another pyranoanthocyanin, the malvidin 3-glucoside−4-vinylphenol adduct, was first detected on membranes used for cross-flow microfiltration of Carignane red wines (*7*) and later isolated and structurally identified through comparison with a synthetic sample (*8*). The occurrence of similar reaction products, namely, the 4-vinylcatechol, 4-vinylguaiacol, and 4-vinylsyringol derivatives, in red wines from *Vitis vinifera* cv. Shiraz and in grape skin extracts has been postulated on the basis of data obtained by nano-electrospray tandem mass spectrometric analysis (*9*). However, the tiny amounts of pigments present in wine did not allow isolation and structural characterization. Most recently, we were able to isolate pinotin A from red wines made of *Vitis vinifera* cv. Pinotage grapes (*10*). The structure was fully characterized by HPLC-ESI-MS^n in combination with one-/two-dimensional NMR measurements and determined to be the 4-vinylcatechol adduct of malvidin 3-glucoside. Anthocyanin−vinylphenol-related adducts that have been isolated and tentatively assigned are summarized in **Figure 1**.

The formation of the malvidin 3-glucoside−4-vinylphenol adduct is considered to be due to a direct reaction of malvidin 3-glucoside with 4-vinylphenol (**Figure 2**). Obviously, through an enzymatic side activity of *Saccharomyces cerevisiae*, formation of 4-vinylphenol via enzymatic decarboxylation of *p*-coumaric acid takes place during fermentation (*8*). It has been suggested (*9*) that the formation of the 4-vinylcatechol, 4-vinylguaiacol, and 4-vinylsyringol derivatives follows the same mechanism with the respective vinylphenols emerging from the enzymatic decarboxylation of caffeic, ferulic, and sinapic acid.

The aim of the present study was to clarify whether the proposed mechanism is the exclusive pathway for the formation of anthocyanin−vinylphenol adducts and related pigments in red wine.

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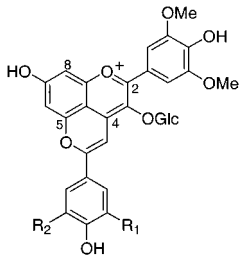

Figure 1. Structure of malvidin 3-glucoside adduct with 4-vinylphenol [R_1 = H, R_2 = H (*8*)], 4-vinylcatechol [pinotin A; R_1 = OH, R_2 = H (*10*), **6**, cf. **Figure 3**], 4-vinylguaiacol [R_1 = OCH_3, R_2 = H (*9*)], and 4-vinylsyringol [R_1 = OCH_3, R_2 = OCH_3 (*9*)].

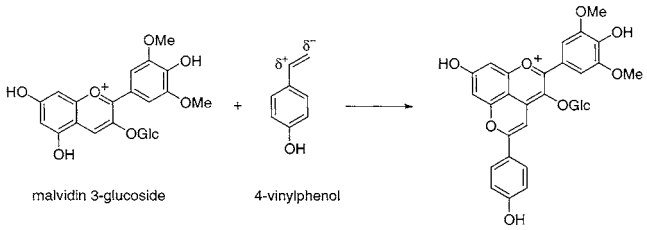

Figure 2. Formation of the malvidin 3-glucoside–4-vinylphenol adduct as proposed by Fulcrand et al. (*8*).

MATERIALS AND METHODS

Chemicals. All solvents were of HPLC quality and all chemicals of p.a. grade.

Synthesis and Reactivity of 4-Vinylcatechol. 4-Vinylcatechol was synthesized according to the method of Bücking (*11*). 3,4-Dihydroxybenzaldehyde (3 g) and malonic acid (4 g) were dissolved in 50 mL of ethanol. Six drops of piperidine were added, and the solution was refluxed for 8 h. Five hundred milliliters of deionized water was added and the solution extracted three times with 300 mL of diethyl ether. The combined ether fractions were evaporated in vacuo. The residue was redissolved in 5 mL of ethanol without further purification and added to 50 mL of a solution of malvidin 3-glucoside (10 mg) in a winelike model solution. This solution was analyzed by HPLC-DAD immediately after the addition of 4-vinylcatechol and after stirring overnight.

Synthesis of Caffeic Acid Methyl Ester. Caffeic acid (3 g) was dissolved in 50 mL of methanol, a few drops of concentrated sulfuric acid were added, and the solution was refluxed for 6 h. Three hundred milliliters of deionized water was added, methanol was evaporated in vacuo, and the solution was freeze-dried. Caffeic acid methyl ester was purified by countercurrent chromatography. The solvent system was *n*-hexane/ethyl acetate/methanol/water (3:5:3:5, v/v/v/v), and the upper layer was used as stationary phase, flow rate = 3.5 mL/min, detection at 323 nm [chromatogram not shown, CCC apparatus as described previously (*12*)]. Organic solvents were evaporated in vacuo from the fraction containing the product, whereupon caffeic acid methyl ester started to crystallize. For complete crystallization, the solution was stored at 5 °C for 12 h. Crystals were removed by filtration, and the remaining solution was lyophilized. Combined crystals were found to be of >99% purity by HPLC-DAD and free from residual caffeic acid. Identity was confirmed by ESI-MS and NMR analyses: ESI-MS (negative ion mode), *m*/*z* 193 $[M - H]^-$; daughter ions (%), *m*/*z* 178 (41), 161 (100), 134 (81); ^{1}H NMR δ 3.75 (3H, s, OCH_3), 6.25 (1H, d, J = 16.0 Hz, H-α), 6.78 (1H, d, J = 8.1 Hz, H-5), 6.93 (1H, dd, J = 8.1, 2.1 Hz, H-6), 7.03 (1H, d, J = 2.1 Hz, H-2), 7.54 (1H, d, J = 16.0 Hz, H-β); ^{13}C NMR δ 51.9 (OCH_3), 114.9 (C-α), 115.2 (C-2), 116.5 (C-5), 122.9 (C-6), 127.8 (C-1), 146.8 (C-β), 146.9 (C-3), 149.5 (C-4), 169.8 (C=O).

Reactivity of Cinnamic Acid Derivatives. Malvidin 3-glucoside of ~90% purity (calculated from the 520 and 280 nm traces of a diode array chromatogram) was isolated from various red wines by countercurrent chromatography according to methods previously described (*12*−*14*) and dissolved in a 0.02 M solution of potassium hydrogen tartrate in deionized water (adjusted to pH 3.2 with hydrochloric acid). To 27 mL of the malvidin 3-glucoside solution (concentration ~ 400 mg/L) was added 3 mL of an ethanolic solution of each of the following *trans*-configured cinnamic acid derivatives: cinnamic acid, *p*-coumaric acid, ferulic acid, caffeic acid, and sinapic acid (~400 mg/L) as well as 4-dimethylaminocinnamic acid, 4-nitrocinnamic acid, and caffeic acid methyl ester (~1200 mg/L). The winelike model solutions were filled into 30 mL amber glass bottles, air in the headspace was replaced with argon, and the solutions were stored in the dark at 15 °C in a climatized room. Samples were analyzed in regular intervals by HPLC-DAD and HPLC-ESI-MS^n for 4 months. Relative reaction rates were determined by HPLC-DAD analyses comparing the concentrations of the newly formed pigments (expressed as pinotin A equivalents; authentic reference standard used for calibration between 0 and 15 mg/L, detection wavelength = 510 nm).

Influence of Oxygen on the Formation of Pinotin A. A winelike model solution was prepared as described above containing malvidin 3-glucoside (353 mg/L), caffeic acid (1326 mg/L), and 10% ethanol. Thirty milliliters of each of the solutions was filled into four glass bottles. The first bottle was ultrasonicated, and argon was bubbled through the solution for 10 min to remove dissolved oxygen. Also, the headspace was filled with argon. The second bottle was not degassed, but the headspace was filled with argon. The third bottle was not degassed nor was the air in the headspace replaced. The fourth solution was saturated with oxygen by bubbling air through it for 10 min. Thus, four solutions containing different amounts of oxygen were created. The bottles were stored for 5 weeks and then analyzed by HPLC-DAD.

HPLC with Diode Array Detection (HPLC-DAD). A PU-980 Intelligent HPLC pump equipped with a DG-980-50 3-line degasser, an LG-980-02 ternary gradient unit, and an MD-1510 multiwavelength detector were used (Jasco). Samples were injected via a Rheodyne 7175 injection valve (Techlab) equipped with a 20 μL loop, and separations were carried out on a Synergi MaxRP-11, 4 μm, 250 × 4.6 mm column (Phenomenex). Solvents were water/formic acid/acetonitrile (87:10:3, v/v/v, solvent A; 40:10:50, v/v/v, solvent B), and the flow rate was 0.5 mL/min. The linear gradient was from 6 to 20% B at 0−20 min, from 20 to 40% B at 20−35 min, from 40 to 60% B at 35−40 min, from 60 to 90% B at 40−45 min, and held at 90% B at 45−50 min.

HPLC with Electrospray Ionization Multiple Mass Spectrometry (HPLC-ESI-MS^n). A Bruker Esquire LC-MS system was used (Bruker Daltonik). The HPLC system consisted of a System 1100 binary pump G1312A (Agilent) and a Rheodyne 7725i injection valve with a 20 μL loop (Techlab). MS parameters were as follows: positive ion mode; dry gas, N_2, 11 L/min, dry temperature, 325 °C; nebulizer, 60 psi; capillary, −2500 V; capillary exit offset, 70 V; end plate offset, −500 V; skimmer 1, 20 V; skimmer 2, 10 V; scan range, *m*/*z* 50−1000, chromatographic conditions as above.

Alternatively, for MS^n experiments the sample solution was delivered directly by a syringe pump 74900 (Cole-Parmer) into the ESI source at a flow rate of 240 μL/h. MS parameters were as follows: positive ion mode; dry gas, N_2, 4.0 L/min; dry temperature, 300 °C; nebulizer, 10 psi; capillary, −3500 V; capillary exit offset, 60 V; end plate offset, −500 V; skimmer 1, 30 V; skimmer 2, 10 V; scan range, *m*/z 50−1000.

NMR. ^{1}H and ^{13}C NMR spectra of caffeic acid methyl ester dissolved in methanol-d_4 were recorded on a Bruker AMX 300 spectrometer (Bruker Biospin) at 300.13 and 75.47 MHz, respectively.

RESULTS AND DISCUSSION

Up to now, the formation of anthocyanin−vinylphenol adducts was considered to take place through the reaction of anthocyanins with the respective 4-vinylphenols, which are formed during fermentation by enzyme-mediated decarboxylation of the corresponding cinnamic acids (*8*, *9*). In the course of a study on the anthocyanin profile of red wines from *V. vinifera* cv. Pinotage, we observed that the concentration of pinotin A (**6**, cf. **Figure 3**) was ~10 times higher in aged wines (5−6 years old) compared to that in very young (<1 year) wine samples. This finding suggests that the reaction between

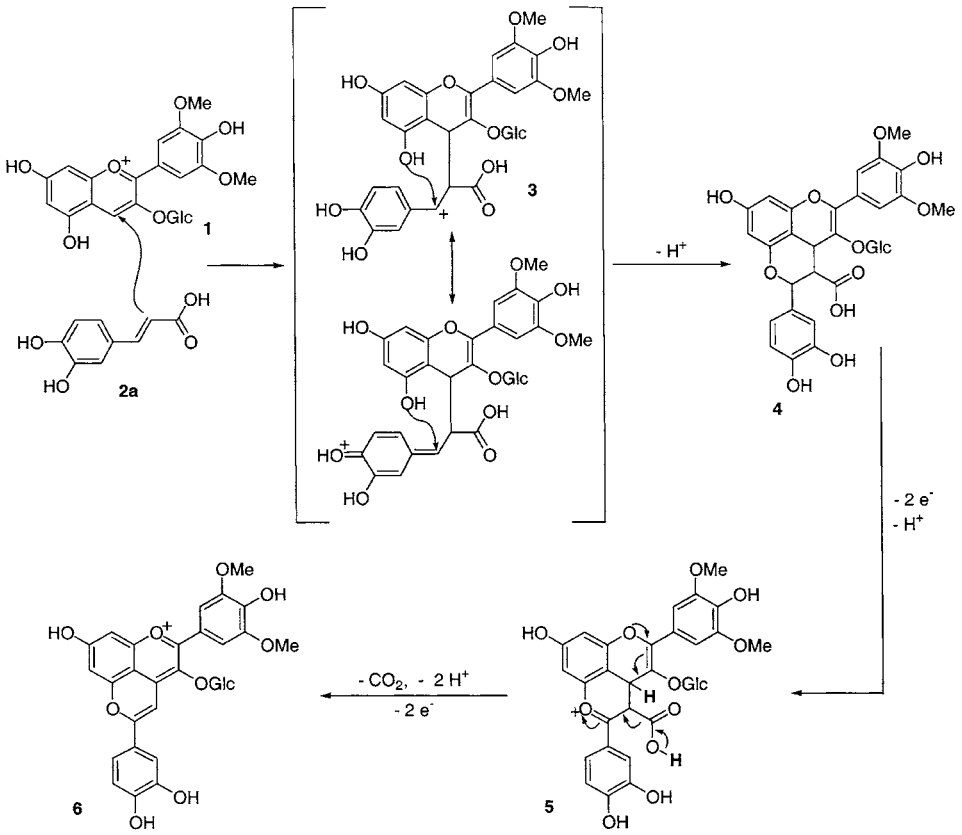

Figure 3. Postulated pathway of pinotin A (**6**) formation involving caffeic acid (**2a**) and malvidin 3-glucoside **1**. (A concerted cycloaddition is also conceivable in the generation of **4**; however, the stepwise addition process delineated here is preferred as the cycloaddition pathway would require **1** to react in an unfavorable keto form.)

4-vinylcatechol and malvidin 3-glucoside proceeds rather slowly and requires years of storage to complete. To verify the slow reaction rate, model experiments with 4-vinylcatechol and malvidin 3-glucoside were carried out. As 4-vinylcatechol is not commercially available, a synthesis was performed according to the method of Bücking (*11*). Synthesized 4-vinylcatechol and malvidin 3-glucoside were reacted in a model wine medium. Subsequent HPLC-DAD and HPLC-ESI-MS^n analyses revealed that malvidin 3-glucoside had been almost quantitatively reacted to a new pigment, the retention time and mass spectral data of which were identical to those of an authentic sample of pinotin A (*10*). It is important to note that our experiment is in line with earlier observations of Fulcrand et al. (*8*) and Sarni-Manchado et al. (*15*), who reported that the reaction of malvidin 3-glucoside and 4-vinylphenol went to completion within hours.

This observation prompted us to search for another explanation for the increase of pinotin A during storage as 4-vinylcatechol, if present at all, would be consumed rapidly and the gradual formation of **6** over prolonged periods could not be rationalized in this way. Our suspicion was strengthened by the fact that despite a comprehensive literature search, we were unable to find evidence that either 4-vinylcatechol or 4-vinylsyringol had ever been detected in red or white wines, whereas the presence of 4-vinylphenol and 4-vinylguaiacol is well documented (*16*−*20*). Although red wines contain much more of the precursors coumaric and ferulic acid, the amount of the corresponding volatile vinylphenols in white wines was determined to be orders of magnitude higher and beyond a certain limit responsible for unpleasant "phenolic" off-flavors. A possible explanation for this phenomenon could be seen in the reaction of anthocyanins with vinylphenols (*8*). However, Chatonnet et al. (*18*) investigated the ability of different strains of *S. cerevisiae* to decarboxylate cinnamic acids and found that certain wine constituents, especially catechin, epicatechin, and oligomeric procyanidins, but not anthocyanins and polymeric constituents (i.e., polymeric procyanidins or other highly condensed macromolecules with a molecular weight >3000), strongly inhibited the decarboxylation of *p*-coumaric acid. The concentration of these inhibitors is much higher in red wines. Hence, it can been concluded that the cinnamate carboxy-lyase is largely inactive during red wine fermentation and that the enzyme-mediated synthesis of 4-vinylphenols in red wine is not significant with regard to anthocyanin−vinylphenol adduct formation. The authors also discovered that all of the yeast strains investigated were able to decarboxylate *p*-coumaric and ferulic acid, but none of them was capable of transforming either caffeic or sinapic or unsubstituted cinnamic acid into 4-vinylcatechol, 4-vinylsyringol, or styrene, respectively.

The decisive hint of a novel pathway of anthocyanin−vinylphenol adduct formation was finally given by the unusually high concentrations of caffeic acid in Pinotage wines. The caffeic acid content in most of the common red wine varieties does not exceed 10 mg/L (*21*−*23*). Only in Pinotage wine were we able to detect as much as 77 mg/L of caffeic acid with a mean value of ∼35 mg/L. Hereupon we started to consider the possibility of a direct reaction between malvidin 3-glucoside and caffeic acid or anthocyanins and cinnamic acids in general. Hence, another experiment was performed with malvidin 3-glucoside and caffeic acid in a winelike model solution. This solution was stored in the dark at 15 °C and analyzed at regular intervals. Just after 5 days we were able to detect the formation of a new pigment with the same retention time and mass spectral properties as pinotin A. The concentration of this peak constantly

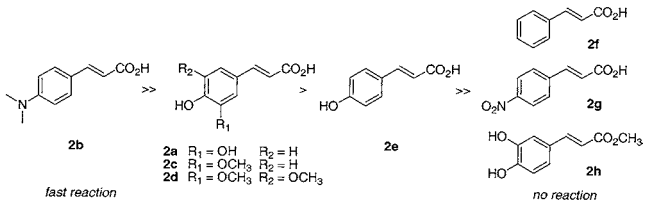

Figure 4. Reactivity of cinnamic acid derivatives with regard to anthocyanin adduct formation with **1**.

Table 1. Mass Spectral Properties of the Generated Malvidin 3-Glucoside Cinnamic Acid Derivatives

malvidin 3-glucoside adduct	molecular ion [M+] (*m*/*z*)	aglycon (*m*/*z*)	elimination masses from aglycon (u)
4-vinylphenol	609	447	16, 32, 44, 61, 89
4-vinylcatechol (pinotin A)	625	463	16, 32, 44, 61, 89
4-vinylguaiacol	639	477	16, 32, 44, 61, 89
4-vinylsyringol	669	507	16, 32, 44, 58, 61, 89
4-dimethylaminostyrene	636	474	16, 33, 44, 61, 89

increased during the following months as shown by HPLC-DAD analyses.

These results showed for the first time that pyranoanthocyanins can be formed directly in a reaction between intact cinnamic acid derivatives and anthocyanin. To obtain a mechanistic rationale for this unprecedented conversion, further experiments were conducted, including investigations on the reactivity of other cinnamic acid derivatives and the possible influence of oxygen on the reaction rate. On the basis of our findings the following reaction sequence can be suggested (**Figure 3**).

The initial bond formation between the C-4 position of malvidin 3-glucoside (**1**) and the C-2 position of caffeic acid (**2a**) is in line with the strongly electrophilic nature of the benzopyrylium unit and the nucleophilicity of the α-carbon atom of acid **2a**. Given the electron-deficient character of the resulting intermediate **3**, it can be expected that electron-donating substituents on the aromatic ring of the cinnamic acid moiety facilitate this reaction due to stabilization of the intermediate carbenium ion **3**. This has indeed been observed in a series of conversion experiments. Whereas *p*-coumaric acid (**2e**), ferulic acid (**2c**), sinapic acid (**2d**), and 4-dimethylaminocinnamic acid (**2b**) could be successfully reacted, no adduct formation at all was observed with 4-nitrocinnamic acid (**2g**) or the parent compound cinnamic acid (**2f**) (**Figure 4**).

Mass spectral properties of the newly formed anthocyanin derivatives and their aglycons were in line with data published for the isolated 4-vinylphenol and 4-vinylcatechol derivatives of malvidin 3-glucoside (*8*, *10*) and the tentatively identified 4-vinylguaiacol and 4-vinylsyringol adducts (*9*). In addition, upon further fragmentation of the aglycons the same elimination masses were observed as by Hayasaka and Asenstorfer, who found this fragmentation pathway to be typical for malvidin-derived pigments (*9*). The *p*-dimethylaminocinnamic acid product has not been reported before (**Table 1**).

The kinetics of reaction of the di- and trisubstituted acids **2a**, **2c**, and **2d** with anthocyanins were moderately enhanced compared to that of **2e** (**Figures 4** and **5**). With the amino moiety in **2b**, however, the rate of formation was accelerated by a factor >100 (data not included in **Figure 5**). These observed reactivities coincide with the expected order of reaction rates for para-substituent effects when electron-deficient transition states are involved, as given by Brown's σ_p^+ constants (*24*, *25*).

It is known that electron-donating substituents can accelerate reactions involving the olefinic double bond of cinnamates.

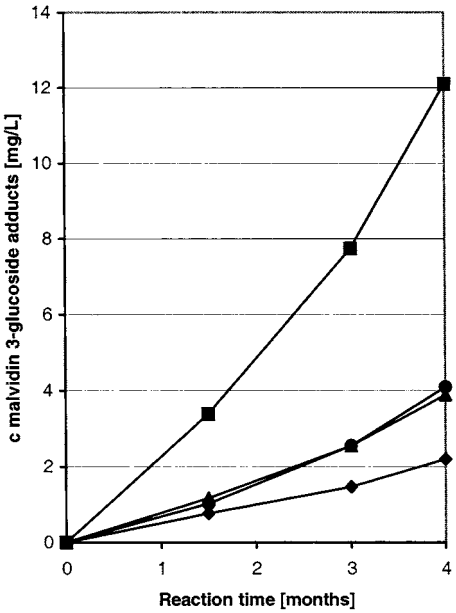

Figure 5. Course of the reaction of malvidin 3-glucoside with coumaric acid (**2e**, ◆), ferulic acid (**2c**, ●), sinapic acid (**2d**, ▲), and caffeic acid (**2a**, ■).

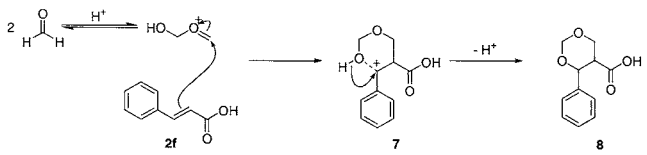

Figure 6. Mechanism of the acid-catalyzed Prins cyclization.

Negative Hammett reaction parameters ρ have been obtained, for example, in the bromate oxidation of cinnamic acids (*26*−*29*). These data confirm that electrophilic attack on cinnamic acids as shown here can be favored over nucleophilic pathways such as conjugate addition to the double bond, which can be accelerated by electron-withdrawing substituents (*30*).

The intermediate carbenium ion **3** can be trapped intramolecularly by the phenolic hydroxy group of **1** to form the pyran ring in **4** (cf. **Figure 3**). Additional support for this cyclization mechanism is drawn from the striking similarity to the extensively studied acid-catalyzed addition of formaldehyde to alkenes, the Prins reaction, where the six-membered intermediate **7** has been postulated (*31*) (**Figure 6**).

Subsequently, the final product **6** is formed via oxidation and decarboxylation of the intermediate **4**. Few details are known about the precise mechanism of pyran oxidation involving reactive benzylic carbon−hydrogen bonds under the conditions used here, but these conversions can generally be achieved with remarkable ease (*32*, *33*). Hydride abstractions (e.g., by unreacted **1**) (*34*−*36*), enzymatic/coenzymatic oxidations (*37*, *38*) and involvement of other known oxidants such as nitrite (*39*) are generally conceivable in authentic wine samples. Radical pathways (autoxidation) in the presence of oxygen and a catalytic species (*40*) are less likely due to the antioxidative properties of phenolic compounds. Correspondingly, the rate of our model reaction exhibited no dependence on the presence of oxygen. The amount of neither malvidin 3-glucoside (mean ± 1.4%) nor pinotin A (mean ± 4.3%) was significantly different in any of the solutions containing different amounts of oxygen, thus making hydride abstraction the most likely pathway under the conditions employed.

There is also ample evidence for oxidative and nonoxidative decarboxylations in the vicinity of a variety of different oxygen

substituents, in both biochemical and synthetic processes (*37*, *39*, *41*, *42*), and the strongly accelerating effect of β-alkoxy groups has been explained in terms of the polar nature of the transition state (*43*). In fact, a facile thermal decarboxylation of a phenyl pyranone-based acid has been noted more than 100 years ago (*44*). The presence of a free acid functionality is essential for decarboxylation; consequently, only traces of the product **6** could be detected upon analysis by HPLC-ESI-MS^n when caffeic acid methyl ester (**2h**) was employed as a substrate (**Figure 4**). On the basis of these data, oxidative generation of a stabilized oxonium intermediate such as **5** can be proposed. Decarboxylation of this intermediate is expected to occur under mild conditions, and further oxidation of the pyran moieties to the aromatic heterocycles results in the final product **6**.

The direct reaction between anthocyanins and hydroxycinnamic acids readily explains the formation of all anthocyanin−vinylphenol-type adducts in red wines. At the time of writing, this is the only experimentally verified mechanism leading to the development of 4-vinylcatechol and 4-vinylsyringol pigments, as the free vinylphenols have neither been detected in wines nor was it possible to generate these compounds via enzymatic decarboxylation using yeasts commonly applied to red wine fermentation (*18*). Small amounts of 4-vinylphenol and 4-vinylguaiacol were detected in experimental Shiraz red wines only if the grape juice was treated with pectic enzymes possessing cinnamoyl esterase activity prior to fermentation, thus increasing the amount of free coumaric and ferulic acids (*20*). However, even in this case the concentrations of the respective vinylphenols were in the low microgram range, and they would have to react quantitatively with malvidin 3-glucoside to generate detectable amounts of the derived pigments. Given the high reactivity of vinylphenols toward other constituents of young red wine, including different anthocyanins, it is extremely unlikely that high levels of anthocyanin−vinylphenol adducts can arise in this way.

Therefore, the pathway starting from free hydroxycinnamic acids is with utmost certainty responsible for the formation of the vast majority of anthocyanin−vinylphenol pigments in red wine. The reaction rate in wines is expected to be slower compared to our model solutions: on the one hand because of the lower concentration of the reactants, and on the other hand because of competing reactions of malvidin 3-glucoside with other wine constituents, leading to the formation of, for example, vitisin A or anthocyanin−flavanol condensation products. This makes the vinylphenol-derived pigments potentially attractive for use as aging indicators for red wines as their concentration will constantly increase during storage as long as free anthocyanins and cinnamic acids are available. During wine storage, the latter can be constantly replenished through slow hydrolysis of the corresponding tartaric esters, which are normally present in higher concentrations than the free acids (*45*, *46*). The fact that unsubstituted cinnamic acid, also present in red wines, can neither directly react with anthocyanins nor be decarboxylated to styrene by wine yeast explains why the occurrence of such a pigment has not been reported.

Both 4-vinylguaiacol and 4-vinylcatechol adducts of other anthocyanins have most likely been generated in several studies on copigmentation in red wines (*47*, *48*) and model solutions (*49*). However, the stabilization of color was solely interpreted by assuming the formation of non-covalent anthocyanin−copigment complexes.

Due to the pure chemical nature of the novel pathway without the need for enzymatic support, the formation of anthocyanin−vinylphenol adducts can take place during years of storage. Research is currently underway to explore the suitability of pinotin A as an aging indicator for Pinotage wines.

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