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A novel synthetic route to substituted pyranoanthocyanins with unique colour properties

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Abstract—Anthocyanins, isolated from natural sources by countercurrent chromatography, were reacted with cinnamic acids bearing at least one electron-donating substituent at the *para*-position. The resulting pyranoanthocyanins obtained by this simple one-step reaction were much less susceptible to pH shifts and retained their original colour over a wide pH-range. Through reaction with *p*-dimethylamino cinnamic acid, synthetic malvidin- and cyanidin-based anthocyanins with a unique violet hue were prepared.
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Anthocyanins are naturally occurring pigments, and along with carotenoids and betalains they are responsible for the characteristic colour of fruits and fruit-derived food products. The observed anthocyanin colour depends on the substitution pattern of the B-ring in the aglycone moiety, and ranges from orange (e.g. strawberries, pelargonidin-based pigments) to bright red (e.g. blackberries, >80% cyanidin 3-*O*-β-D-glucoside) to the bluish-red of young red wines (caused largely by malvidin 3-*O*-β-D-glucoside) (Fig. 1). Bathochromic or hypsochromic shifts of the original colour can occur as a result of copigmentation effects or complexation with metal ions.[1]

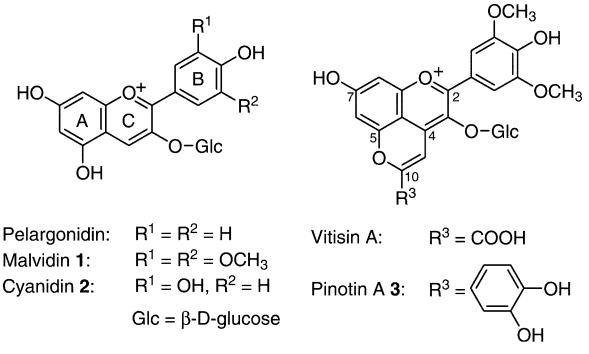

Figure 1. Structures of anthocyanidin 3-glucosides (left) and red wine derived pyranoanthocyanins (right).

Anthocyanin-rich extracts are widely used as food colorants. However, their application is hindered by the fact that anthocyanins exist in several equilibrium structures depending on the pH conditions. The red-coloured flavylium cation exists mainly in acidic solution at pH 1, while at pH 3.6, typical for many fruit juices and red wine, the colourless carbinol base or open chalcone form predominates. Therefore anthocyanins can only be used for colouring of relatively acidic foods. This problem was partly overcome by the discovery of anthocyanins with acylated sugar moieties, from e.g. red cabbage, red radish and purple carrot. The acylated derivatives were found to possess a higher pH stability, attributable to intramolecular copigmentation effects between the anthocyanidin unit and the aromatic acid.[2]

A similar stabilization was observed for pyranoanthocyanins such as the red-wine derived vitisins and pinotins (Fig. 1). The newly introduced pyran ring between C-4 and the hydroxyl group attached to C-5 shields pyranoanthocyanins from nucleophilic attack by water and delays the formation of the colourless carbinol base.

Vitisin A was found to be formed by reaction of malvidin 3-glucoside **1** with pyruvic acid.[3] Pyranoanthocyanins formed by reaction of **1** with vinylphenols were first observed by Fulcrand et al.[4] and later synthesized by Håkansson et al.[5] The 4-vinylphenols were thought to arise during red wine fermentation by enzymatic decarboxylation of the corresponding cinnamic acids.[6]

Keywords: anthocyanins; pyranoanthocyanins; pinotin A; dimethylamino cinnamic acid; CIELab.

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Following the recent isolation of the 4-vinylcatechol adduct, named pinotin A **3**, from Pinotage red wines,[7] a new pathway leading to vinylphenol-derived pyranoanthocyanins was discovered, explaining the formation of these compounds over many years of wine maturation.[8] This new pathway was of a pure chemical nature and did not depend on enzymatic action. It was shown that anthocyanins can react directly with *p*-hydroxy-substituted cinnamic acids in aqueous solution. All of the natural pyranoanthocyanins have a brick-red or orange colour. None of them is available from natural sources in sufficient amounts to be used as a colour additive for food.

So far, multi-step reactions were required to obtain phenyl-substituted pyranoanthocyanins.[5,9] In this paper, we present a novel synthetic approach to pyranoanthocyanins by a simple one-step reaction between anthocyanins and readily available substituted cinnamic acids in aqueous solution.

Optimized reaction conditions for the formation of **3** were explored. The reaction between **1** and caffeic acid was chosen as it proceeded slightly faster than the reaction of **1** with coumaric, ferulic, or sinapic acid (Fig. 2).[8] The rate of formation was monitored in a wine-like model solution (water saturated with potassium hydrogen tartrate) at different ethanol concentrations and various pH values. Between pH 4.2 and 2.9 the reaction rate varied only slightly. At pH 1.6 and 2.2 the reaction proceeded faster, but at the same time we observed a higher degradation of **1**, which considerably slowed down the rate of formation of **3** at pH 1 (Fig. 3). Hence, we performed the following reactions at pH 3 due to the higher stability of educt **1**.

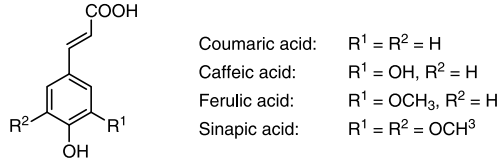


Figure 2. Structures of *trans*-hydroxycinnamic acids participating in pyranoanthocyanin formation in red wine.

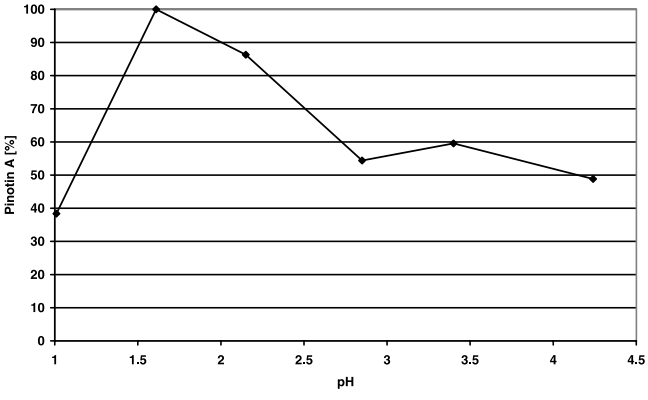


Figure 3. Influence of pH on the rate of formation of **3**.

A higher ethanol content would on one hand increase the solubility of less hydrophilic cinnamic acid derivatives, on the other hand we observed that an ethanol concentration >30% slowed down pyranoanthocyanin formation (Fig. 4). This is consistent with the theory of copigmentation, a phenomenon where anthocyanins and colourless polyphenols associate and stabilize the pigment. This association has been suggested to be the initial step in formation of covalent bindings and it was found that it is hindered by high concentrations of organic solvents.[10]

Malvidin 3-glucoside **1** and cyanidin 3-glucoside **2**, isolated from red wine and blackberries,[11] respectively, were then reacted with *p*-dimethylamino cinnamic acid **4** under the optimized conditions. However, the wine-like model solution was replaced with dilute hydrochloric acid to allow for a simpler work-up. At 15°C in the dark, the reaction took 6 weeks to complete. Alternatively the reaction could be performed at 35°C within approximately 3 days, but then a higher amount of polymeric pigments was formed. The *p*-dimethylaminostyrene adducts of malvidin 3-glucoside **5** and cyanidin 3-glucoside **6** were isolated by preparative HPLC and structurally identified by means of HPLC-ESI-MS, HRMS, and NMR spectroscopy.

The proposed reaction mechanism for **5** (**6**) is shown in Scheme 1. The initial bond formation between the C-4 position of **1** (**2**) and the C-2 position of **4** is consistent with the strongly electrophilic nature of the benzopyrylium unit and the nucleophilicity of the α-carbon of acid **4**. The resulting intermediate **7** is stabilized by the electron-donating amino substituent on **4**. Compound **7** can be trapped intramolecularly by the phenolic hydroxy group of **1** (**2**) to form the new pyran ring. Subsequently, the final product **5** (**6**) is formed via oxidation and decarboxylation.

UV–vis spectra were recorded in a buffer simulating a fruit juice environment. The solutions of **1** and **2** reached maximum colour expression at pH 1.0, and lost approximately 80% in intensity upon an increase in pH to 3.8 while maintaining their hue. A further rise in pH led to formation of the blue quinonoidal base, which dominated at pH 10 (Fig. 5; spectra of **2** were identical

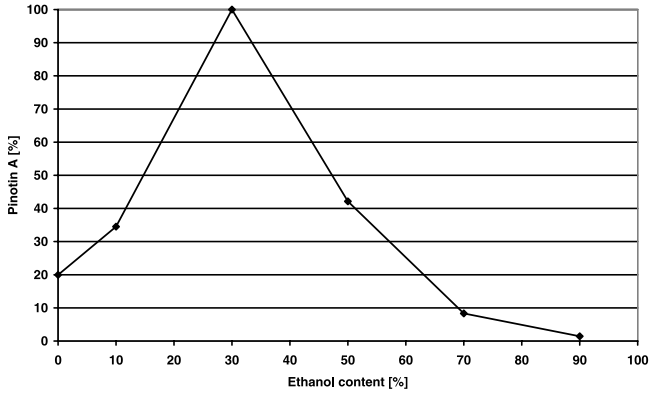
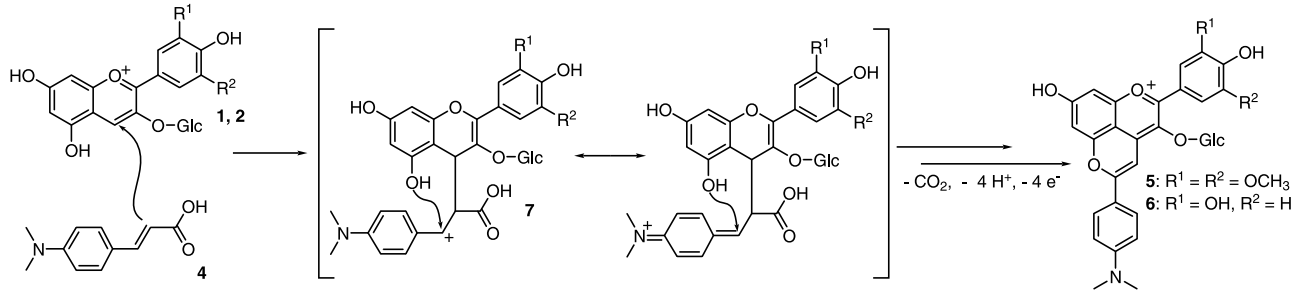


Figure 4. Influence of ethanol on the rate of formation of **3**.



Scheme 1. Reaction pathway for the formation of pyranoanthocyanins from anthocyanins and cinnamic acids.[8]

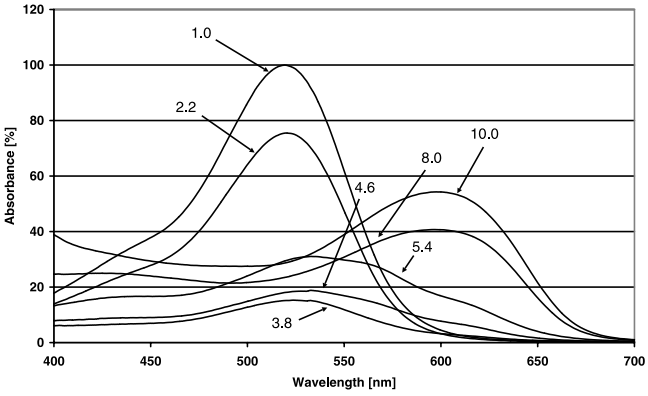


Figure 5. UV–vis spectra of malvidin 3-glucoside **1** at various pH.

except for the slightly different absorbance maxima). In contrast to all naturally occurring pyranoanthocyanins derived from hydroxycinnamic acids, the new dimethylamino substituted pigments exhibited a bathochromic instead of a hypsochromic shift in acidic conditions. UV–vis spectra of **5** are shown in Figure 6, and for compound **6** nearly identical spectra were recorded. The visible absorbance maxima of **5** and **6** were shifted by 35 nm to 555 nm and 545 nm, respectively. In contrast to the underivatized pigments, the solutions of **5** and **6** exhibited maximum colour at pH 2.2. At pH 3.8 the intensity was still very similar to that at pH 1.0 and only 5 and 17% below the maximum absorbance for **5**

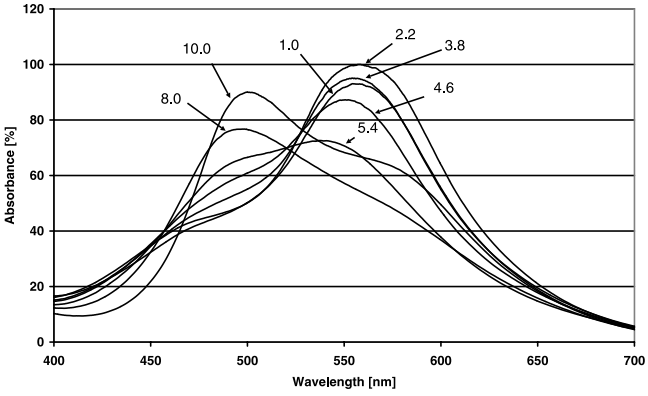


Figure 6. UV–vis spectra of pyranoanthocyanin **5** at various pH.

and **6**, respectively. Formation of equilibrium structures was not significant until pH 5.4. In comparison, the maximum absorbance of the solution of **3** at 505 nm varied only slightly between pH 1.0 and 3.0. At pH 3.8 the loss in intensity was only 8% compared to lower pH (Fig. 7). Only relevant spectra are shown.

CIELab values were recorded in dilute HCl (Table 1). The *p*-dimethylamino substituted compounds were more blue (lower b^* values) compared to the anthocyanins they are based on, and thus appeared violet. The bluish character was more pronounced in the malvidin-based colorants **1**, **5** compared to pigments containing a cyanidin aglycon **2**, **6**. The dihydroxy substituted pinotin A **3** was more yellow ($\Delta b^* = 5{,}6$) compared to **1**, resulting in an orange colour. The impact of the substitution pattern is readily observed in Figure 8.

Experimental

Optimized reaction conditions: 202.4 mg of **1** and 800 mg of caffeic acid were dissolved in 40 ml of ethanol. A wine-like model solution was prepared by saturating water with potassium hydrogen tartrate and also with caffeic acid (to have the same concentration in each model experiment). For the experiments on pH dependency 3 ml of the ethanolic solution were mixed with 27 ml of the model wine and the pH was adjusted with small amounts of either concentrated hydrochloric acid or sodium hydroxide to pH values of 1.0, 1.6, 2.2, 2.9, 3.4 and 4.2. The influence of ethanol was investigated at concentrations of 10, 30, 50, 70, and 90% (v/v). To 3 ml of the ethanolic solution the appropriate amount of ethanol was added and diluted to a final volume of 30 ml with model wine. An ethanol-free solution was prepared by dissolving 15.6 mg of **1** in 30 ml of the model wine. The solutions were stored under argon in 30 ml amber glass bottles in the dark at 15°C and analyzed by HPLC at regular intervals for 4 months.

HPLC analysis: Analyses were carried out on a Synergi MaxRP-12 4 μm 250×4.6 mm column (Phenomenex, Germany) and separation was monitored by diode array detection. 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. Linear gradient from 6–20% B at 0–20 min, 20–40% B at 20–35 min, 40–60% B at 35–40 min, 60–90% B at 40–45 min, 90% B at 45–50 min.

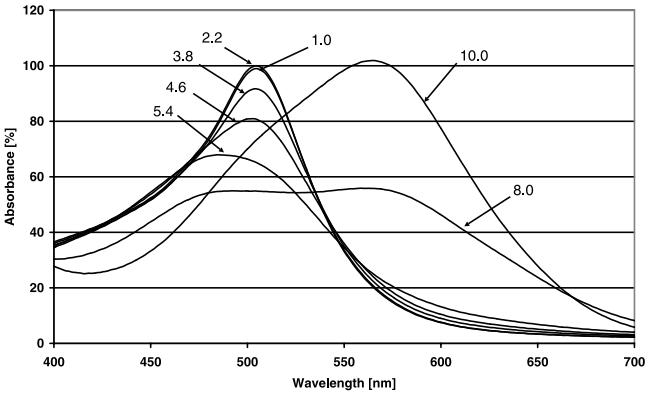


Figure 7. UV–vis spectra of pinotin A **3** at various pH.

Table 1. CIELab values in 0.01 M HCl

Compound	L^*	a^*	b^*
1	88.6	30.3	10.7
2	88.3	33.2	28.3
3	93.8	13.8	16.3
5	52.5	40.6	−32.7
6	77.6	26.1	−17.0

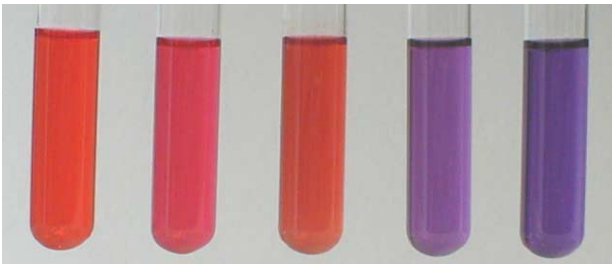


Figure 8. Colours of anthocyanins **2** and **1**, and pyranoanthocyanins **3**, **6**, and **5** in 0.01 M HCl (from left).

Syntheses: Compound **5** was synthesized by dissolving 100 mg of **1** in 35 ml HCl (pH 3.0). 15 ml of a saturated solution of **4** in ethanol was added and the mixture was stored in an amber glass bottle under argon, in the dark, at 15°C for 6 weeks, until the reaction was complete as determined by disappearance of substrate by HPLC analysis. **6** was synthesized accordingly by replacing **1** with **2**. Ethanol was removed by rotary evaporation and the solutions freeze-dried. The products were purified by preparative HPLC.

Preparative HPLC: Purification of compounds **5** and **6** was achieved by isocratic elution with water/acetonitrile/formic acid (68/27/5, v/v/v, 6.0 ml/min) on a Luna RP-18 (Phenomenex, Germany) column (250×10 mm) equipped with a guard column (50×10 mm) of the same material. Detection wavelength was 570 nm.

5: 10-(4-dimethylamino-phenyl)-7-hydroxy-2-(4-hydroxy-3,5-dimethoxyphenyl)-pyrano[4,3,2-*de*]chromen-1-ylium-3-*O*-β-D-glucoside. ^{1}H NMR (300 MHz, CD_3OD/TFA 19:1) $\delta = 8.06$ (H-9, s, 1H), 7.89 (H-2‴/6‴, d, $J = 9.2$ Hz, 2H), 7.57 (H-2′/6′, s, 2H), 6.93 (H-8, d, $J = 1.7$ Hz, 1H), 6.90 (H-6, d, $J = 1.7$ Hz, 1H), 6.72 (H-3‴/5‴, d, $J = 9.2$ Hz, 2H), 4.81 (H-1″, d, $J = 7.7$ Hz, 1H), 3.93 (OCH_3, s, 6H), 3.2–3.7 (glucose resonances, m, 6H), 3.11 (NCH_3, s, 6H). HR-MS (ESI$^+$) m/z calculated for $C_{33}H_{34}O_{12}N$ 636.2081, found 636.2084.

6: 10-(4-dimethylamino-phenyl)-7-hydroxy-2-(3,4-dihydroxy-phenyl)-pyrano[4,3,2-*de*]chromen-1-ylium-3-*O*-β-D-glucoside. ^{1}H NMR (300 MHz, CD_3OD/TFA 19:1) $\delta = 8.07$ (H-9, s, 1H), 7.91 (H-2‴/6‴, d, $J = 9.2$ Hz, 2H), 7.76 (H-6′, dd, $J = 8.3$, 2.1 Hz, 1H), 7.60 (H-2′, d, $J = 2.1$ Hz, 1H), 6.93 (H-8, d, $J = 1.7$ Hz, 1H), 6.91 (H-5′, d, $J = 8.3$ Hz, 1H), 6.90 (H-6, d, $J = 1.7$ Hz, 1H), 6.74 (H-3‴/5‴, d, $J = 9.2$ Hz, 2H), 4.77 (H-1″, d, $J = 7.7$ Hz, 1H), 3.3-3.9 (glucose resonances, m, 6H), 3.11 (NCH_3, s, 6H). HR-MS (ESI$^+$) m/z calculated for $C_{31}H_{30}O_{11}N$ 592.1819, found 592.1829.

UV–vis: Eight McIlvaine buffer solutions of pH 2.2, 3.0, 3.8, 4.6, 5.4, 6.2, 7.0, and 8.0 were prepared by mixing appropriate volumes of solutions of 0.1 M citric acid and 0.2 M disodium phosphate. Solutions of pH 1.0 and 10.0 were prepared by adding concentrated HCl to the buffer with pH 2.2, and concentrated NaOH to the buffer with pH 8.0, respectively. The compounds (1.00 mg of **1**, 0.96 mg of **2**, 1.17 mg of **3**, 1.00 mg of **5**, 1.08 mg of **6**) were dissolved in 1.00 ml of water. 50 μl of these solutions were mixed with 950 μl buffer and UV–vis spectra were recorded in 10 mm quartz cuvettes with a Shimadzu UV-2101PC photometer between 270–800 nm.

Colour measurements: L^*, a^*, b^* values for compounds **1**–**3**, **5**, and **6** were recorded in a 10 mm quartz cuvette at 20 mg/l in 0.01 M HCl on a Varian Cary 100 Conc spectrophotometer.

Acknowledgements

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