Vitis **42** (2), 105–106 (2003)

Research Note

Isolation and structure of Pinotin A, a new anthocyanin derivative from Pinotage wine

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Key words: *Vitis vinifera*, red wine, Pinotage, high-speed countercurrent chromatography, anthocyanins, malvidin, vinylcatechol, pyranoanthocyanin.

Introduction: The color of red wine is composed by monomeric anthocyanins, mainly malvidin-3-O-glucoside, acetylated and coumaroylated derivatives as well as a complex mixture of polymeric pigments which are predominantly present in aged wines. In addition, copigmentation is also known to contribute to the overall color intensity. In recent years, additional anthocyanin derivatives containing a pyran ring between C-4 and the hydroxyl group at position 5 of the aglycon moiety have been characterized. Among these, the vitisin-type pigments are formed by the reaction between malvidin and pyruvic acid (Bakker *et al.* 1997, Fulcrand *et al.* 1998). As further pyranoanthocyanin, the malvidin 4-vinylphenol adduct has been detected by Fulcrand *et al.* (1996). Most recently, by using nanoelectrospray tandem mass spectrometry Hayasaka and Asenstorfer (2002) tentatively identified the 4-vinylcatechol, 4-vinylguaiacol, and 4-vinylsyringol adducts of malvidin-3-glucoside, in red wines and grape skin extracts. However, the tiny amounts did not allow an isolation and characterization.

In this paper we report for the first time the isolation of Pinotin A, a malvidin-3-O-β-D-glucoside 4-vinylcatechol adduct from Pinotage (Pinot Noir ↔ Cinsault) wine by high-speed countercurrent chromatography (HSCCC) and semi-preparative HPLC and its full structural characterization by HPLC-ESI-MS^n and one-/two-dimensional NMR techniques.

Material and Methods: Sample preparation: An anthocyanin-enriched extract from 6 l of a 1997 Pinotage wine (Zonnebloem, Stellenbosch, South Africa) was prepared by solid phase extraction on Amberlite XAD-7 according to the method published by Degenhardt *et al.* (2000).

HSCCC: A CCC-1000 high-speed countercurrent chromatograph (Pharma-Tech Research Corp., USA) with three coils (total volume 850 ml) was used. Revolution speed was set to 1000 rpm. Solvent system I consisted of n-butanol/TBME/acetonitrile/water (2/2/1/5, v/v/v/v, acidified with 0.1 % TFA). Solvent system II was n-butanol/TBME/acetonitrile/water (0.65/3.35/1/5, v/v/v/v, acidified with 0.1 % TFA). Mobile phases (more dense layers) were delivered by a Biotronik BT 3020 HPLC pump (Jasco, Germany) at a flow rate of 3.0 ml·min^{-1}. Separation was monitored with a UV/Vis-detector (Knauer, Germany) at λ = 510 nm. Fractions were collected every 4 min with a Super Frac fraction collector (Pharmacia LKB, Sweden), combined according to the chromatogram and lyophilized prior to HPLC analysis.

Semi-preparative HPLC: Purification was achieved by isocratic elution with water/acetonitrile/formic acid (70/25/5, v/v/v, 6.0 ml·min^{-1}) on a Luna RP-18 (Phenomenex, Germany) column (250 × 10 mm) equipped with a guard column (50 × 10 mm) of the same material. The HPLC-system consisted of a Knauer 64 pump and a variable UV/Vis-detector (λ = 510 nm). Sample was injected through a Rheodyne valve 7125 (USA) with a 200 µl loop.

HPLC-ESI-MS^n: Analyses were done on an Esquire Ion Trap ESI-LC-MS system (Bruker, Germany). Equipment and chromatographic conditions were the same as published in Schwarz *et al.* (2003).

NMR: ^{1}H-, ^{13}C-NMR- and DEPT-spectra were measured on a Bruker AMX 300 spectrometer at 300.1 MHz and 75.4 MHz, respectively. HMQC and HMBC experiments were performed on a Bruker AM 360 instrument. Solvent was a mixture of methanol-d_4 and TFA-d_1 (19:1, v/v).

Results and Discussion: The HPLC chromatogram of the Pinotage wine showed an intense peak with a retention time of 44.7 min, eluting just before the peak of malvidin-3-(6''-*p*-coumaroylglucoside). Molecular mass $[M]^+$ of the unknown compound was determined to be at 625 mass units (in negative mode *m/z* 623 $[M-2H]^-$). Upon fragmentation, a loss of 162 mass units indicated the cleavage of a hexose moiety. The remaining aglycon had a mass of *m/z* 463. Further fragmentation produced losses of 16, 32, 44, 61 and 89, which were in line with the losses of the tentatively identified malvidin-3-O-glucoside 4-vinylcatechol pigment (Hayasaka and Asenstorfer 2002).

For isolation of the target compound, the lyophilized Pinotage XAD-7 extract was subjected first to a CCC clean-up step applying solvent system I. Polymeric pigments eluted first, followed by malvidin-3-O-β-D-glucoside, after which the separation was stopped. The remaining solvent mixture on the coil was pumped out of the CCC system and upper and lower layers were collected separately. Organic solvents were removed by concentration *in vacuo*, and both fractions were freeze-dried. The lyophilizate of the upper layers was again separated by CCC with the less polar solvent system II (Fig. 1). Fraction I contained the target compound in 70 % purity. After freeze-drying, final purification was achieved by semi-preparative HPLC on a Luna C18-column (yield 70 mg).

The ^{1}H-NMR spectrum showed signals for H-6, H-8, H-2', H-6' and two methoxy groups, thus suggesting the structure to be derived from malvidin-3-O-β-glucoside. However, the signal for H-4 was missing and the anomeric glucose proton shifted upfield to 4.81 ppm with a coupling constant of 7.5 Hz, proving β-configuration. The remaining glucose resonances appeared between 3.19 and 3.68 ppm. Fur-

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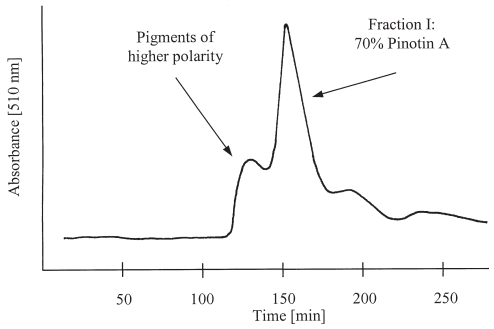

Fig. 1: Isolation of the malvidin-3-O-β-D-glucoside 4-vinylcatechol adduct from Pinotage wine by HSCCC. Solvent system n-butanol/ TBME/acetonitrile/water (0.65/3.35/1/5, v/v/v/v, acidified with 0.1 % TFA).

ther signals observed were a singlet at 7.84 ppm, assigned to H-11, and three additional aromatic proton signals of the catechol ring system. The ^{13}C-NMR spectrum displayed a total of 31 resonances, 6 of them being related to β-D-glucose and another 8 signals to the unchanged B ring of the malvidin-based structure (cf. Table). By means of 2D-NMR techniques (HMQC, HMBC) we were able to assign all ^{1}H- and ^{13}C-NMR connectivities in the molecule. Some of the long-range CH-correlations which are important for structure elucidation are illustrated in Fig. 2. As already expected from the ^{13}C-NMR spectra, a long range CH-correlation starting from the anomeric proton H-1'' (4.81 ppm) to C-3 (134.9 ppm) is clearly indicating the linkage of a glucose moiety to the malvidin aglycon.

Further significant cross peaks were observed from the olefinic proton H-11 (7.84 ppm) to C-3 (134.9 ppm) of the malvidin backbone, to C-12 (169.4 ppm), and to C-1''' (123.1 ppm). Especially a long range correlation of H-11 over 3 bonds to C-10 (108.5 ppm), and as well from H-8 (7.11 ppm) to C-10 are indicative for the proposed structure. Strong correlation signals of protons H-2''' and H-6''' to C-12, confirm the vinylcatechol partial structure from the opposite direction.

NMR data of this newly found pigment in Pinotage red wine - which was named Pinotin A - is in exact accordance with the molecular weight of *m/z* 625 which was determined by means of electrospray-MS measurements in positive and in negative mode.

We are indebted to Dr. OTTENEDER, LUA Trier, for initiating this study, and we thank M. MESSERER and Dr. R. WAIBEL for performing the NMR experiments. The project was financed by the German Ministry of Economy via the Arbeitskreis Industrieller Forschungsvereinigungen/Forschungskreis der Ernährungsindustrie (AIF- FV: 12896N).

BAKKER, J.; BRIDLE, P.; HONDA, T.; KUWANO, H.; SAITO, N.; TERAHARA, N.; TIMBERLAKE, C. F.; 1997: Identification of an anthocyanin occurring in some red wines. Phytochemistry **44**, 1375-1382.

DEGENHARDT, A.; HOFMANN, S.; KNAPP, H.; WINTERHALTER, P.; 2000: Preparative isolation of anthocyanins by high-speed countercurrent chromatography and application of the color activity concept to red wine. J. Agric. Food Chem. **48**, 5812-5818.

FULCRAND, H.; CAMEIRA DOS SANTOS, P. J.; SARNI-MANCHADO, P.; CHEYNIER, V.; FAVRE-BONVIN, J.; 1996: Structure of new anthocyanin-derived wine pigments. J. Chem. Soc. Perkin Trans. **1**, 735-739.

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^{1}H- and ^{13}C-NMR spectral data for Pinotin A measured in ethanol-d_4 and TFA-d_1 (19:1, v/v)

Position	^{1}H [ppm]	J [Hz]	^{13}C [ppm]	DEPT*
2	-	-	162.5	q
3	-	-	134.9	q
4	-	-	108.5	q
5	-	-	154.4	q
6	7.09	d, 2.0	101.1	CH
7	-	-	167.4	q
8	7.11	d, 2.0	101.1	CH
9	-	-	153.6	q
10	-	-	108.5	q
11	7.84	s	98.3	CH
12	-	-	169.4	q
1'	-	-	120.6	q
2', 6'	7.67	s	109.7	CH
3', 5'	-	-	149.1	q
4'	-	-	143.2	q
3'-, 5'-OMe	3.95	s	57.2	CH_3
1''	4.81	d, 7.5	105.6	CH
2''	3.44	nr.	77.9	CH
3''	3.68	nr.	75.8	CH
4''	3.26	nr.	71.6	CH
5''	3.19	nr.	78.7	CH
6''	3.36, 3.68	nr.	62.8	CH_2
1'''	-	-	123.1	q
2'''	7.58	d, 2.5	115.3	CH
3'''	-	-	147.6	q
4'''	-	-	154.1	q
5'''	6.96	d, 8.5	117.4	CH
6'''	7.66	dd, 8.5, 2.5	122.7	CH

* DEPT 90/135; q = quaternary; nr. = not resolved

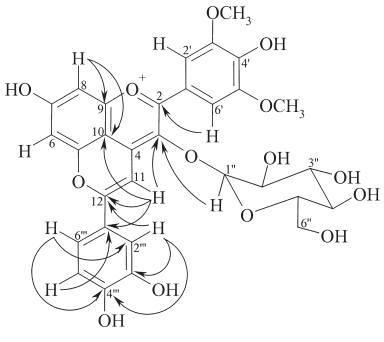

Fig. 2: Structure of Pinotin A with important long-range H-C correlations from HMBC experiment.

FULCRAND, H.; BENABDELJALIL, C.; RIGAUD, J.; CHEYNIER, V.; MOUTOUNET, M.; 1998: A new class of wine pigments generated by reaction between pyruvic acid and grape anthocyanins. Phytochemistry **47**, 1401-1407.

HAYASAKA, Y.; ASENSTORFER, R. E.; 2002: Screening for potential pigments derived from anthocyanins in red wine using nanoelectrospray tandem mass spectrometry. J. Agric. Food Chem. **50**, 756-761.

SCHWARZ, M.; HILLEBRAND, S.; HABBEN, S.; DEGENHARDT, A.; WINTERHALTER, P.; 2003: Application of high-speed countercurrent chromatography to the large-scale isolation of anthocyanins. Biochem. Eng. J. **14**, 179-189.