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Hopeaphenol: The First Resveratrol Tetramer in Wines from North Africa

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Grapes and wines are now known to constitute a rich source of phenolics such as stilbenes and flavonoids. These compounds have been shown to have cancer chemopreventive activity and potential beneficial effects on cardiovascular diseases thanks to their antioxidant and antiplatelet properties. However, because little is known about African wines and their phenolic compositions, we investigated wine samples from North Africa. A three-step method was used for the fractionation of the Merlot variety wine: column chromatography followed by centrifugal partition chromatography and reversed-phase semipreparative high-performance liquid chromatography (HPLC). Six polyphenolic compounds of the Merlot variety (from Algeria) were isolated and identified by NMR spectroscopy, five of which are known (*trans*-resveratrol, *trans*-piceid, *trans*-ϵ-viniferin, pallidol, and astilbin) and one that is reported for the first time in wine, (+)-hopeaphenol, a stilbene tetramer. Furthermore, these molecules were quantified in 10 commercial wines from North Africa by means of an analytical HPLC system coupled with diode array detection. Differences in concentrations were found ranging in mg/L from 4.6 to 45 (*trans*-piceid), 0.66 to 3.45 (*trans*-resveratrol), 0.2 to 1.2 (*trans*-ϵ-viniferin), 0.2 to 9.2 (pallidol), 0.3 to 3.8 (hopeaphenol), and 10.8 to 24.22 (astilbin). Such a high level of pallidol and astilbin has never been recorded in wine. North African wines may contribute to a significant proportion of dietary intake of stilbene and astilbin, which may have health benefits.

KEYWORDS: North African wines; stilbenes; *trans*-resveratrol; *trans*-ϵ-viniferin; *trans*-piceid; pallidol; astilbin; hopeaphenol; NMR; HPLC

INTRODUCTION

According to several epidemiological studies, polyphenols from grapes and wines have significant health benefits, since they decrease the incidence of coronary heart disease (*1*, *2*), reduce platelet aggregation (*3*−*5*), and provide antioxidative and carcinogenic protection (*6*−*8*). Polyphenols are also reported to induce vasorelaxation (*9*) and to have antiinflammatory activity (*8*).

Stilbenes, an important polyphenolic subclass, occur naturally in various plant families, but grapes and related products are considered the most important dietary sources of these substances (*10*). Numerous stilbene compounds have already been reported in wines, such as *trans*- and *cis*-resveratrol, *trans*- and *cis*-piceid (*11*−*15*), and *trans*-astringin (*15*, *16*). Recently, Baderschneider and Winterhalter (*17*) isolated the following compounds from a Riesling wine: 2,4,6-trihydroxyphenantrene-2-*O*-glucoside, resveratrol-2-C-glucoside (*cis* and *trans*), and the dimeric stilbenes ϵ-viniferin diglucosides (*cis* and *trans*) as well as pallidol-3-*O*-glucoside and pallidol-3,3″-diglucoside. Using centrifugal partition chromatography (CPC), Vitrac et al. (*15*) isolated the dimers pallidol and parthenocissin-A from French red wine. In 2002, Landrault et al. (*18*) quantified *trans*-ϵ-viniferin in red and botrytized sweet white wines. Finally, in 2005, δ-viniferin was determined in Brazilian wines (*19*). Among the stilbenes, *trans*-resveratrol has been the most widely studied for its role in human health. It has many marked biological activities with regard to cardiovascular disease and cancer (*6*, *20*), but some other stilbenes also have properties similar to those of *trans*-resveratrol (*21*, *22*). Therefore, monitoring new stilbene derivatives in wine appears to be of particular relevance.

Recently, resveratrol tetramers like hopeaphenol and its isomer isohopeaphenol were identified in the cork of *Vitis vinifera* Kyohou (*23*). Screening of a series of stilbenes against several tumor cell lines showed that hopeaphenol has potent cytotoxicity on the human epidermoid carcinoma of the na-

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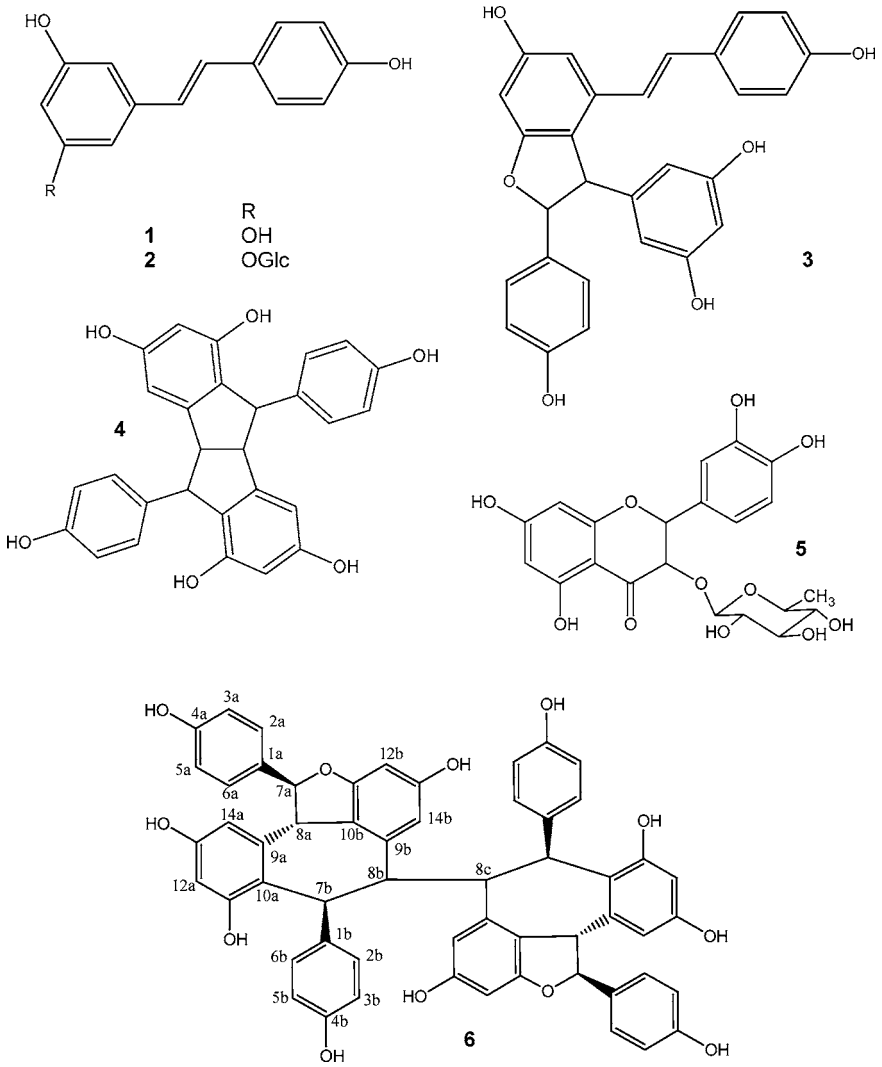

Figure 1. /lp;&-2q;1Chemical structures of the phenolic compounds isolated: *trans*-resveratrol (**1**), *trans*-piceid (**2**), *trans*-ϵ-viniferin (**3**), pallidol (**4**), astilbin (**5**), and hopeaphenol (**6**).

sopharynx with an ED_{50} of 1.2 μg/mL (*24*). It has also been reported to have antimicrobial (*25*), antiinflammatory (*26*), antifungal, and HIV-inhibitory activities (*27*). Up to now, resveratrol tetramers have not been reported in wines.

Flavonoids such as anthocyanins and flavanols are present in high amounts in red wine. However, we recently reported for the first time the isolation of two dihydroflavonols, astilbin and its 5′-hydroxylated derivative in wines, which appear to be rare in plants and food (*15*). Little is known about their occurrence in wine, even though astilbin has several biological activities such as protection of rat red blood cells against oxidative damage (*28*).

This study is the first attempt to analyze North African wines for their polyphenolic content. A red wine of Merlot variety from Algeria was subjected to fractionation using column chromatography followed by CPC. Final purification and identification of polyphenols were done by semipreparative high-performance liquid chromatography (HPLC) and NMR, respectively. Furthermore, all of the compounds isolated in the Merlot variety (**Figure 1**) were quantified in 10 commercial wines from North Africa using an analytical HPLC method coupled with UV detection.

MATERIALS AND METHODS

Reagents. All organic solvents used were of HPLC grade. Methanol and ethanol were purchased from Carlo Erba (Val de Reuil, France), and acetonitrile, ethyl acetate, and hexane were purchased from Scharlau Chemie (Sentmenat, Spain). Water was distilled and filtered through a Millipore membrane (0.22 μm).

Standards. *trans*-Resveratrol was purchased from Sigma (St. Quentin Fallavier, France). *trans*-ϵ-Viniferin, pallidol, astilbin, and hopeaphenol were extracted from Merlot stems. *trans*-Piceid was isolated and purified from *Vitis vinifera* cell suspensions cultures.

Preparation and Fractionation of Algerian Merlot Wine. The Merlot red wine (3 L) from Algiers (ONCV, vintage 2004) was extracted using the protocol described by Vitrac et al. (*15*). Briefly, it was concentrated in vacuo and extracted with EtOAc. The concentrated EtOAc residue was then chromatographed over a cation-exchange resin column (DOWEX, Sigma) and gave 0.85 g of lyophilized solid powder. This residue was submitted to two successive steps of CPC. Fractionation was performed on a Bench scale FCPC 200 apparatus (Kromaton, Angers, France). The solvents were pumped into the column (200 mL) rotating at 1000 rpm with HPLC pumps (Gilson, model 321) at a flow rate of 3 mL/min. Fractions were collected with a fraction collector (Gilson, model FC 204). The solvent system H_2O/EtOH/EtOAc/hexane (*29*) used in the ratio 7/2/8/1 led to four major fractions (A−D) in the ascending mode and three fractions in the descending mode (F−H).

Fraction A (80 mg) was further submitted to CPC using the same quaternary solvent system but in a different ratio 3/3/5/4 (v/v) (*29*). The ascending mode yielded three major fractions (P−R), and the descending mode yielded a single fraction (X, 0.0245 g). Collected fractions were monitored by thin-layer chromatography (TLC) on Polygram silica gel 0.2 mm with fluorescent indicator UV_{254} (Macherey-

Nagel) using $CHCl_3$/MeOH/acetic acid 85/15/3 (v/v) as the mobile phase. Visualization of TLC plates was performed by spraying anisaldehyde reagent (*30*).

Purification of Fractions B, C, Q, R, and X by Semipreparative HPLC. Final purification was performed using semipreparative HPLC (Varian, model ProStar 210 coupled with a diode array detector ProStar 335) with a Bischoff Prontosil 120-5-C18-AQ column (250 mm × 8 mm, 5 μm particle size) at room temperature. The flow rate was set at 3 mL/min with a mobile phase composed of solvent A, 0.025% TFA in water, and solvent B, ACN/solvent A, 80/20 (v/v). The gradient system was as follows: 20−50% B (0−35 min), 50−100% B (35−40 min), 100% B (41−46 min), 100−20% B (46−48 min), and 20% B (48−53 min). Detection was performed at 286 and 306 nm. The isolated compounds were identified on the basis of TLC spots of standards and by coinjection on analytical HPLC. Final verification of the compounds was performed with a matrix-assisted laser desorption/ionization (MALDI) analysis and by comparing their NMR data with literature values.

NMR Spectroscopy. NMR spectra were recorded at 303 K in the Fourier transform mode at 300 MHz on a Bruker AMX 300 spectrometer equipped with a broadband 5 mm probe, using a spectral width of 10 ppm. Chemical shifts were expressed as ppm relative to the deutered methanol signal at δ 3.31 ppm.

MALDI Analysis. MALDI-MS spectra were acquired using a TofSpec MALDI-time-of-flight (TOF) mass spectrometer from Micromass (Manchester, United Kingdom). Spectra were recorded in the positive-ion mode.

HPLC Analysis. Analysis was carried out on an Agilent HPLC 1100 series equipped with a quaternary pump with degasser (model G1354A), an autosampler (model G1313A), thermostated column compartment (model G1316A), and a diode array detector (model G1315B). Analysis was performed with a Bischoff Prontosil 120-5-C18-AQ column (250 mm × 4 mm, 5 μm particle size) thermostated at 25 °C with a flow rate of 1 mL/min. The solvents and the gradient used were the same as described above.

Samples of 100 μL of wine were directly injected into the HPLC system after filtration on a Millipore membrane 0.45 μm. After each analysis, the column was reequilibrated with phase A for 10 min. Detection was carried out at 280, 286, 306, and 330 nm.

UV Spectra−Optical Rotation. UV spectra were recorded on a Hitachi U-2000 spectrophotometer. Optical rotation was measured in MeOH with a Perkin-Elmer 241 polarimeter at 20 °C.

Wine Samples. Ten North African wines (vintages 2003 and 2004) commercially available in France and in Algeria were analyzed as follows: Algerian wines comprised three red (Merlot, Cabernet Sauvignon, and Cuvée du Président,* from ONCV Algiers), one rosé (Gris d'Algérie,* from the area of Medea), and one white (Muscat, from ONCV Algiers) and Moroccan wines comprised four red (Sidi Brahim,* Ksar,* and Guerrouane,* from the area of Meknes; Amjad,* from the area of Oujda), and one Tunisian red wine (Terrale,* from the area of Carthage) (*blended wines).

RESULTS AND DISCUSSION

Isolation and Identification of Polyphenols from Wine. Six components were isolated from Merlot red wine, and their structures were elucidated by one- and two-dimensional (1D and 2D) NMR analysis. Astilbin was the major constituent of fraction B, and piceid was the major component of fraction C. Fraction Q especially contained resveratrol, and fraction R yielded *trans*-ε-viniferin. Fraction X was composed of pallidol and a resveratrol tetramer isolated for the first time in wine: (+)-hopeaphenol (**Figure 1**). Hopeaphenol was isolated by HPLC as a peak collected at t_R = 28 min. Mass spectrometry of this compound gave an $[M + H]^+$ peak at *m*/*z* 907 in a positive ion mode MALDI-TOF, which corresponds to the molecular formula $C_{56}H_{42}O_{12}$ of a resveratrol tetramer. Maxima of absorption in MeOH were observed at 227 (sh) and 283 nm in the UV spectrum. The optical rotation was $[\alpha]_D = +366°$ (*c* = 0.17, MeOH). This was identified by comparing its observed

Table 1. ^1H and ^{13}C NMR Data of (+)-Hopeaphenol in Methanol-d_4

no.	δ_H, *J* (Hz)	δ_C	no.	δ_H, *J* (Hz)	δ_C
1a		129.4	1b		136.1
2(6)a	7.09 *d* (8.6)	130.3	2(6)b	6.89 *d* (8.6)	128.9
3(5)a	6.72 *d* (8.6)	114.2	3(5)b	6.54 *d* (8.6)	113.9
4a		158.7	4b		156.2
7a	5.80 *d* (12.1)	88.1	7b	5.75 *br s*	40.0
8a	4.13 *d* (12.1)	49.9	8b	3.86 *br s*	48.2
9a		141.9	9b		140.2
10a		121.5	10b		118.6
11a		159.3	11b		159.2
12a	6.38 *d* (2.1)	100.3	12b	5.72 *d* (2.1)	94.9
13a		156.3	13b		156.4
14a	6.21 *d* (2.1)	104.8	14b	5.07 *d* (2.1)	110.4

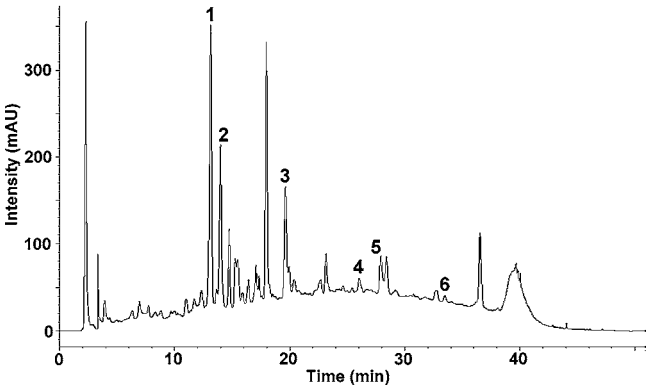

Figure 2. HPLC chromatogram of Merlot red wine (at 286 nm). Key: **1**, *trans*-piceid; **2**, astilbin; **3**, pallidol; **4**, *trans*-resveratrol; **5**, hopeaphenol; and **6**, *trans*-ε-viniferin.

^1H and ^{13}C NMR (**Table 1**). The 1D and 2D NMR data show the presence of four aromatic rings, an ether ring, and two aliphatic protons. 2D correlation spectroscopy, heteronuclear single quantum correlations, and heteronuclear multiple bond correlations led to the determination of the chemical structure of the benzenic rings and the relative position of the other protons. Furthermore, all NMR data were found in full agreement with the structure of the reported hopeaphenol (*23*, *31*).

Polyphenol Levels in North African Wines. We examined 10 wine samples to quantify the isolated compounds. All of the standard solutions showed good linearity ($r^2 > 0.999$), and the quantification of polyphenols was achieved by UV detection. The latter showed good baseline separation for the peaks of interest. **Figure 2** shows an HPLC chromatogram of Merlot red wine. The chromatographic profiles from the other wines were similar to the Merlot chromatogram. The concentrations of the components (**Table 2**) were calculated from the chromatogram peak areas.

As shown in **Table 2**, some compounds were not detected in some wine samples studied. Hopeaphenol was present in significant concentrations in eight of the wines analyzed. Ksar wine presented the highest concentration of hopeaphenol (3.8 mg/L), followed by Muscat (3.06 mg/L), Guerrouane (2.68 mg/L), Merlot (2.1 mg/L), Cabernet Sauvignon (1.48 mg/L), Sidi-Brahim (0.61 mg/L), Amjad (0.34 mg/L), and Gris d'Algérie (0.3 mg/L). Concerning its biosynthetic pathway in Vitaceaeous plants, (+)-hopeaphenol could be formed from resveratrol via the dimer (+)-ε-viniferin by oxidation catalyzed by peroxidases (*32*, *33*).

Regarding *trans*-resveratrol, its levels in North African red wines ranged from 0 to 3.45 mg/L. These levels are in agreement with those reported for wines from Greece (from 0.35 to 1.99 mg/L) (*34*), France (from 0.9 to 3.8 mg/L) (*35*), and Brazil (from

Table 2. Levels of Polyphenols in North African Wines (mg/L)

wines	*trans*-resveratrol	*trans*-ϵ-viniferin	*trans*-piceid	hopeaphenol	astilbin	pallidol	total stilbenes[b]
Merlot	3.4	1.2	14	2.1	10.8	4.1	24.80
Cabernet Sauvignon	2.2	0.69	9.37	1.48	11.8	3.54	17.28
Cuvée du président	3.2	ND[a]	45	ND	10.82	0.2	48.40
Gris d'Algérie	0.77	ND	31.3	0.3	ND	ND	32.37
Muscat	0.72	ND	4.6	3.06	11.4	9.20	17.58
Ksar	0.66	0.49	8.5	3.8	21.4	3.4	16.85
Amjad	2.5	0.2	38.6	0.34	10.82	0.35	41.99
Guerrouane	ND	ND	15	2.68	24.22	4.63	20.36
Sidi-Brahim	3.45	ND	17.6	0.61	ND	5.34	27.00
Terrale	ND	ND	10	ND	ND	7	17.00

[a] ND, not detected. [b] Expressed as the sum of each stilbene compound.

0 to 5.34 mg/L) (*19*). In Muscat wine, the *trans*-resveratrol value (0.72 mg/L) was similar to that reported in Portuguese white wines (*14*).

Concentrations of *trans*-piceid ranged from 4.6 mg/L (Muscat wine) to 45 mg/L (red wine) (**Table 2**). The highest one was detected in Cuvée du Président, which was almost 5-fold greater than in Cabernet Sauvignon and 3-fold greater than in Merlot. This resveratrol glycoside is often found to be the major stilbene in wine and grape juice. In accordance with the literature, considerable amounts of piceid occur in red wine in varying amounts up to 50.8 mg/L in monovarietal red wines from Portugal (*14*) while its level was found to attain 26 mg/L in wines from South Western France (*35*). Concerning Muscat wine, we found a concentration similar to that in white wines from Portugal (*14*).

Pallidol was first isolated from *Cissus pallida* and is a natural constituent of *V. vinifera*, as reported by Waffo-Téguo et al. in grape cell suspension cultures (*36*). In our study, levels of pallidol ranged from 0.2 to 7 mg/L in red wines and attained 9.2 mg/L in Muscat wine. Up to now, this compound has not been found in sweet and dry white wines except in Chardonnay wine (0.3 mg/L) enriched in phenolics by a special winemaking technique (*18*). Levels of pallidol found in this study were higher than those reported in the literature for red wines, ranging from 0.5 mg to 4.8 mg/L (*18*, *35*).

trans-ϵ-Viniferin was present only in four red wines (Merlot, Cabernet Sauvignon, Ksar, and Amjad), with levels ranging from 0.2 to 1.2 mg/L. Viniferins and some other resveratrol dimers are known to be fungal metabolites of resveratrol (*37*). Thus, the occurrence of this compound in wine is possibly due to the oxidation of resveratrol by fungus in the infected berries used for vinification. Grapes and wines containing high levels of ϵ-viniferin could be of health interest. According to Piver et al. (*38*), ϵ-viniferin displayed inhibitory effects for all CYP activities tested, which are involved in the bioactivation of numerous carcinogens.

The highest value of astilbin detected was in Moroccan red wines (Guerrouane, 24.2 mg/L; and Ksar, 21.4 mg/L), i.e., 2-fold higher than in Algerian red wines. These levels are superior to the highest value reported in the literature to date, i.e., 15 mg/L in French red wines (*18*, *35*). The value of astilbin in the North African Muscat white wine (11.4 mg/L) is similar to that found in French white wines, Chardonnay enriched in phenolics (12.9 mg/L), and Sauvignon (9.3 mg/L) (*18*).

Up to now, white wines have been considered to be poor in phenolic compounds since red wines are enriched in polyphenols by a winemaking technique consisting of crushing the grapes with must, seeds, and skins. Surprisingly, in the Muscat wine tested, levels of phenolic compounds expressed in mg/L were found to be 0.72 for *trans*-resveratrol, 4.6 for *trans*-piceid, 11.4 for astilbin, 3.06 for hopeaphenol (approximately the same as some red wines), and 9.2 for pallidol (superior to red wines).

In this study, wines from North Africa (eight red, one rosé, and one white) were analyzed for their concentration in phenolic compounds. The Merlot variety from Algeria was submitted to fractionation and five stilbenes (*trans*-resveratrol, *trans*-piceid, *trans*-ϵ-viniferin, pallidol, and hopeaphenol) and a flavanonol (astilbin) were purified. Hopeaphenol was also found for the first time to be a natural constituent of wine. These findings indicate that African wines contain high levels of resveratrol derivatives and dihydroflavonol in the form of *trans*-piceid and astilbin, respectively, with concentrations of 45 mg/L (Cuvée de Président) and 24.22 mg/L (Guerrouane). Up to now, the highest values reported in the literature have been 50.8 mg/L for *trans*-piceid (*14*) and 15 mg/L for astilbin (*18*, *35*).

The present study suggests that wines from North Africa, even the Muscat variety, can constitute a significant source of daily intake of stilbenes. While relatively high values of phenolic compounds were detected, these findings are not entirely unexpected. A number of factors such as climate, geographical area of cultivation, growing conditions, storage conditions, and winemaking techniques are known to affect the polyphenol content of wines (*39*). Two winemaking conditions could have an important impact on wine stilbene content: first, destalking, because the cluster stems contain many oligostilbenes such as hopeaphenol (*23*; Delaunay, unpublished results), and second, there are the microbial flora involved in alcoholic and malolactic fermentations. Certain wine microorganisms such as yeasts and lactic acid bacteria (*Oenococcus oeni*) can affect the amount of stilbenes detected in wines (*40*).

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