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# Occurrence of Naphtho-Gamma-Pyrones- and Ochratoxin A-Producing Fungi in French Grapes and Characterization of New Naphtho-Gamma-Pyrone Polyketide (Aurasperone G) Isolated from *Aspergillus niger* C-433[1]

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A survey on the occurrence on grape of fungi species in 2001 and their capability to produce ochratoxin A (OTA) and naphtho-gamma-pyrones (NGPs) was conducted in different vineyards from several French viticulture regions. The total numbers of fungal isolates, from setting to harvest, were 732. The *Aspergillus* genus was essentially represented by section *Nigri* (98.53%) and it was predominant (74.72%) when compared to *Penicillium* (25.27%). Approximately one third (30.46%) of the fungal isolates were OTA producers, and 94.17% belong to black aspergilli; *Aspergillus carbonarius* was the main OTA producer. Moreover, 8.33% of isolates (belong to *A. carbonarius* and *A. niger*) were NGP producers. However, none of the *Penicillium* spp. or other *Aspergillus* spp. isolates can produces NGP derivatives under the conditions used. No other study on NGPs production by fungi isolated from grapes has been reported. In the second part, a novel NGP, named aurasperone G (**1**), was isolated from the fermentation broth of the culture extracts of *Aspergillus niger* C-433, strain producer of OTA, along with the known compound aurasperone F (**2**). The chemical structure of the new polyketide was proposed based on complete $^{1}H$ and partial $^{13}C$, COSY, HMQC, 1D NOE NMR spectra as well as UV and MS spectra. This new NGP was not reported before in nature or prepared synthetically.

**KEYWORDS: *Aspergillus niger*; mycotoxin; naphtho-gamma-pyrones; aurasperone G; ochratoxin A**

## INTRODUCTION

Contamination of food and feed with mycotoxins (toxins of fungal origin) represents a high risk for human and animal health. Furthermore, the mycotoxins are responsible for generating huge economic losses to the producing countries (*1*). These toxins are produced, under particular environmental conditions, by filamentous fungi that have been detected in several food commodities. Ochratoxin A (OTA) and naphtho-gamma-pyrones (NGPs) seem to be the most important mycotoxins produced by strains of black aspergilli (or *Aspergillus* section *Nigri*) isolated from grapes (*2*).

In the last few years, OTA has received increasing interest from both scientific communities and food committees because of its nephrotoxic, genotoxic, teratogenic, immunosuppressive, and carcinogenic properties (*3*). Its ingestion by humans, which occurs mainly through different plant-based foods and beverages, could lead to deterioration of liver or kidney function (*4*). IARC (International Agency for Research on Cancer) classified OTA as a possible human carcinogen (group 2 B). Moreover, OTA is suspected to be involved in BEN (Balkan Endemic Nephropathy), a fatal kidney disease occurring in some regions of southeastern Europe and in the high frequency of urinary tract tumors observed in some Balkan areas (*1*). Grapes and derivatives products such as dried vine fruit have been reported

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as potentially contaminated with OTA (*5*). Genera *Aspergillus* and *Penicillium* raised particular attention as the source of OTA (*6*). Among the *Aspergillus*, the section *Nigri* was responsible for OTA production on grapes and its derivatives, and the species *Aspergillus carbonarius* and *Aspergillus niger* aggregate were considered to be particularly important (*7*).

The dimeric NGPs were isolated from a wide variety of fungi belonging to the genera *Aspergillus* (*2, 8, 9*) and *Fusarium* (*10, 11*) as yellow pigments. The interest of many investigators in this class of compounds is due to their broad-range biological actions: antibacterial, (*12, 13*); antifungal (*13*); antimycobacterial (*14*), antimutagenic (*15*); antioxidant (*16*); antiallergic (*17*); hepatoprotective (*18*); antitumor (*19*); anticancer (*20*); cytotoxic (*13, 19, 21*), reversal multidrug resistance of human epidermal KB carcinoma cells (*22*); inhibitor of HMG-CoA reductase (*23*), inhibitor of human breast tumor xenograft MX-1 (*19*), inhibitor of IL-4 signal transduction (*24*), inhibitor of *Taq* DNA polymerase (*11*), inhibitor of xanthine oxidase (*13*), inhibitor of HIV-1 Integrase (*9*), inhibitor of the calmodulin-dependent activity of cAMP phosphodiesterase and NAD-kinase in the presence of CaM (*25*); strong hypotensive activity in cats (*14*); and acute toxicity to mice and rats acting mainly as central nervous system depressant leading to death (*26*).

Previous chemical studies of *A. niger* aggregate revealed the presence of monomeric (*8, 26, 27*), and dimeric (*8, 11, 19, 21, 26–30*) NGP pigments. Moreover, *A. niger* produced a large number of mycotoxins and other secondary metabolites such as nigragillin, aspergillin, asperrubrol, asperyellone, asperenones, kotanins, pyranonigrins, malformins, orobols, tubingensins, yanuthones, and tetra-cyclic compounds (*29, 31–33*).

*A. niger* produced many unknown NGPs which could not be characterized until now (*29, 32*). Furthermore, *A. niger* is the most common species responsible for post-harvest decay of fresh fruits, including apples, pears, peaches, citrus, figs, melons, mangoes, and grapes (*34*). Also, several fungal secondary metabolites produced by *A. niger* are capable of eliciting toxicity in humans and animals (*18*). Until now, no other study on NGPs production by fungi isolated from grapes has been reported.

The principals objectives of the present work were (a) screening for the production of OTA and NGPs by different strains of *Aspergillus* and *Penicillium* isolated from different French vineyards, and (b) the isolation and structure elucidation of a new derivative of NGPs, named aurasperone G, produced by *A. niger* C-433 isolate producer of OTA.

## MATERIALS AND METHODS

**Study Area.** Many vineyards located in different French viticultural regions with different geographical locations and climatic conditions were chosen for this study. Eight varieties were analyzed: Ugni Blanc (UB) from Poitou-Charentes, Riesling (RI) from Alsace, Cinsault (CN) from PACA (Provence-Alpes Côtes d'Azur); and five varieties from Languedoc-Rousillon: Sauvignon (SA), Muscat d'Alexandrie (MA), Syrah (S), Carignan (CA) and Grenache noir (GN). All the varieties analyzed except Ugni Blanc, Riesling and Muscat d'Alexandrie were red vines. For the Syrah variety (S), three parcels were considered, parcels (A) and (B) were treated by the phytochemical agents ("Mikal" (fosetyl-Aluminium and folpet)) and ("Switch" (cyprodinil and fludioxonil)), respectively. However, parcel (C) did not receive any phytochemical treatment (control). One application of the fungicide "Switch" (1.2 Kg/ha) was used 2 weeks before veraison; however, "Mikal" (2.5–3.0 Kg/ha) was applied three times, 3–4 weeks before veraison.

**Grape Samples.** Grape bunches were collected during three growth stages: (green berry (G)), (early veraison (V)), and (ripe berry or harvest time (H)) during the year 2001. The sampling protocol consists of taking 10 bunches from each vineyard and each stage along two crossing diagonals. Bunches were kept in sterile bags and after their rapid transport to the laboratory in refrigerated boxes, the mycological analyses were immediately performed. The remaining samples were frozen at −18 °C for subsequent analysis.

**Fungal Isolation and Identification.** The culture medium used for fungi isolation was DRBC (Dichloran Rose Bengale Chloramphenicol) agar (Oxoid, Basingstoke, Hampshire, England), which contained (per 1 L of distilled water) 10 g of glucose, 5 g of meat peptone (Fisher), 1 g of $KH_2PO_4$, 0.5 g of $MgSO_4.7H_2O$, 25 mg of rose bengal, 2 mg of dichloran, 100 mg of chloramphenicol, and 15 g of agar (Difco). For mycoflora determination, five berries were randomly chosen from each bunch and directly plated onto the surface of the DRBC in Petri dishes (90-mm diameter), and incubated at 25 °C for 7 days. Samples were examined daily with a stereomicroscope, and all *Aspergillus* and *Penicillium* species were reisolated on CZ medium (Czapek Agar, Oxoid), and purified for identification on CYA (Czapek Yeast extract agar). Solid medium CYA contained (per 1 L of distilled water): 30 g of sucrose, 5 g of yeast extract (Difco), 15 g of agar (Difco), 50 mL of solution A, which was composed of (per 0.5 L of distilled water) 20 g of $NaNO_3$, 5 g of KCl, 5 g of $MgSO_4.7H_2O$, and 0.1 g of $FeSO_4.7H_2O$; 50 mL of solution B, which was composed of (per 0.5 L of distilled water) 10 g of $K_2HPO_4$, and 1 mL of trace mineral oligo-elements. The trace mineral solution contained (per 0.1 L): 1 g of $ZnSO_4.7H_2O$ and 0.5 g of $CuSO_4.5H_2O$. The pH of the medium was adjusted to 6.7 prior to autoclaving. These cultures were incubated for 7 days at 25 °C. All fungi isolated were identified by morphological characters according to standard taxonomic systems based on the shape of conidiophores and conidia dimension observed with a binocular microscope with 100× magnification (*35, 36*). The *Aspergillus* species were classified into three groups: *A. japonicus* (uniseriate conidial head and conidia less than 5 $\mu$m in diameter), *A. niger* aggregate (biseriate conidial head and conidia less than 5 $\mu$m diameter), and *A. carbonarius* (biseriate conidial head and conidia more than 7 $\mu$m diameter). Also, several isolates initially identified by Dr. Zofia Lawrence of CABI Bioscience were used for comparison. All strains isolated were preserved as suspension of spores in 25% glycerol solution at −18 °C until use.

**Ability of Isolates to Produce OTA and NGPs.** OTA and NGP production ability of isolates was tested on CYA using the method of Bragulat et al. (*37*). The isolates belonging to *Aspergillus* and *Penicillium* genus were grown in Petri dishes containing CYA medium for 7 days at 25 °C. Three agar plugs (9-mm) were removed from different points of the colony (from the inner, middle, and outer area) for each culture, weighed, and collected into Eppendorf tubes. A volume of 1 mL of methanol was added to each tube, and the tubes were left stationary for 1 h. The Eppendorf tubes were centrifuged three times for 10 min at 13000 rpm, and the extracts obtained were filtered through a 0.45 $\mu$m Millipore filter into vials and then analyzed by HPLC-FLD (high performance liquid chromatography with fluorescence detector).

**High Performance Liquid Chromatography (HPLC).** OTA and NGPs were detected by reversed-phase HPLC. The analysis was performed using a Bio-Tek HPLC System (Milan, Italy) equipped with a solvent delivery system 525, column thermostat 582, auto-sampler 465 with 80 $\mu$L loop, diode array detector 545V, acquisition data Kroma System KS-3000 and Spectra System FL-3000 fluorescence detector with excitation wavelength set at ($\lambda_{ex}$ = 332 nm) and emission wavelength at ($\lambda_{em}$ = 466 nm), and UV detector ($\lambda_1$ = 220 and $\lambda_2$ = 330 nm); calibration with commercial OTA. The analytical column was a $C_{18}$ RP, Zorbax SB, 150 × 4.6 mm, 5 $\mu$m particle size, ODB Uptisphere fitted with a guard column (10 × 4 mm) having the same stationary phase. During analysis, the column temperature was maintained at 30 °C and the injection volume corresponds to 80 $\mu$L. For the first screening and to select strains suspected to produce OTA and/or different NGPs, an isocratic mobile phase mode consisting of a mixture of glacial acetic acid in water 0.2% (A) and acetonitrile (B): (56% (A) and 44% (B)), at a flow rate of 0.8 mL $min^{-1}$ during 10 min was used. Under these conditions OTA was eluted at 5.5 min, and all NGPs were eluted between 3.5 and 4.5 min. The confirmation of production of these mycotoxins (OTA and NGPs) was done with a linear gradient elution at a flow rate of 1 mL $min^{-1}$ over 45 min, starting

**Table 1.** Number of Collected Isolates (Number of OTA Producers, Number of NGPs Producers), Obtained from Each Grape Variety in Different Growth Stages[a]

| | | genera and species | | | | | |
|---|---|---|---|---|---|---|---|
| grape variety[b] | growth stage[c] | *A. carbonarius* | *A. niger* | *A. japonicus* | other *Aspergillus* spp. | *Penicillium* spp. | total |
| SA | G | 00 (00, 00) | 04 (00, 01) | 01 (00, 00) | 02 (00, 00) | 09 (00, 00) | 16 (00, 01) |
| | V | 06 (02, 01) | 11 (00, 01) | 00 (00, 00) | 00 (00, 00) | 03 (00, 00) | 20 (02, 02) |
| | H | 32 (04, 03) | 05 (00, 00) | 00 (00, 00) | 00 (00, 00) | 23 (00, 00) | 60 (04, 03) |
| MA | G | 00 (00, 00) | 08 (00, 00) | 02 (00, 00) | 00 (00, 00) | 05 (00, 00) | 15 (00, 00) |
| | V | 04 (04, 00) | 03 (00, 00) | 01 (00, 00) | 00 (00, 00) | 03 (00, 00) | 11 (04, 00) |
| | H | 67 (61, 05) | 22 (00, 03) | 01 (00, 00) | 00 (00, 00) | 00 (00, 00) | 90 (61, 08) |
| CA | G | 00 (00, 00) | 01 (00, 00) | 00 (00, 00) | 00 (00, 00) | 05 (00, 00) | 06 (00, 00) |
| | V | 01 (01, 00) | 13 (00, 00) | 12 (00, 00) | 00 (00, 00) | 05 (01, 00) | 31 (02, 00) |
| | H | 10 (09, 03) | 19 (00, 05) | 02 (00, 00) | 01 (00, 00) | 10 (01, 00) | 42 (10, 08) |
| CN | G | 00 (00, 00) | 02 (00, 00) | 01 (00, 00) | 01 (00, 00) | 00 (00, 00) | 04 (00, 00) |
| | V | 01 (00, 00) | 06 (00, 01) | 00 (00, 00) | 00 (00, 00) | 07 (00, 00) | 14 (00, 01) |
| | H | — | — | — | — | — | — |
| GN | G | 02 (00, 01) | 17 (00, 00) | 01 (00, 00) | 00 (00, 00) | 00 (00, 00) | 20 (00, 01) |
| | V | 00 (00, 00) | 00 (00, 00) | 19 (00, 00) | 00 (00, 00) | 00 (00, 00) | 19 (00, 00) |
| | H | 17 (11, 03) | 26 (00, 07) | 12 (00, 00) | 01 (01, 00) | 41 (03, 00) | 97 (15, 10) |
| RI | G | 00 (00, 00) | 00 (00, 00) | 00 (00, 00) | 00 (00, 00) | 03 (00, 00) | 03 (00, 00) |
| | V | — | — | — | — | — | — |
| | H | 01 (00, 00) | 00 (00, 00) | 00 (00, 00) | 00 (00, 00) | 00 (00, 00) | 01 (00, 00) |
| UB | G | — | — | — | — | — | — |
| | V | 00 (00, 00) | 02 (00, 00) | 00 (00, 00) | 00 (00, 00) | 03 (00, 00) | 05 (00, 00) |
| | H | 00 (00, 00) | 00 (00, 00) | 00 (00, 00) | 00 (00, 00) | 00 (00, 00) | 00 (00, 00) |
| A | G | 00 (00, 00) | 04 (00, 00) | 03 (00, 00) | 00 (00, 00) | 00 (00, 00) | 07 (00, 00) |
| | V | 03 (02, 01) | 03 (00, 00) | 16 (00, 00) | 01 (00, 00) | 37 (02, 00) | 60 (04, 01) |
| | H | 41 (41, 09) | 11 (01, 02) | 02 (00, 00) | 00 (00, 00) | 02 (00, 00) | 56 (42, 11) |
| B | G | 00 (00, 00) | 04 (00, 00) | 02 (00, 00) | 00 (00, 00) | 00 (00, 00) | 06 (00, 00) |
| | V | 00 (00, 00) | 02 (00, 00) | 00 (00, 00) | 01 (00, 00) | 04 (01, 00) | 07 (01, 00) |
| | H | 32 (29, 01) | 12 (00, 05) | 00 (00, 00) | 00 (00, 00) | 10 (04, 00) | 54 (33, 06) |
| C | G | 00 (00, 00) | 02 (00, 00) | 08 (00, 00) | 00 (00, 00) | 01 (00, 00) | 11 (00, 00) |
| | V | 00 (00, 00) | 03 (00, 00) | 00 (00, 00) | 00 (00, 00) | 08 (00, 00) | 11 (00, 00) |
| | H | 44 (42, 08) | 15 (03, 02) | 00 (00, 00) | 01 (00, 00) | 06 (00, 00) | 66 (45, 10) |

[a] —, Samples not taken. [b] SA, Sauvignon; MA, Muscat d'Alexandrie; CA, Carignan; CN, Cinsault; GN, Grenache noir; RI, Riesling; UB, Ugni Blanc; A, Syrah treated by "Mikal"; B, Syrah treated by "Switch"; C, Syrah non treated by any phytochemical agent (control). [c] G, Green berry; V, Early veraison; H, Ripe berry (harvest time).

from 10 to 50% of B over the first 30 min, followed by a linear gradient to 90% of B from 30 to 35 min, then remained at a steady flow of 90% of B for 8 min, finally reduced to 10% for 2 min. OTA was identified by the retention time according to a standard (Sigma Aldrich, Steinheim, Germany), and its retention time was about 28.5 min. The detection limit was 0.15 ng/mL. Furthermore, the confirmation of the presence of OTA was performed by total degradation of OTA by carboxypeptidase A (E.C.3.4.17.1) leading to α-ochratoxin formation (*38*). The analysis of different NGPs (fonsecin and aurasperones B-G) was done by using the same HPLC system during 45 min. For the detection of NGP derivatives, the crude extract was analyzed by reversed-phase $C_{18}$ and performed using a detector at wavelength 354 nm. The elution was at a flow rate of 1 mL/min with acetonitrile/acetic acid in water (0.2%). The separation linear gradient starting with 30 to 100% acetonitrile for 30 min and then remained at a steady state for 15 min. The retention times were recorded at 17.96 (for fonsecin), 24.28 (for aurasperones C and D), 24.90 (for aurasperone G), 25.30 (for aurasperone F), 27.44 (for aurasperone B) and 28.12 min (for aurasperone E). Moreover, all UV spectra of the known NGPs are very similar to those of aurasperone G. The UV–vis spectra of the NGPs showed absorption maxima at 232, 274, 310, 334, and 406 nm (for fonsecin); 206, 283, 336, and 404 nm (for aurasperone C); 206, 283, 312, 336, and 404 nm (for aurasperone D); 213, 281, 320, 334 and 406 nm (for aurasperone F); 207, 282, 330, and 402 nm (for aurasperone B); and 209, 281, 330, and 403 nm (for aurasperone E).

**Culture Conditions and Extraction of Aurasperone G.** Living spores of the *A. niger* (isolate C-433) were obtained from mycelium grown on CYA medium, at 25 °C, aged 7 days. A suspension of spores was prepared in sterile distilled water containing 0.1% Tween (Tween type 80, Fisher). A Thoma chamber was used to determine final spore concentration (about $10^7$ spores/mL). Before use, spores were carefully washed with physiological water to remove any contaminants or inhibitors of germination. Stock cultures were maintained at −18 °C in a final concentration of 25% glycerol. The suspension of fungus spores (5 mL) was used to inoculate 4 × 1 L Roux bottles, each containing 300 mL of sterile CYA liquid medium. After 10 days of incubation at 25 °C, the mycelium and culture filtrate were extracted with methanol and evaporated to dryness under vacuum at 45 °C to give a black residue (8.1 g).

**Purification of Aurasperone G, and Experimental Procedure.** The purification of aurasperone G was performed on a $C_{18}$ Uptisphere UP15WOD preparative column, φ 7.8 × 300 mm i.d., Interchim, 15 μm. UV–vis spectra were measured in MeOH at 25 °C with a Beckman DU-65 diode array spectrophotometer. The separation linear gradient starting with 30–100% acetonitrile for 30 min and then remained at a steady state for 15 min. Mass spectra (nano-ESI-MS) were recorded on Finnigan LCQ spectrometer equipped with an electrospray ion source (positive and negative ion mode), the scan-range was from 100 to 1000 *m/z*. NMR sample was prepared by dissolving 1.1 mg of compound **1** in 600 μL DMSO-$d_6$. All spectra were recorded on a Bruker AMX400 spectrometer equipped with a 5 mm triple resonance inverse probe operating at 400.13 MHz for $^1$H and 100.61 MHz for $^{13}$C. All chemical shifts for $^1$H and $^{13}$C are relative to TMS using $^1$H (residual) or $^{13}$C chemical shifts of the solvent as a secondary standard. The temperature was set at 298 K in DMSO. The structure of aurasperone G was studied using $^1$H-$^1$H COSY45, $^1$H-$^{13}$C HMQC and $^1$H-$^{13}$C HMBC.

**Aurasperone G (1)** yellow powder (1.1 mg); UV (MeOH) $\lambda_{max}$ 213, 281, 315, 332, 403 nm; $^1$H- and $^{13}$C-NMR data (DMSO-$d_6$), see **Table 2**; nano-ESI-MS/MS (positive-ion mode) *m/z* 589 $[M + H]^+$ (90), 531 (100), 490 (50), 473 (40), 505 (38), 406 (20); nano-ESI-MS/MS (negative-ion mode) *m/z* 587 $[M - H]^-$ (15), 489 (100), 558 (20), 474 (15), 555 (10), 405 (5); HREIMS *m/z* 588.1284 (calcd for $C_{31}H_{24}O_{12}$, 588.1267); HPLC $R_t$ = 24.9 min.

## RESULTS

**Total Fungi Isolated from Grapes.** During 2001, *Aspergillus* sp. and *Penicillium* sp. on French grape berries were isolated; and a total of 732 fungal isolates were obtained and identified

**Table 2.** $^{1}$H- and $^{13}$C-NMR Data for Aurasperone G (**1**) and Aurasperone F (**2**) in DMSO-$d_6$ at 298K ($\delta$ in ppm, $J$ in Hz) [a]

| | aurasperone F | | aurasperone G aurasperone G | | |
|---|---|---|---|---|---|
| Position | $^{13}$C-NMR | $^{1}$H-NMR | $^{13}$C-NMR | $^{1}$H-NMR | HMBC |
| 2 | 169.4 | | 169.1 | | H-3, $CH_3$-2 |
| 3 | 107.6 | 6.21 (1H, s) | 107.0 | 6.21 (1H, s) | $CH_3$-2 |
| 4 | 184.9 | | nd | | |
| 4a | 104.2 | | 103.8 | | H-3 |
| 5 | 162.8 | | nd | | |
| 5a | 108.4 | | 108.4 | | H-7 |
| 6 | 161.6 | | 161.3 | | $OCH_3$-6 |
| 7 | 97.3 | 6.54 (1H, d, 2.0) | 96.8 | 6.55 (1H, d, 2.0) | |
| 8 | 162.0 | | 161.7 | | $OCH_3$-8 |
| 9 | 97.4 | 6.18 (1H, d, 2.0) | 97.1 | 6.16 (1H, d, 2.0) | |
| 9a | 141.0 | | nd | | |
| 10 | 106.4 | | nd | | |
| 10a | 151.1 | | nd | | |
| $CH_3$-2 | 21.0 | 2.15 (3H, s) | 20.8 | 2.15 (3H, s) | |
| OH-5 | | 15.10 (1H, br s) | | 15.10 (1H, br s) | |
| $OCH_3$-6 | 56.9 | 3.93 (3H, s) | 56.1 | 3.93 (3H, s) | |
| $OCH_3$-8 | 56.0 | 3.59 (3H, s) | 55.0 | 3.58 (3H, s) | |
| 2′ | 101.1 | | 169.2 | | H-3′, $CH_3$-2′ |
| 3′ | 48.6 | 2.79/3.24 (2H, AB system, 16.8) | 107.3 | 6.20 (1H, s) | $CH_3$-2′ |
| 4′ | 199.3 | | nd | | |
| 4a′ | 103.9 | | nd | | |
| 5′ | 163.9 | | nd | | |
| 5a′ | 109.4 | | nd | | |
| 6′ | 159.9 | | 158.8 | | $OCH_3$-6′ |
| 7′ | 116.2 | | nd | | |
| 8′ | 160.0 | | nd | | |
| 9′ | 106.4 | 6.93 (1H, s) | nd | | |
| 9a′ | 143.1 | | nd | | |
| 10′ | 102.3 | 6.64 (1H, s) | nd | | |
| 10a′ | 154.3 | | nd | | |
| $CH_3$-2′ | 28.4 | 1.66 (3H, s) | 20.8 | 2.42 (3H, s) | |
| OH-2′ | | 7.11 (1H, s) | | | |
| OH-5′ | | 14.29 (1H, br s) | | 15.00 (1H, s) | |
| $OCH_3$-6′ | 62.2 | 3.34 (3H, s) | 62.1 | 3.36 (3H, s) | |
| OH-8′ | | 10.20 (1H, br s) | | 10.10 (1H, br s) | |
| OH-9′ | | | | 7.22 (1H, br s)[b] | |
| OH-10′ | | | | 7.07 (1H, br s)[b] | |

[a] See **Figure 1** for numbering of H and C atoms, s = singlet, d = doublet, nd = not detected. [b] signals may be interchangeable.

(**Table 1**). *Penicillium* spp. represented only 185 isolates (25.27%), and the species included *P. expansum*, *P. crysogenum*, *P. spinulosum*, *P. oxalicum*, and *P. citrinum*, with other nonidentified *Penicillium* spp. However, the genus *Aspergillus* was predominant: 547 isolates (74.72%), and especially those belonging to the section *Nigri* (black aspergilli) (98.53%). Black aspergilli were represented by three species: the biseriate species (*A. carbonarius* (48.42%) and *A. niger* aggregates (36.17%)) and uniseriate species (*A. japonicus*) (15.39%). *A. carbonarius* was microscopically recognized and distinguished by conidial size (7–10 $\mu$m) and ornamentation. All the other black biseriate aspergilli isolates will be referred as *A. niger* aggregate (conidial size from 3 to 5 $\mu$m). Among the remaining *Aspergillus* spp., we isolated four strains of *A. flavus*, 2 of *A. fumigatus*, 1 of *A. parasiticus*, and 1 of *A. sclerotiorum*.

Indeed, the numbers of *A. carbonarius* and *A. niger* aggregate isolates increased from early veraison (15 and 43 isolates) to maturity stage (244 and 110 isolates), respectively. The number of *A. niger* aggregate at veraison was more than the number of *A. carbonarius* isolates. Meanwhile, the opposite situation is found at the maturation stage. Consequently, the increase of the total number of *Aspergillus* section *Nigri* during the maturation was due to the increasing number of *A. carbonarius* on the grapes. On the other hand, we observed that the total number of fungal isolates increased with the use of the antifungal treatment "Mikal" (123 isolates) but were decreased by using "Switch" (67 isolates), by comparison to the control (88 isolates). Also, we observed that, the number of ochratoxigenic isolates decreased with the use of "Switch" (34 isolates), by comparison to the control (45 isolates). In contrast, the use of "Mikal" cannot reduce the number of OTA-producing isolates (**Table 1**).

**Mycotoxigenic Capacity of Fungal Isolates.** For all isolates, the capacity for producing OTA and NGP derivatives was determined on a solid laboratory medium (CYA) after 7 days at 25 °C. OTA was analyzed and ochratoxigenic potential was expressed as $\mu$g g$^{-1}$ CYA. We observed that 30.46% of the total fungal isolates were ochratoxigenic (206 isolates of *A. carbonarius*, four isolates of *A. niger* aggregate, 12 isolates of *Penicillium* sp. and one isolate of *A. sclerotiorum*). For 261 isolates of *A. carbonarius*, OTA producers represented more than 78%. In contrast, only 2% of *A. niger* aggregate isolates were ochratoxigenic, and no OTA production by *A. japonicus* isolates was detected. Among 206 isolates of *A. carbonarius* which produce OTA, capacity for producing OTA was ranged from 3 to 43 $\mu$g/g CYA, with a maximum of 43.15 $\mu$g/g CYA produced by a strain (*A. carbonarius*: GN738) isolated from the "Grenache noire" variety. By contrast, all ochratoxigenic isolates of *A. niger* never exceeded 0.35 $\mu$g/g of OTA. Furthermore, the numbers of *A. carbonarius* isolates producing OTA increased from early veraison (nine isolates) to maturity stage (197 isolates). The study of development of ochratoxigenic

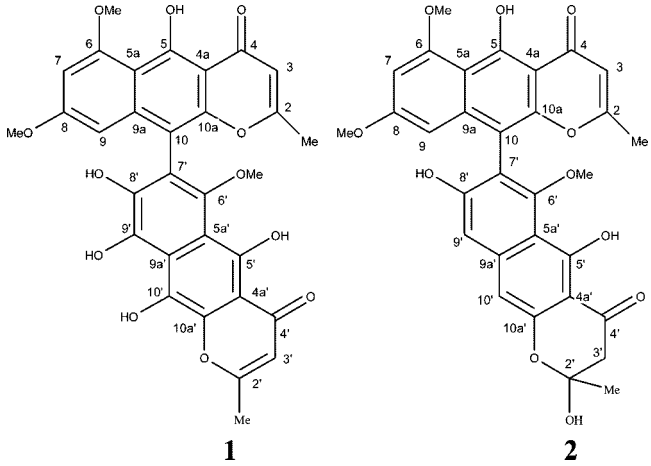

**Figure 1.** Chemical structures of aurasperone G (**1**) and aurasperone F (**2**).

mycobiota of grapes, showed that at the green berry stage, no OTA producing isolates were found. In early veraison, a few OTA producer fungi were recovered and were predominantly *A. carbonarius*. Interestingly, at harvest time, the fungal contamination highly increased.

Moreover, only 8.33% of the total fungal isolates were producers of NGPs (35 isolates of *A. carbonarius* and 27 isolates of *A. niger* aggregate). In contrast, no production by *Penicillium* spp. and other *Aspergillus* spp. was detected. The capacity for producing NGPs was ranged from 1 to 13 $\mu$g/g CYA, with a maximum of 13.15 $\mu$g/g CYA produced by a strain (*A. niger*: C537) isolated from the "Syrah" variety/parcel C.

Strain C-433 was collected from the vineyards of Languedoc-Roussillon region (France) in September 2001. This strain was isolated during the maturation stage from grapes (Syrah variety) nontreated by any phytochemical agents. This strain (C-433) was characterized and identified as *A. niger* van Tieghem Eutotiales anamorph, Ascomycetes. *A. niger* belongs to subgenus *Circumdati*, section *Nigri* (black aspergilli). The strain C-433 was isolated as producer of OTA.

**Characterization of New Derivative of NGPs (Aurasperone G).** The mass spectrum of aurasperone G **(1)** exhibited a molecular ion at *m/z* 587 $[M - H]^-$ by analysis of the negative mode, and a molecular ion at *m/z* 589 $[M + H]^+$ by analysis of the positive mode nano-ESI-MS. The molecular formula of $C_{31}H_{24}O_{12}$ is further confirmed by HREIMS peak matching of molecular ion peaks exhibited by ESI-MS. Aurasperone G **(1)** belongs to the naphtho-pyrone class containing a NGP nucleus as a characteristic structural element. The HPLC retention time was 24.9 min for **1**, and the yield obtained per liter of fermentation broth was 1.4 mg. The UV spectrum of compound **1** ($\lambda_{max}$: 213, 280, 315, 332 and 404 nm (in MeOH)) exhibits the same characteristics to those of aurasperone F (**2**) as described by Bouras et al. (*29*). The structure of 1 was proposed by spectral interpretation as 5,8,9,10,5′-pentahydroxy-6,6′,8′-trimethoxy-2,2′-dimethyl-[7,10′-Bi-4H-naphtho[2,3-b]pyran]-4,4′-dione (**Figure 1**), together with the known compound: aurasperone F (**2**), identified by comparison with the literature spectral analysis (*29*). In the present study, $^1$H and $^{13}$C NMR spectroscopy were used for the characterization of the compound **1**. All the $^1$H and $^{13}$C signals were assigned on the basis of chemical shifts ($\delta$), spin–spin coupling constants (*J*), splitting patterns and signal intensities, and by using $^1$H-$^1$H COSY45, $^1$H-$^{13}$C HMQC, and $^1$H-$^{13}$C HMBC experiments. The $^1$H and $^{13}$C chemical shifts of compound **1** are given in **Table 2**. Due to the low amount available, a complete set of $^{13}$C NMR data could not be obtained for the compound **1**. The $^1$H NMR spectrum revealed two metacoupled aromatic protons ($\delta_H$ 6.55 and 6.16, 2H, $J = 2.0$ Hz), two olefinic protons ($\delta_H$ 6.21 and 6.20, 2H, s), two hydrogen-bonded protons ($\delta$15.10 and 15.0, 2H, s), three methoxy groups ($\delta_H$ 3.93, 3.58, and 3.36, 9H, s), three phenolic hydroxyl protons ($\delta_H$ 10.1, 7.22, and 7.07, 3H, br s), and two methyl groups ($\delta_H$ 2.42 and 2.15, 6H, s). From the HMQC and HMBC spectra, it was possible to discern several sp$^2$-hybridized carbons ($\delta_C$ from 169.4 to 97.3), three methoxy groups ($\delta_C$ 62.2, 56.9, and 56.0) and two methyl groups ($\delta_C$ 28.4 and 21.0). The phenolic hydroxyl protons are very broad, and so many HMBC correlations were missing particularly for quaternary carbons which prevented the complete NMR attribution of compound **1**. However, NMR data in DMSO at 298K showed that compound **1** and aurasperone F are closely related. The structure of compound **1** was thus established through comparison with NMR data obtained for aurasperone F (**Table 2**). Differences between compound **1** and aurasperone F were localized on one of the NGP. The NMR signals of aromatic protons H9′ ($\delta_H$ 6.93) and H10′ ($\delta_H$ 6.64) of aurasperone F were not observed for compound **1** and were most probably replaced by hydroxyl groups (broad signals detected at $\delta_H$ 7.22 and 7.07). Furthermore, methylene signals ($\delta_H$ 2.79/3.24, $\delta_C$ 48.6) of aurasperone F in position 3′ were not observed and were replaced by olefinic signals ($\delta_H$ 6.20, $\delta_C$ 107.3) for compound **1**. The methyl protons $CH_3$-2′ were also shifted from $\delta_H$ 1.66 in aurasperone F to $\delta_H$ 2.42 in compound **1** confirming the presence of the sp$^2$-hybridized carbons C2′ and C3′. This report describes the isolation and structural elucidation of aurasperone G (**1**), which proved to be a novel NGP octaketide.

## DISCUSSION

Fungal growth is one of main causes of food spoilage. It not only generates great economic losses, but also represents a hazard to human and animal health, particularly through the synthesis of mycotoxins. Production of secondary metabolites is not essential to the synthesizing organism but it is regulated by several often interwoven environmental signals (*39*). Numerous studies have shown that molds can be found on grapes from veraison and sometimes even as soon setting. Note that the mold development increases rapidly between veraison and maturation. These results are similar to those found in other studies in France (*40*), Italy (*41*), and Spain (*42*).

In French grapes, we observed the predominance of *Aspergillus* compared to *Penicillium*. Moreover, these genera were frequent in all development stages for all studied vineyards. It is known that *Aspergillus* species are commonly associated with warmer and tropical regions, whereas *Penicillium* species appear more often in temperate and cold climates such as in Northern Europe (*36*, *43*). The dominance of black aspergilli on grapes at the end of maturation has been reported in many others surveys (*40*, *42*, *44*).

Based on the morphology of the conidial head and the shape and the size of conidia, identification to species level was carried out for all *Aspergillus* strains. *Aspergillus* species belonging to the section *Nigri* can cause considerable damage on the yield and the quality of the harvest. Several studies on wine and grape juice (*40*, *41*), but also on dried vine fruit showed that the occurrence of OTA was related to the grape contamination in the vineyard by several OTA-producing species of fungi, especially the black aspergilli, mainly *A. carbonarius* and the members of the *A. niger* aggregates (*7*, *41*, *42*, *44*). Black aspergilli have been reported as the predominant mycobiota of grapes. Our results showed clearly that *A. carbonarius* isolates is the species mainly responsible of OTA contamination of grape berries.

During the growing season, the harvest time was considered as a potential period for fungal development and OTA production. This was also reported by other researchers on Portuguese (*44*), Spanish (*42*) and French grapes (*40*). Acidity decrease, sugar accumulation and cuticle embrittlement in grapes may explain this phenomenon. Although commonly reported in cereal and its derivatives as principal OTA producers (*45, 46*), *A. ochraceus* and *P. verrucosum* (and also by the closely related species *P. nordicum*) have never been isolated on French grapes. In similar studies in other countries, the same results were obtained (*37, 41*).

The phytochemical agent "Switch" could be effective against *Aspergillus* spp. in the field. This observation coincides with that of Tjamos et al. (*47*), who reported that chemical applications with the fungicide "Switch", especially under low to intermediate *Aspergillus* infection of vineyards, could both significantly restrict sour rot severity and reduce the occurrence of OTA-producing *Aspergillus* spp.. In contrast, we found that the application of the fungicide "Mikal" seems to be ineffective in controlling either the number of total fungal isolates or the number of ochratoxigenic isolates. The optimization of concentration and timing of fungicide application was necessary. The nonoptimal (or suboptimal) use of fungicide can reduce the number of fungi but increase mycotoxin production by remaining isolates. Further studies on the stress induced by nonoptimal use of fungicide are needed.

The characterized new molecule aurasperone G **(1)** belonging to naphtho-pyrone class contains a NGP nucleus as a characteristic structural element. The structure of aurasperone G was proposed as 5,8,9,10,5′-pentahydroxy-6,6′,8′-trimethoxy-2,2′-dimethyl-[7,10′-Bi-4H-naphtho[2,3-b]pyran]-4,4′-dione. This new dimeric metabolite was not reported before in nature or prepared synthetically. This compound belongs to the polyketide group and can be formulated as biosynthetically derived from monomeric intermediates by phenol oxidative coupling. Monomeric and dimeric (or bis) naphtho-pyrones are widespread in nature as yellow pigments. These compounds have been isolated from a variety of filamentous fungi (*Aspergillus*, *Cephalosporium*, *Chaetomium*, *Fusarium*, *Penicillium*, and *Trichoderma*) (*2, 19, 29*). Some of them are alpha-pyrone derivatives and most others are gamma-pyrone derivatives (*19*). NGPs have been found in a wide variety of filamentous fungi (*Deuteromycetes*) which belong to the genera *Fusarium* (*F. culmorum*, *F. gramineum* (*8, 9*)), *Aspergillus* (*A. fonsecaus* (*8, 10*), *A. awamori* (*31, 48*), *A. niger* (*2, 11, 22, 24, 26, 27, 29, 30*), *A. vadensis* (*49*), *A. foetidus* (*49*), *A. tubingensis* (*49*), *A. carbonarius* (*49*), *A. brasiliensis* (*33*), *A. costaricaensis* (*33*), *A. piperis* (*33*), *A. sclerotioniger* (*33*)), *Chaetomium* (*C. thielavioideum* (*19*)), *Acremonium butyri* (*21*), and *Claviceps virens* (anamorph: *Ustilaginoidea virens*) (*28*). The NGP derivatives were found to be produced consistently by many strains of *A. niger*, and also by some isolates of *A. carbonarius* isolated in our study. These compounds have a dual characteristics either beneficial, which could be used for pharmacological studies as inhibitors of HIV-1 Integrase (*9*), antibacterial (*12*), antifungal (*13*), or harmful, which could exhibit a serious health hazard to animals and people who might consume contaminated foodstuffs (*14, 26, 27*). Further study is needed to research the possibility of aurasperone G to exhibit other biological activities (antimicrobial, antitumor, antiviral, etc.).

To prevent mycotoxins in foodstuffs, it is necessary to have a rapid and specific method to detect the producing fungi early. Usual identification and quantification methods of food-borne fungi require multiple steps. Morphological and physiological tests were time-consuming and often, mycological expertise was necessary. Nowadays, many chemotaxonomic studies have reported giving more specific, sensitive, and rapid detection. In a large chemotaxonomic investigation of NGPs producing black aspergilli, also including eight isolates of other *Aspergillus* spp. and 185 isolates of *Penicillium* spp., the NGP derivatives are only produced by *A. niger* and *A. carbonarius*, but never by isolates of other *Aspergillus* spp. or *Penicillium* spp.. In addition, many isolates of *A. carbonarius* produced three unknown naphtho-pyrones which are defiantly different from those produced by *A. niger* isolates. Interestingly, the study revealed that only two NGPs producing isolates (C-433 and A232) seem to produce OTA. Furthermore, the new polyketide aurasperone G was produced only by four isolates of *A. niger* (C-433, B935, A432, and MAX44), but never by other isolates or species. The isolate C-433 is a producer of both OTA and NGPs, and this gives us some insight about the relationship between the metabolic pathway of these mycotoxins (both OTA and NGPs are polyketides). According to these findings, we can conclude that *A. niger* C-433 is an important isolate and may be useful in other studies on secondary metabolite pathways. In our study, we observed that OTA is only produced by *A. carbonarius*, *A. niger* aggregate, *A. sclerotiorum*, and also by some strains of *Penicillium* spp., but never by isolates of other *Aspergillus* spp. such as *A. japonicus*, *A. flavus*, *A. fumigatus*, or *A. parasiticus*. Interestingly, many strains isolated from grapes produced a large number of unknown metabolites (*2*). Importantly, all isolates of *A. niger* produce nigragillin, ($R_t$ of 6 min, UV $\lambda_{max}$: 262 nm), and never by other species. Furthermore, some isolates of *A. niger* produced unknown pentaene ($R_t = 36.5$, MW = 538, UV spectrum: $\lambda_{max}$/nm (relative absorbance) 225 (0.17), 310 (0.30), 320 (0.60), 335 (0.98), 353 (1.00)]). Moreover, all *A. japonicus* isolates produced an unknown compound with a $R_t$ of 38 min as a major secondary metabolite produced by this species. Moreover, among all *A. japonicus* tested, we never observed the production of OTA and/or NGPs by any strain of this species under used conditions. Seventeen isolates of *A. japonicus* produced a metabolite which present a similar UV spectra with neoxaline. The results of this study point out that principally black aspergilli are the predominant mycobiota of grapes, where *A. niger* and *A. carbonarius* are considered as the main OTA and NGPs producers that can play a major role in the contamination of grapes and its derivatives and may constitute a risk to human health.

The obtained results would allow the establishment of an adequate program of treatment in the vineyards. This would prevent or limit the fungal contaminations and contribute to getting a harvest with good quality, especially regarding the presence of mycotoxins such as NGPs and OTA in grape berries and their derivatives. More studies are needed to evaluate the presence of different derivatives of NGPs in grapes, must (grape juice), and wine. On the other hand, *A. niger* is the most frequently reported species in the group of black aspergilli used as a production microorganism in industrial fermentations for the production of various substances. This species has often been included in biotechnological processes to produce numerous extracellular enzymes (α-amylase, cellulase, amyloglucosidase, catalase, glucose oxidase, lipase, and pectinase) and citric acid, for the food industry, that are GRAS (generally regarded as safe) by the United States Food and Drug Administration. Interestingly, production of toxic secondary metabolites in *A. niger* is strain-specific and environmental-dependent. New and unknown isolates of black aspergilli should be checked for production of NGPs and OTA, but also for other mycotoxins, before they are developed as production organisms.

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