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Note

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Guaiane Sesquiterpenes from *Biscogniauxia nummularia* Featuring Potent Antigerminative Activity

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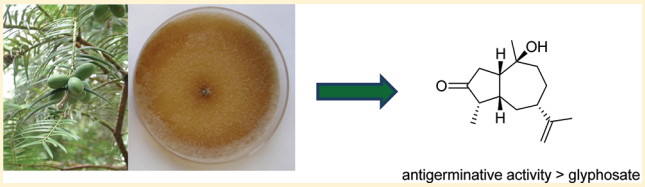
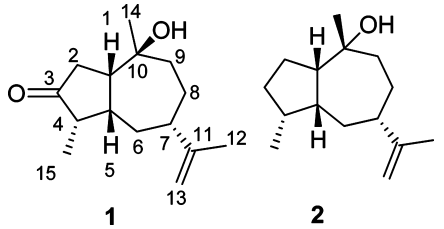
Supporting Information

ABSTRACT: Xylaranone, a previously unreported guaiane sesquiterpene along with the known terpenoid xylaranol B and the two mellein derivatives 3,5-dimethyl-8-methoxy-3,4-dihydroisocoumarin and 3,5-dimethyl-8-hydroxy-3,4-dihydroisocoumarin were isolated from *Biscogniauxia nummularia*. Pogostol was also isolated from this fungus, and in light of our spectroscopic data, its structure was revised and corrected. This fungus, which was isolated as an endophyte from the plum yew *Cephalotaxus harringtonia*, is also suspected of being a pathogen. Interestingly, we report here the potent antigerminative activity of xylaranone and xylaranol B against seeds of *Raphanus sativus* at concentrations comparable to glyphosate, a commonly used herbicide. This effect suggests a role for these metabolites in the latent fungal pathogenesis of *B. nummularia*.



In the course of a program on the structure determination and ecological studies of metabolites from endophytic fungi, we are currently working on *Biscogniauxia nummularia* (Bull.) Kuntze (6E2a). This is an apparent endophytic fungus isolated from the plum yew *Cephalotaxus harringtonia* (Siebold & Zucc.) Koidz. and identified by the species-level molecular marker of fungi, the ITS rDNA sequences. *Biscogniauxia* belongs to the xylariaceous genus represented in Europe by 10 known species growing on the bark of trees and shrubs, preferably on dead or dying branches and more rarely on trunks. It is currently suspected of being pathogenic to certain trees.[1] Although it is usually accepted that endophytes can have profound effects on plant ecology, fitness, and evolution, some examples attest that under certain conditions endophytes act as parasites and cause disease or reduce the fitness of their host plants.[2,3] Indeed, endophytes and pathogens share some virulence factors such as phytotoxic mycotoxins.[4] For example, *Biscogniauxia mediterranea* (De Not.) Kuntz, an endophytic fungus widespread in Sardinian oak forests, is considered as the main causes of cork oak (*Quercus suber* L.) decline. From this fungus, Evidente et al. have isolated and characterized a pyrano derivative, biscopyran, as the main phytotoxic metabolite.[5] Recently, *B. nummularia* has been reconsidered as a pathogenic agent of the European beech (*Fagus sylvatica* L.) in Sicily and Calabria (Italy).[6] Nevertheless, the role of secondary metabolites produced by this ascomycete had not yet been studied with regard to their role in plant decay.

We report here the isolation and characterization, based on extensive 2D-heteronuclear NMR studies, of the new guaiane sesquiterpene **1** along with a previously known terpenoid, xylaranol B,[7] and two mellein derivatives, 3,5-dimethyl-8-hydroxy-3,4-dihydroisocoumarin (5-methymellein)[8] and 3,5-dimethyl-8-methoxy-3,4-dihydroisocoumarin (8-methoxy-5-methylmellein).[9] Moreover, the terpenoid previously reported as pogostol[10−12] was also isolated. In view of our analytical data, we suggest the revised structure **2** for this compound. Moreover, remarkable antigerminative activity against the seeds of *Raphanus sativus* L. was noted for compound **1** and xylaranol B. Such an effect, which is comparable to the herbicide glyphosate, could explain the role of these metabolites in the suspected phytopathogenesis of *B. nummularia*.



Compound **1** was obtained as a colorless oil. Its HRESIMS showed the protonated molecular ion $[M + H]^+$ corresponding to the formula $C_{15}H_{24}O_2$, which implies four degrees of unsaturation. The IR spectrum exhibited absorption bands at ν_{max} 3421 and 1732 cm^{-1} indicative of the presence respectively of a hydroxy and a carbonyl group. The ^{13}C *J*-modulated NMR spectrum (CD_3OD) exhibited the resonances of 15 carbons including three methyls, five methylenes including an sp^2 (=CH_2) at δ_C 108.6, four methines, three quaternary carbons

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including a carbonyl at δ_C 223.8, and an ethylenic carbon at δ_C 153.3 (Table 1). Taking into account the four degrees of unsaturation, the molecule **1** should include two rings. The ^{1}H NMR spectrum displayed typical signals of three methyls, two tertiary (δ_H 1.73, s, H-12 and δ_H 1.15, s, H-14) and one secondary (δ_H 1.02, d, H-15), and an olefinic methylene (δ_H 4.62 and 4.70, m, H-13). The ^{1}H−^{1}H COSY spectrum (CD_3OD) identifies three main substructures, which are represented in Figure 1. The HMBC correlations were indicative of a cyclopentanone ring moiety. Methines H-1 (δ_H 2.57) and H-4 (δ_H 2.01), methylene H-2 (δ_H 2.72 and 2.32), and methyl CH_3-15 (δ_H 1.02) were correlated with carbonyl C-3 at δ_C 223.8. The chemical shift of the quaternary carbon (C-10) at δ_C 75.3 indicated that it was linked to an oxygen. Moreover, it was correlated to the two methines H-1 (δ_H 2.57) and H-5 (δ_H 2.13), the methylenes H-9 (δ_H 1.94; 1.70) and H-2, and the methyl group CH_3-14 at δ_H 1.15. These HMBC correlations with those observed in the ^{1}H−^{1}H COSY allowed the construction of a seven-membered ring. Finally, the olefinic moiety was linked to C-7 because of the correlation between the methine H-7 (δ_H 2.20) and the C-11 at δ_C 153.3. The relative stereochemical assignments of carbons C-1, C-4, C-5, C-7, and C-10 were resolved using the ^{1}H−^{1}H NOESY experiments in CD_3OD and C_5D_5N (Figure 1). These displayed correlations in CD_3OD between H-1 and H-5, indicating a cis ring fusion. Moreover, the spatial correlations of CH_3-14 (δ_H 1.15) with H8-b (δ_H 1.56) and H9-a (δ_H 1.94) clearly indicated that they are on the same face of the molecule. On the other hand, the correlations of H-1 with H-9b (δ_H 1.70) suggested that CH_3-14 and H-1 are placed on opposite faces. Moreover, in C_5D_5N, a correlation between the hydroxy (δ_H 5.74) on C-10 and H-1 confirmed that CH_3-14 and H-1 are on opposite sides. In addition, H-7 (δ_H 2.20) showed a cross-peak with H-8a (δ_H 1.80) and H-9b (δ_H 1.70). These results thereby placed the C-7 isoprenyl group on the α-face. Similarly, NOE correlations between H-4 and H-1 indicated that the methyl CH_3-15 is also on this face. Consequently, the structure of **1** was assigned as depicted and named xylaranone. It is noteworthy that a trans-fused isomer of **1** was synthesized by Piers et al. as an intermediate in the stereoselective synthesis of α-bulnesene.[13]

Table 1. NMR Spectroscopic (CD_3OD and C_5D_5N, 298 K, ^{1}H 400.13 MHz; ^{13}C 75.04 MHz) Data for Xylaranone (1)

	1 in CD_3OD		**1** in C_5D_5N	
position	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	48.0, CH	2.57, ddd (9.7, 4.1)	47.4, CH	2.69, ddd (4.3, 7.3, 9.7)
2a	40.8, CH_2	2.72, ddd (1.9, 4.1, 19.7)	40.3, CH_2	3.14,ddd (1.9, 4.3, 19.6)
2b		2.32, dd (9.7, 19.7)		2.47, dd (9.7, 19.6)
3	223.8, qC		220.8, qC	
4	51.1, CH	2.01, qd (6.5, 1.8)	49.5, CH	1.99, m
5	46.8, CH	2.13, m	45.6, CH	2.15, m
6a	39.5, CH_2	1.99, m	38.3, CH_2	1.94, m
6b		1.52, m		1.47, m
7	46.4, CH	2.20, m	45.3, CH	2.28, m
8a	33.4, CH_2	1.80, m	32.5, CH_2	1.83, m
8b		1.56, m		1.51, m
9a	45.7, CH_2	1.94, ddd (3.8, 4.9)	45.2, CH_2	2.18, ddd (3.9, 4.9)
9b		1.70, m		1.86, m
10	75.3, qC		73.8, qC	
11	153.3, qC		152.4, qC	
12	20.3, CH_3	1.73, s	19.9, CH_3	1.73, s
13a	108.6, CH_2	4.70, qd (1.5)	108.5, CH_2	4.80, qd (1.5)
13b		4.62, br, s		4.72, br, s
14	23.9, CH_3	1.15, s	24.5, CH_3	1.27, s
15	14.1, CH_3	1.02, d (6.5)	14.1, CH_3	1.06, d (6.4)
OH (10)				5.74

The structure of **2** was unambiguously assigned as pogostol by comparison of its spectroscopic data (Supporting Information Table 1) with the literature. Indeed, Hikino et al. previously described pogostol isolated from patchouli *Pogostenum cablin* Benthum in 1968.[10] Pogostol was reisolated from *Alpinia japonica* (Thunb.) Miq.[11] The relative stereochemistry of the pogostol *O*-methyl ether was first suggested by Fleischer et al.[14] and then by Weyerstahl et al. for pogostol.[12] Nevertheless, the racemic synthesis of kessane using a route that also led to the preparation of the reported structure of pogostol was achieved by Booker-Milburn et al.[15] Comparison of the NMR data of the synthetic pogostol with naturally occurring pogostol revealed misassignment in the original structure. The physical data from our compound **2** were identical to those described by Weyerstahl et al. Moreover our extensive 2D-NMR analysis confirmed the bicyclic structure, featuring a bicyclo[5.0.3]decane (Figure 2). The NOE spectra were the basis of the relative configuration assignment of the structure of **2**. Indeed, in C_5D_5N, clear NOE correlations of H-1 were observed with H-4 and H-5, indicating that they are on

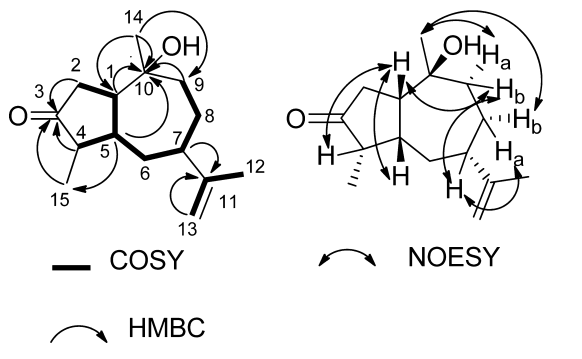

Figure 1. Key ^{1}H−^{1}H COSY, ^{1}H−^{13}C HMBC, and ^{1}H−^{1}H NOESY correlations for compound **1**.

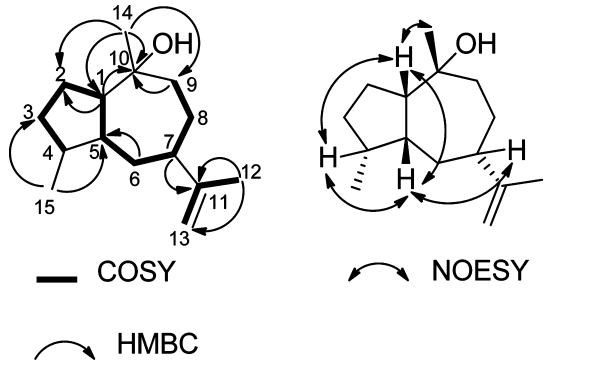

Figure 2. Key ^{1}H−^{1}H COSY, HMBC, and NOESY correlations for compound **2**.

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the same side and that the ring fusion is cis (Figure 2). Similarly, NOE correlations of CH_3-14 (δ_H 1.33) with H-1 (δ_H 2.37) determined their orientation on the same face of the molecule. This was confirmed by the cross-peak between H-4 (δ_H 1.95) and H-5 (δ_H 2.20). Moreover, the NOE correlations of H-5 with H-7 (δ_H 2.68) suggested that proton H-7 is also on the same face. The structure of pogostol **2** was thus determined as depicted in Figure 2.

To investigate the supposed phytopathogenesis of *B. nummularia*, all compounds, except for 8-methoxy-5-methylmellein, which was available only in a small amount, were assayed for their antigerminative activity. They were tested at the maximum concentration of 100 mg/mL, the effective concentration of glyphosate, the active constituent of commonly used weedkillers. As shown in Figure 3, compound

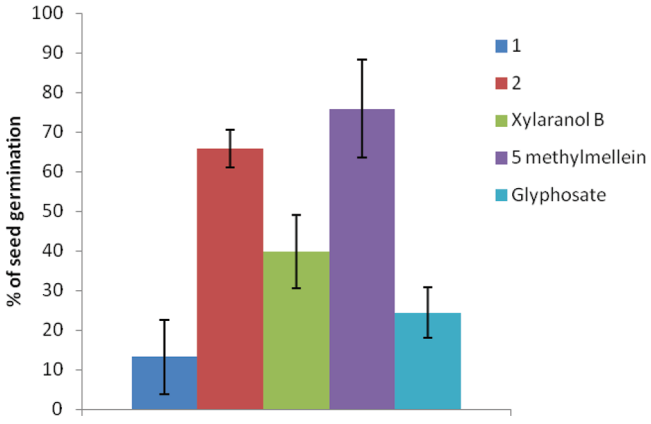

Figure 3. Effect of compounds **1** and **2**, xylaranol B, and 5-methylmellein on radish seed germination at 100 mg/mL.

1 and xylaranol B were the most effective against seed germination, with an inhibition of more than 50% at the tested concentration. It is interesting that compound **1** is more effective (85% inhibition) than the reference glyphosate (75% inhibition). Accordingly, the strong antigerminative activity of compounds **1** and xylaranol B on seeds could be at the source of some of the fungal pathogenesis reported. Current extensive studies on the phytotoxic activity of these products should validate this hypothesis.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured with a Perkin-Elmer model 341 polarimeter, and the $[\alpha]_D$ values are given in deg cm^2 g^{-1}. Mass spectra were recorded on an API Q-STAR PULSAR i from Applied Biosystem. For the CID spectra, the collision energy was 40 eV and the collision gas was nitrogen. IR spectra were recorded with a Shimadzu FTIR 8400S spectrometer, as a film on a NaCl plate The *J*-modulated ^{13}C NMR spectra were recorded on a Bruker AC 300 spectrometer operating at 75.47 MHz. The ^1H and 2D-NMR spectra were recorded at 298 K on a Bruker AVANCE 400 spectrometer operating at 400.13 MHz. For the HMBC experiments the delay (1/2*J*) was 70 ms, and for the NOESY experiments the mixing time was 500 ms.

Fungal Material. The fungus was isolated in October 2008 as an endophyte from a needle of *Cephalotaxus harringtonia* referenced under no. 2686 in the Arboretum de Chèvreloup (MNHN). Both morphological assessment and internal transcribed spacer (ITS) sequencing were performed to characterize 6E2a as *Biscogniauxia nummularia*. It is now maintained at the LCP culture collection (Muséum National d'Histoire Naturelle, Paris) under the number LCP 05669.

Fermentation and Isolation. The fungus *B. nummularia* was maintained in potato dextrose agar at 25 °C. The agar was cut into small plugs and inoculated into eight Erlenmeyer flasks (750 mL) containing V8 medium. After incubation at 27 °C for 5 weeks on a rotary shaker (150 rpm), the culture was centrifuged (7000 rpm, 20 min) to separate the mycelium and the filtrate. The culture filtrate was then extracted by ethyl acetate (3 × 1 L), and the combined organic phases were dried and after removal of the solvent under vacuum gave 605 mg of crude extract. This crude extract was subjected to a column chromatography (CC) on silica gel (cyclohexane–CH_2Cl_2 from 50/50 to 0/100 and then from CH_2Cl_2 to CH_2Cl_2–MeOH, 85/15), which afforded 5-methylmellein (118 mg) and compound **2** (79 mg). Subfraction 14 was further purified by CC (CH_2Cl_2–MeOH, 98/2), which produced 8 methoxy-5-methylmellein (9.5 mg). Subfractions 15 and 18 were further purified by CC (CH_2Cl_2–MeOH) and lead respectively to compound **1** (20 mg) and xylaranol B (16 mg).

Antigerminative Activity. The antigerminative assay was carried out on radish seeds. All compounds were tested at 100 mg/mL in methanol, the effective concentration of glyphosate. A sample of 3 μL of test solution was added on each seed. A paper disk moistened with 100 μL of distilled water was then placed into each well of a 24-well plate, and five seeds were added on this paper disk after evaporation of the solvent. The plates were sealed with Parafilm and incubated at room temperature for 65 h to darkness. Seed germination was scored by counting the number of germinated seeds. Glyphosate and MeOH were used respectively as negative and positive controls. Experiments were performed in triplicate, and the results are expressed as a percentage of germination compared to control.

*Xylaranone (**1**):* $C_{15}H_{24}O_2$; colorless oil; $[\alpha]^{20}_D$ +2 (*c* 0.2, MeOH); IR (NaCl plate) ν_{max} (cm^{-1}) 3421, 2962, 2928, 2870, 1732, 1450; ESI-qTOF *m/z* 237.1853 $[M + H]^+$ (calcd for $C_{15}H_{25}O_2$ 237.1855); ^1H and ^{13}C NMR see Table 1.

*Pogostol (**2**):* $C_{15}H_{26}O$; colorless oil; $[\alpha]^{20}_D$ −21.7 (*c* 0.4, $CHCl_3$); IR (NaCl plate) ν_{max} 3402, 2951, 2928, 2870, 1639; ESI-qTOF *m/z* 223.2055 $[M + H]^+$, 205.1879 $[M + H - H_2O]^+$(calcd for $C_{15}H_{27}O$ 223.2063); ^1H and ^{13}C NMR see Table 1, Supporting Information.

ASSOCIATED CONTENT

Supporting Information

^1H and ^{13}C NMR spectra of compounds **1** and **2**. NMR spectroscopic ($CDCl_3$ and C_5D_5N, 298 K, ^1H 400.13 MHz; ^{13}C 75.04 MHz) data for **2**. This material is available free of charge via the Internet at http://pubs.acs.org

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Notes

The authors declare no competing financial interest.

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