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Isolation and Structural Elucidation of Eight New Related Analogues of the Mycotoxin (−)-Botryodiplodin from *Penicillium coalescens*

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Bioassay-guided fractionation of the organic extract derived from the terrestrial fungus *Penicillium coalescens* led to the isolation of the known mycotoxin (−)-botryodiplodin (**1**) and eight new structurally related analogues (**2**−**9**). The structures of the novel compounds were determined by MS and NMR studies, including 1D and 2D NMR. A likely biogenetic pathway from the aldehydic open form of **1** (C_7 unit, U1) is proposed for these metabolites. Among all the isolated metabolites, only (−)-**1** showed antifungal, antibacterial, and insecticidal activity. This latter activity appears to be a new property attributed to (−)-**1**.

KEYWORDS: ***Penicillium coalescens*; (−)-botryodiplodin; natural products; fungal metabolites; antifungal; antibacterial; insecticidal activity; *Oncopeltus fasciatus; Ceratitis capitata***

INTRODUCTION

Terrestrial fungi are a well recognized source for new bioactive metabolites (*1*), including various mycotoxins (*2, 3*). (−)-Botryodiplodin (**1**, **Figure 1**) was isolated for the first time from *Botryodiplodia theobromae* Pat. (*4*), a fungus responsible for considerable damage in tropical plants (*5*), and was structurally elucidated by Arsenault et al. in 1969 (*6*). Subsequently, (−)-**1** was found in other fungal species such as *P. roqueforti* strains (*5, 7, 8*), *P. stipitatum* (*9, 10*), *Macrophomina phaseolina* (*11*), and others (*12*). In addition, *P. roqueforti* and *M. phaseolina* fungi have been reported as a contaminant of processed food (*8*) and the causal agent of numerous plant diseases (*11*), respectively. (−)-**1** has received attention because of its potent antibiotic (*4*), antileukemic (*13*), and mutagen (*14*) activities as well as the ability to induce protein−DNA cross-links in mammalian cells (*15*−*19*) and to inhibit cell multiplication in growing cultures (*20*). It has been proposed that, biogenetically, (−)-**1** belongs to the polyketide pathway (*21*). Furthermore, several syntheses of (−)-**1** and its derivatives have been reported (*5*, *22*−*34*).

In the course of research aimed at finding new bioactive agents from fungi, the organic extract from the culture broth of the terrestrial fungus *Penicillium coalescens* (*35*) was seen to exhibit potent antifungal, antibacterial, and insecticidal activities. (−)-Botryodiplodin (**1**) and eight new related analogues (**2**−**9**) were isolated and identified by a bioassay-guided fractionation of the fungal extract, in which (−)-**1** was found to be the most abundant compound. The isolation, structural elucidation, and chemical structure relationships for the new analogues are described.

MATERIALS AND METHODS

Chromatographic and Spectroscopic Analysis. TLC was run on silica gel F_{254} precoated plates (Merck), and spots were detected under UV light. Isolation and purification were carried out with a Waters HPLC system with a 600 pump, and both a 2996 Photodiode Array Detector (PDA) and an ELSD 2420 Detector (Milford, MA). ^{1}H, ^{13}C, ^{1}H-^{1}H COSY, and ^{1}H-^{1}H NOESY NMR spectra were recorded on a Bruker AV 300 MHz instrument (Rheinstetten, Germany). Multiplicities of ^{13}C signals were assigned by DEPT experiments. For HSQC, HMBC NMR experiments, and NOEDIFF irradiations, a Bruker 600 MHz (Rheinstetten, Germany) and a Varian Unity-400 MHz spectrometer were used. HRESMS (electrospray) data were collected out on a Micromass Q-TOF micro (Milford, MA). IR spectra were obtained with a 710FT spectrophotometer (Nicolet, Madison, WI). Optical rotations were determined with a Perkin-Elmer 241 Polarimeter (Massachusetts).

Cultivation of *P. coalescens*. The fungal strain *P. coalescens* Quintanilla (CECT 2766) was supplied by the Colección Española de Cultivos Tipo (CECT). The strain of the fungus *P. coalescens* was seeded in Petri dishes containing PDA culture medium and incubated for 7 days at 28 °C. Then, a solution of Tween 80 (0.05%) in sterile

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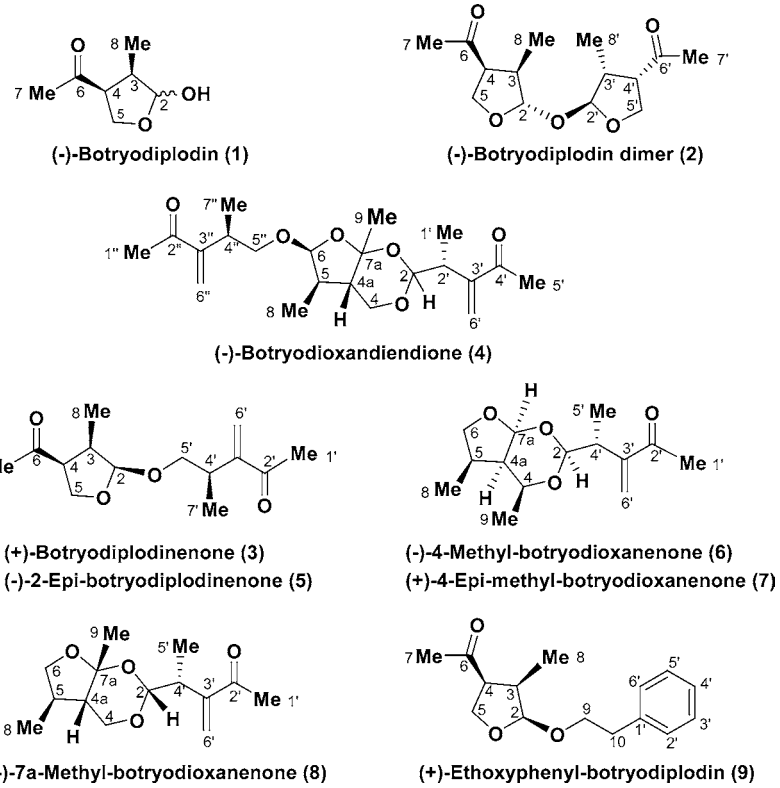

Figure 1. Natural metabolites (**1**–**9**) from *P. coalescens*.

distilled water was used to obtain a suspension containing ~10^6 conidia/mL. This suspension was poured into an Erlenmeyer flask containing antibiotic test broth (1:9, v/v). The mixture was incubated with shaking (200 rpm) for 15 days, in the dark at 25 °C.

Extraction Process and Preliminary Fractionation. After incubation, the mycelium was removed from the culture broth by filtration. Then, the broth (20 L) was partially evaporated in vacuum to 1 L and it was extracted with dichloromethane/ethyl acetate (1:1, v/v) (3 × 1 L). The resulting organic extract was dried (8.1 g) under reduced pressure and partitioned by flash column chromatography on silica gel (230−400 μm, Merck) (1:80, w/w) using stepwise gradient elution from 75% hexane in ethyl acetate, to 100% ethyl acetate, to 100% methanol. The volume eluted in each step was 2 L, and 13 fractions were obtained, evaporated to dryness, and tested for biological activities. The dried mycelium (176 g) was first extracted with dichloromethane (3 × 1 L) to give 0.25 g of dichloromethanic extract, and then with methanol (3 × 1 L) to obtain 13.5 g of methanolic extract.

Isolation and Purification of Secondary Metabolites. Fraction F-IX was subjected to silica gel flash column chromatography using hexane/ethyl acetate (5:5, v/v) as mobile phase, obtaining an anomeric mixture of (−)-botryodiplodin (**1**, 1.5 g, 18.5%). This major compound (−)-**1** was visible neither at 254 nm nor at 365 nm, and cerium (IV) sulfate was employed for its visualization on the TLC plate.

^{1}H NMR spectra of fractions F-II, F-III, F-IV, and F-VI showed their structural similarity to (−)-**1**. The most polar of these fractions, F-VI, was subjected to silica gel flash column chromatography using a gradient from 65% hexane in ethyl acetate to 100% ethyl acetate to give two promising subfractions 7 and 8. A second fractionation by silica gel column chromatography of subfraction 8 afforded compound **2** (19.2 mg, 0.24%) while subfraction 7 yielded **3** (1.7 mg, 0.02%), by using as mobile phase hexane/ethyl acetate (8:2, v/v and 7:3, v/v, respectively).

Fraction F-IV was purified by medium-pressure flash chromatography on a Biotage SP1 instrument coupled to a UV detector at 254 nm, using a gradient from 100% hexane to 100% ethyl acetate. Subfraction 8 was subjected to a second silica gel column (hexane/ethyl acetate, 9:1, v/v) to give compound **4** (8.6 mg, 0.11%). Fraction F-III was purified by silica gel column chromatography (hexane/ethyl acetate, 8:2, v/v) to afford compound **5** (18 mg, 0.22%) in subfraction 3. Analysis of pure compounds was achieved via analytical RP-HPLC using a 250 × 4.6 mm i.d., 4 μm, Tracer Excel ODSB C18 column, eluting with a flow of 0.5 mL/min of acetonitrile/water (6:4, v/v) for **4** (t_R = 22.1 min) and acetonitrile/water (7.5:2.5, v/v) for **5** (t_R = 13.9 min).

Finally, the less polar fraction, F-II, was subjected to silica gel flash chromatography using a gradient from hexane (with 2% triethylamine) to 100% ethyl acetate. Subfractions 2 and 6 were purified by semipreparative RP-HPLC using a 250 × 10 mm i.d., 5 μm, Tracer Excel ODSB C18 column and a flow rate of 2 mL/min. Subfraction 6 was eluted with acetonitrile/water (5:5, v/v) to afford compounds **6** (0.8 mg, 0.01%, t_R = 32.4 min), **7** (0.7 mg, 0.009%, t_R = 28.3 min), and **8** (2.4 mg, 0.03%, t_R = 26.1 min) while subfraction 2 was eluted with acetonitrile/water (7:3, v/v) to give compound **9** (0.5 mg, 0.006%, t_R = 19.8 min).

Characterization of Compounds. *(−)-Botryodiplodin Dimer (2).* $[\alpha]_D^{25}$ −99.1 (*c* 3.3, $CHCl_3$); colorless oil; IR ν_{max} 2965, 2899, 1700, 1521, 1455, 1362 cm^{-1}; HRESMS *m*/*z* 293.1360 $[M + Na]^+$ ($C_{14}H_{22}O_5$-Na calc 293.1365); ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.03 (1H, br s, H-2), 4.28 (1H, t, J = 8.7 Hz, Ha-5), 3.93 (1H, t, J = 8.7 Hz, Hb-5), 3.54 (1H, m, H-4), 2.55 (1H, m, H-3), 2.19 (3H, s, H-7), and 0.86 (3H, d, J = 7.2 Hz, H-8) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 206.19 (C-6), 105.42 (C-2), 67.09 (C-5), 53.39 (C-4), 41.93 (C-3), 30.23 (C-7), and 12.42 (C-8) ppm.

(+)-Botryodiplodinenone (3). $[\alpha]_D^{25}$ +9.0 (*c* 0.67, $CHCl_3$); yellow oil; IR ν_{max} 2955, 2842, 1721, 1674, 1465, 1357 cm^{-1}; HRESMS *m*/*z* 277.1415 $[M + Na]^+$ ($C_{14}H_{22}O_4Na$ calc 277.1416); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.09 (1H, s, Ha-6′), 5.81 (1H, s, Hb-6′), 4.91 (1H, d, J = 5.1 Hz, H-2), 4.25 (1H, dd, J = 9.0, 6.3 Hz, Ha-5), 3.92 (1H, dd, J = 9.0, 7.8 Hz, Hb-5), 3.69 (1H, dd, J = 9.3, 6.0 Hz, Ha-5′), 3.30 (1H, dd, J = 9.3, 6.0 Hz, Hb-5′), 3.11 (2H, m, H-4′), 2.57 (1H, m, H-3), 2.34 (3H, s, H-1′), 2.20 (3H, s, H-7), 1.07 (3H, d, J = 6.9 Hz, H-7′), and 0.93 (3H, d, J = 7.2 Hz, H-8) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 208.57 (C-6), 199.92 (C-2′), 151.74 (C-3′), 125.06 (C-6′), 105.01 (C-2), 71.88 (C-5′), 67.73 (C-5), 54.26 (C-4), 41.41 (C-3), 33.20 (C-4′), 30.05 (C-7), 26.53 (C-1′), 17.28 (C-7′), and 10.25 (C-8) ppm.

(−)-Botryodioxandiendione (4). $[\alpha]_D^{25}$ −39.0 (*c* 10.3, $CHCl_3$); colorless oil; IR ν_{max} 2955, 2934, 1711, 1675, 1455, 1362 cm^{-1}; HRESMS *m*/*z* 403.2103 $[M + Na]^+$ ($C_{21}H_{32}O_6Na$ calc 403.2097); ^{1}H

NMR (600 MHz, $CDCl_3$) δ 6.13 (1H, s, Ha-6′), 6.06 (1H, s, Ha-6″), 6.01 (1H, s, Hb-6′), 5.84 (1H, s, Hb-6″), 4.78 (1H, d, J = 6.0 Hz, H-6), 4.60 (1H, d, J = 3.6 Hz, H-2), 3.88 (1H, dd, J = 12.0, 0.5 Hz, Ha-4), 3.78 (1H, dd, J = 12.0, 3.0 Hz, Hb-4), 3.64 (1H, dd, J = 9.0, 7.2 Hz, Ha-5″), 3.44 (1H, dd, J = 9.0, 6.0 Hz, Hb-5″), 3.09 (2H, m, H-2′, H-4″), 2.54 (1H, m, H-5), 2.34 (6H, s, H-5′, H-1″), 1.44 (3H, s, H-9), 1.37 (1H, m, H-4a), 1.11 (3H, d, J = 6.9 Hz, H-7″), 1.10 (3H, d, J = 6.9 Hz, H-1′), and 1.06 (3H, d, J = 6.9 Hz, H-8) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 199.78 (C-2″), 199.70 (C-4′), 151.47 (C-3″), 149.15 (C-3′), 126.61 (C-6′), 124.43 (C-6″), 111.43 (C-6), 104.19 (C-7a), 98.85 (C-2), 72.67 (C-5″), 63.49 (C-4), 49.22 (C-4a), 39.04 (C-5), 37.53 (C-2′), 33.44 (C-4″), 26.28 (C-5′), 26.24 (C-1″), 20.53 (C-9), 16.93 (C-7″), 14.83 (C-8), and 13.52 (C-1′) ppm.

*(−)-2-epi-Botryodiplodinenone (**5**).* $[\alpha]_D^{25}$ −75.0 (c 1, $CHCl_3$); yellow oil; IR ν_{max} 2970, 2873, 1711, 1680, 1455, 1373 cm^{-1}; HRMS m/z 277.1421 $[M + Na]^+$ ($C_{14}H_{22}O_4Na$ calc 277.1416); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.07 (1H, s, Ha-6′), 5.76 (1H, s, Hb-6′), 4.70 (1H, s, H-2), 4.25 (1H, t, J = 8.4 Hz, Ha-5), 3.87 (1H, t, J = 8.4 Hz, Hb-5), 3.53 (1H, dd, J = 15.9, 8.4 Hz, H-4), 3.44 (1H, dd, J = 9.3, 7.2 Hz, Ha-5′), 3.32 (1H, dd, J = 9.3, 5.7 Hz, Hb-5′), 3.06 (1H, m, H-4′), 2.54 (1H, m, H-3), 2.33 (3H, s, H-1′), 2.17 (3H, s, H-7), 1.05 (3H, d, J = 7.2 Hz, H-7′), and 0.81 (3H, d, J = 7.2 Hz, H-8) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 206.90 (C-6), 199.89 (C-2′), 151.80 (C-3′), 124.64 (C-6′), 109.51 (C-2), 71.66 (C-5′), 66.29 (C-5), 53.85 (C-4), 42.24 (C-3), 33.18 (C-4′), 30.53 (C-7), 26.56 (C-1′), 17.26 (C-7′), and 12.93 (C-8) ppm.

*(−)-4-Methylbotryodioxanenone (**6**).* $[\alpha]_D^{25}$ −6.5 (c 1.4, $CHCl_3$); yellow oil; IR ν_{max} 2925, 1677, 1458, 1172, 1082 cm^{-1}; HRESMS m/z 277.1417 $[M + Na]^+$ ($C_{14}H_{22}O_4Na$ calc 277.1416); ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.0 (1H, s, Ha-6′), 5.81 (1H, s, Hb-6′), 5.36 (1H, d, J = 4.5 Hz, H-7a), 4.75 (1H, d, J = 4.8 Hz, H-2), 4.08 (1H, dd, J = 8.5, 6.6 Hz, Ha-6), 3.40 (1H, m, H-4), 3.36 (1H, m, Hb-6), 3.01 (1H, m, H-4′), 1.86 (1H, m, H-5), 2.26 (3H, s, H-1′), 1.45 (1H, m, H-4a), 1.14 (3H, d, J = 6.0 Hz, H-9), 1.04 (3H, d, J = 7.2 Hz, H-5′), and 0.98 (3H, d, J = 7.2 Hz, H-8) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 200.20 (C-2′), 150.21 (C-3′), 126.19 (C-5′), 100.28 (C-7a), 96.75 (C-2), 73.46 (C-4), 72.59 (C-6), 47.80 (C-4a), 38.02 (C-4′), 35.34 (C-5), 26.71 (C-1′), 20.45 (C-9), 19.80 (C-8), and 14.19 (C-5′) ppm.

*(+)-4-epi-Methylbotryodioxanenone (**7**).* $[\alpha]_D^{25}$ +3.9 (c 0.7, $CHCl_3$); yellow oil; IR ν_{max} 2923, 1733, 1457, 1141 cm^{-1}; HRESMS m/z 277.1420 $[M + Na]^+$ ($C_{14}H_{22}O_4Na$ calc 277.1416); ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.01 (1H, s, Ha-6′), 5.83 (1H, s, Hb-6′), 5.21 (1H, d, J = 3.6 Hz, H-7a), 4.80 (1H, d, J = 4.5 Hz, H-2), 4.19 (1H, t, J = 8.4 Hz, Ha-6), 4.03 (1H, dd, J = 13.5, 6.6 Hz, H-4), 3.41 (1H, t, J = 8.4 Hz, Hb-6), 2.99 (1H, m, H-4′), 2.43 (1H, m, H-5), 2.26 (3H, s, H-1′), 1.33 (1H, m, H-4a), 1.28 (3H, d, J = 6.9 Hz, H-9), 1.03 (3H, d, J = 7.2 Hz, H-5′), and 0.97 (3H, d, J = 6.6 Hz, H-8) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 200.16 (C-2′), 149.92 (C-3′), 126.19 (C-6′), 99.79 (C-7a), 92.74 (C-2), 75.67 (C-6), 68.65 (C-4), 51.88 (C-4a), 38.48 (C-4′), 33.42 (C-5), 26.70 (C-1′), 20.02 (C-9), 16.54 (C-8), and 14.19 (C-5′) ppm.

*(−)-7a-Methylbotryodioxanenone (**8**).* $[\alpha]_D^{25}$ −8.7 (c 0.92, $CHCl_3$); colorless oil; IR ν_{max} 2960, 2929, 2853, 1736, 1496, 1455 cm^{-1}; HRESMS m/z 277.1419 $[M + Na]^+$ ($C_{14}H_{22}O_4Na$ calc 277.1416); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.10 (1H, s, Ha-6′), 5.91 (1H, s, Hb-6′), 4.62 (1H, d, J = 6.0 Hz, H-2), 4.22 (1H, t, J = 9.0 Hz, Ha-6), 3.98 (1H, dd, J = 12.0, 0.5 Hz, Ha-4), 3.82 (1H, dd, J = 12.0, 3.0 Hz, Hb-4), 3.50 (1H, t, J = 9.0 Hz, Hb-6), 3.06 (1H, m, H-4′), 2.67 (1H, m, H-5), 2.34 (3H, s, H-1′), 1.46 (3H, s, H-9), 1.33 (1H, m, H-4a), 1.11 (3H, d, J = 9.0 Hz, H-5′), and 1.08 (3H, d, J = 6.0 Hz, H-8) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 199.72 (C-2′), 149.40 (C-3′), 126.09 (C-6′), 105.41 (C-7a), 98.82 (C-2), 74.19 (C-6), 63.93 (C-4), 50.06 (C-4a), 38.31 (C-4′), 33.31 (C-5), 26.28 (C-1′), 20.14 (C-9), 16.80 (C-8), and 13.80 (C-5′) ppm.

*(+)-Ethoxyphenylbotryodiplodin (**9**).* $[\alpha]_D^{25}$ +13.3 (c 0.75, $CHCl_3$); colorless oil; IR ν_{max} 2955, 2929, 2852, 1721, 1500, 1450 cm^{-1}; HRESMS m/z 271.1301 $[M + Na]^+$ ($C_{15}H_{20}O_3Na$ calc 271.1310); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.24 (5H, m, H-2′, H-6′), 4.93 (1H, d, J = 4.8 Hz, H-2), 4.21 (1H, dd, J = 9.6, 6.6 Hz, Ha-5), 3.98 (1H, m, Ha-9), 3.93 (1H, m, Hb-5), 3.58 (1H, m, Hb-9), 3.09 (1H, m, H-4), 2.88 (2H, t, J = 6.6 Hz, H-10), 2.55 (1H, m, H-3), 2.12 (3H, s, H-7), and 0.95 (3H, d, J = 7.8 Hz, H-8) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 207.82 (C-6), 139.13 (C-1′), 128.98 (C-6′, C-2′), 128.24 (C-5′, C-3′), 126.13 (C-4′), 104.77 (C-2), 68.51 (C-9), 67.39 (C-5), 54.03 (C-4), 41.04 (C-3), 36.28 (C-10), 29.29 (C-7), and 9.82 (C-8) ppm.

Biological Assays. *Insects* Oncopeltus fasciatus Dallas and *Ceratitis capitata* Wiedemann were maintained at 27 ± 1 °C and 50−60% relative humidity and a 16 h/8 h (light/dark) photoperiod on a diet based on sunflower seeds and protein yeast autolysate (Aldrich, Spain) and sucrose in a 1:4 ratio.

Target Microorganisms. Fungicidal activity was measured against 10 phytopathogens: *Verticillium dahliae* (CCM 269), *Aspergillus parasiticus* (CECT 2681), *C. gloecesporoides* (CECT 2859), *Fusarium culmorum* (CECT 2148), *F. oxysporum* ssp. *gladioli* (CCM 233), *F. oxysporum* ssp. *niveum* (CCM 259), *P. italicum* (CECT 2294), *Phytophthora citrophthora* (CECT 2353), *Trichoderma viride* (CECT 2423), and *Trichothecium roseum* (CECT 2410). Six different bacterial strains were used to determine bactericidal activity: *Bacillus cereus* (CECT 148), *Staphylococcus aureus* (CECT 86), *Enterococcus faecalis* (CECT 481), *Salmonella typhi* (CECT 409), *Escherichia coli* (CECT 405), and *Erwinia carotovora* (CECT 225). The strains were provided by the Colección Española de Cultivos Tipo (CECT) or by the Colección de la Cátedra de Microbiología (CMM) of the Biotechnology Department (Universidad Politécnica de Valencia).

Entomotoxicity Activity. The entomotoxicity against *O. fasciatus* was determined basically according to the contact method of Bowers et al. (*36*). It was evaluated by topical application to obtain either acute mortality (%) for the extract (100 μg/nymph) and fractions (25 μg/nymph) or LD_{50} values for the pure compounds. A total of 1 μL of the appropriate dilution in acetone was applied, using a micropipet, on the ventral surface of the abdomen of 10 newly moulted fourth-instar nymphs, which had previously been anesthetized with chloroform. After treatment, nymphs were confined in a 9 cm Petri dish with food and water provided *ad libitum*. Acute toxicity effects were considered according to the number of dead insects after 72 h of exposure to the chemicals. Controls were carried out in parallel and received the same amount of acetone as treated insects. All assays were conducted in triplicate.

The entomotoxicity against *C. capitata* was evaluated by topical application (*37*) to obtain either acute mortality (%) for the extract (100 μg/fly) and fractions (25 μg/fly) or LD_{50} values for the pure compounds. A total of 1 μL of the appropriate dilution in acetone was applied, using a micropipet, on the ventral surface of the abdomen of 2−3-day-old adult flies (five males and five females), which had previously been anesthetized with ice. Controls were similarly grouped, and each fly was treated with 1 μL of acetone. After treatment, the flies were placed into a methacrylate box (10 × 10 × 10 cm^3) that contained a circular hole (6 cm in diameter) covered with a net cloth, and diet and water were provided *ad libitum*. Mortality was assessed at intervals of 24 h for 10 days in triplicate.

Antifungal and Antibacterial Activities. These assays were determined in triplicate by the paper disk−agar diffusion assay according to Cole (*38*). The doses of the assays were at 100 μg/mm^2 (2 mg/disk) for organic extracts, at 50 μg/mm^2 (1 mg/disk) for the fractions, and at 10 μg/mm^2 (0.2 mg/disk) for pure compounds. The fungal strains were seeded in Petri dishes containing PDA culture medium and incubated for 7 days at 28 °C. Then, a solution of Tween 80 (0.05%) in sterile distilled water was used to obtain a suspension containing ~10^6 conidia/mL. 1 mL of this conidia suspension was added to 15 mL of PDA in a Petri dish. After the solidification, four Watman disks (No. 113, 0.5 cm diameter) impregnated with the tested products, at appropriate doses, were added to these Petri dishes. PDA plates containing disks impregnated only with the solvent used to dissolve the tested compounds were used as negative controls, and disks with benomyl (methyl-1-[butylcarbamoyl]-2-benzimidazolecarbamate) (Sigma), at different concentrations according to the fungus species assayed, were used as positive controls. Fungicidal activity was determined by measuring the inhibition zone developed around the paper disk, indicating a zone of no growth.

In the bactericidal tests, cultures of 24 h of each bacterium, maintained in inclined tubes on solid culture medium, were reactivated with a Nutrient Broth (Difco) and were incubated for 24 h at 28 °C or 37 °C, according to the bacterium. Then, 1 mL of this suspension was

Table 1. Biological Activities of the DCM/EtOAc Broth Extract of *P. coalescens*

	fungicidal activity inhibition zone (mm) 72 h (means ± SE)[a]	
fungal strains	broth organic extract[b]	benomyl
F. culmorum	21.33 ± 0.88 A	19.00 ± 0.58 A[c1]
F. oxysporum niveum	7.67 ± 0.33 A	17.33 ± 0.88 B[c2]
F. oxysporum gladioli	11.66 ± 0.33 A	9.33 ± 0.88 A[c1]
V. dahliae	56.33 ± 2.02 B	20.00 ± 1.15 A[c3]
P. citrophthora	38.67 ± 1.33 B	18.67 ± 0.88 A[c4]
C. gloesporoides	29.67 ± 0.88 A	26.67 ± 0.88 A[c5]
T. roseum	11.33 ± 0.33 A	29.67 ± 0.88 B[c2]
T. viride	0 ± 0 A	12.33 ± 0.88 B[c6]
A. parasiticus	7.67 ± 0.33 A	8.33 ± 0.33 A[c6]
P. italicum	10.67 ± 0.33 A	16.67 ± 0.33 B[c7]

	bactericidal activity inhibition zone (mm) 24 h (means ± SE)[a]	
bacterial strains	broth organic extract[b]	tetracycline chlorhydrate[d]
B. cereus	13.33 ± 0.88 A	18.33 ± 0.33 B
S. aureus	19.00 ± 0.57 B	16.67 ± 0.33 A
E. faecalis	28.33 ± 0.88 A	31.67 ± 0.88 A
S. typhii	28.67 ± 0.33 B	18.67 ± 1.20 A
E. coli	21.33 ± 0.33 A	24.00 ± 1.15 A
E. carotovora	35.67 ± 1.33 A	34.67 ± 1.45 A

	insecticidal activity
insects	% acute mortality 72 h
O. fasciatus	100 ± 0[e1]
C. capitata	100 ± 0[e2]

[a] Each value represents the average and the standard error of three independent experiments. Within each line, mean values labeled with the same letter (A or B) do not present statistically significant differences ($P > 0.05$). [b] dose: 2 mg/disk. [c1] dose: 10 μg/disk. [c2] dose: 5 μg/disk. [c3] dose: 0.25 μg/disk. [c4] dose: 1.5 μg/disk. [c5] dose: 0.5 μg/disk. [c6] dose: 1 μg/disk. [c7] dose: 0.2 μg/disk. [d] dose: 0.2 mg/disk. [e1] dose: 100 μg/nymph. [e2] dose: 100 μg /fly.

inoculated in a Petri plate, and 15 mL of culture medium Plate Count Agar (Difco) was added. When the medium was completely solidified, five paper disks loaded with the tested products were placed in the dish. These plates were incubated for 24 h in the dark at 28 °C or 37 °C, according to the bacterium. Plate Count Agar plates containing disks impregnated only with the solvent used to dissolve the tested compounds were used as negative controls, and a positive control with tetracycline chlorhydrate (10 μg/cm^2) was performed to appraise the level of activities. Bactericidal activity was determined measuring the halo developed around the paper disk.

Statistical Analysis. Probit analysis (*39*) was used to determine the LD_{50} values. The results of χ^2 analysis for goodness of fit for the regression equation revealed the existence of considerable homogeneity in the data. Analysis of variance (ANOVA) was performed for fungicidal and bactericidal data (**Table 1**), and the least significant difference (LSD) test was used to compare means (Statgraphics plus 5.1 version).

RESULTS AND DISCUSSION

Elucidation of Metabolites. The molecular formula $C_7H_{12}O_3$ of (−)-**1** was determined by ESMS that showed the ions at *m*/*z* 167.1 $[M + Na]^+$ and 127.1 $[M + 1 - H_2O]^+$, and it was identified as an inseparable anomeric mixture (α/β, 65:35) by comparison of its MS and ^{1}H and ^{13}C NMR data with the data reported previously (*7*). Fraction F-IX was treated with Ac_2O in pyridine, giving in quantitative yield the 2,3-*trans*-botryodiplodin acetate (*6*), confirming the structure of (−)-**1**. It was obtained as a colorless oil, and according to the literature (*13*), (−)-**1** has the property of turning the skin pink after 2−3 h following application.

The (−)-botryodiplodin dimer (**2**) was identified from its ESMS and ^{1}H and ^{13}C NMR data. Its NMR signals practically overlapped with those of (−)-**1**, and its HRESMS gave an $[M + Na]^+$ ion of *m*/*z* 293.1360, indicating a molecular formula containing twice the number of carbon and hydrogen atoms as were observed in its NMR spectra. From these data we deduced that the compound (−)-**2** must be a symmetrical dimer of (−)-**1**. In addition, the acetylation reaction of (−)-**2** was unsuccessful, indicating the absence of a free hydroxyl group. In accordance with the absolute configuration of (−)-**1,** previously determined by crystal X-ray analysis (*7*) and the coupling constant value $J_{2,3} = 0$ Hz, indicating a *trans* relationship between H-2 and H-3 (*5*, *40*), a (2*S*,2′*S*,3*R*,3′*R*,4*S*,4′*S*)-**2** configuration was established.

(−)-2-*epi*-Botryodiplodinenone (**5**) showed a ^{1}H NMR spectra very similar to that of (−)-**1**, and its HRESMS showed an $[M + Na]^+$ ion of *m*/*z* 277.1421, suggesting the presence of the furan core linked to the other C_7 fragment (C-1′ to C-7′). Given that C-6′ (δ 124.64) was correlated in the HSQC spectrum to two proton resonances at δ 6.07 and 5.76, an olefinic methylene contained in this C_7 fragment was proposed. Analysis of the 2D NMR spectra revealed that the linkage between this C_7 moiety and the botryodiplodin core was at the hydroxyl group in C-2, since HMBC correlations were observed from H-2 (δ 4.70) to C-5′ (δ 71.66). Furthermore, assuming that the stereogenic centers of the parent compound (−)-(3*R*,4*S*)-**1** were fixed through the likely biogenetic pathway, the configurations of the carbons C-4, C-3, and C-4′ remain determined. Also, the multiplicity of H-2 as a singlet ($J_{2,3} = 0$ Hz) pointed to a *trans* coupling with H-3 (*5*, *40*). The absolute configuration could be suggested as (2*R*,3*R*,4*S*,4′*R*)-**5**, which could be corroborated by NOESY correlations of H-2 (δ 4.70) to H-8 (δ 0.81), showing that H-2 and H-8 were orientated on the same side of the molecule.

(+)-Botryodiplodinenone (**3**) was elucidated on the basis of the great similarity between its ^{1}H and ^{13}C NMR spectra and those of compound (−)-**5**. In addition, compound (+)-**3** also gave the same $[M + Na]^+$ ion in HRESMS: *m*/*z* 277.1415. The unique major variation in the ^{1}H NMR spectrum of (+)-**3** was the multiplet at δ 3.11 integrating for two protons that through COSY and HSQC experiments was assigned to H-4 and H-4′. The ^{13}C NMR spectrum contained a significant difference in C-2, which suffered a strong shielding (δ 105.01 for (+)-**3** vs 109.51 for (−)-**5**). Also, a doublet (d, $J_{2,3} = 5.1$ Hz) corresponding to the multiplicity of H-2 (δ 4.91) was indicative of a *cis* relationship between H-2 and H-3 (δ 2.57) (*5*, *40*). Therefore, the inspection of the MS and 1D and 2D NMR data showed that compound (+)-**3** is an epimer of (−)-**5** in position 2 with a (2*S*,3*R*,4*S*,4′*R*)-**3** configuration.

HRESMS of (−)-7a-methylbotryodioxanenone (**8**) gave an $[M + Na]^+$ ion *m*/*z* 277.1419 that established the molecular formula as $C_{14}H_{22}O_4Na$. Its resemblance with the ^{1}H and ^{13}C NMR data of (−)-**1** indicated that it was a new member of this group bearing a 3-methylenepentan-2-one moiety, probably from a C_7 unit. COSY and HMBC correlations showed the presence of an AMX system at δ 4.22 (Ha-6) and 3.50 (Hb-6), and 2.67 (H-5) as well as a second ABX system at δ 3.98 (Ha-4) and 3.82 (Hb-4), and 1.33 (H-4a). The characteristic signal of H-4a was correlated in HSQC to the relatively upfield carbon at δ 50.06, pointing out that this methine could be involved in a constrained system such as on the bridge of a fused bicycle. The two highly deshielded sp^3 carbons at δ 105.41 (C-7a) and 98.82 (C-2) were typical of carbons between two oxygen atoms. The correlations provided by HMBC from C-2 (δ 98.82) to H-4′

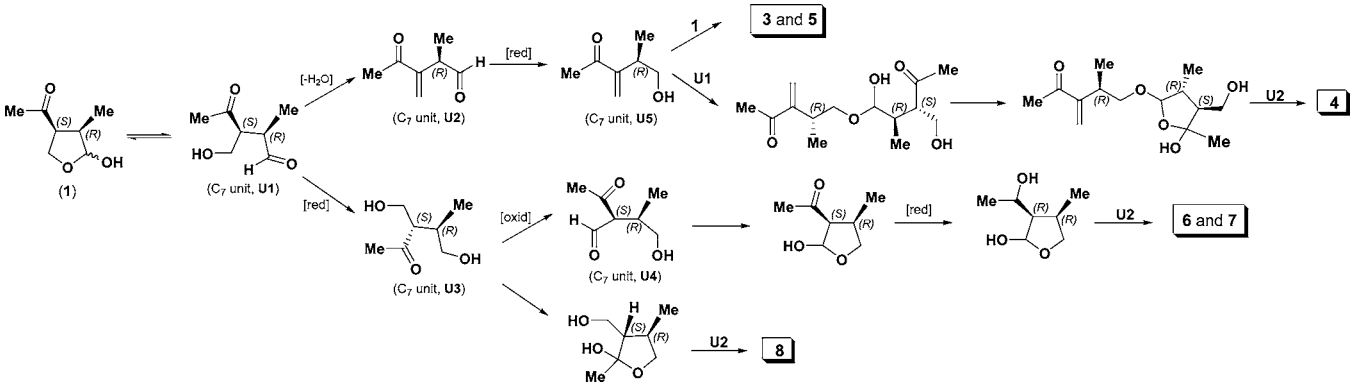

Figure 2. Chemical structure relationships of botryodiplodin analogues **3**–**8**.

(δ 3.06) and H-5′ (δ 1.11), as well as COSY correlations between H-2 and H-4′, indicated that the connectivity of the C_7 unit to the tetrahydrofuro[2,3-d][1,3]dioxane core was on the acetal carbon C-2. The configurations of C-5, C-4a, and C-4′ were suggested as (*R*), (*S*), and (*R*), respectively, according to the biogenetic pathway. In order to determine the stereochemistry of the rest of the stereocenters, NOEDIFF experiments were carried out. Signal enhancement of H-4a (δ 1.33, m) upon irradiation of H-9 (δ 1.46, s) was observed, while irradiation of H-2 (δ 4.62, d) affected H-9 (δ 1.46, s) and H-4′ (δ 3.06, m), suggesting a (2*R*,4a*S*,5*R*,7a*R*,4′*R*)-**8** configuration.

Analysis of spectroscopic data of (−)-botryodioxandiendione (**4**) proved that it was an analogue of compound (−)-**8**, attached to another 5-hydroxy-4-methyl-3-methylenepentan-2-one moiety (C_7 unit). The HMBC experiment showed correlations from the H-6 resonance (δ 4.78) to carbon C-5″ (δ 72.67) revealing the link between C-6 of the tetrahydrofuran core and the terminal alcohol of the newly included C_7 unit. The configurations of C-5, C-4a, C-2′, and C-4″ were set according to its biogenetic pathway as (5*R*,4a*S*,2′*R*,4″*R*), and the coupling constant value $J_{5,6}$ = 6.0 Hz determined a *cis* relationship between H-5 and H-6 (*5*, *40*). Unfortunately, compound **4** was chemically unstable, suffering facile degradation and making NOE studies inaccessible; therefore, the stereogenic centers C-7a and C-2 could not be determined.

The structure of (−)-4-methylbotryodioxanenone (**6**) was elucidated by means of its 1D and 2D NMR spectra. ^{1}H NMR signals and COSY revealed the tetrahydrofuro[2,3-d][1,3]-dioxane core attached to the 4-methyl-3-methylenepentan-2-one framework (C_7 unit). In ^{13}C NMR, both the presence of a unique methylene and the absence of quaternary carbons in the bicyclic system differentiated this new compound from its structural isomer (−)-**8**. Two methine groups highly deshielded at δ 4.75 and 5.36 were detected and assigned to H-2 and H-7a, respectively. COSY correlations from H-4a (δ 1.45) to H-7a (δ 5.36) and H-4 (δ 3.40), from H-5 (δ 1.86) to H-8 (δ 0.98), and from H-4 to H-9 (δ 1.14) were observed, establishing H-9 on position 4 of the 1,3-dioxane ring. The stereochemistry of C-5, C-4a, and C-4′ was fixed through its biogenetic route as (5*R*,4a*R*,4′*R*), while both the coupling constant $J_{7a,4a}$ = 4.5 Hz and the NOESY correlations of H-4a to H-7a determined a *cis* ring junction (*5*, *40*). Finally, the NOESY correlations of H-2 (δ 4.75) to H-4 (δ 3.40), and H-4 (δ 3.40) to H-4a (δ 1.45) showed they were orientated on the same side, suggesting a feasible (2*S*,4*S*,4a*R*,5*R*,7a*S*,4′*R*)-**6** configuration.

In view of the 1D and 2D NMR spectra of (+)-4-*epi*-methylbotryodioxanenone (**7**), a stereoisomer of compound (−)-**6** was proposed. The great difference in the ^{1}H NMR spectra was the deshielding experienced by H-4 (δ 4.03 for (+)-**7** vs 3.40 for (−)-**6**) and H-5 (δ 2.43 for (+)-**7** vs 1.86 for (−)-**6**). Similar to the case of compound (−)-**6**, the biogenetic route, the coupling constant $J_{7a,4a}$ = 3.6 Hz, and the NOESY correlations of H-4a (δ 1.33) to H-7a (δ 5.21) determined a *cis* ring junction and a (5*R*,4a*R*,4′*R*) stereochemistry (*5*, *40*). In addition, NOESY correlations of H-4 (δ 4.03) to H-8 (δ 0.97), H-2 (δ 4.80) to H-7a (δ 5.21) and H-9 (δ 1.28), H-7a (δ 5.21) to H-9 (δ 1.28), and, finally, H-5 (δ 2.43) to H-4a (δ 1.33) allowed us to propose a (2*S*,4*R*,4a*R*,5*R*,7a*S*,4′*R*)-**7** configuration.

1D and 2D NMR spectra of (+)-ethoxyphenylbotryodiplodin (**9**) revealed a molecule of (−)-**1** connected to a phenylethyl fragment on its hydroxyl group. HMBC correlations from C-2 (δ 104.77) to Hb-9 (δ 3.58) confirmed this hypothesis. The multiplicity of H-2 (δ 4.93) as a doublet ($J_{2,3}$ = 4.8 Hz) indicated a *cis* relationship between H-2 and H-3 (δ 2.55) (*5*, *40*). Taking into account the known configuration of (−)-botryodiplodin and the relative stereochemistry between H-2 and H-3, the absolute configuration may be assigned as (2*S*,3*R*,4*S*)-**9**.

Structural Relationship between Metabolites. It was concluded that except for (−)-**2** and (+)-**9**, which are clearly derivatives of **1**, all the newly identified structures (**3**−**8**) seem to be formed from the resultant open form of the hemiacetal (−)-**1** (C_7 unit, U1). This unit U1 might be reactive enough to undergo different reaction sequences (**Figure 2**). In order to determine that compounds **3**−**8** were not formed from (−)-**1** during the purification procedures, it is significant that the initial TLC of the organic extract from the broth already showed the metabolite profile, in which no changes were observed after successive chromatographies. In addition, (−)-**1** was dissolved in ethyl acetate, treated with silica gel (1:80, w/w), and stirred overnight at room temperature, after which both the TLC and ^{1}H NMR of the residue showed no evidence of the botryodiplodin analogues (**3**−**8**).

We propose chemical structure relationships between (−)-**1** and the other metabolites **3**−**8** that might also coincide with a possible biogenetic pathway (**Figure 2**). On the one hand, the aldehydic open form of the hemiacetal (−)-**1**, C_7 unit U1, is able to suffer a reaction sequence of dehydration (U2)/reduction to yield a molecule C_7 unit U5, which could react with the hemicetal (−)-**1**, leading to compounds (+)-**3** and (−)-**5**, or could react with the aldehyde U1, giving an intermediate that undergoes intramoleculat ring closure to obtain compound (−)-**4**. On the other hand, the aldehyde U1 enables a sequence of reduction (U3)/oxidation (U4)/intramolecular ring closure/reduction (ketone function), giving a dihydroxy derivative, which after reaction with an aldehyde U2 can yield the acetal compounds (−)-**6** and (+)-**7**. Also, the unit U3 could undergo intramolecular ring closure to provide another dihydroxy derivative, which could react with an aldehyde U2 to give the

Table 2. Biological Activities of Fraction F-IX and the Pure Compound (–)-Botryodiplodin Isolated from *P. coalescens*

	fungicidal activity inhibition zone (mm) 72 h (means ± SE)[a]		
fungal strains	FIX[b]	(–)-**1**[c]	benomyl
V. dahliae	>25.00	17.31 ± 1.02	14.32 ± 1.11[d1]
P. citrophthora	>25.00	8.21 ± 0.88	17.13 ± 1.22[d2]

	bactericidal activity inhibition zone (mm) 24 h (means ± SE)[a]		
bacterial strains	FIX[b]	(–)-**1**[c]	tetracycline chlorhydrate[c]
S. typhii	22.11 ± 0.12	16.23 ± 0.82	24.21 ± 0.24
S. aureus	18.23 ± 0.75	13.53 ± 0.21	27.32 ± 1.12

	insecticidal activity	
insects	FIX %mortality 72 h	(–)-**1** LD_{50}[f]
O. fasciatus	100 ± 0[e1]	5.55 ± 0.45
C. capitata	93.33 ± 6.66[e2]	12.90 ± 0.66[g]

[a] Each value represents the average and the standard error of three independent experiments. [b] Dose: 1 mg/disk. [c] Dose: 0.2 mg/disk. [d1] Dose: 0.25 μg/disk. [d2] Dose: 1.5 μg/disk. [e1] Dose: 25 μg/nymph. [e2] Dose: 25 μg/fly. [f] Values, in μg/nymph, were determined 72 h after exposure to the chemical. [g] The death of the population was 100% males.

Table 3. Insecticidal Activity of *P. coalescens* Metabolites against *O. fasciatus*

	insecticidal activity[a]				
pure compd	slope	LD_{50} (95% CL)[b]	χ^2	df	*p*
botryodiplodin	6.52 ± 1.65	5.75 (4.60, 6.57)	3.54	4	0.47
botryodiplodin acetate	4.60 ± 1.09	12.19 (9.21, 14.36)	1.56	6	0.95
botryodiplodin dimer		>15.00			
2-*epi*-botryodiplodinenone		>15.00			

[a] Regression analysis, linear model: $y = ax + b$; log dose vs probit mortality. [b] Values, in μg/nymph, were determined 72 h after exposure to the chemical.

compound (−)-**8**. In summary, a common C_7 unit (U1) resulted from the parent compound (−)-**1** may lead *via* a likely biogenetic pathway to a novel family of botryodiplodin analogues.

Biological Assays of the Organic Extracts, Fractions, and Isolated Compounds. Biological assays of the extracts were performed, and only the organic extract from the culture broth exhibited acute antifungal, antibacterial, and insecticidal activities (**Table 1**). Neither the dichloromethane extract nor the methanolic extract of the mycelium showed biological activity. For the antifungal and antibacterial assays, fractions F-I to F-XIII were only tested against those strains that initially had been the most sensitive to the extract: *P. citrophthora*, *V. dahliae*, *S. aureus*, and *S. typhi*. After performing biological assays of all the fractions, fraction F-IX was found to be uniquely bioactive and all the activities were determined for the compound (−)-**1** (**Table 2**). In the course of our research focused on finding new insecticidal agents, the compounds (−)-botryodiplodin dimer (**2**), (−)-2-*epi*-botryodiplodinenone (**5**), and 2,3-*trans*-botryodiplodin acetate were also submitted to tests against *O. fasciatus* (**Table 3**). The other compounds were isolated in an insufficient quantity to perform biological trials.

In conclusion, biological assays showed that (−)-botryodiplodin (**1**) was responsible for the potent antifungal, antibacterial, and insecticidal activities displayed by the organic extract from the fungus *P. coalescens*. The other tested metabolites (**2**, **5**, and botryodiplodin acetate) were not active in the insecticide assays. In accordance with the conclusions of other authors (*5*), it might pointed out that the free hydroxyl function of (−)-**1** seems to be essential for its activity, probably due to its open hemiacetal form with the aldehydic function as the active agent.

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Supporting Information Available: Tabulated 1D and 2D NMR data for compounds **1**−**9**. This material is available free of charge via the Internet at http://pubs.acs.org.

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