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

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ABSTRACT

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**). 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 of all the isolated metabolites, only (-)-**1** showed antifungal, antibacterial and insecticidal activity. According to our knowledge, this latter activity is 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*). 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* (*4*) was seen to exhibit potent antifungal, antibacterial and insecticidal activities. The known mycotoxin (-)-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 (**Figure 1**). (-)-**1** was isolated for the first time from *Botryodiplodia theobromae* Pat. (*5*), a fungus responsible for considerable damage in tropical plants (*6*), and was structurally elucidated by Arsenault et al. in 1969 (*7*). Afterwards, (-)-**1** was found in other fungal species like *P. roqueforti* strain (*6, 8, 9*), *P. stipitatum* (*10, 11*), *Macrophomina phaseolina* (*12*) and others (*13*). In addition, *P. roqueforti* and *M. phaseolina* fungi have been reported as a contaminant of processed food (*9*) and the causal agent of numerous plant diseases (*12*), respectively. (-)-**1** has received attention because of its potent antibiotic (*5*), antileukemic (*14*) and mutagen (*15*) activities as well as the ability to induce protein-DNA cross-links in mammalian cells (*16-20*) and to inhibit cell multiplication in growing cultures (*21*). It has been proposed that biogenetically, (-)-**1** belongs to the polyketide pathway (*22*). Furthermore, several syntheses of (-)-**1** and its derivatives have been reported (*6, 23-35*).

Herein are depicted the isolation, structural elucidation and chemical structure relationships for the new analogues.

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 by a Waters HPLC system with a 600 pump and both a 2996 Photodiode Array Detector (PDA) and ELSD 2420 Detector (Milford, USA). ^{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 carried out on a Micromass Q-TOF micro (Milford, USA). IR spectra were obtained with a 710FT spectrophotometer (Nicolet, Madison, Wisconsin, USA). Optical rotations were determined with a Perkin-Elmer 241 Polarimeter (Massachusetts, USA).

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 ºC. Then, a solution of Tween 80 (0.05%) in sterile distilled water was used to obtain a suspension containing ca. 10^6 conidia/mL. This suspension was poured to 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 ºC.

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 x 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, 100% ethyl acetate to 100% methanol. The volume eluted in each step was 2 L and thirteen fractions were obtained, evaporated to dryness and tested for biological activities. The dried mycelium (176 g) was firstly extracted with dichloromethane (3 x 1 L) to give 0.25 g of dichloromethanic extract, and then with methanol (3 x 1 L) to obtain 13.5 g of methanolic extract.

Isolation and Purification of Secondary Metabolites. The 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 the (-)-botryodiplodin (**1**, 1.5g, 18.52%). This major compound (-)-**1** was not visible neither at λ 254 nm nor λ 365 nm and Cerium (IV) sulfate was employed for its visualization on the TLC plate.

^{1}H NMR spectra of 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 using a gradient from 65% hexane in ethyl acetate to 100% ethyl acetate to give two promising subfractions 7 and 8. Second fractionation by silica gel column of the subfraction 8 afforded compound **2** (19.2 mg, 0.24%) while the 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.

The F-IV was purified by medium pressure flash chromatography (Biotage SP1 coupled to an UV detector 254 λ) using a gradient from 100% hexane to 100% ethyl acetate. The 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%). The F-III was purified by silica gel column (hexane/ethyl acetate, 8:2, v/v) to afford compound **5** (18 mg, 0.22%) in the subfraction 3. Analysis of pure compounds was achieved via analytical RP-HPLC using a Tracer Excel ODSB C18 column, 4 µm (25.0 x 0.46 cm), 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. The subfractions 2 and 6 were purified by semipreparative RP-HPLC using a Tracer Excel ODSB C18 column, 5 µm (25.0 x 1.0 cm) and eluting the mobile phase with a flow of 2 mL/min. The 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 the subfraction 2 was run 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**): $[\alpha]_D^{25}$= -99.1º (*c*. 3.3, $CHCl_3$); colourless oil; IR ν_{max} 2965, 2899, 1700, 1521, 1455, 1362 cm^{-1}; HRESMS *m/z* 293.1360 $[M+Na]^+$ ($C_{14}H_{22}O_5Na$ calc 293.1365). ^1H NMR (300 MHz, $CDCl_3$) δ 5.03 (1H, *brs*, H-2), 4.28 (1H, *t*, *J*=8.7, Ha-5), 3.93 (1H, *t*, *J*=8.7, 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, 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**): $[\alpha]_D^{25}$= +9.0º (*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); ^1H 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, H-2), 4.25 (1H, *dd*, *J*=9.0, 6.3, Ha-5), 3.92 (1H, *dd*, *J*=9.0, 7.8, Hb-5), 3.69 (1H, *dd*, *J*=9.3, 6.0, Ha-5'), 3.30 (1H, *dd*, *J*=9.3, 6.0, 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, H-7'), and 0.93 (3H, *d*, *J*=7.2, 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**): $[\alpha]_D^{25}$= -39.0º (*c*. 10.3, $CHCl_3$); colourless 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); ^1H 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, H-6), 4.60 (1H, *d*, *J*=3.6, H-2), 3.88 (1H, *dd*, *J*=12.0, 0.5, Ha-4), 3.78 (1H, *dd*, *J*=12.0, 3.0, Hb-4), 3.64 (1H, *dd*, *J*=9.0, 7.2, Ha-5"), 3.44 (1H, *dd*, *J*=9.0, 6.0, 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, H-7") 1.10 (3H, *d*, *J*=6.9, H-1'), and 1.06 (3H, *d*, *J*=6.9, 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**): $[\alpha]_D^{25}$= -75.0º (*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); ^1H 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, Ha-5), 3.87 (1H, *t*, *J*=8.4, Hb-5), 3.53 (1H, *dd*, *J*=15.9, 8.4, H-4), 3.44 (1H, *dd*, *J*=9.3, 7.2, Ha-5'), 3.32 (1H, *dd*, *J*=9.3, 5.7, 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, H-7'), and 0.81 (3H, *d*, *J*=7.2, 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-Methyl-botryodioxanenone (**6**): $[\alpha]_D^{25}$= -6.5º (*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). ^1H 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, H-7a), 4.75 (1H, *d*, *J*=4.8, H-2), 4.08 (1H, *dd*, *J*=8.5, 6.6, 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, H-9), 1.04 (3H, *d*, *J*=7.2, H-5'), and 0.98 (3H, *d*, *J*=7.2, 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-methyl-botryodioxanenone (**7**): $[\alpha]_D^{25}$= +3.9º (*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). ^1H 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, H-7a), 4.80 (1H, *d*, *J*=4.5, H-2), 4.19 (1H, *t*, *J*=8.4, Ha-6), 4.03 (1H, *dd*, *J*=13.5, 6.6, H-4), 3.41 (1H, *t*, *J*=8.4, 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, H-9), 1.03 (3H, *d*, *J*=7.2, H-5'), and 0.97 (3H, *d*, *J*=6.6, 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-Methyl-botryodioxanenone (**8**): $[\alpha]_D^{25}$= -8.7º (*c*. 0.92, $CHCl_3$); colourless 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); ^1H 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, H-2), 4.22 (1H, *t*, *J*=9.0, Ha-6), 3.98 (1H, *dd*, *J*=12.0, 0.5, Ha-4), 3.82 (1H, *dd*, *J*=12.0, 3.0, Hb-4), 3.50 (1H, *t*, *J*=9.0, 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, H-5'), and 1.08 (3H, *d*, *J*=6.0, 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.

(+)-Ethoxyphenyl-botryodiplodin (**9**): $[\alpha]_D^{25}$= +13.3º (*c*. 0.75, $CHCl_3$); colourless 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, H-2), 4.21 (1H, *dd*, *J*=9.6, 6.6, 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, H-10), 2.55 (1H, *m*, H-3), 2.12 (3H, *s*, H-7), and 0.95 (3H, *d*, *J*=7.8, 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 ± 1ºC, 50-60% relative humidity and a 16h/8h (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 12 phytopathogens: *Verticillium dahliae* (CCM 269), *Aspergillus parasiticus* (CECT 2681), *C. gloecesporoides* (CECT 2859), *Fusarium culmorum* (CECT 2148), *F. oxysporum* ssp. *gladioli* (CCM 259), *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 carried out 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 appropriated 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 x 10 x 10 cm) 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 dose 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 ºC. Then a solution of Tween 80 (0.05%) in sterile distilled water was used to obtain a suspension containing ca. 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 Wathman disks (nº 113, 0.5 cm diameter) impregnated with the tested products, at appropriate doses, were added in 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-benzymidazolecarbamate; Sigma), at different concentrations according to the fungus species assayed, were used as positive controls. Fungicidal activity was determined 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 ºC or 37 ºC, according to the bacterium. Then, 1 mL of this suspension was inoculated in a Petri plate, and 15 mL of culture medium Plate Count Agar (Difco) were 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 28ºC or 37ºC, 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. Results of χ^2 analysis for goodness of fit for the regression equation revealed 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, ^{1}H and ^{13}C NMR with the data reported (*8*). F-IX was treated with Ac_2O in pyridine giving in a quantitative yield the 2,3-*trans*-botryodiplodin acetate (*7*), confirming the structure of (-)-**1**. It was obtained as a colourless oil and according to precedent literature (-)-**1** has the property of turning the skin pink after 2-3 h following application (*14*).

The (-)-botryodiplodin dimer (**2**) was identified in view of its ESMS, ^{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 (calcd 293.1365 for $C_{14}H_{22}O_5Na$), indicating a molecular formula containing twice the number of carbon and proton 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 (*8*) and the coupling constant value $J_{2,3}$= 0 Hz, indicating a *trans* relationship between H-2 and H-3 (*6, 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 (-)-**1**, and its HRESMS showed an $[M+Na]^{+}$ ion of *m/z* 277.1421 ($C_{14}H_{22}O_4Na$, calc 277.1416) suggesting the presence of the furan core linked to other C_7 fragment (C-1' to C-7'). Given that the 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 into this C_7 fragment was proposed. Analysis of the 2D NMR spectra revealed that the linkage between this C_7 moiety and botryodiplodin core was at hydroxyl group in C-2 since HMBC correlations were observed from the H-2 (δ 4.70) to C-5' (δ 71.66). Furthermore, assuming that the stereogenic centres 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 out a *trans* coupling with H-3 (*6, 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.

(+)-Botryodiplodinenone (**3**) was elucidated on the basis of the great similarity between its ^{1}H and ^{13}C NMR spectra with compound (-)-**5**. In addition, compound (+)-**3** also gave the same $[M+Na]^{+}$ ion in HRESMS, *m/z* 277.1415 ($C_{14}H_{22}O_4Na$, calc 277.1416). The unique major variation in ^{1}H NMR of (+)-**3** was the multiplet at δ 3.11 integrating for two protons that through COSY and HSQC experiments it was assigned to H-4 and H-4'. The ^{13}C NMR 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) (*6, 40*). Therefore, the inspection of the MS, 1D and 2D NMR 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-methyl-botryodioxanenone (**8**) gave an $[M+Na]^+$ ion *m/z* 277.1419 that established the molecular formula as $C_{14}H_{22}O_4Na$ (calc 277.1416). Its resemblance with ^1H and ^{13}C NMR data of (-)-**1** indicated that it was a new membership into this group bearing a 3-methylenepentan-2-one moiety, probably provided 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 like on the bridge of a fused bicycle. Two highly deshielded sp^3 carbons at δ 105.41 (C-7a) and 98.82 (C-2) were suitable to carbons between two oxygen atoms. In view of the correlations provided by HMBC from the C-2 (δ 98.82) to H-4' (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 acetalic carbon C-2. 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 stereocentres, 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 to H-9 (δ 1.46, *s*) and H-4' (δ 3.06, *m*), enabling to suggest a (2*R*,4a*S*,5*R*,7a*R*,4'*R*)-**8** configuration.

Analysis of spectral data of (-)-botryodioxandiendione (**4**) proved that it was an analogue of compound (-)-**8**, attached to another 5-hydroxy-4-methyl-3-methylene-pentan-2-one moiety (C_7 unit). HMBC experiment showed correlations from the H-6 resonance (δ 4.78) to carbon C-5" (δ 72.67) revealed the link between C-6 of 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 (*6, 40*). Unfortunately, compound **4** was chemically unstable suffering facile degradation and making its NOE studies inaccessible, therefore the stereogenic centers C-7a and C-2 could not be determined.

The structure of (-)-4-methyl-botryodioxanenone (**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 on the bicycle differenced this new compound of 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 the 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 the 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 (*6, 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 1D and 2D NMR spectra of (+)-4-epi-methyl-botryodioxanenone (**7**), an steroisomer of compound (-)-**6** was proposed. The great difference in ^{1}H NMR was the deshielding experimented 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 compound (-)-**6**, the biogenetic route, the coupling constant $J_{7a,4a}$=3.6 Hz and 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 (*6, 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 (+)-ethoxyphenyl-botryodiplodin (**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) (*6, 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**.

Chemical Structure Relation Between Metabolites. It was concluded that except for (-)-**2** and (+)-**9**, which are clearly derivatives of **1**, all the new 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 highlighted 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 to the acetalic compounds (-)-**6** and (+)-**7**. Also the unit U3 could undergo intramolecular ring closure to provide other dihydroxy derivative which could react with an aldehyde U2 to obtain the 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 dichloromethanic extract nor the methanolic extract of the mycelium showed biological activity. For the antifungal and antibacterial assays, the 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 the all fractions, the F-IX was found to be the 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 test 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 the 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 to other authors (*6*), 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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FIGURE CAPTIONS

Figure 1. Natural Metabolites (**1-9**) from *P. coalescens*.

Figure 2. Chemical Structure Relationships of Botryodiplodin Analogues **3**-**8**.

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

Fungal strains	**Fungicidal activity** Inhibition zone (mm) 72h (means ± SE)[a]	
	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}
Bacterial strains	**Bactericidal activity** Inhibition zone (mm) 24h (means ± SE)[a]	
	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}
Insects	**Insecticidal activity** % acute mortality 72h	
O.fasciatus	100 ± 0[e1]	
C.capitata	100 ± 0[e2]	

[a] Each value represents the average and the standard error of three independent experiments. Whithin each line mean values labelled with the same superscript (A-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.

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

Fungal strains	**Fungicidal activity** Inhibition zone (mm) 72h (means ± SE)[a]		
	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]
Bacterial strains	**Bactericidal activity** Inhibition zone (mm) 24h (means ± SE)[a]		
	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

Insects	**Insecticidal activity**	
	FIX %mortality 72h	(-)**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.

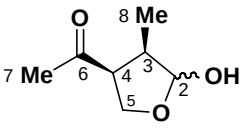
Table 3. Insecticidal Activity of *P. coalescens* Metabolites Against *O. fasciatus*.

Pure Compounds	**Isecticidal activity[a]**				
	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	-	-	-

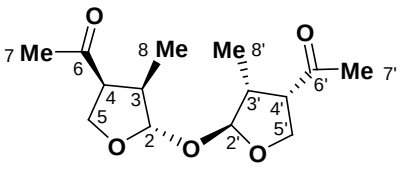
[a] Regression analysis, linear model: $y=ax+b$; log dose vs probit mortality.

[b] Values in μg/nymph, were determined 72 h after exposure to the chemical.

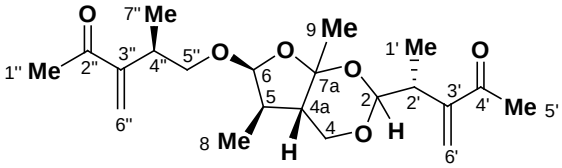
Figure 1.



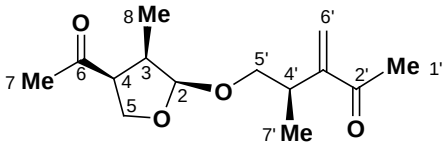
(-)-Botryodiplodin (1)



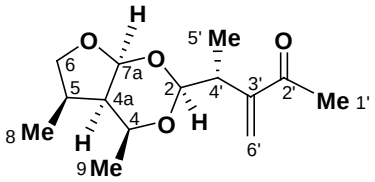
(-)-Botryodiplodin dimer (2)



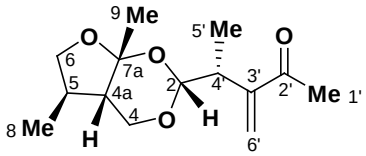
(-)-Botryodioxandiendione (4)



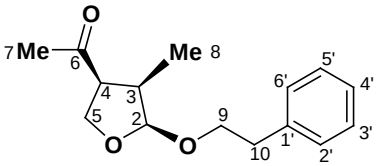
(+)-Botryodiplodinenone (3)
(-)-2-Epi-botryodiplodinenone (5)



(-)-4-Methyl-botryodioxanenone (6)
(+)-4-Epi-methyl-botryodioxanenone (7)

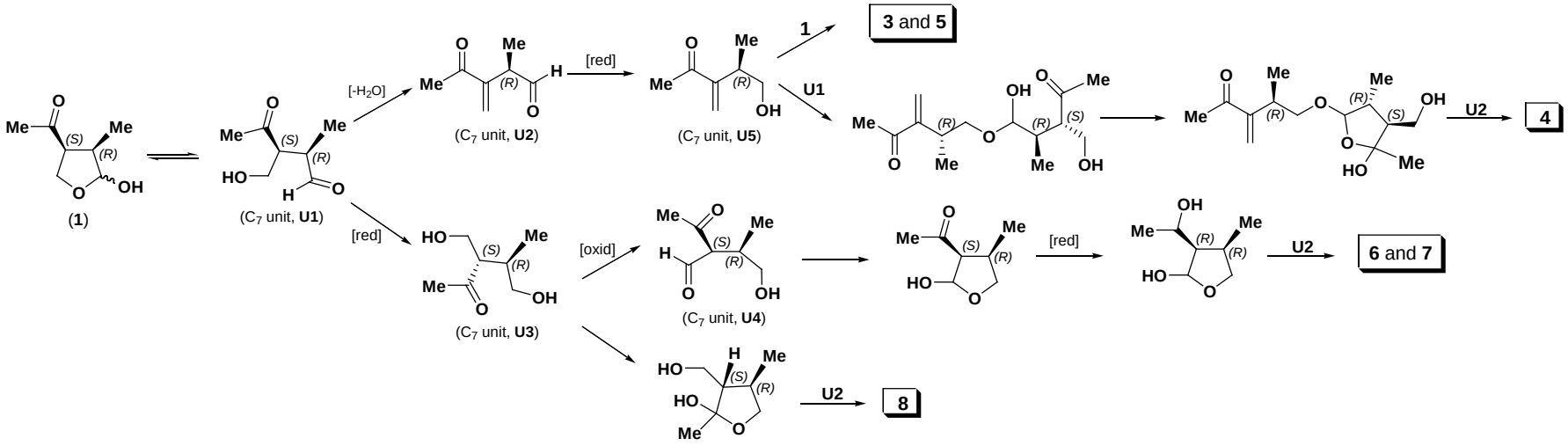


(-)-7a-Methyl-botryodioxanenone (8)



(+)-Ethoxyphenyl-botryodiplodin (9)

Figure 2.


(1)
(C7 unit, U1)
[-H2O]
(C7 unit, U2)
[red]
(C7 unit, U5)
1
3 and 5
U1
U2
4
[red]
(C7 unit, U3)
[oxid]
(C7 unit, U4)
[red]
U2
6 and 7
U2
8