further purified by column chromatography on silica gel eluting with $CHCl_3$ to give the pure aldehyde **5c**, which was redistilled to give an analytical sample: IR (neat) 1740 cm^{-1}; NMR ($CDCl_3$) δ 9.50 (s, 1 H), 3.47 (s, 2 H), 3.30 (s, 6 H).

Anal. Calcd for $C_5H_9BrO_3$: C, 30.48; H, 4.60; Br, 40.55. Found: C, 30.42; H, 4.64; Br, 40.49.

3-Halo-2-oxopropanal (Halomethylglyoxal, 6). A solution of 2,2-dimethoxy-3-halo-1-propanal (**5**; 0.7 mmol) in D_2O (0.5 mL) was placed in an NMR tube. DCl, 10 N, was added to a final concentration of 1 N. ^1H NMR spectra were recorded at time intervals of $T = 0$ (no DCl), 1 h, 24 h, 72 h, and 1 week at room temperature. The extent of deketalization was determined by comparing the ratios of the OMe (ketal) to the OMe (methanol) peak and was found to be complete after 1 week to give 3-halo-2-oxopropanal (halomethylglyoxal, **6**).

Fluoromethylglyoxal (6a): ^1H NMR (D_2O; Me_4Si external standard, $T = 0$) δ 5.07 (d, 1 H, $J = 2$ Hz), 4.50 (d, 2 H, $J = 47$ Hz), 3.27 (s, 6 H); ^1H NMR (D_2O + 1 N DCl, $T = 1$ week) δ 4.95 (d, 1 H, $J = 2$ Hz), 4.36 (d, 2 H, $J = 47$ Hz), 3.23 (s, 6 H); ^{19}F NMR (H_2O) proton coupled, −156.855 ppm (t, $J = 47$ Hz).

Chloromethylglyoxal (6b): ^1H NMR (D_2O; Me_4Si external standard, $T = 0$) δ 5.03 (s, 1 H), 3.58 (s, 2 H), 3.20 (s, 6 H); NMR (D_2O + 1 N DCl, $T = 1$ week) δ 4.73 (s, 1 H), 3.43 (s, 2 H), 3.07 (s, 6 H).

Bromomethylglyoxal (6c): ^1H NMR (D_2O; Me_4Si external standard, $T = 0$) δ 5.20 (s, 1 H), 3.57 (s, 2 H), 3.30 (s, 6 H); NMR (D_2O + 1 N DCl, $T = 1$ week) δ 4.90 (s, partially masked by HDO), 3.43 (s, 2 H), 3.17 (s, 6 H).

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Registry No. **1a**, 433-48-7; **1b**, 3681-17-2; **1c**, 1113-59-3; **2a**, 81371-75-7; **2b**, 55900-23-7; **2c**, 81371-76-8; **3a**, 62741-32-6; **3a**-d_2, 81371-77-9; **3b**, 81371-78-0; **3c**, 81371-79-1; **4a**, 62522-70-7; **4b**, 24423-98-1; **4c**, 38987-72-3; **5a**, 78381-95-0; **5a**-d_1, 81371-80-4; **5b**, 81371-81-5; **5c**, 81371-82-6; **6a**, 78381-94-9; **6b**, 81371-83-7; **6c**, 81371-84-8.

Botryodiplodin, a Mycotoxin Synthesized by a Strain of *P. roqueforti*

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A toxic strain of *Penicillium roqueforti* synthesized the botryodiplodin, a mycotoxin classically elaborated by the *Botryodiplodin theobromae* Pat., whose chemical structure and stereochemistry (obtained by X-ray analysis) are reported.

Toxic metabolites from *Penicillium roqueforti* have been reported by various authors. For example WEI[1] isolated and identified the PR toxin from culture media of the NRRL 849 *P. roqueforti* strain. From this and various other strains we have isolated closely related metabolites such as the eremofortins A–E.[2-4] Alkaloids such as roquefortine,[5] isofumigaclavine,[6] and marcfortine A[7] have also been isolated from the mycelium of this species. Significantly, Lafont[8] showed that the presence of PR toxin could not explain the toxicity of numerous strains of *P. roqueforti*. We have therefore turned our interest towards non-PR-toxin strains of this species, and in this paper we report the isolation and identification of a new toxin from *P. roqueforti*, botryodiplodin.

Results and Discussion

The examination of *P. roqueforti* isolated from various silages enabled us to detect non-PR-toxin producing strains. All were tested for toxicity on mice by intraperitoneal injections of crude chloroform extracts from cultured media. The toxic B-26 strain was chosen for identification of the toxic metabolites.

The chloroform extract of the culture medium was purified by chromatography over silica gel by using a stepwise gradient mixture of methanol–chloroform. The fraction elected by a 2/98 (v/v) mixture was revealed to be toxic.

Table I. Fractional Atomic Parameters

atom	x	y	z
O_1	0.7097	0.9293	−0.1521
O_2	0.4914	1.0112	0.1429
O_3	0.4024	0.8205	0.1466
O_4	0.2685	0.9182	0.2061
C_1	0.4218	0.9307	0.0183
C_2	0.4657	0.8774	−0.1555
C_3	0.5705	0.8541	−0.0310
C_4	0.5863	0.9621	0.1333
C_5	0.4601	0.9859	−0.3277
C_6	0.6455	0.8523	−0.1714
C_7	0.6346	0.7430	−0.3310
C_8	0.3243	0.8310	0.2333
C_9	0.3174	0.7108	0.3665

Further purification was achieved by HPLC. Crystallization from ethyl ether afforded a white crystalline sample of compound 1 (Figure 1).

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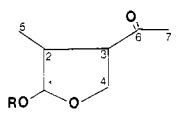

Figure 1. Botryodiplodin and botryodiplodin acetate.

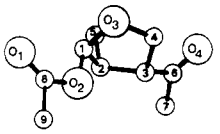

Figure 2. Stereochemistry of botryodiplodin acetate (X-ray diffraction).

Determination of the Structure

The IR spectrum of 1 exhibited a hydroxyl absorption at 3600 and 3400 cm^{-1}. Acetylation of this compound yielded a unique crystalline product, **2**. The structure of **2** was solved by X ray diffraction analysis.

This structure is identical with that of botryodiplodin acetate, a metabolite already characterized.[9] Similarly, the ^1H NMR spectrum is in concordance with the data previously published.[9] The relative stereochemistry is characterized by a *cis* relationship between protons 2 and 3 and a trans relationship between protons 1 and 2. This acetate is a single anomer at the anomeric proton 1.

The structure of the natural compound **1** is therefore the alcoholic derivative. The ^1H NMR spectrum of **1** appeared to be the superposition of two spectra, indicating a mixture of two anomers in solution whose relative amounts vary during the spectral measurement.

Botryodiplodin **1** has been isolated previously from *Botryodiplodia theobromae* and has antibiotic compound. Moulé recently investigated the mutagenic properties of botryodiplodin[11] and the effects on mammalian cells in culture.[12]

We are currently interested in the mode of action of this mutagenic compound.

Experimental Section

Infrared spectra were obtained with a Perkin-Elmer 447 spectrometer. ^1H NMR spectra (60 MHz) and ^{13}C NMR spectra (15,1 MHz) were recorded on a WP-60 Bruker spectrometer. Mass spectra were determined on an AEI Model MS 12 spectrometer for low-resolution and on a Model MS 9 for high-resolution. Ultraviolet spectra were run on a Cary II spectrometer. A Perkin-Elmer 141 polarimeter was used for measurement of specific rotation. All melting points are uncorrected.

Organisms. The B-26 strain used was isolated from contaminated corn silage by J. Pelhate (Brest, France) and was certified as *P. roqueforti* in 1979 by the "Central bureau voor schimmelcultures" of Baarn (Holland). The culture was maintained on potato dextrose agar slants.

Medium. The basal medium consisted of 2% yeast extract (Difco) and 15% sucrose in demineralized water. The medium was sterilized by filtration on membrane filters (0.2-μm, Millipore Corp.).

Cultures. The cultures were maintained in 800-mL Roux bottles containing 150 mL of medium. Each was inoculated with about 10^6–10^8 spores of *P. roqueforti* from potato dextrose agar slants. The Roux bottles were incubated as stationary cultures for 14 days at 25 °C.

Table II. Bond Lengths and Angles

bond	length, Å	bond	length, Å
C_8-O_4	1.186	C_1-O_2	1.403
C_6-O_1	1.194	C_4-O_2	1.448
C_8-O_3	1.352	C_2-C_3	1.556
C_1-O_3	1.471	C_4-C_3	1.53
C_9-C_8	1.528	C_1-C_2	1.51
C_3-C_6	1.547	C_2-C_5	1.576
C_6-C_7	1.519		
bond	**angle, deg**	**bond**	**angle, deg**
C_1-O_3-C_8	116.5	C_4-C_3-C_2	103.7
C_0-C_8-O_4	125.2	O_2-C_1-O_3	108.3
C_9-C_8-O_3	109.4	C_2-C_1-O_2	107.3
C_3-C_6-O_1	122.3	C_2-C_1-O_3	107.8
C_7-C_6-O_1	122.0	C_1-C_2-C_3	99.4
C_7-C_6-C_3	115.7	C_5-C_2-C_3	112.4
C_1-O_2-C_4	108.8	C_5-C_2-C_1	108.3
C_2-C_3-C_6	113.2	C_3-C_4-O_2	106.3
C_4-C_3-C_6	114.1		

Extraction. The cultures were filtered, and the mycelium was carefully washed with demineralized water. The media of 25 bottles were collected together and extracted with chloroform. The chloroform phases were collected and evaporated to dryness to give a crude extract (1.1 g).

Toxicity on Mice. The crude extracts were injected by the intraperitoneal route (500 mg/kg dose dissolved in 0.1 mL of Me_2SO) into three swiss male mice. Death occurred within 40 h. Chromatographic fractions were tested (100 mg/kg doses) in the same manner.

Purification of Botryodiplodin. The crude extract was purified over a silica gel column by elution with a stepwise gradient of methanol–chloroform (v/v: 0/100, 2/98, 5/95, 10/90). Each fraction was tested for toxicity on mice. The 2/98 fraction was revealed to be toxic. TLC plates, eluted with a 5/95 mixture of the same solvents and developed by spraying with sulfuric acid and subsequent heating at 120 °C for 20 min, showed mainly a red spot.

Further purification was achieved by HPLC by using a semipreparative column (50 cm long, 1 cm i.d.) of 5–20-μm Lichroprep (Merck). A 500-mg sample of the toxic fraction was injected and eluted with a mixture ethyl acetate/*n*-hexane (70/30 v/v). The RI detector was used to follow the elution. The major product was eluted after 10 min. Subsequent collection and testing of this fractions revealed it to be the toxin. Crystallization from ethyl ether afforded a white crystalline compound.

Characterization of the botryodiplodin toxin: mp 50–52 °C; mass spectrum, *m/e* 126, 98, 87, 85, 83, 71. High-resolution mass spectrum for *m/e* 126 ($M^+ - H_2O$), *m/e* 126.0688 (calcd for $C_7H_{10}O_2$ 126.06807); IR ($CHCl_3$) 3600, 3400, 1720 cm^{-1}; UV ($CHCl_3$) λ_{max} 278 nm (ϵ 25); ^1H NMR (90 MHz) δ 0.8, 0.9, 1, 1.10 (3 H), 2.2, 2.7 (3 H), 2.8 (1 H, m), complex multiplet between δ 3.3 and 4.4 (4 H), 5.2 (1 H).

Botryodiplodin Acetate. Pyridinic acetylation of botryodiplodin gave a compound which crystallized from ethyl ether: mp 45–47 °C; $[\alpha]_D$ – 104° (c 0.09, $CHCl_3$); mass spectrum, *m/e* 126 (M^+ – ACOH) 98, 87, 85, 83, 71; IR ($CHCl_3$) 1735, 1720 cm^{-1}; UV ($CHCl_3$) λ_{max} 277 nm (ϵ 29); ^1H NMR (60 MHz) δ 0.92 (3 H, d, J = 7.5 Hz, CH_3-5), 2.06 (3 H, s, CH_3-9), 2.23 (3 H, s, CH_3-7), 2.7 (1 H, m, J = 7.5 Hz, H-2), 3.8 (1 H, m, H-3), 4.22 (2 H, m, H-4), 5.95 (1 H, s, H-1); ^{13}C NMR (15.1 MHz) δ 104.2 (d, C-1), 41.07 or 52.8 (d, C-2), 52.8 or 41.7 (d, C-3), 6.77 (t, C-4), 12.2 (q, C-5), 205.4 (s, C-6), 30.3 (q, C-7), 170.3 (s), 21.3 (q, C-9).

X-ray analysis of botryodiplodin acetate: $C_7H_{12}O_3$, monoclinic, $P2_1$, a = 14.192 Å, b = 10.300 Å, c = 6.535 Å, $\alpha = \beta$ = 90°, γ = 102.25°, temperature 123 K; 1643 reflexions were considered in the determination of the structure [λ(Cu Kα) 1.5418 Å].

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