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ISOLATION AND STRUCTURE OF HARZIANUM A: A NEW TRICHOTHECENE FROM *TRICHODERMA HARZIANUM*

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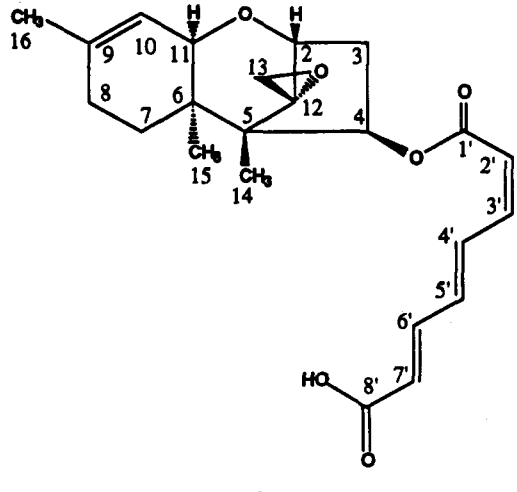
ABSTRACT.—A new trichothecene, harzianum A [**1**], was isolated from the soil-borne fungus *Trichoderma harzianum*. The structure of **1** was determined by extensive spectral analyses including the nmr techniques of PS-COSY, HMQC, HMBC, and NOESY. Harzianum A [**1**] contains a (*Z,E,E*)-2,4,6-octatriendioic acid esterified on the 4β hydroxyl group of trichodermol and is structurally related to the trichoverroids. Harzianum A [**1**] showed no cytotoxicity against baby hamster kidney cells, no activity against Gram-negative and Gram-positive bacteria, but modest antifungal activity at 100 μg/ml.

The trichothecenes are a well-studied class of sesquiterpene-based mycotoxins and, in general, are potent cytotoxins to eukaryotic cells (1,2). The majority of trichothecenes are produced by the genera *Fusarium, Stachybotrys, Verticimonosporium, Myrothecium, Trichothecium,* and *Cephalosporium* (3). To date, only a few trichothecenes have been found in *Trichoderma* species with no reports of trichothecenes isolated from *Trichoderma harzianum*. This species has received attention as a potential biocontrol agent due to its mycoparasitic properties, and diterpenes, peptaibols, butenolides, furanones, pyrones, and pyridones have previously been reported (4–9). In this publication, the isolation and structure determination of the novel trichothecene, harzianum A [**1**], are described. Harzianum A [**1**] is structurally related to the trichoverroids produced by *Myrothecium verrucaria* (10).

Harzianum A [**1**] was isolated as a colorless oil by reversed-phase hplc. The hr fabms of **1** yielded a molecular formula of $C_{23}H_{28}O_6$ indicating ten degrees of unsaturation. The ^{1}H-nmr spectrum of **1** showed the expected resonances for a trichothecene nucleus: a vinyl methyl at δ 1.722 (3H, bs, H-16); a 1,1-disubstituted epoxide AB pattern at δ 2.856 (1H, d, J=4 Hz, H-13β) and δ 3.156 (1H, d, J=4 Hz, H-13α); an olefinic proton at δ 5.422 (1H, dd, J=5.5, 0.8 Hz, H-10); two oxygen-bearing methine signals at δ 3.645 (1H, d, J=5.5 Hz, H-11α) and δ 3.868 (1H, d, J=5.2 Hz, H-2β); and two



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methyl singlets at δ 0.732 (3H, s, H-14) and δ 0.967 (3H, s, H-15). An additional signal in the ^{1}H-nmr spectrum at δ 5.661 (1H, dd, J=7.8, 3.4 Hz, H-4α), which correlated in the one bond proton-carbon experiment HMQC to C-4 at δ 75.2 and correlated in the long-range proton-carbon experiment HMBC to a carbonyl at δ 165.7, indicated an ester linkage at this site. The uv spectrum (λ max=303 nm) was indicative of a triene and was supported by PS-COSY data showing a coupled six-proton olefinic spin system at δ 5.874 (1H, d, J=11.1 Hz, H-2′), δ 6.658 (1H, t, J=11.4 Hz, H-3′), δ 7.948 (1H, ddd, J=15.0, 11.6, 0.7 Hz, H-4′), δ 6.568 (1H, ddd, J=15.4, 11.4, 0.6 Hz, H-5′), δ 7.517 (1H, ddd, J=15.4, 11.4, 0.4 Hz, H-6′) and δ 6.026 (1H, d, J=15.4 Hz, H-7′). The HMBC nmr spectrum established that both ends of the triene terminate with carboxyl moieties by showing H-2′ to C-1′ and H-7′ to C-8′ correlations (Table 1). The HMBC spectral data further showed the C-1′ carboxyl was ester-linked to the C-4 carbon leaving C-8′ as the carboxylic acid observed in the ir spectrum. Inspection of coupling constants and a strong nOe correlation between H-2′ and H-3′ indicated a cis-trans-trans stereochemistry for the 2′,4′,6′-octatrienedioic ester. Strong nOe correlations between H-3′ and H-5′, H-4′ and H-6′, and H-5′ and H-7′ indicated a staggered position as the preferred conformation of the triene in $CDCl_3$. Observed nOe correlations between H-4α and H-3α, H-11α, and the H-15 methyl group pointed to a β-ester configuration which is also consistent with known macrolide trichothecene stereochemistry. Since **1** shares the same trichothecene nucleus as trichodermol, it is likely that they are consistent in absolute stereochemistry (11,12).

Harzianum A [**1**] showed no cytotoxicity against baby hamster kidney cells at concentrations up to 1 μg/ml (13), no

TABLE 1. Carbon, Proton (J in Hertz), HMBC, NOESY and PS-COSY Data for Harzianum A [**1**] ($CDCl_3$).

Position	δ C-13	δ 1-H	HMBC[a]	NOESY	PS-COSY
2β	79.1	3.87 d (5.2)	4, 11, 12	3α, 3β, 13α	3β
3α	36.8	2.59 dd (15.4, 7.8)	2, 5, 12	2β, 3β, 4α, 11α	3β, 4α
3β		2.05 ddd (15.4, 5.2, 3.4)	4	2β, 3α	3α, 2β, 4α
4α	75.2	5.66 dd (7.8, 3.4)	6, 12, 1′	3α, 11α, 15	3α, 3β
5	49.2				
6	40.4				
7α	24.5	1.43 m		7β, 14, 15	7β, 8α, 8β, 11α
7β		1.94 m		7α	7α, 8α, 8β
8α	28.0	2.00 m			7α, 7β, 16
8β		2.00 m			7α, 7β, 16
9	140.4				
10	118.5	5.42 m	6, 8, 11, 16	11α, 16	11α, 16
11α	70.6	3.65 d (5.5)	9, 10, 15	3α, 4α, 10, 15	10, 7α
12	65.5				
13α	47.8	3.16 d (4.0)		2β, 13β	13β
13β		2.86 d (4.0)		13α, 14	13α
14	6.1	0.73 s	4, 5, 6	7α, 13β	
15	16.1	0.97 s	5, 6, 7	4α, 7α	
16	23.2	1.72 bs	8, 9, 10	10	8, 10
1′	165.7				
2′	121.4	5.87 d (11.3)	1′, 4′	3′	3′
3′	142.6	6.66 t (11.3)	1′, 5′	2′, 4′	2′, 4′
4′	136.6	7.95 ddd (15.3, 11.3, 0.6)	2′	3′	3′, 5′
5′	137.3	6.57 ddd (15.3, 11.3, 0.6)	3′	7′	4′, 6′
6′	145.8	7.52 ddd (15.3, 11.3, 0.6)	4′, 8′	4′, 5′	5′, 7′
7′	123.2	6.03 d (15.3)	8′	5′	6′
8′	170.8				

[a]Position of carbon resonances that were long-range correlated with proton.

antimicrobial activity against *E. coli, B. subtilis, M. luteus,* or *S. aureus* at 1 mg/ml, and modest activity against the fungi *C. albicans* and *S. cerevisiae* at 100 μg/ml in a well-diffusion assay.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The uv spectrum was determined on a H/P 8451A diode array spectrophotometer, and the ir spectrum was determined on a Nicolet 800 Ftir instrument. Low- and high-resolution mass spectra were collected on a Finnigan MAT 90 in the positive-ion mode. Nmr spectra were obtained in $CDCl_3$ (δ 7.26 was used as an internal reference) on a Varian Unity 500 MHz spectrometer utilizing a 5mm triple resonance (H-F-C) probe for the proton, carbon, PS-COSY (14) and NOESY (standard Varian sequence, mix=0.8 sec and presat=4 sec) experiments and a 5 mm indirect detection probe for the HMQC and HMBC (J=7 Hz) experiments (15).

FUNGAL MATERIAL.—A sample of micaceous clay was collected from a stream-bed near Windhoek, Namibia in 1988. From this sample a fungus was isolated using potato dextrose agar (Difco Labs., Detroit, MI) supplemented with rose bengal (100 mg/liter) (Allied Chemical & Dye Corp., New York), 2,6-dichloro-4-nitroaniline (5 mg/liter) (Aldrich Chemical, Milwaukee, WI), and streptomycin sulfate (100 mg/liter) (Sigma Chemical Co., St. Louis, MO). The fungus was identified as *Trichoderma harzianum* (16) and has been deposited with the American Type Culture Collection, ATCC 90237.

FERMENTATION CONDITIONS.—Spores of ATCC 90237 were inoculated into 250 ml shake flasks containing 50 ml of a medium consisting of maltrin (10 g/liter), soluble starch (5 g/liter), fish meal (5 g/liter), yeast extract (2.5 g/liter), $MgSO_4 \cdot 7H_2O$ (2 g/liter), KH_2PO_4 (0.5 g/liter), NaCl (0.5 g/liter), $CaCO_3$ (1 g/liter), $ZnSO_4$ (0.005 g/liter), and FeEDTA (0.0184 g/liter). The flask was placed on a rotary shaker at 250 rpm, at 30° for seven days prior to harvesting.

EXTRACTION AND ISOLATION.—A 200 ml aliquot of 0.2 μM filtered fermentation was vacuum-flashed (100 g Baker 40 μM C-18) with four 200 ml fractions collected: load effluent, H_2O-MeOH (7:3), H_2O-MeOH (3:7), and 100% MeOH. The 3:7 fraction was concentrated and hplc chromatographed on a Rainin 22 mm×30 cm Dynamax® 60A C-18 column utilizing the gradient H_2O-MeCN (90:10) (0.05% TFA in both solvents) to 100% MeCN over 60 min at 10 ml/min. Fractions eluting from 24 to 38 min yielded 18 mg of **1** as a colorless oil.

Harzianum A [**1**].—Colorless oil: ir ν max (neat) 3400 br (OH), 1701 vs (C=O), 1630 vs (C=O) cm^{-1}; uv λ max (MeOH) 204 (ε 9,200), 303 nm (ε 21,600) nm; hr fabms *m/z* $[MH]^+$ 401.1993 (calcd 401.1964, Δ 2.9 mmu); ^1H nmr (500 MHz, $CDCl_3$) and ^{13}C nmr (125 MHz, $CDCl_3$) see Table 1. On occasion, ^1H-nmr analysis of **1** in $CDCl_3$ gave broad featureless lines which subsequently sharpened when redissolved in d_4-MeOH. This is likely explained by π-stacking occurring in the octatrienoic acid side-chain.

BIOASSAYS.—Purified **1** was dissolved in DMSO and appropriately diluted so that final test concentrations were 20, 100, 500 and 1000 μg/ml. Two yeasts, *Saccharomyces cerevisiae* (ATCC 2366) and *Candida albicans* (ATCC 10231), three Gram-positive bacteria, *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923), *Micrococcus luteus* (ATCC 9341), and one Gram-negative bacterial species, *Escherichia coli* (ATCC 9723) were spread evenly over the surface of nutrient agar (Difco Labs., Detroit, MI) plates. Wells were cut in the agar using a #2 core borer (6 mm dia.) and 50 μl of sample added. Plates were incubated for 24 h at 30° prior to measuring inhibition.

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