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THE NOVEL DESMETHYLDESTRUXIN B_2, FROM *METARHIZIUM ANISOPLIAE*, THAT SUPPRESSES HEPATITIS B VIRUS SURFACE ANTIGEN PRODUCTION IN HUMAN HEPATOMA CELLS

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ABSTRACT.—We have examined the antiviral activity of a crude extract prepared from the culture medium of the fungus *Metarhizium anisopliae*. Eight active destruxins were identified which showed strong suppressive effect on the production of the hepatitis B surface antigen (HBsAg) in human hepatoma Hep3B cells. One new compound, desmethyldestruxin B_2 [**1**], was isolated from *M. anisopliae*. This structure was determined based on its nmr and mass spectral data.

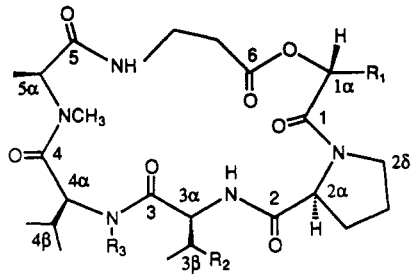
Infection by hepatitis B virus (HBV) frequently results in acute and chronic hepatitis and is also associated with a high risk of developing primary hepatocellular carcinoma in humans (1–4). Although immunization against HBV has been shown to prevent chronic infection (5), effective drugs to eradicate HBV in chronic carriers are still not available and need to be developed.

Recently, we demonstrated that the cyclic depsipeptides, homodestruxin B and destruxin B, from the fungus *Alternaria brassicaea* suppressed hepatitis B virus surface antigen (HBsAg) production in human hepatoma Hep3B cells (6). Inasmuch as the fungus *Metarhizium anisopliae* is the main source of the cyclohexadepsipeptide destruxins (7–9), we extended our search and found that an extract of *M. anisopliae* also suppressed HBsAg production in human hepatoma Hep3B cells. Subsequent bioactivity-directed fractionation resulted in the isolation of a family of destruxins [**1–8**] as the anti-HBsAg principles. More than 20 structurally related destruxins have been isolated to date (10–15), exhibiting a variety of biological activities (12,13,16). In vitro, destruxin E [**6**] disturbs macromolecular syntheses (DNA, RNA, and protein synthesis), displays antiviral activity (17), and modifies the DNA content of leukemia cells (18). In addition, destruxins have also been isolated as phytotoxins from plant pathogenic fungi (19,20).

Here we report the identification of a new cyclic depsipeptide, desmethyldestruxin B_2 [**1**], and seven previously reported destruxins, from the cultured medium of *M. anisopliae*.

RESULTS AND DISCUSSION

The culture broth of *M. anisopliae* was extracted with EtOAc and the crude extract was fractionated into acidic and neutral fractions. The neutral fraction was composed mainly of cyclic depsipeptides, which, on the basis of characteristic ^{1}H-nmr signals, could be identified as destruxins. A combination of Si gel cc and extensive reversed-phase hplc gave eight compounds, the new desmethyldestruxin B_2 [**1**], and previously described destruxins A [**2**], A_2 [**3**], B [**4**], B_2 [**5**], E [**6**], and desmethyldestruxins A [**7**],



1 $R_1=CH_2CH(CH_3)_2$, $R_2=H$, $R_3=H$
2 $R_1=CH_2CH=CH_2$, $R_2=CH_3$, $R_3=CH_3$
3 $R_1=CH_2CH=CH_2$, $R_2=H$, $R_3=CH_3$
4 $R_1=CH_2CH(CH_3)_2$, $R_2=CH_3$, $R_3=CH_3$
5 $R_1=CH_2CH(CH_3)_2$, $R_2=H$, $R_3=CH_3$
6 $R_1=CH_2CH(O)CH_2$, $R_2=CH_3$, $R_3=CH_3$
7 $R_1=CH_2CH=CH_2$, $R_2=CH_3$, $R_3=H$
8 $R_1=CH_2CH(CH_3)_2$, $R_2=CH_3$, $R_3=H$

and B [**8**]. Destruxins **1–8** could be readily identified by ^{1}H-nmr spectroscopy, and by comparison of their other spectral data with reported values (11–15).

The new destruxin [**1**] has a molecular formula of $C_{29}H_{47}N_5O_7$ ($[MH]^+$, *m/z* 566). The ^{1}H-nmr spectrum of **1** contained characteristic signals for one *N*-Me group (2.53 ppm), three amide protons (6.78, 8.26, and 8.63 ppm), and a methyl doublet (1.18 ppm) that belonged to an alanine side-chain. Also, the ^{1}H-nmr spectrum of **1** was similar to

TABLE 1. ^{1}H-Nmr Chemical Shifts for Desmethyldestruxin B_2 [**1**] and Destruxin B_2 [**5**].[a]

Proton(s)		Compound	
		1	**5**
Hia_1	α	4.9 dd (10.1, 3.3)	4.94 dd (9.7, 3.7)
	β	1.43 m, 1.67 m	1.48 m, 1.68 m
	γ	1.75 m	1.77 m
	δ	0.82 dd (6.5, 0.8)	0.90 dd (8.9, 5.7)
Pro_2	α	4.33 d (8.3)	4.38 d (7.4)
	β	1.98 m, 2.13 m	2.05 m, 2.13 m
	γ	1.72 m	1.77 m
	δ	3.40 m, 3.86 t (9.3)	3.48 m, 3.87 t (8.0)
Val_3	NH	6.78 d (9.6)	6.98 d (9.3)
	α	4.54 dd (9.6, 5.2)	4.80 dd (9.3, 5.8)
	β	1.95 m	2.10 m
	γ	0.76 d (6.8), 0.84 d (6.5)	0.81 d (6.8), 0.83 d (6.6)
Val_4	NH/NCH_3	8.63 d (6.5)	3.13 s
	α	4.27 dd (9.6, 6.7)	4.92 d (10.9)
	β	1.96 m	2.20 m
	γ	0.79 d (6.8), 0.98 d (6.8)	0.84 d (6.8), 0.87 d (6.4)
Ala_5	α	5.14 q (6.9)	5.15 q (6.7)
	β	1.18 d (6.9)	1.17 d (6.7)
	NCH_3	2.53 s	2.54 s
β-Ala_6	NH	8.26 dd (9.3, 3.5)	8.01 dd (9.3, 3.5)
	α	2.36 m, 2.69 m	2.38 m, 2.70 m
	β	3.00 br t (11.3), 3.79 m	2.92 t (11.2), 3.83 m

[a]Recorded at 400 MHz in DMSO-d_6 and assigned on the basis of a ^{1}H-^{1}H COSY nmr experiment. Chemical shifts are expressed in δ values, with coupling constants (*J*) in parentheses in Hz.

that of destruxin B_2 [**5**], except for the absence of the *N*-Me group at valine-4 (Table 1). Analysis of its spectroscopic data (ms, nmr) indicated that **1** was desmethyldestruxin B_2. A ^1H-^1H COSY spectrum provided additional support for the proposed structure of **1**.

Cultured Hep3B human hepatoma cells contain one to two copies of integrated HBV genomes and actively secrete HBsAg into the medium (21). The Hep3B cells were plated into a 24-well plate and allowed to attach overnight. The medium was changed to serum-free Dulbecco's Modified Eagle's Medium (DMEM) containing various concentrations of the tested compounds for 48 h. Desmethyldestruxin B_2 [**1**] significantly suppressed HBsAg production by the Hep3B cells with an IC_{50} value of 1.5 μg/ml. The compound itself did not interfere with the enzyme immunoassay of HBsAg determination (data not shown). The suppressive effect of desmethyldestruxin B_2 [**1**] was not due to the cytotoxicity of the compound, since treated cells were still viable and continued to proliferate during the 48-h incubation period (Figure 1). Inhibitory concentrations (IC_{50}, in μg/ml) were obtained for these cyclic destruxins [**1–8**] as summarized in Table 2. None of the destruxins showed toxicity to Hep3B cells.

We have demonstrated that the production of HBsAg in human hepatoma Hep3B cells is very sensitive to various agents (6,22) and can be developed as an effective assay system for screening biologically active natural products. The family of destruxins consists of a series of cyclic depsipeptides with α-hydroxy acid subunits. In order to evaluate the structure-activity relationship of destruxins, it is generally important to know the conformational characteristics of the major compounds, because they should be closely related to the biologically active form. A correlational study of the crystal structure and the ^1H-nmr analysis of the destruxins is currently underway.

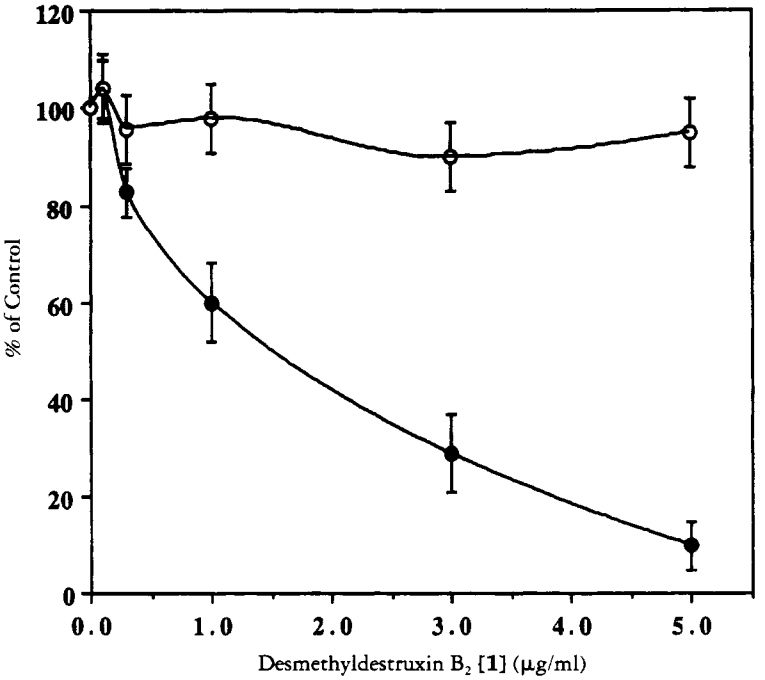


FIGURE 1. Effect of desmethyldestruxin B_2 [**1**] on the growth of production of HBsAg by human hepatoma Hep3B cells. Viable cells (○) and HBsAg (●) titers were measured in Hep3B cell cultures after 2 days of growth in serum-free DMEM containing desmethyldestruxin B_2 [**1**]. The titers obtained without desmethyldestruxin B_2 [**1**] were set for 100%. The bars represent the ranges for the triplicate determinations.

TABLE 2. Effect of Destruxins **1–8** on HBsAg Production in Hep3B Cells.[a]

Compound	IC_{50} (μg/ml)[b]
1	1.5
2	0.1
3	0.4
4	0.2
5	0.3
6	0.05
7	1.2
8	0.6

[a]All compounds at different concentrations were tested in triplicate for their effect on the production of HBsAg by Hep3B cells in 48 h (6,21).

[b]IC_{50} is the concentration of destruxin which affords 50% reduction on HBsAg production in Hep3B cells.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—^{1}H-Nmr data were recorded on a Bruker AM-400 instrument at 400 MHz. Chemical shifts are reported with respect to the solvent peaks (δ 2.49 for DMSO-d_6 at 310°K) as reference. Mass spectral data were obtained on a JEOL JMS-HX 110 mass spectrometer operating in the FAB mode. HBsAg enzyme immunoassay (EIA) kits were purchased from Ever New Corp. (Taipei, Taiwan). Fetal calf serum was obtained from Hyclone (Logan, UT).

CULTURE CONDITIONS.—*Metarhizium anisopliae* (ATCC 26474) was cultured in 2-liter flasks containing 1 liter of 3.9% Czapek-Dox broth (Difco) supplemented with 0.5% peptone (Difco).

EXTRACTION AND ISOLATION.—After 20 days on a gyro-rotary incubator at 120 rpm and 26°, the liquid culture was acidified with HCl, vacuum-filtered to get rid of the mycelia, and extracted three times with EtOAc. The crude extract was neutralized by the addition of $NaHCO_3$ and fractionated repeatedly by Si gel cc employing a stepwise *n*-hexane-to-EtOAc solvent gradient to give four fractions. Further purification was obtained by reversed-phase hplc on a C-18-AR semi-prep. column (5 μm Cosmosil, 6 mm×25 cm) using MeCN-H_2O (50:50, 1.0 ml/min) as the eluent. The following compounds were separated and identified by hplc: destruxins A (R_t 9.40 min), A_2 (R_t 7.98 min), B (R_t 14.48 min), B_2 (R_t 11.64 min), E (R_t 6.74 min), and desmethydestruxins A (R_t 7.50 min), B (R_t 10.69 min), and B_2 (R_t 8.42 min).

Desmethyldestruxin B_2 [**1**].—Fabms *m/z* 566 $[MH]^+$; ^{1}H-nmr data, see Table 1.

CELL CULTURE.—Stock cultures of human hepatoma cells Hep3B were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum and antibiotics (100 units/ml each of penicillin and streptomycin) in a humidified atmosphere containing 5% CO_2 and 95% air at 37°. The cultures were passaged by trypsinization every fourth day. For bioassay, cells were plated in 24-well plates at a density of 1.0×10^5 cells/well in DMEM containing 10% fetal calf serum.

PREPARATION OF TESTED COMPOUNDS.—For bioassay, each compound was first dissolved in EtOH, filtered through a 0.25-μm fluoropore filter (Millipore), and added to cell cultures.

DETERMINATION OF HBsAg.—Hep3B cells were seeded in 24-well plates at a density of 1.0×10^5 cells/well in DMEM containing 10% fetal calf serum. After 24 h of incubation, cells were washed three times with phosphate buffered saline (pH 7.0) and incubated in serum-free DMEM containing various concentrations of test compounds for 48 h. The HBsAg in the culture medium were measured by an enzyme immunoassay (EIA kit). The viability of cells was determined by trypan blue exclusion and counted in a hemocytometer.

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