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SECONDARY METABOLITES FROM *FUSARIUM*. TWO NEW MODIFIED TRICHOTHECENES FROM *FUSARIUM SPOROTRICHIOIDES* MC-72083

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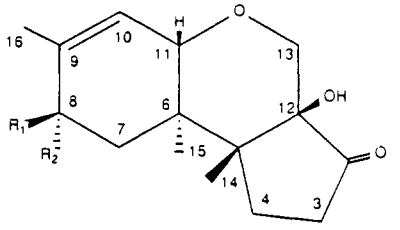
ABSTRACT.—Two new, relatively non-toxic, secondary metabolites characterized as 8α- and 8β-hydroxysambucoin [**1**] and [**2**], isolated from the toxigenic fungus *Fusarium sporotrichioides* MC-72083 are reported. The structural assignments were established by spectral data with ^{1}H-nmr studies (COSY, dnOes) playing a key role in establishing the stereochemistry in **1** and **2**. The isolation of 14 known trichothecenes produced by this fungus is also discussed.

The trichothecene mycotoxins are a growing class of sesquiterpenoid secondary metabolites produced by a variety of fungi, including *Fusarium, Stachybotrys, Verticimonosporium, Myrothecium, Trichothecium,* and *Cephalosporium* (1). Their occurrence is worldwide, and problems resulting from infestation are common. Symptoms resulting from trichothecene toxicosis include vomiting, skin inflammation, weight loss, and death in humans as well as agricultural animals (2).

Trichothecene-producing fungi from the genus *Fusarium* are well known for their pathogenicity to cereal grain for human consumption and to feedstuffs for agricultural use. One of the most severe mycotoxicoses occurred in Russia during the 1940s. In some villages, half the population was afflicted with alimentary toxic aleukia (ATA), which resulted in many deaths. It was postulated that ATA resulted from the consumption of moldy grain from overwintered crops. Eventually it was shown that T-2 toxin, produced by *Fusarium sporotrichioides* and *Fusarium poae,* was the major etiologic agent responsible for the outbreak of ATA (3). In our studies on the toxic fungus *F. sporotrichioides* MC-72083, we have reported several new trichothecene mycotoxins (4-6). We now report the isolation, characterization, and bioassay results of two new modified trichothecenes, 8β-hydroxysambucoin [**1**] and 8α-hydroxysambucoin [**2**]. Both are related to sambucoin [**3**], recently isolated by Tamm and co-workers (7). The isolation of fourteen known trichothecenes from *F. sporotrichioides* MC-72083 is also presented.



	R_1	R_2
1	OH	H
2	H	OH
3	H	H

A large scale work-up of the culture filtrate used a modified method of Burmeister (8). Approximately 800 jars were harvested in batches of 100-200 jars over a period of one year. The corn grits were extracted with $CHCl_3$-Me_2CO (85:15) (400 ml/jar) by blending at high speed until homogenized. The fungal-solvent mixture was allowed to stand overnight and suction filtered. The solid residue was reextracted with Me_2CO, suction filtered, autoclaved, and discarded. The Me_2CO extract was combined with the $CHCl_3$-Me_2CO (85:15) extract and concentrated under vacuum.

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The dark-red oil (ca. 0.5 liters/200 jars) was subjected to a hexane drip to remove the non-polar constituents. This was achieved by dripping the oil into a stirring solution of hexane-Me_2CO (85:15) (ca. 50 ml oil/2 liters solution) and allowing to stand for 24 h. The solvent was decanted and concentrated under vacuum, and the remaining solid residue from the drip was discarded.

The remaining orange-red oil was subjected to florisil column chromatography. The oil was dissolved in C_6H_6-hexane (2:1) to obtain a non-viscous solution (ca. 1:4, oil:solvent). The 100 ml of dissolved oil was applied to the column and washed with 300 ml of C_6H_6-hexane (2:1) and 300 ml of CH_2Cl_2. The trichothecenes were then eluted with 400 ml $CHCl_3$-MeOH (95:5), followed by 400 ml Me_2CO. By analyzing each fraction by tlc, it was determined that T-2 toxin eluted in the $CHCl_3$-MeOH (95:5) and the more polar trichothecenes, such as T-2 tetraol, eluted in the Me_2CO wash.

The majority of the crude T-2 was removed from the oil by crystallization from Me_2CO/hexane. Approximately 300 g of crude T-2 was obtained from the 800 jars.

Multiple runs of 2-g aliquots of crude T-2 (total 100 g) were flash chromatographed (9). The solvent system was toluene-Me_2CO (5:1) and fifty 20-ml fractions were collected for each run. Combining and concentrating the equivalent fractions revealed the presence of other minor metabolites. By tlc analysis (HLF silica plate, C_6H_6-Me_2CO, 3:1), the fractions preceeding T-2 were combined and concentrated. From this, approximately 20 mg of 4-propanoyl HT-2 was obtained (6). Combining and concentrating the fractions after T-2 toxin elution, the known compounds: 8-acetylneosolaniol (ca. 1 mg), NT-1 (ca. 10 mg), 4-acetylscirpentriol (<1 mg), and 3′-hydroxy T-2 (ca. 20 mg) were isolated and identified (4).

The remaining oil, after the crude T-2 was crystallized, was further separated by flash chromatography. Oil (10 g) was applied to a 4×45 cm flash column containing 6-in. flash grade Si gel. The column was eluted first with toluene-Me_2CO (3:1), where three 200-ml fractions were collected, and then eluted with toluene-Me_2CO (1:1), where two 200-ml fractions were collected. Tlc analysis of the five fractions [HLF silica plate, C_6H_6-Me_2CO (3:2), blue spray reagent] showed fraction 1 (ca. 2 g) to contain mainly T-2 (rRf=1.0), fraction 2 (ca. 6 g) containing several spots from HT-2 (rRF=0.32) to T-2, fraction 3 (ca. 1 g) containing spots from HT-2 to neosolaniol (rRf=0.55), fraction 4 (ca. 1 g) containing spots from rRf $\approx$0.2 to rRf $\approx$0.5, and fraction 5 containing spots from T-2 tetraol (rRf=0.15) to rRf $\approx$0.25.

Fraction 1 was subjected to further flash chromatography. Five hundred mg was applied to a 4×45 cm flash column and eluted with toluene-Me_2CO (5:1) and forty 20-ml fractions were collected. Diacetoxyscirpenol (DAS) (40 mg) was collected in the fractions after T-2 toxin and identified by nmr and co-tlc (4).

Fraction 2 proved to be an abundant source of known and new trichothecenes. A linear gradient preparative-hplc run was performed to isolate pure compounds. A typical run entailed injecting 250 mg of sample onto a 3.5×28 cm Si gel preparative column with a flow rate of 20 ml/min and a gradient of 16% Me_2CO against toluene over a 30 min time span. From repeated hplc runs, neosolaniol (ca. 300 mg) was obtained and identified by nmr and co-tlc (4). Trichodiol (2 mg) coeluted with 8β-hydroxytrichothecene (5 mg) (6). The two were separated by preparative rptlc using MeOH-H_2O (7:3). The two compounds sporotrichiol (8 mg) and sporol (4 mg), which also coeluted in the hplc, were purified by preparative rptlc using MeOH-H_2O (7:3). FS-1 (6 mg) was purified by preparative rptlc using MeOH-H_2O (7:3). The new compounds 8β-hydroxysambucoin [**1**] (1.5 mg) and 8α-hydroxysambucoin [**2**] (0.5 mg), which coeluted in the hplc, were purified by preparative tlc using $CHCl_3$-Me_2CO (9:1).

8β-Hydroxysambucoin [**1**] has a molecular formula $C_{15}H_{22}O_4$ (*m/z* 266.149, calcd

TABLE 1. ^{1}H-nmr Data (300 MHz, $CDCl_3$) on 8β-Hydroxysambucoin [**1**], 8α-Hydroxysambucoin [**2**], and Sambucoin [**3**] (7); ^{13}C-nmr Data on 8β-Hydroxysambucoin [**1**] (75.5) MHz, $CDCl_3$) and Sambucoin [**3**] (22.63 MHz, d_5-pyridine) (7)

Atom no.	Compounds				
	1		**2**	**3**	
	^{1}H	^{13}C	^{1}H	^{1}H[a]	^{13}C
2	—	210.2 s	—	—	216.1 s
3	2.50 ddd (2.8, 11.4, 19.9) 2.28 ddd (7.8, 10.2, 19.9)	34.2 t	2.60 ddd (3.3, 11.5, 19.6) 2.32 ddd (8.1, 10.2, 19.6)	2.58 ddd (3, 12, 19.5) 2.29 ddd (8, 10.5, 19.5)	34.7 t
4	2.02 m 1.81 m	27.5 t	2.07 m 1.81 m	2.02 ddd (3, 10.5, 14) 1.77 ddd (8, 12, 14)	27.2 t
5	—	46.3	—	—	46.9 s
6	—	41.1 s	—	—	38.0 s
7	1.45 dd (9.6, 12.9) 1.98 dd (5, 9, 12.9)	37.7 t	1.85 m 1.60 m	1.47-1.9	27.8 t
8	4.13 bm	69.4 d	4.08 m[b]		27.7 t
9	—	136.6 s	—	—	135.2
10	5.33 bs	125.3 d	5.35 bs	5.21 bs	122.9
11	4.03 bs	75.4 d	3.90 bs	3.96 bs	75.9 d
12	—	74.1 s	—	—	74.3 s
13	3.43 d (11.3) 4.21 d (11.3)	66.4 t	3.42 d (11.3) 4.20 d (11.3)	3.45 d (11) 4.21 d (11)	67.4 t
14	1.18 s	16.0 q	1.18 s	1.15 s	16.5 q
15	0.68 s	15.2 q	0.85 s	0.66 s[c]	14.3 q
16	1.76 bs	18.6	1.80 bs	1.64 bs	22.5 q

[a]360 MHz, $CDCl_3$.

[b]Sharpened upon addition of D_2O to broad doublet, J=5.2 Hz, which collapes to a broad singlet upon irradiation of δ 1.85, and also sharpens when δ 1.60 is irradiated.

[c]Misassigned in Mohr *et al.* (7).

266.152). The 300 MHz ^{1}H-nmr spectrum resembled that of sambucoin [**3**] (7) (Table 1) with an additional peak appropriate for a methine bearing oxygen at δ 4.13. COSY (10) and extensive difference nOe spectroscopic experiments (dnOes) were performed and are summarized in Figure 1. This allowed complete proton assignments to be made and established the stereostructure of **1** as depicted. Comparison of the ^{13}C nmr of **1** and **3** (7) confirmed the two compounds are closely related (Table 1) and supported the structure as shown.

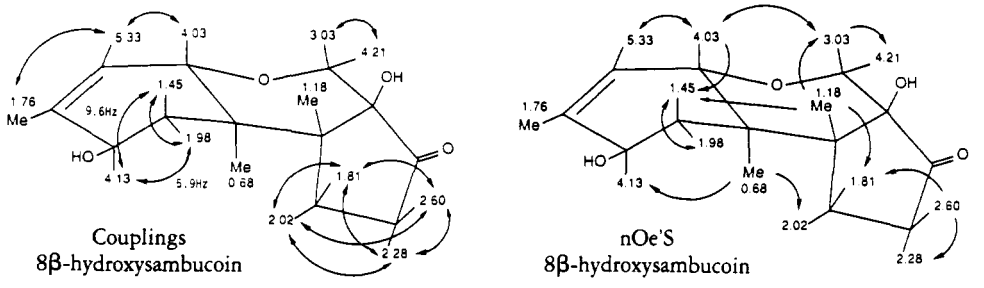

FIGURE 1. Coupling observed by cosy and nOe's derived from dnOes on 8β-hydroxysambucoin [**1**]

8α-Hydroxysambucoin [**2**], $C_{15}H_{22}O_4$, (*m/z* 266.151, calcd 266.152) was clearly an isomer of **1**. Comparison of the ^{1}H-nmr spectra of **1** and **2** also indicated the two compounds were closely related. Key differences are as follows: **1**/**2**; 7-H, δ 1.45, 1.98/δ 1.85, 1.60; 8-H, δ 4.13/δ 4.08; 11-H, δ 4.03/δ 3.90; 15-H, δ 0.68/δ 0.85; 16-H, δ 1.76/δ 1.80. This suggested that **1** and **2** differ by configuration at either C-6, C-8, or C-11. COSY and extensive dnOes results are summarized in Figure 2 and show clearly that **1** and **2** were epimeric at C-8. This led to complete proton and stereochemical assignments of **2**.

The new trichothecenes **1** and **2** were bioassayed for toxicity using the chick embryo inoculation method (11,12). Preliminary results indicate that neither **1** or **2** are very embryotoxic (LD_{50} > 10μg/egg).

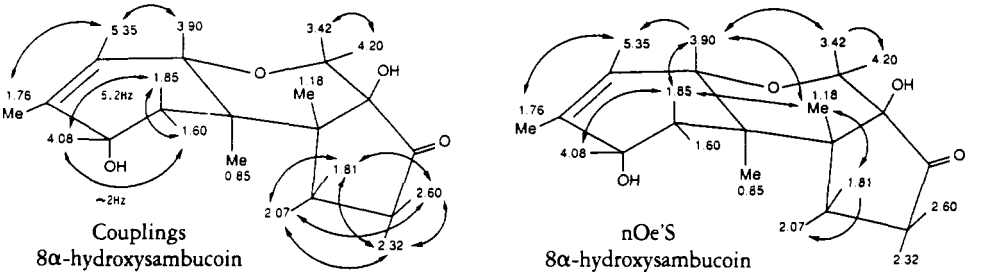

FIGURE 2. Couplings observed by COSY and nOes derived from dnOes on 8α-hydroxysambucoin [**2**]

MATERIALS AND METHODS

PHYSICAL ANALYSES.—All uv spectra were obtained on a Perkin-Elmer 576 ST spectrophotometer. Ir spectra were obtained on a Fourier transform Nicolet 20 DXB. Samples were cast as a film on a NaCl plate.

Mass spectra of the samples were obtained on a Kratos MS-25 mass spectrometer equipped with a DS-55 data system. The instrument was operated in several different modes: low resolution (1/600) electron impact (ei) at 70 eV and 1 sec/decade scanning speed; medium resolution (1/6500) ei at 70 eV and 3 sec/decade for measurement of exact masses. Perfluorokerosene was used for calibration during ei runs. The samples were introduced to the spectrometer source by means of a direct sampling probe. The probe was tem-

perature programmed from 20° to 550° at a rate of 100°/min. The temperature program was started at scan 50 to allow time for a background check and to ensure uniformity between samples.

All nmr experiments were performed on a Nicolet NT-300 WB spectrometer equipped with 5 mm ^1H and ^{13}C probes operating at 300.06 and 75.45 MHz, respectively. All ^1H-nmr chemical shifts were referenced to internal TMS (0.0 ppm), and all ^{13}C-nmr chemical shifts were referenced against the deuterated solvent used ($CDCl_3$=77.0 ppm). In all one-dimensional ^1H-nmr experiments a 90 deg pulse (based on the 180 deg null point) and a delay time of 2.0 sec was used. A 16 K block size with a spectral window of 2,400 Hz (0-8 ppm) was used. To obtain proton-decoupled ^{13}C-nmr spectra for weak samples (<2 mg), a small pulse angle based on the Ernst angle relationship $\cos\alpha=\exp[-\tau/T_1]$, where $\tau=-T_1\ln(\cos\alpha)$, was used to optimize signal-to-noise of quartenary carbon atoms. A 16 K block size with a spectral window of 17,000 Hz (0-225 ppm) was used.

The two-dimensional *J*-correlated experiment COSY (9) used a delay of 3 sec, a 90 deg pulse of 8μs, a 512 block size, 128 increments, quadrature detection in both dimensions, zero filling once in t_1, and an acquisitions multiple of 4. The distortionless enhancement by polarization transfer experiment (DEPT) used the pulse sequence by Doddrell (13). The θ values were varied from $\pi/4,\pi/2$ to $3\pi/4$; the polarization transfer delays were optimized for [$1/(4J)$=1.72 ms].

FUNGAL MATERIALS.—The fungus *F. sporotrichioides* MC-72083 was obtained from Professor John Tuite, Department of Botany and Plant Pathology, Purdue University. The fungus was isolated in 1972 from a wheat sample in Miskolc, Hungary. The wheat had caused mycotoxicoses in hen layers and was shown to be very toxic to rats, chickens, and swine (14). It was originally classified as *F. sporotrichiella* var. *sporotrichioides* by Billay's system and latter reclassified as *F. sporotrichioides* by Booth and Nelson.[2]

CULTURE CONDITIONS.—*F. sporotrichioides* was grown (8) on yeast malt agar plates for 14 days at 25°. Sterilized H_2O was added to the agar plates, and the conidia were scraped into a larger volume of sterilized distilled H_2O (100 ml/agar plate). One-quart Ball® canning jars (100-200) containing 100 g of Quaker Oats® white corn grits were autoclaved for 30 min. Aliquots (2 ml) of the mycelium-H_2O mixture were pipetted into each jar of corn grits, and 33 ml of sterile H_2O was added. The jars were shaken and the lids loosened to allow for respiration. After 24 h of incubation at 10° in darkness, the jars were shaken again to ensure complete dispersal of the mycelium. The jars were incubated for a total of 21 days at 10° in darkness.

ISOLATION.—All solvents used for extractions and florisil chromatography were A.C.S. grade purchased from Fisher. All solvents used for flash chromatography, hplc, and tlc were glass distilled solvents purchased from Burdick & Jackson.

The florisil was Fisher 60-100 mesh and packed by pouring the florisil into a 5×20 cm gravity column (C_6H_6-hexane, 2:1) to a depth of 8 cm. A 2-cm layer of Fisher anhydrous Na_2SO_4 was added on top of the florisil. Flash chromatography (9) used EM® reagent 40-63 micron Kieselgel 60 Si gel.

Normal phase tlc plates were Si gel HLF uniplates®, 250 μm thick, purchased from Analtech. Preparative tlc entailed applying 5-10 mg of material to a 10×20 cm Si gel Analtech HLF uniplate®, 250 μm thick. After development, compounds were identified by their quenching behavior at 254 nm or by spray visualization using a chromogenic reagent. To avoid destruction of compounds, a thin slice in the middle of the tlc plate was removed and used for spray development. The bands of interest were scraped and sonicated with 10 ml Me_2CO for 1 min and allowed to stand for 2 h with occasional stirring. The sample was filtered through a Rainin 0.45 micron nylon-66 filter, concentrated under vacuum, and prepared for nmr analysis using standard procedures. Preparative rptlc plates were Whatman KC18F with 200 μm thickness and were used in the same procedure as described above for preparative tlc.

The *p*-anisaldehyde spray reagent for tlc analysis was prepared with MeOH-HOAc-H_2SO_4-*p*-anisaldehyde (Eastman Organic Chemicals) (85:15:5:0.5 v/v). The blue spray reagent (15) is administered in two parts. Spray #1 [Aldrich 1% 4-(*p*-nitrobenzyl)pyridine in CCl_4-$CHCl_3$, 3:2 v/v] is sprayed liberally on the air-dried plate. The plate is oven-heated at 150° for 30 min, cooled, and sprayed with spray #2 (Aldrich 10% tetraethylenepentamine in CCl_4-$CHCl_3$, 3:2 v/v). The epoxide-containing trichothecenes give a sky-blue color.

Preparative hplc was conducted on a Perkin-Elmer series 3B chromatograph using a linear gradient solvent system. The column was a Si gel 3.5×28 cm Perkin-Elmer 0258-3002. Quantitative hplc was conducted on a Perkin-Elmer series 2 chromatograph using a Waters 3.9 mm×30 cm μBondapak® C-18 column and a Perkin-Elmer LC-85 spectrophotometric detector operating at 195 nm.

BIOASSAY.—The specific-pathogen-free (SPF) fertile chicken eggs, used for the chick embryo bioassay (11,12), were purchased from Larson Lab-Vac Eggs, Inc., P.O. Box 474, Gowrie, Iowa 50543. The in-

[2]J. Tuite, personal communication.

cubators used were the Imperial II produced by Lab-Line Instruments, Inc. and a model 3212-10 produced by National Appliance Company. The temperature was maintained at 37° and the relative humidity was kept at ca. 60%. Embryos were received within 24 h of laying, incubated for 5 days, and candled for viability. Typically, a batch of 150 eggs would yield 120 acceptable eggs. Before dosing, the air cells were circled with a pencil and swabbed with 200 proof EtOH (Midwest Solvents Company of Illinois). An 18 gauge needle, sterilized in 200 proof EtOH, was used to puncture the shell above the encircled air cell. The toxins were dissolved in 200 proof EtOH, serial diluted, and 10 μl injected into the air cell with a microsyringe. The hole was then sealed with Scotch® tape. Into the control eggs 10 μl of 200 proof EtOH was injected. In 4 days, the eggs were candled, and the number of dead embryos were determined for each dose level.

8β-HYDROXYSAMBUCOIN [**1**].—Colorless glass; ir ν max film cm^{-1} 3387 (OH), 1739 (c=O); uv λ max (MeCN) 194 nm (log 3.60) ene $\pi \rightarrow \lambda^*$; eims *m/z* (rel. int.) 266.149[m]$^+$(2) (calcd for $C_{15}H_{22}O_4$: 266.152), 248 (M-H_2O]$^+$ (10), 180 (30), 136 (25), 123 (30), 109 (45), 55 (45), 43 (100); ^{1}H nmr, see Table 1 and Figure 1; ^{13}C nmr, see Table 1.

8α-HYDROXYSAMBUCOIN [**2**].—Colorless glass; ir ν film cm^{-1} 3394 (OH), 1738 (c=O); uv λ max (MeCN) 194 nm (log 3.56) ene $\pi \rightarrow \pi^*$; eims *m/z* (rel. int.) 266.153 [M]$^+$ (25) (calcd for $C_{15}H_{22}O_4$: 266.152), 248 (M-H_2O]$^+$ (4), 222 (15), 136 (20), 123 (23), 109 (37), 55 (50), 43 (100); ^{1}H nmr, see Table 1 and Figure 2.

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