NEW METABOLITES OF *FUSARIUM MARTII* RELATED TO DIHYDROFUSARUBIN

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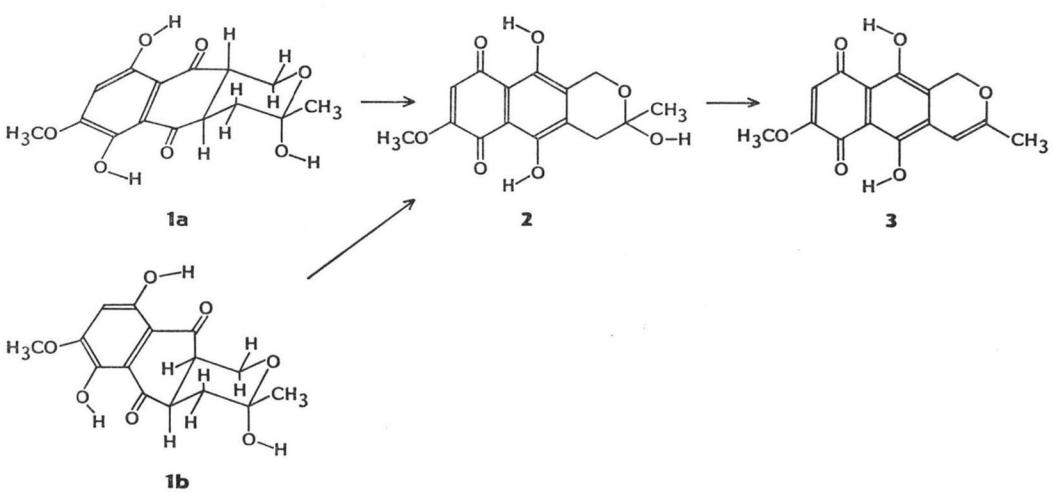
Six strains of *Fusarium martii* produced fusarubin and dihydrofusarubins A and B. Further examination of strains, T-77 and T-127, showed that four additional metabolites were produced. By ^{1}H and ^{13}C NMR spectral analyses, these additional metabolites have been identified as 3-*O*-methyl and 3-*O*-ethyl ethers of fusarubin and dihydrofusarubin A. The methyl ethers are new compounds and showed moderate cytostatic activity against mouse leukemia L1210 culture cells as well as moderate antibiotic activity against Gram-positive bacteria and fungi. 3-*O*-Methyl and 3-*O*-ethyl ethers of dihydrofusarubin A were converted non-enzymically to the respective ethers of fusarubin under alkaline conditions. Since in addition, only dihydrofusarubin derivatives were formed when an acidic pH was maintained in the culture, it can be concluded that only these are true metabolites of *Fusarium martii*.

We have recently reported that *Fusarium solani* produces the diastereoisomeric 4a,10a-dihydrofusarubins A and B (**1a** and **1b**)[1] and that they are non-enzymically oxidized by air to fusarubin (oxyjavanicin) (**2**)[2~7], norjavanicin[7] and neofusarubin under alkaline conditions[8]. Fusarubin is easily converted to anhydrofusarubin (**3**) by non-enzymic dehydroxylation under acidic conditions[16]. Javanicin[3,4], bostrycoidin[9] and (+)-solaniol[10] have also been isolated from the cultures of *F. solani*. Two other metabolites isolated in low yield along with fusarubin and fusarubin 3-*O*-ethyl ether from *F. solani*[11,12] have been identified as dihydrofusarubin A and its 3-*O*-ethyl ether derivative[13].

We now report the isolation of hitherto unknown 3-*O*-methyl ethers, along with the 3-*O*-ethyl

Fig. 1. Non-enzymic conversion of 4a,10a-dihydrofusarubins A (**1a**) and B (**1b**) to fusarubin (**2**) and anhydrofusarubin (**3**).



ethers of fusarubin and dihydrofusarubin A, from the culture of *Fusarium martii*.

Materials and Methods

Organisms

Twelve different strains of *Fusarium martii* var. *pisi* were received from Prof. HANS D. VANETTEN, Department of Plant Pathology, New York State College of Agriculture and Life Sciences, Cornell University, Ithaca, New York, U.S.A.

Culture Medium

One liter of medium contained the following ingredients: maltose 20~60 g, ammonium tartrate 3.4~8.1 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, NaCl 0.1 g, $CaCl_2$ 0.1 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, $ZnSO_4 \cdot 7H_2O$ 8.8 mg, $CuSO_4 \cdot 5H_2O$ 0.4 mg, $MnSO_4$ 0.06 mg, H_3BO_3 0.06 mg, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.04 mg.

Cultivation

Spores and mycelium from cultures grown on malt extract agar for 10~15 days at 27°C were suspended in sterile distilled water and used as inoculum. Each 100 ml portion of culture medium in a 500-ml Erlenmeyer flask was inoculated with 5 ml of the suspension, and incubated at 27°C for 2 days with shaking at 220 rpm. For large scale cultivation, 10 ml of the seed culture was added to 100 ml of the culture medium in a 500-ml Erlenmeyer flask and incubated as above for 1~6 days.

Jar Fermentation

Large scale cultivation was also carried out in a jar fermentor, Model MB-C (Iwashiya Bio-Science Co.). The seed culture (400 ml) was grown as above in 500-ml Erlenmeyer flasks for 2 days, and fermentor cultivation was carried out at 27°C for 5 days with aeration (4 liters/minute) and agitation (250 rpm).

Isolation of Metabolites

The mycelium was removed by filtration and washed with distilled water until free of pigment. The pH of the filtrate was adjusted to 6.5 with 1 N NaOH and extracted with an equal volume of ethyl acetate. The extract was washed twice with distilled water, and concentrated *in vacuo*. A second extraction of the filtrate was carried out as above after the pH had been adjusted to 3.0 with 1 N HCl. In addition, the mycelium was extracted with acetone. After the acetone had been removed by evaporation, the residue was extracted with ethyl acetate, washed with distilled water, and concentrated *in vacuo*.

Purification by Chromatography

The crude ethyl acetate extracts were each loaded on a column of silicic acid (Kieselgel 60, Merck Co.) and eluted with CH_2Cl_2 - ethyl acetate mixtures. Fractions containing colored products were further purified by liquid chromatography (KHLC model c-201-11, Kusano Kagaku-kikai Works Co.) using a prepacked silica gel column (ϕ 22 mm × 300 mm) eluted with hexane - ethyl acetate mixtures.

Analytical Instruments

Electronic absorption spectra were recorded with a Shimadzu model UV-240 UV-visible recording spectrophotometer. IR spectra were recorded with a Hitachi model 285 infrared spectrophotometer. Optical rotations were measured with a Jasco DIP-181 digital polarimeter. Mass spectra were obtained with a Jeol DX-300 high resolution mass spectrometer using an EI ion source. ^1H and ^{13}C NMR spectra were recorded with a Jeol FX-90Q Fourier transform NMR spectrometer.

Bioassay for Antibiotic Activity

The MIC tests were performed by an agar plate dilution procedure based on a standard method[14]. Cultures grown overnight at 37°C in sensitivity test broth (Nissui Seiyaku Co.) were diluted with the same broth to about 1×10^6 cells/ml and streaked on heart infusion agar media containing two-fold serial dilutions of each sample. The cultures were incubated at 37°C for 18 hours. The MIC was

Table 1. Production of dihydrofusarubins of six strains of *Fusarium martii*.

Strain	Broth pH	Mycelium wet weight (g)	Dihydro-fusarubins (mg)
T-76	5.8	1.38	19.2
T-77	3.3	1.06	32.1
T-127	2.8	1.47	17.0
T-129	2.8	1.09	24.7
T-216	2.9	1.42	23.7
K-19-15	3.2	1.37	15.7

Each strain was cultivated at 27°C for 6 days in 100 ml medium containing maltose (3.0 g), ammonium tartrate (0.46 g), minerals and salts. The yield of dihydrofusarubins was estimated from the OD at 390 nm of an ethyl acetate extract at pH 6.5. Data are the average of duplicated experiments; errors were less than ± 10%.

Table 2. Production of metabolites by *Fusarium martii* T-77 and T-127.

Metabolites	Production (mg)	
	T-77	T-127
Fusarubin	46.9	21.2
Dihydrofusarubins	201.3	114.2
Bostrycoidin	9.9	2.7
Javanicin	7.6	2.3
Compound **I**	33.3	34.1
Compound **II**	18.2	Trace
Compound **III**	39.3	55.3
Compound **IV**	11.2	Trace

Each strain was grown in 4 liters of a medium containing maltose (30 g/liter) and ammonium tartrate (4.6 g/liter), minerals and salts. Strain T-77 was cultivated on a rotary shaker (220 rpm) and T-127 in a jar fermentor with aeration (4 liters/minute) and agitation (250 rpm). Metabolites were extracted with ethyl acetate at pH 6.5 from culture broth and purified by column chromatography on silicic acid.

defined as the lowest concentration of sample that prevented development of visible bacterial growth.

Bioassay for Cytostatic Activity[15]

Mouse leukemia L1210 cells were maintained in a suspension culture of RPMI 1640 (Nissui Kayaku Co.) supplemented with 10% heat-inactivated fetal calf serum (Gibco Lab.) and 2 mM L-glutamine (Nissui Kayaku Co.). In a microtiter plate (Becton Dickinson Co.) a 200-μl aliquot of 1×10^5 cells/ml suspension was distributed and 10 μl of two-fold serial dilutions of each sample was added, and incubated for 48 hours at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cytostatic activity was determined by counting cell numbers after staining with 0.4% Trypan blue (Merck Co.).

Results and Discussions

Strains Producing Dihydrofusarubins

Six of the 12 strains of *F. martii*, T-76, T-77, T-127, T-129, T-216 and K-19-15, produced dihydrofusarubins as major metabolites in a medium containing maltose (30 g/liter), ammonium tartrate (4.6 g/liter), minerals and salts (Table 1). Strains T-77 and T-127 showed additional spots on TLC, so that they were further cultivated in large scale.

Production of Metabolites

Strain T-77 was cultivated in 4 liters of medium on a rotary shaker (Table 2). Higher yields of metabolites were obtained than from a culture grown on the same scale in a jar fermentor. Table 2 also gives values for strain T-127 grown in a jar fermentor. The yields reported are of metabolites extracted with ethyl acetate at pH 6.5 and purified by column chromatography. Most (904 mg) of the T-77 metabolites were extracted from the culture broth with ethyl acetate at pH 6.5 but an additional 181 mg extracted at pH 3.0 and 47.1 mg was recovered with acetone from the mycelium. From T-127, ethyl acetate extracted 338 mg at pH 6.5 and 20.1 mg at pH 3.0 while acetone gave 18.9 mg from the mycelium. As shown in Table 2, both strains produced dihydrofusarubin A and fusarubin as major metabolites, and bostrycoidin and javanicin as minor metabolites. Strain T-77 produced four while

Table 3. Physico-chemical characteristics of compounds **I**, **II**, **III** and **IV**.

	I	II	III	IV
MP (°C)	157~158	166~167	134~135	138~139
M^+ (*m/z*)	320.0911	334.1051	322.1038	336.1182
	$C_{16}H_{16}O_7$	$C_{17}H_{18}O_7$	$C_{16}H_{18}O_7$	$C_{17}H_{20}O_7$
$[\alpha]_D^{25}$ (*c* 0.1, acetone)	—	—	+128.5°	+125.8°
UV λ_{max} nm	225 (4.49),	225 (4.44),	242 (4.17),	242 (4.11),
(log ε) in 95% EtOH	302 (3.95),	302 (3.91),	273 (3.79),	273 (3.74),
	470 (3.78),	470 (3.78),	300 (3.70),	300 (3.70),
	495 (3.83),	495 (3.81),	390 (3.82)	390 (3.67)
	532 (3.64)	532 (3.63)		
IR ν_{max} cm^{-1} (KBr)	3425 (m, H-bonded OH), 1600 (s, H-bonded C=O)		3425 (m, H-bonded OH), 1625 (s, H-bonded C=O)	

strain T-127 produced two additional metabolites.

Physico-chemical Characteristics

Compounds **I**~**IV** crystallized from a mixture of $CHCl_3$ - ethanol (1 : 1) as reddish orange needles after purification by column chromatography. The physico-chemical data are summarized in Table 3. Compounds **I** and **II** have similar UV and IR absorption spectra which are also similar to those of fusarubin. On the other hand, the spectra for compounds **III** and **IV** are similar to each other and to those of dihydrofusarubins. Accurate mass measurements demonstrated that compound **I** had one carbon and two protons and compound **II** had two carbons and four protons more than fusarubin; also compound **III** had one carbon and two protons, and compound **IV** had two carbons and four protons more than dihydrofusarubin.

^1H NMR Spectral Analysis

^1H NMR data for fusarubin and compounds **I** and **II** obtained in $CDCl_3$ are summarized in Table 4a, and those for compounds **III** and **IV** in Table 4b. All signals could be easily assigned from previous chemical shift assignments for fusarubin obtained in pyridine-d_5[10], except for two methylene protons at H-1 and H-4. Chemical shifts for these protons could be tentatively assigned from the long-range couplings shown in Table 4a but these assignments might be interchanged. Integral analysis demonstrated that the singlet signal at δ 3.31 ppm in the spectrum of compound **I** had three protons, and the singlet at δ 1.54 ppm had three protons. In the spectrum of compound **II**, the quartet at δ 3.61 ppm had two protons. In addition, spin-spin coupling between the signals at δ 1.55 and 3.61 ppm was proven by decoupling analysis. Thus the ^1H NMR spectra suggested that the 3-OH group was replaced with OCH_3 in compound **I** and with OCH_2CH_3 in compound **II**, and this was also demonstrated by ^{13}C NMR analysis. Derivatization of the 3-OH group explains the evidence that chemical shifts at 3-CH_3, H-4α and H-4β are similar for compounds **I** and **II** but slightly different from fusarubin, while other chemical shifts are the same in all three compounds.

Chemical shift assignments for compounds **III** and **IV** are based on published data for dihydrofusarubins A and B[1]. In the spectrum of compound **III** a singlet was observed at δ 3.23 ppm while signals for the 3-OH is missing. In the spectrum of compound **IV** a triplet was observed at δ 1.38 ppm and a quartet at δ 5.05 ppm. By integral and decoupling analyses, the signal at δ 3.23 ppm was assigned to the OCH_3 group of compound **III**; the signal at δ 5.05 ppm was assigned to the methylene protons and the signal at δ 1.38 ppm to the methyl group of OCH_2CH_3 in compound **IV**.

Table 4a. ^{1}H nuclear magnetic resonance data for fusarubin (**2**), and compounds **I** and **II**.

Compound	H-1α	H-1β	H-4α	H-4β	H-8	3-CH_3
2	3.06 dm $J_{1\alpha,1\beta}$=18.0	2.68 dm	5.01 dm $J_{4\alpha,4\beta}$=18.0	4.76 dm	6.17 s	1.65 s
I	3.04 dd $J_{1\alpha,1\beta}$=18.0 $J_{1\alpha,4\beta}$=2.2	2.64 ddd $J_{1\beta,4\alpha}$=1.7 $J_{1\beta,4\beta}$=2.6	4.90 dd $J_{4\alpha,4\beta}$=18.0	4.53 ddd	6.17 s	1.54 s
II	3.04 dd $J_{1\alpha,1\beta}$=18.0 $J_{1\alpha,4\beta}$=2.2	2.64 ddd $J_{1\beta,4\alpha}$=1.6 $J_{1\beta,4\beta}$=2.6	4.88 dd $J_{4\alpha,4\beta}$=18.0	4.52 ddd	6.17 s	1.55 s

Compound	7-OCH_3	5,10-OH	3-OH	3-OCH_3	3-OCH_2CH_3
2	3.93 s	12.65, 12.91 s s	6.41 s	—	—
I	3.92 s	12.63, 12.91 s s	—	3.31 s	—
II	3.92 s	12.66, 12.93 s s	—	—	3.61, 1.55 q t J=7.0 J=7.0

Spectra were recorded with a Jeol FX-90Q (89.55 MHz) in $CDCl_3$. Chemical shifts in ppm (± 0.002) referred to $(CH_3)_4Si$; coupling constants $J_{p,q}$ in Hz (±0.2); abbreviations: d=doublet, t=triplet, q=quartet, m=multiplet, b=broad.

Table 4b. ^{1}H nuclear magnetic resonance data for compounds **III** and **IV**.

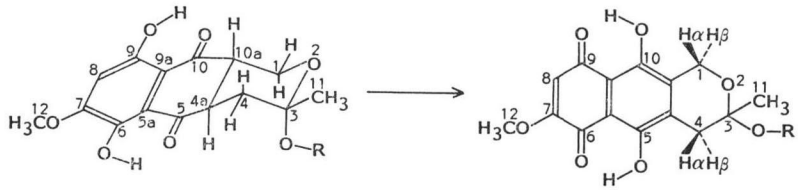
Compound	H-1_{ax}	H-1_{eq}	H-4_{ax}	H-4_{eq}	H-4a	H-10a
III	3.81 dd $J_{1ax,1eq}$=11.7 $J_{1ax,10a}$=10.2	4.22 dd $J_{1eq,10a}$=4.8	1.68 dd $J_{4ax,4eq}$=13.6 $J_{4ax,4a}$=11.5	2.41 dd $J_{4eq,4a}$=3.8	3.43 ddd $J_{4a,10a}$=13.2	2.94 ddd
IV	3.79 dd $J_{1ax,1eq}$=12.5 $J_{1ax,10a}$=7.5	4.34 dd $J_{1eq,10a}$=5.0	1.76 dd $J_{4ax,4eq}$=14.5 $J_{4ax,4a}$=11.4	2.45 dd $J_{4eq,4a}$=3.7	3.42 ddd $J_{4a,10a}$=12.9	2.97 ddd

Compound	H-8	3-CH_3	7-OCH_3	6,9-OH		3-OCH_3	3-OCH_2CH_3	
III	6.65 s	1.41 s	3.95 s	12.02 s	12.17 s	3.23 s	—	—
IV	6.65 s	1.58 s	3.95 s	11.92 s	12.16 s	—	5.05 q J=5.7	1.38 t J=5.7

Spectra were recorded with a Jeol FX-90Q (89.55 MHz) in $CDCl_3$. Chemical shifts in ppm (± 0.002) referred to $(CH_3)_4Si$; coupling constants $J_{p,q}$ in Hz (±0.2); abbreviations: d=doublet, t=triplet, q=quartet, m=multiplet, b=broad.

Although the spectra of dihydrofusarubins A and B were recorded in pyridine-d_5 due to their low solubility in $CHCl_3$[1] and those of compounds **III** and **IV** were in $CDCl_3$, the conformations of compounds **III** and **IV** were concluded to be the same as dihydrofusarubin A and not dihydrofusarubin B for the following reasons; the coupling constants between H-1_{ax} and H-10a in compound **III** (10.2 Hz) and compound **IV** (7.5 Hz) were large, as observed in dihydrofusarubin A (10.8 Hz) while a small

Fig. 2. Non-enzymic conversion of 4a,10a-dihydrofusarubin A (**1**) and its methyl (**III**) and ethyl (**IV**) ethers to fusarubin (**2**) and its methyl (**I**) and ethyl (**II**) ethers.



1 R=H
III R=CH_3
IV R=CH_2CH_3

2 R=H
I R=CH_3
II R=CH_2CH_3

coupling constant was observed in dihydrofusarubin B (3.2 Hz). The coupling constants between H-1_{eq} and H-10a in compound **III** (4.8 Hz), in compound **IV** (5.0 Hz) and in dihydrofusarubin A (5.0 Hz) were similar in magnitude and different from the small coupling constant observed in dihydrofusarubin B (1.4 Hz). However, the coupling constants between H-4_{ax} and H-4a observed in compound **III** (11.5 Hz) and between H-4_{ax} and H-4a in compound **IV** (11.4 Hz) are both larger than for dihydrofusarubins A (3.7 Hz) or B (3.8 Hz). In addition, coupling was clearly observed between H-4_{eq} and H-4a in compound **III** (3.8 Hz) and in compound **IV** (3.7 Hz) whereas no coupling was observed in dihydrofusarubins A and B.

Thus it can be concluded that the conformations of H-1_{ax}, H-1_{eq} and H-10a are the same as in dihydrofusarubin A, but the conformations of H-4_{ax}, H-4_{eq} and H-4a are slightly different from both dihydrofusarubins A and B. Since the coupling constant in compounds **III** (13.2 Hz) and **IV** (12.9 Hz) was large, as in dihydrofusarubin A (13.2 Hz), while a medium sized coupling constant was observed in dihydrofusarubin B (5.4 Hz), the conformation between H-4a and H-10a should be *trans* and not *cis*. Therefore, the difference in coupling constants among H-4_{ax}, H-4_{eq} and H-4a must be due to methyl or ethyl ether formation at the 3-OH position of dihydrofusarubin A. Thus, compound **III** must be the 3-*O*-methyl ether and compound **IV** the 3-*O*-ethyl ether of dihydrofusarubin A.

^{13}C NMR Spectral Analysis

^{13}C NMR spectra of compound **III** with ^{1}H decoupling and nuclear Overhauser enhancement were run in $CDCl_3$, and chemical shifts were assigned from previously published data on anhydrofusarubin[14] (^{13}C NMR spectra were not available for fusarubin or dihydrofusarubin because of their low solubility). The two signals observed at the lowest field (δ 198.79 and 202.85 ppm) were assigned to the C-5 and C-10 carbonyl carbons, respectively, in compound **III**. These signals showed the same ^{1}H-^{13}C coupling constant (5.5 Hz) in compound **III**, whereas C-6 and C-9 showed different ^{1}H-^{13}C coupling patterns in anhydrofusarubin. This provides new evidence for the different position of the carbonyl carbons in the naphthoquinone structure of fusarubin and dihydrofusarubin discussed in our previous paper[1,13].

The signal at δ 146.07 ppm was assigned to C-6 based on ^{1}H-^{13}C couplings with H-8 (7.6 Hz) and with 6-OH (4.6 Hz), and the signal at δ 157.61 ppm was assigned to C-9 based on ^{1}H-^{13}C couplings with H-8 (5.9 Hz) and with 9-OH (5.9 Hz). Four signals observed at δ 106.36, 107.33, 114.11 and 156.74 ppm were assigned, respectively, to C-8, C-9a, C-5a and C-7 from similarities to anhydrofusarubin. A methyl signal at δ 23.16 ppm was unambiguously assigned to C-11. Of the two methoxy group signals, that at δ 56.51 ppm was assigned to C-12 from its similarity in chemical shift to the

Table 5. ^{13}C nuclear magnetic resonance data for anhydrofusarubin (**3**) and compound **III**.

Compound	C-1	C-3	C-4	C-4a	C-5	C-5a	C-6	C-7
3	62.89 t ^1J=151.0	161.51 m	94.54 dq ^1J=171.3 ^3J=4.4	132.85 m	157.50 d ^3J=2.7	110.79 d ^3J=4.6	177.75 dd ^2J=6.7 ^3J=1.2	159.92 dq ^2J=6.5 ^3J=4.1
III	59.55 bt ^1J=147	97.44 m	35.14 bt ^1J=125	43.32 bd ^1J=125	198.79 bd ^2J=5.5	114.11 d ^3J=4.9	146.07 dd ^2J=4.6 ^3J=7.6	156.74 dq ^2J=6.5 ^3J=4.0

	C-8	C-9	C-9a	C-10	C-10a	C-11	C-12	C-13
3	109.84 d ^1J=164.0	182.83 dd ^2J=2.5	107.86 dd ^3J=5.0	157.50 t ^3J=2.7	122.62 bt ^2J=4.5	20.08 qd ^1J=128.6 ^3J=2.7	56.64 q ^1J=146.6	—
III	106.36 dd ^1J=161.7 ^3J=7.6	157.61 dd ^2J=5.9 ^2J=5.9	107.33 dd ^3J=4.9 ^3J=4.9	202.85 bd ^2J=5.5	45.86 bd ^1J=125	23.16 qd ^1J=127.0 ^3J=1.8	56.51 q ^1J=145.9	47.92 qd ^1J=142.2 ^4J=1.2

Chemical shifts in ppm (±0.05) referred to $(CH_3)_4Si$; coupling constants ^nJ in Hz (±0.3); abbreviations: d=doublet, t=triplet, q=quartet, m=multiplet, b=broad. Spectra were recorded with a Jeol FX-90Q (22.5 MHz) in $CDCl_3$.

Table 6. Minimum inhibitory concentrations (μg/ml) of fusarubin (**2**), dihydrofusarubin A (**1a**) and compounds **I**, **II**, **III** and **IV**.

Bacterial strains	**2**	**I**	**II**	**1a**	**III**	**IV**
Staphylococcus aureus FDA209P JC1	50	100	100	12.5	25	50
S. aureus ATCC 25923	100	>100	>100	25	50	50
S. epidermidis ATCC 12228	100	>100	>100	25	100	100
Micrococcus luteus ATCC 9341	>100	>100	>100	50	100	100
Bacillus subtilis ATCC 6633	25	50	50	12.5	12.5	12.5
Streptococcus pyogenes Cook	50	50	100	6.25	25	25
S. pneumoniae DP-1	25	50	50	12.5	12.5	25
S. pneumoniae DP-2	50	50	50	50	25	25
S. pneumoniae DP-3	25	50	50	12.5	12.5	12.5
Candida albicans C-a-21	50	50	50	50	50	50
C. albicans C-a-30	25	50	50	25	25	25
Saccharomyces cerevisiae ATCC 9763	25	50	50	12.5	12.5	12.5

methoxyl of anhydrofusarubin; consequently the signal at δ 47.92 ppm was assigned to C-13. On similar arguments, the methylene signal at δ 59.55 ppm was assigned to C-1 and that at δ 35.14 ppm to C-4. The signal at δ 97.44 ppm was assigned to C-3 because the multiplet splitting observed was like that seen for C-3 in the spectrum of anhydrofusarubin. Two methine signals at δ 43.32 and 45.86 ppm were tentatively assigned to C-4a and C-10a, respectively. However, this assignment could be interchanged.

Table 7. Inhibition effect of fusarubin (**2**), dihydrofusarubin A (**1a**), and compounds **I**, **II**, **III** and **IV** on proliferation of mouse leukemia L1210 culture cells.

Compound	IC_{50} (μg/ml)
2	3.1±0.2
I	5.9±0.5
II	6.2±0.3
1a	1.7±0.2
III	5.4±0.3
IV	5.6±0.4

Antibacterial and Antifungal Activities

MIC values for fusarubin, dihydrofusarubin A, and compounds **I**, **II**, **III** and **IV** are summarized in Table 6. All four compounds showed moderate antibiotic activity against Gram-positive bacteria and fungi. Fusarubin and its derivatives showed relatively less activity than dihydrofusarubin A and its derivatives. In most cases the derivatives showed lower activity than their parent compounds. This decreased activity might be due to their decreased water solubility.

Cytostatic Activity

Cytostatic activity against mouse leukemia L1210 culture cells is summarized in Table 7. As observed for antibacterial and antifungal activity, fusarubin and its derivatives showed slightly lower activity than dihydrofusarubin and its derivatives, also all four derivatives showed less activity than their parent compounds. Again the decreased activity may be due to decreased water solubility.

Stereochemical Identification

When we reported the isolation of 3-*O*-ethyl ethers of dihydrofusarubins, we could not identify the stereochemistry[11~13]. With aid of ^{1}H NMR spectral analysis we have now characterized both the methyl and ethyl ethers. Based on spin-spin couplings among H-1, H-4, H-4a and H-10a, the configuration between H-4a and H-10a is identified as *trans* in both compounds. Therefore, *F. martii* produces 3-*O*-methyl and 3-*O*-ethyl ethers of dihydrofusarubin A. By direct comparison of ethyl ethers isolated from cultures of *F. solani* and *F. martii* the major ethyl ether produced by *F. solani* has

been also identified as the 3-*O*-ethyl ether of dihydrofusarubin A.

Biosynthetic Relationship

The 3-*O*-ethyl ethers of fusarubin and dihydrofusarubins previously isolated from cultures of *F. solani* were suspected to be laboratory artifacts, formed non-enzymically from dihydrofusarubin[13]. However, this possibility is now excluded by the observations presented here. Firstly, ethyl ethers of fusarubin and dihydrofusarubin were isolated from cultures of *F. martii* using procedures that rigorously excluded the use of ethanol or chloroform, which may contain traces of ethanol. Secondly, 3-*O*-methyl ethers were isolated from strain T-77 along with 3-*O*-ethyl ethers, while strain T-127 produced methyl ethers almost exclusively (Table 2). Thirdly, of the six strains that produced dihydrofusarubins, only strains T-77 and T-127 produced methyl and ethyl ethers. Thus the formation of methyl or ethyl ethers is undoubtedly enzymic and not artifactual.

We have pointed out that dihydrofusarubins are true metabolites while fusarubin and norjavanicin are formed non-enzymically from dihydrofusarubins during cultivation[1,8]. Here we also observed the same biosynthetic relationship. The pH of a production medium consisting maltose (30 g/liter) and ammonium tartrate (4.6 g/liter) decreased gradually to 3.0, then increased rapidly to 8.0 as with cultures of *F. solani*[13]. This gave fusarubin and dihydrofusarubin as well as their methyl and ethyl ethers (see Table 2). However, when the production medium contained maltose at 60 g/liter and remained acidic, no fusarubin or fusarubin ethers was observed (data not shown). Therefore, it can be concluded that dihydrofusarubins and their methyl or ethyl ethers are true metabolites, but fusarubin and its derivatives are formed non-enzymically by oxidation under alkaline conditions.

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