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Structure and Conformation of Ophiobolin K and 6-Epiophiobolin K from *Aspergillus ustus* as a Nematocidal Agent.

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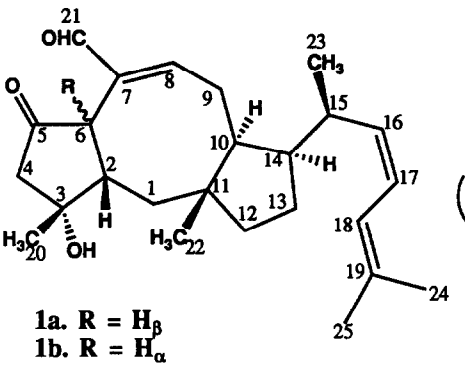
Abstract: *The structure, stereochemistry, solution conformation and nematocidal activity of Ophiobolin K (**1a**) and 6-epiophiobolin K (**1b**) isolated from Aspergillus ustus has been described The molecular structure of these compounds were deduced from extensive use of 2D NMR spectroscopy The stereochemistry and solution conformation was determined in $CDCl_3$ by the application of extensive ^1H-^1H NOE difference spectroscopy Significant difference in the conformation of A/B cis **1a** and its A/B trans isomer **1b** was observed including the folding of the side chain Ophiobolin K showed a unique boat like conformation whereas the B ring of 6-epiophiobolin K must exist in at least two different conformations Ophiobolin K exhibited nematocidal activity (ED_{50} 10 μg/mL) against the free-living nematode Caenorhabditis elegans ,but the A/B trans isomer **1b** was found to be inactive*

INTRODUCTION

Helminths and nematodes are responsible for numerous deadly parasitic diseases in humans, as well as in animals, and represent a serious problem world-wide Search for the cure of such parasitic diseases is the paramount task of many research institutions Among the many programs at Merck directed toward such a goal, one is the discovery of novel natural products *via* a vigorous and systematic screening of fungal and many other microbial extracts using various enzymatic, biochemical and *in vivo* screens During the course of screening fungal extracts against the free-living nematode *Caenorhabditis elegans*[1], we discovered ophiobolin K (**1a**), a new sesterterpenoid of ophiobolane family[2], with nematocidal activity We report herein the isolation, structure elucidation, stereochemistry, solution conformation and nematocidal activity of ophiobolin K (**1a**, ED_{50} 10 μg/mL) along with that of its inactive 6-epi-analog (**1b**)

ISOLATION

Solid state fermentation of *Aspergillus ustus* was repeatedly extracted with methyl ethyl ketone and chromatographed over silica gel column to give an active fraction containing **1a** and **1b** in a ratio of 5 1 (HPLC) This fraction was further purified by reversed phase preparative HPLC (Whatman C-18) with a gradient of CH_3CN-H_2O to yield 6-epiophiobolin K (**1b**) and ophiobolin K (**1a**) in the order of their elution



1a. R = H_β
1b. R = H_α

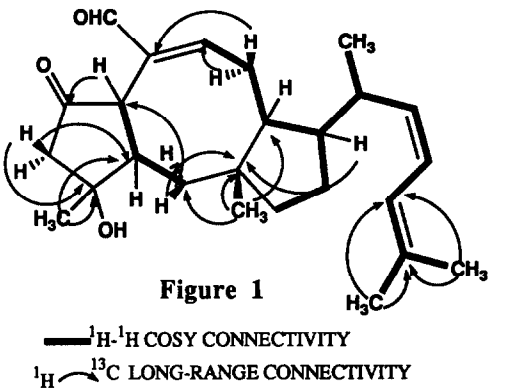


Figure 1

^1H-^1H COSY CONNECTIVITY

^1H ⌒ ^{13}C LONG-RANGE CONNECTIVITY

STRUCTURE ELUCIDATION

High resolution mass spectral analysis of both **1a** and **1b** gave a molecular ion at m/z 384 which was determined to have a molecular formula of $C_{25}H_{36}O_3$ The formula suggests the presence of 8 double bond equivalents (DBE) in the molecule The molecular formula was corroborated by ^{13}C NMR signals (table 1, *vide infra*) Under forcing conditions both **1a** and **1b** formed a mixture of mono and diTMS derivatives. Infrared spectrum indicated the presence of hydroxy (3400), cyclic carbonyl (1737) possibly in a five-membered ring, conjugated carbonyl (approx 1670), and olefinic (approx 1630) moieties Ultra violet spectra of both compounds suggested the presence of an enone type (λ_{max} 240) system

The 300 MHz ^1H NMR spectrum (table 1) of **1a** and **1b** in $CDCl_3$ displayed an aldehyde proton singlet, four olefinic protons, two vinylic methyls, an angular methyl group attached to an oxygen bearing carbon, an angular methyl, and a methyl doublet The ^1H NMR spectra was assigned with the help of 2D ^1H-^1H COSY spectroscopy[3], and the different spin systems are represented in figure 1 (bold lines) One of the isolated spin systems in **1a** was assigned to geminal protons at C-4 whereas the shielded geminal proton (δ 2 42) of **1b** was also correlated to a broad doublet at δ 3 30 (H-6) through a W coupling, which could be easily visualized from Dreiding model ^{13}C NMR spectrum (table 1) of ophiobolin K and its 6-epi analog (**1b**) exhibited 25 carbons and 35 carbon-bound protons as obtained from APT spectrum and revealed the following carbon types a carbonyl group, an aldehyde, six olefinic carbons (four of them bearing hydrogen and two quaternary), an oxygenated quaternary, a quaternary, five methines, five methylenes and five methyl carbons One bond proton-carbon connectivity was determined from 2D ^1H-^{13}C COSY[4] and this spectrum was also helpful in assigning some of the overlapping methylene protons Two carbonyls and 3 olefins account for five DBE's, to satisfy the remaining 3 DBE these molecules must be tricyclic The presence of a conjugated aldehyde (λ_{max} 240), isopropenyl group and a five-membered ring ketone (δ 217) suggested that these sesterterpenoids may be members of the ophiobolane class[2]

Corroboration of the tricyclic ophiobolane skeletal system was further substantiated by a long-range 2D ^1H-^{13}C COSY experiment optimizing for a coupling constant of 7, 8 and 10 Hz This experiment revealed the correlations of H-20 methyl and H-4β to both C-2 and C-3 and H-1β to C-6 and C-11 C-11 was also correlated

to H-22 and H-14 Similarly H-6 was correlated to C-5 carbonyl, H-22 to C-1 and C-10 These (and others see figure 1) two and three bond ^{1}H-^{13}C correlations were in full accord with the ophiobolane skeleton

Table 1: ^{1}H and ^{13}C NMR Assignment of Ophiobolins K and 6-Epiophiobolin K in $CDCl_3$ solutions.

Position		1a	1b	1a	1b
1	Hα	35 08	41 29	1 20,m	1 55,m
	Hβ			1 75,m	1 75,m
2	H	50 28	49 60	2 39,brdt,12 9,3 1	2 16,dd,10 5,4 2
3		76 92	76 72	----	----
4	Hα	54 91	54 94	2 78,d,19.2	2 42,dd,16 8,1 5
	Hβ			2 48,d,19 2	3 09,d,16 8
5		217 70	217 27	-----	-----
6	H	48 54	48 82	3 25, brd,10 5	3 30,brd, 10 8
7		141 32	141 31	-----	-----
8	H	164 07	160 78	7 11,t,8 6	6 87,dd,6 9,2 7
9	Hα	25 44	30 85	2 10,m	2 84,ddd,20,4,2*
	Hβ			2 94,dd,12 6,8 3	2 22,ddd,20,14,2*
10	H	47 13	43 74	2 06,m	2 54,ddd,14 7,10,4*
11		43 85	44 70	-----	-----
12	Hα	26 54	27 71	1 60,m	1 65,m
	Hβ			1 25,m	1 20,m
13	Hα	42 58	45 25	1 40,m	1 45,m
	Hβ			1 40,m	1 45,m
14	H	53 57	54 06	1 56,m	1 87,m
15	H	35 85	32 58	2 70,m	2 53,m
16	H	137 10	135 59	5 17,t,9 6	5 12,t,9 9
17	H	122 44	129 97	6 02,dt,10 5,0 9	6 07,dt,9 8,0 9
18	H	119 95	119 97	5 96,dd,10 5,1 0	6 00,md,9 8,1 2
19		136 06	136 42	-----	-----
20	H	25 65	25 86	1 34,s	1 45,s
21	H	196 29	194 30	9 19,s	9 17,s
22	H	18 73	23 32	0 95,s	0 85,s
23	H	20 40	21 31	0 90,d,6 7	0 98,d,6 6
24	H	18 14	18 22	1 72,brs	1 77,brs
25	H	26 54	26 56	1 80,brs	1 83,brs
OH		---	---	3 34	3 20

*coupling constants were determined from NOE difference spectra

STEREOCHEMISTRY AND CONFORMATIONS

^1H-^1H NOE difference spectroscopy[5] in conjunction with scalar coupling constants were used to deduce the relative stereochemistry and conformation of ophiobolin K (**1a**) and 6-epiophiobolin K (**1b**) with the help of Dreiding model

Ophiobolin K (1a): Irradiation of H-6 (δ 3 25) resulted in significant enhancement of H-2 (δ 2 39) thus establishing a *cis* fusion of rings A/B, it also revealed NOE to H-9β (δ 2 10) and clearly indicating a folded boat type conformation of the eight-membered B ring as depicted in figure 2 This was further substantiated from the additional NOE's from H-22 (δ 0 95) to H-2, H-6 and H-9β (δ 2 10), H-8 (δ 7 11) to H-9α (δ 2 93) and *vice versa* *Syn* relationship of H-8 to CHO group was also apparent from the NOE results NOE's between H-16 and H-17 established the *cis* nature of the C-16, C-17 double bonds NOE's were also observed between H-24 (δ 1 72) and H-17 (δ 6 02,dt), H-25 (δ 1 80) and H-18 (δ 5 96) Most important conformational information regarding the side chain was obtained from the enhancement of H-15 (δ 2 70) after saturation of H-22, and NOE's from H-16 to H-9β and H-9α to H-16 thus placing the side chain close to ring B in the conformation as shown in figure 2

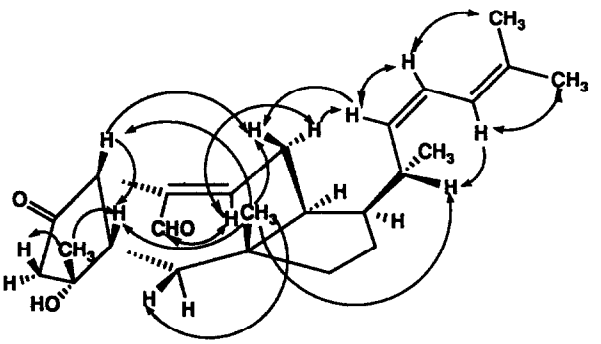


Figure 2

6-Epiophiobolin K (1b): Observation of NOE from H-6 to H-10 and H-20 to H-2 and lack of NOE from H-6 to H-2 gave a clear indication that A/B ring is trans fused and H-2 is on the same side as C-20 methyl group (as in **1a**), whereas H-6 and H-10 are on the opposite side of the molecule Irradiation of both H-20 and H-22 enhanced the proton multiplet assigned to H-1β, therefore, both methyl groups are on the same face of the molecule Interpretation of all the NOE's led us to conclude that **1a** and **1b** differ significantly in their conformations, in order to explain all the measured NOE's, **1b** must exist in at least two different conformations as depicted in figures 3 and 4 In both of these conformations the side chain must remain away from the ring B in contrast to the conformation of **1a**

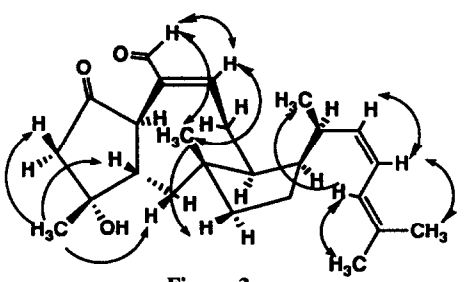


Figure 3

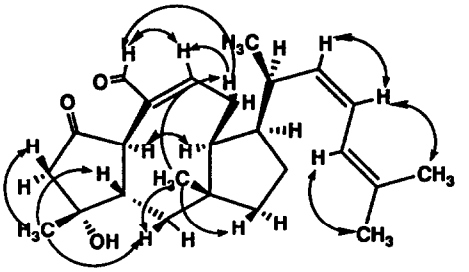


Figure 4

NOE's

The NOE's from H-6 to H-10 and H-22 to H-1β and H-9β can be explained only from conformation in figure 4 whereas NOE's between C-7 aldehyde proton, H-8 and H-22 could be explained from conformation as depicted in figure 3 Therefore, these two conformations exist in equilibrium at least on the NOE measurement time scale The conformation in figure 4 is very similar to that of solid state conformation of ophiobolin G as derived from single crystal x-ray determination and reported[6] from *A ustus* and which may be a dehydration product of **1b** The absolute stereochemical representation is based on ophiobolin I whose absolute stereochemistry was determined[7] from X-ray of p-bromobenzoate derivative and ophiobolin C which has been synthesized[8] The unresolved[6] stereochemistry of C-3 of ophiobolin H, a metabolite reported from *A ustus* could be assigned as in **1a** and **1b**

Many chemical shift differences (table 1) both in ^{1}H and ^{13}C NMR spectra of **1a** and **1b** could be explained by their entirely different conformations Difference in the biological activity could also be attributed to the conformational differences Slow epimerization of **1a** into **1b** was also observed in many solvents including chloroform, this may be due to the less strained and more stable conformations of *trans* isomer **1b** which was found to be reasonably stable in identical conditions The epimerization process precluded the possibility of crystallization of **1a**

BIOLOGY

Ophiobolins have been reported[7] from the pathogenic fungi *Drechslera maydis* , *D oryzae* and *D sorghicola* and are responsible for serious phytotoxicity[9] Ophiobolin G and H have been reported to inhibit the growth of *Bacillus subtilis*[6], but as far as we know, this is first report of the nematocidal activity of any ophiobolins Both ophiobolin K and its 6-epi analog were tested against the free-living nematode *C elegans* at concentrations up to 100 μg/mL Ophiobolin K displayed an ED_{50} of 10 μg/mL, whereas 6-epiophiobolin K was inactive at the highest level tested

EXPERIMENTAL

All the reagents and deuterated solvents were obtained from Aldrich Chemical Company and were used without any purification Yeast extract, malt extract and casamino acids were obtained from Difco, and Junlon was obtained from The Kouyok Trading Co Ltd , Tokyo, Japan E Merck (Darmstadt) silica gel plates (0 25 mm) were used for TLC and developed either with 3% ceric sulfate in 3N H_2SO_4 spray and/or iodine vapors

Stationary phases used for column chromatography were E Merck silica gel (70-230 or 40-63 mesh) Melting points were uncorrected Optical rotation values were recorded using a Perkin-Elmer 241 polarimeter

The IR absorption spectra were obtained with a model 1750 Infrared Fourier Transform Spectrophotometer using a multiple internal reflectance cell (MIR, ZnSe) on neat 10-20 μg samples The UV absorption spectra were measured with a DU-70 Spectrophotometer in MeOH solution Mass spectral data were obtained on a MAT212 instrument by electron impact at 90 eV Trimethylsilyl derivatives were prepared with a 1 1 mixture of BSTFA-pyridine at room temperature Exact mass measurements were made on the same instrument at high resolution by the peak matching method using perfluorokerosene (PFK) as internal standard The FAB spectra were obtained on a MAT731 instrument

NMR data were obtained on a Bruker AM-250 with ASPECT 3000 computer or Varian XL-300 spectrometer at ambient temperature in $CDCl_3$ ^1H NMR chemical shifts in $CDCl_3$ are given relative to the solvent peak at 7 256 ppm ^{13}C NMR chemical shifts in $CDCl_3$ are given relative to the solvent peak at 77 05ppm

^1H-^1H COSY were recorded using the standard pulse sequence of Bax *et al* [3] The 1K-2K data set was accumulated in 512 increments with 32 transients respectively for each value of t_1 for full phase cycling ^1H-^{13}C chemical shift correlation spectra (COSY) were recorded in $CDCl_3$ using the standard pulse sequence of Bax and Morris[4] The 512 x 4K data set was accumulated in 128 increments with 512 transients for each value of t_1 The delay time between transients was 0 5 sec, and the experiment was optimized for $^1J_{CH}$ =150 Hz The corresponding long-range experiment was optimized for a multiple bond carbon-proton coupling constant of 7, 8 and 10 Hz. NOE's were measured using NOE difference[5] in $CDCl_3$, samples were not degassed In NOE difference method, a relaxation delay of 2 0 sec, irradiation time of 0 75-1 0 sec and decoupler power of 40L was used The FID's were substracted before Fourier transformation

Fermentation of Ophiobolin K and 6-Epiophiobolin K:

The fungus *A ustus* (Bainer) Thom & Church (culture number JP118, deposited in Merck culture collection) isolated from *Tamarindus indicus* (commonly known as tamarind) seeds, was obtained from Merck Microbial Resources The composition of seed and production media are shown in table 2 A slant was used to prepare FVMs (frozen vegetative mycelia) in the seed medium (without Junlon) and were frozen at -75°C until use The seed medium was prepared in distilled water and the pH was adjusted to 7 0 before sterilization The medium was dispensed at 54 mL/250 mL plain Erlenmeyer flask, closed with cotton and sterilized at 121°C for 20 minutes Seed cultures were inoculated with a source of *A ustus* and grown on a gyratory shaker (220 rpm) for 72 hours at 25°C A portion of the seed culture (20 mL) was used to inoculate each Medium-4 production flask (table 2, prepared in distilled water at pH 7 0, dispensed at 425 mL/1 L Erlenmeyer flask, closed with cotton and sterilized at 121°C for 20 minutes) This mixture was added to a vermiculite-containing 4L roller jar (1250 cc vermiculite, latex closure sterilized separately from the liquid for 60 minutes at 121°C) and mixed vigorously to coat the vermiculite Roller jars were incubated on a roller machine at 25°C for 28 days

Table 2: Composition of Seed and Production Media.

YMEJ Seed Medium		Medium-4 Production Medium		Trace Elements for Medium-4	
Component	(g/L)	Component	(g/L)	Component	(g/L)
Yeast extract	4 0	Glucose	10 0	$FeCl_3\ 6H_2O$	5 8
Malt extract	10 0	Fructose	15 0	$MnSO_4\ H_2O$	0 1
Dextrose	4 0	Sucrose	40 0	$CoCl_2\ 6H_2O$	0 02
Junlon	1 5	Casamino acids	2 0	$CuSO_4\ 5H_2O$	0 015
		Asparagine	2 0	$NaMoO_4\ 2H_2O$	0 012
		Yeast extract	1 0	$ZnCl_2$	0 02
		Na_2HPO_4	0 5	$SnCl_2\ 2H_2O$	0 005
		$MgSO_4\ 7H_2O$	1 0	H_3BO_3	0 01
		$CaCl_2$	0 5	KCl	0 02
		Trace elements	1 0 mL	HCl (Conc)	2 0 mL/L

Isolation of Ophiobolin K and 6-epiophiobolin K:

The solid state production fermentation (3 roller jars) of *A ustus* was extracted with methyl ethyl ketone (1 5 L each, 4 5 L total) by shaking for 2 hrs The extract was concentrated under reduced pressure and then lyophilized to give a dark solid residue (3 5g) 1g of the solid was chromatographed on a column of silica gel in CH_2Cl_2 and the fraction (500 mg) containing both ophiobolins was eluted with CH_2Cl_2-CH_3OH (49 1) This fraction (500 mg) was rechromatographed on a flash silica gel column Elution of the column with CH_2Cl_2 followed by CH_2Cl_2-CH_3OH (99 1) gave almost clean **1a** (50 mg) and a mixture of **1a** and **1b** (150 mg) This mixture was further chromatographed on a Whatman partisil 10 ODS 3 (22 x 250 mm) column and eluted with a 15 mL/min linear gradient of CH_3CN-H_2O (60 40) to CH_3CN (100) over 40 min to give pure **1a** and **1b** HPLC analysis of the original mixture on a Whatman C-8 column (4 6x250 mm) in CH_3CN-H_2O (50/50) and elution at a flow rate of 1 mL/min gave a ratio 5 1 of **1a/1b** with a retention time **1b** (9 9 min) and **1a** (10 6 min) **1a** mp 80-82°C (powder), $[\alpha]_D^{23}$ 168°, $[\alpha]_{578}$ 175°, $[\alpha]_{546}$ 203°, $[\alpha]_{436}$ 387°, $[\alpha]_{405}$ 495°, $[\alpha]_{365}$ 732° (c=0 4, CH_3OH), UV (λ_{max}) CH_3OH 240 (ε 23470), IR ZnSe(ν_{max}) 3434, 2931, 1737, 1666, 1630, 1457, 1376, 1235,1202, 1157, 1142, 1126, 1102, 1067, 1041, 1022, 980, 931, 907, 879, 840, 823, 808, 757, 735, 702 cm^{-1} HR-FAB (m/z) 385 $(M+H)^+$, HREIMS (m/z) 384 2631 (M^+, calcd for $C_{25}H_{36}O_3$ 384 2664) For ^1H and ^{13}C NMR see table 1 **1b** crystallized from CH_2Cl_2-hexane as plates, mp. 215-17°C, $[\alpha]_D^{23}$ 155°, $[\alpha]_{578}$ 150°, $[\alpha]_{546}$ 175°, $[\alpha]_{436}$ 335°, $[\alpha]_{405}$ 428°, $[\alpha]_{365}$ 568° (c=0 08, CH_3OH), UV (λ_{max}) CH_3OH 239 (ε 21395), IR ZnSe (ν_{max}) 3442, 2932, 2865, 1737, 1681, 1634, 1456, 1379, 1248, 1228, 1209, 1174, 1085, 976, 947, 917, 816, 787, 732 cm^{-1}, HREIMS (m/z) 384 2695 (M^+, calcd for $C_{25}H_{36}O_3$ 384 2664) , for ^1H and ^{13}C NMR refer to table 1

***C. elegans* Motility Assay:**

C elegans, N2 strain was cultivated on NG agar plates covered with a lawn of *Escherichia coli* following Brenner's procedure[10] Worms were rinsed off the agar plates with HEPES (4-(2-hydroxyethyl)-1 piperazineethane sulfonic acid) buffer at 22°C, washed two times by centrifugation at 1,000 x g for two minutes and then resuspended in 25mM HEPES buffer Aliquots of worms (50 µl, approximately 100 worms) were placed into 13 x 100 mm glass test tubes The compounds to be tested were prepared in DMSO and added to the worms in a final volume of 500 µl containing 1% DMSO After 16 hrs incubation at 22°C, the number of worms still motile was determined by examination with a low power dissecting microscope Greater than 90% of the worms continued to swim vigorously in the control tube ED_{50} values represent the concentration of the drug which immotilizes 50% of the worms

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