Pergamon

Bioorganic & Medicinal Chemistry, Vol. 4, No. 4. pp. 531–536, 1996

0968-0896/96 $15.00 + 0.00

S0968-0896(96)00036-3

Ophiobolin M and Analogues, Noncompetitive Inhibitors of Ivermectin Binding with Nematocidal Activity

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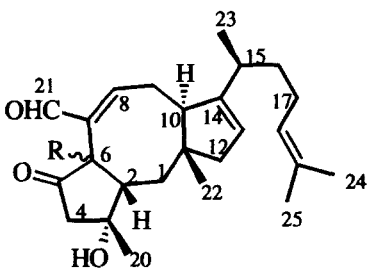
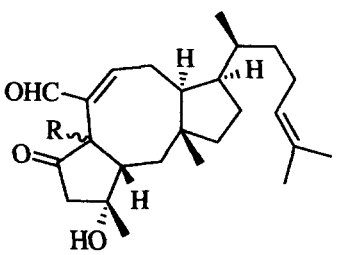
Abstract—A series of ophiobolins were isolated from a fungal extract based on their nematocidal activity. These compounds are non-competitive inhibitors of ivermectin binding to membranes prepared from the free-living nematode, *Caenorhabditis elegans*, with an inhibition constant of 15 μM. The ophiobolins which were most potent in the biological assays, ophiobolin C and ophiobolin M, were also the most potent compounds when evaluated in a *C. elegans* motility assay. These data suggest that the nematocidal activity of the ophiobolins is mediated via an interaction with the ivermectin binding site. The isolation, structure and biological activity of ophiobolins have been described. Copyright © 1996 Elsevier Science Ltd

Introduction

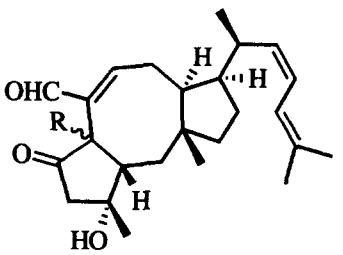
Ophiobolins are a family of naturally occurring sesterterpenes characterized by a tricyclic [5-7-5] ring system. Ophiobolin A (cochliobolin) was originally isolated from *Helminthosporium oryzae* as a toxic principle to rice seedlings.[1–5] The structure and complete stereochemistry was subsequently reported by Nozoe et al.[6] and Canonica et al.[7] A series of ophiobolins have since been isolated from fermentation broths and their chemistry is summarized in several reviews.[8–10] Ophiobolin K and 6-epiophiobolin K were previously reported to have potent nematocidal activity.[11] Two novel ophiobolins, ophiobolin M (**1**) and 6-epiophiobolin M (**2**), have now been isolated from the extracts of *Cochliobolus heterostrophus* and shown to be potent nematocidal agents. In this article we describe the isolation and structure elucidation of the novel ophiobolins and demonstrate that the nematocidal activity of these compounds, as well as that of other ophiobolins (**3**–**7**), correlates with their affinity for the nematode ivermectin receptor. Ivermectin is a widely used anthelmintic agent known to modulate



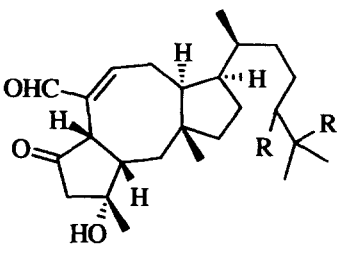
1:R = βH, Ophiobolin M
2:R = αH, 6-Epiophiobolin M



3:R = βH, Ophiobolin C
4:R = αH, 6-Epiophiobolin C



5:R = βH, Ophiobolin K
6:R = αH, 6-Epiophiobolin K



7:R = H, Dihydroophiobolin C
8:R = ^{3}H, Ditritioophiobolin C

an invertebrate specific glutamate-gated chloride channel.[12–14]

Results and Discussion

Purification and identification of ophiobolin analogues

The isolation and structure of ophiobolin K (**5**) and 6-epiophiobolin K (**6**) have been previously reported.[11] The remainder of the natural ophiobolins used in this study were produced in a solid state fermentation of *Cochliobolus heterostrophus*. Extraction with methyl ethyl ketone and size exclusion chromatography gave an ophiobolin mixture active in an assay versus *C. elegans*. Preparative reverse-phase HPLC allowed the isolation of the novel ophiobolin M (**1**) and 6-epiophiobolin M (**2**). In addition, ophiobolins C (**3**) and 6-epiophiobolin C (**4**) were also isolated and characterized on the basis of NMR and mass spectral data and comparison of physical and spectral properties with literature data.[7,15]

The structure of ophiobolins M and 6-epiophiobolin M was elucidated by detailed study of the high resolution NMR spectral data. The molecular formula for each of the two new compounds was established as $C_{25}H_{36}O_3$ by high resolution electron impact mass spectrometry (HREIMS), and corroborated by ^{13}C NMR spectra. The ^{13}C NMR spectrum (APT) of both **1** and **2** revealed 25 carbons (Table 1) and included one ketone, an aldehyde, three olefinic methines, three olefinic quaternaries, one oxygenated quaternary, four methines, six methylenes, and five methyl groups. Both ^1H NMR spectra exhibited two angular methyls, two olefinic methyls, and a secondary methyl. The methyl chemical shifts were typical of ophiobolins having 7,8 and 18,19 double bonds. The structure was completed by interpreting a combination of 2-D ^1H—^1H COSY, HMQC and HMBC spectral data. The remaining double bond was placed between C-13 and C-14 in the C-ring based on HMBC correlations of C-14 to 23-CH_3, 9-H_2, and 12-H_2; as well as correlations of C-13 to 12-H_2. Remaining HMBC correlations of ophiobolin M are listed in Table 1. For further discussions regarding the structural elucidation see ref 11. Ophiobolins and their 6-*epi* (6-αH) isomers can be distinguished from each other by the comparison of ^1H NMR shifts of H-2, H-4, and H-8. In the 6-*epi* (6-αH) isomers, protons at H-2 and H-8 are generally shielded by ca. 0.2–0.3 ppm and the β-proton at C-4 is generally deshielded by about 0.3–0.6 ppm relative to the shifts obtained for the regular (6-βH) series ophiobolins. The same held true for ophiobolin M and 6-epiophiobolin M. These two compounds represent the first examples of ophiobolins with a double bond in the C-ring and as such are Δ^{13-14} ophiobolin C.

Nematocidal activity of ophiobolin M

The purified compound has an LD_{50} value of approximately 13 μM (Table 2) in a *C. elegans* motility assay. The relative nematocidal activity of ophiobolin M as compared to previously known compounds is presented in Figure 1. Ophiobolin M is approximately 150-fold less potent than ivermectin and slightly more active than paraherquamide, levamisole, and thiabendazole (LD_{50} values of 33, 74, and 144 μM, respectively).

Nematocidal activity of ophiobolins

A series of naturally occurring ophiobolin analogues were evaluated for nematocidal activity in a *C. elegans* motility assay (Table 2). Ophiobolin C (**3**) is the most active compound with an LD_{50} value of 5 μM. Ophiobolin K (**5**) and ophiobolin M (**1**) were similarly active with LD_{50} values of 26 and 13 μM, respectively. The six compounds (**1–6**) tested represent three pairs of stereoisomers with the hydrogen at the C-6 position in either an β- (compounds **1**, **3**, **5**, and **7**) or α- (compounds **2**, **4**, and **6**) configuration. In each case the β-stereochemistry results in greater potency (2- to 12-fold).

Ophiobolin inhibition of ivermectin binding to *C. elegans* membranes

Ivermectin binds stereoselectively and with high affinity (K_i 0.27 nM) to *C. elegans* membranes.[14] The effect of ophiobolin C (**3**) on [^3H]ivermectin binding is shown in Figure 2. Ophiobolin C inhibits the binding with an inhibition constant of 15 μM. This inhibition is non-competitive based on the Lineweaver–Burke analysis of the data. Tritiated ophiobolin C was prepared in order to directly determine whether there is a high affinity 'ophiobolin' binding site. However, [^3H-18–19]dihydroophiobolin C (**8**) only binds to the *C. elegans* membranes with low affinity (>500 nM) and no further studies using this radioligand were conducted.

Ivermectin also binds to mammalian brain membranes, although with a lower affinity ($K_d \approx 2$–20 nM) than what is observed with nematode membranes. In order to further evaluate the specificity of ophiobolin activity, we determined the ability of ophiobolin to inhibit [^3H]ivermectin binding to rat brain tissue. Ophiobolin C did not inhibit ivermectin binding at concentrations up to 100 μM.

In summary, several ophiobolin analogues have been identified which have potent nematocidal activity. The correlation between the biological activity and inhibition of ivermectin binding strongly supports the hypothesis that the nematocidal activity is mediated via an interaction of the ivermectin receptor. In nematodes, it has been demonstrated that the ivermectin receptor is an invertebrate specific chloride channel which is also regulated by glutamate. Ophiobolin M is a non-competitive inhibitor of ivermectin binding suggesting that ophiobolin and ivermectin bind to distinct sites on the same molecule. The identification of this family of compounds with nematocidal activity

provides a target for the development of novel anthelmintic agents.

Experimental

Materials

All the reagents and deuterated solvents were obtained from Sigma-Aldrich Chemical Company and were used without further purification. Stationary phases used for liquid chromatography were a Whatman Partisil 5 ODS-3, 4.6 × 250 mm for analytical HPLC, and a Zorbax C-18, 21.2 × 250 mm for preparative HPLC. NMR spectra were obtained using a Varian Unity-500 spectrometer operating at 125 MHz for carbon and 500 MHz for proton, or a Varian XL-300 spectrometer operating at 75 MHz for carbon and 300 MHz for proton.

The avermectins were supplied by Dr H. Mrozik and Dr M. Fisher, Merck Research Laboratories (Rahway, NJ, U.S.A.). [^{3}H]Ivermectin was labeled at the 22,23-position by catalytic hydrogenation with tritium gas to a specific activity of 52.6 Ci/mmol. Purity of the [^{3}H]ivermectin was confirmed using thin-layer chromatography on silica gel 60-F 254 (E. Merck) developing with chloroform:ethyl acetate:methanol:methylene chloride (9:9:1:2); R_f 0.51; the [^{3}H]ivermectin was found to be greater than 95% pure.

Fermentation

A liter of seed medium contained: 5 g of corn steep liquor, 40 g of tomato paste, 10 g of oat flour, 10 g of glucose and 10 mL of trace elements solution, pH adjusted to 6.8 before autoclaving. The trace element solution comprised per liter: 1 g $FeSO_4 \cdot 7H_2O$, 1 g $MnSO_4 \cdot 4H_2O$, 25 mg $CuCl_2 \cdot 2H_2O$, 100 mg $CaCl_2$, 56 mg H_3BO_3, 19 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ and 200 mg $ZnSO_4 \cdot 7H_2O$.

The production medium contained per 250 mL flask: 50 mg yeast extract, 25 mg sodium tartrate, 25 mg potassium phosphate (monobasic), 10 g brown rice, 10 mL deionized water and was used with no pH adjustment.

Seed medium was inoculated from soil preserved *Cochliobolus heterostrophus* (MF 4628a, Merck culture collection). After 3 days of growth with agitation at 220 rpm and temperature maintained at 25 °C the culture

Table 1. NMR assignments of ophiobolin M and 6-epiophiobolin M in $CDCl_3$ solutions

Position	1 δC	1 δH	2 δC	2 δH	1 C→H (HMBC)*
1 Hα	35.4	1.60, m	41.8	1.84, m	H-12, H-22
1 Hβ		1.86, m		1.84, m	
2 H	51.3	2.45, m	49.8	2.10, m	H-1, H-4α, H-6, H-20
3	76.8	—	76.9	—	H-1, H-4α,β, H-6, H-20
4 Hα	54.9	2.82, d, 20	55.1	2.44, dd, 16.5, 1.5	H-20
4 Hβ		2.50, d, 20		3.15, d, 16.5	
5	217.4	—	217.5	—	H-4α,β, H-6
6	48.8	3.30, d, 10	49.0	3.25, d, 10.5	H-4α, H-8
7	142.8	—	142.2	—	H-6, H-9b
8	163.6	7.21, t, 8	158.8	6.90, dd, 7.5, 2.5	H-6, H-9b
9 Hα	23.9	2.60, m	32.5	2.91, ddd, 20, 4, 2.5	—
9 Hβ		2.28, m		2.19, m	
10	57.4	2.20, m	51.3	3.18, m	H-1β, H-9β, H-12β, H-13, H-22
11	47.1	—	44.3	—	H-1β, H-9β, H-12β, H-13, H-22
12 Hα	47.8	2.13, m	51.7	2.36, m	H-1β, H-22
12 Hβ		1.90, m		2.15, m	
13	121.1	5.32, m	120.3	5.28, m	H-12α
14	149.5	—	149.3	—	H-9β, H-12α, H-13, H-23
15	35.4	2.20, m	31.6	2.08, m	H-10, H-17, H-23
16 Hα	32.0	1.20, m	35.2	1.12, m	H-17, H-23
16 Hβ		1.40, m		1.46, m	
17 Hα	25.3	1.90, m	25.1	1.94, m	—
17 Hβ		1.90, m		1.94, m	
18	124.3	5.11, m	124.3	5.10, m	H-17, H-24, H-25
19	131.8	—	131.7	—	H-17, H-24, H-25
20	25.6	1.38, s	25.9	1.45, s	H-4β
21	196.4	9.27, s	194.0	9.23, s	H-6, H-8
22	19.3	0.98, s	22.8	0.91, s	H-12α,β
23	18.3	1.04, d, 6.5	18.1	1.09, d, 6.5	H-15
24	17.7	1.60, brs	17.7	1.60, brs	H-25
25	25.6	1.69, brs	25.7	1.69, d, 1.0	H-24

*"J_{XH} = 7 Hz.

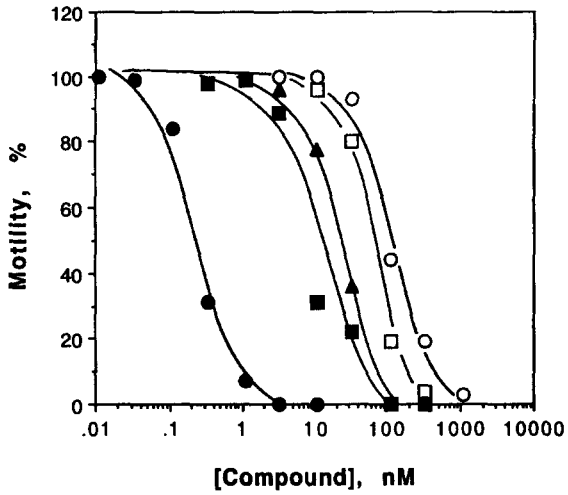


Figure 1. Nematocidal activity of ophobolin M and known anthelmintic agents. *C. elegans* were maintained in the presence of increasing concentrations of ivermectin (●), ophiobolin M (■), paraherquamide (▲), levamisole (□), or thiabendazole (○). After 16 h the percentage of motile worms was determined. This experiment was replicated four times with similar results.

was used to inoculate the production medium and allowed to grow statically for 14 days.

Isolation

To each of 40 Erlenmeyer flasks (250 mL) was added 50 mL of methyl ethyl ketone The solids were suspended and the flasks were shaken at 160 rpm for 30 min. The solids were removed by filtration. The filtrate was concentrated under reduced pressure and the residual water was removed via lyophilization. The residue was taken up in 20 mL methanol and subjected to size exclusion chromatography (column containing 1 L of Sephadex LH-20 gel, flow rate 5 mL/min, 15 mL fractions). Compounds with nematocidal activity were eluted between 0.8 and 0.9 column volumes of eluant. The active fractions were pooled and the solvent removed in vacuo (rotovap). The residue was taken up in 2 mL methanol and purified by preparative HPLC. The column was eluted isocratically with 55% aqueous acetonitrile at a flow rate of 8 mL/min. The compounds of interest were found at t_R 87.9, 107.2, 114.6, and 127.3 min. Fractions from five identical HPLC runs were pooled. Each fraction was concentrated under reduced pressure, until most of the acetonitrile had been removed. The 127.3 min fraction resulted in a precipitate that was filtered off to give 250 mg of an HPLC homogeneous ophiobolin C (**3**), as a white solid. The three other fractions were separately lyophilized to dryness, redissolved in 0.4 mL methanol and rechromatographed as before. Materials were dried down by lyophilization to give HPLC pure: 4 mg of 6-epiophiobolin M (**2**, t_R 87.9 min); 5 mg of 6-epiophiobolin C (**4**, t_R107.2 min); 10 mg of ophiobolin M (**1**, t_R 114.6 min). Ophiobolin M (**1**): IR: ν_{max} (ZnSe): 3391, 2961, 2926, 2852, 1740, 1668, 1629, 1456, 1376, 1229, 1069, 1019, 985, 943, 901, 812, 757 cm^{-1}; HREIMS (*m/z*): 384.2635 (M^+, calcd for $C_{25}H_{36}O_3$: 384.2664); for ^1H NMR and ^{13}C NMR see Table 1. 6-Epiophiobolin M (**2**): IR: ν_{max} (ZnSe): 3409, 2929, 1737, 1684, 1461, 1377, 1201, 1071 cm^{-1}; HREIMS (*m/z*): 384.2632 (M^+, calcd for $C_{25}H_{36}O_3$: 384.2664); for ^1H NMR and ^{13}C NMR see Table 1.

Table 2. Inhibition of [^{3}H]ivermectin binding and biological potencies on *C. elegans* motility in vivo for various ophiobolin analogues

Number	Compound	Binding K_i μM	Motility LD_{50} μM
1	Ophiobolin M	31	13
2	Epiophiobolin M	156	130
3	Ophiobolin C	15	5
4	Epiophiobolin C	180	130
5	Ophiobolin K	130	26
6	Epiophiobolin K	260	>260
7	Dihydroophiobolin C	45	25

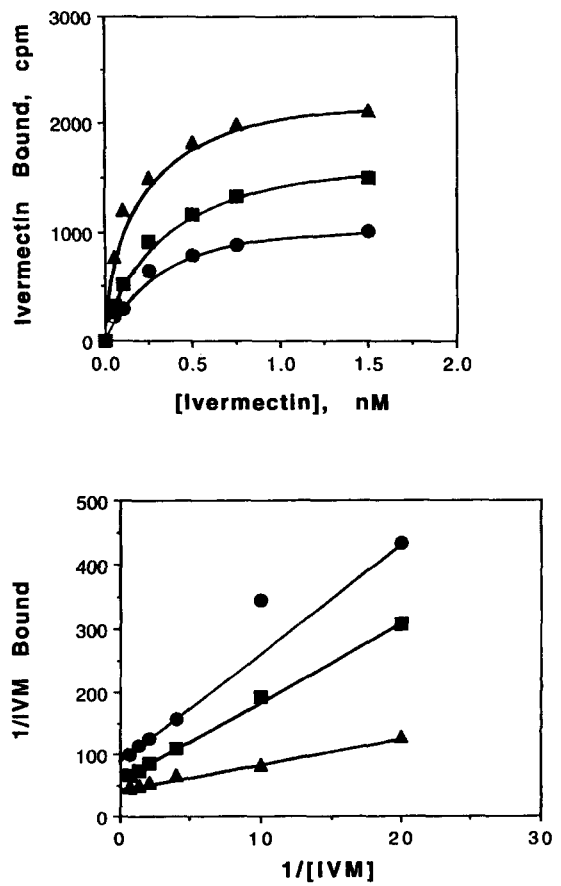


Figure 2. Ophiobolin C inhibition of ivermectin binding to *C. elegans* membranes. (Top) Increasing concentrations of [^{3}H]ivermectin were incubated with *C. elegans* membranes and specific binding was determined in absence (▲) and presence of 0.3 μM (■) and (●) 1 μM ophiobolin C. Each point is the average of four determinations. Replicate experiments gave similar results. (Bottom) Double reciprocal analysis of the data in top. The lines were plotted using linear regression analysis. The inhibition constants were calculated using the formula; y − intercept = $-1/K_d(1+[I]/K_i)$.

Preparation of dihydroophiobolin C and tritiated dihydroophiobolin C

A solution of ophiobolin C (100 mg), Wilkinson's catalyst (10 mg) in 3 mL toluene was hydrogenated for 16 h in a Parr hydrogenation apparatus at 40 psi at room temperature. The progress of hydrogenation was evaluated by analytical HPLC (1 mL/min; 40 °C; 65% aqueous acetonitrile). Ophiobolin C (t_R 17.64 min) was 85% consumed at the end of this time and a new compound at t_R 26.56 min had appeared. Half of the hydrogenation mixture was worked up as follows. The toluene was removed under vacuo, the mixture was filtered through a small bed of silica gel using 50% ethyl acetate–hexane and again dried down in vacuo. The product was taken up in 0.4 mL acetonitrile and fractionated by preparative HPLC (8 mL/min, 65% aqueous acetonitrile). The major compound eluting at 160 min, was dried down to give 25 mg of 18,19-dihydroophiobolin C (**7**) as a white solid. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.79 (d, 3H, J=7 Hz), 0.88 (d, 3H, J=6 Hz), 0.92 (s, 3H), 1.10–1.35 (m, 6H), 1.32 (s, 3H), 1.40–1.60 (m, 6H), 1.62–1.72 (m, 2H), 1.78–1.84 (m, 1H), 2.34–2.38 (m, 1H), 2.38–2.44 (m, 1H), 2.45–2.49 (m, 1H), 2.48 (d, 1H, J=19 Hz), 2.68 (d, 1H, J=19 Hz), 3.12 (s, 1H), 3.28 (d, 1H, J=10 Hz), 7.23 (t, 1H, J=8 Hz), 9.23 (s, 1H). ^{13}C NMR ($CDCl_3$ 75 MHz) δ 16.9, 19.2, 22.8 (2C), 23.5, 25.3, 25.7, 25.8, 28.4, 33.6, 36.4, 37.6, 39.6, 43.0, 44.3, 45.8, 48.9, 51.2, 53.9, 55.3, 77.1, 141.9, 164.9, 196.9, 217.4. EIMS (*m/z*): 388 (M^+).

The hydrogenation reaction was repeated using tritium enriched hydrogen to afford 227.7 mg of [18,19-^{3}H] Dihydroopohiobolin C (**8**) with 16 Ci/mol specific radioactivity and 89% radiochemical purity as determined by HPLC.

Membrane preparation

C. elegans, N2 strain was cultivated on NG agar plates covered with a lawn of *Escherichia coli* as previously described.[16] Worms (all stages) were washed off the plates with 5 mM Trizma base, adjusted to pH 7.2 with HCl. The worms were washed once for 2 min at 1,000 × *g*, resuspended in buffer (approximately 20,000 worms/mL) and then broken up by homogenization in a Braun Homogenizer (Ace Scientific, New Brunswick, NJ, U.S.A.) using 0.5 mm glass beads for 30 s. The homogenate was centrifuged 2 min at 1,000 × *g* and the supernatant centrifuged 20 min at 28,000 × *g*. The resulting pellet was resuspended in buffer and washed three more times by centrifugation at 28,000 × *g* for 20 min in order to dilute cytoplasmic contaminants as well as possible. The final pellet, resuspended in Tris buffer was used immediately.

Membranes from rat brain were prepared as previously described.[17] Male rats (Sprague-Dawley, 250–300 g) were decapitated and the cerebral cortices were quickly removed and homogenized in 10 vol of 50 mM HEPES buffer, pH 7.4, at 4 °C with a glass–glass homogenizer. The homogenate was centrifuged for 5 min at 1,000 *g* and the resulting supernatant fraction was centrifuged for 20 min at 28,000 *g*. The P2 pellet was resuspended in HEPES buffer (1 mg protein/mL) and used immediately to measure [^{3}H]ivermectin binding.

Ivermectin binding

Binding assays were performed as previously described.[16] Briefly, *C. elegans* membranes were incubated with [^{3}H]ivermectin at 22 °C for 15 min in the presence (nonspecific binding) or absence (total binding) of a 500-fold molar excess of unlabeled ivermectin in glass tubes (13 × 100 mm). The incubation was terminated by rapid filtration over Whatman GF/B filters (pre-soaked for 1 h in 0.15% polyethylimine in order to minimize nonspecific binding), and rinsed with 15 mL (3 × 5 mL) of ice-cold Tris buffer. The filters were placed into glass vials containing 10 mL Aquasol II (New England Nuclear, Boston, MA, U.S.A.), and the radioactivity determined by liquid scintillation spectrometry at 62% efficiency. At saturating concentrations of [^{3}H]ivermectin, the nonspecific binding represented approximately 32% of the total counts. Specific binding was determined by subtracting nonspecific from total binding. The K_i values for ophiobolin analogues were determined as described by Chang and Prusoff.[18] The membranes were incubated with 0.2 nM [^{3}H]ivermectin with increasing concentrations of the inhibitor. Under these conditions, >80% of the total [^{3}H]ivermectin binding was displaced by high concentrations of ophiobolin M (100 μM).

Motility assay

Worms were rinsed off the agar plates with Tris buffer at 22 °C, washed two times by centrifugation at 1,000 × *g* for 2 min and then resuspended in Tris buffer. Aliquots of the worms (50 μL, approximately 100 worms) were placed into 13 × 100 mm glass test tubes. The compounds to be tested were added to the worms in a final volume of 500 μL containing 1% dimethyl sulfoxide. After 32 h incubation at 22 °C, the number of motile worms was determined by examination with a low power dissecting microscope. Greater than 90% of the worms continued to swim vigorously in the control tube.

Protein assays

Protein concentrations were determined using the dye staining technique of Bradford.[19]

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(*Received in U.S.A.* 19 *September* 1995; *accepted* 7 *December* 1995)