Nomofungin: A New Microfilament Disrupting Agent

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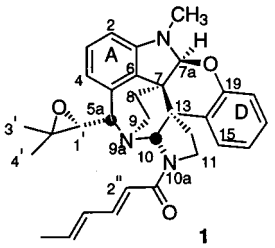
A new alkaloid, nomofungin, has been isolated from the fermentation broth of an unidentified endophytic fungus obtained from the bark of *Ficus microcarpa* L. The structure of nomofungin was determined by application of spectroscopic methods. The absolute stereochemistry of nomofungin was assigned by using the exciton chirality method. Nomofungin disrupts microfilaments in cultured mammalian cells and is moderately cytotoxic with minimum inhibitory concentrations (MICs) of 2 and 4.5 μg/mL against LoVo and KB cells, respectively. The ring system of nomofungin is unprecedented.

Introduction

In the course of a research program aimed at the discovery of natural products with activity against the cytoskeleton, specifically microtubules and microfilaments, we have been examining endophytic fungi as producers of such materials. We became interested in this group of organisms because, in the recent past, several structurally interesting, biologically active compounds have been isolated from laboratory-cultured endophytes and epiphytes, e.g., torreyanic acid,[1] the squalene-synthase inhibitors CP-225,917 and CP-263,114,[2] and the antimicrotubule agent Taxol.[3,4] Strobel has estimated that only a very small fraction of the endophyte biodiversity has been sampled and analyzed with respect to the natural products that may be obtained from this source.[5] The unique flora of Hawaii is of particular interest in that it may harbor fungi that might not be found elsewhere and that may produce novel compounds. We report such an example in the present work.

Results and Discussion

We have isolated an unidentified fungus from the bark of *Ficus microcarpa* L. growing on the Manoa campus of the University of Hawaii. The ethyl acetate extracts of fermentation broths of this organism were highly active in disrupting microfilaments in A-10 cells as shown by an indirect immunofluorescence microscopy assay.[6] As a result of this observation, the broth obtained from this fungus was selected for isolation of the active constituent. Unfortunately, the fungus has since either stopped producing **1** or the producing organism has been outgrown by a contaminant that was introduced accidentally during repeated subculture. In either case, the culture of the producing endophyte must be regarded as lost, a fact that is reflected in the name we have chosen for **1**.



1

Bioassay-guided fractionation of the ethyl acetate extract (480 mg) of the broth by repeated normal-phase chromatography over silica resulted in the isolation of **1** as an optically active, colorless solid in 0.9% yield of extract mass. The chemical structure of **1** was determined by application of spectroscopic methods.

The molecular formula was established by a combination of mass spectrometry and high-resolution NMR spectroscopy (Table 1). Thus, the 125 MHz ^{13}C NMR spectrum of **1** displayed 32 signals of which four were due to methyl, four due to methylene, 15 due to methine, and nine due to quaternary carbon atoms, respectively. Further analysis revealed the presence of one carbonyl group resonating at 168.4 ppm and of 16 signals in the olefinic region, which indicated the presence of eight carbon−carbon double bonds. Inspection of the gHSQC spectrum suggested that all of the protons in **1** were

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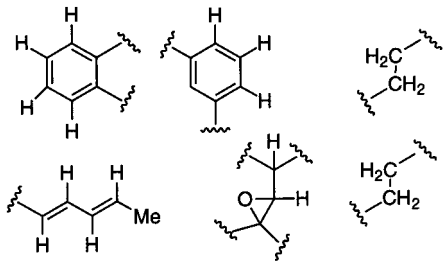
Table 1. ^{1}H and ^{13}C NMR Data for Nomofungine 1 ($CDCl_3$)

position	δ_H (ppm)	multiplicity, *J* (Hz)	δ_C (ppm)	HMBC (H → C)	NOE (H → H)
1			150.5		
2	5.95	d, 7.7	101.9	4, 6	N-Me
3	6.88	dd, 7.7/7.7	128.9	1, 5	
4	6.08	d, 7.7	113.2	2, 5a, 6	5a, 3′, 4′
5			136.6		
5a	4.17	d, 9.0	65.5	5, 9, 2′,1′, 6	4, 10, 3′, 2″
6			132.2		
7			51.4		
7a	4.69	s	82.4	1, 6, 7, 8,19	8, N-Me
8	2.27	m	37.8	6, 7	7a, 12
	2.35	m		6, 7	7a, 12
9	3.40	m	36.0	5a	1′
	3.45	m		5a	1′
10	5.10	s	79.0	5a, 9, 11, 12, 13	5a, 2″
11	3.07	ddd, 12.0/12.0/7.0	44.2		
	3.87	dd, 12.0/8.4		13	
12	2.00	dd, 13.2/7.0	30.4	13,14	
	2.72	m		7, 13, 14	8
13			52.1		
14			132.3		
15	6.65	m	123.4	13, 14	
16	6.66	m	120.5	14	
17	6.88	ddd, 7.0/7.0/2.4	127.4	15	
18	6.67	m	116.8	14	
19			142.6		
N-Me	2.84	s	29.2	1, 7a	2, 7a
1′	2.90	d, 9.0	63.9		9, 4′
2′			59.7		
3′	1.64	s	20.5	1′, 2′, 4′	4, 5a
4′	1.42	s	24.8	1′, 2′, 3′	4, 1′
1″			168.4		
2″	6.54	d, 15.1	121.3	1″, 4″	5a, 10, 4″
3″	7.31	dd, 15.1/10.7	141.8	1″	5″
4″	6.19	dd(*br*), 15.1, 10.7	130.7	6″	2″, 6″
5″	6.10	dq, 15.1, 6.5	137.1	6″	3″, 6”
6″	1.85	d, 6.5	18.6	4″, 5″	4″, 5″

attached to carbon atoms and none to heteroatoms. The molecular ion was found at *m*/*z* 509 in EIMS and HREIMS indicated a molecular formula of $C_{32}H_{35}O_3N_3$ *m*/*z* 509.245 (Δ 3.3 mmu) with seventeen degrees of unsaturation. Given the number of double bonds already accounted for, this meant that **1** contained eight rings.

The IR spectrum (NaCl) showed significant bands at 2925, 1650, 1625, 1595, 1490, 1390, 1280, and 1000 cm^{-1}. The UV/vis spectrum recorded in methanol displayed two absorptions with λ_{max} at 206 nm (log ϵ 4.6) and 269 nm (log ϵ 4.5) with a shoulder at 251 nm. Analysis of these data in conjunction with those from COSY, TOCSY, and gHSQC spectra and consideration of chemical shift information suggested the presence of six fragments as shown below.



These fragments accounted for all but eleven of the hydrogen atoms required by the molecular formula. Six of these were contained in two C-methyl singlets resonating at 1.42 and 1.64 ppm, respectively. Another three were present in the form of a three-proton singlet resonating at 2.84 ppm. This chemical shift and that of the attached carbon atom of 29.2 ppm were suggestive of an *N*-methyl group. The last two hydrogen atoms appeared as singlets resonating at 4.69 and 5.10 ppm, respectively. These chemical shifts, those of the attached carbon atoms of 82.4 and 79.0 ppm, respectively, as well as the absence of exchangeable protons, suggested the presence of acetal/aminal-type functional groups.

Connections between the fragments were established based on the analysis of gHMBC data. The methyl-carrying nitrogen atom was attached to the three-proton aromatic fragment as well as to the C-7a acetal carbon atom based on gHMBC correlations between the resonance for the *N*-methyl protons and the signals due to C-1 and to C-7a. The signal due to H-7a displayed a cross-peak to that for C-19, thereby providing a connection to the aromatic four-proton spin system via the ring C oxygen atom. Moreover, the signal for H-7a correlated to the resonances for the quaternary carbon atoms C-6 and C-7, which suggested the presence of an *N*-methyl indolenine moiety within **1**. The quaternary carbon atom C-7 was connected to one of the two C_2H_4 chains on the basis of gHMBC cross-peaks between the only partially resolved resonances of the protons on C-8 and the absorption for C-7. The second of the C_2H_4 fragments was connected to the aromatic four-proton spin system of ring D because correlations were seen between the signals for the H-12 protons at 2.00 and 2.72 ppm and the resonance for the quaternary aromatic carbon atom C-14. This placement was further supported by a series of correlations, which connected H-11 (3.87 ppm) to the quaternary aliphatic carbon atom C-13, the distal aromatic proton H-18 to the quaternary aromatic carbon atom C-14 and the proximal aromatic hydrogen atom H-15 to C-13. The

critical linkage between the two quaternary centers C-7 and C-13 was based on a long-range correlation of the signal for H-12 at 2.72 ppm to that for C-7. At this stage, five of the eight rings postulated to be present within **1** were accounted for, namely rings A through D and the oxirane.

The epoxide was shown to be part of an isolated C_5 spin system, which contained the two C-methyl groups. The resonances for the latter showed strong gHMBC correlations to those due to C-1′ and C-2′. H-1′ was *J*-coupled to H-5a, which in turn was connected to the three-proton aromatic spin system via a long-range correlation between the resonances for H-4 and C-5a. The signal for the benzylic methine proton H-5a correlated with the resonance for C-9. Conversely, cross-peaks were also visible between the resonances for the H-9 protons and the signal due to C-5a, thereby closing the seven-membered ring. The singlet due to H-10 showed strong gHMBC correlations to absorptions for C-11, C-12, and C-13, which defined ring G, the pyrrolidine. Additional cross-peaks of the H-10 signal with those for C-5a and for C-9 suggested the presence of a piperidine, ring F, containing a trisubstituted nitrogen atom, N-9a. This connection also accounted for the remaining degree of unsaturation.

The stereochemistry of the pentadiene fragment was assigned as all trans on the basis of coupling constant analysis as well as NOE data. Hydrogen atom H-2″ was *J*-coupled to H-3″ with a coupling constant of 15.1 Hz, suggesting trans geometry of this double bond. The resonances for H-4″ and H-5″ overlapped with that for H-4, which prevented straightforward extraction of coupling constant information. However, based on 1D-gTOCSY data $^3J_{\text{H-4″–H-5″}}$ was determined to be 15.1 Hz, a value in accord with trans geometry of the C-4″/C-5″ double bond. This assignment was further supported by NOE data, which showed enhancements of the signal for H-4″, rather than H-3″, upon irradiation of H-6″.

The pentadienyl side chain was attached to the sole carbonyl carbon present within **1** based on a HMBC cross-peak between the resonance for H-2″ and the carbonyl resonance. No C−H gHMBC correlations could be detected in support of the placement of the sorbate moiety on N-10a. Preliminary evidence for this arrangement was provided by NOESY data, which showed a cross-peak between H-10 and H-2″. This was confirmed by means of a $^{15}N-^{1}H$ gHMBC experiment which showed a correlation from H-12 at 2.00 ppm to a ^{15}N signal of $\delta = -243.0$ ppm, somewhat at the downfield end of the typical range of amide ^{15}N chemical shifts.[7]

With the gross structure of **1** in hand, we turned our attention to establishing the relative stereochemistry. This was accomplished by a combination of NOESY and selective NOE experiments. Assembly of molecular models at this stage yielded two potential structures. The first one, "flat", had rings A−D in a more or less planar arrangement with one of the C_2H_4 bridges protruding below and one above the plane. In the second model, "bent", the two bridges were placed on the same side of the molecule and as a result rings A and B were oriented almost at right angles to rings C and D (Figure 1).

Detailed analysis of the NOESY data uncovered several diagnostic correlations, which were explainable only

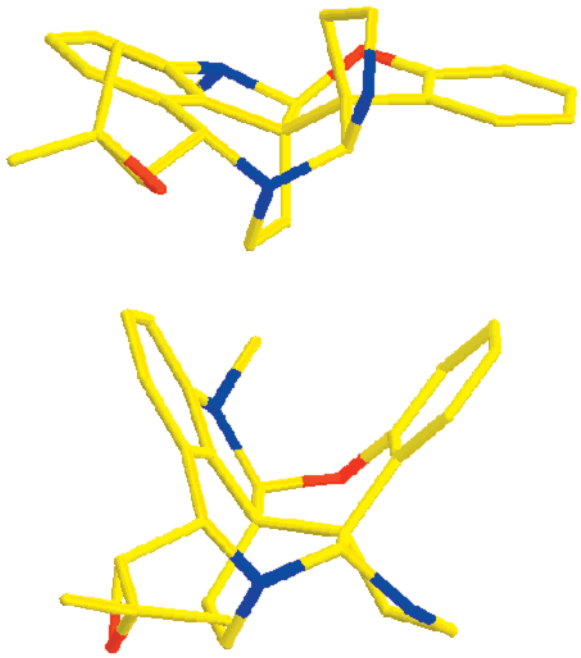


Figure 1. Structures of "flat" (top) and "bent" (bottom) nomofungin (sorbate removed for clarity).

on the basis of the latter arrangement of the two C_2H_4 bridges. Particularly informative were correlations from H-7a to both protons on C-8, which were indicative of cis stereochemistry at the B/C ring junction. Moreover, there was a weak correlation between the signals for the protons on C-8 and the downfield resonance for H-12, which suggested that both bridges were on the same side. This was corroborated by a strong NOE between H-10 and H-5a. Inspection of molecular models readily can account for the cross-peak in the "bent" model as these two protons are placed on the same face of the concave structure, whereas in the "flat" model an NOE between protons on opposite faces of the molecule would have to be invoked.

The relative stereochemistry at C-5a was assigned by interpretation of NOE data. Correlations between the signals for H-5a and H-10 suggested that these hydrogen atoms were placed on the same face of the concave structure and, consequently, that the epoxide bearing side-chain was attached pseudoaxially. The NOE observed between the signal for H-1′ and the protons on C-9 supported this conclusion.

The stereochemistry at H-1′ was determined by application of Murata's *J*-based method[8] in conjunction with NOE data. Substitution by oxygen atoms on vicinal carbon atoms has been shown to reduce the size of the coupling constant between hydrogen atoms attached to these carbon atoms in comparison to those found in hydrocarbons. This rule has recently been shown to hold for halogens, specifically chlorine,[9] as well, and the extension to an amino alcohol, as in the present case, seems justified. Thus, the coupling constant of 9 Hz between H-5a and H-1′ suggested an anti arrangement of these protons in the predominant conformer of **1** around the C-1′/C-5a bond. The observation of NOEs from

(7) Nitromethane ($\delta = 0$ ppm) served as external chemical shift standard.

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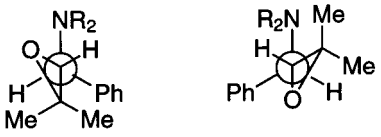
the two methyl groups, C-3′ and C-4′, to H-4 on ring A then led to the conclusion that N-9a and the epoxide oxygen were oriented syn to each other as shown in conformer **A** below. The anti conformer **B**, on the other hand, would not be expected to show NOE's between the methyl protons and H-4 on the aromatic ring. It followed that the relative configuration at C-5a and C-1′ was 5a*S**,1′*R**.



A **B**

Last, we determined the absolute configuration of **1**. The parent compound with its three chromophors gave four strong Cotton effects in methanol solution (see Supporting Information). Since the low energy transition of the anisole and the absorption of the sorbic acid chromophors in the UV spectrum occur at about the same wavelength of 270 nm, assignment of the absolute configuration of **1** based on this absorption would be fraught with considerable uncertainty. The unknown orientation of the sorbate moiety relative to the other chromophors present within **1** exacerbates this problem. Thus, to simplify the analysis, the sorbate side-chain was saturated by catalytic hydrogenation, which resulted in only insignificant changes in the CD spectrum of the product, while altering the appearance of the UV spectrum substantially, as expected. We concluded that the observed split Cotton effects were dominated by the interactions of the anisole (~270 nm) and the aniline chromophors (~246 nm) within **1**. These two absorptions were assigned to the transitions that are polarized along the axes of the chromophors that run through the heteroatom substituent and the para carbon atom of rings A and D, respectively, as has been done in the case of (−)-argemonine,[10] (−)-amurensine,[11] and (+)-calycanthine.[12] According to this analysis, the negative second Cotton effect at 270 nm is due to the ^1L_b band of the anisole chromophore within **1** and the positive third effect at 246 nm to the corresponding transition in the aniline chromophore. The positive first Cotton effect at 292 nm in the CD spectrum of **1** was assigned to a transition (^1L_a) that is orthogonal but in-plane relative to the one giving rise to the band at 246 nm. The UV absorption corresponding to the 292 nm band in the CD spectrum of **1** or its tetrahydro derivative is very weak, and use of such weak transitions is generally discouraged. This rule holds particularly in cases such as the present one, where the symmetry of the chromophore is low.[13]

Thus, the negative and positive signs, respectively, of the Cotton effects at 270 and 246 nm are indicative of negative exciton chirality between the two moieties as shown in Figure 2. Hence, the absolute configuration of **1** was assigned as 5a*S*,7*R*,7a*S*,10*S*,13*R*,1′*R*.

Nomofungin is moderately cytotoxic with MICs of 2 μg/mL and 4.5 μg/mL against LoVo and KB cells, respectively. The cytotoxic effect is achieved by disruption of the microfilament network of mammalian cells. Other fungal metabolites known to disrupt microfilaments are phalloidin[14] and the cytochalasins.[15,16] Some members of the latter class of compounds and phalloidin are orders of magnitude more potent than **1** is.

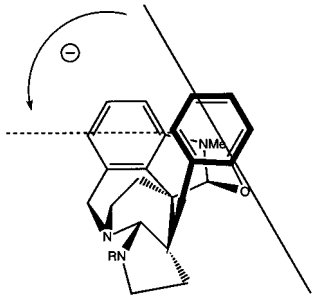


Figure 2. Analysis of the orientation of the ^1L_b transition axes of the anisole and aniline chromophors within **1** for the determination of the absolute stereochemistry of **1** (the epoxide and sorbate side chains have been removed for clarity).

Experimental Section

Spectral Analysis. NMR spectra were determined on an 11.75 T instrument operating at 500 MHz for ^1H and at 125 MHz for ^{13}C. Chemical shifts are referenced to residual $CHCl_3$ (7.26 ppm) in $CDCl_3$; ^{13}C chemical shifts are referenced to the solvent ($CDCl_3$, 77.0 ppm). One-bond $^1H-^{13}C$ connectivities were determined by gHMQC; two- and three-bond connectivities were determined by gHMBC. All chemical shift assignments are based on detailed analysis of COSY, TOCSY, gHMQC and gHMBC analysis. Mass spectra were measured in EI mode. UV- and CD spectra were acquired in spectroscopy grade methanol at 20 °C.

Isolation of the Fungal Strain. The area of bark of *Ficus microcarpa* L. to be sampled was surface sterilized by squirting 70% aqueous ethanol on the bark and letting it evaporate. A piece of bark was removed by means of a flame-sterilized pocket knife and transferred to the laboratory in a sterile polyethylene bag. Within 2 h, the sample was surface-sterilized by repeated treatment (3× for 1 min each) with 70% (v/v) aqueous ethanol and rinsed with sterile water. The sample was divided into several pieces with a dissecting knife or sterile forceps, and individual pieces were incubated at room temperature on Petri dishes containing water−agar. Fungal colonies growing from the bark were removed after 2−3 days and individually subcultured on potato-dextrose agar supplemented with novobiocin (10 μg/mL). Individual colonies were subcultured repeatedly on potato-dextrose agar at 27 °C in the dark until judged to be axenic. Liquid cultures were inoculated by adding agar plugs (Ø ≈ 5 mm) from actively growing cultures.

Cultivation of the Fungus. Preparative-scale fermentation (4 L) was carried out in 2 L Erlenmeyer flasks for 3 weeks as standing cultures at room temperature in the dark. Each flask contained 0.5 L of potato dextrose extract (Difco) in deionized water. After autoclaving for 15 min at 121 °C and cooling, cultures were inoculated from a 1-week-old broth culture in potato dextrose medium by transfer of approximately 5 mL of mycelial suspension.

Isolation of Nomofungin. The mycelium was filtered off and washed with water. The filtrate was extracted with ethyl acetate (4 × 1 L), and the combined organic extracts were

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(16) Buckingham, J. *Dictionary of Natural Products*; Chapman and Hall: New York, 1994; Vol. 1, pp 1260−1263

washed with water (0.5 L) and brine (0.5 L), dried over $MgSO_4$, and evaporated in vacuo to yield 480 mg of a dark orange oil. This was applied to a silica gel column (40 g, 15 mm × 20 cm), which was equilibrated in hexanes, washed with methyl *tert*-butyl ether (MTBE) in hexanes (1:1, 250 mL), and then eluted with pure MTBE (340 mL). Active fractions of the MTBE eluate were combined and evaporated to dryness. The residue (80 mg) was applied to another silica gel column (15 g, 10 mm × 16 cm) equilibrated in hexanes. The column was eluted with a step-gradient of MTBE in hexanes (1:1, 250 mL; 4:1, 140 mL; 6:1, 140 mL). The active material was eluted with MTBE in hexanes (6:1) and active fractions were combined. After evaporation the dry residue (16 mg) was rechromatographed on a silica gel Extract-Clean cartridge (2 g, Alltech) with $CHCl_3$/acetone (7:1) and active fractions were combined (10 mg). Final purification was achieved on a silica gel Extract-Clean cartridge (500 mg, Alltech) eluting with $CHCl_3$. **1** was obtained as a colorless powder (4.2 mg, 0.9% of extract mass).

Nomofungin (1): $[\alpha]_D$ −83 (*c* 0.09, MeOH); UV (MeOH) λ_{max} nm (log ϵ) 206 (4.6), 251 (sh), 269 (4.5); IR (NaCl) ν_{max} 2925, 1650, 1625, 1595, 1490, 1390, 1280, 1000 cm^{-1}; CD (MeOH) $\lambda_{ext.}$ nm ($\Delta\epsilon$) 292 (+18), 270 (−42), 246 (+20), 207 (−39); ^1H and ^{13}C NMR ($CDCl_3$) see Table 1; EIMS *m*/*z* (rel intensity) 509 (M^+, 1), 508 (4), 437 (16), 343 (9), 254 (21), 185 (45), 143 (43), 130 (62), 95 (59), 67 (100); HREIMS *m*/*z* 509.2645 ($C_{32}H_{35}O_3N_3$, Δ 3.3 mmu).

Tetrahydronomofungin. Approximately 0.5 mg of **1** was dissolved in ethanol (0.1 mL) and added to a prehydrogenated suspension of PtO_2 (1 mg) in EtOH (0.1 mL) in a screw cap vial. The mixture was stirred for 4 h at room temperature under a balloon filled with hydrogen. The mixture was filtered through Celite and evaporated to dryness to yield a colorless solid: UV (MeOH) λ_{max} nm (log ϵ) 207 (4.4), 248 (3.8), 270 (3.8); ^1H NMR ($CDCl_3$) δ 7.00 (ddd, J = 7.5, 5.5, 1.3 Hz, 1H), 6.88 (dd, J = 7.7, 7.7 Hz, 1H), 6.71−6.64 (m, 3H), 6.07 (d, J = 7.7, 1H), 5.94 (d, J = 7.7, 1H), 5.03 (s, 1H), 4.69 (s, 1H), 4.09 (d, J = 9.2 Hz, 1H), 3.88 (dd, J = 11.7, 8.5 Hz, 1H), 3.49−3.32 (m, 2H), 3.03−2.97 (m, 1H), 2.84 (s, 3H), 2.90−2.69 (m, 2H), 2.39−2.25 (m, 2H), 1.95 (dd, J = 13.2, 7.3, 1H), 1.76−1.65 (m, 2H), 1.58 (s, 3H), 1.38 (s, 3H), 1.37 (m, 2H), 1.25 (m, 4H), 0.92 (t, J = 6.8 Hz, 3H).

Bioassay. The microfilament immunofluorescence assay was performed as described.[6] Briefly, A10 cells, rat smooth muscle cells, were grown on cover slips in Basal medium Eagle containing 10% fetal calf serum. The cells were treated for 24 h with various dilutions of fractions or with pure **1** dissolved in DMF (final concentration ≤2.5%). Phalloidin and vehicle served as positive and negative controls, respectively. Cells were fixed with 3% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 2 min and then reduced with 1% $NaBH_4$ in PBS (phosphate-buffered saline) three times for 5 min each. The microfilaments were stained with a TRITC−phalloidin conjugate in PBS for 45 min. After repeated washing of the slips, the chromatin was stained with 4,6-diamidino-2-phenylindole. The cover slips were mounted on microscope slides and examined by means of a Zeiss Axioplan fluorescence microscope. Activity was scored semiquantitatively on a + to ++++ scale reflecting the percentage of cells displaying changes in the microfilament network. Cytotoxicity was determined by the sulforhodamine B assay.[17]

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Supporting Information Available: Copies of ^1H, ^{13}C, NOESY, and CD spectra of compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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