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Bacaryolanes A−C, Rare Bacterial Caryolanes from a Mangrove Endophyte

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Supporting Information

ABSTRACT: Caryolanes are known as typical plant-derived sesquiterpenes. Here we describe the isolation and full structure elucidation of three caryolanes, bacaryolane A−C (**1**−**3**), that are produced by a bacterial endophyte (*Streptomyces* sp. JMRC:ST027706) of the mangrove plant *Bruguiera gymnorrhiza*. By 2D NMR, analysis of the first X-ray crystallographic data of a caryolane (bacaryolane C), CD spectroscopy, and comparison with data for plant-derived caryolanes, we rigorously established the absolute configuration of the bacaryolanes and related compounds from bacteria. Bacterial caryolanes appear as the mirror images of typical plant caryolanes. Apparently plant and bacteria harbor stereodivergent biosynthetic pathways, which may be used as metabolic signatures. The discovery of plant-like volatile terpenes in endophytes not only is an important addition to the bacterial terpenome but may also point to complex molecular interactions in the plant−microbe association.

Terpenoids represent the largest group of secondary metabolites, with nearly 400 distinct structural families, which result from mechanistically intriguing enzymatic cyclization and rearrangement reactions.[1−3] Arising from their structural diversity, terpenes exhibit various biological functions, as fragrances, deterrents, hormones, vitamins, and therapeutics for example.[4] Classically, terpenes are signature plant and fungal metabolites. On the other hand, the terpenes geosmin[5] and 2-methylisoborneol,[6] which are responsible for the earthy smell of bacteria, have long been considered as rarities.

It appears that most bacterial terpenes evaded discovery because of low production rates, difficulties in isolation, and the lack of chromophores. However, through genome-mining approaches it has become obvious that genes for terpene synthases and cyclases are widely distributed in bacteria and in particular in actinomycetes.[7−10] It has been impressively demonstrated that knowledge of the biosynthetic potential in combination with modern analytical tools can lead to the discovery of various bacterial terpenes from wild-type microorganisms. As a result, known or new bacterial terpenes are produced by heterologous expression of gene clusters or individual terpene cyclase genes.[11−13] To date, the ecological functions of bacterial terpenoids have remained vague. Yet, it is noteworthy that the steadily growing bacterial "terpenome" includes numerous plant-like terpenoids, which is particularly intriguing in cases where the producing strains were isolated from plants. In order to deduce potential patterns of metabolic profiles, more structural data are required. In the search for natural products from bacteria inhabiting mangrove trees (*Kandelia candel*, *Bruguiera gymnorrhiza*, and *Aegiceras corniculatum*)[14−18] we have identified plant-like germacranes,[19] eudesmenes (kandenols),[20] and a number of diverse indolosesquiterpenes (sespenine, indosespene, xiamycin, and its dimers)[21−24] that are actually produced by bacteria.

Here we report the isolation and structure elucidation of three rare bacterial caryolanes from an endophytic *Streptomyces* sp. (JMRC:ST027706) of *Bruguiera gymnorrhiza*. We present the first crystal structure of a caryolane and corroborate by comprehensive stereochemical analyses that the bacterial terpene skeletons represent the mirror images of plant-derived caryolanes.

RESULTS AND DISCUSSION

Streptomyces sp. JMRC:ST027706 was isolated from the stem of *Bruguiera gymnorrhiza*. After reaction with anisaldehyde−sulfuric acid, TLC (thin-layer chromatography) analysis of

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the crude extract showed interesting spots in the nonpolar region. To elucidate these unusual lipophilic components, the extract from a scaled-up culture (200 L) was subjected to chromatography on Amberchrom 161c resin LC, silica gel, and Sephadex LH-20, yielding **1** (0.5 mg), **2** (7.9 mg), and **3** (2.8 mg). The structures of **1**−**3** (Figure 1) were elucidated by extensive NMR and CD analysis and were supported by X-ray crystallography.

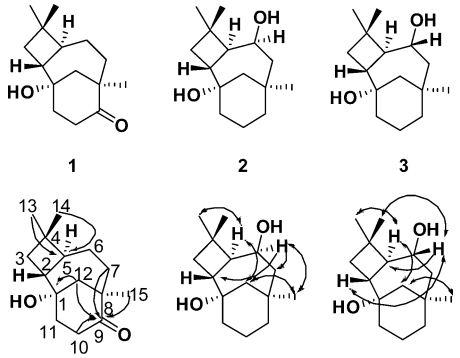

Figure 1. Structures of bacaryolanes A−C (**1**−**3**) and selected 2D NMR correlations; HMBC, normal arrow; NOESY, double-faced arrow.

EIMS measurements indicated molecular masses of 236, 238, and 238 Da for **1**, **2**, and **3**, respectively. The ^{1}H NMR spectrum of **1** exhibited signals for three methyl singlets (δ 0.93, 0.99, 1.10) and other aliphatic proton signals. Its ^{13}C NMR spectrum showed 15 signals for carbons including an oxygenated carbon (δ 69.5) and a carbonyl (δ 216.9). Further analysis by COSY, HSQC, and HMBC spectra identified **1** as a tricyclic sesquiterpene that is identical to 9-oxocaryolan-1-ol (**4**), a metabolite previously isolated from pods of the plant *Sindora sumatrana*.[25] Whereas all NMR data of **1** are in agreement with published data for the plant metabolite, the optical rotation of **1** (+17°) is opposite that of 9-oxocaryolan-1-ol (−62°).[26] Thus, **1** represents the enantiomer of the plant-derived 9-oxocaryolan-1-ol and was named bacaryolane A. The CD spectrum (supplemental data) indicated a weak, positive Cotton effect, which also supports the 8*R* configuration according to the octant rule.

In the ^{1}H NMR spectrum of **2**, three methyl singlets, Me-13 (δ 1.07), Me-14 (δ 1.20), and Me-15 (δ 0.90), remained largely conserved as for **1**. The obvious difference is the appearance of an oxygenated methine (H-6, δ 4.04). According to HSQC and HMBC correlations, the alcohol function is located at the C-6 position. 2D NOESY correlations (Figure 1) between H-12 and H-5/Me-15, H-6 and Me-15, and H-5 and Me-13 established the relative configuration. Thus, **2** was established as a second new caryolane, named bacaryolane B.

^{1}H NMR and ^{13}C NMR data of **3** were very similar to those for **2**, and 2D NMR correlations established the same planar structure as **2**. However, 2D NOESY data indicated correlations between H-6 and H-2/Me-14 (Figure 1). We noted that compound **3** forms fine crystals in CH_2Cl_2−CH_3OH and subjected a sample to X-ray crystallography. The inferred structure (Figure 2) unequivocally confirmed the proposed constitution and the relative configurations. Compound **3** was established as 1,6-dihydroxycaryolane, a recently described bacterial caryolane from another endophytic *Streptomyces* spp.

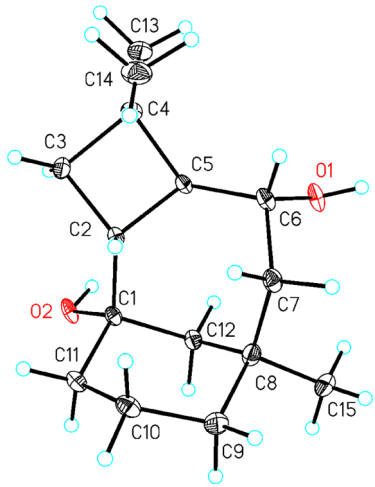

Figure 2. ORTEP diagram showing the atom-numbering scheme and solid-state conformation of **3**.

isolated from *Drymaria diandra*.[27] Thus, **3** was identified as an epimer of bacaryolane B, namely, bacaryolane C.

The isolation of three caryolane congeners from a bacterial culture is remarkable because terpenes with this scaffold are known as typical plant metabolites (Figure 3). Caryolanes are

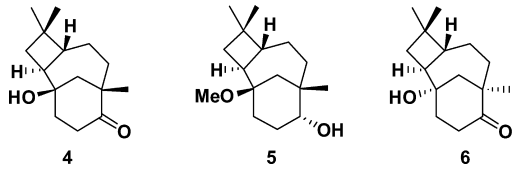

Figure 3. Structures of selected plant-derived caryolanes.

components of essential oils from various medicinal plants, for example, 9α-hydroxy-1β-methoxycaryolanol (**5**) from the Chinese traditional medicine *Sinacalia tangutica*.[28] In addition to 9-oxocaryolan-1-ol (**4**), 9-oxoisocaryolan-1-ol (**6**) was found in pods of *S. sumatrana*.[25,26]

The first caryolanes (Figure 4) reported from bacteria were recently discovered. Caryolane-1,7 α-diol (**7**) was isolated from

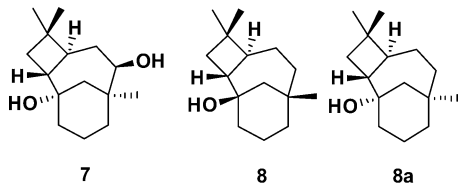

Figure 4. Reported structures of bacterial caryolanes and revised structure of **8** (**8a**).

an endophytic *Streptomyces* sp. from *Drymaria diandra*.[29] Furthermore, heterologous expression of a cryptic sesquiterpene cyclase from *Streptomyces griseus* led to the formation of a bacterial (+)-caryolan-1-ol (**8**).[12] The NMR data for the reported bacterial (+)-caryolan-1-ol were practically identical with the NMR data of synthetic (−)-caryolan-1-ol and showed high similarities to the ones for bacaryolanes A−C. However, in the original publication the structure was mistakenly drawn with an isocaryolane structure. Thus, it should be revised to **8a**.

Having several representatives of each bacterial and plant caryolanes at hand, it appears they result from diverging

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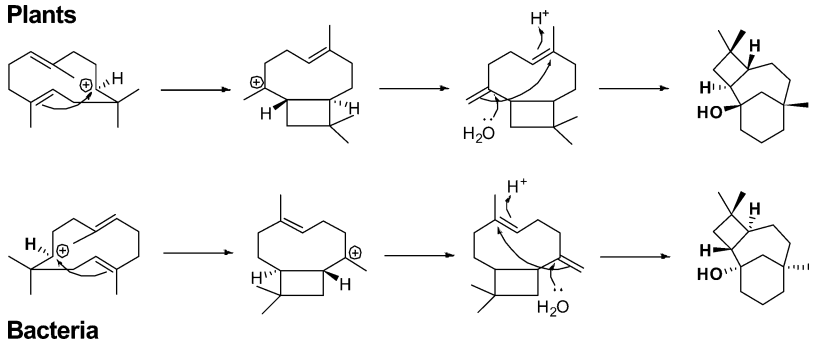
Scheme 1. Proposed Stereodivergent Biosynthetic Pathways for Caryolanes



terpene pathways. All caryolanes are derived from the humulyl cation, a cyclization product of the *E,E*-farnesyl cation. However, further cyclization of the humulyl cation into the caryophyllyl cation may lead to different stereoisomers. At this stage, the pathway diverges, leading to different caryolanes (Scheme 1). Although plant caryolanes have long been known, no enzyme responsible for the biosynthesis of caryolan-1-ol has been identified. However, numerous plant *β*-caryophyllene synthase genes have been characterized,[30] and analysis of a representative from *Arabidopsis thaliana* revealed that the cyclase produces (−)-*β*-caryophyllene.[31] It is conceivable that the downstream cyclization is nonenzymatic or catalyzed by a cyclase that allows for various reaction channels, as one single plant tends to produce caryolanes with different skeletons (e.g., **4** and **6** from pods of *S. sumatrana*). In contrast, in all cases studied thus far, bacterial caryolanes feature a defined carbon skeleton with the same absolute and relative configuration. It is particularly intriguing that the first bacterial caryolane synthase produces (+)-caryolan-1-ol via (+)-*β*-caryophyllene, yielding the enatiomer of typical plant products.[12] Taken together, caryolanes from plants and bacteria have particular stereochemical signatures, and one may use this information to predict the true producer of the terpenes. In this context it is remarkable that the recently isolated caryolanes from the plant *Cirsium souliei* show a typical bacterial motif,[32] which could point toward endophytic metabolites.

Endophytes often play eminent roles in maintaining the fitness of the host plants, as they may produce natural products against plant pathogens or herbivores.[33,34] This concept may also hold true for volatile terpenes that are often involved in induced defense processes in plants.[35] Upon attack of herbivores or plant pathogens, the plant may release a mixture of volatiles containing terpenes, which function as direct defensive compounds or to attract natural enemies of the predator.[36−38] To test whether the bacterial caryolanes could contribute to plant protection by antibiosis, we subjected **1**−**3** to a panel of cell-based antimicrobial and cytotoxicity assays. As only bacaryolane B exhibited weak activity against *Bacillus subtilis* ATCC 6633, the caryolanes likely do not function as antibiotics in the plant−microbe interaction. However, it is remarkable that the only bacterial wild-type producers of caryolanes known to date (**3** and **7**) are endophytes. Previous investigation demonstrated that metabolites secreted by endophytes play a role in defense and competition, but may also be needed for specific interaction and communication with the host plant.[39] In this context it is particularly intriguing that the biosynthetic precursor of caryolanes, (*E*)-*β*-caryophyllene, recruits natural enemies of herbivores in maize plants.[40,41] By analogy, similar scenarios would be conceivable for bacteria-derived caryolane metabolites in mangrove plants. Future research on the mutualism between plants and their endophytes may take into account that also the bacterial terpenome can play a role in multipartner symbiotic interactions. Overall, the synthesis of metabolites by endophytes may contribute to host defense and other types of plant−microbe crosstalk. To this end, it is not surprising that endophytes are a prolific source of small molecules with diverse biological activities.[42]

EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectra were recorded on a Bruker Avance III 500 MHz or Avance III 600 MHz spectrometer. IR spectra were recorded on a Bruker FT-IR (IFS 55) spectrometer. Optical rotation was recorded on a Propol digital automatic polarimeter (Dr. Wolfgang Kernchen GmbH, Seelze, Germany). EI spectra were obtained by a SSQ 710 mass spectrometer (Finnigan MAT). ESIMS data were measured using a triple quadrupole mass spectrometer (Quattro; VG Biotech, Cheshire, UK). Open column chromatography was performed on silica gel 60 (Merck, 0.04−0.063 mm, 230−400 mesh ASTM) and Sephadex LH-20 (Pharmacia). TLC analysis was performed on silica gel plates (Sil G/UV254, 0.20 mm, Macherey-Nagel).

Strain Isolation and Taxonomic Classification. The stems of *Bruguiera gymnorrhiza* were collected in Xiamen, Fujian Province, People's Republic of China, in June 2002 and authenticated by Prof. Peng Lin, Xia Men University, People's Republic of China. A voucher sample of the plant is deposited in the National Research Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing, China. JMRC:ST027706 was isolated from the stems of the plant. It was deposited in the strain collection of the HKI, Jena, Germany. The nucleotide sequence of 16S rDNA was sequenced and deposited under the accession number KR703649 in GenBank (www.ncbi.nlm.nih.gov). It shows a similarity of 99% and 98.3% to *Streptomyces flavovirens* and *Streptomyces flavogriseus*, respectively.

Fermentation. Liquid organic medium 79 (dextrose 10 g, bacto peptone 10 g, casamino acids 1 g, yeast extract 2 g, NaCl 6 g, H_2O 1 L) (2 × 100 mL/flask) was inoculated with a suspension of mycelium and spores (about 1 × 1 cm) of *Streptomyces* sp. JMRC:ST027706 grown on agar slants or agar plates. After incubation for 48 h on a rotary shaker at 28 °C, the culture was transferred to 3200 mL of medium 27 (glucose 20 g, soybean powder 20 g, NaCl 5 g, $CaCO_3$ 3 g, H_2O 1 L, eight 1000 mL scale Erlenmeyer flasks with 400 mL of medium 27 each) and incubated at 28 °C under shaking conditions for 48 h to yield prefermentation culture, which was poured into a 300 L scale fermenter filled with 200 L of medium 27 and fermented for 5 days.

Extraction and Isolation. The fermentation broth of strain JMRC:ST027706 was separated into culture filtrate and mycelia by centrifugation. The fermentation broth was filtered and loaded onto an Amberchrom 161c resin LC column (200 × 20 cm, 6 L). Elution with a linear gradient of H_2O−CH_3OH (from 30% to 100% v/v, flow rate 0.5 L min^{-1}, in 58 min) afforded seven fractions (F1−F7). F1−F5

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Table 1. NMR Data for Compounds 1, 2, and 3 (^{1}H NMR, 500 MHz; ^{13}C NMR, 1, 150 MHz; 2, 3, 125 MHz, $CDCl_3$)

	1		2		3	
no.	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	69.5, C		70.4, C		71.2, C	
2	43.0, CH	1.74, m	36.4, CH	2.57, m	41.6, CH	2.09, m
3	34.4, CH_2	1.63, m	35.3, CH_2	1.51, m	35.8, CH_2	1.57;[a] 1.49, dd (9.4, 8.7)
4	33.6, C		36.1, C		35.4, C	
5	44.9, CH	1.73, m	49.3, CH	1.90, dd (12.6, 4.1)	53.1, CH	1.79, dd (12.0, 8.4)
6	25.6, CH_2	1.43, m; 1.25, m	68.5, CH	4.04, s	72.0, CH	3.80, m
7	38.5, CH_2	2.09, m; 1.04, m	48.2, CH_2	1.56, dd (14.3, 6.2); 1.36, dd (14.5, 4.0)	49.5, CH_2	1.67;[a] 1.26, dd (14.3, 7.6)
8	46.8, C		34.4, C		35.1, C	
9	216.9, C		37.4, CH_2	1.42, m; 1.07, m	38.3, CH_2	1.43, m; 1.41, m
10	34.5, CH_2	2.77, m; 2.35, m	20.6, CH_2	2.05, m; 1.26, m	21.7, CH_2	1.64, m
11	36.9, CH_2	1.97, m; 1.85, m	40.1, CH_2	1.61, m; 1.26, m	39.7, CH_2	1.58, m; 1.29, m
12	49.1, CH_2	2.01, s	48.5, CH_2	1.72, m; 1.07, m	48.5, CH_2	1.91, d (2.9)
13	30.3, CH_3	0.93, s	31.2, CH_3	1.07, s	31.6, CH_3	1.09, s
14	21.7, CH_3	0.99, s	22.8, CH_3	1.20, s	21.6, CH_3	1.12, s
15	31.2, CH_3	1.10, s	35.8, CH_3	0.90, s	36.0, CH_3	0.99, s

[a]Coupling constant could not be deduced due to signal overlapping.

were combined and extracted by ethyl acetate and evaporated to afford a crude extract (8 g). It was first fractionated by silica gel column chromatography with a CH_2Cl_2–CH_3OH gradient to yield four fractions, A–D. Fraction A was fractionated by a Sephadex LH-20 (CH_2Cl_2–50% CH_3OH) column, and six fractions (A1–A6) were obtained. Fraction A1 was further separated by an RP-C_{18} column (CH_3OH–H_2O as gradient), yielding pure **2** (7.9 mg) and **3** (2.8 mg). Fraction B was fractionated by a Sephadex LH-20 column (CH_2Cl_2–50% CH_3OH) to afford pure **1** (0.5 mg).

*Bacaryolane A (**1**):* colorless solid; $[\alpha]_D^{23}$ +17.1 (*c* 0.05, MeOH); CD (*c* 0.30, MeOH) $\Delta\varepsilon_{296}$ 2.98; IR (film) ν_{max} 3419.8, 2924.2, 2857.0, 1697.3, 1455.2, 1378.5, 1328.4, 1259.7, 1159.5, 1103.3, 1039.4, 916.7, 799.1, 756.6, 667.7, 629.5 cm^{-1}; EIMS (14 eV) *m*/*z* 236, 218, 212, 203, 190, 179, 175, 164, 161, 154, 144, 136, 126, 123, 112, 108, 96, 84, 72, 63, 59, 32; HRESIMS *m*/*z* 237.1850 $[M + H]^+$ (calcd 237.1849 for $C_{15}H_{25}O_2$ 274.1085); NMR data, see Table 1.

*Bacaryolane B (**2**):* colorless solid; $[\alpha]_D^{23}$ −1.3 (*c* 0.79, MeOH); IR (film) ν_{max} 3323.9, 2923.2, 2862.5, 1691.1, 1465.1, 1356.0, 1308.6, 1282.5, 1219.7, 1180.3, 1156.8, 1098.2, 1059.3, 988.0, 951.6, 913.9, 884.4, 855.1, 737.3, 677.1, 610.9 cm^{-1}; EIMS (14 eV) *m*/*z* 238, 220, 205, 202, 196, 187, 176, 166, 162, 159, 151, 148, 136, 125, 122, 112, 109, 95, 82, 59; HRESIMS *m*/*z* 239.2004 $[M + H]^+$ (calcd 239.2006 for $C_{15}H_{27}O_2$); NMR data, see Table 1.

*Bacaryolane C (**3**):* colorless solid; $[\alpha]_D^{23}$ −37.4 (*c* 0.28, MeOH); IR (film) ν_{max} 3305.3, 2926.3, 2862.8, 1459.3, 1353.3, 1328.1, 1283.0, 1254.3, 1195.2, 1134.5, 1103.8, 1187.7, 1065.3, 1012.4, 981.9, 947.2, 913.7, 869.3, 851.3, 648.5, 614.2 cm^{-1}; EIMS (14 eV) *m*/*z* 238, 220, 205, 202, 195, 187, 176, 166, 162, 151, 136, 122, 111, 95; HRESIMS *m*/*z* 239.2004 $[M + H]^+$ (calcd 239.2006 for $C_{15}H_{27}O_2$); NMR data, see Table 1.

Crystal Structure Determination. The intensity data were collected on a Nonius KappaCCD diffractometer, using graphite-monochromated Mo Kα radiation. Data were corrected for Lorentz and polarization effects; absorption was taken into account on a semiempirical basis using multiple scans.[43−45] The structure was solved by direct methods (SHELXS)[46] and refined by full-matrix least-squares techniques against F_o^2 (SHELXL-97).[46] All hydrogen atoms were located by difference Fourier synthesis and refined isotropically. XP (SIEMENS Analytical X-ray Instruments, Inc.) was used for structure representations.

*Crystal Data for **3**:* $C_{15}H_{26}O_2$, M_r = 238.36 g mol^{-1}, colorless prism, size 0.106 × 0.098 × 0.080 mm^3, orthorhombic, space group $P2_12_12_1$, *a* = 7.6795(1) Å, *b* = 10.0237(1) Å, *c* = 18.0644(2) Å, *V* = 1390.54(3) Å^3, *T* = −140 °C, *Z* = 4, ρ_{calcd} = 1.139 g cm^{-3}, μ(Mo Kα) = 0.73 cm^{-1}, multiscan, $trans_{min}$ 0.6639, $trans_{max}$ 0.7456, *F*(000) = 528, 11 717 reflections in *h*(−9/9), *k*(−13/12), *l*(−23/23), measured in the range 2.88° ≤ Θ ≤ 27.48°, completeness Θ_{max} = 99.7%, 3174 independent reflections, R_{int} = 0.0263, 3072 reflections with $F_o > 4\sigma(F_o)$, 258 parameters, 0 restraints, R_{1obs} = 0.0379, wR_{2obs} = 0.0979, R_{1all} = 0.0394, wR_{2all} = 0.0995, GOOF = 1.043, Flack parameter 0.1(10), largest difference peak and hole 0.413/−0.160 e Å^{-3}.

Antimicrobial Assays. Sterile filter paper disks were impregnated with 50 μg of the samples using methanol as the carrier solvent. The impregnated disks were then placed on agar plates previously inoculated with *Bacillus subtilis* ATCC 6633, *Mycobacterium vaccae* IMET 10670, *Pseudomonas aeruginosa* K799/61, *Staphylococcus aureus* SG511, *Staphylococcus aureus* 134/94 R9 (methicillin-resistant MRSA), *Enterococcus faecalis* 1528 R10 (vancomycin-resistant VRE), *Escherichia coli* SG458, *Sporobolomyces salmonicolor* SBUG 549, *Candida albicans*, and *Penicillium notatum* JP36. Chloramphenicol and amphotericin were used as positive controls against bacteria and fungi, respectively. MeOH was used as negative control. After the plates were incubated at 37 °C for 24 h, antimicrobial activity was recorded as clear zones (in mm) of inhibition surrounding the disk. The test sample was considered active when the zone of inhibition was greater than 7 mm. Compound **2** exhibited weak activity against *Bacillus subtilis* ATCC 6633 (inhibition zone 10 mm). Chloramphenicol (0.25 μg/paper disk) was used as a positive control against bacteria with strong activity against *Bacillus subtilis* ATCC 6633 (inhibition zone 28 mm). Amphotericin B (0.5 μg/paper disk) was used as a positive control against fungi.

Cytotoxicity Assays. A modified propidium iodide assay was used to determine the cytotoxic activities of compounds **2** and **3** against 12 cell lines derived from solid human tumors. The test procedure has been described elsewhere.[47] Cell lines tested were derived from patient tumors engrafted as a subcutaneously growing tumor in NMRI nu/nu mice or obtained from the American Type Culture Collection (Rockville, MD, USA), National Cancer Institute (Bethesda, MD, USA), or Deutsche Sammlung von Mikroorganismen and Zellkulturen (Braunschweig, Germany). Inhibitory concentrations are provided as 50% inhibition of cell growth (absolute IC_{50}, determined by two-point-curve-fit after plotting compound concentration versus fluorescence intensity). Neither **2** nor **3** exhibited cytotoxic activities (IC_{50} > 30 μM).

Crystallographic data deposited at the Cambridge Crystallographic Data Centre under CCDC-1406840 for **3** contain the supplementary crystallographic data excluding structure factors; this data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (+44) 1223-336-033; or deposit@ccdc.cam.ac.uk).

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■ ASSOCIATED CONTENT

Supporting Information

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NMR spectra of compounds **1**, **2**, and **3** (PDF)

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Notes

The authors declare no competing financial interest.

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