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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 terpeneome 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. Arising from their structural diversity, terpenes exhibit various biological functions, as fragrances, deterrents, hormones, vitamins, and therapeutics for example. Classically, terpenes are signature plant and fungal metabolites. On the other hand, the terpenes geosmin5 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 “terpeneome” 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)14–16 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.

EIMS measurements indicated molecular masses of 236, 238, and 238 Da for 1, 2, and 3, respectively. The 1H NMR spectrum of 1 exhibited signals for three methyl singlets (δ 0.93, 0.99, 1.10) and other aliphatic proton signals. Its 13C 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 8R configuration according to the octant rule.

In the 1H 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-14, 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.

1H NMR and 13C 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 CH2Cl2–CH3OH 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 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
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 \( \beta \)-caryophyllene synthase genes have been characterized, and analysis of a representative from Arabidopsis thaliana revealed that the cyclase produces \((-\beta\)-caryophyllene). It is conceivable that the downstream cyclization is nonenzymatic or catalyzed by a cyclase that allows for various reaction channels, as one single caryolane from plants and bacteria feature a de novo skeleton with the same absolute and relative conﬁguration, 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, 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. This concept may also hold true for volatile terpenes that are often involved in induced defense processes in plants. 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. To test whether the bacterial caryolanes could contribute to plant protection by antibiosis, we subjected a linear gradient of H2O

**Scheme 1. Proposed Stereodivergent Biosynthetic Pathways for Caryolanes**

**Plants**

![Plants pathway diagram]

**Bacteria**

![Bacteria pathway diagram]

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 H2O−CH3OH (from 30% to 100% v/v, flow rate 0.5 L min\(^{-1}\), in 58 min) afforded seven fractions (F1−F7). F1−F5 derived caryolane metabolites in mangrove plants. Future research on the mutualism between plants and their endophytes may take into account that also the bacterial terpeneome 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.
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 CHCl3–CH2Cl2 gradient to yield four fractions, A–D. Fraction A was further separated by an RP-C18 column (CH2Cl2–CH3OH as gradient), yielding pure 2 (7.9 mg) and 3 (2.8 mg). Fraction B was fractionated by a Sephadex LH-20 column (CH2Cl2–50% CH3OH) to afford pure 1 (0.5 mg).

**Bacaryolane A (1):** colorless solid; [α]D23 = +17.1 (c 0.05, MeOH); CD (c 0.30, MeOH) Δα (237 nm) 2.98; IR (κ) νmax 3372.8, 2961.7, 2923.2, 2862.5, 1691.1, 1465.1, 1356.0, 1308.6, 1238.5, 1121.7, 1103.8, 1156.8, 1098.2, 1053.9, 958.3, 915.6, 913.9, 884.4, 855.1, 737.3, 677.1, 610.9 cm–1; 1H NMR (296 MHz) m/z 237.1850 [M + H]+ (calcd 237.1849 for C15H26O2), 239.2004 [M + H]+ (calcd 239.2006 for C16H28O2); 13C NMR (75 MHz) m/z 238.36 g mol–1, 238.36 g mol–1; ρ(MeOH) = 1.42; m; 1.07, m 38.3, CH2 1.43, m; 1.41, m

**Bacaryolane B (2):** colorless solid; [α]D23 = –1.3 (c 0.79, MeOH); IR (κ) νmax 3329.9, 2923.2, 2862.5, 1691.1, 1465.1, 1356.8, 1308.6, 1238.5, 1191.7, 1103.8, 1156.8, 1098.2, 1053.9, 958.3, 915.6, 913.9, 884.4, 855.1, 737.3, 677.1, 610.9 cm–1; 1H NMR (296 MHz) m/z 238.36 g mol–1, 238.36 g mol–1; ρ(MeOH) = 1.42; m; 1.07, m 38.3, CH2 1.43, m; 1.25, m

**Bacaryolane C (3):** colorless solid; [α]D23 = –37.4 (c 0.28, MeOH); IR (κ) νmax 3305.3, 2926.3, 2862.8, 1459.3, 1353.3, 1281.1, 1283.0, 1254.3, 1195.2, 1134.5, 1103.8, 1187.7, 1065.3, 1021.4, 981.9, 947.2, 913.7, 869.3, 851.3, 648.5, 641.2 cm–1; 1H NMR (296 MHz) m/z 238.36 g mol–1, 238.36 g mol–1; ρ(MeOH) = 1.42; m; 1.07, m 38.3, CH2 1.43, m; 1.25, m

Crystal Structure Determination. The intensity data were collected on a Nonius KappaCCD diffractometer, using graphite-monochromated Mo Kα radiation. Data were corrected for Lorentz-polarization effects; absorption was taken into account on a semiempirical basis using multiple scans.33–35 The structure was solved by direct methods (SHELXS)36 and refined by full-matrix least-squares techniques against F2 (SHELX-97).37 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:** C15H26O2, M, = 238.36 g mol–1, colorless prism, size 0.106 × 0.098 × 0.080 mm, orthorhombic, space group P212121, a = 7.6795(1) Å, b = 10.0237(1) Å, c = 18.0644(2) Å, V = 1390.54(3) Å3, T = –140 °C, Z = 4, μ(Mo Kα) = 0.73 cm–1, monoclinic, transax, 0.6639, transax 0.7546, F(000) = 528, 1177 reflections in h(–9/9), k(–13/12), l(–23/23), measured in the range 2.888 ≤ Θ ≤ 27.48°, completeness Θmax = 99.7%, 3174 independent reflections, Rint = 0.0263, 3072 reflections with F0 > 4σ(F0), 258 parameters, 0 restraints, R(1600) = 0.0379, wR2 = 0.0979, Rint = 0.0394, wR2 = 0.0995, GOOF = 1.043, Flack parameter 0.1(10), largest difference peak and hole 0.143/–0.160 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 salmonicola SBUG 549, Candida albicans, and Penicillium notatum JP36. Chloramphenicol and amphotericin B were used as positive controls against bacteria and fungi, respectively. MeOH was used as a 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.37 Cell lines tested were derived from patient tumors engrafted as a subcutaneously growing tumor in NMRU 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 und Zellkulturen (Braunschweig, Germany). Inhibitory concentrations are provided as 50% inhibition of cell growth (absolute IC50, determined by two-point-curve-fit after plotting compound concentration versus fluorescence intensity). Neither 2 nor 3 exhibited cytotoxic activities (IC50 > 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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The Supporting Information is available free of charge on the Journal of Natural Products.

Notes
The authors declare no competing financial interest.

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