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Ribosylhopane, a Novel Bacterial Hopanoid, as Precursor of C_{35} Bacteriohopanepolyols in *Streptomyces coelicolor* A3(2)

Wenjun Liu,[a] Elias Sakr,[a] Philippe Schaeffer,[b] Helen M. Talbot,[c] Janina Donisi,[d] Thomas Härtner,[d] Elmar Kannenberg,[d, e] Eriko Takano,[f] and Michel Rohmer*[a]

Wild-type *Streptomyces coelicolor* A3(2) produces aminobacteriohopanetriol as the only elongated C_{35} hopanoid. The hopanoid phenotype of two mutants bearing a deletion of genes from a previously identified hopanoid biosynthesis gene cluster provides clues to the formation of C_{35} bacteriohopanepolyols. *orf14* encodes a putative nucleosidase; its deletion induces the accumulation of adenosylhopane as it cannot be converted into ribosylhopane. *orf18* encodes a putative transaminase; its deletion results in the accumulation of adenosylhopane, ribosylhopane, and bacteriohopanetetrol. Ribosylhopane was postulated twenty years ago as a precursor for bacterial hopanoids but was never identified in a bacterium. Absence of the transaminase encoded by *orf18* prevents the reductive amination of ribosylhopane into aminobacteriohopanetriol and induces its accumulation. Its reduction by an aldose-reductase-like enzyme produces bacteriohopanetetrol, which is normally not present in *S. coelicolor*.

Introduction

Triterpenoids of the hopane series (biohopanoids) are characteristic metabolites of bacteria.[1] Their molecular fossils (geohopanoids) are ubiquitous in sedimentary deposits and are widely used as biomarkers in organic geochemistry.[2] Hopanoids were first interpreted as biomarkers of specific bacterial taxa. This, however, has been challenged in recent studies on the distribution and the role of hopanoids in bacteria: the hopanoid fingerprints reflect general biological processes in bacterial cells rather than metabolic pathways of specific bacteria.[3] The major hopanoids in bacteria are always compounds with a C_{35} skeleton, the most representative members being bacteriohopanetetrol (**4**) and aminobacteriohopanetriol (**5**; Scheme 1). Incorporation of ^{13}C-labeled acetate into the hopanoids of *Rhodopseudomonas palustris*, *Rhodopseudomonas acidophila*, and *Methylobacterium organophilum* and of ^{13}C-labeled glucose into those of *Zymomonas mobilis* and *M. organophilum* has shed light on the origin of this very peculiar structure. The C_{35} bacteriohopane skeleton results from the formation of a carbon–carbon bond between a C_{30} triterpene moiety and a D-ribose metabolite derived from the non-oxidative pentose phosphate pathway, and linked at its C-5 carbon atom to the hopane skeleton.[4] This unique enzymatic reaction remained a puzzle for a long time. Adenosylhopane (**2**; Scheme 1), a hopanoid that is accumulated in a few bacteria, and ribosylhopane (**3**; resulting from the deadenylation of **2**) were suspected to be intermediates in the hopanoid biosynthetic pathway.[5] Molecular biology techniques recently revealed some clues: identification of the gene for an enzyme responsible for the coupling reaction yielding **2** in *Methylobacterium extorquens*[6] and the gene for a putative transaminase that leads to **5** in *R. palustris*.[7]

Besides very minor amounts of diploptene (**1**; Scheme 1), **5** is the only elongated hopanoid detected in wild-type *Streptomyces coelicolor* A(3)2.[8] Hopanoid biosynthesis in *S. coelicolor* is severely affected by growth conditions. Like all tested *Streptomyces* species,[1a,e] *S. coelicolor* A3(2) does not produce hopanoids, or only minute amounts (a few $\mu g\,g^{-1}$ dry weight) in liquid culture, but synthesizes aminotriol in significant amounts when grown on solid medium and when bacterial cells are sporulating.[8] Mutants defective in the formation of aerial mycelium and unable to sporulate generally do not produce

[a] *W. Liu, E. Sakr, Prof. M. Rohmer*
Université de Strasbourg/CNRS, Institut Le Bel
4 rue Blaise Pascal, 67070 Strasbourg, Cedex (France)
E-mail: mirohmer@unistra.fr

[b] *Dr. P. Schaeffer*
Laboratoire de Biogéochimie Moléculaire
Université de Strasbourg/CNRS, ECPM
25 rue Becquerel, 67200 Strasbourg (France)

[c] *Dr. H. M. Talbot*
School of Civil Engineering and Geosciences
Newcastle University, Drummond Building
Newcastle upon Tyne NE1 7RU (UK)

[d] *J. Donisi, T. Härtner, Dr. E. Kannenberg*
Microbiological Institute, Microbiology/Biotechnology
University of Tübingen
Auf der Morgenstelle 28, 72076 Tübingen (Germany)

[e] *Dr. E. Kannenberg*
Complex Carbohydrate Research Center
University of Georgia
Athens, GA 30602 (USA)

[f] *Prof. E. Takano*
Manchester Institute of Biotechnology
Faculty of Life Sciences, University of Manchester
131 Princess Street, Manchester, M1 7DN (UK)

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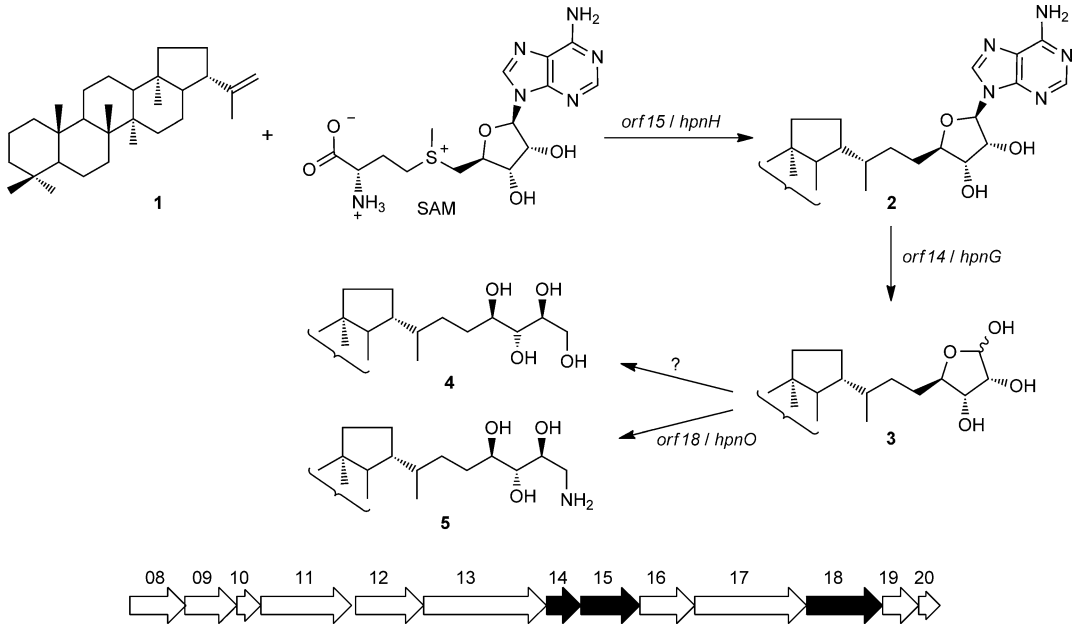

Scheme 1. Hypothetical biosynthesis of aminobacteriohopanetriol in *Streptomyces coelicolor* and isoprenoid/hopanoid biosynthetic genes on cosmid SC6A5. **1**: diploptene; **2**: adenosylhopane; **3**: ribosylhopane; **4**: bacteriohopanetetrol; **5**: aminobacteriohopanetriol; *orfs* 14, 15, and 18 are shown as black arrows in the biosynthesis cluster. Annotation of the genes is according to the SCO numbers and as described by Poralla et al.:[8] *orf08*/SCO6759, phytoene or squalene synthase; *orf09*/SCO6760, phytoene or squalene synthase; *orf10*/SCO6761, unknown function; *orf11*/SCO6762, phytoene dehydrogenase; *orf12*/SCO6763, polyprenyl diphosphate synthase or farnesyl diphosphate synthase; *orf13*/SCO6764, significant similarity also in the conserved motifs to other squalene-hopene cyclases; *orf14*/SCO6765, putative nucleoside phosphorylase or nucleosidase; *orf15*/SCO6766, putative adenosylhopane synthase; *orf16*/SCO6767, putative 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase; *orf17*/SCO6768, putative 1-deoxy-D-xylulose 5-phosphate synthase; *orf18*/SCO6769, putative aminotransferase; *orf19*/SCO6770, DNA-binding protein; *orf20*/SCO677, unknown function.

hopanoids, whereas those that form aerial mycelium but no spores do.[8] This suggests that the membrane-condensing effect of hopanoids protects the cells against water loss through the plasma membrane in the aerial mycelium. A gene cluster comprising 13 open reading frames, seven of which were putative hopanoid biosynthesis genes (deduced from sequence homology with functional hopanoid biosynthesis genes), has been identified in *S. coelicolor* (Scheme 1).[8] In this study, the function of two of these, *orf14* and *orf18*, was investigated. The hopanoid phenotype was determined for mutants defective in either *orf14* or *orf18*. This allowed pinpointing the role of the corresponding genes in elongated hopanoid biosynthesis and, for the first time in a bacterium, identifying **3**, a putative intermediate, whose existence was postulated many years ago,[5a] but has eluded identification.

Results and Discussion

Apramycin insertion mutants for *orf14* and *orf18* were created, and the mutant constructs were confirmed by PCR (see the Experimental Section, data not shown). The most interesting Δ*orf18* mutant accumulated the new hopanoid ribosylhopane (**3**), and was complemented by the corresponding mutated gene. The sporulation and antibiotic production phenotype of the two mutants and the complemented Δ*orf18* mutant did not show any significant differences compared to the wild-type strain when grown on SMMS, R2, DNAgar, R2YE, or SFM medium.

The hopanoid content for both Δ*orf14* and Δ*orf18* strains was first investigated by using the derivatization method: degradation of the side-chain by H_5IO_6 oxidation on whole crude $CHCl_3/CH_3OH$ extract, followed by $NaBH_4$ reduction of the intermediate aldehyde, and final acetylation of the resulting primary alcohol.[1a] Although information about the intact side-chains is lost by this method, it allows easy detection by GC-MS (gas chromatography/mass spectrometry) of all known C_{35} bacteriohopane derivatives with the exception of adenosylhopane. Subsequently, the crude extracts of the two mutant strains were directly acetylated and fractionated in order to unambiguously characterize the intact biohopanoids by NMR spectroscopy.

Hopanoid content of the Δ*orf14* mutant

The Δ*orf14* mutant did not lose the capacity to produce hopanoids: after $H_5IO_6/NaBH_4$ derivatization,[1a] **1** was still present, but no C_{32} hopanoid resulting from the cleavage of a C_{35} tetrafunctionalized bacteriohopanepolyol could be detected by GC-MS (Table 2). This indicated the absence of any C_{35} hopanoid with free vicinal hydroxy groups at C-32 and C-33 (e.g., **5**, the major wild-type hopanoid). Hopanoids with no free hydroxy groups at C-32 and C-33 (e.g., **2**) do not produce the C_{32} bishomohopanol product and cannot be identified by this method. APCI-LCMS^n (atmospheric pressure chemical ionization–liquid chromatography mass spectrometry) was therefore performed on the crude acetylated extract for a directed hopanoid analy-

Table 1. Construction of *S. coelicolor* mutants: list of primers (restriction sites underlined).

	Sequence (5′–3′)
orf14L	GTGAAG GGCAGC TGATGA CCCCGC AGACCG GCCCGT CCCATT CCGGGG ATCCGT CGACC
ORF14r	ACGGAA GGCCGA TATTCC ACCGCG CACGGT GCCGAT CCGTGT AGGCTG GAGCTG CTTC
Redi-F-ORF18	GGACGG ATCGTC ATGCCA GCGCAA GGAGAG AACTGA ATGATT CCGGGG ATCCGT CGACC
Redi-R-ORF18	TCCTTG CCTCAG AGGCAA AAGACC TGGAGG GTGCGC TCATGT AGGCTG GAGCTG CTTC
dig14L	GCTCCT GGGACG AGCCCT ACT
DIG14r	CATGCG CTGCTT GAGCAC ACC
NW-L-ORF18	AGGCAA ATTCCT TGCCTC AG
NW-R-ORF18	GTGGAC CGGCAG TGGAAC
orf14L4	CATATG ACCCCG CAGACC
orf14R4	TTAATT AACATC TGGGCT CAC
orf18L4	CATATG ACCAAG GAGTTC GA
orf18R4	CTCGAG TCAGAG GCAAAA GA

sis. The only C_{35} bacteriohopane derivative found in significant amounts was **2**. Neither **5**, **4**, nor **3** was detected (Scheme 1).

The *orf14* mutant still contains functional copies of the genes encoding the putative aminotransferase (*orf18*) and the unknown enzyme(s) involved in the formation of **4**. This indicates that this mutant is capable of converting **3** into **5** or **4**. However, none of these three compounds was produced by this mutant, thus suggesting that the biosynthesis of **3** is blocked, and that **2** serves as possible precursor of **3**. This interpretation is consistent with the presumed function of the deleted *orf14* gene. The *hpnG* gene in *M. extorquens* has been proposed to encode a putative nucleoside phosphorylase[6] or a nucleosidase in *R. palustris*,[7] which produces either **3** 35-phosphate or free **3**. Deletion of *hpnG* resulted in the accumulation of adenosylhopane in mutants of both bacteria. A BLAST analysis suggested that *S. coelicolor* Orf14 is a homologue of the two reported HpnGs of *M. extorquens* and *R. palustris* (67 and 40% AA identity, respectively).

Hopanoid content of the Δ*orf18* mutant

After $H_5IO_6/NaBH_4$ derivatization of the crude extract and acetylation,[1a] diploptene and bishomohopanol acetate were detected by GC-MS, thus indicating that the Δ*orf18* mutant is capable of synthesizing elongated C_{35} hopanoids (Table 2). In order to identify the intact biohopanoids, the acetylated crude extract of the Δ*orf18* mutant was analyzed by either GC-MS or APCI-LCMS^n, and the intact biohopanoids were isolated for detailed identification by NMR spectroscopy. The tetra-acetate of **4** was easily detected by GC-MS (Table 2); the retention time and the MS spectrum were identical to those of an authentic sample obtained from *M. organophilum*.[9] The ^{1}H NMR spectrum was characteristic of the usual 32*R*, 33*R*, 34*S* configuration of the side chain.[10]

More interestingly, **3** triacetate was detected by direct inlet electron impact ionization mass spectrometry, GC-MS, and APCI-LCMS^n for the first time in a bacterium (see Figures S1–S4 in the Supporting information). As a cyclic hemiketal, **3**, like aldoses, upon acetylation produces two anomeric triacetates[5b] that can be separated by TLC and behave differently with GC-MS. Whereas triacetylated α-**3** was stable under GC-MS conditions and gave a weak *m/z* 670 molecular ion, the β-anomer decomposed and produced a compound with *m/z* 550, corresponding most likely to the loss of two acetic acid moieties (Figure S2C). The retention times and mass spectra of the two anomers isolated from the *S. coelicolor* Δ*orf18* mutant were identical to those of a reference sample. The presence of the two anomers of acetylated **3** was confirmed by APCI-LCMS^n (Figures 3S and S4). For each anomer, $[M+H]^+$ 671, $[M+H\text{-}AcOH]^+$ 611, and $[M+H\text{-}2AcOH]^+$ 551 ions were found in the full mass spectrum in similar abundances. Further fragmentation of the $[M+H\text{-}AcOH]^+$ ion (*m/z* 611) resulted in a MS^2 spectrum in accordance with the MS^3 spectrum of the related diacetate of adenosylhopane after loss of acetyladenine.[11] For definitive identification, the two acetylated 35α and 35β anomers were isolated by reversed-phase HPLC. Their ^{1}H NMR spectra and direct inlet electron impact mass spectra were identical to those of synthetic reference samples.[5b,12]

Finally, as acetylated **2** and **5** are not amenable to GC-MS, the presence of these hopanoids was also analyzed in the acetylated crude extract by APCI-LCMS^n.[11,13] Only **2** triacetate was found in small amounts, together with acetylated **3** and **4**. Acetylated **5**, the only wild-type hopanoid, was not detected in the *orf18* deletion strain. In order to confirm that the mutant phenotype was the result of apramycin insertion into *orf18*, the mutated gene was complemented in the Δ*orf18* mutant by introduction of a hygromycin selective plasmid. The complemented strain produced **5** (no detection in a strain bearing only the empty plasmid).

Although **3** was proposed as a precursor of bacteriohopane derivatives 20 years ago,[5a] it has never been found in a hopanoid-producing bacterium, despite the availability of synthetic reference samples. This is the first report of **3** detected as a natural product. Its isolation from the *S. coelicolor* Δ*orf18* mutant is in agreement with such a precursor role for biohopanoids. As a biosynthetic intermediate, **3** would be expected to have high turnover, to be rapidly transformed into the end products (such as **5** in *S. coelicolor*) and to accumulate only in special circumstances.

Bacteriohopanetetrol (**4**) is the other major hopanoid of the Δ*orf18* mutant. According to the hypothetical biogenetic scheme for hopanoid biosynthesis (Scheme 1), **3** is converted into **4** by reduction and into **5** by reductive amination. When the *orf18* gene is expressed (as in wild-type *S. coelicolor*), **3** is completely converted into **5** by reductive amination, and no other C_{35} hopanoid is found. When this main route is blocked by deletion of *orf18*, intermediates such as **2** and **3** can accumulate to some extent, and another novel hopanoid, **4**, is consequently produced. This tetrol results probably from the reduction of **3** hemiketal, for instance by an aldose-reductase of low specificity and efficiency. This allows the accumulation of both **3** and **4**, but the concentrations of these compounds in the Δ*orf18* mutant were much lower than that of **5** in the wild-type strain. Although **2** was not detected in the wild-type

strain, its presence in trace amounts in the Δ*orf18* mutant is in accordance with an intermediate role in the biosynthesis of C_{35} biohopanoids. The hopanoid phenotype of the Δ*orf18* mutant does not, however, indicate a relationship between **2** and **3**. It might be that **2** is the precursor of **3**, based on the hypothetical biosynthetic pathway (Scheme 1) or it might be derived from **3** by adenylation.

The protein encoded by *hpnO* in *R. palustris* was shown to be required for the biosynthesis of **5**.[7] According to the nucleotide sequence, this gene encodes a homologue of an ornithine-oxo acid aminotransferase. All bacteria that possess a gene homologous to *R. palustris hpnO* and tested for their hopanoid content (i.e., *R. palustris* strains, *Bradyrhizobium* spp., *Beijerinckia indica*, *Nitrosomonas europaea*, *Microcystis aeruginosa*, and *Methylococcus capsulatus*), produce aminobacteriohopanepolyols as major hopanoids.[7] A comparison of the amino acid sequences of *R. palustris* HpnO and *S. coelicolor* Orf18 revealed 52% identity (*E* value: 5e-167; query cover: 98%), thus indicating that *orf18* might be a homologue of *hpnO* and that the corresponding enzyme is most probably an aminotransferase. The hopanoid phenotype/fingerprint of the *S. coelicolor* Δ*orf18* mutant can now be easily interpreted. In the absence of the aminotransferase encoded by the *orf18* gene, **3** (the substrate of this enzyme) is not converted into **5**, and **3** accumulates.

The possible role of *Orf15*

A final gene encoding the enzyme catalyzing the formation of the C_{35} bacteriohopane skeleton in *S. coelicolor* remains to be identified. *hpnH* in *M. extorquens* was found to encode the first enzyme, a putative B_{12} binding radical SAM protein involved in the biosynthesis of the C_{35} biohopanoids.[6] Its deletion in *M. extorquens*[6] and in *R. palustris*[7] resulted in the exclusive production of the C_{30} hopanoids diploptene or diplopterol. The C_{35} compounds **4** and **5**, the main hopanoids in these bacteria, were absent. The amino acid sequence of *S. coelicolor* Orf15[8] was compared with those of the documented HpnH proteins (159 genes in the NCBI database). Significant similarity was found (49–100% AA identity; data not shown). This indicates that *orf15* belongs to the *hpnH* family and might be associated with hopanoid biosynthesis in *S. coelicolor*: encoding the enzyme catalyzing the first step of the biosynthesis of C_{35} biohopanoids to produce adenosylhopane (**2**).

Conclusion

All genes involved in the biosynthesis of aminobacteriohopanetriol (**5**) in *S. coelicolor* have now been characterized, thus confirming the hypothetical biogenetic scheme (Scheme 1) proposed 20 years ago.[5a] The catalytic activities of the gene products remain, however, to be characterized. Most interesting was the hopanoid phenotype of the Δ*orf18* mutant lacking the transaminase producing aminobacteriohopanetriol (**5**) and accumulating ribosylhopane (**3**). This hopanoid, which was hypothesized as a precursor of the C_{35} biohopanoids, was the final missing link in the biogenetic scheme.

Experimental Section

Strains and culture conditions: The wild-type strain was *Streptomyces coelicolor* A3(2).[8,14] The media (SMMS, R2, DNAgar, R2YE, and SFM) and *Streptomyces* cultures were manipulated as described previously.[15]

All cultures on R2YE medium[16] were grown in Petri dishes (10 cm diameter) on cellophane discs at 30 °C. Sterile cellophane was placed on the solid medium, and bacteria were grown on the cellophane surface to allow diffusion of the nutrients without direct contact of bacteria with the solid medium.[15] Apramycin (50 μg mL^{-1}) was added to the culture medium for the cultivation of the Δ*orf14* and Δ*orf18* mutants. Apramycin (50 μg mL^{-1}) and hygromycin (50 μg mL^{-1}) were added for the complementation experiments. Growing times varied depending on the strains: 3 days for wild-type, 7–8 days for Δ*orf18*, 8–10 days for Δ*orf14*, and 16 days for the strains in the complementation experiments. At the end of the culture growth, bacterial cells were harvested together with the cellophane and lyophilized for subsequent lipid extraction.

Inactivation of *orf14* and *orf18*, and complementation of *orf18*: The putative genes *orf14* and *orf18* in the *S. coelicolor* cosmid pSC6A5 were inactivated by using the gene replacement method described by Gust et al.[17] An apramycin-resistant cassette flanked by *orf14*- or *orf18*-specific DNA fragments was used to replace *orf14* or *orf18* in the chromosome. The PCR primers used to construct these cassettes were orf14L and ORF14r for *orf14*, and Redi-F-ORF18 and Redi-R-ORF18 for *orf18* (Table 1). *E. coli* BW25113[17] was used to propagate the recombinant plasmid pIJ790 and the *S. coelicolor* cosmid pSC6A5[18] containing the hopanoid biosynthesis operon. The redirect method was used to replace *orf14* or *orf18* of pSC6A5 by using the cassettes constructed to give apramycin resistance. The replacement of the genes in cosmids pSC6A5 *orf14*::apra (for *orf14*) and pSC6A5 *orf18*::apra (for *orf18*) were confirmed by restriction analysis as well as by PCR with diagnostic primers dig14L and DIG14r, or NW-L-ORF18 and NW-R-ORF18, respectively (Table 1). For conjugation, the *orf14*- and *orf18*-replaced cosmids were transformed into *E. coli* 1257/pUZ8002;[17] plasmid pUZ8002 contained the *tra* genes necessary for the transfer of plasmid DNA into *S. coelicolor*. After conjugative mating of *E. coli* donor and *Streptomyces* recipient cells, apramycin resistance together with kanamycin sensitivity were used to screen for *Streptomyces* with chromosomally inactivated *orf14* or *orf18*. To confirm the mutant constructs, chromosomal DNA was isolated, and gene replacement was analyzed by PCR with the diagnostic primers dig14L and DIG14r, or NW-L-ORF18 and NW-R-ORF18. PCR products of the expected size were further confirmed by restriction analysis.

The *orf14* and *orf18* mutants were complemented by introduction of plasmid pTE299 or pTE296, respectively. The *orf14* or *orf18* fragments were amplified by PCR with orf14L4 and orf14R4, or orf18L4

Table 2. C_{35} hopanoids from *Streptomyces coelicolor* A3(2) and Δ*orf14* and Δ*orf18* mutants.

	Adenosylhopane (**2**)	Ribosylhopane (**3**)	Tetrol (**4**)	Aminotriol (**5**)
S. coelicolor A(3)2	–	–	–	+
Δ*orf14* mutant	+	–	–	–
Δ*orf18* mutant	+	+	+	–

–: not detected, +: detected.

and orf18R4 primer pairs (Table 1), respectively, with genomic DNA as the template. The PCR products were introduced into pGEM-T Easy (Promega) then digested with NdeI/PacI or NdeI/XhoI for *orf14* and *orf18*, respectively. The isolated fragments were then ligated into pIJ10257[19] (also digested with the same restriction enzymes) to construct pTE299 or pTE296. The plasmid constructs were confirmed by restriction analysis, and the PCR amplified products were confirmed by DNA sequencing.

NMR spectroscopy and mass spectrometry: NMR spectra were performed in C^2HCl_3 on a Biospin spectrometer (Bruker) equipped with a cryoprobe (500 MHz for ^1H NMR, 125 MHz for ^{13}C NMR) with $CHCl_3$ ($\delta = 7.260$ ppm) for ^1H NMR and C^2HCl_3 ($\delta = 77.0$ ppm) for ^{13}C NMR.

GC-MS spectra were obtained on a TSQ Quantum mass spectrometer (Thermo Scientific) connected to a Trace GC ultra gas chromatograph (PTV injector, HP-5 MS column, 30 m × 0.25 mm, 0.25 μm film thickness; Thermo Scientific) with the temperature program: 55 °C (3 min), 55–320 °C (10 °C min^{-1}), 320 °C (50 min). Helium was used as the carrier gas (1 mL min^{-1}). Direct inlet mass spectra were recorded on the same spectrometer in electron impact ionization mode (70 eV, 50–800 Da, 0.5 s cycle time, source temperature 230 °C).

APCI-LCMS^n was performed as described previously[11,13] on the total acetylated lipid extract with a Surveyor HPLC system (Thermo Finnigan) fitted with a Gemini C_{18} column (150 × 3.0 mm; 5 μm particle size; Phenomenex, Torrance, CA) and a security guard column cartridge of the same material coupled to a LCQ ion-trap mass spectrometer (Thermo Finnigan) equipped with an APCI source operated in positive ion mode. Chromatographic separation was accomplished at 30 °C (flow rate, 0.5 mL min^{-1}) with the following gradient: 90% A, 10% B (0 min); 59% A, 1% B, 40% C (at 25 min); isocratic to 45 min; returning to the starting conditions in 5 min; stabilizing for 10 min (A: CH_3OH; B: water; C: propan-2-ol; all HPLC grade, Fisher Scientific). APCI was achieved at 155 °C capillary temperature and 490 °C APCI vaporizer temperature with a corona discharge current of 8 μA, sheath and auxiliary gas flows of 40 and 10, respectively (arbitrary units). MS^n analysis was carried out in data-dependent mode with three scan events: SCAN 1: full mass spectrum, *m/z* 300–1300; SCAN 2: data-dependent MS^2 spectrum of the most intense ion from SCAN 1; SCAN 3: data-dependent MS^3 spectrum of the most intense ion from SCAN 2. Detection was achieved at an isolation width of *m/z* 5.0 and fragmentation with normalized collisional dissociation energy 35%, and an activation Q value (parameter determining the *m/z* range of the observed fragment ions) of 0.15.

Identification of aminobacteriohopanetriol (5) from wild-type *S. coelicolor* A(3)2: The lyophilized material from 36 Petri dishes was extracted under reflux with $CHCl_3/CH_3OH$ (2:1 *v/v*, 3 × 150 mL, 45 min). The combined extracts were evaporated to dryness, and the residue was acetylated overnight at room temperature with acetic anhydride/pyridine (1:2 *v/v*, 1 mL). The reagents were removed under vacuum, and the residue was fractionated by flash chromatography (CH_2Cl_2/EtOAc (4:1 *v/v*)).[20] The fractions containing hopanoids according to the methyl signal fingerprint in their ^1H NMR spectra were further purified on preparative TLC silica gel $60F_{254}$ plates (0.25 mm thickness; Merck Millipore) with CH_2Cl_2/CH_3OH (95:5 *v/v*) as eluent, thus yielding **5** tetra-acetate (0.14 mg, $R_f = 0.54$).

Identification of adenosylhopane (2) from the Δ*orf14* mutant: The freeze-dried material from 35 Petri dishes was extracted, and the extract was acetylated as above. Hopanoid detection was performed by APCI-LCMS on the total acetylated lipid extract. Only **2** triacetate was identified, by comparison with published spectra.[11,21]

APCI-MS^2 of the pseudomolecular ion of adenosylhopane triacetate *m/z* 788 $[M+H]^+$: *m/z* 611 ($[M+H\text{-}N\text{-acetyladenine}]^+$, 100%), 509 ($[M+H\text{-}N\text{-acetyladenine-AcOH-}CH_2CO]^+$, 2%), 491 ($[M+H\text{-}N\text{-acetyladenine-2 AcOH}]^+$, 4%), 191 (ring C, cleavage, 1%),[22] 178 ($[N\text{-acetyladenine}+H]^+$, 27%).

Identification of ribosylhopane (3) and bacteriohopanetetrol (4) from the Δ*orf18* mutant: Lyophilized material from 340 Petri dishes was extracted under reflux with $CHCl_3/CH_3OH$ (2:1 *v/v*, 3 × 500 mL, 45 min). The combined extracts were evaporated to dryness, and the residue (1.4 g) was acetylated overnight at room temperature with acetic anhydride/pyridine (1:2 *v/v*, 6 mL). After elimination of the reagents under vacuum, the acetylated extract was fractionated by flash column chromatography, first with CH_2Cl_2 (60 mL) and then with CH_2Cl_2/AcOEt (4:1 *v/v*, 60 mL) to give pink/orange fractions; the last fraction contained hopanoids according to NMR, GC, and GC-MS analysis. This fraction was further separated by liquid chromatography on silica gel and eluting with CH_2Cl_2/AcOEt (5:1 *v/v*), and yielded three subfractions: the first two were considerably enriched in polyacetylated hopanoids **3** and **4**. These were subjected to preparative TLC on $60F_{254}$ silica gel plates (0.25 mm thickness; Merck Millipore) with hexane/EtOAc (5:1 *v/v*) as eluent[23] to yield crude 35β-ribosylhopane triacetate ($R_f = 0.34$), bacteriohopanetetrol tetra-acetate ($R_f = 0.25$), and 35α-ribosylhopane triacetate ($R_f = 0.11$). Final purification of these fractions by reversed-phase HPLC on a Polaris C_{18} column (250 × 4.6 mm, 5 μm; Varian/Agilent Technologies) with CH_3OH/propan-2-ol/H_2O (33:16:1 *v/v/v*, 1 mL min^{-1}) afforded pure 35β-ribosylhopane triacetate (1 mg) and 35α-ribosylhopane triacetate (0.5 mg). Bacteriohopanetetrol tetra-acetate (2.5 mg) was isolated on the same HPLC column with a mixture of CH_3OH/propan-2-ol/H_2O (33:16:1.5 *v/v/v*, 1 mL min^{-1}) as the mobile phase. 35α-Ribosylhopane triacetate, 35β-ribosylhopane triacetate, adenosylhopane triacetate, and bacteriohopanetetrol tetra-acetate were identified in the acetylated crude extract by APCI-LCMS as described above (Figures S3 and S4).

35β-Ribosylhopane (3) triacetate: ^1H NMR (500 MHz, C^2HCl_3): $\delta =$ 6.121 (s, 1H; 35β-H); 5.318 (dd, $J = 1.2$ Hz, 1H; 34-H), 5.181 (dd, $J =$ 6.3, 5.0 Hz, 1H; 33-H), 4.123 (m, 1H, 32-H), 2.111 (s, 3H; -COCH_3), 2.077 (s, 3H; -COCH_3), 2.064 (s, 3H; -COCH_3); 0.945 (s, 6H, 8β and 14α-Me), 0.925 (d, $J = 6.4$ Hz, 3H; 22*R*-Me), 0.842 (s, 3H; 4α-Me), 0.810 (s, 3H; 10β-Me), 0.788 (s, 3H; 4β-Me), 0.690 ppm (s, 3H; 18α-Me); ^{13}C NMR (125 MHz, C^2HCl_3): $\delta = 170.0$, 169.7, 169.5, 98.3, 82.3, 74.8, 73.8, 56.1, 54.3, 50.3, 49.2, 45.9, 44.4, 42.1, 41.8, 41.6, 41.5, 40.3, 37.4, 36.4, 33.6, 33.4, 33.3, 33.2, 31.0, 30.8, 27.5, 23.9, 22.7, 21.6, 21.2, 20.9, 20.7 (2×), 20.0, 18.7, 16.6, 16.5, 15.9 ppm (2×); EI-MS spectrum (direct inlet): *m/z* 670 ($[M]^+$, 1%), 610 ($[M^+ - \text{AcOH}]$, 47%), 595 ($[M^+ - \text{AcOH} - \text{Me}]$, 16%), 550 ($[M^+ - 2\,\text{AcOH}]$, 2%), 449 (ring C cleavage, 5%),[22] 435 (ring C cleavage-Me, 18%), 389 (ring C cleavage-AcOH, 68%), 369 ($[M^+ - \text{side chain}]$, 20%), 329 (16%), 287 (7%), 269 (9%), 191 (ring C cleavage, 100%), 175 (16%), 163 (15%), 149 (22%), 123 (19%), 95 (34%; Figure S2A); GC-MS data: see Figure S2C.

35α-Ribosylhopane (3) triacetate: ^1H NMR (500 MHz, C^2HCl_3): $\delta =$ 6.362 (d, $J = 4.5$ Hz, 1H; 35β-H), 5.207 (dd, $J = 6.8$, 4.5 Hz, 1H; 34-H), 5.041 (dd, $J = 6.8$, 3.6 Hz, 1H; 33-H), 4.193 (m, 1H, 32-H), 2.114 (s, 3H; -COCH_3), 2.103 (s, 3H; -COCH_3), 2.058 (s, 3H; -COCH_3), 0.945 (s, 6H, 8β and 14α-Me), 0.922 (d, $J = 6.4$ Hz, 3H; 22*R*-Me), 0.842 (s, 3H; 4α-Me), 0.811 (s, 3H; 10β-Me), 0.788 (s, 3H; 4β-Me); 0.687 ppm (s,

3H; 18α-Me); EI-MS spectrum (direct inlet): *m/z* 670 ([*M*]$^+$, 3%), 610 ([*M*$^+$−AcOH], 35%), 595 ([*M*$^+$−AcOH−Me], 11%), 550 ([*M*$^+$−2AcOH], 1%), 449 (ring C cleavage, 33%),[22] 389 (ring C cleavage-AcOH, 67%), 369 ([*M*$^+$−side chain], 25%), 329 (5%), 287 (5%), 269 (12%), 191 (ring C cleavage, 100%), 175 (13%), 163 (14%), 149 (21%), 95 (32%; Figure S1A); GC-MS data: seeFigure S1C (degradation product).

(22*R*,32*R*,33*R*,34*S*)-Bacteriohopanetetrol (4) tetra-acetate: ^{1}H NMR (500 MHz, C^2HCl_3): $\delta = 5.259$ (ddd, $J = 6.6$, 5.6, 2.3 Hz, 1H; 34-H), 5.218 (dd, $J = 5.6$, 4.4 Hz, 1H; 33-H), 5.020 (ddd, $J = 9.6$, 4.3, 3.4 Hz, 1H; 32-H), 4.376 (dd, $J = 12.2$, 2.5 Hz, 1H; 35-H_a), 4.139 (dd, $J = 12.1$, 6.6 Hz, 1H; 35-H_b), 2.083 (s, 3H; -COCH_3), 2.077 (s, 3H; -COCH_3), 2.070 (s, 3H; -COCH_3), 2.050 (s, 3H; -COCH_3), 0.926 (s, 6H, 8β and 14α-Me), 0.891 (d, $J = 6.4$ Hz, 3H, 22*R*-Me), 0.832 (s, 3H; 4α-Me), 0.795 (s, 3H; 10β-Me), 0.775 (s, 3H; 4β-Me), 0.665 ppm (s, 3H; 18α-Me); ^{13}C NMR (125 MHz, C^2HCl_3): $\delta = 170.8$ (-*C*OCH$_3$), 170.5 (-*C*OCH$_3$), 170.1 (-*C*OCH$_3$), 169.8 (-*C*OCH$_3$), 71.8 (C-34), 71.5 (C-33), 69.4 (C-32), 62.1 (C-35), 56.0 (C-5), 54.2 (C-17), 50.3 (C-9), 49.1 (C-13), 45.8 (C-21), 44.3 (C-18), 42.0 (C-3), 41.7 and 41.6 (C-8 and C-14), 41.5 (C-19), 40.2 (C-1), 37.3 (C-10), 36.0 (C-22), 33.6 (C-15), 33.4 (C-23), 33.2 and 33.1 (C-4, C-23 and C-7), 30.8 (C-30), 27.5 (C-20), 26.0 (C-31), 23.9 (C-12), 22.7 (C-16), 21.6 (C-24), 21.0 (-COCH_3), 21.0 (-COCH_3×2), 20.8 (-COCH_3×2 and C-11), 19.8 (C-29), 18.6 (C-2 and C-6), 16.5 and 16.4 (C-26 and C-27), 15.9 and 15.8 ppm (C-25 and C-28); EI-MS spectrum (direct-inlet): *m/z* 714 ([*M*]$^+$, 4%), 699 ([*M*$^+$−Me], 4%), 654 ([*M*$^+$−AcOH], 4%), 594 ([*M*$^+$−2AcOH], 1%), 493 (ring C cleavage, 100%),[22] 433 (ring C cleavage-AcOH, 12%), 373 (ring C cleavage-2 AcOH, 10%), 369 ([*M*$^+$−side chain], 10%), 313 (ring C cleavage-3 AcOH, 13%), 253 (ring C cleavage-4 AcOH, 33%), 191 (ring C cleavage, 86%), 175 (41%), 149 (41%), 109 (43%), 95 (62%).

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