Published in final edited form as:

Chembiochem. 2014 September 22; 15(14): 2156–2161. doi:10.1002/cbic.201402261.

Ribosylhopane, a novel bacterial hopanoid, as precursor of C₃₅ bacteriohopanepolyols in *Streptomyces coelicolor* A3(2)

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Abstract

Streptomyces coelicolor A3(2) wild type produces aminobacteriohopanetriol as the only elongated C₃₅ hopanoid. The hopanoid phenotype of two mutants bearing a deletion of genes from a previously identified hopanoid biosynthesis gene cluster provides clues on the formation of C₃₅ bacteriohopanepolyols. *orf14* encodes a putative nucleosidase: its deletion induces the accumulation of adenosylhopane that cannot be converted into ribosylhopane. *orf18* encodes a putative transaminase: its deletion results in the accumulation of adenosylhopane, ribosylhopane and bacteriohopanetetrol. Ribosylhopane was hypothesized 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*.

Keywords

biosynthesis; hopanoids; ribosylhopane; *Streptomyces coelicolor*; terpenoids

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 trend is, however, challenged in recent studies on the distribution and the role of hopanoids in bacteria; the hopanoid fingerprint reflects general biological processes in bacterial cells rather than metabolic pathways of specific bacteria.^[3] The major hopanoids in bacteria are always compounds with a C₃₅ skeleton, the most representative members being bacteriohopanetetrol (**4**) and aminobacteriohopanetriol (**5**) (Scheme 1). Incorporation of ¹³C labeled acetate into the hopanoids of *Rhodopseudomonas palustris*, *Rhodopseudomonas acidophila* and *Methylobacterium organophilum* or ¹³C labeled glucose into those of *Zymomonas mobilis* and *M. organophilum* has shed light on the origin of this very peculiar structure. The C₃₅ bacteriohopane skeleton results from the formation of a carbon/carbon bond between a C₃₀ triterpene moiety and a D-ribose metabolite derived from non-oxidative pentose phosphate pathway and linked via its C-5 carbon atom to the hopane skeleton.^[4] This unique enzymatic reaction remained a puzzle for a long time. Adenosylhopane (**2**), a hopanoid that is accumulated by a few bacteria, and ribosylhopane (**3**) (Scheme 1) resulting from the deadenylation of the former hopanoid, were suspected to represent putative intermediates in the hopanoid biosynthetic pathway.^[5] Molecular biology techniques recently revealed some clues, identifying the genes of an enzyme responsible for the coupling reaction yielding adenosylhopane (**2**) in *Methylobacterium extorquens*^[6] and of a putative transaminase leading to aminobacteriohopanetriol (**5**) in *R. palustris*.^[7]

Besides very minor amounts of diploptene (**1**) (Scheme 1), aminobacteriohopanetriol (**5**) is the only elongated hopanoid detected in wild-type *Streptomyces coelicolor* A(3)2.^[8] Hopanoid biosynthesis is severely affected in *S. coelicolor* by different growth conditions. Like all tested *Streptomyces*,^[1a,e] *S. coelicolor* A3(2) does not produce hopanoids, or only very minute amounts (a few µg.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 hopanoids, whereas mutants, which form an aerial mycelium but no spores, do.^[8] This observation suggests that the membrane condensing effect of hopanoids may protect 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 by their sequence homologies with functional hopanoid biosynthesis genes, has been identified in *S. coelicolor* (Scheme 1).^[8] In the present study, the function of two of these genes, *orf14* and *orf18*, has been investigated. The hopanoid phenotype has been determined in two mutants defective in *orf14* or *orf18*, respectively. This allowed pinpointing the role of the corresponding genes in elongated hopanoid biosynthesis and identifying for the first time in a bacterium ribosylhopane (**3**), a putative intermediate, whose existence was postulated many years ago,^[5a] but escaped all identification attempts.

Results and discussion

The *orf14* and *orf18* apramycin insertion mutants were created and the mutant constructs confirmed by PCR (see material and methods, data not shown). The most interesting *orf18* mutant accumulating the new hopanoid ribosylhopane (**3**). was further complemented by the corresponding mutated gene. The sporulation and antibiotic production phenotype of the two mutants and the complemented *orf18* mutant did not show any significant differences compared to the wild type strain when grown on SMMS, R2, DNAgar, R2YE and SFM media.

The hopanoid content for both *orf14* and *orf18* strains was first investigated using the derivatization method applied to the whole crude $\text{CHCl}_3/\text{CH}_3\text{OH}$ extract by degradation of the side-chain by H_5IO_6 oxidation 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 extract 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 *orf14* mutant

The *orf14* mutant did not lose the capacity to produce hopanoids since, after the $\text{H}_5\text{IO}_6/\text{NaBH}_4$ derivatization,^[1a] diploptene (**1**) (Scheme 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 fact indicated the absence of any C_{35} hopanoid with free vicinal hydroxy groups at C-32 and C-33 such as aminobacteriohopanetriol (**5**), the major hopanoid of the wild type. Hopanoids with no free hydroxy groups at C-32 and C-33, like adenosylhopane (**2**), do not produce the C_{32} bishomohopanol product and cannot be identified by this method. APCI-LCMSⁿ (atmospheric pressure chemical ionization liquid chromatography mass spectrometry) was therefore performed on the crude acetylated extract for a directed hopanoid analysis. Adenosylhopane (**2**) was the only C_{35} bacteriohopane derivative found in significant amounts. Neither aminobacteriohopanetriol (**5**), bacteriohopanetetrol (**4**) nor ribosylhopane (**3**) were 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 bacteriohopanetetrol (**4**). This indicates that this mutant is capable of converting ribosylhopane (**3**) into aminobacteriohopanetriol (**5**) or bacteriohopanetetrol (**4**). However, none of these three compounds were produced by this mutant, suggesting that the biosynthesis of ribosylhopane (**3**) is blocked, and that adenosylhopane (**2**) serves as possible precursor of ribosylhopane. This interpretation is consistent with the presumed function of the deleted *orf14* gene. The *hpnG* gene has been proposed to encode a putative nucleoside phosphorylase in *M. extorquens*^[6] or a nucleosidase in *R. palustris*,^[7] which produces either ribosylhopane 35-phosphate or free ribosylhopane (**3**). Deletion of the *hpnG* resulted in the accumulation of adenosylhopane in mutants of both bacteria. A BLAST analysis suggested

that the *S. coelicolor* Orf14 protein is a homologue of the two reported HpnGs of *M. extorquens* and *R. palustris* with a 67 % AA identity with the *M. extorquens* HpnG and 40 % AA identity with the *R. palustris* HpnG, respectively.

Hopanoid content of the *orf18* mutant

After $\text{H}_5\text{IO}_6/\text{NaBH}_4$ derivatization of the crude extract and acetylation,^[1a] diploptene and bishomohopanol acetate were detected by GC-MS, indicating that the *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 *orf18* mutant was analyzed either by GC-MS, or by APCI-LCMSⁿ, and the intact biohopanoids were later isolated for a detailed identification by NMR spectroscopy. Tetraacetate of bacteriohopanetetrol (**4**) Scheme 1) was easily detected by GC-MS (Table 2). The retention time and the MS spectrum were identical with those of an authentic sample obtained from *Methylobacterium organophilum*.^[9] The ^1H -NMR spectrum was characteristic of the usual 32*R*,33*R*,34*S* configuration of the side chain.^[10]

More interestingly, ribosylhopane (**3**) triacetate was detected by direct inlet electron impact ionization mass spectrometry, GC-MS and APCI-LCMSⁿ for the first time in a bacterium (Supporting information, figures 1S to 4S). As a cyclic hemiketal, ribosylhopane (**3**) (Scheme 1), like aldoses, upon acetylation produces two anomeric triacetates^[5b] that can be separated by TLC and behave differently by GC-MS. Whereas triacetylated α -ribosylhopane was stable under GC-MS conditions and gave a weak M/z 670 molecular ion, the β -anomer decomposed and produced a compound with a M/z 550 molecular ion, corresponding most likely to the loss of two acetic acid moieties (Supporting information, figure 2Sc). Retention times and mass spectra of the two anomers isolated from *S. coelicolor orf18* mutant were identical with those of a reference sample. The presence of the two anomers of acetylated ribosylhopane was confirmed by APCI-LCMSⁿ (Supporting information, figures 3S and 4S). For each anomer, the $[\text{M} + \text{H}]^+$ 671, $[\text{M} + \text{H} - \text{AcOH}]^+$ 611 and $[\text{M} + \text{H} - 2\text{AcOH}]^+$ 551 ions were found in the full mass spectrum in similar abundances. Further fragmentation of the $[\text{M} + \text{H} - \text{AcOH}]^+$ 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 acetyl adenine.^[11] For a definitive identification, the two acetylated 35 α - and 35 β -anomers were isolated by reverse phase HPLC. Their ^1H -NMR spectra and direct inlet electron impact mass spectra were identical with those of synthetic reference samples.^[5b,12]

Finally, as acetylated adenosylhopane (**2**) and aminobacteriohopanetriol (**5**) are not amenable to GC-MS, the presence of these hopanoids was also analyzed in the acetylated crude extract by APCI-LCMSⁿ.^[11,13] Only adenosylhopane (**2**) triacetate was found in small amounts together with acetylated ribosylhopane (**3**) and bacteriohopanetetrol (**4**) (Scheme 1). Acetylated aminobacteriohopanetriol (**5**), the only hopanoid found in the wild type, was not detected in the *orf18* deletion strain in *S. coelicolor*. In order to confirm that the mutant phenotype was the result of the apramycin insertion in *orf18*, the mutated gene was complemented into the *orf18* mutant via a hygromycin selective plasmid. The complemented strain produced aminobacteriohopanetriol whereas this hopanoid was not detected in the strain bearing only the empty plasmid without the *orf18* gene.

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

Bacteriohopanetetrol (**4**) is the other major hopanoid of the *orf18* mutant. According to the hypothetical biogenetic scheme for hopanoid biosynthesis (Scheme 1), ribosylhopane is converted into bacteriohopanetetrol by reduction and into aminobacteriohopanetriol by reductive amination. When the *orf18* gene is expressed as in the wild type of *S. coelicolor*, ribosylhopane (**3**) is completely converted into aminobacteriohopanetriol (**5**) by reductive amination and no other C₃₅ hopanoids are found. When this main route is blocked by deletion of the *orf18* gene, intermediates such as adenosylhopane (**2**) and ribosylhopane (**3**) can be accumulated to some extent, and another novel hopanoid, bacteriohopanetetrol (**4**), is produced in consequence. This tetrol results probably from the reduction of the ribosylhopane (**3**) hemiketal, for instance by an aldose-reductase of low specificity and efficiency. It allows the accumulation of both ribosylhopane (**3**) and bacteriohopanetetrol (**4**) but the concentration of these compounds in the *orf18* mutant is much lower than that of aminobacteriohopanetriol (**5**) in the wild type. Though adenosylhopane (**2**) is not detected in the wild type, its presence in trace amounts in the *orf18* mutant is in accordance with an intermediate role in the biosynthesis of C₃₅ biohopanoids. The hopanoid phenotype of the *orf18* mutant does not, however, indicate a relationship between adenosylhopane (**2**) and ribosylhopane (**3**). Adenosylhopane (**2**) may be the precursor of ribosylhopane, as presumed from the hypothetical biosynthetic pathway (Scheme 1) or may be derived from ribosylhopane (**3**) by adenylation.

The protein encoded by the *hpnO* gene was shown to be required in the biosynthesis of aminobacteriohopanetriol (**5**) in *R. palustris*.^[7] According to the nucleotide sequence, this gene encodes a homologue of an ornithine-oxo acid aminotransferase. All bacteria, which possess a gene homologous to the *R. palustris hpnO* gene and were 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] Comparison of the amino-acid sequences of the *R. palustris* HpnO and the *S. coelicolor* Orf18 resulted in 52 % identity (E value: 5e-167; query cover: 98 %), indicating that the *orf18* may be a homolog of *hpnO* and that the corresponding enzyme is most probably an aminotransferase. The hopanoid phenotype/fingerprint of the *S. coelicolor orf18* mutant is now easily interpreted. In the absence of the aminotransferase encoded by the *orf18* gene, ribosylhopane, the substrate of this enzyme, is not converted into aminobacteriohopanetriol (**5**) and ribosylhopane (**3**) can now be accumulated.

The possible role of *Orf15*

Finally, a last gene coding for the enzyme catalyzing the formation of the C₃₅ bacteriohopane skeleton remains to be identified in *S. coelicolor*. The *hpnH* gene was found as encoding the first enzyme, a putative B₁₂ binding radical SAM protein, involved in the biosynthesis of the C₃₅ biohopanoids in *M. extorquens*.^[6] Its deletion in *M. extorquens*^[6] and in *R. palustris*^[7] results in the exclusive production of the C₃₀ hopanoids diploptene or diplopterol. The C₃₅ bacteriohopanetetrol (**4**) or aminobacteriohopanetetrol (**5**), the main hopanoids in these bacteria, are absent. The amino acid sequence of the *S. coelicolor* Orf15^[8] was compared with those of the documented HpnH proteins (159 genes) in the NCIB database. Significant identities were found from 49 to 100 % AA identity (data not shown). This indicates that the *orf15* gene belongs to the *hpnH* family and may be associated with the hopanoid biosynthesis in *S. coelicolor*, encoding the enzyme catalyzing the first step of the biosynthesis of C₃₅ biohopanoids to produce adenosylhopane (**2**).

Conclusion

All genes involved in the biosynthesis of aminobacteriohopanetriol (**5**) in *S. coelicolor* have now been characterized, confirming the hypothetical biogenetic scheme (Scheme 1) proposed 20 years ago.^[5a] The catalytic activity of the gene products remains, however, to be characterized. Most interesting was the hopanoid phenotype of the *orf18* mutant lacking the transaminase producing aminobacteriohopanetriol (**5**) and accumulating ribosylhopane (**3**). This hopanoid that was hypothesized as a precursor of the C₃₅ biohopanoids was the last missing link in the biogenetic scheme.

Experimental section

Strains and culture conditions

The wild type strain used in this study is *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 allowing the diffusion of the nutrients without direct contact of the bacteria with the solid medium.^[15] Apramycin (50 µg.mL⁻¹) was added to the culture medium for the cultivation of the *orf14* and *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 the wild type, 7-8 days for the *orf18* strain, 8-10 days for the *orf14* strain and 16 days for the strains utilized in the complementation experiments. At the end of the cultures, 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* (open reading frame ORF14) and *orf18* (open reading frame ORF18) and of the *S. coelicolor* cosmid pSC6A5 were inactivated using the gene

replacement method described by Gust *et al.*^[19] An apramycin resistant cassette flanked by *orf14* or *orf18* specific DNA fragments, were used to replace *orf14* or *orf18* in the chromosome. The PCR primers used to construct these cassettes were *orf14*L and ORF14r for *orf14*, and Redi-F-ORF18 and Redi-R-ORF18 for *orf18* (Table 1). *E. coli* 25113 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 the *orf14* or *orf18* of pSC6A5 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 the 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* 12567/pUZ8002; 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 the presence of the gene replacements was analysed by PCR using the diagnostic primers dig14l and DIG14r, or NW-L-ORF18 and NW-R-ORF18. The PCR products of the expected size were further confirmed by restriction analysis.

The *orf14* and *orf18* mutants were further complemented by introduction of plasmid pTE299 or pTE296, respectively. The *orf14* or *orf18* fragments were amplified by PCR using *orf14*L4 + *orf14*R4 and *orf18*L4 + *orf18*R4 primers (Table 1), respectively, with genomic DNA as template. The PCR products were introduced into pGEM-Teasy (Promega) then digested with *Nde*I and *Pac*I or *Nde*I and *Xho*I for *orf14* and *orf18*, respectively. The isolated fragments were then ligated to 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 C²HCl₃ solution on a Bruker Biospin spectrometer equipped with a cryoprobe (500 MHz for ¹H-NMR, 125 MHz for ¹³C-NMR) with CHCl₃ (δ = 7.260 ppm) for ¹H-NMR and C²HCl₃ (δ = 77.0 ppm) for ¹³C-NMR.

GC-MS spectra were obtained on a Thermo TSQ Quantum mass spectrometer connected to a Thermo Trace GC ultra gas chromatograph (PTV injector, HP-5 MS column, 30 m, 0.25 mm internal diameter, 0.25 μm film thickness) with a temperature program (3 min at 55°C, from 55°C to 320°C at 10°C/min, 50 min at 320°C). Helium was utilized as carrier gas (1 mL.min⁻¹). Direct inlet mass spectra were recorded on the same spectrometer in the electron impact ionization mode at 70 eV over a 50-800 Da mass range (0.5 sec cycle time). The source temperature was set at 230°C.

Atmospheric pressure chemical ionization-liquid chromatography mass spectrometry APCI-LCMSⁿ was performed as described previously^[11,13] on the total acetylated lipid extract using a ThermoFinnigan surveyor HPLC system fitted with a Phenomenex Gemini C₁₈ column (150 mm; 3.0 mm internal diameter; 5 μm particle size) and a security guard column

cartridge of the same material coupled to a Finnigan LCQ ion-trap mass spectrometer equipped with an APCI source operated in positive ion mode. Chromatographic separation was accomplished at 30°C with a flow rate of 0.5 mL.min⁻¹ and the following mobile phase solvent 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 and stabilising for 10 min (where A = CH₃OH, B = water and C = propan-2-ol, all Fisher HPLC grade). APCI was achieved at 155°C capillary temperature and 490°C APCI vaporiser temperature with a corona discharge current of 8 μA, sheath and auxiliary gas flow of 40 and 10, respectively (arbitrary units). MSⁿ 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 MS² spectrum of most intense ion from SCAN 1; SCAN 3: data-dependent MS³ spectrum of most intense ion from SCAN 2. Detection was achieved at an isolation width of *m/z* 5.0 and fragmentation with normalised collisional dissociation energy of 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 *S. coelicolor* A(3)2 wild type

The lyophilized material from 36 Petri dishes was extracted under reflux with CHCl₃/CH₃OH (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 fractionated by flash chromatography (CH₂Cl₂, EtOAc, 4:1, v/v).^[20] The fractions containing hopanoids according to the methyl signal fingerprint in their ¹H-NMR spectra were further purified by preparative TLC Merck silica gel 60F₂₅₄ plates (0.25 mm thickness) using CH₂Cl₂/CH₃OH, 95:5, v/v) as eluent, yielding aminobacteriohopanetriol (5) tetraacetate (0.14 mg, R_F = 0.54).

Identification of adenosylhopane (2) from *orf14* mutant

The freeze-dried material from 35 Petri dishes was extracted and the extract acetylated as reported above for the isolation of aminobacteriohopanetriol tetraacetate from wild type. Hopanoid detection was performed by APCI- LCMS on the total acetylated lipid extract. Only adenosylhopane (2) triacetate was identified by comparison with previously published spectra.^[11,21]

APCI-MS² of the pseudomolecular ion of adenosylhopane triacetate *m/z* 788 [M + H]⁺: *m/z* = 611 ([M + H - *N*-acetyladenine]⁺, 100%), 509 ([M + H - *N*-acetyladenine - AcOH - CH₂CO]⁺, 2%), 491 ([M + H - *N*-acetyladenine - 2 AcOH]⁺, 4%), 191 (ring C, cleavage, 1%),^[22] 178 ([N - acetyladenine + H]⁺, 27%).

Identification of ribosylhopane (3) and bacteriohopanetetrol (4) from *orf18* mutant

The lyophilized material from 340 Petri dishes was extracted under reflux with CHCl₃/CH₃OH (2:1, v/v, 3 × 500 mL, 45 min). The combined extracts were evaporated to dryness, and the residue (1.4 g) 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 using first CH₂Cl₂ (60 mL) and then CH₂Cl₂/AcOEt (8:2, v/v, 60 mL) to give pink/orange fractions, the latter fraction containing hopanoids according to NMR, GC and GC-MS analysis. This fraction was

further separated by liquid chromatography on silica gel eluting with CH₂Cl₂/AcOEt (5:1, v/v), and yielded three subfractions, the two first ones being considerably enriched in polyacetylated hopanoids **3** and **4**. These were subjected to preparative TLC on silica gel plates (Merck 60F₂₅₄, 0.25 mm thickness) using hexane/EtOAc (5:1, v/v) as eluent^[23] yielding crude 35 β -ribosylhopane triacetate (R_F = 0.34), bacteriohopanetetrol tetraacetate (R_F = 0.25) and 35 α -ribosylhopane triacetate (R_F = 0.11). Final purification of these fractions by reverse phase HPLC on a C₁₈ column (Varian Polaris C₁₈, 250 mm $\times$ 4.6 mm, 5 μ m) using CH₃OH/propan-2-ol/H₂O (33:16:1, v/v, 1 mL.min⁻¹) afforded pure 35 β -ribosylhopane triacetate (1 mg) and 35 α -ribosylhopane triacetate (0.5 mg). Bacteriohopanetetrol tetraacetate (2.5 mg) was isolated using the same HPLC column and a mixture of CH₃OH/propan-2-ol/H₂O (33:16:1.5, v/v, 1 mL.min⁻¹) as mobile phase. 35 α -Ribosylhopane triacetate, 35 β -ribosylhopane triacetate, adenosylhopane triacetate and bacteriohopanetetrol tetraacetate were identified in the acetylated crude extract by APCI-LCMS as described above (Supplementary figures 3S and 4S).

35 β -Ribosylhopane (**3**) triacetate

¹H-NMR (500 MHz, C²HCl₃): δ /ppm = 6.121 (1H, s, 35 β -H); 5.318 (1H, dd, J = 1.2 Hz, 34-H), 5.181 (1H, dd, J = 6.3, 5.0 Hz, 33-H), 4.123 (1H, m, 32-H), 2.111 (3H, s, -COCH₃), 2.077 (3H, s, -COCH₃), 2.064 (3H, s, -COCH₃); 0.945 (6H, s, 8 β and 14 α -Me), 0.925 (3H, d, J = 6.4 Hz, 22*R*-Me), 0.842 (3H, s, 4 α -Me), 0.810 (3H, s, 10 β -Me), 0.788 (3H, s, 4 β -Me), 0.690 (3H, s, 18 α -Me).

¹³C-NMR (125 MHz, C²HCl₃): δ /ppm = 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 $\times$), 20.0, 18.7, 16.6, 16.5, 15.9 ($\times$ 2).

EI-MS spectrum (direct inlet): m/z = 670 (M⁺, 1%), 610 (M⁺-AcOH, 47%), 595 (M⁺-AcOH - Me, 16%), 550 (M⁺ - 2 AcOH, 2%), 449 (ring C cleavage, 5%),^[22] 435 (ring C cleavage - Me, 18%), 389 (ring C cleavage - AcOH, 68%), 369 (M⁺ - side chain, 20%), 329 (16%), 287 (7%), 269 (9%), 191 (ring C cleavage, 100%), 175 (16%), 163 (15%), 149 (22%), 123 (19%), 95 (34) (supplementary Figure 2S, a).

GC-MS data: supporting information Figure 2S, c.

35 α -Ribosylhopane (**3**) triacetate

¹H NMR (500 MHz, C²HCl₃): δ /ppm = 6.362 (1H, d, J = 4.5 Hz, 35 β -H), 5.207 (1H, dd, J = 6.8, 4.5 Hz, 34-H), 5.041 (1H, dd, J = 6.8, 3.6 Hz, 33-H), 4.193 (1H, m, 32-H), 2.114 (3H, s, -COCH₃), 2.103 (3H, s, -COCH₃), 2.058 (3H, s, -COCH₃), 0.945 (6H, s, 8 β and 14 α -Me), 0.922 (3H, d, J = 6.4 Hz, 22*R*-Me), 0.842 (3H, s, 4 α -Me), 0.811 (3H, s, 10 β -Me), 0.788 (3H, s, 4 β -Me); 0.687 (3H, s, 18 α -Me).

EI-MS spectrum (direct inlet): m/z = 670 (M⁺, 3%), 610 (M⁺-AcOH, 35%), 595 (M⁺-AcOH - Me, 11%), 550 (M⁺ - 2 AcOH, 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%) (supplementary Figure 1S, a). GC-MS data: supporting information, Figure 1S, c (degradation product).

(22*R*,32*R*,33*R*,34*S*)-Bacteriohopanetetrol (4) tetraacetate

¹H NMR (500 MHz, C²HCl₃): δ /ppm = 5.259 (1H, ddd, J = 6.6, 5.6, 2.3 Hz, 34-H), 5.218 (1H, dd, J = 5.6, 4.4 Hz, 33-H), 5.020 (1H, ddd, J = 9.6, 4.3, 3.4 Hz, 32-H), 4.376 (1H, dd, J = 12.2, 2.5 Hz, 35-H_a), 4.139 (1H, dd, J = 12.1, 6.6 Hz, 35-H_b), 2.083 (3H, s, -COCH₃), 2.077 (3H, s, -COCH₃), 2.070 (3H, s, -COCH₃), 2.050 (3H, s, -COCH₃), 0.926 (6H, s, 8 β and 14 α -Me), 0.891 (3H, d, J = 6.4 Hz, 22*R*-Me), 0.832 (3H, s, 4 α -Me), 0.795 (3H, s, 10 β -Me), 0.775 (3H, s, 4 β -Me), 0.665 (3H, s, 18 α -Me).

¹³C NMR (125 MHz, C²HCl₃): δ /ppm = 170.8 (-COCH₃), 170.5 (-COCH₃), 170.1 (-COCH₃), 169.8 (-COCH₃), 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 (-COCH₃), 21.0 (-COCH₃×2), 20.8 (-COCH₃×2 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 (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⁺ - 2 AcOH, 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%).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

WL acknowledges financial support from the China Scholarship Council, and the French embassy in Beijing, ES from the CNRS, and MR from the Alexander von Humboldt Foundation for the collaboration with the University of Tübingen. This research was supported by a grant from the Foundation "Frontier Research in Chemistry" (Strasbourg, France) to MR; EK was supported in part by grant from the United States National Institute of Health (R21 AI76753) and in part by a grant of the United States Department of Energy (DE-FG02-09ER20097) to the Complex Carbohydrate Research Center (University of Georgia, Athens, USA); TH and JD were supported by grants from the Deutsche Forschungsgemeinschaft (SFB 766). HMT thanks the Science Research Infra-structure Fund (SRIF) from HEFCE for funding the purchase of the ThermoFinnigan LCQ ion trap mass spectrometer. Prof. Karl Poralla (Microbiological Institute, Microbiology/Biotechnology, University of Tübingen) is warmly acknowledged for his support to the project and the enthusiastic discussions.

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Scheme 1.

Hypothetical biogenetic scheme for the biosynthesis of aminobacteriohopanetriol in *Streptomyces coelicolor* and isoprenoid/hopanoid biosynthetic genes on cosmid SC6A5. (1) diploptene; (2) adenosylhopane; (3) ribosylhopane; (4) bacteriohopanetetrol; (5) aminobacteriohopanetriol. The Orfs 14, 15, and 18 are highlighted by black arrows in the biosynthesis cluster. Annotation of the genes are 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.

Table 1

Construction of the *S. coelicolor* mutants: list of primers.

	Sequence (5'-3')
orf14L	GTGAAGGGCAGCTGATGACCCCGCAGACCGCCCGTCCCATTCGGGGATCCGTCGACC
ORF14r	ACGGAAGGCCGATATTCCACCGCGCACGGTGCCGATCCGTGTAGGCTGGAGCTGCTTC
Redi-F-ORF18	GGACGGATCGTCATGCCAGCGCAAGGAGAGAACTGAATGATTCCGGGGATCCGTCGACC
Redi-R-ORF18	TCCTTGCCTCAGAGGCAAAAGACCTGGAGGGTGCGCTCATGTAGGCTGGAGCTGCTTC
dig14l	GCTCCTGGGACGAGCCCTACT
DIG14r	CATGCGCTGCTTGAGCACACC
NW-L-ORF18	AGGCAAATTCCTTGCCTCAG
NW-R-ORF18	GTGGACCGGCAGTGGAAC
orf14L4	<u>CATATG</u> ACCCCGCAGACC
orf14R4	<u>TTAATTAA</u> CATCTGGGCTCAC
orf18L4	<u>CATATG</u> ACCAAGGAGTTCGA
orf18R4	<u>CTCGAGT</u> CAGAGGCAAAAGA

Underlined sequences denote for restriction sites.

Table 2

C₃₅ hopanoids from *Streptomyces coelicolor* A3(2) and *orf14* and *orf18* mutants.

	adenosylhopane (2)	ribosylhopane (3)	tetrol (4)	aminotriol (5)
<i>S. coelicolor</i> A(3)2	–	–	–	+
<i>orf14</i> mutant	+	–	–	–
<i>orf18</i> mutant	+	+	+	–

– : not detected; + : detected