Prokaryotic triterpenoids: new hopanoids from the nitrogen-fixing bacteria Azotobacter vinelandii, Beijerinckia indica and Beijerinckia mobilis

Catherine Vilcheze,1 Pierre Llopiz,1 Serge Neunlist,1 Karl Poralla2 and Michel Rohmer1

Author for correspondence: Michel Rohmer.

Three nitrogen-fixing bacteria, Azotobacter vinelandii, Beijerinckia indica and Beijerinckia mobilis, were shown to contain large amounts of triterpenoids of the hopane series. In A. vinelandii, the major compound was a novel bacteriohopanepentol ether accompanied by a similar bacteriohopanetetrol derivative: in both compounds, the hopanoids are linked via an ether bond to a carbapseudopentose moiety often found in bacterial hopanoids. In the two Beijerinckia species, diplopterol and 2β-methyldiplopterol were accumulated in much larger amounts than those usually recorded in hopanoid-producing eubacteria, while 2β-methyldiploptene was isolated for the first time from B. mobilis. Whereas in B. mobilis aminobacteriohopanetriol was the only C35 hopanoid, the simultaneous presence of bacteriohopanetetrol and aminobacteriohopanetriol in B. indica is a rather unusual feature.

**Keywords:** hopanoids, Azotobacter, Beijerinckia

INTRODUCTION

The structural variety and distribution of triterpenoids of the now well-known hopane family is still surprising. In order to try to correlate taxonomy, phylogeny and structures as well as distribution of these unique membrane stabilizers in prokaryotes (Rohmer et al., 1979, 1984), numerous bacterial strains belonging to diverse taxonomic groups are currently being examined for their hopanoid content. As preliminary studies showed that two Azotobacter species were hopanoid producers (Rohmer et al., 1984), the screening was extended to other nitrogen-fixing bacteria. In this paper, we report the characterization of the hopanoids, including identifications of new structures of biogenetic interest (Fig. 1), from three free-living nitrogen-fixing bacteria: Azotobacter vinelandii, Beijerinckia indica and Beijerinckia mobilis.

METHODS

**Culture conditions.** Azotobacter vinelandii DSM 2289 (Deutsche Sammlung von Mikroorganismen, Braunschweig, FRG) cells were obtained from a 10 l culture grown at 30 °C for 4 d in 2 l conical flasks with continuous agitation in DSM culture medium no. 3 (Deutsche Sammlung von Mikroorganismen, 1993) in the presence of calcium carbonate, either in nitrogen-fixing conditions with no nitrogen source or, for comparison, with additional NH4Cl (1 g l⁻¹) (yield: in the presence of NH4Cl about 1 g dry weight l⁻¹, after deduction of calcium carbonate, and in the absence of NH4Cl about 0.5 g l⁻¹). Beijerinckia indica NCIMB 8712 (National Collection of Industrial and Marine Bacteria, Aberdeen, UK) and Beijerinckia mobilis (DSM 2326) cells were obtained from 10 l cultures grown at 30 °C for 3 d in culture medium DSM no. 111 without calcium carbonate (yield 2.2 g dry weight l⁻¹ for each strain). A large-scale culture of B. mobilis was also made in a 200 l fermenter (Giovanaola, Monthey, Switzerland) at 30 °C for 3 d using similar culture conditions (agitation rate 250 r.p.m.; aeration rate 3000 l h⁻¹; yield 270 g dry weight).

**Extraction and isolation of hopanoids.** The cells were harvested by centrifugation (8000 g, 10 min) at 4 °C, freeze-dried and extracted three times for 1 h under reflux using chloroform/methanol (2:1, v/v). After evaporation of the solvent, the crude extract was treated according to one of the following procedures. **Procedure 1.** Periodic acid treatment followed by sodium borohydride reduction, extraction, TLC and acetylation of the alcohol fraction permitted us to determine the diploptene, diplopterol and bacteriohopanopolyol content by GLC (Rohmer et al., 1984). **Procedure 2.** In order to obtain the intact complex hopanoids, extracts were directly acetylated overnight at room temperature using acetic anhydride/pyridine (1:1, v/v) and roughly separated by flash column chromatography using chloroform containing increasing amounts of methanol (Still et al., 1978). In the case of B. indica, the hopanoids and the other lipids had first to be separated from poly-β-hydroxybutyrate by extraction using tetrahydrofuran (Renoux & Rohmer, 1985).
All fractions were monitored by 1H-NMR, and those that showed the typical methyl singlet pattern of the hopane skeleton were further separated by preparative TLC. The fraction containing the acetylated bacteriohopanepolyols of *A. vinelandii* was first separated using chloroform/methanol (95:5, v/v) yielding a mixture of the acetylated polyol ethers (R, Ib, Ia, IIb, IIa, III) which could be separated from one another with difficulty, using ethyl acetate/cyclohexane (7:3, v/v, five migrations) into the hepta-acetate of (III) (R, = 0.35) and the octa-acetate of (IV) (R, = 0.32). The known hopanoids of the two *Beijerinckia* species were obtained as previously described (Rohmer et al., 1984; Renoux & Rohmer, 1985; Neunlist et al., 1988).

**Analytical methods.** GLC was carried out on a Carlo Erba Fractovap 4160 apparatus fitted with a fused silica DB5 capillary column (0.25 mm x 30 m) and an on-column injector. The oven temperature was programmed from 50 °C to 220 °C at 20 °C min⁻¹ and from 220 °C to 310 °C at 6 °C min⁻¹. The flame ionization detector was at 310 °C, and hydrogen was used as carrier gas. The quantity of each product was measured by comparing the peak areas with that of an internal standard of n-dodecane. Direct inlet mass spectrometry was done at 70 eV on a Finnigan TSQ70 apparatus. NMR spectra were recorded on a Brucker AC 250 spectrometer in [1H]chloroform at 300 K. Chloroform was used as internal reference (δ = 7.260 p.p.m.) for 1H spectra (250 MHz) and [1H]chloroform (δ = 7.70 p.p.m.) for 13C spectra (62.9 MHz). Assignments of 13C signals were confirmed by homonuclear 1H/1H correlation (COSY). Distortionless enhancement by polarization transfer (DEPT) was used for the assignment of 13C signals. HPLC was done on Waters 510 apparatus with a Zorbax ODS column (4.6 mm x 15 cm). All fractions were monitored by 'H-NMR, and those that could be separated from one another with difficulty, using methanol/water (95:5, v/v) for dipolopentol (tR = 65 min) and 2P-methylbacteriohopanepolyol ether (tR = 70 min) separation. A Hewlett-Packard HP1090 instrument and a Kromasil C18 column (4.6 mm x 15 cm) were employed with methanol/water (95:5, v/v) for dipolopentol (tR = 32.6 min) and 2P-methylbacteriohopanepolyol ether (tR = 35 min) separation. Relative amounts of dipolopentol (Ia) and 2P-methylbacteriohopanepolyol ether (Ib) were determined by GLC after separation using a shallow temperature programme (from 50 °C to 150 °C at 20 °C min⁻¹, from 150 °C to 180 °C at 1 °C min⁻¹, and from 180 °C to 310 °C at 0.5 °C min⁻¹), those of dipolopentol (Ia) and 2P-methylbacteriohopanepolyol ether (Ib) by 13C-NMR spectroscopy.

**Hopanoid identifications.** Dipolopentol (Ia), dipolopentol ether (IIa), 2P-methylbacteriohopanepolyol ether (Iib), tetrol (V), tetrol ether (Ila, III), etc., were identified by comparison (1H- and 13C-NMR, GLC/MS, GLC and HPLC coelutions) with reference compounds previously obtained from other bacteria in this laboratory or by synthesis (Bisseret et al., 1985; Renoux & Rohmer, 1985; Flesch & Rohmer, 1989; Bisseret & Rohmer, 1989).

2P-Methylbacteriohopanepolyol ether (Iib) isolated from *B. mobilis* was identical (1H- and 13C-NMR, GLC/MS, GLC and HPLC coelutions) with dipolopentol (Iib) isolated from *B. mobilis*.
Table 1. Hopanoid content of nitrogen-fixing bacteria (μg g⁻¹, dry weight)

<table>
<thead>
<tr>
<th>Hopanoid</th>
<th>Agrotobacter vinelandii</th>
<th>Beijerinckia indica</th>
<th>Beijerinckia mobilis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nitrogen source:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NH₄Cl*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N₂*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diploptene (Ia)</td>
<td></td>
<td>17</td>
<td>70</td>
</tr>
<tr>
<td>2β-Methyl diploptene (Ib)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diplopterol (IIa)</td>
<td>250</td>
<td>1000</td>
<td>2900</td>
</tr>
<tr>
<td>2β-Methyl diplopterol (IIb)</td>
<td></td>
<td>3100</td>
<td>1600</td>
</tr>
<tr>
<td>Tetro ether (III)†</td>
<td>700</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentol ether (IV)†</td>
<td>3100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetro (V)†</td>
<td></td>
<td>2600</td>
<td></td>
</tr>
<tr>
<td>Aminotriol (VI)†</td>
<td></td>
<td>1100</td>
<td>2500</td>
</tr>
</tbody>
</table>

-, Not detected.

* As the culture medium contained calcium carbonate, the biomass could not be evaluated. Yields are therefore given in μg g⁻¹ with reference to the culture medium.
† Hopanoids (III)–(VI) were estimated as their polyacetates by weighing.

RESULTS AND DISCUSSION

Diploptene (Ia) is always the least abundant hopanoid in all hopanoid-producing bacteria we have analysed. This hydrocarbon was present in tiny amounts in the two Beijerinckia species, but could not be detected in A. vinelandii (Table 1), in apparent contradiction of previous results indicating that diploptene was present in two other Agrotobacter species (Rohmer et al., 1984). However, GLC detection of such trace amounts is often hampered by other abundant accompanying compounds and makes their correct identification problematic. In B. mobilis, diploptene was accompanied in a 4:1 ratio by a homologue with an additional carbon atom as shown by GLC/MS and the molecular ion at m/z 424. This additional carbon atom could be located on ring A or B as shown by the fragment at m/z 205 arising from the ring C cleavage of the hopane skeleton. The GLC retention time of this compound was only slightly longer than that of diploptene, and as 2β-methyl diploptene was already identified in large amounts in this bacterium, the new compound was suspected to be 2β-methyl diploptene (Ib).

Indeed, after isolation by reverse-phase HPLC, the compound could be definitively identified (¹H- and ¹³C-NMR, GLC/MS, GLC and HPLC coelution) by comparison with a reference sample previously synthesized in this laboratory from 2β-methyl diplopteronol isolated from Methylobacterium organophilum (Stampf et al., 1991; M. Rohmer, unpublished results). Although 2β-methyl diploptene was more or less expected since 2β-methyl diplopterol and 2β-methyl bacteriohopanopolyol has been already found several years ago in bacteria (Bisseret et al., 1985; Simonin, 1993), this is the first report of this simple hopanoid. Further, as in nearly all hopanoid-producing bacteria, diplopteronol (IIa) was present in all three strains. In the Beijerinckia species, it was accompanied by larger amounts of the higher homologue, 2β-methyl diplopteronol (IIb). The high concentrations found for these tertiary alcohols (Table 1) are noteworthy. Until now only Methylobacterium species have been found to contain such amounts of these hopanoids (Knani et al., 1994), which seem to be rather poor membrane reinforcing, at least in the artificial membrane models tested (Bisseret et al., 1983). However, they could efficiently replace cholesterol in a mycoplasma requiring sterols for growth (Kannenberg & Poralla, 1982). The biological significance of this uncommon diplopteronol accumulation is unknown.

Hopanoid contents of the three species analysed were completely different at the level of the C₃₈ bacteriohopane polyol derivatives (Table 1). Whereas A. vinelandii contained only composite hopanoids corresponding to bacteriohopane polyols linked to a carbohydrate derivative, the Beijerinckia species were characterized by very simple hopanoids. In B. mobilis aminobacteriohopanetrol (VI) was nearly the only detected derivative of bacteriohopane. Such a simple composition has only been found in very few bacteria, e.g. Rhodopseudomonas palustris (Neunlist 1985).
et al., 1988) and Streptomyces tendae (P. Simonin & M. Rohmer, unpublished results). In the shake culture of this bacterium, at least three new additional minor hopanoids were present at low concentrations, around 3-10 µg g⁻¹, and could be partially identified by spectroscopic methods. Confirmation of the structures was, however, impossible as these compounds could not be detected in the cells obtained in larger amounts from a fermenter. This illustrates the dependence of the hopanoid composition in bacteria on the growth conditions.

B. indica contained aminobacteriohopanetetrol as well as bacteriohopanetetrol with the usual side-chain stereochemistry derived from D-ribose as determined by NMR-spectroscopy (Bisseret & Rohmer, 1989; Neunlist & Rohmer, 1988). Apart from this Beijerinckiaceae species, only two other bacteria, the cyanobacterium Microcystis aeruginosa (Simonin, 1993) and the obligate methylotroph Methylocystis parus (M. Knani & M. Rohmer, unpublished results), have been found to contain simultaneously these two types of hopanoids, which are not mutually exclusive as first thought. Indeed they might be derived from the same hypothetical precursor, 29-(5'-riboyl)-hopane: the tetrol on the one hand by direct reduction, the aminotetrol on the other hand after reductive amination (Rohmer, 1993). Intensive investigations on B. indica and the two above-mentioned bacteria using synthetic ribosyl-hopane as reference (Bisseret et al., 1994) did not permit us to detect this possible precursor in the bacterial extracts.

In A. vinelandii, free bacteriohopanetetrol could not be detected. Only two composite hopanoids were isolated: a tetrol and a pentol both linked via an ether bond to a carbohydrate derivative. This carbapseudopentofuranose moiety was easily recognized from the ¹H and ¹³C-NMR spectra as well as from the mass spectra of the acetylated derivatives of both hopanoids and seems to be widespread in different taxonomic groups. Indeed bacteriohopanetetrol ether (III) isolated from A. vinelandii is identical with the corresponding tetrol ether from Methylbacterium organophilum (Rohmer et al., 1985) and other Methylbacterium species (Knani et al., 1994), Rhodopseudomonas acidophila (Neunlist et al., 1988) and Zymomonas mobilis (Flesch & Rohmer, 1989). The major component (IV) differed from ether (III) only by the presence of an additional hydroxyl group linked to C-31. GLC analysis of the bacteriohopanopolyol derivatives obtained after H₃BO₃ side-chain cleavage and NaBH₄ reduction indicated that the major component was an acetylated C₃₅ primary alcohol, implying that the corresponding bacteriohopanopolyol had a 1,2-diol group on the C-31 and C-32 positions of the side-chain. The ¹H-NMR spectrum of acetylated compound (IV) showed in the 2 p.p.m. region singlets corresponding to eight acetyl groups. Chemical shifts of the C-35 protons were similar to those of the corresponding protons from the acetylated tetrol ether (III) and indicated that the C-35 carbon atom was linked via an ether bond to the carbapseudopentose moiety. Presence of an additional acetox group at C-31 was further suggested by comparison of the ¹H-NMR spectra of the acetylated tetrol and pentol ethers. In the pentol ether derivative spectrum, a supplementary signal (δ 5.46 p.p.m. corresponding to the proton at C-32. The signal is shifted and modified in comparison with the corresponding H-32 multiplet (δ = 5.04 p.p.m.) in the spectrum of the acetylated tetrol ether. Furthermore, the presence of an additional acetox group at C-31 modifies slightly but significantly the chemical shifts of the 8β and 14α methyl groups: in the ¹H-NMR spectrum of the tetra-acetate of aminotetrol a single signal was observed for the two methyl groups whereas two singlets were found for the aminotetrol penta-acetate (Neunlist & Rohmer, 1985). Similar features were found for polyl ethers (III) and (IV): in the spectrum of acetylated pentol ether the 8β and 14α methyl signals are distinct and found at 0.933 and 0.946 p.p.m. whereas only a single signal is observed at 0.944 p.p.m. in the case of acetylated tetrol ether (III). The pentol-derived side-chain structure could be finally completely deduced and confirmed by ¹H/¹H homonuclear correlation (COSY) giving all proton couplings. Electron impact mass spectrometry of the octa-acetate of (IV) was in accordance with the proposed bacteriohopanopentol ether structure, showing particularly the expected molecular ion at m/z 1059, the ring C cleavage fragments characterizing the hopane skeleton at m/z 191 and 838, as well as the cleavages on both sides of the ether bond at m/z 713 corresponding to the bacteriohopane moiety after cleavage of the C-35/oxygen bond and m/z 330 corresponding to the carbapseudopentose moiety after cleavage of the C-1'/oxygen bond. The same fragmentations were already observed in the mass spectrum of the hepta-acetate of tetrol ether (III) (Renoux & Rohmer, 1985). The stereochemistry of the side-chain of both tetrol and pentol ethers could not be determined, NMR spectroscopy without comparison with reference compounds of known configuration being useless as for most acyclic polyol systems. Concerning the carbapseudopentose moiety, according to the ¹H/¹H coupling constants, the relative configurations are identical for all ethers found up to now in the examined bacteria and correspond to that determined for the Zymomonas mobilis tetrol ether as determined by nuclear Overhauser effects (Smith, 1985).

The C₅ polyhydroxylated side-chain of all bacteriohopanopolysols arises from a D-pentose derivative, most probably D-ribose according to the stereochemistry, linked to the isopropyl group of the hopane skeleton via its C-5 carbon atom (Flesch & Rohmer, 1988; Rohmer et al., 1989). A C₃₅ hopanoid was postulated as a possible precursor for the hopane moiety of the C₃₅ bacteriohopanopolysols (Rohmer, 1993). 2β-Methylbiploptol and 2β-methylbiploptene are apparently metabolic dead-ends. They are accumulated in the Beijerinckiaceae species and apparently not converted, at least not efficiently, into 2β-methylbacteriohopane derivatives, which could not be detected in these bacteria. Indeed careful examination of the ¹³C-NMR spectrum of acetylated aminotriol did not show any signal corresponding to a C-2β methylated homologue. If such a compound were present, it would represent less than 2% of the aminotriol mixture. Similar conclusions could be drawn from the analysis of the hopanoids from cyanobacteria and prochlorophytes: 2β-
methylbacteriohopanoids were quite often found in these prokaryotes, whereas diploptene and/or diploptol were not accompanied by detectable amounts of their 2β-methyl companions.

The presence of hopanoids in nitrogen-fixing bacteria might be related to the nitrogenase function, which is very sensitive to dioxygen. Hopanoid-rich membranes were suggested to protect the enzyme from this oxidizing agent (Berry et al., 1993). Indeed, in another nitrogen-fixing bacterium, Frankia alni, the envelope of the vesicles specialized in nitrogen reduction consisted mainly of bacteriohopanetetrol and bacteriohopanetetrol phenylacetate (Berry et al., 1993). This emphasizes again how dependent is the hopanoid function and concentration on the environmental conditions.

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