The Hopanoids of '*Methylosinus trichosporium*': Aminobacteriohopanetriol and Aminobacteriohopanetetrol

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Triterpenoids belonging to the hopane family are widespread among prokaryotes. The two major hopanoids isolated from the methylotrophic bacterium '*Methylosinus trichosporium*' grown on methane or methanol were identified by spectroscopic methods as the aminotriol 35-aminobacteriohopane-32,33,34-triol and a novel aminotetrol, 35-aminobacteriohopane-31,32,33,34-tetrol. The former hopanoid has been previously isolated from the purple non-sulphur bacterium *Rhodomicrobium vannielii*.

INTRODUCTION

The hopanoids are the most widely distributed triterpenoids among prokaryotes (Rohmer *et al.*, 1979, 1984). The major compounds are always derived from a C₃₅ bacteriohopane skeleton (Förster *et al.*, 1973), which is composed of a C₃₀ pentacyclic triterpene, hopane, linked to a polyfunctionalized C₅ normal acyl chain. By comparison with synthetic hopanoids, it has been shown that the normal acyl chain is linked to the C-30 carbon atom of the hopane side-chain (Rohmer & Ourisson, 1976*a*). Most of the methylotrophic bacteria, growing on either methane or methanol, are good hopanoid producers. In a previous study (Rohmer *et al.*, 1984), we have shown that the methylotroph '*Methylosinus trichosporium*' grown on methane contains at least two bacteriohopane derivatives which release a C₃₂ (III) and a C₃₁ (IV) primary alcohol on treatment by our standard periodic acid/sodium borohydride (H₅IO₆/NaBH₄) side-chain cleavage method (Fig. 1). The C₃₂ alcohol could be derived from the bacteriohopanetetrol already isolated from *Acetobacter aceti* subsp. *xylinum* (Förster *et al.*, 1973; Rohmer & Ourisson, 1976*a*) or from *Bacillus acidocaldarius* (Langworthy & Mayberry, 1976). The C₃₁ alcohol, however, could arise from a pentol or a pentol derivative which has already been isolated in small amounts from the cyanobacterium *Nostoc muscorum* (Rohmer, 1975; Rohmer & Ourisson, 1976*b*), but not in sufficient amounts for a full characterization. This paper describes the isolation and the identification of the hopanoids of '*Methylosinus trichosporium*': 35-aminobacteriohopane-32,33,34-triol (I) and 35-aminobacteriohopane-31,32,33,34-tetrol (II) (Fig. 1).

METHODS

Strain and cultivation. '*Methylosinus trichosporium*' NCIB 11131 was obtained from the National Collection of Industrial Bacteria (Aberdeen, UK) [it was referred to as '*Methylosinus trichosporium*' OB3b in our previous paper (Rohmer *et al.*, 1984)]. The name is placed in quotation marks since it is not on the Approved Lists of Bacterial Names (Skerman *et al.*, 1980) or subsequent validation lists published in the *International Journal of Systematic Bacteriology*. Growth on methane was done as by Whittenbury *et al.* (1970), for 3 d in stoppered 2 litre Erlenmeyer flasks at 30 °C on a rotatory shaker (yield about 240 mg dry wt l⁻¹). Cells were also adapted to methanol growth (Whittenbury *et al.*, 1970; Hou *et al.*, 1978); they were grown in the same conditions and on the same ammonium/mineral salts medium, but with methanol (2%, v/v) as carbon source instead of methane (yield about 300 mg dry wt l⁻¹). The cells were harvested by centrifugation (10000 g, 10 min, 4 °C) and freeze-dried.

Analytical methods. TLC was done on hand-made Merck 7747 Kieselgel 60PF 254 plates (1 mm thickness) or on Merck 5715 HF254 silica gel plates (0.25 mm). The lipids were visualized under 360 nm UV light after spraying

with a 0.1% ethanolic solution of berberine chlorhydrate (Rohmer *et al.*, 1984). The bands containing the hopanoids were scraped off and the triterpenoids recovered with chloroform/methanol (2:1, v/v). HPLC was done with a Waters μ -Bondapak C₁₈ column (300 $\times$ 3.9 mm), methanol/water (93:7, v/v, 2 ml min⁻¹) as solvent and a Waters R401 differential refractometer detector. GC was done with a glass capillary column (OV-1, 25 m) using an on-column injector; the oven temperature was programmed from 60 °C to 220 °C (30 °C min⁻¹), 220 °C to 280 °C (3 °C min⁻¹) and 280 °C to 310 °C (5 °C min⁻¹); the flame-ionization detector was at 310 °C. GC-MS was done at 70 eV on an LKB 9000S spectrometer fitted with a capillary SE-30 column as previously described (Rohmer *et al.*, 1980). Direct inlet mass spectra were recorded at 70 eV on a modified Thomson THN 208 spectrometer using a fast heating technique for less volatile compounds (Dessort *et al.*, 1982). Nuclear magnetic resonance (NMR) spectra were recorded on a Varian T60 or a Bruker W200 instrument using C²HCl₃ and CHCl₃ (δ = 7.265 p.p.m.) as internal standard for ¹H-NMR and C²HCl₃ (δ = 77.0 p.p.m.) for ¹³C-NMR.

Isolation of the hopanoids. The freeze-dried cells (6 g) were extracted three times under reflux for 1 h using chloroform/methanol (100 ml, 2:1, v/v). After filtration, the combined extracts were evaporated to dryness under vacuum and treated according to one of the following procedures. (1) The crude extract was treated first with a solution of H₅IO₆ and then with NaBH₄ (Rohmer *et al.*, 1984). The primary alcohols (III) and (IV) (Fig. 1) arising from the cleavage of the bacteriohopanepolyols were isolated by TLC and acetylated, and their acetates identified by GLC and GLC-MS (Rohmer *et al.*, 1984). (2) The crude chloroform/methanol extract was acetylated overnight at room temperature with an excess of acetic anhydride/pyridine (1:1, v/v). Excess reagent was removed under reduced pressure, and the residue separated by TLC using chloroform/methanol (95:5, v/v) as eluent, giving peracetylated (I) (R_F = 0.36) and peracetylated (II) (R_F = 0.32). These hopanoid polyacetates were further purified by reverse-phase HPLC.

Details of the mass and NMR spectra have been deposited with the British Library Lending Division, Boston Spa, Yorkshire LS23 7BQ, UK, as Supplementary Publication no. SUP 28018 (7 pages). Copies may be obtained from the BLLD on demand; wherever possible, requests should be accompanied by prepaid coupons held by many university libraries and the British Council. The material deposited comprises mass spectra of peracetylated (II), (III) and (IV), including chemical ionization data for the derivative of (II), ¹H- and ¹³C-NMR spectra of peracetylated (I), and the ¹H-NMR spectrum of (II). Tentative assignment of the ¹³C-NMR signals was done by using a J-modulated spin echo technique and data obtained on tetra-acetoxysteroidhopane (J. M. Renoux & M. Rohmer, unpublished results).

RESULTS

A preliminary H₅IO₆/NaBH₄ treatment of a sample of the crude '*Methylosinus trichosporium*' extract released only two primary alcohols (Fig. 1) having a C₃₂ (III) or a C₃₁ (IV) skeleton, in the ratio 70:30. This result shows that two bacteriohopanepolyols with two free hydroxyl groups either at C-32 and C-33 or at C-31 and C-32 were present. The acetate of III was identical (GC and GC-MS) to the derivative obtained from *Acetobacter aceti* subsp. *xylinum* and to synthetic material (Rohmer & Ourisson, 1976a). Furthermore, the methyl region of the ¹H-NMR spectrum of the peracetylated hopanoid (I) as well as its ¹³C-NMR spectrum showed clearly that this compound is a typical bacterial hopanoid with an assumed 22*R* configuration (Rohmer & Ourisson, 1976a; J. M. Renoux & M. Rohmer, unpublished results).

The aminotriol (I) [2.8 mg (g dry wt)⁻¹] was identical (TLC behaviour, ¹H-NMR) to the aminotriol already isolated from the purple non-sulphur bacterium *Rhodospirillum rubrum* (Neunlist *et al.*, 1984, 1985). The amino group may be located at C-35; the signals of the two protons at C-35 in peracetylated (I) were shifted upfield at 3.37 and 3.69 p.p.m., whereas they appeared at 4.14 and 4.39 p.p.m. in the spectrum of the tetra-acetoxysteroidhopane isolated from *Bacillus acidocaldarius* or from *Methylobacterium organophilum* (J. M. Renoux & M. Rohmer, unpublished results). Furthermore, the signal of the C-35 carbon atom was also shifted upfield: it appeared at 39.2 p.p.m. in the ¹³C-NMR spectrum of the acetylated hopanoid (I) and at 62.1 p.p.m. in the spectrum of tetra-acetoxysteroidhopane (J. M. Renoux & M. Rohmer, unpublished results).

Compound (II) differs from the aminotriol (I) by the presence of an additional hydroxyl group. This was already suggested by the direct inlet mass spectrum of the penta-acetate of (II), which showed a molecular ion at m/z 771 and a ring C (Fig. 1) cleavage fragment (Budzikiewicz *et al.*, 1963) at m/z 550. This molecular weight of 771 was confirmed by chemical ionization mass spectrometry using ammonia as reactant gas; ions at m/z 789 and 772 corresponding to $M + NH_4^+$ and $M + H^+$, respectively, as well as a metastable ion at m/z 756 corresponding to the

Fig. 1. The hopanoids of '*Methylosinus trichosporium*' and their $\text{H}_5\text{IO}_6/\text{NaBH}_4$ cleavage products. See text for details.

direct transition from $\text{M} + \text{NH}_4^+$ to $\text{M} + \text{H}^+$ were observed. The presence of the two ions at m/z 771 and 550 suggests also that an oxygen atom is replaced by an NH group in the side chain. The position of the extra hydroxyl group at C-31 is already indicated by the C_{31} skeleton of the H_5IO_6 cleavage product (IV) (Fig. 1). The ^1H -NMR spectrum of peracetylated (II) showed a new signal at 5.20 p.p.m. corresponding to the proton at C-31. Furthermore, the presence of this new acetoxy group at C-31 slightly modified the chemical shifts of some of the methyl groups. The doublet corresponding to the methyl group at C-22 was shielded and appeared at 0.912 p.p.m. whereas it was at 0.901 p.p.m. in the spectrum of peracetylated (I) and the signal corresponding to the methyl groups located at C-8 and C-14 was split. These two methyl groups appeared as two singlets at 0.930 and 0.945 p.p.m. in the spectrum of the penta-acetate of (II), whereas they appeared as a unique singlet at 0.941 p.p.m. corresponding to six protons in the spectra of all other hopanoids lacking a functional group at C-31. The amino group of hopanoid (II) could be localized at C-35 for the same reasons as those described for the localization of the amino group of compound (I).

DISCUSSION

'*Methylosinus trichosporium*' is a good source of the two aminobacteriohopanepolyols (I) and (II) (Fig. 1). The aminotriol (I) was recently identified by us in the photosynthetic purple non-sulphur bacterium *Rhodomicrobium vannielii* (Neunlist *et al.*, 1984, 1985). The isolation of this compound and of a higher homologue, the aminotetrol (II), from a methylotrophic bacterium belonging to an unrelated taxonomic group shows that these derivatives may be far more widespread in prokaryotes than previously expected. In several prokaryotes, the bacteriohopanepolyols are usually not present as free polyols, but are linked to polar moieties. Good examples are *N*-acyl glycosides of bacteriohopanetetrol in *Bacillus acidocaldarius* (Langworthy & Mayberry, 1976), *N*-aminoacyl derivatives of the aminotriol (I) in *Rhodomicrobium vannielii* (Neunlist *et al.*, 1984, 1985) and bacteriohopanetetrol linked to various polyols via an ether linkage in *Methylobacterium organophilum* (J. M. Renoux & M. Rohmer, unpublished results). Such polar derivatives could not be detected in '*Methylosinus trichosporium*'. It seems that only the free aminopolyols (I) and (II) are present, since the amounts of hopanoids detected by two

different procedures are essentially the same. The procedure involving the H_5IO_6 cleavage of the side-chain is able to detect all bacteriohopanepolyols as well as their known polar derivatives, whereas with the alternative procedure some very polar derivatives or those present in very small amounts could remain undetected.

Because of the similarities of the structures and the biosynthesis of sterols and hopanoids, we have postulated that the hopanoids might be the phylogenetic precursors of the sterols (Rohmer *et al.*, 1979; Ourisson & Rohmer, 1982). Experimental support has already been obtained using artificial biological membrane models such as phospholipid monolayers or unilamellar vesicles of phospholipid bilayers. In both cases hopanoids have been shown to induce a condensation of the normal acyl chains above the phase transition temperature of the phospholipids or to reinforce efficiently the bilayer, much like cholesterol (Poralla *et al.*, 1980; Kannenberg *et al.*, 1983; Benz *et al.*, 1983; Bissleret *et al.*, 1983). Furthermore, a physiological effect related to the presence of hopanoids could be observed in the thermoacidophilic bacterium *Bacillus acidocaldarius*: the hopanoid content of the cells increased dramatically with the growth temperature, probably corresponding to the need for more membrane reinforcers (Poralla *et al.*, 1984). Since most of the methane-utilizing bacteria so far described in some conditions contain extensive intracytoplasmic membrane systems (Davies & Whittenbury, 1970; Wolfe & Higgins, 1979), it was reasonable to assume that they would be good hopanoid sources. In fact almost all methylotrophs so far analysed have contained hopanoids, the exceptions being '*Methylomonas clara*' and '*Methylophilus methylotrophus*' grown on an industrial scale by Hoechst and ICI, respectively (Rohmer *et al.*, 1984). '*Methylosinus trichosporium*' is able to utilize either methane or methanol as carbon and energy source (Best & Higgins, 1981). Although one might expect a difference between the methane- and the methanol-grown cells, in fact the hopanoid content was qualitatively and quantitatively the same in the two culture conditions. This result is not too surprising since our '*Methylosinus trichosporium*' cells were harvested in the late deceleration phase. In these conditions the methanol-grown cells, much like the methane-grown cells, contain a large intracytoplasmic membrane system (Best & Higgins, 1981). Since the development of this membrane system is largely dependent on the growth conditions and the age of the culture, a careful control of these factors might reveal a correlation between the hopanoid content of the cells and the extension of the membrane system.

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