(−)-Verrucosan-2β-ol from the phototrophic bacterium Chloroflexus aurantiacus: first report of a verrucosane-type diterpenoid from a prokaryote

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Introduction

Chloroflexus aurantiacus, a filamentous, gliding, thermophilic phototrophic bacterium, is in a number of respects (e.g. ecology, nutrition, photosynthesis, CO2 fixation, bacteriochlorophyll structure) unique among phototrophic bacteria and therefore forms its own taxonomic family, the Chloroflexaceae (Bauld & Brock, 1973; Madigan et al., 1974; Gloe & Risch, 1978; Holo & Sirevåg, 1986; Pierson & Castenholz, 1991). Most of the phototrophic bacteria are Gram-negative, but recently it has been clearly demonstrated that Chloroflexus lacks not only a characteristic cell wall lipopolysaccharide but also a (lipo)protein in the rigid layer and contains L-ornithine as the only diamino acid constituent of the peptidoglycan, properties normally known for Gram-positive cells only (Jürgens et al., 1987; Meissner et al., 1988). These findings for the cell wall composition of C. aurantiacus are exceptional among phototrophic bacteria and underline the isolated genealogical position of Chloroflexus (Fox et al., 1980; Gibson et al., 1985). This is additionally confirmed by 16S ribosomal RNA cataloguing data. The low $S_{AB}$ value ($<0.2$) obtained suggests a very early ($\approx 3.8 \times 10^9$ years) separation of Chloroflexus from other bacteria (Fox et al., 1980; Kandler, 1981).

Chloroflexus occurs naturally in bacterial mats of alkaline hot springs, growing at temperatures between 50 °C and 70 °C. Although a temperate member of this family from freshwater lakes has been described (Gorlenko, 1973, Chloroflexus-like bacteria are also reported as members of the prokaryotic communities from heliothermal, hypersaline ponds such as Solar Lake, Sinai (Boon et al., 1981; Schidlowsky et al., 1984). In natural environments, Chloroflexus is often associated with cyanobacteria.

The ability to form laminated mats with internal structures resembling Precambrian stromatolites (Doemel & Brock, 1974) draws attention to the lipid composition of cyanobacteria and of C. aurantiacus. That Chloroflexus can produce long-chain unsaturated hydrocarbons with a maximum at n-C31:3 and long-chain saturated and unsaturated wax esters in the carbon range C24 to C35 (maximum C34) is well known from studies of cell cultures (Knudsen et al., 1982; Shiea et al., 1991; Hefter, 1992). These compounds were also found in recent sediment samples containing Chloroflexus (Boudou et al., 1986; Dobson et al., 1988; Zeng et al., 1992a, b). Halfen et al. (1972) showed that the carotenoid...
composition of *Chloroflexus* (β-carotene, γ-carotene, OH-γ-carotene-glucoside) is distinct from that of other prokaryotic organisms.

Here we report for the first time the isolation and identification of (−)-verrucosan-2β-ol (Fig. 1) from *C. aurantiacus*. This type of diterpene has been described previously only in liverworts (Hepaticae), a special group of eukaryotic organisms representing an early stage in the evolution of terrestrial plants (Matsuo et al., 1984).

**Methods**

**Organism.** *C. aurantiacus* strain Ok70-fl (DSM 636) was grown photoorganoheterotrophically at 55 °C and 2000 lux in 1 litre screw-cap bottles. The medium (modified after Kaulen & Klemme, 1983) contained, in 1 litre:

- 0.1 g Na₂HPO₄·2H₂O
- 0.1 g MgSO₄·7H₂O
- 0.05 g CaCl₂·2H₂O
- 0.002 g FeSO₄·7H₂O
- 0.4 g glycyglycine and 2 g yeast extract. The pH was adjusted to pH 8.2. Cells were harvested at the stationary growth phase and washed twice with 0.9% NaCl before storage at −20 °C until use.

**Extraction and purification methods.** Freeze-dried cells (10 g) were extracted ultrasonically for 15 min in a mixture of dichloromethane/methanol (1:1, v/v). Solvents were removed by centrifugation (20 min, 2000 r.p.m.) from the remaining cells. Extraction was repeated three times until the solvents were nearly colourless. The combined extracts were evaporated to dryness and separated by column chromatography (2.5 x 50 cm; Merck silica gel, 100 mesh). Sequential elution with hexane, dichloromethane and a mixture of methanol/dichloromethane/water (6:3:1, by vol.) gave three fractions. The first contained the known long-chain unsaturated hydrocarbons and small amounts of a verrucosene, the second fraction consisted of the known mixture of wax esters and additionally the verrucosan-2β-ol, whereas the third fraction was made up mainly of polar lipids. The verrucosene was purified by thin-layer chromatography with impregnated silica gel (Merck) plates (5% AgNO₃) using n-hexane as developer. The verrucosan-2β-ol was separated from the wax esters by thin-layer chromatography (Merck silica gel 60) using a mixture of hexane/dichloromethane (6:4, v/v) as developer. The band containing the verrucosan-2β-ol (Rₛ = 0.27) was scraped off and eluted by dichloromethane from the silica gel for further investigation.

As a control, an extract of the culture medium, prepared using the same methods as for bacteria, was analysed as below. None of the lipids observed in *Chloroflexus* was found in this extract.

**Analytical methods.** The content of hopanoid triterpenes in *Chloroflexus* was examined according to the method described by Rohmer et al. (1984).

GC/MS analyses were performed on a Carlo Erba 4160 gas chromatograph equipped with a fused silica capillary column (DB-5, 30 m x 0.25 mm, J&W Scientific), coupled to a Varian CH7A spectrometer. MS conditions: 70 eV ionization energy; source temperature 250 °C; mass range m/z 50–800; resolution 1000. GC temperature programme: 80 °C, 5 min isothermal; 80–300 °C, 3 °C min⁻¹; 300 °C.

**Table 1.** ¹³C- and ¹H-NMR data for verrucosan-2β-ol from *C. aurantiacus*

<table>
<thead>
<tr>
<th>C-atom</th>
<th>δ¹³C</th>
<th>δ¹H</th>
<th>J (Hz)</th>
<th>δ¹H</th>
<th>J (Hz)</th>
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<tr>
<td>2</td>
<td>73.76</td>
<td>3.55</td>
<td>[9-5; 6-5]</td>
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<tr>
<td>3</td>
<td>23.60</td>
<td></td>
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<tr>
<td>4</td>
<td>18.70</td>
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<td>[8-5; 4-5]</td>
<td>0.17(β)</td>
<td>[4-5; 4-5]</td>
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<tr>
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<td>6</td>
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<td>0.857</td>
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<td>0.723</td>
<td>[1-0; 0-5 (J)]</td>
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Fig. 2. Mass spectra of verrucosan-2β-ol (a), its TMS-derivative (b) and the verrucosene (c), with structural assignments and explanation of some characteristic fragment ions.
was treated with 2760 chemical shifts 20 min isothermal; injection mode, on-column; carrier gas, He. For the NMR spectroscopy was performed on a Bruker AM 400 spectrometer operating at observation frequencies of 400 and 100 MHz for $^1H$ and $^{13}C$ nuclei respectively, and data were recorded at 300 K. The chemical shifts (δ) are reported in p.p.m. from TMS using the solvent (CD$_2$Cl$_2$ δH 5.32, δC 53.84) as internal reference. J values result from first-order interpretations and are usually given to the nearest 0.5 Hz. A NOESY experiment was performed with both relaxation delay and mixing times set at 1.5 s. For inverse long-range $^1H$--$^{13}C$ correlation experiment, the J-selection delay was tuned for $\nu_{CH} = 7$ Hz. The optical rotations were measured in CHCl$_3$ solutions at 24 °C on a Perkin-Elmer 241 MC polarimeter.

Results and Discussion

(−)-Verrucosan-2β-ol isolated from *C. aurantiacus* was identified by NMR studies (′H-NMR, $^{13}C$-NMR, $^1H$ broad band decoupled and DEPT spectra, phase-sensitive $^1H$-$^1H$ COSY and NOESY, inverse one-bond and long-range ($^2J_{CH}$) $^1H$--$^{13}C$ correlation experiments) which also allowed complete assignment of proton and carbon chemical shifts (Table I). Data recorded are in full agreement with those reported in the literature for (−)-verrucosan-2β-ol and related compounds (Eguchi et al., 1982; Kubo et al., 1984; Matsuo et al., 1984; Fukuyama et al., 1988). The absolute configuration of the isolated product and the (−)-verrucosan-2β-ol in liverworts was found to be identical. The measurements of optical rotation gave an [α]$_D$ value of −56° for the compound present in *C. aurantiacus* Matsuo et al. (1984) obtained an [α]$_D$ value of −58° for the liverwort compound.

The mass spectral fragmentation pattern of the verrucosan-2β-ol, its TMS-derivative and of the verrucosane are presented in Fig. 2. Due to the similarities of the mass spectrometric fragmentation patterns of the verrucosene and the verrucosanol, a similar carbon framework of both compounds can be assumed. The molecular ion (m/z 272) of the verrucosene leads to the elemental composition C$_{26}$H$_{32}$, which is in accordance with a verrucosene possessing one double bond. In comparison with the verrucosan-2β-ol, a shift of two mass units in the mass spectrum of the verrucosene can be observed (e.g. 191 → 189, 177 → 175, 149 → 147, 123 → 121), which supports the presence of one double bond in a similar carbon framework. Detailed localization of the double bond in the verrucosene is not possible from the mass spectral analysis alone, because a double bond migration from the isopropyl side-chain into the ring system primary to the fragmentation can occur, analogous to results found for diploptene (Bird et al., 1971).

Therefore, NMR structural elucidation of this compound is in progress. This is, to our knowledge, the first report of verrucosane-type diterpenoids in a prokaryotic organism. The content of verrucosan-2β-ol in *C. aurantiacus* was 1.76 mg per g dry weight of cell material. This is the same order of magnitude as the content of hopanoid-type triterpenes [0-1-2 mg (g dry wt)$^{-1}$; Rohmer et al., 1984] usually occurring in different prokaryotes. The similarity might indicate that the verrucosanol in *Chloroflexus* is a membrane constituent, comparable to hopanoids in other bacteria. Using the method of Rohmer et al. (1984), positive proof for the presence of hopanoid triterpenes in *C. aurantiacus* was not found, nor could steroids be detected during our analyses.

The chemical structure of the verrucosan-2β-ol points to a completely different pathway of lipid biosynthesis, compared to the synthesis of hopanoids in prokaryotes or steroids in eukaryotes (Ourisson et al., 1979). Therefore, verrucosan-2β-ol might be of chemotaxonomic importance reflecting the special phylogenetic position of the *Chloroflexaceae*. However, the absence of hopanoids could reflect an earlier stage of biochemical evolution in comparison to the hopanoid-containing prokaryotes. Thus, the discovery of verrucosan-2β-ol in this organism extends the significance of verrucosane-type molecules as biomarkers for early prokaryotic life forms and chemotaxonomic relationships. Although the biological function of the verrucosene is not clear, it could well represent a biosynthetic intermediate or precursor of the oxygen-functionalized verrucosan-2β-ol.

No reports of such verrucosenes are known from the liverworts or other organisms.

To our knowledge, no reports of verrucosane-type compounds exist from recent or ancient sediments, not even from environments which have been described to contain cells of *Chloroflexus*. Recently, Zeng et al. (1992b) reported an unidentified compound with a comparable retention time as the verrucosanol in an apolar lipid fraction from Octopus Spring cyanobacterial mats containing *Chloroflexus*, but unfortunately, they did not determine the chemical structure of this compound. Further investigations on the biomarker potential of verrucosanes for *Chloroflexaceae* in recent and ancient sediments are in progress.

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References


Verrucosan-2β-ol from Chlorofexus aurantiacus


