Biosynthesis of the Diterpene Verrucosan-2β-ol in the Phototrophic Eubacterium Chloroflexus aurantiacus

A RETROBIOSYNTHETIC NMR STUDY*

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The biosynthesis of verrucosan-2β-ol in the green phototrophic eubacterium Chloroflexus aurantiacus was investigated by in vivo incorporation of singly or doubly 13C-labeled acetate. The 13C labeling of the isolated diterpene was analyzed by one- and two-dimensional NMR spectroscopy. The 13C-labeling patterns of verrucosan-2β-ol were compared with the labeling patterns of intermediary metabolites (acetyl-CoA, pyruvate, and glyceraldehyde 3-phosphate) which were deduced from amino acids and nucleosides by retrobiosynthetic analysis. The results show that verrucosan-2β-ol is synthesized via mevalonate and not via the deoxyxylulose pathway, which was discovered recently in some eubacteria, algae, and plants. A scheme for the formation of the unusual tetracyclic ring system is offered. The cyclization process is initiated by the solvolysis of pyrophosphate from geranyllinaloyl pyrophosphate and the mechanism involves a Wagner-Meerwein rearrangement, a 1,3-hydrate shift, and a cyclopropylcarbinyl to cyclopropylcarbinyl rearrangement.

Chloroflexus aurantiacus is considered as the most basal branch of a photosynthetic organism in eubacterial phylogeny which has been described hitherto (1–3). The bacterium shows unusual characteristics. The fixation of CO2 occurs via a novel cyclic pathway discovered recently (4, 5). The primary step of this pathway is the carboxylation of acetyl-CoA. The resulting malonyl-CoA yields hydroxypropionate, which is converted to propionyl-CoA by a sequence of dehydration and reduction reactions. A further carboxylation reaction of propionyl-CoA leads to dicarboxylic acids, which may be metabolized further to acetyl-CoA and glyoxylate (5).

The cell wall and membrane composition of C. aurantiacus is exceptional among procaryotic organisms. Membrane phospholipids are characterized by the predominance of saturated and unsaturated C18 and C20 fatty acids besides unusual fatty acid residues with a chain length of 17 (6). Monogalactosyldiglyceride, sulfoquinovosyldiglyceride, and most notably, phostidylinositol and diglycosyldiglycerides are found beside phosphatidyglycerol. The glycolipids contain equimolar amounts of glucose and galactose (6, 7). Moreover, the lack of lipopolysaccharides is characteristic for the unusual cell membrane composition (8). L-Ornithine is the only amino acid of the peptidoglycan. Instead of protein, a complex polysaccharide is bound to the peptidoglycan (9). The carotinoid composition is also different from that of other procaryotes (10).

The diterpene verrucosan-2β-ol (compound 1, Fig. 1) has been isolated recently by Hefter et al. (11) from the membrane fraction of C. aurantiacus in yields of 1–2 mg from a 1-g cell mass (dry weight). Previously, this rare diterpene with an unusual 3,6,6,5-tetracyclic ring system had been described only in marine sponges (12) and some liverworts (hepaticae) (13–21), which are considered as early members of the evolution of terrestrial plants. Hapnoids and steroids have not been found in the membrane of C. aurantiacus. Despite its different molecular dimensions, it has been proposed that verrucosan-2β-ol can act as a modulator of membrane fluidity in analogy to hopanoids and steroids in other microorganisms (11).

Current evidence suggests that isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) act as the universal precursors of terpenoid biosynthesis. The two precursors can be interconverted by isopentenyl pyrophosphate isomerase. The biosynthesis of cholesterol from acetate via mevalonate has been studied in detail by Bloch, Lynen, and their co-workers (22). More recently, a second pathway via 1-deoxy-D-xylulose or its 5-phosphate has been discovered in plants and in certain eubacteria by independent work of the groups of Arigoni and Rohmer. More specifically, Arigoni and co-workers reported that 1-deoxyxylulose can serve as precursor for quinoid coenzymes in Escherichia coli and for ginkgolides in Ginkgo biloba. On the other hand, these authors found that sterols are formed in Ginkgo via mevalonate (23–25). Sahm, Rohmer, and their co-workers found that the biosynthesis of hopanoids in the eubacterium Zymomonas mobilis and ubiquinone Q8 in Escherichia coli proceed via the deoxyxylulose pathway (26, 27). Furthermore, the deoxyxylulose pathway has been shown to be operative in the alga Scenedesmus obliquus (28), in cell cultures of the plants Taxus chinensis (29), Catharanthus roseus (30), Rauwolfia serpentina, and Rubia tinctorum (31), and in the plants Lenna gibba, Hordeum vulgare, Daucus carota (32), and Mentha piperita (33).

The pathway of terpenoid biosynthesis in C. aurantiacus is

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¶ The abbreviations used are: IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; NOE, nuclear Overhauser effect.
unknown. Moreover, the biosynthesis of verrucosan type compounds has not been studied in any species to the best of our knowledge. In order to study the biosynthesis of verrucosan-2β-ol, we applied singly and doubly 13C-labeled acetates to growing cultures of C. aurantiacus. The 13C enrichments and the 13C-13C coupling patterns were determined by one- and two-dimensional 13C and 1H NMR spectroscopy. The labeling pattern of cellular amino acids and nucleosides served as a basis for the reconstruction of central metabolic pools by retrobiosynthetic analysis (5). These data clearly show that IPP and DMAPP are formed by the mevalonate pathway in the basis for the reconstruction of central metabolic pools by retrobiosynthetic analysis (5). The isolation of amino acids and nucleosides has been described earlier (5, 36).

**Experimental Procedures**

**Chemicals**—[1-13C]Acetate and [2-13C]acetate were purchased from MSD-Isotopes (IC-Chemikalien, München, Germany) and [1,2-13C2]acetate from Isotec (Miamisburg, OH). Gases were purchased from Linde AG (Hollriegelskreuth, Germany).

**Microorganism—** C. aurantiacus (DSM 636) was obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). Growth conditions and culture media have been described earlier (34).

**Isotope Incorporation Studies—** C. aurantiacus was grown phototrophically under anaerobic conditions as described earlier (34). The feeding experiments with [1,2-13C]acetate or [2-13C]acetate (98%, 13C) have been described (5). Briefly, the sodium salt of the respective 13C-labeled acetate was added as a sterile solution to a final concentration of 10 mM when the culture had reached a value of 30–100 mg of cell protein per liter.

An experiment with [1,2,13C3]acetate was performed with continuous feeding. A sterile solution (32 ml) containing 3.44 mM of sodium [1, 2, 13C3]acetate, 18.6 mM of unlabeled sodium acetate, and 2.4 µCi of [1,2,13C4]acetate was added to the growing culture (2 liters) during a period of 100 h using a peristaltic pump (Braun, Melsungen, Germany). Aliquots were retrieved at intervals, and cell protein was determined by a modified Lowry method (35). The feeding rate was adjusted proportional to the growth rate. The radioactivity of the supernatant was measured by liquid scintillation counting with external standardization. Cells were harvested aerobically when the solution of labeled acetate was consumed.

**Isolation of Verrucosan-2β-ol—**Bacterial cell mass (0.9 g dry weight) from the growth experiments with [1-13C]acetate or [2-13C]acetate was freeze-dried and suspended in a mixture of ethanol/diethyl ether (3:1, v/v, 200 ml). The suspension was boiled under reflux for 90 min. Solids were removed by filtration, and the solvent was evaporated under reduced pressure. The residue was dissolved in 20 ml of a hexane/dichloromethane mixture (1:1, v/v). The solution was placed on a column of Merck Silica Gel 40 (4 × 30 cm). The column was developed with 1000 ml of hexane followed by 1200 ml of dichloromethane and by a mixture (300 ml) of methanol/dichloromethane/water (6:3:1, v/v). Fractions of 30 ml were collected. Verrucosan-2β-ol (1.1 mg) was eluted after 500 ml of dichloromethane and was identified by 1H NMR spectroscopy. Cells grown with [1,2-13C2]acetate (1.4 g dry weight) were suspended in a mixture of methanol/dichloromethane (1:1, v/v, 200 ml). The mixture was ultrasonically treated for 15 min until the supernatant was colorless. Chromatography on silica gel was performed as described above.

The fractions from the experiment with [1,2-13C2]acetate containing verrucosan-2β-ol were subjected to solid phase extraction using a cartridge of C18, reversed phase material (Accu Bond, 5 ml) and methanol as eluent. The eluate was further purified by preparative thin layer chromatography using a Silica Gel 60 TLC plate (Merck) and a mixture of hexane/dichloromethane (6:4, v/v) as solvent. Verrucosan-2β-ol had an Rf value of 0.78. The band containing the diterpene was scraped off. The solid was extracted with 30 ml of dichloromethane for 30 min. Evaporation of dichloromethane under reduced pressure yielded 1.3 mg of verrucosan-2β-ol. The isolation of amino acids and nucleosides has been described earlier (5, 36).

**NMR Spectroscopy**—1H and 13C NMR spectra were recorded at 500 and 125 MHz, respectively, using a Bruker DRX 500 NMR spectrometer. One-dimensional and two-dimensional experiments (COSY, NOESY, INADEQUATE, HMQC, and HMBC) were performed at 8 °C using standard Bruker software (XWINNMR).

**Quantitative Analysis of 13C Enrichment**—Absolute 13C abundance was determined for each carbon atom of verrucosan-2β-ol obtained from the feeding experiments by quantitative NMR spectroscopy (34). Briefly, 1H decoupled 13C NMR spectra of biosynthetic samples and of samples with natural 13C abundance (1.1% 13C) were measured under identical conditions. Relative 13C abundance of individual carbon atoms was then calculated from the integrals of biosynthetic samples by comparison with the natural abundance samples. In order to obtain absolute 13C enrichments, the 13C satellites in the 1H NMR of each metabolite were analyzed yielding absolute 13C-enrichment values for selected carbon atoms. On the basis of these data, the relative 13C abundances were then referenced to absolute values.

**Quantitative Analysis of 13C-13C Coupling**—The fraction of multiply labeled isopropylomers was calculated from 1H-decoupled 13C NMR spectra as the fraction of 1C-13C coupled satellites relative to the integral of the entire 13C signal of the respective carbon atom.

**NMR Spectra Simulation**—13C-Coupling patterns were simulated with NMRSIM software (Bruker).

**NOE Studies**—Build-up rates of NOEs were determined by two-dimensional NOESY experiments using mixing times of 20 ms to 3 s. Interproton distances were calculated by standardization on the distance of the geminal protons at C-6 and C-12 estimated as 1.78 Å. Molecular dynamic simulation of verrucosan-2β-ol was performed using interproton distances from NOESY experiments and a simulated annealing protocol. Energy minimization was performed using the MM+ force field and the software package DISCOVER (Biosym Inc.).

**Results**

A prerequisite for the interpretation of biosynthetic tracer studies by quantitative NMR spectroscopy is the unequivocal assignment of all NMR signals of the target molecule. A 1H and 13C NMR analysis of verrucosan-2β-ol with CD3Cl3 as solvent has been reported by Hefter et al. (11). Our initial NMR analysis using CDCl3 as solvent gave some ambiguities with respect to the published assignments. It was therefore in order to assign all 1H and 13C NMR signals of verrucosan-2β-ol to CD3Cl3 by two-dimensional NMR techniques. The 1H NMR signals were analyzed by two-dimensional COSY and NOESY type experiments (Table I, Fig. 2). The 13C NMR signals were assigned on the basis of DEPT and two-dimensional HMQC and HMBC experiments (Table I). Additional information was gleaned from two-dimensional INADEQUATE experiments using 13C-labeled samples isolated from the growth experiments with 13C-labeled acetate (see below). With the exception of C-3 and C-16 which had to be interchanged, the data were in line with those obtained in CD3Cl3 by Hefter et al. (11).

Interproton distances were calculated from the initial rate of NOE build-up measurements as described under “Experimental Procedures” (Table II). The conformation of verrucosan-2β-ol was analyzed by molecular dynamics simulation. The preferred conformation is shown in Fig. 3. The Rα-methyl group (C-17) is projected between C-14 and C-12 of the five-membered...
TABLE I

| Position | Chemical shift<sup>a</sup> | J<sub>H-H</sub><sup>b</sup> | J<sub>CC</sub><sup>c</sup> | DEPT | NOESY | DQF-COSY | HMBC | INADEQUATE<sup>d</sup> |
|----------|-----------------|-----------------|-----------------|------|------|--------|------|------------------|
| 1        | 46.48 ppm       | 0.74 Hz         | 10.9(14),9.5(2) | CH   | 6β, 4β, 13w, 19w   | 2,14  | 2,6α, 6β, 8α, 13, 14, 19 | 2     |
| 2        | 73.57 ppm       | 3.55 Hz         | 9.5(1)          | CH   | 15,18,19,14, 17w   | 1     | 1,4α, 4β, 14, 18   | 3,1   |
| 3        | 2.33 ppm        | 0.42(α) Hz      | 8.5(5),4(4β)    | CH   | 4β, 5, 18          | 4β   | 2,4α, 4β, 6α, 6β, 18 | 2,18  |
| 4        | 18.46 ppm       | 0.18(β) Hz      | 4(6),5(4α)      | CH   | 4α, 1, 5, 6β       | 4α   | 2,6α, 18          |       |
| 5        | 21.48 ppm       | 0.95 Hz         | ND<sup>f</sup>  | ND   | ND                | ND   | ND               | ND    |
| 6        | 4.50 ppm        | 1.76(α) Hz      | 13.9(6β),9.3(5) | CH   | 6β, 5.8, 19        | 6α, 1β, 1.4β | 5.6α, 1.4β, 1.5β | 6     |
| 7        | 36.96 ppm       | 1.25 Hz         | ND              | C    | 3.4, 9, 32.4       | CH   | 19, 9α, 6β, 11β   | 6β, 1β, 9β, 19 | 6     |
| 8        | 37.55 ppm       | 1.12(α) Hz      | 3.4, 4         | CH   | 8α, 6β, 20         | 8α, 9α, 9β | 11β, 11β, 12β, 20 | 10    |
| 9        | 35.35 ppm       | 1.33(β) Hz      | 1.3           | CH   | 8β, 11β, 8α, 20    | 8α, 8β | 8α, 11β, 11β, 12β, 20 | 10    |
| 10       | 44.27 ppm       | ND              | 5               | C    | ND                | 1.8α, 8β, 9, 11α, 11β, 20 |       |
| 11       | 40.62 ppm       | 0.96(α) Hz      | 2.3, 33.8       | CH   | 14, 12α, 11β       | 9β, 11α, 20 | 11β, 12α, 12β, 20 | 1.2α, 12β, 20 | 9,20  |
| 12       | 22.39 ppm       | 1.42(α) Hz      | 2.9, 33.0       | CH   | 12β, 16, 17, 11α   | 12α, 13, 20, 15, 18 | 11β, 11β, 12α, 19 |       |
| 13       | 43.97 ppm       | 1.98 Hz         | 10.6(14,12),3.9(9,12), 2.6(15) | CH   | 15, 12β, 16, 20, 1w | 12α, 12β, 14 | 9α, 9β, 11β, 13, 15, 20 |       |
| 14       | 47.96 ppm       | 1.22 Hz         | 10.5(1,13)      | CH   | 15, 19, 17, 15, 2, 12β | 11α, 12β, 14 | 12β, 14, 15, 16, 17 |       |
| 15       | 28.52 ppm       | 2.11 Hz         | 6.9(16,17),2.6(13) | CH   | 2,13, 16, 17, 12β, 14 | 12α, 15, 16, 17 | 12β, 14, 15, 16, 17 |       |
| 16 (Si)  | 23.42 ppm       | 0.87 Hz         | 6.8(15)         | CH   | 12α, 13, 15, 12    | 15    | 13, 15, 17        |       |
| 17 (Re)  | 15.08 ppm       | 0.80 Hz         | 6.8(15)         | CH   | 14,12α, 15, 15, 12 | 15    | 13, 15, 16        |       |
| 18       | 25.42 ppm       | 1.17 Hz         | 2.4, 44.7       | CH   | 5, 2, 4α          | 2,4α, 4β, 5 | 3        |       |
| 19       | 19.27 ppm       | 0.83 Hz         | 35.6            | CH   | 14, 8α, 6α, 2.5    | 6β   | 6α, 6β, 8α, 8β, 9 | 7     |
| 20       | 17.83 ppm       | 0.71 Hz         | 36.1            | CH   | 8β, 12β, 13, 9β, 11α | 8β, 9α, 9β, 11β/12α | 10    |       |

<sup>a</sup> Referenced to solvent signals at 7.24 ppm and 77.0 ppm, respectively.
<sup>b</sup> Obtained from 1H NMR spectra; coupling partners in parentheses.
<sup>c</sup> Obtained from 13C NMR spectra of 13C-enriched samples.
<sup>d</sup> Performed with 13C-enriched samples.

"w", weak signal intensity.
<sup>f</sup> ND, not determined due to signal overlapping.

Ring. Both the observed upfield shift of C-17 caused by two γ effects and the coupling constant of 2.6 Hz between H-13 and H-15 are in keeping with the conformation shown in Fig. 3. It should be noted that a similar conformation was found in the crystal structures of 2β,9α-dihydroxyverrucosane and 2β,8β-dihydroxyverrucosane from liverwort species (15, 18).

Growth experiments with [1-13C]acetate and [2-13C]acetate have been reported earlier (5). Briefly, 820 mg (10 mmol) of 13C-labeled acetate and a tracer amount of 14C-labeled acetate (100 kBq) were added as a bolus to a bacterial culture (1 liter) growing phototrophically on a mixture of H2/CO2 (80:20, v/v). The cell mass was extracted with ethanol/ether, and verrucosan-2β-ol was isolated from the supernatant as described under “Experimental Procedures.”

From the solvent-extracted biomass, amino acids and ribonucleosides were obtained after hydrolysis of protein and RNA, and their 13C-labeling patterns were determined by NMR spectroscopy. These data were used to reconstruct the labeling patterns of intermediary metabolites such as acetyl-CoA (compound 3, Fig. 1) and pyruvate (compound 4, Fig. 1). Results are shown in Fig. 4 and Table III. Some of these data have been reported earlier (5). The labeling pattern of glyceraldehyde 3-phosphate (compound 5, Fig. 1) was deduced from C-3 to C-5 of ribose as determined from isolated nucleosides. We have shown repeatedly that C-3 to C-5 of the pentose pool reflect the labeling pattern of C-1 to C-3 of the triose phosphate pool in a variety of organisms (5, 34, 36). The reconstructed 13C enrichments for each carbon atom are shown as bold numbers (percent 13C abundance) in Fig. 4. The quantitative contributions of satellite signals originating from coupling via a single bond to an adjacent 13C atom are indicated by italic numbers and arrows. The arrows point to the observed carbon atoms, and the numbers indicate the fraction of the coupled species referenced to the total signal integral of the observed carbon.

It should be noted that coupling cannot be predicted from enrichment in this study. For example, in pyruvate derived from [1-13C]acetate, the coupling contributions are low despite the enrichment values of 19–28% of the carbon atoms. This reflects the fact that the biosynthetic material is an isotopomer mixture of [1-13C]-, [2-13C]-, and [3-13C]pyruvate. The formation of the single-labeled isotopomers by the carbon fixation cycle (as opposed to a stochastic distribution of 13C atoms which would be conducive to the formation of a substan-
tial fraction of double-labeled isotopomers) has been discussed
earlier (5).

The bolus addition of [1-13C]- and [2-13C]acetate shifted the
growth pattern of the culture to a heterotrophic mode with
efficient acetate utilization (5). The resulting high 13C enrich-
ment of central intermediary metabolites such as acetyl CoA and
pyruvate was favorable for the analysis of the terpene
cyclization (for details see below). On the other hand, flooding
of the acetyl-CoA pool with [1,2-13C2]acetate would have been
conducive to the formation of totally labeled verrucosan-2β-ol
devoid of meaningful biosynthetic information. A growth exper-
iment was therefore performed with slow, continuous addition
of [1,2-13C2]acetate to the growing culture over a period of
100 h. Experimental details are described under “Experimental
Procedures.” Under these conditions, the proffered acetate is
diluted by material obtained from phototrophic CO2 fixation.

Absolute 13C abundance data for verrucosan-2β-ol from
growth experiments with 13C-labeled acetates were determined
by 1H and 13C NMR spectroscopy as described under “Experi-
mental Procedures.” Parts of NMR spectra are shown in Fig. 5.
13C enrichments are summarized in Table IV and Fig. 6.

The labeling patterns of verrucosan-2β-ol samples obtained
from growth with [1-13C]- and [2-13C]acetate followed a recip-
crual pattern (Fig. 6). Eight of the 20 carbon atoms were pre-
dominantly labeled from the carboxylic group of acetate, and
the other 12 carbon atoms were predominantly labeled from
the methyl group of acetate. A statistical analysis is shown in
Table III. The 12 atoms which become highly enriched from
[2-13C]acetate have an average enrichment of 62 ± 4%, and the
8 unlabeled atoms in this sample have an average enrichment
of 3 ± 0.1%.

The expected diversion of precursor atoms to isoprenoid
monomers via the mevalonate pathway and via the deoxyxylu-
lose pathway is summarized in Fig. 7. The mevalonate pathway
implicates the contribution of 3 methyl carbon atoms and 2
carboxyl carbon atoms from acetate. The deoxyxylulose path-
way implicates the incorporation of all 3 carbon atoms of the
putative triose phosphate precursor and both carbon atoms of
activated acetaldehyde, which is derived from pyruvate by de-carboxylation (23, 24, 27). This pathway involves a skeletal rearrangement of 1-deoxyxylulose 5-phosphate conducive to disruption the contiguity of the 3 atoms derived from the triose precursor.

The reciprocal labeling patterns of verrucosan-2β-ol described above are in line with the mevalonate pathway which predicts the diversion of label from the methyl group to 12 carbon atoms of verrucosan-2β-ol and from the carboxyl group of acetate to the other 8 carbon atoms. A detailed analysis which confirms this initial working hypothesis is presented in the Discussion.

In all incorporation experiments, some of the [13C] signals of verrucosan-2β-ol show intense 13C-13C coupling satellites indicating the presence of two or more 13C atoms in adjacent positions (Fig. 5). The location of the 13C atom pairs was obtained from two-dimensional INADEQUATE spectra (Fig. 8). Groups of adjacent 13C atoms are marked in Fig. 6, A-C, by bold lines connecting the respective carbon atoms.

The occurrence of adjacent pairs of 13C atoms is not surprising in the experiment with double-labeled [1,2-13C2]acetate, since the mevalonate pathway is conducive to the incorporation of intact acetate units in each isoprenoid monomer. Eight pairs of 13C atoms would thus be expected for a diterpene, of which seven were observed experimentally (Figs. 6C and 8).

However, adjacent pairs of 13C atoms were also observed in the experiments with single-labeled acetates. Three contiguous pairs of 13C atoms (>60% satellite intensity relative to overall 13C signal intensity) are present in the biosynthetic verrucosan-2β-ol from [1-13C]acetate (Fig. 6A). One pair and a triplet of 13C enrichment of verrucosan-2β-ol and central metabolites from C. aurantiacus suplemented with [13C]labeled acetates.

| Metabolite        | Position | [1-13C]Acetate 13C | [2-13C]Acetate 13C | [1,2-13C]Acetate 13C |
|-------------------|----------|--------------------|--------------------|---------------------|
| Verrucosan-2β-ol  | 2/3/6/7/9/10/12/15 | 37.1 ± 2.3 (6)a   | 3.0 ± 0.1 (6)      | 12.1 ± 1.9 (8)      |
|                   | 14       | 8.1 ± 2.7 (12)     | 62.4 ± 4.1 (12)    | 17.1 ± 1.4 (12)     |
| Acetyl-CoA        | 1        | 54.7 ± 6.2 (6)     | 2.9 ± 0.4 (2)      | 8.2 ± 2.3 (6)       |
|                   | 2        | 8.1 ± 0.9 (5)      | 78.7 ± 8.2 (6)     | 9.8 ± 3.9 (6)       |
| Pyruvateβ/phosphoenolpyruvate | 1   | 14.8 ± 5.2 (4)     | 4.9 ± 0.4 (2)      | 3.2 ± 2.0 (4)       |
|                   | 2        | 18.8 ± 3.4 (10)    | 52.4 ± 4.5 (10)    | 10.4 ± 2.1 (10)     |
|                   | 3        | 28.2 ± 2.9 (8)     | 39.5 ± 4.1 (8)     | 9.1 ± 1.6 (8)       |
| Ribose           | 1        | 19.5 ± 3.5 (3)     | 30.4 ± 1.4 (2)     | 9.2 ± 2.1 (2)       |
|                   | 2        | 19.6 ± 3.4 (3)     | 40.0 ± 5.6 (2)     | 13.9 ± 3.0 (2)      |
|                   | 3        | 11.0 ± 3.5 (3)     | 6.9 ± 0.1 (2)      | 10.1 ± 1.8 (2)      |
|                   | 4        | 16.5 ± 3.0 (3)     | 49.1 ± 8.1 (2)     | 3.4 ± 1.1 (2)       |
|                   | 5        | 19.9 ± 5.6 (3)     | 31.6 ± 7.6 (2)     | 10.9 ± 1.8 (2)      |

*Values in parentheses indicate the number of molecular species or atom positions used for statistical analysis.

Reconstructed from labeling patterns of amino acid by retrobiosynthetic analysis. The data for [1-13C]- and [2-13C]acetate are from Eisenreich et al. (5).
13C atoms are present in the compound from the experiment with [2-13C]acetate (Fig. 6B).

**DISCUSSION**

The generation of a tetracyclic ring system from a linear precursor requires the formation of 4 carbon-carbon bonds. If the linear precursor were biosynthesized from acetate via the mevalonate pathway, each carbon atom derived from the methyl group of acetate should be flanked exclusively by carbon atoms derived from the carboxylic group and vice versa (Fig. 9). Thus, each 13C-enriched carbon would be exclusively bonded to nonenriched neighbor atoms in experiments with [1-13C]- and [2-13C]acetate. Only the feeding of [1,2-13C2]acetate would be conducive to a linear precursor with directly adjacent 13C atoms by incorporation of the double-labeled acetate unit. This suggests that the pairs of directly adjacent 13C atoms observed in the terpene biosynthesized from single-labeled acetate (i.e., [1-13C]- and [2-13C]acetate) are generated by the cyclization reaction. More specifically, the three pairs of adjacent 13C atoms in the experiment with [1-13C]acetate signal the formation of 3 carbon-carbon bonds by the cyclization process (Fig. 6A). The formation of three additional bonds by the cyclization is indicated by the 13C-13C coupling data in the experiment with [2-13C]acetate (Fig. 6B). It follows that a minimum of 6 carbon-carbon bonds were formed de novo during the cycliza-

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**FIG. 5.** Part of 13C NMR spectra of verrucosan-2β-ol. 13C-13C-Coupling patterns as identified by INADEQUATE experiments are indicated on top of the spectra. A, natural 13C abundance; B, from [1-13C]acetate; C, from [2-13C]acetate; D, from [1,2-13C2]acetate.

**TABLE IV**

| Carbon position | Growth with [1-13C]Acetate | Growth with [2-13C]Acetate | Growth with [1,2-13C2]Acetate |
|-----------------|---------------------------|---------------------------|-----------------------------|
|                 | 13C Coupling | 13C Coupling | 13C Coupling |
| 1               | 8.5          | 61.4          | 15.6          |
| 2               | 33.7         | 3.1           | 12.0          |
| 3               | 34.4         | 2.9           | 11.1          |
| 4               | 10.4         | 57.8          | 18.0          |
| 5               | 8.7          | 60.3          | 17.0          |
| 6               | 35.8         | 3.1           | 13.8          |
| 7               | 39.1         | 3.1           | 9.4           |
| 8               | 9.1          | 61.1          | 18.3          |
| 9               | 36.9         | 2.8           | 13.9          |
| 10              | 46.8         | 2.8           | 9.3           |
| 11              | 9.2          | 3.1           | 9.3           |
| 12              | 37.0         | 3.1           | 9.3           |
| 13              | 10.3         | 67.9          | 18.5          |
| 14              | 8.4          | 62.8          | 15.3          |
| 15              | 40.2         | 81.3          | 30.7          |
| 16              | 10.9         | 81.8          | 77.3          |
| 17              | 9.9          | 13.4          | 14.4          |
| 18              | 11.2         | 3.1           | 14.4          |
| 19              | 9.6          | 2.9           | 14.4          |
| 20              | 8.9          | 58.6          | 14.6          |

*ND, not determined due to signal overlapping.*
tion of the linear precursor. Since the formation of a tetracyclic system from a linear precursor requires only four additional bonds, it follows further that a minimum of two carbon carbon bonds must have been broken during the cyclization. Direct evidence is available for the breaking of one carbon carbon bond since the number of $^{13}$C-labeled carbon pairs as determined by the experiment with $[1,2-^{13}$C$_2]$acetate is 7, whereas 8 intact acetate units should have been present in the linear C$_{20}$ precursor (Fig. 9). Thus the reaction mechanism must imply the breaking of a second carbon carbon bond, which does not result in the separation of two carbon atoms transferred jointly from a given acetate molecule.

A possible biosynthetic scheme which fulfills all of these criteria is shown in Fig. 10. Solvolysis of the allylic pyrophosphate group of ($S$)-geranyllinaloyl pyrophosphate (compound 8), triggers off a cyclization process leading via the monocyclic intermediate 9 to the bicyclic ionic intermediate 10. The ($S$)-configuration of the precursor can be predicted from the configuration of the chiral center in intermediate 9 and the known anti-stereochemistry of similar $S$$_{N2}$ bond-making processes (37, 38). Next, ion 10 suffers a 1,2-rearrangement leading to intermediate 11, the cationic center of which then attacks the isopropylidene double bond with formation of the tricyclic ion 12. Saturation of the positive charge in this ion is best accommodated by a 1,5-hydride transfer from the C-2 methylene group which generates the homoallylic intermediate 13. Similar shifts have been observed previously in terpene biosynthesis (39, 40). Collapse of intermediate 13 to the cyclopropylcarbinyl ion 14 is followed by a sigmatropic rearrangement to a new cyclopropylcarbinyl ion 15 and the reaction is terminated by addition of a hydroxyl group from the solvent. The interconversion of the two cyclopropylcarbinyl ions 14 and 15, which may but need not require the intermediacy of a cyclobutyl cation, is well precedented in abiotic and biological systems (41).

The mechanism outlined in Fig. 10 complies with the minimum requirements for bond-making and bond-breaking processes specified by the experimental data, namely the formation of six carbon bonds and the disruption of two carbon bonds. In addition, it provides a rationale for the stereochemical course of the events which lead to the formation of the isopropyl side chain of verrucosan-2$\beta$-ol. The trans arrangement of the two hydrogen atoms at C-13 and C-14 in the final product of the
cyclization requires a boat conformation for the chain segment of the aliphatic precursor which is being used for the formation of the pentacyclic ring (Fig. 11). Inspection of models indicates that in the ionic intermediate 12 issued from this cyclization step one of the lobes of the empty p-orbital is ideally positioned for abstracting a hydride ion from the β-position of the C-2 methylene group; as a consequence, the postulated 1,5-hydride shift predicts a correlation between the (Z)-methyl group of the aliphatic precursor (dotted line in Fig. 11) and the Re-methyl group in the isopropyl chain of the final compound. This prediction is born out by the results of the feeding experiment with [1,2-13C2]acetate, which show that within the isopropyl chain of verrucosan-2β-ol it is the Re-methyl group (responsible for signals at 15.08 ppm in the 13C NMR and at 0.80 ppm in the 1H NMR spectra of the compound) that maintains its bond-labeling to the adjacent atom. Therefore, this group must have been generated specifically from the (Z)-methyl group in the isopropylidene unit of the aliphatic precursor, which is known to correspond to the methyl group of mevalonate (42–44).

On the basis of the proposed cyclization mechanism, the four isoprenoid monomers in verrucosan-2β-ol can now be identified unequivocally. The carbon atoms arising from each monomer are boxed in Fig. 6D. Based on this dissection, it is possible to extract unequivocally the labeling patterns of the isoprenoid monomers from the labeling data of verrucosan-2β-ol shown in

**Fig. 9.** Labeling pattern of linear C20 terpene precursors predicted by the mevalonate pathway. Symbols are the same as in Fig. 6. The conformation of geranylinaloyl pyrophosphate 8 was selected to match the structure of the verrucosane diterpene.

**Fig. 10.** Hypothetical mechanism for the formation of verrucosan-2β-ol in *C. aurantiacus.*

**Fig. 11.** Stereochemical course of the formation of the isopropyl group.

Fig. 6, A–C, and in Table IV. Moreover, the 13C enrichments and also the relative contribution of the coupling satellites can be averaged over all four monomers. The averaged data and their standard deviations are summarized in Table V. The essential features are also shown graphically in the central column of Fig. 12.
The hypothetical labeling patterns of isoprenoids via the mevalonate pathway and via the deoxyxylulose pathway can be predicted on basis of the experimentally determined labeling patterns of acetate, pyruvate, and glyceraldehyde 3-phosphate. The labeling patterns predicted for the mevalonate pathway on basis of the acetyl-CoA pattern are shown in the left column of Fig. 12. The labeling patterns predicted for the deoxyxylulose pathway on basis of the pyruvate and glyceraldehyde 3-phosphate-labeling patterns are shown in the right column, and the experimental data are shown in the center.

The labeling patterns predicted by the mevalonate pathway agree well with the experimental observation. On the other hand, the patterns predicted by the deoxyxylulose pathway do not agree with the experimental data. Specifically, the deoxyxylulose pathway predicts high enrichment of C-3 of IPP formed from [2-13C]acetate in contrast to the virtual absence (3%) observed experimentally. It also predicts extensive 13C-13C coupling in the experiment with [2-13C]acetate which is not observed.

On basis of the data in Fig. 12, the isotope distribution
predicted by the two different pathways can be projected to the entire verrucosan-2β-ol molecule. The labeling predictions for both pathways and the experimental data for the incorporation of [2-13C]acetate are shown in Fig. 13. The agreement between the labeling of intracellular acetyl-CoA is very close. It should be noted that not all observed couplings can be unequivocally and quantitatively assigned to individual pairs of carbon atoms because the coupling constants cluster in a narrow range, and it is not always possible to determine the contributions of individual pairs in the complex isotopomer mixture. Only couplings which can be assigned unequivocally are shown for the structure in the center of Fig. 13.

Using the data of Fig. 13, the 13C NMR signals of verrucosan-2β-ol were simulated. The data (not shown) demonstrate again that only the mevalonate prediction agrees with the experimental data.

Knowledge on the distribution of the two isoprenoid pathways in different species is still limited. The eubacteria E. coli and Z. mobilis have been shown to use the deoxyxylulose pathway (26, 27), but the primitive eubacterium C. aurantiacus uses the mevalonate pathway which is also characteristic of animal metabolism. The deoxyxylulose pathway has been shown to be involved in the biosynthesis of several plant metabolites such as gingkoles in Ginkgo biloba (23), taxoids in the yew (29), loganin in Rauwolfia serpentina (33), and monoterpenoids in G. biloba, Arigoni and his co-workers showed that steroids are formed in the cytoplasm via the mevalonate pathway and gingkoles in plastids via the deoxyxylulose pathway. The presence of different pathways in the cytoplasm and in organelles could be related to the endosymbiotic origin of organelles, but the distribution of the two pathways in the microbial kingdom is as yet insufficiently known to serve as a theoretical basis for evolution arguments. Horizontal gene transfer between distant species may also have to be considered.

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