*Proc. Natl. Acad. Sci. USA*
Vol. 93, pp. 6431–6436, June 1996
Biochemistry

# Studies on the biosynthesis of taxol: The taxane carbon skeleton is not of mevalonoid origin

(NMR spectroscopy/*Taxus chinensis*/plant cell culture/terpene)

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*Contributed by Meinhart H. Zenk, March 4, 1996*

**ABSTRACT** **A cell culture of *Taxus chinensis* was established to produce the diterpene 2α,5α,10β,14β-tetra-acetoxy-4(20),11-taxadiene (taxuyunnanine C) in 2.6% (dry weight) yield. The incorporation of [U-$^{13}C_6$]glucose, [1-$^{13}C$]glucose, and [1,2-$^{13}C_2$]acetate into this diterpene was analyzed by NMR spectroscopy. Label from [1,2-$^{13}C_2$]acetate was diverted to the four acetyl groups of taxuyunnanine C, but not to the taxane ring system. Label from [1-$^{13}C$]glucose and [U-$^{13}C_6$]glucose was efficiently incorporated into both the taxane ring system and the acetyl groups. The four isoprenoid moieties of the diterpene showed identical labeling patterns. The analysis of long-range $^{13}C$ $^{13}C$ couplings in taxuyunnanine C obtained from an experiment with [U-$^{13}C_6$]glucose documents the involvement of an intramolecular rearrangement in the biosynthesis of the isoprenoid precursor. The labeling patterns are inconsistent with the mevalonate pathway. The taxoid data share important features with the alternative pathway of isoprenoid biosynthesis operating in certain eubacteria [Rohmer, M., Knani, M., Simonin, P., Sutter, B. & Sahm, H. (1993) *Biochem. J.* 295, 517–524].**

Isoprenoids are the largest class of secondary compounds found in higher plants and have been studied intensely, but the biosynthesis of the isoprenoid precursor of terpenes is still not clear, despite studies extending over several decades (1, 2). While it is generally agreed that mevalonate can serve as precursor for phytosterols and carotenoids in higher plants (3, 4), mevalonate and acetate are poorly incorporated into the plethora of mono- and diterpenes (1). A variety of hypotheses have been proposed in an attempt to reconcile these findings with the mevalonate hypothesis of terpene biosynthesis. Thus, it has been stated that acetate and mevalonate are poorly translocated in plants and may not reach the intracellular sites of terpenoid synthesis, that the precursors undergo rapid turnover, that they perturbate the normally occurring pathways if fed in necessarily unphysiological concentrations, or that they are degraded to potential enzyme inhibitors. It was also proposed that the exogenous precursors may not equilibrate with enzyme-bound biosynthetic intermediates (1). Recently, however, it was discovered by Sahm, Rohmer, and their coworkers (5, 6) that the classical mevalonate pathway for the formation of isoprenoids does not exist in a number of bacteria and in thylakoids of a cyanobacterium. These authors proposed a novel pathway involving the condensation of a triose phosphate with activated acetaldehyde and a subsequent skeletal rearrangement of the condensation product (6). Tentative evidence for the occurrence of this pathway in higher plants has been reported.§ Thus, it was proposed (quoted in ref. 2, page 196) that embryos of *Ginkgo biloba* synthesize β-sitosterol according to the classical mevalonate pathway, whereas the diterpenoid ginkgolides are formed by condensation of a triose phosphate with a 2-carbon unit arising by decarboxylation of pyruvate (6). It was also proposed that the two different pathways may be separated by compartmentalization.

This paper reports studies on the biosynthesis of the taxane carbon skeleton in higher plants. Taxoid is the generic name of of a large class of diterpenes like taxin (7, 8) which was first isolated from the European yew tree *Taxus baccata*. Two members of this class of compounds, taxol (9) and its semisynthetic analog, Taxotere (10), are used successfully for chemotherapy of solid tumors, especially in cases of resistence against classical cytostatic agents.

It has been claimed that the taxane ring system is formed in *Taxus canadensis* from acetate and mevalonate (11). However, the reported incorporation rates of both radioactive precursors were low (in the range of 0.02–0.12%), and neither the labeled baccatin III nor taxol formed were subjected to chemical degradation.

Impressive progress has been made by the demonstration of taxa-4(5),11(12)-diene as the first committed intermediate in taxol biosynthesis (12) and by the purification and characterization of taxadiene synthase catalyzing the cyclization reaction conducive to the taxane system from the universal diterpene precursor, geranyl–geranyl pyrophosphate, in a single enzymatic step (13).

This paper describes studies with suspension cultures of *T. chinensis* cells on the formation of the terpenoid building blocks from early precursors of the diterpenes. This system has several advantages. Thus, strains producing up to 4% (dry weight) total taxoids have been selected, and the taxoid compounds of this species have been studied in some detail (14). The major diterpene formed by our strain is 2α,5α,10β,14β-tetra-acetoxy-4(20),11-taxadiene (taxuyunnanine C) (15) (Fig. 1*A*), which has the advantage for biosynthetic studies to contain four acetyl residues that can serve as internal reference standards. The biological system is available independent of seasons and lacks the photosynthetic apparatus. Moreover, the cells can grow on glucose as sole carbon source. The data show conclusively that the mevalonate pathway is not involved in the biosynthesis of the taxoid.

## EXPERIMENTAL PROCEDURES

**Plant Cell Cultures.** Suspension cultures of *T. chinensis* were obtained from the culture collection from the Institut für Pharmazeutische Biologie. [U-$^{13}C_6$]Glucose, [1-$^{13}C$]glucose,

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Abbreviations: IPP, isopentenyl pyrophosphate; DMAP, dimethylallyl pyrophosphate; taxuyunnanine C, 2α,5α,10β,14β-tetra-acetoxy-4(20),11-taxadiene.

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§Cartayrade, A., Schwarz, M., Jaun, B. & Arigoni, D., Second Symposium of The European Network on Plant Terpenoids, Strasbourg/Bischenberg, France, Jan. 23–27, 1994, abstr. PI.

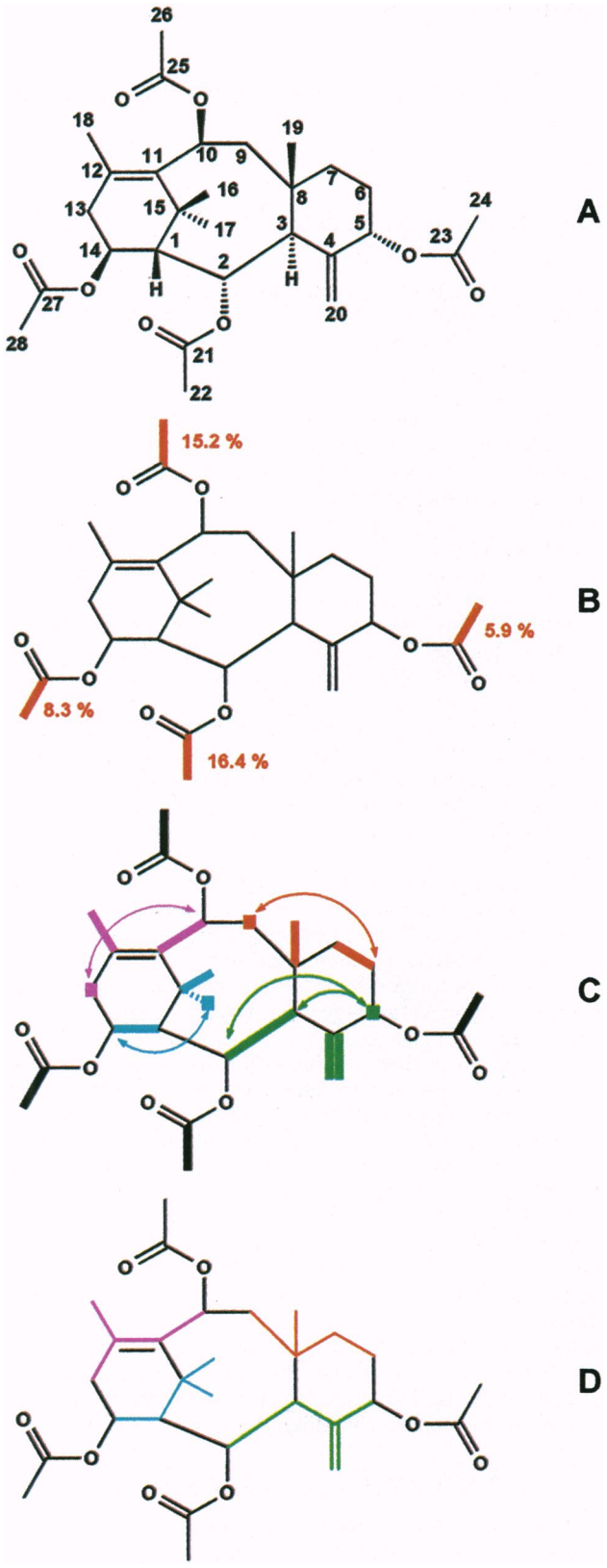


FIG. 1. $^{13}C$ labeling patterns of taxuyunnanine C. (*A*) Structure and carbon numbering of taxuyunnanine C. (*B*) $^{13}C$ labeling pattern of taxuyunnanine C obtained from [1,2-$^{13}C_2$]acetate; contiguous $^{13}C$ pairs are indicated by red lines; the fraction of $^{13}C$ $^{13}C$ coupled isotopomers is indicated. (*C*) Labeling pattern of taxuyunnanine C obtained from [U-$^{13}C$]glucose; pairs of adjacent $^{13}C$ atoms are shown by bold, colored lines; multiple bond $^{13}C$ couplings are shown by arrows. (*D*) Isoprenoid units of the diterpene gleaned from the labeling experiments.

and [1,2-$^{13}C_2$]acetate (99% $^{13}C$ abundance) were purchased from Isotec (Miamisburg OH).

Cells were subjected to cellular cloning (16), and subclones with stable and high production of taxuyunnanine C were selected. Tracer experiments were performed with modified B5 medium containing glucose (30 g/liter) instead of saccharose. Cells (about 3 g wet weight) were suspended in 160 ml of medium in a 500 ml Erlenmeyer flask, and the suspension was shaken at 150 rpm on a gyratory shaker at 24°C. [1,2-$^{13}C$]Acetate was added to the culture medium at a concentration of 1 g/liter. After an incubation period of 14 days, 30 g of glucose and 1 g of [1,2-$^{13}C_2$]acetate were added per liter of culture medium, and incubation was continued for another 30 days. [U-$^{13}C_6$]Glucose (99% $^{13}C$ abundance) was mixed with unlabeled glucose at a ratio of 1:20 (wt/wt). This mixture with an average $^{13}C$ abundance of about 6% (labeled glucose plus natural abundance contribution of the unlabeled glucose) was added to the culture medium at the standard concentration of 30 g/liter. After an incubation period of 14 days, 30 g of the glucose mixture were added per liter of culture, and incubation was continued for another 30 days. [1-$^{13}C$]Glucose (99% abundance) was mixed with unlabeled glucose at a ratio of 1:1 (wt/wt). An incorporation experiment was performed as described above.

**Isolation of Taxoids.** Cells were separated from the medium. About 15 g of wet cell mass were macerated in 150 ml of methanol. The extract was evaporated, and the residue was extracted with ether (500 ml). The aqueous growth medium was also extracted with ether (500 ml). Both organic solutions were combined and were evaporated to dryness. The residue was dissolved in 0.5 ml of methanol, and the solution was applied to a column of silica gel (1.3 × 7 cm, 220–240 mesh). The column was developed with a mixture of hexane/ethylacetate [2:8 (vol/vol), 20 ml]. Colored impurities were retained on the column. The effluent was evaporated to dryness under reduced pressure. The residue (150 mg) was taken up in 0.4 ml of methanol. Aliquots of the methanol solution were subjected to semipreparative HPLC using a column of Lichrosorb (7 μm, Hibar; Merck, 25 × 250 mm). The column was developed with a gradient of acetonitrile. The effluent was monitored photometrically at 227 nm. Taxuyunnanine C was eluted with a retention volume of 500 ml. The yield of taxuyunnanine C was about 68 mg/liter of culture volume.

**NMR Measurements.** $^1H$ and $^{13}C$ NMR spectra of taxuyunnanine C were recorded in $CDCl_3$ by using a Bruker DRX 500 spectrometer equipped with an ASPECT station. $^{13}C$ NMR spectra were measured as follows: 45° pulse (3 μs); repetition time, 3.2 s; spectral width, 29 kHz; data set, 64 kilo-words; temperature, 27°C; zero-filling to 128 kilo-words prior to Fourier transformation, 0.7 Hz line broadening, $^1H$ decoupling by WALTZ 16 during acquisition and relaxation. Two-dimensional experiments and data processing routines were performed according to standard Bruker software (XWINNMR 1.1). All $^{13}C$ NMR signals of taxuyunnanine C were assigned unequivocally on basis of two-dimensional $^1H$ $^{13}C$ correlation experiments in conjunction with $^1H$ homocorrelation experiments. Additional confirmation for $^{13}C$ signal assignments was obtained by two-dimensional INADEQUATE experiments with taxuyunnanine C obtained from an incorporation experiment with [U-$^{13}C_6$]glucose as carbon source. $^{13}C$ abundance in taxuyunnanine C was analyzed by quantitative NMR spectroscopy. $^1H$-decoupled $^{13}C$ NMR spectra of samples from incorporation experiments and of samples with natural $^{13}C$ abundance (1.1% $^{13}C$) were recorded under identical conditions. Relative $^{13}C$ abundance of individual carbon atoms was then calculated by comparison of $^{13}C$ signal integrals between $^{13}C$-labeled and unlabeled taxuyunnanine C. To determine absolute $^{13}C$ abundance, we analyzed $^{13}C$-coupled satellite signals in $^1H$ NMR spectra for H-10 at 6.01 ppm, which is well

separated from other signals. The absolute $^{13}C$ enrichment of C-10 was then used to standardize the relative $^{13}C$ abundance of all carbon atoms. The fraction of multiply labeled isotopomers (% $^{13}C$ $^{13}C$ in Table 1) was calculated from $^{1}H$-decoupled $^{13}C$ NMR spectra as the fraction of $^{13}C$ $^{13}C$-coupled satellites as compared with the integral of the entire $^{13}C$ signal of the respective carbon atom.

## RESULTS

The cell culture of *T. chinensis* used in this study produced a variety of taxoid derivatives. The dominant component was taxuyunnanine C, which could be isolated by HPLC with a yield of about 2.2 mg per g of wet cell mass.

An incorporation experiment with [1,2-$^{13}C_2$]acetate and quantitative NMR analysis of the isolated taxuyunnanine C were performed as above. A part of the resulting $^{13}C$ NMR spectrum is shown in Fig. 2*B*. The overall incorporation of acetate into taxuyunnanine C is low as shown by the absolute $^{13}C$ abundance data in Table 1. Briefly, the carbon atoms of the taxane ring system are in the range of natural $^{13}C$ abundance. More specifically, the average labeling of the taxane carbon atoms is 1.12 ± 0.05. Slightly increased $^{13}C$ abundance values up to about 1.3% were found in the acetyl side chains. The joint incorporation of two $^{13}C$ atoms from acetate can be diagnosed with high sensitivity by the analysis of the satellite signals arising by $^{13}C$–$^{13}C$ coupling. As shown in Fig. 2*B*, $^{13}C$ satellites signaling the incorporation of intact [1,2-$^{13}C_2$]acetate accompany the $^{13}C$ signals of all four acetyl groups of taxuyunnanine C (Fig. 1*B*). On the other hand, acetate was not at all incorporated into the taxane ring system as shown by the virtual absence of coupling satellites; the small satellites of the signals for C-18 and C-19 in Fig. 2*B* represent the natural abundance contribution and do not indicate the incorporation of intact 2-carbon units.

Quantitative analysis of $^{13}C$ $^{13}C$ coupling satellites is summarized in Fig. 1*B*. The methyl $^{13}C$ signals of the acetoxy groups at C-10 and C-14 overlap (Fig. 2*B*), but the respective carbonyl signals are well separated and can be used for unequivocal isotopomer quantitation (Table 1). It should be noted that the fraction of the double-labeled isotopomers differs by more than a factor of 2 between the different acetyl groups. As shown below, these differences are much larger than the experimental error. This could imply that the acetylation of different hydroxyl groups occurs in different compartments.

The failure of acetate to contribute label to the taxane moiety argues against a mevalonate origin of the taxoid system, but is not sufficient to rule it out conclusively. It is still conceivable that the formation of the taxane ring system could proceed in a compartment which is not accessible to exogenous acetate, whereas the acetyl groups are added in a different compartment which is accessible to exogenous acetate. However, the mevalonate pathway can be ruled out conclusively on the basis of experiments with $^{13}C$-labeled glucose isotopomers, which are described below.

In the experiment with [U-$^{13}C_6$]glucose, the labeled precursor was diluted at a ratio of 1:20 with unlabeled (i.e., natural abundance) glucose. Under these experimental conditions, the precursor molecules for the biosynthesis of taxuyunnanine C are derived at random from the labeled or the unlabeled glucose. Consequently, the labeling pattern of the resulting diterpene will be a mosaic of numerous different isotopomers.

Table 1. $^{13}C$ NMR analysis of taxuyunnanine C from *T. chinensis* after growth with [U-$^{13}C$]glucose, [1-$^{13}C$]glucose, or [1,2-$^{13}C_2$]acetate

| Position* | Chemical shift, ppm | [U-$^{13}C$]Glucose | | | | [1-$^{13}C$]-Glucose, | [1,2-$^{13}C_2$]Acetate | |
|---|---|---|---|---|---|---|---|---|
| | | % $^{13}C$ | % $^{13}C$ $^{13}C$ | J Hz | J position† | % $^{13}C$ | % $^{13}C$ | % $^{13}C$ $^{13}C$ |
| 25 | 170.1 | 2.82 | 42.30 | 59.0 | 26 | 1.84 | 1.26 | 15.20 |
| 21 | 169.9 | 2.90 | 42.27 | 59.5 | 22 | 1.68 | 1.29 | 16.72 |
| 27 | 169.8 | 3.05 | 42.49 | 59.5 | 28 | 1.89 | 1.23 | 8.32 |
| 23 | 169.6 | 2.76 | 40.73 | 59.5 | 24 | 1.82 | 1.12 | 5.03 |
| 4 | 142.2 | 4.19 | 56.43 | 74.1 | 20 | 2.32 | 1.12 | |
| 11 | 135.2 | 4.30 | 61.71 | 50.1 | 10 | 1.35 | 1.09 | |
| 12 | 134.6 | 4.69 | 57.79 | 44.6 | 18 | 2.54 | 1.21 | |
| 20 | 116.8 | 4.20 | 57.71 | 74.1 | 4 | 9.06 | 1.15 | |
| 5 | 78.1 | 3.95 | 11.80 | | 2,3 | 2.36 | 1.11 | |
| 14 | 70.4 | 4.26‡ | 61.98‡ | 41.0‡ | 1, 17 | 9.67‡ | 1.16‡ | |
| 2 | 70.4 | | | | 3, 5 | | | |
| 10 | 70.0 | 4.10 | 61.74 | 50.1, 3.5 | 11, 13 | 9.40 | 1.10 | |
| 1 | 58.8 | 4.49 | 61.47 | 41.0 | 14 | 1.41 | 1.22 | |
| 9 | 43.8 | 4.41 | 11.30 | | 6 | 2.41 | 1.09 | |
| 3 | 42.0 | 4.13 | 61.61 | 40.8 | 2, 5 | 1.28 | 1.11 | |
| 8 | 39.6 | 4.30‡ | 58.89 | 36.9 | 19 | 2.45 | 1.12 | |
| 13 | 39.3 | | 10.31 | | 10 | 2.52 | 1.16 | |
| 15 | 37.2 | 4.33 | 56.91 | 36.7 | 16 | 2.21 | 1.03 | |
| 7 | 33.7 | 4.16 | 62.06 | 33.6 | 6 | 1.27 | 1.10 | |
| 17 | 31.7 | 4.29 | 6.75 | | 14 | 2.56 | 1.12 | |
| 6 | 28.8 | 4.10 | 62.22 | 33.6, 2.4 | 7, 9 | 9.12 | 1.10 | |
| 16 | 25.3 | 4.06 | 57.52 | 36.7 | 15 | 8.78 | 1.08 | |
| 19 | 22.4 | 3.99 | 57.80 | 37.2 | 8 | 8.65 | 1.08 | |
| 24 | 21.7 | 2.88 | 41.96 | 59.5 | 23 | 4.15 | 1.23 | 6.76 |
| 22 | 21.4 | 3.02 | 43.05 | 59.5 | 21 | 4.27 | 1.35 | 16.16 |
| 28 | 21.3 | 2.79‡ | 43.60‡ | 59.3‡ | 27 | (3.98)‡ | (1.30)‡ | (12.69)‡ |
| 26 | 21.3 | | | | 25 | | | |
| 18 | 20.8 | 3.96 | 57.12 | 44.9 | 12 | 8.29 | 1.08 | |

*Assignments are based on HMQC, HMQC-DEPT, HMBC, and INADEQUATE experiments.
†Carbon atoms coupled to the respective index carbon as determined by INADEQUATE spectroscopy.
‡Averaged intensities due to signal overlapping.

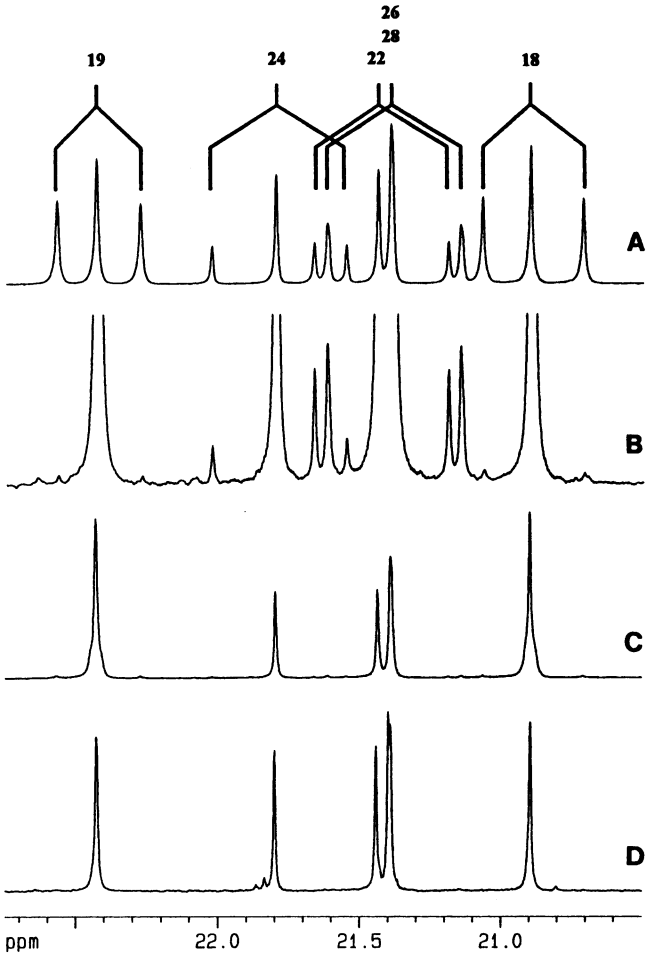


FIG. 2. Partial $^{13}C$ NMR spectra of taxuyunnanine C. (*A*) From [U-$^{13}C_6$]glucose; (*B*) from [1,2-$^{13}C_2$]acetate; (*C*) from [1-$^{13}C$]glucose; (*D*) natural abundance. $^{13}C$ $^{13}C$ coupling patterns are indicated on top of the figure.

Multiply and contiguously $^{13}C$-labeled isotopomers can be diagnosed with high sensitivity and specificity by one-dimensional (Fig. 2*A*) and especially by two-dimensional NMR experiments (Fig. 3). Twelve pairs of contiguously $^{13}C$ labeled carbon atoms shown by bold lines in Fig. 1*C* were observed in taxuyunnanine C obtained from the [U-$^{13}C_6$]glucose experiment by a two-dimensional INADEQUATE experiment. Closer analysis also showed that $^{13}C$ atoms in four of these $^{13}C$ pairs showed long-range couplings (via two or three bonds) to yet another $^{13}C$ atom which was apparently diverted to the diterpene together with the two carbon atoms in the contiguously labeled atom pair. The long-range couplings were diagnosed by INADEQUATE experiments optimized for magnetization transfer via small coupling constants (Fig. 3). For example, the experiment yields direct evidence for the presence of the triple-labeled [2,3,5-$^{13}C_3$]taxuyunnanine C in the complex isotopomer mixture. Approximate quantification of the triple-labeled isotopomer is possible by analysis of the one-dimensional spectra and yield a value around 40%.

The observed long-range couplings are summarized by arrows in Fig. 1*C*. These data indicate that four $^{13}C$ triples have been contributed from the totally labeled glucose. The observation that one carbon atom of the triple is not directly bonded to the $^{13}C$ pair signifies that an intramolecular rearrangement has occurred during the biosynthesis of the isoprenoid precursors of taxuyunnanine C. The four isoprenoid units of the taxoid system are easily gleaned from these data and are shown in Fig. 1*D*. This dissection of the taxoid system is well in line

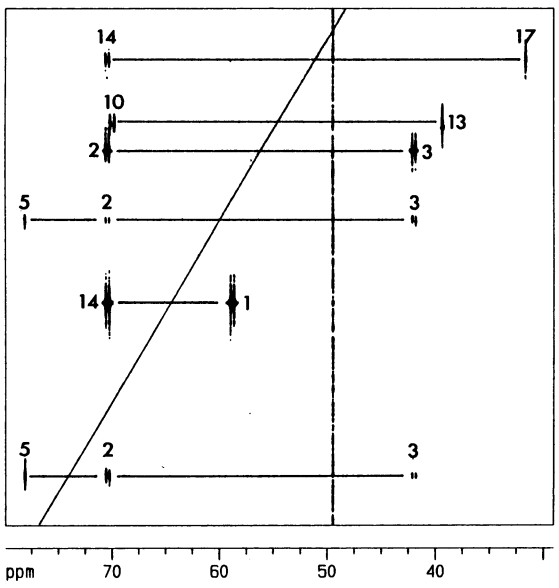


FIG. 3. Partial two-dimensional INADEQUATE spectrum of taxuyunnanine C from [U-$^{13}C_6$] glucose. A mixing time of 100 ms was used to obtain efficient magnetization transfer via small $^{13}C$ $^{13}C$ coupling constants. Other experimental parameters were as follows: 2-s relaxation delay, 2048 × 2048 data matrix (real part), 32 scans per increment, 1000 experiments, calculated in magnitude mode for both dimensions. Passive spins were observed in addition to the double quantum transitions and provide additional confirmation for the incorporation of 3-carbon fragments.

with studies on the enzymatic formation of the taxoid system by cyclization of geranylgeranyl pyrophosphate (13).

An experiment with [1-$^{13}C$]glucose was performed by using a $^{13}C$ enrichment of 50%. The $^{13}C$ enrichment data of taxuyunnanine C are summarized in Table 1. In this experiment, the $^{13}C$ abundance of the taxane system shows wide variation from low values close to natural abundance (1.3%) to values of about 9.5%.

With the isoprenoid precursor dissection in Fig. 1*D* at hand, a statistical analysis of the labeling pattern of the four biosynthetically equivalent isoprene moieties of the taxane system can be performed (Fig. 4 *B* and *C*). Absolute enrichments are shown for all carbon atoms of the averaged isoprenoid precursor. The standard deviations are quite small. Numbers shown in red indicate the fractions of double-labeled and triple-labeled isotopomers. Again, the similarity between the four isoprenoid modules is very high as documented by the small values of the standard deviations.

It appears safe to assume that the established precursor of the taxoid system, geranylgeranyl pyrophosphate (13) is assembled from IPP and dimethylallyl pyrophosphate (DMAP) modules. The strictly identical labeling pattern of all four isoprenoid moieties indicates that DMAP and IPP arise by the same biosynthetic pathway and are interconverted by isomerization.

In contrast to the experiment with [$^{13}C$]acetate, the labeling pattern of the four acetate side chains are closely similar to each other in both experiments with glucose. It should be noted that the $^{13}C$ enrichments for all taxane carbon values are very similar (4.22 ± 0.18%) in the experiment with [U-$^{13}C_6$]glucose. On the other hand, the values for $^{13}C$ enrichment of the acetate groups in this experiment is about 30% lower as compared to the taxane system. Similarly, the fraction of double-labeled acetate is about 30% lower as compared to the contiguous carbon pairs in the taxoid ring. This is surprising since glucose was the only carbon source in this experiment, and one would therefore expect that the carbon atoms of all metabolites should have identical enrichments within the limits of experimental accuracy. The apparent discrepancy of

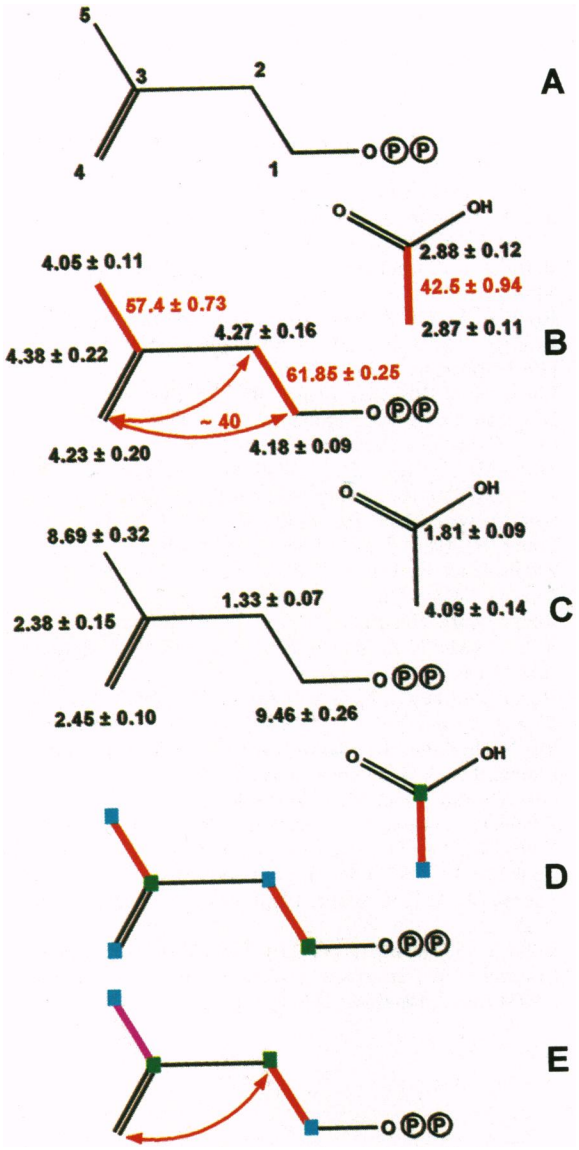


FIG. 4. Averaged labeling patterns of isopentenyl pyrophosphate (IPP) and acetate deduced from taxuyunnanine C. (*A*) Structure and carbon numbering of IPP. (*B*) Experiment with [U-$^{13}C_6$]glucose; $^{13}C$ enrichments and their standard deviations are shown as black numbers; contiguous $^{13}C$ pairs are connected by bold red lines, and long range couplings are indicated by red arrows; the fractions of multiple-labeled isotopomers with their standard deviations are shown in red. (*C*) Experiment with [1-$^{13}C$]glucose; $^{13}C$ enrichments with standard deviations are shown as black numbers. (*D*) Label distribution predicted on basis of the mevalonate pathway; carbon atoms derived from the carboxyl group of acetate are shown in green and carbon atoms derived from the methyl group of acetate are shown in blue; incorporation of intact acetate residues is shown by red lines. (*E*) Label distribution predicted by the alternative pathway proposed by Rohmer *et al.* (6); carbon atoms that should be derived from C-3 of triose phosphate or pyruvate are shown in blue, and carbon atoms derived from C-2 of triose phosphate or acetate are shown in green; the connectivity of the original 3-carbon precursor (prior to the skeletal rearrangment) is shown in red; the carbon atoms supposed to be contributed via activated acetaldehyde are connected by a bold violet line.

the absolute $^{13}C$ enrichment in acetate and the taxane system, respectively, may be due to the fact that the total biomass increased only by a factor of about 2 during the incubation with [U-$^{13}C_6$]glucose. It is therefore conceivable that the acetyl groups were generated to a significant extent by recycling of preformed biomass, whereas the taxane system was mostly formed *de novo* from the proffered glucose. Experiments conducted over different incubation periods will therefore be necessary to analyze the label distribution between isoprenoid and acetyl moieties in more detail.

The mevalonate pathway and the alternative pathway proposed by Rohmer *et al.* (6) both make specific predictions with respect to the labeling pattern of the isoprenoid precursor which can now be checked against the averaged labeling data in Fig. 4 *B* and *C*. In the mevalonate pathway, three carbon atoms of the isoprenoid moiety (i.e., C-2, C-4, and C-5, shown in blue in Fig. 4*D*) are contributed by the methyl group of acetate. Two carbon atoms (C-1 and C-3, shown in green in Fig. 4*D*) are contributed by the carboxyl group of acetate. If taxuyunnanine C were formed by the mevalonate pathway, the carbon atoms of each group should have identical labeling patterns. This predication is clearly violated by the data in Fig. 4. The enrichment values of the carbon atoms 2, 4 and 5, which should all be identical according to the mevalonate hypothesis, vary between 1.3 and 8.7% $^{13}C$ abundance in the experiment with [1-$^{13}C$]glucose. Moreover, the long-range couplings observed in the experiment with [U-$^{13}C_6$]glucose imply the contribution of three carbon atoms via a single intermediate molecule. Since the mevalonate pathway starts with the 2-carbon precursor, acetate, a 3-carbon intermediate cannot be incorporated *in toto* into mevalonate. The mevalonate pathway can therefore be ruled out conclusively.

A similar approach can be used to check the present data against the mechanism proposed by Rohmer *et al.* (6) for the formation of isoprenoids in eubacteria. These authors proposed a condensation of activated acetaldehyde with a compound such as dihydroxyacetone under formation of a 5-carbon product which is subsequently rearranged. Thus, this mechanism involves the incorporation of a 3-carbon precursor and a 2-carbon precursor, but the connectivity of the 3-carbon precursor is disrupted by the proposed rearrangement reaction. The data obtained in this paper are in qualitative agreement with these requirements.

In plants, it can be expected that activated acetaldehyde is formed via pyruvate from the triose phosphate pool. Thus, one would expect that the 2-carbon fragment should reflect the labeling pattern of carbon atoms 2 and 3 of the triose phosphate intermediate, as shown schematically in Fig. 4*E*. The actual labeling pattern of the isoprenoid precursor from *T. chinensis* does not strictly reflect this expected pattern. More specifically, the enrichment values of C-2 and C-3 of the isoprenoid precursor are significantly different in the experiment with [1-$^{13}C$]glucose.

## DISCUSSION

Results of the present studies on the biosynthesis of taxoids in *T. chinensis* can be summarized as follows. (*i*) The four isoprenoid moieties of taxuyunnanine C have virtually identical labeling patterns. (*ii*) A 2-carbon unit and a 3-carbon unit are diverted to taxoid from glucose. (*iii*) The connectivity of the 3-carbon unit is disrupted by a skeletal rearrangement but can still be diagnosed unequivocally by the analysis of long-range $^{13}C$ $^{13}C$ coupling. (*iv*) Exogenous acetate contributes to the acetyl side chains of taxuyunnanine C but not to the taxane ring system.

The data show conclusively that the taxane ring system is not biosynthesized via mevalonate. More specifically, the mevalonate pathway could neither explain the observed contribution of a 3-carbon fragment from glucose to the diterpene nor the label distribution in the isoprenoid moieties.

The assembly of the isoprenoid moiety from a 3-carbon fragment and a 2-carbon fragment is reminescent of the alternative isoprenoid pathway reported by Rohmer, Sahm, and their coworkers in the eubacterium *Zymomonas mobilis* (5, 6). These authors have proposed that the isoprenoid moiety is assembled by

condensation of a triose phosphate-type compound with activated acetaldehyde derived from the decarboxylation of pyruvate. A subsequent skeletal rearrangement has been proposed to disrupt the connectivity of the 3-carbon unit.

The data on taxuyunnanine C obtained from [U-$^{13}C_6$]glucose yield direct proof for the occurrence of an intramolecular rearrangement in the biosynthesis of isoprenoid precursors. However, it remains open whether the taxoid precursor is actually assembled from a triose phosphate type compound and activated acetaldehyde as proposed by Rohmer *et al.* (6). If activated acetaldehyde is derived in plants from the triose pool via pyruvate, one would expect the same labeling patterns for the 2-carbon unit and the biosynthetically equivalent 2-carbon atoms of the 3-carbon unit. This expectation is not completely fulfilled by the experiment with [1-$^{13}$C]glucose.

Thus, the ultimate precursor or precursors of the isoprenoid unit in *T. chinensis* is as yet unknown. In principle, all primary metabolites formed by combination of a 2-carbon fragment with a 3-carbon fragment must be considered as potential candidates. It should be noted that the actual precursor could be a branched-chain compound as suggested by Rohmer *et al.* (6) or a linear compound. In either case, the branched isoprenoid structures could be obtained by appropriate skeletal rearrangements. Thus, the formation of a branched-chain intermediate from a linear precursor (ribulose phosphate) has been documented for the biosynthesis of the riboflavin precursor, 3,4-dihydroxy-2-butanone 4-phosphate (17).

The novel diterpenoid biosynthetic pathway is not restricted to the formation of diterpenes in gymnosperms but occurs also in monoterpenoid biosynthesis of angiosperms (D. Eichinger, W.E., M.H.Z., and A.B., unpublished data), and may be universally present in higher plants.

It should be possible to determine the structure of precursors for IPP/DMAP biosynthesis by comparison of the isoprenoid labeling pattern with the labeling patterns of primary metabolites from *T. chinensis* by using the retrobiosynthetic approach described earlier (18). Such studies using a variety of glucose isotopomers as carbon source are currently underway.

We thank Angelika Kohnle and Astrid König for help with the preparation of the manuscript. This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 369) and the Fonds der Chemischen Industrie.

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