Enzymatic Preparation of $^{14}$C-Labeled Phytoene, Squalene, and Geranylgeranyl Pyrophosphate from [2-$^{14}$C]Mevalonic Acid

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Methods are described for an enzymatic preparation of $^{14}$C-labeled terpenoids. With a cell-free system of a white mutant of Phycomyces blakesleeanus (Mucoraceae) [$^{14}$C]-squalene and [$^{14}$C]-phytoene can be synthesized from [2-$^{14}$C]mevalonate. The application of norflurazon, a phenylpyridazinone herbicide, helps to increase the yield of squalene. Furthermore, the liquid endosperm of Echinocystis lobata (Cucurbitaceae) was used for the formation of either $[^{14}C](-)$kaurene from $[^{14}C]$mevalonic acid or $[^{14}C]$geranylgeranyl pyrophosphate in the presence of Amo 1618.

The hydrocarbons formed were purified by alumina-column chromatography and preparative thin-layer chromatography (TLC). Geranylgeranyl pyrophosphate was separated by DE-column chromatography followed by TLC.

Introduction

The use of radioactively labeled precursors is a prerequisite for the study of terpenoid synthesis in vivo and in vitro. Geranylgeranylpyrophosphate is the common substrate for all C$_{20}$ and C$_{40}$ terpenoids. Its radioactivity can be channeled directly into carotenoids, phytol, gibberellins, and tocopherols in different microorganisms and plants [1, 2]. Squalene, kaurene, and phytoene are the direct precursors for all sterols, gibberellins, and carotenes. Their availability as radioactively labeled substrates is very helpful in biosynthesis studies of the terpenoid groups mentioned [3].

Furthermore, there is a need for [$^{14}$C]geranylgeranylpyrophosphate as a reference compound when the mode of action of growth retardants and herbicides on kaurene synthetase [4] and phytoene synthetase [5], respectively, is studied. None of the substances mentioned is available commercially as a labeled compound.

The present paper describes methods of how labeled squalene, (-)-kaurene, cis-phytoene, and all-trans-geranylgeranyl pyrophosphate can be prepared from [2-$^{14}$C]mevalonic acid with cell-free systems from a fungus and the seeds of a higher plant, both which can be easily cultivated.

Materials and Methods

1. Cultivation of Phycomyces blakesleeanus and Echinocystis lobata

The enzyme system converting [$^{14}$C]mevalonic acid to squalene and phytoene was obtained from a phytoene-accumulating mutant of Phycomyces blakesleeanus [strain C-5-car-B 10(–)]. Growth was carried out as described [6] in a liquid asparagine glucose medium in the light for 3 days on a shaker. Immature seeds of E. lobata were the source of the enzyme extract for geranylgeranyl pyrophosphate and kaurene synthesis. E. lobata (Greene) was cultivated in the Botanical Garden of the Universität Frankfurt. Seedlings were grown from fruits collected at the natural habitat near Ladenburg. Prior to sowing, the seeds were stored at 4°C from autumn to spring.

2. Preparation of $^{14}$C-cis-phytoene and $^{14}$C-squalene using Phycomyces

The enzyme extract for the biosynthesis of [$^{14}$C-cis]-phytoene and [$^{14}$C]-squalene was prepared from 2 g of the freeze-dried mycelium ground in a Dangoumau mill (type 1241, Sovirei, Wiesbaden) with a nylon vessel and korund balls, and 7 ml of 30% glycerol in 0.2 M Tris-HCl buffer [N-tris(hydroxymethyl)aminomethane], pH 8.0, containing 2 mM mercaptoethanol. After centrifugation at 10,000×g for 30 min, the supernatant was used immediately. The reaction was
carried out anaerobically in Thunberg tubes over a period of 5 h at 24 °C. At start, evacuation of the tubes and flushing them five times at least with nitrogen is necessary for a satisfactory yield of squalene. The reaction medium, modified after [5, 7], consisted of 0.1 ml of 0.3 mM DL-[2-14C]malonic acid, sodium salt (CEA France, specific activity 32 mCi/ml); 0.1 ml of 40 mM MgCl₂ and 40 mM MnCl₂; 0.2 ml of cofactor solution (=glutathione, 100 mM, ATP; 50 mM, NADP; 5 mM; NAD, 5 mM; FAD, 5 mM, dissolved in the buffered extraction medium), and 0.6 ml of the enzyme-containing supernatant. The protein content of the 1 ml reaction mixture was 12 to 13 mg. To some incubation samples 10 μM of norflurazon (4-chlor-5-[methylamino]-2-[3-trifluoromethylphenyl]-pyridazin-3-[2H]-one) was added. For the extraction of radioactive compounds 1 ml ofaq. KOH solution (60%, w/v) and 8 ml of ethanol, the latter containing 50 μg of unlabeled carrier phytoene and squalene, were added to the incubate, then the mixture was saponified over night at room temperature or for 1 h at 70 °C. Cis-phytoene was isolated from the same strain of Phycomyces according to [8]. The solution was extracted 3 times with ethyl ether. The organic phase was washed with water, dried with Na₂SO₄, and evaporated in a stream of nitrogen. The residue was dissolved in light petrol (b. p. 40–60 °C) and placed on a column of alumina (1.5×50 cm; Woelm, Eschwege, neutral, grade III). Phytoene and squalene were eluted with petrol (40–60 °C) and purified further by thin-layer chromatography on silica gel G plates (Merck, Darmstadt) with petrol (b. p. 60–80 °C) [7]. The compounds were detected by spraying with a solution of rhodamine 6 G in acetone (1% w/v) and examining the plates under UV light. Squalene separated at Rₜ values between 0.45 and 0.6, phytoene between Rₜ of 0.35 and 0.45. Purity of both labeled compounds was checked by running squalene on silica gel G plates with cyclohexane/chloroform (50:50, v/v) and phytoene on silica gel G plates in a system of 0.5% (v/v) benzene in petrol (b. p. 60–80 °C).

3. Preparation of [14C(-)]kaurene pyrophosphate using Echinocystis lobata

For the kaurene and geranylgeranyl pyrophosphate preparation soft immature seeds were employed which were not yet colored. At this stage of development, the liquid endosperm is not surrounded by the cotyledons. The seeds were cut open and the endosperm was squeezed out and centrifuged for 30 min at 20000×g. The supernatant was used as the (enriched) enzyme extract. The reaction mixture for the assay according to [9] contained: 0.1 ml of 20 mM ATP; 0.1 ml of 20 mM MgSO₄; 0.2 ml of 0.1 M Tris-HCl buffer, pH 7.0; 0.1 ml of [2-14C]malonic acid; and 1 ml of enzyme extract containing 3.5 mg of protein. After a 1 h reaction time at 30 °C saponification, extraction of [14C]kaurene, and separation on alumina columns and by TLC were carried out as described for phytoene and squalene.

4. Preparation of [14C]trans-geranylgeranyl pyrophosphate

The same reaction mixture as for the kaurene preparation was used for geranylgeranyl pyrophosphate synthesis, but additionally 5 mM Amo 1618 [2'-isopropyl-4':(trimethylammonium chloride)-5'-methylphenyl-piperidine-1-carboxylate] was present. After a 3 h reaction time, the whole mixture was boiled for 2 min and centrifuged at 10 000×g for 10 min. The pellet was extracted with some ml of water and the homogenate centrifuged again. The combined supernatants were placed on top of a Whatman DE-52 cellulose column (1.8×12 cm) equilibrated with water [10]. Elution was carried out with a linear gradient of 0 to 0.2 M (NH₄)₂CO₃. The absorbance at 265 nm was recorded and 10 ml fractions were collected. The ATP appeared in samples No. 40 to 45. The following 5 samples were pooled and freeze-dried after adding some Dowex 50 (H⁺) to remove the NH₄⁺ from the solution. [14C]geranylgeranyl pyrophosphate was further purified by TLC on silica gel plates in a solvent system of isopropanol/ammonia (25%)/water (60:30:10, v/v) [11]. The radioactivity of geranylgeranyl pyrophosphate was located in a zone between Rₜ 0.7 and 0.8. For a definite identification of geranylgeranyl pyrophosphate, aliquots were hydrolyzed with alkaline phosphatase [10] and the resulting [14C]geranylgeraniol co-chromatographed with an authentic standard of geranylgeraniol on silica gel plates using benzene/ethylacetate (80:20, v/v) as a solvent [11]. The spots of kaurene, geranylgeraniol, and geranylgeranyl pyrophosphate were detected by staining with iodine vapor. The radioactivity on the plates was traced...
with a thin-layer radioscanner (Berthold, Erlangen, LB 2722). Liquid samples were assayed in a liquid scintillation spectrometer (Packard Tricarb 3320). Protein was determined with the Folin reagent [12].

5. Chemicals

Geranylgeraniol was a gift of Dr. W. Rüdiger, München, (−)-kaurene was a gift of ICI, England. Alkaline phosphatase, ATP, NADP, NAD, FAD, and glutathione were purchased from Boehringer, Mannheim. Other chemicals were from Merck, Darmstadt, unless stated otherwise. Squalene was purchased from Sigma, München, norflurazon was from Sandoz AG, Basle.

Results and Discussion

In this communication we describe the preparation of several radioactively labeled terpenoids. There is a report [13] that extracts from *Phycomyces* can convert geranylgeranyl pyrophosphate to cis-phytoene. The results with strain C5-car-B10(−) of *Phycomyces* show a formation of [14C-cis]phytoene from [2−14C]mevalonic acid with a good yield together with a production of [14C]squalene in the same incubation sample (Table I). The 10:1 ratio in favor of phytoene can be changed toward squalene by addition of the herbicide norflurazon. This compound blocks phytoene synthesis at the dimerization step of geranylgeranyl pyrophosphate to phytoene [5] leading to a doubling of radioactivity accumulation in squalene (Table I). However, strictly anaerobic conditions are necessary to prevent squalene reaction with oxygen forming squalene-2,3-oxide [14]. Both phytoene and squalene are highly pure and free of other labeled compounds (Fig. 1) and show constant specific radioactivity after column chromatography followed by TLC. The radioactivity runs with either added cis-phytoene in petrol/toluene (99.5:0.5, v/v) with an $R_f$ of 0.48, or with squalene in cyclohexane/chloroform (50:50, v/v) with an $R_f$ of 0.78.

Table I. Formation of 14C-labeled cis-phytoene and squalene by a cell-free system from *Phycomyces* C5-car-B10(−).

<table>
<thead>
<tr>
<th>Products</th>
<th>Radioactivity [dpm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>10 μM Norflurazon</td>
</tr>
<tr>
<td>Phytoene</td>
<td>19000</td>
</tr>
<tr>
<td>Squalene</td>
<td>2200</td>
</tr>
</tbody>
</table>

Table II. Formation of 14C-labeled (−)-kaurene and trans-geranylgeranyl pyrophosphate by an enzyme extract from *Echinocystis lobata*.

<table>
<thead>
<tr>
<th>Products</th>
<th>Radioactivity [dpm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>Amo 1618</td>
</tr>
<tr>
<td>(−)-kaurene</td>
<td>385000</td>
</tr>
<tr>
<td>trans-geranylgeranyl pyrophosphate</td>
<td>21000</td>
</tr>
</tbody>
</table>

Fig. 1. Distribution of radioactivity in the squalene and phytoene fraction. A. Thin-layer separation of squalene (S) in the system: cyclohexane/chloroform, 50:50 (v/v). B. Separation of phytoene (P) in the system: benzene/petrol (b. p. 80–80°C), 0.5:99.5 (v/v).

Use of *Echinocystis macrocarpa* for the preparation of [14C]kaurene and geranylgeranyl pyrophosphate was already reported [9, 15]. However, several authors [15, 16] complain about its poor availability. The seeds can only be collected in the coastal areas of Southern California. We have been able to show that *E. lobata*, a wide-spread species that can easily be grown in a botanical garden of our latitudes, is quite as good a source for the kaurene synthetase system (Table II). The overall reaction can be stopped at the pyrophosphate level by addition of Amo 1618. After column chromatography on DE cellulose and TLC a fraction is obtained containing about 60% of the kaurene yielded in the absence of the inhibitor. By comparing the $R_f$ value with the literature [11] and by co-chromatographing of the product formed after alkaline phosphatase treatment...
with cis-,trans- and all-trans-geranylgeraniol, the compound was identified as trans-geranylgeranyl pyrophosphate (Fig. 2).

The biochemical preparation of labeled terpenoids using Phycocyanus and Echinocystis extracts is a rapid procedure to obtain substrate amounts. Another advantage is the immediate availability of the enzyme because either the seeds of Echinocystis lobata or the mycelium powder of Phycocyanus can be stored frozen over months with little loss of activity.

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