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Biosynthesis of the triterpenoids, botryococcenes and tetramethylsqualene in the B race of *Botryococcus braunii* via the non-mevalonate pathway

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Abstract—The green microalga *Botryococcus braunii* race B accumulates two types of triterpenoid hydrocarbons, botryococcenes and tetramethylsqualene. Both triterpenoids are synthesized via the non-mevalonate pathway.

Botryococcus braunii is a unicellular green microalga that produces a substantial amount of liquid hydrocarbons and is classified into three races A, B and L, depending on the types of hydrocarbons produced.[1] The B race produces two types of triterpene hydrocarbons, botryococcenes (C_nH_{2n-10}, $n=30$–37), as the major hydrocarbon component and small amounts of methylated squalenes.[2] This race is promising as resources for renewable fuels or fine chemicals because the total amount of these triterpenes accounts for from 10 to 86% of algal dry weight.[3] There are various homologues of botryococcenes which are derived from the parent C_{30} botryococcene (**1**) by successive methylation up to C_{34} with *S*-adenosylmethionine.[4] Both botryococcene and squalene are derived from the isoprenoid pathway with a common backbone of two C_{15} farnesyl residues. While it is well established that squalene (**2**) is derived from the condensation of two molecules of farnesyl diphosphate (FPP), the actual farnesyl precursors for **1** are not known.[5] Nevertheless the B race of *B. braunii* must have a system to efficiently supply isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), the universal precursors for isoprenoid biosynthesis, to produce such a large amount of triterpenes.

IPP and DMAPP are synthesized either via the classical mevalonate (MVA) pathway or the non-mevalonate pathway in which 2-*C*-methyl-D-erythritol 4-phosphate (MEP) is the first committed intermediate.[6] In higher plants and several algae there is a compartmentation of isoprenoid biosynthesis.[7] The MVA pathway is used to produce triterpenes or sterols in the cytosol, whereas the non-mevalonate pathway is used for the production of isoprenoids like carotenoids and phytol in the plastids. Green algae such as *Scenedesmus obliquus*, *Chlamydomonas reinhardtii* and *Chlorella fusca*, however, generally possess only the non-mevalonate pathway which is utilized to synthesize sterols.[8] *B. braunii* is also a member of green algae. Though the level of incorporation of MVA into botryococcenes was low in an earlier study, it is not known whether this alga utilizes only the non-mevalonate pathway for the mass-production of its specific triterpenes.[9] Thus we investigated which pathway of IPP biosynthesis is utilized for the production of botryococcenes and methylated squalenes. One of the B race of this alga, the Berkeley (Showa) strain, was fed with [1-^{13}C] glucose and the labeling pattern of these triterpenes from the alga was analyzed by means of ^{13}C NMR spectroscopy.[10–12] The labeling pattern of **1** indicated that all carbon atoms derived from C-1 and C-5 of IPP and DMAPP were labeled with a mean isotopic abundance of 5.2%.[13] Such a labeling pattern corresponded to the isoprenoid biosynthesis from [1-^{13}C] glucose via glycolysis and the non-mevalonate pathway (Fig. 1). We also analyzed ^{13}C-labeling pattern of a C_{34} botryococcene (**3**) that was the most abundant component in the Berkeley strain. All carbon atoms derived from C-1 and C-5 of IPP and

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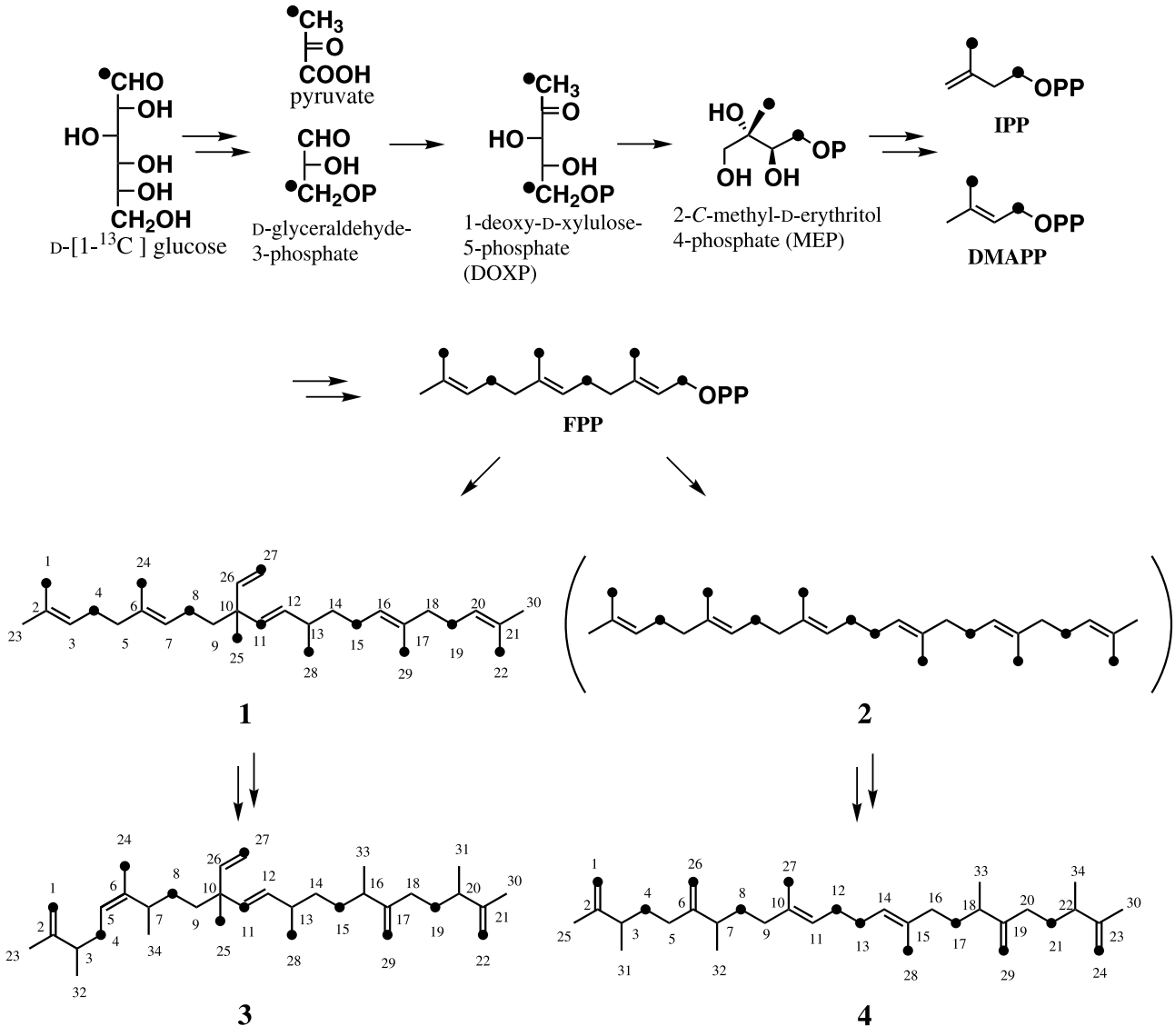

Figure 1. Biosynthesis of botryococcenes and tetramethylsqualene from D-[1-^{13}C] glucose via glycolysis and the non-mevalonate pathway.

DMAPP in this compound were labeled with a mean isotopic abundance of 1.9%.[14] The level of enrichment by [1-^{13}C] glucose found in **3** was rather lower than that in **1**. A possible explanation for this low enrichment is that compound **3** is an end product in this strain that has already accumulated in the algae before the feeding experiment started. Thus the newly synthesized molecules of **3** from [1-^{13}C] glucose represented less of the total **3** purified. We also analyzed ^{13}C-labeling pattern of tetramethylsqualene (**4**). All ^{13}C NMR signals corresponding to C-1 and C-5 of the isoprene units were significantly enhanced with a mean isotopic abundance of 1.8% in a similar way to **3**.[15] The relatively less isotopic abundance could be explained by the same reason as for **3**. Thus, **4** was also synthesized via the non-mevalonate pathway (Fig. 1).

We could not detect contribution of the MVA pathway to the biosynthesis of these triterpenes in the B race of *B. braunii* under the conditions in this study. It is interesting that *B. braunii* supplies all isoprene units to produce such large amounts of triterpenoids by only the non-mevalonate pathway since this pathway is composed of many more reaction steps than the MVA pathway. *B. braunii* might have a particular mechanism in order to produce IPP units efficiently. These IPP units are synthesized from solar energy and environmental carbon dioxide because this alga is a photosynthetic organism. Thus the particular mechanism in this alga to produce IPP units might be very useful for green sustainable chemistry.

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10. Axenic cultures of *B. braunii* Berkeley (Showa) strain were grown for 2 weeks in a modified Chu13 medium containing [1-^{13}C] glucose (1.0% w/v, 30% isotopic abundance) and neomycin (25 mg/100 ml).[11,12] The cultures were grown heterotrophically under low light (30 μE m^{-2} s^{-1}) on a 12L:12D cycle at 25°C, aerated with filter-sterilized air. The freeze-dried algal cells (495 mg) were soaked into *n*-hexane to extract components in the colonial matrix and the extract was centrifuged (3000 rpm×5 min). In order to extract intercellular components, the residue was incubated with 30ml of 1% SDS solution at 60°C for 30 min. The solution was partitioned with Et_2O. The Et_2O fraction was evaporated and dissolved in *n*-hexane. The components from the colonial matrix and the inside of algal cells were combined and subjected to a silica gel column of Wakogel C-300. A hydrocarbon fraction was eluted with *n*-hexane. The hydrocarbon fraction was subjected to normal-phase HPLC on a Develosil Silica 60-3 with *n*-hexane followed by reversed-phase HPLC on a Cosmosil ODS AR-II with MeCN/acetone (7:3). Finally C_{30} botryococcene **1** (0.5mg), C_{34} botryococcene **3** (43.2mg) and tetramethylsqualene **4** (0.8mg) were purified on the same reversed-phase column with MeOH.
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13. The ^{13}C NMR spectra were recorded in $CDCL_3$ with a JEOL ALPHA-600 spectrometer. Assignment of the signals was performed according to published data.[2a] δ (ppm) = 16.0 (C-24, 29, 5.2%), 17.7 (C-1, 22, 5.0%), 21.2 (C-28, 5.3%), 23.1 (C-8, 4.9%), 23.5 (C-25, 5.0%), 25.7 (C-23, 30, 1.6%), 25.8 (C-15, 5.2%), 26.7 (C-4, 19, 5.2%), 36.7 (C-13, 1.1%), 37.4 (C-14, 1.5%), 39.7 (C-5, 18, 1.5%), 41.3 (C-9, 1.4%), 42.0 (C-10, 1.5%), 111.1 (C-27, 4.7%), 124.4 (C-3, 20, 1.1%), 124.7 (C-7, 1.1%), 124.8 (C-16, 1.0%), 131.2 (C-2, 21, 1.0%), 133.7 (C-12, 0.9%), 134.7 (C-6, 17, 1.1%), 135.8 (C-11, 6.0%), 146.7 (C-26, 0.7%).
14. The ^{13}C NMR spectra were recorded as mentioned above.[13] Assignment of the signals was performed according to published data.[2a] δ (ppm) = 18.0 (C-34, 1.3%), 18.9 (C-30, 1.2%), 19.4 (C-23, 1.3%), 19.5 (C-32, 1.5%), 19.7 (C-31, 0.8%), 19.8 (C-24, 1.9%), 20.2 (C-33, 1.2%), 21.2 (C-28, 1.9%), 23.6 (C-25, 2.0%), 29.4 (C-8, 1.9%), 31.6 (C-18, 1.0%), 32.9 (C-4, 1.8%), 33.4 (C-15, 19, 1.9%), 34.5 (C-7, 1.0%), 35.0 (C-14, 1.1%), 37.3 (C-13, 1.0%), 39.5 (C-9, 1.1%), 40.0 (C-16, 1.0%), 41.0 (C-20, 1.0%), 41.6 (C-3, 1.1%), 41.8 (C-10, 1.1%), 107.2 (C-29, 1.8%), 109.1 (C-1, 1.9%), 109.5 (C-22, 1.8%), 112.0 (C-27, 1.7%), 123.9 (C-5, 1.0%), 133.7 (C-12, 1.0%), 135.8 (C-11, 1.8%), 139.3 (C-6, 1.1%), 146.8 (C-26, 1.1%), 149.8 (C-2, 1.1%), 150.0 (C-21, 1.1%), 154.8 (C-17, 1.1%).
15. The ^{13}C NMR spectra were recorded as above mentioned.[13] Assignment of the signals was performed according to published data.[2d] However, the assignment of C-8 and C-17 were substituted for C-9 and C-16 by ^{1}H–^{1}H COSY and HMQC, respectively. δ (ppm) = 16.0 (C-27, 28, 1.8%), 18.9 (C-25, 30, 1.1%), 19.7 (C-31, 34, 1.3%), 20.1 (C-32, 33, 1.3%), 28.2 (C-12, 13, 1.8%), 31.6 (C-5, 20, 1.1%), 33.4 (C-4, 21, 1.7%), 34.0 (C-8, 17, 1.8%), 37.5 (C-9, 16, 1.1%), 39.5 (C-7, 18, 1.1%), 41.0 (C-3, 22, 1.1%), 107.1 (C-26, 29, 1.8%), 109.3 (C-1, 24, 1.7%), 123.9 (C-11, 14, 1.0%), 135.1 (C-10, 15, 1.1%), 149.7 (C-2, 23, 1.2%), 154.6 (C-6, 19, 1.0%).