New Approach to Multiply Deuterated Isoprenoids Using Triply Engineered *Escherichia coli* and Its Potential as a Tool for Mechanistic Enzymology

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Isoprenoids are chemically diverse in nature, ubiquitous in living organisms, and crucial in biological processes. The biosynthesis of such isoprenoids proceeds through mevalonate[1] and non-mevalonate pathways[2] depending upon organisms and cellular organella, isopentenyl diphosphate (IPP) being a key intermediate in both cases. Metabolic engineering and control of these pathways should thus provide new opportunities to study the intriguing chemistry and biochemistry involved and to develop selective chemotherapeutic agents and isoprenoid-related materials. This paper describes a new practical approach for the preparation of highly deuterated isoprenoids, such as zeaxanthin, and their potential for analyzing the biosynthetic mechanism of isoprenoids, based on the metabolic engineering of *Escherichia coli*. Deuterium-labeled compounds are invaluable in biochemical and bioorganic as well as physicochemical research.

The non-mevalonate pathway, but not the mevalonate pathway, is now known to function in *E. coli*. Recently, Seto et al.[3,4] and Boronat et al.[5] described the construction of doubly engineered *E. coli* with the disruption of a gene of a certain enzyme involved in the non-mevalonate pathway and the introduction of the genes responsible for the key enzymes in the mevalonate pathway. These sort of transformant cells can survive only with supplementation of metabolic intermediates in the mevalonate pathway. Importantly, this warrants that all of the IPP formed in the cells *must* be derived from the supplemented intermediate. *E. coli* DK223[6] (pTMV20,[7] pACCAR25ΔcrtX) was constructed in this manner, but was actually triply engineered. That is, the 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*dxr*) gene was disrupted, and a plasmid pTMV20 carrying the genes of three enzymes responsible for the formation of IPP was introduced. In addition, another plasmid pACCAR25ΔcrtX was introduced, which carried the *crtE*, *crtB*, *crtI*, *crtY*, and *crtZ* genes required for the synthesis of zeaxanthin.[8] This strain survived and produced a yellow pigment of zeaxanthin in the presence of exogenous mevalonate.[9]

Fully deuterated mevalonolactone-d_9 (MVL-d_9), which had been synthesized as described previously,[10] was supplemented to the culture of the above triply engineered *E. coli*.[11] The biosynthesized zeaxanthin was extracted and purified to homogeneity by repeated chromatography. The molecular ion (M^+) of the purified product was observed as a cluster of isotopomer ions between *m*/*z* 605−622. No trace of an ion at *m*/*z* 568 (nonlabeled M^+) was detected. All of the zeaxanthin formed was proved to be derived only from the supplemented MVL-d_9. This was the first example of such highly and multiply deuterated zeaxanthin and clearly demonstrates the significant potential of the present approach for the preparation of various isotope-labeled isoprenoids.

As to the biosynthetic mechanism, the fact that zeaxanthin was biosynthesized in the above engineered *E. coli* appears to suggest that the whole mevalonate pathway including IPP isomerase was viable.[12] In addition, the clustered M^+ ion clearly implies variable deuterium content from molecule to molecule. This appears to be due to the exchange of hydrogen at the methyl group of intermediary dimethylallyl diphosphate (DMAPP) during the isomerase reaction and accordingly at the C-4 methylene group of IPP as well. Thus, once a proton is incorporated into the freely rotating C-4 methyl group of DMAPP, the reverse reaction from DMAPP to IPP should afford (*E*)- and (*Z*)-protonated IPP equally as shown in Figure 1.

^{1}H NMR spectra were then taken under deuterium decoupled conditions to look at the zeaxanthin formation more closely (Figure 2).[13] First, no proton incorporation was observed at H-10

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(6) A *dxr*-disruptant (formerly the *yaeM*-disruptant) was previously derived from *E. coli* FS1576 (*Biosci. Biotechnol. Biochem*. **1999**, *63*, 776−778), in which the *dxr* gene was inserted by the kanamycin-resistant gene (Km^r). Then, the disrupted *dxr* gene was transduced to *E. coli* W3110 (wild-type) using bacteriophage P1. The resulting W3110 *dxr*::Km^r strain has now been named as DK223.

(7) A plasmid pTMV20 is a pTTQ18 (Amersham Pharmacia Biotech.) derivative containing the 6.1-kb *SnaBI*-*Sse*8387I fragment (GenBank accession no. AB037666),[4] which contains the genes of mevalonate kinase, phosphomevalonate kinase, and diphosphomevalonate decarboxylase.

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(9) Carotenoid accumulation using doubly engineered *E. coli* based on an approach conceptually different from ours, particularly concerning the source of IPP, has recently been described. Cf. Kajiwara, S.; Fraser, P. D.; Kondo, K.; Misawa, N. *Biochem. J.* **1997**, *324*, 421−426; Wang, C.-W.; Oh, M.-K.; Liao, J. C. *Biotechnol. Bioeng.* **1999**, *62*, 235−241; Albrecht, M.; Misawa, N.; Sandman, G. *Biotechnol. Lett.* **1999**, *21*, 791−795; Matthews, P. D.; Wurtzel, E. T. *Appl. Microbiol. Biotechnol*. **2000**, *53*, 396−400.

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(11) The engineered *E. coli* was grown in LB medium (3 L) supplemented with MVL-d_9 (0.5 g/L), kanamycin (10 mg/L), ampicillin (50 mg/L), and chloramphenicol (30 mg/L) at 30 °C for 48 h. IPTG (238 mg/L) was added to induce production of zeaxanthin. The harvested cells (wet weight 22 g) were extracted with cold acetone to give 248 mg of a crude extract, which was then chromatographed over silica gel with $CHCl_3$−MeOH to afford 22 mg of zeaxanthin. Final purification was carried out by preparative HPLC to yield 5.2 mg of analytical zeaxanthin. Although several UV-positive minor products (.∼15% in total) were detected by HPLC, we focused only on the major product zeaxanthin in the present study.

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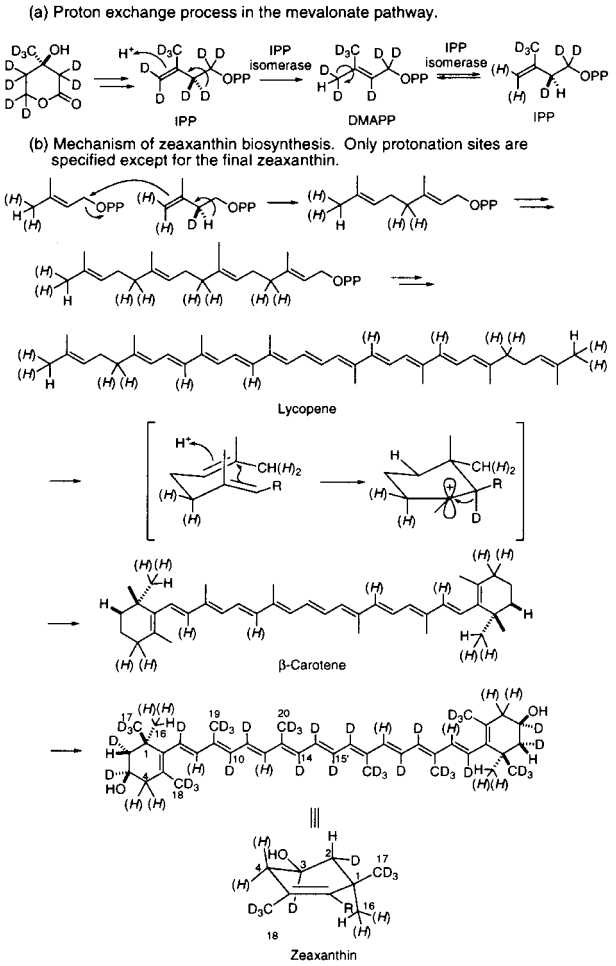

Figure 1.

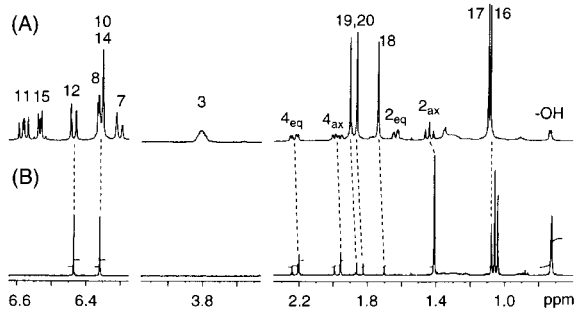

Figure 2. (A) ^{1}H NMR spectrum of nonlabeled zeaxanthin; (B) deuterium decoupled ^{1}H NMR spectrum of labeled zeaxanthin (500 MHz, benzene-d_6).

and H-14 of zeaxanthin,[14] again confirming that no endogenous nonlabeled IPP was formed. This implies that the stereochemistry of deprotonation−reprotonation at C-2 of IPP, catalyzed by the IPP isomerase, and that of deprotonation, catalyzed by prenyl transferase affording geranylgeranyl diphosphate, must be the same. Removal of the *proR* proton on the corresponding methylene group has recently been demonstrated for the *E. coli* IPP isomerase and farnesyl diphosphate synthase by Poulter et al.[15,16]

Since the ring formation from lycopene is triggered by the electrophilic attack of a proton to the terminal double bond, one protium (originated from the medium) must reside at C-2 of the resulting zeaxanthin. Accordingly, the proton signal due to H-2 observed at δ 1.41, together with the hydroxy signal at δ 0.73 (D_2O exchangeable), are logical internal intensity references. The C-18, -19, and -20 methyl signals appeared as a weak singlet of approximately 6% protium incorporation, which correspond well with the original ^{2}H content of the supplemented MVL-d_9. Olefinic signals were observed only for H-8 at δ 6.32 and H-12 at δ 6.47, both of which are singlets with 23% protium-incorporation. The C-4 protons were observed as two singlets of equal intensity at δ 1.96 and 2.20 and a pair of doublets at δ 1.98 and 2.22 ($J = 17$ Hz). Total incorporation was 23%, in which half of the incorporated protium was ascribed to the singlets. These results suggest that the C-4 methylene group of IPP in the metabolic pool of this particular culture was 23% protium-labeled and that no stereochemical differentiation took place. Apparently, the significant equilibrium between IPP and DMAPP afforded a statistically averaged incorporation of protium into the methylene group. Further, despite the fact that the C-17 methyl signal was not identified due to signal overlapping, the C-16 methyl signal was observed as a cluster of three singlets at δ 1.04 (CHD_2, 56% protium-incorporated), 1.06 (CH_2D, 79%), and 1.08 (CH_3, 40%). High turnover between IPP and DMAPP was verified by the extensive incorporation of protium at the C-16 methyl group.

The cryptic stereochemistry of the cyclization of lycopene to β-carotene was elucidated without difficulty (Figure 1). Essentially no protium was incorporated into the equatorial position at C-2, the signal of which should have appeared, if any, at around δ 1.6. Instead, as mentioned above, an intense singlet signal, which was ascribable to the axial proton, was observed at δ 1.41. Further, the protium-incorporated methyl group at C-1 was determined to be axially oriented after comparison with the nonlabeled specimen. It appeared therefore that protonation to the *Si*-face of the C-1/C-2 double bond and the subsequent backside attack of the C-5/C-6 double bond, followed by deprotonation at C-6, gave the well-established β-carotene ring system. These results were completely consistent with that previously reported.[17]

The present study clearly demonstrates the high potential of metabolically engineered microorganisms at least for two aspects. Particularly, the triply engineered *E. coli*, in which the pathway of isoprenoid biosynthesis was switched from the non-mevalonate pathway to the mevalonate pathway and a series of biosynthetic genes were introduced, allowed us to produce useful labeled isoprenoid zeaxanthin. A similar approach using *E. coli* lacking the *crtZ* gene of the final oxidation enzyme appears to conveniently afford highly deuterated and other isotopically labeled β-carotene (provitamin A). Such highly deuterated carotenoids and derived retinoids can stimulate diverse research in areas such as materials science and photochemistry as well as in the biochemistry of this class of isoprenoids.[18] In addition, straightforward stereochemical analysis of isoprenoid biosynthesis was demonstrated by one-shot labeling and ^{1}H NMR spectroscopy.[19] Precise analysis of the simplified proton spectra of highly deuterated isoprenoids, especially under the deuterium decoupled conditions, may well be beneficial for mechanistic enzymology, particularly, for the key transformation involving proton attack and proton quench as observed in the terpene cyclase reactions.

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Supporting Information Available: FAB mass spectra of perdeuterated and nonlabeled zeaxanthin (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) Since zeaxanthin is symmetric in structure, only half of the molecule is discussed in this paper.

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