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Engineering *Escherichia coli* for the Synthesis of Taxadiene, a Key Intermediate in the Biosynthesis of Taxol☆

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Abstract—Taxadiene, the key intermediate of paclitaxel (Taxol) biosynthesis, has been prepared enzymatically from isopentenyl diphosphate in cell-free extracts of *Escherichia coli* by overexpressing genes encoding isopentenyl diphosphate isomerase, geranylgeranyl diphosphate synthase and taxadiene synthase. In addition, by the expression of three genes encoding four enzymes on the terpene biosynthetic pathway in a single strain of *E. coli*, taxadiene can be conveniently synthesized in vivo, at the unoptimized yield of 1.3 mg per liter of cell culture. The success of both in vitro and in vivo synthesis of taxadiene bodes well for the future production of taxoids by non-paclitaxel producing organisms through pathway engineering.

Introduction

Taxadiene [taxa-4(5),11(12)-diene, Fig. 1] is the key intermediate in the biosynthesis of paclitaxel (Taxol), one of the most potent chemotherapeutic agents known, showing excellent activity against a range of cancers including ovarian and breast cancer.[1] The demand for paclitaxel greatly exceeds the supply from both natural sources and semisynthesis, so intensive efforts have been directed toward finding alternate means of production and to synthesize analogues with similar or increased activity. Thus, taxadiene is important for studying the biosynthesis of paclitaxel and other taxoids. However, taxadiene is not readily available, requiring either 750 kg of dry bark from the Pacific yew (*Taxus brevifolia*) for the isolation of only 1 mg of the compound or a 25-step chemical synthesis.[2] Therefore, these procedures are not efficient ways to produce substantial quantities of taxadiene.

The biosynthesis of taxadiene (Fig. 1) from isopentenyl diphosphate (IDP), the universal precursor of all terpenes, requires three enzymes: (a) IDP isomerase for the isomerization of IDP to dimethylallyl diphosphate (DMADP), (b) Geranylgeranyl diphosphate (GGDP) synthase for the condensation of three molecules of IDP with one molecule of DMADP to form GGDP, and (c) taxadiene synthase (TS) for the cyclization of GGDP to taxadiene.[3] Genes encoding all three enzymes have previously been cloned and overexpressed in *Escherichia coli* (see Table 1) but only minute amounts of taxadiene, observed only as radioactive counts or small spots on thin layer chromatography, have been obtained from cell-free systems.[4,5] Better yields have been achieved with the cell-free conversion of IDP to the diterpene hydrocarbon, casbene, in a multi-enzyme system including casbene synthase, as verified by production of sufficient amounts of casbene for analysis by ^{13}C NMR spectroscopy.[6] Apparently, the main difference between the two systems is the solubility of the overproduced cyclases (taxadiene synthase is less soluble than casbene synthase even when produced as a thioredoxin fusion protein[5]), so we felt that the yield of taxadiene in a cell-free system could be improved with a more soluble form of taxadiene synthase. Furthermore, an in vivo system for the de novo synthesis of taxadiene would be attractive, eliminating the need to pre-synthesize any of the substrates. *E. coli*, however, produces GGDP at low levels for use in isoprenoid biosynthesis. In *E. coli*, IDP is derived from deoxyxylulose-5-phosphate (DXP, Fig. 1) rather than via the more common mevalonate pathway,[7] and it has been shown that the addition of deoxyxylulose to a culture of *E. coli* increases the intracellular isoprenoid concentration by a factor of 3–4.[8] Furthermore, overexpression in *E. coli* of the gene encoding DXP synthase from *Bacillus subtilis* or *Synechocystis sp.* 6803 has been shown to increase the levels of carotenoids and isoprenoids.[9] We report here the

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☆Dedicated to Peter Dervan, a pioneer in Chemical Biology.

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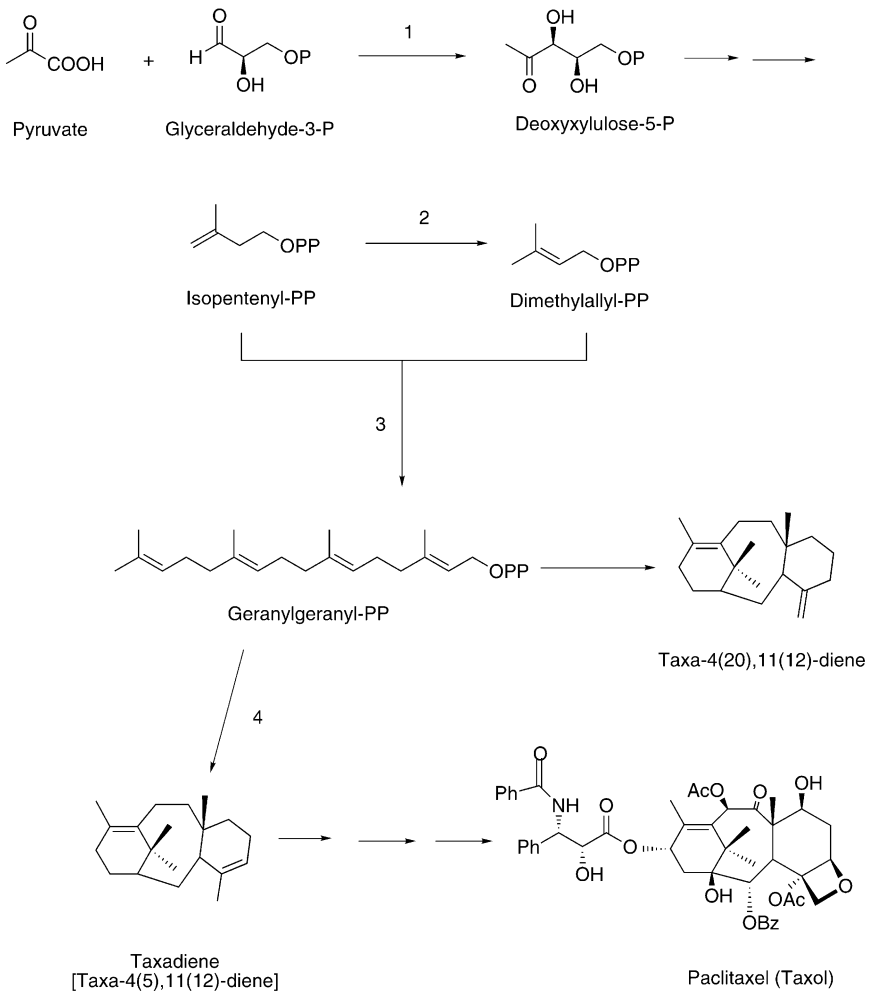

Figure 1. The biosynthetic pathway from pyruvate and glyceraldehyde-3-phosphate to taxadiene. Not all of the intermediates are known and some have been omitted to save space. The enzymes catalyzing the numbered steps are: (1) deoxyxyulose phosphate synthase, (2) isopentenyl diphosphate isomerase, (3) geranylgeranyl diphosphate synthase, and (4) taxadiene synthase.

obtainment of a more soluble form of taxadiene synthase that has resulted in improved yields of taxadiene, both in vitro and in vivo, and demonstrate that, in vivo, overproduction of *E. coli* DXP synthase, in conjunction with the other three enzymes, substantially increases the yields of taxadiene, providing a convenient source of milligram quantities of the target compound.

Results

In vitro production of taxadiene

Our first objective for improved yields of taxadiene was to obtain a more soluble, active form of the enzyme. In the yew tree, taxadiene synthetase is synthesized with a signal peptide, for transport into vacuoles, that is presumably removed during transport. To produce an enzyme without a signal peptide, pcr primers were designed to provide a product having a 5′ *Nco*I restriction site, a gene for taxadiene synthase that eliminates the first 78 amino acids, a stop codon, and a 3′ *Bam*HI restriction site. The pcr product was inserted into pET23H2 to afford pTS79H. We found that removal of the first 78 amino acids to eliminate the signal peptide and form a 'pseudo-mature' enzyme resulted in mostly soluble protein (Fig. 2A, lanes 1–4). While this work was in progress, similar results were reported by Williams et al.[10] The enzyme overproduced in BL21DE3(pTS79H) was active, as demonstrated in small scale incubations containing ^{13}C-IDP and cell-free extracts containing IDP isomerase, GGPD synthase, and the truncated taxadiene synthase (Fig. 3). While it appears that all of the IDP was converted to GGDP, only about 70% of the GGDP was converted to taxadiene, possibly due to hydrolysis of the diphosphate from GGDP or product inhibition of the enzyme. However, attempts to improve the conversion of GGDP to taxadiene by adding fluoride ion as a phosphatase inhibitor, increasing the amount of taxadiene synthase, or adding organic solvent and/or β-cyclodextrin to trap the product were all unsuccessful.

A large scale reaction was performed using crude IPP (derived from 360 mg of isopentenyl tosylate) in a total volume of 2 L. A total of 10 mg of purified taxoid product were obtained from the incubation. Surprisingly, besides the expected taxa-4(5), 11(12)-diene as the major product, a minor product was also formed (13% of the total as calculated by GC–MS), which was shown to be

Table 1. Strains and plasmids used in this study

Plasmids	Relevant characteristics	Source or reference
pACYC184	P15 ori, Cm^R	Chang et al.[14]
pZS*24MCS1	pSC101 ori, Kan^R	Lutz et al.[15]
pET15b	T7 promoter, pBR322 ori, Amp^R	Novagen
pET23a(+)	T7 promoter, pBR322 ori, Amp^R	Novagen
pET23H1	modified pET23	This study
pET23H2	modified pET23	This study
plysS	T7 lysozyme gene in pACYC184	Novagen
pTB42.1	Taxdiene synthase[a]	Wildung et al.[4]
pTS79H	truncated Taxadiene synthase	This study
pIDPI	IDP isomerase gene in pET23	This study
pGGDPS	GGDP synthase gene in pET23H1	This study
pIGS	IDPI-GGDPS fusion in pET23H1	This study
pACYC-IGS	IDPI-GGDPS fusion in pACYC	This study
plysS-IGS	IDPI-GGDPS fusion in plysS	This study
pDXPS	DXP synthase[b] gene in pET23H1	This study
pZS-DXPS	DXP synthase gene in pZS*24MCS1	This study
E. coli strains	Description (enzymes)	Source
TB1	cloning host	Baldwin et al.[16]
BL21DE3	T7 polymerase	Novagen
BL21DE3(plysS)	T7 polymerase, T7 lysozyme	Novagen
JM101(pFMH6)	IDP isomerase[c]	Hahn et al.[17]
JM101(pSM145)	GGDP synthase[d]	Math et al.[18]
BL21DE3(pDXPS)	DXP synthase	This study
BL21DE3(plysS)(pIDPI)	IDP isomerase	This study
BL21DE3(pGGDPS)	GGDP synthase	This study
BL21DE3(pTS79H)	Taxadiene synthase	This study
BL21DE3(pIGS)	IDPI-GGDPS fusion	This study
BL21DE3(pACYC-IGS)	IDPI-GGDPS fusion	This study
BL21DE3(pTS79H)(pACYC-IGS)	IDPI-GGDPS fusion, TS	This study
BL21DE3(pTS79H)(plysS-IGS)(pZS-DXPS)	DXPS, IDPI, GGDPS, TS	This study

[a]Gene isolated from *Taxus brevifolia*.
[b]*dxs* gene isolated from *E. coli*.
[c]*idi*1 gene isolated from *Schizosaccharomyces pombe*.
[d]*crtE* gene isolated from *Erwinia herbicola*.

the isomeric taxa-4(20),11(12)-diene (Fig. 1) by comparison of its NMR spectrum with literature values.[11]

In vivo production of taxadiene

Initial attempts to detect in vivo taxadiene synthesis in strains such as BL21DE3(pTS79H) overproducing only taxadiene synthase were unsuccessful probably because of limited amounts of the substrate, GGDP, in *E. coli*. The first successful production of taxadiene in vivo was accomplished by the simultaneous overproduction of IDP isomerase, GGDP synthase and taxadiene synthase, all from the T7 promoter. To this end, the genes for IDP isomerase and GGDP synthase were moved into the same plasmid compatible with pTS79H as follows: First, a 0.9 kb *Nde*I-*Hin*dIII fragment from pSM145 bearing the *crtE* gene was inserted into pET23H1 to give pGGDPS. Then a pcr product consisting of the *idi*1 gene with a 5′ *Xba*I site and a 3′ *Nco*I site was ligated into pGGDPS resulting in pIGS which bears a *crtE-idi*1 gene fusion and produces a fusion protein ($M_r = 61$ Kd) containing both IDP isomerase and GGDP synthase separated by a 21 amino acid linker including a 6X his-tag. The fusion protein was soluble (Fig. 2B, lanes 1 and 2) and displayed the expected dual activities as demonstrated by the in vitro synthesis of ^{13}C-GGDP from ^{13}C-IDP (not shown). A 2.1 Kb *Bsp*EI fragment from pIGS containing the T7 promoter and the gene for the fusion protein was blunt-ended with T4 DNA polymerase and ligated into the *Eco*RV site of either pACYC184 or plysS to give pACYC-IGS and plysS-IGS, respectively, each with the p15A origin of replication and, in the latter case, a source of T7 lysozyme for tighter regulation expression from the T7 promoter. Transformation of pACYC-IGS into BL21DE3(pTS79H) resulted in a strain that produced both the fusion protein and taxadiene synthase in soluble form (Fig. 2C, lanes 1 and 2). Overproduction of IDP isomerase and GGDP synthase in *E. coli* apparently increases the level of GGDP synthesis since, for the first time, taxadiene has been produced in vivo and isolated with yields approaching 0.5 mg from 1 L of cells from the above engineered strain.

Co-production of DXP synthase with IDP isomerase, GGDP synthase, and taxadiene synthase increases the yield of taxadiene

To study the effect of supplying DXP synthase with the other three enzymes, the *E. coli dxs* gene was cloned and then inserted into a plasmid with yet a third origin of replication. The gene was amplified by pcr using *E. coli* genomic DNA as template and ligated into the *Nde*I-*Bam*HI sites of pET23H1 to afford pDXPS. Soluble DXP synthase was overproduced (Fig. 2B, lane 3) at high levels in strain BL21DE3(plysS)(pDXPS) and its

activity was demonstrated by the synthesis of DXP from ^{13}C-labeled pyruvate and glyceraldehyde-3-phosphate (Fig. 4). To provide an origin of replication compatible with the other vectors already in use, a 2.3 kb *Bsp*EI fragment from pDXPS containing the T7 promoter and the DXP synthase gene was treated with T4 DNA polymerase to provide blunt ends and ligated into pZS*24-MSC1 that had been cleaved with *Bam*HI and blunt ended with T4 DNA polymerase. The resultant plasmid, pZS-DXPS, has the pSC101 origin of replication and a lower copy number but, when transformed into BL21DE3(plysS) produces soluble DXPS at levels equivalent to those observed from pDXPS (Fig. 2B lane 4). pZS-DXPS was transformed into both BL21DE3(pTS79H)(pACYC-IGS) and BL21DE3(pTS79H)(plysS-IGS). The latter strain grew better than the former, presumably due to tighter regulation of expression from the T7 promoter by T7 lysozyme prior to induction, and produced all three proteins with four enzymatic activities (Fig. 2D, lanes 1 and 2). Overproduction of DXP synthase concommitant with IDP isomerase, GGDP synthase, and taxediene synthase in this strain resulted in the isolation of 1.3 mg of taxadiene from a 1 L culture, a 2.6-fold increase over that observed with overproduction of the latter three enzymes only.

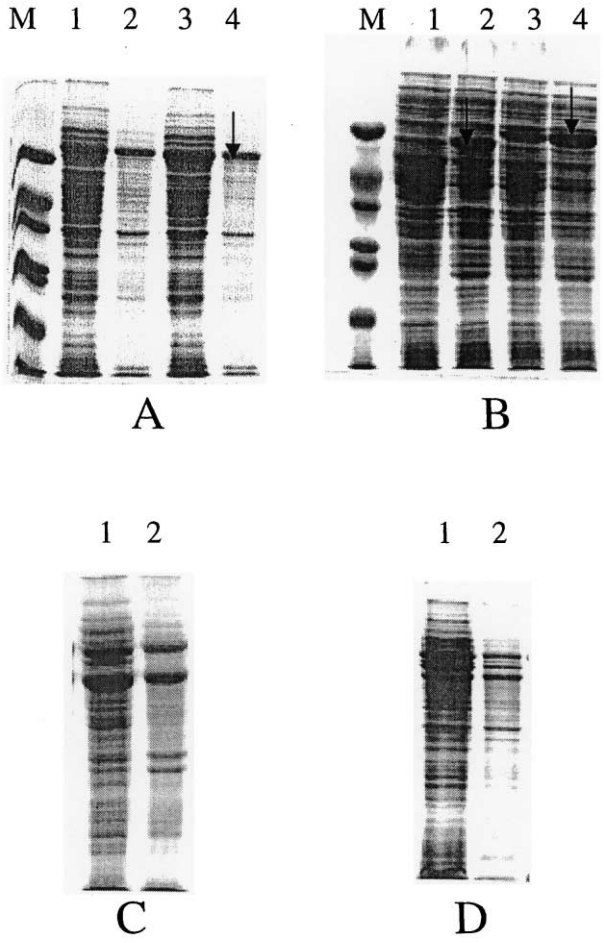

Figure 2. SDS-PAGE of cell-free extracts of *E. coli* strains overproducing enzymes for the biosynthesis of taxadiene: (A) soluble (lanes 1 and 3) and insoluble (lanes 2 and 4) fractions of non-induced (lanes 1 and 2) and IPTG-induced (lanes 3 and 4) BL21DE3(pTS79H) overproducing truncated taxadiene synthase (arrow, $M_r = 90$ Kd); (B) lanes 1 and 2: soluble fractions of BL21DE3(pIGS), BL21DE3(plysS-IGS), overproducing the IDP isomerase-GGPD synthase fusion protein (arrow, $M_r = 61$ Kd); lanes 3 and 4: soluble fractions of BL21DE3(plysS)(pDXPS), and BL21DE3(plysS)(pZS-DXPS) overproducing DXP synthase (arrow, $M_r = 66$ Kd); (C) soluble (lane 1) and insoluble (lane 2) fractions of BL21DE3(pTS79H)(pACYC-IGS); (D) soluble (lane 1) and insoluble (lane 2) fractions of BL21DE3(pTS79H)(plysS-IGS)(pZS-DXPS).

Discussion

We have described the biosynthesis of milligram quantities of taxadiene both in vitro and in vivo. The in vitro system afforded the highest yields of the product (10 mg) but is labor intensive requiring the synthesis of the substrate and the preparation of cell-free extracts. The in vitro system produces not only the true precursor of paclitaxel, but also the taxa-4(20),11(12)-diene isomer. This is the first report of the production of this isomer in a cell-free taxadiene-synthesizing reaction and suggests that there is either an equilibrium between the two isomers or that the last deprotonation by the enzyme is not completely regio-specific. A similar phenomenon is observed with the enzyme pinene synthase which produces both α-pinene and β-pinene from geranyl diphosphate.[12]

The in vivo system is capable of producing milligram quantities of taxadiene from common starting materials such as glucose and glycerol and is, thus, more labor and cost efficient. Overproduction of DXP synthase simultaneously with IDP synthase, GGDP synthase and taxadiene synthase afforded the best yields in vivo presumably due to shunting of the substrates, pyruvate and glyceraldehyde-3-phosphate, down the isoprenoid pathway. DXP, however, is also a precursor of thiamin and pyridoxal phosphate. Experiments are under way to study the effect of engineering the first truly committed enzyme of the pathway, DXP reductoisomerase, which catalyzes the conversion of DXP to C-methyl-4-erythritol-4-phosphate.[13]

Conclusion

E. coli can be genetically engineered for the production of the rare terpenoid intermediate, taxadiene.

Experimental

Bacterial strains and plasmids

The bacterial strains and plasmids used are listed in Table 1.

Chemicals, enzymes, and buffers

4-^{13}C-IDP and unlabeled IDP were synthesized by methods reported earlier.[19] Sodium 3-^{13}C-pyruvate was from Aldrich, Milwaukee, WI, USA. Oligonucleotides were synthesized by the Gene Technology Lab at Texas

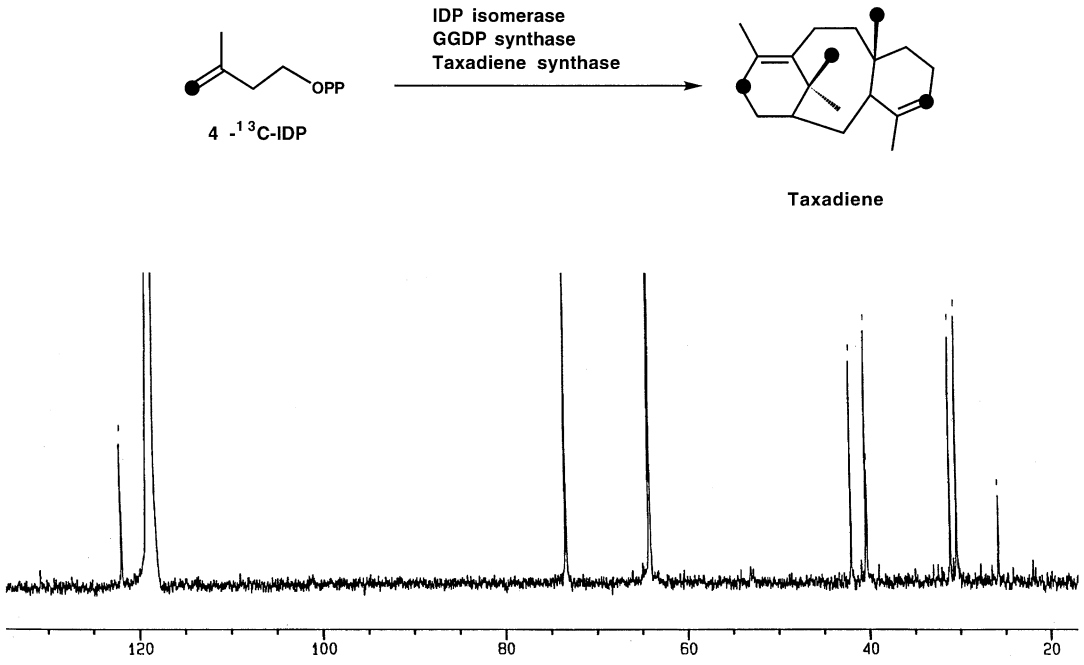

Figure 3. ^{13}C NMR spectrum of the products synthesized from 4-^{13}C-IDP (chemical shift = 111.0 ppm) in a small scale cell-free reaction containing IDP isomerase, GGDP synthase and truncated taxadiene synthase. The signals at 40.2 and 25.8 ppm are from GGDP and the signals at 121.9, 42.0, 31.1 and 30.4 ppm are from taxadiene. The major peaks are from acetonitrile (118.8 ppm) and glycerol (63.9, 73.3 ppm).

A&M University. Molecular biology techniques were performed according to standard protocols[20] using enzymes from New England Biolabs. PCR reactions were performed with *Pfu*Turbo from Stratagene. DNA purification kits were purchased from Qiagen. ^{13}C NMR analyses were performed on a Bruker AM 500 spectrometer. Buffer A: 50 mM phosphate, pH 7.0, 10 mM KF, 1 mM DTT, 5 mM $MgCl_2$; Buffer B: 30 mM HEPES, pH 8.4, 10% glycerol, 5 mM ascorbate, 5 mM $Na_2S_2O_5$, 10 mM KF, 2 mM dithiothreitol, 1 mM β-cyclodextrin.

Preparation of cell-free extracts

E. coli strains were incubated at 37 °C with good aeration in Luria-Bertani broth containing the appropriate antibiotic(s) to an A_{600} of 0.6–1.0, induced by the addition of 50 mg/L IPTG, and incubated overnight at room temperature. Antibiotics were added at the following concentrations when required: ampicillin, 50 μg/mL, kanamycin, 50 μg/mL and chloramphenicol, 34 μg/mL. The cells were pelleted by centrifugation, resuspended in buffer A or B (25 mL/L of cells) and lysed by sonication. The lysate was centrifuged at 10,000 ***g*** for 10 min to remove cell debris, and the cell-free extract used without further purification as the source of enzymes.

DXP synthase activity assay

A mixture containing 1 mg sodium 3-^{13}C-pyruvate, 5 mg DL-glyceraldehyde-3-phosphate, 5 mg thiamine diphosphate and 100 μL of a cell-free extract containing DXP synthase in a total of 1 mL of buffer A was incubated at 37 °C for various time periods. A 400 μL portion of the solution was adjusted to 20% D_2O for ^{13}C NMR analysis.

In vitro synthesis of taxadiene

Small-scale reactions contained, in a total of 5 mL of buffer B, 1 mg of 4-^{13}C-IDP and the following cell-free extracts: 200 μL from strain JM101(pFMH6), 600 μL from strain JM101(pSM145), and 2000 μL from strain BL21DE3(plysS)(pTS79H) containing IDP isomerase, GGDP synthase, and taxadiene synthase, respectively. The solution was incubated overnight at 30 °C during which time a milky white precipitate formed. The precipitate was pelleted by centrifugation at 13,000 ***g*** for 5 min, extracted with 400 μL acetonitrile-d_3 and analyzed by ^{13}C NMR spectroscopy.

Larger syntheses were scaled up to 2 L of buffer B containing 15 mL of IDP-isomerase extract, 60 mL of GGDP-synthase extract and 200 mL of taxadiene synthase extract derived from the above strains. To provide unlabelled substrate, 360 mg of isopentenyl tosylate were converted to IPP by the method of Davisson et al.[19] and added to the cell free extract without further purification. After an overnight incubation at 30 °C, the taxadiene was extracted into hexane, concentrated and purified by chromatography on a silica column with hexane as eluant. The fractions containing taxadiene were pooled and dried, and the amount of taxadiene obtained was determined by weighing.

In vivo synthesis of taxadiene

Strain BL21DE3(pTS79H)(pACYC-IGS) or strain BL21DE3(pTS79H)(plysS-IGS)(pZS-DXPS) was grown at 37 °C in LB medium supplemented with 2% glucose and antibiotics (ampicillin and chloramphenicol for the former-ampicillin, chloramphenicol and kanamycin for the latter) to an A_{600} of 0.6–0.9. The culture was cooled

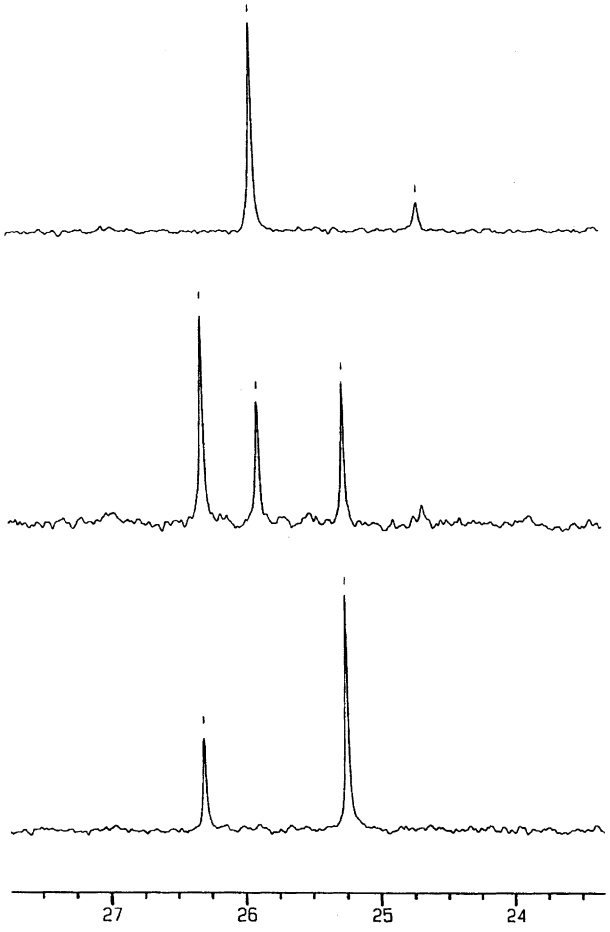

Figure 4. ^{13}C NMR spectra of the products obtained in incubations containing ^{13}C-labeled pyruvate, glyceraldehyde-3-phosphate, and cell-free extracts of BL21DE3(plysS)(pDPXS), as a source of DXP-synthase, after 0 h (top), 3 h (middle), and 16 h (bottom) at 37 °C. The peaks at 25.8 and 26.3 ppm correspond to pyruvate and DXP, respectively. With longer incubations, the DXP is dephosphorylated to deoxyxlulose seen at 25.2 ppm.

to room temperature, induced by adding 50 mg/L of IPTG, and incubated at room temperature for an additional 24 h. The cells were pelleted by centrifugation, resuspended in 100 mL water, lysed by sonication, and the taxadiene extracted into hexane and purified as described above. Yield 1.3 mg/L of culture.

Construction of pET23H1 and pET23H2

Two derivatives of pET23 were constucted to provide plasmids an with N-terminal 6X his-tag and either *Nde*I or *Nco*I sites for inserting pcr products. For construction of pET23H1, the 40 bp *Xba*I-*Nde*I fragment of pET23 was replaced with the 96 bp *Xba*I-*Nde*I fragment from pET15 to providing the sequence rbs-*Nco*I-6X his-*Nde*I-*Nhe*I. For construction of pET23H2, a pcr product derived from pET23H1 was ligated into pET23 to give the sequence rbs-*Nde*I-6X his-*Nco*I-*Nhe*I.

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