STUDIES FOR ENHANCING CARBON FLOW THROUGH THE DXP AND SHIKIMATE PATHWAY

By

Padmesh Venkitasubramanian

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ABSTRACT

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Isoprenoids constitute a diverse family of secondary metabolites found in nature with a broad range of application from the food industry to pharmaceutical industry. The recently discovered 1-deoxy-D-xylulose 5-phosphate pathway for isoprenoid biosynthesis in bacteria and plants provides an avenue for producing a wide range of industrially relevant molecules by metabolic engineering of this pathway. Carbon flow through this pathway was monitored using *Escherichia coli* strains with amplified levels of *dxr*-encoded 1-deoxy-D-xylulose 5-phosphate synthase. Accumulation of 1-deoxy-D-xylulose was examined in *E. coli* DXS2.1, which lacks *dxr*-encoded 1-deoxy-D-xylulose 5-phosphate reductoisomerase; in *E. coli* SP1.1, which express wild-type levels of the reductoisomerase; in *E. coli* SP1.1 cultured in the presence of fosmidomycin, which is an inhibitor of the reductoisomerase; and in *E. coli* SP1.1 carrying the mevalonate pathway genes (*mvk*, *pmd*, *pmk* and *hmgR*) when cultured in the presence of fosmidomycin and mevalonic acid. *E. coli* DXS2.1/pPV4.230 cultures with yeast extract supplementation resulted in the synthesis of 18 g/L of 1-deoxy-D-xylulose in 10% (mol/mol) yield from glucose.

In recent years, the shikimate pathway has been exploited for the microbial synthesis of several industrially applicable chemicals. The factors governing the flow of

carbon through this pathway have been identified and studied in detail for developing suitable *E. coli* biocatalyst. One of the key factors for enhancing the carbon flow through this pathway is the *in vivo* availability of precursors molecules phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate (E4P). Attempts were made at understanding the precursor limitation during microbial synthesis of shikimic acid. The precursors molecules were chemically synthesized and their impact as supplements were evaluated during fed-batch fermentor synthesis of shikimic acid. Chemical synthesis of E4P was accomplished from *cis*-2-butene-1,4-diol. The advantage of this synthesis was the protection of the aldehyde moiety as a 1,3-dioxalane and thus preventing the dimerization and polymerization of E4P during its synthesis. Supplementation of *E. coli* SP1.1/pSP2.054 that had PEP uptake capability with 10 g PEP resulted in the synthesis of 59 g/L of shikimic acid in 20% yield. E4P supplementation was deleterious to the cell growth and did not result in increase in yield and titer of shikimic acid.

To my family

For their love and encouragement

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LIST OF ABBREVIATIONS

Ac	acetyl
ADP	adenosine diphosphate
AMP	adenosine monophosphate
Ap	ampicillin
Arg	L-arginine
Asp	L-aspartic acid
ATP	adenosine triphosphate
bp	base pair
Bu	butyl
BTX	benzene, toluene and xylene
CDP-ME	4-diphosphocytidyl-2-*C*-methyl-D-erythritol
CI	chemical ionization
CIAP	calf intestinal alkaline phosphatase
Cm	chloramphenicol
CMP	cytidine monophosphate
CMP	counts per minute
COMT	catechol-*O*-methyltransferase
CTP	cytidine triphosphate
DAH	3-deoxy-D-*arabino*-heptulosonic acid
DAHP	3-deoxy-D-*arabino*-heptulosonic acid 7- phosphate

DCU	digital control unit
DEAE	diethylaminoethyl
DHAP	dihydroxyacetone 1-phosphate
DHQ	3-dehydroquinate
DHS	3-dehydroshikimic acid
DMAPP	dimethylallyl pyrophosphate
DNA	3-deoxyribonucleic acid
DO	dissolved oxygen
DTT	dithiothreitol
DX	1-deoxy-D-xylulose
DXP	1-deoxy-D-xylulose-5-phosphate
DXR	1-deoxy-D-xylulose 5-phosphate reductoisomerse
EI	electron impact
E4P	D-erythrose 4-phosphate
EPSP	5-enolpyruvylshikimate 3-phosphate
FAB	fast atom bombardment
FBR	feed back resistant
FT	fourier transform
G3P	D-glyceraldehydes 3-phosphate
G6p	D-glucose 6-phosphate
GA	gallic acid
GAP	D-glyceraldehydes 3-phosphate
Glu	L-glutamate

h	hour
His	L-histidine
HMBPP	1-hydroxy-2-methylbut-2-ene pyrophosphate
HRMS	high resolution mass spectrometry
IPP	isopentenyl pyrophosphate
Kan	kanamycin
K_i	inhibition constant
K_m	Michaelis constant
k	rate constant
kg	kilogram
LB	luria broth
Lys	L-lysine
M	molar
Me	methyl
ME	2-*C*-methyl-D-erythritol
MGP	methyl α-D-glucopyranoside
mL	milliliter
mM	millimolar
MOPS	4-morpholinepropanesulfonic acid
MS	mass spectrometry
min	minute
MVA	mevalonate
NAD	nicotinamide adenine dinucleotide, oxidized form

NADH	nicotinamide adenine dinucleotide, reduced form
NADP	nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NMR	nuclear magnetic resonance
OD	optical density
PABA	*p*-aminobenzoic acid
PCA	protocatechuic aid
PEG	polyethylene glycol
PEP	phosphoenolpyruvate
PGT	phosphoglycerate transport
Ph	phenyl
PHB	*p*-hydroxybenzoic acid
PID	proportional-integral-derivative
PCR	polymerase chain reaction
ppm	parts per million
pps	PEP synthase
*i*Pr	isopropyl
PTS	phosphotransferase system
QA	quinic acid
rt	room temperature
rpm	revolutions per minute
SA	shikimic acid
Ser	L-serine

S3P	shikimate 3-phosphate
SDS	sodium dodecyl sulfate
Tc	tetracycline
TBDMS	*t*-butyldimethylsilane
THF	tetrahydrofuran
TM	transmembrane
TMS	trimethylsilyl
TSP	sodium 3-(trimethylsilyl)propionic-2,2,3,3-d_4
UV	ultraviolet
Val	L-valine

CHAPTER 1

Introduction

Chemists have always explored methods for improving a chemical process so that it becomes more economically viable. This involves selective catalysis, using a more viable source of raw material, and modifying the synthetic procedure. The chemical manufacturing industry consumes 7% of the annual US energy output that is mostly derived from petroleum feed stocks.[1] With constantly changing geopolitical situations and tightened environmental regulations, the issue of long-term sustainability due to the high-energy cost is a major challenge for the chemical industry. One way to address the issue of long-term sustainability is by shifting from fossil fuels to a bio-based feedstock.[2]

The use of biological systems in a chemical process brings along with it the added advantage of versatility, regioselectivity, chemoselectivity, enantioselectivity, catalysis at ambient temperature and pressure, and the use of environmentally friendly protocols. This avoids use of toxic reagents, volatile organic solvents, and processing of waste streams that have adverse impact on the environment. Advances in DNA technology, increased knowledge of cell physiology, genetics, and fermentation process development has driven the development of commercially viable processes for the production of chemicals.[3] In order to develop a process from substrate to final product in high yields and large quantities, one needs to establish an appropriate production host and metabolic pathway with a focus on the entire metabolic machinery of the host. This requires identifying the rate-limiting impediments in the pathway, branch points in the pathway and enhancing the carbon flow in the primary metabolic pathway.[3c]

The work carried out in completion of this thesis is an attempt to address these issues of pathway engineering. In Chapter 2 of this thesis the recently discovered 1-deoxy-D-xylulose-5-phosphate (DXP) pathway for isoprenoid biosynthesis has been studied for the identification of potential rate-limiting impediments to the carbon flow through the DXP pathway. Measuring the carbon flow through the DXP pathway can help in designing better biocatalysts for producing a variety of secondary metabolites derived from this pathway. Pathway engineering has been applied to the DXP pathway to develop a biocatalytic process for obtaining novel sugars like 1-deoxy-D-xylulose from D-glucose.

Chapter 3 of this thesis deals with the issue of precursor limitations in the shikimate pathway and looks at a semisynthetic approach as a tool for understanding this problem. Shikimic acid has been used as the model compound in this study. Engineering of shikimate pathway for a source of value-added chemicals has been exemplified with the synthesis of chemicals like vanillin, adipic acid, phenol, and catechol. Understanding the precursor limitations can help in designing better biocatalysts for producing high volumes of industrial chemicals.

The 1-Deoxy-D-Xylulose 5-Phosphate (DXP) Pathway

Isoprenoids constitute a diverse family of secondary metabolites found in nature and are involved in a variety of physiological functions.[4] These molecules participate as structural components of cell membranes (steroids), mediate cellular redox chemistry (ubiquinone, menaquinone), help in protein glycosylation (dolichols), function as photoreceptors in marine organisms (carotenoids), and are also involved in signal transduction (polyprenylated proteins).[5] These natural products have a broad spectrum of applications such as imparting flavor or color to food (e. g. zeaxanthine, astaxanthine)[5] and aroma to various products (e. g. linalool).[6] They also serve as disinfectants (e. g. camphor, α-pinene), anticarcinogenic agents (e. g. taxol),[7] antioxidants (e. g. lutein, β-carotene),[8] and as nutritional supplements (e. g. vitamins A, E, K).[9] These commodity compounds are either isolated from plant sources or are chemically synthesized.[10]

Biosynthesis of all isoprenoids begins with one or both of the two C_5 building blocks of the pathway: isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). In eukaryotes and plants, the biosynthesis of isopentenyl pyrophosphate (IPP) occurs via the well-known mevalonate (MVA) pathway (Figure 1).[11a] Discovered in the early 1950's the biosynthesis of the isoprenoid precursors, IPP and DMAPP, begins with condensation of three acetyl-CoA molecules to give 3-hydroxy-3-methylglutaryl-CoA. 3-Hydroxy-3-methylglutaryl-CoA is then reduced to MVA.[11b] Two sequential phosphorylations of mevalonic acid followed by a phosphorylation-assisted decarboxylation of mevalonate 5-pyrophosphate yields IPP. DMAPP is then derived from IPP by the action of an IPP isomerase.[11c]

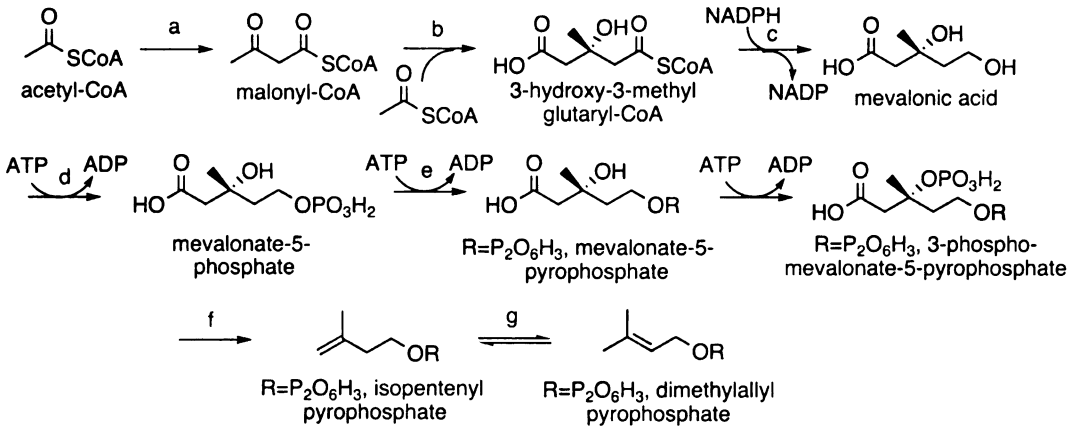


Figure 1. Mevalonate pathway for isoprenoid biosynthesis.
Key: Enzymes (genetic loci) a) acetylcoenzyme A synthetase (*acsA*); b) 3-hydroxy-3-methylglutaryl coenzyme A synthase (*hmgs*); c) 3-hydroxy-3-methylglutaryl coenzyme A reductase (*hmgr*), d) mevalonate kinase (*mvk*); e) phosphomevalonate kinase (*pmvk*); f) mevalonate-5-diphosphate decarboxylase (*pmd*); g) isopentenyldiphosphate isomerase (*idi*).

In plant chloroplast, algae, cyanobacteria, and most bacteria, an alternative pathway known as 1-deoxy-D-xylulose 5-phosphate (DXP) pathway produces the isoprenoid precursors IPP and DMAPP (Figure 2).[12] This pathway uses pyruvate and D-glyceraldehyde-3-phosphate as the initial precursors rather than acetyl-CoA.[12b,c] The initial step in this pathway involves formation of 1-deoxy-D-xylulose 5-phosphate by the condensation of pyruvate with the C_1 aldehyde moiety of D-glyceraldehyde-3-phosphate.[12] This thiamine-dependant reaction is catalyzed by 1-deoxy-D-xylulose-5-phosphate synthase (DXS) which is encoded by the *dxs* gene.[13] In *Arabidopsis*[14] and photosynthetic bacteria *Rhodobacter capsulatus*,[15] DNA sequences with homologies to the *dxs* gene have been identified. The presence of these additional *dxs* genes elsewhere in the genome and not in the photosynthesis-related gene cluster suggests that these genes may be used in early developmental stages or in specific organelles.

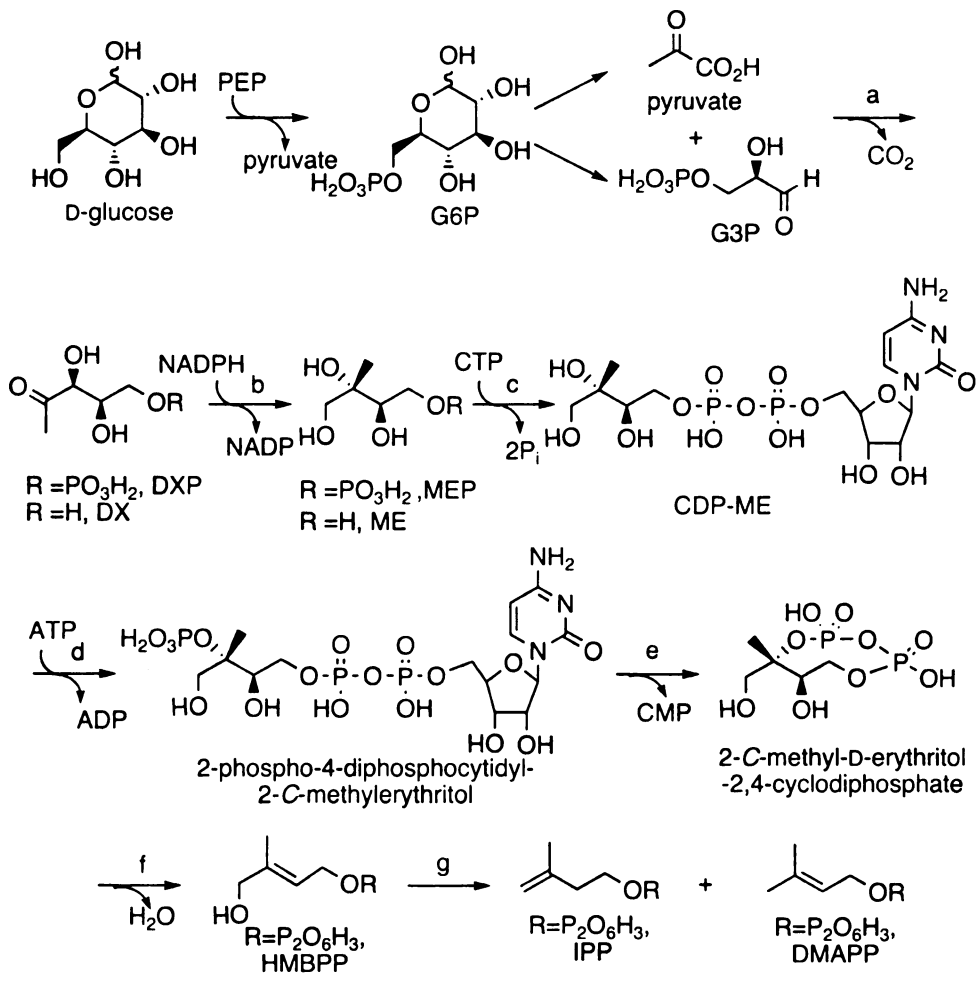


Figure 2. DXP pathway for the biosynthesis of IPP and DMAPP.
Abbreviations: phosphoenolpyruvate (PEP) glucose-6-phosphate (G6P); glyceraldehyde-3-phosphate (G3P); 1-deoxy-D-xylulose (DX)-5-phosphate (DXP); 2-*C*-methyl-D-erythritol (ME)-4-phosphate (MEP); 4-diphosphocytidyl-2-*C*-methyl-D-erythritol (CDP-ME); 1-hydroxy-2-methylbut-2-ene pyrophosphate (HMBPP); isopentenyl pyrophosphate (IPP); dimethyallyl pyrophosphate (DMAPP). Key enzymes (genetic loci): a) 1-deoxy-D-xylulose synthase (*dxs*); b) 1-deoxy-D-xylulose-5-phosphate reductoisomerase (*dxr*); c) 4-diphosphocytidyl-2-*C*-methylerythritol synthetase (*ispD*); d) 4-diphosphocytidyl-2-*C*-methylerythritol kinase (*ispE*); e) 2-*C*-methylerythritol 3,4-cyclophosphate synthase (*ispF*); f) 1-hydroxy-2-methylbut-2-ene pyrophosphate synthase (*ispG*); g) 1-hydroxy-2-methylbut-2-ene pyrophosphate reductase (*ispH*).

In the second step of the pathway, the enzyme 1-deoxy-D-xylulose-5-phosphate reductoisomerase encoded by the gene *dxr* (also known as *ispC* or *yaeM*)[16] converts 1-deoxy-D-xylulose-5-phosphate into 2-*C*-methyl-D-erythritol 4-phosphate (MEP) by an intramolecular rearrangement followed by NADPH reduction. 2-*C*-Methylerythritol 4-

phosphate is then converted into a cytidine derivative by the *ispD* gene product to give 4-diphosphocytidyl-2-*C*-methyl-D-erythritol (CDP-ME).[17] ATP-dependent phosphorylation of the 2-hydroxyl moiety of CDP-ME by the *ispE*-encoded kinase[18] gives 2-phospho-4-diphosphocytidyl-2-*C*-methyl-D-erythritol. The *ispF* gene product catalyzes the intramolecular displacement of cytidine monophosphate (CMP) to yields 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate.[19] The product 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate has been reported to be generated by bacteria in response to oxidative stress.[20] In the subsequent steps of the DXP pathway, the 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate is converted to 1-hydroxy-2-methyl-2-butene-4-pyrophosphate by the *ispG* encoded 1-hydroxy-2-methyl-2-butene-4-pyrophosphate synthase.[21] In the final step, 1-hydroxy-2-methyl-2-butene-4-pyrophosphate is converted to IPP and DMAPP by a Fe-S dependant reductase encoded by the *ispH* gene.[22] The 5:1 ratio of IPP to DMAPP is different compared to MVA pathway. In the latter case *idi*-encoded isomerase converts the derived IPP to DMAPP. Although the *idi* gene has been identified in bacteria, it is not necessary for the biosynthesis of IPP and DMAPP.[23]

DXP is not only an intermediate of the DXP pathway but is also involved in the biosynthesis of vitamins B_1 and B_6.[24] DXP is incorporated in the thiazole ring of vitamin B_1 (Figure 3)[25] and in the pyridine ring of vitamin B_6 (Figure 4).[26] The presence of the DXP pathway in pathogenic bacteria and mycobacteria has provided an avenue for development of new therapeutics. Human Vg9/Vd2 T cells are considered to play an important role in immune response against various microbial pathogens. It has been reported that the intermediates of the DXP pathway are responsible for Vg9/Vd2 T cell activation in response to infection.[27] The DXP pathway has also been observed in

Plasmodium falciparum and has been projected as a route for developing new anti-malarial agents.[28]

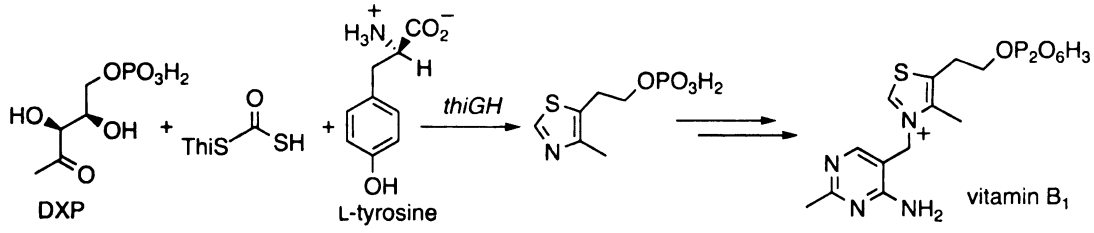


Figure 3. Incorporation of DXP into the thiazole ring of vitamin B_1.

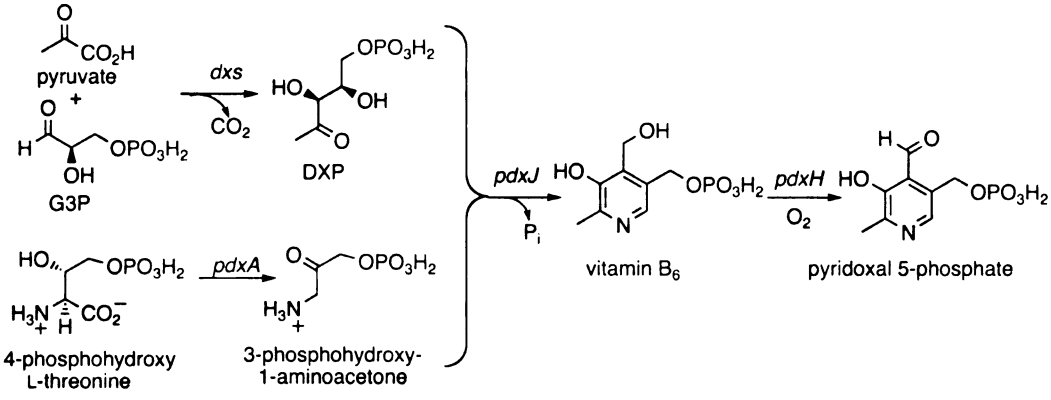


Figure 4. Biosynthesis of vitamin B_6.

With the complete elucidation of DXP pathway and the recent understanding of mechanisms of the enzymes involved in the pathway, a new route is now available for the discovery of novel compounds for pharmaceutical and industrial application.[29] The use of the DXP pathway as a source for value-added chemicals requires identification of the rate-limiting impediments to the carbon flow through the pathway. This systematic understanding of the carbon flow will further our knowledge and aid in the development of a better biocatalyst for the production of value-added chemicals like nutritional supplements, carotenoids, and antioxidants.

The Shikimate Pathway

In plants and microorganisms, the shikimate pathway (Figure 5) serves as a route for the biosynthesis of aromatic amino acids and aromatic vitamins.[30] The carbohydrate precursor molecules are transformed by seven enzyme-catalyzed reactions into carbocyclic intermediates culminating with the biosynthesis of chorismic acid (Figure 5). This metabolic sequence from carbohydrates to chorismate is often referred to as the common pathway of aromatic amino acid biosynthesis. Chorismic acid serves as a common pathway intermediate in the biosynthesis of the aromatic amino acids L-phenylalanine, L-tyrosine, and L-tryptophan. In addition to the aromatic amino acids, several thousand secondary metabolites are derived from this pathway. This includes various isoprenoid quinones, enterochelin, and folic acid coenzyme derivatives.[30] These secondary metabolites are required in essential cellular functions such as electron transport (quinones) and iron uptake (enterochelin). Coenzymes derived from folic acid are involved in the biosynthetic transfer of one carbon atom.

The common pathway for aromatic amino acids begins with the irreversible condensation of phosphoenolpyruvate (PEP) with D-erythrose 4-phosphate (E4P) resulting in 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) that is catalyzed by the enzyme DAHP synthase (Figure 5).[30] In *Escherichia coli*, *aroF*, *aroG*, and *aroH* genes encode for three isozymes of this enzyme. Dehydroquinate (DHQ) synthase encoded by the *aroB* gene converts DAHP into carbocyclic DHQ in a reaction that is catalytic in NAD.[31] The *aroD*-encoded DHQ dehydratase catalyzes the elimination of a molecule of water from DHQ resulting in the formation of dehydroshikimate (DHS).[32]

NADPH dependant reduction of the ketone moiety in DHS by *aroE* encoded shikimate dehydrogenase affords shikimic acid (SA).[33]

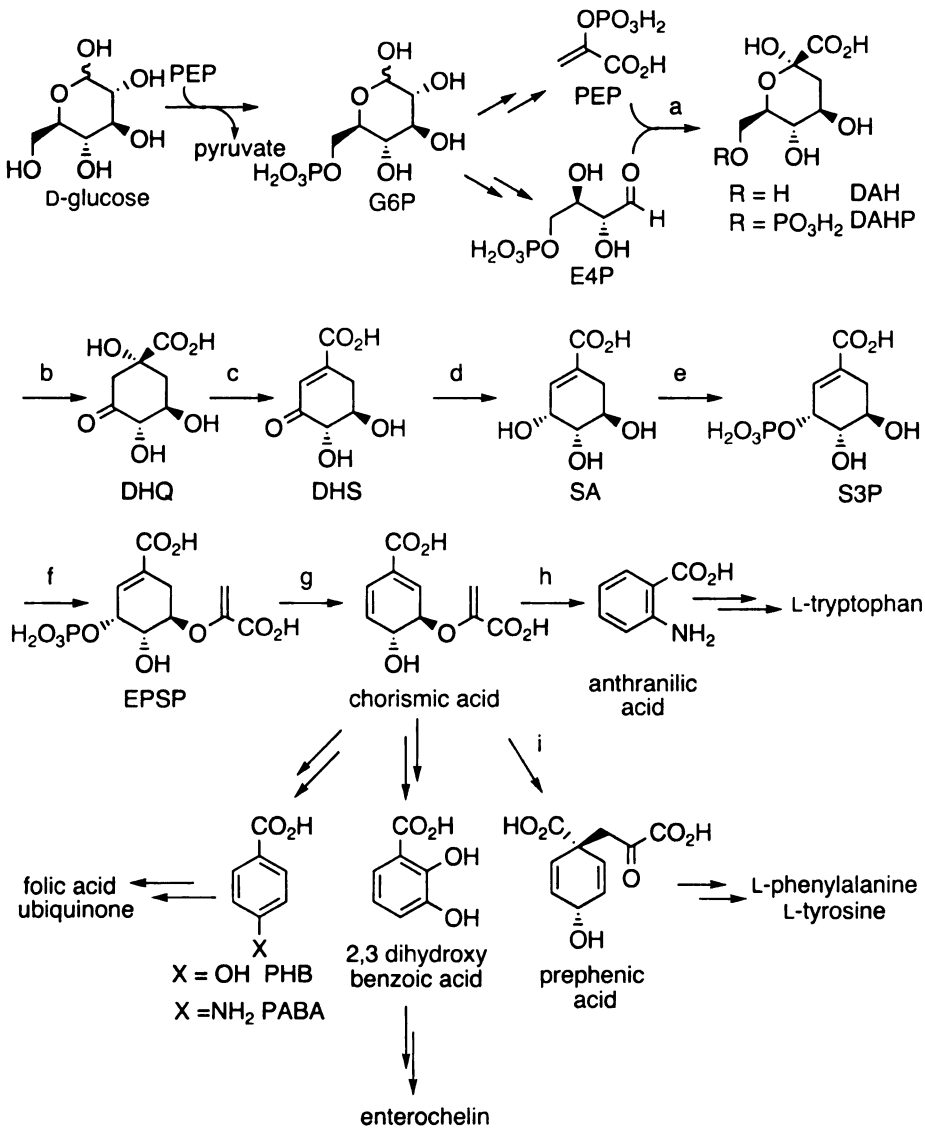


Figure 5. Biosynthesis of aromatic amino acid by the shikimate pathway.
Key: Intermediates (abbreviation); D-glucose 6-phosphate (G6P), Phosphoenolpyruvate (PEP), D-erythrose 4-phosphate (E4P), 3-deoxy-D-*arabino*-heptuosonic acid (DAH) 7-phosphate (DAHP), 3-dehydroquinic acid (DHQ), 3-dehydroshikimic acid (DHS), shikimic acid (SA), shikimate-3-phosphate (S3P), enolpyruvoylshikimate 3-phosphate (EPSP) *p*-hydroxybenzoic acid (PHB) *p*-aminobenzoic acid (PABA). Key enzymes (genetic loci): a) DAHP synthase (*aroF*, *aroG*, *aroH*), b) DHQ synthase (*aroB*), c) DHQ dehydratase (*aroD*), d) shikimate dehydrogenase (*aroE*), e) shikimate kinase (*aroK*, *aroL*), f) EPSP synthase (*aroA*), g) chorismate synthase (*aroC*), h) anthrinilate synthase (*trpE*), i) chorismate mutase/prephenate dehydrogenase (*pheA*).

Phosphorylation of the 3-hydroxy group in shikimic acid by *aroL*[34] and *aroK*[35] encoded shikimate kinase isozymes affords shikimate-3-phosphate (S3P). Condensation of shikimate-3-phosphate with PEP to yield enolpyruvoylshikimate-3-phosphate (EPSP) in a reaction catalyzed by *aroA*-encoded ESPS synthase.[36] In the final step, *aroC* encoded chorismate synthase converts EPSP to chorismic acid by the loss of inorganic phosphate.[37] Chorismic acid serves as the branch point for the biosynthesis of aromatic amino acids and other secondary metabolites derived from this pathway.

Microbial Synthesis of Value-Added Chemicals

Plants and microrganisms produce a wide range of primary and secondary metabolites. Harnessing of this source for producing value-added chemicals has led to the development of biocatalysis as an alternative technology for the synthesis of bulk chemicals, chiral synthons, and food ingredients. The term biocatalysis encompasses both biotransformations of organic molecules as well as fermentation technology.[38] Biotransformation involves conversion of a precursor organic molecule to product by either an enzyme or a microbial host. The term fermentation describes a process that involves synthesis of a desired product, by incorporating a single enzyme or a biosynthetic pathway in a microbial host, from a renewable source of carbon.[38] The use of biocatalysis for commercial production of chemicals has been exemplified with the microbial synthesis of L-amino acids,[39] L-ascorbic acid,[40] indigo,[41] and 1,3-propanediol.[42] This section will focus on examples of biocatalysis for developing a route for value-added chemicals derived from the shikimate and DXP pathway. These

commodity chemicals are currently being isolated from their natural source or obtained via organic synthesis.

Value-Added Chemicals Derived from the Shikimate Pathway.

Aromatic compounds and bulk chemicals derived by subsequent chemical modification of aromatic compounds constitute a majority of the products produced by the chemical industry.[43a] Most of these chemicals are derived from the benzene, toluene, and xylene (BTX) fraction of petroleum refining or from coal. Exposure to benzene has been linked to both acute leukemia and non-Hodgkin's lymphoma.[43b-d] In addition to the fact that all aromatic starting materials are derived from non-renewable sources, benzene, toluene and xylenes are highly volatile and require expensive measures to reduce release into to the environment.[43e,f] Benzene is a hazardous organic air pollutant whose emission must be reduced as mandated by the Chemical and Manufacturing Rule issued by the U. S. Environmental Protection Agency.[43g,h] Manipulation of the shikimate pathway for producing the aromatic compounds and other value-added chemicals opens an avenue for reducing the dependence on fossil feedstocks.

3-Dehydroshikimic acid

3-Dehydroshikimic acid (DHS) is a key hydroaromatic intermediate in the shikimate pathway (Figure 6). It can be used as a starting material for the chemical or biocatalytic synthesi of a variety of industrial chemicals. DHS has been reported to be a potent antioxidant.[44] Chemical oxidation of DHS affords gallic acid.[44a] Gallic acid is also produced bicatalytically from glucose via the intermediacy of DHS and protocatechuic acid (PCA).[45] DHS dehydratase encoded by the *aroZ* locus of *Klebsiella*

pneumonia, converts DHS into PCA. Hydroxylation of PCA by a mutant *p*-hydroxybenzoate hydoxylase isolated form *Pseudomonas fluorescens* affords gallic acid.[45] Gallic acid serves as the precursor to trimethoprim, an antibacterial agent and pyrogallol. Pyrogallol is used in photographic developing solutions. In addition, DHS also serves as the precursor for the syntheses of catechol,[46c] adipic acid,[51] and vanillin (Figure 6).[55]

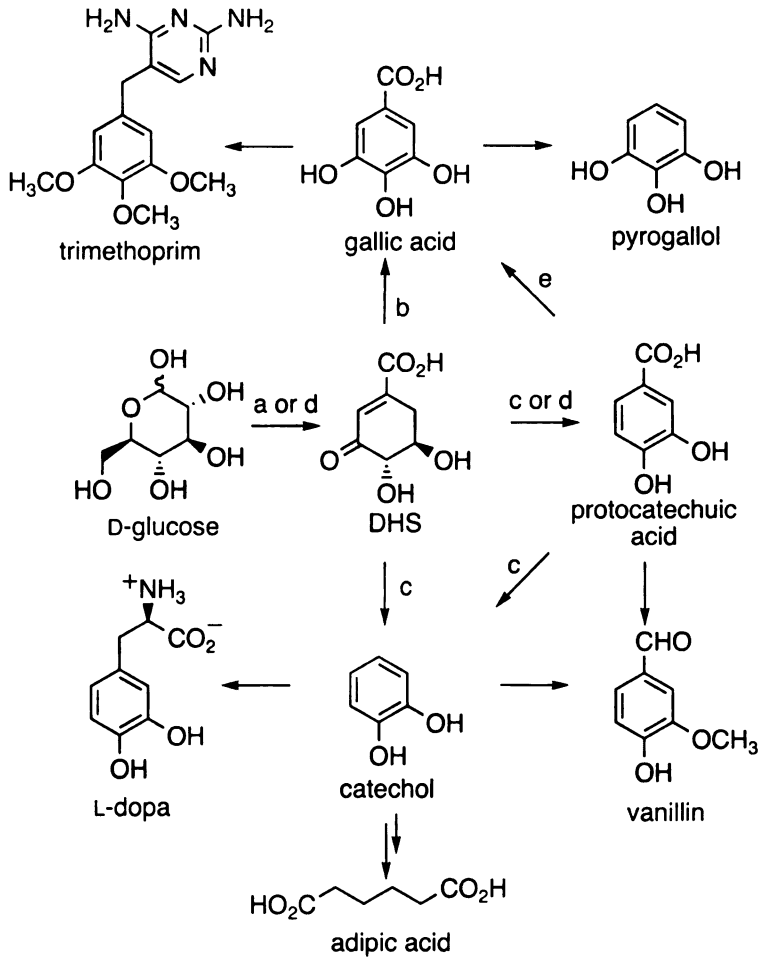


Figure 6. Biosynthesis of 3-dehydroshikimic acid and its various synthetic applications.
Key a) *E. coli* KL3/pJY1.216 ; b) O_2, H_2O or Cu^{+2} Zn^{+2} H_2O_2; c) near-critical water, CO_2[47c] ; d) *E. coli* KL3/pWL2.46B[47c]; e) *p*-hydroxybenoate hydroxylase.

E. coli KL3 carries a mutation in the *aroE* locus and is incapable of converting DHS into shikimic acid.[46a] Introducing a copy of the *aroB* gene in the *serA* locus prevented accumulation of DAHP in the culture medium. Production of DHS was enhanced by transformation of *E. coli* KL3 with plasmid pJY1.216A which carries P_{aroF}-*aroF*^{FBR}, the *serA* locus, *tktA*[61a] and P_{tac}*pps* inserts.[46b] Cultivation of KL3/pJY1.216A under glucose-rich fed-batch fermentation conditions gave a titer of 69 g/L of DHS with a 35% (mol/mol) yield from glucose (Figure 6).[46b] A biocatalytic route for producing PCA and catechol from D-glucose has also been developed using DHS as an intermediate.[46c]

Adipic Acid

Adipic acid is an important chemical intermediate in the manufacture of numerous industrial and consumer products including nylon-6,6, polyurethane, lubricants, and plasticizers.[47] Global production of adipic acid exceeds 2.2 x 10^9 kg/year, and it is mostly produced from benzene. Hydrogenation of benzene to cyclohexane, followed by air oxidation, affords a mixture of cyclohexanol and cyclohexanone. Exhaustive oxidation of the cyclohexyl intermediates with nitric acid yields adipic acid (Figure 7).[48] At one time the manufacture of adipic acid accounted for 10% of the global nitrous oxide generated from anthropogenic sources.[49a] Nitrous oxide is 200 times more potent as a greenhouse gas than carbon dioxide and contributes to ozone depletion and global warming.[49]

Acinetobacter calcoaceticus can catabolize catechol via the keto-adipate pathway. The *catA* encoded catechol 1,2-dioxygenase of *A. calcoaceticus* catalyzes the oxidation of catechol to *cis,cis*-muconic acid.[50] Microbial synthesis of *cis,cis*-muconate from D-

glucose has been accomplished by incorporation of the *catA* gene into the *E. coli aroE* auxotroph WN1.[51a] *E. coli* WN1 harbors a *aroBaroZ* cassette in the genomic *serA* locus and *tktAaroZ* cassette in the genomic *lacZ* locus. Overexpresssion of the plasmid-based $aroF^{FBR}$ along with two genomic copies of *aroB* and two genomic copies of *tktA* increases carbon flow directed towards DHS synthesis. The double genomic copies of *aroZ* convert DHS into PCA. Plasmid pWN2.248, harboring *aroY*-encoded PCA decarboxylase from *K. pneunoniae* and $P_{tac}catA$ genes, converted PCA into *cis,cis*-muconate. The construct WN1/pWN2.248 afforded 37 g/L of *cis,cis*-muconic acid from D-glucose with 23% yield (mol/mol). The clarified fermentation broth was hydrogenated with 10% Pt/C (5% mol /mol) at 50 psi H_2 to afford adipic acid in 97% yield from *cis,cis*-muconic acid (Figure 7).[51] Bridging biocatalysis with chemical catalysis has helped in developing a route for adipic acid synthesis from carbohydrate feedstocks.

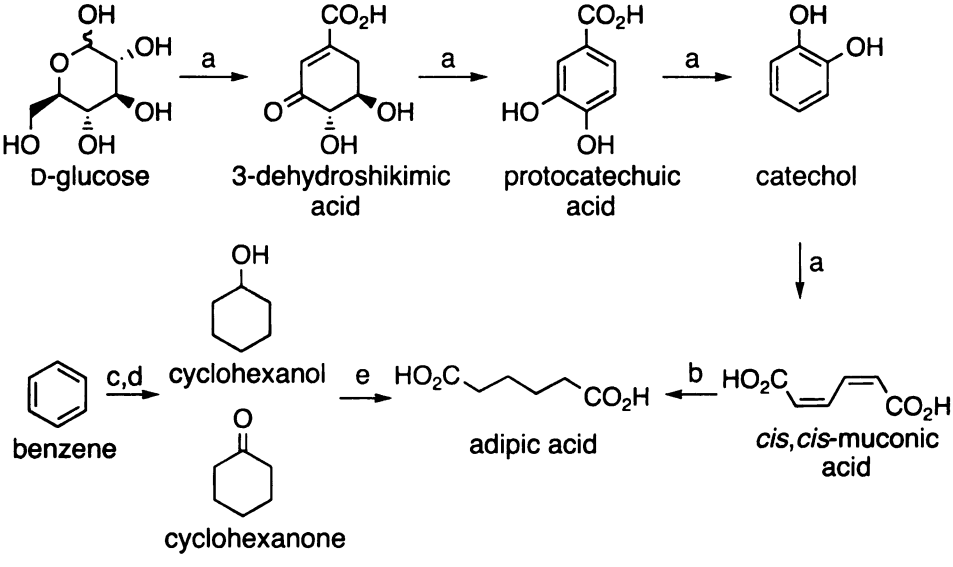


Figure 7. Synthesis of adipic acid from benzene and D-glucose.
Key: a) *E. coli* WN1/pWN2.248, 37°C; b) 10% Pt/C, H_2, 50 psi, rt; c) Ni-Al_2O_3, H_2, 370-800 psi, 150-250°C; d) Co, O_2, 120-140 psi., 150-160 °C; e) Cu, NH_4VO_3, 60% HNO_3, 60-80 °C.

Vanillin

Vanillin, is a flavor used in food and beverages and in fragrances. It is also used by the pharma industry.[52] Natural vanilla obtained from the dried pods of the orchid *Vanilla planifolia* accounts for only 0.2% of the world flavor market (20 tons/year out of 1.2 x10^5 tons/year) and the remainder is supplemented by chemical synthesis of vanillin from guaiacol.[53] Vanillin is also obtained from the waste sulfite lye obtained during wood pulping operation. Condensation of guaiacol with glyoxalic acid affords mandelic acid, which upon oxidation and subsequent decarboxylation, yields vanillin (Figure 8). Synthetic vanillin sells at $12/kg while "natural" vanilla flavour obtained from vanilla pods containing 2% (w/w) vanillin sells for $30 - 120 /kg. The high price for natural vanilla flavour is attributed to the labour-intensive cultivation and processing of the vanilla pods, climatic, economic, and political factors. An increase in the number of health and nutrition conscious customers has boosted the demand for developing biochemical routes for vanillin synthesis and thus retaining its "natural" tag.[53b]

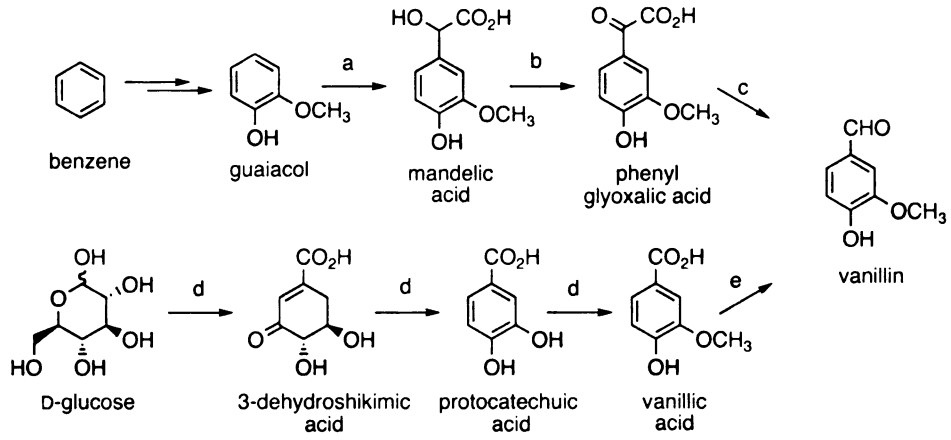


Figure 8. Chemical and biocatalytic synthesis of vanillin.
Key: a) $HCOCO_2H$; b) O_2; c) H^+; d) KL7/pKL5.26A; e) *N. crassa* arylaldehyde dehydrogenase.

The biocatalytic synthesis of vanillin from D-glucose takes advantage of the intermediacy of 3-dehydroshikimic acid. The *E. coli aroE* auxotroph KL7, was created by insertion of an *aroBaroZ* cassette into the genomic *serA* locus.[54] The genomic *aroBaroZ* cassette converts the accumulating 3-dehydroshikimic acid in KL7 into protocatechuic acid by the *aroZ* encoded 3-dehydroshikimate dehydratase. The enzyme catechol-*O*-methyltransferase encoded by the gene *COMT* of rat liver catalyzes the methylation of protocatechuic acid to vanillic acid and isovanillic acid. Plasmid pKL5.26A harbors $P_{aroF}aroF^{FBR}$, $P_{tac}COMT$ and the *serA* locus. Cultivation of KL7/pKL5.26A under fed-batch fermentation conditions with L-methionine supplementation yielded 4.9 g/L of vanillic acid from D-glucose. *In vitro* reduction of vanillic acid to vanillin was accomplished by the enzyme aryl-aldehyde dehydrogenase purified from the fungus *Neurospora crassa* (Figure 8).[55] The biocatalytic synthesis of vanillin from D-glucose using a single microbe remains to be accomplished.

Quinic acid and Hydroquinone.

Quinic acid isolated from *Chichona* bark,[30] finds application as a useful chiral synthon in numerous synthetic schemes. Quinic acid can be used as a precursor in the synthesis of hydroquinone. Hydroquinone finds application in the field of photography development, as an antioxidant in the rubber and food industry, and as polymerization inhibitor.[56] The current industrial synthesis of hydroquinone involves either Hock oxidation of 1,4-diisopropylbenzene or acid-catalyzed hydroxylation of phenol with hydrogen peroxide (Figure 9).[56]

An *E. coli* biocatalyst has been designed to produce quinic acid by the reduction of 3-dehydroquinic acid using *aroE*-encoded dehydroshikimate dehydrogenase. *E. coli aroD* auxotroph QP1.1, harboring an additional copy of *aroB* in the *serA* locus can accumulate 3-dehydroquinic acid. Plasmid pKD12.138A has $aroF^{FBR}$, *serA*, P_{tac}*aroE* and *tktA* genes localized. Culturing of QP1.1/pKD12.138A under fed-batch fermentation conditions produced 49 g/L of quinic acid with 20% yield (mol/mol) from glucose.[57]

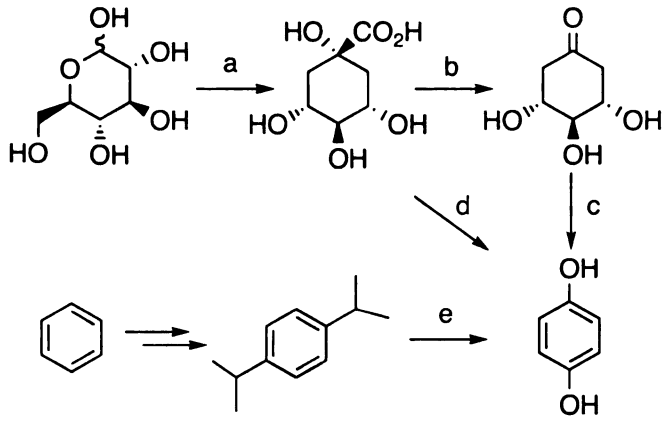


Figure 9. Synthesis of hydroquinone from glucose and benzene.
Key: a) *E. coli* QP1.1/pKD12.138; b) i) NaOCl; ii) isopropanol, H^+; c) Δ; d) Ag_3PO_4(10 mol%), $K_2S_2O_8$, 50°C then reflux; e) O_2, NaOH, 90-100°C.

The fermentation broth containing quinic acid was deproteinized and passed through a cation exchange resin to remove ammonium ions. The clarified broth when treated with commercial bleach followed by heating to reflux affords hydroquinone in 87% isolated yield. Oxidation of quinic acid with 10 mol% of Ag_3PO_4 and $K_2S_2O_8$ as the stoichiometric co-oxidant at 50°C followed by reflux gave hydroquinone in 85% yield.[57b]

Carotenoids

Carotenoids are natural pigments that are produced by plants and microorganisms. Most carotenoids are 40-carbon polyenes of conjugated double bonds. Head to head

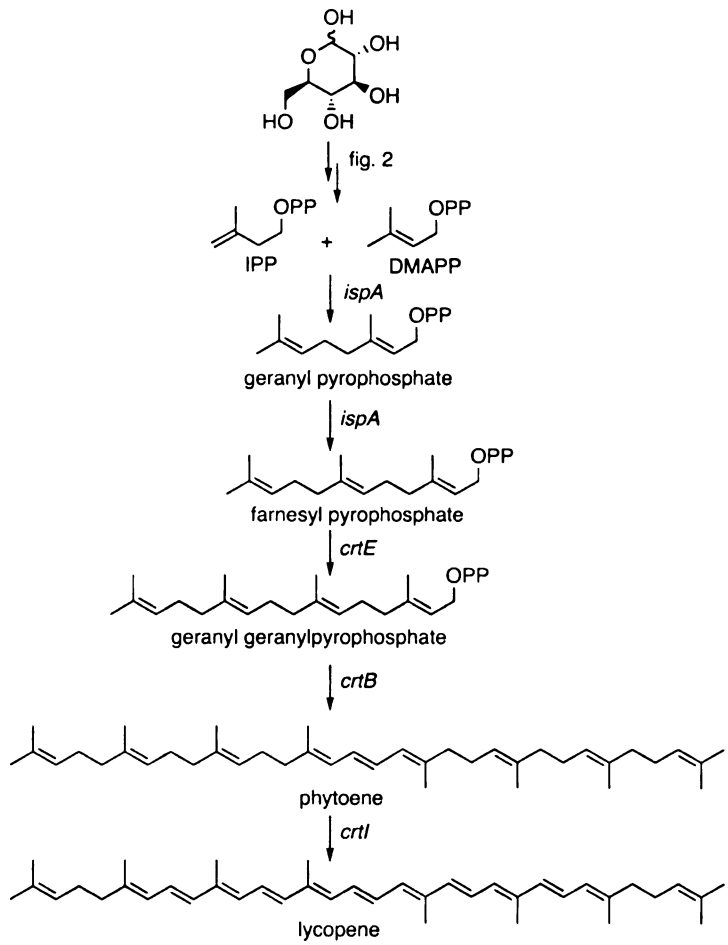


Figure 10. Biosynthesis of lycopene using recombinant *E. coli.*

condensation of two geranylgeranyl pyrophosphate precursors results in the formation of acyclic symmetrical $C_{40}H_{56}$ basic structure (Figure 8).[58a] About 600 natural carotenoids are derived from this basic molecule by various enzymatic reactions. This class of compounds have applications that range from food colorant (e.g. zeaxanthine, astaxanthine), animal feed supplements (e. g. lycopene, b-carotene), and pharmaceuticals.[58a,b]

Out of the 600 known carotenoids, only a few are produced commercially by either chemical synthesis or by fermentation or by extraction from natural sources.[58] The world market for carotenoids was US $ 786 million in 1999 and is projected to be $1

billion by 2005.[58d] With the discovery of a therapeutic role of these compounds in prevention of cancer and other chronic diseases, the demand for these molecules has dramatically increased.[58c] Though most of the market demand for carotenoids is met by chemical synthesis, in recent years research has been focused on metabolic engineering to enhance product formation.[58c]

Lycopene production has been demonstrated in a non-carotenogenic host like *E. coli* by introducing carotenogenic genes from *Erwinia uredovora*.[59a] The *E. coli* TOPO10 F' was used as the host along with plasmid pTAC-ORF1 that had *dxs* gene expressed from a *tac* promoter. Plasmid pACCRT-*E1B* had the carotenoid synthesis genes, *crtE*-encoded geranylgeranyl pyrophosphate synthase, *crtB*-encoded phytoene synthase, and *crtI*-encoded phytoene desaturase. Using this two-plasmid system, a four to ten fold increase in lycopene production was observed in strains that had *dxs* overexpressed.[59a] Production of various other carotenoids like phytoene, zeaxanthine, astaxanthine and β-carotene have been accomplished in a similar manner.[59b,c] The yields in most of these processes are in the range of μg of product per g of dry cell weight.

Higher yields can be achieved by increasing the carbon flow, balancing the precursor pool, optimization of the expression levels of carotenoid genes, and selection of a suitable host system that can store these lipophilic carotenoids. Systematic analysis of carotenoid biosynthesis, including identification, regulation and removal of rate-limiting impediments has not yet been carried out.[59d] Understanding these biochemical mechanisms will provide an avenue for successful metabolic engineering of the carotenoids.

Metabolic Engineering of *Escherichia coli* to Optimize Titer, Yield and Productivity

In order for a biocatalytic route to be competitive with a current chemical process, the cost effectiveness for the development of the process plays a critical role. Many of the current commercialized process encounter the problem of inefficient use of the carbohydrate feedstock. In addition to the synthesis of the desired product, carbon source is also utilized in biosynthesis of biomass and maintenance of cellular functions. As a result, final product titer, product yield, and volumetric productivity are affected.[60] For increasing the titer, yield, and productivity of a microbe-synthesised chemical, the increased carbon flow directed into a given pathway must be delivered without significant loss of the end of the pathway. In this section example of metabolic engineering of common pathway for aromatic amino acid biosynthesis to enhance carbon flow through the pathway will be presented.

Altering the central metabolism in *E. coli* to enhance carbon flow through the common pathway of aromatic amino acid biosynthesis has been the focus of several research groups.[61] As the first enzyme of this pathway, the expression levels of DAHP synthase dictate the amount of carbon flow into the pathway. The *in vivo* activity of DAHP synthase is controlled by transcriptional repression and feedback inhibition. The genes *aroF, aroG* and *aroH* encode, respectively for the tyrosine-sensitive, phenylalanine-sensitive, and tryptophan-sensitive isozymes.[62] Several methods have been explored to remove transcriptional repression of DAHP synthase for increasing the enzyme activity. The *tyrR* regulon modulates the *in vivo* activity of AroF and AroG isozymes.[62] In the presence of excess cytoplasmic concentrations of tyrosine and

phenylalanine, the repressor protein encoded by the *tyrR* gene binds to the promoter region of the *aroF* and *aroG* and prevents transcription. Mutational inactivation of the *tyrR* enhances the *in vivo* activity of the DAHP synthase as transcription of the genes is not affected by the aporepressor.[62] Alternatively, the cellular concentration of TyrR can be titrated away by including an extra copy of the *aroF* gene or the its promoter sequence on a multi-copy plasmid.[46a] Replacing the native promoter (P_{aroF}) of *aroF* gene with other strong promoters like P_{tac} give the same result.[46a,61a] Increased expression of DAHP synthase does not always translate into higher product formation due to feedback inhibition of the enzyme by the biosynthetic end products.[63] Feedback-insensitive mutants of all three isozymes of DAHP synthase have been isolated.[46a,64] Alleviation of the feedback inhibition by the aromatic amino acids leads to improved *in vivo* catalytic activity of DAHP synthase.

Ultimately, DAHP synthase catalytic activity reaches a point where further amplification of feedback-insensitive DAHP synthase has no incremental effect on the synthesis of aromatic amino acids or their precursors.[46a,61a] Therefore, attempts were made to increase the in vivo availability of PEP and E4P which are the substrates for DAHP synthase. Traditionally, *in vivo* PEP concentration has been considered as a limiting factor in the biosynthesis of shikimate pathway intermediates. In addition to two moles of PEP utilized by the common pathway to synthesize chorismate (Figure 5), PEP is also a substrates for a variety of enzymes including pyruvate kinase, PEP carboxylase, and the phosphoenolpyruvate:carbohydrate phosphotransferase (PTS) system. Mutational inactivation of pyruvate kinase[65a] and PEP carboxylase[61c,65b] has not been observed to afford any significant improvement in aromatic amino biosynthesis.

In 1990, Frost and coworkers reported that the *in vivo* concentration of E4P plays a critical role in limiting the DAHP synthase activity when glucose is used as carbon source.[62a,e] Although E4P is known to exist as a pentose phosphate pathway intermediate, E4P has never been isolated or detected in any living organisms.[66]

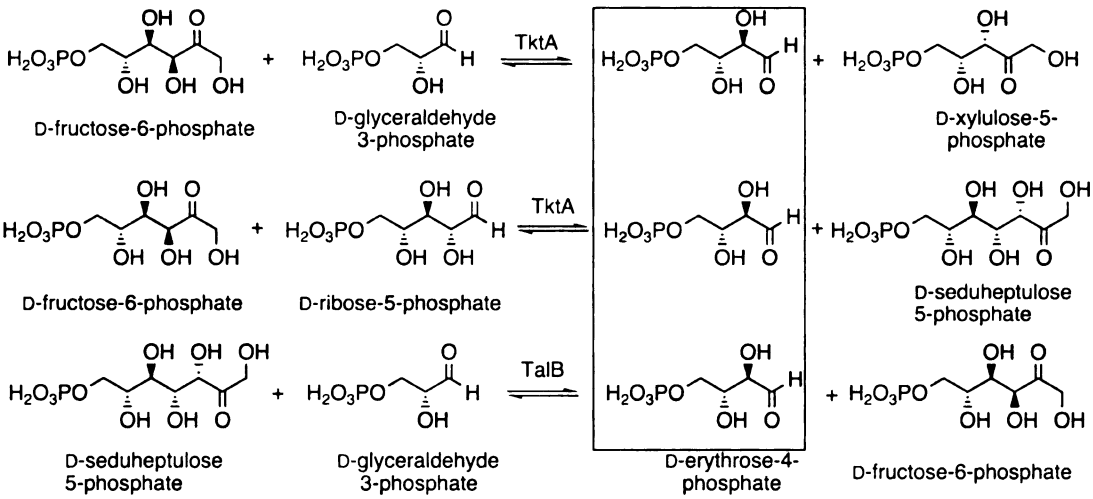


Figure 11. Biosynthesis of D-erythrose-4-phosphate via the non-oxidative pentose pathway.

As an aldose phosphate that cannot exist in solution in a cyclic form, E4P is prone to dimerization, trimerization, and polymerization.[67] *In vivo*, the rate of E4P synthesis might closely match its rate of utilization, thus maintaining a low steady-state concentration. The low steady-state concentration of E4P could be nature's way of favoring the monomeric form of E4P. The low levels of E4P can affect its availability and in turn limit the *in vivo* activity of DAHP synthase. The analysis of the enzymes involved in the biosynthesis of E4P led to the non-oxidative branch of pentose pathway and identification of the enzyme transketolase encoded by the *tktA* gene.[62a] The transketolase enzyme catalyzes the formation of E4P from the coupling of D-fructose 6-phosphate with either D-glyceraldehyde 3-phosphate or D-ribose 5-phosphate

(Figure 9). A second enzyme, transaldolase, also catalyzes the synthesis of E4P. This enzyme however utilizes D-sedoheptulose 7-phosphate, which is generated as a by-product during the transketolase catalyzed formation of E4P. Amplification of transketolase expression levels resulted in increased channeling of E4P into the common pathway. Overexpression of *tktA* along with amplified expression of the feedback insensitive *aroG* in *E. coli aroB* auxotroph led to twofold increase in 3-deoxy-D-*arabino*-heptulosonic acid (DAH) accumulation relative to that observed when only *aroG* was amplified. The twofold accumulation of DAH was interpreted as enhanced carbon flow into the common pathway due to increased in vivo E4P availability.[61a,e]

Alleviating the E4P limitation led to the reexamination of PEP availability as a factor limiting carbon flow directed into the shikimate pathway. PEP synthase catalyses the phosphorylation of pyruvate by ATP resulting in the formation of PEP, adenosine monophosphate (AMP) and inorganic phosphate (Figure 10). The impact of enhanced levels of PEP synthase on the carbon flow through the common pathway was first reported using *E. coli aroB* construct.[61d] Under amplified levels of a feedback insensitive isozyme of DAHP synthase, tranketolase and PEP synthase, a twofold increase in DAH accumulation was observed.[61e] An increase in the yields of shikimate pathway intermediates has also been recently reported with optimized levels of PEP synthase under fed-batch fermentation conditions.[46b,68c]

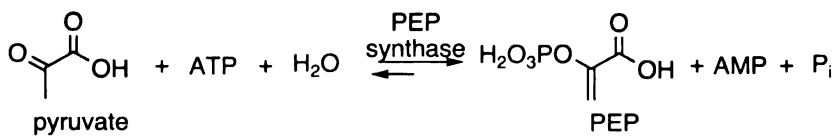


Figure 12. Reaction catalysed by PEP synthase.

In *E. coli*, the transport of glucose and numerous other carbohydrates is mediated by the phosphoenolpyruvate:carbohydrate phosphotransferase (PTS) system.[67] In addition to transport and phosphorylation of large numbers of carbohydrates, the PTS system is also involved in chemotaxis and in the regulation of a number of metabolic pathways.[67] For microbes utilizing the PTS system, for every mole of D-glucose transported into the cytoplasm and phosphorylated to form D-glucose 6-phosphate, one mole of PEP is converted into pyruvate. The resulting pyruvate instead of being recycled to PEP is oxidized to CO_2. As a result the shikimate pathway intermediates and glucose transport compete for the same intracellular PEP.

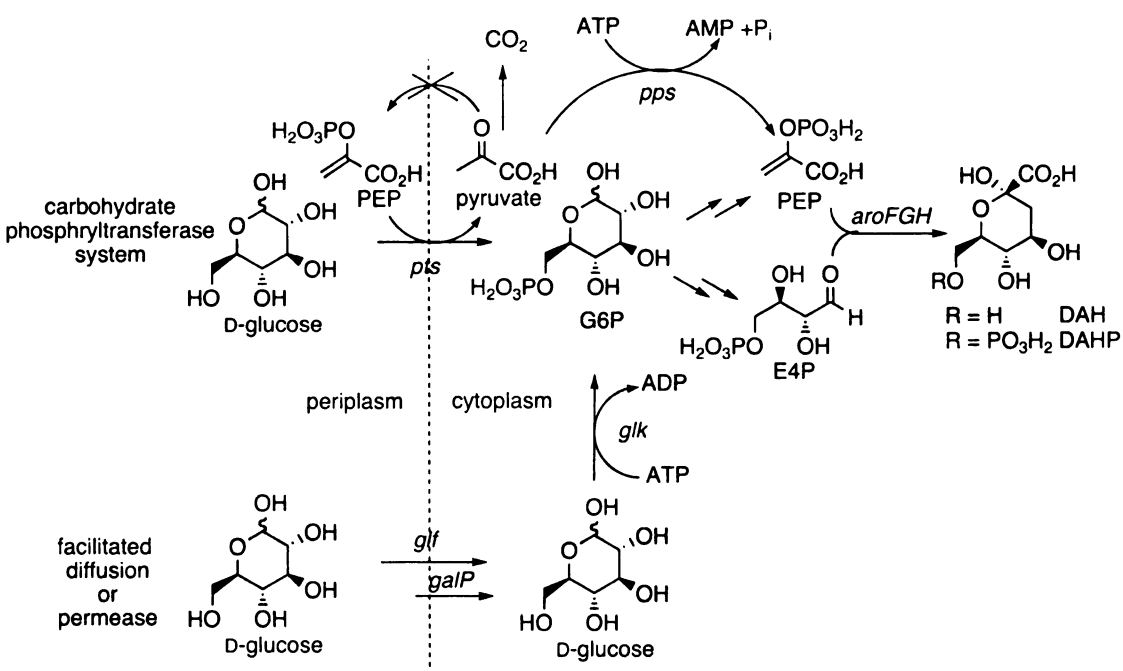


Figure 13. Various methods for alleviating PEP limitation when D-glucose is employed as carbon source.

One strategy to remove PEP limitation involves the use of pentoses like D-xylose and L-arabinose as carbon sources, as these molecules are transported by an ATP-based permease system. This circumvents the loss of PEP during glucose transport mediated by

the PTS system.[61e,68a] Alternatively, replacing or supplementing the PTS system with hetrologous expression of a glucose facilitator (*glf*) from *Zymomonas mobilis*[68b,d] or with native *E. coli galP*-encoded galactose permease alleviates the PEP limitation.[68c,d] All of these methods have led to an increase in carbon flow directed into the shikimate pathway. The maximum theoretical yield of shikimate pathway products synthesized with reliance on the phosphoenolpyruvate:carbohydrate phosphotransferase system is 43% (mol/mol) from D-glucose.[46a] Using D-xylose or L-arabinose as the sole source of carbon increases the theoretical yield to 71%.[69a] Recycling of pyruvate back to PEP or the recruitment of a glucose transport system which does not expend PEP increases the theoretical maximum yield to 86 % (mol/mol) from D-glucose.[46b, 68b.d]

Another important consideration for enhancing yields and titer is the culture conditions. Two different culture conditions, shake-flask and fed-batch fermentation, have been reported for examining the impact of increased carbon flow through the common pathway. Under typical shake-flask conditions, cultures are initially grown in rich medium followed by resuspension in minimal salts medium. Glucose in minimal medium is then converted to the desired molecule with limited or no growth. Although this type of culturing has been widely employed for evaluation the carbon flow in a given pathway, there are inherent problems associated with such experimental procedures. The initial growth in the rich medium prior to resuspension in minimal medium makes analysis complicated, as glucose is not the only source of carbon for growth and product formation. Yields exceeding the theoretical maximum have been reported during the synthesis of DAH by *E. coli aroB* constructs.[62d,e] Under shake-flask cultivation conditions, oxygenation levels, solution pH, and glucose concentration are difficult to

control. The cultures encounter glucose-rich conditions in the beginning of cultivation and glucose-starvation when the carbon source is depleted. This exposes the culture to a wide range of physiological conditions over the course of cultivation under shake flask conditions. Accumulation of pyruvate and acetate in the culture medium along with poor growth characteristics are also problematic.

The results reported in this thesis for synthesis of 1-deoxy-D-xylulose and shikimic acid have all been obtained under fed-batch fermentor conditions using minimal salts medium. Under these culture conditions, the microbial growth and biosynthesis of products occur simultaneously and are all derived from the carbohydrate source. This leads to an accurate appraisal of product yields. Plasmids can be maintained by nutritional pressure in minimal salts medium which circumvents the need for antibiotics, which would otherwise be required to avoid plasmid loss. Finally, under fed-batch culture conditions, growth parameters such as oxygenation levels, temperature, solution pH, and the steady-state concentration of glucose can be maintained and manipulated on line.

CHAPTER 2

IDENTIFICATION OF RATE-LIMITING IMPEDIMENTS TO CARBON FLOW THROUGH THE DXP PATHWAY

Background

Biosynthesis of all isoprenoids begins with one or both of the two C_5 building blocks of the pathway: isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). In eukaryotes and plants the biosynthesis of isopentenyl pyrophosphate (IPP) occurs via the well-characterized mevalonate (MVA) pathway that uses acetyl-CoA as a precursor and proceeds via hydroxymethylglutaryl-CoA and mevalonate.[11] In plant chloroplasts, algae, cyanobacteria and bacteria, IPP and DMAPP are biosynthesized by the 1-deoxy-D-xylulose 5-phosphate (DXP) pathway. The first step in this pathway involves a thiamine-dependant condensation of pyruvate with D-glyceraldehyde 3-phosphate to give DXP. The DXP is then converted into a 5:1 ratio of IPP and DMAPP by six enzymes-catalyzed reactions (Figure 14).[29] The first intermediate, 1-deoxy-D-xylulose 5-phosphate, also serves as a precursor in the biosynthesis of vitamin B_1 and vitamin B_6.[24,25]

Production of industrial chemicals like amino acids (lysine, glutamate),[69] adipic acid,[51] vanillin,[54] phenol,[108] and 1,3-propanediol[70] has been achieved by metabolic engineering of microbial pathways. With the discovery of the DXP pathway, a new avenue is now available for the producing a range of chemicals of industrial and pharmaceutical importance.[71] Enhancing the carbon flow into the DXP pathway requires the understanding of the regulatory mechanisms associated with this pathway. The regulatory mechanisms may include identification of the rate-limiting enzymes, feedback

inhibition, transcriptional regulation, and also the *in vivo* availability of precursor molecules.

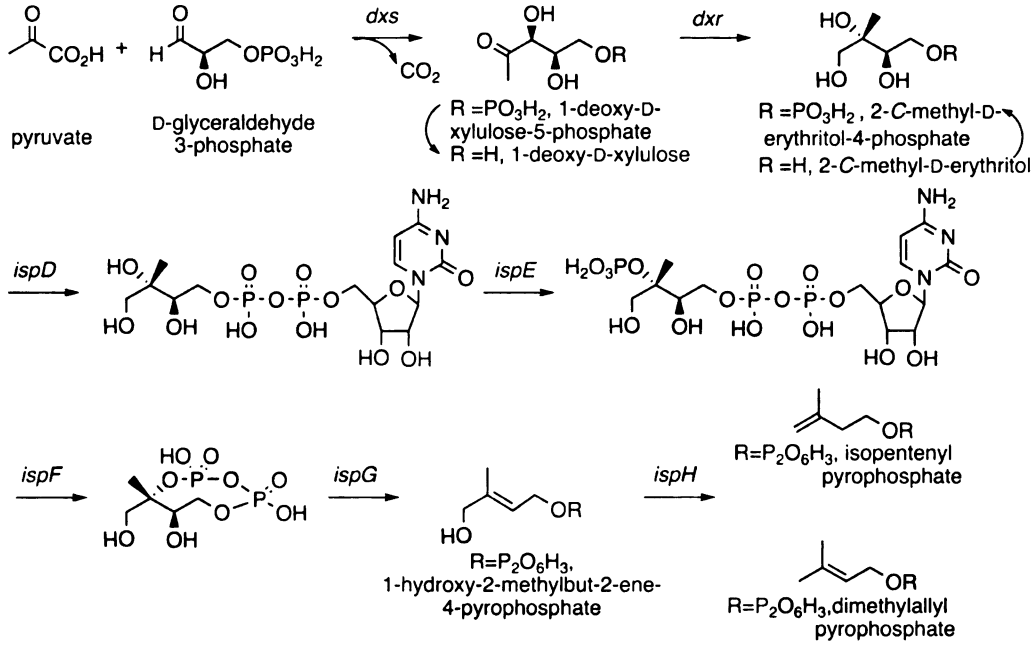


Figure 14. The DXP pathway for the biosynthesis of isoprenoid precursors IPP and DMAPP.
Enzymes (encoding gene): 1-deoxy-D-xylulose 5-phosphate synthase (*dxs*); 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*dxr*); 4-diphosphocytidyl-2-*C*-methylerythritol synthase (*ispD*); 4-diphosphocytidyl-2-*C*-methylerythritol kinase (*ispE*); 2-*C*-methylerythritol 3,4-cyclophosphate synthase (*ispF*); 1-hydroxy-2-methylbut-2-ene pyrophosphate synthase (*ispG*); 1-hydroxy-2-methylbut-2-ene pyrophosphate reductase (*ispH*)

Alleviation of these regulatory impediments may result in increase carbon flow in the DXP pathway. This will help in designing a better biocatalyst for the production of value-added chemical like ubiquinone, carotenoids, tocopherols and plant terpenoids. In this chapter, the measure of carbon flow through this pathway as evidenced by the accumulation of 1-deoxy-D-xylulose and 2-*C*-methyl-D-erythritol has been addressed using *E. coli* constructs that differed in the *in vivo* activity of *dxr*-encoded 1-deoxy-D-xylulose 5-phosphate reductoisomerase. The activity of this enzyme in these constructs

has been altered either by genetic or by chemical methods. Problems associated with culturing and supplementing *dxr* auxotroph under fed-batch fermentor conditions are also addressed. The measure of carbon flow in the pathway has also been elaborated by suppressing the DXR activity with simultaneous heterologous expression of the MVA pathway genes for IPP and DMAPP synthesis.

Synthesis of DXP Pathway Intermediates

Because the in vivo activity of the first enzyme in a pathway often dictates the amount of carbon flow directed into that pathway. The overexpression of *dxs* might be reasonably expected to enhance the carbon flow into the DXP pathway. The increase in carbon flow may result in the accumulation of pathway intermediates and structurally-related metabolites due to the rate-limiting nature of individual pathway enzymes. This would further our understanding about the impediments to the carbon flow into this pathway and aid in designing a better biocatalyst for the production of variety of value-added chemicals such as carotenoids, ubiquinone and α-tocopherol. To simplify identification of products formed during fed-batch fermentation, authentic samples of 1-deoxy-D-xylulose, 1-deoxy-D-xylulose 5-phosphate and 2-*C*-methyl-D-erythritol were chemically synthesized.

The enzyme 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) catalyzes the conversion of 1-deoxy-D-xylulose 5-phosphate (DXP) into 2-*C*-methylerythritol 4-phosphate (Figure 14). Purification and mechanistic enzymology of DXR required availability of DXP.

The measure of carbon flow into a pathway can be studied by creation of chromosomal disruption. Chromosomal disruption of *dxr* and other genes in the DXP pathway has been reported to be lethal.[29] The disruption of the DXP pathway would prevent the production of essential polyprenols that are needed for cell wall synthesis and cellular functions. The *dxr* auxotrophy can be rescued by supplementation with 2-*C*-methyl-D-erythritol.[29] 2-*C*-Methyl-D-erythritol was chemically synthesized for this purpose.

Synthesis of 1-Deoxy-D-Xylulose:

The chemical synthesis of 1-deoxy-D-xylulose was accomplished by modification of literature procedures (Figure 15).[72] One-pot protection of D-(-)tartaric acid as 2,3-isopropylidine dimethyl tartrate followed by reduction of methyl esters with lithium aluminium hydride afforded (-)2,3-isopropylidene-D-threitol **1** in quantitative yields. Monosilylation of the C_2 symmetric acetonide with chloro-*t*-butyldimethylsilane followed by Dess-Martin oxidation of the primary alcohol gave aldehyde **3** in high yield. Reaction of CH_3MgBr with aldehyde **3** gave a mixture of diastreomeric secondary alcohols that on oxidation with Dess-Martin reagent yielded fully protected 1-deoxyxylulose **4.** Deprotection of **4** with AcOH/H_2O/THF(3:1:1 v/v) afforded 1-deoxy-xylulose in high yields. The aqueous solutions of 1-deoxy-D-xylulose consisted of 3:1 ratio of acyclic to cylic isomers.

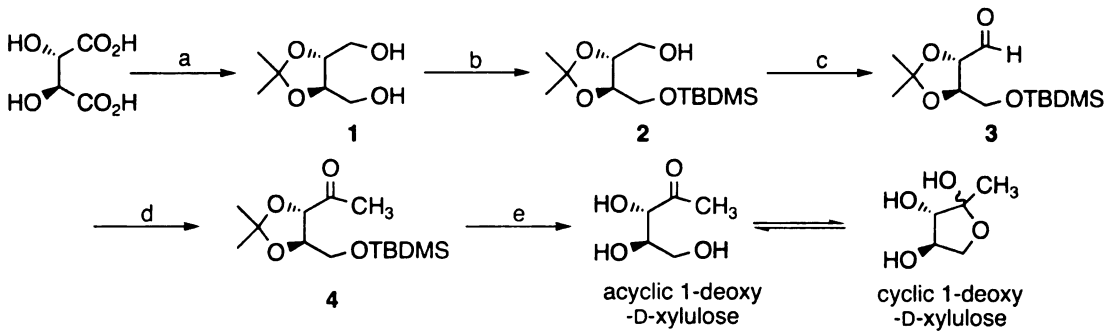


Figure 15. Synthesis of 1-deoxy-D-xylulose.
Key: a) i) CH_3OH, $(CH_3)_2C(OCH_3)_2$, C_6H_{12}, *p*TSA, reflux, 95%, ii) $LiAlH_4$, Et_2O, 0°C to reflux, 95%; b) TBDMSCl, NaH, THF, 0°C to rt, 80%; c) Dess-Martin, CH_2Cl_2, 95%; d) i) CH_3MgBr, THF/Et_2O, -78°C to rt, ii) Dess-Martin, CH_2Cl_2, 95%; e) AcOH: H_2O:THF(3:1:1), 70°C, 80%.

Synthesis of 1-Deoxy-D-Xylulose 5-Phosphate:.

Chemical synthesis of DXP (Figure 16) involved benzylation of D-(-)diethyltartrate followed by reduction of the diester with lithium aluminium hydride to afford 2,3-dibenzyl-D-threitol **6**.[72b] Phosphorylation of 2,3-dibenzyl-D-threitol **6** with tetrabenzyl pyrophosphate yielded a mixture of monophosphate **7**, diphosphate and starting material. Separation of monophosphate **7** from the mixture by radial chromatography followed by oxidation with Dess-Martin reagent afforded the aldehyde **8** in 80% yield. The aldehyde **8** was treated with methylmagnesium bromide at –78°C for 3 h and purified by radial chromatography to give a mixture of alcohols in 50% yield. Subsequent oxidation of the racemic alcohols with Dess-Martin reagent to yield benzyl-protected DXP **9**. Catalytic hydrogenation of **9** with 20% $Pd(OH)_2/C$ afforded 1-deoxy-D-xylulose 5-phosphate in quantitative yields.

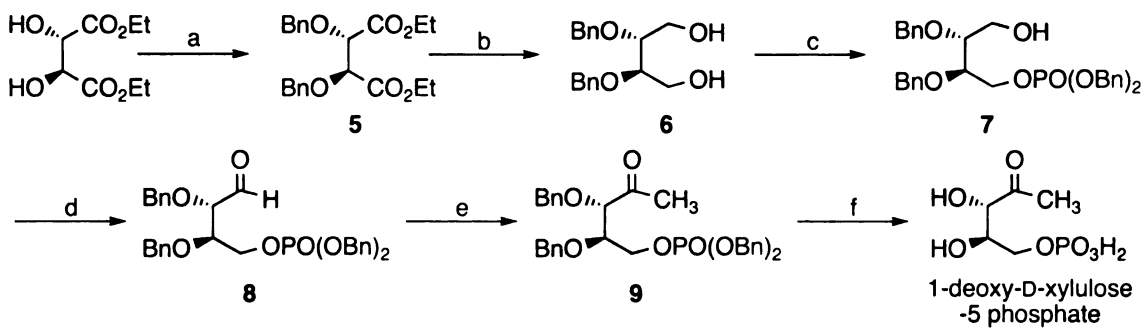


Figure 16. Synthesis of 1-deoxy-D-xylulose 5-phosphate.
Key: a) i) BnBr, NaH, $(C_4H_9)_4N^+I^-$, 18-*C*-6, THF, 32%; b) $LiAlH_4$, Et_2O, 0°C, 50%; c) $[(BnO)_2P(O)]_2O$, NaH, THF, 0°C to rt, 50%; d) Dess-Martin, CH_2Cl_2, 60%; e) i) CH_3MgBr, THF/Et_2O, -78°C, 3 h , 50%, ii) Dess-Martin, CH_2Cl_2, 50%; f) H_2, $Pd(OH)_2$, EtOH, 100%.

Synthesis of 2-*C*-Methyl-D-Erythritol:

The chemical synthesis of 2-C-methyl-D-erythritol was achieved by modification of a literature procedure (Figure 17).[78] Reduction of 3-methyl-2(*5H*)furanone (Aldrich) with $LiAlH_4$ gave a mixture of desired unsaturated diol and saturated diol in a 7:1 ratio. The mixture was treated pyridine/acetic anhydride (1:1, v/v) to give a mixture of saturated and unsaturated diacetate **10**. Sharpless enantioselective dihydroxylation[73] of the diacetate **10** using commercially available AD-mix-β afforded a mixture of 2-*C*-methylerythritol diacetate that was deprotected with K_2CO_3 in methanol to give the free tetrol in 80% yield.

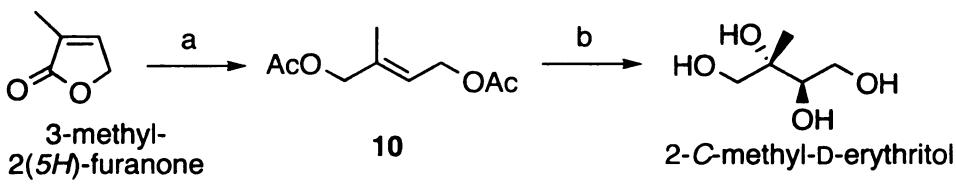


Figure 17. Synthesis of 2-*C*-methyl-D-erythritol.
Key: a) i) $LiAlH_4$, Et_2O, 1 h, 0°C, ii) Ac_2O, pyridine, 3 h, rt, 55%; b) i) AD-mix-β, $CH_3SO_2NH_2$, *t*-BuOH: H_2O (1:1), 2 days, 0°C, 80% (ii) K_2CO_3, MeOH, 2 h, rt, 100%.

Host Design and Plasmid Overview

Host design.

E. coli JWF1 and *E. coli* SP1.1 were used as the host strains for 1-DX synthesis. Both of the strains are derivatives of *E. coli* RB791. *E. coli* JWF1 was constructed by inactivating the *serA* gene of *E. coli* RB791 by homologous recombination. The *serA* locus encodes for 3-phosphoglycerate dehydrogenase which is the first of the three enzymes responsible for L-serine biosynthesis. Microbial growth of *serA* mutants in minimal medium without L-serine supplementation requires stable maintenance of the plasmid-localized *serA* gene.

E. coli SP1.1 (RB791 *serA::aroB aroL478*::Tn10 *aroK17*::Cm^R) is a strain developed for synthesis of shikimic acid.[74] The construction of this strain will be discussed in detail in Chapter 3. Due to disruption of the shikimate kinase-encoding *aroK* and *aroL* loci, SP1.1 is unable to synthesize its own aromatic amino acids and essential aromatic vitamins. Growth of SP1.1 in minimal medium therefore requires supplementation with L-phenylalanine, L-tyrosine, and L-tryptophan, *p*-hydroxybenzoic acid, *p*-aminobenzoic acid, and 2,3-dihydroxybenzoic acid.

E. coli DXS2.1 is a derivative of *E. coli* SP1.1 where the chromosomal *dxr* gene has been disrupted by introduction of an aminoglycoside phosphotransferase gene encoding for kanamycin resistance. *E. coli* DXS2.1 was created by P1 transduction of *dxr::kan* locus from *E. coli* DMY1[75a] into SP1.1. DXS2.1 was selected based on the following phenotype: no growth on M9 medium containing serine; no growth on M9 containing L-phenylalanine, L-tyrosine, L-tryptophan, *p*-hydroxybenzoic acid, potassium *p*-aminobenzoate, and 2,3-dihydroxybenzoic acid and serine; growth on M9 containing L-

phenylalanine, L-tyrosine, L-tryptophan, *p*-hydroxybenzoic acid, potassium *p*-amino benzoate, and 2,3-dihydroxybenzoic acid, serine, and 0.01% 2-*C*-methyl-D-erythritol; no growth on LB containing tetracycline (Tc), chloramphenicol (Cm) and kanamycin (Kan); growth on LB containing Tc, Cm and Kan and 0.01% 2-*C*-methyl-D-erythritol.

Addition of aromatic amino acids L-phenylalanine, L-tyrosine, and L-tryptophan to fed-batch fermentation runs of *E. coli* SP1.1 has been reported to completely inhibit native levels of DAHP synthase activity as reflected by the absence of detectable biosynthesis of shikimate pathway products.[74b] Evaluation of carbon flow in the DXP pathway, using *E. coli* SP1.1 and DXS2.1 as the host strain, hence required addition of aromatic amino acids. L-Phenylalanine (1.4 g), L-tyrosine (1.4 g), and L-tryptophan (0.7 g) were added to the culture medium at the beginning of oxygen sensor-controlled (oxygen sensor-controlled phase) phase of fermentation runs to inhibit the native levels of 3-deoxy-D-*arabino*-heptulosonic acid-7-phosphate (DAHP) synthase activity and prevent accumulation of shikimate pathway intermediates.

Plasmid Construction Overview.

In order to study the impact of carbon flow in the DXP pathway, the *dxs* gene was amplified by PCR and cloned into a plasmid having a strong promoter. The plasmid also harbored a copy of the *serA*, which provided a convenient method for plasmid maintenance during cultivation of strains under fed-batch fermentation conditions. For the controlled expression of the *dxs* gene from the P_{N25} or *tac* promoter, the *lacI*^{Q} gene encoding for the lac repressor was localized on the plasmid. The expression of the *dxs*

gene from the promoter can be induced by addition of β-isopropyl-thiogalactoside (IPTG).

Construction of plasmid pPV2.248.

Plasmid pJF118EH[76] is a derivative of cloning vector pKK223-3 with a pMB1 origin of replication. It has a strong *tac* promoter, a ribosome binding site that can be utilized for the expression of gene inserts and a $lacI^Q$ gene for the controlled expression of the *tac* promoter. The 1.9-kb *dxs* open reading frame was amplified by PCR from *E. coli* RB791 genomic DNA with *Eco*RI ends and ligated to *Eco*RI site of pJF118EH to give pPV2.173 (Figure 18). The *dxs* gene in plasmid pPV2.173 is transcribed in the same direction as the *tac* promoter. Plasmid pPV2.173 was digested with *Bam*HI and treated with Klenow fragment. Blunt-end ligation of a 1.9-kb *Dra*I/*Eco*RV fragment encoding *serA* obtained from pD2625 into the *Bam*HI site of pPV2.173 afforded pPV2.248 (Figure 19). The *serA* gene is transcribed in the opposite direction relative to the *tac* promotor.

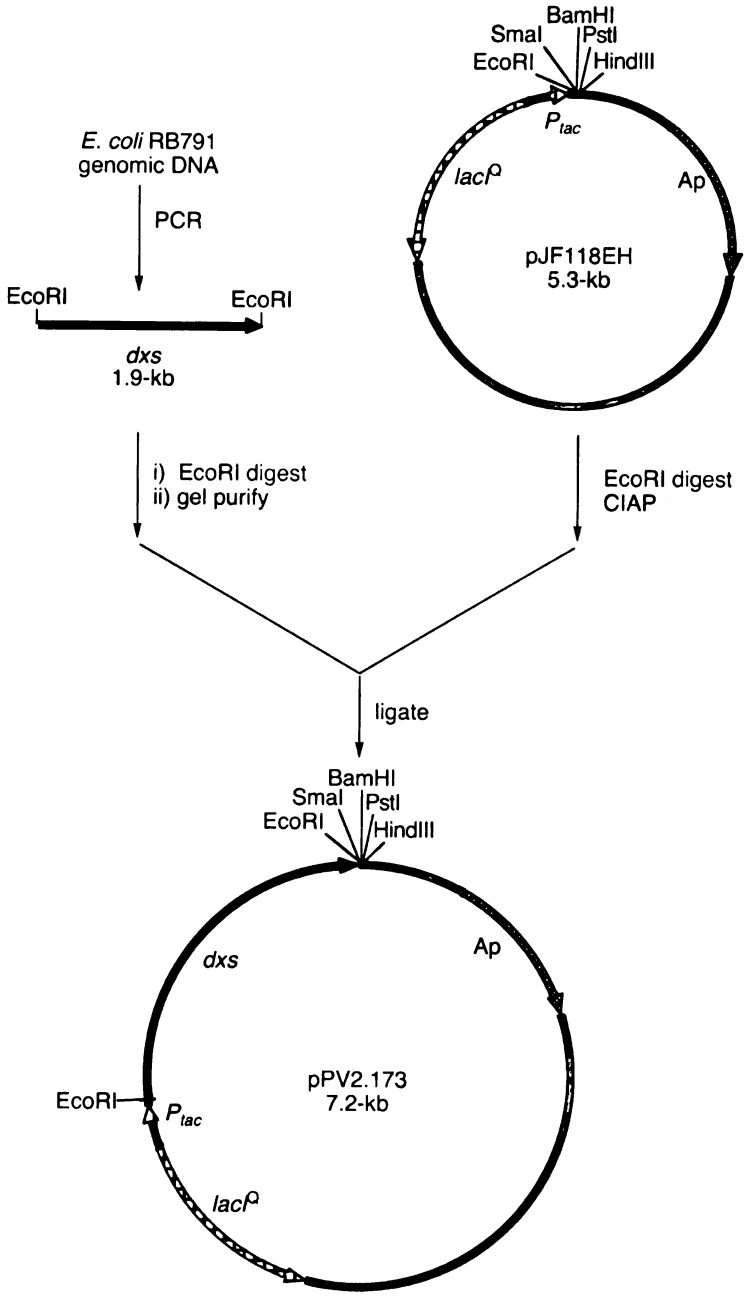


Figure 18. Construction of plasmid pPV2.173.

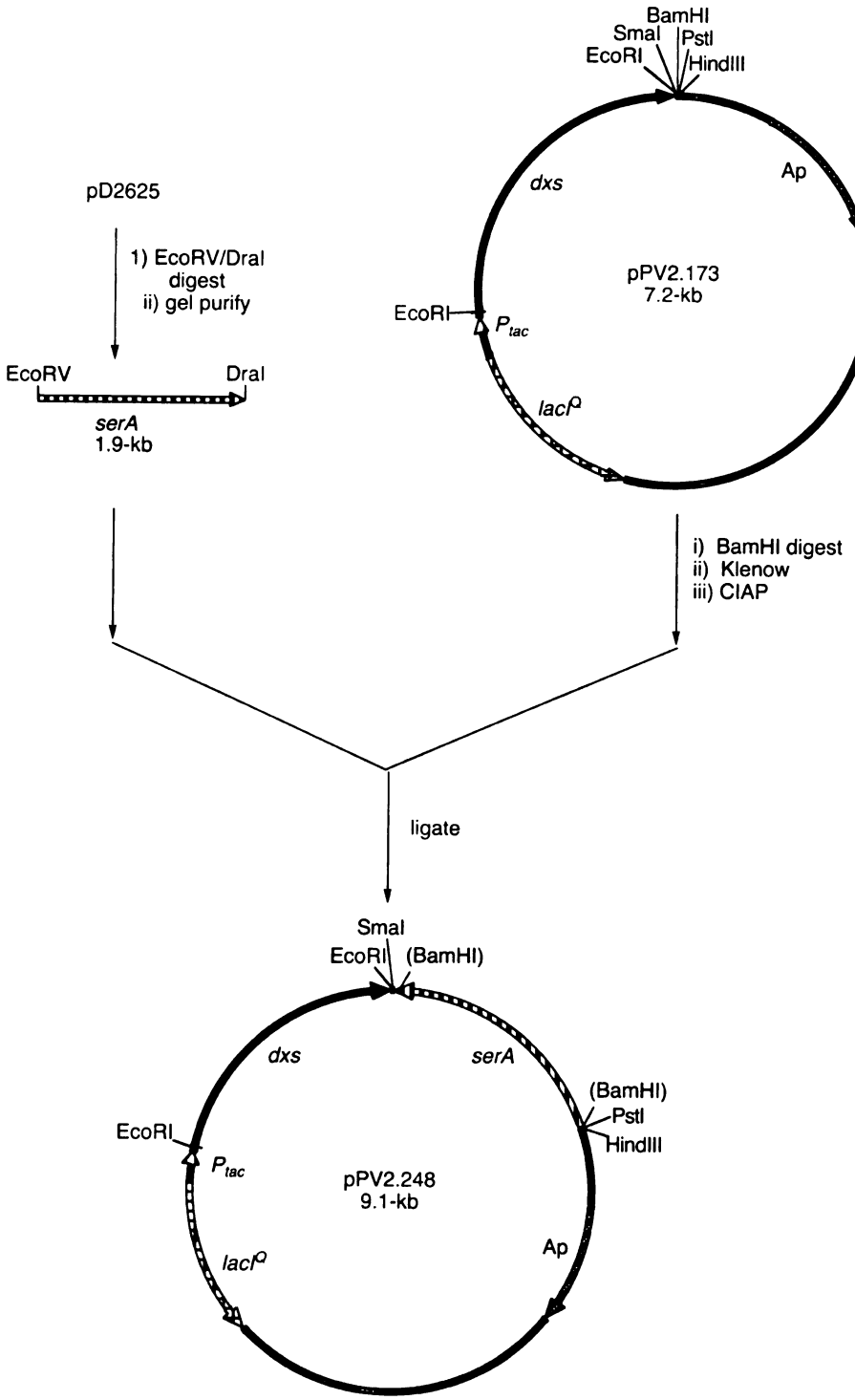


Figure 19. Construction of plasmid pPV2.248.

Construction of Plasmid pPV4.230

When JWF1/pPV2.248 was cultured under glucose-limited fed-batch fermentation conditions, although 1-deoxy-D-xylulose accumulated in the culture supernatant, the assayable activities of the DXS enzyme could not be detected. In order to increase the specific activity of DXS a new plasmid was designed based on plasmid pQE30. Plasmid pQE30 is cloning vector used for high-level expression and purification of proteins. The pQE30-based plasmids have P_{N25} promoter from coliphage *T5* that is recognized by *E. coli* RNA polymerase.[77,78] The pQE30-based plasmids contain a regulatable promoter/operator element $P_{N25/03/04}$[79,80] that can be repressed by the plasmid-localized *lac* repressor and induced by the addition of IPTG. Cultivation of an *E. coli* host carrying pQE30-based plasmids requires plasmid-localized $lacI^Q$ gene or a host that harbors the $lacI^Q$ mutation (e. g. *E. coli* JM109, XL1 Blue) to produce enough lac repressor. The *dxs* locus without the start codon was amplified by PCR from plasmid pPV2.173 with primers containing terminal *Bam*HI recognition sequences. The start codon ATG for transcription of the insert DNA was provided by the vector. This 1.9-kb *dxs* fragment was then ligated into the *Bam*HI site of pQE30 to give pPV4.176 (Figure 20). The orientation of the *dxs* gene is in the same direction as the P_{N25} promoter. For the controlled expression of the P_{N25} promoter the $lacI^Q$ gene was amplified by PCR from plasmid pTrc99A using primers containing *Pst*I recognition sequences and ligated into the *Pst*I site of pPV4.176 to give pPV4.222 (Figure 21). The *serA* gene was then excised from pRC55B by *Sma*I digest and ligated to the pPV4.222 digested with *Sma*I to give pPV4.230. The orientation of *dxs, serA* and the $lacI^Q$ was in the same direction as the P_{T5} promoter (Figure 22).

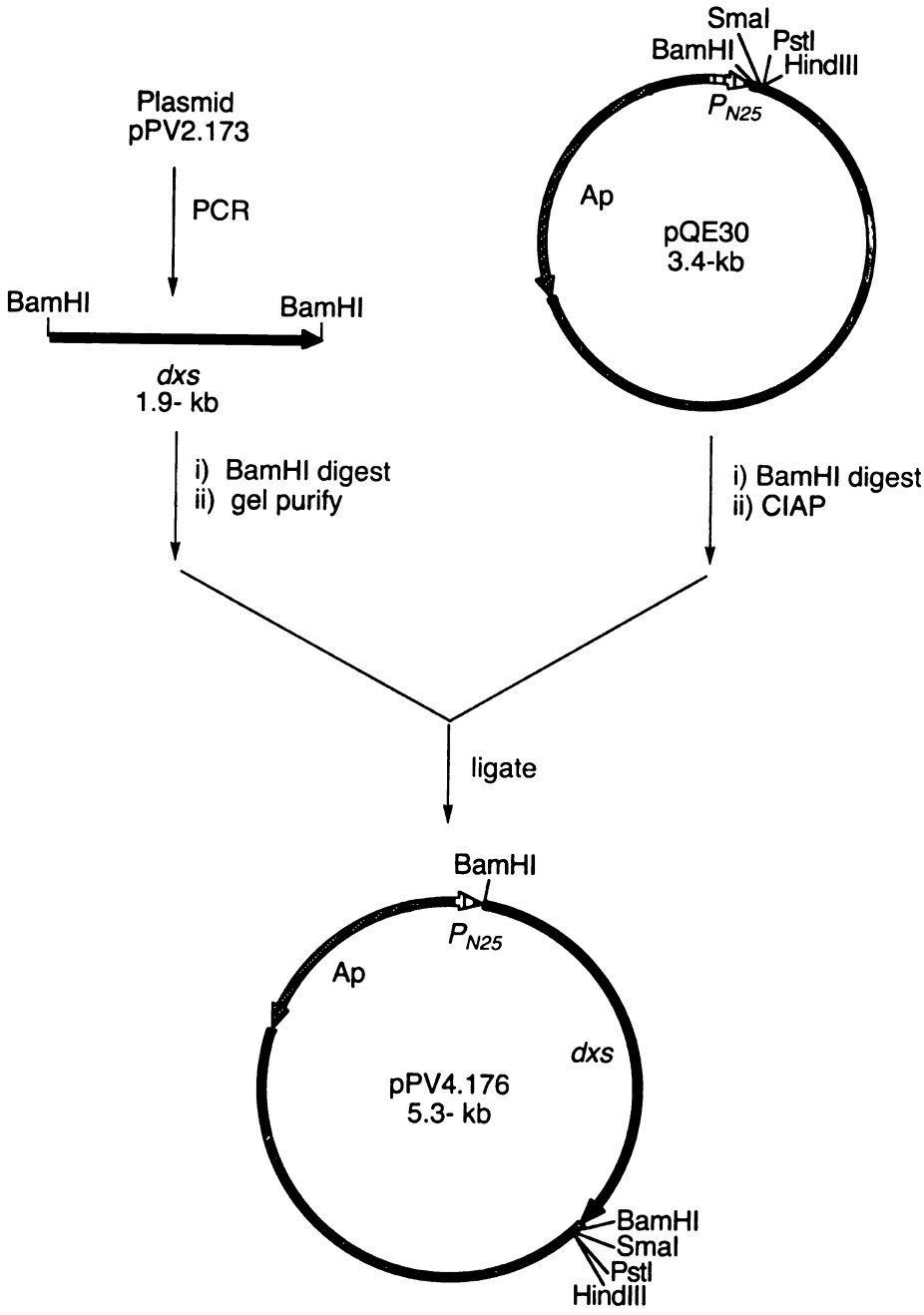


Figure 20. Construction of plasmid pPV4.176.

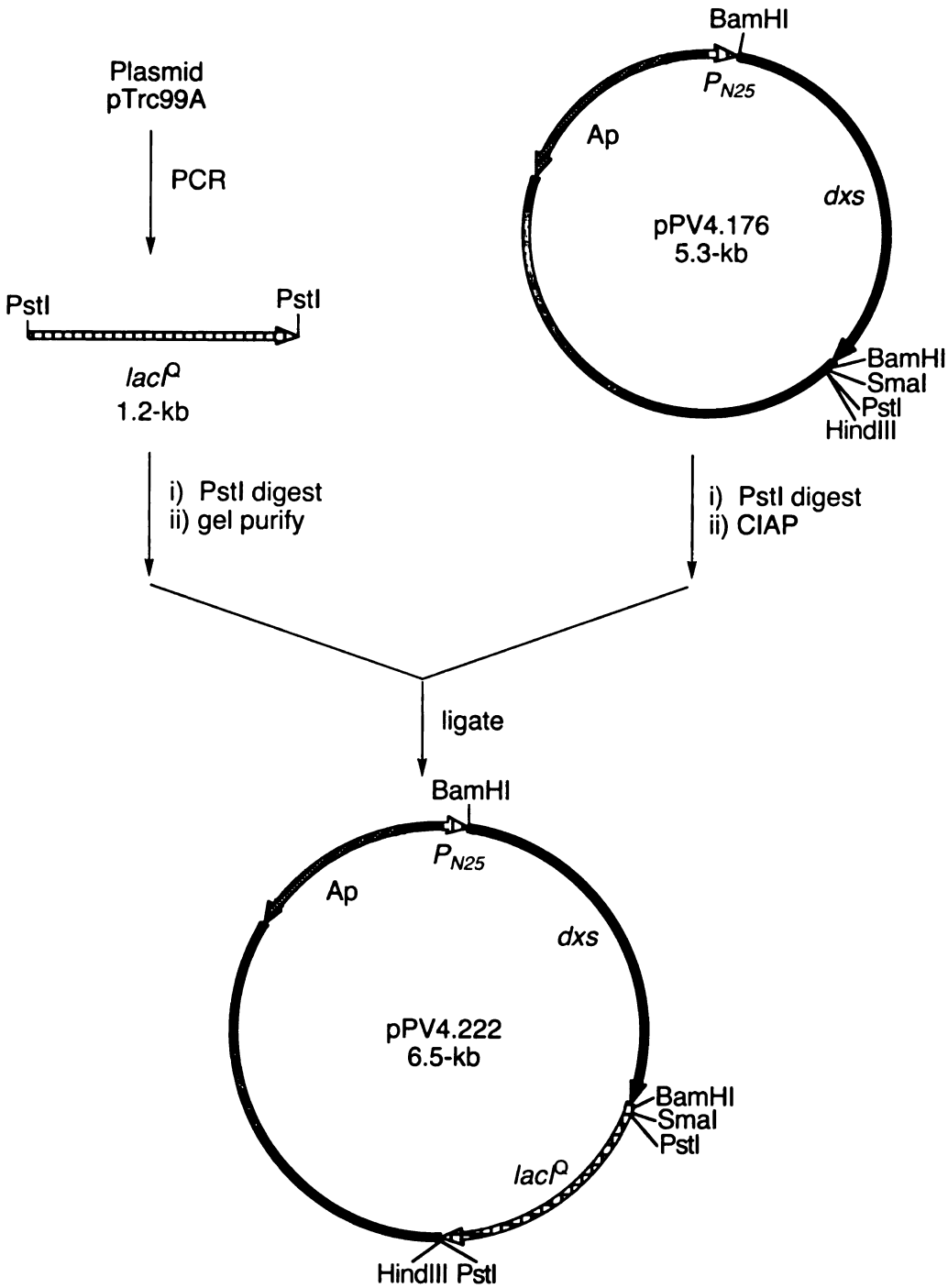


Figure 21. Construction of plasmid pPV4.222.

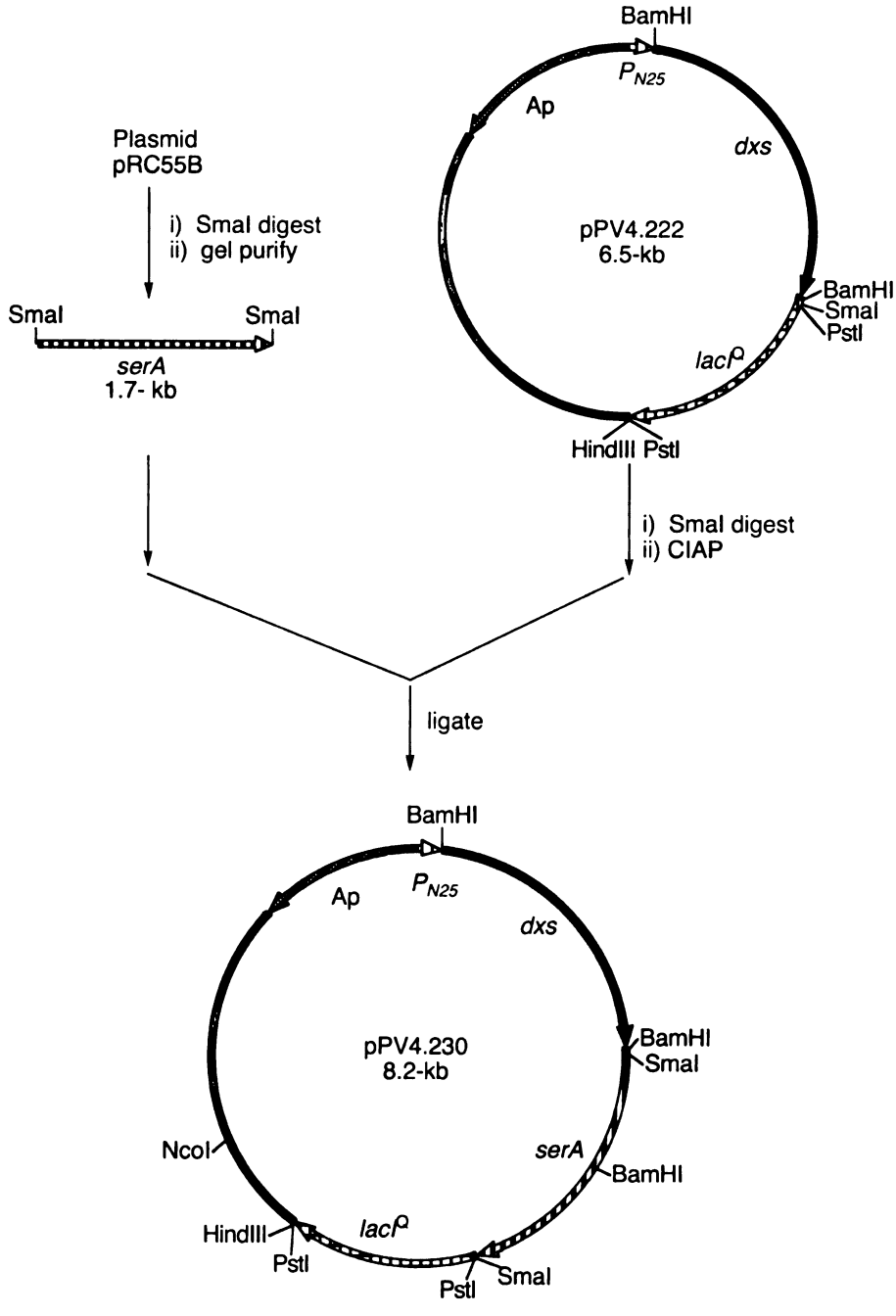


Figure 22. Construction of plasmid pPV4.230

Fed-Batch Fermentations

Studies to measure carbon flow through the DXP pathway were carried out under two different sets of fed-batch fermentation conditions, that differed in the steady state concentration of D-glucose in the culture medium. A steady state concentration of 0.2 mM was employed in glucose-limited conditions while a concentration of 55-170 mM was employed for glucose-rich conditions. Concentrations of the metabolites in the culture supernatant were determined by comparison of the integrals corresponding to each compound with the integral corresponding to an internal standard in the ^{1}H NMR.

Glucose-Limited Fermentor Conditions

Three staged methods were used to maintain D.O. levels at 10% air saturation during the course of fermentor run. With the airflow at an initial setting of 0.06 L/L/min, D.O. concentration was maintained by increasing the impeller speed from its initial set point of 50 rpm to its preset maximum of 1100 rpm. When the impeller reached its preset maximum, the mass flow controller then maintained D.O. levels by increasing the airflow rate from 0.06 L/L/min to a preset maximum of 1.0 L/L/min. At constant impeller speed and constant airflow rate also known as the third stage, D.O. levels were finally maintained at 10% air saturation for the remainder of the fermentation by oxygen sensor-controlled glucose feeding. PID control parameters were set to 0.0 (off) for the derivative control (t_D), and 999.9 s (minimum control action) for integral control (t_I). X_p was set to 950.0% to achieve a K_c of 0.1. The strain was cultured in the fermentation vessel for a total of 48 h. IPTG was first added to the culture when the oxygen sensor-controlled

feeding was initiated and then subsequently added every six hours until the fermentation run was complete.

Glucose-Rich Fermentor Conditions

For fermentations that employed glucose-rich conditions, a stainless steel baffle cage consisting of four 1/2" x 5" baffles was placed in the fermentation vessel. Three staged methods were used to maintain the D.O. concentration at 10% of air saturation. With the airflow at an initial setting of 0.06 L/L/min, the D.O. concentration was maintained by increasing the impeller speed from its initial set point of 50 rpm to a preset maximum of 900 rpm. With the impeller rate constant at 900 rpm, the mass flow controller then maintained the D.O. concentration by increasing the airflow rate from 0.06 L/L/min to a preset maximum of 1.0 L/L/min. After the preset maxima of 900 rpm and 1.0 L/L/min were reached, the oxygen sensor controlled stage of the fermentation was initiated in which glucose (65% w/v) was added to the vessel at a rate sufficient to maintain a glucose concentration of 20-30 g/L for the remainder of the run. Airflow was maintained at 1.0 L/L/min, and the impeller was allowed to vary in order to maintain the D.O. concentration at 10% of air saturation. The impeller speed typically varied from 400 to 1600 rpm during the remainder of the run. IPTG was first added to the culture at he beginning of oxygen sensor-controlled phase of fermentation and then subsequently added every six hours until the fermentation run was complete.

Monitoring Carbon Flow Through the DXP Pathway

Microbial Synthesis of 1-Deoxy-D-Xylulose under Amplified Levels of DXS.

Overexpression of the *dxs* gene could lead to increased carbon flow in the pathway. Depending on rate-limiting impediments in the DXP pathway, the increase in the carbon flow could result in the accumulation of phosphorylated metabolites in the cytoplasm. Hydrolysis and export would result inn the accumulation of dephosphorylated metabolite in the culture medium. Identifying the accumulating metabolites and determining their concentration can result in identification of individual DXP pathway enzymes that are impediments to the carbon flow.

E. coli JWF1/pPV2.248 was the first biocatalyst designed to study the impact of carbon flow when *dxs* gene was overexpressed. The expression of the *dxs* gene from the *tac* promotor was induced by addition of IPTG (5 mg) at the beginning of the oxygen sensor-controlled phase of fermentation and at every 6 h thereafter. Aliquots of the culture medium were removed every 6 h after induction and analyzed for metabolite production by ^{1}H NMR. Cultivation of *E. coli* JWF1/pPV2.248 under glucose-limited conditions for 48 h resulted in accumulation of 6 g/L of 1-deoxy-D-xylulose in a yield of 4% (mol/mol) along with 1 g/L of 2-*C*-methyl-D-erythritol and 2 g of acetic acid (Figure 23). The accumulation of 1-deoxy-D-xylulose in the culture supernatant results when carbon flow in the DXP pathway is increased to a point where 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) is unable to convert 1-deoxy-D-xylulose 5-phosphate into 2-*C*-methyl-D-erythritol 4-phosphate at a rate sufficient enough to prevent hydrolysis of 1-deoxy-D-xylulose 5-phosphate. Hydrolysis of 1-deoxy-D-xylulose 5-phosphate leads to 1-deoxy-D-xylulose which accumulates in the culture supernatant. Varying the

amounts of IPTG for enhancing expression of DXS from 5 mg to15 mg and 48 mg did not have any significant effect on the concentration of accumulated product in the culture supernatant. In both cases approximately 6 g/L of 1-deoxy-D-xylulose and 2 g/L of 2-*C*-methyl-D-erythritol accumulated in the culture supernatant. Reducing the amount of IPTG (< 5 mg) for induction led to a complete absence of products in the culture supernatant.

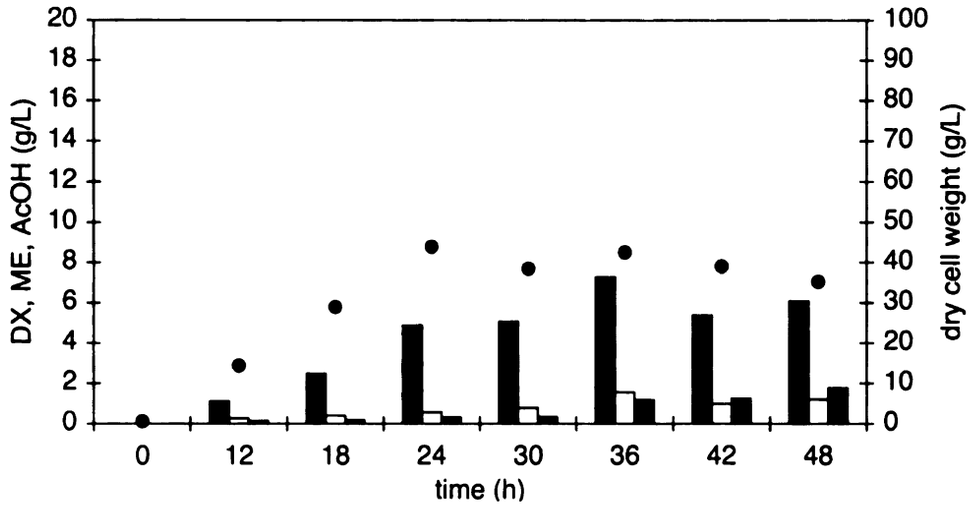


Figure 23. Cultivation of JWF1/pPV2.248 (*dxs* expressed from a P_{tac} promoter) under glucose-limited conditions. Key: filled circles, dry cell weight; black bars, 1-deoxy-D-xylulose; open bars, 2-methyl-D-erythritol; gray bars, acetic acid.

A coupled enzyme assay was developed for measuring DXS activity during the course of fermentation runs JWF1/pPV2.248. The condensation of pyruvate and D-glyceraldehyde 3-phosphate by DXS gave DXP that was converted to 2-*C*-methyl-D-erythritol 4-phosphate by DXR in a NADPH-dependent reaction. Since in a catalytic cycle the enzyme DXR oxidizes one mole of NADPH for one mole of 1 deoxy-D-xylulose-5-phosphate consumed, the rate of NADPH oxidation will be proportional to the rate of DXP synthesized by DXS during the coupled enzyme assay. The oxidation of NADPH was monitored spectrophotometrically at 340 nm. Assayable levels of DXS activity could not be detected from JWF1/pPV2.248 cultures.

In order to increase the specific activity of 1-deoxy-D-xylulose 5-phosphate synthase, a new plasmid pPV4.176 was created where the *dxs* gene was expressed from a P_{N25} promoter. Cloning of the $lacI^Q$ and *serA* genes in pPV4.176 afforded plasmid pPV4.230 (Figure 22). Cultivation of JWF1/pPV4.230 under glucose-limited fed-batch fermentation conditions yielded 7 g/L of 1-deoxy-D-xylulose and 2 g/L of 2-*C*-methyl-D-erythritol after 54 h (Figure 24). The product accumulation decreased when JWF1/pPV4.230 was cultivated beyond 54 h. The DXS enzyme activities measured at 18 h, 24 h, 36 h and 48 h of the fermentation run were 0.02, 0.10, 0.15 and 0.15 μmol/min/mg respectively.

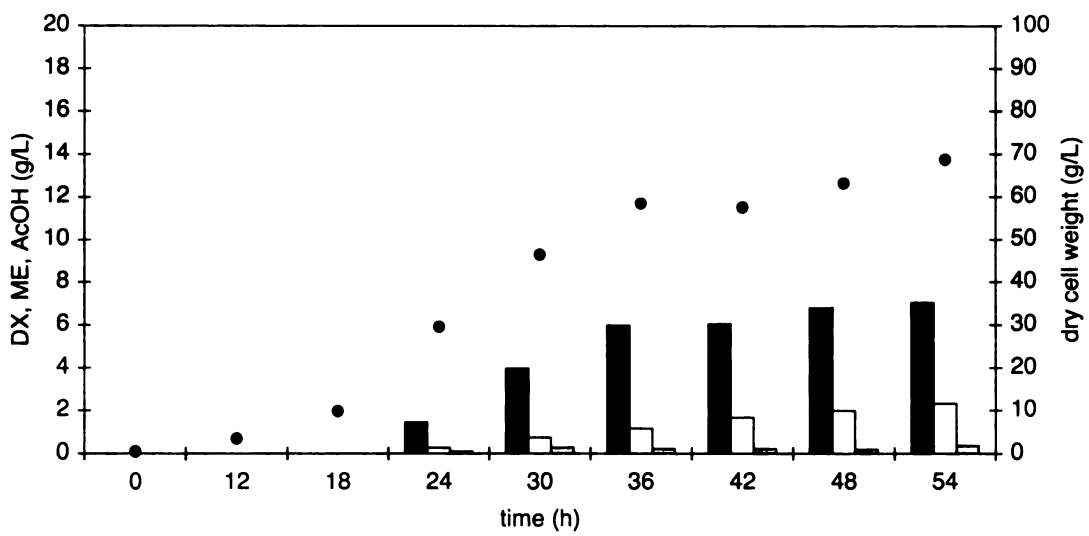


Figure 24. Cultivation of JWF1/pPV4.230 (*dxs* expressed from a P_{N25} promoter) under glucose-limited conditions. Key: filled circles, dry cell weight; black bars, 1-deoxy-D-xylulose; open bars, 2-methyl-D-erythritol; gray bars, acetic acid.

Cultivation of shikimate pathway biocatalysts under glucose-rich fed-batch fermentor condition led to accumulation of higher titers of shikimate pathway products in the culture supernatant.[74b] Based on this precedence, it was decided to cultivate JWF1/pPV4.230 under glucose-rich fed-batch fermentation conditions. Under these

cultivation conditions *E. coli* JWF1/pPV4.230 synthesized 13 g/L of 1-deoxy-D-xylulose in 6% yield (mol/mol) from glucose at the end of 60 h (Figure 25). At 18 h, 30 h, 48 h, and 60 h of cultivation of JWF1/pPV4.230 under glucose-rich conditions, the specific activity of *dxs*-encoded 1-deoxy-D-xylulose 5-phosphate synthase was 0.02, 0.15, 0.13, and 0.10 μmol/min/mg, respectively. By the completion of this fermentor run at 60 h (Figure 25), 2 g/L of 2-*C*-methyl-D-erythritol and 18 g/L of acetic acid had been synthesized.

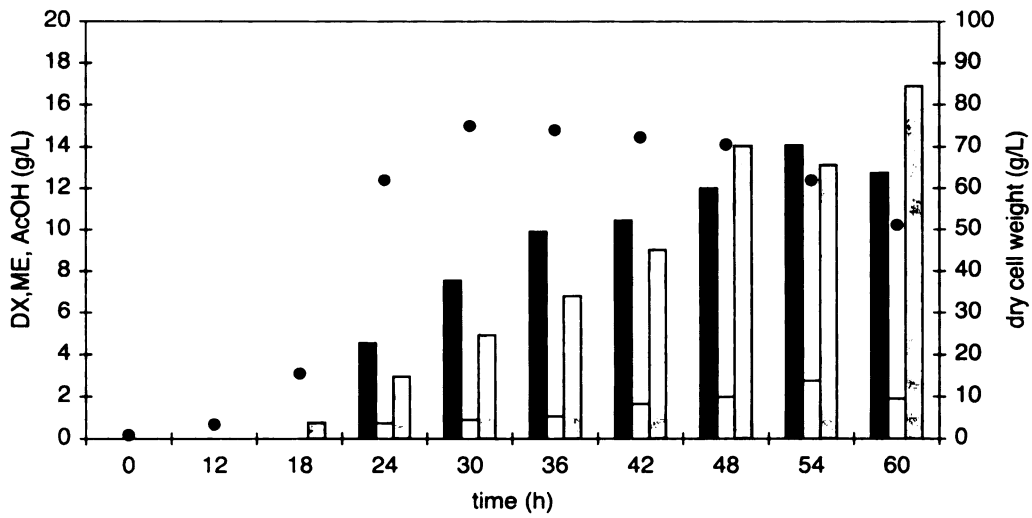


Figure 25. Cultivation of JWF1/pPV4.230 (*dxs* expressed from a P_{N25} promoter) under glucose-rich conditions. Key: filled circles, dry cell weight; black bars, 1-deoxy-D-xylulose; open bars, 2-methyl-D-erythritol; gray bars, acetic acid.

Under both glucose-rich and glucose-limited culture conditions, *E. coli* JWF1/pPV4.230 grew to densities of around 70 g/L (dry cell weight). Fermentations routinely performed in our laboratory with auxotrophs of the shikimate pathway produced cell masses around 25-30 g/L. As a result, the amount of biomass produced by JWF1/pPV4.230 during fed-batch fermentation was considered excessive. High levels of acetic acid are known to have a negative impact on growing cultures of *E. coli*.[81] Hence,

the amount of acetic acid (17 g/L) accumulating in the glucose-rich fermentation was assumed to have a deleterious impact on the product formation. Therefore for the synthesis of 1-deoxy-D-xylulose and other DXP pathway intermediates, requires a suitable host that produces lower amount of acetic acid and biomass.

In order to investigate if the amounts of cell mass and acetic acid had affected carbon flow in the pathway, plasmid pPV4.230 was transformed into the auxotroph *E. coli* SP1.1. *E. coli* SP1.1 has been used in the microbial synthesis of shikimic acid and is known to produce 25-30 g/L of biomass and small amounts of acetic acid during its cultivation under fed-batch fermentor conditions.[74] Cultivation of SP1.1 under fed-batch fermentor conditions required addition of the aromatic amino acids to inhibit native levels of DAHP synthase activity and to prevent accumulation of shikimate pathway intermediates due to disruption in the *aroK* and *aroL* loci. When the aromatic amino acids L-phenylalanine (0.7 g), L-tyrosine (0.7 g), and L-tryptophan (0.35 g) were added at 18 h and 30 h during the fermentation run of SP1.1/pPV4.230, high levels of biomass similar to JWF1/pPV4.230 were observed. Small amounts of shikimate pathway intermediates also accumulated in the culture medium. Reduced formation of biomass (25 g/L) was observed when the aromatic amino acids were added in one part at the beginning of the oxygen sensor-controlled phase instead of adding in two portions. Growth of SP1.1/pPV4.230 under glucose-rich conditions with aromatic amino acids supplementation was characterized by greatly reduced production of acetic acid relative to JWF1/pPV4.230. SP1.1/pPV4.230 synthesized 15 g/L of 1-deoxy-D-xylulose in 8 % yield (mol/mol) at the end of 60 h along with 2 g/L of 2-*C*-methyl-D-erythritol, and 1 g/L of acetic acid (Figure 26). The specific activities for DXS measured at 24 h, 36 h, 48 h

and 60 h were 0.1, 0.15, 0.15 and 0.1 μmol/min/mg, respectively. The 1-deoxy-D-xylulose produced can be extracted from the fermentation broth by ultrafiltration of cell-free fermentation broth through an Amicon membrane (10,000 MW) followed by continuous liquid-liquid extraction with ethyl acetate.

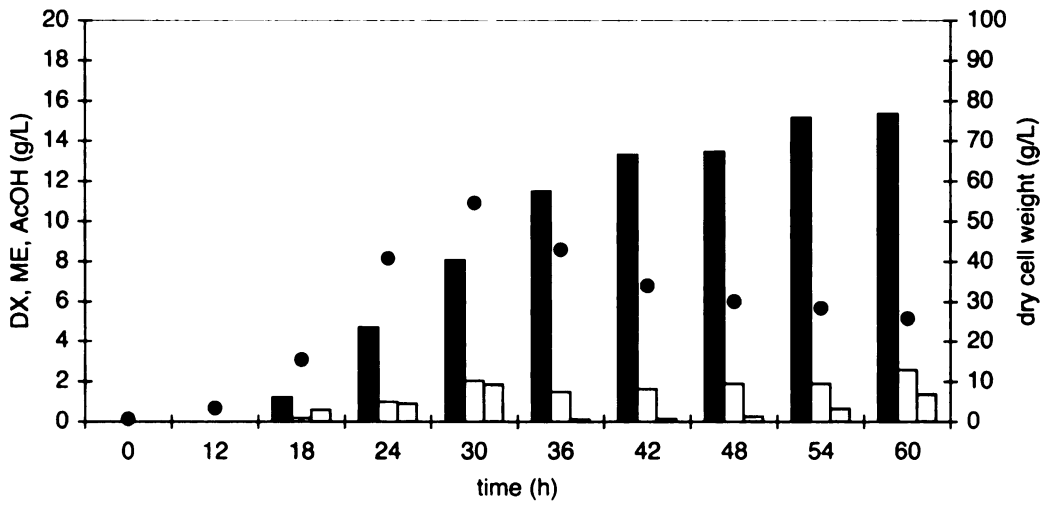


Figure 26. Cultivation of SP1.1/pPV4.230 (*dxs* expressed from a P_{N25} promoter) under glucose-rich conditions supplemented with aromatic amino acids. Key: filled circles, dry cell weight; black bars, 1-deoxy-D-xylulose; open bars, 2-methyl-D-erythritol; gray bars, acetic acid.

Synthesis of 2-C-methyl-D-erythritol under Amplified Levels of DXS and DXR

In order to enhance 1-deoxy-D-xylulose production, a chromosomal disruption downstream of *dxs* in the isoprenoid biosynthetic pathway was required. This would also provide information regarding the measure of carbon flow through the DXP pathway. *E. coli* DXS2.1(SP1.1 *dxr*::Kan^R) was created by disruption of the *dxr* gene in SP1.1 by bacteriophage P1 transduction of *dxr*::Kan^R locus from strain DMY1 (FS1576 *dxr*::Kan^R). Cultivation of *E. coli* DXS2.1 required supplementation of the cultures with 2-*C*-methyl-D-erythritol.[75] This second intermediate in the DXP pathway was chemically

synthesized.[82] A drawback with this synthesis is the 80% *ee* of 2-*C*-methyl-D-erythritol product. This would lead to accumulation of 2-*C*-methyl-L-erthritol in the culture supernatant, which becomes a contaminant of the desired 1-deoxy-D-xylulose. Contamination by 2-*C*-methyl-L-erthritol can be eliminated by synthesizing 2-*C*-methyl-D-erythritol from *E. coli* overexpressing *dxs* and *dxr* genes and using it as a supplement.

For this purpose, plasmid pPV6.044 was assembled. The *dxr* gene without the start codon was amplified by PCR and cloned into plasmid pQE30 to give plasmid pPV3.23 (Figure 27). The orientation of the *dxr* gene was in the same direction as the T5 promoter. The P_{N25} *dxr* fragment was then excised out of pPV3.23 by a *Ssp*I enzyme digest and ligated into pPV2.248 digested with *Sma*I to afford plasmid pPV6.044. The orientation of P_{N25} *dxr* was opposite to P_{tac} *dxs* (Figure 28).

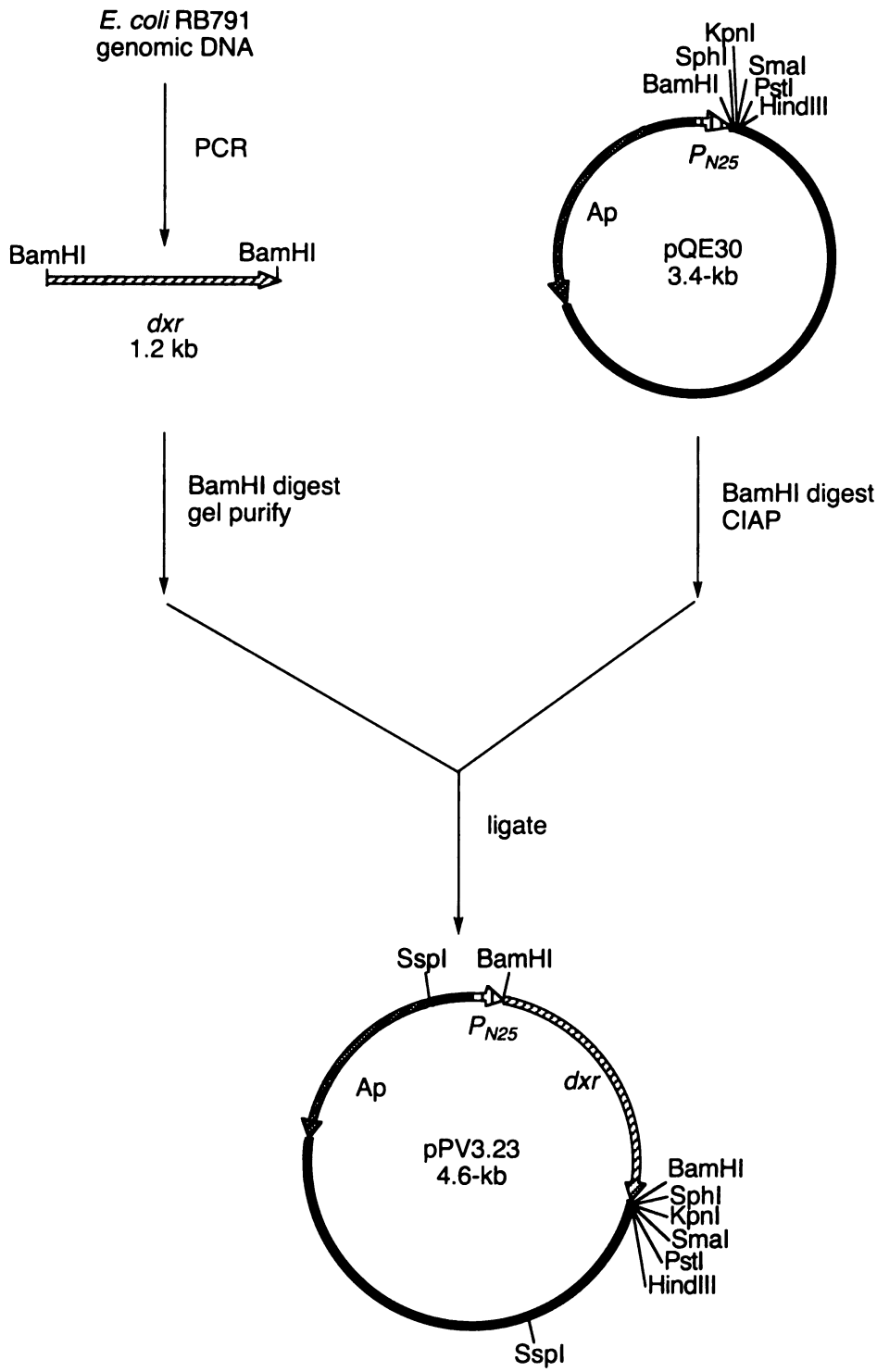


Figure 27. Construction of plasmid pPV3.23.

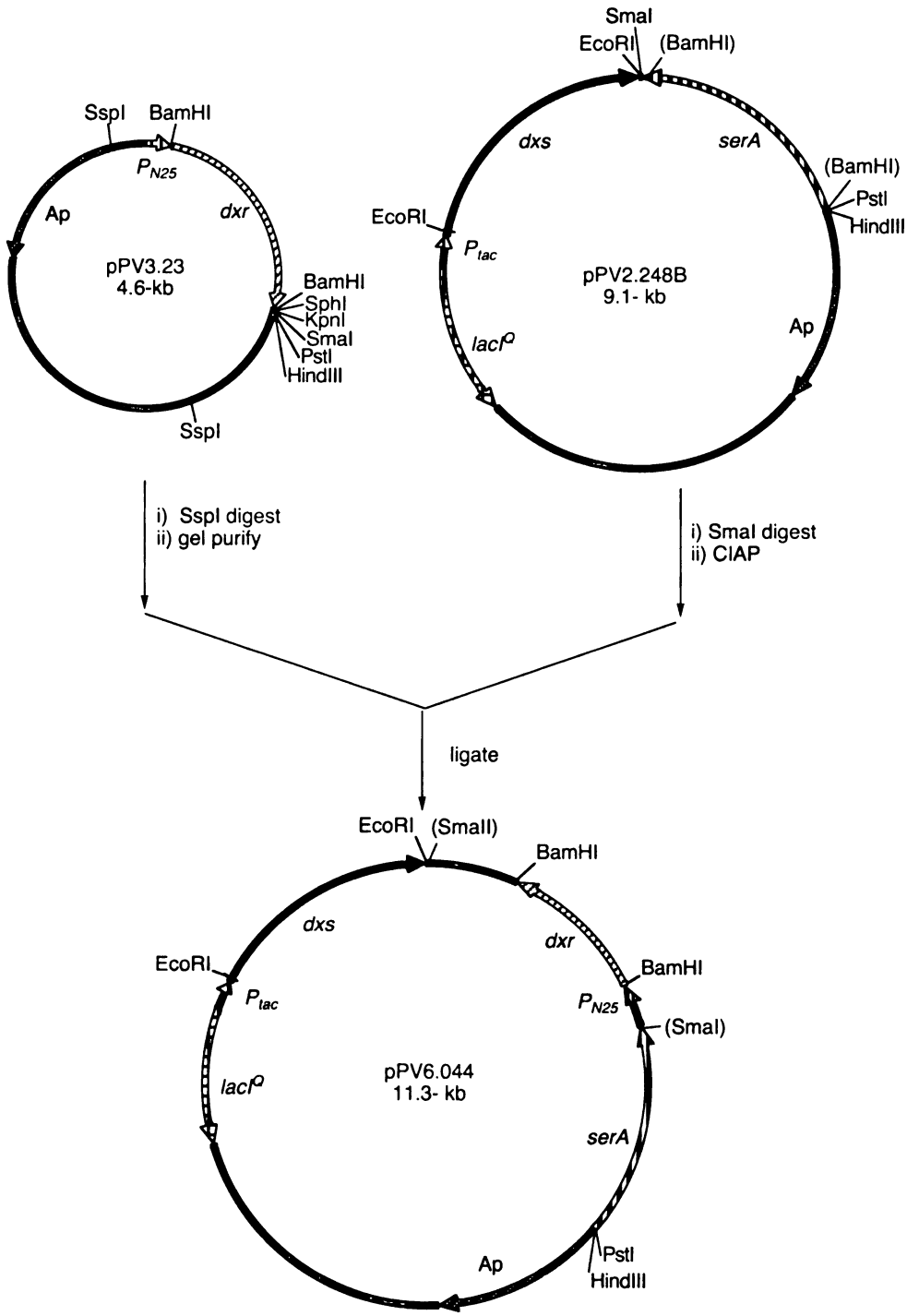


Figure 28. Construction of plasmid pPV6.044.

Cultivation of *E. coli* SP1.1/pPV6.044 (P_{tac} *dxs*, P_{N25} *dxr*, *ser*A, *and lacI*^{Q}) under glucose-rich fed-batch fermentation conditions for 60 h led to the accumulation of 18 g/L of 2-*C*-methyl-D-erythritol in 8% yield (mol/mol). This was the only metabolite that was observed in the culture supernatant (Figure 29). The absence of other downstream pathway intermediates in the culture supernatant of *E. coli* SP1.1/pPV6.044 may be attributed to hydrolysis of the DXP pathway intermediates and export of the resulting 2-*C*-methyl-D-erythritol (Figure 14).

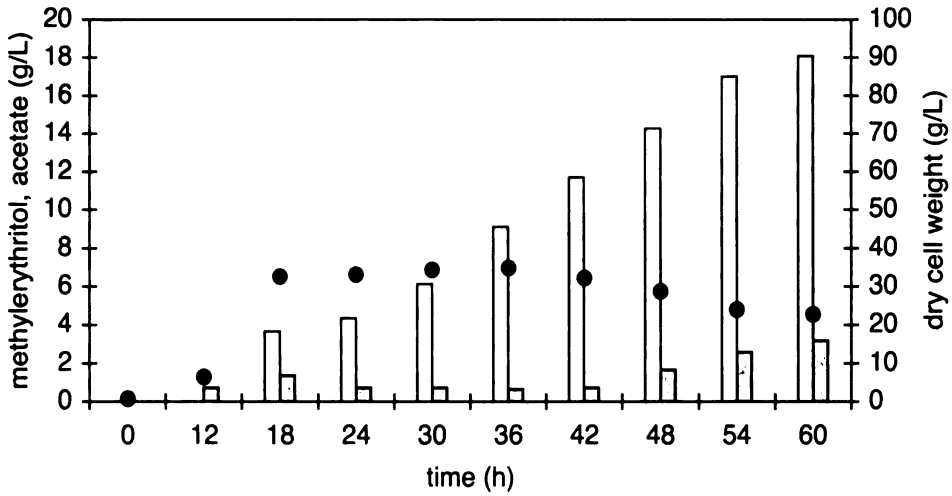


Figure 29. Cultivation of SP1.1/pPV6.044 (*dxs* expressed from a P_{tac} promoter and *dxr* expressed from P_{N25} promoter) under glucose-rich conditions supplemented with aromatic amino acids. Key: filled circles, dry cell weight; open bars, 2-*C*-methyl-D-erythritol; gray bars, acetic acid.

The 2-*C*-methyl-D-erythritol produced can be extracted from the clarified fermenatation broth by continuous liquid-liquid extraction with ethyl acetate. The extracted residue can be further purified by peracetylation followed by silica gel chromatography. Deprotection of the acetylated 2-*C*-methyl-D-erythritol with potassium carbonate in methanol followed by passing the solution through a column of Dowex-50 (H^+ form) affords pure 2-*C*-methyl-D-erythritol. The purification of 2-*C*-methyl-D-

erythritol was necessary as cultures of DXS2.1 grew slowly when supplemented with 2-*C*-methyl-D-erythritol without purification.

Microbial Synthesis of 1-Deoxy-D-Xylulose by *E. coli* DXS2.1/pPV4.230.

E. coli DXS2.1 is a derivative of *E. coli* SP1.1 where the chromosomal *dxr* gene has been disrupted by introduction of an aminoglycoside phosphotransferase gene encoding for kanamycin resistance. DXS2.1 mutants require supplementation of 2-*C*-methyl-D-erythritol (0.01%) for growth even in rich medium. Initial experiments with DXS2.1/pPV4.230 were performed to ascertain the concentration of 2-*C*-methyl-D-erythritol supplementation required for growth during fed-batch fermentations. In the minimal salts medium used for fed-batch fermentation with 2-*C*-methyl-D-erythritol supplementation (0.01%), cultures of DXS2.1/pPV4.230 were slow growing and did not enter the oxygen sensor-controlled phase of fermentation even after 48 h. Increasing the amount of 2-*C*-methyl-D-erythritol supplementation to 0.1-0.3% gave the same result. As a consequence, the culturing conditions for DXS2.1/pPV4.230 were slightly modified. The DXS2.1/pPV4.230 (supplemented with 0.2% 2-*C*-methyl-D-erythritol) was cultured under glucose-rich fermentation conditions. The glucose feed was initiated after 30 h of growth followed by induction of the *dxs* gene. Using these slow-growing cultures and with induction of the *dxs* gene with 5 mg/L IPTG at 30 h, 10 g/L 1-deoxy-D-xylulose accumulated in the culture supernatant after 90 h in 3% (mol/mol) yield from D-glucose (Figure 30).

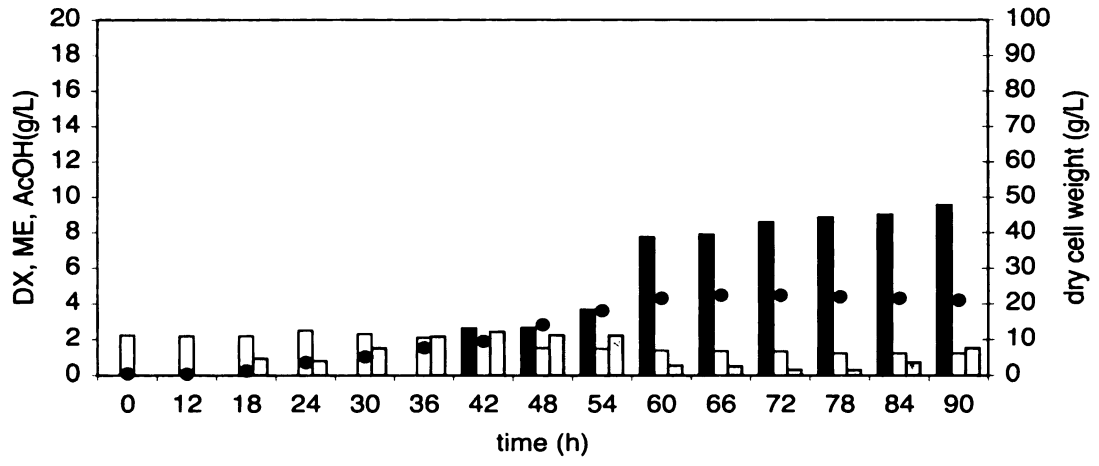


Figure 30. Cultivation of DXS2.1/pPV4.230 (*dxs* expressed from a P_{N25} promoter) under glucose-rich conditions supplemented with aromatic amino acids. Key: filled circles, dry cell weight; filled bars, 1-deoxy-D-xylulose; open bars, 2-*C*-methyl-D-erythritol; gray bars, acetic acid.

Based on these results it was decided to enhance the growth rate by addition of yeast extract to the culture medium. Addition of yeast extract to cultures is known to enhance the growth rate.[83] After analyzing several growth conditions, it was found that addition of 2 mg/mL yeast extract to the starting liquid seed cultures was sufficient to get good growth of cultures in the fed-batch fermentation. In this case no yeast extract was added to the fermentation medium. In the absence of yeast extract, the seed cultures of DXS2.1/pPV4.230 grew five times slower. This was ascertained by measuring OD_{600}. After 12 h of growth, liquid seed cultures supplemented with yeast extract had OD_{600} =1.2-1.5 while those without supplementation had OD_{600} = 0.2-0.3. The liquid seed cultures without yeast extract supplementation took approximately 24 h to achieve an OD_{600} =1.2-1.5. In the absence of 2-*C*-methyl-D-erythritol the cultures of DXS2.1 did not grow even when supplemented with yeast extract. When *E. coli* DXS2.1/pPV4.230 was cultured under glucose-rich fed-batch fermentation conditions with yeast extract (added only to liquid seed culture), 2-*C*-methyl-D-erythritol (0.1%) and aromatic amino

acid supplementation, 18 g/L of 1-deoxy-D-xylulose in 10% yield (mol/mol) accumulated in the culture supernatant after 60 h (Figure 31). The concentration of 2-*C*-methyl-D-erythritol added as supplement did not decrease over the course of fermentation. Based on these results it needed to be ascertained whether the mutant is leaky or there existed a *dxr* isozyme that cause the slow buildup of 2-*C*-methyl-D-erythritol. The slow uptake of 2-*C*-methylerythritol by the cell can be attributed to slow transport rates into *E. coli* or limiting kinase activities of the kinase that phosphorylates 2-*C*-methyl-D-erythritol into 2-*C*-methyl-D-erythritol 4-phosphate.

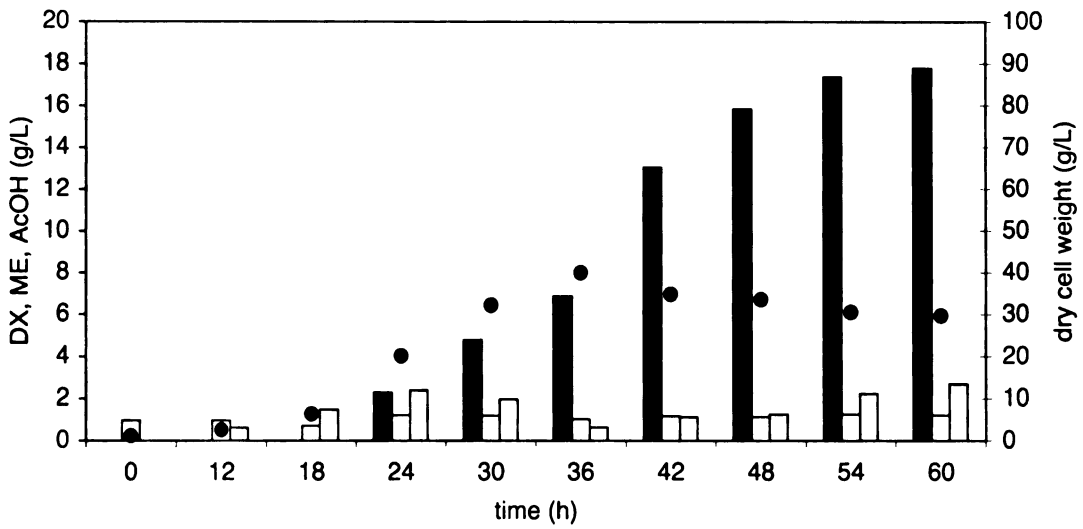


Figure 31. Cultivation of DXS 2.1/pPV4.230 (*dxs* expressed from a P_{N25} promoter) under glucose-rich conditions supplemented with aromatic amino acids and with yeast extract added to seed culture. Key: filled circles, dry cell weight; filled bars, 1-deoxy-D-xylulose; open bars, 2-*C*-methyl-D-erythritol; gray bars, acetic acid.

Alternative Routes for Measuring Carbon Flow Through the DXP Pathway

Inhibition of the Native Levels of DXR by Fosmidomycin.

Fosmidomycin (Figure 32) is a natural product that is produced in small amounts in growing cultures of *Streptomyces lavendula.*[84] This compound was formerly under development as an antibacterial agent. As a phosphonic acid derivative with potent activity against gram-negative bacteria, it was used in treatment of urinary tract infections in the early 1980's but was ineffective against recurrent infections.[85] With the discovery of the DXP pathway, fosmidomycin was found to act as a potent inhibitor of 1-deoxy-D-xylulose-reductoisomerase thereby preventing the biosynthesis of isoprenoids in plants and bacteria.[86] It has also been reported to be effective as an anti-malarial agent against *Plasmodium falciparum.*[87] DXR catalyzes the conversion of 1-deoxy-D-xylulose 5-phosphate to 2-*C*-methylerythritol 4-phosphate by rearrangement followed by reduction. Fosmidomycin was thought to act as a transition state analogue of DXR by mimicking the intermediate 2-*C*-methyl-D-erythrose 4-phosphate. However, recent structural analysis of fosmidomycin bound to DXR seems to suggest that fosmidomycin mimics the substrate 1-deoxy-D-xylulose 5-phosphate and not the intermediate 2-*C*-methylerythrose 4-phosphate.[88]

Using *E. coli* SP1.1/pPV4.230 grown to its maximum cell density, addition of fosmidomycin and subsequent inhibition of 1-deoxy-D-xylulose-reductoisomerase offered an alternate strategy to measure carbon flow directed into the DXP pathway. This would also provide a route to yield 1-deoxy-D-xylulose devoid of 2-*C*-methyl-D-erythritol contamination. This technique circumvented the need for inactivating the

chromosomal *dxr* locus and the attendant need for 2-*C*-methyl-D-erythritol supplementation.

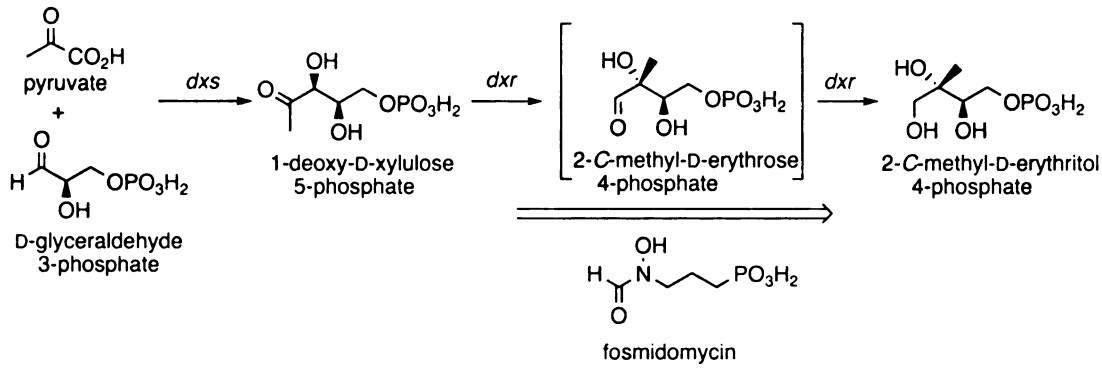


Figure 32. Inhibition of 1-deoxy-D-xylulose reductoisomerase by fosmidomycin.

Fosmidomycin was chemically synthesized according to a literature procedure (Figure 33).[89] Diethyl-(3-bromopropyl) phosphonate **11** was synthesized by an Arbuzov reaction of triethyl phosphite with an excess of 1,3-dibromopropane. Treatment of the phosphonate **11** with hydroxylamine hydrochloride gave diethyl(*N*-hydroxylamino)-propylphosphonate **12**, which was hydrolyzed to 3-(*N*-hydroxylamino)-propylphosphonic acid **13** by refluxing in 40% HCl:AcOH (2:1 v/v). Formic acid and acetic anhydride were mixed to generate reactive formyl acetate mixed anhydride. Formylation of 3-(*N*-hydroxylamino)propylphosphonic acid with the mixture of formic acid and acetic anhydride the afforded fosmidomycin in 70% yield (Figure 33).

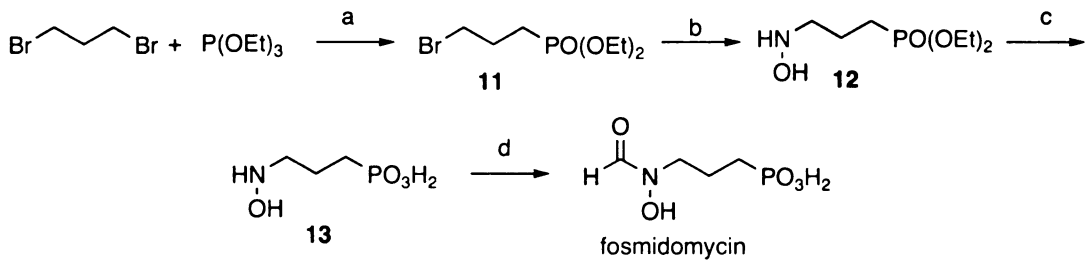


Figure 33. Synthesis of fosmidomycin.
Key: a) 150°C, 30 min, 64% ; b) $NH_2OH{\cdot}HCl$, aq. NaOH, MeOH 0°C to 45°C, 50%; c) 40% aqueous HCl: AcOH (2:1 v/v), reflux, 68%; d) HCOOH, Ac_2O, 0°C to rt. 70%.

E. coli SP1.1/pPV4.230 was cultured under glucose-rich fermentation conditions for 60 h. Fosmidomycin (0.1 g/L) was added to the culture medium at 22 h after the start of the fermentation. The effect of fosmidomycin on the growth rate was observed within 2 h. A pronounced reduction in the stirring rate indicated that fosmidomycin was inhibiting the DXR and depriving the cells of isoprenoids required for growth. Using these non-growing cultures and inducing the *dxs* gene with IPTG, 12 g/L of 1-deoxy-D-xylulose accumulated in the culture supernatant after 60 h in 4.5 % yield (Figure 34). The 20 g/L of acetic acid produced exceeds the amount of this unwanted byproduct previously observed during synthesis of 1-deoxy-D-xylulose using SP1.1/pPV4.230 (Figure 26) and is comparable to that produced by JWF1/pPV4.230 under glucose-rich conditions (Figure 25). The high levels of acetic acid produced is indicative of the *E. coli* strain to be under metabolic stress leading to poor channelling of the carbon source towards the production of 1-deoxy-D-xylulose. No 2-*C*-methyl-D-erythritol is detected in the culture medium at the end of the fermentation run (Figure 34).

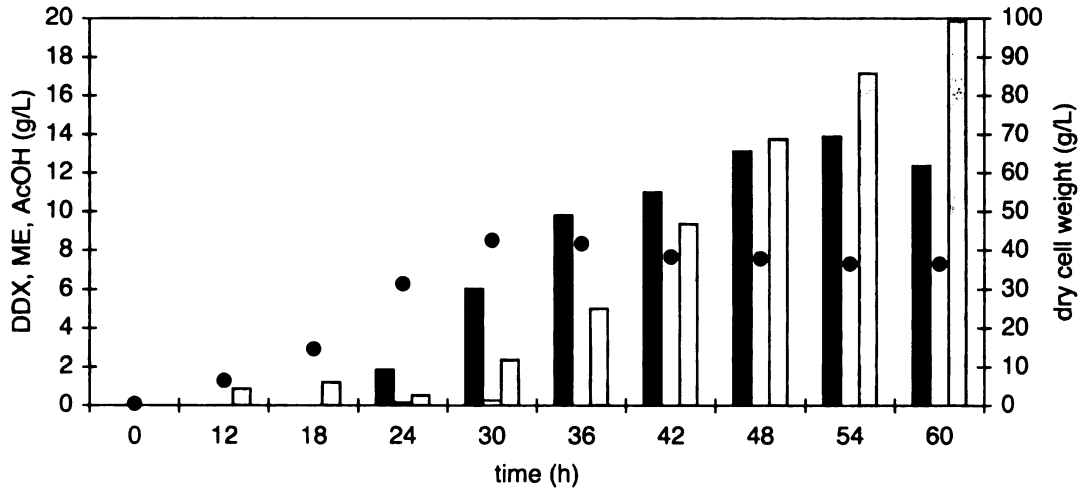


Figure 34. Cultivation of SP1.1/pPV4.230 (*dxs* expressed from a P_{N25} promoter) under glucose-rich conditions supplemented with aromatic amino acids and fosmidomycin added. Key: filled circles, dry cell weight; filled bars, 1-deoxy-D-xylulose; open bars, 2-*C*-methyl-D-erythritol; gray bars, acetic acid.

Recruitment of the Mevalonate Pathway along with Suppression of DXR Activity

An alternative to 2-*C*-methyl-D-erythritol supplementation in *dxr* auxotrophs is to use mevalonic acid supplementation along with heterologous expression of the MVA pathway genes that enables *E. coli* to use mevalonic acid as precursor for IPP. *E. coli* produces its isoprenoid precursors IPP and DMAPP via the DXP pathway and does not have the MVA pathway. Disruption of the DXP pathway along with expression of the MVA pathway genes with mevalonic acid supplementation provides an alternative route for the synthesis of 1-deoxy-D-xylulose. This approach of using the mevalonate pathway has been applied in the production of isoprenoids.[90]

In order to utilize the mevalonate pathway in *E. coli* SP1.1, plasmid pPV6.213 was assembled. Plasmid pPV6.213 is a derivative of plasmid pPV4.230, and it harbors the mevalonate pathway gene cluster from *Streptomyces* sp. strain CL190[91] expressed from a P_{aroF} promoter (Figure 37). To ensure their constitutive expression, mevalonate

pathway genes in *E. coli* SP1.1/pPV4.230 needed to be expressed from a promoter that was not repressed by the LacI. Since the Lac repressor does not affect the expression from the P_{aroF} promoter, this promoter was used for the expression of the mevalonate pathway genes. Promoter P_{aroF} was amplified by PCR from plasmid pKL5.17A and cloned into pTrc99A to give plasmid pPV6.185 (Figure 35). The MVA genes were excised out of the plasmid pUMV20 (obtained from Professor T. Kuzuyama)[91] by a *Xba*I/*Hind*III and ligated to pPV6.185 (digested with *Xba*I/*Hind*III) to give plasmid pPV6.196 (Figure 36). The 6.2-kb P_{aroF}MVA fragment was excised out of pPV6.196 by a *Hind*III digest followed by a *Nco*I partial digest. Plasmid pPV4.230 was digested with *Nco*I followed by a partial *Hind*III digest. Ligation of the 6.2-kb fragment with pPV4.230 gave the 13.8-kb plasmid pPV6.213 (Figure 37). In order to evaluate the activity of P_{aroF}MVA gene products, complementation studies were done with *E. coli* DXS2.1. *E. coli* DXS2.1 transformants carrying plasmid pPV6.213 grew only with mevalonic acid supplementation. An additional line of evidence was obtained from *E. coli* SP1.1 transformants carrying the plasmid pPV6.213 that were capable of growth in the presence of fosmidomycin upon mevalonic acid supplementation. The slow growth rate (5-6 days) of *E. coli* DXS2.1/pPV6.213 in the minimal medium starter cultures (5 mL) with mevalonic acid supplementation hindered its evaluation under fed-batch fermentation condition.

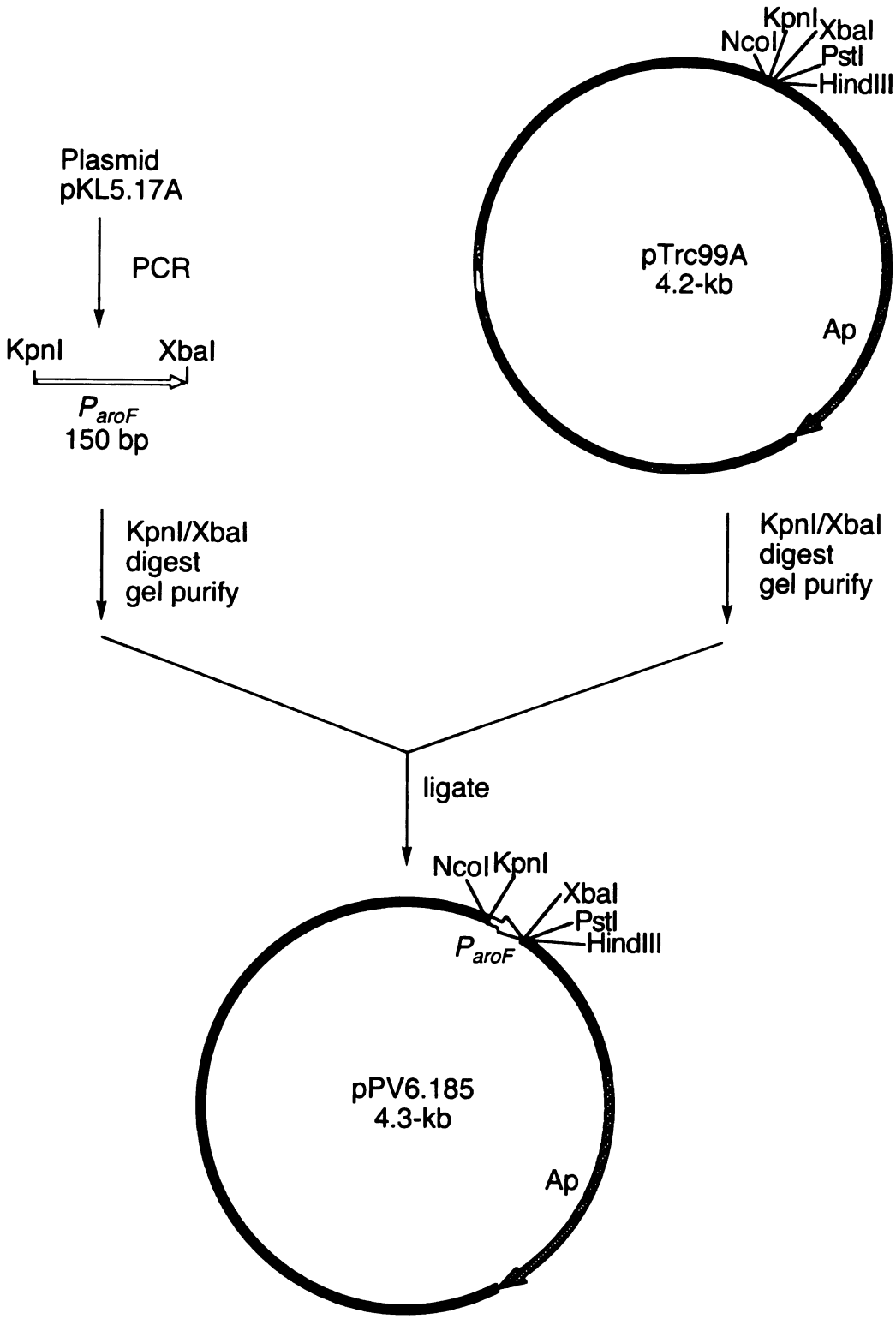


Figure 35. Construction of plasmid pPV6.185.

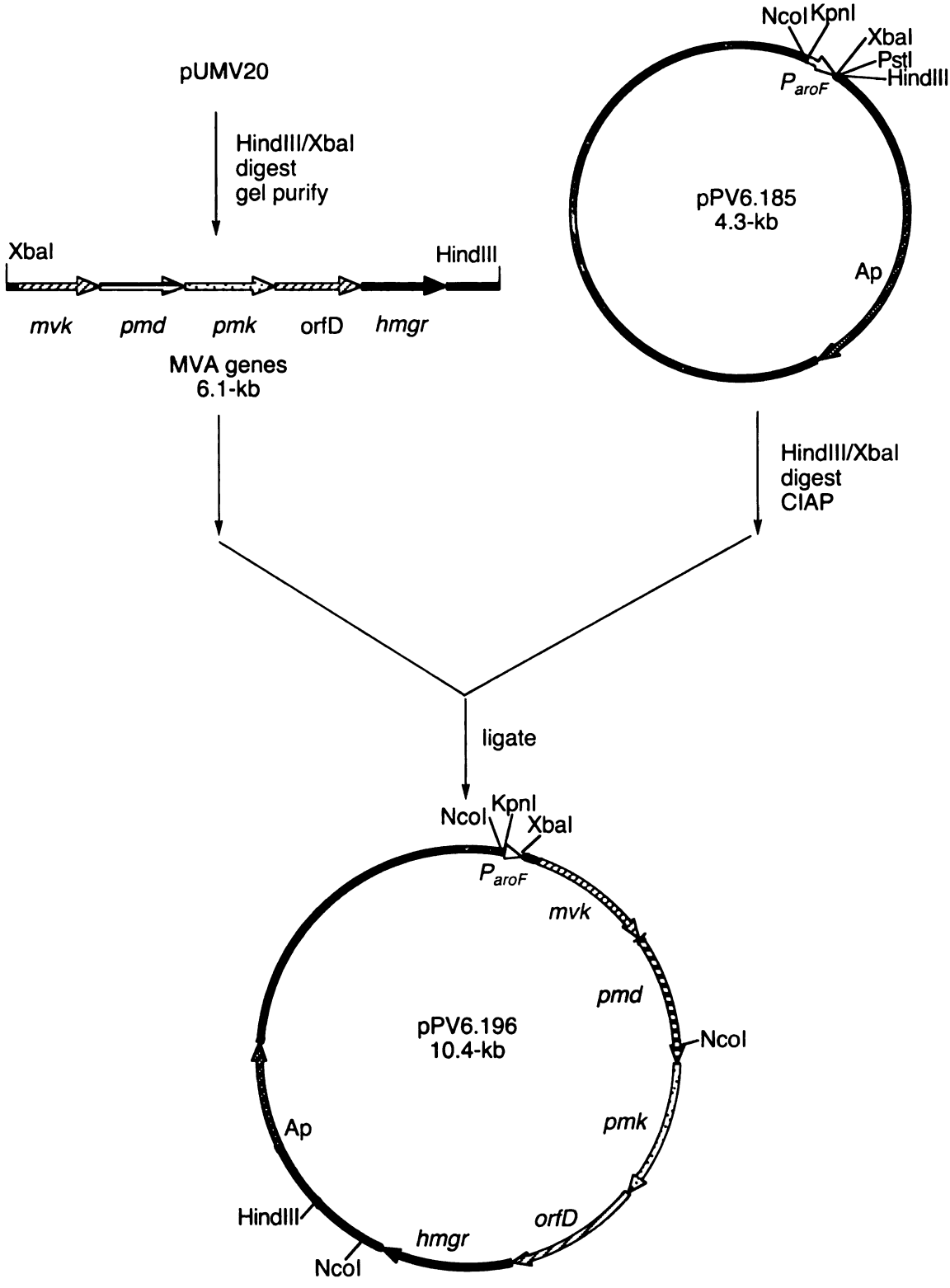


Figure 36. Construction of plasmid pPV6.196.

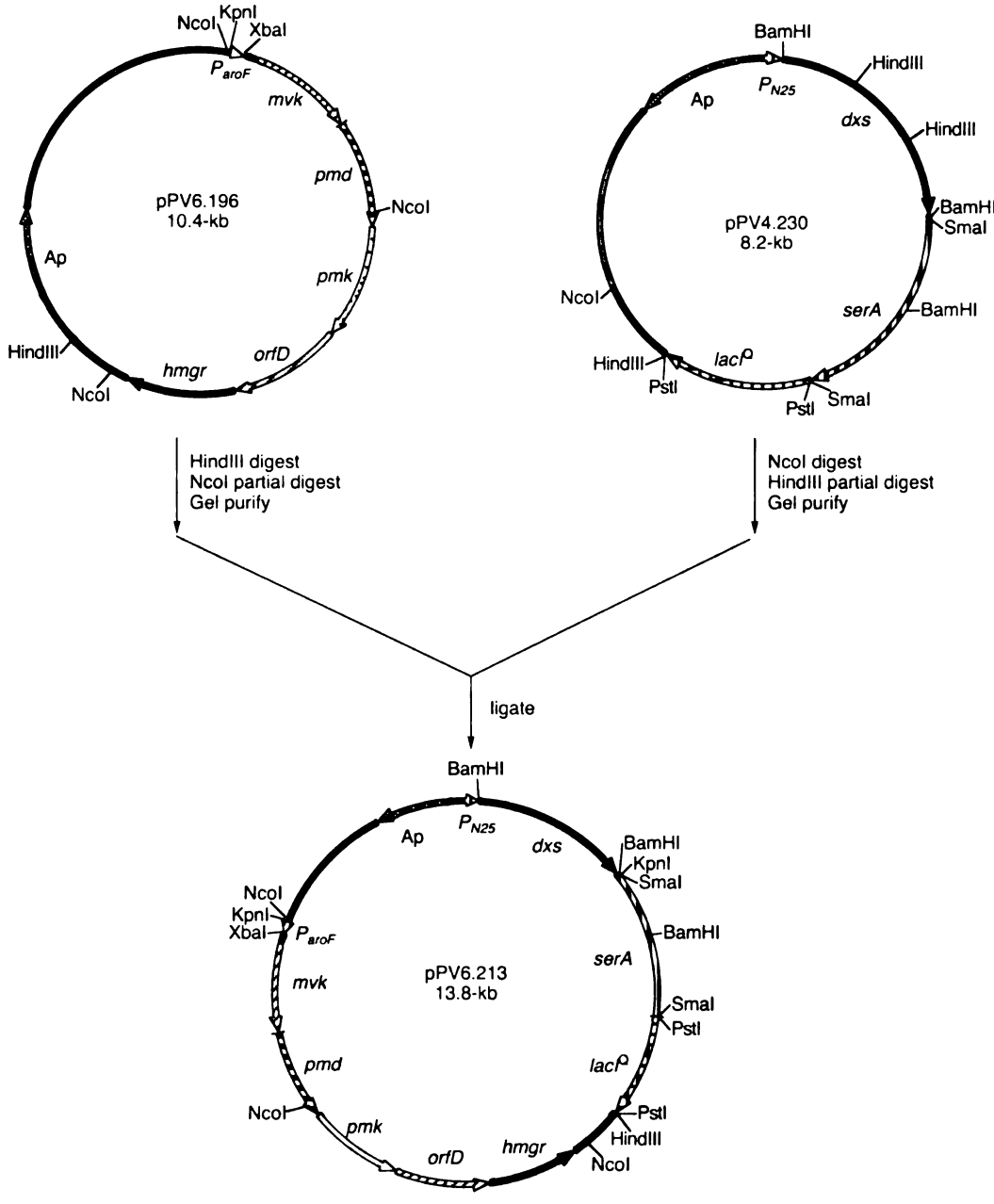


Figure 37. Construction of plasmid pPV6.213.

E. coli SP1.1 was transformed with plasmid pPV6.213 and plated on M9 medium with glucose as the carbon source with aromatic amino acid and aromatic vitamin

supplementation. Single colonies were then inoculated into 5 mL M9 medium with glucose, aromatic amino acids, aromatic vitamins, antibiotics, yeast extract (2 mg/mL), mevalonic acid (0.15 g/L), and fosmidomycin (0.1 g/L) followed by cultivation at 37°C. yeast extract (2 mg/mL) was added to these cultures to enhance the growth rate. This starter culture was then used as the inoculant for 100 mL of growth medium, which was subsequently used as the inoculant for the fermentor run. The fermentation medium contained fosmidomycin (0.1 g/L) and mevalonic acid (0.15 g/L). Under these glucose-rich fed-batch fermentation conditions, *E. coli* SP1.1/pPV4.230 synthesized 10 g/L of 1-deoxy-D-xylulose in 5 % (mol/mol) yield along with 8 g/L of acetic acid after 72 h of cultivation (Figure 38).

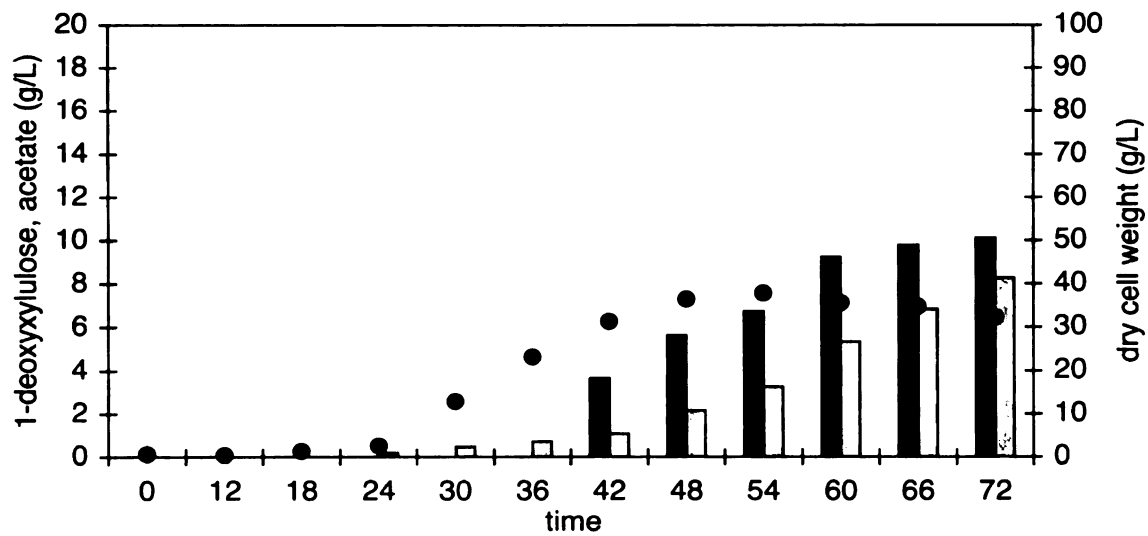


Figure 38. Cultivation of SP1.1/pPV6.213 (*dxs* expressed from a P_{N25} promoter and mevalonate pathway genes) under glucose-rich conditions supplemented with aromatic amino acids, fosmidomycin and mevalonic acid. Key: filled circles, dry cell weight; filled bars, 1-deoxy-D-xylulose; gray bars, acetic acid.

The slow growth of SP1.1/pPV4.230, as indicated by the 36-42 h required to reach the oxygen sensor-controlled phase of fermentation, may be due to poor transport of mevalonic acid into the *E. coli* cytoplasm. Alternatively, expression of the

Streptomyces mevalonate pathway genes may constitute a metabolic burden interfering with SP1.1/pPV4.230 growth and metabolism resulting in lower yields. The production of high levels of acetic acid in these fermentations may be due to the reduced pool of ubiquinone and other quinones that are needed for efficient functioning of several oxidoreductases in the cytoplasm.[92]

Discussion

The DXP pathway for isoprenoid biosynthesis offers a vast new area for the discovery of new therapeutic compounds,[93] production of antioxidants, and provides a new source for compounds of industrial and pharmaceutical importance. With the discovery of the DXP pathway, a new avenue is now available to enhance the production of the carotenoids.[94] Carotenoids production by metabolic engineering of the isoprenoid biosynthetic pathway has been carried out in plants and microbes.[95,96] Understanding the rate-limiting impediments of this pathway is essential for enhancing the carbon flow down the pathway.

Metabolic engineering for enhancing carotenoid production in microbes by manipulation of the DXP pathway has been done by several groups.[96] It has been shown in plants and bacteria that DXS is one of the rate-limiting enzymes of this pathway.[97] Altering the levels of DXS enzyme is known to increase levels of plant plastidic isoprenoids such as chlorophyll, α–tocopherols as well as carotenoids like lycopene and astaxanthin in bacteria.[96]

We have investigated the impact of carbon flux in the DXP pathway under conditions where solution pH, dissolved oxygen levels and glucose feed rates could be

controlled. In order to identify the rate-limiting impediments, we designed *Escherichia coli* constructs with increased *in vivo* catalytic activity of 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and studied the surge of carbon flow through this pathway under fed-batch fermentation conditions. Increase in the *in vivo* catalytic activity of DXS synthase creates a metabolic situation where individual DXP pathway enzymes become rate-limiting. Due to the rate-limiting nature of these enzymes, their substrates are not converted into product at a sufficient rapid rate to avoid hydrolysis and export into the culture supernatant. ^{1}H NMR analysis of the culture supernatant has been employed as a method for identification of the rate-limiting impediments of the DXP pathway. By modulating the expression levels of the *dxr* gene, unique sugars such as 1-deoxy-D-xylulose and 2-*C*-methyl-D-erythritol could be synthesized by fed-batch fermentation. To our knowledge this is the first example of microbial synthesis of these compounds. The surge of the carbon flow through the DXP pathway along with increased in vivo catalytic levels of DXS led to the accumulation of 15 g/L of 1-deoxy-D-xylulose and 2 g/L of 2-*C*-methyl-D-erythritol in culture supernatant of *E. coli* SP1.1/pPV4.230. Introduction of plasmid pPV6.044 that encodes for DXR along with DXS in *E. coli* SP1.1 resulted in the disappearance of 1-deoxy-D-xylulose and the accumulation of 18 g/L of 2-*C*-methyl-D-erythritol in the culture supernatant. The data was interpreted as indicative of DXR being one of the rate-limiting impediments to the carbon flow through the DXP pathway. The absence of other downstream pathway intermediates in the culture supernatant of *E. coli* SP1.1/pPV6.044 was attributed to dephosphorylation of the downstream cytidine intermediates (Figure 14) followed by export of 2-*C*-methyl-D-erythritol into the culture supernatant.

The cultures of DXS2.1/pPV4.230 were slow-growing and the uptake of 2-*C*-methyl-D-erythritol in these cultures over the period of time was relatively slow. The slow growth can be explained by poor transport of 2-*C*-methyl-D-erythritol into the cytoplasm of *E. coli* or due to sluggish kinase activity catalyzing formation of intermediate 2-*C*-methyl-D-erythritol 4-phosphate. Addition of yeast extract to the culture medium enhances growth rate, but does not eliminate the need for 2-*C*-methyl-D-erythritol supplementation. For supplementation of DXS2.1 cultures, 2-*C*-methyl-D-erythritol must either be chemically synthesized or purified from the fermentation broth. Enhancing the uptake of this metabolite will require identification of the transporter system and/or the kinase that phosphorylates 2-*C*-methyl-D-erythritol. Cultures of *Pseudomonas schuylkilliensis* have been reported to produce increased levels of ubiquione when supplemented with prenyl alcohols like isopentenyl alcohol, dimethylallyl alcohol or geraniol.[98] Based on this precedence, cultures of DXS2.1/pPV4.230 were tested for growth in the presence of isopentenyl and dimethylallyl alcohol as supplements. Replacing 2-*C*-methyl-D-erythritol with isopentenyl and dimethylallyl alcohols failed to lead to the growth of DXS2.1 cultures. A similar observation has also been reported by Cunningham *et. al.*[99] Identification of the putative transporter for isopentenyl alcohol from *P. schuylkilliensis* and incorporating it into *E. coli* DXS2.1 could potentially solve the supplementation issue for the auxotrophs in the DXP pathway. Thus, adequate supplementation when the DXP pathway is disrupted remains an issue.

For enhancing the carbon flow in the DXP pathway, various aspects still need to be addressed. One of the important questions is the amount of isoprene that is released

by the microorganism when carbon is channeled in this pathway. Wild-type *E. coli* is known to produce isoprene in small amounts.[100] Attendant monitoring of isoprene emission or IPP and DMAPP formation in intact cells when the DXP pathway genes are overexpressed may provide significant information regarding allosteric regulation in this pathway. Miller *et.al* have addressed the issue of allosteric regulation in this pathway by quantifying the DMAPP formed in intact cells when *dxs* and *dxr* were overexpressed.[101] The authors conclude that only *dxs* is the rate-limiting factor in this pathway and overexpression of *dxr* did not have any effect on the amount of DMAPP produced. To the contrary, we have observed DXR to be one of the rate-limiting enzyme of this pathway when *dxs* is overexpressed in constructs cultured under fed-batch fermentor conditions.

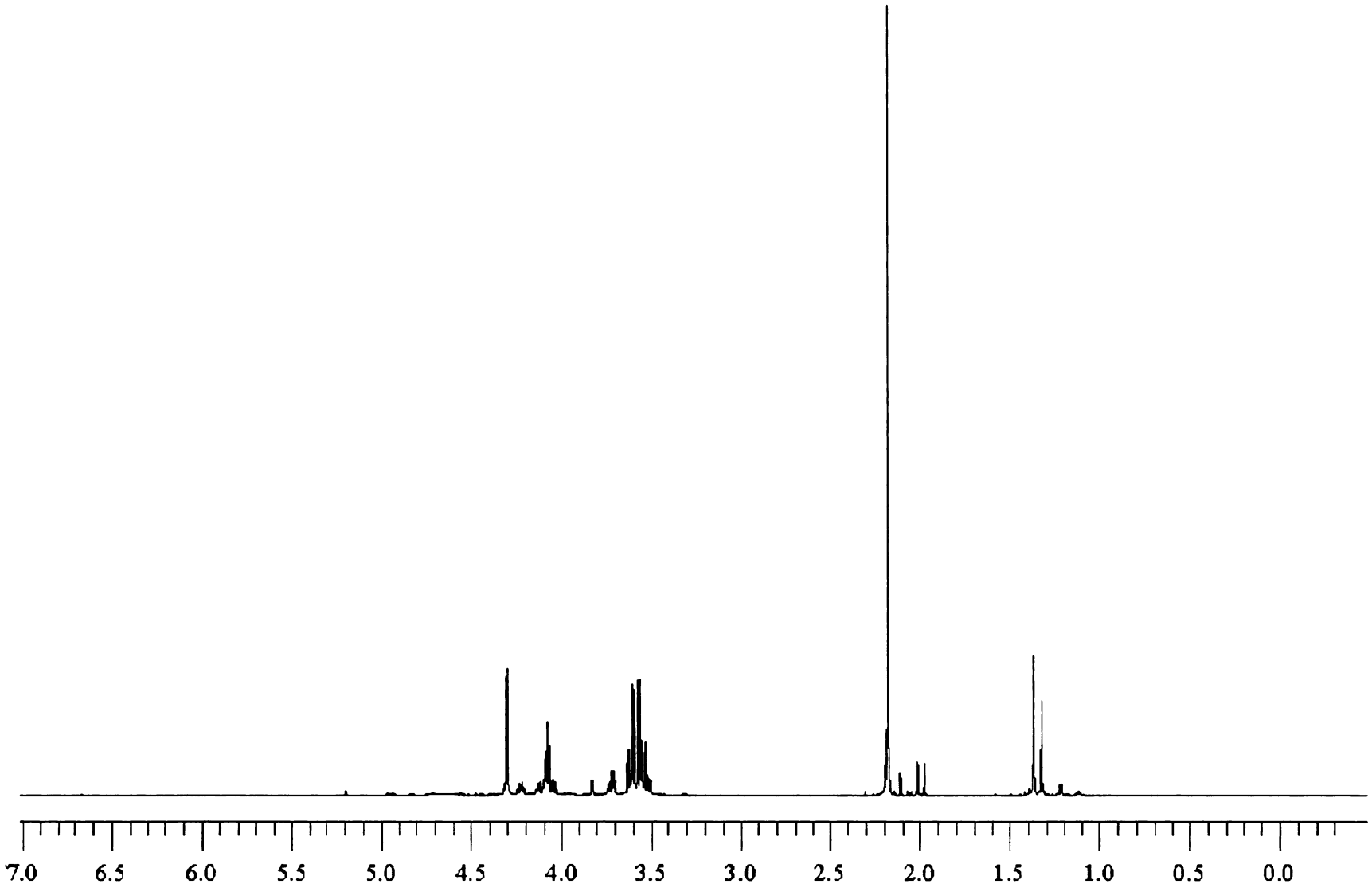
A surge in carbon flow in the DXP pathway resulting from overexpression of *dxs* leads to increased cytoplasmic concentrations of 1-deoxy-D-xylulose 5-phosphate which are hydrolysed and exported into the culture supernatant. Farmer and Liao have addressed the issue of D-glyceraldehyde-3-phosphate and pyruvate availability limiting carbon flow directed into the DXP pathway. The authors have suggested that the availability of D-glyceraldehyde-3-phosphate (GAP) as one of the limiting factor in this pathway.[96c] D-Glyceraldehyde-3-phosphate is an intermediate in the Embdem-Meyerhof pathway for glucose metabolism.[102] Triose-phosphate isomerase catalyzes the interconversion of GAP to dihydroxyacetone 1-phosphate (DHAP). The DHAP/GAP equilibrium for triose-phosphate isomerase is displaced in favor of DHAP (96:4).[103] Due to this unfavorable ratio the *in vivo* availability of GAP may be limiting 1-deoxy-D-

xylulose synthesis. Plasmid-based expression of *talB* gene encoding for translaldolase can potentially alleviate this impediment.

In conclusion we have designed *E. coli* strains where modulating the expression levels of *dxr* leads to the differential accumulation of 1-deoxy-D-xylulose and 2-*C*-methyl-D-erythritol. Further optimization of this process is still needed. This involves identification of feedback inhibition in this pathway, chromosomal disruption of vitamin B_1 and vitamin B_6 pathways that also utilizes DXP as precursor and substrate availability. Identification and optimization of the process can help in designing a better biocalalyst for the production of value-added chemicals.

Figure 39. Control ^{1}H NMR (500 MHz) of chemically synthesized 1-deoxy-D-xylulose.

^{1}H NMR (500 MHz, D_2O) Acyclic 1-deoxy-D-xylulose: δ 4.25 (d, J = 3 Hz, 1 H), 4.03 (m, 1 H), 3.52 (dd, J = 7, 7 Hz, 2 H), 3.46(dd, J = 6, 6 Hz 2 H), 2.12 (s, 3 H). Cyclic 1-deoxy-D-xylulose: δ 1.37 (s, 3 H), 1.31 (s, 3 H).


7.0
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4.0
3.5
3.0
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2.0
1.5
1.0
0.5
0.0

Figure 40. Control ^{1}H NMR (500 MHz) of chemically synthesized 2-*C*-methyl-D-erythritol.

^{1}H NMR (500 MHz, D_2O) δ 3.83 (dd, J = 9, 3 Hz, 1 H), 3.68 (dd, J = 6, 3 Hz, 1 H), 3.60 (d, J = 7 Hz, 1 H); 3.5 (dd, J = 7, 3 Hz, 1 H), 3.47 (d, J = 7 Hz, 1 H), 1.1 (s, 3 H). Singlet at 1.92 ppm is the CH_3 of acetic acid.

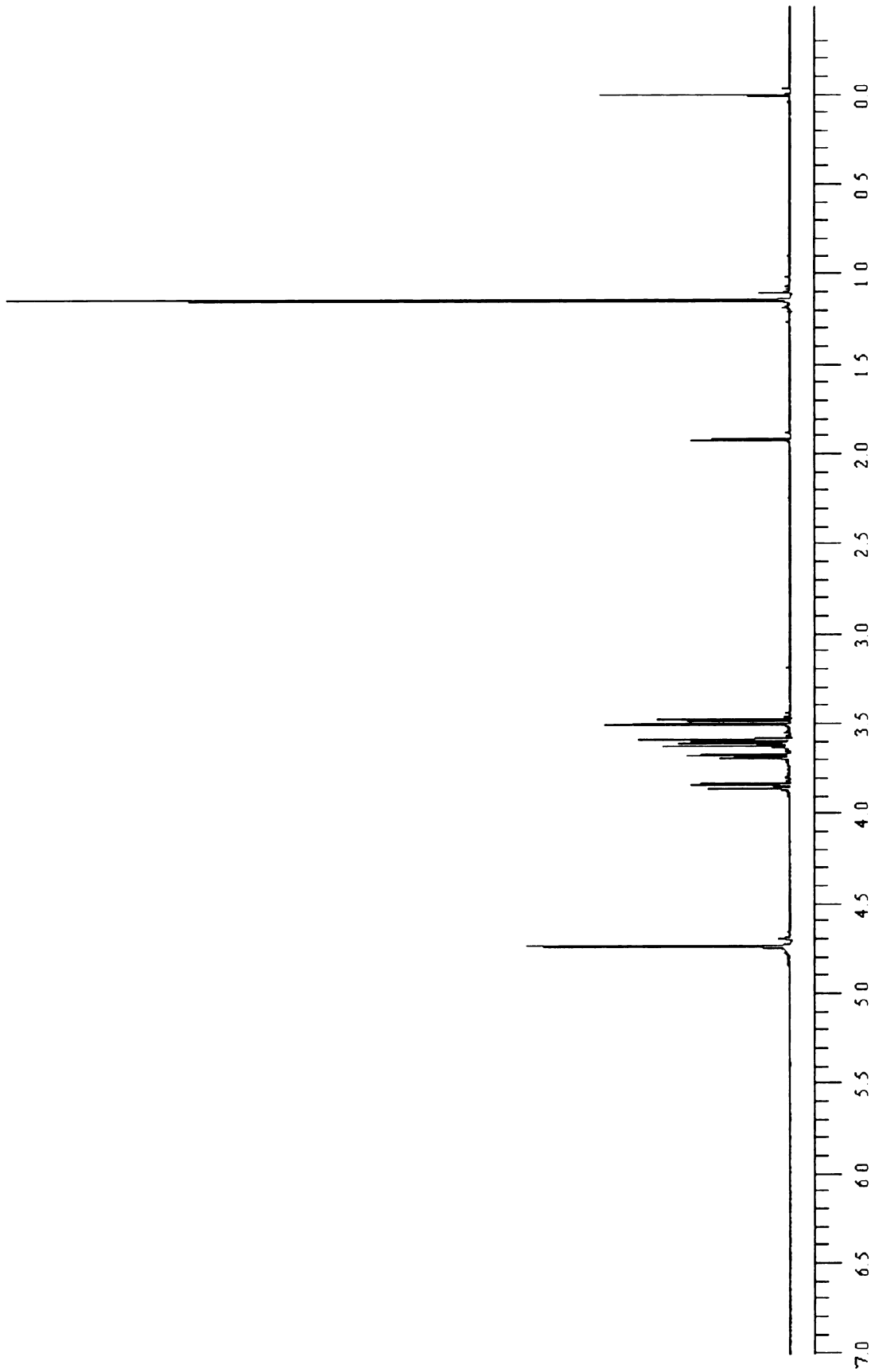
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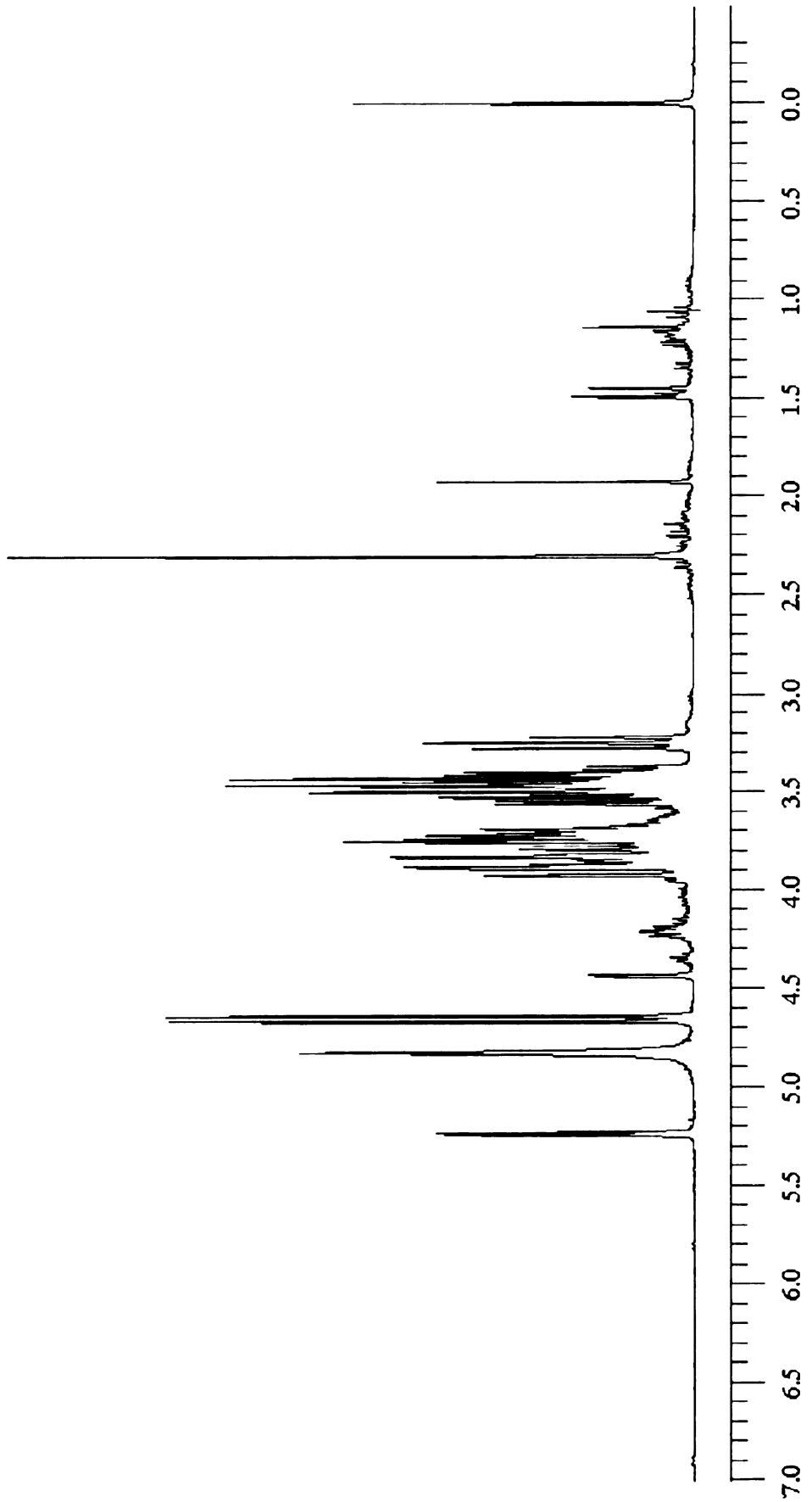
Figure 41. ^{1}H NMR (300 MHz, D_2O) of the culture supernatant of SP1.1/pPV4.230 after 60 h.

Resonances corresponding to Acyclic 1-deoxy-D-xylulose: δ 4.43 (d, J = 2 Hz, 1 H), 2.3 (s, 3 H); Cyclic 1-deoxy-D-xylulose: δ1.49 (s, 3 H), 1.44 (s, 3 H). Resonance corresponding to 2-*C*-methyl-D-erythritol: δ 1.13 (s, 3 H). Resonances corresponding to D-glucose: δ 5.2 (d, J = 8 Hz, 1 H), 4.82 (d, J = 4 Hz, 1 H). Resonances corresponding to acetic acid: δ 1.92 (s, 3 H).


7.0
6.5
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5.5
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3.0
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2.0
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1.0
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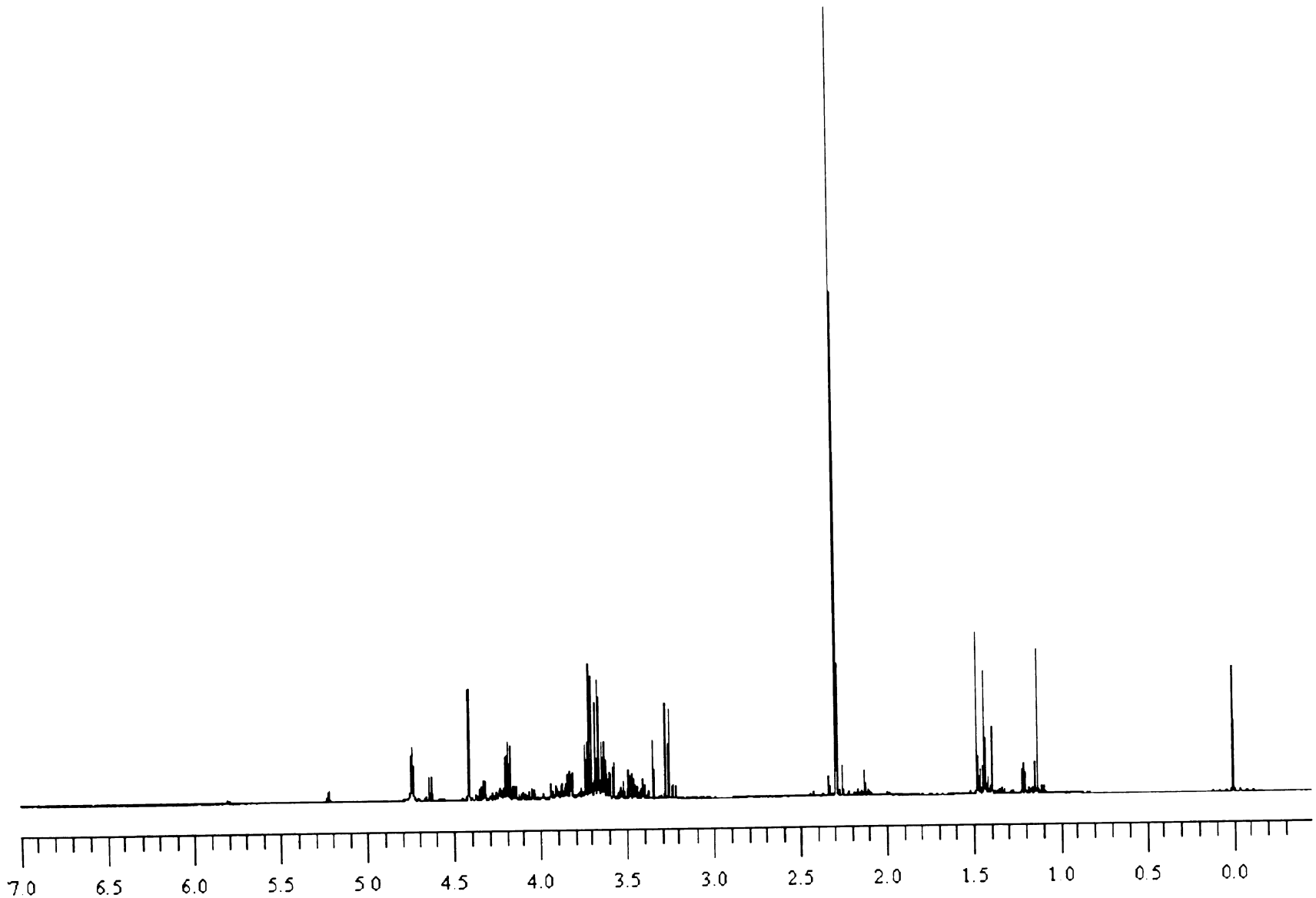
0.0

Figure 42. ^{1}H NMR (500 MHz, D_2O) of the ethyl acetate extract of SP1.1/pPV4.230 fermentation broth.

Resonances corresponding to Acyclic 1-deoxy-D-xylulose: δ 4.43 (d, J = 3 Hz, 1H), 2.3 (s, 3 H); Cyclic 1-deoxy-D-xylulose: δ 1.49 (s, 3 H), 1.44 (s, 3 H). Resonance corresponding to 2-*C*-methyl-D-erythritol: δ 1.13 (s, 3 H).


7.0
6.5
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5.0
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3.0
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2.0
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0.5
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Figure 43. ^{1}H NMR (300 MHz, D_2O) of the culture supernatant of SP1.1/pPV6.044 after 60 h.

Resonance corresponding to 2-*C*-methyl-D-erythritol: δ 1.13 (s, 3 H). Resonances corresponding to D-glucose: δ 5.2 (d, J = 8 Hz, 1 H), 4.82 (d, J = 4 Hz, 1 H). Resonances corresponding to acetic acid: δ 1.92 (s, 3 H).

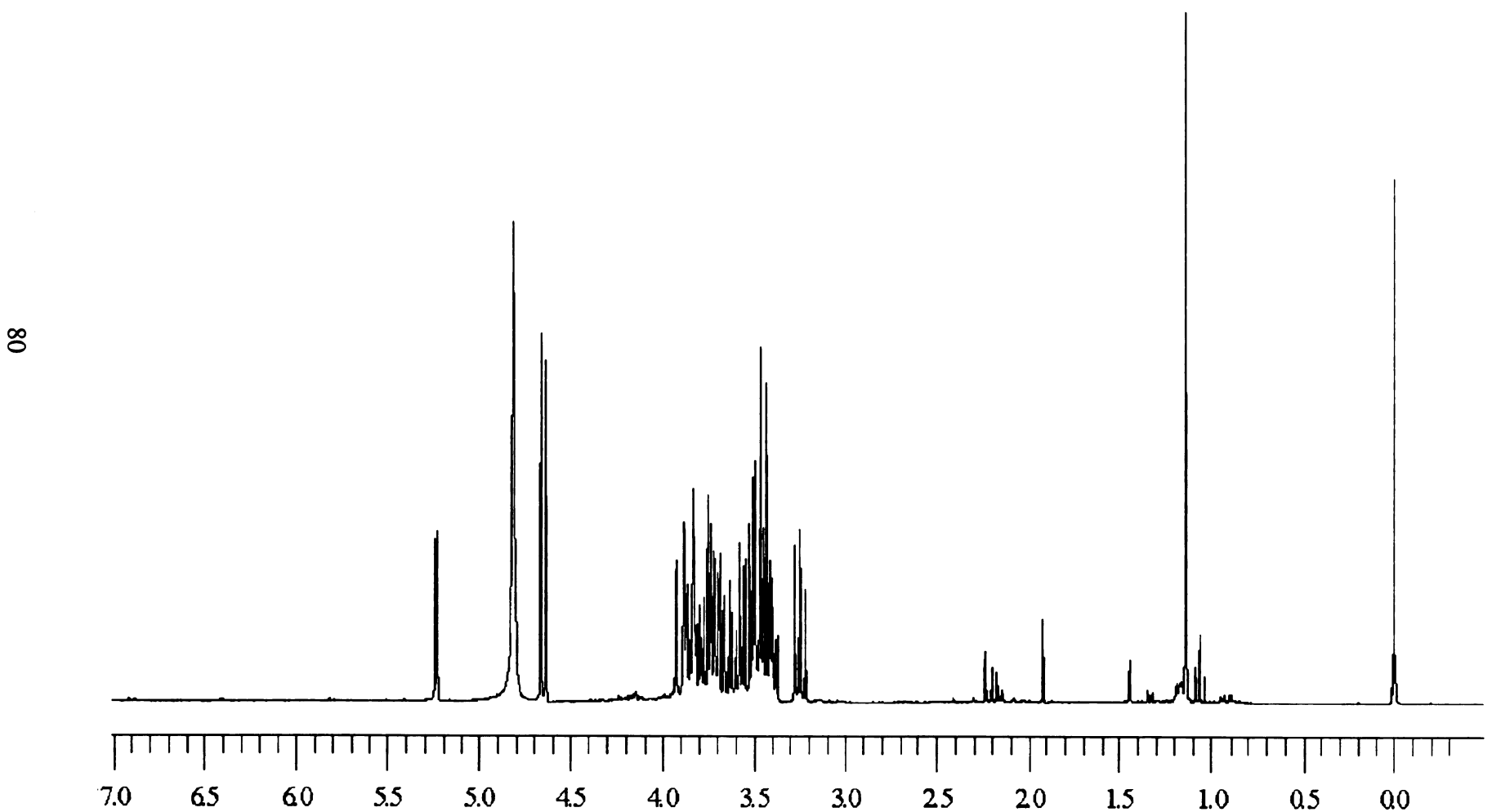
80
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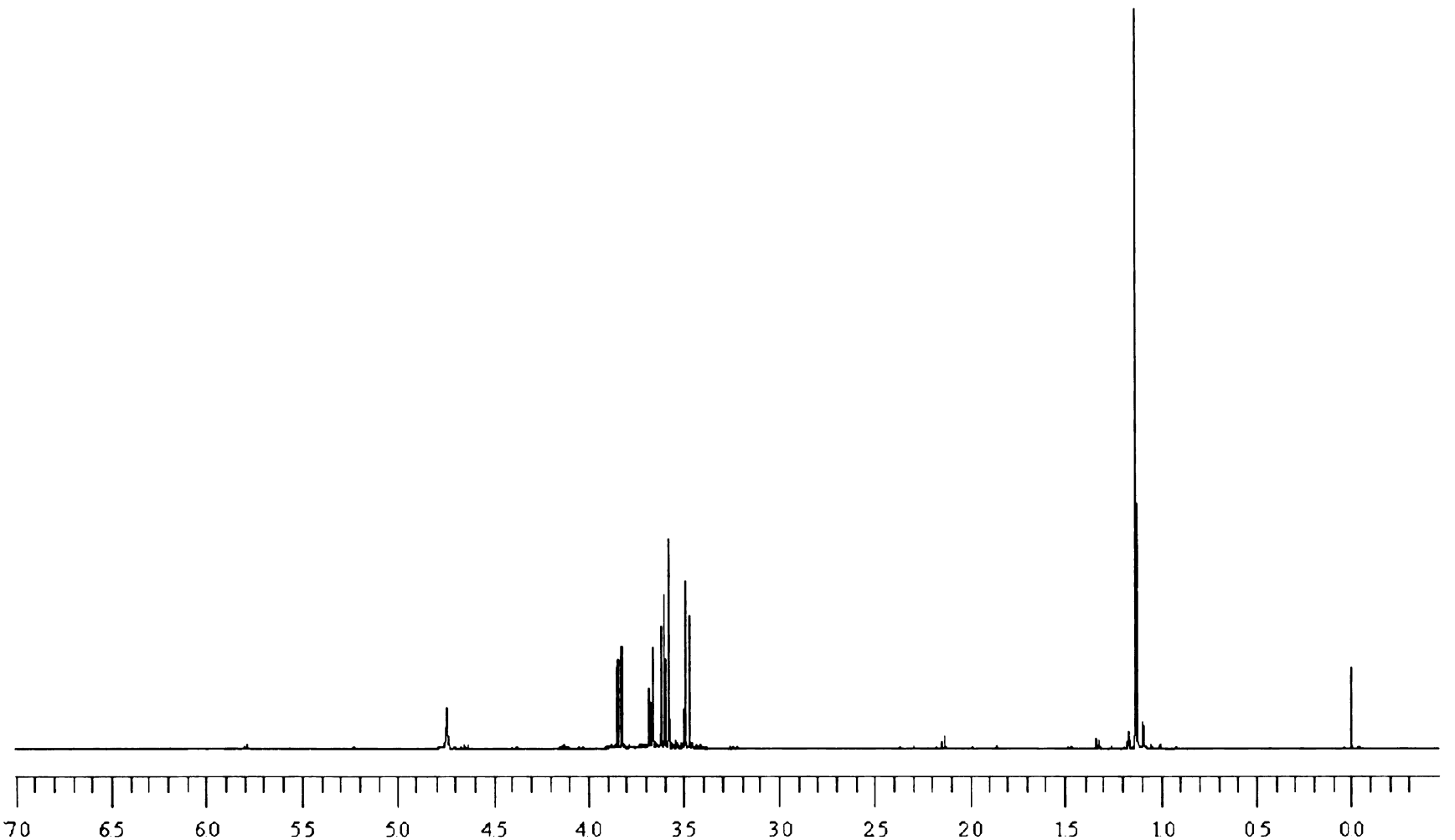
Figure 44. ^{1}H NMR (500 MHz) of the ethyl acetate extract of SP1.1/pPV6.044 fermentation broth.

^{1}H NMR (500 MHz, D_2O) δ 3.83 (dd, J = 9, 3 Hz, 1 H), 3.68 (dd, J = 6, 3 Hz, 1 H), 3.60 (d, J = 7 Hz, 1 H); 3.5 (dd, J = 7, 3 Hz, 1 H), 3.47 (d, J = 7 Hz, 1 H), 1.1 (s, 3 H).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CHAPTER 3

UNDERSTANDING PRECURSOR LIMITATIONS IN THE SHIKIMATE PATHWAY USING A SEMISYNTHETIC APPROACH

Background

Shikimic acid is a hydroaromatic intermediate in the common pathway of aromatic amino acid biosynthesis. This molecule is isolated from the fruits of the plant *Illicium spp* at a cost of approximately $10,000/kg.[104] The diverse array of stereochemistry on this carbocyclic intermediate presents it as a useful advanced chiral synthon for chemical synthesis.[105] This has been exemplified by using shikimic acid as the precursor in the synthesis of the neuraminidase inhibitor GS4104 (Figure 45).[106] This orally active anti-influenza drug is being marketed by Hoffman-LaRoche under the trade name Tamiflu.[107] Recently, a route for the synthesis of commodity chemical phenol has been established by reaction of shikimic acid in near-critical water (Figure 45).[108] High titers and yields of shikimic acid are needed for supplanting benzene with D-glucose as the starting material for phenol. Successful manipulation of the shikimate pathway has been accomplished for the production of pathway intermediates (e. g. 3-dehydroshikimic acid),[46a] secondary metabolites (e. g. aromatic amino acids, *p*-hydroxybenzoic acid)[109] and industrial chemicals (e. g. vanillin,[54] adipic acid,[51] hydroquinone[57]). An *E. coli* biocatalyst has been designed to produce high titers of shikimic acid from a carbohydrate feedstock providing a viable option for the use of shikimic acid as an industrial starting material.[74a] One of important problem associated with all of the processes is the *in vivo*

availability of precursor molecules in order to efficiently convert carbon source to desired product. Supplementing culture medium of strains synthesizing shikimate pathway products with precursor molecules and analyzing the culture supernatant for enhancement in product formation can be used as a diagnostic tool for evaluating the *in vivo* availability of these precursor molecules.

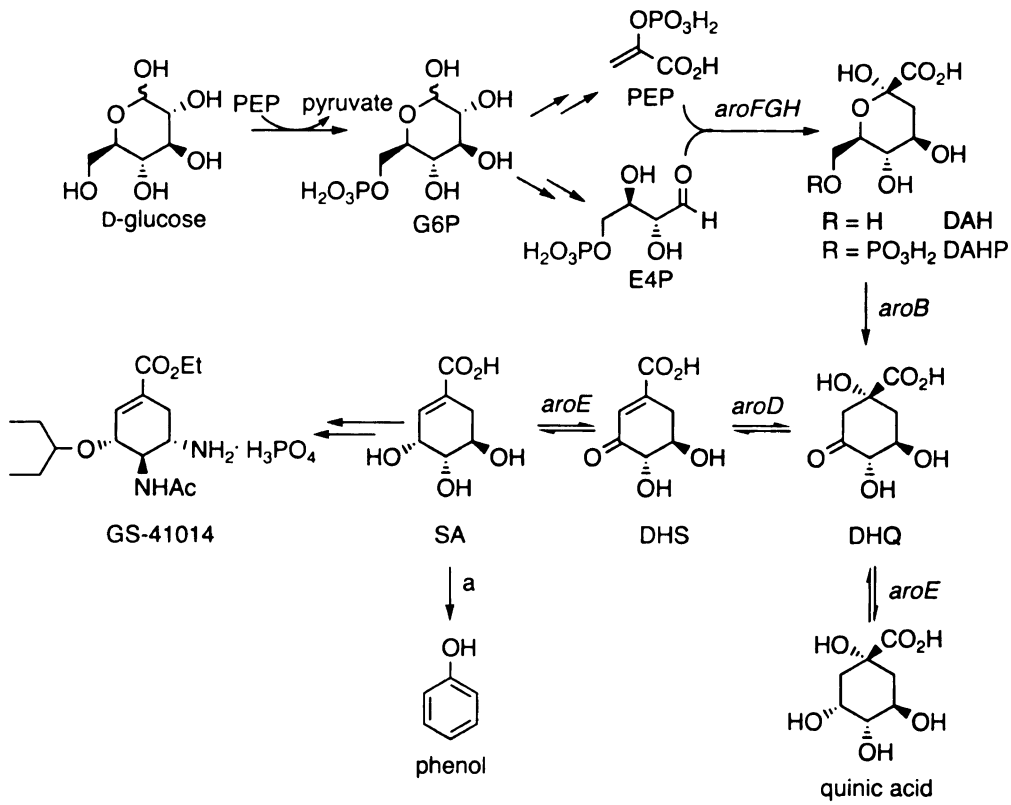


Figure 45. Biosynthesis of shikimic acid and quinic acid from D-glucose and the application of shikimic acid.
Key: a) 1) H_2O, 350°C, 2) Cu^0, H_2O, 350°C.

As the first enzyme in the pathway of aromatic amino acid biosynthesis, the *in vivo* activity of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) synthase ditermines the amount of carbon flow directed into this pathway.[110] Transcriptional repression and feedback inhibition regulates the *in vivo* activity of this enzyme.[62] With upregulation of feed-back insensitive DAHP synthase, direction of additional carbon flow

into the common pathway is dictated by cytoplasmic availability of this enzyme substrates: phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate (E4P). The expression levels of transketolase dictate the intracellular concentration of E4P.[61a] The enzyme transaldolase is also known to be involved in the biosynthesis of E4P, but its overexpression does not offer any advantage over amplified expression of transketolase.[111] PEP is involved in the transport of glucose and also in the synthesis of the shikimate pathway intermediates.[67] As a consequence, the synthesis of pathway intermediates and glucose transport compete for the same intracellular PEP. This results in a maximum theoretical yield of 43% (mol/mol) for the synthesis of shikimate pathway intermediates from glucose.[46a] To achieve higher yields of shikimic acid and other intermediates in this pathway PEP availability must be increased. Strategies like non-PTS mediated uptake of glucose, use of D-xylulose or L-arabinose as carbon sources or recycling of PTS-generated pyruvic acid back to PEP leads to a maximum theoretical yield of 86% (mol/mol) for the microbe-catalyzed syntheses of shikimic acid or 3-dehydroshikimic acid from glucose.[68c,d]

Succinic acid, a tricaboxylic acid cycle intermediate has been used a masked precursor of PEP. When succinic aicd was added as supplement to the culture medium of *E. coli* KL3/pKL6.218A, a 6% increase in total hydroaromatics was observed.[112] Based on this precedence, direct inclusion substrate molecules PEP and E4P as supplements can be used as a diagnostic tool of evaluating the *in vivo* availability of these precursor molecules during the microbial syntheses of shikimic pathway products. Direct transport of PEP and E4P in *Escherichia coli* has not been reported, even though transport of PEP has been observed in closely related *Salmonella typhimurium*.[113]

In this chapter the *E. coli* constructs possessing the ability to transport PEP into their cytoplasm were constructed and the impact od this transported PEP on the synthesis of shikimic acid by *E. coli* was determined under fed-batch fermentor condition. The synthesis of D,L-erythrose 4-phosphate and its impact on the synthesis of shikimic acid when added to the culture medium of *E. coli* was also investigated. The heterologous expression of the *Salmonella* phosphoglycerate transport system in *E. coli* and its impact on the shikimic acid synthesis is also been examined.

Bacterial Tansporters

Carrier proteins that catalyze the translocation of solutes across biological membrane are essential features of cellular metabolism. The membrane-bound proteins are not only involved in transport of substrate and essential nutrients from the surrounding medium but also play an important role in signal transduction, cell motility and export of metabolites from the cytoplasm.[114] There are different kinds of transport process that occurs in the bacterial membranes and can be categorized according to the structural, kinetic and energetic aspects.[115] Based on the utilization of energy sources, the transports can be classified as follows.

1). Primary bacterial transport: This mechanism involves conversion of light or chemical energy into electrochemical energy during transport of solutes. Examples include photosynthetic electron transport, respiratory electron chains, and ATP driven transporters.

2). Secondary transport: This kind of transport depends on the concentration gradient of a given solute. The electrochemical energy of a given solute based on its concentration is utilized to drive the transport of another solute against its own

concentration gradient. The *E. coli* b-galactoside: H^+ symport (LacY, lactose permease) and melibiose carrier (MelB) are prominent examples of this type of transport.

3). Group translocation involves chemical modification of the solute during the transport across the membrane barrier. The most common example of this transport mechanism is the bacterial phosphoenolpyruvate:carbohydrate phosphotransferase (PTS) system.

4). The fourth group involves channels and pores through which solutes are transported across the membrane. Examples of this kind of transport include *E. coli* LamB porin which is specific for transport of maltose and the *E. coli* glycerol facilitator (*glpF*).

During the transport of organic anions, bacteria exploit a variety of transport proteins called antiport carriers,[116] of which the best described are those using phosphate (P_i) or sugar phosphate as substrate. In *E.coli*, the P_i linked antiport carrier UhpT mediates the transport of hexose 6-phosphates and is a member of the Major Facilitator Super family (MFS).[117] Although the members of the MFS show a great diversity in their substrate specificity and kinetic mechanism, they all share a common structural theme characterized by the presence of approximately 12 transmembrane segments (TM) which are thought to transverse the membrane in alpha helical conformation.[118] Indirect genetic approaches like second-site suppressor analysis, site-directed and cysteine-scanning mutagenesis have been applied to obtain structural information, helix relationship and helix function in these TM. Using site directed mutagenesis and second-site suppressor

analysis, an intrahelical salt bridge involving ion pair Asp^{388} and Lys^{391} of UhpT TM11 was identified.[119a] Further studies[119d] of this ion pair revealed that if the anionic partner Asp^{388} is replaced by a cysteine residue, the derived membrane protein has a substrate selectivity bias towards compounds such as PEP that carry an additional anionic charge.[119d,e] Multiple alignment of the amino acid sequences of *uhpT* and related families of transporters revealed that some residues were highly conserved within TM11.[119d] However residues in position 388 and 391 were not conserved in the case of *uhpT*.[119d] Most family members had aliphatic (Val or Ala) and polar residues (Ser, Thr, or Asn) at these positions instead of two polar residues, as in the case of *uhpT*.[119d] In the case of PEP transporter PgtP of *Salmonella,* Val^{388} and Arg^{391} occupy these position. The authors argue the "gain-of function" phenotype for the mutant *uhpT* (D388C) was due to the presence of uncompensated positive charge (lysine) at position 391 and not the presence of cysteine, a proton donor at position 388. An equivalent result was observed after replacing the Asp^{388} with valine, the amino acid occupying the corresponding position in PgtP.[119d] It was also discovered that a positive charge at position 388 or 391 influences the substrate specificity, as does a lone negative center at position 388, thereby strengthening the argument that residues at position 388 and 391 on the TM11 of UhpT are important to selectivity during organophosphate transport.[119e] Incorporation of this mutant hexose phosphate transporter in *E. coli* SP1.1 provided an avenue to study the aspect of PEP limitation during fed-batch fermentation synthesis of shikimic acid.

Host Design and Plasmid Traits

E. coli SP1.1 has been used as a host in the synthesis of shikimic acid.[74] Construction of SP1.1 began with the homologous recombination of the *aroB* gene into the *serA* locus of *E. coli* RB791 resulting in RB791 *serA::aroB*. RB791 *serA::aroB* was subjected to two successive P1 phage-mediated transductions to transfer the *aroL478*::Tn*10* and *aroK17*::Cm^R loci of ALO807 onto the genome eliminating the shikimate kinase activity.[74] Strain SP1.1 lacks the capability of *de novo* aromatic amino acid and aromatic vitamins biosynthesis and hence requires supplementation with L-phenylalanine, L-tyrosine, L-tryptophan, *p*-hydroxybenzoic acid, *p*-aminobenzoic acid, and 2,3-dihydroxybenzoic acid for growth in minimal medium. Culturing of SP1.1 in minimal medium also required L-serine supplementation or *serA* plasmid insert due to inactivation of the genomic *serA* locus.

DAHP synthase catalyzes the condensation of E4P with PEP to give DAHP, the first committed intermediate in the common pathway for aromatic amino acid biosynthesis.[30] Elevated expression levels of DAHP synthase are therefore critical for high-yielding synthesis of shikimic acid. The *in vivo* activity of DAHP synthase is determined by feedback inhibition and transcriptional regulation. Therefore shikimate-producing strains utilize a plasmid-localized mutant isozyme of DAHP synthase, designated as $aroF^{FBR}$,[65] which is insensitive to feedback inhibition by L-tyrosine. However, increased carbon flow directed into the common pathway due to increased activity levels of DAHP synthase results in accumulation of DAH since *aroB*-encoded native DHQ synthase activity were now inadequate to convert DAHP to DHQ at a rate fast enough to avoid DAHP accumulation. Hydrolysis and export results in the

accumulation of DAH in the culture supernatant.[120] This problem was circumvented by site-specific insertion of an additional copy of *aroB* into the genomic *serA* locus of *E. coli* strain via homologous recombination.[110] The *serA* locus encodes 3-phosphoglycerate dehydrogenase, an enzyme required for L-serine biosynthesis. Localization of the *serA* gene on all plasmids provided a convenient method for plasmid maintenance in *E. coli* host strains having disrupted *serA* locus during fed-batch fermentor cultivation. Amplified levels of *aroB* led to accumulation of 3-dehydroshikimic acid in the culture supernatant. Along with *aroB* levels, the feedback inhibition of *aroE*-encoded shikimate dehydrogenase by shikimic acid was identified as an impediment to carbon flow in the common pathway.[110] Localization of P_{tac}*aroE* on the plasmid increased shikimate dehydrogenase, improved shikimic acid yields and lowered 3-dehydroshikimic acid concentrations in the culture supernatant. In addition to allosteric and transcriptional regulation, the cytoplasmic availability of substrates E4P and PEP also influence the *in vivo* DAHP synthase activities. The overexpression of *tktA*-encoded transketolase increased E4P availability and increased carbon flow into the shikimate pathway.[61a,74]

Plasmid pKD12.138A carries $aroF^{FBR}$ under the control of its native promoter, *aroE* locus under the control of the *tac* promoter, *serA* gene, and a copy *tktA*.[74] The plasmid pKD12.138A had been previously constructed in the laboratory.[74] Plasmid pTrc(HisC_6S)-D388C has the altered hexose phosphate transporter *uhtP*(D388C) expressed from the P_{trc} promoter.[119d] The plasmid pTrc(HisC_6S)-D388C is a derivative of plasmid pTrc(HisC_6S) that encoded for a histidine-tagged cysteine-less UhpT where all the six cysteines (in wild type UhpT) were replaced with serine residues.[119a] The

$P_{trc}uhpT$ (D388C) fragment was amplified by PCR from plasmid pTrc(HisC6S)-D388Cand was digested with *Sma*I. Plasmid pKD12.138A was linearized with *Nco*I, treated with Klenow fragment and then ligated with *Sma*I digested $P_{trc}uhpT$ (D388C) fragment to give plasmid pSP2.054 (Figure 46).

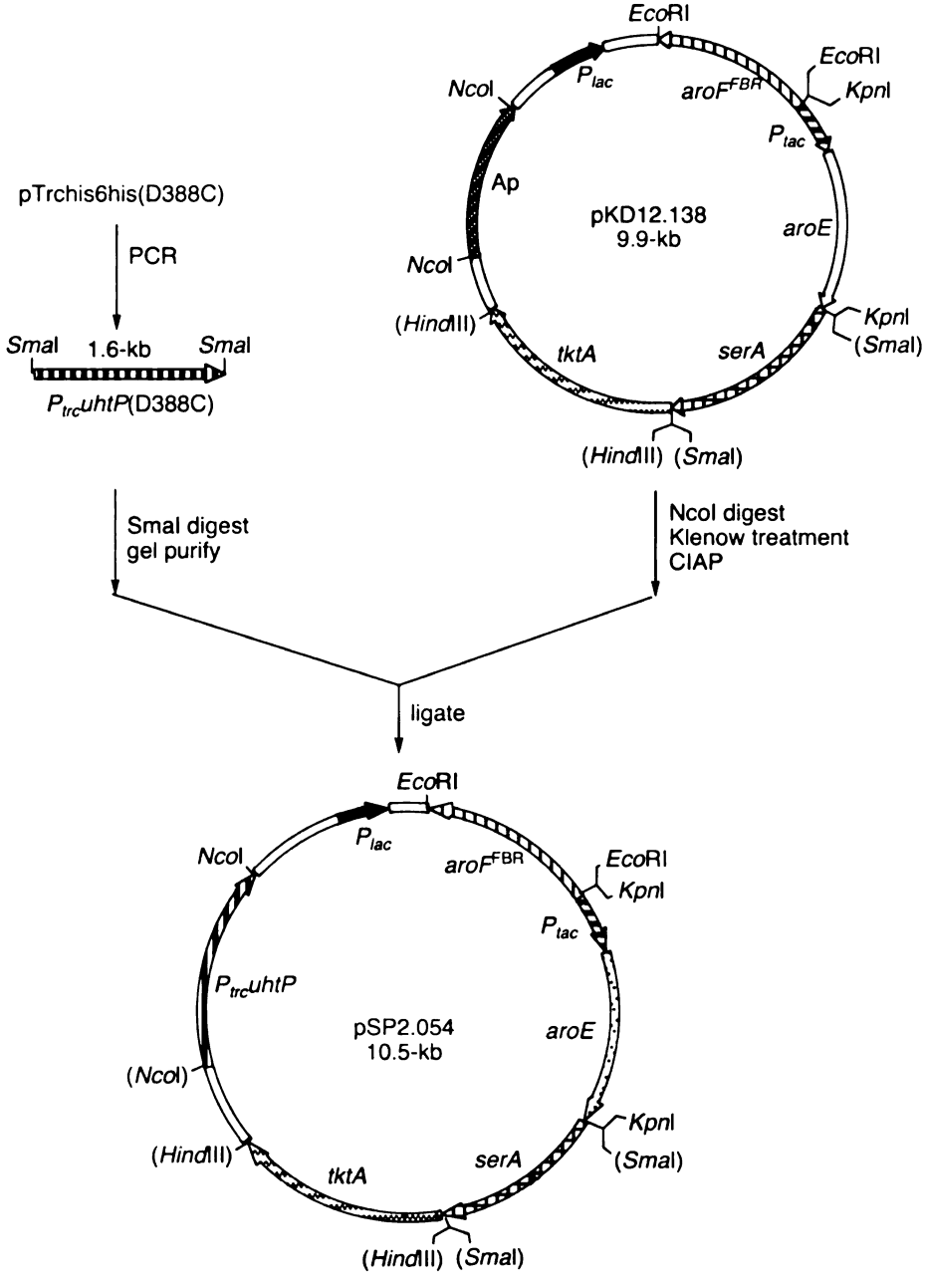


Figure 46. Construction of plasmid pSP2.054

Impact of Phosphoenolpyruvate Supplementation with Altered Hexose 6-Phosphate Uptake System

Synthesis of PEP

Evaluation of the impact of PEP supplementation during the microbial synthesis of shikimic acid by fed-batch fermentation requires multigram quantities of PEP. The synthesis of PEP was accomplished by following the procedure of Clark and Kirby as modified by Hirschbien *et. al.*[121] Pyruvic acid was brominated to 3-bromopyruvic acid **14** in 90% yield. Phosphorylation of **14** with trimethyl phosphite yielded the dimethyl phosphoenolpyruvic acid **15** which upon hydrolysis of the phosphate ester and precipitation with ethanol gave the mono-potassium salt of phosphoenolpyruvate in moderate yields. Prior to addition to the fermentation medium, the synthesized PEP was dissolved in water and adjusted to pH 7 with aq. KOH.

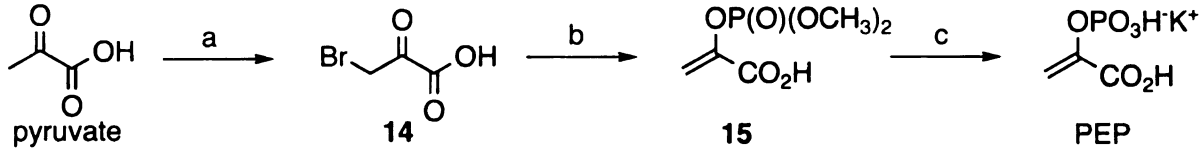


Figure 47. Synthesis of phosphoenolpyruvate.
Key: (a) conc. H_2SO_4, Br_2, CH_2Cl_2, 90%; (b) $P(OCH_3)_3$, ether, reflux; (c) i) H_2O, ii) KOH, pH 2.8, iii) EtOH, 70%.

Fed-Batch Fermentation (Glucose-Limited Conditions)

Culturing of *E. coli* SP1.1/pKD12.138 has been previously examined under glucose-limited fed-batch fermentation conditions.[84b] When cultivated under these conditions, *E. coli* SP1.1/pKD12.138 synthesized shikimic acid, quinic acid and

3-dehydroshikimic acid in a molar ratio of 1.4:1.0:0.68. Use of shikimic acid as an industrial starting material required removal of the accumulating byproducts. Accumulation of 3-dehydroshikimic acid in the culture supernatant is due to the feedback inhibition of *aroE*-encoded shikimate dehydrogenase by shikimic acid.[117] The 3-dehydroshikimic acid can be removed from the fermentation broth by acidification, heating and adsorption of the resulting protocatechuic acid on activated carbon. The accumulation of quinic acid in the culture supernatant is due to the transport of the accumulating shikimic acid into the microbial cytoplasm followed by microbe-catalyzed equilibration (Figure 45). Transport of shikimic acid into the microbial cytoplasm followed by its oxidation to 3-dehydroshikimic acid and subsequent hydration by *aroD*-encoded dehydroquinate dehydratase results in accumulation of 3-dehydroquinic acid. Reduction of accumulating 3-dehydroquinic acid by shikimate dehydrogenase results in the formation of quinic acid.[74a] Separation of contaminating quinic acid from shikimic acid by crystallization required reduction in the concentration of quinic acid which was accomplished by the addition of the glucose mimic methyl-α-D-glucopyranoside to fermentor runs.[74b] Although, *E. coli* can transport and phosphorylate methyl-α-D-glucopyranoside, it cannot metabolize this molecule.[113,122] The presence of this glucose mimic in the culture supernatant during glucose-limited fed-batch fermentations resulted in catabolic repression of all *E. coli* systems employed for shikimate transport.[74b,123] *E. coli* SP1.1/pKD12.138A cultivated under glucose-limited fed-batch fermentation conditions for 48 h with 1 mM methyl-α-D-glucopyranoside, synthesized 29 g/L of shikimic acid in 15% yield (mol/mol) from glucose (Figure 48). In addition to shikimic acid, 7 g/L of 3-dehydroshikimic acid and 4 g/L of quinic acid also accumulated in the

culture medium. The total yield of the hydroaromatic intermediates shikimic acid, quinic acid and 3-dehydroshikimic acid was 18% (mol/mol) from glucose. Although quinic acid synthesis was not completely eliminated, less quinic acid was formed than 3-dehydroshikimic acid. The molar ratio of accumulated shikimate/quinate/3-dehydroshikimate was 8:1:2. Quinic acid can be separated by fractional recrystallization of the crude shikimic acid product after the removal of 3-dehydroshikimic acid.

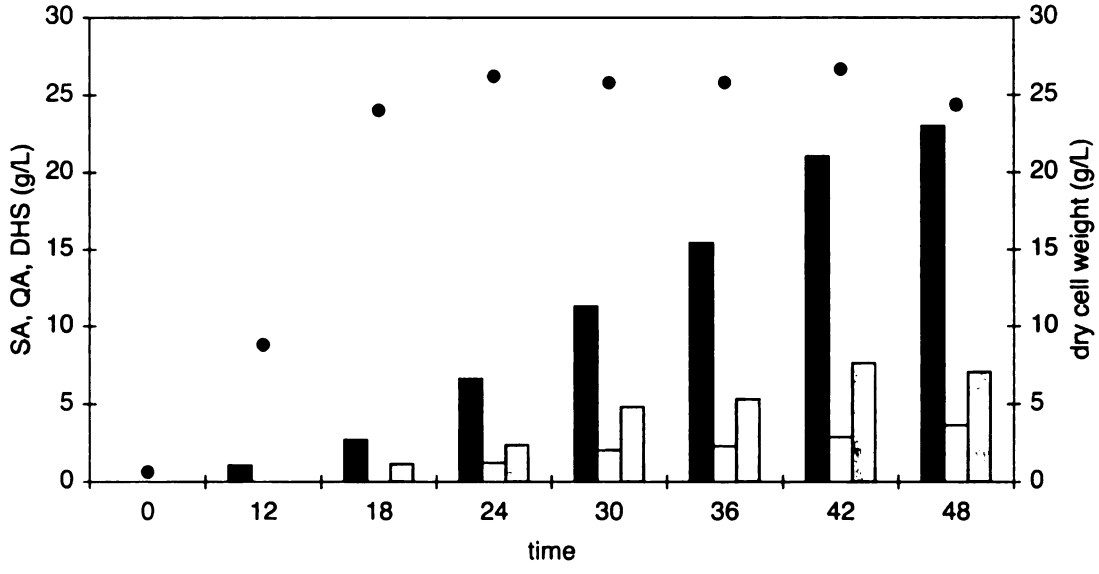


Figure 48. Cultivation of SP1.1/pKD12.138A under glucose-limited fed-batch fermentation conditions with 1 mM methyl-α-D-glucopyranoside. Key: filled circles, dry cell weight; filled bars, shikimic acid; open bars, quinic acid; gray bars, 3-dehydroshikimic acid.

E. coli SP1.1/pSP2.054 was then examined under glucose-limited fed-batch fermentation conditions with 1 mM methyl-α-D-glucopyranoside added to the culture medium. After 42 h, 20 g/L of shikimic acid accumulated in the culture medium in a yield of 11% (mol/mol). 3-Dehydroshikimic acid (10 g/L) and quinic acid (13 g/L) also accumulated in the culture medium (Figure 49). The total yield of the hydroaromatics

was 20% (mol/mol) from glucose. The molar ratio of shikimate/quinate/3-dehydroshikimate in the culture supernatant was 2:1:0.7. High levels of quinic acid impeded the removal of this contamination from crude shikimic acid. Higher titers of hydroaromatics have been reported to be biosynthesized by *E. coli* SP1.1/pKD12.138A under glucose-rich fed-batch fermentor conditions (52 g/L of shikimic acid) relative to glucose-limited fed-batch fermentor conditions (35 g/L of shikimic acid).[74b] The yields of shikimic acid and the total yields of hydroaromatics obtained under the two set of culturing conditions were the same.[74b] This led to the observation that accumulation of high titers of hydroaromatics in the culture supernatant does not always result in higher yields. This impeded evaluating the impact of PEP supplementation during glucose-limited fed-batch fermentation of SP1.1/pSP2.054. Evaluating the impact of PEP supplementations required controlling hydroaromatic equilibration.

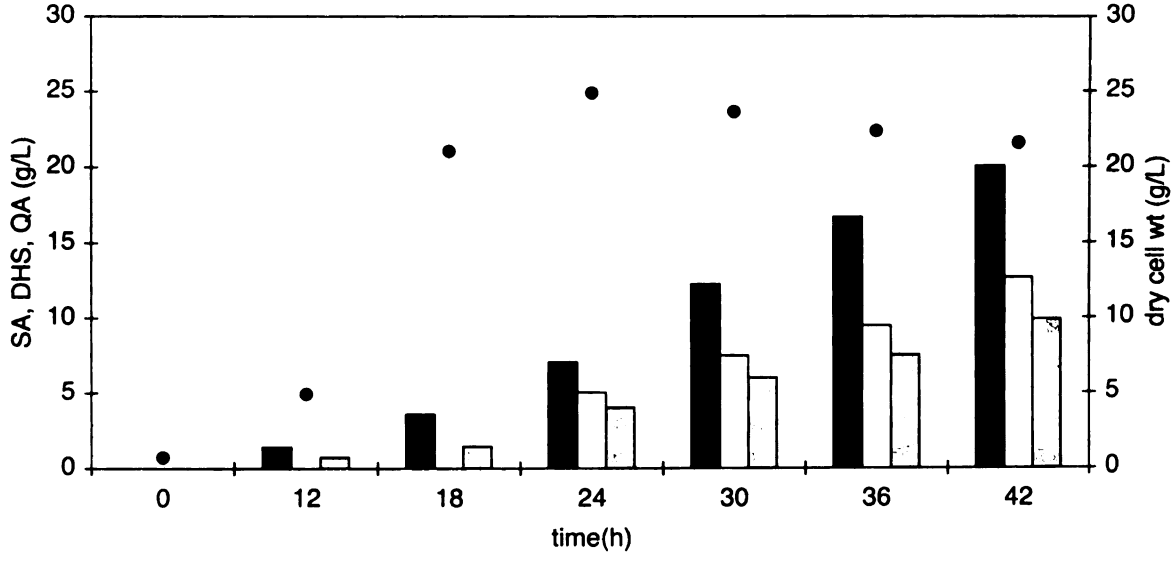


Figure 49. Cultivation of SP1.1/pSP2.054 under glucose-limited fed-batch fermentation conditions with 1 mM methyl-α-D-glucopyranoside. Key: filled circles, dry cell weight; filled bars, shikimic acid; open bars, quinic acid; gray bars, 3-dehydroshikimic acid.

Fed-Batch Fermentation (Glucose-Rich Conditions)

D-Glucose is frequently used by microbes such as *E. coli*, when a mixture of carbon sources in addition to D-glucose are available for growth.[122] Based on this precedence, a steady state concentration of 56-140 mM of D-glucose was maintained in the culture medium. This resulted in the repression of shikimic acid transport into the microbial cytoplasm.[74b] *E. coli* SP1.1/pKD12.138A cultured under glucose-rich fed-batch fermentation conditions for 60 h produced 49 g/L of shikimic acid in 16 % (mol/mol) yield. The amount of quinic acid and 3-dehydroshikimic acid were 4 g/L and 16 g/L. The total yield (mol/mol) was determined to be 22% from glucose. The molar ratio of shikimic acid to quinic acid was 12:1 (Figure 50).

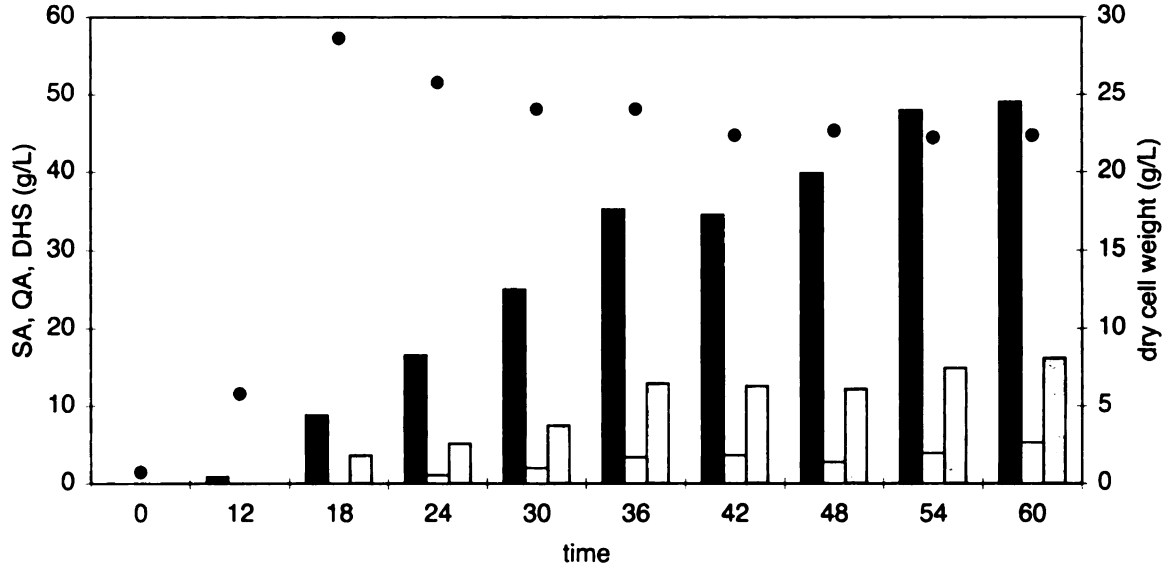


Figure 50. Cultivation of SP1.1/pKD12.138A under glucose-rich fed-batch fermentation conditions. Key: filled circles, dry cell weight; filled bars, shikimic acid; open bars, quinic acid; gray bars, 3-dehydroshikimic acid.

E. coli SP1.1/pSP2.054 when cultured under glucose-rich fed-batch fermentation condition for 60 h synthesized 49 g/L of shikimic acid in yield of 16% (mol/mol). Quinic

acid (5 g/L) and 3-dehydroshikimic acid (16 g/L) were also detected in the culture supernatant (Figure 51, Table 2, entry 1). The total yield was 22% (mol/mol) from glucose. The molar ratio of shikimic acid to quinic acid was 11:1. This showed that the inclusion of P_{trc}*uhpT*D388C did not have any adverse effect on the production of hydroaromatics under glucose-rich condition. The specific activities of DAHP synthase during cultivation of SP1.1/pKD12.138A and SP1.1/pSP2.054 under fed-batch fermentor conditions were comparable (Table 1).

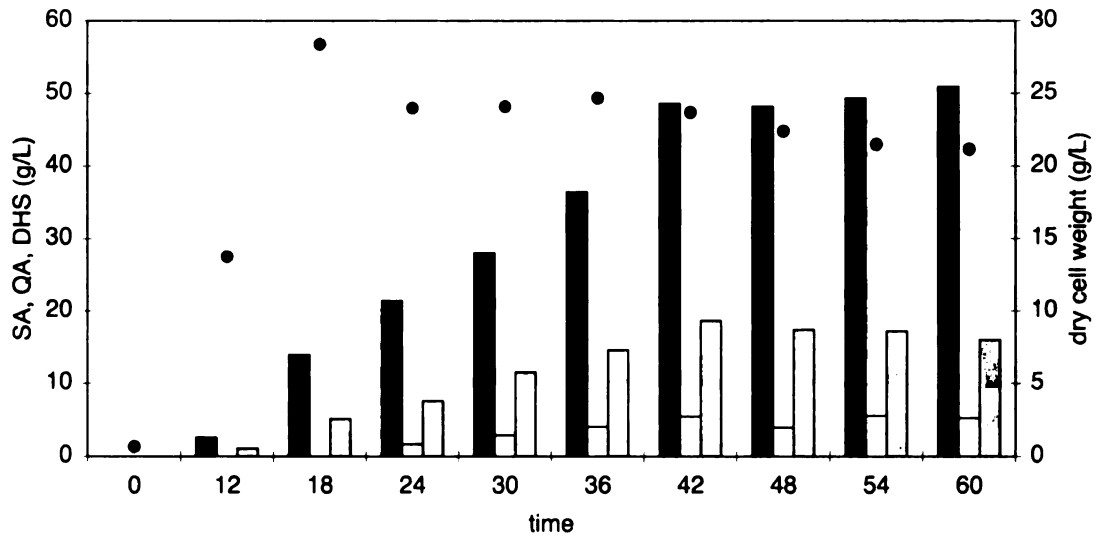


Figure 51. Cultivation of SP1.1/pSP2.054 under glucose-rich fed-batch fermentation conditions. Key: filled circles, dry cell weight; filled bars, shikimic acid; open bars, quinic acid; gray bars, 3-dehydroshikimic acid.

Table 1. DAHP synthase specific activities (μmol/min/mg) under glucose-rich fed-batch fermentor conditions.

Entry no	Strain	[SA] g/L	DAHP synthase activities			
			12 h	24 h	36 h	48 h
1	SP1.1/pKD12.138A	49	0.06	0.15	0.13	0.09
2	SP1.1/SP2.054	49	0.05	0.14	0.14	0.08
3	SP1.1/pKD12.138 + 10 g/L D,L-E4P	32	0.05	0.04	0.06	0.07

The impact of PEP supplementation was then examined in glucose-rich fed-batch fermentation runs of SP1.1/pSP2.054. Poor cell growth along with lower concentration of products was observed in cultures of *E. coli* SP1.1/pSP2.054 where PEP was added at the beginning of the fermentation run or with the glucose feed. As a result, PEP was added at the beginning of the oxygen sensor-controlled phase (12-14 h after the inoculation of the culture medium) during all fed-batch fermentation. ^{1}H NMR analysis revealed rapid uptake of PEP during the log phase of the cell growth, while the rate of uptake of PEP was significantly reduced when the fermentation run reached the stationary phase. Addition of a total of 35 g of PEP in 5 g increments every 6 h from the beginning of the oxygen sensor-controlled phase of the fermentation led to accumulation of PEP in the culture supernatant after 30 h of growth (Figure 52). At the end of 60 h of cultivation, 43 g/L of shikimic acid in 14 % yield (mol/mol glucose+ mol PEP) was obtained. 15 g of PEP still remained in the culture medium at the end of the fermentor run.

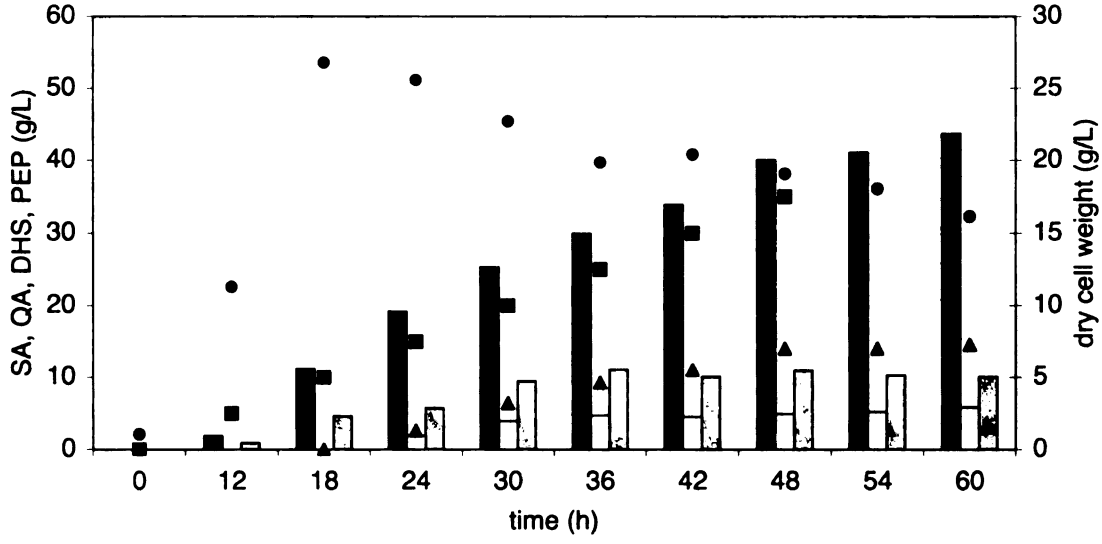


Figure 52. Cultivation of SP1.1/pSP2.054 under glucose-rich fed-batch fermentation conditions with PEP added at every 6 h. Key: filled circles, dry cell weight; filled bars, shikimic acid; open bars, quinic acid; gray bars, 3-dehydroshikimic acid, triangles, phosphoenolpyruvate remaining in the broth; squares, total PEP added.

^{1}H NMR analysis of the aliquots of the culture supernatant removed at various time intervals of fermentation revealed poor uptake of PEP during the stationary phase (24 h and later of the fermentation run). Hence it was decided to add a known amount of PEP at the beginning of oxygen sensor-controlled phase to the culture medium. Addition of 5 g PEP to *E. coli* SP1.1/pSP2.054 fed-batch cultures gave 44 g/L of shikimic acid (Table 2, entry 2). When compared to SP1.1/pSP2.054 without PEP supplementation the titer were low, but the yield of shikimic acid was the same 16% (mol/mol). The total yields were also comparable (Table 2, entry 3). When 10 g PEP was supplemented to culture medium at the initiation of the oxygen sensor-controlled phase, a significant increase in titer and yield of shikimic acid, 59 g/L and 20% (mol/mol) respectively was observed. There was also a corresponding increase in the amounts of quinic acid (9 g/L) and 3-dehydroshikimic acid (16 g/L) produced. The total yield for the hydroaromatics produced was 28% (mol/mol) from glucose (Figure 53).

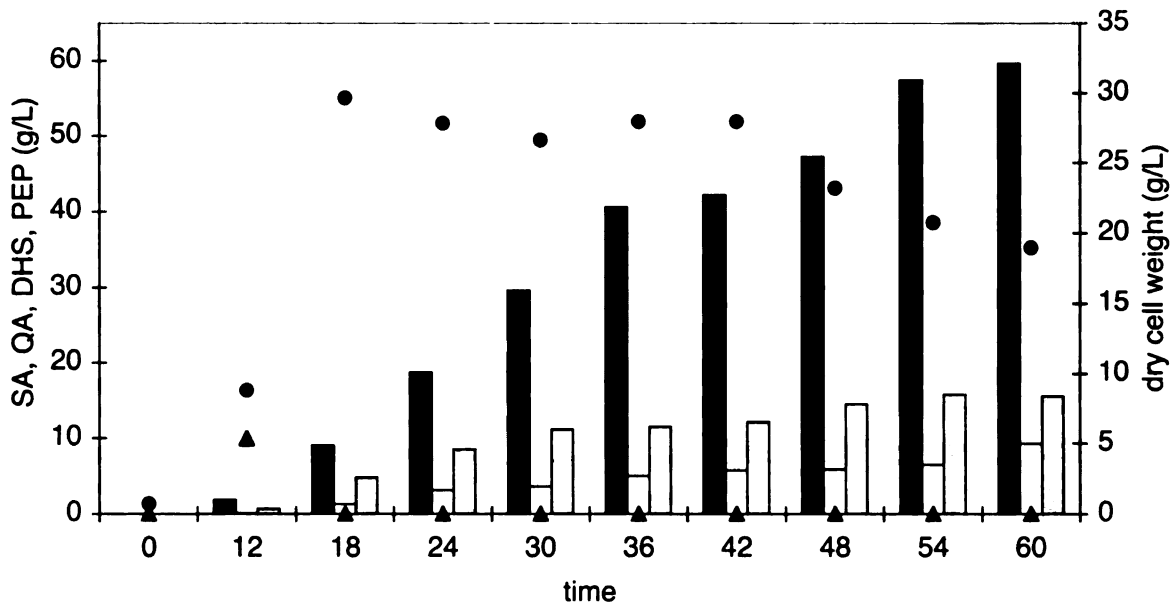


Figure 53. Cultivation of SP1.1/pSP2.054 under glucose-rich fed-batch fermentation conditions with 10 g PEP added at 12 h. Key: filled circles, dry cell weight; filled bars, shikimic acid; open bars, quinic acid; gray bars, 3-dehydroshikimic acid; triangles, phosphoenolpyruvate.

Addition of 20 g and 35 g PEP resulted in rapid uptake of PEP during the log phase and a significant reduction of PEP uptake during the stationary phase, but titers and yields of shikimic acid decreased in both cases (Table 2, entry 4 and entry 5). After analyzing a range of conditions for optimum PEP uptake, we found that 10 g of PEP was the maximum amount that could be added to the culture medium to observe a significant impact on the yield and titer of shikimic acid synthesized. Further increase in the amounts of PEP added led to the synthesis of lower concentration of hydroaromatics.

Table 2. Yields and concentration of metabolites synthesized by *E. coli* SP1.1/pSP2.054 when supplemented with PEP

Entry no	PEP [g/L][a]	[SA] g/L	SA yield[b]	[QA] g/L	[DHS] g/L	Total yield[c]
1	0	49	16	5	16	22
2	5	44	16	5	9	20
3	10	59	20	9	16	28
4	20	41	16	5	9	21
5	35	43	14	7	11	20

Abbreviation: shikimic acid (SA), quinic acid (QA), 3-dehydroshikimic acid (DHS). [a] PEP supplemented to the culture medium. [b](mol SA)/(mol glucose+mol PEP), [c](mol SA+DHS+QA)/(mol glucose + mol PEP).

In order to validate that the results observed during SP1.1/pSP2.054 fermentations was due to the transport of PEP by the UhpT(D388C) and not an artifact of the culture conditions, it was decided to add a known amount of PEP to SP1.1/pKD12.138 fermentation. *E. coli* SP1.1/pKD12.138A lacks PEP transport and hence supplementation with PEP should not have any effect on the yield and titer of synthesized shikimic acid. When 10 g PEP was added to fed-batch cultures of SP1.1/pKD12.138A, a slow uptake of PEP was observed (Figure 54). Although the yields and titers of hydroaromatics

synthesized by SP1.1/pKD12.138A with and without PEP addition were comparable, the transport of PEP was intriguing, as *E. coli* has been reported to be devoid of PEP transport capability.[113]

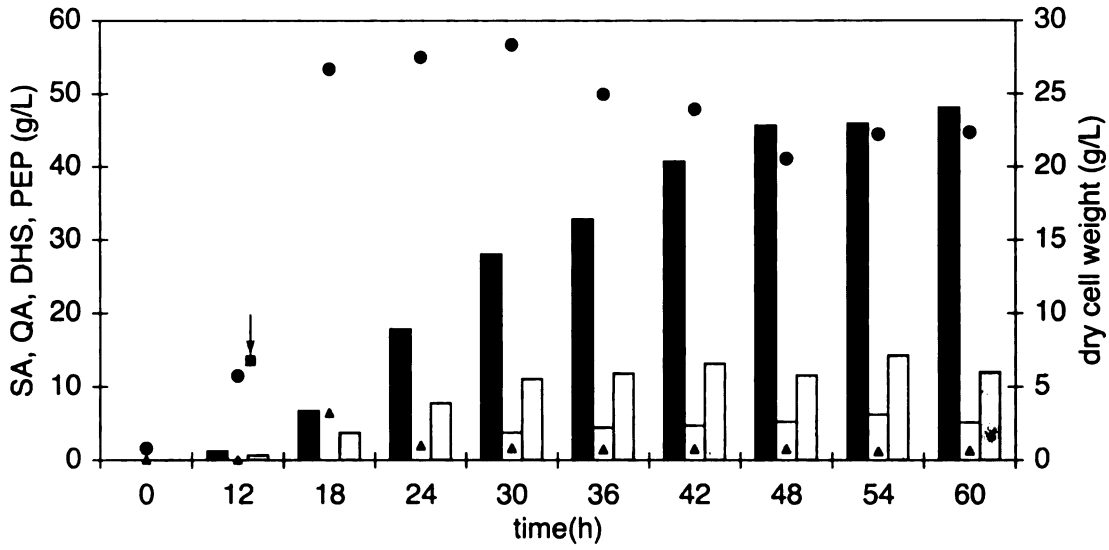
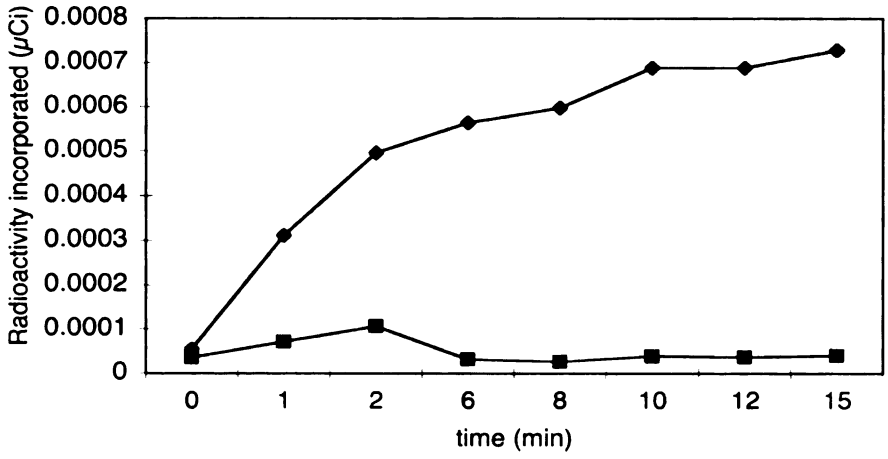


Figure 54. Cultivation of SP1.1/pKD12.138A under glucose-rich fed-batch fermentation conditions with 10 g PEP added at 13 h. Key: filled circles, dry cell weight; filled bars, shikimic acid; open bars, quinic acid; gray bars, 3-dehydroshikimic acid; triangles, phosphoenolpyruvate. Arrow indicates 13 h of growth; Square 10 g PEP added.

As a second line of evidence for PEP transport by the UhpT(D388C), transport assays were performed using [2-^{14}C]-PEP on cell samples collected from fermentor runs of SP1.1/pKD12.138A and SP1.1/pSP2.054 (Figure 55).

The transport assays were performed according to a reported procedure.[119d] The amount of radioactivity incorporated in the cells was quantified by a scintillation counter.

1.



2.

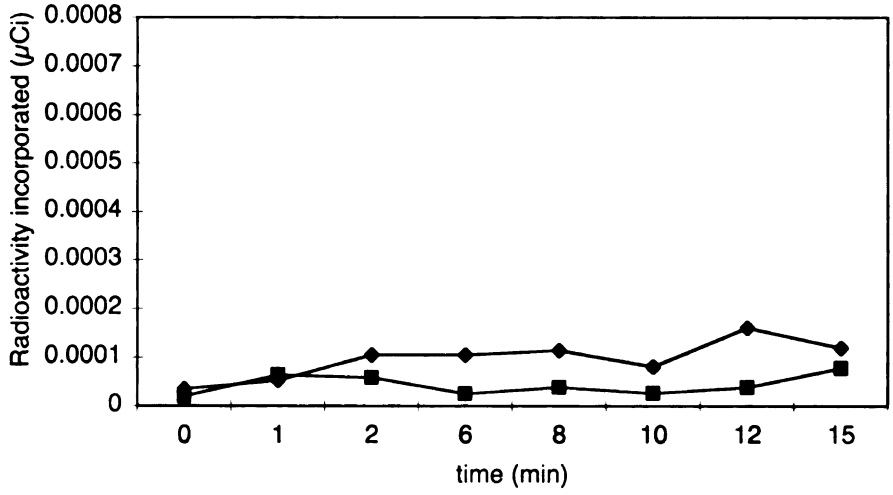


Figure 55. Amount of radioactivity incorporated (μCi) in intact cell when transport assay using [2-^{14}C]-PEP were performed on cell samples obtained at various stages of fed-batch fermentor runs of SP1.1/pSP2.054 and SP1.1/pKD12.138A. Box 1: 12 h of growth. **Box 2:** 24 h of growth. Legends: Squares SP1.1/pKD12.138A, diamonds SP1.1/pSP2.054.

The transport assays were consistant with the titers and yield of shikimic acid synthesized under fed-batch fermentation conditions. The transport of PEP during fed-batch fermentation was due to the incorporation of the altered hexose phosphate transporter *uhpT*(D388C). *E. coli* SP1.1/pSP2.054 transported PEP by during log phase (12-18h) of growth while at stationary stages of cell growth (24 h) the transport rate was significantly reduced. *E. coli* SP1.1/pKD12.138A did not transport PEP into its

cytoplasm during the log phase(12 h) or at the stationary phase (24 h) of its growth (Figure 55) across the cell membrane. The limited transport of PEP during the stationary phase (where rate of shikimic acid production is at its maximum) of *E. coli* SP1.1/pSP2.054 may be the reason why larger improvements in the titer of shikimic acid was not observed during PEP supplementation.

Impact of PEP Supplementation using *Salmonella* Phosphoglycerate Transport System

The inducible phosphoglycerate transport of *Salmonella typhimurium* LT2 catalyses the transport of 2-phosphoglycerate, 3-phosphoglycerate and phosphoenolpyruvate.[119] It has also been shown that this transport system allows energy-depleted cells to use external phosphoenolpyruvate for phosphorylation of methyl-α-glucopyranoside.[124] Incorporation of the phosphoglycerate transport system in a shikimate-producing host was an alternative approach explored, which was aimed at examining the impact of PEP supplementation on the synthesis shikimic acid by *E. coli.*

The *pgt* system for the phosphoglycerate transport consists of four genes in the order *pgtP, pgtC, pgtB* and *pgtA* (Figure 56). The *pgtP* gene encodes for the transporter and is transcribed right to left (Figure 56).[125] The *pgtA* gene[126] encodes an activator for the PgtP while the genes *pgtB and pgtC* encode for proteins involved in the modulation of PgtA activity.[127] The *pgtC, pgtB* and *pgtA* loci are transcribed from left to right and their products are membrane bound. Using deletion and complementing analysis of the *pgtPCBA* system, it was determined that the activity of PgtP depended on the availability of the *pgtA* gene product and deletion of *pgtCB* led to constitutive expression of *pgtP*

only in the presence of *pgtA*.[127] Hence only *pgtP* and *pgtA* gene are needed for evaluating the impact of PEP supplementation during microbe-catalyzed synthesis of shikimic acid.

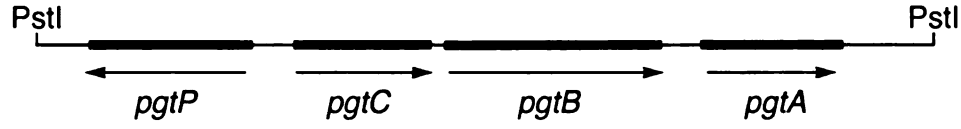


Figure 56. The phosphoglycerate transport system of Salmonella.

Plasmid pPV6.074 was constructed, by the localization of the phosphoglycerate transport genes *pgtP and pgtA* from *Salmonella choleraesuis* LT2 in plasmid pKD12.138A. The phosphoglycerate transport gene *pgtP* was amplified by PCR from genomic DNA of *S. choleraesuis* LT2 and digested with *Eco*RI and *Kpn*I restriction enzymes. The phosphoglycerate activator gene *pgtA* was amplified by PCR from the genomic DNA of *S. choleraesuis* LT2 and digested with *Kpn*I and *Bam*HI restriction enzymes. Plasmid pBR322 was linearized by digestion with *Eco*RI and *Bam*HI double digest. The two PCR DNA fragments and vector were ligated together to give plasmid pPV5.188 (Figure 57). The activity of the *pgtP* transporter gene was confirmed by examining transport of [2-^{14}C]-PEP.[119d] A 1.3-kb DNA fragment encoding for kanamycin resistance (Kan^R) was excised out of plasmid pKAD62A[110] by a *Bam*HI/*Sph*I double digest. This DNA fragment was ligated to pPV5.188 digested with *Bam*HI/*Sph*I to give 7.9-kb plasmid pPV6.030 (Figure 58). The *pgtPpgtA*Kan^R fragment was excised out of pPV6.030 by a *Sca*I/*Psh*AI double digest. Plasmid pKD12.138A was linearized by *Sca*I digestion. Ligation of the two blunt end fragments gave plasmid pPV6.074 (Figure 59).

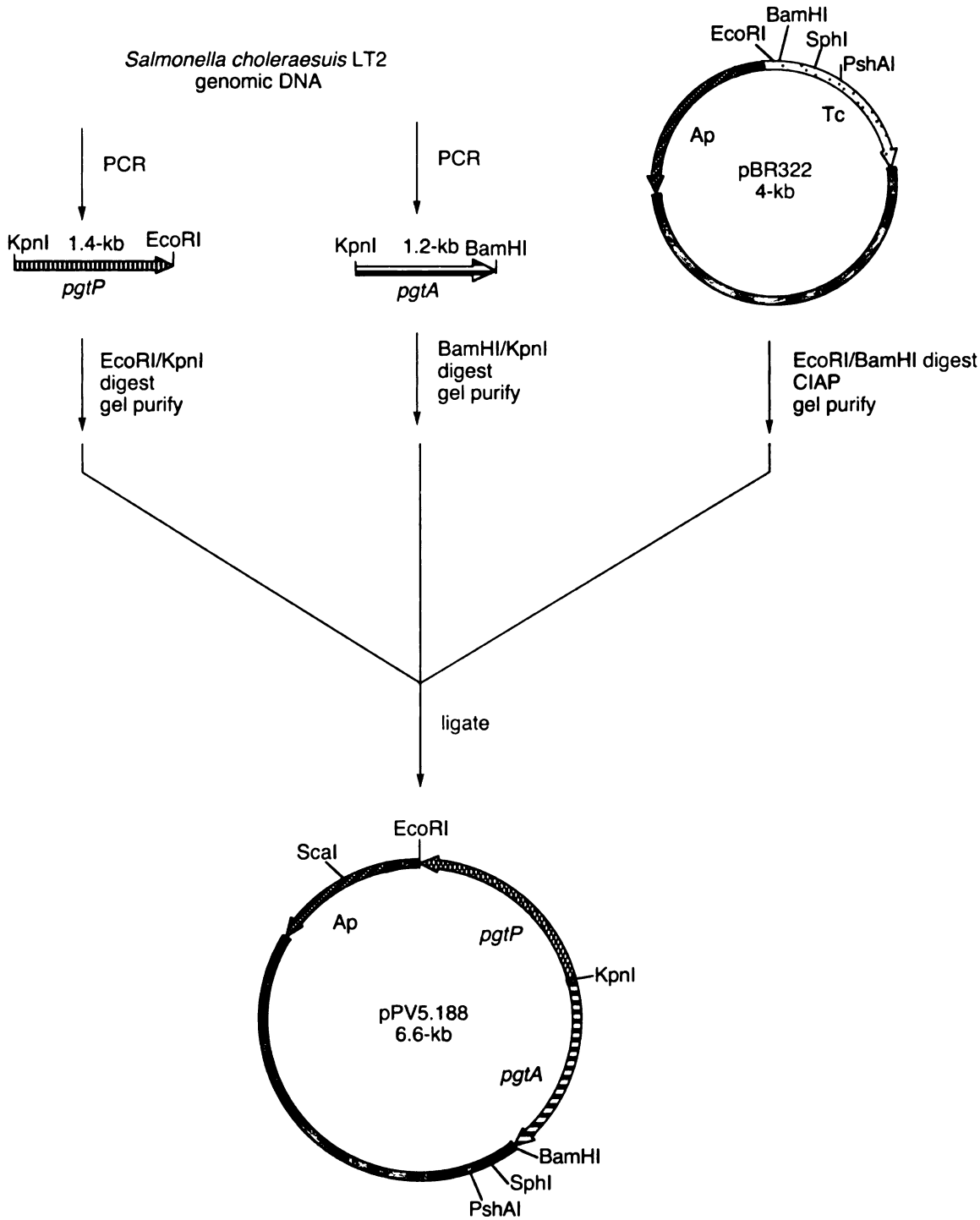


Figure 57. Construction of plasmid pPV5.188

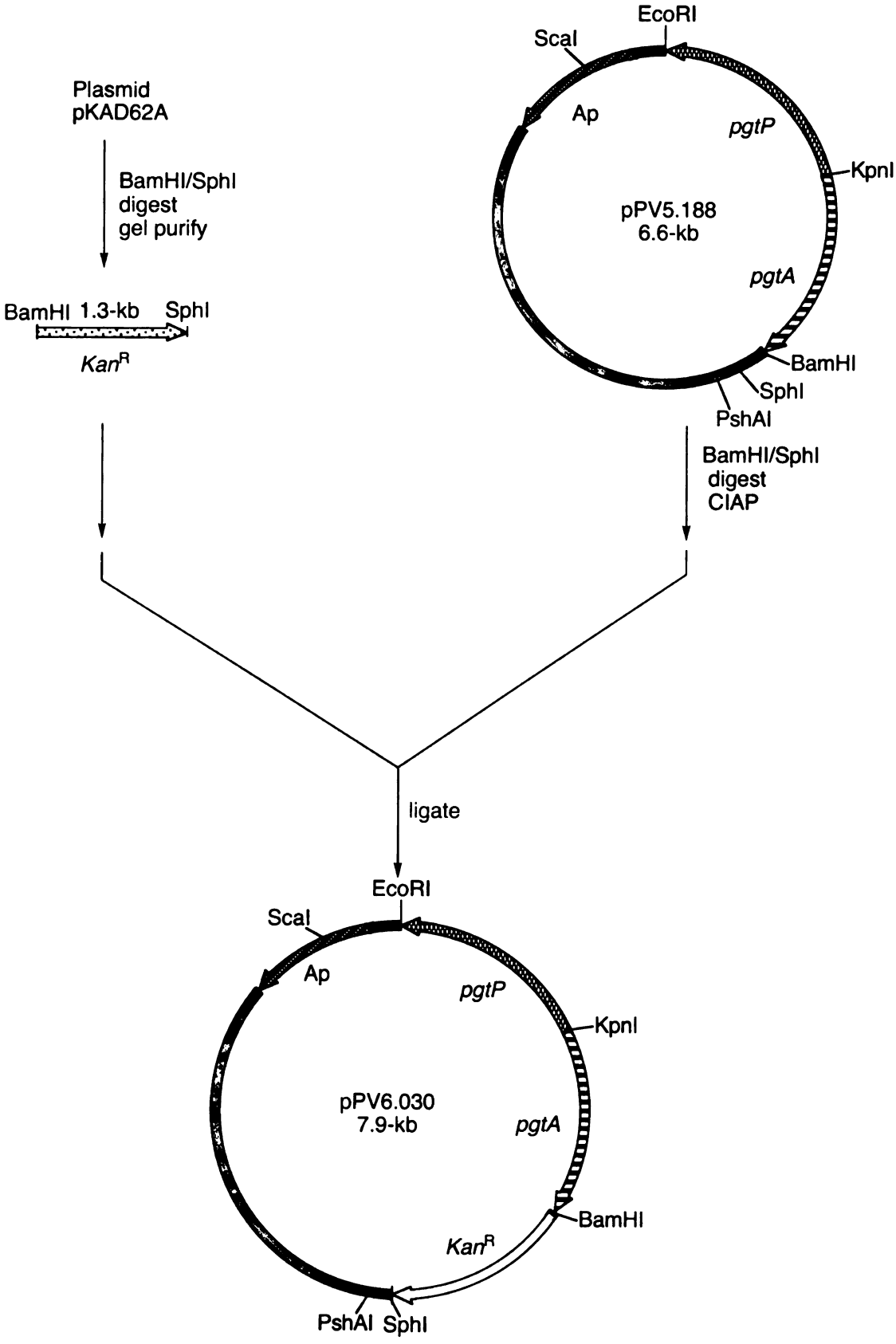


Figure 58. Construction of plasmid pPV6.030.

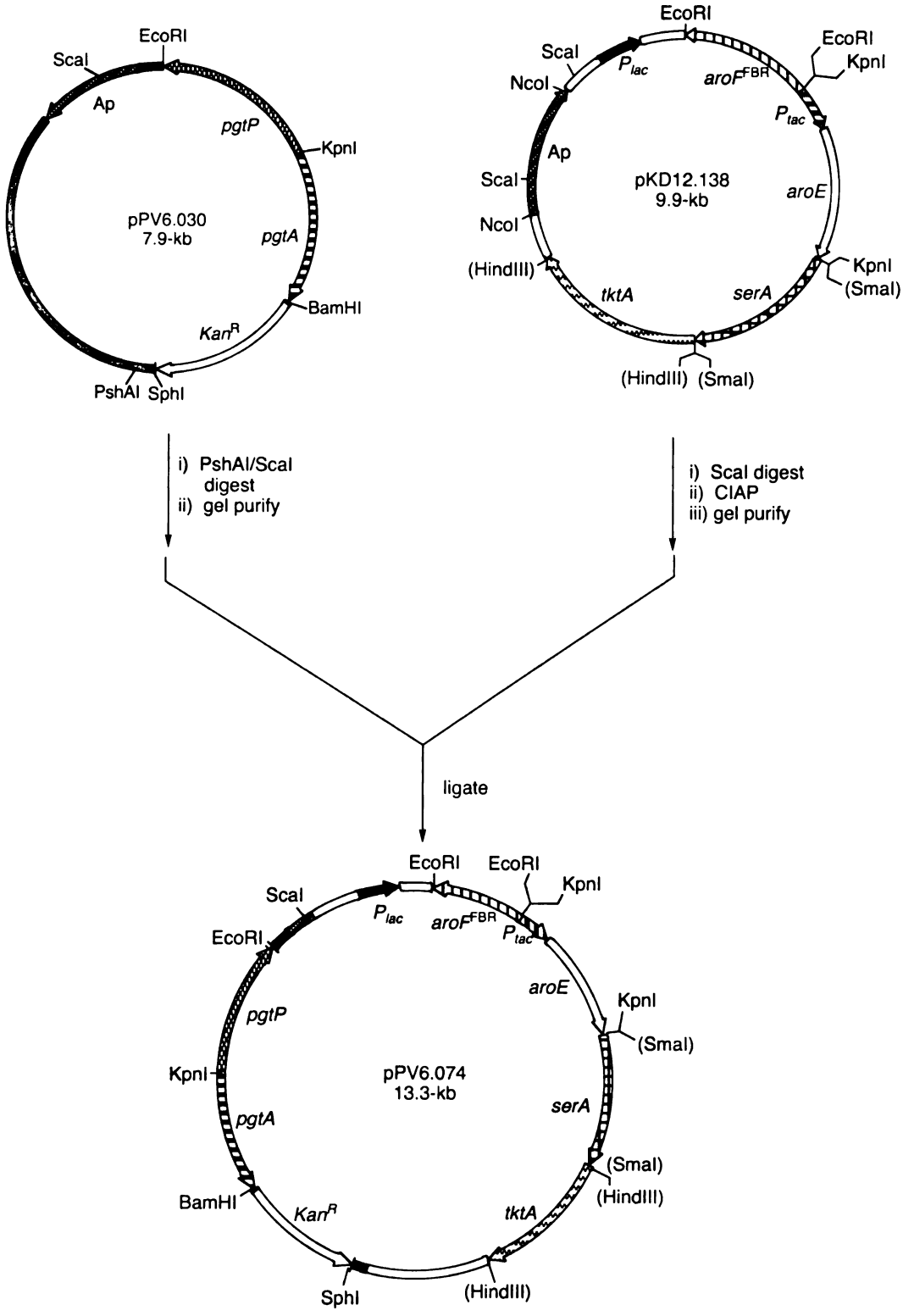


Figure 59. Construction of plasmid pPV6.074.

The impact of PEP supplementation on the fed-batch fermentation synthesis of shikimic acid using the phosphoglycerate transport system was studied using *E. coli* SP1.1/pPV6.074. The range of PEP concentrations analyzed was similar to the concentrations used during SP1.1/pSP2.054 fermentations. Addition of PEP to the fed-batch fermentation of SP1.1/pPV6.074 showed a similar trend (Table 3) of hydroaromatic production as observed in the case of SP1.1/pSP2.054. When 10 g PEP was added to the fermentation medium, 47 g/L of shikimic acid was produced in 22% yield (mol/mol) from glucose. Quinic acid (3 g/L) and 3-dehydroshikimic acid (10 g/L) were also synthesized. The total yield of hydroaromatic produced was 24% (mol/mol) (Figure 60).

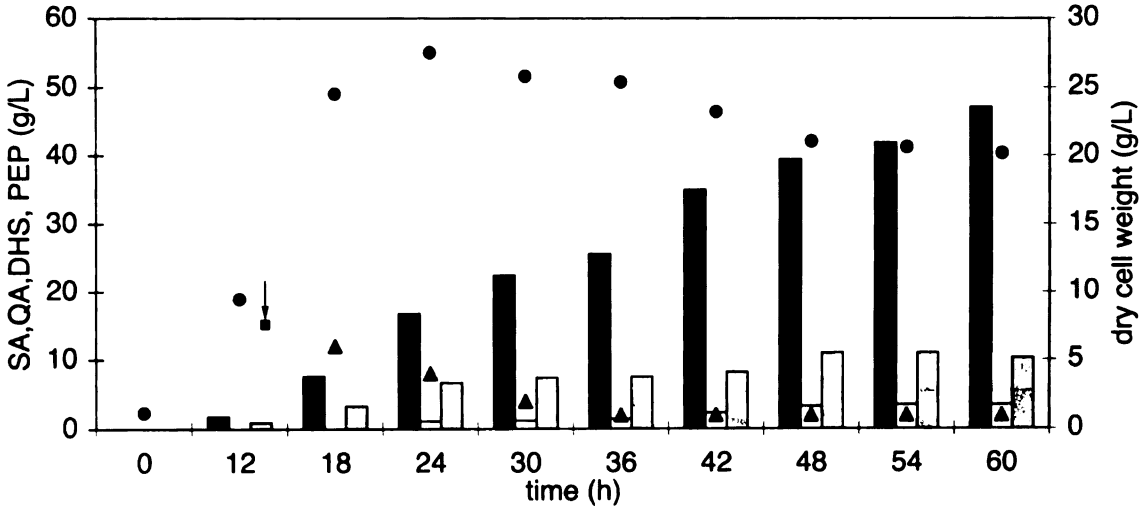


Figure 60. Cultivation of SP1.1/pPV6.074 under glucose-rich fed-batch fermentation conditions with 10 g PEP added at 13 h. Key: filled circles, dry cell weight; filled bars, shikimic acid; open bars, quinic acid; gray bars, 3-dehydroshikimic acid; triangles, phosphoenolpyruvate; Arrow indicates 10 g PEP added at 13 h.

The optimum amount of PEP required for observing significant increase in the yields and titer of shikimic acid was 10 g (Table 3). This was similar to the results obtained from *E. coli* SP1.1/pSP2.054 fermentation. The incorporation of the phosphoglycerate transport system in *E. coli* seemed to impose a metabolic burden on the

host. This observation was based on the lower titers of hydroaromatic intermediates that accumulated during *E. coli* SP1.1/pPV6.074 fermentations relative to that produced in the case of *E. coli* SP1.1/pKD12.138A or *E. coli* SP1.1/pSP2.054 when PEP was not supplemented. Even though the yield of shikimic acid and total yields of hydroaromatic synthesized by *E.coli* SP1.1/pPV6.074 (Table 3) with PEP supplementation were comparable to that produced by *E. coli* SP1.1/pSP2.054 (Table 2), the incorporation of the phosphoglycerate transport system in *E. coli* does not provide any additional advantage for the synthesis of shikimic acid relative to the altered *uhpT* gene.

Table 3. Yields and concentrations of metabolites synthesized by *E. coli* SP1.1/pPV6.074 upon supplementation with PEP.

Entry no	PEP [g/L][a]	[SA] g/L	SA yield[b]	[QA] g/L	[DHS] g/L	Total yield[c]
1	0	42	17	2	9	22
2	5	43	18	3	7	22
3	10	47	22	3	10	24
4	20	38	16	4	6	20
5	35	31	14	5	10	20

Abbreviation: shikimic acid (SA), quinic acid (QA), 3-dehydroshikimic acid (DHS). [a]PEP added to the fermentation medium. [b]mol(SA)/(mol glucose + mol PEP), [c]mol(SA+DHS+QA)/(mol glucose + mol PEP)

Impact of E4P Supplementation on Shikimate Fermentation

As the first enzyme in the pathway of aromatic amino acid biosynthesis, the activity of DAHP synthase dictates the direction of carbon flow in this pathway.[117] Transcriptional repression, feedback inhibition and availability of substrates E4P and PEP regulates the *in vivo* activity of this enzyme.[62] It has been shown that alleviation of feedback inhibition of DAHP synthase along with amplified expression of *tktA*-encoded transketolase leads to a pronounced effect in yield and titer of hydroaromatic production.[61a,74] *E. coli* KL3/pJY1.216(*aroF*^{FBR}, *serA*, *tktA*, P_{aroF} *lacI*^{Q}, and P_{tac}*pps*) has been used as a biocatalyst for the microbial synthesis of 3-dehydroshikimic acid and produces 69 g/L of 3-dehydroshikimic acid in 35% yield (mol/mol).[46b] Recent transcriptome analysis of *E. coli* KL3/pJY1.216 has shown elevated levels of mRNA transcript for *aroF*^{FBR} (constituting approximately 10% of the total mRNA) at the end of fed-batch fermentation.[128] Also, the mRNA transcript levels of *tktA* constituted 3% of the total mRNA transcript. This high level of transcript for *aroF*^{FBR} does not necessarily translate into higher enzyme activity and more products synthesized. On the contrary, the enzyme activities of DAHP synthase declined over the course of fermentation run.[128] This was also due to the fact that *aroF*-encoded DAHP synthase is more proteolytically labile. The *aroG*-encoded phenylalanine-sensitive isozyme of DAHP synthase is known to be more stable than the *aroF*-encoded tyrosine sensitive isozyme of DAHP synthase. Replacing *aroF*^{FBR} feedback-insensitive allele with the *aroG*^{FBR} gene gave similar yields and titers for 3-dehydroshikimic acid when cultured under glucose-rich fed-batch fermantor conditions.[128]

The plasmid-localized *tktA*-encoded transketolase expressed from its native promoter, provided increased the availability of E4P. With amplified expression of the feedback-insensitive DAHP synthase, the issue was raised as to whether E4P availability was once again limiting carbon flow directed into the common pathway. One method to diagnose this problem is to supplement the culture medium with E4P. If E4P is transported into the microbial cytoplasm and a significant increase in the yield and titer of shikimic acid production is observed, evidence will be provide that the intracellular availability of E4P is inadequate.

Few literature syntheses of E4P are available and most of them use chemical degradation of carbohydrate source to generate either E4P or erythrose, which is then phosphorylated.[129] A commonly used method for preparation of E4P is the oxidation of D-glucose 6-phosphate with lead tetraacetate.[129b] This oxidation affords a mixture of E4P, D-glyceraldehyde 3-phosphate, and D-glucose 6-phosphate. Complete removal of acetic acid from this mixture is not possible, as solutions of E4P tend to polymerize when concentrated.[67] A chemical synthesis of E4P free of acetic acid contamination was thus pursued, as several grams of this compound were required for evaluating the impact of E4P on shikimic acid production during fed-batch fermentations. The advantage of the elaborated synthesis (Figure 59) was that it circumvented the issue of dimerization and polymerization of E4P as the aldehyde group was protected as a 1,3-dioxalane. The product E4P was obtained by hydrolysis of the 1,3 dioxalane by mild acid treatment prior to the use of E4P as a supplement.

Synthesis of Erythrose 4-Phosphate.

Synthesis of E4P (Figure 61) began with the protection of *cis*-2-butene-1,4-diol as benzylidine followed by the reduction of the acetal **16** with $LiAlH_4/AlCl_3$ to give 4-benzyloxy-*syn*-but-2-en-1-ol **17** in 79% overall yield. Oxidation of **17** with pyridinium chlorochromate occurs simultaneously with the isomerization to the trans double bond yielding 4-benzyloxy-*trans*-but-2-en-1-al **18** in 69% yield.[130,131] The aldehyde was sequestered as 1,3 dioxalane **19**, and treated with *m*CPBA to give 4-benzyloxy-2,3-epoxy-butane-1,3-dioxalane **20** in 73% overall yield (for the two steps).

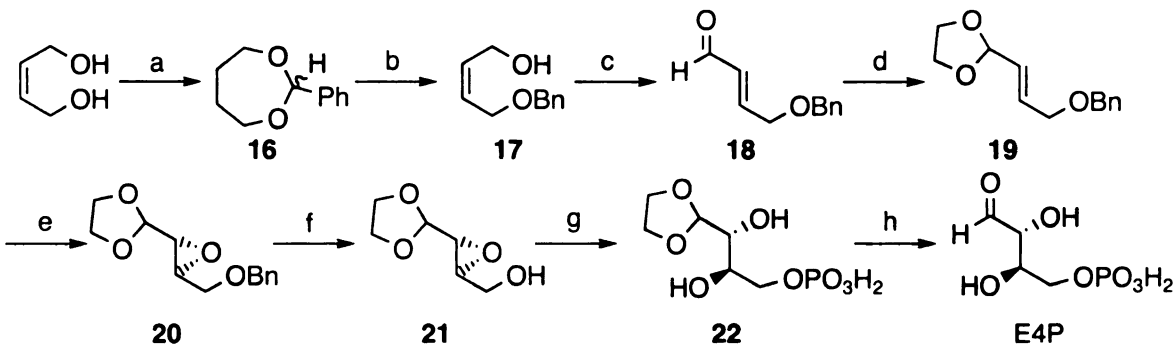


Figure 61. Synthesis of D,L-erythrose 4-phosphate from *cis*-2-butene-1,4-diol.
Key: a) PhCHO, *p*TSA, C_6H_6, Δ 80%; b) $LiAlH_4$, $AlCl_3$,, Et_2O, 98%; c) PCC, CH_2Cl_2, 69%; d) $C_2H_6O_2$, PPTS, C_6H_6, 85%; e) *m*CPBA, CH_2Cl_2,, 85%; f) H_2, Pd/C, 100%; g) K_3PO_4, H_2O:dioxane(v/v, 1:1), reflux, 80% or NaOH, H_2O:dioxane(v/v, 1:1), reflux, 80%; h) Dowex-50 (H^+), H_2O, 70°C, 100%.

Catalytic hydrogenation of **20** with Pearlman's catalyst gave 2,3-epoxy-butane-1,3-dioxalane **21** in quantitative yields. Compound **21** when treated with inorganic phosphate underwent a base catalyzed ring opening *via* a Payne rearrangement giving the protected E4P **22** in 80% yields. The excess inorganic phosphate was precipitated using chilled methanol and the product was purified on an anion exchange resin. The final product E4P was obtained by gentle heating of the protected phosphate monoester with

Dowex-50 (H^+) in water. The product E4P is known to exist as an equilibrium of monomer and dimers in very dilute solutions and as a polymeric mixture at higher concentration. Thus while all the intermediates were characterized by traditional organic techniques, the final product was confirmed by DAHP synthase assay[132] and transketolase assay.[133]

Synthesized E4P was used as a supplement in fed-batch fermentation of *E. coli* SP1.1/pKD12.138A and SP1.1/pSP2.054 and the impact on the yield and titer of shikimic acid synthesized these constructs when cultured under fed-batch fermentor conditions was studied. Synthesized E4P was neutralized to pH 7.0 and was added at the beginning of the oxygen sensor-controlled phase of the fed-batch fermentation. The addition of E4P had a deleterious effect on the fermentation that was reflected by the slower rate of cell growth and lower titers of shikimic acid synthesized (Table 4, entry 3 and entry 4). The specific activity of DAHP synthase measured during the course of fermentation runs of *E. coli* SP1.1/pKD12.138 with 10 g D,L-E4P supplementation was lower by one order of magnitude compared to control (Table 1, entry 3). Though low titers of shikimic acid were produced in these fermentations, the yields of shikimic acid and other hydroaromatics produced were comparable when compared to the control runs. In order to examine the impact of supplementation with both of phosphoenolpyruvate and E4P, 10 g of phosphoenolpyruvate and 10 g of D,L-E4P was added to the *E. coli* SP1.1/pSP2.054 fermentation at the beginning of oxygen sensor-controlled phase of fermentation run. The addition of the two precursor molecules was accompanied by a slow growth rate and lower titers of shikimic acid (Table 4, entry 5).

Table 4. Yields and concentration of metabolites synthesized during E4P supplementation

Entry no	Construct	Additions	[SA] g/L	SA yield [a]	[QA] g/L	[DHS] g/L	Total yield [b]
1	SP1.1/pKD12.138A	-	49	16	5	16	22
2	SP1.1/pSP2.054	-	49	18	5	16	26
3	SP1.1/pKD12.138A	10 g D,L-E4P	32	14	3	9	19
4	SP1.1/pSP2.054	10 g D,L-E4P	42	15	3	9	20
5	SP1.1/pSP2.054	10 g D,L-E4P + 10 g PEP	40	15	5	9	20
6	SP1.1/pKD12.138A	10 g D,L-erythrose	45	16	5	11	21

Abbreviation: E4P (E4P), phosphoenolpyruvate (PEP), shikimic acid (SA), quinic acid (QA), 3-dehydroshikimic acid (DHS). [a]mol(SA)/(mol glucose + mol E4P + mol PEP), [b](mol SA + mol DHS + mol QA)/(mol glucose + mol E4P + mol PEP).

To ascertain if E4P was transported into the cytoplasm, [^{33}P]-E4P was synthesized by using radiolabel orthophosphate in the synthesis of **22**. Transport assays performed on cell samples obtained from various stages SP1.1/pKD12.138A and SP1.1/pSP2.054 fermentor runs using [^{33}P]-E4P showed low level of E4P transport by both construct (Figure 62). The low titers of shikimate and other hydroaromatic intermediates produced were attributed to the excess organic phosphate present in the culture medium.

In order to examine whether D,L-erythrose 4-phosphate was deleterious to the cell growth D,L-erythrose was synthesized. Treatment of epoxide **21** (Figure 61) with NaOH followed by deprotection of the 1,3-dioxalane gave D,L-erythrose in 80% yield. Addition of erythrose instead of E4P to *E. coli* SP1.1/pKD12.138A cultures under fed-batch fermentor conditions gave 45 g/L of shikimic acid in 16% (mol/mol) yield and total yield of 21% (mol/mol) for all of the synthesized hydroaromatic intermediates (Table 4, entry 6). Normal cell growth was observed in this fermentation although increase in synthesis

of shikimic acid was not observed. Poor uptake of E4P and erythrose may be due to the absence of an efficient transport system for these metabolities in *E. coli*.

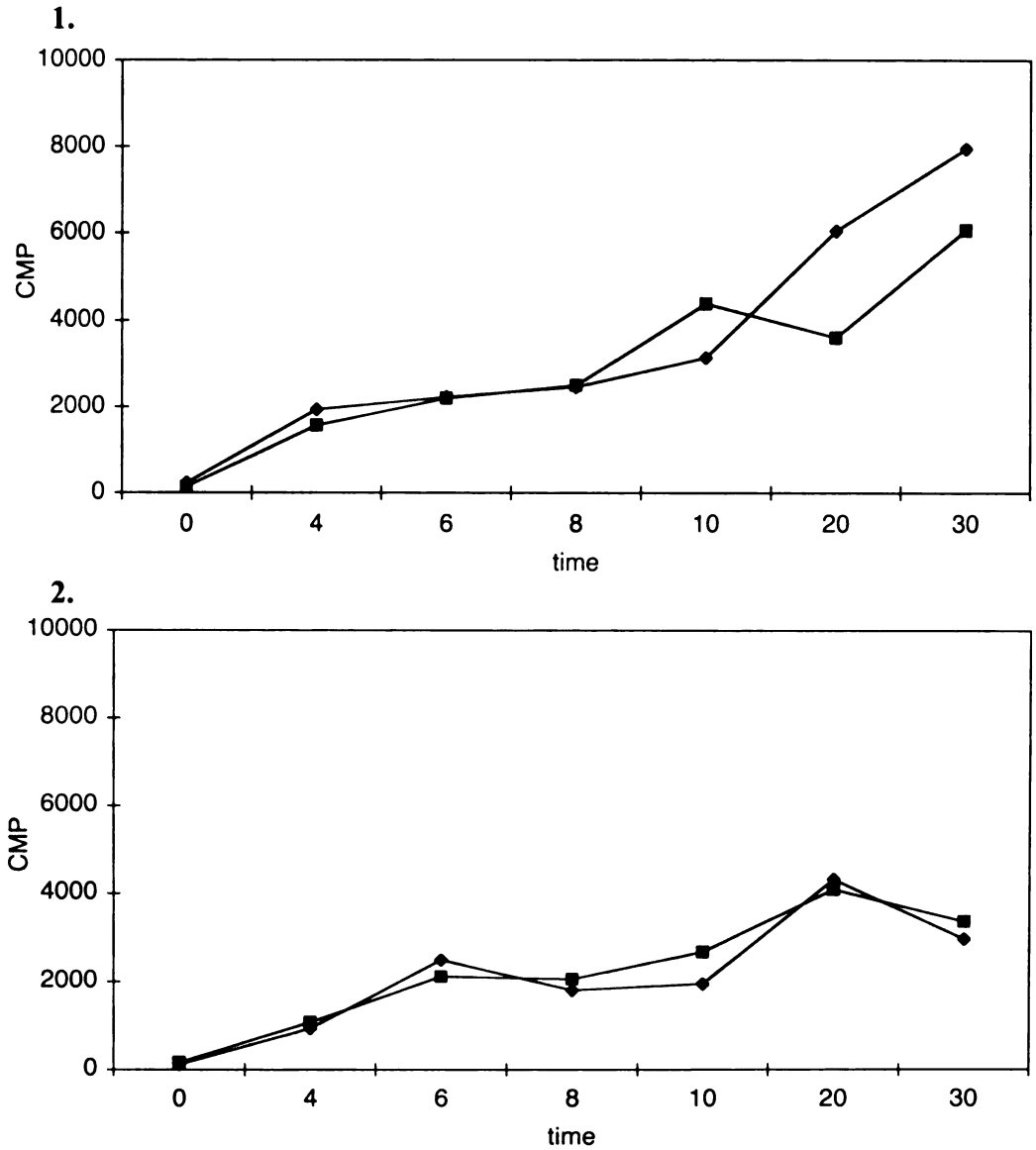


Figure 62. Amount of radioactivity incorporated (μCi) in intact cell when transport assay using [^{33}P]-E4P were performed on cell samples obtained at various stages of fed-batch fermentor runs of SP1.1/pSP2.054 and SP1.1/pKD12.138A. 1: 12 h of growth. **2:** 24h of growth. Legends: Diamonds SP1.1/pKD12.138A, Squares SP1.1/pSP2.054.

DISCUSSION

A) Comparison of Titers and Yields

A biocatalyst can be evaluated by comparing the yield of a synthesized product to the theoretical maximum yield (mol of products produced/mol of glucose consumed). For determining the theoretical maximum yield in the case of shikimic acid synthesis balancing the E4P and PEP inputs with the products and byproducts is required which gives equation (1). The input of E4P and PEP required for synthesis shikimic acid is then equated with the amount of glucose required to form these substrates (Eq. (2a)). In order to account for the operation of the PTS-mediated glucose transport system, a pyruvic acid term is added (Eq. (2a)). This leads to a coefficient of 2.33 required to balance the number of carbon atoms in the glucose input with the number of carbon atoms formed in PEP, E4P and pyruvic acid (Eq. (2b)). Hence, synthesis of 1 mole of shikimic acid requires the consumption of 2.33 moles of glucose resulting in a theoretical maximum yield of 43% (mol of shikimate produced/ mol of glucose consumed).[46a]

(1) PEP + E4P $\longrightarrow$ $2H_3PO_4 + H_2O$ + SA

(2a) x glucose $\longrightarrow$ PEP + E4P + x pyruvic acid

(2b) x 6 (C) $\longrightarrow$ 3 (C) + 4(C) + x 3(C)

Use of the modified hexose phosphate transport, or the phosphoglycerate transport, reduces the dependence of PEP. Use of 1 mol PEP as substrate in the equation (2b) leads to the coefficient of 1.33 and hence a maximum theoretical yield for shikimate

acid of 75% (mol of shikimic acid produced/mol of glucose+ mol PEP consumed) when PEP is used as a supplement along with D-glucose.

In order to quantify carbon flow directed into the common pathway during the synthesis of shikimic acid, the amounts of quinic acid and 3-dehydroshikimic acid should also be taken into account. *E. coli* SP1.1/pKD12.138A which overexpresses $AroF^{FBR}$ and TktA synthesized skimic acid in 49 g/L in 16% (mol/mol) yield. The total yield taking into account synthesized quinic acid and 3-dehydroshikimic acid was 22% (mol/mol). *E. coli* SP1.1/pSP2.054 with 10 g PEP supplementation increased shikimic acid yield (20%) and titer (59 g/L) (Table 2, entry 3). The total yield for all the hydroaromatics produced was 28% (mol/mol). This was the highest yield and titer obtained during PEP supplementation. The 6% increase in total hydroaromatics produced in *E. coli* SP1.1/pSP2.054 is comparable to increase in total hydroaromatic produced in case of 3-dehydroshikimate producing strain *E. coli* KL3/pKL6.218A where tricarboxylic acid cycle intermediate succinic acid was used as a masked precursor for PEP.[112]

B) Impact of Increased PEP and E4P Availability.

The intracellular concentrations of E4P and PEP have been established to be limiting factors for directing more carbon into the common pathway upon amplified expression of feedback-insensitive DAHP synthase. Metabolic engineering of the shikimate pathway for the biocatalytic synthesis of DHS and shikimic acid has demonstrated the pronounced effect of *tktA* overexpression even in the absence of *pps* overexpression.[46,74] It has also been established that overexpression of *pps* improves carbon flow into the common pathway only in presence of amplified *tktA* expression, but

the reverse does not hold true.[46,134b,c] Hence, in a biocatalytic environment possessing ample PEP concentrations and overexpressed, feedback-insensitive DAHP synthase, E4P availability is a critical factor limiting aromatic amino acid biosynthesis. All studies done thereafter utilized amplified expression of *tktA*-encoding transketolase.

Although many enzymes including PEP carboxylase, PEP kinase, PEP carboxykinase and 3-deoxy-D-*manno*-ocutosolunate 8-phosphate synthase utilize PEP as substrate, one of the biggest drains on PEP availability for aromatic amino acid biosynthesis, is the PTS system for glucose transport.[67] Under the fermentation conditions employed, the cells remain under a very glucose-rich environment throughout the course of the fermentation. Hence, the amount of PEP available for the synthesis of the shikimate pathway intermediates is relatively low as the synthesis of the pathway intermediates competes with PEP required for the transport of glucose. Usage of the altered hexose phosphate transport system along with PEP supplementation can alleviate this limitation of PEP.

E. coli strains harboring the modified *uhpT* gene were used to examine the impact of PEP supplementation on shikimate production. The studies showed a moderate increase in the yield and titers of shikimic acid produced when *E. coli* SP1.1/pSP2.054 was supplemented with 10 g of PEP. Increasing the amounts of PEP supplementation did not give any increase in yields and titers of shikimic acid. ^{1}H NMR examination of the culture supernatant showed rapid transport of PEP during the log phase (12-20 h) of the cell growth and while the transport rate of PEP diminished during stationary phase (24-60 h). The significant difference in transport rate of PEP can be attributed to the change in the electrochemical potential within the cellular cytoplasm.[119d] The wild type UhpT

protein, which being an antiporter protein, selects monovalent P_i to use in an electroneutral exchange with divalent glucose-6-phosphate, with an overall 2-for-1 stoichiometry.[114,116] A similar 2-for-1 stoichiometry during the heterologous P_i/PEP antiport is therefore expected to be electrogenic, carrying a negative charge inside the cell, since PEP is trianionic at pH 7.[119] This has to occur against the cell membrane potential that is negative inside the cell. During the log phase of growth, the cells are rapidly dividing and any change in the cellular potential is rapidly dissipated by the cell division, while in the stationary phase the uptake of PEP is against the cell membrane potential. So this may explain the difference in uptake rate for PEP during the various stages of fermentation. The production rate for shikimic acid was maximum (2 g/h/h) between 24-36 h, but the PEP transport rate was maximum during the log phase and diminished during the stationary phase. This resulted in a limited impact in shikimic acid production during PEP supplementation under fed-batch fermentor conditions.

When E4P was added to the culture medium, a decrease in growth rate and shikimic acid production was observed. This may be due to the presence of excess organic phosphate that was deleterious to the growth of cells. When E4P was replaced by D-erythrose as the culture supplement, normal growth was observed, although there was no increase in product yield and titer. The poor transport of E4P can be attributed to absence of an appropriate transporter. Recruitment of a transport system for D-erythrose or E4P in *E. coli* could solve this problem. Alternatively using genetic engineering techniques for directed evolution, mutant *uhpT*(D388C) gene might be altered such that it becomes specific for E4P transport.

Figure 63. Control ^{1}H NMR of microbially synthesized shikimic acid.
^{1}H NMR (300 MHz, D_2O) δ 6.49 (t, J =2 Hz, 1 H), 4.40 (t, J =4 Hz 1H), 3.99 (m, 1 H), H_3 3.78 (dd, J = 4, 4 Hz, 1 H), 2.73 (ddd, J = 2, 1.5, 1 Hz, 1 H), 2.20 (m, 1 H);

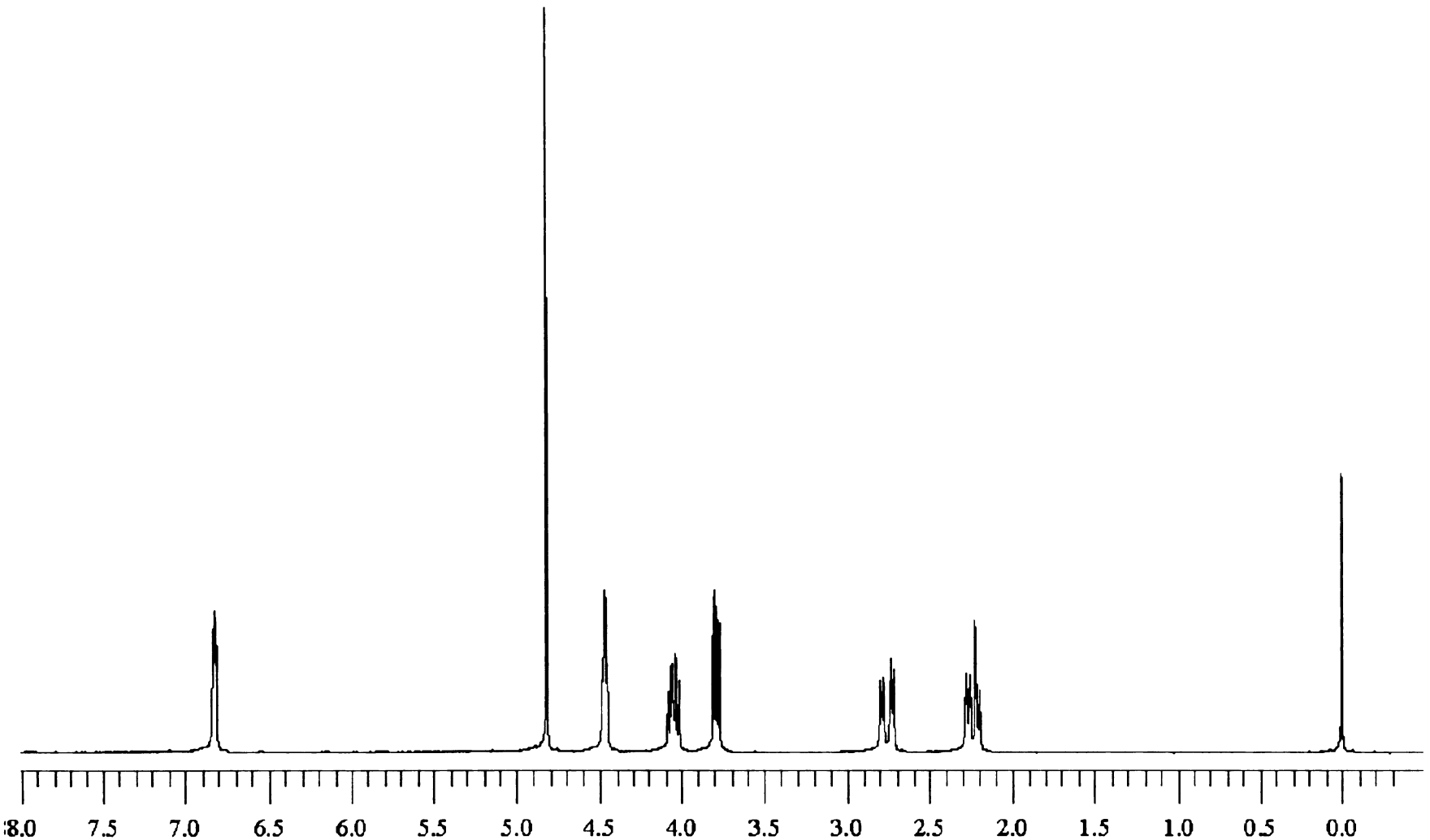

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Figure 64. Control ^{1}H NMR of quinic acid (Aldrich).
^{1}H NMR (300 MHz, D_2O) δ 4.16 (t, J = 3.6 Hz, 1 H), 4.04 (m, 1 H), 3.55 (dd, J = 6, 3 Hz, 1 H), 2.11 (m, 3 H) 1.92 (dd, J= 10, 3 Hz, 1H);

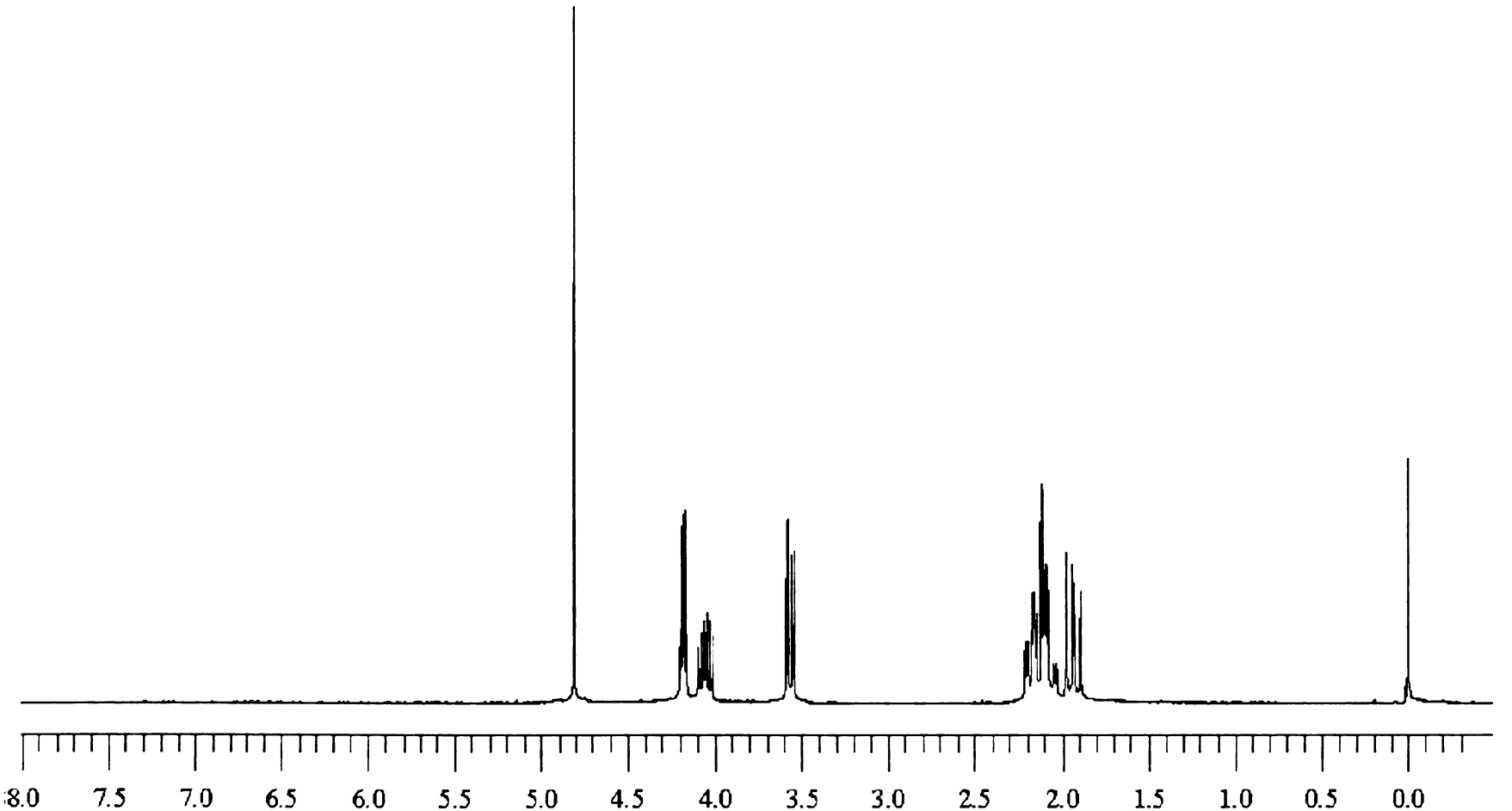
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Figure 65. Control ^{1}H NMR of microbially synthesized 3-dehydroshikimic acid.
^{1}H NMR (300 MHz, D_2O) δ 6.42(d, J = 3 Hz ,1 H) 4.28 (d, J = 11 Hz ,1 H), 4.00 (m, 1 H), 3.14 (dd, J = 13, 5 Hz, 1 H), 2.66 (m, 1H).

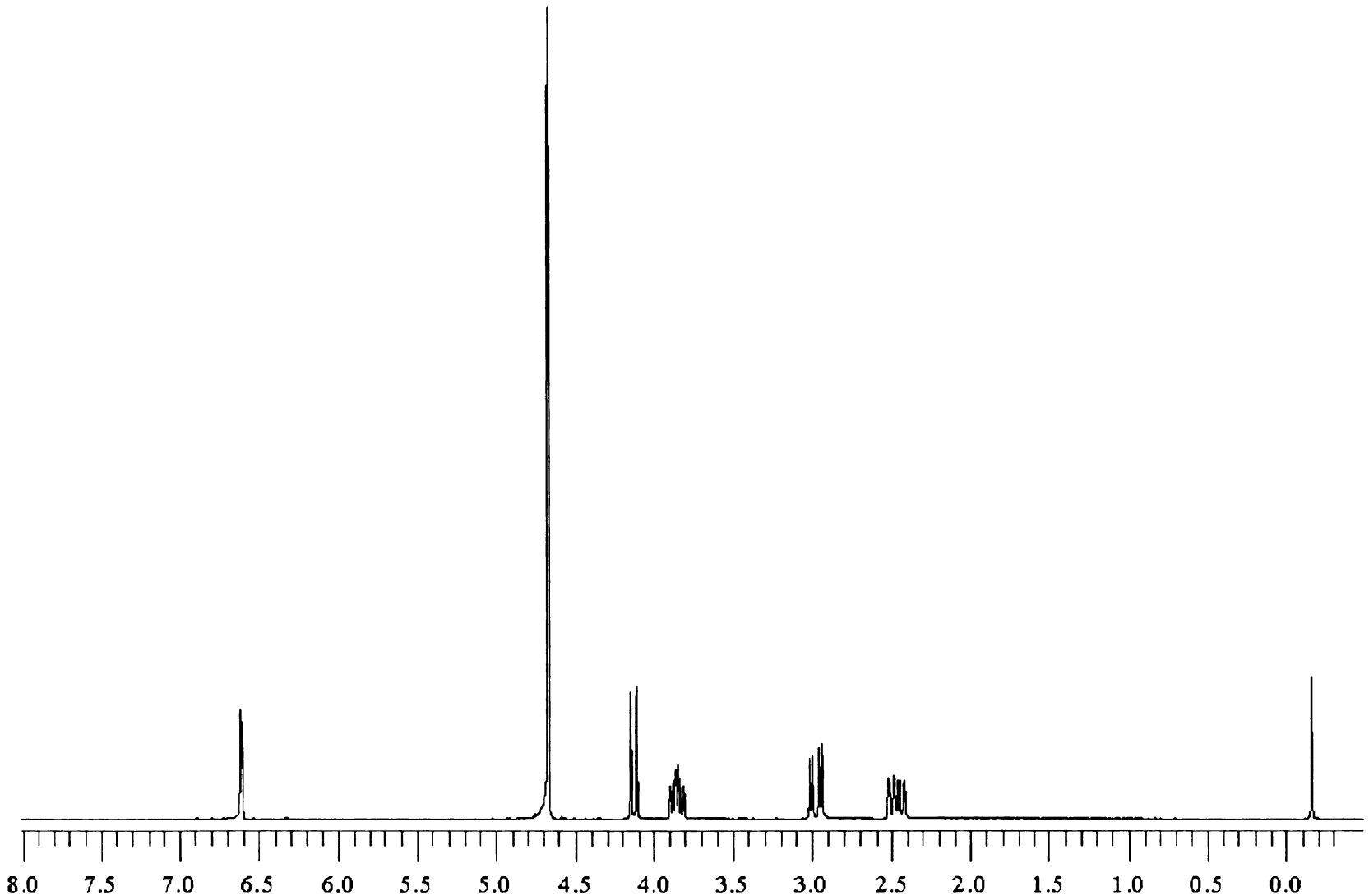
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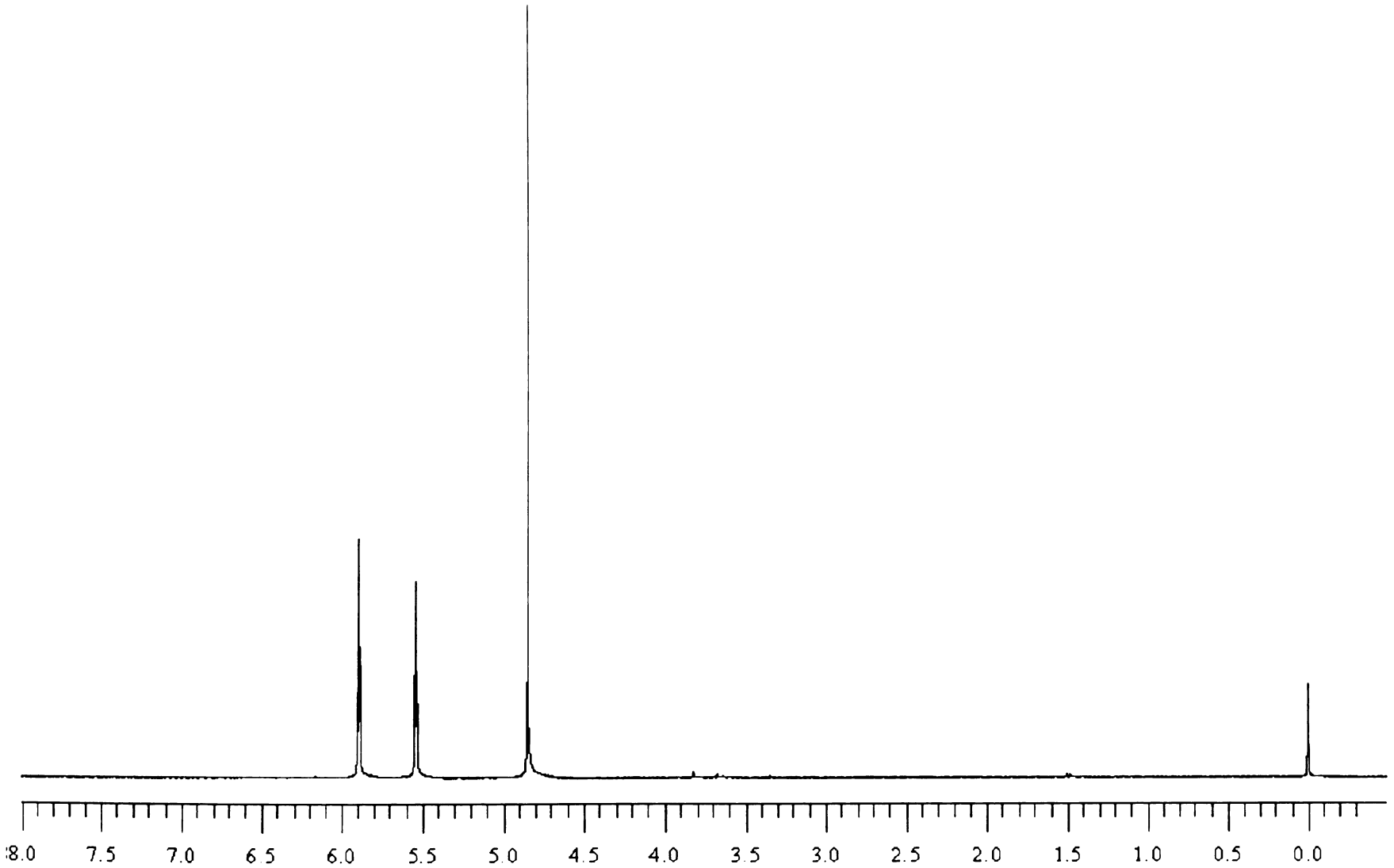
Figure 66. Control ^{1}H NMR of chemically synthesized phosphoenolpyruvate.
^{1}H NMR (300 MHz, D_2O) δ 5.86 (t, 1 H), 5.07 (t,1 H).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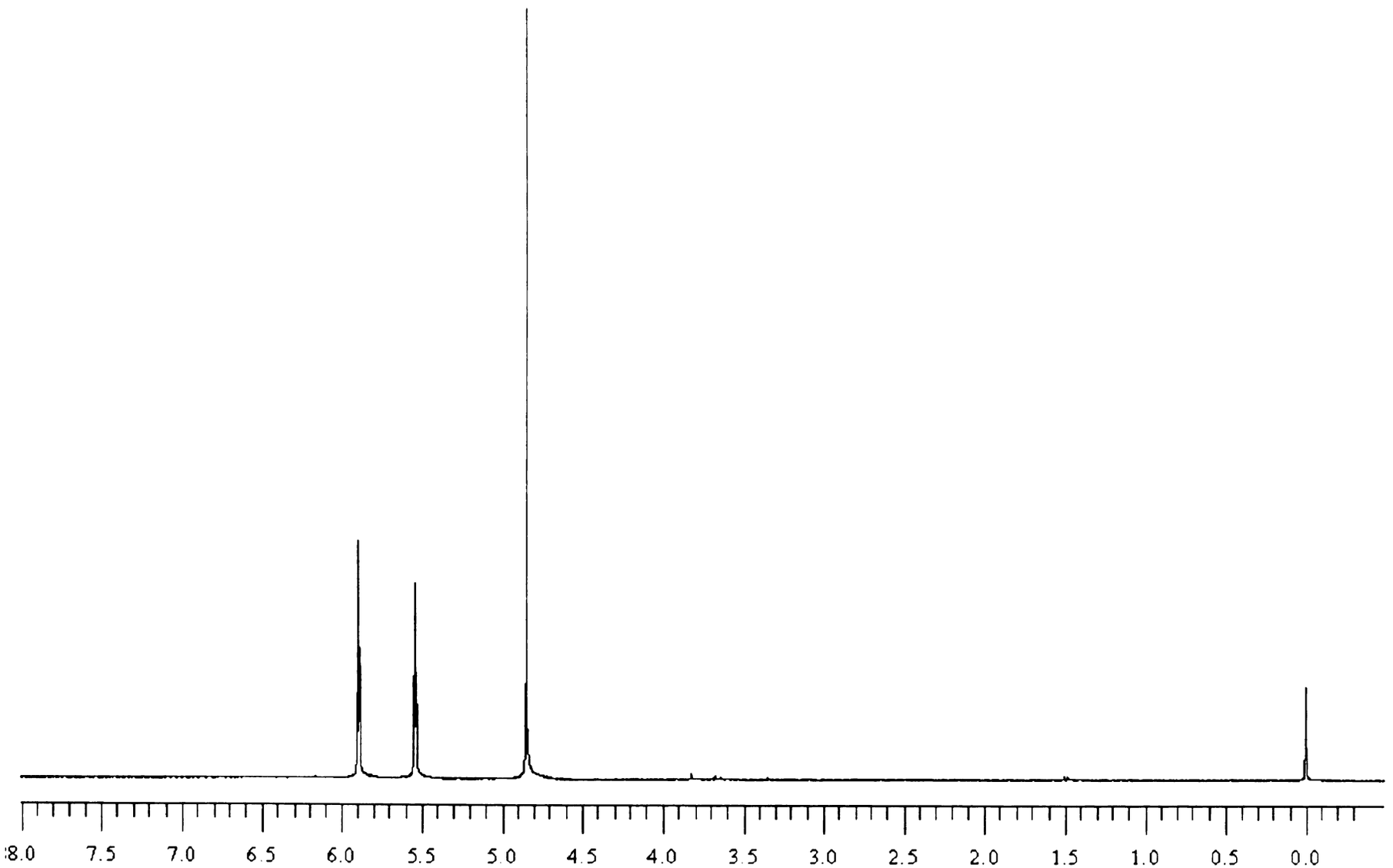
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Figure 67. ^{1}H NMR (300 MHz, D_2O) of the culture supernatant of *E. coli* SP1.1/pKD12.138A after 60 h of cultivation under glucose-rich fed-batch fermentation conditions.

Resonances for shikimic acid include: δ 4.40 (t, J =4 Hz, 1 H). Resonances for quinic acid include: δ 4.16 (t, J = 3.6 Hz, 1 H). Resonances for 3-dehydroshikimic acid include δ 4.28 (d, J = 11 Hz ,1 H). Resonances for acetic acid include δ 1.92 (s, 3 H).

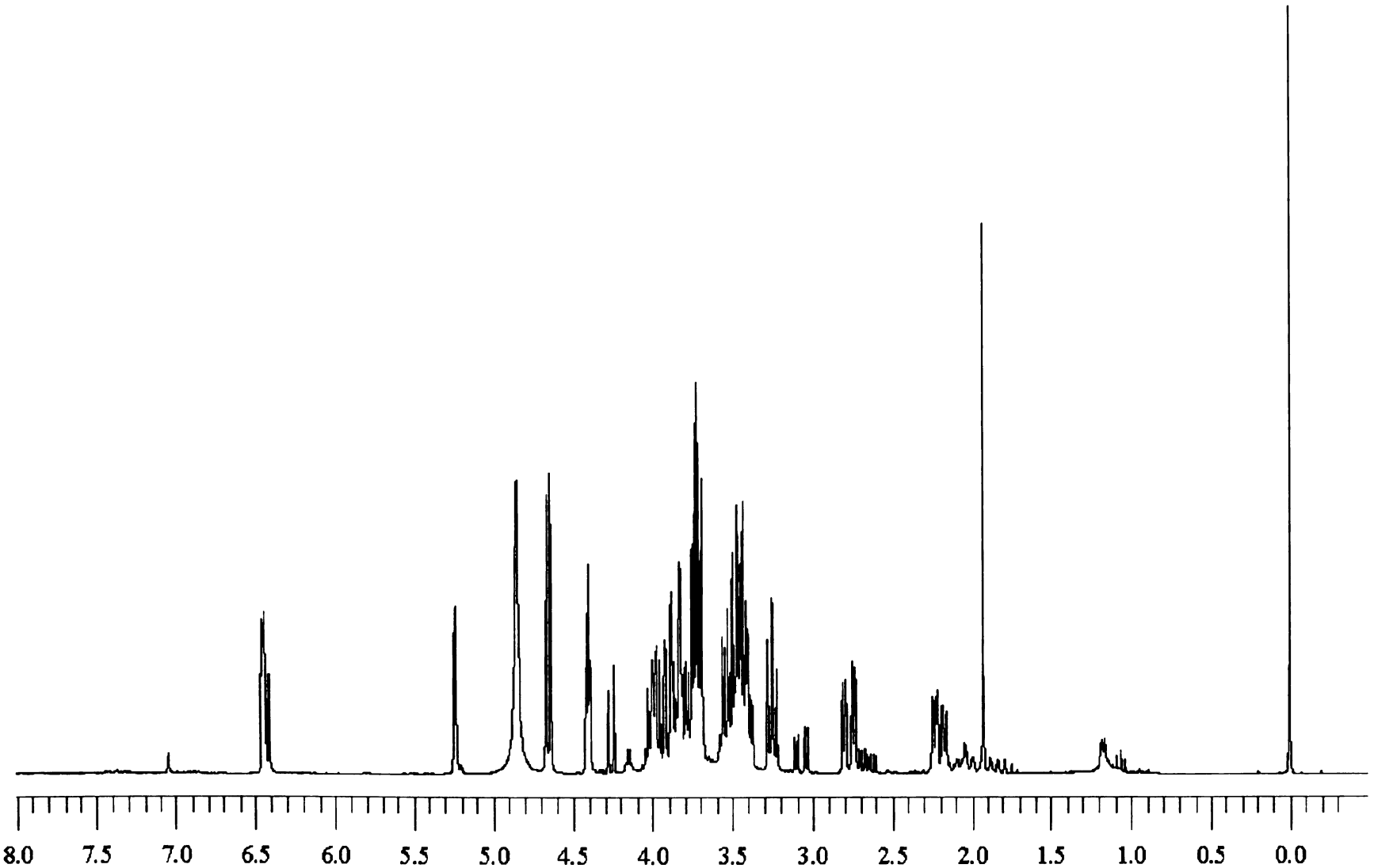
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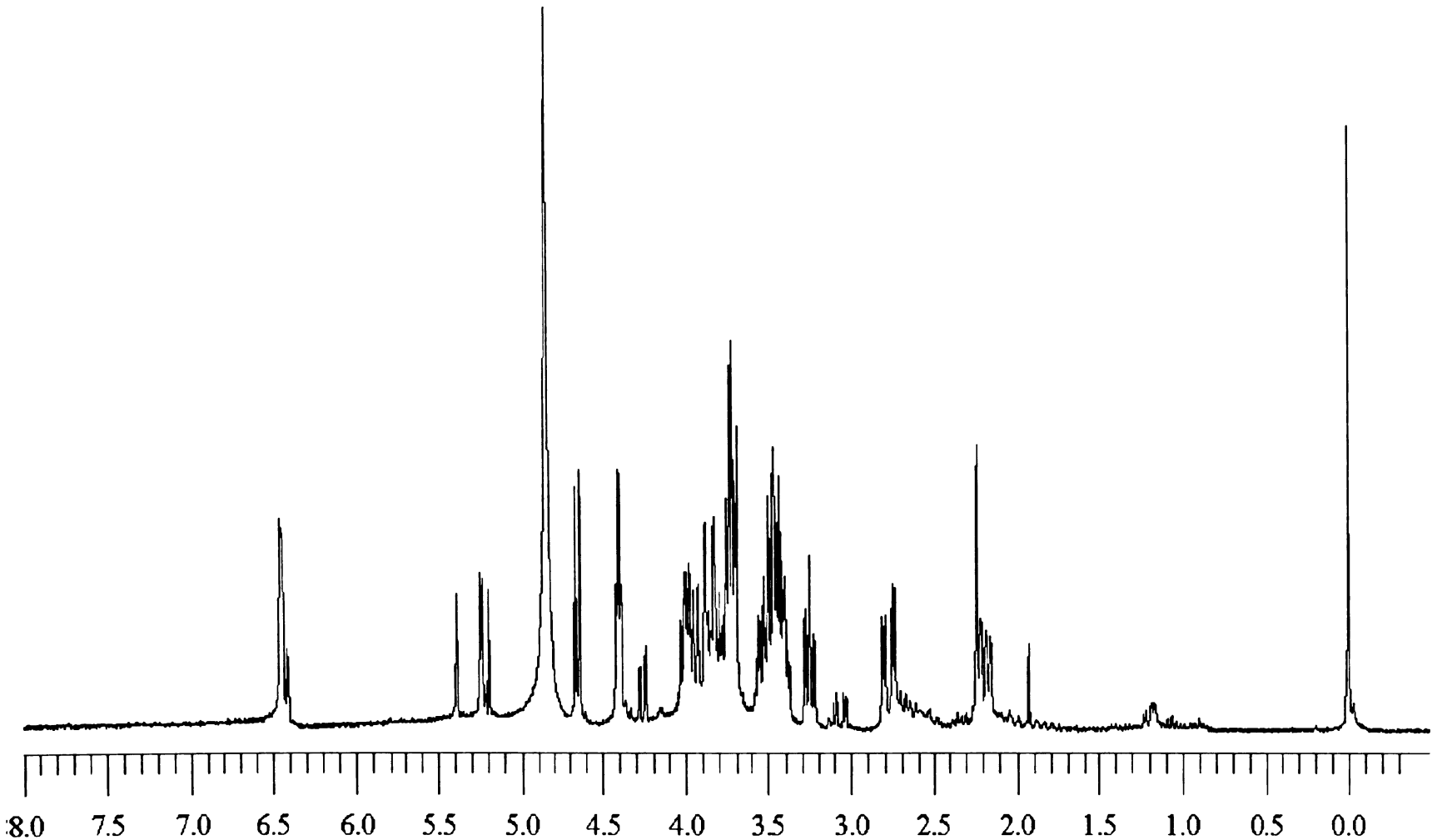
Figure 68. ^{1}H NMR (300 MHz, D_2O) of the culture supernatant of SP1.1/pSP2.054 with PEP added at phase change.

Resonances for shikimic acid include: δ 4.40 (t, J =4 Hz, 1 H). Resonances for quinic acid include: δ4.16 (t, J = 3.6 Hz, 1H). Resonances for 3-dehydroshikimic acid include δ 4.28 (d, J = 11 Hz ,1 H). Resonances for acetic acid include δ 1.92 (s, 3 H). Resonances for PEP include δ 5.86 (t, 1H), 5.07 (t, 1 H).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CHAPTER 4

EXPERIMENTAL

General Methods

General Chemistry

All reactions sensitive to air and moisture were carried out in oven and/or flame dried glassware under positive argon pressure. Air or moisture sensitive reagents and solvents were transferred to reaction flasks fitted with rubber septa via syringes or cannula. Unless otherwise specified, all reactions were carried out at room temperature. Solvents were removed using either a Büchi rotary evaporator at water aspirator pressure or under high vacuum (0.5 mm Hg).

Reagents and Solvents

Tetrahydrofuran and diethyl ether were distilled under nitrogen from sodium/benzophenone. Methylene chloride, benzene, triethylamine and pyridine were distilled over calcium hydride before use. Organic solutions of products were dried over $MgSO_4$. Most chemicals were purchased from Aldrich. Sodium salt of 3-(trimethylsilyl) propionic-*2,2,3,3-d*$_4$ acid (TSP) was purchased from Lancaster Synthesis Inc. [2-^{14}C]-PEP (50 μCi) and [^{33}P] H_3PO_4 (1 mCi) was obtained from New England Nuclear Labs. Distilled deionized water was used for all purposes. Charcoal (Darco® G-60 ~100 mesh) was used for decolorization at a final concentration of 0.1 g/mL. (-)-2,3-*O*-Isopropylidenedimethyl-D-threitol was synthesized from D-tartaric acid

according to the reported procedure of Mash.[72a] Dess-Martin reagent was synthesized according to the procedure of Boeckman.[135] Pyridinium chlorochromate (PCC) was synthesized according to the procedure of Corey and Suggs.[130] D-Erythrose-4-phosphate required for enzyme assays,[136] fosmidomycin[89] and PEP[121] were prepared according to the published procedures. 1-Deoxy-D-xylulose,[72b] DXP,[72c] and 2-*C*-methyl-D-erythritol[82] were synthesized by modification of published procedures.

Chromatography

Radial chromatography was carried out with a Harrison Associates Chromatotron using 1, 2 or 4 mm layers of silica gel 60 PF_{254} containing gypsum (E. Merck). Silica gel 60 (40-63 μm, E. Merck) was used for flash chromatography. Analytical thin-layer chromatography (TLC) utilized precoated plates of silica gel 60 F-254 (0.25 mm, Whatman). TLC plates were visualized by immersing in phosphomolybdic acid stain (7% phosphomolybdic acid in ethanol) followed by heating.

Diethylaminoethyl cellulose (DEAE) was purchased from Whatman, Dowex 50 (H^+) from Sigma, AG-1X8 from Bio-Rad and Ni-NTA agrose from Qiagen. TEAB buffer was prepared by bubbling CO_2 gas through an aqueous solution of Et_3N until the pH reached 7.5 at 0 °C. Dowex 50 (H^+) was cleaned before use by treatment with bromine. An aqueous suspension of resin was adjusted to pH 14 by addition of solid KOH. Bromine was added to the solution until the suspension turned a golden yellow color. Additional bromine was added (1-2 mL) to obtain a saturated solution. The mixture was left to stand at room temperature overnight and the Dowex 50 resin was collected by filtration and washed exhaustively with water followed by 6 N HCl. Dowex

50 (H^+) was stored at 4°C. AG-1X8 was cleaned and prepared for use by sequential washing with 2 N NaOH (4 to 6 column volumes), water (10 column volumes), high end buffer (4 column volume), water (10 column volume) and appropriate low end buffer (2 column volume). All aqueous chromatographic purifications were carried out at 4°C.

Spectroscopic and Analytical Measurements

^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian VX-300 FT-NMR spectrometer. Chemical shifts for ^{1}H NMR spectra are reported in parts per million (ppm) relative to internal tetramethylsilane (Me_4Si, δ 0.0 ppm) with $CDCl_3$ as the solvent and relative to sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ (TSP, δ 0.0 ppm) when D_2O was the solvent. The following abbreviations are used to describe spin multiplicity: s (singlet), d (doublet), t (triplet), m (unresolved multiplet) and dd (doublet of doublets). Chemical shifts for ^{13}C NMR spectra are reported in ppm relative to $CDCl_3$ (δ 77.0 ppm) or internal standard acetonitrile (CH_3CN, δ 3.69 ppm) in D_2O. Fast atom bombardment (FAB) mass spectra were obtained on a double-focusing Kratos MS50 mass spectrometer employing glycerol as the matrix. UV and visible spectra were recorded on a Perkin-Elmer Lambda 3B spectrometer or a Hewlett Packard 8452A diode array spectrometer with an attached thermostat control. Radioactivity was measured using Wallac Liquid Scintillation Counter.

Bacterial Strains and Plasmids

E. coli DH5α [*F' endA1 hsdR17*($r^-_K m^+_K$) *supE44 thi-1 recA1 gyrA relA1 Ø80lacZΔM15* Δ*(lacZYA-argF)*$_{U169}$], RB791 (W3110 *lacL8I*^{q}) and JM109 [*e14-(McrA-)*

recA1 endA1 gyrA96 thi-1 hsdR17($r^-_K m^+_K$) supE44 relA1 Δ(lac-proAB) [F' *traD36 proAB lacI^{Q}ΔM15*]] were obtained previously in this laboratory. The construction of *E. coli* JWF1 (RB791 *serA*),[137] SP1.1 (RB791 *serA*::*aroB aroL478*::Tn*10 aroK17*::Cm^R),[138] has been previously described. *E. coli* M15[pREP4] and plasmid pQE30 were obtained from Qiagen. *E. coli* DMY1 (FS1576 *dxr::kan*) and plasmid pUMV20 was a gift from Dr. T. Kuzuyama (University of Tokyo).[75a] Plasmid pJF118EH was obtained from Professor M. Bagdasarian of Michigan State University.[76] Plasmid pTrc99A was obtained from Amersham Biosciences. Plasmid pBR322 was obtained from Invitrogen. Plasmid pD2625 was obtained from Genencor Inc. Plasmid pTrc(HisC6S)-D388C was obtained from the laboratory of Prof. P. C. Maloney.[119d] Plasmids pKD12.138,[74] pKAD62A,[139] pKL5.17A,[46a,140] and pRC55B[68d] were previously constructed in this laboratory. *Salmonella choleraesuis* LT2 genomic DNA was obtained from ATCC.

Storage of Bacterial Strains and Plasmids

All bacterial strains were stored at -78 °C in glycerol. Plasmids were transformed into either DH5a, JM109 or JWF1 for storage. Glycerol freezes were prepared by adding 0.75 mL of culture to 0.25 mL of sterile 80% (v/v) glycerol. The solutions were mixed, allowed to stand at room temperature for 2 h and stored at -78 °C.

Culture Medium

All solutions were prepared in distilled, deionized water. LB medium[141] (1 L) contained Bacto tryptone (10 g), Bacto yeast extract (5 g), and NaCl (10 g). M9 salts (1 L) contained Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NH_4Cl (1 g), and NaCl (0.5 g). L- broth[141]

contained Bacto tryptone (10 g), Bacto yeast extract (5 g), and NaCl (10 g) D-glucose (2 g) and L-cysteine (0.06 g) diluted in 1 L water and adjusted to pH 7 with 1 N NaOH before sterilization. M9 salts[141] (1L) contained Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NaCl (0.5 g) and NH_4Cl (1 g). M9 medium contained D-glucose (10 g), $MgSO_4$ (0.12 g), and thiamine hydrochloride (0.001 g) in 1 L of M9 salts. M9 medium (1 L) was supplemented where appropriate with L-phenylalanine, L-tyrosine, L-tryptophan, and L-serine to a final concentration of 40 μg/mL and with *p*-hydroxybenzoic acid, potassium *p*-aminobenzoate, and 2,3-dihydroxybenzoic acid to a final concentration of 10 μg/mL. Antibiotics were added were appropriate to the following final concentration: ampicillin (Ap), 50 μg/mL; tetracycline (Tc), 15μg/mL; chloramphenicol (Cm), 20μg/mL; and kanamycin (Kan), 50 μg/mL. Solutions of inorganic salts, D-glucose, and $MgSO_4$ solutions were autoclaved separately. Isopropyl β-D-thiogalactopyranoside (IPTG), 2-*C*-methyl-D-erythritol, fosmidomycin, amino acid supplements, aromatic vitamins, mevalonic acid, thiamine hydrochloride, ampicillin and kanamycin stock solutions were sterilized through 0.22-μm membranes (Millipore). Chloroamphenicol (Cm) stock solution were prepared in 95% ethanol and tetracycline (Tc) stock solutions were prepared in a 50% aqueous ethanol and used without sterilization. Solutions of supplemental yeast extract (0.2 g/mL) were sterilized through 0.22-μm membranes. Solid medium was generally prepared by addition of 1.5% (w/v) Difco agar to the liquid medium. L-Broth plates contained 1.0% (w/v) Difco agar. Soft agar contained (per L) Bacto tryptone (10 g), Bacto yeast extract (5 g), and Difco agar (5.5 g).

Fermentation medium (1 L) contained K_2HPO_4 (7.5 g), citric acid monohydrate (2.1 g), ammonium iron (III) citrate (0.3 g), and concentrated H_2SO_4 (1.2 mL). The

fermentation medium was adjusted to pH 7.0 by the addition of concentrated NH_4OH prior to autoclaving. Before inoculation of the fermentation medium, the following supplements were added to the fermentation medium (1 L): D-glucose (20 g or 30g), $MgSO_4$ (0.24 g), and trace minerals including $(NH_4)_6(Mo_7O_{24})\cdot 4H_2O$ (0.0037 g), $ZnSO_4 \cdot 7H_2O$ (0.0029 g), H_3BO_3 (0.0247 g), $CuSO_4 \cdot 5H_2O$ (0.0025 g), and $MnCl_2 \cdot 4H_2O$ (0.0158 g). Culturing of SP1.1 and DXS2.1 required addition of L-phenylalanine (0.7 g), L-tyrosine (0.7 g), and L-tryptophan (0.35 g) aromatic vitamins *p*-hydroxybenzoic acid (0.01 g), potassium *p*-aminobenzoate (0.01 g), and 2,3-dihydroxybenzoic acid (0.01 g) to the fermentation medium (1 L). The aromatic amino acids were added to the fermentation medium prior to autoclaving, while sterilized solution of aromatic vitamins were added before inoculation of the fermentation medium. 2-*C*-Methyl-D-erythritol (0.01%) was added to all cultures of DXS2.1/pPV4.230 with the exception of the fermentation medium where 2-*C*-methyl-D-erythritol (0.1 %) was added. Solutions of D-glucose and $MgSO_4$ were autoclaved separately while the trace minerals, 2-*C*-methyl-D-erythritol and aromatic vitamins were filtered through sterile 0.22-μm membranes (Millipore).

Protein Purification and Enzyme Assays

General Information

Cells were harvested at 4000*g* for 5 min at 4°C. Cells lysis was achieved by two passes through a French pressure cell (SLM Aminco) at 16,000 psi. Cellular debris was separated from the lysate by centrifugation (48,000*g*, 20 min, 4°C). Protein purification was a carried out at 4°C. Protein solutions were concentrated by ultrafiltration using

either PM-10 Diaflo membranes (10,000 MWCO) or Centricon concentrators from Amicon. Concentrations of phosphorous containing compounds were determined by the methods developed by separately by Avila and Ames.[142]

Protein concentrations were determined using the Bradford dye-binding method.[143] Protein assay solution was purchased from Bio-Rad. The assay solution was prepared by diluting 20 mL of the Bio-Rad concentrate to 100 mL with water followed by gravity filtration of the resulting solution. Assay solution (5 mL) was added to an aliquot of protein containing solution (diluted to 0.1 mL) and the sample was vortexed. After allowing the color to develop for 5 min, the absorbance at 595 nm of the solution was measured. Protein concentrations were determined by comparison to a standard curve prepared using bovine serum albumin. Protein assay solution was prepared fresh every two days.

Purification of 1-Deoxy-D-Xylulose-5-Phosphate Reductoisomerase

1-Deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) was purified as a 6-His tagged protein from M15[pREP4][pPV3.23A]. Overexpression and purification of DXR has been reported by Kuzuyama.[144] *E. coli* M15 [pREP4][pPV3.23A] was grown at 37°C in 1 L LB (4 x) containing ampicillin and kanamycin. The cells were induced at an OD_{600} of 0.5 by addition of IPTG to a final concentration of 0.01mM. After an additional 4 hr growth at 37°C, the cells were harvested, resuspended in Buffer A [100 mM Tris.Cl, 0.4 mM β-mercaptoethanol, 1.2 mM PMSF, pH 7.5]. The cells were disrupted by passage through a French press, the crude cellular lysate was centrifuged and supernatant was separated from cell debris. A 50% slurry (w/v) of Ni-NTA agarose resin was added

to the crude supernatant (1 mL resin per 4 mL of crude lysate), and the mixture was stirred at 0°C for an hour. The lysate resin slurry was collected in a Pharmacia glass column (100 mL) and washed with a solution containing 25 mM imidazole in 100 mM Tris.Cl (pH7.5, 4 x 25 mL). The 6-His tagged protein was eluted from the column by washing with a solution of 200 mM imidazole in 100 mM Tris.Cl (pH 7.5, 2 x 50 mL). The eluted protein was concentrated to 20 mL using an Amicon ultrafiltration membrane (10,000 MWCO) and dialyzed against 100 mM Tris.Cl (pH 7.5, 1 L). The dialysis was carried out for 24 h with three buffer changes at 1 h, 6 h, and 12 h after the start of the dialysis and finally against 50% glycerol:100 mM Tris.Cl (v/v, pH 7.5, 1 L, 2 h). The enzyme was frozen in liquid N_2 and stored at -80°C for further use.

DAHP Synthase Assay

DAHP synthase activities were measured using the procedure reported by Schoner.[132] Harvested cells were resuspended in 50 mM potassium phosphate (pH 6.5) containing 10 mM PEP and 0.05 mM $CoCl_2$. After the cells were disrupted by passage through a French press, the cellular debris was removed by centifugation. A 1:10 or 1:20 dilution of the lysate was prepared by diluting the cellular lysate in a solution of 50 mM potassium phosphate (pH 7.0), 0.5 mM PEP and 250 mM 1,3-propanediol. A diluted solution of E4P was concentrated to 12 mM, and the pH was adjusted to 7.0 with KOH. Two solutions were prepared and incubated separately at 37 °C for 5 min. The first solution (1 mL, pH 7.0) contained 6 mM E4P, 12 mM PEP, 1 mg ovalbumin and 25 mM potassium phosphate. The second solution was 0.5 mL of diluted lysate. The two solutions were mixed (time = 0) and incubated at 37°C. At regular intervals, 0.15 mL samples were removed from the reaction mixture and quenched with 0.1 mL of 10%

trichloroacetic acid (w/v). Precipitated protein was removed by centrifugation and the DAHP level in each sample was determined using the thiobarbiturate assay.[145]

To a 0.1 mL aliquot of DAHP containing sample was added 0.1 mL of 0.2 M $NaIO_4$ in 8.2 M H_3PO_4, and the mixture was incubated at 37 °C for 5 min. The reaction was quenched by addition of 0.8 M $NaAsO_2$ in 0.5 M Na_2SO_4 and 0.1 M H_2SO_4 (0.5 mL) and vortexed until a dark brown color appeared and dissappeared. This solution was finally treated with 3 mL of 0.04 M thiobarbituric acid in 0.5 M Na_2SO_4 (pH 7.0) and heated at 100 °C for 15 min. The samples were cooled and the pink color was extracted into 4 mL of cyclohexanone. The aqueous and organic layers were separated by centrifugation (2000*g*, 15 min) and the absorbance of the organic layer was measured at 549 nm (ε = 68000 L mol^{-1} cm^{-1}). One unit of DAHP synthase activity was defined as the formation of 1 μmol of DAHP per min at 37 °C.

Transketolase assay

Transketolase activity was assayed using the method of Paoletti.[133] Harvested cells were resuspended in 50 mM potassium phosphate (pH 7.5), 1 mM $MgCl_2$, and 0.2 mM DTT, lysed using a French press and centrifuged to remove cellular debris. A dilute solution of E4P was concentrated to 10 mM and the pH adjusted to 7.0 with KOH. The assay mixture (1 mL) contained 200 mM triethanolamine (pH 7.6), 5 mM $MgCl_2$, 0.1 mM thiamine pyrophosphate, 0.4 mM NADP, 0.4 mM β-hydroxypyruvate, 0.2 mM E4P, 8 units glucose-6-phosphate dehydrogenase, and 4 units phosphoglucose isomerase. The solution was allowed to equilibrate at room temperature for 5 min and the absorbance at 340 nm was monitored for several minutes. After all the unreacted D-glucose 6-

phosphate from the E4P synthesis had reacted, an aliquot of the diluted lysate was added and the reaction monitored at 340 nm for 20-30 min. One unit of transketolase activity is defined as the formation of 1 μmol of NADPH (ε = 6220 L mol^{-1} cm^{-1}) per min.

DXR Enzyme Assay

DXR was assayed in a 1 mL solution of 100 mM Tris.Cl (pH 7.5) containing 1 mM $MnCl_2$, 3 mM of chemically synthesized 1-deoxy-D-xylulose-5-phosphate and 0.15 mM NADPH.[17] The reaction was initiated by addition of enzyme solution to the complete reaction mixture. The oxidation of NADPH (ε = 6220 L mol^{-1} cm^{-1}) was monitored at 340 nm at 37°C. One unit of DXR is defined as the amount of enzyme that causes oxidation of 1 μmol of NADPH per min at 37°C.

1-Deoxy-D-Xylulose-5-Phosphate Synthase Assay

A coupled enzyme assay was employed for measuring DXS activity.[146] The cells were harvested, resuspended in 100 mM Tris.Cl (pH 7.5) containing 5 mM DTT, 0.5 mM thiamine pyrophosphate, 2.5 mM $MgCl_2$ and 1.2 mM PMSF and lysed by passing twice through a French press. The crude lysate was centrifuged to separate cellular debris and the supernatant was used as such for enzymatic analysis. The assay mixture (1mL) consisted of 100 mM Tris.Cl (pH 7.5), 5 mM DTT, 0.5 mM TPP, 2.5 mM $MgCl_2$, 1.2 mM PMSF, 50 mM pyruvate, 100 mM D, L-glyceraldehyde-3-phosphate, 1 mM $MnCl_2$, 0.15 mM NADPH, purified DXR (3 U), and crude DXS lysate. The reaction was initiated by addition of DXS and loss of NADPH was monitored spectrophotometrically at 340 nm at 37°C.

Fed-Batch Fermentations

Fermentations were conducted in a B. Braun M2 culture vessel with a 2 L working capacity. A B. Braun Biostat MD controlled by a DCU-1 supplied environmental conditions. Data was acquired on Dell Optiplex Gs+ 5166M personal computer utilizing B. Braun MFCS/Win software. PID control loops were used to control temperature, pH, and glucose addition. The temperature was maintained at 33 °C, and the pH was maintained at 7.0 by addition of NH_4OH or 2 N H_2SO_4. Except for the initial charging of the fermentor, glucose was added as a 65% (w/v) solution for all fermentation runs. Dissolved oxygen (D.O.) was monitored using a Mettler-Toledo 12 mm sterilizable O_2 sensor fitted with an Ingold A-type O_2 permeable membrane. D.O. was maintained at 10% air saturation throughout the course of the fermentations. Antifoam (Sigma 204) was pumped into the vessel manually as needed.

A typical inoculant was started by introduction of a single colony of the strain under investigation into 5 mL of complete M9 medium with antibiotics and grown at 37 °C with agitation for 12-24 h until the culture was turbid. After this time, the starter culture was transferred to 100 mL of complete M9 medium with antibiotics and incubated at 37 °C and 250 rpm. After an appropriate OD_{600} was reached (2.0-3.0), the inoculants were transferred to the fermentation vessel.

Glucose-Limited Fermentor Conditions

The initial glucose concentration in the fermentation medium was 20 g/L. Three staged methods were used to maintain D.O. levels at 10% air saturation during the course of the run. With the airflow at an initial setting of 0.06 L/L/min, D.O. concentration was

maintained by increasing the impeller speed from its initial set point of 50 rpm to its preset maximum of 1100 rpm. When the impeller reached its preset maximum, the mass flow controller then maintained D.O. levels by increasing the airflow rate from 0.06 L/L/min to a preset maximum of 1.0 L/L/min. At constant impeller speed and constant airflow rate, D.O. levels were finally maintained at 10% air saturation for the remainder of the fermentation by oxygen sensor-controlled glucose feeding. This stage was called the third stage of fermentation. At the beginning of this stage, D.O. levels fell below 10% air saturation due to residual initial glucose in the medium. This lasted 0.5 to 1.5 h before glucose feeding started. PID control parameters were set to 0.0 (off) for the derivative control (τ_D), and 999.9 s (minimum control action) for integral control (τ_I). X_p was set to 950.0% to achieve a K_c of 0.1. The strain was cultured in the fermentation vessel for a total of 48 h. IPTG was first added to the culture when oxygen sensor-controlled feeding was initiated and then subsequently added every six hours until the fermentation run was complete.

Glucose-Rich Fermentor Conditions

The initial glucose in the fermentation medium was 30 g/L. A stainless steel baffle cage consisting of four 1/2" x 5" baffles was placed in the fermentation vessel. Three staged methods were used to maintain the D.O. concentration at 10% air. With the airflow at an initial setting of 0.06 L/L/min, the D.O. concentration was maintained by increasing the impeller speed from its initial set point of 50 rpm to a preset maximum of 900 rpm. With the impeller rate constant at 900 rpm, the mass flow controller then maintained the D.O. concentration by increasing the airflow rate from 0.06 L/L/min to a

preset maximum of 1.0 L/L/min. After the preset maxima of 900 rpm and 1.0 L/L/min were reached, the third stage of the fermentation was initiated in which glucose (65% w/v) was added to the vessel at a rate sufficient to maintain a glucose concentration of 20-30 g/L for the remainder of the run. Airflow was maintained at 1.0 L/L/min, and the impeller was allowed to vary in order to maintain the D.O. concentration at 10% air saturation. The impeller speed typically varied from 900 to 1600 rpm during the remainder of the run. IPTG was first added to the culture at the beginning of oxygen sensor-controlled phase of fermentation and then subsequently added every six hours until the fermentation run was complete

Analysis of Fermentation Broth

Samples (5 mL) of fermentation broth were taken at indicated intervals. Cell densities were determined by dilution of fermentation broth with water (1:100) followed by measurement of absorption at 600 nm (OD_{600}). Dry cell weight (g/L) was obtained using a conversion coefficient of 0.43 g/L/OD_{600}. The remaining fermentation broth was centrifuged to obtain cell-free broth. Solute concentrations in the cell-free broth were determined using ^{1}H NMR. Solutions were concentrated to dryness under reduced pressure, concentrated to dryness one additional time from D_2O, and then redissolved in D_2O containing a known concentration of the sodium salt of 3-(trimethylsilyl) propionic-*2,2,3,3-d*$_4$ acid (TSP). Concentrations were determined by comparison of the integrals corresponding to each compound with the integral corresponding to TSP (δ 0.0 ppm) in the ^{1}H NMR. Compounds were quantified using the following resonances: acyclic 1-deoxy-D-xylulose (δ 4.43, d, 1.5 Hz, 1 H); cyclic 1-deoxy-D-xylulose, (δ 1.49, s, 3 H);

cyclic 1-deoxy-D-xylulose, (δ 1.44, s, 3 H); 2-*C*-methyl-D-erythritol (δ 1.13, s, 3 H); and acetate (δ 1.92, s, 3 H). The concentration of 1-deoxyD-xylulose was obtained by application of the following equation $[DX\ (mM)]_{actual} = 0.82 \times [DX\ (mM)]_{NMR} + 6.8$. This equation was obtained by as follows: A known quantity of 1-deoxy-D-xylulose was obtained by drying a sample of 1-deoxy-D-xylulose under vacuum. This weighed sample was dissolved in 10 mL of D_2O to obtain a stock solution. Various known volumes of the stock solution were concentrated under reduced pressure, redissolved in 1 mL of D_2O containing 10 mM TSP and their ^1H NMR spectra was recored. The total concentration of 1-deoxy-D-xylulose (sum total of concentration (mM) corresponding to each isomer) in each sample was estimated from ^1H NMR and was plotted against the calculated concentration of 1-deoxy-D-xylulose for that sample resulting in the calibration curve. No calibration curve was need in case of 2-*C*-methyl-D-erythritol. Metabolites accumulating in fermentation runs of Chapter 3 were shikimic acid (δ 4.4, t, 1 H), DHS (δ 4.3, d, 1 H), quinic acid (δ 4.16, m, 1 H). Concentrations of shikimic acid and quinic acid derived from their respective ^1H NMR integral values tended to be overestimated and precise concentrations of shikimic acid and quinic acid were calculated by application of the following formulae: $[SA\ (mM)]_{actual} = 0.53 \times [SA\ (mM)]_{NMR} + 0.97$; $[QA\ (mM)]_{actual} = 0.58 \times [QA\ (mM)]_{NMR} + 0.60$. The calibration curves of shikimic acid and quinc acid were obtained in a similar manner as used to obtain the calibration curve of 1-deoxy-D-xylulose. No calibration curve was need in case of DHS.

Genetic Manipulations

General Information

Recombinant DNA manipulations were performed according to procedures outlined by Sambrook.[147] Restriction endonucleases were purchased from Gibco BRL and New England Biolabs. T4 DNA ligase, Klenow fragment and dNTP's were purchased from Invitrogen while calf intestinal alkaline phosphatase was purchased from Roche Molecular Biochemicals. Depending on the selection available, *E. coli* DH5α, JM109 or JWF1 were used for all plasmid constructions. Electrophoresis grade agarose was purchased from Invitrogen. Wizard PCR Prep DNA Purification Kit was obtained from Promega and was used as described by the manufacturer. Prior to use, phenol was distilled and mixed with 0.1% (w/v) 8-hydroxyquinoline. This was followed by extraction with 1 M Tris.HCl (pH 8.0) (twice) and finally extraction with 0.1 M Tris.HCl (pH 8.0) until the pH of the aqueous layer was greater than 7.6. Phenol was stored under an equal volume of 0.1 M Tris.HCl (pH 8.0) at 4°C. SEVAG was prepared by mixing chloroform and isoamyl alcohol (24:1 v/v). TE buffer contained 10 mM Tris.HCl (pH 8.0) and 1 mM Na_2EDTA (pH 8.0). Endostop solution (10X concentrated) was made up of 50% glycerol (v/v), 0.1 M Na_2EDTA, pH 7.5, 1% sodium dodecyl sulfate (SDS) (w/v), 0.1% bromophenol blue (w/v) and 0.1% xylene cyanole FF (w/v) and stored at 4 °C. Prior to use, 1 mL of 10X Endostop was mixed with 0.12 mL of DNase-free RNase. DNase-free RNase was prepared by dissolving 10 mg RNase in 1 mL of 10 mM Tris·HCl (pH 7.5) and 15 mM NaCl. The solution was heated at 100°C for 15 min to inactivate the DNase and stored at –20°C. PCR amplifications were performed as described by Sambrook.[147] Each reaction (0.1 mL) contained 10 mM KCl, 20 mM Tris-

HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, dATP (0.2 mM), dCTP (0.2 mM), dGTP (0.2 mM), dTTP (0.2 mM), template DNA (0.02 μg or 1 μg), 0.5 μM of each primer, and 2 units of Vent polymerase. Primers were synthesized by the Macromolecular Structure Facility at Michigan State University.

E. coli Host Construction

Transduction with P1 phage were carried out using a method modified from Miller.[141] P1 phage lysate was prepared by propagation of phage in the donor strain using the following procedure. Serial dilutions of P1 phage stock (0.1 mL, 10^{-1} to 10^{-5}) in LB were prepared in sterile test tubes (13 x 100 mm). To each tube was added an aliquot (0.1 mL, approx. 5 x 10^8 cells/mL) of overnight culture of the donor strain. Sterile molten soft agar (4 mL, 45 °C) was added, each tube was vortexed, and the contents were poured onto pre-warmed L-broth plates. After the agar had solidified, the plates were incubated at 37 °C until confluent lysis had occurred. Because the multiplicity of the infection is critical to phage generation, confluent lysis was generally observed on only one or two of the plates. L-broth (4 mL) were added to these plates, which was then stored overnight at 4 °C to allow phage particle to diffuse into the supernatant. The L-broth was collected from the plate, transferred to a centrifuge tube and vortexed with chloroform (4 mL) to ensure cell lysis. The solution was centrifuged (2000*g*, 5 min) and the aqueous layer was aliquoted into 1.5 mL microfuge tubes containing 0.2 mL of $CHCl_3$ and stored at 4 °C.

Infection of the recipient strain with phage lysate proceeded as follows. Cells were collected from overnight cultures (5 mL) of the recipient strain by centrifugation

(microfuge, 30 s, 4 °C) and the supernatant was discarded. The cells were resuspended in 2.5 mL of 100 mM $MgSO_4$ and 5 mM $CaCl_2$ and shaken (200 rpm) at 37°C for 20 min to promote aeration of the cells. In the meantime, 0.1 mL serial dilutions (10^{-1} to 10^{-6}) of the phage lysate were prepared in LB in sterile microfuge tubes. An aliquot (0.1 mL) of the aerated recipient cells was added to each of the phage dilution, gently mixed and then incubated at 37°C for 20 min without shaking. Sodium citrate (1 M, 0.2 mL, pH 7.0) was added to each sample to quench absorption of the phage. The cells were harvested (microfuge, 30 s) and resuspended in 0.2 mL of LB medium containing 100 mM sodium citrate. The samples were then incubated at 37 °C for 1 h and plated on LB medium containing the appropriate antibiotic. A sample of cells without added phage and one with phage only were carried through the procedure as a controls.

Large Scale Purification of Plasmid DNA

Purification of plasmid DNA on a large scale followed a modified alkaline alkaline lysis procedure described in Sambrook.[147] LB (500 mL in 2 L Erlenmeyer flask) containing appropriate antibiotics, was inoculated with a single plasmid-containing strain and shaken (250 rpm) at 37°C for 14 h. In cases where the plasmid did not possess an antibiotic marker, 500 mL of M9 minimal medium was used and the culture was shaken (250 rpm) at 37°C for 24 h. The cells were harvested by centrifugation (4000*g*, 5 min, 4 °C) and resuspended in 10 mL of cold GETL solution [50 mM glucose, 20 mM Tris·HCl (pH 8.0), 10 mM Na_2EDTA (pH 8.0)] into which 50 mg of lysozyme had been added immediately prior to use. The suspension was kept at room temperature for 5 min after which it was treated with 20 mL of 1% sodium dodecyl sulfate (w/v) in 0.2 N NaOH.

The thick suspension was gently mixed, kept on ice for 15 min and then treated with 15 mL of cold 3 M KOAc (prepared by mixing 60 mL of 5 M KOAc, 11.5 mL of glacial acetic acid and 28.5 mL of water). The mixture was shaken vigorously, resulting in a thick white precipitate. The sample was stored for 10 min on ice. The cellular debris was removed by centrifugation (48000*g*, 20 min, 4 °C) and the supernatant transferred equally to two clean centrifuge tubes. Isopropanol (0.6 volume) was added to each tube to precipitate DNA. After the samples were stored at room temperature for 15 min, the DNA was recovered by centrifugation (20000*g*, 20 min, 4 °C). The DNA pellet was then rinsed with 70% ethanol and dried.

The isolated DNA pellet was dissolved in 3 mL TE and transferred to a 15 mL Corex tube. To this solution was added 3 mL of cold 5 M LiCl and after thorough mixing, the high molecular weight RNA was removed by centrifugation (12000*g*, 10 min, 4 °C). The clear supernatant was transferred to a fresh Corex tube, treated with equal volume of isopropanol (6 mL) followed by gentle mixing. The precipitated DNA was collected by centrifugation (12000*g*, 10 min, 4 °C), rinsed with 70% ethanol and dried. The dried DNA pellet was dissolved in 0.5 mL of TE containing 10 μg of DNase-free RNase, transferred to a 1.5 mL microcentrifuge tube and stored at room temperature for 30 min. To this solution was added 0.5 mL of 13% PEG-8000 (w/v) in 1.6 M NaCl. The solution was mixed well and centrifuged (microcentrifuge, 5 min, 4 °C) to recover the precipitated DNA. The supernatant was discarded, and the pellet was dissolved in 0.4 mL of TE. The sample was sequentially extracted with phenol (0.4 mL), phenol and SEVAG (0.4 mL each) and finally SEVAG (0.4 mL). After the extraction was complete, the aqueous portion was mixed with 0.1 mL of 10 M NH_4OAc followed by addition of 1

mL of 95% ethanol. The sample was mixed well and left at room temperature for 5 min, resulting in precipitation of DNA. After recovery of the DNA by centrifugation (microcentrifuge, 5 min, 4°C), the pellet was dissolved in 0.2-0.4 mL of TE.

In order to determine the DNA concentration, an aliquot (10 μL) of the DNA solution was diluted to 1 mL in TE and the absorbance at 260 nm was measured relative to TE. The DNA concentration was calculated based on the fact that the absorbance at 260 nm of a 50 μg mL^{-1} of plasmid DNA is 1.0.

Small Scale Purification of Plasmid DNA

A single colony was inoculated into 5 mL of LB containing the appropriate antibiotics and grown overnight at 37 °C. In cases where the plasmid did not possess an antibiotic marker, 5 mL of M9 minimal medium was used and the culture grown for 24 h. Cells were harvested from 3 mL of culture in a 1.5 mL microcentrifuge tube by centrifugation. The cell pellet was resuspended in 0.1 mL of cold GETL solution into which lysozyme (5 mg mL^{-1}) was added immediately before use. The suspension was incubated on ice for 10 min and was then treated with 0.2 mL of 1% sodium dodecyl sulfate (w/v) in 0.2 N NaOH. The mixture was shaken gently and kept on ice for 5-10 min. To this sample was added 0.15 mL of cold 3 M KOAc solution, and the mixture shaken vigorously, resulting in formation of a thick white precipitate. The sample was stored on ice for 5 min, after which the precipitate was removed by centrifugation (microcentrifuge, 20 min, 4°C). The supernatant was transferred to a fresh microcentrifuge tube and extracted with phenol and SEVAG (0.25 mL each). The aqueous layer was transferred to a fresh microfuge tube and mixed well with 1 mL of

95% ethanol. The mixture was kept at room temperature for 5 min, and the precipitated DNA was centrifuged (15 min, room temperature) into a pellet. The DNA pellet was rinsed with 70% ethanol, dried well and redissolved in 50-100 μL of TE.

Purification of Genomic DNA

The strain from which the genomic DNA was to be isolated was cultured at 30°C with shaking at 250 rpm for approximately 12 h. The cells were harvested by centrifugation (4000*g*, 5 min, 4 °C), resuspended in 9.5 mL of TE and transferred to a small (45 mL) centrifuge bottle. SDS (0.5 mL, 10 % w/v) and freshly prepared proteinase K (0.05 mL, 20 mg/mL) were added to the resuspended cells and the sample was incubated at 37 °C for 1 h. Aqueous NaCl (5 M, 1.8 mL) was added to the cell suspension, mixed thoroughly and 1.5 mL of CTAB/NaCl solution (aqueous solution containing 0.041 g/mL of NaCl and 0.1 g/mL of hexadecyltrimethylammonium bromide) was added. After mixing thoroughly, the sample was incubated at 65°C for 20 min. The sample was transferred to two Corex tubes and extracted with an equal volume of SEVAG. After centrifugation (6000*g*, 10 min, 4 °C), the clear, aqueous portion was transferred to fresh Corex tubes and the DNA was precipitated by the addition of 0.6 volumes of isopropanol. All transfers of aqueous layers were carried out using large bore pipette tips to minimize shearing of the genomic DNA. After storage for 2 h at room temperature, threads of DNA were spooled onto a flame-sealed Pasteur pipette and transferred to a single Corex tube containing 70% ethanol. The ethanol was removed, the DNA was dried and resuspended in 1 mL of TE. To this solution, 10 mL of RNase (10 μg/mL) was added and the solution was stored at room temperature for 4 h. After a

combined extraction with phenol (1 mL) and SEVAG (1 mL), 0.1 mL of 3 M NaOAc (pH 5.2) was added to the aqueous layer and mixed thoroughly. DNA was precipitated by adding 95% ethanol (3 mL) and stored at room temperature for 1.5 h. The DNA threads were spooled onto a flame-sealed Pasteur pipette and dipped into 1 mL of 70% ethanol. The spooled DNA was transferred to a 1.5 mL microfuge tube, dried, and redissolved in 0.5 mL of TE. DNA was quantified by measuring absorbance at 260 nm.

Restriction Enzyme Digestion of DNA

Restriction enzyme digests were performed in buffers provided by the enzyme suppliers. A typical restriction enzyme digest contained approximately 1 μg of DNA (in 10 μL of TE), 2 μL of restriction enzyme buffer (10X concentration), 1 μL of bovine serum albumin (BSA) (2 mg/mL), 1 μL of restriction enzyme and 6 μL TE. After incubating the sample at 37 °C for 1-2 h, 2.0 μL of 10X Endostop was added. Restriction fragments were analyzed by agarose gel electrophoresis. For cloning experiments, the reaction was terminated by addition of 1 μL of 0.5 M Na_2EDTA (pH 8.0), followed by extraction with phenol and SEVAG (0.1 mL each). DNA was precipitated by addition of 0.1 volume of 3 M NaOAc (pH 5.2) followed by thorough mixing and addition of 3 volumes of 95% ethanol. Samples were mixed and kept at -78 °C for 3 h. Precipitated DNA was recovered by centrifugation (15 min, 4 °C), treated with 0.1 mL of 70% ethanol and again centrifuged (15 min, 4 °C). DNA was dried and redissolved in TE.

Alternatively the DNA was isolated from the reaction mixture using Zymoclean DNA Clean and Concentrate Kit (Zymo Research) using the manufacturer's protocol.

Agarose Gel Electrophoresis

Agarose gels were run in TAE buffer made up of 40 mM Tris base, 20 mM acetate and 1 mM EDTA (pH 8.0). Gels typically contained 0.7% agarose (w/v) in TAE buffer. Addition of ethidium bromide (0.5 $\mu g\ mL^{-1}$) to the agarose allowed for visualization of DNA under ultraviolet exposure. Two sets of DNA size markers were used to determine the size of DNA fragments. λ DNA digested with *Hind*III resulted in bands of 23.1-kb, 9.4-kb, 6.6-kb, 4.4-kb, 2.3-kb, 2.0-kb, and 0.6-kb. λ DNA digested with *Hind*III and *Eco*RI provided fragments of 21.2-kb, 5.1-kb, 5.0-kb, 4.3-kb, 3.5-kb, 2.0-kb, 1.9-kb, 1.6-kb, 1.4-kb, 0.9-kb, and 0.8-kb.

Isolation of DNA from Agarose

Two methods were used for isolating DNA from agarose gels. The first method involved cutting out the band of agarose containing the desired DNA from the gel and chopping it thoroughly with a razor. The agarose was then transferred to a 0.5 mL microfuge tube packed tightly with glass wool and having an 18 gauge hole at the bottom. The tube was centrifuged for 5 min using a Beckman microfuge to extrude the DNA solution from the agarose into a 1.5 mL microfuge tube. The DNA was precipitated using 3 M NaOAc (pH 5.2, 0.1 vol) and 95% ethanol (2-3 vol) as previously described.

In a second method the Zymoclean DNA Isolation Kit (Zymo Research) was used as indicated by the manufacturer.

Treatment of DNA with Klenow fragment

DNA fragments with recessed 3' termini were modified to blunt end fragments by treatment with the Klenow fragment of *E. coli* DNA polymerase I. Digested DNA (0.8-2 μg) was treated with 2 μL solution containing 25 mM of each of the four dNTP's and 1-2 units of Klenow fragment followed by incubation at rt for 30 min. Since the Klenow fragment works well in the common restriction enzyme buffers, there was no need to purify the DNA after restriction digestion and prior to filling recessed 3' termini. Klenow reactions were quenched by extraction with equal volumes of phenol and SEVAG. DNA was recovered by precipitation as described previously and redissolved in TE. Klenow reactions were quenched by addition of 2 μL of 10X Endostop in cases where the DNA fragments needed to be purified by agarose gel electrophoresis,

Treatment of Vector DNA with Calf Intestinal Alkaline Phosphatase

Following restriction enzyme digestion, plasmid vectors were dephosphorylated to prevent self-ligation. Digested vector DNA was dissolved in TE (88 μL). To this sample was added 10 μL of dephosphorylation buffer (10X concentration) and 2 μL of calf intestinal alkaline phosphatase (2 units). The reaction was incubated at 37 °C for 1 h. The phosphatase was inactivated by the addition of 1 μL of 0.5 M EDTA (pH 8.0) followed by heat treatment (65 °C, 20 min). The sample was extracted with phenol and SEVAG (100 μL each) to remove the protein, and the DNA was precipitated as previously described.

Ligation of DNA

Molar ratios of insert to vector were typically maintained at 3 to 1 during ligations. A typical ligation reaction contained 0.1 μg vector DNA, 0.05 to 0.2 μg insert in a total volume of 7 μL. To this was added 2 μL of T4 ligation buffer (5X concentration) and 1 μL of T4 DNA ligase (2 units). The reaction was incubated at 16 °C for at least 10 h and then used to transform competent cells.

In an alternative method the Fast-link™ DNA Ligation Kit (Epicentre Technologies) was used according to the manufacturer's protocol.

Preparation and Transformation of Competent Cells

Competent cells were prepared using a procedure modified from Sambrook.[152] A single colony was inoculated into 5 mL LB containing the necessary antibiotics. After overnight growth, 1 mL of the culture was transferred to a 500 mL Erlenmeyer flask containing 100 mL LB with the necessary antibiotics. The cells were grown in a gyratory shaker (250 rpm, 37 °C) until they reached the mid-log phase of growth (OD_{600} = 0.4). All manipulation were carried out on ice during the remaining portion of the procedure. The entire culture was transferred to a sterile centrifuge bottle and the cells were harvested by centrifugation (4000*g*, 5 min, 4 °C). The cells were washed with 100 mL of cold 0.9% NaCl (w/v) harvested by centrifugation and then resuspended in 50 mL of cold 100 mM $CaCl_2$. The suspension was kept on ice for 30 min and the cells were then collected by centrifugation (4000*g*, 5 min, 4 °C). The cells were resuspended in 4 mL of cold 15% glycerol in 100 mM $CaCl_2$ (v/v) and aliquots (0.25 mL) were dispensed into sterile microfuge tubes and immediately frozen with liquid nitrogen. Competent

cells were stored at -78 °C and used over a period of at least six months without any noticeable loss in transformation efficiency.

In order to perform a transformation, frozen competent cells were allowed to thaw on ice for 5 min immediately prior to use. Plasmid solution (1 to 10 μL) was added to 0.1 mL aliquot of competent cells and the solution was gently mixed. After standing on ice for 30 min, the cells were heat shocked at 42 °C for 2 min and placed on ice for 1 min. LB (0.5 mL) without antibiotics was added to the cells and the sample incubated (no agitation) at 37°C for 1 h. Cells were harvested by microcentrifugation, resuspended in 0.1 mL of LB and plated on a LB plate containing the appropriate antibiotics. If the transformation was to be plated onto minimal medium plates, the cells were washed once with a solution of M9 inorganic salts (0.5 mL). After resuspension in fresh solution of M9 inorganic salts (0.1 mL), the cells were spread onto the plates. Competent cells, that were not transformed with any DNA were also subjected to the same transformation protocol to confirm the viability of the competent cells.

Transformation was also performed by electroporation using electrocompetent cells. In order to prepare electrocompetent cells, a 500 mL culture of the strain of interest was grown. The cells were grown in a gyratory shaker (250 rpm, 37°C) until they reached an absorbance of 0.6-0.8. The cells were harvested by centrifugation (4000*g*, 5 min, 4°C), washed twice with cold water (350 mL followed by 150 mL) and resuspended in 20 mL of cold aqueous 10% glycerol (v/v). The cells were centrifuged (4000*g*, 5 min, 4°C) and the cell pellet was slowly resuspended with 5 mL of cold aqueous 10% glycerol. The cells were dispensed into sterile microfuge tubes in 0.25 mL aliquots, immediately frozen using liquid nitrogen and stored at –78 °C.

The electroporation was performed in Bio-Rad Gene Pulser cuvettes with an electrode gap of 0.2 cm. The cuvettes were chilled on ice for 5 min prior to use. Frozen electrocompetent cells were thawed on ice for 5 min. Plasmid DNA diluted in sterile water (1-2 μL) was mixed with 0.1 mL of thawed cells in a sterile microfuge tube and the mixture was transferred to the chilled cuvette. The Bio-Rad Gene Pulser was set at 2.5 Kvolts, 25 μF and 200-400 Ohms. The outside surface of the cuvette was wiped clean and it was placed in the sample chamber. A single pulse was applied, the cuvette was removed and 0.5 mL of LB was added to it. The contents of the cuvette were transferred to a 1.5 mL microfuge tube. The cells were incubated at 37°C for 1 h and plated on appropriate selective medium.

CHAPTER 2

Genetic Manipulations

E. coli DXS2.1

E. coli SP1.1 was subjected to P1 phage mediated transduction to transfer the *dxr*::Kan^R locus of *E. coli* DMY1(*11*) into the genome. P1 phage was propagated from *E. coli* DMY1 on L-broth plates containing 0.01% 2-*C*-methyl-D-erythritol. SP1.1*dxr*::Kan^R was selected based on the following phenotype: no growth on M9 medium containing serine; no growth on M9 containing L-phenylalanine, L-tyrosine, L-tryptophan, *p*-hydroxybenzoic acid, potassium *p*-aminobenzoate, and 2,3-dihydroxybenzoic acid and serine; growth on M9 containing L-phenylalanine, L-tyrosine, L-tryptophan, *p*-hydroxybenzoic acid, potassium *p*-aminobenzoate, and 2,3-dihydroxybenzoic acid,

serine, and 0.01% 2-*C*-methyl-D-erythritol; no growth on LB containing tetracycline (Tc), chloramphenicol (Cm) and kanamycin (Kan); growth on LB containing Tc, Cm and Kan and 0.01% 2-*C*-methyl-D-erythritol. SP1.1*dxr*::Kan^R was renamed DXS2.1.

Plasmid pPV2.173 (7.2-kb)

A 1.9-kb fragment encoding the *E. coli dxs* gene was amplified from RB791 genomic DNA using the following primers containing *EcoR*I terminal restriction sequences: 5'-GGAATTCCTGATGAGCTTCGATATTGC and 5'-GGAATTCAGGAG-TGGAGTAGGGATTAT. Ligation of the resulting *dxs* PCR fragment into the *Eco*RI site of pJF118EH afforded 7.2-kb plasmid pPV2.173 in which *dxs* is transcribed in the same orientation as the vector-encoded *tac* promoter.

Plasmid pPV2.248 (9.1-kb)

This 9.1-kb plasmid was constructed by ligation of a 1.9-kb fragment encoding the *E. coli serA* gene into the *BamH*I site of pPV2.173. The 1.9-kb *serA* fragment was isolated from a *Dra*I/*EcoR*V digest of pD2625. After isolation the 1.9-kb fragment by gel purification, the fragment was ligated into pPV2.173 digested with *Bam*H1 followed by filling in the 5' overhangs by treatment with Klenow fragment. The *serA* gene in pPV2.248 is oriented in the opposite direction relative to the *tac* promoter.

Plasmid pPV4.176 (5.3-kb)

The *dxs* locus without its native start codon was amplified by PCR from plasmid pPV2.173 with primers containing terminal *Bam*HI recognition sequence: 5'-

GGGGGATCCAGCTTCGATATTGC and 5'-GGGGGATCCAGGAGTGGAGTAG-GGATTAT. Digestion of the 1.9-kb PCR fragment with *Bam*HI followed by ligation into the *Bam*HI site of pQE30 gave plasmid pPV4.176. The orientation of the *dxs* is in the same direction as the P_{N25} promoter.

Plasmid pPV4.222 (6.5-kb)

The $lacI^Q$ gene was amplified by PCR from plasmid pTrc99A with primers containing *Pst*I recognition sequence: 5'-AACTGCAGATTTACGTTGACACCATCG and 5'-AACTGCAGTTAATTGCGTTGCGCTCAC. The 1.2-kb fragment was then cloned into the *Pst*I site of pPV4.176 to create plasmid 6.5-kb plasmid pPV4.222. The $lacI^Q$ gene is transcribed in the same direction as the *dxs* gene.

Plasmid pPV4.230 (8.2-kb)

This 8.2-kb plasmid was constructed by ligation of 1.7-kb *Sma*I fragment encoding the *serA* gene from pRC55B into the *Sma*I site of pPV4.222. The *ser*A gene is oriented in the same direction as the *dxs* gene.

Plasmid pPV3.23 (4.6-kb)

The *dxr* locus without its native start codon was amplified by PCR from KL3 genomic DNA with the following primers containing terminal *Bam*HI restriction sequences: 5'-GGGGGATCCAAGCAACTCACCATTCTGGGC and 5'-GGGGGATCCGCTTGCGA- GACGCATCACATC. This 1.2-kb PCR fragment was then ligated into the *Bam*HI site of pQE30 to give 4.6-kb plasmid pPV3.23. The orientation of the *dxr* is in the same direction as the P_{N25} promoter.

Plasmid pPV6.044 (11.3-kb)

The 2.2-kb P_{T5} *dxr* fragment was excised from pPV3.23 by *Ssp*I digest and ligated to the *Sma*I site of pPV2.248B. The 11.3-kb plasmid had the P_{T5} *dxr* fragment transcribed opposite to P_{tac}*dxs*.

Plasmid pPV6.185 (4.3-kb)

The P_{aroF} promoter region was amplified by PCR from plasmid pKL5.17A with the following primers containing *Kpn*I and *Xba*I restriction sequences, respectively: 5'-GCGGTACCGAATTCAAAGGGAGTCTA and 5'-GCTCTAGACCTCAGCGAGGAT-GACGT. The 150-bp sequence was digested with *Kpn*I/*Xba*I and ligated into the plasmid pTrc99A digested *Kpn*I/*Xba*I with give pPV6.185 (4.3-kb).

Plasmid pPV6.196 (10.4-kb)

The 6.1-kb fragment harboring the mevalonate genes (*mvk*, *pmd*, *pmk*, orfD and *hmgR*) cluster (MVA) was excised from plasmid pUMV20 by *Hind*III/*Xba*I digest. The 6.1-kb *Hind*III/*Xba*I fragment was then ligated to plasmid pPV6.185 digested with *Hind*III/ *Xba*I to afford a 10.2-kb plasmid pPV6.196.

Plasmid pPV6.213 (13.8-kb)

Plasmid pPV6.196 was digested first with *Hind*III and then partially digested with *Nco*I. The 6.2 kb fragment containing P_{aroF} and MVA was purified by gel electrophoresis. Plasmid pPV4.230 was digested with *Nco*I and partially digested with

*Hind*III and purified by gel electrophoresis. The 6.2-kb P_{aroF} MVA fragment was then ligated to 7.6-kb fragment of pPV4.230 to give plasmid pPV6.213.

Synthetic Procedures

Synthesis of 1-Deoxy-D-Xylulose

(2*R*, 3*R*)-4-[(*t*-Butyldimethylsilyl)oxy-]-2,3-*O*-isopropylidene-D-threitol (2)[72b]

A solution of 2,3-*O*-isopropylidene-D-threitol **1** (36 g, 0.2 mol)[72a] in 200 mL THF was added to a solution of NaH (60% in mineral oil, 9 g, 0.2 mol) in 400 mL dry THF at 0°C over a 5 min period. The reaction was stirred at 0°C for 10 min, warmed up to room temperature and subsequently stirred for an additional 45 min. A solution of *tert*-butyldimethylsilyl chloride (34 g, 0.2 mol) in 200 mL dry THF was slowly added over a period 20 minutes. The reaction mixture was stirred at room temperature for 3 h and poured into a solution of saturated sodium bicarbonate (500 mL). The aqueous layer was separated and extracted with ethyl acetate (3 x 200 mL). The combined organic layers were dried and concentrated to 61 g of yellow oil (80 % yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.02-3.63 (m, 6 H), 1.42 (s, 3 H), 1.40 (s, 3 H), 0.9 (s, 9 H), 0.08 (s, 6 H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 109.0, 80.1, 78.1, 63.6, 62.7, 26.9, 26.8, 25.8, 18.2, -5.5.

(2*R*, 3*R*)-4-[(*t*-Butyldimethylsilyl)oxy-]-2,3-*O*-isopropylidene-D-threose (3)

A solution of **2** (33 g, 0.12 mol) in methylene chloride (300 mL) was added to a solution of Dess-Martin reagent (56 g, 0.13 mol) in methylene chloride (300 mL). The reaction mixture was stirred at room temperature and monitored by TLC for the loss of starting material. After consumption of starting material, the reaction mixture was

poured into a solution of saturated sodium thiosulfate buffered with saturated sodium bicarbonate. The aqueous layer was then extracted with ethyl acetate (3 x 200 mL). The combined organic layers were dried and concentrated to give 29 g yellow oil which was used directly in the next reaction (95% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.77 (d, J =1.5 Hz, 1 H), 4.33 (dd, J = 6 Hz, 1.5 Hz, 1 H), 4.1 (m, 1 H), 3.8 (d, J = 4.5 Hz, 2 H), 1.48 (s, 3 H), 1.42 (s, 3 H), 0.9 (s, 9 H), 0.08 (s, 6 H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 200.8, 111.4, 81.9, 77.5, 62.8, 26.7, 26.2, 25.8, 25.6, 18.2, -5.4.

(3*R*,4*R*)-5-[(*t*-Butyldimethylsilyl)oxy-]-3,4-*O*-isopropylidene-2-pentanone (4)

To a solution of aldehyde **3** (30 g, 0.11 mol) in 550 mL dry THF was added 146 mL of methyl magnesium bromide (3.0 M solution in ether, 0.43 mol) at -78°C. The reaction mixture was slowly allowed to warm to room temperature. The reaction mixture was quenched by slow addition of saturated NH_4Cl (300 mL), the aqueous layer was extracted with ethyl acetate and the combined organic layers were dried and concentrated. The residue was passed through a plug of silica gel and eluted with ethyl acetate/hexane (3:7 v/v), which was concentrated to a yellow oil and carried for further reaction. Dess-Martin reagent (48 g, 0.11 mol) was added to a solution of racemic alcohol (30 g, 0.10 mol) in methylene chloride over a period of 15 minutes. The reaction was stirred overnight, and poured into a solution of sodium thiosulfate buffered with saturated sodium bicarbonate. The aqueous layer was extracted twice with ethyl acetate. The combined organic layers were dried, concentrated and purified by flash chromatography (1:20, ethyl acetate/hexane, v/v) to give 28 g of **4** as a colorless oil (95% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.3 (d, J = 7.8 Hz, 1 H), 4.05 (dt, J = 7.8 Hz, 7.5

Hz) 3.88 (dd, J = 7.5 Hz, 3.3 Hz), 3.76 (dd, J = 7.5 Hz, 3.3 Hz), 2.28 (s, 3 H), 1.4 (s, 3 H), 1.42 (s, 3 H), 0.9 (s, 9 H), 0.08 (s, 6 H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 208.6, 110.7, 81.4, 78.7, 62.9, 26.6, 26.5, 26.3, 25.8, 18.3, -5.3, -5.4.

1-Deoxy-D-xylulose

A solution of the protected ketone **4** (18 g, 0.062 mol) in AcOH/THF/H_2O (3:1:1 v/v/v, 100 mL) was heated at 80°C for 3 h. The reaction was concentrated to dryness, dissolved in 100 mL water and the organic residue was removed by liquid-liquid extraction with ethyl acetate. The aqueous layer was then concentrated to dryness to give 6.9 g of colorless oil (80% yield). ^{1}H NMR (300 MHz, D_2O) Acyclic isomer δ 4.25 (d, J = 3 Hz, 1 H), 4.03 (m, 1 H), 3.52 (m, 2 H), 2.12 (s, 3 H) Cyclic isomer δ 1.37 (s, 3 H), 1.31 (s, 3 H). ^{13}C NMR (75 MHz, D_2O) Acyclic isomer δ 215.8, 79.9, 74.3, 65.0, 28.5. Cyclic isomer, δ 121.9, 105.5, 83.9, 83.5, 78.8, 77.7, 73.3, 26.4, 23.5.

Synthesis of 1-Deoxy-D-Xylulose 5-Phosphate

(2*R*, 3R)-2,3-Dibenzyl-diethyl-D-tartrate (5)

A solution of diethyl-D-tartrate (10 g, 0.048 mol) in 125 mL THF was added to a solution of NaH (60% in mineral oil, 3.8 g, 0.096 mol) in 125 mL dry THF at 0°C over a 5 min period. The reaction was stirred at 0°C for 1 h and $(C_4H_9)_4N^+I^-$ (3.5 g, 0.0096 mol), 18-crown-6 (76 mg, 0.3 mol), and benzyl bromide (11.5 mL, 0.096 mmol) were added. The reaction was quenched with water, extracted with ethyl acetate, dried and concentrated. The residue was purified on a silica gel column (1:9 ethyl acetate/hexane, v/v) to give 6 g of colorless oil (32% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.29 (m, 10

H), 4.87 (d, J = 12.1 Hz, 2 H), 4.45 (d, J = 12.1 Hz, 2 H), 4.39 (s, 2 H), 4.12 (m, 4 H), 1.18 (t, J = 7.1 Hz, 6 H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.1, 136.9, 128.3, 128.2, 127.9, 78.3, 73.1, 61.3, 14.0.

(2*R*, 3*R*)-2,3-Dibenzyl-D-threitol (6)

To a slurry of $LiAlH_4$ (1.3 g, 0.03 mol) in dry THF (80 mL) at 0°C under argon atmosphere was added a solution of **5** (6 g, .015mol) in dry THF (75 mL). The reaction was warmed, stirred for 3 h at room temperature, cooled to 0°C and quenched by slow addition of saturated sodium sulfate till a white granular precipitate developed. The precipitate was filtered, resuspended in with ethyl acetate and filtered again. The combined organic layers were dried and concentrated to afford a yellow oil. The oil was purified by flash chromatography (4:1 ethyl acetate/hexane, v/v) to give 4.3 g of **6** as colorless oil (50% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.34 (m, 10 H), 4.66 (s, 4 H), 3.75 (m, 6 H), 2.57 (br, s, 2 H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 137.8, 128.5, 128.0, 127.9, 78.7, 72.5, 60.7.

(2*R*, 3*R*)-2,3-Dibenzyl-4-dibenzylphospho-D-threitol (7)

To a solution of NaH (60% in mineral oil, 0.4 g, 0.01mol) in 10 mL dry THF at 0°C under argon atmosphere was added a solution of **6** (3.19 g, 0.01 mol) in dry THF (15 mL). The reaction was stirred at 0°C for 30 min and a solution of tetrabenzylpyrophosphate (5.6 g, 0.01 mol) in dry THF was added slowly. After stirring for 10 min at 0°C a white solid precipitated out of the reaction mixture. The reaction mixture was filtered and the precipitate was washed with ether. The combined organic

layers were concentrated and purified by radial chromatography (4 mM silica gel plate, 3:7 ethyl acetate/CH_2Cl_2,v/v) to give 2.9 g of a colorless oil (50 % yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32 (m, 20 H), 5.04 (m, 4 H), 4.61 (m, 4 H), 4.29 (m, 1 H), 4.16 (m, 1 H), 3.77 (m, 2 H), 3.61 (m, 2 H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 137.8, 137.7, 135.67 (d J = 5.7 Hz), 128.5, 128.4, 128.3, 128.0, 127.8, 127.7, 78.1, 77.7, 77.6, 73.0, 72.7, 69.3 (d, J = 5.7 Hz), 66.8 (d, J = 5.7 Hz), 61.1.

(2*S*, 3*R*)-2,3-Dibenzyl-4-dibenzylphospho-D-threose (8)

To a solution protected threitol **7** (1.2 g, 2.13 mmol) in methylene chloride was added Dess-Martin reagent (1.3 g, 3.2 mmol). The reaction was stirred for 5 h at room temperature and poured into a solution of sodium thiosulfate buffered with saturated sodium bicarbonate. The aqueous layer was extracted with ethyl acetate (3 x 50 mL), the combined organic layers was dried concentrated and purified by flash column chromatography (3:7 ethyl acetate/CH_2Cl_2, v/v) to give 0.69 g of colorless oil (60% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.60 (d, J = 1 Hz, 1 H), 7.31 (m, 20 H), 5.01 (m, 4H), 4.55 (m, 4 H), 4.15 (m, 2 H), 3.92 (m, 1 H), 3.84 (dd, J = 3.8 Hz, J = 1 Hz, 1 H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 202.3, 137.0, 136.7, 135.6 (d, J = 6.8 Hz), 128.6, 128.5, 128.4, 128.3, 128.1, 128.0, 127.9, 81.8, 77.1, 76.5, 73.5, 73.1, 69.4 (d, J = 5.7 Hz), 64.8 (d, J = 5.7 Hz). ^{31} P NMR (121 MHz, $CDCl_3$) δ -0.11

(3*S*, 4*R*)-3,4-Dibenzyl-5-dibenzylphospho-2-pentanol

To a solution of aldehyde **8** (0.69 g, 1.22 mmol) in dry THF (6 mL) at -78°C was added methyl magnesium bromide (0.57 mL, 3.0 M solution in ether, 1.72 mmol). The reaction was stirred for 3 h at -78°C, quenched by slow addition of saturated NH_4Cl (5 mL), and the aqueous layer was extracted with ethyl acetate (3 x 10mL). The combined organic layers were dried, and concentrated. The residue was purified by radial chromatography (2 mm silica gel, 1:4 ethyl acetate/CH_2Cl_2, v/v) to give 0.35 g of yellow oil (50% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.27 (m, 20 H), 5.05 (m, 4 H), 4.58 (m, 4 H), 4.21 (m, 2 H), 3.90 (m, 2 H), 3.33 (m, 1 H), 1.19 (d, J = 6.3 Hz, 3 H), 1.12 (d, J = 6.4 Hz, 3 H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.0, 138.0, 137.9, 137.5, 135.8 (d, J = 6.8 Hz), 128.7, 128.7, 128.67, 128.65, 128.60, 128.57, 128.53, 128.50, 128.36, 128.27, 128.13, 128.1, 128., 128.0, 127.9, 127.9, 81.8, 80.8, 78.3 (d, J = 5.7 Hz), 77.9 (d, J = 5.7 Hz), 74.82, 73.66, 73.31, 73.17, 69.50 (d, J = 5.7 Hz), 69.41, 67.35, 67.27 (d, J = 5.6 Hz), 67 (d, J = 5.6 Hz), 66.88, 20.35, 19.91.

(3*S*, 4*R*)-3,4-Dibenzyl-5-dibenzylphopho-2-pentanone (9)

To a solution of racemic alcohol (0.34 g, 0.59 mmol) in methylene chloride (5 mL) was added Dess-Martin reagent. The reaction was stirred at room temperature for 1.5 h and poured into a solution of sodium thiosulfate buffered with saturated sodium bicarbonate. The aqueous layer was extracted with ethyl acetate (3 x 10 mL). The combined organic layers were dried, concentrated and purified by radial chromatography (2 mm silica gel, 1:4 ethyl acetate/CH_2Cl_2, v/v) to give 0.3 g of **9** as a colorless oil (50% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.31 (m, 20 H), 4.99 (m, 4 H), 4.48 (m, 4 H), 4.09

(m, 2H), 3.92 (m, 1 H), 3.85 (d, J = 3.4 Hz, 1 H), 2.09 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 210.30, 137.43, 137.02, 135.85 (d,J = 6.8 Hz), 128.84, 128.73, 128.59, 128.49, 128.39, 128.20, 83.74, 78.40 (d, J = 5.7 Hz), 74.09, 73.73, 69.63, 65.50 (d, J = 5.7 Hz), 27.96.

1-Deoxy-D-xylulose-5-phosphate

To a degassed solution of fully protected phosphate **9** (0.3 g, 0.6 mmol) in ethanol, was added 20% $Pd(OH)_2/C$ (120 mg). The reaction was stirred overnight under an atmosphere of hydrogen. The reaction was filtered through celite and the celite pad was washed with ethanol (3 x 20 mL). The combined organic filtrate was concentrated to a thin film (0.128 g, 100%). ^{1}H NMR (300 MHz, D_2O) δ 4.27 (d, 1 H), 4.19 (t, 1 H, J=6.1 Hz.), 3.81 (m, 2 H), 2.11 (s, 3 H). ^{13}C NMR (75 MHz D_2O) δ 212.08, 76.12, 69.33 (d, J = 5.5 Hz) 65.17 (d, J = 5.6 Hz), 25.15. ^{31}P NMR (121 MHz, D_2O) δ 1.05.

Synthesis of 2-*C*-Methyl-D-Eythritol

3-Methyl-2-butene-1,4-diacetate (10)

A solution of 3-methyl-2(5*H*)-furanone (7 g, 0.071 mol) in dry ether (180 mL) was added to slurry of $LiAlH_4$ (5.4 g, 0.14 mol) in dry ether (180 mL) at 0° C under argon atmosphere. The reaction was stirred at 0°C until all of the starting material had been consumed. The reaction was quenched by slow addition of saturated sodium sulfate until a white granular precipitate formed. The slurry was stirred for 30 min and filtered. The precipitate was resuspended in ether, stirred for 15 min and filtered. The combined organic layers were dried and concentrated to a colorless oil. The oil was dissolved in

pyridine (20 mL) and acetic anhydride (20 mL, 0.21 mol) was added at 0°C. The reaction was stirred at 0°C for 3 h and then concentrated to dryness. The residue was purified by flash column chromatography (1:1, ethyl acetate/hexane, v/v) to yield 7.3 g of a colorless oil (55% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.56 (t, J = 6.9, 1 H), 4.63 (d, J = 6.6 Hz, 4 H), 2.06 (s, 3 H), 2.05 (s, 3 H), 1.81 (s, 3 H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.2, 136.2, 123.8, 62.6, 60.2, 21.4, 20.9, 20.8.

Dihydroxy-2-methyl-butanediacetate

To a solution of diacetate **10** (7.3 g, 0.04 mol) in 400 mL *t*-butanol-water (1:1 v/v) at 0°C was added 55 g of AD-mix-β and methanesulfonamide (3.2 g, 0.04 mol). The reaction was stirred at 0°C for 48 h and quenched by addition of solid sodium sulfite. The aqueous layer was extracted with ethyl acetate (3 x 200 mL). The combined organic layers were dried, concentrated and purified by flash column chromatography (4:1 ethyl acetate/hexane, v/v) to give 7 g of colorless oil (80% yield) as a mixture of 1,4 and 1,3 diacetate. (^{1}H NMR (300 MHz, $CDCl_3$) δ 4.38 (dd, J =9.6 Hz, 2.4 Hz, 1 H), 4.26 (d, J =11.4, 1 H), 4.136 (m,1 H), 4.01 (d, J =11.7 Hz, 1 H), 3.77 (dd, J = 5.7 Hz, 2.7 Hz, 1 H), 2.14 (s, 3H), 2.13 (s, 3 H), 2.11(s, 3 H), 2.05 (s, 3 H) 2.04 (s, 3 H), 1.22 (s, 3 H) 1.11 (s, 3 H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.5, 171.4, 113.8, 73.1, 72.7, 68.3, 65.4, 20.9, 20.8, 19.6.

2-*C*-Methyl-D-erythritol

A solution of the protected diacetates (7 g, 0.03 mol) in methanol was stirred with potassium carbonate (20 g, 0.14 mol) for 2 h at room temperature. The reaction mixture

was filtered and passed through a 100 mL column of Dowex-50 (H^+form). The column was washed with methanol and the combined organic layers were concentrated to dryness to syrup (4 g 100%). ^{1}H NMR (300 MHz, D_2O) δ 3.83 (dd, J = 9 Hz, 2.3 Hz, 1 H), 3.6 (m, 4 H), 1.1 (s, 3 H). ^{13}C NMR (75 MHz, D_2O) δ 77.7, 76.9, 69.0, 64.7, 21.1.

Extraction of 1-Deoxy-D-Xylulose and 2-*C*-Methyl-D-Erythritol from Fermentation Broth

The fermentation broth was centrifuged at 14000*g* for 20 min, and the cells were discarded. The supernatant was filtered through an Amicon ultrafiltration membrane (10000 MWCO), and the clarified filtrate was used for extraction. Clarified filtrate (1200 mL) containing 1-deoxy-D-xylulose (17 g) and 2-*C*-methyl-D-erythritol (2 g) of was concentrated to 600 mL, combined with 100 mL of absolute ethanol and transferred to a liquid/liquid continuous extractor. The aqueous phase was extracted with ethyl acetate (1500 mL) for 24 h, during which time the mixture was stirred to ensure efficient mixing of the phases. After 24 h, the phases were separated, the extractor was charged with fresh ethyl acetate (1500 mL), and 100 mL of ethanol was again added to the aqueous phase. The extraction was carried out for an additional 24 h. After completion of the extraction, the two organic phases were worked up separately. Each was concentrated to dryness, yielding red-brown oil, which was dissolved in water and concentrated to dryness again. Each product was characterized and quantified by ^{1}H NMR. The first extraction yielded a 70% recovery of 1-deoxy-D-xylulose while the second extraction afforded a 28% recovery of 1-deoxy-D-xylulose. The extracts were combined (16.3 g), dissolved in methanol (250 mL), and treated with activated charcoal. The slurry was stirred overnight

at room temperature and filtered through celite. The celite pad was washed with methanol (3 x 50 mL) and the conbined filtrate was concentrated to give a red oil containing 16 g of 1-deoxy-D-xylulose and 1.2 g of 2-*C*-methyl-D-erythritol (98 % recovery).

2-*C*-Methyl-D-erythritol (24 g) was extracted from the fermentation broth of SP1.1/pPV6.044 by the aforementioned procedure for extracting 1-deoxy-D-xylulose. The first extraction yielded 62% recovery of 2-*C*-methyl-D-erythritol while the second extraction afforded a 29% recovery of product. The combined extract containing 22 g of product was dissolved in methanol, treated with activated charcoal, filtered and concentrated to a yellow oil containing 21 g of 2-methyl-D-erythritol (95 % recovery)

Purification of 2-*C*-methyl-D-erythritol

The extracted 2-*C*-methyl-D-erythritol (21 g, 154 mmol) was dried under vacuum overnight, dissolved in dry pyridine (100 mL) and stirred under argon at 0°C. Acetic anhydride (100 mL) was slowly added via an addition funnel at 0°C. The reaction mixture was removed from the ice bath and stirred at room temperature for 3 h. The solvent was removed *in vacuo* and the brown residue was purified by flash column chromatography (4:1 ethyl acetate/hexanes, v/v) to give 40 g of triacetylated methylerythritol as pale yellow oil (95%). The oil was then dissolved in 100 mL of methanol and solid K_2CO_3 (15 g) was added to the stirred solution. The slurry was stirred at room temperature for 3 h and filtered to remove solid. The filtrate was passed through a 300 mL column of Dowex-50 (H^+ form) at 4°C. The column was eluted with 600 mL of methanol and the combined methanolic eluent was concentrated to a red-brown oil.

The residue was dissolved in 50 mL of water and concentrated to dryness. The residue was redissolved in water (100 mL) and adjusted to pH 7. ^{1}H NMR was used to determine the approximate concentration of 2-*C*-methyl-D-erythritol.

Synthesis of Fosmidomycin[89]

Diethyl-(3-bromopropyl)phosphonate (11)

A mixture of triethyl phosphite (50 mL, 0.3 mol) and 1,3-dibromopropane (52.2 mL, 1.5 mol) was stirred at 150°C for 30 min. The bromoethane produced during the course of the reaction was collected via a Dean-Stark trap. The reaction vessel was cooled to room temperature and the reaction mixture was purified by vacuum distillation to give 50 g of diethyl-(3-bromopropyl)phosphonate (64% yield) (bp = 100°C, 0.7 mm Hg). Excess 1,3-dibromopropane was recovered and used in future reactions. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.12 (m, 4 H), 3.48 (t, J =6.3 Hz, 2 H), 2.15 (m, 2 H), 1.9 (m, 2 H), 1.33 (t, J =7 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 61.5 (d, J =6.8 Hz), 33.4 (d, J =18.2 Hz), 25.8 (d, J = 4.5 Hz), 25.1, 23.2, 16.3 (d, J = 5.7 Hz). ^{31}P (121 MHz, $CDCl_3$) δ 31.7

Diethyl(*N*-hydroxylamino)-propylphosphonate (12)

Aqueous NaOH (1.6 N, 150 mL) was slowly added via an addition funnel to a stirred solution of hydroxylamine hydrochloride (109 g, 1.56 mol) in water (200 mL) at 0°C. After addition was complete, 150 mL of methanol was added. The mixture was stirred (10 min) and **11** (50 g, 0.19 mol) was slowly added to the reaction mixture at 0°C. After the addition was complete, the reaction was heated at 45°C for 3 h during which the

reaction mixture clarifies. Methanol was removed *in vacuo* and the solution was adjusted to pH 8 by addition of solid $NaHCO_3$. The solution was washed with benzene (1 x 300 mL, 2 x 200 mL) and then extracted with chloroform (3 x 300 mL). The combined chloroform extracts were dried and concentrated to give 20 g of **12** as a yellow oil (50% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.3 (bs, 2 H), 4.1 (m, 4 H), 3.0 (t, J = 6.3Hz, 2H), 1.8-1.9 (m, 4 H) 1.3 (t, J = 7Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 61.5 (d, J=6.8 Hz), 53.3 (d, J = 16Hz), 23.8, 21.9, 19.8, 16.3 (d, J = 5.8Hz). ^{31}P (121 MHz, $CDCl_3$) δ 32.3

3-(*N*-hydroxylamino)propylphosphonic acid (13)

A solution of **12** (20 g, 0.094 mol) in glacial acetic acid (100 mL) and 40% aqueous HCl (200 mL) was heated to reflux under an argon atmosphere for 12 h. The reaction mixture was cooled to room temperature and concentrated *in vacuo*. The residue was dissolved in water (100 mL) and the solution was adjusted to pH 3.5 with $NaHCO_3$ where upon a white precipitate developed. The precipitate was collected by filtration and dried under vacuum to give 9 g of **13** as an off-white solid (67 % yield). ^{1}H NMR (300 MHz, D_2O) δ 3.35 (t, J = 6.3 Hz, 2 H), 1.98 (m, 2 H), 1.7 (m, 2 H). ^{13}C NMR (75 MHz, D_2O) δ 54.5 (d, J = 16.2), 28.8, 27.0. MP = 159-160 °C

Fosmidomycin

A dry 100 mL round bottom flask equipped with an addition funnel was charged with formic acid (5.5 mL) and chilled to 0°C under argon atmosphere. Acetic anhydride (11 mL, 0.11 mol) was added dropwise into the chilled solution over a period of 15 minutes. After the addition was complete, the reaction mixture was warmed up to 45°C

for 20 min and then cooled to 0°C. A solution of **13** (9 g, 0.063 mol) in formic acid (16.5 mL) was added dropwise into the reaction mixture via a cannula and stirred at 0°C for 20 minutes. The reaction mixture was warmed up to room temperature and stirred for 2 h. The reaction mixture was concentrated to dryness, dissolved in 1 N KOH (55 mL) and concentrated once again. The residual oil was chilled to –20°C to obtain crystals of fosmidomycin (7 g, 70% yield). ^{1}H NMR (300 MHz, D_2O) δ 8.08 (s, 1/2 H) 7.75 (s, 1/2 H), 3.39 (t, J = 6.6 Hz, 2 H), 1.68 (m, 2 H), 1.35 (m, 2 H). ^{13}C NMR (75 MHz, D_2O) δ 159.9, 51.2 (d, J=7.2 Hz), 25.3, 23.6. ^{31}P (121 MHz, $CDCl_3$) δ 25.6. MP = 179-181 °C.

CHAPTER 3

Transport Assays

Cells were harvested (4000*g*, 5 min at 4°C) and the supernatant was discarded. The harvested cells were washed twice by resuspending the cells in a solution of potassium-3-(*N*-morpholino)propanesulfonate (MOPS) buffer (100 mM, pH 7), containing 50 mM K_2SO_4 and 1 mM $MgSO_4$) followed by centrifugation (4000*g*, 5 min at 4°C). The cells were finally resuspended again in the aforementioned buffer to give a final concentration of 2 x 10^9 cells/mL. The cells were equilibrated in this buffer for 10 min at room temperature and the transport was initiated by adding labeled substrate.

For PEP transport assays,[124d] the final concentration of [2-^{14}C]PEP was 50 μM. The assays were performed at room temperature. Aliquots (0.1 mL) were withdrawn at appropriate time intervals, filtered on a 0.45 μm nitrocellulose membrane (Millipore) and washed twice with 5 mL of MOPS buffer (100 mM, pH 7), containing 50 mM K_2SO_4.

The membranes were dried on heat block (70°C) and the amount of radioactivity incorporated into the cells was quantified by a scintillation counter.

Transport assay using labelled [^{33}P]E4P were performed by modification of aforementioned procedure. A 10 mL cell suspension containing 2 x 10^9 cells/mL was added to 25 mL sterile flask and incubated for 10 min at 37°C in water shaker. Assays were initiated by adding labeled [^{33}P]E4P to the cell suspension. Aliquots (0.8 mL) were withdrawn at appropriate time intervals, filtered on a 0.45 μm nitrocellulose membrane (Millipore) and washed twice with 5 mL of buffer without $MgSO_4$. The membranes were dried and the amount of radioactivity incorporated into the cells was quantified by a scintillation counter.

Genetic Manipulation

Plasmid pSP2.054 (10.5-kb)

A 1.6-kb DNA fragment encoding the P_{trc}*uhpt*(D388C) gene sequence was amplified by PCR from plasmid pTrc(hisC_6S-uhpt-D388C) using following primers with *Sma*I recognition sequences: 5'-TCCCCCGGGAGGTCGTAAATCACTGCATAA and 5'-TCCCCCGGGTCACCA GTTACGTTTATGCCA. Plasmid pKD12.138 was digested with *Nco*I and treated with Klenow fragment. The PCR product was digested with *Sma*I and ligated to linearized pKD12.138 to afford a 10.5-kb pSP2.054.

Plasmid pPV5.188 (6.6-kb)

Phosphoglycerate transport genes *pgtP* and *pgtA* were amplified by PCR from *Salmonella choleraesuis* LT2 genomic DNA. The 1.4-kb *pgtP* gene was amplified using

primers 5'-CGCGGTACCAACCTTTAAATTCACCCAAT and 5'-CGAATTCTTATT GCGCGTCC with *Kpn*I and *Eco*RI restriction ends. The 1.2-kb *pgtA* gene was amplified using primers 5'-GGGGTACCGATGAATGTTCTATTTTGCTGA and 5'-CGCGG ATCCAGTAACTGTAATATCAGAA with *Bam*HI and *Kpn*I restriction ends. The PCR products *pgtP* and *pgtA* were digested with *Eco*RI/*Kpn*I and *Kpn*I/*Bam*HI respectively and ligated to pBR322 digested with *Eco*RI and *Bam*HI to give plasmid pPV5.188. The *pgtP* and *pgtA* gene are oriented in the opposite direction.

Plasmid pPV6.030 (7.9-kb)

1.3-kb DNA fragment encoding the kanamycin resistance gene was excised out of pKAD62A by *Bam*HI/*Sph*I digest. Plasmid pPV5.188 digested with *Bam*HI/*Sph*I. Ligation of the two DNA fragments afforded plasmid pPV6.030. The kanamycin gene is oriented in the same direction as the *pgtA* gene.

Plasmid pPV6.074 (13.3-kb)

The 4.3-kb fragment harboring *pgtP, pgtA* and kan^R genes was excised from plasmid pPV6.030 by *Sca*I/*Psh*AI digest. Plasmid pKD12.138 was digested with *Sca*I and purified by gel electrophoresis to give a 9-kb fragment. Ligation of the two blunt end fragments gave plasmid pPV6.074.

Synthetic Procedures

Synthesis of Phosphoenolpyruvate

3-Bromopyruvic acid (14).

A 3-neck flask equipped with a reflux condenser, overhead stirrer and additional funnel was charged with CH_2Cl_2 (190 mL), pyruvic acid (158 ml, 2.27 mol) and sulfuric acid (8 drops). Bromine (115 mL, 2.27 mol) was added dropwise to the stirred solution and the liberated HBr was removed from the system by passing a constant stream of N_2 into a trap containing saturated sodium thiosulfate. After addition of the bromine was complete, the reaction mixture was stirred for an additional hour where upon an oily residue developed. The oil was cooled in an icebath and 40 mL of cyclohexene and 200 mL of ligorin was added. The mixture was stirred for a hour and the supernatant was discarded. The residue was washed with 200 mL ligorin and dried under vacuum (322 g, 90% yield based on pyruvic acid).

Mono-potassium salt of Phosphoenolpyruvate

A 5 L 3-neck flask equipped with an addition funnel and condenser was charged with anhydrous ether (1.8 L) and trimethylphosphite (258 mL, 2.1 mol) under argon atmosphere. A solution of 3-bromopyruvic acid (323 g, 1.9 mol) in ether (555 mL) was added at a rate sufficient to maintain ether at reflux. The reaction mixture was stirred overnight and concentrated to brown oil. The residue dimethylphosphoenolpyruvic acid **15** was dissolved in water (720 mL) and stirred for 15 h during which the methyl esters hydrolyzed. The solution was cooled in an ice-bath and then solid KOH (115 g, 2.1 mol) was added to adjust the solution to pH 2.5. Chilled absolute ethanol (1.5 L) was then

added to precipitate PEP. The precipitate was collected by filtration and dried under vacuum to give the mono potassium salt of PEP as a white, fluffy powder (180 g, 60% yield based on 3-bromopyruvic acid) ^{1}H NMR (300 MHz, D_2O) δ: 5.85 (m, 1 H). 5.51 (t, J = 2.1 Hz, 1 H). ^{13}C NMR (75 MHz, D_2O) δ: 169.5 (d, J = 7 Hz), 147.5 (d, J = 7 Hz), 111.9 (d, J = 4.3 Hz). ^{31}P NMR (121 MHz, D_2O) δ: -3.6 .

Synthesis of Erythrose 4-Phosphate

(*E*)-2-(3-Benzyloxy-propenyl)-[1,3]dioxalane (19)

A solution of (*E*)-4-benzyloxy-2-butenal **18** (89 g, 0.5 mol), benzene (1.6 L, dried over 3Å molecular sieves for 12 h), ethylene glycol (31 mL, 0.55 mol) and pyridinium-*p*-toluenesulfonic acid (75 mg) was refluxed under argon atmosphere for 18 h with continuous azeotropic removal of water. The reddish brown mixture was cooled to room temperature and washed with 10% $NaHCO_3$ (2 x 1 L), water (2 x 1 L), and dried. Removal of solvent left a dark brown residue which was vacuum-distilled to give 70 g of pure **19** (85% yield). (bp =141°C, 0.1 mm Hg). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.25 (m, 5 H), 6.02 (dt, J = 15.6 Hz, 6.3 Hz, 1 H), 5.77 (dt, 15 Hz, 9 Hz, 1 H), 5.27 (d, 6.3 Hz, 1 H), 4.51 (s, 2 H), 4.06 (dd, J = 3.6 Hz, 1.2 Hz, 2 H), 3.94 (m, 2 H), 3.88 (m, 2 H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 137.9, 132.6, 128.2, 128.1, 127.6, 127.5, 127.4, 103.1, 72.1, 69.2, 64.8. HRMS (FAB) calculated for $C_{13}H_{16}O_3$ 220.1099, found 220.1102.

2-(3-Benzyloxymethyl-oxirnyl-)-[1.3]dioxolane (20)

A solution of alkene **19** (23 g, 0.1 mol) in methylene chloride (150 mL) at 0°C was added a solution of *m*CPBA (29 g, 0.17 mol) in methylene chloride (150 mL) over a

period of 15 min. The reaction was stirred at room temperature over a period of 2 days during which *m*-chlorobenzoic acid, precipitates out as a white solid. The reaction was filtered through celite, washed with 10% $Na_2S_2O_3$ (2 x 100 mL), 10% $NaHCO_3$ (2 x 100 mL) water (2 x 100 mL) and finally with brine (2 x 100 mL). The organic layer was dried and concentrated, and the residue was purified by flash column chromatography (7:3 ethylacetate/hexane, v/v) to give 18 g of **20** as colorless oil (85% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.3 (m, 5 H), 4.89 (d, J = 3.9 Hz, 1 H), 4.59 (d, J = 4.5 Hz, 2 H), 4.02 (m, 2 H), 3.9 (m, 2 H), 3.81 (dd, J = 9 Hz, 3 Hz, 1 H), 3.5 (m, 1 H), 3.24 (m, 1 H), 3.02 (dd, J = 2.1 Hz, 2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ 137.7, 128.3,128.7, 113.8, 102.4, 73.1, 69.1, 65.4, 65.2, 54.5, 53.8. HRMS (FAB): calculated for $C_{13}H_{16}O_4$ 236.1049, found 236.1053.

4-Hydroxy-2,3-epoxy-butanal-1,3-dioxalane (21)

A mixture of **20** (18 g) and 20% $Pd(OH)_2$/C (100 mg) in absolute ethanol (50 mL) was shaken under an atmosphere of hydrogen at 50 psi for 20 min. The mixture was filtered through a celite pad and rinsed with ethanol (3 x 30 mL). The filtrate was concentrated to pure colorless oil (100%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.8 (d, J = 3.9 Hz, 1 H), 3.9 (m, 5 H), 3.66 (dd, J = 8.7 Hz, 4.2 Hz, 1 H), 3.18 (m, 1 H), 3.11 (m, 1H). ^{13}C NMR δ 102.4, 65.4, 65.2, 60.7, 55.2, 54.4. HRMS (FAB) calculated for $C_6H_{10}O_4$ 146.0579, found 146.0585

(3-[1,3]Dioxanyl-2,3-dihydroxypropylphosphate (22)

A solution of oxirane **21** (7.3 g, 50 mmol) and potassium phosphate (58 g, 275 mmol) in 500 mL water : dioxane (1:1 v/v) was heated to reflux for 3 h. The solution was cooled and poured dropwise to stirred solution of methanol (1 L). The solution was kept at -20°C for 2 days and precipitated inorganic phosphate was filtered away and the filtrate was concentrated under reduced pressure to 200 mL. The solution was loaded onto a 400 mL AG-1X8 column (washed with 2 L of 2 N NaOH and then with 4 L of water) and eluted under a linear gradient of (0-1 M) water: triethylammonium bicarbonate (2 L) at 4°C. The fractions that tested positive for inorganic phosphate were excluded. The fractions that tested positive for organic phosphate were combined, lyophilized to a syrup, redissolved in water (25 mL) and passed through a small column of Dowex-50 (H^+) at 4°C and washed with water. The eluent was lyophilized to a colorless syrup (80% yield). ^{1}H NMR (300 MHz, D_2O) δ 4.9 (d, J = 1.8 Hz, 1 H) 3.88-3.75 (m, 6 H), 3.4 (m, 1 H), 3.5 (dd, J = 6.3 Hz, 2.1 Hz, 1 H). ^{13}C NMR (75 MHz, D_2O) δ 104.8, 73.03 (d, J= 8 Hz), 72.2, 68.8(d, J = 4.9 Hz), 68.08, 65.2. ^{31}P NMR δ 1.07

D,L-Erythrose 4-phosphate. A solution of protected erythrose-4-phosphate **22** and Dowex-50 (H^+ form) in water was heated at 50°C for 3 h. The progress of the reaction was monitored by transketolase assay. The solution was cooled, filtered and the residue was washed with water. The filtrate was concentrated to desired amount and the concentration of erythrose 4-phosphate was then determined by transketolase assay. The authenticity of the product was also confirmed by DAHP synthase assay.

[^{33}P]D,L-Erythrose 4-Phosphate. To a stirred solution of oxirane **21** (0.2 g, 1.36 mmol) and K_3PO_4 (1.45 g, 6.8 mmol) in 14 mL water : dioxane (1:1 v/v) was added [^{33}P]H_3PO_4 (0.1 mL, 1 mCi) and the mixture was heated to reflux for 3 h. The solution was cooled and poured dropwise in a stirred solution of methanol (100 mL). The solution was kept at -20°C for 2 days and the precipitated inorganic phosphate was filtered and the filtrate was distilled. The solution was loaded onto a 50 mL AG-1X8 column and eluted with water (100 mL) followed by 100 mL elution of 0.1 M, 0.2 M, 0.5 M and finally 1 M triethylammonium bicarbonate. The fractions that tested positive for organic phosphate were combined, lyophilized to a syrup and redissolved in water (10 mL). The solution was stirred with 2 g Dowex-50 (H^+ form) in a ice-bath, filtered, washed with water and lyophilized to colorless syrup. The protected labelled E4P was dissolved in water (10 mL) and heated with 2 g Dowex-50 (H^+ form) in a 20 mL vial following the aforementioned procedure. The concentration of E4P was measured by transketolase assay. The exact concentration of the radioactivity incorporated could not be ascertained due to lack of [^{33}P] standard.

References.

[1] Thomas, S. M.; DiCosimo, R.; Nagarajan, A. *Trends Biotechnol.* **2002**, *20*, 238-242.

[2] *Biobased Industrial Products: Priorites for Research Commercialization.* National Academy Press. **2000**.

[3] a) Sanford, K.; Soucaille, P.; Whited, G.; Chotani, G. *Curr. Opin. Microbiol.* **2002**, *5*, 318-322. b) Rohlin, L.; Oh, M. K.; Liao, J. C. *Curr. Opin. Microbiol.* **2001**, *4*, 330-335. c) Chotani, G.; Dodge, T.; Hsu, A.; Kumar, M.; LaDuca, R.; Trimbur, D.; Weyler, W.; Sanford, K. *Biochim. Biophys. Acta-Protein Struct. Molec. Enzym.* **2000**, *1543*, 434-455.

[4] Porter, J. W.; Spurgeon, S. L. *Biosynthesis of Isoprenoid Compounds*; Wiley: New York, 1981

[5] a) Mann, V.; Harker, M.; Pecker, I.; Hirschberg, J. *Nat. Biotechnol.* **2000**, *18*, 888-892. b) Lagarde, D.; Beuf, L.; Vermaas, W. *Appl. Environ. Microbiol.* **2000**, *66*, 64-72.

[6] a) Lewinsohn, E.; Schalechet, F.; Wilkinson, J.; Matsui, K.; Tadmor, Y.; Nam, K. H.; Amar, O.; Lastochkin, E.; Larkov, O.; Ravid, U.; Hiatt, W.; Gepstein, S.; Pichersky, E. *Plant Physiol.* **2001**, *127*, 1256-1265. b) Lucker, J.; Bouwmeester, H. J.; Schwab, W.; Blaas, J.; van der Plas, L. H. W.; Verhoeven, H. A. *Plant Journal* **2001**, *27*, 315-324.

[7] Wheeler, A. L.; Long, R. M.; Ketchum, R. E.; Rithner, C. D.; Williams, R. M.; Croteau, R. *Arch. Biochem. Biophys.* **2001**, *390*, 265-278.

[8] a) Sies, H.; Stahl, W. *Am. J. Clin. Nutr.* **1995**, *62*, s1315-s1321. b) van Poppel, G.; Goldbohm,R. A. *Am. J. Clin. Nutr.* **1995**, *62*, s1393-s1402.

[9] Knudsen, C. A.; Tappel, A. L.; North, J. A. *Free Radic. Biol. Med.* **1996**, *20*, 165-173.

[10] Cofrancesco, A. J. In *Kirk-Othmer Encyclopedia of Chemical Technology*, 4th ed.; Kroschwitz, J. I., Howe-Grant, M., Eds.; Wiley: NY, 1997; Vol. 8, p. 801-803.

[11] a) Bach, T. J. *Lipids* **1995**, *30*, 191-202. b) Bach, T. J. *Lipids* **1986**, *21*, 82-88. c) Bach, T. J.; Boronat, A.; Campos, N.; Ferrer, A.; Vollack, K. U. *Crit. Rev. Biochem. Mol. Biol.* **1999**, *34*, 107-122.

[12] a) Rohmer, M.; Knani, M.; Simonin, P.; Sutter, B.; Sahm, H. *Biochem. J.* **1993**, *295*, 517-524. b) Schwender, J.; Seemann, M.; Lichtenthaler, H. K.; Rohmer, M. *Biochem J.* **1996**, *316 (1)*, 73-80. c) Rohmer, M.; Seemann, M.; Horbach, S.; BringerMeyer, S.; Sahm, H. *J. Am. Chem. Soc.* **1996**, *118*, 2564-2566.

[13] a) Lois, L. M.; Campos, N.; Putra, S. R.; Danielsen, K.; Rohmer, M.; Boronat, A. *Proc Natl Acad Sci U S A* **1998**, *95*, 2105-2110. b) Sprenger, G. A.; Schörken, U.; Wiegert, T.; Grolle, S.; De Graaf, A. A.; Taylor, S. V.; Begley, T. P.; Bringer-Meyer, S. Sahm, H. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 12857-12862. c) Lange, B. M., Wildung, M. R., McCaskill, D. Croteau, R. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 2100-2104. d) Kuzuyama, T.; Takagi, M.; Takahashi, S.; Seto, H. *J. Bacteriol.* **2000**, *182*, 891-897.

[14] Araki, N.; Kusumi, K.; Masamoto, K.; Niwa, Y.; Iba, K. *Plant Physiol.* **2000**, *108*, 19-24. b) Estevez, J. M.; Cantero, A.; Romero, C.; Kawaide, H.; Jimenez, L. F.; Kuzuyama, T.; Seto, H.; Kamiya, Y.; Leon, P. *Plant Physiol.* **2000**, *124*, 95-103. c) Estevez, J. M.; Cantero, A.; Reindl, A.; Reichler, S.; Leon, P. *J Biol Chem* **2001**, *276*, 22901-22909.

[15] Hahn, F. M.; Eubanks, L. M.; Testa, C. A.; Blagg, B. S.; Baker, J. A.; Poulter, C. D. *J. Bacteriol.* **2001**, *183*, 1-11.

[16] a) Takahashi, S.; Kuzuyama, T.; Watanabe, H.; Seto, H. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 9879-9884. b) Kuzuyama, T.; Takahashi, S.; Watanabe, H.; Seto, H. *Tetrahedron Lett.* **1998**, *39*, 4509-4512. c) Kuzuyama, T; Takahashi, S; Takagi, M.; Seto, H. *J. Biol. Chem.* **2000**, *26*, 19928-19932.

[17] a) Rohdich, F., Wungsintaweekul, *J.*, Fellermeier, M., Sagner, S., Herz, S., Kis, K., Eisenreich, W., Bacher, A. Zenk, M. H. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 11758-117563. b) Kuzuyama, T.; Takagi, M.; Kaneda, K.; Dairi, T.; Seto, H. *Tetrahedron Lett.* **2000**, *41*, 703-706. c) Richard, S. B.; Bowman, M. E.; Kwiatkowski, W.; Kang, I.; Chow, C.; Lillo, A. M.; Cane, D. E.; Noel, J. P. *Nat. Struct. Biol.* **2001**, *8*, 641-648.

[18] a) Kuzuyama, T.; Takagi, M.; Kaneda, K.; Watanabe, H.; Dairi, T.; Seto, H. *Tetrahedron Lett.* **2000**, *41*, 2925-2928. b) Rohdich, F.; Wungsintaweekul, J.; Luttgen, H.; Fischer, M.; Eisenreich, W.; Schuhr, C. A.; Fellermeier, M.; Schramek, N.; Zenk, M. H.; Bacher, A. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 8251-8256.

[19] a) Takagi, M.; Kuzuyama, T.; Kaneda, K.; Watanabe, H.; Dairi, T.; Seto, H. *Tetrahedron Lett.* **2000**, *41*, 3395-3398. b) Herz, S.; Wungsintaweekul, J.; Schuhr, C. A.; Hecht, S.; Luttgen, H.; Sagner, S.; Fellermeier, M.; Eisenreich, W.; Zenk, M. H.; Bacher, A.; Rohdich, F. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 2486-2490. d) Richard, S. B.; Ferrer, J. L.; Bowman, M. E. ; Lillo, A. M.; Tetzlaff, C. N.; Cane, D. E.; Noel, J. P.. *J. Biol. Chem.* **2002**, *277*, 8667-8672.

[20] a) Ogrel, O. D.; Fegeding, K. v.; Kharatian, E. F. Sudarikov, A. B.; Ostrovsky, D. M. *Curr. Microbiol.* **1996**, *32*, 225-228. b) Ostrovsky, D.; Diomina, G.; Lysak, E.; Matveeva, E.; Ogrel, O.; Trutko, S. *Arch Microbiol* **1998**, *171*, 69-72. c) Lysak, E. I.;

Demina, G. R.; Matveeva, E. V.; Ogrel, O. D.; Trutko, S. M.; Ostrovskii, D. N. *Microbiology* **1999**, *68*, 1-9.

[21] a) Hecht, S.; Eisenreich, W.; Adam, P.; Amslinger, S.; Kis, K.; Bacher, A.; Arigoni, D.; Rohdich, F. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 14837-14842. b) Seemann, M.; Bui, B. T. S.; Wolff, M.; Tritsch, D.; Campos, N.; Boronat, A.; Marquet, A.; Rohmer, M. *Angew. Chem.-Int. Edit.* **2002**, *41*, 4337-4339.

[22] a) Rohdich, F.; Hecht, S.; Gartner, K.; Adam, P.; Krieger, C.; Amslinger, S.; Arigoni, D.; Bacher, A.; Eisenreich, W. *Proc. Nat. Acad. Sci. USA* **2002**, *99*, 1158-1163. b) Adam, P.; Hecht, S.; Eisenreich, W. G.; Kaiser, J.; Grawert, T.; Arigoni, D.; Bacher, A.; Rohdich, F. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 12108-12113. c) Rohdich, F.; Zepeck, F.; Adam, P.; Hecht, S.; Kaiser, J.; Laupitz, R.; Grawert, T.; Amslinger, S.; Eisenreich, W.; Bacher, A.; Arigoni, D. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 1586-1591.

[23] Hahn, F. M.; Hurlburt, A. P.; Poulter, C. D. *J. Bacteriol.* **1999**, *181*, 4499-4504.

[24] a) Hill, R. E.; Sayer, B. G.; Spenser, I. D. *J. Am. Chem. Soc.* **1989**, *111*, 1916-1917. b) Therisod, M.; Fischer, J. C.; Estramareix, B. *Biochem. Biophys. Res. Commun.* **1981**, *98*, 374-379.

[25] a) Begley, T. P. *Nat. Prod. Rep.* **1996**, *13*, 177-185. b) Begley, T. P.; Downs, D. M.; Ealick, S. E.; McLafferty, F. W.; Van Loon, A.; Taylor, S.; Campobasso, N.; Chiu, H. J.; Kinsl , C.; Reddick, J. J.; Xi, J. *Arch. Microbiol.* **1999**, *171*, 293-300.

[26] a) Kennedy, I. A.; Hill, R. E.; Pauloski, R. M.; Sayer, B. G.; Spenser, I. D. *J. Am. Chem. Soc.* **1995**, *117*, 1661-1662 b) Hill, R. E.; Himmeldirk, K.; Kennedy, I. A.; Pauloski, R. M.; Sayer, B. G.; Wolf, E.; Spenser, I. D. *J. Biol. Chem.* **1996**, *271*, 30426-30435. c) Zhao, G.; Winkler, M. E. *FEMS Microbiol. Lett.* **1996**, *135*, 275-280. d) Drewke, C.; Klein, M.; Clade, D.; Arenz, A.; Müller, R.; Leistner, E. *FEBS Lett.* **1996**, *390*, 179-182.

[27] a) Jomaa, H.; Wiesner, J.; S erbr , S.; Altincicek, B.; Weidemeyer, C.; Hintz, M.; Turbachova, I.; Eberl, M.; Zeidler, J.; Lichtenthaler, H. K.; Soldati, D.; Beck, E. *Science* **1999**, *285*, 1573-1576. b) Jomaa, H.; Feurle, J.; Luhs, K.; Kunzmann, V.; Tony, H. P.; Herderich, M.; Wilhelm, M. *FEMS Immunol. Med. Microbiol.* **1999**, *25*, 371-378. c) Altincicek, B.; Moll, J.; Campos, N.; Foerster, G.; Beck, E.; Hoeffler, J. F.; Grosdemange-Billiard, C.; Rodriguez-Concepcion, M.; Rohmer, M.; Boronat, A.; Eberl, M.; Jomaa, H. *J. Immunol.* **2001**, *166*, 3655-3658.

[28] a) Jomaa, H.; Wiesner, J.; S erbr , S.; Altincicek, B.; Weidemeyer, C.; Hintz, M.; Turbachova, I.; Eberl, M.; Zeidler, J.; Lichtenthaler, H. K.; Soldati, D.; Beck, E.. *Science* **1999**, *285*, 1573-1576. b) Lell, B.; Ruangweerayut, R.; Wiesner, J.; Missinou, M. A.; Schindler, A.; Baranek, T.; Hintz, M.; Hutchinson, D.; Jomaa, H.; Kremsner,

P. G. *Antimicrob. Agents Chemother.* **2003**, *47*, 735-738. c) Lell, B.; Ruangweerayut, R.; Wiesner, J.; Missinou, M. A.; Schindler, A.; Baranek, T.; Hintz, M.; Hutchinson, D.; Jomaa, H.; Kremsner, P. G. *Antimicrob. Agents Chemother.* **2003**, *47*735-738.

[29] Rodriguez-Concepcion, M.; Boronat, A. *Plant Physiol* **2002**, *130*, 1079-1089.

[30] a) Haslam, E. *The Shikimate Pathway*; John Wiley Sons: New York, 1974. b) Bentley, R. *Crit. Rev. Biochem. Mol. Biol.* **1990**, *25*, 307. c) Herrman, K. M. In *Amino Acids: Biosynthesis Genetic Regulation*; Herrmann, K. M.; Somerville, R. L., Eds.; Addison-Wesley: Reading, 1983: p. 301.

[31] Srinivasan, P. R.; Rothchschild, J.; Sprinson, D. B. *J. Biol. Chem.* **1963**, *238*, 3176.

[32] a) Haslem, E.; Smith, B. W.; Turner, M. J. *J. Chem. Soc., Perkin Trans. 1* **1995**, 52. b) Duncan, K.; Chaudhuri, S.; Campbell, M. S.; Coggins, J. R. *Biochem. J.* **1986**, *238*, 475-486.

[33] a) Anton, I. A.; Cogins, J. R. *Biochem. J.* **1988**, *249*, 319-326. b) Chaudhuri, S.; Coggins, J. R. *Biochem. J.* **1985**, *226*, 217-233.

[34] a) Defeyter, R. C.; Pittard, J. *J. Bacteriol.* **1986**, *165*, 226-232. b) Defeyter, R. C.; Pittard, J. *J. Bacteriol.* **1986**, *165*, 331-336. c) Defeyter, R. C.; Pittard, J. *J. Bacteriol.* **1986**, *165*, 233-239.

[35] Løbner-Olesen, A.; Marinus, M. G. *J. Bacteriol.* **1992**, *174*, 525.

[36] Duncan, K.; Coggins, J. R. *Biochem. J.* **1986**, *234*, 49-57. b) Duncan, K.; Lewendon, A.; Coggins, J. R. *FEBS Lett.* **1984**, *165*, 121-127.

[37] White, P. J.; Millar, G.; Coggins, J. R. *Biochem. J.* **1988**, *251*, 313-322.

[38] Held, M.; Schmid, A.; van Beilen, J. B.; Witholt, B. *Pure Appl. Chem.* **2000**, *72*, 1337-1343.

[39] a) Ikeda, M. *Adv. Biochem. Eng. Biotechnol.* **2003**, *79*, 1-36. b) Kimura, E. *Adv. Biochem. Eng. Biotechnol.* **2003**, *79* 37-58. c) Pfefferle, W.; Mockel, B.; Bathe, B.; Marx, A. *Adv. Biochem. Eng. Biotechnol.* **2003**, *79* 59-112. d) Muller, U.; Huebner, S. *Adv. Biochem. Eng. Biotechnol.* **2003**, *79* 137-172.

[40] a) Saito, Y.; Ishii, Y.; Hayashi, H.; Imao, Y.; Akashi, T.; Yoshikawa, K.; Noguchi, Y.; Soeda, S.; Yoshida, M.; Niwa, M.; Hosoda, J.; Shimoura, K. *Appl. Environ. Microbiol.* **1997**, *63*, 454-460. b) Saito, Y.; Ishii, Y.; Hayashi, H.; Yoshikawa, K.; Noguchi, Y.; Yoshida, S.; Soeda, S.; Yoshida, M. *Biotechnol. Bioeng.* **1998**, *58*, 309-315. c) Estell, D. A.; Light, D. R.; Rasteter, W. H.; Lazarus, R. A.; Miller, J. V. *U. S Patent* 4757012, **1985** d) Anderson, S.; Lazarus, R. A.; Miller, H. I.; Stafford, R. K.

U. S Patent 5032514 **1991**.

[41] a) Ensley, B. D.; Ratzkin, B. J.; Osslund, T. D.; Simon, M. J.; Wackett, L. P.; Gibson, G. T. *Science* **1983**, *222*, 167-169. b) Murdock, D.; Ensley, B. D.; Serdar, C.; Thalen, M. *Biotechnology* **1993**, *11*, 381-386. c) *U. S. Patent* 5866396.

[42] a) Cameron, D. C.; Altaras, N. E.; Hoffman, M. L.; Shaw, A. J. *Biotechnol. Prog.* **1998**, *14*, 116-125. b) *WO* 9821229.

[43] a) Weissermel, K.; Arpe, H.-J. *Industrial Organic Chemistry, 3rd ed.*; VCH; New York, 1997: p. 310-335. b) O' Connor, S. R.; Farmer, P. B.; Lauder, I. *J. Pathol.* **1999**, *189*, 448. c) Farris, G. M.; Everitt, J. I.; Irons, R.; Popp, J. A. *Fundam. Appl. Toxicol.* **1993**, *20*, 503. d) Huff, J. E.; Haseman, J. K.; DeMarini, D. M. *Environ. Health Perspect.* **1989**, *82*, 125. e) *Trade Secrets, A Moyer Report*; http://www.pbs.org/tradesecrets/transcript.html. f) Noah, T. *Wall Street J.* **1994**, B2 (March 2); g) *Washington Post* **1994**, A3 (March 2); h) Ember, L. *Chem. Eng. News* **1994**, *72*, 4.

[44] a) Richman, J. E.; Chang, Y. C.; Kambourakis, S.; Draths, K. M.; Almy, E.; Snell, K. D.; Strasburg, G. M.; Frost, J. W. *J. Am. Chem. Soc.* **1996**, *118*, 11587-11591. b) Chang, Y.-C.; Almy, E. A.; Blamer, G. A.; Gray, J. I.; Frost, J. W.; Strasburg, G. M.; *J. Agric. Food Chem.*; **2003**; In press

[45] a) Kambourakis, S.; Draths, K. M.; Frost, J. W. *J. Am. Chem. Soc.* **2000**, *122*, 9042-9043. b) Kambourakis, S.; Frost, J. W. *J. Org. Chem.* **2000**, *65*, 6904-6909.

[46] a) Li, K.; Mikola, M. R.; Draths, K. M.; Worden, R. M.; Frost, J. W. *Biotechnol. Bioeng.* **1999**, *64*, 61-73. b) Yi, K.; Li, K.; Draths, K. M.; Frost, J. W. *Biotechnol. Prog.* **2002**, *18*, 1141-1148. c) Li, W.; Xie, D. M.; Frost J. W. Unpublished results.

[47] Davis, D.D.; Kemp, D. R. In *Kirk-Othmer Encyclopedia of Chemical Technology*, 4th ed.; Kroschwitz, J. I., Howe-Grant, M., Eds.; Wiley: New York, 1991; Vol. 1 pp 466-493.

[48] Stadehofer, J. W. *Industrial Aromatic Chemistry*; Springer Verlag: New York ,1988

[49] a) Thiemens, M. H.; Trogler, W. C. *Science* **1991**, *251*, 932-934. b) Dickinson, R. E.; Cicerone, R. J. *Nature* **1986**, *319*, 109-115. c) Shimizu, A.; Tanaka, K.; Fujimori, M. *Chemosphere-Glo. Change Sci.* **2000**, *2*(3-4), 425-434.

[50] Neidle, E. L.; Ornston, L. N. *J. Bacteriol.* **1986**, *168*, 815-820.

[51] a) Niu, W.; Draths, K. M.; Frost, J. W. *Biotechnol. Prog.* **2002**, *18*, 201-211. b) Draths, K. M.; Frost, J. W. *J. Am. Chem. Soc.* **1994**, *116*, 399-400.

[52] Esposito, L.; Formanek, K.; Kientz, G.; Mauger, F.; Maureux, V.; Robert, G.; Truchet, F. In *Kirk-Othmer Encyclopedia of Chemical Technology*, 4th ed.; Kroschwitz, J. I., Howe-Grant, M., Eds.; Wiley: New York, 1997; Vol. 24, p. 812.

[53] a) Clark, G. S. *Perfum. Flavor.* **1990**, *15*, 45-54. b) Priefert, H.; Rabenhorst, J.; Steinbuchel, A. *Appl. Microbiol. Biotechnol.* **2001**, *56*, 296-314.

[54] Li, K.; Frost, J. W. *J. Am. Chem. Soc.* **1998**, *120*, 10545-10546.

[55] a) Gross, G. G.; Bolkart, K. H.; Zenk, M. H. *Biochem. Biophys. Res. Commun.* **1968**, *32*, 173. b) Gross, G. G.; Zenk, M. H. *Eur. J. Biochem.* **1969**, *8*, 413-419. c) Gross, G. G. *Eur. J. Biochem.* **1972**, *11*, 585-592. d) Zenk, M. H.; Gross, G. G. *Recent. Adv. Phytochem.* **1972**, *4*, 87.

[56] Krumenacker, L.; Costantini, M.; Pontal, P.; Sentenac, J. In *Kirk-Othmer Encyclopedia of Chemical Technology*; Kroschwitz, J. I.; Howe-Grant, M., Eds.; Wiley: New York, 1995; Vol. 13, p. 996.

[57] a) Draths, K. M.; Ward, T. L.; Frost, J. W. *J. Am. Chem. Soc.* **1992**, *114*, 9725-9726. b) Ran, N. Q.; Knop, D. R.; Draths, K. M.; Frost, J. W. *J. Am. Chem. Soc.* **2001**, *123*, 10927-10934.

[58] a) Johnson, E. A.; Schroeder, W. A.; Adv. Biochem. Eng. **1995**, *53*, 120-178. b) Teisserire, P.J. In *Chemistry of Fragrant Substances* **1994**, VCH Publ. New York. c) S mann, G. *Arch. Biochem. Biophys.* **2001**, *385*, 4-12. d) Lee, P. C.; Schmidt-Dannert, C. *Appl. Microbiol. Biotechnol.* **2002**, *60*, 1-11.

[59] a) Matthews, P. D.; Wurtzel, E. T. *Appl. Microbiol. Biotechnol.* **2000**, *53*, 396-400. b) Albrecht, M.; Takaichi, S.; Misawa, N.; Schnurr, G.; Boger, P.; S mann, G. *J Biotechnol* **1997**, *58*, 177-185. c) Ruther, A.; Misawa, N.; Boger, P.; S mann, G. *Appl Microbiol Biotechnol* **1997**, *48*, 162-167. d) S mann, G. *Trends Plant Sci.* **2001**, *6*, 14-17.

[60] Chotani, G.; Dodge, T.; Hsu, A.; Kumar, M.; LaDuca, R.; Trimbur, D.; Weyler, W.; Sanford, K. *Biochim. Biophys. Acta.* **2000**, *1543*, 434-455.

[61] a) Draths, K. M.; Pompliano, D. L.; Conley, D. L.; Frost, J. W.; Berry, A.; Disbrow, G. L.; Staversky, R. J.; Lievense, J. C. *J. Am. Chem. Soc.* **1992**, *114*, 3956-3962. b) Gubler, M.; Jetten, M.; Lee, S. H.; Sinskey, A. J. *Appl. Environ. Microbiol.* **1994**, *60*, 2494-2500. c) Miller, J. E.; Backman, K. C.; O'Connor, M. J.; Hatch, R. T. *J. Ind. Microbiol.* **1987**, *2*, 143. d) Patnaik, R.; Liao, J. C. *Appl. Environ. Microbiol.* **1994**, *60*, 3903-3908. e) Patnaik, R.; Spitzer, R. G.; Liao, J. C. *Biotechnol. Bioeng.* **1995**, *46*, 361-370. e) Draths, K. M.; Frost, J. W. *J. Am. Chem. Soc.* **1990**, *112*, 1657-1659

[62] Pittard, A. J. Biosynthesis of Aromatic amino acids In *Escherichia coli and Salmonella Typhimurium*; Neidhardt, F. C., Ed.; American Society for Microbiology: Washington, DC, 1987; Vol. 1, p 368.

[63] Ogino, T.; Garner, C.; Markley, J. L.; Herrmann, K. M. *Proc. Natl. Acad. Sci. USA* **1982**, *79*, 5828-5832.

[64] a) Weaver, L. M.; Herrmann, K. M. *J. Bacteriol.* **1990**, *172*, 6581-6584. b) Ray, J. M.; Yanofsky, C.; Bauerle, R. *J. Bacteriol.* **1988**, *170*, 5500-5506. c) Shio, I; Sugimoto, S.; Nakagawa, M. *Agric. Biol. Chem.* **1975**, *39*, 627-635. d) Mikola, M. R.; Widman, M. T.; Worden, R. M. *Appl. Biochem. Biotechnol.* **1998**, *70*, 905-918.

[65] a) Mori, M.; Yokota, A.; Sugitomo, S.; Kawamura, K. Patent JP 62,205,782, 1987. b) Backman, K. C. U.S. Patent 5,169,768, 1992.

[66] a) Paoletti, F.; Williams, J. F.; Horecker, B. L. *Anal. Biochem.* **1979**, *95*, 250-253. b) William, J. F.; Blackmore, P. F.; Duke, C. C.; Macleod, J. F. *Int. J. Biochem.* **1980**, *12*, 339-344. c) Blackmore, P. F.; Williams, J. F.; Macleod, J. F. *FEBS Lett.* **1976**, *64*, 222. d) Duke, C. C.; Macleod, J. F.; Williams, J. F. *Carbohydr Res.* **1981**, 95, 1-26.

[67] Postma, P. W.; Lengeler, J. W.; Jacobson, G. R. Phosphoenolpyruvate: Carbohydrate Phosphotransferase Systems. In *Escherichia coli and Salmonella Typhimurium: Cellular and Molecular Biology*, 2nd ed.; Neidhardt, F. C., Ed.; ASM Press: Washington, DC, **1996**; pp 1149-1174.

[68] a) Li, K.; Frost, J. W. *Biotechnol. Prog.* **1999**, *15*, 876-883. b) Gibson, J. M.; Thomas, P. S.; Thomas, J. D.; Barker, J. L.; Chandran, S. S.; Harrup, M. K.; Draths, K. M.; Frost, J. W. *Angew. Chem.-Int. Edit.* **2001**, *40*, 1945-1948. c) Chandran, S. S.; Jian Y.; Draths, K. M.; von Daeniken, R.; Weber, W.; Frost, J. W. *Biotechnol. Prog.* **2003**, In press. d) Jian Y.; Draths, K. M.; Li, K.; Frost, J. W *Biotechnol. Prog.* **2003** Accepted for publication.

[69] a) Kimura, E.. *Adv. Biochem. Eng. Biotechnol.* **2003**, *79*, 38-57. b) Pfefferle, W.; Möckel, B.; Bathe, B.; Marx, A. *Adv. Biochem. Eng. Biotechnol.* **2003**, *79*, 60-112.

[70] Cameron, D. C.; Altaras, N. E.; Hoffman, M. L.; Shaw, A. J. *Biotechnol. Prog.* **1998**, *14*, 116-125. e)

[71] Barkovich, R.; Liao, J. C. *Metabol. Eng.* **2001**, *3*, 27-39.

[72] a) Mash, E. A. Nelson, K. A. Deusen, S. V. Hemperly, S. B. *Org. Synth.* **1990**, *68*, 92-98. b) Blagg, B. S. Poulter, C. D. *J. Org. Chem.* **1999**, *64*, 1508-1511. c) Taylor, S. V.; Vu, L. D. Begley, T. P. *J. Org. Chem.* **1998**, *63*,2375-2377.

[73] Kolb, H. C.; VanNieuwenhze, M. S.; Sharpless, K. B. *Chem. Rev.* **1994**, *94*, 2483-

2547.

[74] a) Draths, K. M.; Knop, D. R.; Frost, J. W. *J. Am. Chem. Soc.* **1999**, *121*, 1603-1604. b) Knop, D. R.; Draths, K. M.; Chandran, S. S.; Barker, J. L.; von Daeniken, R.; Weber, W.; Frost, J. W. *J. Am. Chem. Soc.* **2001**, *123*, 10173-10182.

[75] a) Kuzuyama, T., Takahashi, S. Seto, H. *Biosci. Biotechnol. Biochem.* **1999**,*63*,776-778. b) Rodriguez-Concepcion, M.; Campos, N.; Maria Lois, L.; Maldonado, C.; Hoeffler, J. F.; Grosdemange-Billiard, C.; Rohmer, M.; Boronat, A. *FEBS Lett* **2000**, *473*, 328-332.

[76] Fürste, J. P.; Pansegrau, W.; Frank, R.; Blocker, H.; Scholz, P.; Bagdasarian, M.; Lanka, E. *Gene* **1986**, *48*, 119-131.

[77] Gentz, R.M.; Bujard, H. *J. Bacteriol.* **1985**, *164*, 70–77.

[78] Bujard, H., Gentz, R., Lanzer, M., Stueber, D., Mueller, M., Ibrahimi, I., Haruptle, M.-T.; Dobberstein, B. *Methods Enzymol.* **1987**, *155*, 416–433.

[79] Lanzer, M.; Bujard, H. *Proc. Natl. Acad. Sci. U. S. A.* **1988**, *85*, 8973-8977.

[80] Stüber, D.; Matile, H.; Garotta, G. In *Immunological Methods* Lefkovits, I. Prenis, B. eds Academic press, New York, **1990**. vol IV, pp 121-152.

[81] Farmer, W. R.; Liao, J. C. *Appl. Environ. Microbiol.* **1997**, *63*, 3205-3210.

[82] Duvold, T.; Cali, P.; Bravo, J. M.; Rohmer, M. *Tetrahedron Lett.* **1997**, *38*, 6181-6184.

[83] a) Li, X. L.; Robbins, J. W.; Taylor, K. B. *J. Ind. Microbiol.* **1990**, *5*(2-3), 85-93. b) Konishi, Y.; Yoshida, S.; Asai S. *Biotechnol. Bioeng.* **1998**, *58*(6), 663-667.

[84] a) Okuhara, M.; Kuroda, Y.; Goto, T.; Okamoto, M.; Terano, H.; Kohsaka, M.; Aoki, H.; Imanaka, H. *J Antibiot.* **1980**, *33*, 24-28. b) Kojo, H.; Shigi, Y.; Nishida, M. *J. Antibiot.* **1980**, *33*, 44-48.

[85] a) Kuemmerle, H. P.; Murakawa, T.; Soneoka, K.; Konishi, T. *Int. J. Clin. Pharmacol. Ther. Toxicol.* **1985**, *23*, 515-520. b) Kuemmerle, H. P.; Murakawa, T.; Sakamoto, H.; Sato, N.; Konishi, T.; De Santis, F. *Int. J. Clin. Pharmacol. Ther. Toxicol.* **1985**, *23*, 521-528.

[86] Kuzuyama, T.; Shimizu, T.; Takahashi, S.; Seto, H. *Tetrahedron Lett.* **1998**, *39*, 7913-7916.

[87] a) Jomaa, H.; Wiesner, J.; S erbr , S.; Altincicek, B.; Weidemeyer, C.; Hintz, M.; Turbachova, I.; Eberl, M.; Zeidler, J.; Lichtenthaler, H. K.; Soldati, D.; Beck, E. *Science* **1999**, *285*, 1573-1576. b) Lell, B.; Ruangweerayut, R.; Wiesner, J.; Missinou, M.A.; Schindler, A.; Baranek, T.; Hintz, M.; Hutchinson, D.; Jomaa, H.; Kremsner, P. G. *Antimicrob. Agents Chemo.* **2003**, *47*, 735-738.

[88] Steinbacher, S.; Kaiser, J.; Eisenreich, W.; Huber, R.; Bacher, A.; Rohdich, F. *J. Biol. Chem.* **2003**, *278*, 18401-18407.

[89] a) Kamiya, T.; Hemmi, K.; Takeno, H.; Hashimoto, M. *Tetrahedron Lett.* **1980**, *21*, 95-98. b) Hemmi, K.; Takeno, H.; Hashimoto, M.; Kamiya, T. *Chem. Pharm. Bull.* **1981**, *29*, 646-650. c) Kamiya, T; Hashimoto, M.; Hemmi, K.; Takeno, H *U. S. Patent* 4182758, **1980**.

[90] Kakinuma, K.; Dekishima, Y.; Matsushima, Y.; Eguchi, T.; Misawa, N.; Takagi, M.; Kuzuyama, T.; Seto, H. *J. Am. Chem. Soc.* **2001**, *123*, 1238-1239.

[91] Takagi, M.; Kuzuyama, T.; Takahashi, S.; Seto, H. *J. Bacteriol.* **2000**, *182*, 4153-4157.

[92] Gennis, R. B.; Stewart, V. Respiration In *Escherichia coli and Salmonella Typhimurium: Cellular and Molecular Biology*, 2nd ed.; Neidhardt, F. C., Ed.; ASM Press: Washington, DC, 1996; pp 217-261.

[93] a) Jomaa, H.; Feurle, J.; Luhs, K.; Kunzmann, V.; Tony, H. P.; Herderich, M.; Wilhelm, M. *FEMS Immunol Med Microbiol* **1999**, *25*, 371-378. b) Altincicek, B.; Moll, J.; Campos, N.; Foerster, G.; Beck, E.; Hoeffler, J. F.; Grosdemange-Billiard, C.; Rodriguez-Concepcion, M.; Rohmer, M.; Boronat, A.; Eberl, M.; Jomaa, H. *J. Immunol* **2001**, *166*, 3655-3658.

[94] a) Sandmann, G. *Arch Biochem Biophys* **2001**, *385*, 4-12. b) Sandmann, G. *Chembiochem* **2002**, *3*, 629-635.

[95] Eisenrich, W.; Rohdich, F.; Bacher, A. *Trends Plant Sci.* **2001**, *6*, 78-84

[96] a) Matthews, P. D.; Wurtzel, E. T. *Appl. Microbiol. Biotechnol.* **2000**, *53*, 396-400. b) Kim, S. W.; Keasling, J. D. *Biotechnol. Bioeng.* **2001**, *72*, 408-415. c) Farmer, W. R.; Liao, J. C. *Biotechnol. Prog.* **2001**, *17*, 57-61.

[97] a) Estevez, J. M.; Cantero, A.; Reindl, A.; Reichler, S.; Leon, P. *J. Biol. Chem.* **2001**, *276*, 22901-22909. b) Lois, L. M.; Rodriguez-Concepcion, M.; Gallego, F.; Campos, N.; Boronat, A. *Plant J.* **2000**, *22*, 503-513. c) Harker, M.; Bramley, P. M. *FEBS Lett.* **1999**, *448* 115-119. d) Kuzuyama, T.; Takagi, M.; Takahashi, S.; Seto, H. *J. Bacteriol.* **2000**, *182*, 891-897.

[98] Kawada, I.; Uchida, K.; Aida, K. *Agric. Biol. Chem.* **1980**, *44*, 407-411.

[99] Cunningham, F. X., Jr.; Lafond, T. P.; Gantt, E. *J. Bacteriol.* **2000**, *182*, 5841-5848.

[100] a) Kuzuma, J.; Nenecek-Marshall, M.; Pollack, W.; Fall, R. *Curr. Microbiol.* **1995**, *30*, 97-103. b) Wagner, W. P.; Helmig, D.; Fall, R. *J. Nat. Prod.* **2000**, *63*, 37-40.

[101] a) Miller, B.; Heuser, T.; Zimmer, W. *FEBS Lett* **1999**, *460*, 485-490. b) Miller, B.; Heuser, T.; Zimmer, W. *FEBS Lett* **2000**, *481*, 221-226.

[102] Fraenkel, D. A. Glycolysis In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd ed.; Neidhardt, F. C., Ed.; ASM Press: Washington, DC, 1996; pp 189-198.

[103] a) Veech, R. L.; Rajman, L.; Dalziel, K.; Krebs, H. H. *Biochem. J.* **1969**, *115*, 837-842. b) Hahn, F. M.; Eubanks, L. M.; Testa, C. A.; Blagg, B. S.; Baker, J. A.; Poulter, C. D. *J. Bacteriol* **2001**, *183*, 1-11.

[104] a) Haslam, E. *The Shikimate Pathway*; John Wiley Sons: New York, 1974. b) Bentley, R. *Crit. Rev. Biochem. Mol. Biol.* **1990**, *25*, 307. c) Herrman, K. M. In *Amino Acids: Biosynthesis and Genetic Regulation*; Herrmann, K. M.; Somerville, R. L., Eds.; Addison-Wesley: Reading, 1983: p. 301. d) Sigma-Aldrich catalogue 2003.

[105] a) Tan, D. S.; Foley, M. A.; Shair, M. D.; Schreiber, S. L. *J. Am. Chem. Soc.* **1998**, *120*, 8565-8566. b) Tan, D. S.; Foley, M. A.; Stockwell, B. R.; Shair, M. D.; Schreiber, S. L. *J. Am. Chem. Soc.* **1999**, *121*, 9073-9087.

[106] Kim, C. U.; Lew, W.; Williams, M. A.; Zhang, L.; Liu, H.; Swaminathan, S.; Bischofberger, N.; Chen, M. S.; Tai, C. Y.; Mendel, D. B.; Laver, W. G.; Stevens, R. C. *J. Am. Chem. Soc.* **1997**, *119*, 681-6902. b) Rohloff, J. C.; Kent, K. M.; Postich, M. J.; Becker, M. W.; Chapman, H. H.; Kelly, D. E.; Lew, W.; Louie, M. S.; McGee, L. R.; Prisbe, E. J.; Schultze, L. M.; Yu, R. H.; Zhang, L. *J. Org. Chem.* **1998**, *63*, 4545-4550.

[107] Federspiel, M.; Fischer, R.; Hennig, M.; Mair, H.-J.; Oberhauser, T.; Rimmler, G.; Albiez, T.; Bruhin, J.; Estermann, H.; G ert, C.; Göckel, V.; Götzö, S.; Hoffmann, U.; Huber, G.; Janatsch, G.; Lauper, S.; Röckel-Stäbler, O.; Trussardi, R.; Zwahlen, A. G. *Org. Process Res. Dev.* **1999**, *3*, 266-274. b) Karpf, M.; Trussardi, R. *J. Org. Chem.* **2001**, *66*, 2044-2051.

[108] Gibson, J. M.; Thomas, P. S.; Thomas, J. D.; Barker, J. L.; Ch ran, S. S.; Harrup, M. K.; Draths, K. M.; Frost, J. W. *Angew. Chem. Int. Ed.* **2001**, *40*, 1945-1948.

[109] a) Barker, J. L.; Frost, J. W. *Biotechnol. Bioeng* **2001**, *76*, 376-390. b) Konstantinov, K. B.; Nishio, N.; Yoshida, T. *J. Ferment. Bioeng.* **1990**, *70*, 253-260.

[110] Dell, K. A.; Frost, J. W. *J. Am. Chem. Soc.* **1993**, *115*, 11581-11589.

[111] Lu, J.-L.; Liao, J. C. *Biotechnol. Bioeng.* **1997**, *53*, 132-138. b

[112] Li, K.; Frost, J. W. *J. Am. Chem. Soc.* **1999**, *121*, 9461-9462.

[113] Saier, M. H.; Wentzel, D. L.; Feucht, B. U.; Judice, J. J. *J. Biol. Chem.* **1975**, *250*, 5089-5096.

[114] a) Kramer, R. *Biochim. Biophys. Acta.* **1994**, *1185*, 1-34. b) Driessen, A. J.; Rosen, B. P.; Konings, W. N. *Trends Biochem. Sci.* **2000**, *25*, 397-401.

[115] Mitchell, P. In *Comprehensive Biochemistry* Florkin, M.; Stotz, E. H., Ed.; **1967**, *22*, pp167-197.

[116] a) Maloney, P. C.; Ambudkar, S. V.; Anatharam, V.; Sonna, L. A.; Varadhachary, A. *Microbiol. Rev.* **1990**, *54*, 1-17. b) Maloney, P. C. *J. Bioenerg. Biomemberane* **1990**, *22*, 509-523.

[117] Pao, S. S.; Paulsen, I. T.; Saier Jr, M. H. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 1-34.

[118] a) Maiden, M. C. J.; Davis, E. O.; Balwin, S. A.; Moore, D. C. M.; Henderson, P. J. F. *Nature*, **1987**, *325*, 641-643. b) Maloney, P. C. *Res. Microbiol.* **1990**, *141*, 309-316.

[119] a) Yan, R. T.; Maloney, P. C. *Cell* **1993**, *75*, 37-44. b) Yan, R. T.; Maloney, P. C. *Proc. Natl. Acad. Sci. U S A* **1995**, *92*, 5973-5976. c) Fann, M.; Davies, A. H.; Varadhachary, A.; Kuroda, T.; Sevier, C.; Tsuchiya, T.; Maloney, P. C. *J. Member. Biol.* **1998**, *164*, 187-195. d) Hall, J. A.; Fann, M. C.; Maloney, P. C. *J. Biol. Chem.* **1999**, *274*, 6148-6153. e) Hall, J. A.; Maloney, P. C. *J. Biol. Chem.* **2001**, *276*, 25107-25113.

[120] a) Draths, K. M.; Frost, J. W. *J. Am. Chem. Soc.* **1990**, *112*, 1657-1659. b) Draths, K. M.; Frost, J. W. *J. Am. Chem. Soc.* **1990**, *112*, 9630-9632.

[121] a) Clark, V. M.; Kirby, A. J. *Biochem. Prep.* **1966**, *11*, 101-104. b) Hirschbien, B. L.; Manzenod, F. P.; Whitesides, G. M. *J. Org. Chem.* **1982**, *47*, 3765-3766.

[122] a) Grenier, F. C.; Waygood, E. B.; Saier, M. H., Jr. *J. Cell. Biochem.* **1986**, *31*, 97-105. b) Meadow, N. D.; Roseman, S. *J. Biol. Chem.* **1982**, *257*, 14526-14537. c) Stock, J. B.; Waygood, E. B.; Meadow, N. D.; Postma, P. W.; Roseman, S. *J. Biol. Chem.* **1982**, *257*, 14543-14552.

[123] Saier, M. H., Jr.; Ramseier, T. M.; Reizer, J. Regulation of Carbon Utilization. In *Escherichia coli Salmonella: Cellular Molecular Biology*, 2nd ed.; Neidhardt, F. C., Ed.; ASM Press: Washington, DC, **1996**; pp1325-1343.

[124] Saier, M. H.; Feucht, D. L. *J. Bacteriol.* **1980**, *141*, 611-617.

[125] Yang, Y. L.; Goldrick, D.; Hong, J. S. *J. Bacteriol.* **1988**, *170*, 4299-4303.

[126] Yu, G. Q.; Hong, J. S. *Gene* **1986**, *45*, 51-57.

[127] a) Jiang, S. Q.; Yu, G. Q.; Li, Z. G.; Hong, J. S. *J. Bacteriol.* **1988**, *170*, 4304-4308. b) Yang, Y. L.; Goldrick, D.; Hong, J. S. *J. Bacteriol.* **1988**, *170*, 4299-4303.

[128] a) Yi, J.; Frost, J. W. Unpublished results.

[129] a) Simpson, F. J.; Perlin, A. S.; Sieben, A. S. *Meth. Enzymol.* **1966**, *9*, 35-38. b) Sieben, A. S.; Perlin, A. S.; Simpson, F. J. *Can. J. Biochem.* **1966**, *44*, 663-669. c) Ballou, C. E.; Fischer, H. O. L.; MacDonald, D. L. *J. Am. Chem. Soc.* **1955**, *77*, 5967-5970.

[130] Corey, E. J.; Suggs, J. William *Tetrahedron Lett.* **1975**, *31*, 2647-2650.

[131] Danishefsky, S.; Berman, E. M.; Ciufolini, M.; Etheredge, S. J. Segmuller, B. E. *J. Am. Chem. Soc.* **1985**, *107*,3891.

[132] Schoner, R.; Herrmann, K. M. *J. Biol. Chem.* **1976**, *251*, 5440-5447.

[133] Paoletti, F.; Williams, J. F.; Horecker, B. L. *Anal. Biochem.* **1979**, *95*, 250.

[134] a) Li, K.; Frost, J. W. *Biotechnol. Prog.* **1999**, *15*, 876-883. b) Liao, J. C.; Hou, S. Y.; Chao, Y. P. *Biotechnol. Bioeng.* **1996**, *52*, 129-140.

[135] Boeckman, R. K. Jr.; Shao, P.; Mullins, J. J. *Org. Synth.* **1999**, *77*, 141.

[136] a) Sieben, A. S.; Perlin, A. S.; Simpson, F. J. *Can. J. Biochem.* **1966**, 44, 663-669. b) Draths, K. M. Ph. D. Dissertation, Stanford University, 1991.

[137] a)Hansen, C. A.; Dean, A. B.; Draths, K. M.; Frost, J. W. *J. Am. Chem. Soc.* **1999**, *121*, 3799-3800. b) Hansen, C. A. Ph. D. Dissertation, Michigan State University, 2002.

[138] Chandran, S. S. Ph. D. Dissertation, Michigan State University, 2001.

[139] Snell, K. D.; Draths, K. M.; Frost, J. W. *J. Am. Chem. Soc.* **1996**, *118*, 5605.

[140] a) Li, K.; Mikola, M. R.; Draths, K. M.; Worden, R. M.; Frost, J. W. *Biotechnol. Bioeng.* **1999**, *64*, 61-73. b) Li, K. Ph. D. Dissertation Michigan State University, 1999.

[141] Miller, J. H. *Experiments in Molecular Gentics*; Cold Spring Harbor Laboratory: Cold Spring Harbor, 1972.

[142] a) Ames, B. N. *Methods Enzymol.* **1966**, *8*, 115. b) Avila, L. Z. Ph. D. Dissertation, Stanford University, 1990.

[143] Bradford, M. M. *Anal. Chem.* **1976**, *72*, 248-254.

[144] Kuzuyama, T.; Takahashi, S.; Watanabe, H.; Seto, H. *Tetrahedron Letters* **1998**, *39*, 4509.

[145] Gollub, E.; Zalkin, H.; Sprinson, D. B. *Methods Enzymol.* **1971**, *17A*, 349-350.

[146] Altincicek, B.; Hintz, M.; S erbr , S.; Wiesner, J.; Beck, E.; Jomaa, H. *FEMS Microbiol. Lett.* **2000**, *190*(2), 329-333.

[147] Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory: Plainview, NY, 1990.