(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
3 April 2014 (03.04.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/052054 A1

(51) International Patent Classification:
C12P 5/02 (2006.01) *C12P 7/04* (2006.01)
C12P 5/00 (2006.01)

(21) International Application Number:
PCT/US2013/059857

(22) International Filing Date:
16 September 2013 (16.09.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/705,191 25 September 2012 (25.09.2012) US
61/727,788 19 November 2012 (19.11.2012) US

(71) Applicant: **ALBERT EINSTEIN COLLEGE OF MEDICINE OF YESHIVA UNIVERSITY** [US/US]; 1300 Morris Park Avenue, Bronx, NY 10461 (US).

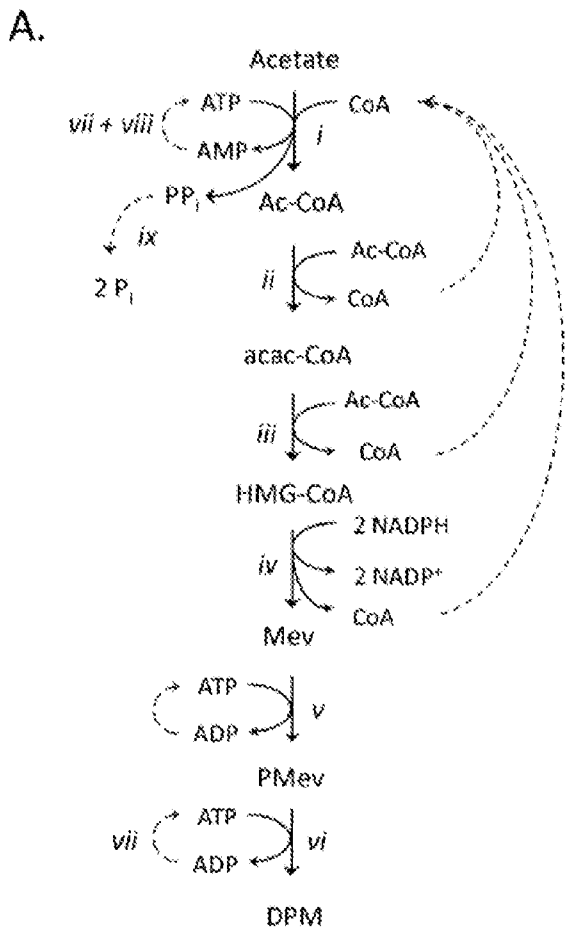
(72) Inventor: **LEYH, Thomas, S.**; 20 Oak Road, Katonah, NY 10536 (US).

(74) Agents: **MILLER, Alan D.** et al.; Amster, Rothstein & Ebenstein LLP, 90 Park Avenue, New York, NY 10016 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,

[Continued on next page]

(54) Title: ENZYMATIC PLATFORM FOR THE SYNTHESIS OF ISOPRENOID PRECURSORS AND USES THEREOF



(57) Abstract: Provided are method of synthesizing (R)-5-diphosphomevalonate (DPM), isopentenyl 5-pyrophosphase (IPP), dimethylallyl 5 -pyrophosphate (DMAPP) and isoprenoid using a one-pot synthesis.

WO 2014/052054 A1

OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

ENZYMATIC PLATFORM FOR THE SYNTHESIS OF ISOPRENOID PRECURSORS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority of U.S. Provisional Patent Application No. 61/727,788, filed on November 19, 2012 and U.S. Provisional Patent Application No. 61/705,191, filed on September 25, 2012, the contents of which are incorporated by reference.

STATEMENT OF GOVERNMENT SUPPORT

[0002] This invention was made with government support under grant number AI068989 awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

[0003] The present invention generally relates to methods of synthesizing (R)-5-diphosphomevalonate (DPM), isopentenyl 5-pyrophosphase (IPP), dimethylallyl 5-pyrophosphate (DMAPP) and isoprenoid using a one-pot synthesis.

BACKGROUND OF THE INVENTION

[0004] Throughout this application various publications are referred to in parenthesis. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications are hereby incorporated by reference in their entireties into the subject application to more fully describe the art to which the subject application pertains.

[0005] Improved access to large numbers of pure proteins, and a rapidly increasing repertoire of well characterized enzymes, isoenzymes and mutants have substantially increased the potential to utilize metabolic pathways, or concatenated enzymatic reactions, in the synthesis of complex natural and synthetic products. Enzymes have been honed over evolutionary time to accomplish specific catalytic tasks (Albery and Knowles 1976; Burbaum et al. 1989). Many are extremely efficient, regio-selective catalysts, while others exhibit broad substrate specificities that provide flexibility in synthetic schemes. Indeed, significant efforts are underway to develop enzymes whose catalytic properties have been altered to

achieve specific synthetic goals (Eijsink et al. 2005; Gerlt and Babbitt

Enzymatic synthesis, used to produce numerous valuable compounds (Cheng et al. 2007; Huang et al. 2006; Kajiwara et al. 2006; Kuberan et al. 2003; Lefurgy et al. 2010; Melnick et al. 2003; Ohdan et al. 2007; Schultheisz et al. 2008; Sugiyama et al. 2007; Van Den Heuvel et al. 2001; Yamaguchi et al. 2007), often provides significant enhancements in yield, purity, production time and cost when compared to traditional chemical synthetic methods (Koeller and Wong 2001; Schmid et al. 2001). These remarkable macromolecular machines are a valuable adjunct to mainstream synthesis particularly when the objective is to build complex natural products.

[0006] The isoprenoid family of natural compounds is estimated to contain approximately 65,000 unique structures including medicines, fragrances, and biofuels (Oldfield and Lin 2012). The medicinal values of isoprenoids have been documented as early as 168 BC (Arsenault et al. 2008; Hsu 2006). Biotechnology companies are attempting to synthesize isopreonoid-based medicines, cosmetics (Osbourn et al. 2011), flavors (Puri et al. 2011), fragrances (Caputi and Aprea 2011) and biofuels (Peralta-Yahya et al. 2011; Rude and Schirmer 2009; Zhang et al. 2011) by genetically engineering plants and bacteria to produce desired isoprenoids in commercial quantities (Dugar and Stephanopoulos 2011; Farhi et al. 2011; Rude and Schirmer 2009; Wu et al. 2011). Recent efforts along these lines include attempts to genetically engineer organisms to produce artemesinin (an antimalarial) at costs that will significantly expand third-world access to the drug (Hale et al. 2007; Lacaze et al. 2011) and to produce isoprenoid-based fuels (Peralta-Yahya et al. 2011; Zhang et al. 2011).

[0007] The present invention addresses the need for simplified, efficient, single-pot syntheses to produce isoprenoids in high yield.

SUMMARY OF THE INVENTION

[0008] The invention is directed to methods of synthesizing (R)-5-diphosphomevalonate (DPM) comprising the steps of: i) synthesizing acetyl-Coenzyme A (Ac-CoA) from acetate, Coenzyme A (CoA) and adenosine-5'-triphosphate (ATP) in the presence of acetyl-CoA synthetase; ii) synthesizing acetoacetyl-CoA (acac-CoA) from Ac-CoA in the presence of acetoacetyl-coA thiolase; iii) synthesizing hydroxymethylglutaryl-CoA (HMG-CoA) from acac-CoA and Ac-CoA in the presence of hydroxymethylglutaryl-CoA synthase; iv) synthesizing (R/S)-mevalonate (Mev) from HMG-CoA and reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of hydroxymethylglutaryl-CoA reductase; v) synthesizing phosphorylated Mev (PMev) from

Mev and ATP in the presence of mevalonate kinase; and vi)synthesizin

and ATP in the presence of phosphomevalonate kinase. Also provided are methods of synthesizing isopentenyl 5-pyrophosphase (IPP), dimethylallyl 5-pyrophosphate (DMAPP) and isoprenoid.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] FIG. 1A-1B. Schematics for the in-situ enzymatic synthesis of DPM and its isotopomers. Panel A. The enzymatic synthesis of DPM from acetate and CoA. The synthesis occurs in six steps (*i* - *vi*). CoA is consumed at reaction *i*, and regenerated at steps *ii-iv*. To prevent product inhibition and thermodynamically bias the system toward DPM formation, ADP (*vii*) and AMP (*vii* and *viii*) are recycled and pyrophosphate is hydrolysed (*ix*). Panel B. The incorporation of acetate into DPM. Acetate fragments are enzymatically concatenated to form the 6-carbon skeleton of DPM. Isotopic labels can be introduced at various points in the DPM synthesis to achieve a particular labeling outcome. The enyzmes used in the synthesis are as follows: *i*, acetyl-CoA synthetase; *ii, acetoacetyl-coA thiolase; iii,* hydroxymethylglutaryl-CoA synthase; *iv*, hydroxymethylglutaryl-CoA reductase; *v,* mevalonate kinase; *vi*, phosphomevalonate kinase; *vii*, pyruvate kinase; *viii*, adenylate kinase; *ix*, inorganic pyrophosphatase.

[0010] FIG. 2. The isotopomers of (R)-diphosphomevalonate. Dots (●, [^{13}C]) and asterisks (*, [^{2}H]) mark the positions of heavy atoms in the synthesized compounds. Each mark represents a separate, singly-labeled compound. A triply-labeled compound, enriched at all of the [^{2}H]-positions, was also synthesized.

[0011] FIG. 3. Tautomerization of acetoacetyl-CoA. Tautomerization allows H/^{2}H-exchange at the C_2-position of the 3-oxobutyryl-moiety of acac-CoA, and thus provides the precursor for the synthesis of [4-$^{2}H_2$]DPM.

[0012] FIG. 4A-4B. ^{1}H and ^{13}C NMR spectra of (R)-diphosphomevalonate isotopomers. Spectra indicate specificity and efficiency of labeling. Panel A. ^{1}H NMR spectra of labeled and unlabeled DPM. The efficiency and specificity of [$^{2}H_2$]-labeling were estimated at > 98% and > 95%, respectively. Panel B. ^{13}C NMR spectrum of [1-^{13}C]DPM. The resonance at 181 ppm corresponds to C_1. The efficiency of labeling at C_1 is estimated at > 92% (see *Results and Discussion*). Based on the absence of non-C_1 signals and the S/N, the labeling specificity is calculated at > 98%. Asterisks indicate instrumental artifacts.

[0013] FIG. 5. DPM and IPP synthesis at high reactant concentration. DPM and IPP were synthesized in separate, single-pot reactions. Reactions were initiated from acetate, or

(R,S)-mevalonate. The conditions are described in *Materials and*

initiated with acetate yielded 69% conversion of acetate to IPP (○), or 63% conversion of acetate to DPM (●). Reactions starting with (R,S)-mevalonate yielded 77% conversion of (R)-mevalonate to IPP (□), or 73% conversion of (R)-mevalonate to DPM (■). The data points represent the average of results from three independent experiments.

[0014] FIG. 6. ^{1}H NMR spectra of DPM isotopomers. The specificity and efficiency of labeling of the isotopomers were estimated based on the *integration* of the ^{1}H *signals*. The results were as follows: [4-$^{2}H_2$]DPM (96%, 95%), [6-$^{2}H_3$]DPM (97%, 95%) and [2, 4, 6-$^{2}H_7$]DPM (96%, 97%).

[0015] FIG. 7. ^{31}P NMR spectrum of DPM. The resonance positions, splitting pattern and nearly identical integrated intensities of the α- and β-resonances indicate an intact pyrophosphoryl-moiety. The asterisk identifies the resonance of phosphocreatine, which was added as an internal standard. The absence of a peak at ~ 5 ppm indicates that phophomevalonate is undetectable.

DETAILED DESCRIPTION OF THE INVENTION

[0016] The invention is directed to a method of synthesizing (R)-5-diphosphomevalonate (DPM) comprising the steps of:

i) synthesizing acetyl-Coenzyme A (Ac-CoA) from acetate, Coenzyme A (CoA) and adenosine-5'-triphosphate (ATP) in the presence of acetyl-CoA synthetase;

ii) synthesizing acetoacetyl-CoA (acac-CoA) from Ac-CoA in the presence of acetoacetyl-coA thiolase;

iii) synthesizing hydroxymethylglutaryl-CoA (HMG-CoA) from acac-CoA and Ac-CoA in the presence of hydroxymethylglutaryl-CoA synthase;

iv) synthesizing (R/S)-mevalonate (Mev) from HMG-CoA and reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of hydroxymethylglutaryl-CoA reductase;

v) synthesizing phosphorylated Mev (PMev) from Mev and ATP in the presence of mevalonate kinase; and

vi) synthesizing DPM from PMev and ATP in the presence of phosphomevalonate kinase.

[0017] Preferably, CoA generated during any of steps ii), iii) or iv) is used in the synthesis of Ac-CoA. Preferably, pyrophosphate generated in step i) is hydrolyzed to phosphate ions by inorganic pyrophosphatase. Preferably, adenosine-5'-monphosphate

(AMP) generated in step i) is converted to adenosine-5'-diphosphat
kinase. Preferably, adenosine-5'-diphosphate (ADP) generated in step v) or vi) is converted to adenosine-5'-triphosphate (ATP) by pyruvate kinase.

[0018] Preferably, the mevalonate kinase used in step v) is from *S. aureus*.

[0019] An isotopic label can be introduced in the DMP synthesis. For example, the label can be a carbon label or a hydrogen label. The isotopic label can be introduced in the DMP synthesis using, for example, labeled acetate or labeled acetyl-CoA.

[0020] The invention further provides a method of synthesizing isopentenyl 5-pyrophosphase (IPP) comprising synthetizing (R)-5-diphosphomevalonate (DPM) by any of the methods disclosed herein and converting DPM to IPP.

[0021] The invention further provides a method of synthesizing dimethylallyl 5-pyrophosphate (DMAPP) comprising synthetizing isopentenyl 5-pyrophosphase (IPP) by any of the methods disclosed herein and converting IPP to DMAPP. Conversion of IPP to DMAPP can be by an isomerase.

[0022] The invention further provides a method of synthesizing an isoprenoid comprising synthetizing IPP by any of the methods disclosed herein and synthetizing DMAPP by any of the methods disclosed herein, and forming a carbon backbone of the isoprenoid using IPP and DMAPP. Trepenoid is another term for isoprenoid.

[0023] Preferably, the synthesis reactions disclosed herein can be carried out as a one-pot synthesis. As used herein, a one-pot synthesis is synthesis of compounds whereby a reactant is subjected to successive chemical reactions in just one reactor. This procedure avoids lengthy separation processes and purification of intermediate chemical compounds.

[0024] The invention further provides a (R)-5-diphosphomevalonate (DPM) or an isopentenyl 5-pyrophosphase (IPP) or dimethylallyl 5-pyrophosphate (DMAPP) or an isoprenoid produced any of the methods disclosed herein.

[0025] Abbreviations used herein include: *acac-CoA, acetoacetyl-CoA;* Ac-CoA, acetyl-CoA; ACS, acetyl-CoA synthetase; *ACT, acetoacetyl-CoA thiolase*; β-ME, β-mercaptoethanol; *DTNB,* 5, 5'-Dithio-bis(2-nitrobenzoic acid); DPM, (R)-5-diphosphomevalonate; DPM-DC, diphosphomevalonate decarboxylase; DTT, dithiothreitol; HMG-CoA, hydroxymethylglutaryl-CoA; HMGR, hydroxymethylglutaryl-CoA reductase; HMGS2, hydroxymethylglutaryl-CoA synthase; IPTG, Isopropyl-1-thio-β-D-galactopyranoside; LDH, lactate dehydrogenase; Mev, (R/S)-mevalonate; MK, myokinase; MVK, mevalonate kinase; PEP, phosphoenolpyruvate; PK, pyruvate kinase; PMK,

phosphomevalonate kinase; PMSF, phenylmethylsulfonyl fluoride; *acetoacetyl-CoA thiolase*/hydroxymethylglutaryl-CoA reductase; Units (U) μmols product min^{-1} at a saturating substrate.

[0026] This invention will be better understood from the Experimental Details, which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims that follow thereafter.

EXPERIMENTAL DETAILS

Introduction

[0027] The carbon backbones of isoprenoids are assembled from two fundamental building blocks, isopentenyl 5-pyrophosphate (IPP) and dimethylallyl 5-pyrophosphate (DMAPP) - the endproducts of either the mevalonate or non-mevalonate pathway (Hemmerlin et al. 2003; Rohmer 1999; Thulasiram et al. 2008; Wouters et al. 2003). By linking the HMG-CoA reductase pathway, which produces mevalonate, to the mevalonate pathway, these building blocks can be enzymatically assembled from acetate, ATP, NAD(P)H, and CoA (Fig. 1). Here, enzyme reactions are strategically employed to accomplish efficient, single-pot syntheses of the intermediates and endproducts of the mevalonate pathway in high yield (> 85%) (Andreassi et al. 2004; Hedl et al. 2002; Middleton 1974; Miziorko 2011; Miziorko and Lane 1977; Sutherlin and Rodwell 2004). Labeling strategies that regio-specifically position carbon and hydrogen isotopes into the building-block backbone are developed and used to synthesize and purify isotopomers of the immediate endproduct of the mevalonate pathway, diphosphomevalonate (DPM, Fig 2) (Miziorko 2011). Finally, the enzymatic system is shown to be robust and capable of producing pathway end-products in simple, aqueous solutions at levels that match or exceed the highest reported levels, which are only achieved using high-density fermentation.

Materials and Methods

[0028] *Materials.* Lactate dehydrogenase (rabbit muscle), pyruvate kinase (rabbit muscle), and inorganic pyrophosphatase (Baker's yeast) were purchased from Roche Applied Science. (*R, S*)-[$^{2}H_3$]methyl-mevalonolactone, (*R, S*)-mevalonolactone, acetyl-CoA, glutamate dehydrogenase (bovine liver), acetyl-CoA synthetase (Baker's yeast), myokinase (rabbit muscle) and lysozyme (bovine) were purchased from Sigma. Sodium acetate (^{13}C, 99%), sodium acetate (^{2}H, 99%) and D_2O (99%) were purchased from Cambridge Isotope Laboratories, Inc. All other chemical reagents were of the highest grade available. Plasmids

pET28efTR (encodes a bi-functional enzyme, *Enterococcus faec* thiolase/HMG-CoA reductase), pET28efS2 A100G (encodes *Enterococcus faecalis* HMG-CoA synthase), and pET28-efR (encodes *Enterococcus faecalis* HMG-CoA reductase) were provided by Prof. V. W. Rodwell (Sutherlin and Rodwell 2004). Mevalonate kinase (*Staphylococcus aureus*), phosphomevalonate kinase (*Streptococcus. pneumoniae*), and diphosphomevalonate decarboxylase (*Streptococcus. pneumoniae*) were expressed and purified as described previously (Andreassi et al. 2004; Pilloff et al. 2003).

[0029] *Enzyme expression and purification.* 37 °C LB/ampicillin media was inoculated with *E. coli* BL21(DE3) freshly transformed with the expression plasmid of interest. The cells were then cultured to an OD_{595} of 0.8, protein expression was then induced by the addition of IPTG (0.75 mM), and the incubation was continued for 4 h at 37 °C. The culture temperature was then shifted to 18 °C and incubation was continued for 16 h. The cells were then harvested by centrifugation (30 min, RCF 5,000 g, 4 °C). The MVK (Andreassi et al. 2004), PMK (Andreassi et al. 2004) and DPM-DC (Andreassi et al. 2004) expression vectors fuse a His_9-GST-tag to the N-terminus of the enzyme; whereas, the *acetoacetyl-CoA thiolase* (Hedl et al. 2002), HMG-CoA synthase (Sutherlin et al. 2002) and HMG-CoA reductase (Hedl et al. 2002) vectors fuse a His_6-tag to the N-terminus. Dual-tag proteins were purified using a GST resin followed by a His resin. All buffers and columns were equilibrated at 4 °C prior to use. Purification began by suspending cell pellets (5.0 ml/g cell paste) in Buffer A [H_2KPO_4 (50 mM), NaCl (140 mM), KCl (2.7 mM), pH 7.3] supplemented with lysozyme (0.10 mg/ml), PMSF (290 μM), and pepstatin A (1.5 μM). EDTA (1.0 mM) was added to Buffer A when purifying dual-tag systems. After suspension for 1 hr at 4 °C, cells were disrupted by sonication and debris was removed by centrifugation (50 min, RCF 15,000 g, 4°C). Supernatants containing dual-tag proteins were loaded onto a Glutathione Sepharose 4 FF column equilibrated with Buffer A + EDTA, the column was then washed with three column volumes of the same buffer, and protein was eluted using Tris/Cl (250 mM, pH 8.0), KCl (500 mM) and reduced glutathione (10 mM). Supernatants containing singly tagged proteins, or GST-resin eluants containing dual-tag proteins, were loaded onto a *Ni*-NTA column equilibrated with Buffer B [H_2KPO_4 (50 mM), NaCl (300 mM), imidazole (10 mM), pH 8.0]. The column was washed with Buffer C [H_2KPO_4 (50 mM), NaCl (300 mM), imidazole (20 mM), pH 8.0], and fusion protein was eluded with Buffer D [H_2KPO_4 (50 mM), NaCl (300 mM), imidazole (300 mM), β-mercaptoethanol (10 mM), pH 8.0]. Glycerol was then added to the singly-tagged eluants (5% v/v) and stored (see below). Tags were

removed from the dual-tag proteins by incubation with PreScission prc
al. 2002) during overnight dialysis at 4 °C against Hepes/K^+ (50 mM, pH 8.0) containing DTT (10 mM) and KCl (100 mM). Following proteolysis, the dialysate was passed over a GSTrap column to remove the GST-tagged protease. The purity of the single- and double-tags proteins was estimated, using SDS-PAGE, at > 85 and > 95%, respectively. Eluants containing purified proteins were frozen rapidly and stored at - 80 °C.

[0030] *Enzymatic assays*. To establish conditions for the synthesis of DPM, the activity of each enzyme was assessed under the synthesis conditions. Apparent kinetic constants were extracted from reaction progress curves (Lefurgy et al. 2010) and were in good agreement with published values (Table I). *Acetyl-CoA synthetase activity* was monitored by coupling the production of AMP to the oxidation of NADH (Pilloff and Leyh 2003). The assay conditions were as follows: inorganic pyrophosphatase (4.0 U/ml), myokinase (4.0 U/ml), PK (4.0 U/ml), LDH (8.0 U/ml), NADH (3.0 mM, ε_{398} = 0.136 mM^{-1} cm^{-1}), acetate (2.0 mM), CoA (2.0 mM), ATP (4.0 mM), PEP (6.0 mM), $MgCl_2$ (1.0 mM + [nucleotide]), KCl (50 mM), β-ME (10 mM). *Acac-Coa thiolase activity* was monitored by following the appearance of acac-CoA at 302 nm (Clinkenbeard et al. 1975). The conditions were: Ac-CoA (6.0 mM), $MgCl_2$ (2.0 mM). *HMG-CoA synthase activity* was monitored at 386 nm ($\varepsilon_{386}^{NADPH}$ = 0.61 mM^{-1} cm^{-1}) by coupling the production of 3-hydroxy-3-methyl glutaryl-CoA to the oxidation of NADPH using HMG-CoA reductase. The conditions were: HMG-CoA reductase (1.0 μM), acac-CoA (1.0 mM), Ac-CoA (1.0 mM), NADPH (1.5 mM), KCl (50 mM), β-ME (10 mM). *HMG-CoA reductase activity* was monitored by following oxidation of NADPH. The conditions were: 3-hydroxy-3-methyl glutaryl-CoA (0.50 mM), NADPH (0.20 mM), KCl (50 mM), β-ME (10 mM). *Mevalonate kinase activity* was monitored by coupling the production of ADP to the oxidation of NADH (Andreassi et al. 2004; Pilloff et al. 2003). The conditions were: PK (4.0 U/ml), LDH (8.0 U/ml), NADH (200 μM, ε_{339} = 6.22 mM^{-1} cm^{-1}), PEP (7.0 mM), mevalonate (135 μM), ATP (5.0 mM), $MgCl_2$ (1.0 mM + [nucleotide]), KCl (50 mM), β-ME (10 mM). *Phosphomevalonate kinase activity* was monitored by coupling the production of ADP to the oxidation of NADH (Andreassi et al. 2004; Pilloff et al. 2003). The conditions were identical to those used for mevalonate kinase except phosphomevalonate (50 μM) replaced mevalonate. *DPM Decarboxylase activity* was monitored by coupling the production of ADP to the oxidation of NADH (Andreassi et al. 2004; Pilloff et al. 2003). The conditions were identical to those used for mevalonate kinase

except diphosphomevalonate (50 μM) replaced mevalonate. In all buffered with Hepes/K^+ (50 mM), pH 8.0, and T = 25 ± 2 °C.

[0031] *The synthesis of (R)-diphosphomevalonate.* DPM was synthesized in a one-pot reaction using the following conditions: Ac-CoA synthetase (2.0 *μM*), *acac-CoA thiolase (2.0 μM),* HMG-CoA Synthase (4.0 *μM*), HMG-CoA reductase (2.0 *μM*), mevalonate kinase (2.0 μM), phosphomevalonate kinase (3.0 μM), pyruvate kinase (5.0 U/mL), myokinase (2.0 U/ml), inorganic pyrophosphatase (2.0 U/ml), ATP (5.0 mM), PEP (10 mM), acetate (12 mM), CoA (5.0 mM), NADPH (10 mM), KCl (50 mM), *$MgCl_2$ (1.0 mM + [ATP])*, β-ME (10 mM), Hepes/K^+ (50 mM), pH 8.0, T = 25 ± 2 °C. Reactions progress was monitored by following the oxidation of NADPH associated with the HMG-CoA reductase reaction. DPM formation was assayed by adding an aliquot the DPM-synthesis reaction into a DPM decarboxylase assay mixture (DPM-DC (0.10 μM), PK (4.0 U/ml), LDH (8.0 U/ml), NADH (200 μM), PEP (4.0 mM) ATP (2.0 mM), $MgCl_2$ (1.0 mM + [nucleotide]), KCl (50 mM), β-ME (10 mM), Hepes/K^+ (50 mM), pH 8.0, T = 25 ± 2 °C) and monitoring NADH oxidation at 340 nm. The assay-reaction dilution was sufficient (330-fold dilution) to prevent the HMG-CoA reductase reaction from contributing significantly to the measurement. The reactions yielded essentially quantitative conversion of acetate to the endproduct, DPM.

[0032] *Synthesis of labeled acetyl-CoA precursors.* The synthesis of regiospecifically labeled DPM requires appropriately labeled Ac-CoA. Labeled Ac-CoA precursors were synthesized using the following conditions: acetyl-CoA synthetase (2.0 *μM*), pyrophosphatase (2.0 U/ml), labeled acetate (4.0 mM), CoA (4.0 mM), ATP (4.0 mM), $MgCl_2$ (5.0 mM), Hepes/K^+ (50 mM), pH 8.0. The reactants were mixed gently for 10 hr at T = 25 ± 2 °C. Reaction progress was monitored by assaying aliquots of the reaction for AMP synthesis using the Ac-CoA synthetase assay described above. The conversion of CoA to labeled Ac-CoA was > 95%.

[0033] *Synthesis of acac-CoA.* The synthesis of acac-CoA was achieved using the conditions identical to those described for the synthesis of Ac-CoA with the exception that acac-CoA thiolyase (2.0 *μM)* and DNTB (10 mM) were present. DNTB reacts with CoA and was used to draw the acac-thiolase reaction forward. The DTNB reaction was monitored at 412 nM (Kredich and Tomkins 1966). Acac-CoA formation was monitored at 302 nm (*see, Enzymatic assays, Materials and Methods*). The reaction reached completion after approximately 17 hr, after which > 98% acetyl-CoA had converted to acac-CoA. The reaction

was filtered (10 kDa membrane) to remove enzymes prior to us subsequent syntheses.

[0034] *Synthesis of [1-^{13}C]DPM or [2-$^{2}H_2$]DPM.* Labeled Ac-CoA (^{13}C or ^{2}H) was prepared from CoA and labeled acetate as described above (see, *Synthesis of labeled acetyl-CoA precursors, Materials and Methods*). Labeled DPM was synthesized by adding the following reagents to the labeled Ac-CoA reaction mixture: PK (10 U /mL), HMG-CoA synthase (4.0 *μM*), HMG-CoA reductase (2.0 *μM*), MVK (2.0 μM), PMK (1.0 μM), PEP (10 mM), NADPH (5.0 mM), unlabelled acac-CoA (2.0 mM), ATP (5.0 mM), KCl (50 mM), and β-ME (10 mM). The unlabeled acac-CoA was prepared as describe above (see, *Synthesis of acac-CoA, Materials and Methods*). The reaction was stirred gently overnight (~16 h, 25 ± 2 °C), at which point > 97% of the labeled Ac-CoA had been incorporated into DPM. The quantitation of DPM is described above (see, *Synthesis of (R)-diphosphomevalonate*).

[0035] *Synthesis of [4-$^{2}H_2$]DPM.* The synthesis of [4-$^{2}H_2$]DPM was accomplished in two steps. First, the tautomerization of acac-CoA was used to exchange protons at the C_2-position of the 3-oxobutyryl-moiety of acac-CoA with solvent deuterons (Fig. 3). This was accomplished by synthesizing acac-CoA in D_2O (99%) under the following conditions: Ac-CoA (3.0 mM), *acac-CoA thiolase (2.0 μM), $MgCl_2$ (6.0 mM)* and Hepes/K^+ (50 mM) pH 8.0, T = 25 ± 2 °C. After three hours, the reaction reached completion, ~ 70% of the Ac-CoA had been converted to acac-CoA and the methylene protons had exchanged fully with solvent (confirmed by ^{1}H NMR). In the second step, *acac-CoA thiolase* was removed by ultrafiltration (10-kDa cutoff membrane) to prevent formation of unlabelled acac-CoA in the subsequent reactions. The synthesis of labeled DPM was initiated by adding the following reactants to the strained solution containing [^{2}H]-acac-CoA (1.0 mM) in D_2O: HMG-CoA synthase (4.0 *μM*), HMG-CoA reductase (2.0 *μM*), MVK (2.0 μM), PMK (1.0 μM), PK (10 U /mL), PEP (5.0 mM), NADPH (3.0 mM), Ac-CoA (1.5 mM), ATP (5.0 mM), KCl (50 mM), and β-ME (10 mM). Following 6 hr of gentle stirring at T = 25 ± 2 °C, the reaction reached completion and > 95% the [$^{2}H_2$]-acac-CoA had been converted to [4-$^{2}H_2$]DPM. The quantitation of DPM is described above (see, *Synthesis of (R)-diphosphomevalonate*).

[0036] *Synthesis of [2, 4, 6-^{2}H] DPM.* The following one-pot reaction mixture was used for this synthesis: *acac-CoA thiolase (2.0 μM),* HMG-CoA Synthase (4.0 *μM*), HMG-CoA reductase (2.0 *μM*), MVK (2.0 μM), PMK (1.0 μM), PK (10 U/mL), PEP (10 mM), [^{2}H]Ac-CoA (5.0 mM), NADPH (3.5 mM), ATP (6.0 mM), *$MgCl_2$ (7.0 mM),* KCl (50 mM),

β-ME (10 mM), Hepes/K^+ (50 mM), pH 8.0, T = 25 ± 2 ºC. The incubated for ~8 hrs, until reaction was completed.

[0037] *Synthesis of [6-$^2H_3$]DPM.* Mevalonolactone was linearized for enzymatic conversion to DPM by suspending 385 μmoles (50 mg) of (*R, S*)-[$^2H_3$]methyl-mevalonolactone in 200 μl of water containing five lactone-equivalents of KOH, and the solution was incubated at 37 °C for 1 h. The pH was adjusted to 7.5 with 1.0 M HCl, and Hepes/K^+ (1.0 M, pH 7.5) as added to 50 mM. The concentration of the *R*-isomer (50% of the racemate) was determined by enzymatic assay (see, *Enzymatic Assays*). [6-$^2H_3$]DPM was synthesized in a one-pot-reaction under the following conditions: (*R, S*)-[6-$^2H_3$]mevalonate, MVK (2.0 μM), PMK (1.0 μM), PK (10 U/ml), ATP (5.5 mM), PEP (10 mM), β-ME (10 mM), $MgCl_2$ (6.5 mM) and Hepes/K^+ (50 mM, pH 8.0), T = 25 ± 2 ºC. The reaction achieved > 97% conversion of (*R*)-[6-$^2H_3$]mevalonate to DPM, which was quantitated as described above (see, *Enzymatic Assays*).

[0038] *Synthesis of DPM from acetate at high concentration.* DPM synthesis was accomplished in a one-pot reaction using the following conditions: Ac-CoA synthetase (5.0 *μM*), *acac-CoA thiolase (7.0 μM),* HMG-CoA Synthase (10 μM), HMG-CoA reductase (*7.0 μM*), mevalonate kinase (5.0 μM), phosphomevalonate kinase (3.0 μM), pyruvate kinase (10 U/mL), myokinase (5.0 U/ml), inorganic pyrophosphatase (5.0 U/ml), ATP (100 mM), PEP (800 mM), acetate (340 mM), CoA (5.0 mM), NADPH (300 mM), *$MgCl_2$ (110 mM)*, β-ME (10 mM), Hepes/K^+ (50 mM), pH 8.0, T = 25 ± 2 ºC. Reaction progress was monitored as described above (see, Enzymatic Assays). Sixty-three percent of the acetate was converted to DPM.

[0039] *Synthesis of IPP from acetate at high concentration.* IPP synthesis was accomplished in a one-pot reaction using the following conditions: Ac-CoA synthetase (7.0 *μM*), *acac-CoA thiolase (10 μM),* HMG-CoA Synthase (12 *μM*), HMG-CoA reductase (*10 μM*), mevalonate kinase (7.0 μM), phosphomevalonate kinase (5.0 μM), diphosphomevalonate decarboxylase (3.5 μM), pyruvate kinase (20 U/mL), myokinase (7.0 U/ml), inorganic pyrophosphatase (7.0 U/ml), ATP (200 mM), PEP (800 mM), acetate (340 mM), CoA (5.0 mM), NADPH (300 mM), *$MgCl_2$ (220 mM),* β-ME (10 mM), Hepes/K^+ (50 mM), pH 8.0, T = 25 ± 2 ºC. The conversion of acetate to IPP yields nine IPP-equivalents of pyruvate. Pyruvate was quantitated by adding an aliquot of the IPP-synthesis reaction to a lactate dehydrogenase assay mixture: (LDH (8.0 U/ml), NADH (200 μM), KCl (50 mM), β-ME (10 mM), Hepes/K^+ (50 mM), pH 8.0, T = 25 ± 2 ºC). Dilution of the synthesis reaction

was sufficient (> 500-fold) to prevent enzymes from the reacti significantly to the pyruvate measurements. Sixty-nine percent of the acetate was converted to DPM.

[0040] *Synthesis of DPM from (R/S)-mevalonate at high concentration.* DPM synthesis was accomplished in a reaction using the following conditions: mevalonate kinase (5.0 μM), phosphomevalonate kinase (3.0 μM), pyruvate kinase (10 U/mL), (*R/S*)-mevalonate (370 mM), ATP (50 mM), PEP (350 mM), *$MgCl_2$ (60 mM),* β-ME (7.0 mM), Hepes/K^+ (50 mM), pH 8.0, T = 25 ± 2 °C. Reaction progress was monitored as described above (see, Enzymatic Assays). Seventy-one percent of the (R)-mevalonate in the (*R/S*)-mixture was converted to DPM.

[0041] *Synthesis of IPP from (R/S)-mevalonate at high concentration.* IPP synthesis was accomplished in a reaction using the following conditions: mevalonate kinase (5.0 μM), phosphomevalonate kinase (3.0 μM), diphosphomevalonate decarboxylase (1.6 μM), pyruvate kinase (10 U/mL), (*R/S*)-mevalonate (375 mM), ATP (50 mM), PEP (450 mM), *$MgCl_2$ (60 mM),* β-ME (7.0 mM), Hepes/K^+ (50 mM), pH 8.0, T = 25 ± 2 °C. The conversion of (*R/S*)-mevalonate to IPP was monitored by following the formation of pyruvate using lactate dehydrogenase (see, *Synthesis of IPP from acetate at high concentration*). Seventy-seven percent of the (R)-mevalonate in the (*R/S*)-mixture was converted to IPP.

[0042] *Purification (R)-diphosphomevalonate.* To maximize the purity and recovery of DPM, PEP (which chromatographs near DPM) was converted to pyruvate by adding one PEP-equivalent of ADP to the synthesis reaction mixture. Small and large molecules were separated by ultrafilitration (10-kDa cutoff). The small-molecule filtrate was passed through a 35 mL bed of anion exchange resin (AG MP-1) equilibrated with Hepes/K^+ (10 mM, pH 7.5), and the column was "washed" with five volumes of equilibration buffer. The compounds were eluted using a 750 ml, linear salt gradient (0 - 1.0 M KCl) at 2.0 mL/min. DPM eluted at 0.32 mM KCl and contained < 1% nucleotide. To remove excess KCl and concentrate the DPM, the purified compound was loaded onto a 5.0 ml bed of AG MP-1 equilibrated with NH_4HCO_3 (10 mM, pH 7.5). The column was then "washed" with five volumes of NH_4HCO_3 (10 mM, pH 7.5) before eluting the DPM with 1.8 volumes of NH_4HCO_3 (350 mM, pH 7.5). Excess NH_4HCO_3 was removed by rotary evaporation at 45 °C. The desalted compounds were dissolved in ultra pure water (2.0 mL) and the solution was adjusted to pH 7.5 with KOH. NH_4HCO_3 in the desalted, purified DPM was measure using an enzymatic assay that couples the reduction of $NADP^+$ to the synthesis of glutamate from NH_4^+ and

α-ketoglutarate (Ozer 1985). The assay conditions were as follows mM), $NADP^+$ (0.20 mM), glutamate dehydrogenase (14 U/mL), Hepes/K^+ (45 mM) pH 8.0 at T = 25 ± 2 °C. The NH_4^+/DPM stoichiometry was ~ 4:1. The DPM concentration and purity, presence of mevalonate and phosphomevalonate, were determined spectrophotometrically using the assay described above. (see, *Enzymatic assay*), and the purified compounds were stored in Hepes/K^+ (10 mM, pH 8.0) at -80 °C.

[0043] *NMR protocols.* One dimensional NMR was used to confirm the structure and isotopic labeling of the DPM isotopomers. A Bruker DRX 300MHz spectrometer equipped with a 5 mm broadband probe was used to acquire data. Sample temperature was 25 ± 2 °C. Proton spectra were the average of 32 scans (64K points each) acquired over 20 ppm using a 1.0 s recycle delay. The residual water signal was suppressed by presaturation of the HOD resonance. Spectra were processed with 1.0 Hz line broadening, and proton chemical shifts were referenced to 3-(trimethylsilyl) propionate (Wishart et al. 1995). Proton-decoupled carbon spectra were the average of 100 scans (61K points each) acquired over 315 ppm using a 3.0 s recycle delay. Spectra were processed with a 1.5 Hz line broadening, and chemical shifts were referenced indirectly (Wishart et al. 1995). Proton-decoupled phosphorus spectra were the average of 256 scans (64K points each) acquired over 50 ppm using a 6.0 s recycle delay. Spectra were processed with a 3.0 Hz line broadening, and chemical shifts were referenced to phosphocreatine (Gorenstein 1984).

Results and Discussion

[0044] *The enzymatic synthesis of DPM.* Diphosphomevalonate is synthesized from acetate, ATP and NADPH in six consecutive enzymatic reactions (*i* - *vi*, Fig 1A). The first four reactions produce mevalonate from 3 acetate, 3 ATP, and 2 NADPH (Cornforth et al. 1974; Cornforth et al. 1960; Popjak 1970). CoA, which acts as an acetyl-carrier, is consumed in reaction *i*, and regenerated in reactions *ii*, *iii* and *iv* (see, dashed arrows on right side of Fig 1A). Reactions *v* and *vi* are catalyzed by kinases that phosphorylate mevalonate to produce the pyrophosphoryl-group of DPM. To bias the reactions toward the endproduct and avoid product inhibition, ADP and AMP were recycled to ATP using pyruvate kinase and myokinase, and PP_i was hydrolyzed to P_i using inorganic pyrophosphatase. In total, nine enzymes were used in the synthesis (Andreassi et al. 2004; Hedl et al. 2002; Middleton 1974; Miziorko 2011; Miziorko and Lane 1977; Sutherlin and Rodwell 2004).

[0045] Enzymes *ii* - *vi* were cloned, expressed in *E. coli* and purified (see, *Materials and Methods)*; *i* and *vii* - *ix* were obtained from commercial sources. The purified enzymes

were 80 – 95% pure, as judged by Comassie staining (Neuhoff V.
(Shapiro et al. 1967) gels, and were obtained in yields of 30 - 40 mg pure protein/liter of *E. coli*. The kinetic constants of the purified enzymes were determined under the conditions used for the synthesis, and were in good agreement with literature values (Table I). The assays are described in *Enzymatic Assays* (see, *Materials and Methods*). The enzymes showed no significant loss of activity over an 8 month period when frozen rapidly and stored at - 80 °C in Hepes (50 mM, pH 8.0), 150 mM KCl, 5% glycerol (v/v).

[0046] The relative enzyme concentrations used in the DPM syntheses were determined empirically by adjusting concentrations such that flux through the pathway was not rate-limited by any single step. This was accomplished by setting PMK (*vi*) at a fixed concentration and titrating each preceding enzyme successively until the DPM-synthesis rate was 80 – 90% of the maximum rate achievable at each step. For example, MVK (*v*) was titrated at a fixed concentration of PMK until the rate of DPM synthesis became independent of MVK concentration – the maximum rate. The MVK concentration was then adjusted to allow 80-90% of the maximum rate, and an analogous procedure was performed with HMG-CoA reductase (*iv*). The procedure was performed in succession for each enzyme in the pathway to determine the relative enzyme concentrations to be used in the synthesis. Once the relative concentrations were established, the absolute concentrations were set to achieve the desired reaction times, which ranged from 8 – 72 hr. Mevalonate kinase from *S. aureus* was selected because, unlike the *S. pneumoniae* enzyme, it is not allosterically inhibited by DPM (Lefurgy et al. 2010).

[0047] Substrates were set at saturating, sub-inhibiting concentrations. ATP, a substrate for five of the enzymes (*i*, *v*, *vi*, *vii* and *viii*), was set at 5.0 mM, which ranges from 5.8 - 68 x K_m. Typical substrate concentrations of the other reactants were as follows: acetate (12 mM, 42 x K_m); CoA (2.0 mM , 8.0 x K_m); NADPH (10 mM, 320 x K_m); and PEP (10 mM, 250 x K_m). Under these conditions, and using the absolute enzyme concentrations detailed in *Synthesis of (R)-diphosphomevalonate* (see, *Materials and Methods*), acetate was converted to DPM in a single-pot reaction, with a yield of ~ 98%.

[0048] *The incorporation of isotopes into DPM.* The six-carbon backbone of DPM is constructed in the first three enzymatic steps (*i – iii*), each of which adds a single acetate to the CoA thioester R-group. The pattern of acetate incorporation into the R-group, and ultimately DPM, is shown in Figure 1B. Acetate is first esterfied onto the CoA thiol, and subsequent two-carbon units are added by forming carbon-carbon bonds with the existing R-group. Isotopes can be incorporated into specific positions in DPM (Fig 2) using labeled

acetate or acetyl-CoA, or *via* solvent exchange with exchange-se
Achieving certain labeling patterns required removal of enzymes by ultrafiltration at intermediate stages of the synthesis, and/or that reactions were run in D_2O (see below).

[0049] *The synthesis of [2, 4, 6,-$^2H_7$]- and [6-$^2H_3$]DPM.* The compounds were synthesized in approximately 50 mg quantites in one-pot reactions using commercial [2-$^2H_3$]acetate or (*R, S*)-[6-$^2H_3$]mevalonolactone as starting material. Reactions were complete after 22 hrs, and virtually quantitative conversion of starting material to DPM was achieved in all cases. Approximately ~ 86% of the maximum theoretical maximum yield of DPM was obtained after purification (see, *Material and Methods*). The labeling of DPM was confirmed using ^{1}H NMR (Fig. 6).

[0050] *Synthesis of [4-$^2H_2$]DPM.* The synthesis of [4-$^2H_2$]DPM was carried out in several steps. First, unlabeled Ac-CoA was synthesized using acetyl-CoA synthetase (*i*) and inorganic pyrophosphatase (*ix*) (see, *Synthesis of acetyl-CoA, Materials and Method*). Acetyl-CoA thiolyase (*ii*) and DTNB (in excess over Ac-CoA) were then added to form acac-CoA. DTNB reacts quantitatively with CoA (Kredich and Tomkins 1966) and was used to draw the unfavorable acac-CoA-forming reaction to completion (Hedl et al. 2002). Acac-CoA tautomerizes (Cederstav 1994), and its enol-form exchanges protons with solvent (Fig. 3). To streamline the synthesis, both enzymatic reactions were run in D_2O. ^{1}H NMR confirmed that exchange was complete and occurred exclusively at the C_4-position of DPM (Fig. 6). It is notable that this exchange suggests the possibility of using equilibrium isotope exchange to produce Ac-CoA in which the methyl-protons have been exchanged with solvent. To attach the third acetate without forming unlabelled acac-CoA, which would dilute isotopic enrichment, the Ac-CoA thiolyase was removed by ultrafiltration before adding the reactants that complete the synthesis of DPM (*Synthesis of [4-$^2H_2$]-DPM, Materials and Methods*). The reactions were essentially quantitative and the production of DPM was ~ 96% of the theoretical maximum.

[0051] *The synthesis of [1-^{13}C]- and [2-$^2H_2$]DPM.* The strategy used to synthesize these compounds was similar to that used in the synthesis of [4-$^2H_2$]DPM. Unlabeled acac-CoA was synthesized, the enzymes used in the synthesis were "strained" from the reaction by ultrafiltration, labeled Ac-CoA was then added along with the enzymes, and reagents needed to complete the synthesis of DPM (see, *Materials and Methods*). Care was taken to ensure that the Ac-CoA remaining in the acac-CoA synthesis reaction was ~ 0.2% of the labeled Ac-CoA used in the subsequent conversion to DPM. The yield was quantitative; DPM levels reached ~ 95% of the theoretical maximum.

[0052] *The synthesis of [2, 4, 6,-$^2H_7$]- and [6-$^2H_3$]DPM.* The r
detailed in *Materials and Methods*. The labeling of DPM was confirmed using ^{1}H NMR (Fig. 6).

[0053] *Synthesis of [4-$^2H_2$]DPM.* ^{1}H NMR confirmed that exchange was complete and occurred exclusively at the C_4-position of DPM (Fig. 6). It is notable that this exchange suggests the possibility of using equilibrium isotope exchange to produce Ac-CoA in which the methyl-protons have been exchanged with solvent.

[0054] A typical ^{31}P NMR spectrum of synthesized DPM is shown in Figure 7. The chemical shifts and splitting patterns are consistent with literature values for the pyrophoryl-moiety (Gorenstein 1984). The purified DPM isotopomers were checked for contamination with mevalonate and/or phosphomevalonate using mevalonate and phosphomevalonate kinase (see, *Enzymatic Assays, Materials and Methods*). These contaminants are detected by coupling their phosphorylation to the oxidation of NADH (Andreassi et al. 2004; Pilloff et al. 2003). Contamination with either species was < 2%.

[0055] *Confirming the structure and labeling of the compounds.* The specificity and efficiency of isotopic labeling were assessed using ^{1}H and ^{13}C NMR. Deuterium incorporation at a given position was assessed by quantitating the loss of proton signal at that position. The ^{1}H NMR spectra of the synthesized compounds are compiled in Figures 4A and 6. In all cases, proton signal at the targeted position(s) was below detection (i.e., > 97% incorporation efficiency) and the integrated intensities of the remaining proton peaks were identical within error (± 3%); thus, deuterium did not incorporate significantly into positions other than the target sites. Comparison of the ^{1}H spectra of [1-^{13}C]- and natural abundance C_1-DPM reveals that the AB quartet associated with the C_2 of DPM (2.41 ppm) is split to into an ABX pattern by the incorporation of ^{13}C (Fig 4A). This splitting is consistent only with ^{13}C incorporation at C_1. If the synthesis had resulted in a significant fraction of natural abundance C_1-DPM, the AB and ABX resonances are expected to overlap. Close inspection of the upfield doublet of the ABX pattern gives no indication of the AB species (Fig 4 inset) indicating that the incorporation efficiency is quite high (> 95%). The labeling specificity of [1-^{13}C]DPM is given by the ^{13}C spectrum (Fig 4B), which shows the expected C_1-resonance (Levy and Nelson 1972) and no detectible signal at the positions associated with the other carbon atoms in the molecule (dotted arrows).

[0056] *The synthesis of highly concentrated DPM and isopentenyldiphosphate.* Given the considerable societal value of isoprenoids, the difficulties obtaining them, and the current efforts to bio-synthesize these compounds at commercial scale, it was of interest to assess the

potential of the *in-situ* enzymatic synthesis to produce large quantitie

this end, the velocity of the acetate-to-DPM conversion was studied as a function of initial-reactant concentration with the goal of determining the highest, useful concentrations. The system proved remarkably robust. Only slight inhibition (~ 30%) was observed at 0.50 M acetate. PEP and NADPH could be increased to near saturation (~ 500 and 200 mM, respectively) without significant decrease in velocity, and ATP could be added to 0.15 M without inhibition or noticeable precipitation. The concentration-optimized system contained acetate, ATP, PEP and NADPH at 0.35, 0.10, 0.40, 0.30 M, respectively, and yielded DPM and IPP at 22 and 18 g/liter, respectively – 63% and 69% conversions of acetate to product (Fig 5). Product formation in these reactions was limited by the solubility of nucleotide. To assess whether the enzymatic system was capable of producing even higher product concentrations, DPM and IPP synthesis was initiated from mevalonate (see, *Materials and Methods*). Reactions contained (R/S) mevalonate, ATP, and PEP at 0.370, 0.05, 0.35 M, respectively, and yielded DPM and IPP at 42 and 35 g/liter, respectively – 73% and 76% product yields (Fig 5).

[0057] Attempts to genetically engineer plants and bacteria to produce commercial quantities of isoprenoids have met with variable success. Artemisinin, a potent antimalarial, is currently isolated from the *quinghao* plant (*Artemisia annua*) at ~ 3 mg/g dry weight. In contrast, genetically engineered tobacco produces artemisinin at ~ 0.8 mg/g dry weight (Fox 2011), and transgenic yeast secrete artemisinic acid (an artemisinin precursor (Brown 2010) at 100 mg/liter (Ro et al. 2006). Using *E. coli* as the host, pathway optimization has yielded ~ 0.3 g/liter of artemisinic acid in shaking flasks (Anthony et al. 2009; Wu et al. 2011), while high-density batch fermentation of engineered *E. coli* has produced yields as high as 23g/liter (Tsuruta et al. 2009). Similar efforts in *E. coli* have produce taxadiene (a precursor of Taxol, an anticancer therapeutic) at ~ 1g/liter in shaking flasks (Ajikumar et al. 2010). Production of farnesol, a relatively simple isoprenoid and potential biofuel (Rude and Schirmer 2009), has reached 130 mg/liter in engineered *E. coli* grown in shaker flasks (Wang et al. 2010).

[0058] While these efforts have helped define the complexities associated with expressing and controlling the isoprenoid biosynthetic pathway in living organisms, only high-density fermentation in conjunction with genetic engineering is yielding product quantities required for successful commercial application. The cell-free approach described here yields product quantities that are comparable to, or exceed those achieved in high-density fermentation and have the advantage that product is formed in a simple, aqueous system from which it can be recovered readily.

Table I. Enzymes used in the synthesis of DPM.

[a]Enzyme	EC #	Gene	Source	Substrate	[d]K_m (mM)	[d]k_{cat} (sec^{-1})
[b]ACS	6.2.1.1	*Acs1*	*S. cerevisiae*	Acetate CoA	0.28 0.24	10
[c]*ACT*	2.3.1.9	*mvaE*	*E. faecalis*	Ac-CoA	0.60	2.3
[c]HMGS2	2.3.3.10	*mvaS*	*E. faecalis*	acac-CoA Ac-CoA	0.015 0.35	1.0
[c]ThRed	1.1.1.34	*mvaE*	*E. faecalis*	HMG-CoA	0.023	0.55
[c]HMGR	1.1.1.34	*mvaE*	*E. faecalis*	HMG-CoA	0.020	0.67
[c]MVK	2.7.1.36	*mvaK 1*	*S. aureus*	Mev	0.027	19
[c]PMK	2.7.4.2	*mvaK 2*	*S. pneumoniae*	P-mev	0.0042	5.0
[b]PK	2.7.1.40	*PKM2*	*O. cuniculus*	PEP	0.040	160
[b]MK	2.7.4.3	*AK1*	*O. cuniculus*	AMP	[e]0.50	[e]410
[b]PP_iase	3.6.1.1	*Ppa1*	*S. cerevisiae*	PP_i	[e]0.0050	[e]260

[a]Abbreviations: ACS, acetyl-CoA synthetase; ACT, acetoacetyl-CoA thiolase; HMGS2, HMG-CoA synthase; ThRed, acetoacetyl-CoA thiolase/ HMG-CoA reductase (dual-function enzyme); HMGR, HMG-CoA reductase; MVK, mevalonate kinase; PMK, phosphomevalonate kinase; PK, pyruvate kinase; MK, myokinase; PP_iase, inorganic pyrophosphatase. [b]Obtained from commercial sources. [c]Expressed in *E. coli* and purified. [d]Standard errors are < 5% in all cases (see *Materials and Methods*). [e]Parameters taken from literature (MK (Noda 1958; Noda 1962), PP_iase (Pohjanjoki et al. 1998; Zyryanov et al. 2002)).

REFERENCES

Ajikumar PK, Xiao WH, Tyo KE, Wang Y, Simeon F, Leonard E, Mucha O, Phon TH, Pfeifer B, Stephanopoulos G. 2010. Isoprenoid pathway optimization for Taxol precursor overproduction in Escherichia coli. Science 330(6000):70-4.

Albery WJ, Knowles JR. 1976. Evolution of enzyme function and the development of catalytic efficiency. Biochemistry 15(25):5631-40.

Andreassi JL, 2nd, Dabovic K, Leyh TS. 2004. Streptococcus pneumoniae isoprenoid biosynthesis is downregulated by diphosphomevalonate: an antimicrobial target. Biochemistry 43(51):16461-6.

Anthony JR, Anthony LC, Nowroozi F, Kwon G, Newman JD, Keasling JD. 2009. Optimization of the mevalonate-based isoprenoid biosynthetic pathway in Escherichia

coli for production of the anti-malarial drug precursor amorp
Eng 11(1):13-9.

Arsenault PR, Wobbe KK, Weathers PJ. 2008. Recent advances in artemisinin production through heterologous expression. Curr Med Chem 15(27):2886-96.

Brown GD. 2010. The biosynthesis of artemisinin (Qinghaosu) and the phytochemistry of Artemisia annua L. (Qinghao). Molecules 15(11):7603-98.

Burbaum JJ, Raines RT, Albery WJ, Knowles JR. 1989. Evolutionary optimization of the catalytic effectiveness of an enzyme. Biochemistry 28(24):9293-305.

Caputi L, Aprea E. 2011. Use of terpenoids as natural flavouring compounds in food industry. Recent Pat Food Nutr Agric 3(1):9-16.

Cederstav AK. 1994. Investigations into the Chemistry of Thermodynamically Unstable Species. The Direct Polymerization of Vinyl Alcohol, the Enolic Tautomer of Acetaldehyde. Journal of the American Chemical Society 116(9):4073-4076.

Cheng Q, Xiang L, Izumikawa M, Meluzzi D, Moore BS. 2007. Enzymatic total synthesis of enterocin polyketides. Nat Chem Biol 3(9):557-8.

Clinkenbeard KD, Sugiyama T, Lane MD. 1975. Cytosolic acetoacetyl-CoA thiolase from chicken liver. Methods Enzymol 35:167-73.

Cornforth JW, Phillips GT, Messner B, Eggerer H. 1974. Substrate stereochemistry of 3-hydroxy-3-methylglutaryl-coenzyme A synthase. Eur J Biochem 42(2):591-604.

Cornforth RH, Fletcher K, Hellig H, Popjak G. 1960. Stereospecificity of enzymic reactions involving mevalonic acid. Nature 185:923-4.

Dugar D, Stephanopoulos G. 2011. Relative potential of biosynthetic pathways for biofuels and bio-based products. Nat Biotechnol 29(12):1074-8.

Eijsink VG, Gaseidnes S, Borchert TV, van den Burg B. 2005. Directed evolution of enzyme stability. Biomol Eng 22(1-3):21-30.

Farhi M, Marhevka E, Ben-Ari J, Algamas-Dimantov A, Liang Z, Zeevi V, Edelbaum O, Spitzer-Rimon B, Abeliovich H, Schwartz B and others. 2011. Generation of the potent anti-malarial drug artemisinin in tobacco. Nat Biotechnol 29(12):1072-4.

Fox JL. 2011. Interest groups jostle to influence PDUFA V. Nat Biotechnol 29(12):1062.

Gerlt JA, Babbitt PC. 2009. Enzyme (re)design: lessons from natural evolution and computation. Curr Opin Chem Biol 13(1):10-8.

Gorenstein DG. 1984. Phosphorus-31 NMR : principles and applications. Orlando, Fla.: Academic Press. xiv, 604 p. p.

Hale V, Keasling JD, Renninger N, Diagana TT. 2007. Microbially biotechnology solution to the global problem of access to affordable antimalarial drugs. Am J Trop Med Hyg 77(6 Suppl):198-202.

Hedl M, Sutherlin A, Wilding EI, Mazzulla M, McDevitt D, Lane P, Burgner JW, 2nd, Lehnbeuter KR, Stauffacher CV, Gwynn MN and others. 2002. Enterococcus faecalis acetoacetyl-coenzyme A thiolase/3-hydroxy-3-methylglutaryl-coenzyme A reductase, a dual-function protein of isopentenyl diphosphate biosynthesis. J Bacteriol 184(8):2116-22.

Hemmerlin A, Rivera SB, Erickson HK, Poulter CD. 2003. Enzymes encoded by the farnesyl diphosphate synthase gene family in the Big Sagebrush Artemisia tridentata ssp. spiciformis. J Biol Chem 278(34):32132-40.

Hsu E. 2006. The history of qing hao in the Chinese materia medica. Trans R Soc Trop Med Hyg 100(6):505-8.

Huang KT, Wu BC, Lin CC, Luo SC, Chen C, Wong CH. 2006. Multi-enzyme one-pot strategy for the synthesis of sialyl Lewis X-containing PSGL-1 glycopeptide. Carbohydr Res 341(12):2151-5.

Kajiwara Y, Santander PJ, Roessner CA, Perez LM, Scott AI. 2006. Genetically engineered synthesis and structural characterization of cobalt-precorrin 5A and -5B, two new intermediates on the anaerobic pathway to vitamin B12: definition of the roles of the CbiF and CbiG enzymes. J Am Chem Soc 128(30):9971-8.

Koeller KM, Wong CH. 2001. Enzymes for chemical synthesis. Nature 409(6817):232-40.

Kredich NM, Tomkins GM. 1966. The enzymic synthesis of L-cysteine in Escherichia coli and Salmonella typhimurium. J Biol Chem 241(21):4955-65.

Kuberan B, Lech MZ, Beeler DL, Wu ZL, Rosenberg RD. 2003. Enzymatic synthesis of antithrombin III-binding heparan sulfate pentasaccharide. Nat Biotechnol 21(11):1343-6.

Lacaze C, Kauss T, Kiechel JR, Caminiti A, Fawaz F, Terrassin L, Cuart S, Grislain L, Navaratnam V, Ghezzoul B and others. 2011. The initial pharmaceutical development of an artesunate/amodiaquine oral formulation for the treatment of malaria: a public-private partnership. Malar J 10:142.

Lefurgy ST, Rodriguez SB, Park CS, Cahill S, Silverman RB, Leyh TS. 2010. Probing ligand-binding pockets of the mevalonate pathway enzymes from Streptococcus pneumoniae. J Biol Chem 285(27):20654-63.

Levy GC, Nelson GL. 1972. Carbon-13 nuclear magnetic resonance New York,: Wiley-Interscience. xiii, 222 p. p.

Melnick JS, Sprinz KI, Reddick JJ, Kinsland C, Begley TP. 2003. An efficient enzymatic synthesis of thiamin pyrophosphate. Bioorg Med Chem Lett 13(22):4139-41.

Middleton B. 1974. The kinetic mechanism and properties of the cytoplasmic acetoacetyl-coenzyme A thiolase from rat liver. Biochem J 139(1):109-21.

Miziorko HM. 2011. Enzymes of the mevalonate pathway of isoprenoid biosynthesis. Arch Biochem Biophys.

Miziorko HM, Lane MD. 1977. 3-Hydroxy-3-methylgutaryl-CoA synthase. Participation of acetyl-S-enzyme and enzyme-S-hydroxymethylgutaryl-SCoA intermediates in the reaction. J Biol Chem 252(4):1414-20.

Neuhoff V. SR, Eibl, H. 1885. Clear background and highly sensitive protein staining with Coomassie Blue dyes in polyacrylamide gels: A systematic analysis. Electrophoresis 6(9):427-448.

Noda L. 1958. Adenosine triphosphate-adenosine monophosphate transphosphorylase. III. Kinetic studies. J Biol Chem 232(1):237-50.

Noda L. 1962. Nucleoside triphosphate-nucleoside monophosphokinases. In: Boyer PD, Lardy, H. and Myrbäck, K., editor. The Enzymes 2nd ed. New York: Academic Press. p 139-149.

Ohdan K, Fujii K, Yanase M, Takaha T, Kuriki T. 2007. Phosphorylase coupling as a tool to convert cellobiose into amylose. J Biotechnol 127(3):496-502.

Oldfield E, Lin FY. 2012. Terpene biosynthesis: modularity rules. Angew Chem Int Ed Engl 51(5):1124-37.

Osbourn A, Goss RJ, Field RA. 2011. The saponins: polar isoprenoids with important and diverse biological activities. Nat Prod Rep 28(7):1261-8.

Ozer N. 1985. A new enzyme-coupled spectrophotometric method for the determination of arginase activity. Biochem Med 33(3):367-71.

Peralta-Yahya PP, Ouellet M, Chan R, Mukhopadhyay A, Keasling JD, Lee TS. 2011. Identification and microbial production of a terpene-based advanced biofuel. Nat Commun 2:483.

Pilloff D, Dabovic K, Romanowski MJ, Bonanno JB, Doherty M, Burley SK, Leyh TS. 2003. The kinetic mechanism of phosphomevalonate kinase. J Biol Chem 278(7):4510-5.

Pilloff DE, Leyh TS. 2003. Allosteric and catalytic functions of the PPi-binding motif in the ATP sulfurylase-GTPase system. J Biol Chem 278(50):50435-41.

Pohjanjoki P, Lahti R, Goldman A, Cooperman BS. 1998. Evoluti
enzymatic catalysis: quantitative comparison of the effects of mutation of aligned residues in Saccharomyces cerevisiae and Escherichia coli inorganic pyrophosphatases on enzymatic activity. Biochemistry 37(7):1754-61.

Popjak G. 1970. Stereospecificity of Enzymic Reactions. In: Boyer PD, editor. The Enzymes. 3rd ed. New York: Academic Press, Inc. p 115-215.

Puri M, Sharma D, Tiwari AK. 2011. Downstream processing of stevioside and its potential applications. Biotechnol Adv 29(6):781-91.

Ro DK, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho KA, Eachus RA, Ham TS, Kirby J and others. 2006. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440(7086):940-3.

Rohmer M. 1999. The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. Nat Prod Rep 16(5):565-74.

Romanowski MJ, Bonanno JB, Burley SK. 2002. Crystal structure of the Streptococcus pneumoniae phosphomevalonate kinase, a member of the GHMP kinase superfamily. Proteins 47(4):568-71.

Rude MA, Schirmer A. 2009. New microbial fuels: a biotech perspective. Curr Opin Microbiol 12(3):274-81.

Schmid A, Dordick JS, Hauer B, Kiener A, Wubbolts M, Witholt B. 2001. Industrial biocatalysis today and tomorrow. Nature 409(6817):258-68.

Schultheisz HL, Szymczyna BR, Scott LG, Williamson JR. 2008. Pathway engineered enzymatic de novo purine nucleotide synthesis. ACS Chem Biol 3(8):499-511.

Sen S, Venkata Dasu V, Mandal B. 2007. Developments in directed evolution for improving enzyme functions. Appl Biochem Biotechnol 143(3):212-23.

Shapiro AL, Vinuela E, Maizel JV, Jr. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem Biophys Res Commun 28(5):815-20.

Sugiyama M, Hong Z, Liang PH, Dean SM, Whalen LJ, Greenberg WA, Wong CH. 2007. D-Fructose-6-phosphate aldolase-catalyzed one-pot synthesis of iminocyclitols. J Am Chem Soc 129(47):14811-7.

Sutherlin A, Hedl M, Sanchez-Neri B, Burgner JW, 2nd, Stauffacher CV, Rodwell VW. 2002. Enterococcus faecalis 3-hydroxy-3-methylglutaryl coenzyme A synthase, an enzyme of isopentenyl diphosphate biosynthesis. J Bacteriol 184(15):4065-70.

Sutherlin A, Rodwell VW. 2004. Multienzyme mevalonate pathway Bioeng 87(4):546-51.

Thulasiram HV, Erickson HK, Poulter CD. 2008. A common mechanism for branching, cyclopropanation, and cyclobutanation reactions in the isoprenoid biosynthetic pathway. J Am Chem Soc 130(6):1966-71.

Tsuruta H, Paddon CJ, Eng D, Lenihan JR, Horning T, Anthony LC, Regentin R, Keasling JD, Renninger NS, Newman JD. 2009. High-level production of amorpha-4,11-diene, a precursor of the antimalarial agent artemisinin, in Escherichia coli. PLoS One 4(2):e4489.

Van Den Heuvel RH, Fraaije MW, Laane C, van Berkel WJ. 2001. Enzymatic synthesis of vanillin. J Agric Food Chem 49(6):2954-8.

Wang C, Yoon SH, Shah AA, Chung YR, Kim JY, Choi ES, Keasling JD, Kim SW. 2010. Farnesol production from Escherichia coli by harnessing the exogenous mevalonate pathway. Biotechnol Bioeng 107(3):421-9.

Wishart DS, Bigam CG, Yao J, Abildgaard F, Dyson HJ, Oldfield E, Markley JL, Sykes BD. 1995. 1H, 13C and 15N chemical shift referencing in biomolecular NMR. J Biomol NMR 6(2):135-40.

Wouters J, Oudjama Y, Barkley SJ, Tricot C, Stalon V, Droogmans L, Poulter CD. 2003. Catalytic mechanism of Escherichia coli isopentenyl diphosphate isomerase involves Cys-67, Glu-116, and Tyr-104 as suggested by crystal structures of complexes with transition state analogues and irreversible inhibitors. J Biol Chem 278(14):11903-8.

Wu T, Wu S, Yin Q, Dai H, Li S, Dong F, Chen B, Fang H. 2011. [Biosynthesis of amorpha-4,11-diene, a precursor of the antimalarial agent artemisinin, in Escherichia coli through introducing mevalonate pathway]. Sheng Wu Gong Cheng Xue Bao 27(7):1040-8.

Yamaguchi S, Komeda H, Asano Y. 2007. New enzymatic method of chiral amino acid synthesis by dynamic kinetic resolution of amino acid amides: use of stereoselective amino acid amidases in the presence of alpha-amino-epsilon-caprolactam racemase. Appl Environ Microbiol 73(16):5370-3.

Zhang F, Rodriguez S, Keasling JD. 2011. Metabolic engineering of microbial pathways for advanced biofuels production. Curr Opin Biotechnol 22(6):775-83.

Zyryanov AB, Shestakov AS, Lahti R, Baykov AA. 2002. Mechanism by which metal cofactors control substrate specificity in pyrophosphatase. Biochem J 367(Pt 3):901-6.

What is claimed is:

1. A method of synthesizing (R)-5-diphosphomevalonate (DPM) comprising the steps of:

i) synthesizing acetyl-Coenzyme A (Ac-CoA) from acetate, Coenzyme A (CoA) and adenosine-5'-triphosphate (ATP) in the presence of acetyl-CoA synthetase;

ii) synthesizing acetoacetyl-CoA (acac-CoA) from Ac-CoA in the presence of acetoacetyl-coA thiolase;

iii) synthesizing hydroxymethylglutaryl-CoA (HMG-CoA) from acac-CoA and Ac-CoA in the presence of hydroxymethylglutaryl-CoA synthase;

iv) synthesizing (R/S)-mevalonate (Mev) from HMG-CoA and reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of hydroxymethylglutaryl-CoA reductase;

v) synthesizing phosphorylated Mev (PMev) from Mev and ATP in the presence of mevalonate kinase; and

vi) synthesizing DPM from PMev and ATP in the presence of phosphomevalonate kinase.

2. The method of claim 1, wherein CoA generated during any of steps ii), iii) or iv) is used in the synthesis of Ac-CoA.

3. The method of claim 1 or 2, wherein pyrophosphate generated in step i) is hydrolyzed to phosphate ions by inorganic pyrophosphatase.

4. The method of any of claims 1-3, wherein adenosine-5'-monphosphate (AMP) generated in step i) is converted to adenosine-5'-diphosphate (ADP) by adenylate kinase.

5. The method of any of claims 1-4, wherein adenosine-5'-diphosphate (ADP) generated in step v) or vi) is converted to adenosine-5'-triphosphate (ATP) by pyruvate kinase.

6. The method of any of claims 1-5, wherein the mevalonate kinase used in step v) is from *S. aureus*.

7. The method of any of claims 1-6, wherein an isotopic label is introduced in the DMP synthesis.

8. The method of claim 7, wherein the label is a carbon label or a hydrogen label.

9. The method of any of claims 1-8, wherein an isotopic label is introduced in the DMP synthesis using labeled acetate or labeled acetyl-CoA.

10. A method of synthesizing isopentenyl 5-pyrophosphase (IPP) comprising synthetizing (R)-5-diphosphomevalonate (DPM) by the method of any of claims 1-9 and converting DPM to IPP.

11. A method of synthesizing dimethylallyl 5-pyrophosphate (DMAPP) comprising synthetizing isopentenyl 5-pyrophosphase (IPP) by the method of claim 10 and converting IPP to DMAPP.

12. A method of synthesizing an isoprenoid comprising synthetizing IPP by the method of claim 10 and synthetizing DMAPP by the method of claim 11, and forming a carbon backbone of the isoprenoid using IPP and DMAPP.

13. The method of any of claims 1-12, where the synthesis reactions are carried out as a one-pot synthesis.

14. A (R)-5-diphosphomevalonate (DPM) produced by the method of any of claims 1-9 or 13.

15. An isopentenyl 5-pyrophosphase (IPP) produced by the method of claim 10 or 13.

16. A dimethylallyl 5-pyrophosphate (DMAPP) produced by the method of claim 11 or 13.

17. An isoprenoid produced by the method of claim 12 or 13.

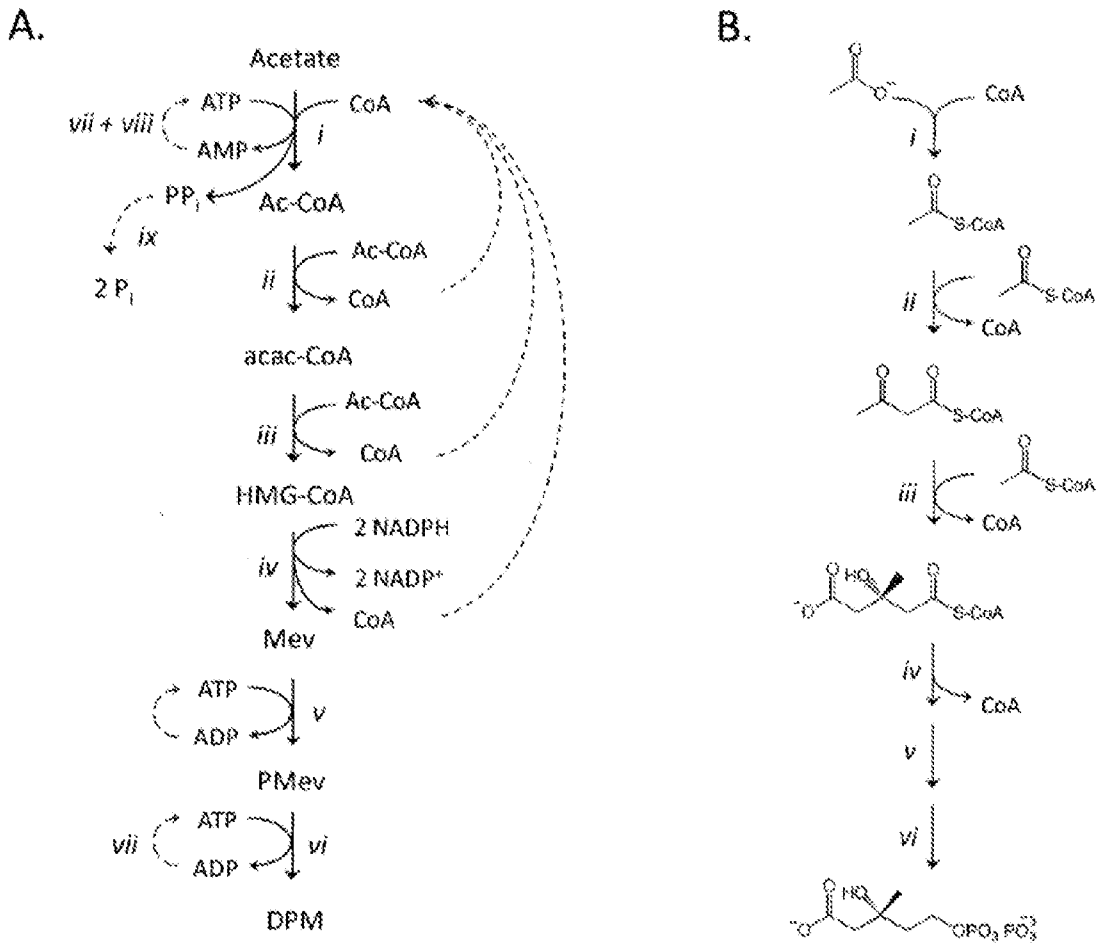

A.
Acetate
ATP
CoA
vii + viii
i
AMP
PP$_i$
Ac-CoA
ix
2 P$_i$
ii
Ac-CoA
CoA
acac-CoA
iii
Ac-CoA
CoA
HMG-CoA
2 NADPH
iv
2 NADP+
CoA
Mev
ATP
v
ADP
PMev
ATP
vii
vi
ADP
DPM
B.
CoA
i
S-CoA
ii
S-CoA
CoA
S-CoA
iii
S-CoA
CoA
HO
S-CoA
iv
CoA
v
vi
HO
OPO$_3$ PO$_3^{2-}$

FIGURE 1A-1B

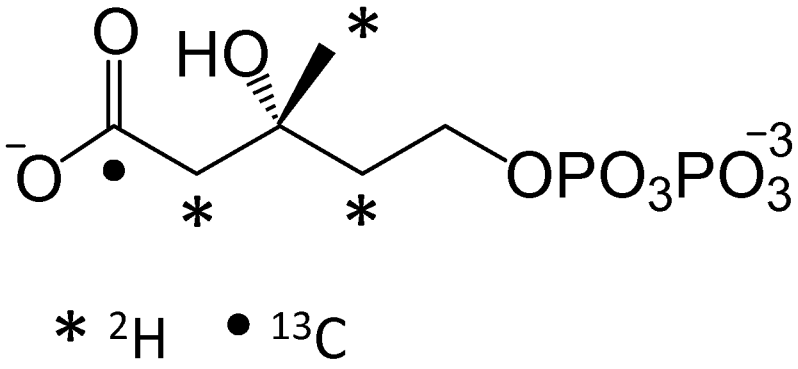


FIGURE 2

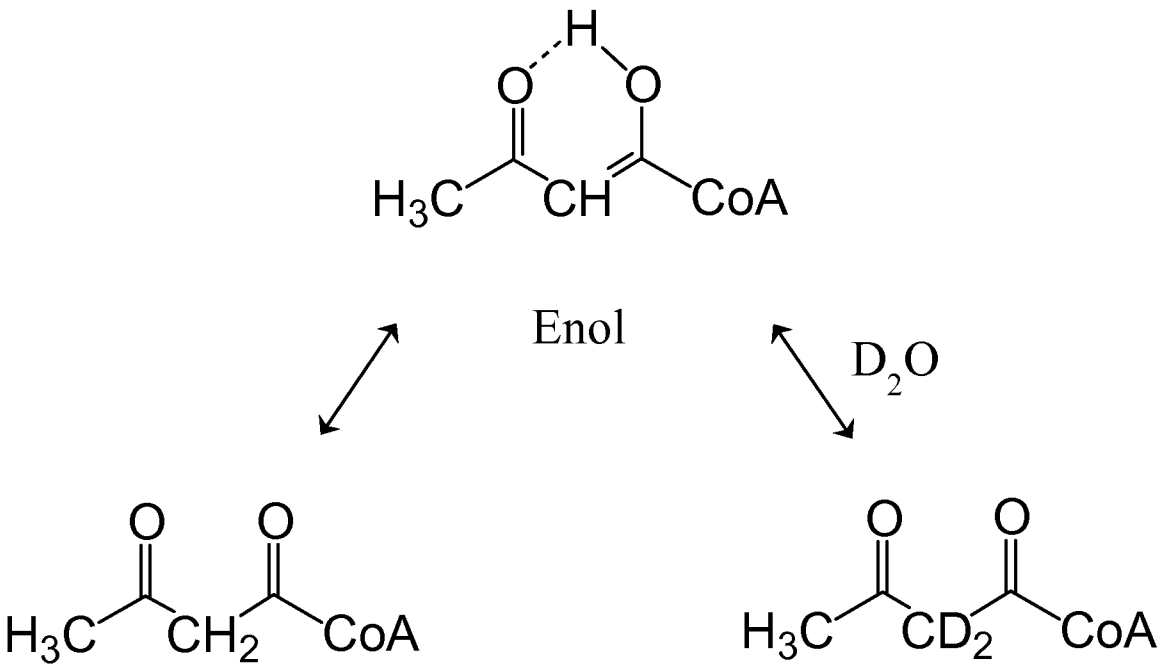


FIGURE 3

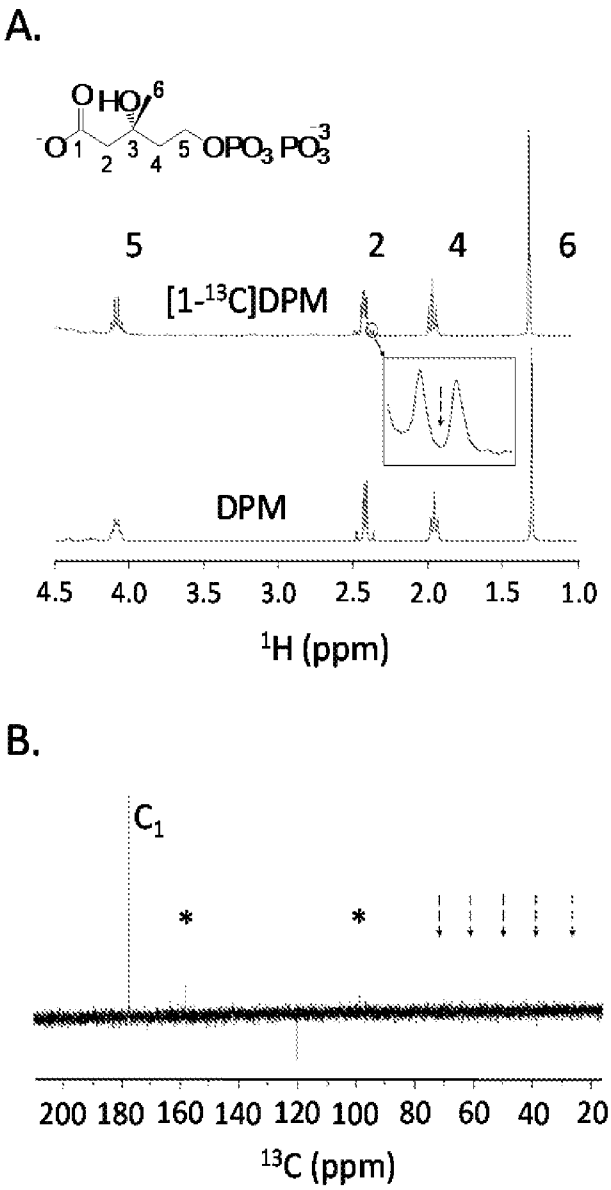

A.
5
2
4
6
[1-13C]DPM
DPM
4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0
1H (ppm)
B.
C1
*
*
200 180 160 140 120 100 80 60 40 20
13C (ppm)

FIGURE 4A-4B

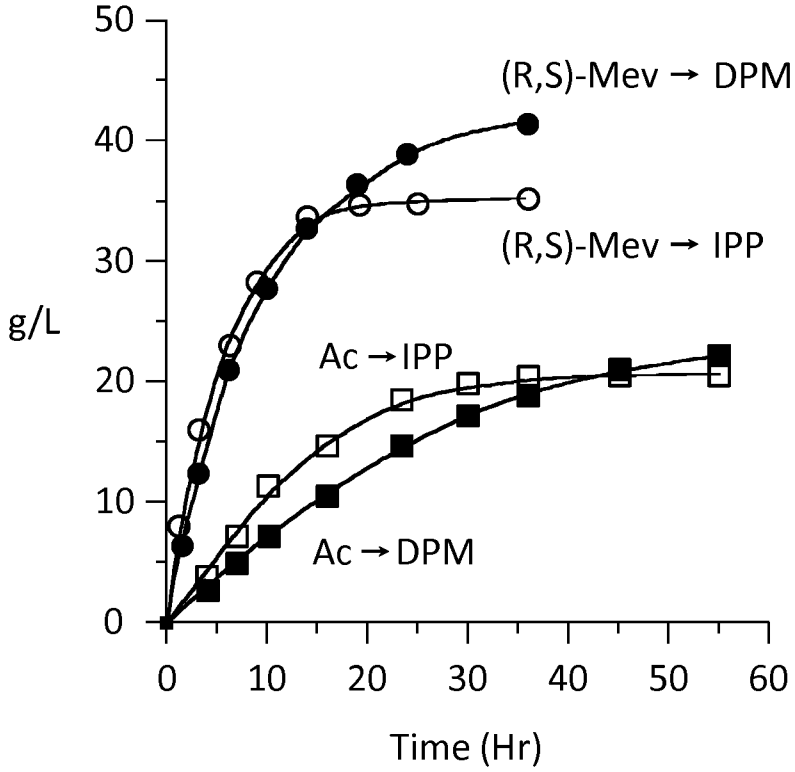

50
40
30
20
10
0
g/L
0
10
20
30
40
50
60
Time (Hr)
(R,S)-Mev → DPM
(R,S)-Mev → IPP
Ac → IPP
Ac → DPM

FIGURE 5

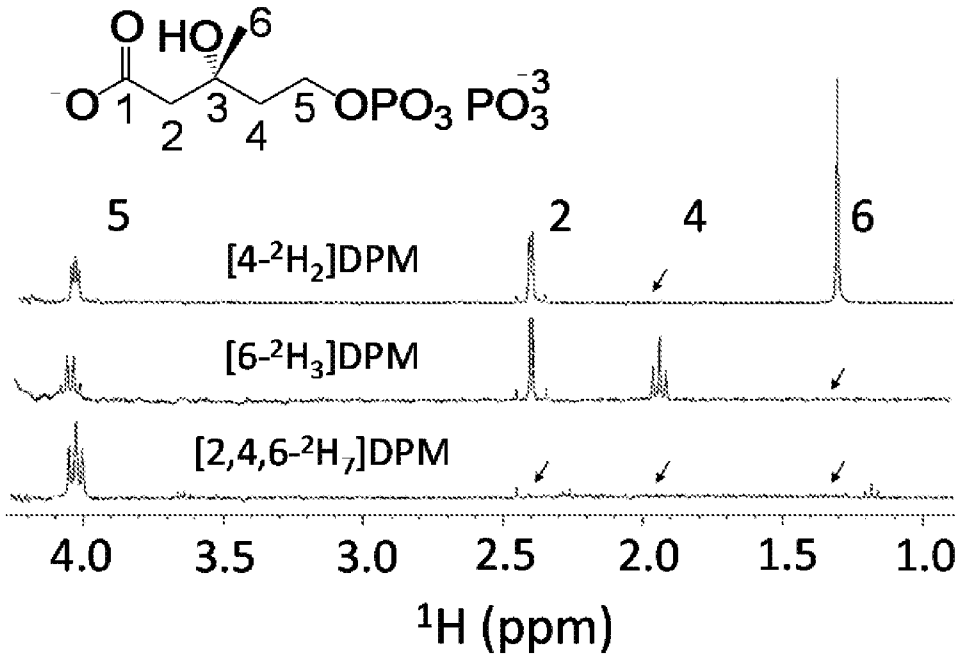

O HO 6
O 1 2 3 4 5 OPO3 PO3 -3
5
2
4
6
[4-2H2]DPM
[6-2H3]DPM
[2,4,6-2H7]DPM
4.0
3.5
3.0
2.5
2.0
1.5
1.0
1H (ppm)

FIGURE 6

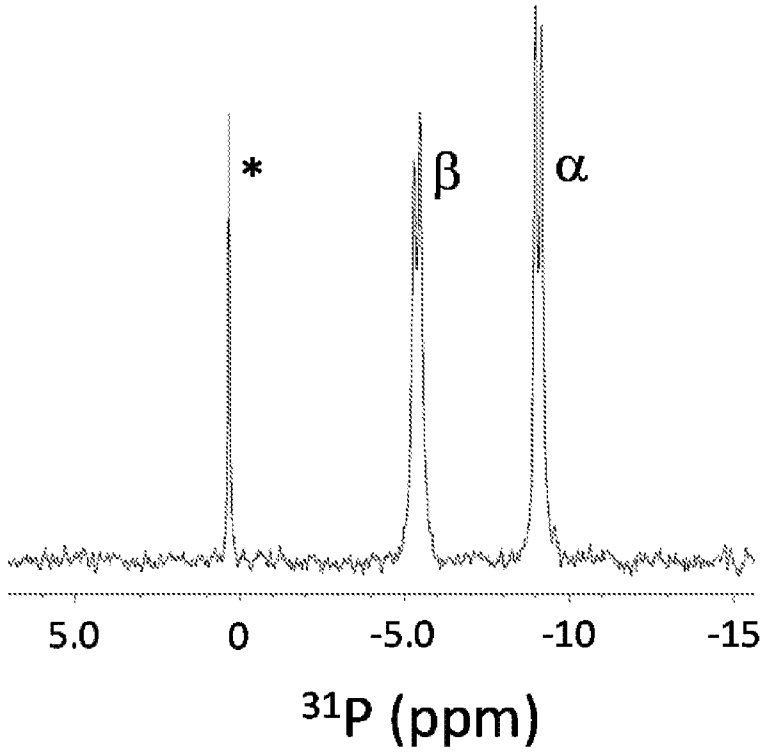


FIGURE 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US13/59857

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12P 5/02, 5/00, 7/04 (2013.01)
USPC - 435/132, 136, 146
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C12P 5/02, 5/00, 7/04, 7/00 (2013.01)
USPC: 435/167, 166, 41, 132, 136, 146

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Proquest Dialog (Pharmaceutical & Biomedical); Google/Google Scholar; Pubmed/Pubmed central; Synthesis, Production, Biosynthesis, Diphosphomevalonate, Pyrophosphomevalonate, mevalonate-5-pyrophosphate, mevalonate, pathway, acetyl-CoA regeneration, inorganic pyrophosphatase

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WOODYER, R et al. Cofactor Regeneration for Biocatalytic Applications. In Enzyme Technology. Chapter 5, pp. 83-101, Asiatech Publishers Inc., New Delhi, India, 2004. [Retrieved from the internet 13 December 2013] <URL: http://www.scs.illinois.edu/~zhaogrp/publications/HZ21.pdf>	1, 2, 3/1, 3/2
Y	US 2005/0266518 A1 (BERRY, A et al.) December 1, 2005; abstract; figure 1A; paragraphs [0031], [0071]	1, 2, 3/1, 3/2
Y	US 5436143 A (HYMAN, E) July 25, 1995; column 17, lines 5-9	3/1, 3/2
A	WO 2000/001649 A1 (MILLIS, J et al.) January 13, 2000; entire document	1, 2, 3/1, 3/2
A	SUTHERLIN, A et al. Multienzyme Mevalonate Pathway Bioreactor. Biotechnol Bioeng. 20 August 2004, Vol. 87, No. 4, pp.546-551; entire document; DOI: 10.1002/bit.20157	1, 2, 3/1, 3/2

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 December 2013 (19.12.2013)	30 DEC 2013
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US13/59857

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-17
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.