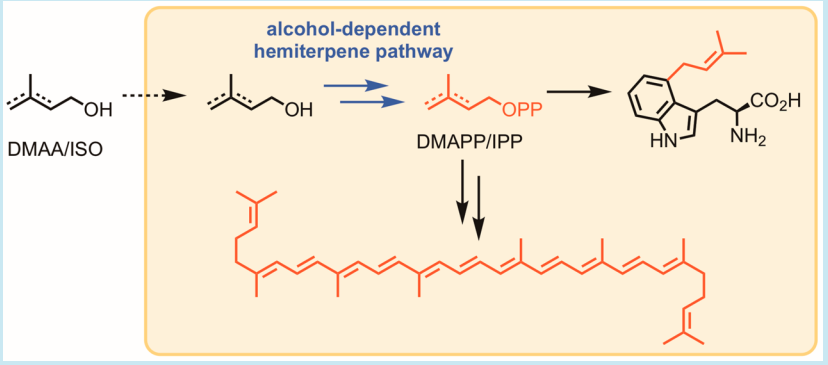
ACS Synthetic Biology
Cite This: ACS Synth. Biol. 2019, 8, 232−238
Letter
pubs.acs.org/synthbio

An Artificial Pathway for Isoprenoid Biosynthesis Decoupled from Native Hemiterpene Metabolism

Sean Lund,[†,#] Rachael Hall,[†] and Gavin J Williams*[,†,§]

[†]Department of Chemistry, and [§]Comparative Medicine Institute, North Carolina State University, Raleigh, North Carolina 27695, United States

Supporting Information



ABSTRACT: Isoprenoids are constructed in nature using hemiterpene building blocks that are biosynthesized from lengthy enzymatic pathways with little opportunity to deploy precursor-directed biosynthesis. Here, an artificial alcohol-dependent hemiterpene biosynthetic pathway was designed and coupled to several isoprenoid biosynthetic systems, affording lycopene and a prenylated tryptophan in robust yields. This approach affords a potential route to diverse non-natural hemiterpenes and by extension isoprenoids modified with non-natural chemical functionality. Accordingly, the prototype chemo-enzymatic pathway is a critical first step toward the construction of engineered microbial strains for bioconversion of simple scalable building blocks into complex isoprenoid scaffolds.

KEYWORDS: *isoprenoids, terpenoids, hemiterpene, kinase, lycopene, prenyltransferase*

Isoprenoids comprise >55 000 natural products for which methods to access and diversify their structures are in high demand.[1−3] Ultimately, the isoprene motif plays a critical role in modulating the biological activity of isoprenoids, determines their utility as tools to study and treat human diseases, and provides the basis to develop new fuels and chemicals.[4−7] Notably, although several valuable isoprenoids have been accessed *via* heterologous expression,[8−11] our ability to diversify isoprenoids is extremely limited largely due to critical limitations imposed by native isoprenoid biosynthesis. First, only the mevalonate (MEV) and 1-deoxy-D-xylulose-5-phosphate (DXP) pathways (Figure 1A) are known to produce the universal hemiterpene isoprenoid diphosphate building blocks, dimethylallyl pyrophosphate (DMAPP), and isopentenyl pyrophosphate (IPP).[12,13] The negatively charged hemiterpenes are not cell permeable, thus preventing feeding them or analogues thereof into cultures. The MEV and DXP pathways involve at least six enzymatic steps[13] each with stringent substrate specificity and therefore offer little opportunity to diversify the structures of isoprenoids through feeding in non-natural precursors.[14] As a result, while precursor-directed biosynthesis has proven a powerful approach to access diverse structures of natural products[15,16]—especially polyketides[17−21]—by feeding non-natural building blocks, this approach has not yet been applied to isoprenoids. Furthermore, late-stage biosynthetic modification of isoprenoid scaffolds is typically limited to oxidations, often catalyzed by P450s.[11,22] Second, terpene metabolism is highly regulated and is a burden to the carbon supply on the cell.[23] For example, the MEV pathway uses three molecules of phosphate donor (ATP) and two reducing equivalents (NADPH) for each DMAPP/IPP, while the DXP pathway requires two phosphate donors (ATP and CTP) and two reducing equivalents (NADPH) (Figure 1A).[13] Third, given that native terpenes are typically essential for maintenance of the cell, genetic modification of native hemiterpene pathways would likely be lethal.[24] Together, these limitations could be overcome by supplying a membrane-permeable carbon building block dedicated for a designer pathway that would function independent of native isoprenoid metabolism. A potential strategy for hemiterpene biosynthesis could start with isopentenol (ISO) and dimethylallyl alcohol (DMAA) which are converted to the required diphosphates *via* stepwise

Received: September 11, 2018
Published: January 16, 2019

DOI: 10.1021/acssynbio.8b00383
ACS Synth. Biol. 2019, 8, 232−238

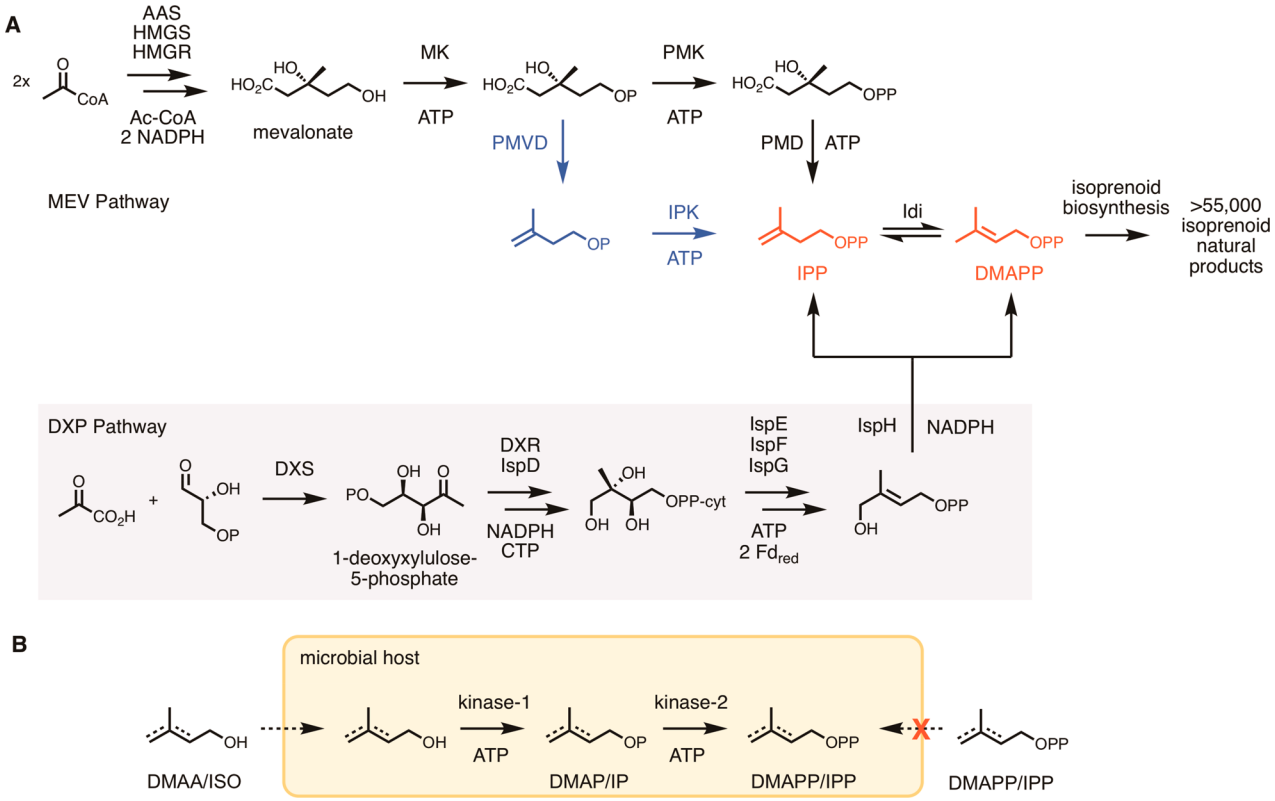


Figure 1. Natural and engineered hemiterpene biosynthetic pathways. (A) The MEV and DXP (gray box) pathways. A branch of the MEV pathway, the archaeal MEV I pathway, is shown in blue. For full names of enzymes, see ref 12. (B) An artificial alcohol-dependent hemiterpene (ADH) pathway completely decoupled from native isoprenoid metabolism.

phosphorylation catalyzed by two independent kinases (Figure 1B). This proposed alcohol-dependent hemiterpene (ADH) pathway is completely orthogonal to the endogenous DMAPP/IPP biosynthetic machinery, such that non-natural precursors are not expected to inhibit endogenous enzymatic machinery. In addition, the ADH pathway requires only two equivalents of ATP and no other cofactors. Furthermore, an artificial pathway designed "bottom-up" as a replacement for natural hemiterpene biosynthesis could leverage naturally or engineered promiscuous enzymes. In this way, the ADH pathway could enable a broad panel of easily scalable and accessible alcohols to be converted to the corresponding diphosphate, thus providing a simple strategy to probe the plasticity of downstream isoprenoid biosynthesis *in vivo* or *in vitro*. In this study, as a necessary first step to realizing this goal, we report the design and development of a prototype ADH pathway that is completely orthogonal to native hemiterpene biosynthesis. The ability of this pathway to access isoprenoids is demonstrated by coupling the ADH pathway to two different isoprenoid biosynthetic systems.

Inspired by the observation that several mammalian cell lines convert farnesol and farnesol analogues to the corresponding disphosphates,[25−27] it was first determined whether *E. coli* harbors suitable enzymatic machinery that could convert exogenously provided ISO or DMAA into a pool of hemiterpenes for isoprenoid production. To test this, a previously reported reporter system that leverages lycopene biosynthesis was used that includes genes from the *CrtEBI* operon, a geranylgeranyl diphosphate synthase (CrtE or IspA Y80D), and the isopentenyl diphosphate isomerase *ipi* (Figure 2A).[28] In *E. coli*, this reporter system expresses genes that use pools of hemiterpenes to generate the lycopene pigment, enabling quantification.[28,29] The intensity of the absorbance at 450−470 nm is directly associated with an increase in hemiterpene production given that the reporter system itself is not rate limiting.[30−33]

The native DXP pathway in the *E. coli* reporter strains supports production of lycopene independently of exogenously added DMAA/ISO. Because of this, fosmidomycin (Fs), an inhibitor of the first dedicated step in hemiterpene biosynthesis (Figure 1A),[34,35] was leveraged to knock-down endogenous lycopene production in order to determine whether any endogenous machinery could support conversion of DMAA/ISO to hemiterpenes (Figure 2A). Fs was added to the culture medium at sufficient concentration (0.5 μM) to inhibit the DXP pathway[34] but at low enough concentration growth was not significantly suppressed. In this way, the goal was to employ Fs to prevent accumulation of excess DXP-dependent hemiterpene, forcing production of lycopene solely from the exogenously fed precursors *via* potential unknown endogenous enzymes. Following the addition of DMAA and ISO (each at 2.5 mM) and Fs (0.5 μM) to an overnight culture of *E. coli* BL21Tuner(DE3) harboring the lycopene reporter plasmids pCDFDuet-GGPP + pACYCDuet-Lyc, lycopene production was quantified by visual examination of the culture broth. However, no difference in lycopene production was observed upon comparison of the *E. coli* strain to an otherwise identical culture prepared in the absence of DMAA/ISO (Supplementary Figure S1). Thus, the *E. coli* native metabolism does not support conversion of the ISO/DMAA mixture to lycopene at a rate greater than the inhibited DXP-pathway. Thus, our efforts shifted to identification of enzymes that could be

DOI: 10.1021/acssynbio.8b00383
ACS Synth. Biol. 2019, 8, 232−238

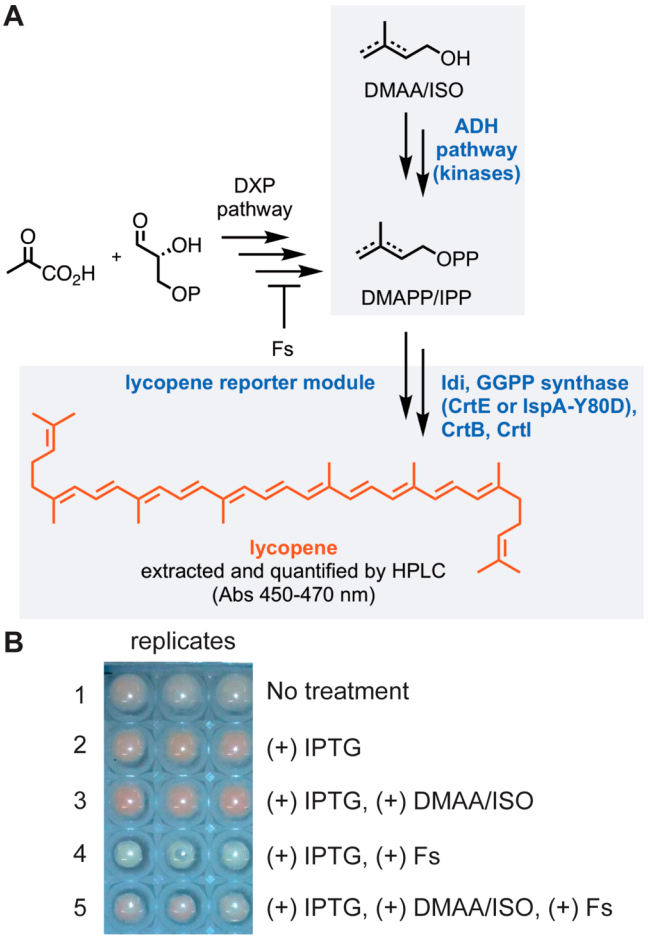


Figure 2. Lycopene reporter system and preliminary characterization of IPK. (A) The reporter system module generates lycopene from cellular hemiterpenes. Fs is expected to inhibit native hemiterpene biosynthesis *via* the DXP pathway but not an artificial alcohol-dependent hemiterpene pathway. (B) *E. coli* BL21Tuner(DE3) harboring pCDFDuet-GGPP, pACYCDuet-Lyc, and pETDuet-IPK in wells of a microplate were treated with combinations of IPTG, DMAA/ISO, and Fs and visualized.

heterologously expressed in *E. coli* and function as kinase-2 in the proposed ADH pathway.

A protein recently found in archaea, isopentenyl phosphate kinase (IPK),[36,37] is responsible for the generation of IPP from isopentenyl phosphate (IP) and forms a branch of the MEV pathway called the Archaeal MEV Pathway I (Figure 1A). Overexpression of IPK in *E. coli* could lead to improved production of lycopene from exogenously added ISO if (1) an endogenous kinase (kinase-1, Figure 1B) can convert ISO to the corresponding monophosphate, and (2) the obligatory second phosphorylation (Figure 1B) contributes to the rate-limiting step in the conversion of ISO to lycopene in *E. coli*. To test these assumptions, a codon-optimized gene sequence for *ipk* from *Thermoplasma acidophilum* (Supplementary Table S1) was expressed in a strain of *E. coli* harboring a lycopene reporter module and was incubated either in the presence or absence of supplementary DMAA/ISO, IPTG, and Fs. The expression of IPK clearly resulted in increased lycopene (compare 1−3, Figure 2B). In addition, the presence of Fs almost completely inhibited lycopene production (2 vs 4, Figure 2B). However, an increase in lycopene production was observed when the Fs-treated *E. coli* cultures that expressed IPK were supplemented with DMAA/ISO (Figure 2B). This increase in lycopene production that was dependent on IPK and DMAA/ISO validates the assumption that an endogenous kinase is capable of providing IPP from DMAA/ISO and that IPK supports the second required phosphorylation.

It was reasoned that overexpression of the *E. coli* gene product putatively responsible for DMAA/ISO phosphorylation would result in improved production of hemiterpenes. In a preliminary attempt to identify a suitable enzyme that could act as "kinase-1" (Figure 1B), a set of 12 soluble alcohol kinases from *E. coli*, *S. cerevisiae*, and *A. thaliana*, in addition to PhoN, a class-A nonspecific acid phosphatase from *Shigella flexneri* (Supplementary Table S2) were cloned, expressed in *E. coli*, subjected to immobilized-metal affinity chromatography, and analyzed by LC−MS analysis for their ability to phosphorylate DMAA/ISO. PhoN was included in this set as it has previously been shown to phosphorylate various alcohols *in vitro*.[38,39] Notably, while none of the kinases displayed the desired activity (data not shown), mass ions consistent with DMAPP (calculated 165.0317 m/z, $[M\text{-}H]^-$; observed 165.0318 m/z, $[M\text{-}H]^-$) and IPP (calculated 165.0317 m/z, $[M\text{-}H]^-$; observed 165.0317 m/z, $[M\text{-}H]^-$) were detected in the presence of PhoN. Another potential candidate for kinase-1 is a membrane-associated diacylglycerol kinase (DGK) from *Streptococcus mutans* which is known to display undecaprenol-kinase activity.[40] Given that DGK is membrane-bound, it was tested *in vivo*. In parallel, PhoN was also tested *in vivo* to ensure that its activity could contribute to isoprenoid biosynthesis. Accordingly, PhoN and DGK (Supplementary Table S1) were each cloned into pETDuet-IPK and tested for their ability to support isoprenoid production in an *E. coli* lycopene strain by extraction and HPLC-based quantification of the pigment (Figure 3A and Supplemental Figure S2). Remarkably, the prototype PhoN-IPK system was capable of converting DMAA/ISO to lycopene in titers of ∼150 mg/L in *E. coli* after 12 h postinduction (Figure 3A). These titers are comparable to that of an optimized engineered DXP pathway (24 mg/L)[41] and heterologous production of the MEV pathway (102 mg/L) in *E. coli*.[31] As expected, the activity was largely dependent on the presence of PhoN given that 17-fold less lycopene was produced at 26 h postinduction when PhoN is absent. In addition, wild-type DGK could support lycopene production in good yields, although these were 4-fold lower than that with PhoN after 24 h.

Next, to determine whether the lycopene titers were dependent on the concentration of the exogenously provided alcohol substrates, a series of lycopene assays were carried out at various concentrations of DMAA/ISO using the strain harboring pETDuet-PhoN-IPK/pAC-LYCipi in the presence of Fs (Figure 3B). After 24 h, the prototype strain produced ∼60 mg/L lycopene at 0.5 mM each of DMAA/ISO, ∼100 mg/L at 2.5 mM each of DMAA/ISO, and ∼190 mg/L at 5 mM each of DMAA/ISO (Figure 3B). At concentrations of 10 mM, DMAA/ISO supported a maximum lycopene titer of ∼20 mg/L after 24 h.

As an additional test of the ability of the designed prototype strain to provide an isoprenoid building block, the PhoN-IPK pathway was used to provide DMAPP which was then transferred to L-Trp using the prenyltransferase (PTase) FgaPT2[42] by providing an additional plasmid that expressed the PTase (Figure 4A). First, an *in vitro* reaction using purified FgaPT2 and a DMAPP standard confirmed production of a single product by HPLC and LC-MS analysis of the product

DOI: 10.1021/acssynbio.8b00383
ACS Synth. Biol. 2019, 8, 232−238

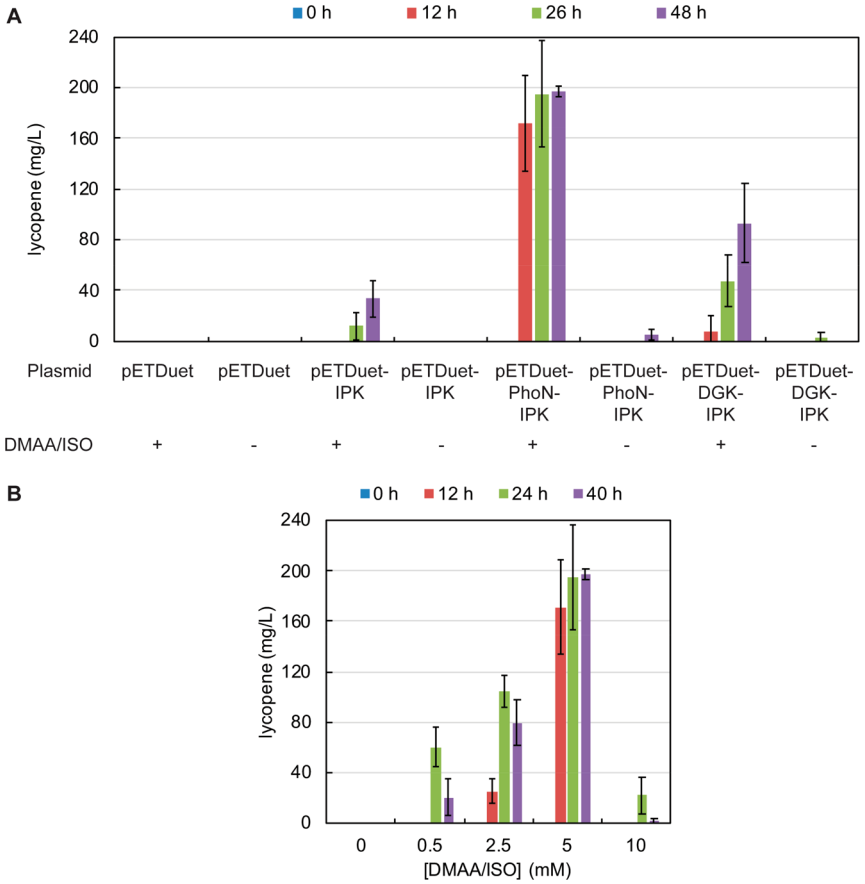


Figure 3. Lycopene titers supported by engineered *E. coli* strains. (A) Lycopene titers are shown at 0, 12, 26, or 48 h post-induction, in the presence or absence of DMAA/ISO, using *E. coli* NovaBlue(DE3) harboring pAC-LYCipi and the indicated plasmid with Fs treatment. Values are the average of three replicates. Error bars are the standard deviation. (B) Substrate dependence of lycopene titers determined at 0, 12, 26, or 40 h post induction with the strain harboring pETDuet-PhoN-IPK + pAC-LYCipi with Fs treatment. Values are the average of three replicates. Error bars are the standard deviation.

mixture (Figure 4B), the mass of which (273.1591 *m/z*, $[M + H]^+$) was consistent with the expected regioselectively monoalkylated product, dimethylallyl-L-Trp (DMAT, calculated 273.1598 *m/z*, $[M + H]^+$). In addition, when purified PhoN, IPK, and FgpaPT2 was used in a one-pot reaction, the same DMAT product was detected when DMAA was included in the *in vitro* reaction mixture (Figure 4B), confirming the ability of the ADH pathway to produce the required prenyl donor for FgaPT2. To test the ability of the *in vivo* system to provide the hemiterpene and couple it with the PTase, DMAA/ISO were fed into *E. coli* that harbored the ADH module (pETDuet-PhoN-IPK) in conjunction with a plasmid that harbored the PTase (pCDFDuet-FgaPT2), and after protein expression, HPLC confirmed the presence of the expected product (DMAT) in the culture media with an elution time indistinguishable from that produced by both *in vitro* reactions (Figure 4B). As expected from the *in vitro* reactions, an extracted-ion chromatogram of the culture media revealed a single peak corresponding to the mass ion for DMAT (Figure 4C). By using the *in vitro* FgaPT2-catalyzed conversion of L-Trp and DMAPP to DMAT as a calibration standard (the reaction went to completion), the ADH-FgaPT2 pathway in *E. coli* was judged to support the production of DMAT at ~20 mg/L. Furthermore, according to HPLC analysis, DMAT was not detected in negative controls that lacked either the alcohol or FgaPT2. Consistent with this observation, HR-MS analysis revealed that DMAT was present in 100-fold lower in abundance in the negative controls as compared to the positive reaction (data not shown). Together, this data confirms that the artificial ADH module is required for high-level production of the prenylated product.

In summary, an artificial hemiterpene biosynthetic pathway dependent on the exogenous addition of DMAA/ISO was developed. The prototype ADH pathway performed similarly to previously established routes that depend on building blocks from primary metabolism. It is expected that ribosome binding site and/or promoter engineering can be leveraged to optimize the productivity of the pathway further. Notably, although previous synthetic biology efforts have for example constructed a new entry point into the DXP pathway[43] and provided new routes to DXP,[44,45] to the best of our knowledge, the ADH pathway described here is the first to transform scalable simple precursors directly into the required pyrophosphates and couple them to isoprenoid biosynthesis. This provides a simple strategy to provide isoprenoids in good yields given that only two enzymes and DMAA/ISO need to be provided. Indeed, in the absence of ISO/DMAA, there was insufficient endogenous DMAPP in *E. coli* to support high level production of the prenylated tryptophan, even when Fs was not used to inhibit the native DXP pathway. This strategy has been designed primarily as a future discovery tool that potentially enables for the first time the *in vivo* biosynthesis of hemiterpene analogues, and by extension, non-natural isoprenoids. For example, PhoN displays a broad specificity *in vitro*, and this is expected to

DOI: 10.1021/acssynbio.8b00383
ACS Synth. Biol. 2019, 8, 232−238

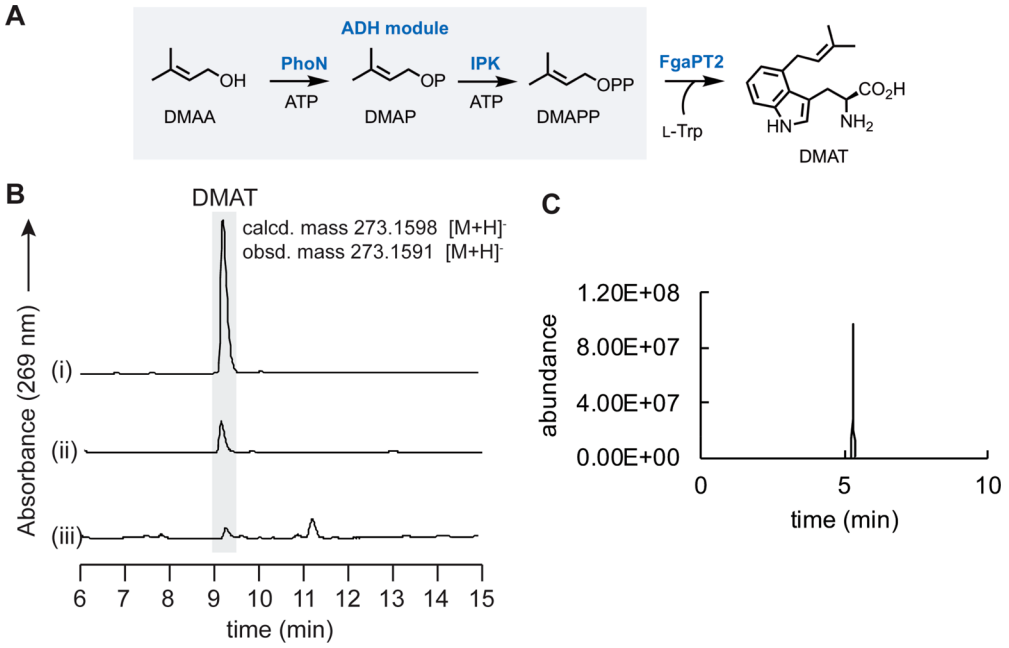


Figure 4. Tryptophan prenylation *via* the artificial alcohol-dependent hemiterpene pathway. (A) Reaction scheme of the ADH pathway coupled to the prenyltransferase, FgaPT2. (B) HPLC chromatograms showing peaks corresponding to DMAT: (i) *in vitro* FgaPT2 reaction with synthetic DMAPP; (ii) *in vitro* reaction with purified PhoN, IPK, and FgaPT2; (iii) culture media from *in vivo* bioconversion with the ADH module. The chromatograms are scaled equally. (C) Extracted-ion chromatogram showing a single peak corresponding to DMAT in the culture media of *E. coli* Rosetta(DE3)pLysS + pETDuet-PhoN-IPK + pCDFDuetFgaPT2 after treatment with DMAA/ISO, and IPTG.

extend to the *in vivo* system here. Furthermore, several features of PhoN have been previously targeted by enzyme engineering, including shifting its pH optima for neutral media[46] and improving its kinase activity with concomitant reduction in phosphatase activity.[47,48] Similarly, although the promiscuity of IPK is largely underexplored, its substrate interacts with the enzyme active site through electrostatic forces dictated by the phosphate portion of the substrate, while the remaining alkyl portion of the substrate is simply sterically accommodated.[49] Indeed, the substrate specificity of IPK has been expanded to include geranyl- and farnesylphosphate.[50] An expanded set of non-natural hemiterpenes provided by the prototype or engineered ADH pathway could be coupled with downstream enzymes to probe the promiscuity and utility of isoprenoid biosynthesis. For example, it is expected that this precursor-directed approach to non-natural isoprenoids will be readily extendible to natural product scaffolds that include L-Trp and/or other aromatics given the previously reported promiscuity of aromatic PTases.[51−54] Subsequently, the ADH pathway may enable the production of prenylated and terpene natural products with non-natural alkyl groups expanding upon the limited chemical diversity afforded by nature.

METHODS

General. All plasmids were verified by DNA sequencing. Purifications of all DNA were performed with kits from BioBasic. Lycopene standard was purchased from Sigma-Aldrich. Synthetic oligonucleotides were purchased from IDT (Coralville, IA, USA). Plasmid pAC-LYCipi was purchased from Addgene (Plasmid #53279, Addgene, Cambridge, MA, USA). Restriction enzymes were purchased from New England Biolabs (Ipswich, MA, USA). Polymerase chain reactions were conducted using Phire Hot Start II DNA Polymerase from ThermoFisher Scientific (Waltham, MA, USA).

Lycopene Quantification. *E. coli* NovaBlue (DE3) containing pAC-LYCipi and various pETDuet constructs were grown in 250 mL of LB supplemented with ampicillin (100 μg/mL) and chloramphenicol (35 μg/mL) at 37 °C overnight with shaking at 250 rpm after inoculation with 0.25 mL of the starter culture. After 5 h the OD_{600} of the culture was ∼0.2 at which point combinations of DMAA/ISO (in DMSO), IPTG, and Fs were added to give final concentrations of 5 mM, 1 mM, and 0.5 μM, respectively. In controls that lacked DMAA/ISO, DMSO was added to give the equivalent volume. At various time points, 600 μL of culture was removed, and lycopene was extracted and quantified (see the Supplemental Methods).

***In Vitro* FgaPT2 Assay.** FgaPT2 reactions were run at pH 7.5 in 200 μL containing 50 mM Tris-HCl, 5 mM $CaCl_2$, 1 mM L-tryptophan, 2 mM DMAPP, and 40 μg of FgaPT2. The reactions were incubated at 37 °C for 1 h and then quenched by the addition of an equal volume of methanol. Reactions with PhoN-IPK generated DMAPP contained 25 mM Tris-HCl, 5 mM magnesium chloride, 1 mM L-tryptophan, 1.8 mM ATP, 30 mM DMAA, 270 ng/μL FgaPT2, 20 ng/μL IPK, and 87 ng/μL PhoN at pH 8.0 in a total volume of 50 μL. The reactions were incubated at 37 °C overnight and then quenched by the addition of an equal volume of methanol. For analytical-scale HPLC analysis, FgaPT2 reactions were followed at 269 nm using a Phenomenex Kinetex 5u EVO C18 column (250 × 4.6 mm; 100 Å) at a flow rate of 1 mL/min. A linear gradient of 20−70% acetonitrile in 0.1% aqueous trifluoroacetic acid over 20 min was used.

***In Vivo* FgaPT2 Assay.** A 3 mL culture of *E. coli* Rosetta(DE3) pLysS pETDuet-PhoN-IPK + pCDFDuetFgaPT2 was grown overnight at 37 °C and 250 rpm in LB media containing ampicillin (100 μg/mL), chloramphenicol (35 μg/mL), and streptomycin (100 μg/mL). An aliquot (100 μL) of overnight culture was used to inoculate 10 mL cultures

DOI: 10.1021/acssynbio.8b00383
ACS Synth. Biol. 2019, 8, 232−238

in TB media containing the same antibiotics as before, and those cultures were grown at 30 °C for 6 h at 250 rpm until induction with 0.5 mM IPTG (final concentration) and addition to a final concentration of 5 mM DMAA, 5 mM ISO, and 10 mM Trp. The cultures were grown for 48 h after induction. The culture supernatant was diluted 1:1 in methanol before analysis by HPLC and LC−MS.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.8b00383.

Supplementary tables, supplemental figures, and detailed methods that describe cloning candidate kinase genes; screening of kinases for phosphorylation of ISO and DMAA; synthesis of DMAPP/IPP; preliminary colorimetric screening of IPK; cloning of IPK, PhoN, DGK, and FgaPT2; expression and purification of PhoN, IPK, and FgapT2; extraction and quantification of lycopene; and lycopene standard curve (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: gjwillia@ncsu.edu.

ORCID

Gavin J Williams: 0000-0003-2172-9063

Present Address

[#]Amyris, 5885 Hollis St Ste. 100, Emeryville, California 94608, United States.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was supported in part by National Institutes of Health Grant GM104258 (G.J.W.). All high resolution LC−MS measurements were made in the Molecular Education, Technology, and Research Innovation Center (METRIC) at NC State University.

■ REFERENCES

(1) Christianson, D. W. (2008) Unearthing the roots of the terpenome. *Curr. Opin. Chem. Biol. 12*, 141−150.

(2) Vickers, C. E., Williams, T. C., Peng, B., and Cherry, J. (2017) Recent advances in synthetic biology for engineering isoprenoid production in yeast. *Curr. Opin. Chem. Biol. 40*, 47−56.

(3) Chang, M. C., and Keasling, J. D. (2006) Production of isoprenoid pharmaceuticals by engineered microbes. *Nat. Chem. Biol. 2*, 674−681.

(4) Alhassan, A. M., Abdullahi, M. I., Uba, A., and Umar, A. (2014) Prenylation of aromatic secondary metabolites: a new frontier for development of novel drugs. *Trop. J. Pharm. Res. 13*, 307−314.

(5) Wollinsky, B., Ludwig, L., Hamacher, A., Yu, X., Kassack, M. U., and Li, S. M. (2012) Prenylation at the indole ring leads to a significant increase of cytotoxicity of tryptophan-containing cyclic dipeptides. *Bioorg. Med. Chem. Lett. 22*, 3866−3869.

(6) George, K. W., Alonso-Gutierrez, J., Keasling, J. D., and Lee, T. S. (2015) Isoprenoid drugs, biofuels, and chemicals−artemisinin, farnesene, and beyond. *Adv. Biochem. Eng./Biotechnol. 148*, 355−389.

(7) Beller, H. R., Lee, T. S., and Katz, L. (2015) Natural products as biofuels and bio-based chemicals: fatty acids and isoprenoids. *Nat. Prod. Rep. 32*, 1508−1526.

(8) Ajikumar, P. K., Xiao, W. H., Tyo, K. E., Wang, Y., Simeon, F., Leonard, E., Mucha, O., Phon, T. H., Pfeifer, B., and Stephanopoulos, G. (2010) Isoprenoid pathway optimization for Taxol precursor overproduction in *Escherichia coli*. *Science 330*, 70−74.

(9) Tsuruta, H., Paddon, C. J., Eng, D., Lenihan, J. R., Horning, T., Anthony, L. C., Regentin, R., Keasling, J. D., Renninger, N. S., and Newman, J. D. (2009) High-level production of amorpha-4,11-diene, a precursor of the antimalarial agent artemisinin, in *Escherichia coli*. *PLoS One 4*, No. e4489.

(10) Leonard, E., Ajikumar, P. K., Thayer, K., Xiao, W. H., Mo, J. D., Tidor, B., Stephanopoulos, G., and Prather, K. L. (2010) Combining metabolic and protein engineering of a terpenoid biosynthetic pathway for overproduction and selectivity control. *Proc. Natl. Acad. Sci. U. S. A. 107*, 13654−13659.

(11) Chang, M. C. Y., Eachus, R. A., Trieu, W., Ro, D. K., and Keasling, J. D. (2007) Engineering Escherichia coli for production of functionalized terpenoids using plant P450s. *Nat. Chem. Biol. 3*, 274−277.

(12) Zhao, L., Chang, W. C., Xiao, Y., Liu, H. W., and Liu, P. (2013) Methylerythritol phosphate pathway of isoprenoid biosynthesis. *Annu. Rev. Biochem. 82*, 497−530.

(13) Frank, A., and Groll, M. (2017) The methylerythritol phosphate pathway to isoprenoids. *Chem. Rev. 117*, 5675−5703.

(14) Kudoh, T., Park, C. S., Lefurgy, S. T., Sun, M., Michels, T., Leyh, T. S., and Silverman, R. B. (2010) Mevalonate analogues as substrates of enzymes in the isoprenoid biosynthetic pathway of *Streptococcus pneumoniae*. *Bioorg. Med. Chem. 18*, 1124−1134.

(15) Kalkreuter, E., Carpenter, S. M., and Williams, G. J. (2018) Chapter 11 Precursor-directed Biosynthesis and Semi-synthesis of Natural Products, In *Chemical and Biological Synthesis: Enabling Approaches for Understanding Biology*, pp 275−312, The Royal Society of Chemistry.

(16) Kalkreuter, E., and Williams, G. J. (2018) Engineering enzymatic assembly lines for the production of new antimicrobials. *Curr. Opin. Microbiol. 45*, 140−148.

(17) Walker, M. C., Thuronyi, B. W., Charkoudian, L. K., Lowry, B., Khosla, C., and Chang, M. C. (2013) Expanding the fluorine chemistry of living systems using engineered polyketide synthase pathways. *Science 341*, 1089−1094.

(18) Lowell, A. N., DeMars, M. D., Slocum, S. T., Yu, F., Anand, K., Chemler, J. A., Korakavi, N., Priessnitz, J. K., Park, S. R., Koch, A. A., Schultz, P. J., and Sherman, D. H. (2017) Chemoenzymatic total synthesis and structural diversification of tylactone-based macrolide antibiotics through late-stage polyketide assembly, tailoring, and C-H functionalization. *J. Am. Chem. Soc. 139*, 7913−7920.

(19) Gregory, M. A., Petkovic, H., Lill, R. E., Moss, S. J., Wilkinson, B., Gaisser, S., Leadlay, P. F., and Sheridan, R. M. (2005) Mutasynthesis of rapamycin analogues through the manipulation of a gene governing starter unit biosynthesis. *Angew. Chem., Int. Ed. 44*, 4757−4760.

(20) Moss, S. J., Carletti, I., Olano, C., Sheridan, R. M., Ward, M., Math, V., Nur, E. A. M., Brana, A. F., Zhang, M. Q., Leadlay, P. F., Mendez, C., Salas, J. A., and Wilkinson, B. (2006) Biosynthesis of the angiogenesis inhibitor borrelidin: directed biosynthesis of novel analogues. *Chem. Commun. (Cambridge, U. K.)*, 2341−2343.

(21) Jacobsen, J. R., Hutchinson, C. R., Cane, D. E., and Khosla, C. (1997) Precursor-directed biosynthesis of erythromycin analogs by an engineered polyketide synthase. *Science 277*, 367−369.

(22) Zhang, X., and Li, S. (2017) Expansion of chemical space for natural products by uncommon P450 reactions. *Nat. Prod. Rep. 34*, 1061−1089.

(23) Gruchattka, E., Hadicke, O., Klamt, S., Schutz, V., and Kayser, O. (2013) In silico profiling of *Escherichia coli* and *Saccharomyces cerevisiae* as terpenoid factories. *Microb. Cell Fact. 12*, 84.

(24) Mori, H., Baba, T., Yokoyama, K., Takeuchi, R., Nomura, W., Makishi, K., Otsuka, Y., Dose, H., and Wanner, B. L. (2015) Identification of essential genes and synthetic lethal gene combinations in *Escherichia coli* K-12. *Methods Mol. Biol. 1279*, 45−65.

DOI: 10.1021/acssynbio.8b00383
ACS Synth. Biol. 2019, 8, 232−238

(25) Palsuledesai, C. C., and Distefano, M. D. (2015) Protein prenylation: enzymes, therapeutics, and biotechnology applications. *ACS Chem. Biol. 10*, 51−62.
(26) DeGraw, A. J., Palsuledesai, C., Ochocki, J. D., Dozier, J. K., Lenevich, S., Rashidian, M., and Distefano, M. D. (2010) Evaluation of alkyne-modified isoprenoids as chemical reporters of protein prenylation. *Chem. Biol. Drug Des. 76*, 460−471.
(27) Onono, F. O., Morgan, M. A., Spielmann, H. P., Andres, D. A., Subramanian, T., Ganser, A., and Reuter, C. W. (2010) A tagging-via-substrate approach to detect the farnesylated proteome using two-dimensional electrophoresis coupled with Western blotting. *Mol. Cell. Proteomics 9*, 742−751.
(28) Cunningham, F. X., Jr., Lee, H., and Gantt, E. (2007) Carotenoid biosynthesis in the primitive red alga *Cyanidioschyzon merolae*. *Eukaryotic Cell 6*, 533−545.
(29) Wang, H. H., Isaacs, F. J., Carr, P. A., Sun, Z. Z., Xu, G., Forest, C. R., and Church, G. M. (2009) Programming cells by multiplex genome engineering and accelerated evolution. *Nature 460*, 894−898.
(30) Alper, H., and Stephanopoulos, G. (2008) Uncovering the gene knockout landscape for improved lycopene production in *E. coli*. *Appl. Microbiol. Biotechnol. 78*, 801−810.
(31) Yoon, S. H., Lee, Y. M., Kim, J. E., Lee, S. H., Lee, J. H., Kim, J. Y., Jung, K. H., Shin, Y. C., Keasling, J. D., and Kim, S. W. (2006) Enhanced lycopene production in *Escherichia coli* engineered to synthesize isopentenyl diphosphate and dimethylallyl diphosphate from mevalonate. *Biotechnol. Bioeng. 94*, 1025−1032.
(32) Farmer, W. R., and Liao, J. C. (2001) Precursor balancing for metabolic engineering of lycopene production in *Escherichia coli*. *Biotechnol. Prog. 17*, 57−61.
(33) Alper, H., Miyaoku, K., and Stephanopoulos, G. (2005) Construction of lycopene-overproducing *E. coli* strains by combining systematic and combinatorial gene knockout targets. *Nat. Biotechnol. 23*, 612−616.
(34) Armstrong, C. M., Meyers, D. J., Imlay, L. S., Freel Meyers, C., and Odom, A. R. (2015) Resistance to the antimicrobial agent fosmidomycin and an FR900098 prodrug through mutations in the deoxyxylulose phosphate reductoisomerase gene (dxr). *Antimicrob. Agents Chemother. 59*, 5511−5519.
(35) Kuzuyama, T., Shimizu, T., Takahashi, S., and Seto, H. (1998) Fosmidomycin, a specific inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase in the nonmevalonate pathway for terpenoid biosynthesis. *Tetrahedron Lett. 39*, 7913−7916.
(36) Dellas, N., Thomas, S. T., Manning, G., and Noel, J. P. (2013) Discovery of a metabolic alternative to the classical mevalonate pathway. *eLife 2*, No. e00672.
(37) Chen, M., and Poulter, C. D. (2010) Characterization of thermophilic archaeal isopentenyl phosphate kinases. *Biochemistry 49*, 207−217.
(38) van Herk, T., Hartog, A. F., Babich, L., Schoemaker, H. E., and Wever, R. (2009) Improvement of an acid phosphatase/DHAP-dependent aldolase cascade reaction by using directed evolution. *ChemBioChem 10*, 2230−2235.
(39) Tanaka, N., Hasan, Z., Hartog, A. F., van Herk, T., Wever, R., and Sanders, R. J. (2003) Phosphorylation and dephosphorylation of polyhydroxy compounds by class A bacterial acid phosphatases. *Org. Biomol. Chem. 1*, 2833−2839.
(40) Lis, M., and Kuramitsu, H. K. (2003) The stress-responsive dgk gene from *Streptococcus mutans* encodes a putative undecaprenol kinase activity. *Infect. Immun. 71*, 1938−1943.
(41) Kim, S. W., and Keasling, J. D. (2001) Metabolic engineering of the nonmevalonate isopentenyl diphosphate synthesis pathway in *Escherichia coli* enhances lycopene production. *Biotechnol. Bioeng. 72*, 408−415.
(42) Unsold, I. A., and Li, S. M. (2005) Overproduction, purification and characterization of FgaPT2, a dimethylallyltryptophan synthase from *Aspergillus fumigatus*. *Microbiology 151*, 1499−1505.
(43) King, J. R., Woolston, B. M., and Stephanopoulos, G. (2017) Designing a new entry point into isoprenoid metabolism by exploiting fructose-6-phosphate aldolase side reactivity of *Escherichia coli*. *ACS Synth. Biol. 6*, 1416−1426.
(44) Kang, A., Meadows, C. W., Canu, N., Keasling, J. D., and Lee, T. S. (2017) High-throughput enzyme screening platform for the IPP-bypass mevalonate pathway for isopentenol production. *Metab. Eng. 41*, 125−134.
(45) Kirby, J., Nishimoto, M., Chow, R. W., Baidoo, E. E., Wang, G., Martin, J., Schackwitz, W., Chan, R., Fortman, J. L., and Keasling, J. D. (2015) Enhancing terpene yield from sugars via novel routes to 1-deoxy-d-xylulose 5-phosphate. *Appl. Environ. Microbiol. 81*, 130−138.
(46) Makde, R. D., Dikshit, K., and Kumar, V. (2006) Protein engineering of class-A non-specific acid phosphatase (PhoN) of *Salmonella typhimurium* modulation of the pH-activity profile. *Biomol. Eng. 23*, 247−251.
(47) Mihara, Y., Utagawa, T., Yamada, H., and Asano, Y. (2000) Phosphorylation of nucleosides by the mutated acid phosphatase from *Morganella morganii*. *Appl. Environ. Microbiol. 66*, 2811−2816.
(48) Tasnadi, G., Zechner, M., Hall, M., Baldenius, K., Ditrich, K., and Faber, K. (2017) Investigation of acid phosphatase variants for the synthesis of phosphate monoesters. *Biotechnol. Bioeng. 114*, 2187−2195.
(49) Mabanglo, M. F., Schubert, H. L., Chen, M., Hill, C. P., and Poulter, C. D. (2010) X-ray structures of isopentenyl phosphate kinase. *ACS Chem. Biol. 5*, 517−527.
(50) Mabanglo, M. F., Pan, J. J., Shakya, B., and Poulter, C. D. (2012) Mutagenesis of isopentenyl phosphate kinase to enhance geranyl phosphate kinase activity. *ACS Chem. Biol. 7*, 1241−1246.
(51) Yu, H. L., Liebhold, M., Xie, X. L., and Li, S. M. (2015) Tyrosine O-prenyltransferases TyrPT and SirD displaying similar behavior toward unnatural alkyl or benzyl diphosphate as their natural prenyl donor dimethylallyl diphosphate. *Appl. Microbiol. Biotechnol. 99*, 7115−7124.
(52) Liebhold, M., Xie, X., and Li, S. M. (2012) Expansion of enzymatic Friedel-Crafts alkylation on indoles: acceptance of unnatural beta-unsaturated allyl diphospates by dimethylallyltryptophan synthases. *Org. Lett. 14*, 4882−4885.
(53) Elshahawi, S. I., Cao, H. N., Shaaban, K. A., Ponomareva, L. V., Subramanian, T., Farman, M. L., Spielmann, H. P., Phillips, G. N., Thorson, J. S., and Singh, S. (2017) Structure and specificity of a permissive bacterial C-prenyltransferase. *Nat. Chem. Biol. 13*, 366.
(54) Chen, R. D., Gao, B. Q., Liu, X., Ruan, F. Y., Zhang, Y., Lou, J. Z., Feng, K. P., Wunsch, C., Li, S. M., Dai, J. G., and Sun, F. (2017) Molecular insights into the enzyme promiscuity of an aromatic prenyltransferase. *Nat. Chem. Biol. 13*, 226−234.

DOI: 10.1021/acssynbio.8b00383
ACS Synth. Biol. 2019, 8, 232−238