

1 **Metabolic engineering of *Escherichia coli* for production of valerenadiene**

2 S. Eric Nybo^{1,†}, Jacqueline Saunders¹, Sean P. McCormick²

3 ¹ Ferris State University, College of Pharmacy, Department of Pharmaceutical Sciences, Big Rapids, MI
4 49307, USA

5 ² Ferris State University, Department of Physical Sciences, Big Rapids, MI 49307, USA

6 † Corresponding Author: EricNybo@ferris.edu

7

8 **Abstract**

9 *Valeriana officinalis* is a medicinal herb which produces a suite of compounds in its root tissue useful for
10 treatment of anxiety and insomnia. The sesquiterpene components of the root extract, valerenic acid and
11 valerena-1,10-diene, are thought to contribute to most of the observed anxiolytic of Valerian root
12 preparations. However, valerenic acid and its biosynthetic intermediates are only produced in low
13 quantities in the roots of *V. officinalis*. Thus, in this report, *Escherichia coli* was metabolically engineered
14 to produce substantial quantities of valerena-1,10-diene in shake flask fermentations with decane overlay.
15 Expression of the wildtype valerenadiene synthase gene (pZE-*wvds*) resulted in production of 12 µg/mL
16 in LB cultures using endogenous FPP metabolism. Expression of a codon-optimized version of the
17 valerenadiene synthase gene (pZE-*cvds*) resulted in 3-fold higher titers of valerenadiene (32 µg/mL). Co-
18 expression of pZE-*cvds* with an engineered methyl erythritol phosphate (MEP) pathway improved
19 valerenadiene titers 65-fold to 2.09 mg/L valerenadiene. Optimization of the fermentation medium to
20 include glycerol supplementation enhanced yields by another 5.5-fold (11.0 mg/L valerenadiene). The
21 highest production of valerenadiene resulted from engineering the codon-optimized valerenadiene
22 synthase gene under strong P_{trc} and P_{T7} promoters and via co-expression of an exogenous mevalonate
23 (MVA) pathway. These efforts resulted in an *E. coli* production strain that produced 62.0 mg/L
24 valerenadiene (19.4 mg/L/OD₆₀₀ specific productivity). This *E. coli* production platform will serve as the
25 foundation for the synthesis of novel valerenic acid analogues potentially useful for the treatment of
26 anxiety disorders.

27 **Keywords:** *Valeriana*, valerenic acid, metabolic engineering, sesquiterpene, *Escherichia coli*

28

29 Introduction

30 *Valeriana officinalis* is a medicinal wild herb indigenous to many habitats, and the root of this
31 plant is used as a nutraceutical preparation (Valerian) that is currently used for the treatment of anxiety
32 and insomnia (Bent et al., 2006). The roots of *V. officinalis* produce a suite of compounds, including
33 valepotriate alkaloids and sesquiterpenes (Bos et al., 1996). Notably, the sesquiterpene components of
34 Valerian root extract are hypothesized to exhibit many of the beneficial anti-anxiety and anti-insomnia
35 effects. Of the sesquiterpenes, valerenic acid is the most potent GABA-A agonist in these extracts, while
36 valerenal, valerenol, and valerena-1,10-diene (valerenadiene) also modulate GABA-A activity to varying
37 extents in zebrafish and mouse models (Del Valle-Mojica and Ortíz, 2012; Takemoto et al., 2014). Most
38 importantly, the anxiolytic effect of valerian has been demonstrated in human clinical studies in recent
39 years (Anderson et al., 2005; Barton et al., 2011).

40 Valerenic acid in particular has demonstrated nanomolar binding affinity for the GABA-A receptor
41 (Benke et al., 2009). Recently, the putative binding site of valerenic acid has been determined via
42 docking studies and site-directed mutagenesis (Luger et al., 2015). Luger et al. modeled valerenic acid in
43 the GABA-A receptor in a distinct cleft near the β 2/3N265 transmembrane residue. The valerenic acid C-
44 12 carboxyl group is predicted to have important hydrogen-bonding interactions with residues β 3N265
45 and β 1S265. Furthermore, the C-13, C-14, and C-15 methyl groups of the valerenane skeleton are
46 predicted to have significant hydrophobic interactions within the pocket at residues within this binding
47 pocket (Figure 1) (Luger et al., 2015). In summation, these observations have renewed interest in
48 development of novel valerenic acid analogues for structure activity relationship studies.

49 Despite these advances, valerenic acid is produced as a minor constituent in root tissue of
50 *Valeriana officinalis* (0.7-0.9% DW) (Bos et al., 1998). The low production of valerenic acid hinders further
51 attempts at structure activity relationship and biological activity studies. Synthetic routes toward valerenic
52 acid derivatives are also expensive and ecologically unsuitable. Recently, Ricigliano et al. have
53 engineered *V. officinalis* hairy roots for enhanced production of valerenic acid, which lends credence to a
54 metabolic engineering approach for availing these molecules (Ricigliano et al., 2016). Despite this
55 progress, the hairy root system still produces valerenic acid at very lower titers.

56 As an alternative approach, metabolic engineering of microbial organisms represents a green,
57 cost-effective approach for large-scale production of sesquiterpene pharmaceuticals (Zhang et al., 2011).
58 For example, *Escherichia coli* is one such model host that affords several advantages over plant-based
59 systems due to its fast growth kinetics and capacity to produce high-value chemicals via fermentation on
60 simple carbon sources (Lee, 1996). Furthermore, *E. coli* boasts considerable genetic tools, including
61 multiple promoters and expression vectors that establish it as an ideal host for metabolic engineering.
62 Additionally, terpenes are attractive molecules for metabolic engineering, due to their use as fragrances,
63 flavors, and advanced biofuels (Peralta-Yahya et al., 2011; Sowden et al., 2005). Subsequently, these

64 observations established *E. coli* as a suitable platform for engineering of valerenic acid in this present
65 study.

66 Two specialized isoprenoid biosynthetic pathways exist for production of C₅ isoprenyl phosphate
67 precursors, dimethyl allyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP). Many bacteria,
68 algae, and plant chloroplasts employ the methyl erythritol phosphate pathway (MEP), which fluxes
69 glyceraldehyde-3-phosphate and pyruvate towards IPP and DMAPP (Figure 1) (Rohmer, 1999). Fungi
70 and non-plant eukaryotes utilize the mevalonate pathway (MVA) to convert acetyl-coenzyme A (acetyl-
71 CoA) to IPP and DMAPP via eight enzymatic steps (Figure 1) (Martin et al., 2003). Subsequently, IPP
72 and DMAPP are concatenated into progressively longer C₁₀, C₁₅, or C₂₀ molecules by prenyltransferase
73 enzymes. For sesquiterpene metabolism, farnesyl pyrophosphate synthase condenses 2 IPP units and 1
74 DMAPP unit to produce farnesyl pyrophosphate (FPP). Subsequently, FPP can be cyclized by a variety
75 of sesquiterpene hydrocarbons by cognate terpene synthases, such as valerenadiene synthase (VDS)
76 (Figure 1).

77 However, *E. coli* generates only a finite pool of FPP, and while introduction of a sesquiterpene
78 synthase results in detectable production of sesquiterpenes (Martin et al., 2001), overproduction of the
79 molecule requires redirecting substantial carbon flux to the limited substrate FPP. For example,
80 amorphadiene is a sesquiterpene intermediate in the biosynthesis of the antimalarial drug artemisinin.
81 Martin and co-workers discovered that heterologous expression of the mevalonate isoprenoid pathway
82 from yeast in *E. coli* leads to unregulated carbon flux towards FPP and amorphadiene, which can be
83 semi-synthetically converted to artemisinin (Martin et al., 2003). In using this substrate-engineering
84 approach, Keasling and co-workers have produced amorphadiene in *E. coli* at yields of 500 mg L⁻¹ in
85 shake flask (Redding-Johanson et al., 2011) and 27 g L⁻¹ in bioreactors (Tsuruta et al., 2009). This
86 generalized approach lends itself to the microbial synthesis of other sesquiterpenes via introduction of
87 variant terpene synthases.

88 In this report, a metabolic engineering platform was developed for synthesis of valerenadiene in
89 *E. coli* in three steps. Because plant terpene synthase genes are poorly expressed in *E. coli*, owing to
90 different codon usage, first an *E. coli* codon-optimized version of the valerenadiene synthase gene (*cvds*)
91 was synthesized. This led to a three-fold higher production of valerenadiene over expression of the
92 wildtype plant gene (*wvds*). Secondly, we enhanced carbon flux to FPP by designing a construct that
93 overexpressed several rate-limiting steps of the native methyl erythritol phosphate (MEP) pathway.
94 Thirdly, we cloned *cvds* under the control of P_{trc} and P_{T7} promoters to further enhance terpene synthase
95 protein levels and we co-expressed these constructs with a heterologous mevalonate pathway from
96 baker's yeast. These efforts resulted in a high-level production strain that synthesized 62.0 mg L⁻¹
97 valerenadiene (19.4 mg/L/OD₆₀₀ specific productivity) in shake flask cultures.

98 **Methods and Materials**

99 *Bacterial strains and growth conditions*

100 *E. coli* JM109 (New England Biolabs) was used as the host for all routine cloning manipulations,
101 and *E. coli* DH5 α Z1 (Expressys, Germany) and *E. coli* BL21(DE3) (ThermoFisher) were used as hosts for
102 sesquiterpene production (Supplementary Table 1). *E. coli* DH5 α Z1 overexpresses a copy of the lacI
103 repressor on the chromosome for efficient repression of the lac operator (Lutz and Bujard, 1997).
104 Chemically competent *E. coli* were generated with the *E. coli* Mix and Go Transformation Kit (ZYMO
105 Research) and were transformed using standard molecular methodologies (Sambrook and W Russell,
106 2001). *E. coli* strains were grown in LB agar or LB broth at 37 °C for routine maintenance. For
107 production of sesquiterpenes, *E. coli* DH5 α Z1 derivatives were grown in 2xYT with supplemented glycerol
108 at 30 °C. For production of valerenadiene. For expression of the mevalonic acid pathway, bacterial
109 growth media was buffered with phosphate buffered saline using a stock solution of 10x PBS (Sambrook
110 and W Russell, 2001). Strains were supplemented with ampicillin (100 μ g mL⁻¹), chloramphenicol (35 μ g
111 mL⁻¹), and kanamycin (50 μ g mL⁻¹) as necessary. When multiple plasmids were co-expressed, the
112 chloramphenicol and kanamycin concentrations were adjusted to one-half these amounts.

113 *Cloning of vds and engineered MEP pathway constructs*

114 Oligonucleotide primers were synthesized by IDT-DNA (Supplementary Table 2), and sequences
115 were verified by sequencing analysis (ACGT, Inc.). Polymerase chain reaction was carried out using
116 Primestar® HS Polymerase (Takara Bio.) by following the manufacturer's protocols. The wildtype
117 valerenadiene synthase gene (*wvds*) gene was amplified via polymerase chain reaction from the
118 pET28a(+)-VoTPS1 (hereafter referred to as pET-*wvds*) construct as described previously (Yeo et al.,
119 2013). The *cvds* gene was codon-optimized for expression in *E. coli*, synthesized, and spliced into
120 cloning vector pUC57-*cvds* (GenScript). The *cvds* gene was amplified via polymerase chain reaction, and
121 both the *wvds* and *cvds* genes were cut and spliced into the *EcoRI/BamHI* restriction sites of pZE12MCS
122 under the control of the intermediate strength P_{LacO1} promoter (Expressys, Germany) to afford constructs
123 pZE-wVDS and pZE-cVDS, respectively (Lutz and Bujard, 1997). For insertion under the stronger P_{trc}
124 promoter, *cvds* was PCR amplified, cut, and spliced into the *BamHI/EcoRI* sites of pTrcHisA (Thermo
125 Fisher Scientific) to afford pTrcHis-*cvds*. In this construct, *cvds* is fused to the N-terminal hexahistidine
126 and Xpress™ epitope tags. For comparisons involving the P_{T7} promoter, the *cvds* gene was cloned into
127 the *NcoI* and *EcoRI* sites of pET28a(+) to afford pET-*cvds*. pET-*wvds* features *wvds* fused to the P_{T7}
128 promoter and was included in experiments to reflect baseline production of valerenadiene from T7-
129 polymerase driven expression of the wildtype gene.

130 The *dxs-idi-fps* genes were synthesized as a polycistron as previously described in vector
131 pUC57-operon 3 (Genscript) (Bell et al., n.d.). The *dxs-idi-fps* genes were digested with *EcoRI/BamHI*
132 and were spliced into the *EcoRI/BamHI* sites of pSTV28 to afford pSTV-*dxs-idi-fps*.

133 *Cloning and expression of mevalonate pathways*

134 pBbA5c-MevT(CO)-MBIS(CO, ispA) was a gift from Jay Keasling & Taek Soon Lee (Addgene
135 plasmid # 35151). For co-expression of valerenadiene synthase and the mevalonate pathway, *E. coli*
136 competent cells were co-transformed with the relevant constructs and plated on LB agar supplemented
137 with antibiotics. For terminal endpoint assays, *E. coli* strains were grown in 1 mL of LB supplemented
138 with antibiotics for 12-16 hours until the strains were in stationary phase growth, and then they were
139 inoculated into 5 mL 2x YT medium containing 3% glycerol with an overlay of 1 mL of decane. For time
140 course assays, *E. coli* strains were grown in 50 mL 2x YT medium containing 3% glycerol with an overlay
141 of 10 mL of decane. Time points were collected at 12, 24, 36, 48, 72, and 96 hours of 100 microliters of
142 culture broth (for OD₆₀₀ measurements) and 100 microliters of decane overlay for valerenadiene
143 determination.

144 *GC-MS analysis of valerenadiene*

145 Valerenadiene production by the various strains was measured by GC-MS via selective ion
146 monitoring for the molecular ion (204 *m/z*), the 189 *m/z* ion, and the 69 *m/z* ion, as described previously
147 (Martin et al., 2003). Cells were grown in LB or 2x YT medium with 3% glycerol and induced with 200
148 micromolar IPTG to express the *wvds* or *cvds* gene and either the engineered MEP operon or the
149 mevalonate pathways. Cultures were covered with a 20% (v/v) decane overlay to trap volatile
150 sesquiterpenes. 100 microliters of decane overlay was diluted in 900 microliters of hexane, and 1
151 microliter of sample was subjected to GC-MS analysis. Samples were analyzed at the Ferris State
152 University Shimadzu Core Laboratory for Academic Research Excellence (FSU-SCLARE) on a Shimadzu
153 QP-2010 Ultra GC mass spectrometer. Valerenadiene in experimental samples was compared to
154 authentic valerenadiene standard. Valerenadiene concentration was converted to beta caryophyllene
155 equivalents using a caryophyllene standard curve and the relative abundance of 69 *m/z*, 189 *m/z*, and
156 204 *m/z* mass fragments. Valerenadiene standard was isolated as described previously (Yeo et al.,
157 2013).

158 **Results**

159 *Valerenadiene production in E. coli and gene optimization*

160 To evaluate the production of valerenadiene synthase in *E. coli*, the gene encoding wildtype
161 valerenadiene synthase (*wvds*) from *V. officinalis* was cloned under the control of the IPTG-inducible
162 P_{LlacO1} promoter of high copy number expression plasmid pZE12MCS to afford pZE-wVDS. Previously,
163 other groups have reported that sesquiterpene production in *E. coli* is limited by poor expression of
164 cognate plant terpene synthases (Martin et al., 2001; Wang et al., 2011). We surmised that the *wvds*
165 gene may encode several rare codons that inhibit optimal translation in *E. coli*. Therefore, we
166 synthesized a codon-optimized version of the valerenadiene synthase gene for expression in *E. coli*
167 (GenScript) and fused it to the P_{LlacO1} to afford pZE-cVDS. pZE-wVDS, pZE-cVDS, and empty
168 pZE12MCS vector were transformed in *E. coli*. The *E. coli* lines were grown in 5 mL of LB medium

169 overlaid with decane for 48 hours and induced with IPTG. A major valerenadiene peak was detected in
170 extracts of pZE-wVDS and pZE-cVDS cultures that eluted at the same retention time and exhibited
171 identical mass fragmentation pattern to valerenadiene standard (Supplementary Figure 1). This peak was
172 not detected in pZE12MCS control strains, which confirmed low production of valerenadiene in the VDS-
173 engineered lines. *E. coli* pZE-wVDS produced 8 µg/L and 12 µg/L valerenadiene at 24 and 48 hours,
174 respectively, whereas *E. coli* pZE-cVDS produced 16 and 32 µg/L valerenadiene at 24 and 48 hours,
175 respectively (Figure 2). This results suggests that the codon-optimized valerenadiene synthase is more
176 efficiently expressed than the wildtype version, which results in a 3-fold improvement in valerenadiene
177 titer.

178 However, the yields of valerenadiene achieved were quite low, and we surmised this was likely
179 due to low levels of prenyl phosphate precursors (IPP, DMAPP, and FPP). The endogenous MEP
180 pathway of *E. coli* produces a small pool of FPP that is the precursor for *trans*-octaprenyl diphosphate
181 (ODP) in production of ubiquinone and *cis*, *trans*-undecaprenyl diphosphate (UDP) for production of
182 peptidoglycan (Asai et al 1994, Bouhss et al 2008, Okada et al 1997). However, the production of FPP
183 by the endogenous pathway is limited due to regulation of several key steps (Estévez et al., 2001).
184 Therefore, we designed a construct that would overexpress rate-limiting steps of the MEP pathway to
185 augment greater carbon flux from primary metabolism to FPP. In our previous work, we engineered
186 *Rhodobacter capsulatus* to produce high levels of the triterpene botryococcene, which is biosynthesized
187 from two molecules of FPP via squalene synthase-like enzymes (SSL-1 and SSL-3), via expression of the
188 deoxy-xylulose phosphate synthase (*dxs*), isopentenyl diphosphate isomerase (*idi*) genes from *E. coli*,
189 and farnesyl pyrophosphate synthase (*fps*) gene from *Gallus gallus* (Khan et al., 2015). Fusion of the
190 *dxs-idi-fps* genes to the SSL-1+3 chimeric botryococcene synthase and expression of the resulting
191 construct generated 5 mg/gDW production of botryococcene in the *R. capsulatus* host. In a similar
192 fashion, we fused the *dxs-idi-fps* genes under the control of the IPTG-inducible P_{lacUV5} promoter of
193 pSTV28 to generate construct pSTV-*dxs-idi-fps*. This construct was co-transformed with pZE-*wvds* and
194 pZE-*cvds*, and the lines were grown in 5 mL LB medium with decane overlay at 30°C for 48 hours and
195 induced with IPTG. Co-expression of pSTV-*dxs-idi-fps* with pZE-wVDS and pZE-cVDS resulted in a 14-
196 fold increase (0.17 mg/L) and a 65-fold increase (2.09 mg/L) in production of valerenadiene, respectively
197 (Figure 2). These results demonstrate that expression of the engineered MEP pathway greatly
198 augmented carbon flux to FPP and valerenadiene.

199 *Effect of glycerol supplementation on valerenadiene production*

200 With the engineered MEP pathway construct in hand, we hypothesized that optimization of the
201 production medium could further enhance sesquiterpene yield. In fact, Zhang et al. reported that glycerol
202 supplementation of culture medium increased sabinene production to >40 mg/L in *E. coli* engineered with
203 sabinene synthase and a heterologous mevalonate pathway (Zhang et al., 2014). Furthermore, Morrone

204 and co-workers engineered higher levels of C₂₀ abietadiene production and increased biomass when
205 glycerol supplemented media was used (e.g. increasing abietadiene specific productivity from 1
206 mg/L/OD₆₀₀ without glycerol supplementation to 2.5 mg/L/OD₆₀₀ with glycerol supplementation) (Morrone
207 et al., 2010). *E. coli* pZE-wVDS/pSTV-*dxs-idi-fps* and *E. coli* pZE-cVDS/pSTV-*dxs-idi-fps* were grown in 5
208 mL of 2x YT medium supplemented with 0 to 5% glycerol. The *E. coli* pZE-wVDS/pSTV-*dxs-idi-fps*
209 valerenadiene production increased 15-fold from 0.11 mg/L to 1.5 mg/L when supplemented with glycerol,
210 however, further production was possibly stymied by poor heterologous expression of valerenadiene
211 synthase. In contrast, *E. coli* pZE-cVDS/pSTV-*dxs-idi-fps* demonstrated better responsiveness to the
212 glycerol supplementation, as valerenadiene productivity increased from 5.1 mg/L to 11.0 mg/L when
213 supplemented with 3% glycerol (Figure 3). Therefore, 3% glycerol was used for all further experiments.

214 To observe production of valerenadiene over time, we conducted a time course of *E. coli* pZE-
215 wVDS/pSTV-*dxs-idi-fps* and *E. coli* pZE-cVDS/pSTV-*dxs-idi-fps* in 2x YT+3% glycerol media over 72
216 hours. The results of the time course replicated the observations in the glycerol supplementation
217 experiment. *E. coli* co-expressing the engineered MEP pathway and the *wvds* gene reached maximal
218 growth (OD₆₀₀= 9.0) and valerenadiene production (3 mg/L) at 24 hours (Figure 4). The *E. coli* line co-
219 expressing the engineered MEP pathway and *cvds* gene reached also reached both its greatest level of
220 growth (OD₆₀₀ =9.0) and valerenadiene production (10.6 mg/L) at 24 hours. Valerenadiene concentration
221 decreased in both lines from 48 to 72 hours, likely due to volatilization of the sesquiterpene.

222 *Engineering of vds under the P_{trc} promoter*

223 We hypothesized that low-level expression of the terpene synthase from the P_{LacO1} promoter
224 might be a metabolic rate-limiting factor in the turnover of FPP substrate. Based on the previous
225 experiment, expression of the *cvds* gene resulted in 5-10-fold higher quantities of valerenadiene than
226 expression of *wvds*, depending on glycerol supplementation, which was inferred to be the direct result of
227 higher concentration of terpene synthase catalyst in the *E. coli* cell. Therefore, the *cvds* gene was fused
228 to the strong P_{trc} promoter to afford construct pTrcHis-*cvds*. This construct was expressed alone and co-
229 expressed with pSTV-*dxs-idi-fps* in *E. coli* DH5αZ1 cells in triplicate 5 mL 2xYT+3% glycerol
230 fermentations for 48 hours. The strain harboring pTrcHis-*cvds* produced 0.046 mg/L valerenadiene,
231 whereas the strain harboring pTrcHis-*cvds* and pSTV-*dxs-idi-fps* produced approximately 20.3 mg/L
232 (Supplementary Figure 2). This represents a 440-fold improvement over the pTrcHis-*cvds* only
233 expression line. Furthermore, expression of the *cvds* gene by the P_{trc} promoter (e.g. by strain *E. coli*
234 DH5αZ1/(pTrcHis-*cvds*)/(pSTV-*dxs-idi-fps*)) represented a 40% increase in valerenadiene titers as
235 compared to expression from P_{LacO1} (e.g. by strain *E. coli* DH5αZ1/(pZE-*cvds*)/(pSTV-*dxs-idi-fps*)).

236 Recently, the MEP pathway has been demonstrated to be subject to endogenous regulation by *E.*
237 *coli*, possibly due to feedback inhibition of the metabolite methylerythritol cyclodiphosphate (MEcDP)
238 (Banerjee and Sharkey, 2014). We rationalized that FPP enhancement might be limited via the MEP

239 pathway, therefore we co-expressed an exogenous MVA pathway to greatly augment FPP precursor
240 levels along with pTrcHis-*cvds*. In a previous study, Peralta-Yahya and co-workers engineered a
241 heterologous MVA pathway for production of the sesquiterpene biofuel bisabolene (Peralta-Yahya et al.,
242 2011). Expression of the construct pBbA5c-MevT(CO)-MBIS(CO, *ispA*) along with the codon-optimized
243 AgBis gene resulted in production of 586±65 mg/L bisabolene. pBbA5c-MevT(CO)-MBIS(CO, *ispA*)
244 features a codon-optimized, eight gene MVA pathway driven by the P_{trc} promoter on a medium copy
245 number plasmid (Supplementary Table 1). The three upstream gene products (*atoB*, *hmgs*, *thmgr*)
246 convert acetyl-CoA to mevalonic acid, and the five downstream gene products (*erg12*, *erg8*, *mvd1*, *idi*,
247 *ispA*) convert mevalonic acid to farnesyl pyrophosphate (Figure 1). Similarly, *E. coli* DH5αZ1 was
248 transformed with pTrcHis-*cvds* and pBbA5c-MevT(CO)-MBIS(CO, *ispA*) and grown in triplicate 5 mL
249 2xYT+3% glycerol fermentations for 48 hours. This strain produced 42.5 mg/L valerenadiene, a 1000-fold
250 increase over the *cvds*-expressing line and a 2-fold increase over the engineered MEP pathway
251 (Supplementary Figure 2). This result demonstrated that the mevalonic acid pathway efficiently coupled
252 the microbial synthesis of FPP to valerenadiene synthase to produce significant levels of valerenadiene.
253 Thus, the mevalonic acid pathway was used for further experiments. These results were further confirmed
254 in a time course experiment of the same strain, which achieved a titer of 35 mg/L valerenadiene at 24
255 hours (Figure 5).

256 *Co-expression of vds with the T7 RNA polymerase promoter and MVA pathway*

257 We observed that the P_{trc}-*cvds* gene constructs demonstrated more robust production of
258 valerenadiene than the comparatively weaker P_{LacO1}-*cvds* gene constructs, and we inferred that levels of
259 VDS catalyst may be rate-limiting in this latter system. Therefore, we decided to compare the expression
260 of the *wvds* and *cvds* genes from a “weak” promoter system (P_{LacO1}), an “intermediate/strong” promoter
261 system (P_{trc}), and a strong promoter system (P_{T7}) along with the mevalonic acid pathway. We
262 hypothesized that the P_{LacO1} promoter might not be able to drive sufficient expression of VDS to efficiently
263 turnover the abundant FPP substrate synthesized via the mevalonic acid pathway. *E. coli* DH5αZ1 pZE-
264 *wvds* and *E. coli* DH5αZ1 pZE-*cvds* were grown in triplicate cultures as before and produced minimal
265 amounts of valerenadiene (Supplementary Figure 3. When pBbA5c-MevT(CO)-MBIS(CO, *ispA*) was co-
266 expressed in these lines, they produced 2.8 mg/L (0.497 mg/L/OD₆₀₀) and 7.3 mg/L (1.03 mg/L/OD₆₀₀),
267 respectively, which strongly suggested that the amount of VDS catalyst was limited (Supplementary
268 Figure 3). Expression of *cvds* from the P_{trc} promoter with the mevalonic acid pathway increased
269 production 6-fold to 42.5 mg/L (6.07 mg/L/OD₆₀₀) (Supplementary Figure 3).

270 Subsequently, the *wvds* and *cvds* genes were cloned into pET28a for expression under the strong
271 T7 RNA polymerase promoter. The resulting constructs, pET-*wvds* and pET-*cvds* were introduced into *E.*
272 *coli* BL21(DE3) cells to utilize the IPTG-inducible T7 RNA polymerase system. These plasmids were
273 transformed alone or in combination with the mevalonic acid pathway and grown in triplicate 2xYT + 3%

274 glycerol cultures for 48 hours. The pET-*wvds*-only line produced 0.345 mg/L valerenadiene, whereas the
275 pET-*cvds*-only line produced 1.09 mg/L valerenadiene (Figure 6). Co-expression of pET-*wvds* and
276 pBbA5c-MevT(CO)-MBIS(CO, *ispA*) resulted in production of 10.5 mg/L valerenadiene (3.27 mg/L/OD₆₀₀)
277 (Figure 7). Most importantly, when pET-*cvds* was introduced with the mevalonic acid pathway, the
278 highest-yielding valerenadiene strain was achieved with production of 62.0 mg/L valerenadiene (19.4
279 mg/L/OD₆₀₀ specific productivity). These experiments demonstrated that the amount of VDS catalyst and
280 FPP substrate *in vivo* are both limiting factors for production of valerenadiene, which can be remedied via
281 expression of *cvds* from a strong P_{trc}/P_{T7} promoter and heterologous expression of the mevalonic acid
282 pathway.

283 Discussion

284 Valerenic acid is the most potent sesquiterpenoid component produced in the roots of *Valeriana*
285 *officinalis* that exhibits GABA-A activity. Development of this compound as a novel anxiolytic drug is
286 hindered by the low production of valerenic acid by the native plant. As a first step towards the
287 development of a production platform for valerenic acid analogues, we have employed *E. coli* as a
288 workhorse metabolic host for engineering of terpene chemicals. In this study, valerenadiene was
289 produced by introducing a codon-optimized valerenadiene synthase gene into *Escherichia coli* along with
290 an engineered MEP pathway or *Saccharomyces cerevisiae*-based mevalonic acid pathway. The codon-
291 optimized valerenadiene synthase gene was found to be a critical component for engineering high
292 valerenadiene titers, as it resulted in 3-fold higher production of valerenadiene as compared to the
293 wildtype sequence. This very likely indicates that translation of the codon-optimized form of the gene is
294 very efficient. Further improvements in productivity were realized by the overexpression of the *dxs*, *idi*,
295 and *fps* genes to augment carbon flux through the MEP pathway. This strategy resulted in a 65-fold
296 increase in production of valerenadiene (2.09 mg/L). This was improved a further 6-fold by optimizing the
297 fermentation medium to 2xYT with 3% glycerol supplementation (11.0 mg/L). Next, the *cvds* gene was
298 fused to the stronger P_{trc} promoter, which resulted in approximately 40% increase in production of
299 valerenadiene when pTrcHis-*cvds* was co-expressed with the engineered MEP pathway (20 mg/L). This
300 result demonstrated the importance of driving expression of valerenadiene synthase from strong
301 promoters to ensure ample availability of terpene synthase catalyst.

302 Notably, the valerenadiene synthase exhibits similar kinetic properties (e.g. K_m for FPP = 7.2 μM,
303 $k_{cat} = 5.7 \times 10^{-3} \text{ s}^{-1}$) to other published terpene synthases (Yeo et al., 2013). For example, the well-
304 characterized amorphadiene synthase exhibits a two-fold lower K_m for FPP (i.e. K_m = 3.3 μM and $k_{cat} =$
305 $6.8 \times 10^{-3} \text{ s}^{-1}$) (Picaud et al., 2005). Further efforts to improve the catalytic functionality of valerenadiene
306 synthase could incorporate rationale modifications to the enzyme. Alternatively, further improvements in
307 terpene synthase activity have been realized via expression of a terpene synthase as a gene fusion with
308 farnesyl pyrophosphate synthase to achieve substrate channeling of FPP to the active site of VDS. Wang

309 and co-workers reported that expression of *ispA* and apple farnesene synthase (*aFS*) from the same
310 construct with the mevalonic acid pathway resulted in production of 57.6 mg/L of α -farnesene in *E. coli*
311 (Wang et al., 2011). Fusing of *ispA-aFS* as a single gene construct with a (GGGS)₂ amino acid linker
312 increased production to 87.8 mg/L of α -farnesene. Additionally, Niehaus et al. achieved substrate
313 channeling by fusing the triterpene synthases SSL-1 and SSL-3 genes to form a single gene fusion, SSL-
314 1+SSL-3, which increased production from 20 mg/L botryococcene to 50 mg/L botryococcene in yeast
315 (Niehaus et al., 2011).

316 However, the greatest increases in valerenadiene yield involved heterologous expression of the
317 *Saccharomyces cerevisiae* mevalonic acid pathway. Expression of the MVA pathway resulted in an
318 unregulated metabolic flux towards FPP, which could be efficiently coupled to valerenadiene synthase for
319 production of 62.0 mg/L valerenadiene in culture tubes with a specific productivity of 19.4 mg/L/OD₆₀₀.
320 This study showed that *E. coli* is capable of high-level production of valerenadiene with relatively minimal
321 optimization of the production media and commercially available expression vectors, such as pBBa5c-
322 MevT-MBIS(CO,*ispA*). Further efforts to improve valerenadiene yield will require optimization of the
323 upstream and downstream genetic components of the MVA pathway and subsequent scale-up
324 fermentation in bioreactors. In addition, we are conducting rational attempts to engineer CYP450s to
325 oxidize and functionalize the valerenadiene skeleton to generate a library of potential GABA-A ligands.

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335

336 **Figure 1** Biosynthesis of valerenadiene via the methyl erythritol phosphate pathway (MEP, pathway A)
337 and the mevalonic acid pathway (MVA, pathway B). IPP and DMAPP are synthesized via the MEP or
338 MVA pathways via different upstream routes. The MEP pathway is initiated via condensation of
339 glyceraldehyde 3-phosphate and pyruvate from glycolysis via 1-deoxy-D-xylulose 5-phosphate synthase
340 (DXS), and the MVA pathway is initiated via condensation of two molecules of acetyl-CoA. Two
341 molecules of IPP and one molecule of DMAPP are condensed into C₁₅ farnesyl pyrophosphate (FPP).
342 FPP is cyclized into valerenadiene via valerenadiene synthase (VDS). The abbreviations of the enzymes
343 are as follows: DXS, 1-deoxy-D-xylulose 5-phosphate synthase; atoB, thiolase; HMGS, hydroxy-
344 methylglutaryl-CoA synthase; HMGR, truncated hydroxy-methylglutaryl-CoA reductase; ERG12,
345 mevalonate kinase; ERG8, phosphomevalonate kinase; MVD1, diphosphomevalonate decarboxylase;
346 IDI, isopentenyl diphosphate isomerase; FPS, farnesyl pyrophosphate synthase from *Gallus gallus*; IspA,
347 farnesyl pyrophosphate synthase from *Escherichia coli*; VDS, valerenadiene synthase.
348

349 **Figure 2** Engineering of wildtype (pZE-*wvds*) and codon-optimized (pZE-*cvds*) valerenadiene synthase.
350 **(A)** *E. coli* engineered with empty vector control, wildtype, and codon optimized VDS genes was grown
351 for 24 (white bars) and 48 hours (black bars) and yield of valerenadiene was determined. **(B)** Growth of
352 engineered *E. coli* lines was determined by measuring optical density (OD₆₀₀) at 24 (white bars) and 48
353 hours (black bars), respectively. **(C)** *E. coli* strains were co-transformed with empty vector control,
354 wildtype, or codon-optimized *vds* and pSTV-*dxs-idi-fps* for enhancement of the endogenous MEP
355 pathway. **(D)** Growth of engineered *E. coli* lines was determined by measuring optical density (OD₆₀₀).
356 Strains were inoculated in 5 mL cultures of LB media with 1 mL decane overlay and experiments were
357 carried out in triplicate. Error bars indicate standard error of the mean.
358

359 **Figure 3** Effect of glycerol conditions on production of valerenadiene. *E. coli* strains engineered with
360 pZE-*wvds* and pSTV-*dxs-idi-fps* or pZE-*cvds* and pSTV-*dxs-idi-fps* were grown in 5 mL 2xYT media with
361 0%-4% glycerol and 1 mL decane overlay for 48 hours and valerenadiene yield was determined.
362 Experiments were carried out in triplicate and error bars indicate standard error of the mean.
363

364 **Figure 4** Time course of *E. coli* expressing an engineered MEP pathway and valerenadiene synthase.
365 *E. coli* DH5αZ1 strains harboring the engineered MEP construct pSTV-*dxs-idi-fps* were co-expressed with
366 the wildtype (pZE-*wvds*, solid line) or codon-optimized (pZE-*cvds*, dotted line) valerenadiene synthase
367 gene. Cultures were grown in 50 mL 2x YT+3% glycerol with 10 mL decane overlay for 72 hours. (Left
368 panel) Valerenadiene yield (mg L⁻¹) and (right panel) biomass (OD₆₀₀) were determined from double
369 triplicate experiments. Error bars indicate the standard error of the mean.
370

371 **Figure 5** Time course experiment of *E. coli* co-expressing pTrcHis-*cvds* and the *Saccharomyces*
372 *cerevisiae* MVA pathway. *E. coli* DH5αZ1 strains harboring the pBBa5c-MevT+MBIS(CO,IspA) and
373 pTrcHis-*cvds* constructs were grown in 50 mL 2x YT+3% glycerol with 10 mL decane overlay for 72
374 hours. (Left panel) Valerenadiene yield (mg L⁻¹) and (right panel) biomass (OD₆₀₀) were determined from
375 double triplicate experiments. Error bars indicate the standard error of the mean.
376

377 **Figure 6** Production of valerenadiene using the T7 RNA polymerase to drive expression of *vds*. *E. coli*
378 BL21(DE3) strains harboring empty vector control, pET-*wvds*, and pET-*cvds* were expressed with and
379 without the *Saccharomyces cerevisiae* MVA pathway (pBBa5c-MevT-MBIS(CO, IspA)). Strains were
380 grown in triplicate 5 mL 2xYT media + 3% glycerol fermentations with 1 mL decane overlay for 48 hours.
381 (Left panel) valerenadiene volumetric production (mg/L) was determined after 48 hours. (Right panel)
382 Valerenadiene production was normalized by dividing volumetric production by the biomass after 48
383 hours (mg/L/OD₆₀₀).

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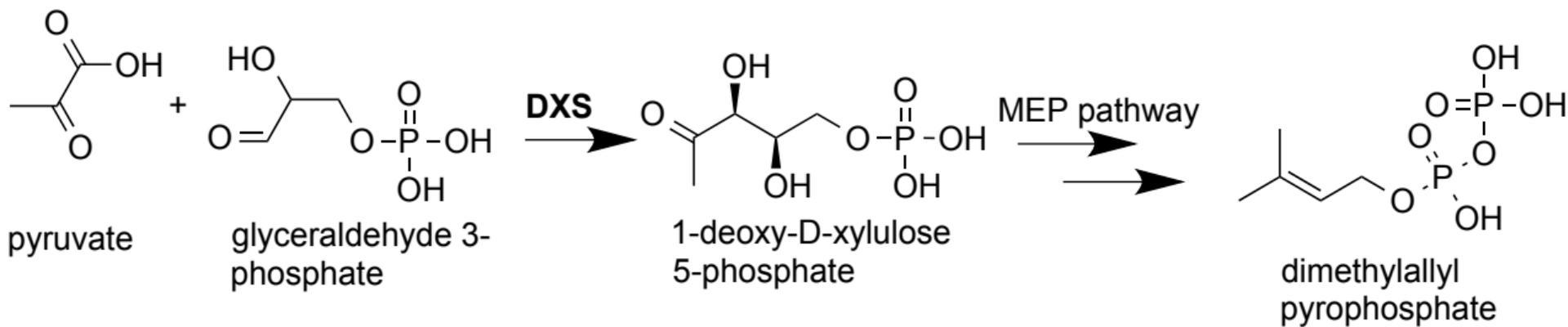
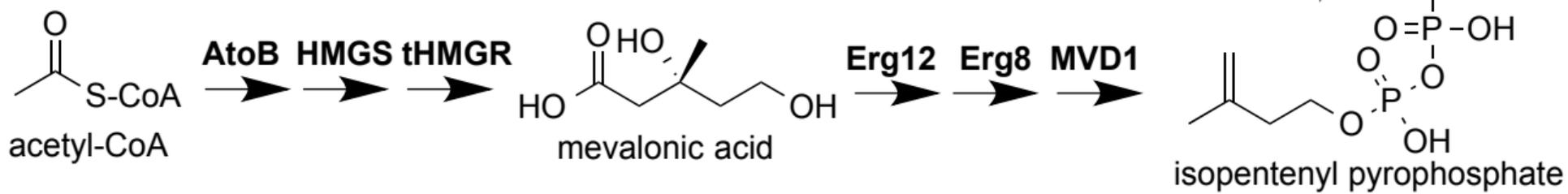
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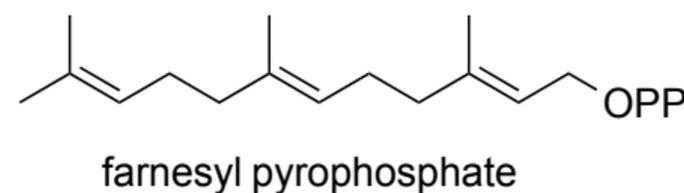
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Methyl erythritol phosphate (MEP)

A**B**

Mevalonic acid pathway (MVA)

**FPS
IspA****VDS**