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This is a pre print version of the following article:

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1681518> since 2019-06-11T11:45:00Z

Published version:

DOI:10.1038/s41589-018-0166-5

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Synthesis of 11-carbon terpenoids in yeast

Engineering the biosynthesis of a non-canonical building block expands the chemical space accessed
by terpenoids

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Abstract:

One application of synthetic biology is the re-design of existing biological systems to acquire new functions. In this context, expanding the chemical code underlying key biosynthetic pathways will lead to the synthesis of compounds with new structures and potentially new biological activities. Terpenoids are the largest group of specialized metabolites with numerous applications. Yet, being synthesized from 5-carbon units, they are restricted to distinct classes that differ by 5 carbon atoms (C₁₀, C₁₅, C₂₀, etc.). To expand the diversity of terpenoid structures, we engineered yeast cells to synthesize a non-canonical building block with 11 carbons, and produced 40 C₁₁ terpene scaffolds that can form the basis for an entire terpenoid class. By identifying a single-residue switch that converts C₁₀ plant monoterpene synthases to C₁₁-specific enzymes, we engineered dedicated synthases for C₁₁ terpene production. This approach will enable the systematic expansion of the chemical space accessed by terpenoids.

29 For centuries, specialized metabolites, the small molecules made by plants and microorganisms to
30 protect themselves and to communicate with their environment, provide humankind with
31 pharmaceuticals, flavors, fragrances and colorants. Despite the immense diversity of specialized
32 metabolites found in nature, the pathways responsible for their biosynthesis are based on a handful of
33 basic building blocks derived from primary metabolism¹. As a result, the chemical space accessed by
34 specialized metabolites is restricted to the structural complexity that can be obtained using the specific
35 building blocks. This is particularly evident in the case of terpenoids, which are synthesized from
36 prenyl diphosphate precursors involved in the synthesis of sterols and other membrane lipids. The core
37 terpenoid biosynthetic pathway is built up by the successive addition of 5-carbon (C₅) units,
38 consequently giving rise to a small set of building blocks that differ by 5 carbon atoms. In turn, each of
39 these building blocks generates a distinct class of terpenoid compounds, i.e. monoterpenoids (C₁₀),
40 sesquiterpenoids (C₁₅), diterpenoids (C₂₀), etc^{2,3}. The use of a small number of building blocks, in
41 combination with the modular structure of the downstream pathways⁴, establishes a chemical code for
42 terpene biosynthesis (Fig. 1). According to this code, biosynthetic activities involving initially terpene
43 synthases and subsequently decorating enzymes (cytochrome P450s, dehydrogenases, O-
44 methyltransferases, O-acetyltransferases, etc.) act in a sequential manner on prenyl diphosphate
45 building blocks of defined size to yield a plethora of structurally complex molecules (Fig. 1). This
46 general scheme is very strict and only few exceptions have been reported⁵⁻⁹. Expanding the chemical
47 code of terpenoid biosynthesis will provide numerous structures with unexplored properties and
48 potentially new pharmacological or industrial applications.

49 To introduce additional layers in the stratified terpenoid chemical space, we aimed to bypass the
50 strict dependence of terpenoid biosynthesis on building blocks of specific size. **Previous studies using**
51 **recombinant enzymes suggested that it may be possible for a terpene synthase to convert non-standard**
52 **substrates**^{10,11}. To enable a systematic approach, we set out to develop a complete biosynthetic system
53 for novel terpenoids by engineering the yeast *Saccharomyces cerevisiae* to produce alternative terpene
54 scaffolds. To this end, we searched for enzymatic activities that can be used to synthesize non-
55 canonical prenyl diphosphates. Certain bacteria produce a unique volatile, 2-methylisoborneol (2MIB,
56 **1**), which contributes to the musty-earthly fragrant notes of Brie and Camembert cheeses¹². 2MIB is an
57 11-carbon molecule synthesized by a unique mechanism in which the universal monoterpene precursor
58 geranyl diphosphate (GPP) becomes methylated at C-2 by a dedicated methyltransferase and
59 subsequently the 2-methyl-GPP (2meGPP) substrate is cyclized by a specific synthase to yield 2MIB
60 (inset in right panel of Fig. 1 and references^{13,14}). This mechanism appears to be specific for the
61 biosynthesis of 2MIB and the related compound 2-methylenebornane (2MB, **2**), since no other C₁₁

62 compound has been reported as a major metabolite in bacteria¹⁵⁻¹⁷. In addition, all gene clusters
63 containing the combination of a methyltransferase with a terpene synthase characterized to date have
64 been found to produce either 2MIB or 2MB as the main product^{13,14,18-20}. Thus, we explored the
65 possibility to use 2meGPP as an alternative substrate for the systematic synthesis of non-canonical
66 terpenes in yeast.

67

68 **Results**

69 **Establishing efficient 2meGPP synthesis.** To engineer yeast cells to produce 2meGPP, we introduced
70 the GPP methyltransferase from the cyanobacterium *Pseudanabaena limnetica* (*PIGPPMT*)¹⁹ into a
71 strain engineered for terpenoid production (strain AM94; Table S1). To monitor synthesis of 2meGPP,
72 we also introduced the second enzyme in the pathway, *P. limnetica* 2MIB synthase (*PIMIBS*)¹⁹, and
73 applied solid-phase micro extraction (SPME) of the head-space above yeast liquid cultures to analyze
74 the volatile compounds produced by the engineered cells. The 2MIB degradation products 2MB, 1-
75 methylcamphene (1MC, **3**) and 2-methyl-2-bornene (2M2B, **4**) were clearly detected in the head-space
76 above cells co-expressing *PIGPPMT* and *PIMIBS*, confirming synthesis of 2meGPP and production of
77 2MIB (Fig. 1, 2a, and Table S2). 2MIB is unstable and readily converts to an approximately 1:1 ratio of
78 2MB:1MC and traces of 2M2B²¹. The identity of these compounds was confirmed by comparison with
79 authentic 2MIB and its degradation products, while control experiments confirmed that these
80 compounds were not produced by a yeast enzyme or any of the two *P. limnetica* enzymes alone (Fig.
81 **2a** and Fig. S1).

82 Establishing 2meGPP synthesis in yeast opened the possibility to use this alternative building
83 block to produce non-canonical terpenoids. To facilitate this effort, we first aimed to improve 2meGPP
84 production. In yeast, GPP is synthesized by Erg20p, which is a bi-functional enzyme that subsequently
85 converts it to farnesyl diphosphate (FPP). The mechanism of Erg20p is distributive, allowing GPP to be
86 released and taken up by *PIGPPMT* to synthesize 2meGPP. Thus, as shown in the scheme in Fig. **2b**,
87 Erg20p competes with *PIGPPMT* for GPP. To facilitate *PIGPPMT* in obtaining access to GPP, a series
88 of interventions were implemented and the *PIMIBS* products were used as readout to evaluate system
89 performance in each step. **Protein fusions between enzymes catalyzing successive steps have**
90 **previously been shown to improve heterologous terpenoid production²².** Thus, N-terminal and C-
91 terminal fusions of *PIGPPMT* with Erg20p were constructed and tested. Expression of the Erg20p-
92 *PIGPPMT* fusion resulted in a 15-fold increase in *PIMIBS*-produced C₁₁ compounds compared to
93 expression of the non-fused Erg20p and *PIGPPMT* proteins (Fig. **2c**). A lower 6.1-fold increase was
94 observed when the reverse fusion was used (Fig. **2c**). **Evaluation of the *PIGPPMT* protein levels by**

95 western blotting revealed that the observed improvements in C₁₁ production could be attributed to the
96 higher levels of the *P/GPPMT* protein when fused to Erg20p (Fig. S2). Subsequently, to further
97 decrease competition for GPP by Erg20p, we introduced a mutant form of Erg20p that functions as a
98 GPP synthase. Erg20p(N127W) had previously been developed in our lab as a variant that specifically
99 synthesizes GPP and at the same time inhibits FPP synthesis by wild-type Erg20p²³⁻²⁵. By
100 incorporating the N127W mutation into the Erg20p-*P/GPPMT* fusion an additional 4.5-fold
101 improvement in C₁₁ production was obtained (Fig. 2d). To further improve C₁₁ synthesis, the level of
102 wild-type Erg20p was reduced by heterozygous deletion of *ERG20*²⁶. Strain MIC1 (Table S1),
103 generated by replacing one of the two *ERG20* alleles with the gene encoding *P/GPPMT*, was 5.3-fold
104 more efficient than the parent strain, AM94, when *P/MIBS* and the Erg20p(N127W)-*P/GPPMT* fusion
105 were overexpressed (Fig. 2e; Table S3).

106 We subsequently explored whether the performance of *P/GPPMT* could be improved. We
107 constructed a model of *P/GPPMT* based on the structure of the closely related GPPMT from
108 *Streptomyces coelicolor* (PDB id: 3VC2; reference²⁷) (Fig. S3) and searched for residue substitutions
109 that could facilitate the association of the GPP substrate. A total of 16 residues were selected (Y36,
110 M160, Y161, I202, Y206, C208, I210, Y240, W241, R244, V250, V253, F257, Y261, F266, and Y268;
111 Fig. S3) and a library of 44 different mutants of the non-fused form of the *P/GPPMT* enzyme was
112 constructed (Table S5). Screening this library in yeast using the *P/MIBS* products as readout revealed
113 two substitutions, V250A and F266H, which improved performance by approximately 2- and 3-fold,
114 respectively (Fig. 2f and Table S4). The most efficient substitution, F266H, was subsequently
115 introduced into the Erg20p(N127W)-*P/GPPMT* fusion and a 2-fold improvement in production was
116 obtained (Table S3). The combination of all the above interventions amounted in an overall 760-fold
117 improvement in C₁₁ yield from the starting strain (Table S3 and Fig. 2g), reaching 23.5 mg/L.
118 Consistent with these findings, analysis of the intracellular prenyl diphosphates revealed a 1.94±0.29:1
119 ratio of 2meGPP:GPP in the most efficient strain.

120 The new building block does not interfere with yeast metabolism. First, we confirmed that
121 2meGPP was not taken up by Erg20p to produce larger prenyl diphosphates. We set up *in vitro*
122 reactions using bacterially produced Erg20p (Fig. S4) and found that although the recombinant enzyme
123 could readily produce FPP from GPP or DMAPP (and IPP), it was not active with 2meGPP (online
124 methods). We also analyzed the extracts of Erg20p(N127W)-*P/GPPMT*-expressing yeast cells and
125 found no additional Erg20p-derived product beyond the regular sterol, squalene or sesquiterpenol
126 compounds (Fig. S5). We were also unable to detect any 2meGPP-derived products of Erg20p in yeast
127 cell extracts supplemented with 2meGPP and IPP (online methods). We also confirmed that *P/GPPMT*

128 could not methylate other diphosphate substrates than GPP. For this, we tested recombinant *PI*GPPMT
129 (Fig. S4) in *in vitro* reactions with other prenyl diphosphate substrates (DMAPP, IPP, FPP and GGPP)
130 and were not able to identify any new products (online methods). In combination with the overall
131 observation that yeast growth properties were unaffected by *PI*GPPMT expression (Fig. S6), we
132 concluded that the system developed here is orthogonal to the yeast metabolism.

133 **The 2meGPP building block can provide several C₁₁ terpenes beyond 2MIB and 2MB.**

134 Having established efficient synthesis of 2meGPP, we questioned whether this building block can yield
135 other C₁₁ terpene scaffolds. Initially, we evaluated the range of products obtained by other microbial
136 enzymes by expressing the 2MB synthases from *Micromonospora olivasterospora* (*Mo*MBS)¹³ and
137 *Pseudomonas fluorescens* (*Pf*MBS)¹⁸. The volatile profiles produced by *Mo*MBS or *Pf*MBS were
138 clearly dominated by 2MB, 1MC and 2M2B which in combination reached 93.8% and 67.1% of total
139 C₁₁ terpene products, respectively (Table S2). However, several other C₁₁ terpenes could also be
140 detected in lower amounts (Fig. S7). These included 2-methylmyrcene (5), 2-methyllimonene (6), 2-
141 methylinalool (7), 2-methyl- α -terpineol (8), 2-methylgeraniol (9) (Fig. 1 and Fig. S8), and 17
142 additional C₁₁ products (Fig. S7, Tables S2 and S6) identified by characteristic molecular fragment ions
143 in mass-spectrometry (m/z 150 for C₁₁ terpene hydrocarbons or 168 for C₁₁ terpene alcohols). The
144 structure of the 17 minor products could not be characterized at this stage because of their low
145 quantities and the complexity of the mixture. Re-examination of the *PI*MBS product profile using the
146 improved yeast platform revealed that some of these molecules could also be detected as minor
147 products of *PI*MBS (Tables S2 and S6). Several of the minor C₁₁ compounds (2-methylmyrcene, 2-
148 methyllimonene, 2-methylinalool, 2-methyl- α -terpineol) had previously been identified in *in vitro*
149 assays using the studied enzymes^{18,28} or in minute amounts in the headspace of bacterial cultures^{16,29},
150 where, in agreement with present findings, they were considered to be side-products of a promiscuous
151 terpene synthase rather than the main product of a specific enzyme. Our engineered yeast platform also
152 produces GPP, and we observed that all three 2MIB/2MB synthases were also able to accept GPP as a
153 substrate and produce C₁₀ terpenes (Fig. 3 and S7). *Mo*MBS was the least efficient with GPP,
154 producing only 8.9% of C₁₀ terpenes, while *PI*MBS produced 10.9% C₁₀ compounds (Fig. 3). *Pf*MBS
155 was the most promiscuous enzyme of the three, producing an almost equal amount of the two product
156 groups (56.2% C₁₁ and 43.8% C₁₀ terpenes; Fig. 3). These results strongly suggested that 2meGPP can
157 give several other products beyond 2MIB/2MB and that there is likely a broad chemical diversity of
158 C₁₁ structures that can be obtained from this building block, which could be similar in complexity to
159 that obtained by the canonical monoterpene substrate GPP (Fig. 1). However, since the bacterial
160 synthases identified so far are restricted to the 2MIB/2MB bouquet (2MIB, 2MB, 2M2B, 1MC)¹³,

161 tapping into the unexplored C₁₁ diversity would require mining or engineering suitable terpene
162 synthases.

163 **Expanding the diversity of C₁₁ terpenes.** The ability of the 2MIB and 2MB synthases to
164 accept both GPP and 2meGPP, albeit with varying efficiency, suggested that these two building blocks
165 could serve as alternative substrates for the same enzyme. Thus, to access the C₁₁ diversity, we
166 investigated whether enzymes characterized as canonical terpene synthases could accept 2meGPP to
167 yield C₁₁ products. To this end, we turned to the rich resource of plant monoterpene synthases and
168 selected 7 different enzymes with varied product specificity. Expression of the 1,8-cineole synthase
169 from *Salvia fruticosa* (*SfCinS1*)³⁰ together with the Erg20p(N127W)-*PlGPPMT*(F266H) fusion
170 resulted in the production of 19 C₁₁ compounds, alongside the characteristic C₁₀ monoterpenes
171 produced by this enzyme from GPP. The main C₁₁ products were 2MB (58.5% of C₁₁), 2-
172 methylmyrcene (19.4% of C₁₁), a yet unknown C₁₁ alcohol (11.2% of C₁₁), and 2-methyllimone (2.1%
173 of C₁₁) (Fig. 4a, Tables S2 and S6). Still, wild-type *SfCinS1* produced predominantly C₁₀ terpenes
174 (73.9% of total C₁₀ and C₁₁ production) in the yeast system (Fig. 3). The second plant monoterpene
175 synthase tested, pinene synthase from *Pinus taeda* (*PtPinS*)³¹, converted 2meGPP to 29 different C₁₁
176 compounds accounting for almost 26% of the product blend from both substrates (Fig. 3 and 4b, Tables
177 S2 and S5). 2MB, 2-methylinalool, 1MC and 2-methylmyrcene were among the most abundant C₁₁
178 products of *PtPinS* (Table S2). Sabinene synthase from *S. pomifera* (*SpSabS*)³⁰ produced a complex
179 blend of 17 C₁₁ volatiles with 2-methylmyrcene as the main C₁₁ product (45% of C₁₁) (Fig. 3 and 4c,
180 Tables S2 and S6). Myrcene synthase from *Ocimum basilicum* (*ObMyrS*)³² converted 2meGPP mainly
181 to 2-methylmyrcene (59.7% of C₁₁) and 2-methylinalool (24.1% of C₁₁) (Fig. 3 and Tables S2 and S6).
182 Geraniol synthase from *O. basilicum* (*ObGerS*)³³ produced 19 different C₁₁ terpenes (Fig. 3 and Tables
183 S2, S6), three of which were made only by this enzyme. *ObGerS* produced 2% C₁₁ compounds in the
184 yeast system. The highly specific limonene synthase from *Citrus limon* (*CLimS*)³⁴ produced a C₁₁
185 blend dominated by 2-methyllimonene (70% of C₁₁; Fig. 3, Tables S2 and S6). *Solanum elaeagnifolium*
186 camphene synthase (*SeCamS*)³⁵ produced 14 C₁₁ compounds, including 2-methylmyrcene (55.2% of
187 total C₁₁), 2M2B, 1MC, 2MB, traces of 2-methyllimonene and 9 additional C₁₁ molecules, amounting
188 to 6% of total terpenes produced (Fig. 3 and 4d, Tables S2 and S6).

189 Overall, the 7 plant monoterpene synthases tested produced 36 different C₁₁ terpenes beyond
190 the 2MIB/2MB blend (2MIB, 2MB, 2M2B and 1MC; Tables S2 and S5). Although all plant enzymes
191 exhibited relative preference for GPP, their efficiency with 2meGPP in the yeast system ranged from
192 approximately 25% C₁₁ in the case of *SfCinS* and *PtPinS* to 1% in *CLimS1* (Fig. 3). Many C₁₁
193 compounds were synthesized exclusively by the plant **terpene synthases**. These results suggested that it

194 would be possible to expand the terpene universe in a predictable and systematic manner using an
195 alternative substrate and terpene synthases from plants or other organisms. But since all the plant
196 enzymes tested here still produced preferentially the canonical monoterpene products in the yeast
197 system, we set out to develop dedicated C₁₁ synthases by protein engineering.

198 **Developing C₁₁-specific terpene synthases.** Due to the availability of a crystal structure³⁰,
199 *SfCinS1* was selected as the starting point to generate variants with improved selectivity towards
200 2meGPP. We examined the structure of *SfCinS1* in conjunction with that of the closely related
201 limonene synthase from *Mentha spicata* in the presence of substrate analogues³⁶. The early steps of the
202 monoterpene synthase mechanism involve the binding and ionization of the GPP substrate, followed by
203 the *syn*-migration of the diphosphate moiety to C-3 to yield linalyl diphosphate (LPP) in the transoid
204 conformation (Fig. 5a). Subsequent rotation of the C-2,3 bond converts LPP to the cisoid conformer,
205 which brings C-6 and C-1 at a position competent for cyclization (closed conformation). Another
206 ionization event promotes cyclization to generate the α -terpinyl cation, which further isomerizes to the
207 final products. Recent evidence from experiments with 2-fluoro-GPP and 2-fluoro-LPP suggested that
208 initial substrate binding occurs at an extended conformation, as shown in Fig. 5c left panel, and not in
209 the catalytically competent closed conformation (Fig. 5d right panel)³⁶. This has been considered to be
210 an essential requirement for the transoid to cisoid transition, because the required rotation around the
211 C-2,3 bond would be forbidden by the position of the 6,7-double bond in the closed conformer of LPP
212³⁶. As a result, the closed form is believed to be adopted only subsequently to the conversion of the
213 transoid to the cisoid conformer.

214 Since the bulkier 2-propenyl substituent of 2-methyl-LPP would pose an even greater difficulty
215 in the rotation of the C-2,3 bond, it is possible that canonical C₁₀ terpene synthases could be less
216 efficient with 2meGPP because the shape of their active site would be too narrow to fit an adequately
217 extended configuration of 2meGPP. Thus, we selected two sites at the bottom of the active site cavity
218 of *SfCinS1*, namely N338 and I451 (Fig. 5c, d), which if substituted could provide the additional space
219 required to facilitate the binding of the extended substrate conformation. A series of site-directed
220 mutants were constructed to test this hypothesis (Table S7). Among the N338 variants, *SfCinS1*(N338S)
221 resulted in an approximately 66% improvement in total C₁₁ levels and a shift to 2-methylmyrcene as
222 the main product. Another N338 variant, *SfCinS1*(N338A), was equally efficient with wild-type in
223 overall C₁₁ synthesis but was three times more specific for 2-methylmyrcene compared to other
224 products (Table S7). Substitution of I451 by A, V or S resulted in improved or comparable C₁₁
225 production and a shift in specificity towards 2-methylmyrcene in all cases (Table S7). In one of the
226 I451 variants, *SfCinS1*(I451A), 2-methylmyrcene reached 72% of the total C₁₁ production. The two

227 most successful of these substitutions were combined and the double variant *SfCinS1(N388S-I451A)*
228 showed significant improvement in C₁₁ yield and increased C₁₁-product specificity, producing almost
229 exclusively (88% of C₁₁) 2-methylmyrcene. As a result, in *SfCinS1(N388S-I451A)* 2-methylmyrcene
230 became the main product among both C₁₀ and C₁₁ compounds (Fig. 6a). Kinetic analysis of
231 recombinant *SfCinS1(N388S-I451A)* suggested that the observed increase in C₁₁ conversion by this
232 enzyme was the result of a significant improvement in 2meGPP binding affinity, manifested by an 8-
233 fold decrease in the apparent K_M (7.07 μM for the mutant vs. 57.7 μM for the wild-type; Table S8).
234 This was in agreement with the hypothesis that the specific mutation would facilitate binding of the
235 bulkier 2meGPP substrate. However, the overall catalytic efficiency of *SfCinS1(N388S-I451A)* was
236 significantly compromised (Tables S8, S9), prompting us to explore alternative approaches for the
237 development of synthases suitable for C₁₁ production in yeast.

238 **Identification of a C₁₁-specific single-residue switch.** Further examination of the available
239 structural information highlighted one residue in *SfCinS1*, F571, which could be involved in the
240 conversion from the open to the closed substrate conformation. As shown in Fig. 5b, F571 is positioned
241 in the trajectory of the transoid to cisoid transition and at a short distance from C-1 of LPP in the closed
242 form and could play a stabilizing or regulatory role in the extensive conformational changes undergone
243 by the substrate³⁶. We substituted F571 in *SfCinS1* with different side chains and found that variants
244 F571H and F571Y shifted production towards 2meGPP-derived compounds in yeast (Fig. 6a, 6d). In
245 *SfCinS1(F571H)*, this substitution improved specificity for 2MB, which comprised 84.7% of C₁₁
246 products (Fig. 6a; Table S10), while in *SfCinS1(F571Y)* it shifted product specificity to 2-methyl- α -
247 terpineol (58% of total C₁₁; Table S10). To understand the molecular basis of these changes, we carried
248 out kinetic analysis of recombinant *SfCinS1(F571Y)* and found that the substitution resulted in a 6-fold
249 improvement in the affinity for 2meGPP and a significant decrease in the $k_{\text{cat}}^{\text{app}}$ for GPP by 17-fold.
250 Although there was also an 8-fold decrease in 2meGPP turnover, the apparent catalytic efficiency
251 ($k_{\text{cat}}^{\text{app}}/K_{\text{M}}^{\text{app}}$) for 2meGPP surpassed that for GPP (Table S8).

252 Based on this finding, we examined an amino acid sequence alignment of *SfCinS1* with the
253 other plant terpene synthases included in this study and found that residues analogous to F571 were
254 typically an F or an H in the other enzymes (Fig. S9). We targeted these residues for site-directed
255 mutagenesis (Table S5). Three limonene synthase variants, *CiLimS(H570V)*, *CiLimS(H570L)* and
256 *CiLimS(H570I)* became 2-methyllimonene synthases producing 70-95% C₁₁ compounds in yeast, of
257 which 79-88% was 2-methyllimonene (Fig. 6c, 6d and Table S10). In *PtPinS*, all variants tested
258 switched specificity to become C₁₁ synthases (between 60-88% of C₁₁ products). *PtPinS(F607I)*
259 synthesized mainly 2MB, while *PtPinS(F607L)* produced predominantly 2-methylinalool (Fig. 6b, 6d

260 and Table S10). Moreover, in *ObMyrS*, two variants, F579V and F579I, switched their preference to
261 2meGPP and produced 55% C₁₁ terpenes giving rise to specific 2-methylinalool synthases (Fig. 6d and
262 Table S10). In *SpSabS*, variants H561Y, V or L became promiscuous C₁₁ synthases (60-70% C₁₁
263 production) synthesizing 2MB as the main compound (Fig. 6d and Table S10). *SeCamS*(H583L) was
264 also selective for 2meGPP producing mainly 1MC (Fig. 6d and Table S10).

265 To further understand the observed shifts in specificity, we analyzed the kinetic parameters of
266 *C/LimS* variants H570L, V and I, and *SeCamS* variant H583L, and found that the H570/H583
267 substitutions resulted in strong decreases in the $k_{\text{cat}}^{\text{app}}$ for GPP but only small changes in the $k_{\text{cat}}^{\text{app}}$ for
268 2meGPP (Table S8). As a result, the catalytic efficiency of the mutants shifted in favor of 2meGPP. To
269 further corroborate these findings, we carried out *in vitro* substrate competition experiments using
270 recombinant *SfCinS1*(F571Y) and *C/LimS*(H570L). We confirmed that at equal GPP and 2meGPP
271 concentrations, both variants produced higher amounts of C₁₁ than C₁₀ products (Fig. S10). At a 2:1
272 2meGPP:GPP ratio, corresponding to the determined ratio in our engineered yeast cells, both
273 *C/LimS*(H570L) and *SfCinS1*(F571Y) produced >75% C₁₁ products (Fig. S10), explaining the
274 dominance of C₁₁ products in the product blend of yeast cells expressing these variants (Fig. 6d). These
275 findings suggest that F571 in *SfCinS1*, and related residues in other terpene synthases, likely perform a
276 critical function in the reaction of canonical terpene synthases with GPP, which is, however, not
277 equally critical for 2meGPP turnover. Thus, this residue can serve as a single-residue switch to change
278 the substrate specificity of existing synthases and derive C₁₁-specific enzymes.

279 Overall, we obtained several dedicated C₁₁ synthases specific for products other than those
280 made by the bacterial 2MIB/2MB synthases. These include the 2-methylimonene synthases
281 *C/LimS*(H570V, L or I), the 2-methyl- α -terpineol synthase *SfCinS1*(F571Y), the 2-methylinalool
282 synthases *ObMyrS*(F579V) and *PtPinS*(F607L), and the 2-methylmyrcene synthase *SfCinS1*(N388S-
283 I451A). Although some of these variants were less efficient in C₁₁ synthesis than their wild-type
284 counterparts (yeast titer ranging from 56% to 14% of the corresponding wild-type enzyme, Table S9),
285 having now established the principles for substrate selectivity, further protein engineering will enable
286 the development of optimized C₁₁ synthases for the highly efficient and specific synthesis of products
287 desirable for industrial applications.

288

289 Discussion

290 The biosynthetic pathways of specialized metabolism are based on a handful of basic building blocks
291 (e.g. aromatic amino acid-derived C₆-C₃ or C₆-C₁ blocks, acetyl CoA-derived C₂ blocks, isoprenoid C₅
292 blocks, etc.). This restricts the chemical space that can be accessed by specialized metabolites to the

293 complexity that can be generated using these basic scaffolds. Expanding specialized biosynthesis using
294 additional building blocks can help explore uncharted areas of the chemical space and provide
295 molecules with new or improved properties. To address this challenge, we focused on terpenoids
296 because they are the most numerous group of plant natural products and have wide-spread applications
297 as pharmaceuticals (e.g. taxol, artemisinin), flavors and fragrances (e.g. ambroxan), sweeteners (e.g.
298 steviosides), nutraceuticals and colorants. To establish proof-of-principle, we started from the simplest
299 group, the monoterpenoids, and hijacked a biosynthetic mechanism dedicated to the synthesis of
300 specific microbial compounds (2MB/2MIB) to produce a new terpenoid precursor in yeast cells. We
301 established a modular platform to harvest the chemical diversity that can be produced from this new
302 building block in a systematic and predictable manner by acquiring parts from canonical monoterpene
303 biosynthesis and engineering them to become specific for the new substrate. This chemical diversity
304 can now be expanded further by introducing the subsequent modules of terpene biosynthesis, such as
305 P450-driven oxidations, reductions, acetylations etc., to yield a whole class of non-canonical terpenoids.
306 The efficient yeast production system established here will facilitate analysis of the properties of the
307 new compounds and enable the development of new industrial products. Furthermore, the parts and
308 know-how developed in yeast can be transferred to other organisms, such as bacteria or plants, to help
309 investigate *in vivo* the potential biological functions of the novel compounds. The monoterpene
310 building block GPP is also involved in the biosynthesis of other natural products, such as iridoids,
311 monoterpene indole alkaloids, cannabinoids, and other prenylated aromatic compounds. Thus,
312 establishing a yeast platform that synthesizes 2meGPP also opens up the possibility to engineer the
313 synthesis of non-canonical variants of many other GPP-containing specialized metabolites, several of
314 which belong to pharmacologically important groups (e.g. the monoterpene indole alkaloid anticancer
315 agents vinblastine and vincristine).

316 This effort makes the first step towards creating a far broader diversity of non-canonical
317 terpenoid structures. Larger terpenoids, such as sesqui-, di- and tri-terpenoids, have higher inherent
318 complexity due to their larger size. Having established proof-of-concept with the production of C₁₁
319 terpenes, the same tools and overall approach can now be extended to the other terpenoid classes to
320 achieve the production of larger-size non-canonical terpenes. This can be achieved by applying gene
321 mining and protein engineering to establish methylation of larger building blocks in the terpene
322 biosynthetic ladder, such as FPP and GGPP. In combination with the increased potential for complex
323 decoration of the larger scaffolds, this approach will result in an expansion of novel structures.

324 These findings also raise important questions regarding the extent of C₁₁ terpene biosynthesis in
325 bacteria or other organisms and the process of evolution of specialized metabolic pathways. Although

326 our results suggest that it is possible to produce a large number of C₁₁ scaffolds from 2meGPP and to
327 develop specific synthases for several C₁₁ compounds from existing parts, such synthases seem to be
328 absent from bacteria while the volatile bouquet of all microbial cultures studied to date is strictly
329 dominated by 2MIB or 2MB. It is possible that the present situation is a snapshot of evolution taken at
330 an early stage of the development of a new pathway. It would be very informative to analyze the
331 continuously expanding microbial genomic information for clusters containing combinations of terpene
332 synthase and methyltransferase genes and to study their evolution.

333 Our work used terpenoid biosynthesis to show that synthetic biology approaches can be
334 successful in expanding the diversity of **natural** biosynthetic pathways. The same concept can now be
335 applied to other groups of specialized metabolites, **beyond terpenoids**.

336

337 **Online methods**

338 **Chemicals and enzymes.** Standards used include: 2-methylisoborneol (Sigma, M3933-1ML), 1,8-
339 cineole (Aldrich, C8,060-1), α -pinene (Aldrich, P-7408), γ -terpinene (Aldrich, T2134), β -myrcene (M-
340 0382) and a 70% sabinene solution (kindly donated by VIORYL S.A., Athens, Greece). In-house
341 standards of 2-methyllimonene, 2-methylmyrcene, 2-methyl- α -terpineol were obtained by extraction
342 and partial purification of yeast cultures expressing *PfMBS*. 2-methyllinalool and 2-methylgeraniol
343 were obtained by hydrolysis under acidic conditions of the products of *in vitro* reactions of
344 recombinant *PIGPPMT* protein with SAM (A2408-Sigma) and GPP (G6772-Sigma). Phusion High-
345 Fidelity DNA Polymerase (New England BioLabs, M0530S) and MyTaq DNA polymerase (BIO-
346 21105, Biorline) were used in PCR amplifications. QIAquick Gel Extraction Kit (#28704, Qiagen) was
347 used for gel extraction and DNA purification. NucleoSpin Plasmid Kit (740588.250, Macherey-Nagel)
348 was used for plasmid DNA purification. **Anti-c-Myc antibody from Sigma-Aldrich (cat no: M4439).**

349 **Yeast media.** D-(+)-glucose monohydrate (16301, Sigma); D-(+)-galactose (G0625, Sigma); raffinose
350 pentahydrate (R1030, US Biological); Yeast Nitrogen Base w/o AA (Y2025, US Biologicals);
351 Complete Minimal (CM) medium was composed of 0.13% (w/v) dropout powder (all essential amino
352 acids), 0.67% (w/v) yeast nitrogen base w/o AA, 2% glucose; For galactose based medium, glucose
353 was substituted with 2% galactose, 1% raffinose.

354 **Gene cloning and expression in yeast.** All primers used for cloning purposes are listed in the Table
355 S5. *Cloning of the P. limnetica GPPMT in an inducible yeast expression:* The codon optimized version
356 of the GPP methyltransferase from *P. limnetica* (GenBank HQ630882.1)¹⁹ was kindly provided by
357 Professor David Cane (Brown University, USA) in bacterial expression plasmid pET28a(+) and was
358 subcloned into an appropriate yeast expression vector. An inducible expression plasmid (pYESmyc)

359 was employed in order to minimize any adverse effects in yeast growth due to the activity of the
360 methyltransferase. The pYESmyc vector allows for the galactose-inducible expression of an N-
361 terminally myc-tagged version of the inserted gene. The *PIGPPMT* insert was PCR amplified using
362 pET28a(+)/*PIGPPMT* as a template. PCR primers (GPPMT5-MfeNdeI and GPPMT3-NotXhoI) were
363 designed to incorporate *MfeI* and *NdeI* restriction sites immediately upstream of the ATG and *NotI* and
364 *XhoI* sites right after the stop codon to allow for further subcloning of the amplified product. The PCR
365 product was inserted into the pCRII-TOPO vector using the TOPO cloning methodology (Invitrogen
366 Inc.) and insert-containing plasmids were selected by the release of an 800 bp fragment upon *EcoRI*
367 restriction digestion. The pCRII-TOPO/GPPMT plasmid was then digested with *MfeI* and *XhoI*
368 restriction enzymes to release the GPPMT (~800 bp) insert, creating compatible ends to allow
369 subcloning into the *EcoRI* and *XhoI* digested yeast expression vector pYESmyc. Following ligation, the
370 construct was confirmed by restriction digestion and sequencing.

371 *Fusion of the PIGPPMT protein with ERG20*: Digestion of pYESmyc/ERG20-GS²³ with *EcoRI* and
372 *XhoI* restriction enzymes generated a linearized fragment. The *PIGPPMT* gene was released from the
373 pCRII-TOPO-*PIGPPMT* plasmid through restriction digestion with compatible enzymes, *MfeI* and
374 *XhoI*. The obtained fragments of pYESmyc/ERG20-GS and *PIGPPMT* were ligated to generate
375 pYESmyc/ERG20-*PIGPPMT* construct.

376 *Cloning of the terpene synthases in compatible yeast and bacteria expression vectors*: Yeast codon
377 optimized version of *Citrus limon* (+)-S-limonene synthase (*CLimS*) was kindly provided as pCEV-
378 G2-Ph/*CLimS* (GenBank AF514287.1) by Dr. Claudia E. Vickers, University of Queensland,
379 Australia. The ORF was PCR amplified using primers LimS-BGL and LimS-XHOstop to introduce
380 *BglII* and *XhoI* restriction sites at the 5' and 3' ends, respectively. The PCR product was initially
381 introduced into vector pCRII-TOPO by TOPO cloning. The generated construct, pCRII-TOPO/*CLimS*
382 was then digested using *BglII* and *XhoI* enzymes and the *CLimS* gene was excised and subcloned into
383 vector pESC-TRP (Agilent Technologies, Cat. #217453) restricted with *BamHI* and *XhoI*. In this
384 construct, *CLimS* was expressed under the control of the P_{GAL1} promoter. Yeast codon optimized
385 versions of *P. limnetica* 2-methylisoborneol synthase (*PIMIBS*) (GenBank ADU79148.1), *M.*
386 *olivasterospora* 2-methylenebornene synthase (*MoMBS*) (GenBank BAK26793.1), *P. fluorescens*
387 pfl_1841 encoding for a 2-methylenebornene synthase (*PfMBS*) (GenBank WP_011333305), *O.*
388 *basilicum* myrcene synthase (*ObMyrS*) (GenBank Q5SBP1.1), *P. taeda* α -pinene synthase (*PtPinS*)
389 (GenBank Q84KL3.1), *O. basilicum* geraniol synthase (*ObGerS*) (GenBank Q6USK1.1) were obtained
390 by gene synthesis bearing flanking regions containing specific restriction sites and a generic sequence
391 compatible for USER cloning. Using the generic primes USER-Gen-FP and USER-Gen-RP, the above

392 genes were amplified and cloned by USER cloning into a generic backbone introducing *Bam*HI and
393 *Eco*RI sites at the 5' end, and *Not*I, *Bg*III and *Xho*I sites at the 3' end. The constructs were confirmed
394 by sequencing. Subsequently, the genes of interest were excised from the generic USER vector by
395 *Bam*HI and *Xho*I digestion and ligated into pESC-TRP vector (Agilent Technologies, Cat. #217453)
396 linearized with *Bam*HI and *Xho*I restriction enzymes. This approach enabled subcloning of the genes of
397 interest under P_{GAL1} promoter and resulted into construction of the following plasmids: pESC-
398 TRP/*P*MIBS, pESC-TRP/*Mo*MBS, pESC-TRP/*Pt*MBS, pESC-TRP/*Ob*MyrS, pESC-TRP/*Pt*PinS,
399 pESC-TRP/*Ob*GerS. Constructs of *S. fruticosa* 1,8-cineole synthase (*Sf*CinS1) (GenBank
400 ABH07677.1), *S. pomifera* sabinene synthase (*Sp*SabS) (GenBank DQ785794.1) and *Se*CamS in
401 appropriate yeast vectors were available from previous work^{23,30,35,37}.

402 For expression in bacteria the genes encoding *P*MIBS, *Pt*PinS, *Ci*LimS, *Ob*MyrS and *Se*CamS were
403 cloned into pRSETa (Invitrogen). *P*MIBS, *Ob*MyrS and *Pt*PinS were digested from pESC-
404 TRP/*P*MIBS, pESC-TRP/*Ob*MyrS and pESC-TRP/*Pt*PinS with *Bam*HI and *Xho*I. *Ci*LimS was then
405 digested using *Bg*III and *Xho*I enzymes from pCRII-TOPO/*Ci*LimS. *Se*CamS was amplified from
406 pCRII-TOPO/*Se*CamS³⁵ using primers CamS-BamHI FP and CamS-SalI RP to introduce *Bam*HI and
407 *Sal*I restriction sites. Following TOPO cloning, *Se*CamS insert was excised with *Bam*HI and *Sal*I
408 restriction enzymes. The following inserts were produced: *P*MIBS (*Bam*HI/*Xho*I), *Ob*MyrS
409 (*Bam*HI/*Xho*I), *Pt*PinS (*Bam*HI/*Xho*I), *Ci*LimS (*Bg*III/*Xho*I) and *Se*CamS (*Bam*HI/*Sal*I). The resulted
410 inserts were ligated into pRSETa vector digested with *Bam*HI and *Xho*I to generate the corresponding
411 bacterial expression constructs: pRSETa/*P*MIBS, pRSETa/*Ob*MyrS, pRSETa/*Pt*PinS,
412 pRSETa/*Ci*LimS, pRSETa/*Se*CamS. Selective mutations were introduced by USER mutagenesis³⁸
413 using pRSETa/*Ci*LimS (H570V, H570L and H570I) and pRSETa/*Se*CamS (H583L) as templates. The
414 constructs pRSETa/*Sf*CinS1, pRSETa/*Sf*CinS1(N388S-I451A), pRSETa/*Sf*CinS1(F571Y) and
415 pRSETa/*Erg*20p were already available from previous work^{23,30}.

416 **Strain AM94.** In *S. cerevisiae*, heterozygous deletions lead to 50% decrease in the level of the
417 corresponding protein in the majority of yeast genes tested²⁶. Strain AM94 (see Table S1), the basis
418 strain used in this study, is a diploid strain that contains a heterozygous deletion in the squalene
419 synthase gene *ERG9*, which is essential for sterol biosynthesis. This deletion helps reduce the drain of
420 isoprenoid substrates and boost the heterologous production of terpenoids³⁹.

421 **Development of the MIC1 yeast strain.** To integrate *PI*GPPMT in one of the two *ERG20* alleles of
422 the diploid yeast strain AM94 the protocol previously described³⁷ was used. The *PI*GPPMT gene was
423 digested from the pCRII-TOPO/*PI*GPPMT plasmid with *Mfe*I and *Xho*I restriction enzymes and cloned
424 into *Eco*RI-*Xho*I restriction sites of the plasmid construct COD7 (GPPMT-CYC1t, LoxP-*HIS5*-LoxP)

425 ³⁹. The COD7/*PIGPPMT* construct was PCR amplified using primers ERG20F and ERG20OL3, which
426 incorporate flanking sequences complementary to the 5' and 3' end of the *ERG20* gene respectively.
427 Following selection marker excision, strain MIC1 was obtained.

428 **Yeast growth assay.** Selected engineered yeast strains were grown in corresponding glucose-based
429 media until OD₆₀₀ ~0.5-0.7. The obtained cells were collected and washed twice with sterile water
430 followed by dilution (in water) to OD₆₀₀ ~0.1. Subsequently, the obtained cell suspensions were used to
431 serial dilution of 1:10, 1:100 and 1:1000. The obtained dilutions of selected strains were plated (20 µL)
432 on glucose-based or galactose/raffinose-based agar media and incubated at 30 °C for 3 days (Fig. S6a).
433 Similarly, yeast cells grown in glucose media were washed twice with sterile water and resuspended in
434 galactose/raffinose containing media to induce protein expression. Subsequently, the obtained cultures
435 were diluted to OD₆₀₀ ~0.1 (considered time 0) and 25 mL were incubated at 30 °C with 150 rpm
436 shaking. Measurements of OD₆₀₀ were taken every 2 h to monitor the curve of yeast growth (Fig. S6b).
437 For OD₆₀₀ values higher than 1, samples were diluted accordingly.

438 **Terpene quantification and extraction from yeast cells.** Selected *S. cerevisiae* strains were cultivated
439 in 2 mL liquid media using 20 ml glass vials with magnetic screw cap (Mikrolab Aarhus A/S,
440 Denmark). Solid Phase Microextraction (SPME) was applied for measuring the volatile terpenes
441 produced in yeast cells, using 2 cm-50/30um DVB/Carboxen™/PDMS StableFlex™ Fiber followed by
442 GC-TQ and GC-qTOF analysis as previously described ³⁷. For quantification, terpene extraction was
443 performed using 1% (w/v) Diaion HP20 (Supelco, Bellefonte, PA) as adsorbent resin or dodecane
444 extraction following the protocols previously described ^{23,40}. Samples were analyzed in triplicates and
445 the error calculated as the mean absolute deviation (MAD) around the mean.

446 **Protein expression in bacteria.** Erg20p, *PIGPPMT* and *PIMIBS* were expressed in *E. coli* EXPRESS
447 DUOs BL21 [DE3] (Lucigen, Cat. #60401-2) cells. *PtPinS*, *CILimS*, *SfCinS1* and *SeCamS* were
448 expressed in *E. coli* BL21 [DE3, pLANT(3)/RIL ⁴¹]. Expression and purification of Erg20p was
449 performed as described in ²³. *PIGPPMT* and *PIMIBS* was expressed and purified following the
450 protocol described in ¹⁹. Bacterial expression of *SfCinS1*, *PtPinS*, and *CILimS* was performed as
451 described in ³⁰ with the following modifications. Bacterial cells carrying the corresponding plasmids
452 were grown at 23 °C until OD₆₀₀ reached 0.5-0.7, then induced with 0.1 mM isopropylthio-β-
453 galactoside (IPTG), after which cell growth was continued for 20 h at 19 °C. The His-tagged proteins
454 were then purified by Ni²⁺ affinity chromatography as described in ³⁰. The resulting protein fractions
455 are shown in Fig. S4. Production of SpSabS1, *ObMyrS* and their mutants resulted in the production of
456 insoluble protein aggregates at all conditions tested and was not pursued further. Despite obtaining

457 soluble *PtPinS*, the resulting protein preparations showed very low specific activity that precluded
458 kinetic evaluation.

459 **Total yeast extracts.** Yeast cells expressing Erg20p(N127W)-*PIGPPMT* were resuspended in
460 enzymatic reaction buffer (10 mM MOPS (pH 7.0), 5 mM MgCl₂, 1 mM DTT, 0.1 mg/mL BSA)
461 supplemented with 1 mM PMSF, protease inhibitor cocktail (Roche) and lysozyme (0.1 mg/mL) and
462 disrupted by sonication. The lysate was centrifuged at 13,000g at 4 °C for 20 min and the resulted
463 supernatant was collected and stored at -80 °C until further use.

464 **Enzymatic assays and kinetic analysis.** Erg20p activity was assayed in 0.2 mL reactions as described
465 in ²³, using as co-substrates 10 μM IPP with 70 μM DMAPP, GPP or 2meGPP. Reactions also included
466 50 ng purified Erg20p or 50 μL of extract of yeast cells expressing Erg20p(N127W)-*PIGPPMT*.
467 Reactions were incubated for 1 h at 30 °C and then terminated by the addition of an equal volume (0.2
468 mL) of 2N HCl in 83% ethanol and 0.2 mL hexane. Following 20 min incubation at 37 °C to allow
469 diphosphate hydrolysis, reactions were neutralized with 0.2 mL of 10% NaOH and the hexane phase
470 was analyzed by GC-MS using the conditions described below.

471 Enzymatic activity of *PIGPPMT* was assayed in 0.2 mL reactions as previously described ^{14,19} using as
472 co-substrates 120 μM SAM and 70 μM of GPP, DMAPP, IPP, FPP or GGPP. The reactions were
473 incubated 18 h at 30 °C and terminated by addition of 0.2 mL 2N HCl in 83% ethanol and 0.2 mL
474 hexane. Acid hydrolysis of the diphosphates was carried for 20 min at 37 °C and the reactions were
475 neutralized and analyzed as above.

476 The kinetic parameters of different terpene synthases (wild-type and mutants) were determined as
477 previously described ³⁰ using 20 mM MOPS, 20 mM MgCl₂, 0.02 mM MnCl₂, 1 mM DTT, 0.01 % (v/v)
478 BSA, 50 ng enzyme and varying concentrations (0-150 μM) of substrates, GPP or 2meGPP. The
479 reaction were overlaid with hexane containing 1 ppm nonane and 1 ppm dodecane as internal standards
480 and incubated for 1 h with mild shaking at 30 °C. Extraction and GC-MS analysis were performed as
481 previously described ³⁰. Kinetic analysis was performed using WinCurveFit 1.1.8 (Kevin Raner
482 software).

483 All enzymatic assays and kinetic analysis experiments were carried out in triplicates.

484 **Analysis of yeast sterols and squalene.** Yeast cells resulting from 4 mL cultures induced to express
485 Erg20p(N127W)-*PIGPPMT* (or containing the empty pYESmyc vector) were treated with butylated
486 hydroxytoluene (BHT) in a ratio of 2:1 per pellet weight, resuspended in 500 μL 10% KOH in 80%
487 EtOH solution and incubated at 70 °C for 1-2 h. Samples were cooled at room temperature and overlaid
488 with 500 μL of hexane followed by vigorous vortexing prior to collecting the hexane phase. This step
489 was repeated twice. The pooled hexane (~1.5 mL) was washed with water (equal volume) three times.

490 The collected hexane phase was evaporated down to 100 μ L and derivatized with 150 μ L trimethylsilyl
491 cyanide (Sigma, Cat. #212849) for 30 min at 60 $^{\circ}$ C prior to GC-MS analysis ⁴².

492 **Determination of intracellular prenyl diphosphates.** The ratio of intracellular GPP and 2meGPP was
493 determined with the method described in reference ²³. Briefly, 50 mL cultures of the selected MIC1
494 cells expressing the Erg20p(N127W)-*P/GPPMT*(F266H) fusion were grown to mid-log phase and the
495 cells were harvested washed twice with 10 mL H₂O. Following resuspension in 1 mL of H₂O and
496 disruption by glass beads, the cell extract was centrifuged at 13 000 for 10 min and 0.5 mL of the
497 supernatant was mixed with 0.5 mL 2 N HCl in 83% ethanol and overlaid with 1 mL of hexane. The
498 reactions were incubated at 37 $^{\circ}$ C for 20 min and neutralized by adding 0.35 mL of 10% NaOH. The
499 mixtures were extracted with hexane and analyzed by GC-MS. The ratio of 2meGPP:GPP was
500 determined by quantification of the linalool and 2-methyllinalool peaks that result from their hydrolysis.

501 **GCMS analysis conditions.** GCMS analysis was carried on a DB-5 column using hydrogen as a
502 carrier gas with a constant velocity of 30 cm/sec. Samples resulting from incubation of the SPME fiber
503 for 30 min over the head-space of 10 mL yeast cultures producing monoterpenes, C₁₁ terpenes or
504 sesquiterpenols or 2 μ L of the hexane phase overlaying the *in vitro* enzymatic reactions were analyzed
505 using the following temperature program: initial temperature 40 $^{\circ}$ C, ramp to 80 $^{\circ}$ C with a rate of
506 3 $^{\circ}$ C/min, ramp to 110 $^{\circ}$ C with a rate of 30 $^{\circ}$ C/min, ramp to 130 $^{\circ}$ C with a rate of 3 $^{\circ}$ C/min 280 $^{\circ}$ C with
507 a rate of 30 $^{\circ}$ C/min, hold for 3 min. Sample resulting from non-saponifiable lipid extraction were
508 analyzed for squalene and sterols using the following temperature program: initial temperature 60 $^{\circ}$ C,
509 ramp to 300 $^{\circ}$ C with a rate of 10 $^{\circ}$ C/min, ramp to 320 $^{\circ}$ C with a rate of 3 $^{\circ}$ C/min, hold for 3.5 min.

510 **Chemical synthesis of 2meGPP.** 2meGPP was chemically synthesized by the general procedure of
511 Wang and Cane ¹⁴, but including the following modifications (synthesis scheme shown in Fig. S11).

512 **General chemical synthesis procedures.** All reactions were monitored by TLC on aluminium sheets
513 coated with silica gel 60F254 (0.2 mm thickness, Merck) and the components present were detected by
514 charring with 10% H₂SO₄ in MeOH. Column chromatography was carried out using silica gel 60
515 (particle size 0.040-0.063 mm, 230-400 mesh ASTM, Merck). Solvent extracts were dried with
516 anhydrous MgSO₄ unless otherwise specified. The ¹H and ¹³C NMR spectra were recorded on a Bruker
517 Avance 400 spectrometer at 400 and 101 MHz, respectively. CDCl₃ was used as solvent (unless
518 otherwise indicated), δ H values are relative to internal TMS and δ C values are referenced to the solvent
519 [δ C (CDCl₃) = 77.0].

520 **Ethyl (*E/Z*)-2,3,7-trimethylocta-2,6-dienoate (43).** (By using the LiCl/DBU System ⁴³ but solvent-
521 free). A neat mixture of LiCl (0.80 g, 18.9 mmol), triethyl 2-phosphopropionate (**41**) (3.0 mL, 14.0
522 mmol) and DBU (1,8-diazabicyclo[5,4,0]undec-7-ene)(2.3 mL, 15.6 mmol) was stirred at room

523 temperature for 1.0 h under Ar followed by the addition of 6-methyl-5-hepten-2-one (**42**) (2.3 mL, 15.6
524 mmol). Stirring was continued at room temperature for 24 h. The reaction was quenched with water (50
525 mL) and the reaction mixture was extracted with Et₂O (200 mL). The extract was washed with brine
526 and dried (MgSO₄). The solvent was evaporated and chromatographed on silica (100 g) with 0-10 %
527 Et₂O in n-pentane to afford compound **42** as colorless oil (2.6 g, 88 %) *E/Z*-mixture.

528 **Ethyl (*E*)-2,3,7-trimethylocta-2,6-dien-1-ol (**44**)**. (Using DIBAL-H as the reducing agent⁴⁴). A stirred
529 solution of Ethyl (*E/Z*)-2,3,7-trimethylocta-2,6-dienoate (**43**) (2.4 g, 11.4 mmol) in diethyl ether (50 mL)
530 and cooled to -78 °C. DIBAL-H (1.0 M in toluene) (27.7 mL, 27.7 mmol) was added dropwise and the
531 reaction mixture was stirred at -78 °C for 2 h and then quenched by addition of MeOH (5.0 mL). The
532 reaction mixture was warmed gradually to 0 °C and diluted with Et₂O (100 mL) followed by the
533 addition of saturated aqueous solution of Rochelle salt (100 mL), water (100 mL) and vigorously
534 stirred. Once the Et₂O layer became clear, it was separated, dried over MgSO₄, filtered, and
535 concentrated on a rotary evaporator under reduced pressure. Chromatographic purification of the
536 resulting oil on silica (100 g) with 20 % Et₂O in n-pentane afforded the separation of the pure (*E*-
537 isomer **44** colorless oil (0.40 g, colorless oil, 21 %). ¹H and spectral data for **44** were identical with
538 those reported¹⁴.

539 **(*E*)-2-Methylgeranyl pyrophosphate (2meGPP)**^{14,45}. (The Ph₃P/CBr₄ system⁴⁶ was used instead). To
540 a stirred solution of ethyl (*E*)-2,3,7-trimethylocta-2,6-dien-1-ol (**44**) (0.10 g, 0.60 mmol) in dry benzene
541 (5.0 mL) and dry CH₂Cl₂ (1.0 mL) was added CBr₄ (0.41 g, 1.20 mmol) and Ph₃P (0.32 g, 1.20 mmol)
542 at 0 °C under a nitrogen atmosphere. After the solution was stirred at the same temperature for 2 h, n-
543 pentane (50 mL) was added to the reaction mixture. The reaction mixture was filtered to remove
544 triphenylphosphine oxide. The filtrate was concentrated under reduced pressure to afford the crude (*E*-
545 2-methylgeranyl (0.14 g, quant.) as a yellow oil which was immediately subjected to the following
546 pyrophosphorylation reaction without further purification. A solution of the crude bromide in dry
547 CH₃CN (15 mL) was added dropwise to a stirred solution of [(n-Bu₄N)]₃HP₂O₇ (0.81 g, 0.9 mmol, 1.5
548 eq) in dry CH₃CN (15 mL) at 0 °C under Ar. The mixture was stirred at room temperature for 24 h and
549 concentrated on a rotary evaporator below 35 °C. The obtained residue was transferred to two
550 centrifuge tubes with 10 mL of acetone and concentrated NH₄OH (0.5-1 mL) was added to each. The
551 precipitated ammonium salts, isolated by centrifugation (4000 rpm for 10 min), were washed twice by
552 resuspension in 5-mL aliquots of acetone containing 0.01 N NH₄OH. The combined supernatants of the
553 acetone containing NH₄OH were rota-evaporated at below 35 °C. The resulting crude 2meGPP as its
554 ammonium salt was pure enough to be used for the enzymatic studies. However, a pure sample of

555 **2meGPP** was accomplished by preparative TLC-plates using the eluent system MeOH/CH₂Cl₂/0.01 N
556 NH₄OH (1: 1.2: 0.3 v/v). When the synthesized organic diphosphate was subjected to acid hydrolysis,
557 the recovered alcohol was compared with authentic samples by TLC and GC. In each case the
558 recovered alcohol was essentially identical with the corresponding (*E*)-2-methylgeranyl alcohol (44).

559 **Molecular graphics** were performed with the UCSF Chimera package. Chimera is developed by the
560 Resource for Biocomputing, Visualization, and Informatics at the University of California, San
561 Francisco (supported by NIGMS P41-GM103311) ⁴⁷.

562 **Mutagenesis.** Site-directed mutagenesis of *PIGPPMT*, *SfCinS1*, *SpSabS*, *SeCamS* was performed with
563 the Quickchange method (Stratagene) and mutagenesis of *ObMyrS*, *PtPinS*, *CIlimS* was performed by
564 USER mutagenesis ³⁸ using the primers listed in Table S5.

565 **Construction of the *PIGPPMT* model.** The structural model of *PIGPPMT* was constructed using the
566 SWISS-MODEL server ⁴⁸ and 3VC2 ²⁷ as template.

567 **Data availability statement.** All data generated or analysed during this study are included in this
568 published article (and its supplementary information files

569

570 **Acknowledgments**

571 We would like to thank Prof. David Cane and Dr. Wayne Chou (Brown University, USA) for providing
572 the bacterial construct pET28a(+)/*PIGPPMT*, Dr. Claudia E. Vickers, (University of Queensland,
573 Australia) for providing construct pCEV-G2-Ph/*CIlimS*, Assist. Prof. Fernando Geu-Flores
574 (University of Copenhagen, Denmark) for critical reading of the manuscript, and Morten Raadam for
575 technical assistance. We are grateful to Dr. David Ian Pattison and Eleni Lazaridi from the PLEN
576 metabolomics platform for their support. This work was supported by the Greek General Secretariat of
577 Research and Technology (GSRT) grant 11ΣΥΝ_3_770 (to AMM and SCK) and the Novo Nordisk
578 Foundation grants NNF16OC0019554 (to CI) and NNF16OC0021760 (to SCK).

579

580 **Author contributions**

581 SCK conceived the project and designed experiments; CI designed experiments, engineered MIC1
582 strain, expressed and analyzed C₁₀ and C₁₁ wild-type and mutant synthases in yeast and bacteria,
583 performed mutagenesis of *PIGPPMT*, *PtPinS*, *SpSabS*, *ObMyrS*, *CIlimS*, *SeCamS*, produced the
584 purified proteins, conducted in vitro enzymatic assays for the determination of the kinetic parameters;
585 MP performed expression and analysis of *PIGPPMT*, *SfCinS1* wt and mutants, *SpSabS* wt and
586 conducted yeast optimization for 2meGPP production, MSM performed the chemical synthesis of

587 **2meGPP**, MEM and AMM assisted in data analysis, SCK and CI analyzed the data and drafted the
588 manuscript.

589

590 **Competing financial interests**

591 The authors declare no competing financial interest.

592

593 **Supplementary Materials**

594 Table S1 – S10

595 Fig S1 – S11

596

597

598 **Figure legends**

599

600 **Figure 1. Terpenoid biosynthetic code.** The biosynthesis of terpenoids is a modular process that
601 follows an established pattern. In module 1, C₅ isoprenoid units are put together by prenyltransferase
602 enzymes to produce universal diphosphate substrates with sizes that differ by 5-carbon increments. In
603 module 2, each of these substrates forms the basis for a different terpenoid class (monoterpenoids,
604 sesquiterpenoids, etc.), being converted to the different terpene backbones by one of the many terpene
605 synthase enzymes. **The** basic terpene skeletons become decorated in **subsequent** modules involving
606 different decorating enzymes, i.e. cytochrome P450s, O-acetyltransferase, O-methyltransferases,
607 dehydrogenases, etc. The use of few building blocks and the modularity of the pathway establish a code
608 for terpene biosynthesis. In this work, we hijacked the 2MIB pathway (inset in the right panel) to
609 establish a parallel universe of C₁₁ terpene scaffolds (right panel).

610

611 **Figure 2. Production of 2meGPP in yeast. a.** Expression of *PIGPPMT* and *PI*MIBS in yeast resulted
612 in the production of the 2MIB degradation products 2MB, 1MC and 2M2B (blue), identified by
613 comparison with the authentic 2MIB standard (pink). None of these compounds was produced by any
614 of the two enzymes alone (green, brown) or by yeast cells carrying the two empty vectors (red). **b.**
615 Diagram showing the synthesis of GPP, 2meGPP and derived isoprenoids in yeast. Synthesis of
616 canonical monoterpenes (C₁₀; green) or non-canonical C₁₁ terpenes (blue) also require GPP (**TPS:**
617 **terpene synthase**). **c.** *PIGPPMT* was fused with Erg20p. The N-terminal fusion, Erg20p-*PIGPPMT*,
618 enabled a 15-fold increase. **d.** Introduction of the Erg20p(N127W) variant in the Erg20p-*PIGPPMT*
619 fusion resulted in a 4.8-fold increase over the previous step. **e.** Strain MIC1 was derived by

620 chromosomal integration of *PIGPPMT* in strain AM94. Both strains were engineered to overexpress
621 *PI*MIBS and the Erg20p(N127W)-*PIGPPMT* fusion and their performance was compared. **f.**
622 Mutagenesis of *PIGPPMT* (non-fused form) identified two variants, V250A and F266H, with improved
623 performance when introduced into AM94 together with *PI*MIBS. **g.** Bar chart showing a summary of
624 the yield improvement achieved in each of the previous steps (shown in this figure in panels c, d, e and
625 f). In the experiments shown in panels c, d, e and f, samples were analyzed in triplicates and the error
626 bars represent the mean absolute deviation (MAD) around the mean.

627

628 **Figure 3. Percentage of C₁₁ and C₁₀ terpene scaffolds produced by the different wild-type terpene**
629 **synthases in yeast.** Samples were analyzed in triplicates, yield of each compound was averaged, and
630 the percentages of C₁₁ and C₁₀ compounds were calculated.

631

632 **Figure 4. Production of C₁₁ terpenoids in yeast by plant monoterpene synthases.** **a.** Co-expression
633 of *SfCinS1* with Erg20p(N127W)-*PIGPPMT* (blue) resulted in the production of a blend of C₁₁
634 terpenoids. Compound **30**, which accounted for 11.2% of the total C₁₁ production, displays a 168 m/z
635 fragment (Table S6) characteristic of C₁₁ terpene alcohols. **b.** *PtPinS* co-expressed with
636 Erg20p(N127W)-*PIGPPMT* (blue) produced 29 C₁₁ molecules representing almost 26% of the total
637 terpene production. Compounds **31** – **35** are specific to plant enzymes. **c.** In the presence of
638 Erg20p(N127W)-*PIGPPMT*, *SpSabS* (blue) produced a blend of C₁₁ compounds dominated by 2-
639 methylmyrcene and 2MB. **d.** *SeCamS* (blue) showed preference for 2-methylmyrcene (55%) and the 2-
640 MIB dehydration products. Yeast samples of single transformations expressing the either the
641 corresponding monoterpene synthase alone (green), Erg20p(N127W)-*PIGPPMT* alone (brown), or the
642 two empty vectors (red) are shown as controls.

643

644 **Figure 5. a.** Proposed GPP cyclization mechanism. Initially, the substrate binds in the extended
645 conformation and the transition from the transoid extended conformation to the catalytically competent
646 cisoid closed conformation is essential for the reaction (according to ³⁶). **b.** Proposed mechanism of
647 2meGPP cyclization. **c.** Model of the active site of *SfCinS1* with the “extended” form of 2-fluorolinalyl
648 diphosphate (2F-LPP) superimposed. The extended conformation is believed to be achieved by on-
649 enzyme conversion of 2-fluorogeranyl diphosphate (2F-GPP) in the crystal. The structure of the
650 substrate analog in the extended conformation is considered to resemble that of the early steps of the
651 mechanism. **d.** Model of the active site of *SfCinS1* with the “closed” structure of 2-fluorolinalyl

652 diphosphate (2F-LPP) superimposed. This analogue adopts the closed cisoid conformation that is
653 competent for cyclization. Extensive conformational changes are needed for the substrate to modify its
654 structure, and F571 is positioned in the heart of this transition. The residues selected for mutagenesis
655 are indicated. Graphic produced with UCSF Chimera ⁴⁷.

656

657 **Figure 6. Engineering dedicated C₁₁ terpene synthases.** **a.** Mutagenesis of *SfCinS1*. The double
658 mutant N338S-I451A lost its ability to synthesize 1,8-cineole and became a dedicated 2-
659 methylmyrcene synthase, while substitution of F571 with H resulted in a 2MB-specific synthase
660 (shown in blue). The product profile of the wild-type enzyme is shown in green. The asterisk (*)
661 denotes a yeast-produced non-terpene compound. **b.** Substitution of H570 in *C/LimS* with V or L
662 (shown in blue) changed the specificity of this enzyme from limonene to 2-methyllimonene. **c.**
663 Mutagenesis of *PtPinS*. The product profile of the wild-type enzyme is shown in green. Variant F607L
664 became a dedicated 2-methylinalool synthase, while variant F607I turned into a 2MB synthase (blue).
665 Peaks marked a star correspond to non-terpenoid compounds. **d.** Bar chart showing the specificity of
666 the different plant monoterpene synthases and their variants for C₁₀ (green) or C₁₁ terpene production in
667 yeast (blue). In panel D, samples were analyzed in triplicate and the mean value of total C₁₀ and C₁₁
668 products is shown. All panels show results from the yeast system.

669

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