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# Semisynthesis of Plant-Derived Englerin A Enabled by Microbe Engineering of Guaia-6,10(14)-diene as Building Block

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#### Supporting Information Placeholder

ABSTRACT: Herein, we report the semisynthetic production <sup>35</sup> of the potent transient receptor potential canonical (TRPC) <sup>36</sup> channel agonist englerin A (EA), using guaia-6,10(14)-diene as the starting material. Guaia-6,10(14)-diene was systematically 38 engineered in Escherichia coli and Saccharomyces cerevisiae using the CRISPR/Cas9 system and produced with high titers. This opened the possibility for a very short semisynthesis of EA and the two related guaianes oxyphyllol and orientalol E. Q The potentially scalable approach combines the advantages of synthetic biology and chemical synthesis and provides an efficient and economical method for producing EA and its ana-logues.

From the beginnings of mankind, extracts from Nature have been used to deliver medical benefit to those in need.<sup>1</sup> As 39 therapeutics moved from mixtures to constitutionally and ste- 40 reochemically defined molecular entities, there has been a 41 growing demand to access active pharmaceutical ingredients 42 in high purity and on scale. Extraction from the natural host has traditionally been in competition with chemical synthesis. While extraction from plants may be harmful to the environ-ment or suffer from low titers, multistep chemical synthesis may require large amounts of potentially harmful solvents and toxic reagents. In recent years, the progress in molecular biol-ogy and the development of the CRISPR/Cas9 system have al-lowed for the engineering of microbial pathways to produce biopharmaceuticals.<sup>2</sup> However, biotechnological processes are often optimized towards defined products and usually do not allow the flexibility that chemical synthesis offers. There-fore, a biomimetic semisynthesis can provide a viable alterna-tive by combining the benefits of synthetic biology and chem-ical synthesis.3 Terpenoid natural products are prime examples for such a 

strategy as they represent some of the most potent modulators
 of biological processes and their biosynthesis is rather
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straightforward. The initial cyclization of oligoprenyls is followed by oxidation and optional post-functionalization processes. Each step in this sequence adds a layer of complexity towards the optimal binding to the protein of interest (**Fig. 1**).



**Figure 1. Assembly of bioactive terpenoids. a)** Nature's blueprint for complex terpenoid synthesis. **b)** Examples of bioactive natural products with layered complexity.

Baran and coworkers successfully mimicked the sequential execution of cyclase and oxidase phases in their concise syntheses of ingenol and other terpenoid natural products.<sup>4</sup> This strategy becomes even more intriguing if the core structure is derived from biological processes and synthetic chemists need to handle only the oxidation and post-functionalization phase, as successfully applied for the commercial production of paclitaxel by using leaf extracted baccatin III as the starting core material.5 The efficiency of this concept is further demonstrated by the production of microbe-engineered artemisinic acid which enabled the semisynthesis of artemisinin and analogous,<sup>6</sup> and the semisynthesis of morphinan drugs from microbe-overproduced thebaine.3c, 3d, 7 Herein, we describe the fusion of biotechnology with chemical synthesis to provide a straightforward access to the potent transient receptor potential canonical (TRPC) channel agonist (-)-englerin A (EA).

by overloading.9

 

using synthetic biology produced guaia-6,10(14)-diene via the MVA pathway. b) Guaia-6,10(14)-diene produced by E. coli G1-G5 in shaking flasks. c) Efficiency of yeast precursor-providing platform YZL141 and JCR27. d) Increased titer of guaia-6,10(14)-diene in JCR27 step by step. e) Guaia-6,10(14)-diene production of E. coli G5 in 5-L fed-batch fermentation. f) 1 production of S. cerevisiae YLo6 in 5-L fed-batch fermentation. Abbreviations: C1 (E. coli BL21(DE3)/pMH1/pFZ81), G1 (E. coli C1/pGB218), G2 (E. coli C1/pSC52), G3 (E. coli C1/ pSC54), G4 (E. coli C1/ pSC56), G5 (E. coli C1/ pSC61). YZL141: S. cerevisiae CEN.PK2-1D::\Dgal1,7,10::tHMG1; JGH20: 5. cerevisiae YZL141::\DLEU2, FgJ02895, ERG20; JGH18: 5. cerevisiae JCR27::\DLEU2, FgJ02895, ERG20; JCR27: S. cerevisiae CEN.PK2-1D:: \Delta ChrXII-2, Cas9, \Delta ChrXI-3, ERG8, tHMG1, ERG12, gRNA\_ChrXI-3, \Delta ChrXI-3, ERG13, tHMG1, gRNA\_ChrX-3, AChrXII-4, IDI, ERG10, MVD1, gRNA\_ChrXII-4; JGH22: S. cerevisiae JCR27:: ALEU2, FgJ02895-Sc, ERG20; YL01: S. cerevisiae JGH22::: URA3, FgJ02895-Sc, tHMG1; YL03: S. cerevisiae YL01:: LOHIS3, FgJ02895-Sc; YL05: S. cerevisiae YLo3::ΔYPRCdelta15, FgJo2895-Sc, gRNA\_YPRCdelta15; YLo6: S. cerevisiae YLo5::ΔGAL80, URA3.

Intrigued by its promising bioactivity and appealing mo-108 lecular architecture, more than 20 different total<sup>10</sup> or formal109 syntheses<sup>n</sup> of EA have been developed. However, these multi-110 step syntheses are often laborious and cannot meet the de-111 mand for a large-scale production. Therefore, a concise sem-112 isynthetic approach towards EA, starting from a biotechnolog-113 ically derived advanced intermediate presents an attractive al-114 ternative. 

In 2009, Beutler and coworkers isolated EA from the root 64

bark of the East African plant Phyllanthus engleri and found 65

that it selectively inhibits the growth of renal cancer cells with 66

nanomolar activity.8 The cellular target of EA was identified as 67

Since the enzymes involved in the biosynthetic pathway of 116 EA in P. engleri remain unclear, it is important to first identify 117 the building blocks for EA. It has been reported that sesquit-118 erpene cyclase STC5 from the filamentous fungus Fusarium119 *fujikuroi* can produce guaia-6,10(14)-diene (1).<sup>12</sup> However, the120 low isolation yield of 4% would make it impossible to use this 121 compound as a starting material for a scalable semisyntheis of 122 EA. Synthetic access has only been described for (±)-1 with 123 similarly low yields in a multistep process.13 Nevertheless, the 124 structural features of 1 would be very suitable for the use as a 125 building block for EA, if a sufficient availability of 1 is ensured. 126 In recent years, we have developed a platform for the bio-127 synthesis of terpenoids, that allows for the production of large 128 amounts of farnesene, taxadiene, and lycopenes, as well as for 129 the genome mining of novel sesqui-, di-, and sesterterpenes.<sup>14</sup>130 This technology was applied to screen for guaia-6,10(14)-diene131 cyclases with high catalytic activity. Sesquiterpene cyclases of 132 filamentous fungi were systematically evaluated by phyloge-133 netic analysis and multiple sequence alignment. FgJ02895,134 FpN62905 and FmM7560, which located into the same clade135 

with STC5, were selected for functional verification (Supplementary Figs. 1 and 2). Escherichia coli C1 (E. coli BL21(DE3)/ pMH1/pFZ81), harboring an engineered mevalonate (MVA) pathway, has been widely used for efficient overproduction and genome mining of terpenoids.14-15 This platform was selected for functional verification of terpene cyclase activity (Fig. 2a). Plasmids pGB218, pSC52, pSC54, and pSC56, which harbor the farnesyl pyrophosphate synthase (FPPS), sesquiterpene cyclases, and an additional copy of Idi, were constructed and transformed into E. coli C1 to generate mutants E. coli G1-G4 (Supplementary Fig. 4). Subsequently, in vivo fermentation was carried out and the production of 1 was detected by GC/MS analysis. The data showed that in addition to STC5, three sesquiterpene cyclases, FgJ02895, FpN62905, and FmM7560 from F. graminearum J1-012, F. proliferatum NRRL 62905, and F. mangiferae MRC 7560, respectively, were potent guaia-6,10(14)-diene cyclases. Finally, the production of 1 was further confirmed by *in vitro* assay and NMR analysis (Supplementary Figs. 3, 6a-g and Supplementary Table 4)<sup>12</sup>. Additionally, E. coli G1 showed the highest catalytic efficiency with a titer of 48.8 mg/L at the shaking flask fermentation level, indicating that FgJ02895 is a suitable candidate for further engineering (Fig. 2b).

the non-selective TRPC ion channels 1/4/5. EA selectively

binds to the externally exposed site of those channels and in-

duces a Ca2+- or Na+-ion influx that finally leads to cell death

e 32-1

In order to acquire a sufficient amount of 1, the upstream MVA pathway and downstream guaia-6,10(14)-diene forming pathway were balanced by integrating an additional copy of FqJ02895 into pGB218 to generate plasmid pSC61. The resulting data showed that 1 was increased by 1.28-fold with a titer of 62.3 mg/L in E. coli G5. Finally, production of 1 was further

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increased by 9.6-fold and up to 468 mg/L in 5-L fed-batch fer- 49 1 mentation of *E. coli* G<sub>5</sub> (Fig. 2e). Based on these results, o.8 g 2

3 of 1 were purified for chemical synthesis of (–)-EA.

To meet the potential high demand for guaia-6,10(14)-4 5 diene (1) for a scalable synthesis of EA, S. cerevisiae was selected for further engineering. The MVA pathway was system-6 7 atically engineered, a series of mutants were constructed 8 (Supplementary Fig. 5), and the titer of 1 was gradually in-10 9 creased. First, as a proof of concept, downstream ERG20 (FPP 11 10 synthase) and FgJ02895 were overexpressed in the previously 12 11 engineered terpenoid precursor-providing platform S. cerevisiae YZL141,<sup>16</sup> and 1 was produced by the resulting strain 12 13 JGH20 with a titer of 7.68 mg/L in shaking flasks. To increase 13 14 14 the metabolic flux towards terpene synthesis, and based on 15 15 previous work related to metabolic engineering of the MVA 16 pathway,<sup>6a, 15</sup> additional copies of ERG10 (acetyl-CoA acetyl-16 17 17 transferase from S. cerevisiae), ERG13 (ERG13, 3-hydroxy-3-18 18 methylglutaryl CoA synthase), tHMG1 (truncated version of 3-19 19 hydroxy-3-methylglutaryl CoA reductase), ERG12 (mevalonate 20 kinase), ERG8 (phosphomevalonate kinase), MVD1 (mevalo-20 21 nate diphosphate decarboxylase), and IDI (isopentenyl di-21 22 phosphate isomerase) in the MVA pathway were systemati-22 cally engineered by the CRISPR/Cas9 system, and the efficient 23 23 50 24 precursor-providing platform S. cerevisiae JCR27 was estab-24 25 lished. Next, downstream ERG20 and FgJ02895 were inte- 51 25 26 grated into JCR27 and the titer of 1 was increased by 5.4-fold 52 26 27 to 41.4 mg/L in the resulting strain JGH18. In parallel, strain 53 27 28 JGH22 (with codon optimized FqJ02895-Sc and ERG20) was 54 28 29 constructed and the titer was further increased to 67.5 mg/L 55 29 30 (Fig. 2c). Considering that tHMG1 is the rate-limiting step in 56 the MVA pathway  ${}^{\scriptscriptstyle 15}$  and an increase in the copy number of  $\,57$ 31 30 32 FqJ02895 can increase the titer of 1, strain YL01 (JGH22 with an 58 31 33 additional copy of FqJ02895-Sc and tHMG1) was generated 32 with a titer of 132.8 mg/L. One and two additional copies of 59 34 33 FgJ02895-Sc were sequentially integrated and strains YL03 and 6035 34 YL05 were constructed with titers of 166.9 and 170.2 mg/L, re- 61 36 35 37 spectively. Finally, to decrease production costs, GAL80 in 62 36 38 YL05 was deleted to eliminate the requirement for galactose 63 37 39 and the resulting strain YLo6 was generated with a titer of 64 292.9 mg/L (Fig. 2d). The production of 1 was further in- 65 40 38 creased up to 0.8 g/L in 5-L fed-batch fermentation of S. cere- 66 41 39 67 42 visiae YLo6 (Fig. 2f). 40

The efficient production of guaia-6,10(14)-diene (1) ena-68 43 bled the development of a short synthetic route towards EA. 69 44 70 45 Following Shenvi's protocol<sup>17</sup> using catalytic amounts of co-46 balt-catalyst 2 and phenylsilane the exocyclic double bond of 71 47 diene 1 was selectively isomerized to give the trisubstituted 72 73 48 olefin 3 in 73% yield (Scheme 1). 74





Conditions: (a) 2 (5 mol%), PhSiH<sub>3</sub> (5 mol%), PhH, 23 °C, 18 h, 73%; (b) K<sub>2</sub>[OsO<sub>2</sub>(OH)<sub>4</sub>] (10 mol%), (DHQD)<sub>2</sub>PHAL (20 mol%), K<sub>3</sub>[Fe(CN)<sub>6</sub>], K<sub>2</sub>CO<sub>3</sub>, MeSO<sub>2</sub>NH<sub>2</sub>, t-BuOH, H<sub>2</sub>O (1:1), 23 °C, 3 d, 83%; (c) 5, pyridine, CH<sub>2</sub>Cl<sub>2</sub>, 23 °C, 1.5 h, 96%, (d) DMDO, CHCl<sub>3</sub>, 23 °C, 16 h, then AcOH, 62 °C, 17 h, 78%; (e) cinnamic acid, 2,4,6-trichlorobenzoyl chloride, Et<sub>3</sub>N, 4-DMAP, CH<sub>2</sub>Cl<sub>2</sub>, 23 °C, 5 h; (f) TBAF, AcOH (40 mol%), THF, 23 °C, 1.5 h, 85% over 2 steps.

Dihydroxylation using Upjohn conditions<sup>18</sup> as performed on a similar intermediate in the englerin synthesis by López et al.<sup>101</sup> was difficult, as their substrate possessed a bulky silylether at the C4-position which guided the regio- and stereoselectivity. Fortunately, this issue was solved by performing Sharpless asymmetric dihydroxylation with (DHQD)<sub>2</sub>PHAL<sup>19</sup> as a chiral ligand to afford diol 4 in 83% yield as a single diastereomer.

One of the major issues in our first-generation synthesis of the unnatural enantiomer (+)-EA was the low diastereoselectivity in the epoxidation of the trisubstituted olefin with m-CPBA (2.3:1 d.r.).10a This issue was overcome in this secondgeneration synthesis by installing a (tert-butyldimethyl)silyl ether at C<sub>9</sub>, resulting in an increased diastereomeric ratio (5.4:1 d.r.).<sup>10b</sup> To avoid additional protecting group manipula-75 tions, glycolate 6 was synthesized using acid chloride 5 and subsequently screened with different epoxidizing agents. Di-76 methyldioxirane (DMDO) in chloroform<sup>20</sup> gave epoxide 7 as a single diastereomer. A direct one-pot transannular opening of epoxide 7 was achieved by adding acetic acid and heating to 80 62 °C to afford the tricyclic intermediate 8 in 75% yield. The use of standard DMDO solution in acetone led to longer reac-82 tion times and lower yields. Yamaguchi esterification of alcohol 8 with cinnamic acid followed by desilylation using a buffered TBAF solution afforded (-)-EA in 85% yield in 2 steps. In 85 conclusion, this approach allows for a concise synthesis of (-)-EA in only 6 steps from guaia-6,10(14)-diene with an overall yield of 38%.

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The potential for diversification at the chemical stage was 49 1 further demonstrated by the synthesis of two other guaianes 50 2 3 starting from 1. (-)-Oxyphyllol (10) and (+)-orientalol E (11) 51 were isolated from Phyllanthus oxyphyllus<sup>21</sup> and Alisma orien- 52 4 5 talis,22 respectively, but their biological activities have not 53 been studied, in detail. In the context of their englerin synthe- 54 6 ses, Sun and Lin et al.,10i Wang et al.,10n and Metz et al.23 re- 55 7 8 ported syntheses or formal syntheses of these two natural 56 9 products.

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10 A two-step synthesis of (-)-oxyphyllol was achieved by 58 11 epoxidizing 1 using an excess of DMDO (Scheme 2). The bi- 59 sepoxides **9a** and **9b** were obtained in 75% yield with a dia- 60 12 stereomeric ratio of 1.7:1 for the exocyclic epoxide, while the 61 13 14 trisubstituted olefin was epoxidized with high diastereoselec- 62 tivity. Opening of the exocyclic epoxide with LiAlH<sub>4</sub> and acidic 15 workup gave (-)-oxyphyllol in 81% yield. Applying a three-step 63 16 17 sequence like reported by Sun and Lin et al.10i and 18 Wang et al.<sup>10n</sup> allowed for the conversion of (-)-oxyphyllol 64 19 (10) into (+)-orientalol E (11) by acetylation, C-H-oxidation, 65 and saponification in 44% yield. 20 66

Scheme 2. Synthesis of (-)-Oxyphyllol and (+)-Orientalol
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 Conditions: (a) DMDO, acetone, o °C, 30 min, 75% (1.7:1 d.r.);

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 (b) LiAlH<sub>4</sub>, Et<sub>2</sub>O, o °C, 1 h, then HCl<sub>aq</sub>, o °C, 15 min, 81%; (c)
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 Ac<sub>2</sub>O, Et<sub>3</sub>N, DMAP, 23 °C, 16 h; (d) CrO<sub>3</sub>, Bu<sub>4</sub>NIO<sub>4</sub>, CH<sub>2</sub>Cl<sub>2</sub>,
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 MeCN, -30 °C → o °C, 45 min; (e) KOH, MeOH, 50 °C, 3 h, 44%
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 over 3 steps.
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29 In summary, we report a conceptionally new synthetic 84 30 route towards EA involving synthetic biology and chemical 85 31 synthesis. Guaia-6,10(14)-diene cyclase from filamentous fungi was employed as an alternative to introduce the missing en- 86 32 zymes and provide a building block for the semisynthesis of 87 33 EA. In the biotechnological processes, overproduction of 1 in  $\frac{1}{88}$ 34 35 E. coli and S. cerevisiae were realized with titers of 0.5 and 0.8 89 g/L in fed-batch fermentation, respectively. Compared to our 90 36 37 previously developed S. cerevisiae YZL141, we systematically 92 38 engineered the upstream mevalonate pathway and the down-93 stream guaia-6,10(14)-diene forming pathway in S. cerevisiae, 39 94 improved the titer of guaia-6,10(14)-diene by a 100-fold to 95 40 gram per liter levels. The production of sufficient starting ma- $\frac{1}{96}$ 41 42 terial enabled a highly efficient synthesis of (-)-EA in only 7 43 steps with 38% overall yield, representing the shortest and 98 highest yielding synthesis to date. The efficiency for this route 99 44 can be further optimized via enzymatic engineering to en-100 45 hance the catalytic efficiency of FgJ02895, and produce the al- $\frac{101}{100}$ 46 ternative cyclization product, guaia-6,9-diene, to further 102 47 shorten the chemical synthesis. Additionally, this route allows  $\frac{100}{104}$ 48 105

for short syntheses of two related sesquiterpenoids, (–)-oxy-phyllol and (+)-orientalol E, showcasing unfunctionalized terpenes to constitute a versatile platform for unlocking the chemical space of oxidized terpenes and beyond.

The interplay between synthetic biology and chemical synthesis was key in providing a straightforward and scalable access to EA and two other guaiane terpenes. Synthetic biology offered a direct entry into the three-dimensional carbon framework using the power of terpene cyclases. Synthetic chemistry on the other hand allowed for a rapid scaffold diversification by adding additional layers of complexity onto the carbocyclic core. We anticipate that this synergistic approach will become a general tool to cast the chemical space of biologically active terpene natural products.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

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<sup>⊥</sup>T.S., Z.W. and G.B. contributed equally.

#### 76 Notes

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The authors declare no competing financial interests.

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