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# *Synthetic Biology-Driven Heterologous Biosynthesis of Cis-abienol in Escherichia coli*

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***Abstract.*** Cis-abienol is a labdane-type diterpenoid compound that can be used as a valuable precursor for ambergris-like fragrance compounds and for flavoring agents in tobacco. Conventional supply is based on plant extraction, low yield and high land cost, seasonal variation. However, microbial cell factories have shown to be a promising alternative, but the limitation in precursors, particularly the common C5 isoprene units isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), must be overcome. In this review, the heterologous biosynthesis of cis-abienol in engineered Escherichia coli, by using three different upstream pathways for precursor supply, namely the native MEP pathway, the heterologous MVA pathway and the synthetic isopentenol utilization pathway (IUP), is discussed. IPP and DMAPP are elongated into FPP and GGPP, which are subsequently cyclized by SsTPS and AbCAS to form cis-abienol in the downstream diterpene-synthesis module. Across three engineering rounds, direct DMAPP supplementation proved infeasible at scale owing to substrate cost and poor membrane permeability; simultaneous operation of the native MEP and heterologous MVA pathways increased theoretical precursor supply but imposed a strong metabolic burden, reaching only approximately 220 mg/L under high-cell-density fermentation; and the two-step IUP salvage pathway relieved cofactor pressure and achieved the highest reported titre, approximately 311.8 mg/L in shake-flask culture and 1375.7 mg/L in 1.3-L fed-batch fermentation, albeit at the cost of externally supplied DMAOH. These results point to the key design principles of evaluating the precursor generating capacity and host physiological fitness and indicate that dynamic regulation and enzyme engineering are key for future industrial translation.

***Keywords:*** synthetic biology, cis-abienol, microbial cell factory, metabolic engineering, isopentenol utilization pathway

## 1. Introduction

Synthetic biology is an interdisciplinary science that uses the principles of engineering to design, build and redesign biological systems. Its underlying principle is to change biology from a "discovery science" to an "engineering science": The rational programming of the behaviour of cells through standardised biological parts (BioBricks), modular metabolic pathways and predictable genetic circuits. In contrast, biomanufacturing is a manufacturing paradigm that relies on living cells

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or enzymes as catalysts to yield chemicals, materials, pharmaceuticals and energy via fermentation or biotransformation.

The high degree of cross fertilization between the two is bringing the world of manufacturing from the "black-box fermentation" to an "era of cell factory" with rational design based on data. Synthetic biology builds on the idea of systematic design, high-throughput construction and automatic screening and allows the reconstruction of metabolic networks at the whole genome scale, which results in a product spectrum widening and yield improvement that cannot be obtained by conventional breeding. These capabilities are particularly transformative for high-value natural products whose plant-derived supply is limited; terpenoids—the largest class of natural products—exemplify this opportunity [1, 2]. Microbial cell factories, and in particular engineered Escherichia coli, offer a sustainable alternative: by introducing heterologous biosynthetic pathways into this well-characterized host, cis-abienol can be produced from inexpensive carbon sources under controlled fermentation [1, 3]. For example, cis-abienol is a labdane-type diterpenoid which is used as a precursor for ambergris-like fragrance compounds and for tobacco flavoring agents [4]. Natural cis-abienol is obtained from tobacco trichomes and balsam fir oleoresin, however, it is present in small amounts in the plant and the land cost is high and the concentration of the plant extract varies from year to year [5]. An alternative is provided by microbial cell factories, and in particular by the introduction of the heterologous biosynthetic pathway into the well-characterized E. coli host, which can be cultured under controlled fermentation conditions using inexpensive carbon sources to produce cis-abienol [1, 3]. The main challenge is the universal C5 isoprene building blocks IPP and DMAPP. These precursors can be produced through three pathways: the native MEP pathway, the heterologous MVA pathway and the IUP [6-8].

This review focuses on the synthetic biology approach of biomanufacturing in the context of the production of cis-abienol in a heterologous E. coli strain. The biosynthetic pathway is subdivided into two modules: an upstream module for the provision of the precursor and a downstream module for the synthesis of the diterpenes, and the three upstream pathways are compared regarding precursor supply, cofactor burden and substrate cost. Three rounds of strain improvements are then tracked, showing the central trade-off between the ability to generate precursors and the physiological fitness of the host. Finally, future optimization directions to cost competitive biomanufacturing are discussed.

## 2. Synthesis method

Cis-abienol is a labdane-type diterpenoid with high commercial value as a precursor for ambergris-like fragrance compounds and tobacco flavoring agents [4]. To achieve heterologous biosynthesis of cis-abienol in Escherichia coli, the biosynthetic pathway is rationally divided into two functional modules, as illustrated in Figure 1: an upstream precursor-supply module responsible for generating the universal C5 isoprene units—IPP and its isomer DMAPP—and a downstream diterpene-synthesis module that assembles these precursors into cis-abienol [4]. Three pathways can supply IPP and DMAPP with different resource-consumption patterns. In this study, the MEP pathway serves as the native route, the MVA pathway as the heterologous pathway, and the IUP pathway as the alternative salvage route. They are compared primarily on three criteria: precursor supply, cofactor burden, and substrate cost.

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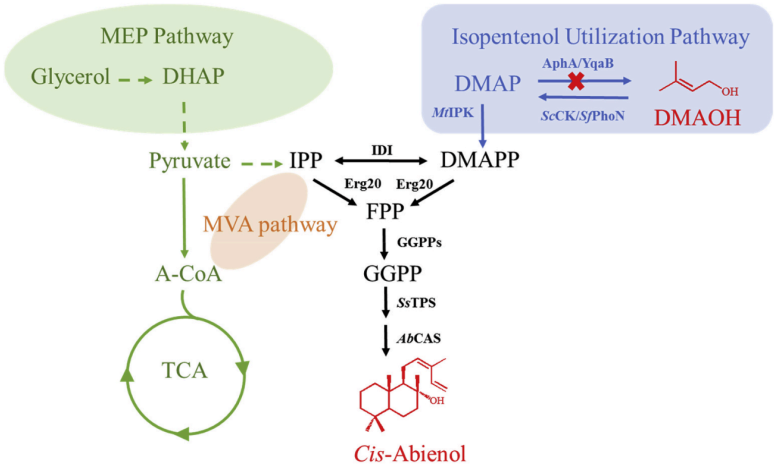



Figure 1. Schematic overview of the cis-abienol biosynthetic pathway in engineered E. coli. The upstream precursor-supply module (MEP, MVA and IUP pathways) generates IPP and DMAPP; the downstream diterpene-synthesis module converts them into cis-abienol via FPP, GGPP, SsTPS and AbCAS

## 2.1. Upstream precursor-supply module

The endogenous 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway is native to E. coli and uses glycerol as the primary carbon source. It should be noted that the representation "glycerol → DHAP → pyruvate → IPP" is a simplified carbon-flow schematic rather than a direct stoichiometric conversion. In reality, glycerol is phosphorylated and oxidized to dihydroxyacetone phosphate (DHAP), which enters glycolysis and is converted to pyruvate. Pyruvate and glyceraldehyde-3-phosphate are then condensed by 1-deoxy-D-xylulose-5-phosphate synthase (DXS) to form 1-deoxy-D-xylulose-5-phosphate (DXP), followed by a cascade of seven enzymatic steps (DXR, IspD, IspE, IspF, IspG and IspH) that ultimately yield IPP and DMAPP [4]. The principal advantage of the MEP pathway is that it requires no heterologous gene insertion, minimizing the genetic load on the host. However, the native MEP pathway is tightly regulated by cellular feedback mechanisms and its intracellular flux is inherently limited. Consequently, the IPP/DMAPP pool generated by the MEP pathway alone is often insufficient to support high-level diterpenoid production [4].

The MVA pathway is a heterologous route originating from acetyl-CoA. It proceeds through a well-characterized sequence of core enzymatic reactions: acetoacetyl-CoA thiolase (AtoB) condenses two molecules of acetyl-CoA; HMG-CoA synthase (HMGS) and HMG-CoA reductase (HMGR) convert the product to mevalonate; and mevalonate kinase (MK), phosphomevalonate kinase (PMK) and diphosphomevalonate decarboxylase (PMD) successively phosphorylate and decarboxylate mevalonate to yield IPP, which is finally isomerized to DMAPP by isopentenyl diphosphate isomerase (IDI) [6]. The MVA pathway can significantly boost the intracellular IPP/DMAPP pool when fully expressed. However, this enhanced precursor supply comes at a substantial energetic cost: the MVA pathway consumes approximately three molecules of ATP and two molecules of NADPH per IPP molecule produced, diverting cellular resources away from growth and maintenance. This cofactor drain can manifest as metabolic burden, retarded growth and reduced final biomass, particularly when the MVA pathway is operated in parallel with the native MEP pathway [7].

The IUP is a synthetic two-step salvage route that decouples precursor generation from central carbon metabolism [8]. The pathway involves two distinct phosphorylation steps. First, DMAOH is

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phosphorylated to dimethylallyl phosphate (DMAP), a reaction catalyzed by a choline kinase from Saccharomyces cerevisiae (ScCK) or a phosphatase from Shigella flexneri (SfPhoN). Second, DMAP is further phosphorylated to DMAPP by an isopentenyl phosphate kinase from Methanocaldococcus jannaschii (MtIPK). By bypassing the long multi-enzyme cascades of both the MEP and MVA pathways, the IUP route dramatically reduces cofactor consumption and metabolic pressure on the host chassis. Nevertheless, the IUP pathway remains dependent on externally supplied DMAOH, which introduces a substrate-cost consideration that must be weighed against its physiological benefits [8].

Table 1 summarizes the key differences among the three precursor-supply pathways in terms of precursor supply, cofactor burden, substrate cost and reported titre.

Table 1. Comparison of the three IPP/DMAPP precursor-supply pathways evaluated in this study

| Pathway | Precursor supply | Cofactor burden | Substrate cost | Reported titre (fermentation) |
|---|---|---|---|---|
| MEP (native) | Low (native flux) | Moderate (CTP, NADPH) | None (glycerol) | Not industrially sufficient |
| MVA (heterologous) | High | High (≈3 ATP + 2 NADPH per IPP) | None (acetyl-CoA) | ≈220 mg/L (high-cell-density) |
| IUP (salvage) | Moderate | Low (two phosphorylation steps) | High (DMAOH added) | ≈311.8 mg/L (shake-flask); 1375.7 mg/L (1.3-L fed-batch) |

### 2.2. Downstream diterpene-synthesis module

Once IPP and DMAPP are available, the downstream module converts these C5 precursors into cis-abienol through a defined sequence of chain-elongation and cyclization reactions, as shown in Figure 1. First, one molecule of DMAPP condenses with two molecules of IPP to form farnesyl diphosphate (FPP, C15), catalyzed by the farnesyl diphosphate synthase ScErg20 from S. cerevisiae. FPP is then extended by an additional IPP unit to generate geranylgeranyl diphosphate (GGPP, C20), mediated by a GGPP synthase from Taxus canadensis (TcGGPPS). The final conversion of GGPP to cis-abienol requires two sequential plant-derived diterpene synthases: SsTPS catalyzes the initial cyclization of GGPP into a labdadienyl diphosphate intermediate, and AbCAS—a bifunctional cis-abienol synthase originally identified in Abies balsamea—completes the structural rearrangement to yield cis-abienol [9]. Because none of these downstream enzymes are native to E. coli, all corresponding genes must be heterologously expressed, typically from multi-copy plasmids with carefully tuned promoter strengths to balance enzyme expression and avoid accumulation of toxic intermediates [1, 10].

## 3. Detection method

Accurate quantification of cis-abienol is required to assess the efficiency of each modified strain. Given the highly hydrophobic nature of cis-abienol (a nonpolar diterpenoid), most of the product will be in the organic phase during two-phase fermentation. The liquid-liquid extraction and HPLC-UV quantification protocol used here follows the methods reported in previous cis-abienol studies [4, 5].

For sample preparation, the fermentation culture was first mixed with isopropyl myristate in a volume ratio of 4:1 (culture medium : organic solvent) and then vortexed vigorously to ensure complete extraction of cis-abienol into the organic phase. Then, at 14,000 rpm for 10 minutes, the

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mixture was centrifuged to separate the phases; then, the upper organic layer was taken out and filtered through a 0.22-μm PTFE syringe filter to remove any remaining cell debris. High-performance liquid chromatography with a UV-Vis detector (HPLC-UV, Agilent 1260, Agilent Technologies, Santa Clara, CA, USA) was used to analyse the filtered organic extract. A 4.6 mm × 250 mm Eclipse XDB-C18 reversed-phase column (5 μm particles, Agilent) was employed for separation. Ethanol, methanol and 0.1% (v/v) aqueous acetic acid are used as the mobile phase at a volume ratio of 71:18:11, and the flow rate is 0.8 mL/min. Set the temperature of the column to 30 °C and, at a detection wavelength of 237 nm, measure the characteristic absorbance peak of the conjugated diene part of cis-abienol.

A standard calibration curve was prepared using an external standard of actual cis-abienol, and quantification was performed. All experiments were conducted in four independent biological replicates, and the results are shown as the mean ± standard deviation (mean ± SD). Biological replicates show the natural fluctuations in plasmid maintenance, induction efficiency and fermentation behaviour among different cultures, as opposed to technical replicates [4].

HPLC-UV was chosen over other options such as gas chromatography-mass spectrometry (GC-MS) for practical reasons; it has a lower operating cost and higher throughput, and is more suitable for the iterative optimisation workflow in this study. A single-wavelength detector will only provide some structural information and cannot simultaneously track low-level by-products resulting from the extensive activity of diterpene synthase. GC-MS or LC-MS/MS can be used to conduct whole-metabolite profiling [1].

## 4. Iterative improvement

The construction of a high-performance microbial cell factory is rarely achieved in a single design cycle. Each round of genetic modification addresses a specific bottleneck of the previous strain while potentially introducing new physiological constraints, such as metabolic burden or substrate-cost barriers. The cis-abienol-producing E. coli platform underwent three major rounds of iterative engineering.

### 4.1. Round 1: Direct substrate supplementation and the cost barrier

The first approach to a proof of concept was to simply supplement the IPP and DMAPP directly to the downstream unit. However, this proved to be successful and it was found that the SsTPS and AbCAS, expressed in E. coli, were active and yielded detectable amounts of cis-abienol. But, for two reasons, this approach could be problematic on the industrial scale. For one, DMAPP is a specialty biochemical product which is costly; if the titres are to be used on a commercial scale, the cost of the substrate alone would be prohibitive. Second, externally supplied diphosphate intermediates are poorly membrane permeable and their effective substrate utilization and longevity are limited by rapid degradation by extracellular phosphatases [4]. These considerations drove the first major engineering decision that was made - to allow for the intracellular autonomous synthesis of DMAPP, by implementing the heterologous MVA pathway, which makes no use of externally supplied phosphorylated precursors.

### 4.2. Round 2: MEP–MVA dual pathway and the metabolic burden problem

In the second version, the entire MVA pathway was imported into E. coli, now allowing for the production of DMAPP in the cell using acetyl-CoA. The native MEP pathway was also boosted by

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over-expression of the rate-limiting enzyme DXS and was run in parallel with the heterologous MVA pathway, a dual-pathway strategy that has been widely investigated for terpenoid metabolic engineering [1]. The idea was that two independent pathways to IPP/DMAPP would allow the production of more precursor and drive more carbon flux towards cis-abienol.

The dual-pathway strategy, in practice however, resulted in limited improvement in cis-abienol titre [4]. One is an energetically costly process that uses a considerable amount of ATP and NADPH, and both pathways force the host cell to sacrifice growth and cell maintenance for the biosynthesis of precursors. This can be manifested by the reduction of growth rates, decrease in final biomass and, in certain instances, plasmid instability [7]. It is important to note that this is an interpretation in a framework of known principles regarding metabolic burden; it would have to be demonstrated through measurement of intracellular ATP/NADPH levels and growth kinetics to prove the causal link. The important take home message was that adding pathways on top of each other was not beneficial if the added metabolic burden reduced the fitness of the host organism and spurred it to take a more parsimonious pathway to DMAPP production.

### 4.3. Round 3: IUP pathway introduction and cofactor relief

In the third iteration, the long MEP–MVA cascades were replaced with the synthetic IUP pathway to supply DMAPP from DMAOH via only two phosphorylation steps, which are provided by ScCK (or SfPhoN) and MtIPK [8]. The simplicity of this architecture carries two benefits, in that it avoids the use of central carbon metabolism (the ATP and NADPH cost of the MVA pathway), and in that it is only a two enzyme cassette, as opposed to the full MVA operon, which is quite cumbersome and prone to plasmid instability. As expected, previous work that employed an IUP-based approach achieved a cis-abienol titre of around 311.8 mg/L in shake-flask culture (1375.7 mg/L in a 1.3-L fed-batch bioreactor), compared with around 220 mg/L for the MVA-pathway strain under high-cell-density fermentation, representing a ~1.4-fold improvement in shake-flask titre and restored cell growth [4, 5].

However, the IUP strategy also has certain drawbacks. It still uses DMAPP as raw material, which is less expensive than DMAOH but not included in the completely glucose based processes. Furthermore, the ability of the phosphorylation reaction to be efficient can be limiting at a high DMAOH concentration, and the intermediate DMAP may be able to feedback inhibit the upstream kinase (ScCK/MtIPK). Thus, further optimization is needed for industrial viability of the process, including engineering of the enzymes, ScCK and MtIPK, for enhanced catalytic activity, dynamic control of the expression of the IUP gene, and/or in vivo production of the prenol-type substrates [5].

### 4.4. Summary of iterative engineering lessons

These are the main takeaways from the iterative engineering lessons. The same pattern holds true for all three: every engineering decision busts a single issue while creating another. Direct supplementation with substrate achieves proof of concept, but not at cost; dual-pathway overexpression provides more theoretical precursor supply, but also imposes a cost of metabolic burden; IUP pathway relieves cofactor pressure but again imposes a trade-off of substrate dependency. It is an iterative problem–solution–new-problem process, highlighting the core compromise of synthetic biology engineering: precursor-synthesis capacity, and host physiological fitness. Future studies should be made to incorporate the dynamic regulatory circuits that regulate

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pathway flux based on cell state and remove the need to add DMAOH, for sustainable and cost competitive cis-abienol biomanufacturing process [5].

## 5. Conclusion

This review has outlined the evolution of an Escherichia coli cell factory for cis-abienol production, through successive iterative engineering, centred on two functional modules – the upstream module for precursor supply and the downstream module for diterpene assembly. The overarching theme that comes from the three rounds of engineering is that capacity to generate precursors and the physiological fitness of the host must be balanced and not maximised separately. The results showed that direct DMAPP supplementation was simple, but expensive; that activation of the native MEP pathway in parallel with activation of the heterologous MVA pathway increased the availability of precursors in theory, but had a high ATP and NADPH cost and thus limited the growth of the cells; and that the 2-step IUP salvage pathway yielded the highest reported titre (shake-flask) of around 311.8 mg/L and (fed-batch) 1375.7 mg/L, but required an external source of DMAOH.

Three restrictions must be underscored. First, the IUP route still requires the use of a relatively costly alcohol substrate, and future work should include de novo conversion of an inexpensive carbon source to DMAOH or include IUP with an optimized native flux of MEP. Second, the downstream diterpene synthases SsTPS and AbCAS are not optimised; protein engineering to enhance their catalytic efficiency and decrease their tendency to be product inhibited may be able to provide further gains. Third, dynamic pathway regulation (e.g. switching the precursor supply on only after the biomass phase) would reduce the growth burden that reduces constitutive MVA overexpressing. In addition to cis-abienol, the general principles of these trade-offs apply to the production of other high-value terpenoids from microbes, and highlight the need to use modular pathway design and iterative chassis engineering in synthetic biology-driven biomanufacturing.

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