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A Modular Approach for Facile Biosynthesis of Labdane-Related Diterpenes

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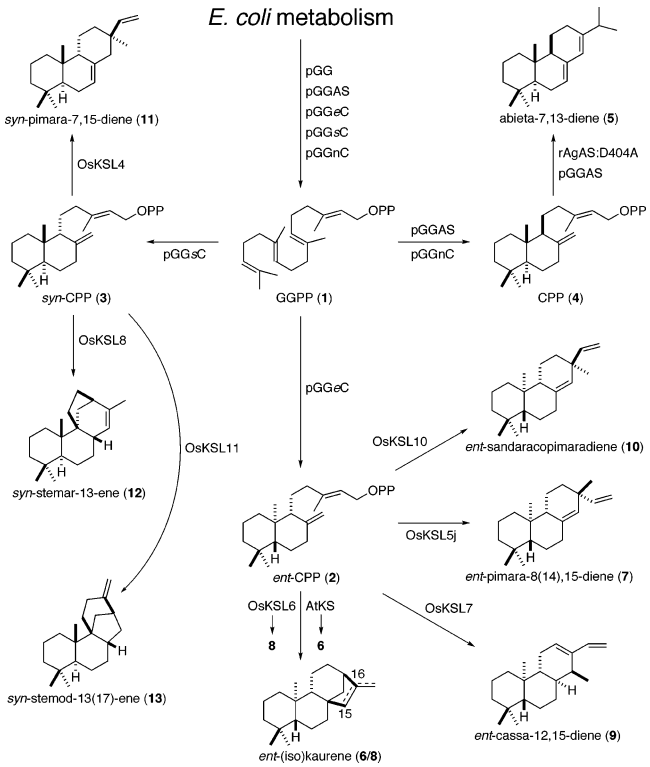
Labdane-related diterpenoids comprise a large group of over 5000 known natural products defined as minimally containing the fused bicyclic hydrocarbon structure found in the labdane family of diterpenoids. This characteristic core structure results from the unusual biosynthetic origins of these compounds, which is uniquely initiated by a sequential pair of terpene synthase catalyzed reactions. In particular, cyclization of the universal diterpenoid precursor (*E*,*E*,*E*)-geranylgeranyl diphosphate (GGPP, **1**) to a specific stereoisomer of labdadienyl/copalyl diphosphate (CPP, e.g., **2**−**4**) in a carbon−carbon double bond protonation-initiated reaction catalyzed by class II diterpene cyclases. This core bicyclic structure is then further cyclized and/or rearranged in diphosphate ionization initiated reactions catalyzed by more typical class I, but generally CPP stereospecific, labdane-related diterpene synthases, as in the related/derived structural families (e.g., kauranes, abietanes, and pimaranes).[1]

Included in the group of labdane-related diterpenoids are the gibberellin phytohormones required for normal growth and development in all higher plants, which has provided a ready reservoir of biosynthetic genes whose duplication has led to the widespread occurrence of such natural products throughout the plant kingdom. However, the vast majority of these compounds are secondary metabolites only found in a subset of species, in limited quantities, and often only under certain conditions. Thus, although several such compounds are utilized in industry, research, and medicine (e.g., resin acids and forskolin), similar uses for the bulk of this group of natural products have not been well explored. The use of recombinant microbial hosts for production of specific isoprenoid/terpenoid natural products has been recently reviewed.[2] To provide a foundation for broader investigations of labdane-related diterpenoid natural products we report here development of a modular approach that facilitates recombinant bacterial expression of various combinations of the sequentially acting class II and class I diterpene synthases, to produce hydrocarbon diterpene precursors to literally thousands of known natural products.

Because diterpene biosynthesis in plants occurs in plastids, recombinant microbial expression of the associated enzymes requires construction of pseudo-mature genes missing the N-terminal plastid targeting peptide sequence that interferes with proper protein folding.[3] This was recently accomplished for the *ent*-CPP synthase from *Arabidopsis thaliana* (AtCPS),[4] providing a guide for construction of pseudomature versions of other strictly class II diterpene cyclases and, thus, enabling the studies reported here.

While all organisms produce terpenoids/isoprenoids, *E. coli* does not typically produce GGPP, hence it was necessary to introduce a GGPP synthase (GGPS), which was done through the use of a previously reported recombinant pseudomature GGPS from grand fir (*Abies grandis*), rAgGGPS.[5] To minimize the number of plasmids required for these metabolic engineering efforts, rAgGGPS was introduced into the dual gene expression vector pACYCDuet, creating a base pGG plasmid (see Supporting Information). For initial proof-of-concept studies a previously reported pseudomature version of the bifunctional class II/I diterpene cyclase from grand fir, abietadiene synthase (rAgAS)[6] was added to pGG to create pGGAS. Transformation with pGGAS yielded recombinant bacteria that produce the expected abietadiene (**5**) diterpene upon induction of the encoded enzymatic genes (Scheme 1).

Scheme 1. Modular Approach to Labdane-Related Diterpene Biosynthesis



From this initial success we were encouraged to attempt the expression of more typical separate class II and class I labdane-related diterpene synthases. In particular, because we have recently reported characterization of a pseudomature version of AtCPS,[4] this gene was incorporated into pGG to create pGG*e*C, which should enable bacterial production of *ent*-CPP (**2**). We also have reported functional recombinant expression of tagged (e.g., with glutathione-*S*-transferase, GST) versions of class I labdane-related diterpene synthases,[7] including that for the *ent*-CPP specific kaurene synthase (KS) from *Arabidopsis thaliana* (AtKS),[8] using pET based expression vectors (e.g., pDEST), which can be co-transformed with pACYCDuet and, hence, the derived pGG and pGG*e*C. Co-transformation of pGG*e*C with a pDEST15/AtKS vector encoding a GST-AtKS fusion protein yielded recombinant bacteria that produce the expected *ent*-kaur-16-ene (**6**) (Figure 1).

More detailed analysis of this kaurene (**6**) producing strain was carried out to optimize diterpene production. Somewhat surprisingly,

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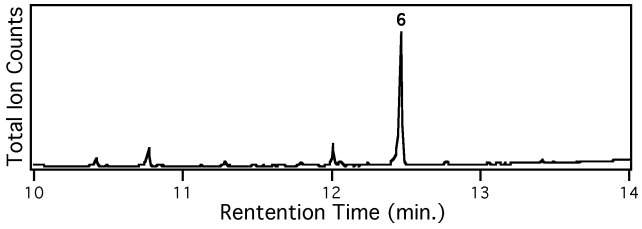

Figure 1. Production of *ent*-kaur-16-ene (**6**) by *E. coli* transformed with pGGeC and pDEST15/rAtKS. Total ion chromatogram from GC−MS analysis of organic solvent eluant of Diaion HP-20 beads from a mixed phase culture (see Supporting Information).

∼90% of the hydrocarbon product was secreted to the media, which had not been previously reported.[9] Similar secretion was observed with all the labdane-related diterpenes we have tested. Accordingly, it is possible to trap these hydrocarbon compounds on hydrophobic (Diaion HP-20) beads under mixed phase liquid−solid (i.e., media-beads) growth conditions,[10] and easily isolate the produced diterpenes (e.g., Figure 1). With the optimized conditions it was possible to obtain ∼100 μg of kaurene (**6**)/L mixed phase bacterial culture, similar to previously reported terpenoid yield from *E. coli* that has not been engineered for increased isoprenoid precursor supply.[11]

The vast majority of labdane-related diterpenoid natural products are derived from three of the four possible stereoisomers of CPP, specifically, *ent*-(**2**), *syn*-(**3**), or normal (**4**) CPP.[1] The pGG*e*C construct provides access to *ent*-CPP derived diterpenes through coexpression of class I labdane-related diterpene synthases. To provide similar access to *syn*-CPP (**3**) derived diterpenes we utilized the *syn*-CPP diterpene cyclase from rice (*Oryza sativa*) that we have previously identified (OsCPS4)[12] and, based on our work with rAtCPS,[4] constructed a pseudomature version suitable for recombinant bacterial expression (rOsCPS4, missing amino acids 2−70). Insertion of rOsCPS4 into pGG created pGG*s*C. To provide access to normal CPP (**4**) derived diterpenes, we returned to abietadiene synthase, which produces normal CPP (**4**) as an intermediate, and for which a D621A mutant (rAgAS:D621A) that no longer has class I activity (i.e., only produces **4** from GGPP (**1**)] has been reported.[13] Thus, rAgAS:D621A was inserted into pGG to create pGGnC.

To demonstrate the ability of these three pGGxC constructs to provide access to various stereoisomers of CPP for the production of a range of CPP derived diterpenes, pGG*e*C and pGG*s*C were individually co-transformed with previously described class I enzymes specific for the corresponding stereoisomer of CPP,[7] while pGGnC was co-transformed with a D404A mutant of rAgAS (rAgAS:D404A) that only catalyzes the production of abietadiene (**5**) from **4** (i.e., only has class I activity).[13] In each case this resulted in recombinant bacteria that produce the expected labdane-related diterpene (Table 1). Eight different hydrocarbon skeletal structures, with one as distinct double bond isomers, were produced, proving the utility of our modular approach for facile biosynthesis of labdane-related diterpenes (Scheme 1). Notably, more than 1000 natural products are derived from kaurene, with almost as many being derived from abietadiene, as well as ∼500 from various pimaradienes.[14] Thus, the diterpenes produced in this study are precursors to literally thousands of known compounds, and these results establish a foundation for biosynthetic production of elaborated labdane-related diterpenoid natural products. Both in providing substrates for characterization of downstream biosynthetic steps and for further extension of metabolic engineering efforts. In addition, the three pGGxC constructs reported here will be useful for characterization of novel class I labdane-related diterpene synthases. Finally, we expect that a similar approach will provide access to labdane-related diterpenes and diterpenoid natural products derived from other class II intermediates such as clerodadienyl diphosphate (i.e., by incorporating class II diterpene cyclases that produce such compounds[15] into pGG), which will further broaden the reach of the modular approach to labdane-related diterpene biosynthesis demonstrated here.

Table 1. Recombinant Bacteria Characterized in This Study[a]

plasmid(s)	product
pGGAS	abieta-7,13-diene (**5**)
pGGeC + pDEST15/rAtKS	*ent*-kaur-16-ene (**6**)
pGGeC + pDEST15/OsKSL5j	*ent*-pimara-8(14),15-diene (**7**)
pGGeC + pTH1/OsKSL6	*ent*-isokaur-15-ene (**8**)
pGGeC + pDEST15/OsKSL7	*ent*-cassa-12,15-diene (**9**)
pGGeC + pDEST15/OsKSL10	*ent*-sandaracopimaradiene (**10**)
pGGsC + pDEST14/OsKSL4	*syn*-pimara-7,15-diene (**11**)
pGGsC + pTH8/OsKSL8	*syn*-stemar-13-ene (**12**)
pGGsC + pDEST15/OsKSL11	*syn*-stemod-13(17)-ene (**13**)
PGGnC + pDEST14/rAgAS:D404A	abieta-7,13-diene (**5**)

[a] *E. coli* containing the indicated plasmid(s) produce the indicated labdane-related diterpene, as identified by GC−MS based comparison to authentic standards, with production levels ranging from 5 to 100 μg diterpene/L mixed phase culture (see Supporting Information).

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Supporting Information Available: Plasmid construction and characterization of recombinant bacteria. This material is available free of charge via the Internet at http://pubs.acs.org.

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