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March 13th, 2019

Dear Dr. Weldon,

In response to your kind invitation (Manuscript Number JoVE58850), we here submit for possible publication in *JoVE* a manuscript entitled "*A scalable approach for the enzymatic production and purification of diterpenoid natural products*.", authored by Katherine M. Murphy, Siwon Chung, Shruti Fogla, Hana B. Minsky, Karen Yong Zhu and Philipp Zerbe.

The article describes protocols for the enzymatic production of diterpenoid natural products via multi-gene co-expression in *Escherichia coli* or *Nicotiana benthamiana* as host systems, and the purification of target compounds using silica chromatography and optional HPLC. The described protocols provide inexpensive, customizable approaches that can be readily implemented in most laboratories and enable the production of diterpenoid natural products for a range of downstream analysis and applications, such as NMR structural analyses, bioactivity studies, and enzyme functional studies, thus offering a community resource to facilitate a broader investigation of the chemical and functional diversity of plant diterpenoid metabolism.

We are respectfully suggesting reviewers with considerable expertise and a track record of publications relevant to the research area of this submission, including **Dr. Dana Morrone** (St. Louis College of Pharmacy), **Dr. Reuben Peters** (Iowa State University), **Dr. Trent Northen** (Laurence Berkeley National Laboratory), and **Dr. Hiroshi Maeda** (University of Wisconsin - Madison).

Sincerely,

Philipp Zerbe
Assistant Professor of Plant Biology, UC Davis

TITLE:

A Customizable Approach for the Enzymatic Production and Purification of Diterpenoid Natural Products

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KEYWORDS:

diterpenoid biosynthesis, co-expression, diterpene synthase, cytochrome P450 monooxygenase, *Nicotiana benthamiana*, *Zea mays*, plant specialized metabolism

SUMMARY:

Here we present easy to use protocols for producing and purifying diterpenoid metabolites through the combinatorial expression of biosynthetic enzymes in *Escherichia coli* or *Nicotiana benthamiana*, followed by chromatographic product purification. The resulting metabolites are suitable for various studies including molecular structure characterization, enzyme functional studies, and bioactivity assays.

ABSTRACT:

Diterpenoids form a diverse class of small molecule natural products that are widely distributed across the kingdoms of life and have critical biological functions in developmental processes, interorganismal interactions, and environmental adaptation. Due to these various bioactivities, many diterpenoids are also of economic importance as pharmaceuticals, food additives, biofuels, and other bioproducts. Advanced genomics and biochemical approaches have enabled a rapid increase in the knowledge of diterpenoid-metabolic genes, enzymes, and pathways. However, the structural complexity of diterpenoids and the narrow taxonomic distribution of individual compounds in often only a single species remain constraining factors for their efficient production. Availability of a broader range of metabolic enzymes now provide resources for producing diterpenoids in sufficient titers and purity to facilitate a deeper investigation of this important metabolite group. Drawing on established tools for microbial and plant-based enzyme co-expression, we present an easily operated and customizable protocol for the enzymatic

production of diterpenoids in either *Escherichia coli* or *Nicotiana benthamiana*, and the purification of desired products via silica chromatography and semi-preparative HPLC. Using the group of maize (*Zea mays*) dolabralexin diterpenoids as an example, we highlight how modular combinations of diterpene synthase (diTPS) and cytochrome P450 monooxygenase (P450) enzymes can be used to generate different diterpenoid scaffolds. Purified compounds can be used in various downstream applications, such as metabolite structural analyses, enzyme structure-function studies, and in vitro and in planta bioactivity experiments.

INTRODUCTION:

Diterpenoids comprise a chemically diverse group of more than 12,000 predominantly polycyclic 20-carbon natural products that play critical roles in many organisms[1]. Fungi and plants produce the largest diversity of diterpenoids, but bacteria have also been shown to form bioactive diterpenoids (see reviews[2-5]). Rooted in their vast structural diversity, diterpenoids serve a multitude of biological functions. A few diterpenoids, such as gibberellin growth hormones, have essential functions in developmental processes[5]. However, the majority of diterpenoids serve as mediators of chemical defense and interorganismal interactions. Among these, diterpene resin acids in the pest and pathogen defense of coniferous trees and species-specific blends of antimicrobial diterpenoids in major food crops such as maize (*Zea mays*) and rice (*Oryza sativa*) have been most extensively studied[6,7]. These bioactivities provide a rich chemical repository for commercial applications, and select diterpenoids are used as important pharmaceuticals, food additives, adhesives, and other bioproducts of everyday modern life[8-10]. To advance research on the natural diversity and biological functions of diterpenoids and ultimately promote broader commercial applications, tools for the cost-efficient preparation of pure compounds are required. Large-scale isolation from plant material has been established for a few diterpenoid bioproducts, such as diterpene resin acids that are produced as a byproduct of the pulp and paper industry[8]. However, accumulation of diterpenoids in only specific tissues and under tight regulation by environmental stimuli often limits isolation of sufficient product amounts from the natural producer[2]. In addition, the structural complexity of diterpenoids hampers their production through chemical synthesis, although such approaches have been successful in several cases[11,12]. With the availability of advanced genomic and biochemical technologies, enzymatic production platforms have gained increasing attention for producing a range of diterpenoid compounds (see reviews[13-18]).

All terpenoids, including diterpenoids, are derived from two isomeric isoprenoid precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP)[19] that, in turn, are formed through the mevalonate (MVA) or the methylerythritol-5-phosphate (MEP) pathway. Terpenoid biosynthesis proceeds through the MEP pathway in bacteria and the MVA pathway in fungi, whereas plants possess a cytosolic MVA and a plastidial MEP pathway, with the latter being the primary route toward diterpenoid formation[20]. Condensation of IPP and DMAPP by prenyl transferases yields the central 20-carbon precursor to all diterpenoids, geranylgeranyl diphosphate (GGPP)[20]. Downstream of GGPP formation, two enzyme families, terpene synthases (TPSs) and cytochrome P450 monooxygenases (P450s) largely control the formation of the vast chemical diversity of terpenoid metabolism[21,22]. Diterpene synthases (diTPSs) catalyze the committed carbocation-driven cyclization and rearrangement of GGPP to form various

stereospecific bi-, poly-, or macro-cyclic diterpene scaffolds[1,3,23,24]. Oxygenation and further functional decoration of these scaffolds is then facilitated by P450 enzymes and select other enzyme families[22,25]. TPSs and P450s commonly exist as species-specific, multi-gene families that can form modular biosynthetic networks, where combining different enzyme modules along a common blueprint enables the formation of a broad range of compounds[2,26]. The rapid discovery of functionally distinct enzymes operating in modular terpenoid pathways in recent years has provided expanding opportunities for their use as a versatile parts list for metabolic engineering of partial or complete pathways in both microbial and plant-based production platforms. For example, yeast (*Saccharomyces cerevisiae*) has been applied successfully to engineer multi-enzyme pathways for the manufacture of terpenoid bioproducts, such as the antimalarial drug artemisinin[27], the sesquiterpenoid biofuels bisabolene and farnesene[28], but also select diterpenoids[29-31]. Likewise, engineered *Escherichia coli* platforms for the industrial-scale manufacture have been established for a few diterpenoid metabolites, including the Taxol precursor taxadiene used as an anti-cancer drug and the diterpene alcohol, sclareol, used in the fragrance industry[13,32-34]. Advances in genetic engineering and transformation technologies also have made plant host systems increasingly viable for producing plant natural products[9,14,35,36]. In particular, the close tobacco relative, *Nicotiana benthamiana*, has become a widely used chassis for terpenoid pathway analysis and engineering, due to the ease of *Agrobacterium*-mediated transformation of multiple gene combinations, efficient biosynthesis of endogenous precursors, and high biomass[14,35,36].

Drawing on these established platforms for terpenoid biosynthesis, we describe here easy-to-use and cost-efficient methods for the enzymatic production of diterpenoids and the purification of single compounds. The presented protocols illustrate how *E. coli* and *N. benthamiana* platforms engineered for enhanced diterpenoid precursor biosynthesis can be utilized for the combinatorial expression of different diTPSs and P450 enzymes to generate desired diterpenoid compounds. Application of this protocol to produce and purify structurally different diterpenoids is shown by example of specialized diterpenoids from maize (*Zea mays*), termed dolabralexins, endogenous biosynthesis of which recruits two diTPS and one P450 enzyme. Purification of different dolabralexins ranging from olefins to oxygenated derivatives is then achieved by combining separatory funnel extraction with large-scale silica column chromatography and preparative high-pressure liquid chromatography (HPLC). The described protocols are optimized for the production of diterpenoids, but can also be readily adapted for related terpenoid classes, as well as other natural products for which enzyme resources are available. Compounds produced using this approach are suitable for various downstream applications, including but not limited to, structural characterization via nuclear magnetic resonance (NMR) analysis, use as substrates for enzyme functional studies, and a range of bioactivity assays.

PROTOCOL:

CAUTION: The protocols described here include the use of hazardous chemicals, sharp objects, electrical devices, hot objects, and other hazards that may result in injury. Appropriate personal

protective equipment should be worn, and the appropriate safety procedures, including safety trainings, should be followed.

1. Preparation of materials and solutions

1.1. Prepare and autoclave lysogeny broth medium (LB) for 30 min: Per 1 L of medium, mix 10 g of tryptone, 5 g of bacterial yeast extract, and 10 g of NaCl and dissolve in 1 L of deionized (DI) water.

1.2. For 1 L co-expression cultures, prepare and autoclave Terrific Broth (TB) medium for 30 min: In a 2.8 L Erlenmeyer flask mix 12 g of tryptone, 24 g of bacterial yeast extract, and 40 mL of 10% (v/v) glycerol and dissolve in 860 mL of DI water. Prepare and autoclave 1.3 L of 10x phosphate buffer [1.3 L of DI water, 30.03 g of monopotassium phosphate (KH_2PO_4), and 163.02 g of dipotassium phosphate (K_2HPO_4)] and add to the above medium.

1.3. Prepare Super Optimal broth with Catabolite repression (SOC) media.

1.3.1. Prepare and autoclave for 30 min a solution containing 2% (w/v) tryptone, 0.5% (w/v) bacterial yeast extract, 8.5 mM NaCl, and 2.5 mM KCl.

1.3.2. Add sterile $MgSO_4$ and glucose both with a final concentration of 20 mM. Use 1 M NaOH to adjust to pH 7.0. Store the SOC media at -20 °C.

1.4. Prepare 1 L of 1 M sodium pyruvate. Autoclave for 15 min and store at 4 °C.

1.5. Prepare 20 mL of 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) in sterile DI water. Store 1–2 mL aliquots at -20 °C.

1.6. Prepare infiltration buffer: 10 mM MES (1.952 g) and 10 mM $MgCl_2$ (2.033 g) dissolved in 1 L of DI water. Store at 4 °C.

1.7. Prepare and autoclave LB agar for 30 min: Per 100 mL of DI water, add 1 g of tryptone, 0.5 g of bacterial yeast extract, 1 g of NaCl, and 1.5 g of agar. Once LB agar is cool enough to handle the bottle, add antibiotics as required for the desired plasmid combinations. Pour agar plates using petri dishes and store at 4 °C.

1.7.1. See **Table 1** for specifications regarding compound production in *E. coli* and *N. benthamiana*, respectively. Wrap plates containing rifampicin in foil to prevent degradation upon storage.

[Place **Table 1** here]

2. Production of diterpenoid metabolites in *E. coli*

NOTE: The protocol described here for producing diterpenoid metabolites in *E. coli* has been adapted from a previously reported enzyme co-expression platform developed by the group of Dr. Reuben J. Peters (Iowa State University, IA, USA)[13,32].

2.1. Transformation of competent cells with plasmid combinations.

2.1.1. Thaw chemically competent *E. coli* cells on ice (BL21DE3-C41 cells were used in this protocol).

2.1.2. Add 1 µL of a 100 ng/µL solution of each construct used for co-expression to 25 µL of competent cells in a 1.5 mL microtube. Do not vortex or mix by pipetting.

NOTE: For optimal expression and activity of TPS and P450 enzymes, several sequence modifications need to be considered. For TPS, removal of the N-terminal plastidial transit peptide is often essential. Specifically, plastidial mono- and di-TPS typically require removal of the predicted transit peptide (using common prediction algorithms[37]), whereas cytosolic sesqui-TPS can usually be used as full-length genes. With regards to P450s, codon optimization as well as removal or replacement with the leader sequence MAKKTSSKGK of the N-terminal transmembrane domain has proven effective in many cases[38,39]. In addition, when co-expressing P450 enzymes a cytochrome P450 reductase (CPR) should be included to ensure sufficient P450 activity.

2.1.3. Incubate the mixture on ice for 30 min. Mix every 10 minutes by gently scraping the tube across a microtube rack.

2.1.4. Pre-incubate SOC media and LB agar plates at 37 °C containing antibiotics as required for the desired combination of constructs.

NOTE: Each construct to be co-transformed must have a distinct antibiotic resistance, as well as distinct origins of replication to ensure optimal protein expression.

2.1.5. Heat shock the cell mixture at 42 °C for 1 minute, and then incubate on ice for at least 2 min.

2.1.6. Add 200 µL of warm SOC media.

2.1.7. Shake the cell mixture for 1 h at 37 °C and 200 rpm.

2.1.8. Add approximately 10 autoclaved glass beads to the warmed LB agar plate. Add 100 µL of the cell mixture and replace the lid. Shake plate horizontally with the lid on to distribute the cells evenly. Remove the glass beads by tapping them off into a waste container. Alternatively, use other preferred plating methods.

2.1.9. Incubate the LB agar plate at 37 °C overnight with the coated surface face down. The plate with transformed *E. coli* colonies can be used the following day or stored at 4 °C sealed in paraffin film for up to 2 weeks.

2.2. Preparation of inoculation cultures

2.2.1. On the following day, prepare a solution of LB medium with antibiotics required for the transformed plasmid combination using the concentrations provided in **Table 1**.

2.2.2. In a sterile hood, transfer 5 mL of LB medium to a 15 mL sterile glass test tube with a plastic breathable cap. Prepare one small culture tube for each desired large (1 L) culture.

2.2.3. Select individual *E. coli* colonies from the LB agar plate using a pipette tip. Inoculate each tube of LB with an *E. coli* colony by ejecting one pipette tip containing a selected colony into each tube.

2.2.4. Cap each inoculation culture test tube with a breathable plastic cap. Place the capped *E. coli* small cultures in a 37 °C shaking incubator for 12–24 h.

2.3. Preparation and induction of co-expression cultures

2.3.1. On the following day, add 100 mL of prepared 10x phosphate buffer to 900 mL of prepared TB for a final phosphate buffer concentration of 1x. Add necessary antibiotics with concentrations according to **Table 1**.

2.3.2. Shake at 140 rpm at 37 °C until warm (approximately 30 min).

2.3.3. Inoculate each flask of media for 1 L cultures with 5 mL of the inoculation culture. Retain the pipette tip used for inoculation culture inoculation in the inoculation culture tube so that, upon extraction with organic solvent in subsequent steps, there are no extracted plastic contaminants.

2.3.4. Incubate with shaking at 200 rpm until the optical density at 600nm (OD_{600}) reaches 0.6, approximately 3 h. To measure the OD_{600} with a spectrophotometer, use a mixture of sterile TB with phosphate buffer as a blank.

2.3.5. At the desired OD_{600}, set the incubator settings to 16 °C.

2.3.6. When co-expressing P450s, freshly prepare riboflavin and aminolevulinic acid, which are essential for sufficient P450 co-factor production. For every experiment, make 4 g/L riboflavin and 150 g/L aminolevulinic acid. Keep solution wrapped in foil until use, as riboflavin is light sensitive.

2.3.7. After the incubator has reached 16 °C (approximately 30 min), add 1 mL of 1 M IPTG, 1 mL of 4 g/L riboflavin, and 1 mL of 150 g/L aminolevulinic acid to each culture. For diterpenoid production, 25 mL of 1 M sodium pyruvate should be added to each culture to assure sufficient precursor formation.

NOTE: All constructs used in this assay were under the same IPTG-inducible promoter. Different promoters can be used as desired.

2.3.8. Incubate at 16 °C and 140 rpm for 72 h. Add 25 mL of sodium pyruvate each subsequent day after induction if producing diterpenoids. Immediately use cultures are immediately used for separatory funnel extraction of metabolites; do not harvest or store cultures.

3. Separation and purification of metabolites

3.1. Separatory funnel extraction of metabolites

NOTE: It is important to use only glassware and glass pipettes when using organic solvents to prevent plasticizer contaminations.

3.1.1. In a fume hood, secure the separatory funnel onto a ring stand. Place a waste beaker underneath the separatory funnel.

3.1.2. Pour 500 mL of 50/50 (v/v) ethyl acetate/hexanes into the separatory funnel.

NOTE: Solvent mixture should be adjusted based on the solubility and polarity of the targeted metabolites. Water-miscible solvents should be avoided to ensure suitable phase separation.

3.1.3. Add 500 mL of the *E. coli* culture to the separatory funnel and place on the glass stopper.

3.1.4. Shake the funnel to mix the culture with the extraction solvent, approximately 5–10x. Frequently de-gas the funnel by opening the spigot while the funnel is held upside down and pointed into the fume hood to release pressure. Repeat the shaking and de-gas procedure 2x.

3.1.5. Place the funnel upright in the ring stand and wait until the solvent layer (top) has separated from the aqueous (culture) layer (bottom), approximately 1 min.

NOTE: When a large amount of bubbles is observed in the interphase, addition of a small volume of 5–10 mL EtOH can be added to improve phase separation.

3.1.6. Remove the stopper. Drain the *E. coli* layer into a waste beaker, retaining the solvent layer in the funnel.

3.1.7. Repeat the procedure using the remaining 500 mL of *E. coli* culture and the same 500 mL solvent used for the first extraction.

3.1.8. Drain the solvent containing the extracted metabolites into a clean flask. Avoid contamination with *E. coli* culture.

3.2. **Rotary Evaporation Concentration**

3.2.1. Prepare the rotary evaporation (rotovap) equipment: fill the water bath and set the temperature to 25 °C. For heat sensitive compounds, use a lower temperature setting or add ice to the water bath. Fill the condensing chamber with dry ice and set the rotating speed to 60–80 rpm.

3.2.2. Add approximately 700 mL of extracted metabolites to a 1 L evaporating flask, attach to the rotovap, and lower into the water bath. Turn the water bath heater on and set to 25 °C.

3.2.3. Start rotation of the evaporating flask, turn on the vacuum system, and gradually increase the suction to avoid rapid boiling of the metabolite solution into the waste flask. Evaporated solvent should begin condensing and dripping into the condensate-collecting (waste) flask.

3.2.4. When only a few mL of the metabolite solution remains in the evaporating flask, stop rotations and turn off the vacuum system. Raise the evaporating flask and depressurize by closing the vacuum line. Retain concentrated metabolite solution remaining in the evaporating flask. Dispose of the waste in the waste flask.

3.2.5. Continue rotary evaporation by adding up to 700 mL of additional extracted metabolite solution to the evaporating flask. Repeat process until all of the extracted metabolite solution has been concentrated.

3.2.6. Remove the concentrated metabolites from the evaporating flask by transferring with a glass pipette to new test tube. Rinse evaporating flask with 5 mL of 50/50 (v/v) ethyl acetate/hexanes or desired solvent mixture two times, transferring the rinsing solution to the test tube.

3.2.7. Store concentrated metabolites at -20 °C or -80 °C (depending on product stability) until further use.

3.3. Silica Column Purification

3.3.1. Prepare glassware by rinsing a 1 L beaker, glass funnel, 50 mL test tubes, and 3.2 L chromatography column (equipped with a glass fret) once with hexane and once with ethyl acetate. Label the 50 mL test tubes, which will be used to collect fractions.

3.3.2. Add 2 L of silica gel (230–400 mesh, grade 60) to a 3.2 L chromatography column with a 2 L reservoir capacity and a fritted disk, then load sand to form a 5 cm layer at the top of the column.

3.3.3. Prepare the column for chromatography by flushing it thoroughly with 2 L of hexane. At all times, there should be a thin (~0.5 cm) layer of the solvent liquid above the sand layer of the column to ensure the column does not dry out or acquire air pockets. A glass inlet adaptor connected to an air hose can be used to gently increase the flow rate through the column rather than gravity alone.

3.3.4. Load the concentrated metabolite extract (see section 3.2) onto the column. Rinse the bottle that contained the sample 3x with hexane and add to the column to ensure all of the sample has been transferred.

3.3.5. Using the following gradient, load 100 mL at a time and collect 50 mL fractions in labeled test tubes: 100% hexanes 3x, 10% (v/v) ethyl acetate in hexanes 3x, 12.5% (v/v) ethyl acetate in hexanes 3x, 15% (v/v) ethyl acetate in hexanes 3x, 20% (v/v) ethyl acetate in hexanes 3x, 40% (v/v) ethyl acetate in hexanes 3x, 60% (v/v) ethyl acetate in hexanes 3x, and 100% ethyl acetate 4x.

NOTE: Gradient should be adjusted based on compound size and polarities and desired separation.

3.3.6. Using a glass pipette, transfer 1 mL from each fraction into a labeled GC vial. Analyze each sample via GC-MS to determine which fractions contain the desired metabolites and their level of purity.

NOTE: The GC-MS method suitable for the metabolites produced in this method has been described in Mafu et al. 2018[39]. In brief, all analyses were performed on an GC with an XL MS detector using an HP-5MS column (see **Table of Materials**), a sample volume of 1 µL, and oven temperature ramp from 50 °C to 300 °C at 20 °C min^{-1}.

3.3.7. After determining which fractions contain the compound(s) of interest, combine all fractions that contain the same compound. Properly dispose of fractions that do not contain any compounds into a waste container. Repeat the rotary evaporation procedure if necessary to concentrate the purified metabolites.

3.3.8. If additional purification is necessary, use (semi-)preparative HPLC to improve product purity. HPLC protocols should be adapted based on individual equipment specifications and compounds of interest.

3.3.8.1. Resuspend dried silica chromatography samples in 1 mL hexane (diterpene hydrocarbons) or acetonitrile (oxygenated diterpenoids), and filter through a filter syringe to prevent contamination with small particles.

3.3.8.2. For non-polar diterpenoids, use a CN column (see **Table of Materials**) with a recommended flow rate of 1 mL/min and a hexane:ethyl acetate gradient, with fractions collected every 1 minute.

3.3.8.3. For polar diterpenoids, use a C18 column (see **Table of Materials**) with a recommended flow rate of 3 mL/min and an acetonitrile:water gradient, with fractions collected every 1 minute.

3.3.8.4. Dry all HPLC purified fractions under a nitrogen stream and resuspend in 1 mL hexane before GC-MS analysis to assess product abundance and purity.

4. Production of diterpenoid metabolites using *N. benthamiana*

NOTE: The protocol described here for producing diterpenoid metabolites in *N. benthamiana* has been adapted from previously reported studies[35,36,40,41]. The below protocol is specific to syringe-infiltration of *N. benthamiana* leaves. Other infiltration methods, such as vacuum infiltration are equally suitable. Binary T-DNA vector systems, such as pCAMBIA130035Su (pLIFE33) or pEAQ-HT[40-42], that enable propagation in *E. coli* and *A. tumefaciens* and gene expression in plant hosts are suitable for this protocol.

4.1. Planting *Nicotiana benthamiana*

4.1.1. Fill one 750 mL pot with potting soil and water the pot. Add ~20 tobacco seed and gently tap with finger so that they are in the soil. Do not cover with soil.

NOTE: Seeds can be generated through self-pollination for seed propagation. Seeds for this experiment were obtained from the UC Davis Department of Plant Biology, and are publicly available through USDA germplasm repository (https://npgsweb.ars-grin.gov/gringlobal/accessiondetail.aspx?id=1450450).

4.1.2. Leave pot in a growth chamber with the following conditions, watering every other day to ensure the soil does not dry out. Grow plants at 26 °C and 60% humidity with 16:8 h day:night cycle for all steps in this protocol.

4.1.3. After 1 week, fill ~20 pots with potting soil and water the pots. Using forceps, grasp a tobacco seedling by the stem and gently remove from the source pot, placing one seedling in each new pot and carefully burring the roots. Do not damage the leaves or roots.

4.1.4. Water the plants every other day by placing water in the tray the pots are in (also called "bottom watering"). Every fourth watering, include generic fertilizer in the water according to package instructions.

4.2. Freeze-thaw transformation of *Agrobacterium tumefaciens* strain GV3101 competent cells

4.2.1. Thaw *Agrobacterium tumefaciens* strain GV3101 (or other preferred strain) competent cells on ice (~1 h). Pre-chill 0.2 mL microcentrifuge tubes on ice. Warm SOC media at 28 °C.

4.2.2. Gently mix cells with the pipet tip (do NOT pipet up and down) and aliquot 15 µL of cells for each transformation into the chilled 1.5 mL microtubes.

4.2.3. Add 1-5 µL (~400 ng) of DNA to each tube of competent cells and mix gently by scraping tube along a tube rack.

NOTE: Desired constructs need not have different antibiotic resistances because they are each being transformed separately and will be mixed before infiltration.

4.2.4. Place microtubes in liquid nitrogen for 5 min.

4.2.5. Remove microtubes from liquid nitrogen and place the tubes on the room-temp rack. Thaw tubes for 5 min in 37 °C incubator.

4.2.6. Add 250 µL of the pre-warmed (28 °C) SOC to each microtube. Shake at 28–30 °C, 225 rpm, for 3 h.

4.2.7. Plate 50 µL of transformed cells onto LB agar plates containing the necessary antibiotics described in **Table 1** using sterile glass beads, as described in step 2.1.8.

4.2.8. Incubate plates inverted at 28–30 °C for 48 h. Growth within the 1st day of incubation may be a sign of contamination. Transformed *A. tumifaciens* can be used following the 2-day incubation or stored at 4 °C sealed in paraffin film for up to 2 weeks.

4.3. *Agrobacterium*-mediated transient enzyme co-expression in *Nicotiana benthamiana*

4.3.1. Prune 4-week-old *Nicotiana benthamiana* plants 2 days before infiltration by removing lower leaves. Leave 4 uppermost leaves. Water the plants. Do not water the plants 24 h before infiltration in order to allow for open stomata and easier infiltration.

4.3.2. Add 10 mL of LB with working concentrations of the appropriate antibiotics for chosen constructs and *Agrobacterium* strain (described in **Table 1**) to a 50 mL sterile glass test tube with a foil lid.

4.3.3. Inoculate using a pipette tip to swab a single colony of transformed *Agrobacterium* and eject the tip into the LB media. Each *Agrobacterium* transformant should have at least 2 small cultures.

4.3.4. Incubate overnight at 28 °C and 220 rpm.

4.3.5. Measure optical density at 600 nm (OD_{600}) of the overnight cultures using a spectrophotometer. Dilute the overnight cultures to an OD_{600} of 1.

NOTE: Optimal OD_{600} values may vary when using other *Agrobacterium* strains.

4.3.6. Distribute 10 mL of diluted overnight culture into 50 mL conical tubes.

4.3.7. Harvest the bacteria by centrifugation at 3500 rpm for 15 min at room temp. Pour off and discard the supernatant.

4.3.8. Re-suspend the cultures in 10 mL infiltration buffer by gently shaking the tube and rolling on a tube rack. The OD_{600} should equal 1 for each construct.

4.3.9. Generate the combinations desired for infiltrations by combining equal volumes of each transformed cell line. The OD_{600} should equal 1 for each infiltration. Estimate 5 mL of infiltration solution per leaf, 2 leaves per plant.

4.3.10. Attach tubes horizontally to a rocker and rock gently for 2 h at room temperature.

4.3.11. Infiltrate ~2 leaves per *N. benthamiana* plant using approximately 5 mL of infiltration mixture per leaf. Infiltrate healthy leaves with a needleless syringe on the underside of the leaves while exerting a counter-pressure with a finger on the top side of the leaf to ensure infiltration solution suffuses the leaf tissue.

4.3.12. Mark all infiltrated leaves with a black marker or other indicator. Place infiltrated plants in the growth chamber for 5 days. Keep plants well-watered.

NOTE: An incubation time of five days has proven sufficient for reaching diterpenoid levels sufficient for most downstream analyses, while maintaining time-efficiency of product formation. Longer incubation periods can be tested, where higher product amounts are required.

4.4. Metabolite extraction and purification from transformed *Nicotiana benthamiana*

4.4.1. Harvest infiltrated leaves from plants by clipping them from the plant. Add ~100 mL of liquid nitrogen and a single leaf to a mortar, then grind using a mortar and pestle or tissue mill until obtaining a fine powder.

4.4.2. Add powdered tissue to a GC-MS vial to the 500 µL demarcation. Add 1.5 mL of 50/50 (v/v) ethyl acetate/hexane or desired solvent mixture to the vial and cap tightly.

4.4.2.1. For expressions that have been tested to provide the desired products, pool ground tissue into a larger flask or test tube, then add ~2x the amount of solvent than tissue volume and shake overnight. Proceed to step 4.4.5 for purification.

4.4.3. Place all vials in a microtube rack and tape down tightly to secure. Extract under vigorous shaking overnight at room temperature.

4.4.4. Transfer 400 µL of extract and 600 µL of hexane into a fresh GC-MS vial. Do not aliquot any leaf tissue. Analyze samples using GC-MS using the method described in step 3.3.6.1.

4.4.5. After analysis via GC-MS for presence of desired metabolites, pool leaf extracts together and proceed through rotary evaporation, silica column chromatography, and HPLC to obtain pure compounds as described in sections 3.2 and 3.3.

REPRESENTATIVE RESULTS:

Schematic workflow for diterpenoid production using *E. coli*

Figure 1 illustrates the described workflow for diterpenoid production. The protocol outlined here has been adapted from a previously described *E. coli* platform for diterpenoid biosynthesis[13,32] for use of larger-volume cultures and purification of desired diterpenoid products via silica chromatography. To demonstrate the use of this protocol, we used a recently identified dolabralexin pathway from maize that comprises two diTPSs, ZmAN2 (Zm00001d029648) and ZmKSL4 (Zm00001d032858), a multi-functional P450 (CYP71Z18, Zm00001d014134), and a cytochrome P450 reductase (ZmCPR2, Zm00001d026483) (**Figure 2**). In brief, *E. coli* BL21DE3-C41 competent cells were pre-transformed with the pCDFDuet:IRS and pACYC-Duet:GGPPS/ZmAN2 plasmids[13,32]. The pCDFDuet:IRS plasmid contains key enzymes for diterpenoid precursor production, including 1-deoxy-*D*-xylulose-5-phosphate synthase (*dxs*), 1-deoxy-*D*-xylulose-5-phosphate reductase (*dxr*), and isopentenyl diphosphate isomerase (*idi*), and was shown to increase diterpenoid formation in *E. coli*[13]. The pACYC-Duet:GGPPS/ZmAN2 plasmid contains the maize *ent*-copalyl diphosphate synthase ZmAN2 and a GGPP synthase from *Abies grandis*. Enzymes catalyzing the committed reactions in dolabralexin biosynthesis were then co-transformed as plasmids pET28b:ZmKSL4 and pETDUET:ZmCPR2/ZmCYP71Z18. For details on sequences and plasmid constructs see Mafu et al. 2018[39].

A GC-MS chromatogram of the extracted enzyme products is shown in **Figure 3A**, illustrating the formation of three dolabralexin compounds, namely dolabradiene (1.2 ± 0.25 mg/L culture), epoxydolabrene (0.65 ± 0.2 mg/L culture), and epoxydolabranol (11.4 ± 1.1 mg/L culture) as quantified based on a standard curve using the diterpenoid sclareol. Sclareol was used as a reference standard, due to its similar structure and chemical properties as compared to dolabralexins. Typically observed minor byproducts include chloramphenicol, the indole derivatives oxindole and indole-5-aldehyde, and the precursor geranylgeranyl diphosphate (GGPP) (**Figure 3**). Indole commonly represents the primary byproduct, but is not shown here, due to its retention time shorter than the set solvent delay of 7 min to preserve the integrity of the GC-MS instrument.

Schematic workflow of diterpenoid production using *N. benthamiana*

Figure 4 depicts an overview of the expression of the dolabralexin pathway in *N. benthamiana*. For the products described here, the following constructs were transformed separately into *A.*

tumefaciens strain GV3101: pLife33:p19 (expressing the p19 gene silencing suppressor protein), pLife33:ZmCYP71Z18, pLife33:ZmAN2, pLife33:ZmKSL4. Full-length native sequences of the maize dolabralexin pathway genes were used in the binary T-DNA vector pLife33[41] with kanamycin resistance for propagation in *E. coli* and *A. tumefaciens.* Co-expression of upstream terpenoid pathway genes is optional, since the precursor geranylgeranyl diphosphate is endogenously formed in *N. benthamiana.* However, several studies have successfully employed such approaches to increase diterpenoid formation in *N. benthamiana*[14,36,41]. As illustrated in **Figure 3**, co-expression successfully produced dolabradiene and 15,16-epoxydolabrene. Unlike enzyme co-expression in *E. coli*, 15,16-epoxydolabranol was not detected in metabolite extracts.

Presence of 15,16-epoxydolabrene in leaf extracts demonstrated the activity of CYP71Z18 in *N. benthamiana*. As 15,16-epoxydolabranol was shown to be stable after extraction from microbial cultures (**Figure 3**) as well as after isolation from maize root tissues in previous studies[39], it appears plausible that the hydroxylated product is glycosylated by endogenous glycosyltransferases and subsequently sequestered in the vacuole, rendering it inaccessible to extraction with the organic solvent mixtures used here for extraction[36,43-46]. Similar undesired product modifications in the context of pathway engineering in *N. benthamiana* have been reported in previous studies[47]. As shown for co-expression in *E. coli*, transient expression *in N. benthamiana* results in the extraction of several byproducts, including linear alkanes of different chain length as based on comparison to reference mass spectra databases. Compound titers extracted from leaf material were found to be on average 2.4 +/- 0.5 mg dolabradiene and 0.9 +/- 0.3 mg 15,16-epoxydolabrene per g dry leaf tissue. These titers cannot be compared directly to the *E. coli* co-expression system given the different experimental set ups.

Diterpenoid purification

Diterpenoid purification was achieved using silica column chromatography and subsequent semi-preparative HPLC. Metabolite extracts from 12 L of pooled *E. coli* cultures were purified using silica column chromatography to separate the three focal dolabralexin compounds (**Figure 3A**). Silica chromatography is ideal for achieving high purity of the target compounds, since it enables simple separation of diterpene olefins and oxygenated derivatives, and readily removes the major contaminant, oxindole, which is retained on the silica matrix (**Figure 3A**).

FIGURE AND TABLE LEGENDS:

Figure 1: Workflow for diterpenoid production in *E. coli* and metabolite purification from liquid bacterial cultures. Dashed boxes depict optional steps where additional purification is required. (**A**) Representative image of extracted *E. coli* culture using a separatory funnel. (**B**) Representative image of metabolite extract purification using silica chromatography.

Figure 2: Dolabralexin biosynthetic pathway and gene constructs used in this study.

Figure 3: GC-MS results. Shown are representative GC-MS chromatograms of purified diterpenoid products obtained using enzyme co-expression assays in (**A**) *E. coli* and (**B**) *N. benthamiana*. Product identifications are based on comparisons to authentic standards and reference mass spectra of the National Institute of Standards and Technology (NIST) mass

spectral library. 1, oxindole; 2, indole-5-aldehyde; 3, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-; 4, 6-O-Acetyl-1-[[4-bromophenyl]thio]-a-d-glucoside S,S-dioxide; 5, dolabradiene; 6, 15,16-epoxydolabrene; 7, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-; 8, 15,16-epoxydolabranol; 9 and 12, unknown; 10, chloramphenicol; 11, 3,7,11,15-tetramethyl-2-hexadecen-1-ol; 13-16, alkanes.

Figure 4: Diterpenoid production in *N. benthamiana.* (**A**) Workflow of diterpenoid production in *N. benthamiana* and metabolite purification from leaf material. (**B**) Representative image of *N. benthamiana* plants ready for infiltration experiments, before pruning. (**C**) Representative images of *N. benthamiana* plants after pruning. (**D**) Image of syringe-infiltrated leaves. Darker areas have been infiltrated.

Table 1: Antibiotic concentrations for plasmid co-expression in *E. coli* or *N. benthamiana*.

DISCUSSION:

Broader investigation and application of diterpenoid natural products necessitates simple, inexpensive protocols to synthesize and purify sufficient quantities of desired compounds. The rapid increase in the number of available diterpenoid-metabolic enzymes from a broad range of species now provides an expansive inventory for the enzymatic production of diterpenoids using microbial and plant-based host systems. In addition, the modular architecture of many diterpenoid pathways enables the use of enzymes from the same or different species in 'plug & play' combinatorial engineering approaches to generate an array of natural and new nature-like diterpenoid natural products[2,14,26,35].

E. coli is a preferred microbial host for natural product biosynthesis due to its robustness, ease of scalability, limited chemical complexity for reduced byproduct contamination, and the wealth of available tools for DNA assembly and expression optimization. In our experience, the platform described here is well-suited for producing product yields of up to several hundred mg of diterpene olefins and alcohols, which is suitable for many downstream applications including those proposed here. While not meeting industrial scale, the production platform described here can serve as a foundation for further pathway, host, and fermentation optimization as has been successfully demonstrated for related diterpenoids such as taxadiene and sclareol[33,34]. Over-expression of rate-limiting MVA or MEP pathway genes has been successfully established to overcome yield-limiting factors for diterpenoid biosynthesis, such as insufficient precursor supply and precursor flux into competing pathways[13,32,33,39]. Although proven successful in several studies, poor expression and catalytic activity of terpenoid-metabolic eukaryotic P450s and other membrane-bound enzymes in *E. coli* is a likely limiting factor[33,39,48-52]. Use of codon-optimized sequences and protein modifications, such as removal of the endoplasmic reticulum signal peptide or introduction of a plastidial signal peptide, have proven useful to increase soluble P450 expression[14,38,49,50,53]. Such modifications were also employed for the microbial co-expression of maize CYP71Z18[39] used as an example pathway in this study. The protocols described are based on the use of plasmids carrying one or two genes per construct, all under the same inducible promoter. Where larger-scale gene combinations are desired, it is advisable to use various available multi-gene cassettes or gene stacking systems to mitigate reduced transformation

efficiency and culture growth due to the use of multiple plasmids and antibiotics[13].

With the broader availability of genetics and genomics resources, plant host systems also become increasingly suitable for the manufacture of natural products. Advantages include the ability of plants to produce the required natural precursors powered by photosynthesis, thus enabling product formation without the need to supplement precursor molecules[54,55]. *N. benthamiana* is already widely used for *in vivo* functional characterization and combinatorial expression of terpenoid and other natural product pathways[14,35,36,40]. Notable advantages of using *N. benthamiana* as a host system include the endogenous production of diterpenoid precursors, use of native gene sequences, simplified expression of eukaryotic P450s, ease of combinatorial gene transformation (as separate antibiotics are not required for transient co-transformation), and simple extraction of target products from leaf material. Where needed, diterpenoid production can be enhanced through co-expression of key MEP pathway genes to increase precursor supply[36,41]. Constraints for scalable diterpenoid production in *N. benthamiana* are more complex as compared to liquid microbial cultures due to the need for generating sufficient plant biomass, more labor-intensive product purification from chemically complex plant tissue, and possible undesired metabolization of target products through, for example, oxidation, glycosylation or dephosphorylation by endogenous enzymes[36,43-47]. However, this procedure can be scaled up to mg product quantities by increasing the number of plants used for agroinfiltration[56].

The product extraction and purification protocols described here are compatible with *E. coli* and *N. benthamiana*, as well as *S. cerevisiae* and other plant or microbial host systems, and provide a cost-efficient approach that is easy to set up in both biology and chemistry laboratories and does not require expensive purification equipment. Metabolite extraction using a separatory funnel is well-suited for efficient extraction and phase separation prior to chromatographic purification. Funnel sizes can be readily adjusted to allow for larger culture volumes and reduce experimental time needed to extract from large cultures. We found the use of a hexane/ethyl acetate gradient to be ideal for extracting diterpenoids of different polarity as demonstrated here for the group of dolabralexins that comprise both hydrocarbon and oxygenated compounds (**Figure 3**). Depending on the properties of target products, other solvent mixtures may be advantageous. However, solvents must not be miscible with water to ensure successful extraction and phase separation using the separatory funnel technique. In addition, product loss through evaporation must be taken into account when using this approach for producing volatile organic compounds (VOCs), such as lower molecular weight mono- and sesqui-terpenoids and other VOCs. Chromatographic separation of diterpenoids of different levels of oxygenation using a larger-scale (~2 L) silica column has been advantageous in our experience, since it provides improved product separation and minimizes the need for iterative purification steps when using smaller column volumes. Column volumes and matrices can be adjusted as needed for the desired culture volume and the type of natural product. The purity of target products that can be achieved using this protocol is suitable for many downstream applications, such as bioactivity assays or for use in enzyme activity analyses. However, where higher purity levels are required, such as structural analyses via NMR, product purity can be efficiently enhanced by additional purification using (semi)-preparative HPLC.

This protocol described here has been optimized for the production of diterpenoid natural products, but can also be readily customized to related mono-, sesqui- and tri-terpenoids, as well as other natural product classes simply by generating the desired enzyme modules for combinatorial expression[14,57]. However, modifications of the procedures for product extraction and purification must be taken into consideration for compounds with higher volatility, such as mono- and sesqui-terpenoids, or higher polarity and functional modification as exemplified by glycosylation of many triterpenoids, phenylpropanoids, and other natural product classes.

Although industrial-scale platforms for the manufacture of natural products are available, the protocols described here offer an inexpensive, customizable tool that can be readily set up in most laboratories. As demonstrated by the production of maize dolabralexins here and elsewhere[39], the product quantities and purity that can be achieved using this approach are typically sufficient to facilitate various downstream analysis and uses, including, but not limited to, various bioactivity studies, analysis of interactions between organisms, as well as for use as enzyme substrates or as starting material for semi-synthesis approaches.

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DISCLOSURES:

The authors declare they have no competing financial interests.

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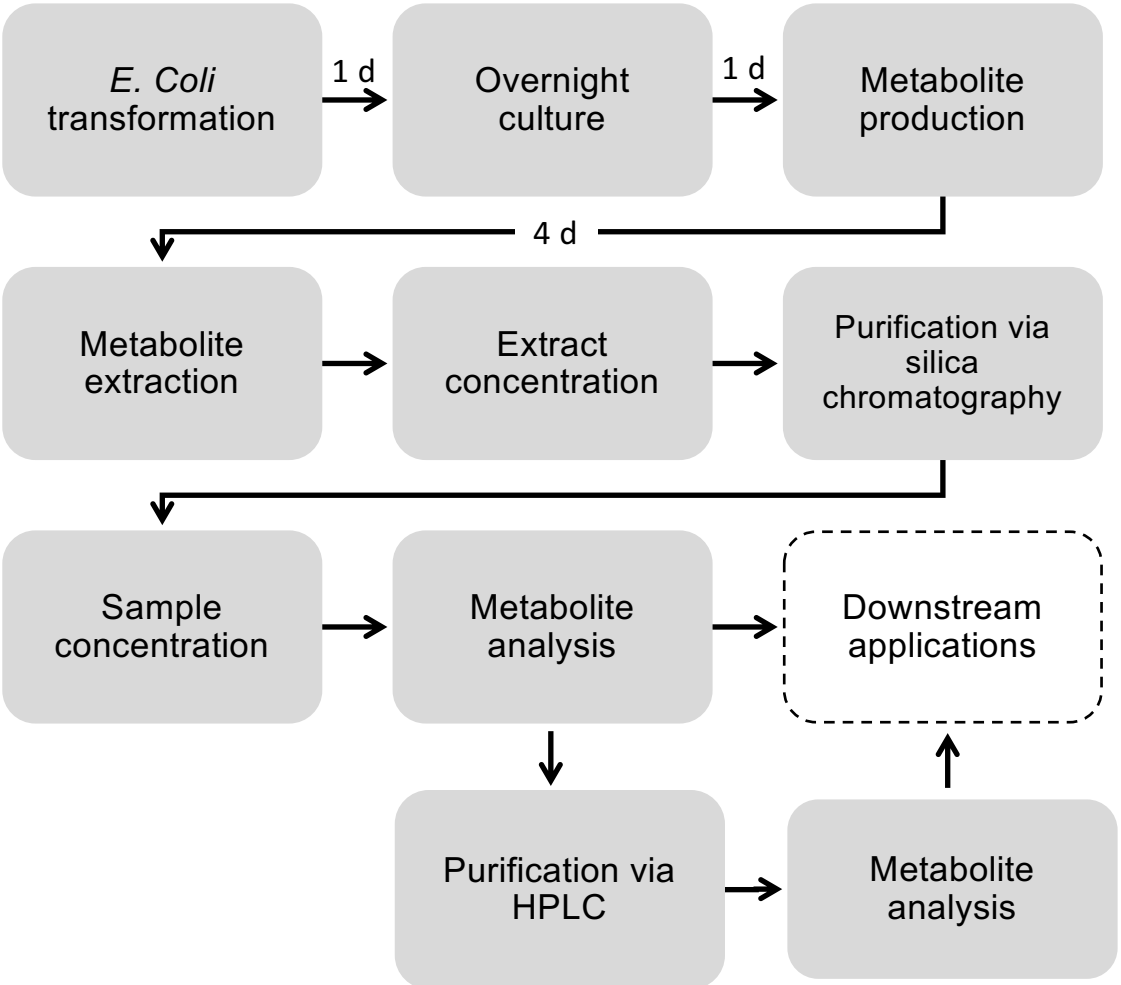
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Overnight culture
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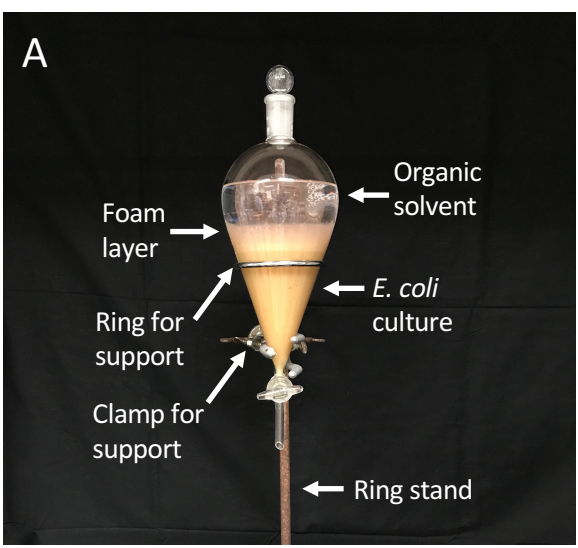
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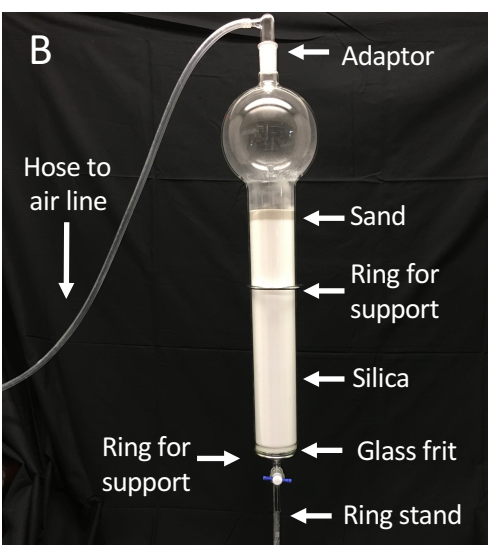
Clamp for support
Ring stand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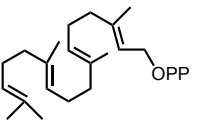

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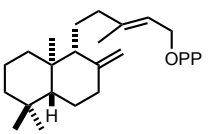

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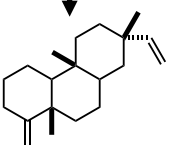
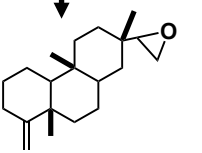


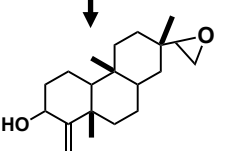
Dolabradiene

ZmCYP71Z16/18

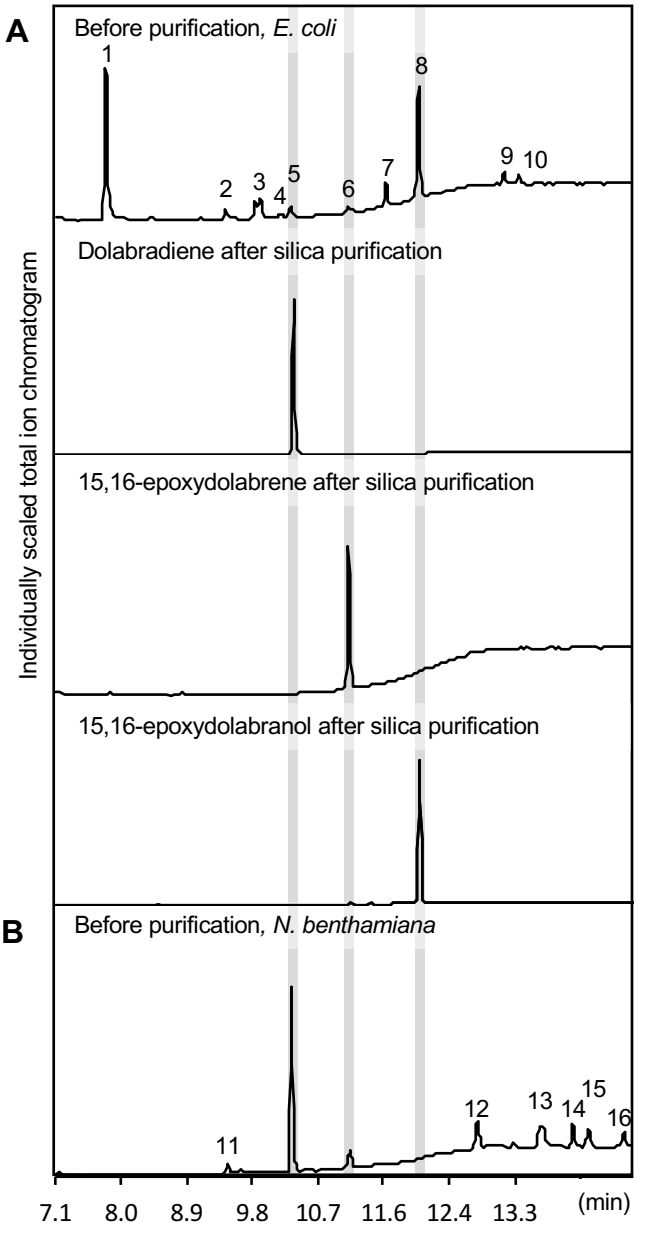


15,16-epoxydolabrene

ZmCYP71Z16/18



15,16-epoxydolabranol


A
Before purification, E. coli
1
2
3
4
5
6
7
8
9
10
Dolabradiene after silica purification
15,16-epoxydolabrene after silica purification
15,16-epoxydolabranol after silica purification
Individually scaled total ion chromatogram
B
Before purification, N. benthamiana
11
12
13
14
15
16
7.1
8.0
8.9
9.8
10.7
11.6
12.4
13.3
(min)

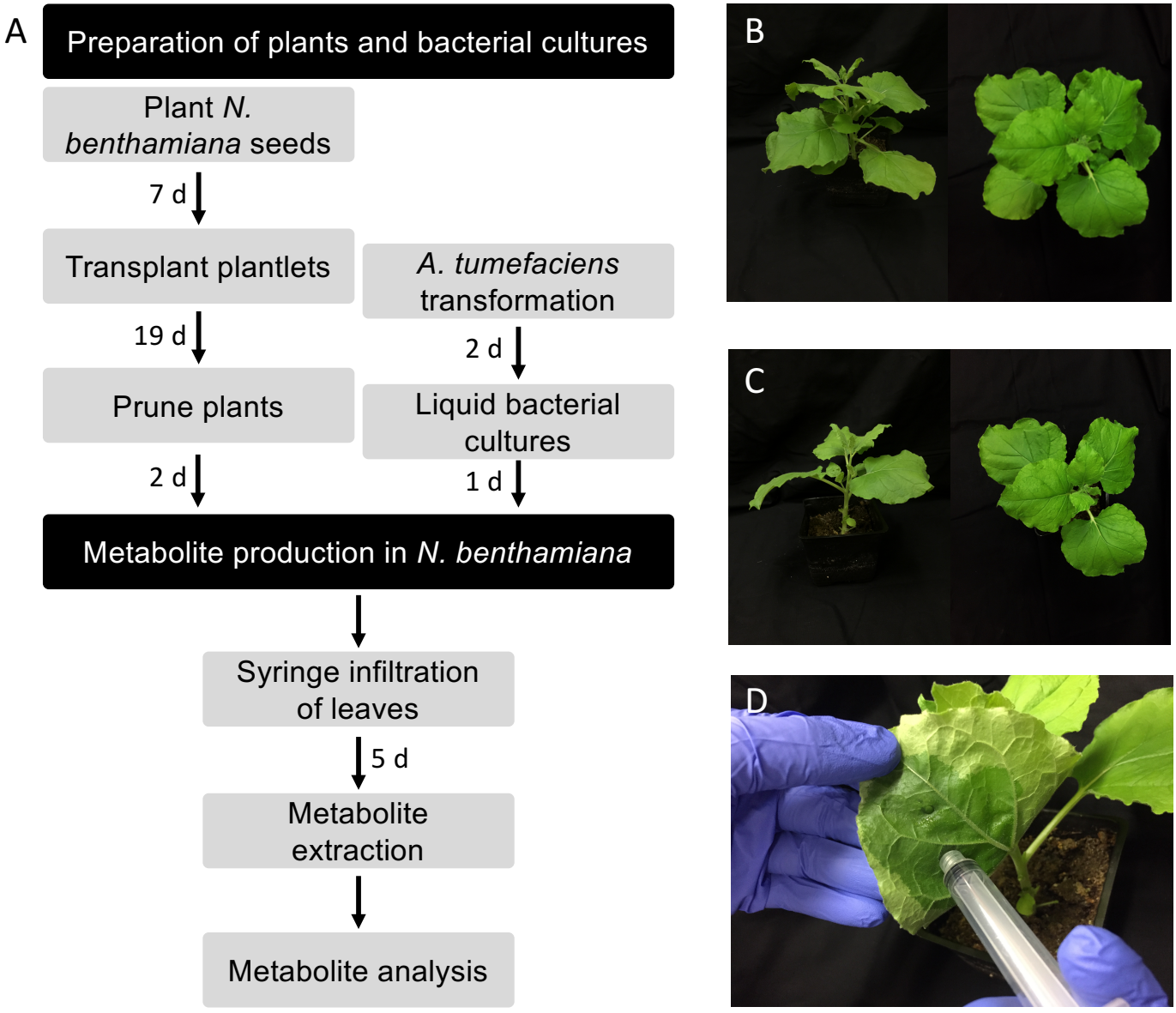

A
Preparation of plants and bacterial cultures
Plant N. benthamiana seeds
7 d
Transplant plantlets
19 d
Prune plants
2 d
A. tumefaciens transformation
2 d
Liquid bacterial cultures
1 d
Metabolite production in N. benthamiana
Syringe infiltration of leaves
5 d
Metabolite extraction
Metabolite analysis
B
C
D

Table 1

Antibiotic	Stock (mg/mL)	Solvent	Working concentration (µg/mL)		
			1 plasmid	2 plasmids	3 or 4 plasmids
Carbenicillin	50	H_2O	50	25	20
Chloramphenicol	30	EtOH	30	20	20
Kanamycin	50	H_2O	50	25	20
Spectinomycin	30	H_2O	30	25	20
Gentamycin	50	H_2O	30		
Rifampicin	10	MeOH	10		

Table of Materials

Click here to access/download;Table of Materials;JoVE59992_Table_of_Materials.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
1020 Trays	Greenhouse Megastore	CN-FLHD	
2-(N-morpholino)ethanesulfonic	Sigma	M8250-500g	MES
4" Tech Square Pot	McConkey Wholesale Grower's Supply	JMCTS4	
5977 Extractor XL MS	Agilent	-	
7890B GC	Agilent	-	
Acetonitrile	Sigma	271004	
Agar	Fisher	BP1423-2	
Bacterial yeast extract	Fisher	BP9727-2	
Beaker	CTechGlass	BK-2001-015B	
Cap, 9 mm blue screw, PFTE	Agilent	5185-5820	GC vial cap
Carbenicillin	Genesee	25-532	Carb
Chloramphenicol	Fisher	50247423	Chlor
Chromatography column	CTechGlass	CL-0015-022	
Clear humidity dome	Greenhouse Megastore	CN-DOME	
ColiRollers Plating Beads	Sigma	71013	Glass beads
CoorsTek Porcelain Mortars	Fisher	12-961A	mortar
CoorsTek Porcelain Pestles	Fisher	12-961-5A	pestle
Delta-Aminolevulinic acid hydrochloride	Sigma	50981039	Aminoleuvolinic acid
Ethanol	Fisher	A962-4	EtOH
Ethyl acetate	Fisher	E1454	
Falcon 50 mL Conical Centrifuge Tubes	Fisher	14-432-22	Falcon tubes
Fisherbrand Disposable Cuvettes	Fisher	14-955-127	cuvette
Fisherbrand Petri Dishes with Clear Lid	Fisher	FB0875713	petri dish

Fisherbrand Polypropylene Microtube Storage Racks	Fisher	05-541	microtube rack
Glucose	Sigma	G7021	
Glycerol	Fisher	G33-500	
Hexanes	Fisher	H292-4 (CS)	
HP-5MS	Agilent	19091S-433	GC column
Inlet adapter	CTechGlass	AD-0006-003	glass inlet adapter
Isopropyl β-D-1-thiogalactopyranoside	Fisher	BP1755-100	IPTG
Kanamycin	Fisher	BP9065	Kan
KIM-KAP Caps, Disposable, Polypropylene, Kimble Chase	VWR	60825-798	breathable test tube lids
Magnesium chloride	Acros	223210010	$MgCl_2$
Magnesium sulfate	Sigma	M7506-500g	$MgSO_4$
Miracle-Gro Water Soluble All Purpose Plant Food	Miracle-Gro	2756810	
Mixer Mill MM 200	Retsch	20.746.0001	tissue mill
Nalgene Fernbach culture flask	Sigma	Z360236	2.8 L flask
New Brunswick I26	Eppendorf	M1324-0000	Shaking incubator
Nicotiana benthamiana seed	USDA Germplasm Repository	Accession TW16	*N. benthamiana*
OverExpress C41(DE3) Chemically Competent Cells	Lucigen	60442	C41-DE3 cells
Parafilm M wrapping film	Fisher	S37440	Parafilm
Potassium chloride	Sigma	P-9541	KCl
Potassium phosphate dibasic	Fisher	P288-3	Dipotassium phosphate
Potassium phosphate monobasic			Monopotassium
rimless	Sigma	CLS9944516	test tubes
Pyruvate Acid Sodium Salt	Fisher	501368477	Sodium pyruvate
Retort Ring Stands	CTechGlass	ST00	ring stand

Riboflavin	Amresco	0744-250g	
Rifampicin	Sigma	R7382	Rif
Rotovap			
Sand, 50-70 mesh particle size	Sigma	274739-1KG	
Silica	Fisher	AC241660010	silica gel
Sodium chloride	Fisher	5271-3	NaCl
Sodum hydroxide	Fisher	SS266-1	NaOH
Spectinomycin	Fisher	501368607	Spec
Squibb Separatory Funnel	CTechGlass	FN-1060-006	Separatory funnel
Sunshine Mix #1	Sungro Horticulture		Potting soil
Thermo Scientific Snap Cap Low Retention Microcentrifuge Tubes	Fisher	21-402-902	microtube
Triangle funnel	CTechGlass	FN-0035	funnel
Tryptone	Fisher	BP14212	
Vial, screw, 2 mL, amber, WrtOn	Agilent	5182-0716	GC vial
visible spectrophotometer, V-1200	VWR	634-6000P	spectrophotometer
ZORBAX Eclipse XDB-C18	Agilent	990967-202	HPLC column
ZORBAX Eclipse XDB-CN	Agilent	990967-905	HPLC column

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Author(s):	Katherine M. Murphy, Siwon Chung, Shruti Fogla, Hana B. Minsky, Karen Yong Zhu, Philipp Zerbe

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Signature:	[signature]
Date:	3/4/2019

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Dear Dr. Steindel,

On behalf of all authors, I would like to thank you and the reviewers for their detailed review of our manuscript and the overall positive evaluation of our work. To improve the manuscript in response to the reviewers' recommendations, we have carefully revised our manuscript as outlined below in the specific comments sections.

Sincerely,
Philipp Zerbe

Editorial comments:

General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Author's response: We have carefully revised our manuscript to remove any spelling and grammar errors.

2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

Author's response: The manuscript has been formatted accordingly.

Protocol:
1. The structure of the protocol section is a bit confusing-it looks like there is a preparation section (1) and 3 broad procedures-production of diterpenoid metabolites in E. coli, purification of diterpenoid metabolites, and production of diterpenoid metabolites. If that is the case, please make those sections number 2, 3, and 4 in the protocol and change the remainder to sub-sections (e.g., the current section 3 would become 2.2, with the substeps 2.2.1-2.2.4).

Author's response: The individual protocol numbering has been revised as suggested by the reviewer.

2. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Author's response: We have carefully addressed each step in the manuscript to have no more than 2-3 actions or four sentences per step. We have added notes into substeps to improve the readability.

Figures:
1. Please number figures in the order they are cited in the text-currently, Figure 4 is cited before Figures 2 and 3.

Author's response: The figure order and numbering has been corrected.

References:
1. Please do not abbreviate journal titles.

The references have been edited to include full journal titles.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Author's response: The table of materials has been updated to include all material and equipment in the manuscript.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The authors describe a scalable approach to generate and purify diterpenes from the heterologous host E. coli and Nicotiana benthamiana. The Manuscript describes the necessary steps involved very well and detailed and will allow the reader to repeat the protocol for a diterpene molecule of their choosing.

Major Concerns:
I do not have any major concerns.

Minor Concerns:
The authors focus on diterpenes, however in principle the method could also be used for terpenes with higher carbon numbers or Sesquiterpenes, with the later the only limit should be volatility of the products. The manuscript might benefit from a short section in the discussion that would point to this.

Author's response: We thank the reviewer for the very positive overall evaluation and helpful critique of our paper. In response to their important addition of the applicability of this method, we have added a paragraph to the discussion section detailing how this method could be applied to other specialized metabolites, including sesquiterpenes, and the limitations therein.

Reviewer #2:

Manuscript Summary:

Plant secondary metabolites or natural products have immense value to humans due to their economic importance as pharmaceuticals, food additives, biofuels and other bioproducts. However, efficient production of important natural products is constrained by their low abundance and limited distribution. In this manuscript, authors demonstrate an easily operated and customizable protocol for the enzymatic production of important diterpenoids in both microbial (Escherichia coli) and plant (Nicotiana benthamiana) host systems using combinations of maize diterpene synthase (diTPS) and cytochrome P450 monooxygenase (P450) enzymes. Further, they show that the compounds produced from both systems can be purified in reasonable amounts. Finally, they propose that this method can be efficiently adopted for overproduction of different diterpenoid scaffolds that can be used in various downstream applications. Overall, the methodology is presented in detail and would be very useful for the researchers working on secondary metabolism.

Author's response: We appreciate the reviewer's critical review of our work and have taken the reviewer's suggestions under serious consideration.

Major Concerns:

Scalable means what?. Is it adoptable for industrial application?

Author's response: The described protocol is designed for laboratory-scale diterpenoid production with compound yields in the milligram range that are suitable for many different downstream analyses. While upscaling of the described expression system to achieve industrial production levels can be envisioned it would require larger-scale infrastructure for microbial cultures or plant cultivation and further system/host optimization. We have clarified this in the discussion section.

Minor Concerns:

Line 173: It is not clear regarding the TPS and CYP constructs designed for this specific work. More detail about their source is required.

Author's response: Additional details on the specific constructs used in this study have been included.

Line 177 to 296: Why the text is highlighted in yellow?

Author's response: This text is highlighted to denote the portion of the manuscript to be filmed for the JoVE video, as requested in the journal's author guidelines.

Line 182: "Add 1 µL of each construct" - specify the concentration of DNA used.

Author's response: DNA concentrations used are now included.

Line 487: Isn't the OD of 1 too much?

Author's response: In our experience using the described experiments an OD of 1 leads to effective Agrobacterium infiltration, as demonstrated by our results here. Optimal OD levels might vary across different Agrobacterium strains.

Line 499: "5 days" - how this duration was arrived, was it standardized?. Some protocols say that secondary metabolites are better produced in much longer incubations.

Author's response: In our experience optimizing the described protocols, an incubation time of five days was sufficient to reach product levels for downstream analysis. While longer incubation times might further increase product yield, it also extends the time needed for product synthesis. We have addressed this in the manuscript.

Line 548: "Figure 4A" - should be Figure 3A.

Author's response: The figure numbering has been corrected.