A cell-free platform for the prenylation of natural products and application to cannabinoid production

Meaghan A. Valliere1, Tyler P. Korman1, Nicholas B. Woodall2, Gregory A. Khitrov1, Robert E. Taylor1, David Baker2 & James U. Bowie1

Prenylation of natural compounds adds structural diversity, alters biological activity, and enhances therapeutic potential. Because prenylated compounds often have a low natural abundance, alternative production methods are needed. Metabolic engineering enables natural product biosynthesis from inexpensive biomass, but is limited by the complexity of secondary metabolite pathways, intermediate and product toxicities, and substrate accessibility. Alternatively, enzyme catalyzed prenyl transfer provides excellent regio- and stereo-specificity, but requires expensive isoprenyl pyrophosphate substrates. Here we develop a flexible cell-free enzymatic prenylating system that generates isoprenyl pyrophosphate substrates from glucose to prenylate an array of natural products. The system provides an efficient route to cannabinoid precursors cannabigerolic acid (CBGA) and cannabigerovarinic acid (CBGVA) at >1 g/L, and a single enzymatic step converts the precursors into cannabidiolic acid (CBDA) and cannabidivarinic acid (CBDVA). Cell-free methods may provide a powerful alternative to metabolic engineering for chemicals that are hard to produce in living organisms.

1 Department of Chemistry and Biochemistry, Molecular Biology Institute, UCLA-DOE Institute, University of California, Los Angeles 90095 CA, USA.
2 Department of Biochemistry, Institute for Protein Design, University of Washington, Seattle 98105 WA, USA. Correspondence and requests for materials should be addressed to J.U.B. (email: bowie@mbi.ucla.edu)
Prenylated natural products are a large class of bioactive molecules with demonstrated medicinal properties. Examples include prenyl-flavonoids, prenyl-stilbenoids, and cannabinoids (see Fig. 1). Cannabinoids in particular show immense therapeutic potential with over 100 ongoing clinical trials as antiemetics, anticonvulsants, antidepressants, and analgesics. Nevertheless, despite the therapeutic potential of prenyl-natural products, their study and use is limited by the lack of cost-effective production methods. Plant-derived prenyl compounds are difficult to isolate due to the structural similarity of contaminating molecules, and the variable composition between crops. These challenges are further exacerbated when attempting to isolate low abundance compounds. Many chemical syntheses have been developed to address the challenges associated with making prenylated natural products, but they are generally impractical for drug manufacturing due to the degree of complexity and low yields.

Microbial production is a useful alternative to natural extraction for prenylated natural products, but comes with many challenges such as the need to divert carbon flux from central metabolism and product toxicity to name a few. For example, prenyl-natural products like prenylvanilin, prenly-resveratrol, and cannabidiol (CBD) are derived from a combination of the metabolic pathways for fatty acid, isoprenoid, and polyketide biosynthesis. So, high-level production requires efficient rerouting of long, essential and highly regulated pathways. Despite the challenges, many groups have engineered microbes to produce unprenylated polyketides, like naringenin, resveratrol, and cannabinoids (see Fig. 1). Cannabinoids in particular show immense therapeutic potential with over 100 ongoing clinical trials as antiemetics, anticonvulsants, antidepressants, and analgesics. Much recent effort has focused on alternative methods for cannabinoid production. Two groups have produced the polyketide cannabinoid intermediate, olivetolic acid (OA) at low levels in yeast (0.5 mg/L) or E. coli (80 mg/L), but did not prenylate OA or produce a cannabinoid from the biosynthesized OA. In other work, tetrahydrocannabinolic acid was produced in cell extracts from either exogenously added GPP and OA in a two enzyme pathway or from cannabigerolic acid (CBGA) using a single enzyme. However, it is unclear how GPP or CBGA could be obtained at sufficient levels for economical production due to the high cost of these molecules.

Here, we propose an alternative biological approach to prenylated natural product biosynthesis using a cell-free enzymatic platform we call synthetic biochemistry, which has shown great promise for the production of bio-based molecules. The synthetic biochemistry approach frees us from worrying about the toxicity of products and intermediates, affords rapid design-build-test cycles, precise control of all system components, and complete flexibility in pathway design. Nevertheless, building highly complex systems involving dozens of enzymes, associated cofactors and myriad metabolites on a large scale outside the context of the cell is an enormous challenge. One of the keys to making commercially viable cell-free systems is reducing enzyme costs by employing stable enzymes that can last for long periods of time. Recently, Zhang and co-workers converted maltodextrin into inositol at a 20,000 L scale in a five enzyme system using thermophilic enzymes purified by simple heating step, demonstrating that at least simple cell-free systems can reach industrial scale. Another key requirement is designing systems that effectively generate and recycle high energy cofactors (ATP, NAD(P)H) so that they can be used many times. We have previously reported a flexible enzymatic purge valve and rheostat for regulating the supply of reducing equivalents and ATP, allowing us to build systems that run for many days and produce high titers of isobutanol and terpenes. Here, we employ these concepts to develop cell-free production of a variety of prenylated compounds. We use glucose as a feedstock to produce GPP and optimize the system for the high-titer production of the cannabinoid compounds CBGA and cannabigerovarinic acid (CBGVA).
Results
Construction of the cell-free prenylation pathway. Our synthetic biochemistry approach is outlined in Fig. 1 (detailed in Supplementary Figure 1) and expands on a system we developed previously for terpene production26. First, glucose is broken down via a modified glycolysis pathway to produce high-energy cofactors ATP and NADPH in addition to the carbon building block, acetyl-CoA using an alternative pyruvate oxidation pathway26. The acetyl-CoA is then assembled into the prenyl-donor compound, GPP, via the mevalonate pathway using the ATP and NADPH produced from glycolysis. Importantly, a purge valve26 introduced into the glycolysis pathway balances NADPH production and consumption while maintaining carbon flux. The prenylation module then uses the GPP to prenylate exogenously added substrate to yield the desired prenylated product. To expand the capabilities of our synthetic biochemistry platform we developed a prenylating system that employs a nonspecific prenylating enzyme such as NphB, AtaPT, or NovQ to produce an array of prenyl-compounds derived from glucose29–31. We then further engineered NphB using Rosetta to specifically prenylate OA.

As a first test of the system, we built the full cell-free system (23 enzymes) to generate GPP from glucose and employed wild-type NphB to prenylate its preferred substrate 1,6 dihydroxynapthalene (1,6 DHN; added exogenously). 1,6 DHN was added at the beginning of the reaction along with glucose. Up to ~400 mg/L (1.3 mM) of prenylated product was obtained from 2.5 mM 1,6 DHN. However, increasing the 1,6 DHN concentration from 2.5 to 5 mM, decreased final titers ~2-fold suggesting that 1,6 DHN inhibited one or more enzymes (Fig.2a). Enzyme assays revealed that pyruvate dehydrogenase (PDH) was inhibited by 1,6 DHN, as well as olivetol, resveratrol, and olivetolate (Fig. 2b). Therefore, to engineer a general prenylation system, we sought to eliminate PDH.

To remove the need for PDH, we implemented a PDH bypass (Fig. 1). In the PDH bypass, pyruvate is converted to acetyl-CoA using a pyruvate oxidase (PyOx) to produce acetyl-phosphate.
followed by the action of acetyl-phosphate transferase (PTA). The PDH bypass has two advantages. First, PDH is a large enzyme complex that is difficult to work with, so bypassing PDH streamlines enzyme production. More importantly, initial experiments revealed that the bypass is not subject to the inhibition seen at higher concentrations of 1,6 DHN. Once we confirmed the PDH bypass improved 1,6 DHN titers, we began to optimize the system as a general prenylation system. We varied co-factor concentrations, protein levels, and environmental conditions such as temperature and pH to identify the ideal set of conditions. Throughout this process we found that ATP, NADP as temperature and pH to identify the ideal set of conditions.

For CBGA production, we undertook a systematic redesign of NphB. NphB is the only known functional CBGA synthase, and is responsible for the formation of CBGA from OA and GPP. The first step in the reaction is the conversion of OA to geranyl ester, followed by the action of acetyl-phosphate transferase (PTA). The PDH bypass has two advantages. First, PDH is a large enzyme complex that is difficult to work with, so bypassing PDH streamlines enzyme production. More importantly, initial experiments revealed that the bypass is not subject to the inhibition seen at higher concentrations of 1,6 DHN. Once we confirmed the PDH bypass improved 1,6 DHN titers, we began to optimize the system as a general prenylation system. We varied co-factor concentrations, protein levels, and environmental conditions such as temperature and pH to identify the ideal set of conditions. Throughout this process we found that ATP, NADP as temperature and pH to identify the ideal set of conditions.

In order to improve CBGA production through NphB redesign, we decided to focus on the previously identified hot-spot residues. The residues at positions 271, 286, and 288 appeared to be critical for the activity of NphB. We therefore, sought to improve CBGA production by enhancing the activity and specificity of NphB by design.

Briefly, OA was docked into the active site of the NphB crystal structure (Fig. 3a)32, then Rosetta was used to predict mutations that would improve OA binding. We narrowed the Rosetta results to a 22 construct library (see Supplementary Tables 2 and 3), and screened for CBGA production (Supplementary Table 4). We made several key observations during the initial screen, shown in Supplementary Figure 2: (1) Y288A (M1) and Y288N (M2) by themselves dramatically enhanced activity, as predicted by computation; (2) the presence of Y288N in any construct decreased the enzyme yield suggesting Y288N may be a destabilizing mutation (making Y288A the preferred mutation); (3) the addition of G286S in the Y288N (M10) background appeared to improve activity further over Y288N (M2), suggesting that G286S could be another favorable mutation; (4) we noted an activity improvement of Y288A/F213N/A232S (M15) over Y288A/F213N (M5) suggesting that A232S may also be a favorable mutation. From these initial observations we constructed a focused library with all but one of the constructs in the second library exhibiting activity at least 100-fold higher than WT NphB in an endpoint assay (Fig. 3b). The best two constructs, M23 and M31, exhibited dramatically improved activity and specificity. Both had kcat values 1000-fold higher than WT NphB and both produce only the correct prenylated isomer, CBGA. As shown in Fig. 3c, WT NphB produces CBGA, but the dominant product is a prenylated side-product, 2-O-geranyl olivetolate, whereas M23 makes CBGA almost exclusively. Overall, the designed enzyme is a much more active and specific CBGA synthase than WT NphB, and is easier to work with than the natural cannabis prenyltransferase, which is an integral membrane protein16,33. Our soluble, CBGA synthase (M23) could potentially be applied in both cell-free and in vivo systems to improve cannabinoid production.

**Redesign of NphB to improve CBGA synthesis.** Although the system produced CBGA, there were two problems. First, the initial reaction rate of the prenyltransferase NphB for CBGA production is extremely poor (kcat = 0.0021 ± 0.0008 min−1, Supplementary Table 5). Second, prenylation of OA by NphB is highly nonspecific, generating a major side-product, 2-O-geranyl olivetolate16. We therefore, sought to improve CBGA production by enhancing the activity and specificity of NphB by design.

**Improved production of cannabinoids.** With our designed CBGA synthase in hand (M23), we tested the ability to produce CBGA directly from glucose and OA using the full synthetic biochemistry system, including the PDH bypass (Fig. 1). The

**Fig. 3 Engineering NphB to improve CBGA production.** a A model of olivetolate in the active site of WT NphB (1ZB6). Residues highlighted in yellow and purple were allowed to vary during the design process. The residues in yellow had the largest effects on activity with OA and were the positions targeted in the focused library. b The results of an activity assay to determine the approximate activity of NphB mutants with olivetolate as the substrate. The fold improvement is an average of triplicate reactions with GPP (2.5 mM), olivetolate (5 mM), MgCl2 (5 mM), and 1 mg/mL of WT or mutant NphB (biological replicates, n = 3, error range is standard deviation). c GC-MS chromatograms of the full pathway reaction products using M23 or WT NphB compared to a CBGA standard. Source data for Fig. 3b, c are provided as a Source Data file.
initial productivity of the system using M23 was 67 mg L\(^{-1}\) h\(^{-1}\) with a final titer of 744 ± 34 mg/L CBGA—100-fold faster and 21-fold higher titer than CBGA production using WT NphB (Fig. 4a). We noted that with the mutant NphB enzyme, maximum titers were reached within 24 h, after which production spontaneously stopped. In contrast, the system with the wild-type enzyme ran continuously for up to 4 days, suggesting enzymes and cofactors remain active and viable for longer periods of time, consistent with prior work\(^{26}\). So what is stopping the reaction at the higher titers? We observed that reactions turned cloudy once ~500 mg/L CBGA was produced. We collected the precipitate and identified a mix of enzymes in the precipitate by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Supplementary Figure 3), indicating high-levels of CBGA in solution may be causing enzymes to precipitate. We, therefore, sought to continually remove the product in situ during the reaction (a capability difficult to implement in living systems).

Initially a fixed volume nonane overlay was used for each reaction to extract CBGA. Unfortunately, CBGA is more soluble in water than nonane, limiting the amount of CBGA that can be extracted with a simple overlay. We therefore designed a flow system that would capture CBGA from the nonane layer and trap it in a separate buffered reservoir (Fig. 4b). By implementing this flow system we hoped to maintain a lower concentration of CBGA in the reaction vessel to mitigate enzyme precipitation. The flow system indeed improved the final titers to 1.25 ± 0.07 g/L, however, enzyme precipitation still occurred at about 24 h.

We next evaluated the system flexibility by replacing OA with diarvinic acid (DA) to produce the precursor of many rare cannabinoids, CBGVA. We first tested whether our designed enzymes would be active with DA as the substrate. Kinetic analysis (Table S5) indicated that M31 effectively prenylates DA, with catalytic efficiencies 15-fold higher than M23 and 650-fold higher than WT NphB. We, therefore, utilized M31 to produce CBGVA from glucose and DA. As shown in Fig. 4a, CBGVA was produced at a maximum productivity of ~107 mg L\(^{-1}\) h\(^{-1}\) and reached a final titer of 1.74 ± 0.09 g/L, converting 92% of the added DA to CBGVA. The nonane-flow system was not needed for the production of CBGVA because CBGVA was less potent in precipitating enzymes.

**Production of additional cannabinoids.** To illustrate the production of other cannabinoids from the central cannabinoids CBGA and CBGVA, we employed CBDA synthase to convert CBGA into CBDA and CBGVA into CBDVA. Conversion of CBGA into CBDA has been demonstrated by several groups\(^{17,34-36}\). In our case, we simply transferred the nonane overlay containing CBGA to an aqueous solution containing CBDA synthase, and indeed we were able to convert CBGA into CBDA at a constant rate of 14.4 ± 0.8 mg L\(^{-1}\) h\(^{-1}\) mg total protein\(^{-1}\) over the course of 4 days converting 25% of the CBGA added to CBDA (Fig. 4c). To our knowledge it is not known whether CBGVA can be converted into the rare cannabinoid CBDVA using the CBDA synthase. So we added CBGVA, extracted from the cell-free system, to a reaction containing CBDA synthase. CBDA was produced (Supplementary Figure 4) by CBDA synthase at a rate of 7.1 ± 0.1 mg L\(^{-1}\) h\(^{-1}\) mg total protein\(^{-1}\) for 24 h. We note that the cannabinoid acids can undergo spontaneous decarboxylation or heat induced decarboxylation to ultimately form additional bioactive cannabinoids CBD and cannabidivarin (CBDV). Thus, our system provides opportunities for ultimately producing a wide-variety of cannabinoids.

**Discussion**

Our results demonstrate the power and flexibility of a cell-free approach, not only for the production of pure, therapeutically relevant cannabinoids and other prenylated natural products, but...
for bio-derived chemicals in general. Freedom from worries about cell viability allowed us to focus on pathway optimization rather than minimizing GPP toxicity, while the lack of a cell membrane barrier freed us to design a system with added aromatic molecules, which would not be possible in cells. Moreover, we could flexibly change the input from OA to DA to target rare cannabinoids without redesigning an entire pathway. Finally, it was straightforward to identify and focus our efforts on fixing the bottleneck steps. When we started this project we were only able to produce 9 mg/L of CBGA using the monoterpene pathway developed by Korman et al.25, 26. By introducing the PDH bypass and optimizing for cofactors, enzymes and environmental factors we were able to increase those titers to 132 mg/L. To improve titers further we engineered the NphB prenyltransferase, which further increased titers to 600 mg/L of CBGA. The final bottleneck was enzyme stability in the presence of CBGA, so by limiting the CBGA in the reaction vessel, we increased the titer to 1.25 g/L of CBGA, nearly a 140-fold improvement. Solutions were quickly implemented due to speedy design-build-test cycles, quickly yielding results that far exceed published results using 1 mg/L of CBGVA, respectively) were altered slightly. Optimization of the cannabinoid pathway showed that the same titers could be achieved with less glucose, so we reduced the glucose concentration to 150 mM (we did not test lower glucose concentrations). Additionally, increasing the NADP+ concentration to 6 mM and decreasing the ATP concentration from 1 mM led to higher titers. The oligovolate concentration was set at 5 mM. The amount of NphB added to the reaction was variable. The data shown in Fig. 2c utilized 1.5 mg/mL NphB, and the reactions were quenched at ~4, 8, 14, 24, 48, 72, and 96 h. The data shown in Fig. 4a was achieved with 0.5 mg/mL of WT NphB and M23 and M31 (for dianotic acid) and reactions were quenched at ~6, 9, 12, 24, 48, 72, and 96 h.

The conditions were identical to the method above with the following exceptions, the final concentration of the aromatic substrates was 1 mM and the initial glucose concentration was 150 mM. Additionally, the final concentration of the prenyl-transferase was 1 mg/mL, and we tested AtaPt, NodQ, and NphB with apigenin, daidzein, genistein, and resveratrol. We also tested NphB with olivetol, olivetolate, and 1,6 DHN. The reactions were quenched at 24 h.

### Methods

**Chemicals and reagents.** Yeast hexokinase and Corynebacterium glutamicum catalase were purchased from Sigma Aldrich. *Aerococcus viridians* pyruvate oxidase was purchased from A.G. scientific. All cofactors and reagents were purchased from either Sigma Aldrich or Thermo Fisher Scientific, with the exception of OA, which was purchased from Santa Cruz Biotechnology and dianotic acid, which was purchased from Toronto Research Chemicals.

**Cloning and purification of enzymes.** The NphB gene was purchased as a gene block from IDT DNA, and cloned into a pET 28(+) vector using the Gibson Assembly method. The gene block sequences were amplified in Supplementary Table 1. The remaining enzymes were amplified from genomic DNA or a plasmid, and cloned into pET28(+) using the same Gibson assembly method. The primer sequences used for amplification in this work are listed in Supplementary Table 6. All plasmid stocks were transformed into BL21 (DE3) Gold, and enzymes expressed in LB media with 50 µg/mL kanamycin. One litre cultures were inoculated in a 3 mL of saturated culture in the same media, and grown to an OD600 of 0.5 at 37 °C. The cultures were induced with 1 mM IPTG, and expressed at 18 °C for 16 h. The cells were harvested by centrifugation at 20,000×g and resuspended in ~20 mL lysis buffer: 50 mM Tris pH 8.0, 150 mM NaCl, and 10 mM imidazole. The cells were lysed using an Emulsiflex instrument. The lysate was clarified by centrifugation at 20,000×g, and the supernatant was batch bound to 1 mL NiNTA resin for 30 min at 4 °C. The resin was transferred to a gravity flow column. The column was washed with 10 column volumes of wash buffer: 50 mM Tris [pH 8.0], 150 mM NaCl, and 10 mM imidazole. The protein was then eluted with 2 column volumes of elution buffer: 50 mM Tris [pH 8.0], 150 mM NaCl, 250 mM imidazole, and 30% (v/v) glycerol. Enzymes were then frozen in elution buffer using liquid N2, and the enzyme stocks were stored at −80 °C.

**PDH cell-free reactions.** The PDH reactions were assembled in two parts. First the cofactors and substrates were combined in one tube, and the enzymes were combined in another. The reactions were initiated by mixing the enzymes and aliquots of the final volume of 200 µL. The reactions were set up as follows: 500 mM glucose, 1 mM 1.6 fructose bisphosphoglucone, 4 mM ATP, 0.5 mM 2,3 bisphosphoglycerate, 0.5 mM NAD+, 1.5 mM CoA, 3 mM mM NADP+, 0.5 mM TPP, 6 mM MgCl2, 10 mM KCl, 50 mM Tris, and 50 mM phosphate buffer [pH 8.0]. The amount of enzyme added to each reaction is detailed in Supplemental Table 1. The cofactors and enzymes were mixed to initiate the reaction, and a 500 µL nanomeasure overlay was added to the top. The reactions were incubated at room temperature shaking gently on a gel shaker.

When the aromatic substrate was the varied component 0.5–5 mM of the aromatic substrate was added to the reaction, and the reactions were quenched at 24 h. When time was the varied component, 5 mM of 1,6 DHN was added, and separate reactions were quenched at ~12, 24, 48, and 72 h.

The conditions for the oligovolate were divergent from the conditions for CBGVA and CBGVA, respectively) were altered slightly. Optimization of the cannabinoid pathway showed that the same titers could be achieved with less glucose, so we reduced the glucose concentration to 150 mM (we did not test lower glucose concentrations). Additionally, increasing the NADP+ concentration to 6 mM and decreasing the ATP concentration from 1 mM led to higher titers.

**Quantification of products.** The reactions were fractionated by reverse phase chromatography on a C18 column (4.6 x 100 mm) using a Thermo Ultimate 3000 HPLC. The column compartment temperature was set to 40 °C, and the flow rate was 1 mL/min. The compounds were separated using a gradient elution with water +0.1% TFA (solvent A) and acetonitrile +0.1% TFA (solvent B) as the mobile phase. Solvent B was held at 20% for the first min. Then solvent B was increased to 95% B over 4 min, and 95% B was then held for 3 min. The column was then re-equilibrated to 20% B for 3 min, for a total run time of 11 min. The cannabinoid samples (CBGA, CBDA, and CBDV) were quantified using an external calibration curve derived from an analytical standard purchased from Sigma-Aldrich. The 5-p-1,6-DHN and CBGVA, nuclear magnetic resonance (NMR) samples were used to generate an external calibration curve because authentic standards were not available (see below). A known concentration of the standard was dissolved in water, and then extracted using the method detailed above.

**Quantify prenyl-products without authentic standards.** Due to the lack of authentic standards for the prenyl-products prenyl-apigenin, prenyl-daidzein, prenyl-naringenin, prenyl-genistein, prenyl-resveratrol, and prenyl-olivetol, we developed a method on substrate-attached standard to generate a standard curve, serial dilutions of each aromatic substrate were subjected to the reaction mix, but to prevent product formation the prenyl-transferase was left out.
We used liquid chromatography–mass spectrometry to quantify the amount of substrate consumed by the reaction compared to the standard curve. We used liquid chromatography–mass spectrometry to quantify the amount of substrate consumed by the reaction compared to the standard curve.

NMR spectroscopy. NMR spectroscopy was used to identify prenyl-products, and quantify $5\cdot$1.6-DHN. The PyOx/PTA cell-free system was used to produce prenyl-DHN. A total of 200 µL reactions were pooled, and extracted three times with an equivalent amount of methanol and then the nonane was evaporated. The product of the reactions was suspended in 500 µL of deuterated methanol (CD$_3$OD), with 2 mM 1,3,5-trimethoxybenzene (TMB) as an internal standard. Spectra were collected on an AV400 Bruker NMR spectrometer. The amount of the prenylated compound in the sample was determined with reference to the internal TMB standard. We compared the proton signal from TMB (3H, singlet) at 6.05 ppm with an aromatic proton corresponding to 5-1.6-DHN (1H, doublet) at 7.27 ppm.

NMR was also used to identify the product of the enzymatic system with divarinic acid as the aromatic substrate. The PyOx/PTA system was set up as described above, and reactions were run with the substrate at 24h. The reaction was allowed to react for 1 h, and then the nonane was evaporated. The products were extracted as described above (under the subheading: Quenching Reactions), and analyzed on the HPLC. There was a new major peak at 6.7 min that we predicted to be the prenylated divarinic acid. We HPLC purified the peak, removed the solvent, and redissolved the pure compound in 600 µL of CD$_3$OD. A proton spectrum collected on an AV400 Bruker NMR spectrometer was compared with a proton spectrum published by Shoyama et al. [37] for CBGVA to confirm that CBGVA was the main product. Based on the report by Shoyama et al. the study by Bohmann et al. [38], we conclude that the prenylation of divarinic acid occurs at the C3 carbon of divarinic acid. Shoyama et al. [37] published the chemical shifts of CBGVA in CD$_3$OD, so by direct comparison of our NMR spectra to the published chemical shifts we conclude that we produced CBGVA. This is further supported by the work conducted by Bohmann, which suggests that if the prenylation occurred at the C3 site, we would observe a proton with a chemical shift around 5.8 ppm, which we did not observe, Supplementary Figure 11.

Rosetta design to modify the binding pocket of NphB. We placed olivetol in the active site of NphB in six different starting positions denoted as Olivetolate P1–6 in Table S2. We ran ROSETTA 5 times for each olivetolate position for a total of 30 designs. The mutations predicted in each design are listed in Table S2. For each olivetolate position we chose a consensus set of mutations (i.e., the most frequently chosen or evaluate further: Consensus Group through F (Table S2)). We then sought to evaluate the relative importance of each ROSETTA suggested mutation. For each Consensus Group, we set the mutations back to WT (see Table S2). We then sought to evaluate the relative importance of each ROSETTA suggested mutation. For each Consensus Group, we set the mutations back to WT residue, one at a time, and used ROSETTA to calculate the change in energy score (see Table S2). Those that caused the largest change in energy were deemed to be the most important mutants to include in the library for experimental testing.

To model the OA, we took the 4MX.sdf 3-D structure of olivetolate from the 5B09 crystal structure and added hydrogen atoms to the structure assuming pH 7.27 ppm. The OA was manually placed into the co-crystal structure of NphB with GST and DHN (1ZB6) with the DHN and crystallographic waters removed using pymol. The OA was manually placed into the co-crystal structure of NphB with GST and DHN (1ZB6) with the DHN and crystallographic waters removed using pymol. The OA was manually placed into the co-crystal structure of NphB with GST and DHN (1ZB6) with the DHN and crystallographic waters removed using pymol. The OA was manually placed into the co-crystal structure of NphB with GST and DHN (1ZB6) with the DHN and crystallographic waters removed using pymol. The OA was manually placed into the co-crystal structure of NphB with GST and DHN (1ZB6) with the DHN and crystallographic waters removed using pymol.

The enzymes were assayed under the following conditions: 2.5 mM geranylpyrophosphate, 5 mM olivetol, 5 mM MgCl$_2$, 50 mM Tris pH 8.0, and ~1 mg/mL of NphB enzyme in a final volume of 100 µL. The reactions were incubated at room temperature for 1 h. A total of 40 µL of each reaction was quenched in 80 µL of acetonitrile. The samples were centrifuged for 5 min to remove unreacted proteins. The supernatant was analyzed using HPLC as described above.

Enzyme kinetic parameters. The reactions were set up under the following conditions: 50 mM Tris pH 8.0, 2.5 mM GPP, 5 mM MgCl$_2$, ~27 µM enzyme, and olivetol or divarinic acid was varied from 0.1 to 6 mM in a final volume of 200 µL. A total of 40 µL of the reaction was quenched in 80 µL acetonitrile +0.1% TFA, at the time intervals detailed below. The reactions were centrifuged for 5 min at 13,000–16,060× g to pellet the protein, and the supernatant was analyzed using the HPLC method detailed above. The initial rate was plotted versus the concentration of substrate, and fit with the Michaelis–Menten equation to determine the kinetic parameters $K_{cat}$ and $K_{m}$ (OriginPro). Each Michaelis–Menten curve was performed in triplicate. The average and standard deviation of the kinetic parameters are reported. The time courses with olivetolate as the substrate were as follows: for WT, M1, M10, and M30 the time course was 3, 6, 9, and 12 min. For M25 the reactions were quenched at 1, 2, 4, and 8 min, and for M31 the reactions were quenched at 1, 2, 4, and 6 min.

The conditions were altered slightly to characterize the constructs with divarinic acid as the substrate. For M31, the time course was 0.5, 1, 1.5, and 2 min. For M23, the time course was 5, 10, 15, and 20 min, and for WT NphB the time course was 8, 16, 24, and 32 min. The enzyme concentration for the mutants was ~27 µM, and the amount of WT NphB was ~35 µM.

GC–MS characterization of isomer profile for WT NphB and M23. Samples were dissolved in 200 µL of ethyl acetate. Gas chromatography–mass spectrometry (GC–MS) measurement was carried out on an HP5 capillary column with dimensions 30 m × 0.25 mm. Ultra High Purity Grade He (Airegas) was used as carrier gas with the flow set to 1.1 mL/min in constant flow mode. The initial oven temperature was set to 120 °C for 1 min followed by a 20 °C/min ramp to a final temperature of 300 °C which was maintained for 4 min. A 3.0 min solvent delay was used. EI energy was set to 15 eV. The MSD was set to scan the 50–500 m/z range. Data collection and analysis were performed using Mass Hunter Acquisition and Qualitative Analysis software (Agilent).

Due to the increased temperature of the GC inlet, CBGAs undergoes spontaneous decarboxylation as described by Radwan et al. [41], resulting in an m/z ion at 316 m/z. The retention time corresponding to the 316 m/z ion for the CBGA standard was 18.68 min.

Nonane-flow system for the extraction of CBGs from solution. A PyOx/PTA reaction was set up as described above. A 500 µL nonane overlay was added to the reaction in a 2 mL glass vial which was covered with 2 layers of breathable cell culture film. Two 18-gauge needles were inserted into a 15 mL Falcon tube at the ~750 µL mark and the 3.5 mL mark. Luer locks to tubing connectors were connected to the needles and Viton tubing was connected to the other end of the luer lock, with 18-gauge needles were connected to the other end of the luer lock via a luer lock connector and inserted through the mesh covering so they were only touching the nonane layer and not the reaction. In total, 2 mL of Tris buffer was used.
[pH 8.5] was added to the 15 mL conical tube, and 6 mL of nonane was added. The nonane was pumped through the system using a peristaltic pump (~1 mL/min) such that the nonane flowed from the top of the reaction, through the buffered solution (~18 cm tubing). The nonane pumped into the reservoir separated into the top layer of the 15 mL conical tube. The nonane from the top of the 15 mL conical tube was pumped into the top of the reaction vial (~55 cm tubing). This essentially diluted the CBGAs throughout the system driving the diffusion of CBGA into the nonane layer and out of the reaction.

Cloning CBDAS. A gene block of cannabidiolic acid synthase (CBDAS) with the signaling peptide was ordered from IDT codon optimized for Pichia pastoris. The signal sequence was removed by PCR amplifying from the 28th residue of the mature sequence (NPREN–) through the end of the protein, with overhangs compatible with the pPICZa vector. The PCR product was cloned into the pPICZa vector digested with EcoRI and XbaI using the Gibson cloning method. The product of the assembly reaction was transformed into BL21 Gold (DE3) cells, and a clone with the correct sequence was isolated. The plasmid was digested with Pmel for 2 h, and then purified using the Qiagen PCR purification protocol. The plasmid was transformed into Pichia pastoris X33 using electroporation. Immediately following electroporation, the cells were incubated in 1 mL of cold 1 M sorbitol and 1 mL of YPD media without shaking for 2 h. The cells were plated on YPDS plates with 500 µg/mL of zeocin. Colonies were screened using PCR for the presence of the CBDAS gene between the AOX1 promoter and terminator. For screening, the colonies were resuspended in 15 µL of sterile water and 5 µL of the resuspended culture from three clones (~300 mL total), was collected to obtain CBDAS colonies were resuspended in 15 µL of sterile water and 5 µL of the resuspended colony was transferred into a PCR tube with 0.2% SDS. The samples were heated for 10 min at 99 °C, and then 1 µL was used as the template for PCR. Six colonies with positive colony PCR hits were screened for the expression of CBDAS.

CBDAS-expression test. The six colonies were grown overnight at 30 °C in 25 mL of buffered complex glycerol medium (BMGY) to obtain a saturated culture. The overnight cultures were used to inoculate a 25 mL culture in BMGY media and grown to an OD of ~2. The cells were harvested by centrifugation at 2000 × g for 20 min at 4 °C. The cell pellet was resuspended in 90 mL of buffered minimal methanol yeast extract media, and incubated at 30 °C for 5 days. Each day, 1 mL of the culture was removed for SDS–PAGE analysis, and 500 µL of methanol was added to the remaining culture. On day 3 the cultures were screened for CBDAS activity. The 1 mL culture samples were centrifuged to pellet the cells (16,000 × g, 5 min). A 50 µL of the media was used in an subsequent activity assay, and the remainder of the media was stored at ~80 °C in addition to the cell pellet. The assay conditions were as follows: 100 µL of 200 mM citrate buffer, 100 µM CBGA, 5 mM MgCl2, 5 mM KCl, 1 mM FAD, and 50 µL of the expression media in a final reaction volume was 200 µL, with 50 mM Hepes [pH 7.0], 5 mM MgCl2, 5 mM KCl, 25 µM FAD, 0.1 mg/mL (total protein) of CBDAS. The assay mixture was preincubated to 80 °C in addition to the cell. The assay mixture was cooled to 4 °C and 25 µL of preincubated cell-free extract was added. The reactions were incubated overnight at room temperature and then extracted 3 times with 200 µL of ethyl acetate. The ethyl acetate extractions were pooled for each sample, and removed using a vacuum centrifuge. The samples were resuspended in 200 µL of methanol and analyzed by HPLC. All clones produced active CBDAS. The culture from three clones (~300 mL total), was collected to obtain CBDAS activity. The cells were pelleted by centrifuging at ~3000 × g for 20 min at 4 °C. Then the supernatant was passed through a 22 µm filter. The filtrate was concentrated and buffer exchanged into 100 mM citrate buffer pH 5.0 using a 50,000 molecular weight cut-off protein concentration from Millipore.

Production of CBDVA and CBDA. To convert the precursors CBGA and CBGVA into CBDVA and CBDA, respectively, a secondary reaction was set up with CBDAS. To produce CBDVA, a Pwo/KF/PTA enzymatic system was set up as detailed above to produce CBGA. After 24 h 200 µL of the nonane overlay from the CBGA reaction was transferred to a CBDAS reaction vessel. In the aqueous layer: 50 mM Hepes [pH 7.0], 5 mM MgCl2, 5 mM KCl, 25 µM FAD, 0.1 mg/mL CBDAS. The reaction was incubated at 30 °C with gentle shaking. Reactions were quenched at 12, 24, 48, 72, and 96 h.

To produce CBDVA, HPLC purified CBGA was converted to CBDVA by using CBDAS. The final reaction volume was 200 µL with 50 mM Hepes [pH 7.0], 5 mM MgCl2, 5 mM KCl, 25 µM FAD and 0.1 mg/mL (total protein) of CBDAS. A 200 µL nonane overlay was added, and the reactions were incubated at 30 °C with gentle shaking. The reactions were quenched at ~24, 48, 72, and 96 h.

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All data generated or analyzed in this study are in this published article and its Supplementary materials. The authors will make every reasonable effort to provide data and materials described herein upon request. A reporting summary for this article is available as a Supplementary Information file. The source data underlying Figs. 2A–D, 3B, C, 4A, C, Supplementary Figures 2 and 4 and Supplementary Table 5 are provided as a Source Data file.

Received: 13 November 2018 Accepted: 10 January 2019
Published online: 04 February 2019
30. Chen, R. et al. Molecular insights into the enzyme promiscuity of an aromatic prenyltransferase. *Nat. Chem. Biol.* 13, 226 (2016).
31. Ozaki, T., Mishima, S., Nishiyama, M. & Kuzuyama, T. NovQ is a prenyltransferase capable of catalyzing the addition of a dimethylallyl group to both phenylpropanoids and flavonoids. *J. Antibiot.* 62, 385 (2009).
32. Kuzuyama, T., Noel, J. P. & Richard, S. B. Structural basis for the promiscuous biosynthetic prenylation of aromatic natural products. *Nature* 435, 983 (2005).
33. Fellermeier, M. & Zenk, M. H. Prenylation of olivetolate by a hemp transferase yields cannabigerolic acid, the precursor of tetrahydrocannabinol. *FEBS Lett.* 427, 283–285 (1998).
34. Zirpel, B., Kayser, O. & Stehle, F. Elucidation of structure–function relationship of THCA and CBDase synthase from Cannabis sativa L. *J. Biotechnol.* 284, 1–25 (2018).
35. Taura, F. et al. Cannabidiolic-acid synthase, the chemotype-determining enzyme in the fiber-type Cannabis sativa L. *FEBS Lett.* 581, 2929–2934 (2007).
36. Taura, F., Morimoto, S. & Shoyama, Y. Purification and characterization of cannabidiolic-acid synthase from Cannabis sativa L.: biochemical analysis of a novel enzyme that catalyses the oxidocyclization of cannabigerolic acid to cannabidiolic acid. *J. Biol. Chem.* 271, 17411–17416 (1996).
37. Shoyama, Y., Hirano, H., Makino, H., Umekita, N. & Nishio, I. The isolation and structures of four new propyl cannabinoid acids, tetrahydrocannabivarinc acid, cannabidivarin acid, cannabichromivarin acid and cannabigerovarin acid, from Thai Cannabis, ‘Meao Variant’. *Chem. Pharm. Bull.* 25, 2306–2311 (1977).
38. Bohmann, F. & Hoffmann, E. Cannabigerol-ähnliche verbindungen aus Helichrysum umbraeculigerum. *Phytochemistry* 18, 1371–1374 (1979).
39. O’Boyle, N. M. et al. Open Babel: an open chemical toolbox. *J. Cheminf.* 3, 33 (2011).
40. Kothiwale, S., Mendenhall, J. L. & Meiler, J. BCL: Conf: small molecule conformational sampling using a knowledge based rotamer library. *J. Cheminform.* 7, 47 (2015).
41. Radwan, M. M. et al. Isolation and characterization of new cannabis constituents from a high potency variety. *Planta Med.* 74, 267–272 (2008).

**Acknowledgments**

The authors thank members of the Bowie lab for helpful comments. This work was supported by DOE grants DE-FC02-02ER63421 and DE-AR0000556 to J.B., and an NIH National Research Service Award 5T32GM008496 to M.V.

**Author contributions**

M.V., T.K., and J.B. conceived of the project. M.V. performed the bulk of the experiments along with N.W., M.V., T.K., and D.B. developed enzyme designs. G.K. and R.T. assisted with mass spectrometry and NMR experiments and analysis, respectively. All authors contributed to data interpretation and writing the manuscript.

**Additional information**

**Supplementary Information** accompanies this paper at https://doi.org/10.1038/s41467-019-08448-y.

**Competing interests:** T.K. and J.B. are founders of a company, Invizyne Technologies, that seeks to develop cell-free chemical production. The remaining authors declare no competing interests.

**Reprints and permission** information is available online at http://npg.nature.com/reprintsandpermissions/

**Journal peer review information:** *Nature Communications* thanks Olive Kayser and the other anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

**Publisher’s note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

---

**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019