Enzymatic Menthol Production: One-Pot Approach Using Engineered *Escherichia coli*

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Ⓢ *Supporting Information*

ABSTRACT: Menthol isomers are high-value monoterpenoid commodity chemicals, produced naturally by mint plants, *Mentha* spp. Alternative clean biosynthetic routes to these compounds are commercially attractive. Optimization strategies for biocatalytic terpenoid production are mainly focused on metabolic engineering of the biosynthesis pathway within an expression host. We circumvent this bottleneck by combining pathway assembly techniques with classical biocatalysis methods to engineer and optimize cell-free one-pot biotransformation systems and apply this strategy to the mint biosynthesis pathway. Our approach allows optimization of each pathway enzyme and avoidance of monoterpenoid toxicity issues to the host cell. We have developed a one-pot (bio)synthesis of (1R,2S,5R)-(−)-menthol and (1S,2S,5R)-(+)-neomenthol from pulegone, using recombinant *Escherichia coli* extracts containing the biosynthetic genes for an "ene"-reductase (NtDBR from *Nicotiana tabacum*) and two menthone dehydrogenases (MMR and MNMR from *Mentha piperita*). Our modular engineering strategy allowed each step to be optimized to improve the final production level. Moderate to highly pure menthol (79.1%) and neomenthol (89.9%) were obtained when *E. coli* strains coexpressed NtDBR with only MMR or MNMR, respectively. This one-pot biocatalytic method allows easier optimization of each enzymatic step and easier modular combination of reactions to ultimately generate libraries of pure compounds for use in high-throughput screening. It will be, therefore, a valuable addition to the arsenal of biocatalysis strategies, especially when applied for (semi)-toxic chemical compounds.

KEYWORDS: *menthol production, one-pot biosynthesis, recombinant biosynthetic pathways, Escherichia coli*
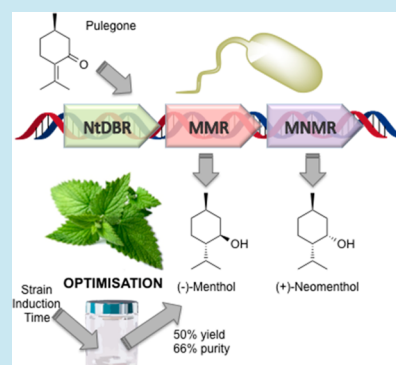
L imonene enantiomers are the most abundant monocyclic monoterpenoids in nature; (−)-limonene is found in herbs such as *Mentha* spp. (e.g., peppermint and spearmint), whereas the (+)-enantiomer is the major oil constituent of orange and lemon peels.[2] Natural limonene derivatives are known to be important precursors in the production of several pharmaceutical and commodity chemicals, such as fragrances, perfumes, and flavors. For example, essential oils of mint contain a variety of limonene derivatives (Scheme 1), such as menthol isomers, pulegone, and methanofuran in peppermint (*Mentha piperita*) and carveol/carvone in spearmint (*Mentha spicata*). Menthol isomers, (1R,2S,5R)-(−)-menthol, (1R,2S,5S)-(+)-isomenthol, (1S,2S,5R)-(+)-neomenthol, and (1R,2R,5R)-(+)-neoisomenthol, and carvone are used as additives in oral hygiene products and flavors in food and beverages. Carveol is found in cosmetics, whereas pulegone is commonly found as a flavor in perfumery and aromatherapy products. Carvone and carveol are also known to have anticancer properties, whereas menthol has antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*.[3]

There is a high demand for limonene and derivatives (e.g., menthol oil ca. 3000 t/$373−401 US million pa), which are traditionally obtained from natural sources (*Mentha* spp.) due to the flavor/fragrance industries demanding so-called natural sources that are compatible with use in food products. However, the production of natural menthol relies heavily on ca. 0.29 million hectares of arable land and requires expensive steam-distillation and filtration processes.[4,5] Given the volatility of mint crop prices due to unpredictable harvest yields and the environmental footprint of intensive mint cultivation, alternative clean (bio)synthetic routes to these compounds are commercially attractive.[6−8]

An alternative "natural" route to highly pure complex organic compounds utilizes microorganisms as biological factories. They are built from existing or *de novo* biosynthetic pathways, incorporated into rapidly growing, cost-effective, and even food-compatible microorganisms grown on non-petroleum-based renewable feedstock.[9] For example, a precursor of Taxol (paclitaxel), a potent anticancer drug found naturally in *Taxus brevifolia* (Pacific yew tree), has been successfully produced in *E. coli*.[9] Several reports describe the production of limonene and other terpenoids in *E. coli*, *Saccharomyces cerevisiae*, and

Scheme 1. Monoterpenoid Biosynthesis Pathways in Different *Mentha* Species[1,a]
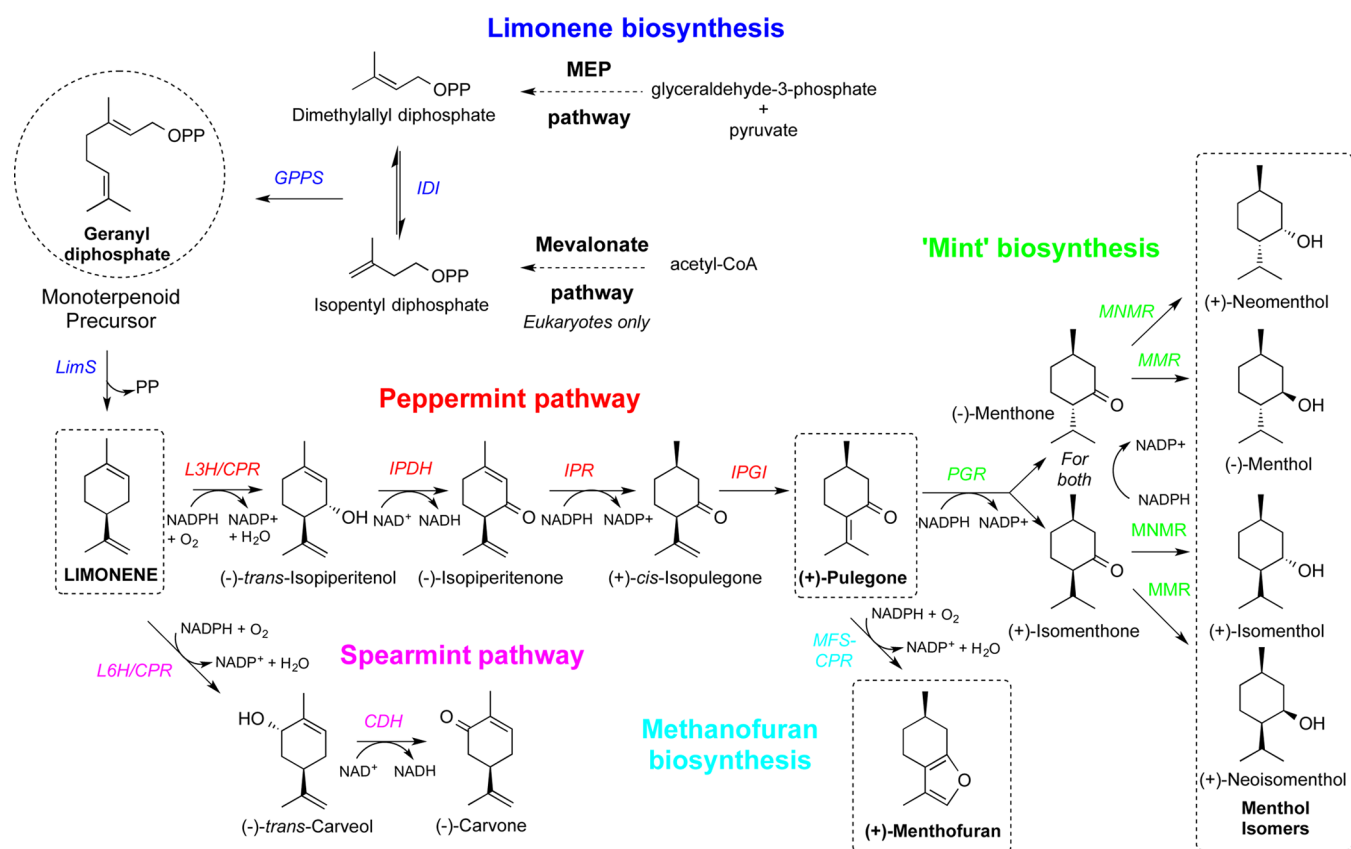
[a]IDI = isopentenyl-diphosphate delta-isomerase; GPPS = geranyl diphosphate synthase; LimS = (−)-limonene synthase; L3H = (−)-limonene-3-hydroxylase; CPR = cytochrome P450 reductase; IPDH = (−)-*trans*-isopiperitenol dehydrogenase; IPR = (−)-isopiperitenone reductase; IPGI = (+)-*cis*-isopulegone isomerase; PGR = (+)-pulegone reductase; MMR = (−)-menthone:(−)menthol reductase; MNMR = menthone:(+)-neomenthol reductase; MFS = (+)-menthofuran synthase; L6H = (−)-limonene-6-hydroxylase; CDH = (−)-*trans*-carveol dehydrogenase.

cyanobacteria by incorporating genes encoding plant terpene synthases and ones that upregulate isoprene precursor production.[6,10−14] The semisynthetic industrial-scale production (ca. 35 tonnes per annum) of artemisinin (major active ingredient in modern malarial treatments) by Sanofi has shown commercial success, in comparison to traditional extractions/purifications from natural sources (sweet wormwood).[15] In all of these examples, the optimization of terpene yields was conducted *in vivo*, i.e., in the production cell. One-pot biosynthesis of complex chemical compounds is not common and may be difficult to achieve due to technical challenges in successfully completing a production process.[16]

We have demonstrated, as proof of principle, the successful one-pot biosynthesis of menthol isomers from pulegone[17] by incorporating part of the peppermint biosynthetic pathway into *E. coli*. This hybrid approach integrates both pathway engineering and classical biocatalysis strategies, where the best performing enzymes (according to factors such as catalytic rate, enantiospecificity, soluble expression) were identified and assembled into a functional biocatalytic cascade. Further optimization for production was conducted by host strain selection, varying the pathway enzyme expression strength and increasing the coenzyme (NADPH) availability to increase *in vitro* production. Our approach combines enzyme selection and validation, followed by seamless PCR-based operon assembly and one-pot biotransformations using *E. coli* extracts. We demonstrate the effectiveness of this approach in generating

moderately to highly pure menthol isomers from recombinant *E. coli* cell extracts. This alternative one-pot biocatalytic method, which avoids the bottlenecks of host optimization and *in vivo* metabolic engineering, has the potential to be applied as a general strategy for optimizing (semi)toxic chemical compounds production, based on its ease of optimization of each enzymatic step and the possibility of using the resulting engineered *in vitro* pathways in high-throughput screening.

■ **RESULTS AND DISCUSSION**

Product Synthesis. A synthetic route was designed to give access to all menthone and menthol isomers required for biotransformations, starting from menthone (Scheme 2). This was motivated by the fact that isomenthone and neoisomenthol are not commercially available, whereas the remaining menthols can be derived from the purification of essential oil mixtures. Further details of the synthesis, purification, and identification of the isomers are described in the Supporting Information. Our chemical synthetic approach has provided routes to obtain diastereomerically pure isomers of menthone and menthol for the first time, overcoming existing limitations in the commercial supply of some these compounds.

Enzyme Production and Validation. Our cloning strategies enabled the rapid generation and manipulation of multigene expression constructs from individual components, including the presence of repeated sequences of relatively high

Scheme 2. Chemical Synthesis of Isomenthone and All Four Menthols from Menthone


DBAD: Di-*tert*-butyl azodicarboxylate
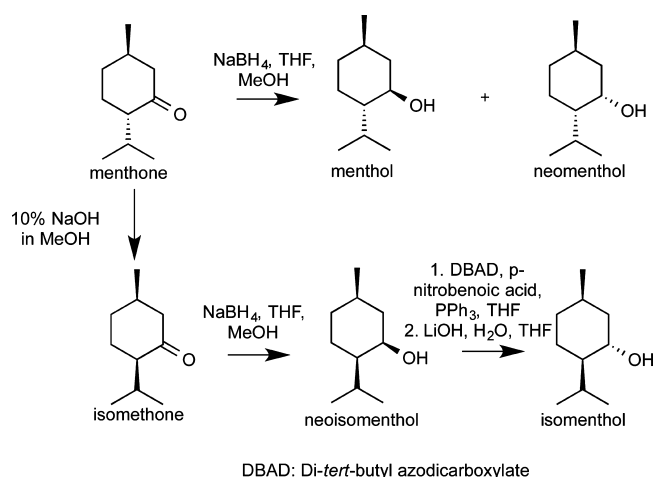
GC content. However, to design the best production pathway, it is necessary to characterize the individual enzyme activity and to use the most active enzymes for the desired reaction. Initially, the three recombinant enzymes (NtDBR, MMR, and MNMR) were purified, and preliminary biocatalytic ability of each was assessed to test their functionality when produced in a bacterial system. The three genes encoding these plant enzymes were first codon-optimized for *E. coli* and separately cloned into pET21b to generate C-His$_6$-tagged recombinant enzymes. After production and purification, each enzyme was tested for activity under steady-state conditions, via indirect NADPH oxidation

monitoring. The ene-reductase activity of NtDBR-His$_6$ with (5R)-pulegone was relatively low (<0.1 s^{-1}); however, this is known to be a poor kinetic substrate for this enzyme.[18] Ketoreductase activity of MMR-His$_6$ (1.63 and 2.00 s^{-1}) and the double tagged His$_6$-MNMR-His$_6$ (0.96 and 0.20 s^{-1}) was determined with both (2S,5R)-menthone and (2R,5R)-isomenthone, respectively. This shows that MMR has a similar rate with both isomers, whereas MNMR has a preference for menthone over isomenthone. Further characterization studies are currently being performed and will be the focus of a later report.

Initial *in vitro* biotransformation reactions were performed with each enzyme, as well as an equimolar combination of all three (2 μM), in the presence of an externally added cofactor recycling system (GDH/glucose/NADP$^+$) to identify the yields and ratio of each monoterpenoid product formed (Supporting Information Table 2). For NtDBR, the reduction of pulegone yielded near equivalent amounts of menthone and isomenthone, similar to that with the non His$_6$-tagged recombinant enzyme.[19] This differs from previous studies with the His$_6$-tagged enzyme where the ratio was nearly 1:2.[18] Prior studies suggested that variations can be obtained under different reaction conditions.[19] Interestingly, under biotransformation conditions (24 h; cofactor recycling system), MMR appears to generate more products from menthone than isomenthone. This differs from the kinetic studies, where the rates slightly favored the reaction with isomenthone. Product ratios agree with prior studies[17] where MMR has a preference for menthol over neomenthol from menthone and neoisomenthol over isomenthol with isomenthone, whereas the opposite is true for
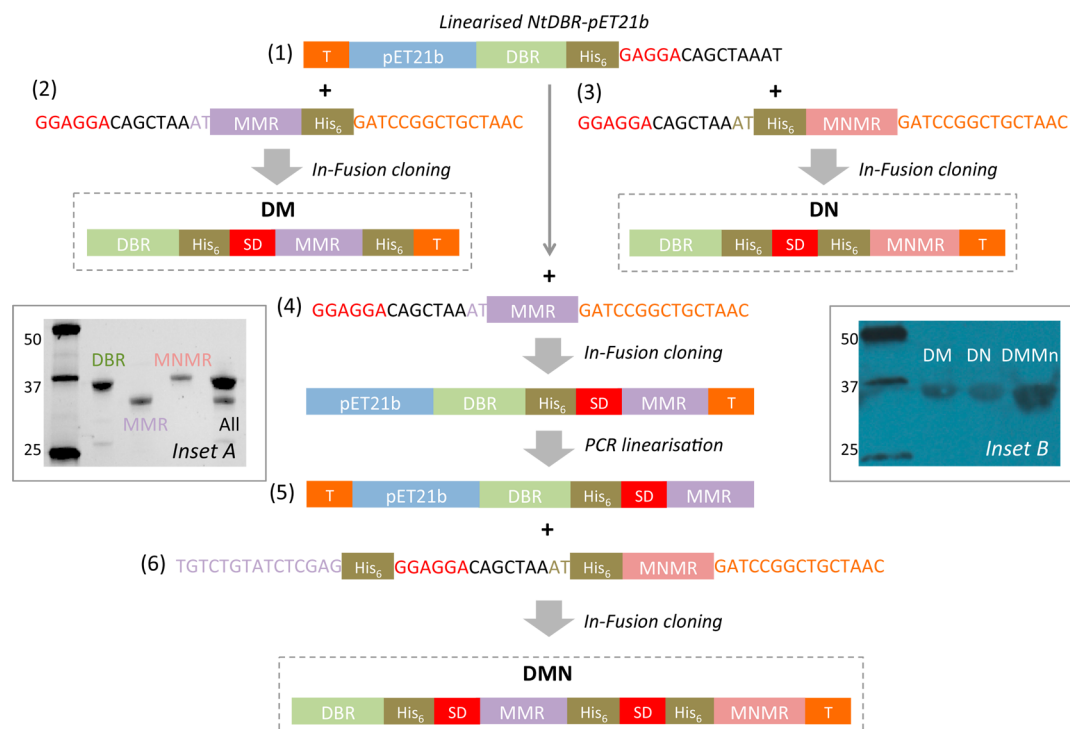
Figure 1. Method of generating multigene expression constructs with a single promoter using In-Fusion cloning. Sequences are color-coded to show the overlap with the next gene. Large overlaps (e.g., MNMR 5′ region) were generated using 2 to 3 overlapping forward PCR primers within one PCR reaction. Red = Shine−Dalgarno sequence (SD); orange = T7 terminator region of pET21b (T). The numbers in parentheses refer to the PCR steps and correlate with the oligos in Supporting Information Table 5. Inset A shows the SDS-PAGE analysis of the three purified enzymes (20−30 pmol each). Inset B is a western blot (anti-His$_6$) of soluble protein extracts from whole cells expressing the three multigene constructs.
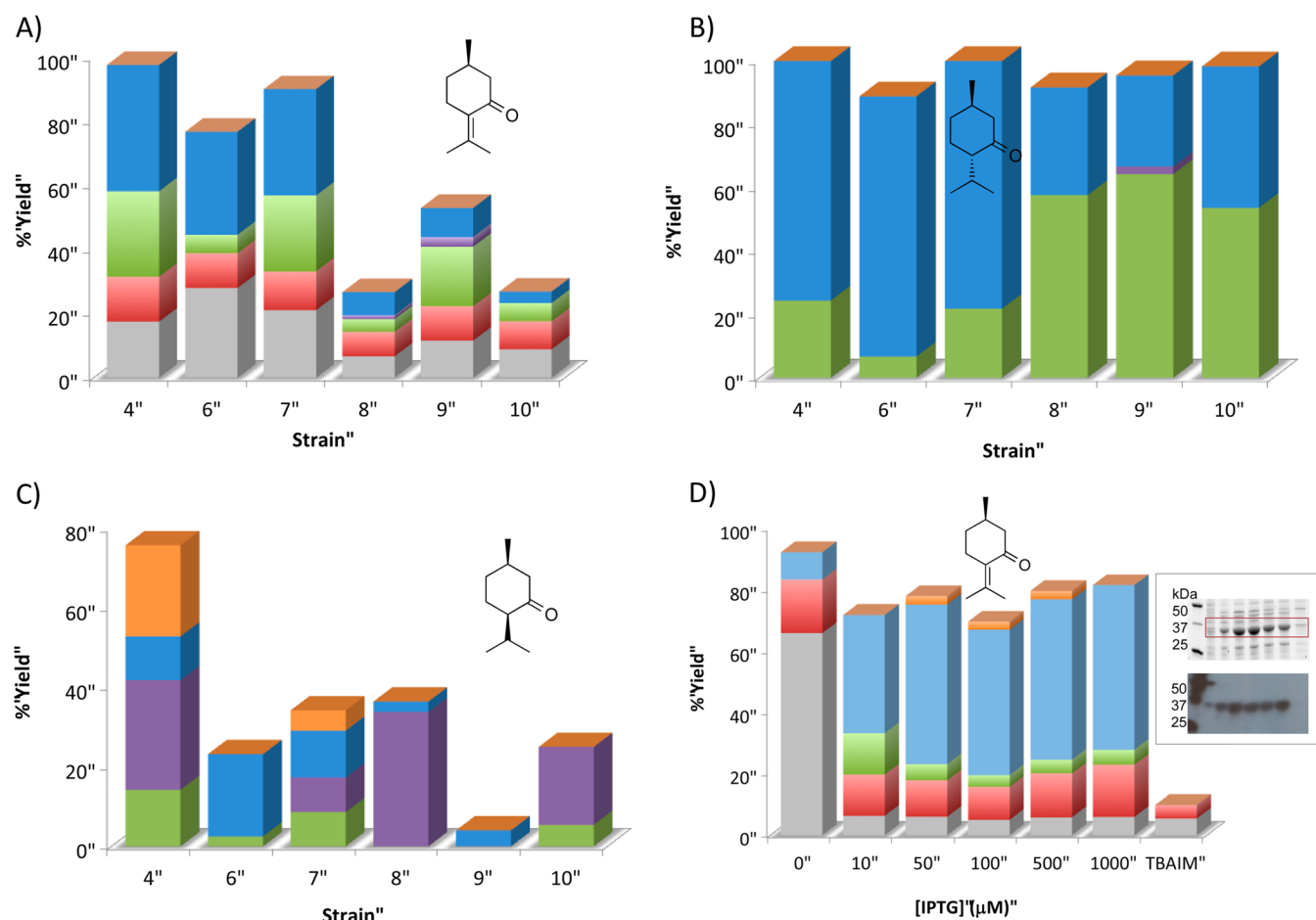
Figure 2. Ratio of products formed during biotransformations of DMN in 6 strains with substrates (A) pulegone, (B) menthone, and (C) isomenthone. (D) Products formed during biotransformations of DMN cell extracts in strain 4 at different IPTG concentrations and TBAIM media. Control reactions (strain 1 with empty pET21b) yielded no products. Inset: SDS-PAGE and western blot analysis of the cell extracts from the biotransformations. Reactions (2 mL) were performed in buffer (50 mM Tris, pH 7.0) containing monoterpenoid (1 mM), cell extracts (0.5 mL), $NADP^+$ (10 μM), glucose (15 mM), and GDH (10 U). The reactions were agitated at 30 °C for 24 h at 130 rpm. Product yields were determined by GC analysis using a DB-WAX column. Data point colors: menthone = gray; isomenthone = red; menthol = green; neoisomenthol = purple; neomenthol = blue; isomenthol = orange. Strain identification is found in the Methods section.

MNMR. This shows these His_6-tagged-enzymes have comparable activity to that of the native biocatalysts in *M. piperita*.

Small amounts of the nonstandard products (e.g., neomenthol from isomenthone) with both enzymes were obtained due to the presence of approximately 5% of the opposite substrate enantiomer contaminating the reactions. Reactions with a mixture of equivalent amounts of each enzyme show that all of the menthone generated by NtDBR has been consumed to form menthol and neomenthol, whereas activity utilizing isomenthone was poor. This shows that the recombinant enzymes were all active *in vitro* and display previously determined activities. In a stoichiometric mixture of all three enzymes, MMR activity is biased over that of MNMR, as expected from the steady-state kinetics of each enzyme.

Operon Construction. As each recombinant enzyme shows the required activities, a synthetic operon was designed (Figure 1) to enable the coexpression of each gene within *E. coli* under the control of one promoter (T7*lac*). The initial construct encoding NtDBR, MMR, and MNMR (pDMN) was designed to include a Shine−Dalgarno (SD) sequence between successive genes and to maintain the His_6-tags (N-His_6 for MNMR). Our initial attempts to construct this vector using standard PCR and cloning methods were complicated due to

the presence of a His_6-tag next to the SD sequence with a repeating unit of 43 bp and high G/C content (T_m = 73 °C). To overcome this issue, we designed a cloning protocol (Figure 1) based on the In-Fusion recombination system, avoiding the use of PCR primers annealing to the His_6-tag when the repeating unit was present. This sequential approach was based on the following rules: (i) the vector containing the first gene is opened by reverse PCR, maintaining the C-His_6-tag and incorporating the SD sequence at the 3′ end; (ii) each inserted gene contains a 5′ SD sequence, and all except the last gene are amplified without their His_6-tag at the 3′ end; (iii) the third and successive gene(s) contain the missing His_6-tag of the previously inserted gene at the 5′ end before the SD sequence; (iv) the 3′ overlaps of the inserted genes anneal to the terminator region of the vector; and (v) each PCR-amplified product contains a 15 bp overlap annealing to the PCR product with which it will be recombined.

This general protocol can be used for assembly of pathways to enable multiple genes (up to 5) to be added in one reaction in the correct order, providing that the 15 bp overlaps are specific for the next gene and not the His_6-tag/SD region (5′ region of all except the first gene insert contains the His_6-tag, SD region and 15 bp overlap with the previous gene). Only the

Table 1. Comparative Yields of Each of the Six Products from Pulegone Biotransformations Catalyzed by Cell Extracts of DM, DN, and DMN in *E. coli* Strain NiCO$_2$(DE3)[a]

	product yield (μM)					
	DM		DN		DMN	
product	glucose[b]	cofact.[c]	glucose[b]	cofact.[c]	glucose[b]	cofact.[c]
menthone	121 ± 2	144 ± 3	107 ± 2	213 ± 5	108 ± 1	192 ± 3
isomenthone	82 ± 1	ND	73 ± 2	143 ± 3	84 ± 1	144 ± 2
menthol[d]	109 ± 1	339 ± 2	ND	ND	37 ± 2	132 ± 2
neoisomenthol[d]	ND	ND	ND	ND	ND	ND
neomenthol[e]	ND	ND	132 ± 4	247 ± 1	103 ± 1	149 ± 4
isomenthol[e]	ND	ND	ND	28 ± 1	ND	ND
total % yield	312 (352)	483 (820)	312 (543)	632 (766)	332 (404)	618 (705)

[a]Reactions (2 mL) were performed in buffer (50 mM Tris pH 7.0) containing pulegone (1 mM), cell extracts (0.5 mL), and glucose (15 mM) ± cofactor recycling system (10 μM NADP$^+$ and 10 U GDH). The reactions were agitated at 30 °C for 24 h at 130 rpm. Product yields were determined by GC analysis using a DB-WAX column. Data in parentheses are % conversion data; ND = none detected [b]Reactions containing glucose and NADP+ but relying on native *E. coli* cofactor recycling enzymes. [c]Reactions containing a complete externally added cofactor recycling system. [d]Product from a reaction with menthone. [e]Product from a reaction with isomenthone.

last gene to be inserted contains a 3′ overlap annealing to the terminator region.

The new constructed pathway (DMN; Figure 1) was expressed in *E. coli* (strain 1; BL21(DE3)pLysS; Figure 1, inset A), and expression of the tagged proteins was analyzed by western blot (Figure 1, inset B). Unfortunately, the three enzymes are similar in size (Figure 1 inset A), so, in most cases, only two of the three proteins could be identified. Whole cell soluble protein extracts of *E. coli* strain 1 containing the DMN construct underwent biotransformations with the substrates pulegone, menthone, and isomenthone to determine the activity of each enzyme. The results (Supporting Information Table 3; strain 1) showed the DMN extract had activity of all three enzymes, and no product formation was observed in the control reactions (cell extracts with empty pET21b). In the presence of pulegone, both NtDBR (78 μM menthone and 3.5 μM isomenthone) and MNMR (22 μM neomenthol) activities were detected, but no MMR activity was observed. Reactions with menthone showed primarily MNMR activity (212 μM neomenthol) but also that of MMR (21 μM menthol). However, product yields were poor (20−220 μM), and further optimization was clearly required.

Comparative Cell Extract Biotransformations. The DMN construct (pDMN) was transformed into a further 11 *E. coli* strains to screen for improved expression of each gene. Reactions with pulegone, menthone, and isomenthone were performed with each extract as before but in the presence of an externally added cofactor recycling system for the production of NADPH required by each enzyme. To our surprise, the results showed a wide variation in the product yields and ratios (Figure 2 and Supporting Information Table 3), suggesting that the *E. coli* strain strongly impacts on the expression levels of the individual genes independently. For example, strains NiC-O$_2$(DE3) (4) and Tuner(DE3) (7) showed high activity of each enzyme with pulegone and generated at least 75% yield of neomenthol with menthone (Figure 2a,b). In contrast, many other extracts produced very little product, especially with isomenthone as the substrate (Figure 2c and Supporting Information Table 3). Interestingly, strains 8−10 generated proportionally more menthol over neomenthol, suggesting higher MMR activity over MNMR. These proportions of menthol isomers differ from essential oils of *M. piperita*; menthol yields are typically around 50%, whereas neomenthol is a relatively minor component (<3%).[17] The low yields of

isomenthol from isomenthone is not surprising, as MMR is known to have a 10-fold higher specificity for menthone over isomenthone.[17] No activity was seen with the control reactions, although some minor unidentified byproducts were observed (results not shown).

Given that NtDBR generates nearly equal amounts of menthone and isomenthone (54:46), it was surprising that reactions of strain 4 with pulegone gave a total yield of menthone and subsequent reduction products of 84% (840 μM). Additionally, reactions with isomenthone of this strain showed at least 20% yield of products from menthone reduction (menthol and neomenthol), in spite of there being a maximum of 5% contaminating menthone in the reaction. These findings are not observed in reactions with the purified enzymes, suggesting the *E. coli* extracts contain epimerase activity, where isomenthone is isomerized to menthol. This was confirmed by control reactions (*E. coli* extracts without recombinant enzymes) in the presence of menthone or isomenthone, showing a change from the 95:5 ratio to 60:40 (results not shown). Given that reactions of many strains with menthone generated little or no isomenthol or neoisomenthol, this suggests that the equilibrium strongly favors the direction of menthone formation. Work is currently underway to identify and characterize the *E. coli* epimerase(s).

To further optimize the production level of menthol, the best three strains were identified (4, 6, and 7) based on product yields and the balance of all three activities (Figure 2d and Supporting Information Figure 2). These strains underwent studies to determine the optimal IPTG concentration for protein expression and thereby high activity. Strain 4 showed high NtDBR activity (∼75% yield) in uninduced cells, suggesting high levels of leaky expression of this gene. In contrast, strain 6 (Rosetta2(DE3)pLysS) gave almost no products in the absence of IPTG, consistent with its pLysS phenotype (tight regulation of the T7*lac* promoter). Western blots of each extract (Figure 2d, inset) showed a correlation between expression levels and product yields, with the optimal IPTG concentration being as low as 50 μM.

Construct Optimization. The product ratios from DMN biotransformations suggested that modifications of the multi-gene construct combined with further expression optimization and native *E. coli* epimerization activity may lead to near single product formation. Therefore, two new operons were designed and engineered, namely, NtDBR-MMR (DM) and NtDBR-

Table 2. Optimization of Biotransformations from Cell Extracts of DM and DN in *E. coli* Strain NiCO$_2$(DE3)[a]

construct	product yield (μM)					
	menthone	isomenthone	menthol[b]	neoisomenthol[c]	neomenthol[b]	isomenthol[c]
	(A) Effect of the Enzyme Loading on Product Yields over 24 h					
DM Extract Volume (mL)						
0.6	160 ± 1	106 ± 1	307 ± 1	ND	ND	ND
0.8	165 ± 4	106 ± 2	359 ± 10	ND	ND	ND
1.0	235 ± 17	149 ± 9	489 ± 112	trace	trace	ND
DN Extract Volume (mL)						
0.6	207 ± 8	131 ± 4	ND	ND	265 ± 57	ND
0.8	171 ± 12	107 ± 7	ND	ND	317 ± 7	ND
1.0	160 ± 6	99 ± 3	ND	ND	371 ± 12	ND
(B) Effect of Reaction Time on Product Yields						
DM Reaction Time (h)						
1	37 ± 2	229 ± 7	359 ± 10	ND	ND	ND
2	63 ± 2	203 ± 6	354 ± 9	ND	ND	ND
6	114 ± 3	153 ± 4	351 ± 6	ND	ND	ND
24	117 ± 10	90 ± 6	270 ± 16	ND	ND	ND
DN Reaction Time (h)						
1	25 ± 1	205 ± 2	ND	ND	370 ± 30	ND
2	125 ± 4	163 ± 7	ND	ND	254 ± 16	25 ± 2
6	170 ± 25	134 ± 17	trace	ND	189 ± 27	trace
24	167 ± 26	118 ± 18	ND	ND	180 ± 28	trace

[a]Reactions (2 mL) were performed in buffer (50 mM Tris pH 7.0) containing pulegone (1 mM), cell extracts (0.5 mL), NADP$^+$ (10 μM), glucose (15 mM), and GDH (10 U). The reactions were agitated at 30 °C for 1−24 h at 130 rpm. Product yields were determined by GC analysis using a DB-WAX column. ND = none detected; trace = <20 μM product detected in some cultures. Total % yields were within 1−18% of the % conversion in each case. [b]Product from a reaction with menthone. [c]Product from a reaction with isomenthone.

MNMR (DN; Figure 1). These were designed to enrich the products with menthol and neomenthol, respectively. Comparative biotransformations were performed from the three multigene constructs in strain 4, using pulegone as the substrate (Table 1). The results clearly showed an enrichment of the desired product (menthol or neomenthol) from DM and DN, respectively, compared to that from DMN. However, there were still significant levels of menthone/isomenthone and the original substrate at the end of the reaction, suggesting that further optimization is needed to increase MMR/MNMR activity.

Further studies were performed to see if the production of additional NADPH increases enzyme activity. This was tested by the addition of either glucose, to supply the native *E. coli* cofactor recycling systems, or a full externally provided cofactor recycling system (glucose/NADP$^+$/GDH). Reactions in the presence of an externally added cofactor recycling system showed higher product yields in all cases (0.5−3-fold; Table 1). This suggests the incorporation of cofactor recycling genes to the multigene constructs may increase strain productivity. This was previously reported in an *E. coli* strain expressing a glucose dehydrogenase gene from *Bacillus megaterium*, which showed an increase in chiral alcohol production.[20]

Biotransformation Optimization. To further maximize product yields, biotransformations with constructs DM and DN were performed with different levels of cell extracts. Control reactions were performed where the DN/DM extracts and cofactor recycling system were incubated in the absence of monoterpenoids. There was only a moderate improvement in product yields, with the largest effect seen in the increasing NtDBR activity in DN (Table 2A). In some cases, high concentrations of cell extract in biotransformations inhibited product production (DN reactions), and side product formation was significant. The most abundant side product

(up to 470 μM) detected during reaction optimization studies (Table 2A) was identified as the nonterpenoid ester ethyl propanoate. Therefore, this product has likely resulted from metabolic processes independent of the introduced pathways. Increasing the cell extract quantity generated problems with product extractions and clean ups due to viscosity, so further enzyme loading optimization studies will concentrate on increasing protein expression levels.

Reactions also became significantly cloudy after a few hours of incubation, suggesting protein precipitation and/or extract degradation. Therefore, parallel reactions were performed each construct using pulegone as the substrate, and samples were analyzed at time points 1, 2, 6, and 24 h to determine the optimal reaction time (Table 2B). The results clearly show that long incubations with cell extracts lead to product loss in most cases (10−20%). The exception was menthone formation, where yields increased with time. Menthone gain and isomenthone loss over time may simply be due to epimerization activity.

The loss of menthol isomers over time suggests either product breakdown or utilization via other *E. coli* pathways (side products of 4−43% yield). Interestingly, the reaction between DN and pulegone yielded a small quantity (10 μM) of menthol from menthone (MMR activity). However, studies have shown that MMR and MNMR do not have absolute stereochemistry and can produce both enantiomers from menthone and isomenthone.[17]

To further investigate the catalytic abilities of MMR and MNMR, biotransformations with DM and DN were performed with menthone and isomenthone at high levels of cell extract (1 mL) for 1 h. Complete conversion of menthone with DM was obtained, yielding highly pure menthol (79.1%). However, reactions with isomenthone gave lower product yields (43.1%) with a near equal ratio of menthol and neoisomenthol

Table 3. Menthol Production Biotransformations from Optimization Studies with DM Cell Extracts in *E. coli* Strain NiCO₂(DE3)[a]

condition	menthol yield (mg/g protein)	menthol purity (% total product)	condition	menthol yield (mg/g protein)	menthol purity (% total product)
(A) Medium			(B) IPTG Concentration (μM)		
minimal	1.40 ± 0.09 (ND)	18 ± 1 (ND)	0	ND	ND
LB	0.92 ± 0.25 (1.51)	31 ± 8 (54)	10	0.42 ± 0.27	30 ± 19
2YT	1.33 ± 0.28 (1.60)	37 ± 8 (56)	50	0.46 ± 0.22	28 ± 13
TB	1.39 ± 0.34 (0.51)	45 ± 11 (62)	100	0.91 ± 0.26	42 ± 12
SB	1.31 ± 0.29 (0.93)	56 ± 13 (59)	500	0.32 ± 0.13	14 ± 6
TBAIM	1.61 ± 0.39	56 ± 14	1000	0.17 ± 0.01	7 ± 1
(C) Induction Temperature (°C)			(D) Cell Growth at Induction (OD₆₀₀ₙₘ)		
15	0.91 ± 0.04	58 ± 3	initial (induction)	2.02 ± 0.40	57 ± 11
20	1.32 ± 0.14	57 ± 6	0.3	1.93 ± 0.54	56 ± 16
25	0.82 ± 0.11	42 ± 6	0.7	1.28 ± 0.18	55 ± 8
30	0.60 ± 0.15	19 ± 5	1.1	1.44 ± 0.40	57 ± 16
37	ND	ND	2.1	1.64 ± 0.24	59 ± 9
(E) Induction Length (h)					
3	1.11 ± 0.11	76 ± 7			
5	1.27 ± 0.11	57 ± 5			
16	1.80 ± 0.19	53 ± 6			

[a]Culture growth conditions and extract production protocols for each optimization trial are described in the Methods section. Reactions (1 mL) were performed in buffer (50 mM Tris pH 7.0) containing pulegone (1 mM), cell extracts (0.25 mL), NADP⁺ (10 μM), glucose (15 mM), and GDH (10 U). The reactions were agitated at 30 °C for 2 h at 130 rpm. Product yields were determined by GC analysis using a DB-WAX column. Menthol yields are expressed as the milligrams produced per gram of protein in the cell extracts. Purity is the percent menthol produced compared to the total product yields. Minimal = minimal medium; LB = lysogeny broth; 2YT = 2YT broth; TB = Terrific broth; SB = Super broth; TBAIM = Terrific broth autoinduction medium; ND = none detected. Menthol yields in parentheses represents the activity detected in uninduced culture extracts. Full data showing other products produced are shown in Supporting Information Table 4.

Scheme 3. Possible Mechanisms of the Enzymatic (a) Neomenthol Oxidation to Menthone by MNMR and (b) Menthone/Isomenthone Epimerization[a]

[a]The epimerization reaction is based on the current mechanism of glutamate racemase.[21]

(16.3:19.7), presumably due to epimerization activity on isomenthone. Similarly, reactions of DN with menthone (735 μM) produced almost entirely neomenthol (89.9% purity), whereas isomenthone reactions showed lower product yields (440 μM) with a ratio of mostly neomenthol and isomenthol (28:11). Therefore, there is potential to generate highly pure menthol or neomenthol by eliminating MMR or MNMR from the operon, respectively.

Given the large consumer and industrial markets for menthol, further expression optimization studies were performed with the DM construct (Table 3). The efficiency of menthol production (mg/g extract protein) and purity (% of total product yield) was assessed (Table 3) based on the total menthol produced, cell mass generated, and relative proportions of DBR vs MMR production/activity (Supporting Information Table 4). The effect of the medium composition showed that, on average, richer media gave higher yields and purity of menthol (Table 3A). Interestingly, uninduced cultures in less rich media generated more menthol than that from induced cells, which is likely related to the observation of a significant reduction in the levels of insoluble protein production (results not shown). Autoinduction media also gave slightly higher menthol yields than those from IPTG-induced cells. Using rich medium (TB), the optimal IPTG concentration for menthol production over menthone/isomenthone was 100 μM, with a postinduction temperature of 20 °C (Table 3B,C and Supporting Information Table 4B,C). Slightly higher yields of menthol were produced when induction occurred within the first couple of hours of culture growth (Table 3D), although the menthol purity was essentially

the same at any induction time (55−59%). Interestingly, the highest purity of menthol (76%) was obtained at the shortest induction length (3 h at 20 °C; Table 3E); however, the overall menthol yield was reduced due to lower cell density.

Side Reactions. An important aspect of designing an optimal pathway is the elimination of any unwanted side reactions. An interesting observation in this context is the apparent correlation between neomenthol loss and menthone gain (Table 2). This suggests the presence of an oxidase acting on neomenthol, converting it back to menthone. We performed reactions where neomenthol was incubated with DN and control *E. coli* extracts for 24 h to check if *E. coli* contained a contaminating neomenthol oxidase. Significant menthone (261 μM) and isomenthone (173 μM) were produced in the DN reactions but not in the control ones, suggesting that the oxidase activity is due solely to the reversibility of the ketoreduction reaction by MNMR (Scheme 3a). This reaction is likely to proceed via a proton abstraction from the hydroxyl group by a nearby basic residue and a simultaneous hydride transfer to NADP$^+$, resulting in oxidation at the 3-position. Prior studies of menthone reductases showed that ketoreduction of menthone by MNMR, but not MMR, was reversible, with a relatively high K_m for neomenthol (1 mM).[17]

Menthone and isomenthone isomerization within *E. coli* extracts (Figure 2) is likely to proceed via a classical glutamate racemase-type mechanism (Scheme 3b). First, a base extraction of an acidic proton alpha to the carbonyl group results in formation of an enolate. This acts as a nucleophile and abstracts a proton from an acidic residue. The proton could potentially be attacked from either face of neomenthol, resulting in reformation of the initial substrate or formation the isomerized product (Scheme 3b).

Conclusions. We have successfully engineered the later stages of the mint production pathway, employing a careful quantitative characterization of all individual building blocks (e.g., selection of active and stereo/enantiomerically suitable biocatalysts) and developing simple unidirectional gene expression systems for codon-optimized genes. In addition, we optimized the production further by assessing the expression of each part within an assembled pathway by incorporating protein identification tags. Other optimization strategies employed were selecting the best production host strain, characterizing the influence of the promoter strength on the concerted enzyme expression and reaction in a pathway, optimizing the cofactor availability, and understanding and decreasing of side reactions (Figure 3). These optimization steps enabled 50% improvement in production of menthol and in another case increased purity to 76% from pulegone, thus greatly improving the production from 0% in the first construct (DMN in strain 1). The optimization steps described in this article are required to achieve the maximum production levels of any compound. By sidestepping the time-consuming host optimization and *in vivo* biosynthesis for most of the optimization pipeline, we could substantially speed up the process.

Our studies have demonstrated the successful integration of chemical synthesis of pure precursor(s) with biochemical analysis of each enzymatic steps to select the best combination of enzymes and powerful, improved pathway assembly methods for easy engineering. In addition, we have demonstrated moderate final production of the end compound(s); this production enabled easy analysis for further high-throughput screening, by deploying one-pot biosynthesis of menthol.
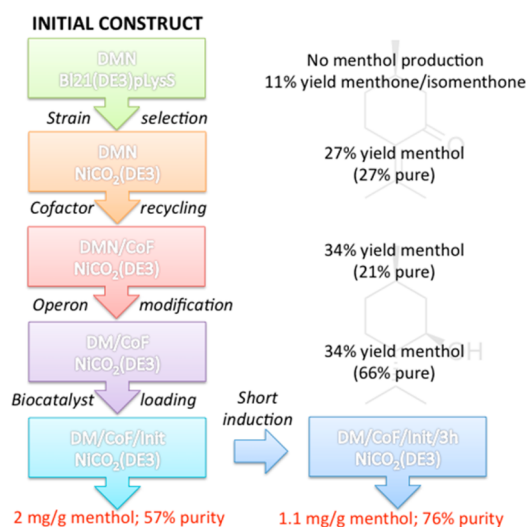
Figure 3. Optimization scheme and achieved menthol production and/or purity using cell extracts of recombinant *E. coli* expressing plant biosynthetic genes (DMN and DM) in a one-pot biosynthesis starting from pulegone. CoF = external cofactor recycling system incorporated in the reaction; Bl21(DE3)pLysS/NiCO₂(DE3) = *E. coli* strains; Init = induction at inoculation. Menthol purity (%) reflects the proportion of menthol vs other monoterpenoid products.

Compared to the use of *in vivo* cells for production, this approach has particularly important benefits when producing (semi)toxic chemical compounds. This approach is also useful when aiming for the easy optimization of individual enzyme steps or their combinations for the production of chemically diverse compound libraries. These benefits are highly relevant when optimizing the production of high-value chemicals in a high-throughput manner.

■ METHODS

General Reagents and Procedures. All chemicals and solvents were purchased from commercial suppliers, except where specified, and were of analytical grade or better. Media components were obtained from Formedium (Norfolk, UK). Gene sequencing and oligonucleotide synthesis were performed by Eurofins MWG (Ebersberg, Germany). Chemical syntheses were monitored by thin-layer chromatography using Merck aluminum foil-coated TLC plates carrying silica gel 60 F254 (0.2 mm thickness). Ultraviolet light, cerium molybdate (12 g of ammonium molybdate, 0.5 g of ceric ammonium molybdate, and 15 mL of concentrated sulfuric acid), and/or phosphomolybdic acid (10 g of phosphomolybdic acid in 100 mL of absolute ethanol) were used to detect compounds. Purification of compounds was carried out using column chromatography (Fluka Analytical high-purity grade silica gel, 60 Å pore size, 220−440 mesh particle size, 35−75 μm particle size). NMR spectra were recorded on a 400 MHz spectrometer and referenced to the solvent. All monoterpenoid compounds were dissolved as stock solutions in absolute ethanol, with a final concentration of 2% (v/v) in the reactions. The ^{1}H and ^{13}C NMR spectra for the synthesized compounds can be found in the Supporting Information.

Synthesis of Isomenthone. Menthone (1.5 g, 9.7 mmol) was dissolved in methanol (15 mL), and a 10% aqueous solution of NaOH (1.5 mL, 10% w/v) was added. The solution was stirred at room temperature for 3 h, followed by solvent removal. The compound(s) were dissolved in ethyl acetate and

washed with water and brine. The organic layer was dried over magnesium sulfate, filtered, and reduced. Isomenthone (451 mg, 30%) was separated from menthone (959 mg, 64%) by column chromatography using gradient elution (hexane/diethyl ether, 98:2 to 90:10, v/v). ^{1}H NMR (400 MHz; CDCl$_3$) δ 2.30 (ddt, 1H, H-2a, J = 13.1, 4.5, 1.2 Hz), 2.11 (dd, 1H, H-2b, J = 13.2, 10.1 Hz), 2.06−1.91 (m, 4H, H-1, H-4, H-5a, H-8), 1.76−1.66 (m, 2H, H-5b, H-6a), 1.52−1.43 (m, 1H, H-6b), 0.99 (d, 3H, H-7a-c, J = 6.6 Hz), 0.93 (d, 3H, H-9a-c/H-10a-c, J = 6.5 Hz), 0.84 (d, 3H, H-9a-c/H-10a-c, J = 6.6 Hz). ^{13}C NMR (101 MHz; CDCl$_3$) δ 57.3 (C4), 48.1 (C2), 34.5 (C1), 29.5 (C6), 27.05 (C8), 27.0 (C5), 21.6 (C7), 21.0, 20.0 (C9, C10).

Synthesis of Menthol and Neomenthol. Sodium borohydride (90 mg, 3.36 mmol) was added portionwise to a stirred solution of menthone (320 mg, 2.077 mmol) in tetrahydrofuran/methanol (10 mL, 9:1, v/v) at 0 °C. The solution was returned to room temperature and stirred for 30 min. The reaction was quenched at 0 °C by slow addition of water (4 mL), followed by addition of 1 M HCl (4 mL). The menthol derivatives were extracted with dichloromethane, and the organic layer was washed with water and brine. The organic layer was then dried over magnesium sulfate, filtered, and reduced. GC analysis was performed on a sample of the crude, which gave the product ratio of menthol/neomenthol/neo-isomenthol as 60:30:4. Menthol (165 mg, 51%), neomenthol (88 mg, 27%), and neoisomenthol (trace) were then separated and isolated by column chromatography using a gradient elution system (hexane/diethyl ether, 49:1 to 9:1, v/v).

Menthol: ^{1}H NMR (CDCl$_3$, 400 MHz) δ 3.39 (td, 1H, H-3, J = 10.5, 4.3 Hz), 2.16 (dtd, 1H, H-8, J = 14.0, 7.0, 2.8 Hz), 1.97−1.92 (m, 1H, H-2a), 1.67−1.56 (m, 3H, OH, H-5a, H-6a), 1.44−1.36 (m, 1H, H-1), 1.09 (ddt, 1H, H-4, J = 12.4, 9.8, 2.8 Hz), 1.04−0.81 (m, 3H, H-2b, H-5b, H-6b), 0.91−0.90 (d, 3H, CH$_3$, J = 6.27 Hz), 0.90−0.88 (d, 3H, CH$_3$, J = 5.83 Hz), 0.79 (d, 3H, CH$_3$, J = 7.0 Hz). ^{13}C NMR (101 MHz; CDCl$_3$) δ 71.6 (C3), 50.2 (C4), 45.1 (C2), 34.6 (C6), 31.7 (C1), 25.9 (C8), 23.2 (C5), 22.3 (C7), 21.1 (C10), 16.2 (C9). Neomenthol: ^{1}H NMR (CDCl$_3$, 400 MHz) δ 4.10−4.09 (d, 1H, H-3, J = 2.85 Hz), 1.85−1.80 (dq, 1H, H-2a, J = 13.72, 3.02 Hz), 1.74−1.63 (m, 3H, H-1, H-5a, H-6a), 1.56−1.47 (ddt, 1H, H-8, J = 13.33, 9.34, 6.67 Hz), 1.37 (s, 1H, OH), 1.28−1.21 (m, 1H, H-5b), 1.11−1.10 (m, 1H, H-2b), 0.96−0.94 (d, 3H, H-7a-c, J = 6.67), 0.92−0.90 (d, 3H, H-9a-c/10a-c, J = 6.65), 0.87−0.85 (d, 3H, H-9a-c/10a-c, J = 6.33), 1.00−0.84 (m, 2H, H-4, H-6b). ^{13}C NMR (101 MHz; CDCl$_3$) δ 67.8 (C3), 48.0 (C4), 42.7 (C2), 35.2 (C6), 29.3 (C8), 25.9 (C1), 24.3 (C5), 22.4 (C7), 21.3 (C9/C10), 20.8 (C9/C10).

Synthesis of Neoisomenthol. Sodium borohydride (45 mg, 1.168 mmol) was added portionwise to a stirred solution of menthone (150 mg, 0.974 mmol) in tetrahydrofuran/methanol (5 mL, 9:1, v/v) at 0 °C. The solution was returned to room temperature and stirred for 30 min. The reaction was quenched at 0 °C by slow addition of water (2 mL) followed by addition of 1 M HCl (2 mL). The menthol derivatives were extracted with dichloromethane, and the organic layer was washed with water and brine. The organic layer was then dried over magnesium sulfate, filtered, and reduced. GC analysis was performed on a sample of the crude, which gave the product ratio of neoisomenthol/menthol/neomenthol/isomenthol as 93:4:1.5:0.6. Neoisomenthol (123 mg, 82%) was separated from the other isomers by column chromatography (hexane/diethyl ether, 99:1 to 95:5, v/v). ^{1}H NMR (CDCl$_3$, 400 MHz) δ 4.02−3.99 (dt, 1H, H-3, J = 6.26, 3.22 Hz), 1.78−1.70 (m,

1H, H-1), 1.68−1.52 (m, 4H, H-8, H-2a, H-2b, H-5a), 1.46−1.36 (m, 4H, H-5b. H-6a, H-6b, OH), 1.15−1.083 (m, 1H, H04), 1.067−1.049 (d, 3H, CH$_3$, J = 7.10 Hz), 0.99−0.98 (d, 3H, CH$_3$, J = 6.63 Hz), 0.92−0.90 (d, 3H, CH$_3$, J = 6.67 Hz). ^{13}C NMR (CDCl$_3$, 101 MHz) δ 70.8 (C3), 47.5 (C4), 39.1 (C2), 31.1 (C6), 28.4 (C1), 27.6 (C8), 21.9 (C5), 21.6 (3 × CH$_3$).

Synthesis of Isomenthol. Neomenthol (80 mg, 0.519 mmol) was dissolved in anhydrous tetrahydrofuran (5 mL). To this solution were added triphenylphosphine (163 mg, 0.622 mmol) and p-nitrobenzoic acid (104 mg, 0.622 mmol). Upon dissolution of these reagents, di-tert-butylazodicarboxylate (143 mg, 0.622 mmol) was added portionwise over 30 min. The reaction was stirred at room temperature overnight. The solvent was reduced, and the diethyl ether was added to the crude, causing triphenylphosphine oxide to precipitate. This was filtered off, the solvent was removed, and the process was repeated until no more triphenylphosphine oxide precipitated. The crude was then purified by column chromatography (hexane/diethyl ether, 98:2, v/v) and the p-nitrobenzoate derivative (107 mg) was isolated in 68% yield. ^{1}H NMR (400 MHz; CDCl$_3$) δ 8.29−8.20 (m, 4H, ArCH), 5.36−5.32 (td, 1H, H-3, J = 6.2, 3.3 Hz), 1.98−1.32 (qd, 1H, H-1, J = 7.4, 3.9 Hz), 1.83−1.73 (m, 2H, H-2a, H-8), 1.69 (dt, 1H, H-5a, J = 8.9, 4.3 Hz), 1.63−1.48 (m, 4H, H-2b, H-4, H-5b, H-6a), 1.28 (dtd, 1H, H-6b, J = 12.6, 8.6, 3.7 Hz), 0.98 (dd, 6H, H-9a-c, H-10a-c, J = 8.0, 6.9 Hz), 0.90 (d, 3H, H-7a-c, J = 6.7 Hz). ^{13}C NMR (101 MHz; CDCl$_3$) δ 164.1 (C=O), 150.5 (ArCNO$_2$), 136.5 (ArC), 130.7 (ArCH), 123.6 (ArCH), 74.0 (C3), 45.7 (C4), 35.7 (C2), 29.9 (C6), 27.8 (C1), 26.5 (C8), 21.4 (C5), 21.01, 20.81, 19.4 (C7, C9, C10).

The p-nitrobenzoate derivative was dissolved in a tetrahydrofuran/water mixture (2 mL, 4:1, v/v), and lithium hydroxide monohydrate (47 mg) was added. The solution was placed in a sealed sample vial and heated at 40 °C for 4 h. Water was then added, and the compound was extracted with diethyl ether. The organic layer was dried over magnesium sulfate, filtered, and reduced. Column chromatography (hexane/diethyl ether, 98:2, v/v) gave isomenthol (46 mg) in an 85% yield. ^{1}H NMR (CDCl$_3$, 400 MHz) δ 3.83−3.78 (td, 1H, H-3, J = 7.89, 3.79), 2.024 (m, 2H, H-1, H-8), 1.65−1.27 (m, 6H, H-2a, H-2b, H-5a, H-5b, H-6a, H-6b), 1.419 (s, 1H, OH), 1.19−1.12 (m, 1H, H-4), 0.953−9.35 (d, 6H, H-9a-c, H-10a-c, J = 7.0), 0.88−0.87 (d, 3H, H-7a-c, J = 6.83 Hz). ^{13}C NMR (101 MHz; CDCl$_3$) δ 68.0 (C3), 49.7 (C4), 40.1 (C2), 30.5 (C6), 27.6, 26.1 (C8, C1), 21.0, 19.9 (C9/C10), 19.5 (C5), 18.1 (C7).

Gene Synthesis and Modifications. The double-bond reductase from *Nicotiana tabacum* (NtDBR-His$_6$ in pET21b; UniProt: Q9SLN8) was prepared as described previously.[18] The protein sequences for the following enzymes were obtained from UniProt (http://www.uniprot.org): (i) (−)-menthone:(−)menthol reductase from *M. piperita* (MMR; UniProt: Q5CAF4) and (ii) (−)-menthone:(+)-neomenthol reductase from *M. piperita* (MNMR; UniProt: Q06ZW2). The respective gene sequences were designed and synthesized by GenScript (USA), incorporating codon optimization techniques of rare codon removal for optimal expression in *E. coli*. The genes were subcloned individually into pET21b (Novagen) via *Nde*I/*Xho*I restriction sites, without a stop codon, to incorporate a C-terminal His$_6$-tag to allow expression monitoring by western blotting. Due to poor expression of the MNMR-His$_6$ construct, the gene was subcloned into pET15b, via *Nde*I/*Xho*I restriction sites, to

Table 5. *E. coli* Strains and Growth Conditions with pET21b Constructs

name	strain	media	antibiotics	growth conditions[a]	source
control[b]	Bl21(DE3)pLysS	TBAIM	Amp + Chl	autoinduction	Novagen
strain 1	Bl21(DE3)pLysS	TB + 0.4% glycerol	Amp + Chl	standard	Novagen
strain 2	Bl21-Codon Plus(DE3)RIPL	TB + 0.4% glycerol	Amp + Chl	standard	Agilent
strain 3	Arctic Express(DE3)	TB + 0.4% glycerol	Amp + Gent	low temperature	Agilent
strain 4	NiCO2 21(DE3)	TBAIM	Amp	autoinduction	NEB
strain 5	Rosetta(DE3)pLysS	TB + 0.4% glycerol	Amp + Chl	standard	Novagen
strain 6	Rosetta2(DE3)pLysS	TB + 0.4% glycerol	Amp + Chl	standard	Novagen
strain 7	Tuner(DE3)	TBAIM	Amp	autoinduction	Novagen
strain 8	Bl21(DE3)	TBAIM	Amp	autoinduction	Novagen
strain 9	Rosetta2(DE3)	TBAIM	Amp + Chl	autoinduction	Novagen
strain 10	Origami2(DE3)	TBAIM	Amp + Strep	autoinduction	Novagen
strain 11	BLR(DE3)	TBAIM	Amp	autoinduction	Novagen
strain 12	HMS174(DE3)	TBAIM	Amp + Rifam	autoinduction	Novagen

[a]Standard expression conditions: Incubate at 37 °C until OD_{600} = 0.5, induce with 0.4 mM IPTG and incubate at 25 °C overnight at 200 rpm shaking. Autoinduction conditions: Incubate at 25 °C for 24 h at 200 rpm shaking. Low temperature = Incubate at 37 °C for 3 h, then 10 min at 12 °C. Induce with 1 mM IPTG and incubate at 12 °C overnight at 200 rpm shaking. [b]Contains empty pET21b. Amp = 100 μg mL^{-1} ampicillin; Chl = 34 μg mL^{-1} chloramphenicol; Gent =20 μg mL^{-1} gentamycin; Strep = 50 μg mL^{-1} streptomycin; Rifam = 200 μg mL^{-1} rifampicin.

generate a N- and C-terminally His$_6$-tagged protein. Each construct was transformed into the *E. coli* strain BL21(DE3)-pLysS (Stratagene) for soluble protein overexpression according to the manufacturer's protocol.

Protein Production and Purification. A general protocol for the production and purification of each individual His$_6$-tagged protein was used, based on the NtDBR method described previously.[18] Cultures of *E. coli* BL21(DE3)pLysS containing expression vectors were grown in (12 × 1 L) Terrific broth (TB; tryptone 12 g L^{-1} and yeast extract 24 g L^{-1}; pH 7.0), supplemented with glycerol (0.4%), ampicillin (100 μg mL^{-1}; 15 μg mL^{-1} kanamycin for MNMR in pET15b) and chloramphenicol (34 μg mL^{-1}). Cultures were incubated at 37 °C until OD_{600nm} reached 0.5, followed by a 16 h induction with isopropyl-β-D-1-thiogalactopyranoside (IPTG; 10 μM) at 25 °C. Cells were harvested by centrifugation at 5000g for 10 min at 4 °C. Cell pellets were resuspended in lysis buffer 1 (50 mM Tris, pH 8.0, containing the EDTA-free complete protease inhibitor cocktail, 1 mM MgCl$_2$, 0.1 mg mL^{-1} DNase I, 0.1 mg mL^{-1} lysozyme, and 10% glycerol) and stirred for 20 min at 4 °C. Cells were disrupted by sonication (Sonics Vibra Cell) followed by extract clarification by centrifugation for 60 min at 26 600g. The clarified supernatants were passaged twice through Ni^{2+} Sepharose, as described previously.[18] Subsequent gel filtration was required for MMR and MNMR on a HiLoad 16/600 Superdex 200pg column (GE Healthcare) column pre-equilibrated in buffer A (50 mM Tris pH 8.0 containing 1 mM 2-mercaptoethanol, 10% sorbitol, 10% glycerol). An isocratic elution of the protein from the column was carried out in the same buffer. Purified enzymes were dialyzed into cryobuffer (10 mM Tris pH 7.0 containing 10% glycerol) and flash frozen in liquid nitrogen for storage at −80 °C. Protein concentration was determined using the Bradford and extinction coefficient methods.[22] In the case of MMR and MNMR, 2-mercaptoethanol (1 mM) was included in all buffers.

Protein Detection and Identification. Purity was assessed by SDS-PAGE, using 10−12% Mini-PROTEAN TGX Stain-Free gels and Precision Plus protein unstained markers (BioRad) according to the manufacturer's instructions. Identification of His$_6$-tagged proteins was performed by western blots using the Trans-Blot Turbo transfer system (PVDF membranes; BioRad) and the Western Breeze chemiluminescent immunodetection kit (alkaline phosphatase; Life Technologies) with mouse (His-tag monoclonal antibody) and alkaline phosphatase-containing (Anti-C-My) primary and secondary antibodies, respectively.

Enzyme Kinetics. The concentration of nicotinamide coenzymes (Melford) was determined by the extinction coefficient method (ε_{340} = 6220 M^{-1} cm^{-1}). Steady-state kinetic analyses were performed on a Cary UV-50 Bio UV/vis scanning spectrophotometer using a quartz cuvette (1 mL; Hellma) with a 1 cm path length. Standard reactions (1 mL) were performed in buffer (50 mM Tris, pH 7.0) containing NADPH (50−100 μM for MMR/MNMR and NtDBR, respectively), monoterpenoid (1 mM), and enzyme (30 nM to 2 μM). Reactions were followed by continuously monitoring NADPH oxidation at 340 nm for 1 min at 25 °C. The standard monoterpenoid substrates used were pulegone for NtDBR (alkene reduction)[18] and menthone/isomenthone for both MMR and MNMR (ketoreduction).[23] Ketoreductase reactions were performed in an alternative buffer (12.5 mM trisodium citrate, 12.5 mM KH$_2$PO$_4$, 12.5 mM K$_2$HPO$_4$, and 12.5 mM CHES) containing dithiothreitol (1 mM; DTT). All steady-state reactions were performed in at least duplicate.

Operon Construction. Coexpressing multigene constructs were prepared in pET21b using the recombination-based In-Fusion HD cloning kit (Takara/Clontech) with sequential gene addition according to the manufacturer's protocols. These protocols (Figure 2) are summarized by the following steps: (i) vector linearization (containing gene 1) and new gene amplification by PCR (a 13 bp Shine−Dalgarno sequence (SD; GGAGGACAGCTAA) was incorporated between the stop and start codons of successive genes to allow expression from one promoter (T7*lac*; Figure 2); (ii) template removal by cloning enhancer (Takara/Clontech) or *Dpn*I (New England Biolabs; NEB) restriction digest; (iii) gel extraction and purification of PCR products; (iv) In-Fusion cloning reaction between the vector and new gene; (v) transformation into an *E. coli* cloning strain (Stellar; Takara/Clontech); (vi) plasmid preparation and sequencing; and (vii) repeating the above steps to incorporate any additional genes. Each PCR product contained a 15 bp overlap (Figure 2) between the vector and insert pairs to facilitate recombination. To generate the long overhangs, overlapping sets of PCR primers were used, with the

outermost oligo at a considerably higher concentration than that of the innermost one (ratio 5:1). The PCR primers used are found in Supporting Information Table 5. In most cases, the PCR reactions, template removal, and gel purification of DNA were performed using the kit protocols and enzymes (CloneAmp PCR premix; cloning enhancer). However, in some cases, alternative enzymes were used, such as Q5 DNA polymerase and *Dpn*I restriction enzyme (NEB). The following three constructs were generated: (i) NtDBR-His$_6$-SD-MMR-His$_6$-SD−His$_6$-MNMR (DMN); (ii) NtDBR-His$_6$-SD-MMR-His$_6$ (DM), and (iii) NtDBR-His$_6$-SD-His$_6$-MNMR (DN). Constructs were transformed into the *E. coli* expression strain BL21(DE3)pLysS, according to the manufacturer's protocols, to check the expression levels of the individual genes. Cell extracts of each culture were obtained and checked for recombinant protein expression by SDS-PAGE, western blotting, and biotransformations.

Optimization of Multigene Expression. The multigene construct DMN was transformed into a further 11 *E. coli* expression strains according to the manufacturers' protocols (Table 5). Small-scale cultures of each strain (1 L) were produced as described in Table 5. A generalized Terrific broth autoinduction medium (TBAIM; Formedium) protocol was chosen, except for strains with the pLysS phenotype and Arctic Express (DE3) cells. However, autoinduction protocols were used for the control cells (Bl21(DE3)pLysS containing pET21b), as no recombinant gene expression is needed. Further optimization was performed by culture growth under the conditions used to generate the individual enzymes, except that the IPTG concentration was varied (10 μM to 1 mM). Cell extracts of each culture were obtained and checked for recombinant protein expression by SDS PAGE, western blotting, and biotransformations.

Biotransformation Reactions. All biotransformation reactions were performed in at least duplicate, and the results are averages of the data. Biotransformations with DBR were performed as described previously.[18] Ketoreductase reactions (1.0 mL) with purified enzymes were performed in biotrans buffer (50 mM Tris, pH 7.0) containing the monoterpenoid (5 mM), NADP$^+$ (10 μM), glucose (15 mM), glucose dehydrogenase (GDH from *Pseudomonas* sp.; 10 U), and enzyme (2 μM). Reactions were shaken at 30 °C for 24 h at 130 rpm and terminated by extraction with ethyl acetate (0.9 mL) containing an internal standard (1% *sec*-butyl-benzene in ethyl acetate). The extracts were dried using anhydrous magnesium sulfate and analyzed by GC. Quantitative analysis was carried out by a comparison of product peak areas to standards of known concentrations. Products were identified by comparison with authentic standards.

Cell pellets of multigene constructs (from 1 L culture; Table 5) were combined with equal volumes (15 mL) of lysis buffer 2 (lysis buffer 1 at pH 7.0 containing 1 mM 2-mercaptoethanol), and clarified extracts were produced as above. Standard biotransformation reactions were performed with modifications (2 mL; 25 mL reaction vials; 25 °C) with cell extracts (0.5 mL per 2 mL reaction, equivalent to around 30 mL of culture) in the presence or absence of an externally added cofactor recycling system. The substrates tested were pulegone, menthone, and isomenthone (1 mM), and all possible products were processed as described above. Control reactions were performed with cell extracts of an *E. coli* construct containing an empty pET21b vector. To test the effect of enzyme loading on product yields, the cell extract volume was varied (0.6−1.0

mL/2 mL reaction). To minimize substrate/product decomposition and/or utilization by other *E. coli* pathways, reactions (1 mL) were performed and analyzed at different times (1, 2, 6, and 24 h).

Latter optimization studies with DM cultures were performed as described above, except that a smaller culture volume was used (50 mL). The different medium compositions trialed (for medium preparations unless stated) were minimal medium (M9 salts containing 10 μM FeSO$_4$, 1 mM MgSO$_4$, 100 μM CaCl$_2$, and 0.4% glycerol), LB, 2YT, TB, SB (35 g/L tryptone, 20 g/L yeast extract, and 5 g/L NaCl), and TB autoinduction medium. Induction parameters varied were IPTG concentration (0−1 mM), induction time (OD$_{600nm}$ = 0.01−2.1), postinduction temperature (15−37 °C), and length (3−16 h). Cell-free extracts and biotransformations (1.0 mL) were performed as described above.

Analytical Procedures. Reaction extracts (1 μL) were analyzed by gas chromatography on an Agilent Technologies 7890A GC system equipped with an FID detector and a 7693 autosampler. A DB-WAX column (30 m; 0.32 mm; 0.25 μm film thickness; JW Scientific) was used to separate the seven substrates and product isomers/enantiomers: pulegone (coelutes with isomenthol), menthone, isomenthone, menthol, neomenthol, and neoisomenthol. In this method, the injector temperature was at 220 °C with a split ratio of 20:1 (1 μL injection). The carrier gas was helium with a flow rate of 1 mL min^{-1} and a pressure of 5.1 psi. The program began at 40 °C with a hold for 1 min followed by an increase of temperature to 210 °C at a rate of 15 °C/min, with a hold at 210 °C for 3 min. The FID detector was maintained at a temperature of 250 °C with a flow of hydrogen at 30 mL/min. To quantify isomenthol yields, the program was modified (Supporting Information Figure 2) to allow the separation of pulegone and isomenthol (increase of temperature to 210 °C at a rate of 10 °C/min, with a hold at 210 °C for 1 min). Unknown products were identified by gas chromatography in combination with mass spectrometry on an Agilent Technologies 7890A GC with an Agilent Technologies 5975C inert XL-EI/CI MSD with triple axis detector and a Zebron ZB-Semi Volatiles column (15 m × 0.25 mm × 0.25 μm film thickness, Phenomenex). In this method, the injector temperature was at 220 °C with a split ratio of 10:1 (1 μL injection). The carrier gas was helium with a flow rate of 1 mL min^{-1} and a pressure of 5.1 psi. The program began at 40 °C with a hold for 3 min followed by an increase of temperature to 210 °C at a rate of 10 °C/min, with a hold at 210 °C for 3 min. The mass spectra fragmentation patterns were entered into the NIST/EPA/NIH 11 (mass spectral library for identification of a potential match.

■ ASSOCIATED CONTENT

⑤ Supporting Information

Additional product synthesis methodology. Table 1: Product distributions for sodium borohydride reduction of menthone and isomenthone. Table 2: Biotransformations of the purified enzymes. Table 3: Biotransformations of cell extracts of DMN in 12 *E. coli* expression strains. Table 4: Biotransformations of cell extracts of DN produced under different growth conditions. Table 5: PCR primers used in the production of multi-gene expression constructs. Figure 1: ^{1}H and ^{13}C NMR spectra for synthesised compounds. Figure 2: Products formed during biotransformations of DMN cell extracts in strains 6 and 7 at different IPTG concentrations and TBAIM media. Figure 3: GC trace showing the separation of seven monoterpenoids on a

DB-WAX column. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.5b00092.

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Notes
The authors declare no competing financial interest.

■ ABBREVIATIONS

NtDBR, double-bond reductase from *Nicotiana tabacum*; MMR, (−)-menthone:(−)menthol reductase from *Mentha piperita*; MNMR, (−)-menthone:(−)neomenthol reductase from *M. piperita*; GDH, glucose dehydrogenase; DTT, dithiothreitol; SD, Shine−Dalgarno sequence; IPTG, isopropyl-β-D-1-thiogalactopyranoside; TB, Terrific broth; TBAIM, Terrific broth autoinduction medium; expression gene clusters: DM, NtDBR and MMR; DN, NtDBR and MNMR; DMN, NtDBR, MMR, and MNMR

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