A ‘Plug and Play’ Platform for the Production of Diverse Monoterpenoid Hydrocarbon Scaffolds in *Escherichia coli*.

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Supporting Information

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Experimental Section

Bacterial strains and media
All *E. coli* strains (Table S1) were routinely grown in Lysogeny Broth (LB) or on LB agar plates including antibiotic supplements as appropriate (ampicillin, 100 μg ml⁻¹; kanamycin, 50 μg mL⁻¹). Cloning and plasmid propagation was performed using *E. coli* α-Select (Bioline).

Construction of plasmids
To construct plasmid pMVA (Figure S1, panel A), the hybrid MVA pathway and *E. coli* idi (*Ecidi*), including 5' PlacUV5 was PCR amplified using primers MVA-F and MVA-R (Table S9) from pJBEI-6410[1]. The p15a origin of replication was similarly amplified using primers p15a-F and p15a-R and a kanamycin resistance cassette was amplified from pBbA1k-RFP[2] using primers kan-F and kan-R. These fragments were phosphorylated and assembled using the ligase cycling reaction with bridging oligos brMBAori, brorikan, crkanMVA (Table S2) as previously described[3].

pBbB2a-trAgGPPS(co)-trMsLS (Figure S1, panel B) was constructed by insertion of *Abies grandis* GPPS2 (GPPS_Ag) and *Mentha spicata* (limS_Ms) into plasmid pBbB2a-RFP[2]. The predicted plastidial signal sequence was removed from both LimS_Ms and GPPS_Ag, as previously reported[1, 4] and were codon-optimised for expression in *E. coli* (GeneArt, Life Technologies) and The Ribosome Binding Site Calculator[5] was used to design bespoke 5’ untranslated regions (UTRs) encoding a RBS sites for each gene (A.U.=15,000) and to remove any potential aberrant, mid-gene translational start sites (A.U.<1,000). The limS_Ms unit (including 5’ UTR) was flanked with HindIII and BamHI restriction sites and both gene units were synthesised together (GeneArt) flanked by EcoRI and XhoI restriction sites, for subcloning into pBbB2a-GFP.

For production of other monoterpenoids the limS_Ms gene was replaced by genes encoding various other monoterpen synthases (Table S3). Synthetic genes encoding monoterpen synthases without plastidial signal sequences were codon-optimised for expression in *E. coli* and sub-cloned into the Ncol-XhoI restriction sites of pETM-11 (a modified version of the pET24-b vector, Novagen), fused to a TEV protease cleavable N-terminal His₆-tag. Monoterpen synthase genes, including RBS, were amplified from the pETM-11 plasmids using primers pETM-11_Fw and pETM-11_Rv (Table S2). PCR fragments were cloned between the HindIII and BamHI sites of the pBbB2a-trAgGPPS(co)-trMsLS, thereby removing the original limS_Ms gene. The 3 geraniol synthase genes (GerS_Ob, GerS_PC and GerS_Ct) were cloned by Infusion (Takara) after PCR amplification (primer pairs trObGES_Fv-over / trObGES_Rv-over, trPcGES_Fv-over / trPcGES_Rv-over, trCtGES_Fv-over / trCtGES_Rv-over, Table S2) into plasmid pGPPSmTC/S15 which was PCR linearized (pLimS_inverse_rev/ pLimS_inverse_Fw, Table S2).

The control plasmid pBbGPPS, without mTC/S was constructed by amplification of the complete tp pSBC000339 plasmid except LimS_Ms using primers pBbGPPS_Fw and pBbGPPS_Rv, the resulting PCR product was digested with HindIII and subsequently self-ligated.
Monoterpene production conditions, product capture and detection

Expression strains were inoculated with freshly transformed colonies into 3 ml terrific broth (TB) supplemented with 0.4 % glucose and antibiotics in 28 ml glass screw capped vials. Cultures were grown for 7 h at 37 °C with shaking at 200 rpm before transferring to 30 °C and induction with 50 μM (isopropyl β-D-1-thiogalactopyranoside) IPTG and 25 nM anhydro-tetracycline (aTet) and overlaid with a 20 % n-nonane layer followed by incubation for 72 h with shaking at 200 rpm.

A₆₀₀ readings were taken of the aqueous phase, and nonane layers were harvested and clarified by centrifugation (14,000 rpm, 3 min, 4°C), dried over anhydrous MgSO₄ and mixed 1:1 with ethyl acetate containing 0.1 % sec-butyl benzene.

Monoterpenes were analysed by GCMS using an Agilent Technologies 7890B GC equipped with an Agilent Technologies 5977A MSD. The products were separated on a DB-WAX column (30 m x 0.32 mm i.d., 0.25 μM film thickness, Agilent Technologies). The injector temperature was set at 240°C with a split ratio of 20:1 (1 μL injection). The carrier gas was helium with a flow rate of 1 mL/min and a pressure of 5.1 psi. The following oven program was used: 50°C (1 min hold), ramp to 68°C at 5°C/min (2 min hold), and ramp to 230°C at 25°C/min (2 min hold). The ion source temperature of the mass spectrometer (MS) was set to 230°C and spectra were recorded from m/z 50 to m/z 250. Compound identification was carried out using authentic standards and comparison to reference spectra in the NIST library of MS spectra and fragmentation patterns.

Monoterpenoids were quantified using authentic standards wherever possible, using experimentally determined relative response factors in relation to the internal standard used. In the absence of an authentic standard concentrations were estimated using a relative response factor of 1.
Figure S1: Plasmid maps of pMVA (A) and pBbB2a-trAgGPPS(co)-trMsLS (B). Figures were prepared using the SnapGene software package.
## Table S1: Strains used in this study

| Strain name          | Strain | Reference     |
|----------------------|--------|---------------|
| **Chassis**          |        |               |
| DH5α (α-Select)      | K-12   | Bioline       |
| MG1655               | K-12   | [6]           |
| W3110                | K-12   | [7]           |
| DH1                  | K-12   | [8]           |
| MDS42 Meta           | K-12   | Scarab Genomics |
| BL21 (λ-DE3)         | B      | New England Biolabs |
| Mach1                | W      | Thermo-Fisher |
| **Production strain**|        |               |
| DH5α (α-Select)      | K-12   | Bioline       |
Table S2: Primers used in this study

| Primer                  | Sequence (5’-3’)                                                                 |
|-------------------------|---------------------------------------------------------------------------------|
| JBEIMVA-F               | ATTTAGAAAAATAAAACATATAGGGGCC                                                  |
| JBEIMVA-R               | TTATTTAAGCTGGTAAATGCA                                                        |
| p15a-F                  | GGATCCAAACTCGAGTAAAGG                                                        |
| p15a-R                  | GTTAACTGTCAGACCAAGTTTAC                                                       |
| kan-F                   | TCGACGTGGAATTGCCGAC                                                          |
| kan-R                   | TCAGAGAAACTCGTCAAGAAGG                                                       |
| brMVAori                | CCCAGAAAACGATTATCTGCAATTACCCAGCTAAAAATGAGATCAAACATCGAGAGAGATCTCCAG          |
| brorikan                | AAAGCGAGCTCGTAACTTGGTACAGTTACCTCAGAAAGAACTCGTCAAGAGGCGATAGAGG              |
| brkanMVA                | CCCAGCTGGCAATTCGACGTCAATTTAGAAAAATAACCAAATAGGGGTCGCCACATTTTC                |
| pETM-11_Fw              | CGCGCGAAGCTCTAATTTTTGAGTTAAC                                                |
| pETM-11_Rv              | ATGGATCCCTTTTGGCTAACAGCCGATCN                                                |
| pBbGPPS_Fw              | CCCAGCTTAAATCTGACGAAATGC                                                     |
| pBbGPPS_Rv              | CCCAGCTTAAATCTGACGAAATGC                                                     |
| pLimS_inverse_rev       | GGCGCCCTGAAATAAAAGAT                                                        |
| pLimS_inverse_Fw        | TAATACGAGCACCACCACC                                                        |
| trObGES_Rv              | CTGGTTTTTTTAACAGCCGAC                                                       |
| trObGES_Fv              | ATGGAAGAACAGCAGCC                                                       |
| trObGES_Fv-over         | ATCTTTATTTTCAGGGGGCCATGGAAGAACAGCAGCAG                                       |
| trObGES_Rv-over         | TGGTGGGCTGGCTTATTACCGTTGTAATTACACGGCC                                          |
| trPcGES_Fv-over         | ATCTTTATTTTCAGGGGGCCATGGAAGAACAGCAGCAG                                       |
| trPcGES_Rv-over         | TGGTGGGCTGGCTTATTACCGTTGTAATTACACGGCC                                          |
| trCtGES_Fv-over         | ATCTTTATTTTCAGGGGGCCATGGAAGAACAGCAGCAG                                       |
| trCtGES_Rv-over         | TGGTGGGCTGGCTTATTACCGTTGTAATTACACGGCC                                          |
Table S3: Monoterpene synthase genes used in this study. All synthetic genes were codon optimised for use in *E. coli*, fused to a cleavable N-terminal His<sub>6</sub>-tag, and, if present, the N-terminal plastid sequence was omitted.

| Name          | Source                          | Main product            | Uniprot nr. | Reference |
|---------------|---------------------------------|-------------------------|-------------|-----------|
| CamS_Ag       | *Abies grandis*                 | Camphene                | Q948Z0      | [9]       |
| CamS_SI       | *Solanum lycopersicum*          | Camphene                | G1JUH1      | [10]      |
| CarS_Pa       | *Picea abies*                   | 3-Carene                | Q84SM8      | [11]      |
| CarS_Ps       | *Picea sitchensis*              | 3-Carene                | F1CK16      | [12]      |
| CinS_Sf       | *Salvia fruticosa*              | 1,8-Cineole             | A6XH05      | [13]      |
| CinS_Cu       | *Citrus unshiu*                 | 1,8-Cineole             | Q5CDB2      | [14]      |
| CinS_At       | *Arabidopsis thaliana*          | 1,8-Cineole             | P0DI76      | [15]      |
| FenS_Ob       | *Ocimum basilicum*              | Fenchol                 | Q5SBP2      | [16]      |
| FenS_Lv       | *Lavandula viridis*             | Fenchol                 | T1RR72      | [16]      |
| GerS_Pc       | *Perilla citriodora*            | Geraniol                | Q4JHG3      | [17]      |
| GerS_Ob       | *Ocimum basilicum*              | Geraniol                | Q6USK1      | [18]      |
| GerS_Ct       | *Cinnamomum tenuipilum*         | Geraniol                | Q8GUE4      | [19]      |
| RLimS_Cl      | *Citrus lemon*                  | (+)-(4R)-Limonene       | Q8L5K3      | [20]      |
| RLimS_La      | *Lavandula angustivola*         | (+)-(4R)-Limonene       | Q2XSC6      | [21]      |
| SLimS_Ms      | *Mentha spicata*                | (-)-(4S)-Limonene       | Q40322      | [22]      |
| RLinS_Aa      | *Artemisia annua*               | (-)-(3R)-linalool       | Q95P0N0     | [23]      |
| SLinS_At      | *Arabidopsis thaliana*          | (+)-(3S)-linalool       | Q84UV0      | [24]      |
| MyrS_Ag       | *Abies grandis*                 | β-Mycene               | Q24474      | [9, 25]   |
| MyrS_Ca       | *Coffee arabica*                | β-Mycene               | R4YXW8      | [26]      |
| MyrS_At       | *Arabidopsis thaliana*          | β-Mycene               | Q9ZUH4      | [27]      |
| OcIS_Cu       | *Citrus unshiu*                 | (E)-β-ocimene           | Q5CDB1      | [14]      |
| OcIS_At       | *Cinnamomum majus*              | (E)-β-ocimene           | Q84NC8      | [28]      |
| PheS_Ag       | *Abies grandis*                 | β-phellandrene         | Q9M7D1      | [9]       |
| PheS_La       | *Lavandula angustivola*         | β-phellandrene         | E9N3U9      | [29]      |
| PheS_SI       | *Solanum lycopersicum*          | β-phellandrene         | C1K5M3      | [30]      |
| (-)aPinS_Ps   | *Picea sitchensis*              | (-)-α-pinene           | Q84K6L      | [31]      |
| (-)aPinS_Pt   | *Pinus taeda*                   | (-)-α-pinene           | Q84K6L      | [32]      |
| (+)aPinS_Pt   | *Pinus taeda*                   | (+)-α-pinene           | Q84K3L      | [32]      |
| (-)bPinS_Aa   | *Artemisia annua*               | (-)-β-pinene           | Q94G53      | [33]      |
| SabS_Ps       | *Picea sitchensis*              | Sabinene               | F1CKJ1      | [32]      |
| SabS_Sp       | *Salvia pomifera*               | Sabinene               | A6XH06      | [33]      |
| gTerS_Cs      | *Coriandrum sativum*            | γ-Terpineene            | A0A059SVE6  | [34]      |
| gTerS_Ov      | *Origanum vulgare*              | γ-Terpineene            | E2E2P0      | [35]      |
| aTerS_Mg      | *Magnolia grandiflora*          | α-Terpineol            | B3TPQ7      | [36]      |
| aTerS_Sa      | *Santalum album*                | α-Terpineol            | B5A434      | [37]      |
| TerS_Ob       | *Ocimum basilicum*              | Terpinolene            | Q5SBP0      | [16]      |
| TerS_Pm       | *Pseudotsuga menziesii*         | Terpinolene            | Q4QSN6      | [38]      |
### Table S4: Plasmids used in this study

| Plasmid reference | Plasmid name | Description (Origin of replication, Antibiotic marker, Reference(s), Promoters and Operons) | Reference(s) |
|-------------------|--------------|------------------------------------------------------------------------------------------|--------------|
| pBEI-6410         | pBBBa5a-MTSAe-T1f-MB1(f)-T1002i-Ptrc-trGPPS(co)-L5 | p15A, Ampr, PlacUV5, MTSA, T1, MB1-f, T1002, Ptrc, trGPPS, L5 | [1]          |
| pSBC000338        | pBBBa5a-MTSAe-T1f-MB1(f)-T1002i | p15A, Kanr, PlacUV5, MTSA, T1, MB1-f, T1002 | This study |
| pSBC000339        | pBBBa2a-trAgGPPS(co)-trMsLS | pBRR, Ampr, Ptet, trAgGPPS(co)-trMsLS | This study |
| pGPPS             | pBBBa2a-trAgGPPS(co) | pBRR, Ampr, Ptet, trAgGPPS(co) | This study |
| pGPPSsmTC/S1      | pBBBa2a-trAgGPPS(co)-trCamS_Ag | pBRR, Ampr, Ptet, trAgGPPS(co)-trCamS_Ag | This study |
| pGPPSsmTC/S2      | pBBBa2a-trAgGPPS(co)-trCamS_SI | pBRR, Ampr, Ptet, trAgGPPS(co)-trCamS_SI | This study |
| pGPPSsmTC/S3      | pBBBa2a-trAgGPPS(co)-trCarS_Pa | pBRR, Ampr, Ptet, trAgGPPS(co)-trCarS_Pa | This study |
| pGPPSsmTC/S4      | pBBBa2a-trAgGPPS(co)-trCarS_Ps | pBRR, Ampr, Ptet, trAgGPPS(co)-trCarS_Ps | This study |
| pGPPSsmTC/S5      | pBBBa2a-trAgGPPS(co)-trCinS_SF | pBRR, Ampr, Ptet, trAgGPPS(co)-trCinS_SF | This study |
| pGPPSsmTC/S6      | pBBBa2a-trAgGPPS(co)-trCinS_Cu | pBRR, Ampr, Ptet, trAgGPPS(co)-trCinS_Cu | This study |
| pGPPSsmTC/S7      | pBBBa2a-trAgGPPS(co)-trCinS_At | pBRR, Ampr, Ptet, trAgGPPS(co)-trCinS_At | This study |
| pGPPSsmTC/S8      | pBBBa2a-trAgGPPS(co)-trFenS_Ob | pBRR, Ampr, Ptet, trAgGPPS(co)-trFenS_Ob | This study |
| pGPPSsmTC/S9      | pBBBa2a-trAgGPPS(co)-trFenS_Lv | pBRR, Ampr, Ptet, trAgGPPS(co)-trFenS_Lv | This study |
| pGPPSsmTC/S10     | pBBBa2a-trAgGPPS(co)-trGerS_Pc | pBRR, Ampr, Ptet, trAgGPPS(co)-trGerS_Pc | This study |
| pGPPSsmTC/S11     | pBBBa2a-trAgGPPS(co)-trGerS_Ob | pBRR, Ampr, Ptet, trAgGPPS(co)-trGerS_Ob | This study |
| pGPPSsmTC/S12     | pBBBa2a-trAgGPPS(co)-trGerS_Ct | pBRR, Ampr, Ptet, trAgGPPS(co)-trGerS_Ct | This study |
| pGPPSsmTC/S13     | pBBBa2a-trAgGPPS(co)-trLimS_CI | pBRR, Ampr, Ptet, trAgGPPS(co)-trLimS_CI | This study |
| pGPPSsmTC/S14     | pBBBa2a-trAgGPPS(co)-trLimS_LA | pBRR, Ampr, Ptet, trAgGPPS(co)-trLimS_LA | This study |
| pGPPSsmTC/S15     | pBBBa2a-trAgGPPS(co)-trLimS_MS | pBRR, Ampr, Ptet, trAgGPPS(co)-trLimS_MS | This study |
| pGPPSsmTC/S16     | pBBBa2a-trAgGPPS(co)-trLipS_Aa | pBRR, Ampr, Ptet, trAgGPPS(co)-trLipS_Aa | This study |
| pGPPSsmTC/S17     | pBBBa2a-trAgGPPS(co)-trLimS_At | pBRR, Ampr, Ptet, trAgGPPS(co)-trLimS_At | This study |
| pGPPSsmTC/S18     | pBBBa2a-trAgGPPS(co)-trMyrS_Ag | pBRR, Ampr, Ptet, trAgGPPS(co)-trMyrS_Ag | This study |
| pGPPSsmTC/S19     | pBBBa2a-trAgGPPS(co)-trMyrS_Ca | pBRR, Ampr, Ptet, trAgGPPS(co)-trMyrS_Ca | This study |
| pGPPSsmTC/S20     | pBBBa2a-trAgGPPS(co)-trMyrS_At | pBRR, Ampr, Ptet, trAgGPPS(co)-trMyrS_At | This study |
| pGPPSsmTC/S21     | pBBBa2a-trAgGPPS(co)-trOciS_Cu | pBRR, Ampr, Ptet, trAgGPPS(co)-trOciS_Cu | This study |
| pGPPSsmTC/S22     | pBBBa2a-trAgGPPS(co)-trOciS_Am | pBRR, Ampr, Ptet, trAgGPPS(co)-trOciS_Am | This study |
| pGPPSsmTC/S23     | pBBBa2a-trAgGPPS(co)-trPheS_Ag | pBRR, Ampr, Ptet, trAgGPPS(co)-trPheS_Ag | This study |
| pGPPSsmTC/S24     | pBBBa2a-trAgGPPS(co)-trPheS_La | pBRR, Ampr, Ptet, trAgGPPS(co)-trPheS_La | This study |
| pGPPSsmTC/S25     | pBBBa2a-trAgGPPS(co)-trPheS_SI | pBRR, Ampr, Ptet, trAgGPPS(co)-trPheS_SI | This study |
| pGPPSsmTC/S26     | pBBBa2a-trAgGPPS(co)-tr(+)JaPinS_Ps | pBRR, Ampr, Ptet, trAgGPPS(co)-tr(+)JaPinS_Ps | This study |
| pGPPSsmTC/S27     | pBBBa2a-trAgGPPS(co)-tr(+)JaPinS_Pt | pBRR, Ampr, Ptet, trAgGPPS(co)-tr(+)JaPinS_Pt | This study |
| pGPPSsmTC/S28     | pBBBa2a-trAgGPPS(co)-tr(+)JaPinS_Pt | pBRR, Ampr, Ptet, trAgGPPS(co)-tr(+)JaPinS_Pt | This study |
| pGPPSsmTC/S29     | pBBBa2a-trAgGPPS(co)-tr(-)JaPinS_Aa | pBRR, Ampr, Ptet, trAgGPPS(co)-tr(-)JaPinS_Aa | This study |
| pGPPSsmTC/S30     | pBBBa2a-trAgGPPS(co)-trSabS_Ps | pBRR, Ampr, Ptet, trAgGPPS(co)-trSabS_Ps | This study |
| pGPPSsmTC/S31     | pBBBa2a-trAgGPPS(co)-trSabS_Sp | pBRR, Ampr, Ptet, trAgGPPS(co)-trSabS_Sp | This study |
| pGPPSsmTC/S32     | pBBBa2a-trAgGPPS(co)-trGerS_CS | pBRR, Ampr, Ptet, trAgGPPS(co)-trGerS_CS | This study |
| pGPPSsmTC/S33     | pBBBa2a-trAgGPPS(co)-trGerS_Ov | pBRR, Ampr, Ptet, trAgGPPS(co)-trGerS_Ov | This study |
| pGPPSsmTC/S34     | pBBBa2a-trAgGPPS(co)-trCamS_Mg | pBRR, Ampr, Ptet, trAgGPPS(co)-trCamS_Mg | This study |
| pGPPSsmTC/S35     | pBBBa2a-trAgGPPS(co)-trCamS_Sa | pBRR, Ampr, Ptet, trAgGPPS(co)-trCamS_Sa | This study |
| pGPPSsmTC/S36     | pBBBa2a-trAgGPPS(co)-trTerS_Ob | pBRR, Ampr, Ptet, trAgGPPS(co)-trTerS_Ob | This study |
| pGPPSsmTC/S37     | pBBBa2a-trAgGPPS(co)-trTerS_Pm | pBRR, Ampr, Ptet, trAgGPPS(co)-trTerS_Pm | This study |
Supporting Results

Figure S2: Limonene production titres in different *E. coli* strains. Final titres of limonene produced by *E. coli* strains (Table S1) bearing plasmid pJBEI-6410 after 72 h growth in a bi-phasic culture (concentrations in 20 % organic phase), as calculated from GC analysis. Average titres from triplicate biological repeats; error bars represent standard deviation. Bar colour denotes *E. coli* strain type; K-12 (green), B (orange) or W (blue).
Figure S3: GCMS analysis of authentic monoterpenoid standards.

A) GCMS traces of authentic monoterpenoid standards. GCMS traces showing the separation of monoterpenoids (1 mg mL\(^{-1}\)) produced in this study on a DB-WAX column. The internal standard used, sec-butylbenzene (0.1%, v/v), has a retention time of 6.10 minutes. Peak 1: \(\alpha\)-pinene (rt: 2.544), 2: camphene (rt: 3.001), 3: \(\beta\)-pinene (rt: 3.525), 4: sabinene (rt: 3.714), 5: 3-carene (rt: 4.141), 6: \(\beta\)-myrcene (rt: 4.395), 7: limonene (rt: 4.996), 8: 1,8-cineole (rt: 5.200), 9: terpinolene (rt: 6.985), 10: linalool (rt: 9.881), 11: fenchol (rt: 10.133), 12: \(\alpha\)-terpineol (rt: 10.788), 13: nerol (rt: 11.208), 14: geraniol (rt: 11.490). Because \(\gamma\)-terpinene (rt: 6.040) (peak 15) and (E)-\(\beta\)-ocimene (rt: 6.226) (peak 17), have similar retention times as sec-butylbenzene (rt: 6.085), limonene (0.1% v/v) was used as internal standard in \(\beta\)-ocimene and \(\gamma\)-terpinene containing samples. The \(\beta\)-ocimene standard contains about 30% (Z)-\(\beta\)-ocimene (rt: 5.815) (peak 16). Method: the injector temperature was set at 240°C with a split ratio of 20:1 (1 µL injection). The carrier gas was helium with a flow rate of 1 mL/min and a pressure of 5.1 psi. The following oven program was used: 50°C (1 min hold), ramp to 68°C at 5°C/min (2 min hold), and ramp to 230°C at 25°C/min (2 min hold). The ion source temperature of the mass spectrometer (MS) was set to 230°C and spectra were recorded from m/z 50 to m/z 250.
B) MS spectra of authentic monoterpenoid standards. Chemical structures are shown as insets.
Figure S4: GCMS analysis monoterpenoid production strains. Representative GCMS traces of \( n \)-nonane overlays obtained from two-phase \( E. \) coli \( \alpha \)-Select cultures containing pmVA and pGPPSmTC/S plasmids are shown. MS spectra of indicated monoterpenoid peaks are shown. Retention times of additional terpenoid peaks are mentioned below. Chemical structures of detected monoterpenoids are shown as insets. Retention times and MS spectra of the detected peaks were compared to retention times and MS spectra of authentic standards wherever possible (See Figure S3). No authentic standards were available for the following monoterpenoids which are produced as by-products: \( \beta \)-phellandrene, 4-carene, \( \delta \)-terpineol, sabinene hydrate, thujene, borneol, Pinan-2-ol, citronellal, neral, geranial, and citronellol. Also no authentic standards were available for the sesquiterpene by-products farnesal, farnesene and (\( E \))-\( \alpha \)-bisabolene. Identification of these compounds was achieved by comparing the obtained MS spectra and fragmentation patterns to the NIST reference library.
Additional terpenoids detected: geranial (rt: 10.955), citronellol (rt: 11.101), nerol (rt: 11.278), geraniol (rt: 11.487), farnesal (rt: 13.225), and farnesol (rt: 13.542). The peak at rt 14.037 is indole.
Additional terpenoids detected: geranial (rt: 10.952), citronellol (rt: 11.101), nerol (rt: 11.278), geraniol (rt: 11.487), farnesal (rt: 13.222), and farnesol (rt: 13.540). The peak at rt 14.034 is indole.
Additional terpenoids detected: geranial (rt: 10.952), citronellol (rt: 11.099), nerol (rt: 11.275), geraniol (rt: 11.484), farnesal (rt: 13.222), and farnesol (rt: 13.540). The peak at rt 14.034 is indole.
Additional terpenoids detected: citronellol (rt: 11.101), nerol (rt: 11.278), geraniol (rt: 11.489), farnesal (rt: 13.222), and farnesol (rt: 13.542). The peak at rt 14.034 is indole.
Additional terpenoids detected: neral (rt: 10.690), geranial (rt: 10.957), citronellol (rt: 11.101), nerol (rt: 11.278), geraniol (rt: 11.490), and farnesol (rt: 13.542). The peak at rt 14.039 is indole.
Additional terpenoids detected: neral (rt: 10.690), geranial (rt: 10.955), citronellol (rt: 11.101), nerol (rt: 11.278), geraniol (rt: 11.487), and farnesol (rt: 13.542). The peak at rt 14.039 is indole.
Additional terpenoids detected: neral (rt: 10.690), geranial (rt: 10.957), citronellol (rt: 11.101), nerol (rt: 11.278), geraniol (rt: 11.487), and farnesol (rt: 13.542). The peak at rt 14.039 is indole.
Additional terpenoids detected: farnesol (rt: 13.542). The peak at rt 14.037 is indole.
Additional terpenoids detected: geranial (rt: 10.955), citronellol (rt: 11.101), nerol (rt: 11.278), geraniol (rt: 11.487), and farnesol (rt: 13.540). The peak at rt 14.037 is indole.
Additional terpenoids detected: citronellal (rt: 9.376), neral (rt: 10.690), geranial (rt: 10.957), citronellol (rt: 11.101), nerol (rt: 11.278), geraniol (rt: 11.487), and farnesol (rt: 13.543). The peak at rt 14.039 is indole.
Additional terpenoids detected: neral (rt: 10.690), geranial (rt: 10.959), citronellol (rt: 11.101), nerol (rt: 11.278), geraniol (rt: 11.489), farnesal (rt: 13.224), and farnesol (rt: 13.543). The peak at rt 14.039 is indole.
Additional terpenoids detected: neral (rt: 10.690), geranial (rt: 10.957), citronellol (rt: 11.101), nerol (rt: 11.278), geraniol (rt: 11.487), and farnesol (rt: 13.542). The peak at rt 14.039 is indole.
Additional terpenoids detected: neral (rt: 10.690), geranial (rt: 10.955), citronellol (rt: 11.101), nerol (rt: 11.278), geraniol (rt: 11.487), and farnesol (rt: 13.542). The peak at rt 14.037 is indole.
Additional terpenoids detected: neral (rt: 10.687), geranial (rt: 10.955), citronellol (rt: 11.098), nerol (rt: 11.275), geraniol (rt: 11.484), and farnesol (rt: 13.540). The peak at rt 14.034 is indole.
Additional terpenoids detected: neral (rt: 10.688), geranial (rt: 10.955), citronellol (rt: 11.101), nerol (rt: 11.278), geraniol (rt: 11.487), and farnesol (rt: 13.540). The peak at rt 14.034 is indole.
Additional terpenoids detected: neral (rt: 10.688), geranial (rt: 10.955), citronellol (rt: 11.101), nerol (rt: 11.278), geraniol (rt: 11.487), and farnesol (rt: 13.540). The peak at rt 14.034 is indole.
Additional terpenoids detected: neral (rt: 10.690), geranial (rt: 10.955), citronellol (rt: 11.101), nerol (rt: 11.278), geraniol (rt: 11.487), and farnesol (rt: 13.542). The peak at rt 14.037 is indole.
Additional terpenoids detected: farnesal (rt: 13.222), and farnesol (rt: 13.543). The peak at rt 14.039 is indole.
Additional terpenoids detected: farnesol (rt: 13.537). The peak at rt 14.032 is indole.
Additional terpenoids detected: neral (rt: 10.687), geranial (rt: 10.952), citronellol (rt: 11.099), nerol (rt: 11.275), geraniol (rt: 11.484), and farnesol (rt: 13.540). The peak at rt 14.034 is indole.
Table S5: Product profiles and monoterpenoid titres of production strains. Product profiles and monoterpenoid titres (mg L<sub>0.1</sub>⁻¹) are determined from two-phase cultures with an n-nonane overlay for each *E. coli* strain containing the MVA pathway and a distinct monoterpenic synthase. Averages of at least 3 biological replicates per mTC/S and the corresponding standard deviations (in brackets) are shown. Highest values for each compound are highlighted in bold.

| Synthase       | Geraniol | (E)-β-pinene | myrcene | limonene | γ-terpinene | α-terpineol | β-phellandrene | terpinene | α-phine | β-phine | fenchol | 1,8-cineole | sabalene | 3-carene | camphene | Other minor | Total product | Geranoids | Camphor | Carvacrol |
|----------------|----------|---------------|---------|----------|-------------|-------------|----------------|------------|----------|---------|--------|-------------|-----------|-----------|----------|------------|---------------|-----------|---------|-----------|
| (-)JaPinS_Pt   | 11.2      | 6.6           | <1      | 7.4       | 517.2       | 94.3        | 2.5            | 2.3        | 3.8      | 3.3     | 642    | 8.1         | 2.4       | 41.9      | (22.3)   |            |               |           |         |           |
| SLimS_Ms       | 7.7       | 530.6         | 2.1     | 5.3       | 50.3        | 9.7         | 0.5            | 3.3        | 554      | 7.7     | 33.2    | 11.5       |           |           |           |            |               |           |         |           |
| RLimS_CI       | 4.3       | 260.0         | <1      |           |             |             |                |            | 265      | 4.4     | 17.9    | (3.3)      |           |           |           |            |               |           |         |           |
| gTerS_Ov       | 3.1       | 197.1         | <1      | 13.3      | 18.5        |              |                |            | 232      | 19.2    | 42.9    | (42.3)     |           |           |           |            |               |           |         |           |
| (+)JaPinS_Pt   | 3.2       |              | <1      | 190.6     | 40.8        |              |                |            | 197      | 35.2    | 40.8    | (6.1)      |           |           |           |            |               |           |         |           |
| Cins_Sf        | 3.2       | 1.9           | 11.4    | 7.1       |              |              |                |            | 183      | 12.3    | 25.9    | (3.2)      |           |           |           |            |               |           |         |           |
| (-)JbPinS_Aa   | 3.0       | 2.3           | 2.1     | 12.1      | 118.2       |              |                |            | 174      | 48.0    | 41.1    | (7.0)      |           |           |           |            |               |           |         |           |
| GerS_Pc        | 162.6     | 17.3          | 4.4     | 1.9       |              |              |                |            | 163      | 189.9   | 30.7    | (15.6)     |           |           |           |            |               |           |         |           |
| Cins_At        | <1        | 15.5          | 4.4     | 1.9       |              |              |                |            | 111      | 47.4    | 16.6    | (7.4)      |           |           |           |            |               |           |         |           |
| FenS_Lv        | 2.7       | 6.1           | 20.7    | 1.6       |              |              |                |            | 82.1     | 79.0    | 17.6    | (1.4)      |           |           |           |            |               |           |         |           |
| SabS_Sp        | 1.3       | 1.5           |         |           |              |              |                |            | 76.6     | 34.5    | 19.2    | (12.0)     |           |           |           |            |               |           |         |           |
| aTerS_Mg       | 1.9       | 3.3           | 3.2     | 4.9       |              |              |                |            | 77.9     | 28.8    | 13.2    | (7.3)      |           |           |           |            |               |           |         |           |
| OcIs_Am        | 57.3      | 37.9          | <1      | 4.9       |              |              |                |            | 65.5     | 28.8    | 13.2    | (7.3)      |           |           |           |            |               |           |         |           |
| CamS_SI        | 10.3      | 3.4           | 18.2    | 9.9       |              |              |                |            | 40.0     | 28.7    | 8.1     | (6.2)      |           |           |           |            |               |           |         |           |
| CinS_Cu        |            | 3.6           | 18.2    | 9.9       |              |              |                |            | 28.7     | 24.7    | 8.6     | (6.2)      |           |           |           |            |               |           |         |           |
| (-)JaPinS_Ps   | <1        | 18.8          | 12.2    |           |              |              |                |            | 19.7     | 65.9    | 26.6    | (8.6)      |           |           |           |            |               |           |         |           |
| GerS_Ob        | 14.3      | 10.8          | <1      | 15.9      |              |              |                |            |          |         |           |            |           |           |           |            |               |           |         |           |
| GerS_Ct        | 14.3      | 6.5           | 14.3    |           |              |              |                |            |          |         |           |            |           |           |           |            |               |           |         |           |
| RLimS_Aa       | 1.3       | 2.1           | 2.1     |           |              |              |                |            |          |         |           |            |           |           |           |            |               |           |         |           |

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Table S6: Overview of all linear monoterpenoids produced. Highest titres observed and the mTC/S responsible are shown. Averages of at least 3 biological replicates and the corresponding standard deviations are shown.

| Linear monoterpenoids | Structure | Titre (mg L\textsubscript{org}^{-1}) | Strain + mTC/S |
|-----------------------|-----------|--------------------------------------|---------------|
| geraniol              | ![Structure](geraniol.png) | 162.6 ± 98.2 | GerS_Pc |
| (E)-β-ocimene         | ![Structure](ocimene.png) | 57.3 ± 56.9 | OciS_Am |
| β-myrcene             | ![Structure](myrcene.png) | 17.3 ± 15.5 | CinS_At |
| linalool              | ![Structure](linalool.png) | 0.8 ± 1.4 | CarS_Pa |
| nerol                 | ![Structure](nerol.png) | 73.2 ± 61.8 | GerS_Pc |
| geranial              | ![Structure](geranial.png) | 56.1 ± 35.0 | GerS_Pc |
| citronellol           | ![Structure](citronellol.png) | 35.1 ± 19.7 | GerS_Pc |
| neral                 | ![Structure](neral.png) | 16.8 ± 7.2 | GerS_Pc |
| citronellal           | ![Structure](citronellal.png) | 10.7 ± 2.0 | GerS_Pc |
| iso-geraniol          | ![Structure](iso-geraniol.png) | 1.2 ± 1.4 | GerS_Pc |
| (Z)-β-ocimene         | ![Structure](z-ocimene.png) | 0.8 ± 1.7 | OciS_Am |
Table S7: Overview of all monocyclic monoterpenoids produced. Highest titres observed and the mTC/S responsible are shown. Averages of at least 3 biological replicates and the corresponding standard deviations are shown.

| Target monocyclic compounds                      | Structure | Titre (mg L<sub>org</sub>⁻¹) | Strain + mTC/S |
|--------------------------------------------------|-----------|-------------------------------|---------------|
| (-)-{(4S)}-limonene*                              |           | 530.6 ± 102.3                 | SLimS_Ms      |
| (+)-{(4R)}-limonene*                              |           | 260.6 ± 122.2                 | RLimS_CI      |
| γ-terpinene                                       |           | 197.1 ± 119.7                 | gTerS_Ov      |
| α-terpineol                                       |           | 37.9 ± 36.3                   | aTerS_Mg      |
| β-phellandrene                                    |           | 7.4 ± 3.5                     | (-)aPinS_PT   |
| terpinolene                                       |           | 3.4 ± 0.5                     | CamS_SL       |
| Additional monocyclic compounds                   |           |                               |               |
| δ-terpineol                                       |           | 0.7 ± 1.3                     | CinS_At       |

* Stereoisomers identified based on published product profiles
Table S8: Overview of all bicyclic monoterpenoids produced. Highest titres observed and the mTC/S responsible are shown. Averages of at least 3 biological replicates and the corresponding standard deviations are shown.

| Bicyclic monoterpenoids |
|--------------------------|
| **Target bicyclic compounds** | **Structure** | **Titre (mg L<sub>org</sub>⁻¹)** | **Strain + mTC/S** |
| (-)-α-pinene* | | 517.2 ± 132.7 | (-)aPinS_Pt |
| (+)-α-pinene* | | 190.6 ± 31.9 | (+)aPinS_Pt |
| (-)-β-pinene* | | 145.0 ± 33.5 | (-)bPinS_Aa |
| 1,8-cineole | | 118.2 ± 153.6 | CinS_Sf |
| sabinene | | 76.6 ± 74.0 | SabS_Sp |
| fenchol | | 68.6 ± 30.9 | FenS_Lv |
| 3-carene | | 15.9 ± 3.6 | CarS_Pa |
| camphene | | 12.2 ± 3.4 | CamS_SI |
| **Additional bicyclic compounds** | | | |
| 4-carene | | 18.5 ± 12.7 | gTerS_Ov |
| sabinene hydrate | | 2.7 ± 4.1 | CinS_Sf |
| thujene | | 0.5 ± 0.1 | CinS_Sf |
| borneol | | 1.8 ± 0.1 | CamS_SI |
| pinan-2-ol | | 0.8 ± 0.7 | (-)aPinS_Pt |

* Stereoisomers identified based on published product profiles
**Table S9: Overview of all additional terpenoids produced.** Highest titres observed and the mTC/S responsible are shown. Averages of at least 3 biological replicates and the corresponding standard deviations are shown.

| Sesquiterpenoids     | Structure | Titre (mg L$_{org}$⁻¹) | Strain + mTC/S |
|----------------------|-----------|-------------------------|----------------|
| farnesol             | ![farnesol](image) | 46.6 ± 38.9             | PheS_Sl        |
| farnesal             | ![farnesal](image) | 3.7 ± 2.1               | SLimS_Ms       |
| (E)-α-bisabolene     | ![bisabolene](image) | 1.4 ± 2.4               | CinS_At        |
| farnesene            | ![farnesene](image) | 0.5 ± 0.9               | CinS_At        |
Figure S5: Titres produced in the platform vs. previously published titres. Monoterpenoid titres produced using our platform are shown in coloured bars, previously published titres are in grey bars. Published values: geraniol: 182.5 mg L\(^{-1}\) \[^{[29]}\]; \(\beta\)-myrcene: 58.2 mg L\(^{-1}\) \[^{[40]}\]; linalool: 0.1 mg L\(^{-1}\) \[^{[41]}\]; limonene: 435 mg L\(^{-1}\) \[^{[1]}\]; pinene: 32 mg L\(^{-1}\) \[^{[42]}\]; 1,8-cineole: 21 mg L\(^{-1}\) \[^{[43]}\]. Error bars represent the standard deviation of at least 3 biological replicates.
Figure S6: Monoterpenoid, geranoid and farnesol titres detected for each strain. Monoterpenoid titres produced using our platform are shown in blue bars, geraniol and derivatives in red bars, and farnesol and derivatives in green bars. Error bars represent the standard deviation of at least 3 biological replicates.
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