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In vitro Applications of the Terpene Mini-Path 2.0

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In 2019 four groups reported independently the development of a simplified enzymatic access to the diphosphates (IPP and DMAPP) of isopentenol and dimethylallyl alcohol (IOH and DMAOH). The former are the two universal precursors of all terpenes. We report here on an improved version of what we call the terpene mini-path as well as its use in enzymatic cascades in combination with various transferases. The goal of this study is to demonstrate the *in vitro* utility of the TMP in, i) synthesizing various natural terpenes, ii) revealing the product selectivity of an unknown terpene synthase, or iii) generating unnatural cyclobutylated terpenes.

Introduction

It has been shown recently that a new synthetic terpene biopath could reduce the number of enzymes from 18 to 2 to access the universal precursors of all terpenes *i.e.* isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP).[1–4,5] Indeed, starting from the corresponding two commodity alcohols isopentenol (IOH) and dimethylallyl alcohol (DMAOH), instead of glucose for the natural pathways, a two steps enzymatic phosphorylation, using ATP as phosphorylating agent, efficiently generated various terpenoids, both *in vitro* and *in vivo* (Scheme 1).

The first phosphorylation step of the starting C5 alcohols was initially catalyzed either by a kinase[1,4] or by an acid phosphatase[2,3] thanks to the substrate promiscuity of these two types of enzyme, while the second phosphorylation step was, in the four cases, catalyzed by an archaeal isopentenyl phosphate kinase (IPK).[1–4]

We initially developed what we call the terpene mini-path (TMP) by using a phosphatase for the first phosphorylation step but noticed the adverse hydrolytic effect on the mono-, di- and tri-phosphate esters present in the reaction medium (IP, DMAP, IPP, DMAPP, ADP, ATP) of this family of enzymes.[3] A design of experiments allowed nevertheless a huge optimization of the reaction conditions, leading to complete prenylation of brevianamide F (BF) into tryprostatin B (TB), catalyzed by prenyl transferase FtmPT1 from *Aspergillus fumigatus* (FtmPT1$_{Af}$), at 10 mM in 20 h (Scheme 2).[3]

In 2018, Wang *et al.* described the promiscuous kinase activity of the hydroxyethylthiazole kinase from *E. coli* (ThiM$_{Ec}$ kinase) on DMAOH.[6] This finding and the fact that IOH was also a substrate of this enzyme, stimulated the use of ThiM$_{Ec}$ as a catalyst for the mono-phosphorylation of DMAOH and IOH in the context of the TMP, either *in vivo* or *in vitro*, to generate various terpenoids.[4,7] Within this contribution we wish to report i) the *in vitro* improvement of the TMP using the ThiM$_{Ec}$ kinase instead of a phosphatase in the first phosphorylation step, ii) the extension of the available terpene chemical space thanks to the simplicity of the TMP allowing the use of a structural equivalent of DMAOH as a starter unit and iii) the relevance of the TMP to identify the product specificity of a yet uncharacterized sesquiterpene synthase without the need to chemically synthesize farnesyl diphosphate (FPP).

Results and Discussion

TMP 2.0 assisted in vitro synthesis of various terpenoids

Following the description of the first version of the TMP, we were interested in testing a real kinase instead of the phosphatase we used initially which acted as both alcohol kinase and (di)phosphate ester hydrolase. Based on our benchmark reaction (Scheme 2), *i.e.* the DMAPP dependent prenylation of brevianamide F (BF) into tryprostatin B (TB), we proved that replacing the phosphatase from *Xanthomonas transluscens* (PhoN$_{Xt}$) by the ThiM$_{Ec}$ kinase allowed a total transformation of BF (10 mM) into TB in 4 h instead of 20 h as previously

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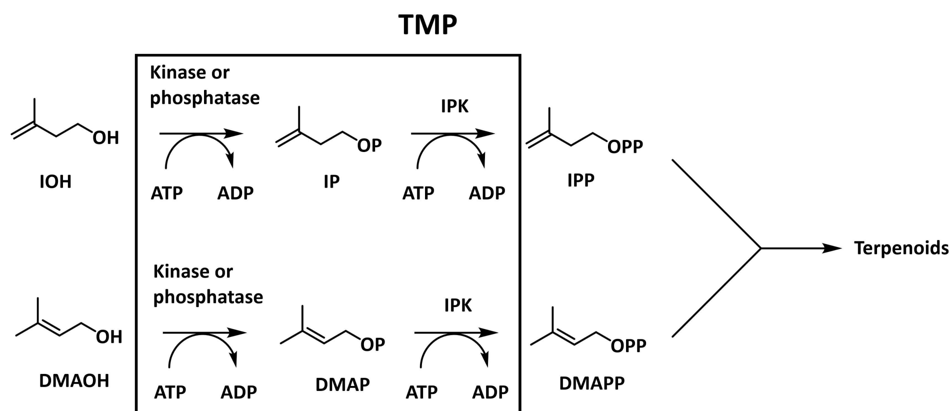
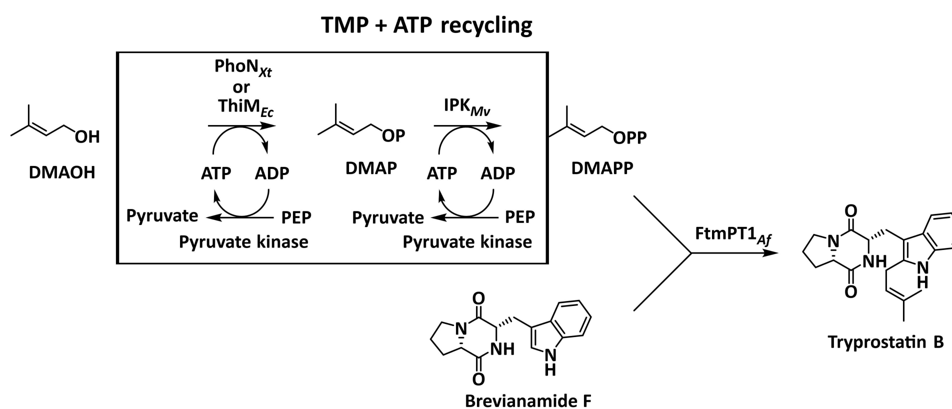


Scheme 1. Simplified enzymatic access to the universal terpene precursors.



Scheme 2. *In vitro* one pot – three steps enzymatic synthesis of tryprostatin B using the TMP 1.0 (phosphatase, PhoN$_{Xt}$) or 2.0 (kinase, ThiM$_{Ec}$).

described, the two other enzymes (IPK$_{Mv}$ and FtmPT1$_{Af}$) being unchanged (Figure 1).[3]

Furthermore, if an ATP recycling system is used (pyruvate kinase/phosphoenol pyruvate: PEP) the time required to totally transform BF into TB dropped to 1 h allowing to reach a 92% final isolated yield of TB (Figure 1). As expected, the incorporation of a real kinase instead of a phosphatase allowed to complete the prenylation of BF quicker, here twenty times, without impacting the final yield in TB. We thus selected in the following the ThiM$_{Ec}$ kinase as the first enzyme of what we have called the TMP 2.0.

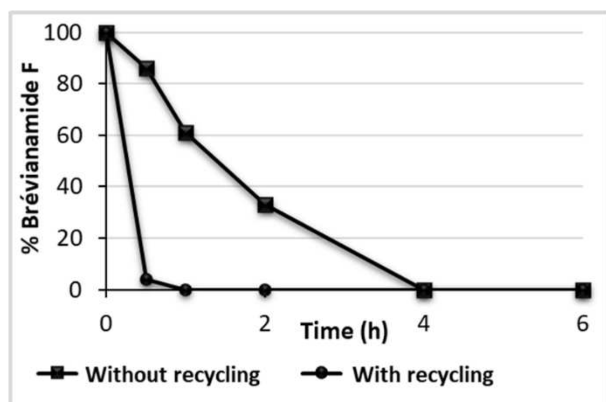


Figure 1. One pot – three steps enzymatic conversion of brevianamide F using the TMP 2.0 and FtmPT1$_{Af}$ with (▪) or without (•) an ATP recycling system.

Thanks to this large improvement we then turned our attention to the generation of sesquiterpenes. To this end, we first incorporated to the TMP 2.0, the farnesyl diphosphate synthase from *Geobacillus stearothermophilus* (FPPS$_{Gs}$), a known stable and highly active FPPS,[8] in order to access FPP in a one pot – three steps cascade (Scheme 3). For note, a similar reaction was conducted by the group of Allemann, using two steps (firstly, synthesis of DMAPP and IPP then synthesis of FPP), instead of the one pot cascade presented here.[7]

An optimization of FPP synthesis was conducted using design of experiments (see Supporting Information). A 2.5 mM concentration of DMAOH (*vs* 5 mM), a 15 mM concentration of Mg^{2+} (*vs* 10 mM), a pH of 7.5 (*vs* 8) and the absence of Mn^{2+} and NH_4^+ were the most beneficial conditions among the tested ones. By combining all these values in a single experiment, we ended-up with a 54% yield of isolated FPP as its tris-ammonium form, something comparable with a previous

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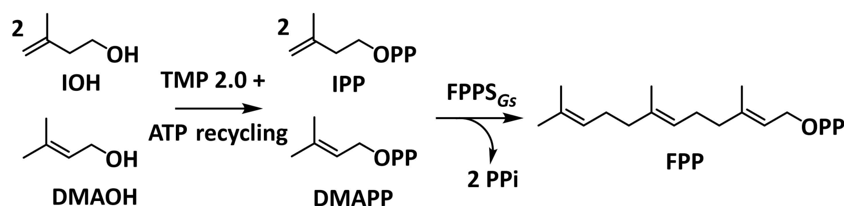


Scheme 3. One pot – three steps enzymatic farnesyl diphosphate synthesis using the TMP 2.0 and the FPPS$_{Gs}$ with an ATP recycling system.

report,[7] but at a larger concentration. The use of the TMP 2.0 coupled to an efficient FPPS is thus a real alternative to the chemical synthesis of FPP, avoiding the cumbersome generation of activated farnesol as halide derivatives and the need of tris(tetrabutylammonium) hydrogen diphosphate as diphosphorylating agent.[9]

TMP 2.0 assisted in vitro synthesis of unnatural cyclobutylated terpenoids

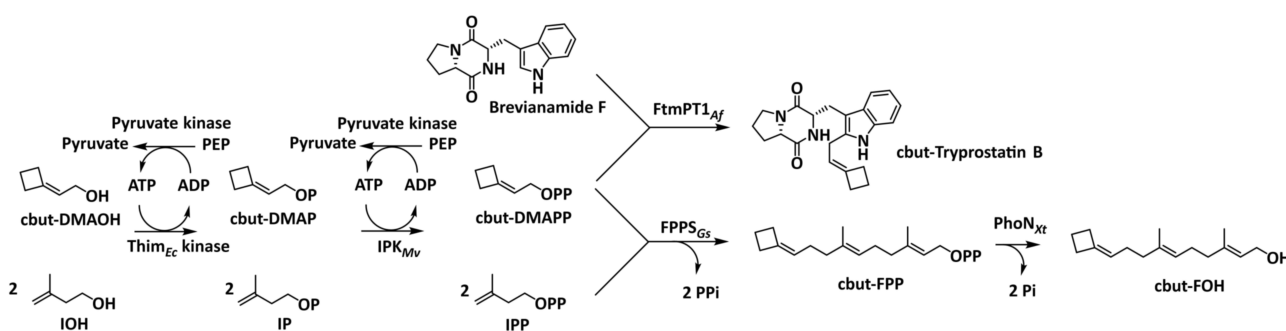
We then envisioned to use derivatives of DMAOH (see ref. [7] for a first report) to access non-natural terpenes. This point is of particular interest since the synthesis of the diphosphate analogs could be run enzymatically thanks to the TMP, instead of chemically.[10] We focused here on the cyclobutyl equivalent of DMAOH (cbut-DMAOH) since this structure has, to the best of our knowledge,[10] never been tested to generate terpene analogs. The cbut-DMAOH proved to be a good substrate of ThiM$_{Ec}$ (92% relative activity as compared to IOH activity taken as 100%) and thus tested in the three enzymes cascade used previously to access TB (Scheme 4).

The cbut diphosphate derivative proved to be a good prenylating unit as compared with DMAPP since BF was nearly totally consumed after 4 h of reaction and totally consumed after 24 h. It appeared thus clearly that replacing the *gem*-dimethyl structure in DMAOH by a cyclobutyl structure was quite well tolerated by the three enzymes of the cascade and that cbut-DMAP and cbut-DMAPP were substrates of IPK$_{Mv}$ kinase and FtmPT1$_{Af}$ prenyl transferase, respectively. Then, we ran a preparative scale reaction affording cbut-TB in 72.4% isolated yield (44.7 mg) starting from 50 mg of BF (10 mM). As tryprostatin B has been categorized as a cytotoxic agent,[11] we were also interested in comparing TB and cbut-TB for cytotoxicity. Indeed, it has been demonstrated that BF prenylation was the key structural feature leading to TB biological activity,[12] BF itself showing no activity. We tested four different cellular lineages (A2780, MCF7, PC3 and HUVEC) in an antiproliferative assay and found that substituting the *gem*-dimethyl unit of TB by a cyclobutyl one did not changed the biological activity in the worst case and divided by two the observed IC_{50} (lineage MCF7) in the best one (see Supporting Information Figure S1).

The easiness to introduce a structural modification using the TMP prompted us to also test cbut-DMAOH as a starter unit for the synthesis of the cyclobutyl equivalent of FPP (Scheme 5) with the idea in mind to access modified sesquiterpenes. Using the same reaction conditions as for the synthesis of FPP we recovered cbut-FPP in 44% yield as tris ammonium salt, the structure of which was assessed by NMR (see Supporting Information).

TMP 2.0 assisted in vitro determination of sesquiterpene synthase product specificity

In order to also demonstrate the potential of the TMP 2.0 to determine product specificity of unknown sesquiterpene synthases, we retrieved from Mycocosm (https://mycocosm.jgi.doe.gov) the gene of a potential sesquiterpene synthase (STS$_{Lm}$, protID 671455) from a polypore, *Leiotrametes menziesii* (fungus, basidiomycete). The *E. coli* production of the 6His-STS$_{Lm}$ was optimized, the enzyme purified and used in a 4 enzymes



Scheme 4. One pot – three steps enzymatic synthesis of cbut-TB and cbut-farnesyl diphosphate using the TMP 2.0 and either of the 2 prenyl transferases FtmPT1$_{Af}$ or FPPS$_{Gs}$ with ATP recycling.

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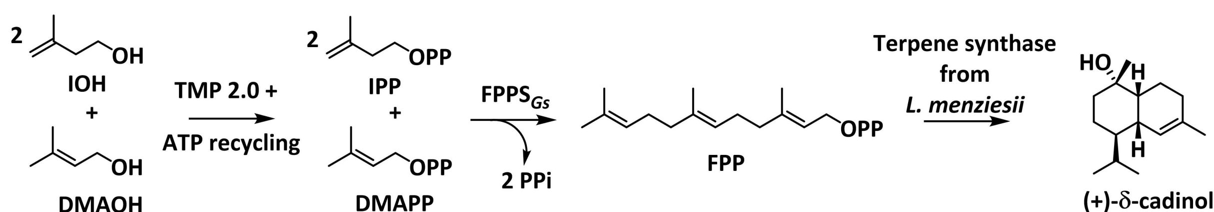

Scheme 5. One pot – four steps enzymatic synthesis of (+)-δ-cadinol synthesis using the TMP 2.0 with an ATP recycling system, the FPPS$_{Gs}$ and an unknown terpene cyclase from basidiomycete *Leiotrametes menziesii.*

cascade at analytical scale (Scheme 5). After 24 h reaction and pentane extraction, a GC analysis revealed a major single peak in the expected zone for a sesquiterpenic alcohol, the structure of the product being predicted as cadinol after GC–MS analysis. The same compound was obtained when chemically synthesized FPP was tested as substrate of the purified STS$_{Lm}$. Ten mg scale synthesis allowed a detailed NMR analysis of the purified product, the structure of which was unambiguously assigned as δ-cadinol, further refined to (+)-δ-cadinol after polarimetry analysis.[13]

Thus, coupling the TMP 2.0 to a prenyl transferase, here a FPPS but other transferases such as a geranyl diphosphate synthase or geranylgeranyl diphosphate synthase can also be considered, offerings the possibility to readily characterized product specificity of unknown terpene synthases without the need for either buying or chemically synthesizing any diphosphates.

Conclusion

In conclusion, during this work we studied the replacement of the initially used phosphatase in the first version of the TMP by the ThiM$_{Ec}$ kinase whose capacity to phosphorylate DMAOH and IOH was recently discovered. We showed that this 2.0 version of the TMP allowed, when coupled to the FtmPT1$_{Af}$ prenyl transferase, an extremely efficient *in vitro* chemo-enzymatic synthesis of TB (10 mM scale, 1 h, 92% yield), dividing by 20 the time required to reach a total bioconversion of starting BF. When coupled to the FPPS$_{Gs}$ prenyl transferase, the TMP 2.0 proved to be efficient enough to consider the enzymatic synthesis of FPP as an alternative to the chemical one, the quantity of formed FPP depending only on the available quantity of each purified enzymes. We were also interested to show that the TMP 2.0 coupled to the FPPS$_{Gs}$ could also be useful to reveal the product specificity of unknown terpene synthases. Here we identified quite simply a new basidiomycete (+)-δ-cadinol synthase, by adding the STS as a fourth enzyme to the enzymatic cascade. We really believe that the discovery of new terpene cyclase/synthase activities could thus be made easier in the future thanks to the TMP. Finally, due to the simplified cascade of the TMP, generating non-naturel equivalent of terpene precursors could now be envisioned at the level of the starting alcohols. The equivalents of DMAOH and IOH are to be synthesized chemically, leaving the TMP in charge of the enzymatic synthesis of the corresponding diphosphates, which was not the case previously since the diphosphates had to be chemically synthesized.[10] In that context the cyclobutyl equivalent of DMAOH proved to be a good substrate of the TMP 2.0 as well as of FPPS$_{Gs}$ and FtmPT1$_{Af}$ allowing the synthesis of the cyclobutyl equivalent of both TB and FPP. The later results open the way to an enlargement of the already huge described terpene chemical space, something not achievable by the use of the natural mevalonate and methyl erythritol phosphate pathways.

Experimental Section

cbut-DMAOH synthesis

This synthesis was adapted from Arai *et al.*[14]

Cyclobutylidene ethyl acetate: Cyclobutanone (938 mg, 13.4 mmol) was added to a 100 mL three-neck round-bottom flask, dissolved in benzene (10 mL) and brought to reflux. Then, a solution of 4.67 g (13.4 mmol) of (carbethoxymethylene)triphenylphosphorane in 25 mL of benzene was slowly added, and the reaction mixture left under agitation and reflux conditions for 2 h. The evolution of the reaction was analyzed by TLC (solvent: pentane/diethyl ether, 7/3). Then the reaction mixture was evaporated and the liquid residue recovered in a minimum of 8/2 pentane/diethyl ether mixture for purification by silica gel flash chromatography using the same solvent system. After purification and evaporation of organic solvents, 996 mg (7.1 mmol) of cyclobutylidene ethyl acetate were obtained corresponding to a 53% isolated yield.

Cyclobutylidene ethyl acetate: ^{1}**H NMR** (300 MHz, $CDCl_3$), δ (ppm) 5.66 (m, 1H), 4.22 (q, $J=7.15$ Hz, 2H), 3.17–3.26 (m, 2H), 2.86–2.95 (m, 2H), 2.16 (quint, $J=7.7$ Hz, 2H), 1.34 (t, $J=7.15$ Hz, 3H) ^{13}**C NMR** (75 MHz, $CDCl_3$), δ (ppm) 167.5, 166.6, 112.4, 59.5, 33.8, 32.3, 17.7, 14.3.

cbut-DMAOH: A solution of 996 mg (7.1 mmol) of cyclobutylidene ethyl acetate in 10 mL of dichloromethane was put in a 100 mL three-neck round-bottom flask under agitation and inert atmosphere (N_2) and cooled to −80 °C. Then 15.6 mL of a 1 M DiBAl–H solution in dichloromethane was slowly added (reaction evolution followed by TLC, solvent system: pentane/diethyl ether, 8/2). After addition completion, the reaction mixture was left under agitation until room temperature was reached. Then, the reaction mixture was transferred to an ice cooled Erlenmeyer flask under agitation, followed by the careful addition of 20 mL of methanol and 20 mL of Rochelle salt solution (sodium potassium tartrate tetrahydrate) and left under agitation for 30 min before filtration over Celite®

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(washed with diethyl ether and water). The resulting filtrates were pooled, the aqueous phase was extracted three times with 100 mL of diethyl ether and washed with a saturated NaCl solution before drying step over anhydrous sodium sulfate. After filtration and removal of the solvents under reduced pressure, the liquid residue was purified over silica gel (pentane/diethyl ether, 1/1) affording 157 mg (1.6 mmol) of cyclobutylidenethanol corresponding to a 22.5% isolated yield.

Cyclobutylidenethanol: ^{1}**H NMR** (300 MHz, $CDCl_3$), δ (ppm) 5.22 (m, 1H), 3.90 (dq, *J*=7.2 Hz, 1.3 Hz, 2H), 2.83 (s, 1H), 2.63 (m, 4H), 1.88 (m, 2H); ^{13}**C NMR** (75 MHz, $CDCl_3$), δ (ppm) 144.58, 119.25, 58.97, 31.01, 29.21, 17.10.

Tryprostatin B enzymatic synthesis

Analytic scale: Tryprostatin B (TB) was enzymatically synthesized from DMAOH and chemically synthesized brevianamide F (BF) in 1.5 mL Eppendorf® tubes in a VWR thermomixer. DMSO stock solutions of BF and DMAOH were prepared in order to reach a final concentration of 10 and 20 mM respectively as well as a final DMSO concentration of 10% (v/v). Other reagents and enzymes were prepared in 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5. ATP was added alone (final concentration 40 mM) or included in a recycling system based on the use of ATP 4 mM (final concentration), PEP 40 mM (final concentration) and 50 μL of pyruvate kinase (Sigma-Aldrich, 30–50 U). These reagents were incubated with 0.2 U each of purified $ThiM_{Ec}$, IPK_{Mv} and $FtmPT1_{Af}$ in 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5 buffer in a total volume of 1 mL at 37 °C, with shaking at 800 rpm for 24 h. Samples were regularly withdrawn and analyzed by TLC (ethyl acetate/methanol, 9/1) and HPLC for BF disappearing.[5] HPLC analysis were performed after dilution of a 100 μL sample with 200 μL stock solution of indole (internal standard) in acetonitrile (5 mM).

Large-scale synthesis: the reaction was conducted in a 50 mL round-bottom flask under the same conditions as described above. TB was synthesized starting from 50 mg of BF, with the previously described ATP recycling system, 0.2 U/mL of each enzyme in a total volume of 17 mL. After 2 h, the reaction mixture was extracted three times with 50 mL of ethyl acetate, the organic phases were washed with water and dried over anhydrous sodium sulfate. After filtration and evaporation, TB was purified over silica gel (ethyl acetate/methanol, 9/1) affording 58.4 mg of a white powder corresponding to a 92% isolated yield.

Tryprostatin B: ^{1}**H NMR** (300 MHz, $CDCl_3$), δ (ppm) 8.22 (br s, 1H), 7.45 (d, *J*=7.4 Hz, 1H), 7.27 (d, *J*=7.5 Hz, 1H), 7.09 (m, 2H), 5.66 (br s, 1H), 5.28 (tt, *J*=7.2, 1.0 Hz, 1H), 4.33 (dd, *J*=11.1, 3.0 Hz, 1H), 4.01 (t, *J*=7.4 Hz, 1H), 3.71–3.40 (m, 5H), 2.94 (dd, *J*=15.0, 11.1 Hz, 1H), 2.35–2.23 (m, 1H), 2.04–1.79 (m, 3H), 1.75 (s, 3H), 1.72 (s, 3H); ^{13}**C NMR** (75 MHz, $CDCl_3$), δ (ppm) 169.34, 165.85, 136.55, 135.55, 135.23, 128.47, 121.79, 119.89, 119.84, 117.74, 110.82, 104.60, 59.25, 54.69, 45.4, 28.34, 25.7, 25.17, 22.59, 17.96.

Farnesyl diphosphate enzymatic synthesis

Large-scale synthesis of FPP: (2*E*-6*E*)-Farnesyl diphosphate (FPP) was synthesized from DMAOH and IOH (stock solutions in DMSO, final concentration of 5 and 10 mM respectively) in a 1:2 ratio. DMSO concentration was fixed at 3.4% (v/v), other reagents and enzymes were prepared in 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5. DMAOH and IOH were incubated with an ATP recycling system corresponding to ATP 4 mM, PEP 40 mM and 50 μL of pyruvate kinase (Sigma-Aldrich, 30–50 U), 0.2 U/mL of $ThiM_{Ec}$ and IPK_{Mv} and 0.3 U/mL of $FPPS_{Gs}$. $MgCl_2$ concentration was adjusted to 15 mM and the total reaction volume to 11 mL by the use of 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5 buffer. The reaction mixture was placed in eleven 1.5 mL Eppendorf® tubes and left for 24 h at 37 °C, shaking at 800 rpm. Under these conditions, farnesyl diphosphate precipitated and after 24 h was first isolated by centrifugation (13000 rpm, 5 min) and withdrawal of the supernatant. The solids were then wash thrice with 1 mL of water followed by centrifugation. The washed white solids were recovered in each tube with 0.7 mL of a 25 mM ammonium carbonate solution, the solutions mixed and then cation-exchanged over NH_4^+-DOWEX-50WX8 pre-equilibrated with the same solution of ammonium carbonate. Fractions containing the FPP ammonium salt were pooled, lyophilized and the diphosphate purified over RP-18 silica gel pre-equilibrated with the solvent mixture water/tetrahydrofuran 90/10. FPP was recovered using a stepwise THF gradient in water (10/90, 25/75, 50/50, 75/25 and 100/0). After evaporation and lyophilization, 12.5 mg of FPP corresponding to a 54% isolated yield were obtained.

(2*E*-6*E*)-FPP: ^{1}**H NMR** (300 MHz, $D_2O.NH_4OD$), δ (ppm) 5.62 (t, *J*=5.9 Hz, 1H), 5.33 (t, *J*=6.8 Hz, 1H), 5.28 (t, *J*=6.4 Hz, 1H), 4.63 (t, *J*=5.9 Hz, 2H), 2.19 (m, 8H), 1.89 (s, 3H), 1.82 (s, 3H), 1.77 (s, 3H), 1.75 (s, 3H); ^{31}**P NMR** (121 MHz, D_2O. NH_4OD), δ (ppm) – 5.75 (d, *J*=20.9 Hz), – 9.72 (d, *J*=20.9 Hz).

cbut-TB enzymatic synthesis

Analytical scale: The cyclobutyl derivative of TB was synthesized from the corresponding cyclic analog of DMAOH and BF (stock solutions in DMSO, final concentration of 20 and 10 mM respectively) with the same ATP recycling system as previously described (4 mM ATP/40 mM PEP, 50 μL/30–50 U of pyruvate kinase). In these experiments, DMSO concentration was fixed at 10% (v/v), other reagents and enzymes were prepared in 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5. These reagents were incubated with 0.2 U each of purified $ThiM_{Ec}$, IPK_{Mv} and $FtmPT1_{Af}$ in 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5 buffer in a total volume of 1 mL at 37 °C, with shaking at 800 rpm (VWR thermomixer) for 24 h. Samples were regularly withdrawn and analyzed by TLC (ethyl acetate/methanol, 9/1) and HPLC for BF disappearing. HPLC analysis were performed after dilution of a 100 μL sample with 200 μL stock solution of indole (internal standard) in acetonitrile (5 mM).

Large-scale synthesis of cbut-TB: cbut-TB was synthesized starting from 50 mg of chemically synthesized BF (10 mM, stock solution in DMSO) and cbut-DMAOH (20 mM, stock solution in DMSO), with the previously described ATP recycling system, 0.2 U/mL of each enzyme in a total volume of 17 mL. After 24 h, the reaction mixture was extracted three times with 50 mL of ethyl acetate, the organic phases were washed with water and dried over anhydrous sodium sulfate. After filtration and solvent removal under reduced pressure, cbut-TB was purified over silica gel (ethyl acetate/methanol, 9/1) affording 44.7 mg corresponding to a 72.4% isolated yield.

cbut-TB: ^{1}**H NMR** (600 MHz, $CDCl_3$), δ (ppm) 8.00 (br s, 1H), 7.47 (br d, *J*=7.9, 1H), 7.32 (br d, *J*=8.1, 1H), 7.16 (ddd, *J*=8.1, 7.1, 1.1 Hz, 1H), 7.09 (ddd, *J*=7.9, 7.1, 1.1 Hz, 1H), 5.64 (br s, 1H), 5.24 (br tquint, *J*=7.2, 2.4 Hz, 1H), 4.37 (ddd, *J*=11.5, 4.0, 1.2 Hz, 1H), 4.06 (br t, *J*=7.8 Hz, 1H), 3.68 (dd, *J*=15.2, 4.0 Hz, 1H), 3.68 (m, 1H), 3.59 (ddd, *J*=12.0, 9.2, 3.2 Hz, 1H), 3.37 (ddquint, *J*=16.1, 7.4, 1.5 Hz, 1H), 3.33 (ddquint, *J*=16.1, 6.8, 1.5 Hz, 1H), 2.94 (dd, *J*=15.2, 11.5 Hz, 1H), 2.72 (m, 4H), 2.34 (m, 1H), 2.06 (m, 1H), 2.03 (m, 1H), 2.00 (br quint, *J*=8.0 Hz, 2H), 1.91 (m, 1H); ^{13}**C NMR** (150 MHz, $CDCl_3$), δ (ppm) 169.5, 166.0, 144.7, 136.3, 135.6, 128.1, 122.1, 120.1, 117.9, 115.8, 110.9, 105.0, 59.4, 54.7, 45.6, 31.1, 29.5, 28.5, 25.7, 25.2, 22.9, 17.1.

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cbut-FPP and cbut-FOH enzymatic synthesis

cbut-(2*E*-6*E*)-Farnesyl diphosphate: cbut-FPP was synthesized from cbut-DMAOH and IOH (stock solutions in DMSO, final concentration of 5 and 10 mM respectively) in a 1:2 ratio. DMSO concentration was fixed at 3.4% (v/v), other reagents and enzymes were prepared in 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5. DMAOH and IOH were incubated with an ATP recycling system corresponding to ATP 4 mM, PEP 40 mM and 50 μL of pyruvate kinase (Sigma-Aldrich, 30–50 U), 0.2 U/mL of $ThiM_{Ec}$ and IPK_{Mv}, 0.3 U/mL of $FPPS_{Gs}$. $MgCl_2$ concentration was adjusted to 15 mM and the total reaction volume to 11 mL using 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5 buffer. The reaction mixture was placed in 11 1.5 mL Eppendorf® tubes and left for 24 h at 37 °C, shaking at 800 rpm. Under these conditions, diphosphates precipitated and after 24 h were first isolated by centrifugation at 13000 rpm and withdrawal of the supernatant. The solids were then wash thrice with 1 mL of water followed by centrifugation. The washed white solids were recovered in each tube with 0.7 mL of a 25 mM ammonium carbonate solution, the solutions mixed and then cation-exchanged over NH_4^+ -DOWEX-50WX8 pre-equilibrated with the same solution. Fractions containing the FPP ammonium salt were pooled, lyophilized and the diphosphate purified over RP-18 silica gel pre-equilibrated with the solvent mixture water/tetrahydrofuran 90/10. cbut-FPP was recovered using a stepwise THF gradient in water (10/90, 25/75, 50/50, 75/25 and 100/0). After evaporation and lyophilization, 10.7 mg of cbut-FPP corresponding to a 44% isolated yield were obtained.

cbut-FPP: ^{1}**H NMR** (300 MHz, D_2O-NH_4OD), δ (ppm) 5.77 (t, *J*= 6.6 Hz, 1H), 5.51 (t, *J*=6.6 Hz, 1H), 5.40 (m, 1H), 2.93 (t, *J*=7.7 Hz, 4H), 2.28–2.52 (m, 8H), 2.15–2.28 (m, 2H), 2.03 (s, 3H), 1.92 (s, 3H); ^{31}**P NMR** (121 MHz, D_2O-NH_4OD), δ (ppm) −6.00 (d, *J*=22.4 Hz), −10.09 (d, *J*=22.4 Hz).

The protons of the methylene group bearing the diphosphate functionality were hidden by the solvent peak. Suppression of the latter led to the apparition of a triplet at 4.77 ppm.

cbut-(2*E*-6*E*)-Farnesol: cbut-FOH was obtained by treatment of cbut-FPP with phosphatase $PhoN_{Xt}$.[5] Briefly, the recovered white solid of cbut-FPP was hydrolyzed in a Tris-HCl buffer (50 mM, pH 7.5) with 0.2 U/mL of purified $PhoN_{Xt}$ in a total volume of 1 mL (1.5 mL Eppendorf® tube, 800 rpm, 37 °C, 24 h). The reaction mixture was then extracted with diethyl ether and the formed cbut-FOH purified through silica column (solvent: pentane/diethyl ether, 1/1).

cbut-FOH: ^{1}**H NMR** (300 MHz, $CDCl_3$), δ (ppm) 5.42 (tq, *J*=6.6, 1.3 Hz, 1H), 5.42 (t, *J*=5.7 Hz, 1H), 5.02 (m, 1H), 4.16 (d, *J*=6.6 Hz, 2H), 2.63 (t, *J*=7.7 Hz, 4H), 1.86–2.17 (m, 10H), 1.69 (s, 3H), 1.59 (s, 3H).

(+)-δ-Cadinol enzymatic synthesis

Using the TMP 2.0: The product of STS_{Lm} was synthesized starting from DMAOH and IOH (stock solutions in DMSO) in a 1:2 ratio corresponding to 5- and 10-mM final concentration respectively. DMSO concentration was fixed at 3.4% (v/v), other reagents and enzymes were prepared in 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5. Alcohols were incubated with an ATP recycling system (ATP 4 mM, PEP 40 mM, 50 μL of pyruvate kinase Sigma-Aldrich, 30–50 U), 0.2 U of $ThiM_{Ec}$, IPK_{Mv} and STS_{Lm} and 0.3 U of $FPPS_{Gs}$. $MgCl_2$ concentration was adjusted to 10 mM and the total reaction volume to 1 mL with Tris-HCl buffer (50 mM, 5 mM $MgCl_2$, pH 7.5). The reaction mixture was placed in an Eppendorf® tube and left for 24 h at 37 °C, shaking at 800 rpm. After 24 h, the reaction mixture was extracted with 500 μL of methyl tertiobutyl ether (MTBE), vortexed and centrifuged 1 min at 12500 rpm. The organic phase was recovered and analyzed by GC, revealing a major product, RT=23.5 min.

Using chemically synthesized FPP: A 20 mM (2*E*,6*E*)-FPP solution in 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5 was incubated with 0.2 U/mL of purified STS_{Lm} in 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5 buffer and a total volume of 500 μL at 37 °C shaking at 800 rpm for 24 h (VWR thermomixer). The reaction mixture was extracted by 300 μL of MTBE, vortexed and centrifuged 1 min at 12500 rpm. The organic phase was then recovered and analyzed by GC, revealing the same major peak corresponding to a sesquiterpene alcohol, RT= 23.5 min.

A large-scale synthesis was realized on a 23 mL scale, in a 50 mL round-bottom flask at the same temperature and agitation conditions. FPP (49.2 mg, 5 mM final concentration) was suspended in 15.6 mL of Tris-HCl buffer 50 mM pH 7.5 containing $MgCl_2$ (10 mM) and NaCl (300 mM). The reaction was initiated by the addition of purified STS_{Lm} (7 mL in the same buffer) reaching a final 0.2 U/mL activity concentration. After 24 h, the reaction mixture was extracted three times with 20 mL of MTBE, the organic phases were washed with water and dried over anhydrous sodium sulfate. After filtration and evaporation, the resulting product was purified over silica gel (pentane/diethyl ether, 1/1) affording 8 mg of δ-cadinol (31% isolated yield).

(+)-δ-cadinol: $[\alpha]_D^{25}=+37$ (c=0.73, $CHCl_3$); ^{1}**H NMR** (600 MHz, $CDCl_3$), δ (ppm) 5.62 (dq, *J*=5.1, 1.7 Hz, 1H), 2.02 (1H, m), 1.99 (2H, m), 1.97 (1H, m), 1.90 (1H, m), 1.66 (3H, brs), 1.57 (1H, m), 1.56 (1H, m), 1.51 (1H, m), 1.50 (1H, m), 1.31 (1H, m), 1.30 (3H, s), 1.10 (qd, *J*= 13.1, 4.3 Hz, 1H), 0.88 (d, *J*=6.9 Hz, 3H), 0.81 (d, *J*=6.9 Hz, 3H); ^{13}**C NMR** (150 MHz, $CDCl_3$), δ (ppm) 134.2. 124.8, 72.7, 45.7, 44.2, 36.9, 35.3, 31.3, 28.2, 26.8, 23.8, 21.8, 21.7, 18.6, 15.6; **HR–MS** : 222.1984 Da (expected 222.1984 Da).

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: cyclobutyl terpenoid derivatives · farnesyl diphosphate · terpene mini-path · tryprostatin B · (+)-δ-cadinol

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