Supporting Information for:

Edaxadiene: a new bioactive diterpene from *Mycobacterium tuberculosis*

Francis M. Mann,[†] Meimei Xu,[†] Xiaoming Chen,[†,§] D. Bruce Fulton,[†] David G. Russell,[‡] and Reuben J. Peters[†,*]

[†]Iowa State University, Department of Biochemistry, Biophysics, & Molecular Biology, Ames, Iowa 5001; [‡]Cornell University, College of Veterinary Medicine, Department of Microbiology & Immunology, Ithaca, New York 14853

Materials and Methods:

General: Unless otherwise noted, all chemicals were obtained from Fisher Scientific (Loughborough, Leicestershire, UK) and molecular biology reagents from Invitrogen (Carlsbad, CA, USA). GC-MS analysis was carried out with a Saturn 3900 Gas Chromatographer coupled to a Saturn 2100T Mass Spectrometer (Varian Industries, Palo Alto, CA, USA) run in electrospray ionization (70 eV) mode using a VF-1ms column. For this purpose, samples (1 μL) were injected in splitless mode at 50 °C and, after holding for 3 min., the oven temperature was raised at a rate of 14 °C/min to 300 °C, where it was held for an additional 3 min. MS data was collected from 90 to 600 *m/z* starting 12 min. after injection until the end of the run. High-resolution mass spectrometry (HRMS) was carried out by the HRMS facility at the University of Iowa using an AutoSpec (Micromass Inc.) in electron ionization mode.

Construct Assembly: Rv3377c and Rv3378c were cloned from *Mycobacterium tuberculosis* strain H37Rv genomic DNA (a kind gift from Dr. John Bannantine, USDA-NADC) based on public sequence information (Tuberculist, Pasteur Institute). Both genes were inserted into the Gateway expression system (pENTR), verified by complete sequencing, then transferred via directional recombination to pTH1,[1] which results in expression as fusion proteins with the maltose binding protein (MBP) at the N-terminus.

Biosynthesis of Edaxadiene: Protein was expressed by growing recombinant *E. coli* to OD_{600} of 0.8-1.0 and inducing with 0.5 mM IPTG for 16 hours, which yielded approximately 5 mg/L of protein. Cells were lysed via sonication on ice and the MBP tagged proteins purified over 1 mL bed volume of amylose resin as directed by the manufacturer (New England Biolabs, Ipswich, MA, USA), but in the presence of 10 mM $MgCl_2$ for MBP-Rv3377c and 10 mM $MnCl_2$ for MBP-MtEDS. Geranylgeranyl diphosphate was obtained from Isoprenoids, LC (Tampa, FL, USA) and converted to halimadienyl diphosphate (133 μM GGPP, 75 mM HEPES pH 6.8, 1 mM $MgCl_2$, 15 mM KCl, 10% Glycerol, and 250 μg MBP-Rv3377c). This reaction was incubated for 4 hours at 30 °C to ensure complete turnover. The resulting halimadienyl diphosphate was converted to edaxadiene by the addition of $MnCl_2$ to 10 mM and MBP-MtEDS (in 100 μg increments per hour for 10 hours). Final yield for the overall reaction was limited to 10-15% by competing non-enzymatic hydrolysis of the diphosphate to the corresponding primary alcohol tuberculosinol (**2**) (as determined by comparison to control reactions without the addition of MBP-MtEDS). Edaxadiene (**3**) was extracted from the reaction with hexane, the resulting organic solvent overlay removed, evaporated under nitrogen, resuspended in 50% acetontrile (500 μg/mL), and purified using an HPLC (1100 Series HPLC, Agilent Technologies,

Santa Clara, CA, USA) equipped with a 150 × 4.6 mm C8 column (100-5C8; Kromasil, Bohus, Sweden) with detection at 215, 222, 254, 280, and 300 nm wavelengths. After sample injection (0.5 mL), the column was washed with 1 mL 50% acetonitrile, then a 50-100% acetonitrile gradient was applied over 2.5 mL, and edaxadiene eluted with 8 mL of 100% acetonitrile. Complete purification required a second pass of the initially purified material over the column using the same protocol with a slower flow rate during edaxadiene elution. Product purity was analyzed via GC-MS, as described above.

Semi-synthesis of edaxadiene: Halimadienyl diphosphate (**1**) was enzymatically produced as above. Tuberculosinol (**2**) was generated by enzymatic dephosphorylation with Calf Intestinal Alkaline Phosphatase (NEB) in a final concentration of 100 U/mL, extracted with hexane, and dehydrated via a pseudourea intermediate.[2] Specifically, the hexane was removed by evaporation under nitrogen, the tuberculosinol (**2**) dissolved in acetone, then stirred under nitrogen with 2 molar equivalents of dicyclohexylcarbo-diimide (DCC) in the presence of 0.1 molar equivalents of $CuCl_2$ for 4-8 hours at room temperature. The resulting products were quantified by GC-FID, analyzed by GC-MS, and the produced edaxadiene (**3**) purified by HPLC as described above. Notably, the resulting loss during purification led to reduction in the final yield of fully purified edaxadiene (**3**) from tuberculosinol (**2**) being <10%. The purified edaxadiene (**3**) was characterized by NMR; first to confirm equivalence to the enzymatic product, followed by more extensive analyses to determine the corresponding structure.

Structural Data: NMR spectra for the edaxadiene (**3**) product were recorded at 25 °C on a Bruker Avance 700 equipped with a probe with cryogenic detection for ^{1}H and ^{13}C. For 1D ^{13}C spectra 15 mM chromium (III) acetylacetonate was added to the sample as a relaxation enhancement agent. Structural analysis was undertaken using 1D ^{1}H, 1D ^{13}C, DQF-COSY, HSQC, multiplicity-edited HSQC, HMBC, HMQC-COSY and ROESY spectra acquired at 700 MHz using standard experimental protocols. In addition a ROESY spectrum was acquired at 10 °C. Chemical shifts were referenced to TMS. Connections between protonated carbons were obtained from DQF-COSY and HMQC-COSY correlations (Fig. S2b). The structural fragments derived from the COSY data were connected through unsaturated carbons by use of HMBC correlations (Fig. S2c). The proposed six membered ring formed by carbons 7, 8, 9, 11, 12, and 13 (numbering as given in Fig. S2a) is flexible and exhibits intermediate chemical exchange effects in the ^{1}H NMR spectra. This resulted in overlap of the ^{1}H frequencies for protons at positions 11 and 12. Due to this flexibility the ROESY spectra recorded at 10 and 25 °C provided no useful correlations for this part of the molecule. Thus it was not possible to unambiguously assign the stereochemical configuration at carbon 13.

Biological Assays: Analysis of phagosomal maturation was performed employing ratio fluorescent measurements as detailed previously.[3] In brief, phagosomal pH was measured with IgG-coated silica particles, labeled with the pH-sensitive fluorochrome carboxyfluorescein-SE.[4] Phagosomal proteolysis was quantified with IgG-coated silica beads carrying the substrate DQ Green Bodipy BSA and the calibration fluorochrome Alexa 594.[3] β-galactosidase activity was measured with IgG-opsonized, C18-silica beads coated in the substrate C12-fluorescein-di-galactopyranoside and the calibration fluorochrome octadecyl rhodamine B.

The particles for measuring pH and proteolysis were complexed with de-fatted BSA and were loaded with edaxadiene (**3**) by suspending the particles in 500 ml PBS in a sonicating waterbath at 37 °C. 1-10 μg of **3** in 5 μl hexane was added to the aqueous solution and sonicated

for 2 min. The particles were washed and used immediately. Particles for measurement of β-galactosidase activity were loaded with edaxadiene through addition of the isoprenoid (1-10 μg) in hexane to the lipid suspension in $CHCl_3$ prior to evaporation of the solvent and re-hydration of the particles in PBS in a sonicator waterbath at 37 °C for 2 min. Particles were washed and used immediately.

Particle suspensions were adjusted to give a dose of approximately 3 particles per cell prior to addition to macrophage monolayers as detailed.[3-5] The particles were bound for 3 min. at ambient temperature prior to washing and transfer to cuvettes in the environmental chamber of the PTI QM4 SE spectrofluorometer pre-equilibrated to 37 °C. Measurements were acquired and processed as previously described.[3-5] Cell viability was assessed at termination of all the assays through exclusion of the non-permeable dye Trypan Blue.[3]

Enzymatic Assays: MBP-MtEDS was purified as described above to >90% purity and assessed for activity. Enzymatic activity was optimized at 100 nM enzyme for 5 min. in the presence of 10 mM $MnCl_2$ or other divalent cation. The divalent cation was optimized via addition of 1, 5, or 10 mM of the relevant divalent cation to the reaction mixture described above. MtEDS was then added in 100-250 nM equivalents. Due to the instability of MBP-MtEDS, it was not dialyzed, but all samples were tested against a 'negative' control or baseline divalent cation concentration of <0.25 mM $MnCl_2$. After determination of the optimal divalent cation (Mn^{2+}) and pH (7.5), purification was done in the presence of 10 mM $MnCl_2$. Assays were conducted in assay buffer containing 50 mM HEPES pH 7.5, 10% glycerol, 1 mM KCl, 10 mM $MnCl_2$, and concentrations of halimadienyl diphosphate (**1**) ranging from 0.5 μM to 65 μM. Assays were conducted for 5 min. at 30 °C and stopped with 110 μL of 20 mM N-ethyl-maleimide (NEM) and incubated for 5 min. at 75 °C. The remaining NEM was neutralized with excess DTT. An internal standard of geranylgeraniol was inoculated into each individual assay at concentrations of 2.5 μg. The assay was then extracted three times with ~1 mL hexanes, dried under N_2 gas to complete dryness, then resuspended in 50 uL fresh hexanes and quantified via GC-FID by comparison of edaxadiene (**3**) peak area to internal standard peak area. All reported data points are averages from duplicate assays with the error bars corresponding to the corresponding standard deviation. Curve fits were carried out with Kaleidagraph (Synergy Software). For all curve fits $R^2 > 90\%$.

Mutagenesis and Enzymatic Assays of MtEDS Mutants: Mutants of MtEDS were constructed via site directed mutagenesis of MtEDS/pENTR with single site mutant primers of approximately 18 bp. Mutants were confirmed via sequence analysis. The genes were then transferred to the pTH1 vector for expression as fusion to MBP as described above for wild type MtEDS. The mutant enzymes also were purified as described above, and then assayed with 15 or 30 μM halimadienyl diphosphate (**1**) and 1 μg enzyme for periods of 5 minutes, 30 min., 1 hour, or 24 hours. Assays were stopped, extracted, and quantified as described above.

Figure S1: GC-MS analysis of edaxadiene (**3**) produced by MtEDS from halimadienyl diphosphate (**1**). (a) Selected ion chromatogram (a) and MS (b).

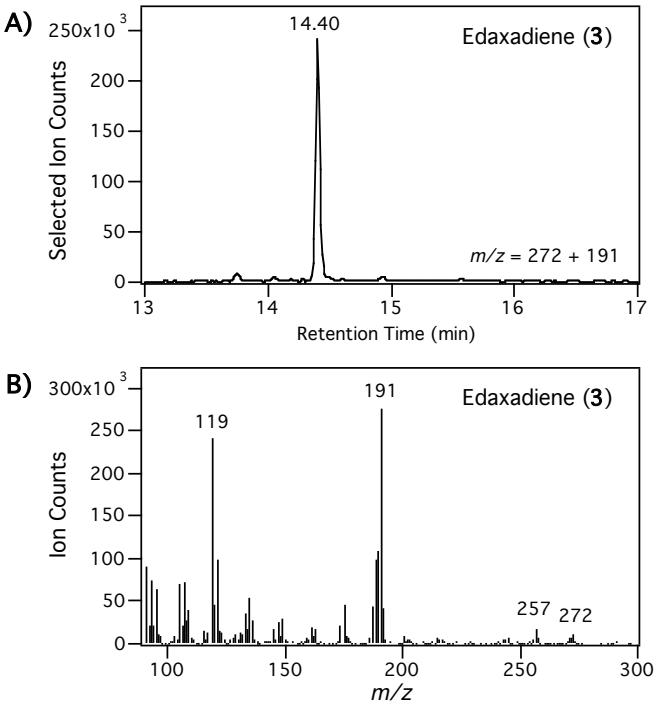


Figure S2: Divalent Cation Optimization of MtEDS (relative rate). Mn^{2+} performed slightly better than Mg^{2+}. Though Zn^{2+} gave the most optimal activity, free Zn^{2+} is not believed to be biologically relevant.

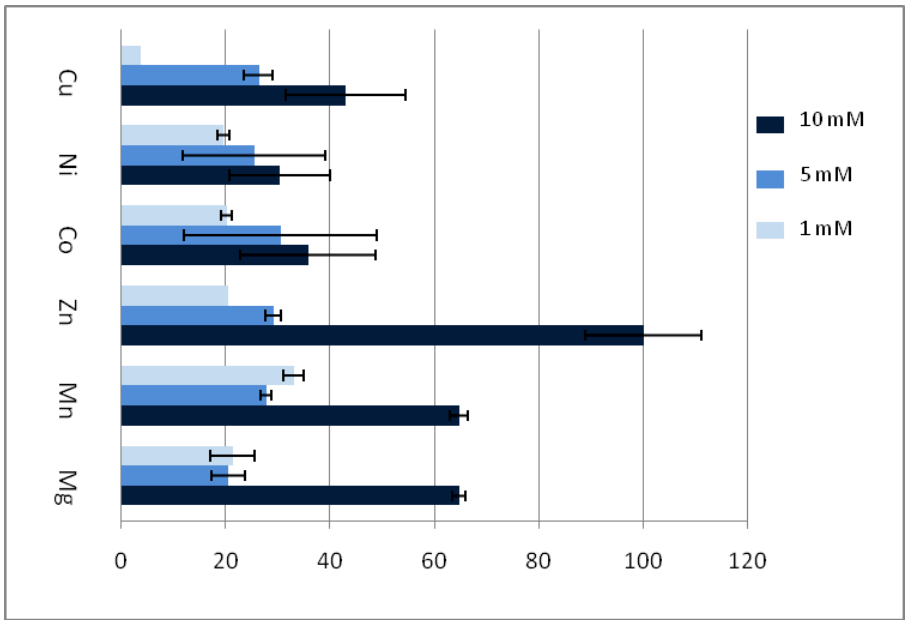


Figure S3: Edaxadiene (**3**). (a) Numbering. (b) COSY connections. (c) HMBC correlations.

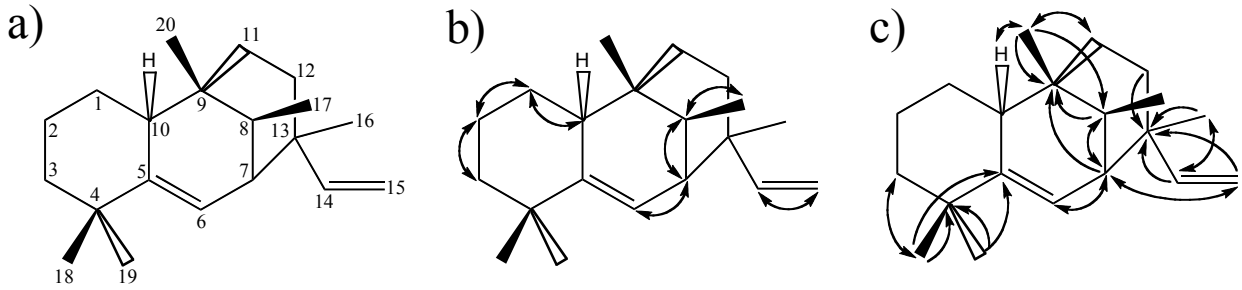


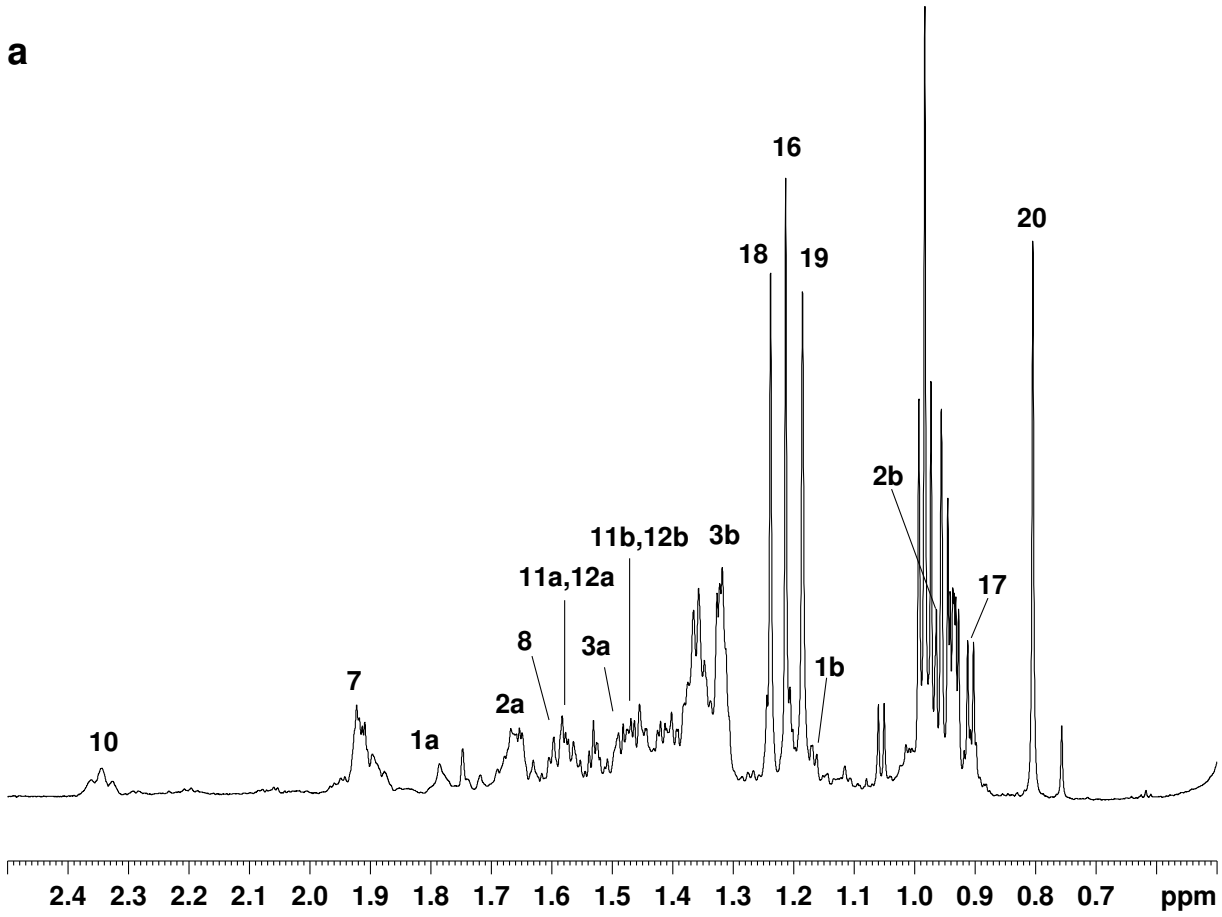
Table S1: NMR chemical shift data for **3**.

Carbon Position	$\delta^{13}C$ (ppm)	$\delta^{1}H$ (ppm)
1	27.53	1.894 (t) 14.0; 1.164 (t) 12.5
2	22.48	1.651 (m), 0.957 (m)
3	41.15	1.481 (d) 12.4; 1.359 (t) 12.5
4	36.13	
5	145.80	
6	116.69	5.643 (m)
7	31.84	1.920 (m)
8	33.53	1.580 (m)
9	36.14	
10	40.07	2.346 (t) 12.4
11	30.24	1.561 (m); 1.453 (m)
12	35.35	1.549 (m); 1.473 (m)
13	72.90	
14	145.60	5.871 (dd) 17.3, 10.8
15	111.49	5.273 (dd) 17.7, 4.2; 5.051 (d) 10.6
16	27.55	1.211 (s)
17	15.20	0.909 (d) 7.1
18	29.97	1.239 (s)
19	29.22	1.186 (s)
20	16.42	0.806 (s)

Figure S4: 700 MHz ^{1}H NMR spectrum of **3** recorded in benzene-d6 at 25°C. Chemical shift assignments for overlapped regions were obtained from ^{1}H-^{13}C HSQC spectrum. (a) Upfield region of ^{1}H NMR spectrum. (b) Downfield region of ^{1}H NMR spectrum. (on page S6)

Figure S5: ^{13}C NMR spectrum of **3** recorded in benzene-d6 at 25° C with chromium (III) acetylacetonate added as a relaxation enhancement agent. (a) Expanded upfield region. (b) Full spectrum. (on page S7)

Figure S4


a
16
20
18
19
2b
11b,12b
3b
11a,12a
17
8
3a
1b
7
2a
10
1a
2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 1.0 0.9 0.8 0.7 ppm

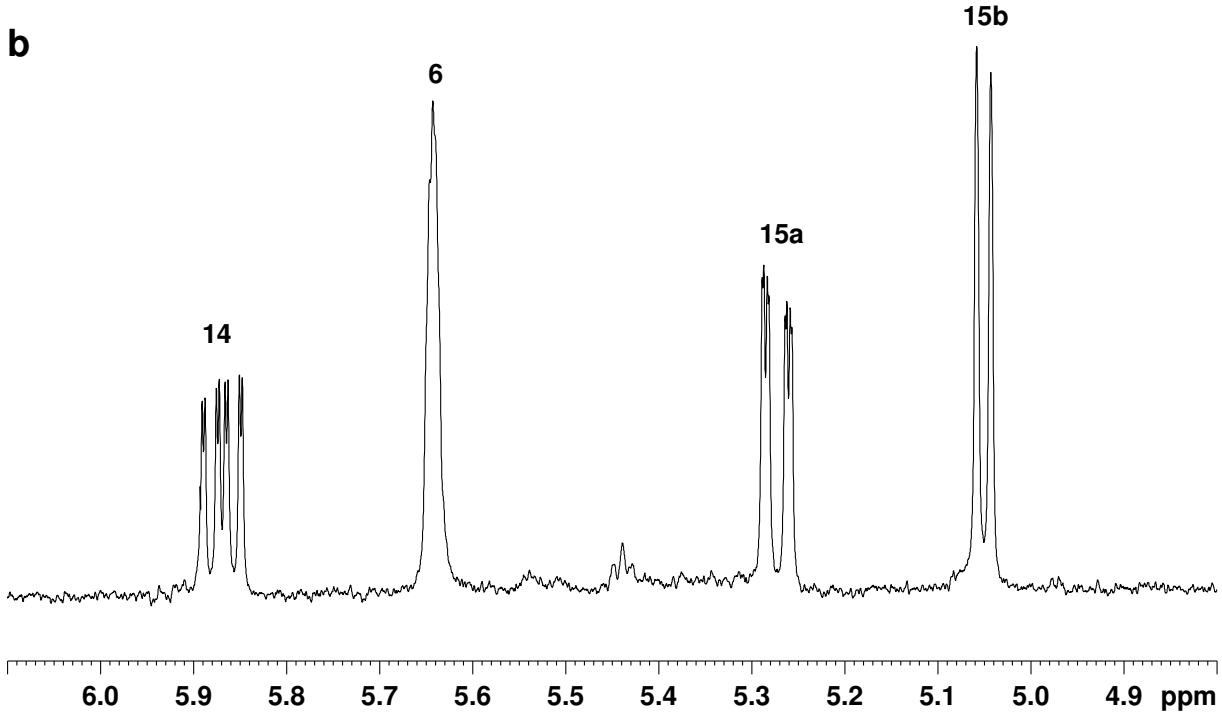
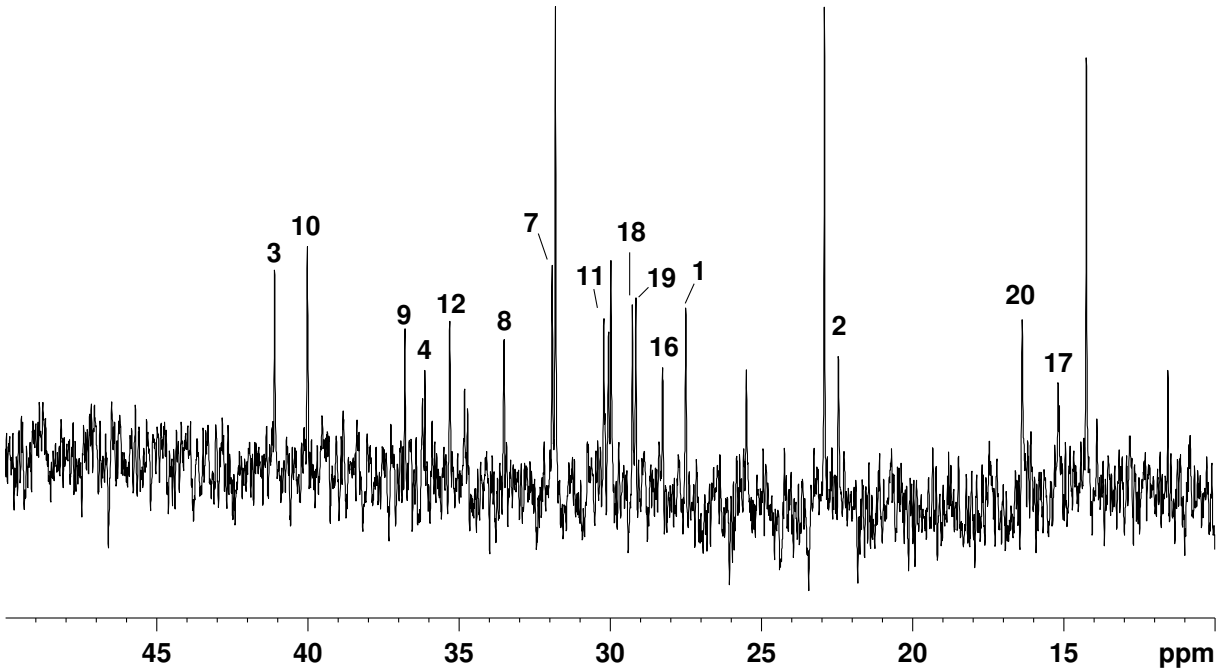

b
15b
6
15a
14
6.0 5.9 5.8 5.7 5.6 5.5 5.4 5.3 5.2 5.1 5.0 4.9 ppm

Figure S5

a


3
10
9
4
12
8
7
11
18
19
16
1
2
20
17
45
40
35
30
25
20
15
ppm

b

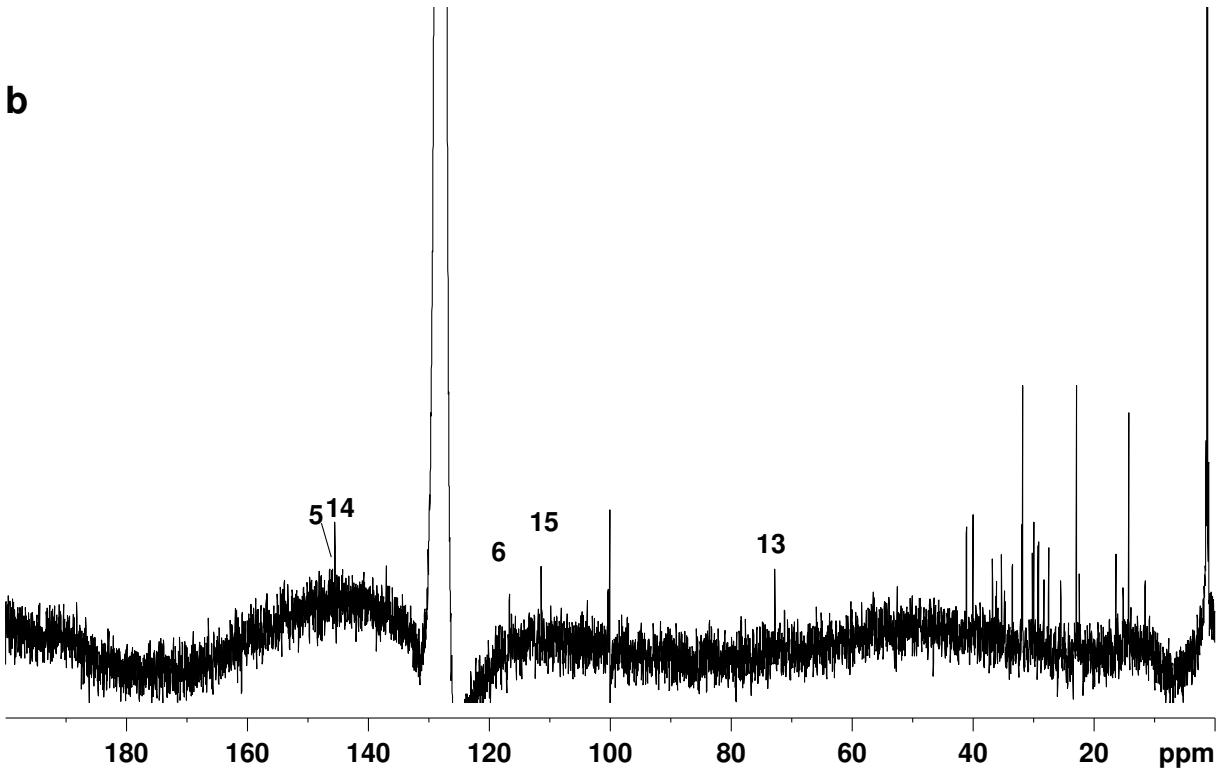

5
14
6
15
13
180
160
140
120
100
80
60
40
20
ppm

Figure S6: Alignment of MtEDS with other bacterial diterpene synthases. The conserved DDXXD and otherwise conserved NDXX(S/T)XXXE motifs are underlined. SgTS, terpentetriene synthase from *Streptomyces grisea*[6]; SsPS, pimaradiene synthase from *Streptomyces* sp. strain KO-3988[7]; BjKS, kaurene synthase from *Bradyrhizobium japonicum*[8].

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                1                                                                 80
  SsPS    (1) ---MRARHRVALKVLADLRSWAAEYPQVLEATPIEALAISTAAIS-PWRGANELRLSAPDVRCGPTPLDDHVEQNVRS-L
  SgTS    (1) ---MPDAIEFEHEGRRNPNSAEAESAYSSIIAALDLQESDYAVISGHSRIVGAAALVYPDADAETLLAASLWTACLIV-N
  BjKS    (1) ------MIQTERAVQQVLEWGRSLTGFADEHAVEAVRGGQYILQRIHPSLRGTSARTGRDPQDETLIVTFYRELALLFWL
 MtEDS    (1) MNLVSEKEFLDLPLVSVAEIVRCRGPKVSVFPFDGTRRWFHLECNPQYDDYQQAALRQSIRILKMLFEHGIETVISPIFS

                81                                                               160
  SsPS   (76) DELDDLFGRCEAIVRGGDRDDGHPLLASLSGWQSALERAPH------YPKLAGLWGDRFAEALRGERYDWTAGLARDRGE
  SgTS   (77) DDRWDYVQEDGGRLAPGEWFDG--VTEVVDTWRTAGPRLPDPFFELVRTTMSRLDAALGAEAADEIGHEIKRAITAMKWE
  BjKS   (75) DDCNDLGLISPEQLAAVEQALGQGVPCALPGFEGCAVLRAS----LATLAYDRRDYAQLLDDTRCYSAALRAGHAQAVAA
 MtEDS   (81) DDLLDRGDRYIVQALEGMALLAN--DEEILSFYKEHEVHVLFYGDYKKRLPSTAQGAAVVKSFDDLTISTSSNTEHRLCF

                161                                                              240
  SsPS  (150) GPSDPQEYLTYAASSNAWITHFP---------RWATSDR-DDLLDGLPVLDNALEAIEVAVRLSNDLATFERER-AEPGQ
  SgTS  (155) GVWNEYTKKTSLATYLSFRRGYCTMDVQVVLDKWINGGRSFAALRDDPVRRAIDDVVVRFGCLSNDYYSWGREKKAVDKS
  BjKS  (151) ERWSYAEYLHNGIDSIAYANVFCC--------LSLLWGLDMATLRARPAFRQVLRLISAIGRLQNDLHGCDKDRSAGEAD
 MtDTS  (159) GVFGNDAAESVAQFSISWNETHG-----------------KPPTRREIIEGYYGEYVDKADMFIGFGRFSTFDFPLLSSG

                241                                                              318
  SsPS  (219) NNILMYDTSPDWVHDELDRHSRKAQEQLDPLATAGFPPAVELLRLLDWSVTFYSGADFRGWGSDRDLTGPSGLPSDM-
  SgTS  (235) NAVRILMDHAGYDESTALAHVRDDCVQAITDLDCIEESIKRSGHLGSHAQELLDYLACHRPLIYAAATWPTETNRYR-
  BjKS  (223) NAVILLLQRYPAMPVVEFLNDELAGHTRMLHRVMAEERFPAPWGPLIEAMAAIRVQYYRTSTSRYRSDAVRGGQRAPA
 MtEDS  (222) KTSLYFTVAPSYYMTETTLRRILYDHIYLRHFRPKPDYSAMSADQLNVLRNRYRAQPDRVFGVGCVHDGIWFAEG---
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Figure S7: Kinetic parameters of MtEDS in the presence of 10 mM $MnCl_2$. The apparent K_M for halimadienyl diphosphate (HPP, **1**) is 12 μM, and k_{cat} is 0.13 s^{-1} (catalytic efficiency is then 1.1 x 10^4 s^{-1} M^{-1}).

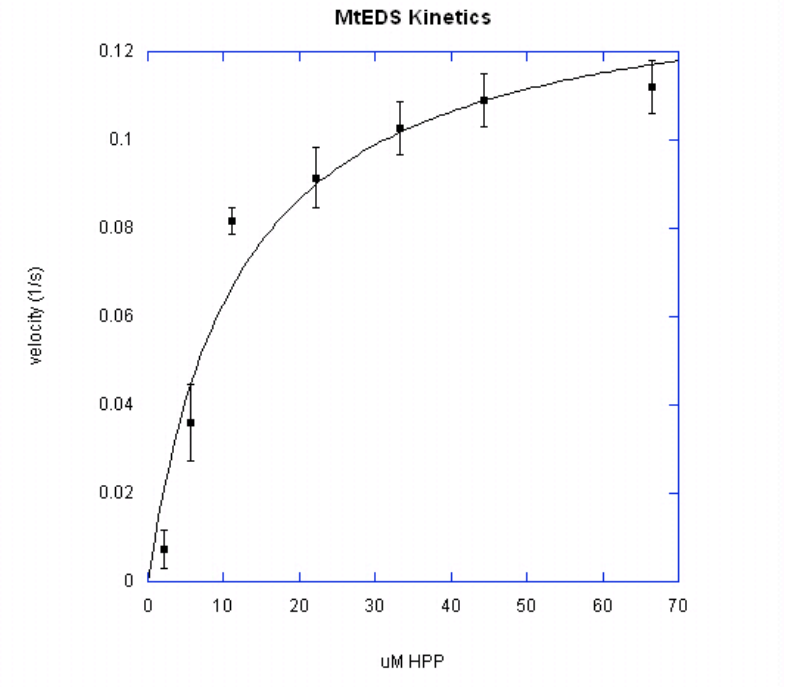

Table S2: Rate analysis of MtEDS DDXXD mutants.

MtEDS Mutant	k_{cat} (min^{-1})	std
Wt	7.8	0.1
D81A	3.1×10^{-4}	2×10^{-5}
D82A	0.055	0.005
D85A	4.3×10^{-4}	4×10^{-5}

References:

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