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Structure, function and inhibition of *ent*-kaurene synthase from *Bradyrhizobium japonicum*

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Correspondence and requests for materials should be addressed to R.-T.G. (guo_rt@tib.cas.cn) or E.O. (eoldfiel@illinois.edu)

* These authors contributed equally to this work.

Wenting Liu^{1*}, Xinxin Feng^{2*}, Yingying Zheng^{1*}, Chun-Hsiang Huang¹, Chiaki Nakano⁴, Tsutomu Hoshino⁴, Shannon Bogue², Tzu-Ping Ko³, Chun-Chi Chen¹, Yunfeng Cui¹, Jian Li¹, Iren Wang³, Shang-Te Danny Hsu³, Eric Oldfield² & Rey-Ting Guo¹

¹Industrial Enzymes National Engineering Laboratory, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China, ²Department of Chemistry, University of Illinois, Urbana, IL 61801, USA, ³Institute of Biological Chemistry, Academia Sinica, Taipei 115, Taiwan, ⁴Department of Applied Biological Chemistry, Niigata University, Niigata 950-2181, Japan.

We report the first X-ray crystal structure of *ent*-kaur-16-ene synthase from *Bradyrhizobium japonicum*, together with the results of a site-directed mutagenesis investigation into catalytic activity. The structure is very similar to that of the α domains of modern plant terpene cyclases, a result that is of interest since it has been proposed that many plant terpene cyclases may have arisen from bacterial diterpene cyclases. The *ent*-copalyl diphosphate substrate binds to a hydrophobic pocket near a cluster of Asp and Arg residues that are essential for catalysis, with the carbocations formed on ionization being protected by Leu, Tyr and Phe residues. A bisphosphonate inhibitor binds to the same site. In the kaurene synthase from the moss *Physcomitrella patens*, 16- α -hydroxy-*ent*-kaurane as well as kaurene are produced since Leu and Tyr in the *P. patens* kaurene synthase active site are replaced by smaller residues enabling carbocation quenching by water. Overall, the results represent the first structure determination of a bacterial diterpene cyclase, providing insights into catalytic activity, as well as structural comparisons with diverse terpene synthases and cyclases which clearly separate the terpene cyclases from other terpene synthases having highly α -helical structures.

Terpenes (or terpenoids) represent the largest class of small molecules on the planet¹ and most are synthesized by enzymes that contain one or more of six main types of fold: α , β , γ , δ , ϵ and ζ ². About 20% of terpenes are diterpenes, molecules that contain a core of 20 carbon atoms, and most of these are made by plants and fungi. Diterpenes have, however, also been found in bacteria^{3–11}, and several of these compounds have activity as anti-infective and anti-cancer drug leads, virulence factors, as well as plant growth hormones. There are two different classes of terpene cyclases, Class I and Class II, defined by the amino acid motifs that are essential for catalysis^{12–14}. Class I cyclases contain two conserved motifs, DDXXD, and (N,D)DXX(S,T,G)XXX(E,D) (hereafter referred to as NTE), in which the boldface residues generally bind to the three Mg²⁺ that facilitate ionization of an isoprenoid diphosphate group, generating a reactive carbocation intermediate. In contrast, Class II cyclases typically contain only a DXDD motif in which the boldface aspartic acid protonates an isoprenoid double bond or an oxirane to, once again, generate a reactive carbocation intermediate. In some cases, both Class I as well as Class II motifs are present and these molecules are bi-functional or in one reported case, tri-functional¹⁵. For example, abietadiene synthase from *Abies grandis* (AgABS) is bi-functional and converts geranylgeranyl diphosphate (GGPP) to copalyl diphosphate (CPP) in a Class II reaction, then CPP is converted to abietadiene in a Class I reaction, Figure 1a. Similarly, the moss *Physcomitrella patens* utilizes a tri-functional enzyme to convert GGPP to *ent*-CPP in a Class II reaction, then *ent*-CPP is converted to *ent*-kaur-16-ene (20%) and 16 α -hydroxy-*ent*-kaurane (80%) via a Class I reaction with, in the case of kauranol, carbocation quenching¹⁶, Figure 1b.

Here, we report the first structure of a (Class I) bacterial diterpene cyclase, *ent*-kaurene synthase, from the soil bacterium *Bradyrhizobium japonicum* (BjKS; gene # blr2150)⁴. BjKS catalyzes the cyclization of *ent*-CPP which is produced by a separate (Class II) enzyme, *ent*-copalyl diphosphate synthase (*ent*-CPPS; blr2149), from GGPP (Figure 1c), and one possible mechanism of action¹⁷ for the *ent*-kaurene synthase reactions is shown in Figure 1c. The *ent*-KS reactions have been proposed to proceed via initial diphosphate loss from *ent*-CPP (catalyzed by Mg²⁺)

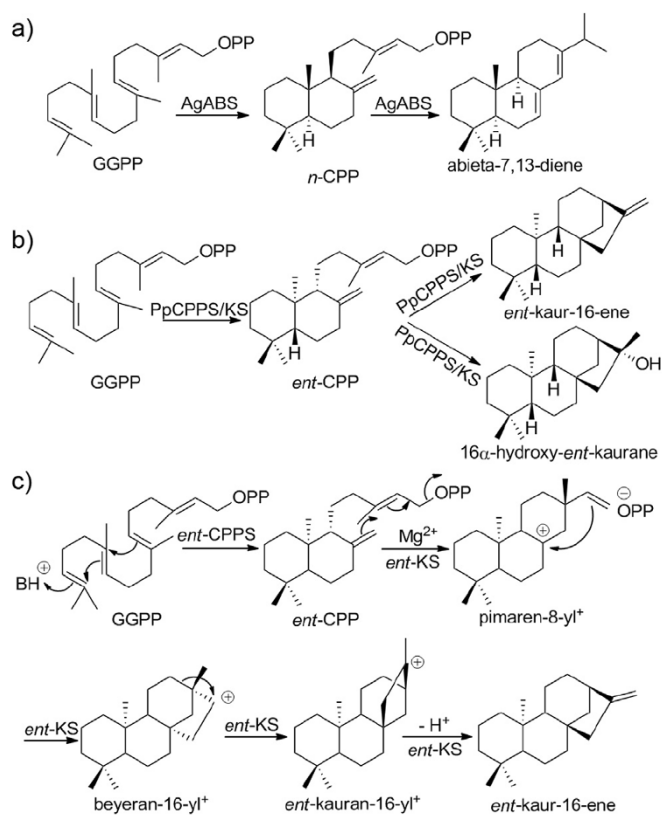


Figure 1 | Reactions catalyzed by AgABS, PpCPPS/KS and BjKS, and proposed catalytic mechanism for the BjKS reaction. (a) Abietadiene is formed from GGPP by a bi-functional terpene cyclase abietadiene synthase, in *Abies grandis*. (b) Formation of *ent*-CPP, *ent*-kaurene and kauranol from GGPP catalyzed by *Physcomitrella patens* CPPS/KS. (c) Two proteins, *ent*-CPPS and *ent*-KS, are used to produce *ent*-kaurene in the soil bacterium, *Bradyrhizodium japonicum*. Proposed carbocation intermediates are shown.

followed by cyclization to form the 8-carbonium ion, the pimaren-8-yl cation, which then undergoes secondary cyclization with Wagner-Meerwein migration of C-12 to C-16 to form the *ent*-kauran-16-yl cation. This cation can then lose a proton to form *ent*-kaur-16-ene, or in *P. patens* the *ent*-kauranyl cation can also be quenched by a water molecule to form 16- α -hydroxy-*ent*-kaurane, Figure 1b¹⁶.

Structurally, Class I proteins contain a highly α -helical catalytic α domain while Class II proteins contain two domains, $\beta\gamma$, and with bi-functional species such as abietadiene synthase, all three domains ($\alpha\beta\gamma$) are present¹⁸. In previous work¹⁹ we proposed that these $\alpha\beta\gamma$ domain proteins might have arisen by the fusion of the genes of $\alpha + \beta\gamma$ proteins in ancestral, perhaps soil dwelling, bacteria, so determining the structures of bacterial α (and $\beta\gamma$) cyclases is of interest not only from a mechanism of action perspective, but also from an evolutionary one. Is, for example, the structure of BjKS (a predicted¹⁹ α domain Class I protein) very similar to that of the α domains seen in modern plant terpene cyclases? How does *ent*-CPP bind? To investigate these and other questions we determined the structure of BjKS in the presence of its *ent*-CPP substrate, as well as apo- and inhibitor-bound structures, and we used site-directed mutagenesis to probe catalytic activity. In addition, we compared the BjKS structure with the structures of diverse other terpene cyclases and synthases, to see to what extent there were structural similarities among the different species.

Results and Discussion

Structure of apo-*ent*-kaurene synthase. We cloned, expressed and purified *ent*-kaurene synthase from *B. japonicum* then crystallized it

and solved its structure using a selenomethionine-substituted (SeMet) protein (Supplementary Table S1). Full data acquisition and crystallographic details for two native (i.e. apo) crystals are given in Table 1. These crystals were found in the same drop. The final models were refined at a resolution of 1.9–2.0 Å. There are four molecules (chains A, B, C, D) in the asymmetric unit of the apo-1 crystal and two in the asymmetric unit of the apo-2 crystal. Chain A and chain B in the apo-1 structure contained the most complete densities (residues 3–290 for chain A, 3–281 for chain B), while there was a missing loop (residues ~211–221) in both chains C and D in the apo-1 structure, and this loop was also missing (i.e. disordered) in all other structures (Table S2). The structures of the monomers of apo-1 and apo-2 are very similar with a 0.52–0.77 Å C α rmsd for any apo-1/apo-2 chain comparison. A stereo-view of chain A in the apo-1 structure (PDB ID code 4KT9) is shown in Figure 2a, from which it can be seen that the structure consists primarily of twelve α helices, as found in many other terpene cyclases, as well as synthases such as farnesyl diphosphate synthase. Both apo-1 and apo-2 crystal structures contain dimers (two dimers/4 chains in apo-1, one dimer in apo-2). In the apo-1 chain A, B structure (the most complete structure) there is a cluster of side-chains of hydrophobic residues (M235, Y232 and F240, cyan spheres) from both chains at one end of the dimer interface, Figure 2b, with a buried surface area of ~1000 Å³. Further up (green spheres), the E244 side-chain interacts with the N-terminus of the helix starting at Y155 in the other subunit in the dimer, and salt bridges exist between the E157(A) and R250(B) side-chains, and between E157(B) and R250(A) (purple spheres). These interactions all stabilize the dimer, at least in the crystal. To determine whether dimers are present in solution, we used size exclusion chromatography coupled with multi-angle light scattering (SEC/MALS). The SEC/MALS result (Figure 2c) indicates a molecular weight of 66.4 ± 0.3 kDa for the symmetric elution peak with a poly-dispersity value of 1.000, indicating that BjKS forms (and is presumably active as) a dimer in solution (the theoretical dimer MW is 66.76 kDa). However, as can be seen in Figure 2b the predicted catalytic motifs (DDXXD and NTE) are ~20 Å from the dimer interface and unlike some other prenyl synthases²⁰, there is no chain inter-digitation. This suggests that the presence of a dimer is not essential for catalysis.

Comparisons between kaurene synthase and other terpene cyclases/synthases. In previous work¹⁹, we used purely computational methods to predict the structure of (α -domain) BjKS (as well as $\beta\gamma$ domain BjCPPS) which led to the idea that modern plant terpene cyclases might have arisen by the fusion of the genes of ancestral (bacterial) α and $\beta\gamma$ domain proteins. It is thus of particular interest to now compare the actual BjKS structure with those of other, modern terpene cyclases (and synthases, which also have highly α -helical structures).

The BjKS structure is generally similar to that found in the α domains (α_c ¹⁹) of terpene cyclases from bacteria, fungi as well as plants: some representative examples are shown in Figure 3. Figure 3a shows a superposition between BjKS (red; PDB ID 4KT9) and pentalenene synthase (green; PDB ID 1HM7)²¹ from the bacterium *Streptomyces* UC5319 where there is a 2.7 Å C α rmsd (root mean square deviation) between C α atoms over 204 residues; Figure 3b shows a superposition with 1, 8-cineole synthase (PDB ID 2J5C) from the flowering plant *Salvia fruticosa* (Sf-CinS1, blue), an “ $\alpha\beta$ ” domain protein¹⁹ in which the C α rmsd is 2.4 Å over 195 residues, while Figure 3c shows a superposition with abietadiene synthase from the gymnosperm *Abies grandis* (AgABS; PDB ID 3S9V), an $\alpha\beta\gamma$ domain protein¹⁸.

As can be seen in Figure 3c, the BjKS structure (red) is similar to the α domain of AgABS (yellow), having a 2.6 Å C α rmsd over 224 residues. The C α rmsd value for the three other $\alpha\beta\gamma$ domain proteins: taxadiene synthase (PDB ID 3P5R); *ent*-copalyl diphosphate



Table 1 | Data collection and refinement statistics for KS crystals

	Apo-1	Apo-2	D75C + CPP	WT + BPH-629
Data collection				
Resolution (Å)	25.0 – 2.0 (2.1 – 2.0)	25.0 – 1.9 (2.0 – 1.9)	25.0 – 1.8 (1.9 – 1.8)	25.0 – 2.0 (2.1 – 2.0)
Space group	$P2_1$	$P4_1$	$P4_1$	$P4_1$
Unit-cell a, b, c (Å)	65.0, 130.1, 66.4	65.8, 65.8, 135.2	65.8, 65.8, 136.2	65.8, 65.8, 135.7
β (°)	95.6			
No. of unique reflections	72234 (6728)	43643 (4390)	51614 (5123)	38697 (3888)
Redundancy	3.0 (2.7)	5.6 (5.8)	4.9 (4.4)	3.4 (3.3)
Completeness (%)	98.0 (91.5)	99.8 (100.0)	99.5 (98.9)	99.4 (100.0)
Mean $I/\sigma(I)$	18.1 (3.1)	42.9 (4.5)	42.3 (3.6)	29.2 (3.3)
R_{merge} (%)	5.6 (20.7)	5.9 (46.7)	3.5 (37.2)	3.9 (46.3)
Refinement				
No. of chain(s)	4	2	2	2
No. of reflections	70362 (6036)	42679 (3982)	50837 (3310)	37623 (3499)
R_{work} (95% of data)	0.176 (0.230)	0.191 (0.251)	0.206 (0.28)	0.197 (0.279)
R_{free} (5% of data)	0.220 (0.276)	0.232 (0.287)	0.239 (0.309)	0.235 (0.291)
R.m.s.d. bonds (Å)	0.020	0.010	0.008	0.010
R.m.s.d. angles (°)	1.7	1.3	1.2	1.3
Ramachandran plot (%)				
Most favored (%)	97.3	96.7	97.5	96.6
Allowed (%)	2.2	2.8	2.1	2.8
Disallowed (%)	0.5	0.5	0.4	0.6
Mean B (Å ²)/atoms				
Protein atoms	27.1/8766	38.0/4310	39.8/4129	46.4/4300
Ligand atoms			90.0/58	154.0/30
Water molecules	38.2/651	51.2/624	50.8/415	57.4/360
PDB ID code	4KT9	4W4R	3WBV	4W4S

Values in parentheses are for the outer-most resolution shells.

synthase (PDB ID 3PYB) and bisabolene synthase (PDB ID 3SDV) are 2.6 Å/201 residues, 3.5 Å/169 residues, and 2.9 Å/226 residues for taxadiene synthase, *ent*-copalyl diphosphate synthase and (the ses-

quiterpene) bisabolene synthase, respectively, and the closest similarity to abietadiene synthase is likely due to the fact that AgABS produces a C₂₀ species that is structurally more similar to *ent*-kaur-

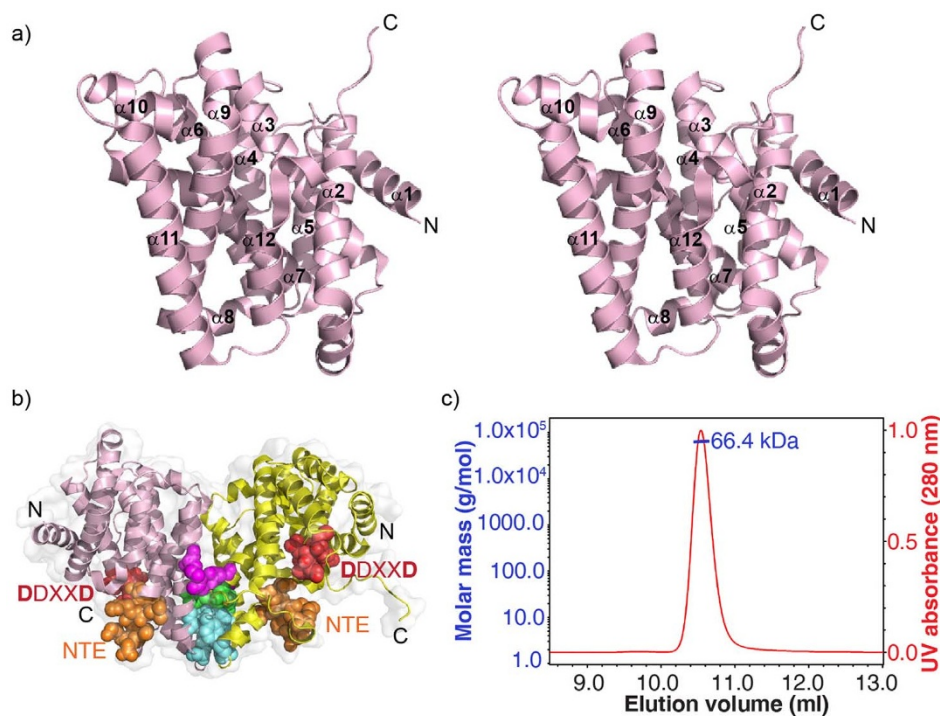


Figure 2 | Structure of BjKS. (a) Stereoview of (apo-1) BjKS (PDB ID code 4KT9) showing highly α -helical structure. (b) Dimer interface in *B. japonicum* *ent*-kaurene synthase. Hydrophobic residues (M235, Y232 and F240, cyan spheres) form a hydrophobic core; E244 side-chains interact with the N-terminus of the helix starting at Y155 in the other subunit (green spheres); salt bridges exist between the E157(A) and R250(B) side-chains, and between E157(B) and R250(A) (purple spheres). The two catalytic motifs (DDXXD, red and NTE, orange) are also shown. (c) SEC-MALS results.

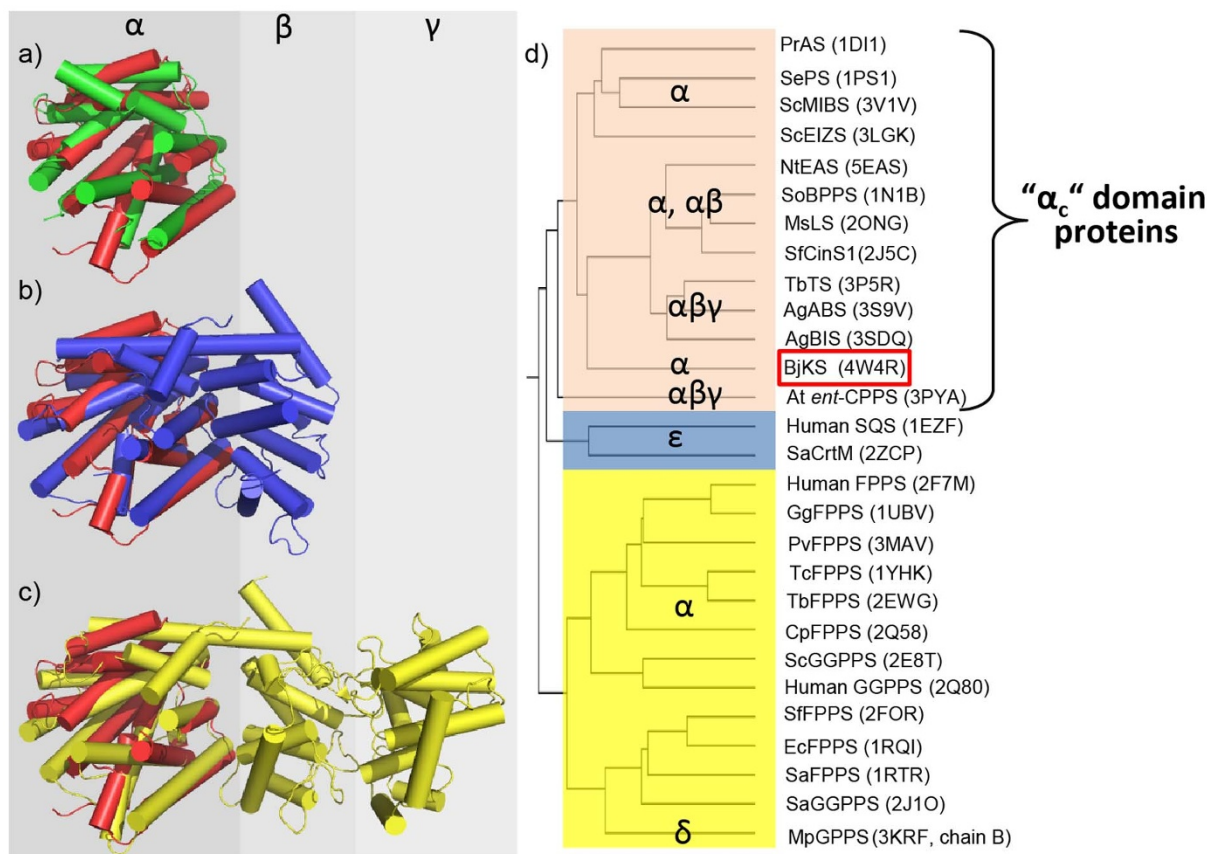


Figure 3 | Superimposition of the overall structure of BjKS (red; PDB ID 4KT9) with other terpene cyclases. (a) Superimposition of BjKS with pentalenene synthase from *Streptomyces* UC5319 which has only an α domain (α , green; 1HM7); (b) Superimposition of BjKS with 1, 8-cineole synthase from *Salvia fruticosa* which has two domains ($\alpha\beta$; blue; 2J5C); (c) Superimposition of BjKS with abietadiene synthase from *Abies grandis* which has three domains ($\alpha\beta\gamma$; yellow; 3S9V); (d) Homology tree (based on α domain Q-scores^{22,23}) showing clustering of BjKS with the plant α domain proteins. Note that the α domains in the terpene cyclase (top) are distinct from the α domains in synthases such as FPPS, and have previously been referred to as “ α_c ” domains¹⁹.

ene than are the products of the three other $\alpha\beta\gamma$ proteins. Remarkably, however, there is only a 17% sequence identity between BjKS and AgABS.

We next sought to see how the BjKS structure compared with a broader range of α , β , γ , δ , ϵ , and ζ -fold proteins by using their Q-scores²². The Q-score attempts to minimize difficulties that may arise by use of e.g. just C α rmsd values when the numbers of residues in target and query proteins, are different. The Q-score is defined in Equation (1)^{22,23}:

$$Q = \frac{N_{\text{align}}^2}{(1 + (\text{rmsd}/R_0)^2) * N_{\text{res1}} * N_{\text{res2}}} \quad (1)$$

where N_{align} is the number of aligned residues, determined here by using the secondary structure matching (SSM) program²²; N_{res1} and N_{res2} are the total numbers of residues in the two aligned proteins whose structures are to be compared, rmsd in the C α root mean square deviation of the C α residues in the two proteins, and R_0 is an empirical parameter, set (by the program) to 3 Å^{22,23}. The Q-score is 1 if two structures are identical, but obviously decreases as the rmsd increases, N_{align} decreases, or if N_{res1} or N_{res2} are much larger than N_{align} . As expected, Figure S1, the use of complete structures results in a clustering of BjKS with other bacterial and fungal cyclases (which are all small proteins, as is BjKS), while the plant $\alpha\beta$ or $\alpha\beta\gamma$ proteins are very distant because of the large N_{res} in these systems. The Q-scores used to construct Figure S1 are shown in Table S3. However, since we are primarily interested in structural comparisons between α domains, we next constructed a second tree using just the highly α -

helical domains in a broad range of terpene cyclases and synthases. As shown in Figure 3d (data in Table S4), we now see that BjKS clusters with the plant α domains (in $\alpha\beta\gamma$ proteins), while the fungal cyclases are separate, as are other α proteins such as farnesyl diphosphate synthase and geranylgeranyl diphosphate synthase (yellow, Figure 3d). The $\alpha\beta\gamma$ domain protein *ent*-CPPS from the plant *Arabidopsis thaliana* is more distant from the three other $\alpha\beta\gamma$ domain plant proteins due, we propose, to the fact that the α domain in *ent*-CPPS is not catalytic in this protein, so its structure is less likely to be conserved. The BjKS structure thus most closely resembles the α domain structures in modern plant terpene cyclases, and as noted previously^{4,19}, in *B. japonicum*, the *ent*-CPPS and *ent*-KS genes overlap by a single nucleotide⁴, a potential route to the origin of bi-functional species. Indeed, very primitive plants such as mosses do contain such bi- (or tri-) functional species, as discussed below.

Site-directed mutagenesis and enzyme inhibition. We next sought to investigate the catalytic mechanism of BjKS. We first used the BjKS and AgABS sequences to generate a set of 354 homologous proteins (using the JPRED3 program²⁴) which was then used as input to the SCORECONS server²⁵ which ranks residues in terms of their essential nature: 1.000 is essential, 0 is non-essential. The top 10 most essential residues are shown in Figure 4a. We show in Figure 4b the locations of the top 10 non-structural (i.e. not Gly or Pro) residues that are most conserved, from which it can be seen that D75, D76, D79, R204 and D208 (red spheres) are close to the catalytic site seen in other terpene cyclases while the other residues are more distant. We elected to mutate D75, D79 and R204 (to D75A, D75C,

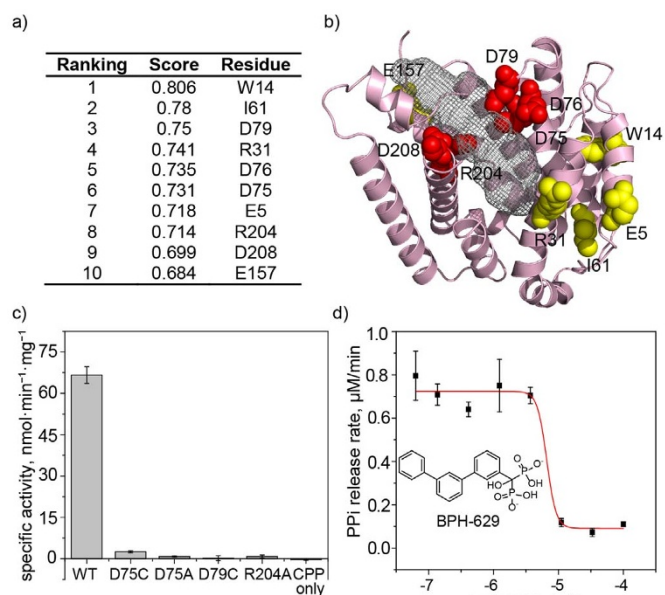


Figure 4 | Essential residues, catalysis and inhibition. (a) SCORECONS²⁵ results. (b) Highly conserved residues; residues in red were mutated to A or C. (c) Activity of wild-type and mutant proteins. (d) Inhibition of BjKS by the bisphosphonate BPH-629.

D79C and R204A) to test whether they were essential for catalysis since D75 and D79 are part of the highly conserved (Figure S2) DDXD motif found in many Class I cyclases, and R204 is also highly conserved, as part of the RLX(N,D)DXX(S,TR,G)XXX(E,D) (Figure S2) motif. As can be seen in Figure 4c and Table S5, mutation of D75, D79 or R204 (to Ala or Cys) greatly reduced activity to, on average, $1.7 \pm 1.6\%$.

We also sought to obtain a BjKS inhibitor, since such compounds might be leads for plant growth hormones targeting gibberellin biosynthesis, as well as providing information on the structure of the active site. The structures of several bisphosphonates bound to the α

domain in the $\alpha\beta\gamma$ protein bisabolene synthase have been reported²⁶, so we chose to investigate a bisphosphonate inhibitor, BPH-629 (Figure 4d), which has a C₁₉O side-chain, comparable in size to the side-chain in *ent*-CPP. We found that BPH-629 inhibited BjKS with an IC₅₀ = 9.5 μ M, Figure 4d and we report its structure in the following section.

Substrate and ligand-bound structures. In other articles²⁷ there have been reports of the binding of CPP isosteres (containing nitrogen) to terpene cyclases, but there have been no reports of *ent*-CPP (or CPP) bound to a terpene cyclase (or synthase). We therefore next determined the structure of the substrate *ent*-CPP (Figure 1) bound to the BjKS D75C mutant, in addition to determining the structure of the bisphosphonate inhibitor, BPH-629. Full data acquisition and crystallographic details are given in Table 1.

We obtained an *ent*-CPP structure by soaking apo-1 crystals with *ent*-CPP, while BPH-629 bound crystals were obtained by co-crystallization. Electron density results (for L71, D/C75, R204 and N207 residues) in all three structures are shown in Figures 5a–c (F_o-F_c omit maps, contoured at 2 σ). These results clearly indicate that *ent*-CPP and BPH-629 and not e.g. tartrate or PEG from the crystallization buffer, bind to the protein. More complete ligand densities (F_o-F_c omit maps, contoured at 1 σ (grey) and 3 σ (red)) are shown in Figures 5d, e. The cyclic structures are well defined, although density is weaker for the diphosphate/bisphosphonate moieties, due perhaps to some phosphate/phosphonate group disorder, since Mg²⁺ was not bound.

The complete BjKS structure with *ent*-CPP bound is shown in Figures 6a, and in Figure 6b, the BjKS-*ent* CPP structure is shown superimposed on that of 2-F-geranylgeranyl diphosphate (FGG) bound to the α domain of taxadiene synthase (TXS; PDB ID 3P5R). The 3 Mg²⁺ are from the taxadiene synthase structure. Three Asp residues (D76, D79 and D208) in BjKS are located in very similar positions to the corresponding Asp residues in TXS (C α rmsd of 0.8 Å for these 3 Asp residues), and might be expected to coordinate to a [Mg²⁺]₃ cluster which was, however, not observed in our BjKS crystals. This may be due to use of a D75C mutant, or to the high tartrate concentration (~1 M) used in BjKS crystallization,

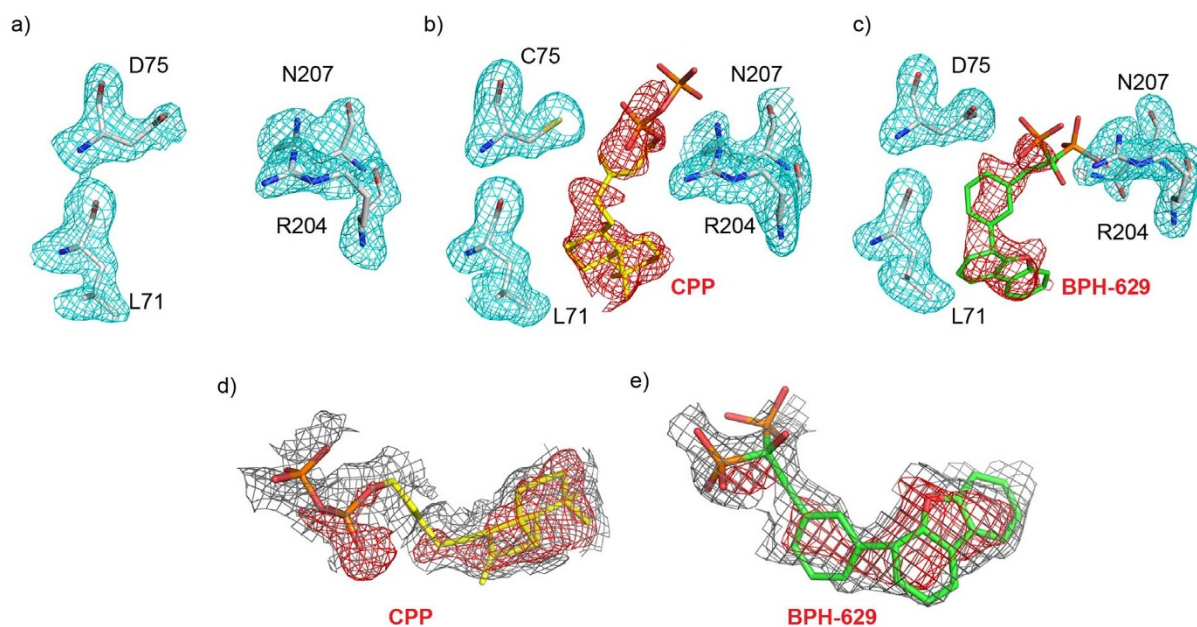


Figure 5 | Electron density results for BjKS. In (a)–(c) both protein residues (L71, D/C75, R204 and N207) as well as the ligands (*ent*-CPP, BPH-629) are shown as F_o-F_c omit maps, contoured at 2 σ . (a) apo-BjKS. (b) *ent*-CPP soaked. (c) BPH-629 co-crystal. (d) *ent*-CPP F_o-F_c map, contoured at 1 σ (grey) and 3 σ (red). (e) as (d) but BPH-629 ligand.

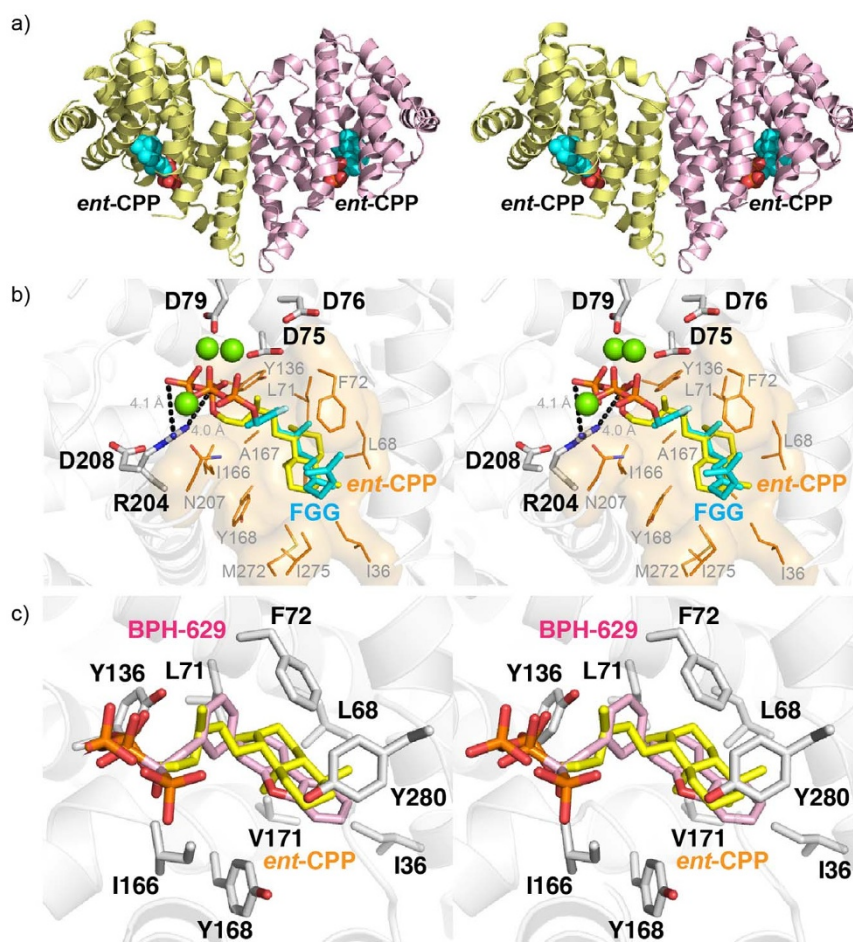


Figure 6 | BJKS *ent*-CPP and BPH-629 structures. (a) Stereo-view of *ent*-CPP bound structure (PDB ID 3WBV). (b) Stereo-view of the structure of *ent*-CPP bound to BjKS superimposed on 2-F-geranylgeranyl diphosphate (FGG, cyan) bound to the α domain of taxadiene synthase (TXS) (PDB ID code 3P5R). The highly conserved “DDXXD” Asps of BjKS are indicated. D75 is constructed based on C75 in the mutant protein. The 3 Mg²⁺ are from the TXS structure and are not observed in BjKS. The hydrophobic side-chain of *ent*-CPP is buried in a hydrophobic pocket composed of I36, L68, L71, F72, Y136, Y168 and N207, shown as an orange surface. (c) Stereo-view of *ent*-CPP (yellow) and BPH-629 (pink) in the BjKS active site. Hydrophobic residues surround the diterpene’s decahydronaphthalene and the bisphosphonate’s dibenzofuran side-chains.

since tartrate can chelate Mg²⁺. The hydrophobic side-chain of *ent*-CPP is buried in a hydrophobic pocket composed of I36, L68, L71, F72, Y136, I166, A167, Y168 and N207, shown as the orange surface in Figure 6b. In this conformation, the *exo*-methylene group is poised (~4 Å distance) to interact with the carbocation formed on diphosphate ionization, forming the first initial reactive intermediate, the pimaren-8-yl ion (Figure 1).

We also obtained the structure of BPH-629 bound to BjKS at 2.34 Å resolution (PDB ID code 4W4S), finding that the inhibitor occupies the same site as does *ent*-CPP, Figure 6c. Full data acquisition and crystallographic details are given in Table 1. The bisphosphonate has hydrogen bonds from the bisphosphonate group to D75, S165, R204 and N207, but just as with the *ent*-CPP complex, there is no Mg²⁺ observed. The lack of a [Mg²⁺]₃ cluster in this case cannot be due to use of a D75C mutant (as used with the *ent*-CPP structure) since wild type protein was employed for the bisphosphonate structure, so again it seems likely that the very high tartrate levels in the crystallization buffer may be responsible for Mg²⁺ chelation.

Structural basis for formation of *ent*-kaurene and kauranol in *P. patens*. The results described above delineate the active site in BjKS together with several residues (D75, D79 and R204) that are essential for catalysis. These residues comprise part of the conserved DDXXD/NTE motifs involved in diphosphate loss to form carbocation transition states/reactive intermediates (Figure 1c) that need to be

protected from solvent attack in BjKS, raising the question: which residues provide this protection? As noted above, in the moss *Physcomitrella patens*, there is in fact solvent attack in the PpCPCS/KS protein¹⁶ which results in the formation of only 20% *ent*-kaurene but 80% 16- α -hydroxy-*ent*-kaurane, and Kawaide *et al.*¹⁵ identified a key residue (A710, corresponding to L140 in BjKS) that enabled water access, quenching the kauranyl cation. A comparison between the two protein structures would be informative since it might help clarify the BjKS mechanism of action, but the structure of the *P. patens* protein has not been reported. There is, however, a 45% sequence identity between PpCPCS/KS and the *A. grandis* abietadiene synthase, and a Phyre2²⁸ structure prediction of the tri-functional *P. patens* protein is shown in Figure 7a. As expected, the protein is predicted to have an $\alpha\beta\gamma$ domain structure and the 45% conserved residues, shown in red in Figure 7a, are distributed over all three domains. When the *P. patens* active site structure prediction is superimposed on the BjKS structure (with *ent*-CPP from the BjKS structure), Figure 7b it is clear that BjKS contains larger residues (Y136 and L140) adjacent the *ent*-CPP ligand-binding site than those found in *P. patens* (which has Ala instead of Leu, and Leu instead of Tyr), and these larger residues (Figure 7b) prevent water from quenching the kauranyl cation (Figure 1). The increase in side-chain volume is expected to be ~100 Å³ (based on amino-acid volumes), and such steric differences might be particularly important in the presence of Mg²⁺, because Class I terpenoid synthases typically

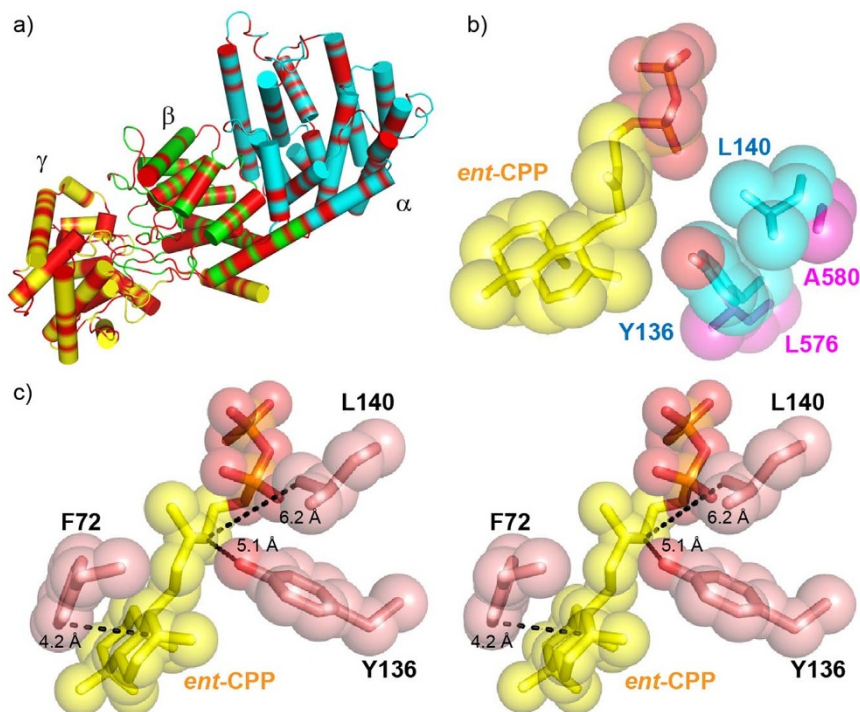


Figure 7 | Comparisons between BjKS structure and the tri-functional *P. patens* CPPS/KS structural model. (a) Phyre2²⁸ homology model of *Physcomitrella patens* ent-CPPS/ent-KS showing the 45% of residues (in red) that are identical to those in abietadiene synthase. The conserved residues are uniformly dispersed over the α , β and γ domains. (b) ent-CPP (yellow) bound to BjKS (PDB ID 3WBV) superimposed on corresponding *P. patens* KS residues. The large BjKS residues adjacent ent-CPP are in cyan. The smaller *P. patens* residues are in magenta and permit water quenching of the kauranyl-16-yl cation. (c) Stereo view of residues that are proposed to stabilize the carbocation intermediates in BjKS (PDB ID 3WBV). Distances between ent-CPP C12 and Y136 O, ent-CPP C12 and L140 C ^{δ} , ent-CPP C8 and F72 C are 5.1 Å, 6.2 Å and 4.2 Å, respectively.

undergo a structural transition from an open to a closed active-site conformation after Mg²⁺ binding to the substrate's diphosphate group, helping protect the reactive carbocation intermediates from premature quenching by bulk solvent.

In addition to Y136 and L140 protecting the carbocation intermediates in BjKS, we propose that F72 plays a similar role since F72 is very close to the ent-CPP substrate, as shown in Figure 7c. The phenyl group in F72 could also be involved in cation- π stabilization of one or more reactive intermediates, a widely used catalytic strategy adopted by terpenoid cyclases^{29–31}. Based on the x-ray structural results and structure comparison, it thus appears that Y136 and L140 can protect carbocations from solvent attack on one side of the intermediate(s), while F72 protects from solvent attack from the other face, as shown in Figure 7c.

Methods

Cloning, expression and purification of ent-kaurane synthase from *Bradyrhizobium japonicum* (BjKS). The gene encoding KS from *Bradyrhizobium japonicum* USDA 110 DNA was amplified by polymerase chain reaction (PCR) with a forward primer 5'-GGTATTGAGGGTCGCGAGAATCTTTATTTTCAGGG-CGCTGGTGCTGGTGCTATGAT-3' and a reverse primer 5'-AGAGGAGAGTTA-GAGCCGTTATTAGCCGCGCGCGCTGCCCTCCCCTCAC-3', then cloned into the pET32 Xa/LIC vector. A tobacco etch virus protease (TEV protease) cutting sequence (ENLYFQG) was added before the KS gene. The recombinant plasmids were transformed to *E. coli* BL21

SeMet KS was carried out as described previously³² and the purification procedures were the same as those used for the wild-type enzyme, but the DEAE column was omitted. The protein was then concentrated to 6.4 mg/mL in 25 mM Tris, pH 7.5 and 150 mM NaCl.

Size exclusion chromatography-multi-angle light scattering (SEC/MALS). The molecular weight of KS was determined by static light scattering (SLS) using a Wyatt Dawn Heleos II multi-angle light scattering detector (Wyatt Technology). This was coupled to an AKTA Purifier UPC10 FPLC protein purification system equipped with a WTC-030S5 size-exclusion column (Wyatt Technologies). 2.5 mg/mL KS (100 μ L) was applied to the size-exclusion column with a buffer containing 25 mM Tris (pH 7.5), 150 mM NaCl, 10 mM DTT and 0.02% NaN₃ using a flow rate of 0.5 mL/min. BSA (2 mg/mL) was used for the system calibration. The molecular weights of the individual peaks in the size-exclusion chromatogram were determined from the SLS results in conjunction with refractive index measurements (Wyatt Optilab rEX, connected downstream of the LS detector). A standard value of the refractive index, $dn/dc = 0.185$ mL/g, was used for the proteins and the buffer viscosity $\eta = 1.0408$ cP at 25°C, was used. The value of the reference refractive index, 1.3462, was taken directly from the measurement of the Wyatt Optilab rEX when only buffer was passing through the reference cell.

Crystallization, data collection and structure determination of BjKS. The wild-type KS protein containing 1 mM MgCl₂ and 10 mM DTT was first crystallized by using the Index Kit (Hampton Research, Aliso Viejo, CA) and the sitting-drop vapor diffusion method. The reservoir solution (No. 26) contained 1.1 M ammonium tartrate dibasic, pH 7.0. Better crystals were obtained by optimizing the reservoir composition to 0.7 M ammonium tartrate dibasic, pH 7.0 and 2–4% w/v PEG4000. Two different crystals having different space groups (P2₁ for apo-1; P4₁ for apo-2) appeared in the same crystallization condition drop. Crystallization conditions for SeMet KS were 0.8 M ammonium tartrate dibasic, pH 7.0. For the inhibitor BPH-629 complex, the KS protein solution containing 1 mM MgCl₂ and 10 mM DTT was co-crystallized with 2.5 mM BPH-629 under the same conditions as the wild-type protein. To obtain the KS-CPP structure, the D75C mutant crystal was prepared under the same crystallization conditions and then soaked with the cryo-protectant containing 5 mM CPP for 5 hours. All crystals were prepared at 4°C for 12 hours and were then transferred to room temperature. They reached suitable size for X-ray diffraction in 2–3 days. The cryo-protectant was 1.0 M ammonium tartrate dibasic, pH 7.0, 10% w/v PEG4000 and 20% glycerol.

X-ray diffraction data sets were processed by using the HKL2000 program³³. Prior to structure refinement, 5% randomly selected reflections were set aside for cal-



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Author contributions

R.T.G. and E.O. designed and directed the research. W.L., Y.Z., Y.C. and J.L. purified and crystallized the proteins. C.H.H., T.P.K. and C.C.C. determined the crystal structures. C.N. and T.H. synthesized *ent*-CPP. X.F. and S.B. performed activity assays. R.T.G., T.P.K. and E.O. analyzed structures. I.W. and S.-T.D.H. performed the SEC-MALS analysis. E.O. wrote the manuscript with input from co-authors. All authors reviewed the manuscript.

Additional information

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