Communication

Cannabinoid Biosynthesis Using Noncanonical Cannabinoid Synthases

Maybelle Kho Go [1,2,3], **Tingting Zhu** [1,2,3], **Kevin Jie Han Lim** [1,2,3], **Yossa Dwi Hartono** [1,2,3,4], **Bo Xue** [1,2,3], **Hao Fan** [2,4] **and Wen Shan Yew** [1,2,3,*]

1 Synthetic Biology for Clinical and Technological Innovation, National University of Singapore, 14 Medical Drive, Singapore 117599, Singapore
2 Synthetic Biology Translational Research Programme, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 119077, Singapore
3 Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, 8 Medical Drive, Singapore 117597, Singapore
4 Bioinformatics Institute, A*STAR, 30 Biopolis Street, Matrix #07-01, Singapore 138671, Singapore
* Correspondence: wenshanyew@nus.edu.sg

Abstract: We report enzymes from the berberine bridge enzyme (BBE) superfamily that catalyze the oxidative cyclization of the monoterpene moiety in cannabigerolic acid (CBGA) to form cannabielsoin (CBE). The enzymes are from a variety of organisms and are previously uncharacterized. Out of 232 homologues chosen from the enzyme superfamily, four orthologues were shown to accept CBGA as a substrate and catalyze the biosynthesis of CBE. The four enzymes discovered in this study were recombinantly expressed and purified in *Pichia pastoris*. These enzymes are the first report of heterologous expression of BBEs that did not originate from the *Cannabis* plant that catalyze the production of cannabinoids using CBGA as substrate. This study details a new avenue for discovering and producing natural and unnatural cannabinoids.

Keywords: cannabinoid biosynthesis; cannabinoid synthase; berberine bridge enzyme; cannabielsoin; cannabigerolic acid; *Pichia pastoris*

1. Introduction

Cannabis sativa L. is an annual herbaceous plant that has been widely used due to its industrial [1], ornamental [2], and pharmaceutical [3] applications. *Cannabis sativa* contains at least 113 cannabinoid compounds; these cannabinoids exhibit a range of unique pharmacological and therapeutic properties. Despite the report of biosynthesis of tetrahydrocannabinolic acid (THCA) **(2)** and cannabidiolic acid (CBDA) **(3)** using yeast [4], the biosynthetic pathways of most of these cannabinoids remain largely unknown, making it difficult for medicinal cannabinoids to be produced in a stable and sustainable manner [5]. Chemical synthesis of these cannabinoids remains a challenge due to their complex chemical structures. Most of the well-known and studied cannabinoids are derived from cannabigerolic acid (CBGA) **(1)**. Five of the products, THCA **(2)**, CBDA **(3)**, cannabicyclolic acid **(4)**, cannabichromenic acid **(5)**, and cannabicitranic acid **(6)**, are structural isomers ($C_{22}H_{30}O_4$); only cannabielsoic acid B (CBEA) **(7)** is unique, due to an additional oxygen atom ($C_{22}H_{30}O_5$) (Scheme 1).

The biosynthesis of cannabinoids from CBGA **(1)** are catalyzed by cannabinoid synthases. The enzymes are characterized as flavin adenine dinucleotide (FAD)—dependent berberine bridge enzymes that catalyze the oxidative cyclization of the monoterpene moiety in CBGA **(1)**. The structure of THCA synthase (Uniprot ID Q8GTB6, PDB ID 3VTE) was elucidated by Kuroki and his associates in 2012 [6]. Cannabidiolic acid synthase was functionally expressed in yeast by Stehle and his associates in 2017 [7]. The known cannabinoid synthases share approximately 80% sequence identity.
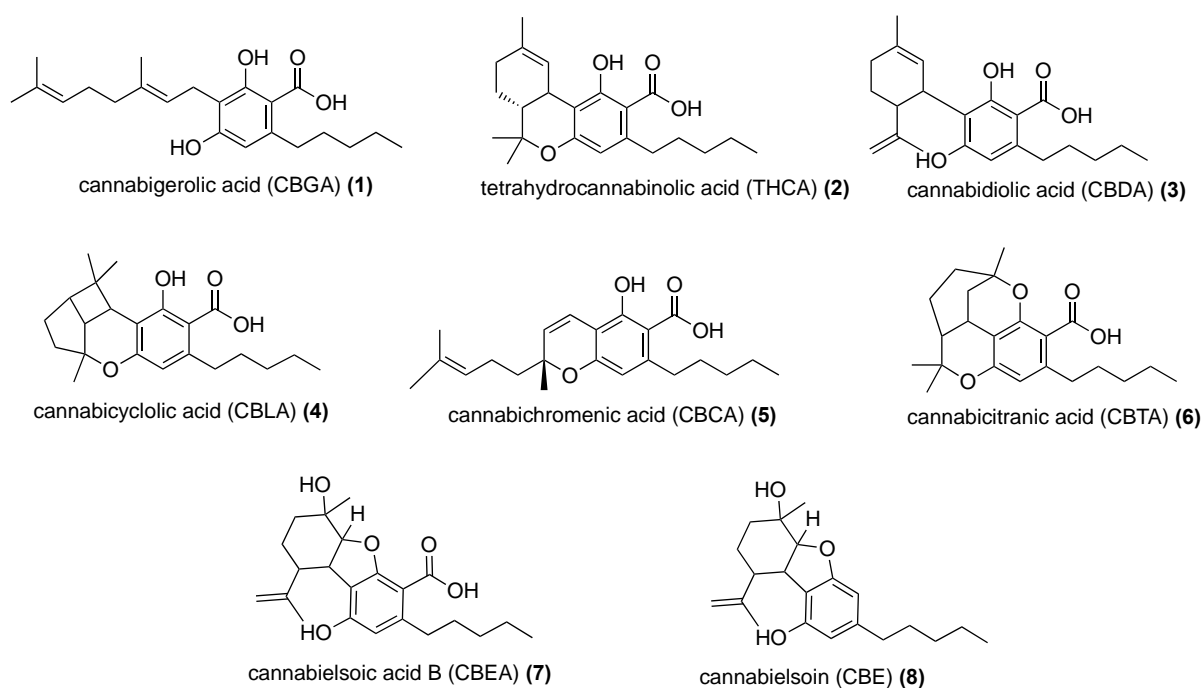
Scheme 1. Cannabinoid products **(2–8)** derived from CBGA **(1)**.

Q8GTB6 is an enzyme of the berberine bridge enzyme (BBE) family (IPR012951). The family contains about 4500 members, of which 37 are experimentally annotated and reviewed. Using the EFI-EST tool to generate a sequence similarity network (SSN) of this family [8], it is possible to segregate the proteins into iso-functional clusters. This will place uncharacterized enzymes in sequence–function context with proteins that have been previously characterized from reliable experiments [9,10]. Using this information, it is possible to find potential enzymes from other organisms that will catalyze the oxidative cyclization of the monoterpene moiety in CBGA **(1)**. In this study, we selected 232 homologues from the generated SSN and exploited their potential in accepting CBGA **(1)** as a substrate and catalyzing an FAD-dependent oxidative cyclization reaction to produce novel cannabinoid compounds.

2. Results

2.1. Sequence Similarity Network of Berberine Bridge Enzymes

The SSN generated approximately 4500 nodes with 300,000 edges (Figure 1). A small number of homologues (232 homologues) were chosen based on either their similarity to Q8GTB6 or their difference. The goal is to create a small yet chemically diverse library to discover novel enzyme activity. The hypothesis of the study follows that those enzymes "similar" to Q8GTB6 may catalyze the production of cannabinoids such as THCA **(2)**; enzymes "different" from Q8GTB6 may catalyze the production of novel cannabinoids. Scheme 2 is an illustration of the workflow for the screening of orthologs for cannabinoid synthase activity using CBGA **(1)** as substrate.
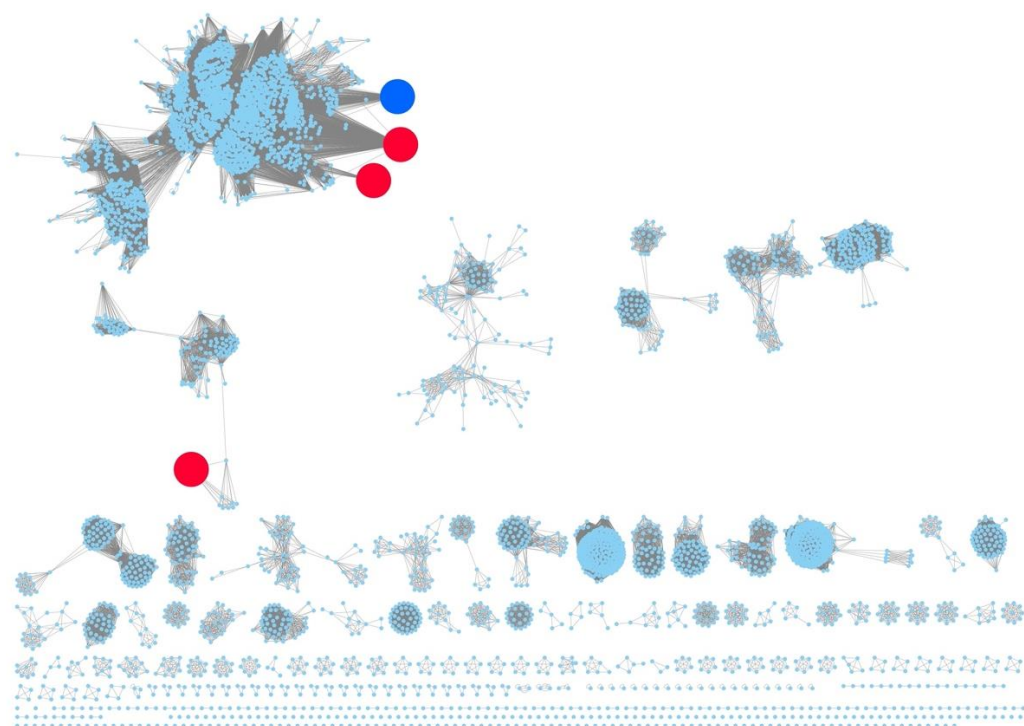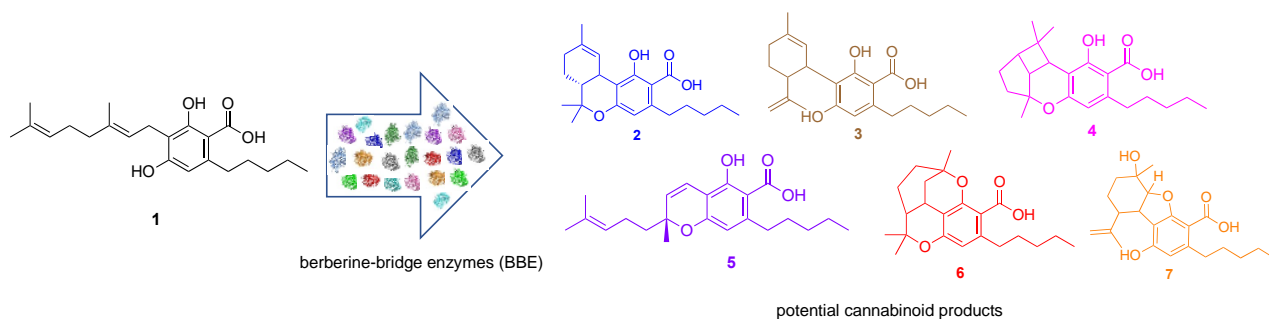
Figure 1. Full SSN of BBEs (IPR012951). The three orthologues capable of catalyzing the production of cannabinoids are denoted by the red circles. Q8GTB6 is denoted by the blue circle. Image generated using Cytoscape [11].

berberine-bridge enzymes (BBE)

potential cannabinoid products

Scheme 2. Schematic of workflow for the screening of orthologs for cannabinoid synthase activity using CBGA **(1)** as substrate.

2.2. Production of Cannabinoids Catalyzed by Potential Cannabinoid Synthase Orthologs

Annotated enzymes from *C. sativa* did not produce detectable amounts of a cannabinoid; it is possible that the signal peptide impeded the heterologous over-expression in *S. cerevisiae* [7]. Most of the homologues chosen (98% of 232 sequences) did not produce any detectable amounts of a cannabinoid, which may indicate that the active sites of BBEs are not as promiscuous as other enzyme superfamilies. Since the organisms chosen for this study are all eukaryotes, it is also possible that the annotated sequences contain introns, which may render the synthesized genes inactive. Three orthologues were discovered to utilize CBGA **(1)** to catalyze the production of CBE **(8)**, the decarboxylated product from CBEA **(7)** (Scheme 1). Table 1 shows the Uniprot IDs of orthologues and the organisms they originated from.

Table 1. Berberine bridge enzymes that catalyze the production of cannabinoids.

Uniprot ID	Organism
M4DIE5	*Brassica rapa* subsp. *pekinensis*
A0A1J6KPK0	*Nicotiana attenuata*
P93479	*Papaver somniferum*

M4DIE5 and A0A1J6KPK0 are uncharacterized enzymes. The sequences are annotated as BBE-like enzymes based on sequence homology. P93479 from *Papaver somniferum* was also detected at the transcript level and is proposed to be involved in the formation of (*S*)-scoulerine via the oxidative cyclization of the *N*-methyl moiety of (*S*)-reticuline [12]. The orthologs share about 35–40% sequence identity to Q8GTB6.

The three orthologues were expressed in *P. pastoris* according to previous work on the expression of Q8GTB6 using the same host [13,14]. The purified enzymes were used to determine the kinetic parameters of cannabinoid synthase activity using CBGA **(1)** as substrate (Table 2). When compared to the THCA synthase Q8GTB6 also expressed in *P. pastoris* [13,14], the orthologues are approximately 10-fold slower.

Table 2. Kinetic parameters of cannabinoid synthase orthologues.

Uniprot ID	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})
M4DIE5	$1.8 \pm 0.8 \times 10^{-2}$	6.4 ± 3.4	2.7×10^3
A0A1J6KPK0	$2.0 \pm 0.8 \times 10^{-2}$	8.1 ± 3.8	2.5×10^3
P93479	N.S.	N.S.	$1.3 \pm 0.1 \times 10^3$

N.S.—not saturating.

2.3. Mass and NMR Analysis of Cannabielsoin

The structure of CBE **(8)** is shown in Scheme 3; HR-MS (ESI, negative mode): [M-H]-, m/z = 329.2134 (experimental); m/z = 329.2122 (theoretical). ^{1}H NMR (CD$_3$OD, ppm): H$_1$ (1H) 3.3; H$_2$ (1H, *m*) 2.5; H$_3$ (2H, broad) 1.5; and H$_4$ (2H, broad) 1.6; H$_6$ (1H, *m*) 3.9; H$_7$ (3H, *s*) 1.7; H$_9$ (2H, *m*) 4.5 and 4.6; H$_{10}$ (3H, s) 2.0; H$_{3'}$ (1H) 6.1; H$_{5'}$ (1H) 6.1; H$_{1''}$, H$_{2''}$, H$_{3''}$, H$_{4''}$ (8H, *m*) 1.3; H$_{5''}$ (3H, *t*) 0.9, J_{Ha-Hb} = 7.5 Hz. ^{13}C NMR (CD$_3$OD, ppm): C$_1$ 49.8; C$_2$ 35.7; C$_3$ 31.2; C$_4$ 31.7; C$_5$ 110.3; C$_6$ 68.3; C$_7$ 17.9; C$_8$ 153.5; C$_9$ 68.3; C$_{10}$ 26.7; C$_{1'}$ 108.3; C$_{2'}$ 160.3; C$_{3'}$ 143.6; C$_{4'}$ 148.4; C$_{5'}$ 117.1; C$_{6'}$ 163.2; C$_{1''}$ 40.6; C$_{2''}$ 35.7; C$_{3''}$ 34.4; C$_{4''}$ 30.9; C$_{5''}$ 13.0.
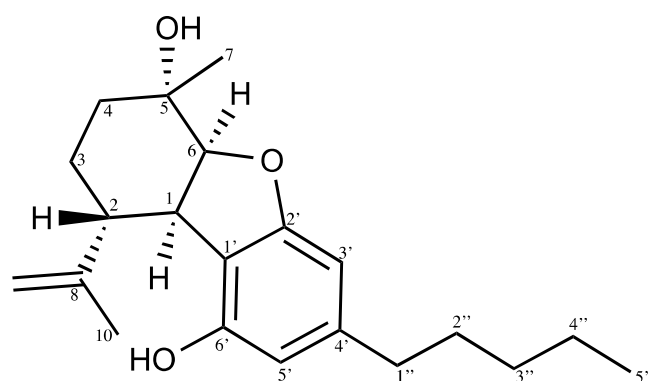
Scheme 3. Structure of CBE **(8)**.

3. Discussion

Five residues in Q8GTB6 are critical for its activity—two FAD-anchoringresidues, His-114 and Cys-176, which are covalently bonded to FAD, and three catalytic residues, His-292, Tyr-417, and Tyr-484 [6]. Table 3 and Figure 2 show critical residues in Q8GTB6 and the structurally aligned residues in the AlphaFold models of the orthologues. A0A1J6KPK0 contains both the FAD-anchoring residues and the corresponding Tyr-484 residue, whilst

His-292 and Tyr-417 are not conserved. The orthologues do not retain amino acid chemical properties for the following positions: His-292 is altered to a hydrophobic residue (Val-291 or Leu-286) and Tyr-417 is changed to an Asn residue (Asn-211, Asn-408, and Asn-394, respectively).

Table 3. Critical residues in THCA synthase (Q8GTB6) with the corresponding analogous residues in the orthologues. The proteins are identified by their UNIPROT ID. Residues are labelled using the single letter amino acid code.

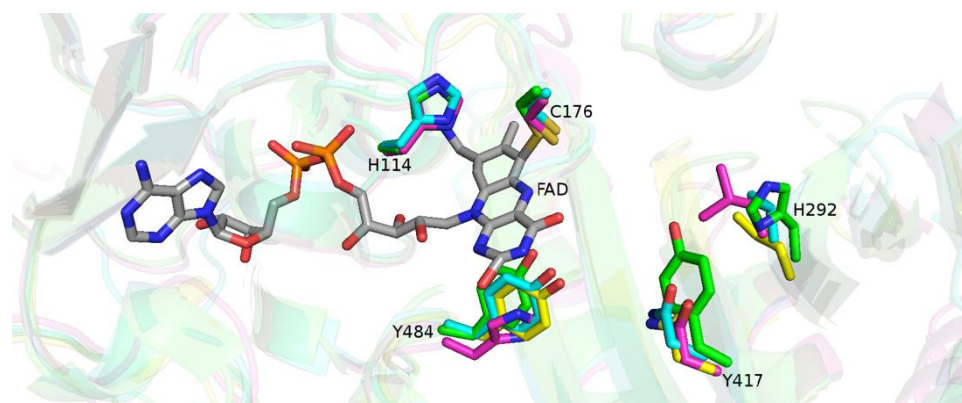| Q8GTB6	M4DIE5	A0A1J6KPK0	P93479
H114	-	H112	H108
C176	-	C176	C170
H292	L98	V291	L286
Y417	N211	N408	N394
Y484	Y278	Y477	H463

Figure 2. Alignment of critical residues in THCA synthase and the orthologues.

Surprisingly, M4DIE5 lacks both FAD-anchoring residues, only Tyr-484 is conserved. Comparing M4DIE5 AlphaFold model with Q8GTB6 experimental structure (PDB 3VTE), M4DIE5 lacks a whole subdomain that includes the two missing FAD-anchoring residues. M4DIE5 is shorter in sequence compared to the rest and has an RMSD value 1.9 Å over 328 atoms that are structurally aligned to Q8GTB6 (A0A1J6KPK0: 1.7 Å over 496 atoms; P93479: 1.7 Å over 488 atoms). Nevertheless, M4DIE5 has detectable cannabinoid synthase active to catalyze the production of CBE **(7)**.

The three enzymes discovered in this study are the first reported heterologously expressed BBEs that do not originate from the *Cannabis* plant yet can catalyze the production of cannabinoids using CBGA **(1)** as substrate. It is recognized that the study only explored a very limited portion of this enzyme family (232 out of 4500; 5%); the corollary expectation follows that those other enzymes within this family may accept either CBGA **(1)** or/as well as the shorter analogs (with *n*-butyl, *n*-propyl, ethyl, and methyl side chains) as substrates. This study delineates a new avenue for the discovery and biosynthesis of natural and unnatural cannabinoids.

CBEA **(7)** is different from the rest of the carboxylated products in Scheme 1 due to an additional oxygen atom; CBE **(8)** is the decarboxylated product of CBEA **(7)** and has previously been reported as an oxidative product from CBDA **(3)** [15]. Analogous to previously determined oxidative cyclization mechanisms of CBGA **(1)**, we propose that it begins with the formation of the carbocation in the monoterpene moiety and the corresponding reduction of FAD to FADH$_2$ (Scheme 4). The secondary carbocation that is formed rearranges to a more stable tertiary carbocation. Then, a proton is extracted from a terminal methyl group of the octadienyl chain by a general base, and a cascade of electron pair movements forms the cyclohexyl ring. Thereafter, we propose a nucleophilic

attack of the first carbocation intermediate (presumably by a nucleophile derived from a H_2O molecule) to form 2,4-dihydroxy-3-[2-hydroxy-2-methyl-5-(2-propenyl)-cyclohexyl]-6-pentyl-benzoic acid (2,4-OH-6PBz) **(9)**. A second carbocation forms with the corresponding reduction of a second FAD molecule to $FADH_2$ and a final cyclization step produces CBEA **(7)**. We propose that a spontaneous decarboxylation occurs to form the final product, CBE **(8)**.
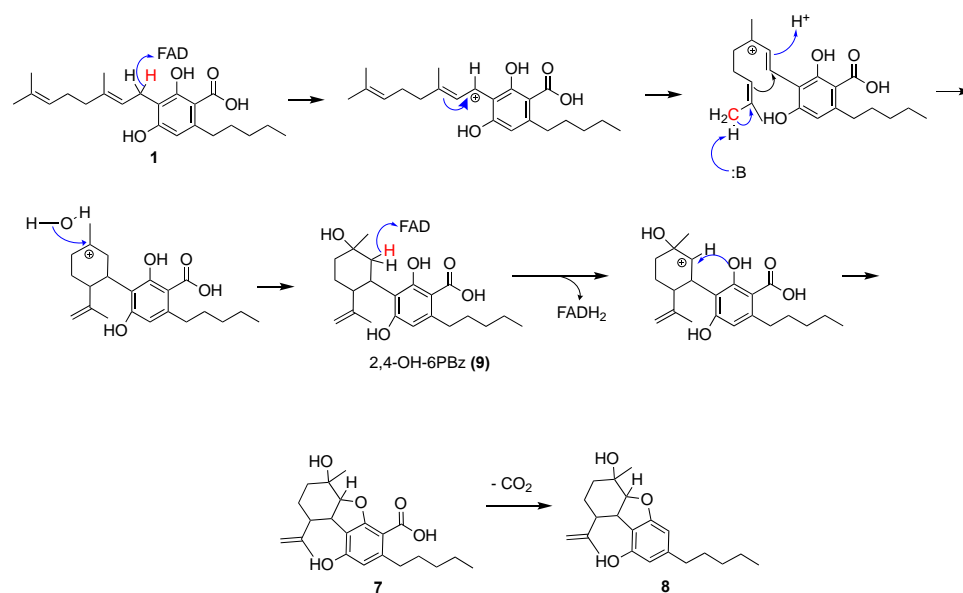
Scheme 4. Proposed oxidative cyclization mechanism of CBE **(8)** formation from CBGA **(1)**.

4. Materials and Methods

CBGA **(1)** and CBE **(8)** were purchased from Cayman Chemicals. All other chemicals used are of the highest purity that is required for the different experiments. The 232 homologues chosen from the SSN were codon optimized for yeast expression, synthesized, and cloned into pYES2-CT vector (Thermofisher).

The sequence of Q8GTB6 was used as a query to determine the superfamily the enzyme belongs to (http://www.ebi.ac.uk/interpro/ (accessed on 2 January 2018)) [16]. Thereafter, the EFI-EST tool (https://efi.igb.illinois.edu/efi-est/ (accessed on 2 January 2018)) was used to generate the SSN. A small number of homologues (232 homologues) were chosen to test if CBGA **(1)** can be used as a substrate for these enzymes to catalyze the biosynthesis of a cannabinoid. The homologues were chosen using the following criteria: (1) Orthologues found in *C. sativa* and its related organisms such as *Nicotiana* sp.; (2) Orthologues that are experimentally shown to exist either at the protein or transcript level; (3) Orthologues from other plants that share less than 40% sequence identity to Q8GTB6.

The cloned genes were transformed into *Saccharomyces cerevisiae* BY4741 by chemical transformation [17–19]. The transformed cells were plated onto SC-URA-glucose plates and incubated for two days at 30 °C. Three single colonies were picked and grown in SC-URA-glucose media for 24 h. The cells were harvested and resuspended in SC-URA-galactose media and incubated for another 24 h. Thereafter, cells were harvested and resuspended in 100 mM citrate, pH 5.5, and 1 mM $MgCl_2$. The cell wall was digested by the addition of lyticase (0.5 mg) for 1 h at 37 °C. Glass beads (425–600 μm) were added and the cells were broken using a tissue homogenizer. The cell supernatant was clarified by centrifuge and used for the biosynthetic activity screen.

A 50-μL reaction mixture was prepared for the cannabinoid biosynthesis activity screen. The mixture contains 0.4 mM CBGA, 1.5 mM FAD, and 48 μL cell lysate. The reaction was incubated for 24 h under ambient conditions. The compounds were extracted using ethyl acetate, dried, and re-dissolved in acetonitrile. The presence of the cannabinoids was analyzed using the Agilent RapidFire 365 High-Throughput system coupled with the

Agilent 6495 TQ. As mentioned previously, the potential cannabinoid products using CBGA
(1) have two different molecular formulas—$C_{22}H_{30}O_4$ and $C_{22}H_{30}O_5$ with m/z values (ESI,
negative mode) of 357.2 and 373.2, respectively. Corresponding control experiments in the
absence of CBGA **(1)** were also prepared.

Orthologues that were determined to produce the cannabinoid compounds in *S.
cerevisiae* were expressed in *P. pastoris* according to published protocols with some modifications [13,14]. A0A1J6KP0 and P93479 were annotated to contain a signaling peptide
using the SingalP 5.0 bioinformatic tool. Thus, the signaling peptide was removed and
a Strep-tag II and a 6x-histidine tag were added on the C-terminus for purification purposes [20]. The modified genes were cloned into the pPICZα A plasmid (Invitrogen). Cells
were transformed by electroporation using 1 mg of *Pme*I linearized plasmid DNA following
published protocols (Invitrogen). Transformants were selected by plating the cells on YPD
agar plates containing zeocin (100 mg mL^{-1}). Clones containing the integrated gene were
confirmed by following published protocols (Invitrogen).

The orthologue M4DIE5 does not contain a signaling peptide. Thus, the gene was
modified by adding a Strep-tag II on the C-terminus for purification purposes [20]. The
gene was cloned into the pPICZα A plasmid (Invitrogen). Cells were transformed and the
presence of the gene was confirmed using protocols mentioned previously.

Recombinant *P. pastoris* cells containing the orthologues were grown in BMGY media
at 30 °C and 190 rpm for 24 h. Afterwards, cells were harvested by centrifugation at $3000\times g$
for 5 min and resuspended in modified BMMY (mBMMY) media to an OD_{600} of 2. Pichia
cells were cultivated at 15 °C and 90 rpm and supplemented with 1% (v) methanol and
0.02% (w/v) riboflavin every 24 h for induction of protein expression. Cells were harvested
and resuspended in 100 mL of binding buffer. Cells were cracked using a homogenizer
(M110P Microfluidizer®®) and debris was removed by centrifugation at 20,000 rpm and
4 °C for 30 min. The cleared lysate was loaded onto a Ni^{2+} chelating sepharose column (GE
Healthcare) or a Strep-Tactin®® XT column (IBA Lifesciences). For orthologues purified
using a Ni^{2+} chelating sepharose column, enzymes were eluted using increasing amounts
of imidazole (4 mM to 300 mM imidazole). For orthologues purified using a Strep-Tactin®®
XT column, enzymes were eluted using 50 mM D-biotin. Fractions containing the protein
were pooled, concentrated, and dialyzed vs. 100 mM citrate, pH 5.5, and 0.2 mM FAD.
Purified samples were frozen and stored at −80 °C.

Kinetic parameters of the orthologs were determined using an end-point assay under
initial rate conditions. A 100-mL reaction containing 100 citrate, pH 5.5, 0.2 mM FAD,
0.2 to 2 mM CBGA, 5 mM CBS orthologue was prepared for each orthologue. A 20-μL
aliquot was stopped by the addition of acetonitrile every 10 min. Samples were filtered
and analyzed using the Agilent 1290 Infinity HPLC coupled with the Agilent 6550 iFunnel
Q-TOF high resolution mass spectrometer.

The purified proteins were used to catalyze the production of cannabinoids on a
larger scale to produce enough compounds for ^{1}H-nuclear magnetic resonance (NMR) and
^{13}C-NMR analysis. The steps performed are analogous to the smaller scale experiment
mentioned previously. Samples were dissolved in CD_3OD and the spectra were recorded
using a Bruker AVANCE 500 MHz NMR spectrometer at the Department of Chemistry,
National University of Singapore.

AlphaFold models of the orthologues are taken from their respective UniProt entry pages.

5. Conclusions

We have discovered that enzymes from the berberine bridge enzyme family (IPR012951)
can catalyze the oxidative cyclization of the monoterpene moiety in CBGA **(1)** to form CBE
(8). This is the first report of enzymes that did not originate from the Cannabis plant that
catalyze the production of cannabinoids. This study demonstrated the attractive potential
of curating SSNs to discover uncharacterized enzymes and curate them based on sequence
and structure. Further exploration may include expanding the screen towards the rest of

the ~4500 sequences in the BBE family, as well as using the lesser-known analogs of CBGA (**1**) as substrates. We believe that this discovery will aid in the production of cannabinoids in a stable and sustainable manner. This report provides new enzymes that can advance the molecular production of cannabinoid molecules that exist in nature, but for which there are no known molecular tools or enzymes to biosynthesize them. Conceptually, the work also advances the approach to biosynthesize natural products that otherwise are refractory to bioproduction due to the lack of suitably identified enzymes in the biosynthetic pathways.

6. Patents

Go, MK; Yew, WS. Biosynthesis of cannabinoids from cannabigerolic acid using novel cannabinoid synthases. WO 2021/071438, 2021.

References

1. Karche, T.; Singh, M. The application of hemp (*Cannabis sativa* L.) for a green economy: A review. *Turk. J. Bot.* **2019**, *43*, 710–723. [CrossRef]
2. Hesami, M.; Pepe, M.; Baiton, A.; Salami, S.A.; Jones, A.M.P. New Insight into Ornamental Applications of Cannabis: Perspectives and Challenges. *Plants* **2022**, *11*, 2383. [CrossRef] [PubMed]
3. Sharma, D.; Bhushan, S.; Agrawal, D.C.; Dhar, M.K.; Kaul, S. Cannabis as a Potent Therapeutic Agent for Pharmaceutical Drugs: Recent Advancement in Drug Discovery and Human Healthcare. In *Cannabis/Marijuana for Healthcare*; Agrawal, D.C., Kumar, R., Dhanasekaran, M., Eds.; Springer Nature: Singapore, 2022; pp. 77–99.
4. Luo, X.; Reiter, M.A.; d'Espaux, L.; Wong, J.; Denby, C.M.; Lechner, A.; Zhang, Y.; Grzybowski, A.T.; Harth, S.; Lin, W.; et al. Complete biosynthesis of cannabinoids and their unnatural analogues in yeast. *Nature* **2019**, *567*, 123–126. [CrossRef] [PubMed]
5. Hesami, M.; Pepe, M.; Baiton, A.; Jones, A.M.P. Current status and future prospects in cannabinoid production through in vitro culture and synthetic biology. *Biotechnol. Adv.* **2023**, *62*, 108074. [CrossRef] [PubMed]
6. Shoyama, Y.; Tamada, T.; Kurihara, K.; Takeuchi, A.; Taura, F.; Arai, S.; Blaber, M.; Shoyama, Y.; Morimoto, S.; Kuroki, R. Structure and function of Δ1-tetrahydrocannabinolic acid (THCA) synthase, the enzyme controlling the psychoactivity of *Cannabis sativa*. *J. Mol. Biol.* **2012**, *423*, 96–105. [CrossRef] [PubMed]
7. Zirpel, B.; Degenhardt, F.; Martin, C.; Kayser, O.; Stehle, F. Engineering yeasts as platform organisms for cannabinoid biosynthesis. *J. Biotechnol.* **2017**, *259*, 204–212. [CrossRef]
8. Gerlt, J.A.; Bouvier, J.T.; Davidson, D.B.; Imker, H.J.; Sadkhin, B.; Slater, D.R.; Whalen, K.L. Enzyme Function Initiative-Enzyme Similarity Tool (EFI-EST): A web tool for generating protein sequence similarity networks. *Biochim. Biophys. Acta* **2015**, *1854*, 1019–1037. [CrossRef] [PubMed]
9. Gerlt, J.A. Genomic Enzymology: Web Tools for Leveraging Protein Family Sequence-Function Space and Genome Context to Discover Novel Functions. *Biochemistry* **2017**, *56*, 4293–4308. [CrossRef] [PubMed]
10. Zallot, R.; Oberg, N.; Gerlt, J.A. The EFI Web Resource for Genomic Enzymology Tools: Leveraging Protein, Genome, and Metagenome Databases to Discover Novel Enzymes and Metabolic Pathways. *Biochemistry* **2019**, *58*, 4169–4182. [CrossRef] [PubMed]

11. Shannon, P.; Markiel, A.; Ozier, O.; Baliga, N.S.; Wang, J.T.; Ramage, D.; Amin, N.; Schwikowski, B.; Ideker, T. Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Res.* **2003**, *13*, 2498–2504. [CrossRef] [PubMed]

12. Facchini, P.J.; Penzes, C.; Johnson, A.G.; Bull, D. Molecular characterization of berberine bridge enzyme genes from opium poppy. *Plant Physiol.* **1996**, *112*, 1669–1677. [CrossRef] [PubMed]

13. Lange, K.; Schmid, A.; Julsing, M.K. Δ(9)-Tetrahydrocannabinolic acid synthase production in Pichia pastoris enables chemical synthesis of cannabinoids. *J. Biotechnol.* **2015**, *211*, 68–76. [CrossRef] [PubMed]

14. Zirpel, B.; Stehle, F.; Kayser, O. Production of Δ9-tetrahydrocannabinolic acid from cannabigerolic acid by whole cells of Pichia (Komagataella) pastoris expressing Δ9-tetrahydrocannabinolic acid synthase from *Cannabis sativa* L. *Biotechnol. Lett.* **2015**, *37*, 1869–1875. [CrossRef] [PubMed]

15. Seo, C.; Jeong, M.; Lee, S.; Kim, E.J.; Rho, S.; Cho, M.; Lee, Y.S.; Hong, J. Thermal decarboxylation of acidic cannabinoids in Cannabis species: Identification of transformed cannabinoids by UHPLC-Q/TOF–MS. *J. Anal. Sci. Technol.* **2022**, *13*, 42. [CrossRef]

16. Paysan-Lafosse, T.; Blum, M.; Chuguransky, S.; Grego, T.; Pinto, B.L.; Salazar, G.A.; Bileschi, M.L.; Bork, P.; Bridge, A.; Colwell, L.; et al. InterPro in 2022. *Nucleic Acids Res.* **2022**, *51*, D418–D427. [CrossRef] [PubMed]

17. Winston, F.; Dollard, C.; Ricupero-Hovasse, S.L. Construction of a set of convenient saccharomyces cerevisiae strains that are isogenic to S288C. *Yeast* **1995**, *11*, 53–55. [CrossRef]

18. Baker Brachmann, C.; Davies, A.; Cost, G.J.; Caputo, E.; Li, J.; Hieter, P.; Boeke, J.D. Designer deletion strains derived fromSaccharomyces cerevisiae S288C: A useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **1998**, *14*, 115–132. [CrossRef]

19. Bergkessel, M.; Guthrie, C. Chemical transformation of yeast. *Methods Enzym.* **2013**, *529*, 311–320.

20. Schmidt, T.G.M.; Skerra, A. The Strep-tag system for one-step purification and high-affinity detection or capturing of proteins. *Nat. Protoc.* **2007**, *2*, 1528–1535. [CrossRef] [PubMed]