The biosynthetic origin of psychoactive kavalactones in kava

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Kava (Piper methysticum) is an ethnomedicinal shrub native to the Polynesian islands with well-established anxiolytic and analgesic properties. Its main psychoactive principles, kavalactones, form a unique class of polyketides that interact with the human central nervous system through mechanisms distinct from those of conventional psychiatric drugs. However, an unknown biosynthetic machinery and difficulty in chemical synthesis hinder the therapeutic use of kavalactones. In addition, kava also produces flavokavains, which are chalconoids with anticancer properties structurally related to kavalactones. Here, we report de novo elucidation of the key enzymes of the kavalactone and flavokavain biosynthetic network. We present the structural basis for the evolutionary development of a pair of paralogous styrylpyrone synthases that establish the kavalactone scaffold and the catalytic mechanism of a regio- and stereo-specific kavalactone reductase that produces a subset of chiral kavalactones. We further demonstrate the feasibility of engineering styrylpyrone production in heterologous hosts, thus opening a way to develop kavalactone-based non-addictive psychiatric therapeutics through synthetic biology.

For millennia, humans have used plants for medicinal purposes. However, our limited understanding of plant biochemistry hinders the translation of such ancient wisdom into modern pharmaceuticals. The Piper (pepper) genus, consisting of about 2,000 pantropically distributed species, is recognized as a remarkable source of bioactive specialized metabolites. The dried fruits of black pepper (Piper nigrum) are used worldwide as a spice having high content of alkaloids piperine and chavicine responsible for their pungent taste. The Indian long pepper (P. longum), one of the oldest herbs described in Ayurvedic medicine, biosynthesizes the phenolic amide piperlongumine, which was recently shown to exhibit selective anticancer activities. Leaves of the betel plant (P. betle) are commonly used as a wrapper for chewing psychoactive areca nut in South Asia. Betel leaves accumulate several specialized allylphenols, including chavibetol and hydroxychavicol, which may act as antimutagens that counteract the carcinogenic effect of the areca nut while enhancing its psychoactive effect. Act as antimutagens that counteract the carcinogenic effect of the allylphenols, including chavibetol and hydroxychavicol, which may act as antimutagens that counteract the carcinogenic effect of the areca nut while enhancing its psychoactive effect.

The kava plant (P. methysticum; Fig. 1a) is native to the Polynesian islands, together with species under the subgenus Macropiper, forming a South Pacific clade of Piper plants phylogenetically distant from Piper plants from Asia. Throughout the Polynesian indigenous cultures, kava root is prepared as a beverage and consumed for its unique sedative and anesthetic effects in religious and cultural rituals. Its anxiolytic and analgesic properties are supported by over 3,000 years of traditional use, as well as numerous recent clinical trials. The bioactive ingredients of kava are phenolic polyketides collectively known as kavalactones (labelled here as 1–20; Supplementary Fig. 1a). Kava roots can contain up to 15% kavalactones by dry weight, whereby a person can ingest several grams of kavalactones during one traditional kava drinking session. Although some concerns were raised in the past about potential hepatotoxicity of kava, it is now generally accepted that the several reported toxicity cases arose from other causative factors rather than kavalactones. Due to its unique anxiolytic effect and generally safe nature, kava drinking has gained popularity worldwide in recent years. Over 100 kava bars are now operating throughout the United States and some athletes have embraced kava as a safe and non-addictive remedy for pain management. Kavalactones interact with the human central nervous system through mechanisms distinct from those of common prescription psychiatric drugs, such as benzodiazepines or opioids, and therefore show promise to be developed into pharmaceuticals for treating anxiety, insomnia and pain.

Despite the worldwide consumption of kava as a sedative beverage and the potential of kavalactones to be developed as anxiolytic medicines, the biosynthesis pathway of kavalactones is unknown. Here, we find key enzymes involved in the biosynthesis of structurally diverse kavalactones in kava and trace their evolutionary origins in other, more conserved, metabolic pathways. We also show it is feasible to use these newly discovered catalysts to engineer microbes to produce styrylpyrones as an alternative means to access these high-value natural products.

The biosynthetic proposal for kavalactones. To investigate kavalactone biosynthesis, we first surveyed the taxonomic distribution of kavalactones in the Piper genus by untargeted metabolomics. Total methanolic extracts from kava and four other Piper species were analysed by liquid chromatography–high-resolution accurate-mass mass-spectrometry (LC–HRAM-MS). While kavalactones are among the most abundant metabolites present in kava extract, they are absent in P. nigrum, P. betle, P. auritum and P. sarmentosum (Fig. 1b), suggesting that kavalactone biosynthesis probably emerged after kava diverged from other Piper species. In addition to kavalactones, we also detected flavokavains A, B and C in kava, three previously characterized chalconoids with reported anticancer properties (21–23; Fig. 1b and Supplementary Fig. 1b).

Considering the structural relationship between kavalactones and flavokavains, which feature the styrylpyrone and chalcone backbones, respectively, we hypothesized that kavalactone biosynthesis probably involves styrylpyrone synthase (SPS), a polyketide synthase (PKS).
related to chalcone synthase (CHS). CHS catalyses the first committed enzymatic step in flavonoid biosynthesis and is ubiquitously present in land plants. Like CHS, SPS would accept a 4-coumarate-3′-hydroxylase (3′H), 4′-hydroxylase (4′H); C4H, 4-coumarate 3-hydroxylase; SAM, S-adenosyl-l-methionine; SAH, S-adenosyl-l-homocysteine.

Kava contains two styrlypyrone synthases. Kava is a decaploid (2n = 10x = 130 chromosomes), rendering biosynthetic enquiry via conventional genetic approaches infeasible. We therefore turned to a multomics-guided candidate gene approach. We first identified three CHS-like genes in a kava transcriptome assembled de novo from leaf and root tissues (Supplementary Table 1). Phylogenetic analysis of these genes in the context of other land-plant CHSs suggests that two of the three genes were probably derived from recent gene duplication events specific to kava (Fig. 2a). In vitro enzyme assays using heterologously expressed and purified enzymes established that these two CHS-like genes encode functional SPSs. Both enzymes exclusively produce the triketide lactone bisnoryangonin (25; Supplementary Note 1) from p-coumaroyl-CoA (Fig. 2b) and are therefore named PmSPS1 and PmSPS2 hereafter (where Pm signifies P. methysticum). The assay also suggested that the third CHS-like gene encodes a bona fide CHS, as it was the only enzyme capable of producing the expected triketide-derived naringenin chalcone (27) and we refer to it as PmCHS hereafter (Fig. 2b). It is noted that in comparison to naringenin chalcone, PmCHS produced substantially more p-coumaroylpiraneric acid lactone (CTAL; 28) and bisnoryangonin (25) in vitro. Although such derailment products have been reported for a number of previously characterized CHSs (refs. 26,27), PmCHS seems to be atypical in the sense that CTAL and bisnoryangonin are its major products in vitro, while naringenin chalcone is a minor product.
To assess the in vivo function of the three newly identified kava PKSs, we expressed each of them as stable transgenes in the *Arabidopsis thaliana* CHS-null mutant *tt4-2* background. Whereas *PmCHS* restored flavonoid biosynthesis in *tt4-2*, neither *PmSPS1* nor *PmSPS2* did, consistent with their respective in vitro biochemical activities (Supplementary Fig. 2). However, we could not detect bisnoryangonin or other styrylpyrones in transgenic *A. thaliana* and *N. benthamiana* plants expressing *PmSPS1* or *PmSPS2*. Similarly, *Agrobacterium*-infiltration-mediated transient expression of *PmSPS1* or *PmSPS2* in the leaves of *Nicotiana benthamiana* also failed to yield any styrlypyrone compounds. On careful examination, we instead identified a series of benzalacetone-type metabolites that accumulated in transgenic *A. thaliana* and *N. benthamiana* plants expressing *PmSPS1* (Supplementary Note 2). We conclude that styrlypyrones generated by heterologously expressed *PmSPS1* are rapidly turned over by unknown enzymes present in *A. thaliana* and *N. benthamiana* to yield benzalacetones as breakdown products.

To probe the mechanistic basis for SPS neofunctionalization from the ancestral CHS, we solved the apo structure of *PmCHS* and the holo structure of *PmSPS1* in complex with *p*-coumaroyl-CoA.
by X-ray crystallography (Supplementary Table 2; ligand omit maps in Supplementary Fig. 3). Both proteins are homodimers and share the canonical αβαβαβ thiolase fold typical for plant type III PKSs 21 (Fig. 2c). Evolutionary analysis using the Mixed Effects Model of Evolution (MEME)30 on the ancestral branch of kava SPSs (Fig. 2a) detected six amino acid residues under episodic diversifying selection (P < 0.05) (Supplementary Fig. 4). Three of these substitutions, S133C, T198N and Q213L, are mapped to the enzyme active site (Fig. 2d). Notably, the T198N substitution was previously reported as one of the three mutations sufficient to convert CHS into a 2-pyrone synthase (2PS; ref. 31). We also observed an unusual insertion of threonine (T194) in a loop region abutting the active site, together with the S133C substitution, causing a reduction of the PmSPS1 active site volume compared to PmCHS in the direction along the polyketide chain elongation (Fig. 2e). In comparison, the previously reported Rheum palmatum benzalacetone synthase (RpBAS), which catalyses only a single round of ketide elongation32, features a further shortened active site compared to PmSPS1 (Fig. 2e). These observations therefore support the notion that subtle changes of active-site volume and shape of type III PKSs dictate the iterative cycles of polyketide elongation and alternative cyclization mechanisms33. In addition, we observed two different conformations of T194 among the six protomers present in the asymmetric unit, indicating the dynamic nature of the underlying loop and its potential role in the catalytic cycle of PmSPS1 (Fig. 2f). One of the protomers in our PmSPS1–p-coumaroyl-CoA complex structure also captures a p-coumaroyl-monoketide intermediate covalently bound to the catalytic cysteine (Fig. 2f), providing a rare glimpse of the active-site configuration after the starter substrate loading step of the PKS catalytic cycle33. Homology modelling and sequence analysis of PmSPS2 suggest that its active site contains a similar set of amino acid substitutions as observed in PmSPS1, except for the T194 insertion (Supplementary Figs. 4 and 5).

Although PmSPS1 and PmSPS2 seem redundant in their in vitro activity, we proposed that they may exhibit differences in substrate preference. In vitro assays in the presence of equimolar mixture of six different acyl-CoAs as starter substrates revealed substantial differences in substrate preference among the three kava PKSs (Supplementary Note 3). Whereas PmSPS1 preferentially produces 11-methoxy-bisnoryangonin (39) and 4-hydroxy-6-styryl-2-pyrene...
Regio-specific methylation by two kava OMTs. Structures of naturally occurring kavalactones implicate rich O-methylation reactions during their biosynthesis (Supplementary Fig. 1a). We hypothesized that kavalactone-biosynthetic OMTs might have been recruited from more conserved OMTs involved in plant phenylpropanoid metabolism; for example, the caffeic acid O-methyltransferase in ligulin biosynthesis\(^1\). Combining phyllogenomics and expression analysis, we identified six putative OMT candidate genes from the kava transcriptome (Supplementary Table 1). Since kavalactones contain four possible O-methylation sites (-OH groups at the C4, C10, C11 or C12 positions), we established a coupled enzymatic system using a 4CL enzyme cloned from kava (designated Pm4CL1) and PmSPS1 to produce five styrylpyrones featuring different aromatic ring modification patterns derived from five hydroxycinnamonic acids, namely cinnamic, p-coumaric, caffeic, ferulic and umbellic acid (Supplementary Note 4). Subsequent OMT activity assays using recombinant kava OMTs revealed that two of the six kava OMT candidates showed activity against various styrylpyrones (Fig. 3a) and were thus named kava O-methytransferases 1 and 2 (PmKOMT1 and PmKOMT2), respectively. PmKOMT1 is one of the most highly expressed enzymes according to the abundance of its corresponding transcript in the kava transcriptome (Supplementary Table 1) and is capable of methylating the -OH groups at the C4, C11 or C12 positions of the styrylpyrone backbone. On the other hand, PmKOMT2 activity is specific to the -OH group attached at the C10 position (Fig. 3a). Transient co-expression of PmKOMT1 together with PmSPS1 or PmSPS2 in N. benthamiana resulted in the production of yangonin, a kavalactone with the 5,6-olefin reduced to alkane (Supplementary Fig. 7a). On the other hand, PmKOMT1 is responsible for the same styrylpyrone substrates, but is unable to reduce the 5,6-olefin can only accept the unmethylated styrylpyrone precursor in vivo. Subsequent in vitro enzyme assays further corroborated this observation (Supplementary Fig. 7b). On the other hand, PmKOMT1 is permissive towards styrylpyrone substrates regardless of whether the 5,6-olefin is reduced or not (Supplementary Fig. 7b). We therefore conclude that PmKOMT1 competes with PmKOMT1 for the same styrylpyrone substrates, but is unable to reduce the 5,6-olefin once the O-methylation has occurred, providing an explanation to the fact that only a fraction of the major kavalactones contain the reduced C5–C6 bond (Supplementary Fig. 1a).

Phylogenetic analysis of PmKLR1 together with several related reductases from A. thaliana suggests that PmKLR1 probably originated from an ancestral cinnamoyl-CoA reductase (Fig. 4c). To further dissect the structural basis for the reaction mechanism and regio- and stereo-selectivity of PmKLR1, we solved its holo structure in complex with nicotinamide adenine dinucleotide phosphate (NADP\(^+\)) by X-ray crystallography (Supplementary Table 2; ligand 5-hydroxylunularic acid and phloretin, respectively\(^{35,36}\). To test this, we performed coupled Pm4CL1–PmSPS1 in vitro enzyme assays using phloretic acid and phenylpropanoic acid as starter substrates, both of which contain a reduced α–β bond. These assays resulted in the production of the corresponding 7,8-saturated kavalactones, suggesting that Pm4CL1 and PmSPS1 can use substrates derived from dihydro-hydroxycinnamic acids with a reduced α–β bond (Supplementary Fig. 6). Although dihydro-hydroxycinnamic acids are widespread in plants\(^{37,38}\), the mechanism underlying (hydroxy) cinnamic acid α–β olefin reduction is yet to be characterized.

Biosynthesis of 7,8-saturated kavalactones. The styrylpyrone backbones produced by SPs from hydroxycinnamic acids contain two olefins (C5=C6 and C7=C8), whereas a number of naturally occurring kavalactones possess alkane at either position (for example, kavain with C5–C6 or 7,8-dihydroxykavain with C7–C8) or both positions (for example, 7,8-dihydrokavain; Supplementary Fig. 1a). We proposed that the 7,8-saturated kavalactones could be derived from dihydro-hydroxycinnamoyl-CoA precursors already containing a reduced α–β bond. This proposal was provoked by previous reports that two type III PKSs in the genera Pinus\(^24\) from feruloyl-CoA and cinnamoyl-CoA, respectively, PmSPS2 produces mostly dihydrobisdinoyngonin (35) and bisbonyangonin (25) from p-dihydrocoumaroyl-CoA and p-coumaroyl-CoA, respectively. No tetraketide products were observed in the PmSPS1 and PmSPS2 assays. In contrast, PmCHS produces both tetraketide and tetraketide products, preferentially derived from feruloyl-CoA and cinnamoyl-CoA. This is unusual as most of the previously characterized CHS orthologues accept p-coumaroyl-CoA as the preferred starter substrate\(^{35}\).
omit maps in Supplementary Fig. 8) and computationally docked a styrylpyrone substrate, 4-hydroxy-6-styrlyl-2-pyrene (24), into its active site (Fig. 4d). The PmKLR1 structure reveals features of the substrate-binding pocket probably responsible for the regio- and stereo-selective reduction of the styrylpyrone 5,6-olefin (Fig. 4c). The docked styrylpyrone substrate is coordinated by several

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**Diagram a**

- **Cinnamic acid**
- **4CL1**
- **SPS1**
- **KL1**
- **KOMT1**
- **(24) 4-hydroxy-6-styrlyl-2-pyrene**
- **215.079 m/z**

**Diagram b**

- **Relative abundance (%)**
- **Retention time (min)**
- **Kavain standard**
- **Desmethoxyyangonin standard**
- **4CL1 + SPS1 + KLR1 + KOMT1**
- **4CL1 + SPS1 + KOMT1**
- **4CL1 + SPS1 + KLR1**
- **4CL1 + SPS1**
- **AtCCR1 cinnamoyl-CoA reductase 1**
- **AtCCR2 cinnamoyl-CoA reductase 2**
- **AtKPR1 tetraketide alpha-pyrone reductase 1**
- **AtKPR2 tetraketide alpha-pyrone reductase 2**
- **AtDFRA dihydroflavonol 4-reductase**
- **AtBAN anthocyanidin reductase**

**Diagram c**

- **AtCCR1**
- **AtCCR2**
- **AtKPR1**
- **AtKPR2**
- **AtDFRA**
- **AtBAN**
- **PmKLR1**
- **AtKOMT1**
- **AtKLR1**
- **AtSPS1**
- **At4CL1**

**Diagram d**

- **Pm KLR1**
- **(24) 4-hydroxy-6-styrlyl-2-pyrene**
- **NADP^+**

**Diagram e**

- **Thr 129**
- **Ser 128**
- **Tyr 194**
- **Lys 169**
- **Kavain standard**
- **Desmethoxyyangonin standard**
- **4CL1 + SPS1 + KLR1 + KOMT1**

**Diagram f**

- **Relative abundance (%)**
- **m/z**
- **231.102**
- **230.2478**
- **232.1052**
- **233.0784**
- **234.1337**
- **229.1799**
- **231.1016**

**Diagram g**

- **Ser 128**
- **Lys 169**
- **HOH**
- **H^+**
- **NADPH**
- **R_1 = H, OH**
- **R_2 = Adenine dinucleotide phosphate**
active-site-lining residues to adopt a specific binding pose, and indicates that the hydride from the nicotinamide group of NADPH is transferred to C5. Lys 169 and Tyr 165 coordinate the binding of NADPH by hydrogen bonding with its ribosyl-OH groups. While Lys 169 probably also serves as a proton donor that relays its side-chain amine proton through Ser 128 to C6 of the styrylpyrone substrate exclusively from one side of the lactone ring (Fig. 4e). As the 5,6-olefin is reduced to alkane, a new chiral centre at C6 is formed. The intricate hydrogen bonds between the styrylpyrone 4-OH, 5,6-olefin is reduced to alkane, a new chiral centre at C6 is formed.

Methysticin synthase installs a methylenedioxy bridge. Methysticin-like kavalactones, which carry a methylenedioxy bridge at the C11—C12 position (Supplementary Fig. 1a), exhibit strong modulatory effects on human liver cytochromes P450 and thus contribute to the complex pharmacology of kava. The CYP719 family of plant P450 enzymes is known to catalyse the formation of the methylenedioxy bridge moiety from vicinal methoxy and hydroxy groups; for example, in the biosynthesis of berberine and etoposide. We identified a single gene of the CYP719 family in kava, PmCYP719A26 (Fig. 5a) and suggested that it may catalyse the formation of methylenedioxy bridge using ferulic acid-derived styrylpyrone precursors carrying vicinal 11-methoxy and 12-hydroxy groups (Fig. 5b). Indeed, co-expression of PmSPS1, PmKOMT1 and PmCYP719A26 in N. benthamiana resulted in the transgenic production of a compound with the molecular mass and retention time identical to that of methysticin (Fig. 5c). On the basis of the tandem mass spectrometry (MS/MS) fragmentation pattern comparison with a methysticin standard, we tentatively assigned this compound as 5,6-dehydro-7,8-dihydromethysticin (47; Supplementary Note 6). We therefore designated PmCYP719A26 as methysticin synthase 1 (PmMTS1).

Flavokavain biosynthesis uses the same KOMTs. Through OMT activity screen, we found that PmKOMT1 and PmKOMT2 also exhibit O-methylation activities against naringenin chalcone produced by CHS (Supplementary Note 7). Using enzyme assays and heterologous expression in N. benthamiana, we confirmed that PmKOMT1 methylates the hydroxy groups at the C4 and C6 positions on the chalcone scaffold, whereas PmKOMT2 methylates the hydroxy group at the C2 position (Supplementary Fig. 11). We thus established that the three known flavokavains (Supplementary Fig. 1b) can be produced by the combined activities of PmCHS, PmKOMT1 and PmKOMT2.

Heterologous production of styrylpyrones in microorganisms. As a proof of concept, we examined the feasibility of kavalactone production in microbial hosts through the means of metabolic engineering. Using an operon-like construct co-expressing Pm4CL1 together with PmSPS1 or PmSPS2 under the constitutive pGAP promoter, we could reconstitute the biosynthesis of the kavalactone precursor, bisnoryangonin, from supplemented p-coumaric acid in Escherichia coli under shake flask conditions (Supplementary Fig. 12a). Similarly, plasmid-based expression of the same combination of enzymes under the constitutive pTEF promoter in the yeast Saccharomyces cerevisiae also resulted in bisnoryangonin production from supplemented p-coumaric acid under shake flask conditions (Supplementary Fig. 12b). Future efforts in combining kavalactone-biosynthetic genes with previously developed background bacterial or yeast strains over-producing p-coumaroyl-CoA can potentially further increase the titre of kavalactone production in these hosts.

Discussion

Plant type III PKSs descended from the ancestral 3-ketoacyl-acyl carrier protein synthase III (KAS III) enzymes involved in fatty acid metabolism. They have radiated during land plant evolution to give rise to a diverse class of specialized metabolic enzymes responsible for producing a battery of important polyketide scaffolds, including chalcones, stilbenes (for example, resveratrol), benzalacetones and others. Polyketide natural products stemmed from these scaffolds contribute to the fitness of their plant hosts amid unique selective...
pressures present in diverse terrestrial environmental niches. Plant natural products that carry the styrylpyrone structural motif have been known for decades but our study presents molecular cloning and functional characterization of two dedicated SPSs from a styrylpyrone-producing plant. Notable SPS activity was observed in PmCHS, which represents the extant version of the evolutionary...
progenitor that gave rise to PmSPS1 and PmSPS2. The latent SPS activity of PmCHS probably resulted in the initial serendipitous accumulation of styrlypyrones at appreciable amount in the ancestors of kava, which kick-started the natural selection process that further shaped the evolutionary trajectory of these newly emerged metabolic traits. Mainly through the mechanism of gene duplication followed by neo-functionalization, two dedicated SPSs as well as additional kavalactone-biosynthetic tailoring enzymes evolved from progenitor enzymes in pre-existing pathways. These evolutionarily new enzymes assembled into a metabolic network (Fig. 6) to produce the full repertoire of kavalactones as observed in extant kava cultivars today. Although the biosynthetic origin of kavalactones as delineated in this study is unique to kava, it epitomizes a common evolutionary process underpinning the rapid expansion of specialized metabolism in the plant kingdom.

Given the psychoactive properties of kavalactones, evolutionarily exploration of the variety of naturally occurring kavalactones probably conferred protection in kava against herbivorous animals. Consistent with this notion, folk stories about small rodents paralyzed by chewing on the roots of kava plants are widespread in the Pacific islands. It is also likely that the thousands of years of consumption and cultivation of kava plants by Polynesian islanders have led to human artificial selection of kava plants that produce higher content and specific composition of kavalactones.

In animals, kavalactones display complex pharmacology, including modulation of GABA, and cannabinoid (CB) receptors, blockade of voltage-gated sodium ion channels, reduced release and reuptake of neurotransmitters and interactions with monoamine oxidase B (refs. 11,12). In particular, kavain was shown to act as a positive allosteric modulator of the GABA<sub>B</sub> receptor through a binding site different from that of benzodiazepine psychiatric drugs. The capability to produce structurally diverse kavalactones and their derivatives by metabolic engineering of the biosynthetic enzymes described herein will help delineate the structure–function relationships of kavalactones and potentially lead to biomedical applications. In light of several major challenges facing modern society, such as anxiety, opioid crisis and the scarcity of effective treatments for psychiatric disorders, kavalactones and their derivatives present a promising class of psychoactive molecules to potentially answer these unmet needs.

Methods

Chemicals and reagents. Kavalactone standards (yangonin, methysticin, desmethoxyyangonin, 7,8-dihydrokavain and 7,8-dihydroxythymysticin) were obtained from AvaChem Scientific. Cinnamoyl-CoA, p-coumaryl-CoA, p-dihydrocinnamoyl-CoA, caffeoyl-CoA, feruloyl-CoA and sinapoyl-CoA were obtained from MicroCombiChem. Naringenin was obtained from AK Scientific. Isopropanol-d<sub>6</sub> was obtained from Cambridge Isotope Laboratories. (±)-Kavain, malonyl-CoA, trans-cinnamic acid-d<sub>6</sub>, d-glucose-1-d<sub>4</sub>, and other cofactors and reagents were obtained from Sigma–Aldrich.

RNA extraction and complementary DNA template preparation. Total RNA was extracted separately from kava root and leaf tissue using the RNeasy Plant Mini Kit (QIAGEN). First-strand cDNAs were synthesized by PCR with reverse transcription from the total RNA samples as templates using the SuperScript III First-Strand Synthesis System with the oligo(dT)₁₂ primer (Thermo Fisher Scientific).

Transcriptome sequencing and assembly. The transcriptome library preparation and sequencing were performed at the Beijing Genomics Institute using the standard BGISEQ-500 RNA sample preparation protocol. The libraries were sequenced as 50 × 50 base pair (bp) (PE-50) reads on the BGISEQ-500 platform. Sequence reads (FASTQ files) were obtained after trimming of low quality reads using the Trimmomatic package. The transcriptome dataset was assembled using Transdecoder. The transcripts were annotated using GoTools and NCBI BLAST search service. Gene expression statistics were determined using RSEM. Transcripts and predicted protein sequences were annotated with transcript-per-million (TPM) values and closest BLAST hits from the UniprotKB/Swiss-Prot database using in-house scripts. Transcriptome mining was performed on a local BLAST server. In addition, two kava root RNA-seq datasets previously published by the PhytoMetaSyn Project (NCBI SRA accessions SRX202785 and SRX202184) were assembled and used as complementary sources of candidate enzyme sequences. Existing RNA-seq datasets of P. aeratum, P. betle and P. nigrum (NCBI SRA accessions ERX2099199, SRX691517 and SRX890122) were also assembled into de novo transcriptomes to obtain their corresponding CHS sequences.

Sequence alignment and phylogenetic analyses. Sequence alignments were performed using the MUSCLE algorithm in MEGA7 (ref. 10). Evolutionary histories were inferred by using the maximum-likelihood method on the basis of the JTT matrix-based model (ref. 10). Bootstrap values were calculated using 1,000 replicates. All phylogenetic analyses were conducted in MEGA7 (ref. 10).

Evolutionary analysis. The MEME analysis (ref. 10) was performed via the Hypothesis Testing using Phylogenies (HyPhy) package, starting from codon-aligned CHS nucleotide sequences and the corresponding phylogenetic tree. The P-value threshold was set to 0.05.

Cloning of candidate genes from cDNA. Phusion High-Fidelity DNA Polymerase (Termo Fisher Scientific) was used for PCR amplifications from kava cDNA. Gibson assembly was used to clone the amplified genes into target vectors. Restriction enzymes and Gibson assembly reagents were purchased from New England Biolabs. Oligonucleotide primers were purchased from Integrated DNA Technologies. All primers used for cloning are listed in Supplementary Table 4.

Protein expression and purification. Candidate genes were cloned into the pHis8-4, a bacterial expression vector containing an N-terminal 8x His tag followed by a tobacco etch virus (TEV) cleavage site for recombinant protein production in E. coli. Proteins were expressed in the BL21(DE3) E. coli strain cultivated in terrific broth (TB) and induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) overnight at 18°C. For the OMT enzyme screen, crude protein extracts were prepared from E. coli cultures using Bacterial Protein Extraction Reagent (Termo Fisher Scientific). For protein purification, E. coli cells were harvested by centrifugation, resuspended in 150 mL lysate buffer (50 mM Tris buffer pH 8.0, 500 mM NaCl, 30 mM imidazole, 5 mM dithiothreitol) and lysed by five passages through an M-110L microfluidizer (Microfluidics). The resulting crude protein lysate was clarified by centrifugation (19,000g, 1 h) before QIAVEN nickel-nitriolactric acid (Ni–NTA) gravity flow chromatographic purification. After loading the clarified lysate, the Ni–NTA resin was washed with 20 column volumes of lysate buffer and eluted with 1 column volume of elution buffer (50 mM Tris buffer pH 8.0, 500 mM NaCl, 300 mM imidazole, 5 mM dithiothreitol). Then 1 mg of His-tagged TEV protease was added to the eluted protein, followed by dialysis at 4°C for 16 h in dialysis buffer (50 mM Tris buffer pH 8.0, 500 mM NaCl, 5 mM dithiothreitol). After dialysis, protein solution was passed through Ni–NTA resin to remove uncleaved protein and His-tagged TEV. The recombinant proteins were further purified by gel filtration on an AKTA fast protein liquid chromatography (FPLC) system (GE Healthcare Life Sciences). The principal peaks were collected, verified by SDS polyacrylamide gel electrophoresis and dialysed into a storage buffer (12.5 mM Tris buffer pH 8.0, 500 mM NaCl, 5 mM dithiothreitol). Finally, proteins were concentrated to >10 mg mL<sup>−1</sup> using Amicon Ultra-15 Centrifugal Filters (Millipore). The PmSPS1, PmSPS2 and PmCHS proteins purified as homodimers, while PmCQL1, PmKOMT1, PmKOMT2 and PmKLR11 purified as monomers.

PmKLR11 site-directed mutagenesis. Two overlapping gene fragments were separately amplified by PCR from the plasmid containing PmKLR11 as template using primers containing the desired single-amino-acid substitutions (see Supplementary Table 4). The two fragments were subsequently cloned into the pHis8-4 target vector using Gibson assembly (ref. 21).

In vitro enzyme assays. Enzyme assays were performed in 50 mM potassium phosphate buffer, pH 7.6 containing 5 mM MgCl<sub>2</sub>, 5 mM adenosine triphosphate, 1 mM oceonzyme A, 3 mM malonyl-CoA and 0.25 mM initial substrate (typically, cinnamic acid or p-coumaric acid). Recombinant enzymes were added to final concentration of 10 μg mL<sup>−1</sup>. For OMT assays, additional 3 mM S-acetyl-oximethione was added. For reductase assays, additional 5 mM NADPH was added. Reactions were incubated at 30°C until completion (typically overnight), followed by the addition of methanol to 50% final concentration. Samples were centrifuged (13,000g, 20 min) and supernatants were collected for LC–MS analyses.

Stereo-specific deuterium labelling using NADPH isotopic analogues. Enzyme assays using site specifically deuterium-labelled NADPH isotopic analogues were performed as described above as additional compounds were added to produce the desired NADPH isotopic analogue. (R)-NADPH was produced by adding 1 mM NADP<sup>+</sup>, 10 mM isopropanol-d<sub>1</sub>, 1% (v/v) alcohol dehydrogenase (Sigma–Aldrich catalogue no. 49641) and 1 mM semicarbazide. (S)-NADPH was produced by adding 1 mM NADP<sup>+</sup>, 2 mM d-glucose-1-d<sub>5</sub>, 5 mM MgCl<sub>2</sub>, 4 mM ATP, 1 unit hexokinase and 1 unit glucose-6-phosphate dehydrogenase.
Plant metabolite extraction. Approximately 100 mg of plant leaf tissue was dissected, transferred into grinding tubes containing ~15 zirconia/silica disruption beads (2 mm diameter; Research Products International) and snap-frozen in liquid nitrogen. The frozen samples were homogenized twice on a Tissuelyser II (QiAGEN). Metabolites were extracted using 5–10 volumes (w/v) of 50% methanol at 55 °C for 1 h. Extracts were centrifuged twice (13,000g, 20 min) and supernatants were collected for LC–MS analysis.

LC–MS analysis. LC was conducted on a Dionex UltiMate 3000 UHPLC system (Thermo Fisher Scientific), using water with 0.1% formic acid as solvent A and acetonitrile with 0.1% formic acid as solvent B. Reverse phase separation of analytes was performed on a Kinetex C18 column (2.6 μm particle size; Phenomenex). Chiral chromatography was performed on a Lux–Amyleose-1 column, 250 × 4.6 mm, 5 μm particle size (Phenomenex). The column oven was held at 30 °C. Most injections were eluted with a gradient of 5–60% B for 9 min, 95% B for 3 min and 5% B for 3 min, with a flow rate of 0.7 ml min⁻¹. The initial analysis of Piper species leaf extracts was performed on a gradient of 5–80% B for 40 min, 95% B for 4 min and 5% B for 1 min, with a flow rate of 0.4 ml min⁻¹. Chiral chromatography was performed on a gradient of 5–95% B for 30 min, 95% B for 5 min and 5% B for 10 min, with a flow rate of 1 ml min⁻¹. MS analyses for the OMT enzyme screen and for chiral chromatography were performed on a TSQ Quantum Access MAX mass spectrometer (Thermo Fisher Scientific) operated in positive ionization mode with full scan range of 100–1000 m/z and top five data-dependent MS/MS scans. Raw LC–MS data were analysed using XCalibur (Thermo Fisher Scientific), MZmine 2 (ref. 64) and MetaboAnalyst4. The SIRIUS tool was used to interpret MS/MS spectra.55–57 Identified metabolites are listed in Supplementary Table 3.

X-ray crystallography. The purified PmSPS1 protein (17.1 mg ml⁻¹) was incubated with 4 mM p-coumaryl-CoA for 1 h before setting crystal trays. Crystals of PmSPS1 were obtained after 4 d at 21 °C in hanging drops containing 0.8 μl of protein solution and 0.8 μl of reservoir solution (50 mM HEPES buffer pH 7.5, 10% w/v polyethylene glycol (PEG) 8000, 4% v/v ethylene glycol). Crystals were frozen in reservoir solution with ethylene glycol concentration increased to 15% (v/v). PmSPS1 X-ray diffraction data were collected on the 24-ID-C beam line equipped with a Pilatus-6 MF pixel array detector (Advanced Photon Source, Argonne National Laboratory) and processed using the online software HKL-2000. Crystals of the purified PmKLR1 protein (14 mg ml⁻¹) were obtained from a screening plate after 4 d at 4 °C in sitting drops containing 150 nl protein solution and 150 nl of reservoir solution (100 mM HEPES pH 7.0, 10% w/v PEG 6000). Crystals of the purified PmKLR1 protein (14 mg ml⁻¹) were obtained after 4 d at 21 °C in hanging drops containing 0.8 μl of protein solution (including 1 mM NADP⁺) and 0.8 μl of reservoir solution (100 mM HEPES pH 7.5, 25% w/v PEG 3350, 200 mM ammonium acetate NH₄Ac). Crystals were frozen in reservoir solution with PEG 3350 concentration increased to 34% (w/v). PmCHS and PmKLR1 X-ray diffraction data were collected on the 24-ID-E beam line equipped with an Eiger-16M pixel array detector (Advanced Photon Source, Argonne National Laboratory). Diffraction intensities were indexed and integrated with SCALA58 and scaled with SCALA. Initial phases were determined by molecular replacement using Phaser59 in PHENIX, using search models generated from protein sequences on the Phyre2 server.70 Subsequent structural building and refinements were conducted in PHENIX. Coot was used for graphical map inspection and manual tuning of atomic models. Root mean square deviation (RMSD) of PmSPS1 and PmCHS structures was calculated using the structure comparison function in PHENIX. Crystallographic statistics are listed in Supplementary Table 2. The average B-factor in the PmKLR1 structure was higher than usual due to a number of flexible or disordered regions present in chains A and D of the structure (residues 91–97, 133–143, 212–226, 243–250 and 256–292).

Protein structure modelling, docking and rendering. The PmSPS2 structure model was generated on the Phyre2 server using the PmSPS1 crystal structure as a modelling template.71 The active-site pocket in the PmSPS1 structure was determined using KVFinder.72 Ligand docking calculations were performed with AutoDock Vina1.1.2.1 (ref. 73). Molecular graphics were rendered with PyMOL 2.0.7 (Schrodinger).

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Author contributions
T.P. and J.-K.W. designed experiments. T.P. performed most of the experiments. M.P.-S. assisted with cloning and crystallography. T.R.F. assisted with transcriptome assembly and LCM–MS analyses. A.D.A. cloned genes and purified proteins. C.H.S. constructed expression vectors. T.P. analysed data. T.P. and J.-K.W. wrote the paper.

Competing interests
T.P. and J.-K.W. have filed a patent application on metabolic engineering of kavalactones and flavokavains using the enzymes discovered in this study. J.-K.W. is a co-founder, a member of the Scientific Advisory Board and a shareholder of DoubleRainbow Biosciences, which develops biotechnologies related to natural products. All other authors have no competing interests.

Additional information
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Data collection: No specific software was used to collect data

Data analysis: MZmine 2.31, Metaboanalyst 4.0, SIRIUS 4.0, Trinity 2.6.5, Trimmomatic 0.36, Transdecoder 5.0.2, sequenceserver 1.0.9, PHENIX 1.12, CCP4 7.0.53, Coot 0.8.8, PyMOL 2.0.7, GraphPad Prism 7, Microsoft Excel

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The sequences of the genes reported in this article have been deposited in NCBI GenBank (accessions MK058492-MK058514). Protein expression plasmids are available from Addgene. The raw sequencing reads have been submitted to NCBI SRA (accession PRJNA494686) and the de novo assembled transcriptome to NCBI
Raw metabolomic LC-MS datasets have been uploaded to the GNPS-MassIVE database (accessions MSV000083272 and MSV000083274–MSV000083277). Protein structures have been deposited in Protein Data Bank (accessions 6OP5, 6CQB, and 6NBR).

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Life sciences

Study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | A single kava plant was used for the initial RNA sequencing and cloning from cDNA, since no quantitative differential analyses were performed. |
| Data exclusions | No data exclusions in this study. |
| Replication | Nicotiana benthamiana expression experiments were performed in 3-4 replicates. In vitro enzyme assays were repeated multiple times with different combinations of enzymes, as shown in individual figures. Results from different replicates were consistent. |
| Randomization | No randomization was performed, because this study does not contain any statistical analyses that would require randomization. |
| Blinding | No blinding required, because no clinical experiments were performed. |

Materials & experimental systems

Policy information about availability of materials

| n/a | Involved in the study |
| --- | --- |
| [ ] | Unique materials |
| [ ] | Antibodies |
| [ ] | Eukaryotic cell lines |
| [ ] | Research animals |
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Unique materials

Obtaining unique materials Kava (Piper methysticum) plants were obtained from Hawaiian Kava Center, LLC (www.hawaiiankava.com)

Method-specific reporting

| n/a | Involved in the study |
| --- | --- |
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