Characterization of the kaurene oxidase CYP701A3, a multifunctional cytochrome P450 from gibberellin biosynthesis

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KO (kaurene oxidase) is a multifunctional cytochrome P450 catalysing three sequential oxidations in gibberellin phytohormone biosynthesis. These serve to transform the C4a methyl of ent-kaurene olefin intermediate into the carboxylic acid moiety of ent-kauren-19-oxic acid. To investigate the unknown catalytic mechanism and properties of KO, we have engineered the corresponding CYP701A3 from Arabidopsis thaliana (AtKO) for functional recombinant expression in Escherichia coli, involving use of a fully codon-optimized construct, along with additional N-terminal deletion and modification. This recombinant tKO (rAtKO) was used to carry out 18O2 labelling studies with ent-kaurene, and the intermediates ent-kaurenol and ent-kaurenal, to investigate the multifunctional reaction sequence; revealing catalysis of three hydroxylation reactions, which further requires dehydration at some stage. Accordingly, following initial hydroxylation, ent-kaurenol must then be further hydroxylated to a gem-diol intermediate, and our data indicate that the subsequent reactions proceed via dehydration of the gem-diol to ent-kaurenal, followed by an additional hydroxylation to directly form ent-kaurenoic acid. Kinetic analysis indicates that these intermediates are all retained in the active site during the course of the reaction series, with the first hydroxylation being rate-limiting. In addition, investigation of alternative substrates demonstrated that ent-beyerene, which differs in ring structure distal to the C4a methyl, is only hydroxylated by rAtKO, indicating the importance of the exact tetracyclic ring structure of kaurene for multifunctional KO activity. Thus the results of the present study clarify the reaction sequence and enzymatic mechanism of KO, as well as substrate features critical for the catalysed multiple reaction sequence.

Key words: cytochrome P450, gibberellin, hormone metabolism, mono-oxygenase catalysis, substrate specificity.

INTRODUCTION

The importance of gibberellins as endogenous plant growth regulators has resulted in intensive studies on their metabolism, which has now been almost completely elucidated [1]. Notably, pioneering studies by West and co-workers demonstrated the intermediacy of ent-kaur-16-en-16-ene (1) in gibberellin biosynthesis [2], along with its conversion into ent-kaur-16-en-19-oxic acid (4) via the action of cytochromes P450 (P450s) [3]. They later reported inhibition studies further indicating that the production of 4 from 1 might be catalysed by a single such enzyme that is specifically targeted by the plant growth inhibitor ancydol [4], which suggests that this enzymatic activity might represent a control point in gibberellin biosynthesis. More recently, the gene for the corresponding P450, CYP701A3, was cloned from Arabidopsis thaliana [5]. Subsequent recombinant expression in Saccharomyces cerevisiae demonstrated that this single P450, KO (kaurene oxidase), does catalyse the conversion of 1 into 4, with the oxidized intermediates ent-kaur-16-en-19-ol (2) and ent-kaur-16-en-19-al (3) also found [6].

P450s are nearly ubiquitous haem-thiolate enzymes that reduce molecular dioxygen with concomitant substrate oxidation, and typically obtain the necessary electrons from a separate NADPH-specific reductase. P450s typically catalyse the insertion of oxygen into an unactivated substrate along with the production of water, leading to the common mono-oxygenase designation. The generalized mixed-function oxidation catalysed by P450s can be summarized as:

\[ \text{O}_2 + \text{R} + \text{NADPH} + \text{H}^+ \rightarrow \text{RO} + \text{H}_2\text{O} + \text{NADP}^+ \]

In eukaryotic organisms, their substrates are generally lipophilic, reflecting the integral membrane nature of these ‘microsomal’ P450s. P450s operate via a catalytic cycle consisting of substrate binding, one electron reduction of the haem-iron, O2 binding, a second single-electron reduction, protonation and oxygen bond cleavage to release water, and terminal oxygen insertion mediated by the resulting perferryl species [7,8]. However, the details of the P450 catalytic cycle and enzymatic mechanism remain an active area of investigation. In addition, while often associated with hydroxylation mediated by insertion of oxygen into a carbon–hydrogen bond, P450s have been found to catalyse a wide range of reactions [9]. For example, these reactions include desaturation, epoxidation, dealkylation, coupling reactions, decarboxylation, ring contraction, ester cleavage and methyl transfer. Thus there may be a variety of mechanisms by which P450s operate.

A number of P450s catalyse sequential reactions, using more than one molecule of O2 and iteration of the catalytic cycle to carry out multiple oxidations of a single compound, which includes the KO from gibberellin biosynthesis described above. Work with P450-containing microsomal preparations from plants demonstrated the use of multiple molecules of O2 for the conversion of 1 into 4, with the expected incorporation of stable isotope labelled oxygen from 18O2 into the initially produced ent-kaur-16-en-19-ol (2) [10]. However, although genes encoding KO have been cloned from several plant species [5,11–14], the
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Scheme 1 Role of KO in gibberellin phytohormone (e.g. GA) biosynthesis

(a) Gibberellin biosynthetic pathway, highlighting KO-catalysed conversion of ent-kaurene (1) into ent-kaurenoic acid (4). (b) Potential reactions catalysed by KO. Insertion of oxygen in hydroxylation reactions indicated by + O, dehydrogenation reactions indicated by − H₂, with hydration and dehydration reactions also indicated.

catalytic properties of this multifunctional P450 have not been investigated in any detail.

Although previous ¹⁸O₂ labelling with KO demonstrates that the first step is the expected hydroxylation to ent-kaur-16-en-19-ol, the remaining steps are unclear [10]. In particular, formally 2 could undergo dehydrogenation to ent-kaur-16-en-19-al (3), although it seems more likely to be hydroxylated to the corresponding gem-diol (3a). Similarly, this hydrate (3a) could undergo dehydrogenation to directly produce 4, but is more likely to either be further hydroxylated to the ortho-acid hydrate (4a) that undergoes dehydration to 4, or be dehydrated to the aldehyde (3), which could then undergo a hydroxylation reaction to directly produce 4 (Scheme 1). Furthermore, while a preliminary investigation of KO indicated some substrate or inhibitor plasticity, the extent of this tolerance and the substrate determinants for multifunctional oxidation remain unknown [10].

To enable biochemical investigation of KO, we have developed a modified CYP701A3 construct rAtKO [recombinant AtKO (A. thaliana KO)] which exhibits good activity and stability upon recombinant expression in Escherichia coli. Using this rAtKO, in the present paper we report the results of kinetic analyses and ¹⁸O₂ incorporation with 1, 2 or 3 as substrate, which provide insight into the catalysed enzymatic reaction sequence, as well as investigation of alternative substrates that provide insight into the substrate structure–activity relationship underlying the multifunctional nature of KO catalysis.

EXPERIMENTAL

General

Unless otherwise stated, molecular biology reagents were purchased from Invitrogen and chemicals from Fisher Scientific. All compounds reported were verified by comparison with authentic standards via GC with MS detection, and quantified by FID (flame ionization detection), both carried out as described previously [15]. Cloning procedures for the Gateway expression system (Invitrogen) were conducted according to the manufacturer’s recommended conditions.

Cloning, engineering, AtKO and AtCPR1 expression

The open reading frames for AtCPR1 (GenBank® accession number X66016) (CPR is cytochrome P450 reductase) and AtKO/CYP701A3 (GenBank® accession number AF047719) were cloned from A. thaliana cv Columbia by PCR amplification from a cDNA library, inserted into pENTR/SD/D-TOPO vectors (Invitrogen), sequence verified and then recombined into pDEST14 vectors for recombinant expression in E. coli strain C41 (Lucigen). AtKO was also recombined into pYES-DEST52 for recombinant expression in yeast. A synthetic version of AtKO, codon-optimized for expression in E. coli, was obtained from Celtek, and cloned as above (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/431/bj4310337add.htm for the corresponding nucleotide sequence). N-terminally modified AtKO constructs, as depicted in Figure 1, were constructed by PCR, verified by complete sequencing, and cloned as described above.

Expression and harvest of native AtKO in the yeast strain WAT11 was carried out as described previously [6,15,16]. Briefly, yeast cells were transformed by a lithium acetate protocol and selected in uracil-deficient SCU medium and plates, with the presence of AtKO in the resulting colonies verified by PCR. Recombinant 50 ml cultures were grown at 30°C, induced by the addition of galactose to 2% (w/v) of the SCU medium when at a D₆₀₀ of 0.4, then cultured for an additional 16 h. Negative control cultures (WAT11 transformed with pYES-DEST52 carrying a different P450, CYP76M7) failed to oxidize kaurene, whereas AtKO-expressing cell cultures fed 1 µg of kaurene/ml for 1 h exhibited enzymatic activity, oxidizing kaurene. Microsomes were prepared by glass bead lysis following the method described by Pompon et al. [16].

Spheroblasts and microsomes from recombinant E. coli were both easier to prepare than from yeast, and provided a more
robust, reliable source of full KO activity, although SDS/PAGE analysis indicates that notable overexpression was not achieved, even with the optimal rAtKO construct (Supplementary Figure S2 at http://www.BiochemJ.org/bj/431/bj4310337add.htm). KO activity was increased by co-expression with the GroEL/GroES chaperones using pGro7 (Takara). Recombinant E. coli C41 cultures, containing pDEST14/rAtKO and pGro7, were grown in TB (terrific broth) medium with 50 mg/l carbenicillin and 34 mg/l chloramphenicol at 37 °C to a $D_{600}$ of 1.0, whereupon the temperature was lowered to 28 °C and cultures supplemented with $\delta$-amino-levulinic acid (75 mg/l), and GroEL/GroES expression was induced with arabinose (0.5 g/l). After 1 h at 28 °C, the cultures were further induced with 1 mM IPTG (isopropyl-1-D-thiogalactopyranoside), and cultured for an additional 40 h. Spheroblast preparation consisted of cell harvest by centrifugation at 5000 g for 10 min, followed by centrifugation at 5000 g for an additional 40 min. Spheroblast preparation consisted of cell harvest by centrifugation at 5000 g for 10 min, followed by centrifugation at 5000 g for 10 min to obtain spheroplasts, which were resuspended in 10 ml of MP buffer [10 mM Tris/HCl (pH 7.2), 0.5 mM EDTA, 20% (v/v) glycerol, 1 mM DTT (dithiothreitol) and 1× Sigma protease inhibitor cocktail]. Lysozyme was added to 0.1 mg/ml and the resuspended cells stirred at 4 °C for 10 min, followed by centrifugation at 5000 g for 10 min to obtain spheroplasts, which were resuspended in 10 ml of MP buffer and subject to brief, mild sonication (3×3 s bursts, 30% output). Micromesomes could be further prepared by centrifugation of the spheroblast preparation at 10000 g for 10 min, and the resulting supernatant ultracentrifuged at 41000 rev/min (Beckman Ti70.1) for 30 min, all at 4 °C. Homogenization of the resulting (typically red) microsomal pellet in MP buffer did yield active micromosomal preparations suitable for CO difference spectra, conducted by reduction with sodium dithionite and subsequent immersion in CO-saturated TE buffer [10 mM Tris/HCl (pH 8.0) and 1 mM EDTA]. Nevertheless, it was found that the spheroplasts were easier to harvest and yielded higher enzymatic activity, perhaps due to reduced handling/preparation. Negative control preparations, from E. coli expressing a different P450 (CYP76M7), did not react with the diterpenes used here. Expression and harvest of AtCPR1 was as described for AtKO above, with the exception of 1 mM thiamine and 0.2 mg/l riboflavin being added to the cultures instead of $\delta$-amino levulinic acid, and the duration of expression lasting 24 h. Solubilization was performed with spheroplasts by drop-wise addition of Triton X-100 to 0.5% at 4 °C with stirring over 30 min. The solubilized fraction was separated from the membranes by centrifugation at 15000 g for 10 min, with the resulting supernatant then frozen in liquid nitrogen and stored at −80 °C, as AtCPR1 was found not to undergo significant loss of activity over time under these conditions.

### In vitro assays

To verify and quantify reductase activity, AtCPR1 assays were conducted using solubilized aliquots to reduce cytochrome c, as measured by optical absorbance at 550 nm in TE buffer in the presence of 50 $\mu$M NADPH, 5 $\mu$M FAD, 5 $\mu$M FMN, 1 mM DTT and a NADPH-regenerating system consisting of 1 mM glucose-6-phosphate and 1 mM NADP+ with yeast glucose-6-phosphate dehydrogenase. Negative controls consisted of solubilized untransformed E. coli spheroplasts as well as heat-denatured AtCPR1-solubilized spheroplasts. AtCPR1 reductase concentrations capable of reducing 40 $\mu$mol of cytochrome c in less than 1 min were found to be sufficient for optimal rAtKO activity in the presence of an NADPH-regeneration system. Assays of rAtKO consisted of harvested spheroplasts reconstituted with AtCPR1 and the NADPH-regeneration system. It was found that spheroplasts could be effectively diluted into TE buffer [1:10 (v/v)] and maintain activity. Catalysis was initiated by the addition of concentrated diterpene substrate, dissolved in 50:50 (v/v) methanol and DMSO to make stock solutions of 5 mg/ml. Product analysis was conducted by lowering the pH to ≤ 2, followed by organic extraction with an equal volume of ethyl acetate and two subsequent extractions each with an equal volume of hexane. Control extractions with authentic standards demonstrated no effect of pH on product stability. The organic extract was evaporated under a gentle stream of nitrogen and redissolved in diazomethane-saturated hexane to generate the methyl ester for GC-MS analysis. Diazomethane was generated by drop-wise addition of KOH to diazald and collected in a hexane trap [17]. The methylated diterpene products, exhibiting a characteristic molecular ion with $m/z$ of 316, were identified by comparison with authentic standards using GC-MS. To quantify the most effective AtKO expression construct and conditions for E. coli, 50 ml recombinant cultures were grown and spheroplasts prepared in parallel. Equal volumes (0.2 ml) of these preparations were then used in 2 ml assays with 50 $\mu$M kaurene substrate and a stock AtCPR1-regeneration solution, with product formation measured after 5 and 10 min. These assays were performed in duplicate, and the results are presented without normalization for spheroblast protein content (in order to measure overall functional expression level), with normalization (percentage) to the best performing construct and conditions.

### $^{18}$O2 labelling assays

For $^{18}$O2 labelling assays, all buffers, minus supplemented reagents, were degassed by boiling for 10 min while sparging with
nitrogen. Then, 5 ml serum vials with balch-type stoppers were evacuated, as determined by reduced weight (-13 mg), flushed with nitrogen, and re-evacuated. Degassed buffers were added to evacuated vials, which were then re-evacuated and pressurized by saturation with 18O2. Fresh microsomal preparations of rAtKO fully reconstituted with AtCPR1 were then added [1:10 (v/v)] to these flasks. ent-Beyerene, which is only converted into ent-beyeranol, served as an internal control to determine the maximum extent of labelled oxygen incorporation. Reactions were initiated with the addition of 20 \( \mu \)M substrate and 10 \( \mu \)M beyerene and allowed to continue for several hours, by which time the instability of rAtKO (E. coli spheroblast) conversion of ent-kaurene (\( \text{ent}\)-Beyerene, which is only converted into \( \text{ent}\)-beyeranol, served as an internal control to determine the maximum extent of labelled oxygen incorporation. Reactions were initiated with the addition of 20 \( \mu \)M substrate and 10 \( \mu \)M beyerene and allowed to continue for several hours, by which time the instability of rAtKO typically led to complete loss of activity. The vials were slowly heated to 65°C to fully terminate the reaction, whereupon the vials were opened and products extracted, methylated and analysed by GC-MS.

Kinetic analysis

Kinetic assays for all substrates were performed much as described previously [15]. Following spheroblast preparation from 2 litre expression cultures, the initial velocity was determined with the various substrate concentrations using 5 ml assays, with removal of 1 ml at time points (0.5, 1, 3, 5 and 10 min) to ensure linearity. Steady-state kinetic measurements were made using duplicate assays run in parallel for 1, 2 and 3, and using duplicate series of assays for 5, 7 and 9. Assays were terminated by the addition of 1 ml of 1 M HCl to the 1 ml time point aliquots removed from the kinetic assays, followed by immediate vortex mixing, with the resulting solution then extracted and methylated as above. Comparison with heat-denatured/uncoupled spheroblasts indicated no degradation of oxidized diterpenoid products or substrates. Products from the assays were analysed by GC-MS for definitive product identification, and then subjected to GC-FID analysis for more sensitive and accurate quantification. Quantification was based upon generation of a standard curve from an authentic standard of methyl kaurenoate extracted from negative control reactions. Protein concentrations were determined by 1% (v/v) trichloroacetic acid precipitation and the Bradford-based Bio-Rad protein assay.

RESULTS

Development of a fully functional recombinant AtKO

Reconstitution of P450 activity typically requires the appropriate CPR, two of which have been identified from Arabidopsis [18,19]. Thus both AtKO (CYP701A3) and one of the CPRs (AtCPR1) were cloned from an A. thaliana cDNA library. As previously reported [18], it was possible to recombinantly express AtCPR1 in E. coli, including retention of activity (i.e. NADPH-dependent reduction of cytochrome c) following solubilization with 0.5% Triton X-100. Also as previously reported, using the WAT11 strain of S. cerevisiae in which the endogenous CPR has been replaced by AtCPR1 [19], it was possible to detect the conversion of 1 into 4 upon expression of AtKO [6]. However, microsomal preparations from these recombinant yeast cells, even when supplemented with endogenous solubilized AtCPR1, were not able to carry out the full multiple reaction sequence.

More recently, it has been reported that the methylotropic yeast Pichia pastoris co-expressing the rice KO (CYP701A6) and a fungal CPR provides a source for fully active microsomal preparations [20]. However, our own parallel attempts to develop a more robust recombinant expression system for AtKO/CYP701A3 focused on E. coli. While expression and activity of full-length native AtKO was very limited, it has been reported that N-terminal modification significantly improves recombinant expression of eukaryotic P450s in bacteria [21]. Based on previously reported such modifications [21–23], we designed and tested several AtKO constructs (Figure 1). The most successful, based on modifications used for bacterial expression and crystallization of CYP2B sub-family members [23], involved replacement of the first 49 amino acids by a leader peptide, which offered significantly improved KO activity. Furthermore, as codon-optimization has been shown to enhance heterologous P450 expression [24], we had a completely recoded version of AtKO synthesized, with codon usage optimized for expression in E. coli, which also was then similarly modified and found to exhibit robust activity. Accordingly, this codon-optimized and modified rAtKO was used for the present study. Further optimization demonstrated that use of the C41 strain of E. coli grown in TB medium, with co-expression of the GroEL/GroES chaperones, resulted in sufficient KO activity for in vitro assays. In particular, assays carried out with spheroblast preparations from rAtKO-expressing E. coli and supplemented with endogenous solubilized AtCPR1 in the presence of an NADPH-regeneration system led to good conversion of 1 into 4, with minimal appearance of intermediates 2 or 3 (Figure 2), and it also was possible to efficiently convert either 2 or 3 into 4.

Analysis of the KO-catalysed multiple reaction sequence

To investigate the series of reactions catalysed by rAtKO, stable 18O2 isotope labelling studies examining oxygen incorporation were conducted with 1, 2 and 3 (Figure 3 and Supplementary
increases as the number of requisite reactions 

To briefly summarize the results, assays with 

Table S1 at http://www.BiochemJ.org/bj/431/bj4310337add.htm). Briefly, while the \( k_{\text{cat}} \) values for 1, 2 and 3 differed less than 4-fold, the calculated \( k_{\text{cat}}/K_m \) values varied up to 20-fold, increasing as the number of requisite reactions 

Table 1 rAtKO kinetic parameters for 1–3

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( K_m (\mu M) )</th>
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<tr>
<td>ent-Kaurene (1)</td>
<td>1.8 ± 0.6</td>
<td>( (8 ± 3) \times 10^{-3} )</td>
<td>4 ± 1</td>
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<td>ent-Kaureno (2)</td>
<td>6 ± 1</td>
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<td>ent-Kaurenol (3)</td>
<td>1.9 ± 0.2</td>
<td>( (170 ± 20) \times 10^{-3} )</td>
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Table 2 rAtKO kinetic parameters for 5 and 9

<table>
<thead>
<tr>
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<td>ent-Isokaurene (5)</td>
<td>10 ± 2</td>
<td>( (35 ± 7) \times 10^{-3} )</td>
<td>3.6 ± 0.8</td>
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<td>ent-Beyerene (9)</td>
<td>28 ± 7</td>
<td>( (15 ± 4) \times 10^{-3} )</td>
<td>0.5 ± 0.1</td>
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Figure 3 Incorporation of \(^{18}\text{O} \) into ent-kaurenoic acid (4) indicated by the change in \( m/z \) of the molecular ion in the MS for the derived MeKA generated by the rAtKO reaction with the indicated substrate in the presence of \(^{18}\text{O}_2\).

Scheme 2 rAtKO-catalysed reactions with alternative substrates

(a) Conversion of ent-isokaur-15-ene (5) into ent-isokaurenoic acid (6). (b) Conversion of ent-atiser-16-ene (7) into ent-atiserenoic acid (8). (c) Conversion of ent-beyer-15-ene (9) only into ent-beyer-15-ol (10). The number of arrows indicates the expected number of catalysed oxidation reactions in each case.

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decreased (i.e. \( k_{\text{cat}} / K_m \) for 3 > 2 > 1), such that the catalytic efficiency (\( k_{\text{cat}}/K_m \)) is more than 15-fold greater for 3 than for 1 or 2.

Analysis of the substrate structure–activity relationship for full KO activity

The critical role of gibberellins in promoting plant growth has led to intensive studies on their metabolism and signalling mechanism [1,26,27]. Intriguingly, a number of plant growth inhibitors

DISCUSSION
have been shown to target gibberellin biosynthesis [28], with ancymidol having been demonstrated to specifically inhibit the KO investigated in the present study [4], suggesting a potential control/regulatory role for KO in such phytohormone metabolism.

The multifunctional nature of KO and its role in hormone biosynthesis evoke some parallels with the multifunctional P450s involved in sterol/steroid hormone biosynthesis [29]. However, rather than the carbon–carbon bond cleavage reactions typically catalysed by these P450s, KO catalyses conversion of a methyl into a carboxylic acid. While there are identified plant P450s that catalyse similar terpenoid carboxylic-acid-forming reactions, the amorphadiene oxidase CYP71AV1 and the abietadien-ol/al oxide CYP720B1 [30–32], neither is thought to actually catalyse such a full reaction series in planta [30,33], nor has either been studied in great mechanistic detail.

Through development of an AtKO (CYP701A3) construct for fully functional recombinant expression in E. coli, involving whole-gene codon-optimization and N-terminal modification, we have been able to biochemically investigate the multifunctional enzymatic activity of KO. Because steady-state kinetic analysis demonstrated approximately equivalent $K_m$ pseudo-binding constants for the initial ent-kaurene (1) substrate and intervening ent-kaurenol (2), ent-kaurenal (3) intermediates (Table 1), yet ent-kauren-16-en-19-oic acid (4) can be produced without appreciable build-up of either intermediate (Supplementary Figure S5 at http://www.BiochemJ.org/bj/431/bj4310337add.htm), it seems that KO retains the stable intermediates in its active site between the catalysed sequential reactions. The oxygen labelling studies are further consistent with this hypothesis, as it is possible to observe retention of labelled oxygen through the second intermediate (3a/3), which would be expected to exchange oxygen atoms with water during the interconverting dehydration/hydration of 3a and 3 if this intermediate were released and exposed to bulk solvent. In addition, retention of such stable intermediates appears to be a fairly common feature in the enzymatic mechanism of other such multifunctional P450s [34,35].

Previous labelling results demonstrated the expected incorporation of oxygen from O$_2$ in the hydroxylation of ent-kaurene (1) to ent-kaurenol (2) [10]. Our oxygen labelling studies, focused on incorporation in the formation of ent-kaurenoic acid (4), clarifies the subsequently catalysed series of reactions. The incorporation of $^{18}$O into 4 when using ent-kaurenal (3) as the substrate indicates that the last reaction is either hydroxylation of 3 directly to 4, or of the gem-diol 3a to the ortho-acid hydrate 4a, which then undergoes dehydration to 4, although this later then further implies selective retention of the last oxygen to be incorporated. The implication of the 1:1 mixture of singly and doubly labelled 4 resulting from reaction with ent-kaurenol (2) is less obvious. Incorporation of two atoms of $^{18}$O indicates that the conversion of 2 into 4 involves two hydroxylation reactions, along with dehydration. Combined with the previously noted retention of the last oxygen that is incorporated, the loss of one of the incorporated oxygen atoms in half of the 4 formed from 2 suggests that dehydration occurs with the initially formed gem-diol 3a to produce 3, followed by hydroxylation to directly form 4. In particular, rather than ortho-acid hydrate (4a) formation with loss of either of the two original hydroxyl groups (but not that just added), it seems more likely that non-selective dehydration of the gem-diol 3a would lead to the observed approximately equimolar distribution of singly and doubly labelled 4. Accordingly, it seems that the KO-catalysed sequence of reactions consists of three hydroxylations, with an intervening dehydration between the second and third/last hydroxylation reactions (Scheme 3).

Given the active-site retention of the intermediate products noted above, the relative reaction rates ($k_{cat}$; Table 1) indicate that the initial hydroxylation of 1 into 2 is the rate-limiting step.

The series of reactions hypothesized here for KO is also consistent with the already known catalytic mechanisms of P450s. The formation of gem-diols by P450s appears rather common [34,36–40], whereas P450-catalysed dehydrogenation of an alcohol does not appear to have been reported (although there are examples of P450 dehydrogenation reactions, including those of oxygenated substrates, these tend to generate desaturated products, rather than aldehydes or carboxylates [29]). Beyond the catalysis of such consecutive hydroxylation reactions, intervening dehydration has been seen in the reaction series catalysed by other multifunctional P450s [35]. Indeed, previous work has demonstrated stereoselective removal of the C19 pro-$R$ hydrogen of 2 in formation of ent-kaurenal (3) [41], and it seems possible that the functional group necessary to interact with the hydroxy group of 2 to fix its position within the active site may further assist dehydration of the initially formed 3a. Furthermore, carboxylic acid formation has been examined with other P450s. In the case of CYP2C29, which is capable of converting the aldehyde in 11-oxo-$\Delta^2$-tetrahydrocannabinol into a carboxylic acid, experimental evidence indicates that it probably proceeds through direct hydroxylation [42], similar to the reaction hypothesized here for KO. By contrast, CYP52A3, which catalyses carboxylate formation with an alkane substrate, is suggested to catalyse formation of an ortho-acid hydrate, with some stereospecific in subsequent dehydration [37]. Nevertheless, the relatively slow exchange rate observed with 3 in the presence of H$_2$O (results not shown), in combination with the ability of rAtKO to readily convert 3 into 4, argues against specific catalysis of the alternative 1 $\rightarrow$ 2 $\rightarrow$ 3a $\rightarrow$ 4a $\rightarrow$ 4 series of reactions by KO.

The robust activity exhibited by rAtKO further enabled investigation of the substrate structure–activity relationship underlying the enzymatic specificity for catalysis of the full multiple reaction sequence. Of particular interest is the difference in rAtKO reactivity with ent-atiser-16-ene (7), which is converted into the corresponding ent-atiser-16-en-19-oic acid (8), relative to ent-beyer-15-ene (9), which is only hydroxylated to

![Scheme 3 Proposed mechanism for KO conversion of ent-kaurene (1) into ent-kaurenoic acid (4), consisting of hydroxylation of 1 into ent-kaurenol (2), further hydroxylation of 2 to the gem-diol 3a, which undergoes dehydration to ent-kaurenal (3), followed by hydroxylation to 4 (insertion of oxygen in hydroxylation reactions indicated by $^+$O)](image)
Characterization of kaurene oxidase

ent-beyer-15-en-19-ol (10). Comparison of their chemical structures with that of 1 demonstrates that this pattern of reactivity arises from the effect of their different distal (C/D) ring structure on the corresponding molecular ‘envelope’ (Figure 4). In particular, although there is a difference in C/D ring regiochemistry between 1 and 7, they actually have a very similar overall configuration. By contrast, the differing regiochemistry of the C/D ring in 9 results in a clear change in the position of its C-12 relative to either 1 or 7, which is apparently sufficient to prevent additional reactivity beyond initial hydroxylation.

Note that, although the initial hydroxylation of 1 to 2 in the native reaction sequence appears to be rate-limiting given the active-site retention of the stable intermediates, the 3-fold increased Kᵦ pseudo-binding constant for 2 relative to either 1 or 3 indicates that the initially formed hydroxy group is not well accommodated in the KO active site. In combination with the relatively poor binding of 9, which already appears to bind 10-fold less well than 1 (Kᵦ of 28 compared with 2 μM respectively; Tables 1 and 2), this effect presumably precludes binding of 10, at least with catalytically relevant positioning, providing a consistent rationale for the observed selectivity. In addition, such poor accommodation of hydroxy groups is consistent with necessary removal of one such group from the gem-diol product of the second hydroxylation reaction prior to catalysis of the final hydroxylation reaction.

In conclusion, the present study provides insight into the interesting and somewhat unusual sequence of reactions catalysed by KO in gibberellin phytohormone biosynthesis. Specifically, that KO catalyses three hydroxylation reactions in the conversion of ent-kaurene (1) into ent-kaurenoic acid (4) through formation and subsequent dehydration of a gem-diol intermediate (3a) inbetween the second and third/last hydroxylation reactions, such that the catalysed series of reactions is 1→2→3a→3→4. In this native reaction sequence, KO seems to retain the intermediate products in its active site, with the first hydroxylation reaction then representing the rate-limiting step. Furthermore, the substrate structure–activity relationship study with rAtKO highlights a critical role for the specific tetracyclic ring structure of ent-kaurene in enabling catalysis of the full multiple reaction sequence, as this can be derailed at the apparently relatively poorly bound initially formed hydroxy intermediate stage by even relatively subtle changes in structure distal to the targeted carbon.

Figure 4 Comparison of the configurations of (a) ent-kaurene (1), with the alternative substrates (b) ent-atisene (7) and (c) ent-beyerene (9), including (d) a three-dimensional overlay of 1 and 9 demonstrating the difference in C-12 position between 1 and 9, as indicated (1, 12 compared with 9, 12).

REFERENCES

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SUPPLEMENTARY ONLINE DATA

Characterization of the kaurene oxidase CYP701A3, a multifunctional cytochrome P450 from gibberellin biosynthesis

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Figure S1  Optimized gene sequence for AtKO/CYP701A3

Figure S2  SDS/PAGE analysis of preparations from recombinant E. coli expressing rAtKO

The molecular mass in kDa is indicated on the left-hand side.

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Figure S3  Steady-state kinetic plots for rAtKO reaction with ent-kaurene (1), ent-kaurenol (2) or ent-kaurenal (3) as substrate

Initial velocity, determined from the linear portion of the reaction progress curve, is plotted against relevant substrate concentration and fit to the Michaelis–Menten equation to determine the kinetic parameters reported in Table 2 of the main text.

Figure S4  Steady-state kinetic plots for rAtKO reaction with ent-isokaurene (5) or ent-beyerene (9) as substrate

Initial velocity, determined from the linear portion of the reaction progress curve, is plotted against relevant substrate concentration and fit to the Michaelis–Menten equation to determine the kinetic parameters reported in Table 3 of the main text.

Figure S5  Partial conversion of 1 into 4, demonstrating no accumulation of 2 or 3
**Table S1**  
Percentage incorporation of labelled oxygen, $^{18}$O, into methyl kaurenoate ($m/z$ 316) from indicated substrates with rAtKO

ent-Beyerene (9), which is only converted into the alcohol entbeyerenol (10) in a single hydroxylation reaction, was used as an internal control with intermediates 2 and 3 to determine the percentage incorporation of labelled oxygen under assay conditions.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$m/z$ 316</th>
<th>$m/z$ 318</th>
<th>$m/z$ 320</th>
<th>Beyerenol internal control $m/z$ 290</th>
</tr>
</thead>
<tbody>
<tr>
<td>ent-Kaurene (1)</td>
<td>2%</td>
<td>3%</td>
<td>95%</td>
<td>–</td>
</tr>
<tr>
<td>ent-Kaurenol (2)</td>
<td>6%</td>
<td>46%</td>
<td>48%</td>
<td>92%</td>
</tr>
<tr>
<td>ent-Kaurenal (3)</td>
<td>7%</td>
<td>87%</td>
<td>6%</td>
<td>93%</td>
</tr>
</tbody>
</table>

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