Plant Biosynthetic Pathways | Hot Paper

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Minimum Set of Cytochromes P450 for Reconstituting the Biosynthesis of Camalexin, a Major *Arabidopsis* Antibiotic**

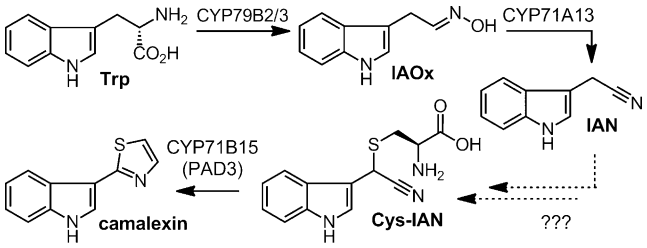
*Andrew P. Klein, Gülbenk Anarat-Cappillino, and Elizabeth S. Sattely**

Camalexin is an archetype of the nitrogen- and sulfur-containing phytoalexins from the crucifer family, which includes important crop plants.[1] These antimicrobial natural products are known to protect plants against fungal infection and confer health-promoting properties in humans (e.g. $EC_{50} = 34$–183 μM against fungal pathogen *Alternaria brassicicola*;[2] and $IC_{50} = 2.7$ μM against human SKBr3 breast cancer cell line[3]). The diversity of the heteroatom-rich phytoalexin scaffolds has prompted us to study the biosynthetic enzymes responsible for unusual reactions, such as carbon–sulfur (C–S) bond formation in the camalexin pathway. Despite the importance of camalexin as the primary phytoalexin in the model plant *Arabidopsis thaliana*, the enzyme that catalyzes a key step (coupling Trp- and Cys-derived fragments) has remained elusive. Here we elucidate the missing oxidative step in the camalexin biosynthetic pathway. We show that three cytochrome P450 enzymes (P450s) are necessary and sufficient to reconstitute the biosynthesis of camalexin in vitro, and uncover a novel mechanism for C–S bond formation.

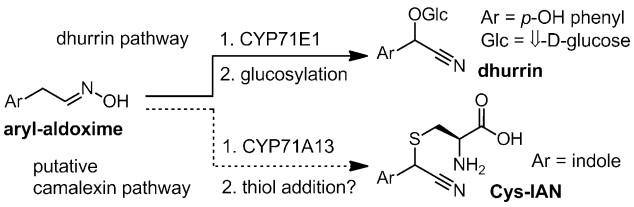
Prior efforts have demonstrated that three P450s function are involved in the camalexin pathway[1] (Scheme 1). This partial pathway exemplifies a common motif in plant secondary metabolism: unusual oxidations performed by noncanonical P450s to generate modified amino acid scaffolds.[4] As an example, CYP71B15 catalyzes an oxidative decarboxylation and heterocyclization to form the thiazole ring of camalexin.[5] Similarly, we hypothesized that oxidative chemistry could promote coupling of IAN to a Cys derivative (e.g. glutathione).

Halkier, Glazebrook, and co-workers[6] previously showed that CYP71A13, heterologously expressed in *Escherichia coli* or *Nicotiana benthamiana*, catalyzes the non-oxidative dehydration of indole-3-acetaldoxime (IAOx) to indole-3-acetonitrile (IAN) in vitro (Scheme 1).[7] We began our search for an enzyme that converts IAN to the Cys–IAN conjugate (Cys–IAN) with the knowledge that camalexin biosynthetic genes are highly co-expressed in *Arabidopsis* following treatment with pathogens (Figure S1, Supporting Information). An analysis of publicly available gene expression data showed that several P450 enzymes and flavoenzymes co-express with known camalexin pathway genes; however, camalexin was produced at normal levels in *Arabidopsis* homozygous mutants deficient in each candidate gene (unpublished study, E. S. Sattely, N. K. Clay, Y. A. Millet, F. M. Ausubel).

We next turned to the chemical analysis of related pathways for clues to camalexin biosynthesis and noted that the P450 CYP71E1 from the dhurrin pathway in *Sorghum bicolor* catalyzes both the dehydration and benzylic hydroxylation of an aldoxime.[8] The activity of CYP71E1 suggested that an analogous benzylic oxidation could provide a mechanism for the C–S bond formation in camalexin biosynthesis (Scheme 2).



Scheme 1. Camalexin biosynthesis in *Arabidopsis*.



Scheme 2. Comparison of the cyanogenic glycoside dhurrin pathway in *Sorghum bicolor* and the camalexin pathway.

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Since there are no enzymes in *Arabidopsis* closely related to CYP71E1, we hypothesized that CYP71A13 might catalyze the oxidative coupling of Cys and a Trp intermediate, in addition to oxime dehydration. To explore this possibility, we used a *Saccharomyces cerevisiae* WAT11 heterologous expression system to produce recombinant CYP71A13 together with the *Arabidopsis* P450 reductase ATR1; these membrane-associated enzymes were isolated in the microsomal fraction. We examined the in vitro activity of recombinant CYP71A13 with IAOx using HPLC coupled to high-

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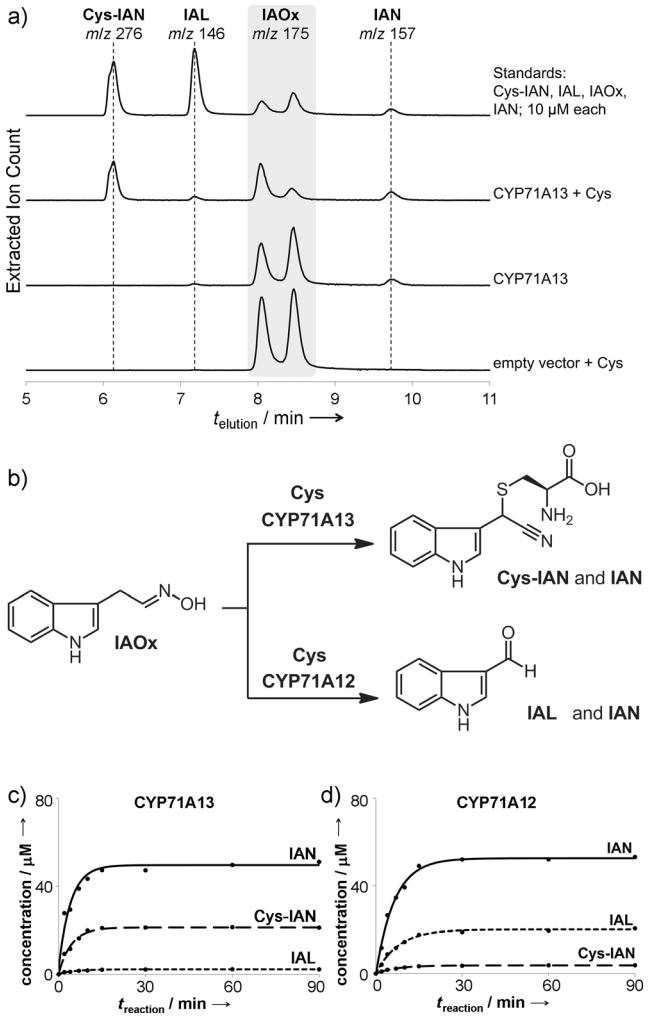

Figure 1. a) LC-MS extracted ion chromatograms of endpoint assays of CYP71A13 with IAOx (resolved as *E*- and *Z*-oxime isomers). Note that the concentration of each standard is 10 μM, therefore, the peak heights of each standard reflects differences in compound ionization by ESI. b) Scheme depicting the in vitro catalytic activity of CYP71A13 and CYP71A12 with IAOx in the presence of Cys. c, d) Time course of the product formation from the conversion of IAOx by CYP71A13 (C) or CYP71A12 (D). Concentrations of products are calculated by integration of HPLC peaks (absorbance at 280 nm).

resolution mass spectrometry (LC-MS). Similar to previous studies,[6] our preliminary assays indicated that IAN is the major product of CYP71A13 catalysis (Figure 1 A). However, we also observed a minor product ($\approx$3% of the total) that appears concurrently with IAN, identified as indole-3-carboxyaldehyde (IAL). Although present in low abundance, we suspected that IAL is the result of CYP71A13-catalyzed oxidation of IAN followed by loss of hydrogen cyanide. Notably, the formation of both IAN and IAL is strictly dependent on the presence of both CYP71A13 and the cofactor NADPH. The proposed α-hydroxy-IAN intermediate is analogous to the labile cyanohydrins part of cyanogenic glycoside biosynthesis in many higher plants.

The observed oxidative activity of CYP71A13 led us to test whether the addition of a thiol donor to the enzymatic reaction would result in the formation of products on pathway to camalexin. When Cys was included in the assay, a new compound appeared, which we assigned as Cys–IAN based on co-elution with a synthetic standard and ion mass ($[M+H]^+$ *m/z* 276.0801, calcd 276.0801, Figure 1 A,B). Using glutathione as an alternative thiol donor resulted in a product with MS and MS/MS properties that matched those of a glutathione–IAN conjugate ($[M+H]^+$ *m/z* 462.1438, calcd 462.1442, Figures S5 and S6).

These data establish that CYP71A13 catalyzes the conversion of IAOx to an electrophile capable of accepting the thiol group of L-cysteine or glutathione, and provides a mechanism for the missing key step in camalexin biosynthesis. Steady-state kinetic constants for CYP71A13 were measured by monitoring the disappearance of starting material by HPLC (Figure S9); the apparent K_m of IAOx is 21.4 ± 2.7 μM, with a V_{max} of 723 ± 38 pmol min^{-1} mg^{-1}.

The *Arabidopsis* P450 CYP71A12 shares 89.5% amino acid identity with CYP71A13, and has also been linked to camalexin biosynthesis.[9] To determine if these two P450s are functionally redundant in vitro, we characterized the activity of CYP71A12 expressed in yeast. Although CYP71A12 turned over IAOx at a rate comparable to that of CYP71A13, the final products IAL and Cys–IAN accumulated in different ratios with these two enzymes (Figure 1 C,D, Figure S3). Conversion to oxidized products at the assay endpoint is illustrative: CYP71A13 produced Cys–IAN (20%) and IAL (2%), while CYP71A12 produced Cys–IAN (3%) and IAL (18%). The difference in the distribution of oxidized products suggests that CYP71A12 is less effective at promoting the formation of the Cys–IAN adduct en route to camalexin than CYP71A13, despite high sequence identity.

The persistent production of the dehydration product IAN alongside oxidized products from IAOx led us to examine whether IAN is an intermediate en route to camalexin or an off-pathway product. When IAN was used as a substrate by either CYP71A13 and CYP71A12 (along with Cys as thiol donor), detectable levels of Cys–IAN and IAL were observed (Figure S4), although the rate of formation of these oxidized products was 10^3 times slower than when IAOx was used as substrate (Table S3). No products were observed when IAN was incubated with empty-vector microsomes and Cys or glutathione as thiol donor. The slow rate of the reaction made it impractical to measure steady-state kinetic constants of CYP71A13 or CYP71A12 with IAN, but the K_m values are estimated to be higher than 0.1 mM. These data suggest that IAOx is likely the in vivo substrate of both P450s.

Relatively few types of mechanisms for C–S bond formation in natural product biosynthesis are known. Those characterized include enzymes involved in ovothiol (Fe^{II}-dependent oxidase),[10] gliotoxin (proposed P450 and dedicated GST),[11] and glucosinolate (P450 and proposed GST)[12] biosynthetic pathways. To elucidate the mechanism of C–S bond formation in the camalexin pathway, we asked two key questions: 1) is canonical P450-catalyzed oxygen incorporation involved in the oxidation of IAOx, and 2) does CYP71A13 directly catalyze C–S bond formation in camalexin biosynthesis?

To address (1) and formulate a mechanism for Cys–IAN production, we looked for evidence of the putative cyanohy-

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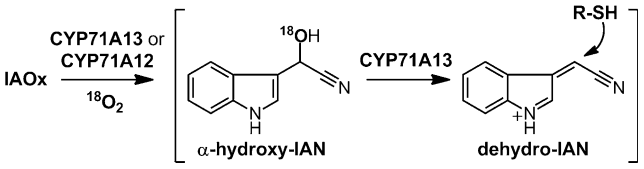

Scheme 3. Putative enzymatic intermediates.

drin intermediate (α-hydroxy-IAN in Scheme 3) that would result from classical P450-catalyzed monooxygenation of IAOx by taking advantage of the fact that IAL is formed along with the on-pathway product Cys–IAN through CYP71A13 or CYP71A12 catalysis. We reasoned that if IAL is a result of direct collapse of α-hydroxy-IAN (rather than water addition to an intermediate such as dehydro-IAN, Scheme 3), then we should observe ^{18}O incorporation in IAL if the assay is performed under an atmosphere of $^{18}O_2$. With 97% $^{18}O_2$, both CYP71A13 and CYP71A12 catalyze incorporation of the isotopic label into IAL (74% and 81% ^{18}O incorporation, respectively, Figure S10), providing clear evidence for an α-hydroxy-IAN intermediate. This result establishes that CYP71A13 and CYP71A12 possess a function and regiospecificity similar to P450s involved in cyanogenic glycoside biosynthesis, and suggests that a novel catalytic activity was acquired in these *Arabidopsis* P450s for the biosynthesis of camalexin. To further probe the mechanism of C–S bond formation and address question (2) regarding the enzymatic catalysis of the coupling, we varied the identity and concentration of the thiol donor at biologically relevant concentrations ($\approx$1 mM)[13] in our in vitro assays. However, the rate of IAOx consumption with CYP71 A13 was not significantly different with the following thiol donors: 0.01–1 mM L-Cys or glutathione, and 1 mM D-Cys (Table S3).

Taken together, the above data indicate that these P450s do not directly catalyze C–S bond formation, and that α-hydroxy-IAN is a precursor to Cys–IAN. We propose that Cys–IAN is not directly formed from α-hydroxy-IAN released from the enzyme. Instead, we suggest that the enzyme dehydrates α-hydroxy-IAN to a reactive electrophile (e.g. dehydro-IAN, Scheme 3) that can be captured by a free thiol. The difference in product distribution (Cys–IAN vs. IAL) observed with CYP71A13 versus CYP71A12 suggests that CYP71A13 more efficiently generates this reactive intermediate. Dehydro-IAN is an iminium species akin to intermediates proposed in the glucosinolate[12] and gliotoxin[11] pathways; here we suggest that sulfur is incorporated at the carbon atom in γ position to the nitrogen atom rather than at the α position.

Since CYP71A13 converts IAOx to Cys–IAN, we reasoned that the addition of CYP79B2 and CYP71B15 (Scheme 1) to our assay should be sufficient to reconstitute camalexin biosynthesis in vitro. These two *Arabidopsis* P450s were individually expressed in yeast and isolated as for CYP71A13. Incubation of Trp, Cys, and NADPH with the three P450s and reductase partner produced a compound with an ion mass ($[M+H]^+$ *m/z* 201.0483, calcd 201.0481), UV absorption spectrum, and elution time matching a synthetic camalexin standard (Figure 2). Substituting CYP71A12 in

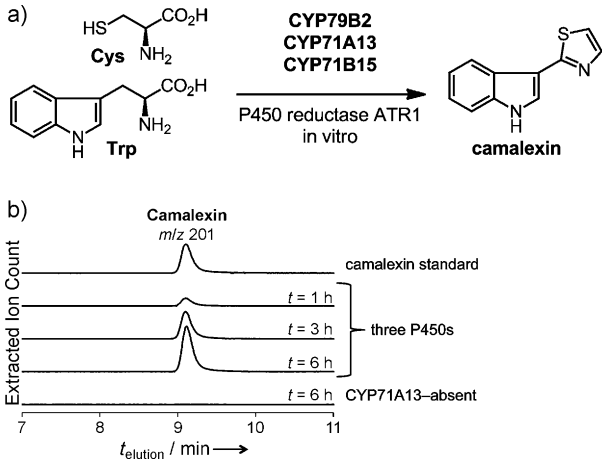

Figure 2. a) In vitro reconstitution of camalexin biosynthesis requires only three cytochromes P450 and the native P450 reductase. b) LC-MS extracted ion chromatograms of in vitro camalexin biosynthesis reconstitution assays.

place of CYP71A13 also resulted in camalexin production, albeit at lower levels (Figure S11). This is the first report of the reconstitution of camalexin biosynthesis in vitro, and demonstrates the set of three P450s that is necessary and sufficient to synthesize this key *Arabidopsis* phytoalexin. This study supports the recent report by Halkier and co-workers[14] that expression of these three P450s results in the production of camalexin in tobacco, and addresses the outstanding question of whether additional enzymes are needed to complete the biosynthetic pathway.

We cannot rule out the possibility that other proteins associate with CYP71A13 in plants to direct the addition of a thiol donor (e.g. glutathione) to dehydro-IAN, such as the glutathione-*S*-transferase GSTF6 implicated in camalexin biosynthesis.[15] Such an association may be required to protect reactive biosynthetic intermediates. This problem is mitigated in the dhurrin pathway by a stable association between CYP71E1 and the glycosyl transferase UGT85B1, which channels the labile cyanohydrin toward the stable cyanogenic glycoside (Scheme 2).[16] Although several cyanogenic glucosides are known, one derived from Trp has not been observed in nature. It is tempting to speculate that the camalexin and cyanogenic glucoside pathways are evolutionarily related, although CYP71E1 and CYP71A13 are only 33.1% identical on the amino acid level. We have tried to cross the two pathways in vitro; however, attempts to trap α-hydroxy-IAN using recombinant UGT85B1 purified from *E. coli* and UDP–glucose were unsuccessful (data not shown).

In conclusion, this study demonstrates the minimal set of enzymes required for in vitro biosynthesis of camalexin, a major plant antibiotic. We also have elucidated a mechanism for C–S bond formation mediated by a multifunctional P450, setting the stage for heterocyclization. This series of events represents a new pathway for thiazole biosynthesis that is orthogonal to the well-studied nonribosomal peptide synthases and cyclases in microbes. We propose that CYP71A13 differs from classical P450 monooxygenases by catalyzing two additional dehydration events to generate the reactive

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dehydro-IAN from IAOx. Subsequent coupling of this electrophile with glutathione could be facilitated in vivo by a dedicated or broadly acting GST followed by γ-glutamyl peptidase GGP1 or GGP3 and a yet-unidentified carboxypeptidase to yield Cys–IAN.[17] The sequential action of a P450 and GST is reminiscent of a conserved detoxification mechanism utilized here for natural product biosynthesis. P450s have acquired a stunning diversity of catalytic capacities through evolution, and protein-engineering efforts have shown how this plasticity can have industrial importance in biocatalysts. CYP71A13, as CYP79B2 and CYP71B15 in the camalexin pathway, highlights the often multifunctional activities of P450s that are especially prevalent in plant biosynthetic pathways.

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