HHS Public Access

Author manuscript

Angew Chem Int Ed Engl. Author manuscript; available in PMC 2019 May 22.

Published in final edited form as:

Angew Chem Int Ed Engl. 2018 May 22; 57(21): 6082–6085. doi:10.1002/anie.201713403.

Traveling two diverging roads, cytochrome-P450 catalyzed demethylation and γ-lactone formation in bacterial gibberellin biosynthesis

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Abstract

Biosynthesis of the gibberellin plant hormones evolved independently in plants and microbes, but the pathways proceed via similar transformations. The combined demethylation and γ-lactone ring forming transformation is of significant mechanistic interest, yet remains opaque. The relevant CYP112 from bacteria was probed via activity assays and $^{18}O_2$ labeling experiments. Notably, the ability of *tert*-butyl hydroperoxide to drive this transformation indicates use of the ferryl-oxo (Compound I) from the CYP catalytic cycle for this reaction. Together with the confirmed loss of C-20 as CO_2, this necessitates two catalytic cycles for carbon-carbon bond scission and γ-lactone formation. The ability of CYP112 to hydroxylate the δ-lactone form of GA_{15} shown by the labeling studies is consistent with the implied use of a further oxygenated heterocycle in the final conversion of GA_{24} to GA_9, with the partial labeling of GA_9 demonstrating that CYP112 partitions its reactants between two diverging mechanisms.

Graphical Abstract

Gibberellins are important phytohormones, requiring complex biosynthetic processes that independently evolved in bacteria and fungi as well as plants. The requisite coupled demethylation and γ-lactone ring formation, catalyzed by the bacterial cytochrome P450 CYP112, was mechanistically probed, providing insight into this complex transformation.

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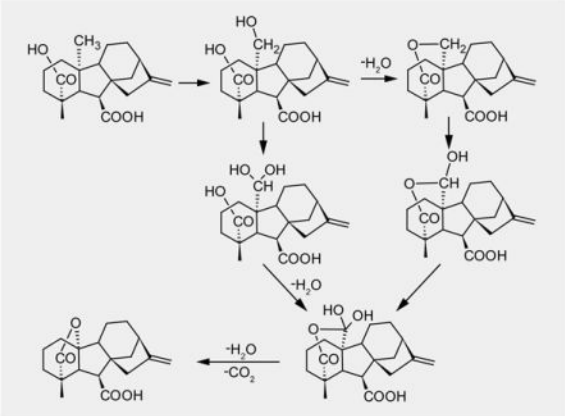

Keywords

enzyme mechanism; oxygenase; lactol; decarboxylation; anhydride

Gibberellins (GAs) are essential hormones in plants, which has led some plant-associated fungi and bacteria to produce GA to manipulate their host plants. The biosynthetic steps of the individual pathways are essentially identical in plants and bacteria, and differ from the fungal pathway only in the order of occurrence of one peripheral (C-3β) hydroxylation step. Yet on the enzyme level it is evident from the low sequence identity that GA biosynthesis evolved separately in plants, fungi and bacteria.[1] One hallmark transformation in GA biosynthesis is the conversion of 20-carbon GAs to the 19-carbon GAs, involving the combined loss of a methyl group and formation of an intramolecular γ-lactone bridge. In plants this complex multi-step reaction is catalyzed by iron/α-ketoglutarate-dependent dioxygenases termed GA 20-oxidase (GA20ox), while it is catalyzed by cytochrome P450 (CYP) monooxygenases in fungi (CYP68B) and bacteria (CYP112).[1a] Although fungi and bacteria both use CYPs, these fall into different families that share less than 15% sequence identity. Nonetheless the reactions catalyzed by the three individual enzymes appear to be identical, and involve the stepwise oxidation of C-20 from a methyl group in GA_{12} in plants and bacteria, and GA_{12} as well as the 3β-hydroxy derivative GA_{14} in fungi, to the corresponding alcohols GA_{15} or GA_{37} and further oxidation to the aldehyde function, yielding GA_{24} or GA_{36}, respectively. The final step is combined loss of the oxidized methyl group and formation of a γ-lactone bridge between carbon C-19 and C-10, producing GA_9 or GA_4, respectively (Scheme 1). While the fungal enzyme does not react with the intermediates GA_{15}/GA_{37} and GA_{24}/GA_{36}, the bacterial and plant enzymes can use GA_{15} and GA_{24} as substrates to produce GA_9.[1a, 1b] Plants and fungi also produce the C-20 carboxylic acids GA_{25} or GA_{13} as side products, since neither the plant GA20ox nor the fungal CYP68B further convert GA_{25}/GA_{13} to GA_9/GA_4 (Scheme 1).[2] For the fungal CYP68B very little is known about the catalytic mechanism, only that C-20 appears to be lost as CO_2.[3] With one plant GA20ox catalyzing this multi-step reaction some artificial substrates have been tested, and it has been demonstrated that C-20 also is lost as CO_2.[4] The combination of the apparently straightforward first two reactions with the complex last

reaction, and the known differences between plants and fungi, makes the catalytic mechanism of CYP112 of significant interest, which was investigated here.

Previously, isoforms of CYP112 were characterized by addition of GA_{12} to recombinant *Escherichia coli* cultures.[1b, 1d] This however did not permit detailed investigation of the catalytic mechanism. Here the isoform from *Erwinia tracheiphila* (*Et*CYP112), chosen as it is in the same Enterobacteriaceae family as *E. coli*, was His-tagged, purified and found to exhibit full activity in a reconstituted system with purified ferredoxin reductase from *E. tracheiphila* (*Et*FdR) and spinach ferredoxin (Fd), converting GA_{12} to GA_9 (Figure 1). Under optimal conditions (i.e., with a molar excess of NADPH), no intermediates were detected, even when turnover was not yet complete, indicating their retention in the active site. It was possible to observe the expected C-20 hydroxylated intermediate GA_{15} and aldehyde intermediate GA_{24} under NADPH-limited conditions. These assays also produced the C-20 carboxylate derivative GA_{25} (Figures 1 and S1). However, while GA_{15} and GA_{24} were readily converted to GA_9, GA_{25} is not (Figure S2).

The form in which C-20 is released by *Et*CYP112 was examined by $^{18}O_2$ labeling, using the reconstituted enzymatic system with an excess of NADPH under an $^{18}O_2$ atmosphere, with GC-MS analysis of the head-space. In the absence of substrate only unlabeled CO_2 was found, while addition of GA_{12} resulted in production of CO_2 with one or two ^{18}O labels (Figure 2). The observed loss of label presumably originates from the interconversion of CO_2 with bicarbonate during the incubation period of the enzyme assay.[5] Thus, *Et*CYP112 clearly releases C-20 as CO_2. In addition, the resulting GA_9 product was extracted, methylated and analyzed by GC-MS. Surprisingly, 44% had incorporated one ^{18}O (Figures 3 and S2, and Table S1). This was found not only in the molecular ion, but also the fragments at m/z = 298/299 and 270, which retain the γ-lactone ring,[6] wherein the ^{18}O label must have been inserted.

^{18}O incorporation during the transformation of GA_{12} to GA_9 was further probed with limiting NADPH, enabling examination of the intermediates GA_{15} and GA_{24}. While this reduced the amount of ^{18}O incorporated into GA_9 (to 14%), a substantial proportion of the observed GA_{24} accumulated two ^{18}O (37%). The remainder of the GA_{24} contained a single ^{18}O. GA_{15} is observed in the closed δ-lactone form, and was essentially fully labeled with a single ^{18}O. This demonstrated that GA_{12} is converted to GA_{15} via hydroxylation rather than direct formation of the δ-lactone ring. Analogous labeling experiments were carried out with GA_{15} (open lactone), GA_{15} (closed δ-lactone) or GA_{24} as substrate (Figure 3 and Table S1). GA_9 produced from these intermediates did not contain ^{18}O. However, GA_{24} produced from the closed (δ-lactone) form of GA_{15} was fully labeled with ^{18}O. Thus, *Et*CYP112 readily hydroxylates the δ-lactone form of GA_{15} to the lactol form of GA_{24}. Given that GA_{24} is observed in its aldehyde form,[7] the incorporated ^{18}O is retained by direct opening of the lactol. Starting from the open form of GA_{15}, 67% of GA_{24} was labeled with ^{18}O, indicating that *Et*CYP112 produces a *geminal*-diol, which upon dehydration loses ~50% of the label and forms the observed GA_{24} aldehyde. The greater amount of labeled species observed here originates from both a slight bias towards removal of the unlabeled hydroxyl-group,[8] but presumably largely from partial closure of the open GA_{15} to its δ-lactone form in the assay buffer before conversion by *Et*CYP112.

To further investigate formation of the lactol form of GA_{24}, the C-7 and C-19 carboxylate groups were methylated in GA_{12}, GA_{15}, and GA_{24}. This was expected to block lactone formation in GA_{15} and GA_9. However, C-19 methylation seems to be unstable in GA_{15}, and is quickly lost by conversion to the δ-lactone upon purification in organic solvents. The instability of the C-19 methyl ester in GA_{15} is also reflected in assays with the enzymatic system. In particular, $MeGA_{12}$ was efficiently converted into GA_9 and, under an $^{18}O_2$ atmosphere, all GA_9 product contains one ^{18}O label. $MeGA_{15}$ (closed δ-lactone) also is transformed into GA_9, but does not incorporate any ^{18}O label, while $MeGA_{24}$ is not acted upon by *Et*CYP112 (Figures 4 and S3, and Table S1).

It has been suggested that GA_{24} can be converted into GA_9 in a single catalytic cycle via the atypical use of a ferric-peroxo activated group,[2a] and similar carbon-carbon bond cleavage reactions catalyzed by CYP1A2 and CYP17A1 have been suggested to utilize superoxo or peroxo states of the heme-iron.[9] In CYPs these early stages of the catalytic cycle can be bypassed by using H_2O_2 or *tert*-butyl hydroperoxide (*t*BuOOH) as the reducing agent instead of molecular oxygen and electrons from NADPH (Scheme S2).[10] However, while the overall activity of *Et*CYP112 with H_2O_2 is significantly lower, limiting conversion of GA_{12} to GA_{15} and GA_{24}, it is able drive the conversion of GA_{15} to GA_{24} and GA_9, as well as GA_{24} to GA_9. *t*BuOOH also can drive conversion of GA_{24} to GA_9 (Figure S4).

The incorporation of ^{18}O into a significant proportion of the GA_9 γ-lactone ring indicates that one of the oxygens incorporated by *Et*CYP112 is transferred to the C-19 carboxylate. This must stem from the initial hydroxylation of GA_{12} to the open form of GA_{15} that then undergoes dehydration to the δ-lactone ring form.[7b] Indeed, the essentially complete labeling of the GA_{15} produced from GA_{12} demonstrates that this reaction invariably involves hydroxylation. The ability of *Et*CYP112 to catalyze hydroxylation of the δ-lactone form of GA_{15} to the lactol form of GA_{24} is demonstrated by both the complete ^{18}O labeling of the GA_{24} produced from the closed (lactone) form of GA_{15}, and the significant double-labeling of the GA_{24} produced from GA_{12}. *Et*CYP112 can also hydroxylate the open form of GA_{15} to the *geminal*-diol form of GA_{24}, as demonstrated by the partial labeling of the resulting GA_{24}.

The most interesting transformation is however the conversion of GA_{24} into GA_9, requiring both carbon-carbon bond scission and γ-lactone ring formation. While MacMillan has proposed several reaction mechanisms,[2a] these were focused on the plant dioxygenases. Nevertheless, CYP monooxygenases also use an iron co-factor to bind molecular oxygen, and both of these oxygenases generally utilize activated ferryl-oxo complexes (Compound I) for catalysis.[10–11] Thus, the proposed mechanisms are adaptable to CYPs (Schemes S3 and S4). In addition, CYPs are known to catalyze reactions via ferryl-hydroxo (Compound II), ferric-superoxo, ferric-peroxo,[9] or ferric-hydroperoxo species (Compound 0) as well (Scheme S2).[12] However, the ability of *Et*CYP112 to use H_2O_2 or *t*BuOOH to drive this transformation precludes the use of either the superoxo or peroxo complexes, or Compound 0, respectively. Thus, *Et*CYP112 uses Compound I in the transformation of GA_{24} to GA_9, which is consistent with the implied use of Compound I for the hydroxylation reactions shown here for the two preceding transformations, as well as the mechanism proposed for the similar reaction catalyzed by CYP19A1.[13] However, release of C-20 as CO_2 rules out

transformation of GA_{24} to GA_9 via a single oxidation cycle, even with consideration of alternative reactivity with Compound II, despite the inability of the C-20 carboxylate GA_{25} to serve as a substrate. It has been suggested that the plant GA20ox also uses two oxidation cycles, but proceeds via a covalent intermediate.[4b] However, there is no evidence for such a mechanism with *Et*CYP112. On the other hand, the intermediacy of a C-20 *geminal*-diol cyclic anhydride equivalent may explain why the open C-20 carboxylate GA_{25} is not a substrate. Consistent with a requirement for a cyclic anhydride intermediate is the inability of *Et*CYP112 to use $MeGA_{24}$ as a substrate, as it cannot form such a heterocycle.

The results presented here indicate that, similar to CYP170A1 in albaflavenone biosynthesis,[14] *Et*CYP112 utilizes two diverging routes in transformation of GA_{12} to GA_9. This is demonstrated by the partial labeling of the GA_9 produced from GA_{12}, and contrast with the complete labeling observed with $MeGA_{12}$, which is biased towards formation of the closed (δ-lactone) form of GA_{15}. Accordingly, rather than choose, *Et*CYP112 partitions the travel of its reactants between two mechanistic roads that diverge at GA_{15} (Scheme 2). Initial hydroxylation to the open form of GA_{15} can be followed by dehydration to the δ-lactone form, with subsequent hydroxylation of either producing the *gem*-diol or lactol forms of GA_{24}, respectively (Scheme S4). These distinct intermediates can then undergo separate conversion to GA_9 via two sequential oxidation reactions. This presumably involves formation of the C-20 *gem*-diol cyclic anhydride equivalent in both cases, reflecting either direct hydroxylation of δ-lactol, or cyclization of *gem*-diol GA_{24} mediated by the ability of Compound II to abstract an additional hydrogen. The resulting anhydride *gem*-diol then undergoes coupled carbon-carbon bond scission and γ-lactone ring formation, with release of CO_2, again relying on abstraction of another hydrogen by Compound II. Altogether, despite their independent evolution, plants, fungi and bacteria catalyze the same series of reactions in the coupled demethylation and γ-lactone ring formation transformation step of gibberellin biosynthesis.[3–4] The C-20 carboxylate equivalent GA_{25} does not serve as a productive intermediate for any of the relevant oxygenases, and the C-20 *gem*-diol cyclic anhydride equivalent indicated here for the bacterial CYP112 has also been suggested for plants.[2a] Thus, despite their independent evolutionary origins, these oxygenases seem to have converged on similar mechanistic routes, perhaps reflecting the chemical constraints of this complex transformation as well as biochemical constraints of the utilized iron-dependent oxygenases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the NIH (GM109773) and NSF (CHE-1609917) to R.J.P., along with a postdoctoral fellowship to R.N. from the Deutsche Forschungsgemeinschaft (DFG) NA 1261/1-2. We wish to thank ISU Chemical Instrumentation Facility staff member Steve Veysey for assistance with the Waters GCT GC-MS analyses, and Prof. Peter Hedden (Rothamsted Research) for GA_{12} and GA_{25} standards.

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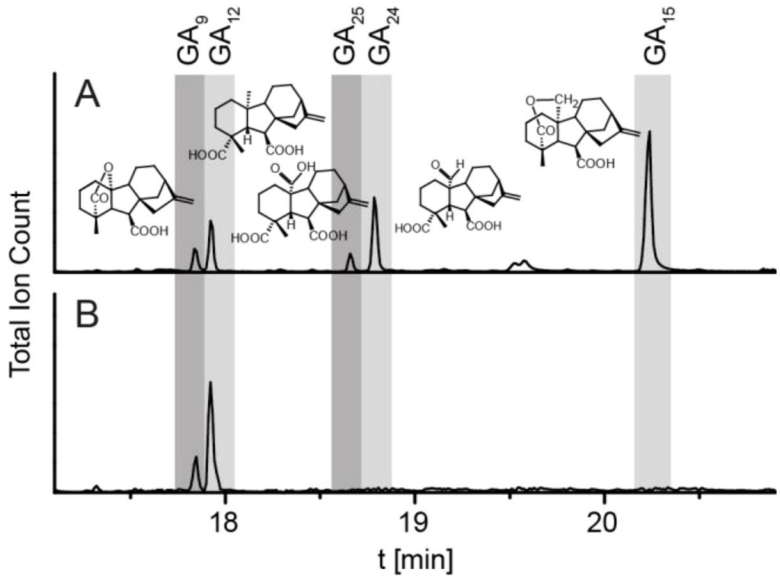


Figure 1.

In vitro enzyme activity of *Et*CYP112. GC-MS chromatograms of assays with purified *Et*CYP112, spinach Fd and *Et*FdR under limiting NADPH concentrations (A), or NADPH in excess stopped after 30 min (B), with GA12 as substrate.

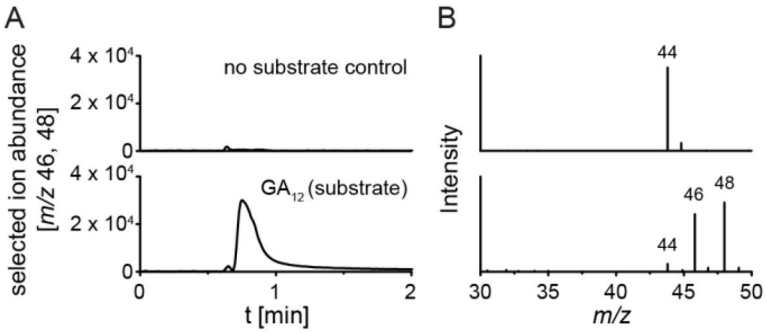


Figure 2.

Figure CO_2 loss during γ-lactone formation. (A) GC-MS chromatograms of enzyme assays with purified *Et*CYP112, spinach Fd, *Et*FdR, NADPH in excess and either no substrate or GA_{12} as the substrates under an $^{18}O_2$ atmosphere. (B) MS-spectra of the CO_2 peak.

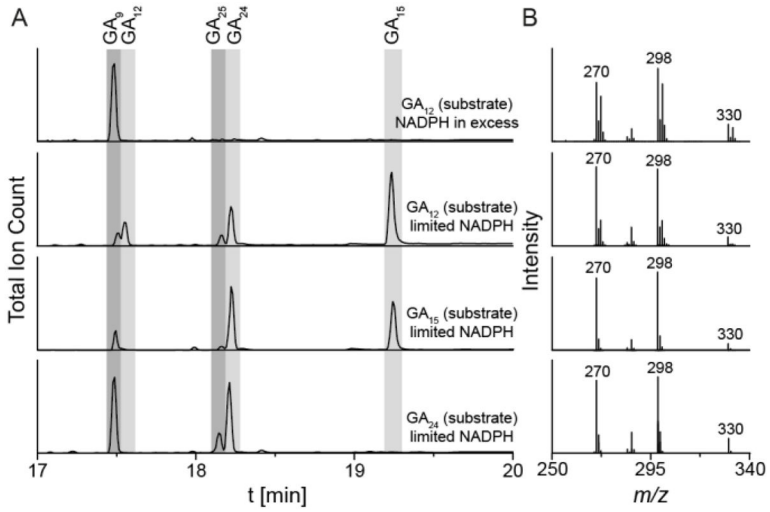


Figure 3.

In vitro enzyme activity of *Et*CYP112 under an $^{18}O_2$ atmosphere. (A) GC-MS chromatograms of enzyme assays with purified *Et*CYP112, spinach Fd and *Et*FdR with an excess of NADPH and GA_{12} or under limiting NADPH concentrations with GA_{12}, GA_{15} (closed lactone) or GA_{24} as the substrate under an $^{18}O_2$ atmosphere. (B) MS-spectra of GA_9 showing the molecular ion at *m/z* 330 or 332 and two larger fragments corresponding to the loss of the methylated acid at C-7.

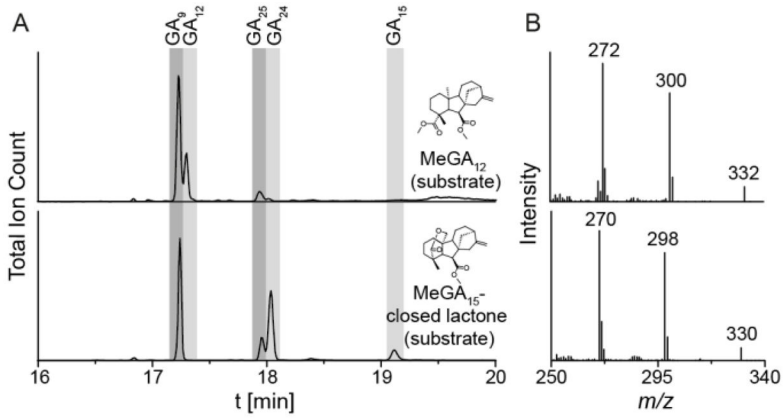
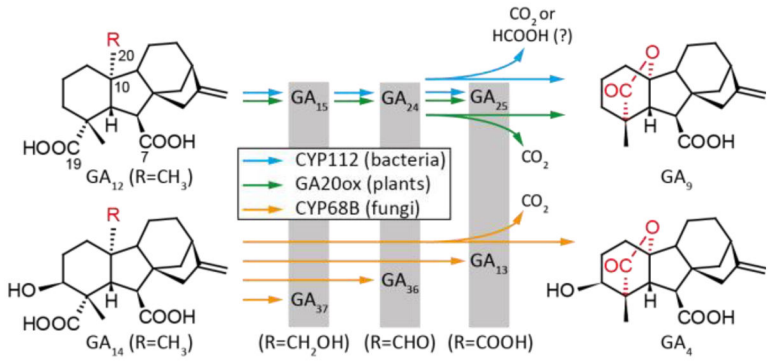


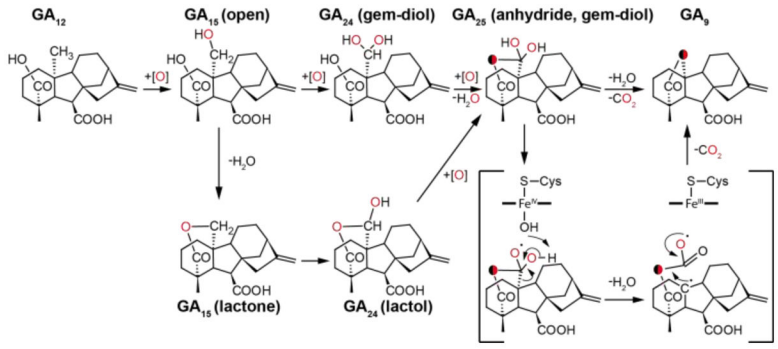
Figure 4.

In vitro enzyme activity of *Et*CYP112 with $MeGA_{12}$ and $MeGA_{15}$ under an $^{18}O_2$ atmosphere. (A) GC-MS chromatograms of enzyme assays with purified *Et*CYP112, spinach Fd and *Et*FdR under limiting NADPH concentrations with $MeGA_{12}$ or $MeGA_{15}$ (closed lactone) as the substrate under an $^{18}O_2$ atmosphere. (B) MS-spectra of GA_9 showing the molecular ion at *m/z* 330 or 332 and two larger fragments corresponding to the loss of the methylated acid at C-7.



Scheme 1.

Reactions catalyzed by CYP112, CYP68B and GA20ox in GA biosynthesis from bacteria, fungi and plants, respectively. Sequential oxidation and elimination of C-20, while the plant and fungal enzyme release CO_2 upon elimination of C-20, this was unknown for the bacterial CYP112.



Scheme 2.

Proposed bifurcation and reconversion of pathways from GA_{12} to GA_9 catalyzed by *Et*CYP112.