Labeling Studies Clarify the Committed Step in Bacterial Gibberellin Biosynthesis

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Supporting Information

ABSTRACT: Bacteria have evolved gibberellin phytohormone biosynthesis independently of plants and fungi. Through 13C-labeling and NMR analysis, the mechanistically unusual “B” ring contraction catalyzed by a cytochrome P450 (CYP114), which is the committed step in gibberellin biosynthesis, was shown to occur via oxidative extrusion of carbon-7 from ent-kaurenoic acid in bacteria. This is identical to the convergently evolved chemical transformation in plants and fungi, suggesting a common semipinacol rearrangement mechanism potentially guided by carbon-4α carboxylate proximity.

Gibberellins (GAs) are phytohormones that play important roles in plant growth, development, and interactions with microbes.1,2 These diterpenoid-derived compounds are characterized by a 6-5-6-5 fused ring structure, termed the ent-gibberellane carbon skeleton. However, GAs are produced via ent-kaurane precursors, which have a 6-6-6-5 carbon skeleton (see Scheme 1 for numbering and ring nomenclature).

Accordingly, the committed step in GA biosynthesis is contraction of the “B” ring from a cyclohexane to cyclopentane. This occurs via oxidation of ent-kaurenoic acid (1; ent-kaur-16-en-19-oic acid), first to 7β-hydroxy-ent-kaurenoic acid (2; ent-7α-hydroxykaurenoic acid), and then to GA12-aldehyde (3), the latter of which involves oxidative extrusion of an endocyclic “B” ring carbon (Scheme 1). The cytochrome P450 mono-oxygenases (CYPs) catalyzing this mechanistically unusual and challenging reaction are termed ent-kaurenoic acid oxidases (KAOs).

In addition to their endogenous production by plants, GAs are also produced by certain plant-associated fungi and bacteria, wherein the relevant biosynthetic pathways have independently evolved.3,4 Plant and fungal GA biosynthesis has been extensively studied, and it has been directly demonstrated that the carbon extruded from the “B” ring is C-7 in fungal biosynthesis,5–7 and convincing indirect evidence has been presented that plants also extrude C-7 (i.e., retention of the C-6α proton from 1 during the “B” ring contraction reaction).8,9 Moreover, 2 has been shown to be a bona fide intermediate in both plant and fungal GA biosynthesis. By contrast, 6β,7β-dihydroxy-ent-kaurenoic acid (4; ent-6α,7α-dihydroxy-kaurenoic acid), which is also produced in the KAO-catalyzed oxidation of 1 by plants and fungi, is not,10–14 thus, implicating a mechanism in which C-7, but not C-6, is hydroxylated prior to ring contraction.5,15

The GA biosynthetic pathway in bacteria has only recently been elucidated.4,16 In particular, the role of each enzyme from a CYP-rich gene cluster/operon in symbiotic rhizobia has now been functionally identified, showing that they act to produce GA9 (Scheme S2). While rhizobia only express these enzymes
and produce GA after differentiation into their nodule-residing bacteroid form, it was possible to observe activity with the individual enzymes upon recombinant expression. Notably, “B” ring contraction requires not only a CYP (CYP114) but also the ferredoxin (Fd_{GA}) found within the operon, which presumably acts as an electron donor. This is distinct from plant and fungal KAOs, which simply utilize an archetypical cytochrome P450 reductase for their activity.

When expressed alone, CYP114 only converts 1 to 2, while coexpression of CYP114 and Fd_{GA} enables full conversion of 1 to 3. This suggests that endogenous ferredoxins from the recombinant host support partial CYP114 activity and indicates a unique role for Fd_{GA} in facilitating full activity, presumably through its interaction with CYP114. Although recombinantly coexpressed CYP114 and Fd_{GA} are not able to convert 2 to 3, nodule-extracted rhizobial bacteroids can use 2 as a GA precursor, implicating this as an intermediate in bacterial GA biosynthesis as well.

Though the intermediacy of 2 might be taken to suggest that C-7 also will be extruded during the “B” ring contraction reaction catalyzed by the convergently evolved bacterial enzymes, it is still plausible that C-6 might be extruded instead (e.g., via a pinacol ring rearrangement mechanism). The extrusion of C-7 during fungal GA biosynthesis was shown by feeding (2-^{13}C)mevalonolactone (5; the δ-lactone form of mevalonate) to cultures of Fusarium fujikuroi (the anamorph of Gibberella fujikuroi), which leads to specific labeling of C-7 in 1, followed by NMR analysis of the resulting GA_{12}-aldehyde. This approach was enabled, at least in part, by the high titers of bacterial KAO activity. Thus, the KAOs from all three biological kingdoms could serve as an intermediate in bacteria as well, implying C-7/δ-hydroxylation prior to ring contraction. It is known for plants and fungi that the 6β-hydrogen of 1 is removed prior to rearrangement/ring contraction, although 4 does not serve as an intermediate and seems to be a side product of the corresponding CYPs in both kingdoms. Interestingly, closer analysis of incubations of 1 in cells coexpressing CYP114 and Fd_{GA} showed that a trace amount of 4 is produced (Figure S5).

However, feeding 4 to bacterial cultures recombinantly coexpressing CYP114 and Fd_{GA} does not result in further conversion (Figure S5), suggesting that 4 is a side product of bacterial KAO activity. Thus, the KAOs from all three biological kingdoms not only extrude C-7 in “B” ring contraction but also exhibit a conserved order of chemical transformations, with conversion of 1 to 3 via 2, but apparently not 4.

Although it is possible to produce 13C-labeled 1 by simply feeding (2-^{13}C)-5 to E. coli engineered to produce 1 from 5 (i.e., via coexpression of the necessary nine enzymes; see Scheme S3, Figures S1 and S2). As expected, this enabled isolation of 1 with 13C enrichment at four positions, as initially confirmed by gas chromatography–mass spectroscopy (GC-MS), with comparison to an authentic standard (Figure S2). The expected incorporation of 13C at carbons 1, 7, 12, and 18 (Scheme 2)\(^6,7\) was verified by 13C NMR analysis with comparison to unlabeled 1 (Figure S3; Tables S1 and S2).

To investigate the origin of the extruded carbon, 13C-enriched 1 was fed to bacterial cultures recombinantly coexpressing CYP114 and Fd_{GA}. This allowed isolation of 3 enriched at four positions, as confirmed by GC-MS comparison to an authentic standard (Figure S4). Following purification,

\[\text{Scheme 2. } ^{13}\text{C Label from (2-^{13}C)-5 Is Specifically Incorporated into 1 via Metabolically Engineered Bacteria}^a\]

\[\text{Figure 1. Comparison of the } ^{13}\text{C-labeled 3 }^{13}\text{C NMR spectrum to that of the unlabeled standard (800 MHz, CDCl}_3\text{ for each) reveals that C-7 is extruded during the ring contraction from ent-kaurenic acid 1 to GA}_{12}\text{-aldehyde 3. The } ^{13}\text{C-enriched carbons in the labeled substrate are indicated with asterisks (*).} \]
these previously been reported in other investigations of bacterial GA biosynthesis.\textsuperscript{4,14} To further evaluate the possibility of the “B” ring contraction reaction proceeding through this type of intermediate, kaurenolide and 7β-hydroxykaurenolide (see Figure S6 for chemical structures) were fed to bacterial cultures recombinantly coexpressing CYP114 and Fd\textsubscript{GA}. However, these were not converted in this system (Figure 2) and likely are not intermediates in bacterial GA biosynthesis, similar to what has been reported for plants and fungi.\textsuperscript{27}

All three biological kingdoms have convergently evolved KAOs that carry out this committed step in GA biosynthesis.\textsuperscript{3} In each case the KAO is a member of the CYP superfamily, but falls within phylogenetically distinct families specific to each biological kingdom, as bacterial KAOs come from the CYP114 family, the fungal KAOs from the CYP68 family, and those from plants from the CYP88 family.\textsuperscript{14,28} However, the results reported here show that these convergently evolved KAOs all catalyze extrusion of the same carbon, using a conserved order of central chemical transformations (i.e., from 1 to 3 via 2) in each case.\textsuperscript{6} This suggests a physical/chemical restraint for the “B” ring contraction reaction.

Intriguingly, there appears to always be a free C-4α carboxylate (C-19) present. While CYPs typically catalyze radical based reactions, it has been previously suggested that this “B” ring contraction reaction might proceed via a carboxylation based mechanism instead, with transfer of the unpaired electron from the initially formed C-6 radical of 2 to the heme-iron.\textsuperscript{29,30} Notably, the C-4α carboxylate is ideally positioned to offer anchimeric assistance to formation of this putative C-6 carboxyl, potentially guiding the observed oxidative extrusion of C-7 via a semipinacol rearrangement mechanism (Scheme 3, path A).\textsuperscript{31} Alternatively, hydroxylation of 2 to form 4 may argue against the classic pinacol mechanism, it is possible that suboptimal expression of CYP114 and/or Fd\textsubscript{GA} prevented turnover here.

Consistent with a role for anchimeric assistance by the free C-4α carboxylate, neither the methyl ester of 1 nor ent-kaurenal (which in the predominant diol form sterically resembles 1) is further transformed by recombinantly expressed CYP114 (either with or without coexpression of Fd\textsubscript{GA}). Additional support for the proposed mechanism stems from the use of pinacol-like intermediates to achieve “B” ring contraction in the chemical synthesis of GA.\textsuperscript{28} Thus, it seems likely that the independently evolved CYPs catalyzing the characteristic “B” ring contraction in gibberellin biosynthesis in all three biological kingdoms may have converged on a common (semi)pinacol rearrangement mechanism to selectively carry out this unusual and challenging reaction.

\textbf{Scheme 3. Proposed Reaction Mechanisms for “B” Ring Contraction during GA Biosynthesis That Proceed Either through a Semipinacol (Path A) or Classic Pinacol (Path B) Rearrangement}

of 2 to form 4 as a transient intermediate would enable rearrangement via a classical pinacol mechanism. If protonated in the CYP114 active site (pK\textsubscript{a} \approx 4.6), the C-4α moiety might then provide anchimeric assistance by acting as an acid to protonate the 6β-hydroxyl group, leading to specific extrusion of C-7 (Scheme 3, path B). While the lack of enzymatic conversion of 4 may argue against the classic pinacol mechanism, it is possible that suboptimal expression of CYP114 and/or Fd\textsubscript{GA} prevented turnover here.

Consistent with a role for anchimeric assistance by the free C-4α carboxylate, neither the methyl ester of 1 nor ent-kaurenal (which in the predominant diol form sterically resembles 1) is further transformed by recombinantly expressed CYP114 (either with or without coexpression of Fd\textsubscript{GA}). Additional support for the proposed mechanism stems from the use of pinacol-like intermediates to achieve “B” ring contraction in the chemical synthesis of GA.\textsuperscript{28} Thus, it seems likely that the independently evolved CYPs catalyzing the characteristic “B” ring contraction in gibberellin biosynthesis in all three biological kingdoms may have converged on a common (semi)pinacol rearrangement mechanism to selectively carry out this unusual and challenging reaction.

\textbf{ASSOCIATED CONTENT}

\textbf{Supporting Information}

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b02569.

Experimental methods, Supplemental figures and tables (PDF)

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\textbf{Notes}

The authors declare no competing financial interest.

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