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Spiro-Ring Formation is Catalyzed by a Multifunctional Dioxygenase in Austinol Biosynthesis

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Supporting Information

ABSTRACT: Austinol, a fungal meroterpenoid derived from 3,5-dimethylorsellinic acid, has a unique chemical structure with a remarkable spiro-lactone ring system. Despite the recent identification of its biosynthetic gene cluster and targeted gene-deletion experiments, the process for the conversion of protoaustinoid A (**2**), the first tetracyclic biosynthetic intermediate, to the spiro-lactone preaustinoid A3 (**7**) has remained enigmatic. Here we report the mechanistic details of the enzyme-catalyzed, stereospecific spiro-lactone ring-forming reaction, which is catalyzed by a non-heme iron-dependent dioxygenase, AusE, along with two flavin monooxygenases, the 5′-hydroxylase AusB and the Baeyer−Villiger monooxygenase AusC. Remarkably, AusE is a multifunctional dioxygenase that is responsible for the iterative oxidation steps, including the oxidative spiro-ring-forming reaction, to produce the austinol scaffold.

The fungal meroterpenoids comprise structurally diverse natural products with a wide range of important biological activities,[1] as exemplified by pyripyropene A, a potent acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, and the andrastins, which are protein farnesyltransferase inhibitors.[2−4] The genetic and molecular bases for the biosyntheses of these complex meroterpenoids, including pyripyropene A,[5,6] paxilline,[7−9] austinol/dehydroaustinol,[10,11] and terretonin,[12−14] have recently been elucidated by the identification of their biosynthetic gene clusters, targeted gene deletions, complementation experiments, and reconstitutions of their biosynthetic pathways in heterologous expression hosts. Among them, austinol, dehydroaustinol, and terretonin are members of the 3,5-dimethylorsellinic acid (DMOA)-derived meroterpenoid family, which includes a quite large number of structurally diverse meroterpenoids (Figure 1).[15] These molecules are all derived from the farnesylated aromatic tetraketide DMOA but differ as a result of variations in the cyclization and tailoring reactions. Therefore, the chemical processes and mechanisms that generate the remarkable structural diversity of this class of natural products have long attracted keen interest.

The biosynthetic gene clusters for austinol (**1**) and dehydroaustinol in *Aspergillus nidulans* are located in two separate regions in the genome, and 14 genes in the clusters are involved in the biosynthesis of **1**.[11] The biosynthetic pathway up to protoaustinoid A (**2**), the first tetracyclic biosynthetic intermediate, was elucidated by gene disruption and reconstitution in a fungal expression system[11,13] and involves a

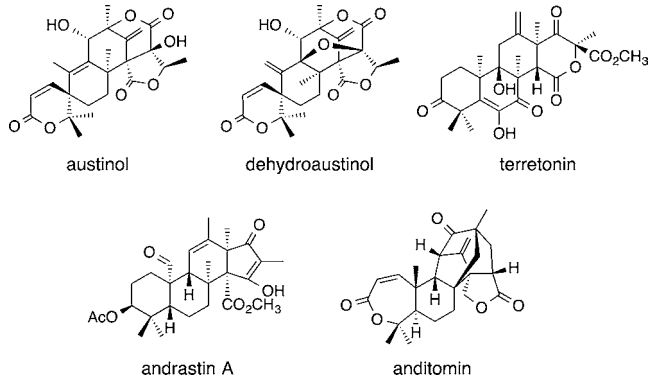

Figure 1. Fungal meroterpenoids derived from DMOA.

polyketide synthase (AusA), a farnesyltransferase (AusN), a methyltransferase (AusD), a flavin-dependent monooxygenase (FMO) (AusM), and a terpene cyclase (AusL). The transformation of the spiro-lactone preaustinoid A3 (**7**) into **1** has also been proposed. It is presumably achieved by five enzymes, including two hypothetical proteins (AusJ and AusH), a ketoreductase (AusK), and two P450 monooxygenases (AusI and AusG).[11] However, the biosynthetic process for the conversion of **2** into **7** has remained a missing link. A key feature of the biosynthesis of **7** is the spiro-lactone ring formation, which makes these compounds quite unique, since this type of spiro system is found in only a few natural products, such as andibenins[16,17] and carapolide G.[18] Although a reaction mechanism for spiro-lactone formation was previously proposed for the biosynthesis of an austinol derivative,[19] the mechanistic details of the enzyme-catalyzed process remain to be clarified.

Previously reported gene-disruption experiments suggested that an FMO, designated as AusB, accepts **2** as a substrate. In addition, two other genes designated as *ausC* and *ausE*, encoding another putative FMO and a non-heme iron-dependent dioxygenase, respectively, were also suggested to be involved in the biosynthesis of **1**.[11] However, since neither the *ausC*- nor *ausE*-targeted gene-deletion mutant of *A. nidulans* yielded any biosynthetic intermediates, their roles in the biosynthesis have yet to be elucidated. Considering that multiple oxidation steps, such as hydroxylation and dehydrogenation, would be required for the formation of spiro-lactone **7** from **2**, we hypothesized that these three oxygenases, AusB, AusC, and AusE, are involved in the generation of **7**. We now describe this previously unknown

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pathway in meroterpenoid biosynthesis and the characterization of these enzymes, including a multifunctional non-heme iron-dependent dioxygenase that catalyzes the iterative oxidation steps and the stereospecific oxidative spiro-ring-forming reaction to produce the austinol scaffold.

To characterize the functions of these three oxygenases, we constructed a heterologous fungal expression system in *Aspergillus oryzae* NSAR1, which is a quadruple auxotrophic mutant strain (*niaD*$^{-}$, *sC*$^{-}$, Δ*argB*, *adeA*$^{-}$).[20] In the expression system, the sequences of *ausB* and *ausC* in the database (AN8379.4 and AN8381.4, respectively) were manually revised to exclude apparently mispredicted intron sequences [Figure S1 in the Supporting Information (SI)]. Each transformant was cultured in induction medium supplemented with **2**, and the compounds that accumulated in the culture supernatant were analyzed.

When AusB alone was expressed in *A. oryzae*, **2** was successfully converted into the single new product **3**, which was not found in the control transformant harboring the empty vectors (Figure 2, lanes i and ii). Interpretations of the ^{1}H and ^{13}C NMR spectra confirmed **3** as berkeleyone A (Table S4),[21] a C5′-hydroxylated derivative of **2**. This experiment revealed that AusB is responsible for the C5′ hydroxylation of **2**. Contrary to the previous proposal that AusB could be a Baeyer−Villiger (BV) monooxygenase that forms the seven-membered lactone A-ring,[11] no such compound was obtained from the transformant expressing AusB. Likewise, **3** was also produced by the transformant expressing both AusB and AusC (Figure 2, lane iii), indicating that AusC is not the enzyme that accepts **3** as its substrate.

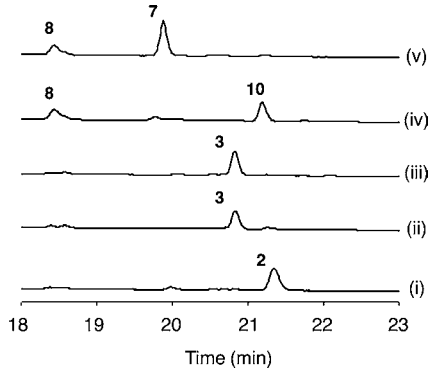

Figure 2. HPLC profiles of culture supernatant extracts from transformants incubated with **2**. The transformants harbored (i) only empty vectors; (ii) *ausB*; (iii) *ausB* and *ausC*; (iv) *ausB* and *ausE*; and (v) *ausB*, *ausC*, and *ausE*. The chromatograms were monitored at 190 nm.

On the other hand, the transformant expressing both AusB and AusE converted **2** into two major products **8** and **10** (Figure 2, lane iv) along with two minor products **4** and **9** having HPLC retention times close to that for **10** (Figure S2A). The structures of all four products were confirmed by one- and two-dimensional NMR spectroscopy. **4** was identified as the known trione preaustinoid A (Table S5),[22] while **8**−**10** were novel compounds. Compound **8** was found to be a C5-hydroxylated derivative of **3**, which we named 5-hydroxyberkeleyone A (Table S1 and Figures S4 and S5). Compound **9**, designated as preaustinoid C, is a dehydrogenated form of **4** with a $\Delta^{1,2}$-conjugated double bond (Table S2 and Figures S6 and S7), and compound **10**, designated as austinoid C, has a spiro-cyclopentenone structure that is probably derived from oxidative carbon rearrangement of **9** (Table S3 and Figures S8 and S9). On the basis of the four different products, including **10** with the spiro system, AusE probably catalyzes not only the oxidative spiro-ring formation but also the multistep oxidation processes. Finally, the transformant expressing all three enzymes AusB, AusC, and AusE successfully converted **2** into spiro-lactone **7** (Figure 2, lane v, and Table S7), suggesting that AusC catalyzes the BV reaction to insert an O atom between C3 and C4 of the A-ring. Concurrently, preaustinoid A2 (**6**)[23] was also detected as a minor product in the transformant expressing all three oxygenases (Figure S2B and Table S6), implying that the BV reaction occurs prior to the spiro-ring formation.

To obtain deeper insight into the AusE-catalyzed spiro-ring-forming reaction, we performed in vitro assays using a recombinant AusE heterologously expressed in *Escherichia coli*. A bioinformatic analysis revealed that AusE shares sequence similarity with the members of the phytanoyl-CoA dioxygenase (PhyH) superfamily. PhyH requires Fe(II) and α-ketoglutarate (α-KG) for its activity.[24] Therefore, when AusE was incubated with $FeSO_4$, α-KG, and substrate **3**, we obtained **10** as well as **4**, **8**, and **9** (Figure 3, lane iv). The enzymatic reaction proceeded in the absence of exogenous Fe(II), probably by means of residual enzyme-bound ferrous ions (Figure 3, lane ii), but the reaction was completely abolished by either the presence of 1 mM EDTA or the absence of α-KG (Figure 3, lanes i and iii). These results indicated that AusE requires Fe(II) and α-KG for the catalytic activity and that AusE accepts **3** as a substrate to catalyze three consecutive oxidation reactions, including the stereospecific spiro-cyclopentenone-forming reaction, to produce **10**.

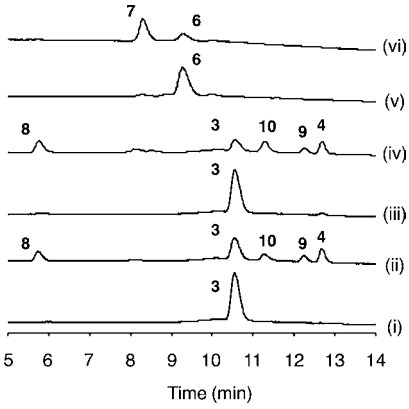

Figure 3. HPLC profiles of the products of reactions of AusE with (i−iv) **3** or (v, vi) **6**: (i) without α-KG; (ii) without Fe(II); (iii) with EDTA; (iv) complete reaction with **3**; (v) without AusE; (vi) complete reaction with **6**. The chromatograms were monitored at 190 nm.

Furthermore, the in vitro assay revealed that AusE accepts **6** as a substrate to generate spiro-lactone **7** (Figure 3, lanes v and vi). The observation that AusE produces both **7** and **10** in turn evoked the question about the timing and the substrate of the BV oxidation reaction catalyzed by AusC. To clarify the complete biosynthetic pathway, we performed bioconversion experiments; five compounds (**2**, **3**, **4**, **9**, and **10**) were incubated with the transformant expressing both AusE and AusC. As a result, **3** and **4** were successfully converted into **7**, whereas **9** was transformed into spiro-cyclopentenone **10** and **2** and **10** remained unchanged (Figure S3). These results clearly indicated that AusC accepts **4** to produce preaustinoid A1 (**5**)[23] with a seven-membered lactone A-ring and that **5** is further oxidized to give enone **6** by

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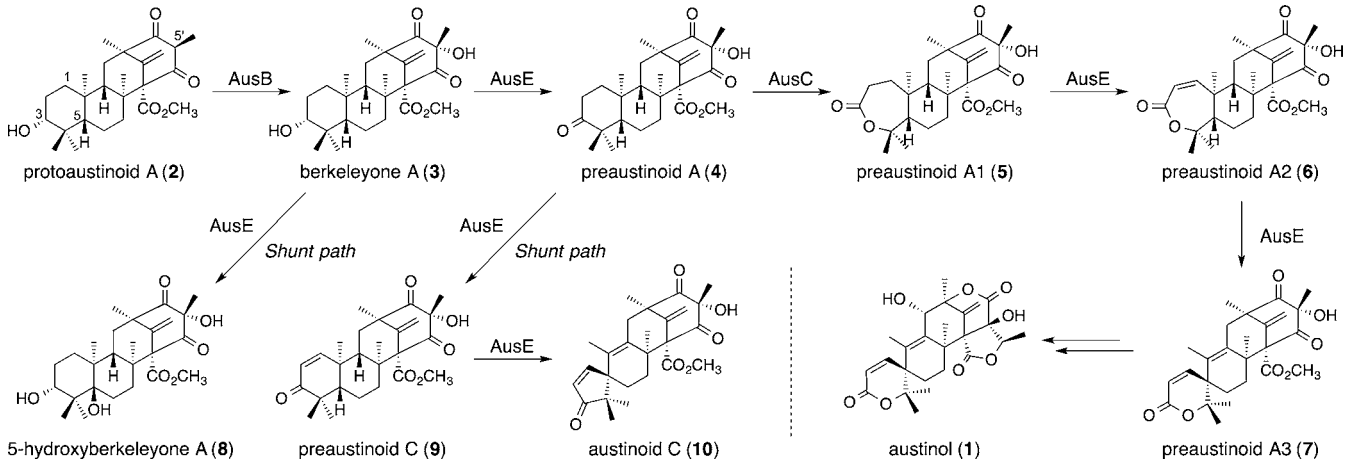
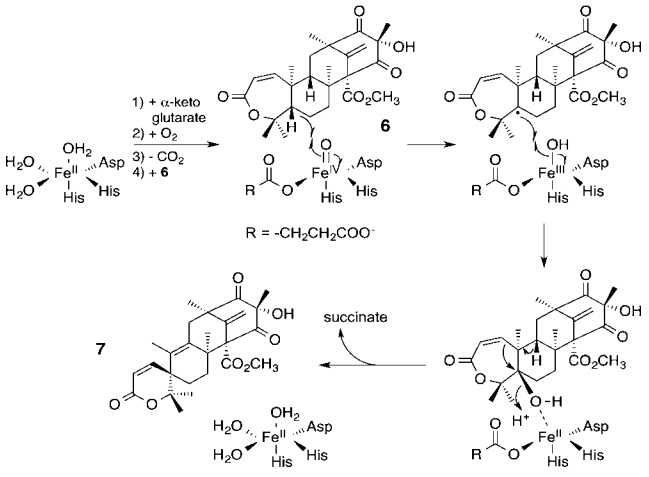
Scheme 1. Proposed Biosynthetic Pathway Leading to Preaustinoid A3 (7)



AusE (**5** was not isolated). Considering these results, we propose the complete biosynthetic pathway for the conversion of **2** into **7** (Scheme 1), in which AusE iteratively catalyzes multistep oxidation reactions, including the spiro-ring formation, both "before" and "after" the BV lactone formation by AusC.

A sequence comparison revealed that AusE has a conserved 2-His-1-carboxylate iron-binding facial triad (H130, D132, H214).[25] An iron bound to this site could play a central role in the reaction catalyzed by AusE, and thus we propose the following mechanism for the oxidative spiro-ring formation (Scheme 2). The initial binding of α-KG and O_2 would lead to

Scheme 2. Proposed Reaction Mechanism for Spiro-Ring Formation



oxidative decarboxylation of α-KG, generating the active Fe(IV)−oxo species. This species would hydroxylate enone **6** at C5, forming the hydroxylated intermediate bound to an Fe(II) species. Elimination of the hydroxyl group followed by rearrangement of the carbon skeleton of the resulting carbocationic intermediate would generate spiro-lactone **7**. Similar carbaspirocycle-forming reactions were previously achieved by synthetic chemistry; steroids with a 4β,5β- or 5α,6α-epoxide were converted into spiro compounds by treatment with formic acid or boron trifluoride, respectively.[26,27] These reactions suggested that the generation of the C5 carbocationic species would spontaneously lead to spiro-ring formation, corroborating the proposed reaction mechanism for AusE.

The Fe(II)/α-KG-dependent dioxygenase family proteins catalyze various reactions in nature, such as the insertion of a C atom into the penicillin molecule in cephalosporin biosynthesis[28] and the formation of tropolone in stipitatic acid biosynthesis.[29] Clavaminate synthase 2,[30] another member of this family, remarkably catalyzes three oxidation reactions in clavulanic acid biosynthesis, as observed for AusE. To the best of our knowledge, however, this is the first report of oxidative carbaspirocycle formation by this class of enzymes. On the other hand, a mutated sesquiterpene synthase reportedly produced a compound with a spiro-ring system by means of only carbon skeletal rearrangement without an oxidation process.[31] Therefore, this study has also provided insights into the biosynthetic mechanisms involved in the production of other spiro-ring-containing natural products.

In conclusion, we identified and characterized three oxygenases involved in the biosynthesis of austinol, including the multifunctional and oxidative spiro-ring-forming dioxygenase AusE. AusE catalyzes various oxidation reactions in addition to spiro-ring formation and works iteratively in the biosynthetic process. This study has defined the complete pathway of austinol biosynthesis by revealing the missing link between **2** and **7**. Furthermore, in the course of our study, we isolated three novel austinol derivatives (**8**−**10**) as well as four known compounds (**3**, **4**, **6**, and **7**). Notably, except for **7**, none of these were isolated in the previous gene-disruption study.[11] This highlights the significant advantage of the reconstitution approach to obtain biosynthetic intermediates of natural products. Finally, the spiro-ring moiety of austinol also exists in a few other natural products. This study has provided insights suggesting that AusE-like dioxygenases may be involved in the biosynthesis of these carbaspirocycle-containing compounds in general as well as clues for manipulating the biosynthetic pathway to create novel and structurally distinct molecular scaffolds for drug discovery.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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