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Biosynthesis of Shearinine: Diversification of a Tandem Prenyl Moiety of Fungal Indole Diterpenes

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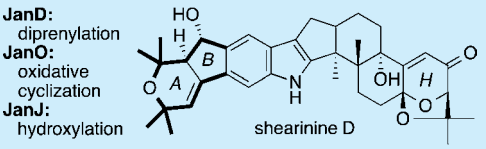
Supporting Information

ABSTRACT: The late-stage biosynthetic pathway of the indole diterpene shearinine involving four enzymatic reactions (JanQDOJ) was elucidated by an efficient heterologous expression system using *Aspergillus oryzae*. Key oxidative cyclization, forming a characteristic A/B bicyclic shearinine core by flavoprotein oxidase, was studied using a substrate analogue and a buffer containing $H_2{}^{18}O$. These experimental data provided evidence that JanO catalyzes two-step oxidation via a hydroxylated product and that the JanO reaction involves the hydride-transfer mechanism.



Prenylated indole alkaloids such as indole diterpenes, ergot alkaloids, and cyclic dipeptides frequently occur in filamentous fungi.[1] Indole prenylation and further modification is an important transformation for diversification of these metabolites. Although their core structures such as chanoclavine,[2] communesin,[3] and cyclopiazonic acid[4] are further modified by oxidative cyclizations, detailed mechanisms of these conversions have not been elucidated by in vitro analysis.

We recently successfully applied a heterologous expression system, using *Aspergillus oryzae*, to total biosynthesis of a number of fungal metabolites, including polyketides, terpenes, meroterpenoids, and cyclic peptides.[5] Using this system, we have reported a core construction mechanism of fungal indole diterpene (IDT) synthesis of paxilline (**1**) (six genes, four transformations)[6] and diversification mechanisms of aflatrem (seven genes, two transformations)[7] and a highly elaborated congener penitrem requiring 17 genes.[8] These data showed that the *A. oryzae* system is highly reliable and suitable for reconstitution of target IDTs gene clusters. In IDT family members, one of the most unique structural features is a diprenylation moiety, which is frequently modified by further oxidations to install a bicyclic system (Figure 1). Although we found one of the diversification mechanisms using the oxidation−prenylation sequence in the study of penitrem, it remains elusive whether the same mechanism can be applied to other IDTs.

Janthitremane IDTs, having a characteristic A/B-bicyclic system, are produced by *Penicillium* and its teleomorph *Eupenicillium* species.[9] In view of their core skeletons, members of this family are divided into two groups, paxilline and paspalinine type, which only differ in the oxidation level of ring H (Figure S1). Five strains have been reported to produce either paxilline- or paspalinine-type janthitremanes. Among these, only *Eupenicillium* sp. produces both types of janthitremanes (Figure S1).[9c] In this paper, we have elucidated the late four-step biosynthesis of janthitrem using reconstitution of its gene cluster in *A. oryzae*. We have also explored a biosynthetic mechanism for formation of the A/B bicyclic system in janthitremane IDTs.

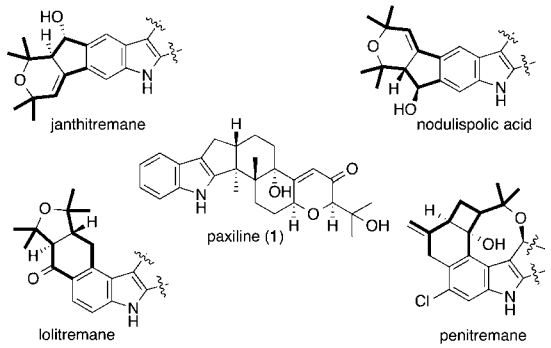


Figure 1. Representative indole diterpenes installing an additional bicyclic system by diprenylation−oxidation.

In a recent study involving one of us, a gene cluster responsible for shearinine biosynthesis was reported (Figure S2).[10] This cluster consists of four putative biosynthetic genes for paspaline core assembly (*janGCMB*) and two for additional oxidative transformations (cytochrome P450 monooxygenase, *janPQ*). Homologues of these genes are found in the IDT gene clusters for paxilline, aflatrem, lolitrem, and penitrem (Scheme 1).[11] In addition, the remaining three genes in the *jan* cluster, *janDOJ* (*janJ* = *PJ-13*) are proposed to be responsible for construction of the *A*/*B* bicyclic system of shearinines. Based on the function of PaxD[12] (JanD homologue), JanD is likely to catalyze diprenylation, suggesting that the mechanism of shearinine bicyclic core synthesis differs from that of penitrem although homologous enzymes PtmE and PtmO are involved.[8]

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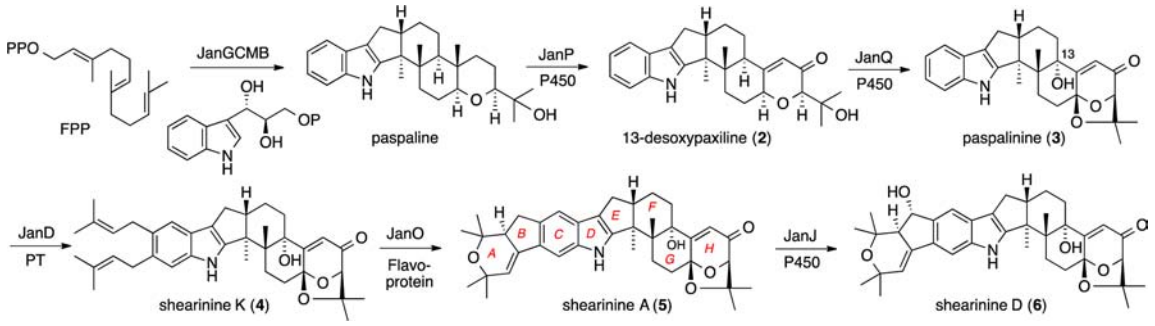
Scheme 1. Proposed Biosynthetic Pathway of Shearinines[a]



[a]FPP: farnesyl diphosphate. PT: prenyltransferase. P450: cytochrome P450.

To determine the function of the shearinine-specific four-gene *janQDOJ*, we prepared plasmids (pUARA2-*janQ*, pAdeA2-*janDO*, and pUSA2-*janJ*) and introduced them into *A. oryzae* NSAR1 with different combinations of plasmids to obtain the transformants AO-*janQ*, AO-*janQDO*, and AO-*janQDOJ*. When we incubated the transformant AO-*janQ* with 13-desoxypaxilline (**2**),[6] we detected a relatively unstable new metabolite (*m*/*z* 434 $[M+H]^+$) in the HPLC analysis (Figure 2). Its MW and ^{1}H NMR

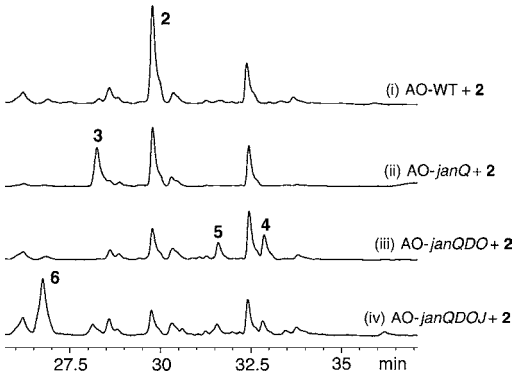


Figure 2. HPLC analytical data: (i) *A. oryzae* NSAR1 (wild type); (ii) metabolites produced by transformant AO-*janQ* with **2**; (iii) AO-*janQDO* with **2**; (iv) AO-*janQDOJ* with **2**.

spectrum were identical to that of paspalinine (**3**). We could not find a peak corresponding to **1**, indicating that JanQ, just like AtmQ,[7] specifically gave **3** (Scheme 1).

Incubation of AO-*janQDO* with **2** gave two new products: **4** (*m*/*z* 570.3625, $C_{37}H_{47}NO_4$) and **5** (*m*/*z* 584.3393, $C_{37}H_{45}NO_5$), the molecular formulas of which were determined by HR-MS (Figure 2). The difference in molecular formulas ($C_{10}H_{16}$) between **3** and **4** and the ^{1}H NMR data of **4** showed that **4** is a diprenylation product of **3** [δ_H 5.30 (br t, 6.5 Hz), 5.29 (br t, 6.5 Hz), 3.39 (d, 7.1 Hz)]. The increased oxidation level of the diprenyl moiety and ^{1}H NMR data of **5** [disappearance of characteristic signals of diprenyl moiety and newly appeared signals at δ_H 5.90 (d, 3.0 Hz)] suggested **5** corresponds to the cyclization product. Thus, a comparison of their ^{1}H NMR spectra enabled us to determine the structures of **4** and **5** as shearinines K[9d] and A,[9c] respectively (Scheme 1).

Finally, when we incubated transformant AO-*janQDOJ* with **2**, we found a prominent product **6** in the HPLC analysis of the extract (Figure 2). Its ^{1}H NMR spectrum showed a characteristic oxymethine signal at δ_H 4.96 (br.t, 6.0 Hz), suggesting that addition of *janJ*, encoding a P450 monooxygenase, resulted in hydroxylation of **5**. The structure of **6** was confirmed by a comparison with the published ^{1}H NMR data of shearinine D[9d] (Scheme 1). Together with the results of three transformants, we identified the individual functions of *janQDOJ*. These results clearly eliminated the possibility that JanJ involves cyclization and showed that a single flavoprotein oxidase JanO catalyzed sequential oxidations to convert the diprenylated product **4** into the cyclization product **5**.

For investigation on the detailed mechanism of the cyclization, we employed in vitro analysis of JanD and JanO. The JanD cDNA was cloned into the pMAL-c4E vector for protein expression in *Escherichia coli* BL21. Maltose binding protein−tagged JanD recombinant enzyme was successfully expressed in a soluble form. The JanO cDNA was cloned into the pQE30 vector for protein expression in *E. coli* M15. His-tagged JanO recombinant enzyme showed a yellow color, thus indicating binding of a flavin cofactor (Figure S3).

Next, we examined the substrate specificity of the diprenyltransferase JanD and its homologue PaxD (65% identity), which is present in the paxilline biosynthetic gene cluster (*pax*) in *Penicillium paxilli*.[13] Previously, we reported that the recombinant PaxD catalyzed a stepwise regular-type diprenylation and the structural difference of substrates significantly affected regioselectivity in the PaxD prenylation.[12] In the incubation of **3** with recombinant JanD in the presence of Mg^{2+} and DMAPP, we observed a clean conversion of **3** into **4** (Figure 4). In contrast, incubation of JanD with **1** gave a mixture of diprenylation products **7a** and **7b**. Compound **7b** is a product in the reaction of prenyltransferase AmyD (a homologue of LtmE)[14] and is one of the putative intermediate in lolitrem biosynthesis. On the other hand, PaxD converted **1** into diprenylpaxilline (**7a**) quantitatively, while **3** was a good substrate of PaxD to afford **4** and a small amount of a regioisomer.

The *pax* cluster contains two genes, *paxD* and *paxO*, that are poorly expressed, encoding JanD and JanO homologues.[13] Interestingly, the orientation of all the genes and the positions of introns in both the *pax* and *jan* clusters are the same except the presence of *janJ* and overall sequence identities are very high,[10] suggesting that these genes have similar functions (Figure S2). Based on the experimental evidence described here, we speculate that the *pax* cluster might be responsible for the biosynthesis of janthitremanes with paxilline core and the *jan* cluster is for shearinines with paspalinine core. However, this hypothesis needs to be clarified experimentally.

To obtain experimental data on the cyclization mechanism, we employed the in vitro analysis of JanO. Incubation of **4** with the recombinant JanO gave a single product **5** (Figure 3), suggesting that the second step of this two-step oxidation proceeded rapidly and no intermediate was detected. After many unsuccessful

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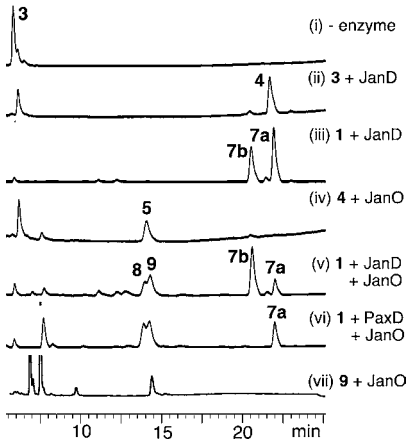


Figure 3. HPLC analytical data of various enzymatic reactions: (i) without enzyme(s); (ii) reaction of **3** with JanD; (iii) **1** with JanD; (iv) **4** with JanO; (v) **1** with JanD/JanO; (vi) **1** with PaxD/JanO; (vii) **9** with JanO.

attempts, we found that the JanD−JanO coupled reaction with **1** and DMAPP gave two products with poor yields by HPLC analysis. LC−MS data of these peaks suggested that these are cyclization product (**8**, *m*/*z* 585) and its related product (**9**, *m*/*z* 587). In this reaction, only **7a** was converted into products, but **7b** remained intact (Figure 3). Large-scale JanO-mediated oxidation of **7a**, by enzymatic prenylation of **1** with PaxD, afforded **8** and **9** in sufficient amounts for NMR measurement. The structure of **8** was readily determined as shearinine B (deoxydehydrojanthitrem E)[9c] by comparison of ^{1}H NMR data. The molecular formula of **9** ($C_{37}H_{49}NO_5$) from HR-MS (*m*/*z* 570.3622 [M + H − H_2O]$^+$) and its ^{1}H NMR data showed that one of the prenyl groups in **7a** was converted into an allyl alcohol [*trans*-olefin: δ_H 5.94 (d, 16.1 Hz) and 6.80 (d, 16.1 Hz). The structure of **9**, designated as protoshearinine, was determined by extensive NMR analysis as shown in Scheme 2 (Figure S4). Incubation of **9** with JanO did not convert it into **8**, suggesting that **9** is a shunt product.

Phylogenetic analysis of JanO and its related flavoprotein oxidases showed that JanO belongs to the vanillyl alcohol oxidase (VAO) family (Figure S5).[15] These members in the family oxidize the substrate by a two electron transfer and have a characteristic flavin adenine dinucleotide (FAD) binding domain in N-terminal half of the proteins (Figure S6). X-ray crystallographic analysis of the family members including VAO,[16] tetrahydrocannabinolic acid synthase (THCAS),[17] and reticuline oxidase (BBE)[18] were reported, and detailed reaction mechanisms have been discussed by their structures. Some of them, THCAS, BBE, and

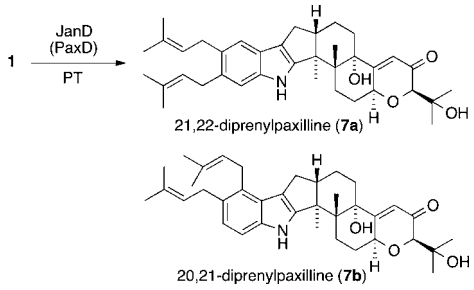


Figure 4. Prenylation of paxilline (**1**) with JanD or PaxD.

solanapyrone synthase,[19] are known to catalyze C−C bond formation.

Recently, we clarified the intriguing cyclization mechanism of bicyclo[3.2.0]heptane formation catalyzed by prenyltransferase PtmE and flavoprotein oxidase PtmO.[8] In shearinine biosynthesis, JanD and JanO, homologues of PtmE and PtmO, catalyzed an oxidative A/B ring formation in a different manner. To elucidate the reaction mechanism of flavoprotein oxidases PtmO/JanO, we examined the origin of oxygen atom at prenyl moiety and the electron acceptor of flavin.

PtmO reaction with **11** was performed in the buffer containing 30% $H_2{}^{18}O$. In the ESI-MS of the oxidation product **12**, we observed an enhancement of the 2 m.u.-shifted molecular ion peak at *m*/*z* 506 ([M + H]$^+$) (Figure 5). This indicated that the

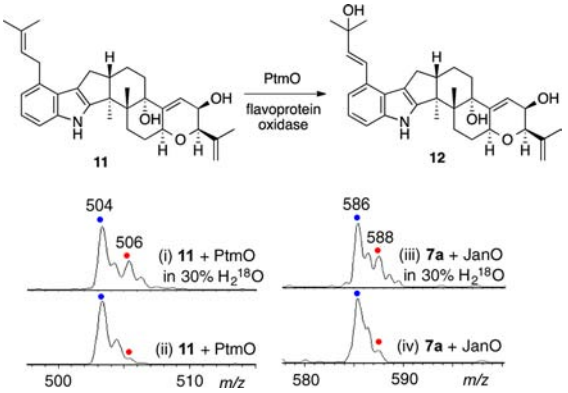
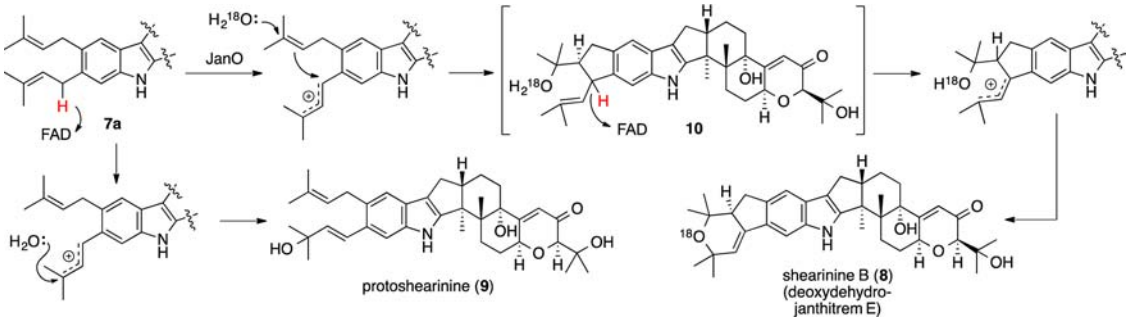


Figure 5. ESI-MS data: (i) incubation of **11** with PtmO in $H_2{}^{18}O$; (ii) **11** in H_2O; (iii) incubation of **7a** with JanO in $H_2{}^{18}O$; (iv) **7a** in H_2O.

oxygen atom of allylic alcohol, derived from the prenyl moiety, originated from $H_2{}^{18}O$ and not from O_2 and that a carbocation intermediate is involved in hydride transfer of the benzylic position. This finding is consistent with the mechanism of other

Scheme 2. Proposed Oxidative Cyclization Mechanism of Shearinine B



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VAO family members. ESI-MS data of a similar experiment on the JanO reaction with **7a** showed an enhancement of the 2 m.u.-shifted molecular ion peak for **8** at m/z 588. These results indicate that the oxygen atom of the ring A in **8** is derived from $H_2{}^{18}O$. When we monitored H_2O_2 production using Amplex Red assay, time-dependent formation of H_2O_2 (2 mol) was observed (Figure S7). This result supported involvement of O_2 as an electron acceptor.

Based on the coproduction of **9** and the origin of the oxygen atom in **8**, we propose an oxidative cyclization mechanism for the formation of the A/B rings of janthitremane shearinines as shown in Scheme 2. This pathway involves a JanO-catalyzed two-step oxidation via a putative intermediate **10**. Interestingly, the green alga *Caulerpa racemosa* produces two metabolites, caulerprenylols A and B (Scheme S1).[20] The structural similarity of **10** and B provides circumstantial support for **10** being an intermediate. Accordingly, we propose a biosynthetic pathway for caulerprenylols, which involves an oxidation of a diprenylated *p*-xylene into an allyl alcohol A and a deoxy analogue of B (Scheme S1). To our knowledge, this is the first example of in vitro analysis of an oxidative C−C bond formation of a prenylated indole.

Similar modifications may be involved in the biosynthesis of the structurally related nodulisporic acid (Scheme S2).[21] In this case, the JanD homologue affords essentially the same 21,22-diprenylated indole core precursors as **7a**. Starting from this substrate, the JanO homologue oxidizes the alternative prenyl group but by different folding of the A/B rings. A similar mechanism may be applied to the construction of the tricyclic system in ergot alkaloids. In the biosynthesis of chano-clavine,[22] the flavoprotein oxidase EasE, a JanO homologue (38% identity), in collaboration with EasC catalyzes the oxidative cyclization of the prenyl group attached to the indole ring and Trp side chain (Scheme S3). The role of the catalase EasC is not clear, but our observations suggest that EasC may eliminate 2 mol of H_2O_2 generated in the sequential oxidation to avoid degradation of a putative H_2O_2-sensitive intermediate.

In view of the diversification of the diprenyl moiety in the IDT core skeleton, we previously reported that flavoprotein catalyzed oxidation of the prenyl moiety and the subsequent prenyltransferase catalyzed cyclization to give bicyclo[3.2.0]heptane in penitrem biosynthesis.[8] In this study, we have found that essentially the same set of homologous enzymes catalyze diprenylation−flavoprotein oxidative cyclization in shearinine biosynthesis. For construction of the bicyclic core of lolitrem, a third mode, diprenylation-P450-catalyzed oxidative cyclization, was proposed on the basis of gene inactivation data.[23] Our investigation on the IDT biosynthesis revealed that fungi use unexpected diverse mechanisms for construction of the bisprenylation−oxidation sequence (Figure 1).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b02482.

Experimental procedures; analytical and spectral data (PDF)

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Notes

The authors declare no competing financial interest.

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