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Citation	Bioscience biotechnology and biochemistry, 80(3), 426-431 https://doi.org/10.1080/09168451.2015.1104234
Issue Date	2016-03-04
Doc URL	https://hdl.handle.net/2115/64655
Rights	This is an Accepted Manuscript of an article published by Taylor & Francis in Bioscience biotechnology and biochemistry on 2016.3.4, available online: http://www.tandfonline.com/DOI:10.1080/09168451.2015.1104234 .
Type	journal article
File Information	15BBB-solanapyrone-revised-HUSCAP.pdf



Reconstitution of biosynthetic machinery of fungal polyketides: Unexpected oxidations of biosynthetic intermediates by expression host

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Abstracts

Reconstitution of whole biosynthetic genes in *Aspergillus oryzae* has successfully applied for total biosynthesis of various fungal natural products. Heterologous production of fungal metabolites sometimes suffers unexpected side reactions by host enzymes. In the studies on fungal polyketides solanapyrone and cytochalasin, unexpected oxidations of terminal olefin of biosynthetic intermediates were found to give one and four by-products by host enzymes of the transformants harboring biosynthetic genes. In this paper, we reported structure determination of by-products and described a simple solution to avoid the undesired reaction by introducing the downstream gene in the heterologous production of solanapyrone C.

Keywords

biosynthesis; heterologous expression; *Aspergillus oryzae*; solanapyrone; cytochalasin

Heterologous expression of entire biosynthetic gene cluster of fungal natural products has become a practical method to study biosynthetic pathway and to produce natural products.¹ We successfully applied *Aspergillus oryzae* expression system to total biosynthesis of diterpene,² indole diterpenes,³ polyketide⁴ and to genome mining of novel metabolite.⁵ During the projects on fungal metabolites, we have found unexpected reactions catalyzed by host, resulting in formation of by-products derived from intermediates accumulated in the transformants harboring the biosynthetic genes of two polyketide metabolites cytochalasin K (**1**) and solanapyrone A (**2a**). Here, we describe structure determination of modified intermediates and a method for suppressing undesired products.

Tang and co-workers reported identification of the biosynthetic gene cluster of cytochalasin consisting of 7 enzyme genes *ccsA-G*.⁶ And they proposed the cytochalasin biosynthetic pathway as shown in Scheme 1. Recently, we have succeeded to isolate a linear intermediate analog **4** from the *A. oryzae* transformant AO-*ccsAC* harboring polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS) and trans-acting enoyl reductase genes *ccsA* and *ccsC*.⁷ **4** is most likely derived by 2-step reductions of **3** with reductases in *A. oryzae* although many other possibilities can not be excluded. This strongly suggested that CcsA is capable for constructing the octaketide connected with phenylalanine in collaboration with CcsC and that the CcsA R domain catalyzes reductive cleavage of thio-tethered PKS-NRPS product. In this study, HPLC analysis of AO-*ccsAC* extracts also showed a large peak of structurally related metabolite **4A** (Figure 1). UV and ¹H NMR spectra were very similar to **4**. HR-MS data of **4A** showed molecular formula C₂₈H₄₁NO₄ and in its ¹H NMR spectrum, a hydroxymethyl signal was observed at 4.23 ppm in place of allylic methyl group at 1.58 ppm in **4**, suggesting that **4A** is an oxidation product of terminal methyl group. This was further supported by

extensive 2D-NMR data. As we did not introduce any oxidation enzyme gene, **4A** is most likely derived by oxidation of **4** with unknown monooxygenase in *A. oryzae*.

In parallel to cytochalasin biosynthesis, we investigated total biosynthesis of phytotoxins solanapyrones to examine versatility of *A. oryzae* expression system. Solanapyrones (**2a**, **2b**) were isolated as phytotoxins of a causal fungus *Alternaria solani* of potato early blight disease.⁸ And later it was found that **2a** showed a specific inhibitory activity against DNA polymerase β .⁹ Based on co-isolation of chiral [4+2] cycloadducts **2a** (exo) and **2b** (endo), involvement of Diels-Alderase has been proposed (Scheme 2).¹⁰ Extensive studies with feeding experiments established the biosynthetic pathway via Diels-Alder reaction of prosolanapyrone III (**8**) giving **2a** and **2b**,¹¹ and detection of the enzymatic activity and purification of the enzyme solanapyrone synthase have been employed.¹² Recently, a biosynthetic gene cluster of solanapyrone has been identified.¹³ This study showed involvement of four genes *sol1* (PKS), *sol2* (methyltransferase), *sol6* (P450 monooxygenase) and *sol5* (FAD-dependent oxidase, solanapyrone synthase), and functional analyses of two enzymes Sol1 and Sol5 were performed.¹³ Based on this background, we started heterologous expression of four genes to produce solanapyrones.

Initial introduction using pTAex3 harboring *sol1* in *A. oryzae* NSAR1 has successfully gave transformants AO-*sol1*. AO-*sol1* produced desmethyl-prosolanapyrone I (**5**) in 120 mg/kg whose methylation product (Me₂SO₄, acetone) was identical to the synthetic prosolanapyrone I (**6**) (Figure 2).¹⁴ To the AO-*sol1*, we introduced the second gene *sol2* with a plasmid pPTRI-*sol2*. The resultant AO-*sol1/2* produced **6** and significant amount of by-products **11a-11d** (Figure 3). Based on their characteristic UV spectra, we speculated that all of them possessed 2-pyrone moiety derived from **6**. Molecular formula C₁₈H₂₆O₅ of a major product **11a** suggested that **11a** had the same carbon framework of **6**. Compared its ¹H NMR spectrum with that of **6**, up-field shifted doublet methyl signal (1.13 ppm), and two oxymethine signals (3.85, 4.01 ppm) were newly observed in place of a terminal propenyl moiety in **6**, suggesting

that the terminal olefin in **6** was oxidized to a vicinal diol in **11a**. Structure of **11a** was further confirmed by several 2D-NMR data as shown in Figure 3. Similarly, structures of three additional metabolites were determined in a similar manner as shown in the Figure 3. Length of the side chain on the pyrone ring in **11b** (C7), **11c** (C5) and **11d** (C3) suggested that putative C9-product derived by the oxidative cleavage of *vic*-diol in **11a** further degraded by β -oxidation to give **11b-11d**. Thus we speculated that host monooxygenase oxidized terminal olefin in **6** to epoxide **10** in which hydrolysis underwent to give diol **11a** as in the case of the total biosynthesis of paxilline.^{3a} Further oxidations were also likely catalyzed by oxidases in *A. oryzae*. Again, we faced a similar problem which occurred in the cytochalasin project.

To avoid the side reaction, we decided to introduce the downstream gene *sol6*. Our initial attempt to introduce *sol6* to the AO-*sol1/2* failed to produce prosolanapyrone II (**7**).¹⁴ When we reexamined DNA sequence of gene cluster with a program 2ndFind which has recently developed for rapid identification of natural product gene cluster, we found the previous prediction of *sol6* intron was not correct.¹³ Based on this observation, we prepared the plasmids pUSA-*sol2* and pUSA2-*sol2/6* which were subsequently introduced into AO-*sol1* to yield AO-*sol1/2* and AO-*sol1/2/6*. We gratifyingly found that the AO-*sol1/2/6* gave the desired product **7** (12.5 mg/kg) as a single product which was identical to the authentic sample (Figure 2). AO-*sol1/2* gave **6** and **11a** (26 and 9.0 mg/kg) and prolonged fermentation increased the amount of **11a**.

Finally, we introduce the last gene *sol5* with pAdeA-*sol5* and the resultant AO-*sol1/2/6/5* afforded a new product (47.3 mg/kg) (Figure 2). Its molecular formula $C_{19}H_{25}NO_4$ did not matched to those of **2a** and **2b** but solanapyrone C (**9**).⁸ ¹H NMR data of the new product was identical to **9** (Scheme 2). Previous report showed treatment of **2a** with ethanolamine gave **9** in quantitative yield.^{8b} Based on this result, we speculated relatively abundant ethanolamine present in *A. oryzae* reacted with **2a** non-enzymatically to yield **9**.

Our reconstitution study on solanapyrone biosynthetic genes in *A. oryzae*

unambiguously confirmed the pathway proposed by incorporation of isotopically labeled precursors¹¹ and the subsequent gene cluster identification.¹³ Previously, with the synthetic standard, we attempted to detect the intermediates **6-8** in the extracts from large scale fermentation. We failed to detect any intermediate in the *A. solani*. This strongly suggested that the enzyme system for the solanapyrone biosynthesis extremely efficient to convert it to downstream end product solanapyrones. As in the case of *A. solani*, no intermediate was found in the all transformants of *A. oryzae* (Figure 2). Not like chemical synthesis, titer of intermediates increased in later steps (**5**: 120 mg/kg; **6**: 26 mg/kg; **7**: 12.5 mg/kg; **9**: 47.3 mg/kg). The reason of this change is not obvious at this stage.

In this report, we described undesired oxidation of biosynthetic intermediate by the expression host *A. oryzae* in biosynthesis of fungal polyketides cytochalasin and solanapyrone. Similar oxidation products **12A** and **13A** have been reported in the heterologous production in *A. oryzae* of desmethylbassianin (**13**)¹⁵ and PKS-NRPS product **13** from *Magnaporthe grisea*.¹⁶ In all cases including **1** and **2a/2b**, oxidations occurred at the terminal of linear polyketide precursors **12** and **13** (Figure 4). Intriguingly, in the solanapyrone production, similar oxidation did not occur in structurally similar **5** and **7** but did in **6**, suggesting that the corresponding monooxygenase in the formation of **11a** showed strict substrate specificity in the pyrone moiety. In general, it is likely that several oxidation enzymes are responsible for the oxidations at terminal olefin of polyketide intermediates.

Regarding increasing importance of the *A. oryzae* expression system for producing the secondary metabolites,¹⁷ the undesired oxidations found in our two projects could be a potential problem on expressing PKS related genes. Therefore, we attempted to identify enzyme (most likely cytochrome P450) responsible for the oxidation in the transformant AO-*sol1/2* using *A. oryzae* P450 library expressing in yeast which covered 121 out of putative 155 genes.¹⁸ In these experiments, we found two yeast strains carrying CYP52H3 and CYP584G1 were able to give three new products from **6** in the

LC-MS analysis (Figure S1). Intriguingly, CYP52H3 and CYP584G1 showed significant sequence similarity. LC-MS analysis of the extracts from the transformants showed that molecular ion peaks of the new products did not match that of both epoxide and the corresponding diol **11a** (Figure S2). Based on these data, we speculated other monooxygenases in *A. oryzae* is responsible for the undesired oxidations.

In the solanapyrone biosynthesis, we faced a problem that accumulated intermediate **6** in AO-*sol1/2* was oxidized into shunt products **11a-11d**. This problem has been overcome by simply introducing the downstream gene *sol6*. Similar phenomena were observed in the biosynthesis of pyripyropenes^{1a} and paxilline^{3a} (Scheme 3). In these cases, the epoxide intermediates **14/15** were readily converted into the corresponding diols **14A/15A** by a host hydrolase. Introduction of downstream cyclase gene *paxB/pyr4* completely suppressed the undesired diol formation. In the case of conversion of intermediate **16** in the indole diterpene penitrem biosynthesis,^{3c} unidentified degradation of the putative intermediate **17** was suppressed in the transformant having downstream gene *ptmI*. Heterologous production of fungal metabolites sometimes suffered unexpected side reactions by host enzymes^{17b,17c}. In this report, we have introduced a simple solution to avoid the undesired reaction by introducing downstream gene. We are currently working on identification of downstream gene of *ccsA/ccsC* in the cytochalasin project, and its introduction to the AO-*ccsAC* for elucidating the biosynthetic pathway and the production of cytochalasin.

Acknowledgement

This work was supported by MEXT research grant on innovative area 22108002 to H. Oikawa.

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Caption

Scheme 1 Proposed biosynthetic pathway of cytochalasin and undesired oxidation by *A. oryzae*

Scheme 2 Reconstitution of solanapyrone biosynthetic gene cluster in *A. oryzae*

Scheme 3 Undesired side reactions found in heterologous expression of biosynthetic gene clusters in *A. oryzae*

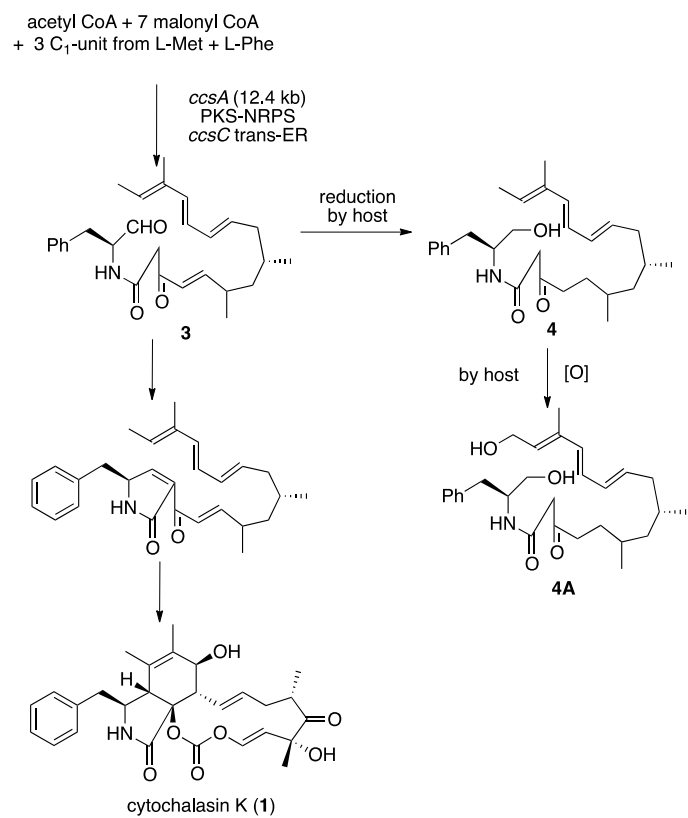
Figure 1 HPLC profile of the extracts from *A. oryzae* transformant AO-*ccsAC*

Figure 2 HPLC profiles of the extracts from *A. oryzae* transformants; i) AO-*sol1*; ii) AO-*sol1/2*; iii) AO-*sol1/2/6*; iv) AO-*sol1/2/6/5*

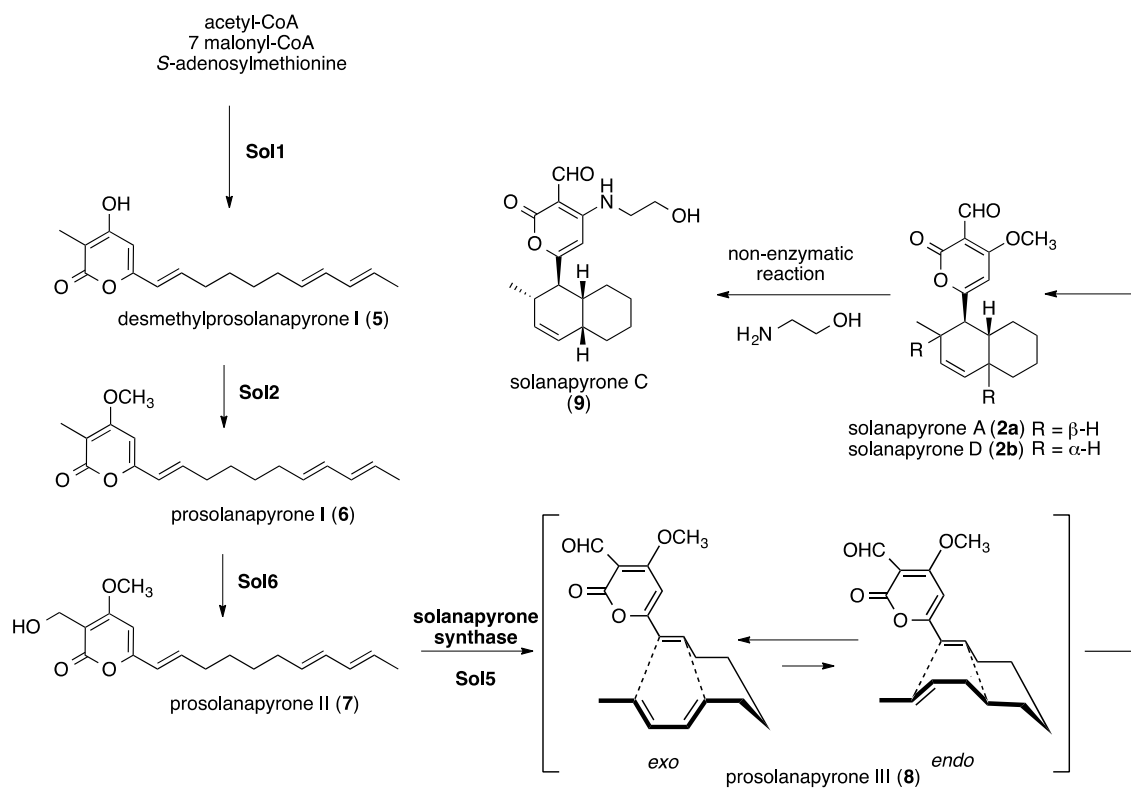
Figure 3 Undesired oxidative transformation of prosolanapyrone I (**6**) in the AO-*sol1/2*

Figure 4 Undesired oxidations of fungal metabolites found in heterologous expression of biosynthetic gene clusters in *A. oryzae*

Scheme 1



Scheme 2



Scheme 3

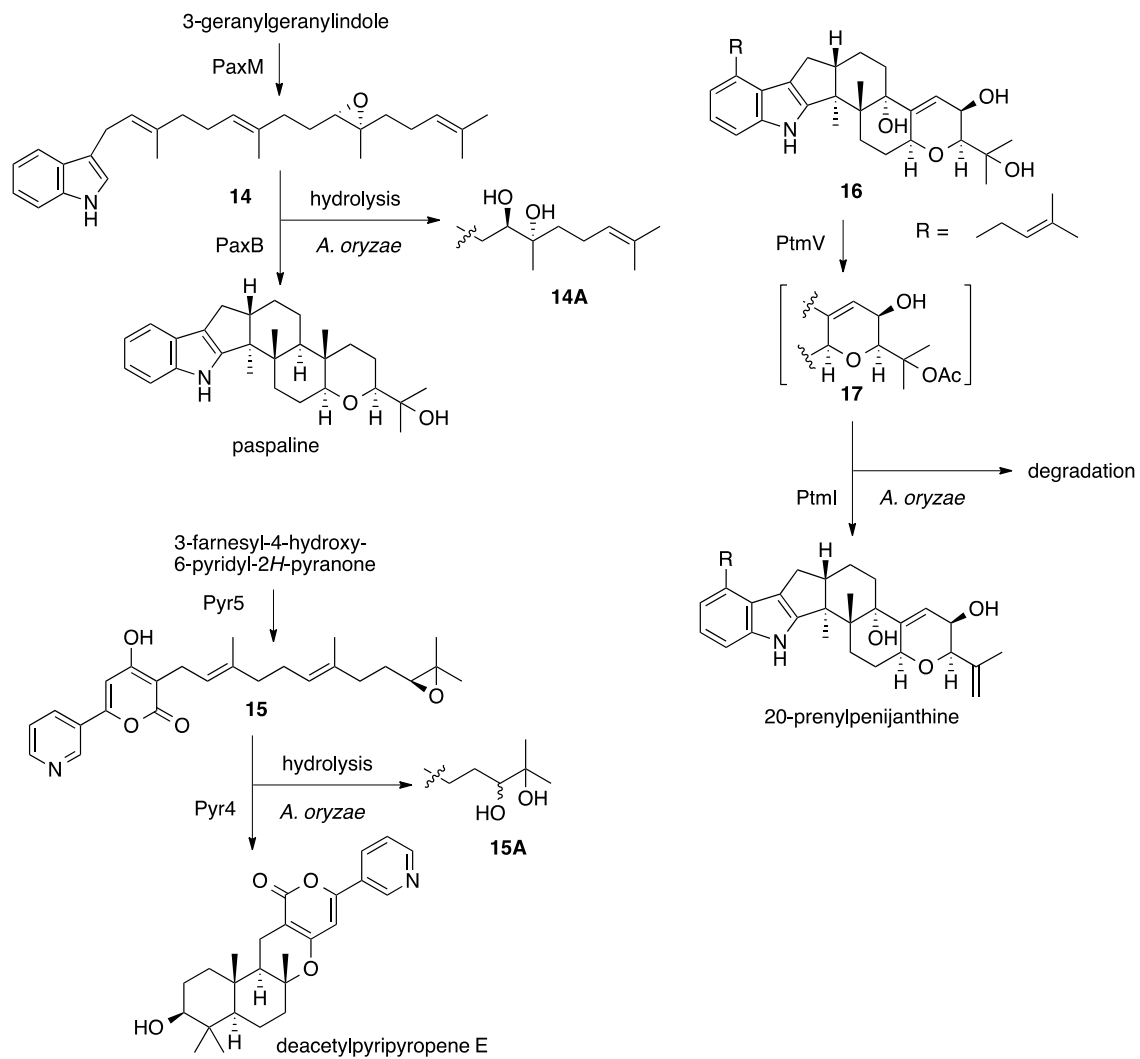


Figure 1

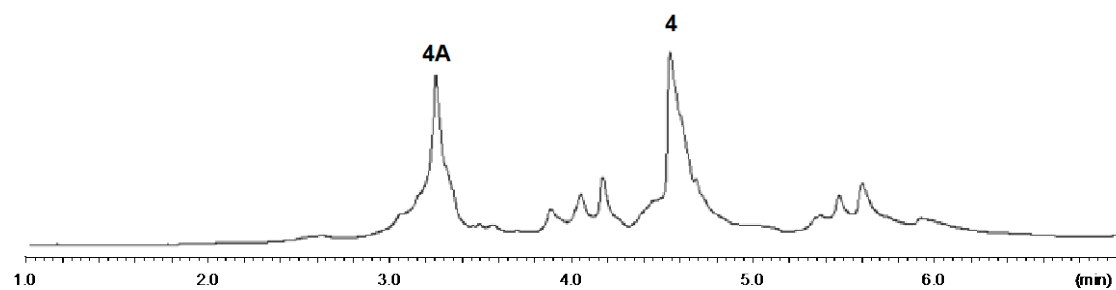


Figure 2

(i) AO-sol1



(ii) AO-sol1/2



(iii) AO-sol1/2/6



(iv) AO-sol1/2/6/5

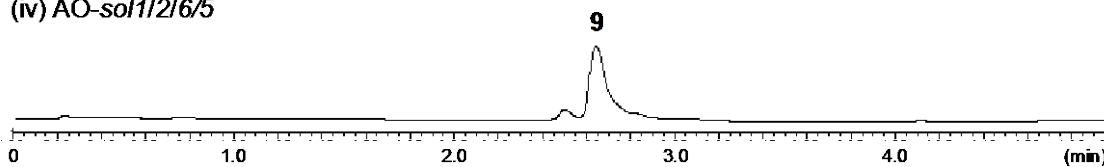


Figure 3

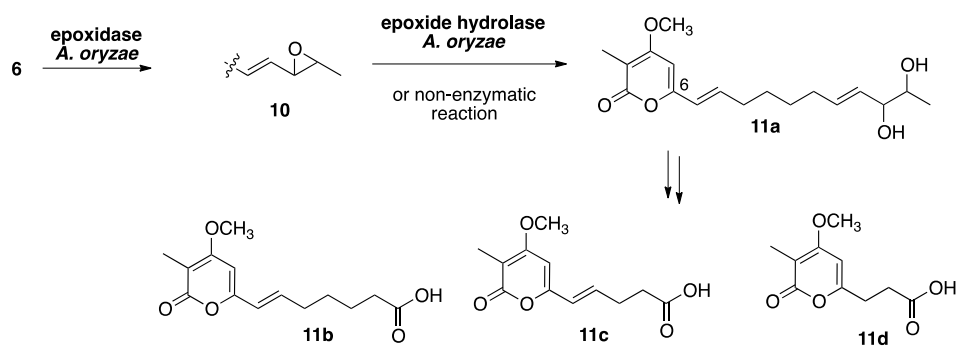


Figure 4

