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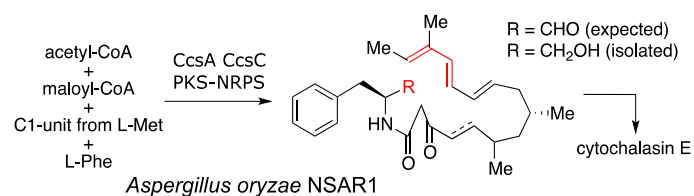
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Biosynthetic Assembly of Cytochalasin Backbone

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Ryuya Fujii, Atsushi Minami, Katsuya Gomi and Hideaki Oikawa



Biosynthetic Assembly of Cytochalasin Backbone

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ABSTRACT

Cytochalasins are an important class of fungal natural products in view of structural diversity and biological activities. Although their biosynthetic studies have been examined extensively, the detailed molecular assembly mechanism remained to be solved. We have succeeded to heterologously express the cytochalasin polyketide synthase-non-ribosomal peptide synthetase (PKS-NRPS) hybrid gene *ccsA* and the trans-acting enoyl-CoA reductase gene *ccsC* in *A. oryzae*. The resultant transformant produced a novel metabolite possessing the cytochalasin backbone. This established that CcsA is capable of constructing the octaketide connected with phenylalanine in collaboration with CcsC, and that CcsA R domain catalyzes reductive cleavage of the thio-tethered PKS-NRPS product.

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1. Introduction

A family of cytochalasan members represented by cytochalasin A¹ (Fig. 1) is well known mycotoxins widely distributed in various fungi.² Its potent inhibition of the actin polymerization affects a wide range of cellular events and thus cytochala synthetically sins are important biochemical tools for

studying fundamental cellular processes.² Molecular diversity of cytochalasan family members such as cytochalasin, chaetoglobosin, aspochalasin is derived from three parts; 1) a pyrrolidinone consisting of distinct amino acid; 2) a cyclohexane with functionalities derived from epoxide; 3) a macrocycle including 11- and 13-membered carbocycles, a lactone and a carbonate with various oxidative decorations (Fig. 1).²⁻⁴

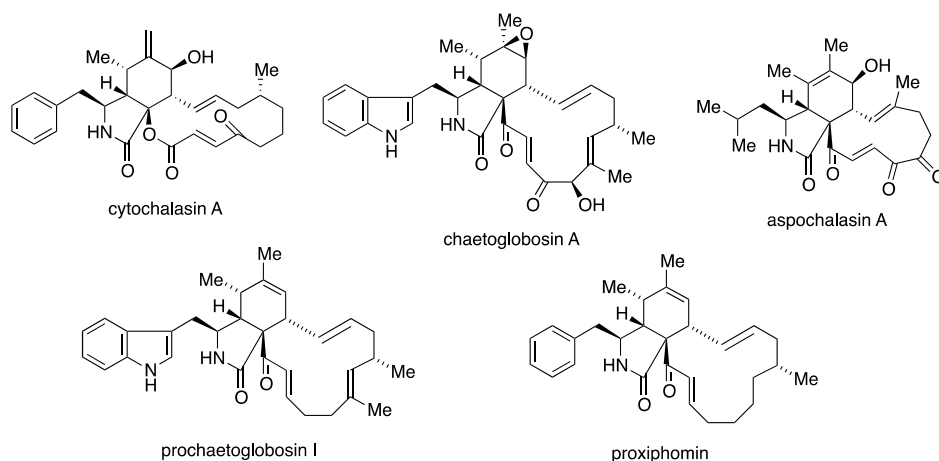
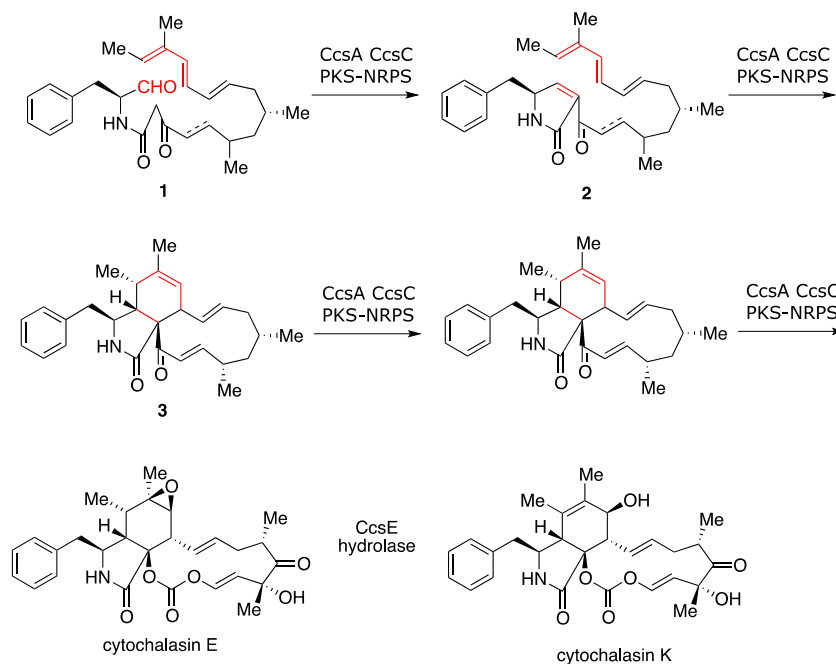


Figure 1. Structures of representative cytochalasin family metabolites from various fungi.

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Scheme 1. Proposed late step biosynthetic pathway of cytochalasins E and K in *Aspergillus clavatus* NRRL1.

The biosynthetic origins of cytochalasan molecular skeleton were established by a number of feeding experiments with isotope-labeled precursors such as acetate and amino acids.^{3,4} Although involvement of the intramolecular Diels-Alder reaction (IMDA) was proposed for construction of the characteristic perhydro-isoindolone with macrocyclic system in the cytochalasin biosynthesis, it was difficult to define the reaction sequence in the late stage modifications.^{2,4} In the biosynthetic study of the structurally related chaetoglobosin, we succeeded to determine the origin of oxygen atoms⁵ and to obtain a series of less oxidized analogs⁶ including prochaetoglobosin I by treatment of the producer strain with the specific P450 inhibitors (Fig. 1). These results enabled us to propose IMDA of the pyrrolinone with linear polyketide chain to give prochaetoglobosin I.⁵ In 2007, using an RNA silencing method, Schumann and Hertweck identified the first cytochalasan biosynthetic gene cluster⁷ consisting of a polyketide synthase-non-ribosomal peptide synthetase (PKS-NRPS) hybrid and oxidative modification enzymes which are expected in our proposal.

Recently, Tang and co-workers successfully identified the biosynthetic gene cluster of cytochalasin E⁸ in *Aspergillus clavatus* NRRL1.⁹ This cluster consisted of eight genes for the backbone construction (*ccsA*: PKS-NRPS, *ccsC*: trans-acting enoyl-CoA reductase (trans-ER)), modification enzymes (*ccsBDG*: oxidative modifications, *ccsE*: hydrolase), and transcription factor (*ccsR*) whose functions are reasonably assigned except hypothetical protein (*ccsF*) (Schemes 1, 2). These proposals are essentially same as the proposed biosynthetic pathway of chaetoglobosins.^{5,7} Our continuous interest on a Diels-Alderase prompted us to examine the molecular assembly mechanism of cytochalasin. Herein, we describe novel metabolites from the transformants by introducing two genes (*ccsA* and *ccsC*) in a heterologous host *Aspergillus oryzae* and discuss the construction mechanism of the cytochalasin core scaffold.

2. Results and Discussion

To date, only three PKS-NRPS hybrids such as TenS (tenellin),¹⁰ CpaS (cyclopiazonic acid),¹¹ ApdA (aspyridone)¹² were successfully expressed in heterologous hosts and studied in

detail. TenS and ApdA require trans-ER (TenC, ApdC) for complementation of the inactive modular ER. To examine the function of the cytochalasin PKS-NRPS CcsA, we expressed *ccsA* and the trans acting ER gene *ccsC*. For this purpose, we used the auxotrophic mutant *A. oryzae* NSAR-1¹³ aiming to the reconstitution of cytochalasin machinery as in the case of other fungal natural products.¹⁴ After preparing the expression plasmids pTAex3¹⁵-*ccsA*, and pUSA¹⁶-*ccsC*, the resultant plasmids were transformed into the *A. oryzae* NSAR-1 in a stepwise manner. The *ccsA/ccsC* transformant was cultured in CD medium supplemented with maltose to induce target gene expression.^{14a} HPLC analysis of the partially purified fraction from the mycelial extracts showed a new peak that were not found in a control culture of the wild-type strain (Fig. 3). To determine their structures, the mycelial acetone extract of the *ccsA/ccsC* double transformant obtained from large-scale incubation using solid medium was partitioned with hexane-acetonitrile. The resultant acetonitrile extract was purified by silica gel column chromatography and by reverse-phase HPLC to give the major product **4** (3.0 mg/kg medium).

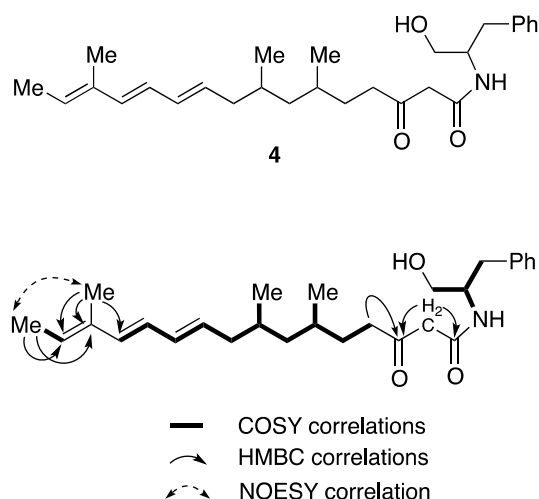


Figure 2. Structure and NMR data of metabolite 1 from *ccsA/ccsC* double transformants.

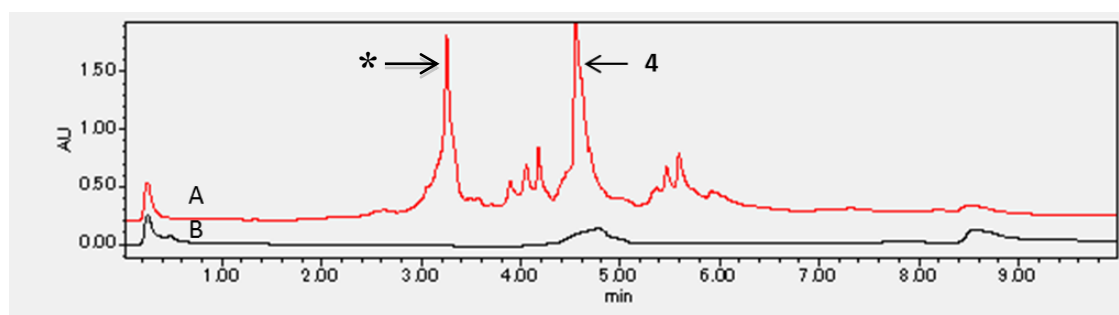


Figure 3. HPLC analysis of mycelial extracts from *A. oryzae* NSAR1. A) Partially purified extracts from a transformant carrying *ccsA* and *ccsC* genes; B) extracts from wild type strain. * is an unidentified peak that is neither **1** nor **2** (ESI-MS analysis). Crude extracts were analyzed by a Water UPLC system including a ACQUITY UPLC BEH C18 (1.7 μ m, ϕ 2.1 mm x 150 mm). A gradient of water and acetonitrile solvent system was used. 10 μ L of the extract solution was injected for each round 15 minutes HPLC program (0 min, 20% CH₃CN; 5 min, 90% CH₃CN, 10 min, 90% CH₃CN, 12 min, 20% CH₃CN, 15 min, 20% CH₃CN)

Table 1. NMR data of the compound **4** (500 MHz, C₆D₆)

Carbon No.	δ_C	δ_H	(mult, <i>J</i> in Hz)
1	167.2		
2	50.2	2.78	(d, 16.7)
		2.85	(d, 16.7)
3	207.5		
4	42.4	1.93	(t, 7.5)
5	32.3	1.42	(m)
		1.21	(m)
6	31.0	1.68	(m)
7	45.5	1.08	(m)
		1.17	(m)
8	32.1	1.29	(m)
9	42.7	2.07	(dd, 13.8, 6.3)
		1.96	(dd, 13.8, 7.3)
10	133.2	5.68	(dt, 14.6, 7.3)
11	134.3	6.22	(dd, 14.6, 7.0)
12	127.9	6.31	(m)
13	137.5	6.32	(m)
14	136.3		
15	127.9	5.52	(q, 7.1)
16	15.1	1.58	(d, 7.1)
17	20.7	0.69	(d, 6.5)
18	20.4	0.85	(d, 6.5)
19	13.3	1.70	(s)
1'	65.5	3.47	(dd, 10.9, 3.8)
		3.37	(dd, 10.9, 5.3)
2'	54.8	4.23	(m)
3'	38.4	2.70	(d, 7.5)
4'	139.6		
5'	130.8	7.26	(m)
6'	129.9	7.27	(m)
7'	129.7	7.20	(m)
8'	129.9	7.27	(m)
9'	130.8	7.26	(m)
NH		6.90	(d, 7.0)

HR-EIMS of the isolated **4** indicated a molecular formula of C₂₈H₄₁NO₃ (unsaturation: 9). ¹H- and ¹³C-NMR spectral data (Table 1) indicated the presence of phenyl group (δ_H 7.20-7.27, m, 5H), two ketone (δ_C 207.5) and amide (δ_C 167.18) carbonyl groups, suggesting that its structure is closely related with that of cytochalasin. Extensive NMR data analysis, including COSY, HSQC, and HMBC, enabled us to determine the structure of **4** as shown in Fig. 2. Observed NOEs between the terminal (C16) and the branched (C19) methyl groups established the stereochemistry of trisubstituted olefin as *E*. The structure thus

determined is nearly identical to a proposed octaketide intermediate **1** consisting of a conjugated triene, three branched methyl group and a β -ketoamide connected with phenylalanine. For formation of putative pyrrolinone precursor **2**, Hertweck and co-workers proposed two routes; route A proceeds via Claisen condensation, reduction and dehydration; route B via reduction and Knoevenagel condensation (Scheme 2).^{2b} The structure of **4** suggested that the CcsA reductase (R) domain reductively liberated the putative thioester intermediate appended to the CcsA thiolation (T) domain to give **1**. Isolation of proxiphomin¹⁷ and procheatoglobosin I⁶ (Fig. 1), non oxidatively modified putative precursors in the corresponding cytochalasin producing fungi, provides the circumstantial evidence that the first free linear intermediate has an enone moiety.

Isolation of acyclic alcohol **4** clearly indicated that the PKS-NRPS CcsA is responsible for the cytochalasin backbone assembly in collaboration with the trans-ER CcsC, and also provided the first direct evidence for involvement of the linear intermediate before the putative Diels-Alder reaction and the Bayer-Villiger oxidation(s) (Schemes 1 and 2). Our preliminary examination failed to detect putative Diels-Alder adduct **3** in the extracts of the *ccsA/ccsC* transformant. This suggests that the *ccsF* whose function is not assigned catalyzes both Knoevenagel condensation and [4+2] cycloaddition. The reduced product **4** can be converted into putative intermediate analogs dihydro-**1** and pyrrolinone dihydro-**2** via oxidation of primary alcohol and the subsequent Knoevenagel condensation. Therefore, isolation of **4** opens a door to investigate the intriguing IMDA in vitro using the candidate enzyme including CcsF.

3. Conclusion

In summary, we have succeeded to heterologously express the cytochalasin PKS-NRPS *ccsA* and the trans aciting ER *ccsC* in *A. oryzae*. The resultant transformant produced metabolite **4** possessing the cytochalasin backbone. This established 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. The product obtained in this study may enable a rapid access to the IMDA substrate. Currently, we are working on in vitro study of the putative cytochalasin Diels-Alderase most likely CcsF.

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