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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 remains 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 *Aspergillus 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.

Introduction

A family of cytochalasan members represented by cytochalasin A[1] (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 cytochalasins are important biochemical tools for studying fundamental cellular processes.[2] Molecular diversity of cytochalasan family members such as cytochalasin, chaetoglobosin, and 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]

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 cheatoglobosin, we succeeded to determine the origin of oxygen atoms[5] and to obtain a series of less oxidized analogs[6] 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 cluster[7] consisting of a polyketide synthase–non-ribosomal peptide synthetase (PKS–NRPS) hybrid and oxidative modification enzymes which are expected in our proposal.

Recently, Tang et al., successfully identified the biosynthetic gene cluster of cytochalasin E[8] 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 the hypothetical protein (*ccsF*) (Schemes 1 and 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.

Results and discussion

To date, only three PKS–NRPS hybrids such as TenS (tenellin),[10] CpaS (cyclopiazonic acid),[11] and ApdA (aspyridone)[12] were

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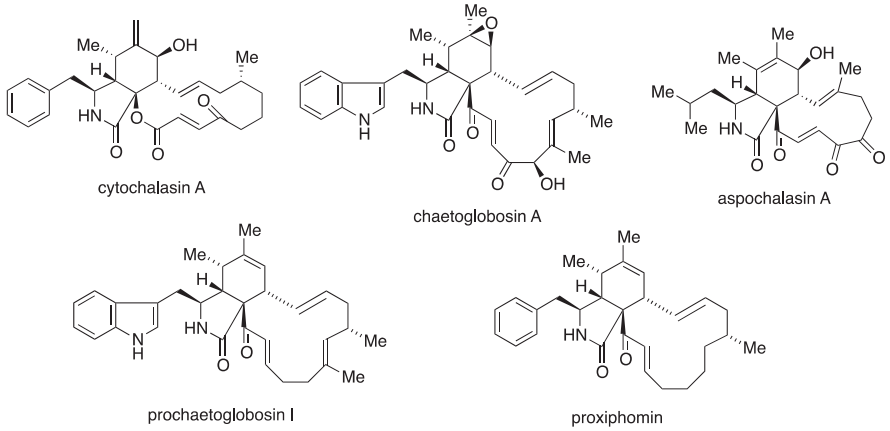
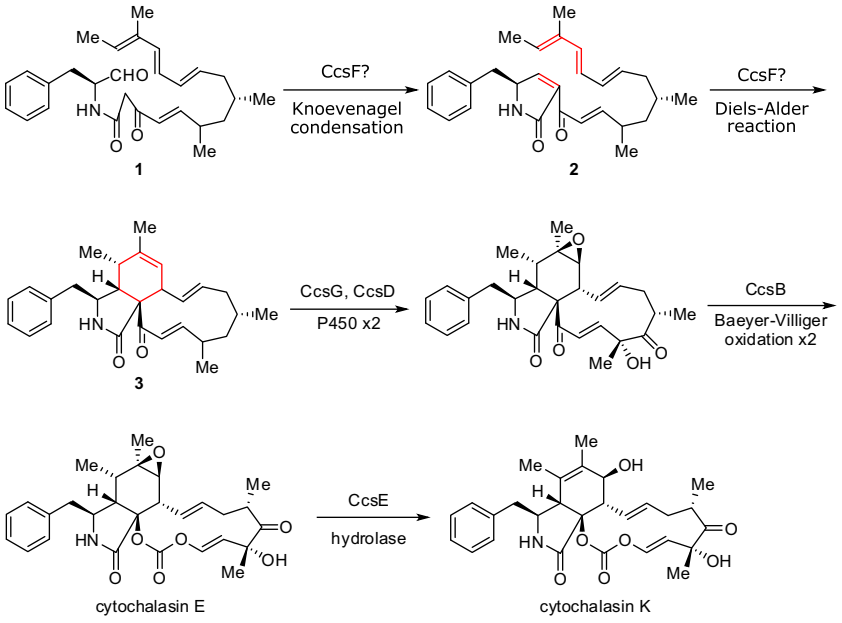


Figure 1. Structures of representative cytochalasan family metabolites from various fungi.



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

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* NSAR1[13] aiming to the reconstitution of cytochalasin machinery as in the case of other fungal natural products.[14] After preparing the expression plasmids pTAex3[15]-*ccsA* and pUSA[16]-*ccsC*, the resultant plasmids were transformed into the *A. oryzae* NSAR1 in a stepwise manner. The *ccsA*/*ccsC* transformant was cultured in the 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 was not found in a control culture of the wild-type strain (Fig. 2). To determine their structures, the mycelial acetone extract of the *ccsA*/*ccsC* double transformant obtained from large-scale incubation using a 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).

HR-ESI-MS of the isolated **4** indicated a molecular formula of $C_{28}H_{41}NO_3$ (unsaturation: 9). ^1H and ^{13}C NMR spectral data (Table 1) indicated the presence of a 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 Figure 3. 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 groups, and a β-ketoamide connected with phenylalanine. For the formation of putative pyrrolinone precursor **2**, Hertweck et al., proposed two routes; route A proceeds via Claisen condensation, reduction, and dehydration;