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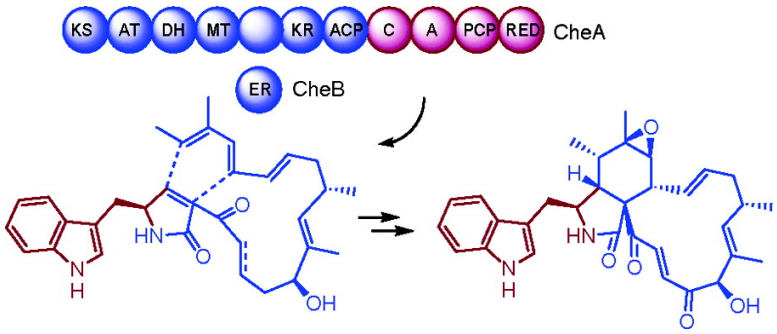
J. Am. Chem. Soc., **2007**, 129 (31), 9564-9565• DOI: 10.1021/ja072884t • Publication Date (Web): 18 July 2007



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Published on Web 07/18/2007

Molecular Basis of Cytochalasan Biosynthesis in Fungi: Gene Cluster Analysis and Evidence for the Involvement of a PKS-NRPS Hybrid Synthase by RNA Silencing

Julia Schümann and Christian Hertweck*

Leibniz-Institute for Natural Product Research and Infection Biology, HKI, Beutenbergstrasse 11a, 07745 Jena, Germany, and Friedrich-Schiller-University, Jena, Germany

Received April 25, 2007; E-mail: christian.hertweck@hki-jena.de

Cytochalasans comprise a diverse group of structurally intriguing fungal metabolites that are well-known for their distinctive biological activities.[1,2] Several members of this family have been identified as antibiotic, antiviral, antiinflammatory, and antitumoral agents. At higher concentrations they interfere with glucose transport and with the release of thyroid and growth hormones. The most important properties of the cytochalasans, however, is the specific binding to actin filaments.[1,2] While mitosis remains unaffected, cytochalasans efficiently inhibit cytokinesis. As a result, giant cells with multiple nuclei or denucleized cells emerge, which is reflected by the greek terms kytos = cell, and chalasis = relaxation. Since their discovery cytochalasans have emerged as important chemical tools in cell and molecular biology and represent ambitious targets for total synthesis.[3] Common to all members of this group is the isoindolone moiety fused to a macrocycle. Isotope labeling experiments revealed that cyctochalasan biosynthesis involves the formation of an acetate and methionine-derived octa- or nonaketide chain and the attachment of an amino acid[4−6] (Figure 1). Depending on the type of metabolites, phenylalanine (cytochalasins, zygosporins), tryptophan[7] (cytochalasins, chaetoglobosins), or leucine (aspochalasins) is incorporated.

It has long been a matter of speculation if this intriguing tricyclic ring system is generated by a Diels−Alder-type reaction.[8−12] However, to date there has not been a single report on the molecular basis of cytochalasan biosynthesis. To shed more light onto the biogenesis we sought to clone the responsible genes. For this purpose we generated a genomic cosmid library of chaetoglobosin (A and C) producer *Penicillium expansum*. Since the polyketide moiety of the cytochalasins implies the involvement of a reducing and C-methylating polyketide synthase, the 5000 clone library was screened by PCR using degenerated C-methyltransferase primers[13,14] and by dot blot hybridization with a heterologous probe, containing CMeT, KR, and ACP of the *Fusarium venenatum* fusarin C synthase.[15] Three overlapping cosmids were identified that harbor genes coding for a rare fungal PKS-NRPS hybrid synthase. Two clones appeared to contain the whole chaetoglobosin (*che*) biosynthesis gene cluster (Figure 2). Shotgun sequencing and primer walking led to the contiguous sequence of the *che* gene locus. Database searches revealed that the deduced gene products of the chaetoglobosin gene cluster are most similar to a multidomain PKS-NRPS hybrid synthase (ApdA) involved in aspyridone biosynthesis in *Aspergillus nidulans*.[16] The flanking regions encode an enoyl reductase (CheB) and three putative oxygenases, CheD, CheE, and CheG. Chaetoglobosin biosynthesis is likely regulated by the two putative C6 transcription factors CheC and CheF (Table S1).

Correlating the *che* gene cluster with chaetoglobosin biosynthesis proved to be a challenging task. It is known that genetic manipulation of *Penicillium* can be cumbersome, and in fact all attempts to generate a targeted gene knock-out in *P. expansum* failed. Likewise,

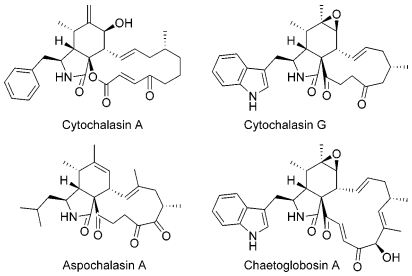

Figure 1. Structures of representative cytochalasans.

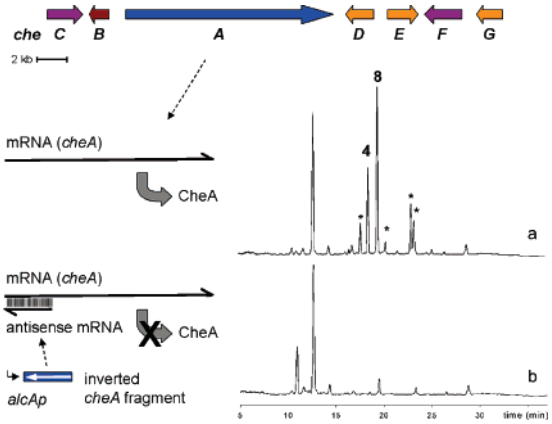

Figure 2. Organization of the *che* biosynthesis gene cluster. Deduced functions of gene products: CheA, PKS-NRPS; CheB, enoyl reductase; CheC, CheF, regulators; CheD, CheE, CheG, oxygenases. HPLC profiles of extracts from (a) *P. expansum* wild-type producing chaetoglobosin A and C (**4** and **8**) and (b) mutant with silenced cytochalasan synthase. Asterisks indicate peaks of unidentified chaetoglobosin derivatives.

we were not successful in the heterologous expression of the entire or partial gene cluster in *Aspergillus*, possibly due to different splicing and regulation mechanisms. As the last possible alternative, we tested RNA-mediated gene silencing.[17] A 2.9 kb fragment of the PKS-NRPS hybrid gene, containing the KS and part of the DH domain, was cloned in inverted direction into vector pAL4 downstream of the inducible alcohol dehydrogenase promotor *alcAp*.[18] This silencing construct was transferred into an expression vector containing the acetamidase gene *amdS* as selection marker. Transforming the *Penicillium expansum* wild-type strain with the resulting plasmid resulted in several acetamide resistant mutant strains. By Southern blotting we could identify several mutants containing ectopic integrations of the silencing construct. Although RNA silencing should significantly reduce target mRNA concentration it is well-known that inactivation may only be partial and not necessarily lead to a null phenotype like a gene knockout.[17] However, inactivation of CheA in *Penicillium expansum* gave a surprisingly clear result (Figure 2b). Production of cheatoglobosins

10.1021/ja072884t CCC: $37.00

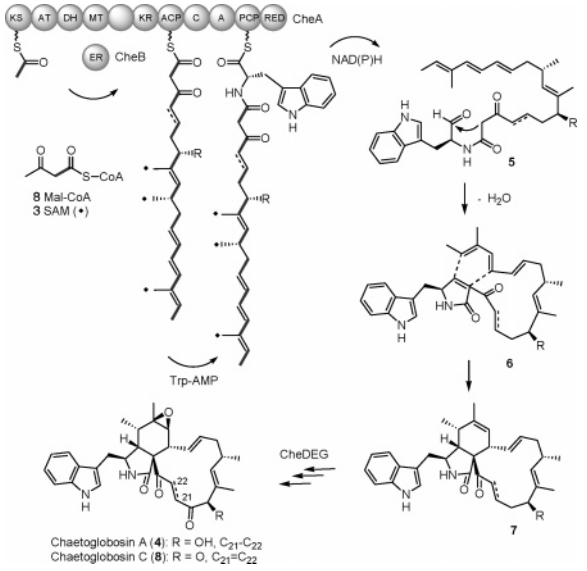

Figure 3. Architecture of the cytochalasan synthase CheA and model for the biosynthesis of chaetoglobosin A.

was nearly completely abolished under inductive silencing conditions, unequivocally proving that CheA correlates with cheatoglobosin biosynthesis. The catalytic activities encoded by the *che* cluster together with classical isotope labeling studies[7] now provided the ground for a new biosynthetic model. CheA is closely related to pyrrolinone synthases involved in the fusarin,[19] equisetin,[20] tenellin,[21] and aspyridone[16] pathways. This information provides valuable insights into the mode of backbone assembly in the cytochalasan pathway. First, the nonaketide moiety is generated in an iterative fashion by the PKS part of the hybrid synthase (Figure 3). Because this module lacks a functional ER domain (blank globe), ER CheB is likely used *in trans* as in lovastatin biosynthesis.[22] Once the polyketide chain assembly is accomplished, the thioester is passed on to the NRPS and condensed with an activated tryptophan (Table S2). The presence of the *C*-terminal reductase domain implies a reductive downloading mechanism. The resulting aminoaldehyde **5** would be trapped by a Knoevenagel condensation, yielding a disubstituted 1,5-dihydro-pyrrol-2-one **6**. This key subunit is in fact a potent dienophile, as demonstrated by biomimetic syntheses using related compounds.[23−30] Nonetheless, considering the rather harsh reaction conditions required for chemical synthesis, the cytochalasan synthase obviously promotes the cyclization reaction, either through enzyme catalysis or by acting as an entropic trap. Interestingly, CheA is closely related to the lovastatin[22] and equisetin[20] synthases, both of which have been implicated in pathways involving Diels−Alder reactions. However, the topology of the cyclization reaction differs in cytochalasan biosynthesis, which affords a macrocylization.

Since no epimerization domain is present in the NRPS part of the cytochalasin synthase, the L-Trp building block remains unaltered, setting the stereochemistry of the resulting pyrrol-2-one. Consequently, the absolute and relative configuration of the isoindolone **7** can be rationalized by the predicted diastereofacial selectivity of the cycloaddition (Figure 3). In conclusion, CheA promotes or at least sets the stage for a novel type of natural Diels−Alder reaction in the synthesis of isoindolones.[9,10,31] The putative Cyt P450 monooxygenases CheD and CheG and the FAD-dependent monooxygenase CheE are the best candidates to catalyze the oxidative polyketide tailoring reactions of **7** to yield **4** and **8**. This is in full accord with earlier Cyt P450 inhibitor studies[32] and exemplifies the generation of structural diversity by the PKS-NRPS and associated modifying enzymes.

In summary, we have cloned and sequenced the first gene cluster coding for the biosynthesis of a cytochalasan. We have established a specific RNA silencing method for analyzing cytochalasan biosynthesis in *Penicillium*. The successful inactivation provided direct evidence that the fungal PKS-NRPS hybrid synthase CheA plays an essential role in chaetoglobosin formation. According to its architecture the multifunctional enzyme catalyzes polyketide assembly as well as the attachment of L-Trp and reductive downloading. The latter sets the stage for the immediate formation of the 1,5-dihydro-pyrrol-2-one dienophile, which is capable of reacting with the polyene portion in a Diels−Alder fashion. This work allows the first insight into the remarkable cytochalasan pathway and provides the ground for engineering analogues.

Acknowledgment. This project has been financially supported by the EU (EUKETIDES). We thank K. Scherlach (HKI) for assistance in HPLC analysis and C. Lazarus (Bristol) for the kind gift of the FUSS gene probe.

Supporting Information Available: Table of deduced gene functions and all experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA072884T