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Production of New Cladosporin Analogues by Reconstitution of the Polyketide Synthases Responsible for the Biosynthesis of this Antimalarial Agent

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Abstract: *The antimalarial agent cladosporin is a nanomolar inhibitor of the Plasmodium falciparum lysyl-tRNA synthetase, and exhibits activity against both blood- and liver-stage infection. Cladosporin can be isolated from the fungus Cladosporium cladosporioides, where it is biosynthesized by a highly reducing (HR) and a non-reducing (NR) iterative type I polyketide synthase (PKS) pair. Genome sequencing of the host organism and subsequent heterologous expression of these enzymes in Saccharomyces cerevisiae produced cladosporin, confirming the identity of the putative gene cluster. Incorporation of a pentaketide intermediate analogue indicated a 5+3 assembly by the HR PKS Cla2 and the NR PKS Cla3 during cladosporin biosynthesis. Advanced-intermediate analogues were synthesized and incorporated by Cla3 to furnish new cladosporin analogues. A putative lysyl-tRNA synthetase resistance gene was identified in the cladosporin gene cluster. Analysis of the active site emphasizes key structural features thought to be important in resistance to cladosporin.*

Cladosporin (**1**; asperentin) is a tricyclic octaketide that is produced by several fungal species, including *Cladosporium*,[1–4] *Chaetomium*,[5] *Penicillium*,[6] *Eurotium*,[7,8] and *Aspergillus*.[9] Cladosporin exhibits interesting bioactivity, including antifungal, antibiotic, and plant-growth inhibitory properties and anti-inflammatory responses in mouse lung tissue.[10] Recently, cladosporin has been shown to be a nanomolar inhibitor of *Plasmodium falciparum* blood- and liver-stage proliferation.[11] Although many antimalarial agents currently exist, endoperoxides are the only class of molecules for which resistance has not significantly developed, and even these do not inhibit the asymptotic liver-stage infection. The bioactivity of cladosporin represents a promising lead for the treatment of malaria, and several studies on this topic have been published.[12,13]

Previously, we investigated the biosynthesis of several related fungal polyketides that belong to the resorcylic acid lactone (RAL) and dihydroxyphenylacetic acid lactone (DAL) containing polyketides, including hypothemycin (RAL type),[14] radicicol (RAL type),[15] and dehydrocurvularin (DAL type).[16–19] Biosynthesis of these polyketides requires cooperative action of two iterative type I polyketide synthases (PKSs): a highly reducing (HR) PKS and a non-reducing (NR) PKS. Based on structural similarities, we hypothesized that cladosporin is also biosynthesized by a HR and an NR PKS. Our early work assigned the absolute stereochemistry of cladosporin.[2,3] More recently, the total synthesis of cladosporin and its diastereomer, isocladosporin, has been reported.[20,21] To better understand PKS assembly and enable analogue production by synthetic biology, we sought to heterologously express and reconstitute cladosporin expression in *Saccharomyces cerevisiae*. Hence, we sequenced the genome of the producer, *Cladosporium cladosporioides* UAMH 5063. This resulted in 30 Mb of genomic information over a total of 764 contigs. The genomic data was annotated using Antibiotics & Secondary Metabolite Analysis Shell (antiSMASH v2.0).[22] The software identified 50 putative secondary-metabolite gene clusters in the genome of *C. cladosporioides*, seven of which encode type I iterative PKSs. One gene cluster possessed high sequence homology to those of hypothemycin and zearalenone. Spliced gene sequences contained within this gene cluster were identified using the hidden Markov model (HMM) based software FGENESH (Softberry),[23] and the resulting intron-less sequences were analyzed individually by BLAST (NCBI; Figure 1).

The HR and NR PKS contained within the gene cluster, Cla2 and Cla3, respectively, were cloned and expressed in *S. cerevisiae* BJ5464-NpgA (see the Supporting Information). The proteins were expressed from single transformants, and with minimal optimization, cladosporin was isolated from double transformants at a titer of $10\,mg\,L^{-1}$ (see the Supporting Information). Its identity was confirmed by LC-ESI-MS, using combined retention time matching with accurate mass matching, and NMR analysis (see the Supporting Information). This confirmed the identification of the cladosporin gene cluster in *C. cladosporioides*. As *S. cerevisiae* is a well-studied organism for the heterologous production of other natural products, such as artemisinic acid[24] and lovastatin,[25] this result constitutes a significant step towards the large-scale production of cladosporin.

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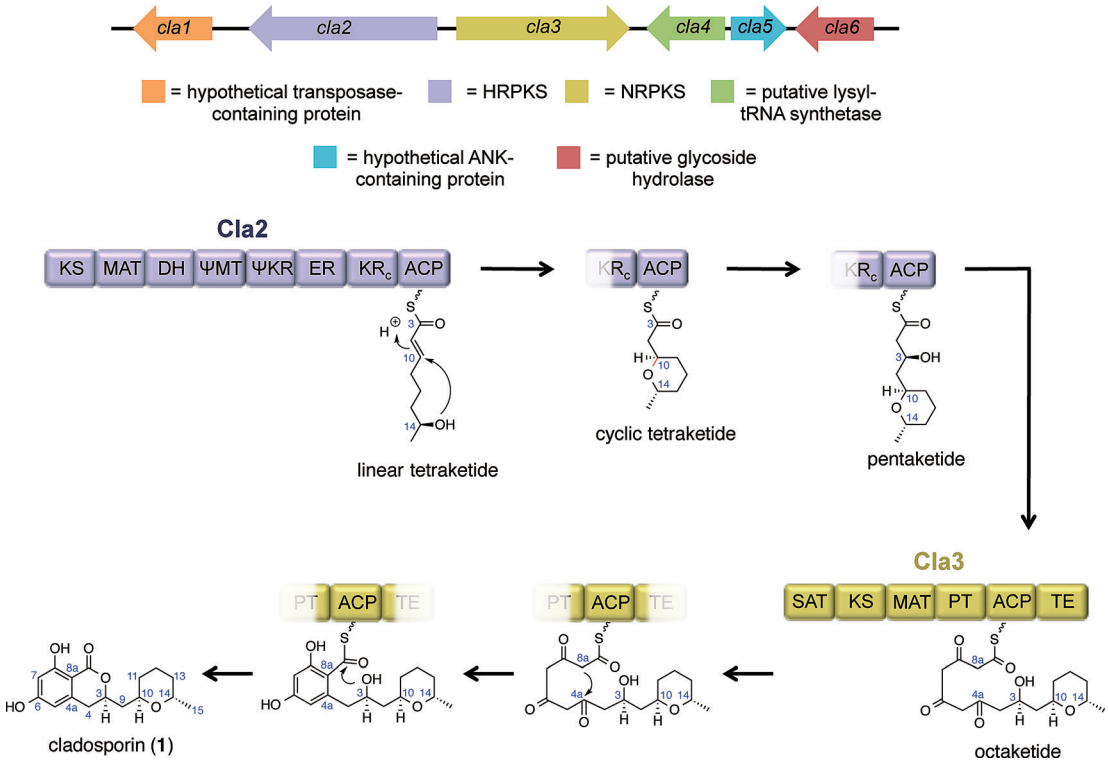
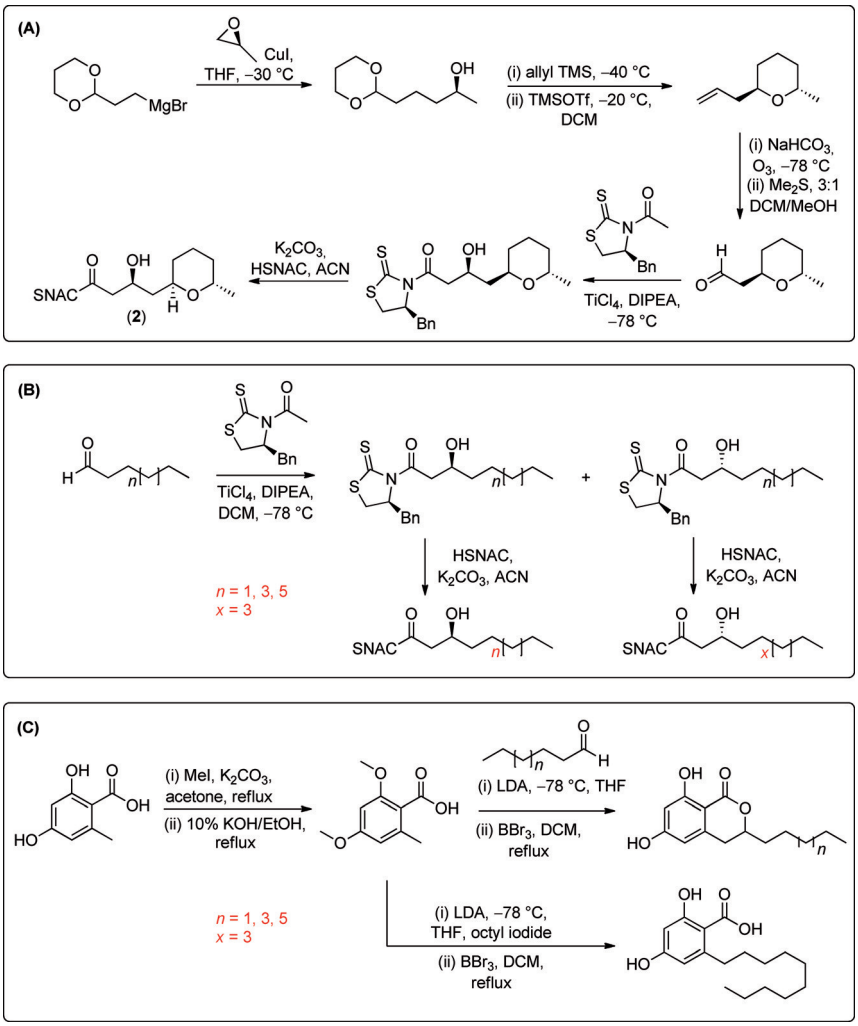


Figure 1. Cladosporin gene cluster in *Cladosporium cladosporioides* and putative biosynthesis by the HR PKS Cla3 and NR PKS Cla2. KS: ketosynthase; MAT: malonyl-CoA:ACP acyltransferase; DH: dehydratase; ψMT: pseudo C-methyltransferase; ψKR: structural ketoreductase; ER: enoylreductase; KRc: catalytic ketoreductase; ACP: acyl carrier protein; SAT: starter unit:ACP transacylase; PT: product template; TE: thioesterase.

To probe the biosynthesis of cladosporin in vivo, we conducted advanced precursor feeding studies. The necessary reduction of the C3 position of cladosporin suggested that the HR PKS, Cla2, is responsible for biosynthesis up to the pentaketide stage, including of the THP ring (see the Supporting Information),[3] whereas the three subsequent ketide extensions with no reduction are catalyzed by Cla3. In accord with observations on other NR PKSs that require an HR PKS partner,[14] Cla3 is not able to load and produce the product on its own from malonyl-CoA. Hence, the proposed pentaketide intermediate was synthesized as an *N*-acetylcysteamine (SNAC) thioester (**2**; Supporting Information) using a modified literature procedure[20] (Scheme 1 A), and fed to purified Cla3 with malonyl-CoA. After incubation for 24 hours, metabolites were extracted and analyzed by LC-ESI-MS (see the Supporting Information). The identification of cladosporin (**1**), by combined retention time matching with accurate mass matching, confirmed that the pentaketide intermediate analogue **2** is recognized by Cla3. It is likely the final product of the HR PKS Cla2, which remains covalently bound as a thioester until transfer to the SAT domain of the NR PKS Cla3 occurs. This "5+3" ketide assembly of cladosporin represents the first example of its type, with other DAL- and RAL-type polyketides assembled in a "4+4" (dehydrocurvularin),[17–19] "6+3" (hypothemycin, zearalenone),[14,26] and "5+4" (radicicol)[15] fashion. Thus THP ring formation appears to be catalyzed by Cla2, this oxa-Michael-type cyclization onto an unsaturated thioester intermediate at the tetraketide stage would be highly favored (Figure 1).[3] Recent work has highlighted the ability of nature to catalyze such reactions using PKSs.[27,28]

We synthesized additional substrate analogues as SNAC thioesters, along with their expected products, as shown in Scheme 1 B,C (see also the Supporting Information). β-Hydroxy-substituted substrate analogues were synthesized by titanium-catalyzed aldol reactions followed by direct coupling to HSNAC. The product standards were made starting from global protection of orsellinic acid. Saponification of the ester furnished the common intermediate acid, which then underwent reactions with either aliphatic aldehydes or octyl iodide. Boron tribromide catalyzed deprotection afforded the product standards. The results of the feeding studies with Cla3 are shown in Table 1. Surprisingly, Cla3 accepts unnatural analogues that do not contain the tetrahydropyran ring. The presence of a hydroxy group is vital but its stereochemical configuration is not important for recognition. However, carbon chains longer than ten carbon atoms are not accepted, suggesting a hydrophobic binding pocket of limited size in the enzyme. The promiscuity of Cla3 could allow for the semi-synthesis of new antimalarial agents.

We next conducted homology modeling of the product template (PT) domain using I-TASSER[29–31] and docking studies using AutoDock Vina[32] to better understand how the THP ring fits into the active site (Figure 2). The most suitable homology used by I-TASSER as the top threading template was the crystal structure of the PT domain from PksA, the NR PKS responsible for the biosynthesis of aflatoxin B1 in *Aspergillus parasiticus* (PDB ID 3HRQ).[33] Our homology



Scheme 1. A) Synthesis of pentaketide analogue **2**. B) Synthesis of β-hydroxy analogues. C) Synthetic route to product standards. ACN = acetonitrile, DCM = dichloromethane, LDA = lithium diisopropylamide, DIPEA = diisopropylethylamine, Tf = trifluoromethanesulfonyl, TMS = trimethylsilyl.

model indicates that the THP ring can be readily accommodated in the PT active site. As the ketide is extended in the PT domain, it appears to curve around on itself up until the octaketide stage where the C8a atom is now in close enough proximity to the C4a atom for cyclization to occur. The resulting aromatized intermediate could then be transferred to the TE domain, where hydrolysis and/or lactone formation would produce cladosporin (**1**).

Cladosporin's proposed antimalarial mode of action is inhibition of the *P. falciparum* lysyl-tRNA synthetase (KRS1).[11,12,34] Interestingly, a putative lysyl-tRNA synthetase gene, *cla4*, is contained within the cladosporin cluster. Resistance genes can often be found close to the biosynthetic machinery of natural products. It is possible that *Cla4* may infer cladosporin resistance. Hoephner et al. found that the *S. cerevisiae* lysyl-tRNA synthetase KRS1 is not inhibited by cladosporin, and that this resistance is related to two active-site residues, Gln_{324} and Thr_{340}.[11] Replacement of Gln_{324} with a hydrophobic valine led to a 5.7-fold increase in cladosporin sensitivity, whereas replacement of Thr_{340} with a less bulky serine increased sensitivity by a factor of 10.4. The corresponding double mutant was 38.7-fold more sensitive to cladosporin. Therefore, it appears that a prerequisite of cladosporin resistance is the presence of a polar group at position 324 and a bulky group at position 340, a requirement that is met by the analogous residues in *Cla4*. This is supported by the co-crystal structure of *P. falciparum* KRS1 and cladosporin, wherein the isocoumarin moiety of cladosporin has a similar orientation as the adenine of ATP, with its aromatic ring in a hydrophobic interaction with Val_{328} (see the Supporting Information). The THP ring of cladosporin is adjacent to Ser_{344}, where any increase in steric bulk would clash with the methyl substituent on the THP ring. The *C. cladosporioides* genome contains three putative lysyl-tRNA synthetases, Cla4, Lys2, and Lys3. Neither Lys2 nor Lys3 contain the Gln-Thr pair, which appears to be necessary for cladosporin resistance, in contrast to Cla4.

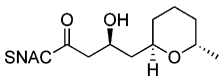
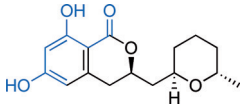
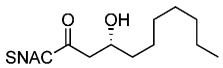
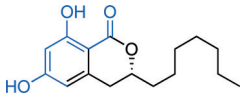
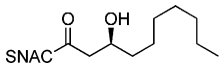
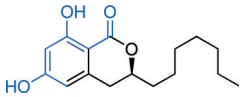
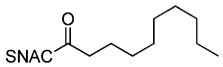
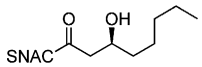
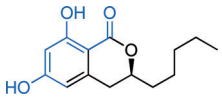
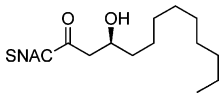
We suggest that Cla4 may not be inhibited by cladosporin, thereby imparting cladosporin resistance to *C. cladosporioides*. It is likely that *cla4* is under the control of the same regulation as *cla2* and *cla3*, and when cladosporin biosynthesis is switched on, transcription of *cla4* will then be necessary for continued protein synthesis in *C. cladosporioides*.

In conclusion, we have presented the identification and expression of the HR and NR PKS responsible for cladosporin production in *Cladosporium cladosporioides* and demonstrated its functional activity. Synthesis of advanced-intermediate analogues probed the promiscuity of the NR PKS, Cla3, and led to the production of several unnatural cladosporin analogues. We have also identified a putative lysyl-tRNA synthetase encoded in the cladosporin gene cluster. This likely indicates a probable resistance mechanism in *Cladosporium cladosporioides*.

Experimental Section

Genomic sequencing, protein expression, and in vivo production of cladosporin: DNA was isolated from a 7 day culture of *Cladosporium cladosporioides* UAMH 5063 and sent to Ambry Genetics for sequencing using Illumina HiSeq 2000 technology. GenBank Accession numbers KT037691-KT037693 and KT250989-KT250991. Cla2 and Cla3 were cloned into two 2μ-based yeast–*E. coli* shuttle vectors, pKOS518-120A and pXK30 (see the Supporting Information). pKOS518-120A and pXK30 contain *TRP1* and *URA3* as auxotrophic markers, respectively. The proteins were expressed with a His tag in *Saccharomyces cerevisiae* BJ5464-NpgA, and purified using nickel

Table 1: Conversion of natural and unnatural substrate analogues by Cla3. Extensions that are formed by Cla3 catalysis are highlighted in blue.

Intermediate	Product
	
	
	
	[a]
	
	[a]

[a] Expected product not observed.

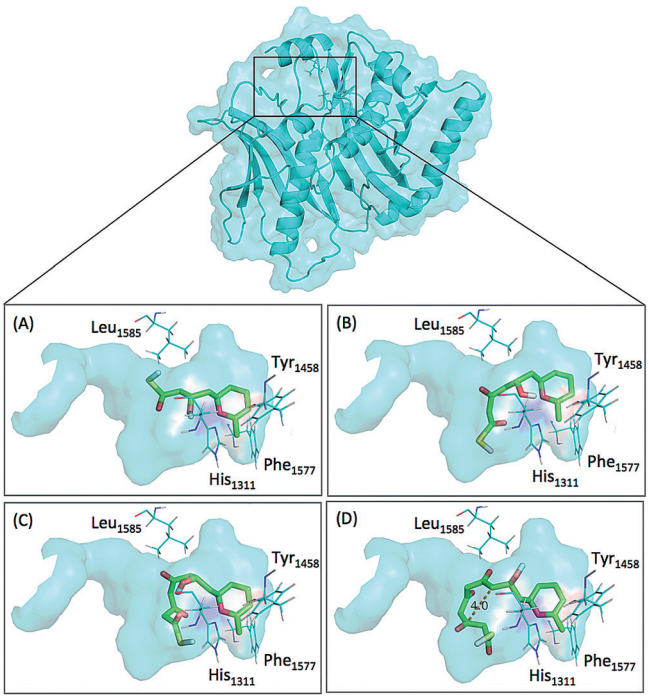


Figure 2. Homology model of Cla3 PT showing the predicted double hot dog (DHD) fold, and active-site cavity of Cla3 PT with docked thioacid analogues. Residues analogous to Leu_{1585}, Tyr_{1458}, and catalytic His_{1311} are conserved among all RAL-type PKSs. A) The pentaketide analogue adopts a relatively linear conformation in the active site. B) The hexaketide starts to adopt a bent conformation. C) A constricted heptaketide analogue forms a loop structure. D) Uncyclized octaketide thioacid analogue. C8a is now only 4 Å away from C4a.

affinity chromatography (see the Supporting Information). Cladosporin could be isolated from double transformants at a titer of 10 mg L^{-1} with addition of cyclopentanone and NaOAc to increase production (see the Supporting Information).

In vitro assays with advanced intermediates: Purified Cla3 was dissolved in Buffer A (50 mM Tris-HCl, pH 7.9, 2 mM EDTA, 2 mM DTT, 10% glycerol) to a final concentration of 10 μM. Malonyl-CoA (2 mM) and the advanced intermediate (2 mM) were added to this solution (1 mL), and the reaction was incubated overnight at 20 °C. In parallel, two negative controls were performed in either the absence of Cla3 or in the absence of pentaketide **2**. The reactions were quenched and extracted with an equal volume of an EtOAc/AcOH (99:1) solution, concentrated, and analyzed by LC-MS using an Agilent 1200 SL HPLC System with a Kinetex 2.6 μ XB-C18 100 Å, 100 × 2.1 mm reverse-phase column with guard (Phenomenex, Torrance, USA), thermostated at 55 °C, and an Agilent 6220 accurate-mass TOF HPLC/MS system (Santa Clara, CA, USA) with a dual sprayer. The presence of each expected product in the samples was confirmed by NMR and LC-ESI-MS analysis using combined retention time matching with accurate mass matching to chemically synthesized authentic standards (see the Supporting Information).

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