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Guest Articles

Biosynthetic Potential and metabolites of the Bioherbicide *Colletotrichum spinosum*

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Introduction

C. spinosum was first found and reported in Queensland, Australia, as *Colletotrichum* sp.¹ and later discovered in New South Wales.² In the late 1940s, this fungus caused severe damage (50–80% mortality) in the *Xanthium spinosum* population.^{3,4} *C. spinosum* was recently identified as a species within the *C. orbiculare* complex,⁴ and has previously been investigated as a targeted bioherbicide for *X. spinosum* based on its ability to kill 6-week-old plants in 14 days using spore concentrations of 107 mL⁻¹.⁵ Since there have been no reports on secondary metabolites (natural products) isolated from *C. spinosum*, the aim of this study was to discover the chemical potential of *C. spinosum* CBS 515.97. In this study, we performed genome mining on the publicly available genome 6 while also screening chemical extracts obtained from different cultivation conditions. The detailed isolation and structural elucidation of 3"-demethylthielavin M (**1**), a polyketide of the tridepside family, and seven other known compounds (**2-8**) that were unknown to be produced by *C. spinosum* was recently published. In addition, detailed bioinformatics analysis enabled us to identify a putative biosynthetic gene cluster (BGC) encoding the biosynthesis of **1**.⁷

Genome Mining

Using fungiSMASH version 7.1.0,⁸ on the publicly available genome (NCBI accession number QAPG000000000.1), we determined that *C. spinosum* CBS 515.97 possesses 74 BGCs likely to encode secondary metabolites. Of the 74 BGCs identified, 15 encode Type I polyketide synthases (PKSs), one encodes a Type III PKS, 17 encode non-ribosomal peptide synthetases (NRPS) or NRPS-like enzymes, 16 are identified as potentially encoding ribosomally synthesized and post-translationally modified peptides (fungal RiPPs) or fungal RiPP-like enzymes, and the remainder are comprised of hybrid combinations, as well as seven miscellaneous BGC types. Most of the BGCs detected by fungiSMASH show little similarity to characterized BGCs from other fungi, with only four of these BGCs sharing significant homology to known BGCs (Table 1).

Table 1. Bioinformatic overview of BGCs present within *C. spinosum* CBS 515.97 including fungiSMASH-reported cluster homologs and their percent similarity with query clusters. Miscellaneous includes: five indole, one isocyanide, and one NRPS-like/betalactone BGC(s).

Biosynthetic Gene Cluster Type	Count	Associated Homolog(s) ^a
Type I PKS	15	alternapyrone (100%)
Type III PKS	1	
NRPS	11	chrysogine (100%)
NRPS-like	6	microperfuraneone (100%), choline (100%)
Terpene	9	
Fungal-RiPP, fungal-RiPP-like ^b	16	
Type I PKS/NRPS	5	
Type I PKS/terpene	1	
Type I PKS/fungal-RiPP-like ^b	3	
Miscellaneous	7	

[a] FungiSMASH identifies associated homologs as the percentage of genes in the analyzed BGCs that match known products from the Minimum Information about a Biosynthetic Gene Cluster (MIBiG) database,⁹ with at least 30% sequence identity to any gene in the query BGC. Associated homologs were only included in this table if the fungiSMASH calculated similarity was greater than or equal to 70%.

[b] These RiPP and RiPP-like BGCs encoded one or more DUF3328 protein, implicated in RiPP biosynthesis¹⁰, however, leader peptide signals were not detected via fungiSMASH.

Metabolites of *C. spinosum*

Although no natural products have been reported from *C. spinosum* CBS 515.97, this fungus clearly has the genetic potential to produce a wide variety of secondary metabolites. Therefore, we proceeded with establishing cultivation and fermentation conditions to study metabolite production. The ethyl acetate micro-extracts obtained from cultivation of *C. spinosum* CBS 515.97 in four media (CM, DPY, OM, and CD)

were analyzed using LC-PDA-MS. LCMS traces from extracts prepared from cultivation in DPY and CM indicated production of several unknown secondary metabolites, therefore these media were chosen for large-scale fungal fermentation. The final purification of all compounds using semi-preparative HPLC resulted in amorphous powders except 2 which resulted in yellow oil. The structures of compounds 1 - 8 (Figure 1) were confirmed using spectroscopic methods (MS, 1D and 2D NMR) and comparison with reference compounds and literature data.

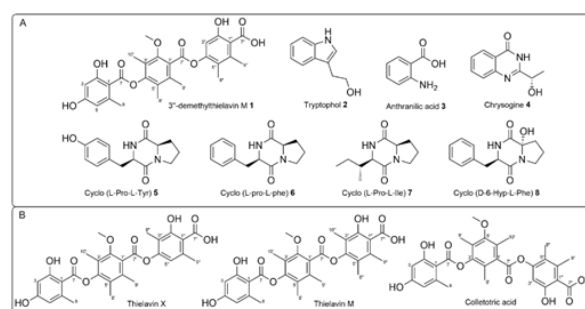


Figure 1. A) Compounds isolated from *Colletotrichum spinosum* CBS 515.97 during this study, which include the polyketide-derived depside 1, primary metabolites tryptophol 2 and anthranilic acid 3, non-ribosomal peptide 4, and cyclodipeptides or diketopiperazines (DKPs) 5-8. B) Reported structures closely related to 3''-demethylthiellavin M.

Anthranilic acid (3) is a precursor to both tryptophan and chrysogine (4) biosynthesis.^{11,12} The yellow pigment, chrysogine (2-(1-hydroxyethyl)-4(3H)-quinazolinone) was first isolated from filamentous fungus *Penicillium chrysogenum* in 1973.^{13,14} The BGC and biosynthetic pathway of 4 have been characterized and elucidated, explaining the co-isolation of 3 seen in this study.^{11,15} As is typically the case in the highly conserved chrysogine BGCs that have been elucidated thus far, strong similarity can be seen in the subsequent BGC and clinker analysis (Figure 4).

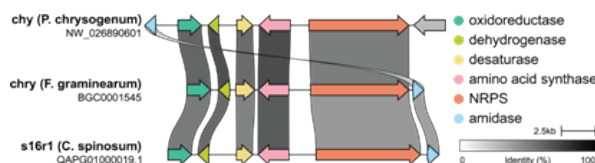


Figure 4. Synteny analysis of the chrysogine BGCs from *Penicillium chrysogenum*¹¹ and *Fusarium graminearum* PH-1.^{9,15}

The pure compounds of 5-8 were subjected to ¹H, ¹³C, and HRESIMS analysis and the proposed structures of these compounds corresponded to four cyclic dipeptides also known as diketopiperazines (DKPs). The DKPs identified were cyclo (L-Pro-L-Tyr) (5), cyclo (L-Pro-L-Phe) (6), cyclo (L-Pro-L-Ile) (7) and cyclo (D-6-Hyp-L-Phe) (8) (Figure 1). The absolute configuration of amino acid moiety of cyclo-dipeptides was determined by Marfey's analysis.

Proposed Biosynthesis of 3''-demethylthielavin M

Depsides are a large family of polyketides, consisting of orsellinic acid (OA) and its derivatives which are polymerized via ester linkages, typically arising from between two to six OA monomers. Biosynthesis of depsides is achieved through the action of a Type I iterative, non-reducing polyketide synthases (NRPKS), which in addition to the essential ketosynthase (KS), acyltransferase (AT) and product template (PT) domains often include multiple acyl carrier protein (ACP) domains, based on studies into the biosynthesis of lecanoric acid¹⁶ and duricamidepside.¹⁷ Studies into the biosynthesis of these compounds have found differing mechanisms for depside bond formation, through either the starter-unit acyltransferase (SAT)¹⁷ or thioesterase (TE)¹⁸ domain.

Thielavins are a sub-class of depsides which are highly c-alkylated and typically feature diverse methylation patterns within the polyketide chain and in the post-translational methylation of hydroxy groups. Thielavins discovered so far demonstrate diverse bioactivities, including antibacterial, anticancer, anti-hyperglycemic, and herbicidal activities.¹⁹⁻²² Thielavins have been reported from various microorganisms including marine-derived fungi,²³ endophytic fungi,²⁴ and lichens.²⁵

Examining the structure of 1 it appears to be biosynthesized from three different OA-derived subunits, each containing one, three, or two methyl groups respectively, with specific methyl groups being introduced by a dedicated C-methyltransferase (CMeT) domain, common in fungal PKS enzymes. Furthermore, the methoxy group at position 2' of the second ring indicates that in addition to an NRPKS containing a CMeT domain, an O-methyltransferase enzyme is also required for the biosynthesis of 1. Of the

15 Type I PKS BGCs detected by fungiSMASH, seven encode non-reducing PKSs, and only two of these contain a CMeT domain: BGCs [15.1] and [20.1]. Both BGCs also encode an O-methyltransferase required for methylation, typically after post-polyketide assembly.

Recently, the Matsuda group investigated the biosynthesis of the tridepside thielavin A which requires just two enzymes: the NRPKS ThiA and O-methyltransferase ThiX.¹⁸ Through extensive biochemical investigations, the programming of ThiA was thoroughly investigated and it was shown that this NRPKS is capable of synthesizing two different OA analogues and assembling three OA units via depside bond formation catalyzed by the TE domain.¹⁸ Furthermore, the tandem ACP domains of ThiA were individually inactivated and shown to alter the methylation pattern observed in the tridepside product, inferring an unusual influence of the tandem ACPs in methylation pattern.

To further narrow to the most likely possibility between the two candidate BGCs, phylogenetic analyses were performed on the PT, TE, and SAT domains of the candidate clusters alongside depside and non-depside NRPKSs. Analysis of PT domains placed BGCs [15.1] and [20.1] nearest to clades I and VI, respectively, which both contain tetraketide products thus not definitively ruling out either candidate²⁶. Additional analyses of SAT domains, as previously described in the literature¹⁷, and TE domains revealed that only the SAT and TE domains of [15.1] claded with depsides, making this BGC the most likely candidate. Furthermore, the NRPKS encoded by BGC [15.1] also contains two tandem ACP domains as well as the domain organization of SAT-KS-AT-PT-ACP-ACP-TE-CMeT, seen and noted as unusual in the NRPKS ThiA.¹⁸ The elucidated thi BGC was compared with the putative depside BGC [15.1] in *C. spinosum* CBS 515.97, resulting in a 68.3% similarity score on the O-methyltransferase and 60.8% similarity score on the NRPKS (Figure 5). Interestingly, BGC [15.1] also encodes two additional polyketide synthases although the NRPKS lacks a CMeT domain indicating it is unlikely to have a role in the biosynthesis of 1.

We therefore propose that the biosynthesis of 1 proceeds as follows; first the NRPKS synthesizes a linear tetraketide lacking any S-adenosylmethionine (SAM)-derived methyl groups. This first tetraketide unit is

cyclized and aromatized by the PT domain before being transferred to the TE domain, where it is not released but instead remains tethered. Next, a linear tetraketide containing SAM-derived methyl groups at positions C'-4 and C'-6 is synthesized, cyclized and aromatized, remaining attached to the ACP domain. Nucleophilic attack by the 4'-OH towards the TE-bound C-7 thioester results in depside formation with the intermediate still tethered to one of the ACP domains, before being transferred to the TE domain. A third linear tetraketide with a C''-6 SAM-derived methyl group is synthesized, cyclized, aromatized, and remains tethered to one of the ACP domains. A second nucleophilic attack by the 4''-OH of the ACP-bound polyketide towards the C'-7 thioester results in tridepside formation. The tridepside is transferred to the TE domain and released via hydrolysis. Finally, the O-methyltransferase catalyzes site-selective methylation of the hydroxyl group of ring B (Scheme 1).

Conclusions

A putative BGC encoding 3''-demethylthielavin M was identified by genome mining and a biosynthetic pathway is proposed that explains the incorporation of three different OA derivatives. Based on the high number of BGCs identified within *C. spinosum* CBS 515.97, there are likely more secondary metabolites produced under specific environmental conditions that may explain the host specificity of *C. spinosum* towards *X. spinosum*.

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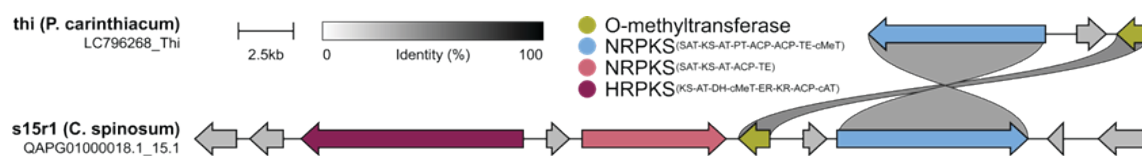
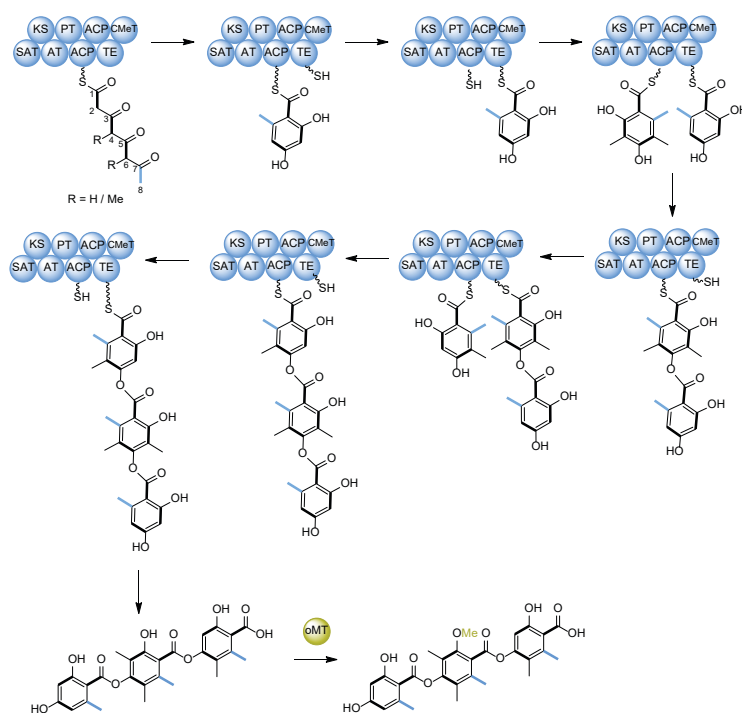


Figure 5. Synteny analysis of the thielavin X BGC in *Parahaetomium carinthiacum* and the putative 3''-demethylthielavin M BGC in *C. spinosum*. Abbreviations not yet described in the text: HRPKS = highly reducing polyketide synthase; DH = dehydratase; ER = enoylreductase; KR = ketoreductase; cAT = carnitine acyltransferase.



Scheme 1. Proposed biosynthesis of **1** with depside bond formation through the TE domain of the NRPKS.

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