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Genome Mining of a Prenylated and Immunosuppressive Polyketide from Pathogenic Fungi

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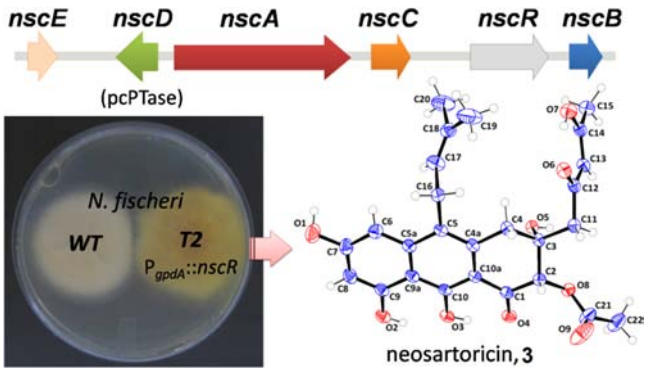
ABSTRACT



Activation of the polycyclic polyketide prenyltransferase (pcPTase)-containing silent clusters in *Aspergillus fumigatus* and *Neosartorya fischeri* led to isolation of a new metabolite neosartoricin (3). The structure of 3 was solved by X-ray crystallography and NMR to be a prenylated anthracenone. 3 exhibits T-cell antiproliferative activity with an IC_{50} of 3 μM, suggestive of a physiological role as an immunosuppressive agent.

Genome mining of sequenced microorganisms has yielded new natural products with interesting activities.[1] Mining of pathogenic species may yield new virulence factors or compounds active against human targets that are not produced in routine laboratory culturing conditions. As part of our efforts to understand fungal polyketide biosynthesis, we recently identified a group of polycyclic prenyltransferases (pcPTases) that catalyze the Friedel–Crafts transfer of C5 and C10 prenyl groups to aromatic polyketides. Phylogenetic analyses showed that the homologous pcPTase genes formed a distinct clade from the common indole prenyltransferase.[2] The pcPTases are found mostly in the aspergillosis- and keratitits-causing *Aspergillus fumigatus* and *Neosartorya fischeri* and the seven genome-sequenced arthrodermataceous dermatophytes.[3]

Interestingly, all these pcPTase genes are associated with a three-gene ensemble that makes polycyclic aromatic polyketides such as anthracenones (e.g., asperthecin) and

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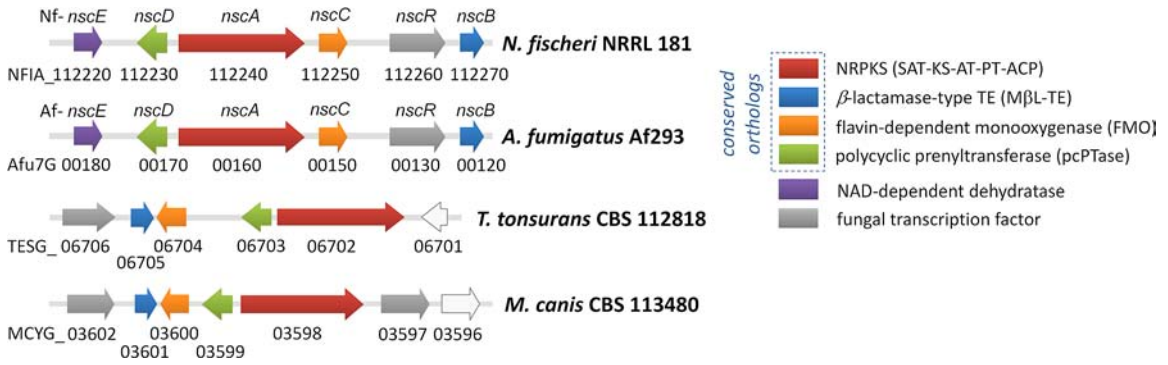
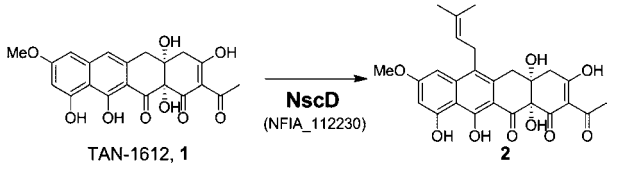


Figure 1. Evolutionary conserved pcPTase-containing gene clusters in *N. fisheri*, *A. fumigatus*, *T. tonsurans*, and *M. canis*.

napthacenediones (e.g., TAN-1612 **1**, viridicatumtoxin).[4,5] The ensemble includes genes that encode a nonreducing polyketide synthase (NR-PKS), a flavin-dependent monooxygenase (FMO), and a metallo-β-lactamase-like thioesterase (MβL-TE) (Figure 1). The prevalence of these pcPTase-encoding gene clusters among human and animal-associated fungi has invited the speculation that they may encode for production of a group of structurally related prenylated polycyclic polyketides that have a common ecological role. When cloned and assayed in vitro, pcPTases from *N. fischeri*, *M. canis*, and *T. tonsurans* were confirmed to be dimethylallyltransferases that can utilize naphthacenedione substrates including **1** (Scheme 1), supporting the hypothesis that prenylated polycyclic compounds may be produced by these organisms.

Scheme 1. Conversion of **1** to **2** by pcPTase NscD



Using the *N. fischeri* pcPTase (NFIA_112230, Nf-NscD) as a lead, we aim to identify the compound(s) produced by the gene cluster (designated as *nsc* gene cluster). We expect the highly homologous and syntenic *nsc* cluster (Afu7g00120-170) in *A. fumigatus* to encode for identical metabolite(s) as in *N. fischeri* (>90% protein identities, Table S2). Under our laboratory culturing conditions, neither *N. fischeri* NRRL 181 nor *A. fumigatus* Af293 produced any detectable prenylated polyaromatics, consistent with the lack of such compounds associated with strains in literature.

Reverse transcriptase-PCR (RT-PCR) from the total RNA of *N. fischeri* did not detect transcription of the *nsc* cluster genes (Figure S1A), suggesting that the cluster is transcriptionally silent. To activate the pathway, we overexpressed a putative pathway-specific $Zn(II)_2Cys_6$ transcriptional factor (FTF) encoded by NFIA_112260 (Nf-*nscR*), a strategy that has been successfully used in various fungi recently.[4,6] Transformation of *N. fischeri* with pBGP-Nf112260, which places *nscR* under the regulation of *A. nidulans gpdA* promoter (P_{gpdA}) and contains the *bar* resistance gene, resulted in several glufosinate-resistant colonies that exhibit yellow pigmentation on the reverse

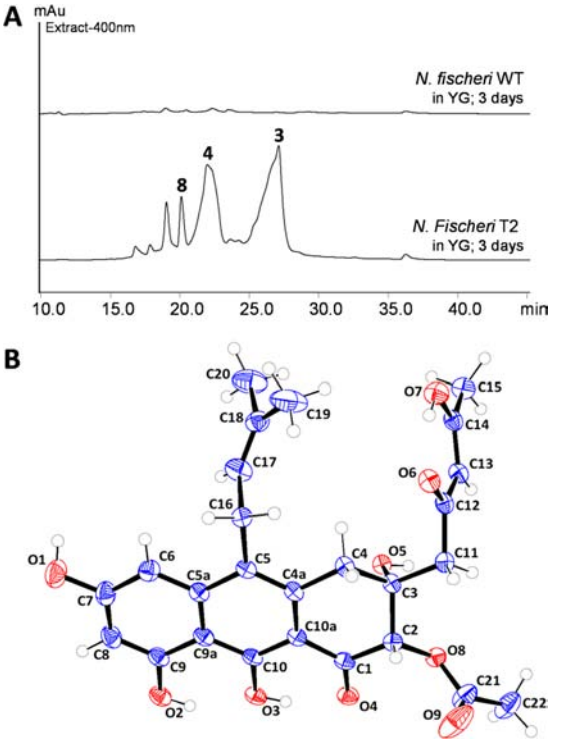


Figure 2. Activation of a silent pathway in *N. fischeri*. (A) Metabolic profile of *N. fisheri* T2 (*nscR* overexpression) compared to WT. (B) Perspective drawing of the molecular confirmation of neosartoricin **3**.

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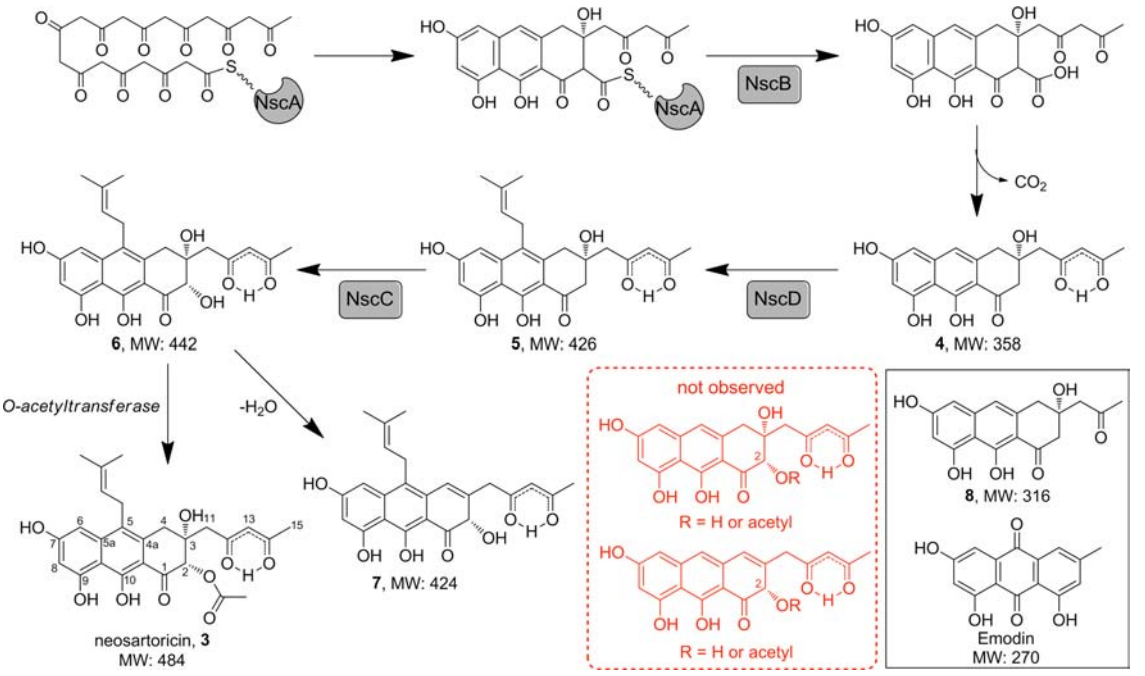
Scheme 2. Proposed Pathway for Biosynthesis of **3**[a]



[a] See Figure S2 and Supportion Information for more details.

side on agar plates (Figure S1B). Diagnostic PCR confirmed the integration of *nscR*-overexpression cassette in transformant T2, while RT-PCR analysis showed that the *nsc* genes (*nscA*-E) were indeed upregulated (Figure S1A).

The transformant T2 cultured in stationary liquid GMM culture was extracted after 3 days. LC/MS analysis of the extract indicates production of multiple new compounds that are not found in the wild type *N. fischeri* (Figure 2A). All the new compounds exhibit similar UV spectra and λ_{max} between 395 and 410 nm, while the MW range from 358 to 484. We reasoned that the higher MW peaks, including **3** (m/z 485 $[M + H]^+$) and **7** (m/z 425 $[M + H]^+$) at later retention times, are likely to be prenylated, while the compounds at earlier retention times are unprenylated intermediates and shunt products, i.e. **4** (m/z = 359 $[M + H]^+$) and **8** (m/z = 317 $[M + H]^+$) (Figures 2A and S2). To investigate if the same set of metabolites is produced by *A. fumigatus* upon transcriptional activation of the syntenic *nsc* cluster (Figure 1), an overexpression cassette encoding the putative FTF Afu7G00130 (p402-Af0130) with the resistance marker $phleo^R$ for selection was randomly integrated into the genome. In identical fashion to *N. fischeri*, *A. fumigatus* incorporating the cassette exhibits yellow pigmentation. The metabolite profile of *A. fumigatus* T1 is indeed identical to *N. fischeri* T2 based on UV spectra, retention time, and m/z values of major compounds **3**, **4**, **7**, and **8** during LC-MS analysis (Figure S3). Thus, both clusters in *N. fischeri* and *A. fumigatus* have been activated to produce the same set of new metabolites.

Approximately 35 mg of **3** (as bright yellow, amorphous powder) were successfully purified from 2 L of the *N. fischeri* T2 culture. Orange platelet-like crystals were obtained in an EtOAc/*n*-hexane solvent system, and the atom connectivity of **3** was resolved by X-ray diffraction (Figure 2B). **3** apparently cocrystallized with residual DMSO-d_6 from NMR analyses. Anomalous scattering of the X-rays by the sulfur atom in DMSO allowed elucidation of the absolute 3-D structure of **3**. The X-ray structure assisted in the assignment of the NMR spectra (Table S2). The structure of **3** consists of a tricyclic 3,4-dihydroanthracen-1(2*H*)-one core with a 2,4-keto-enol pentyl side chain extending from a decaketide (C_{20}) backbone, a dimethylallyl side chain at C5, and a C2-acetoxy substitution *syn* to a C3-hydroxyl. The 2,4- keto-enol pentyl chain tautomerizes in organic solvent, where the β-keto-enol form is dominant over the β-diketo form as observed in the ^1H, ^{13}C and 2D NMR spectra (Table S2; Figures S5−S8). **3** was named neosartoricin, based on the common distribution in both *N. fisheri* and *A. fumigatus* (given the Teleomorph name *Neosartorya fumigata*).[7] Besides **3**, the desacetyl derivative **7** (overlapped with **3** in Figure 2A) was isolated in small quantity from the *N. fisheri* T2. Due to low titer (< 1 mg/L) and instability upon purification, only ^1H NMR and LCMS data can be obtained for **7**. Spectra analysis and comparison to **3** led to assignment of the structure in Scheme 2 (Table S3). **4** and **8** are nonprenylated anthracenones derived from a decaketide and a nonaketide (C_{18}) respectively. Both compounds were

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isolated and characterized from yeast expressing the NR-PKS and FMO pair from the *A. niger ada* pathway for **1** and *A. nidulans apt* pathway for asperthecin, previously.[4]

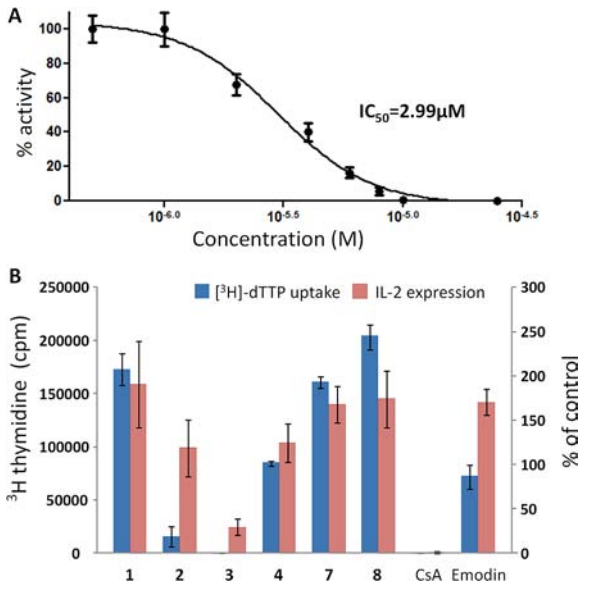

Figure 3. Effect of **3** on murine T-cell proliferation. (A) Dose–response curve of **3** on [^{3}H]-thymidine uptake by murine T-cells (% of DMSO control). (B) Comparison of the activity of **3** with **1**, **2**, **4**, **7**, **8**, cyclosporine A (CsA), and emodin at 10 μM concentration.

A pathway for biosynthesis of **3** was proposed based on analysis of the intermediates (Scheme 2 and Figure S2). As NscA-C are highly homologous to AdaA-C in *A. niger*, the biosynthesis of the anthracenones portion of **3** is likely to follow a parallel pathway for that of **1**: NscA synthesizes and cyclizes the decaketide backbone; NscB mediates the product release through hydrolysis followed by spontaneous decarboxylation to afford **4**; and NscC is responsible for the stereospecific hydroxylation at C2. The NscD catalyzes the addition of the dimethylallyl group to the aromatic C5, consistent with the previously determined regioselectivity (Scheme 1 and Figure S4). The timing of NscC, which was proposed to occur after prenylation of **4** to yield **5**, likely directs the formation of the anthracenone present in **6** instead of the naphthacenedione in **1**, although an alternative pathway via a Baeyer–Villiger cleavage of the fourth ring, such as that observed in mithramycin pathway,[8] cannot be completely ruled out (see Supporting Information). There is no gene encoding *O*-acetyltransferase in the *nsc* gene cluster; thus, the 2-*O*-acetylation of **6** to **3** may be catalyzed by an unidentified *O*-acetyltransferase in *N. fisheri*, while off-pathway dehydration of **6** affords **7**.

3 Does not exhibit significant inhibitory activity against either Gram positive or negative bacteria, nor the yeasts *Saccharomyces cerevisiae* and *Candida albicans* (> 64 μg/mL). We tested **3** for immunosuppressive activity in a cell-based in vitro assay, which showed that **3** exhibits antiproliferative activity on anti-CD3/CD28-activated murine spleenic T-cells with an IC_{50} of 2.99 μM (1.45 μg/mL) (Figure 3A). The effect of **3** on [^{3}H]-thymidine uptake by murine T-cells is dosage-dependent. Cyclosporin A (CsA) was used as a control in the assay and was shown to exhibit an IC_{50} of 26 nM, which is comparable to the value reported previously.[9] Next, we included **1**, **2**, **4**, **7**, and **8** in a structure–activity relationship study along with emodin, which has been previously shown to exhibit moderate immunosuppressive activity.[10] The data show that **3** exhibits a higher inhibitory activity compared to all the analogs tested including emodin (Figure 3B) and that the dimethylallyl group and *O*-acetyl is required for its activity. **3** was then assayed for cytotoxicity against HeLa and HFF cells, which shows that **3** is less toxic to the two cell lines compared to **1** and **2** at up to 50 μM, suggesting that the antiproliferative activity of **3** is relatively specific to T-cells (Figure S9).

We next examined the possible role of **3** in establishing primary infection in human hosts with a ^{51}Cr release cell damage assay on A549 lung epithelial cells and RAW 264.7 macrophage cells. **3** does not appear to cause any damage to both cell types up to 10 μM (< 5% specific release). The extent of A549 cell damage caused by the *nsc* pathway-overexpressing *A. fumigatus* T1 in vitro was also investigated, but no statiscally significant differences in cell damage were observed compared to wild type *A. fumigatus* (Figure S10). Thus, we concluded that the *nsc* pathway compounds including **3** are not involved in primary virulence but may facilitate infection through suppressing the host adaptive immunity.[11]

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Supporting Information Available. NMR and MS characterization of compounds and additional experimental information and discussion. Crystallographic Information File of **3** (CCDC 866028). This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.