Natural Products

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Cytotoxic Pheofungins from an Engineered Fungus Impaired in Posttranslational Protein Modification**

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Fungi produce a multitude of natural products that have a major impact on ecology, agriculture, and health.[1,2] While traditional natural product discovery has provided a vast array of chemically and functionally diverse compounds, it appears that most of the biosynthetic potential is still hidden and has thus been overlooked. Indeed, analyses of fully sequenced fungal genomes revealed that the number of encoded biosynthetic pathways by far exceeds the range of metabolites observed under standardized cultivation in the laboratory.[3–6] The reason for this observation is that most biosynthesis genes remain silent, and a major challenge is to explore ways to activate these cryptic pathways. A growing body of data illustrates that the variation of culture conditions and environmental factors including microbial interactions may dramatically influence the metabolite patterns of microorganisms.[4,7,8] To control the production of secondary metabolites at certain developmental stages or under specific environmental conditions, fungi employ a finely tuned system of global and specific regulatory mechanisms.[9–12] For example, the putative nuclear transcriptional regulator LaeA controls secondary metabolite production in *Aspergillus*, probably by defining the chromatin landscape, thus influencing the activation or silencing of gene transcription.[13] Furthermore, epigenetic regulatory processes such as histone deacetylation and DNA methylation direct the transcription of fungal genes. It has been demonstrated that the deletion of genes (*hdaA*) that encode an *Aspergillus nidulans* histone deacetylase (HDAC) as well as the treatment of fungal cultures with HDAC inhibitors cause transcriptional activation of secondary metabolite gene clusters and production of several natural compounds.[14–17] It has also been shown that manipulation of the COP9 signalosome, a crucial regulator of ubiquitin ligases, results in an altered transcriptional and metabolic response.[18]

While searching for novel ways to tap the metabolic potential of fungi, we turned to engineering mutants impaired in posttranslational protein modification. As one of the prime targets, we focused on the acetylation of target proteins,[19] a process that is required for a correct function of several enzymes and cellular proteins.[20] We hypothesized that a modulation of protein acetylation would influence the complex regulation of diverse cellular processes. As a consequence, this influence would interfere with signaling pathways that activate or repress the transcription of genes that code for secondary metabolite biosynthetic enzymes. Herein, we describe the first successful application of this new strategy by using the fungal model organism *Aspergillus nidulans*, and report the discovery of a previously unknown type of heterocyclic fungal pigments that are remarkably similar to red hair pigments in humans.

We initially generated a number of *A. nidulans* mutant strains that lack genes that encode putative N-acetyltransferases, and investigated these strains with regard to changes in phenotype and secondary metabolite production.[21] Our attention was drawn to a slowly growing strain that lacks the gene AN2745. This strain showed a change in color from yellow (wild type) to red/orange mycelium (mutant; Figure 1 A). An NCBI BLAST database search with the protein coding sequence of AN2745 revealed homology to the NatB type N-acetyltransferases Nat3[22] and hNatB[19] of *Saccharomyces cerevisiae* and humans, respectively. We therefore renamed the gene to *nnaB* (nidulans N-acetyltransferase B). In addition to the obvious phenotypic changes, the strain also showed a significantly increased production of secondary metabolites compared to the wild type when it was cultured in malt medium (Figure 1 B). This effect was clearly correlated to the absence of *nnaB* because the complemented strain *nnaB*^{c} fully restored the wild-type phenotype as well as the metabolic fingerprint (Figure 1 B).

Profiling of the Δ*nnaB* culture extract by HPLC-HRESI-MS and dereplication with in-house standards and databases revealed not only the formation of several orsellinic acid derived phenolic compounds **5**–**10**, but also small amounts of previously unknown heterocyclic products **1**–**4**, which displayed an intensely red color (see Figure 1 and Scheme 1). Compounds **1**–**4** were isolated from an upscaled culture by a combination of different chromatographic techniques, and their structures were elucidated by UV, MS, and 1D and 2D NMR analyses.

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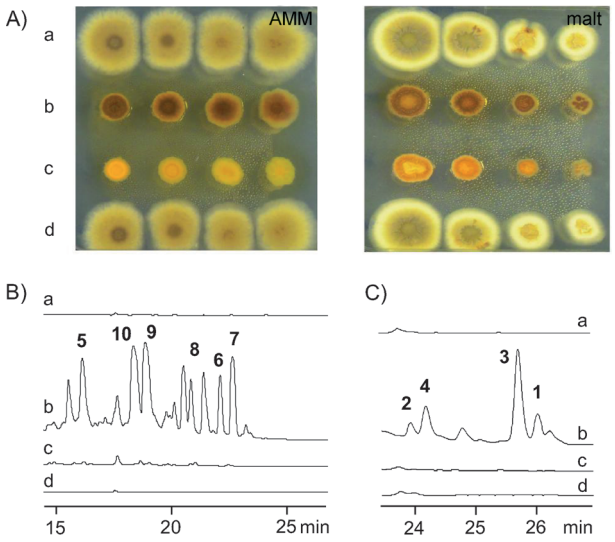
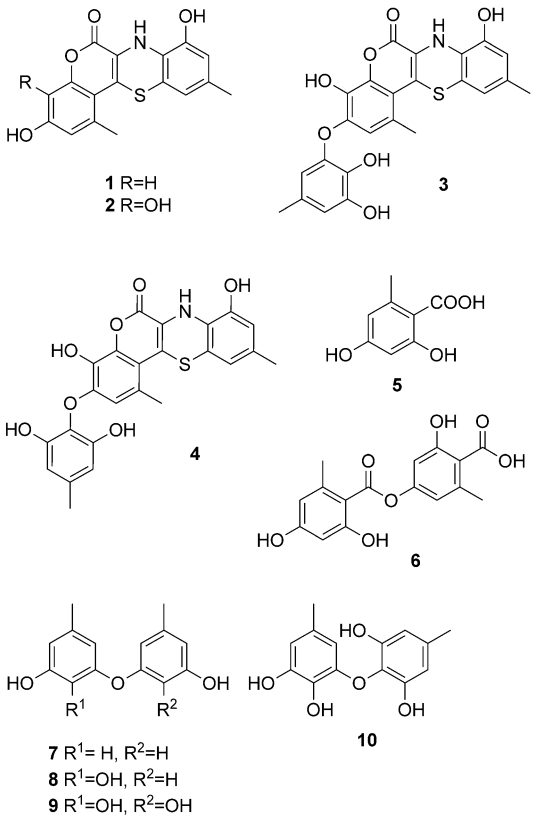


Figure 1. Phenotypes of a) wild type (wt), b) Δ*nnaB* mutant, c) Δ*nnaB*-Δ*orsA* double mutant, and d) complemented mutant strain (*nnaB*^{c}). A) Serial 1:10 dilutions on solid minimal medium (AMM) and full medium (malt) for 3 days at 37 °C, lower site of the plate is shown. B) HPLC profiles of extracts from cultures grown in malt medium. C) HPLC profile of 10× concentrated extracts to amplify the 24–26 min area in (B).



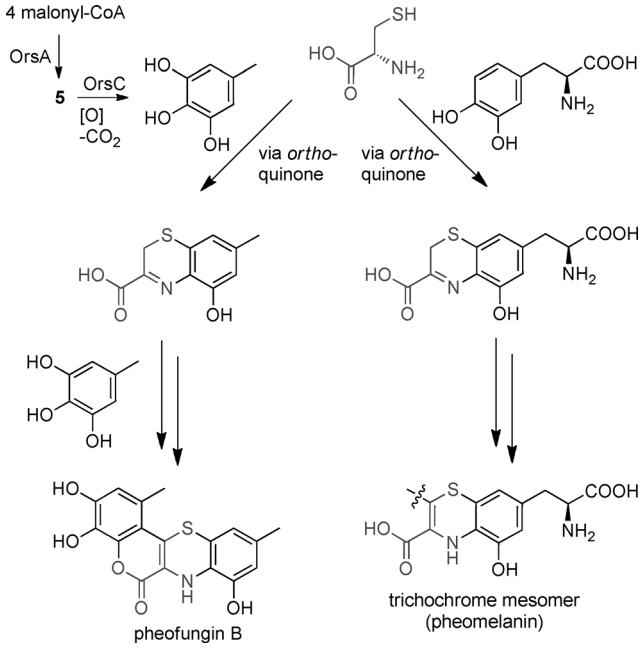
Scheme 1. Structures of characterized metabolites. Pheofungin A (**1**), pheofungin B (**2**), pheofungin C (**3**), pheofungin D (**4**), orsellinic acid (**5**), lecanoric acid (**6**), diorcinol (**7**), cordyol C (**8**), violaceol I (**9**), and violaceol II (**10**).

The molecular composition of compound **1** was determined as $C_{17}H_{13}NO_4S$ by HRESI-MS. The ^{1}H NMR spectrum showed the presence of two hydroxy and four aromatic methine protons as well as an NH group and two methyl groups. The coupling constant ($J = 2.5$ Hz) of H-2 ($\delta =$ 6.59 ppm) and H-4 ($\delta = 6.57$ ppm) indicated a *meta* relationship of the aromatic protons. Couplings of these protons with C-3 and C-12b and a coupling of the methyl protons at C-13 ($\delta = 2.74$ ppm) with C-2 and C-12b in the HMBC spectrum established the substitution pattern of the aromatic ring as a chromene substructure. Cross-peaks of the proton of the secondary amine with C-6, C-8, C-11a, and C-12a in the HMBC spectrum as well as a chemical shift of $\delta = 114.0$ and 122.9 ppm for C-11a and C-12a, respectively, suggested the presence of a benzothiazinone moiety. Couplings of the hydroxy proton 8-OH with C-7a, C-8, and C-9 and a coupling of the methyl function H-14 with C-9, C-10, and C-11 in the HMBC spectrum finally established the structure of pheofungin A (**1**; see Figure S1 in the Supporting Information).

Compound **2** has a molecular formula of $C_{17}H_{13}NO_5S$, as determined by HRESI-MS, and was found to be closely related to compound **1**. The proton spectrum showed an additional hydroxy proton instead of the aromatic proton H-4 in **1**. Furthermore, a downfield shift of C-4 to $\delta = 130.7$ ppm disclosed the position of the OH group, thus establishing the structure of pheofungin B (**2**). Compounds **3** and **4**, which give signals at m/z 464 ($[M-H]^-$) in ESI-MS measurements, possess a similar chromophore to **1** and **2**, as deduced from their UV spectra. The molecular compositions ($C_{24}H_{19}NO_7S$ for both), the ^{13}C NMR data, and the missing hydroxy group proton 3-OH ($\delta = 10.34$ ppm in **1** and $\delta = 9.79$ ppm in **2**) suggested a substitution with an additional aromatic ring. For compound **3**, the signals of C-4′ and C-6′ appear at $\delta =$ 104.8 ppm and $\delta = 110.4$ ppm, respectively, whereas both carbon atoms show the same chemical shift ($\delta = 108.2$ ppm) for compound **4**. Analysis of the HMBC data unequivocally established the unique structures of pheofungins C (**3**) and D (**4**; see Scheme 1, and Figure S1 in the Supporting Information).

To the best of our knowledge, the benzopyrano–benzothiazinone structure, which accounts for the red color of the fungal pigments, has never been described in natural products. Interestingly, the unusual heterocyclic core is reminiscent of the well-known reddish-brown pheomelanins found in mammalian red hair and red bird feathers. The chromophoric components of this sulfur-containing subgroup of melanins are the trichochromes with a protonated 2,2′-bis-[1,4]benzothiazinylidene skeleton with several mesomeric structures. These pigments are associated with the increased UV susceptibility typically observed for the familiar red hair phenotype of Celtic origin.[23] While details on red pigment production have yet to be elucidated, the current understanding of pheomelanin biosynthesis provides valuable hints to the formation of the pheofungins in *A. nidulans*. Similar to the black eumelanins, the pheomelanins are formed through a tyrosinase-catalyzed oxidation of tyrosine leading to dopaquinone. At elevated levels of cysteine, the pathway is then channeled to pheomelanin production by the reaction of dopaquinone and cysteine leading to *S*-cysteinyldopa. Further

oxidative cyclization and rearrangement reactions finally produce a series of benzothiazines.[23] Instead of 3,4-dihydroxyphenylalanine (DOPA), Δ*nnaB* seems to utilize the abundant orsellinic acid derived phenolic compounds as precursors for the heterocyclic structures of pheofungins. As in the pheomelanin pathway, the pheofungin sulfur atom is most likely derived from the incorporation of cysteine. Indeed, we found that supplementation of the culture medium with cysteine resulted in an increased pheofungin formation, yet the minute amounts of the compounds produced by the fungus and the sole production in a complex medium prevented feeding experiments with labeled cysteine precursors (Scheme 2).



Scheme 2. Structural similarity of the pheomelanin and pheofungin chromophores and plausible model for pheofungin biosynthesis.

To gain insight into the molecular basis of pheofungin biosynthesis, we performed full-genome microarray analyses and quantitative real-time PCR (qRT-PCR) experiments. Bioinformatic analysis of the array data revealed an upregulation of central genes of two secondary metabolite gene clusters in the Δ*nnaB* mutant, (Figure 2), the designated *ors* cluster that codes for the biosynthesis of orsellinic acid,[8] and a putative polyketide synthase gene cluster that spans around AN2032 and AN2035 (see Figure S11 in Supporting Information). However, an involvement of AN2032 and AN2035 in pheofungin formation could be fully ruled out by targeted gene knockouts, which did not affect the phenotype. In contrast, deletion of *orsA* in the Δ*nnaB* strain fully abolished red-pigment formation (Figure 1). HPLC analysis of the culture extracts verified that the production of the phenolic compounds and the pheofungins is fully abolished in the Δ*nnaB*/Δ*orsA* double mutant. This result clearly shows that the orsellinic acid synthase OrsA is essential for the formation of the heterocyclic compounds (Figure 1). Another important

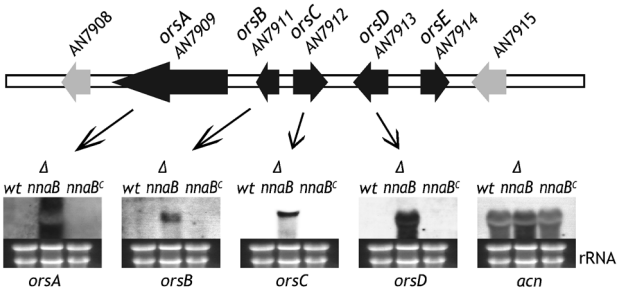


Figure 2. Secondary metabolite gene cluster activation in Δ*naaB* strain. Northern blot analysis of the *ors* gene cluster genes in wild type (wt), Δ*nnaB* mutant and complemented strain incubated for 29 h in malt medium. Numbers above the arrows indicate annotated ORFs. The actin gene (*acn*) was used as a control/reference.

result is that a gene (*orsC*) that codes for a tyrosinase is significantly upregulated in the Δ*nnaB* mutant. Taken together, the orsellinic acid synthase OrsA produces the precursors of the phenolic compounds, which would be oxidized by the tyrosinase, and high amounts of phenolic precursors and intracellular cysteine would set the stage for pheofungin formation in analogy to mammalian pheomelanin biosynthesis.

Deletion of the N-acetyltransferase gene *nnaB* and complementation of the mutant provided evidence that the formation of the red pigments specifically results from impaired protein acetylation. N-terminal acetylation is a highly abundant modification of eukaryotic proteins, and occurs on 40 % of all yeast proteins and on more than 80 % of the human proteome.[19] NatB specifically acetylates proteins with methionine followed by aspartate, glutamate, or asparagine at the N terminus, and this cotranslational modification is required for the proper functioning of numerous proteins.[20] For the induction of pheofungin biosynthesis in the *A. nidulans* Δ*nnaB* mutant, two scenarios are conceivable (Figure 3). One possibility would be that NnaB directly acetylates and thus activates a repressor of genes coding for pheofungin biosynthesis. In this case, these genes would be upregulated in the Δ*nnaB* mutant because of a reduced activity of the putative repressor. A similar mechanism was shown for Tfs1, a repressor of kinase activity, which requires NatB-mediated acetylation for full activity.[20] Another, yet more likely, explanation for the metabolic switch in the Δ*nnaB* mutant might be the fungal response to intracellular stress caused by a reduced functionality of central proteins without proper acetylation. Nearly 10 % of all *A. nidulans* proteins start with the putative target sequences of NnaB. Consequently, a pool of around 1000 fungal proteins are candidates for impaired activity in the mutant, thus resulting in a global stress response. Several observations support this hypothesis. It has been reported that efficient binding of tropomyosin to actin is inhibited without N-terminal acetylation catalyzed by NatB, thus leading to impaired transport processes and, therefore, stress conditions in yeast.[18,24] In a *natB*-deficient strain, a global change in protein phosphorylation levels was observed that results directly or indirectly from protein acetylation. Furthermore, deletion of CsnE, which likely plays

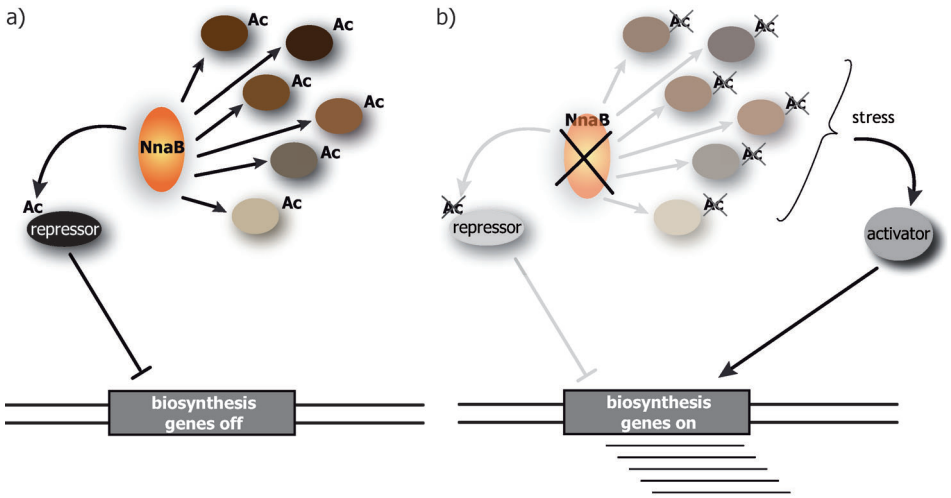


Figure 3. Model for inducing pheofungin biosynthesis in the Δ*nnaB* mutant. Mechanism (a): Repressor function requires acetylation by NnaB, lack of acetylation leads to activation gene expression. Mechanism (b): Loss of NnaB activity leads to a global stress reaction because N-terminal acetylation of target proteins is essential for proper function of cell processes. Stress response is accompanied by induction of phenolic compounds and pigment formation.

a role in protecting *A. nidulans* from oxidative stress, led to the formation of violaceols.[18]

To test whether the lack of NnaB induces a stress response in *A. nidulans*, we used qRT-PCR analysis to study the expression of several genes that encode stress-related proteins. We noted a significantly altered expression of various genes that code for enzymes that are essential for coping with intracellular stress (see Figure S12 in the Supporting Information). For example, genes AN9339 and AN7388, which code for homologues of proteins involved in oxidative stress response,[25–27] exhibit a significantly differential expression in the Δ*nnaB* mutant. Furthermore, we observed an altered transcription rate of genes AN7577 and AN10507 in the deletion strain. The deduced products of these genes are assigned as an ubiquitin-conjugating enzyme and a small heat shock protein, respectively, and are required for correct protein degradation and protein folding.[28] These data indicate that the production of pheofungins and other orsellinic acid derivatives are, at least, a result of a global stress response. The retarded growth of the mutant on agar plates and the antioxidant activity of the polyphenolic compounds further corroborate this hypothesis. For example, we found that lecanoric acid is a strong inhibitor of horseradish peroxidase (data not shown), thus implying the ability to decrease the formation of reactive oxygen species.

Finally, we were intrigued to test whether the novel fungal metabolites are pharmacologically active. We found that the major metabolite pheofungin C (**3**) effectively inhibits the proliferation of HUVEC ($GI_{50}=7.5$ μM) and K-562 human leukemia cell lines ($GI_{50}=2.4$ μM). Furthermore, **3** shows cytotoxic effects against HeLa cells with an IC_{50} value of 10 μM. In this respect, it is interesting that related synthetic benzothiazinones have been shown to possess antimicrobial[29] and estrogenic[30] activities.

In conclusion, we have reported a new approach to trigger metabolite production in a eukaryote. By deleting a gene that encodes the N-acetyltransferase NnaB in the model fungus *A. nidulans*, we induced a metabolic switch to allow the isolation and structural elucidation of unparalleled fungal metabolites named pheofungins A–D. We provided a body of evidence that these red pigments are the fungal response to global stress caused by impaired posttranslational modification. The structures of the pheofungins represent benzopyrano–benzothiazinones that are fully unprecedented for fungi. However, the pheofungin chromophore is remarkably similar to pheomelanins, the red pigments in human hair of Celtic origin. Transcription analysis and gene knockout experiments indicated that pheofungins result from the condensation of orsellinic acid derived phenolic compounds with cysteine in analogy to the pathway for red hair pigments.[31] Thus, this study describes a new avenue to structurally intriguing metabolites and illustrates that modulation of fungal regulatory systems can promote the discovery of natural products with potential therapeutic applications.

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