Biosynthesis of Hexahydroxyperylenequinone Melanin via Oxidative Aryl Coupling by Cytochrome P-450 in Streptomyces griseus

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Dihydroxyphenylalanine (DOPA) melanins formed from tyrosine by tyrosinases are found in microorganisms, plants, and animals. Most species in the soil-dwelling, gram-positive bacterial genus Streptomyces produce DOPA melanins and melanogenesis is one of the characteristics used for taxonomy. Here we report a novel melanin biosynthetic pathway involving a type III polyketide synthase (PKS), RppA, and a cytochrome P-450 enzyme, P-450mel, in Streptomyces griseus. In vitro reconstitution of the P-450mel catalyst with spinach ferredoxin-NADP+ reductase/ferredoxin revealed that it catalyzed oxidative biryl coupling of 1,3,6,8-tetrahydroxynaphthalene (THN), which was formed from five molecules of malonyl-coenzyme A by the action of RppA to yield 1,4,6,7,9,12-hexahydroxyperylene-3,10-quinone (HPQ). HPQ readily autopolymerized to generate HPQ melanin. Disruption of either the chromosomal rppA or P-450mel gene resulted in abolishment of the HPQ melanin synthesis in S. griseus and a decrease in the resistance of spores to UV-light irradiation. These findings show that THN-derived melanins are not exclusive in eukaryotic fungal genera but an analogous pathway is conserved in prokaryotic streptomycete species as well. A vivid contrast in THN melanin biosynthesis between streptomycetes and fungi is that the THN synthesized by the action of a type III PKS is used directly for condensation in the former, while the THN synthesized by the action of type I PKSs is first reduced and the resultant 1,8-dihydroxynaphthalene is then condensed in the latter.

The genus Streptomyces comprises gram-positive, soil-dwelling, filamentous bacteria with a complex life cycle similar to that of fungi. In addition to the complex morphological differentiation, Streptomyces is also characterized by its ability to produce a wide variety of secondary metabolites, including pharmaceutically useful compounds, such as antibiotics, antitumor agents, and immunosuppressants (3). Melanins, which are high-molecular-weight dark-brown to black pigments, are secondary metabolites produced by diverse species of streptomycetes and are used for the taxonomy of this genus (24, 25). Various organisms synthesize a so-called dihydroxyphenylalanine (DOPA) melanin, which is a nonenzymatically or laccase-mediated polymerized product from DOPA. DOPA is formed from tyrosine by the oxidative action of tyrosinases. The representative of the tyrosinases responsible for the formation of DOPA melanin in Streptomyces is MelC2 (5), which is a copper-containing monooxygenase that catalyzes the o-hydroxylation of monophenols and the oxidation of o-diphenols to yield o-quinones using molecular oxygen (2). Because tyrosine occurs universally in living organisms, tyrosinases are also responsible for browning in plants and melanization in animals (2).

We previously found that a mutation in rppA, encoding a type III polyketide synthase (PKS), caused the host, Streptomyces griseus, to show an “albino” phenotype (8). Although S. griseus produces DOPA melanin, its production is apparent only when copper is added to the medium (5). RppA catalyzes condensation of five molecules of malonyl-coenzyme A (CoA) to form 1,3,6,8-tetrahydroxynaphthalene (THN) (8). These findings suggested the presence of a novel melanin biosynthetic pathway, via not DOPA but THN, in S. griseus (8), although the details of the presumptive pathway remained unknown.

For elucidation of the predicted melanin biosynthetic pathway, we focused on an open reading frame, named P-450mel, as a neighbor of rppA (Fig. 1), since functionally related genes are often organized in operons. P-450mel is a member of the cytochrome P-450 family, which are a superfamily of hemoproteins catalyzing monooxygenation of a wide range of compounds (13, 14). Because P-450mel constitutes an operon with rppA not only in S. griseus but also in several other Streptomyces species (7), we expected that P-450mel might be involved in the formation of a novel melanin by modifying THN in some way.

As expected, P-450mel was found to catalyze aryl coupling of THN to yield 1,4,6,7,9,12-hexahydroxyperylene-3,10-quinone (HPQ) in an in vitro-reconstituted system with spinach ferredoxin/ferredoxin reductase. By analyzing the intermediates accumulated in rppA and/or P-450mel mutants, we also confirmed that HPQ is formed in vivo by the action of P-450mel from the THN that has been formed from malonyl-CoA by the action of RppA. HPQ polymerized nonenzymatically to yield a brownish pigment. Because the spores of the P-450mel mutant showed decreased resistance to UV irradiation, we named the brownish pigment HPQ melanin. The biosynthesis of HPQ melanin by a type III PKS and P-450 represents a novel melanin biosynthetic pathway, although THN-derived melanin is also produced by fungi. However, the THN used for melanin biosynthesis in fungi is a product of type I PKSs, which are evolutionarily distinct from type III PKSs.

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FIG. 1. Effects of rppA and P-450mel on melanin production by S. griseus. (A) Gene organization of the S. griseus chromosomal region including the rppA and P-450mel loci and the DNA fragments on the plasmids used in this study. The ΔP-450mel mutant contains an in-frame deletion from Gln-84 to Tyr-250. OrfA is similar to transcriptional factors of the AraC family. The orfBCDE operon constitutes an ABC transporter, because of the similarity of OrfB to substrate-binding domains and OrfCD to membrane integral subunits. OrfA and OrfCD to membrane integral subunits, and ABC transporter, because of the similarity of OrfB to substrate-binding domains, OrfCD to membrane integral subunits, and ABC transporter, because of the similarity of OrfB to substrate-binding domains. The absence of undesired alterations during PCR was checked by monitoring the region from Met-1 of P-450mel to the end of its 3′-flanking region was the result of PCR with primer VII, 5′-CCGGAATTTCGAGGTTCTCTCCGTCTTGAGAAC-3′ (the italic letters indicate an EcoRI site and the boldface letters represent the start codon of rppA). The HindIII-EcoRI fragment excised from the amplified DNA fragment was cloned between the HindIII and EcoRI sites of pUC19, resulting in pUC19-RP450-N. The HindIII-EcoRI fragment from pUC19-RP450-C was ligated via the common BglII site and cloned between the HindIII and EcoRI sites of pUC19, resulting in pUC19-RP450. pUC19-RP450 was denatured with NaOH and introduced by protoplast transformation into S. griseus FI013350.

Transformants containing pUC19-RP450 in the chromosome as a result of single crossover were selected among kanamycin-resistant colonies. One of the kanamycin-resistant colonies was grown for a week on YMDP agar (10) without kanamycin. Spores recovered were spread on YMDP agar without kanamycin. From these colonies, one ΔP-450mel mutant, in which the P-450mel gene on the chromosome was in-frame deleted, was isolated as a kanamycin-sensitive colony. The correct replacement was confirmed by Southern hybridization.

Construction of pKU209-RB6. A 1.6-kb DNA fragment containing the promoter and P-450mel coding sequence was amplified with primer V, 5′-GGGTTCTCCTCGTTCTCTGGA-3′ (the italic letters indicate a HindIII site, and the underline indicates the HindIII site and XhoI sites, respectively). The amplified fragment was cloned between the HindIII and EcoRI sites of pUC19, resulting in pUC19-RB6. The HindIII-BglII fragment from pUC19-RB6 was excised from pUC19, ligated via the common BglII site, and inserted between the NdeI and BamHI sites of pNF1. pET16b-NF1 was cloned between the NdeI and BamHI sites of pIJ6021, resulting in pET16b-NF1. The NdeI-BamHI fragment excised from pEC1 into pNF1 was cloned between the NdeI and BamHI sites of pNF1, resulting in pNF1.

For construction of pNF2 containing P-450mel under the control of the thiostrepton-inducible tipA promoter in pJH6021 (Fig. 1A), an NdeI site was introduced at the start codon of P-450mel by PCR with primer VII, 5′-GGCCGAATTTCGAGGTTCTCTCCGTCTTGAGAAC-3′ (the italic letters indicate an EcoRI site and the boldface letters represent the start codon of rppA). The HindIII-EcoRI fragment excised from the amplified DNA fragment was cloned between the HindIII and EcoRI sites of pUC19, resulting in pUC19-RP450-N. The HindIII-EcoRI fragment from pUC19-RP450-N and the BglII-EcoRI fragment from pUC19-RP450-C were ligated via the common BglII site and cloned between the HindIII and EcoRI sites of pUC19, resulting in pUC19-RP450. pUC19-RP450 was denatured with NaOH and introduced by protoplast transformation into S. griseus FI013350.

High-performance liquid chromatography analysis of in vivo products. S. lividans TK21 harboring pNF1, pNF2, pNF3, or pJH6021 was grown at 30°C for 24 h in YEME medium. Thiostrepton was added at a final concentration of 5 μg/ml to induce the tipA promoter and the culture was continued for 36 h. The material in the culture broth was extracted with ethyl acetate, dried, and applied to high-performance liquid chromatography (HPLC). The HPLC conditions were an OD-802S column (Shodex) with a linear gradient from 5 to 40% CH3CN in water (each containing 2% acetic acid) over 30 min and then 100% CH3CN within 10 min at a flow rate of 0.8 ml/min. UV absorbance was detected at 254 nm and 440 nm. In vivo products of wild-type, ΔpapA, and ΔΔP-450mel S. griseus FI013350 strains were similarly analyzed, except that these strains were resistant to thiostrepton.
produce a final concentration of 5 μg/ml for induction of the tetP promoter and the culture was continued for a further 24 h. The culture broth was adjusted to pH 1.0 with 6 M HCl and 500 ml of ethyl acetate was added and vigorously mixed. The mixture was then passed through a pad of Celite to remove the emulsion and the organic layer was collected. The Celite was washed five times with 100 ml of ethyl acetate and twice with 50 ml of acetone. The organic layers were combined, washed with brine, and dried with Na₂SO₄. After evaporation to dryness, the residue was methylated by trimethylsilyl-diazomethane in methanol.

The resultant mixture was separated by silica gel flash chromatography using ethyl acetate containing 1% acetic acid as an eluant, and the major compound was further purified by reversed-phase preparative HPLC (Docosil B [C₂₂], 20 by 250 mm) by a linear gradient of 80 to 100% CH₃CN in water (each containing 1% acetic acid) at a flow rate of 6 ml/min to provide 2 mg of 4,9-dihydroxy-1,6,7,12-tetramethoxyphenylene-3,10-quinone as a red solid.

HPLC patterns of compounds produced by S. lividans TK21 harboring the vector pIJ6021 was also analyzed. Both THN and flavinol were identified by their comigration with authentic samples. (B) HPLC patterns of compounds produced by S. lividans TK21 expressing P-450mel and P-450mel rppA harboring the vector pIJ6021. As a negative control, the culture broth of S. lividans harboring the vector pIJ6021 was also analyzed. Both THN and flavinol were identified by their comigration with authentic samples. (B) HPLC patterns of compounds produced by S. griseus wild-type (IFO1350), ΔP-450mel, and ΔrppA strains.

Isolation and identification of polyketides produced by S. lividans harboring pNF1. S. lividans TK21 harboring pNF1 was grown at 30°C in 1 liter of YEME medium containing 5 μg/ml of kanamycin. After 24 h, thiostrepton was added to grown at 28°C for 4 days on R5 agar medium (11) and the material produced was extracted by ethyl acetate after homogenization of the agar.

Production and purification of P-450mel. The nucleotide sequence (TGACGA) covering the TGA stop codon of P-450mel was changed to CTTCGAG to create an Xhol site by PCR with primer X, 5'-GGGAGTCGATCTCGAGACACC-3' (the italic letters indicate a HindIII site, the underline indicates an Ndel site), and the boldface letters indicate the start codon of P-450mel, and primer XL, 5'-GGGAGTCGATCTCGAGACACC-3' (the italic letters indicate an EcoRI site and the underline indicates an Xhol site). The amplified 1.2-kb fragment was cloned between the HindIII and EcoRI sites of pUC19, resulting in pUC19-P-450mel-Xho. The Ndel-Xhol fragment excised from pUC19-P-450mel-Xho was cloned between the Ndel and Xhol sites of pET2b.

In vitro reconstitution of P-450mel activity. The reactions, containing 100 mM sodium phosphate (pH 7.3), 1 mM EDTA, 10 mM glycerol, 1 mM NADPH, 0.5 U of spinach ferredoxin-NADP⁺ reductase, 40 μg of spinach ferredoxin, 23.4 μg of P-450mel, and 400 μM of THN, were performed in a total volume of 500 μl. Ferredoxin and ferredoxin-NADP⁺ reductase from spinach were purchased from Sigma. THN was synthesized according to the method of Iino et al. (9). The reactions were carried out at 30°C for 30 min and terminated by adding 50 μl of 6 M HCl, and extracted with 200 μl of ethyl acetate. The organic layer was collected and evaporated and the residual material was dissolved in 20 μl of methanol for HPLC analysis. Conditions of HPLC were as follows: ODS-80Ts column (4.6 by 150 mm, Tosoh), maintained at 40°C, eluted with 25 mM KH₂PO₄ (pH 4.7) containing 13% CH₃CN at a flow rate of 1 ml/min.

Spore survival after UV irradiation. Approximately 9 × 10⁷ spores of wild-type IFO1350, ΔP-450mel, and ΔrppA S. griseus strains were suspended in 20 ml of 10% glycerol, stirred at room temperature, and then irradiated with 254-nm UV light at a distance of 30 cm. A portion (0.1 ml) of the UV-irradiated spores of appropriate dilutions at different time intervals was spread on R5 medium. Surviving spores formed visible colonies after incubation at 30°C for 1 day, and colonies were counted the next day. Survival was the ratio of the number of colonies that appeared after administration of an appropriate dose of UV irradiation to that of colonies that appeared without UV irradiation.

Nucleotide sequence accession number. The nucleotide sequence of the 12.1-kb BamHI fragment including P-450mel has been deposited in the DDBJ database with accession number AB218878.
RESULTS

Involvement of P-450mel and rppA in melanogenesis of S. griseus.
The mycelium and spores of S. griseus IFO13350 are brownish and
greenish, respectively, when grown on routine YMPD and
R5 media. We previously found that disruption of the chromo-
somal rppA gene in S. griseus caused the host strain to show
an albino phenotype (8); the mycelium and spores of the
/H9004
rppA
mutant remained colorless. The DNA databases re-
valed that in some actinobacteria, such as
Streptomyces coeli-
color
A3(2),
Streptomyces avermitilis,
and
Saccharopolyspora
erythraea,
rppA appeared to form an operon with a gene en-
coding a P-450 enzyme (7). These observations led us to hy-
pothesize that the THN formed from malonyl-CoA by the
action of RppA would be further modified by the P-450s to
yield a precursor for melanin.

We inactivated the chromosomal P-450mel gene by means
of in-frame deletion to examine possible involvement of this
gene, as a neighbor of rppA, in pigmentation (Fig. 1A). Correct
deletion was checked by Southern hybridization with the 6.5-kb
BamHI fragment as the 32P-labeled probe against the chromo-
somal DNA digested with BamHI (data not shown). The
\Delta P-450mel mutant constructed in this way had a deletion of
the region encoding Glu-84 to Tyr-250. Because THN is readily
converted into flavin by auto-oxidation, we expected that the
\Delta P-450mel mutant would be red due to accumulation of fla-
vin. In fact, the mycelium and spores of mutant \Delta P-450mel
were light red, which were apparently different from those of
the wild-type strain IFO13350 and mutant \Delta rppA (Fig. 1B).
Introduction of pKU209-RB6 containing P-450mel alone into
mutant \Delta P-450mel caused the host to produce the same brown-
ish pigment, as did strain IFO13350 (data not shown). The
red-brown pigment accumulated in mutant \Delta P-450mel
was identified to be flavin, which was nonenzymatically derived
from THN, a product from malonyl-CoA by the action of
RppA. These data suggested that the brownish pigment, which
we later named HPQ melanin, was formed as a result of po-
lymerization of an unstable compound derived from malonyl-
CoA via THN.

Identification of HPQ as a precursor of melanin in S. griseus.
We constructed three plasmids, pNF1 carrying both P-450mel and
rppA, pNF2 carrying rppA alone, and pNF3 carrying P-450mel
alone (Fig. 1A), by using pIJ6021, and introduced them by pro-
toplast transformation into *S. lividans* TK21. These genes were all under the control of a strong, thiostrepton-inducible *tipA* promoter. After the *S. lividans* cells had been grown in the presence of thiostrepton, cell extracts were prepared and analyzed by HPLC (Fig. 2A). *S. lividans* harboring pNF2 accumulated THN and flaviolin (Fig. 3A), as we detected both compounds as products from the in vitro reaction of RppA on malonyl-CoA (8). Flaviolin was probably produced mainly as a result of auto-oxidation of THN and as a result of oxidation of THN by MomA (7). *S. lividans* harboring pNF1 produced a dark green pigment, which was later identified to be HPQ (see below), whereas no HPQ was produced by *S. lividans* harboring pNF2 or the vector pIJ6021 (Fig. 2A).

Structural elucidation of the dark green pigment accumulated in *S. lividans* harboring pNF1 was difficult because the pigment was unstable and rapidly underwent polymerization. Hence, we treated the cell extract with trimethylsilyl-diazomethane since the pigment was presumed to be derived from THN, which possesses phenolic hydroxyl groups that can be replaced by methyl ethers. The methyl-substituted pigment was identified as 4,9-dihydroxy-1,6,7,12-tetramethoxyperylene-3,10-quinone (Fig. 3A) by proton and carbon NMR spectra, with the aid of heteronuclear multiple bond correlation (HMBC) analysis, and HRESI+/TOF-MS. From the structure of the methylated compound, we deduced the structure of the green pigment to be HPQ (1,4,6,7,9,12-hexahydroxyperylene-3,10-quinone). This deduction was confirmed by observing a [M – H]− ion peak of HPQ at m/z 377.02980 (calculated for C20H9O8, 0.06 mmu error) by HRESI+/TOF-MS.

HPQ was also detected in the extract of *S. griseus* grown on agar medium (Fig. 2B), although its amount gradually decreased during prolonged cultivation (data not shown), perhaps due to polymerization of the HPQ produced. As described above, no HPQ formation was observed in the ΔrppA or ΔP-450mel mutant, whereas the ΔP-450mel mutant accumulated flaviolin as a shunt product. As described below, P-450mel did not form HPQ from flaviolin in vitro. These in vivo observations suggested that HPQ was an intermediate of melanin biosynthesis in *S. griseus* and that P-450mel was responsible for the synthesis of HPQ, catalyzing aryl coupling of the THN that had been produced from malonyl-CoA by RppA (Fig. 3A).

**In vitro analysis of aryl coupling of THN by P-450mel.** For convenient purification of P-450mel, we placed its coding sequence under the control of the T7 promoter in pET26b and introduced it in *E. coli* BL21 (DE3). Plasmid pET26b-P-450mel thus constructed would direct the synthesis of P-450mel–Leu–His–His, P-450mel purified from the soluble fraction using histidine-binding resin gave a single protein band of about 42 kDa, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. P-450mel produced in *E. coli* showed an absorbance at 450 nm on reduction by sodium dithionite and subsequent bubbling with CO, which suggested that the enzyme was active (results not shown).

For reconstitution of the P-450mel activity in vitro, we used the ferredoxin and ferredoxin-NADP+ reductase from spinach, as was successfully used for reconstitution of a soluble cytochrome P-450<sub>om</sub> from *S. griseus* (20). Incubation of the reconstituted system with THN gave two products, as analyzed by HPLC (Fig. 4A). The product at retention time 9 min was identified as HPQ by its comigration with authentic HPQ, which was prepared from *S. lividans* harboring pNF1 (Fig. 4B).

The product at retention time 6.2 min appeared to be pseudo-HPQ (Fig. 3A), a dimeric form of THN, because it had a molecular mass of 382, which is equal to [2M<sub>om</sub> – 2] Da of THN (M<sub>om</sub> 192), as revealed by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry analysis (data not shown). The in vitro formation of HPQ from THN by the action of P-450mel was consistent with the in vivo observation that simultaneous overexpression of *rppA* and P-450mel in *S. lividans* led to accumulation of HPQ.
Insignificant amounts of HPQ and pseudo-HPQ were observed in the control incubations with a boiled enzyme (Fig. 4C) and without the redox partners (Fig. 4D), indicating that these products were also formed nonenzymatically as a result of free radical-induced oxidation of THN, but at a lesser rate. We then performed the reaction using pseudo-HPQ which was recovered from the reaction of THN as a substrate to clarify whether both steps of sequential oxidation of THN leading to HPQ were catalyzed by P-450mel. P-450mel rapidly consumed pseudo-HPQ to yield HPQ (Fig. 4E), although pseudo-HPQ was extremely unstable and underwent spontaneous intramolecular aryl coupling to yield HPQ in a control reaction containing a boiled P-450mel enzyme (Fig. 4F). These results suggest that P-450mel catalyzes sequential oxidation of THN, which is intramolecular aryl coupling of THN and intramolecular aryl coupling of the resultant pseudo-HPQ, to yield HPQ (Fig. 3A).

**Protective efficacy of HPQ melanin against UV radiation.**

The melanin polymer has many interesting properties, the most conspicuous of which is its wide spectral absorbance, including the UV region, due to the high degree of conjugation in the molecule. We determined the viability of melanized and nonmelanized spores after different doses of UV irradiation. The protective efficacy of HPQ against UV radiation was demonstrated in the biosynthesis of a benzylisoquinoline alkaloid at the cell-free level (26), P-450 enzymes catalyzing aryl-aryl coupling have not been characterized until the recent discovery of OxyC, which is perhaps involved in the last oxidative phenol coupling in vancomycin biosynthesis (17). However, no in vitro analysis of OxyC, which shares 32% identity in amino acid sequence to P-450mel, was conducted because its physiological substrate was unknown and unavailable. Very recently, Zhao et al. (27) solved the crystal structure of CYP158A2 from *S. coelicolor* A3(2) and showed that it catalyzes aryl coupling of two molecules of flavilin (Fig. 3A), as predicted by Cortés et al. (4). Therefore, these two enzymes are the same in their catalytic properties, although CYP158A2 and P-450mel share only 43% identity in amino acid sequence. In fact, we used flavilin as a substrate for P-450mel and detected a probable dimer of flavilin as the product (data not shown). Since disruption of the CYP158A2 gene or an *rppA* homologue in *S. coelicolor* A3(2) (10) or in *S. lividans* TK21 (our unpublished data) caused no effects on pigmentation, these genes appear to be involved in the biosynthesis of a secondary metabolite other than HPQ melanin.

**DISCUSSION**

The present study has demonstrated that P-450mel mediates the biaryl coupling of THN without inserting an oxygen atom into the product, HPO. The reaction mechanism is best explained by a radical coupling as follows. One-electron oxidation of THN affords a phenolate radical, and subsequent intramolecular radical coupling furnishes the aryl-aryl bond in a regiospecific manner. The resultant pseudo-HPQ is subjected to further intramolecular aryl coupling that elaborates HPQ (Fig. 3A). However, a cationic mechanism, which is an attack of an anion from THN on a cation of another THN molecule, cannot be excluded.

Although the aryl-aryl bond formation by a P-450 catalyst was first demonstrated in the biosynthesis of a benzylisoquinoline alkaloid at the cell-free level (26), P-450 enzymes catalyzing aryl-aryl coupling have not been characterized until the recent discovery of OxyC, which is perhaps involved in the last oxidative phenol coupling in vancomycin biosynthesis (17). However, no in vitro analysis of OxyC, which shares 32% identity in amino acid sequence to P-450mel, was conducted because its physiological substrate was unknown and unavailable. Very recently, Zhao et al. (27) solved the crystal structure of CYP158A2 from *S. coelicolor* A3(2) and showed that it catalyzes aryl coupling of two molecules of flavilin (Fig. 3A), as predicted by Cortés et al. (4). Therefore, these two enzymes are the same in their catalytic properties, although CYP158A2 and P-450mel share only 43% identity in amino acid sequence. In fact, we used flavilin as a substrate for P-450mel and detected a probable dimer of flavilin as the product (data not shown). Since disruption of the CYP158A2 gene or an *rppA* homologue in *S. coelicolor* A3(2) (10) or in *S. lividans* TK21 (our unpublished data) caused no effects on pigmentation, these genes appear to be involved in the biosynthesis of a secondary metabolite other than HPQ melanin.

Perylenequinones form a relatively small but expanding group of biologically active pigments obtainable from natural sources (23). Although HPQ was chemically synthesized for evaluation of its antiviral activity (22), this is the first report of isolation of HPQ from natural sources. We have shown that HPQ melanin in *S. griseus* is synthesized by the condensation of malonyl-CoA to yield THN by the action of RppA and the
We showed that the HPQ melanin of the ascomycete *Daldinia concentrica* (1). The only difference in the biosynthetic pathway between *S. griseus* and *D. concentrica* is that the ascomycete synthesizes the perylenequinone by symmetrical oxidative coupling of two molecules of DHN instead of THN. This analogy is remarkable because the analogous pathway is conserved between two distinct organisms, prokaryotic streptomycetes and eukaryotic ascomycetes. It is noteworthy that DHN melanin is derived from 4,9-dihydroxy-3,10-perylenequinone, which is synthesized by PKS1, a type I PKS (6). Thereafter, a series of reductions and dehydrations take place, leading to DHN (2). The last step is polymerization of DHN, yielding DHN melanin.

It is interesting that 4,9-dihydroxy-3,10-perylenequinone, which is derived from 4,5,4'-5'-tetrahydroxy-1,1'-diphenyl, was isolated as a precursor of perylenequinone-related melanin from the ascomycete *Daldinia concentrica* (1). The only difference in the biosynthetic pathway between *S. griseus* and *D. concentrica* is that the ascomycete synthesizes the perylenequinone by symmetrical oxidative coupling of two molecules of DHN instead of THN. This analogy is remarkable because the analogous pathway is conserved between two distinct organisms, prokaryotic streptomycetes and eukaryotic ascomycetes. It is noteworthy that DHN melanin is associated with virulence and pathogenicity on infection of animal and plant hosts with DHN melanin producers (15). We showed that the HPQ melanin of *S. griseus* enhances protection from UV irradiation, which supports the idea that HPQ melanin plays a role in protection of the host cell from an environmental stress.

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