Grixazone contains a phenoxazinone chromophore and is a secondary metabolite produced by *Streptomyces griseus*. In the grixazone biosynthesis gene cluster, *griF* (encoding a tyrosinase homolog) and *griE* (encoding a protein similar to copper chaperons for tyrosinases) are encoded. An expression study of *GriE* and *GriF* in *Escherichia coli* showed that *GriF* activated *GriF* by transferring copper ions to *GriF*, as has been observed for a *Streptomyces* metabolism network system in which the MelC1 copper chaperon transfers copper ions to MelC2 tyrosinase. In contrast with tyrosinases, *GriF* showed no monophenolase activity, although it oxidized various *o*-aminophenols as preferable substrates rather than catechol-type substrates. Deletion of the *griEF* locus on the chromosome resulted in accumulation of 3-amino-4-hydroxybenzaldehyde (3,4-AHBAL) and its acetylated compound, 3-acetylamino-4-hydroxybenzaldehyde. *GriF* oxidized 3,4-AHBAL to yield an *o*-quinone imine derivative, which was then non-enzymatically coupled with another molecule of the *o*-quinone imine to form a phenoxazinone. The coexistence of *N*-acylcysteine in the *in vitro* oxidation of 3,4-AHBAL by *GriF* resulted in the formation of grixazone A, suggesting that the –SH group of *GriF* is conjugated to the o-quinone imine formed from 3,4-AHBAL and that the conjugate is presumably coupled with another molecule of the *o*-quinone imine. *GriF* is thus a novel *o*-aminophenol oxidase that is responsible for the formation of the phenoxazinone chromophore in the grixazone biosynthetic pathway.

We have long studied the A-factor regulatory cascade that leads to secondary metabolite formation and morphological differentiation in *Streptomyces griseus* (1, 2). The A-factor (2-isocapryloyl-3-AHBAL by GriF) triggers the synthesis of almost all the secondary metabolites produced by this species. One of the secondary metabolites under the control of the A-factor is grixazone. Grixazone is a yellow pigment and actually a mixture of grixazones A and B (compounds 1a and 1b) (see Fig. 2C) (3). Grixazone A is a novel compound, and grixazone B has been reported to show a parasiticide activity (4).

Grixazones contain a phenoxazinone chromophore. The phenoxazine skeleton is common to actinomycin D produced by *Streptomyces griseus* (5), michigazone produced by *Streptomyces antibioticus* (6), texazone produced by *Streptomyces* sp. WRAT-210 (7), exfoliazone produced by *Streptomyces exfoliatus* (8), and 4-deethoxymichigazone produced by *Streptomyces halstedii* (9). Hsieh and Jones (10) reported a phenoxazinone synthase in *S. antibioticus* that catalyzes the six-electron oxidative coupling of *o*-aminophenol compounds derived from tryptophan through 3-hydroxyanthranilic acid. However, disruption of the phenoxazinone synthase gene in *S. antibioticus* does not affect actinomycin D synthesis, showing that the phenoxazinone skeleton in actinomycin D is biosynthesized *in vivo* by a still unknown enzyme or non-enzymatically (11). On the other hand, michigazone with a hydroxymethyl group at the 8-position of the phenoxazinone and texazone with a carboxyl group at this position are assumed to be synthesized from a precursor(s) different from that of actinomycin D with no functional group at this position (7). No study on the precursor(s) or the biosynthetic enzyme for the phenoxazinone skeleton of these compounds has so far been reported. Because grixazones A and B contain an aldehyde and a carboxyl group, respectively, at the 8-position, the biosynthesis of the phenoxazinone skeleton in grixazones should be the same as those in michigazone and texazone. The phenoxazinones of exfoliazone and 4-deethoxymichigazone (which both contain a hydroxymethyl group at this position) are also presumed to be synthesized in a similar way.

We cloned the grixazone biosynthesis gene cluster and revealed the organization of the genes. Because all the biosynthesis and regulatory genes for a certain secondary metabolite are usually contained within the gene cluster, we expected that one of the gene products encoded in this gene cluster is responsible for the formation of the phenoxazinone skeleton of grixazones. Because some tyrosinases show an additional activity to use *o*-aminophenol as a substrate and yield phenoxazinones (12, 13), we aimed at two gene products (GriE and GriF) that are similar to the MelC1 and MelC2 proteins in streptomycetes, respectively. MelC2 is a tyrosinase and MelC1 is a copper chaperon for MelC2. Tyrosinase is a copper-containing monooxygenase that catalyzes two types of reaction, the *o*-hydroxylation of monophenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinone (diphenolase activity) using molecular oxygen. Tyrosinase is a member of the type 3 copper protein family, which also includes hemocyanins and catechol oxidases. The type 3 copper proteins contain a binuclear copper active site that is composed of two closely spaced copper atoms, each coordinated by three histidine residues. Although the binuclear copper active site is highly conserved, as determined by its characteristic spectroscopic signatures, by sequence homologies, and by the crystal structures of several hemocyanins and a catechol oxidase, their functions in catalysis are different. Catechol oxidases oxidize diphenols to the corresponding quinones but lack tyrosine hydroxylase activity. Hemocyanins serve as oxygen carriers and oxygen storage proteins in arthropods and mollusks. Tyrosinases are responsible for melanin pigmentation in streptomycetes, browning in plants, and melanization in animals. In
addition to the intrinsic tyrosinase activities, the tyrosinases from *Neurospora crassa* (12), mushroom (13), and *Streptomyces glaucescens* (12) convert 2-aminophenols into the corresponding o-quinone imines in *vitro* at a lesser rate, which are then non-enzymatically converted into phenoxazinones.

In this study, we determined the plausible substrate of GriF by structural elucidation of an intermediate (3-amino-4-hydroxybenzaldehyde) from a extract, which are then non-enzymatically converted into phenoxazinones in *Streptomyces*. We have thus concluded that GriF is an o-aminophenol oxidase responsible for the *in vivo* formation of the phenoxazine none chromophore in the *grixazone biosynthetic pathway*.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Media, and Materials**—*S. griseus* strain IFO13350 was obtained from the Institute of Fermentation (Osaka, Japan). *S. griseus* strains were grown at 26.5 or 30 °C in TYP medium (0.2% yeast extract, 0.4% Bacto-peptone, 0.05% NaCl, 0.2% MgSO₄·7H₂O, 1% glucose, and 0.5% glycine, pH 7.2) and SMM medium (0.9% glucose, 0.9% asparagine, 0.2% (NH₄)₂SO₄, 0.24% Trizma (Tris base), 0.1% NaCl, 0.05% K₂SO₄, 0.02% MgSO₄·7H₂O, 0.01% CaCl₂, 0.003% (0.25 mxt) KH₂PO₄, and 1% trace element solution (14), pH 7.2). The thionestrase resistance plasmid pIJ702, with its copy number of 40–300 (14), was used for expression of griR. *E. coli* strains JM109 and TOP10 (Invitrogen) and plasmids pUC19 and pCR4Blunt-TOPO (Invitrogen), pET-26b (Novagen) and plasmids pCR4Blunt-TOPO and sequenced to confirm the absence of errors during PCR, the DNA ligase, Pyrobest DNA polymerase, and other DNA-modifying enzymes were purchased from Takara Biochemicals. DNA was manipulated in *Streptomyces* (14) and *E. coli* (15) as described. L-Tyrosine, L-3,4-dihydroxyphenylalanine, and benzoic compounds except 3,4-AHBAL were purchased from Wako Chemicals. 3,4-AHBAL (compound 3,4-AcAHBAL) was obtained from the Institute of Fermentation (Osaka, Japan).

**Identification of 3,4-AHBAL and 3-Acetylamino-4-hydroxybenzaldehyde**—The 3,4-AHBAL (compound 2a) and 3-AcAHBAL (compound 2b) were identified on the basis of the following spectroscopic parameters (see Fig. 2C). 3,4-AHBAL (compound 2a): 1H NMR (500 MHz, Me₂SO-d₆) δ 9.63 (s, 1H, CHO), 7.60 (d, 1H, J = 2.5 Hz, ArH), 7.03 (dd, 1H, J = 7.5 Hz, ArH), and 6.81 (d, 1H, J = 8.0 Hz, ArH). 13C NMR (125 MHz, Me₂SO-d₆) δ 191.6 (C-7), 150.7 (C-4), 137.6 (C-3), 129.3 (C-1), 122.4 (C-6), 114.0 (C-2), and 112.5 (C-5); high resolution electron-spray ionization time-of-flight mass spectrum, m/z 137.90312 [M]+ (calculated for C₇H₇N₁O₂, 2.39 mmu error). 3,4-AcAHBAL (compound 2b): 1H NMR (500 MHz, Me₂SO-d₆) δ 11.12 (s, 1H, OH), 8.976 (s, 1H, CHO), δ 9.34 (s, 1H, NH), 8.41 (d, 1H, J = 2.0 Hz, ArH), 8.73 (dd, 1H, J = 8.5 Hz, 2.0, ArH), 8.70 (d, 1H, J = 8.0 Hz, ArH), and δ 8.12 (s, 3H, CH₃). 13C NMR (125 MHz, Me₂SO-d₆) δ 193.1 (C-7), 169.2 (C-8), 153.6 (C-4), 128.3 (C-3), 127.7 (C-6), 127.2 (C-2), 121.2 (C-5), 115.4 (C-4), and 23.8 (C-9); high resolution electrospray ionization time-of-flight mass spectrum, m/z 180.06632 [M + H]+ (calculated for C₇H₆O₂N, 0.25 mmu error).

**o-Aminophenol Oxidase Catalyzing Phenoxazinone Formation**

The o-aminophenol oxidase (griF) was expressed with PCR with two primers, 5′-CACATTGCTCAGCTAGGACG-3′ (with the start codon of griF in italic letters and the Sphl site underlined) and 5′-TCTGAATCTTACGCGAGCCGCC-3′ (with the stop codon of griF in italic letters and the EcoRI site underlined). After the amplified fragment had been cloned into pCR4Blunt-TOPO and sequenced to confirm the absence of PCR-generated errors, the griF sequence was excised with Sphl plus EcoRI and cloned between the Sphl and EcoRI sites of a pIJ702-derived plasmid in which the melC-melC2 sequence was replaced by a shorter linker containing Spht, BglIII, and EcoRI sites, resulting in pIJ702-griR. S. griseus mutant ΔgriF-containing pIJ702-griF was cultivated at 26.5 °C with rotary shaking for 3 days in SMM medium supplemented with 10 μg/ml thiorstrene. The culture broth was cleared by centrifugation at 6000 × g for 20 min and treated with hexane for liquid-liquid distribution. The materials in the aqueous layer were extracted with ethyl acetate and dried by evaporation. The crude material was dissolved in Me₂SO and subjected to reversed-phase HPLC using a Waters 600 HPLC system equipped with a Waters 996 photodiode array detector. Compounds 2a and 2b (see Fig. 2C) were collected. HPLC conditions were as follows: column, CAPCELL PAK C18 (10 × 250 mm; Shinseido Fine Chemicals); flow rate, 3 ml/min; solvent A, 0.1% (v/v) trifluoroacetic acid in water; and solvent B, 0.1% (v/v) trifluoroacetic acid in 90% (v/v) acetonitrile in water. After 10 μl of the crude material had been injected into the column equilibrated with 5% solvent B, the column was initially developed isocratically for 3 min, followed by elution with a linear gradient from 5 to 100% solvent B over 13 min. 3,4-AHBAL (compound 2a) and 3-AcAHBAL (compound 2b) were identified on the basis of the following spectroscopic parameters (see Fig. 2C). 3,4-AHBAL (compound 2a): 2H NMR (500 MHz, Me₂SO-d₆) δ 9.63 (s, 1H, CHO), 7.60 (d, 1H, J = 2.5 Hz, ArH), 7.03 (dd, 1H, J = 7.5 Hz, ArH), and 6.81 (d, 1H, J = 8.0 Hz, ArH). 13C NMR (125 MHz, Me₂SO-d₆) δ 191.6 (C-7), 150.7 (C-4), 137.6 (C-3), 129.3 (C-1), 122.4 (C-6), 114.0 (C-2), and 112.5 (C-5); high resolution electron-spray ionization time-of-flight mass spectrum, m/z 137.90312 [M]+ (calculated for C₇H₇N₁O₂, 2.39 mmu error). 3,4-AcAHBAL (compound 2b): 2H NMR (500 MHz, Me₂SO-d₆) δ 11.12 (s, 1H, OH), 8.976 (s, 1H, CHO), δ 9.34 (s, 1H, NH), 8.41 (d, 1H, J = 2.0 Hz, ArH), 8.73 (dd, 1H, J = 8.5 Hz, 2.0, ArH), 8.70 (d, 1H, J = 8.0 Hz, ArH), and δ 8.12 (s, 3H, CH₃). 13C NMR (125 MHz, Me₂SO-d₆) δ 193.1 (C-7), 169.2 (C-8), 153.6 (C-4), 128.3 (C-3), 127.7 (C-6), 127.2 (C-2), 121.2 (C-5), 115.4 (C-4), and 23.8 (C-9); high resolution electrospray ionization time-of-flight mass spectrum, m/z 180.06632 [M + H]+ (calculated for C₇H₆O₂N, 0.25 mmu error).

Expression of griE and griF in *E. coli*—The *griE* and *griF* gene sequences were amplified by PCR with two primers, 5′-CACATTGCTCAGCTAGGACG-3′ (with the start codon of *griF* in italic letters and the Ndel site underlined) and 5′-TCTGAATCTTACGCGAGCCGCC-3′ (with the Xhol site underlined). After the amplified fragment had been cloned into pCR4Blunt-TOPO and sequenced to confirm the absence of PCR errors, the *griF* sequence was excised with Ndel and Xhol and cloned into pET-26b (+). The *griF* sequence, together with the lacI gene, was excised from the plasmid by digestion with Bsp1102I and Bst1107I and transferred onto pET-17b digested with Bsp1102I and Bst1107I, resulting in pET-griE, which contained *griF* under the control of the T7 promoter.
o-Aminophenol Oxidase Catalyzing Phenoxazinone Formation

T7/lac promoter. The griE sequence was amplified by PCR with primers 5’-CATATGGCCATGAAAGGGGAG-3’ (with the start codon of griE in italic letters and the Ndel site underlined) and 5’-CTCGAGTCAGAGTGGCGTGAGCTG-3’ (with the stop codon of griE in italic letters and the XhoI site underlined). The amplified fragment was cloned into pCR4Blunt-TOPO and sequenced to confirm the absence of errors during PCR. The griF sequence was excised with Ndel and Xhol and cloned in pET-17b, resulting in pET-griE, which contained griE under the control of the T7 promoter. For construction of plasmid containing both griE and griF, pET-griE was digested with Sphl and treated with T4 DNA polymerase to produce blunt ends, followed by BgIII digestion. To this linear plasmid with a BgIII site at one end and a blunt end at the other end, a 0.9-kb fragment containing the griE expression cassette excised from pET-griE by digestion with SspI and BgIII was ligated, resulting in pET-griEF, which contained griE under the control of the T7 promoter and griF under the control of the T7/lac promoter. These plasmids were used to transform E. coli BL21(DE3)/pLysS cells. Transformant cells were cultured at 26.5°C for 10 h in LB medium supplemented with 50 μg/ml ampicillin, 34 μg/ml chloramphenicol, and 1% (w/v) lactose, allowing constant expression of the T7 and T7/lac promoters.

Purification of Recombinant GriF—All operations were carried out at 4°C. Cells (3.6 g, wet weight) were harvested by centrifugation and suspended in 6 ml of buffer A (50 mM sodium phosphate, pH 8.0, 0.5 M NaCl, and 10% glycerol) containing 10 mM imidazole and 2 mg/ml lysozyme. After incubation of the mixture on ice for 30 min, the cell suspension was sonicated for 3 min and then centrifuged at 10,000 × g for 10 min to remove cell debris. Polyethyleneimine was added to the supernatant to give a final concentration of 0.1% (w/v), followed by centrifugation of the mixture at 20,000 × g for 20 min. The supernatant was a crude enzyme solution used for some analyses. For further purification of GriF, the supernatant was applied to a Cu²⁺-charged HiTrap chelating HP column (1 ml; Amersham Biosciences) equilibrated with buffer A containing 10 mM imidazole. The column was successively washed with buffer A containing 10 mM imidazole and buffer B (50 mM sodium phosphate, pH 8.0) containing 50 mM imidazole, and then the materials were eluted with buffer B containing 200 mM imidazole. The eluate was applied to a HiTrap Q column (Amersham Biosciences) equilibrated with buffer B containing 200 mM imidazole, and the column was washed with the same buffer. The flow-through and wash fractions were pooled, and ammonium sulfate was added to 20% saturation. The enzyme solution was subjected to fast protein liquid chromatography with a Resource PHE column (1 ml; Amersham) by fast protein liquid chromatography with isocratic elution in 20 mM sodium phosphate (adjusted to pH 7.0, 5 mM substrate, and an enzyme sample. The mixture without enzyme was preincubated at 30°C in a cuvette, and the reaction was started by the addition of the enzyme. GriF activity was assayed by monitoring the formation of 2-aminophenoxazin-3-one (APO; ε₄₃₃ = 3600 m⁻¹ cm⁻¹) from o-aminophenol as a substrate by measuring the absorbance at 433 nm on a spectrometer (Spectra Max Plus, Molecular Devices Corp.). The initial velocity was used for the calculation of activity, which was defined as the amount (moles) of substrate decreased per s at 30°C. The tyrosinase activity of GriF and the mushroom tyrosinase was assayed by monitoring the formation of dopachrome (ε₆₇₅ = 3600 m⁻¹ cm⁻¹) from l-tyrosine or l-3,4-dihydroxyphenylalanine as a substrate by measuring the absorbance at 475 nm (16). One unit of GriF activity was defined as the amount of enzyme that decreased 1 nmol of substrate/s at 30°C.

Identification of the Reaction Products from o-Aminophenol, o-Aminophenol Plus Catechol, and 3,4-AHBAL—The products were analyzed by reversed-phase HPLC. HPLC conditions were as follows: column, Senshu Pak DOCOSIL-B (4.6 × 250 mm, Senshu Kagaku); column temperature, 30°C; and flow rate, 1 ml/min. After 10 μl of the reaction mixture had been injected into the column equilibrated with solvent A, the column was initially developed isocratically for 3 min, followed by elution with a linear gradient from 0 to 100% solvent B over 15 min. The reaction products from o-aminophenol, o-aminophenol plus catechol, and 3,4-AHBAL were identified as APO (compound 3a) (see Fig. 2C), 2-hydroxyphenoxazin-3-one (HPO; compound 3b), and 2-aminophenoxazin-3-one-8-aldehyde (APOAL; compound 3c), respectively, on the basis of the following spectroscopic parameters. APO (compound 3a): ¹H NMR (500 MHz, Me₂SO-d₆) δ 7.69 (dd, 1H, J = 8.0, 1.5, ArH), δ 7.49 (dd, 1H, J = 8.3, 1.5, ArH), δ 7.45 (td, 1H, J = 7.0, 1.5 Hz, ArH), δ 7.38 (td, 1H, J = 7.5, 1.5, ArH), δ 6.63 (s, 1H, ArH), and δ 6.35 (s, 1H, ArH); ¹3C NMR (125 MHz, Me₂SO-d₆) δ 180.2 (C-3), δ 149.1 (C-10a), δ 148.1 (C-4a), δ 147.6 (C-2), δ 141.9 (C-5a), δ 133.4 (C-9a), δ 128.8 (C-7), δ 127.7 (C-9), δ 125.3 (C-8), δ 116.0 (C-6), δ 103.5 (C-1), and δ 98.1 (C-4); high resolution electrospray ionization time-of-flight mass spectrum, m/z 213.06874 [M + H]⁺ (calculated for C₉H₈N₂O₃, 234.34 mnu error). HPO (compound 3b): ¹H NMR (500 MHz, Me₂SO-d₆) δ 8.71 (d, 1H, J = 7.0, ArH), δ 7.59 (t, 1H, J = 7.5 Hz, ArH), δ 7.54 (d, 1H, J = 8.0 Hz, ArH), δ 7.44 (t, 1H, J = 7.5, ArH), δ 6.69 (s, 1H, ArH), and δ 6.43 (s, 1H, ArH); ¹3C NMR (125 MHz, Me₂SO-d₆) δ 180.3 (C-3), δ 155.7 (C-2), δ 149.1 (C-10a), δ 148.6 (C-4a), δ 142.6 (C-5a), δ 133.2 (C-9a), δ 131.1 (C-7), δ 128.9 (C-9), δ 125.5 (C-8), δ 116.2 (C-6), δ 106.7 (C-1), and δ 104.2 (C-4); high resolution electrospray ionization time-of-flight mass spectrum, m/z 214.06998 [M + H]⁺ (calculated for C₉H₈N₂O₄, 4.25 mnu error). APOAL (compound 3c): ¹H NMR (500 MHz, Me₂SO-d₆) δ 10.05 (s, 1H, CHO), δ 8.20 (d, 1H, J = 2.0 Hz, ArH), δ 7.93 (dd, 1H, J = 8.5, 1.5 Hz, ArH), δ 7.66 (d, 1H, J = 8.0, ArH), δ 6.43 (s, 1H, ArH), and δ 6.36 (s, 1H, ArH); ¹3C NMR (125 MHz, Me₂SO-d₆) δ 191.8 (CHO), δ 180.5 (C-3), δ 149.2 (C-10a), δ 148.6 (C-4a), δ 147.8 (C-2), δ 146.0 (C-5a), δ 133.9 (C-9a), δ 133.2 (C-8), δ 130.1 (C-7), δ 128.1 (C-9), δ 117.1 (C-6), δ 104.3 (C-1), and δ 98.2 (C-4); high resolution electrospray ionization time-of-flight mass spectrum, m/z 241.09174 [M + H]⁺ (calculated for C₈H₇N₂O₄, 30.42 mnu error).

RESULTS

GriF as a Tyrosinase Homolog—We cloned and sequenced the whole gene cluster for the biosynthesis of grixazone from S. griseus IFO13350 (Fig. 1A).² The gene cluster contains 13 open reading frames. Of the 13 gene products, two proteins (GriF and GriG) are similar to the MelC1 and MelC2 proteins, respectively, both of which are responsible for melanin biosynthesis in streptomyces (17–21). MelC2 tyrosinase belongs to the type 3 copper protein family, and MelC1 serves as a copper chaperon for MelC2. Although tyrosinases from various organisms from bacteria to human have different secondary and tertiary structures and domain structures, they all contain two copper-binding sites, each formed by three histidine residues (Fig. 1B). The two copper-binding sites of GriF are also highly conserved. Therefore, GriF was
expected to contain two copper atoms. The two closely spaced copper atoms, each coordinated by three histidine residues, must compose a binuclear copper active site of GriF, as is found for the type 3 copper proteins. Thus, because of the high end-to-end similarity (~50% identity) to MelC2, GriF was expected to show monophenolase and/or diphenolase activity by using molecular oxygen as an oxidizing agent.

GriE shows end-to-end similarity to MelC1 in streptomycetes and has a probable twin arginine translocation–type N-terminal signal peptide for protein secretion (data not shown), as does MelC1 (22). The amino acid sequences of MelC1 in streptomycetes are conserved at a moderate level (40–50% identity) and are always encoded as a neighbor of melC2 (17, 20). GriE was therefore expected to be involved in the transfer of copper ion to the apo form of GriF via binary complex formation (23, 24).

**FIGURE 1.** Organization of the grixazone biosynthesis gene cluster (A) and alignment of amino acid sequences covering the two copper-binding sites (CuA and CuB) of GriF and tyrosinase family members (B). A, the 1.1-kb-region covering the grif–grif region was deleted by double reciprocal crossover. The correct deletion was checked by Southern hybridization with the 0.5-kb fragment as a 32P-labeled probe. wt, wild type; A, the three histidine residues (H) responsible for copper binding by each copper-binding site and the position (H) corresponding to Phe261 of SgMelC2 (18). S. castaneoglobisporus (ScMelC2; accession number A24089 (18)), S. antibioticus (SaMelC2; accession number AAP33665 (20)), S. glaucescens (SgMelC2; accession number A24089 (18)), Streptomyces lavendulae (SlMelC2; accession number 2113331B (21)), N. crosa (NcTYR; accession number YRNC (17)), mushroom (Agaricus bisporus; AbTYR; accession number CAA59432 (38)), and Homo sapiens (HsTYR; accession number NP_000363 (39)).

**Accumulation of 3,4-AHBAL and 3,4-AcAHBAL in S. griseus Mutant Δgrif**—Because some tyrosinases show the ability to convert o-aminophenols into the corresponding phenoxyazinone moieties, in addition to their intrinsic tyrosinase activity (12, 13), we expected that a mutation in grif would result in accumulation of o-aminophenol(s) as a substrate of GriF for the formation of the phenoxyazinone chromophore of grixazones. We deleted the grif locus on the chromosome of *S. griseus*IFO13350 to determine the chemical structure of a possible substrate for GriF or an intermediate in the grixazone biosynthetic pathway (Fig. 1A). As a result of a double-reciprocal crossover, a 1.1-kb grif locus (corresponding to the region from Met1 of GriE to His226 of GriF) was deleted, which was confirmed by Southern hybridization with the 0.5-kb PmaCI-SphI fragment (shown in Fig. 1A) as a 32P-labeled probe against the chromosomal DNA digested with SphI (data not shown).

HPLC analysis of the culture broth of mutant Δgrif revealed the accumulation of some compounds different from grixazones. After 3 days of cultivation, mutant Δgrif accumulated two major compounds (2a and 2b) that were not seen in the culture broth of the parental strain when detected by absorbance at 254 nm (Fig. 2, A and B). After 5 days of cultivation, the parental strain accumulated grixazone A (compound 1a) when detected by absorbance at 433 nm, characteristic for phenoxyazinones, whereas no accumulation of compound 1a was observed for mutant Δgrif (Fig. 2, A and B).

For experimental convenience in purification and structural determination of the compounds that accumulated in mutant Δgrif, we introduced grir under the control of a foreign promoter on the high copy number plasmid pIJ702 into mutant Δgrif because GriR (the pathway-specific transcriptional activator for the grixazone biosynthesis genes) activates the transcription of all the grixazone biosynthesis genes and because overexpression of grir leads to enhancement of the yield of grixazones. The plasmid we constructed was pIJ702-grir, in which grir under the control of the melC promoter would be expressed in the late exponential and stationary phases. The copy number of pIJ702 is 40–300/chromosome (14). *S. griseus* ΔgrifEΔgrif harboring pIJ702-grir produced compounds 2a and 2b in larger amounts during 3–5 days of cultivation. The amounts of these compounds were ~5-fold as large as those produced by mutant Δgrif. After purification of compounds 2a and 2b by HPLC, their structures were determined to be 3,4-AHBAL (compound 2a) (Fig. 2C) and 3,4-AcAHBAL (compound 2b) by 1H and 13C NMR and high resolution electrospray ionization time-of-flight mass spectrometry. These findings suggest that compound 2a or 2b or both serve as a substrate of GriF, as we had expected.

**Production of GriE and GriF in E. coli**—The apo forms of MelC2 tyrosinases responsible for melanin production in *S. antibioticus* (22) and *Streptomyces castaneoglobisporus* (16), forming a stable complex with MelC1, are usually copurified with MelC1. For production of the copper-containing active form of GriF, we constructed pET-grif, which contained grif under the control of the T7 promoter and grir under the control of the T7/lac promoter, and pET-grif, which contained grir under the control of the T7/lac promoter. We also constructed pET-grir as a negative control, which contained grir under the control of the T7 promoter. The GriF protein encoded by these plasmids would have the structure of GriF-Leu-Glu-His6. Both the T7 and T7/lac promoters in *E. coli* BL21(DE3)/pLysS were inducible by lactose in the medium. Crude lysates of *E. coli* harboring pET-grif and pET-grir showed the activity to convert o-aminophenol into APO (compound 3a) (Fig. 2C), whereas no such activity was detected in a crude lysate prepared from *E. coli* harboring pET-grir. The structural elucidation of APO, which was produced by an in vitro reaction of GriF with o-aminophenol, will be described below. The reaction yielding APO

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from o-aminophenol is shown in Fig. 3B. The specific activities of the lysates from E. coli harboring pET-griEF and pET-griF were 0.060 ± 0.003 and 0.0035 ± 0.0003 units/mg of protein, respectively.

We purified GriF from the soluble fraction of E. coli BL21(DE3)/pLysS harboring pET-griEF by following the activity to convert o-aminophenol into APO (compound 3a) by three steps of chromatography (see below). The purified sample (specific activity of 63.3 ± 6.6 units/mg of protein) still contained a small amount of GriE of 13 kDa, as determined by SDS-PAGE (data not shown). GriE contained no histidine tag. The ratio of GriF to GriE in the final sample, even after Resource PHE column chromatography, was ∼10:1. This is perhaps due to the property of GriF to form a complex with GriE, as is found for MelC2 and MelC1. Consistent with this idea, the activity eluted from a Superdex 200 gel filtration column at positions corresponding to 23 kDa (GriF) and 38 kDa (GriF-GriE complex).

Because of the contamination of GriE in the GriF preparation, we examined, as described below, GriF by measuring APO formation from o-aminophenol as a substrate and N-acetylcysteine (NAC) as an -SH group-containing compound.

chromatography, on the assumption that histidine-tagged GriF would bind to the Cu2+ -bound affinity column through the histidine tag, incorporate Cu2+ into apo-GriF to form the active form, and be eluted by 200 mM imidazole, as is observed for the tyrosinase of S. castaneoglobisporus (16). As expected, the Cu2+ -bound HiTrap column chromatography step extraordinarily increased the specific activity (Table 1). The GriF enzyme thus purified gave a single protein band of 36 kDa upon SDS-PAGE (Fig. 4A). Because the GriF preparation showed a specific activity of 65.8 ± 2.6 units/mg of protein, which was almost same as that of the purified enzyme from E. coli harboring pET-griEF, we used this sample for further study.

General Properties of GriF—We determined the general properties of GriF by measuring APO formation from o-aminophenol as a substrate, although 3,4-AHBAL was the best substrate among the various phenols we examined, as described below. GriF was active over a wide pH range of 6.0–11.0, with a maximum activity at pH 8.5–10.5 (Fig. 4C, panel a), and was most stable at pH 6.5–8.5 (panel b). This suggests that alkaline conditions are suitable for the GriF reaction. The optimum temperature for GriF at pH 7.0 was 55 °C (Fig. 4C, panel b). The activation energy was calculated to be 14 kcal/mol on the basis of Arrhenius plots of the data. When the enzyme sample was kept at pH 7.0 for 20 min at various temperatures, it remained active below 55 °C (Fig. 4C, panel d).

We determined the effects of metals on GriF activity. Our attempts to determine the copper content using atomic absorption were hampered...
TABLE 1

| Purification step                                      | Total protein | Specific activity | Yield | Purification |
|--------------------------------------------------------|---------------|------------------|-------|--------------|
| Crude extract                                          | 64            | 0.0035           | 100   | 1            |
| Cu²⁺-charged HiTrap chelating chromatography           | 1.3           | 11               | 6400  | 3100         |
| HiTrap Q chromatography                                | 0.42          | 17               | 3300  | 5000         |
| Resource PHE FPLC                                      | 0.16          | 66               | 4700  | 19,000       |

Because apo-GriF bound Cu²⁺ and became active during Cu²⁺-charged HiTrap chelating column chromatography, the specific activity was dramatically increased after this step.

FPLC, fast protein liquid chromatography.

Substrate Specificity of GriF—As described above, GriF used o-aminophenol as a substrate, yielding APO. The kinetic parameters for various substrates are summarized in Table 2. When o-aminophenols, with the exception of 2-amino-4-methylphenol and 3,4-AHBAL, were used as substrates, the products with a phenoxazinone chromophore were followed by measuring the absorbance at 433 nm, characteristic for phenoxazinone derivatives. When 2-amino-4-methylphenol was used as a substrate, the quinone imine produced was dimerized to yield an unknown compound of 14 carbons, but not a phenoxazinone derivative, as determined by 13C NMR analysis. We did not characterize this compound any further. Production of this compound was followed by measuring the absorbance at 433 nm. When 3,4-AHBAL was used as a substrate, the dimerization of the quinone imine produced was very slow, and no dimerization product was detected during the reaction period. Production of the quinone imine was also followed by measuring the absorbance at 433 nm. The molar extinction coefficient (ε) of the quinone imine was calculated to be 7400 by analyzing the products (a mixture of the substrate and product) by HPLC and estimating the amount of the product from the amount of the substrate decreased. Similarly, ε values of the dimers produced from 3-amino-4-hydroxybenzoic acid (3,4-AHBA; 2-aminophenoxyazin-3-one-8-carboxylate (AOPC); compound 3d), o-aminophenol (APO; compound 3a), and 3-amino-4-methylphenol were calculated to be 30,000, 9600, and 7000, respectively. On the other hand, we tentatively used a value of 30,000 calculated for APOC for ε of the dimer from 4-amino-3-hydroxybenzoic acid (4,3-AHBA) because it was not efficiently used as a substrate. When catechol was used, a yellow product, perhaps o-quinone, was obtained, and its ε value was calculated to be 2400. We used an ε value of 2400 also for 3,4-dihydrobenzaldehyde and protocatechuic acid. Thus, we measured the amounts of the o-quinones from o-diphenol derivatives, the quinone imine from 3,4-AHBAL, and some dimerization products from other o-aminophenols as products of the GriF reaction. Although it is not known why the dimerization of the quinone imine from 3,4-AHBAL was very slow, we assume that dimerization of the quinone imine occurs non-enzymatically, as discussed below. Therefore, to compare the kinetic parameters of GriF for different substrates, we defined GriF activity on the basis of the amount of the substrate decreased (–d[S]/dt), but not the amount of the product (d[P]/dt). Even though the amounts of the products were measured using the calculated or estimated ε value, the kinetic parameters in Table 2 do not cause serious problems in discussion of the substrate specificity of GriF.

GriF showed the highest activity toward 3,4-AHBAL to yield the corresponding quinone imine, which, in turn, dimerized to form APOAL (compound 3c), as was expected from the finding that mutant ΔgriEF accumulated this compound. The formation of APOAL from two molecules of the quinone imine (in which the –CHO group on one of the
o-Aminophenol Oxidase Catalyzing Phenoxazinone Formation

In Vitro Production of Grixazone A by GriF—In the oxidation of catechin by tyrosinases and peroxidases in the presence of -SH compounds (e.g. glutathione), the o-quinones produced are non-enzymatically conjugated to glutathione, resulting in the formation of catechin-glutathione conjugates (26). We therefore supposed that, in the grixazone biosynthetic pathway, the N-acetylcysteine moiety would be introduced in the quinone imine produced from 3,4-AHBA by GriF by non-enzymatic conjugation via the -SH group. In the reaction of GriF with 5 mM 3,4-AHBA, peak 4 in Fig. 5B (panel b) gradually increased until 30 min, and peak 3c appeared thereafter. The compound in peak 3c was identified to be APOAL (compound 3c) by [1H and 13C NMR and mass spectrometry. The peak in peak 4 was presumed to be the quinone imine derived from 3,4-AHBA because its m/z was 135.0334 [M+H]+, as determined by high resolution electrospray ionization time-of-flight mass spectrometry (calculated for C10H13N2O2, 1.31 mmu error). The quinone imine was detected in the culture broth of S. griseus, excluding the possibility that this compound is an artifact produced by the in vitro reaction. When 1 mM N-acetylcysteine was present in the GriF reaction with 3,4-AHBA, a new peak 1a appeared, in addition to peaks 3c and 4 (Fig. 5B, panel c). The compound in peak 1a had the same retention time upon HPLC and the same UV-visible spectrum as grixazone A (compound 3a) (Fig. 5B, panel a).

Heterodimerization—As described above, GriF produced APO when o-aminophenol was used as a substrate. We determined whether heterogenous dimerization occurs when catechol is present in the GriF reaction with o-aminophenol. In the presence of o-aminophenol alone, GriF gave a single product of APO (compound 3a) (Fig. 5A, panel a). However, the coexistence of catechol yielded an additional product (compound 3b) (Fig. 5A, panel b), which was identified to be HPO (Fig. 2C) by 1H and 13C NMR and mass spectrometry. The coexistence of catechol in the GriF reactions with 3,4-AHBA, 3-amino-4-methylphenol, 3,4-AHBA, and 3,4-AHBA also yielded a probable heterogeneously dimerized product, in addition to the homogeneously dimerized product (data not shown). However, the coexistence of phenol or aniline did not yield any product other than APO in the GriF reaction with o-aminophenol, as determined by following the absorbance from 210 to 490 nm by HPLC (Fig. 5A, panels c and d).

### TABLE 2

Kinetic parameters for GriF activity

| Substrate                      | \( k_{\text{cat}} \) | \( K_m \) | \( k_{\text{cat}}/K_m \) | Relative \( k_{\text{cat}}/K_m \) | \( K_i \) |
|--------------------------------|------------------|--------|----------------|----------------------------|--------|
| **o-Aminophenol derivatives**  |                  |        |                |                            |        |
| o-Aminophenol                  | 20 ± 3           | 3.5 ± 0.6 | 5800 ± 170 | 100                        | 160    |
| 3,4-AHBA                       | 14 ± 1           | 0.58 ± 0.08 | 24,000 ± 1400 | 420                        | 0.10   |
| 3,4-AcAHBAL                     | 18 ± 1           | 0.75 ± 0.06 | 23,000 ± 1200 | <0.01                      | <0.01  |
| 2-Amino-4-methylphenol          | ND               | ND     | 190 ± 5       | 3.2                        | 0.04   |
| 4,3-AHBA                        | ND               | ND     | 7.3 ± 0.1     | 0.13                       | 0.08   |
| 3-Amino-4-hydroxybenzensulfonic acid | ND   | ND     |                |                            |        |
| **o-Diphenol derivatives**      |                  |        |                |                            |        |
| 3,4-Dihydroxybenzaldehyde      | 0.80 ± 0.09      | 0.41 ± 0.06 | 970 ± 40     | 17                         | 0.21   |
| Catechol                       | 12 ± 1           | 19 ± 2  | 320 ± 3       | 5.6                        | 2500   |
| Protocatechuic acid            | ND               | ND     | 6.3 ± 0.3     | 0.11                       | 5.9    |
| 1-DOPA                         | 0.066 ± 0.016    | 5.5 ± 1.8 | 12 ± 1       | 0.21                       | 100    |
| **Other compounds**            |                  |        |                |                            |        |
| L-Tyrosine                     | <0.01            | <0.01  | 3.2 ± 0.6     |                            |        |
| p-Hydroxybenzaldehyde         | <0.01            | <0.01  | 1.9 ± 0.2     |                            |        |
| 4-Hydroxy-3-nitrobenzaldehyde | <0.01            | <0.01  | 3.9 ± 0.3     |                            |        |
| o-Nitrophenol                  | <0.01            | <0.01  | 6.1 ± 0.6     |                            |        |
| Aniline                        | <0.01            | <0.01  | 14 ± 1        |                            |        |
| Phenol                         | <0.01            | 12     | 21 ± 3        |                            |        |
DISCUSSION

Functions of GriF and GriE—GriF encoded within the grixazone biosynthesis gene cluster has been found to be responsible for the formation of the phenoxazinone chromophore of grixazone by oxidative coupling of o-aminophenols. GriF is incapable of oxidation of t-tyrosine, even though it shows ~50% identity in amino acid sequence to the tyrosinases responsible for melanin production in streptomycetes. Like other MelC2 tyrosinases in streptomycetes, GriF perhaps accepts copper ions from GriE, a homolog of the MelC1 copper chaperon. This is the first report to describe the enzyme system responsible for the in vivo formation of a phenoxazinone skeleton. Streptomycetes produce a variety of secondary metabolites with a phenoxazinone chromophore, and some of the tyrosinase homologs are supposed to be responsible for the formation of phenoxazinones. Because the biosynthesis genes for a given secondary metabolite are usually organized as a gene cluster, we can predict that a tyrosinase homolog encoded within the gene cluster for a secondary metabolite containing a phenoxazinone chromophore is involved in the formation of the phenoxazinone. We can safely say that the phenoxazinone chromophores of michigazone, 4-demethoxymichigazone, exfoliazone, and texazone (all with a functional group at the 8-position) are formed from o-aminophenol derivatives by the action of GriF homologs.

GriF as a Novel o-Aminophenol Oxidase Catalyzing Phenoxazinone Formation

GriF as a Novel o-Aminophenol Oxidase—Tyrosinase is a member of the type 3 copper protein family, to which hemocyanins and catechol oxidases also belong. On the basis of the catalytic properties of tyrosinases and x-ray crystallography of the binuclear copper active sites of hemocyanin (27) and catechol oxidase (28), detailed mechanisms of the tyrosinase reaction as both a monophenolase and a diphenolase have been proposed (29, 30). Some tyrosinases seem to have generous substrate specificity; the tyrosinases from N. crassa (12), mushroom (13), and S. glaucescens (12) catalyze the oxidation of aromatic amines, o-dia-

mines, and o-aminophenols, but at a low rate, in addition to phenol and catechol. This study has revealed a unique substrate specificity of GriF, which we can classify as an o-aminophenol oxidase. First, GriF prefers o-aminophenol to catechol as a substrate, whereas tyrosinases prefer catechol-type substrates (o-diphenols) to o-aminophenols. The $k_{cat}/K_m$ value for 3,4-dihydroxybenzaldehyde is 25 times smaller than that for 3,4-AHBAL, solely due to the decrease in the $k_{cat}$ value for 3,4-dihydroxybenzaldehyde. Concerning very low rates of aromatic amine and o-aminophenol oxidation by tyrosinases, Gasowska et al. (13) pointed out that o-diphenols are deprotonated more readily than aryldiamines due to a large difference in the $pK_a$ value between phenols ($pK_a = 10$) and aryldiamines ($pK_a = 27$). In addition, GriF had a rather small $k_{cat}$ value (14.0 ± 1.1 s$^{-1}$) for the best substrate (3,4-AHBAL) compared with that (390 ± 16 s$^{-1}$) of the tyrosinase for catechol. We therefore assume that GriF shows high specificity for o-aminophenols by decreasing the catalytic activity on o-diphenols.

Second, GriF shows no monophenolase activity, whereas tyrosinases show monophenolase activity. Like GriF, catechol oxidase shows diphenolase activity, but not monophenolase activity (28, 31, 32). On the basis of a paramagnetic study of the interaction between the monophenolic inhibitor p-nitrophenol and the tyrosinase from S. antibioticus, together with x-ray crystallography of a plant catechol oxidase, Tepper et al. (32) proposed that the phenyl ring of Phe261 of catechol oxidases prevents the ortho-protons of the substrate phenolic ring from its direct approach toward the catalytic center. In fact, an amino acid alignment of various catechol oxidases and tyrosinases shows that a Phe residue analogous to Phe261 of catechol oxidases is absent in tyrosinases. Interestingly, the amino acid corresponding to Phe261 in GriF is Tyr, which also has a phenyl ring as a side chain (Fig. 1B). The absence of monophenolase activity in GriF may be ascribed to the blocking activity of this Tyr residue.

Third, GriF activity to oxidize o-aminophenols depends on the functional group at the para-position to the phenolic hydroxyl group. The $k_{cat}/K_m$ value for 2-amino-4-methylphenol (which contains a –CH$_3$ group at this position) was similar to that for 3,4-AHBAL (which contains a –CHO group), whereas that for o-aminophenol (which contains no functional group at this position) was decreased by 4.1-fold mainly due to an increase in the $K_m$ value. Furthermore, the $k_{cat}/K_m$ value for 3,4-AHBA (which contains a –COOH group at this position) was decreased by 130-fold compared with that for 3,4-AHBAL. GriF showed no activity for 3-amino-4-hydroxybenzenesulfonic acid (which contains an –SO$_3$H group at this position). The kinetic parameters for diphenols showed a tendency similar to those for o-aminophenols; the $k_{cat}/K_m$ values for 3,4-dihydroxybenzaldehyde (which contains a –CHO group at the corresponding position) were 3 and 150 times larger than those for catechol (which contains no functional group at this position) and that for protocatechuc acid (which contains a –COOH group), respectively. Therefore, the functional group at the para-position with respect to the hydroxyl group of o-aminophenol is important in binding to GriF, and a –CHO group at this position is optimum for the GriF reaction. GriF appears to dislike compounds containing a negatively charged...
group at this position. X-ray crystallography of GriF as well as a tyrosinase will shed light on these unique properties in the substrate specificity and catalysis of GriF.

Putative Model for the Coupling of o-Aminophenols—The phenoxazinone synthase (an oligomeric multi-copper oxidase) from S. antibioticus catalyzes the oxidative condensation of two molecules of 4-methyl-3-hydroxyanthranilic acid to actinomycin in vitro, the phenoxazone chromophore of actinomycin D (33). The mechanism for the reaction was studied by blocking the reaction at various stages with substituted o-aminophenols (33). According to this mechanism, o-aminophenols are converted by two-electron oxidation to the highly electrophilic quinone imines, which then conjugate to another molecule of the o-aminophenols in the catalytic site of the enzyme. The coupling is initiated by a nucleophilic attack on the carbon at the para-position with respect to the ketone group of the quinone imine by the amino group of the o-aminophenol. The conjugates are further oxidized non-enzymatically by two electrons to yield the p-quinone imines. The second intramolecular conjugation of the p-quinone imine, followed by a final two-electron oxidation to give the phenoxazinone chromophore, occurs non-enzymatically out of the active site. On the other hand, Simandi et al. (34) analyzed the kinetics and mechanism of the ferroxime(II)-catalyzed biomimetic oxidation of o-aminophenol by dioxygen as a functional phenoxazinone synthase model. Because a 4-substituted 2-aminophenoxy free radical was detected using the ESR technique as a reaction intermediate, they proposed a model in which two molecules of the quinone imine produced from o-aminophenol were coupled. Do quinone imines react with o-aminophenols or another molecule of quinone imines in the formation of phenoxazinones through the oxidation of o-aminophenols by GriF? If the coupling of o-aminophenol by GriF is initiated by the nucleophilic attack on the carbon at the para-position with respect to the ketone group of the quinone imine by the amino group of the o-aminophenol, the presence of aniline in the GriF reaction with o-aminophenol would yield an aniline-quinone imine conjugate (Fig. 3B). If the coupling is initiated by the electrophilic attack on the carbon at the para-position with respect to the hydroxy group of o-aminophenol by the imino group of the quinone imine, the presence of phenol in the GriF reaction with o-aminophenol would yield phenoxazin-3-one and/or its reaction intermediate (Fig. 3B). However, no such conjugates were produced. On the other hand, the presence of catechol (a substrate of GriF) resulted in the formation of a conjugate, HPO (compound 3b) (Fig. 3B). We therefore speculate that the coupling of two molecules of o-aminophenol in the GriF reaction occurs between two molecules of the quinone imine produced (Fig. 3A). GriF catalyzes only the oxidation of o-aminophenols to yield the corresponding quinone imines, and the coupling perhaps occurs non-enzymatically out of the active site, unlike the phenoxazinone synthase. Consistent with this idea, when 3,4-AHBAL was used as a substrate, an overwhelmingly larger number of quinone imine molecules compared with GriF molecules were produced during an early stage of the reaction, and then the phenoxazinone began to be produced (Fig. 5B).

The oxidation of 3,4-AHBAL and 3,4-AHBA by GriF gave APOAL and APOC, respectively. In this reaction, the –CHO or –COOH group of one of the two substrate molecules was lost. A similar observation was reported by Hughes et al. (35): 4,3-AHBA in a minimal medium was oxidized non-enzymatically to yield 2-aminophenoxy-3-one-7-carboxylic acid with the loss of a –COOH group. We also observed the formation of APOC when 10 mM 3,4-AHBA in 50 mM sodium phosphate buffer, pH 7.0, was shaken vigorously at 30 °C for 12 h (data not shown), showing that the formation of APOC from 3,4-AHBA accompanied by the loss of the –COOH group of one of the two 3,4-AHBA molecules can occur non-enzymatically. The formation of APOAL from 3,4-AHBAL is also presumed to occur non-enzymatically, although the reaction is much slower.

Role of GriF in Grixazone Biosynthesis—S. griseus mutant ΔgriEF accumulates 3,4-AHBAL, which is the substrate of GriF. We have identified GriCD to be responsible for the reduction of 3,4-AHBA to yield 3,4-AHBA. (2) These findings support the prediction of Gould et al. (36) that the phenoxazinones of michigazona, exfoliazone, and 4-demethoxygrixazone might be synthesized from 3,4-AHBA. Our in vitro synthesis of grixazone A from N-acetylcysteine and 3,4-AHBAL in the presence of GriF (Fig. 5B, panel e) gives us a hint to establish the biosynthetic pathway of grixazone (Fig. 3C) on the basis of the organization of the grixazone biosynthesis genes. Because grixazone A is not formed from N-acetylcysteine and APOAL in the presence of GriF (Fig. 5B, panel e), the addition of N-acetylcysteine to the quinone imine derived from oxidation of 3,4-AHBAL by two electrons occurs before its dimerization with another molecule of the quinone imine. In the oxidation of catechin by tyrosinases and peroxidases in the presence of –SH compounds (e.g., glutathione), the o-quinones produced are non-enzymatically conjugated to glutathione, resulting in the formation of catechin-glutathione conjugates (26). We therefore assume that, in the grixazone biosynthetic pathway, the quinone imine produced from 3,4-AHBAL by GriF is non-enzymatically conjugated by the –SH group of N-acetylcysteine and is then dimerized with another quinone imine, resulting in the formation of grixazone A. The non-enzymatic conjugation of N-acetylcysteine to the quinone imine is consistent with the fact that, within the grixazone biosynthesis gene cluster, there is no plausible gene encoding an enzyme able to attach N-acetylcysteine to the phenoxazinone skeleton.

Grixazone B (compound 1b) (Fig. 2C) was not formed from N-acetylcysteine and 3,4-AHBA in the presence of GriF, although APOC (compound 3d) was formed (data not shown). The coupling of the quinone imines produced from 3,4-AHBA by the action of GriF is rather rapid, although that of the quinone imines produced from 3,4-AHBAL is slow. This may facilitate the introduction of N-acetylcysteine to the quinone imine from 3,4-AHBA. Therefore, the conversion from 3,4-AHBA to 3,4-AHBAL is important in grixazone biosynthesis. The substrate specificity of GriF (unfavorable to compounds with a negatively charged substituent at the para-position with respect to the hydroxyl group of o-aminophenols) supposedly restrains the formation of a shunt product (APOC) as a result of oxidation of 3,4-AHBA by GriF in the biosynthesis of grixazone.

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