Novel Tryptophan Metabolism by a Potential Gene Cluster That Is Widely Distributed among Actinomycetes*

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Background: Potential gene clusters involving indole prenyltransferase are widely distributed in actinomycetes.
Results: One gene cluster was found to be responsible for 5-dimethylallylindole-3-acetonitrile biosynthesis.
Conclusion: The biosynthetic route leading to 5-dimethylallylindole-3-acetonitrile represents a novel tryptophan metabolism.
Significance: This result provides insight into the biosynthesis of prenylated indole derivatives that have been purified from actinomycetes.

The characterization of potential gene clusters is a promising strategy for the identification of novel natural products and the expansion of structural diversity. However, there are often difficulties in identifying potential metabolites because their biosynthetic genes are either silenced or expressed only at a low level. Here, we report the identification of a novel metabolite that is synthesized by a potential gene cluster containing an indole prenyltransferase gene (SCO7467) and a flavin-dependent monooxygenase (FMO) gene (SCO7468), which were mined from the genome of Streptomyces coelicolor A3(2). We introduced these two genes into the closely related Streptomyces lividans TK23 and analyzed the culture broths of the transformants. This process allowed us to identify a novel metabolite, 5-dimethylallylindole-3-acetonitrile (5-DMAIAN) that was overproduced in the transformant. Biochemical characterization of the recombinant SCO7467 and SCO7468 demonstrated the novel 1-tryptophan metabolism leading to 5-DMAIAN. SCO7467 catalyzes the prenylation of 1-tryptophan metabolism to form 5-dimethylallyl-1-tryptophan (5-DMAT). This enzyme is the first actinomycetes prenyltransferase known to catalyze the addition of a dimethylallyl group to the C-5 of tryptophan. SCO7468 then catalyzes the conversion of 5-DMAT into 5-dimethylallylindole-3-acetaldoxime (5-DMAIAOx). An aldoxime-forming reaction catalyzed by the FMO enzyme was also identified for the first time in this study. Finally, dehydration of 5-DMAIAOx presumably occurs to yield 5-DMAIAN. This study provides insight into the biosynthesis of prenylated indoles that have been purified from actinomycetes.

Natural products have been an important resource for drug discovery and development over the last 3 decades. Actinomycetes are a rich source of natural products, and a wide variety of these chemicals have been used as medicinal drugs (1, 2). Recently, however, the screening of bioactive compounds from microorganisms has often resulted in the identification of known compounds. The decreasing hit rate for identifying new compounds has reduced the advantages of screening natural products. However, genome sequencing of actinomycetes has highlighted numerous potential areas with metabolic diversity and revealed the existence of a number of potential gene clusters with unknown function (3–5). In fact, the number of potential gene clusters is significantly more than the number of metabolites observed under standard fermentation conditions employed in laboratories (6).

To uncover the function of potential gene clusters that might code for the biosynthesis of secondary metabolites, genome sequence-guided identification of metabolites has been performed in combination with heterologous expression, gene knock-out and complementation analyses, and silent gene activation studies (7, 8). Normally, it is difficult to elucidate the function of a potential gene cluster and to identify the metabolite(s) associated with the cluster unless an appropriate system for heterologous expression is available (9), the gene clusters are highly expressed under normal conditions (8), or a pathway-specific regulator is identified (7). In this study, we characterized a gene cluster by employing a relatively simple strategy that is based on a gene dosage effect. We hypothesized that this introduction of some structural genes from a targeted gene cluster into a heterologous host using a plasmid vector would allow us to detect the production of a targeted product that has never been detected.

We focused on a selected group of putative gene clusters including an indole prenyltransferase IptA homolog because it is widely distributed among actinomycetes (Fig. 1). We classified the gene clusters into two types on the basis of their constitutent genes; in addition to the indole prenyltransferase gene, one gene cluster contained a tryptophanase gene, whereas the other contained a flavin-dependent monooxygenase (FMO).
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**Gene clusters containing the indole prenyltransferase gene.** The Type A gene clusters contain tryptophanase genes. This type of cluster is associated with the biosynthesis of 6-dimethylallylindole-3-carbardehyde. The Type B gene clusters contain flavin-dependent monooxygenase genes. The function of the Type B clusters has not been identified.

**EXPERIMENTAL PROCEDURES**

Reagents, Bacterial Strains, Plasmids, and Culture Conditions—Tryptophan, indole-3-pyruvic acid, NADPH, NADH, FAD, and FMN were purchased from Sigma-Aldrich. Indole-3-acetonitrile (IAN) and hydroxylammonium chloride were purchased from Tokyo Chemical Industry. 2-Picoline-borane was purchased from Junsei Chemical (Tokyo, Japan). A mixture of sym- and anti-isomers of indole-3-acetaldoxime (IAOx) was a gift from Dr. Hiroyuki Kasahara (RIKEN, Yokohama, Japan). The bacterial strains, plasmids, and primers used in this study are listed in Table 1. The *Streptomyces-Escherichia coli* shuttle vector pSE101 (11) was used for the heterologous expression of genes in *S. lividans* TK23. The genes cloned downstream of the *lac* promoter in pSE101 are efficiently expressed in *S. lividans* TK23 (12). The transformants of *S. lividans* TK23 cultures were routinely grown at 30 °C on TSB agar plates (3% tryptone soya broth (Oxoid, England), 1.5% agar). For the analysis of metabolites produced by the transformants, a scrape of the mycelium on the culture plate was inoculated into a test tube containing 10 ml of TSB tsr medium (3% tryptone soya broth and 30 μg ml⁻¹ thiostrepton) for 2 days at 30 °C on a reciprocal shaker at 300 rpm. Two milliliters of the preculture was inoculated into 500-ml baffled flasks containing 100 ml of TSB tsr medium, and the culture was incubated for 4 days at 27 °C on a rotary shaker at 160 rpm. *Escherichia coli* was grown at 37 °C in LB broth or Terrific broth (13). Vector pHi58 (14) was used for the heterologous expression of genes in *E. coli* BL21(DE3). Ampicillin (100 μg ml⁻¹), or kanamycin (50 μg ml⁻¹) was added for the selection of plasmid-containing *E. coli* cells.

**Construction of Heterologous Expression Plasmids**—The *SCO7467* and *SCO7468* genes were amplified from genomic DNA of *S. coelicolor* A3(2), using the Expand High Fidelity PCR system (Roche Diagnostics Japan, Tokyo, Japan) and the primers listed in Table 1. Primers were designed to amplify each gene from the upstream region that included a ribosomal binding site. A PCR fragment containing the *SCO7468* gene was cloned downstream of the *lac* promoter in pSE101 to give pSCO102. Similarly, a PCR fragment containing the *SCO7467* gene was cloned into pSE101 to give pSCO101 and into pSCO102 to give pSCO103. Each plasmid was introduced into *S. lividans* TK23 by a polyethylene glycol-mediated protoplast method (15).

**LC-MS/MS Analysis of the Metabolite Produced by *S. lividans* TK23 Transformants**—The culture broths of the transformants were extracted twice with ethyl acetate. The ethyl acetate fraction was concentrated in vacuo and then dissolved in methanol. The resultant methanol solution was analyzed on an LC-MS/MS system equipped with a CAPCELL PAK C₁₈ IF.
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TABLE 1

| Strains, plasmids, and primers used in this study |
|--------------------------------------------------|
| **Strains**                                   | **Description** | **References** |
| S. coelicolor A3(2)                           | SCO7467 and SCO7468 were cloned from this strain |
| S. lividans TK23                              | host strain for heterologous expression |
| E. coli DH5α                                  | host strain for gene cloning |
| E. coli BL21(DE3)                             | host strain for protein expression |

| **Plasmids**                                | **Description** | **References** |
|---------------------------------------------|-----------------|----------------|
| pT7Blue T-vector                            | general cloning vector with TA-cloning site | Novagen 14 |
| pHi58                                       | T7-RNA polymerase-dependent recombinant protein expression vector; the N-terminal 8-histidine tag allows protein purifcation by Ni-affinity column chromatography. |
| pHIS8_SCO7467                                | The SCO7467 fragment was inserted into Ncol and BamHI sites of pHIS8. |
| pHIS8_SCO7468                                | The SCO7468 fragment was inserted into Ncol and HindIII sites of pHIS8. |
| pSE101                                      | Streptomyces-Esherichia coli shuttle vector |
| pSCO101                                     | The SCO7467 fragment was inserted into HindIII and SphI sites of pSE101. |
| pSCO102                                     | The SCO7468 fragment was inserted into SphI and XbaI sites of pSE101. |
| pSCO103                                     | The SCO7467 fragment was inserted into HindIII and SphI sites of pSCO102. |

| **Primers**                                 | **Description and sequence** |
|---------------------------------------------|-----------------------------|
| p7467H18Fw and p7467H18Rv                   | were used for the amplification of SCO7467 for pHIS8_SCO7467. |
| p7467H18Fw                                  | 5'-GGGGCATGCGCAGGCCGCGTCGACGGGCGCGG-3' (Ncol site underlined) |
| p7467H18Rv                                 | 5'-GGGGCATGCGCAGGCCGCGTCGACGGGCGCGG-3' (BamHI site underlined) |
| p7468H18Fw and p7468H18Rv                   | were used for the amplification of SCO7468 for pHIS8_SCO7468. |
| p7468H18Fw                                  | 5'-GGGGCATGCGCAGGCCGCGTCGACGGGCGCGG-3' (Ncol site underlined) |
| p7468H18Rv                                 | 5'-GGGGGATTCCTCAGCGCCGACGACGCGTGTTGGC-3' (BamHI site underlined) |
| p7467PSEFw and p7467PSEFv                   | were used for the amplification of SCO7467 for pSCO101 and pSCO103. |
| p7467PSEFw                                  | 5'-GGGGGATTCCTCAGCGCCGACGACGCGTGTTGGC-3' (BamHI site underlined) |
| p7467PSEFv                                 | 5'-GGGGGATTCCTCAGCGCCGACGACGCGTGTTGGC-3' (BamHI site underlined) |
| p7468PSEFw and p7468PSEFv                   | were used for the amplification of SCO7468 for pSCO102. |
| p7468PSEFw                                  | 5'-GGGGGATTCCTCAGCGCCGACGACGCGTGTTGGC-3' (BamHI site underlined) |
| p7468PSEFv                                 | 5'-GGGGGATTCCTCAGCGCCGACGACGCGTGTTGGC-3' (BamHI site underlined) |

Isolation of SCO7467 and SCO7468—Two liters of culture broth of the transformant was extracted with an equal volume of acetone and then concentrated to remove the solvent. The remaining water fraction was extracted twice with an equal volume of ethyl acetate, and the organic layer was then concentrated in vacuo. The remaining residue was resolved in methanol and passed through Sep-Pak Plus C18 Cartridges (Waters, Ireland). The eluted fraction was again concentrated in vacuo to give 300 mg of a residue. The concentration extract was separated by preparative HPLC on a Senshu Pak PEGASIL ODS column (20 × 250 mm, Senshu Scientific, Tokyo, Japan) and an isocratic elution of 75% methanol at a flow rate of 8 ml min⁻¹. The column eluate was monitored at 271 nm. Finally, 8.9 mg of 5-DMAIAN was obtained.

Structural Analysis—The structures of the unidentified products were analyzed using ¹H NMR spectroscopy and ¹³C NMR spectroscopy (600 MHz, JEOL ECA-600; Tokyo, Japan). The high resolution MS apparatus was used to determine the molecular formulas of the reaction products. The MS analysis was performed using electrospray ionization in the positive-ion mode.

NMR and MS Spectral Data of 5-DMAIAN—¹H NMR (DMSO-d6) δ: 1.68 (3H, H-5′), 1.69 (3H, H-6′), 3.36 (d, JH= 6.9 Hz, H-2), 3.97 (s, 2H, H-8), 5.31 (t, JH= 6.9 Hz, H-2′), 6.93 (dd, JH= 1.4, 8.2 Hz, H-1′), 7.26 (d, JH= 2.1 Hz, H-1′, H-2′), 7.28 (d, JH= 8.2 Hz, 1H, H-7), 7.32 (s, 1H, H-4), 10.99 (s, 1H, H-1). ¹³C NMR (DMSO-d6) δ: 13.8 (C-8), 18.2 (C-4′), 26.1 (C-5′), 34.6 (C-1′), 103.7 (C-3), 112.2 (C-7), 117.4 (C-4), 120.1 (C-9), 123.1 (C-6), 124.6 (C-2′), 125.1 (C′-2′), 126.7 (C-3a), 131.3 (C-3′), 132.4 (C-5), 135.3 (C-7a). The molecular formula was established as C₁₅H₁₇N₂ by high resolution MS (m/z 225.1385 [M+H]⁺; calculated molecular weight for C₁₅H₁₇N₂, 225.1386).

Expression and Purification of N-terminal His-tagged Proteins—A PCR fragment containing SCO7467 and SCO7468 was amplified from the genomic DNA of S. coelicolor A3(2) using the Expand High Fidelity PCR system and the primers listed in Table 1. After the E. coli BL21(DE3) that harbored pHi58_SCO7467 or pHi58_SCO7468 was cultured overnight in LB-kanamycin, then Terrific broth containing 50 µg ml⁻¹ kanamycin was inoculated with the resultant cells and cultured
at 37 °C. After a 2-h culture, isopropyl 1-thio-β-d-galactopyranoside was added to a final concentration of 0.5 mM. After overnight culture at 18 °C, cells were harvested by centrifugation and frozen at −80 °C. All subsequent steps were performed at 4 °C. After being thawed on ice, cells were suspended in a lysis buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20 mM imidazole, 20% (w/v) glycerol, and 1% Tween 20). The cell suspension was sonicated with a Branson Sonifier 250 (Emerson Japan, Tokyo, Japan). To separate the cellular debris from the soluble protein, the lysate was centrifuged at 17,000 rpm at 4 °C for 20 min. The supernatant was applied to a Ni-nitrilotriacetic acid column (Qiagen, Tokyo, Japan) that was equilibrated with the lysis buffer and washed with a buffer containing 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20 mM imidazole, and 20% (w/v) glycerol. Then, recombinant protein was eluted using the wash buffer containing 250 mM imidazole.

To investigate the multimeric status of the recombinant proteins, a gel filtration analysis was performed. Before analysis, the column was equilibrated with a buffer containing 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl, and SCO7467 was then applied to a Superdex 75 column (GE Healthcare), which was calibrated with conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and ribonuclease A (13.7 kDa). In the case of SCO7468, a Superdex 200 column (GE Healthcare) was used. Before analysis, the column was equilibrated with a buffer containing 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl, and SCO7468 was then applied to the equilibrated column, which was calibrated with aldolase (158 kDa), conalbumin, ovalbumin, carbonic anhydrase, and ribonuclease A.

Prenyltransferase Assay of SCO7467—The SCO7467 assay was performed in 100 mM Tris-HCl, pH 8.0, containing 2 mM MgCl₂, 2 mM IAN, 2 mM DMAPP, and up to 1 mg ml⁻¹ SCO7467, which was added in two consecutive steps over 4 h. The reaction mixture was incubated at 30 °C for 16 h and then extracted three times with 10 ml of ethyl acetate. The organic layer was evaporated in vacuo, and the residue was dissolved in 1 ml of methanol. The enzymatic reaction product was purified by preparative HPLC with a PEGASIL ODS column (20 × 250 mm; Senshu Scientific) and isocratic elution of 65% MeOH + 0.1% acetic acid at a flow rate of 7.0 ml min⁻¹; the column eluate was monitored at 223 nm.

_NMR and MS Spectral Data of 2-DMMAIN—1H NMR (DMSO-d₆) δ: 1.67 (s, 3H, H-5'), 1.71 (s, 3H, H-4'), 3.44 (d, J₁H = 6.8 Hz, 2H, H-1'), 3.93 (s, 2H, H-8), 5.26 (t, J₁H = 6.8 Hz, 1H, H-2'), 6.97 (dd, J₂H = 7.6, 7.6 Hz, 1H, H-5), 7.02 (dd, J₁H = 7.6, 7.6 Hz, 1H, H-6), 7.27 (d, J₁H = 7.6 Hz, 1H, H-7), 7.47 (d, J₁H = 7.6 Hz, 1H, H-4), 10.9 (s, 1H, H-1). 13C NMR (DMSO-d₆): 12.5 (C-8), 18.3 (C-4'), 25.1 (C-1'), 26.0 (C-5'), 99.3 (C-3), 111.5 (C-7), 117.8 (C-4), 119.4 (C-5), 119.9 (C-9), 121.1 (C-2'), 121.4 (C-6), 127.6 (C-3a), 133.3 (C-3'), 135.7 (C-7a), 137.1 (C-2') The molecular formula was established as C₁₅H₁₈N₂O₂ by high resolution MS (m/z 273.1600 [M+H]+; calculated molecular weight for C₁₅H₁₈N₂O₂ 273.1598).

The large scale preparation of the SCO7467-catalyzed reaction product was performed in a total volume of 10 ml with IAN as the substrate. The reaction was performed in 100 mM Tris-HCl, pH 8.0, containing 2 mM MgCl₂, 2 mM IAN, 2 mM DMAPP, and up to 1 mg ml⁻¹ SCO7467, which was added in two consecutive steps over 4 h. The reaction mixture was incubated at 30 °C for 16 h and then extracted three times with 10 ml of ethyl acetate. The organic layer was evaporated in vacuo, and the residue was dissolved in 1 ml of methanol. The enzymatic reaction product was purified by preparative HPLC with a PEGASIL ODS column (20 × 250 mm; Senshu Scientific) and isocratic elution of 65% MeOH + 0.1% acetic acid at a flow rate of 7.0 ml min⁻¹; the column eluate was monitored at 223 nm.

_NMR and MS Spectral Data of 6-DMMAIN—1H NMR (DMSO-d₆) δ: 1.68 (s, 3H, H-5'), 1.68 (s, 3H, H-4'), 3.35 (d, J₁H = 7.6 Hz, 2H, H-1'), 3.96 (s, 2H, H-8), 5.30 (t, J₁H = 7.6 Hz, 1H, H-2'), 6.85 (d, J₂H = 8.2 Hz, 1H, H-5), 7.12 (s, 1H, H-7), 7.22 (brs, 1H, H-2'), 7.43 (d, J₁H = 8.2 Hz, 1H, H-4), 10.92 (s, 1H, H-1). 13C NMR (DMSO-d₆): 13.8 (C-8), 18.2 (C-4'), 26.1 (C-5'), 34.5 (C-1'), 104.0 (C-3), 111.3 (C-7), 118.4 (C-4), 120.0 (C-9), 120.6 (C-2'), 124.0 (C-2'), 124.7 (C-2'), 124.7 (C-3a), 131.6 (C-3'), 135.5 (C-5), 137.2 (C-7a) The molecular formula was established as C₁₅H₁₉N₂ by HRESI-MS (m/z 225.1382 [M+H]+; calculated molecular weight for C₁₅H₁₉N₂ 225.1386).

_Steady-state Kinetics Study of SCO7467—A spectrophoto metric SCO7467 assay using a coupled system with a pyrophosphate reagent (Sigma-Aldrich) was used to study the steady-state kinetics of SCO7467 because the SCO7467 prenyltransferase forms a pyrophosphate anion co-product during catalysis. This assay was essentially performed as described previously (15). Prenyltransferase activity was assayed in 100 mM Tris-HCl, pH 8.0, containing 2 mM MgCl₂, 1-tryptophan, DMAPP, and 267 µl of the pyrophosphate reagent in a total volume of 800 µl. When the concentration of DMAPP was fixed at 1 mM, the concentrations of tryptophan were varied at 15, 30, 60, and 120 µM. When the concentration of tryptophan was fixed at 1 mM, the concentrations of DMAPP were varied at 10, 20, 40, 80, and 160 µM. After the reaction mixture containing no enzyme was incubated at 30 °C for 5 min, the reaction was started by adding 67 µg of SCO7467. The SCO7467-depen-
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The enzymatic activity of SCO7468—The SCO7468 assays were performed in 100 mM Tris-HCl, pH 8.0, containing 50 μM 5-DMAT or tryptophan, 1 mg ml⁻¹ 5-DMAIAOx, and 1 mM NADPH or NADH. The reaction mixtures were incubated at 30 °C for 10 min. After the incubation, the reaction was quenched by the addition of an equal volume of methanol and mixing by vortexing. The mixture was centrifuged at 15,000 rpm for 1 min to remove protein. The supernatant was subjected to LC-MS/MS analysis under the same conditions as described above. The large scale preparation of the SCO7468-catalyzed reaction product was performed in a total volume of 10 ml. The reaction was performed in 100 mM Tris-HCl, pH 8.0, containing 1 mM 5-DMAT, 1 mM NADPH, and up to 1 mg ml⁻¹ SCO7468, which was added in two consecutive steps over 4 h. The reaction mixture was incubated at 30 °C for 16 h and then extracted twice with 10 ml of ethyl acetate. After drying over Na₂SO₄, the organic layer was evaporated in vacuo, and the residue was dissolved in 1 ml of methanol. The enzymatic reaction product was purified by preparative HPLC with a PEGASIL ODS column (20 × 250 mm; Senshu Scientific) and isocratic elution of 80% MeOH at a flow rate of 8.0 ml min⁻¹; the column eluate was monitored at 275 nm.

NMR and MS Spectral Data of 5-DMAI-3-acetaldoxime (5-DMAIAOx)—¹H NMR (CDCl₃) δ: 7.14 (s, 3H, H-5'), 1.75 (s, 3H, H-4'), 3.48 (d, J₉/₉ = 7.1 Hz, 2H, H-1'), 3.64 (d, J₉/₉ = 6.2 Hz, 2H, H-8 (anti)), 3.83 (d, J₉/₈₁ = 5.0 Hz, 2H, H-8 (syn)), 3.58 (t, J₉/₈₁ = 7.1 Hz, 1H, H-2'), 6.94 (t, J₉/₉ = 5.0 Hz, 1H, H-9 (syn)), 7.01 (brs, 1H, H-2 (anti)), 7.04 (brs, 1H, H-2 (syn)), 7.04 (m, 1H, H-6'), 7.05 (m, 1H, H-6'), 7.27 (d, J₉/₉ = 7.2 Hz, 1H, H-7'), 7.29 (d, J₉/₉ = 7.7 Hz, 1H, H-7'), 7.37 (brs, 1H, H-4'), 7.38 (brs, 1H, H-4'), 7.60 (t, J₉/₉ = 6.2 Hz, 1H, H-9 (anti)), 7.95 (s, 1H, H-1). ¹³C NMR (CDCl₃): δ: 18.0 (C-4'), 21.6 (C-8 (syn)), 25.9 (C-5'), 26.0 (C-8 (anti)), 34.6 (C-1'), 110.3 (C-3 (anti)), 110.7 (C-3 (syn)), 111.2 (C-7), 117.7 (C-4'), 117.8 (C-4'), 122.4 (C-6'), 122.5 (C-6'), 123.4 (C-2'), 124.5 (C-2'), 127.5 (C-3a), 131.8 (C-3'), 133.3 (C-5), 134.9 (C-7a), 151.0 (C-9 (anti)), 151.9 (C-9 (syn)). (Asterisks indicate that we were unable to assign syn or anti for these peaks.) The molecular formula was established as C₁₅H₁₉N₂O₃ by high resolution MS (m/z 243.1495 [M+H]⁺; calculated molecular weight for C₁₅H₁₉N₂O₃, 243.1492).

Identification of N-Hydroxytryptophan as a Reaction Intermediate of SCO7468—N-Hydroxytryptophan was synthesized by reductive amination of indole-3-pyruvic acid. Indole-3-pyruvic acid oxime, which was derived from indole-3-pyruvic acid and hydroxyammonium chloride, was reduced by 2-picolineborane according to the method described in the literature (17, 18). The SCO7468 assay with N-hydroxytryptophan was performed in 100 mM Tris-HCl, pH 8.0, containing 1 mM N-hydroxytryptophan, 1 mg ml⁻¹ SCO7468, and 1 mM NADPH. The reaction mixture was incubated at 30 °C for 60 min. After the incubation, the reaction was quenched by the addition of an equal volume of methanol and mixed by vortexing. The resulting mixture was centrifuged at 15,000 rpm for 1 min to remove protein. The supernatant was subjected to LC-MS/MS analysis under the same conditions as described above.

Steady-state Kinetics Study of SCO7468—SCO7468 kinetic assays were performed in a reaction mixture containing 100 mM Tris-HCl, pH 8.0, 20 μM FAD, and 1 mM NADPH. The reactions with 5-DMAT were carried out in 0.2 ml of buffer, and the reactions with tryptophan were carried out in 0.1 ml of buffer. The concentrations of 5-DMAT were 62.5, 125, 250, 500, and 1000 mM, whereas the concentrations of tryptophan were 0.625, 1.25, 2.5, 5.0, and 10 mM. After the reaction mixture containing no enzyme was incubated at 30 °C for 1 min, the reaction was started by adding SCO7468 at a final concentration of 0.1 mg ml⁻¹. After the 40-s reaction with 5-DMAT, the reaction mixture was extracted three times by an equal amount of ethyl acetate and dried in vacuo. The dried residues were dissolved in 40 μl of methanol and analyzed by LC-MS/MS. The 8-min reactions with 0.625, 1.25, and 2.5 mM 5-DMAT and the 2-min reactions with 5.0 and 10 mM 5-DMAT were quenched by the addition of an equal volume of methanol. The resultant methanol solutions were analyzed directly using an LC-MS/MS system equipped with a CAPCELL PAK C₁₈ IF column (2.0 × 50 mm; column temperature, 40 °C) under the following conditions: mobile phase A, water + 0.1% formic acid; mobile phase B, acetonitrile + 0.1% formic acid; 50% B for 5 min at a flow rate of 0.4 ml min⁻¹. The amount of the product was calculated from a standard curve, which was obtained from LC-MS/MS fragmentation analysis using the multiple reaction monitoring mode. The peak area of a characteristic product ion resulting from each precursor ion corresponding to 5-DMAIAOx (m/z 243.1) or IAOx (m/z 175.1) was used for quantification; a characteristic product ion (m/z 187.1) at 1.38 min was selected for 5-DMAIAOx, and a characteristic product ion (m/z 158.1) at 0.61 min was selected for IAOx.

RESULTS

Indole Prenyltransferase Genes Are Widely Distributed in Actinomycetes—Takahashi et al. (10) reported the presence of IptA indole prenyltransferase homologs in S. coelicolor A3(2) and Streptomyces ambofaciens ATCC23877, but no mention has been made of the distribution of IptA indole prenyltransferase homologs in other actinomycetes. Therefore, to determine the distribution of IptA-containing gene clusters in other actinomycetes, we performed a BLAST search against the National Center for Biotechnology Information (NCBI) database using an amino acid sequence of IptA as a query. Surprisingly, the database searches retrieved >10 IptA homologs in various actinomycetes (Fig. 1). In addition, the gene organizations harboring the indole prenyltransferase gene were very similar to each other. All of these gene clusters had a four-gene cassette including the sensor-like histidine kinase gene, two hypothetical genes, and an ATP/GTP-binding protein gene. This gene cassette is known as a “conservon,” which is a gene cassette conserved among actinomycetes (4, 19–21), some of which have an additional cytochrome P-450 gene. The database...
search results allowed us to classify these gene clusters into two types (here, we designate them Type A and Type B) based on differences in their constituent genes; the Type A gene cluster includes a tryptophanase gene, whereas the Type B gene cluster includes a FMO gene (Fig. 1). Although Takahashi et al. have demonstrated that one of the Type A gene clusters is responsible for the biosynthesis of 6-DMAI-3-carbaldehyde, no functions of the Type B gene cluster have been elucidated to date.

**Heterologous Expression of the Prenyltransferase Gene and the FMO Gene**—To identify the metabolite originating from the expression of the S. coelicolor A3(2) Type B gene cluster containing SCO7467 and SCO7468, we performed heterologous expression of SCO7467 and SCO7468 using S. lividans TK23 as a host strain. Because S. lividans TK23 possesses a gene cluster identical to that of S. coelicolor A3(2), we expected a gene dosage effect on the production of an unidentified metabolite caused by the introduction of SCO7467 and SCO7468 into S. lividans TK23.

For heterologous expression, the plasmid pSCO101 (containing SCO7467, pSCO102 (containing SCO7468), or pSCO103 (containing SCO7467 and SCO7468) was individually introduced into S. lividans TK23 (Fig. 2A). As a control strain, S. lividans TK23 harboring the empty vector pSE101 was used. Introduction of SCO7467 did not cause apparent changes in the metabolic profile, whereas introduction of SCO7468 caused the production of an unidentified product that showed an indole-like UV spectrum. LC-MS/MS analysis unambiguously identified this product as IAN (Fig. 2, C and D). A transformant that harbored both SCO7467 and SCO7468 also produced 1. In addition to 1, this transformant produced unidentified product 2, which showed a UV spectrum similar to 1 but with a longer chromatographic retention time than that of 1 (Fig. 2B). Although this product 2 was also produced at a negligible level by S. lividans TK23 transformants harboring the empty vector or SCO7467 alone, introduction of both SCO7467 and SCO7468 increased the production of the unidentified IAN-like product. The large-scale preparation of 2 allowed us to deduce its structure to be 5-DMAIAN (2) (Fig. 2D) on the basis of NMR and high resolution MS spectral data.

SCO7467 displayed 59% amino acid sequence identity to lptA, which catalyzes a dimethylallyl group transfer to the C-6 position of tryptophan (3). In addition, the introduction of SCO7467 into S. lividans TK23 increased the production of 2 (IAN with a dimethylallyl group at C-5) in this transformant. Both facts suggest that SCO7467 is involved in the attachment of a dimethylallyl group to the C-5 of 3 to yield 5-DMAT (4). Next, SCO7468 presumably uses 4 as a substrate in the formation of 2. To verify this hypothesis, we next performed *in vitro* analysis with the recombinant SCO7467 and SCO7468 prepared from the E. coli transformants.

**Expression and Purification of Recombinant SCO7467 and SCO7468**—The N-terminal His$_{6}$-tagged SCO7467 and SCO7468 were overexpressed in E. coli, and the recombinant proteins were purified to homogeneity by a Ni-nitrilotriacetic acid column. The molecular mass of SCO7467 was estimated to be 40 kDa by SDS-PAGE and gel filtration chromatography, suggesting that SCO7467 is likely a monomer (Fig. 3A). The molecular mass of SCO7468 was estimated to be 45 kDa by SDS-PAGE and 90 kDa by gel filtration chromatography, suggesting that SCO7468 is likely a dimer (Fig. 3B). Purified SCO7468 was yellow, suggesting that the enzyme contained a flavin co-factor and was successfully expressed as an active enzyme. Denaturation of SCO7468 with methanol followed by reverse-phase HPLC analysis showed a major peak identical to FAD, indicating that FAD existed in the recombinant SCO7468 protein as a tightly bound form (Fig. 3C).

**In Vitro Analysis of Recombinant SCO7467—SCO7467** showed 59% amino acid sequence identity to 1.-tryptophan-di-
methyltransferase IptA, which appends a dimethylallyl group at the C-6 position of \( \text{3} \) to form 6-DMAT. This high amount of shared identity led us to expect that SCO7467 would accept \( \text{3} \) as a substrate. To assess enzyme activity, we incubated SCO7467 with \( \text{3} \) in the presence of DMAPP. The formation of one product with a dimethylallyl group was readily detected by HPLC (Fig. 4A). Large scale incubation of SCO7467 with \( \text{3} \) and DMAPP produced a sufficient amount of the product to permit its structural elucidation using both MS and NMR analyses. The product possessed a single dimethylallyl chain at C-5 and was identified as 5-DMAT (4). This SCO7467-catalyzed reaction indicates that SCO7467 is capable of converting \( \text{3} \) to 4.

Similar to IptA, SCO7467 also catalyzed the prenylation of \( \text{3} \) in the absence of Mg\(^{2+}\) and maintained its activity even in the presence of 5 mM EDTA, indicating that the SCO7467-catalyzed prenylation reaction is Mg\(^{2+}\)-independent (Fig. 4A). We then performed steady-state kinetic analysis, which demonstrated that the SCO7467 reaction followed Michaelis-Menten kinetics. The apparent \( K_m \) values were 80 ± 6 \( \mu M \) for \( \text{3} \) and 50 ± 2 \( \mu M \) for DMAPP at fixed saturating concentrations of DMAPP (1 mM) and \( \text{3} \) (1 mM), respectively. The turnover number \( k_{cat} \) of the reaction was 0.40 ± 0.1 s\(^{-1}\) (Fig. 4C). Meanwhile, the in vivo results mentioned above also suggested the possibility that SCO7467 appends a dimethylallyl group to the C-5 of \( \text{1} \) to give \( \text{2} \). To verify this possibility, we incubated SCO7467 with \( \text{1} \) in the presence of DMAPP. This reaction resulted in the formation of several products (Fig. 4B). We isolated two of the products and determined the structure of each compound by NMR and HRESI-MS. The structure of \( \text{5} \) was determined to be 2-dimethylallylindole-3-acetonitrile (2-DMAIAN, 5), whereas \( \text{6} \) was determined to be 6-dimethylallylindole-3-acetonitrile (6-DMAIAN, 6) (Fig. 4B). This in vitro result was obviously inconsistent with the finding that no other indole compounds, with the exception of \( \text{1} \) and \( \text{2} \), were produced in the heterologous expression experiment. We thus excluded the possibility that SCO7467 appends a dimethylallyl group to the C-5 of \( \text{1} \) to give \( \text{2} \).
and concluded that SCO7467 encodes a 5-dimethylallyltryptophan synthase (L-tryptophan:5-dimethylallyltransferase).

In Vitro Analysis of Recombinant SCO7468—The bioinformatic analyses of SCO7468 revealed that SCO7468 had 34% amino acid sequence identity to dimethylaniline monooxygenase (N-oxide-forming) (accession number EHA99148), suggesting that this enzyme is involved in the formation of an N-oxide from an amine. To assess enzyme activity, 4 was used as a substrate in the SCO7468 reaction because SCO7467 catalyzes the prenylation of 3 to give 4. HPLC analysis of the reaction mixture readily revealed the enzyme-dependent formation of an unknown product 7 (Fig. 5A). After a 10-min incubation, SCO7468 converted all of 4 (50 μM) to 7. The enzymatic activity was strictly dependent on NADPH; no activity was detected in the presence of NADH. Large scale incubation of SCO7468 with 4 and DMAPP produced a sufficient amount of 7 to permit its structural elucidation using both MS and NMR analyses. The structure was determined to be a mixture of syn- and anti-isomers of 5-DMAIAOx (7).

Meanwhile, 1 accumulated when SCO7468 was expressed in S. lividans (Fig. 2B), which suggests that SCO7468 is involved in the formation of 1 in vivo. Therefore, to further verify the function of SCO7468, we incubated SCO7468 with 3 in the presence of β-NADPH. However, the 10-min incubation of SCO7468 with 50 μM 3 resulted in almost no substrate depletion and almost no product formation (Fig. 4B). In contrast, the prolonged incubation (2 h) of SCO7468 with a higher concentration of 3 (1 mM) resulted in the clear formation of IAOx (8) (Fig. 5C). The SCO7468-catalyzed formation of IAOx (8) from tryptophan (3) is similar to that of p-hydroxyphenylacetaldoxime from L-tyrosine in cyanogenic glucoside biosynthesis, where cytochrome P-450TYR likely catalyzes two successive N-hydroxylations of L-tyrosine followed by nonenzymatic decarboxylation to form p-hydroxyphenylacetaldoxime (22). To investigate whether SCO7468 catalyzes the aldoxime formation through the same reaction mechanism as that of cytochrome P-450TYR, we synthesized N-hydroxytryptophan (9), a possible intermediate of the SCO7468 reaction, and evaluated whether 9 could be converted into 8. N-Hydroxytryptophan (9) was incubated with SCO7468 in the presence of NADPH. The incubation revealed that 8 was produced concomitantly with the complete consumption of 9 (Fig. 6), which unambiguously demonstrates that 9 is a reaction intermediate of SCO7468.

We then determined the kinetic parameters of SCO7468 toward 4 and 3. \( K_m \) and \( k_{cat} \) values for 4 were estimated to be \( 0.57 \pm 0.1 \mu M \) and \( (8.2 \pm 0.7) \times 10^{-3} \text{ s}^{-1} \), respectively, whereas \( K_m \) and \( k_{cat} \) values for 3 were estimated to be \( (3.3 \pm 0.7) \times 10^{3} \mu M \) and \( (5.2 \pm 0.4) \times 10^{-3} \text{ s}^{-1} \), respectively (Fig. 5D). This notable difference clearly indicates that 4 is a physiological substrate of SCO7468.
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In this study, we demonstrated that S. coelicolor A3(2) and S. lividans TK23 have the ability to biosynthesize a novel natural product, 2. Although similar gene clusters responsible for the biosynthesis of 2 are widely distributed in actinomycetes, this specific function has not yet been elucidated. The heterologous expression in S. lividans of the structural genes SCO7467 and SCO7468 enabled us to identify 2. S. lividans harboring the empty vector alone also produced 2 but at a negligible level (Fig. 2). These results indicate that introduction of exogenous SCO7467 and SCO7468 into S. lividans effectively increases the production of 2. The present study demonstrated that a simple strategy such as heterologous expression is widely applicable for the identification of unidentified secondary metabolites synthesized by uncharacterized gene clusters that are buried in the public genome database. Identification of such metabolites would contribute to our knowledge of the structural diversity of natural products.

We also elucidated the biochemical functions of SCO7467 and SCO7468 in the biosynthesis of 2. SCO7467 catalyzes the initial reaction in the biosynthesis and transfers a dimethylallyl group to C-5 of an indole nucleus of 3. Despite high sequence similarity between SCO7467 and IptA, their regiospecificities of prenylation are different. IptA transfers a dimethylallyl group to C-6 of an indole nucleus of 3. However, fungal 5-dimethylallyltryptophan synthase and SCO7467 exhibit a low level of overall sequence similarity, although they catalyze the same reaction. Crystallographic analysis would be necessary to understand the mechanism of the regiospecificity of these prenyltransferases.

After the prenylation catalyzed by SCO7467, the FMO enzyme SCO7468 readily catalyzes the conversion of 4 to 7, as its $K_m$ value (0.57 μM) toward 4 is quite low. SCO7468 also accepts 3 as the substrate, although its $K_m$ value (3.3 x 10^3 μM) toward 3 is much higher than that for 4. In fact, prolonged incubation of SCO7468 with the higher concentration of 3 resulted in the formation of 8, which might explain the significant accumulation of 3 in the culture broth of the S. lividans TK23 transformant harboring pSCO102. In addition, a low in vivo concentration of DMAPP, one of the two substrates of SCO7467, might also explain the significant accumulation of 1. If the concentration of DMAPP is limited in vivo, the SCO7467-catalyzed dimethylallyl transfer to 3 only occurs to a minimum extent, allowing the SCO7468-catalyzed conversion of 3 to 8 to take precedence in vivo. Meanwhile, the notable difference of kinetic parameters described above clearly indicates that 4 is a physiological substrate of SCO7468. We thus conclude that the FMO enzyme SCO7468 catalyzes the conversion of 4 to 7 and is responsible for the second step in the biosynthesis of 2. In the final step of the biosynthesis of 2, an unidentified enzyme(s) such as a promiscuous dehydratase, presumably dehydrates both 7 and 8 to give 2 and 1, respectively, both of which were detected as final products in the S. lividans transformant harboring both SCO7467 and SCO7468.

A precedent for the conversion of 3 to 8 has been demonstrated in the biosynthesis of the plant hormone auxin in Arabidopsis thaliana. However, in the plant, the conversion is catalyzed by the action of the cytochrome P-450 enzyme CYP79B2 (24, 25). The finding that actinomycetes and plants utilize different types of oxygenases for the formation of 8 from 3 led us to investigate the reaction mechanism. We then identified 9 as a reaction intermediate of the FMO enzyme SCO7468. This identification suggests that the aldolase formation from L-tryptophan catalyzed by SCO7468 proceeds via N,N-dihydroxytryptophan.
droxytryptophan (Fig. 6). Presumably, N,N-dihydroxytryptophan nonenzymatically dehydrates to yield 3-(1H-indol-3-yl)-2-nitrosopropanoic acid, which successively decarboxylates to produce 8. SCO7468 likely converts 4 to 7 by the same reaction mechanism. A precedented reaction mechanism has been presumed in the formation of p-hydroxyphenylacetaldoxime from L-tyrosine catalyzed by cytochrome P-450TYR (also known as CYP79A1) from the plant *Sorghum bicolor* (22). However, an aldoxime-forming reaction by a FMO has been identified only in the metabolism of primary amines (26, 27). Thus, SCO7468 represents an unprecedented FMO that catalyzes two successive N-hydroxylation reactions of tryptophan-related amino acids to form the corresponding aldoximes.

BLAST searches against the NCBI database revealed a wide distribution of the indole prenyltransferase-containing gene cluster among various actinomycetes. Some prenylated indole derivatives have also been isolated from *Streptomyces* species (10, 28–30). However, no studies on the biosynthesis of prenylated indoles have been reported other than the identification of an L-tryptophan:6-dimethylallyltransferase responsible for the biosynthesis of 6-DMAI-3-carboxylic acid (10). The gene cluster involved in the biosynthesis of 6-DMAI-3-carboxylic acid contains a tryptophanase. The presence of a tryptophanase gene in the cluster may explain the biosynthesis of 6-prenylindole isolated from *Streptomyces* sp. TP-A0595 (30). 6-Prenylindole is likely an intermediate in the biosynthesis of 6-DMAI-3-carboxylic acid. This aldehyde moiety may be further oxidized to carboxylic acid as observed for the 5-DMAI-3-carboxylic acid in *Streptomyces* sp. MS239 (28). 6-DMAIAN has been isolated from the mycelium of *Streptomyces* sp. (BL-49-58-005) during a screening program for cytotoxic compounds (29). This strain produces 6-DMAIAOx and 6-prenyltryptophol as well as 6-DMAIAN. Despite the differences in their prenylated positions, 2 and 6-DMAIAN might be synthesized through similar pathways where indole prenyltransferase first appends a dimethylallyl group to 3 and FMO then catalyzes the conversion of DMAT into 5- or 6-DMAIAOx, followed by conversion into 5- or 6-DMAIAN by an unidentified dehydratase. *Streptomyces* sp. (BL-49-58-005) may have both a nitrilase and a reducing enzyme that act to metabolize 6-DMAIAN into 6-prenyltryptophol. Thus, the present study provides insight into the biosynthesis of prenylated indoles that have been purified from actinomycetes. The proposed biosynthetic pathway of the prenylated indoles is summarized in Fig. 7.

Prenylated indole derivatives are widely distributed in nature and show diverse biological and pharmacological activities including cytotoxicity and antifungal activity. In addition, because the potential for prenylated indole biosynthesis is widely distributed in soil actinomycetes, the prenylated indoles identified in the present study might possess yet unknown and important biological activities. Particularly intriguing is the structural similarity between 2 and indole-3-acetic acid (auxin), a major plant hormone. Recently, transkingdom signaling between *Pseudomonas aeruginosa* and *A. thaliana* was reported (31). *P. aeruginosa* produces diketopiperazine derivatives of cyclodepsipeptides that are involved in plant growth promotion. Considering that actinomycetes mainly live in soil, which is a rhizosphere of plants, prenylated indoles such as 2 might contribute to an uncharacterized signaling pathway between actinomycetes and plants. Such signaling might involve the conservon that is always located upstream of the structural biosynthetic genes of 2 and resembles the eukaryotic G protein-coupled regulatory sys-

![Diagram](image-url)
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tem. Investigation of the biological activity of 2 against A. thaliana is currently underway in our laboratory.

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