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ORIGINAL ARTICLE

Isolation, structural elucidation and biosynthesis of 3-hydroxy-6-dimethylallylindolin-2-one, a novel prenylated indole derivative from *Actinoplanes missouriensis*

Ryutaro Satou[1,4], Miho Izumikawa[2,4], Yohei Katsuyama[1], Misato Matsui[1], Motoki Takagi[2], Kazuo Shin-ya[3] and Yasuo Ohnishi[1]

Many prenylated indole derivatives are widely distributed in nature. Recently, two *Streptomyces* prenyltransferases, IptA and its homolog SCO7467, were identified in the biosynthetic pathways for 6-dimethylallylindole (DMAI)-3-carbaldehyde and 5-DMAI-3-acetonitrile, respectively. Here, we isolated a novel prenylated indole derivative, 3-hydroxy-6-dimethylallylindolin (DMAIN)-2-one, based on systematic purification of metabolites from a rare actinomycete, *Actinoplanes missouriensis* NBRC 102363. The structure of 3-hydroxy-6-DMAIN-2-one was determined by HR-MS and NMR analyses. We found that *A. missouriensis* produced not only 3-hydroxy-6-DMAIN-2-one but also 6-dimethylallyltryptophan (DMAT) and 6-DMAI when grown in PYM (peptone-yeast extract-$MgSO_4$) medium. We searched the complete genome of *A. missouriensis* for biosynthesis genes of these compounds and found a gene cluster composed of an *iptA* homolog (*AMIS_22580*, named $iptA_{\text{-Am}}$) and a putative tryptophanase gene (*AMIS_22590*, named $tnaA_{\text{-Am}}$). We constructed a $tnaA_{\text{-Am}}$-deleted ($\Delta tnaA_{\text{-Am}}$) strain and found that it produced 6-DMAT but did not produce 6-DMAI or 3-hydroxy-6-DMAIN-2-one. Exogenous addition of 6-DMAI to mutant $\Delta tnaA_{\text{-Am}}$ resulted in the production of 3-hydroxy-6-DMAIN-2-one. Furthermore, *in vitro* enzyme assays using recombinant proteins produced by *Escherichia coli* demonstrated that 6-DMAI was synthesized from tryptophan and dimethylallyl pyrophosphate in the presence of both $IptA_{\text{-Am}}$ and $TnaA_{\text{-Am}}$, and that $IptA_{\text{-Am}}$ preferred tryptophan to indole as the substrate. From these results, we concluded that the $iptA_{\text{-Am}}$–$tnaA_{\text{-Am}}$ gene cluster is responsible for the biosynthesis of 3-hydroxy-6-DMAIN-2-one. Presumably, tryptophan is converted into 6-DMAT by $IptA_{\text{-Am}}$ and 6-DMAT is then converted into 6-DMAI by $TnaA_{\text{-Am}}$. 6-DMAI appears to be converted into 3-hydroxy-6-DMAIN-2-one by the function of some unknown oxidases in *A. missouriensis*.
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Keywords: *Actinoplanes*; dimethylallyl pyrophosphate; prenylated indole; prenyltransferase; tryptophan; tryptophanase

INTRODUCTION

Many prenylated indole derivatives have been isolated from *Streptomyces*,[1–4] fungi,[5–7] and plants.[8,9] Examples of prenylated indole derivatives isolated from *Streptomyces* are 6-dimethylallylindole (DMAI)-3-carbaldehyde,[4] 6-prenylindole[3] and 6-prenyltryptophol.[2] Recently, Takahashi *et al.*[4] identified the IptA protein as an enzyme catalyzing the transfer of a dimethylallyl group to the C-6 position of an indole ring in the biosynthesis of 6-DMAI-3-carbaldehyde. IptA homologs are distributed among actinomycetes including *Streptomyces*, *Saccharomonospora*, *Saccharopolyspora*, *Micromonospora*, *Kitasatospora* and *Actinosynnema* (Figure 1).[4,10] They are phylogenetically distinct from both bacterial aromatic, and fungal indole prenyltransferases.[4] Interestingly, these IptA homolog genes are always accompanied by a tryptophanase gene or a flavin-dependent monooxygenase (FMO) gene. They are located in the vicinity of a 'conservon', a four-gene cassette conserved among actinomycetes and composed of a sensor-like histidine kinase gene, two hypothetical protein genes and an ATP/GTP-binding protein gene (Figure 1).[11–13] These four proteins encoded by a conservon are suggested to comprise a membrane-associated heterocomplex that resembles a eukaryotic G protein-coupled regulatory system.[12] Very recently, Ozaki *et al.*[10] reported that the *SCO7467–SCO7468* gene cluster, located in the vicinity of a conservon in *Streptomyces coelicolor* A3(2), and encoding an IptA-homolog and FMO, directed the biosynthesis of

[1]Department of Biotechnology, Graduate School of Agricultural and Life Science, The University of Tokyo, Tokyo, Japan; [2]Japan Biological Informatics Consortium (JBIC), Tokyo, Japan and [3]National Institute of Advanced Industrial Science and Technology (AIST), Tokyo, Japan
[4]These two authors contributed equally to this work.
Correspondence: Professor Y Ohnishi or Dr K Shin-ya, Department of Biotechnology, Graduate School of Agricultural and Life Science, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan.
E-mail: ayasuo@mail.ecc.u-tokyo.ac.jp or k-shinya@aist.go.jp
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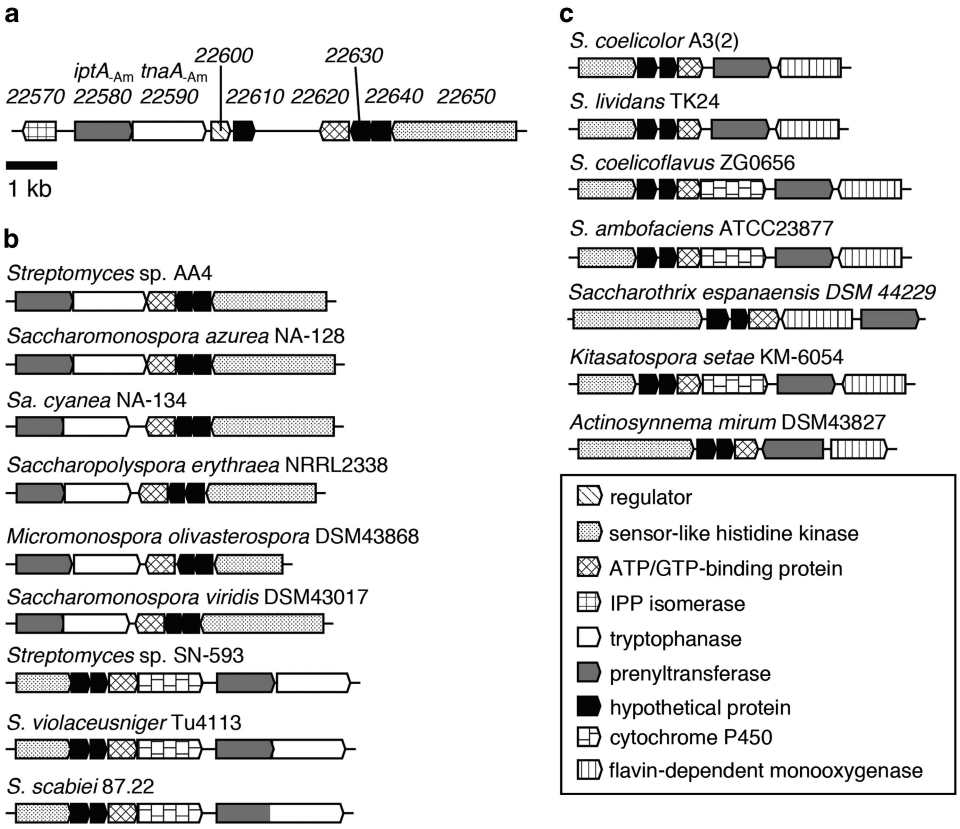

Figure 1 Gene clusters involved in the biosynthesis of prenylated indole derivatives. (**a**) The *iptA*$_{Am}$–*tnaA*$_{Am}$ locus in *A. missouriensis*. (**b**) Gene clusters containing an *iptA* homolog and a tryptophanase gene. (**c**) Gene clusters containing an *iptA* homolog and an FMO gene.

5-DMAI-3-acetaldoxime, which was converted into 5-DMAI-3-acetonitrile in the heterologous host *Streptomyces lividans*. These studies by Takahashi *et al.*[4] and Ozaki *et al.*[10] suggest that gene clusters containing an *iptA* homolog and a tryptophanase or FMO gene are involved in the biosynthesis of prenylated indole derivatives in actinomycetes.

Filamentous actinomycetes other than streptomycetes are often called rare actinomycetes. Rare actinomycetes are known as producers of many useful anti-infective agents, such as rifamycin produced by *Amycolatopsis mediterranei*, teicoplanin by *Actinoplanes teichomyceticus*, vancomycin by *Amycolatopsis orientalis* and gentamicin by *Micromonospora purpurea*.[14] Rare actinomycetes have therefore received increasing attention as potential producers of bioactive compounds. Recently, the complete genome sequence of *Actinoplanes missouriensis* NBRC 102363 was determined.[15] Although *A. missouriensis* ATCC 23342 produces actaplanin, a glycopeptide antibiotic,[16] and *A. missouriensis* A-5987 produces 5-azacytidine, a cytosine analog used as an epigenetic modifier,[17] the secondary metabolites produced by *A. missouriensis* NBRC 102363 have not been well studied; only 6-alkyl-4-*O*-dihydrogeranyl-2-methoxyhydroquinones derived from $C_{16–18}$ fatty acids have been isolated.[18] Therefore, we performed a systematic isolation of secondary metabolites from *A. missouriensis* NBRC 102363.

In this study, we isolated a novel prenylated indole derivative from *A. missouriensis* NBRC 102363 and elucidated its structure as 3-hydroxy-6-dimethylallylindolin (DMAIN)-2-one. In addition, we discovered 6-dimethylallyltryptophan (DMAT) and 6-DMAI (Figure 2) as possible biosynthesis intermediates of the compound. We then focused our attention to the gene cluster *AMIS_22580–AMIS_22590*, which encodes an IptA homolog (IptA$_{Am}$) and a putative tryptophanase (TnaA$_{Am}$) and which is located in the vicinity of a conservon. Gene disruption experiments and *in vitro* enzyme assays revealed that the gene cluster is responsible for the biosynthesis of 6-DMAI, and that 6-DMAI is the genuine biosynthetic intermediate of 3-hydroxy-6-DMAIN-2-one. Possible biosynthetic pathways for prenylated indole derivatives in actinomycetes and putative biological function of the compounds are discussed.

RESULTS

Isolation and structure elucidation of 3-hydroxy-6-DMAIN-2-one from *A. missouriensis* NBRC 102363

A. missouriensis NBRC 102363 was cultivated in a production medium at 27 °C for 5 days. The fermentation broth (6 l) was centrifuged, and the mycelial cake was extracted with acetone. Following concentration *in vacuo*, the aqueous concentrate was extracted with ethylacetate. The extract was dried over Na_2SO_4 and evaporated to dryness. The residue (1.86 g) was subjected to normal-phase medium-pressure liquid chromatography. The fractions containing compound **1** were further purified by preparative reversed-phase HPLC to yield **1** (1.4 mg).

Compound **1** gave an $[M+Na]^+$ ion at *m/z* 240.1009 with high-resolution ESI-MS. This spectrum was consistent with a molecular formula of $C_{13}H_{15}NO_2$ (calcd. for $C_{13}H_{15}NO_2Na$, 240.1000). The ^1H and ^{13}C NMR spectral data for **1** are shown in Table 1. The structure of **1** was elucidated as 3-hydroxy-6-DMAIN-2-one (Figure 2) by a

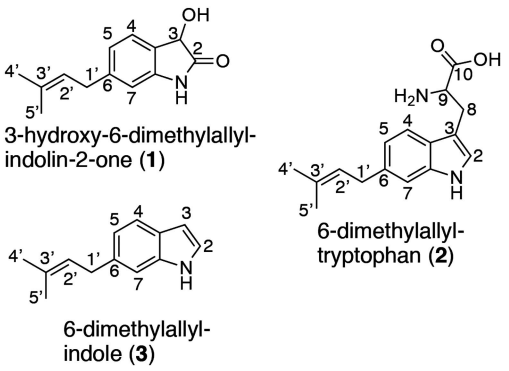

Figure 2 Structures of 3-hydroxy-6-DMAIN-2-one (**1**), 6-DMAT (**2**) and 6-DMAI (**3**).

Table 1 ^{13}C and ^{1}H NMR data for 1

Position	δ_H	δ_C
1	10.16 (s)	
2		178.4
3	4.75 (s)	69.2
3a		126.9
4	7.15 (d, J=7.2 Hz, 1H)	124.9
5	6.75 (d, J=7.2 Hz, 1H)	121.5
6		142.8
7	6.57 (s, 1H)	109.6
7a		142.6
1′	3.25 (d, J=7.5 Hz, 2H)	34.1
2′	5.24 (t, J=7.5 Hz, 1H)	123.4
3′		132.1
4′	1.69 (s, 3H)	25.7
5′	1.67 (s, 3H)	17.9

^{13}C (125 MHz) and ^{1}H (600 MHz) NMR spectra were recorded in DMSO-d_6 on a Varian 600 NB CL NMR System (Varian, Palo Alto, CA, USA). The solvent peak was used as an internal standard (δ_C 39.7, δ_H 2.49).

series of two-dimensional NMR analyses, including HSQC, DQF-COSY and constant time–HMBC (see Methods). In addition, the specific rotation value of **1** ($[\alpha]^{25}_D + 4.2$, *c* 0.1, MeOH) is similar to that of (*R*)-3-hydroxyindolin-2-one ($[\alpha]^{25}_D + 7$, *c* 1.0, MeOH).[19] Thus, the absolute configuration of **1** was concluded to be *R*. Although dioxindoline has been reported as a biosynthetic intermediate of anthranilic acid, produced by *Bradyrhizobium japonicum*,[20] there has been no report of a dimethylallyl-conjugated dioxindole moiety.

Production of 6-DMAT and 6-DMAI by *A. missouriensis* NBRC 102363

From biosynthetic studies of 6-DMAI-3-carbaldehyde[4] and 5-DMAI-3-acetonitrile,[10] we speculated that 6-DMAT and 6-DMAI were possible biosynthetic intermediates of 3-hydroxy-6-DMAIN-2-one. These compounds were not detected, at least in a large amount, in the extract of our 5-day culture of *A. missouriensis*. Analysis using LC-MS (liquid chromatography mass spectrometry) of a 2-day culture in PYM (peptone-yeast extract-$MgSO_4$) medium, however, indicated that *A. missouriensis* produced not only 3-hydroxy-6-DMAIN-2-one (**1**) but also 6-DMAT (**2**) and 6-DMAI (**3**) (Figure 3a). To identify compound **3**, authentic 6-DMAI was chemically synthesized from 6-indoleboronic acid and dimethylallylbromide by Suzuki–Murayama coupling using $Pd_2(dba_3)$ as a catalyst. The structure of synthetic 6-DMAI was confirmed by ^{1}H and ^{13}C NMR analyses (Supplementary Table S1 in Supplementary Information). By comparison of the retention time, mass and UV spectral data with the synthetic standard, we identified compound **3** as 6-DMAI. In a similar way, we also identified compound **2** as 6-DMAT by using authentic 6-DMAT, prepared with a large-scale *in vitro* reaction of the IptA homolog from *A. missouriensis* as described below.

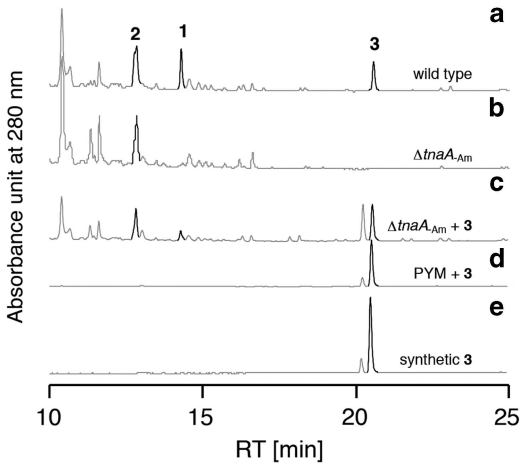

Figure 3 UV chromatograms from the LC-MS analysis of metabolites produced by the wild-type and Δ*tnaA*$_{\text{-Am}}$ strains of *A. missouriensis*. (**a**) The wild-type strain produces three prenylated indole derivatives; 3-hydroxy-6-DMAIN-2-one (**1**), 6-DMAT (**2**) and 6-DMAI (**3**). (**b**) The Δ*tnaA*$_{\text{-Am}}$ mutant produces **2**, but does not produce **1** and **3**. (**c**) When **3** is supplemented to the Δ*tnaA*$_{\text{-Am}}$ mutant, **1** is detected. (**d**) **3** is not oxidized to **1** in a PYM medium without cells. (**e**) Synthetic standard of 6-DMAI (**3**).

A gene cluster encoding an IptA homolog and a putative tryptophanase on the *A. missouriensis* chromosome

We searched the complete genome of *A. missouriensis* for the biosynthesis genes of 3-hydroxy-6-DMAIN-2-one. In a BLAST search using IptA as a query, we found a gene cluster composed of an *iptA* homolog (*AMIS_22580*, named *iptA*$_{\text{-Am}}$) and a putative tryptophanase gene (*AMIS_22590*, named *tnaA*$_{\text{-Am}}$) (Figure 1a). This gene cluster is located close to a conservon (*AMIS_22620-AMIS_22650*). IptA$_{\text{-Am}}$ and TnaA$_{\text{-Am}}$ show high amino-acid sequence similarities (36% and 58% identity) to IptA and its cognate tryptophanase homolog, respectively, from *Streptomyces* sp. SN-593, which produces 6-DMAI-3-carbaldehyde.[4] From these data we proposed that this gene cluster should be involved in the biosynthesis of 3-hydroxy-6-DMAIN-2-one.

Involvement of *tnaA*$_{\text{-Am}}$ in the biosynthesis of 6-DMAI and 3-hydroxy-6-DMAIN-2-one

The functional role of the putative tryptophanase, encoded in the vicinity of *iptA* in 6-DMAI-3-carbaldehyde biosynthesis in *Streptomyces* sp. SN-593, has not yet been examined experimentally. Therefore, we generated a *tnaA*$_{\text{-Am}}$-deleted mutant of *A. missouriensis* (Supplementary Figure S2 in Supplementary Information). As expected, the Δ*tnaA*$_{\text{-Am}}$ mutant did not produce either 6-DMAI (**3**) or 3-hydroxy-6-DMAIN-2-one (**1**), but did produce 6-DMAT (**2**) (Figure 3b). This indicated TnaA$_{\text{-Am}}$ is involved in the biosynthesis of 6-DMAI (**3**) and 3-hydroxy-6-DMAIN-2-one (**1**). Tryptophanases catalyze the elimination of pyruvate and ammonia from tryptophan to

produce indole. We postulate that TnaA$_{\text{-Am}}$ catalyzes the same reaction and uses 6-DMAT as a substrate to produce 6-DMAI.

Conversion of 6-DMAI into 3-hydroxy-6-DMAIN-2-one by the *A. missouriensis* Δ*tnaA*$_{\text{-Am}}$ mutant

As described above, the Δ*tnaA*$_{\text{-Am}}$ mutant did not produce 6-DMAI (**3**) or 3-hydroxy-6-DMAIN-2-one (**1**). To examine whether 6-DMAI (**3**) is the biosynthetic intermediate of 3-hydroxy-6-DMAIN-2-one (**1**), we added 6-DMAI (**3**) to the culture medium of the Δ*tnaA*$_{\text{-Am}}$ mutant. In this culture, a small but distinct amount of 3-hydroxy-6-DMAIN-2-one (**1**) was produced (Figure 3c). When 6-DMAI (**3**) was added to the culture medium without addition of the microorganism, conversion of 6-DMAI (**3**) to 3-hydroxy-6-DMAIN-2-one (**1**) was not observed (Figure 3d). These results indicate that oxidation of 6-DMAI (**3**) is likely to be catalyzed by an oxidase(s) present in *A. missouriensis*. Because no oxidase gene is encoded in the vicinity of the *iptA*$_{\text{-Am}}$–*tnaA*$_{\text{-Am}}$ gene cluster, it is very difficult to predict which oxidase(s) may be responsible for oxidation of 6-DMAI (**3**) to 3-hydroxy-6-DMAIN-2-one (**1**).

In vitro analysis of TnaA$_{\text{-Am}}$ and IptA$_{\text{-Am}}$

To further elucidate the biosynthetic pathway of 3-hydroxy-6-DMAIN-2-one (**1**), we produced recombinant TnaA$_{\text{-Am}}$ and IptA$_{\text{-Am}}$ proteins in *Escherichia coli*. These were fused with a histidine tag at their N-terminus, and purified by Ni^{2+} affinity chromatography. When recombinant TnaA$_{\text{-Am}}$ and IptA$_{\text{-Am}}$ were incubated with L-tryptophan and dimethylallyl pyrophosphate (DMAPP) in the presence of Mg^{2+} ions and pyridoxal-5′-phosphate, small but distinct amounts of 6-DMAI (**3**) were produced (Figure 4b). This *in vitro* study showed that these two enzymes are sufficient for the biosynthesis of 6-DMAI (**3**). In this reaction, another compound (**2**) was produced in a large amount (Figure 4b). This compound exhibited an $[M+H]^+$ ion at *m/z* 273.3 suggesting it should be 6-DMAT. Compound **2** was prepared in a large-scale enzymatic reaction and purified by size exclusion chromatography using LH-20 and HPLC equipped with an octadecylsilyl (ODS) column. Ultimately, compound **2** was identified as 6-DMAT by comparing the ^1H and ^{13}C NMR spectra with those of previously reported data (Supplementary Table S2 in Supplementary Information).[4] When TnaA$_{\text{-Am}}$ was not included in the reaction, only formation of 6-DMAT (**2**) was observed (Figure 4c). When IptA$_{\text{-Am}}$ was excluded from the reaction, formation of neither 6-DMAT (**2**) nor 6-DMAI (**3**) was observed and only trace amounts of indole were produced (Figure 4d). This indicates that, at least *in vitro*, TnaA$_{\text{-Am}}$ accepts not only 6-DMAT (**2**) but also tryptophan. It was difficult to know from this *in vitro* reaction, which is the preferable substrate of TnaA$_{\text{-Am}}$, tryptophan or 6-DMAT (**2**). This is because TnaA$_{\text{-Am}}$ activity appears to be very low. Next, we examined the substrate specificity of IptA$_{\text{-Am}}$ *in vitro*. IptA$_{\text{-Am}}$ was found to use L-tryptophan much more efficiently than indole; only trace amounts of 6-DMAI were formed from indole (Figure 4e). This result and the observation that a large amount of 6-DMAT (**2**) was produced by the Δ*tnaA*$_{\text{-Am}}$ mutant indicate that the first step of 3-hydroxy-6-DMAIN-2-one (**1**) biosynthesis is dimethylallylation of L-tryptophan by IptA$_{\text{-Am}}$, and the subsequent reaction, catalyzed by TnaA$_{\text{-Am}}$, results in the formation of 6-DMAI (Figure 5). Biosynthesis of 3-hydroxy-6-DMAIN-2-one (**1**) requires further two oxidation steps (Figure 5).

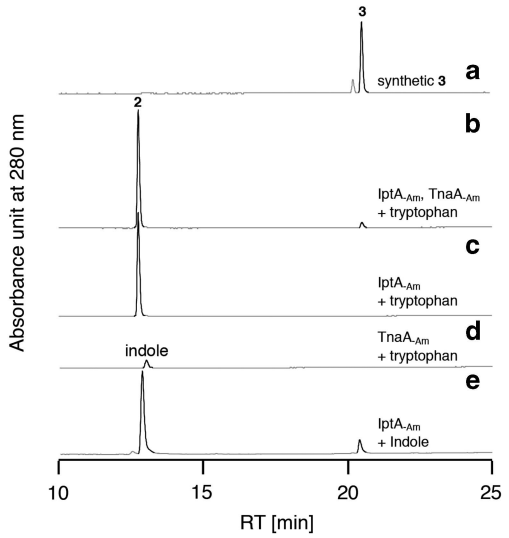

Figure 4 UV chromatograms from the LC-MS analysis of the products of the *in vitro* enzyme reaction using recombinant IptA$_{\text{-Am}}$ and TnaA$_{\text{-Am}}$. (**a**) Synthetic standard of 6-DMAI (**3**). (**b**) **2** and **3** are produced from tryptophan in the presence of both IptA$_{\text{-Am}}$ and TnaA$_{\text{-Am}}$. (**c**) **2** is produced from tryptophan by IptA$_{\text{-Am}}$. (**d**) Indole is produced from tryptophan by TnaA$_{\text{-Am}}$. (**e**) A trace amount of 6-DMAI is produced from indole by IptA$_{\text{-Am}}$.

DISCUSSION

In this study, we isolated the novel prenylated indole derivative 3-hydroxy-6-DMAIN-2-one (**1**) from an extract of *A. missouriensis* NBRC 102363. *In vivo* and *in vitro* experiments showed that the *iptA*$_{\text{-Am}}$–*tnaA*$_{\text{-Am}}$ gene cluster is involved in the biosynthesis of **1**. IptA homologs are widely distributed among actinomycetes and their involvement in the biosynthesis of prenylated indole derivatives has already been suggested.[4,10] Interestingly, IptA homolog genes are always accompanied by a tryptophanase gene or a FMO gene. Ozaki *et al.*[10] characterized such a FMO, SCO7468, from *S. coelicolor* A3(2) and found that the enzyme catalyzed the conversion of 5-DMAT into 5-DMAI-3-acetaldoxime. To date, no experimental work has been carried out on tryptophanase genes that are associated with *iptA* homologs. This study has therefore given the first experimental evidence that a tryptophanase associated with an IptA homolog is involved in the biosynthesis of prenylated indole derivatives. Because IptA$_{\text{-Am}}$ preferred tryptophan over indole as a substrate, it is probable that tryptophan is first converted into 6-DMAT (**2**) by IptA$_{\text{-Am}}$ (Figure 5), as indicated in the biosynthesis of

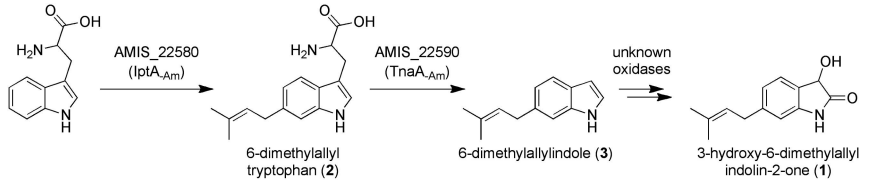

Figure 5 Proposed biosynthesis pathway of 3-hydroxy-6-DMAIN-2-one (**1**).

6-DMAI-3-carbaldehyde.[4] TnaA$_{\text{-Am}}$ is then responsible for the conversion of 6-DMAT (**2**) into 6-DMAI (**3**) (Figure 5).

We found that 6-DMAI (**3**) is a biosynthetic intermediate of 3-hydroxy-6-DMAIN-2-one (**1**) (Figure 3c). Conversion of **3** into **1** requires two oxidation steps, which can be catalyzed by several kinds of oxidases; cytochrome P450, FMO, α-ketoglutarate-dependent dioxygenase and metal-dependent oxidase. Because there are many possible candidates, it was difficult to predict which genes are responsible for these oxidations in the genome sequence of *A. missouriensis*. In addition, these oxidation steps may be catalyzed, as a side reaction, by oxidases involved in the metabolism of other aromatic compounds. Activity-based purification of the enzymes responsible for these oxidations is required for further study.

Various microorganisms, such as *E. coli*, *Klebsiella oxytoca*, *Providencia stuartii*, *Citrobacter koseri*, *Morganella morganii*, *Haemophilus influenzae* type b strain and *Fusobacterium nucleatum*, produce indole. It has been predicted that indole acts as a signal molecule that regulates biofilm formation.[21,22] Martino *et al.*[22] reported that a tryptophanase inhibitor, oxiindolyl-L-alanine, decreased biofilm formation of indole-producing bacteria. A recent study also revealed that indole and some indole derivatives including indole-3-carboxaldehyde and indole-3-acetic acid are important for virulence of pathogenic *E. coli*.[23] Prenylated indole derivatives may have important functions as signal molecules also in actinomycetes, because the set of genes responsible for the synthesis of prenylated indole derivatives is widely conserved among actinomycetes. Interestingly, the biosynthesis genes of prenylated indole derivatives are always accompanied by a conservon (Figure 1). It has been suggested that four intrinsic proteins encoded by a conservon, comprise a membrane-associated heterocomplex that resembles a eukaryotic G protein-coupled regulatory system.[12] The neighboring conservon could be involved in signal transduction mediated through prenylated indole derivatives. Conservons sometimes have an additional cytochrome P450 gene, the function of which remains to be elucidated. We speculate that additional cytochrome P450s encoded in the vicinity of the biosynthesis genes of prenylated indole derivatives are responsible for the modification of prenylated indole derivatives. Elucidation of the biological function of prenylated indole derivatives in actinomycetes is an interesting topic and certainly warrants further study.

METHODS

Isolation of 3-hydroxy-6-dimethylallylindolin-2-one

A. missouriensis NBRC 102363 was cultivated in 50 ml test tubes containing 15 ml of the seed medium consisting of yeast extract (BD Biosciences, San Jose, CA, USA) 4.0%, malt extract (Nacalai Tesque, Kyoto, Japan) 10% and glucose 4.0%, pH 7.2 (adjusted before sterilization). The test tubes were shaken on a reciprocal shaker (355 r.p.m.) at 27 °C for 2 days. Aliquots (2.5 ml) of the broth were transferred to 60 × 500 ml baffled Erlenmeyer flasks containing 100 ml of a production medium consisting of β-cyclodextrin (Kanto Chemical, Tokyo, Japan) 4.0%, glycerin 0.5%, pharmamedia (Traders Protein, Lubbock, TX, USA) 2.0%, $CuSO_4$ 5 mg, $MnCl_2$ 5 mg and $ZnSO_4$ 5 mg, pH 7.2 (adjusted before sterilization), and cultured on a rotary shaker (180 r.p.m.) at 27 °C for 5 days. The fermentation broth (6 l) was centrifuged, and the mycelial cake was extracted with acetone (1.2 l). After *in vacuo* concentration, the aqueous concentrate was extracted with ethylacetate (3 × 300 ml). The extract was dried over Na_2SO_4 and evaporated to dryness. The residue (1.86 g) was subjected to medium-pressure liquid chromatography (Purif-Pack SI–60, Shoko Scientific Co., Yokohama, Japan), and successively eluted with a stepwise solvent system of $CHCl_3$-MeOH (0, 1, 2, 4, 10, 20, 50 and 100% MeOH). The fractions eluted with 4 and 10% MeOH (175 mg) were chromatographed by normal-phase medium-pressure liquid chromatography with a gradient solvent system of $CHCl_3$–MeOH (3–16% MeOH). The fractions eluted with 3–4% MeOH (41 mg) were purified by preparative reversed-phase HPLC using a CAPCELL PAK C18 MGII column (5.0 μm, 20 i.d. × 150 mm; Shiseido, Tokyo, Japan) with 60% MeOH–H_2O containing 0.1% formic acid (flow rate 10 ml min^{-1}) to yield **1** (1.4 mg, retention time 19.2 min).

Structure elucidation of 3-hydroxy-6-dimethylallylindolin-2-one

Compound **1** was isolated as a colorless oil ($[\alpha]^{25}_{D}$ + 4.2, c 0.1, MeOH) that gave an $[M+Na]^+$ ion at *m/z* 240.1009 on the HR-ESIMS. This spectrum was consistent with a molecular formula of $C_{13}H_{15}NO_2$ (calcd. for $C_{13}H_{15}NO_2Na$, 240.1000). Compound **1** displayed the following IR and UV spectra: IR (KBr) ν_{max} = 1716 cm^{-1}; UV (MeOH) λ_{max} (ε) = 247 (4200) and 309 (1100) nm. The ^1H and ^{13}C NMR spectral data for **1** are shown in Table 1. The structure of **1** was elucidated by a series of 2D NMR analyses, including HSQC, CT-HMBC and DQF-COSY (Supplementary Figure S1 in Supplementary Information).[24] The ^1H–^{13}C long-range couplings from the amino proton 1-H (δ_H 10.16) to an amide carbonyl carbon C-2 (δ_C 178.4), an oxymethine carbon C-3 (δ_C 69.2) and aromatic quaternary carbons C-3a (δ_C 126.9) and C-7a (δ_C 142.6), and from an oxymethine proton 3-H (δ_H 4.75) to the amide carbonyl carbon C-2 and the aromatic quaternary carbons C-3a and C-7a established a 3-hydroxy-γ-lactam moiety. An *ortho*-coupling between 4-H (δ_H 7.15) and 5-H (δ_H 6.75) was observed in the DQF-COSY spectrum. Together with the strong ^1H–^{13}C *meta* couplings from 4-H to C-6 (δ_C 142.8) and C-7a (δ_C 142.6), from 5-H to C-7 (δ_C 109.6) and C-3a (δ_C 126.9) and from 7-H (δ_H 6.57) to C-5 (δ_C 121.5) and C-3a, they revealed a 1,2,4-trisubstituted benzene ring moiety. The low-field chemical shift of C-7a and ^1H–^{13}C *peri*-coupling between the aromatic proton 4-H and the oxymethine carbon C-3 established a 3-hydroxyindolin-2-one moiety. Two singlet methyl protons 4′-H (δ_H 1.69) and 5′-H (δ_H 1.67) were ^1H–^{13}C long-range coupled to each other (δ_C 25.7 and 17.9) and commonly coupled to an olefinic quaternary carbon C-3′ (δ_C 132.1) and an olefinic methine carbon C-2′ (δ_C 123.4), of which the proton was ^1H spin-coupled to a methylene proton 1′-H (δ_H 3.25). These results indicated the presence of a dimethylallyl moiety. Finally, the connection between the 3-hydroxyindolin-2-one and the dimethylallyl moieties was elucidated by the ^1H–^{13}C long-range correlations from the methylene proton 1'-H to the aromatic carbons C-5, C-6 and C-7. The structure of **1** therefore was determined to be 3-hydroxy-6-DMAIN-2-one (Figure 2). All of the NMR spectra are shown in supporting information (Supplementary Figures S3–S7).

Construction of the Δ*tnaA*$_{\text{-Am}}$ mutant

An in-frame *tnaA*$_{\text{-Am}}$ deletion mutant was constructed by eliminating the region encoding the central part of TnaA$_{\text{-Am}}$ (from Thr-57 to Asp-300) according to the method described by Awakawa *et al.*[18] The correct deletion and replacement were confirmed by PCR using primers 5′-GAGAGACATATGT CCGCTGACCAGATCCCG-3′ and 5′-GAGAAAGCTTTCAGAGGGGCGCCAA CCGC-3′ (Supplementary Figure S2 in Supplementary Information).

LC-MS analysis of metabolites produced by the wild-type and Δ*tnaA*$_{\text{-Am}}$ strains of *A. missouriensis* NBRC 102363

The wild-type and Δ*tnaA*$_{\text{-Am}}$ strains of *A. missouriensis* NBRC 102363 were inoculated into a PYM medium (0.5% peptone, 0.3% yeast extract, 0.1% $MgSO_4 \cdot 7\,H_2O$, pH 7.0) and incubated at 26 °C for 2 days. The cell culture was extracted with ethyl acetate. The organic layer was evaporated to dryness and the residual materials were dissolved in methanol for LC-ESIMS analysis. LC-ESIMS was carried out using an Agilent 1100 series with a high-capacity trap plus (HCT) system (Bruker Daltonics, Billerica, MA, USA) and equipped with a MonoBis column (2 × 100 mm; Kyoto Monotech Corporation, Kyoto, Japan). The compounds were eluted using a linear gradient of water and acetonitrile with 0.1% formic acid as the mobile phase.

Synthesis of 6-DMAI (3)

One hundred and sixty milligrams of indole-6-boronic acid, 90 mg of dimethylallylbromide, 930 mg of K_2CO_3 and 54 mg of $Pd_2(dba_3)$ were dissolved in 10 ml of tetrahydrofuran and refluxed for 2 h. The solvent was removed by evaporation, and the residual material was dissolved in water. The compound was extracted with ethylacetate. The organic layer was evaporated to dryness and 6-DMAI (**3**) was purified by preparative silica-gel thin layer

chromatography (PLC silica gel 60 F254, 1 mm, Merck, Darmstadt, Germany) using a mobile phase of 100% chloroform. The structure of 6-DMAI (**3**) was confirmed by one- and two-dimensional NMR.

In vitro analysis of $IptA_{-Am}$ and $TnaA_{-Am}$

A reaction mixture containing 0.05 mg ml^{-1} DMAPP, 100 mM Tris-HCl buffer (pH 8.0), 2 mM $MgCl_2$, 1 mM tryptophan, 100 mM pyridoxal phosphate, 1 μg of recombinant $IptA_{-Am}$ and 5 μg of recombinant $TnaA_{-Am}$, in a total volume of 20 μl, was incubated at 30 °C for 1 h. The reaction was quenched by adding 20 μl of methanol. Five microliters of reaction mixture was applied to LC-ESIMS after removing the precipitate by centrifugation. LC-ESIMS was carried out using Agilent 1100 series as previously described. The compounds were eluted with a linear gradient using water and acetonitrile containing 0.1% formic acid as the mobile phase.

Large-scale production of 6-DMAT (2)

For large-scale production, we used synthetic DMAPP, which was prepared and purified according to the method reported previously.[25,26] Although the purity of this synthetic DMAPP is low compared with the pure, commercially available material, it showed sufficient activity for a large-scale reaction. $IptA_{-Am}$ was incubated with 2 mM tryptophan and approximately 0.2 mg ml^{-1} DMAPP in buffer containing 2 mM $MgCl_2$, 100 mM Tris-HCl (pH 8.0) in a total volume of 20 ml. Following overnight incubation at 30 °C, the solution was acidified using formic acid and directly applied to a Sep-Pak C18 column (Waters, Milford, MA, USA). The column was washed with 20 ml of water, 20% methanol and 50% methanol containing 0.1% formic acid. 6-DMAT (**2**) was then eluted with 100% methanol containing 0.1% formic acid. The organic layer was removed by evaporation and the residual material was further purified using LH-20. The compound was confirmed as 6-DMAT by comparing the ^{1}H and ^{13}C NMR data with that reported in the literature.[4]

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