Identification and characterization of a bacterial cytochrome P450 monooxygenase catalyzing the 3-nitration of tyrosine in rufomycin biosynthesis

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Rufomycin is a circular heptapeptide with anti-mycobacterial activity and is produced by Streptomyces atratus ATCC 14046. Its structure contains three non-proteinogenic amino acids, N-dimethylallyltryptophan, trans-2-crotylglycine, and 3-nitrotyrosine (3NTyr). Although the rufomycin structure was already reported in the 1960s, its biosynthesis, including 3NTyr generation, remains unclear. To elucidate the rufomycin biosynthetic pathway, we assembled a draft genome sequence of S. atratus and identified the rufomycin biosynthetic gene cluster (ruf cluster), consisting of 20 ORFs (rufA–rufJ). We found a putative heptamodular nonribosomal peptide synthetase encoded by rufF, a putative tryptophan N-dimethylallyltransferase encoded by rufP, and a putative trimodular type I polyketide synthase encoded by rufE. Moreover, the ruf cluster contains an apparent operon harboring putative cytochrome P450 (rufO) and nitric oxide synthase (rufN) genes. A similar operon, txtDE, is responsible for the formation of 4-nitrotryptophan in thaxtomin biosynthesis; the cytochrome P450 TxtE catalyzes the 4-nitration of Trp. Therefore, we hypothesized that RufO should catalyze the Tyr 3-nitration. Disruption of rufO abolished rufomycin production by S. atratus, which was restored when 3NTyr was added to the culture medium of the disrupted Recombinant RufO protein exhibited Tyr 3-nitration activity both in vitro and in vivo. Spectroscopic analysis further revealed that RufO recognizes Tyr as the substrate with a dissociation constant of ~0.1 μM. These results indicate that RufO is an unprecedented cytochrome P450 that catalyzes Tyr nitration. Taken together with the results of an in silico analysis of the ruf cluster, we propose a rufomycin biosynthetic pathway in S. atratus.

Natural products have been found in a wide range of organisms, including plants, fungi, microorganisms, and mammals. Because these compounds exhibit various physiological activities applicable to medical and pharmaceutical products, animal drugs, and agricultural chemicals, they have been used to facilitate and improve human life. From a biochemical point of view, these compounds display diverse chemical structures, and many studies have focused on their biosynthetic mechanisms.

The nitro group is not an abundant functional group in natural products, but it has been found in some important bioactive compounds, including chloramphenicol, aureothin, and thaxtomin (1, 2). Although the mechanism for generating the nitro group in natural products is not fully understood, two possible nitration mechanisms have been reported. One is the oxidation reaction of an amino group found in chloramphenicol, aureothin, and pyrrolnitrin biosynthesis. In the former two cases, the p-amino group of p-aminophenylalanine and p-aminobenzoic acid are oxidized to p-nitro groups using molecular oxygen as a substrate by the non-heme di-iron–containing monooxygenases CmlM (3, 4) and AurF (5–7), respectively. In pyrrolnitrin biosynthesis, PrnD, which contains a Rieske iron–sulfur cluster, catalyzes the oxidation of aminopyrrolnitrin to complete the biosynthetic pathway (8). Recently, CreD, a flavin-dependent monooxygenase that is involved in crememycin biosynthesis, was reported to catalyze nitro group formation by oxidizing the amino group of Asp (9).

The other mechanism is direct nitration catalyzed by TxtE, a bacterial cytochrome P450 involved in thaxtomin biosynthesis in plant-pathogenic Streptomyces strains, including Streptomyces scabies (10–12). TxtE uses nitric oxide (NO) supplied by a nitric oxide synthase (NOS)3 encoded by txtD as its substrate,

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The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBankTM/EMBL Data Bank with accession number(s) LC257593.

This article contains supplemental Tables S1–S3 and Figs. S1–S3.

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3 The abbreviations used are: NOS, nitric oxide synthase; DEANO, diethylamino NONOate; 3NTyr, 3-nitrotyrosine; NRPS, nonribosomal peptide synthetase; MT, methyltransferase; A, adenylation; T, thiolation; PKS, polyketide synthase; TE, thioesterase; KS, ketosynthase; AT, acyltransferase; ACP, acyl carrier protein; DH, dehydratase; KR, ketoreductase; ER, enoylreductase; TSb, tryptic soy broth; ISP2, International Streptomyces Project 2; MG2, modified G-2; TB, terrific broth; PAM, protospacer adjacent motif; ESI, electrospray ionization; IPTG, isopropyl β-D-1-thio-β-galactopyranoside.
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and it catalyzes the selective nitration of Trp to generate 4-nitrotryptophan. The \textit{txtE} and \textit{txtD} genes are located next to each other, and they form a putative operon in the thaxtomin biosynthetic gene cluster (\textit{txt} cluster) (13).

In addition to these compounds, pyrrolomycins were reported to possess a nitro group in the pyrrole ring (14–18), but the nitration mechanism has not been elucidated in detail (19, 20). NOS is not considered to be involved in this mechanism, because NOS inhibitors do not affect the nitration of pyrrole (20). Instead, putative nitrate reductases are encoded by its biosynthetic gene cluster, suggesting that NO is synthesized by the reduction of nitrate (19).

Rufomycin (21, 22), also known as ilamycin (23–27), is a circular heptapeptide with anti-mycobacterial activity, which was isolated from \textit{Streptomyces atratus} ATCC 14046. Although its chemical structure was reported in the 1960s (26, 27), its biosynthetic mechanisms are unclear. Rufomycin contains three non-proteinogenic amino acids, \textit{N}-dimethylallyltryptophan, \textit{trans}-2-crotylglycine, and 3-nitrotyrosine (3NTyr). The formation of \textit{N}-dimethylallyltryptophan was found in cyclomarin biosynthesis, in which the tryptophan \textit{N}-dimethylallyltransferase CymD catalyzes the \textit{N}-prenylation of free Trp (28, 29). In contrast, the mechanisms for generating \textit{trans}-2-crotylglycine and 3NTyr are unknown. Although 2-crotylglycine is an intermediate in the degradation pathway of aromatic rings in \textit{Pseudomonas putida} (30), this pathway is unlikely to be used for 2-crotylglycine synthesis in rufomycin biosynthesis, and no enzymes for 3NTyr formation have been reported. Therefore, elucidating the rufomycin biosynthetic pathway will shed light on novel enzymes involved in the biosynthesis of 2-crotylglycine and 3NTyr.

Here we report the identification and \textit{in silico} characterization of the rufomycin biosynthetic gene cluster (\textit{ruf} cluster). We also report genetic and biochemical analyses of a cytochrome P450 enzyme, RufO, which catalyzes the 3-nitration of Tyr. According to these analyses, we propose a probable rufomycin biosynthetic pathway.

Results

Draft genome analysis of \textit{S. atratus} to identify the \textit{ruf} cluster

To elucidate the mechanisms of rufomycin biosynthesis and 3NTyr generation, identifying the rufomycin biosynthetic gene cluster is indispensable. Therefore, we first determined a draft genome sequence of \textit{S. atratus} with the HiSeq 2000 system (Illumina) using a paired-end sequencing strategy. Then we searched the draft genome sequence for the rufomycin biosynthetic gene cluster \textit{in silico}. Based on the rufomycin structure, we assumed that the rufomycin biosynthetic gene cluster should include a nonribosomal peptide synthetase (NRPS) gene(s) for peptide assembly, a tryptophan transferase gene for the synthesis of \textit{N}-dimethylallyltransferase, and a gene(s) for the nitro group synthesis. As expected, we found a gene cluster that satisfied all of these requirements (see below) and named it the \textit{ruf} cluster.

In \textit{silico} analysis of the \textit{ruf} cluster

Here we describe the results of our \textit{in silico} analysis of several important enzyme-encoding genes for rufomycin biosynthesis. NRPS—Because rufomycin harbors three non-proteinogenic amino acids, we assumed that it should be synthesized by an NRPS. In the \textit{ruf} cluster, we found a large gene (\textit{rufT}) encoding a heptamodular NRPS, the domain component of which was consistent with the chemical structure of rufomycin (Fig. 1B).
The substrate of each adenylation (A) domain was predicted by NRPS predictor 2 (supplemental Table S1) (31, 32). Although most of the predictions were not consistent with the structure of rufomycin, the A domain of the loading module was predicted to recognize N-dimethylallyltryptophan with high reliability. This result indicates that peptide assembly is initiated from N-dimethylallyltryptophan. The location of two methyltransferase (MT) domains at modules 1 and 4 also supports this notion. Modules 1 and 4 correspond to the second and fifth amino acid residues, respectively, in the rufomycin assembly pathway. When N-dimethylallyltryptophan is considered to be the first amino acid residue, the second and fifth amino acid residues are both N-methylleucine, which agree with the presence of MT domains at modules 1 and 4. Meanwhile, the A domain of module 2 was predicted to incorporate Tyr. This module corresponds to the third amino acid residue of rufomycin (i.e. 3NTyr), assuming that N-dimethylallyltryptophan is the first amino acid residue. If this prediction is correct, Tyr should be loaded on the thiolation (T) domain of module 2, and 3-nitration should occur during or after peptide assembly. However, because there is no report of A domains recognizing 3NTyr, it is impossible to predict 3NTyr as a substrate of any A domains. Therefore, it is also possible that 3NTy, instead of Tyr, is incorporated into the NRPS.

**Tryptophan N-dimethylallyltransferase**—In cyclomarin biosynthesis, CymD catalyzes the N-dimethylallylation of free Trp (28, 29). In the ruf cluster, rufP encodes a CymD homolog (44% identity). We speculate that N-dimethylallyltryptophan is synthesized by RufP.

**Type I polyketide synthase (PKS) for the synthesis of trans-2-crotylglycine**—PKS genes (rufE and rufF) were found in the ruf cluster. RufE consists of three modules (loading module and modules 1 and 2), and RufF is a stand-alone thioesterase (TE) (Fig. 2A), and the RufEF PKS is most likely responsible for trans-2-crotylglycine biosynthesis. The loading module contains keto synthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains. Whereas KS domains generally catalyze decarboxylative Claisen condensation for C–C bond formation, a group of KSs called KSQ3, in which the Cys residue for substrate binding is substituted by Gln, catalyze only decarboxylation (33). The KS domain of the loading module is classified as KSQ2 (Fig. 2B), suggesting that it catalyzes only the decarboxylation of malonyl-CoA to provide the acetyl starter unit. Module 1 consists of KS, AT, dehydratase (DH), ketoreductase (KR), and ACP domains, and module 2 consists of KS, AT, DH, enoylreductase (ER), KR, and ACP domains. RufF is a TE domain. Via such a domain organization, the RufEF PKS is expected to synthesize 4-hexenoic acid (Fig. 2A). In general, the cis-trans stereochemistry of a double bond formed by a DH domain can be predicted by analyzing the KR domain of the same module. KR domains can be classified into A, B, and C types based on the presence or absence of the conserved XXD motif and Trp residue, and the stereochemistry of their products can be predicted according to this classification (34). The KR domain of module 1 belongs to the B type, suggesting that the trans C=C bond is synthesized by module 1 (Fig. 2C). Thus, we speculate that RufEF produces trans-4-hexenoic acid, which is a compatible precursor for trans-2-crotylglycine biosynthesis. Hydroxylation and oxidation of the C2 position of trans-4-hexenoic acid can form trans-2-oxo-4-hexenoic acid, and transamination of the keto group can form trans-2-crotylglycine (Fig. 2A). We speculate that these reactions are catalyzed by a cytochrome P450 enzyme (RufC; see below) and an aminotransferase (RufF).

**NOS and cytochrome P450 for the 3-nitration of Tyr**—In the thaxtomin biosynthetic gene cluster, txtD and txtE (encoding NOS and cytochrome P450, respectively) form a putative operon. We also found a putative operon consisting of NOS, P450, and tryptophan N-dimethylallyltransferase genes (rufN, rufO, and rufF, respectively) in the ruf cluster. RufO and RufN show 26 and 58% amino acid sequence identities with TxtE and TtxD, respectively. Although the sequence identity between RufO and TxtE was not high, we assumed that RufO and RufN should be responsible for the nitration of Tyr.

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**Figure 2. Putative pathway for trans-2-crotylglycine biosynthesis by the RufEF PKS.** A, trans-4-hexenoic acid is synthesized by the type I PKS consisting of RufE and RufF, and then it is hydroxylated and oxidized by the cytochrome P450 RufC to trans-2-oxo-4-hexenoic acid. Finally, trans-2-crotylglycine is generated by the transamination catalyzed by RufF. B, alignment of partial amino acid sequences of KS and KSQ domains from type I PKSs. Nid, KSQ2 for niddamycin (AF016585); Ty1, KSQ3 for tylosin (U78289); Ery, KSQ1 for erythromycin (X62569); Ruf, KSQ3 of the loading module for rufomycin (in this study). The key residue for distinguishing KS and KSQ2 is indicated by an arrow. C, alignment of partial amino acid sequences of KR domains from type I PKSs. Ery KR1B and KR2A, B-type and A-type KRs for erythromycin (L07626 and X62569), respectively; RufKR1 and RufKR2, KRs of module 1 and module 2, respectively, for rufomycin (in this study). The key residues for distinguishing cis- and trans-type KRs are indicated by arrows.
Cytochrome P450 monooxygenases—The cytochrome P450 enzymes except for RufO encoded in the ruf cluster (RufC, RufM, and RufS) may catalyze occasional oxidative modification reactions, such as the epoxidation of N-dimethylallyltryptophan and the hydroxylation and oxidation of Leu, to synthesize other rufomycin derivatives. Although this biosynthetic process is hypothetical, we predict the roles of three P450 monooxygenases. Using a BLAST search with the primary sequence of RufC as query, we discovered several cytochrome P450 monooxygenases (~50% identity with RufC) whose genes form an operon with PKS genes (supplemental Fig. S1A). Interestingly, these operons also encode an aminotransferase. In addition, the domain organization of the PKS discovered from Streptomyces aidingensis strain CGMCC 4.5739 is identical to that of RufE. Thus, RufC is most likely to be involved in trans-2-crotylglycine biosynthesis, and this trans-2-crotylglycine biosynthesis pathway seems to be distributed to other natural product biosynthetic pathways. Similarly, we predict the functions of RufM and RufS. Among the characterized enzymes, RufM and RufS show the highest identity with CYP107Z4 (46%) and TbtJ1 (45%), respectively. CYP107Z4 and TbtJ1 catalyze the hydroxylation of avermectin to produce 4'-oxo-avermectin (35) and the hydroxylation of Phe or Leu of the thiopseud peptide thiomuracin (36, 37), respectively. This result implies that RufS should catalyze the hydroxylation of Leu; therefore, RufM is considered to play the remaining role, which is epoxidation of the dimethylallyl group.

Gene disruption of rufO

To confirm the involvement of the ruf cluster in rufomycin biosynthesis and to examine the contribution of the candidate P450 gene (rufO) to 3NTyr synthesis, we disrupted rufO using the CRISPR/Cas9 system (38). A disruption plasmid was designed so that the rufO coding region could be cut by Cas9 and removed during the repair of the region, guided by the recombination template cassette introduced into the plasmid. After multiple trials, we isolated the rufO deletion strain (ΔrufO) (supplemental Fig. S2).

We cultivated the wild-type and ΔrufO strains and compared their metabolic profiles in three nutrient-rich media, tryptic soy broth (TSB), International Streptomyces Project 2 (ISP2), and modified G-2 (MG2). After extracting metabolites with n-butanol, the organic phase was analyzed by LC-MS.

In the wild-type strain cultivated in MG2, we clearly detected the production of four major compounds whose m/z values were identical to those of rufomycin derivatives reported previously (Fig. 3 and supplemental Table S2) (39). This indicates that these compounds are the rufomycin derivatives. Three of the four congeners (compounds 1, 2, and 3 in Fig. 3) were also produced in ISP2 medium, whereas the rufomycin derivatives were hardly produced in TSB (data now shown). In contrast, in the ΔrufO strain, we did not detect any rufomycin derivatives or shunt products in any of the media. However, when 3NTyr was added to the culture medium of the ΔrufO strain, rufomycin production was fully recovered (Fig. 3). From these results, we conclude that the ruf cluster is responsible for rufomycin biosynthesis. In addition, the recovery of rufomycin production in the ΔrufO mutant denied the possibility that the mutant lost heptapeptide production because of the unexpected inactivation of rufP caused by the possible polar effect of the rufO deletion; rufP appears to form an operon, rufNOP, with rufN and rufO. Inactivation of rufP must inhibit the peptide synthesis, because rufP presumably encodes a tryptophan N-dimethylallyltransferase essential for producing the first amino acid (N-di-dimethylallyttryptophan) of the rufomycin peptide. We think that the substrate specificity of the A domain in module 2 of the RufT NRPS should be strict and that only 3NTyr can be accepted by RuT as the third amino acid residue in the peptide assembly line. Thus, nitration seems to occur prior to peptide assembly.

UV-visible spectroscopic study of RufO

To investigate the function of RufO in vitro, the recombinant RufO protein with a His6 tag at its C terminus was produced in Escherichia coli and purified by nickel chelate affinity chromatography (Fig. 4). The color of the obtained solution ranged from yellow to red, which is typical of cytochrome P450 solutions. The purified RufO protein was subjected to carbon monoxide (CO)-binding and NO-binding spectral analyses (40). Bubbling of CO into the RufO solution resulted in the Soret band shifting from ~420 to ~450 nm (Fig. 5A). In the case of NO, a shift to ~440 nm was observed (Fig. 5B). These physicochemical properties are consistent with those of typical P450 enzymes. All of these results indicate that the recombinant RufO was purified in an active form.

Substrate-binding analysis of RufO

The substrate specificity of RufO was examined by investigating substrate-binding spectra (41). We used five
compounds: Tyr, Phe, Trp, 4-hydroxyphenylpyruvate (the proximate precursor of Tyr biosynthesis), and 4-aminophenylalanine. The addition of Tyr resulted in the typical type I binding spectra, a Soret band shift from ~420 to ~390 nm (41), suggesting that Tyr is recognized as a substrate (Fig. 5C). The dissociation constant ($K_d$) was estimated to be ~0.1 μM (Fig. 5D). In contrast, the other four compounds did not give apparent binding spectra, suggesting that they are not recognized as substrates or that their binding affinity is too low to be detected.

**Examination of the enzyme activity of recombinant RufO protein**

To investigate the ability of RufO to nitrate Tyr, we conducted an *in vitro* analysis using the purified RufO protein. Because bacterial cytochrome P450s require a ferredoxin and a ferredoxin reductase as the redox system, putidaredoxin (CamB) and putidaredoxin reductase (CamA), which are involved in camphor biosynthesis in *P. putida* (42), were also prepared as reported previously (Fig. 4). RufO was incubated with Tyr and diethylamine NONOate (DEANO), a chemical NO generator, in the presence of CamA, CamB, and NADH. After incubating the mixtures, the reactions were quenched with HCl and then subjected to an LC-MS analysis. As a result, the formation of 3NTyr was clearly detected, indicating that RufO catalyzes the nitration reaction of free Tyr (Fig. 6). However, regardless of repeated trial and error, we could not
increase the amount of 3NTyr produced in this in vitro reaction, which hampered the kinetic analysis of RufO (see “Discussion”). We also examined whether Phe, Trp, 4-hydroxyphenylpyruvate, and 4-aminophenylalanine could be used as a substrate of RufO in this in vitro reaction. Consistent with the results of the substrate-binding spectrum analysis described above, these four compounds were not recognized as a substrate of RufO, and no nitrated products were detected (data not shown).

Production of 3-(N-acetyl)aminotyrosine by a recombinant E. coli strain expressing rufO and camAB

To further confirm the Tyr nitration activity of RufO, we attempted to produce 3NTyr by a recombinant E. coli strain. We used a Tyr-overproducing strain, AN219 (43), as the host to express rufO and camAB. The recombinant strain was cultivated, expression of rufO and camAB was induced, and then DEANO was added to the culture. After a further 24-h incubation, the culture supernatant was analyzed with LC-MS. As a result, 3-(N-acetyl)aminotyrosine was detected, indicating that Tyr was converted to 3NTyr by RufO in this strain, because we confirmed that the nitro group of 3NTyr was efficiently reduced and N-acetylated to yield 3-(N-acetyl)aminotyrosine by the E. coli BL21(DE3) resting cells (supplemental Fig. S3). In E. coli, two major nitroreductases, NfsA and NfsB, were reported to have wide substrate specificity (44), and therefore they may reduce 3NTyr. E. coli also has an aroylamine N-acetyltransferase (45, 46), which seems to be responsible for the acetylation of 3-aminotyrosine. Taken together with the results of all other experiments in this study, we conclude that RufO catalyzes 3-nitration of free Tyr.

Discussion

In this study, we identified the rufomycin biosynthetic gene cluster (ruf cluster) in S. atratus. Rufomycin harbors a 3NTyr moiety in its structure, and we demonstrated that 3NTyr is synthesized from Tyr by a cytochrome P450 enzyme, RufO.

Based on the bioinformatic analysis of the ruf cluster and genetic and biochemical studies of RufO, we propose the biosynthetic pathway of rufomycin as follows (Fig. 7). In the first step, three non-proteinogenic amino acids are synthesized individually. (i) Trp is converted to N-dimethylallyltryptophan by RufP. (ii) RufE and RufF, which form a trimodular type I PKS, produce trans-4-hexenoic acid. A cytochrome P450 monooxygenase RufC introduces a keto group into the C2 position of trans-4-hexenoic acid to generate trans-2-oxo-4-hexenoic acid. Finally, a transamination reaction catalyzed by RufI results in trans-2-crotylglycine (trans-2-amino-4-hexenoic acid). (iii) RufO catalyzes the 3-nitration of Tyr, with NO produced by RufN, to form 3NTyr. In the second step, the heptamodular NRPS RufT assembles seven amino acids using N-dimethylallyltryptophan as the first amino acid to synthesize the heptacyclic peptide rufomycin B. During the peptide elongation, two N-methyl groups are introduced by the two MT domains located in modules 1 and 4. Of course, further experiments are required to confirm this proposed biosynthetic pathway.

Regarding the proposed biosynthetic pathway for trans-2-crotylglycine, a similar pathway has been predicted for the Tyrosine nitration in rufomycin biosynthesis

Figure 6. In vitro analysis of RufO. A, UV chromatogram of the RufO reaction. Compounds were separated by reverse-phase liquid chromatography. Top, authentic Tyr and 3NTyr; middle, in vitro reaction with RufO; bottom, in vitro reaction without RufO. The sample with enzymes was injected and analyzed prior to that without enzymes and authentic standards. B, the mass spectrum of the reaction product. 3NTyr (calculated m/z ([M + H]10) = 227.1) was clearly detected in the RufO reaction mixture. C, the tandem mass spectrum of the reaction product was identical with that of authentic 3NTyr.
biosynthesis of (4R)-4-[(E)-2-butenyl]-4-methyl-L-threonine, which is a building block of cyclosporin A (47–49). In this predicted pathway, the polyketide intermediate is hydroxylated by a P450 monooxygenase and further oxidized by a dehydrogenase. However, the ruf cluster contains no putative dehydrogenase gene. Therefore, we speculate that the P450 monooxygenase RufC should catalyze two sequential oxidations (hydroxylation and dehydrogenation) to generate the keto group, similar to how CYP170A1 catalyzes the two-step oxidation of epi-isozizaene to produce albaflavenone in Streptomyces coelicolor A3(2) (50).

During rufomycin biosynthesis, the timing of Tyr nitration is of great interest. In the biosynthetic pathway of natural products, many P450 enzymes that catalyze post-assembly reactions (called tailoring reactions) have been reported. In addition, 3NTyr is non-proteinogenic, and it may be toxic to cells. Therefore, nitration of the Tyr residue after peptide assembly was regarded as reasonable. There was also a possibility that the nitration of Tyr occurs during peptide assembly. For example, Tyr or some intermediate peptides attached to a T domain could be the substrate of the nitration enzyme. However, all of our experimental data indicate that 3NTyr is produced from free Tyr and incorporated into the peptide as a substrate of the RufT NRPS. First, the rufO mutant produced neither rufomycin nor its denitrated derivatives, and it restored rufomycin production following the exogenous addition of 3NTyr. Second, recombinant RufO catalyzed the nitration of Tyr in vitro. Third, 3-(N-acetyl)aminotyrosine, the reduced and N-acetylated derivative of 3NTyr, was produced by the recombinant E. coli cells expressing rufO and camAB. From these three results, we conclude that Tyr nitration by RufO occurs prior to peptide assembly. It should be noted that no rufomycin deriv-
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atives lacking the nitro group on Tyr have been found, although various patterns of modification have been observed within rufomycin (39). This also strongly supports our conclusions that 3NtYr is produced from free Tyr by RufO and that only 3NtYr is incorporated into the peptide assembly by the RufT NRPS because of high substrate specificity of the A domain in module 2.

Many cytochrome P450 enzymes have been studied extensively, but only TxtE and its close homologs were reported to catalyze the direct nitration of aromatic compounds (10–12). The catalytic mechanism of the nitration catalyzed byTxtE was proposed by Barry et al. (10) as follows. After substrate binding and heme reduction, TxtE combines NO with molecular oxygen to generate a ferric peroxynitrite intermediate, which then undergoes either homolytic cleavage to yield NO2 and an Fe(IV)=O species (compound II) or heterolytic cleavage initiated by protonation to yield NO3 and an Fe(III)-OH species. In the former case, the nitration of enzyme-bound Trp should occur via NO2 addition and hydrogen atom abstraction by compound II, resulting in the formation of an Fe(III)-OH species. In the latter case, it should occur via electrophilic aromatic substitution. In both cases, protonation of the Fe(III)-OH species is required for the regeneration of Fe(III)-OH2 to restore the resting state of the enzyme. Crystal structure analyses ofTxtE and an investigation of substrate analogs indicated the substrate recognition mechanisms of TxtE and the substrate properties required for catalysis (11, 12). In addition, a recent study discovered that a single mutation of His-176 in the F/G loop of TxtE completely shifts the enzyme’s regioselectivity from the C4 to the C5 position of Trp (51). The F/G loop is a flexible loop connecting the F and G helices, and it is involved in the open-to-closed transitions of the substrate-binding pocket; His-176 also plays a key role in gating these transitions. However, despite these studies, detailed molecular mechanisms of Trp nitration, including the formation of a ferric peroxynitrite intermediate, by TxtE remain to be elucidated. Moreover, the reason why TxtE does not show conventional monoxygenase activity toward Trp is of great interest. RufO is the first cytochrome P450 revealed to catalyze the nitration of Tyr; therefor, it will provide new clues to solve the aforementioned difficulty in the analysis of these nitrating cytochrome P450 enzymes and be utilized by respective NRPS systems. Studies of these RufO homologs, as well as further structural analyses and mutagenesis studies of RufO, will provide important insights into the mechanisms of the nitration of aromatic compounds, which will extend our knowledge of cytochrome P450 enzymes.

Experimental procedures

Strains, media, and culture conditions

S. atratus ATCC 14046 was obtained from the NITE Biological Resource Center (NBRC) (Chiba, Japan) and was cultivated in mannitol soya flour, TSB, ISP2, and MG2 media. G-2 is a medium optimized for rufomycin production (36). Mannitol soya flour medium consists of mannitol (2%) and soya flour (2%). TSB medium consists of tryptic soy broth (3%). ISP2 medium consists of yeast extract (0.4%), malt extract (1%), and glucose (0.4%) (pH 7.2). MG2 medium consists of potato dext- trin (5%), glucose (3%), glycerol (1%), molasses (1%), soluble starch (0.5%), soybean meal (0.5%), corn steep liquor (0.5%), MgSO4·7H2O (0.05%), CaCO3 (0.3%), Fe(NH4)2(SO4)2·6H2O (0.01%), ZnCl2 (0.01%), and MnSO4 (0.01%). For solid culture, agar powder (2%) was added to solidify the medium. E. coli strains JM109 and ET12567/pUZ8002, used for plasmid construction and conjugation, respectively, were cultivated at 37 °C in Luria-Bertani (LB) medium with or without ampicillin (100 mg/liter) and apramycin (50 mg/liter). E. coli strains BL21(DE3) and BLR(DE3), used for heterologous gene expression, were cultivated at various temperatures in LB medium or terrific broth (TB). Trace element solution consists of 50 mM FeCl3·6H2O, 20 mM CaCl2·2H2O, 10 mM MnCl2·4H2O, 10 mM ZnSO4·7H2O, 2 mM CoCl2·6H2O, 2 mM CuCl2·2H2O, 2 mM NiSO4·6H2O, 2 mM NaMoO4·2H2O, 2 mM Na2SeO3, and 2 mM H2BO3. Specific modifications of media are described below. Unless mentioned otherwise, all chemicals were purchased from Tokyo Chemical Industry (Tokyo, Japan), Wako Pure Chemicals (Osaka, Japan), or Nacalai Tesque (Kyoto, Japan).

Enzymes and plasmids

cPRISPoymes-2 (38) was purchased from Addgene (Cambridge, MA). BbsI was purchased from New England Biolabs (Ipswich, MA). Other restriction enzymes and In-Fusion enzymes were purchased from Takara Bio (Shiga, Japan).

Construction of plasmids for rufO disruption

A sequence containing a protospacer and a protospacer adjacent motif (PAM) was chosen in the reverse complementary sequence of rufO. Primers containing this sequence, PAM-F/R (supplemental Table S3), were annealed with each other, and the product was introduced into the BbsI site of pCRISPoymes-2, resulting in pCRISPoymes-2/ΔrufO-PAM. The ~1-kb fragments of the 5′- and 3′-flanking regions of the target sequence to be disrupted were amplified with the primer pairs DruFO5′-F/R and DruFO3′-F/R (supplemental Table S3), respectively. After digestion of pCRISPoymes-2/ΔrufO-PAM with XbaI, both of the amplified fragments were simultaneously introduced into the plasmid using In-Fusion reaction to yield the gene disruption plasmid pCRISPoymes-2/ΔrufO.
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Disruption of rufO

Transformation of *S. atratus* was carried out by a conjugation method with *E. coli* ET12567/pUZ8002 (53). The *E. coli* cells harboring pCRISPlomysces-2/ΔrufO were cultivated in LB medium until the OD₆₀₀ reached ~0.5. The cells were harvested by centrifugation (5,000 × g, 15 min, room temperature), washed twice with fresh LB medium, and resuspended in fresh LB medium. Spores of *S. atratus* were harvested from the culture on ISP2 solid medium incubated for 7 days at 30 °C. The spores were suspended in TSB medium (0.5 ml) and incubated at 50 °C for 10 min. The spores and *E. coli* cells were mixed and inoculated on mannitol soya flour solid medium containing MgCl₂ (10 mM). After incubation for 16 h at 30 °C, the culture was overlaid with sterilized water (1 ml) containing nalidixic acid (0.75 mg) and apramycin (0.75 mg). After a further cultivation for several days, a transformant colony was picked up and inoculated onto ISP2 solid medium. Then the transformant was repeatedly cultivated on ISP2 solid medium without apramycin until the cells lost apramycin resistance. Gene disruption was confirmed by PCR using the primer pair DrfuO-F/R (supplemental Table S3 and supplemental Fig. S2).

Comparison of metabolic profiles and rufomycin production

*S. atratus* cells were cultivated in TSB, ISP2, and MG2 media at 28 °C for 7 days. n-Butanol saturated with water was added to the medium at a ratio of 1:1 (v/v). The organic layer was concentrated in *vacuo*, and the residual materials were dissolved in methanol and subjected to an LC-electron spray ionization MS (LC-ESI-MS) analysis in an 1100 series spectrometer (Agilent Technologies) coupled to high-capacity Trap Plus system (Bruker Daltonics) equipped with a COSMOCORE 2.6 C₁₈ column (2.1-mm inner diameter × 150 mm; Nacalai Tesque). The compounds were separated with a linear gradient of water and acetonitrile containing formic acid (0.1%) at a flow rate of 0.4 ml min⁻¹.

Production and purification of the recombinant RufO, CamA, and CamB proteins

The coding region of the *rufO* gene was first amplified from the genomic DNA of *S. atratus* by PCR using the primer pair rufO-F/R (supplemental Table S3). During the PCR, an eight-histidine tag-coding sequence was incorporated into the 3' end of *rufO*. pET16b was applied to inverse PCR using the primer pair 16b-inv-F/R (supplemental Table S3), and the original N-terminal 10-histidine tag coding sequence was removed during the PCR. The resultant two fragments were applied to the In-Fusion reaction, and the plasmid obtained (pET16b-rufO) was introduced into *E. coli* BLR(DE3) after confirming the absence of unintended mutations. The transformant was cultivated at 37 °C in TB with 5-aminolevulinic acid (80 mg/liter), Fe(NH₄)₂(SO₄)₂ (40 mg/liter), trace element solution (200 μl/liter), and ampicillin (100 mg/liter) until the OD₆₀₀ reached ~0.5. After cooling the medium to room temperature, isopropl 1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.1 mM to induce gene expression, and cells were cultivated for a further 20 h at 15 °C. Then cells were harvested, resuspended in lysis buffer (20 mM Tris-HCl (pH 8.0) containing 200 mM NaCl and 10% (v/v) glycerol), and disrupted by sonication. After centrifugation (20,000 × g, 20 min, 4 °C), His60 Ni Superflow resin was added to the soluble cell extracts and mixed gently at 4 °C for 30 min. After the His-tagged proteins were eluted with lysis buffer containing 0.5 m imidazole, the buffer was exchanged for 25 mM Tris-HCl (pH 8.0) containing 0.1 mM DTT and 0.1 mM EDTA using a PD-10 column (GE Healthcare).

The pET28b-camA and pET28b-camB plasmids (54) were individually introduced into *E. coli* BL21(DE3). The transformants were cultivated at 37 °C in LB medium with kanamycin (50 mg/liter) until the OD₆₀₀ reached ~0.5. FeCl₃ was added to the culture medium for the camA expression at a final concentration of 0.1 mM. After cooling the medium to room temperature, IPTG was added to a final concentration of 0.1 mM to induce expression, and cells were cultivated for a further 12 h at 28 °C. Cells were harvested, resuspended in lysis buffer, and disrupted by sonication. After centrifugation (20,000 × g, 20 min, 4 °C), His60 Ni Superflow Resin was added to the soluble cell extracts and mixed gently at 4 °C for 30 min. After the His-tagged proteins were eluted with lysis buffer containing 0.5 mM imidazole, the buffer was exchanged for 25 mM Tris-HCl (pH 8.0) containing 0.1 mM DTT and 0.1 mM EDTA using a PD-10 column.

The protein concentrations were determined using Nano-Drop (Thermo Fisher Scientific) with a millimolar coefficient of 29.1 for RufO, 38.8 for CamA, and 10.3 for CamB.

UV-visible spectroscopic analysis

The RufO Fe(II)–CO complex was investigated as follows. The protein solution (5 μM RufO in 25 mM Tris-HCl (pH 8.0) containing 0.1 mM DTT and 0.1 mM EDTA) was prepared, and sodium hydrosulfite (Sigma-Aldrich) was added. The solution was divided into two cuvettes, A and B, and carbon monoxide (CO) gas was bubbled into cuvette A for 2–3 min. UV-visible spectra of both the cuvettes were measured, and the CO difference spectrum was obtained by the subtraction of the spectrum of cuvette B from that of cuvette A.

The RufO Fe(III)–NO complex was investigated as follows. The protein solution (5 μM RufO in 25 mM Tris-HCl (pH 8.0) containing 0.1 mM DTT and 0.1 mM EDTA) was prepared. DEANO (Sigma-Aldrich) solution was prepared by dissolving DEANO into the same buffer. The protein solution was divided into two cuvettes, A and B. An equivalent volume of the DEANO solution was added to cuvette A, whereas the buffer was added to cuvette B. UV-visible spectra of both cuvettes were measured, and the NO difference spectrum was obtained by the subtraction of the spectrum of cuvette B from that of cuvette A.

Substrate-binding analysis

The protein solution (3.4–5 μM RufO in 25 mM Tris-HCl (pH 8.0) containing 0.1 mM DTT and 0.1 mM EDTA) was prepared and divided into two cuvettes, A and B. The potential substrate was added to cuvette A (0.1–5 μM), and the same volume of the buffer was added to cuvette B. The solutions were mixed well. After 2 min, UV-visible spectra of both cuvettes were measured. The spectrum of cuvette B was subtracted from that of cuvette A, resulting in the difference spectrum. For determination of the dissociation constant (Kᵣ) with Tyr, the difference in
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absorbance of each spectrum at 386 and 422 nm was calculated in triplicate, and the average was plotted against the substrate concentration. The \( K_v \) value was calculated by fitting the data with a hyperbolic curve.

In vitro analysis using the recombinant proteins

In vitro enzymatic reaction mixtures contained 3.4 \( \mu \text{M} \) recombinant RufO, 1.6 \( \mu \text{M} \) recombinant CamA, 3.2 \( \mu \text{M} \) recombinant CamB, 1 mm Tyr, 1 mm NADH, and 1 mm DEANO in 25 mm Tris-\( \text{HCl} \) (pH 8.0) containing 0.1 mm DTT and 0.1 mm EDTA. When the substrate specificity of RufO was examined, Tyr was replaced with Phe, Trp, 4-hydroxyphenylpyruvate, or 4-aminophenylalanine. The reactions were carried out at 28 °C for 3 h with reciprocal shaking at 1,500 rpm. After the addition of HCl (0.15 M) to the mixtures to solubilize Tyr and 3NTyr and denature proteins, the mixtures were centrifuged (20,000 \( \times \) g, 5 min, room temperature) and applied to LC-ESI-MS analysis. The compounds were separated using COSMOSIL 5PYE column (2.0-mm inner diameter \( \times \) 150 mm) (Nacalai Tesque) with a linear gradient of water and acetonitrile containing formic acid (0.1%) at a flow rate of 0.4 ml min\(^{-1}\).

Generation of 3NTyr by a recombinant \textit{E. coli} strain expressing \textit{rufO} and \textit{camAB}

The \textit{camA} and \textit{camB} genes were introduced into pCDF-Duet-1. The coding region of \textit{camA} was amplified from pET28b-\textit{camA} by PCR using the primer pair camA-F/R (supplemental Table S3). pCDF-Duet-1 was applied to inverse PCR using the primer pair pCDFinv-F/R (supplemental Table S3). The resultant two amplified fragments were applied to the In-Fusion reaction, resulting in pCDFDuet-1-\textit{camA}. The coding region of \textit{camB} was amplified from pET28b-\textit{camB} by PCR using the primer pair camB-Nde-F/R (supplemental Table S3). This fragment and Ndel-digested pCDFDuet-1-\textit{camA} were applied to the In-Fusion reaction, resulting in pCDFDuet-1-\textit{camAB}. After confirming the absence of unintended mutations, pCDFDuet-1-\textit{camAB} and pET16b-\textit{rufO} were introduced together into the \textit{Tyr}-overproducing \textit{E. coli} strain AN219. The transformant was cultivated at 37 °C in TB with 5-aminolevulinic acid (80 mg/liter), Fe(NH\(_4\))\(_2\)(SO\(_4\))\(_2\) (40 mg/liter), trace vitamins, \( \frac{1}{10} \) yeast extract, ampicillin (100 mg/liter), kana- mycin (50 mg/liter), and streptomycin (50 mg/liter) until the OD\(_{600}\) reached \( \approx 0.5 \). Then IPTG was added to a final concentration of 0.1 mm to induce gene expression, and cells were cultivated for a further 24 h at 15 °C. DEANO was then added to a final concentration of 0.1 mm, and cells were further cultivated for an additional 24 h at 15 °C. After centrifugation (20,000 \( \times \) g, 5 min, room temperature) to remove the cells, HCl (0.15 M) was added to the culture supernatant, followed by the second centrifugation (20,000 \( \times \) g, 5 min, room temperature) to remove the precipitation. The supernatant was analyzed with LC-ESI-MS as described above.

Author contributions—H. T. designed the study, performed experiments, analyzed data, and wrote the manuscript. Y. K. designed the study, analyzed data, and wrote the manuscript. H. M. contributed to the experiment shown in supplemental Fig. S3. Y. O. directed the research, analyzed data, and wrote the manuscript.

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