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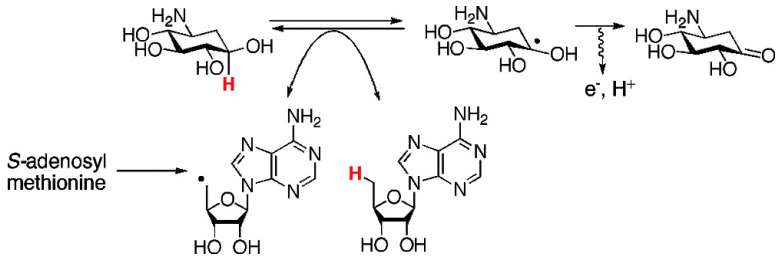

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Characterization and Mechanistic Study of a Radical SAM Dehydrogenase in the Biosynthesis of Butirosin

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Abstract: BtrN encoded in the butirosin biosynthetic gene cluster possesses a CXXXCXXC motif conserved within the radical *S*-adenosyl methionine (SAM) superfamily. Its gene disruption in the butirosin producer *Bacillus circulans* caused the interruption of the biosynthetic pathway between 2-deoxy-*scyllo*-inosamine (DOIA) and 2-deoxystreptamine (DOS). Further, in vitro assay of the overexpressed enzyme revealed that BtrN catalyzed the oxidation of DOIA under the strictly anaerobic conditions along with consumption of an equimolar amount of SAM to produce 5′-deoxyadenosine, methionine, and 3-amino-2,3-dideoxy-*scyllo*-inosose (amino-DOI). Kinetic analysis showed substrate inhibition by DOIA but not by SAM, which suggests that the reaction is the Ordered Bi Ter mechanism and that SAM is the first substrate and DOIA is the second. The BtrN reaction with [3-^{2}H]DOIA generated nonlabeled, monodeuterated and dideuterated 5′-deoxyadenosines, while no deuterium was incorporated by incubation of nonlabeled DOIA in the deuterium oxide buffer. These results indicated that the hydrogen atom at C-3 of DOIA was directly transferred to 5′-deoxyadenosine to give the radical intermediate of DOIA. Generation of nonlabeled and dideuterated 5′-deoxyadenosines proved the reversibility of the hydrogen abstraction step. The present study suggests that BtrN is an unusual radical SAM dehydrogenase catalyzing the oxidation of the hydroxyl group by a radical mechanism. This is the first report of the mechanistic study on the oxidation of a hydroxyl group by a radical SAM enzyme.

Introduction

2-Deoxystreptamine (DOS) containing aminoglycosides constitute the largest subgroup of aminoglycoside antibiotics. This group of aminoglycosides includes kanamycin, neomycin, and gentamicin, which are clinically important antibiotic agents used against bacterial and some protozoal infections.[1] In addition to these classical roles they are also attracting attention for their RNA and DNA recognition ability, which can be applied for anti-plasmids[2,3] and anti-HIV agents.[4,5] Increased attention to the biological activity of aminoglycosides makes their biosynthetic machinery more attractive for creating structurally diverse substances by engineered biosynthesis.

The biosynthetic gene cluster of butirosin was the first of the DOS-containing aminoglycosides to be identified,[6,7] which led to the identification of several other DOS-containing aminoglycoside gene clusters.[8−12] Comparative genetics of the open reading frames (ORFs) in those gene clusters, combined with gene disruption experiments and in vitro assays of overexpressed enzymes, led to reasonable assignments of each ORF in the butirosin biosynthetic gene cluster to each biosynthetic reaction.[8,13] However, the functions of several ORFs still remain uncharacterized.

BtrN, a functionally unknown enzyme encoded in the butirosin biosynthetic gene cluster, has an amino acid sequence motif (CXXXCXXC), which is typical of the radical *S*-adenosyl methionine (SAM) superfamily.[14] The radical SAM superfamily is an emerging group of enzymes that catalyze a wide range of

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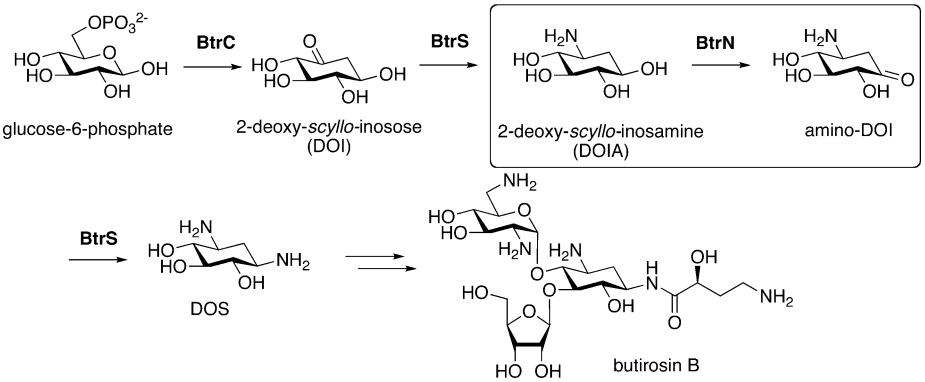
Scheme 1. Biosynthetic Pathway of DOS in the Butirosin Producer *Bacillus circulans*



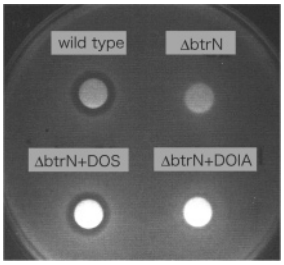


Figure 1. Feeding experiments against the *btrN* deletion mutant.

radical reactions.[15] The common mechanism found in all radical SAM enzymes is a reductive cleavage of SAM to generate the 5′-deoxyadenosyl radical, which is subsequently used for radical reactions such as activation of other enzymes through generating protein radical, sulfur insertion, C−C bond rearrangement, and amino group rearrangement.[16] Bioinformatics analysis has identified more than 600 genes as members of the radical SAM superfamily with a common CXXXCXXC sequence motif.[14] They are widely distributed in various vital metabolic pathways including biosynthesis of heme, thiamine, molybdopterin, and many famous secondary metabolites such as mitomycin C, fosfomycin, and erythromycin.[14] However, their high sensitivity to oxygen precluded the study of radical SAM enzymes, and relatively few of them have been enzymatically characterized.[16,17] Radical SAM enzymes in secondary metabolism have especially been underinvestigated. DesII, an enzyme catalyzing deoxygenation of the C-4 position of an aminosugar during the biosynthesis of the desosamine part of erythromycin, is the only one defined at the enzymatic level.[18] Since secondary metabolites are structurally diverse, radical SAM enzymes involved in secondary metabolism appear to catalyze various unique and chemically important radical reactions. Uncovering these reaction mechanisms will expand our knowledge of radical reactions in biological systems.

It is thus interesting how BtrN participates in the butirosin biosynthesis as a radical SAM protein. In this report, using a combination of gene disruption and in vitro enzyme reaction studies, BtrN was found to be a radical SAM dehydrogenase catalyzing oxidation of a hydroxyl group of 2-deoxy-*scyllo*-inosamine (DOIA, Scheme 1).

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Materials and Methods

Construction of the His-Tagged BtrN Expression Vector pETbtrN. The *btrN* gene was amplified by PCR with primers, btrN-His (AATTT*CCATGG*ATAAGCTGTTTTCG) and btrN-C (CAAA*GAATTC*AAACGTACACCCCC). PCR reaction was performed under conditions as follows: 94 °C, 2 min for denature, 30 cycles of 94 °C, 15 s; 56 °C, 30 s; 68 °C, 1 min for extension of DNA using KOD-Plus-DNA polymerase (TOYOBO, Japan). PCR product was digested by *Eco*RI and *Nco*I and subcloned into the *Eco*RI-*Nco*I site of LITMUS 28 to obtain LITMUSbtrN-His. After confirmation of the sequence, the *Eco*RI-*Nco*I fragment of LITMUSbtrN-His was inserted into pET30b (Novagen) vector to obtain pETbtrN-His, which was subsequently introduced into *E. coli* BL21(DE3).

Overexpression and Purification of N-Terminally His-Tagged *btr*N. *E. coli* BL21(DE3) carrying pETbtrN-His was grown in 800 mL of LB medium containing 30 μg/mL of kanamycin at 37 °C until OD_{600} 0.6−1.0. The expression was induced by addition of 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG), and cultivation was continued at 15 °C for 18 h. The cells were harvested by centrifugation at 7000 rpm for 10 min, washed with 50 mM HEPES−NaOH (pH 8.0), and stored at −30 °C until use. The wet cells (4 g) were suspended in buffer A (50 mM HEPES−NaOH, pH 8.0, 300 mM NaCl, 50 mM imidazole) and anaerobically disrupted by sonication at 0 °C for 1 min (5 times). All further steps were carried out under anaerobic conditions. All solutions were saturated with Ar prior to use. Cell debris was removed by centrifugation at 15 000 rpm, for 30 min, at 4 °C. The supernatant was loaded onto His-bind resin (Novagen) column, which had been previously equilibrated with buffer A. The column was washed with 15 mL of buffer A to remove unbound proteins. BtrN was eluted using buffer B (50 mM HEPES−NaOH pH 8.0, 300 mM NaCl, 200 mM imidazole) at a flow rate of 1.3 mL/min. The yellowish protein was collected and desalted with a 5 mL HiTrap desalting column (Amersham Pharmacia Biotech AB) equilibrated with buffer C (50 mM HEPES−NaOH, pH 8.0).

Reconstitution of BtrN [4Fe-4S] Cluster. The purified BtrN was treated with 5 mM dithiothreitol (DTT) at room temperature for 15 min. $Fe^{II}(NH_4)_2(SO_4)_2$ (1 mM) and Na_2S (1 mM) were then added, and the mixture was incubated at room temperature for 1 h. The BtrN sample was then desalted by passage through a 5 mL HiTrap desalting column (Amersham Pharmacia Biotech AB) equilibrated with buffer C. The obtained reconstituted BtrN was reduced by sodium dithionite for 1 h at room temperature prior to use. UV−visible absorption spectra were determined by a Shimadzu UV-2450 spectrophotometer. For EPR measurements, the reduced BtrN was transferred to an EPR tube and flash frozen in liquid nitrogen. EPR spectra were recorded with a JEOL JES-FA300 ESR spectrometer (9.02 GHz). Recording conditions are indicated in the legend of Figure 2. The concentration of unpaired spin was determined using Cu(II)−EDTA as a standard. Protein concentrations were determined with the Bradford assay (Bio-Rad) with bovine

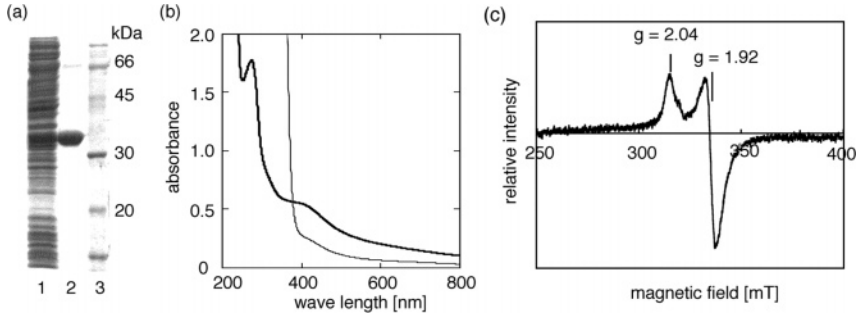

Figure 2. (a) SDS-PAGE of the purified BtrN. Lane 1: cell-free extract of *E. coli* overexpressing His-tagged BtrN. Lane 2: purified BtrN. Lane 3, marker. (b) UV−vis spectra of the reconstituted BtrN (bold line) and the reconstituted BtrN reduced with sodium dithionite (thin line). (c) EPR spectrum of the reconstituted BtrN reduced with sodium dithionite. Recording conditions: temperature of 10 K, microwave power of 1 mW, and modulation of 0.6 mT.

plasma gamma globulin as a standard. Enzymes were freshly prepared for each set of experiments.

Assay of DOIA Oxidation by BtrN. The reduced BtrN (20 μM) was incubated with 2 mM DOIA, 2 mM SAM in 50 mM HEPES−NaOH (pH 8.0) in the presence of 10 mM sodium dithionite at 28 °C for 12 h. The reaction solution was analyzed for 3-amino-2,3-dideoxy-*scyllo*-inosose (amino-DOI) formation by HPLC after treatment with acetic anhydride and *O*-(*p*-nitrobenzyl)-hydroxylamine as previously described.[19]

For 5′-deoxyadenosine detection, the enzyme reaction mixture was quenched with 10% trichloroacetic acid and then centrifuged at 10 000 rpm for 1 min to remove the precipitated enzyme. An aliquot (5 μL) of the solution was injected into a HPLC (Hitachi) equipped with a PEGASIL ODS column (4.6 × 250 mm, Senshu). Elution was performed with 20% aqueous methanol at a flow rate 0.9 mL/min at room temperature and monitored at 254 nm.

Methionine was detected as a dinitrophenyl derivative. The enzyme reaction mixture (20 μL) was derivatized with 20 μL of 5% 2,4-dinitrofluorobenzene in methanol and 0.1 g/mL $NaHCO_3$ at room temperature for 3 h. After addition of 2 M NaOH aq. (10 μL) and H_2O (300 μL), the solution was washed with ethyl acetate (500 μL). The aqueous layer (300 μL) was acidified with 2 M HCl (50 μL) and extracted with ethyl acetate (800 μL). The organic layer (700 μL) was then evaporated, and the residue was dissolved in 50 μL of methanol. An aliquot (5 μL) of the solution was injected into the same HPLC system as described above. Elution was performed with 20% aqueous methanol at a flow rate 0.9 mL/min at room temperature and monitored at 350 nm.

Time Course of DOS and 5′-Deoxyadenosine Production. The reconstituted BtrN was reduced with 10 mM sodium dithionite at room temperature for 1 h prior to use. The reduced BtrN (21 μM) was incubated with 1 mM DOIA, 0.9 mM SAM, 10 mM DTH, 16 μM BtrS, 10 mM glutamine, and 0.5 mM PLP in 50 mM HEPES−NaOH (pH 8.0) at room temperature. The reaction was started by addition of DOIA and quenched with the same volume of ethanol at specific periods of time (5, 30, 60, 120, 180, 240, 360, 480, 600 min). The produced SAM, DOS, and 5′-deoxyadenosine were quantified by HPLC. The reactions were run in duplicate. 5′-Deoxyadenosine was detected as mentioned above. The standard curves were generated with the authentic 5′-deoxyadenosine so as to correlate peak area with the amount of 5′-deoxyadenosine.

DOS was detected by the previously reported procedure[19] with a slight modification. The enzyme reaction was quenched by 50% EtOH, and an aliquot (20 μL) of the mixture was treated with 20 μL of 5% 2,4-dinitrofluorobenzene in methanol, 10 μL of DMSO, and 2 μL of 2 M NaOH at 60 °C for 1 h. The reaction mixture was extracted with 500 μL of ethyl acetate. The ethyl acetate layer (400 μL) was successively diluted with 3 mL of hexane, and the solution was passed through the Sep-Pak Plus Silica (Waters). The cartridge was washed with 7 mL of 50% ethyl acetate in hexane, and the product, bisdinitrophenyl DOS, was eluted with 5 mL of 20% methanol in ethyl acetate. After removal of the solvent by a centrifugal evaporator, the residue was dissolved in 100 μL of methanol. An aliquot (4 μL) of the solution was injected into the same HPLC system as described above and eluted with 70% aqueous methanol at a flow rate 0.9 mL/min at room temperature. Elution was monitored at 350 nm. The standard curves were generated with the authentic DOS treated with dinitrofluorobenzene in an identical manner to that for other samples so as to correlate peak area with DOS amount.

For the SAM detection, the enzyme reaction was quenched with 50% ethanol and centrifuged at 10 000 rpm for 1 min to remove the precipitated enzyme. SAM concentration was determined by HPLC with the previously reported procedure.[20] The standard curves were generated with the authentic SAM (∼90% purity, MP Biochemicals) so as to correlate peak area with the amount of SAM.

Kinetic Analysis of the BtrN Reaction. Kinetic measurements were carried out in 50 mM HEPES−NaOH (pH 8.0) in the presence of 10 mM sodium dithionite in a final volume of 200 μL at 28 °C for 30 min. The initial velocities were determined by quantifying the generated 5′-deoxyadenosine by HPLC as mentioned above. The reduced BtrN was prepared as described above except that the sodium dithionite reduction was prolonged for 3 h.

For determination of the mechanism of the substrate inhibition by DOIA, 2.9 μM of the reduced BtrN was incubated with varied concentrations of SAM from 0.1 to 0.5 mM at different concentrations of DOIA (0.4, 0.8, 1.5, 3 mM). Reactions were run in duplicate. For determination of the kinetic parameters with respect to DOIA, 0.18−0.25 μM of the reduced BtrN was incubated with 3 mM SAM and varied concentrations of DOIA from 0.01 to 0.2 mM. Reactions were run in triplicate, and the data were fitted to the generic equation for uncompetitive substrate inhibition (v/[BtrN] = k_{cat}[DOIA]/(K_{MDOIA} + [DOIA] + [DOIA]^2/K_s)) by the least-squares method. For determination of the kinetic parameters with respect to SAM, 0.22−0.54 μM of the reduced BtrN was incubated with 0.1 mM DOIA and varied concentration of SAM from 0.08 to 5.0 mM. Reactions were run in duplicate, and the data were fitted to the Michaelis−Menten equation (v/[BtrN] = k_{cat}*[SAM]/(K_M + [SAM]) by the least-squares method.

Results

A gene-disruption mutant of *btrN* was constructed by standard homologous recombination. The resultant mutant completely lost its ability to produce antibiotic, which suggested that *btrN* is required for biosynthesis of butirosin. Feeding experiments of biosynthetic intermediates were further performed to identify the interrupted biosynthetic reaction. The mutant recovered its

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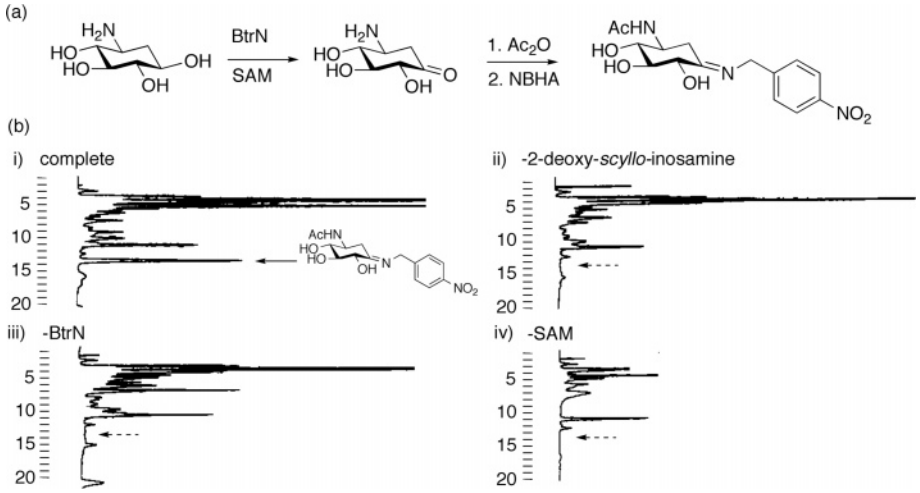

Figure 3. (a) Amino-DOI derivatization with Ac_2O and NBHA. (b) HPLC charts of in vitro activity assay of BtrN detected by the absorption at 254 nm: (i) 0.1 mM BtrN, 2 mM SAM, 2 mM DOIA, 10 mM sodium dithionite, 50 mM HEPES-NaOH pH 8.0, (ii) without DOIA, (iii) without BtrN, and (iv) without SAM.

butirosin productivity with DOS supplementation but not with DOIA (Figure 1). Since the transamination of amino-DOI into DOS is catalyzed by BtrS,[21,22] these results indicate that BtrN is involved in the oxidation of DOIA to give amino-DOI in the butirosin producer *B. circulans*.

For enzymatic analysis, BtrN was overexpressed as an N-terminal His-tagged protein and anaerobically purified by Ni nitriloacetic acid (NTA) affinity chromatography, since other radical SAM enzymes were reported to be sensitive to oxygen. The purified BtrN was anaerobically incubated with DOIA, SAM, and some other presumed cofactors such as flavin cofactors, nicotinamide cofactors, and $Fe^{II}(NH_4)_2(SO_4)_2$. Sodium dithionite was used to maintain strict anaerobic conditions. When $Fe^{II}(NH_4)_2(SO_4)_2$ was added to the enzyme reaction solution, a small amount of amino-DOI formation was observed. This result suggested that the purified BtrN had an incomplete iron−sulfur cluster and reconstitution of the 4Fe-4S cluster seemed to be necessary for activity. Thus, reconstitution of the 4Fe-4S cluster was examined. The anaerobically purified BtrN was thus treated with $Fe^{II}(NH_4)_2(SO_4)_2$ and Na_2S in the presence of dithiothreitol. After removal of the unbound ferrous ion and sulfide ion by gel filtration, the reconstituted BtrN was obtained as a dark brown solution. The reconstituted BtrN showed absorption maxima at 420 nm, which is characteristic for the $[4Fe\text{-}4S]^{2+}$ cluster containing radical SAM proteins (Figure 2b). The reconstituted BtrN was then reduced with 20 mM sodium dithionite at room temperature for 1 h. The absorption at 420 nm was bleached (Figure 2b) as reported for other radical SAM enzymes.[23,24] The EPR spectrum of the reduced BtrN showed *g* values of 1.92 and 2.04, which are characteristic for the $[4Fe\text{-}4S]^{+}$ cluster of radical SAM enzymes (Figure 2c). These results suggested the generation of $[4Fe\text{-}4S]^{+}$ cluster in the BtrN active site. The spin quantitative analysis revealed that the reduced BtrN contained 0.67 spins per polypeptide chain. Thus, about 70% of BtrN was successfully reconstituted to obtain the $[4Fe\text{-}4S]^{+}$ cluster under these conditions.

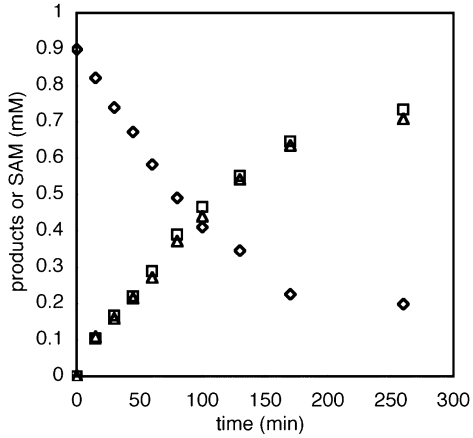

Figure 4. Time dependence of DOS (Δ) and 5′-deoxyadenosine (□) formations and SAM (◇) consumption. BtrN (0.030 mM) was incubated with 1 mM DOIA and 0.9 mM SAM in the presence of 10 mM sodium dithionite and BtrS system as described in Materials and Methods.

The reduced BtrN was, then, incubated with DOIA and SAM. HPLC analysis of the oxime derivative of the reaction product revealed that amino-DOI was efficiently formed as shown in Figure 3. Production of amino-DOI was not detected when BtrN, DOIA, or SAM was omitted. The enzyme reaction product was further confirmed by isolation of amino-DOI as nitrobenzyloxime derivative, which was identical in all respects to an authentic standard.[21] These results clearly proved that BtrN itself catalyzes the oxidation of DOIA into amino-DOI in the presence of SAM. Further analysis of the other reaction products showed that 5′-deoxyadenosine and methionine were formed by incubation of SAM with BtrN and DOIA (see Supporting Information).

Time-course experiments of the amino-DOI and 5′-deoxyadenosine formation are shown in Figure 4. The amount of amino-DOI was estimated after conversion to DOS by aminotransferase BtrS since quantitative analysis of amino-DOI was not practical. Thus, DOS was detected as its 2,4-dinitrophenyl derivative. The BtrN reaction was found to be extremely slow ($0.2\ min^{-1}$) under these conditions. The first-order rate constant of the BtrS reaction with DOI and L-glutamine ($1.6 \times 10\ min^{-1}$, unpublished result) was sufficiently faster than that for the BtrN

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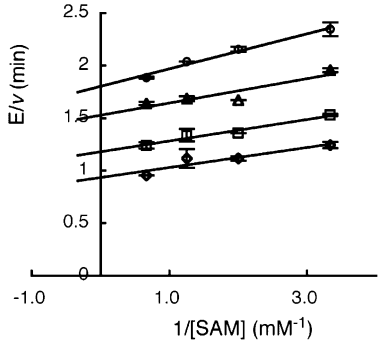

Figure 5. Substrate inhibition by DOIA. DOIA concentration was fixed at 0.4 (◇), 0.8 (□), 1.5 (Δ), and 3 mM (o), while SAM concentration was varied between 0.1 and 0.5 mM. Each data point represents the average of two replicates.

reaction, which allowed the coupling assay of BtrN with BtrS to be used for quantitative analysis of the amino-DOI formation. Equimolar amounts of 5′-deoxyadenosine and DOS were formed along with consumption of the corresponding amount of SAM during the BtrN-catalyzed reaction as shown in Figure 4. Thus, these results clearly prove that one molecule of SAM is reductively cleaved into methionine and 5′-deoxyadenosine along with oxidation of one molecule of DOIA.

Kinetic analysis was then performed by detection of 5′-deoxyadenosine as a product. In the kinetic analysis with varying concentrations of DOIA, DOIA displayed substrate inhibition. The inhibition mechanism was investigated by an initial velocity study with varying concentrations of SAM at different fixed concentrations of DOIA (Figure 5). The Lineweaver−Burk plots in the conditions where DOIA acts as an inhibitor gave parallel lines, thereby suggesting the uncompetitive substrate inhibition of DOIA. Then the initial velocity data obtained by reactions of varying concentrations of DOIA (10−200 μM) with saturating SAM (3 mM) were fitted to the equation for uncompetitive substrate inhibition (eq 1), where k_{cat} is the first-order rate constant, $K_{M\ DOIA}$ is a Michaelis constant for DOIA, and K_s is the substrate inhibition constant

$$v/[\mathrm{BtrN}] = k_{cat}[\mathrm{DOIA}]/(K_{M\ DOIA} + [\mathrm{DOIA}] + [\mathrm{DOIA}]^2/K_s) \quad (1)$$

The obtained data fit well to eq 1 (Figure 6a) and gave kinetic values of $k_{cat} = 2.3 \pm 0.22$ min^{-1}, $K_{M\ DOIA} = 0.022 \pm 0.004$ mM, and $K_s = 0.16 \pm 0.01$ mM. On the other hand, when SAM concentrations were varied from 0.08 to 5.0 mM with fixed concentration of DOIA (0.1 mM), the data were best fitted to a simple hyperbolic curve, which indicated that SAM does not behave as an inhibitor (Figure 6b). The apparent kinetic constants at 0.1 mM of DOIA were $K_{M\ SAM\ app} = 0.46 \pm 0.10$ mM and $k_{cat\ app} = 1.2 \pm 0.10$ min^{-1}. The substrate inhibition by DOIA but not by SAM suggests an Ordered Bi Ter mechanism for this reaction in which SAM is the first and DOIA is the second substrate.[25]

The substrate recognition of BtrN was then investigated using several cyclitols and sugars as substrate analogues (Table 1). Although it is not clear whether or not the whole catalytic cycle is operative with these substrate analogues since the relative activities were estimated by the initial velocities of 5′-dexyadenosine formation, BtrN was found to be highly specific for DOIA and able to strictly distinguish sugars from cyclitols, which further supports its biological function in cells.

From a mechanistic point of view, the 5′-deoxyadenosyl radical generated through the reductive cleavage of SAM by the $[4Fe\text{-}4S]^+$ cluster was presumed to be involved in the abstraction of a substrate hydrogen atom to produce a radical intermediate. To investigate this hypothesis, 2-deoxy-*scyllo*-[3-^{2}H]inosamine ([3-^{2}H]DOIA) was prepared and subjected to the BtrN reaction. [3-^{2}H]DOIA (97% atom D) was prepared from amino-DOI by reduction of the keto group with NaB^2H_4 (see Supporting Information).

The enzyme reaction was conducted using [3-^{2}H]DOIA (10 mM) as a substrate in the presence of BtrN, SAM (3 mM), and sodium dithionite. HPLC quantitative analysis revealed that 2.5 mM 5′-deoxyadenosine was formed in this reaction. The generated 5′-deoxyadenosine was then purified and analyzed by NMR spectroscopy and FAB-MS spectrometry (Figures 7 and 8). The ^{2}H NMR data clearly showed incorporation of deuterium atom into the methyl group of 5′-deoxyadenosine (Figure 7). In the ^{1}H NMR data, in addition to the nondeuterated doublet methyl signal, 0.016 and 0.032 ppm upfield doublet signals caused by the geminal ^{2}H isotope effect were observed in the generated 5′-deoxyadenosine. These signals can be assigned as monodeuterated (CH_2D) and dideuterated (CHD_2) methyl groups as shown in Figure 8a. Further, observation of molecular ions at *m*/*z* 252.1, 253.1, and 254.1 in the FAB-MS spectrum also suggested the generation of the non, mono, and dideuterated 5′-deoxyadenosines (Figure 8b). According to the ^{1}H NMR and FAB-MS results the ratio of the three 5′-deoxyadenosines was determined to be CH_3:CH_2D:CHD_2 = 1.0:1.8:0.87. In addition, the kinetic isotope effect (KIE) in this reaction was estimated to be 1.3 ± 0.1 (Figure 9), which suggests that hydrogen-atom abstraction is involved in the oxidation reaction but is not the rate-determining step. Furthermore, the reaction was carried out in a deuterium oxide buffer (99.8% D) using nonlabeled DOIA. No incorporation of the deuterium was observed in 5′-deoxyadenosine (see Supporting Information). This suggests that the reaction proceeds through direct hydrogen-atom abstraction from DOIA by the 5′-deoxyadenosyl radical, not via the protein radical produced on a cysteine or a tyrosine residue, since such a proton is exchangeable with solvent.

The above results revealed that the protium incorporated into 5′-deoxyadenosine in the reaction of BtrN with [3-^{2}H]DOIA was not from the solvent. Thus, generation of nondeuterated and dideuterated 5′-deoxyadenosine in the reaction with [3-^{2}H]-DOIA proves the reversibility at the hydrogen abstraction step. The mechanism of the generation of these 5′-deoxyadenosines is illustrated in Figure 10. At first, SAM is reductively cleaved in the presence of DOIA and transformed into the 5′-deoxyadenosyl radical, which directly abstracts the substrate deuterium atom. The generated substrate radical then has a chance to abstract the protium atom on 5′-deoxyadenosine to form nonlabeled DOIA and the deuterated 5′-deoxyadenosyl radical, which would then react with methionine to give the monodeuterated SAM. Reaction of the produced nonlabeled DOIA with nonlabeled SAM produces nonlabeled 5′-deoxyadenosine (Figure 10b). On the other hand, monodeuterated SAM will also

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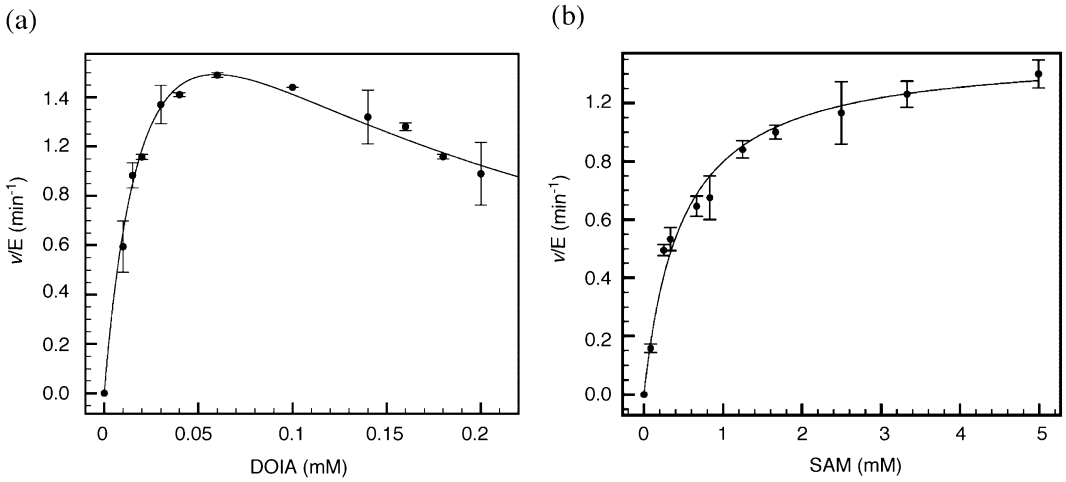
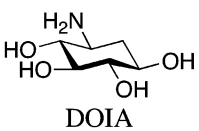
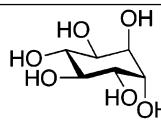
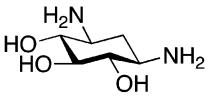
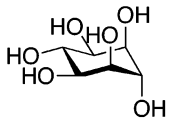
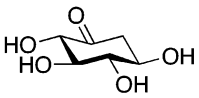
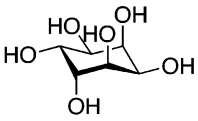
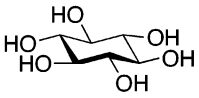
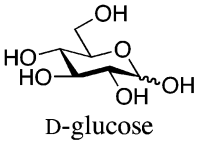
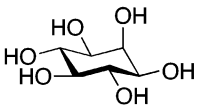
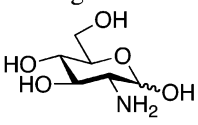
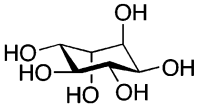
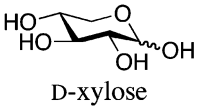


Figure 6. Substrate concentration dependence of the initial velocity. (a) DOIA concentration was varied from 10 to 200 μM, while SAM concentration was fixed at 3 mM. Each data point represents the average of three replicates. The line is the nonlinear root-mean-square fit to eq 1. (b) SAM concentration was varied from 0.08 to 5.0 mM, while DOIA concentration was fixed at 0.1 mM. The line is the nonlinear root-mean-square fit to the Michaelis−Menten equation: $v/[\mathrm{BtrN}] = k_{\mathrm{cat}}[\mathrm{SAM}]/(K_{\mathrm{M}} + [\mathrm{SAM}])$.

Table 1. Relative Activity against Substrate Analogues[a]

substrate	relative activity	substrate	relative activity
 DOIA	100	 1L-*chiro*-inositol	n.d.
 DOS	14.4 ± 0.9	 *muco*-inositol	trace
 DOI	n.d.	 *allo*-inositol	n.d.
 *scyllo*-inositol	3.3 ± 1.0	 D-glucose	n.d.
 *myo*-inositol	0.9 ± 0.4	 D-glucosamine	n.d.
 1D-*chiro*-inositol	n.d.	 D-xylose	n.d.

[a] n.d.: activity was not detected.

react with the deuterated DOIA to generate dideuterated 5′-deoxyadenosine (Figure 10c).

Discussion

BtrN was proved to be an enzyme catalyzing the oxidation of DOIA to form amino-DOI based on the combination of gene disruption experiments and in vitro assay of the overexpressed enzyme. However, this profile was unexpected because an NAD-dependent dehydrogenase, NeoA, was reported to catalyze the same oxidation of DOIA in the DOS biosynthesis in the neomycin producer *Streptomyces fradiae*.[19] An NAD-dependent dehydrogenase, BtrE, is actually encoded in the *btr* gene cluster and thus was believed to catalyze the oxidation of DOIA. However, several attempts to detect the DOIA dehy-

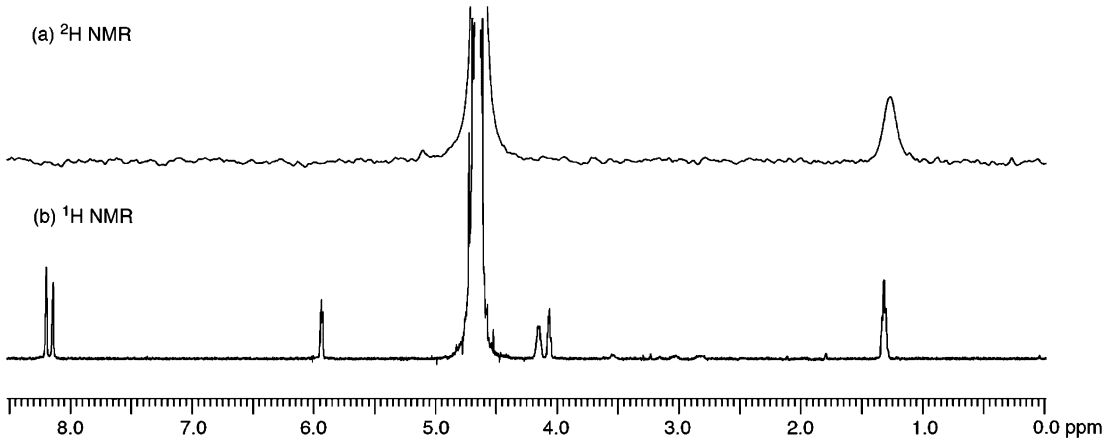


Figure 7. (a) ^{2}H NMR (61 MHz, H_2O) and (b) ^{1}H NMR (400 MHz, D_2O) of 5′-deoxyadenosine generated by BtrN reaction with [3-^{2}H]DOIA.

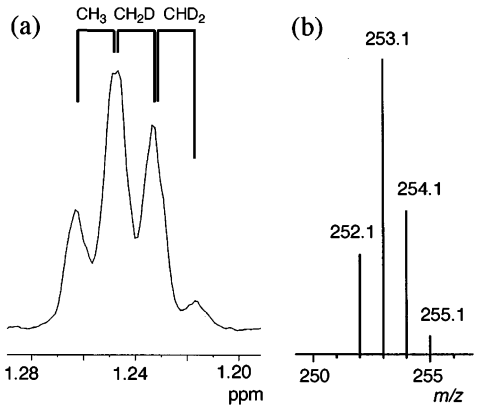


Figure 8. (a) ^{2}H-decoupled ^{1}H NMR (500 MHz, D_2O) and (b) FAB-MS (positive) of the isolated 5′-deoxyadenosine generated by reaction with [3-^{2}H]DOIA. Nonlabeled 5′-deoxyadenosine: *m/z* $[M + H]^+$ 252.1.

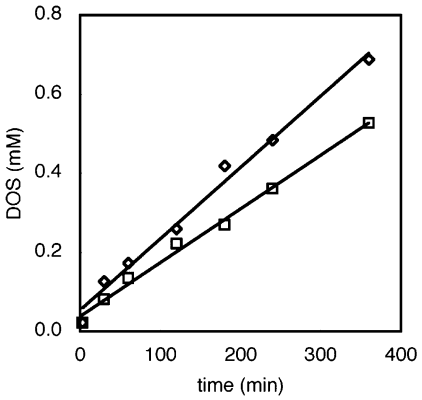


Figure 9. Time dependence of DOS formation in the reaction with nonlabeled (◇) or [3-^{2}H]DOIA (□).

drogenase activity failed in our laboratory (data not shown). As we suggested in the previous report, BtrE lacks two important zinc binding motifs and thus may have a different type of enzymatic activity.[19] Combining those genetic information and our experimental results, it is clear that *Bacillus circulans* has a different system for DOS biosynthesis from that of the neomycin producer, *S. fradiae*. Functional analysis of BtrE is still underway.

The UV−vis absorption spectra and the EPR spectrum of the reconstituted BtrN confirmed that BtrN is a [4Fe-4S] cluster containing radical SAM enzyme. The proposed catalytic mechanism of the BtrN reaction is shown in Figure 11. The 5′-deoxyadenosyl radical is generated by reductive cleavage of SAM using the $[4Fe\text{-}4S]^+$ cluster as a reductant. The 5′-deoxyadenosyl radical then abstracts a hydrogen atom from DOIA to generate the radical intermediate, which is subsequently transformed into amino-DOI upon transferring one electron, possibly to the $[4Fe\text{-}4S]^{2+}$ cluster to regenerate the $[4Fe\text{-}4S]^+$ cluster.

Observation of the deuterium-atom transfer from [3-^{2}H]DOIA to 5′-deoxyadenosine clearly proved that the reaction involves hydrogen-atom abstraction by the 5′-deoxyadenosyl radical. Further, no deuterium was incorporated into the 5′-deoxyadenosine upon the BtrN reaction in a deuterium oxide buffer, which suggests that the reaction proceeds through direct abstraction of the substrate hydrogen atom by the 5′-deoxyadenosyl radical but not via the protein radical found in several radical-dependent enzymes.[26] Direct abstraction of a substrate hydrogen atom by 5′-deoxyadenosyl radical has also been proposed in biotin synthase.[27] The substrate-bound crystal structure of biotin synthase indicated direct hydrogen-atom abstraction.[28] The present study clearly shows that BtrN also catalyzes the reaction by direct abstraction of the substrate hydrogen atom by the 5′-deoxyadenosyl radical.

Formation of nondeuterated and dideuterated 5′-deoxyadenosines by incubation of BtrN with [3-^{2}H]DOIA suggests the re-abstraction of a hydrogen atom on 5′-deoxyadenosine by the substrate radical intermediate (Figure 10). This type of re-abstraction is necessary for enzymes that utilize the 5′-deoxyadenosyl radical in a catalytic manner, such as adenosyl cobalamin-dependent enzymes[29] and several radical SAM enzymes.[16] Transfer of the deuterium atom from the deuterated substrate to 5′-deoxyadenosine was reported for two radical SAM enzymes, biotin synthase[27] and pyruvate formate lyase-activating enzyme (PFL-AE).[30] However, in both cases, only a single incorporation of a deuterium to 5′-deoxyadenosine was observed.[27,30] On the other hand, the BtrN reaction using [3-^{2}H]-DOIA generated nondeuterated and dideuterated 5′-deoxyadenosines, which suggests that the hydrogen-atom abstraction is reversible. The reversibility of the hydrogen-atom abstraction

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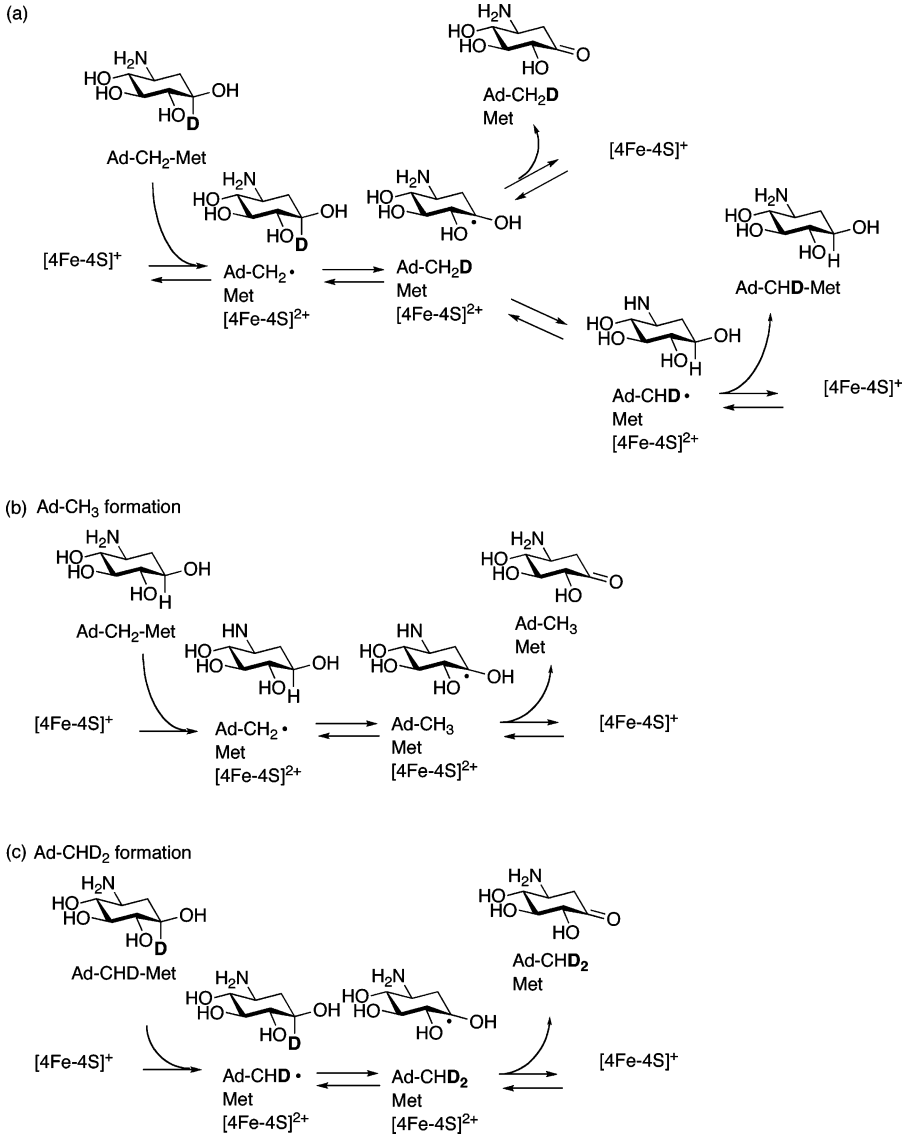

Figure 10. Mechanism of (a) monodeuterated 5′-deoxyadenosine (Ad-CH_2D), (b) nondeuterated 5′-deoxyadenosine (Ad-CH_3), and (c) dideuterated 5′-deoxyadenosine (Ad-CHD_2) formation by reaction with [3-^{2}H]DOIA.

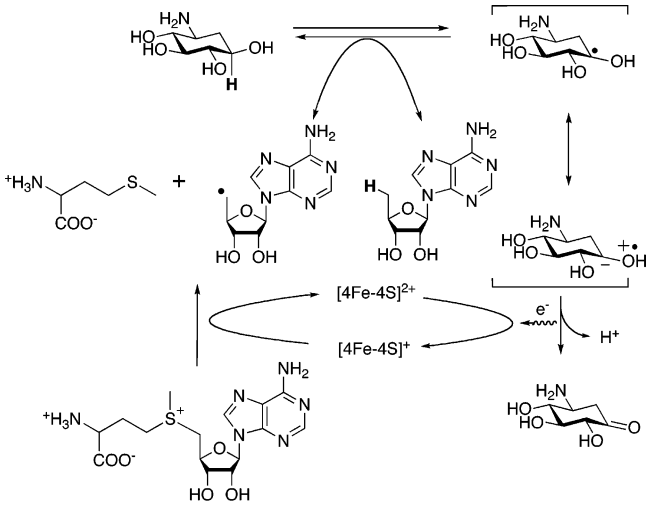

Figure 11. Proposed reaction mechanism of BtrN.

and the small KIE value suggest that the subsequent ketone formation is slower than the deuterium-atom abstraction, which enables the substrate radical intermediate to re-abstract a protium on 5′-deoxyadenosine. Thus, ketone formation might be the rate-determining step.

Each catalytic cycle requires one molecule of SAM and generates one molecule of methionine and 5′-deoxyadenosine, which reveals that the subsequent ketone formation from the substrate radical intermediate is not catalyzed by another 5′-deoxyadenosyl radical. Therefore, one electron of the substrate radical intermediate must be somehow transferred to an electron acceptor to complete the ketone formation. However, no extra electron acceptor was present in the reaction solution. On the other hand, the $[4Fe\text{-}4S]^{2+}$ cluster of BtrN must be reduced to $[4Fe\text{-}4S]^{+}$ for the next catalytic cycle. Although reduction of the $[4Fe\text{-}4S]^{2+}$ cluster back to $[4Fe\text{-}4S]^{+}$ cluster by sodium dithionite cannot be ruled out, it is reasonable to conclude that the electron released from the substrate radical intermediate is somehow transferred to the $[4Fe\text{-}4S]^{2+}$ cluster to produce amino-DOI and regenerate the $[4Fe\text{-}4S]^{+}$ cluster for the next catalytic cycle. Since the midpoint redox potential of the $[4Fe\text{-}4S]^{1+/2+}$ clusters of radical SAM enzymes is extremely negative (~ -500 mV), reduction of the oxidized $[4Fe\text{-}4S]^{2+}$ cluster under

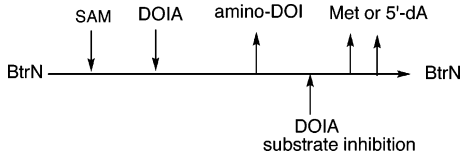

Figure 12. Cleland's shorthand notation of the BtrN reaction.

physiological conditions seems to be difficult. However, binding of SAM to oxidized $[4Fe\text{-}4S]^{2+}$ cluster was shown to significantly elevate the reduction potential,[31] and reduction of the $[4Fe\text{-}4S]^{2+}$ cluster by flavodoxin appeared to occur by further binding of a substrate.[32] Thus, in the case of BtrN, direct reduction of the $[4Fe\text{-}4S]^{2+}$ cluster by the radical intermediate seems to be possible when coordination of 5′-deoxyadenosine, methionine, or the substrate radical intermediate to a sphere of the $[4Fe\text{-}4S]^{2+}$ cluster causes a conformational change of the active site and elevates the reduction potential of the cluster. In this case, the electron might be directly transferred from the radical intermediate to the oxidized cluster, although the existence of a kind of internal electron sink mechanism such as a modified amino acid side chain and a disulfide bond cannot be excluded. Further mechanistic analysis of ketone formation is currently underway.

The oxygen-independent coproporphyrinogen-III oxidase, HemN, is currently the only radical SAM enzyme that is proved to require one electron-transfer step. However, in this case, an uncharacterized electron acceptor in a cell-free extract of *E. coli* is required to complete the reaction.[33] Thus, we propose that BtrN is the first example of a self-reactivating radical SAM enzyme.

Kinetic analysis gave further insights into the mechanism of the BtrN reaction. Importantly, we observed uncompetitive substrate inhibition by DOIA at concentrations greater than 0.08 mM, while SAM did not inhibit the reaction. Since the same amounts of amino-DOI and 5′-deoxyadenosine were formed in the BtrN reaction and no hydrogen atom from solvent was incorporated into the produced 5′-deoxyadenosine, we conclude that inhibition by DOIA is caused by a simple uncompetitive substrate inhibition but not by inactivating side reactions. Thus, the results of the kinetic studies suggest that the BtrN reaction proceeds by an ordered Bi Ter mechanism in which DOIA is the second substrate incorporated into the active site and amino-DOI is the first product released as shown in Figure 12. The Ordered mechanism is consistent with the mechanism proposed in Figure 11. Further, because the BtrN reaction was found to be slow and the just prior amino transfer reaction catalyzed by BtrS is known to be relatively fast and reversible,[12] the fact that DOIA inhibits the BtrN reaction by uncompetitive substrate inhibition may imply that BtrN controls the overall production rate of butirosin.

A similar oxidation reaction by a radical SAM enzyme is known for the reaction of anaerobic sulfatase maturating enzymes, which post-translationally oxidizes a serine or cysteine residue of sulfatases to formyl glycine.[34,35] Since the formyl glycine formation requires SAM and the radical SAM (CXXX-CXXC) motif, involvement of the 5′-deoxyadenosyl radical was proposed.[34,35] However, no further mechanistic investigation was reported.

Enzymatic oxidations of hydroxyl groups generally proceed through hydride transfers catalyzed by nicotinamide- or flavin-dependent enzymes. Several examples are known for radical oxidations of hydroxyl groups using oxygen as an electron acceptor in biological systems. P_{450} oxidoreductases are considered to catalyze oxidation of a hydroxylated carbon center to produce a ketone or carboxylate.[36,37] Galactose oxidase also catalyzes the unusual oxidation of the C-6 hydroxyl group of galactose into an aldehyde using Cu^{I} for oxygen activation.[38] However, no report has been made for the oxygen-independent oxidation of a hydroxyl group by a radical mechanism, except for the aforementioned reaction by sulfatase maturating enzymes. Our results described here elucidate the mechanism of anaerobic radical oxidation of hydroxyl groups for the first time. This type of oxidation suggests the use of SAM as an oxidant substitute, instead of molecular oxygen, in anaerobic metabolisms.

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Supporting Information Available: Experimental procedures including gene disruption study, all spectroscopic data, and HPLC charts. This material is available free of charge via the Internet at http://pubs.acs.org.

JA072481T

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