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ORIGINAL ARTICLE

Substrate specificity of radical *S*-adenosyl-L-methionine dehydratase AprD4 and its partner reductase AprD3 in the C3′-deoxygenation of aminoglycoside antibiotics

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A radical *S*-adenosyl-L-methionine dehydratase AprD4 and an NADPH-dependent reductase AprD3 are responsible for the C3′-deoxygenation of pseudodisaccharide paromamine in the biosynthesis of apramycin. These enzymes are involved in the construction of the characteristic structural motif that is not modified by 3′-phosphotransferase in aminoglycoside-resistant bacterial strains. AprD4 catalyzes the C3′-dehydration of paromamine via a radical-mediated reaction mechanism to give 4′-oxolividamine, which is then reduced by AprD3 with NADPH to afford lividamine. In the present study, the substrate specificity of this unique combination of enzymes has been investigated. AprD4 was found to recognize paromamine, neamine, kanamycin C, and kanamycin B to afford 5′-deoxyadenosine as one of products during the C3′-dehydration of aminoglycosides, but not 2′-*N*-acetylparomamine and paromomycin. Only paromamine and kanamycin C were converted to the corresponding C3′-deoxygenated compounds by AprD4 and AprD3. AprD3 recognizes the 4′-oxolividamine moiety, including the pseudotrisaccharide kanamycin C, and seems to reject the amino group at C6′ of neamine and kanamycin B. Chirally deuterium-labeled NADPH was used to identify that that AprD3 transfers the pro-*S* hydrogen atom of NADPH when reducing 4′-oxolividamine to give lividamine.
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INTRODUCTION

The majority of clinically important aminoglycoside antibiotics contain an aminocyclitol 2-deoxystreptamine (2DOS) as central aglycon in pseudo-oligosaccharide structure (Figure 1).[1,2] The C4-hydroxy group of 2DOS is generally glycosylated with D-glucosamine to construct the common pseudodisaccharide biosynthetic intermediates paromamine **1** and neamine **2**. These intermediates are further glycosylated leading to a structurally diverse family of 2DOS-containing aminoglycosides including kanamycin, tobramycin, apramycin, neomycin and lividomycin.[2] Among these, apramycin, tobramycin and lividomycin lack the C3′-hydroxy group of the paromamine/neamine moiety, and these antibiotics are not processed by the aminoglycoside 3′-phosphotransferase in aminoglycoside-resistant bacterial strains.[3] The deoxygenation of the C3′-position of biosynthetic intermediates is considered to be an important modification reaction to improve the value of aminoglycoside antibiotics against aminoglycoside antibiotic-resistant bacteria. Dibekacin (3′,4′-dideoxykanamycin B) is a clinically important semisynthetic aminoglycoside antibiotic that has been designed based on the deoxygenated motif of gentamicin.[4]

Comparison of the biosynthetic gene clusters responsible for the biosynthesis of structurally similar aminoglycosides (apramycin and lividomycin) revealed two common genes that code for a putative NAD/NADP-dependent oxidoreductase AprD3/LivY and a putative radical *S*-adenosyl-L-methionine (SAM) enzyme AprD4/LivW. These characteristic proteins were predicted to be responsible for the deoxygenation of the C3′ of the biosynthetic intermediates containing paromamine/neamine moieties.[2,5] Another C3′-deoxygenated aminoglycoside is tobramycin. The tobramycin biosynthetic gene cluster lacks corresponding genes. However, tobramycin-producing strains *Streptomyces tenebrarius* and *Streptoalloteichus hindustanus* also produce apramycin and it appears that AprD3/AprD4 are shared for the C3′-deoxygenation biosynthesis of both apramycin and tobramycin. Presumably, a putative common biosynthetic intermediate lividamine (3′-deoxyparomamine) is involved in both tobramycin and apramycin biosynthetic pathways. Liu and coworkers[6] have recently shown that both AprD4 and AprD3 are responsible for the C3′-deoxygenation of **1** to give lividamine (**7**) using *in vitro* enzymatic analysis (Figure 2). They observed that the H4′ of **1** is abstracted by a 5′-deoxyadenosyl radical generated from SAM to initiate this unique radical-mediated dehydration reaction (Supplementary Figure S1).

Inactivation of the *aprD4* gene by Xia and coworkers[7] resulted in the loss of the production of carbamoyltobramycin

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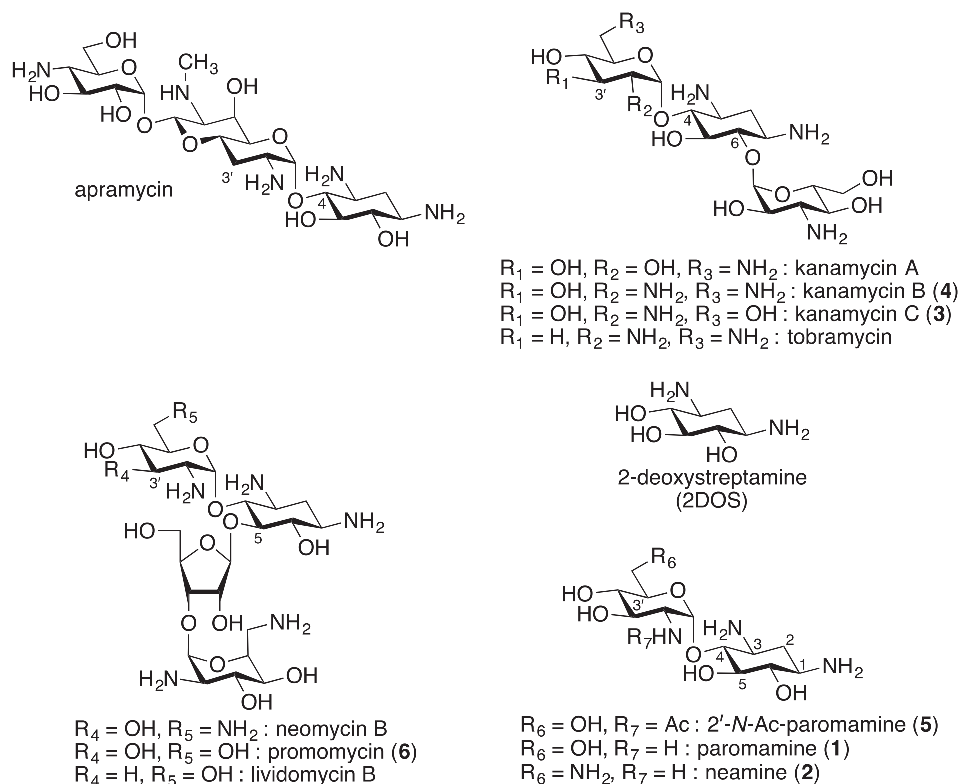

Figure 1 Structures of 2-deoxystreptamine (2DOS) and aminoglycoside antibiotics containing 2DOS.

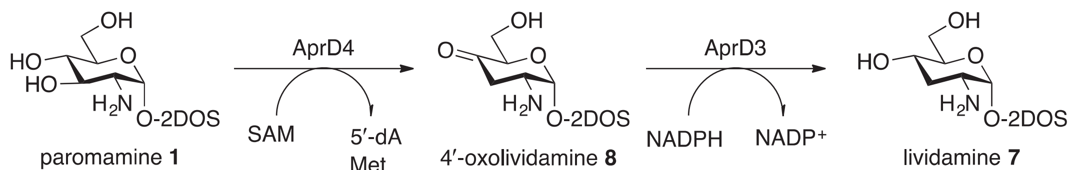

Figure 2 C3′ deoxygenation by a radical SAM dehydratase AprD4 and NADPH-dependent reductase AprD3. Met, L-methionine; SAM, *S*-adenosyl-L-methionine; 5′-dA, 5′-deoxyadenosine.

(6‴-*O*-carbamoyl-3′-deoxykanamycin B) supporting the involvement of AprD4 in the C3′-deoxygenation reaction. However, the *aprD3* knockout mutant still produced carbamoyltobramycin and only the radical SAM dehydratase AprD4 appears critical for the C3′-deoxygenation. The *aprD3* knockout mutant suffered from considerably lowered apramycin production and AprD3 is presumably also involved in the C3′-deoxygenation during normal apramycin formation. In the present study, we investigated the substrate specificity of AprD4 and AprD3 *in vitro* to understand the intriguing C3′-deoxygenation transformation in aminoglycoside biosynthesis. Accumulating the enzymatic properties of this unique combination of enzymes should be useful in the rational design and engineered biosynthesis of C3′-deoxygenated aminoglycoside antibiotics. We also describe the stereochemistry of the reduction of 4′-oxolividamine (**8**) by AprD3 using chirally deuterium-labeled NADPH as the reducing reagent.

MATERIALS AND METHODS

Preparation of AprD4 and AprD3

The *aprD4* and *aprD3* genes were amplified by polymerase chain reaction using genomic DNA of *S. hindustanus* JCM 3268[8] as the template. For the *aprD4* gene, aprD4-N: 5′-TGAACATATGCGACGCATGCGGCTC-3′ and aprD4-C: 5′-GGCGAatTCGTGTGGGTCCCTCTCG-3′, and for the *aprD3* gene, aprD3-N: 5′-GTTCCATATGACGGCGGCGGG-3′ and aprD3-C: 5′-GTAAG CTTTCAGCGCCGCGTCGCCC-3′ primers (Fasmac, Kanagawa, Japan) were used in the polymerase chain reaction. PrimeSTAR GXL DNA polymerase (Takara, Otsu, Japan) was used for the polymerase chain reaction, and the reaction solutions contained 5×PrimeSTAR GXL buffer 2 μl, 2.5 mM dNTP mixture 0.8 μl, 10 μM of primers 0.3 μl each, genomic DNA solution 0.5 μl, dimethyl sulfoxide 0.5 μl, sterilized water 5.4 μl and 1.25 U μl^{-1} polymerase 0.2 μl. The polymerase chain reaction conditions were 10 s of denaturation at 98 °C and 30 s of extension at 68 °C for 30 cycles. The obtained DNA fragments were reacted with Ex Taq polymerase (Takara) with dNTP mixture at 70 °C for 2 min. The obtained DNA fragments were then ligated with pGEM-T-easy (Promega, Madison, WI, USA) using the DNA ligation kit version 2.1 (Takara) and introduced into *Escherichia coli* DH5α competent cells. The presence of plasmids containing the desired *aprD4* gene (*aprD4*/pGEM) and the *aprD3* gene (*aprD3*/pGEM) was confirmed by the sequence analysis. The plasmid *aprD4*/pGEM was digested with *Nde*I and *Eco*RI, and the insert DNA was introduced into the corresponding site of the pColdI expression vector and transformed into *E. coli* DH5α. The *aprD4-Chis* gene was amplified with the primer aprD4-N and the primer aprD4-Chis: 5′-CTCGAGTCCG

GCGTCGCTGGTC-3′ using *aprD4*/pColdI as the template to obtain a gene for C-terminal His-tag AprD4. This gene was cloned into the *Nde*I/*Xho*I site of pET30a to obtain *aprD4-Chis*/pET30. The *aprD4-Chis*/pET30 expression plasmid was then introduced into *E. coli* C41(DE3) with a pRKSUF017 plasmid to co-express the *suf* gene cluster derived from *E. coli*.[9,10] Similarly, the *aprD3* gene was cloned into the pColdI expression vector and expressed in *E. coli* BL21 (DE3).

The *aprD4-Chis*/pET30/pRKSUF017/C41(DE3) strain was cultured in Luria–Bertani medium containing 50 μg ml^{-1} of kanamycin and 5 μg ml^{-1} of tetracycline at 37 °C, and shaken at 200 r.p.m. until the OD_{590} reached 0.6. Isopropyl 1-thio-β-D-galactopyranoside (final concentration 0.2 mM), 0.2 mM $FeSO_4(NH_4)_2SO_4$ and 0.2 mM L-cysteine were added, and the culture continued at 28 °C and 100 r.p.m. overnight. The *E. coli* cells were harvested by centrifugation at 4100 *g* for 20 min, and stored at −30 °C until use. The cells were placed in a glovebox and suspended in 50 mM HEPES-NaOH/200 mM KCl/10% glycerol (pH 8.0, buffer A). The cell suspension was lysed with a Q55 sonicator (Qsonica, WAKENBTECH, Kyoto, Japan) to obtain a cell-free extract. The supernatant was centrifuged at 23 180 g for 20 min, loaded onto a Talon resin (Takara) column and washed with buffer A containing 10 mM imidazole. The AprD4 protein was eluted with buffer A containing 200 mM imidazole. The AprD4 containing eluent was passed through a PD-10 column (GE Healthcare, Buckinghamshire, UK). The purified AprD4 was treated with 5 mM dithiothreitol at room temperature for 15 min. $FeSO_4(NH_4)_2SO_4$ (0.5 mM) and Na_2S (0.5 mM) were added and the mixture was incubated at room temperature for 30 min. The resulting brownish AprD4 solution was passed through a PD-10 column. The reconstituted AprD4 showed absorption at 410 nm, confirming the presence of a characteristic $[4Fe\text{-}4S]^{2+}$ cluster (Supplementary Figure S7). The reconstituted AprD4 was concentrated with an Amicon Ultra-0.5 mL Centrifugal Filter (Ultracel-10 K, 10 000 MWCO, Millipore, Darmstadt, Germany) at 9300 *g* at 10 °C and then reduced with sodium dithionite (10 mM) for 20 min at room temperature. The absorption around 410 nm was bleached to indicate that the cluster was in the reduced form $[4Fe\text{-}4S]^{+}$ (Supplementary Figure S2). The reduced form of $[4Fe\text{-}4S]^{+}$ in AprD4 was confirmed by electron paramagnetic resonance spectroscopic analysis using the methods of our previous report regarding NeoN protein[9] (Supplementary Figure S8). SDS polyacrylamide gel electrophoresis (12.5%) was performed to confirm the size of the recombinant AprD4 (predicted size 51.6 kDa; Supplementary Figure S9). The protein concentration for AprD4 (A_{280} 1 A cm^{-1} = 1.245 mg ml^{-1}) was estimated using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

The *aprD3*/pColdI/BL21(DE3) strain was cultured in Luria–Bertani medium containing 50 μg ml^{-1} of ampicillin at 37 °C shaken at 200 r.p.m. until the OD_{590} reached 0.6. Isopropyl 1-thio-β-D-galactopyranoside (final concentration 0.2 mM) was added, and the culture continued at 15 °C and 200 r.p.m. overnight. The *E. coli* cells containing AprD3 were harvested by centrifugation at 4100 *g* for 20 min and stored at −30 °C until use. The recombinant AprD3 (predicted size 27.5 kDa) was also prepared under the same methods as used for AprD4 preparation except for the reconstitution steps to investigate anaerobic enzymatic reactions (Supplementary Figure S9). The protein concentration of AprD3 (A_{280} 1 A cm^{-1} = 2.128 mg ml^{-1}) was estimated using a Nanodrop spectrophotometer.

Enzymatic reactions with AprD4 and AprD3

Enzymatic reactions were carried out with 7.0 μM of AprD3, 36 μM of AprD4, 1 mM substrates (2′-*N*-acetylparomamine, paromamine, neamine, kanamycin C, kanamycin B and paromomycin), 2 mM SAM and 2 mM NADPH at room temperature overnight. NADH was not utilized because of its low binding affinity to AprD3 (data not shown). Each reaction mixture was quenched with one equivalent of ethanol. The supernatant (5 μl) of each reaction was used to detect the 5′-deoxyadenosine (5′-dAdo) formation by HPLC analysis. The amount of 5′-dAdo was determined from the calibration curve with the authentic sample. A part of the quenched enzymatic solution was treated with 2,4-dinitro-1-fluorobenzene according to the previous report.[9] The 2,4-dinitrophenyl (DNP) derivatives were analyzed by HPLC and LC-ESI-MS according to the neomycin C 5‴-epimerase NeoN analysis.[9] HPLC analysis was undertaken on a Hitachi system (Hitachi L7100 pump, L-7405-UV detector, L-7300 column oven or L-6250 pump, L-4000 UV detector, L-7300 column oven) equipped with a PEGASIL ODS column (5 μm, 4.6 × 250 mm, Senshu, Tokyo, Japan) and the chromatographic data were recorded with Chromato-PRO (Run Time Instruments, Sagamihara, Japan). Elution was performed with 25% CH_3OH for 20 min at a flow rate of 0.8 ml min^{-1} at 40 °C, monitored at 254 nm for the analysis of 5′-dAdo formation. Elution was performed with 70% CH_3OH for 10 min and then a linear gradient to 90% for 10 min at a flow rate

of 0.8 ml min^{-1} at 40 °C, monitored at 350 nm for the analysis of DNP derivatives of paromamine, kanamycin C, paromomycin and deoxygenated products. Elution was performed with 60% CH_3OH for 10 min and then a linear gradient to 90% for 10 min for the analysis of 2′-*N*-acetylparomamine. Elution was performed with 75% CH_3OH for 10 min and then a linear gradient to 90% for 10 min for the analysis of neamine and kanamycin B.

Isolation of lividamine from AprD4 and AprD3 reaction with paromamine

AprD3 (21–28 μM), AprD4 (56–74 μM), SAM 1 mM, and NADPH 1 mM were mixed with 1 mM of paromamine (1.6 mg) in a 5 ml solution and left at room temperature overnight. If the reaction was not completed, enzymes and cofactors were added for an additional 1–2 times. This reaction was repeated a total of three times. Lividamine was purified by ion-exchange chromatography according to the neomycin B purification method.[9] A total of 2.0 mg of lividamine (sulfate salt) was obtained from 4.8 mg of paromamine (HCl salt). ^{1}H NMR (500 MHz, D_2O): δ 1.74 (q, *J* = 12.6 Hz, 1H, H-2-ax), 1.88 (q, *J* = 11.7 Hz, 1H, H-3′-ax), 2.21 (dt, *J* = 11.7, 4.4 Hz, 1H, H-3′-eq), 2.39 (dt, *J* = 12.6, 4.1 Hz, 1H, H-2-eq), 3.22 (m, 1H, H-1), 3.37 (m, 1H, H-3), 3.51 (t, *J* = 9.7 Hz, 1H, H-6), 3.54–3.59 (m, 2H, H-5, H-2′), 3.61–3.67 (m, 2H, H-4′, H-6′), 3.71–3.76 (m, 2H, H-4, H-5′), 3.82 (dd, *J* = 12.0, 2.2 Hz, 1H, H-6′), 5.40 (d, *J* = 3.5 Hz, 1H, H-1′). ^{13}C NMR (125 MHz, D_2O): δ 29.1, 30.1, 48.3, 49.0, 49.9, 60.2, 63.3, 72.7, 74.8, 74.8, 81.4, 95.5.

Enzyme reaction with chirally deuterium-labeled NADPH

(4*S*)-[4-^{2}H]NADPH and (4*R*)-[4-^{2}H]NADPH were prepared according to literature methods.[11] Briefly, 5 mM NADP$^+$ and 5 mM D-[1-^{2}H]glucose (98%D, Cambridge Isotope Laboratories, Tewksbury, MA, USA) were reacted with 1 U ml^{-1} of glucose dehydrogenase derived from *Bacillus* sp. (bGDH; Wako) in 100 μl at 28 °C for 2 h to obtain (4*S*)-[4-^{2}H]NADPH. 5 mM NADP$^+$ and 5 mM D-[1-^{2}H]glucose were reacted with 50 μM of glucose dehydrogenase from *Thermoplasma acidophilum* in 100 μl at 28 °C for 2 h to obtain (4*R*)-[4-^{2}H]NADPH. Crude chirally deuterium-labeled NADPH solutions were utilized for the AprD4 and AprD3 reaction after the estimation of the labeled NADPH concentration by absorbance at 340 nm. Enzymatic reactions were undertaken with 18 μM of AprD3, 25 μM of AprD4, 1 mM paromamine, 2 mM SAM, and 1.35 mM of (4*S*)-[4-^{2}H]NADPH or 1.85 mM of (4*R*)-[4-^{2}H]NADPH in 50 μl. After overnight incubation at room temperature, the reaction mixtures were quenched with one equivalent of ethanol and the products were converted to DNP derivatives according to the above-mentioned method. The isotopic patterns of the products were measured by a Shimadzu LCMS-2020 mass spectrometer equipped with LC-20AD pump and SPD-M20A UV detector equipped with a TSK-GEL ODS-100Z column (3 μm, 2.0 × 150 mm, TOSOH, Tokyo, Japan). Elution was performed with 75% CH_3OH at a flow rate of 0.1 ml min^{-1}, monitored at 350 nm. The negative mode was selected to detect DNP derivatives of aminoglycosides.

Isolation of deuterium-labeled lividamine

A large amount of (4*S*)-[4-^{2}H]NADPH was prepared to obtain deuterium-labeled lividamine. NADP$^+$ (10 mM) and 10 mM D-[1-^{2}H]glucose were reacted with 1 U ml^{-1} of bGDH in 1 ml at 28 °C for 2 h. Then 0.1 mM paromamine (0.8 mg), 15–41 μM of AprD3, 23–51 μM of AprD4 and 1 mM SAM were added to the solution, and incubated at room temperature overnight five times. The free form of deuterium-labeled lividamine was isolated by cation-exchange chromatography and analyzed by NMR spectroscopy.

Enzyme reaction in the presence of deuterium oxide

The buffer of the AprD4 and AprD3 solution was exchanged by ultracentrifugation in the concentration process with buffer prepared in D_2O. HEPES-NaOH buffer (50 mM, pD 8.4) with 200 mM KCl was prepared with D_2O (99.8, %D, Kanto Chemical, Tokyo, Japan) and used for ultracentrifugation five times. All other operations were as mentioned above. Consequently, 1.2 mg of lividamine (sulfate salt) was prepared from 4.0 mg of paromamine (HCl salt).

RESULTS AND DISCUSSION

Substrate specificity of AprD4 and AprD3

The radical SAM enzyme AprD4 contains a [4Fe-4S] cluster at the active site and reductively cleaves the carbon–sulfur bond of SAM with the reduced form $[4Fe\text{-}4S]^+$ to generate 5′-deoxyadenosyl radical (5′-dAdo•) or most likely its organometallic adduct with the [4Fe-4S] cluster (Supplementary Figure S1).[12] Liu and coworkers showed that the H4′ atom of **1** is abstracted by 5′-dAdo• to give 5′-deoxyadenosine (5′-dAdo) as one of products in addition to the dehydrated product (Figure 2).[6] The reaction of AprD4/AprD3 with **1**, **2**, kanamycin C (**3**) and kanamycin B (**4**) produced similar amounts of 5′-dAdo, whereas only a small amount of 5′-dAdo was detected with 2′-*N*-acetylparomamine (**5**) and paromomycin (**6**) (Figure 3 and Table 1). Without substrate, only trace amount of 5′-dAdo formation was detected (data not shown). These results indicate that the radical SAM enzyme AprD4 can recognize the paromamine/neamine moiety, even in kanamycin-type pseudotrisaccharides, and abstract a hydrogen atom of the substrates to give 5′-dAdo. However, AprD4 does not accept the 2′-*N*-acetyl group of **5** or substrates with a substituent at C5 of the 2DOS moiety such as **6**.

We next analyzed the C3′-deoxygenated products from reactions of aminoglycosides with AprD4 and AprD3 by HPLC and LC-ESI-MS after dinitrophenyl (DNP) derivatization (Figure 3 and Table 1). Reaction of **1** with AprD3/AprD4 gave the expected deoxygenated product. The deoxygenated product from this reaction was further isolated by ion-exchange chromatography and analyzed by NMR spectroscopy to confirm its structure as **7** (Supplementary Figures S2 and S3). AprD4/AprD3 also efficiently converted **3** to the corresponding deoxygenated compound. Small amounts of the deoxygenated products from **2** and **4** were also detected by LC-ESI-MS analysis (Supplementary Figure S4). These results indicate that AprD4 could react with various paromamine/neamine-containing molecules to give the corresponding dehydrated ketones. Only the paromamine

Table 1 Summary of the AprD4/AprD3 reaction

Substrate (aminoglycoside)	*5′-dA production yield (mM)*	*Conversion ratio in the C3′-deoxygenation (%)*
Paromamine (**1**)	0.49	57
Neamine (**2**)	0.46	5
Kanamycin C (**3**)	0.39	52
Kanamycin B (**4**)	0.47	11
2′-*N*-acetylparomamine (**5**)	0.02	ND
Paromomycin (**6**)	0.01	ND

Abbreviation: ND, not determined.
1 mM substrates (2′-*N*-acetylparomamine, paromamine, neamine, kanamycin C, kanamycin B and paromomycin), 2 mm SAM, and 2 mM NADPH were reacted with 7.0 μM of AprD3 and 36 μM of AprD4 at room temperature overnight.

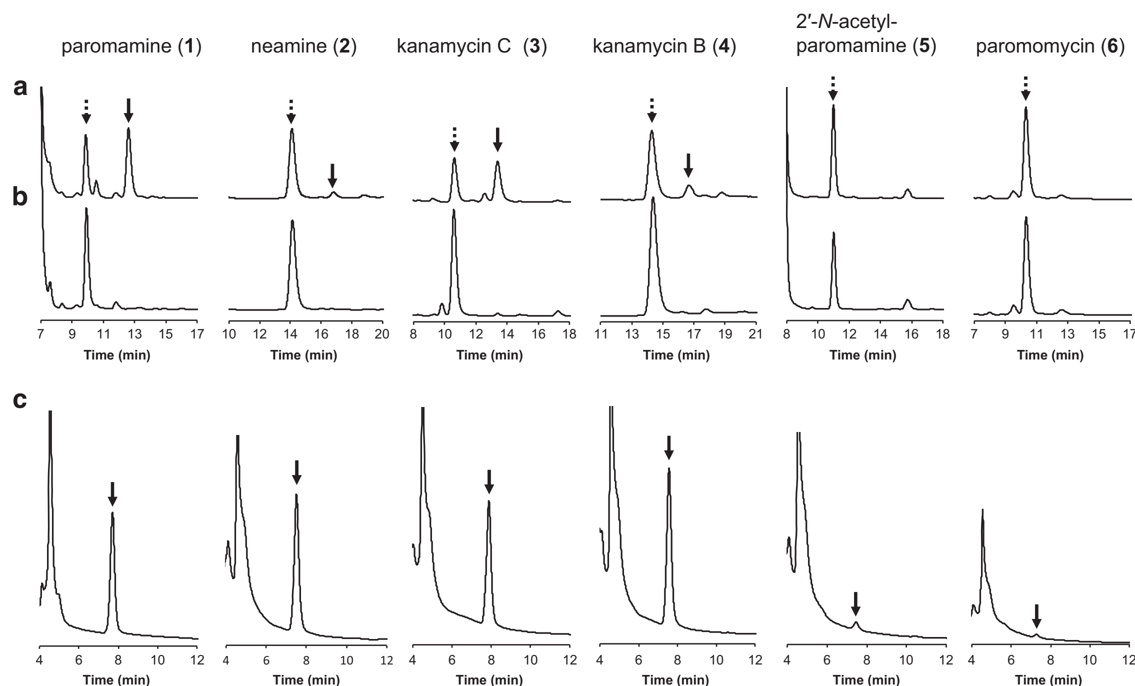

Figure 3 HPLC analysis of 5′-dA formation and DNP derivatives of C3′-deoxygenated products from AprD4/AprD3 reaction. Upper charts (**a**) are HPLC traces for DNP derivatives of C3′-deoxygenated products from paromamine (**1**), neamine (**2**), kanamycin C (**3**), kanamycin B (**4**), 2′-*N*-acetylparomamine (**5**) and paromomycin (**6**). Solid arrows indicate the enzymatic reaction products and dashed arrows indicate substrates. Middle charts (**b**) are negative control reactions without SAM. Bottom charts (**c**) are HPLC traces for 5′-dA formation. 5′-dA production (0.49 mM) was estimated from the peak integration in the reaction with **1**.

type compounds, such as 4′-oxolividamine (**8**), were reduced by AprD3, but neamine type compounds, such as 4′-oxonebramine (4′-oxo-3′-deoxyneamine) that contain a 6′-amino group, seem to be unreactive. Therefore, substrate specificity of reductase AprD3 seems to be critical to afford C3′-deoxygenated aminoglycosides, whereas AprD4 has more tolerant substrate specificity. AprD3 is essential for the formation of **7**, which is a biosynthetic intermediate of both apramycin and tobramycin. However, carbamoyltobramycin is produced even in the absence of AprD3 in the *aprD3* knockout strain.[7] Presumably, AprD4 catalyzes the C3′-dehydration of kanamycin B/kanamycin C to give the corresponding C3′-dehydrated 4′-oxo intermediates, which are then reduced by a reductase that is encoded in the genome of the producer strain to give carbamoyltobramycin as a dead-end product. AprD3 type oxidoreductases widely exist in microbial genomes from standard BLAST search. Thus, such AprD3 orthologs might be involved in the reduction of the presumed C4′-oxo biosynthetic intermediates resulting the production of carbamoyltobramycin, but cannot reduce **8** to give **7**.

We further investigated whether the C4′ moiety of **8** is selectively reduced by reductase AprD3. Clear incorporation of a ^{2}H atom into **7** was detected by LC-ESI-MS analysis when the AprD4/AprD3 reaction was carried out with (4*S*)-[4-^{2}H]NADPH, but not with (4*R*)-[4-^{2}H] NADPH (Figure 4). We also isolated the ^{2}H-labeled **7** and confirmed that the ^{2}H atom was incorporated at the C4′ position (Supplementary Figure S5). This labeling confirmed that **8** is generated from **1** by AprD4 and then is reduced by AprD3 with the pro-*S* hydrogen atom of NADPH to give **7**. AprD3 shows 26.6% homology to BtrF, which is an NADPH-dependent reductase involved in the epimerization of ribostamycin to give xylostasin in the butirosin A biosynthesis.[11] In

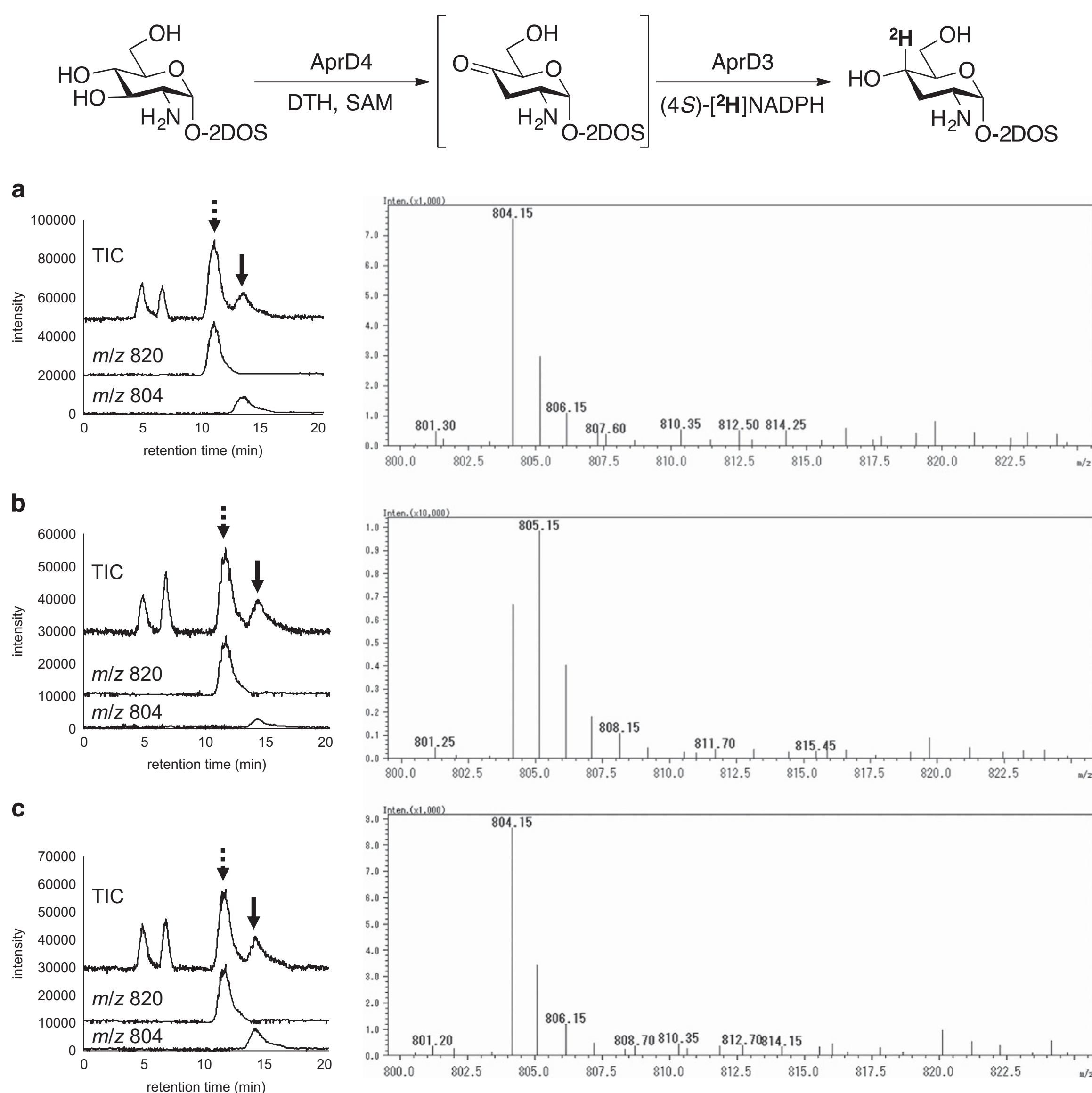

Figure 4 LC-ESI-MS analysis (negative mode) of AprD4/AprD3 reaction product from paromamine with deuterium-labeled NADPH. DNP derivatives of paromamine (*m/z* 820) and lividamine (*m/z* 804) in the AprD4/AprD3 reaction with (**a**) non-labeled NADPH, (**b**) (4*S*)-[4-^{2}H]NADPH and (**c**) (4*R*)-[4-^{2}H] NADPH. Upper traces are total ion chromatography (TIC). Middle traces are selected ion monitoring (SIM) with *m/z* 820 corresponding to [M-H]$^{-}$ of tri-DNP derivative of paromamine. Bottom traces are SIM with *m/z* 804 corresponding to [M-H]$^{-}$ of tri-DNP derivative of lividamine. Combined mass spectra from the retention time 13.8–14.5 min are shown on the right.

this epimerization, BtrE oxidizes the C3″ moiety of ribostamycin to give 3″-oxoribostamycin, which is stereoselectively reduced by BtrF to afford xylostasin. The stereoselectivity of the NADPH-dependent oxidoreductase BtrF is the same as that of AprD3.[11] The substrate specificity and stereoselectivity of the AprD3/BtrF family of NADPH-dependent reductases appear to be critical in determining the chemical structures of dead-end aminoglycosides from common biosynthetic intermediates such as paromamine and ribostamycin. It is necessary to understand the enzymatic properties of reducing enzymes such as AprD3 and BtrF in addition to the oxidative enzymes such as AprD4 and BtrE to utilize these enzymes in the rationally engineered biosynthesis of aminoglycosides.

The catalytic reaction mechanism of radical SAM dehydratase AprD4 is intriguing, because the proposed radical intermediates are reduced with one electron (Supplementary Figure S1). Several dehydration mechanisms from the C4′-radical intermediate have been proposed by Liu and coworkers.[6] Any reaction mechanisms must introduce one hydrogen atom into the C3′ moiety of 4′-oxolividamine. When we conducted the AprD4/AprD3 reaction with **1** in the presence of D_2O, a deuterium atom was incorporated into the equatorial position at C3′ of **7** (Supplementary Figure S6). This result indicates that hydrogen atom incorporation at the C3′ position would be enzymatically catalyzed with strict selectivity during the dehydration. Mutational analysis is in progress to understand the dehydration mechanism of AprD4 in detail.

In summary, we showed substrate specificity of radical SAM dehydratase AprD4 and NADPH-dependent reductase AprD3 in the C3′-deoxygenation of aminoglycosides. AprD4 recognizes the paromamine/neamine moiety of various aminoglycosides, but cannot accept compounds with a 2′-*N*-acetyl group on 2′-*N*-acetylparomamine and the substituent at C5 of the 2DOS moiety of paromomycin. However, AprD3 strictly recognizes only 4′-oxolividamine-containing molecules, and rejects compounds with an amino group at C6′ of the pseudodisaccharide moiety such as neamine and kanamycin B. Detailed substrate recognition mechanisms of both enzymes should be understood in addition to the dehydration mechanism of unique radical SAM dehydratase AprD4 to enable rationally engineered biosynthesis with these characteristic C3′-deoxygenation enzymes.

Note

During reviewing process of this manuscript, Lv and coworkers reported the same C3-deoxygenation catalyzed by AprD4 and AprD3.[13]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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