

## Biosynthesis of 2-Deoxystreptamine by Three Crucial Enzymes in *Streptomyces fradiae* NBRC 12773

Fumitaka Kudo, Yasuhito Yamamoto, Kenichi Yokoyama, Tadashi Eguchi, Katsumi Kakinuma

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**Abstract** NeoA, B, and C encoded in the neomycin biosynthetic gene cluster have been enzymatically confirmed to be responsible to the formation of 2-deoxystreptamine (DOS) in *Streptomyces fradiae*. NeoC was functionally characterized as 2-deoxy-*scyllo*-inosose synthase, which catalyzes the carbocycle formation from D-glucose-6-phosphate to 2-deoxy-*scyllo*-inosose. Further, NeoA appeared to catalyze the oxidation of 2-deoxy-*scyllo*-inosamine (DOIA) with NAD(P)<sup>+</sup> forming 3-amino-2,3-dideoxy-*scyllo*-inosose (amino-DOI). Consequently, NeoA was characterized as 2-deoxy-*scyllo*-inosamine dehydrogenase. Finally, amino-DOI produced by NeoA from DOIA was transformed into DOS by NeoB. Since NeoB (Neo6) was also reported to be L-glutamine:2-deoxy-*scyllo*-inosose aminotransferase, all the enzymes in the DOS biosynthesis were characterized for the first time.

**Keywords** 2-deoxy-*scyllo*-inosose synthase, L-glutamine:2-deoxy-*scyllo*-inosose aminotransferase, 2-deoxy-*scyllo*-inosamine dehydrogenase, 2-deoxystreptamine, neomycin biosynthetic gene cluster

### Introduction

Aminoglycosides are among clinically important antibiotics for long time and include kanamycin, neomycin, gentamicin, streptomycin *etc.* It has been shown that

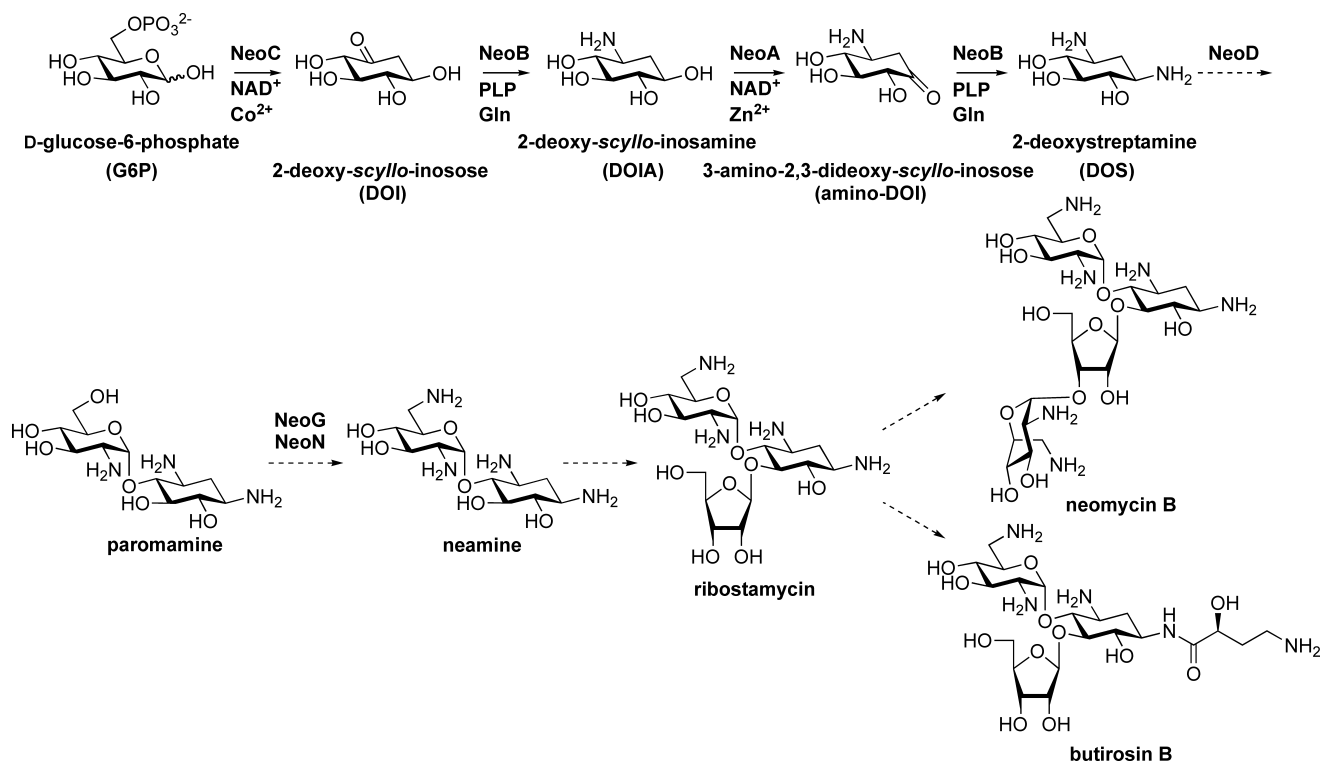
aminoglycosides specifically bind to the bacterial ribosomal RNA and inhibit the bacterial protein biosynthesis [1]. Based on the specific interaction with RNA molecules, many applications such as anti-HIV infection [2, 3] and anti-plasmid [4, 5], are continuously investigated [6–8]. Thus, the creation of a library for structurally diverse aminoglycoside molecules is strongly desired. Biosynthetic approach appears to be promising for this purpose, so that the detailed studies will be necessary for construction of an efficient biosynthetic system.

Concerning the biosynthesis of 2-deoxystreptamine (DOS)-containing aminoglycosides, the biosynthetic study of butirosin in our laboratory leads on the genetic and enzymatic level. 2-Deoxy-*scyllo*-inosose synthase (DOIS), which catalyzes the first crucial carbocycle formation from D-glucose-6-phosphate in the DOS biosynthesis, has been purified from the butirosin producer *Bacillus circulans* and its gene *btrC* was identified based on the N-terminal sequence [9, 10]. Subsequently, the butirosin biosynthetic gene (*btr*) cluster was identified [11, 12]. Also, BtrS and BtrD in the cluster were successfully characterized as a doubly functional L-glutamine:2-deoxy-*scyllo*-inosose (DOI) aminotransferase [13–15] in the DOS biosynthesis and a secondary metabolism specific TDP-glucosamine synthase [16], respectively.

During the recent two years, several groups reported the similar DOS-containing aminocyclitol biosynthetic gene clusters from actinomycetes including kanamycin, gentamicin, tobramycin, ribostamycin, and neomycin

**T. Eguchi** (Corresponding author): Department of Chemistry & Materials Science, Tokyo Institute of Technology, 2-12-1, O-okayama, Meguro-ku, Tokyo 152-8551, Japan.  
E-mail: eguchi@cms.titech.ac.jp

**F. Kudo, Y. Yamamoto, K. Yokoyama, K. Kakinuma:** Department of Chemistry, Tokyo Institute of Technology, 2-12-1, O-okayama, Meguro-ku, Tokyo 152-8551, Japan



**Scheme 1** Biosynthesis of neomycin and butirosin.

Solid lines: The elucidated biosynthetic steps on the enzyme reaction level. Broken lines: Not elucidated yet.

producers, and the functions of the genes have been proposed [17~23]. Among those, three genes *neoA* (*neo5*), *neoB* (*neo6*), and *neoC* (*neo7*) in the neomycin biosynthetic gene cluster have been recently proposed to be responsible to the formation of DOS by comparative analysis of the genes [22]. In the present paper, we clarified the functions of three genes *neoA*, *neoB*, and *neoC*, using the separately expressed proteins derived from the independently identified genes in *Streptomyces fradiae* NBRC 12773.

## Results and Discussion

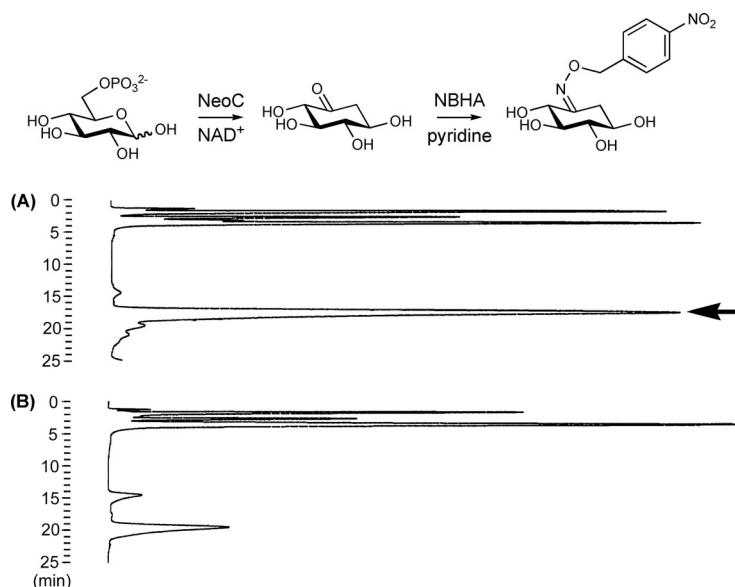
At first, the neomycin biosynthetic (*neo*) gene cluster was obtained from *S. fradiae* NBRC 12773 by screening of the genomic library using a probe of the L-glutamine:DOI aminotransferase gene [24]. The DNA sequence data of the *neo* gene cluster in *S. fradiae* NBRC 12773 has been deposited to the DDBJ under accession number AB211959. The present identified *neo* genes were basically same as those recently reported by Spencer group [22]. Obvious differences were found in the size of *neoD*, *neoE*, and *neoO* corresponding to *neo8*, *neo9*, and *neo19*, respectively.

As Spencer group has reported [22], a comparative analysis of all known DOS-containing antibiotic

**Table 1** Six conserved genes for the neamine biosynthesis

<i>neo</i> gene cluster	<i>btr</i> gene cluster	Functions
<i>neoA</i> ( <i>neo5</i> )	<i>btrE</i>	DOIA dehydrogenase
<i>neoB</i> ( <i>neo6</i> )	<i>btrS</i>	Gln:DOI aminotransferase
<i>neoC</i> ( <i>neo7</i> )	<i>btrC</i>	DOI synthase
<i>neoD</i> ( <i>neo8</i> )	<i>btrM</i>	Putative glycosyltransferase
<i>neoG</i> ( <i>neo11</i> )	<i>btrQ</i>	Putative dehydrogenase
<i>neoN</i> ( <i>neo18</i> )	<i>btrB</i>	Putative aminotransferase

biosynthetic gene clusters by sequence similarity of the encoding proteins, clearly showed the six conserved genes (Table 1); *i.e.* DOIS gene (*btrC*, *neoC*); L-glutamine:DOI aminotransferase gene (*btrS*, *neoB*); a putative glycosyltransferase gene (*btrM*, *neoD*); a putative aminotransferase (similar to glutamate-1-semialdehyde 2,1-aminomutase) gene (*btrB*, *neoN*); a putative alcohol dehydrogenase gene (*btrE*, *neoA*); another putative dehydrogenase (similar to chorine dehydrogenase and related flavoproteins) gene (*btrQ*, *neoG*). These six genes thus are now considered to be involved in the biosynthesis of neamine, which is the core structure of DOS-containing



**Fig. 1** DOIS activity of NeoC (HPLC analysis).

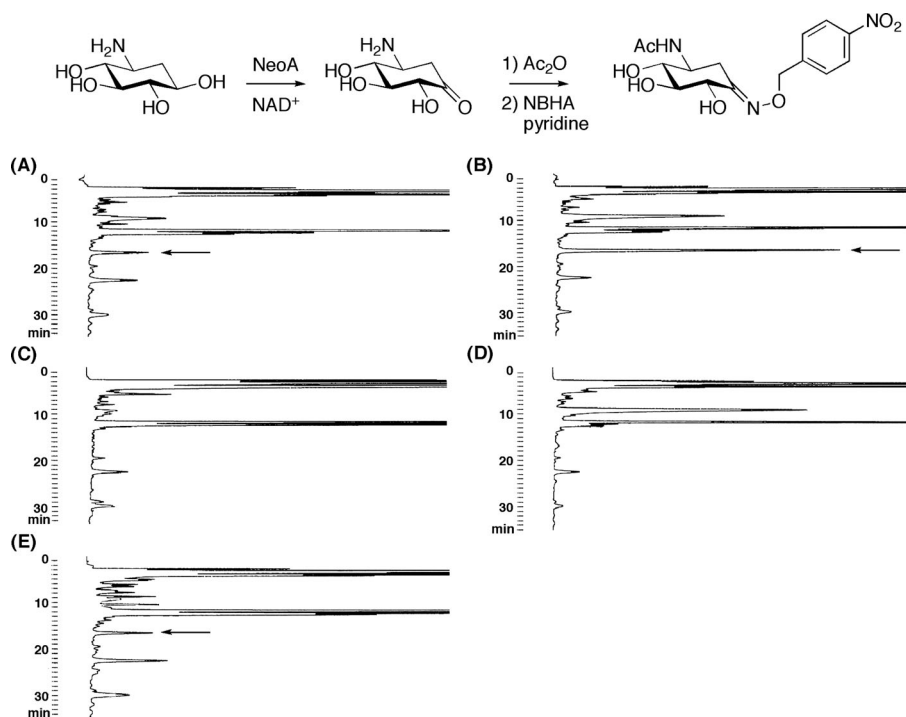
Analytical HPLC traces showing the NeoC catalyzed formation of DOI, which was converted to the nitrobenzyl oxime derivative using 4-nitrobenzylhydroxylamine and pyridine at 60°C for 1 hour. Arrow shows the peak for the DOI-nitrobenzyl oxime derivative (absorbance at 262 nm), which was confirmed by co-injection with the authentic compound [26]. (A) NeoC reaction with D-glucose-6-phosphate (G6P) and NAD<sup>+</sup>. (b) Control reaction without G6P. Some unidentified peaks were observed in the HPLC traces, which may be derived from impurities in the partially pure protein solution.

aminocyclitol antibiotics (Scheme 1). The functions of NeoB, NeoC, and NeoD in the biosynthesis of neomycin were easily assigned based on our previous reports about the functions of the corresponding *btr* genes. In fact, NeoB (Neo6) was clearly reported as L-glutamine:DOI aminotransferase on the enzyme reaction level, although the forward reaction of the second transamination in the DOS biosynthesis has not been examined yet [22]. The *neoA* gene is to encode a zinc-containing alcohol dehydrogenase, which generally uses NAD<sup>+</sup>/NADP<sup>+</sup> as a cofactor. Since the addition of 2-deoxy-*scyllo*-inosamine (DOIA) and NAD<sup>+</sup> into the cell free system of *S. fradiae* was reported to increase the DOS production [25], it seems likely that NeoA could catalyze the oxidation of DOIA because it is the only NAD(P)<sup>+</sup> dependent dehydrogenase among the conserved proteins. The remaining steps from D-glucose-6-phosphate into neamine are the transformations of the hydroxy group at C-6 of the glucosamine moiety in paromamine. NeoG and NeoN have been thus assigned as a dehydrogenase and an aminotransferase in the oxidation and transamination at the position, respectively (Scheme 1).

To confirm whether three continuous enzymes, NeoA, NeoB, and NeoC are responsible to the formation of DOS or not, these were separately expressed in *Escherichia coli*. In the case of NeoC, although the expressed protein was mostly observed in the insoluble pellet, the soluble NeoC

was partially purified by the DEAE ion exchange chromatography and the enzyme activity was analyzed according to the previously reported method [26]. As shown in Fig. 1, NeoC clearly turned out to have DOIS activity in the DOS biosynthesis. Although the C-terminus peptide of NeoC, which is relatively longer than other DOISs, appears to be unnecessary for the activity, the precise function is not unclear at the moment.

The *neoA* gene was also expressed in *E. coli* and the resulting product NeoA was examined without purification for the oxidation of DOIA with either NAD<sup>+</sup> or NADP<sup>+</sup> in the presence of Zn<sup>2+</sup> ion. The formation of the expected enzyme reaction product, 3-amino-2,3-dideoxy-*scyllo*-inosose (amino-DOI), can be analyzed on HPLC by conversion to the *N*-acetyl nitrobenzyl oxime derivative as previously reported [15]. Thus, after the NeoA enzyme reaction, the mixture was treated *in situ* with Ac<sub>2</sub>O for *N*-acetylation and the resulting *N*-acetamide was further converted to the nitrobenzyl oxime derivative with 4-nitrobenzylhydroxylamine (NBHA). After the partial purification using a cartridge column, the resulting samples were analyzed by HPLC (Fig. 2). As a result, the crude cell free extract of *E. coli* containing the expressed NeoA protein appeared to produce amino-DOI, but not in a control reaction without NeoA protein (derived from *E. coli* harboring the pET30 vector). Also, both NAD<sup>+</sup> and



**Fig. 2** Amino-DOI formation from DOIA by NeoA (HPLC analysis).

Arrows at 17 minutes show the peak for the *N*-acetyl-nitrobenzyl oxime derivative of amino-DOI. (A) NeoA reaction with DOIA and NAD<sup>+</sup>. (B) Co-injection with the authentic compound in addition to (A). (C) Control reaction using the cell free extract of *E. coli* containing pET30 vector only with DOIA and NAD<sup>+</sup>. (D) Without DOIA. (E) NADP<sup>+</sup> instead of NAD<sup>+</sup>. A peak at 9 minutes was the degradation product from 4-nitrobenzylhydroxylamine (NBHA), which was not sometimes removed by Sep-Pak purification.

NADP<sup>+</sup> cofactor were found to be effective for the NeoA catalyzed oxidation. These results thus clearly confirmed that NeoA catalyzed the oxidation of DOIA using NAD(P)<sup>+</sup> in the DOS biosynthesis.

Sequence alignment of NeoA, BtrE and the structurally elucidated similar zinc-containing NAD(P)<sup>+</sup> dependent alcohol dehydrogenases (ADHs), BaKR (Ketose reductase, *Bemisia argentifolii*, 1E3J) [27], PaADH (ADH, *Pseudomonas aeruginosa*, 1LLU) [28], htADH (ADH, *B. stearothermophilus*, 1RJW) [29], is shown in Fig. 3. Based on the crystal structural studies, two zinc coordination motifs for catalytic (+ in Fig. 3) and structural (# in Fig. 3) zincs were shown to be important to construct the active site and are highly conserved in this family. Most of these amino acids are conserved in NeoA and thus NeoA appears to be a typical zinc-containing ADH. On the other hand, BtrE does not contain any residue of these. Therefore, it is hypothesized that BtrE may be inactive in the antibiotic biosynthesis or catalyze the reaction by a different manner.

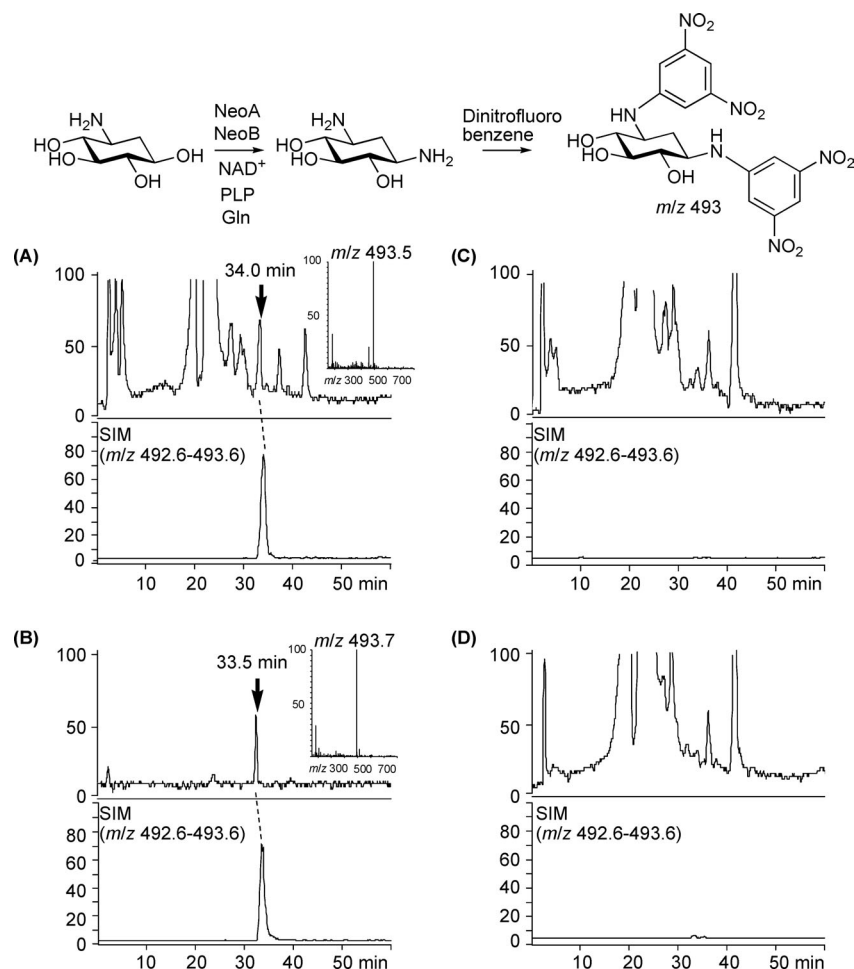
NeoB (Neo6) was reported to catalyze the reverse reaction of the transamination of amino-DOI to DOS as well as BtrS in the butirosin biosynthesis [15, 22]. However, its forward reaction has never been examined due

to the difficulty of the preparation of unprotected amino-DOI. Thus, amino-DOI produced by the NeoA reaction was continuously used as substrate for the natural forward transamination catalyzed by NeoB in the DOS biosynthesis. The continuous conversion of DOIA into DOS consisting of the oxidation of DOIA and the transamination of amino-DOI was thus examined using the cell free extracts containing NeoA and NeoB (Fig. 4). As expected, DOS was produced by the combined use of NeoA and NeoB with DOIA, NAD<sup>+</sup>, PLP and L-glutamine (Fig. 4A). Since the cell free extract of *E. coli* harboring the expression plasmid of NeoB did not produce DOS at all, it was obvious that the expressed NeoA protein was absolutely required for this two-step enzymatic conversion as DOIA dehydrogenase. Therefore, the present study confirmed that NeoA is responsible to the NAD(P)<sup>+</sup>-dependent dehydrogenation of DOIA in the DOS biosynthesis. Also, this is the first report showing the transamination of amino-DOI using the expressed protein. Consequently, DOS biosynthesis from D-glucose-6-phosphate was established at the enzyme reaction level for the first time as shown in Scheme 1.

In summary, we have identified the neomycin biosynthetic gene cluster in *S. fradiae* NBRC12773 and NeoA, B, and C







**Fig. 4** DOS formation from DOIA by NeoA and NeoB (LC-MS analysis).

LC-MS spectra: TOP shows a trace of LC (relative abundance of  $A_{350}$ ). Bottom shows a trace of the selected ion monitoring (SIM) between  $m/z$  492.6~493.6. ESI-MS was analyzed by the negative mode. The scale was normalized for all spectra. (A) All components containing NeoA, NeoB, DOIA,  $NAD^+$ , PLP and Gln. (B) The authentic sample of DOS. (C) Without DOIA. (D) Without NeoA. Arrows at 33~34 minutes show the peak for the bisdinitrophenyl DOS derivative and the offset mass spectra were derived from the peak.

fragments. *E. coli* BL21(DE3) was used for expression of the *neo* genes. The plasmid pUC119 and LITMUS28 (New England Biolabs) were routinely used as a plasmid vector for subcloning and sequencing. The pT7-blue T vector (Novagen) was routinely used as a plasmid vector for the subcloning of PCR amplified DNA. The cosmid vector pOJ446 was used for construction of a cosmid library [31]. *E. coli* containing plasmid were cultured in Luria-Bertani (LB) medium with appropriate antibiotics at 37°C for over night to extract plasmids. Purification of plasmids was carried out with GFX micro Plasmid Prep Kit (Amersham Bioscience). A 100  $\mu$ g/ml of ampicillin or 30  $\mu$ g/ml of kanamycin was added to LB medium.

Oligonucleotides were purchased from Sigma Genosys. Restriction enzymes and modification enzymes were purchased from TaKaRa. PCR was performed by GeneAmp

PCR System 9700 (Perkin Elmer Applied Biosystems) using KOD-plus-DNA polymerase (TOYOBO) or LA-Taq DNA polymerase (TaKaRa). DNA sequence analysis was carried out with a LONG READER 4200 (Li-Cor) and a Thermo Sequenase Cycle Sequencing Kit according to the manufacturer's protocol. HPLC was performed on a Hitachi L-6250 Intelligent Pump equipped with a L-4000H UV Detector and a D-2500 Chromato-Integrator for the enzyme assay. A LCQ mass spectrometer (Finnigan) connected with NANOSPACe HPLC and SE-1 UV detector (Shiseido, Japan) equipped with a RP-18 GP column (Kanto Chemical, Japan) was used for the LC-MS analysis. A FPLC (Pharmacia Biotech.) was used for protein purification. Other chemicals were of highest grade commercially available.

### Identification of the *neo* Gene Cluster in *S. fradiae*

A large amount of chromosomal DNA from *S. fradiae* NBRC 12773 was extracted by the standard protocol from the cells cultured in a 200 ml medium for 8 days [32]. The chromosomal DNA was partially digested with *Sau3AI* for 10 minutes and the restriction enzyme was denatured with the phenol-chloroform extraction. After the ethanol precipitation, the digested DNA fragments were treated with Calf intestine alkaline phosphatase (CIAP) at 50°C for 30 minutes and CIAP was denatured with the phenol-chloroform extraction. A cosmid vector pOJ446 was separately digested with *HpaI*, and after the treatment of CIAP, followed by digestion with *BamHI*. After the phenol-chloroform extraction and the ethanol precipitation, the resulting DNA was dissolved with TE buffer. The digested pOJ446 and the partially digested chromosomal DNA were ligated using DNA Ligation kit Ver.2 (TaKaRa). After the ethanol precipitation, the ligated DNA was dissolved with TE buffer. The resulting ligation mixture was packaged into  $\lambda$  phage followed by phage transfection to *E. coli* XL1 Blue MRF' by Gigapack III Gold Packaging Extract (Stratagene) according to the manufacturer's protocol. The host strain *E. coli* XL1 Blue MRF' was cultured in 3 ml of LB containing 10 mM MgSO<sub>4</sub> and 0.2% maltose by OD<sub>600</sub> 1-2. 1 ml of the culture was centrifuged and the resulting wet cells were suspended in a 10 mM MgSO<sub>4</sub> up to OD<sub>600</sub> 1 for transfection.

The cosmid library of 2,000 clones in *E. coli* was directly screened by hybridization with a Dig labeled DNA probe, which was made from the PCR amplified DNA fragments for the partial gene of L-Gln:DOI aminotransferase by the DIG DNA Labeling Kit (Roche). PCR conditions were 94°C, 5 minutes for denature, 30 cycles of 94°C, 30 seconds, 62°C, 30 seconds, 72°C, 30 seconds for extension of DNA in 5  $\mu$ l of 2 $\times$ GC buffer II, 1.6  $\mu$ l of dNTP (2.5 mM each), 0.2  $\mu$ l of neoSP-F 5'-TGGGTCGCCTCCGGCTCCACCG-3' (50  $\mu$ M), 0.2  $\mu$ l of neoSP-R 5'-GCAGCGTTGCTGCCCATCAGC-3' (50  $\mu$ M), 0.1  $\mu$ l of LA-Taq DNA polymerase, 1  $\mu$ l of template solution (10 ng/ $\mu$ l of chromosomal DNA), and 1.9  $\mu$ l of water. The hybridization was carried out by standard manner and the hybridized DNA was detected by DIG Nucleic Acid Detection Kit with NBT/BCIP solution (Roche) according to the manufacturer's protocol.

Positive 16 clones were cultured and the cosmids were extracted by standard protocol. The cosmids were examined for whether they contained the L-Gln:DOI aminotransferase gene by PCR under the conditions as mentioned above followed by Southern hybridization with the same probe for screening after *KpnI* digestion. Among the positive cosmids, the cosmid cfra10 was randomly

sequenced by a shotgun sequence method on double-stranded DNA templates (Hitachi Instruments Service). All gaps in the draft sequence were separately cloned from the cosmid cfra10 and the DNA sequence was determined by a DNA sequencer. As a result, 39 kbp of DNA sequence was determined. The ORFs were determined by FramePlot analysis [33] ([www.nih.go.jp/~jun/cgi-bin/frameplot.pl](http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl)) and blast homology search through web considering a possible Shine-Dargano sequence. The DNA sequence data of the *neo* gene cluster in *S. fradiae* NBRC 12773 has been deposited to the DDBJ, EMBL, and GenBank databases under accession number AB211959.

### Expression and Functional Analysis of the *neoC* Gene Encoding DOI Synthase

The *neoC* gene was amplified by PCR from the cosmid cfra10 with primers neoC-F: 5'-GCCATATGCAGAC-CACCCGC-3' and neoC-R: 5'-GCGGGAAGCTTGCCGAC-3'. PCR conditions were 94°C, 2 minutes for denature, 25 cycles of 94°C, 15 seconds, 52°C, 30 seconds, 68°C, 70 seconds for extension of DNA in 1  $\mu$ l of 10 $\times$ KOD buffer, 1  $\mu$ l of dNTP (2.0 mM each), 0.4  $\mu$ l of MgSO<sub>4</sub> (25 mM), 0.5  $\mu$ l of DMSO, 0.1  $\mu$ l of neoC-F (50  $\mu$ M), 0.1  $\mu$ l of neoC-R (50  $\mu$ M), 0.2  $\mu$ l of KOD-plus polymerase, 0.6  $\mu$ l of cfra10 (50 ng/ $\mu$ l), and 6.1  $\mu$ l of water. The amplified PCR product was digested with *HindIII* and the resulting DNA fragment was subcloned into the LITMUS28 previously digested with *EcoRV* and *HindIII* to obtain LITMUS28-*neoC*. After confirmation of the DNA sequence, the appropriate plasmid was digested with *NdeI* and *HindIII* and the resulting DNA fragment was inserted into the corresponding site of pET30b (Novagen) to obtain pET-*neoC*. The pET-*neoC* was introduced into *E. coli* BL21(DE3) by a standard chemical transformation. The *E. coli* harboring pET-*neoC* was grown in a LB medium supplemented with 30  $\mu$ g/ml of kanamycin at 37°C by OD<sub>600</sub> 0.7 and a final 0.25 mM of isopropyl  $\beta$ -D-thiogalactoside was added for induction of overexpression. The culture was continued at 18°C for over night and the cells were harvested by centrifugation (6,000 rpm $\times$ 30 minutes). The wet cells (2 g) were suspended in 20 ml of 50 mM Tris buffer (pH 7.5) containing 1 mM of CoCl<sub>2</sub> and 10% glycerol and disrupted by French Press (Thermo IEC). After centrifugation (12,000 rpm $\times$ 30 minutes), the supernatant was loaded onto a DEAE-Sepharose Fast Flow column (2.5 $\times$ 10 cm) previously equilibrated with the same buffer. NeoC was eluted with a linear gradient of 0~0.3 M of NaCl in the same buffer. The fractions containing NeoC were combined and concentrated by ultrafiltration (VIVASPIN 20, Vivascience, Germany). DOI synthase reaction was carried out in 5 mM of glucose-6-phosphate,

5 mM of  $\text{NAD}^+$  with the partially purified NeoC, at 46°C, for 2 hours. The product DOI was analyzed by previously reported method [26].

#### Expression of the *neoA* and *neoB* Genes

The expression systems for the *neoA* and *neoB* gene were constructed by same method for functional analysis of the *neoC* gene, except primers (neoA-F: 5'-GCCATATGAAGGCTCTGGTG-3' and neoA-R: 5'-GGGCAAGCTTTCCTGAGGG-3' for the *neoA* gene, neoB-F: 5'-GCCATATGGTCTCCCCGTTG-3' and neoB-R: 5'-GCATGAAGCTTCCCGTCC-3' for the *neoB* gene). The *neoA* and *neoB* gene were separately expressed by the same conditions mentioned above. The wet cells (2 g) harboring the *neoA* and *neoB* gene expression plasmid were independently suspended in 10 ml of 50 mM Tris buffer (pH 8.0) containing 0.5 mM of  $\text{ZnCl}_2$  and disrupted by French Press (Thermo IEC). After centrifugation (12,000 rpm×30 minutes), the supernatants were independently used for enzyme reactions as the cell free extracts.

#### Detection of the Amino-DOI Formation from DOIA by NeoA

The enzyme reaction was performed using the cell free extracts of NeoA with 5 mM of DOIA, 10 mM of  $\text{NAD}^+$  in total 200  $\mu\text{l}$  of 50 mM Tris buffer (pH 8.0) containing 0.5 mM of  $\text{ZnCl}_2$  at 28°C for 12 hours. To the resulting mixture (100  $\mu\text{l}$ ) was added 300  $\mu\text{l}$  of acetic anhydride, and then reacted at 37°C for 3 hours. After removal of solvent by a centrifugal evaporator, the residue was dissolved with 200  $\mu\text{l}$  of methanol, and followed by addition of 100  $\mu\text{l}$  of 4-nitrobenzylhydroxylamine solution in pyridine (10 mg/ml), and then reacted 60°C for 3 hours. The residue was dissolved with 2.1 ml of 5% methanol in chloroform and the resultant solution was passed through the Sep-Pak® Plus Silica (Waters). The cartridge was washed with 6 ml of 6.3% methanol in chloroform and the expected product, *N*-acetyl nitrobenzyl oxime, was eluted with 5 ml of 17% methanol in chloroform. After concentration of the solution, the residue was dissolved with 100  $\mu\text{l}$  of methanol. An aliquot of the solution (5  $\mu\text{l}$ ) was injected into a HPLC (Hitachi) equipped with a PEGASIL ODS column (4.6 mm×250 mm, Senshu) and eluted with 30% aqueous methanol at a flow rate 1 ml/minute at 40°C. The elution was monitored by 254 nm.

#### DOS Formation from DOIA by NeoA and NeoB

The enzyme reaction was performed using the cell free extracts of NeoA and NeoB with 5 mM of DOIA, 5 mM of  $\text{NAD}^+$ , 0.5 mM of PLP, and 10 mM of glutamine at 28°C

for 12 hours. The resulting mixture (50  $\mu\text{l}$ ) was reacted with 25  $\mu\text{l}$  of 5% 2,4-dinitrofluorobenzene in methanol, 12.5  $\mu\text{l}$  of DMSO, and 2.5  $\mu\text{l}$  of 2 M NaOH at 60°C for 1 hour. After removal of the solvent by a centrifugal evaporator, the residue was dissolved with ethyl acetate and water, and the ethyl acetate layer was separated and concentrated. The residue was dissolved with 2.1 ml of 5% ethyl acetate in hexane. The resultant solution was passed through the Sep-Pak® Plus Silica (Waters). The cartridge was washed with 6 ml of 10% ethyl acetate in hexane and the expected product, bisdinitrophenyl DOS, was eluted with 5 ml of ethyl acetate. After concentration of the solution, the residue was dissolved with 100  $\mu\text{l}$  of methanol. An aliquot of the solution (5  $\mu\text{l}$ ) was injected into a HPLC connected with LCQ mass spectrometer and eluted with 10% aqueous methanol for 10 minutes, and 62% aqueous methanol for 40 minutes at a flow rate 40  $\mu\text{l}$ /minute. The elution was monitored by 350 nm and analyzed by the negative mode. LC-ESI-MS (negative): 33~34 minutes,  $m/z$  493.5 ( $\text{M}-\text{H}^-$ ); calcd for  $\text{C}_{18}\text{H}_{17}\text{N}_6\text{O}_{11}$ : 493.382.

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