Identification of L-Glutamine : 2-Deoxy-*scyllo*-inosose Aminotransferase Required for the Biosynthesis of Butirosin in *Bacillus circulans*

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Using inverse PCR, two new genes (*btrN* and *btrS*) were identified upstream of the putative glycosyltransferase gene *btrM* in the butirosin-biosynthetic *btr* gene cluster of *Bacillus circulans*. The upstream gene *btrS* showed significant homology with *stsC* of *Streptomyces griseus*, which encodes L-glutamine:*scyllo*-inosose aminotransferase in the biosynthesis of streptomycin. The function of BtrS was further confirmed by heterologous expression in *Escherichia coli* and chemical identification of the conversion of 2-deoxy-*scyllo*-inosose into 2-deoxy-*scyllo*-inosamine. The identification of BtrS as L-glutamine : 2-deoxy-*scyllo*-inosose aminotransferase is the first report of the aminotransferase gene responsible for 2-deoxystreptamine biosynthesis.

Aminoglycosides have been one of the most clinically-important groups of antibiotics for some time. Based on their chemical structures, aminoglycosides can be classified into two major groups. One has as aglycone a fully-substituted aminocyclitol which is believed to be synthesized from *myo*-inositol. Streptomycin, fortimicin, and spectinomycin are among this group, and genetic as well as enzymic analyses of their biosynthesis have been reported.[1~3] The other group of aminoglycosides has the common aglycone of 2-deoxystreptamine (DOS), and, numerous clinically important compounds are included in this category, such as neomycin, kanamycin, ribostamycin, butirosin, gentamicin, tobramycin and sisomicin. The biosynthetic relationships between these two classes has been long recognized. However, no evidence to substantiate this has been presented on the enzymic or genetic level.

The biosynthesis of DOS-containing aminoglycosides has been studied mostly by the use of isotope-tracer technology with whole cell systems of producing organisms or their mutants.[4~8] The biosynthetic pathway of butirosin, produced by a *Bacillus* species is proposed as shown in Fig. 1.[6] However, a biosynthetic gene of DOS-containing aminoglycosides has only recently been identified. Genetic analysis had been carried out on the butirosin-producer *Bacillus circulans* and a region around the self-resistance gene (*butA*) was analyzed.[9] While a gene for butirosin-transport (*butB*) was found in this particular region, no biosynthetic gene was found. With respect to enzymes involved in the biosynthesis of these antibiotics only transamination had been detected.[10,11] In our laboratory, efforts were focused on the study of 2-deoxy-*scyllo*-inosose (DOI) synthase, the key enzyme for the initial step of DOS biosynthesis using isotope-labeled substrates or substrate analogues.[12~17] DOI synthase is thought to be essential for the biosynthesis of DOS-containing aminoglycosides.

DOI synthase from the butirosin-producer *Bacillus circulans* has been purified.[18] The enzyme is composed of two subunits with molecular mass of 40 and 20 kDa, respectively. Subsequently, the gene for the larger subunit (*btrC*) was identified and over-expressed in *Escherichia coli* as confirmation of its catalytic function;[19] a butirosin-biosynthetic gene cluster containing *btrC* identified.[20] These results are the only report of the biosynthetic genes of DOS-containing aminoglycoside to date.

In the present study, we analyzed the genes upstream of *btrM* (putative glycosyltransferase gene)[20] in the gene cluster, and found two additional ORFs. One of these, *btrS*, was cloned and expressed in *E. coli*, and its function as

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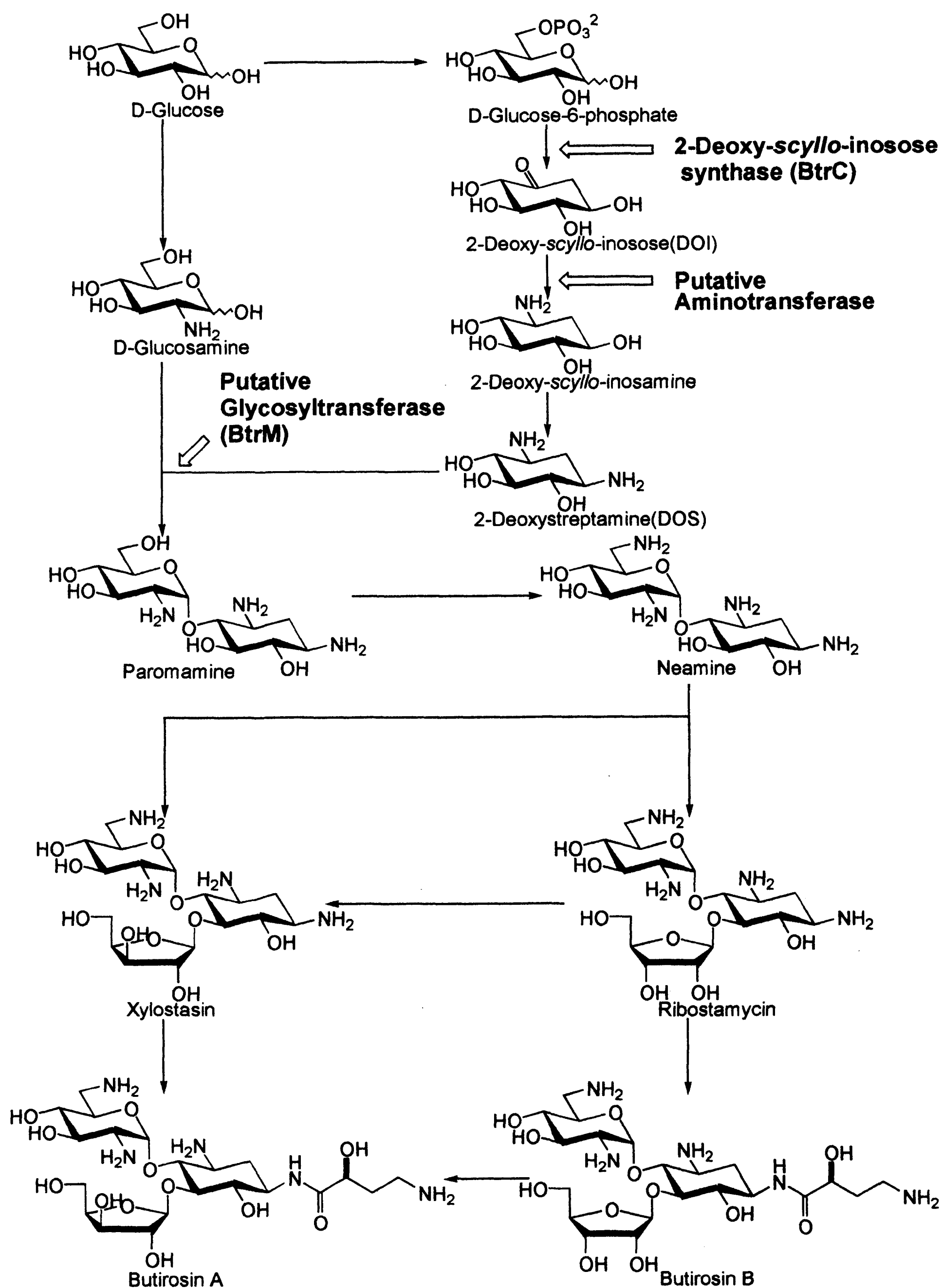
Fig. 1. The proposed biosynthetic pathway of butirosin.



L-glutamine : DOI aminotransferase was confirmed.

Materials and Methods

Organisms

B. circulans SANK72073 was cultured as described previously.[18] *E. coli* JM109 was grown in LB medium or on LB-agar containing 50 μg/ml of ampicillin, as necessary.

Cloning of the Upstream Region of *btrM* by Inverse PCR

Based on the sequence determined previously,[20] two oligonucleotide primers, HH5-1 (GTCATTGTGCCAG-ATATCCGAAATATGCT) and btrMf-1 (GGTTTGATC-

CGATGGGCGGCATGCA), were designed. *Dra* I-digested chromosomal DNA of *B. circulans* was extracted with phenol-chloroform and the mixture was subjected to self-ligation. The resulting circular DNAs were used as template for amplification. The PCR conditions were; 1 cycle at 95°C for 10 minutes, followed by 30 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 5 minutes, and then 72°C for 7 minutes, using AmpliTaq Gold DNA polymerase (Applied Biosystems). The PCR product was subcloned into pT7-blue T vector (Novagen), and the resulting plasmid (pDS16) was sequenced. A minimum of 3 individual clones were so analyzed.

Expression of *btrS* in *E. coli*

Primers btrS-f (TTCATATGACCATTCCATTTGACCA) and btrS-r (AAGGATCCTTCGTTAATGAGTCACG) were designed and used for PCR; 1 cycle at 98°C for 5 minutes, followed by 30 cycles of 98°C for 10 seconds, 55°C for 1 minute, and 72°C for 1.5 minutes, using Pyrobest DNA polymerase (TaKaRa, Japan). The PCR product was 5′-phosphorylated, and subcloned into the *Sma* I-site of pUC119 to yield pUCbtrS. *E. coli* JM109 carrying pUCbtrS was grown in LB medium containing 50 μg/ml of ampicillin to OD_{600} 0.6, and, isopropyl β-D-thiogalactopyranoside added to a final concentration of 1 mM. After 4 hours, cells were harvested by centrifugation (10,000×*g*, 10 minutes) and stored at −30°C until use.

Enzyme Assay

Cells of *E. coli*/pUCbtrS and *E. coli*/pUC119 (as a control) were separately suspended in 50 mM BES-NaOH (pH 7.7) buffer (5 g of wet cells in 50 ml), and the suspensions sonicated using a Branson sonifier Type-250 at 0°C for 2 minutes×10 times in an ice-water bath. The sonicates were centrifuged at 15,000×*g* for 30 minutes and the resulting cell-free extract used for enzyme analysis. Reaction mixtures contained 50 mM BES-NaOH (pH 7.7), 25 mM L-glutamine, 0.5 mM pyridoxal phosphate, 5 mM DOI and 100 μl cell-free extract in a total volume of 200 μl. The mixtures were incubated at 37°C for 3 hours, and filtered through a Microcon YM-10 (Millipore, USA) to remove protein (>10,000 MW).

Dansyl chloride (10 mg/ml in CH_3CN, 75 μl) and 2 N NaOH (15 μl) were added to an aliquot (25 μl) of each enzyme reaction mixture. Derivatization was carried out at 37°C for 1.5 hours, and 5 μl of the reaction mixture injected into a HPLC system equipped with a Senshu Pak ODS 1251N column (4.6 mm i.d.×25 cm, Senshu Scientific, Japan). The eluent was 20% CH_3CN in 0.2 M sodium acetate with a flow rate of 1 ml/minute. HPLC was performed on a Hitachi L-7100 pump equipped with a Hitachi L-7485 FL-detector, a Hitachi column oven L-7300 and a Hitachi D-2500 integrator. Elution was monitored by fluorescence detection (excitaion; 331 nm and fluorescence emission; 523 nm).

Large-scale Preparation of 2-Deoxy-*scyllo*-inosamine

A mixture of a cell-free extract of *E. coli*/pUCbtrS (10 ml), 0.5 mM pyridoxal phosphate, 25 mM L-glutamine, 5 mM DOI in 50 mM BES-NaOH buffer, pH 7.7, in a total volume of 20 ml was incubated at 37°C for 3 days. The reaction product was chromatographed on Dowex 50W X-8 (H^+) with gradient elution from 0.1 N HCl to 0.7 N HCl to give 1.3 mg of purified 2-deoxy-*scyllo*-inosamine as hydrochloride salt; ^{13}C-NMR (D_2O): δ 33.3, 50.7, 69.5, 73.8, 75.2, 77.1.

Reagents and Materials

DNA manipulation was performed as described in the literature[21]. DNA sequencing was carried out with a LONG READIR 4200 (Li-Cor) according to the manufacturer's protocol. PCR was performed using a GeneAmp PCR System 9700 (Amersham Pharmacia). Oligo DNAs for PCR primer were custom-synthesized by Amersham Pharmacia. Purification of plasmids was carried out with the GFX Micro Plasmid Prep Kit (Amersham Pharmacia). Restriction and modification enzymes were purchased from TaKaRa (Japan). Genomic DNA extractions were carried out with Dr. GENTLE (TaKaRa). Genetic analysis was performed with GENETYX-WIN ver. 3 (Software Development, Japan), and database searches were carried out by FASTA and BLAST2 on the Internet. DOI was synthesized as described previously[22]. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a JEOL AL-400 spectrometer. NMR chemical shifts were reported in δ value based on internal TMS (0 ppm) or solvent signal ($CDCl_3$ δ_C=77.0; D_2O δ_H=4.65; CD_3OD δ_H=3.30, δ_C=49.0) as references. When the solvent was D_2O, dioxane was used as an internal standard (δ_C=67.4). Column chromatography was carried out with Merck Kieselgel 60 Art. Nr. 7734. Preparative thin layer chromatography was carried out with Merck Kieselgel 60 F_{254} plates Art. Nr. 5744. All other reagents were of the highest grade commercially available.

Synthesis of (±)-2-Deoxy-*scyllo*-inosamine

All reactions, except for catalytic hydrogenation reaction, were carried out in an inert (Ar or N_2) atmosphere.

DL-1,2,4/3,5-Tetrabenzyloxycyclohexan-1-ol (**2**)

To a solution of *O*-tetrabenzyl-2-deoxy-*scyllo*-inosose **1**[22] (217 mg, 0.416 mmol) in dry THF (3 ml) was added Super-Hydride™ (1.0 M THF sol. 0.70 ml, 0.64 mmol) slowly at −78°C. The mixture was stirred for 2 hours at −78°C. The mixture was then diluted with water and 2 ml of 2 N HCl, and then extracted with EtOAc (50 ml×2). The combined organic extract was washed with NaCl-saturated water and dried over anhydr. $MgSO_4$. After filtration and removal of the solvent, the residue was purified by silica gel column chromatography (hexane - EtOAc, 7 : 1) to afford 202 mg of **2** (93% yield): IR ($CHCl_3$) cm^{-1} 3066, 2927, 1454, 1365, 1070, 1028; ^{1}H-NMR ($CDCl_3$) δ 1.34 (1H, t, J=10 Hz), 2.35 (1H, dt, J=4.0 and 10 Hz), 2.44 (OH, br), 3.48 (2H, m), 3.83 (1H, t, J=9.6 Hz), 3.94 (1H, ddd, J=4.8, 9.2 and 12 Hz), 4.11 (1H, m), 4.80 (8H, m, PhCH_2O–), 7.33 (20H, aromatic); ^{13}C-NMR ($CDCl_3$) δ 32.4, 65.8, 72.7, 72.8, 75.6, 76.6, 81.5, 82.7, 85.7, 127.4, 127.4, 127.7, 127.8, 127.9, 128.2, 128.2, 128.4, 137.9, 138.6, 138.7, 138.8. *Anal.* Calcd. for $C_{34}H_{36}O_5$: C, 77.84; H, 6.92. Found: C, 78.04; H 6.92.

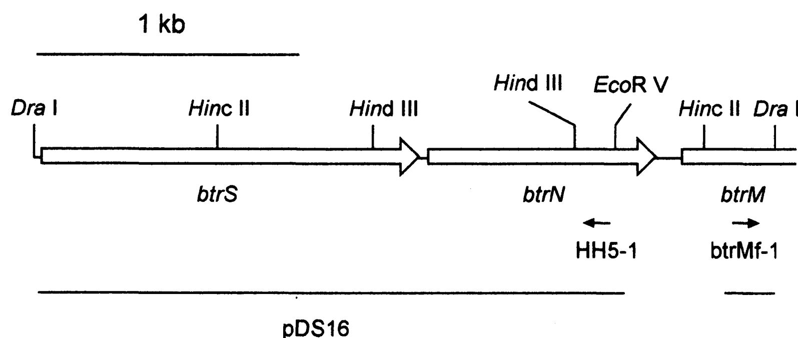
DL-1,2,4/3,5-Tetrabenzyloxycyclohexyl Methanesulfonate (**3**)

To a solution of **2** (151 mg, 0.288 mmol) in pyridine (2.5 ml) were added DMAP (10 mg, 0.082 mmol) and methanesulfonyl chloride (MsCl, 49 μl, 0.63 mmol). The mixture was stirred for 19 hours at room temperature. Additional MsCl (25 μl, 0.32 mmol) and pyridine (1.5 ml) was added to the mixture and stirring was continued for 3 hours. The mixture was diluted with water at 0°C and extracted with EtOAc (40 ml×2). The combined organic extract was washed with 2 N HCl, sat. aq. $NaHCO_3$ and NaCl-saturated water, and then dried over anhydr. $MgSO_4$. After filtration and removal of the solvent, the residue was crystallized from hexane - EtOAc to afford 127 mg of **3** (73% yield): mp 76~78°C; IR ($CHCl_3$) cm^{-1} 2927, 2871, 1454, 1363, 1173; ^{1}H-NMR ($CDCl_3$) δ 1.54 (1H, td, J=2.0 and 12 Hz), 2.47 (1H, dt, J=4.4 and 12 Hz), 2.97 (3H, s), 3.51 (1H, t, J=9.6 Hz), 3.53 (1H, t, J=9.6 Hz), 3.80 (1H, t, J=9.2 Hz), 3.90 (1H, ddd, J=4.4, 9.2 and 12 Hz), 4.68 (4H, m, PhCH_2O–), 4.87 (4H, m, PhCH_2O–), 5.16 (1H, m) 7.31 (20H, aromatic); ^{13}C-NMR ($CDCl_3$) δ 32.4, 38.7, 72.8, 73.2, 75.8, 76.4, 76.5, 80.3, 80.8, 85.1, 127.3, 127.4, 127.5, 127.5, 127.6, 127.7, 127.8, 127.9, 128.1, 128.1, 128.2, 137.0, 137.9, 138.1, 138.3. *Anal.* Calcd. for $C_{35}H_{38}O_7S$: C, 69.79; H, 6.35; S, 5.32. Found: C, 69.54; H, 6.09; S, 5.16.

DL-1,3,5/2,4-1-Azido-2,3,4,5-tetrabenzyloxycyclohexane (**4**)

To a solution of **3** (127 mg, 0.210 mmol) in DMF (3 ml) was added sodium azide (141 mg, 2.17 mmol). The mixture was stirred for 4 hours at 50°C and then at 70°C for 14 hours. Additional sodium azide (52.7 mg, 0.811 mmol) was added to the mixture and stirring was continued for 5 hours at 70°C. The mixture was then diluted with water and extracted with EtOAc (30 ml×2). The combined organic extract was washed with NaCl-saturated water, and then dried over anhydr. $MgSO_4$. After filtration and removal of the solvent, the residue was purified by silica gel column chromatography (hexane~hexane - EtOAc, 20 : 1) to afford 109 mg of **4** (94% yield): mp 73~76°C; IR ($CHCl_3$) cm^{-1} 2873, 2103, 1454, 1363, 1068, 1027; ^{1}H-NMR ($CDCl_3$) δ 1.34 (1H, t, J=10 Hz), 2.35 (1H, dt, J=4.0 and 10 Hz), 2.44 (OH, br), 3.48 (2H, m), 3.83 (1H, t, J=9.6 Hz), 3.94 (1H, ddd, J=4.8, 9.2 and 12 Hz), 4.11 (1H, m), 4.80 (8H, m, PhCH_2O–), 7.33 (20H, aromatic); ^{13}C-NMR ($CDCl_3$) δ 33.9, 61.2, 73.8, 77.9, 78.2, 78.5, 78.7, 84.9, 85.6, 86.3, 128.7, 128.8, 128.9, 128.9, 129.0, 129.3, 129.5, 129.5, 129.5, 138.8, 139.2, 139.4, 139.5. *Anal.* Calcd. for

Fig. 2. Genetic map of the region upstream of *btrM*.



Arrows indicate positions of primers for inverse PCR.

Fig. 3. Deduced sequence of BtrS and alignment with StsC of *S. griseus* (Y08763).

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BtrS     1:M----TI---P-F-DH-WPEWPQHSDRTRRKIEEVFQSNRWAIS-GYWTGEESMERKFAK  49
StsC     1:MDSSLAISGGPRLSNREWPRWPQPGDRALKSLEDVLTSGRWTISCAY-QGRDSYERQFAS  59

BtrS    50:AFADFNGVPYCVPTTSGSTALMLALEALGIGEGDEVIVPSLTWIATATAVLNVNALPVFV 109
StsC    60:AFADYCGSAMCVPISTGTASLAIALEACGVGAGDEVIVPGLSWVASASAVLGINAVPVLV 119

BtrS   110:DVEADTYCIDPQLIKSAITDKTKAIIPVHLFGSMANMDEINEIAQEHNLFVIEDCAQSHG 169
StsC   120:DVDPATYCLDPAATEAAITERTRAITVVHAYSAVADLDALLDIARRHGLPLIEDCAHAHG 179

BtrS   170:SVWNNQRAGTIGDIGAFSCQQGKVLTAGEGGIIVTKNPRLFELIQQLRADSRVYCDDSSE 229
StsC   180:AGFRGRPVGAHGAAGVFSMQGSKLLTCGEGGALVTDDADVALRAEHLRADGRVVRREPVG 239

BtrS   230:LMHGDMQLVKKGDIQGSNYCLSEFQSAILLDQLQELDDKNAIREKNAMFLNDALSKIDGI 289
StsC   240:-V-GEMELEETGRMMGSNACLSEFHAAVLLDQLELLDGQNARRTRAADHLTDRLSEL-GM 296

BtrS   290:KVMKRPPQVSRQTYYGYVFRFDPVKFGGLNADQFCEILREKLNMGTFYLHPPYLPVHKNP 349
StsC   297:TAQATAPGTTARAYYRYLVRLPDEVLAVAPVERFAHALTAEL--G-FAVTQTHRPLNDNP 353

BtrS   350:LFCPWTKNRYLKSVRKTEAYWRG-LHYPVSERASGQSIVIHHAILLAEPSHLSLLVDAVA 408
StsC   354:LNRPSSRRRFATDARYLERVDPSRFDLPAAKRAHESVVSFSHEVLLAPLDAIDDIARAFR 413

BtrS   409:E-LARKFCVTH                                                 418
StsC   414:KVLDNVREVSR                                                 424
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Fig. 4. The function of BtrS (A) and StsC (B).

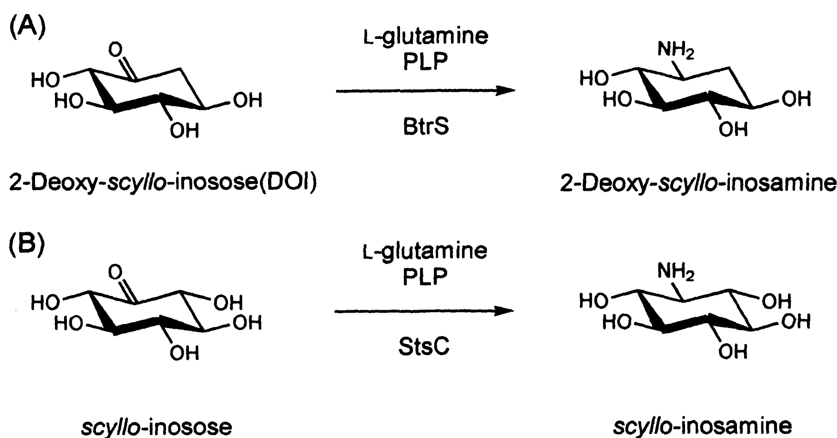
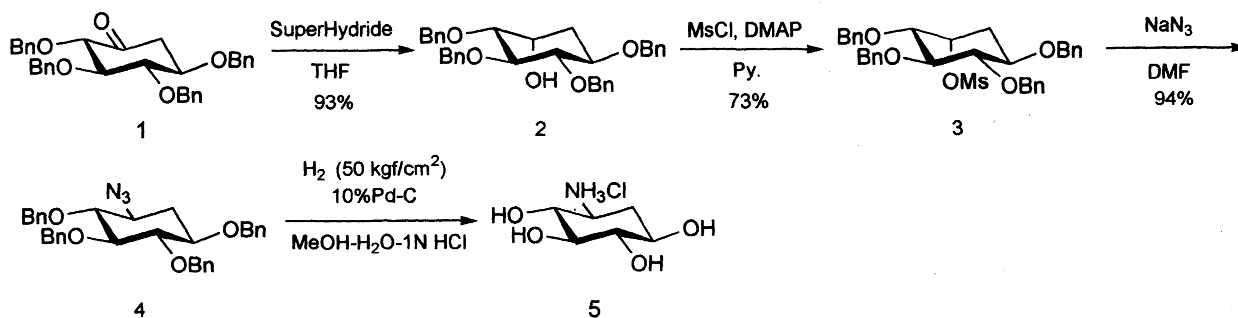


Fig. 5. Chemical synthesis of 2-deoxy-*scyllo*-inosamine.



$C_{34}H_{35}O_4N_3$: C, 74.79; H, 6.42; N, 7.64. Found: C, 74.01; H, 6.49; N, 7.65.

(±)-2-Deoxy-*scyllo*-inosamine HCl (**5**)

To a solution of **4** (304 mg, 0.553 mmol) dissolved in a minimal volume of EtOAc were added MeOH - H_2O - 1 N HCl (1.0 ml - 0.39 ml - 0.15 ml) and 1.11 g of 10% Pd-C (Aldrich, USA) and the mixture was placed in a pressure-reactor. The atmosphere was first replaced with hydrogen gas then filled with pressurized hydrogen gas (50 kgf/cm^2). The mixture was vigorously stirred under these conditions at room temperature for 4 days. After renewal of catalyst, the same procedure was repeated. The catalyst was then removed by filtration, and the filtrate was evaporated *in vacuo* to give pale yellow solid **5**. The solid was then suspended in acetone and filtered. The precipitate was further washed with MeOH to give **5** as a white solid (108 mg, 98%): ^{1}H-NMR (400 MHz, D_2O) δ 1.45 (1H, ddd, J=12.4, 12.4 and 4.4 Hz), 2.14 (1H, ddd, J=12.4, 4.4 and 4.4 Hz), 3.06 (1H, ddd, J=12.4, 10.6 and 4.4 Hz), 3.07 (1H, q, J=9.6 Hz), 3.16 (2H, m), 3.32 (1H, m), 3.47 (1H, m); ^{13}C-NMR (D_2O) δ 33.2, 50.8, 69.4, 73.7, 75.2, 77.0.

Accession Number

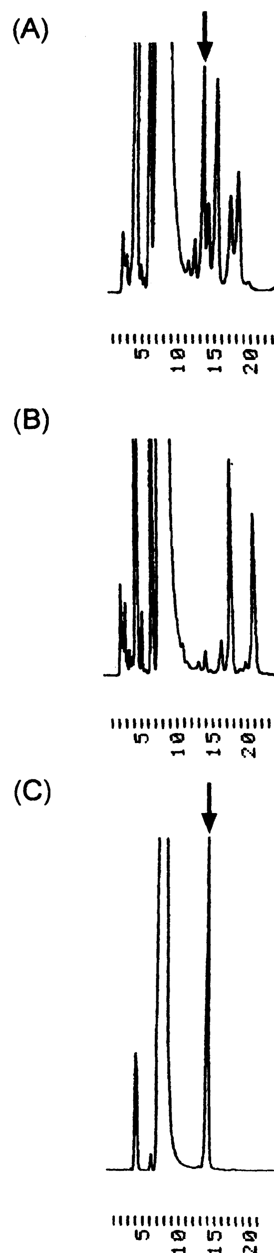
The sequence determined in the present study has been deposited in DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number of AB066276.

Results and Discussion

Identification of the Upstream Region of *btrM*

The upstream region of *btrM* (putative glycosyltransferase gene)[20] was analyzed by inverse PCR (Fig. 2). The *btrN* gene located immediately upstream of *btrM*, showed no similarity to any known sequences by FASTA or BLAST2 searches. Further upstream of *btrN*, the gene *btrS* was found. The latter showed significant homology to *stsC* of *Streptomyces griseus*, the gene encoding L-glutamine : *scyllo*-inosose aminotransferase in the biosynthesis of streptomycin[3], as shown in Fig. 3 (40% identity by FASTA). *BtrS* appeared to be a component of the butirosin-biosynthetic gene cluster, and, the function of BtrS was deduced to be L-glutamine : DOI aminotransferase, catalyzing the reaction shown in Fig. 4. Previously, transamination activity of ketocyclitols from L-glutamine was described in cell-free extracts of several DOS-containing aminoglycoside producers[10,11,23,24]. However, neither the enzymes nor the corresponding genes were identified.

Fig. 6. HPLC profiles of the dansyl derivatives from enzyme reactions using cell-free extracts from (A) *E. coli*/pUCbtrS or (B) *E. coli*/pUC119, and (C) authentic dansylated 2-deoxy-*scyllo*-inosamine (C).



Arrows indicate identifying peaks for 2-deoxy-*scyllo*-inosamine.

Confirmation of L-Glutamine : DOI Aminotransferase Activity

To establish the function of BtrS, the proposed product, authentic (±)-2-deoxy-*scyllo*-inosamine was synthesized

(Fig. 5). The amine was derivatized into a dansyl amide, used for fluorescence detection. The *btrS* gene was expressed using *E. coli*. The corresponding DNA fragment was amplified by PCR and inserted into an expression vector pUC119, and transformed into *E. coli* JM109. L-Glutamine : DOI aminotransferase activity of the cell-free extract from *E. coli*/pUCbtrS was examined as described in Materials and Methods. The enzyme reaction product was derivatized into a dansyl amide, and was compared with the authentic dansyl derivative of synthetic (±)-2-deoxy-*scyllo*-inosamine by HPLC as shown in Fig. 6. Both the enzyme reaction product and the authentic sample eluted similarly at about 14 minutes, no peak was observed using the cell-free system of *E. coli*/pUC119. Furthermore, a large-scale incubation was also carried out as described in Materials and Methods. The 2-deoxy-*scyllo*-inosamine product was identified by ^{1}H- and ^{13}C-NMR as well as FAB-MS (*m/z* 164 $[M+H^{+}]$). All the data were identical to those of the authentic standard. These results clearly showed that BtrS is the L-glutamine : DOI aminotransferase required for the biosynthesis of butirosin.

In the present study, we have identified the L-glutamine : DOI aminotransferase gene (*btrS*) of the butirosin-biosynthetic gene cluster of *B. circulans*, and confirmed its enzymatic activity with expressed protein. Because the transamination reaction to ketocyclitols is unique in the biosynthetic pathways of aminoglycoside antibiotics, similar genes should be expected in other DOS-containing aminoglycoside-producers. Consequently, this gene would appear to be a useful genetic marker for the screening of aminoglycoside-producers.

Acknowledgements

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