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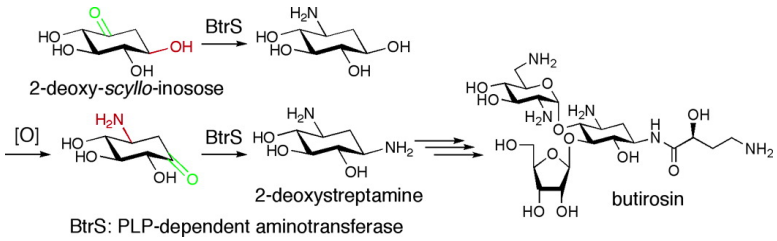

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Stereochemical Recognition of Doubly Functional Aminotransferase in 2-Deoxystreptamine Biosynthesis

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Abstract: The doubly functional aminotransferase BtrS in the 2-deoxystreptamine (DOS) biosynthesis, in which two transaminations are involved, was characterized by a genetic as well as a chemical approach with the heterologously expressed enzyme. The gene disruption study clearly showed that BtrS is involved, in addition to the previously confirmed first transamination, in the second transamination as well. This dual function of BtrS for the DOS biosynthesis was further confirmed by the structural determination of the reverse reaction product from DOS. Enantiospecific formation of the reverse reaction product from DOS clearly showed that BtrS distinguishes the enantiotopic amino groups of DOS, but in contrast, both enantiomers of 2-deoxy-*scyllo*-inosose (DOI) were efficiently accepted by BtrS to give a racemic product. This unique stereochemical recognition of DOI chirality and DOS prochirality by BtrS is mechanistically explained by a specific hydrogen-bond donating force in the enzyme active site as a particular feature of this doubly functional enzyme.

Aminocyclitols, represented by streptidine and 2-deoxystreptamine (**1**, DOS), are key aglycons of clinically important aminoglycoside antibiotics and are biosynthesized from the corresponding inososes through two steps of aminotransferase reaction (Scheme 1). The responsible enzymes and their corresponding genes for the biosynthesis of streptidine are known, in which two distinct aminotransferases are independently involved.[1−3] A different scenario has been suggested for the biosynthetic pathway for DOS. Walker et al. previously showed the involvement of aminotransferase for DOS biosynthesis in certain aminoglycoside producers and further suggested recently by using an enzyme system derived from gentamicin producing *Micromonospora purpurea* that both the first transamination and the reverse of the second could be catalyzed by the same enzyme.[4,5] It seems, therefore, that a single aminotransferase can catalyze two almost enantiotopic chemical conversions from 2-deoxy-*scyllo*-inosose (**2**, DOI) and a putative aminoketone intermediate (**4**) in the DOS biosynthesis. To precisely address this intriguing character of this class of aminotransferase on the molecular level, we took advantage of genetic as well as stereochemical approaches using a heterologously overexpressed aminotransferase.

Recently, we successfully identified the gene *btrS* as an aminotransferase in the butirosin biosynthetic gene cluster from *Bacillus circulans* SANK72073 and heterologously overexpressed in *Escherichia coli*.[6] BtrS is known to catalyze the first transamination from **2** to 2-deoxy-*scyllo*-inosamine (**3**) in the presence of L-glutamine and a PLP cofactor in the DOS biosynthesis (Scheme 1).[6,7]

Our first concern was focused on the roles of *btrS* in the DOS biosynthesis, since no other appropriate gene was envisioned for the second transamination within the gene cluster.[6,8] We undertook disruption of the *btrS* gene and observed as expected that a *btrS* disruptant was unable to produce any antibiotics including butirosin. Supplementation of **1** to the disruptant culture recovered the production of antibiotics, mostly comprising neamine. By feeding **3** to the culture of disruptant, we were unable to rescue the antibiotic production at all. These results clearly indicated on the genetic basis that BtrS is in fact involved, in addition to the first transamination, in the second transamination which converts amino-dideoxy-*scyllo*-inosose, (2*R*,3*S*,4*R*,5*S*)-5-amino-2,3,4-trihydroxycyclohexanone (**4**), into **1** (Scheme 1). This was well-coincided with an independent observation by Huang et al. that **1** was taken up as an amino donor for the reverse transamination to pyruvate.[7] The present results also agreed with those reported previously by Walker and Suzukake.[5,9]

Prior to the further mechanistic studies, we undertook the chemical identification of the product of the reverse reaction

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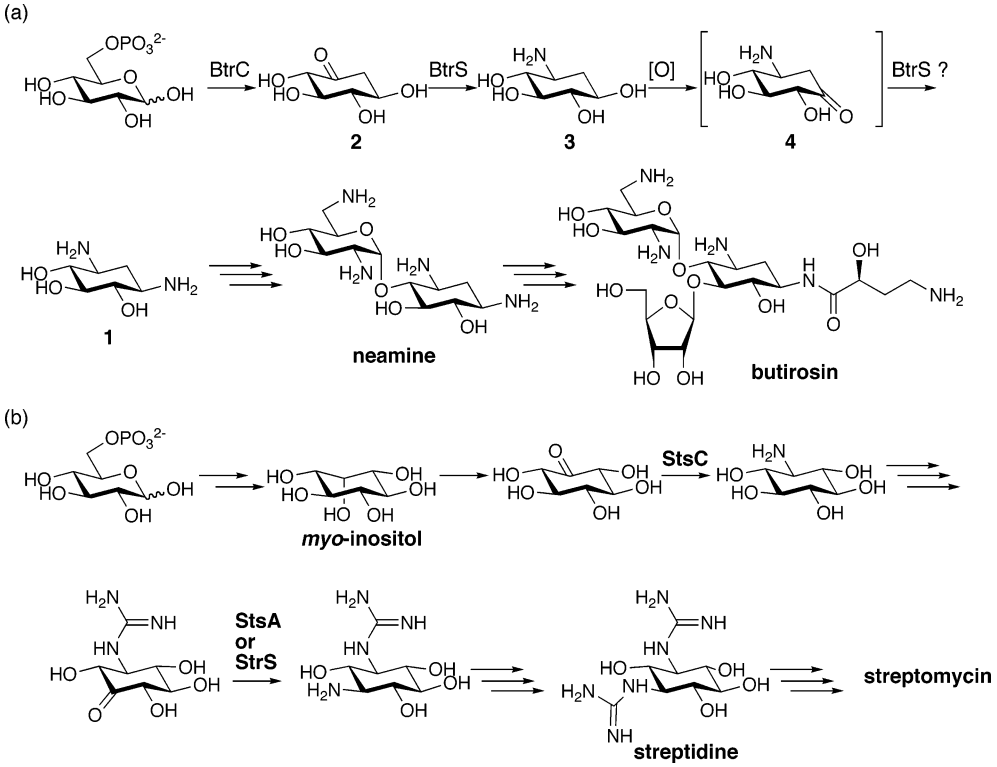
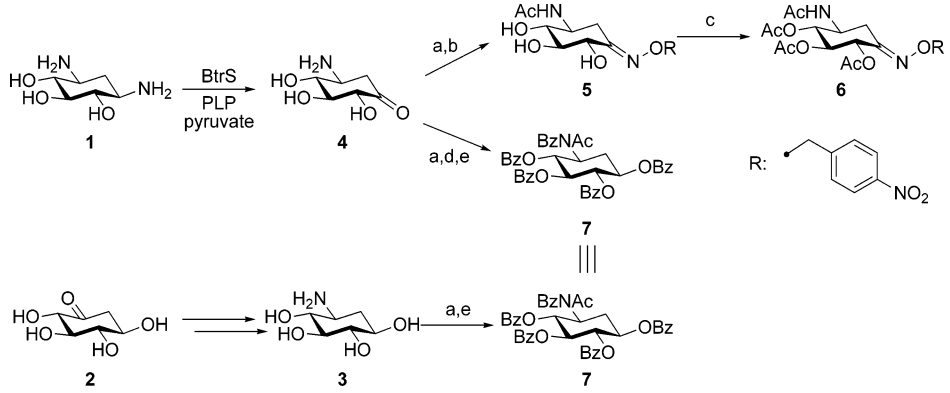
(1) Ahlert, J.; Distler, J.; Mansouri, K.; Piepersberg, W. *Arch. Microbiol.* **1997**, *168*, 102−113.
(2) Walker, J. B.; Walker, M. S. *Biochemistry* **1969**, *8*, 763−770.
(3) Walker, J. B. *Methods Enzymol.* **1975**, *43*, 429−470.
(4) Chen, Y. M.; Walker, J. B. *Biochem. Biophys. Res. Commun.* **1977**, *77*, 688−692.
(5) Lucher, L. A.; Chen, Y.-M.; Walker, J. B. *Antimicrob. Agents Chemother.* **1989**, *33*, 452−459.
(6) Tamegai, H.; Nango, E.; Kuwahara, M.; Yamamoto, H.; Ota, Y.; Kuriki, H.; Eguchi, T.; Kakinuma, K. *J. Antibiot.* **2002**, *55*, 707−714.
(7) Huang, F.; Li, Y.; Yu, J.; Spencer, J. B. *Chem. Commun.* **2002**, 2860−2861.
(8) Ota, Y.; Tamegai, H.; Kudo, F.; Kuriki, H.; Koike-Takeshita, A.; Eguchi, T.; Kakinuma, K. *J. Antibiot.* **2000**, *53*, 1158−1167.
(9) Suzukake, K.; Tokunaga, K.; Hayashi, H.; Hori, M.; Uehara, Y.; Ikeda, D.; Umezawa, H. *J. Antibiot.* **1985**, *38*, 1211−1218.

Scheme 1. Two Steps of Transaminaion in the Aminocyclitol Biosynthesis[a]



[a] (a) Biosynthesis of 2-deoxystreptamine containing aminoglycoside antibiotics. **1**, 2-deoxystreptamine; **2**, 2-deoxy-*scyllo*-inosose; **3**, 2-deoxy-*scyllo*-inosamine; **4**, amino-dideoxy-*scyllo*-inosose. BtrC, 2-deoxy-*scyllo*-inosose synthase that catalyzes carbocycle formation from glucose-6-phosphate. BtrS, L-glutamine:2-deoxy-*scyllo*-inosose aminotransferase. (b) Relevant enzymatic reactions involved in the biosynthesis of streptidine in streptomycin. StsC, L-glutamine:*scyllo*-inosose aminotransferase. StsA or StrS catalyzes the second transamination of the keto intermediate.

Scheme 2. Identification of BtrS Reaction Intermediates **4**[a]



[a] Reagents: (a) Ac_2O; (b) pyridine, NBHA; (c) pyridine, Ac_2O; (d) $NaBH_4$; (e) pyridine, DMAP, BzCl.

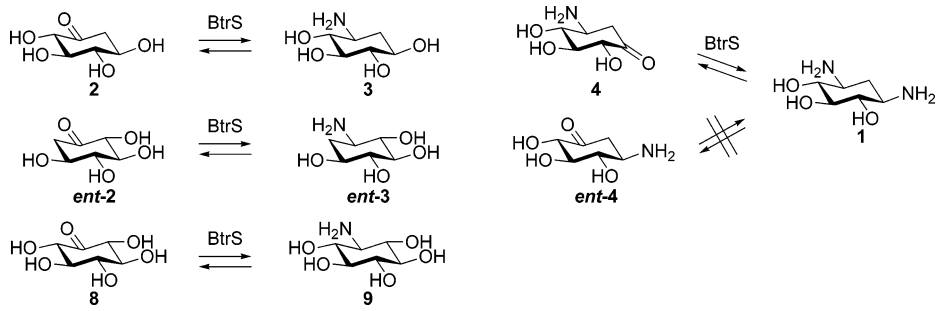
from **1** and pyruvate using purified BtrS (Scheme 2). After the enzyme reaction, the mixture was treated in situ with Ac_2O for N-acetylation, and the resulting *N*-acetamide was further derivatized to nitrobenzyl oxime with 4-nitrobenzyl hydroxylamine (NBHA).[10] LC−MS of the oxime product showed a molecular ion $(M + H)^+$ at *m*/*z* 353.9 corresponding to the expected derivative (**5**). The reaction yield was not necessarily good because most of the added DOS was recovered unchanged. The product (**5**) was further subjected to O-acetylation, and the fully protected product was purified by silica gel chromatography and was characterized by ^1H NMR, ^{13}C NMR, and MS analysis, which allowed us to unambiguously identify it as the expected derivative (**6**) of the biosynthetic intermediate amino-dideoxy-*scyllo*-inosose (**4**).

Our attention was next turned to the mechanistic issue of the BtrS reaction, particularly concerning the stereochemistry of the first and second transamination products, to see how the enzyme recognizes chirality of the substrates. Although Walker showed previously that chemically biased chiral N^3-methyl-2-deoxystreptamine was an amino donor in the reverse reaction by a native aminotransferase,[5,11] it appeared to be important to examine the chirality recognition toward unbiased achiral DOS. In fact, the above-mentioned nitrobenzyl oxime-*N*,*O*-tetraacetate (**6**) was optically active; $[\alpha]_D$ +28.0 (*c* 1.09, $CHCl_3$). Therefore,

(10) Yamauchi, N.; Kakinuma, K. *J. Antibiot.* **1992**, *45*, 774−780.

(11) Walker, J. B. *Appl. Environ. Microbiol.* **2002**, *68*, 2404−2410.

Scheme 3. BtrS-Catalyzed Reactions[a]



[a] Racemic DOI **2** and ***ent*-2** were converted to **3** and ***ent*-3**, respectively, and *scyllo*-inosose **8** was converted to **9** by BtrS. Isomer **4** was specifically formed from DOS **1** by BtrS in the presence of pyruvate and pyridoxal phosphate.

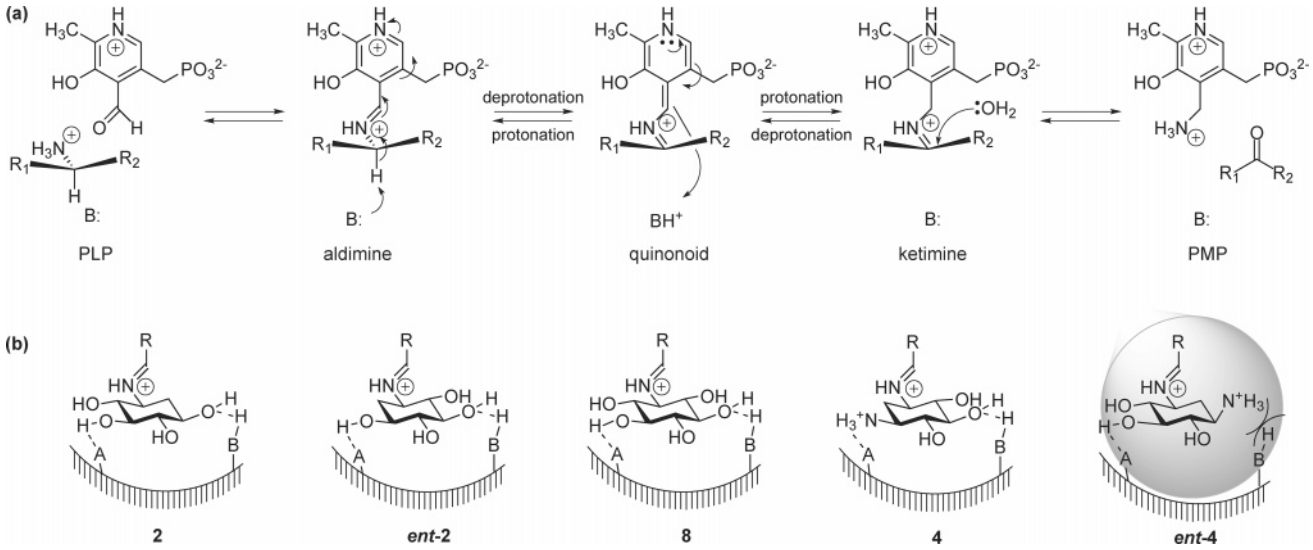


Figure 1. Reaction mechanism and substrate recognition by BtrS. (a) The first half-reaction catalyzed by BtrS. L-Glutamine (R_1: CO_2^-, R_2: CH_2CH_2-$CONH_2$) is utilized as an amino donor in the BtrS-catalyzed transamination. The aldimine intermediate between PLP and L-glutamine is initially formed. The Cα proton in the intermediate is stereospecifically abstracted by the active site amino acid, yielding the quinonoid intermediate. Reprotonation of the cofactor by the same amino acid residue generates the ketimine intermediate, which is subsequently hydrolyzed to release α-keto-glutaramate, leaving the enzyme in the PMP form. A complete BtrS catalytic cycle involves subsequent reaction with *scyllo*-inososes to give **3** and **1** and regenerates the PLP form of the holo-enzyme. PLP: pyridoxal 5′-phosphate, PMP: pyridoxamine 5′-phosphate. (b) Compounds **2**, ***ent*-2**, **8**, and **4** are acceptable intermediates. Unfavorable interaction of unacceptable intermediate ***ent*-4** is shaded.

it appears that BtrS obviously distinguishes the two enantiotopic amino groups of DOS. To confirm the absolute stereochemistry of **6**, we prepared *N*-acetyl-*N*-benzoyl-*O*-tetrabenzoate derivative (**7**) of stereochemically pure 2-deoxy-*scyllo*-inosamine (**3**) as the authentic standard, which was prepared from **2** produced enzymatically from D-glucose by DOI synthase BtrC in vivo.[6,12] Separately, the initial product (**4**) from **1** by BtrS reverse reaction was first N-acetylated, and the residual ketone was reduced with $NaBH_4$ to give a mixture of diastereoisomeric tetraols. After protection of all the hydroxy groups with benzoyl chloride, the resulting benzoates of diastereoisomeric 2-deoxyinosamine were separated by preparative thin-layer chromatography (TLC). As a result, both the authentic 2-deoxy-*scyllo*-inosamine derivative (**7**) derived from **2** and one of the diastereomers derived from the BtrS reverse reaction product were completely identical in every respects including optical activity. These results clearly demonstrate that BtrS distinguishes the two enantiotopic amino groups of DOS (**1**) and stereospecifically converts it to **4**.

To figure out the above-mentioned doubly functional character of BtrS, we examined the first transamination step from **2** to **3** in the DOS biosynthesis. Thus, the BtrS reaction was carried out with racemic DOI to investigate whether the natural enantiomer was selectively recognized. Surprisingly, the BtrS reaction product 2-deoxy-*scyllo*-inosamine was racemic (Scheme 3). Although the reaction kinetics has not been analyzed, BtrS does accept both enatiomers of DOI with the same efficiency. Thus, BtrS cannot distinguish the DOI enantiomers. The particular feature of doubly functional BtrS may be basically ascribable to the lack of recognition of DOI enantiomers.

An emerging question then was the mechanism of recognition by BtrS for DOS prochirality. Generally in the transamination reactions, a PLP or PMP coenzyme is incorporated in a specific manner at the enzyme active site prior to the incorporation of substrate so that the orientation of the initially formed aldimine or ketimine bond is rather fixed and the direction of protonation/deprotonation is stereospecific (Figure 1a).[13,14] R_1 and R_2 group of substrates are strictly distinguished so that L-glutamine is utilized as an amino source in the BtrS-catalyzed transamination, but not D-glutamine (data not shown). In the transamination to DOI, both DOI enantiomers are acceptable as substrate, which

(12) Kakinuma, K.; Nango, E.; Kudo, F.; Matsushima, Y.; Eguchi, T. *Tetrahedron Lett.* **2000**, *41*, 1935−1938.

(13) Eliot, A. C.; Kirsch, J. F. *Annu. Rev. Biochem.* **2004**, *73*, 383−415.
(14) Soda, K.; Yoshimura, T.; Esaki, N. *Chem. Rec.* **2001**, *1*, 373−384.

strongly suggests that any structural element making DOI chiral is not important at all for the substrate recognition (Figure 1b). In other words, both a hydroxy and deoxy functionality of the α-positions to the DOI carbonyl group is not the determinant of substrate recognition, but rather a plausible hydrogen-bonding interaction between hydroxy groups at the *β*-position to the carbonyl, and certain functionality of the enzyme should be significant. We confirmed that *scyllo*-inosose (**8**) was smoothly converted to the corresponding *scyllo*-inosamine (**9**) by BtrS.[5,11] This observation may now rationalize on the molecular level the well-documented historical successes of mutasynthesis or mutational biosynthesis using D^- (DOS negative) idiotrophic mutants of *M. purpurea*.[15−18] While the idiotroph produced gentamicin C upon supplementation with 2-deoxy-*scyllo*-inosose (**2**), 2-hydroxygentamicin C was produced as well when *scyllo*-inosose (**8**) was supplemented. Thus, it appears that the BtrS homologue in *M. purpurea* recognizes **8** as well as **2** for the transamination in the biosynthesis of gentamicin C.

As to the reverse deamination reaction from DOS, its prochirality was recognized by BtrS, and the enantiotopic amino groups were completely distinguished (Scheme 3, **4** and ***ent*-4**). The initially formed two putative aldimines are enantiomeric to each other, and one enantiomer appears to be preferentially formed through favorable recognition. As discussed above, the functionality at the *β*-positions to the carbonyl is important, and the same should be true for the formation of an aldimine intermediate in the DOS deamination reaction. The hydroxy and amino groups at the *β*-positions can be distinguished by such interactions as hydrogen-bonding or electrostatic interaction. Thus, a proper approach of DOS to PLP in the holo-enzyme appears to be enhanced by hydrogen-bond donating interaction from the enzyme to the hydroxy group of DOS as illustrated in the Figure 1b (**4**), but an unfavorable entry of DOS to the active site may encounter a repulsive interaction between the amino group (ammonium) of DOS and the hydrogen-bond donating residue of the enzyme (***ent*-4**). It seems, therefore, that even a single-point interaction may be enough for specific recognition of prochirality. Significance of such a single-point interaction for chiral recognition has been described in the reports about the different stereospecificities shown by the same protein hold of two tropinone reductases involved in the biosynthesis of tropane alkaloids.[19,20]

In conclusion, the doubly functional aminotransferase in the DOS biosynthesis in fact catalyzes the two almost enantiomeric transaminations, and the second transamination may probably be controlled by specific hydrogen-bond donating force in the enzyme active site to the substrate.

Experimental Section

General. *B. circulans* SANK 72073 was used as the source strain for butirosin biosynthetic genes and the construction of *btr*S gene disruption mutants. *E. coli* JM 109 and DH5α were used as host strain for *btr* genes cloning. *E. coli* BL21(DE3) was used for gene expression. *Bacillus subtilis* PCI 219 was used as an antibiotic test strain. The plasmids for gene disruption were constructed with the *E. coli*−*Bacillus* shuttle vector pHB201 (Tanaka et al., unpublished). The plasmid pUC 119 was routinely used as a vector for subcloning and sequencing and pT7-blue T vector (Novagen, USA) was routinely used as a vector for subcloning after PCR. Oligonucleotides were purchased from Sigma Genosis. Restriction enzymes were purchased from Takara. PCR was performed by GeneAmp PCR System 9700 (Perkin-Elmer Applied Biosystems) using ULTma DNA polymerase (Roche). DNA sequence analysis was carried out with a LONG READER 4200 (Li-Cor) 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. FPLC (Pharmacia Biotech.) was used for protein purification. Melting point (mp) data were measured with a Yazawa BY-1 micro melting point apparatus and were uncorrected. Optical rotations were recorded on a JASCO DIP-360 digital polarimeter. IR spectra were taken on a Horiba FT-710 Fourier transform infrared spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded with a JEOL AL-400 spectrometer or a Bruker DRX-500 spectrometer. Deuteriochloroform (Merck, 99.8 atom % enriched), deuterium oxide (Merck, 99.9 atom % enriched), deuteriomethanol (Acros, 99.5 atom % enriched), or deuteriopyridine (Merck, 99.8 atom % enriched) were used as NMR solvent. Chemical shifts are reported in δ ppm based on internal TMS (0 ppm) or the solvent signal ($CDCl_3$ $\delta_C = 77.0$; D_2O $\delta_H = 4.65$; pyridine-d_5 $\delta_H = 7.55$) as reference. When D_2O was used, dioxane was used an internal standard ($\delta_C = 66.5$). FAB-MS was taken by a JMS-700 spectrometer (JEOL). Column chromatography was carried out with a Merck silica gel 60 (70−230 mesh, 0.063−0.20 mm Merck) or a Merck silica gel 60 (230−400 mesh, 0.040−0.063 mm for a flash chromatography, Merck). TLC was carried out with a Merck silica gel 60 F_{254} (0.25-mm thick). Other chemicals were of the highest grade commercially available.

***btr*S Gene Disruption.** A fragment containing 5′-terminus of *btr*S gene was amplified by PCR with a primer btrS-d1 T*GGATCC*GGCGGGAGAAAAGCCGGAGTGTG and a primer btrS-d2 T*ATCGAT*CGTACGATCGGAGTGTTGAGGCC. A fragment containing 3′ terminus of *btr*S gene was amplified by PCR with a primer btrS-d3 C*ATCGAT*TGTCGCTTCTGGTCGATGCCGTAGCC and a primer btrS-d4 A*GAATTC*CCTGCCGGACTCCTTGATCGGCC. PCR conditions were 95 °C, 10 min for denature, 30 cycles of 95 °C, 1 min, 55 °C, 1 min, 72 °C, 1 min for extension of DNA. Each PCR product was subcloned into pT7-blue to obtain pT7dbtrS−N and pT7dbtrS−C. After confirmation of both sequences, a BamHI−ClaI fragment derived from pT7dbtrS−N was inserted into pHSG398 vector (Takara). The resultant plasmid was digested with ClaI and introduced into a ClaI site of pT7dbtrS−C. After confirmation of the direction of inserted DNA in the resulting plasmid, its BamHI-EcoRI fragment was introduced into pHB201 to obtain pHB201dbtrS, which lacked a part of the *btr*S gene. *B. circulans* SANK72073 was transformed with pHB201dbtrS by electroporation, and a *btr*S gene disruptant was constructed by homologous recombination according to the previous method.[8] Disruption of *btr*S was confirmed by colony direct PCR, under the conditions of 94 °C, 5 min for denature, 40 cycles of 94 °C, 1 min, 55 °C, 1 min, 72 °C 4.5 min for extension of DNA with primer btrSdt-F CACGGATTGTGCGTAGAAGCAGA and primer btrSdt-R GGTCCCGAAGTTGTAAGGTTCGG. The *btrS* disruptant was cultured in a glycerol-supplemented nutrient broth medium,[21] and an aliquot of the culture was collected every day for 10 days. The supernatant of each specimen was tested for antibiotic activity by a paper diffusion method. Antibiotic production by the *btr*S mutant was further examined by supplementation of DOS (final 50 mg/L) or 2-deoxy-*scyllo*-inosamine (final 50 or 250 mg/L) on the first and fourth day of culture. After 10 days, each cultured medium was centrifuged, and the supernatant was neutralized with aqueous HCl. The solution was separately loaded onto an Amberlite IRC50 column (NH_4^+ form, 3 cm × 10 cm). The column was washed

(15) Daum, S. J.; Lemke, J. R. *Annu. Rev. Microbiol.* **1979**, *33*, 241−265.
(16) Rinehart, K. L., Jr. *J. Antibiot.* **1979**, *32* (Suppl.) S32−S46.
(17) Daum, S. J.; Rosi, D.; Goss, W. *J. Am. Chem. Soc.* **1977**, *99*, 283−284.
(18) Daum, S. J.; Rosi, D.; Goss, W. A. *J. Antibiot.* **1977**, *30*, 98−105.
(19) Nakajima, K.; Yamashita, A.; Akama, H.; Nakatsu, T.; Kato, H.; Hashimoto, T.; Oda, J.; Yamada, Y. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 4876−4881.
(20) Nakajima, K.; Kato, H.; Oda, J.; Yamada, Y.; Hashimoto, T. *J. Biol. Chem.* **1999**, *274*, 16563−16568.
(21) Kudo, F.; Hosomi, Y.; Tamegai, H.; Kakinuma, K. *J. Antibiot.* **1999**, *52*, 81−88.

with 400 mL of water and 400 mL of 0.1 M aqueous NH_4OH and eluted with 500 mL of 1 M aqueous NH_4OH. The bioactive fractions were combined, and the solution was concentrated. The concentrated solution was loaded onto an Amberlite CG50 column (NH_4^+ form, 1.2 cm × 50 cm), and the column was washed with 0.3 M aqueous NH_4-OH. Aminoglycosides were eluted with 0.3−0.8 M aqueous NH_4OH in a linear gradient mode. The bioactive fractions were combined and concentrated. The residue was dissolved in 1 mL of water, and an aliquot of the solution (50 μL) was applied onto a TLC plate. The TLC plate was developed with $CHCl_3$/MeOH/aqueous NH_4OH/EtOH = 4:6:7:1. After being dried, the TLC plate was placed over an agar medium containing *B. subtilis* PCI 219 for 1 day at 4 °C. After the plate was removed, the bacteria were cultured at 37 °C for 1 day.

Preparation of BtrS. The plasmid pUCbtrS containing the *btr*S gene[6] was digested with NdeI and EcoRI, and the NdeI-EcoRI fragment containing *btrS* gene was introduced into the NdeI-EcoRI site of pET30b to give pETbtrS, which was subsequently introduced into *E. coli* BL21-(DE3). The transformant harboring pETbtrS was incubated in LB medium containing 30 mg/mL of kanamycin at 37 °C. The expression was induced at $OD_{600} \approx 0.6-1.0$ by adding final 1.0 mM of isopropyl thio-β-D-galactoside (IPTG), and the culture was continued for an additional 4 h. The cells were harvested by centrifugation at 7000 rpm for 15 min at 4 °C and washed with a 50 mM Tris buffer (pH 8.0). The wet cells (14 g) were suspended in 25 mL of 50 mM Tris buffer (pH 8.0) and then disrupted by a French Press (Thermo IEC). After removal of cell debris by centrifugation at 15 000 rpm for 30 min at 4 °C, the supernatant was loaded onto a DEAE-Sepharose fast flow column (2.5 cm × 10 cm) previously equilibrated with a 50 mM Tris buffer (pH 8.0). BtrS was eluted with a linear gradient of 0.05−0.5 M of NaCl in the same buffer. The fractions containing BtrS were combined and concentrated by ultrafiltration (VIVASPIN 20, Vivascience, Germany). The concentrated solution was loaded onto a Superdex Hi-load 75 gel filtration column (Amersham Pharmacia) equilibrated with a 50 mM Tris buffer containing 100 mM of NaCl. The fractions containing BtrS were combined and concentrated. The concentration of protein was estimated by the method of Lowry using bovine serum albumin as a standard.

Identification of BtrS Reverse Reaction Product from DOS. The BtrS reverse reaction was carried out with 157 mg of purified BtrS, 50 mM sodium pyruvate, 100 mM DOS, and 0.5 mM pyridoxal 5′-phosphate in 39 mL of 50 mM Tris buffer (pH 8.0). After the reaction at 37 °C for 3 h, BtrS was removed by ultrafiltration. The resultant solution was mixed with 60 mL of Ac_2O and was stirred for 3 h at room temperature. After concentration, 40 mL of methanol, 28 mL of pyridine, and 105 mg of *O*-(*p*-nitrobenzyl)-hydroxylamine hydrochloride (NBHA) were added, and the solution was stirred for 4 h at 60 °C. After removal of the solvent, the residue was purified by silica gel column chromatography (100 g of silica gel, $CHCl_3/CH_3OH$ = 5:1) to afford 94 mg of crude product. Because of the poor solubility of the product, a part of the crude product was further purified by HPLC equipped with a Senshu Pak ODS 1251N column (4.6 mm × 250 mm, Senshu Scientific, Japan) with 30% aqueous methanol at a flow rate of 1 mL/min. Elution was monitored by 254 nm, and appropriate fractions were combined and concentrated to give compound **5** (20 mg). ^{1}H NMR (400 MHz, pyridine-d_5): δ 2.07 (s, 3H), 2.48 (dd, J = 11.0, 14.2 Hz, 1H), 4.10 (dd, J = 5.0, 14.3 Hz, 1H), 4.25 (m, 2H), 4.71 (m, 2H), 5.29 (s, 2H), 7.50 (m, 2H), 8.09 (m, 2H).

LC−MS analysis of **5** was performed by 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) with 10% aqueous methanol containing 0.1% TFA for 10 min, a linear gradient of 10−60% aqueous methanol containing 0.1% TFA for 35 min, and a linear gradient of 60−100% aqueous methanol containing 0.1% TFA for 15 min at a flow rate 40 μL/min, and elution was monitored by 262 nm. LC−ESI-MS (positive): 40.8 min, m/z 353.9 $(M + H)^+$; calcd for $C_{15}H_{20}N_3O_7$: 354.1301.

A solution of **5** (20 mg) in 2.8 mL of pyridine was mixed with 0.27 mL of Ac_2O, and the solution was stirred for 12 h at room temperature. After evaporation, the residue was purified by preparative TLC (ethyl acetate) to afford 24 mg of **6** (88%) as colorless solid. mp 181−182 °C; $[\alpha]^{24}_D$ +28.0 (*c* 1.09, $CHCl_3$); IR ($CHCl_3$): 1755, 1683, 1522, 1373, 1346, 1223, 1038 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.92 (dd, J = 12.0, 15.2 Hz, 1H), 1.95 (s, 3H), 2.05 (s, 3H), 2.06 (s, 3H), 2.07 (s, 3H), 3.68 (dd, J = 5.2, 15.2 Hz, 1H), 4.19 (dddd, J = 5.2, 8.4, 10.4, 12.0 Hz, 1H), 5.03 (dd, J = 8.6, 10.6 Hz, 1H), 5.18 (s, 2H), 5.26 (t, J = 8.8 Hz, 1H), 5.33 (d, J = 8.8 Hz, 1H), 5.74 (d, J = 8.4 Hz, 1H), 7.47 (m, 2H), 8.22 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 20.49, 20.55, 20.65, 23.22, 27.49, 47.91, 71.13, 71.47, 73.24, 75.11, 123.7, 128.5, 144.6, 147.6, 148.3, 169.0, 169.4, 169.7, 171.5; HRMS (FAB) m/z 480.1608 $(M + H)^+$; calcd for $C_{21}H_{26}N_3O_{10}$: 480.1618.

***N*-Acetyl-*N*-benzoyl-3,4,5,6-tetra-*O*-benzoyl-2-deoxyinosamine (7).** 2-Deoxy-*scyllo*-inosamine hydrochloride **3** was prepared by the previously reported synthetic method[6] from **2**. To a solution of **3** (32 mg) in water (3 mL) was added 9 mL of Ac_2O at 0 °C, and the mixture was stirred for 36 h. After concentration in vacuo, the crude product was successively passed through columns of Dowex 50WX8 (H^+ form, 1.8 cm × 10 cm) and Dowex AG1X8 (OH^- form, 1.8 cm × 10 cm), and the product was eluted with water. The solvent was removed in vacuo to afford 24 mg of *N*-acetyl-2-deoxy-*scyllo*-inosamine (72%) as a colorless syrup. IR (neat): 3301, 2885, 1635, 1558 cm^{-1}; ^{1}H NMR (400 MHz, D_2O): δ 1.24 (q, J = 12.2 Hz, 1H), 1.85 (s, 3H), 1.94 (dt, J = 12.6, 4.4 Hz, 1H), 3.17 (m, 3H), 3.44 (ddd, J = 5.3, 9.0, 15.3 Hz, 1H), 3.61 (ddd, J = 4.0, 9.5, 13.0 Hz, 1H); ^{13}C NMR (100 MHz, D_2O); δ 22.0, 34.6, 48.2, 69.1, 74.4, 74.9, 76.5, 173.8.

To a solution of *N*-acetyl-2-deoxy-*scyllo*-inosamine (24 mg, 0.115 mmol) in pyridine (6 mL) were added 4-(dimethylamino)pyridine (DMAP) (8.0 mg, 0.065 mmol) and benzoyl chloride (0.41 mL, 3.5 mmol) at 0 °C. The mixture was warmed to room temperature and was stirred for an additional 15 h. The reaction was quenched with water, and the mixture was extracted with ethyl acetate. The organic layer was washed with $NaHCO_3$ aq, water, and brine and dried over $MgSO_4$. After filtration and evaporation, the residue was purified by flash silica gel column chromatography (8 g of silica gel, hexane/ethyl acetate = 2:3) to afford 69 mg of the almost pure product (83%). The product was recrystallized from $CHCl_3$/hexane to afford 52 mg of *N*-acetyl-3,4,5,6-tetra-*O*-benzoyl-2-deoxy-*scyllo*-inosamine as white powder: mp 240−241 °C; $[\alpha]^{28}_D$ −31.3 (*c* 1.09, $CHCl_3$); IR ($CHCl_3$): 1730, 1664, 1450, 1278, 1111, 1070, 1025 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.85 (q, J = 12.4, 1H), 1.87 (s, 3H), 2.82 (dt, J = 12.6, 4.6 Hz, 1H), 4.56 (m, 1H), 5.46 (dd, J = 9.5, 10.8 Hz, 1H), 5.51 (ddd, J = 4.9, 9.3, 11.5 Hz, 1H), 5.86 (m, 3H), 7.27 (m, 4H), 7.40 (m, 6H), 7.51 (m, 2H), 7.79 (m, 2H), 7.83 (m, 2H), 7.93 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 23.22, 33.18, 47.80, 69.63, 71.40, 73.14, 74.09, 128.17, 128.20, 128.30, 128.42, 128.69, 128.75, 129.13, 129.52, 129.60, 129.63, 129.85, 133.10, 133.14, 133.16, 133.60, 165.20, 165.28, 165.61, 167.18, 169.66; Anal. Calcd for $C_{36}H_{31}NO_9$: C, 69.56; H, 5.03; N, 2.25. Found: C, 69.31; H, 4.74; N, 1.97.

To a solution of *N*-acetyl-3,4,5,6-tetra-*O*-benzoyl-2-deoxy-*scyllo*-inosamine (30 mg, 0.048 mmol) in pyridine (2.4 mL) were added DMAP (9 mg, 0.074 mmol) and benzoyl chloride (0.35 mL, 3.0 mmol) at 0 °C. The reaction mixture was warmed to room temperature and was stirred for 17 h. The reaction was quenched with water, and the mixture was extracted 3 times with ethyl acetate. The combined organic layer was washed with $NaHCO_3$ aq, water, and brine and dried over $MgSO_4$. After filtration and removal of the solvent, the crude product was recrystallized from $CHCl_3$/hexane to afford 23 mg of **7** (66%) as white powder: mp 249−251 °C; $[\alpha]^{28}_D$ +12 (*c* 0.991, $CHCl_3$); IR ($CHCl_3$): 1730, 1666, 1450, 1279, 1107, 1070, 1026 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, room temperature): δ 1.81 (s, 3H), 2.59 (dt, J = 12.4, 4.4 Hz, 1H), 2.97 (br, 1H), 5.03 (br, 1H), 5.44 (br, 1H), 5.79 (t, J = 9.8 Hz, 1H), 5.98 (t, J = 10.2 Hz, 1H), 6.47 (t, J = 10.0 Hz, 1H), 7.26 (m, 3H), 7.36 (m, 8H), 7.53 (m, 6H), 7.80 (d, J = 8.0 Hz, 2H),

7.85 (t, J = 8.0 Hz, 4H), 7.95 (d, J = 8.0 Hz, 2H); ^{1}H NMR (400 MHz, $CDCl_3$, 80 °C): δ 1.83 (s, 3H), 2.57 (dt, J = 12.4, 4.6, Hz, 1H), 2.99 (q, J = 12.3, 1H), 4.97 (m, 1H), 5.42 (ddd, J = 5.0, 10.2, 11.5 Hz, 1H), 5.75 (t, J = 9.9 Hz, 1H), 5.93 (t, J = 10.0, 1H), 6.43 (t, J = 10.1 Hz, 1H), 7.22 (m, 4H), 7.35 (m, 8H), 7.48 (m, 3H), 7.55 (m, 2H), 7.77 (m, 2H), 7.80 (m, 2H), 7.84 (m, 2H), 7.93 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 27.65, 29.57, 70.44, 71.95, 72.39, 72.89, 128.13, 128.16, 128.28, 128.33, 128.70, 128.75, 128.81, 128.96, 129.04, 129.58, 129.67, 133.02, 133.19, 133.24, 165.10, 165.39, 165.46, 165.56; Anal. Calcd for $C_{43}H_{35}NO_{10}$: C, 71.16; H, 4.86; N, 1.93. Found: C, 70.88; H, 4.86; N, 1.76.

Stereochemistry of the BtrS Reverse Reaction Product from DOS. The BtrS reverse reaction was carried out with 30 mg of purified enzyme, 50 mM sodium pyruvate, 100 mM DOS, and 0.5 mM pyridoxal-5′-phosphate in 7.5 mL of 50 mM Tris buffer (pH 8.0). After the reaction at 37 °C for 3 h, BtrS was removed by ultrafiltration. The resultant solution was mixed with 60 mL of Ac_2O and was stirred for 3 h at room temperature. After the removal of solvent, the residue was dissolved in methanol, and the solution was mixed with 116 mg of $NaBH_4$. After being stirred for 2 h at 0 °C, the reaction was quenched with water and methanol was removed. The resultant mixture was loaded onto Dowex AG1X8 column (OH^- form), and the product was eluted with water. The obtained solution was neutralized with HCl and was evaporated. After being dried, the residue was redissolved in 9 mL of pyridine and the solution was mixed with DMAP (8 mg, 0.065 mmol) and benzoyl chloride (0.41 mL, 3.5 mmol) at 0 °C. The solution was warmed to room temperature and was stirred for an additional 15 h. The reaction was quenched with water, and the mixture was extracted with ethyl acetate. The organic layer was washed with $NaHCO_3$ aq, water, and brine and dried over $MgSO_4$. After filtration and evaporation in vacuo, the product was purified by standard silica gel column chromatography (70 g of silica gel, hexane/ethyl acetate = 1:1) and was further purified by preparative TLC (ethyl acetate) to separate **7** and its 3-*epi* isomer to give **7** as colorless solid (12 mg). **7**: mp 249−251 °C; $[\alpha]^{27}_D$ +13 (*c* 0.79, $CHCl_3$); IR ($CHCl_3$): 1728, 1666, 1450, 1277, 1106, 1070, 1026 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$); δ 1.81 (s, 3H), 2.59 (dt, J = 12.4, 4.4 Hz, 1H), 2.97 (br, 1H), 5.03 (br, 1H), 5.44 (br, 1H), 5.79 (1H, t, J = 9.8 Hz), 5.98 (1H, t, J = 10.2 Hz), 6.47 (1H, t, J = 10.0 Hz), 7.26 (m, 3H), 7.36 (m, 8H), 7.53 (m, 6H), 7.80 (d, J = 8.0 Hz, 2H), 7.85 (t, J = 8.0 Hz, 4H), 7.95 (d, J = 8.0 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$); δ 27.65, 29.57, 70.44, 71.95, 72.39, 72.89, 128.13, 128.16, 128.28, 128.33, 128.70, 128.75, 128.81, 128.96, 129.04, 129.58, 129.67, 133.02, 133.19, 133.24, 165.10, 165.39, 165.46, 165.56; Anal. Calcd for $C_{43}H_{35}NO_{10}$: C, 71.16; H, 4.86; N, 1.93. Found: C, 70.89; H, 4.58; N, 1.90.

Stereochemistry of the BtrS Reaction Product from Racemic DOI. Racemic DOI was synthesized from *myo*-inositol by a previously reported procedure.[22] The BtrS reaction (final 40 mL) was carried out with 30 mg of purified enzyme, 50 mM L-glutamine, 25 mM racemic DOI, and 0.5 mM pyridoxal 5′-phosphate in a 50 mM Tris buffer (pH 8.0). After incubation at 37 °C for 3 h, the protein was removed by ultrafiltration. The resultant solution was applied onto a Dowex 50WX8 column (H^+ form, 1.8 cm × 7 cm), and the product was eluted with 0.1−0.7 M HCl aq linear gradient (600 mL). Fractions containing 2-deoxy-*scyllo*-inosamine were combined, and the solvent was evaporated to give a mixture of 2-deoxy-*scyllo*-inosamine and 2-amino-2-hydroxymethyl-1,3-propandiol (Tris). The residue was dissolved in a mixture of 7 mL of water and 28 mL of Ac_2O. The mixture was stirred for 2 h at 4 °C and allowed to be warmed to room temperature. After being stirred for an additional 10 h, the solvent was evaporated and the residue was dissolved in 10 mL of water. The solution successively passed through columns of Dowex 50WX8 (H^+ form, 1.5 cm × 7 cm) and Dowex AG1X8 (OH^- form, 1.4 cm × 7 cm), and the product was eluted with water. Appropriate fractions were combined and evaporated to give 58 mg of *N*-acetyl-2-deoxy-*scyllo*-inosamine and acetylated Tris. The residue was then dissolved in pyridine (13 mL), and the solution was mixed with DMAP (16 mg) and benzoyl chloride (2.0 mL) at 0 °C. The mixture was allowed to be warmed to room temperature and was stirred for 20 h. The reaction was quenched with water, and the product was extracted with ethyl acetate. The organic layer was washed with $NaHCO_3$ aq, water, and brine and dried over $MgSO_4$. After filtration and evaporation, the product was purified by a silica gel column chromatography (hexane/ethyl acetate = 2:3) to obtain 25 mg of *N*-acetyl-tetra-*O*-benzoyl-2-deoxyinosamine as colorless solid: mp 250−252 °C; $[\alpha]^{21}_D$ +0.1 (*c* 1.27, $CHCl_3$); IR ($CHCl_3$): 1728, 1659, 1450, 1279, 1111, 1070, 1025 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.85 (q, J = 12.4 Hz, 1H), 1.87 (s, 3H), 2.82 (dt, J = 12.9, 4.6 Hz, 1H), 4.56 (m, 1H), 5.46 (t, J = 10.0 Hz, 1H), 5.52 (m, 1H), 5.86 (m, 2H), 7.27 (m, 4H), 7.40 (m, 6H), 7.51 (m, 2H), 7.79 (m, 2H), 7.83 (m, 2H), 7.93 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 23.22, 33.18, 47.80, 69.63, 71.40, 73.14, 74.09, 128.17, 128.20, 128.30, 128.37, 128.42, 129.69, 128.75, 129.13, 129.52, 129.60, 129.63, 129.85, 133.10, 133.14, 133.16, 133.60, 165.20, 165.28, 165.61, 167.18, 169.66; Anal. Calcd for $C_{36}H_{31}NO_9$: C, 69.56; H, 5.03; N, 2.25. Found: C, 69.30; H, 4.77; N, 1.99.

BtrS Reaction Using *scyllo*-Inosose (8). *scyllo*-Inosose was prepared from 2,3,4,5,6-penta-*O*-benzyl-*scyllo*-inosose.[23] Briefly, to a solution of 2,3,4,5,6-penta-*O*-benzyl-*scyllo*-inosose (44 mg) in CH_3OH/H_2O/AcOH = 5:1:1 (4 mL) was added 10% Pd/C (10 mg), and the mixture was stirred for 3 days under H_2 atmosphere. After removal of the catalyst, the solvent was evaporated to obtain 5 mg of *scyllo*-inosose. The BtrS reaction (1.2 mL) was carried out with purified enzyme (1.0 mg), L-glutamine (50 mM), *scyllo*-inosose (20 mM), and pyridoxal-5′-phosphate (0.5 mM) in a 50 mM Tris buffer (pH 8.0). The solution was incubated at 32 °C for 3 h. After removal of the protein by ultrafiltration, the resultant solution was subsequently applied onto a Dowex AG1X8 column (OH^- form, 0.5 cm × 6 cm), and the product was eluted with water. Ninhydrin-positive fractions were collected and applied onto an Amberlite CG50 column (NH_4^+ form, 0.5 cm × 4 cm). The product was eluted with a stepwise elution 0.1−0.7 M NH_4-OH aq. *scyllo*-Inosamine was eluted with 0.4 M NH_4Cl aq. The appropriate fractions were combined and applied onto an Amberlite CG50 column (H^+ form, 0.5 cm × 4 cm, eluted by 1 N HCl aq), giving *scyllo*-inosamine hydrochloride **9**: ^{1}H NMR (400 MHz, D_2O): δ 2.86 (t, J = 10.0 Hz, 1H), 3.19 (t, J = 9.2 Hz, 1H), 3.26 (t, J = 9.2 Hz, 2H), 3.30 (t, J = 9.6 Hz, 2H).

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Supporting Information Available: All spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(22) Yamauchi, N.; Kakinuma, K. *J. Antibiot.* **1992**, *45*, 756−766.

(23) Martin, S. F.; Josey, J. A.; Wong, Y.-L.; Dean, D. W. *J. Org. Chem.* **1994**, *59*, 4805−4820.