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Characterization of L-glutamine:2-deoxy-*scyllo*-inosose aminotransferase (*tbmB*) from *Streptomyces tenebrarius*

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Abstract—2-Deoxystreptamine (DOS)-containing aminoglycoside–aminocyclitol (AmAc) antibiotics represent the majority of clinically important AmAcs. Biosynthetic investigations of formation of DOS in actinomycetes are limited to the characterization of 2-deoxy-*scyllo*-inosose synthase, the first step enzyme of the DOS biosynthetic pathway. A gene encoding L-glutamine:2-deoxy-*scyllo*-inosose aminotransferase (*tbmB*) from the tobramycin producer *Streptomyces tenebrarius* was expressed heterologously in *Escherichia coli*. The conversions of 2-deoxy-*scyllo*-inosose to 2-deoxy-*scyllo*-inosamine and *scyllo*-inosose to *scyllo*-inosamine with the activity of TbmB were determined in vitro. The results indicate that *tbmB* catalyzes the second step of the DOS biosynthetic pathway during the biosynthesis of 2-deoxystreptamine, a subunit of tobramycin, in *S. tenebrarius*.

Aminoglycoside–aminocyclitol (AmAc) antibiotics are among the oldest antibiotics in human therapeutic usage. These antibiotics are primarily produced by the actinomycetes and have been using for the treatment of the infections caused by Gram positive (pseudomonas) and Gram negative (bacillus) pathogens. Despite the emergence of several AmAcs-resistant pathogens, the synergistic effect of AmAcs with other antibiotics on action has sustained their clinical utilities, particularly for treating the nosocomial infections.[1–3] Most of these compounds have an aglycon (cyclitol subunit) with two amino groups. Glycosylations of hydroxyl groups of this aminocyclitol with other sugar subunits lead to the formation of AmAc antibiotics. Based on the aminocyclitol present, these antibiotics can be divided into two groups; one with streptamine and the other with 2-deoxystreptamine (DOS) subunit. The latter includes the most of the clinically important AmAc antibiotics (gentamicin, kanamycin, ribostamycin, tobramycin, neomycin, etc.) whereas the former includes spectinomycin, bluensomycin, streptomycin, etc.

Genetic and biochemical investigations on the biosynthesis of the DOS-containing AmAcs have begun with the isolation of the butirosin biosynthetic gene cluster from *Bacillus circulans*.[4] 2-Deoxy-*scyllo*-inosose synthase (*btrC*) catalyzes a redox reaction resulting in the formation of 2-deoxy-*scyllo*-inosose (DOI) (**3**, Scheme 1), and introduces the intracellular metabolite flux to the DOS biosynthetic route in *B. circulans*.[5] The stereochemistry of carbocyclization, and the crucial role of some amino acid residues of BtrC have been determined.[6,7] BtrS transaminates the product of BtrC (DOI) to form 2-deoxy-*scyllo*-inosamine (**4**) utilizing L-glutamine (an amino donor) in the presence pyridoxal phosphate.[8,9] Dehydrogenation of *scyllo*-inosamine and the subsequent transamination of the product are the predicted biosynthetic reactions to generate DOS (Scheme 1). Because the bacteria belonging to actinomycetes are the major AmAcs producers, we have extended the genetic and biochemical investigations of AmAcs production to these organisms isolating biosynthetic gene clusters for tobramycin from *S. tenebrarius* (AJ579650), gentamicin from *Micromonospora echinospora* (AJ575934) and kanamycin from *Streptomyces kanamyceticus* (AJ582817). Recently, Yanai and Murakami have also reported a cluster for kanamycin biosynthesis independently.[10]

Despite a good structural similarity between streptamine and DOS, the genetic and biosynthetic investigations of their formation to date have indicated two separate biosynthetic routes (Scheme 1). Organization of

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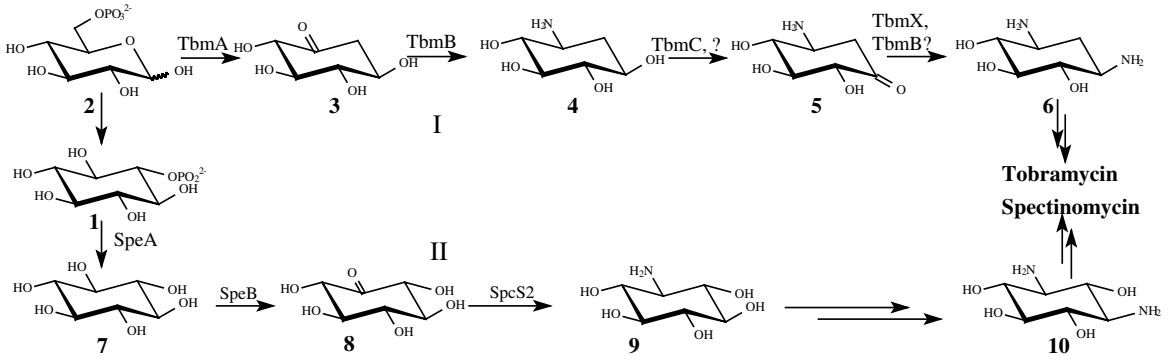
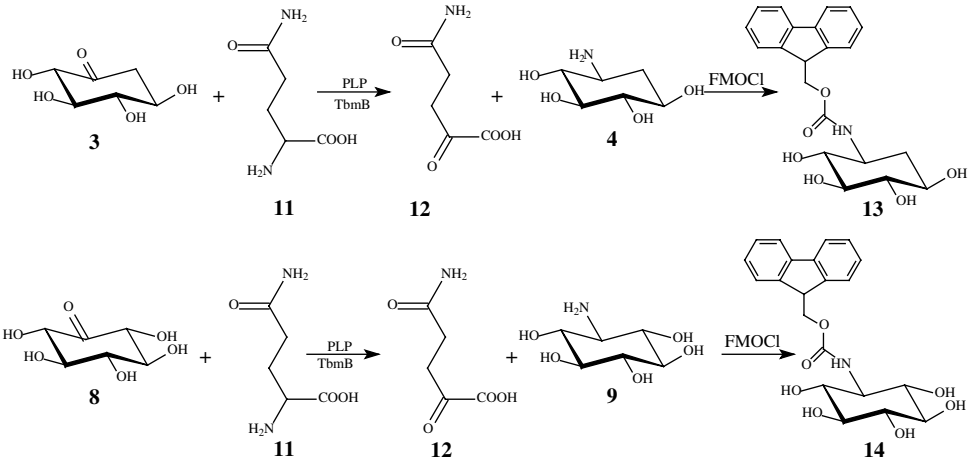


Scheme 1. Proposed pathways for the biosyntheses of 2-deoxy-streptamine (pathway **I**) and streptamine (pathway **II**). Tobramycin and spectinomycin are the typical antibiotics with 2-deoxystreptamine and actinamine (methylated streptamine) subunits. **1**: *myo*-Inositol-6-phosphate; **2**: glucose-6-phosphate; **3**: 2-deoxy-*scyllo*-inosose; **4**: 2-deoxy-*scyllo*-inosamine; **5**: 3-amino-2-deoxy-*scyllo*-inosose; **6**: 2-deoxystreptamine; **7**: *myo*-inositol; **8**: *scyllo*-inosose; **9**: *scyllo*-inosamine; **10**: streptamine.

streptamine biosynthetic genes in a cluster has been reported in spectinomycin producer *Streptomyces spectabilis*.[11] Biosynthesis of streptamine begins with the formation of *myo*-inositol-1-phosphate (**1**) from G-6-P (**2**) by *myo*-inositol-1-phosphate synthase. The product undergoes dephosphorylation to yield *myo*-inositol (**7**) with the activity of *myo*-inositol monophosphatase (SpeA). Dehydrogenation of *myo*-inositol with *myo*-inositol dehydrogenase (SpeB) to form *scyllo*-inosose (**8**) and its subsequent transamination by an aminotransferase (SpcS2) have been determined as actinamine (a methylated derivative of streptamine) biosynthetic steps. Transamination of *scyllo*-inosose with StsC from *Streptomyces griseus* has been determined as one of the streptidine biosynthetic steps.[12] However, the biosynthesis of DOS in actinomycetes has been largely circumstantial and is limited to the characterization of DOI synthase in *S. tenebrarius*.[13] In this communication, we report the characterization of 2-deoxy-*scyllo*-inosose aminotransferase (*tbmB*) from the tobramycin producer *S. tenebrarius*.

The deduced amino acid sequence encoded by *tbmB* is 74% and 60% identical to putative 2-deoxy-*scyllo*-inosose aminotransferases from *S. kanamyceticus* (KanB, protein_id CAE46938.1) and *M. echinospora* (GtmB, protein_id CAE06513.1), respectively. Lesser amino acid identities of TbmB to recently characterized BtrS (BAC41204.1) from *B. circulans* were also found. The latter protein represents the sole 2-deoxy-*scyllo*-inosose aminotransferase with the functional identification reported to date.[8,9]

To determine *tbmB* encodes a 2-deoxy-*scyllo*-inosose aminotransferase, the gene was amplified by PCR with a set of primers (AMTT1: 5′-AGGAATTCTCGACCATGCCCGTCCA-3′ and AMTT2: 5′-CAAAGCTTGGAGCGGGCGCTCAG-3′) and cloned into *Eco*RI and *Hin*dIII site of pET32a(+) vector to form pTBMB. The cloned DNA was transformed into competent *E. coli* BL21(DE3) cells following the standard protocol. The transformant was cultured in Luria Bertani (LB) medium. Overexpression of the *tbmB* was observed on SDS-PAGE by inducing the culture (at OD_{600} 0.5) with 0.4 mM IPTG at 20 °C for 20 h. Expression of *btrC* and enzyme assay of the product were carried out as previously described.[9] Coupled assays of TbmB with BtrC and *myo*-inositol dehydrogenase (Sigma) were carried out in 1 mL phosphate buffer (50 mM, pH 7.5) containing 5 mM G-6-P, 2.5 mM NAD^+, 5 mM L-glutamine and 0.6 mM pyridoxal 5′-phosphate (PLP). The reaction was initiated by the addition of 6 mg of each enzyme and quenched by heating at 90 °C following the incubation at 37 °C for 20 h. The protein was removed by centrifugation and the resulting mixture was acidified to pH 3 with 50 mM H_2SO_4. An aliquot (50 μL) was taken for the ESI-MS analysis. Water was used in place of G-6-P for a reference reaction. Two distinct peaks were observed in the reaction mixture as expected. A peak (*m*/*z* 164) displayed the MW of the expected 2-deoxy-*scyllo*-inosamine (**4**, Scheme 2), while the other corresponded to the formation of α-ketoglutarate (*m*/*z* 146.3) according to the Scheme 2 (data is not shown). To confirm the formation of 2-deoxy-*scyllo*-inosamine, the pH of the reaction mixture was raised to 6, passed through the ion exchange column (IRC-50), washed the impurities with water, and the compound was eluted with 5 mL of 2 M ammonia solution. The solvent was evaporated and concentrated the sample to 1 mL under reduced pressure. UV–vis derivative of 2-deoxy-*scyllo*-inosamine (**13**) was prepared following the previous report of Stead and Richards with slight modifications (Scheme 2).[14] To the concentrated effluent (500 μL), 9-fluorenylmethyl chloroformate (FMOCl) (20 μL, 20 μg/mL) was added and heated the mixture at 37 °C for 3 h. The mixture was filtered through a membrane (0.25 μm pore diameter) and taken for HPLC analysis at 260 nm. A new peak was detected while separating the components in C-18 column (Mightysil RP-18 Gp, Japan) with a linear gradient of acidified water (with 0.1% trifluoroacetic acid) and acetonitrile from 100% to 0%, and comparing the chromatogram with that of the reference sample. LC–MS analysis revealed peaks with MW 385.6 and 386.6, which correspond to the $[M^+]$ and $[M+H^+]$ for the amide derivative of 2-deoxy-*scyllo*-inosamine (**13**) (Fig. 1).



Scheme 2. Reactions catalyzed by TbmB. **3**: 2-deoxy-*scyllo*-inosose; **4**: 2-deoxy-*scyllo*-inosamine; **8**: *scyllo*-inosose; **9**: *scyllo*-inosamine; **11**: L-glutamine; **12**: α-keto-glutamate; **13**: amide derivative of **4**; **14**: amide derivative of **9**; FMOCl: 9-flourenylmethyl chloroformate; PLP: pyridoxal 5′-phosphate.

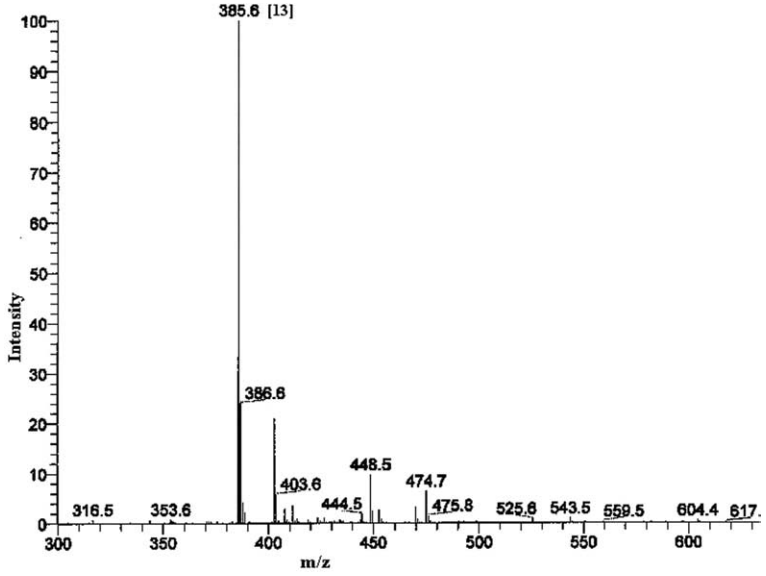


Figure 1. ESI-MS of the amide derivative of 2-deoxy-*scyllo*-inosamine (**15**) where the peaks at 385.6 and 386.6 stand for the corresponding [M^+] and [$M+H^+$] values.

TbmB also displays 55% identity of its amino acid residues to StsC (CAA70012.1) from *S. griseus*. The latter protein catalyzes the transamination of *scyllo*-inosose to *scyllo*-inosamine utilizing L-glutamine in the presence of PLP. To clarify whether TbmB accepts the substrate of StsC or not, a coupled assay was carried out using *myo*-inositol dehydrogenase (mdh) (Sigma, USA) in 1 mL incubation mixture where BtrC and G-6-P were replaced with 5 mM of *myo*-inositol and 2-unit of mdh, respectively. The LC-MS analyses of the FMOCl-derivatized products revealed a peak with MW of 401.5 and 402.6, which correspond to the [M^+] and [$M+H^+$] for the amide derivative of *scyllo*-inosamine (**14**), respectively (Fig. 2).

The results clearly indicate that *tbmB* encodes a protein (TbmB) that involves in the formation 2-deoxy-*scyllo*-inosamine, an intermediate in the DOS biosynthetic pathway, in the *S. tenebrarius*. Here, it is emphasized that this is the first report of identification of an aminotransferase involved in DOS biosynthesis from *Streptomyces*. Conversion of *scyllo*-inosose to *scyllo*-inosamine with the TbmB is the other interesting feature. Walker and co-workers have given an indirect evidence for the involvement of an aminotransferase in the both of the transamination reactions of the DOS biosynthetic pathway by carrying out the mimic reactions using a pure enzyme isolated from cell-free extract of *M. purpurea*.[15] Instead, the presences of *tbmB* and *tbmX*, *gtmB* and *gtmD*, and *kanB* and *kanD* in tobramycin, gentamicin, and kanamycin biosynthetic gene clusters support for 'one enzyme one step transamination' hypothesis for DOS biosynthetic steps. However, it is yet to be seen whether TbmB catalyzes both

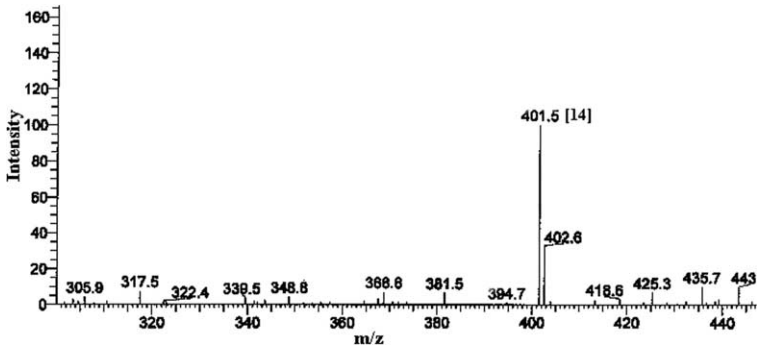


Figure 2. ESI-MS of the amide derivative of *scyllo*-inosamine (**14**) where the peaks at 385.6 and 386.6 stand for the corresponding [M^+] and [$M+H^+$] values.

transaminations or not. Provided that an idiotroph of *M. echinospora* requiring exogenous DOS to produce gentamicin yielded a mixture of hydroxygentamicin derivatives with broad-spectrum activities when the culture was fed with streptamine,[16] construction of a gene cassette expressing DOS and its subsequent transformation into streptamine-containing AmAcs producers would generate new DOS-containing AmAc derivatives. In this particular aspect, the substrate flexibility of TbmB can be exploited for replacing DOS with streptamine and vice versa to generate unusual aminocyclitols.

Acknowledgements

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