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Molecules and Cells



# Heterologous Production of Paromamine in *Streptomyces lividans* TK24 Using Kanamycin Biosynthetic Genes from *Streptomyces kanamyceticus* ATCC12853

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**The 2-deoxystreptamine and paromamine are two key intermediates in kanamycin biosynthesis. In the present study, pSK-2 and pSK-7 recombinant plasmids were constructed with two combinations of genes: *kan*ABK, and *kan*ABKF and *kac*A respectively from kanamycin producer *Streptomyces kanamyceticus* ATCC12853. These plasmids were heterologously expressed into *Streptomyces lividans* TK24 independently and generated two recombinant strains named *S. lividans* SK-2/SL and *S. lividans* SK-7/SL, respectively. ESI/ MS and ESI-LC/MS analysis of the metabolite from *S. lividans* SK-2/SL showed that the compound had a molecular mass of 163 $[M + H]^+$, which corresponds to that of 2-deoxystreptamine. ESI/MS and MS/MS analysis of metabolites from *S. lividans* SK-7/SL demonstrated the production of paromamine with a molecular mass of 324 $[M + H]^+$. In this study, we report the production of paromamine in a heterologous host for the first time. This study will evoke to explore complete biosynthetic pathways of kanamycin and related aminoglycoside antibiotics.**

## INTRODUCTION

Aminoglycoside-aminocyclitol (AmAc) antibiotics containing 2-deoxystreptamine (DOS) are among the most clinically important broad-spectrum antibiotics mainly produced by *Streptomyces* and *Micomonospora spp* (Mai et al., 2005). These antibiotics can be divided into two major classes: 4,5-disubstituted DOS antibiotics, such as ribostamycin, butirosin and neomycin, and 4,6-disubstituted DOS antibiotics, like kanamycin, gentamicin and tobramycin. Glycosylation of DOS with a variety of sugars at various positions has brought structural diversity to the AmAc antibiotics (Karimi and Ehrenberg, 1994).

The cloning of DOS biosynthetic gene clusters from aminoglycoside antibiotics as kanamycin, tobramycin, gentamicin, ribostamycin, butirosin, and neomycin was carried out in previous studies, and some of the genes responsible for DOS biosynthesis have been characterized (Hirayama et al., 2006; Huang et al., 2005; Kharel et al., 2004a; Kudo et al., 1999; 2005; Subba et al., 2005; Zhao et al., 2005). However, the gene function information currently available for aminoglycoside biosynthesis is still inadequate for application in combinatorial biosynthesis (Nagaya et al., 2005). A biosynthetic study of kanamycin was recently begun with the isolation of its biosynthetic gene cluster (Kharel et al., 2004b). The heterologous expression of the pSKC2 cosmid in *Streptomyces venezuelae* YJ003 indicated that all of the genes responsible for kanamycin production are located in the pSKC2 cosmid (Thapa et al., 2007), even though the biosynthesis of each intermediate and final kanamycin has never been performed, except for the characterization of *kanA* involved in 2-deoxy-*scyllo*-inosamine (DOIA) synthesis (Mai et al., 2005). Reviews of the biosynthesis of AmAc antibiotics and related compounds containing DOS provided direction for the pathway of kanamycin biosynthesis (Flatt and Mahmud, 2006; Llewellyn and Spencer, 2006).

During kanamycin biosynthesis, D-glucose-6-phosphate (G6P) is converted into 2-deoxy-*scyllo*-inosose (DOI) in the presence of 2-deoxy-*scyllo*-inosose synthase (DOIS) and eventually yields DOS *via* keto-2-DOIA with the involvement of two enzymes: 2-DOI-keto-DOIA aminotransferase and 2-DOIA dehydrogenase (Kudo et al., 2005; Ota et al., 2000). The results of further investigation into the enzymes BtrM and BtrD suggest that *N*-acetyl-D-glucosamine (GlcNAc) is added to DOS to give 2′-*N*-acetylparomamine and that the acetyl group would be removed by the deacetylase enzyme to yield paromamine (Llewellyn and Spencer, 2006). The remainder of the pathway leading from paromamine (Fig. 1A) to kanamycin derivatives (kanamycin A, B and C) still awaits experimental elucidation. Therefore, we are trying to explore the exact route of kanamycin biosynthesis by expressing the various gene cassettes *in vivo*.

In the present study, we constructed two recombinant plasmids : pSK-2 and pSK7. Both plasmids were heterologously expressed in *S. lividans* TK24, separately, and the analysis of secondary metabolites showed the production of DOS and paromamine from these recombinant strains, respectively. This is the first report concerning the production of paromamine in a

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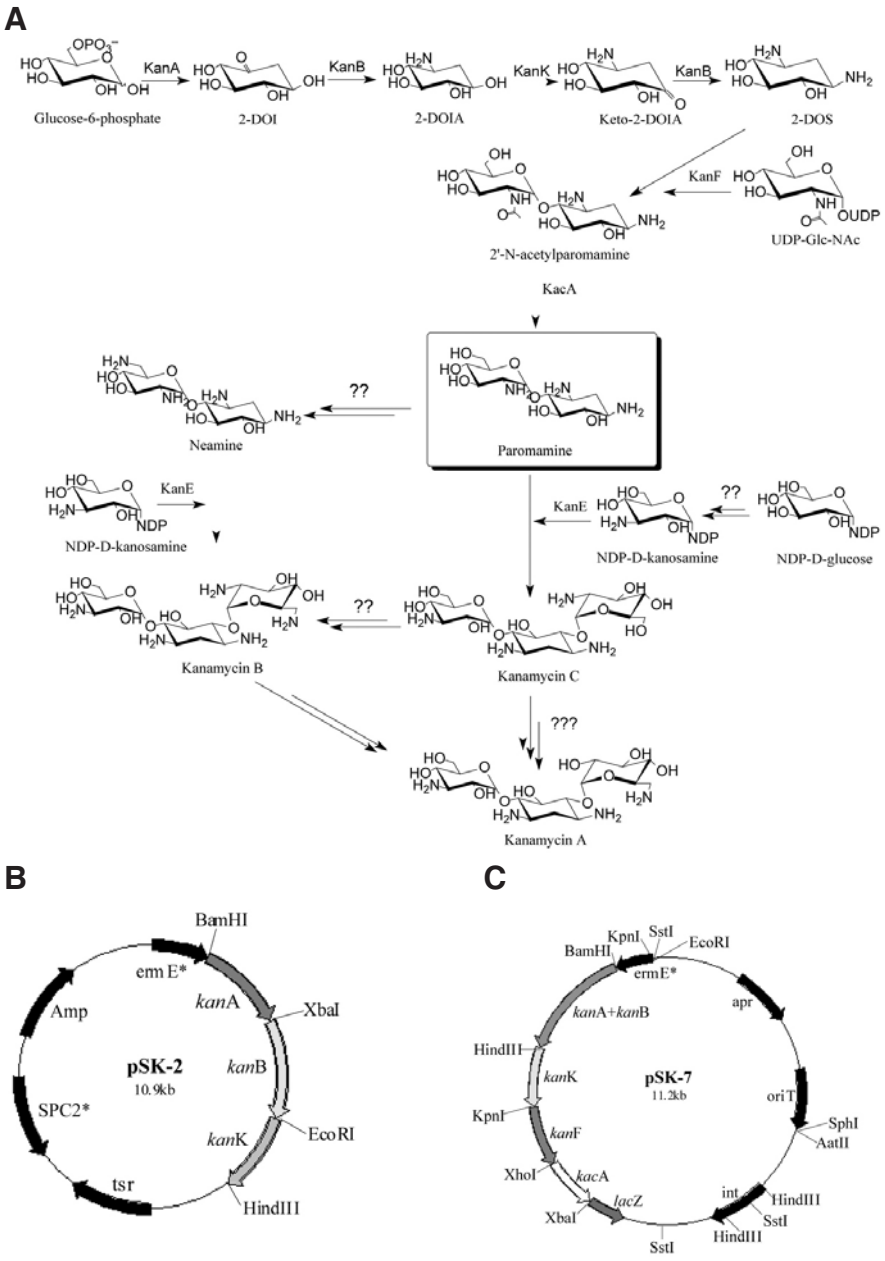


**Fig. 1.** Biosynthetic pathway of kanamycin via its intermediates **(**A). Maps of pSK-2 (B), and pSK-7 (C) recombinant plasmids.

heterologous host.

## MATERIALS AND METHODS

### Microorganisms, culture conditions and vectors

*S. lividans* TK24 was grown in R2YE medium at 28°C in shaking incubators (Hopwood et al., 1985). The organism was grown in solid plates for protoplast regeneration. Liquid R2YE was used for product isolation from the wild-type or recombinant strains by adjusting the pH 7.0 prior to sterilization. *Escherichia coli* XL1-Blue (Stratagene, USA) were used as a cloning host for DNA manipulation and cultured as described (Rosemary et al., 2008). The polymerase chain reaction (PCR) products were cloned into pGEM-T Easy vector (Promega, USA). Cosmid pSKC2 (Kharel et al., 2004b) was used to amplify the *kanA*, *kanB*, *kanK*, *kanF* and *kacA* genes. The pIBR25 (Ghimire et al., 2008; Sthapit et al., 2004) was used as an expression vector to generate pSK-2, and pSET152 was used as integration vector to generate pSK-7 (Table 1).

### DNA manipulations and insilico analysis

Routine manipulations, such as genomic and plasmid DNA isolation, restriction endonuclease digestion, alkaline phosphate treatment, and DNA ligation, were performed according to standard procedures (Basnet et al., 2006; Pageni et al., 2008; Sambrook and Russell, 2001). Oligonucleotide primers were synthesized at Geno-Tech. (Korea), and the enzymes were purchased from TAKARA (Japan). All chemicals used were of molecular biology grade and commercially available. Database searches were performed using the BLAST, FASTA and CLUSTAL W programs.

**Table 1.** List of plasmids and Strains used in this study

| Strains or plasmids | Description | Source or reference |
|---|---|---|
| *S. kanamyceticus* | Wild type produced kanamycin A, B and C | ATCC |
| *S. lividans* TK24 | Heterologous host | Hopwood et al. (1985) |
| *E. coli* XL1 blue | Cloning host | Stratagene PBL |
| *E. coli ET12456* | Cloning host (demethylation host) | Stratagene (USA) |
| *S. lividans* SK-2/SL | Transformant resulted from transformation of pSK-2 in *S. lividans* TK24 | This study |
| *S. lividans* SK-7/SL | Transformant resulted from transformation of pSK-2 in *S. lividans* TK24 | This study |
| pSET152 | *Streptomyces* integration vector containing *erm*E promotor and thiostreption selection marker | Hopwood et al. (2000) |
| pIBR25 | *Streptomyces* integration vector containing *erm*E promotor and thiostreption selection marker | Basundhara et al. (2004) |
| pGEM-T easy vector | *E. coli* general cloning vector AmpR | Promega (USA) |
| pGEM7f (+) Z | *E. coli* subcloning vector | Promega (USA) |
| pSKC2 | Kanamycin cosmid containing kanamycin gene cluster used to amplify *kan*A, *kan*B, *kan*K, *kan*F and *kac*A genes | Kharal et al. (2004b) |
| pSK-2 | Recombinant plasmid containing *kan*A, *kan*B, and *kan*K genes in pIBR25 | This study |
| pSK-7 | Recombinant plasmid containing *kan*A, *kan*B, *kan*K, *kan*F and *kac*A genes in pIBR25 | This study |

**Designing primers and PCR amplification**

Three sets of primers for *kanA*, *kanB* and *kanK* were designed for the construction of the DOS-producing plasmid, whereas four sets of primers for *kanA*/*kanB*, *kanK*, *kanF* and *kacA* were designed for the construction of the paromamine-producing plasmid (Table 2). In this study, the PCR products of two genes, *kanA* and *kanB*, were obtained using a single set of primers as they are located together and in same direction in gene cluster. PCR was carried out as follows: denaturation at 94°C for 7 min, 30 cycles of annealing at 60-70°C for 1 min and polymerization at 72°C, followed by one stage of gap filling at 72°C for 7 min.

**Construction of 2-DOS and paromamine producing plasmids**

For the construction of a DOS-producing plasmid, an *E. coli-Streptomyces* shuttle vector, pIBR25, which has a strong *ermE** promoter and thiostrepton resistant gene as a selection marker, was digested with *Eco*RI/*Hin*dIII, and *kanK* was ligated at the same restriction sites to form pSK-1. pSK-1 was digested with *Bam*HI and *Eco*RI, whereas the two genes, *kanA* and *kanB*, were digested and purified with *Bam*HI/*Xba*I and *Xba*I/*Eco*RI, respectively. Ligation was immediately carried out with purified pSK-1 to generate pSK-2 (Fig. 1B). The *Xba*I/*Bam*HI fragment containing five genes obtained from pGEM 7f (+) Z were cloned into *Xba*I/*Bam*HI digested pSET152 integration plasmid to construct pSK-7 (Fig. 1C).

**Protoplast preparation and transformation**

Protoplast preparation, transformation and the selection of antibiotic-resistant transformants were performed as described previously (Kieser et al., 2000). The pSK-2 and pSK-7 recombinant plasmids were transformed into freshly prepared protoplasts. After incubation at 28°C for 26 h, each plate was overlaid with 4 ml Bacto agar (0.3%) supplemented with 50 μg/ml thiostrepton for pSK-2 recombinant plasmids and the same concentration of apramycin was used for the pSK-7. Five days of incubation yielded some thiostrepton- and apramycin-resistant colonies, which were designated *S. lividans* SK-2/SL and *S. lividans* SK-7/SL, respectively. As a control, only vectors pIBR25 and pSET152 were transformed into *S. lividans* TK24 and designated as *S. lividans* IBR25 and *S. lividans* SET152, respectively. Transformation was confirmed by PCR, followed by respective restriction analysis.

**Product isolation and analysis**

For product analysis, four sets of recombinant strains viz; *S. lividans* SK-2/SL, *S. lividans* SK-7/SL, *S. lividans* IBR25 and *S. lividans* SET152, were cultured in R2YE media for 72 h at 28°C. 50 μg/ml thiostrepton and 50 μg/ml apramycin antibiotics were used for plasmid maintenance for respective recombinant strains. Compounds were isolated following procedures as described previously (Nepal et al., 2009). Electrospray ionization-liquid chromatography/mass spectrometries (ESI-LC/MS) of the isolated compounds were carried out for the identification of molecular weight. The UV-visible derivatives of isolated compounds were prepared according to the method described by Stead and Richards, with slight modification (Stead and Richards, 1997). During derivatization, the compounds were mixed with 9-fluorenylmethyl chloroformate (FMOC-Cl). The pH of the reaction sample was adjusted to approximately 9.5 using borate buffer (pH 10 and 0.37 mM), and the mixture was heated at 37°C for 90 min. The reaction was quenched by 0.1 M glycine. The sample was centrifuged for about 20 min and filtered by 0.2-μm nylon membrane filters (Whatman®, England). Liquid chromatography/mass spectrometry (LC/MS) analysis was carried out at 260 and 280 nm using a C18 column (Mightysil RP-18 Gp, Japan).

## RESULTS

**Sequence analysis of paromamine biosynthetic genes**

Analysis of secondary metabolite aminotransferase (SMAT) enzyme sequences showed that the G-D-E-x-77-E-D-x10-G-x3-G-x8-S-x4-K-x5-6-(E/G)-G-G motif is firmly conserved among the members of the SMAT family (Popovic et al., 2006). The amino acid sequence analysis of KanB with other aminotransferae showed that this enzyme conserved all the required motifs (Fig. 2A). Elucidation of the structure of zinc-containing NAD(P)-dependent alcohol dehydrogenase showed that the two zinc coordination motifs associated with catalytic and struc-

**Table 2.** List of primers used in this study

| Gene | Primer sequence (5′-3′) | Restriction site |
|---|---|---|
| For pSK-2 plasmid | | |
| *kan*K | F : ACG**GAATTC**AAAGGAGCAGACACCGTG | *Eco*RI |
| | R : CTA**AAGCTT**GGTGCCGGACAGGCCCTA | *Hin*dIII |
| *kanB* | F : GCG**TCTAGA**TTGGAGGTATGTGTCATG | *Xba*I |
| | R : GAA**GAATTC**TGTGACGGCTTCGGCTCA | *Eco*RI |
| *kan*A | F : GCC**GGATCC**CAGGATTGGGGACAGACC | *Bam*HI |
| | R : TTA**TCTAGA**CCCGTGGCGCCGCCGTCA | *Xba*I |
| For pSK-7 plasmid | | |
| *kac*A | F : CAT**CTCGAG**CTACCGGGAGATCGGGCT | *Xho*I |
| | R : CGG**TCTAGA**ACAGCGCCAGCTTCTTGC | *Xba*I |
| *kanF* | F : ATT**GGTACC**GAGTTCGACGCGGTGGGC | *Kpn*I |
| | R : ATA**CTCGAG**ACGTCGGGTGTCGTACGG | *Xho*I |
| *kan*K | F : ACT**AAGCTT**AAAGGAGCAGACAACGTG | *Hin*dIII |
| | R : ATA**GGTACC**GCCGGACAGGCCCTA | *Kpn*I |
| *kan*AB | F : ACT**GGATCC**TTGGAGGTATGTGTCATG | *Bam*HI |
| | R : ATAT**AAGCTT**GTGGCGCCGCCGTCA | *Hin*dIII |

Restriction sites are in bold.

tural zincs are important for the construction of the active site that are highly conserved in this family. Most of these aminoacids are conserved in Neo5 and thus Neo5 appears to be a typical zinc-containing alcohol dehydrogenase. KanK has 66% sequence similarity with Neo5 (GenBank accession no. AJ843080) from neomycin producer *S. fradiae.* Alignment of the deduced amino acid sequence of KanK showed that all of these motifs were conserved, as in Neo5. Thus, it appears to have the same enzymatic activity as Neo5 (Fig. 2B). The Sequence analysis of KanF showed 63% similarity with Neo8 (GenBanK accession no. CAH 58691) followed by 57% with RbmD (GenBank accession no. CAG34717) and 54% with GtmG (GenBank accession no. CAE06514). All of these enzymes showed its activity as 2-*N*-acetlyglycosyltransferase (Fan et al., 2008; Nepal et al., 2009; Park et al., 2008; Yokoyama et al., 2008). These results suggested that GlcNAc is added to DOS by KanF during paromamine biosynthesis. The amino acid sequence of KacA also contains the entire conserved domain as present in BtrD (Truman et al., 2007) and its function has been characterized as deacetylase (Park et al., 2008)

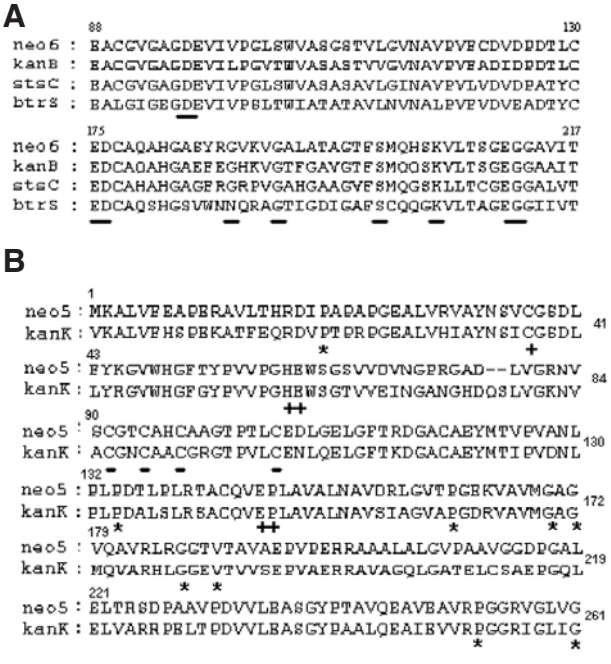


**Fig. 2.** Multiple alignment of KanB with Neo6, StsC and BtrS from *Streptomyces fradiae, Streptomyces griseus* and *Bacillus circulans* respectively. Black solid line represents the conserved region. The numbers are given from KanB amino acid sequence (A). Multiple alignment of KanK amino acid sequence with Neo5 from *Streptomyces fradiae*. The conserved amino acid residues are marked with asterisk. +, for catalytic zinc-ion coordination residue; -, structural zinc coordination cysteins. The numbers are given from KanK amino acid sequence (B).

**Construction and heterologous expression of pSK-2 and pSK-7**

*S. lividans* TK24 was chosen as a heterologous host due to its high transformation efficiency and rapid growth (mycelia can be harvested in a short period of time). Therefore, it is also considered to be a good heterologous host. Two recombinant plasmids, pSK-2 (*kan*A, *kan*B and *kan*K genes in pIBR25 expression vectors) and pSK-7 (*kan*A, *kan*B, *kan*K, *kan*F and *kac*A genes in pSET152 integration vector) were constructed. Both recombinant plasmids were transformed into *S. lividans* TK24 independently to generate *S. lividans* SK-2/SL and *S. lividans* SK-7/SL. *S. lividans* IBR25 and *S. lividans* SET152 were also generated as described in “Materials and Methods”.

**Product isolation and analysis**

All recombinant strains were cultured in R2YE medium in order to analyze the secondary metabolites. After the proper growth of mycelia, the compound was isolated separately from each culture broth as described in “Materials and Methods”. Electrospray ionization/mass spectrometry (ESI/MS) analysis of the compound isolated from *S. lividans* SK-2/SL showed that it had

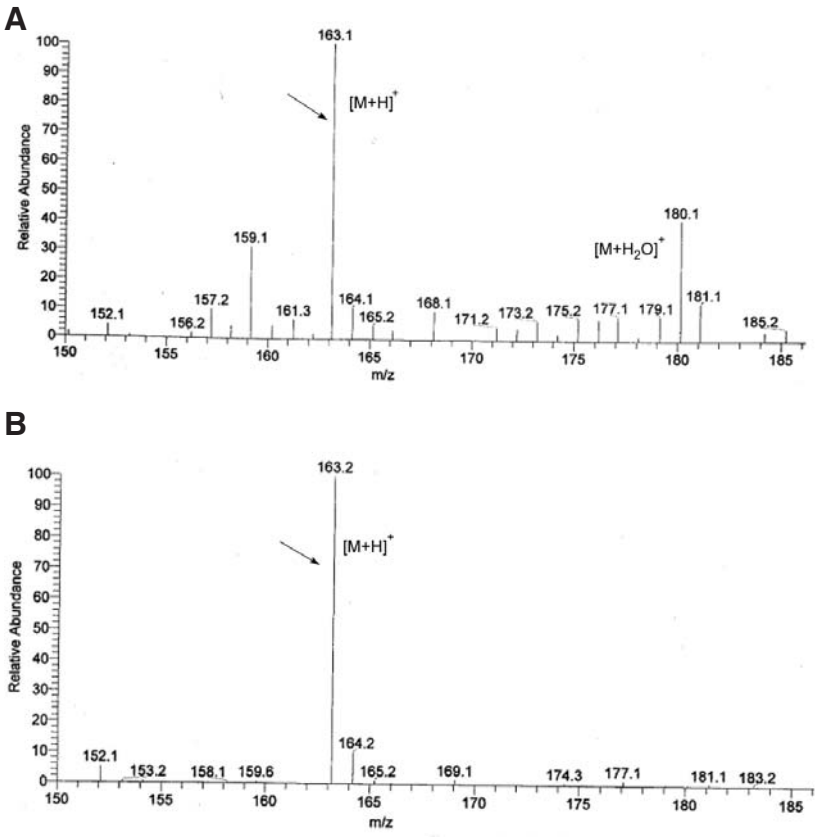


**Fig. 3**. ESI/MS of isolated compound from the extract of *S. lividans* SK-2/SL (A) and authentic 2-DOS (B).

a mass of *m/z* = 163 $[M + H]^+$, which was identical to the authentic DOS (Figs. 3A and 3B). The UV-visible derivative of the isolated compound was prepared as described in the materials and methods. LC/MS analysis of the derivatized compound from *Streptomyces lividans* SK-2/SL and standard DOS showed a mass of *m/z* = 385 $[M + H]^+$ (Figs. 4A and 4B) at the same retention time. However, both of these profiles were not observed in the control (data not shown). This indicated that 2-DOS was produced by the combined activity of *kanA*, *kanB* and *kanK*. Meanwhile, a feeding experiment was carried out with D-glucose for the overproduction of DOS from *S.lividans* SK-2/SL. 0.6% of D-glucose was fed into the culture broth at 48 h and the compounds were subsequently isolated. The production was slightly enhanced which made easy to carry out mass analysis but insufficient to quantify the production level.

The compound isolated from *S. lividans* SK-7/SL was purified and subjected to ESI/MS analysis for the detection of paromamine production. It showed a mass of *m/z* = 324 $[M + H]^+$, which was identical to authentic paromamine (Fig. 5A). ESI/MS/MS fragmentation of this compound with *m/z* = 324 produced a pattern with two major fragments. One fragment was at 163 amu, corresponding to the mass of DOS, and the other fragment was at 162 amu, corresponding to the mass of the amino sugar. In addition, two major fragments were obtained at 307 amu and 289 amu (Fig. 5B). These findings were consistent with previously analyzed data (Kotretsou and Kokotou, 1998; Park et al., 2007) and also with the proposed fragmentation pattern (Fig. 5C). Another fragment was also observed at 279 amu, which was not consistent with the previously reported data. Therefore, we assumed that this peak might be attributable to high collision energy.

## DISCUSSION

Sequence analysis of *kanB* with other genes, such as *neo6*, *stsC* and *btrS*, and *kanK*, with *neo5* showed that all of the conserved domains required for each function were present in *kanB* and *kanK*. It was previously proposed that the *kanA*, *kanB*, *kanC* and *kanD* genes are responsible for the production of DOS: *kan*B would act amination in one position, and *kan*D act at another position, while *kan*C would act as dehydrogenase (Kharel et al., 2004b). However, the results of the present study demonstrated that two transamination reactions are catalyzed by a single KanB enzyme as shown by the action of BtrS, an aminotranfserase from the butirosin producer *Bacillus circulans* (Yokoyama et al., 2007). We have generated the *neo*6 disrupted mutant of *Streptomyces fradiae* which completely failed to produce the neomycin. This mutated strain was complemented with *kan*B. Secondary metabolites analysis of complementary strain showed the restoration of neomycin production (unpublished data). This result further supports the function of *kan*B as doubly aminotransferase in 2-DOS biosynthesis. Thus, *kan*D would act at a later stage of biosynthesis and *kan*K gene acts as a dehydrogenase in the same pathway. Similarly, sequence analysis of *kanF* with *btrM* and *neo8*, and *kac*A with *btr*D provided the route from DOS to paromamine. Therefore, we integrated *kanF* and *kacA* along with DOS-producing genes (pSK-2 plasmid) to facilitate further expression of the genes. Similarly, the detection of paromamine in *S. lividans* SK-7/SL culture broth showed that two genes, *kan*F (glycosyltransferase) and *kac*A (deacetylase), are responsible for the addition of the primary metabolite UDP-*N*-acetylglucosamine at the C-4 position to produce 2′-*N*-acetylparomamine and its deacetylation activity, respectively.

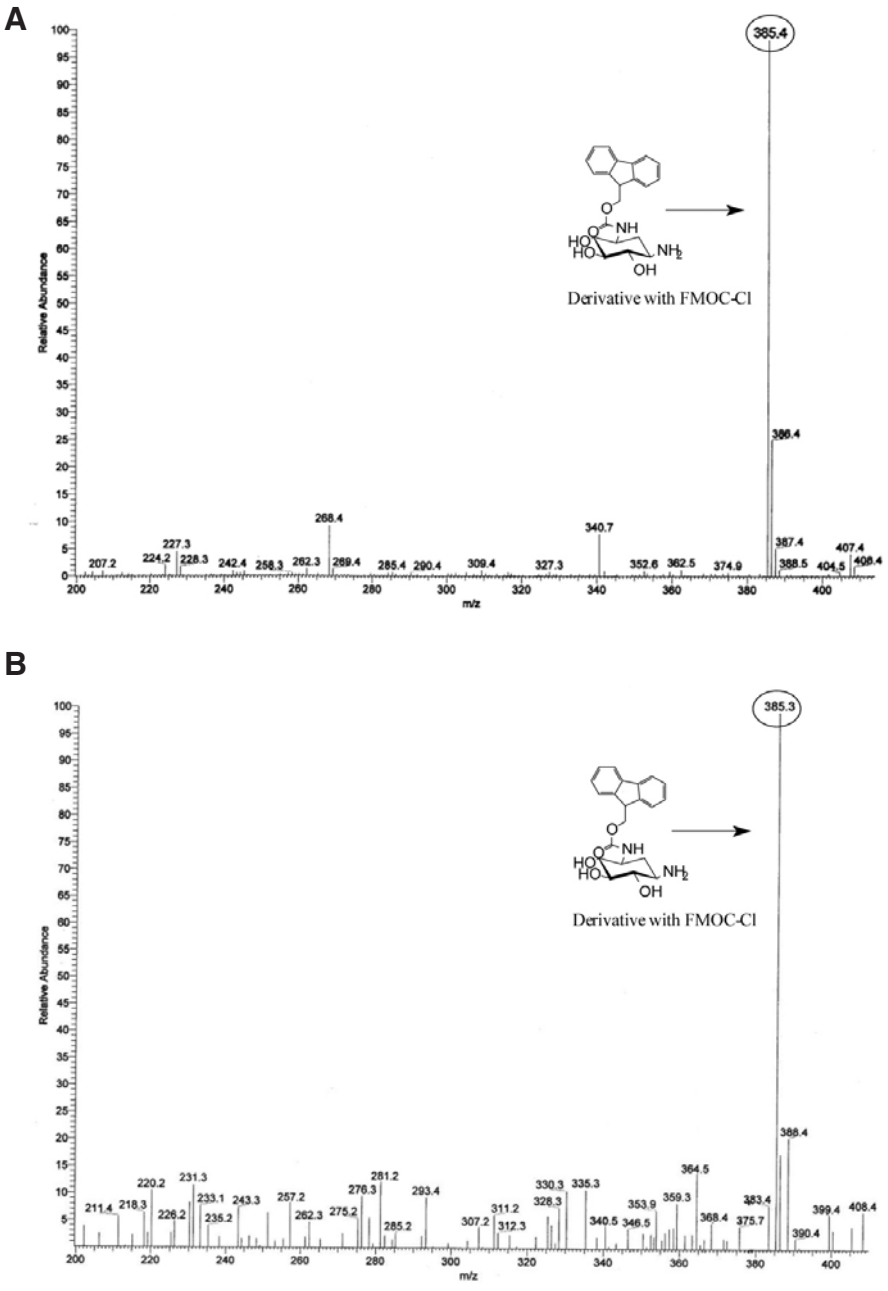


**Fig. 4**. ESI-LC/MS of DOS (A) and the isolated compound from the extract of *S. lividans* SK-2/SL (B) after derivatization with FMOC-Cl.

During product isolation, we tried to isolate the compound in different glucose-rich production media, including ISP2, R2YE and N-Z amine. Among the tested media, R2YE was found to be the best media for the growth of mycelia but the level of production was too low even difficult to analysis by mass. D-glucose is the precursor to DOS biosynthesis. Thus, a feeding experiment with D-glucose was carried out during the peak hours of culture (48-52 h) and showed D-glucose feeding culture broth produced a bit higher amount (enough to mass analysis) of DOS and paromamine (but not quantified in this study) when it was harvested between 130 and 150 h.

Comparing with authentic 2-DOS, ESI/MS and LC/MS analysis of secondary metabolites from *S. lividans* SK-2/SL showed the production of 2-DOS. Similarly, ESI/MS/MS profiles of the metabolites from *S. lividans* SK-7/SL showed the production of paromamine and fragmentation patterns were consistent with the previously analyzed data (Park et al., 2007).

We transformed pSK-2 recombinant plasmid in *S. venezuelae* YJ003 and generated *S. venezuelae* SK-2/SV recombinant strain. The 2-DOS was detected from the isolated product of *S. venezuelae* SK-2/SV which was verified by ESI/MS and LC/MS as compared with authentic 2-DOS (data not shown). Detection of 2-DOS and characterization of KacA enzyme as deacetylase was carried out by Park et al. (2008) using *S. venezuelae* YJ003 as a heterologous host. KanF involved in paromamine biosynthesis as glycosyltransferase which transfers UDP-GlcNac as sugar donor and 2-DOS as a acceptor to form 2-*N*-acetylparomamine which eventually is converted to paromamine by the activity of KacA enzyme. Production of 2-DOS from both of recombinant strains (*S. venezuelae* SK-2/SV and *S. livi-*

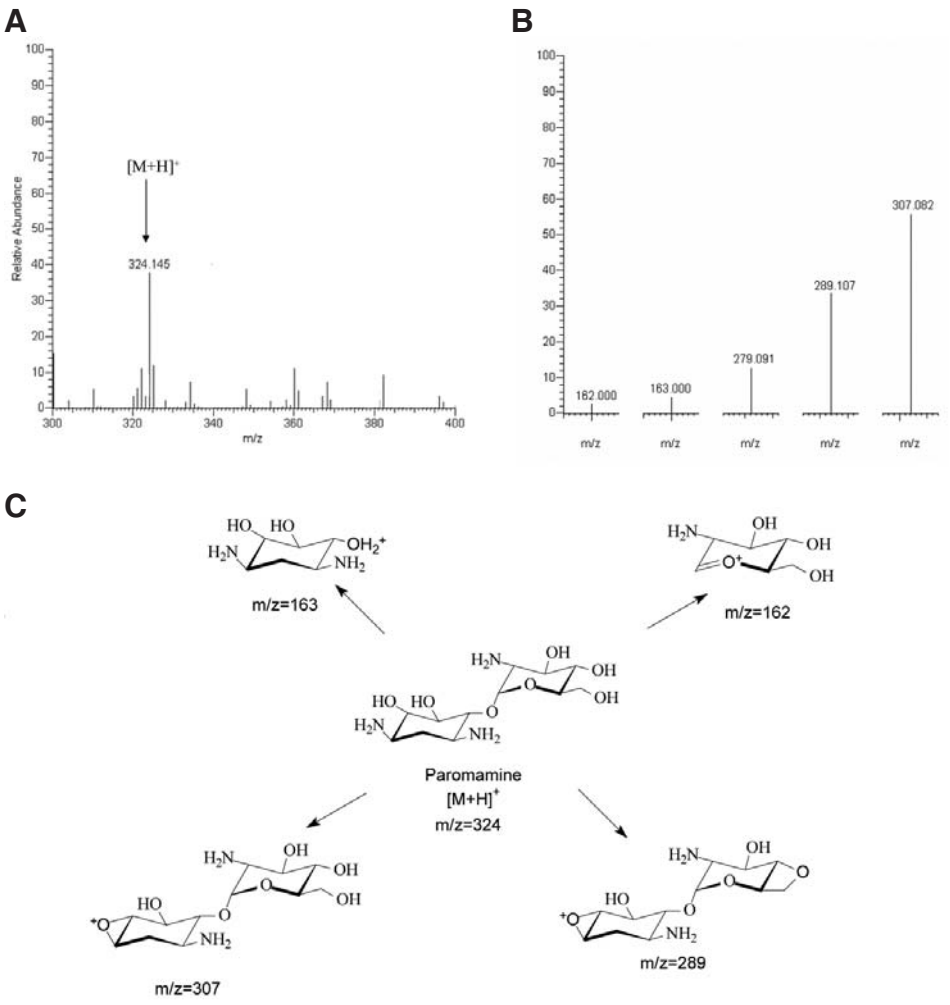


**Fig. 5.** ESI/MS (A) and ESI/MS-MS (B) spectra from the compound isolated from *S. lividans* SK-7/SL. Proposed fragmentation pattern of paromamine (C).

*dans* SK-2/SL) and null production of 2-DOS and paromamine from *S. lividans* IBR25 and *S. lividans* SET152 clearly demonstrated that there was no any enzymatic role of *S. lividans* TK24 host on biosynthesis of both of the intermediates.

The paromamine intermediate is important in kanamycin biosynthesis because it is still unclear whether the kanamycin A biosynthesis follows a path from paromamine to kanamycin C to kanamycin B or it follows the kanamycin B route *via* the neamine route. There are two possible pathways for the formation of kanamycin B complex: first formation of kanamycin C at fist by the attachment of NDP-D-kanosamine to paromamine and modification on it and second, paromamine first converted to neamine and attachment of NDP-D-kanaosamine with neamine by glycosyltransferase enzyme and formed final kanamycin B. One more possibility is that kanamycin B may be formed via neamine route concurrently with kanamycin C route as well (Fig. 1A). Thus, biosynthesis of kanamycin derivatives is still awaiting for experimental elucidation and expected that present study will give the gateway for further researches on aminoglycosides antibiotics.

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