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Biosynthesis of Ribostamycin Derivatives by Reconstitution and Heterologous Expression of Required Gene Sets

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Abstract Ribostamycin is a 4,5-disubstituted 2-deoxystreptamine (DOS)-containing aminoglycoside antibiotics and naturally produced by *Streptomyces ribosidificus* ATCC 21294. It is also an intermediate in the biosynthesis of butirosin and neomycin. In the biosynthesis of ribostamycin, DOS is glycosylated to generate paromamine which is converted to neamine by successive dehydrogenation followed by amination, and finally ribosylation of neamine gives ribostamycin. Here, we report the biosynthesis of 6′-deamino-6′-hydroxyribostamycin (a ribostamycin derivative or pseudoribostamycin) in *Streptomyces venezuelae* YJ003 by reconstructing gene cassettes for direct ribosylation of paromamine. A trace amount of pseudoribostamycin was detected with ribostamycin in the isolates of ribostamycin cosmid heterologously expressed in *Streptomyces lividans* TK24. It has also indicated that the ribosyltransferase can accept both neamine and paromamine. Thus, the present in vivo modification of ribostamycin could be useful for the production of hybrid compounds to defend against bacterial resistance to aminoglycosides.

Keywords Aminoglycoside · Ribostamycin derivatives · Biosynthesis · Ribosyltransferase · Heterologous expression · *Streptomyces*

Introduction

Aminoglycosides are clinically important broad-spectrum antibiotics [1] and are useful drugs against human immunodeficiency virus [2, 3]. Ribostamycin is a 4,5-disubstituted 2-deoxystreptamine (DOS)-containing aminoglycoside antibiotic produced by *Streptomyces ribosidificus* [4]; in contrast, butirosin and xylostasin, which belong to the same class of ribostamycin, are produced by *Bacillus* species. Despite the distinct taxonomic and/or

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morphological differences of the producing strains, the biosynthetic pathways of these antibiotics appear to be closely related since mutants of butirosin-producing *Bacillus circulans* also produced ribostamycin or xylostasin [5]. Ribostamycin is composed of three subunits: DOS (ring I), neosamine (ring II), and ribose (ring III), which are also common subunits of neomycin and butirosin (Fig. 1). The C1-NH_2 in butirosin is conjugated with (2S)-4-amino-2-hydroxy butyric acid (AHBA), and neomycin contains one additional neosamine sugar (Fig. 1).

Bacterial resistance to aminoglycosides has been a serious health threat [6] in the past several years. To overcome this problem, several enzymatic and semisynthetic modifications of existing compounds have been carried out. Some semisynthetic compounds like amikacin or arbekacin, in which the AHBA side chain is attached to kanamycin or tobramycin, have shown improved biological activity [7]. Recently, some researchers have also focused on enzymatic synthesis of AHBA–aminoglycoside conjugants [8]. In addition to this, modification of the existing compounds by exchanging the sugars or altering specific functional groups within the sugar moiety via genetic manipulation can be an effective measure to conquer the bacterial resistance.

The biosynthetic gene clusters for aminoglycosides have been identified recently. In 2008, Park et al. reported the minimal biosynthetic genes for a gentamicin intermediate, gentamicin $A_{2,}$ which corresponds to 4,6-disubstituted DOS-containing aminoglycoside [9], but the complete biosynsthetic pathway is still under investigation. Their study proved that three genes (*gtmA–gtmB–gacH*) were necessary and sufficient for DOS formation in *Streptomyces venezuelae* YJ003. Two genes, *gtmG–gtmM* (glycosyltransferase and deacetylase), added an *N*-acetylglucosamine to the DOS, resulting in paromamine. Furthermore, *gtmE* added a xylose to the paromamine, resulting in gentamicin A2. In addition to this, they also expressed three resistance genes (*gtmF–gtmK–gtmL*) to protect host cells against the activity of the produced antibiotics. The biosynthetic gene cluster for

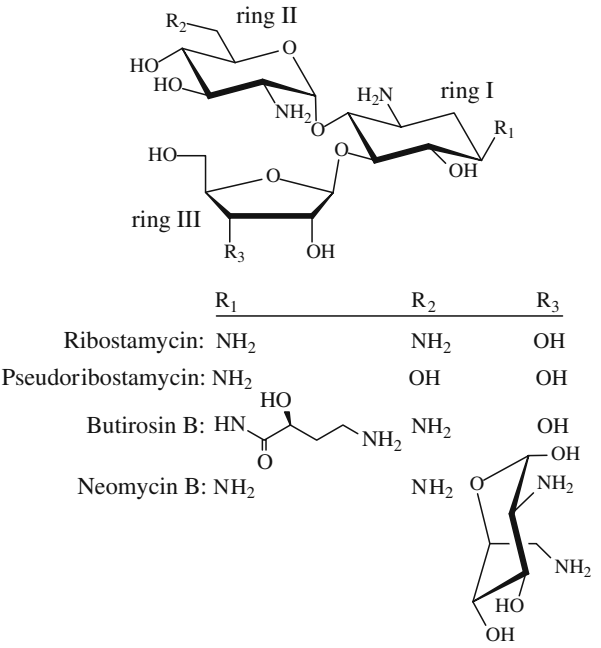

Fig. 1 Structure of ribostamycin-related aminoglycosides

ribostamycin was previously isolated from *S. ribosidificus* [10]. When heterologously expressed in *Streptomyces lividans* TK24, the isolated cosmid produced ribostamycin [11], demonstrating that the cosmid contained all the genes required for its biosynthesis. Genetic analysis of ribostamycin gene cluster shows that there are three genes (*rbmA–rbmB–rbmC*) analogous to *gtmA–gtmB–gacH* from gentamicin or *neo7–neo6–neo5* from neomycin assigned for the formation of DOS. Complementation study of *rbmD* (glycosyltransferase in ribostamycin) in glycosyltransferase (*neo8*) mutant of *Streptomyces fradiae* restored neomycin production [12]. Analysis also shows that there is a deacetylase (*racJ*) homologous to *neo16* (neomycin) and *brtD* (butirosin) required for formation of paromamine. Furthermore, two genes *rbmG* and *rbmH* show high homology with *neo11* (glucosaminyl-6′-oxidase) and *neo18* (aminotransferase) from neomycin gene cluster and the respective functional genes *btrQ* and *btrB* from butirosin gene cluster. The two genes have been characterized to function during conversion of paromamine to neamine in the biosynthesis of both neomycin and butirosin [13]. Ribostamycin is presumed to be produced by addition of a ribose to neamine by a ribosyltransferase (*racK*) and a phosphatase (*racH*). However, it is also possible to ribosylate on paromamine by the enzyme BtrL (ribosyltransferase of butirosin) [14]. Assuming that it is possible to make a modified ribostamycin (6′-deamino-6′-hydroxyribostamycin or pseudoribostamycin; Fig. 2) by direct ribosylation of paromamine, we cloned the minimal required gene sets and heterologously expressed them in *S. venezuelae* YJ003 [15]. For this, three genes (*neo5*, *neo6*, and *neo7*) that are responsible for DOS formation [16] were taken from the neomycin gene cluster, and other genes were taken from the ribostamycin. Since there is no report of target-modifying resistance gene in ribostamycin gene cluster, to protect the heterologous host, we have used *kmr* from kanamycin which acts by binding to ribosome without modification of antibiotics [17]. Step-by-step transformation of gene sets and

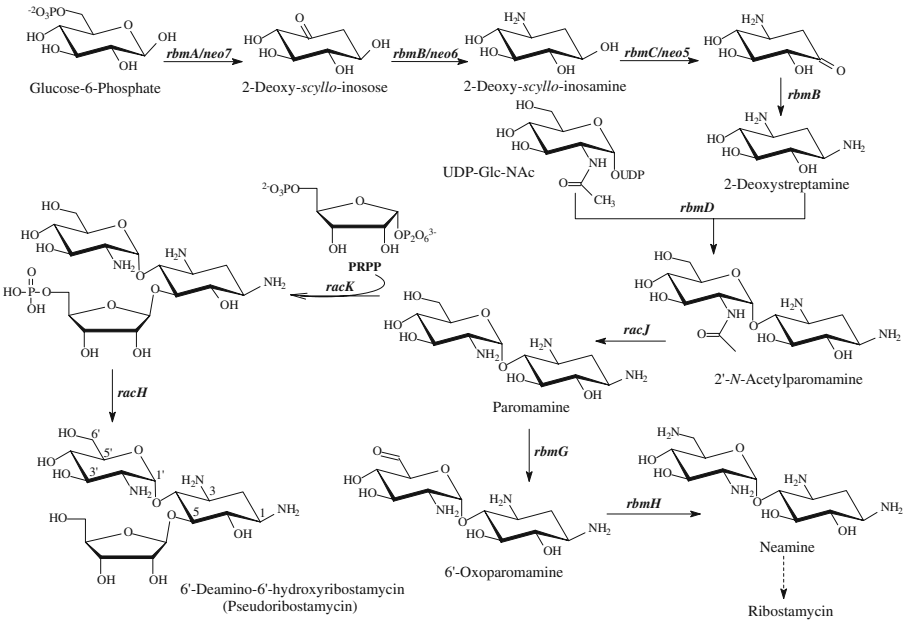

Fig. 2 Proposed biosynthetic pathway of ribostamycin and 6′-deamino-6′-hydroxyribostamycin (pseudoribostamycin)

analysis of the product showed that there is a formation of pseudoribostamycin along with other intermediates. The result revealed the possibility of synthesizing modified aminoglycoside by in vivo manipulation of the genetic organization.

Materials and Methods

Bacterial Strains, Plasmids, Media, and Culture Conditions

All bacterial strains, vectors, and recombinant plasmids used in this study are listed in Table 1. The standard strains of *Escherichia coli* XL1-Blue MRF and *E. coli* ET12567 (Stratagene) were used for cloning and demethylation. pGEM-T Easy and pGEM-7Zf(+) (Promega, USA) were used for DNA sequencing and subcloning. All *E. coli* strains were grown at 37 °C in an incubator for 6–12 h in Luria Bertani supplemented with ampicillin (100 μg/ml), tetracycline (25 μg/ml), chloramphenicol (100 μg/ml), and apramycin (100 μg/ml) when required.

S. venezuelae YJ003 was used for integration of DOS-producing genes, and the integrated host was then used to express other genes. pSET152ermE* modified from pSET152 and pIBR25 were used as integration and expression vectors, respectively. *Streptomyces* strains were cultured in R2YE medium or N–Z amine medium [18] supplemented with 500 μg/ml thiostrepton and/or apramycin for selection. All *Streptomyces* strains were cultured at 28 °C in an incubator for 4–6 days (liquid culture) and 6–10 days (plates). Restriction enzymes were purchased from TaKaRa (Shiga, Japan). Standard-quality media and chemicals were used throughout the experiments. DNA isolation and manipulation were performed according to standard protocols [19].

Table 1 Strains and plasmids used in this study.

Strains or plasmids	Description	Reference(s)
Strains		
S. ribosidificus	Ribostamycin producer	Wild type
S. fradiae	Neomycin producer	Wild type
S. venezuelae YJ003	Sugar gene deleted mutant of pikromycin producer	[15]
S. venezuelae YJ3DOS152	*S. venezuelae* YJ003 integrated with pNDOS-152	This study
S. venezuelae YJ3DOS152/pIBR-RmDJ	Paromamine producer	This study
S. venezuelae YJ3DOS152/pIBR-RmDJKHEr	Pseudoribostamycin producer	This study
S. venezuelae YJ003/pIBR25	For control strain	[20]
Plasmids		
pIBR25	*Streptomyces* expression vector	[21]
pSET152-ermE*	*Streptomyces* integration vector	[22]
pGEM-7Zf(+)	Subcloning vector	Promega (USA)
pNDOS-152	pSET152-ermE* containing DOS genes	This study
pIBR-RmDJ	pIBR25 containing rbmD and racJ	This study
pIBR-RmDJKHEr	pIBR25 containing rbmDE, racJKH, and kmr	This study

Cloning and Construction of Recombinant Plasmids

A recombinant pNDOS-152 (supplementary Fig. S1) was constructed by using two pairs of primers to amplify the three genes responsible for DOS. Two genes, *neo5* (dehydrogenase) and *neo6* (L-glutamine: 2-deoxy-*scyllo*-inosone aminotransferase), were obtained from the Nem37 cosmid DNA template (neomycin-cosmid unpublished) using the primers Neo56-F and Neo56-R. Similarly, *neo7* (2-deoxy-*scyllo*-inosone synthase) was obtained with the primers Neo7-F2 and Neo7-R. The *Streptomyces* integration vector pSET152-*ermE** was used for cloning. The expression recombinants, pIBR-RmDJ (supplementary Fig. S2) and pIBR-RmDJKHEr (supplementary Fig. S3), were constructed in pIBR25 by using primers designed to amplify the genes that are assigned to their respective functions (Table 2). All of the primers used in this study are listed in supplementary Table S1. The ribostamycin cosmid, pRMB4 [10], was used as a template and the polymerase chain reaction (PCR) was performed as follows: denaturation at 94 °C for 1 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 to 70 °C for 1 min, and polymerization at 72 °C for 1 min; and finally gap filling at 72 °C for 1 min. The PCR products were purified and cloned in pGEM-T Easy vector for DNA sequencing.

Transformation

The recombinants were introduced into *S. venezuelae* YJ003 by protoplast transformation method as described previously [20]. The integration of pNDOS-152 into the chromosome of *S. venezuelae* YJ003 was confirmed by PCR, and the correct transformants were named as *S. venezuelae* YJ3DOS152. Other recombinants were introduced in *S. venezuelae* YJ3DOS152 by the same protocol. Again, the transformation was confirmed by PCR and restriction enzyme digestion. The correct exconjugants were named as *S. venezuelae* YJ3DOS152/pIBR-RmDJ and *S. venezuelae* YJ3DOS152/pIBR-RmDJKHEr, respectively, and kept in stock for further uses.

Fermentation, Extraction, and Analysis of Ribostamycin Derivatives

S. venezuelae YJ3DOS152 and *S. venezuelae* YJ3DOS152/pIBR-RmDJKHEr were cultured in 50 ml of seed media containing the required antibiotics. After 36-h incubation at 28 °C, these seed cultures were transferred to 400 ml of main media in Erlenmeyer flasks. The culture flasks

Table 2 Functional assignment of the required gene sets for biosynthesis of pseudoribostamycin.

Gene	Proposed role of gene product	Source strain
neo7	DOI synthase	*S. fradiae*
neo6	Aminotransferase	*S. fradiae*
neo5	Dehydrogenase	*S. fradiae*
rbmD	Glycosyltransferase	*S. ribosidificus*
racJ	Deacetylase	*S. ribosidificus*
racK	Phosphoribosyltransferase	*S. ribosidificus*
racH	Phosphoribosyl phosphatase	*S. ribosidificus*
rbmE	Efflux pump	*S. ribosidificus*
kmr	RNA methylase for resistance to host	*S. kanamyceticus*

were incubated for 5 days at 28 °C. The target compound was isolated using Amberlite IRC-50 ion-exchange resin (Acros Organics, NJ, USA) according to the previous protocol [20] and partially purified by cold methanol precipitation method. Then the molecular weight of the compounds was observed by electrospray ionization mass spectrometry (ESI-MS). The compounds were further identified by liquid chromatography coupled to an electrospray ionization mass spectrometer (LC-ESI/MS) after derivatization with fluorenylmethyl chloroformate (FMOC-Cl) as described previously [20]. Also, the identification of compounds was carried out by mass-to-mass fragmentation analysis (ESI-MS/MS).

Results and Discussion

Analysis of Biosynthetic Gene Cluster for Ribostamycin Derivatives

Three genes (*neo5*, *neo6*, and *neo7*) from *S. fradiae* that are responsible for formation of DOS have already been characterized in vitro and in vivo [16, 20]. Here, we cloned these genes in order to produce DOS in a heterologous host; we also cloned other putative genes functionally assigned to the formation of paromamine and pseudoribostamycin. The *rbmD* can replace *neo8* for the first glycosylation step in the biosynthesis of neomycin [12]. In silico analysis has shown that *racJ* plays the role of a deacetylase homologous to the well-characterized *btrD* or *neo16* in butirosin or neomycin biosynthesis, respectively, to form paromamine [23, 24]. Similarly, RacK shares high homology with other phosphoribosyl transferases (supplementary Fig. S4); for example, BtrL has been characterized in the biosynthesis of butirosin as *O*-ribosyltransferase that accepts neamine or paromamine and 5-phosphoribosyl-1-diphosphate (PRPP) as substrates [14]. RacK and its homologs from other ribostamycin-containing aminoglycoside biosynthetic gene clusters have a distinctive PRPP binding motif that is also found in other phosphoribosyl transferases. A previous study showed that the adjacent Asp-Asp, or Glu-Asp, residues within this motif play key catalytic roles and are responsible for divalent metal binding [25]. In RacK, the conserved residues correspond to D239 and D240 which is indicated by white letters on dark background (supplementary Fig. S4). The RacH shows high homology with BtrP, which acts as a phosphatase in butirosin biosynthesis [14]. Similarly, the RbmE protein presumably acts as an efflux pump [10] and *kmr* of kanamycin provides self-resistance for the host [17].

Biosynthesis of Pseudoribostamycin and Other Derivatives in *S. venezuelae*

The recombinant pNDOS-152 derived from the integration vector was introduced into *S. venezuelae* YJ003. Integration of DOS genes into the heterologous host was confirmed by PCR, and the transformant was named *S. venezuelae* YJ3DOS152. Similarly, the recombinant pIBR-RmDJ derived from the expression vector, bearing a glycosyltransferase and a deacetylase gene, *rbmD* and *racJ*, was introduced into *S. venezuelae* YJ3DOS152. Gene transfer was again confirmed by PCR and restriction enzyme digestions, and the transformant was named *S. venezuelae* YJ3DOS152/pIBR-RmDJ. Both *S. venezuelae* YJ3DOS152 and *S. venezuelae* YJ3DOS152/pIBR-RmDJ were cultured in R2YE liquid medium. The products from the cultures were DOS and paromamine, respectively, as shown by ESI-MS and LC-MS data (supplementary Fig. S5a, b, and c), whereas isolates from *S. venezuelae* YJ003/pIBR25 did not produce these mass peaks (data not shown).

Ribostamycin is thought to be produced via the paromamine to neamine pathway, as shown in analogous pathways of butirosin and neomycin biosynthesis [14, 24]. Here, we

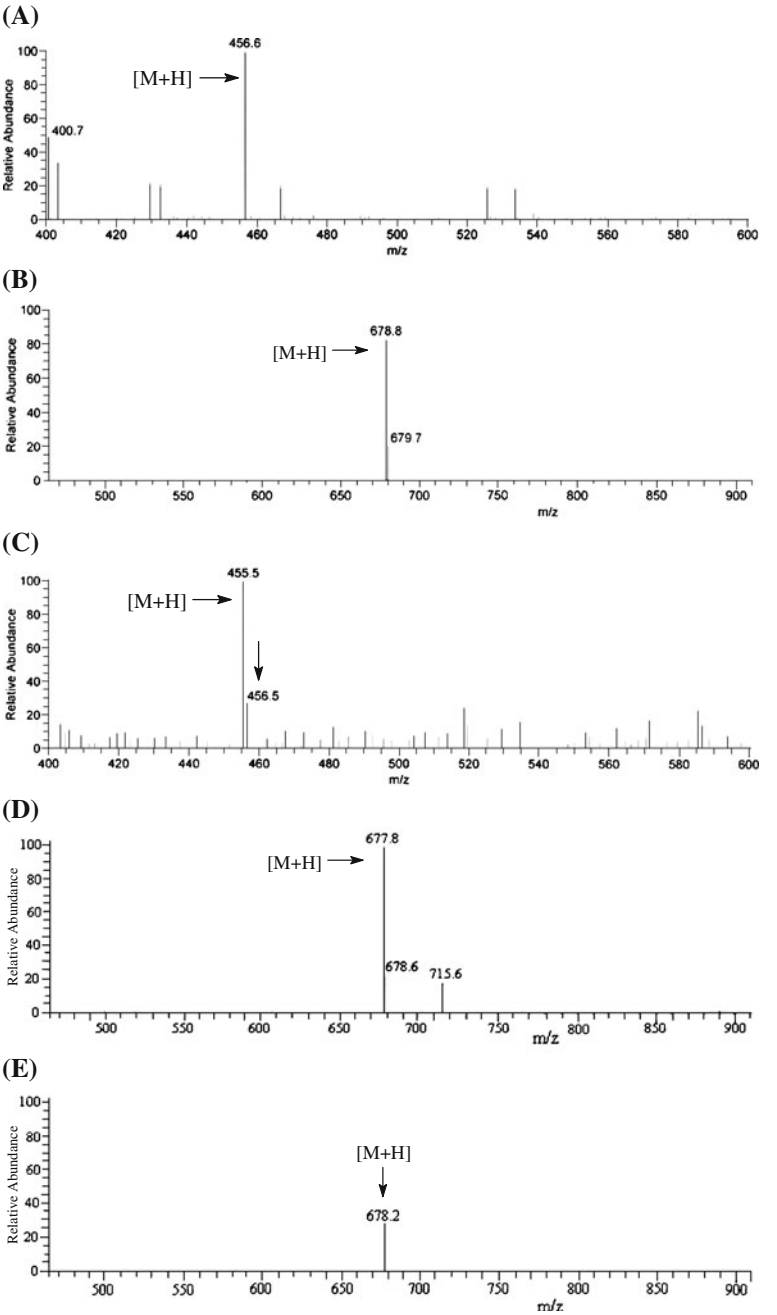

Fig. 3 MS analysis of the ribostamycin derivatives. **a** ESI-MS of extracted compound from pIBR-RmDJKHEr/*S. venezuelae* YJ3DOS152 showing pseudoribostamycin $[M+H]^+ = 456$, **b** LC-ESI/MS profile of pseudoribostamycin+FMOC-Cl: $[M+H]^+ = 678$, **c** ESI-MS of extracted compound from *S. lividans* TK24/pRMB4 showing ribostamycin derivatives: $[M+H]^+ = 455$ (ribostamycin) and $[M+H]^+ = 456$ (pseudoribostamycin), **d** LC-ESI/MS profile of ribostamycin+FMOC-Cl: $[M+H]^+ = 677$, and **e** LC-ESI/MS profile of pseudoribostamycin+FMOC-Cl: $[M+H]^+ = 678$

have eliminated biosynthetic steps involved in the amination of paromamine and directly cloned ribosylation genes, bypassing the neamine pathway for rapid biosynthesis of a ribostamycin derivative. For this, the recombinant pIBR-RmDJKHEr, bearing the minimal gene set for pseudoribostamycin, was transformed into *S. venezuelae* YJ3DOS152. The correct transformant which was named *S. venezuelae* YJ3DOS152/pIBR-RmDJKHEr was able to produce pseudoribostamycin as shown by ESI-MS with $[M+H]^+$=456 and by LC-MS with a derivative of FMOC-Cl $[M+H]^+$ = 678 (at RT=12.2 min; Fig. 3a, b); in contrast, *S. venezuelae* YJ003/pIBR25 failed to produce this compound (data not shown). The extracted compound was partially purified by precipitation in cold methanol, and the whitish precipitate was dried. Approximately 0.5 mg/l of partially purified pseudoribostamycin was produced from the heterologous host. It was dissolved in 200 μl of pure water and kept frozen until its analysis. Further identification of the compound was carried out by ESI-MS/MS analysis with reference to standard compound or ribostamycin (Sigma; Fig. 4a, b). A paper disk containing 10 μl of the partially purified compound was tested against *Bacillus subtilis*. The produced compound had little bioactivity, but similar treatment with the product of *S. venezuelae* YJ3DOS152/pIBR-RmDJ did not show any such activity (Supplementary Fig. S6). The compound was also extracted from the culture broth of *S. lividans* TK24/

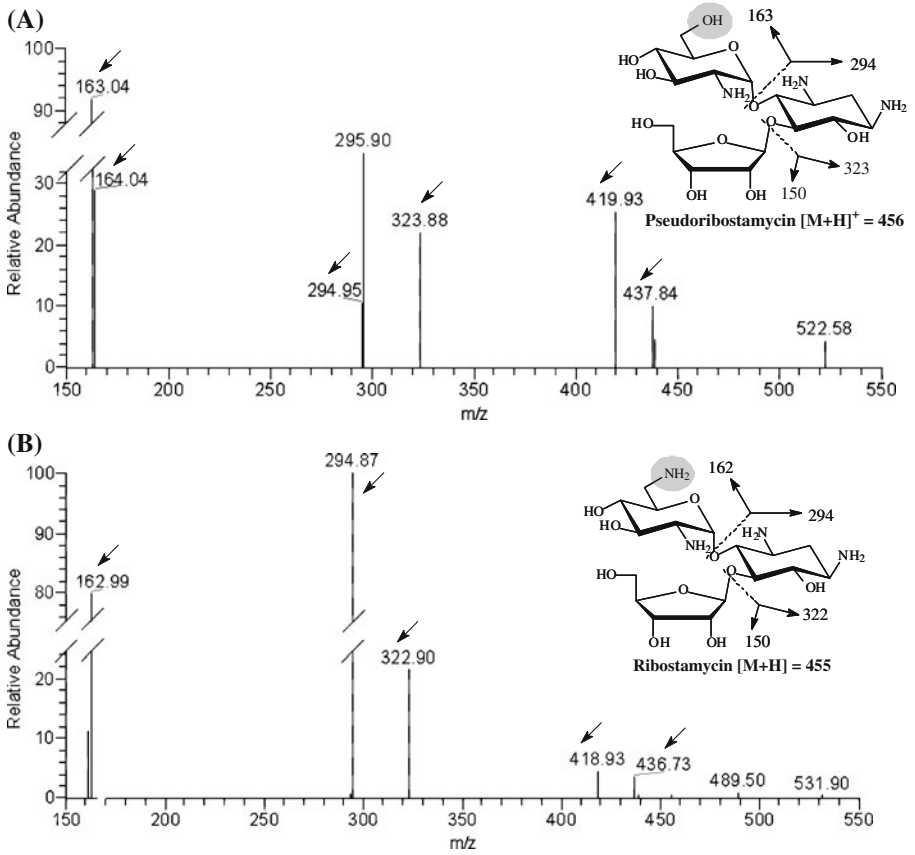

Fig. 4 ESI-MS/MS analysis. **a** Pseudoribostamycin produced by pIBR-RmDJKHEr/*S. venezuelae* YJ3DOS152 and **b** standard ribostamycin

pRMB4 [11] and analyzed by ESI-MS and LC-MS; the extract contained ribostamycin (derivative of FMOC-Cl $[M+H]^+$ = 677 at RT=13.9 min) as the major product and a trace amount of pseudoribostamycin (derivative of FMOC-Cl $[M+H]^+$ = 678 at RT=12.1 min; Fig. 3c, d). The result showed that ribosylation is possible on neamine as well as on paromamine. Although the neamine is the favored substrate for ribosylation in the usual pathway of ribostamycin biosynthesis, paromamine ribosylation cannot be ruled out as shown by substrate flexibility of BtrL [14].

The emergence of resistance to aminoglycosides which normally occurs by enzymatic inactivation or modification [26, 27] has compelled researchers to accelerate the search of novel antibiotics or engineer the existing ones. Indeed, many structural analogs of aminoglycosides have been synthesized over the past decades [6, 28]. Some of them showed improved antibacterial activities [28].

The neosamine sugar of ribostamycin contains an NH_2 group at the C-6 position, and this is one of the susceptible groups that can be modified by aminoglycoside-modifying enzymes such as acetyltransferase AAC (6′) [1]. Thus, our goal was to replace this group with OH by direct ribosylation of paromamine using a phosphoribosyltransferase, the RacK. Despite the importance of NH_2 group for RNA binding activity, we have generated a novel aminoglycoside analog by replacing this group with hydroxyl group to achieve the improved potency to overcome certain bacterial resistance. During the biosynthesis of ribostamycin, ribosylation takes place after formation of neamine, but an in vitro study has shown that ribosylation is also possible in paromamine [14]. Taking advantage of such flexible ribosyltransferase, we designed the experiment for ribosylation on paromamine and produced pseudoribostamycin (6′-deamino-6′-hydroxyribostamycin). These results reveal the biosynthetic enzymes responsible for the formation of ribostamycin derivatives and also indicate a convenient method for generating hybrid analogs through simple genetic manipulation within the host gene cluster.

Conclusion

Based on previous studies and homology search, we identified the minimal required gene sets for pseudoribostamycin (a ribostamycin derivative) and other intermediates of ribostamycin biosynthesis. Those genes were cloned in *Streptomyces* integration and expression vectors using the genes of neomycin and ribostamycin gene cluster. Upon expression in a heterologous host of *S. venezuelae*, we were able to produce pseudoribostamycin and the intermediate products. The results also prove the flexibility of ribostamycin ribosyltransferase (RacK) for accepting neamine as well as paromamine as a substrate to produce ribostamycin and pseudoribostamycin, respectively.

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