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ORIGINAL PAPER

Production of rosamicin derivatives in *Micromonospora rosaria* by introduction of D-mycinose biosynthetic gene with ΦC31-derived integration vector pSET152

Yojiro Anzai · Yohei Iizaka · Wei Li · Naoki Idemoto · Shu-ichi Tsukada · Kazuo Koike · Kenji Kinoshita · Fumio Kato

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Abstract Some of the polyketide-derived bioactive compounds contain sugars attached to the aglycone core, and these sugars often impart specific biological activity to the molecule or enhance this activity. Mycinamicin II, a 16-member macrolide antibiotic produced by *Micromonospora griseorubida* A11725, contains a branched lactone and two different deoxyhexose sugars, D-desosamine and D-mycinose, at the C-5 and C-21 positions, respectively. The D-mycinose biosynthesis genes, *mycCI*, *mycCII*, *mycD*, *mycE*, *mycF*, *mydH*, and *mydI*, present in the *M. griseorubida* A11725 chromosome were introduced into pSET152 under the regulation of the promoter of the apramycin-resistance gene *aac(3)IV*. The resulting plasmid pSETmycinose was introduced into *Micromonospora rosaria* IFO13697 cells, which produce the 16-membered macrolide antibiotic rosamicin containing a branched lactone and D-desosamine at the C-5 position. Although the *M. rosaria* TPMA0001 transconjugant exhibited low rosamicin productivity, two new compounds, IZI and IZII, were detected in the ethylacetate extract from the culture broth. IZI was identified as a mycinosyl rosamicin derivative, 23-*O*-mycinosyl-20-deoxo-20-dihydro-12,13-deepoxyrosamicin (MW 741), which has previously been synthesized by a bioconversion technique. This is the first report on production of mycinosyl rosamicin-derivatives by a engineered biosynthesis approach. The integration site ΦC31*attB* was identified on *M. rosaria* IFO13697 chromosome, and the site lay within an ORF coding a pirin homolog protein. The pSETmycinose could be useful for stimulating the production of "unnatural" natural mycinosyl compounds by various actinomycete strains using the bacteriophage ΦC31 *att*/*int* system.

Keywords Combinatorial biosynthesis · D-mycinose · Rosamicin · Bacteriophage ΦC31 · *attB* site

Y. Anzai (✉) · Y. Iizaka · W. Li · N. Idemoto · S. Tsukada · K. Koike · F. Kato
Faculty of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan
e-mail: yanzai@phar.toho-u.ac.jp

K. Kinoshita
School of Pharmaceutical Sciences, Mukogawa Women's University, 11-68 Kyuban-cho, Koshien, Nishinomiya 663-8179, Japan

Introduction

Actinomycetes produce various polyketide bioactive compounds, including therapeutically important antibiotics (e.g., erythromycin), antifungals (e.g., amphotericin B), antiparasitics (e.g., avermectin), and antitumor drugs (e.g., doxorubicin). Some of these compounds contain sugars attached to the aglycone core (moiety), and these sugars often impart specific biological activity to the molecule or enhance this activity. Most of these sugars belong to the group of 6-deoxyhexoses, which are synthesized from nucleoside diphosphate-activated hexoses (mainly glucose) via a 4-keto-6-deoxy intermediate. The biosynthesis of this intermediate is catalyzed by two ubiquitous enzymes, dNTP-D-hexose synthase and dNTP-D-hexose-4,6-dehydratase. The intermediate is modified by various modification enzymes, and the resultant deoxysugars are transferred to the corresponding aglycone by glycosyltransferases [22]. Almost all of the genes encoding deoxysugar biosynthetic and transfer enzymes are located in the biosynthetic gene cluster that

encodes the corresponding polyketide bioactive compounds on the chromosome of the producer. Some of these genes and their combinations were introduced into cells of heterologous host actinomycetes, and the resultant strains were found to produce novel glycosylated derivatives of natural products [19, 21, 27].

In general, protoplast and electroporation techniques have been used for actinomycete transformation, but the efficiency of these techniques is very low. Especially the transformation by plasmid DNA has often proved to be only slightly effective for industrially important antibiotic-producing streptomycete strain antibiotics and has not been developed for many other representatives of the order Actinomycetales. As an alternative, an *Escherichia coli–Streptomyces* spp. intergeneric conjugation system has recently been developed to introduce plasmid DNA into not only streptomycete cells, but also non-*Streptomyces* actinomycete cells. Transconjugation vectors, possessing a *Φ*C31 *int* gene and *attP* site, site-specifically insert into the *Φ*C31 *attB* attachment site of a host chromosome. The *attB* site is distributed widely throughout streptomycete strains, but there are few reports about the *attB* site of non-*Streptomyces* actinomycetes [6, 12]. *Saccharopolyspora erythraea*, which produces erythromycin, does not possess the *Φ*C31 *attB* site on its chromosome; the site was artificially introduced into the chromosome for antibiotic production by using a combinatorial biosynthesis technique [20].

Mycinamicin II is a 16-member macrolide antibiotic produced by *Micromonospora griseorubida* A11725 (Fig. 1). It comprises a branched lactone and two different deoxyhexose sugars, D-desosamine and D-mycinose, at the C-5 and C-21 positions, respectively, and exhibits strong antimicrobial activity against gram-positive bacteria [24]. The nucleotide sequence of the complete mycinamicin biosynthetic gene cluster has been reported [4]. A set of genes involved in D-desosamine biosynthesis and transfer (*mydA–mydG* and *mycB*) is located immediately downstream of the *mycAI–mycAV* genes; these genes encode polyketide synthetase (PKS), catalyzing the synthesis of the aglycone protomycinolide IV. In this gene set, *mydA* and *mydB* encode dNTP-D-hexose synthase and dNTP-D-hexose-4,6-dehydratase, respectively, which are responsible for the biosynthesis of dTDP-4-keto, 6-deoxy-D-glucose (an intermediate in the biosynthesis of two different deoxyhexose sugars). On the other hand, the D-mycinose biosynthetic genes *mycCI*, *mycCII*, *mycD, mycE*, *mycF*, and *mydI* are located upstream of the mycinamicin PKS gene, and *mydH* lies between *mydA* and *mydB*. The functions of the products of these genes have been proposed through chemical, genetic, and enzymatic analysis [3, 4, 26]. *MycCI* and *mycCII* encode the cytochrome P450 enzyme and ferredoxin, respectively, which mediate the hydroxylation of

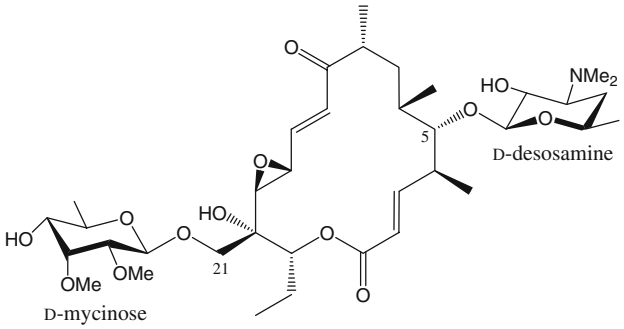

Fig. 1 Mycinamicin II produced by *M. griseorubida* A11725

mycinamicin VIII at the C-21 methyl group in combination with ferredoxin reductase. On completion of this hydroxylation reaction, MycD transfers 6-deoxy-D-allose to the C-21 hydroxyl group by using dTDP-6-deoxy-D-allose, which is synthesized from dTDP-4-keto, 6-deoxy-D-glucose by MydH and MydI as a substrate. The methyltransferases MycE and MycF convert the resulting compound mycinamicin VI to mycinamicin IV, which has D-mycinose at the C-21 position.

Rosamicin (**4** in Fig. 2) is a 16-membered macrolide antibiotic produced by *M. rosaria* IFO13697 (= NRRL 3718) [29]. The antibiotic and its analogues are also produced by other species of the genus *Micromonospora*, including *M. chalcea* var. *izumensis* ATCC21561, *M. capillata* MCRL0904, and *M. fastidious* BA06108 [10, 11, 13]. Moreover, the nucleotide sequence of the complete rosamicin biosynthetic-gene cluster in *M. carbonacea* was determined (7, accession no. AX697977). Rosamicin contains a branched lactone and D-desosamine at the C-5 position, but no other sugar residue is attached to the lactone ring. In this study, D-mycinose biosynthetic genes, whose transcription was controlled by the promoter of the apramycin resistant gene *aac(3)IV*, were introduced into *M. rosaria* IFO13697 by using pSETmycinose derived from the *E. coli–Streptomyces* conjugated shuttle vector pSET152. The transconjugant, *M. rosaria* TPMA0001, produced a mycinosyl rosamicin derivative IZI, which was identified as 23-*O*-mycinosyl-20-deoxo-20-dihydro-12,13-deepoxyrosamicin (**7** in Fig. 2). The integration site of pSETmycinose was identified on the *M. rosaria* TPMA0001 chromosome.

Materials and methods

Strains, media, and culture conditions

The wild-type strain *M. rosaria* IFO13697 was purchased from the Institute of Fermentation, Osaka, Japan. *M. rosaria* strains were incubated at 27°C in trypticase soy broth (TSB; Becton, Dickison and Company, Franklin Lakes, NJ)

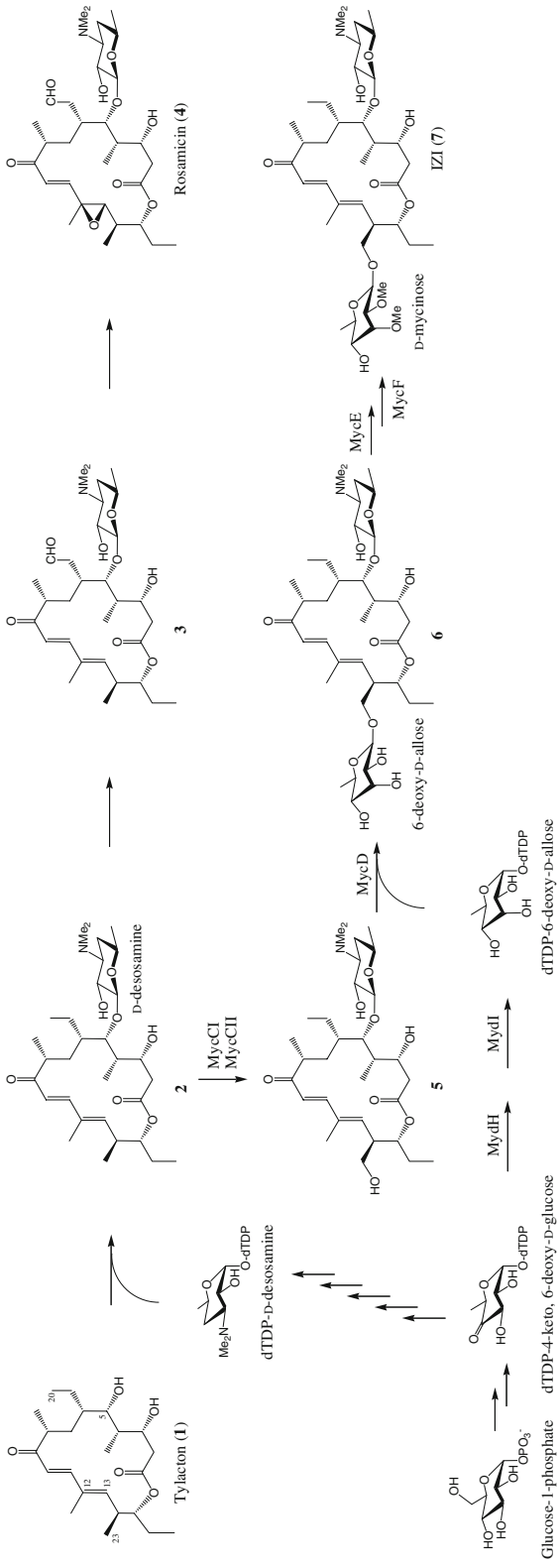

Fig. 2 The proposed biosynthetic pathway of rosamicin and IZI in *M. rosaria* TPMA0001

and on trypticase soy agar (TSA) plates. *E. coli* JM109 was used as the general subcloning host. *E. coli* ET12567/pUZ8002 was used as the donor strain for intergeneric conjugation [8]. *Micrococcus luteus* ATCC9341 was purchased from the American Type Culture Collection (Manassas, VA). The media were supplemented with the appropriate antibiotics (100 μg ml^{-1} ampicillin, 50 μg ml^{-1} apramycin, or 30 μg ml^{-1} nalidixic acid) as required.

Vectors and DNA manipulation

The vectors pUC18 and pLITMUS 28 (New England BioLabs, Ipswich, MA) were the routine cloning vectors, and the pDrive cloning vector (QIAGEN, Valencia, CA) was used for cloning the DNA fragment amplified by polymerase chain reaction (PCR). The vector pSET152 [15] was used for intergeneric conjugation from *E. coli* to *M. rosaria*. Plasmid and genomic DNA amplification, restriction enzyme digestion, fragment isolation, and cloning were performed by using the standard procedures [23].

Construction of pSETmycinose

The D-mycinose biosynthesis plasmid, pSETmycinose (Fig. 2), was constructed by using restriction fragments (1.4-kb *Nco*I–*Apa*I and 3.2-kb *Apa*I fragments) derived from pMC01 [4], which contains some D-mycinose biosynthesis genes upstream of the mycinamicin polyketide synthase (PKS) gene *mycAI*, the PCR-amplified fragment, and pSET152 as the vector plasmid. Using total DNA extracted from *M. griseorubida* or some cosmid clones as the template, *mycF* and *mydH* as well as a part of *mycD* were amplified by PCR under standard conditions by using the following primers: mycF-F-Ps, 5′-CTGCAGTCAGCATCTCGGGTTCGGAC-3′, and mycF-R-Hd, 5′-AAGCTTCGCCCACCAGGCAAGGAGGT-3′ for *mycF*; mydH-F-Ps, 5′-CTGCAGGTTGCTCGACTGGAAGGGGG-3′, and mydH-R-Xb, 5′-TCTAGATCACGCCCGACGGTCGGGGG-3′ for *mydH*, and mycDend-F-Ap 5′-GGTCGACGGGCCCGACGTGC-3′, and mycDend-R-Hd 5′-AAGCTTCTACCCGGTGAGCGTCCTGG-3′ for partial *mycD*. The restriction sites (underlined in the preceding) *Pst*I (CTGCAG), *Hin*dIII (AAGCTT), *Xba*I (TCTAGA), and *Apa*I (GGGCCC) were used for cloning. Further, the promoter region of the apramycin-resistant gene, *acc(3)VI*p, in the cosmid vector pKC505 [15] was amplified by PCR with the primers Apr-F-EV, 5′-GATATCGGTTCATGTGCAGCTCCATC-3′, and Apr-R-Nc, 5′-GCTGATGCCATGGGTCGATC-3′ (the underlined restriction sites *Eco*RV and *Nco*I were used for cloning). All the amplified DNA fragments were cloned into pDrive by using the TA cloning system, and the sequences of the cloned DNA fragments were determined.

Conjugation procedure

The intergeneric conjugation from *E. coli* ET12567/pUZ8002 to *M. rosaria* IFO13697 was performed by using a protocol similar to our previous procedure [1]. An overnight culture of the *E. coli* donor strain was diluted in fresh medium and incubated for 3–5 h. The cells were harvested, washed twice, and concentrated tenfold in TSB. *M. rosaria* IFO13697 culture grown in TSB for 5 days was harvested by centrifugation, washed, and resuspended in TSB in the ratio 2:1 (v/v). *M. rosaria* IFO13697 recipient cells were mixed with *E. coli* donor cells in the ratio 2:1 (v/v), and 150 µl of the mixture was spread on R2YE medium [15]. The plates were incubated at 27°C for 20 h and then overlaid with 1 ml water containing 500 µg nalidixic acid for inhibiting further growth of *E. coli* and 1 mg apramycin for selecting the *M. rosaria* exconjugants. The plates were then reincubated at 27°C for 2–3 weeks for growth of the exconjugants.

Fermentation, isolation, and identification

M. rosaria TPMA0001 was cultured in eight 15-ml test tubes, each containing 5 ml of TSB. The tubes were incubated on a rotary shaker (150 rpm) at 27°C for 3 days. Further, 0.8 ml of the culture was transferred into 32 500-ml Sakaguchi flasks, each containing 300 ml of TSB, and these flasks were incubated on a rotary shaker (120 rpm) at 27°C for 6 days. The broth filtrate (9.6 l) was adjusted to pH in the range of 9–11 with 28% ammonia solution and extracted twice with an equal volume of ethyl acetate (EtOAc); the extract was then concentrated in vacuo. The combined crude extracts were applied to a silica gel column (Merck 60; 3 × 25 cm, 0.063–0.100 mm) and eluted with a chloroform–methanol–28% ammonia solution (100:10:1). The fractions containing antibacterial metabolites were applied to a preparative HPLC system [YMC-Pack Pro C18, i.d. = 150 × 20 mm; MeCN-0.06% TFA (35:65); flow rate, 5 ml min^{-1}] for further purification. The purified antibacterial metabolite designated as IZI (7.3 mg; **7** in Fig. 2) was characterized by ^{1}H-NMR (600 MHz) and ^{13}C NMR (150 MHz) spectroscopy (JEOL JNM-ECA600) and mass spectrometry (JEOL JMS-T100LC). The ^{1}H-NMR and ^{13}C-NMR spectra of the product were assigned by a combination of ^{1}H–^{1}H COSY, DEPT, and ^{1}H–^{13}C HETCOSY spectroscopy: ^{1}H-NMR (500 Hz, $CDCl_3$): 7.30 (1H, d, *J* = 15.5, H-11), 6.27 (1H, d, *J* = 15.5, H-10), 5.87 (1H, d, *J* = 10.4, H-13), 4.96 (1H, td, *J* = 9.4, 2.3, H-15), 4.58 (1H, d, *J* = 7.8, Myc-1), 4.26 (1H, d, *J* = 7.3, Des-1), 3.99 (1H, dd, *J* = 9.6, 3.9, Ha-23), 3.76 (1H, overlapped, H-3), 3.76 (1H, overlapped, H-5), 3.75 (1H, t, *J* = 3.1, Myc-3), 3.61 (1H, s, Myc-8), 3.54 (1H, dd, *J* = 9.6, 6.5, Hb-23), 3.50 (1H, m, Myc-5), 3.50 (1H, m, Dec-5), 3.49 (1H, s, Myc-7), 3.26 (1H, overlapped, Des-2), 3.26 (1H, overlapped, Des-3), 3.18 (1H, dd, *J* = 9.4, 3.1, Myc-4), 3.02 (1H, dd, *J* = 7.8, 2.7, Myc-2), 2.95 (1H, m, H-14), 2.73 (1H, m, H-8), 2.49 (1H, dd, 10.3, 6.5, Ha-2), 2.32 (1H, br.s, Des-7), 2.32 (1H, br.s, Des-8), 1.96 (1H, br.d, *J* = 16.5, Hb-2), 1.87 (1H, dqd, *J* = 7.0, 7.1, 2.7, Ha-16), 1.78 (1H, d, *J* = 1.0, H-22), 1.68 (1H, m, H-4), 1.65 (1H, m, Ha-7), 1.65 (1H, m, Ha-19), 1.61 (1H, dqd, *J* = 3.0, 7.1, 1.9, Hb-16), 1.53 (1H, dqd, *J* = 5.0, 11.2, 4.8, Hb-7), 1.26 (1H, d, *J* = 6.2, Myc-6), 1.24 (1H, overlapped, H-6), 1.24 (2H, overlapped, Des-4), 1.22 (1H, d, *J* = 6.2, Des-6), 1.18 (1H, d, *J* = 6.9, H-21), 1.09 (1H, d, *J* = 6.9, H-18), 0.93 (1H, t, *J* = 7.1, H-17), 0.93 (1H, overlapped, Hb-19), and 0.86 (1H, t, *J* = 7.1, H-20). ^{13}C-NMR (500 Hz, $CDCl_3$): 204.2 (C-9), 174.5 (C-1), 147.3 (C-11), 135.1 (C-12), 119.0 (C-10), 104.5 (Des-1), 101.1 (Myc-1), 82.0 (Myc-2), 79.9 (C-5), 79.9 (Myc-3), 75.2 (C-15), 72.7 (Myc-4), 70.7 (Myc-5), 70.5 (Des-2), 69.3 (Des-5), 69.1 (C-23), 67.7 (C-3), 65.9 (Des-3), 61.8 (Myc-8), 59.7 (Myc-7), 45.0 (C-14), 44.8 (C-8), 40.4 (C-4), 40.3 (Des-7), 40.3 (Des-8), 39.8 (C-2), 38.2 (C-6), 34.0 (C-7), 29.7 (Des-4), 25.4 (C-16), 21.2 (C-19), 21.0 (Des-6), 17.8 (Myc-6), 17.6 (C-21), 13.0 (C-22), 12.1 (C-20), 9.7 (C-17), and 9.1 (C-18). The antibacterial activity of the fermentation products and purified compounds was assayed against *M. luteus* ATCC9341.

Southern hybridization

Hybridization followed the standard phototope-detection protocol (New England BioLabs) with the biotin-labeled probe. The 0.5-kb PCR fragment containing the *attP* site on pSET152 and 0.4-kb *Sal*I fragment containing the partial *attB* site on *M. rosaria* were labeled with biotin by using the NEBlot Phototope Kit (New England BioLabs). The 0.5-kb PCR fragment containing the *attP* site was amplified from pSET152 by using oligonucleotide primers pSET152.3485F, 5′-ACGAACCCTTTGGCAAAATC-3′, and pSET152.4037R, 5′-AATGCCCGACGAACCTGAAC-3′.

Results

Isolation of *M. rosaria* TPMA0001 producing mycinosyl rosamicin derivative

The D-mycinose biosynthesis genes (*mycCI*, *mycCII*, *mycD*, *mycE*, *mycF*, *mydH*, and *mydI*) are located in the mycinamicin biosynthesis gene cluster in the chromosomal DNA of the mycinamicin-producing strain *M. griseorubida* A11725; *mydA* and *mydB*, whose products are responsible for the biosynthesis of dTDP-4-keto, 6-deoxy-D-glucose, are also located in this gene cluster. On the other hand, rosamicin has D-desosamine at the C-5 position of the lactone moiety, suggesting that the D-desosamine biosynthesis genes, including the two genes encoding dNTP-D-hexose synthase

and dNTP-D-hexose-4,6-dehydratase, are present on the chromosome of *M. rosaria* IFO13697, and that dTDP-4-keto, 6-deoxy-D-glucose is produced by the cells. By using an intergeneric conjugation system, we directly introduced two DNA fragments (one containing *mycCI*, *mycCII*, *mycD*, *mycE*, *mycF*, and *mydI*, which are located upstream of the mycinamicin PKS gene, and the other containing *mydA*, *myd B*, *and mydH*, which are located downstream of the mycinamicin PKS gene) into *M. rosaria* IFO13697. However, the transconjugant could not produce any mycinosyl rosamicin derivative (data not shown). To account for the negative results, we speculated that some of the promoters responsible for the expression of the introduced DNA fragments did not function in transconjugant cells.

The novel plasmid pSETmycinose was constructed from pSET152 (Fig. 3). The region extending from *mycCI* and covering a part of *mycD* was subcloned from pMC01, and *mycF* and *mydH* as well as a part of *mycD* were amplified by PCR. Moreover, the promoter region of the apramycin-resistant gene, *acc(3)VI*p, carried by the cosmid vector pKC505 was also amplified by PCR. These subcloned and amplified D-mycinose biosynthetic genes were introduced into pSET152 under the control of *acc(3)VI*p. The resulting plasmid (i.e., pSETmycinose) was introduced into *M. rosaria* IFO13697 cells, and some apramycin-resistant transconjugants were isolated. *M. rosaria* TPMA0001 and the wild-type strain *M. rosaria* IFO13697 were cultured at 27°C in 5 ml of TSB for 1 week, and EtOAc extracts obtained from the culture broth were compared by using HPLC (Fig. 4). Rosamicin (3.6 mg l^{-1} of broth) was detected at 9.1 min in the EtOAc extract obtained from the *M. rosaria* IFO13697 culture (Fig. 4b). Although rosamicin productivity of the *M. rosaria* TPMA0001 culture was lower (1.4 mg l^{-1} of broth), two new peaks appeared at 11.4 and 3.8 min (designated as IZI and IZII, respectively) in the chromatogram recorded at 285 nm (Fig. 4a). IZI was isolated and purified from 9.6 l of the *M. rosaria* TPMA0001 culture broth to obtain 7.3 mg of purified IZI. Purified IZI was characterized by ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) spectroscopy and mass spectrometry. From the NMR chemical shift and its molecular weight (MW 741) of IZI was determined as a mycinosyl rosamicin derivative, i.e., 23-*O*-mycinosyl-20-deoxo-20-dihydro-12,13-deepoxyrosamicin (**7** in Fig. 2). This derivative has already been reported as a bioconversion compound, which was converted from 20-deoxo-20-dihydro-12,13-deepoxy-12,13-dihydrorosaranolide by the mycinamicin non-producing mutant GS-9001 [17]. The molecular weight of IZII was determined to be 757 (i.e., 16 units more than that of IZI). We hope to purify IZII and determine its molecular structure by using NMR spectroscopy in our future studies.

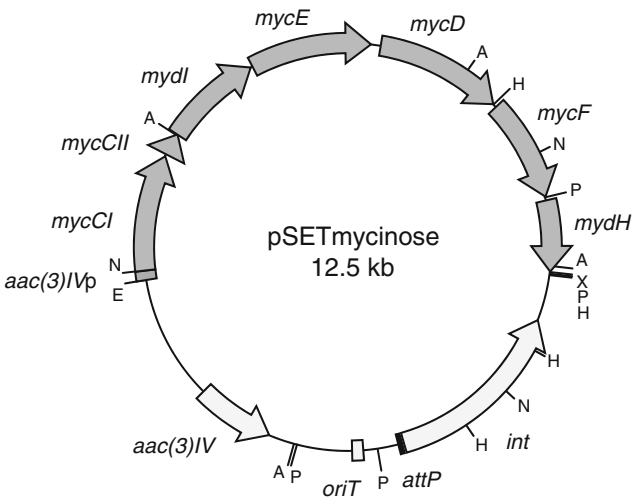

Fig. 3 The structural map of pSETmycinose, *A Apa*I, *E Eco*RV, *H Hin*dIII, *N Nco*I, *P Pst*I, and *X Xba*I

Identification of *attB* site on *M. rosaria* chromosome

The D-mycinose biosynthesis plasmid, pSETmycinose, was constructed from the site-specific integration vector pSET152, containing *Φ*C31 *int*, *attP*, *oriT* of RK2, and apramycin-resistant gene *aac(3)IV*. To confirm the integration of pSETmyinose on the chromosome, a 0.5-kb PCR fragment including *attP* on pSET152 was used as a probe for Southern blot hybridization. Hybridized bands at 0.4 and 1.6 kb were detected in the total DNA of *M. rosaria* TPMA0001 digested with *Sal*I (data not shown). The 0.4-kb *Sal*I fragment was cloned from the *Sal*I fragment library prepared from *M. rosaria* TPMA0001 total DNA. The nucleotide sequence (397 bp) of the cloned fragment was determined, and the *attR* site, which was one of the franking arms of the insertion site on the *M. rosaria* TPMA0001 chromosome, was identified. A half sequence of *attP* on pSET152 was included in the *attR* site, and a partial sequence of the *attB* site, which is recognized by *Φ*C31 integrase, flanked the half part of *attP*. By using the 0.4-kb *Sal*I fragment as a probe, 0.5- and 0.4-kb hybridized bands appeared in *Sal*I-digested total DNAs of *M. rosaria* IFO13697 and TPMA0001, respectively (Fig. 5b). The nucleotide sequence of the 0.5-kb *Sal*I fragment cloned from the *Sal*I DNA library of *M. rosaria* IFO13697 was determined. The partial open reading frame (ORF) of the pirin homolog protein, which is highly conserved among mammals, plants, fungi, and prokaryotes, was coded in the determined 528-base sequence (the sequence is available from GenBank, EMBL, and DDBJ under the accession no. AB481387), and a single *attB* site was also recognized in the sequence. The *attB* site sequence of *M. rosaria* IFO13697 was aligned with those of actinomycetes, including rare actinomycetes (Fig. 6), and the identities between

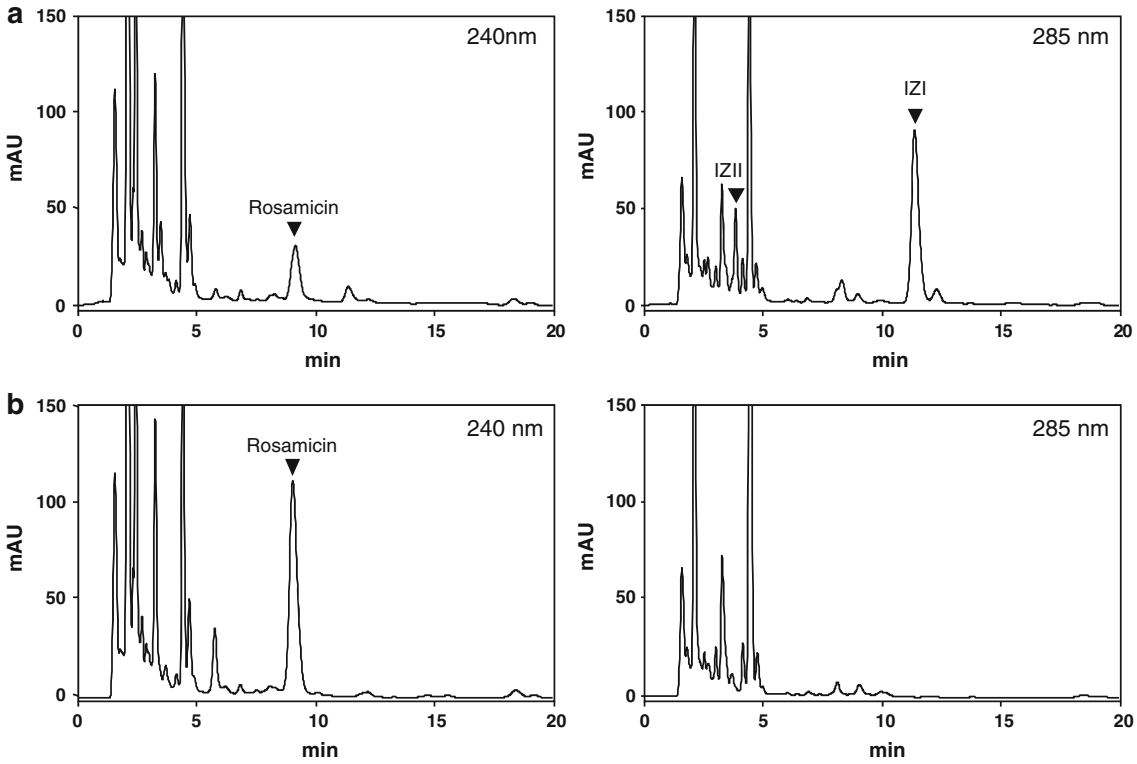

Fig. 4 HPLC chromatograms of the EtOAc extract obtained from *M. rosaria* TPMA0001 (**a**) and *M. rosaria* IFO13697 (**b**). *IZI* compound IZI, *IZII* compound IZII

M. rosaria and *Streptomyces* (88.2–90.2%) were found to be higher than those of *M. rosaria* and non-*Streptomyces* actinomycetes (78.4–86.3%). However, the determined 528-nucleotide sequence of *M. rosaria* IFO13697 showed the highest homology (88.4% identity) to the nucleotide sequence coding the pirin homolog protein of *Actinoplanes teichomyceticus*, and the partial amino acid sequence deduced from *M. rosaria* pirin homolog ORF also showed the highest homology (85.6% identity) to that from *A. teichomyceticus* pirin homolog ORF.

Discussion

In this study, we isolated the new transconjugant *M. rosaria* TPMA0001, containing the D-mycinose biosynthesis plasmid pSETmycinose, and producing the mycinosyl rosamicin derivative, 23-*O*-mycinosyl-20-deoxo-20-dihydro-12,13-deepoxyrosamicin (**7** in Fig. 2). This rosamicin derivative has previously been synthesized by a bioconversion technique [17]. Moreover, various glycosylated polyketide compounds have also been produced by the bioconversion technique using blocked mutants [2, 14, 18]. However, this is the first report on the production of a mycinosyl rosamicin derivative by using a combinatorial biosynthesis approach. The proposed biosynthetic pathway of rosamicin and IZI in *M. rosaria* TPMA0001 is shown in Fig. 2 (4 and 7, respectively). The biosynthetic pathway from tylactone (**1** in Fig. 2) to rosamicin (**4** in Fig. 2) was referred from the tylosin biosynthetic pathway, because the structure of rosamicin is very similar to that of the tylosin intermediate *O*-mycaminosyltylonolide [5, 9]. The structure of rosamicin aglycone is the same as that of tylactone, which is the first intermediate of tylosin. In the tylosin biosynthetic pathway, mycaminose was bonded at the C-5 position of tylactone by the glycosyltransferase TylB, and the resulting intermediate, 5-mycaminosyl tylacone, was converted to 23-deoxy-*O*-mycaminosyltylonolide by C-20 oxidase TylI. If desosamin bonded at the C-5 position of tylactone, the rosamicin intermediate 5-desosaminyl tylacone (**2** in Fig. 2) would have been produced, and then 5-desosaminyl tylacone would have been converted to rosamicin via 12,13-deepoxy-12,13-dehydrorosamicin (**3** in Fig. 2) with C-20 oxidation and C-12/13 epoxidation. It has been estimated from the sequence of the rosamicin biosynthetic-gene cluster in *M. carbonacea* patented by Farnet et al. [7] that tylactone is synthesized by products from the PKS gene; then, two kinds of cytochrome P450 genes, which are responsible for C-20 oxidation and C-12/13 epoxidation, and the glycosyltransferase gene, which is similar to *tylB*, modify tylactone to rosamicin. In the case of *M. rosaria* TPMA0001, the C-23 position of 5-desosaminyl

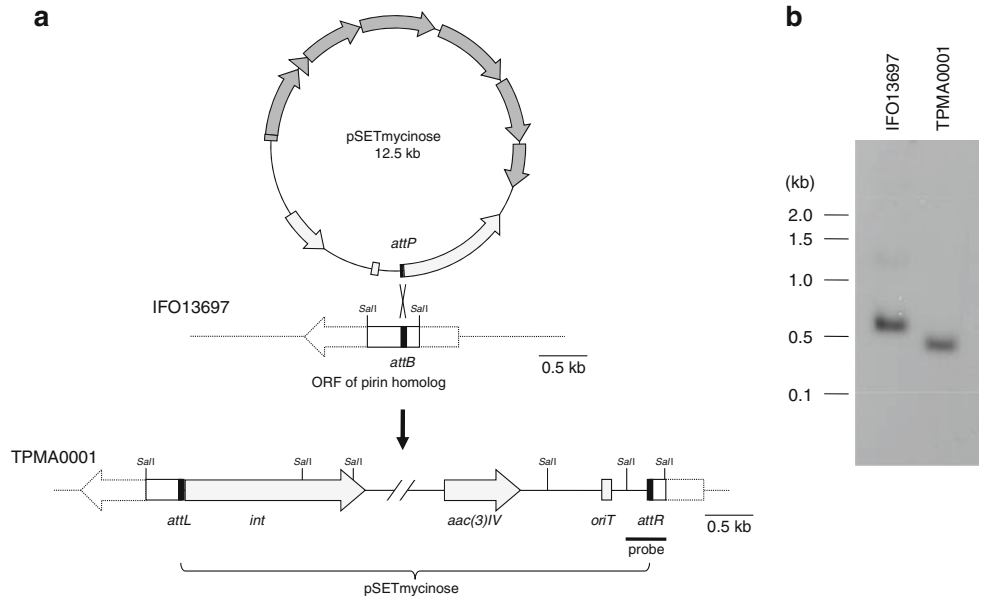

Fig. 5 The *attP*/*B*-mediated site-specific integration of pSETmycinose into the *M. rosaria* IFO13697 chromosome. **a** Scheme of the *attP*/*B*-mediated site-specific integration and physical maps of chromosomal DNA comprising the ORF of pirin homologin in *M. rosaria* IFO13697 (wild type) and the pirin homolog ORF integrating pSETmycinose in *M. rosaria* TPMA0001 (IFO13697/pSETmycinose). Probe, 0.4-kb *Sal*I fragment used for Southern hybridization. **b** Southern hybridization analysis of chromosomal DNA from *M. rosaria* IFO13697and TPMA0001. Total DNA from the corresponding strain was digested with *Sal*I, separated by electrophoresis in 0.8% (w/v) agarose gel, and transferred on Hybond N (Amersham). Hybridization followed the standard phototope-detection protocol (New England BioLabs) using the biotin-labeled 0.4-kb *Sal*I fragment, including the *attR* site. The 500-bp DNA Ladder (Takara, Japan) was used as the standard size. The 1.2-kb weak hybridized bands of *M. rosaria* IFO13697 appeared as non-specific

Fig. 6 Alignment of the *attB* site sequences among *M. rosaria* and other actinomycetes. *S. coe Streptomyces coelicolor* A3(2) (AL939117), *S. amb Streptomyces ambofaciens* ATCC15154 (AB306970), *S. liv Streptomyces lividans* 66 (X60952), *S. ave Streptomyces avermitilis* MA-4680 (BA000030), *S. gri Streptomyces griseus* subsp. *griseus* NBRC13350 (AP009493), *M. ros M. rosaria* IFO13697 (this study, AB481387), *F. aln Frankia alni* ACN14A (CT573213), *A. tei Actinoplanes teichomyceticus* NBRC13999 (AB361274), *K. set Kitasatosporia setae* KM-6054 (AB116267)

tylacone was hydroxylated by mycinamicin P450 MycCI with the redox partners, mycinamicin ferredoxin MycCII and *M. rosaria* unidentified ferredoxin reductase. Then, 6-deoxy-D-allose was transferred to the hydroxyl radical at the C-23 position of compound **5** (Fig. 2) by MycD using dTDP-6-deoxy-D-allose, which is synthesized from dTDP-4-keto, 6-deoxy-D-glucose by MydH and MydI. Finally, mycinamicin methyltransferases MycE and MycF converted compound **6** to IZI.

As shown in Fig. 5a, pSETmycinose was integrated into the *M. rosaria* IFO13697 chromosome by the *Φ*C31 *att*/*int* system. The sequence and location of the chromosomal integration site *Φ*C31*attB* of *M. rosaria* IFO13697 was identified in this study. To our knowledge, this is the first report of the *Φ*C31*attB* site for the genus *Micromonospora* containing many kinds of pharmaceutically important antibiotic-producing strains. The *att*B site overlapping the $tRNA^{Phe}$ gene was found on the chromosome of *M. halophytica* var. *nigra* ATCC33088, but the integration system using the *attB* site was not the *Φ*C31 att/int system [28]. The *M. rosaria attB* site was found to lie within an ORF coding a homolog of pirin, similar to the previous reports on several actinomycete species [12]. Pirin is highly conserved among mammals, plants, fungi, and prokaryotes. It

acts as a transcriptional cofactor or an apoptosis-related protein in mammals, and is involved in seed germination and seedling development in plants. In prokaryotes, the *Serratia marcescens* pirin regulates pyruvate metabolism [25]. However, the destruction of these pirin genes is not lethal for the tested organisms, and especially the destruction with pSET152 integration has no effect on morphological differentiation, antibiotic productivity, and cell replication of several actionomycete strains [6, 12]. In this study, integration of pSETmycinose did not appear to affect the growth of the exconjugant (data not shown), and total antibiotic productivity was not decreased by the integration. Therefore, the conjugal transfer system with *attP/B*-mediated site-specific integration could be used as a suitable method for gene transfer in *M. rosaria*.

Many genes encoding the enzymes involved in deoxysugar biosynthesis and transfer have been cloned from actinomycete strains. Some of these genes and their combinations have been cloned into some kinds of plasmid vectors, and the actinomycete strains into which these expression vectors were subsequently introduced were able to produce novel glycosylated derivatives of natural products [19, 21, 27]. However, a D-mycinose biosynthesis plasmid has never been constructed. The antibacterial activity of mycinamicin IV, which is a mycinosyl–mycinamicin intermediate, was higher than that of mycinamicin VIII, which is a non-mycinosyl–mycinamicin intermediate [16]. The D-mycinose biosynthesis genes were inserted into the *E. coli–Streptomyces* shuttle vector pSET152 for conjugation, under the regulation of *acc(3)VI*p. As the apramycin-resistant gene *acc(3)VI* has been used as a marker in DNA recombination experiments on many actinomycete strains, pSETmycinose would be useful for stimulating the production of "unnatural" natural mycinosyl compounds in the actinomycete strains using the bacteriophage *Φ*C31 att/int system.

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References

1. Anzai Y, Ishii Y, Yoda Y, Kinoshita K, Kato F (2004) The targeted inactivation of polyketide synthase *mycAV* in the mycinamicin producer, *Micromonospora griseorubida*, and a complementation study. FEMS Microbiol Lett 238:315–320
2. Anzai Y, Kinoshita K, Seki T, Kato F (2004) Hybrid biosynthesis by targeted inactivation of polyketide synthases in the mycinamicin producer, *Micronionospora griseorubida*. J Antibiot 57:819–822
3. Anzai Y, Li S, Chaulagain MR, Kinoshita K, Kato F, Montgomery J, Sherman DH (2008) Functional analysis of MycCI and MycG, cytochrome P450 enzymes involved in biosynthesis of mycinamicin macrolide antibiotics. Chem Biol 15:950–959. doi:10.1016/j.chembiol.2008.07.014
4. Anzai Y, Saito N, Tanaka M, Kinoshita K, Koyama Y, Kato F (2003) Organization of the biosynthetic gene cluster for the polyketide macrolide mycinamicin in *Micromonospora griseorubida*. FEMS Microbiol Lett 218:135–141. doi:10.1111/j.1574-6968.2003.tb11509.x
5. Baltz RH, Seno ET (1981) Properties of *Streptomyces fradiae* mutants blocked in biosynthesis of the macrolide antibiotic tylosin. Antimicrob Agents Chemother 20:214–225
6. Choi SU, Lee CK, Hwang YI, Kinoshita H, Nihira T (2004) Intergeneric conjugal transfer of plasmid DNA from *Escherichia coli* to *Kitasatospora setae*, a bafilomycin B1 producer. Arch Microbiol 181:294–298. doi:10.1007/s00203-004-0654-8
7. Farnet CM, Staffa A, Yang X (2003) Genes and proteins for the biosynthesis of rosaramicin. World Intellectual Property Organization, Geneva. Pat WO 03010193-A:39
8. Flett F, Mersinias V, Smith CP (1997) High efficiency intergeneric conjugal transfer of plasmid DNA from *Escherichia coli* to methyl DNA-restricting streptomycetes. FEMS Microbiol Lett 155:223–229. doi:10.1111/j.1574-6968.1997.tb13882.x
9. Fouces R, Mellado E, Díez B, Barredo JL (1999) The tylosin biosynthetic cluster from *Streptomyces fradiae*: genetic organization of the left region. Microbiology 145:855–868
10. Funaishi K, Kawamura K, Satoh F, Hiramatsu M, Hagiwara M, Okanishi M (1990) New analogues of rosaramicin isolated from a *Micromonospora* strain. I. Taxonomy, fermentation, isolation and physico-chemical and biological properties. J Antibiot 43:938–947
11. Furumai T, Maezawa I, Matsuzawa N, Yano S, Yamaguchi T, Takeda K, Okuda T (1977) Macrolide antibiotics m-4365 produced by micromonospora. 1. Taxonomy, production, isolation, characterization and properties. J Antibiot 30:443–449
12. Ha HS, Hwang YI, Choi SU (2008) Application of conjugation using *ϕ*C31 *att/int* system for *Actinoplanes teichomyceticus*, a producer of teicoplanin. Biotechnol Lett 30:1233–1238. doi:10.1007/s10529-008-9671-z
13. Hatano K, Higashide E, Shibata M (1976) Studies on juvenimicin, a new antibiotic. 1. Taxonomy, fermentation and antimicrobial properties. J Antibiot 29:1163–1170
14. HJung WS, Han AR, Hong JS, Park SR, Choi CY, Park JW, Yoon YJH (2007) Bioconversion of 12-, 14-, and 16-membered ring aglycones to glycosylated macrolides in an engineered strain of *Streptomyces venezuelae*. Appl Microbiol Biotechnol 76:1373–1381. doi:10.1007/s00253-007-1101-y
15. Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hoopwood DA (2000) *Practical Streptomyces genetics*. John Innes Centre, Norwich
16. Kinoshita K, Imura Y, Takenaka S, Hayashi M (1989) Mycinamicins, new macrolide antibiotics. XI. Isolation and structure elucidation of a key intermediate in the biosynthesis of the mycinamicins, mycinamicin VIII. J Antibiot 42:1869–1872
17. Lee BK, Puar MS, Patel M, Bartner P, Lotvin J, Munayyer H, Waitz JA (1983) Multistep bioconversion of 20-deoxo-20-dihydro-12, 13-deepoxy-12, 13-dehydrorosaranolide to 22-hydroxy-23-O-mycinosyl-20-deoxo-20-dihydro-12, 13-deepoxyrosaramicin. J Antibiot 36:742–744
18. Olano C, Lomovskaya N, Fonstein L, Roll JT, Hutchinson CR (1999) A two-plasmid system for the glycosylation of polyketide antibiotics: bioconversion of epsilon-rhodomycinone to rhodomycin D. Chem Biol 6:845–855. doi:10.1016/S1074-5521(00)80004-6
19. Perez M, Lombo F, Baig I, Brana AF, Rohr J, Salas JA, Mendez C (2006) Combinatorial biosynthesis of antitumor deoxysugar pathways in Streptomyces griseus: Reconstitution of "unnatural natural gene clusters" for the biosynthesis of four 2, 6-d-dideoxyhexoses. Appl Environ Microbiol 72:6644–6652. doi:10.1128/AEM.01266-06
20. Rodriguez E, Hu Z, Ou S, Volchegursky Y, Hutchinson CR, McDaniel R (2003) Rapid engineering of polyketide overproduction by gene transfer to industrially optimized strains. J Ind Microbiol Biotechnol 30:480–488. doi:10.1007/s10295-003-0045-1

21. Rodriguez L, Aguirrezabalaga I, Allende N, Brana AF, Mendez C, Salas JA (2002) Engineering deoxysugar biosynthetic pathways from antibiotic-producing microorganisms: A tool to produce novel glycosylated bioactive compounds. Chem Biol 9:21–729. doi:10.1016/S1074-5521(02)00154-0
22. Salas JA, Mendez C (2007) Engineering the glycosylation of natural products in actinomycetes. Trends Microbiol 15:219–232. doi:10.1016/j.tim.2007.03.004
23. Sambrook J, Russell DW (2001) Molecular Cloning: A Laboratory Manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
24. Satoi S, Muto N, Hayashi M, Fujii T, Otani M (1980) Mycinamicins, new macrolide antibiotics. 1. Taxonomy, production, isolation, characterization and properties. J Antibiot 33:364–376
25. Soo PC, Horng YT, Lai MJ, Wei JR, Hsieh SC, Chang YL, Tsai YH, Lai HC (2007) Pirin regulates pyruvate catabolism by interacting with the pyruvate dehydrogenase E1 subunit and modulating pyruvate dehydrogenase activity. J Bacteriol 189:109–118. doi:10.1128/JB.00710-06
26. Suzuki H, Takenaka S, Kinoshita K, Morohoshi T (1990) Biosynthesis of mycinamicins by a blocked mutant of *Micromonospora griseorubida*. J Antibiot 43:1508–1511
27. Tang L, McDaniel R (2001) Construction of desosamine containing polyketide libraries using a glycosyltransferase with broad substrate specificity. Chem Biol 8:547–555. doi:10.1016/S1074-5521(01)00032-1
28. Hosted TJ Jr, Wang T, Horan AC (2005) Characterization of the *Micromonospora rosaria* pMR2 plasmid and development of a high G + C codon optimized integrase for site-specific integration. Plasmid 54:249–258. doi:10.1016/j.plasmid.2005.05.004
29. Wagman GH, Weinstei MJ, Waitz JA, Testa RT, Oden EM, Murawski A, Marquez J (1972) New *Micromonospora*-produced macrolide antibiotic, rosamicin. J Antibiot 25:641–652