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METABOLIC ENGINEERING AND SYNTHETIC BIOLOGY

Production of a hybrid 16-membered macrolide antibiotic by genetic engineering of *Micromonospora* sp. TPMA0041

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Abstract Some polyketide-derived bioactive compounds contain sugars attached to the aglycone core, and these sugars often enhance or impart specific biological activity to the molecule. 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. We previously engineered an expression plasmid pSETmycinose containing the D-mycinose biosynthesis genes from *M. griseorubida* A11725. This plasmid was introduced into *Micromonospora* sp. FERM BP-1076 cells, which produce the 16-membered macrolide antibiotic izenamicin. The resulting engineered strain TPMA0041 produced 23-*O*-mycinosyl-20-deoxy-izenamicin B_1 and 22-*O*-mycinosyl-izenamicin B_2. 23-*O*-mycinosyl-20-deoxy-izenamicin B_1 has been produced by the engineered strain *M. rosaria* TPMA0001 containing pSETmycinose as 23-*O*-mycinosyl-20-deoxo-20-dihydro-12,13-deepoxyrosamicin (=IZI) in our recent study, and 22-*O*-mycinosyl-izenamicin B_2 has previously been synthesized as a macrolide antibiotic TMC-016 with strong antibacterial activity. The production of 22-*O*-mycinosyl-izenamicin B_2 (=TMC-016) was increased when propionate, a precursor of methylmalonyl-CoA, was added to the culture broth.

Keywords Combinatorial biosynthesis · D-mycinose · Izenamicin · Methylmalonyl-CoA · Propionate · *Micromonospora* · Bacteriophage ϕC31 *attB* site

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Introduction

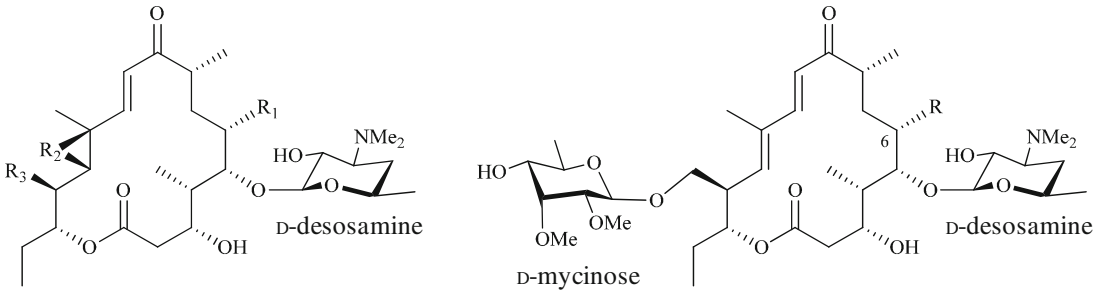
Several of the most important clinically used antibiotics are derivatives of macrolides, which are compounds containing deoxysugars attached to a polyketide macrolactone. These sugars often impart specific biological activity to the molecule or enhance this activity. Many genes involved in the biosynthesis of macrolide antibiotics have been cloned and sequenced; in addition, the functions of many proteins encoded by macrolide biosynthetic genes have been elucidated. With this information and experimental results, manipulation of the polyketide macrolactone synthase and deoxysugar biosynthetic pathways to create novel macrolide antibiotics has become possible [23]. Therefore, a combined approach that employs genes involved in the biosynthesis of macrolactone rings and deoxysugars, and in the glycosylation of macrolactone rings has been used to modify the macrolide structure [24].

Mycinamicin II is a 16-membered macrolide antibiotic produced by *Micromonospora griseorubida* A11725. 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. The nucleotide sequence of the complete mycinamicin biosynthetic gene cluster has been determined, and the

biosynthetic genes for the above-mentioned sugars are included in the cluster [3]. The plasmid pSETmycinose contains the D-mycinose biosynthesis genes (*mycCI*, *mycCII*, *mycD*, *mycE*, *mycF*, *mydH*, and *mydI*) from *M. griseorubida* A11725 [1]. *MycCI* and *mycCII* encode cytochrome P450 enzyme and ferredoxin, respectively, which mediate the hydroxylation of mycinamicin VIII at the C-21 methyl group along with ferredoxin reductase. Upon 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 substrates. The methyltransferases MycE and MycF convert the resulting compound mycinamicin VI to mycinamicin IV, which has a D-mycinose at the C-21 position. pSETmycinose was introduced into *M. rosaria* IFO13697, which produces the 16-membered macrolide antibiotic rosamicin (**1**). This antibiotic contains a branched lactone and D-desosamine at the C-5 position, and the resulting engineered strain *M. rosaria* TPMA0041 produces mycinosyl rosamicin derivatives of rosamicin [1, 4].

Izenamicins are 16-membered macrolide antibiotics produced by *Micromonospora* sp. FERM BP-1076 [13]. They contain branched lactone and the deoxyhexose sugar D-desosamine at the C-5 position, but no other sugar residue is attached to the lactone ring. *Micromonospora* sp. FERM BP-1076 produced at least seven izenamicins A_1, A_2, A_3, B_1, B_2, B_3, and B_4 (**1**–**7**) (Fig. 1). Among these, izenamicins A_1 (**1**), A_2 (**2**), B_1 (**4**), and B_4 (**7**) correspond to the natural products rosamicin, juvenimicin A_4, juvenimicin B_1, and juvenimicin B_4, respectively. On the other hand, izenamicins A_3 (**3**), B_2 (**5**), and B_3 (**6**) are reported as semisynthetic antibiotics prepared by chemical transformations from a 16-membered macrolide antibiotic tylosin. In this study, D-mycinose biosynthetic genes were introduced into FERM BP-1076 using pSETmycinose, and the resulting engineered strain TPMA0041 produced mycinosylizenamicins, 23-*O*-mycinosyl-20-deoxy-izenamicin B_1 (**9**) and 22-*O*-mycinosyl-izenamicin B_2 (**8**) (Fig. 1). In our recent study, 23-*O*-mycinosyl-20-deoxy-izenamicin B_1 (**9**) was identified as 23-*O*-mycinosyl-20-deoxo-20-dihydro-12,13-deepoxyrosamicin (IZI, **9**), which was produced by the engineered *M. rosaria* TPMA0001 [1]. 22-*O*-mycinosyl-izenamicin B_2 (**8**) has previously been synthesized as a macrolide antibiotic TMC-016 (**8**) by utilizing Wilkinson's catalyst $(Ph_3P)_3RhC1$, and it has strong antibacterial activity [8].

The *Escherichia coli–Streptomyces* spp. intergeneric conjugation system can be used to introduce DNA into both streptomycete and non-*Streptomyces* actinomycete cells. Transconjugation vectors possessing a ϕC31 *int* gene and *attP* site can insert into the ϕC31 *attB* attachment site of a host chromosome in a site-specific manner. The *attB* site is widely distributed throughout streptomycete strains; however, only few reports of the *attB* site of non-*Streptomyces* actinomycetes have been previously published [5, 6, 9]. In our previous study, the integration site ϕC31 *attB* was identified on the *M. rosaria* IFO13697 chromosome [1], and some ϕC31 *attB* sites were identified in the genomic sequences of *Micromonospora* spp. In this study, we identified ϕC31 *attB* sites on the chromosome of *Micromonospora* sp. FERM BP-1076 and performed a cluster analysis among the nucleotide sequences of the ϕC31 *attB* site of actinomycetes.



	R_1	R_2	R_3
izenamicin A_1 (rosamicin, **1**)	CH_2CHO	O	CH_3
izenamicin A_2 (**2**)	CH_2CH_2OH	O	CH_3
izenamicin A_3 (**3**)	CH_2CHO	O	CH_2OH
izenamicin B_1 (**4**)	CH_2CH_2OH	-	CH_3
izenamicin B_2 (**5**)	CH_3	-	CH_2OH
izenamicin B_3 (**6**)	CH_2CHO	-	CH_2OH
izenamicin B_4 (**7**)	CH_2CH_2OH	-	CH_2OH

	R
22-*O*-mycinosyl-izenamicin B_2 (TMC-016, compound C, **8**)	CH_3
23-*O*-mycinosyl-20-deoxy-izenamicin B_1 (IZI, compound D, **9**)	CH_2CH_3

Fig. 1 Structures of izenamicins and their mycinosyl derivatives

Materials and methods

Strains, media, and culture conditions

Micromonospora sp. FERM BP-1076 was purchased from the National Institute of Advanced Industrial Science and Technology, Japan. *Micromonospora* sp. FERM BP-1076 and TPMA0041 were grown at 27 °C in MR0.1S broth [2], FMM broth [27], trypticase soy broth (TSB; Becton, Dickinson and Company, USA), and on Yeast Starch agar (YSA) plates. The composition of YSA plate was 0.2 % Bacto^{TM} Yeast Extract (Becton, Dickinson and Company), 1 % soluble starch (Wako Pure Chemical Industries, Ltd., Japan), and 1.5 % agar (Wako Pure Chemical Industries, Ltd.) (pH 7.3). The media were supplemented with appropriate antibiotics (100 μg l^{-1} ampicillin, 50 μg ml^{-1} apramycin, or 30 μg ml^{-1} nalidixic acid) as required. These antibiotics were purchased from Wako Pure Chemical Industries, Ltd.

Vectors, DNA manipulation, and PCR

The pUC19 vector was used for routine cloning, and the pDrive cloning vector (QIAGEN, USA) was used for cloning the DNA fragment amplified by polymerase chain reaction (PCR). TaKaRa Ex Taq® (TaKaRa, Japan) was used for amplification of DNA fragments by PCR. Plasmid and genomic DNA amplification, restriction enzyme digestion, fragment isolation, cloning, and DNA fragment amplification were performed according to standard procedures [25]. Southern-blot analysis was performed according to our previous method [27].

Conjugation procedure

The intergeneric conjugation from *E. coli* S17-1 to *Micromonospora* sp. FERM BP-1076 was performed using a protocol essentially as we have in our previous study [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. *Micromonospora* sp. FERM BP-1076 culture grown in MR0.1S broth for 5 days was harvested by centrifugation, washed, and resuspended in TSB in the ratio 2:1 (v/v). *Micromonospora* sp. FERM BP-1076 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 a YSA plate. The plates were then incubated at 27 °C for 20 h and overlaid with 1 ml of water containing 500 μg nalidixic acid to inhibit further growth of *E. coli*, and 1 mg of apramycin to select the *Micromonospora* exconjugants. The plates were then reincubated at 27 °C for 7–10 days for growth of the exconjugants.

Analysis of macrolide antibiotics in culture of the izenamicin producer

The culture broth of *Micromonospora* sp. FERM BP-1076 and *Micromonospora* sp. TPMA0041 was adjusted to pH 9–11 with 28 % ammonia solution and extracted with an equal volume of ethyl acetate (EtOAc). Further, an equal volume of 0.1 % trifluoroacetic acid (TFA) was added to the organic layer. The water layer containing macrolide antibiotics was adjusted to pH 9–11 with 28 % ammonia solution and extracted with an equal volume of EtOAc. The organic layer was concentrated in vacuo, and each residue was dissolved in methanol for HPLC analyses with an L-2450 diode array detector (Hitachi, Japan), and liquid chromatography-mass spectrometry (LC–MS) analyses with an LCMS-2010 liquid chromatograph mass spectrometer (Shimadzu, Japan).

Fermentation, isolation, and identification of TMC-016

Micromonospora sp. TPMA0041 was cultured in 300-ml Erlenmeyer flasks, each containing 40 ml of MR0.1S broth. The flasks were incubated at 27 °C in a shake-incubator rotating at 120 rpm for 5–7 days. Further, 1 ml of the culture was transferred into 300-ml Erlenmeyer flasks, each containing 50 ml of FMM broth, and these flasks were incubated on a rotary shaker (135 rpm) at 27 °C. One milliliter of 0.5 M propionic acid adjusted to pH 7.2 with NaOH was added to the culture broth after 1 day and 3 days. After 5 days of cultivation, the broth (7.8 l) was centrifuged at 4,000 rpm for 10 min. The supernatant adjusted to pH 9–11 with 28 % ammonia solution was extracted twice with an equal volume of EtOAc. The mycelia were extracted with triethylamine-EtOAc (1:25–40). The combined crude extracts, which were concentrated in vacuo, were dissolved with EtOAc, after which an equal volume of 0.1 % TFA was added. The water layer containing TMC-016 (**8**) was adjusted to pH 9–11 with 28 % ammonia solution and extracted with an equal volume of EtOAc. After concentrating the organic layer in vacuo, the extracts were applied to a silica gel column (25 × 3 cm, silica gel 60; Merck, USA) and eluted with a chloroform/methanol/28 % ammonia solution (100:10:1). The fractions containing antibacterial metabolites (24.9 mg) were applied to a preparative HPLC system (Shim-Pack PREP-ODS, i.d. = 250 × 20 mm; MeCN-0.06 % TFA [35:65]; flow rate, 20 ml min^{-1}) for further purification. The purified TMC-016 (3.32 mg, **8**) 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 TMC-016 (**8**) were compared with those of the synthesized TMC-016 (**8**) [8].

Antibacterial activity assay

Staphylococcus aureus ATCC 25923, *Micrococcus luteus* ATCC 9341, *Salmonella enterica* serovar Typhimurium ATCC 14028, and *E. coli* ATCC 25922 were purchased from American Type Culture Collection (ATCC), Manassas, VA, USA. *Streptococcus pneumoniae* NBRC 102642 and *Enterococcus faecalis* NBRC 100480 were purchased from the National Institute of Technology and Evaluation, Japan. *Staphylococcus aureus* IID 1677 and *Streptococcus pneumoniae* IID 544 were purchased from the Institute of Medical Science, The University of Tokyo, Japan. The antibiotic activity of mycinosyl izenamicins was assayed by the micro-broth dilution method using Mueller–Hinton broth [14]. For the culture of *S. pneumoniae*, 5 % defibrinated horse blood (Nippon Biotest Laboratories, Japan) was added to the broth. After addition of test components, the strains were cultured at 37 °C for 20–24 h under aerobic conditions except for *S. pneumoniae*. Two strains of *S. pneumoniae* were cultured under anaerobic conditions using AneroPack® (Mitsubishi Gas Chemical Company, Japan). The lowest antibiotic concentration that prevented the growth of a given test organism was determined as the minimal inhibitory concentration (MIC). Erythromycin was purchased from Wako Pure Chemical Industries, Ltd.

Identification of the *attB* site on the chromosome of *Micromonospora* sp. FERM BP-1076

A 0.5-kb PCR fragment including *attP* on pSET152 was used as a probe for Southern-blot hybridization, and 2.2 and 0.1-kb hybridized bands were detected in total DNA of TPMA0041 digested with *Sal*I (TaKaRa, Japan). The 2.2-kb *Sal*I fragment was cloned from the *Sal*I fragment library prepared from total DNA of TPMA0041. Several hundred nucleotide sequences of both ends of the 2.2-kb *Sal*I fragment were determined. Partial sequence of the *int* gene on pSET152 was encoded by one of the ends of the *Sal*I fragment, and the nucleotide sequence (593 bp) of the other end of the *Sal*I fragment was typical of an actinomycete chromosome, because the percentage GC content was very high (73.5 %). By using the 2.2-kb *Sal*I fragment as a probe, 1.2-kb hybridized bands appeared in the *Sal*I-digested total DNA of *Micromonospora* sp. FERM BP-1076 (Fig. S1). Total DNA of *Micromonospora* sp. FERM BP-1076 was digested with *Sal*I, and 1.0–1.5 kb *Sal*I fragments isolated using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) were circularized with T4 DNA ligase (TaKaRa, Japan). We used the circularized DNA as the template to amplify 0.8-kb DNA fragments containing the *attB* site by standard PCR with the following primers: 2411_invF, 5′-GCGCTGATCTCTCGGATGTG-3′, and 2411_invR, 5′-ATCACGAACGGCCCGTAATG-3′ (Fig. S2). The nucleotide sequence of the 0.8-kb amplified fragment was aligned with 593 nucleotide sequences on the 2.2-kb *Sal*I fragment from total DNA of TPMA0041. The partial open reading frame (ORF) of pirin-like protein was encoded in the aligned 1,139-base sequence (the sequence is available from GenBank, EMBL and DDBJ under Accession no. AB720112), and a single *attB* site (50 bp) was identified in the sequence of the pirin-like protein gene (Fig. S2).

Results and discussion

The plasmid pSETmycinose containing the D-mycinose biosynthesis genes from *M. griseorubida* A11725 was derived from the *E. coli–Streptomyces* conjugal shuttle vector pSET152, which possesses the ϕC31 *int* gene and *attP* site, and can site-specifically insert into the ϕC31 *attB* attachment site of a host chromosome. pSETmycinose was transferred from *E. coli* to *Micromonospora* sp. FERM BP-1076 by conjugation, and the resulting apr^r transconjugant TPMA0041 was isolated. This plasmid was introduced into the ϕC31 *attB* site on the chromosome of *Micromonospora* sp. FERM BP-1076 (Fig. S1), and a 50-bp sequence of the *attB* site was identified in the partial ORF of a pirin-like protein (Fig. S2).

The transconjugant TPMA0041 and the wild strain FERM BP-1076 were cultured in 50 ml of FMM broth at 27 °C for 6 days, and EtOAc extracts obtained from these culture broths were compared by using HPLC and LC–MS. In the EtOAc extract obtained from the FERM BP-1076 culture, compound A (MW, 583; UV_{max}, 286 nm) and compound B (MW, 567; UV_{max}, 286 nm) were detected at 3.2 and 7.1 min, respectively (Fig. 2). Based on these properties, compound A and B were estimated to be izenamicin B_4 (**7**) and izenamicin B_1 (**4**), respectively [13]. On the other hand, compound A and B were not detected in the TPMA0041 extract, and a new major peak (compound D: MW, 741; UV_{max}, 286 nm) was observed on the chromatogram at 13.8 min (Fig. 2). The major peak was a mycinosyl izenamicin derivative IZI (**9**), which has been previously produced by the engineered strain *M. rosaria* TPMA0001 containing pSETmycinose as 23-*O*-mycinosyl-20-deoxo-20-dihydro-12,13-deepoxyrosamicin [1] or synthesized by a bioconversion technique [18]. Moreover, another new small peak (compound C: MW, 727; UV_{max}, 285 nm) was also detected at 9.9 min in the TPMA0041 extract (Fig. 2). From the molecular weight and UV spectrum, compound C was inferred to be 22-*O*-mycinosyl-izenamicin B_2 (**8**), which was identical to the synthesized macrolide antibiotic TMC-016 (**8**) [8].

The carbons of the lactone ring in macrolide antibiotics are derived from short-chain fatty acids (i.e., acetate, propionate, butyrate, and other simple carboxylic acids) or

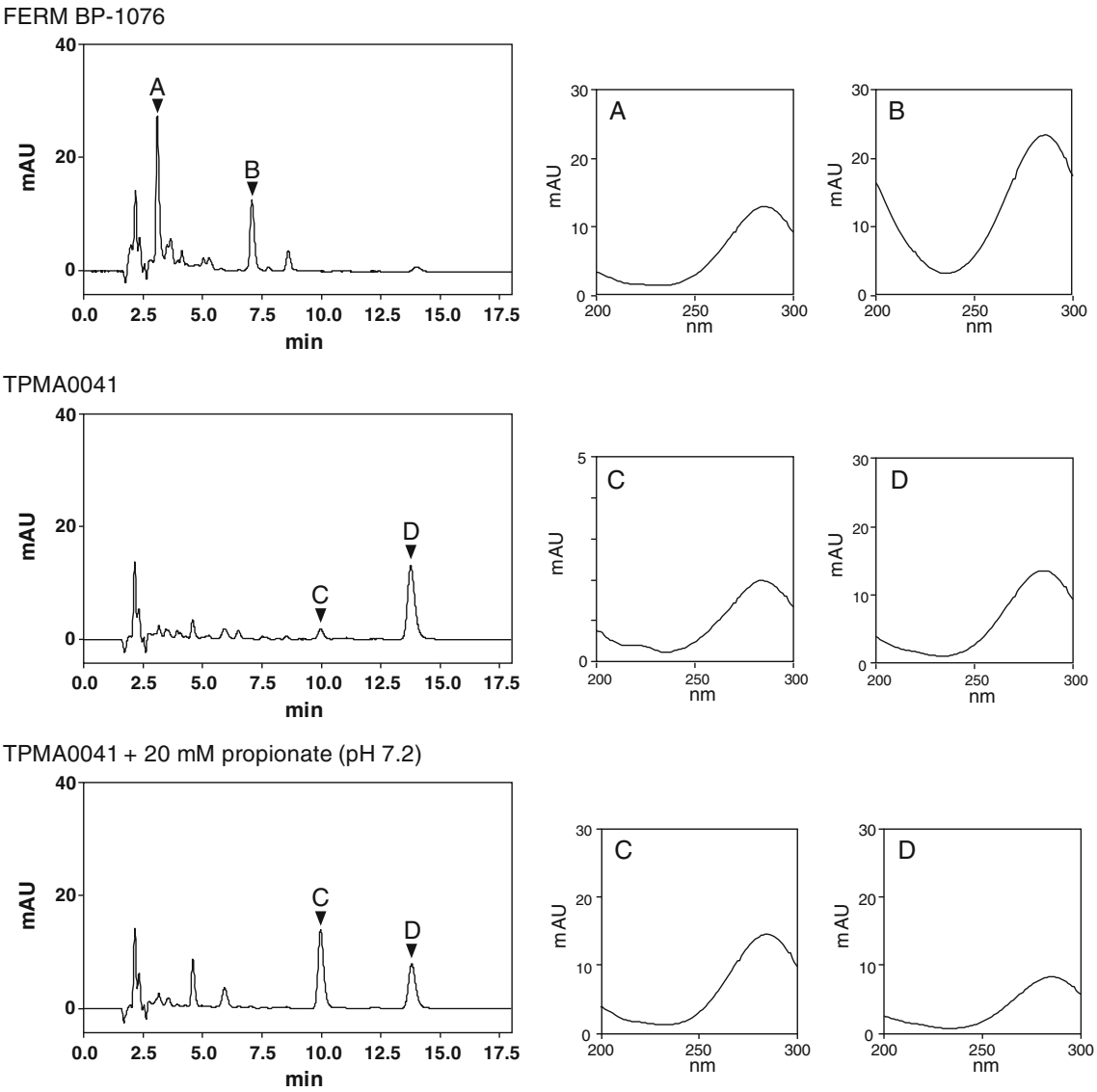


Fig. 2 HPLC chromatograms (280 nm) of EtOAc extracts obtained from the culture broths of *Micromonospora* sp. FERM BP-1076 and *Micromonospora* sp. TPMA0041, and UV spectrograms (200–300 nm) of compound A [izenamicin B_4 (**7**), estimated], compound B [izenamicin B_1 (**4**), estimated], compound C (TMC-016, **8**), and compound D (IZI, **9**) on these chromatograms. *Micromonospora* sp. FERM BP-1076 and TPMA0041 were cultured in 50 ml of FMM broth at 27 °C for 6 days, and 10 mM propionate (pH 7.2) was added to the culture broths of *Micromonospora* sp. TPMA0041 after 1 day and 3 days, as necessary

their derivatives such as acyl coenzyme A (CoA) and 2-carboxyacyl-CoA. The CoA-containing compounds are used as substrates by polyketide synthase (PKS) to synthesize the lactone ring [12]. The precursor of the C-6 ethyl group of IZI was estimated to be ethylmalonyl-CoA, whereas the precursor of the C-6 methyl group of TMC-016 (**8**) was estimated to be methylmalonyl-CoA. To increase the production of compound C, propionate was added to the culture broth of TPMA0041. When 20 mM propionate (pH 7.2) was added to the culture broth, the productivity of compound C was increased, and the production was approximately ninefold higher than that of the culture broth without propionate (Fig. 2).

Following isolation and purification from 7.8 l of the *Micromonospora* sp. TPMA0041 culture broth supplemented with 20 mM propionate (pH 7.2), we obtained 3.32 mg of purified compound C and characterized it by ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) spectroscopy. From the NMR chemical shift (Table S1) and its molecular weight (MW, 727), compound C was identified as 22-*O*-mycinosyl-izenamicin B_2 (TMC-016, **8**), which corresponds to the synthesized TMC-016 (**8**) identified by Fujiwara et al. [8]. We compared the ^{1}H-NMR data of TMC-016 with that of IZI, and we found that the methylene proton H-19 (1.65 ppm, m and 0.93 ppm, overlapping) found in IZI was not found in TMC-016. Moreover, a

Table 1 Antibacterial activity of mycinosyl izenamicins and erythromycin

Microorganism	MIC (μg ml^{-1})		
	TMC-016 (22-*O*-mycinosyl-izenamicin B_2, **8**)	IZI (23-*O*-mycinosyl-20-deoxy-izenamicin B_1, **9**)	Erythromycin
Staphylococcus aureus ATCC 25923	0.20	0.20	0.098
Staphylococcus aureus IID 1677[a]	>100	>100	>100
Streptococcus pneumoniae NBRC 102642	1.6	1.6	0.024
Streptococcus pneumoniae IID 544	3.1	3.1	0.098
Micrococcus luteus ATCC 9341	0.024	0.024	0.024
Enterococcus faecalis NBRC 100480	6.3	3.1	25
Escherichia coli ATCC 25922	>100	50	3.1
Salmonella enterica serovar Typhimurium ATCC 14028	>100	100	25

[a] Methicillin-resistant *Staphylococcus aureus* (MRSA)

methyl proton was observed at H-19 (1.07 ppm, d) in TMC-016 and at H-20 (0.86 ppm, t) in IZI. In the ^{13}C-NMR spectrum, TMC-016 contained methyl carbon C-19 (17.6 ppm), and IZI contained methylene carbon C-19 (21.2 ppm) and methyl carbon C-20 (12.1 ppm). Because of the presence of the side chain at C-6, we found differences in the chemical shifts of C-5, C-6, and C-7 between TMC-016 (85.9, 31.7, and 34.9 ppm) and IZI (79.9, 38.2, and 34.0 ppm). These NMR data indicate that TMC-016 and IZI contain a methyl group and an ethyl group at C-6, respectively. On the other hand, the ^{13}C-NMR and ^{1}H-NMR data for TMC-016 were almost the same as those for the synthesized TMC-016 [8].

Antibacterial activities of TMC-016 (**8**) were compared with those of IZI (**9**) and erythromycin, which is a clinical important 14-membered macrolide antibiotic (Table 1). The antibacterial activities of TMC-016 (**8**) were very similar to those of the synthesized TMC-016 (**8**) [8]. Both mycinosyl-izenamicins were effective against Gram-positive bacteria and had low activity against Gram-negative bacteria. MICs of TMC-016 (**8**) were 0.20, 0.024, 1.6, and 3.1 μg ml^{-1} against *S. aureus* ATCC 25923, *M. luteus* ATCC 9341, *S. pneumoniae* NBRC 102642, and *S. pneumoniae* IID 544, respectively. The antibacterial activity of TMC-016 (**8**) was similar to that of IZI (**9**), and both compounds and erythromycin had no activity against *S. aureus* IID 1677 (methicillin-resistant *S. aureus*; MRSA) at 100 μg ml^{-1}. On the other hand, antibacterial activities of both mycinosyl-izenamicins were lower than that of erythromycin in this antibacterial activity assay, and it has been shown in the previous report by Fujiwara et al. [8] that in vitro antibacterial activity of the synthesized TMC-016 (**8**) against *S. aureus*, *M. luteus*, and *S. pneumoniae* was lower than that of erythromycin. However, it was described by Fujiwara et al. [8] that the antibacterial activity of the synthesized TMC-016 (**8**) against *Mycoplasma pneumoniae* and anaerobic bacteria were higher than that of erythromycin. Moreover, in vivo antibacterial activity of the synthesized TMC-016 (**8**) against *S. aureus*, *S. pneumonia, and Streptococcus pyogenes* was higher than that of erythromycin [8]. The D-mycinose moiety imparts higher antibacterial activity to 16-membered macrolide antibiotics. Antibacterial activity of mycinamicin IV (Fig. S3), which is a intermediate in mycinamicin biosynthesis and has the highest antibacterial activity among mycinamicins, was higher than that of mycinamicin VIII (Fig. S3), which lacked mycinose moiety of mycinamicin IV [15]. Moreover, in our unpublished study, MICs of IZI biosynthetic precursor 20-deformyl-12,13-deepoxyrosamicin (Fig. S3), which was isolated from rosamicin producer *M. rosaria* as a rosamicin intermediate, against *S. aureus* ATCC 25923 and *M. luteus* ATCC 9341 were both 3.1 μg ml^{-1}. On the other hand, MICs of IZI (**9**) against *S. aureus* ATCC 25923 and *M. luteus* ATCC 9341 were 0.20 and 0.024 μg ml^{-1}, respectively (Table 1), and antibacterial activity of IZI (**9**) was higher than that of 20-deformyl-12,13-deepoxyrosamicin. Therefore, the attachment of mycinose moiety enhances antibacterial activity of 16-membered macrolide antibiotics and the genetic engineering with the mycinose biosynthetic gene would be expected to generate more effective macrolide antibiotics.

The lactone rings in macrolide antibiotics are synthesized by modular type I PKSs, which are large multifunctional enzymes harboring from one to many modules. Each module contains a set of at least three domains—a ketosynthase, an acyltransferase (AT), and an acyl carrier protein—that catalyze a 2-carbon extension of the growing polyketide chain. The choice of extender unit—acetate, propionate, butyrate, and other simple carboxylic acids in the form of CoA thioesters—is determined by the substrate specificity of the AT domain in each domain [12]. In this study, we found that the ethyl group at the C-6 position of IZI (**9**) was derived from ethylmalonyl-CoA, and the methyl group at the C-6 position of TMC-016 (**8**) was

derived from methylmalonyl-CoA. Ethylmalonyl-CoA and methylmalonyl-CoA were introduced into the lactone ring of izenamicins by the AT domain on module 5 of an izenamicin PKS. Therefore, the AT domain on module 5 of an izenamicin PKS appears to be able to recognize ethylmalonyl-CoA and methylmalonyl-CoA as substrates. Generally, methylmalonyl-CoA can be supplied to macrolide biosynthesis via three accepted pathways: (1) isomerization of succinyl-CoA, catalyzed by methylmalonyl-CoA mutase; (2) carboxylation of propionyl-CoA carboxylase or methylmalonyl-CoA transcarboxylase; (3) catabolism of valine [21]. The expression of methylmalonyl-CoA mutase gene *mutB* from the erythromycin producer *Saccharopolyspora erythraea* in the FK506 producer *Streptomyces clavuligerus* CKD1119 increased in the methylmalonyl-CoA accumulation and FK506 production, respectively [19]. Erythromycin macrolactone 6-deoxyerythrolide B was overproduced by heterologous expression of the propionyl-CoA carboxylase genes *pccB* and *accA1* from *Streptomyces coelicolor* in an engineered *E. coli* strain expressing erythromycin PKS [20]. Moreover, there are other pathways that can supply methylmalonyl-CoA to macrolide biosynthesis. For example, the production of epothilone was increased by the heterologous expression of propionyl-CoA synthetase, which converts propionate into propionyl-CoA [10]. In this study, addition of propionate to the culture broth containing TPMA0041 significantly increased the production of TMC-016 (**8**) in *Micromonospora* sp. TPMA0041, as leads to the robust production of methylmalonyl-CoA in the cells. We found that the C-6 position of izenamicin B_2 produced by the wild strain FERM BP-1076 has a methyl group derived from methylmalonyl-CoA. In contrast, ethylmalonyl-CoA was introduced into the corresponding position in other izenamicins. *Micromonospora chalcea* var. *izumensis* T-1124 produces the 16-membered macrolide antibiotic juvenimicins, which have one or two carbon atoms at the C-6 position as a side chain juvenimicin [11, 16]. This implies that the AT domain on module 5 of juvenimicin PKS must recognize both ethylmalonyl-CoA and methylmalonyl-CoA as substrates. The substrate specificities of AT domains on module 5 of PKSs that synthesize izenamicin and juvenimicin therefore appear to be relaxed, and it would be interesting for the elucidation of the flexibility of substrate utilization on these AT domains to compare among the amino acid sequences of these AT domains on PKSs.

As shown in Figure S1, pSETmycinose was integrated into the *Micromonospora* sp. FERM BP-1076 chromosome by the ϕC31 *att*/*int* system. The sequence and location of the chromosomal integration site ϕC31 *attB* of *Micromonospora* sp. FERM BP-1076 was identified in this study. The 50-nucleotide sequence encoding the *attB* site of *Micromonospora* sp. FERM BP-1076 showed the highest homology (94 % identity) to those of pirin-like protein genes of *Micromonospora aurantiaca* ATCC 27029 (Accession no. CP002162) and *Micromonospora* sp. L5 (Accession no. CP002399). The *Micromonospora* sp. FERM BP-1076 *attB* site was found to lie within an ORF coding a pirin-like protein. The 786-nucleotide sequence encoding a partial pirin-like protein gene of *Micromonospora* sp. FERM BP-1076 showed the highest homology (89 % identity) to that of *M. aurantiaca* ATCC 27029. In actinomycetes, the target genes containing the ϕC31 *attB* site encode pirin-like proteins, and these genes are highly conserved within *Streptomyces* and some other actinomycete strains [5]. Cluster analysis based on 45 sequences of ϕC31 *attB* site from *Micromonospora* sp. FERM BP-1076 and other actinomycetes was performed using the

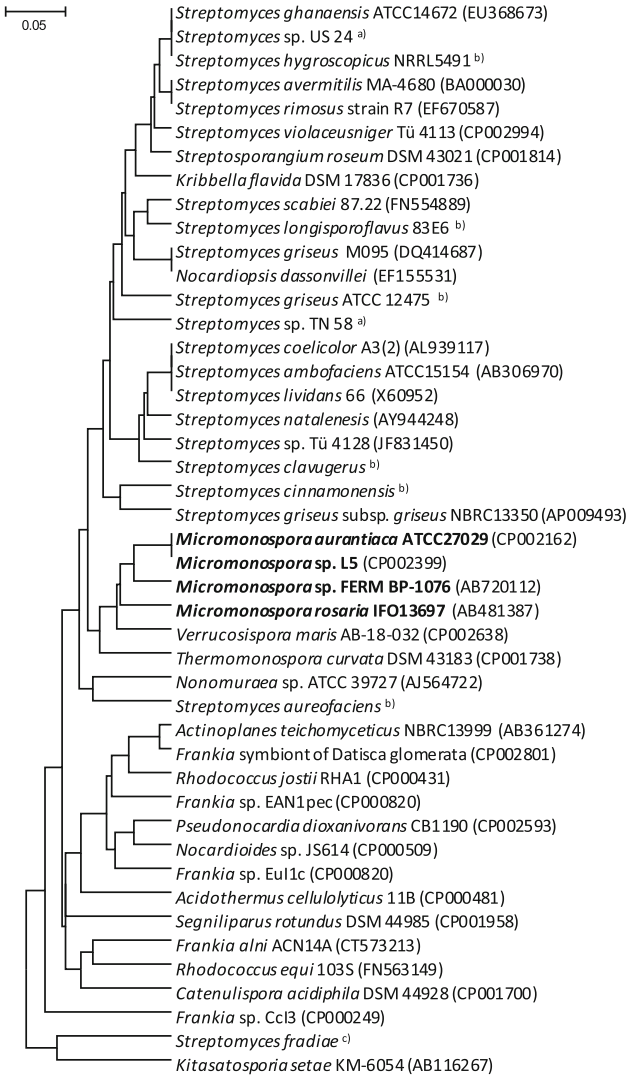


Fig. 3 Phylogenetic tree based on ϕC31 *attB* core sequences (50 bp) from *Micromonospora* sp. FERM BP-1076 and other actinomycetes. Accession numbers for the sequences are in parentheses. a) [26], b) [7], c) [17]

CLUSTALW program (Fig. 3). In this cluster analysis, we used 34 *attB* sequences, which were recognized in the sequence of the pirin-like protein gene and had high homology to the 50-nucleotide sequence encoding the *attB* site of *Micromonospora* sp. FERM BP-1076 as per BLAST analysis, and ten additional sequence data, which were previously reported as the ϕC31 *attB* site of actinomycetes [1, 7, 17, 26]. It was interesting that all strains of the genus *Micromonospora* and almost all *Streptomyces* strains constituted independent clusters. *Micromonospora* sp. FERM BP-1076 was placed in the cluster of *Micromonospora* strains, and *Verrucosispora maris* AB-18-032 belonged to the family *Micromonosporaceae* [22]. Nineteen *Streptomyces* strains constituted a cluster, and this cluster also included non-*Streptomyces* strains, *Streptosporangium roseum* DSM 43021, *Kribbella flavida* DSM 17836, and *Nocardiopsis dassonvillei*. On the other hand, *Streptomyces aureofaciens* and *S. fradiae* were more divergent, and instead clustered with *Nonomurea* sp. ATCC 39727 and *Kitasatospora setae* KM-6054, respectively. In this cluster analysis, only 50-nucleotide sequences of *attB* site were used to describe a phylogenetic tree. However, a cluster of *Micromonospora* was independently constituted because the sequences encoding the *attB* site of *Micromonospora* strains were very similar to each other. In summary, our present study shows that introduction of DNA into cells of *Micromonospora* strains using the bacteriophage ϕC31 *att/int* system is a powerful approach for novel antibiotic biosynthesis.

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