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A new mycinosyl rosamicin derivative produced by an engineered *Micromonospora rosaria* mutant with a cytochrome P450 gene disruption introducing the D-mycinose biosynthetic gene

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Abstract Genetic engineering of post-polyketide synthase-tailoring genes can be used to generate new macrolide analogs through manipulation of the genes involved in their biosynthesis. Rosamicin, a 16-member macrolide antibiotic produced by *Micromonospora rosaria* IFO13697, contains a formyl group and an epoxide at C-20 and C-12/13 positions which are formed by the cytochrome P450 enzymes RosC and RosD, respectively. The D-mycinose biosynthesis genes in mycinamicin II biosynthesis gene cluster of *Micomonospora guriseorubida* A11725 were introduced into the *rosC* and *rosD* disruption mutants of *M. rosaria* IFO13697. The resulting engineered strains, *M. rosaria* TPMA0054 and TPMA0069, produced mycinosyl rosamicin derivatives, IZIV and IZV, respectively. IZIV was identified as a novel mycinosyl rosamicin derivative, 23-*O*-mycinosyl-20-deoxo-20-dihydrorosamicin.

Introduction

Biosynthesis of macrolide antibiotics begins with the assembly of a macrolactone ring by a modular polyketide synthase (PKS) and continues with a series of post-PKS modifications such as glycosylation and oxidation. Post-PKS modifications contribute to structural diversification and serve to improve the bioactivity profiles of natural products. Many genes involved in the biosynthesis of macrolide antibiotics have been cloned and sequenced; in addition, the functions of many enzymes encoded by the biosynthetic genes have been elucidated. Based on these findings, new macrolide analogs can be generated using genetic engineering and combinatorial biosynthesis techniques [13, 15].

Rosamicin is a 16-membered macrolide antibiotic produced by *Micromonospora rosaria* IFO 13697 (=NRRL 3718), and it contains a branched lactone and deoxyhexose sugar D-desosamine at the C-5 position [17]. The plasmid pSETmycinose, which contains the D-mycinose biosynthetic genes (*mycCI*, *mycCII*, *mycD*, *mycE*, *mycF*, *mydH*, and *mydI*) from the producer of a 16-membered macrolide antibiotic mycinamicin II, was introduced into *M. rosaria* IFO 13697, and the resulting engineered strain *M. rosaria* TPMA0001 produced mycinosyl rosamicin derivatives, IZI, IZII, and IZIII (Fig. 1) [1, 3]. Structural differences among these mycinosyl rosamicin derivatives were found at C-20 and C-12/13. Recently, we demonstrated that the C-20 formyl group and C-12/13 epoxide ring of rosamicin are formed by the cytochrome P450 enzymes, RosC and RosD [7]. In particular, the C-20 formyl group of the rosamicin macrolactone ring is formed with a two-step oxidation—hydroxylation and dehydrogenation—catalyzed by RosC. Moreover, we proposed that *M. rosaria* IFO 13697 has two branching pathways from the first desosaminyl rosamicin intermediate, 20-deoxo-20-dihydro-12,13-deepoxyrosamicin (RS-E), in the rosamicin post-PKS biosynthetic pathway (Supplementary Fig. S1). The structures of IZI, IZII, and IZIII are the same as those of rosamicin biosynthetic intermediates (RS-E; RS-C, 20-dihydro-12,13-deepoxyrosamicin; and RS-A, 20-dihydrorosamicin, respectively)

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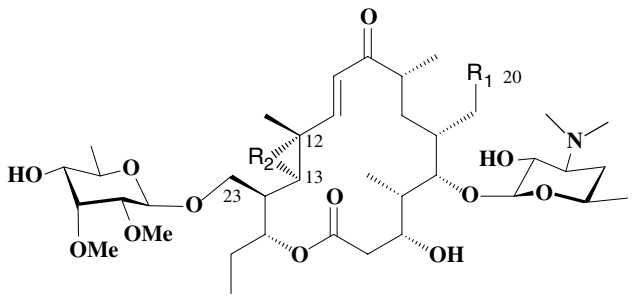

	R1	R2
IZI	CH_3	-
IZII	CH_2 OH	-
IZIII	CH_2 OH	O
IZIV	CH_3	O
IZV	CHO	-

Fig. 1 Structures of IZI to IZV

with D-mycinose attached at C-23 of these intermediates. However, the compounds that have D-mycinose attached to C-23 of the rosamicin intermediates 20-deoxo-20-dihydrorosamicin (RS-B) and 12,13-deepoxyrosamicin (RS-D) have never been detected in the culture broth of TPMA0001. On the other hand, in our previous study [7], we showed that the amount of RS-B produced by the *rosC* disruption mutant *M. rosaria* TMPA0050 was approximately 20-fold higher than that of RS-B produced by the wild-type strain IFO 13697. In addition, RS-D, which was not detected in the culture broth of the wild-type strain IFO 13697, appeared in the HPLC chromatogram of the ethyl acetate (EtOAc) extracts obtained from the culture broth of the *rosD* disruption mutant *M. rosaria* TPMA0055. Here, we have performed that hybrid 16-membered macrolide antibiotics were produced by coupling of generation of *M. rosaria* mutants blocked expression of cytochrome P450 genes involved in rosamicin biosynthesis and expression of the D-mycinose biosynthetic genes in *M. rosaria* mutants.

Materials and methods

Strains, media, and culture conditions

The strains used in this study are shown in Table 1. The culture conditions of *M. rosaria* strains and *Escherichia coli* have been described previously [1]. Trypticase soy broth (TSB; Becton, Dickinson and Company, Sparks, MD, USA), 172F medium (56 mM glucose, 10 mM $MgSO_4 \cdot 7H_2O$, 8 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 % yeast extract, 1 % soluble starch, 0.5 % Bacto^{TM} casitone, pH 7.2) and MR0.1S agar medium [8] were used for fermentation of *M. rosaria*. The media were supplemented with appropriate antibiotics (50 μg ml^{-1} apramycin or 25 μg ml^{-1} nalidixic acid) as required. These antibiotics were purchased from Wako Pure Chemical Industries, Ltd.

Conjugation procedure

Intergeneric conjugation between *E. coli* S17-1 and *M. rosaria* was performed using a modified protocol based on a previously described procedure [1]. A mixture of the *E. coli* donor cells and the *M. rosaria* recipient cells was spread on trypticase soy agar (TSA) plates. The plates were incubated at 32 °C for 20–24 h and were then overlaid with 1 ml of water containing 500 μg of nalidixic acid to inhibit growth of *E. coli* and 1 mg of apramycin for selecting

Table 1 Strains used in this study

Strain	Description	Source or reference
M. rosaria		
IFO 13697	Wild type, rosamicin producer	Institute of Fermentation, Osaka (IFO)
TPMA0001	*M. rosaria* IFO13697 harboring pSETmycinose	[1]
TPMA0050	*rosC* deletion derivative of *M.rosaria* IFO13697	[7]
TPMA0054	*M. rosaria* TPMA0050 harboring pSETmycinose	This study
TPMA0055	*rosD* deletion derivative of *M.rosaria* IFO13697	[7]
TPMA0069	*M. rosaria* TPMA0055 harboring pSETmycinose	This study
E. coli		
ATCC 47055	Conjugation donor (*E. coli* S17-1)	American Type Culture Collection (ATCC)
ATCC 25922	Test strain for antibacterial assay	ATCC
S. aureus		
ATCC 25923	Test strain for antibacterial assay	ATCC
IID 1677	Test strain for antibacterial assay	The Institute of Medical Science, The University of Tokyo (IID)
M. luteus		
ATCC 9341	Test strain for antibacterial assay	ATCC

M. rosaria exconjugants. The plates were then reincubated at 32 °C for 2–3 weeks for growth of the exconjugants. The genetic status of the exconjugants was confirmed by PCR.

Analysis of rosamicins in the *M. rosaria* culture

TPMA0001, TPMA0054, and TPMA0069 were cultured in 5 ml of TSB at 27 °C for 7 days. The broth was adjusted to a pH of 9–11 with 28 % ammonia solution and extracted twice with an equal volume of EtOAc. The extract was then concentrated in vacuo. The crude extracts were dissolved with EtOAc, and then an equal volume of 0.1 % trifluoroacetic acid (TFA) was added. The water layer containing rosamicins was adjusted to a pH of 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 (MeOH) for HPLC analysis with a diode array detector model L-2450 (Hitachi, Japan) and liquid chromatography-mass spectrometry (LC–MS) analysis with a liquid chromatograph mass spectrometer model LCMS2010 (Shimadzu, Japan).

Fermentation, isolation, and identification of IZIV and IZV

To isolate and purify IZIV, TPMA0054 was cultured in 300-ml Erlenmeyer flasks containing 40 ml of TSB medium. The flasks were incubated on a rotary shaker (120 rpm) at 27 °C for 3–5 days. TPMA0054 culture (0.8 ml) was transferred to each of the fourteen 500-ml Sakaguchi flasks, each containing 300 ml of TSB medium, and these flasks were incubated on a rotary shaker (120 rpm) at 27 °C for 14 days. The culture filtrate was passed through a column of HP20 sorbent (Mitsubishi Chemical, Tokyo, Japan). After rosamicin derivatives were washed with water, they were eluted from the column with MeOH, and the eluate was concentrated to a pasty residue in vacuo. This residue was resuspended in 300 ml of water, and the pH of this suspension was adjusted to 9–11 with 28 % ammonia solution and extracted twice with equal volumes of EtOAc. The organic extracts were combined and concentrated to dryness in vacuo. The concentrated extracts, including rosamicin derivatives derived from culture products of TPMA0054, were dissolved with EtOAc, after which an equal volume of 0.1 % TFA was added. The water layer containing rosamicins was adjusted to a pH of 9–11 with 28 % ammonia solution and extracted with an equal volume of EtOAc, and the organic layer was concentrated in vacuo. The residue was dissolved in MeOH and applied to a preparation HPLC system with a Shim-pack PREP-ODS column (20 mm i.d. × 250 mm; Shimadzu, Japan) using the mobile phase acetonitrile (MeCN):0.06 % TFA (35:65) (flow rate, 20 ml min^{-1}) to collect IZIV. The fraction containing IZIV was adjusted to a pH of 9–11 with 28 % ammonia solution and extracted with an equal volume of EtOAc. After the organic solution was concentrated to dryness in vacuo, IZIV (15.1 mg) was obtained.

To isolate and purify IZV, 100 μl of TSB culture broth of TPMA0069 was spread on 15-ml MR0.1S agar plates. After 2–3 weeks, the agar from 80 culture plates was homogenized and extracted with a twofold volume of EtOAc containing 1 % triethylamine at 50 °C. The combined crude extracts, which were concentrated in vacuo, were dissolved with EtOAc, and then an equal volume of 0.1 % TFA was added. The water layer containing rosamicins was adjusted to a pH of 9–11 with 28 % ammonia solution and extracted with an equal volume of EtOAc. The organic layer was concentrated in vacuo. The residue dissolved with MeOH was applied to a preparation HPLC system (Shim-Pack PREP-ODS, 20 mm i.d. × 250 mm; MeCN:0.06 % TFA [35:65]; flow rate, 15 ml min^{-1}) to collect IZV. The fraction containing IZV was adjusted to a pH of 9–11 with 28 % ammonia solution, and the fraction was extracted with an equal volume of EtOAc. After concentrating the organic solution to dryness in vacuo, IZV (5.5 mg) was obtained.

The purified IZIV and IZV were characterized by ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) spectroscopy (JEOL JNM-ECA500) and mass spectrometry (JEOLJMS-T100LP).

Antibacterial activity

The antibacterial activity was determined using the microbroth dilution method in Mueller–Hinton medium (Becton, Dickinson, and Company). After the addition of test compounds, the Gram-positive bacteria *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* IID 1677 and *Micrococcus luteus* ATCC 9341 and the Gram-negative bacterium *E. coli* ATCC 25922 were grown at 37 °C for 20–24 h. The minimum inhibitory concentration (MIC) was defined as the lowest concentration that inhibited 80 % of the growth.

Results and discussion

The plasmid pSETmycinose was introduced into TPMA0050 and TPMA0055 by intergenetic conjugation using our previous procedure [1]. The resulting apramycin-resistance transconjugants TPMA0054 and TPMA0069 were isolated. Integration of pSETmycinose into the *ϕ*C31 *attB* site on the chromosome of TPMA0054 and TPMA0069 was confirmed by PCR (data not shown). TPMA0001, TPMA0054, and TPMA0069 were cultured in 5 ml of TSB medium at 27 °C for 7 days, and EtOAc extracts obtained from these culture broths were compared by using HPLC and LC–MS (Fig. 2). In HPLC analysis,

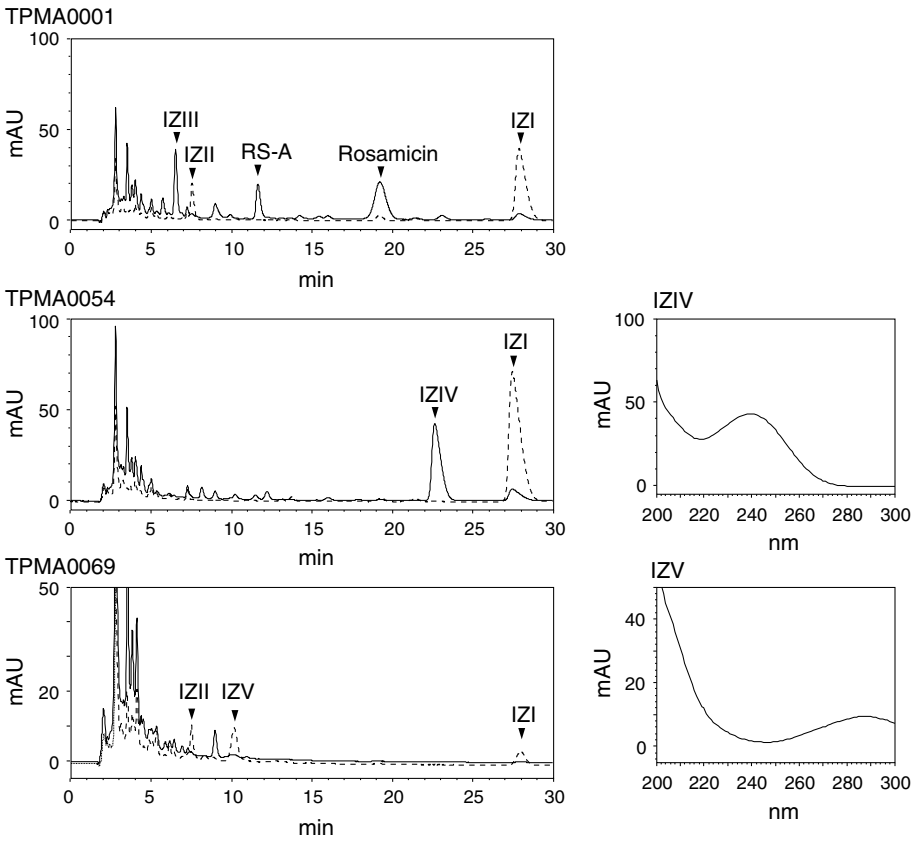

Fig. 2 HPLC chromatograms of the EtOAc extracts obtained from the culture broths of TPMA0001 (IFO 13697/pSET-mycinose), TPMA0054 (Δ*rosC*/pSETmycinose) and TPMA0069 (Δ*rosD*/pSETmycinose) as well as the UV spectra of IZIV and IZV. HPLC analysis was performed on the following condition: column, ODS-80TM, 4.6 mm i.d. × 150 mm (Toso Co., Japan); mobile phase, MeCN:0.06 % TFA (30:70); flow rate, 0.8 ml min^{-1}; UV wavelength, 200–300 nm. *Solid line*, at 240 nm; *dashed line*, at 285 nm

IZI was detected at 27.49 min and recorded at 285 nm in the chromatograms of all strains, TPMA0001, TPMA0054, and TPMA0069. Moreover, the new peak IZIV, which was not detected in the culture broth of TPMA0001 or TPMA0069, was observed at 22.67 min in the chromatogram of TPMA0054 and recorded at 240 nm. On the HPLC chromatogram of TPMA0069 recorded at 285 nm, the peak IZII and the other additional peak IZV appeared at 7.51 and 10.16 min, respectively. LC–MS analysis was performed with the extract to measure the molecular weights of IZIV and IZV, and those signals appeared at *m/z* 758 $(M+H)^+$ and *m/z* 756 $(M+H)^+$, respectively. IZIV (15.1 mg) was isolated and purified from 4.2 l of TPMA0054 culture broth. IZV (5.5 mg) was isolated and purified from 80 × 15 ml MR0.1S culture plates of TPMA0069.

The molecular formula of IZIV was established as $C_{39}H_{67}NO_{13}$ on the basis of HR-ESI–MS data (*m/z* 758.46766; calculated for $C_{39}H_{68}NO_{13}$, *m/z* 758.46906). The UV λ_{max} of IZIV is 240 nm, based on the UV spectrum data of the HPLC analysis. The structure of IZIV was elucidated by NMR spectroscopic studies. Chemical-shift assignments for IZIV were determined on the basis of the DQF-COSY, HMQC, HMBC, and NOESY spectral data. The ^{1}H- and ^{13}C-NMR data for IZIV were compared with those of rosamicin and IZI (Supplementary Table S1) [1, 10]. We found that the ^{1}H- and ^{13}C-NMR spectral data of IZIV resemble those of IZI, with the exception of the proton and carbon signals of C-12 and C-13. The proton and carbon chemical shifts of C-12 and C-13 of IZIV coincide with those of rosamicin, which has an epoxy ring at C-12/13. On the basis of those results, the structure of IZIV was determined to be 23-*O*-mycinosyl-20-deoxo-20-dihydrorosamicin, which has a structure of D-mycinose attached to the rosamicin biosynthetic intermediate RS-B (Fig. 1).

The UV λ_{max} of IZV [*m/z* 756 $(M + H)^+$] is 287 nm, based on the UV spectrum data of the HPLC analysis. To determine the structure of IZV, the ^{1}H- and ^{13}C-NMR data of IZV were compared with those of rosamicin and IZI (Supplementary Table S1) [1, 10]. The ^{1}H- and ^{13}C-NMR data of IZV are considerably similar to those of IZI, except that IZV does not contain the proton and carbon signals of a methyl group at C-20 as seen in IZI. Instead, the C-20 chemical shift of IZV corresponds with those of rosamicin, which has an aldehyde group at C-20. From these results, the structure of IZV was determined to be 23-*O*-mycinosyl-12,13-deepoxyrosamicin, which has a structure of D-mycinose attached to the rosamicin intermediate RS-D (Fig. 1). This compound has previously been synthesized as 4′-deoxy-demycarosyl tylosin from demycarosyl tylosin by Tanaka et al. [16]. However, this is the first report on the fermentative production of 23-*O*-mycinosyl-12,13-deepoxyrosamicin by using a combinatorial biosynthesis approach.

Table 2 Antibacterial activities of rosamicins and their mycinosyl derivatives

Microorganism	MIC (μg ml^{-1})										
	RS-B[a]	IZIV	RS-D[a]	IZV	RS-E[a]	IZI[b]	RS-C[a]	IZII[b]	RS-A[a]	IZIII[b]	Rosamicin[a]
S. aureus ATCC 25923	3.12	0.39	0.39	0.39	3.12	0.19	6.25	0.39	12.5	1.56	0.19
M. luteus ATCC 9341	3.12	0.048	0.39	0.048	3.12	0.048	3.12	0.097	6.25	0.19	0.048
E. coli ATCC 25922	100	50.0	6.25	50.0	>100	50.0	100	>100	100	>100	3.12

[a] Data from Iizaka et al. [7]

[b] Data from Anzai et al. [3]

In our previous study, when pSETmycinose was introduced into the wild-type strain IFO 13697, IZIV and IZV were not detected in the culture broth of the resulting engineered strain TPMA0001, which produced other mycinosyl rosamicin derivatives, IZI, IZII, and IZIII. Moreover, we confirmed that the cytochrome P450 enzymes RosC and RosD could recognize the first desosaminyl rosamicin intermediate, RS-E, as a substrate in bioconversion studies of RS-E with *E. coli* TPMB0002 and TPMB0003 expressing RosC and RosD, respectively [7]. However, IZI, which has a structure of D-mycinose attached to RS-E, was not converted into any other derivative by TPMB0052 and TPMB0053 (data not shown). Therefore, it was estimated that the oxidation by RosC and RosD occurred before the addition of D-mycinose in the mycinosyl rosamicin derivatives biosynthetic pathway. In the biosynthetic pathway of two other 16-member macrolides, tylosin and spiramycin, the oxidation by the cytochrome P450 enzymes TylI and Srm13 also occurred before the addition of the second sugar, mycinose and forosamine, respectively [4, 11]. On the other hand, it has been reported that the addition of the second sugar, mycinose, to the C-21 in the biosynthetic pathway of 16-membered macrolide mycinamicin II is essential to the epoxidation at C-12/13 by the cytochrome P450 enzyme MycG [2]. Thus, it was exhibited that post-PKS tailoring steps are greatly different even if it is the enzymes which catalyzed epoxidation of 16-membered ring macrolide such as RosD and MycG.

In the recent report, 5-*O*-desosaminyl erythronolide A, a part precursor of ketolides, was produced by the expression of *pikC* encoding a cytochrome P450 enzyme in an *eryBV* deletion mutant of *Saccharomyces erythraea* [5]. Moreover, disruption of the genes related to TDP- D-chalcose biosynthesis and heterologous expression of TDP—D-desosamine biosynthesis genes for the dihydrochalcomycin producer *Streptomyces* sp. KCTC 0041BP led to production of novel 16-membered macrolide 5-*O*-desosaminyl-20-*O*-mycinosyl-dihydrochalconolide [12]. It was shown in our previous study that rosamicin biosynthetic intermediates RS-B and RS-D were effectively accumulated in the culture broth of *M. rosaria* by disruption of the *rosC* and *rosD* genes, respectively. It was succeeded in this study that a novel compound IZIV, which was not detected in the culture broth of the previous engineered strain TPMA0001, was produced by the new generation engineered strain TPMA0054, and IZV, which was not also detected in the culture broth of TPMA0001, was accumulated in the culture broth on the other engineered strain TPMA0069. Although post-PKS modifications of macrolide antibiotics contribute to structural diversification, amount of each biosynthesis intermediate is not enough. However, our present study showed that genetic engineering of post-PKS tailoring genes based on the detailed analysis of the macrolide biosynthetic pathway leads the effective production of novel macrolide compound.

The antibacterial activities of IZIV and IZV, which were tested on *S. aureus* ATCC 25923, *M. luteus* ATCC 9341, and *E. coli* ATCC 25922, were compared with those of the other mycinosyl rosamicin derivatives (IZI, IZII, and IZIII), rosamicin biosynthetic intermediates (RS-A, RS-B, RS-C, RS-D, and RS-E), and rosamicin (Table 2) [3, 7]. The antibacterial activities of IZV are similar to those of the same compound reported by Tanaka et al. [16]. IZIV and IZV showed inhibition against gram-positive bacteria and had low activity against gram-negative bacteria, which correlates with other mycinosyl rosamicin derivatives. The antibacterial activities of mycinosyl rosamicin derivatives against *S. aureus* ATCC 25923 and *M. luteus* ATCC 9341 are higher than those of rosamicin biosynthetic intermediates. Kinoshita et al. [9] reported that the antibacterial activity of mycinamicin IV, which is a mycinosyl mycinamicin intermediate, is higher than that of mycinamicin VIII, which lacks the D-mycinose moiety of mycinamicin IV. Therefore, attachment of the D-mycinose moiety enhances antibacterial activity of 16-membered macrolide antibiotics against gram-positive bacteria. The antibacterial activities of IZIV and IZV were also tested on *S. aureus* IID 1677 (methicillin-resistant *S. aureus*; MRSA), and MICs of IZIV and IZV were 25 and 50 μg ml^{-1} against the MRSA strain, respectively. The antibacterial activities of IZIV and IZV against *S. aureus* IID 1677 were higher than that of a macrolide antibiotic TMC-016, of which MIC was more than 100 μg ml^{-1} against *S. aureus* IID 1677 [14], with strong antibacterial activity against *Mycoplasma*

pneumonia [6]. These results showed that combinations of macrolactone rings and deoxysugars affect the antibacterial activity of macrolide antibiotics. Genetic manipulations of the polyketide macrolactone and deoxysugar biosynthetic pathways and these combinations would be useful for the production of novel and effective macrolide antibiotics.

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