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Hybrid Biosynthesis by Targeted Inactivation of Polyketide Synthases in the Mycinamicin Producer, *Micromonospora griseorubida*

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Mycinamicin, composed of a branched lactone and two sugars, desosamine and mycinose, at the C-5 and C-21 positions, is a 16-membered macrolide antibiotic produced by *Micromonospora griseorubida* A11725, which shows strong antimicrobial activities against Gram-positive bacteria. Structural[1~3] and biosynthetic[4,5] studies of mycinamicin have previously been reported. Recently, details of the DNA sequence for mycinamicin biosynthetic genes (*ca.* 65 kb) of the *M. griseorubida* genome, in which there are 22 open reading frames, including the resistance gene *myrB*, have been completely determined.[6]

Mycinamicins have interesting features such as the presence of α,β-unsaturated lactone and a lack of aldehyde moiety in the polyketide 16-membered macrolactone, and have shown higher antibacterial activities than other clinically used 16-membered macrolide antibiotics. The deformylation derivatives of the aldehyde moiety of desmycosin (**6**), 19-deformyl-desmycosin (**8**) and 19-deformyl-4′-deoxydesmycosin, have been synthesized by utilizing Wilkinson's catalyst $(Ph_3P)_3RhC1$.[7] Apart from α,β-unsaturated lactone, these macrolides have very similar structural features and antimicrobial activity *in vitro* to those of mycinamicins. Bioconversion of 19-deformy1-5-*O*-desosaminyl-tylonolide to 19-deformyl-4′-deoxydesmycosin has been performed by the mycinamicin producer, *M. griseorubida*.[8]

The double bond between C-2 and C-3 of mycinamicin is a unique structure in comparison with general 16-macloride antibiotics such as tylosin and leucomycins. The double bond formation is carried out by module 7 including dehydrase (DH) and ketoreductase (KR) domain in polyketide synthase MycAV.[6] We have already obtained a mutant strain *M. griseorubida* M7A21 using the targeted inactivation of *mycAV*.[9] Here we report the characterization of a mutant strain *M. griseorubida* M7A21 by the bioconversion of mycinamicin biosynthetic intermediates and desmycosin derivatives toward combinatorial biosynthetic approaches in *M. griseorubida* A11725.

M. griseorubida M7A21 only lacked productivity of mycinamicin biosynthetic intermediate, protomycinolide IV (**1**), but the functions of deoxysugar biosynthesis by MydA to I, and the glycosylation and other modifications of protomycinolide IV (**1**) by MycB to G remained in this strain. When protomycinolide IV (**1**) was fed into culture plate of the mutant, the productivity of mycinamicin II (**4**) with the bioconversion was the same as that of mycinamicin II (**4**) in wild-type strain.[9] Moreover, in this study, mycinamicin VII (**2**) were efficiently converted to mycinamicin I (**3**) and II (**4**, see Figure 1). HPLC analysis provided clear evidence for the production of mycinamicins and their ratio is shown in Table 1. These results suggested that the bioconversion of macrolide intermediate using *M. griseorubida* M7A21 might be suitable to produce new macrolide antibiotics. Previously, Ōmura's group isolated some of new 16-membered macrolide antibiotics with the hybrid biosynthesis method which utilized to feed protylonolide into culture of several *Streptomyces* strains producing macrolide antibiotics.[10~12]

As shown in Figure 1, 5-*O*-mycaminosyl-tylonolide (**5**) was efficiently converted to desmycosin (**6**). The hybrid compounds obtained from 19-deformyl-5-*O*-mycaminosyl-tylonolide (**7**) with *M. griseorubida* M7A21 was determined to be 19-deformyldesmycosin (**8**) with a further oxidation product similar to that of mycinamicins (**3**, **4**), 19-Deformyl-12,13-epoxydesmycosin (**9**) on the basis of HPLC analysis (Table 1). It is suggested that the M7A21 strain is also able to attach mycinose at C-21 carbon on 5-*O*-mycaminosyltylonolide derivatives, which have structural differences from mycinamicin at the C-2, C-3 and C-12 positions of aglycone. Antibiotic activity of 19-deformyl-12,13-epoxydesmycosin (**9**) against *Micrococcus luteus* was almost the same level as that of compound **8** in paper disk diffusion assay (data not shown).

As the starting material for the biosynthesis of 19-deformyldesmycosin analogues, we prepared 19-deformyl-5-*O*-mycaminosyltylonolide (**7**) by organic synthesis. 19-Deformyldesmycosin (**8**) was derived from intact incorporation of compound **7** by the genetically manipulated mutant strain *M. griseorubida* M7A21. Moreover, compound **9** was also detected in the HPLC chromatography as a minor component, which was

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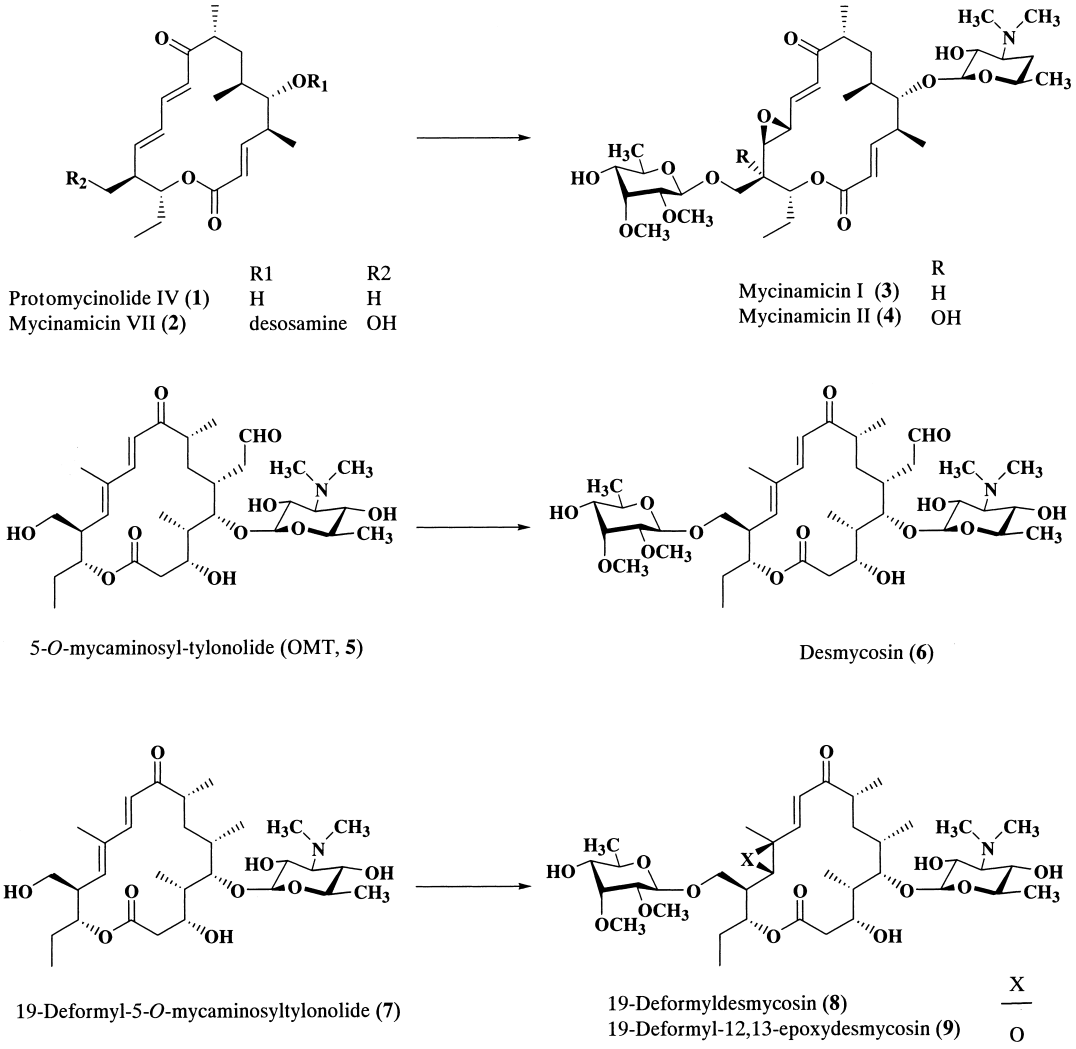
Fig. 1. Bioconversion of mycinamicin biosynthetic intermediates and tylonolide analogues by the engineered strain *M. griseorubida* M7A21.



Protomycinolide IV (**1**) was obtained by fermentation using *M. griseorubida* A11725 wild strain,[2] mycinamicin VII (**4**) was prepared by mild acidic hydrolysis of mycimamicin IV,[3] as described in previous papers. It is well known in the production of desmycosin (**6**) that mild acidic hydrolysis of tylosin causes the removal of mycarose.[15] More vigorous hydrolysis yielded 5-*O*-mycaminosyl-tylonolide (**5**) accompanied with the removal of mycinose.[16] Synthesis of 19-deformyl-desmycosin (**8**) was performed by utilizing Wilkinson's catalyst $(Ph_3P)_3RhCl$.[7]

Table 1. Bioconversion yeild of mycinamicin biosynthetic intermediated and tylonolide analogues by *M. griseorubida* M7A21.

Compound	Feeding (μmol/plate)	Products (μmol/plate)					Total yield (%)
		3	**4**	**6**	**8**	**9**	
Mycinamicin VII (**2**)	0.96	0.13	0.27				42
5-*O*-Mycaminosyl-tylonolide (OMT, **5**)	0.84			0.04			5
19-Deformyl-OMT (**7**)	0.88				0.36	0.03	44

epoxidated between positions 12 and 13 as predicted, but unexpectedly, hydroxyl analogues of 19-deformyldesmycosin at position 14 were not isolated in this bioconversion study.

The hydroxylation of mycinamicin IV at position 14 to mycinamicin V and the epoxidation of mycinamicin IV to mycinamicin II (**4**) are the final steps in the pathway to convert protomycinolide IV (**1**) to mycinamicin II (**4**)[9] which is catalyzed by the cytochrome P450 oxidase MycG.[13] As previously reported, the epoxidation of mycinamicin IV to mycinamicin I (**3**) is a shunt metabolite in the post-PKS mycinamicin biosynthetic pathway, since the hydroxylation reaction at the C-14 position must occur before the epoxidation reaction at the C-12 to C-13 position.[5] Therefore, this suggested that the hydroxylation of 19-deformyl-12,13-epoxydesmycosin (**9**) is difficult to identify from the bioconversion of compound **7** by the engineered strain *M. griseorubida* M7A21. The hydroxylation of compound **8** at carbon C-14 probably occurs due to hindered configuration of the methyl moiety of 19-deformyltylactone at C-12 position in the active site of MycG oxidase.

Experimental

General Precursor Feeding and Extraction Conditions

M. griseorubida M7A21[9] was inoculated into 5 ml of MR0.1S broth in a 15-ml test tube. After incubation for 4 days at 27°C on a reciprocal shaker, 150 μl of seed culture was inoculated into 15-ml MR0.1S medium. Simultaneously, each plate was then overlaid with 1 ml of precursor solution (*ca.* 0.9 μmol) in 30% DMSO/water. After an additional 8 days, each agar was homogenized and extracted with EtOAc (3×30 ml) at 50°C. The organic extract was concentrated to 5 ml *in vacuo*, extracted with 1 ml of 0.2 N HCl. The aqueous layer was added to 100 μl of conc. NH_4OH, extracted with 1 ml of EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Each residue was redissolved in 100 μl of MeOH for HPLC analysis.

Analysis of Fermentation Products and Identification

Fermentation products were analyzed using reverse-phase HPLC, using a 4.6 mm i.d.×150 mm C18 column (ODS-80TM, Toso Co., Japan), and a UV detector at 280, 240 and 220 nm. The flow rate of the mobile phase was 1.0 ml/minute, and the column was operated at room temperature. The mobile phase consisted of acetonitrile/0.1% trifluoroacetic acid, 35 : 65. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-ECA600 600 MHz NMR spectrometer. Chemical shifts are reported in ppm relative to tetramethylsilane, and *J* values are in Hz. A JEOL JMS-T100LC (AccuTOF LC system) mass spectrometer was used to obtain high-resolution mass spectra (reported as *m*/*z*) by the technique of electrospray ionization mass spectrometry (ESI-MS).

Preparation of 19 - Deformyl - 5 - *O* - maycaminosyl - tylonolide (**7**)

5-*O*-mycaminosyl-tylonolide (**5**, 607 mg, 1.0 mmol) and $(Ph_3P)_3RhCl$ (1.03 g, 1.1 mmol) were dissolved in 20 ml of dry benzene and refluxed for 5 hours under an argon atmosphere. The reaction mixture was evaporated and washed with acetone, then the resulting yellow precipitate was filtered off and the filtrate was evaporated *in vacuo*. The residue was dissolved in 5 ml of benzene and extracted with 0.2 N aqueous hydrochloric acid (3×10 ml). The extracts were combined and adjusted to pH 9 by adding conc. ammonia, then extracted with chloroform (3×20 ml). The organic solution was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to afford 19-deformyl-5-*O*-mycaminosyl-tylonolide (**10**, 440 mg) of white powder.[14]

Preparation of 19-Deformyl-12,13-epoxydesmycosin (**9**)

The retention time on HPLC and molecular weight of the bioconversion product **9** was identical with the synthetic compound **9**, which was prepared with the following synthetic method. For structural elucidation and antibiotic activity test, the synthetic compound **9** was used.

To a stirred solution of 100 mg (0.13 mmol, 1.0 eq) of 19-deformyldesmycosin (TMC-014, **7**) in 2 ml of CH_2Cl_2 at room temperature was added 92 mg (77% purity, 0.41 mmol, 3.16 eq) of m-CPBA in 1 ml of CH_2Cl_2 in one portion. The resulting mixture stood at room temperature overnight before stopping with 2 ml of ethanol and 120 mg of sodium hydrosulfite in 1 ml of H_2O at 0°C, and extracting with $CHCl_3$ (3×5 ml). The organic extract was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was subjected to initial purification by SiO_2 column chromatography (*ca.* 1 g of in a Pasteur pipette) with $CHCl_3$/MeOH/conc.NH_4OH (20 : 1 : 0.1). The relevant fraction thus obtained was further purified by analytical TLC plate (6 cm×10 cm; Merck silica gel 60 F_{254}) using $CHCl_3$/MeOH/conc.NH_4OH (10 : 1 : 0.1) as an eluent. The product band was eluted with MeOH. The product **9** was characterized by ^1H (600 MHz) and ^{13}C NMR (150 MHz) spectroscopy (JEOL JNM-ECA600) and mass spectrometry (JEOL JMS-T100LC). The ^1H and ^{13}C NMR spectra of

products were assigned by a combination of ^{1}H-^{1}H COSY, DEPT and ^{1}H-^{13}C HETCOSY spectroscopy: Rf 0.38 ($CHCl_3$/MeOH/conc.NH_4OH=10:1:0.1); ^{1}H-NMR (600 MHz, $CDCl_3$): δ 6.54 (1H, d), 5.32 (1H, td), 4.55 (1H, d), 4.28 (1H, d), 4.14 (1H, dd), 3.79 (1H, d), 3.76 (1H, t), 3.64 (1H, dd), 3.61 (3H, s), 3.56 (1H, d), 3.54 (3H, s), 3.54~3.46 (3H, m), 3.28 (1H, m), 3.20 (1H, dd), 3.14 (1H, d), 3.12~3.07 (2H, m), 2.67 (1H, m), 2.59 (1H, dd), 2.54 (6H, s), 2.43 (1H, m), 2. 07 (1H, d), 1.91 (1H, m), 1.73 (1H, m), 1.70~1.62 (2H, m), 1.56 (1H, m), 1.42 (3H, s), 1.28 (3H, d), 1.26 (3H, d), 1.14 (3H, d), 1.06 (3H, d), 1.04 (3H, d), 0.88 (3H, t); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 201.15 (s), 174.01 (s), 150.27 (d), 123.47 (d), 104.36 (d), 101.01 (d), 93.14 (s), 85.56 (d), 81.98 (d), 79.70 (d), 74.19 (d), 73.25 (d), 72.73 (d), 71.17 (d), 70.84 (d), 70.61 (d), 67.40 (t), 64.18 (d), 61.78 (q), 59.73 (d), 59.52 (q), 45.40 (d), 43.58 (d), 41.81 (q), 39.57 (t), 24.86 (t), 17.97 (q), 17.82 (q), 17.73 (q), 17.34 (q), 15.81 (q), 9.63 (q), 9.28 (q); HR-MS (ESI-TOF, $[M+H]^+$): Calculated for $(C_{38}H_{66}NO_{14})^+$: 760.4483, Found: 760.4490.

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