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 and biosynthetic 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.

Mycinamicins have interesting features such as the presence of α,β-unsaturated lactone and a lack of aldehyde moiety in the polyketide 16-membered macro lactone, 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 (Ph₃P)₃RhCl. 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-deformyl-5-O-desosaminyl-tylonolide to 19-deformyl-4'-deoxydesmycosin has been performed by the mycinamicin producer, *M. griseorubida*. The double bond between C-2 and C-3 of mycinamicin is a unique structure in comparison with general 16-macrolide 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. We have already obtained a mutant strain *M. griseorubida* M7A21 using the targeted inactivation of *mycAV*. 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. 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 tytonolide into culture of several *Streptomyces* strains producing macrolide antibiotics.

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-mycaminosyltylonolide (7) with *M. griseorubida* M7A21 was determined to be 19-deformyl-desmycosin (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-deformyl-desmycosin analogues, we prepared 19-deformyl-5-O-mycaminosyltylonolide (7) by organic synthesis. 19-Deformyl-desmycosin (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
Fig. 1. Bioconversion of mycinamicin biosynthetic intermediates and tylonolide analogues by the engineered strain *M. griseorubida* M7A21.

Table 1. Bioconversion yield of mycinamicin biosynthetic intermediates and tylonolide analogues by *M. griseorubida* M7A21.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Feeding (µmol/plate)</th>
<th>Products (µmol/plate)</th>
<th>Total yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycinamicin VII (2)</td>
<td>0.96</td>
<td>0.13 0.27</td>
<td>42</td>
</tr>
<tr>
<td>5-O-Mycaminosyl-tylonolide (OMT, 5)</td>
<td>0.84</td>
<td>0.04</td>
<td>5</td>
</tr>
<tr>
<td>19-Deformyl-OMT (7)</td>
<td>0.88</td>
<td>0.36 0.03</td>
<td>44</td>
</tr>
</tbody>
</table>
epoxidated between positions 12 and 13 as predicted, but unexpectedly, hydroxyl analogues of 19-deformyl-desmycosin 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 M7A219) was inoculated into 5ml of MR0.1S broth in a 15-ml test tube. After incubation for 4 days at 27°C on a reciprocal shaker, 150μl of seed culture was inoculated into 15-ml MR0.1S medium. Simultaneously, each plate was then overlaid with 1ml of precursor solution (ca. 0.9μmol) in 30% DMSO/water. After an additional 8 days, each agar was homogenized and extracted with EtOAc(3~30ml) at 50°C. The organic extract was concentrated to 5ml in vacuo, extracted with 1ml of 0.2N HCl. The aqueous layer was added to 100μl of conc. NH₄OH, extracted with 1ml of EtOAc. The organic layer was dried over anhydrous Na₂SO₄ and concentrated in vacuo. Each residue was redissolved in 100μl of MeOH for HPLC analysis.

Analysis of Fermentation Products and Identification

Fermentation products were analyzed using reverse-phase HPLC, using a 4.6mm i.d.×150mm C18 column (ODS-80TM, Tosco Co., Japan), and a UV detector at 280, 240 and 220nm. The flow rate of the mobile phase was 1.0ml/minute, and the column was operated at room temperature. The mobile phase consisted of acetonitrile/0.1% trifluoroacetic acid, 35 : 65. ¹H and ¹³C 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-mycaminosyl-tylonolide (7)

5-O-mycaminosyl-tylonolide (5, 607mg, 1.0mmol) and (Ph₃P)₃RhCl (1.03g, 1.1mmol) were dissolved in 20ml 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 5ml of benzene and extracted with 0.2 N aqueous hydrochloric acid (3×10ml). The extracts were combined and adjusted to pH 9 by adding conc. ammonia, then extracted with chloroform (3×20ml). The organic solution was dried over anhydrous Na₂SO₄ and concentrated in vacuo to afford 19-deformyl-5-O-mycaminosyl-tylonolide (10, 440mg) 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 100mg (0.13mmol, 1.0 eq) of 19-deformyl-desmycosin (TMC-014, 7) in 2ml of CH₂Cl₂ at room temperature was added 92mg (77% purity, 0.41mmol, 3.16eq) of m-CPBA in 1ml of CH₂Cl₂ in one portion. The resulting mixture stood at room temperature overnight before stopping with 2ml of ethanol and 120mg of sodium hydrosulfite in 1ml of H₂O at 0°C, and extracting with CHCl₃ (3×5ml). The organic extract was dried over anhydrous Na₂SO₄ and concentrated in vacuo. The residue was subjected to initial purification by SiO₂ column chromatography (ca. 1g of in a Pasteur pipette) with CHCl₃/MeOH/conc.NH₄OH (20 : 1 : 0.1). The relevant fraction thus obtained was further purified by analytical TLC plate (6cm×10cm; Merck silica gel 60 F₂₅₄) using CHCl₃/MeOH/conc.NH₄OH (10 : 1 : 0.1) as an eluent. The product band was eluted with MeOH. The product 9 was characterized by ¹H (600 MHz) and ¹³C NMR (150 MHz) spectroscopy (JEOL JNM-ECA600) and mass spectrometry (JEOL JMS-T100LC). The ¹H and ¹³C NMR spectra of
products were assigned by a combination of $^1$H-$^1$H COSY, DEPT and $^1$H-$^{13}$C HETCOR spectroscopy: Rf 0.38 (CHCl$_3$/MeOH/conc.NH$_4$OH = 10:1:0.1); $^1$H-NMR (600 MHz, CDCl$_3$): $\delta$ 6.54 (1H, d), 5.32 (1H, td), 4.55 (1H, d), 4.28 (1H, d), 3.79 (1H, d), 3.76 (1H, t), 3.64 (1H, dd), 3.61 (3H, s), 3.56 (1H, d), 3.54 (1H, 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$_3$): $\delta$ 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.

References


