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Biotransformation of Rosamicin Antibiotic into 10,11-Dihydrorosamicin with Enhanced *In Vitro* Antibacterial Activity Against MRSA

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A biotransformation approach using microbes as biocatalysts can be an efficient tool for the targeted modification of existing antibiotic chemical scaffolds to create previously uncharacterized therapeutic agents. By employing a recombinant *Streptomyces venezuelae* strain as a microbial catalyst, a reduced macrolide, 10,11-dihydrorosamicin, was created from rosamicin macrolide. Its chemical structure was spectroscopically elucidated, and the new rosamicin analog showed 2–4-fold higher antibacterial activity against two strains of methicillin-resistant *Staphylococcus aureus* compared with its parent rosamicin. This kind of biocatalytic approach is able to expand existing antibiotic entities and can also provide more diverse therapeutic resources.

Keywords: Enhanced anti-MRSA activity, microbial catalyst, reduced macrolide antibiotic, *Streptomyces venezuelae*, 10,11-dihydrorosamicin

The emergence and rapid spread of antibiotic-resistant pathogens has rekindled interest in the discovery of new antibiotics. A semisynthetic or mutational biosynthetic approach using microbes as biocatalysts can be an efficient tool for the targeted modification of existing antibiotic chemical scaffolds to create the next generation of antibiotics, as well as previously uncharacterized therapeutic agents [4]. The macrolide antibiotics are a group of polyketides, whose activity is derived from the presence of a large macrocyclic lactone ring. They are a structurally diverse class of natural products that show a wide variety of bioactive properties [12]. Rosamicin, which was first isolated from a culture of the soil bacterium *Micromonospora rosaria* [13], is a 16-membered macrolide antibiotic (Fig. 1). It displays fair and broad-spectrum antibacterial properties, although it is more potent against gram-positive pathogens [3]. There have been some reports regarding the microbial biotransformation of this antibiotic [1, 2, 5, 6, 11]. Supplementation of the chemically de-epoxidized aglycone of rosamicin into a randomly mutated strain of mycinamicin-producing *Micromonospora polytrota* provided new derivatives of rosamicin, such as mycinosyl deoxysugar-attached rosamicins [6]. Recent studies have reported that the above mycinosyl rosamicin derivative could be produced by a genetically engineered recombinant of *M. rosaria* in which the gene set responsible for the biosynthesis and attachment of mycinose was introduced [1, 2, 5]. In addition, structural modification of a biosynthetic precursor of rosamicin, M-4365 G2, using various macrolide-producing microorganisms has been reported, with attachment of the acetyl group at the C3 hydroxyl position and reduction of the carbonyl function at the C9 position [11]. However, none of the above-described rosamicin analogs exhibited higher antibacterial activity than the parent rosamicin. On the other hand, chemical hydrogenation of unsaturated macrolides could be done by employing both a metal catalyst such as rhodium chloride or palladium on carbon and hydrogen gas, but this semisynthetic approach to the reduction of unsaturated macrolides appears to be still time- and energy-consuming, as well as nonspecific for the target double bond [14].

We recently reported the unique and region-specific hydrogenation activity towards a series of macrolides (12-, 14-, 16-, and 26-membered) of a recombinant strain of

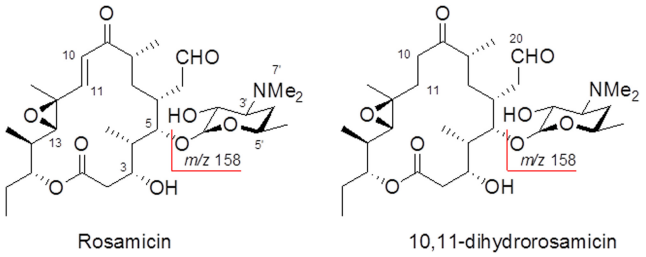

Fig. 1. Rosamicin and its bioconverted analog 10,11-dihydrorosamicin.

Typical product ion produced by electrospray ionization-tandem mass spectroscopy analyses of each macrolide is shown at *m/z* 158, representing D-desosamine moiety equality.

Streptomyces venezuelae and its application to the generation of unnatural macrolides [7, 8]. In addition, by employing this strain as a microbial catalyst, the novel reduced acyclic polyketide 2,3-dihydrotrichostatin A was created from trichostatin A [9]. The microbial hydrogenation system could recognize the specific structural elements around the target double bond; that is, a carbonyl functional group on the neighboring carbon, and a methyl group on another adjoining carbon in the polyketide backbone.

In this study, an attempt was made to expand the applicability of this unique biohydrogenation activity of *S. venezuelae* towards other unsaturated macrolides such as rosamicin antibiotic, in which the above-described structural elements are well conserved. The structure of reduced rosamicin, namely 10,11-dihydrorosamicin, was elucidated by HPLC-ESI-MS/MS and NMR analyses, and its antibiotic potential was evaluated *in vitro* against two strains of methicillin-resistant *Staphylococcus aureus* (MRSA).

The recombinant strain *S. venezuelae* YJ028 [7], in which the pikromycin polyketide synthase-encoding gene and desosamine biosynthetic genes had been deleted, was provided with rosamicin (Sigma; 5 μg/ml in MeOH as the final concentration). The recombinant strain was cultivated with this non-native macrolide in 50 ml of SCM medium (1.5% soluble starch, 2.0% soytone, 0.01% $CaCl_2$, 0.15% yeast extract, and 1.0% MOPS) in baffled Erlenmeyer flasks at 30°C for 2 days, followed by further cultivation for 3 days, leading to an approximate 22% conversion of rosamicin to the corresponding reduced rosamicin analog (Fig. 2). After organic solvent partition using EtOAc, analyses of rosamicin and its bioconverted analog were performed on a reversed-phase XTerra MS C_{18} column (Waters, Milford, MA, USA; 50 × 2.1 mm, 3.5 μm) interfaced with a Finnigan LXQ ion-trap MS (Thermo Finnigan, San Jose, CA, USA) tracing by MS/MS, using an isocratic

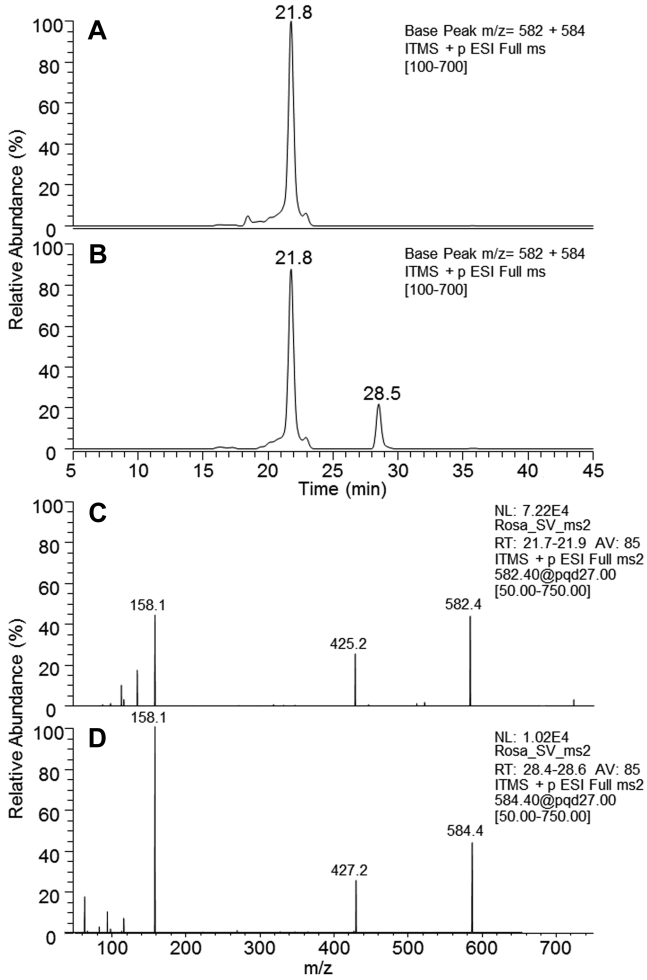

Fig. 2. Representative chromatograms and HPLC-ESI-MS/MS spectra.

(**A**) Extracts of blank medium fed with rosamicin and (**B**) extract obtained from *S. venezuelae* fermentation fed with rosamicin; (**C**) MS/MS fragmentation pattern of rosamicin peak in the trace of (**B**); and (**D**) the MS/MS fragmentation pattern of the bioconverted rosamicin analog in the trace of (**B**).

elution of 90% aqueous MeOH with 0.1% formic acid. In previous *in vivo* studies using pikromycin as a native substrate of *S. venezuelae* [7], the conversion rate into its dihydro form was approximately 85%. In an attempt to increase the rosamicin conversion yield, we extended the cultivation time after feeding rosamicin up to a week. However, no further increased conversion was observed after day 3, as in the case of pikromycin. We also investigated using a cell-free extract made from the recombinant strain using the same procedure described previously, but there was no significant increase in the conversion yields, suggesting that the biocatalytic activity

of *S. venezuelae* towards the non-native unsaturated macrolide is limited compared with its natural substrate. The organic extracts obtained from several batch cultures (450 ml) of strain YJ028 fed with exogenous rosamicin (total 2.25 mg) were pooled. The bioconverted rosamicin analog was purified by chromatographic isolation, using a preparative reversed-phase Watchers 120 ODS-BP (Daiso Watchers, Japan; 250 × 10.0 mm, 5.0 μm) with subsequent ESI-MS analyses of each fraction (5 ml/fraction) collected. Bioconverted rosamicin analog was obtained as a white powder (0.38 mg; production yield 17%). The MS/MS spectrum of rosamicin at *m/z* 582, representing its protonated molecular ion, indicated two main fragment ions at *m/z* 158 and 425, which arise from the cleavage of a desosamine moiety attached at the C5 position. The MS/MS spectrum of the rosamicin analog demonstrated a main fragment ion at *m/z* 158 common to rosamicin. However, there was a characteristic product ion at *m/z* 427 (Fig. 2), which is different by 2 Da from a fragment ion at *m/z* 425 typical to rosamicin, implying the disappearance of a double bond in the polyketide scaffold.

The chemical structure of the biotransformed analog was further confirmed by ^{1}H- and ^{13}C-NMR spectroscopies by comparing its chemical shifts with those of rosamicin. NMR samples were prepared by dissolving in 200 μl of $CDCl_3$, and placing the solution in a 5 mm Shigemi advanced NMR microtube (Sigma, St. Louis, USA) matched to the solvent. The ^{1}H and ^{13}C NMR spectra were acquired using a Varian INOVA 500 spectrometer at 298 K, with chemical shifts reported in ppm using TMS as an internal reference. All NMR data processing was performed using Mnova Suite 5.3.2 software.

Rosamicin; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.96 (t, 17-H), 1.02 (d, 23-H), 1.06 (d, 18-H), 1.20 (m, 7-H), 1.21 (d, 6′-H), 1.23 (d, 21-H), 1.40 (s, 22-H), 1.42 (m, 16-H), 1.44 (m, 7-H), 1.50 (m, 4′-H), 1.55 (m, 16-H), 1.75 (m, 4′-H), 2.01 (m, 6-H), 2.05 (m, 4-H), 2.23 (m, 19-H), 2.26 (d, 2-H), 2.28 (s, 7′-NCH_3), 2.48 (m, 19-H), 2.50 (d, 13-H), 2.52 (m, 8-H), 2.53 (dd, 2-H), 2.56 (m, 14-H), 2.80 (m, 5-H), 2.82 (m, 3′-H), 3.77 (m, 5′-H), 3.84 (br d, 3-H), 3.88 (dd, 2′-H), 3.93 (dd, 15-H), 5.03 (d, 1′-H), 6.31 (dd, 10-H), 7.03 (dd, 11-H), 9.71 (s, 20-CHO); ^{13}C NMR (125 MHz, $CDCl_3$): δ 202.4, 201.2, 171.2, 143.4, 124.1, 104.1, 81.9, 78.4, 73.4, 70.1, 67.3, 66.4, 65.7, 56.9, 45.3, 44.1, 43.2, 43.1, 40.3, 37.3, 32.9, 32.5, 31.3, 24.3, 22.8, 22.3, 15.6, 11.8, 8.9, 8.2.

10,11-dihydrorosamicin; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.94 (t, 17-H), 1.04 (d, 18-H), 1.06 (d, 23-H), 1.16 (d, 21-H), 1.20 (d, 6′-H), 1.31 (s, 22-H), 1.37 (ddd, 11-H), 1.43 (m, 16-H), 1.49 (m, 4′-H), 1.53 (m, 7-H), 1.57 (m, 16-H), 1.62 (m, 11-H), 1.72 (m, 4′-H), 1.78 (m, 7-H), 2.02 (m, 6-H), 2.06 (m, 4-H), 2.23 (m, 19-H), 2.25 (d, 2-H), 2.28 (s, 7′-NCH_3), 2.43 (ddd, 10-H), 2.45 (d, 13-H), 2.47 (t, 10-H), 2.49 (m, 19-H), 2.51 (dd, 2-H), 2.52 (m, 8-H), 2.55 (m, 14-H), 2.81 (d, 5-H), 2.83 (m, 3′-H), 3.74 (m, 5′-H), 3.83 (br d, 3-H), 3.89 (dd, 2′-H), 3.94 (dd, 15-H), 5.02 (d, 1′-H), 9.72 (s, 20-CHO); ^{13}C NMR (125 MHz, $CDCl_3$): δ 211.1, 202.1, 171.3, 104.0, 81.7, 78.2, 73.8, 73.5, 70.1, 66.7, 65.3, 57.5, 45.4, 44.4, 44.0, 43.4, 43.0, 37.2, 32.9, 32.6, 31.9, 31.3, 31.1, 24.3, 22.7, 22.5, 15.4, 11.8, 8.8, 8.3.

The most obvious difference in the ^{1}H-NMR spectra of rosamicin and its reduced analog was the absence of signals at 6.31 and 7.03 ppm, which are typical of olefinic protons (H-10,11) in rosamicin. The upfield shift of the C-10 and C-11 signals of the parent compound (from 124.1 and 143.4 ppm to 31.9 and 31.3 ppm, respectively) also corroborated the distinction found in the above ^{1}H-NMR data, confirming that rosamicin was reduced at the C-10,11 double bond. These results show that the unique bio-hydrogenation of *S. venezuelae* is able to regiospecifically reduce the diverse macrolide polyketide rings.

The antimicrobial activities of rosamicin and 10,11-dihydrorosamicin against gram-positive MRSA were determined using the microdilution method [10]. Two type strains of MRSA, *Staph. aureus* ATCC 33592 and 43300, were obtained from the American Type Culture Collection (Manassas, USA). The growth of the test strains in Mueller-Hinton broth was monitored at 600 nm using a Labsystems Bioscreen C reader (Labsystems Corp., Finland), and the minimum inhibitory concentration (MIC) was determined to be the lowest concentration of the macrolides diluted in a broth medium that inhibited the growth of the test microorganisms. Reduced macrolide 10,11-dihydrorosamicin showed a 2-fold to 4-fold increase (MIC 8 and 128 μg/ml, respectively) in antibacterial activity against the two strains of MRSA compared with the parent rosamicin (MIC 32 and 256 μg/ml, respectively). This result shows that the lack of

Table 1. Comparison of MIC (μg/ml) data for rosamicin and its analog, 10,11-dihydrorosamicin, against two strains of MRSA.

MRSA strain	Rosamicin	10,11-Dihydrorosamicin
Staphylococcus aureus ATCC 33592	32	8
Staphylococcus aureus ATCC 43300	256	128

a C10,11-double bond in rosamicin had no adverse effect on antibiotic activity, but instead, enhanced the antibacterial effect on MRSA (Table 1).

A novel preparation of rosamicin analog is thus reported *via* microbial biotransformation, rather than extant total or semisynthetic procedures. The regiospecific biohydrogenation activity of *S. venezueale* was found to reduce unsaturated macrolide rings with specific structural elements around the target double bond, demonstrating the expandability of the biohydrogenation activity of *S. venezuelae* to develop more diverse therapeutic resources with the possibility of improved therapeutic effects.

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