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NOTE

Generation of reduced macrolide analogs by regio-specific biotransformation

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The emergence of antibiotic-resistant pathogens has rekindled interest toward the discovery of new antibiotics. Semi-synthetic or biosynthetic approach using microbe as a biocatalyst 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.[1] 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, including anticancer, antifungal, immuno-suppressive and anti-aging activity.[2] Oligomycin A, which was first isolated from a culture of soil bacterium Streptomyces diastatochromogenes in 1954, is a major component of oligomycins as a series of 26-membered macrocyclic lactones (Figure 1).[3] This antibiotic has also been reported to possess some potential antitumor activity.[4] Tilmicosin, a semi-synthetic tylosin that occurs as a 16-membered macrolide in the fermentation of S. fradiae, has use as a veterinary antibiotic (Figure 1).[5] We recently reported the unique and regio-specific hydrogenation activity toward unsaturated 12- (methymycin) and 14-membered (pikromycin) macrolides by S. venezuelae, and its application to the generation of unnatural 16-membered macrolides.[6] This bacterial reduction 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 onto another adjoining carbon in the polyketide backbone (depicted as faded rectangles in Figure 1).

In this study, an attempt was made to expand the applicability of the unique bio-hydrogenation activity of S. venezuelae toward other unsaturated macrolide antibiotics (natural oligomycin A and semi-synthetic tilmicosin) that possess the above mentioned identical catalytic scaffold, and the structure and antibiotic potential of two previously uncharacterized macrolides, 2,3-dihydro-oligomycin A (1) and 10,11-dihydro-tilmicosin (2), isolated from a S. venezuelae culture

supplemented with oligomycin A and tilmicosin, respectively, were evaluated.

A recombinant strain of S. venezuelae YJ028,[6] which had both the pikromycin polyketide synthase-encoding gene and desosamine biosynthetic genes deleted, was provided with oligomycin A or tilmicosin. The organism was cultivated in SCM media (1.5% soluble starch, 2.0% soytone, 0.01% $CaCl_2$, 0.15% yeast extract, and 1.0% MOPS) in baffled Erlenmeyer flasks. After 2 days growth at 30 °C, the cultures were supplemented with either macrolides oligomycin A (Sigma, St Louis, MO, USA) and tilmicosin (Sigma) at a final concentration of 5 µg ml^{-1}, and then incubated for additional 3 days. The whole cultures were extracted and partitioned using EtOAc, and then the organic extracts were concentrated under vacuum. The dried residues were dissolved in 200 µl of MeOH and a portion of the solvent was subjected to HPLC-ESI-MS (Waters, Milford, MA, USA) analysis.[6] Consequently, supplementation of each non-native macrolide led to ∼67 and 30% conversion yields for the corresponding reduced macrolides 1 and 2, respectively (see Supplementary Figure S1). To isolate and structurally characterize both converted macrolides 1 and 2, crude extracts containing 1 were prepared from 150 ml (three batch cultivations) of the fermentation broth from YJ028 strain fed with oligomycin A (total 0.75 mg), whereas crude extracts containing 2 were from 250 ml (five batches) culture broth from the same strain supplemented with tilmicosin (1.25 mg). These new macolides in the crude extracts were purified by chromatographic isolation using a preparative reversed-phase HPLC on a Watchers 120 ODS-BP (250×10.0 mm, 5.0 µm, Daiso, Osaka, Japan)[6] and subsequent ESI-MS analyses of each fraction (5 ml per fraction) collected. Compound 1 was obtained as a white powder (0.41 mg), which analyzed for the molecular formula $C_{45}H_{76}O_{11}$ by the HR-ESI-MS ([M+H]$^+$ m/z 793.5409, calcd. 793.5421) using LCT-premier XE mass spectrometer (Waters; see Supplementary Figure S2). The MS/MS spectrum of 1 at

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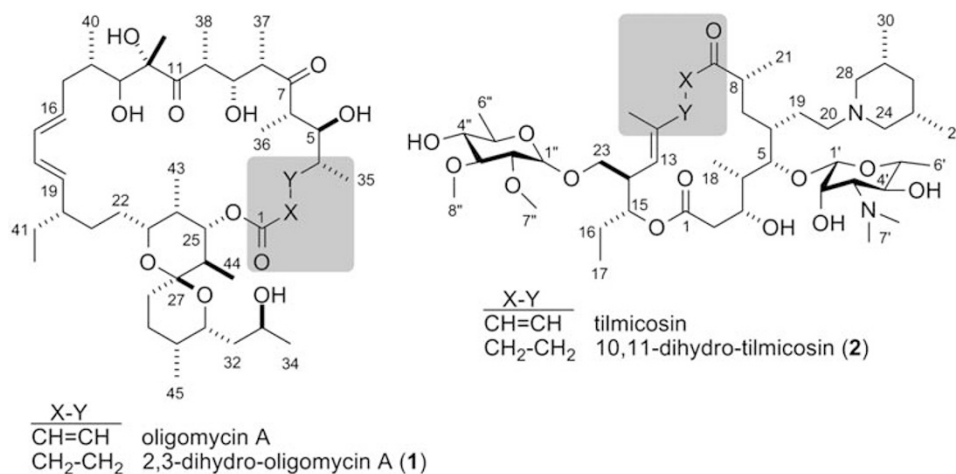E-mail: joonyoon@ewha.ac.kr

Figure 1 Structure of the macrolides. The faded boxes show the regio-specific functions.

m/z 793, which represents its protonated molecular ion, showed the main fragment ion at *m/z* 447, that is identical to that of oligomycin A,[7] suggesting that the structural modification is likely to occur in the polyketide chain spanning from C1 to C12 position (see Supplementary Figure S3). On the other hand, HR-ESI-MS of **2** (light yellow powder; 0.30 mg) gave an *m/z* of 871.5835 as $[M+H]^+$, which corresponds to the calculated molecular formula for $C_{46}H_{82}N_2O_{13}$ (871.5850; see Supplementary Figure S2). The MS/MS spectrum of tilmicosin at *m/z* 869 indicated two fragment ions at *m/z* 174 and 696, which arise from the cleavage of a mycaminose moiety attached at C5 position, whereas MS/MS spectrum of **2** showed a similar fragment at *m/z* 174 as in case of tilmicosin, but the characteristic product ion at m/z 698 that is different by 2 Da from a fragment ion at *m/z* 696 typical to tilmocosin,[8] implying the disappearance of a double bond in the polyketide scaffold (see Supplementary Figure S3). Their chemical structures were further elucidated by ^{1}H- and ^{13}C-NMR spectroscopic analyses. The NMR samples were prepared by dissolving each macrolide in 200 µl of CDCl₃ in a 5 mm Shigemi advanced NMR microtube (Sigma) matched to the solvent. Their ^{1}H- and ^{13}C-NMR spectra were acquired using a Varian INOVA 500 NMR spectrometer (Varian, Inc., Palo Alto, CA, USA) at 298 K. The chemical shifts of each converted macrolide were assigned by a comparison with those of the parent macrolides oligomycin A and tilmicosin. Compounds **1** and **2** were identified as 2,3-dihydro-oligomycin A and 10,11-dihydro-tilmicosin, respectively (see Supplementary Tables S1 and S2).

The most obvious change observed by a comparison of the ^{1}H-NMR spectroscopic data of oligomycin A and its reduced product **1** was the absence of signals at 5.83 and 6.88 p.p.m., which are typical for olefinic protons (H-2,3) in oligomycin A. The upfield shift of the C-2 and C-3 signals of the parent compound (from 121.5 and 147.6 p.p.m. to 30.6 and 25.3 p.p.m., respectively) also support the distinction found in the above ^{1}H-NMR data, demonstrating that **1** is a reduced form of oligomycin A with an absent C-2,3 double bond (see Supplementary Table S1, Figures S4 and S6). Similarly, a noticeable difference between tilmicosin and its reduced form **2** was observed at the C-10,11 position. Indeed, the findings of both the absence of a signal downfield of 6.00 p.p.m. in the ^{1}H-NMR spectroscopic data of **2**, and the upfield shift of signals designating C-10 and C-11 positions in tilmicosin, indicated **2** to also be a reduced form of semi-synthetic tilmicosin (see Supplementary Table S2, Figures S5 and S7). These results show that the reduced macrolides **1** and **2** are transformed by

Table 1 Comparison of the MIC data for compounds 1, 2, and the corresponding oligomycin A and tilmicosin

	Oligomycin A	1	Tilmicosin	2
MIC (µg ml⁻¹)ᵃ	25.0	12.5	1.56	1.56

Abbreviations: **1**, 2,3-dihydro-oligomycin A; **2**, 10,11-dihydro-tilmicosin.
ᵃOligomycin A and **1** were tested against *Saccharomyces cerevisiae*, whereas tilmicosin and **2** against *Bacillus subtilis*.

S. venezuelae, which contains regio-selective activity for the bio-hydrogenation[6] of diverse ring macrolides. A recent study reported the chemical modification of oligomycin A, yielding pyrazolo (1, 5-a) pyridine-conjugated oligomycin A.[9] However, there are no reports on the generation of oligomycin analogs by biotransformation.

As each macrolide oligomycin A and tilmicosin was initially discovered as an antifungal and antibacterial (especially against Gram-positive bacteria) antibiotic, respectively,[3,5] the antimicrobial activities of compounds **1** and **2** against *Saccharomyces cerevisiae* and *Bacillus subtilis*, respectively, were determined using the micro-dilution method recommended by the Clinical and Laboratory Standard Institute (formerly National Committee for Clinical Laboratory Standards).[10] Briefly, *S. cerevisiae* ATCC 9763 and *B. subtilis* ATCC 23857 were grown in Antibiotic 19 (Difco, BD Biosciences, San Jose, CA, USA) and Mueller–Hilton broth (Difco), respectively. Oligomycin A and its corresponding **1** were tested against *S. cerevisiae*, whereas tilmicosin and **2** was tested against *B. subtilis*. The growth of test strains was monitored at 600 nm using a Labsystems Bioscreen C reader (Labsystems Corp., Helsinki, Finland), and the 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 **1** showed a twofold increase in activity against *S. cerevisiae* (MIC ∼ 12.5 µg ml⁻¹) compared with oligomycin A (MIC ∼ 25.0), and compound **2** was as active as tilmicosin (Table 1). Therefore, the lack of these double bonds in both macrolides has no adverse effects on their antibiotic activities. Evidently, this is the first report on the microbe-derived biotransformation of oligomycin A and tilmicosin, even though the antibacterial activity of the reduced macrolide **2** was not improved. In our previous works, we determined that the hydrogenation activity exerted by *S. venezuelae* is not common among the representative streptomycetes, and also found

that this unique bio-hydrogenation function is independent of reductive enzymatic domains (that is, enoyl reductase) in the modular-type polyketide synthase system including the innate pikromycin polyketide synthase.[6] Recent discoveries of *trans*-acting enoyl reductase function in several microorganisms could be a clue to identify this unusual reductive enzyme in *S. venezuelae*.[11–13] In summary, diverse macrolide scaffolds, which include 26-membered ring macrolide as well as 12-, 14-, and 16-membered ones,[6] could be produced by exploiting nature's chemical diversity, demonstrating the expandability of the bio-hydrogenation activity of *S. venezuelae* to develop more diverse therapeutic resources. An attempt to expand the applicability of this unique biocatalyst toward acyclic polyketides, in which the described catalytic scaffolds are well conserved, is currently in progress.

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