Exploiting the natural metabolic diversity of *Streptomyces venezuelae* to generate unusual reduced macrolides†

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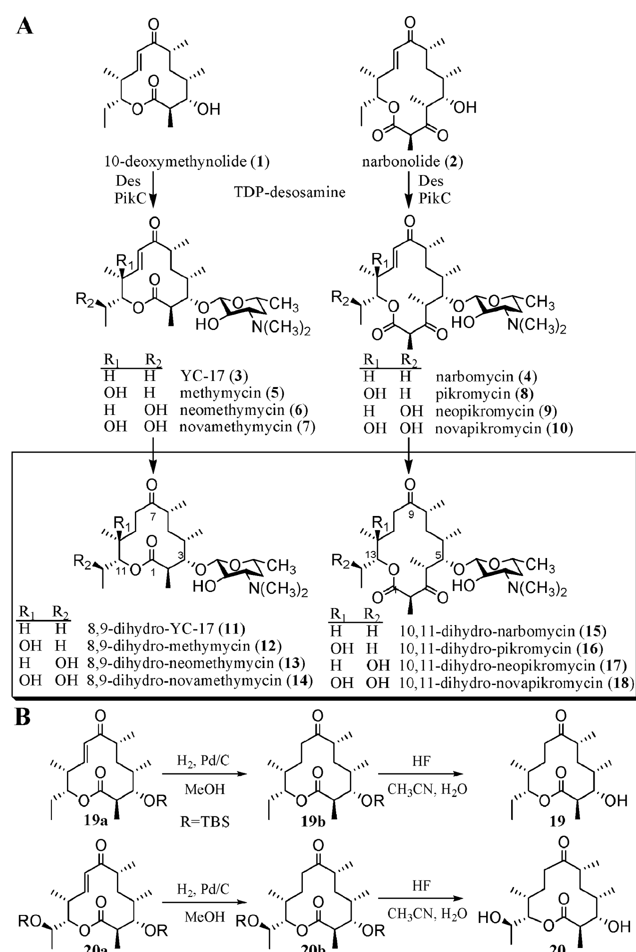
An unusual set of reduced macrolide antibiotics was discovered by combination of organic synthesis and a biosynthetic approach using the unique metabolic diversity of *Streptomyces venezuelae*; two unnatural 16-membered ring macrolides are also created by employing this bio-catalyst.

Even though natural products from microbes have historically been an important source of therapeutic agents, there has been a slow decline in pharmaceutical research for such natural products. Indeed, it has been reported that the majority of the natural products have not been tested for biological activity,[1] therefore an effective way to expand the impact of natural chemical diversity on drug discovery would be to evaluate the minor components. To exploit nature's diversity extensively, the minor components which have not been structurally and biologically characterized must be identified and then prepared in quantities that allow their characterization. Furthermore, the generation of unnatural natural products by mimicking natural diversity is an effective tool for expanding the currently available natural product libraries.

In the soil bacterium *Streptomyces venezuelae* ATCC 15439, the pikromycin (*pik*) modular polyketide synthase (PKS) system catalyzes the biosynthesis of two macrolactones with different ring sizes; a 12-membered ring known as 10-deoxymethynolide (**1**) and a 14-membered ring narbonolide (**2**).[2] These two aglycones are then further modified into a structurally diverse series of macrolides *via* action of post-PKS tailoring enzymes, desosaminyl glycosyltransferase (DesVII) and cytochrome P450 hydroxylase (PikC)[3] (Scheme 1A). Of these macrolides, the predominant metabolites are pikromycin (**8**) and (neo)methymycin (**5**, **6**). Recent studies conducted to identify additionally hydroxylated novamethymycin (**7**) as well as neo- and nova-pikromycin (**9**, **10**) produced by the wild-type *S. venezuelae* have prompted us to utilize this biocatalyst for the discovery and generation of structural variations of macrolides.[4]

Large-scale fermentation of *S. venezuelae* ATCC 15439 and analysis using HPLC-ESI-MS/MS[4b] revealed a peak that likely corresponded to the known reduced pikromycin derivative, 10,11-dihydropikromycin (**16**),[5] as well as seven different trace peaks that were suspected to be the reduced macrolide analogues corresponding to YC-17 (**3**), and narbomycin (**4**), **5–7**, **9** and **10** (ESI†). The MS/MS spectra of these compounds clearly indicated the presence of a desosaminyl moiety at *m*/*z* 158.1 and fragmentation patterns of the analogues appeared to be very similar to those obtained from known unreduced macrolides, differing by only two mass units. Furthermore, none of the identified analogues were capable



Scheme 1 (A) Structures and proposed biosynthetic pathway of reduced macrolides produced by *S. venezuelae* ATCC15439. (B) Synthesis of unnatural macrolactones **19** and **20**.

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of absorbing UV, which indicates that the double bond in their PK backbones had been reduced. The newly detected macrolides were prepared in amounts large enough to elucidate their structures using the following strategies. First, both natural macrolactone (**2**)[6] and unnatural reduced 8,9-dihydro-10-deoxymethynolide (**19**) and 8,9-dihydroneomethynolide (**20**) were synthesized. These unnatural macrolactones **19** and **20** were made from TBS-protected **19a** and **20a**, respectively (Scheme 1B, ESI†). Next, an engineered strain of *S. venezuelae* DHS2001, that had the entire *pik* PKS gene deleted but still contained the genes responsible for post-PKS tailoring,[7] was provided with **2**, **19** or **20**. Supplementation of the native macrolactone **2**, into *S. venezuelae* DHS2001 cultivated in SCM media at 30 °C for 2 d, followed by further cultivation for 3 d led to a conversion of <20% of the macrolactone into the reduced 14-membered macrolides. However, the addition of reduced forms of macrolactone, **19** or **20**, into the *S. venezuelae* DHS2001 culture resulted in >85% conversion of the aglycones into the corresponding 12-membered reduced macrolides of interest. Feeding the above macrolactones into several batch cultures of DHS2001 strain resulted in the generation of seven novel reduced macrolides (**11–15**, **17** and **18**) in quantities sufficient to allow structure elucidation. These macrolides were purified using preparative reversed-phase HPLC and subsequent ESI-MS analyses of each fraction collected, and identified as the following 12-membered rings: 8,9-dihydro-YC-17 (**11**), 8,9-dihydromethymycin (**12**), 8,9-dihydroneomethymycin (**13**), and 8,9-dihydronovamethymycin (**14**); and 14-membered rings: 10,11-dihydronarbomycin (**15**), 10,11-dihydroneopikromycin (**17**), and 10,11-dihydronovapikromycin (**18**). Their chemical structures were then further elucidated by NMR analysis. The 12-membered ring macrolides were found to have H-2 at around 2.8 ppm as a quartet of doublets and methyl group on the nitrogen atom as a singlet at 2.2–2.3 ppm, which indicated the presence of macrolactone aglycone and desosamine sugar moieties, respectively. The 14-membered ring macrolides also possessed characteristic similar signals at around 3.3 ppm as a quartet and at 2.2–2.3 ppm as a singlet. However, major differences between the known macrolides and their new reduced forms were observed at the C-8,9 and C-10,11 positions for the 12- and 14-membered ring macrolides, respectively. Furthermore, no signals downfield of 6.0 ppm, which are typical for olefinic protons (H-8,9 and H-10,11) in the known 12- and 14-membered macrolides were observed. This lack of downfield signals clearly demonstrated that none of the novel macrolides identified here contained any double bonds, indicating that they were reduced. Bioconversion of synthetic reduced macrolactones **19** and **20** into the corresponding macrolides demonstrated that the native post-PKS enzymes DesVII and PikC in *S. venezuelae* are still flexible to these structurally altered saturated substrates To determine if the unusual catalytic activity on these macrolides is only occurred in *S. venezuelae*, the following five species of *Streptomyces* were incubated with **8**; three macrolide producers including avermectin-producing *S. avermitilis*, rapamycin-producing *S. hygroscopicus* and tylosin-producing *S. fradiae*, as well as the representative streptomycetes *S. coelicolor* and *S. lividans*.[8] However, no conversion of the supplemented **8** into **16** was detected in their cultures, which indicates that the hydrogenation activity exerted by *S. venezuelae* is not ubiquitous among the species. In addition, conversion tests using **8** as a precursor were conducted in differently engineered strains of *S. venezuelae* including YJ003, in which the desosamine biosynthetic genes (*des*) in the *pik* gene cluster have been deleted, and YJ028, in which both the *pik* PKS and the *des* genes have been deleted.[9] **16** was detected in both cultures, indicating that this reducing system operates independently of the *pik* gene cluster (ESI†).

The production level of each reduced macrolide generated in the culture appeared to be proportional to those of the corresponding unreduced forms, which suggests that this reducing system could recognize both 12- and 14-membered macrolides equivalently. Furthermore, the accumulation of a series of reduced 12-membered macrolides in the DHS2001 culture that was supplemented with **19** and **20** confirmed the substrate–product relationship between macrolides and corresponding reduced forms (ESI†). The absence of reduced forms of macrolactone in the fermentation of the wild-type *S. venezuelae* and failure of the bioconversion of both **1** and **2** into the corresponding saturated macrolactones **19** and 10,11-dihydronarbonolide, respectively, infers that this microbial system cannot catalyze the reduction of macrolactones.

In vitro reactions using cell-free extract (CFE) prepared by glass bead homogenization were carried out.[8] Initially, we examined CFE obtained from the DHS2001 strain for its ability to catalyze the biosynthesis of **16** from **8**. Approximately 85% of **8** was reduced into **16**, whereas a control using boiled CFE did not produce **16**. These findings confirm that enzyme-directed hydrogenation occurred (ESI†). Interestingly, when we conducted CFE experiments using synthetic macrolactone **19** as a substrate, small amounts of the known unreduced macrolides, **5** and **6**, were detected (<3% of total products) in addition to the expected reduced macrolides. This finding suggests that this reductive reaction could be reversible and dependent on substrate availability (ESI†). In a previous study characterizing the *pik* gene cluster in *S. venezuelae*, the presence of another modular PKS gene cluster, in addition to the *pik* gene cluster, was found.[2a] This proposes that some PKS modules from another modular PKS system in *S. venezuelae* contain reductive enzymatic domains (*i.e.* enoyl reductase, ER) that might play roles as hydrogenation catalysts in a collaborative manner with *pik* PKS. However, direct interaction between modular PKS proteins located in two different clusters is unprecedented. To determine if this reductive modification was caused by the action of another PKS system, the effect of the PKS inhibitor, cerulenin,[10] on the above CFE reaction was examined using **8** as a substrate. Cerulenin had no adverse effect on the production of **16**, which excludes the possible involvement of a PKS module containing an ER domain in generating these unusually saturated macrolides. A *trans*-acting ER for phthiocerol dimycocerosate and phenolglycolipid biosynthesis was recen-tly reported in *Mycobacterium tubeculosis*,[11] which suggests the possibility that a similar independent enzyme is involved in the reduction reactions of *S. venezuelae*. However, a gene encoding an independent ER-like enzyme was not identified by sequence analyis of the region surrounding *pik* cluster; therefore further investigations are required to identify this unique reductive enzyme.

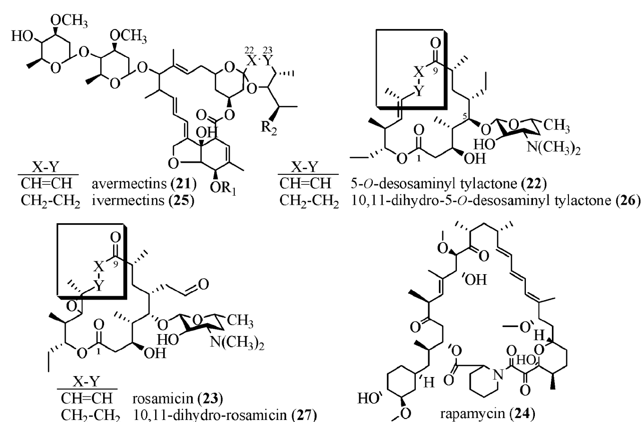

Fig. 1 Structure of heterologous macrolides examined in this study. Boxes depict the regiospecific functions adjacent to the catalytic site on the macrolide scaffold.

The flexibility of this reducing biocatalyst to both 12- and 14-membered ring macrolides encouraged us to examine its activity toward the 16-membered ring macrolides, avermectin (**21**), 5-*O*-desosaminyl tylactone (**22**), and rosamicin (**23**): and the 31-membered ring, rapamycin (**24**) (ESI†). Bioconversion of these four macrolides using YJ028 revealed the presence of two reduced analogues in the extracts of culture each fed with 16-membered macrolides **22** and **23**. However, there appeared to be no conversion of **21** and **24** (Fig. 1). The structures of the novel macrolides isolated were elucidated by assigning NMR data based on those from their corresponding macrolides. The analogues were shown to be 10,11-dihydro-5-*O*-desosaminyl tylactone (**26**) and 10,11-dihydrorosamicin (**27**), respectively; the absence of any signals downfield of 6.0 ppm in the ^1H NMR spectra indicated that these macrolides did not contain any double bonds. Inspection of the chemical structures of the new reduced macrolides revealed an interesting perspective on the structural elements that this *S. venezuelae* reduction system can recognize. Specifically, the same functional groups were located at identical positions in the vicinity of the modification site of the substrate. These included a carbonyl functional group on one carbon next to the target double bond and a methyl group attached onto another neighboring carbon (Scheme 1A and Fig. 1).

Two studies[5,12] on the reduced macrolides **16** and 2,3-dihydroalbocycline demonstrated that the reduced macrolides exhibited weak or inactivated antimicrobial activities. The antibacterial activities of all macrolide analogues obtained were determined against *Bacillus subtilis* using the agar diffusion method as described by the NCCLS.[13] Interestingly, antibacterial activities of reduced macrolides were not different from those of the corresponding macrolides (ESI†). Although we cannot explain this apparent discrepancy with previous reports,[5,12] our results indicate that the saturation of macrolides has no adverse effects on their biological activities.

This study shows a unique bio-hydrogenation activity toward unsaturated macrolides by *S. venezuelae* that has not been observed during routine cultivation and analytical procedures. Synthesis of macrolactones as precursors followed by their supplementation into the cultures of engineered strains of *S. venezuelae* facilitated the production of seven novel saturated macrolides in quantities enough to allow their chemical structures and antibacterial activities to be elucidated. The application of *S. venezuelae* as a microbial catalyst for the hydrogenation of other heterologous macrolides generated two unnatural reduced macrolide antibiotics. Taken together, these results demonstrate that expanding the metabolic diversity of a microbe by digging minor components and exploiting Nature's chemical diversity to generate natural product mimics by biosynthetic methods can offer new therapeutic resources.

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