Biosynthesis of Desosamine: Molecular Evidence Suggesting β-Glucosylation as a Self-Resistance Mechanism in Methymycin/Neomethymycin Producing Strain, *Streptomyces venezuelae*

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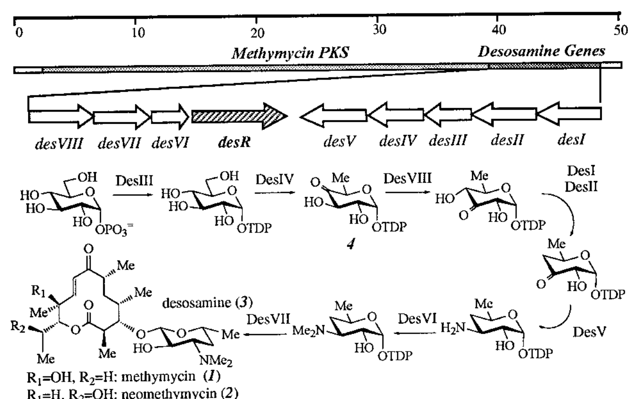
Many macrolide antibiotics contain sugar components that are essential for their biological activity. While our knowledge of the genetics of such microbial secondary metabolites has grown considerably in recent years, most of the early genetic work has focused on the polyketide synthase (PKS) genes,[1] and little is known about the biosynthesis of the appended unusual sugars.[2] Presently, results from sequencing beyond the PKS region have provided critical evidence revealing that the sugar biosynthetic genes are also part of the overall antibiotic biosynthetic gene cluster.[2] However, unlike the PKS genes, which are composed of clustered modules, the sugar biosynthetic genes are scattered at both ends of the PKS cluster and, hence, impose a challenge in distinguishing them from those for regulatory or aglycone modification enzymes that are also interspersed in the same region. Furthermore, in macrolides with more than one attached sugar moiety, the assignment of these genes to the appropriate sugar biosynthetic pathway can be quite difficult. Since methymycin (**1**) and neomethymycin (**2**),[3] two closely related macrolide antibiotics produced by *Streptomyces venezuelae*, contain desosamine (**3**) as their sole sugar component, the organization of the sugar biosynthetic genes in *S. venezuelae* is expected to be less complicated. Thus, this system was chosen for the study of the biosynthesis of desosamine which also exists in the erythromycin structure.[4]

To study the formation of this unusual sugar, we have recently cloned the entire methymycin/neomethymycin cluster.[5,6] Sequence analysis of this cluster led to the identification of nine open reading frames (ORFs) downstream of the PKS genes (Scheme 1).[7] On the basis of sequence similarities to other sugar biosynthetic genes, especially those derived from the erythromycin cluster,[8] eight of these nine ORFs are believed to be involved in the biosynthesis of TDP-D-desosamine, and their functions have been tentatively assigned. However, the presence of *desR*, which shows strong sequence homology to β-glucosidases (as high as 39% identity and 46% similarity),[9] within the desosamine gene cluster is puzzling. To investigate the function of DesR relative to the biosynthesis of methymycin/neomethymycin, a disruption plasmid (pBL1005) derived from pKC1139 (containing an apramycin resistance marker)[10] was constructed in which a 1.0 kb *Nco*I/*Xho*I fragment of the *desR* gene was deleted and replaced by the thiostrepton resistance (*tsr*) gene (1.1 kb)[11] via blunt-end ligation. This plasmid was used to transform *Escherichia coli* S17-1, which serves as the donor strain to introduce the pBL1005 construct through conjugal transfer into the wild-type *S. venezuelae*.[10] The double crossover mutants in which chromosomal *desR* had been replaced with the disrupted gene were selected according to their thiostrepton-resistant and apramycin-sensitive characteristics. Southern blot hybridization analysis was used to confirm the gene replacement.

Scheme 1



The desired mutant was first grown at 29 °C in seed medium for 48 h and then inoculated and grown in vegetative medium for another 48 h.[12] After the fermentation broth was centrifuged to remove cellular debris and mycelia, the supernatant was adjusted to pH 9.5 with concentrated KOH and extracted with chloroform. The organic layer was dried over sodium sulfate and evaporated to dryness. The amber oil-like crude products were first subjected to flash chromatography on silica gel using a gradient of 0−40% methanol in chloroform, followed by HPLC purification on a C_{18} column eluted isocratically with 45% acetonitrile in 57 mM ammonium acetate (pH 6.7). In addition to methymycin (**1**) and neomethymycin (**2**), two new products that differ from **1** and **2** by an extra hexose were isolated.[13] The chemical nature of these two new compounds were later elucidated to be C-2′ β-glucosylated methymycin and neomethymycin (**5** and **6**, respectively) by extensive spectral analysis.[14,15] The

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(6) A DNA library was constructed by partially digesting the genomic DNA of *S. venezuelae* (ATCC 15439) with *Sau*3A I into 35−40 kb fragments which were ligated into the cosmid vector pNJ1 (Tuan, J. S.; Weber, J. M.; Staver, M. J.; Leung, J. O.; Donadio, S.; Katz, L. *Gene* **1990**, *90*, 21−29). The recombinant DNA was packaged into bacteriophage λ which was used to transfect *E. coli* DH5α. The resulting cosmid library was screened for desired clones using the *tylA1* and *tylA2* genes from the tylosin biosynthetic cluster as probes (Merson-Davies, L. A.; Cundliffe, E. *Mol. Microbiol.* **1994**, *13*, 349−355). These two probes are specific for sugar biosynthetic genes, since they encode α-D-glucose-1-phosphate thymidylyltransferase (TylA1) and TDP-D-glucose 4,6-dehydratase (TylA2), respectively, catalyzing the first two steps universally followed by all 6-deoxyhexoses studied thus far.

(7) The *des* sequence has been deposited in the GenBank databases under the accession no. AF079762.

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(13) The yield of **5** and **6** was each in the range of 5−10 mg/L of fermentation broth. However, **1** and **2** remained to be the major products.

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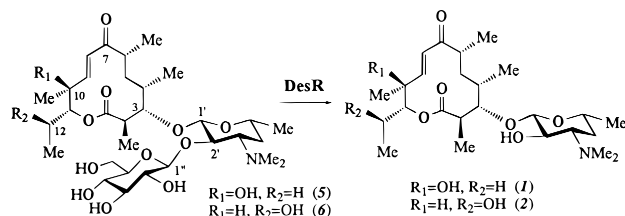
antibiotic activity of **5** and **6** against *Streptococcus pyogenes* was examined by separately applying 20 μL of each sample (1.6 mM in MeOH) to sterilized filter paper disks which were placed onto the surface of *S. pyogenes* grown on Mueller−Hinton agar plates.[16] After being grown overnight at 37 °C, the plates of the controls (**1** and **2**) showed clearly visible inhibition zones. In contrast, no such clearings were discernible around the disks of **5** and **6**. Evidently, β-glucosylation at C-2′ of desosamine in methymycin/neomethymycin renders these antibiotics inactive.

It should be noted that similar phenomena involving inactivation of macrolide antibiotics by glycosylation are known.[17] For example, it was found that when erythromycin was given to *Streptomyces lividans*, which contains a macrolide glycosyltransferase (MgtA), the bacterium was able to defend itself by glycosylating the drug.[18] Such a macrolide glycosyltransferase activity has been detected in 15 out of a total of 32 actinomycete strains producing various polyketide antibiotics.[17c] Interestingly, the coexistence of a macrolide glucosyltransferase (OleD) capable of deactivating oleandomycin by glucosylation,[19] and an extracellular β-glucosidase capable of removing the added glucose from the deactivated oleandomycin in *Streptomyces antibioticus*[20] has led to the speculation of glycosylation as a possible self-resistance mechanism in *S. antibioticus*. Although the genes of the aforementioned glycosyltransferases have been cloned in a few cases, such as *mgtA* of *S. lividans* and *oleD of S. antibioticus*, the whereabouts of those macrolide β-glycosidase genes remain obscure.[21] Thus, the discovery of *desR*, a macrolide β-glucosidase gene, within the desosamine gene cluster is significant, and the accumulation of deactivated **5**/**6** after *desR* disruption provides direct molecular evidence indicating that a similar self-defense mechanism via glycosylation/deglycosylation may also be operative in *S. venezuelae*. However, because a significant amount of methymycin and neomethymycin also exist in the fermentation broth of the mutant strain, glucosylation of desosamine may not be the primary self-resistance mechanism in *S. venezuelae*. Indeed, an rRNA methyltransferase gene found upstream from the PKS genes in this cluster may confer the primary self-resistance protection.[5] Thus, our results are consistent with the fact that antibiotic producing organisms generally have more than one defensive option.[22] In light of this observation, it is conceivable that methymycin/neomethymycin may be produced in part as the inert diglycosides (**5**/**6**), and the macrolide β-glucosidase encoded by *desR* is responsible for transforming methymycin/neomethymycin from their dormant state to their active form. Supporting this idea, the translated *desR* gene has a leader sequence characteristic of secretory proteins,[23] thus DesR may be transported through the cell membrane and hydrolyze the modified antibiotics (**5**/**6**) extracellularly to activate them (Scheme 2).

Scheme 2



In summary, the identification of *desR* as the first macrolide β-glucosidase gene in this study offers a unique opportunity to further study glycosylation/deglycosylation as a microbial self-resistance mechanism. More importantly, this gene can be used as a probe to search for possible homologues in other antibiotic biosynthetic pathways. Deletion of the corresponding macrolide glycosidase gene in these pathways may lead to the accumulation of the glycosylated products which may be used as prodrugs with reduced cytotoxicity. Glycosylation also holds promise as a tool to regulate and/or minimize the potential toxicity associated with new macrolide antibiotics produced by genetically engineered microorganisms. Therefore, the availability of a number of macrolide glycosidases, which would be used for the activation of newly formed antibiotics that have been deliberately deactivated by engineered glycosyltransferases, could be a valuable part of an overall strategy for the development of novel antibiotics using the combinatorial biosynthetic approach.[1,24]

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Note Added in Proof. The gene (*oleR*) encoding the glycosidase involved in oleandomycin modification has recently been found in *S. antibioticus* (Quiros, L. M., et al. *Mol. Microbiol.* **1998**, *28*, 1177−1185).

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(14) Spectral data (J values are in hertz) for **5**: ^{1}H NMR (acetone-d_6) δ 6.56 (1H, d, J = 16.0, 9-H), 6.46 (1H, d, J = 16.0, 8-H), 4.67 (1H, dd, J = 10.8, 2.0, 11-H), 4.39 (1H, d, J = 7.5, 1′−H), 4.32 (1H, d, J = 8.0, 1″−H), 3.99 (1H, dd, J = 11.5, 2.5, 6″−H), 3.72 (1H, dd, J = 11.5, 5.5, 6″−H), 3.56 (1H, m, 5′−H), 3.52 (1H, d, J = 10.0, 3-H), 3.37 (1H, t, J = 8.5, 3″−H), 3.33 (1H, m, 5″−H), 3.28 (1H, t, J = 8.5, 4″−H), 3.23 (1H, dd, J = 10.5, 7.5, 2′−H), 3.15 (1H, dd, J = 8.5, 8.0, 2″−H), 3.10 (1H, m, 2-H), 2.75 (1H, 3′−H, burried under H_2O peak), 2.42 (1H, m, 6-H), 2.28 (6H, s, NMe_2), 1.95 (1H, m, 12-H), 1.9 (1H, m, 5-H), 1.82 (1H, m, 4′−H), 1.50 (1H, m, 12-H), 1.44 (3H, d, J = 7.0, 2-Me), 1.4 (1H, m, 5-H), 1.34 (3H, s, 10-Me), 1.3 (1H, m, 4-H), 1.25 (1H, m, 4′−H), 1.20 (3H, d, J = 6.0, 5′-Me), 1.15 (3H, d, J = 7.0, 6-Me), 0.95 (3H, d, J = 6.0, 4-Me), 0.86 (3H, t, J = 7.5, 12-Me); ^{13}C NMR (acetone-d_6) δ 206.1 (C-7), 176.2 (C-1), 150.8 (C-9), 125.6 (C-8), 108.4 (C-1″), 104.1 (C-1′), 85.1 (C-3), 83.0 (C-2′), 78.3 (C-3″), 78.2 (C-5″), 76.8 (C-2″), 76.6 (C-11), 74.5 (C-10), 71.9 (C-4″), 69.3 (C-5′), 66.0 (C-3′), 63.8 (C-6″), 46.3 (C-6), 44.6 (C-2), 40.8 (NMe_2), 34.4 (C-5), 34.3 (C-4), 30.5 (C-4′), 21.8 (C-12), 21.5 (5′-Me), 19.4 (10-Me), 17.9 (6-Me), 17.7 (4-Me), 17.6 (2-Me), 11.3 (12-Me); high-resolution FAB-MS calcd for $C_{31}H_{54}NO_{12}$ $(M + H)^+$ 632.3646, found 632.3686. Spectral data (J values are in hertz) for **6**: ^{1}H NMR (acetone-d_6) δ 6.69 (1H, dd, J = 16.0, 5.5 Hz, 9-H), 6.55 (1H, dd, J = 16.0, 1.3, 8-H), 4.71 (1H, dd, J = 9.0, 2.0, 11-H), 4.37 (1H, d, J = 7.0, 1′−H), 4.31 (1H, d, J = 8.0, 1"-H), 3.97 (1H, dd, J = 11.5, 2.5, 6″−H), 3.81 (1H, dq, J = 9.0, 6.0, 12-H), 3.72 (1H, dd, J = 11.5, 5.0, 6″−H), 3.56 (1H, m, 5′−H), 3.50 (1H, bd, J = 10.0, 3-H), 3.36 (1H, t, J = 8.5, 3″−H), 3.32 (1H, m, 5″−H), 3.30 (1H, t, J = 8.5, 4″−H), 3.23 (1H, dd, J = 10.2, 7.0, 2′−H), 3.13 (1H, dd, J = 8.5, 8.0, 2″−H), 3.09 (1H, m, 2-H), 3.08 (1H, m, 10-H), 2.77 (1H, ddd, J = 12.5, 10.2, 4.5, 3′−H), 2.41 (1H, m, 6-H), 2.28 (6H, s, NMe_2), 1.89 (1H, t, J = 13.0, 5-H), 1.83 (1H, ddd, J = 12.5, 4.5, 1.5, 4′−H), 1.41 (3H, d, J = 7.0, 2-Me), 1.3 (1H, m, 4-H), 1.25 (1H, m, 5-H), 1.2 (1H, m, 4′−H), 1.20 (3H, d, J = 6.0, 5′-Me), 1.17 (6H, d, J = 7.0, 6-Me, 10-Me), 1.12 (3H, d, J = 6.0, 12-Me), 0.96 (3H, d, J = 6.0, 4-Me); ^{13}C NMR (acetone-d_6) δ 204.1 (C-7), 175.8 (C-1), 148.2 (C-9), 126.7 (C-8), 108.3 (C-1″), 104.2 (C-1′), 85.1 (C-3), 83.0 (C-2′), 78.2 (C-3″), 78.1 (C-5″), 76.6 (C-2″), 76.4 (C-11), 71.8 (C-4″), 69.3 (C-5′), 66.1 (C-12), 66.0 (C-3′), 63.7 (C-6″), 46.2 (C-6), 44.4 (C-2), 40.8 (NMe_2), 36.4 (C-10), 34.7 (C-5), 34.0 (C-4), 29.5 (C-4′), 21.5 (5′-Me), 21.5 (12-Me), 17.9 (6-Me), 17.7 (4-Me), 17.2 (2-Me), 9.9 (10-Me); high-resolution FAB-MS calcd for $C_{31}H_{54}NO_{12}$ $(M + H)^+$ 632.3646, found 632.3648.

(15) The coupling constant (d, J = 8.0 Hz) of the anomeric hydrogen (1″−H) of the added glucose and the magnitude of the downfield shift (11.8 ppm) of C-2′ of desosamine are all consistent with the assigned C-2′ β-configuration (Seo, S.; Tomita, Y.; Tori, K.; Yoshimura, Y. *J. Am. Chem. Soc.* **1978**, *100*, 3331−3339).

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(21) Interestingly, the recently released *eryBI* sequence as part of the erythromycin biosynthetic cluster is highly homologous to *desR* (55% identity).[8a] It is likely that the EryBI protein may assume a similar role as DesR, participating in a secondary self-defense mechanism.

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