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Biosynthesis of Desosamine: Construction of a New Macrolide Carrying a Genetically Designed Sugar Moiety

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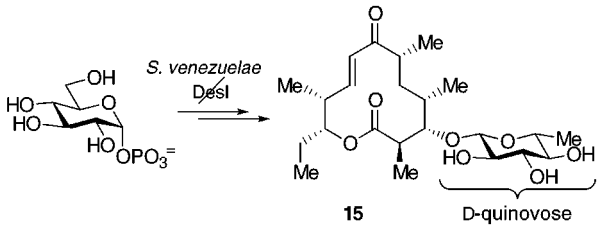
ABSTRACT



The appended sugars in macrolide antibiotics are indispensable to the biological activities of these important drugs. In an effort to generate a set of novel macrolide derivatives, we have created a new analogue of methymycin and neomethymycin, antibiotics produced by *Streptomyces venezuelae*. This analogue 15 carrying a different sugar, D-quinovose, instead of D-desosamine, was constructed by taking advantage of targeted gene deletion combined with a specific pathway-independent C-3 reduction capability of the wild type *S. venezuelae*.

With more and more bacteria becoming resistant to many commonly used antibiotics, the development of new antimicrobial drugs has become more urgent.[1] Naturally occurring metabolites continue to be an important source for providing the lead compounds in this quest. However, as the modular nature of the biosynthetic machinery that produces many natural products is being discovered,[2] the idea of manipulating the existing biosynthetic genes to produce new nonnatural compounds is becoming increasingly attractive. Particularly highlighted by the recent advances made on the biosynthesis of polyketides, the approach based on combinatorial biology for the creation of novel structures has shown great promise. Yet, many of the naturally occurring polyketides also contain unusual sugars as an essential component. Without the appended sugar(s), the biological activities of these clinically important secondary metabolites are either completely lost or dramatically decreased. Therefore, studying the formation of these critical sugar residues is important in order to fully realize the potential of generating novel antibiotics by genetic manipulation of the existing pathways.

As a part of our effort to study the biosynthesis of unusual sugars, we have recently cloned and sequenced the entire macrolide biosynthetic gene cluster in *Streptomyces venezuelae*, the producer of the 12-membered polyketides methymycin (**1**) and neomethymycin (**2**)[3] and the 14-membered co-metabolites pikromycin and narbomycin. All of these macrolide antibiotics contain the single 3-(dimethylamino)-

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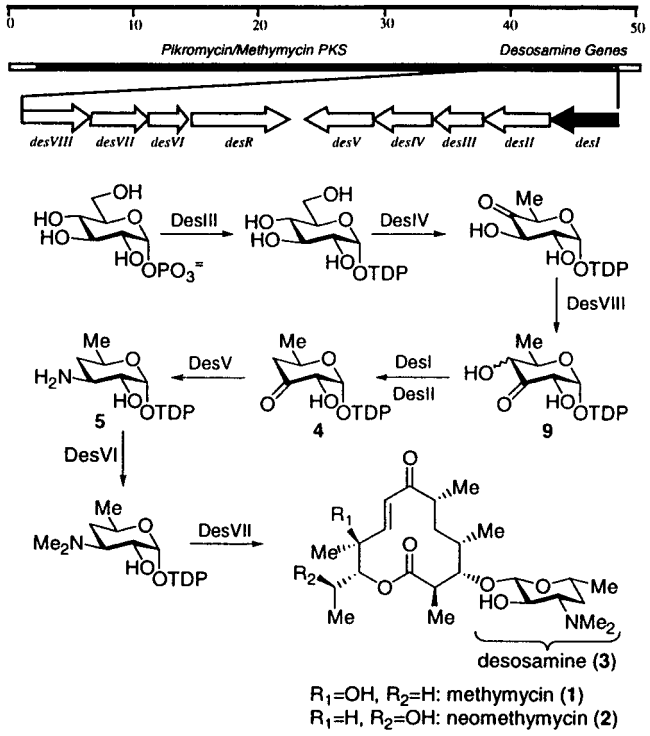
3,4,6-trideoxy sugar desosamine (**3**), which also exists in erythromycin[4] and a few other macrolide antibiotics. On the basis of sequence similarities to other sugar biosynthetic genes,[5] especially those derived from the erythromycin cluster,[6] eight open reading frames, *desI*−*desVIII* (*des* region), in the pikromycin/methymycin cluster have been assigned as genes involved in desosamine biosynthesis (Scheme 1).[7] Since these sugar genes are clustered, they are

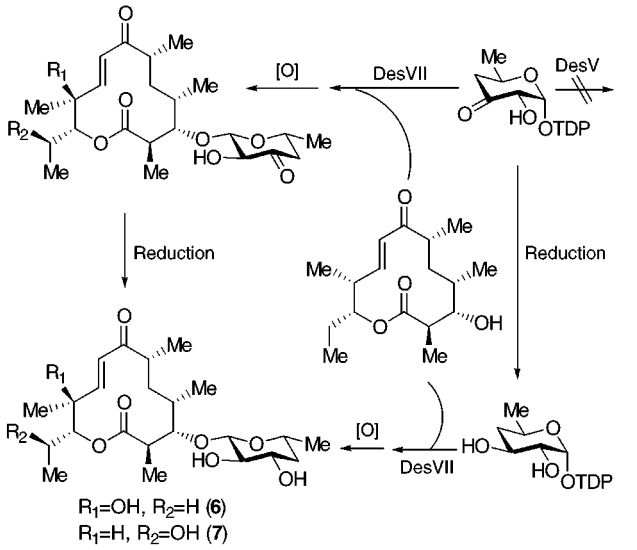
Scheme 1



readily amenable to direct genetic manipulations. Therefore, the *des* cluster, as part of the pikromycin/methymycin biosynthetic machinery, represents an ideal system to probe the feasibility of making new polyketides carrying modified sugar components in vivo.

Initial success was achieved in a recent experiment, in which a mutant of *S. venezuelae* (KdesV-41) was constructed that had the *desV* gene disrupted.[7d] Since *desV* encodes the 3-aminotransferase that catalyzes the conversion of the 3-keto sugar **4** to the corresponding amino sugar **5**, deletion of this gene should prevent C-3 transamination, resulting in the accumulation of **4**. It was expected that if the glycosyltransferase (DesVII) of this pathway is capable of recognizing and processing the keto sugar intermediate **4**, the macrolide product(s) produced by the KdesV-41 mutant should have an attached 3-keto sugar. Surprisingly, the two products isolated in this experiment were the methymycin/neomethymycin analogues **6** and **7**, each carrying a 4,6-dideoxyhexose (Scheme 2).[7d] While this result nicely demonstrated a relaxed

Scheme 2



specificity for the glycosyltransferase toward its sugar substrate, it also indicated the existence of a pathway-independent reductase in *S. venezuelae* that can stereospecifically reduce the C-3 keto group of the sugar metabolite.

With information on the genetic organization of the pikromycin/methymycin biosynthetic machinery and the existence of the specific pathway-independent reductase in *S. venezuelae* in hand, we decided to explore the possibility of generating a mutant capable of synthesizing new macrolides of this class containing an engineered sugar. The *desI* gene, which has been proposed to encode the dehydrase responsible for the C-4 deoxygenation in the biosynthesis of desosamine,[7d] was chosen as our target in this gene deletion experiment, with the prediction that it would lead to the incorporation of D-quinovose (**8**; Figure 1), also known as 6-deoxy-D-glucose, into the final product(s). The rationale of our experimental design and prediction is based on the following. (1) Desosamine biosynthesis will be "terminated" at the C-4 deoxygenation step due to *desI* deletion and, thus, should result in the accumulation of 3-keto-6-deoxyhexose **9** (Scheme 1). (2) By taking advantage of the existence of a 3-ketohexose reductase in *S. venezuelae*, the sugar intermediate **9** is expected to be reduced stereospecifically to D-quinovose (**8**). (3) The glycosyltransferase (DesVII), with

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(5) (a) Liu, H-w.; Thorson, J. S. *Annu. Rev. Microbiol.* **1994**, *48*, 223. (b) Kirschning, A.; Bechthold, A. F.-W.; Rohr, J. In *Bioorganic Chemistry Deoxysugars, Polyketides and Related Classes: Synthesis, Biosynthesis, Enzymes*; Rohr, J., Ed.; Springer: Berlin, 1997; pp 1−84. (c) Johnson, D. A.; Liu, H.-w. In *Comprehensive Chemistry of Natural Products Chemistry*; Barton, D., Nakanishi, K., Meth-Cohn, O., Eds.; Pergamon: New York, 1999; Vol. 3, pp 311−365.

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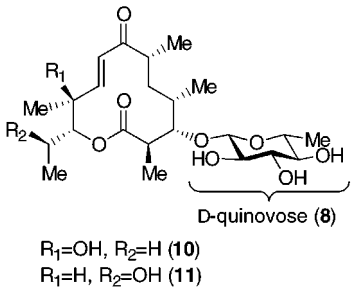

Figure 1.

its relaxed specificity toward the sugar substrate, should catalyze the coupling of **8** to the macrolactones to give new macrolides **10** and **11** containing the engineered sugar D-quinovose (Figure 1).

To test our idea, a disruption plasmid, pDesI-K, derived from pKC1139 that contains an apramycin resistant marker, was constructed in which *desI* was replaced by the neomycin resistance gene, which also confers resistance to kanamycin. This construct was then introduced into wild type *S. venezuelae* by conjugal transfer using *Escherichia coli* S17-1 as the donor strain.[8] Several double crossover mutants were identified on the basis of their phenotypes of kanamycin resistant (Kan^R) and apramycin sensitive (Apr^S). One mutant, KdesI-80, was selected and grown at 29 °C in seed medium (100 mL) for 48 h and then inoculated and grown in vegetative medium (5 L) for another 48 h.[9] The fermentation broth was centrifuged to remove cellular debris and mycelia, and the supernatant was adjusted to pH 9.5 with concentrated potassium hydroxide solution. The resulting solution was extracted with chloroform, and the pooled organic extracts were dried over sodium sulfate and evaporated to dryness. The yellow oil was subjected to flash chromatography on silica gel using a gradient of 0−12% methanol in chloroform, and the isolated products were further purified by HPLC using a C_{18} column eluted isocratically with 50% acetonitrile in water. As expected, no methymycin or neomethymycin was detected; instead, 10-deoxymethynolide **12** was found as the major product (approximately 600 mg). Significant quantities of methynolide **13** (approximately 40 mg) and neomethynolide **14** (approximately 2 mg) were also isolated.[10] Most importantly, a new macrolide **15** containing D-quinovose (3.2 mg) was produced by this mutant. Its structure was fully established by spectral analyses.[11] Compounds **12**−**15** are shown in Figure 2.

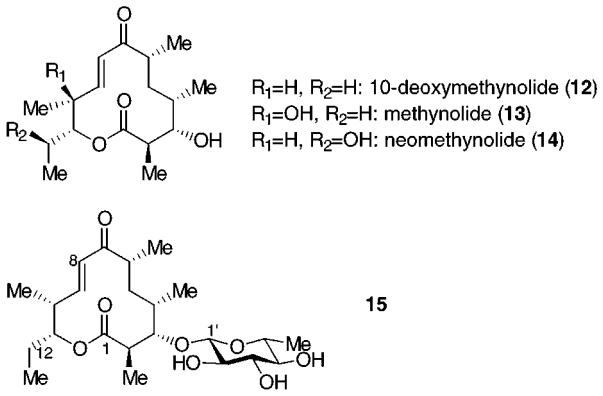

Figure 2.

The fact that macrolide **15** containing D-quinovose is indeed produced by the *desI* mutant is significant. First, the formation of quinovose as predicted further corroborates the presence of a pathway-independent reductase in *S. venezuelae* that reduces the 3-keto sugars. Interestingly, this reductase is able to act on the 4,6-dideoxy sugar **4** as well as the 6-deoxy sugar **9**, suggesting that it is oblivious to the presence of a hydroxyl group at C-4. However, it is not clear at this point whether the reduction occurs on the free sugar or after it is appended to the aglycone. Second, the retention of the 4-OH in quinovose as a result of *desI* deletion provides strong evidence supporting the assigned role of *desI* to encode a C-4 dehydrase. This finding also lends credence to the proposed biosynthetic pathway in which C-4 deoxygenation precedes C-3 transamination as depicted in Scheme 1.[7c,d] An alternative pathway[6a,b] where the order of these two steps is reversed is incompatible with the current observation. Moreover, our results again show that the glycosyltransferase (DesVII) of this pathway can recognize alternative sugar substrates whose structures are considerably different from the original amino sugar substrate desosamine (**3**).[12] While

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(10) Compounds were identified by comparison of their 500 MHz ^1H NMR spectra with literature data for **12** (Lambalot, R. H.; Cane, D. E. *J. Antibiot.* **1992**, *45*, 1981), **13** (Oikawa, Y.; Tanaka, T.; Yonemitsu, O. *Tetrahedron Lett.* **1986**, *27*, 3647 and Ditrich, K. *Liebigs Ann. Chem.* **1990**, *8*, 789) and **14** (Inanaga, J.; Kawanami, Y.; Yamaguchi, M. *Bull. Chem. Soc. Jpn.* **1986**, *59*, 1521).

(11) Spectral data (*J* values are in hertz) for **15**: ^1H NMR ($CDCl_3$) δ 6.76 (1H, dd, J = 16.0, 5.5, 9-H), 6.43 (1H, d, J = 16.0, 8-H), 4.97 (1H, ddd, J = 8.4, 5.9, 2.5, 11-H), 4.29 (1H, d, J = 8.0, 1′-H), 3.62 (1H, d, J = 10.5, 3-H), 3.49 (1H, t, J = 9.0, 3′-H), 3.36 (1H, dd, J = 9.0, 8.0, 2′-H), 3.32 (1H, dq, J = 8.5, 5.5, 5′-H), 3.23 (1H, dd, J = 9.0, 8.5, 4′-H), 2.82 (1H, dq, J = 10.5, 7.0, 2-H), 2.64 (1H, m, 10-H), 2.55 (1H, m, 6-H), 1.70 (1H, m, 12a-H), 1.66 (1H, bt, J = 12.5, 5b-H), 1.56 (1H, m, 12b-H), 1.40 (1H, dd, J = 12.5, 4.5, 5a-H), 1.35 (3H, d, J = 7.0, 2-Me), 1.31 (3H, d, J = 5.5, 5′-Me), 1.24 (1H, bdd, J = 10.0, 4.5, 4-H), 1.21 (3H, d, J = 7.0, 6-Me), 1.11 (3H, d, J = 6.5, 10-Me), 1.00 (3H, d, J = 7.0, 4-Me), 0.92 (3H, t, J = 7.5, 12-Me); ^{13}C NMR ($CDCl_3$) δ 205.0 (C-7), 174.7 (C-1), 146.9 (C-9), 125.9 (C-8), 102.9 (C-1′), 85.4 (C-3), 76.5 (C-3′), 75.5 (C-4′), 74.7 (C-2′), 73.9 (C-11), 71.6 (C-5′), 45.0 (C-6), 43.9 (C-2), 37.9 (C-10), 34.1 (C-5), 33.4 (C-4), 25.2 (C-12), 17.7 (6-Me), 17.5 (5′-Me), 17.4 (4-Me), 16.2 (2-Me), 10.3 (12-Me), 9.6 (10-Me); high-resolution FAB-MS calculated for $C_{23}H_{38}O_8$ $(M + H)^+$ 443.2644, found 443.2661.

(12) A similar relaxed specificity toward sugar substrates has been reported for the glycosyltransferases in the biosynthesis of glycopeptide antibiotics (Solenberg, P. J.; Matsushima, P.; Stack, D. R.; Wilkie, S. C.; Thompson, R. C.; Baltz, R. H. *Chem. Biol.* **1997**, *4*, 195) and in daunorubicin (Madduri, K.; Kennedy, J.; Rivola, G.; Inventi-Solari, A.; Filippini, S.; Zanuso, G.; Colombo, A. L.; Gewain, K. M.; Occi, J. L.; MacNeil, D. J.; Hutchinson, C. R. *Nature Biotech.* **1998**, *16*, 69), erythromycin (Gaisser, S.; Bohm, G. A.; Doumith, M.; Raynal, M.-C.; Dhillon, N.; Cortés, J.; Leadlay, P. F. *Mol. Gen. Genet.* **1998**, *258*, 78), and methymycin/neomethymycin[7b,c] pathways.

the incorporation of quinovose is important, another noteworthy, albeit unexpected, result was the fact that the aglycone of the isolated macrolide **15** was 10-deoxymethynolide **12** instead of methynolide **13** and neomethynolide **14**. It is possible that the cytochrome P450 hydroxylase (PikC), which catalyzes the hydroxylation of 10-deoxymethynolide at either its C-10 or C-12 position,[13] is sensitive to structural variations in the appended sugar. It could be argued that the presence of the 4-OH group in the sugar moiety is somehow responsible for decreasing or preventing hydroxylation of the macrolide.

In conclusion, the work presented here demonstrates the feasibility of combining pathway-dependent genetic manipulations and pathway-independent enzymatic reactions to engineer a sugar of designed structure. It is conceivable that the pathway-independent enzymes could also be used in concert with the natural biosynthetic machinery to generate further structural diversity, which can provide an array of random compounds. With the continually expanding knowledge in cloning, characterization and expression of the genes involved in unusual sugar biosynthesis, the goal of constructing sets of diverse nucleotide sugars to be used for glycosylation may soon be realized. It is hoped that the experience gained from the ongoing research of unusual sugar biosynthesis will facilitate the development of novel drugs against bacterial infections based on a combinatorial biosynthetic approach.

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(13) This enzyme can accept both 12- and 14-membered-ring macrolide substrates and is active on both C-10 and C-12 of the 12-membered ring (Xue, Y.; Wilson, D.; Zhao, L.; Liu, H.-w.; Sherman, D. H. *Chem. Biol.* **1998**, *5*, 661).