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COMMUNICATIONS

Published on Web 03/28/2007

Engineered Biosynthesis of Macrolide Derivatives Bearing the Non-Natural Deoxysugars 4-*epi*-D-Mycaminose and 3-*N*-Monomethylamino-3-Deoxy-D-Fucose

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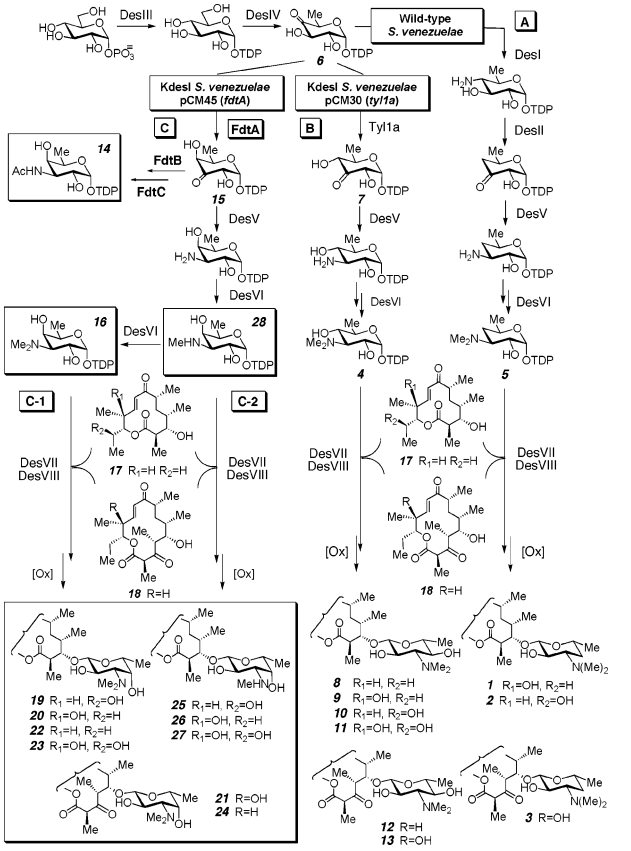
Received November 17, 2006; E-mail: h.w.liu@mail.utexas.edu

A growing appreciation of the essentiality of deoxysugars for the physiological functions of cell surface polysaccharides[1] and the biological activities of many secondary metabolites[2] has led to a surge of investigations of the biosynthesis of deoxysugars.[3] These studies have facilitated the rational manipulation of the deoxysugar biosynthetic machinery to generate a diverse array of new glycoconjugates with potential clinical applications. The success of these engineering strategies hinges on the identification and exploitation of the substrate flexibilities of pathway enzymes. Interestingly, the substrate flexibilities of several natural product glycosyltransferases have been demonstrated and subsequently exploited for the biosynthesis of a variety of natural product glycoforms.[4] Recent in vitro and in vivo investigations of unusual sugar biosynthesis and work on construction of hybrid deoxysugar biosynthetic pathways in engineered hosts suggest that relaxed substrate specificity is a general trait among secondary metabolite sugar biosynthetic enzymes.[4,5] Hence, we envisioned taking advantage of the substrate flexibility of deoxysugar biosynthetic enzymes to assemble pathways for the construction of sugar structures that have not yet been found in nature, thus increasing the diversity of sugar donors available for natural product glycodiversification. Herein we report an example of an engineered biosynthetic pathway which yielded non-natural sugar-bearing macrolide derivatives in vivo.

Macrolide antibiotics, such as methymycin (**1**), neomethymycin (**2**), and pikromycin (**3**), produced by *Streptomyces venezuelae* (Scheme 1, path A) comprise an important class of compounds, many of which are effective antibacterial agents. As part of our efforts to alter the glycosylation patterns of macrolide antibiotics, we have successfully carried out manipulation of the well-characterized TDP-D-mycaminose (**4**) and TDP-D-desosamine (**5**) biosynthetic pathways to generate methymycin/pikromycin analogues with altered sugar structures.[6] We have also found that replacement of DesI, the C-4 aminotransferase which acts on TDP-4-keto-6-deoxy-D-glucose (**6**) in the desosamine pathway, with Tyl1a, which converts **6** to TDP-3-keto-6-deoxy-D-glucose (**7**), switches the desosamine pathway to an efficient mycaminose biosynthesis pathway,[6a] producing mycaminosylated macrolide derivatives **8**−**13** in the resulting *S. venezuelae* mutant (Scheme 1, path B). FdtA, a TDP-4-keto-6-deoxy-D-glucose 3,4-ketoisomerase involved in the biosynthesis of 3-*N*-acetyl-3-deoxy-D-fucose (**14**), a precursor of the *S*-layer polysaccharides in *Aneurinibacillus thermoaerophilus*, shows moderate sequence identity with Tyl1a (33% identity),[7] and like Tyl1a uses **6** as the substrate, yet forms the C-4 epimer of the Tyl1a product, TDP-3-keto-D-fucose (**15**). The stereochemical divergence of these two related enzyme-catalyzed reactions offered a unique opportunity to convert our engineered D-mycaminose biosynthetic pathway into a system making the non-naturally occurring TDP-4-*epi*-D-mycaminose (**16**) by the replacement of Tyl1a with FdtA (Scheme 1, path C). The

Scheme 1



product **16** may serve as the sugar donor for glycosyl transfer of 4-*epi*-D-mycaminose to the endogenous aglycones 10-deoxymethynolide (**17**) and narbonolide (**18**) (Scheme 1, path C-1). The success of this endeavor depends both on the efficient expression of FdtA encoded by highly AT-rich *A. thermoaerophilus* DNA in the high-GC Gram-positive *S. venezuelae* and on the ability of the desosamine biosynthetic enzymes DesV, DesVI, DesVII, and DesVIII to process substrates with an axial C-4 hydroxyl group.

In order to construct this pathway, *fdtA* was amplified by colony PCR from *A. thermoaerophilus* with an engineered ribosome binding site and cloned into the *S. venezuelae* expression vector pCM1d.[8] The resulting construct, pCM45, was expressed in the KdesI mutant of *S. venezuelae*.[8] TLC analysis of small-scale chloroform extracts of this mutant revealed several new polar spots with R_f values indicative of glycosylated macrolide products. Subsequently, a large-scale culture (3 L) of the KdesI/pCM45 mutant was grown in vegetative media under previously reported standard conditions[8] to obtain more of the new compounds. Separation of the crude extracts by silica gel chromatography ($CHCl_3$/MeOH) and analysis of the resulting fractions by ^{1}H NMR

10.1021/ja068254t CCC: $37.00

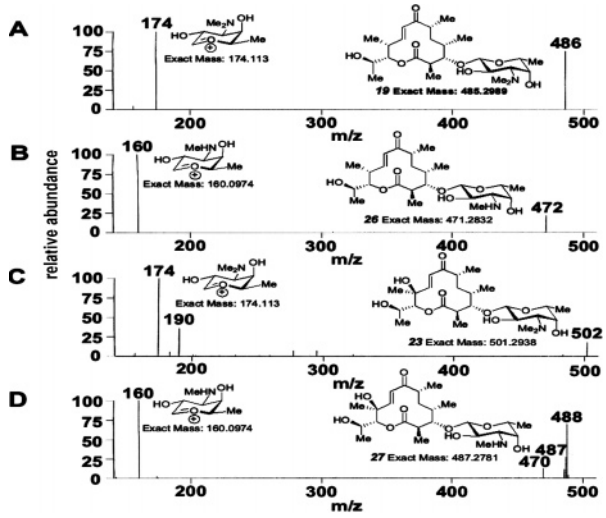


Figure 1. Confirmation of the identity of the deoxysugar 3-*N*-monomethyl-3-deoxy-D-fucose in **26** (B) and **27** (D) by comparison of positive mode ESI-MS/MS fragmentation patterns of the parent peaks of **26** and **27** to those of **19** (A) and **23** (C), respectively.

spectroscopy revealed the presence of three major glycosylated compounds. Interestingly, seven additional minor glycosylated macrolide species were also discernible. Together, these glycosylated compounds accounted for about 55% of the total macrolide produced.[9] Further separation by silica gel chromatography and reverse-phase HPLC[8] allowed purification of one of the three major compounds, which was structurally characterized by ^{1}H, ^{13}C, COSY, HSQC, HMBC, and NOESY NMR spectroscopies and high-resolution CI^{+}-MS, unambiguously identifying it as the non-natural sugar-bearing macrolide 4-*epi*-D-mycaminosyl neomethynolide (**19**).[8] The other two major compounds were identified by ^{1}H NMR spectroscopy and high-resolution CI^{+}-MS as 4-*epi*-D-mycaminosyl methynolide (**20**) and 4-*epi*-D-mycaminosyl pikronolide (**21**).[8]

Six of the remaining minor compounds were purified and characterized by high-resolution CI^{+}-MS. Three were found to have masses and polarities consistent with 4-*epi*-D-mycaminosyl 10-deoxymethynolide (**22**), 4-*epi*-D-mycaminosyl novamethynolide (**23**), and 4-*epi*-D-mycaminosyl narbonolide (**24**). Surprisingly, the other three compounds displayed masses consistent with desmethyl analogues of **19**/**20** and **23**. Reasoning that these compounds could bear 3-*N*-monomethylated sugars, ESI-MS/MS fragmentation analysis was carried out on the purified desmethyl analogues of **19** and **23** to determine whether the aglycone or the sugar lacked a methyl group. Comparison of the ESI-MS/MS fragmentation patterns of **19** and **23** (Figure 1A,C, respectively) and their corresponding desmethyl analogues (Figure 1B,D, respectively) clearly showed that the sugar moiety of each of these analogues lacks a methyl group. These results strongly suggest that these analogues are new 3-*N*-monomethyl-3-deoxy-D-fucosyl derivatives of neomethynolide/methynolide (**25**/**26**) and novamethynolide (**27**, Scheme 1). The presence of these compounds was unexpected, as no macrolides bearing 3-*N*-monomethylated derivatives of desosamine or mycaminose have ever been detected in the wild-type or engineered *S. venezuelae* strains. The production of these compounds by the KdesI/pCM45 mutant likely results from the interception of a portion of TDP-3-*N*-monomethyl-3-deoxy-D-fucose (**28**), the product of the first DesVI-catalyzed methyl transfer reaction, by the glycosyltransferase DesVII (Scheme 1, path C-2) before it can be converted to TDP-4-*epi*-D-mycaminose (**16**) by DesVI. A plausible explanation for this phenomenon is that the kinetics of the second DesVI-catalyzed methylation step might be slowed due to the change in substrate C-4 configuration, allowing sufficient accumulation of **28** for DesVII to couple it to 10-deoxymethynolide (**17**), leading to the formation of **25**/**26** and **27**.

These results are significant for three reasons. First, they demonstrate the feasibility of designing and assembling a pathway for the biosynthesis and attachment of a non-natural deoxysugar, TDP-4-*epi*-D-mycaminose (**16**), resulting in the new macrolides **19**−**24**. Second, the ability of four desosamine pathway enzymes, DesV, DesVI, DesVII, and DesVIII, to tolerate substrates with altered C-4 stereochemistry was revealed and was crucial for a successful outcome. Third, this engineering work serendipitously led to the creation of three additional new macrolide derivatives (**25**−**27**) bearing the non-natural sugar, 3-*N*-monomethyl-3-deoxy-D-fucose. Formation of **25**−**27** relied on subtle differences in the proficiencies of two desosamine pathway enzymes, DesVI and DesVII, for turnover of non-natural substrates. These differences were only brought to light after interrogation of these enzymes with non-natural substrates generated by pathway engineering, highlighting the potential of exploiting the influence of subtle enzymological effects on the outcome of engineered pathways.

With the increasingly rapid discovery of sugar pathway-encoding genes in both natural product and polysaccharide biosynthesis, more new components for pathway construction are becoming available to the biosynthetic engineer. These new "glycosyl tools" expand the number of feasibly constructed sugar structures, making it possible to assemble pathways to make sugars that do not exist in nature, such as **16** and **28**. The nine non-natural sugar-bearing compounds (**19**−**27**) generated in this work demonstrate the feasibility of using this approach to generate non-natural sugar-bearing secondary metabolites. Efforts are underway to fully exploit the potential of such a combinatorial biosynthesis strategy.

Acknowledgment. We thank Dr. Paul Messner at the University of Natural Resources and Applied Life Sciences, Vienna, Austria, for his generous gift of the *A. thermoaerophilus* cell stock, and Drs. Mehdi Moini and Yasushi Ogasawara for valuable discussions. This work was supported by the National Institutes of Health Grants GM35906 and GM54346.

Supporting Information Available: Experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) See Supporting Information for details.
(9) Both the estimated amounts of **19**−**27** in crude extracts and isolated yields of **19**−**21** and **23**−**27** are provided in Supporting Information.

JA068254T