Biosynthesis of Desosamine: Construction of a New Methymycin/Neomethymycin Analogue by Deletion of a Desosamine Biosynthetic Gene

Lishan Zhao,[†] David H. Sherman,[‡] and Hung-wen Liu*,[†]

Department of Chemistry, University of Minnesota Minneapolis, Minnesota 55455
Department of Microbiology and Biological Process Technology Institute
University of Minnesota, Minneapolis, Minnesota 55455

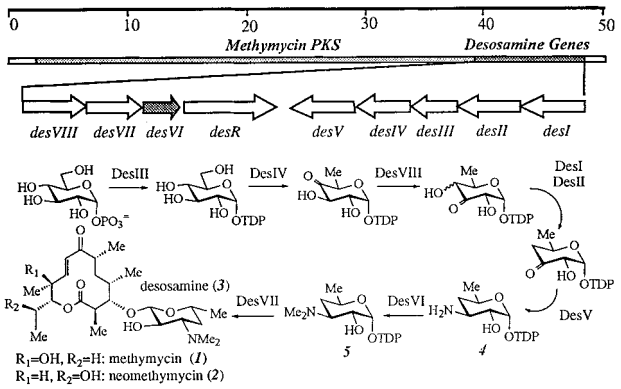
Received May 22, 1998

The emergence of pathogenic bacteria resistant to many commonly used antibiotics poses a serious threat to human health and has been the impetus of the present resurgent search for new antimicrobial agents.[1] Since the first report on using genetic engineering techniques to create "hybrid" polyketides,[2] the potential of manipulating the genes governing the biosynthesis of secondary metabolites to create new bioactive compounds, especially macrolide antibiotics, has received much attention.[3] This class of clinically important drugs consists of two essential structural components, a polyketide aglycone and the appended deoxy sugars.[4] The aglycone is synthesized via sequential condensations of acyl thioesters catalyzed by a highly organized multienzyme complex, polyketide synthase (PKS).[5] Recent advances in our understanding of polyketide biosynthesis have allowed recombination of the PKS genes to construct an impressive array of novel skeletons.[3,5,6] Without the sugar components, however, these new compounds are usually biologically impotent. Hence, if one plans to make new macrolide antibiotics by a combinatorial biosynthetic approach, two immediate challenges must be overcome, assembling a repertoire of novel sugar structures and then having the capacity to couple these sugars to the structurally diverse macrolide aglycones.

Unfortunately, our knowledge of the formation of the unusual sugars in these antibiotics remains limited.[7] Part of the reason for this comes from the fact that the sugar genes are generally scattered at both ends of the PKS genes. Such an organization within the macrolide biosynthetic gene cluster makes it difficult to distinguish the sugar genes from those encoding regulatory proteins or aglycone modification enzymes that are also interspersed in the same regions. The task can be made even more formidable if the macrolides contain multiple sugar components. In view of the "scattered" nature of the sugar biosynthetic genes, the antibiotic methymycin (**1**) and its cometabolite, neomethymycin (**2**), of *Streptomyces venezuelae* present themselves as an attractive system to study the formation of deoxy sugars.[8] First, they carry D-desosamine (**3**), a prototypical aminodeoxy sugar that also exists in erythromycin. Second, since desosamine is the only sugar attached to the macrolactone of **1** and **2**, identification of the sugar biosynthetic genes within the methymycin/neomethymycin gene cluster should be possible with much more certainty. Thus, we have cloned and sequenced the methymycin/neomethymycin gene cluster, which is about 60 kb in length.[9] Interestingly, a 13 kb stretch of DNA downstream from the PKS genes was found to harbor the entire desosamine biosynthetic gene cluster. Among the nine open reading frames (ORFs) mapped in this segment, eight are believed to be involved in desosamine formation, while the remaining one, *desR*, encodes a macrolide β-glycosidase that may be involved in a glycosylation−deglycosylation self-protection mechanism.[10] Their identities, shown in Scheme 1, are assigned on the basis of sequence similarities to other sugar biosynthetic genes, especially those derived from the erythromycin cluster.[11] While part of the scheme is still ambiguous because the function of several genes and part of the reaction sequence cannot yet be clearly defined, the incorporation of the initial and the final few steps in the proposed pathway is well-founded on literature precedent and mechanistic intuition for the construction of aminodeoxy sugars.[7]

With these genes in hand, we decided to explore whether new methymycin/neomethymycin analogues carrying modified sugars could be generated by altering the desosamine biosynthetic genes. The *desVI* gene, which has been predicted to encode the *N*-methyltransferase,[11] was chosen as our initial target for two reasons. First, deletion of *desVI* should have little polar effect[12] on the expression of other desosamine biosynthetic genes because

Scheme 1



[†] Department of Chemistry.
[‡] Department of Microbiology and Biological Process Technology Institute.

(1) (a) Box, R. P. *Clin. Infect. Dis.* **1997**, *24*, S151. (b) Davies, J. *Nature* **1996**, *383*, 219−220. (c) Service, R. F. *Science* **1995**, *270*, 724−727.

(2) Hopwood, D. A.; Malpartida, F.; Kieser, H. M.; Ikeda, H.; Duncan, J.; Fujii, I.; Rudd, B. A.; Floss, H. G.; Omura, S. *Nature* **1985**, *314*, 642−644.

(3) (a) Kramer, P. J.; Khosla, C. *Annu. N. Y. Acad. Sci*. **1996**, *799*, 32−45. (b) Khosla, C.; Zawada, R. J. *Trends Biotechnol*. **1996**, *14*, 335−341.

(4) *Macrolide Antibiotics, Chemistry, Biology, and Practice*, Omura, S., Ed.; Academic Press: New York; 1984.

(5) (a) Hopwood, D. A.; Sherman, D. H. *Annu. Rev. Genet.* **1990**, *24*, 37−66. (b) Katz, L.; Donadio, S. *Annu. Rev. Microbiol*. **1993**, *47*, 875−912. (c) Hutchinson, C. R.; Fujii, I. *Annu. Rev. Microbiol*. **1995**, *49*, 201−238. (d) Carreras, C. W.; Pieper, R.; Khosla, C. In *Bioorganic Chemistry Deoxysugars, Polyketides & Related Classes: Synthesis, Biosynthesis, Enzymes*; Rohr, J. Ed.; Springer: Berlin; 1997, 85−126.

(6) (a) Epp, J.; Huber, M. L. B.; Tuner, J. R.; Goodson, T.; Schoner, B. E. *Gene* **1989**, *85*, 293−301. (b) Donadio, S.; McAlpine, J. B.; Sheldon, P. J.; Jackson, M.; Katz, L. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 7119−7123. (c) Arisawa, A.; Kawamura, N.; Takeda, K.; Tsunekawa, H.; Okamura, K.; Okamoto, R. *Appl. Environ. Microbiol*. **1994**, *60*, 2657−2660. (d) Jacobsen, J. R.; Hutchinson, C. R.; Cane, D. E.; Khosla, C. *Science* **1997**, *277*, 367−369. (e) Marsden, A. F. A.; Wilkinson, B.; Cortés, J.; Dunster, N. J.; Staunton, J.; Leadlay, P. F. *Science* **1998**, *279*, 199−201.

(7) (a) Liu, H.-w.; Thorson, J. S. *Annu. Rev. Microbiol*. **1994**, *48*, 223−256. (b) Kirschning, A.; Bechthold, A. F.-W.; Rohr, J. In *Bioorganic Chemistry Deoxysugars, Polyketides & Related Classes: Synthesis, Biosynthesis, Enzymes*; Rohr, J. Ed.; Springer: Berlin; 1997, 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; in press.

(8) (a) Donin, M. N.; Pagano, J.; Dutcher, J. D.; McKee, C. M. *Antibiot. Annu*. **1953**−**1954**, *1*, 179−185. (b) Djerassi, C.; Zderic, J. A. *J. Am. Chem. Soc*. **1956**, *78*, 6390−6395.

(9) Xue, Y., Zhao, L.; Liu, H.-w.; Sherman, D. H. *Proc. Natl. Acad. Sci. U.S.A.*, in press.

(10) (a) Zhao, L.; Sherman, D. H.; Liu, H.-w. *J. Am. Chem. Soc*., in press. (b) Quiros, L. M.; Aquirrezabalaga, I.; Olano, C.; Méndez, C.; Salas, J. A. *Mol. Microbiol.* **1998**, *28*, 1177−1185. (c) Salah-Bey, K.; Doumith, M.; Michel, J.-M.; Haydock, S.; Cortés, J.; Leadlay, P. F.; Raynal, M.-C. *Mol. Gen. Genet*. **1998**, *257*, 542−553.

(11) (a) Gaisser, S.; Bohm, G. A.; Cortés, J.; Leadlay, P. F. *Mol. Gen. Genet*. **1997**, *256*, 239−251. (b) Summers, R. G.; Donadio, S.; Staver, M. J.; Wendt-Pienkowski, E.; Hutchinson. C. R.; Katz, L. *Microbiology* **1997**, *143*, 3251−3262.

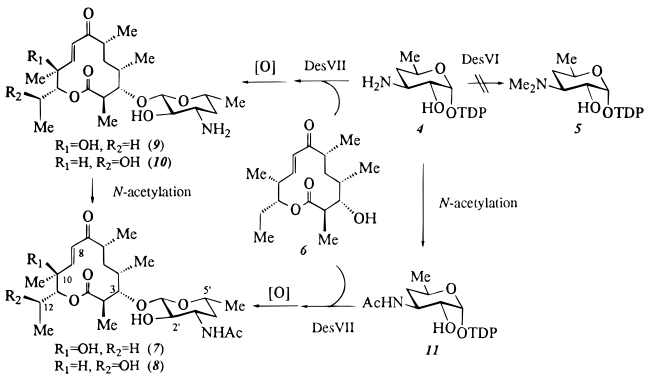
(12) Lin, E. C. C.; Goldstein, R.; Syvanen, M. *Bacteria, Plasmids, and Phages, An Introduction to Molecular Biology*; Harvard University Press: Cambridge; 1984; p 123.

S0002-7863(98)01782-X CCC: $15.00 © 1998 American Chemical Society
Published on Web 09/17/1998

the ORF (*desR*) lying immediately downstream from *desVI* is not directly involved in desosamine formation,[10a] and those lying further downstream are transcribed in the opposite direction. Second, since N,N-dimethylation is almost certainly the last step in the desosamine biosynthetic pathway,[7,11] perturbing this step may lead to the accumulation of **4**, which stands the best chance among all of the other intermediates of being recognized by the glycosyltransferase (DesVII) for successful linkage to the macrolactone **6**.[13] Thus, a plasmid pBL3001, in which *desVI* was replaced by the thiostrepton resistance gene (*tsr*),[14] was constructed and introduced into wild type *S. venezuelae* by conjugal transfer using *Escherichia coli* S17-1.[15] Two identical double crossover mutants, KdesVI-21 and KdesVI-22 with phenotypes of thiostrepton resistance (Thio^R) and apramycin sensitivity (Apm^S) were obtained. Southern blot hybridization using *tsr* or a 1.1 kb *Hin*cII fragment from the *desVII* region further confirmed that the *desVI* gene was indeed replaced by *tsr* on the chromosome of these mutants. The KdesVI-21 mutant was first grown at 29 °C in seed medium (100 mL) for 48 h and then inoculated and grown in vegetative medium (3 L) for another 48 h.[16] The fermentation broth was centrifuged to remove the cellular debris and mycelia, and the supernatant was adjusted to pH 9.5 with concentrated KOH, followed by extraction with chloroform. No methymycin or neomethymycin was found; instead, the 10-deoxymethynolide **6** (350 mg)[17] and two new macrolides containing an N-acetylated amino sugar, **7** (20 mg) and **8** (15 mg), were isolated. Their structures were determined by spectral analyses and high-resolution MS.[18]

The fact that compounds **7** and **8** bearing modified desosamine are produced by the *desVI*-deletion mutant is an exciting discovery.[13] However, this result is also somewhat surprising since the sugar component in the products is expected to be the aminodeoxy hexose **4**. As illustrated in Scheme 2, it is possible that **7** and **8** are derived from the predicted **9** and **10**, respectively, by a postsynthetic nonspecific acetylation of the attached aminodeoxy sugar. It is also conceivable that N-acetylation of **4** occurs first, followed by coupling of the resulting sugar **11** to the 10-deoxymethynolide **6**. Although the exact sequence of events remains to be determined, the lack of N-methylation of the sugar component in these new products provides convincing evidence, sustaining the assignment of *desVI* as the *N*-methyltransferase gene. Most significantly, the production of **7** and **8** by the *desVI*-deletion mutant attests to the fact that the glycosyltransferase (DesVII) in methymycin/neomethymycin pathway is capable of recognizing and processing sugar substrates other than TDP-desosamine (**5**). Since both **7** and **8** are new compounds synthesized in vivo by the *S. venezuelae* mutant strain, the observed N-acetylation might be a necessary step for self-protection.[19,20] Indeed, purified **7** and **8** are inactive against *Streptococcus pyogenes* grown on Mueller−Hinton agar plates,[21] while the controls (**1** and **2**) show clearly visible inhibition zones.

Scheme 2



It should be pointed out that a few glycosyltransferases involved in the biosynthesis of antibiotics have been shown to have relaxed specificity toward modified aglycones.[6d,e,22] However, a similar relaxed specificity toward sugar substrates has only been reported for the glycosyltransferases in the daunorubicin[23] and erythromycin[11c,24] pathways, in which these enzymes are able to recognize modified sugars and catalyze their coupling to the respective aglycones. Thus, the fact that the methymycin/neomethymycin glycosyltransferase can also tolerate structural variants of its sugar substrate is an encouraging result. If such a property holds true for glycosyltransferases from other antibiotic biosynthetic pathways, the promise of creating biologically active hybrid natural products by genetic engineering may be one step closer to being realized.[25]

Acknowledgment. This work was supported in part by the National Institutes of Health grants GM35906, GM54346 (both to H.-w.L.), and GM48562 (to D.H.S.). D.H.S. also thanks the Office of Naval Research for a grant.

Supporting Information Available: Characterization data of the new compounds **7** and **8**, including ^{1}H and ^{13}C NMR spectra, high-resolution FABMS results, and the complete spectral assignments (6 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

JA981782N

(13) It is well-known that deletion and/or disruption of a single biosynthetic gene often affects the pathway at more than one specific step. In fact, disruption of *eryCVI*, the *desVI* equivalent in the erythromycin cluster, which has been predicted to encode a similar *N*-methylase to make desosamine in erythromycin,[11] led to the accumulation of an intermediate devoid of the entire desosamine moiety.[11a,b] Thus, targeting the *desVI* gene in this study should not be perceived as a conservative approach, especially considering the potential toxicity of any new products toward the producing microorganism.

(14) Bibb, M. J.; Bibb, M. J.; Ward, J. M.; Cohen, S. N. *Mol. Gen. Genet.* **1985**, *199*, 26−36.

(15) Bierman, M.; Logan, R.; O'Brien, K.; Seno, G.; Nagaraja, R.; Schoner, B. E. *Gene* **1992**, *116*, 43−49.

(16) Cane, D. E.; Lambalot, R. H.; Prabhakaran, P. C.; Ott, W. R. *J. Am. Chem. Soc.* **1993**, *115*, 522−526.

(17) Lambalot, R. H.; Cane, D. E. *J. Antibiot.* **1992**, *45*, 1981−1982.

(18) See the Supporting Information for details.

(19) Cundliffe, E. C. *Annu. Rev. Microbiol.* **1989**, *43*, 207−233.

(20) This result clearly pointed out that how to minimize the potential toxicity associated with new macrolide antibiotics produced by the genetically engineered microorganisms and how to activate the newly formed antibiotics that have been deactivated (either deliberately or not) during production should be part of the overall strategy for the development of novel antibiotics using the combinatorial biosynthetic approach. Experiments toward these goals are being actively pursued.

(21) Mangahas, F. R. M.S. Thesis, University of Minnesota, 1996.

(22) (a) Weber, J. M.; Leung, J. O.; Swanson, S. J.; Idler, K. B.; McAlpine, J. B. *Science* **1991**, *252*, 114−117. (b) Decker, H.; Haag, S.; Udvarnoki, G.; Rohr, J. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1107−1110.

(23) 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*, 69−74.

(24) Gaisser, S.; Bohm, G. A.; Doumith, M.; Raynal, M.-C.; Dhillon, N.; Cortés, J.; Leadlay, P. F. *Mol. Gen. Genet.* **1998**, *258*, 78−88.

(25) A relaxed substrate specificity has also been noted for the glycosyltransferases involved 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*, 195−202).