Engineering a Methymycin/Pikromycin−Calicheamicin Hybrid: Construction of Two New Macrolides Carrying a Designed Sugar Moiety

Lishan Zhao,[†] Joachim Ahlert,[§] Yongquan Xue,[‡] Jon S. Thorson,[§] David H. Sherman,[‡] and Hung-wen Liu*,[†]

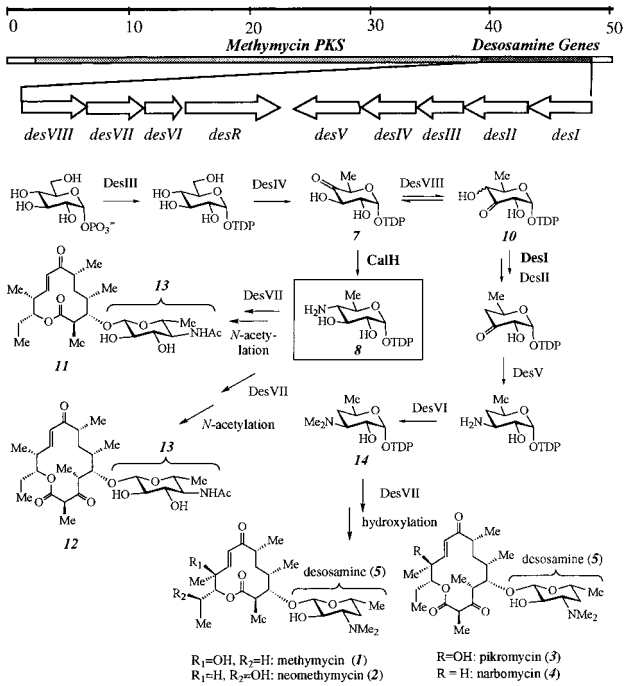
Departments of Chemistry and Microbiology and Biological Process Technology Institute University of Minnesota, Minneapolis, Minnesota 55455 Laboratory for Biosynthetic Chemistry Memorial Sloan-Kettering Cancer Center 1275 York Avenue, Box 309, New York, New York 10021

Received August 6, 1999

Nature continues to be the inspiration for most pharmaceutical drug leads, and given the synthetic challenge posed by many complex secondary metabolites, the emerging field of combinatorial biosynthesis has become a rich new source for modified non-natural scaffolds.[1] Yet, many naturally occurring bioactive secondary metabolites possess unusual carbohydrate ligands, which serve as molecular recognition elements critical for biological activity.[2] Without these essential sugar attachments, the biological activities of most clinically important secondary metabolites are either completely abolished or dramatically decreased. We and others have demonstrated that glycosyltransferases, responsible for the final glycosylation of certain secondary metabolites, show a high degree of promiscuity toward the nucleotide sugar donor.[3,4] These discoveries have opened the door to the possibility of manipulating the corresponding biosynthetic pathways for modifying the crucial glycosylation pattern of natural, or non-natural, secondary metabolite scaffolds in a combinatorial fashion. To date, the genetic manipulation of the carbohydrate appendage for any given metabolite has been limited to alterations and/or knock-outs of the small subset of genes required to construct and attach each desired carbohydrate moiety. However, a significant expansion of the saccharide structure diversity obtained by these methods might be accomplished via the recruitment and collaborative action of sugar genes from a variety of biosynthetic pathways to construct composite clusters with the potential to make and attach non-natural sugars.

To test this possibility, we selected the *Streptomyces venezuelae* methymycin/pikromycin gene cluster as the parent system and a gene from the calicheamicin biosynthetic gene cluster (from *Micromonospora echinospora* spp. *Calichensis*) as the foreign collaborator gene. The parent cluster encodes the biosynthetic enzymes for methymycin (**1**), neomethymycin (**2**), pikromycin (**3**), and narbomycin (**4**), all of which are macrolides containing desosamine (**5**) as the sole sugar component crucial for antibiotic activity.[5] Eight open reading frames (*desI*−*desVIII*) in this cluster have been assigned as genes involved in desosamine biosynthesis (Scheme 1). The antitumor agent calicheamicin (**6**) contains four unique sugars crucial to tight DNA binding ($K_a \approx 10^6-10^8$), one of which (**9**) is derived from 4-amino-4,6-dideoxyglucose (**8**) and is part of the unusually restricted N−O connection between sugars A and B (Scheme 2).[6] Compound **8** is anticipated to be derived from the corresponding 4-ketosugar **7** via a transamination reaction, and recent work has led to the assignment of a gene (*calH*) as encoding the desired C-4 aminotransferase (Scheme 2).[7] Interestingly, the proposed substrate for CalH, **7**, is also an intermediate in the desosamine pathway and is expected to exist

Scheme 1



[†] Department of Chemistry, University of Minnesota.

[‡] Department of Microbiology and Biological Process Technology Institute, University of Minnesota.

[§] Memorial Sloan-Kettering Cancer Center.

(1) (a) Katz, L.; Donadio, S. *Annu. Rev. Microbiol.* **1993**, *47*, 875−912. (b) Hutchinson, C. R.; Fujii, I. *Annu. Rev. Microbiol.* **1995**, *49*, 201−238. Jacobsen, J. R.; Hutchinson, C. R.; Cane, D. E.; Khosla, C. *Science* **1997**, *277*, 367−369. (d) Cane, D. E.; Walsh, C. T.; Khosla, C. *Science* **1998**, *282*, 63−68. (e) Marsden, A. F. A.; Wilkinson, B.; Cortés, J.; Dunster, N. J.; Staunton, J.; Leadlay, P. F. *Science* **1998**, *279*, 199−201. (f) McDaniel, R.; Thamchaipenet, A.; Gustafsson, C.; Fu, H.; Betlach, M.; Betlach, M.; Ashley, G. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1846−1851.

(2) (a) *Macrolide Antibiotics, Chemistry, Biology and Practice*; Omura, S. Ed.; Academic Press: New York; 1984. (b) Weymouth-Wilson, A. C. *Nat. Prod. Rep*. **1997**, *14*, 99−110.

(3) Targeted deletion/disruption of the *desVI*, *desV*, or *desI* genes in the methymycin/pikromycin cluster has led to the production of methymycin/pikromycin derivatives carrying, instead of desosamine, 3-acetamido-3,4,6-trideoxyglucose, 4,6-dideoxyglucose, or 6-deoxyglucose (quinovose) as the appended sugar by the respective mutants [(a) Zhao, L.; Sherman, D. H.; Liu, H.-w. *J. Am. Chem. Soc*. **1998**, *120*, 10256-10257. (b) Zhao, L.; Que, N. L. S.; Xue, Y.; Sherman, D. H.; Liu, H.-w. *J. Am. Chem. Soc.* **1998**, *120*, 12159−12160. (c) Borisova, S. A.; Zhao, L.; Sherman, D. H.; Liu, H.-w. *Org. Lett.* **1999**, *1*, 133−136].

(4) A similar relaxed specificity toward sugar substrates has also been reported for the glycosyltransferases in the biosynthesis of a few other secondary metabolites (see the following examples: Weber, J. M.; Leung, J. O.; Swanson, S. J.; Idler, K. B.; McAlpine, J. B. *Science* **1991**, *252*, 114−117. Decker, H.; Haag, S.; Udvarnoki, G.; Rohr, J. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1107−1110. Sasaki, J.; Mizoue, K.; Morimoto, S.; Omura, S. *J. Antibiot.* **1996**, *49*, 1110−1118. Solenberg, P. J.; Matsushima, P.; Stack, D. R.; Wilkie, S. C.; Thompson, R. C.; Baltz, R. H. *Chem. Biol.* **1997**, *4*, 195−202. 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 Biotechnol.* **1998**, *16*, 69−74. Salah-Bey, K.; Doumith, M.; Michel, J.-M.; Haydock, S.; Cortes, J.; Leadlay, P. F.; Raynal, M.-C. *Mol. Gen. Genet.* **1998**, *257*, 542−553. Gaisser, S.; Bohm, G. A.; Doumith, M.; Raynal, M.-C.; Dhillon, N.; Cortés, J.; Leadlay, P. F. *Mol. Gen. Genet.* **1998**, *258*, 78−88. Wohlert, S.-E.; Blanco, G.; Lombo, F.; Fernandez, E.; Brana, A. F.; Reich, S.; Udvarnoki, G.; Mendez, C.; Decker, H.; Frevert, J.; Salas, J. A.; Rohr, J. *J. Am. Chem. Soc.* **1998**, 120, 10596−10601).

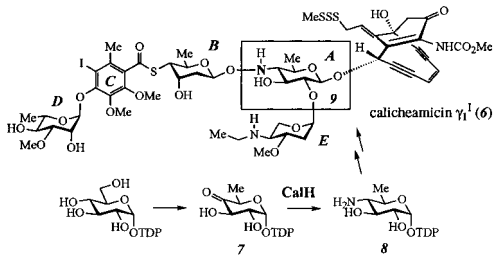
(5) (a) Xue, Y.; Zhao, L.; Liu, H.-w.; Sherman, D. H. *Proc. Natl. Acad. Sci. U.S.A.*. **1998**, *95*, 12111−12116. (b) Zhao, L.; Sherman, D. H.; Liu, H.-w. *J. Am. Chem. Soc*. **1998**, *120*, 9374−9375.

(6) (a) Ding, W.-d.; Ellestad, G. A. *J. Am. Chem. Soc*. **1991**, *113*, 6617. (b) Drak, J.; Iawasawa, S.; Danishefsky, S.; Crothers, D. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 7464. (c) Walker, S.; Yang, D.; Kahne, D.; Gange, D. *J. Am. Chem. Soc.* **1991**, *113*, 4716−4717. (d) Ellestad, G. A.; Ding, W.-D.; Zein, N.; Townsend, C. A. In *Calicheamicins: DNA-Cleaving Properties of Calicheamicin* γ_1^I; Borders, D. B., Doyle, T. W., Eds.; Marcel Dekker: New York, 1995; pp 137−160.

(7) Ahlert, J.; Biggins, J. B.; Thorson, J. S. Unpublished results.

10.1021/ja992810k CCC: $18.00
Published on Web 10/06/1999

Scheme 2



in a tautomerase (DesVIII)-mediated equilibrium with **10**.[8] Thus, it is conceivable that **7** might accumulate in a *desI* or *desVIII* disruption/deletion *S. venezuelae* mutant strain. Heterologous expression of *calH* in this mutant may reconstitute a hybrid pathway toward new methymycin/pikromycin derivatives which carry the 4-amino-4,6-dideoxyglucose derived from **6**.

To test this idea, the 1.2 kb *calH* gene was amplified by polymerase chain reaction (PCR) from pJST1192$_{Kpn7.0Kb}$, a subclone containing a 7.0 kb *Kpn*I fragment of cosmid 13a.[9] The amplified gene was cloned into the *Eco*RI/*Xba*I sites of the expression vector pDHS617, which contains an apramycin resistance marker.[10] The resulting plasmid, pLZ-C242, was introduced by conjugal transfer using *Escherichia coli* S 17−1[11] into a previously constructed *S. venezuelae* mutant (KdesI),[3c] in which *desI* was replaced by the neomycin resistance gene that also confers resistance to kanamycin. The pLZ-C242-containing *S. venezuelae*-KdesI colonies were identified on the basis of their resistance to apramycin antibiotic (Apr^R). One of the engineered strains, KdesI/calH-1, was first grown in 100 mL of seed medium at 29 °C for 48 h and then inoculated and grown in vegetative medium (5 L) for another 48 h.[12] 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 chloroform extraction. The crude products (700 mg) were subjected to flash chromatography on silica gel using a gradient of 0−20% methanol in chloroform. A major product, 10-deoxymethynolide (ca. 400 mg), and a mixture of two minor macrolide compounds were obtained. The two macrolides were further purified by HPLC on a C_{18} column using an isocratic mobile phase of acetonitrile/H_2O (1:1). They were later identified as **11** (11.0 mg) and **12** (1.5 mg) by spectral analyses.[13]

The observed production of macrolides **11** and **12** by the KdesI/calH-1 has vast implications. First, the appended hexose (**13**), which indeed carries the predicted amino group at C-4, provides indisputable support for the *calH* gene assignment as encoding the TDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose 4-aminotransferase of the calicheamicin pathway. Second, the successful expression of the CalH protein in *S. venezuelae* by the newly constructed expression vector highlights the potential of using this system to express other foreign genes in this strain, a prerequisite for developing more elaborate combinatorial biosynthetic strategies. Moreover, this result also reveals that the glycosyltransferase (DesVII) of this pathway can recognize alternative sugar substrates (such as **8**) whose structures are considerably different from the original amino sugar substrate, TDP-D-desosamine (**14**).[3] While the sugar component in the products is expected to be the aminodeoxy hexose **8**, the 4-amino group of the attached sugar component in **11** and **12** is *N*-acetylated. It is not clear at this point whether the acetylation occurs on the free sugar or after it is appended to the aglycone. Since both **11** and **12** are new compounds synthesized in vivo by the *S. venezuelae* mutant strain, the observed *N*-acetylation might be a necessary step for self-protection.[14] Indeed, purified **11** and **12** are inactive against *Streptococcus pyogenes* grown on Mueller−Hinton agar plates,[15] while the controls (methymycin and pikromycin) show clearly visible inhibition zones.

Another noteworthy, albeit unexpected result was the fact that the aglycon of the isolated macrolide **11** was 10-deoxymethynolide instead of methymycin and neomethymycin analogues that are hydroxylated. Interestingly, the aglycon of **12** was the 14-membered narbonolide that is also devoid of hydroxylation. It is possible that the cytochrome P450 hydroxylase (PikC), which catalyzes the hydroxylation of 10-deoxymethynolide and narbonolide,[16] is sensitive to structural variations on the appended sugar. Indeed, no aglycon hydroxylation was discernible when **11** and **12** were incubated with purified PikC in vitro. A similar observation was also noted in the case where desosamine was replaced by quinovose.[3c] It could be argued that the presence of a substituent (either hydroxyl or amino group) at C-4 in the sugar moiety is responsible, at least in part, for decreasing or preventing hydroxylation of the macrolide.

In conclusion, the work presented is proof of principle that non-natural secondary metabolite glycosylation patterns can be engineered through a rational selection of heterologous gene combinations. This demonstrated ability to engage foreign enzymes in concert with the natural biosynthetic machinery offers a tremendous potential to generate further structural diversity. By extension of the present study, the construction of diverse nucleotide sugar glycosylation precursor pools may soon substantially enhance current novel drug discovery through combinatorial biosynthesis efforts.

Acknowledgment. This work was supported by grants from the National Institutes of Health (GM35906 and GM54346 to H.-w.L., GM58196 to J.S.T., and GM48562 to D.H.S.), the Rita Allen Foundation (J.S.T.), and the Mizutani Foundation for Glycoscience (J.S.T.). H.-w.L. also thanks the National Institute of General Medical Sciences for a MERIT Award. L.Z. is a recipient of a Graduate School Dissertation Fellowship of the University of Minnesota, and J.A. is a postdoctoral fellow of the Charles A. Dana and Norman and Rosita Winston Foundations.

JA992810K

(8) A similar tautomerization has been implicated in the mycaminose biosynthetic pathway (Chen, H.; Yeung, S.-M.; Que, N. L. S.; Müller, T.; Schmidt, R. R.; Liu, H.-w. *J. Am. Chem. Soc.* **1999**, *121*, 7166−7167).

(9) Thorson, J. S.; Shen, B.; Whitwam, R. E.; Liu, W.; Li, Y.; Ahlert, J. *Bioorg. Chem.* **1999**, *27*, 172−188.

(10) The plasmid pDHS617 is derived from pOJ446,[11] and a promoter sequence from the methymycin/pikromycin cluster[5a] was incorporated to drive the expression of foreign genes in *S. venezuelae*.

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

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

(13) Spectral data of **11**: ^{1}H NMR (500 MHz, $CDCl_3$, *J* in hertz) δ 6.75 (1H, dd, *J* = 16.0, 5.5, 9-H), 6.44 (1H, dd, *J* = 16.0, 1.2, 8-H), 5.34 (1H, d, *J* = 8.0, N−H), 4.96 (1H, m, 11-H), 4.27 (1H, d, *J* = 7.5, 1′-H), 3.66 (1H, dd, *J* = 9.5, 8.0, 4′-H), 3.60 (1H, d, *J* = 10.5, 3-H), 3.50 (1H, t, *J* = 9.5, 3′-H), 3.4 (1H, m, 5′-H), 3.4 (1H, m, 2′-H), 2.84 (1H, dq, *J* = 10.5, 7.5, 2-H), 2.64 (1H, m, 10-H), 2.53 (1H, m, 6-H), 2.06 (3H, s, Me−C═O), 1.7 (1H, m, 12-H), 1.66 (1H, m, 5-H), 1.56 (1H, m, 12-H), 1.4 (1H, m, 5-H), 1.36 (3H, d, *J* = 7.5, 2-Me), 1.25 (3H, d, *J* = 6.5, 5′-Me), 1.24 (1H, m, 4-H), 1.21 (3H, d, *J* = 7.5, 6-Me), 1.10 (3H, d, *J* = 6.5, 10-Me), 0.99 (3H, d, *J* = 6.0, 4-Me), 0.91 (3H, t, *J* = 7.2, 12-Me); ^{13}C NMR (125 MHz, $CDCl_3$) δ 205.3 (C-7), 175.1 (C-1), 171.9 (Me−*C*═O), 147.1 (C-9), 126.1 (C-8), 103.0 (C-1′), 85.8 (C-3), 75.8 (C-5′), 75.8 (C-3′), 74.1 (C-11), 70.8 (C-2′), 57.6 (C-4′), 45.3 (C-6), 44.0 (C-2), 38.1 (C-10), 34.2 (C-5), 33.6 (C-4), 25.4 (C-12), 23.7 (*Me*−C═O), 18.1 (C-6′), 17.9 (6-Me), 17.6 (4-Me), 16.4 (2-Me), 10.5 (12-Me), 9.8 (10-Me). High-resolution FAB-MS calculated for $C_{25}H_{42}NO_8$ (M + H$^+$) 484.2910, found 484.2903. Spectral data of **12**: ^{1}H NMR (500 MHz, $CDCl_3$, *J* in hertz) δ 6.69 (1H, dd, *J* = 16.0, 6.0, 11-H), 6.09 (1H, dd, *J* = 16.0, 1.5, 10-H), 5.35 (1H, d, *J* = 8.5, N−H), 4.96 (1H, m, 13-H), 4.36 (1H, d, *J* = 7.5, 1′-H), 4.19 (1H, m, 5-H), 3.83 (1H, q, *J* = 6.5, 2-H), 3.68 (1H, dt, *J* = 10.0, 8.5, 4′-H), 3.52 (1H, t, *J* = 8.5, 3′-H), 3.50 (1H, m, 5′-H), 3.42 (1H, t, *J* = 7.5, 2′-H), 2.92 (1H, dq, *J* = 7.0, 5.0, 4-H), 2.81 (1H, m, 8-H), 2.73 (1H, m, 12-H), 2.06 (3H, s, Me−C═O), 1.8 (1H, m, 6-H), 1.6 (1H, m, 14-H), 1.55 (1H, m, 7-H), 1.37 (3H, d, *J* = 6.5, 2-Me), 1.32 (3H, d, *J* = 7.0, 4-Me), 1.3 (1H, m, H-14), 1.27 (3H, d, *J* = 6.5, 5′-Me), 1.25 (1H, m, 7-H), 1.12 (3H, d, *J* = 6.0, 8-Me), 1.11 (3H, d, *J* = 6.5, 12-Me), 1.07 (3H, d, *J* = 6.0, 6-Me), 0.91 (3H, t, *J* = 7.2, 14-Me); high-resolution FAB-MS calculated for $C_{28}H_{46}NO_9$ (M + H$^+$) 540.3172, found 540.3203.

(14) (a) Cundliffe, E. In *Self-Protection Mechanisms in Antibiotic Producers*; Cundliffe, E., Ed.; Wiley: Chichester, 1992; pp 199−214. (b) McManus, M. C. *Am. J. Health-Syst. Pharm.* **1997**, *54*, 1420−1433.

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

(16) Xue, Y.; Wilson, D.; Zhao, L.; Liu, H.-w.; Sherman, D. H. *Chem. Biol.* **1998**, *5*, 661−667.