Engineering a Hybrid Sugar Biosynthetic Pathway: Production of L-Rhamnose and Its Implication on Dihydrostreptose Biosynthesis

Hiroshi Yamase, Lishan Zhao, and Hung-wen Liu*

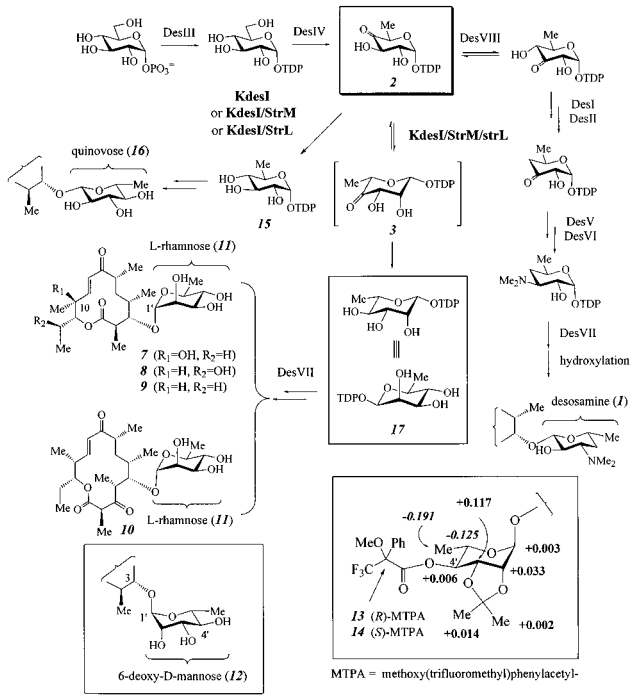
Department of Chemistry, University of Minnesota Minneapolis, Minnesota 55455
Division of Medicinal Chemistry, College of Pharmacy Department of Chemistry and Biochemistry University of Texas, Austin, Texas 78712

Received August 31, 2000

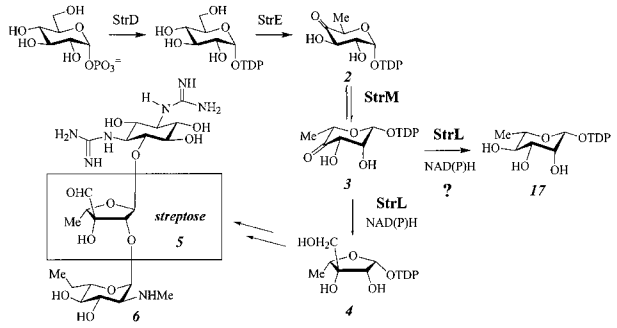
While many methods have been developed to find pharmaceutical drug leads, the combinatorial biology approach, which involves direct manipulation of the biosynthetic machinery of secondary metabolites, has emerged as a promising means to gain access to new chemical entities.[1] Indeed, disruption of the sugar biosynthetic genes in the methymycin/pikromycin gene cluster of *Streptomyces venezuelae*[2] and recruitment of genes from other pathways into the cluster[3] have succeeded in the generation of new macrolides carrying modified sugars. However, all of the carbohydrate appendages produced so far by genetic manipulation of the *S. venezuelae* system are D-sugars, whose global structures still resemble that of D-desosamine found in the parent compounds. A significant expansion of the saccharide structural diversity can thus be achieved if various L-sugars are incorporated into these macrolides. To assess the feasibility of this idea, *S. venezuelae* strains containing selected genes from the L-dihydrostreptose pathway were constructed and studied. Reported herein are the results of these experiments and the biosynthetic implications deduced for the dihydrostreptose pathway.

S. venezuelae is a producer of two 12-membered polyketides, methymycin and neomethy-mycin, and two 14-membered co-metabolites, pikromycin and narbomycin, all of which contain D-desosamine (**1**) as the sole sugar component.[4] Eight open reading frames, *desI*−*desVIII*,[5] in the methymycin/pikromycin cluster have been assigned as genes involved in desosamine biosynthesis (Scheme 1). Interestingly, the 6-deoxy-4-hexulose **2** in this pathway has also been suggested as a biosynthetic intermediate for TDP-L-dihydrostreptose (**4**), the precursor of streptose (**5**) found in the antibiotic streptomycin (**6**) of *Streptomyces griseus* (Scheme 2).[6] With the tentative assignment of genes in the streptomycin cluster,[7] a biosynthetic pathway for TDP-L-dihydrostreptose has been postulated. As illustrated in Scheme 2, the *strM* gene may encode a 3,5-epimerase responsible for the conversion of **2** to **3**, while the product of *strL* gene is speculated

Scheme 1



Scheme 2



to catalyze the ring contraction of **3** to give **4**.[7] Since the proposed substrate for StrM, **2**, is also an intermediate in the desosamine pathway, heterologous expression of StrM, StrL, or StrM/StrL in the *S. venezuelae desI*-mutant in which **2** accumulates, may reconstitute hybrid pathways toward new methymycin/pikromycin derivatives carrying an L-pyranose or an L-furanose.

In these experiments, the *strM* (0.8 kb) and *strL* (1.0 kb) genes were separately amplified by polymerase chain reaction (PCR) from the genomic DNA of *S. griseus*. The amplified *strM* gene was cloned into the *Eco*RI/*Nsi*I sites of the expression vector pDHS702,[8] which contains a thiostrepton resistance marker. The *strL* gene was cloned into the *Eco*RI/*Xba*I sites of the vector pDHS617,[3] which has an apramycin resistance marker. Each plasmid was transformed into *Escherichia coli* S 17−1[9] and then introduced separately by conjugal transfer into the previously constructed mutant *S. venezuelae* KdesI.[2c] The resulting strains, KdesI/strM and KdesI/strL, were identified on the basis of their resistance to the corresponding antibiotics. Using the same strategy, the *strL*-containing plasmid was further engineered into

(1) Katz, L.; Donadio, S. *Annu. Rev. Microbiol.* **1993**, *47*, 875−912. (b) Hutchinson, C. R.; Fujii, I. *Annu. Rev. Microbiol.* **1995**, *49*, 201−238. (c) 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) 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.

(3) Zhao, L.; Ahlert, J.; Xue, Y.; Thorson, J. S.; Sherman, D. H.; Liu, H.-w. *J. Am. Chem. Soc.* **1999**, *121*, 9881−9882.

(4) 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.

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

(6) Ortmann, P.; Matern, U.; Grisebach, H.; Stadler, P.; Sinnwell, V.; Paulsen, H. *Eur. J. Biochem.* **1974**, *43*, 265−271. (b) Wahl, P.; Matern, U.; Grisebach, H. *Biochem. Biophys. Res. Commun.* **1975**, *64*, 1041−1045. (c) Maier, S.; Matern, U.; Grisebach, H. *FEBS Lett.* **1975**, *49*, 317−319. (d) Wahl, H. P.; Grisebach, H. *Biochim. Biophys. Acta* **1979**, *568*, 243−252.

(7) Pisowotzki, K.; Mansori, K.; Piepersberg, M. *Mol. Gen. Genet.* **1991**, *231*, 113−123. (b) Distler, J.; Mansouri, K.; Mayer, G.; Stockmann, M.; Piepersberg, W. *Gene* **1992**, *115*, 105−111.

(8) Xue, Y.; Sherman, D. H. *Nature* **2000**, *403*, 571−575.

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

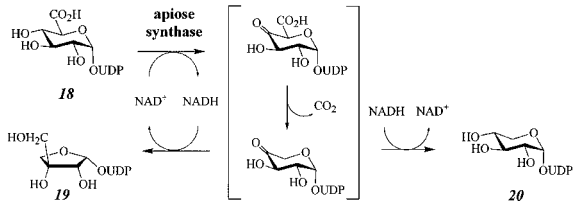
10.1021/ja003246c CCC: $19.00
Published on Web 11/23/2000

the KdesI/strM mutant to produce the recombinant strain KdesI/strM/strL, which confers resistance to both apramycin and thiostrepton. One such strain, KdesI/strM/strL-8, was chosen to grow in 150 mL of seed medium at 29 °C for 48 h, and then inoculated and grown in vegetative medium (6 L) for another 48 h.[10] The fermentation broth was centrifuged, and the supernatant was extracted with chloroform. After concentration, the residual yellow oil (1.5 g) was subjected to flash chromatography on silica gel using 10% methanol in chloroform as eluent. The crude products were further purified by HPLC on a C_{18} column eluted with a linear gradient of 0−50% acetonitrile in water over 20 min to yield four new macrolide derivatives, **7** (31.1 mg), **8** (6.3 mg), **9** (3.0 mg), and **10** (3.9 mg).

Spectral analysis of these compounds revealed that **7**−**9** are 12-membered macrolide derivatives, while **10** is a 14-membered macrolide.[11] Interestingly, the stereochemistry of the linkage between the aglycone and the appended sugar in **7**−**10** was established to be α ($J_{1'2'} = 0-0.5$ Hz), which is distinct from the β-glycosidic linkage (d, $J_{1'2'} = 6.5-7.5$ Hz) found in the wild-type structures. While these new compounds all carry an identical 6-deoxyhexose, the NMR data could not distinguish whether the appended sugar is L-rhamnose (**11**) or its enantiomer, 6-deoxy-D-mannose (**12**). To unambiguously identify the newly incorporated sugar, **7** was treated with dimethoxypropane followed by derivatization with (*S*)- or (*R*)-MTAP chloride to generate the corresponding Mosher's esters (**13** and **14**). Since the orientation of the phenyl ring of MTAP is different in these two diastereomers, the protons adjacent to MTAP will experience differential shielding, depending on their spatial relationship with respect to the anisotropic cone of the aryl group.[12] On the basis of this well documented phenomenon, the absolute stereochemistry of the chiral center (C-4′) can be deduced from the difference in the chemical shifts measured as $\Delta\delta = \delta_{(S)-\text{MTPA ester}} - \delta_{(R)-\text{MTPA ester}}$. As shown in the bottom of Scheme 1, positive $\Delta\delta$ values were observed for 1′−H, 2′−H, 3′−H, 4′−H, and the two methyl signals of the acetonide group, while negative values were recorded for 5′−H and 5′-Me. These findings are indicative of an *S* configuration at C-4′, allowing the attached sugar in **7**−**10** to be assigned as α-L-rhamnose.

With the identification of L-rhamnose (**11**) as the sugar component of metabolites **7**-**10** produced by the engineered KdesI/strM/strL strain, the assignment of StrM to be a 3,5-epimerase converting **2** to **3** can now be considered as definitive.[13] The corresponding methymycin/pikromycin derivatives carrying a β-linked D-quinovose (**16**, 6-deoxy-D-glucose) were produced by the KdesI/strL strain (Scheme 1). These quinovose-containing macrolides were also found as metabolites of the host strain, *S. venezuelae* KdesI[2c] and the KdesI/strM strain. Since the substrate of StrL is expected to be **3**, in the absence of StrM to catalyze the necessary D-/L-conversion of **2** to provide **3**, it is not surprising that both KdesI/strL and KdesI strains produce the same macrolide compounds as observed. The fact that no new macrolide products were found in the broth of the KdesI/strM strain may be attributed to the instability of **3** in vivo, or the inability of the glycosyltransferase DesVII to process **3** as a substrate.[14] Apparently, the host strain of *S. venezuelae* KdesI contains a pathway-independent D-hexulose reductase that can reduce **2** to TDP-D-quinovose (**15**),[2c] but lacks an L-hexulose reductase of its own to reduce **3**. Since L-rhamnose is formed only in the *strL*-containing strain, one can conclude that, in addition to its putative function as dihydrostreptose synthase, StrL could also serve as a sugar reductase capable of reducing an L-6-deoxy-4-hexulose such as **3** to TDP-L-rhamnose (**17**).

Scheme 3



It should be noted that the mechanism of the ring contraction step in the dihydrostreptose pathway is remarkably similar to that proposed for the biosynthesis of UDP-D-apiose (**19**), which is derived from UDP-D-glucuronic acid (**18**) catalyzed by apiose synthase (Scheme 3).[15] This synthase has been assigned to have dual functions, possessing both 4-hexulose reductase and ring-contraction activities, since UDP-D-xylose (**20**) is a byproduct of the catalysis of apiose synthase.[15] Thus, the fact that StrL resembles apiose synthase having hexulose reductase activity lends strong credence for an analogous role of StrL as the catalyst for the ring contraction step in the dihydrostreptose pathway. The failure to detect the incorporation of **4** into the macrolide structures may simply reflect the limitation of DesVII to accommodate a furanose in its active site.[16]

The results described here present a rare example of a glycosyltransferase that recognizes both D- and L-sugar as substrates.[17] The established versatility of this glycosyltransferase (DesVII) on substrate selection[2,3] highlights its potential as a catalyst in the construction of new macrolides carrying a broad range of modified sugars, a prerequisite for developing more exquisite combinatorial biosynthetic strategies for new antibiotics. This work once again demonstrates the feasibility of engineering secondary metabolite glycosylation through a rational selection of gene combinations. Although the actual roles of StrM and StrL remain to be further characterized, our current data have revealed new complication of the ring contraction step in the biosynthesis of dihydrosteptose and have also helped to establish the close resemblance of the dihydrostreptose and apiose pathways.

Acknowledgment. We thank Professor David Sherman for kindly providing us the plasmids pDHS617 and pDHS702. This work was supported in part by the National Institutes of Health grants GM35906 and GM54346. H.-w.L. also thanks the National Institute of General Medical Sciences for a MERIT Award. L.Z. was a recipient of a Graduate School Dissertation Fellowship of the University of Minnesota.

Supporting Information Available: Characterization data of the new compounds **7**−**10**, including ^{1}H and ^{13}C NMR spectra, high-resolution FABMS results, and the complete spectral assignments (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA003246C

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

(11) See the Supporting Information for details.

(12) Ohtani, I.; Kusumi, T.; Kashman, Y.; Kakisawa, H. *J. Am. Chem. Soc.* **1991**, *113*, 4092−4096. (b) Ferreiro, M. J.; Latypov, S. K.; Quinoa, E.; Riguera, R. *J. Org. Chem.* **2000**, *65*, 2658−2666.

(13) While the ultimate proof of its function must await the isolation of StrM protein to confirm its activity, a number of StrM homologues have been unambiguously established to be hexose 3,5-epimerases. The best known example is RfbC in the rhamnose biosynthetic pathway (Graninger, M.; Nidetzky, B.; Heinrichs, D. E.; Whitfield, C.; Messner, P. *J. Biol. Chem.* **1999**, *274*, 25069; Giraud, M.-F.; Leonard, G. A.; Field. R. A.; Berlind, C.; Naismith, J. H. *Nat. Struct. Biol.* **2000**, *7*, 398; Tanner, M. E.; Kenyon, G. L. In *Comprehensive Biological Catalysis*; Sinnott, M., Ed.; Academic Press: San Diego, 1998; Vol. II, p 7−41, and references therein).

(14) The StrM-catalyzed epimerization is expected to be reversible. Thus, in the presence of a D-hexulose reductase, the equilibrium between **2** and **3** in the KdesI/strM strain will be shifted toward **2**, which after reduction gives quinovose as observed in the product.

(15) Kelleher, W. J.; Baron, D.; Ortmann, R.; Grisebach, H.; *FEBS Lett.* **1972**, *22*, 203−204. (b) Gebb. C.; Baron, D.; Grisebach, H.; *Eur. J. Biochem.* **1975**, *54*, 493−498. (c) Watson, R. R.; Orenstein, N. S. *Adv. Carbohydr. Chem. Biochem.* **1975**, *31*, 135−184. (d) Matern, U.; Grisebach, H. *Eur. J. Biochem.* **1977**, *74*, 303−312. (e) Wahl. H. P.; Grisebach, H. *Adv. Carbohydr. Chem. Biochem.* **1978**, *35*, 81−126.

(16) The StrL-catalyzed ring contraction is NAD^+-dependent and the equilibrium has been suggested to be driven by the reduction of the formyl side chain at C-3 after ring contraction. Thus, the overall catalysis is an intramolecular redox reaction and is expected to be irreversible.

(17) Wohlert, S. E.; Blanco, G.; Lombó, F.; Fernández, 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.