DOI: 10.1002/cbic.200800155

Glycosylation of Acyclic and Cyclic Aglycone Substrates by Macrolide Glycosyltransferase DesVII/DesVIII: Analysis and Implications

Svetlana A. Borisova, Hak Joong Kim, Xiaotao Pu, and Hung-wen Liu*[a]

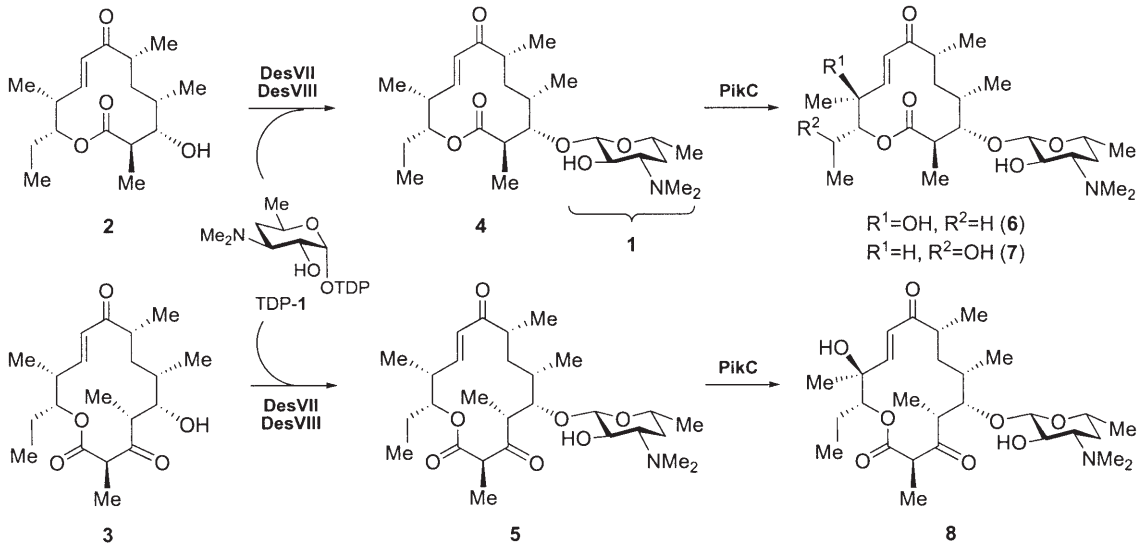
Macrolides target the bacterial protein translation machinery and are one of the most prescribed classes of antibiotics to treat bacterial infection.[1] However, emerging bacterial resistance to macrolides necessitates the development of new analogues that can potentially be used as drugs. The structures of macrolides are characterized by a macrolactone, which is commonly glycosylated with one or more deoxysugars.[1] Extensive experiments revealed a relaxed specificity of the responsible macrolide glycosyltransferases (GTs) toward both the sugar and aglycone substrates.[3] Thus, exploiting the catalytic flexibility of these GTs offers an attractive means for creating new macrolide derivatives.[2]

Among the growing number of in vitro studies of related GTs, DesVII, which catalyzes the attachment of D-desosamine (**1**) to 10-deoxymethynolide (**2**) or narbonolide (**3**) in the biosynthesis of methymycin (**6**), neomethymycin (**7**), narbomycin (**5**), and pikromycin (**8**) in *Streptomyces venezuelae*, is one of the most extensively studied macrolide GTs (Scheme 1).[3d,4] Previous studies showed that DesVII needs an auxiliary protein, DesVIII, for in vitro activity.[4a,5] The detailed in vitro analysis of sugar substrate specificity of DesVII/DesVIII had been carried out and our results showed that both L-sugars and D-sugars are recognized as substrates and variant substitutions at C-3 and C-4 are tolerated, but deoxygenation at C-6 is required.[3d]

It was also demonstrated in a preliminary study that the linear precursor of macrolactone **2**, as its *N*-acetylcysteamine thioester form (**9**), can be glycosylated by DesVII/DesVIII.[4b] Although we could not determine at the time which of the three potential glycosylation sites present in **9** were modified, TLC and MS analysis suggested that only one monoglycosylated product was formed. To further exploit the catalytic capability of DesVII/DesVIII in biosynthetic applications, we have carried out detailed studies to explore the range of aglycone substrate flexibility of this enzyme pair. Reported herein are the experimental results and the assessment of the biosynthetic potential of DesVII/DesVIII.

Glycosylation of linear precursor 9

A reaction mixture containing **9** (17 mg) and a 1.2-fold excess of TDP-**1** was incubated with DesVII/DesVIII.[6] TLC analysis of the reaction mixture revealed the presence of at least four



Scheme 1. Reactions catalyzed by DesVII/DesVIII.

[a] *Dr. S. A. Borisova, H. J. Kim, Dr. X. Pu, Prof. H.-w. Liu*
Division of Medicinal Chemistry, College of Pharmacy
and Department of Chemistry and Biochemistry
University of Texas at Austin
Austin, TX 78712 (USA)
Fax: (+1) 512-471-2746
E-mail: h.w.liu@mail.utexas.edu

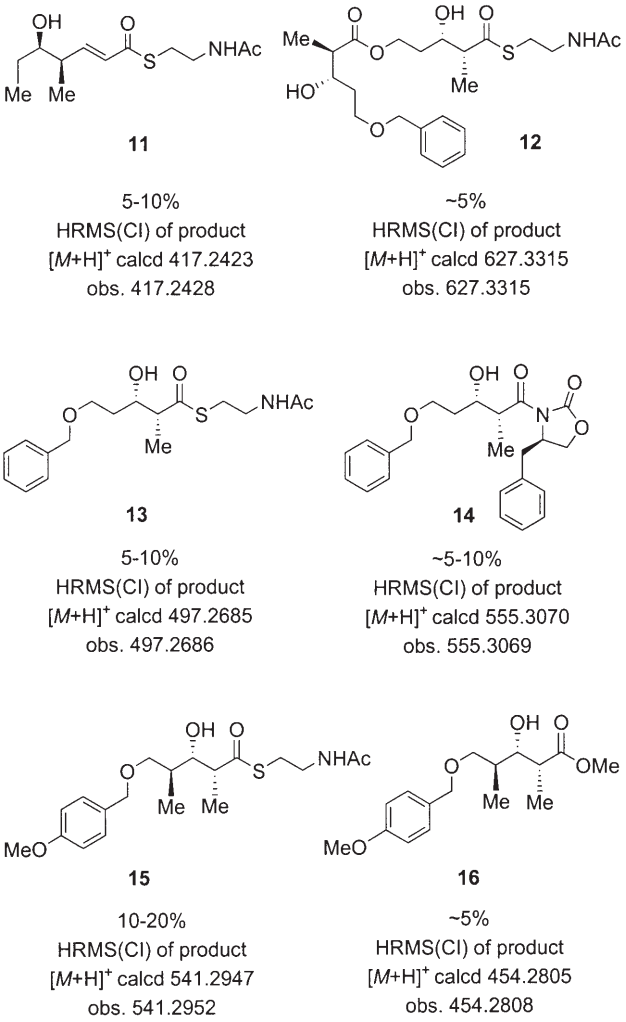
Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

polar compounds (R_f = 0.04, 0.19, 0.32, and 0.37) in addition to **9** (R_f = 0.51). The overall conversion of **9** to the more polar products was estimated to be nearly 50%. After separation by flash silica gel chromatography, compound **10** was isolated as the major component (R_f = 0.19), which accounts for 80–90% of all polar compounds produced. MS (CI^+) analysis showed that **10** ($[M+1]^+$ = 575) is a singly desosaminylated derivative of **9**. Further purification of **10** by preparative TLC led to a

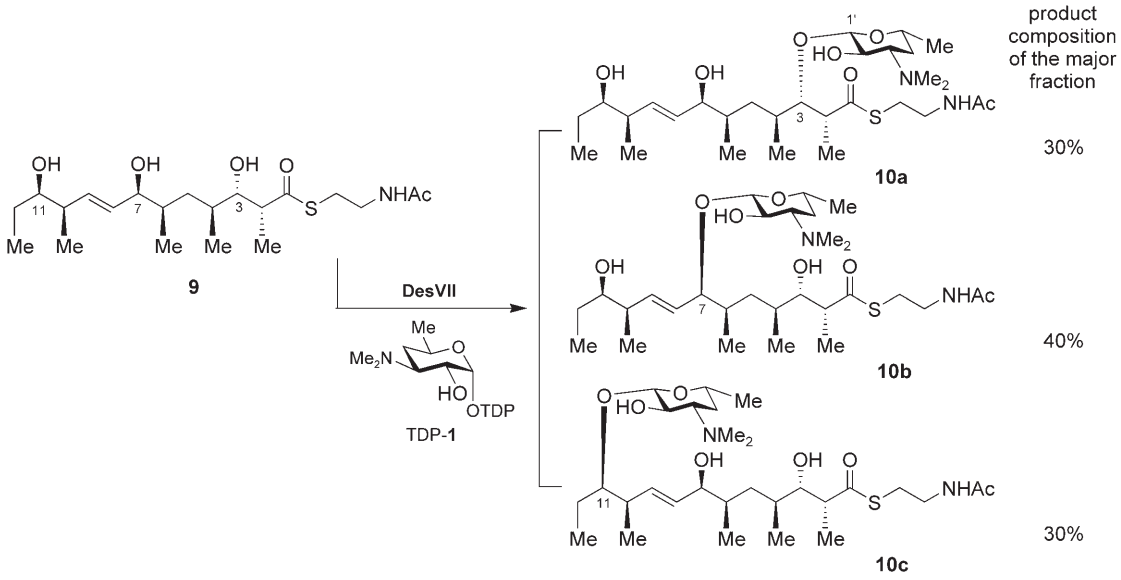
purer sample (0.4 mg), which was shown by extensive NMR analysis to be a mixture of three monodesosaminylated products (**10a**, **10b**, and **10c**, Scheme 2).[6,7] Attempts to further purify **10** by HPLC (C_{18}) were unsuccessful. Nevertheless, the three 1′-H (δ 4.31, 4.35, 4.36) and C-1′ (δ 99.5, 103.0, 103.5) signals of the attached sugars are clearly discernible. Analysis of the HMBC spectra revealed correlations between H-1′ (δ 4.36) and C-3 (δ 82.5) in **10a**, and between H-1′ (δ 4.31) and C-7 (δ 81.6) in **10b**. The 4–6 ppm downfield shift of the C-3 and C-7 peak in each respective compound is indicative of glycosylation at the designated locus.[6] Although no cross-peak between H-1′ (δ 4.35) and C-11 (δ 84.0) in **10c** was detected, the downfield shift of C-11 from δ 76.5 to 84.0 is consistent with the structure assignment. Integration of the H-1′ signals allowed an estimation of a ratio of 3:4:3 for products **10a**–**c**. It appears that all three OH groups of **9** can be glycosylated with little discrimination. Clearly, there is no regiospecificity of DesVII/DesVIII in reaction with **9**.

Glycosylation of short acyclic aglycones

A series of linear compounds/intermediates **11**–**16** (Scheme 3) was prepared and tested as substrates for DesVII/DesVIII. The structure of compound **11** resembles the glycosylation site at 11-OH in **9**, and the -(C=O)-CHMe-CHOH- fragment in compounds **12**–**16** models the glycosylation site at 3-OH in **2**/**9**, and at 5-OH in **3**. Compounds **15** and **16** are designed to be better mimics of **2**, **3**, and **9**, as they include a longer fragment -(C=O)-CHMe-CHOH-CHMe- as found in **2**/**3**/**9**. To explore if the *N*-acetylcysteamine thioester moiety is important for recognition, two sets of substrates differing in the ester functionality (**13**/**14** and **15**/**16**) were prepared. Interestingly, all these compounds could be converted by DesVII/DesVIII to the respective desosaminylated products which were verified by high resolution MS analysis.[6] Compound **12** contains two possible modification sites, but the product is only monodesosaminylated



Scheme 3. Desosaminylation of short acyclic substrates.



Scheme 2. Desosaminylation of the linear polyketide substrate **9**.

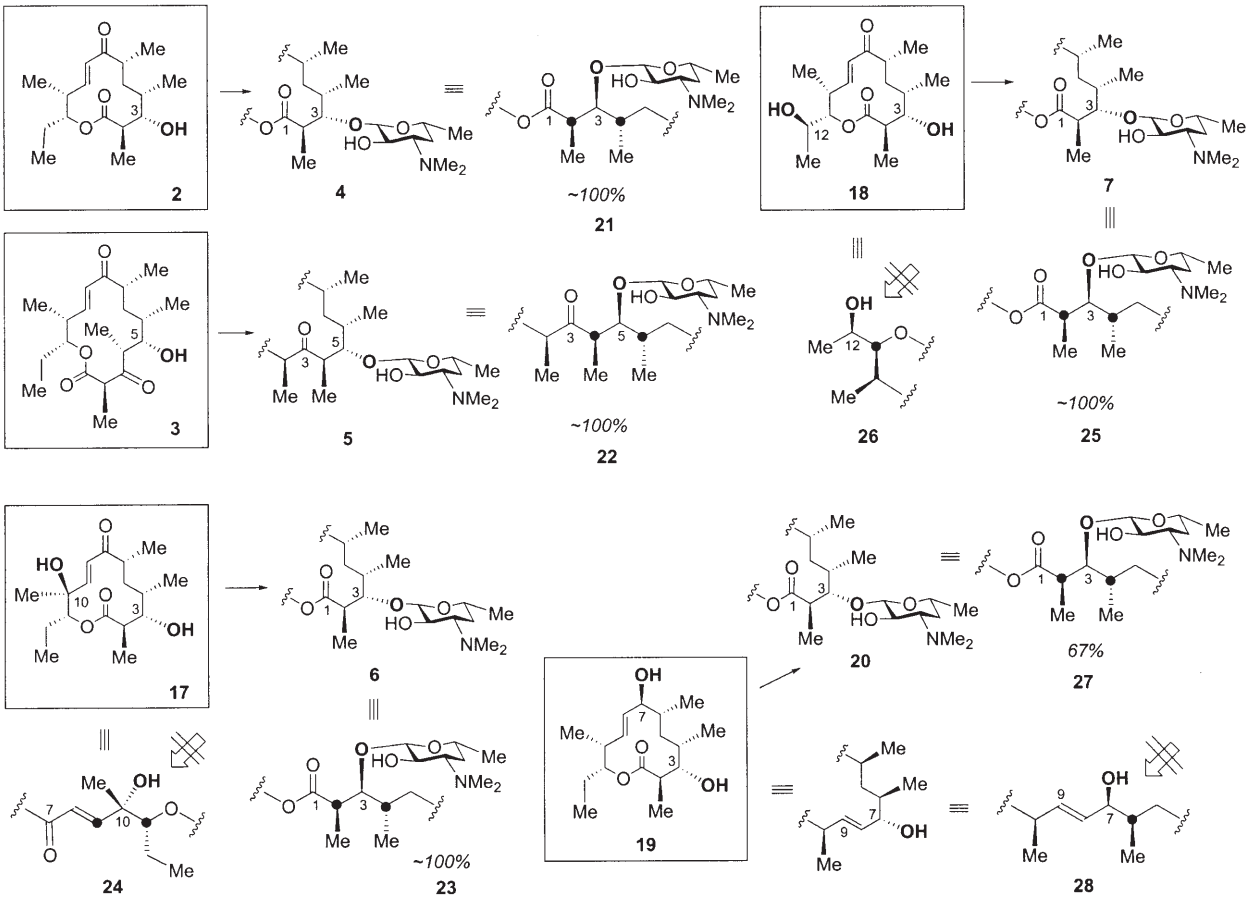
based on MS results. Similarly to the situation found for **10**, the product of **12** is very likely a mixture of two singly glycosylated compounds. It is evident that the ability of DesVII/DesVIII to glycosylate linear substrates is not limited by the length of the substrate backbone, the presence of protecting groups, or the nature of the ester functionality. This enzyme pair may therefore be regarded as a general purpose glycosyltransferase that works on a wide variety of acyclic substrates.

Glycosylation of cyclic aglycones

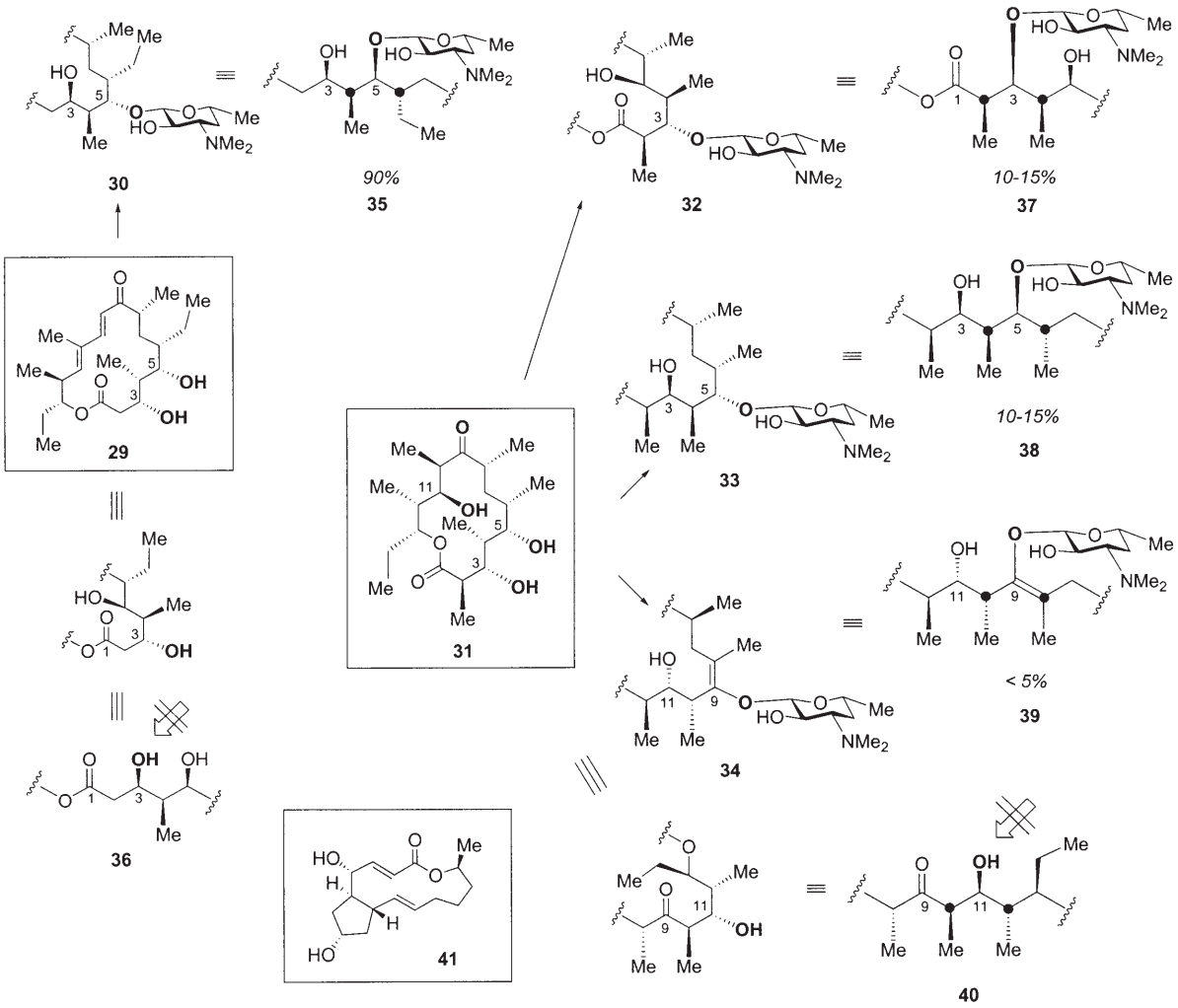
As shown in Scheme 4, DesVII/DesVIII is responsible for adding desosamine (**1**) to **2** and **3** to give **4** and **5**, respectively. It could also desosaminylate methynolide (**17**) and neomethynolide (**18**),[3d] which are produced by hydroxylation of **2** by a P450 enzyme known as PikC, to yield **6** and **7**, respectively. Although there is more than one hydroxyl group in **17** and **18**, glycosylation in both cases occurs only at the 3-OH site. The steric hindrance around the hydroxyl groups at C-10 and C-12 may have prevented their modification by DesVII/DesVIII (see **24** and **26**). Interestingly, incubation of this enzyme pair with the 7-keto-reduced **19** and TDP-**1** also led to a monoglycosylated product **20**.[4b] Comparing the motifs of **21**/**22**/**23**/**25**/**27** with that of **28**, it seems that the lack of an alkyl substituent at C_{n+1} (C-8) to the 7-OH group in **19** and/or the stereochemistry of the methyl substituent at C_{n-1} are detrimental to recognition. It is also possible that the sp^2 configuration of C_{n+1} (C-8) in **19** might result in a conformational change around 7-OH, preventing it from being glycosylated. However, this putative conformational change must be alleviated when the ring is opened, as the corresponding acyclic analogue **9** can be glycosylated by DesVII/DexVIIII to give **10b** (compare **10b** and **28**). Because only 3-OH is glycosylated in these cases, the results, which are in contrast to those observed with acyclic substrates, show that the reaction catalyzed by DesVII/DesVIII is regiospecific if cyclic substrates are used.

When the 16-membered tylactone (**29**), the precursor of tylosin produced by *Streptomyces fradiae*, was subjected to DesVII/DesVIII in the presence of TDP-D-desosamine (TDP-**1**), a single glycosylated product **30** was produced in high yield (Scheme 5).[3d] The high regioselectivity of 5-OH as the modification site is consistent with the close resemblance of the motif adjacent to 5-OH in **29** (see **35**) to 3-OH in **2** and 5-OH in **3** (see **21** and **22**). However, the reason why 3-OH in **29** is not acted upon by DesVII/DesVIII is not immediately apparent.

6-Deoxyerythronolide B (**31**), the aglycone of erythromycin produced by *Saccharopolyspora erythraea*, closely resembles **3**, but carries three secondary hydroxyl groups, which may be glycosylated by DesVII/DesVIII. Two of them, 5-OH and 11-OH, have the same core motif as the 3-OH in **2** and 5-OH in **3**. For



Scheme 4. Desosaminylation of native substrates and derivatives.



Scheme 5. Desosaminylation of cyclic polyketide substrates.

all three sites, the C_{n-2} is oxygen-linked as either an ester carbonyl (C_{n-2} to 3-OH) or a secondary alcohol (C_{n-2} to 5-OH) or a ketone (C_{n-2} to 11-OH, see **37**, **38**, and **40** in Scheme 4). Incubation of **31** with TDP-D-desosamine (TDP-**1**) and DesVII/DesVIII generated two major and one minor products, with a total of 20–30% conversion after the overnight reaction. The two major products, **32** and **33**, were identified by NMR and HRMS to be monodesosamylated at 3-OH and 5-OH, respectively. To our surprise, the 11-hydroxyl group was not glycosylated. Instead, the minor compound was found to be **34**, resulting from glycosylation of the hydroxyl group of the enol form of the 9-keto group in **31** (see **39**). Thus, unlike the cases of other cyclic aglycones examined, glycosylation of **31** catalyzed by DesVII/DesVIII is regioselective but not regiospecific, as modification takes place at three different sites, but with only one site being modified at a time.

Interestingly, upon incubation with TDP-**1** and DesVII/DesVIII, the bicyclic compound **41** could also be glycosylated, albeit with low efficiency (~5% conversion overnight). This compound contains two hydroxyl groups, none of which resembles the glycosylation site in the natural substrates (**2** and **3**). The product is singly glycosylated ($[M+H]^+$ calcd 438.2856, obs. 438.2857), however the site of modification or if one or two singly glycosylated compounds were formed was not determined because of small quantities of the material. Nevertheless, the formation of glycosylated product(s) in this case implies an even more permissive active site of DesVII than previously thought.

In summary, the study reported here clearly demonstrates that the macrolide glycosyltransferase, DesVII/DesVIII, can recognize and process not only cyclic substrates of different ring size, but also a variety of linear substrates albeit with reduced, but measurable activities. When multiple hydroxyl groups are present, essentially no regiospecificity is observed for the linear substrates. Interestingly, only singly glycosylated products were generated in all cases. In contrast, when cyclic substrates with multiple glycosylation sites were tested, the enzyme displayed excellent regiospecificity in most cases (3-OH of **17**–**19**, and 5-OH of **29**). Whereas the regiospecificity seems to be relaxed for **31**, the reaction is still not totally random as glycosylation at 11-OH was not detected. Factors governing the glycosylation outcome for the cyclic substrates may be multifold, including the substitution pattern near the glycosylation site and the ring conformation. Also, the specifici-

ty/selectivity may be sensitive to the substituents distant from the glycosylation site. Taken together, the substrate flexibility of DesVII/DesVIII is apparently extended to a wide range of linear as well as cyclic substrates. A similar capability is expected for other macrolide glycosyltransferases. Although the yield of product(s) is notably reduced when unnatural substrates are used, once a desired activity is identified, the catalytic efficiency of these enzymes may be fine-tuned by protein engineering. The substrate flexibility of glycosyltransferases expands the opportunities for glycodiversification to generate new glycoforms of synthetic compounds and macrolide analogues, which may help augment our battery of effective agents against the ever-increasing number of drug-resistant organisms.

Acknowledgements

This work was supported by the National Institute of Health Grant GM54346. We also thank Professor Jon Thorson for providing 6-deoxyerythronolide B.

Keywords: antibiotics · biosynthesis · carbohydrates · glycosylation · macrolides

[1] For an overview, see: a) *Macrolide Antibiotics: Chemistry,Biology, and Practice*, (Eds: S. Omura), Academic, San Diego, CA, 2002; b) C. Walsh in *Antibiotics: Actions, Origins, Resistance*, ASM, Washington, D.C., **2003**.

[2] For relevant reviews, see: a) C. Méndez, J. A. Salas, *Trends Biotechnol.* **2001**, *19*, 449–456; b) W. R. Strohl, *Metab. Eng.* **2001**, *3*, 4–14; c) E. Rodriguez, R. McDaniel, *Curr. Opin. Microbiol.* **2001**, *4*, 526–534; d) C. T. Walsh, *ChemBioChem* **2002**, *3*, 124–134; e) J. D. Kittendorf, D. H. Sherman, *Curr. Opin. Biotechnol.* **2006**, *17*, 597–605; f) C. Thibodeaux, C. E. Melançon III, H.-w. Liu, *Nature* **2007**, *446*, 1008–1016.

[3] Few representative examples are: a) L. M. Quirós, R. J. Carbajo, A. F. Braña, J. A. Salas, *J. Biol. Chem.* **2000**, *275*, 11713–11720; b) A. Minami, R. Uchida, T. Eguchi, K. Kakinuma, *J. Am. Chem. Soc.* **2005**, *127*, 6148–6149; c) M. Yang, M. R. Proctor, D. N. Bolam, J. C. Errey, R. A. Field, H. J. Gilbert, B. G. Davis, *J. Am. Chem. Soc.* **2005**, *127*, 9336–9337; d) S. A. Borisova, C. Zhang, H. Takahashi, H. Zhang, A. W. Wong, J. S. Thorson, H.-w. Liu, *Angew. Chem.* **2006**, *118*, 2814–2819; *Angew. Chem. Int. Ed.* **2006**, *45*, 2748–2753.

[4] a) S. A. Borisova, L. Zhao, C. E. Melançon 3rd, C.-L. Kao, H.-w. Liu, *J. Am. Chem. Soc.* **2004**, *126*, 6534–6535; b) C.-L. Kao, S. A. Borisova, H. J. Kim, H.-w. Liu, *J. Am. Chem. Soc.* **2006**, *128*, 5606–5607.

[5] A similar requirement has since been observed for other glycosyltransferases involved in the biosynthesis of macrolides a) C. E. Melançon III, H. Takahashi, H.-w. Liu, *J. Am. Chem. Soc.* **2004**, *126*, 16726–16727; b) Y. Yuan, H. S. Chung, C. Leimkuhler, C. T. Walsh, D. Kahne, S. Walker, *J. Am. Chem. Soc.* **2005**, *127*, 14128–14129, and anthracyclines; c) W. Lu, C. Leimkuhler, G. J. J. Gatto, R. G. Kruger, M. Oberthur, D. Kahne, C. T. Walsh, *Chem. Biol.* **2005**, *12*, 527–534.

[6] See the Supporting Information for details.

[7] MS analysis of fractions containing the minor compounds with $R_f = 0.32$ and 0.37 revealed the presence of dehydrated **10** (m/z 557 corresponding to $[M+1-18]$). None of the fractions (or the original crude extracts) contained doubly glycosylated compounds (expected $[M+1] = 732$).

[8] A. R. Gandecha, S. L. Large, E. Cundliffe, *Gene* **1997**, 184, 197–203.

Received: March 11, 2008
Published online on June 11, 2008

