Pure Appl. Chem., Vol. 79, No. 4, pp. 785–799, 2007.
doi:10.1351/pac200779040785

Manipulating nature's sugar biosynthetic machineries for glycodiversification of macrolides: Recent advances and future prospects*

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Abstract: Changing the sugar structures and glycosylation patterns of natural products is an effective means of altering the biological activity of clinically useful drugs. Several recent strategies have provided researchers with the opportunity to manipulate sugar structures and to change the sugar moieties attached to these natural products via a biosynthetic approach. In this review, we explore the utility of contemporary in vivo and in vitro methods to achieve natural product glycodiversification. This study will focus on recent progress from our laboratory in elucidating the biosynthesis of D-desosamine, a deoxysugar component of many macrolide antibiotics, and will highlight how we have engineered the D-desosamine biosynthetic pathway in *Streptomyces venezuelae* through targeted disruption and heterologous expression of the sugar biosynthetic genes to generate a variety of new glycoforms. The in vitro exploitation of the substrate flexibility of the endogenous D-desosamine glycosyltransferase (GT) to generate many non-natural glycoforms will also be discussed. These experiments are compared with recent work from other research groups on the same topics. Finally, the significance of these studies for the future prospects of natural product glycodiversification is discussed.

Keywords: biosynthesis; glycodiversification; unusual sugars; macrolides; antibiotics.

INTRODUCTION

Most clinically useful anticancer and anti-infectious agents are either isolated from natural sources or are synthetic compounds, which are frequently inspired by natural products [1]. The remarkable structural diversity of natural products is greatly enhanced by glycosylation—modifications which have significant effects on both the physical properties and chemical reactivity of the parent compounds [2]. Some well-known examples of pharmaceutically important glycosylated natural products include the macrolide antibiotics, aromatic polyketides, glycopeptides, indolocarbazoles, aminoglycosides, and cardiac glycosides. In these compounds, the sugar moieties are often essential for the observed biological activity. Thus, changing the structures and/or substitution patterns of the sugar appendages on the aglycone scaffolds, a process known as glycodiversification, could potentially generate new glyco-

Pure Appl. Chem.* **79, 467–823 (2007). An issue of reviews and research papers based on lectures presented at the 25th International Symposium on Chemistry of Natural Products (ISCNP-25) and 5th International Conference on Biodiversity (ICOB-5), held jointly in Kyoto, Japan, 23–28 July 2006, on the theme of natural products.
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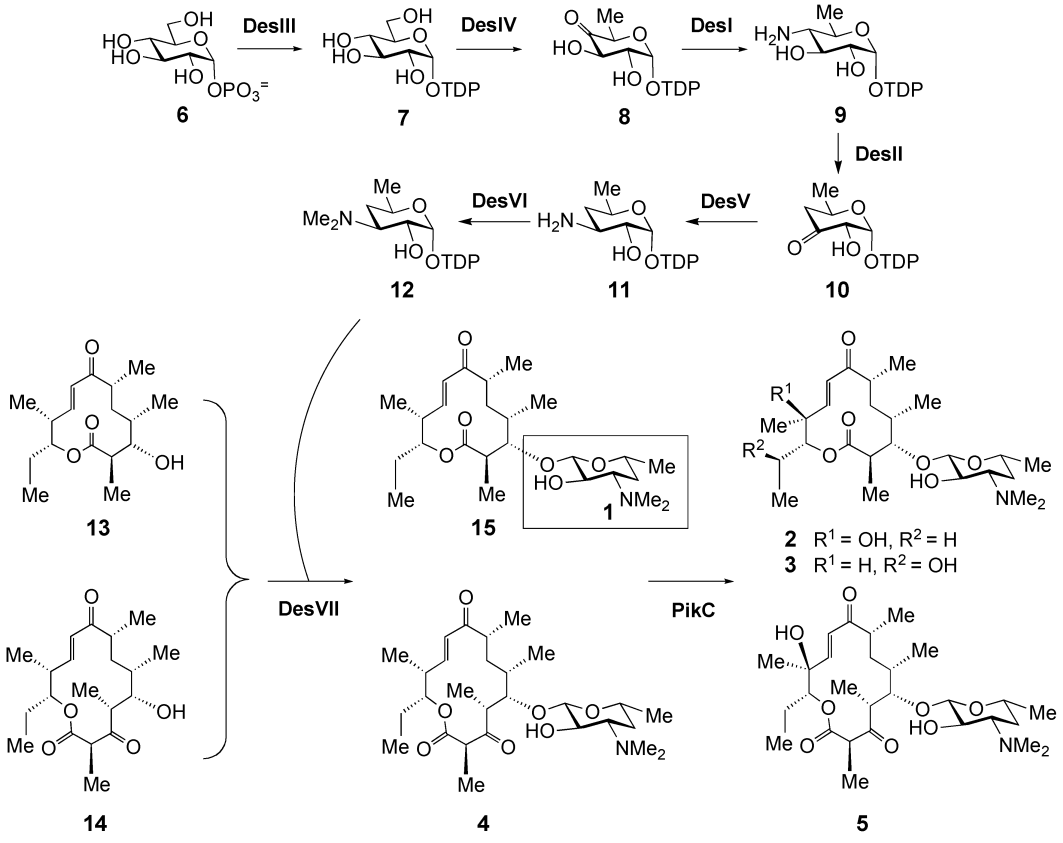
conjugates with enhanced or altered biological activities [1,3,4]. Therefore, glycodiversification may ultimately lead to new antibiotics against drug-resistant infectious bacteria, improved cytotoxic agents for the treatment of cancer, or potent chemicals for combating other ailments.

Nature's strategies for constructing and assembling glycoforms are elegant and efficient, and the responsible biosynthetic machineries are adaptable to genetic, chemical, and biochemical manipulations [1,3,4]. Thus, understanding the biosynthesis of glycosylated natural products is an important step for the development of efficient methods for glycodiversification. In recent years, the availability of biosynthetic gene sequence information and biochemical studies of these gene products have enabled scientists to establish the field of combinatorial biosynthesis [5–8]. In this approach, designed compounds can be assembled in vivo by combining and expressing genes from different biosynthetic pathways in a single host organism. These techniques allow the chemical structures of sugars and the glycosylation patterns of aglycone scaffolds to be manipulated. Successful implementation of this pathway engineering approach to achieve glycodiversification ultimately relies on the ability of the host organism to link together diverse sugar and aglycone substrate combinations—a feat performed by glycosyltransferases (GTs). Importantly, recent studies have shown that certain GTs exhibit remarkable flexibility with both sugar and/or aglycone substrates and, as such, are invaluable tools for glycodiversification efforts.

In addition to their essential role in pathway engineering in vivo, substrate-flexible GTs can be used to alter the glyco-patterns of natural products in vitro. Specifically, GT activity can be screened with libraries of both natural and unnatural substrates under defined reaction conditions in order to generate novel glycoforms that may not be accessible in vivo [9–11]. These in vitro GT studies not only present a means to achieve structural diversity of glycoforms, but also provide more detailed information on the mechanism of substrate recognition by GTs. Such information, combined with structural studies, may eventually lead to the custom design of GTs—engineered to catalyze specific reactions. In this review, we will highlight recent progress from our laboratory, along with relevant examples from other groups, that show our advances in understanding the biosynthesis of deoxysugars, the in vivo engineering of macrolide sugar structures, and the in vitro testing of GT substrate flexibility, with the ultimate goal of natural product glycodiversification. These (and other) studies illustrate the potential of both in vivo and in vitro engineering techniques for the generation of new glycosylated natural product derivatives.

BIOSYNTHESIS OF D-DESOSAMINE FROM *STREPTOMYCES VENEZUELAE*

D-Desosamine (**1**, Scheme 1) is a 3-dimethylamino-3,4,6-trideoxysugar found in several macrolide antibiotics including methymycin (**2**), neomethymycin (**3**), narbomycin (**4**), and pikromycin (**5**) from *S. venezuelae*. It is also found in erythromycin from *Saccharopolyspora erythraea*, oleandomycin from *Streptomyces antibioticus*, and in mycinimicin and rosarimicin, both from *Micromonospora* species [12]. Macrolide antibiotics target the prokaryotic ribosome, and inhibit protein synthesis by binding to the 23S ribosomal subunit. The attachment of D-desosamine to its respective aglycone is critical for the antibiotic activity [1,13]. Sequence analysis of the methymycin/pikromycin gene cluster from *S. venezuelae* [14–16] revealed the presence of eight open reading frames (designated *desI-desVIII*, see Scheme 1) that may encode D-desosamine biosynthetic enzymes based on sequence similarity to known sugar biosynthetic genes in the databank [17,18]. Functional assignment of these genes was subsequently verified by gene knockout experiments and by in vitro biochemical characterization of the encoded proteins.



Scheme 1 TDP-D-desosamine biosynthesis and attachment in *S. venezuelae*. TDP-D-desosamine (**12**) is synthesized from glucose-1-phosphate (**6**) and is added onto the *S. venezuelae* aglycones 10-deoxymethynolide (**13**) and narbonolide (**14**) by the action of DesVII/DesVIII to give the YC-17 (**15**) and narbomycin (**4**), respectively. Compounds **15** and **4** can undergo subsequent hydroxylation by the cytochrome P450 enzyme PikC to give methymycin (**2**), neomethymycin (**3**), and pikromycin (**5**).

The *desIII* and *desIV* genes encode α-D-glucose-1-phosphate thymidylyltransferase and TDP-glucose-4,6-dehydratase, respectively. These enzymes catalyze the first two steps in the desosamine pathway (**6** → **7** → **8**), and homologs of these enzymes are found in nearly every deoxysugar biosynthetic pathway. Nucleotidylyltransferases transfer a nucleotide monophosphate moiety from a nucleotide triphosphate (thymidine triphosphate, TTP, in the case of desosamine) to the sugar-1-phosphate to generate a nucleotide diphosphate (NDP)-sugar and pyrophosphate [19,20]. The well-characterized 6-deoxygenation step catalyzed by DesIV and its homologs is the first committed step to deoxysugar biosynthesis [4]. In this interesting enzyme, the 4-OH group of **7** is first oxidized to a keto group by a tightly bound nicotinamide adenine dinucleotide (NAD^+) cofactor. This lowers the pK_a of the C5 proton, allowing elimination of the C6-OH and the formation of an α,β-unsaturated enone intermediate. The NADH formed in the first step then transfers a hydride back to C6, generating **8**. In contrast to its typical role, NAD^+ is used as a cofactor (regenerated at the end of each catalytic cycle) rather than as a co-substrate in the 4,6-dehydratase reaction [4].

The next transformation is C4-deoxygenation, which proceeds in two steps catalyzed by the *desI* and *desII* gene products (**8** → **9** → **10**). DesI catalyzes the C-4 transamination of **8** to generate TDP-4-amino-4,6-dideoxy-D-glucose (**9**) in the presence of pyridoxal 5'-phosphate (PLP) and L-glutamate [16]. Sequence analysis revealed that DesII contains a [4Fe-4S] consensus motif (**C**xxx**C**xx**C**) and that it be-

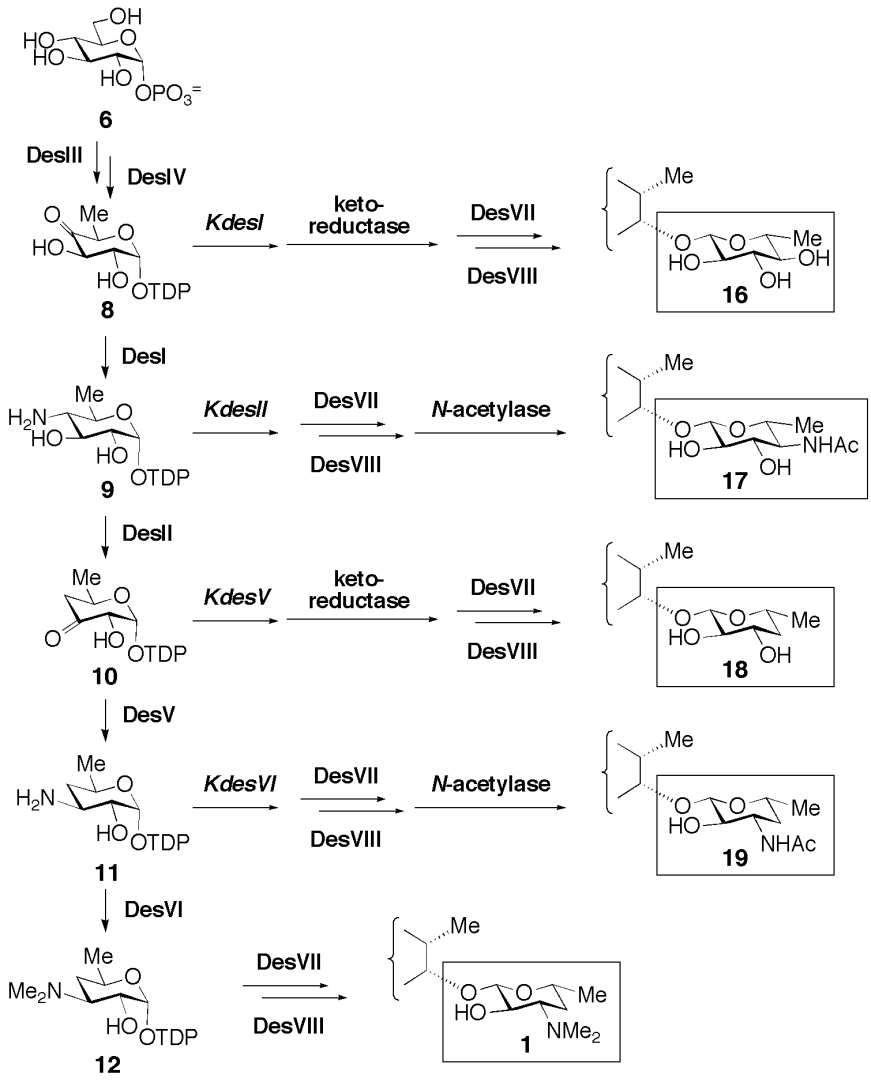
longs to the SAM (*S*-adenosylmethionine) radical enzyme superfamily [21,22]. Homologs of DesII are rare, but they are involved in the biosynthesis of D-desosamine in several other pathways (including the erythromycin, oleandomycin, mycinamycin, and narbomycin pathways) and also in the biosynthesis of the D-chalcose moiety of chalcomycin [23]. Biochemical characterization of DesII revealed that it indeed catalyzes the oxidative deamination of **9** to **10** in the presence of SAM and a reduced $[4Fe\text{-}4S]^{1+}$ cluster [22]. The mechanistic details of this C-4 deoxygenation are still under investigation.

Immediately following the C-4 deoxygenation reaction is the C-3 transamination step (**10** → **11**) catalyzed by DesV. This enzyme catalyzes a similar reaction to DesI and shares considerable amino acid sequence similarity with TylB, a well-characterized PLP-dependent transaminase from the D-mycaminose biosynthetic pathway in *Streptomyces fradiae* [24]. Further support for this 3-aminotransferase functional assignment was obtained when the heterologous expression of *tylB* in a *S. venezuelae desV* knockout strain was shown to restore methymycin (**1**) and pikromycin (**5**) production [25]. Subsequent in vitro characterization of DesV fully established it as the expected C-3 aminotransferase. Homologs of *tylB* and *desV* are found widely in the deoxysugar biosynthetic pathways [26–28]. Together, these genes represent a family of transaminases specific for the biosynthesis of aminosugars [29]. The final step in the desosamine pathway involves two successive N-methylations (**11** → **12**) catalyzed by DesVI. The DesVI protein was thoroughly characterized in vitro, showing that it sequentially incorporates two SAM-derived methyl groups onto the 3-amino-group of the DesV product (**11**) [30,31].

After the completion of TDP-D-desosamine biosynthesis, the sugar is coupled to the *S. venezuelae* aglycones (**13** or **14**) through the combined action of DesVII and DesVIII [32]. Although sequence analysis revealed DesVII to be the GT, the function of DesVIII has, until recently, remained enigmatic. Because DesVIII is only found in the biosynthetic pathways of deoxyamino sugars, it was originally thought to be involved in the regulation of the incorporation of aminosugars. However, recent gene knockout, heterologous expression, and biochemical studies have shown that efficient glycosyl transfer by DesVII requires DesVIII [32]. Although the exact function of DesVIII is still unclear, it appears to be involved in the activation of DesVII, perhaps enabling the nascent GT to fold into or adopt a proper conformation for catalysis. The requirement for this auxiliary protein has also been established for other DesVII/DesVIII homologs including AknS/AknT from the aclacinomycin A pathway [33] and EryCII/EryCIII from the erythromycin pathway [34]. Studies investigating the substrate flexibility of DesVII/DesVIII, their homologs from other deoxysugar biosynthetic pathways, and the use of these GT/auxiliary protein pairs in glycodiversification will be discussed in the following sections.

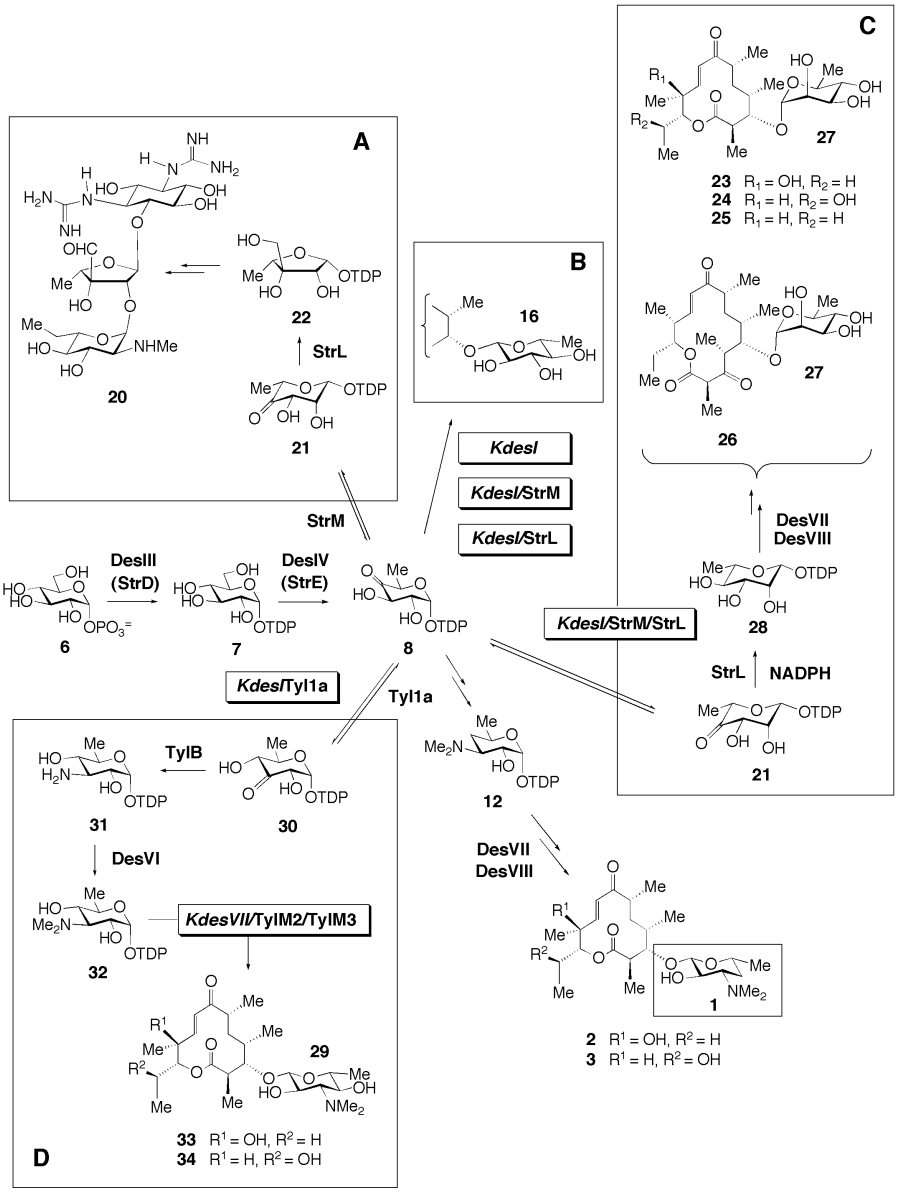
GLYCODIVERSIFICATION BY IN VIVO PATHWAY ENGINEERING

During the elucidation of the desosamine biosynthetic gene cluster, it was found that disrupting individual des genes (*desI*, *II*, *V*, and *VI*) led to the production of methymycin and neomethymycin derivatives in the mutant strains that carried new sugar moieties (Scheme 2). Instead of desosamine, the new macrolide compounds contained a 6-deoxyglucose (**16**) [35], a 4-*N*-acetylamino-4,6-dideoxyglucose (**17**) [16], a 4,6-dideoxyglucose (**18**) [36], or a 3-*N*-acetylamino-3,4,6-trideoxyglucose (**19**) [37], depending on which gene was knocked out. Apparently, DesVII and DesVIII are capable of coupling the accumulated deoxysugar biosynthetic intermediate in each mutant to the macrolide aglycones. The N-acetylation in **17** and **19** and the 4-ketoreduction in **16** and **18** occur via enzyme activities not encoded in the methymycin/pikromycin gene cluster.



Scheme 2 In vivo glycodiversification of *S. venezuelae* aglycones through gene disruption experiments. In separate experiments, individual TDP-D-desosamine biosynthetic genes (*desI*, *desII*, *desV*, or *desVI*) were disrupted, leading to the accumulation of **8**, **9**, **10**, and **11**, respectively, in the mutant strains. In each mutant, a new sugar was attached to the *S. venezuelae* aglycones by the desosaminyl transferase/auxiliary protein pair, DesVII/DesVIII. The reduction of the 4- and 3-keto groups of **8** and **10**, respectively, give **16** and **18** following glycosyltransfer. The amino groups of **9** and **11** are acetylated to give **17** and **19**, respectively. The ketoreductase and *N*-acetyltransferase activities are derived from enzymes not encoded in the *pik* cluster.

Heterologous expression of two genes (*strM/L*) from the streptomycin (**20**) biosynthetic pathway of *Streptomyces griseus* in the *S. venezuelae KdesI* mutant further demonstrated the substrate flexibility of DesVII/DesVIII [38]. The *strM/L* genes were proposed to encode a 3,5-epimerase and dihydrostreptose synthase, respectively (Scheme 3A) [39,40], which together convert **8** to TDP-dihydrostreptose (**22**) in *S. griseus*. Since the 4-keto-6-deoxysugar intermediate (**8**) is found in both desosamine and dihydrostreptose biosynthetic pathways, it was proposed that inclusion of these two *str* genes in the *KdesI* knockout strain could redirect this common intermediate to a new deoxysugar (**22**), which could then be attached to the *S. venezuelae* aglycones by the substrate-flexible DesVII/DesVIII.



Scheme 3 In vivo glycodiversifivation of *S. venezuealae* aglycones through gene disruption and heterologous expression. (A) Proposed biosynthesis (**6** → **7** → **8** → **21** → **22**) of TDP-dihydrostreptose, the precursor of the streptose moiety of the aminoglycoside streptomycin (**20**) from *S. griseus*. (B) Heterologous expression of the single *S. griseus* genes *strM* or *strL* in the *S. venezuelae KdesI* mutant led to the production of **16**, which was also generated in the *KdesI* mutant lacking *str* genes. (C) Heterologous expression of both *strM* and *strL* in the *KdesI* mutant yielded four new compounds (**23–26**), each carrying an L-rhamnose moiety (**27**). The biosynthesis of TDP-L-rhamnose in this mutant likely occurs via **8** → **21** → **28** prior to the coupling of the non-natural sugar to the *S. venezuelae* aglycones by DesVII/DesVIII. (D) Heterologous expression of D-mycaminose biosynthetic genes (*tyl1a*, *tylB*, *tylM2*, and *tylM3*) from the tylosin biosynthetic pathway of *S. fradiae* in the *S. venezuelae KdesI/KdesVII* double mutant. Tyl1a and TylB convert the *KdesI* accumulation product (**8**) into the aminosugar **31**, which is subsequently *N*,*N*-dimethylated by DesVI to give **32** and then coupled to the *S. venezuelae* aglycones by the *S. fradiae* GT/auxiliary protein pair TylM2/TylM3 to give **33** and **34**—both harboring a D-mycaminose moiety (**29**).

Expression of *strM* or *strL* alone in *KdesI* only led to the production of methymycin/neomethymycin derivatives carrying D-quinovose (**16**, Scheme 3B). Since these same compounds were obtained in the *KdesI* mutant without any *S. griseus* genes (Scheme 2), DesVII/DesVIII are apparently intolerant of the epimerized StrM product, **21**. However, when both *strM* and *strL* were heterologously expressed (Scheme 3C), four new products (**23–26**)—each containing an L-rhamnose moiety (**27**)—were obtained [38]. Thus, TDP-L-rhamnose (**28**) is most likely being formed in this mutant via the route **8** → **21** → **28**. These experiments not only demonstrated the ability of DesVII/DesVIII to accept L-deoxysugars, but also revealed a novel TDP-4-keto-6-deoxy-L-glucose reductase activity of StrL.

In addition to exploring the in vivo glycosylation activities of DesVII/DesVIII, progress was also made on the heterologous gene expression of DesVII/DesVIII homologs to generate macrolide compounds carrying various unusual sugars [41]. For example, four D-mycaminose (**29**) biosynthetic genes (*tyl1a*, *tylB*, *tylM2*, and *tylM3*) from *S. fradiae* were heterologously expressed in the *S. venezuelae KdesI/KdesVII* double mutant. The tandem action of Tyl1a and TylB convert **8** into the aminosugar (**31**) which is then *N*,*N*-dimethylated by DesVI to give TDP-D-mycaminose (**32**) (Scheme 3D) [41]. Because the desosaminyl GT (DesVII) is disrupted in this strain, the coupling of mycaminose to the *S. venezuelae* aglycones (**13** and **14**) relies on the ability of the heterologously expressed *S. fradiae* GT (TylM2) and its auxiliary protein (TylM3) to recognize non-natural aglycone substrates. Indeed, only in the presence of TylM3, was TylM2 shown to generate 3-*O*-mycaminosyl-methynolide, 3-*O*-mycaminosyl-neomethynolide (**33** and **34**, respectively), and the corresponding narbonolide derivatives. These results showed that GTs such as TylM2, whose natural algycone substrate is a 15-membered ring tylactone, is capable of using different aglycone substrates. Not only did these experiments result in the production of new hybrid macrolide derivatives, but they also firmly established the requirement of DesVII and its homologs (such as TylM2) for their auxiliary proteins (DesVIII and TylM3, respectively).

Similar in vivo pathway engineering studies have led to the glycodiversification of other classes of natural products. For urdamycin A (**35**, Fig. 1), an angucycline-type polyketide antibiotic and anticancer agent produced by *S. fradiae* Tü2717, the targeted inactivation of GT and sugar biosynthetic genes have allowed for the determination of catalytic roles for various GTs involved in the pathway, the demonstration of the relaxed substrate specificity of these endogenous GTs, and the generation of a variety of new urdamycin derivatives [42–44]. One of these derivatives showed improved anticancer activity over the parent compound, urdamycin A (**35**). For the anthracyclin-like polyketide ellaromycin (**38**) from *Steptomyces olivaceus* Tü2353, heterologous expression of cosmid *16F4* (which contains most of the ellaromycin aglycone and deoxysugar biosynthetic genes) in the urdamycin (**35**) producer *S. fradiae* and in the mithramycin (**40**) producer *S. argillaceus*, led to the production of many new ellaromycin derivatives carrying varied sugar appendages [45]. In these derivatives, the ellaromycin anthracyclin core, 8-demethyltetracenomycin C (8-DMTC, **44**)—encoded by cosmid *16F4*—was glycosylated by the alternative sugars (**36**, **37**, **42**, and **43**) instead of the typical permethylated L-rhamnosyl moiety (**39**) of ellaromycin. Control experiments established that the GT responsible for the attachment of the urdamycin and mithramycin sugar moieties (**36/37** and **42/43**, respectively) to 8-DMTC was encoded by *elmGT*, a gene in the heterologously expressed *16F4* cosmid [45,46], rather than by an endogenous GT in the expression hosts, *S. fradiae* or *S. argillaceus*. The authors later cloned *elmGT* into the chromosome of *Streptomyces albus*, a strain which does not produce any macrolide antibiotics [47]. The mutant *S. albus* strain was transformed with plasmids that encoded the production of different deoxysugars, and was subsequently fed **44**. In addition to the compounds already reported, 8-DMTC derivatives containing **45** and **46** were also produced. Cumulatively, the conceptually simple experiments outlined in this section have not only revealed the substrate flexibility of several antibiotic GTs, but have also demonstrated the feasibility of in vivo glycodiversification through both targeted gene disruption and heterologous gene expression. Furthermore, these studies underscore the potential power of manipulating Nature's biosynthetic machinery for engineering new natural products with desired sugar structures.

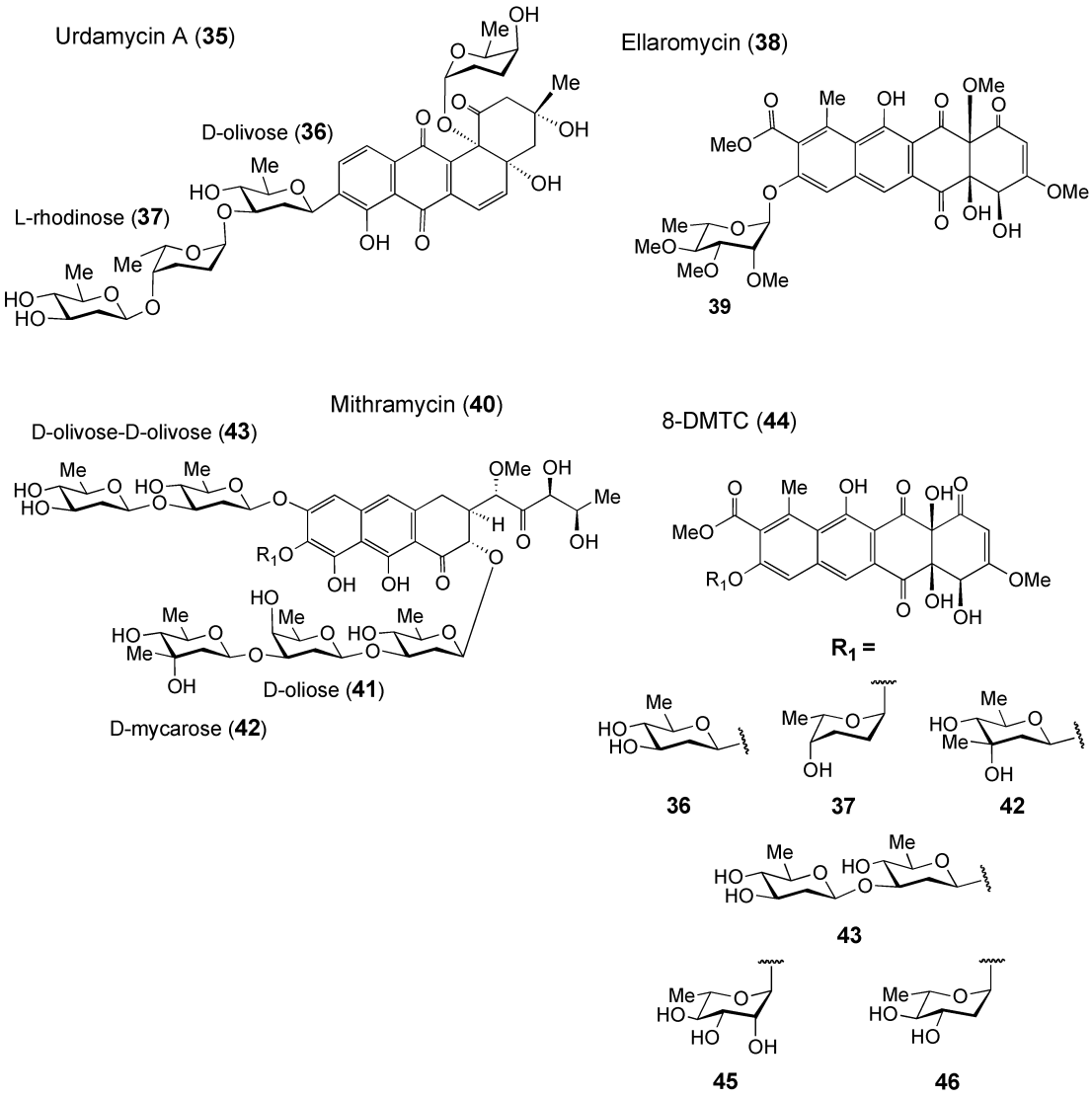
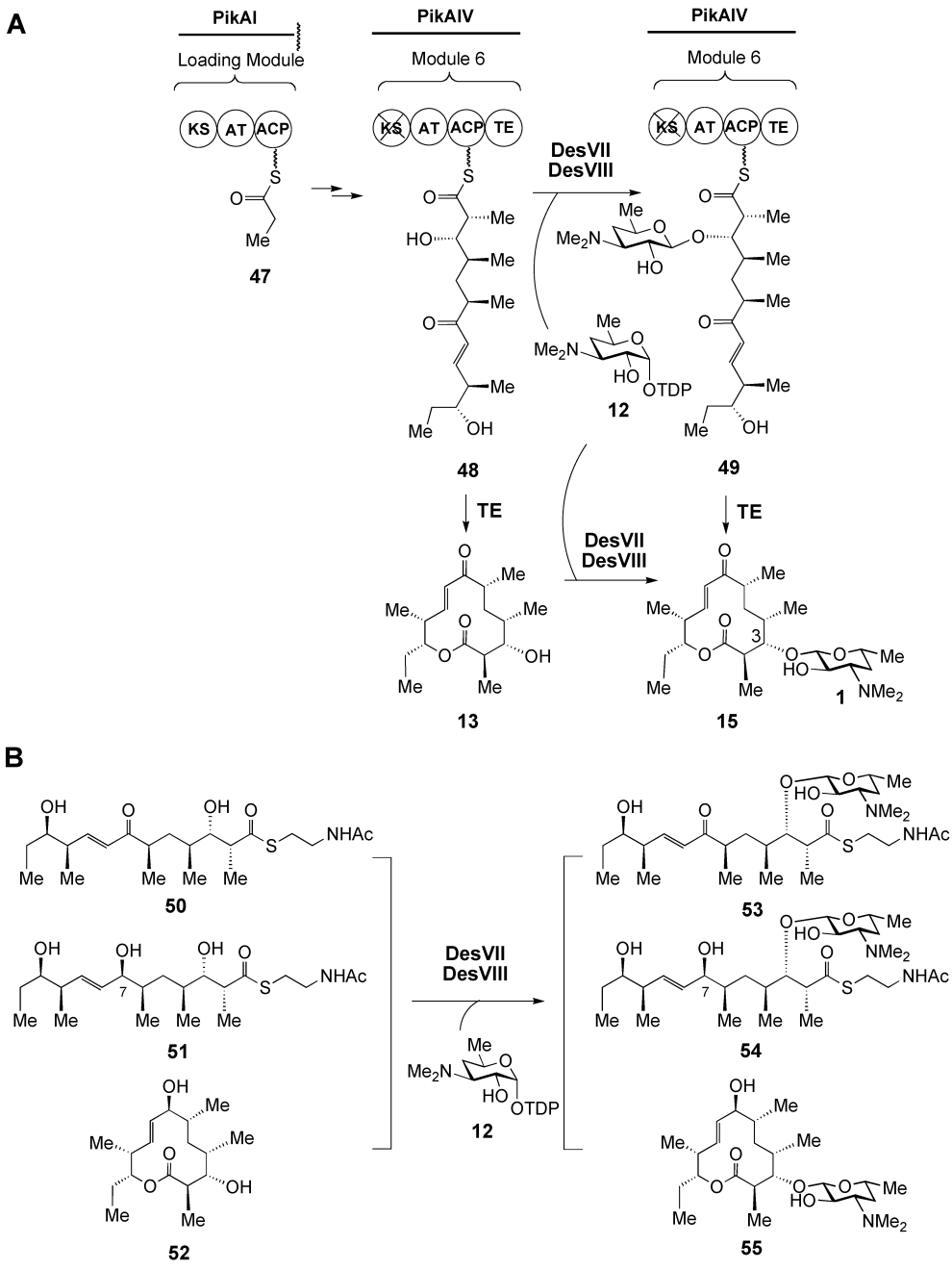


Fig. 1 Representative aromatic polyketides and the in vivo glycodiversification of the ellaromycin aglycone precursor, 8-demethyltetracenomycin (8-DMTC, **44**). The heterologous expression of cosmid *16F4* (containing part of the ellaromycin biosynthetic pathway of *S. olivaceus* Tü2353) in several organisms that produce aromatic polyketides led to novel glycosylated derivatives of 8-DMTC. The expression of *16F4* in the urdamycin A producer, *S. fradiae* Tü2717, led to **44** containing the urdamycin sugars **36** or **37**. Expression of the same cosmid in the mithramycin producer, *S. argillaceus,* led to the attachment of the mithramycin sugars **42** and **43**. The substrate flexible ellaromycin GT, ElmGT, was later cloned out of *16F4* and into the chromosome of *S. albus,* along with plasmids encoding production of **45** and **46**. When these mutants were fed **44**, 8-DMTC derivatives containing either **45** or **46** were obtained.

EXPLORING DESVII/DESVIII SUBSTRATE FLEXIBILITY AND MACROLIDE GLYCODIVERSIFICATION IN VITRO

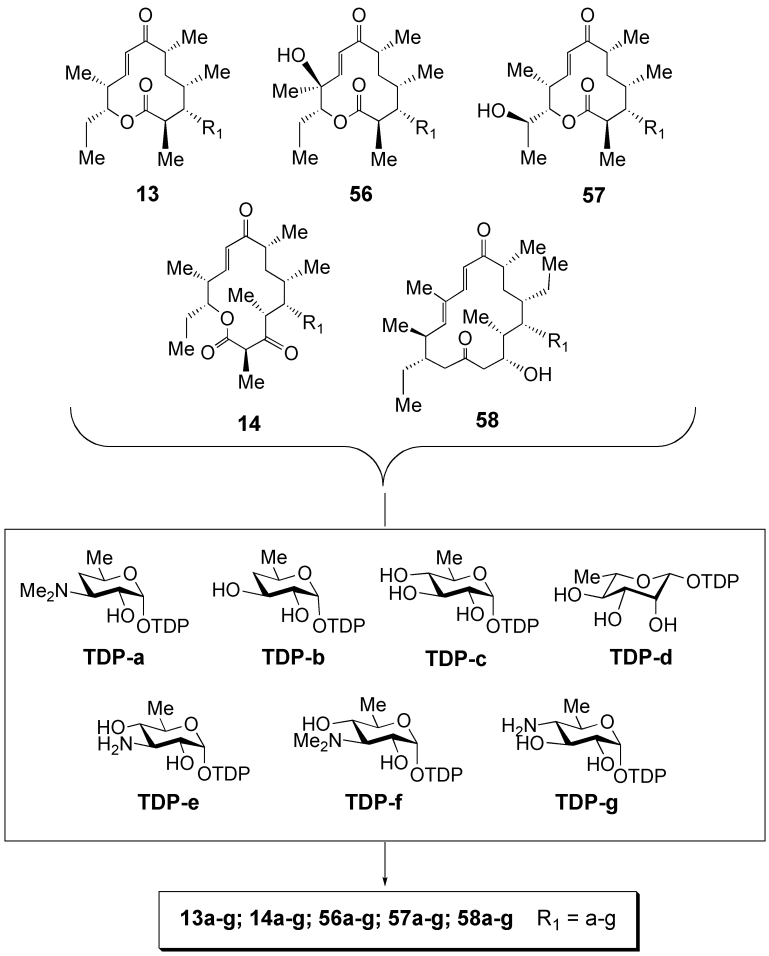
Having established the substrate flexibility and in vivo engineering potential of DesVII/DesVIII, we next sought to explore their flexibility toward unnatural sugar and aglycone substrates in vitro. Toward this goal, we have investigated whether DesVII/DesVIII could glycosylate linear polyketide derivative of the normal cyclic aglycone substrate, **13**. The *S. venezuelae* polyketide aglycone **13** is assembled from **47** via the sequential condensation of acyl thioesters by the modular polyketide synthase (PKS) encoded by the *pikAI-pikAIV* genes of the methymycin/pikromycin cluster (Scheme 4A) [15]. A thioesterase (TE) domain in the last PKS module (module 6), catalyzes the cyclization of the linear ACP-linked intermediate, **48**, to give **13**. Though it has been established that polyketide GTs couple NDP-sugars to cyclized aglycones (e.g., **13**), it is not known whether the linear PKS-aglycone intermediates (e.g., **48**) can also be processed by GTs.

To test the ability of DesVII/DesIII to glycosylate linear aglycones, we chemically synthesized **50**, **51**, and **52**, which are the *N*-acetylcysteamine (NAC) thioester analogs of **48**, a derivative of **50** with the C7 ketone group reduced, and the cyclized form of **51**, respectively (Scheme 4B) [48]. It was found that all three linear aglycone analogs were glycosylated with TDP-D-desosamine (**12**) by DesVII/DesVIII, yielding products **53**, **54**, and **55**. The regiospecific glycosylation suggests that the identity of the functional groups around the glycosylation site are likely important for molecular recognition by DesVII/DesVIII. Although both linear aglycones **50** and **51** were accepted as DesVII/DesVIII substrates, their corresponding cyclic forms (**13** and **52**, respectively) were better substrates, suggesting that the preferred biosynthetic sequence in vivo proceeds with cyclization followed by glycosylation (Scheme 4A, **48** → **13** → **15**). However, these in vitro studies do not exclude the possibility that DesVII/DesVIII is capable of glycosylating a linear aglycone precursor in vivo (**48** → **49**), or that the TE domain of the PKS can cyclize the linear glycosylated intermediate (**49** → **15**). While experiments to resolve these biosynthetic ambiguities are ongoing, the above study clearly expands the potential for the glycodiversification of linear aglycones by DesVII/DesVII and, by extension, other substrate-flexible GTs.



Scheme 4 (A) The biosynthesis of the *S. venezuelae* polyketide aglycone (**13**) occurs via the sequential condensation of acyl thioesters catalyzed by the modular PKS encoded by the *pikAI-pikAIV* genes of the methymycin/pikromycin cluster. The physiological substrate of DesVII/DesVIII could be either the cyclized aglycone (**13**) or a linear ACP-linked intermediate (**48**). (B) DesVII/DesVIII catalyzes the regiospecific glycosylation of **50–52** in vitro. Compound **50** is the NAC thioester analog of **48**, compound **51** is a derivative of **50** with a hydroxyl group at C7, and compound **52** is the cyclized form of **51**. Although **13**, **50**, **51**, and **52** were all glyosylated by DesVII/DesVIII, the cyclic forms (**13** and **52**) were better substrates than their corresponding linear forms (**50** and **51**, respectively), lending credence to the biosynthetic pathway **48** → **13** → **15**.

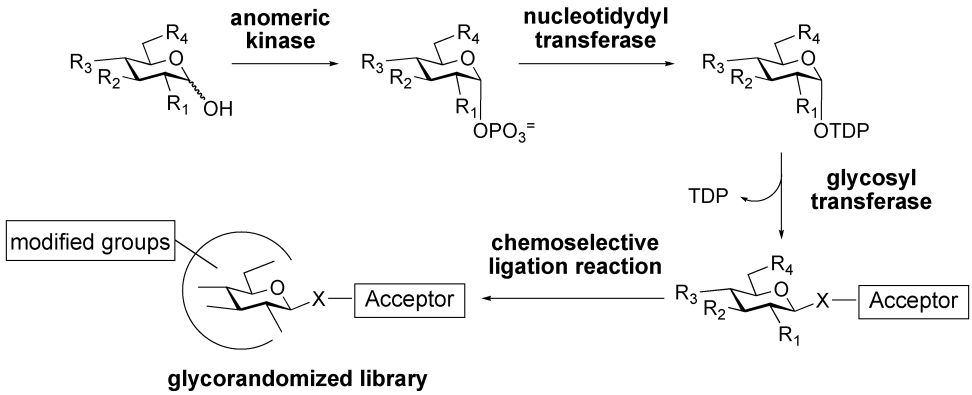
The in vitro aglycone and sugar substrate specificity of DesVII/DesVIII were further explored in another study [49]. Here, 24 TDP-sugars were tested as alternative substrates in the DesVII/DesVIII-catalyzed reaction that couples a TDP-sugar to 10-deoxymethynolide (**13**). Of the sugars tested, seven (**TDP-a–TDP-g**, Scheme 5) were accepted as substrates. These results revealed that DesVII/ DesVIII only accepts 6-deoxysugars with the correct anomeric configuration at C1. Also, C3 alkylation and C2 deoxygenation of the sugar structure appear to hamper the recognition by DesVII/DesVIII, whereas D-, L-, deoxy, amino, and *N*-alkylamino groups at C3 and C4 were tolerated by the coupling enzyme. The seven TDP-deoxysugar substrates (which includes the natural substrate TDP-D-desosamine, **a**) were next tested as substrates for glycosylation of various aglycones (**14**, **56–58**). Interestingly, not only were all seven of these sugars coupled to **13**, but they could also be cou-



Scheme 5 In vitro glycodiversification of various aglycones catalyzed by DesVII/DesVIII. Several natural *S. venezuelae* aglycones including 10-deoxymethynolide (**13**), narbonolide (**14**), and their respective hydroxylated derivatives **56** and **57**, can all be glycosylated by DesVII/DesVIII with seven different TDP-sugars (**TDP-a–g**) to give the glycodiversified library (**13a–g**, **14a–g**, **56a–g**, and **57a–g**). In addition, the non-natural DesVII/DesVIII aglycone substrate, tylactone (**58**), can also be glycodiversified in vitro with **TDP-a–g** to give **58a–g**. All TDP-sugars are in the α-configuration, except TDP-L-rhamnose (**TDP-d**), which is in the β-configuration. DesVII/DesVIII catalyzes glycosylation of the aglycones with inversion of stereochemistry at the anomeric position. Thus, the rhamnose substituents (R_1) in **13d**, **14d**, **56d**, **57d**, and **58d** all have the α-anomeric configuration, while all of the other sugars in the glycosylated products have the β-configuration.

pled to the other three aglycones—narbonolide (**14**), methynolide (**56**), and neomethynolide (**57**) to give **14a–g**, **56a–g**, and **57a–g**, respectively. The relaxed substrate specificity of DesVII/DesVIII for **13** and its hydroxylated derivatives (**56** and **57**) suggests that there is probably some flexibility as to the timing of the glycosylation and aglycone hydroxylation events during methymycin/neomethymycin biosynthesis in vivo. Finally, the seven TDP-deoxysugar substrates for DesVII/DesVIII could also be regiospecifically coupled to the C5 hydroxyl of the 16-membered ring of tylactone (**58**) to yield **58a–g**. In total, 19 new glycosylated macrolides were generated in these experiments. This study established unequivocally that DesVIII is absolutely required for the efficient glycosyl transfer by DesVII in all the reactions examined.

Another useful strategy that employs the substrate-flexible GTs for in vitro glycodiversification of natural products is known as chemoenzymatic glycorandomization (Scheme 6) [9]. Here, natural products that differ only in the identity of their sugar substituent are generated in one-pot reactions through enzymatic synthesis. This approach involves chemical synthesis of a series of unactivated sugars, which are then converted to NDP-sugars enzymatically. Using a substrate-flexible GT, the members of this NDP-sugar library are subsequently coupled to a given aglycone acceptor to yield an array of glycorandomized products. In these studies, Thorson and his coworkers successfully demonstrated the feasibility of chemoenzymatic glycorandomization by creating a library of NDP-sugars using a substrate-flexible anomeric kinase and a substrate-flexible nucleotidylyl transferase [50]. These two enzymes were generated through directed evolution and structure-based engineering of naturally occurring sugar-1-kinase and thymidylyltransferase enzymes [51–55]. Using a GT from the vancomycin biosynthetic pathway (a glycopeptide antibiotic produced by *Amycolatopsis orientalis*), which had previously been shown to be substrate flexible, they were able to generate 31 vancomycin derivatives in a single reaction. One of these new vancomycin derivatives contained an azidosugar allowing further chemoselective diversification to afford 39 additional new vancomycin derivatives. Two of these new compounds exhibited better antibiotic activity than the parent compound against pathogenic *Staphylococcus* and *Enterococcus* strains.



Scheme 6 The concept of chemoenzymatic glycorandomization. This method employs substrate-flexible enzymes to activate and attach the members of a sugar library to a given acceptor molecule in a series of one-pot reactions. First, the library of saccharides is phosphorylated by an anomeric kinase. Next, the sugar-1-phosphates are converted into activated NDP-sugars by a nucleotidylyl transferase. Finally, the NDP-sugar donors are coupled to a given acceptor by a substrate-flexible GT. The "glycorandomized library" is composed of acceptor molecules differing only in the identity of their glycosyl moieties. If appropriate substituents (R_1–R_4) are present on the sugar rings, further diversification of the library can be obtained through chemoselective ligation reactions.

Recently, several antibiotic GTs were shown to catalyze reversible reactions in vitro [56]. This seemingly simple discovery has profound implications for glycodiversification of natural products and for glycobiology research in general. In this work, Thorson and his coworkers showed that CalG1, a GT involved in calicheamicin (CLM) biosynthesis, could exchange the 3-*O*-methyl-L-rhamnose moiety of several different CLM derivatives with many different TDP-sugars. In addition to these "sugar exchange" reactions, they also performed "aglycon exchange" reactions, where a sugar is removed from one aglycone by a reverse GT reaction and then coupled to a separate aglycone by the same GT. This methodology was extended to a 2-GT aglycone exchange reaction, in which a single sugar was exchanged between two structurally unrelated aglycones through the actions of two separate GTs. Furthermore, they demonstrated that NDP-sugars could be easily obtained by removing the sugar from a glycosylated compound with the appropriate GT in the presence of excess NDP. This is an important application of GT reversibility because it offers easy access to NDP-sugars, which are frequently difficult to synthesize by chemical and/or enzymatic methods [3]. Finally, for natural products which contain multiple sugar moieties whose attachments are catalyzed by different GTs, the reverse GT reactions could be used to identify which GT is responsible for the addition of which sugar, and also to elucidate the order of glycosylation events. The general utility of the sugar exchange, the 1 and 2-GT aglycone exchange, and the NDP-sugar synthesis reactions was further explored by kinetic simulation models [57]. These studies indicated that, in most cases, relatively high yields of the desired glycoconjugates can be obtained through proper adjustment of the reactant concentrations. Thus, these simple but practical methodologies will likely enjoy widespread use in future glycodiversification and natural product engineering studies.

CONCLUSION

The examples presented here illustrate the contemporary strategies for glycodiversification of natural products through both in vivo pathway engineering and by various in vitro methods. These glycodiversification strategies represent emerging approaches in our efforts to develop new and improved glycoforms to combat antibiotic-resistant pathogens. With the ever-increasing number of genomes and biosynthetic clusters sequenced, pathway engineering is rapidly becoming a versatile and convenient means to assemble new and very useful biosynthetic machineries. Successful implementation of these sugar-engineering studies relies on our understanding of the substrate flexibilities of the enzymes involved in sugar biosynthesis. Thus, studies that elucidate the mechanisms and biochemical properties of sugar biosynthetic enzymes are of paramount significance and will undoubtedly facilitate glycodiversification research in the future. Importantly, the accumulated results described in this review also reveal the inherent substrate flexibility of several natural product GTs, which ultimately determine the feasibility of enzymatic glycodiversification. Much more knowledge regarding the range of substrate recognition by these and other GTs will be needed before the full potential of this biosynthetic approach can be realized. Clearly, the success of enzymatic glycodiversification will dependent on continuous progress in the discovery of new deoxysugar biosynthetic pathways and the characterization of the inherent or engineered promiscuity of the corresponding GTs. The examples presented in this review suggest a promising future for natural product glycodiversification in the search for new or improved drugs.

ACKNOWLEDGMENT

This work was supported in part by the National Institutes of Health Grants (GM35906 and GM54346).

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