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Natural Product Sugar Biosynthesis and Enzymatic Glycodiversification**

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Abstract

Many biologically active small molecule natural products produced by microorganisms derive their activities from sugar substituents. Changing the structures of these sugars can have a profound impact on the biological properties of the parent compounds. This realization has inspired attempts to derivatize the sugar moieties of these natural products through exploitation of the sugar biosynthetic machinery. This approach requires an understanding of the biosynthetic pathway of each target sugar and detailed mechanistic knowledge of the key enzymes. Scientists have begun to unravel the biosynthetic logic behind the assembly of many glycosylated natural products, and have found that a core set of enzyme activities is mixed and matched to synthesize the diverse sugar structures observed in nature. Remarkably, many of these sugar biosynthetic enzymes and glycosyltransferases also exhibit relaxed substrate specificity. The promiscuity of these enzymes has prompted efforts to modify the sugar structures and/or alter the glycosylation patterns of natural products via metabolic pathway engineering and/or enzymatic glycodiversification. In applied biomedical research, these studies will enable the development of new glycosylation tools and generate novel glycoforms of secondary metabolites with useful biological activity.

Keywords

Biosynthesis; Unusual Sugars; Enzyme Catalysis; Glycodiversification; Enzyme Mechanism

1. Introduction

Glycosylation is one of the most common and important reactions in biological systems and the resulting glycoconjugates have diverse functions, including information storage and transfer, energy storage, maintenance of cell structural integrity, molecular recognition, signaling, virulence, and chemical defense. Several human diseases are associated with aberrant protein glycosylation patterns,[1,2] and initiation of viral infections often involves recognition of specific cell surface protein glycoforms.[3] Likewise, bacterial virulence is related to cell surface polysaccharides,[4] and many bacteria use glycosylated small molecules as chemical weapons to gain a selective advantage, or as signaling molecules for intra- and inter-species communication.[5] A significant number of these glycosylated small molecules are clinically useful for the treatment of bacterial and fungal infections, cancer, and other human diseases. This class of small molecule glycoconjugates will be the focus of this review. Changes in the structures of the sugar moieties of glycosylated compounds can have profound effects

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on their activities, selectivities, and pharmacokinetic properties.[6,7] For all of these reasons, it is desirable to understand the biochemical processes for the formation of glycoconjugates.

Common glycosylated biomolecules include nucleic acids, polysaccharides, proteins, lipids, and secondary metabolites. The biosynthesis of D-ribose (**1**), 2-deoxy-D-ribose (**2**) (Figure 1), and nucleosides will not be covered in this review. Surprisingly, eukaryotic glycoproteins and glycolipids are synthesized from only nine nucleotide sugar donors (**3**–**11**, Figure 1).[8] Although several enzymatic tailoring modifications can occur on these sugars after glycosyltransfer, most eukaryotic glycan structural diversity results from variation in the number and type of the sugar moieties, and in the linkages between the sugar components of oligosaccharides. Conversely, prokaryotic polysaccharides and glycosylated natural products contain more than one hundred different sugars, many of which are deoxygenated and highly functionalized. Therefore, prokaryotic glycoconjugates derive most of their structural diversity from the identities of their unusual sugar moieties.

Because these unusual sugar appendages are important for the bioactivities of many bacterial natural products, there has been much interest in developing strategies to alter the sugar structures of these glycoconjugates via biosynthetic engineering approaches.[9] This requires a sound understanding of both the organization of the native biosynthetic machinery and the mechanisms of the encoded enzymes. The advent of modern molecular biological techniques has led to the discovery and sequencing of the biosynthetic gene clusters for many natural 4 products and unusual sugars, and this has made comparative genomic approaches to functional assignment of the encoded enzymes feasible. This, in turn, has enabled the genetic and biochemical characterization of a number of sugar biosynthetic pathways. A key finding from these studies is that many unusual sugar biosynthetic enzymes and glycosyltransferases (GTs, the enzymes that couple activated sugars to an acceptor molecule) have broad substrate specificity, allowing their use both in vivo and in vitro for the attachment of alternative sugars to natural product acceptors (a process termed glycodiversification). In vitro glycodiversification relies on utilizing a GT with broad specificity to couple chemically or enzymatically synthesized non-native sugar donors to acceptor molecules. Gene disruption and heterologous expression of foreign sugar biosynthetic genes has also enabled the manipulation of endogenous sugar biosynthetic pathways in vivo through metabolic pathway engineering and combinatorial biosynthesis. Both in vitro and in vivo strategies have proven effective in generating natural product analogues with modified sugar structures.

In this review, we summarize the current knowledge of the biosynthesis and glycosyltransfer of unusual sugars found in biologically active small molecule natural products of bacterial origin (Section 2). Only those pathways that have been genetically and/or biochemically verified will be discussed in detail. Next, we discuss the catalytic mechanisms of several sugar biosynthetic enzymes, focusing on common themes employed by Nature to generate sugar structural diversity (Section 3). We will also highlight several unusual and not well-understood sugar modifications that merit further investigation. The structure and mechanisms of glycosyltransferases will be presented in Section 4, with a focus on glycosyltransferases involved in bacterial secondary metabolism. Finally, recent attempts to change the sugar components of natural products through enzymatic glycoengineering will be discussed (Section 5). Together, these studies have not only illuminated Nature's stunning ingenuity in using diverse chemical mechanisms and natural combinatorial biosynthetic processes to drive glycodiversity, but have also enabled the development of methods to manipulate sugar biosynthetic machinery in the hope of generating clinically useful agents.

2. Biosynthesis of Unusual Sugars Found in Natural Products

2.1. Sugar Activation

Monosaccharides must first be activated as either nucleotide monophosphate (NMP)- or nucleotide diphosphate (NDP) derivatives so that they can be used by the biosynthetic enzymes and GTs within the cell. Examples of adenylyldiphosphate (ADP)-, thymidylyldiphosphate (TDP)-, guanylyldiphosphate (GDP)-, uridylyldiphosphate (UDP)-, cytidylyldiphosphate (CDP)-, and cytidylylmonophosphate (CMP)-activated monosaccharides are known. The phosphonucleotidyl moiety has dual purposes: it serves as a recognition element for enzymes involved in the biosynthetic pathways, and it functions as a good leaving group during the glycosyltransfer reaction. The glycolytic intermediates, glucose-6-phosphate (**12**) and fructose-6-phosphate (**13**), are the sources for most nucleotide sugars (Scheme 1). Fructose-6-phosphate (**13**) is converted to mannose-6-phosphate (**14**) by phosphomannoisomerase (PMI) in the biosynthesis of GDP-sugars, and to glucosamine-6-phosphate (**15**) by glucosamine-6-phosphate synthase (GlmS) in the formation of UDP-sugars. Alternatively, UDP-sugars can be derived from galactose (**16**) via the Leloir pathway, which ultimately leads to UDP-glucose (**3**). Glucose-6-phosphate (**12**) is also a biosynthetic precursor of many UDP-sugars, but is more commonly used in the biosynthesis of TDP- and CDP-sugars. In all cases, the sugar-6-phosphates **12**, **14**, and **15** are converted to the corresponding sugar-1-phosphates (**17**, **18**, and **19**, respectively) by distinct, but related phosphohexose mutases prior to nucleotidylyl transfer.[10] In eukaryotes, salvage pathways that utilize sugars generated via catabolic routes (such as glycoprotein degradation) as biosynthetic precursors also exist for several common sugars such as *N*-acetylglucosamine, *N*-acetylgalactosamine, mannose, and fucose.[8] These salvage pathways involve either direct anomeric phosphoryltransfer or 6-phosphorylation followed by a mutase-catalyzed 6→1 migration to yield the sugar-1-phosphate products. The biosynthetic details for the formation of each group of nucleotide sugars will be discussed below.

Transfer of nucleotide monophosphates to sugar-1-phosphate substrates is catalyzed by nucleotidylyltransferase enzymes, and this activation reaction usually occurs early in sugar biosynthetic pathways. A notable exception is that nucleotidylyltransfer occurs late in the biosynthesis of CMP-sugars (such as CMP-sialic acid).[11–13] The majority of nucleotidylyltransferases identified so far share modest to high sequence similarity. However, it is not yet possible to reliably predict nucleotide specificity of these enzymes solely based on amino acid sequence, although phylogenetic analysis has had limited success in the identification of subgroups that roughly correlate with nucleotide specificity. The utility of anomeric sugar kinases and nucleotidylyltransferases in the construction of NDP-sugar libraries for in vitro glycoengineering will be discussed below (Section 5.2.1).

2.2. Naturally Occurring TDP-sugars

TDP-activated sugars are the most structurally diverse class of nucleotide sugars found in nature. In addition to their uses as building blocks for many bacterial polysaccharides, TDP-sugars are also the preferred sugar donors in the biosynthesis of bacterial glycosylated natural products. Nearly all known TDP-sugars are 6-deoxyhexoses, and many are also deoxygenated at C-2, C-3, or C-4 of the pyranose ring. In fact, TDP-sugars are the only known class of NDP-sugars yet discovered that can be deoxygenated at C-2 or C-4. The combination of deoxygenation at one or more positions and the wide variety of other modifications, many of which are not found in other NDP-sugar classes, leads to the rich diversity of TDP-sugar structures seen in nature.

All natural product TDP-sugars whose biosyntheses have been studied are derived from glucose-1-phosphate (**17**), which is converted to TDP-D-glucose (**20**) by a thymidylyltransferase and then to TDP-4-keto-6-deoxy-D-glucose (**21**) by TDP-D-glucose 4,6-

dehydratase (Scheme 2). Because **21** is a key intermediate in the biosynthesis of most bacterial deoxysugars, most natural product biosynthetic gene clusters contain genes encoding a dedicated thymidylyltransferase and 4,6-dehydratase, but examples of clusters lacking these genes are not rare. It is presumed that in these latter cases, the enzymes are shared with polysaccharide biosynthesis. To date, the biosynthetic pathways for more than thirty unusual TDP-sugars have been reported. Most of these pathways are proposed based on gene cluster sequence information, and less than half of these pathways are supported by experimental data. However, correlation of phenotypes with specific gene disruptions and/or biochemical characterization of heterologously expressed enzymes have enabled the detailed elucidation of several pathways. These studies have provided an important framework for understanding the molecular logic behind the reaction sequences for the biosynthesis of unusual sugars which has, in turn, allowed better prediction of other pathways based on gene sequence information.

Summarized in Scheme 3 is a nearly comprehensive collection of natural product TDP-sugar biosynthetic pathways, which has been assembled on the basis of at least some biochemical and genetic data. These pathways are divided into three groups based mainly on the degree of deoxygenation. One remarkable aspect of these pathways is that the primary structural differences in the final TDP-sugar products are generated by the action of only five enzyme reaction types, illustrating Nature's economical use of a "combinatorial biosynthesis" strategy to create structural diversity. The mechanistic details of some of these "common" enzymatic activities are discussed in Section 3.1.

2.2.1. Group I – 6-Deoxy-, 3-Amino-3,6-dideoxy-, and 4-Amino-4,6-dideoxysugars—The D-fucose (see **22**) and D-digitalose (**23**) residues of the antitumor compound chartreusin produced by *Streptomyces chartreusis* and the D-fucofuranose (see **24**) residue of the antibiotic gilvocarcin V produced by *Streptomyces griseoflavus* are believed to be derived from TDP-D-fucose (**22**), which is in turn derived from **21** via ketoreduction (Scheme 3a). Compound **22** is also a building block for the capsular polysaccharides in *Aneurinibacillus actinomycetemcomitans*.[14, 15] The ketoreduction step is likely catalyzed either by ChaS3, [16] a homologue of the ketoreductase Fcd in *A. actinomycetemcomitans,*[17] or by the short-chain dehydrogenase/reductase (SDR) enzyme ChaS4. D-Fucose is then *O*-methylated by the methyltransferase ChaM after glycosidic coupling to form D-digitalose (**23**). In gilvocarcin biosynthesis, GilL and GilU (both of the SDR family) are candidates for catalyzing the conversion of **21** to **22** and the subsequent ring contraction step to form TDP-D-fucofuranose (**24**), although this is speculative.[18]

Biosyntheses of sugars **25–35** are proposed to share a 3,5-epimerization step converting **21** to TDP-4-keto-6-deoxy-L-mannose (**31**). Enzymes catalyzing this reaction are homologues of RmlC involved in TDP-L-rhamnose (**34**) biosynthesis in *Salmonella enterica*.[17,[19]–21] The sugar L-nogalose (**25**) is present in the anthracycline antibiotic nogalamycin made by *Streptomyces nogalater*. Formation of **25** was proposed to proceed by a sequential 3,5-epimerization reaction (SnogF), 3-*C*-methylation (SnogG2), and 4-ketoreduction (SnogC) to afford TDP-6-deoxy-3-*C*-methyl-L-mannose (**32**), which is likely the substrate used in the glycosyltransfer reaction.[22] Methylations of the 2-, 3-, and 4-hydroxyl groups by the methyltransferases SnogL, SnogM, and SnogY, to give L-nogalose (**25**) are presumed to be post-glycosylation events.

The unusual sugar D-streptose (**26**), found in the aminoglycoside antibiotic streptomycin, is produced by several *Streptomyces* species, most notably *S. griseus*. Early biochemical work demonstrated that the immediate donor of the streptose moiety is TDP-D-dihydrostreptose (**33**),[23] which is formed in two steps from **21**: 3,5-epimerization to form **31** followed by NADPH-dependent ring contraction to give **33**.[24,25] The streptomycin gene cluster was later identified in *S. griseus*, and the epimerization and ring contraction reactions were assigned to

be catalyzed by StrM, a RmlC homologue, and StrL, a SDR superfamily member, respectively.[26] Heterologous expression of *strL* and *strM* together in a mutant of the methymycin producer *S. venezuelae*, which accumulates **21**, resulted in the production of methymycin derivatives bearing L-rhamnose (see **34**).[27] Although no dihydrostreptose was produced, the fact that **21** was converted to TDP-L-rhamnose (**34**) in this recombinant strain provided strong support that StrM is a 3,5-epimerase and that StrL has 4-ketoreductase activity.[27] The proposed formation of both furanose (**33**) and pyranose (**34**) products from **31** by StrL is reminiscent of the reaction catalyzed by UDP-apiose synthase ecoded by *AXS1* in *Arabidopsis thaliana*.[28–30] The ring contraction of **22** to **24** in gilvocarcin biosynthesis may also follow a similar route.

Various *O*-methylated L-rhamnose moieties exist in nature, such as **27** and **28** found in the macrolide compounds spinosyn and butenylspinosyn, both produced by *Saccharopolyspora spinosa*,[31] the aromatic polyketide elloramycin produced by *Streptomyces olivaceus*,[32] and the enediyne calicheamicins of *Micromonospora echinospora*.[33] The genes *spn/busH*, *spn/busI*, and *spn/busK* encode the *O*-methyltransferases in the spinosyn/butenylspinosyn pathways, whereas *calS11* encodes the 3-*O*-methyltransferase used in calicheamicin biosynthesis. Interestingly, the genes required for the formation of **34** are absent in the gene clusters of spinosyns/butenylspinosyns. Instead, they are located in other regions of the genome in *S. spinosa*, and they likely function both in cell wall biosynthesis and in the formation of spinosyns.[34]

TDP-4-*N*,*N*-dimethylamino-4-deoxy-5-*C*-methyl-L-rhamnose (**29**) and TDP-L-noviose (**30**) are the predicted sugar donors for the biosynthesis of the enediyne antibiotic C-1027[35] and the aminocoumarin antibiotics novobiocin,[36] clorobiocin,[37] and coumermycin,[38] respectively. These sugars have a 5,5-*gem*-dimethyl moiety formed by *C*-methylation at C-5. Their biosynthesis from **21** involves either 3,5- or 3-epimerization catalyzed by RmlC homologues to form **31** or **36**, respectively, followed by 5-*C*-methyltransfer to give TDP-4-keto-6-deoxy-5-*C*-methyl-L-mannose (**35**). Results obtained from coupled assays of the purified epimerase NovW and 5-*C*-methyltransferase NovU from the novobiocin pathway,[39] along with gene disruption studies of *cloU* from the clorobiocin biosynthesis[40] suggested that the biosynthesis of **30** involves 3,5-epimerization rather than 3-epimerization. However, a recent in vitro study showed that the epimerase NovW is kinetically competent only as a 3-epimerase.[41] The final step of the biosynthesis of **30** is the C-4 reduction of **35** catalyzed by NovS/CloS/CumU.[39] Formation of **29** in C-1027 biosynthesis has been proposed to involve 3,5-epimerization by SgcA2, *C*-methyltransfer by SgcA3, C-4 aminotransfer by SgcA4, and 4-*N*,*N*-dimethyltransfer by SgcA5.

Several post-glycosylation tailoring steps on the L-noviose (see **30**) moiety of the aminocoumarin antibiotics have been characterized via gene disruption and in vitro biochemical methods.[42–45] In novobiocin biosynthesis, the carbamoyltransferase NovN modifies the C-3 hydroxyl group of L-noviose, after which the *O*-methyltransferase NovP acts at the C-4 hydroxyl group to produce the fully elaborated sugar.[44] In clorobiocin and coumermycin biosynthesis, 4-*O*-methylation catalyzed by CloP/CouP is thought to occur first. The 5-methyl-2-pyrrolylcarbonyl moiety is then transferred from the peptidyl carrier protein (PCP) 11 CloN1/CouN1 to the 3-position of the pendant 4-*O*-methyl-L-noviose by the acyltransferase CloN7/CouN7.[43,45]

The sugars D-mycinose (**38**) and D-mycaminose (see **41**) are found in the structures of several macrolide antibiotics, including tylosin, chalcomycin, dihydrochalcomycin, and mycinamicin. Tylosin carries both sugars, whereas chalcomycin, dihydrochalcomycin, and mycinamicin contain **38**. The biosynthetic gene clusters for these compounds have been sequenced,[46–50] and recent genetic and biochemical studies performed on the tylosin and dihydrochalcomycin systems have fully established the pathways for the formation of these

two sugars.[47,[51]–55] The key intermediate, TDP-6-deoxy-D-allose (**37**), in the pathway of **38** is synthesized from **21** via C-3 epimerization by the RmlC homologues GerF/TylJ/ChmJ/MydH followed by C-4 ketoreduction by GerKI/TylD/ChmD/MydI. In a recent in vitro study, **37** was confirmed to be the sole product formed in incubations of **21** with the purified dihydrochalcomycin biosynthetic enzymes GerF and GerKI.[47] A similar reaction sequence likely occurs in the tylosin, chalcomycin, and mycinamicin pathways. The *O*-methylation of the two hydroxyl groups occurs after glycosyltransfer, and is catalyzed by GerMII,MIII and Tyl/Chm/MycE,F. During tylosin biosynthesis, TDP-D-mycaminose (**41**) is constructed in three steps from **21**: 3,4-ketoisomerization by Tyl1a to form TDP-3-keto-6-deoxy-D-glucose (**39**), aminotransfer by TylB to form **40**, and *N*,*N*-dimethylation by TylM1 to form **41**. The functions of Tyl1a,[54] TylB,[53] and TylM1[52] have all been verified biochemically with purified enzymes.

2.2.2. Group II – 4,6-Dideoxy-, 3-Amino-3,4,6-trideoxy-, and 3-Amino-2,3,6-trideoxysugars—The sugars D-chalcose (**42**) and D-desosamine (see **43**) are constituents of many macrolide antibiotics (Scheme 3b). Of those whose gene clusters have been sequenced, lankamycin,[56] chalcomycin,[50] and dihydrochalcomycin[46] contain D-chalcose, while erythromycin,[57] oleandomycin,[58–60] mycinamicin,[49] methymycin/pikromycin,[61] and megalomicin[62, 63] contain D-desosamine. Early gene disruption experiments carried out with the erythromycin producer *Saccharopolyspora erythraea* led to several possible pathways for TDP-D-desosamine (**43**) formation.[57, [64]–66] Later genetic and biochemical studies of the methymycin/pikromycin system from *Streptomyces venezuelae* clearly showed that **43** is biosynthesized from **21** in four steps.[67–70] As delineated in Scheme 3b, the reaction is initiated with C-4 aminotransfer catalyzed by DesI to give **44**, followed by oxidative deamination by DesII to yield **45**, C-3 transamination by DesV to afford **46**, and 3-*N*,*N*-dimethylation by DesVI to furnish **43**.[67–69] The reaction catalyzed by DesII (**44** → **45**), which is a member of the radical-SAM superfamily, is unique in sugar biosynthesis. Together, DesI and DesII carry out C-4 deoxygenation of **21** to form TDP-3-keto-4,6-dideoxy-D-glucose (**45**).[70–72]

Homologues of DesI, DesII, DesV, and DesVI are found in the erythromycin, oleandomycin, mycinamicin, and megalomicin pathways, and are presumed to catalyze the equivalent reactions in the biosynthesis of D-desosamine (**43**) in each pathway. Although the biosynthesis of **43** has now been fully elucidated, that of chalcose (**42**) remains unexplored. However, genes encoding homologues of DesI and DesII are present in the lankamycin, chalcomycin, and dihydrochalcomycin gene clusters, suggesting that C-4 deoxygenation in chalcose (**42**) formation occurs in a manner analogous to that of desosamine biosynthesis (**21** → **44** → **45**). Conversion of **45** to TDP-4,6-dideoxy-D-glucose (**47**) requires a 3-ketoreductase. An NDP-sugar ketoreductase gene, *lkm42*, exists in the lankamycin gene cluster, but is absent in the chalcomycin and dihydrochalcomycin clusters. The corresponding gene in the latter cases may be encoded elsewhere in the *Streptomyces bikiniensis* or *Streptomyces* sp KCTC 0041BP genomes, respectively. *O*-Methylation at C-3 to form **42** likely happens after glycosyltransfer, and may be catalyzed by ChmCI/Lkm45/GerMI in chalcomycin, lankomyicn, and dihydrochalcomycin biosynthesis, respectively.

The aminoglycoside antibiotic spectinomycin produced by *Streptomyces flavopersicus* and *Streptomyces spectabilis* contains an unusual 3-keto-4,6-dideoxy-glucose moiety, known as actinospectose (**49**). Partial gene clusters for spectinomycin biosynthesis[73] have been isolated from these two strains. Both clusters contain glucose-1-phosphate thymidylyltransferase and TDP-glucose-4,6-dehydratase genes (*spcK* and *spcJ*, respectively, in *S. flavopersicus* and *spcD* and *spcE*, respectively, in *S. spectabilis*). The activity of SpcE has been verified in vitro,[73] implicating TDP-glucose as the precursor in the actinospectose pathway. Although the mechanism of C-4 deoxygenation is not obvious, both spectinomycin

clusters encode a putative radical-SAM enzyme (ScpY in *S. flavopersicus* and SpeY in *S. spectabilis*), which may play a role in generating TDP-actinospectose (**45**). Thus, a pathway involving 4-ketoreduction of **21** to **48** by the SDR enzyme SpcI/SpeI, followed by oxidative dehydroxylation by ScpY/SpeY is conceivable for the biosynthesis of **49**. The proposed mechanism (**21** → **48** → **45**) parallels that of the C-4 deoxygenation step carried out by DesI/DesII during D-desosamine biosynthesis. Interestingly, SpcY and SpeY share no detectable sequence identity with DesII. Their functions clearly warrant further investigation.

Compounds **50–58** are representatives of 3-amino-2,3,6-trideoxy sugars, whose gene clusters have been sequenced. Each gene cluster encodes a 2,3-dehydratase and a 3-aminotransferase, which catalyze the respective C-2 deoxygenation of **21** to give TDP-3,4-diketo-2,6-dideoxy-D-glucose (**59**) and the subsequent C-3 aminotransfer to generate TDP-3-amino-4-keto-2,3,6-trideoxy-D-glucose (**60**). After **60**, each individual pathway adopts a distinct combination of epimerization, stereospecific C-4 ketoreduction, and *C*- and/or *N*-methyltransfer steps to produce the TDP-sugar product. For example, the key intermediate (**61**) in the biosynthesis of 3-*N*-methyl-4-*O*-methyl-L-ristosamine (**50**) -the sugar component of the indolocarbazole antibiotic staurosporine -is formed via a StaE-catalyzed C-5 epimerization of **60** followed by StaK-catalyzed C-4 ketoreduction. Transfer of L-ristosamine to the aglycone by StaG is the next step, which is followed by crosslinking between C-5 of ristosamine and the indole nitrogen of the aglycone mediated by StaN, a P450 enzyme. The final 3-*N*-methylation and 4-*O*-methylation reactions to give staurosporine result from the action of StaMA and StaMB, respectively.[74] Evidence supporting the proposed pathway for **50** comes from the successful reconstitution of staurosporine biosynthesis in heterologous hosts.[74,75]

The biosynthesis of L-megosamine (see **51**) in the macrolide antibiotic megalomicin is predicted to be analogous to TDP-L-ristosamine (**61**), involving C-5 epimerization of **60** (MegDIV), C-4 ketoreduction of the resulting L-sugar (MegDV), and 3-*N*,*N*-dimethylation of intermediate **61** (MegDIII) to give TDP-L-megosamine (**51**).[63] Interestingly, megalomicin contains two 3-*N*,*N*-dimethylamino sugars, D-desosamine (see **43**) and L-megosamine, yet the gene cluster has only one aminotransferase (*megDII*) and one dimethyltransferase (*megDIII*) gene. The encoded enzymes likely catalyze the corresponding steps in both sugar biosynthetic pathways.[62]

The sugars L-nogalamine (**52**),[22] L-daunosamine (see **53**),[76] and L-rhodosamine (see **54**) [77] are found in the anthracycline antibiotics nogalamycin, daunorubicin, and aclarubicin, respectively. Their common precursor is **60**, which undergoes 3,5-epimerization and stereospecific ketoreduction in each pathway. The C-4 hydroxyl group of TDP-L-acosamine (**62**), produced by the tandem action of SnogF and SnogG, is equatorial, whereas that of TDP-L-daunosamine (**53**) is axial. These sugars can be 3-*N*,*N*-dimethylated to produce TDP-2-deoxy-L-nogalamine (**63**), the sugar donor in nogalamycin formation, or TDP-L-rhodosamine (**54**), the sugar donor in aclarubicin and rhodomycin biosynthesis. Once transferred to the aglycone, crosslinking of C-5 of 2-deoxy-L-nogalamine (**63**) to the aglycone and re-hydroxylation at C-2 are proposed to generate the final compound.[22] The identity of these tailoring enzymes, as well as the logic for having to deoxygenate and then re-hydroxylate at C-2 of the sugar moiety is not clear.

The 3-amino-2,3,6-trideoxysugars, TDP-4-oxo-L-vancosamine (**55**) and TDP-L-eremosamine (**56**), are intermediates in the biosynthesis of the vancomycin-type antibiotics balhimycin[78] and chloroeremomycin,[79] respectively. TDP-3-*N*,*N*-dimethyl-L-eremosamine (**57**) along with TDP-D-angolosamine (**58**) are the two sugar donors in the biosynthesis of hedamycin. [80] Sugar **58** is also involved in the biosynthesis of the benzoisochromanequinone antibiotic medermycin.[81] The complete biosynthetic pathway for **56**, starting from **21**, has been elucidated through the biochemical analysis of the pathway enzymes.[82] The key intermediate **55**, the substrate for glycosyltransfer in the balhimycin pathway, is derived from **60** by C-3

methylation followed by 5-epimerization. Subsequent C-4 ketoreduction of **55** results in **56**, the sugar donor in chloroeremomycin biosynthesis. It is unusual for a ketosugar, such as **55**, to be a substrate for a glycosyltransferase. However, inspection of the balhimycin gene cluster shows an inactive 4-ketoreductase gene (*dvaE*), which at one point likely catalyzed the conversion of **55** to **56** in the balhimycin producing strain. This, combined with the extensive conservation observed between balhimycin and chloroeremomycin clusters, suggests a close evolutionary relationship between the two pathways.[83]

L-Vancosamine, the C-4 epimer of L-eremosamine (see **56**), is a component of the glycopeptide antibiotic vancomycin. Although analysis of the L-vancosamine biosynthetic genes has not been reported, formation of TDP-L-vancosamine is presumed to be identical to that of **56** except that the stereochemistry of C-4 ketoreduction is reversed. Likewise, TDP-3-*N*,*N*-dimethyl-L-eremosamine (**57**), involved in hedamycin biosynthesis, can be made in an identical manner to that of **56** by the respective *Hed* biosynthesis enzymes, but with an additional dimethylation step catalyzed by HedO to convert **56** to **57**.[80] TDP-D-angolosamine (**58**), whose genes have been identified in both the hedamycin and medermycin gene clusters, is predicted to be made in two steps from **60**: 4-ketoreduction by Med14/HedN, and 3-*N*,*N*-dimethyltransfer by Med15/HedH.[80,81]

2.2.3. Group III – 2,6-Dideoxy-, 4-Amino-2,4,6-trideoxy-, 2,3,6-Trideoxy-, and 4-Amino-2,3,4,6-tetradeoxysugars—TDP-2,6-dideoxysugars and their derivatives, which are formed by 2,3-dehydration of **21** and subsequent 3-ketoreduction, account for the majority of TDP-sugars used in natural product biosynthetic pathways (Scheme 3c). The enzymes catalyzing 2,3-dehydration of **21** to form **59** in each of these pathways are homologous to those catalyzing the same reaction in the biosynthesis of 3-amino-2,3,6-trideoxysugars depicted in Scheme 3b. This group of TDP-sugars can be further divided into two subgroups depending on the configuration of their 3-OH group (see **64** and **73**). Interestingly, enzymes catalyzing the axial and equatorial 3-ketoreduction are all NAD(P)H-dependent reductases, but share no detectable sequence similarity, making their coding genes readily distinguishable.

TDP-D-vicenisamine (**67**), TDP-D-digitoxose (**68**), 4-*O*-acetyl-L-arcanose (**70**), TDP-L-mycarose (**71**), and L-cladinose (**72**), all have an axial 3-OH group and each is derived from TDP-4-keto-2,6-dideoxy-D-allose (**64**), which is formed from **21** by 2,3-dehydration followed by 3-ketoreduction. The enzymes catalyzing these two steps (**21** → **59** → **64**) in the biosynthesis of **71** (TylX3 and TylC1, respectively) have been characterized in vitro.[84] Compound **66**, the sugar donor in the avermectin biosynthetic pathway,[85] is produced from **64** in three steps: 5-epimerization by AveBV (AvrF), 4-ketoreduction by AveBIV (AvrE), and 3-*O*-methylation by AveBVII (AvrH). Heterologous expression of the complete set of the biosynthetic enzymes supports the proposed pathway of **66**.[86] Although the exact order of these steps remains unknown, current data suggest that 3-*O*-methyltransfer occurs at the TDP-L-olivose (**65**) stage (**65** → **66**), rather than as a separate tailoring step.[87]

TDP-D-vicenisamine (**67**), the sugar donor for the biosynthesis of the macrolactam antibiotic vicenistatin in *Streptomyces halstedii*, is proposed to be derived from **64** via C-4 transamination by VinF followed by *N*-monomethylation by VinG.[88] Sugar **67** is the only 4-amino-2,4,6-trideoxysugar whose biosynthetic genes have been identified, and is a rare example of an *N*-monomethylated aminosugar. The gene cluster encoding the formation of lipomycin, which contains D-digitoxose (see **68**), has been located in *Streptomyces aureofaciens*.[89] This unusual sugar is formed by C-4 ketoreduction of **64** by LipDig4. The sugars 4-*O*-acetyl-L-arcanose (**70**), TDP-L-mycarose (**71**), and the *O*-methylated L-mycarose derivative L-cladinose (**72**) are biosynthesized from **64** via similar routes. The biosynthetic pathway for **71**, part of the tylosin pathway of *Streptomyces fradiae*, has been fully characterized in vitro.[90–92] Compound **64** is 3-*C*-methylated by the SAM-dependent methyltransferase TylC3. Next, TylK

epimerizes C-5 and TylC2 reduces C-4 to form **71**. In erythromycin biosynthesis, L-cladinose (**72**) is produced by 3-*O*-methylation of L-mycarose by EryG after it has been transferred from **71** to the macrolactone. The homologues of **71** biosynthetic enzymes found in the erythromycin pathway must catalyze identical reactions as their counterparts in the tylosin pathway.

The biosynthetic pathway for 4-*O*-acetyl-L-arcanose (**70**), which is found in the macrolide antibiotic lankamycin produced by *Streptomyces rochei*, is expected to be analogous to that of **71**.[56] Indeed, genes with high sequence identity (40–75%) to those involved in the biosynthesis of **71** are found in the lankamycin cluster, consistent with a pathway in which all reactions (except the final 4-ketoreduction step) are the same as those found in the biosynthesis of **71**. The 4-ketoreduction by Lkm41 would give the C-4 epimer of **71**, TDP-L-axenose (**69**), which is a reasonable substrate for glycosyltransfer. Tailoring reactions involving 3-*O*-methylation, possibly by Lkm28, and 4-*O*-acetylation by an unknown enzyme would complete the biosynthesis of **70**.

The sugars 2-deoxy-L-fucose (see **75**), L-oleandrose (**77**), L-digitoxose (see **78**), D-olivose (see **79**), 4-*O*-carbamoyl-D-olivose (**80**), D-oliose (see **81**), 4-*O*-acetyl-D-oliose (chromose D, **82**), 4-*O*-methyl-D-oliose (chromose A or olivomose, **83**), D-mycarose (**85**), L-chromose B (or olivomycose, **87**), and 2-deoxy-D-evalose (**90**) are all 2,6-dideoxysugars, most carrying an equatorial 3-OH group. They are biosynthesized from TDP-4-keto-2,6-dideoxy-D-glucose (**73**), which is derived from **21** via 2,3-dehydration followed by stereospecific 3-ketoreduction. Compound **73** has been suggested to be the substrate for glycosyltransfer in the biosynthesis of mithramycin, an antitumor agent, and granaticin, a benzoisochromanequinone antibiotic. Granaticin contains an unusual aryl-*C*-L-olivosyl moiety (**74**), which is likely formed using **73** as the sugar donor followed by oxidative crosslinking between the aglycone and the C-4 carbonyl carbon of the sugar appendage.[93]

Interestingly, mithramycin derivatives bearing a 4-keto-2,6-dideoxy-D-glucose moiety (presumably derived from **73**) in place of D-olivose (see **79**) were produced by a *Streptomyces argillaceus* mutant in which a *C*-methyltransferase gene (*mtmC*) was inactivated. Curiously, heterologous expression of *mtmC* in trans in this mutant restored mithramycin production.[94] In a later study, the authors proposed that the MtmC protein may need to be present in order to interact with a 4-ketoreductase (either MtmTI or MtmTII) also encoded in the cluster.[95] They proposed that this 4-ketoreductase may reduce **73** following its transfer to the mithramycin aglycone.

2-Deoxy-L-fucose (see **75**) is a sugar component of the anthracycline antibiotics aclarubicin (aclacinomycin) and rhodomycin, and is presumably synthesized as TDP-2-deoxy-L-fucose (**75**) in two steps from **73**: 3,5-epimerization and 4-ketoreduction. Although the gene clusters for both aclarubicin[77] and rhodomycin[96] have been partially sequenced, genes for these activities have not been assigned in either cluster. L-Oleandrose (**77**) is found in the macrolide antibiotic oleandomycin produced by *Streptomyces antibioticus* and in avermectin produced by *Streptomyces avermitilis*. Interestingly, L-oleandrose is constructed via different routes in these two pathways. It was shown via heterologous expression of the oleandomycin biosynthetic genes[97] that **77** is formed from **73** by 3,5-epimerization and 4-ketoreduction catalyzed by OleL and OleU, respectively, resulting in TDP-L-olivose (**76**), which is the donor for glycosyltransfer. 3-*O*-Methylation by OleY has been confirmed in vitro to occur after sugar attachment.[98] This is in contrast to the biosynthesis of L-oleandrose in the avermectin pathway, where TDP-Loleandrose (**66**) is generated from **64** via 5-epimerization, followed by 4-ketoreduction and 3-*O*-methylation on the nucleotide sugar prior to glycosyltransfer.[86]

TDP-L-digitoxose (**78**) is the precursor for the L-digitoxose unit found in the antibiotics jadomycin and kijanimicin produced by *Streptomyces venezuelae* ISP5230 and *Actinomadura*

kijaniata, respectively. Studies of purified *A. kijaniata* sugar biosynthetic enzymes have fully established the TDP-L-digitoxose (**78**) pathway. The conversion of **21** to **73** involves KijB1 and KijD10, and that of **73** to **78** is catalyzed by the 5-epimerase KijD11 and the 4-ketoreductase KijC2.[99] The same roles are predicted for the KijD11 and KijC2 counterparts, JadU and JadV, respectively, in the biosynthesis of jadomycin.[100] TDP-D-olivose (**79**) is a common sugar donor used in the biosynthesis of a variety of natural products, including landomycin,[101] urdamycin,[102] mithramycin,[103] chromomycin,[104] chlorothricin,[105] avilamycin,[106] and concanamycin.[107] The biosynthetic gene clusters for these compounds have been identified. Genes encoding enzymes for the conversion of **21** to **79** have been found in each cluster except that of mithramycin. In the concanamycin cluster, a putative carbamoyltransferase, Con7, catalyzing 4-*O*-carbamoylation of TDP-D-olivose (**79**) to make **80** has also been assigned. This reaction could occur prior to or after glycosyltransfer.

TDP-D-oliose (**81**) is the presumed precursor for the D-oliose moiety in mithramycin, and the chromose D and olivomose moieties in chromomycin. Results of gene disruption studies in the mithramycin producer *Streptomyces argillaceus* provided indirect evidence that MtmU functions as the 4-ketoreductase converting **73** to **81**.[94] However, MtmU shares sequence identity (~ 50%) with sugar 3-ketoreductases rather than 4-ketoreductases. If the proposed activity for MtmU is correct, it would be an interesting example of "regio-promiscuity" of a sugar biosynthetic enzyme. The chromomycin gene cluster encodes two 4-ketoreductase homologues, CmmUI and CmmUII, one of which should catalyze the conversion of **73** to **81**. The 4-*O*-acetylation and 4-*O*-methylation of the two D-oliose moieties of chromomycin to form chromose D (**82**) and olivomose (**83**) may be catalyzed by CmmA and CmmMIII, respectively, and likely occur after glycosyltransfer.[104]

Mithramycin and chromomycin also contain D-mycarose (**85**) and olivomycose (**87**), both of which are derived from 3-*C*-methylation of **73**. The methyltransferase MtmC has been assigned this role through gene disruption studies in *S. argillaceus*.[94] The homologous CmmC encoded in the chromomycin cluster likely functions in the same capacity. The resulting compound, TDP-4-keto-D-mycarose (**84**) can be used as the substrate in the glycosyltransfer reaction in the mithramycin pathway, since disruption of the gene encoding the C-4 reductase MtmTIII resulted in mithramycin derivatives carrying a 4-keto-D-mycarose moiety (see **84**) in place of **85**.[95] The olivomycose (**87**) unit of chromomycin is predicted to be constructed from **84** by 5-epimerization and 4-ketoreduction to give TDP-L-chromose (**86**), followed by glycosyltransfer and 4-*O*-acetylation. It is possible that the 4-*O*-acetylation reaction is also catalyzed by CmmA, as in the proposed pathway for **82**.[104]

The 2-deoxy-D-evalose moiety (**90**) of the heptasaccharide chain of avilamycin A is believed to come from **73** via 3-*C*-methylation by AviG2 to generate **88**. This methylation step is identical to the TylC3/EryBIII/Lkm27/MtmC/CmmC reaction (discussed above). However, in the AviG2-catalyzed reaction, the stereochemistry of the 3-OH group is retained, whereas it is inverted in the TylC3/EryBIII/Lkm27/MtmC/CmmC-catalyzed reactions. Following *C*-methylation, 4-ketoreduction by either AviZ1 or AviZ2 is expected to produce TDP-2-deoxy-D-evalose (**89**).[106] After glycosyltransfer, an orthoester linkage is formed between the 2-deoxy-D-evalose moiety and the adjacent D-olivose residue. This step may be catalyzed by one of the three non-heme iron dependent enzymes (AviO1, AviO2, and AviO3) encoded in the avilamycin cluster.

The 2,3,6-trideoxysugars, such as TDP-L-amicetose (**92**) and TDP-L-rhodinose (**94**), and the 4-amino-2,3,4,6-tetradeoxysugar, TDP-D-forosamine (**100**), are another subset of TDP-sugars derived from **73**. The key step in their biosynthesis is the C-3 deoxygenation of **73** to form TDP-4-keto-2,3,6-trideoxy-D-glucose (**91**) as an intermediate.[108] Compound **92** is predicted to be the sugar donor in the biosynthesis of the terpene antibiotic phenalinolactone, which

carries a 4-*O*-methyl-L-amicetose (**93**) moiety. A pathway consisting of 3-deoxygenation by PlaA1 to form **91**, 5-epimerization by PlaA8, and 4-ketoreduction by PlaA7 likely generates **92**. *O*-Methyltransfer by PlaM1, which is assumed to occur after glycosyltransfer, will give **93**.[109]

L-Rhodinose (see **94**), the C-4 epimer of L-amicetose (see **92**), is found in urdamycin,[102] landomycin,[101] aclarubicin (aclacinomycin),[77] rhodomycin,[96] and granaticin,[93] all of whose gene clusters have been sequenced. TDP-L-rhodinose (**94**) is biosynthesized from **91** via 5-epimerization and 4-ketoreduction. Evidence for the functions of the 5-epimerase (UrdZ1) and the 4-ketoreductase (UrdZ3) in the biosynthesis of **94** was obtained when their corresponding genes were individually disrupted in the urdamycin producer *Streptomyces fradiae*, which subsequently failed to produce urdaymcin derivatives containing L-rhodinose moieties.[102] Genes encoding enzymes for these steps have been assigned in the landomycin, and granaticin gene clusters, but neither was identified in the rhodomycin or aclacinomycin cluster. The epimerase gene is also not found in the nanchangmycin cluster. These activities may be encoded elsewhere in the genome, or may be carried out by the promiscuous L-rhodosamine biosynthetic enzymes in the case of rhodomycin and aclacinomycin. In the polyether natural product nanchangmycin, L-rhodinose is methylated after attachment by NanM, giving 4-*O*-methyl-L-rhodinose (**95**).[110]

The major products of aclacinomycin biosynthesis, aclacinomycin A and B, contain L-cinerulose (**96**) and L-cinerulose B (**98**), respectively, while aclacinomycin N contains L-rhodinose (see **94**) and is only a minor compound. The available evidence suggests that extracellular oxidases rapidly convert the L-rhodinose moiety to **96**, which is further oxidized to L-aculose (**97**), and finally to **98** outside the cell. An intracellular system for reducing these intermediates back to **96** also exists, but the purpose for these interconversions is not clear.[111] The 4-amino-2,3,4,6-tetradeoxysugar, D-forosamine (see **100**), is found in the macrolide antibiotics spinosyn, butenyl spinosyn, and spiramycin. To date, it is the most highly deoxygenated sugar found in nature. The biosynthesis of TDP-D-forosamine (**100**) in the spinosyn producing strain, *Saccharopolyspora spinosa*, has been fully elucidated in vitro. In this work, SpnQ was shown to be the 3-dehydrase converting **73** to **91**,[112] and SpnR was identified as the 4-transaminase converting the SpnQ product **91** to TDP-4-amino-2,3,4,6-tetradeoxy-D-glucose (**99**).[113] The *N*,*N*-dimethylation of **99** is catalyzed by SpnS. Interestingly, unlike its homologue E_1 (see Section 3.1.5 for a mechanistic discussion), SpnQ does not have a dedicated reductase partner encoded in the gene cluster, but instead uses general cellular reductases such as ferredoxin and/or flavodoxin for electron transfer.[112–114]

2.3 UDP-sugars

A variety of UDP-sugars exist in nature, including six of the nine common eukaryotic sugar donors and many sugar donors used in the synthesis of bacterial cell surface polysaccharides. Biosynthetically, UDP-activated sugars fall into two groups (Scheme 4): those derived from α-D-glucose-1-phosphate (**17**) via the glycolytic intermediate α-D-glucose-6-phosphate (**12**), and those derived from fructose-6-phosphate (**13**) via UDP-*N*-acetyl-α-D-glucosamine (**5**). Compound **17** is converted to UDP-α-D-glucose (**3**) by α-D-glucose-1-phosphate uridylyltransferase (UGP), an essential enzyme for all organisms. In contrast, the four Leloir pathway enzymes, galactose mutarotase (GMR), galactokinase (GK), galactose-1-phosphate uridylytransferase (G1PUT), and UDP-galactose 4-epimerase (GalE) are responsible for the conversion of β-D-galactose (**101**) to **3**.

UDP-α-D-glucuronic acid (**9**) is formed from **3** by the NAD^+-dependent UDP-glucose dehydrogenase (UDPGlcDH). This UDP-sugar is a building block for capsular polysaccharides, which are critical to bacterial virulence.[115] Recently, the activity of a UDP-glucose dehydrogenase, CalS8, was demonstrated to be involved in the synthesis of the

deoxyaminopentose moiety (**104**) of calicheamicin.[116] The formation of the UDP-dideoxyaminopentose (**103**) used for calicheamicin (Cal) and AT2433 (Atm) biosynthesis was proposed to start with the oxidation of **3** by Cal/AtmS8 to form **9**, followed by the oxidative decarboxylation of **9** to form **102** by Cal/AtmS9. This is followed by C-2 deoxygenation (Cal/AtmS14), C-3 ketoreduction (Cal/AtmS12), C-4 transamination (Cal/AtmS13), and 4-*N*-monomethylation (Cal/AtmS10).[117] Interestingly, the first two steps of this proposed pathway (**3** → **9** → **102**) have no precedent in TDP-sugar pathways, while the last four steps bear close similarity to reactions which are common in TDP-sugar biosynthesis but are unique for UDP-sugar formation. It was proposed that some (such as CalS8) or all of the enzymes in this pathway are pyrimidine indiscriminant, accepting both UDP- and TDP-sugars as substrates. This is supported by an in vitro analysis of CalS8, which demonstrated that while UDP-glucose (**3**) is the preferred substrate, TDP-glucose can also be efficiently oxidized.[116]

For the biosynthesis of the L-lyxose-derived moiety (**107**) of avilamycin in *Streptomyces viridochromogenes*, compound **9** is converted to UDP-D-xylose (**10**) by the short-chain dehydrogenase/reductase (SDR) enzyme, AviE2, which is a UDP-glucuronate decarboxylase (or UDP-xylose synthase) homologue.[118] Compound **10** is the common xylose donor used in the biosynthesis of cell wall polysaccharides in plants and fungi, cell surface polysaccharides in bacteria, and for protein glycosylation in animals. Interestingly, with the exception of AviE2, enzymes catalyzing the formation of **10** have not been found in any other secondary metabolite biosynthetic pathways in actinomycetes.[118] Formation of **106** is thought to proceed from **10** by sequential C-4 and C-3 epimerization reactions. These reactions may be catalyzed by two of the three SDR family enzymes (AviQ1, AviQ2, or AviQ3) with unknown functions that are encoded in the cluster.[118] This family of enzymes (discussed in Section 3.2) is known to catalyze the epimerization of hydroxyl groups at unactivated C-2, C-4, and C-6 positions of various NDP-sugar substrates. The involvement of these putative SDR enzymes in the formation of **106** has not yet been established.

A second group of UDP-sugars used in various biosynthetic reactions is derived from fructose-6-phosphate (**13**) via UDP-*N*-acetyl-D-glucosamine (**5**, Scheme 4). The first step in this process is the conversion of **13** to glucosamine-6-phosphate (**15**) catalyzed by glucosamine-6-phosphate synthase (GlmS). In bacteria, **15** is converted to glucosamine-1-phosphate (**19**) by phosphoglucosamine mutase (GlmM).[119] This is followed by *N*-acetyltransfer, catalyzed by the *C*-terminal domain of GlmU, to generate **108**.[120] The final step, resulting in UDP-Glc*N*Ac (**5**), is catalyzed by the *N*-terminal domain of GlmU. Recently, a nucleotidyltransferase (BtrD) that catalyzes either uridylylation or thymidylylation of **19** to give **109** was discovered in the biosynthetic pathway of butirosin, an aminoglycoside antibiotic produced by *Bacillus circulans*.[121] Acetylation of **109** could provide an alternative biosynthetic route to **5** in some bacteria.

Most aminoglycoside antibiotics containing 2-deoxy-*scyllo*-inosose or *myo*-inositol-derived aglycones are decorated with structurally diverse aminosugars (Figure 2).[122] The biosynthetic gene clusters for several members of these classes of aminoglycosides have been identified. These include butirosin, kanamycin, apramycin, lividomycin, paromomycin, neomycin, tobramycin, gentimycin, ribostamycin, fortimicin, and kasugamycin.[122] Since nucleotidylyltransferase genes are absent from most of these gene clusters, the NDP-sugar precursors (such as **3** or **5**,Scheme 4) used for the biosynthesis of these sugars are likely derived from the common NDP-sugar pool.[121,122] As expected, genes encoding NAD(P)-dependent dehydrogenases, oxidoreductases, and PLP-dependent aminotransferase enzymes are abundant in these clusters, and are likely involved in introducing amino groups into the sugar products via various oxidation/transamination reactions. At this point, however, the biosynthesis of most of these sugars is poorly understood, and in most cases it is not clear

whether the biosynthetic enzymes perform their reactions on NDP-sugar substrates, or whether they are tailoring reactions after glycosyl coupling. One notable exception is the kasugamine moiety (**110**) of kasugamycin, whose biosynthetic gene cluster encodes several enzymes with high homology to established UDP- and TDP-sugar modifying enzymes.[123] The aminoglycoside sugars are rich in atypical structural features (Figure 2), such as the unusual patterns of deoxygenation observed in tobramycin, gentamycins, and fortimycins, the unusual *C*-methyl branches in the gentamycins and fortimicins, and the axial stereochemistry of the C-5 aminomethyl groups of neomycin B, lividomycin A, and paromomycin I. The proposed mechanisms of some of these modifications are discussed in more detail in Section 3.3.

2.4. GDP-sugars

Although GDP-activated sugars (Scheme 5) are generally involved in the biosynthesis of bacterial cell surface polysaccharides and eukaryotic glycans, GDP-mannose (**111**) is the suggested precursor of the sugar moieties in the polyene macrolide natural products nystatin, amphotericin, pimaricin, and candicidin (which each contain D-mycosamine **112**), the aminoglycoside antibiotic hygromycin A (which contains 5-dehydro-α-L-fucofuranose **113**), and the antitumor drug bleomycin (which contains both L-gulose **114** and 3-*O*-carbamoyl-D-mannose **115**).[124–126] GDP-α-D-mannose (**111**) is derived from fructose-6-phosphate (**13**) by the action of three enzymes: phosphomannose isomerase (PMI) catalyzes the reversible interconversion of **13** and D-mannose-6-phosphate (**14**); phosphomannomutase (PMM) catalyzes the reversible interconversion of **14** and α-D-mannose-1-phosphate (**18**), and mannose-1-phosphate guanylyltransferase (also known as GDP-mannose pyrophosphorylase, GMP) catalyzes the GTP-dependent formation of GDP-α-D-mannose (**111**) from **18**. Compound **111** is then converted to GDP-4-keto-6-deoxy-α-D-mannose (**116**) by GDP-mannose 4,6-dehydratase (GM-4,6-D), a member of the SDR superfamily, which catalyzes essentially the identical reaction as its counterparts in ADP-, CDP-, UDP-, and TDP-sugar biosyntheses.[127] The GM-4,6-D genes (NysDIII/CanM/AmphDIII/PimJ) have been located in the gene clusters of nystatin, candicidin, amphotericin, and pimaricin. Following the conversion of **111** to **116**, a 3,4-sugar ketoisomerase, which has not yet been identified, is predicted to convert **116** to **117**. Subsequent C-3 transamination catalyzed by an aminotransferase encoded in each gene cluster leads to GDP-D-mycosamine (**118**),[124] which is the likely sugar donor in the biosynthesis of these compounds.

Analysis of the recently sequenced hygromycin A gene cluster has resulted in a proposed biosynthetic route for its 5-dehydro-α-L-fucofuranose moiety (**113**).[125] The pathway starts with the conversion of **111** to **116** by Hyg5, followed by 3,5-epimerziation and C-4 reduction to GDP-L-fucose (**8**) by Hyg23, an SDR enzyme. As in the biosynthesis of the D-fucofuranose (**24**) and D-streptose (**26**) residues of gilvocarcin V and streptomycin (Scheme 3a), the mechanism for the ring contraction of **8** to **119** is unknown, but the authors proposed that this step could be mediated by Hyg20, which shares sequence identity (31%) with transglucosylases. Though it is not clear how this enzyme would function, a Hyg20 homologue (Ata16) is also present in the gene cluster of the structurally related antibiotic A201A.[125] Following formation of the furanose ring, Hyg26 is predicted to oxidize the C-5 hydroxy group to give **120**, which is then coupled to the hygromycin aglycone by Hyg16.

The L-gulose (**114**) and 3-*O*-carbamoyl-D-mannose (**115**) moieties of bleomycin, a hybrid polyketide/non-ribosomal peptide antitumor agent from *Streptomyces verticillus*, are also derived from GDP-mannose.[126] In addition to the nucleotidylyl transferase (*blmC*) and glycosyltransferase genes (*blmE,F*), putative carbamoyl transferase (*blmD*) and NDP-sugar epimerase (*blmG*) genes are present in the gene cluster. BlmD likely carbamoylates **111** directly to give **121**. BlmG is closely related to the GDP-mannose-3,5-epimerases (GME), which catalyze 3-, 5-, or 3,5-epimerization of GDP-mannose.[128] A 5-epimerization of GDP-

mannose (**111**) would generate GDP-L-gulose (**122**), which could then be coupled to the aglycone. Finally, putative GDP-mannose-4,6-dehydratase genes (*aviE3* and *evrD*) are also present in the biosynthetic gene clusters for avilamycin A[106] and evernimicin[129] produced by *Streptomyces viridochromogenes* Tü57 and *Micromonospora carbonacea var africana*, respectively. It is not known, however, which of the seven sugar residues in each of these heptasaccharide antibiotics are derived from GDP-mannose derivatives, as each cluster also contains a TDP-glucose-4,6-dehydratase gene (*aviE1* and *evrW*).

2.5. CDP-sugars

CDP-activated sugars are rare, and are used mostly in the biosynthesis of 3,6-dideoxyhexoses found in the cell wall lipopolysaccharides of certain Gram-negative bacteria, where they are known to be important antigenic determinants. Much like TDP- and UDP-sugars, CDP-sugars are derived from glucose-6-phosphate (**12**), which is converted to glucose-1-phosphate (**17**) by phosphoglucomutase (Scheme 6),[130] and then to CDP-α-D-glucose (**123**) by α-D-glucose-1-phosphate cytidylyltransferase (E_p, DdhA).[131,132] Interestingly, a DdhA homologue (StrQ) was found in the streptomycin (**124**) gene cluster of *Streptomyces glaucescens*, and its cytidylyltransferase activity was verified in vitro.[133] However, TDP-glucose has been implicated as the precursor of the streptose moiety (**26**) of streptomycin (see also Scheme 3a).[122] Thus, StrQ may participate in the biosynthesis of the *N*-methyl-L-glucosamine (**125**) moiety of streptomycin.

The production of **125** in *S. glaucescens* has been observed and pathways for its biosynthesis have been proposed, but have not yet been studied in much detail.[122,134] After the formation of **123** by StrQ (Scheme 6), oxidation of the 4-OH group by StrP, StrT, or StrU could yield **126**. These enzymes are all NAD(P)-dependent oxidoreductases/dehydrogenases. The subsequent 3,5-epimerization to **127** is likely carried out by StrX, which has several homologues in TDP- and CDP-sugar biosynthesis including StrM, the TDP-4-keto-6-deoxy-α-D-glucose-3,5-epimerase involved in the biosynthesis of the D-streptose moiety (**26**) of streptomycin. Following reduction of the 4-keto group of **127** to give **128** by one of the aforementioned NAD(P)-dependent enzymes, the C-2 amine group is likely incorporated by the combined action of the oxidoreductase, StrT, and the PLP-dependent aminotransferase, StrS, to give **129**. Interestingly, the *strT/S* genes are located in tandem in all of the streptomycin and bluensomycin (a closely related antibiotic) gene clusters that have been sequenced.[122] Next, *N*-monomethylation of **129** to give **130** in *S. glaucescens* could be carried out by a homologue of StsG, an *N*-monomethyltransferase which is found in the streptomycin gene cluster of *Streptomyces griseus*, but which is absent in the *S. glaucescens* cluster. Coupling of **130** to the D-streptose (**26**) moiety of **131** may be catalyzed by StrF, which is part of a conserved cassette including the *strFGH* genes in all streptomycin clusters. Expression of a DNA fragment containing *strFG* and part of the *strH* gene led to the restoration of streptomycin production in a *Streptomyces bikiniensis* mutant strain that otherwise accumulated **131**.[135] Interestingly, analysis of the gene clusters for streptomycin and bluensomycin biosynthesis suggest that the pathways for formation of **125** are likely different between the producing strains.[122] Clearly, more work is needed to fully elucidate the biosynthetic pathway for formation of **125**.

2.6. Summary of NDP-sugar Biosynthetic Pathways

Through a combination of genetic, biochemical, and bioinformatic efforts, significant advances have been made in our understanding of natural product NDP-sugar biosynthesis. Although many of the steps proposed to occur in the pathways have not been experimentally verified, the following general principles have been gleaned from the work performed on these pathways. First, excluding the few sugars that are not 6-deoxyhexoses such as the *N*-methyl-L-glucosamine moiety (**125**) of streptomycin, 4,6-dehydration occurs as the first step after

nucleotidylyl transfer in all pathways studied thus far, and is a requisite step for all subsequent reactions. Indeed, many of the following enzymatic modifications (discussed in Section 3) in these pathways either occur directly at the 4-keto site (such as 4-ketoreduction and 4-transamination) or they rely on the activation provided by the 4-keto group to lower the pK_a of the C-3 and C-5 protons (3-, 5-, or 3,5-epimerization, 3- and 5-*C*-methylation, 3,4-ketoisomerization, 3- and 2-dehydration). Second, in all 2,6-dideoxysugar pathways, C-2 deoxygenation occurs after C-6 deoxygenation (**21** → **59**, Schemes 3b and 3c), and is followed by either 3-ketoreduction (**59** → **64** or **59** → **73**, Scheme 3c) or 3-aminotransfer (**59** → **60**, Scheme 3b). The C-3 ketoreductases giving equatorial (**59** → **73**) or axial (**59** → **64**) products can be distinguished by amino acid sequence alignments. In the case of 2,3,6-trideoxysugars, C-3 deoxygenation occurs after the C-2 deoxygenation/C-3 ketoreduction step (**21** → **59** → **73** → **91**, Scheme 3c). For the 4,6-dideoxysugars (e.g., D-desosamine and D-chalcose), C-4 deoxygenation requires prior 4-aminotransfer, and occurs after C-6 deoxygenation (**21** → **44** → **45**, Scheme 3b). Thus, the order of deoxygenation steps is C-6→C-2 for 2,6-deoxysugars, C-6→C-2→C-3 for 2,3,6-trideoxysugars, and C-6→C-4 for 4,6-dideoxysugars.

Further modifications, such as ketoreduction, *C*-methylation, epimerization, and transamination (except before C-4 deoxygenation) seem to occur subsequent to all deoxygenation reactions. The C-4 ketoreduction and *N*-methylation reactions generally occur at late stages of these pathways, while *O*-methylation usually happens after the TDP-sugar donor has been coupled to its aglycone acceptor. Cumulatively, insight gained from these studies can be used as guidelines for gene cluster-assisted or *de novo* prediction of natural product sugar biosynthetic pathways. However, this type of sequence-based functional prediction should be performed with caution. In many cases, biochemical characterization of the encoded proteins and mechanistic studies of the key enzymes involved remains necessary to unambiguously establish the overall biosynthetic pathways.

3. The Chemistry of NDP-Sugar Biosynthetic Enzymes

Despite the number of unusual sugar structures present in bacterial secondary metabolites (see Section 2), only five common enzyme reaction types are used by Nature to generate most of this structural variation. Table 1 (supporting information) lists these common reactions along with an illustration of the reaction type and the names of representative enzymes that are known to catalyze these reactions either in vitro or in vivo through gene disruption or heterologous expression experiments. Since several comprehensive reviews on the enzyme chemistry related to deoxysugar biosynthesis are available,[136–139] this section will only highlight the common themes employed by these enzymes to generate sugar diversity.

Because the great majority of natural product sugars are 6-deoxyheoxes, a particularly prevalent theme observed in deoxysugar biosynthesis is the intermediacy of NDP-4-keto-6-deoxyhexose (see **21**, Scheme 7) in the pathways. Accordingly, most of the subsequent transformations such as ketoreduction, transamination, epimerization, isomerization, methylation, dehydration, and deoxygenation, have taken advantage of the activation provided by the 4-keto group of this intermediate. The mechanisms of several of the enzymes involved in these transformations will be discussed in Section 3.1. The essential nature of keto-group installation to deoxysuagr biosynthesis is further underscored by the presence of short-chain dehydrogenase/reductase (SDR) enzymes in many sugar biosynthetic pathways. This versatile group of enzymes uses a tightly-bound NAD^+ coenzyme to generate a transient NDP-keto-sugar intermediate, which is then further processed within the same active site to achieve a desired chemical transformation. The proposed mechanisms for several selected sugar-modifying SDR enzymes are discussed in Section 3.2. In the final topic of this section, we will investigate several unusual modifications observed in some natural product deoxysugars,

whose mechanisms of formation are not well understood. These unusual modifications are also partly responsible for the vast number of different final sugar structures.

3.1. The General Reaction Types of Sugar Biosynthetic Enzymes

3.1.1. Reduction—Ketoreductases are the most widely distributed group of enzymes in deoxysugar biosynthesis, and a number of their functions have been biochemically established (see Table 1). The ketoreducatses found in NDP-sugar biosynthetic pathways catalyze the NAD(P)H-dependent hydride reduction of 3- and 4-ketosugars to yield the corresponding secondary alcohol. Both 3- and 4-ketoreduction can occur with either stereochemistry. Many 2,6-dideoxysugar biosynthetic gene clusters encode a 3-ketoreductase, whose activity is required to reduce the unstable NDP-3,4-diketosugar produced by the 2-dehydratase-catalyzed reaction (see Section 3.1.5). Another possible explanation for the large number of ketoreductases in these pathways is simply that the biosynthesis of most deoxysugars involves ketosugar intermediates, which are essential for the enzyme-catalyzed reactions described below. Following the necessary chemical transformations, ketoreductases (many of which are believed to act at late stages in NDP-deoxysugar biosynthesis) may serve to stabilize the final NDP-sugar product. Interestingly, multiple amino acid sequence alignments of established and putative NDP-sugar ketoreductases indicate evolutionary divergence between the 3- and 4-ketoreductases, as the two groups do not share any significant sequence similarity. In addition, within the 3-ketoreductase group, enzymes that generate axial and equatorial C-3 hydroxyl groups can be readily distinguished, whereas the stereochemistry of the 4-ketoreductase-catalyzed reaction is more difficult to predict based on amino acid sequence alone. Due to the variety of C-2, C-3, C-4, and C-5 substituents that must be accommodated by individual ketoreductases during their catalyzed reactions, detailed structural and substrate specificity studies of these enzymes should help to assess their usefulness for in vitro NDP-sugar synthesis and glycoengineering applications.

3.1.2. Epimerization/Isomerization—RmlC, which catalyzes the conversion of TDP-4-keto-6-deoxy-α-D-glucose to TDP-4-keto-6-deoxy-L-mannose (**21** → **31**, Scheme 7a) in the biosynthesis of L-rhamnose in bacteria, is one of the most studied sugar epimerases/isomerases and can serve as the prototype for other NDP-ketosugar-3-, 5-, and 3,5-epimerases. Structural and mechanistic studies of *Pseudomonas aeruginosa* RmlC led to a mechanism in which both 3- and 5-epimerization of **21** proceed with deprotonation at C-3 and C-5 by His65, which forms a catalytic dyad with a conserved Asp171 residue.[19] The resulting enolate intermediates (**132**, **135**) are stabilized by Lys74, and the subsequent protonation is mediated by Tyr140 (or possibly a water molecule for the C-3 epimerization) to complete each epimerization step. Deuterium exchange studies have shown that epimerization at C-5 is much more facile than at C-3, and thus likely occurs first. After C-5 epimerization, a ring-flipped intermediate (**133**) in the $^1C_4$ conformation typical of L-sugars, is proposed to form in order to avoid steric clashes between the 5-methyl group, the O1 atom and His65. Intermediate **133** is likely in equilibrium with a twist boat conformation (**134**), in which the C-3 hydrogen is orthogonal to the plane of the 4-keto group to facilitate C-3 proton abstraction. While most other RmlC homologues involved in natural product biosynthesis (Scheme 3a, the enzymes that catalyze **21** → **31**) are not as well characterized as RmlC from *Pseudomonas aeruginosa*, sequence alignments show that all these enzymes share the conserved His-Lys-Tyr catalytic machinery, so that they likely operate by a similar mechanism.

TDP-4-keto-6-deoxyglucose (**21**) is also the substrate for Tyl1a (Scheme 7b), the TDP-4-keto-6-deoxy-3,4-ketoisomerase from *Streptomyces fradiae*, which catalyzes the conversion of **21** to **39** in the D-mycaminose pathway.[54, 55] While few genes encoding Tyl1a homologues are found in natural product biosynthetic gene clusters, they are abundant in the biosynthetic gene clusters for bacterial outer membrane polysaccharides. Among these, FdtA from

Aneurinibacillus thermoaerophilus L420-91^T, has recently been structurally and mechanistically characterized.[140] A conserved histidine pair (His49 and His51) in FdtA is proposed to catalyze the isomerization (Scheme 7b), with His49 being responsible for C-3 deprotonation and His51 mediating the proton transfer between O3 and O4. Subsequent protonation at C-4 by His49 results in the formation of **136**. The corresponding residues His63 and His65 in Tyl1a are expected to play similar roles in the conversion of **20** to **39**, as shown in Scheme 7b.

3.1.3. Transamination—Another common enzymatic reaction used in these biosynthetic pathways is the pyridoxal 5'-phosphate (PLP)/pyridoxamine 5'-phosphate (PMP)-dependent transamination reaction. The crystal structures of several sugar aminotransferases have been solved, including those of the 4-aminotransferase, DesI,[72] and the 3-aminotransferase, DesV, involved in D-desosamine biosynthesis in *Streptomyces venezuelae*.[141,142] The structure of DesI, in the presence of PLP and the aminosugar product **44** (Scheme 7c), revealed an external aldimine intermediate (**137**) where Lys220, the residue that normally anchors PLP in the active site via a Schiff base linkage, is in close proximity to both C-4' of PLP (3.4 Å) and the C-4 atom of the sugar substrate (3.0 Å). It likely plays a role in mediating the proton transfers that occur during the transamination. Interestingly, when compared to the structure of PseC from *Helicobacter pylori*,[143] a 4-aminotransferase that introduces an axial amino group into a 4-ketoketosugar, the hexose moiety observed in DesI is flipped about 180°. This major difference in hexose orientation is likely responsible for the opposite stereochemistry of amino group incorporation catalyzed by these two enzymes.[141]

3.1.4. Methylation—The 3-*C*-methyl transfer reaction catalyzed by TylC3 in the biosynthesis of the L-mycarose moiety of tylosin in *Streptomyces fradiae* was the first NDP-sugar *C*-methyltransferases to be characterized in vitro (Scheme 7d).[90] This enzyme, like many other *C*-, *O*-, and *N*-methyltransferases, requires an *S*-adenosylmethionine (SAM) cosubstrate for catalysis. Similar to the reactions catalyzed by 3,5-epimerases and 3,4-ketoisomerases, catalysis is initiated by the abstraction of the C-3 proton from **64**, which may need to adopt a twisted conformation (similar to the conversion of **133** → **134** in Scheme 7a) to faciliate the deprotonation step by an active site base. The nascent enediolate intermediate (**138**) then reacts with the electrophilic methyl group of SAM to generate **139** with net inversion of the 3-OH stereochemistry. Interestingly, no metal ion is required for this transformation, suggesting that the TylC3 active site stabilizes the enediolate intermediate (**138**) mainly by electrostatic interactions. The activities of a few other NDP-sugar C-3 and C-5 methyltransferases have also been verified in vitro (Table 1). They are all believed to employ a similar mechanism to that of TylC3.[39, 40, 82, 94]

3.1.5. Deoxygenation—The 2,6-dideoxysugars depicted in Schemes 3b and 3c represent the largest group of unusual sugars found in natural products. All of these sugars require a 2-deoxygenation step catalyzed by 2-dehydratase enzymes at an early stage of their biosynthesis. Gra Orf27 from the granaticin pathway of *Streptomyces violaceoruber* Tü22 and the accompanying 3-ketoreductase (Gra Orf26) were the first enzymes involved in NDP-sugar 2-deoxygenation to be studied.[144] Shortly after this initial report, studies on TylX3 and TylC1, the corresponding 2-dehydratase and 3-ketoreductase from the L-mycarose pathway of *Streptomyces fradiae*, provided additional insights into the mechanism for 2-deoxygenation.[84] It was shown that TylX3 activity required a Zn^{2+} ion, which is most likely involved in activating a water molecule to serve as the base for C-3 deprotonation or in stabilizing the enolate intermediate (**140**). Following β-elimination of the 2-OH group, the nascent enol product (**141**) is ketonized to **59** with a solvent hydrogen stereospecifically incorporated into the equatorial position at C-2. Subsequent reduction of the 3-keto group by the NADPH-dependent TylC1 gives **64** with an axial 3-OH group. In the biosynthesis of granaticin, the 3-

ketoreducatse Gra Orf26 transfers the NADPH-derived hydride to the opposite side of the 3-ketohexose (**59**), resulting in an equatorial 3-OH.

The mechanism of 3-deoxygenation further demonstrates the diverse transformations in NDP-deoxysugar biosynthesis involving 4-keto-6-deoxy-α-D-glucose. This reaction requires two enzymes and the mechanism was originally established for CDP-4-keto-6-deoxy-D-glucose-3-dehydrase (E_1) and its reductase (E_3) in the ascarylose biosynthetic pathway from *Yersinia pseudotuberculosis*.[145–148] E_1 is homologous to PLP-dependent aminotransferases, but contains PMP instead of PLP as the coenzyme and possesses a histidine in place of the conserved Schiff base-forming lysine found in all aminotransferases. E_1 also contains a catalytically essential [2Fe-2S] cluster and requires a [2Fe-2S]-containing flavodoxin-$NADP^+$ reductase partner, E_3, for activity. The E_1 mechanism begins with Schiff base formation between PMP and the 4-keto group of the substrate to form **142** (Scheme 7f). Next, the C-4' proton of PMP is abstracted, which triggers expulsion of the 3-OH group to form the $\Delta^{3,4}$-glucoseen intermediate **143**. This intermediate is then reduced to **144** by two sequential one-electron transfers from the NADH reduced E_3-bound FAD via the [2Fe-2S] cluster of E_3 and the [2Fe-2S] cluster of E_1. Subsequent hydrolysis gives product **145** and regenerates PMP.

Recently, the 3-dehydrase activity of SpnQ from the TDP-D-forosamine (**100**) biosynthetic pathway of *Saccharopolyspora spinosa* was verified in vitro.[112] No E_3 homologue is present in the *spn* gene cluster, and efficient conversion of **59** → **91** (Scheme 3c) was observed only in the presence of various cellular enzymatic reducing systems suggesting that SpnQ, like E_1, most likely employs a general reductase from the cellular pool to complete the 3-deoxygenation process. Interestingly, the 3-dehydrase (ColD) from the L-colitose (see **149**) biosynthetic pathway of *Yersinia pseudotuberculosis*, is also a PMP-dependent enzyme, but it lacks the [2Fe-2S] cluster present in E_1.[149] The first half of ColD catalysis was shown to mimic the E_1 reaction, with Schiff base formation and dehydration to give an intermediate similar to **143** (**146**, Scheme 7g). The second half of the ColD reaction involves hydrolysis of the $\Delta^{3,4}$-amino-mannoseen intermediate (**146**) to give PLP and an enamine sugar (**147**), which then undergoes tautomerization followed by hydrolysis to form the 4-keto-3,6-dideoxymannose product (**148**), releasing ammonia in the process. In contrast to the E_1 reaction, where PMP is regenerated by sequential one-electron reduction from E_3, the PMP coenzyme in ColD must be regenerated from PLP after each catalytic cycle by a transamination reaction in the presence of glutamate. The combined deoxygenation-transamination activity makes ColD a unique enzyme.

3.2. The Versatile Roles of SDR Family Enzymes in Unusual Sugar Biosynthesis

The importance of ketosugar intermediates in sugar biosynthesis is further underscored by the extensive use of a subfamily of the short-chain dehydrogenase/reductase (SDR) enzymes in many NDP-sugar biosynthetic pathways.[150] These "nucleotide sugar modifying" SDR enzymes have a conserved protein fold and active site geometry, and commonly use an $NAD(P)^+$ cofactor (or occasionally an NAD(P)H cosubstrate) in a variety of reactions, including ketoreduction, oxidation/dehydration, epimerization at unactivated carbon centers, α-epimerization/ketoreduction, and oxidation/decarboxylation. In the first step of most of these reactions, the SDR enzyme in question oxidizes one of the sugar hydroxyl groups to a keto group, thus generating a reactive intermediate that can be further manipulated in the active site to effect numerous chemical transformations.

The SDR-enzyme-catalyzed 4,6-dehydration is one of the most important reactions in sugar biosynthesis. As illustrated in the previous section, the product of this reaction, NDP-4-keto-6-deoxysugar, is a key intermediate in deoxysugar biosynthesis. The mechanism[124–127] and structure[151–154] of several NDP-D-glucose-4,6-dehydratases have been characterized in

great detail. The reaction catalyzed by the 4,6-dehydratases is initiated by deprotonation of the 4-OH group of NDP-glucose (such as TDP-glucose, **20**) by a conserved Tyr residue with concomitant transfer of the 4-H as a hydride to NAD^+ (Scheme 8a). Crystallographic studies of 4,6-dehydratases from *Salmonella enterica*,[151] *Streptococcus suis*,[153] and *Streptomyces venezuelae*[154] as well as several other sugar-modifying SDR enzymes[128, [155]–158] have revealed the presence of conserved Ser/Thr and Lys residues, which likely lower the pK_a of the sugar C4-OH and Tyr hydroxyl groups, respectively. Together, this conserved Ser/Thr-Tyr-Lys triad forms one of the signature motifs in SDR enzymes and is believed to be involved in mediating the hydride transfer steps in all of these enzymes. The dehydration of **150** across the C-5/C-6 bond is facilitated by a Glu residue, which is conserved in NDP-sugar-4,6-dehydratases and which deprotonates C-5, resulting in an enone intermediate (**151**). In many 4,6-dehydratases, such as RmlB of *S. enterica*, an Asp residue is believed to be responsible for protonating the C6-OH group to facilitate dehydration.[153] This Asp residue, however, is absent in the GDP-α-D-mannose-4,6-dehydratases, implying the involvement of a different catalytic acid group in these enzymes. Compound **151** is then reduced at C-6 with the hydride that was originally derived from 4-H and is protonated at C-5 by the Glu residue to yield the product, **21**. Internal hydride return from the transient NAD(P)H to the product is a key mechanistic trait of most SDR enzymes involved in sugar biosynthesis. Thus, in these SDR enzymes, NAD^+ is a tightly-bound coenzyme rather that a cosubstrate.

UDP-xylose (**10**, Scheme 8b) is required for primary metabolism in all organisms, but related pentoses are rare constituents of natural products. In primary metabolism, UDP-xylose results from decarboxylation of UDP-α-D-glucuronic acid (UDP-GlcA, **9**) by the NAD^+-dependent UDP-glucuronate decarboxylase (also known as UDP-xylose synthase). Early mechanistic studies of this enzyme demonstrated that the reaction is initiated by oxidation of the 4-OH group of **9**, followed by decarboxylation and protonation to give **102**.[159] Reduction of **102** with the transiently formed NADH yields UDP-xylose (**10**). Structural studies and amino acid sequence alignments of the ArnA decarboxylase domain of *E. coli* (a related enzyme that catalyzes **9** → **102**) suggest that an Arg/Ser pair that is conserved in this class of enzymes is important in mediating the decarboxylation event.[157] Recently, AviE2, which is involved in the biosynthesis of the L-lyxose-derived moiety (**107**, Scheme 4) of avilamycin, has been shown to be a UDP-GlcA decarboxylase, making it the first enzyme of this type found in an actinomycete natural product sugar biosynthesis pathway.[118] Genes encoding putative UDP-GlcA decarboxylase homologues (CalS9 and AtmS9, respectively) are also present in the gene clusters for the enediyne antibiotic calicheamycin and the indolocarbazole antibiotic AT2433. [33, 117] Although the activities of CalS9/AtmS9 have not yet been biochemically verified, they are believed to catalyze the formation of **102**, rather than **10**, which makes their activities more similar to the ArnA decarboxylase domain than to UDP-xylose synthases.

Formation of the L-lyxose-derived moiety of avilamycin requires epimerization at an unactivated carbon atom. This type of reaction is often catalyzed by a group of SDR enzymes homologous to the well-studied UDP-galactose-4-epimerase (GalE) from the Leloir pathway of primary metabolism.[160] GalE homologues that catalyze epimerization of pyranose hydroxyl groups at C-2, C-4, and C-6 have been characterized.[160–165] Structural studies of GalE from *E. coli* have shown that, following the oxidation of the 4-OH group of UDP-galactose (**4** Scheme 8c), the hexose ring of intermediate **152** rotates along the C_1-O-P bond in the active site.[156] This allows the transfer of the hydride from NADH to the opposite face of **152** at C-4 to form UDP-glucose (**3**) and regenerate NAD^+. With regard to avilamycin biosynthesis, epimerization at both C-3 and C-4 of UDP-xylose (**10**) are required to form **106** (Scheme 4). Two putative SDR-enzymes, AviQ1 and AviQ2, that show homology to GalE are encoded in the avilamycin cluster, and may be responsible for the epimerization reactions. [118] If this is found to be the case, it will be the first example of an SDR enzyme-catalyzed epimerization of an NDP-sugar 3-hydroxyl group.

Interestingly, several SDR 3,5-epimerases also have 4-reductase activity (Scheme 8d). GDP-6-deoxy-4-keto-D-mannose-epimerase/reductase (GMER or GDP-fucose synthase), involved in GDP-fucose biosynthesis in all organisms, is a representative of such a dual function enzyme. The *E. coli* enzyme catalyzes the 3,5-epimerization reaction using a His179/Cys109 pair in the absence of NADPH.[158, [166]–168] The conserved Tyr136 residue of the Ser/Thr-Tyr-Lys motif stabilizes the enolate intermediates (**153** and **154**). In the reductive reaction, Tyr136 protonates the 4-keto group of **155** upon hydride transfer from NADPH. Recently, a GMER homologue (Hyg23) was proposed to catalyze an identical reaction in the hygromycin A biosynthesis pathway of *Streptomyces hygroscopicus* NRRL 2388 (Scheme 5).[125] Interestingly, the related SDR enzyme, GDP-mannose-3,5-epimerase (GME), uses the NAD^+ coenzyme to oxidize the 4-OH group of GDP-D-mannose (**111**) prior to 3,5-epimerization (Scheme 8e).[128] Overall, catalysis by GME is very similar to that of GMER, except that GME uses a Lys/Cys acid/base pair instead of a His/Cys pair. Following 5-epimerization, the intermediate **156** can be reduced at C-4 to give GDP-β-L-gulose (**122**), or epimerized at C-3 and then reduced at C-4 to give GDP-β-L-galactose (**157**). The L-gulose moiety of bleomycin (**114**, Scheme 5) is believed to be generated in an analogous manner, most likely by BlmG.[126]

3.3. Unusual Modifications in Natural Product Sugar Biosynthesis

While most unusual sugar biosyntheses are accomplished by the "common" enzyme activities listed in Table 1, further structural diversification involving modifications such as epimerization and methylation at unactivated carbon centers, sulfurylation, nitro and hydroxylamino group formation, ring contractions, and others, also happen. However, most of these uncommon tailoring modifications have not been experimental investigated.

Recently, Boll *et al*. demonstrated that removing a single gene, *aviX12*, from the avilamycin A gene cluster of *Streptomyces viridochromogenes* Tü57, led to an inactive avilamycin derivative (gavibamycin N1) where a glucose moiety (see **158**) replaced the mannose moiety (see **159**) that is normally present (Figure 3a).[169] This surprising observation suggested that the mannose residue is not directly derived from GDP-mannose as previously thought.[106] Instead, it may be formed via C-2 epimerization of a glucose unit (**158** → **159**) mediated by AviX12, which leads to the final active form of avilamycin A. Examination of the predicted AviX12 amino acid sequence revealed a CxxxCxxC motif, which is characteristic for a radical SAM enzyme.[170] Thus, the reaction catalyzed by AviX12 is proposed to be initiated by hydrogen atom abstraction at C-2 of the glucose unit by the 5′-deoxyadenosyl radical ($AdoCH_2$•) generated by reductive cleavage of SAM with the reduced $[4Fe\text{-}4S]^{1+}$ cluster (Figure 3a), followed by delivery of a hydrogren atom to the opposite side of the sugar ring to give mannose as the product. If verified, this would clearly be an unusual radical initiated epimerization reaction. Interestingly, the aminoglycoside antibiotics neomycin B, lividomycin A, and paromomycin I, all contain a C-5 epimer of neosamine (**160**, Figure 3b), and each gene cluster contains a putative radical SAM enzyme gene, NeoN, LivN, and ParN, respectively.[122] The encoded enzymes may also catalyze AviX12-like epimerization reactions.

A new type of methylation mediated by methylcobalamin-dependent radical SAM enzymes is speculated to be involved in the formation of several antibiotics including moenemycin A of *Streptomyces ghanaensis*,[171] fortimycin A of *Micromonospora olivasterospora*, and gentamycin of *Micromonospora echinospora*.[172] Each of these gene clusters contains a gene encoding a putative methylcobalamin-dependent radical SAM enzyme (MoeK5, ForK, and GntK), respectively), which could introduce a methyl group at an unactivated carbon center of the respective sugar substrate (Figure 3c). These enzymes have not been studied, but a similar enzyme (Fom3) in fosfomycin biosynthesis has been identified and its mechanism has been proposed.[173] The enzyme catalyzes the conversion of 2-hydroxyethylphosphonate (HEP,

161) to (*S*)-2-hydroxypropylphosphonate (HPP, **163**). As shown in Figure 3d, the reduced $[4Fe\text{-}4S]^{1+}$ cluster first generates the 5'-deoxyadenosyl radical ($AdoCH_2$•), which abstracts a hydrogen atom from the substrate (**161**) to generate a radical intermediate **162**. The substrate radical then abstracts Me• from the methylcobalamin to form the methylated product (**163**) and cob(II)alamin. This putative mechanism is metabolically expensive since 2 equivalents of SAM are consumed per cycle. Also the role of methylcobalamin as a methyl radical donor is highly unusual.

Other novel enzyme activities are required for the biosynthesis of unusual sugars, including the thiosugars found in calicheamicin (**164**) and esperamicin (**166**), the hydroxylamine sugars of calicheamycin (**165**), esperamicin (**167**) and viriplanin A (**168**), and the nitrosugars of kijanimicin (**169**), rubradirin (**170**), evernimicin (**171**), cororubicin (**172**), and decilorubicin (**173**) (Figure 3e). To date, the enzymes responsible for these modifications are not known, and only the calicheamicin,[33] evernimicin,[129] rubradirin,[174] and kijanimicin[99] biosynthetic gene clusters have been identified. Notably, the calicheamicin gene cluster encodes a putative cysteine desulfurase (CalS4), which may be involved in the biosynthesis of the calicheamicin thiosugar moiety (**164**).[175] A cytochrome P450 enzyme or a flavin-dependent monooxygenase is expected to be responsible for the formation of the hydroxylamine moiety.[175] The nitrosugars (**169**–**173**) are most reasonably derived from oxidation of the corresponding aminosugars. In fact, the clusters for evernimicin,[129] rubradirin,[174] and kijanimicin[99] each contain a three-gene cassette encoding a NDP-3-*C*-methyltransferase (EvdA/RubN5/KijD1), a NDP-3-aminotransferase (EvdB/RubN4/ KijD2), and a flavin-depedent oxidoreductase (EvdC/RubN8/KijD3) that may be involved in 3-methyl-3-nitrosugar biosynthesis.[99] The *O*-methylcarbamate moiety of kijanose (**169**) is also unusual. A series of *N*-methylation, methyl oxidation to a carboxylate group, and *O*-methylation mediated by KijD8, KijB3 and KijD9, respectively, has been proposed.[99] As highlighted by the thio, nitro, and hydroxylamine sugars, many interesting modifications in natural product sugar biosynthesis remain to be discovered and explored.

Although initial studies have produced significant advances in our understanding of the mechanisms of enzymes catalyzing C-O bond cleavage in deoxysugar biosynthesis,[136–138] our knowledge is far from complete. For example, quite a few deoxygenations, such as those in the formation of the 2,6-diamino-2,3,4,6-tetradeoxysugar unit (**175**) of gentimicin and fortimicin, and the 2,6-diamino-2,3,6-trideoxy neosamine moiety (**176**) in tobramycin (Figure 3f), may proceed via distinct mechanisms. Studies of *Micromonospora olivasterospora* mutants blocked at various stages of fortimicin A biosynthesis revealed the accumulation of various 3,4-dihydroxy-(**174**) and 3,4-dideoxysugar (**175**) intermediates, but no 3- or 4-monohydroxylated compounds.[176] Complementation studies of these mutants using fragments of the fortimicin gene cluster eventually led to identification of the *fms8(forP)* gene product as the possible catalyst for the didehydroxylation step,[177] because when heterologously expressed in *M. olivasterospora*, Fms8 restored the didehydroxylation phenotype.[177,178] How the didehydroxylation occurs and whether other enzymes are needed remain unclear, but phosphorylation of an intermediate in the pathway may be critical, because Fms8 is a homologue of NmrA, a phosphotransferase involved in neomycin B resistance. A similar didehydroxylation mechanism is possible for the formation of gentimicin. The biosynthetic route to the tobramycin sugar (**176**) is also mysterious, as the characterized mechanisms for 3-deoxygenation require the generation of a 4-keto-6-deoxy sugar intermediate by a 4,6-dehydratase, followed by 3-dehydration catalyzed either by an E_1 or a ColD homologue (see Scheme 7f). However, none of the genes for these enzymes are present in the reported tobramycin clusters, suggesting a different mechanism for 3-deoxygenation in the tobramycin pathway.

The attachment of a 5-methylpyrrole-2-carboxyl moiety (**177**) to the 4-*O*-methyl-noviose residue in the antibiotics clorobiocin and coumermycin A_1 is another remarkable tailoring modification (Figure 3g). This modification greatly enhances the ability of these drugs to inhibit the bacterial type-II topoisomerase DNA gyrase. The biosynthesis of **177** and its attachment has been studied both in vivo and in vitro.[43, 45] It was found that **177** is derived from L-proline (**178**), which is activated as an acyl-adenylate and linked to the Clo/CouN5 peptidyl carrier protein (PCP) by Clo/CouN4. Subsequent oxidization of **179** by Clo/CouN3 results in a PCP-linked pyrrole-2-carboxyl substituent (**180**), which is transferred to a separate PCP (Clo/CouN1) by the acyl-ACP-synthase (Clo/CouN2) to give **181**. The final steps include the transfer of **181** to the 4-*O*-methyl-noviose moiety by a thioesterase (Clo/CouN7) to give **182**, and *C*-methylation by a methylcobalamin-dependent radical SAM methyltransferase (Clo/CouN6) to afford **177**. Likewise, the biosynthesis of the C_5N unit (**183**) in moenomycin produced by *Streptomyces ghanaensis* is also intriguing (Figure 3h). An aminolevulinate synthase (MoeA5), an acyl-CoA-ligase (MoeA4), and an amide synthetase (MoeB4), which are located together in the gene cluster, have been proposed to catalyze the conversion of succinyl-CoA and glycine to **183**.[171] Indeed, a *moeA4*$^-$ knockout mutant failed to produce moenomycin bearing **183**.

The methyleurekanate (**184**) residue in avilamycin of *Streptomyces viridochromogenes* also requires several intriguing tailoring steps: the formation of the orthoester linkage at C-1, the incorporation of the methylene unit between O2 and O3, and the attachment of the 4-*C*-acetyl moiety (Figure 3i). A 4-ketosugar (perhaps **185**) is likely the precursor of **184**. Condensation of **185** with the thiamine-pyrophosphate (TPP)-bound acetyl carbanion unit (**186**) would give **184**.[179] The acetyl carbanion unit is likely generated from pyruvate by an AviB1/B2 complex whose subunits share homology with the α and β chains, respectively, of pyruvate dehydrogenase. The enzyme(s) responsible for the incorporation of the methylene unit between O2 and O3, and for the formation of the orthoester linkage are unknown, but as noted earlier, two of the three non-heme iron-dependent enzymes encoded in the avilamycin cluster could be responsible for these tailoring reactions.

3.4. Outlook

The work highlighted in the previous sections has revealed Nature's ingenious and judicious utilization of a small set of core enzyme activities to generate significant sugar structural diversity. Most of these enzymes operate on similar ketosugar substrates, but are able to catalyze distinct reactions using unique active site architecture and cofactor requirements. Future structural and mechanistic studies on sugar biosynthetic enzymes may help clarify the potential of these enzymes as synthetic catalysts for glycodiversification. In particular, members of the SDR family of enzymes represent attractive targets for the rational engineering of enzyme function, as they catalyze many different reactions on diverse sugar substrates using a conserved protein fold and similar, yet distinct active site chemistries. Further elaboration of sugar structures could be achieved by employing enzymes catalyzing unusual transformations and/or tailoring reactions. Together, an understanding and appreciation of the unusual sugar biosynthetic pathways and the mechanisms of the enzymes involved, has contributed to the recent explosion in the use of glycoengineering approaches (the subject of Section 5) to generate new glycoforms of natural products.

4. Natural Product Glycosyltransferases

4.1. The Gatekeepers of Glycodiversity

Glycosyltransferases (GTs) form a critical group of enzymes in biological systems that catalyze the attachment of sugar moieties to acceptor molecules. At present, there are more than 15,800 putative glycosyltransferases in the protein databank, but the functions of most of these GTs

have not been verified.[180] Several hundred GTs are predicted to be involved in the biosynthesis of natural products found in bacteria and plants. These GT-catalyzed reactions reside at a critical juncture in natural product biosynthesis, where the products of the sugar and aglycone biosynthetic pathways meet. Hence, in recent years, GTs have been the subject of many studies aimed at understanding and finetuning their biochemical properties.[181] Ultimately, these enzymes may be useful as tools to catalyze "unnatural" coupling reactions to generate new glycoforms of natural products. These efforts have been focused in two main areas: the exploitation of the broad substrate specificity found in many wild-type GTs and the alteration of GT specificity through genetic engineering. The success of these endeavors relies on a multi-faceted strategy encompassing genetic, genomic, molecular biological, biochemical, and chemical approaches.

For most characterized GTs, the donor substrate is a nucleotide diphosphate (NDP)-sugar. However, nucleotide monophosphate (NMP)-sugar and polyprenyl diphosphate-sugar donors are also substrates for specific GTs. Interestingly, a phosphoribosyltransferase (PRTase), [182,183] which uses 5-phosphoribose diphosphate (PRPP) as a donor for the transfer of 5-phosphoribose to an acceptor substrate was found to be involved in the biosynthesis of the aminoglycoside antibiotic butirosin.[184] This represents the first characterized PRTase involved in natural product biosynthesis.

Like the NDP-sugar donor substrates, the acceptor substrates for natural product GTs are also structurally diverse and include many classes of compounds (Figure 4), such as the polyketide-derived aglycones of pikromycin (**187**), urdamycin A (**188**), calicheamicin (**189**), avilamycin (**190**), and BE-7585A (**191**),[185] the non-ribosomal peptide (NRP)-derived aglycone of vancomycin (**192**), the indolocarbazole aglycone of staurosporine (**193**), the aminocoumarin aglycone of novobiocin (**194**) and many others. The coupling reaction entails the displacement of the anomeric substituent of the sugar donor by a nucleophilic functional group of the acceptor to form the glycosidic linkage. The nucleophile is most commonly a hydroxyl group. However, *N*- and aryl-*C*-glycosidic linkages (as in **193** and **188**, respectively), as well as the unusual orthoester (as in **190**), hydroxylamine (as in **189**), and thio linkages (as in **191**) are also seen in some natural products. The mechanisms for the formation of the latter three types of linkages have not been explored.

4.2. Structures of Glycosyltransferases

Since the first GT crystal structure was reported in 1994,[186] 35 GTs structures have been solved.[187] With the exception of a bifunctional transpeptidase-glycosyltransferase involved in peptidoglycan biosynthesis (which has a novel structure),[188] these GT structures fall into two classes, the GT-A and GT-B families, whose properties have been previously reviewed. [150,[180],[181],[189]–194] The GT-A superfamily is characterized by a single domain with an α/β/α sandwich topology that resembles a Rossmann fold.[191–193] The NDP-sugar-binding region of GT-A enzymes contains a conserved DXD (Asp-X-Asp) motif for binding a divalent metal (usually Mn^{2+}) that anchors the diphosphate moiety of the NDP-sugar,[195,196] and stabilizes the NDP leaving group during turnover.[195–200] Interestingly, there is a recent example of a GT-A enzyme that is metal ion-independent and lacks the DXD motif.[201] In contrast, GT-B superfamily members have two Rossmann fold-like domains with a deep, interdomain cleft where the donor and acceptor substrates bind.[193,194] They are metal-ion independent and lack universally conserved amino acid residues, though the *C*-terminal nucleotide binding domain is more conserved than the *N*-terminal acceptor binding domain. Despite the low sequence identity (<10%) among GTs, the three dimensional structures of GTs within the same superfamily are quite similar.

Almost all bacterial natural product GTs are predicted to be members of the GT-B superfamily. [194] To date, the crystal structures of only a handful of natural prodcut GTs have been

determined.[202–207] The structure of GtfB from the chloroeremomycin pathway was the first reported structure.[202] As is typical for GT-B superfamily members, GtfB has two domains separated by a flexible linker region, forming a deep cleft between the two domains. The *N*-terminal domain contains the aglycone binding site, and the *C*-terminal domain contains the sugar binding site. Since these two domains appeared to be well-separated in GtfB, it was proposed that it may be possible to create chimeric GTs containing donor and acceptor binding domains from separate GTs.[202] The structures of the L-epivancosaminyltransferases (GtfA and GtfD) from the chloroeremomycin and vancomycin pathways, respectively, were later determined in the presence of bound acceptor substrate and TDP.[203,204] GtfA was found to exist in both open and closed conformational states, with few inter-domain contacts in the closed state. The open state was seen when only the acceptor substrate was bound, while the closed state was observed when both acceptor and TDP were bound, suggesting that TDP-sugar binding may trigger the formation of the catalytically active, ternary Michaelis complex. In contrast to GtfA, the structure of GtfD in the closed conformation revealed several critical interdomain contacts. These results indicated that creation of chimeric or engineered GT variants may be more complicated than thought based on the GtfB structure. To date, there are only two examples where engineering of a natural product GT-B enzyme has successfully altered substrate specificity (discussed in Section 5.2.2.),[208–210] whereas rational structure-based engineering efforts in the GT-A family enzymes have been sowewhat more successful.[211]

4.3. Mechanisms of Glycosyltransferases

Understanding the mechanisms of GTs is important for active site engineering strategies to broaden or alter their substrate specificities. Mechanistically, GTs can be classified as inverting or retaining based on the stereochemical course of the glycosyltransfer reaction they catalyze (Figure 5a).[212] Structural, mechanistic, and computational studies on the inverting GTs support an S_N2-like mechanism.[197, 213–217] As shown in Figure 5b, the lone pair electrons from the endocyclic oxygen atom facilitate the formation of an oxocarbenium-like intermediate (or transition state, see **195**) by donating electron density to the σ* orbital of the anomeric C1-O1 bond prior to the attack of the acceptor nucleophile. The retaining GTs were originally thought to proceed by a double displacement mechanism involving the initial formation of a covalent sugar-enzyme intermediate (**196**, Figure 5c),[212] analogous to the well-studied retaining glycosidases.[218] However, structural studies of several retaining GTs failed to identify suitable candidates for the putative enzyme nucleophile.[206, 219–224] A general lack of conserved amino acid residues on the β-face of the anomeric carbon in retaining GTs from the GT-A family has also been noted.[225] Hence, an alternative mechanism was proposed, where the nucleophilic acceptor attacks the anomeric carbon from the same side of the sugar ring as the NDP leaving group in an asynchronous, concerted manner with highly dissociative oxocarbenium-like character (**197**, Figure 5d).[219, 220, 225, 226]

4.4. Summary of Biochemical Work on Natural Product Glycosyltransferases

Despite the importance of GTs in controlling the glycosylation patterns of natural products, surprisingly few natural product GT activities have been verified in vitro, though a number of GT functions have been deduced by gene knockout and heterologous expression experiments. A list of 167 known and putative bacterial small molecule natural product GTs are compiled in Table 2 (see supporting information). The GTs whose functions have been verified are indicated and the corresponding references are provided. The phylogenetic relationships among antibiotic GTs have been previously reviewed,[227] and most of these enzymes fall into glycosyltransferase family 1 (GT-1),[190] which is comprised of GT-B enzymes catalyzing glycosylation with inversion of stereochemistry. The macrolide resistance GT OleD, which uses UDP-glucose as the sugar donor, was the first macrolide-related GT to be characterized in vitro.[228] Studies on OleD suggested an ordered, sequential kinetic

mechanism with the acceptor substrate binding prior to the UDP-sugar to form the ternary Michaelis complex. Following glycosyltransfer, UDP is released from the enzyme prior to the glycosylated product. This kinetic mechanism is supported by the crystallographic studies on other inverting GT-B enzymes, in which a conformational change to a closed state occurs upon binding of NDP to the GT-aglycone complex.[202–205] Although detailed kinetic analyses have not yet been performed on most other natural product GTs, a similar kinetic mechanism is expected to be operative for many inverting GT-B enzymes of the GT-1 family.

It has recently been demonstrated that several macrolide GTs require an auxiliary protein for efficient glycosyltransfer.[229] The genes for the glycosyltransferase and its corresponding auxiliary protein are almost always located next to each other in their respective biosynthetic clusters. The translated gene sequences for these auxiliary proteins share moderate homology with cytochrome P450 enzymes, yet lack the conserved Cys residue that coordinates the heme iron. The requirement of a helper protein for a GT involved in natural product biosynthesis was first established for the desosaminyltransferase, DesVII, and its auxiliary protein, DesVIII, from the methymycin/pikromycin pathway of *Streptomyces venezuelae*.[229] Subsequently, an enhancement of k_{cat} by AknT was observed for the reaction catalyzed by the anthracycline GT, AknS.[230] The authors proposed that AknT could be functioning as a regulatory subunit that transiently interacts with AknS in order to maintain AknS in an active conformation or to stabilize the transition state for glycosyltransfer. More recent studies of the EryCII/EryCIII glycosylation system demonstrated that the erythromycin GT (EryCIII) remains fully active in vitro after removal of its auxiliary protein (EryCII) from the pre-incubation mixture.[231] Experiments with the DesVII/DesVIII system gave similar results.[232] These observations could suggest that the auxiliary proteins have a chaperone-like function to facilitate a one-time conformational change that activates their corresponding GT. Clearly, more work on these systems is required to fully understand the exact role of the GT auxiliary proteins.

Although the vast majority of glycosylated natural products are *O*-glycosides, aryl-*C*-glycosides are also present in bacteria and plants.[233] Two possible mechanisms for *C*-GT catalysis have been proposed (Figure 5e).[80] One mechanism (path A) involves the initial formation of an *O*-glycoside, followed by an intramolecular rearrangement to an *ortho*-*C*-glycoside. The other mechanism (path B) involves the attack of a resonance-stabilized phenolate anion at the anomeric carbon of the NDP-sugar donor to form the *C*-glycosidic linkage. Although neither mechanism has been experimentally verified, direct formation of the *C*-glycosidic linkage is particularly appealing for natural products containing *C*-glycosyl substituents both *ortho*- and *para*-to the activating phenolate group.[233] Several natural products such as gilvocarcin and enterobatin contain only *para*-*C*-glycosides, which would be difficult to form with an *O*-glycosylation/rearrangement sequence.[18,234,235]

Recent studies of UrdGT2, a *C*-GT involved in the biosynthesis of urdamycin in *Streptomyces fradiae* Tü2717, have provided important insights into the mechanism of *C*-glycosylation (Figure 5f). While the *C*-GT activity (**198** → **199**) of UrdGT2 had been previously established, feeding of the alternative aglycone substrate (**200**) to an *S. fradiae* mutant that was deficient in wild-type aglycone biosynthesis, but which still expressed UrdGT2, resulted in the production of the *O*-glycoside, **201**.[236] This study was the first to demonstrate that a natural product GT could synthesize both *C*- and *O*-glycosidic linkages. In addition, these results support the direct *C*-glycoslation mechanism, because initial *O*-glycosylation of **200** followed by O-O rearrangement is not favored. Moreover, the recently solved X-ray crystal structure of UrdGT2 revealed that the anomeric carbon of the NDP-sugar substrate binds in close proximity to C-9 of the aglycone substrate, and is properly positioned for a direct addition to the aromatic ring.[207] Asp137 was also proposed to be the base that deprotonates the aglycone phenolate group via a tightly bound active site water molecule.

A striking discovery from studies of natural product GTs is that many of these enzymes exhibit remarkably broad substrate specificity towards their aglycone and NDP-sugar substrates. For example, VinC, a GT from the vicenistatin biosynthetic pathway of *Streptomyces halstedii* HC34, has been shown to accept the α- and β-anomers of both D- and L-sugars in vitro.[237] GtfE, the GT from the vancomycin pathway is known to accept over 30 different NDP-sugars, which has facilitated in vitro glycodiversification of the vancomycin aglycone.[238] The DesVII/DesVIII system has also been shown to accept numerous cyclized and linear forms of aglycone substrates[239–241] as well as a number of different NDP-sugars.[232] In addition to these in vitro studies, the substrate flexibility of many natural product GTs has been demonstrated in vivo. Cumulatively, these studies have helped fuel the metabolic pathway engineering, combinatorial biosynthesis, and in vitro enzymatic glycodiversification efforts that have resulted in the construction of new natural product derivatives with altered glycosylation patterns. Some of this recent glycoengineering work is highlighted in the next section.

5. Natural Product Glycoengineering

A large portion of biologically active natural products are glycosylated. Since the sugar moieties are often important for bioactivity,[5,7] alteration of the glycosylation patterns of the parent structures (a process known as glycodiversification, glycorandomization, or glycooptimization) has the potential to produce modified molecules with new activities. Accordingly, a number of strategies (both in vivo and in vitro) have emerged in recent years, wherein the biosynthetic machinery (i.e. enzymes) is manipulated to produce new natural product glycoforms. These methods have four advantages over traditional chemical synthesis/derivatization approaches. First, the stereo- and regioselectivity of enzyme-catalyzed reactions generally produce single products with defined stereochemistry. Second, the producing organism is a renewable source of the desired compounds. Third, production of targeted compounds by fermentation is readily scaled up. Finally, both the in vivo and in vitro strategies are amenable to the construction of compound libraries in a combinatorial fashion. The following sections will highlight a few selected in vivo and in vitro experiments designed to alter natural product sugar structures. Several excellent reviews on this topic can be consulted for more information about these methods and their applications.[150,[181],[242]–253]

5.1. In vivo Glycodiversification

Our improved understanding of unusual sugar biosynthesis has significantly impacted biosynthesis-based glycosdiversification efforts aimed at producing natural products with altered sugar structures. In early studies of tylosin biosynthesis in *Streptomyces fradiae*, random mutagenesis yielded *S. fradiae* strains defective in the biosynthesis or the attachment of each of the three tylosin sugars, mycaminose, mycarose and mycinose.[51] The value of these gene disruption experiments and their potential for generating novel glycosylated natural products was soon recognized. Consequently, more sophisticated in vivo glycodiversification strategies involving heterologous expression of genes were developed and applied in metabolic pathway engineering and combinatorial biosynthesis studies.[254–256]

The use of cells as catalysts to carry out chemical reactions on exogenous molecules is referred as biotransformation. Precursor-directed biosynthesis[257] and mutasynthesis[258] are two well established biotransformation processes in which a biosynthetic precursor of a natural product is replaced by a structural analogue through feeding to a wild-type strain (precursor-directed biosynthesis) or to a gene disruption mutant (mutasynthesis). Metabolic pathway engineering[259] and combinatorial biosynthesis[260] are two more recently developed methods. The basic premise of these methods is that genes from different organisms are combined and expressed in a single host strain in an attempt to re-route the biosynthetic intermediates to new final products. These heterologous expression experiments can be carried

out either in the wild type strain, or in knock-out mutant strains, where the mutation allows the accumulation of a specific biosynthetic intermediate by disrupting a downstream step in the pathway. This intermediate can then be processed by the heterologously expressed enzyme(s). The success of these methods relies on the substrate promiscuity of sugar biosynthetic enzymes and GTs. The synthetic potential of these techniques can be further elaborated when performed in conjunction with precursor feeding or bioconversion experiments.

An elegant early example of glycoengineering that utilized a combination of gene disruption and heterologous expression was the creation of 4'-*epi*-daunorubicin (**204**) and 4'-*epi*-doxorubicin (or epirubicin, **205**),[256] which are therapeutically useful analogues of the antitumor agents daunorubicin (**202**) and doxorubicin (**203**), respectively (Figure 6). In this study, the 4-ketoreductase gene *dnmV* involved in the final step of TDP-L-daunosamine (**53**,Scheme 3b) biosynthesis was disrupted in the *Streptomyces peucetius* host[256] and replaced by *avrE* or *eryBIV*, which encode the corresponding epimeric 4-ketoreductase from the L-oleandrose (**66**) and L-mycarose (**71**) pathways, respectively (Scheme 3c). The latter two sugars have an equatorial 4-OH group resulting from the axial reduction of the 4-keto group by AvrE or EryBIV. Substitution of *dnmV* with either of these two reductase genes provided a convienent route to *epi*-daunosamine. This was the first example of a *designed* in vivo biosynthesis of a non-natural sugar -*i.e.*, a sugar that has not heretofore been found in nature.

5.1.1. Erythromycin—The sugar biosynthetic genes in the erythromycin (**206**) cluster from *Saccharopolyspora erythraea* were sequenced, and a number of these genes were individually disrupted and classified as "EryB" or "EryC" genes depending on whether erythronolide B (EB, **207**) or mycarosyl erythronolide B (MEB, **208**) accumulated (Figure 7).[57,65] In addition to the accumulation of EB or MEB, other minor derivatives were also produced in these gene disruption mutants (Figure 7, path A). For example, small amounts of desosaminyl erythronolide B (**209**) were found in an *eryBVI* disruption mutant, indicating that desosaminyltransfer could still occur to some extent in the EryB mutants.[64] Disruption of the 4-ketoreductase gene, *eryBIV*, led to an erythromycin analogue (**210**) having 4-keto-mycarose in place of L-mycarose (**211**),[57] and disruption of the 3-ketoreductase gene, *eryBII*, resulted in several minor compounds (one of which is **212**) carrying a 2,6-dideoxyglucose instead of L-mycarose.[65] Also, disruption of the *C*-methyltransferase gene *eryBIII* gave an erythromycin derivative (**213**) having 3-desmethylmycarose in place of L-mycarose.[66] Several erythromycin analogues obtained in this manner retained bioactivity, albeit with reduced potency in comparison to erythromycin A.

Heterologous expression of foreign glycosyltransferases in various *S. erythraea* mutants was also used to generate new glycosylated forms of macrolides. For example, expression of the gene encoding the desosaminyltransferase (OleG1) from the oleandomyicn pathway in an *S. erythraea* mutant lacking the endogenous desosaminyltransferase (EryCIII) restored erythromycin A (**206**) production,[261] establishing the proposed functions for both EryCIII and OleG1. When the gene encoding the oleandrosyltransferase OleG2 from the oleandomyicn pathway was expressed in an *S. erythraea* mutant lacking the mycarosyltransferase EryBV (Figure 7, path B), new erythronolide derivatives (**214**) bearing an L-rhamnose moiety linked to *O*-3 of the aglycone were formed. Interestingly, the 3-*O*-L-rhamnosyl erythronolide derivatives were also found when OleG2 was expressed in the wild-type strain, indicating that the heterologously expressed OleG2 could compete with the endogenous GT for sugar transfer to the 3-OH position of the aglycone.

In a separate study, the gene encoding the mycaminosyltransferase (TylM2) from the tylosin biosynthetic pathway of *Streptomyces fradiae* was integrated into the chromosome of a triple *S. erythraea* mutant (termed SGT2) that lacked the endogenous glycosyltransferases *eryCIII* and *eryBV*, as well as the polyketide synthase gene *eryA*.[262] When the mutant cell cultures

were fed tylactone (**215**), 5-*O*-desosaminyltylactone (**216**) was produced (Figure 7, path C), revealing that the heterologously expressed TylM2 recognizes and couples the non-native desosamine sugar produced by *S. erythraea* onto its natural aglycone (**215**). Finally, the individual expression of several L-rhamnosyl-*O*-methyltransferases from the spinosyn biosynthetic pathway of *Saccharopolyspora spinosa* in the SGT2 triple mutant, demonstrated that two of these methyltransferases (SpnI and SpnK) could sequentially *O*-methylate the 2′- and 3′-OH groups, respectively, of exogenously fed 3-rhamnosyl erythronolide B (**214**) to give **217** and **218** (Figure 7, path D).[263]

5.1.2. Methymycin/Pikromycin—TDP-D-desosamine (**43**) is the sugar donor used for methymycin and pikromycin (**219**–**221**, **187**) biosynthesis in *Streptomyces venezuelae*. As shown in Figure 8, disruption of the dimethyltransferase gene *desVI* resulted in the accumulation of macrolide analogues carrying 3-*N*-acetylamino-3,4,6-trideoxy-D-glucose (**222**) in place of D-desosamine.[67] Similarly, disruption of the aminotransferase gene *desV* led to analogues bearing 4,6-dideoxy-D-glucose (**223**);[68] disruption of the *desII* gene led to analogues with 4-*N*-acetylamino-4,6-dideoxy-D-glucose (**224**),[72] and disruption of the *desI* gene resulted in analogues carrying 6-deoxy-D-glucose (D-quinovose) (**225**).[264] The ketoreduction at C-4 and C-3 to give the corresponding hydroxyl groups in **223** and **225**, and the acetylation to give the *N*-acetylamino group in **222** and **224** are catalyzed by enzymes not encoded by the *pik* cluster. These enzymes may be part of the cell surface polysaccharide biosynthetic machinery or they could be involved in other natural product pathways in the host. They function when the appropriate "unnatural" intermediates accumulate. Clearly, the opportunistic participation of some enzymes during metabolic pathway engineering further broadens sugar structural diversity.

As described above, TDP-4-keto-6-deoxyglucose (**21**), which is an intermediate in the desosamine pathway, accumulates in the *KdesI S. venezuelae* mutant. When a predicted 4-aminotransferase (CalH) from the calicheamicin producer *Micromonospora echinospora* was expressed in the *KdesI* mutant, derivatives with the 4-*N*-acetyl-4,6-dideoxysugar (**224**) were isolated.[265] In a separate study, the same quinovosyl methynolide derivative (**225**) was obtained when the putative TDP-4-keto-3,5-epimerase (*strM*) and TDP-streptose synthase (*strL*) genes from the streptomycin (**124**) producer *Streptomyces griseus* were expressed individually in the *KdesI* mutant.[27] However, when both genes were expressed together in this mutant, new macrolide derivatives containing an L-rhamnose (**226**) substituent were generated. This study not only revealed an unexpected 4-ketoreductase activity for StrL, but also demonstrated that the desosaminyltransferase DesVII can process both D- and L-sugar donors.

Several new macrolide derivatives were generated when different combinations of D-mycaminose biosynthetic genes (**21** →→ **41**, Figure 8) from the tylosin producer *S. fradiae* were heterologously expressed in *S. venezuelae* mutants.[55] First, the *tylM1/B/M2/M3* genes were expressed in an *S. venezuelae KdesI/KdesVII* mutant, which lacks the desosaminyltransferase (DesVII) and which was predicted to accumulate **21**. These four *tyl* genes were originally believed to comprise a complete set of mycaminose biosynthetic genes. However, when the mutant cultures were fed tylactone (**215**), a 5-*O*-quinovosyl-tylactone derivative was obtained.[56] This result not only reflected the relaxed tolerance of the mycaminosyltransferase (TylM2) for its TDP-sugar donor, but it also suggested that the previously proposed mycaminose pathway was incomplete. An orphan orf in the tylosin gene cluster, *tyl1a*, was subsequently identified and expressed in the *S. venezuelae KdesI/KdesVII* mutant along with *tylM1/B/M2/M3*. When tylactone (**215**) was fed to this strain, a new tylosin derivative (**227**) containing a 5-*O*-mycaminosyl substituent was obtained.[56] Interestingly, when *tyl1a* was expressed individually in the *KdesI* mutant, new methymycin/pikromycin derivatives (such as **228** and **229**) that carried a mycaminosyl moiety were isolated. These

experiments conclusively established the TDP-D-mycaminose pathway (**21** → **39** → **40** → **41**), and revealed the relaxed substrate specificity of DesV, DesVI, and DesVII/DesVIII.

Finally, when *tyl1a* was replaced with *fdtA* (a 3,4-ketoisomerase from *Aneurinibacillus thermoaerophilus* that catalyzes **21** → **136**) in a *KdesI* mutant, new macrolide derivatives bearing either a 4-*epi*-D-mycaminose (**230** and **231**) or a 3-*N*-monomethyl-3-deoxy-D-fucose (**232**) substituent were obtained.[266] As neither of these sugars are naturally occurring, this work again illustrates the potential for constructing novel sugar structures by using selected natural sugar biosynthetic enzymes. In addition, these results reveal that many desosamine pathway enzymes, including DesV, DesVI, and DesVII/DesVIII, tolerate sugar donors with an axial 4-OH group. As is evident from these and other[232,[239],[240],[267],268] studies, the DesVII/DesVIII pair clearly exhibits remarkably relaxed substrate specificity towards its sugar and aglycone substrates.

5.1.3. Elloramycin—The first reported example of in vivo glycodiversification that relied on heterologous expression of biosynthetic genes involved the expression of a cosmid (16F4) that contained most of the elloramycin (**233**, Figure 9a) biosynthetic gene cluster from *Streptomyces olivaceus* in the urdamycin (**188**) producer *Streptomyces fradiae* Tü2717.[254] The resulting strain produced the hybrid elloramycin derivative 8-demethyl-8-β-D-olivosyltetracenomycin C (**234**, Figure 9b). The sugar donor TDP-D-olivose was supplied by the urdamycin pathway, and the aglycone (8-DMTC, **235**) was produced by the heterologously expressed cosmid 16F4. Later experiments established that the substrate flexible GT, responsible for formation of **234**, was ElmGT encoded on cosmid 16F4. In this work, cosmid 16F4 was transformed into a mutant of *Streptomyces fradiae* Tü2717, in which several genes essential for formation of the urdamycin aglycone were deleted (ΔPKS). Cosmid 16F4 was also transformed into the wild-type and a PKS-defective mutant of the mithramycin (**236**) producer *Streptomyces argillaceus*.[269] Expression of cosmid 16F4 in *S. fradiae* ΔPKS led to increased yields of **234** and also to a new hybrid compound (**237**) containing the urdamycin sugar L-rhodinose. When the cosmid was expressed in wild-type or ΔPKS *S. argillaceus*, **234** was again formed along with 8-demethyl-8-β-D-mycarosyltetracenomycin C (**238**) and the disaccharide-containing compound 8-demethyl-8-β-D-olivo-3'-1''-β-D-olivosyltetracenomycin C (**239**). When *S. fradiae* Tü2717/ΔPKS and the *S. argillaceus* strains were fed **235** in the absence of the cosmid 16F4, no glycosylated tetracenomycin derivatives were obtained, firmly establishing that ElmGT (encoded by cosmid 16F4) is the GT responsible for the formation of the tetracenomycin analogues.

ElmGT was subsequently incorporated into the chromosome of *Streptomyces albus*, a non-producing strain. This strain was transformed with several plasmids encoding the production of different NDP-sugars, and each resulting strain was then fed 8-DMTC (**235**).[270] In these experiments, ElmGT was shown to attach L-olivose and L-rhamnose (its natural sugar substrate) onto **235** to generate **240** and **241** (Figure 9c). In a different set of combinatorial biosynthesis studies, cosmid 16F4 was transformed into *Streptomyces lividans* (also a non-producing strain) along with plasmids encoding the production of NDP-L-digitoxose,[271] NDP-4-deacetyl-L-chromose B,[272] and NDP-L-mycarose.[272] Each of these strains produced the corresponding glycosylated 8-DMTC analogue (**242**–**244**, respectively). A glucosylated 8-DMTC compound (**245**) was also obtained, indicating the unusual tolerance of ElmGT for a sugar containing a 6-OH group.[271] Finally, in an impressive experiment, genes from four different deoxysugar biosynthetic pathways were combined on a single vector to generate D- and L-amicetosyl-8-DMTC derivatives (**246** and **247**, respectively) when co-expressed in *S. lividans* 16F4.[273] Since biosynthetic gene clusters for D-amicetose are not available, the above experiment illustrates the power of combinatorial biosynthesis to generate a desired sugar structure based solely on the logic observed in other biosynthetic pathways.

5.1.4. Urdamycin—Urdamycin A (**188**, Figure 10a), produced by *Streptomyces fradiae* Tü2717, is an angucycline type antibiotic and anticancer agent. The urdamycin aglycone has an *O*–linked L-rhodinose residue at C-12b and a *C*–linked D-olivose-L-rhodinose-D-olivose trisaccharide at C-9. To verify the functions of the four GTs encoded in the urdamycin A gene cluster, as well as the order of glycosylation steps, a number of *S. fradiae* mutants were constructed in which individual GTs or combinations of GTs were disrupted. This led to a number of urdamycin derivatives (**248**–**258**) with unnatural glycosylation patterns (Figure 10a).[274, 275] When the *urdGT2* gene was disrupted, several urdamycin shunt metabolites (**248**–**250**) accumulated, all of which lacked the trisaccharide moiety at C-9, suggesting that UrdGT2 is the *C*-GT. Interestingly, **250** showed much better anticancer activity than the parent compound, urdamycin A.[274] In similar knockout experiments, UrdGT1a was identified to be the C-12b-L-rhodinosyl transferase, while UrdGT1c and UrdGT1b were found to be the rhodinosyl- and olivosyltransferases, respectively, responsible for the construction of the trisaccharide. When *urdGT1c* was overexpressed in the *urdGT1c* knockout strain, a second L-rhodinose moiety was incorporated into the trisaccharide chain by UrdGT1c to give **257** and **258**.

In a separate study, several urdamycin A deoxysugar biosynthetic genes were individually disrupted in *S. fradiae*, and this led to even more new derivatives.[102] The *urdZ3*, *urdQ*, and *urdZ1* knockout strains each accumulated urdamycinone B (**254**, see Figure 10a), reflecting the essential roles of these genes in the biosynthesis of L-rhodinose (Figure 10b). Surprisingly, the inactivation of the 4-ketoreducatse (UrdR) needed for TDP-D-olivose (**79**) synthesis, yielded urdamycin M (**259**), which contains a D-rhodinose moiety (see **260**) as opposed to normally produced L-rhodinose (see **94**) attached to C-9 of **248** through a *C*-glycosidic linkage. Thus, it appears that the rhodinosyl 4-ketoreductase (UrdZ3) can reduce a rhodinosyl intermediate (such as **91**) prior to UrdZ1-catalyzed C-5 epimerization. Intermediate **91** may accumulate to unnaturally high levels in the absence of UrdR, leading to increased concentrations of **260**, which can then be coupled to **248** by UrdGT2. These results suggested that UrdGT2 is flexible for its NDP-sugar donor substrate, and is able to accept both TDP-D-olivose (**79**) and TDP-D-rhodinose (**260**). In a subsequent study with the *S. fradiae urdR*$^{-}$ mutant, it was also demonstrated that UrdGT1c could transfer an L-rhodinose moiety to **259** to generate urdamycin R (**261**).[276] This study also revealed that UrdGT2 could attach L-rhodinose (see **94**) to C-9 of **248**. The resulting compound could then be L-rhodinosylated by UrdGT1c to give urdamycin S (**262**). Thus, UrdGT2 is clearly capable of synthesizing *C*-glycosides using both L- and D-rhodinose in vivo.

When heterologously expressed in *Streptomyces argillaceus* strains lacking the native mithramycin (**236**, Figure 9a) glycosyltransferases, UrdGT2 was able to couple the mithramycin deoxysugars, D-olivose and D-mycarose, to the premithramycinone aglycone (to give **263** and **264**, Figure 10c) through *C*-glycosidic linkages at positions of the aglycone that are not normally glycosylated.[277] When UrdGT2 was co-expressed with LanGT1 (a D-olivosyltransferase from the landomycin producer *Streptomyces cyanogenus* S136) in this same *S. argillaceus* strain,[277] a hybrid compound (**265**) was formed. This compound was composed of an *S. argillaceus*-derived aglycone and a disaccharide assembled by the action of both UrdGT2 and LanGT1. In a separate combinatorial biosynthesis study, heterologous expression of LanGT1 and LanGT4 (an L-rhodinosyl transferase) in a *S. fradiae* triple GT mutant (*urdGT1a-/1b-/1c-*) was used to generate hybrid urdamycin/landomycin compounds (such as **266**) that contained a new trisaccharide moiety.[278] Clearly, like DesVII and ElmGT, UrdGT2 accepts a variety of NDP-sugar and aglycone substrates and, thus, UrdGT2 may prove to be a useful tool for enzymatic glycodiversification of aryl-*C*-glycosides.

5.1.5. Indolocarbazoles—The indolocarbazole alkaloid *N*-glycosides, rebeccamycin (**267**) produced by *Saccharothrix aerocolonigenes* and staurosporine (**193**) produced by

several *Streptomyces* species, are antitumor compounds with DNA topoisomerase I and protein kinase inhibition activities, respectively (Figure 11). Heterologous expression of different combinations of *reb* and *sta* genes in the non-producing strain *Streptomyces albus* helped elucidate the biosynthetic pathway for the rebaccamyin and staurosporine aglycones, and also resulted in a number of new derivatives, many of which were *N*-glucosylated by RebG.[279] Recent bioconversion experiments demonstrated that RebG, when expressed in either *E. coli* or *S. lividans*, could *N*-glucosylate a number of exogenously fed indolocarbazole derivatives, including the staurosporine aglycone (**268**).[280] Interestingly, RebG catalysis lacks regioselectivity, since it can glycosylate either of the *N* atoms of the asymmetric indolocarbazoles used in this study.

In a separate study, staurosporine biosynthesis was reconstituted in *S. albus* by co-expressing the biosynthetic genes for the staurosporine aglycone (**268**), along with those for L-ristosamine, and the putative *N*-GT, StaG (Figure 11).[74] The transformed *S. albus* mutant produced holyrine A (**270**), a compound containing an *N*-linked 3-*N*-4-*O*-didemethyl-L-ristosamine moiety in a $^4C_1$ conformation. When *staN* (a putative cytochrome P450 gene) was expressed in this *S. albus* mutant, the cell cultures produced staurosporin, establishing StaN as the enzyme responsible for C5′-*N* bond formation. The substrate flexibility of StaG was then tested by transforming plasmids encoding the production of different deoxysugars (L-rhamnose **34**, L-digitoxose **78**, L-olivose **65**, and D-olivose **79**) into the mutant *S. albus* strain. HPLC analysis showed that each of the strains expressing L-deoxysugar genes produced two new compounds, while the strain expressing the D-olivose genes only produced one new compound. Subsequent MS and NMR analysis revealed that all five deoxysugars tested in this study could be singly linked to the N-13 atom of the staurosporine aglycone (by StaG) to form **269–273**, each with an equatorial *N*-glycosidic bond that places the sugar in the $^4C_1$ conformation. For the L-sugars, the $^4C_1$ conformation is unusual because the bulky substituents at C-3, C-4, and C-5 are in a less favorable axial configuration. The compounds containing L-sugars (**269–272**) could be further processed by StaN to yield the doubly attached staurosporine analogues **274–277**. Interestingly, in the doubly attached compounds, the L-sugars exist exclusively in the $^1C_4$ conformation, suggesting that StaN converts the $^4C_1$ conformation of the L-sugars into a $^1C_4$ conformation prior to the oxidative coupling of C-5′ to the indole N-12.

5.2. In vitro Glycodiversification

Although significant progress has been made towards natural product glycodiversification through in vivo combinatorial biosynthesis and metabolic engineering, there are several inherent disadvantages that limit the applicability of these approaches. First, it is difficult to control the reaction conditions and to prevent undesired side reactions, which lower the efficiency of the desired glycosylation reactions. Also, the newly generated metabolites are potentially toxic to the bacterial strain used as the host for expression of the heterologous genes. Finally, only those aglycone acceptors and sugar donors that can be biosynthesized or fed to the host can be used as potential building blocks, and this ultimately limits the structural diversity of glycoforms that can be generated. To overcome some of these problems, recent efforts have focused on the development of methods for in vitro glycodiversification using purified sugar biosynthetic enzymes and glycosyltransferases. These efforts have benefited from the accumulated body of knowledge on sugar biosynthetic enzymes and the discovery of several substrate flexible anomeric kinases, nucleotidylyltransferases, and glycosyltransferases. These substrate flexible enzymes have been used to generate libraries of NDP-sugars (reviewed in [281]), which can then be tested in vitro as substrates for glycosyltransferases with natural or engineered substrate flexibility. In this section, we will focus only on those glycoengineering efforts which employ purified sugar biosynthetic enzymes and glycosyltransferases to generate glycorandomized natural product libraries. However, the recent development of purely chemical methods for natural product

glycodiversification,[248,[249],[282]–286] will undoubtedly provide researchers with robust, alternative strategies for their glycoengineering efforts.

5.2.1. Engineering Sugar Anomeric Kinases—The major limitation to the enzymatic synthesis of NDP-sugars is the availability of the specific enzymes required for the construction of the desired NDP-sugars. To facilitate the preparation of NDP-sugars, directed evolution and structure-based protein engineering have been used to create sugar biosynthetic enzymes with broader substrate specificity. For example, a single round of random mutagenesis on the galactokinase (*galK*) gene from *E. coli*[287] was sufficient to generate a GalK variant (Y371H) that tolerates substitutions at C-2, C-3, C-5, and C-6 of D-galactose, but which maintains a stringent requirement for the axial 4-OH group. This mutant can also phosphorylate two L-sugars (**278** and **279**,Figure 12). Based on a structural homology model with galactokinase from *Lactococcus lactis*, two conserved residues (Asp37 and Tyr223) in the *E. coli* enzyme were proposed to form hydrogen bonds with the axial 4-OH group.[288] However, mutation of these residues failed to change the C-4 specificity of the *E. coli* GalK. In constrast, the Y385H (equivalent to *E. coli* Y371H) mutant of *L. lactis* GalK could accept D-glucose and a few other D-sugars with equatorial 4-OH groups as substrates.[289] Further analysis of the *E. coli*/*L. lactis* GalK homology model suggested that the Met173 residue in the *E. coli* enzyme (Leu182 in *L. lactis*) may have prevented the *E. coli* enzyme from processing D-sugars with an equatorial 4-OH configuration.[290] Indeed, the *E. coli* M173L mutant was found to accept D-*gluco*-configured sugars. Furthermore, the M173L/Y371H double mutant retained the substrate flexibility observed for each single mutant and, in addition, also recognized azido sugars, which can be further modified by chemoselective ligation reactions. The sugar-1-phosphates synthesized by the wild type and mutant *E. coli* GalK are listed in Figure 12.

5.2.2. Engineering Nucleotidylyltransferases—Preparation of natural and unnatural sugar-1-phosphates represents only the first stage in the synthesis of NDP-sugars. The next challenge is to convert these compounds to the corresponding NDP derivatives. The α-D-glucose thymidylyltransferase from *Salmonella enterica* LT2 (RmlA or E_p), which couples either TMP or UMP to a set of sugar-1-phosphates, is the most extensively studied NDP-sugar synthase.[291] RmlA prefers pyranosyl phosphates in the $^4C_1$ chair conformation, and is less efficient towards 2-deoxysugars. It can also process amino and acetamido sugars.[292] The position of the amino group has no effect on turnover, while bulky acetamido groups are only tolerated at the C-2 and C-3 positions. The crystal structures of RmlA in complex with UDP-glucose or TTP[293] showed that the active-site residue Trp224 interferes with the thymidylylation of sugars that contain bulky substituents at C-6. The Trp224 residue was subsequently mutated to His to alleviate the steric crowding around C-6 of the substrate. This mutation may also introduce a positive charge that facilitates binding of sugars containing a C-6 carboxylate group. The substrate flexibility of RmlA was further enhanced by the mutation of Leu89 to Thr, which relieves steric crowding around C-2 of the substrate.[294] In all, over 30 different sugar-1-phosphates were found to be substrates of RmlA or its variants in these studies.

In the search for alternative nucleotidylyltransferases with broad substrate specificity, a heatstable nucleotidylyltransferase from the archaeal organism *Pyrococcus furiosus* DSM 3638 has been shown to have relatively broad substrate specificity, and can even efficiently uridylylate L-fucose-1-phosphate (see **8**, Figure 1).[295] The enzyme is bifunctional and catalyzes 2-*N*-acetyltransfer to glucosamine-1-phosphate prior to the uridylyltransfer reaction.[296] Using *N*-acetylcysteamine thioesters in place of acetyl-CoA, several new UDP-glucosamine derivatives were synthesized in one pot from glucosamine-1-phosphate by this enzyme. Nucleotidylyl-transferases from twp other thermophilic archaeal organisms have been shown to accept alternative NTP substrates, including both purine and 2'-deoxy-ribonucleotides.[297,298]

In a more recent study, RmlA (which is a thymidylyltransferase) was found to use each of the eight naturally occurring NTPs (UTP, CTP, ATP, GTP, TTP, dCTP, dATP, and dGTP) to activate 10 different sugar-1-phosphate substrates, albeit with drastically different catalytic efficiencies.[299] Steady state kinetic analysis indicated that the thymidine preference of RmlA is primarily attributable to a low K_m for TTP, while both TTP and UTP are processed with much higher k_{cat} values than other NTPs. Mutation of the Gln83 residue of RmlA, which forms hydrogen bonds to the base-pairing face of the uridine/thymidine moiety, to either Asp or Ser resulted in an enzyme favoring purine nucleotides over pyrimidine nucleotides by three orders of magnitude. As more structural information for nucleotidylyltransferases becomes available, it may be possible to alter the substrate specificity for NTPs by protein engineering as a practical means to expand the repertoire of available NDP-sugars for in vitro glycosylation studies.

5.2.3. In vitro Synthesis of NDP-sugars—To facilitate the enzymatic synthesis of highly-modified TDP-sugars, efficient methods for the preparation of TDP-4-keto-6-deoxy-D-glucose (**21**) -a common intermediate in many deoxysugar biosynthetic pathways -have been developed. For example, purified sucrose synthase (SuSy) from potato, TDP-glucose-4,6-dehydratase (RmlB) from *Salmonella typhimurium*, and TMP kinase from yeast were used to synthesize **21** from the inexpensive starting materials sucrose (**280**) and TMP in one-pot (Scheme 9a) with a typical yield of ~70% (relative to TMP).[300] An ATP-regeneration system consisting of pyruvate kinase (PK) and phosphoenol pyruvate (PEP) was included so that only catalytic amounts of ATP were needed. An analogous strategy using SuSy has also been extended to make other NDP-sugars.[281] In a separate biosynthesis-based approach, TMP kinase (TMK), acetate kinase, and glucose-1-phosphate thymidylyltransferase from *E. coli*, along with RmlB from *S. typhimurium*, were expressed in *E. coli* BL21 cells (Scheme 9b). [301] The crude extracts from these cells were incubated with TMP, acetylphosphate, and glucose-1-phosphate to synthesize **21** in 80% yield (from TMP).

To date, only a handful of highly modified natural product TDP-sugars have been synthesized via tandem reactions using purified biosynthetic enzymes. These include TDP-L-mycarose (**71**) of the tylosin pathway,[92] TDP-L-epivancosamine (**56**) of the chloroeremomycin pathway,[82] TDP-D-forosamine (**100**) of the spinosyn pathway,[114] and TDP-L-digitoxose (**78**) of the kijanimicin pathway.[99] To avoid complications, a two-stage one-pot approach was developed for the synthesis of TDP-L-mycarose (**71**) from thymidine and glucose-1-phosphate (**17**, Scheme 9c). The initial reaction mixture contained thymidine, PEP, ATP and four enzymes, thymidine kinase (TK), thymidylate kinase (TMK), nucleoside diphosphate kinase (NDK), and pyruvate kinase (PK). After incubation and subsequent removal of the enzymes by ultrafiltration, the filtrate was then incubated with glucose-1-phosphate, RfbA and RfbB (a thymidylyltransferase and a TDP-glucose-4,6-dehydratase from *Salmonella typhi*, respectively), and the mycarose biosynthetic enzymes (TylX3, TylC1, TylC3, TylK, and TylC2, Scheme 3c), together with NADPH and SAM. The yield of **71** was 16%. Interestingly, there are no apparent incompatibilities within the reaction conditions for the enzymes used in this multienzyme-synthesis, and there is no obvious cross-inhibition caused by substrates or products generated in the course of this one-pot synthetic scheme. The successful enzymatic preparation of various TDP-sugars sets the stage for exploring the glycosylation of secondary metabolites in vitro.

5.2.4. Protein Engineering of Glycosyltransferases—An elegant example of GT engineering was recently reported. UrdGT1b and UrdGT1c from the urdamycin pathway (discussed in Section 5.1.4.), share 91% amino acid sequence identity but have distinct substrate specificities. The domain of each enzyme that confers the UrdGT1b- or UrdGT1c-specific activity was localized to a region consisting of 31 amino acids near the *N*-terminus of both enzymes.[208] When this region in UrdGT1c was replaced with the corresponding region in UrdGT1b, the resulting chimeric enzyme exhibited UrdGT1b-like activity. An analogous

result was observed when the region in UrdGT1b was replaced with the corresponding region in UrdGT1c. Of the 31 amino acids in this region, 18 are different between the two enzymes. Further studies indicated that only 10 of these 18 variable amino acids were critical for conferring either UrdGT1b- or UrdGT1c-like activities.[209] These residues were subsequently mutated and the resulting constructs were screened for GT activity. In addition to mutants that retained either UrdGT1b or UrdGT1c activity, and those that had both parental activities, mutants that catalyzed a new reaction were also found. In this new reaction, a D-olivose residue was transferred onto the D-olivose-L-rhodinose disaccharide moiety of 12b-derhodinosyl urdamycin G (**253**, Figure 13a) to produce a compound with a branched sugar chain (urdamycin P, **281**). Interestingly, some of the mutants with the new activity also retained the normal UrdGT1b and/or UrdGT1c activity. Clearly, such protein engineering efforts have potential to generate new GTs with broader substrate specificities and the ability to catalyze new reaction(s).

The high sequence identity between the two urdamycin GTs and the rational selection of amino acid residues for mutation reduced the size of the mutant GT library in the previous example. However, more typcial engineering experiments based on directed evolution and/or random mutagenesis would generate far more mutants. Thus, the development of high-throughput assays for screening enzyme activities is a critical component of protein engineering efforts.[210,302–304] Recently, the directed evolution of CstII, a sialyltransferase of the GT-A family,[303] and OleD, a macrolide resistance glucosyltrasferase of the GT-B family (that catalyzes **282** → **283**,Figure 13b),[210] have been reported. A library of over 1000 OleD variants was constructed using error prone PCR and the GT activities of the variants were screened using a fluorescent aglycone substrate (**284**,Figure 13b) whose fluorescence is quenched upon glycosylation (**284**→ **285**). Three single-site OleD mutants (Pro67Thr, Ser132Phe, Ala242Val) exhibited enhanced activity for **284** relative to that observed for the wild-type OleD. The corresponding triple mutant was then constructed and its substrate specificity was examined using a library of 22 NDP-sugars with **284** as the acceptor. The triple mutant processed 15 of the 22 sugars, whereas the wild-type OleD used only 3 of the 22 sugars. In addition, the triple mutant exhibited enhanced GT activity for 6 other unnatural acceptors. Interestingly, the Pro67 residue of OleD resides in a hypervariable loop region in the acceptor-binding domain near the *N*-terminus of the protein. Mutation at the equivalent position in the UrdGTs also altered substrate specificity. Finally, in a very recent study, a high-throughput GT assay was developed wherein the proton released from the acceptor nucleophile upon glycosyltransfer is detected by a pH indicator in weakly buffered solutions.[304] This assay is attractive because it can theoretically be applied to any GT (or GT variant) while using native GT substrates instead of chromophoric substrate analogues. Although the engineering of natural product GTs is still in its infancy, the studies with the urdamycin GTs and OleD clearly demonstrate the significant potential of this approach to generate enzymes with enhanced catalytic efficiency, broadened substrate promiscuity, and/or novel activities.

5.2.5. In vitro Natural Product Glycoengineering—With the rapid expansion of the NDP-sugar pools and the availability of promiscuous GTs, it is now possible to derivatize structurally diverse natural product aglycones with a variety of sugar moieties in order to make different glycoforms. This approach has emerged as an exciting method that provides ready access to new glycosylated natural products. This strategy, termed in vitro glycodiversification or glycorandomization,[248,249,286,305] has recently been used to produce a large number of methymycin/pikromycin (**187**, **219**–**221**,Figure 8) derivatives[232] and vancomycin (**192**,Figure 14a) derivatives.[238] The vancomycin aglycone (**286**) is sequentially glycosylated at the 4-hydroxyphenylglycine residue (**286** → **287** → **192**) by the glucosyltransferase, GtfE, and the vancosaminyltransferase, GtfD (Figure 14a).[306] GtfD and GtfE had previously been shown to have a relaxed substrate specificity.[255,306,307] To exploit these properties, a library of TDP-sugars (prepared using chemical synthesis and the GalK and

RmlA mutants described in Sections 5.2.1. and 5.2.2.) was incubated with the vancomycin aglycone and GtfE, and the reaction contents were analyzed by LC-MS (Figure 14b).[238] Twenty-one of the 23 TDP-sugars analyzed in this study were found to be substrates for GtfE, including the TDP-azidosugar, **289**, which could be further modified in the presence of alkynes via the Huisgen cycloaddition reaction to generate 39 additional vancomycin derivatives. [238,308] One of the new compounds displayed improved antibiotic activity against *Staphylococcus aureus* and *Enterococcus faecium*. Following these initial studies on vancomycin glycorandomiztion, several other natural product GTs with relaxed substrate specificity were used for similar in vitro glycodiversification studies.[87,[232],[237],[309]–316]

The versatility of in vitro glycodiversification was recently expanded during a calicheamicin (**189**, Figure 4) glycorandomization study.[310] It was first demonstrated that the calicheamicin GT, CalG1, accepts ten different TDP-sugars as substrates. One of the sugars, TDP-3-deoxy-α-D-glucose (**294**, Figure 15a), was then incubated with CalG1 and the 3-*O*-methylrhamnosylated aglycone (**290**). Since the glycosylation site for CalG1 was already occupied in **290**, no reaction was expected. However, a new product (**292**) carrying a 3-deoxy-α-D-glucose moiety was identified. Interestingly, analysis of the control reactions revealed that CalG1 had catalyzed a reverse GT reaction in the presence of TDP to generate TDP-3-*O*-methyl-β-L-rhamnose (**293**) and a de-glycosylated aglycone (**291**). Glycosylation of **291** by CalG1 could then proceed using the alternative TDP-sugar (**294**) present in the reaction mixture to give **292**. The calicheamicin aminopentosyl transferase (CalG4) and the vancomycin GTs (GtfD and GtfE) were also shown to catalyze reversible reactions in this study, suggesting that reaction reversibility may be a general property of GTs in vitro.

The reversible reactions catalyzed by these GTs were then exploited in a number of glycorandomization applications (Figure 15b–e). Using a set of eight calicheamicin derivatives and the ten established CalG1 TDP-sugar substrates, CalG1 catalyzed several "sugar exchange" reactions, yielding a glycorandomized calicheamicin library of over 70 compounds (Figure 15b). CalG1, CalG4, and GtfD were also individually used in one-enzyme "aglycone exchange" reactions, where the 3-*O*-methyl-β-L-rhamnosyl, aminopentosyl, or vancosaminyl moieties were transferred by CalG1, CalG4, or GtfD, respectively, from one calicheamycin or vancomycin aglycone to another (Figure 15c). Later, a one-pot two-enzyme aglycone exchange reaction was developed, wherein GtfE was used to excise an unnatural azido sugar moiety from a vancomycin aglycone in order to generate a TDP-azidosugar intermediate which was then coupled by CalG1 to a calicheamicin aglycone (Figure 15d). GT reversibility can also be exploited to synthesize NDP-sugars (Figure 15e) and to verify the biological functions of GTs. [87, [252], 317]

6. Summary and Outlook

In this review, we have highlighted our current understanding of unusual sugar biosynthesis. While the final structures of these sugars vary considerably, only a handful enzyme activities are used for their biosynthesis. This process of "natural combinatorial biosynthesis" can account for much of the sugar structural diversity observed in nature. Investigations into the sequence of biosynthetic events and the mechanisms of the pathway enzymes have revealed that most of these enzymes rely on their unique arrangements of catalytic residues, coenzymes, and cofactor requirements in order to carry out specific chemical transformations on chemically similar NDP-ketosugar intermediates. The fact that the family of sugar-modifying SDR enzymes has evolved not only to generate NDP-ketosugar intermediates, but also to manipulate this intermediate in one active site reflects the elegance of Nature's strategy for creating structural diversity. The variation in sugar structure imparted by "natural combinatorial biosynthesis" and SDR enzymes can be further augmented by unusual enzyme activities, many of which remain to be characterized.

One observation that has emerged during studies of natural product glycosylation is that many sugar biosynthetic enzymes and glycosyltransferases exhibit some degree of substrate flexibility towards their NDP-sugar and/or aglycone substrates. The metabolic pathway engineering and combinatorial biosynthesis studies carried out over the past decade have illustrated the potential utility of these enzymes for the generation of new glycoforms. Armed with our understanding of biosynthetic logic and a solid foundation of mechanistic and structural work, we are entering a new era of natural product glycodiversification, where these substrate-flexible enzymes can be further manipulated through protein engineering and used to generate libraries of substrates for in vitro glycosylation reactions, or to rapidy glycorandomize a given natural product scaffold. Such efforts have the potential to produce new compounds that could mitigate the ubiquitous and daunting threat to human health imposed by drug-resistant pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Biographies

Christopher J. Thibodeaux was born and raised in Louisiana (USA), where he earned Bachelor's degrees in both Biochemistry and Plant Biology from the Louisiana State University. He has since joined the research group of Prof. Hung-wen Liu at the University of Texas, Austin, where he is working towards his Ph.D. in Cellular and Molecular Biology. His primary research

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Charles E. (Chad) Melançon grew up in New Orleans, LA (USA), where he earned Bachelor's degrees in both Chemistry and Biology from The University of New Orleans in 2001. Chad did his doctoral work in Biochemistry in Professor Liu's lab at the University of Texas at Austin, where he focused on investigating and engineering macrolide antibiotic sugar biosynthesis and glycosylation pathways of Actinomycetes. In June of 2007, Chad began an NIH postdoctoral fellowship in Dr. Peter Schultz' lab at the Scripps Research Institute in La Jolla, CA, where he works on application of diversity-based strategies to creating engineered organisms with expanded genetic codes.

Hung-wen (Ben) Liu was born in Taipei, Taiwan (ROC). Following his undergraduate, graduate, and postdoctoral studies, he joined the faculty of Chemistry at the University of Minnesota in 1984. In 2000, he moved to the University of Texas, Austin, where he now holds the George H. Hitchings Regents Chair in Drug Design and is a Professor of Medicinal Chemistry, Chemistry, and Biochemistry. His research lies at the crossroads of organic and biological chemistry, with particular emphasis on enzymatic reaction mechanisms, natural product biosynthesis, protein function regulation, inhibitor design and synthesis, and metabolic pathway engineering. He enjoys reading, music, and travelling.

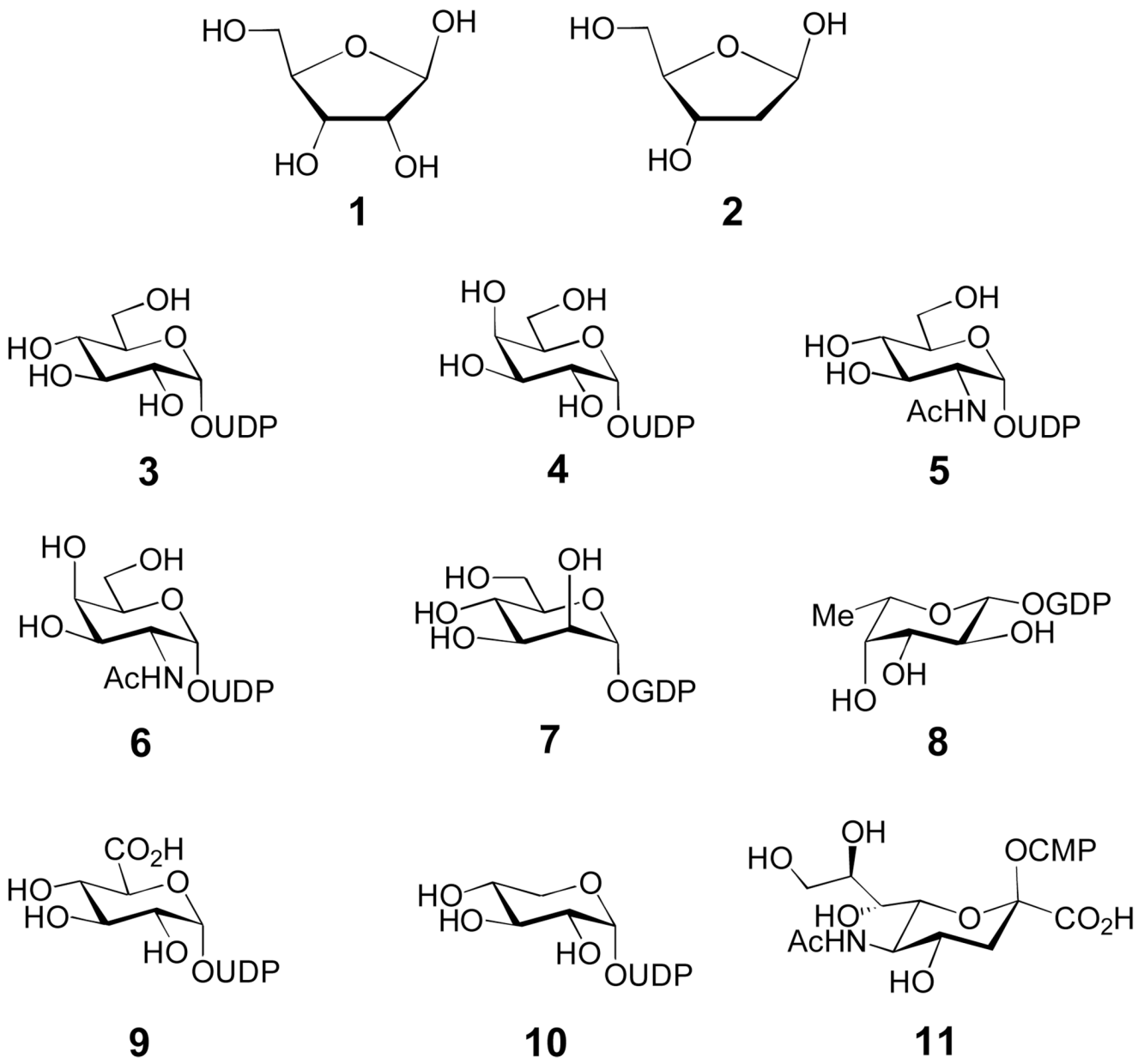


Figure 1. Common sugars of primary metabolism

D-ribose (**1**), 2-deoxy-D-ribose (**2**), UDP-D-glucose (**3**), UDP-D-galactose (**4**), UDP-2-*N*-acetyl-D-glucosamine (**5**), UDP-2-*N*-acetyl-D-galactosamine (**6**), GDP-D-mannose (**7**), GDP-L-fucose (**8**), UDP-D-glucuronic acid (**9**), UDP-D-xylose (**10**), CMP-*N*-acetyl-neuraminic acid (sialic acid, **11**).

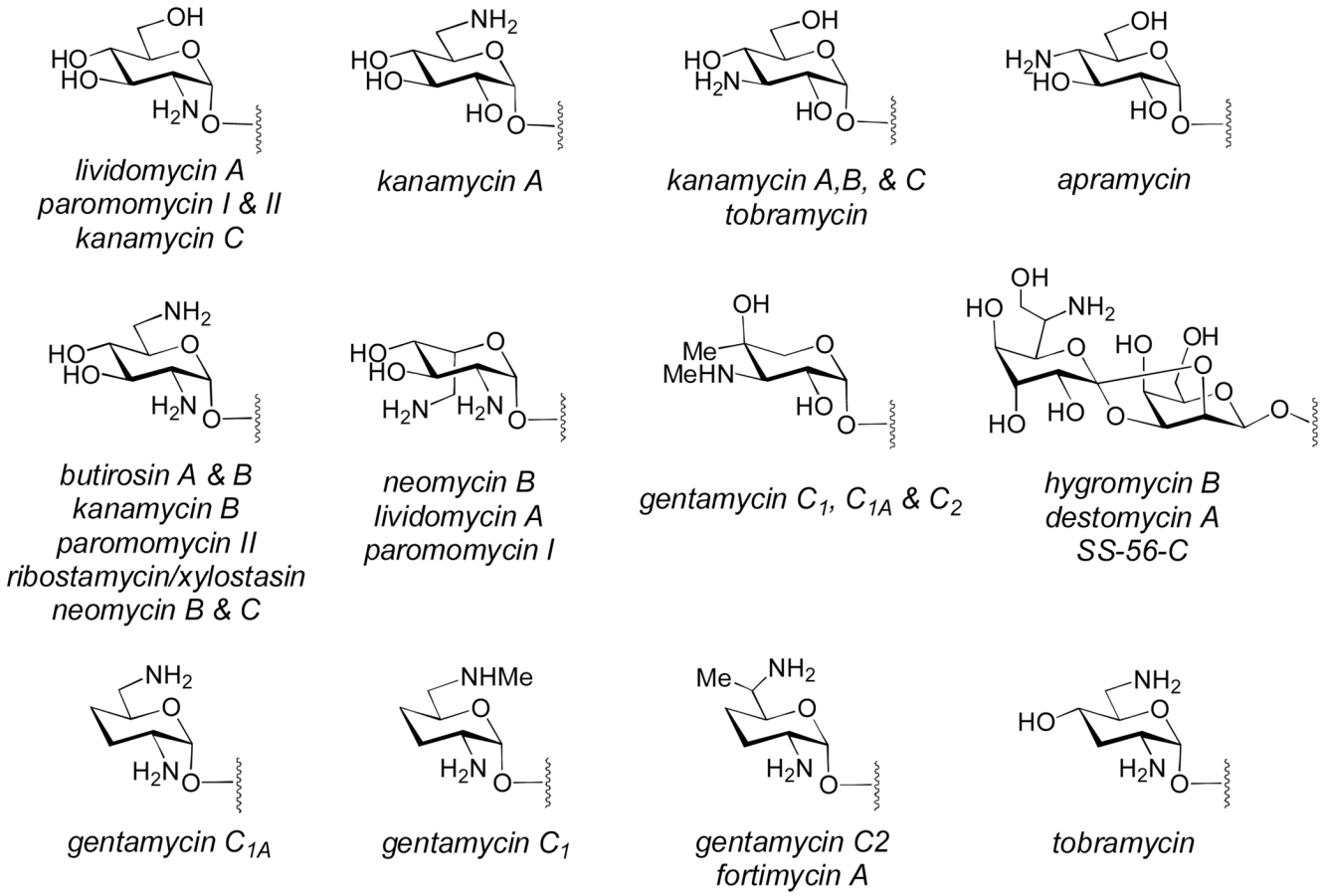


Figure 2. Representative aminoglycoside sugars

The aminosugar substituents of many aminoglycosides contain unusual modifications, whose biosyntheses are not well understood. See text for details.

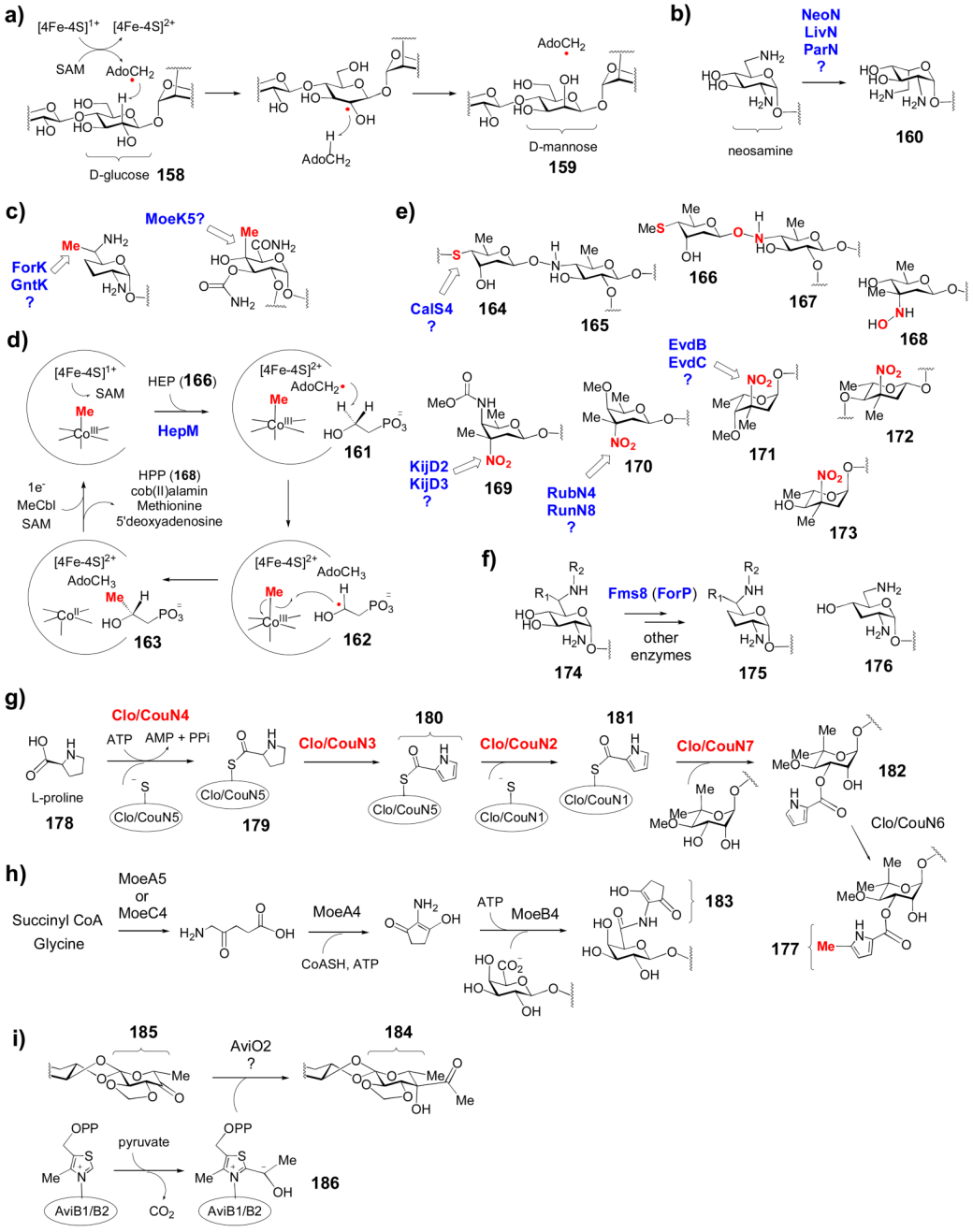


Figure 3. Selected unusual sugar-tailoring modifications

a) Conversion of D-glucose to D-mannose (**158** → **159**) in the biosynthesis of avilamycin by AviX12 – a radical SAM-dependent enzyme. b) Putative function of several radical SAM-dependent enzymes encoded in the neomycin, lividomycin, and paromomycin clusters. c) Methylation at unactivated carbon centers in gentamycin, fortimycin, and moenomycin biosynthesis may be carried out by a group of radical SAM/cobalamin-dependent enzymes. d) Proposed mechanism for Fom3, a radical SAM/cobalamin-dependent enzyme from the fosfomycin biosynthetic pathway of *Streptomyces wedmorensis*. e) Representative thio-, nitro- and hydroxylamine sugars found in several bacterial natural products. f) Putative involvement of Fms8 in 3,4-didehydroxylation of a fortimycin biosynthetic intermediate and the 2,3,6-

trideoxysugar residue (**176**) found in tobramycin. g) Assembly of the 5-methyl-carboxypyrrole substituent (**177**) of cloromomycin and coumermycin using non-ribosomal peptide synthase biosynthetic logic. h) Biosynthesis of the unsual sugar substituent (**183**) in moenomycin. i) Unusual modifications required to synthesize the methyleurekanate moiety (**184**) of avilamycin A.

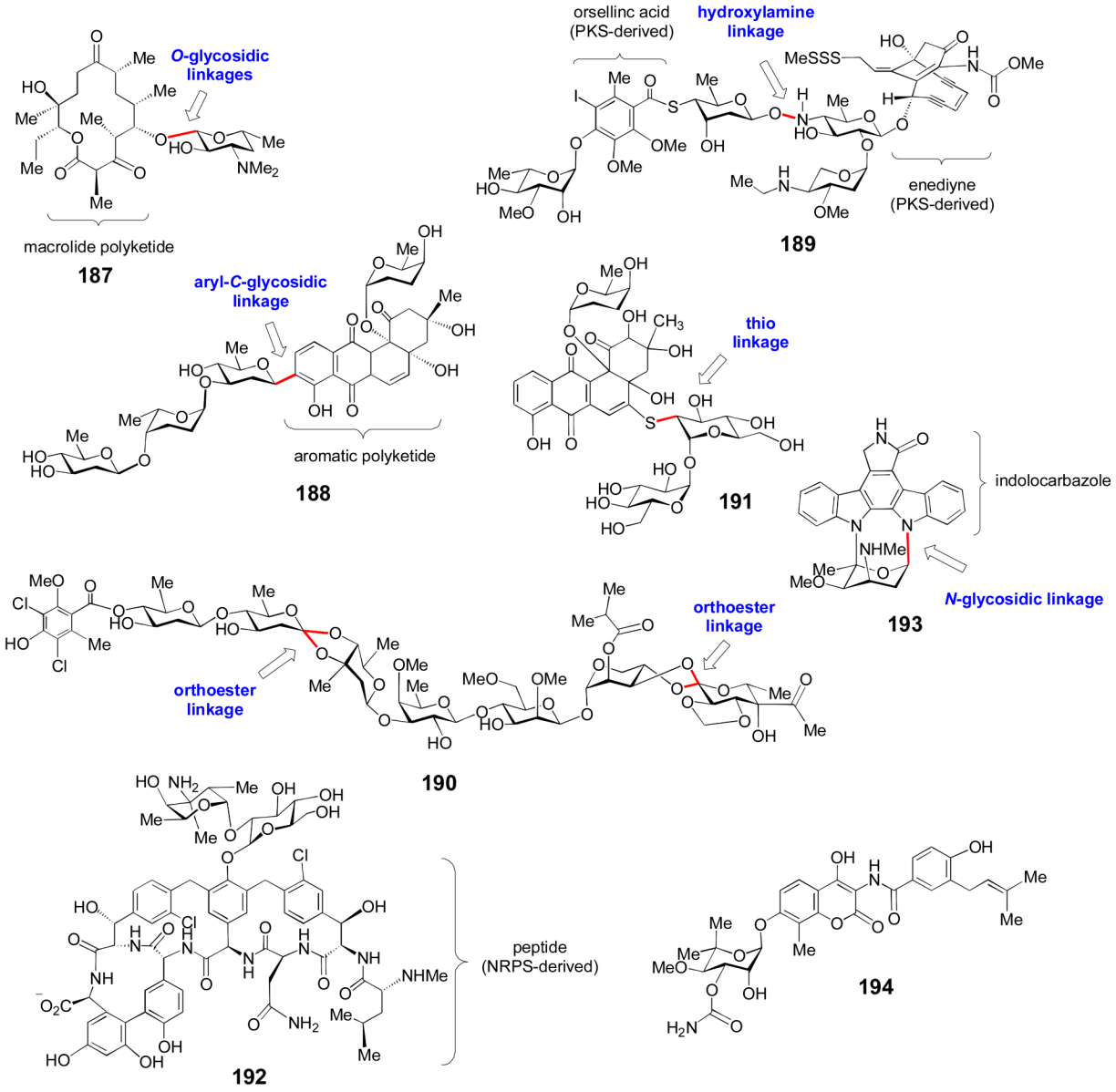


Figure 4. Representative bacterial natural product glycoforms

Examples of glycosylated microbial natural products: pikromycin (**187**), urdamycin A (**188**), calicheamicin (**189**), avilamycin (**190**), BE-7585 (**191**), vancomycin (**192**), staurosporine (**193**), and novobiocin (**194**). Glycosidic linkages are typically *O*-linked, but *C*-glycosides (see **188**), *N*-glycosides (see **193**), hydroxylamine linkages (see **189**), and orthoester linkages (see **190**) also exist in nature. The disaccharide moiety of **191** is coupled to the aromatic aglycone through an unusual thio linkage.

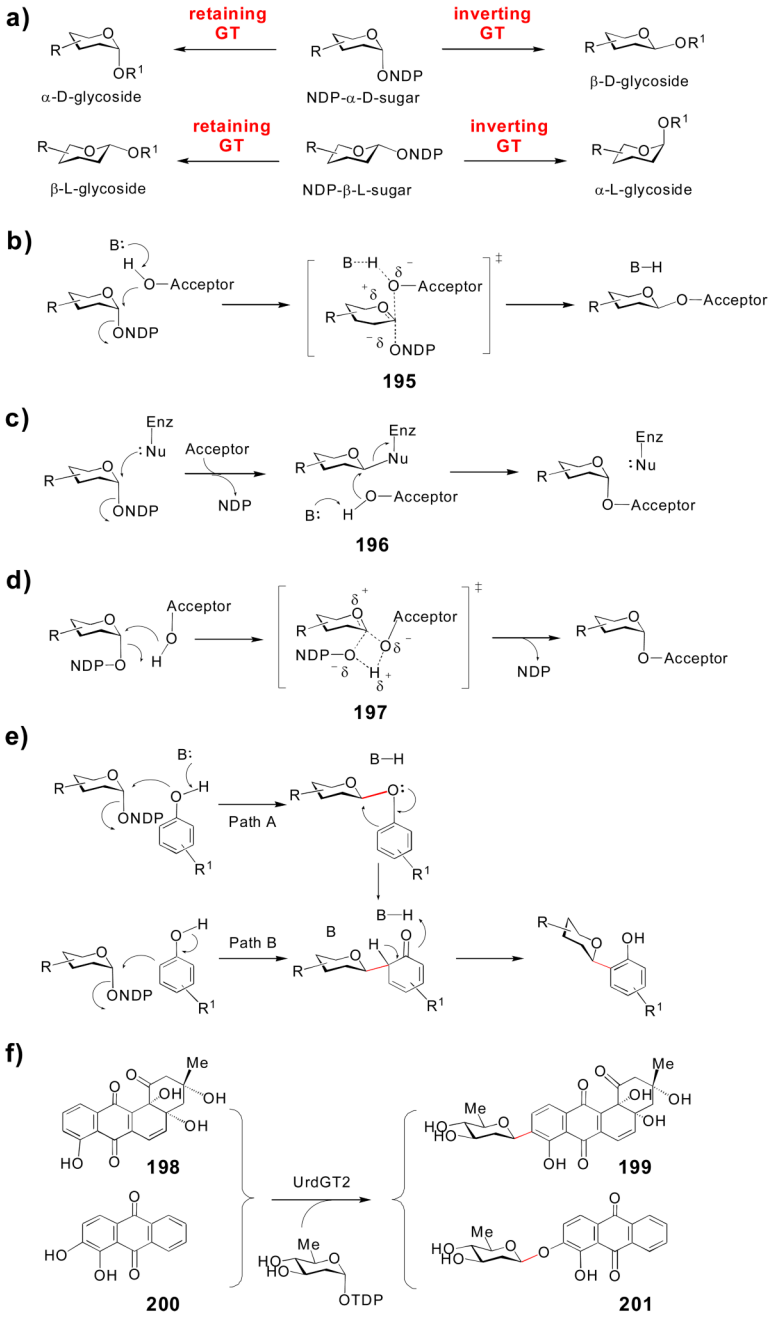


Figure 5. Mechanisms of glycosyltransferases

a) Possible stereochemical outcomes of GT-catalyzed reactions. b) Direct displacement mechanism proposed for inverting GTs. c) Double displacement mechanism proposed for retaining GTs. d) Alternative mechanism for retaining GTs involving nucleophilic attack from the same face of the sugar molecule as leaving group departure. e) Proposed mechanisms for aryl-*C*-glycosidic bond formation. f) UrdGT2 catalyzes the formation of both *C*- and *O*-glycosidic linkages (**198** → **199** and **200** → **201**, respectively).

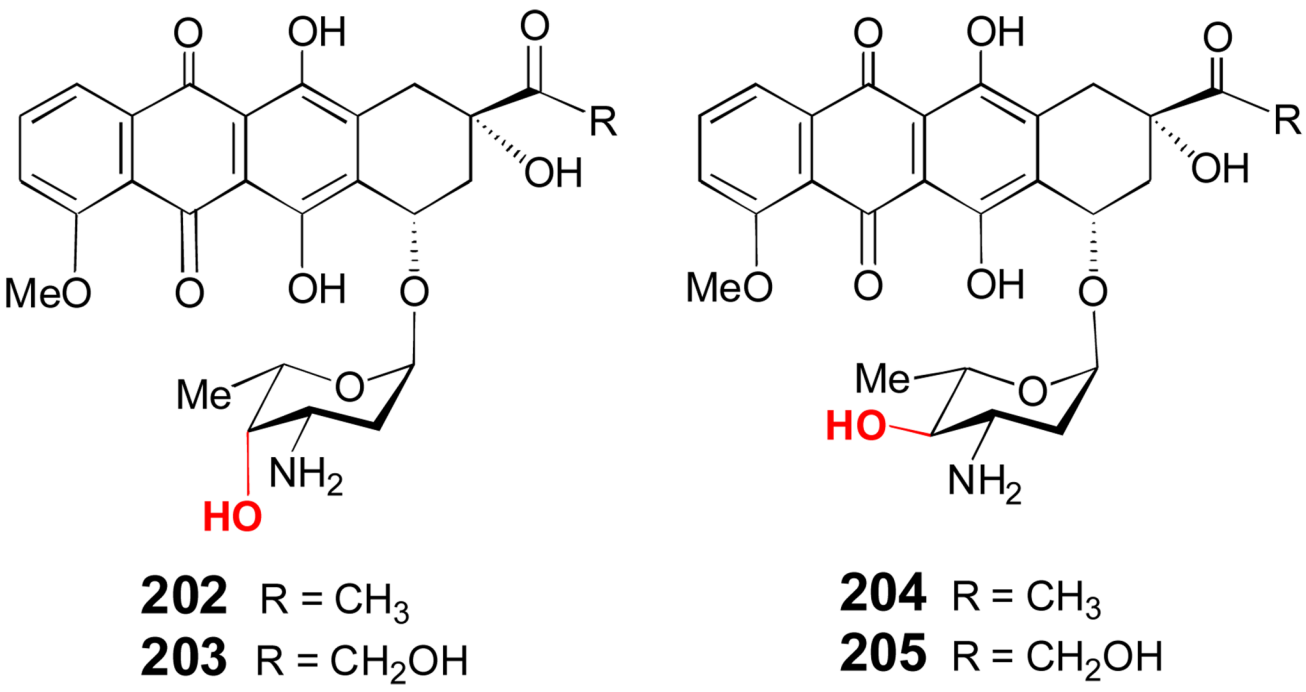


Figure 6. Designed synthesis of an unnatural sugar in *Streptomyces* peucetius

The axial C4-OH stereochemistry (highlighted in red) of the daunosamine moiety of daunorubicin (**202**) and doxorubicin (**203**) was altered to equatorial in **204** and **205** by the replacement of a single *S. peucetius* L-daunosamine biosynthetic gene (*dnmV*) with 4-ketoreductase genes from L-oleandrose (*avrE*) or L-mycarose (*eryBIV*) biosynthesis.

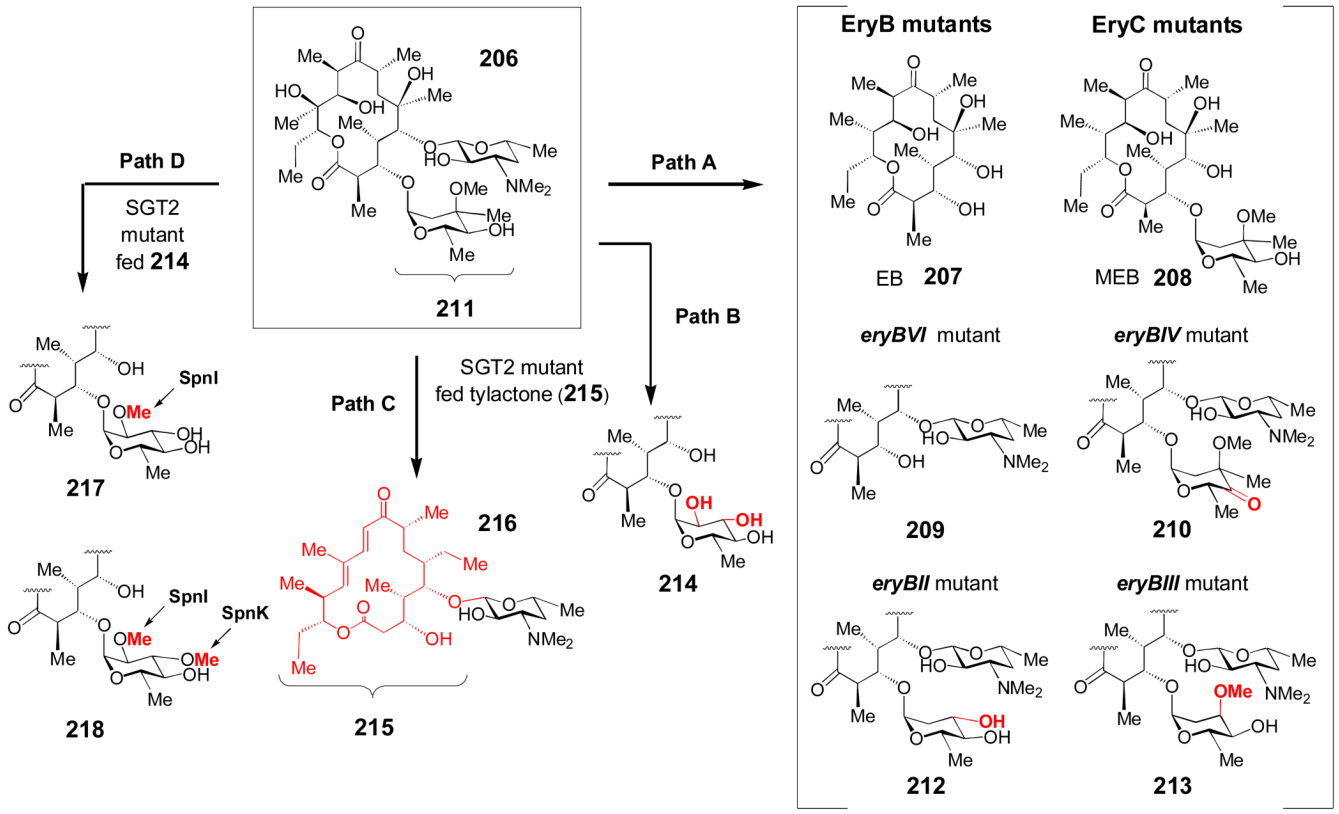


Figure 7. Engineering erythromycin derivatives in *Saccharopolyspora erythraea* with gene disruption, heterologous expression, and feeding experiments

Path A – Disruption of individual L-mycarose (*eryB*) or D-desosamine (*eryC*) biosynthetic genes afforded **207**–**210**, **212**, and **213**. Path B – Heterologous expression of the oleandrosyltransferase (OleG2) from *Streptomyces antibioticus* in an *S. erythraea* mutant lacking the endogenous mycarosyltransferase (EryBV). Path C – Expression of the mycaminosyl transferase (TylM2) from the tylosin pathway of *Streptomyces fradiae* in a triple *S. erythraea* mutant (SGT2) deficient in desosaminyltransfer (*eryCIII*−), mycarosyltransfer (*eryBV*−), and polyketide synthesis (*eryA*−), generated **216** when the strain was fed tylactone (**215**). Path D – Novel erythromycin derivates (**217** and **218**) generated when *O*-methyltransferase genes (*spnI* and *spnK*) from *Saccharopolyspora spinosa* were heterologously expressed in the *S. erythraea* SGT2 mutant.

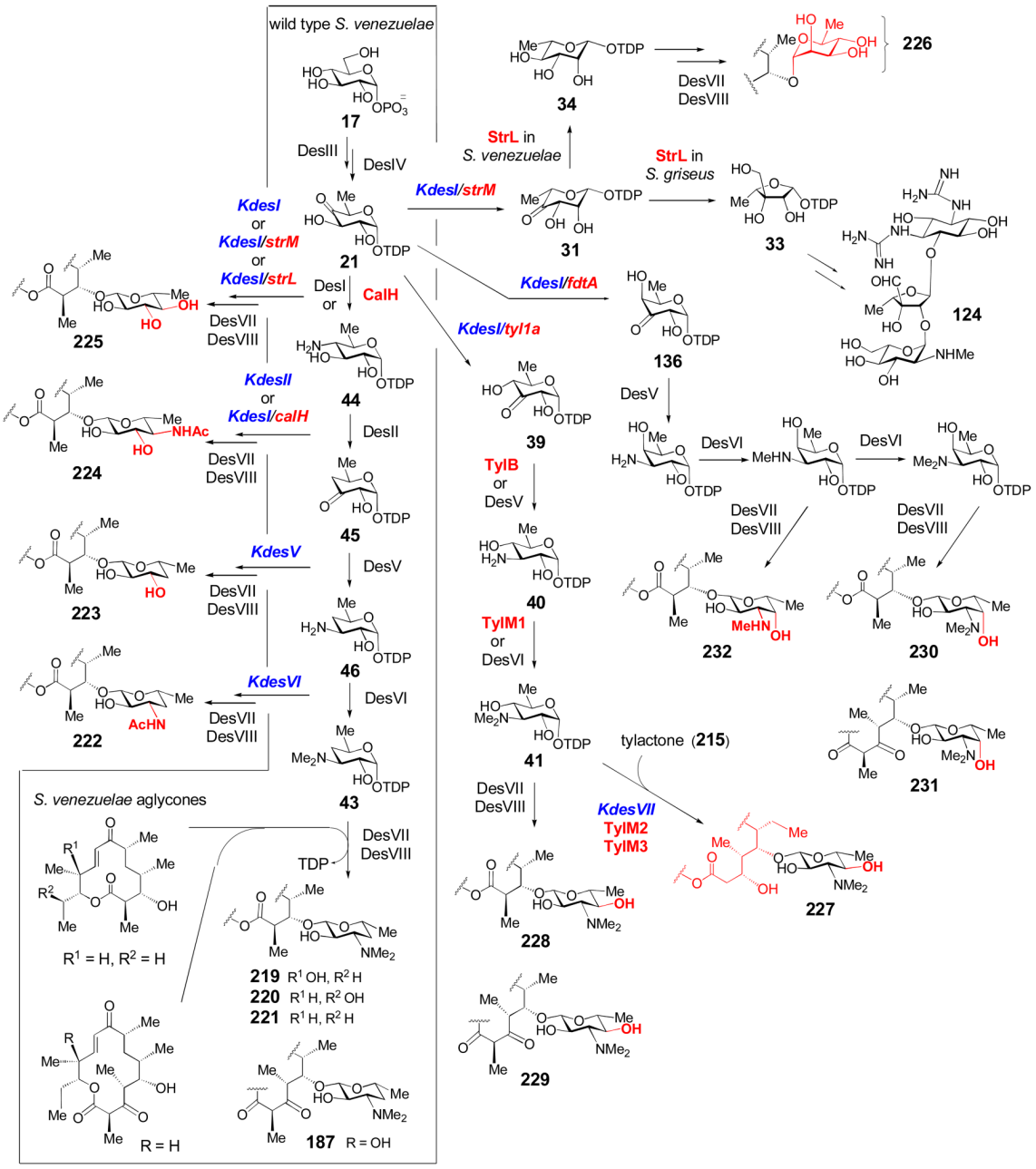


Figure 8. Metabolic pathway engineering in the methymycin/pikromycin producer *Streptomyces venezuelae*

The natural pathway for the biosynthesis of TDP-D-desosamine (**43**) and the glycosylated methymycin/pikromycin derivatives (**187**, **219**–**221**) produced by *S. venezuelae* are shown in the boxed pathway. Disruption of individual *des* genes (highlighted in blue), combined with heterologus expression of foreign genes (highlighted in red) was used to engineer a variety of novel, glycosylated macrolide derivatives (**222**–**232**).

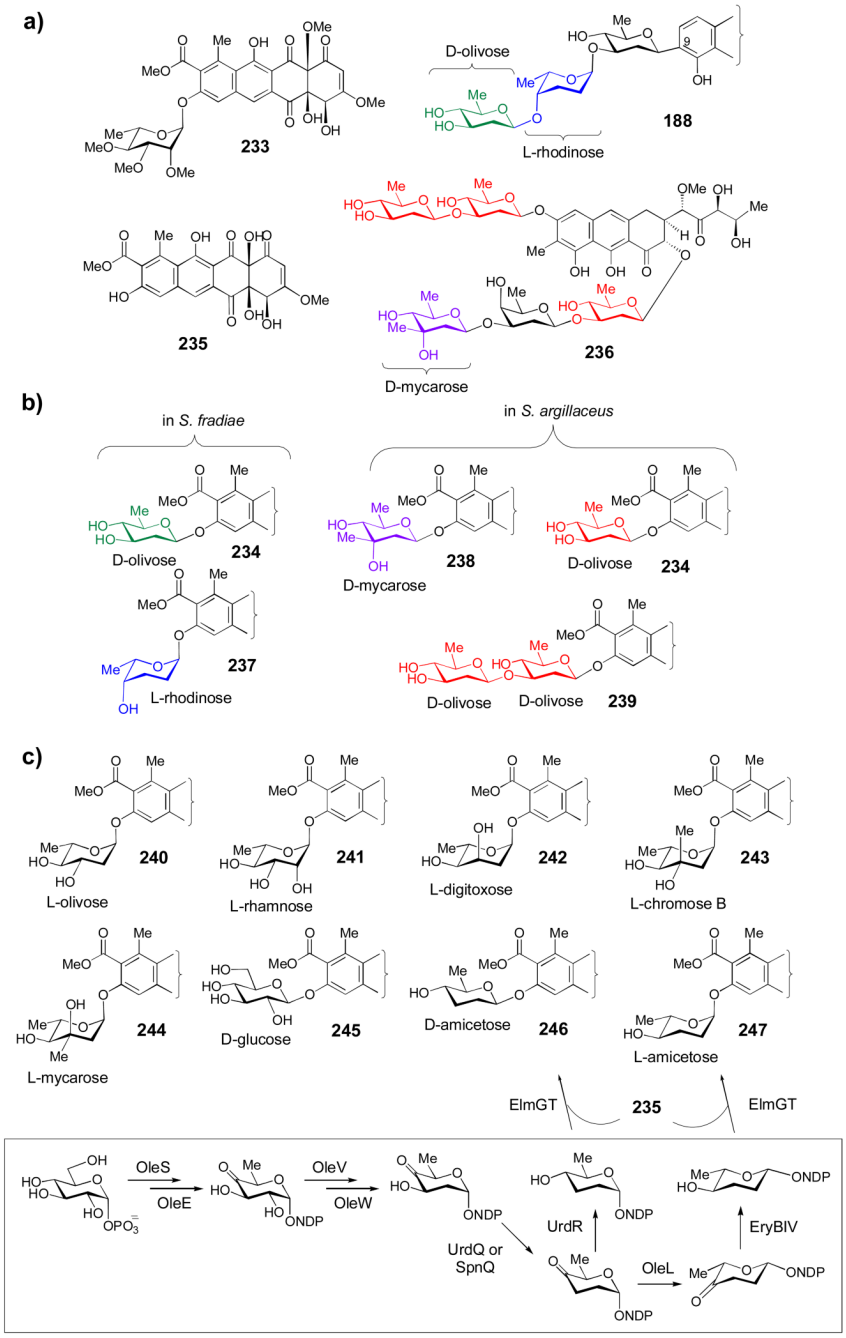
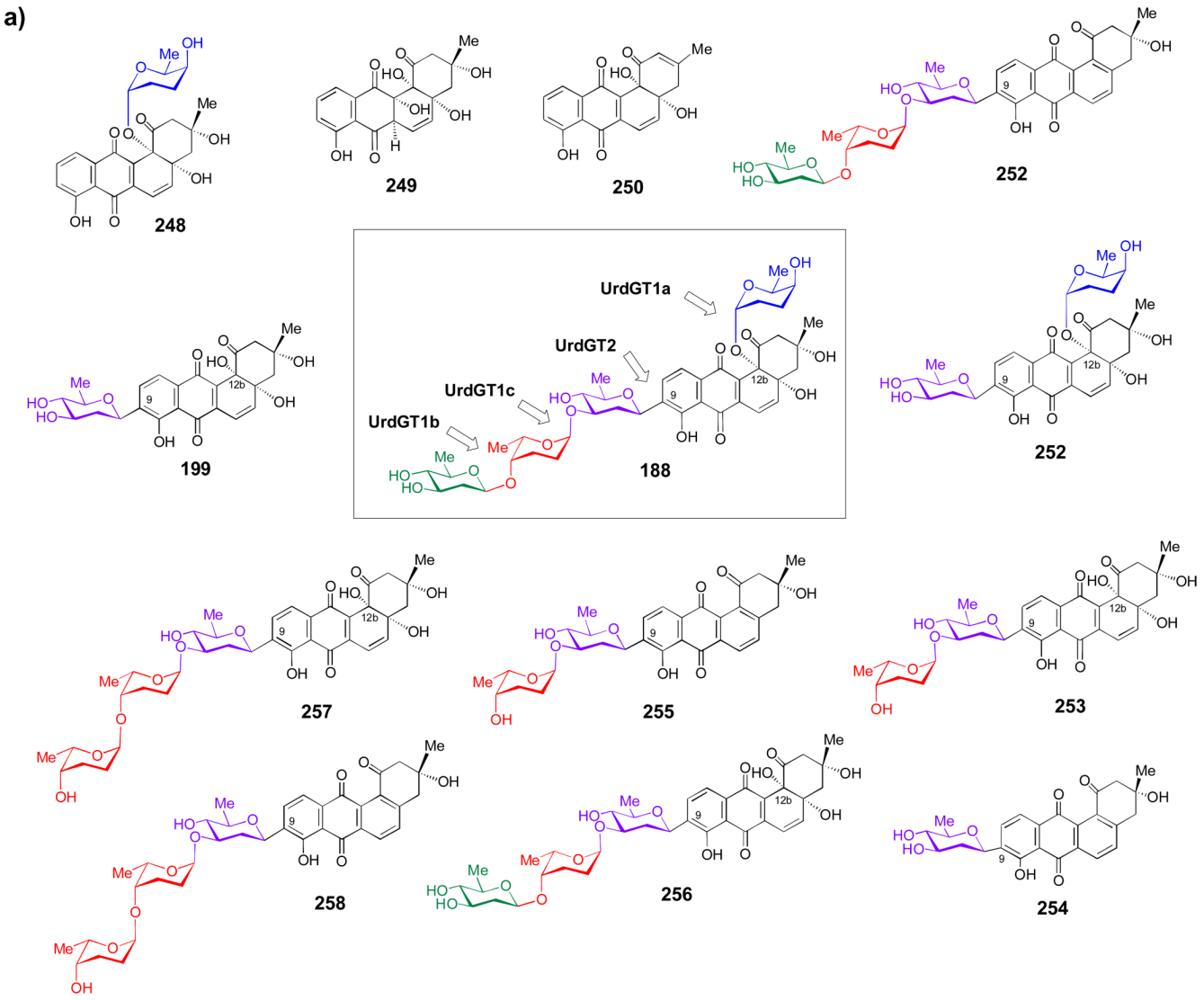


Figure 9. Relaxed NDP-sugar substrate specificity of ElmGT, an L-rhamnosyltransferase involved in elloramycin biosynthesis in *Streptomyces olivaceus*

a) Several naturally occurring aromatic polyketides: urdamycin A (**188**) produced by *Streptomyces fradiae*, elloramycin (**233**) produced by *Streptomyces olivaceus*, mithramycin (**236**) produced by *Streptomyces argillaceus* and the 8-DMTC aglycone (**235**) encoded by the *Streptomyces olivaceus* cosmid 16F4. b) Hybrid aromatic polyketides produced in vivo by the action of ElmGT expressed from cosmid 16F4 in the heterologous hosts *Streptomyces fradiae* (compounds **234** and **237**) and *Streptomyces argillaceus* (compounds **234**, **238**, and **239**). c) Novel aromatic polyketides produced through combinatorial biosynthesis (see text for details).



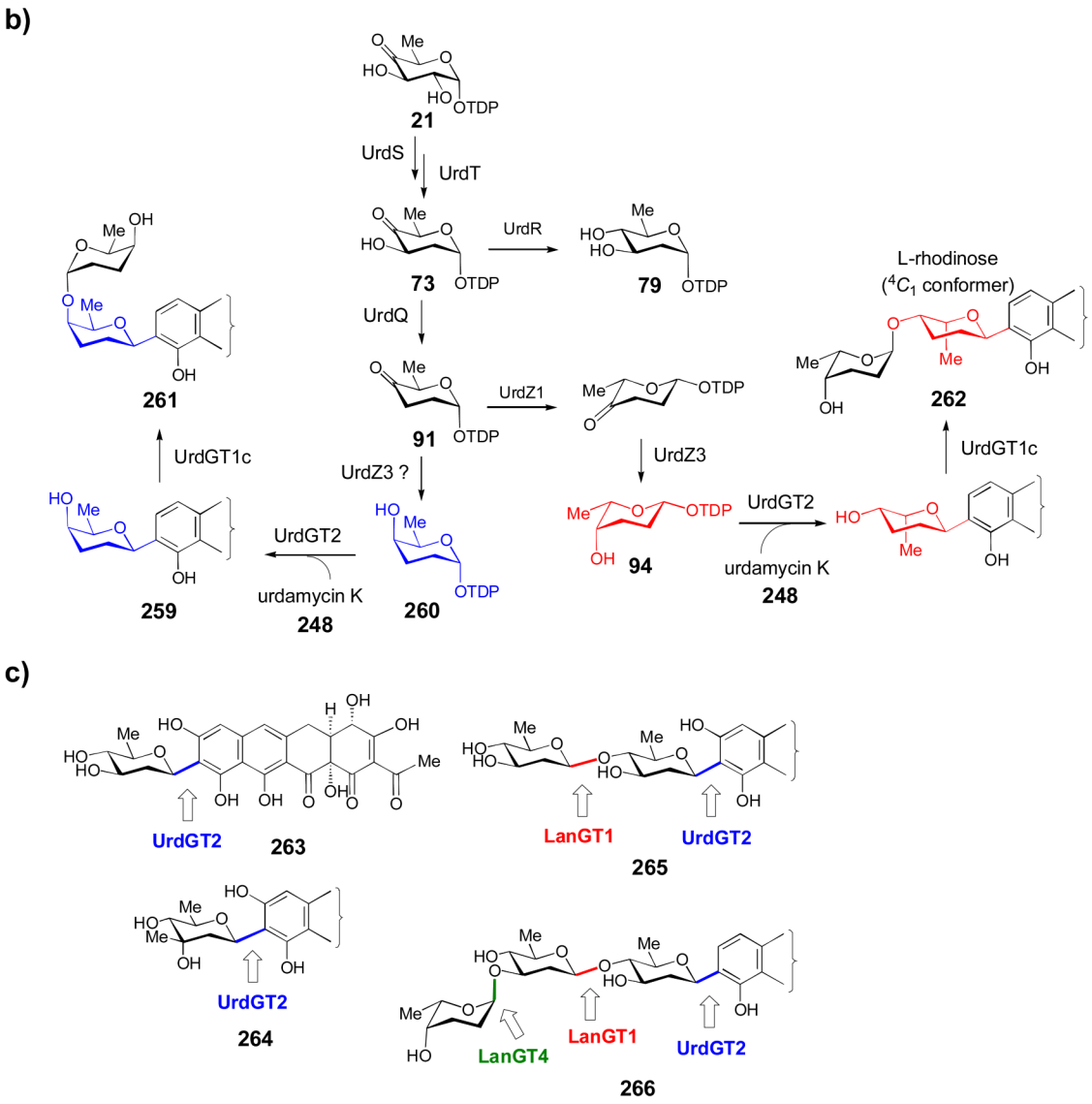


Figure 10. Manipulating urdamycin biosynthesis

a) Acceptor substrate flexibility of the urdamycin GTs revealed in various *Streptomyces fradiae* GT mutants. Different combinations of urdamycin A (**188**) GTs were disrupted, resulting in the production of various glycosylated derivatives (**199**, **248**–**258**) in the corresponding *S. fradiae* GT mutant strains. The sugar residues are color-coded to indicate which urdamycin GT is responsible for glycosyl coupling: blue – UdrGT1a, green – UrdGT1b, red – UrdGT1c, and purple – UrdGT2. b) Products isolated from *Streptomyces fradiae* Tü2717 upon disruption of deoxysugar biosynthetic genes. c) When expressed in glycosyltransferase-deficient mutants of the mithramycin producer *Streptomyces argillaceus*, UrdGT2 catalyzed production of *C*-glycosides (**263** and **264**). Heterologous expression of both UrdGT2 and the D-olivosyltransferase from the landomycin pathway of *Streptomyces cyanogenus* (LanGT1) in this same *S. argillaceus* mutant led to compound **265**. Expression of UrdGT2, LanGT1, and LanGT4 in a glycosyltransferase deficient mutant of *S. fradiae* Tü2717, led to the production of **266**.

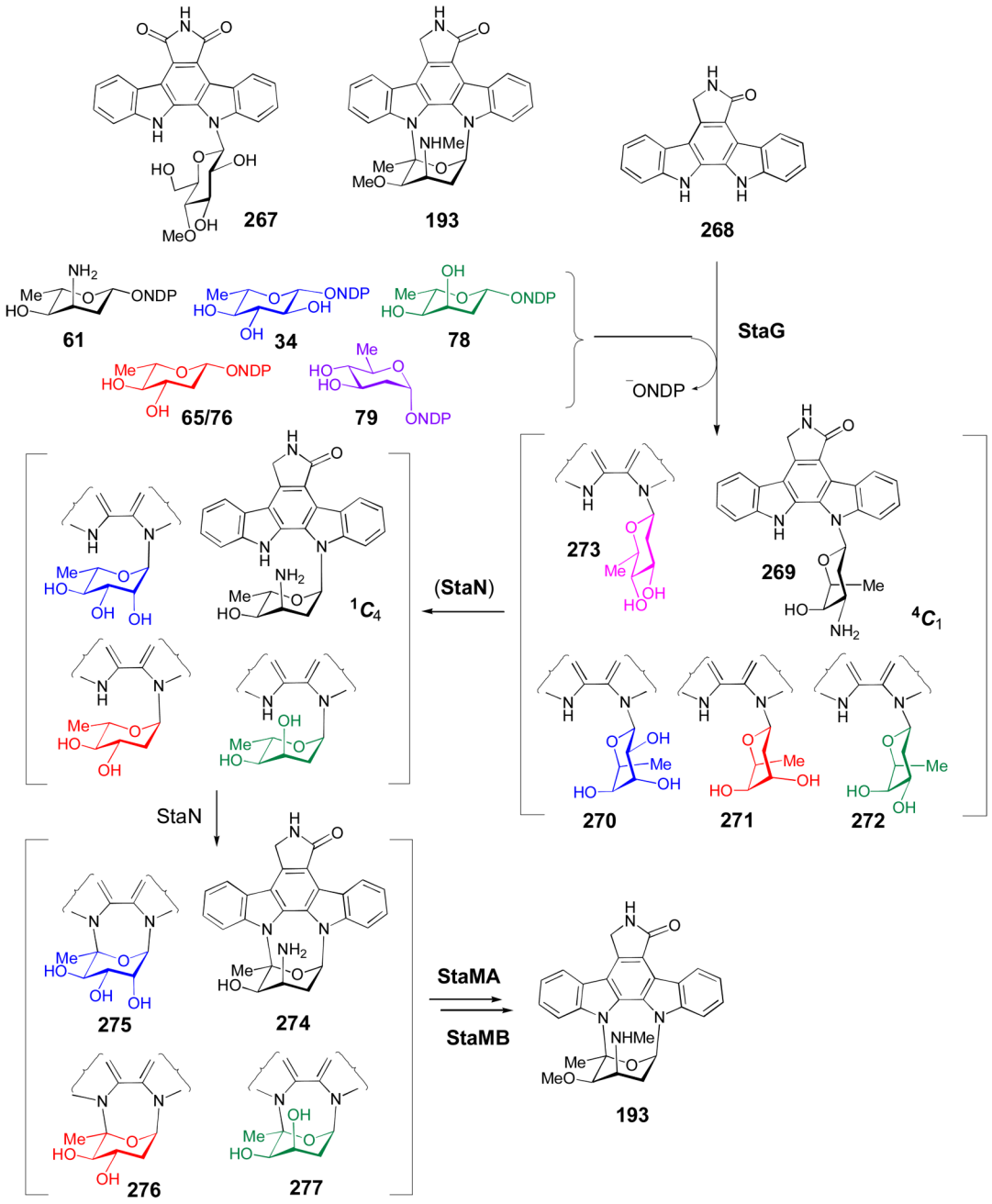


Figure 11. Novel indolocarbazoles generated by combinatorial biosynthesis in *Streptomyces albus*

The indolocarbazoles rebeccamycin (**267**) and staurosporine (**193**) both contain unusual *N*-glycosidic linkages. Staurosporine biosynthesis was reconstituted in *Streptomyces albus* by expressing genes required for the formation of the staurosporine aglycone (**268**), genes encoding production of different deoxysugars (**34**, **61**, **65**, **78**, and **79**), along with the *N*-GT (StaG) and the P450 enzyme (StaN) responsible for the oxidative crosslinking between C5′ of the sugar and the N-12 atom of the aglycone. While StaG coupled both L- and D-sugars to **268**, only the L-sugars could be oxidatively crosslinked by StaN to give **193** and **274**–**277**.

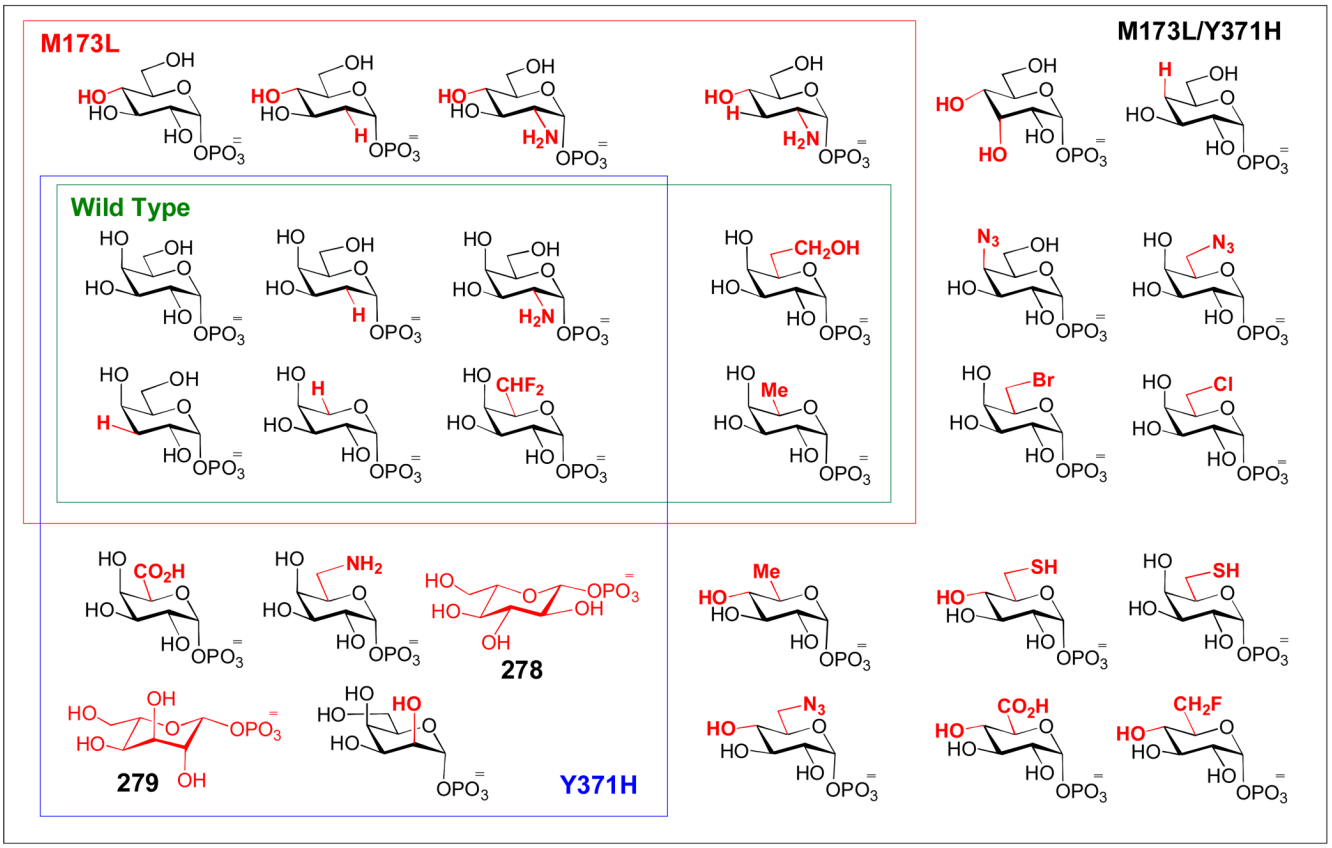


Figure 12. Sugar-1-phosphates produced by wild type and engineered *E. coli* galactokinase (GalK) mutants

The substrate specificity of wild type GalK was broadened by mutation of active site methionine (M173L) and tyrosine (Y371H) residues. The M173L/Y371H double mutant retained the substrate specificity of each single mutant and also accepted a variety of other sugars. The sugar-1-phosphates generated by each enzyme are boxed and the structural deviations from the wild type GalK substrate (D-galactose) are highlighted in red.

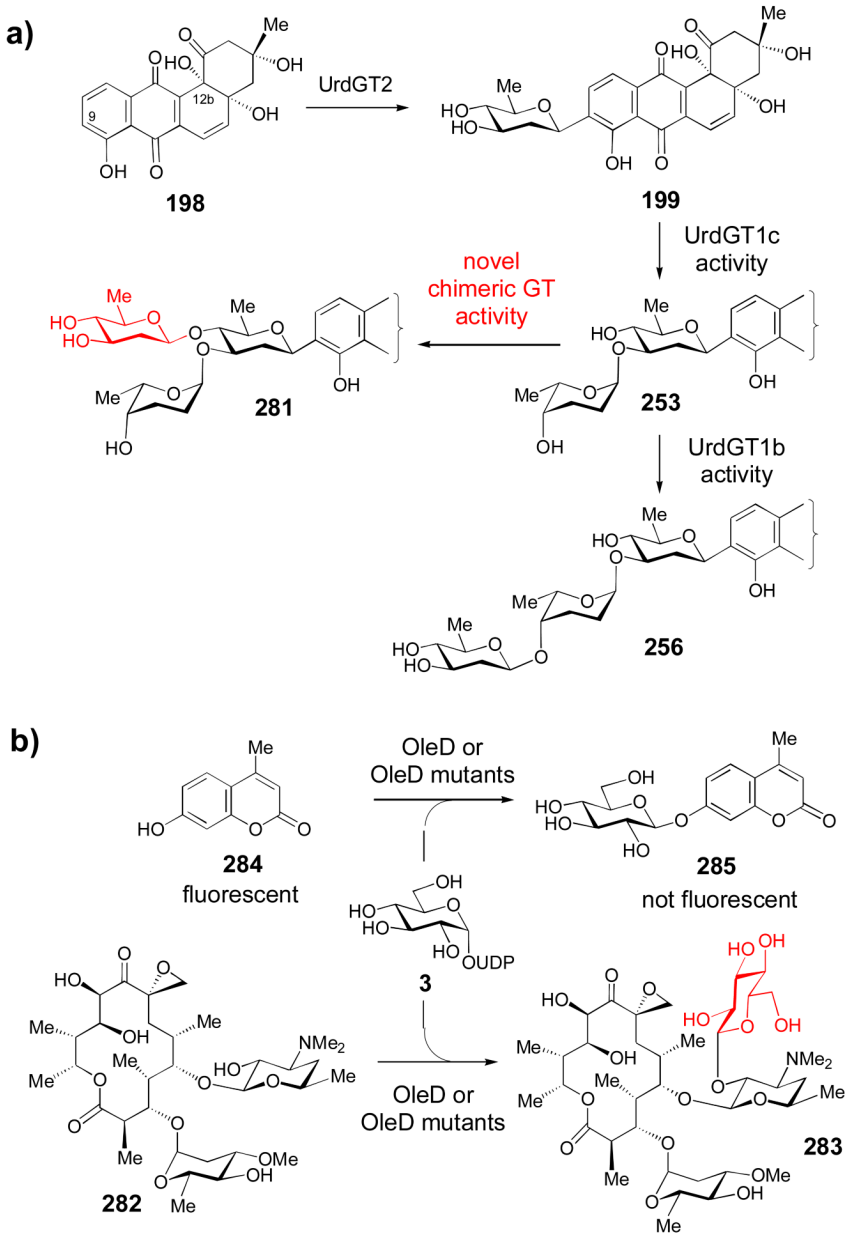


Figure 13. Protein engineering of glycosyltransferases

a) Novel activity of UrdGT1b/1c chimeras. The biosynthesis of the trisaccharide moiety of urdamycin A involves the tandem action of UrdGT2, UrdGT1c, and UrdGT1b (**198** → **199** → **253** → **256**). Several chimeric UrdGT1b/1c enzymes catalyzed a new reaction (**253** → **281**). b) High-throughput screening of GT activity. Random mutagenesis was used to create a library of OleD variants (a macrolide resistance GT that normally catalyzes **282** → **283**). The activity of these variants was then screened in a high-throughput fashion using the fluorescent acceptor **284**, whose fluorescence is quenched upon glycosyltransfer. Several active mutants were identified in this manner, some of which had broadened substrate specificity (see text for details).

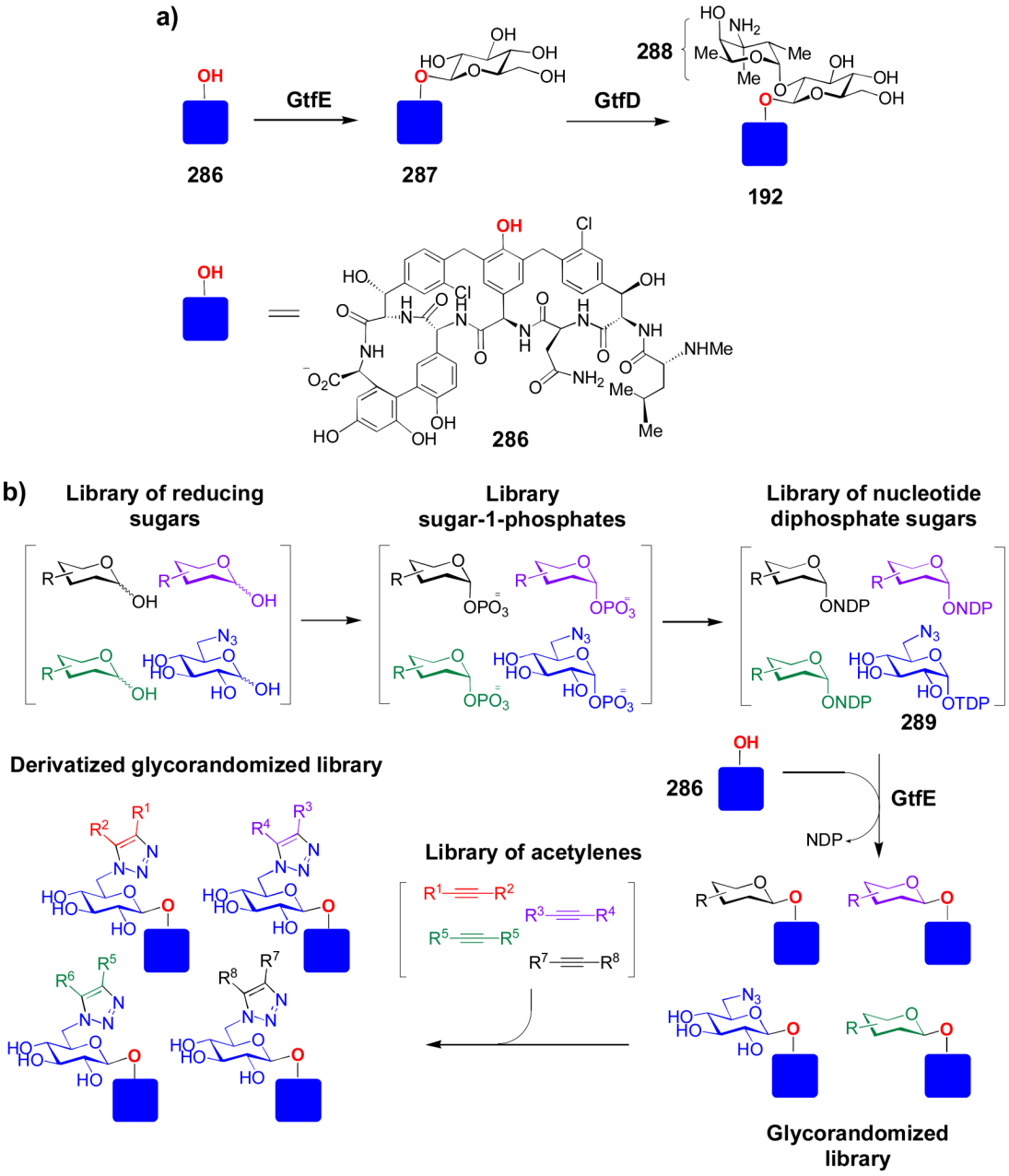


Figure 14. Chemoenzymatic glycorandomization of the vancomycin aglycone

a) The disaccharide moiety of vancomycin (**192**) is constructed by the tandem addition of D-glucose and L-vancosamine residues to **286** by the glycosyltransferases GtfE and GtfD, respectively. b) A library of reducing sugars was converted to a library of sugar-1-phosphates by either chemical synthesis or by incubation with engineered GalK mutants and ATP. This library was, in turn, converted into a library of NDP-sugars using engineered RmlA nucleotidylyltransferase. These NDP-sugars were then screened as substrates for GtfE in vitro, leading to a glycorandomized library of 21 vancomycin analogues. One of these analogues contained an azido sugar (see **289**) that could be further modified by a variety of acetylene compounds using the Cu^{I}-catalyzed Huisgen [3+2] cycloaddition to yield 39 additional vancomycin derivatives.

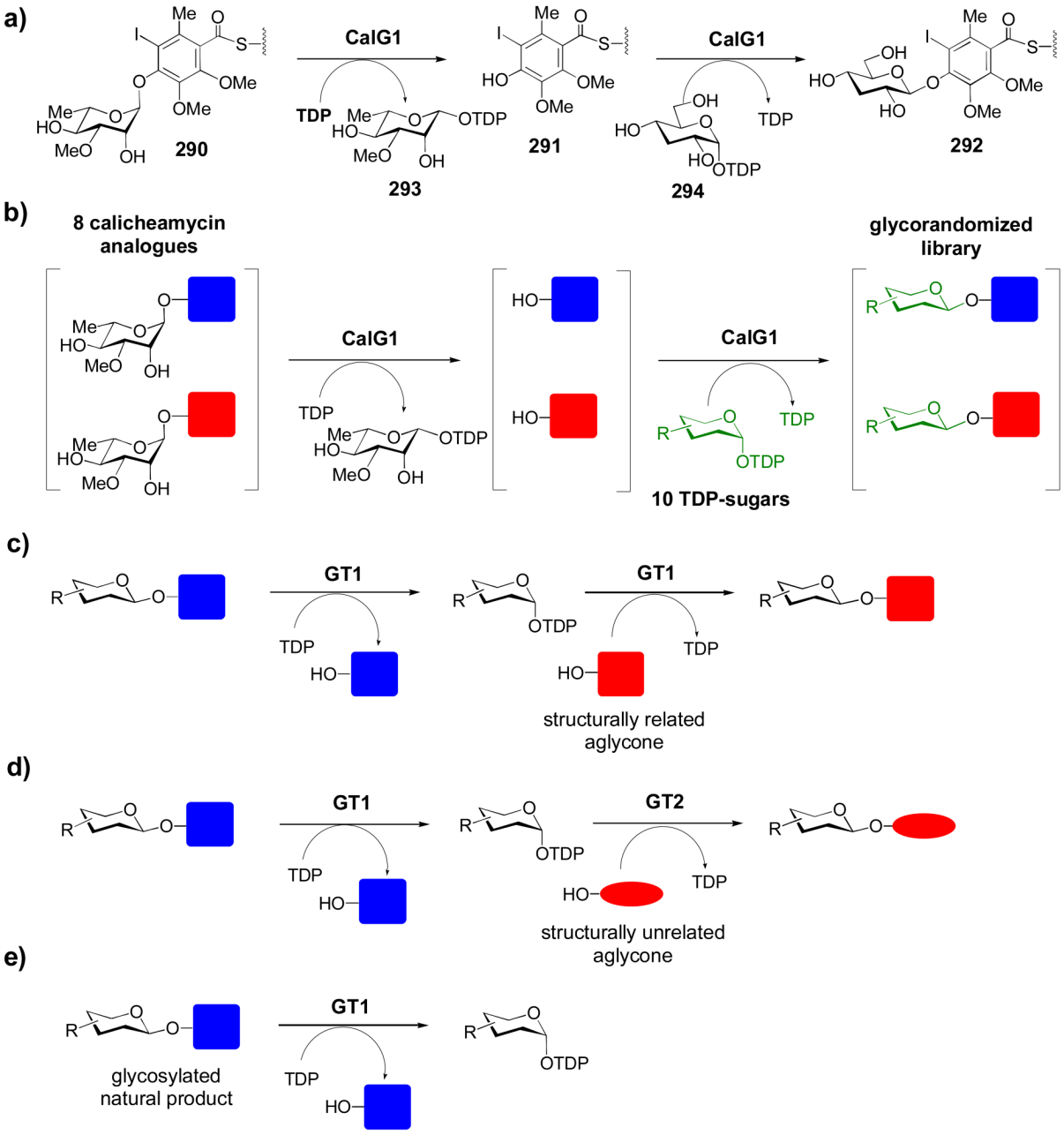
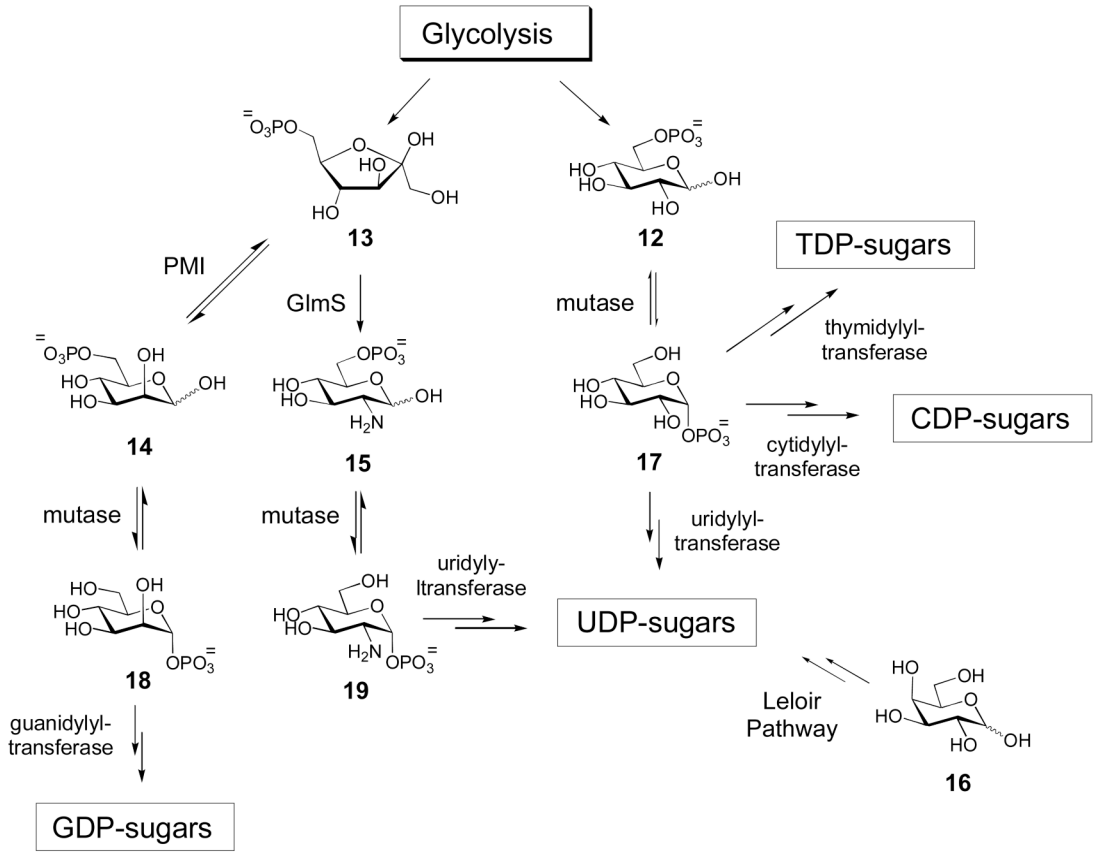


Figure 15. Exploiting GT reversibility in vitro

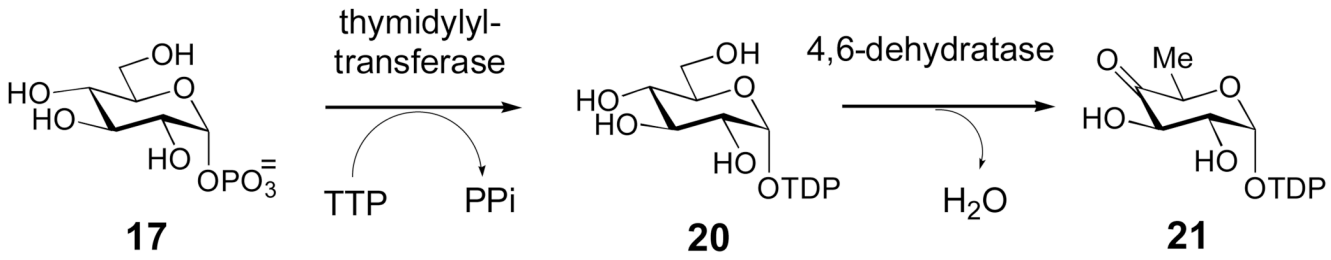
a) The calicheamicin GT, CalG1, was shown to synthesize both **293** and **291** from **290** in the presence of TDP through a reverse glycosyltransfer reaction. Alternative TDP-sugars (such as **294**) present in the reaction mixture could also be coupled to the aglycone (**291**) generated in situ to yield a new glycoside, **292**. b) CalG1 was used in sugar exchange reactions to rapidly glycorandomize 8 different glycosylated calicheamicin analogues with 10 different sugars to generate a library of 72 compounds. c) One-enzyme aglycone exchange reactions involve a single GT, which transfers a sugar moiety from one aglycone to a structurally similar aglycone. d) Using two enzymes that both recognize the same TDP-sugar, a sugar moiety can be moved

from one aglycone to a structurally unrelated aglycone. e) In the presence of excess TDP and a glycosylated natural product, reverse GT catalysis can be used to synthesize TDP-sugars.



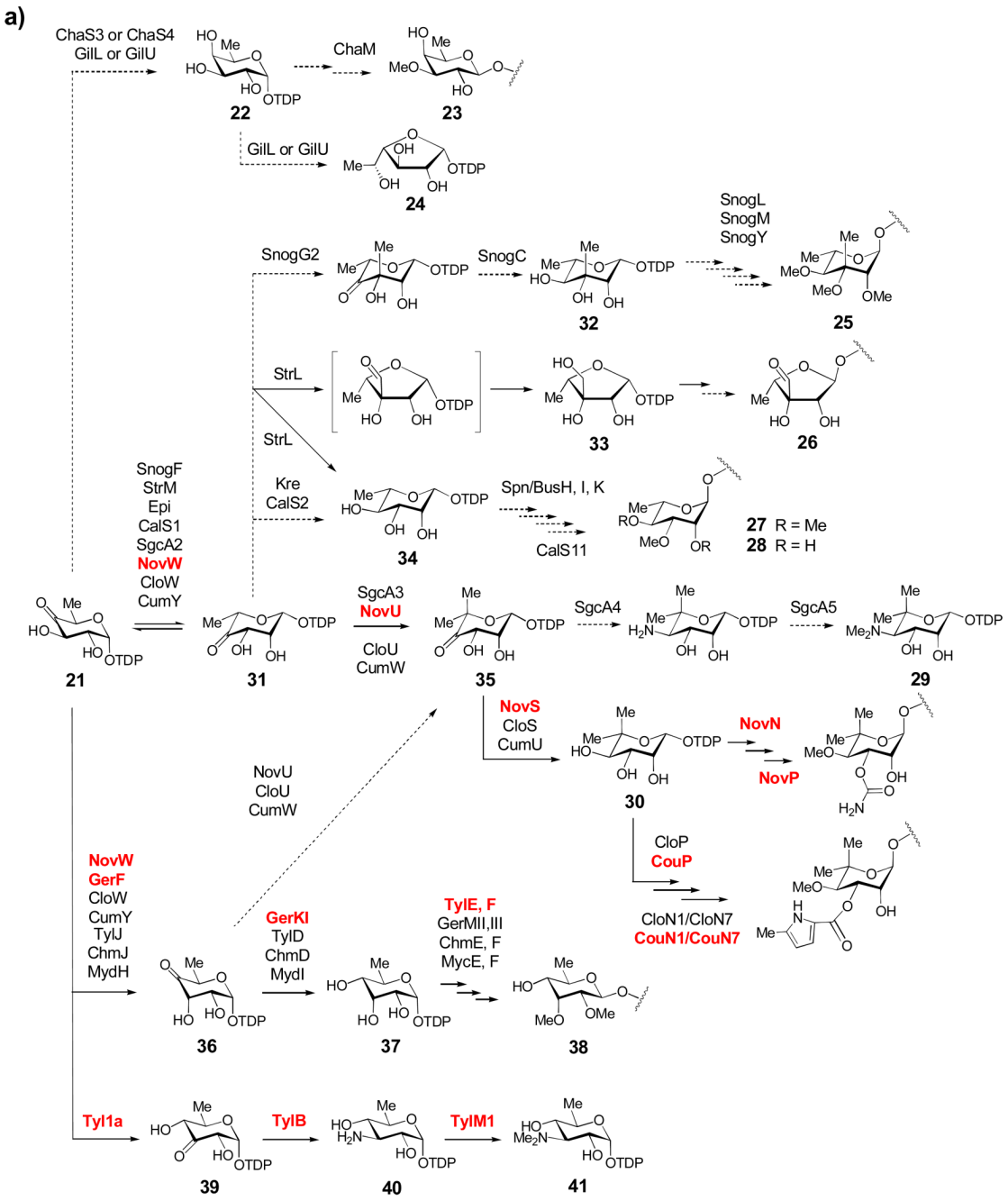
Scheme 1. Biosynthetic origins of NDP-sugars

Most NDP-sugars are derived from glycolytic intermediates glucose-6-phosphate (**12**) and fructose-6-phosphate (**13**) or from galactose (**16**). Eventually, all of these sugars are converted into sugar-1-phosphates, which can then be activated by the appropriate nucleotidylyltransferase.

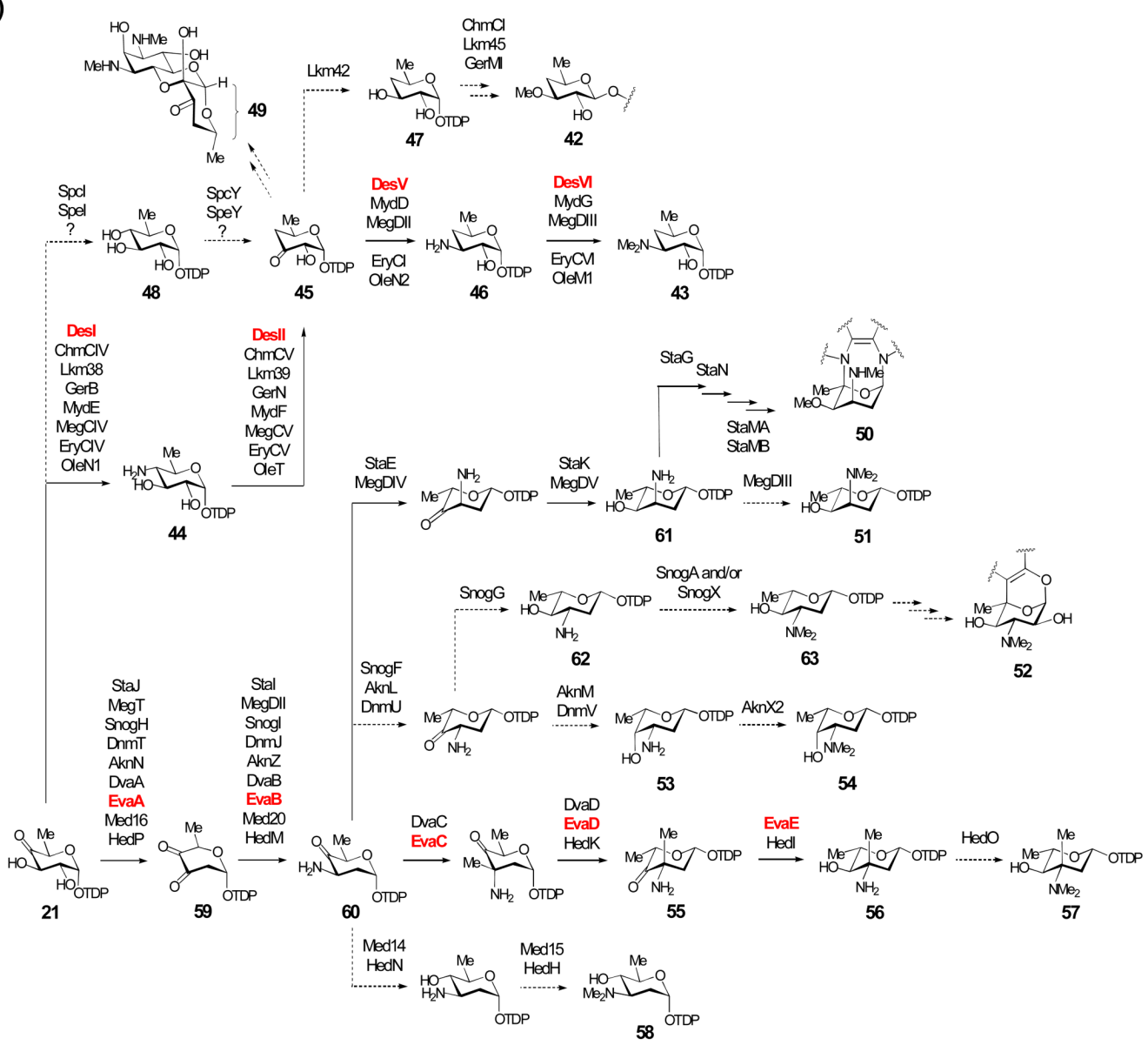


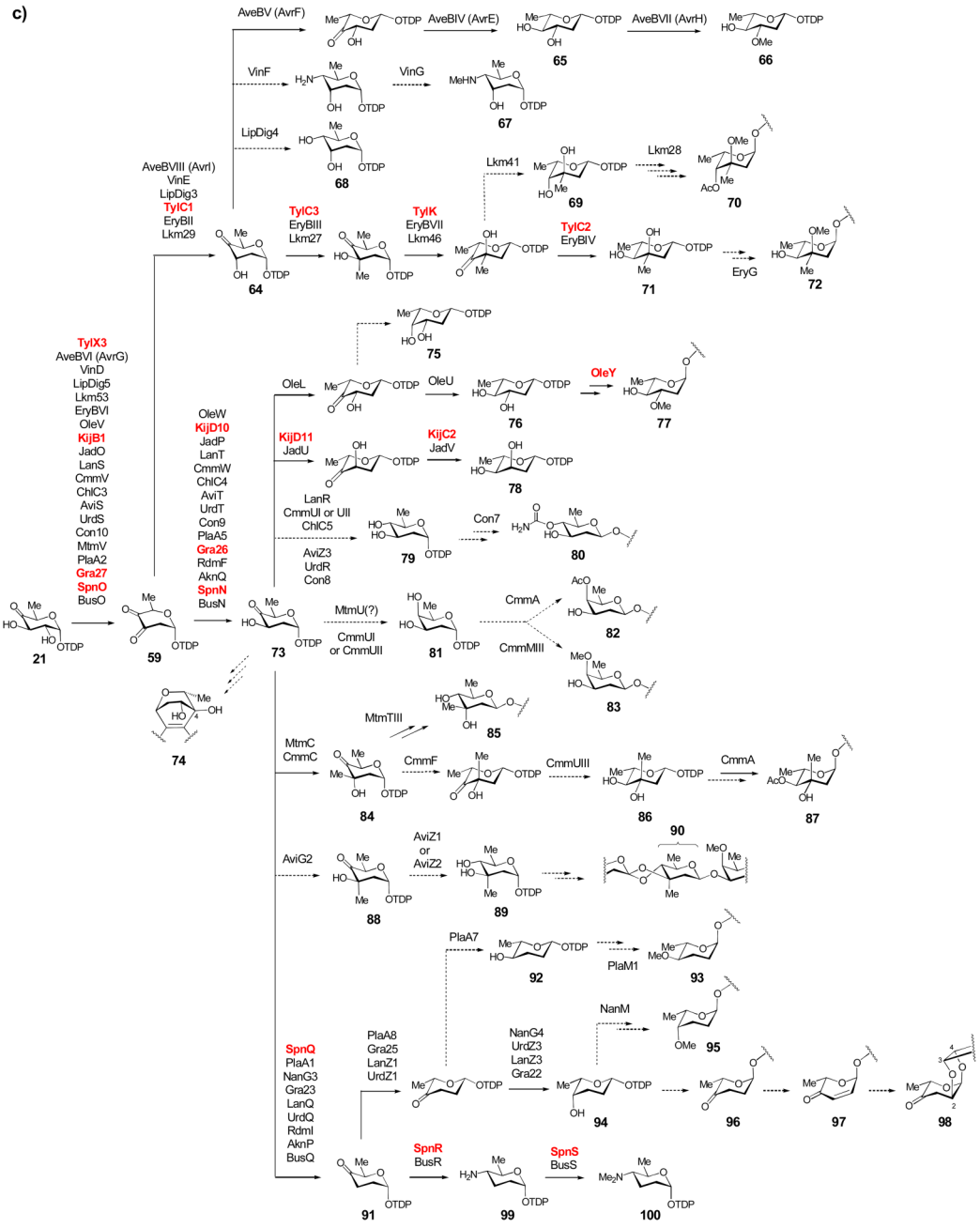
Scheme 2. Entry point into TDP-deoxysugar secondary metabolism in bacteria

Following thymidylylation of α-D-glucose-1-phosphate (**17**) by a thymidylyltransferase, a TDP-glucose-4,6-dehydratase enzyme catalyzes the conversion of TDP-D-glucose (**20**) to TDP-4-keto-6-deoxy-α-D-glucose (**21**) in the committed step to TDP-deoxysugar biosynthesis.



b)





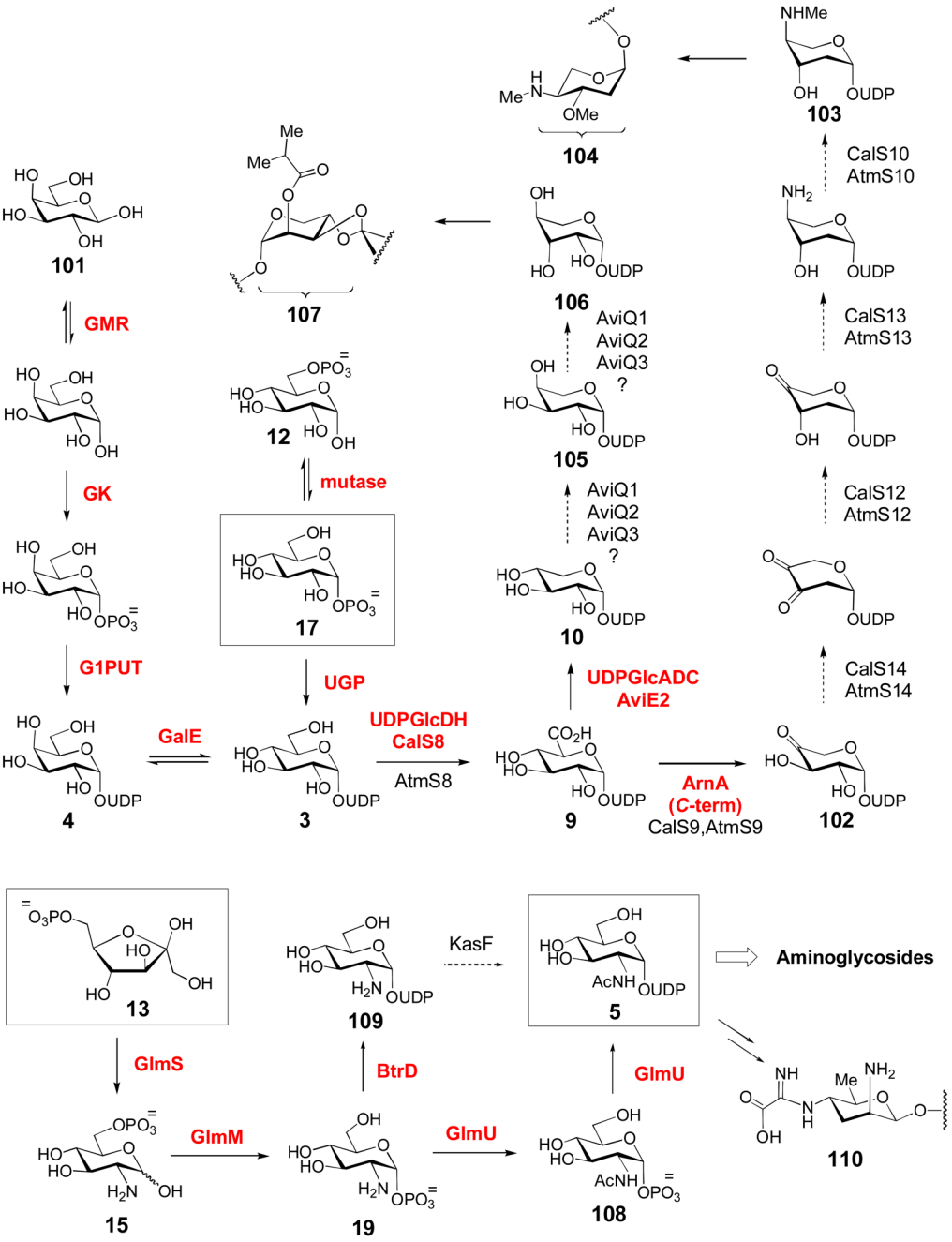
Scheme 3.

Scheme 3a: Biosynthesis of Group I TDP-sugars. This group includes 6-deoxysugars (such as **23–28**, **30** and **38**), as well as the 4-amino-4,6-dideoxysugar (**29**) and the 3-amino-3,6-dideoxysugar (**41**). From the common intermediate TDP-4-keto-6-deoxy-α-D-glucose (**21**), most of the TDP-sugars in this group share an epimerization step (**21** → **31**) early in their biosynthetic pathways. Solid arrows indicate enzyme-catalyzed reactions that have been verified either in vitro through biochemical experiments with purified enzymes or in vivo through gene disruption/heterologous expression experiments. Dashed arrows indicate reactions that have not been experimentally verified, but have been proposed based on

comparison of gene sequences to genes of known function. Names in red indicate enzymes whose functions have been verified biochemically using purified enzymes.

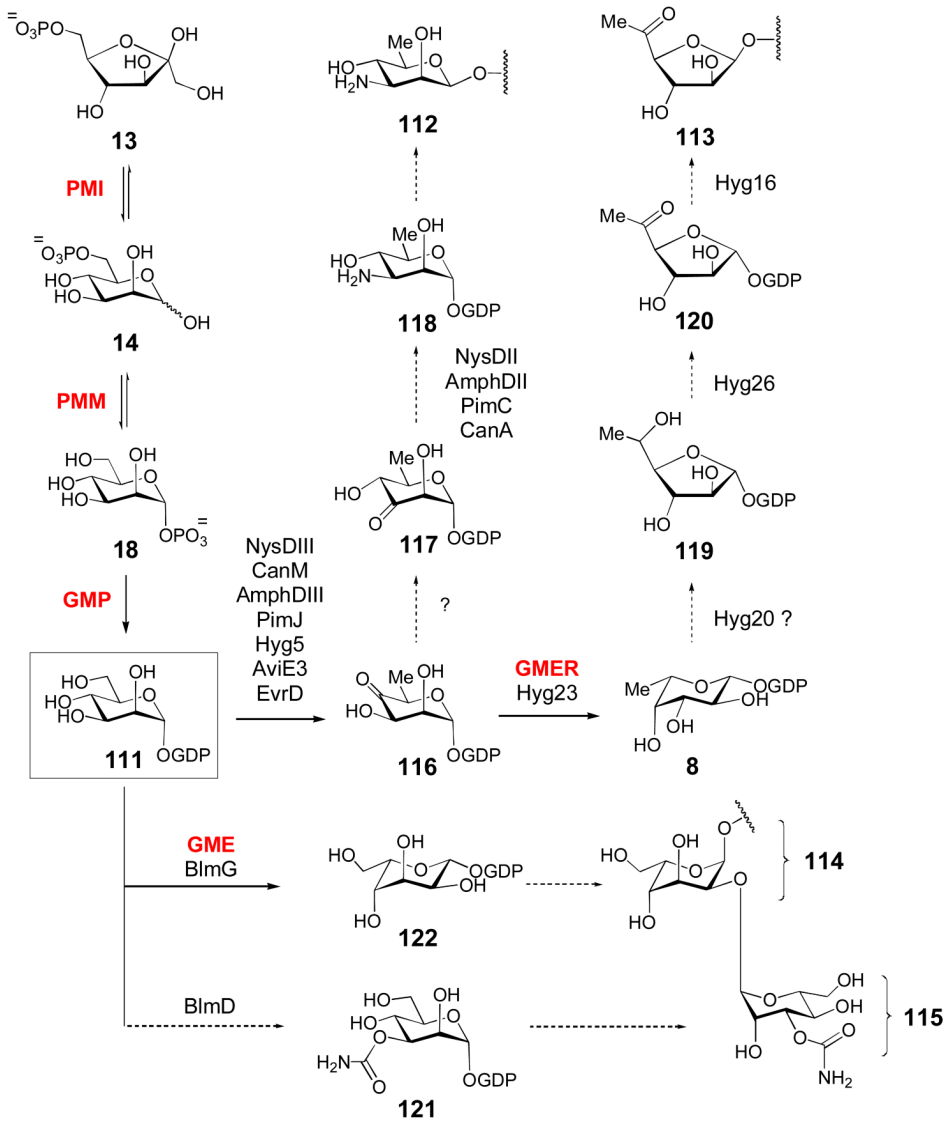
Scheme 3b: Biosynthesis of Group II TDP-Sugars. The extremely rare TDP-4,6-dideoxysugars include TDP-D-desosamine (**43**), TDP-D-chalcomycin (**47**), and actinospectose (**49**). The majority of sugars in Group II are 3-amino-2,3,6-trideoxysugars (**50**–**58** and **60**–**63**) that share a common 2-dehydration/3-aminotransfer reaction sequence (**21** → **59** → **60**).

Scheme 3c: Biosynthesis of Group III TDP-sugars. The largest group of TDP-deoxysugars each share 2-dehydration (**21** → **59**) and 3-ketoreduction steps (**59** → **64** or **59** → **73**) early in their respective biosynthetic pathways. TDP-sugars derived from **64** are proposed to include **66**–**72**. TDP-sugars proposed to be derived from **73** include numerous 2,6-dideoxysugars (see **75**–**90**) as well as 2,3,6-trideoxysugars (see **91**–**98**), and a TDP-4-amino-2,3,4,6-tetradeoxy sugar (**100**).



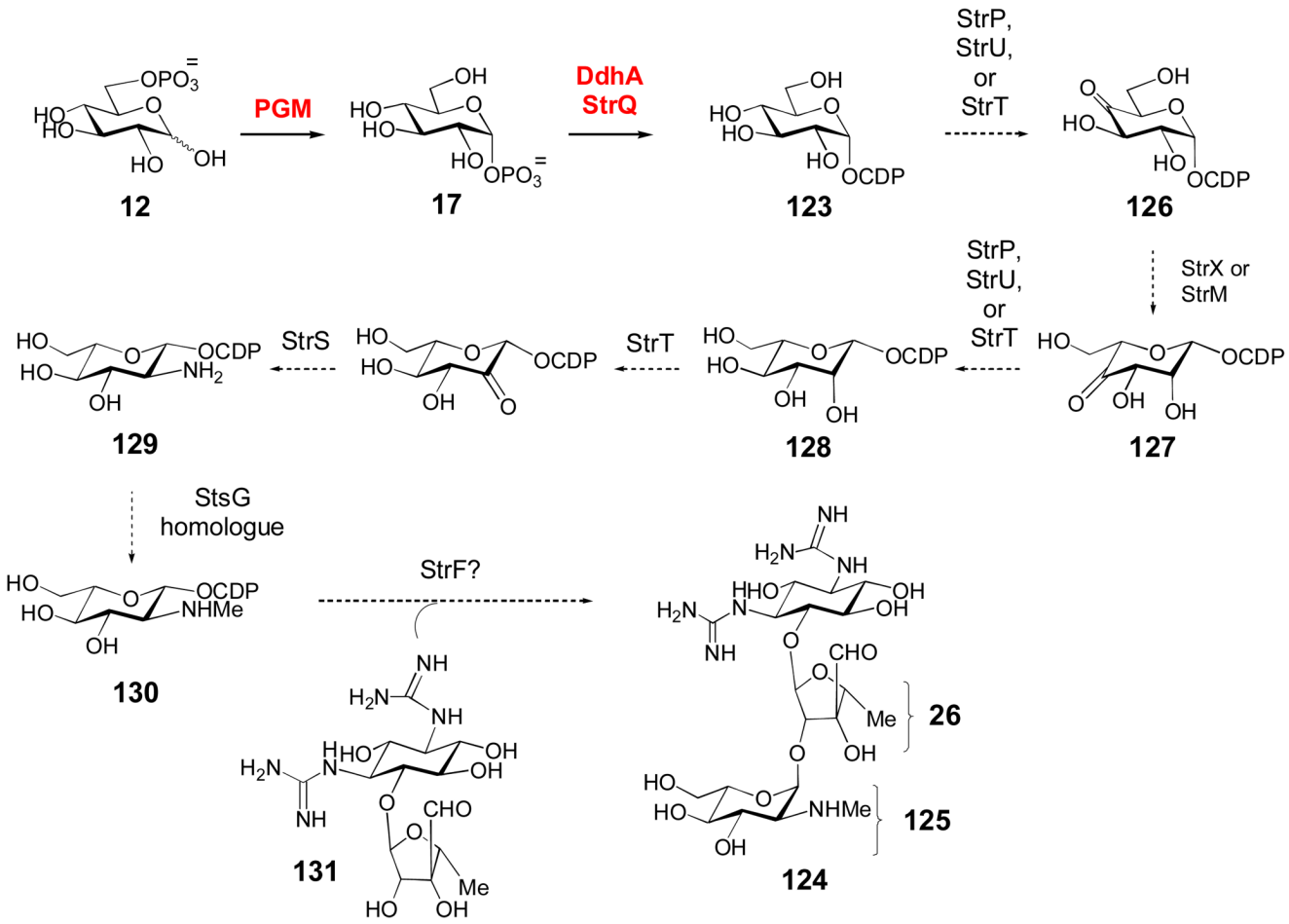
Scheme 4. Biosynthesis of UDP-sugars

a) Biosynthetically, UDP-α-D-glucose (**3**) is derived either from glycolytic intermediate **12**, or from β-D-galactose (**101**) via the Leloir pathway (see text for details). The pentose moieties of calicheamicin (**104**) and avilamycin (**107**) are likely derived from a UDP-α-D-glucose (**3**) precursor. A separate group of UDP-sugars are derived from the glycolytic intermediate fructose-6-phosphate (**13**) via UDP-*N*-acetyl-D-glucosamine (**5**, see text for details). Compound **5** is likely the biosynthetic precursor of many aminoglycoside sugars, many of which are 2-aminosugars (see Figure 2).



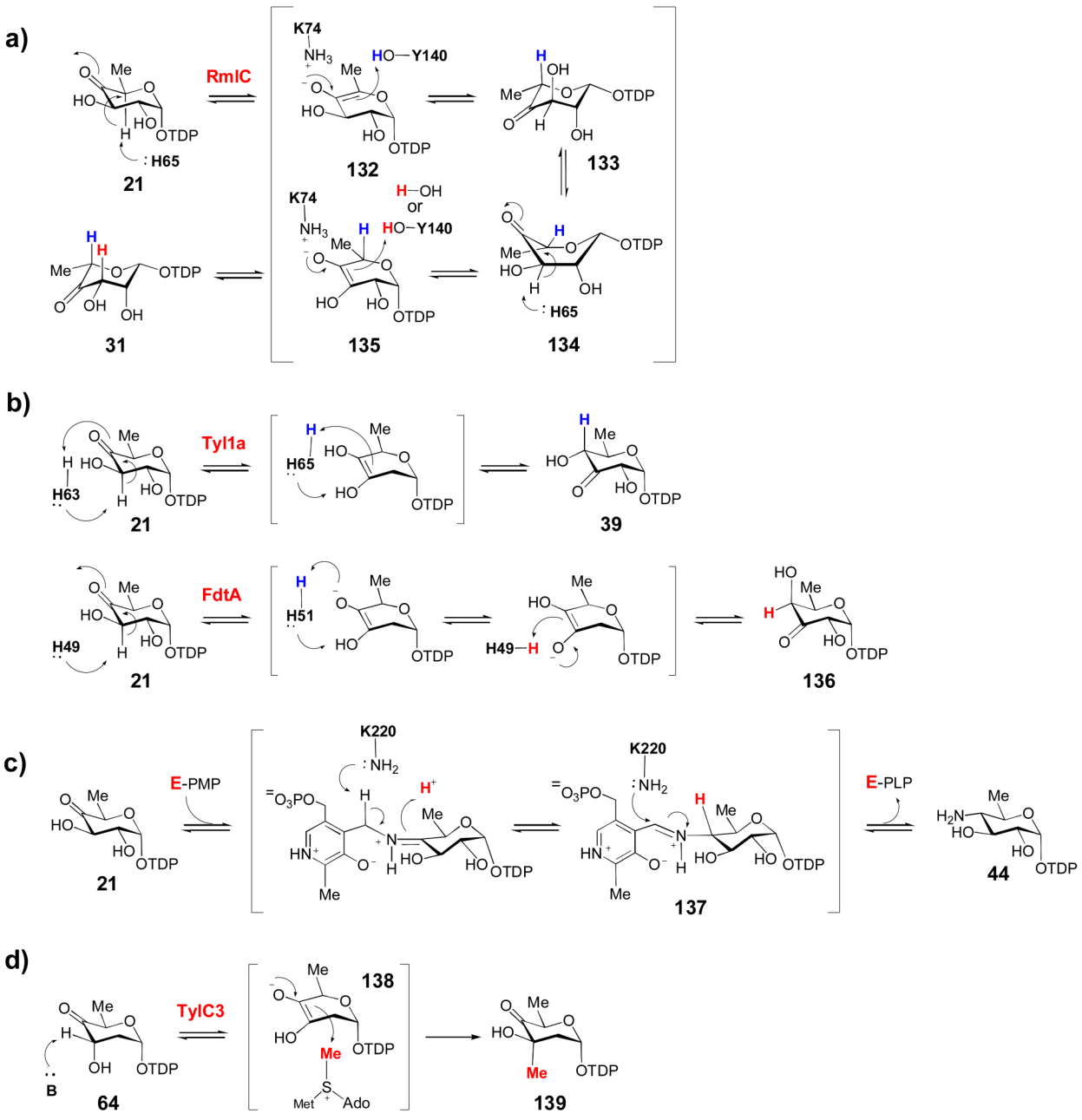
Scheme 5. Biosynthesis of GDP-sugars

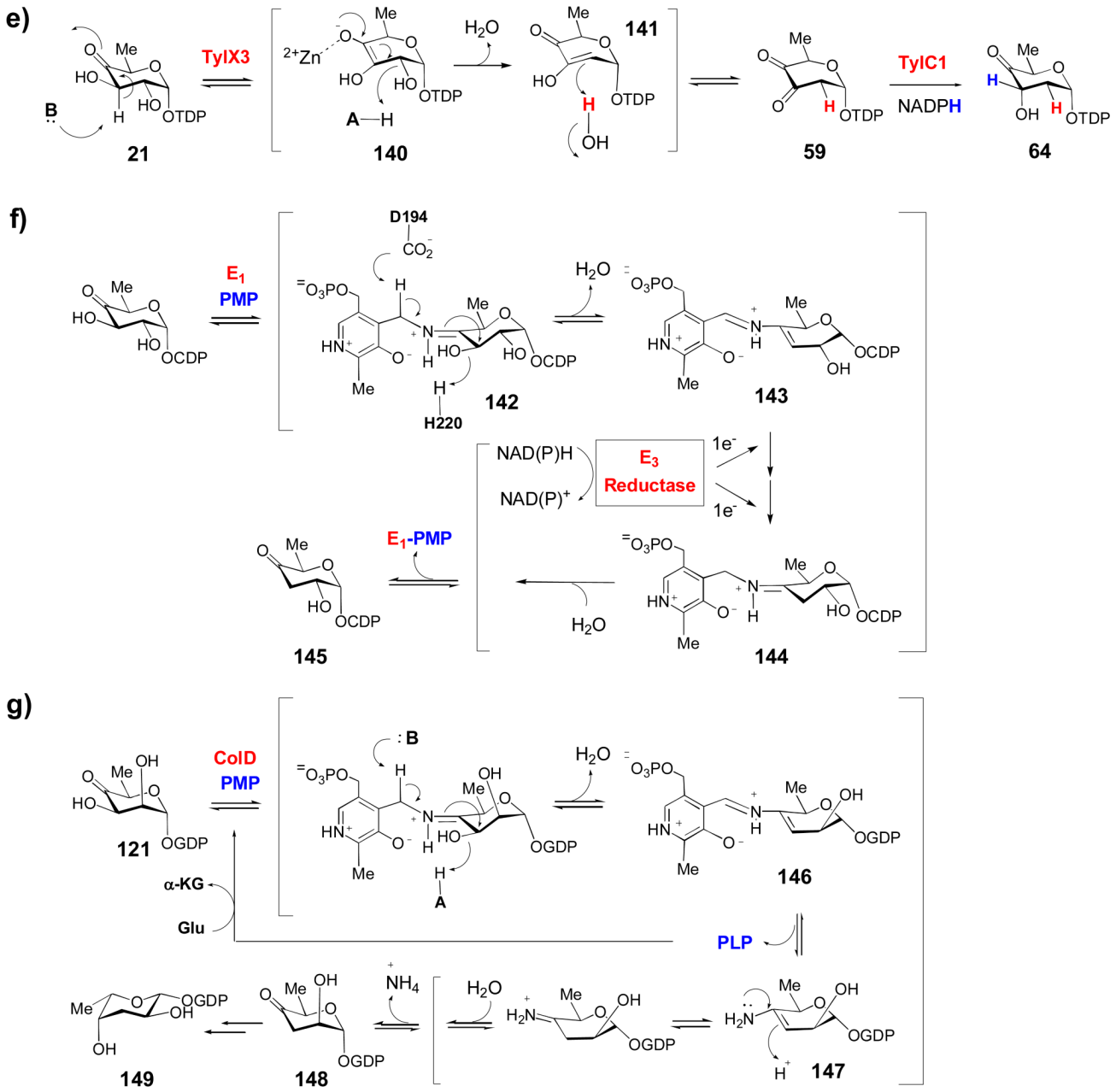
GDP-sugars are derived from GDP-α-D-mannose (**111**), which is in turn derived from fructose-6-phosphate (**13**, see text for details). The biosynthetic gene clusters for nystatin (*nys*), amphotericin (*amph*), pimaricin (*pim*), candicidin (*can*), hygromycin A (*hyg*), avilamycin (*avi*), and evernimicin (*evr*) each encode putative GDP-mannose-4,6-dehydratase genes that are predicted to convert **111** to **116**. The hygromycin A cluster encodes a putative GDP-6-deoxy-4-keto-D-mannose-epimerase/reductase (GMER or GDP-fucose synthase) homologue (Hyg23) -enzymes which are known to convert **116** to **8**. The L-gulose (**114**) and 3-*O*-carbamoyl-D-mannose (**115**) residues of bleomycin are proposed to be synthesized from **111** via the GDP-α-D-mannose-3,5-epimerase (GME) homologue BlmG and the carbamoyltransferase BlmD, respectively.



Scheme 6. A putative biosynthetic pathway for CDP-L-glucosamine in *Streptomyces glaucescens*

The biosynthesis of the *N*-methyl-L-glucosamine moiety (**125**) of the aminoglycoside antibiotics streptomycin and bluensomycin is poorly understood. However, the cytidylyltransferase activity (**17** → **123**) of StrQ encoded by the streptomycin gene cluster of *Streptomyces glaucescens* was verified in vitro, suggesting that **125** may be derived from a CDP-sugar precursor. The absence of StrQ homologues in other streptomycin and bluensomycin clusters, however, suggests that different biosynthetic routes to **125** may exist (see text for details).

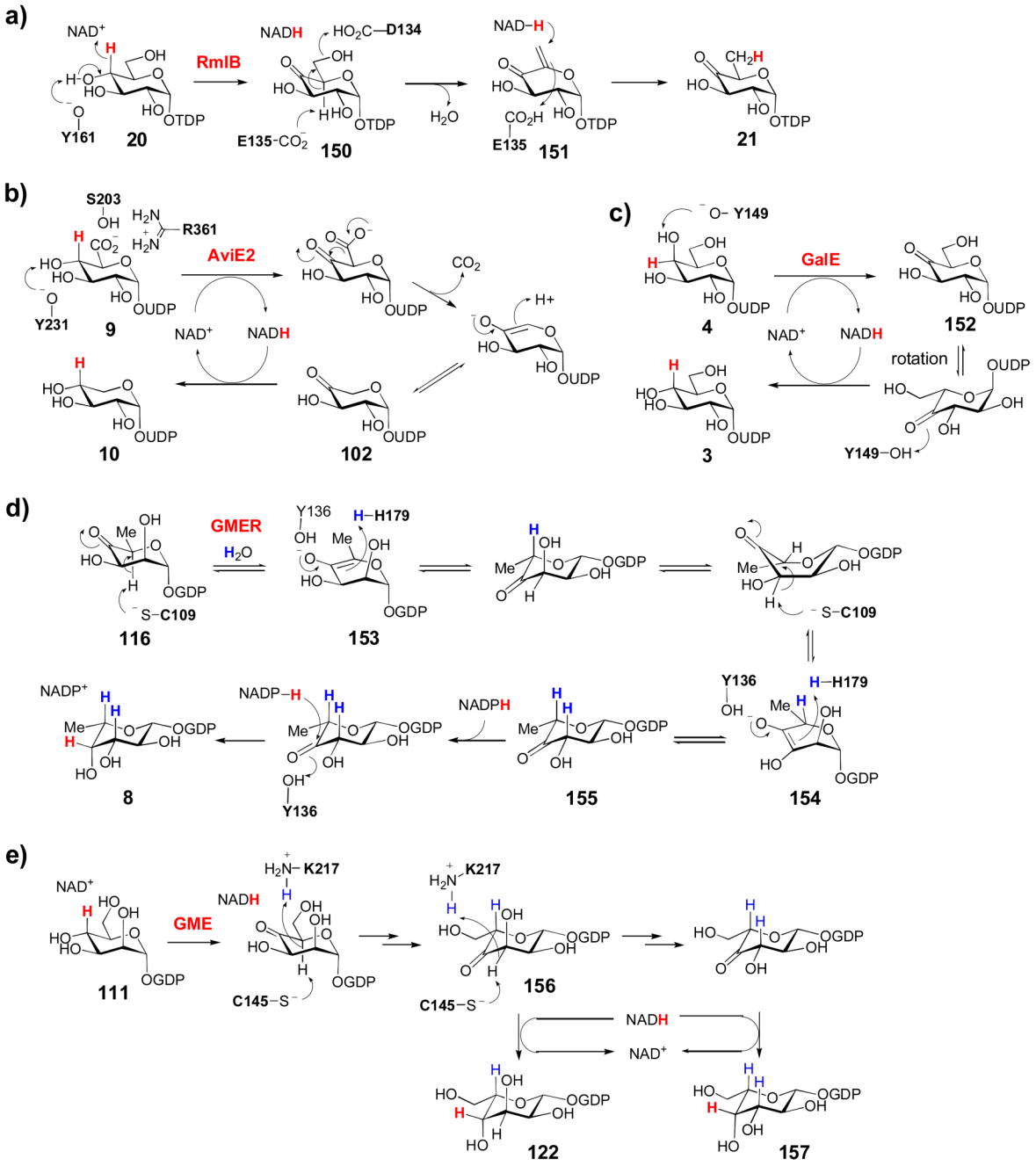




Scheme 7. A common theme in the mechanisms of many deoxysugar biosynthetic enzymes

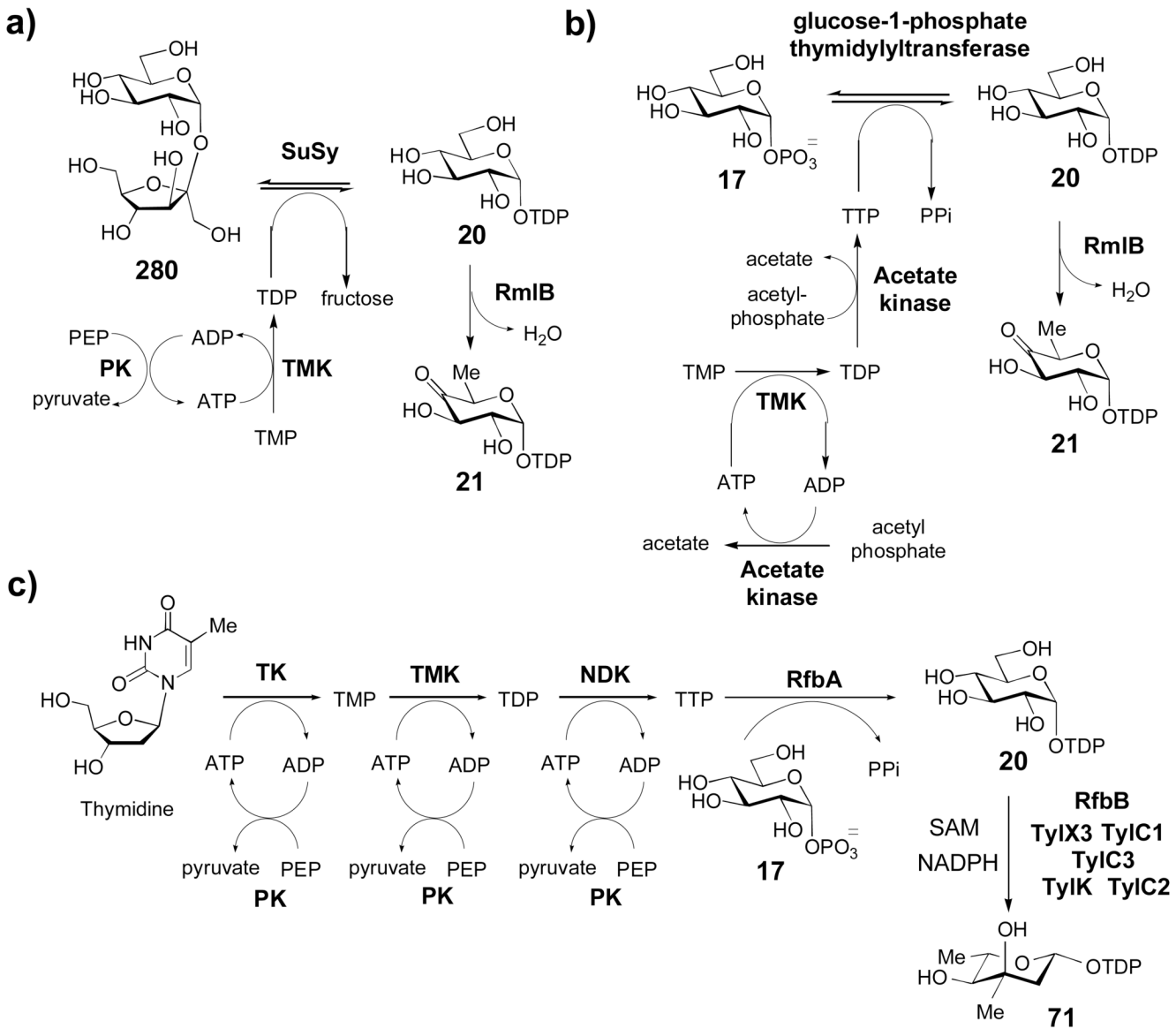
Many deoxysugar biosynthetic enzymes utilize the 4-keto group installed during the first step of deoxysugar biosynthesis (see **20** → **21**, Scheme 2) to catalyze their respective reactions. Some examples include the TDP-4-keto-6-deoxy-D-glucose-3,5-epimerase (RmlC) from *Pseudomonas aeruginosa* (a), the TDP-4-keto-6-deoxy-D-glucose-3,4-ketoisomerases (Tyl1a and FdtA) from *Streptomyces fradiae* and *Aneurinibacillus thermoaerophilus*, respectively (b), the TDP-4-keto-6-deoxy-D-glucose-4-aminotransferase (DesI) from *Streptomyces venezuelae* (c), the TDP-4-keto-2,6-dideoxy-D-glucose-3-*C*-methyltransferase (TylC3) from *Streptomyces fradiae* (d), the TDP-4-keto-6-deoxy-D-glucose-2-dehydratase (TylX3) from *S. fradiae* (e), the CDP-4-keto-6-deoxy-D-glucose-3-dehydrase (E_1) from *Yersinia pseudotuberculosis* (f), and

the GDP-4-keto-6-deoxy-D-mannose-3-dehydrase (ColD) from *Y. pseudotuberculosis* (g). See text for mechanistic details of each reaction.



Scheme 8. Mechanisms of selected sugar-modifying short-chain dehydrogenase/reductase (SDR) enzymes

Proposed mechanisms of TDP-α-D-glucose-4,6-dehydratase (RmlB) from *Salmonella enterica* (a), UDP-α-D-glucuronate decarboxylase (AviE2) from *Streptomyces viridochromogenes* (b), UDP-α-D-galactose-4-epimerase (GalE) from *E. coli* (c), GDP-4-keto-6-deoxy-α-D-mannose-3,5-epimerase-4-reductase (GMER or GDP-fucose synthase) from *E. coli* (d), and GDP-α-D-mannose-3,5-epimerase (GME) from *Arabidopsis thaliana* (e). See text for details of each proposed mechanism.



Scheme 9. Enzymatic synthesis of NDP-sugars

a) One-pot synthesis for the common deoxysugar biosynthetic intermediate (TDP-4-keto-6-deoxy-D-glucose, **21**) from sucrose (**280**) and thymidine monophosphate (TMP) using TMP kinase (TMK), sucrose synthase (SuSy), and RmlB. b) Biosynthesis-based approach for the synthesis of **21** (see text for details). c) Two-stage, one-pot synthesis of TDP-L-mycarose (**71**). In the first stage, thymidine was converted to TTP by thymidylate kinase (TK), TMK, and nucleotide diphosphate kinase (NDK). Following purification of TTP by filtration, **17** was converted to **71** (in 16% yield from **17**) by the combined action of seven enzymes in the presence of *S*-adenosylmethionine (SAM) and NADPH.