Reviews

Neoglycorandomization and Chemoenzymatic Glycorandomization: Two Complementary Tools for Natural Product Diversification[#]

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In an effort to explore the contribution of the sugar constituents of pharmaceutically relevant glycosylated natural products, chemists have developed glycosylation methods that are amenable to the generation of libraries of analogues with a broad array of glycosidic attachments. Recently, two complementary glycorandomization strategies have been described, namely, neoglycorandomization, a chemical approach based on a one-step sugar ligation reaction that does not require any prior sugar protection or activation, and chemoenzymatic glycorandomization, a biocatalytic approach that relies on the substrate promiscuity of enzymes to activate and attach sugars to natural products. Since both methods require reducing sugars, this review first highlights recent advances in monosaccharide generation and then follows with an overview of recent progress in the development of neoglycorandomization and chemoenzymatic glycorandomization.

Introduction

Nature produces a vast pool of small molecule metabolites capable of invoking a broad spectrum of biological activities. In fact, the vast majority of the world's drug leads directly derive from, or are inspired by, natural products. In particular, glycosylated natural products (Figure 1) have served as reliable platforms for the development of many existing front-line drugs. Given carbohydrates are capable of accessing a wide array of unique chemical space,[1] the sugars attached to these metabolites greatly enhance natural product chemical diversity. As a result, such sugar attachments wield remarkable influences that range from modulating pharmacology and pharmacokinetic properties to dictating specificity at the tissue, cellular, and/or molecular level.[2] The following few examples highlight the drastic influence even very subtle sugar variations can render upon a variety of glycosylated natural product structural classes.

Aromatic Polyketides. Among nucleic acid-targeted drugs, the sugars attached to anthracyclines such as doxorubicin (Figure 1, **1**), aclarubicin (Figure 1, **2**), or MEN-10755 (a semisynthetic disaccharide-substituted **1** analogue) are critical to forming the anthracycline−DNA binary and anthracycline−DNA−topoisomerase ternary complexes ultimately leading to DNA damage and cell death.[3] Anthracycline sugar variations (e.g., MEN-10755) reduce both the quinone-dependent generation of reactive oxygen species that mediate acute, reversible arrhythmias and hypotension and the formation of certain anthracycline secondary alcohols that have been attributed to chronic, irreversible cardiomyopathy.[4] Alteration of sugars attached to anthracyclines can also broaden the tumor scope accessible by these pharmaceutically important cytotoxins (e.g., MEN-10755 and **2**),[5] reduce MDR efflux (e.g., **2**),[6] and can even completely abolish activity.[7]

Reduced (Complex) Polyketides. Within the broad array of drugs that inhibit protein synthesis, macrolides specifically inhibit the 50S ribosome via specific binding with the 23S ribosomal subunit and various proteins.[8] The 16-membered macrolides (e.g., tylosin, Figure 1, **3**) generally inhibit peptidyltransferase activity, while the 14-membered macrolides (e.g., erythromycin, Figure 1, **4**) generally inhibit the translocation of peptidyl-tRNA. Corroborated by the recently elucidated three-dimensional structures of macrolide−target complexes, extensive SAR revealed the attached sugars to be absolutely essential to macrolide bioactivity.[9] Remarkably, nature has evolved macrolide glycosylation as a means to both alter the molecular mechanism and inactivate these chemical warfare agents. Specifically, the addition of a single sugar (L-megosamine) to the erythromycins provides the megalomicins (Figure 1, **5**), produced by *Micromonospora megalomicea*, which are uniquely potent inhibitors of protein trafficking in the golgi and may present opportunities for the development of novel antiparasitic and/or antiviral agents.[10] For inactivation, many of the same macrolide producers utilize enzymatic glucosylation of the desosaminyl-2′-OH as a key self-resistance mechanism and also contain specific glucosidases capable of regenerating the active parent compound.[11]

Indolocarbazoles. Among protein-directed signaling modulators, the indolocarbazoles are divided into two major classes dependent upon their structure and mechanism of action.[12] The sugar constituents of the first class, exemplified by rebeccamycin from *Saccharothrix aerocolonigenes* (Figure 1, **6**),[13] are critical for their potent topoisomerase I poisoning effects and/or DNA specificity/affinity. Yet, increasing the DNA affinity of this class via sugar variations

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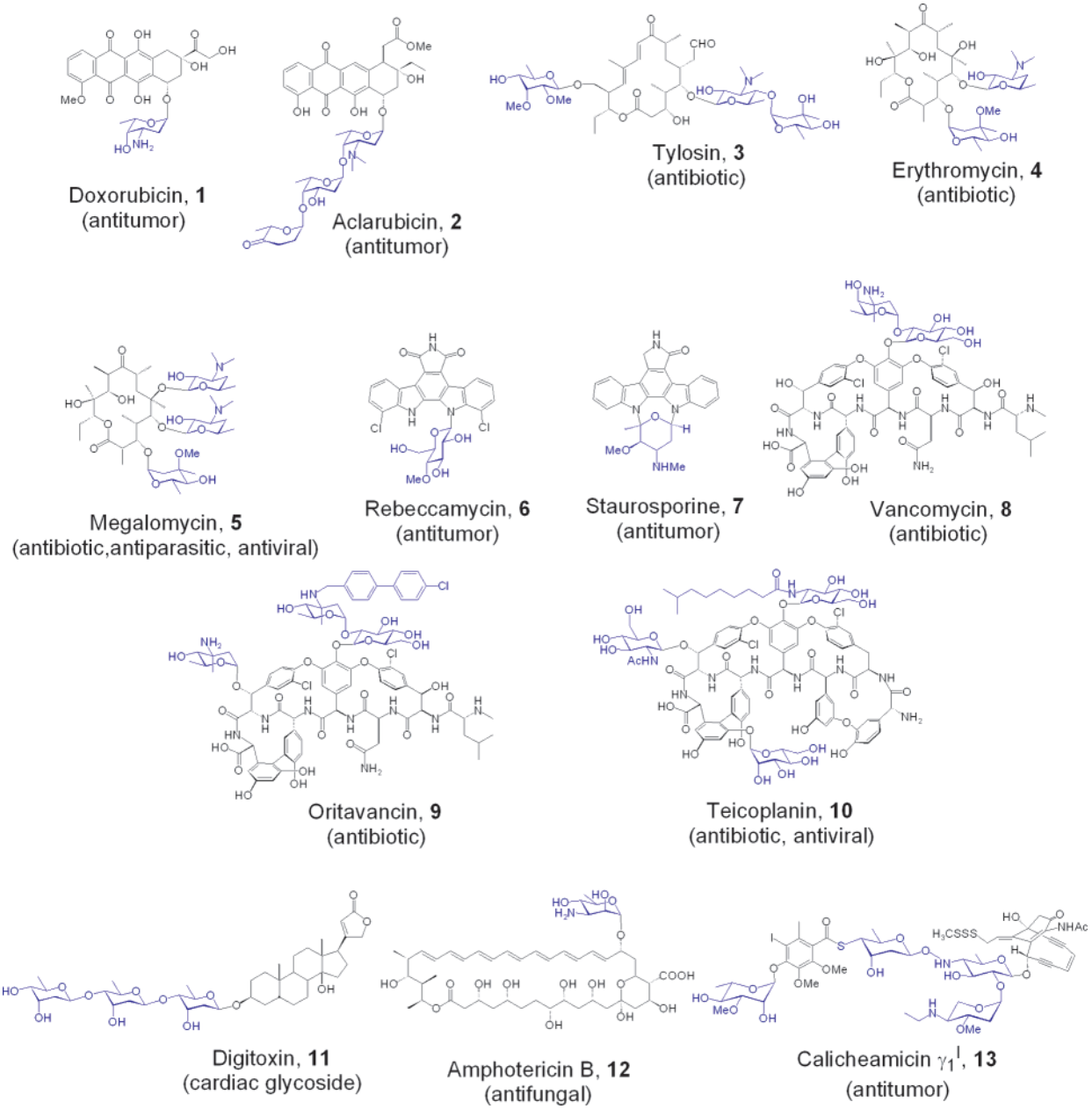

Figure 1. Representative glycosylated natural products. Glycosidic attachments are indicated in blue.

surprisingly abolishes topoisomerase I inihibition while enhancing cytotoxicity.[14] Notably, a recent **6** analogue bearing a 2′,3′-anhydro-*β*-D-glucose led to the specific inhibition of both CDK1/cyclin B (a cyclin-dependent kinase) and CDK5/p25 (a kinase involved in the phosphorylation of neuron cytoskeletons)—a drastic mechanistic switch from the parent natural product.[15] This kinase inhibitory activity is reminiscent of the second structural class of naturally occurring indolocarbazoles, exemplified by staurosporine (*S. staurosporeus*; Figure 1, **7**), which possess indole nitrogens bridged by a single glycosyl moiety at C-1′ and C-5′ and are potent protein kinase C inhibitors.[16]

Nonribosomal Peptides. Within agents targeting cellular structural elements, vancomycin (Figure 1, **8**) from *Amycolatopsis orientalis* is a glycopeptide antibiotic that inhibits bacterial transpeptidases by binding to the *N*-acyl-D-Ala-D-Ala termini of lipid-PP-disaccharide-pentapeptides, thus rendering the organism susceptible to osmotic lysis.[17] Three lines of evidence implicate the critical contribution of the disaccharide **8** to this bioactivity. First, removal of the disaccharide provides an aglycon with markedly reduced antibacterial activity.[18] Second, *N*-alkylation of the terminal vancosamine of **8** with a hydrophobic group dramatically increases activity against **8**-resistant strains and presents analogues that, unlike **8**, do not induce VanB resistance.[19] Finally, certain carbohydrate-modified **8** hydrophobic analogues operate via a mechanism distinct from **8**.[20] While a number of formal hypotheses have been put forth to explain the mechanism of action of these hydrophobic derivatives,[21] compelling evidence suggests the chlorobiphenyl glycopeptide derivatives [e.g., ortivancin-(**9**)-type] circumvent VanA resistance via alternative targeting of the transglycosylase,[21a,f] while long-chain acyl substituted glycopeptide derivatives [e.g., teicoplanin-(**10**)-type] escape VanB resistance via an undefined mechanism.[21d]

Natural Product Glycosylation Methods

These are but a few examples of how subtle shifts in the glycosylation of a natural product drastically alter biological activity, highlighting a potential new strategy for the generation of small molecules with utilities ranging from

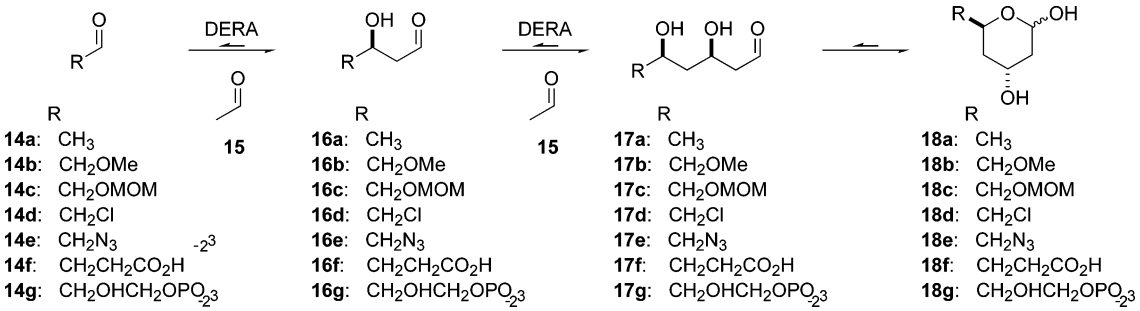

Figure 2. Deoxyribose-5-phosphate aldolase (DERA)-catalyzed sequential aldol reactions between diversely functionalized aldehydes to generate monosaccharides **18**.

tools for chemical genomics to therapeutic leads. Yet, our ability to capitalize upon this potential and/or to clearly understand the contribution of an attached carbohydrate to the biological activity of a given small molecule remains limited by the availability of convenient and effective glycosylation methods. A number of complementary routes for altering the glycosylation of natural products have been reported,[22] but each method is accompanied by its own set of limitations. Arguably the most powerful from the perspective of diversity, total synthesis and semisynthesis are considered nearly limitless in terms of target range.[23] However, the synthesis of complex natural products is extremely labor intensive, often not divergent, nonrenewable, and remains plagued by the restrictions of currently available chemical glycosylation strategies.[2f,24] Alternatively, a number of genetic engineering-based in vivo methods, including pathway engineering (often misrepresented by the term "combinatorial biosynthesis")[22,25] and bioconversion,[26] have also led to glycosylated natural product variants. The key advantage of in vivo strategies is that the engineered metabolites can be generated via fermentation and thus are renewable. However in vivo strategies are currently restricted by difficulties with host strain genetic engineering, the often drastic reduction of engineered variant product yields,[26] the limited diversity available via natural biosynthetic pathways,[27] and even the potential for host death resulting from the inherent toxicity of novel metabolites.

Capitalizing upon the strengths of these existing strategies, chemists have developed two complementary glycosylation methods to generate compound libraries that differ solely via their glycosyl substituents. These "glycorandomization" approaches (which have also occasionally been referred to as "glycodiversification" in the literature)[28] are anticipated to greatly expedite our understanding of the role of sugars in a variety of glycoconjungates and the exploitation of these critical attachments. Two complementary glycorandomization strategies have been described: (a) *neoglycorandomization*, a recently disclosed chemical approach based upon a one-step sugar ligation reaction that does not require prior sugar protection or activation, and (b) *chemoenzymatic glycorandomization*, a biocatalytic approach that relies on the substrate promiscuity of enzymes to activate and attach sugars to natural products. Both glycorandomization strategies rely upon the availability of diverse reducing sugar libraries. Thus, this review begins with a brief highlight of recent advances in monosaccharide synthesis and proceeds with a discussion of the recent progress in the development of neoglycorandomization and chemoenzymatic glycorandomization.

Sugars: The Foundation of Glycorandomization

Not only are monosaccharides the diversity element that defines each glycorandomized library but they are also essential precursors to a variety of other emerging solution-phase and solid-phase glycoconjugate synthetic strategies for oligosaccharides, glycopeptides, and small molecules.[29] Thus, the development of methods to rapidly access diverse reducing sugars will have a remarkable impact upon glycobiology and therapeutic development. Highlighted herein are recent developments that simplify access to these critical building blocks.

Sugar-Pirating. While a wide range of reducing sugars can be obtained from commercial sources, the utility of highly functionalized rare sugars remains untapped due to their rarity. An abundant source of such uncommon sugars are natural products themselves.[27,30] This approach is viable when the glycosylated natural product is inexpensive, when it can be bioproduced in high abundance, and/or when the sugar portion of a natural product molecule accounts for a significant percentage of its overall molecular weight. For example, digitoxin (Figure 1, **11**) is economical and contains 3 equiv of the 2,6-dideoxy sugar digitoxose. Thus, simple hydrolysis of this cardiac glycoside is a viable source of large quantities of this unique dideoxy sugar. However, a universal protocol applicable to sugar appendages representing a broad range of reactivities, such as the three rare sugar appendages of tylosin (Figure 1, **3**), is often difficult to develop.[31] It is also possible to envision isolating unique carbohydrates from bacterial cell walls or antigens,[27] but such strategies would also be limited by the percent mass of target sugar(s) within the overall organism biomass.

Aldolases. Arguably the best enzymes to generate diverse reducing sugars are aldolases.[32,33] Deoxyribose-5-phosphate aldolase (DERA) has been used to perform sequential aldol reactions between three or four substrates, in a single process, to generate 2,4-dideoxyhexoses (Figure 2).[34] Since hemiacetals inhibit elongation, only α-substituted aldehydes that restrict cyclization can be used as the initial acceptor. For example, α-substituted acetaldehydes (**14**) were used in conjunction with acetaldehyde (**15**) to form 2,4-dideoxyhexoses **17** that rapidly cyclize into cyclic hemiacetals **18** to prevent further enzymatic elongation.[34d] In contrast, over 100 different aldehyde acceptors have been accepted by dihydroxyacetone phosphate (DHAP)-dependent aldolases (Figure 3)[35] to generate phosphates such as **21** and **25**, which after hydrolysis provide ketone monosaccharides such as **22** and **26**.[36] By using pentose or hexose phosphate donors, nonnatural high-carbon ketoses can be obtained in the same manner.[37] Isomerases are then used to generate aldoses from these ketose units. For example, when ketoses **22** and **26** were treated with glucose isomerase or fucose isomerase, monosaccharides **23** and **27** were obtained, respectively.

Despite these successful demonstrations, there are limitations to enzymatic methodologies. Aldolases display stringent selectivity toward the dihydroxyacetone donor,

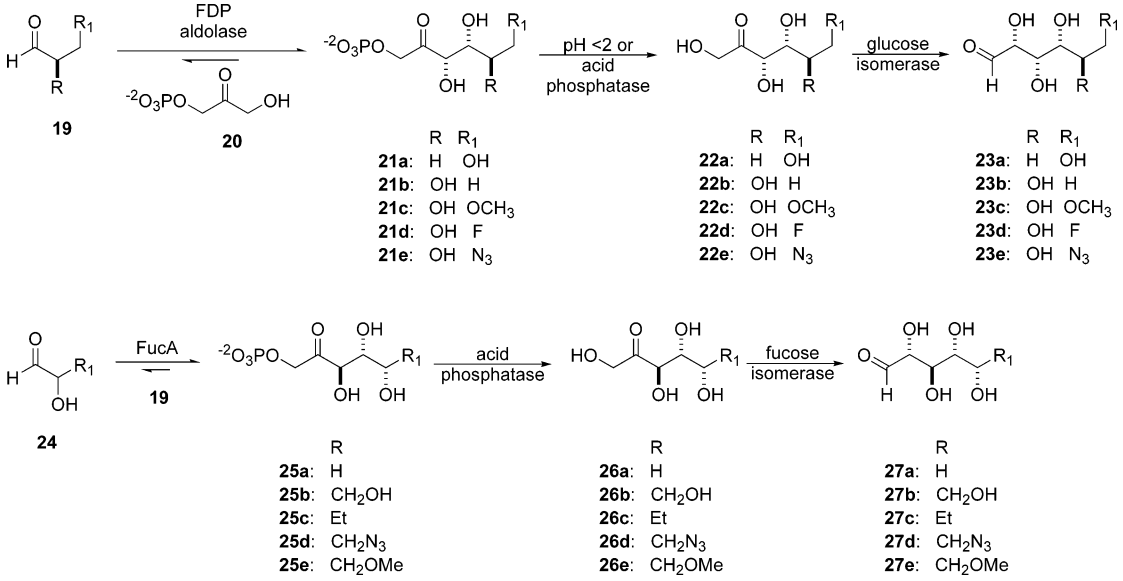

Figure 3. Dihydroxyacetone phosphate (DHAP)-dependent aldolase catalysis to generate monosaccharides.

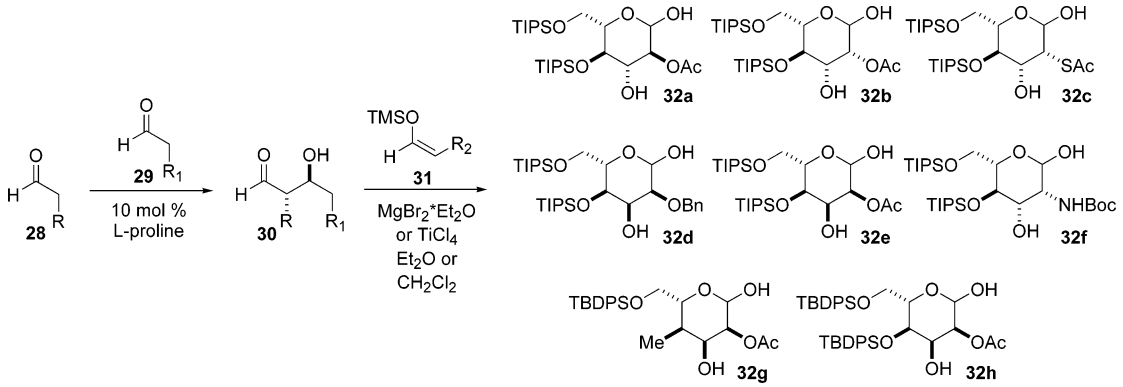

Figure 4. Two-step aldol coupling developed by MacMillan and co-workers that employs proline organocatalysis to ultimately generate diverse monosaccharides.

which limits the structural diversity of sugar products. When racemic aldehydes are used as substrates, separation of diastereomeric products is also necessary, but routes to enantiomerically pure aldehydes are available. In addition, isomerases lead to an equilibrium mixture of ketoses and aldoses, which ultimately require resolution. Finally, DERA is limited to substrates that limit hemiacetal formation as mentioned above. Despite such limitations, both DERA-mediated reactions and DHAP-dependent aldolase-mediated reactions are often quite scalable and have been used to successfully prepare a variety of monosaccharides. While the parallel aldolase-mediated synthesis of a monosaccharide library has not been reported, these methods appear particularly poised to provide in vivo pools of reducing sugars for the in vivo glycorandomization efforts described later in this review.

Organocatalysis. Chemical synthesis has been used to generate monosaccharides de novo[38,39] or from commercially available sugars,[40] but these routes are often nondivergent, challenging, and time-consuming. Recently, chemical methods simple and efficient enough to allow for robust monosaccharide library synthesis have emerged. The first method, a two-step aldol coupling developed by MacMillan and co-workers (Figure 4),[41] is reminiscent of aldolase-mediated strategies wherein L-proline replaces an aldolase as the asymmetric catalyst. In the first step, α-oxyaldehyde or α-alkylaldehyde aldol donors (**28**) and acceptors (**29**) are coupled stereoselectively via L-proline catalysis in excellent yields without further undesired elongation of the aldol products.[41,42] In the next step, a Mukaiyama aldol reaction between enolsilanes **31** and aldehydes **30** afforded putative hexose-oxocarbenium intermediates, which spontaneously cyclized to prevent further elongation and provide cyclic hemiacetals **32a**−**h** in excellent yields and diastereoselectivities. Interestingly, a given sugar diastereomer could often be formed predictably by using certain solvent/Lewis acid combinations. For instance, the use of $MgBr_2 \cdot Et_2O$ provided mannose derivative **32b**, while the same enolsilane and aldehyde reactants provided allose derivative **32e** when $TiCl_4$ was employed. Several features make this reaction sequence promising for the synthesis of reducing sugar libraries. First, fragments **28**, **29**, and **31** can be derivatized independently to provide sugars that contain diverse functional groups, including carbon, nitrogen, and sulfur groups (**32g**, **32f**, and **32c**, respectively). Additionally, the hydroxyl groups of monosaccharides **32a**−**h** can be unmasked sequentially and elaborated to provide further diversity. Finally, product enantiomers are easily obtained using D-proline as the organocatalyst in place of L-proline. Recent studies by Cordova and co-workers show that it is possible to streamline the amino acid-catalyzed generation of monosaccharides even further by forming sugar products directly from aldehyde precursors without the use of preformed enolate equivalents, although currently such procedures lead to diminished yields.[43] While still early in development, synthesizing monosaccharides via organocatalysis appears

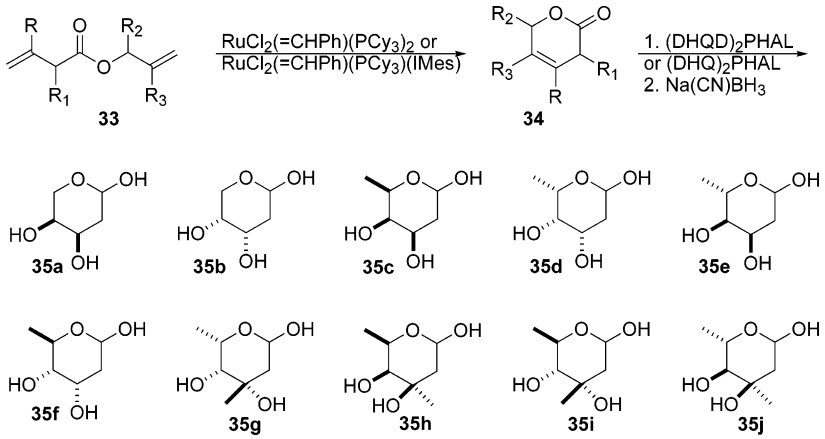

Figure 5. Ring-closing metathesis strategy toward 2,6-dideoxypyranoses, developed by Wang and co-workers.

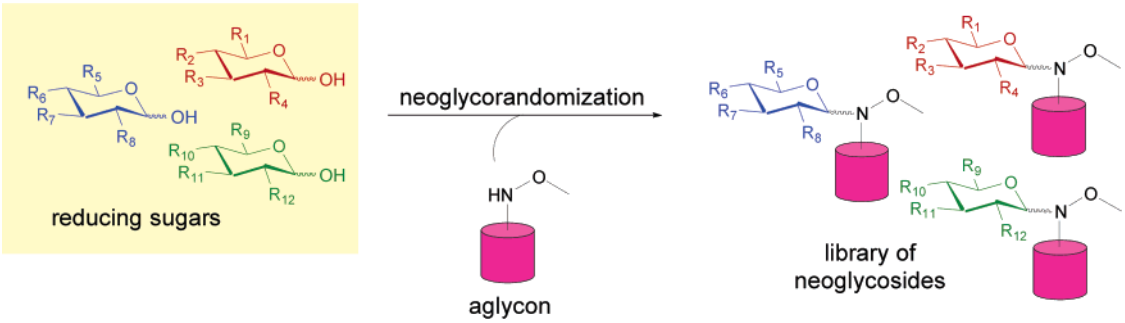

Figure 6. "Neoglycorandomization", developed by Thorson and co-workers, which involves the chemoselective formation of glycosidic bonds between reducing sugars and a secondary alkoxylamine to form a library of neoglycosides.

to present the broadest versatility of currently available chemical or enzymatic strategies.

Ring-Closing Metathesis (RCM). Another synthetic method that may eventually be applied to sugar library synthesis is the ring-closing metathesis-mediated production of 2,6-dideoxypyranoses developed by Wang and co-workers (Figure 5).[44] In this reaction sequence, homoallylic acids were synthesized in one step from commercially available allyl chlorides and coupled to readily available alcohols to provide olefinic ester precursors **33**. With few exceptions, $RuCl_2(-CHPh)(PCy_3)_2$- or $RuCl_2(-CHPh)(PCy_3)$-(IMes)-mediated ring-closing metathesis of ester precursors **33** proceeded in good yields, without appreciable cross-metathesis. Asymmetric dihydroxylation of the resulting δ-lactones (**34**) using commercially available AD mixes, followed by $Na(CN)BH_4$ reduction, provided monosaccharides **35** in an average yield of 62% and 72% average de. This metathesis route potentially could be applied to generate a host of other sugar products by incorporating alternative homoallylic acid and homoallylic alcohol precursors and by applying alternative chemistries to the unsaturated δ-lactones (e.g., epoxide/aziridine formation and subsequent ring opening). A major limitation of this RCM approach derived from the prohibitive cost of allylic alcohol precursors. Wang and co-workers recently reported an alternative economical approach to these precursors from *trans*-4-phenyl-3-buten-2-one.[45]

Neoglycorandomization

Neoglycorandomization is based upon the chemoselective formation of glycosidic bonds between reducing sugars and secondary alkoxyamine-containing aglycons to form a library of "neoglycosides" (Figure 6). In sharp contrast to traditional chemical glycosylation reactions that rely upon tedious sugar donor protection and activation schemes, "neoglycosylation" advantageously utilizes unprotected and nonactivated reducing sugar donors under mild conditions.[46] Such reducing sugars are readily available commercially or via the elegant methods described in the preceding section. In early examples of this chemoselective reaction, sugars and peptides containing secondary alkoxyamines were reacted with reducing sugars to generate oligosaccharide and glycopeptide mimics, respectively.[47,48] These pioneering studies revealed that, unlike primary alkoxyamines, which provide open-chain oxime isomers,[49] secondary alkoxyamines react to form closed-ring neoglycosides (Figure 7). Presumably, such secondary alkoxyamines react with reducing sugars to form an intermediate oxy-imminium species, which then undergoes ring closure with O-5.[50,51]

Among the key neoglycosylation prototype studies,[50] Peri et al. synthesized monomer **36**, containing both a reducing end and a methoxyamine group (masked as an oxime), to allow iterative synthesis of linear oligosaccharide mimics (Figure 8).[52] Monomer **36** underwent condensation with **37** to form disaccharide mimic **38**. Treatment with $NaCNBH_3$ reduced the oxime to a methoxyamino group that could participate in a chemoselective ligation with **36** to generate trisaccharide mimic **39**. Carrasco et al. synthesized Fmoc-protected *N*-methyl-aminooxy amino acid **40** and successfully incorporated it into a small peptide via solid-phase peptide synthesis (Figure 9).[53] The fully deprotected peptide was ligated with D-glucose or D-lactose under aqueous conditions to afford the desired closed-ring neoglycopeptides **43** in good yields. Although the stability of these model neoglycosides was not examined, the distribution of pyranose, and occasionally furanose, anomers in neoglycosides was found to be dependent on the identity of the sugar.[50] Closed-ring neoglycosides were found to display conformational behavior similar to natural *O*-glycosides by NMR studies, molecular dynamics simulations, and ab initio calculations.[54]

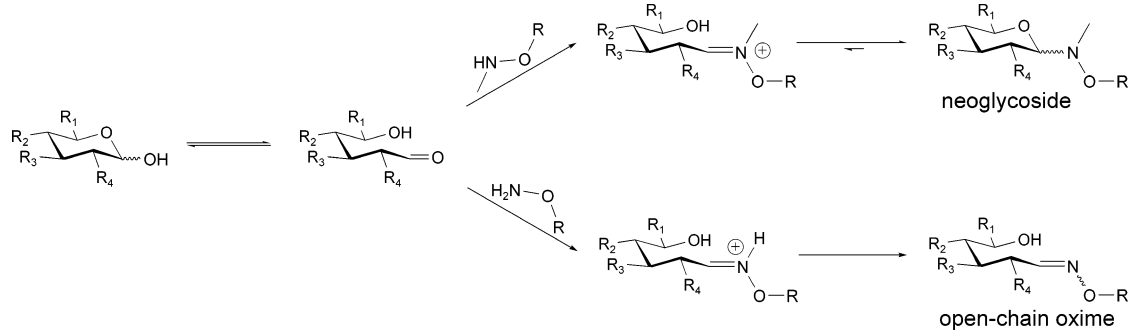

Figure 7. Reactions of primary and secondary alkoxyamines with reducing sugars to form open-chain oximes or closed-ring neoglycosides, respectively.

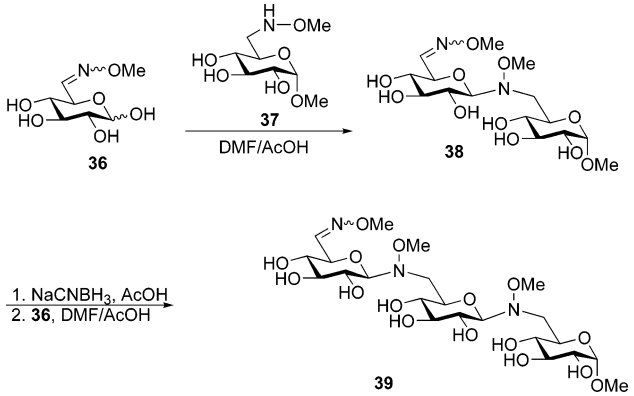

Figure 8. Iterative synthesis of an N(OCH_3)-linked oligosaccharide analogue based on neoglycosylation.

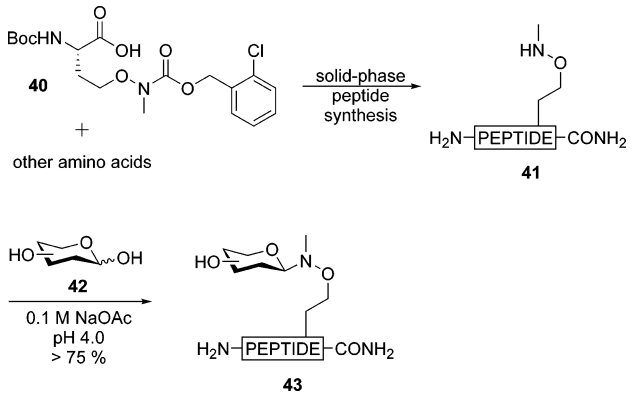

Figure 9. Chemoselective reaction between a methoxyamine-containing peptide and a reducing sugar to generate glycopeptide mimics.

To test the potential of this remarkable glycosylation reaction toward natural product neoglycorandomization, Thorson and co-workers selected digitoxin (**11**) as a model platform.[55] In addition to its well-known cardiac activity, which is mediated by inhibition of the plasma membrane Na^+/K^+-ATPase,[56] digitoxin has demonstrated in vitro anticancer properties,[57] provides protective effects against polyglutamine-based diseases,[58] and inhibits activation of signaling pathways in cystic fibrosis cells.[59] Since the attached sugars are implicated as mediators of the unique spectrum of biological properties exhibited by cardiac glycosides,[57a,60] digitoxin was deemed an appropriate model to examine the general utility of neoglycosylation to efficiently construct a glycorandomized library.

A library of 78 digitoxin derivatives was synthesized and purified in parallel from 39 reducing sugars and aglycons **44**β and **44**α (Figure 10), which were easily obtained from digitoxin (Figure 1, **11**) in three simple chemical steps.[55] A diverse array of reducing sugars was used, including L-sugars, deoxy sugars, dideoxy sugars, disaccharides, and uronic acids, and—in every case—neoglycosides were generated successfully. Neoglycosides with sugars containing reactive handles were also constructed, as exemplified by two members containing a reactive azido group amenable to further diversification via Huisgen 1,3-dipolar cycloaddition (Figure 10, black box).[61] The digitoxin neoglycosides were completely stable over the period of one month under neutral or basic conditions but slowly hydrolyzed under acidic conditions over this same time period. These results represent the largest and most diverse sugar-based library generated to date and clearly highlight the power of neoglycorandomization. Interestingly, LC chromatograms suggested that ~50% of the library members contained >90% of a single product isomer, and for library members containing isomeric mixtures, purified components equilibrated back to a mixture of isomers over a period of several hours.[62] Yet, although it is impossible to isolate pure compounds from neoglycosylation reactions with sugars favoring isomeric mixtures, equilibration between product isomers in these mixtures could actually be beneficial since sequestration by the biological target in vivo could ultimately drive this equilibrium toward the active isomer.

The activity of digitoxin and neoglycoside library members **45**β and **45**α was assessed using a high-throughput cytotoxicity assay on nine human cancer cell lines representing a broad range of carcinomas including breast, colon, CNS, liver, lung, and ovary, and a mouse mammary normal epithelial control line.[55] Digitoxin displayed broad, low-level (average $IC_{50} = 0.44\ \mu M$) cytotoxicity toward all nine cancer cell lines. In contrast, a number of neoglycoside hits were identified with improved cytotoxic properties relative to the parent natural product. Interestingly, these hits all contained sugars with a common structural feature, an *S*-configured C-2′ sugar stereocenter. This C-2′ stereochemistry appears to be of critical importance for cytotoxicity, and changing this stereocenter dramatically abolished this activity. The two most significant hits included one with striking potency and another with excellent selectivity (Figure 10, red box and blue box, respectively). Specifically, the first hit (Figure 10, red box) was a potent cytotoxin against six cancer cell lines and displayed extremely strong potency (~18 nM) against one line. The second hit (Figure 10, blue box) exhibited dramatic selectivity toward a multidrug-resistant line that contains high levels of MDR-1 and P-glycoprotein expression.[63] Given that cardiac glycosides are substrates for P-glycoprotein,[64] such tumor specificity suggests this hit may not serve as a P-glycoprotein substrate and/or may be interacting with a unique cellular target. The latter notion is bolstered by the observation that all identified hits were demonstrated to be significantly less potent Na^+/K^+-ATPase inhibitors in HEK-239 human embryonic kidney cells than digitoxin.[55] This

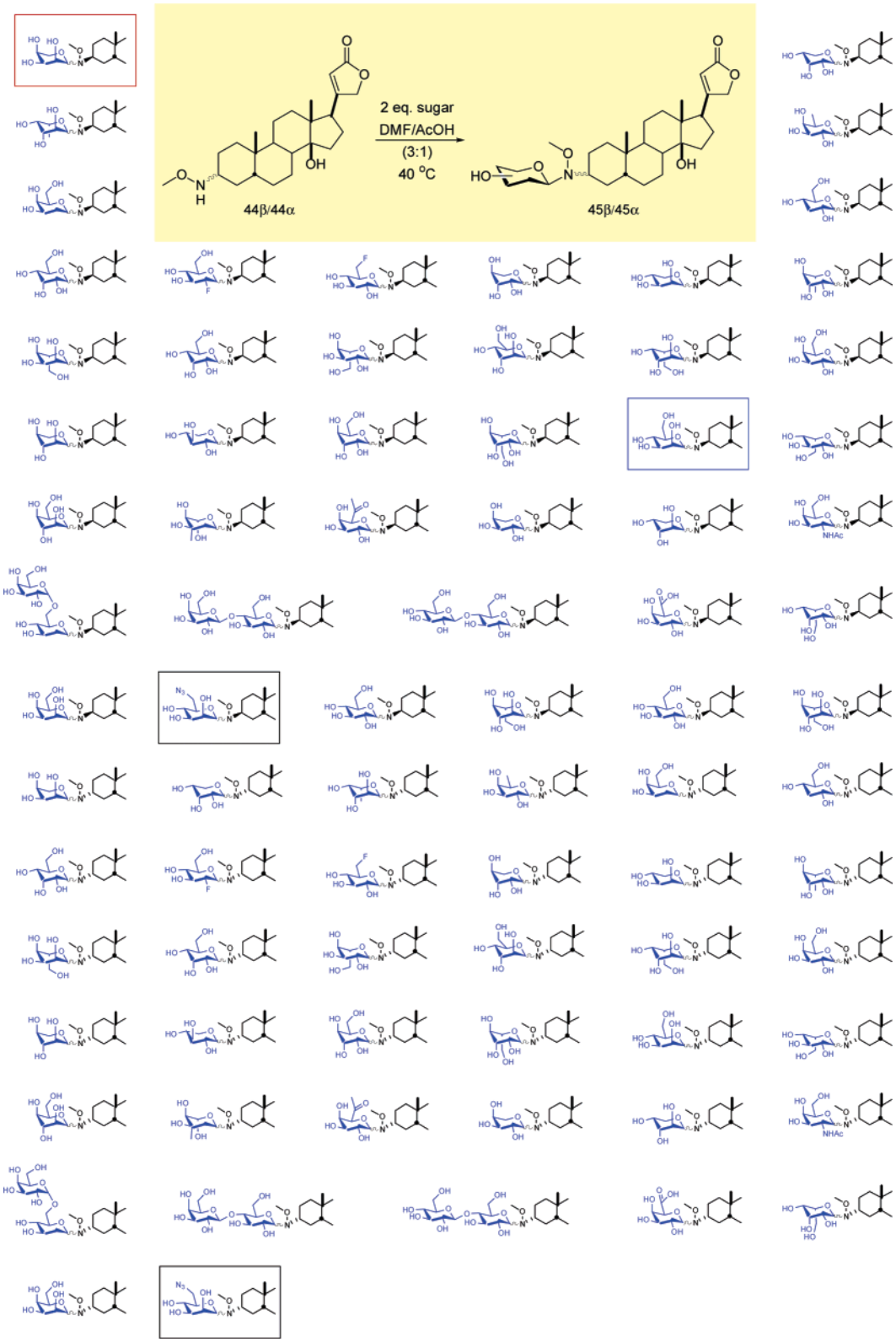

Figure 10. Neoglycorandomized digitoxin library synthesized by Thorson and co-workers.

observation also contrasts the general belief that the cytotoxic activities of cardiac glycosides correlate with Na^+/K^+-ATPase inhibition and is supported by other evidence in the literature.[57]

The neoglycorandomization of digitoxin illustrates the remarkable ease by which the influence a sugar has on a natural product scaffold can be quickly scanned via a simple, mild, and robust reaction with unprotected and nonactivated reducing sugars. This work also clearly illustrates that subtle changes within an appended saccharide can drastically alter the bioactivity of a natural product. Also, alkoxyamines have been recently incorporated into nonribosomal peptides,[65] macrolides,[66] and anthracyclines,[67] and neoglycorandomized libraries based on these scaffolds have been generated, further illustrating the broad applicability of this chemistry.

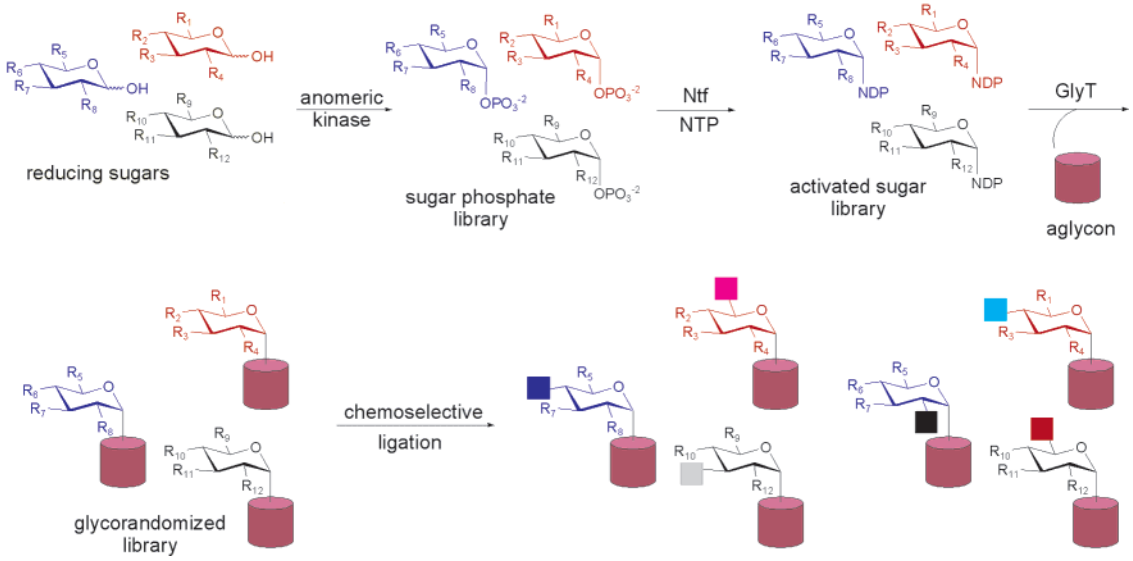

Figure 11. Chemoenzymatic glycorandomization exploits enzymes—anomeric kinases, nucleotidylyltransferases (Ntf), and glycosyltransferases (GlyT)—and chemoselective ligation.

Chemoenzymatic Glycorandomization

Chemoenzymatic glycorandomization employs the inherent or engineered substrate promiscuity of anomeric kinases and nucleotidylyltransferases (Ntf) to provide nucleotide diphosphosugar (NDP sugar) donor libraries to inherently promiscuous natural product glycosyltransferases (GlyT), thereby providing a rapid chemoenzymatic means to glycodiversify natural product-based scaffolds (Figure 11).[68] The main advantages of this approach are twofold. First, an efficient in vitro multienzyme, single-vessel reaction is anticipated to simplify greatly glycorandomized library production in comparison to traditional glycosylation strategies. Second, enzymatic processes are amenable to in vivo applications that should facilitate the process and significantly enhance the ability to scale production. Similar to neoglycorandomization, sugar substrates are also the foundation for chemoenzymatic glycorandomization, and sugars bearing uniquely reactive functional groups can be employed for downstream chemoselective ligation and further library diversification. Recent advances with sugar anomeric kinases, nucleotidylyltransferases, and glycosyltransferases potentially relevant to glycorandomization are highlighted below. In addition, recent progress toward in vivo glycorandomization is also briefly summarized.

Anomeric Kinases. Chemoenzymatic glycorandomization (in vivo or in vitro) starts with sugar-1-phosphates. These sugar phosphates can be accessed via chemical synthesis;[69] however, synthetic routes to these compounds are often plagued by tedious chemistry, low yields, and difficult product resolution. Single-step kinase-mediated routes to sugar phosphates from free sugars would bypass these restrictions and also present a means to generate sugar-1-phosphate precursors in vivo. Unfortunately, the naturally occurring anomeric kinases studied to date display a relatively limited substrate scope. Thus, the utility of sugar anomeric kinases for glycorandomization depends on whether their promiscuity toward monosaccharide substrates can be enhanced via either substrate-based engineering or directed evolution.

Prior to the emergence of the first structure for an anomeric kinase,[70] Thorson and co-workers embarked upon a directed evolution approach using a high-throughput multisugar colorimetric assay[71] to convert *E. coli* galactokinase (GalK)[72] into a flexible sugar anomeric kinase.[73] Because previously studied GalKs exhibited a strict adherence to D-sugars, initial screens focused upon L-sugar variants and also upon diverse C-6 alterations (e.g., deoxy, amino, uronic acid derivatives). From this approach, a particular GalK mutant carrying a single amino acid exchange (Y371H) ~20 Å from the active site displayed a remarkable degree of kinase activity toward sugars as diverse as D-galacturonic acid, D-talose, L-altrose, and L-glucose, all of which failed as wild-type GalK substrates (Figure 12). This landmark mutant also provided enhanced turnover of the small pool of sugars converted by the wild-type enzyme.

A subsequent elucidation of the slightly expanded substrate scope exhibited by wild-type *L. lactis* GalK[74] led Thorson and co-workers to generate a structure−activity model based on the newly available crystal structure of this enzyme.[69] Using their structure−activity template as the basis for an *E. coli* GalK active site homology model, Thorson and co-workers proposed that the active site M173 residue in *E. coli* GalK restricted the flexibility of this enzyme with regard to the C-4 and C-6 positions of sugars.[75] To test this hypothesis, an *E. coli* GalK mutant was generated in which this methionine residue was replaced with a smaller leucine residue. As predicted, the M173L mutant displayed enhanced promiscuity relative to wild-type *E. coli* GalK (Figure 12). Other active site *E. coli* GalK mutants also provided modest improvements.[76,77] When the M173L and Y371H mutations were combined to create a single double mutant enzyme, kinase activity toward a variety of new sugars was observed, while the activity of the corresponding single mutant enzymes was retained (Figure 12).[74] Notably, among the new substrate set are three azido sugars and two thio sugars (Figure 12, black box), setting the stage for downstream chemical modification of glycorandomized natural product libraries via chemoselective strategies.

Nucleotidylyltransferases. The next step for in vitro glycorandomization involves the conversion of sugar phosphates to NDP sugars, which are the activated sugar substrates of glycosyltransferases (GlyTs, Figure 11). NDP sugars can be obtained using chemical synthesis,[78] but such routes suffer from the same difficulties that complicate sugar phosphate synthesis. Single-step nucleotidylyltrans-

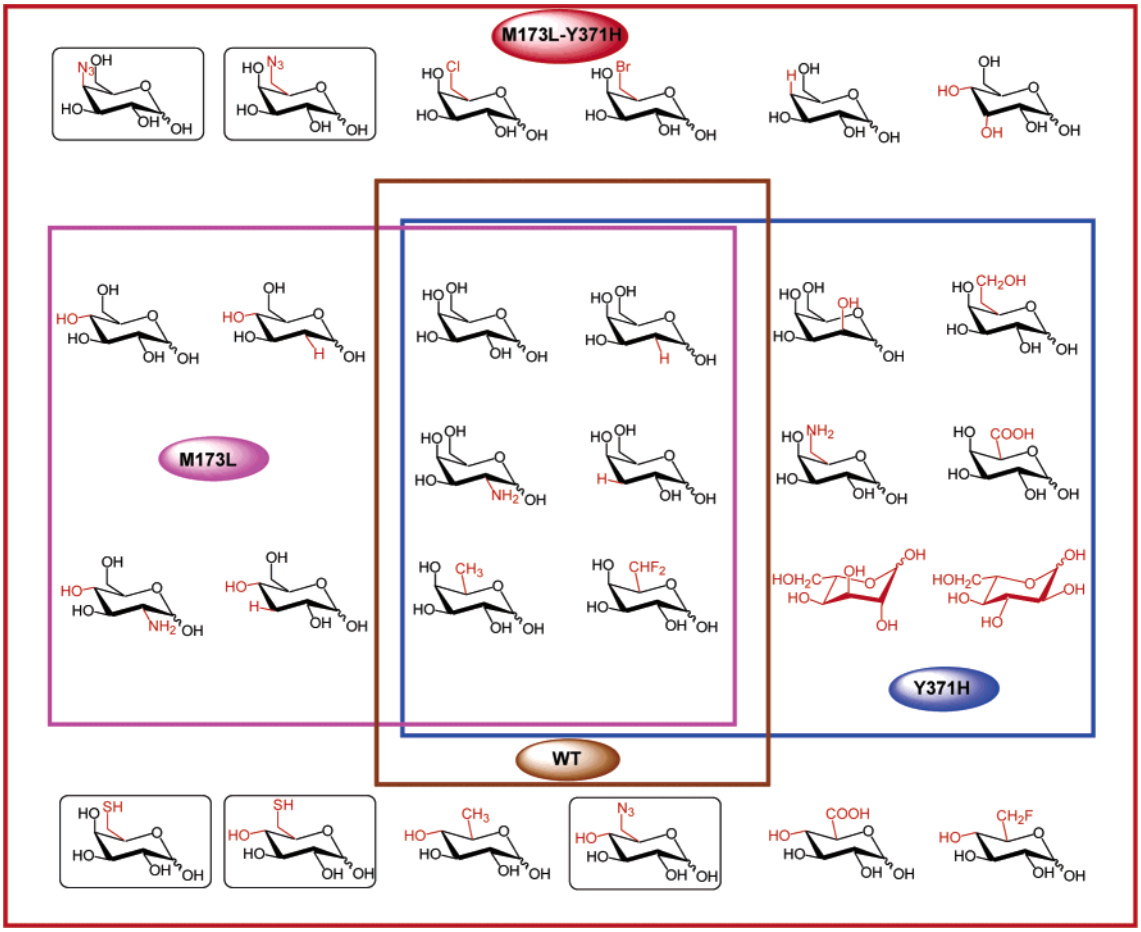

Figure 12. "Natural" and "unnatural" substrates of wild-type GalK and GalK mutants (M173L, Y371H, and M173L-Y371H). The positions deviating from the natural GalK substrate D-galactose are highlighted in red. Reprinted with slight modifications from ref 75 with permission from Elsevier.

ferase (Ntf)-mediated conversion of sugar phosphates to NDP sugars would represent a convenient route to these activated compounds in vitro or in vivo assuming that a Ntf with appreciable sugar phosphate substrate promiscuity could be identified or engineered. After surveying a number of Ntfs, Thorson and co-workers focused upon *S. enterica* LT2 α-D-glucopyranosyl phosphate thymidylyltransferase (also known as RmlA or E_p) as a potential Ntf for chemoenzymatic glycorandomization. E_p displayed a surprisingly large degree of flexibility toward both its nucleotide triphosphate (NTP) and sugar phosphate substrates.[68] Specifically, this enzyme converted a wide array of derivatized α-D-hexopyranosyl and α-D-pentopyranosyl phosphates to the corresponding dTDP sugars (Figure 13); the majority of these sugar phosphates could be converted to UDP sugars as well. Importantly, just as azido and thio sugars were substrates for GalK, E_p could also utilize azido and thio sugar phosphate substrates (Figure 13, boxed). Nikolov and co-workers elucidated the first crystal structure of a thymidylyltransferase (E_p), which was subsequently used as a template to rationally engineered E_p variants capable of using additional sugar phosphates not accepted by wild-type E_p.[79,80] The crystal structures of E_p bound to product (UDP-Glc) and to substrate (dTTP) determined by Nikolov and co-workers[80] revealed the precise molecular details of substrate recognition and substrate specificity of E_p, providing information necessary for enzyme-engineering experiments. Several engineered E_p mutants displayed broadened substrate specificities (Figure 14). For example, the W224H mutation accommodated bulkier substitutions at C-6, and the T201A mutation allowed larger functional groups at C-2 and C-3. The most noteworthy E_p mutant was L89T, which enhanced activity toward sugar phosphate substrates with unique C-2, C-3, and C-4 substitutions, such as members of the α-D-hexose series.[81] These cumulative rational design efforts have lead to the acceptance of ca. 40 functional sugar phosphate substrates, dramatically improving access to NDP sugars for glycorandomization.

Other researchers have also investigated the promiscuity of Ntfs. For instance, a number of Ntfs have been shown to tolerate different gluco-hexopyranosyl phosphates with limited C-2 substitutions, including BtrD from *B. ciculans*.[82] Pohl and co-workers recently revealed an *E. coli* uridylyltransferase to convert three deoxyglucose phosphate substrates, and a carbocyclic analogue of its natural substrate, to the corresponding NDP sugar products, albeit at low efficiencies.[83,84] Pohl and co-workers have also recently reported uridylyltransferases and thymidylyltransferases from *P. furiosus* to accept a few sugar phosphates in addition to their natural glucose-1-phosphate substrates (Figure 15).[85,86] Notably, when glucosamine-1-phosphate was tested as a substrate in these studies, *P. furiosus* glucose-1-phosphate thymidylyltransferase was found to be bifunctional, catalyzing both *N*-acylation and nucleotide transfer. As a result of this unique bifunctional activity, Pohl and co-workers synthesized a small set of *N*-acylglucosamine NDP sugars. These studies highlighted an elegant electrospray-ionization mass spectroscopy kinetic assay and the first characterization of nucleotidylyltransferases from archaeal sources.[83,87] Yet, to date only ca. eight nonnatural sugar phosphates are demonstrated substrates for these enzymes, the bulk of which were previously demonstrated to serve as efficient E_p substrates.[78−80]

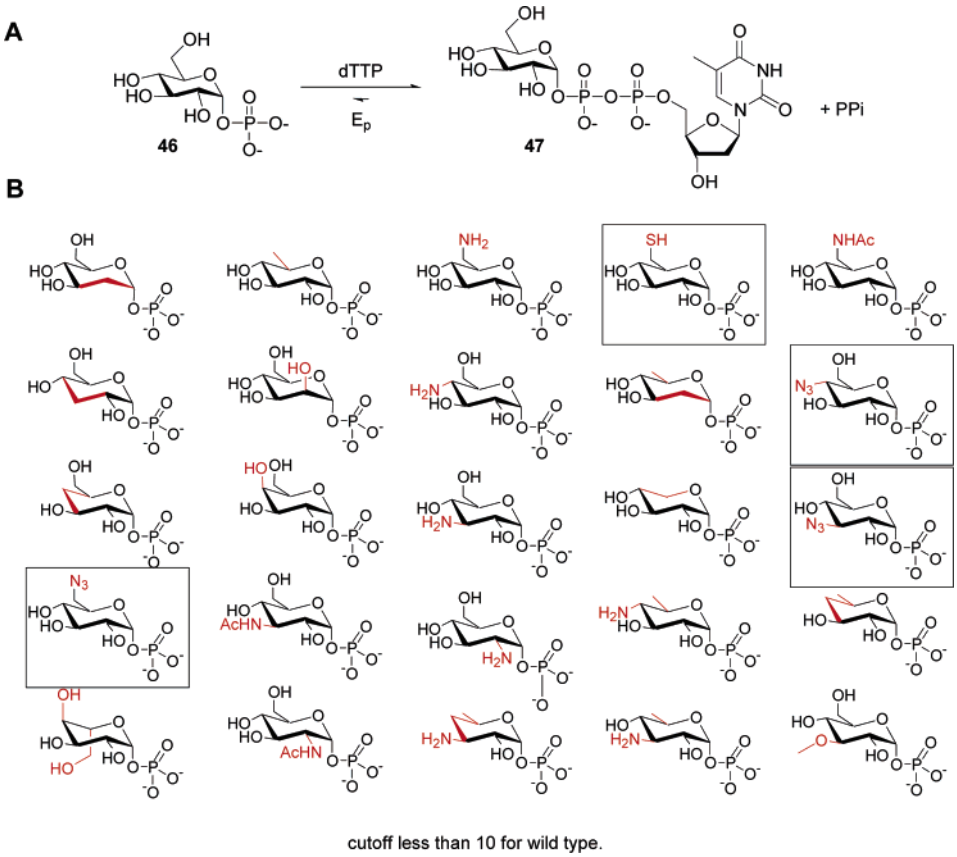

Figure 13. (A) "Natural" reaction catalyzed by E_p. (B) E_p converts a wide array of sugar phosphates to the corresponding dTDP sugars (>10% turnover). The positions deviating from the natural E_p substrate glucose-1-phosphate (**46**) are highlighted in red. Sugars containing reactive azide or thiol handles are boxed.

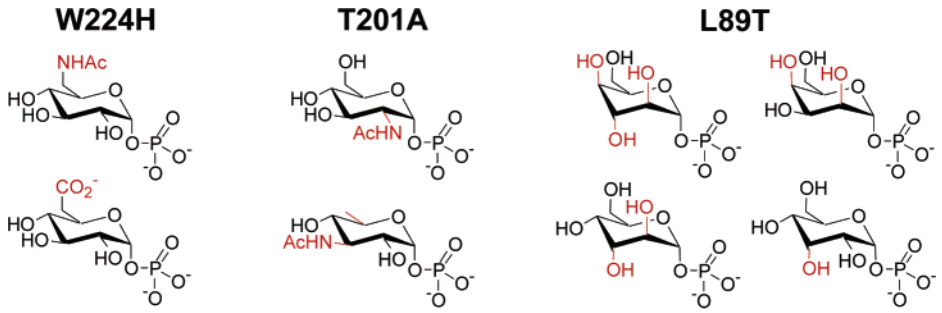

Figure 14. E_p mutants display improved turnovers toward these sugar phosphates; most of these substrates are not viable substrates of wild-type E_p (see Figure 13). The positions deviating from glucose-1-phosphate (**46**) are highlighted in red.

The ability to expand the utility of flexible nucleotidylyltransferases, such as E_p, beyond pyrimidine nucleotide triphosphates would be a major advance. Specifically, rapid access to purine nucleotide sugar libraries would provide versatile glycobiology reagents and present the potential to expand the application of chemoenzymatic glycorandomization into a variety of new areas including cell wall and oligosaccharide antigen biosynthesis as well as glycoprotein engineering. Within this context, Kawarabayasi and co-workers recently reported a unique thermostable thymidylyltransferase from *Sulfolobus tokodaii* that remarkably utilized dTTP, dCTP, dATP, dGTP, and UTP as substrates.[88] With the exception of dTTP, this is the first reported example of a nucleotidylyltransferase capable of efficiently utilizing both pyrimidine and purine dNTPs and, to our knowledge, one of the first reported evaluations of dNTPs (versus NTPs) as substrates for such catalysts. This report should prompt a general reevaluation of dNTP utility among nucleotidylyltransferases and possibly even a reevaluation of the relevance of deoxy- versus ribo-based nucleotides in certain sugar nucleotide biosynthetic pathways.

Glycosyltransferases. The last enzymatic step of in vitro glycorandomization exploits the inherent flexibility of secondary metabolite-associated glycosyltransferases[22] to generate glycosylated natural products from NDP sugar libraries (Figure 11). Most such glycosyltransferases are capable of catalysis as a single polypeptide. However, a pioneering discovery by Liu and co-workers recently revealed the macrolide glycosyltransferase DesVII to require an additional protein, DesVIII, for in vitro and in vivo activity.[89] Shortly thereafter, Walsh and co-workers demonstrated a similar two-component glycosyltransferase relationship between the aclacinomycin AknS/AknT, where the inclusion of AknT enhanced the estimated AknS k_{cat} (0.005 min^{-1}) to 0.22 min^{-1}.[90] Recently, Liu and co-workers further extended their original discovery to include in vivo correlations for tylosin TylM2/M3 and mycinamycin MycB/

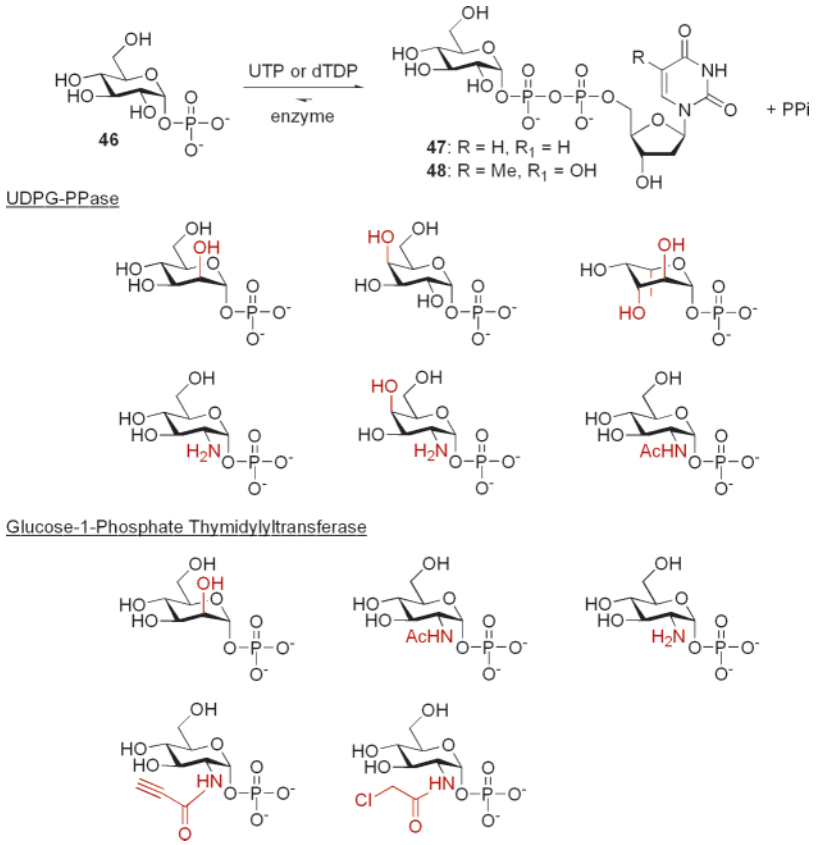

Figure 15. Substrates of two Ntfs investigated by Pohl and co-workers that display modest sugar phosphate flexibility. Positions deviating from the natural glucose-1-phosphate (**46**) substrate of these enzymes are highlighted in red.

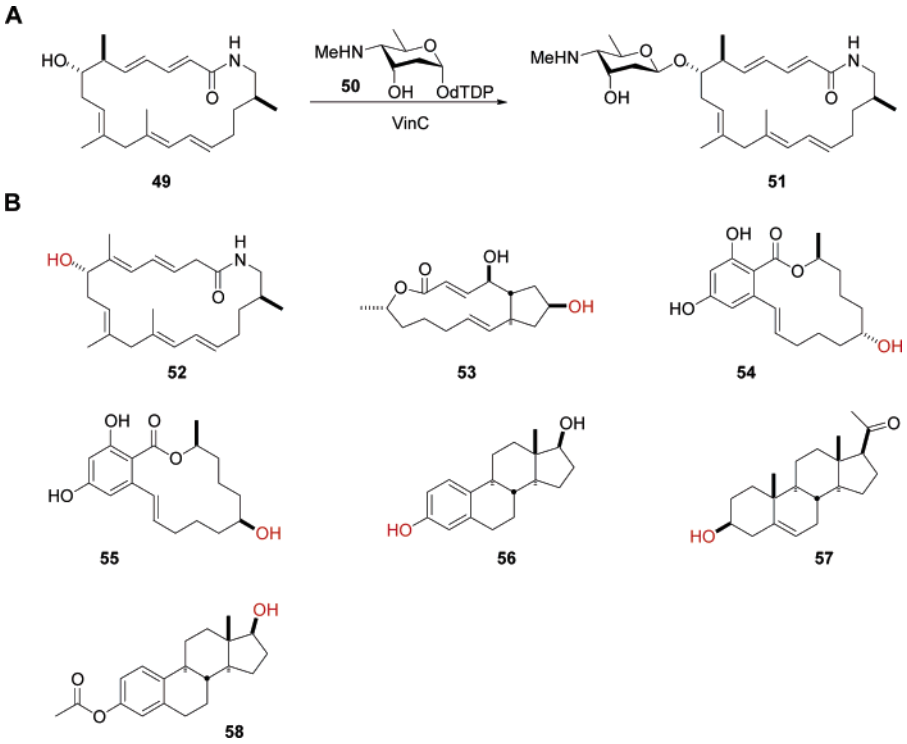

Figure 16. (A) VinC catalyzes the glycosylation of vicenilactam with dTDP-vinceniamine. (B) VinC alternative aglycon substrates. The positions where sugars are attached are highlighted in red.

MydC.[91] The DesVIII homologues within these systems show weak homology to P450s and were proposed by Liu and co-workers to be generally essential for NDP amino-sugar substrates, but the mechanistic role of the unique two-component glycosyltransferase relationship remains unresolved.[88−90]

Secondary metabolite-associated glycosyltransferases display varying degrees of flexibility with respect to the aglycon acceptor.[62,92,93] One recent example of a glycosyltransferase that accepts a number of different aglycons is VinC from *S. halstedii* HC-34.[94] In the final step of vincenistatin (**51**) biosynthesis, VinC catalyzes the glyco-

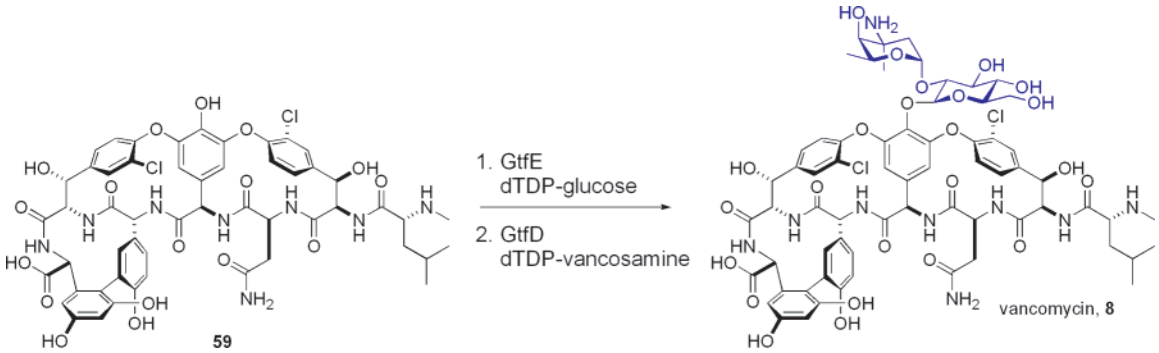

Figure 17. The final step in the biosynthesis of vancomycin is the glycosyltransferase-mediated assembly of the l-vancosaminyl-1,2-D-glucosyl disaccharide. The stepwise glycosylation is mediated by GtfE and GtfD.

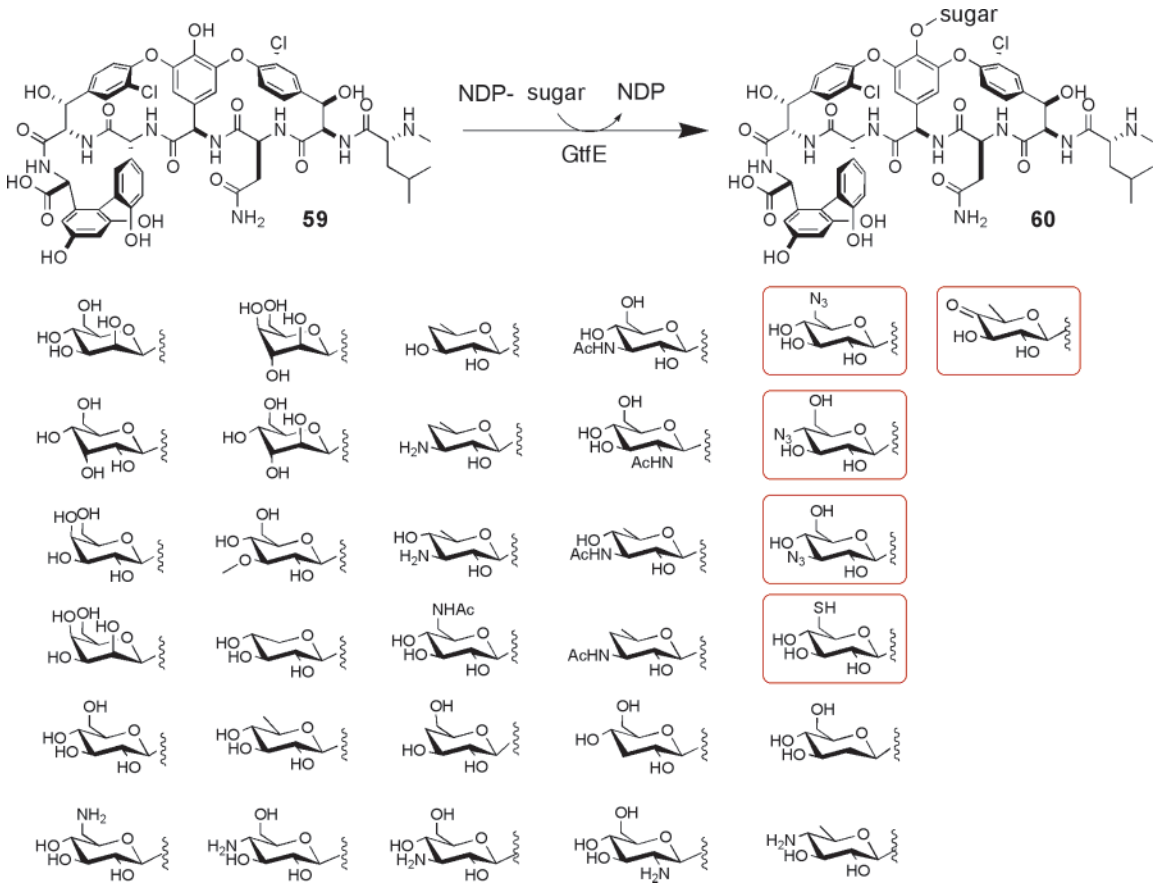

Figure 18. GtfE-catalyzed glycorandomization of vancomycin. Library members containing azido groups (red boxes) are poised for diversification via Huisgen 1,3-dipolarcycloaddition.

sylation of vicenilactam (**49**) using dTDP-viceniamine (**50**) as the nucleotide sugar substrate (Figure 16). Kakinuma and co-workers found that VinC also accepts other hydrophobic aglycons (**52**−**58**), and on the basis of a comparison of **49** and **52**−**58**, the critical elements for aglycon recognition were proposed. Glycosyltransferases involved in secondary metabolism are more often noted for their NDP sugar flexibility.[95] For example, the two vancomycin glycosyltransferases that assemble the l-vancosaminyl-1,2-D-glucosyl disaccharide upon the heptapeptide aglycon (**59**), GtfE and GftD (Figure 17),[96] were shown in early studies to accept several NDP sugar substrates.[88,89] These promising studies, coupled with the fact that modifications on the sugar substituents of vancomycin led to novel antibiotics that are active against vancomycin-resistant enterococci (VRE), prompted Thorson and co-workers to initiate full-fledged glycorandomization efforts on this scaffold using the NDP sugar libraries described above. From a panel of 33 natural and unnatural NDP sugars, 31 were accepted as substrates for GtfE (Figure 18).[61] Prior to this work, the number of monoglycosylated vancomycin analogues in the literature totaled 11 members constructed via multistep chemical synthesis.[97]

A variety of potential routes toward further diversification of nonribosomal peptide glycorandomized libraries exist. Structure-based efforts to engineer glycosyltransferases with altered regiochemistry and/or enhanced substrate promiscuity, particularly based upon their bidomain architecture, have been proposed but remain unsuccessful.[78,98] Alternatively, since unnatural members of the vancomycin monoglycosylated library served as a substrate for the second glycosyltransferase, GtfD (Figure 17),[18a,92] this enzyme, if equally flexible, may be able to glycorandomize some or all of the monoglycosylated variants presented in Figure 18. Finally, glycopeptides bearing reactive handles (Figure 18, red boxes) set the stage for the final step of chemoenzymatic glycorandomization,

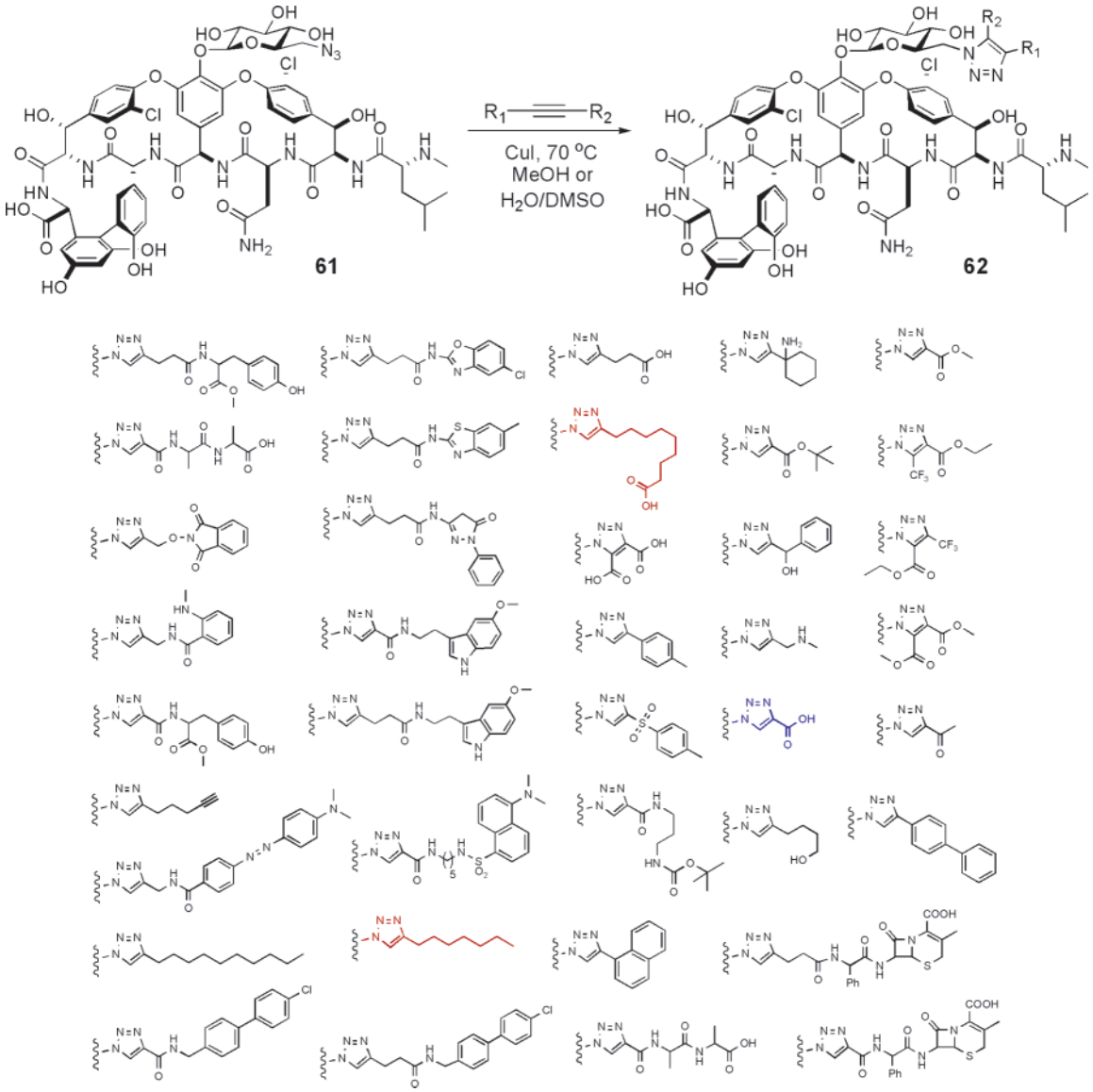

Figure 19. Huisgen 1,3-dipolar cycloaddition-mediated diversification of glycorandomized vancomycin library member **61**. Compounds that displayed antibacterial activity that exceeded (red compounds) or rivaled (blue compounds) that of the parent natural product.

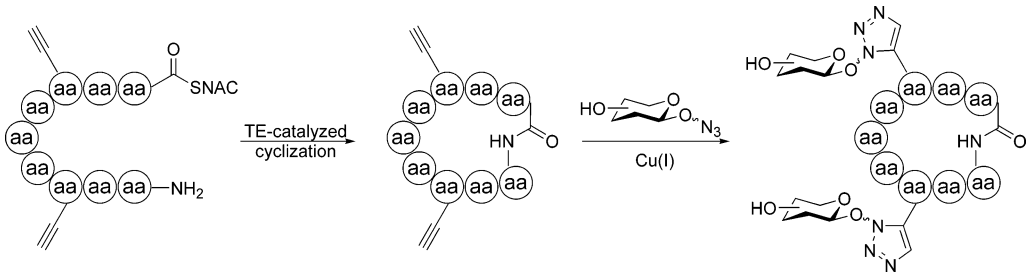

Figure 20. Chemoenzymatic approach to glycopeptide glycoside modification involving the Huisgen 1,3-dipolar cycloaddition reaction.

further diversification via chemoselective modification, as described in the next section.

Downstream Chemical Diversification. Chemoselective ligation reactions are highly efficient and specific covalent bond-forming reactions capable of proceeding in a physiological environment. Chemoselective ligations offer exquisite specificity, similar to enzymatic reactions, but with the significant advantage of accessing a much broader range of coupling partners. These reactions have been used extensively in recent years to generate or modify carbohydrate-containing structures.[99] One such reaction is the Huisgen 1,3-dipolar cycloaddition of azides and acetylenes to provide 1,2,3-triazoles.[100,101] With terminal alkynes, the ratio of 1,4- to 1,5-regioisomers can be controlled using Cu(I) to preferentially generate 1,4-disubstituted 1,2,3-triazoles.[102] Thorson and co-workers pioneered the chemoselective diversification of vancomycin using the monoglycosylated derivative **61**.[61,103] This effort provided 39 additional novel vancomycin library members bearing diverse functionality, albeit in varying cycloaddition yields (Figure 19). Most of these library members were tested for their antibacterial activity against methicillin-resistant *S. aureus*, vancomycin-sensitive *E. faecalis*, and vancomycin-sensitive *E. faecium*. In comparison to the parent natural product, vanco-

mycin (**8**), two analogues (Figure 19, red) displayed slightly more potent activity against one or more of the pathogens, and a third displayed similar activity (Figure 19, blue). These compounds represent the first monoglycosylated vancomycin derivatives that rival the biological activity of the parent natural product[96,104] and clearly support the significance of sugar substitution as a means to enhance the potency of natural products.

Walsh and co-workers have recently followed these studies with two related approaches to glycopeptide glycoside modification. In the first, the tyrocidine synthetase thioesterase (TE) domain was employed to cyclize linear peptide *N*-acetyl cysteamine thioester substrates bearing propargylglycine residues (Figure 20). Following macrocyclization, the glycopeptide alkyne side chains were then conjugated to azido sugars via the Huisgen 1,3-dipolar cycloaddition reaction.[105] Employing the azido sugar as the diversity element may limit this particular approach via the requirement of extensive precursor azido sugar chemical synthesis. Their second approach employed the same TE-catalyzed macrocyclization of linear peptidyl-SNACs bearing glycosylated amino acids, the predominant limitation again deriving from glycosylated amino acid precursor synthesis.[106] Cumulatively, these two studies boast >250 glycopeptide analogues, with two compounds exhibiting a 6-fold increase in therapeutic index compared to that of the parent tyrocidine. Drastic variations in both the TE-catalyzed macrocyclization and the Huisgen 1,3-dipolar cycloaddition reaction were also observed.

In Vivo Glycorandomization. The first step toward constructing short sugar-activation pathways in vivo for natural product glycorandomization was also recently described by Thorson and co-workers. Specifically, an *E. coli* strain overexpressing the M173L-Y371H GalK double mutant was shown to convert 6-azido-6-deoxy-D-galactose and 6-azido-6-deoxy-D-glucose (Figure 12) efficiently in vivo, the analysis of which was simplified through installation of a fluorescent label using the Huisgen 1,3-dipolar cycloaddition chemistry.[76] Recently, this in vivo application has been extended to "unnatural" sugars bearing unique mass signatures and alternative chemoselective ligation handles.[107] In conjunction with the demonstrated promiscuity of nucleotidylyltransferases and glycosyltransferases, this initial in vivo demonstration supports the feasibility of in vivo glycorandomization in both heterologous non-producing organisms (e.g., *E. coli* or *S. lividans*) and native producing hosts (e.g., actinomycetes).

Perspective

In summary, the two glycorandomization methods described in this review each have complementary strengths and limitations. For example, neoglycorandomization is limited by the efficiency and specificity of alkoxyamine handle installation, while in vitro glycorandomization does not require chemical manipulation of aglycons. Neoglycorandomization is scalable, while in vitro chemoenzymatic glycorandomization suffers from scalability problems (although efforts to establish an in vivo glycorandomization system may overcome this limitation). Chemoenzymatic glycorandomization is restricted to natural products for which promiscuous enzymes are available or can be engineered, while neoglycorandomization is robust for virtually any reducing sugar substrate. The critical requirement for both methods remains reducing sugar availability, and both methods are augmented by incorporating reactive chemical handles for downstream chemoselective ligation. Importantly, neoglycorandomization and chemoenzymatic glycorandomization have each led to natural product derivatives with enhanced biological activities in multiple therapeutic areas. Thus, not only do glycorandomization strategies bypass many restrictions imposed by traditional glycosylation strategies, including organic synthesis and pathway engineering, but more importantly, glycorandomization will continue to make powerful contributions to understanding the intricate interplay between natural product glycosylation, molecular recognition, biological activity, pharmacology, and drug discovery.

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