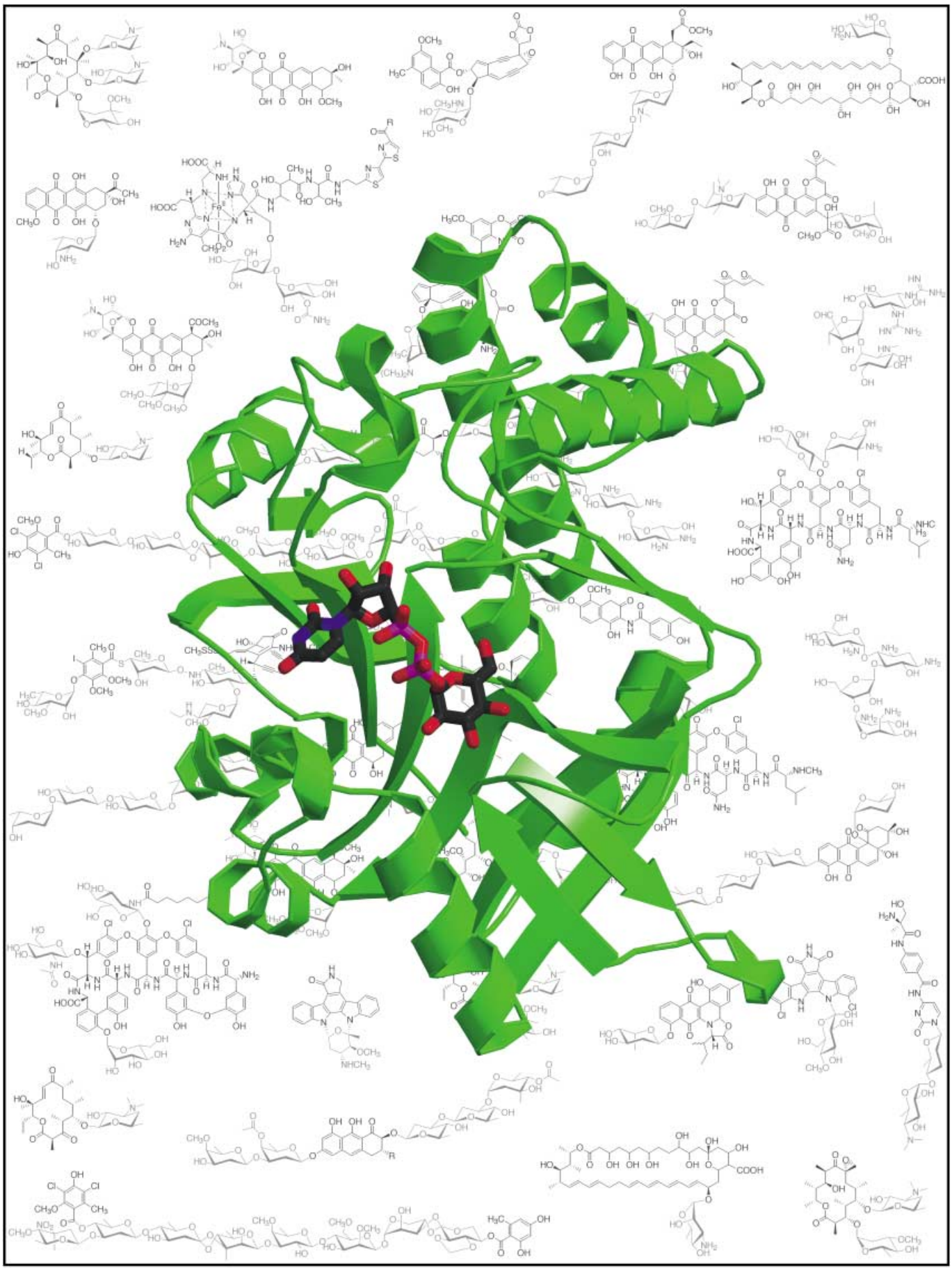

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Structure-Based Enzyme Engineering and Its Impact on In Vitro Glycorandomization

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1. Introduction

A recent estimate suggests that over half of the world's drug leads derive directly from the natural product pool, many of which are glycosylated secondary metabolites (Scheme 1).[1] Natural product sugar ligands have been shown to be critical to DNA recognition (calicheamicin, **1**), inhibition of DNA processing (daunomycin, **2**), RNA recognition (streptomycin, **3**), translation (erythromycin, **4**), cell-wall synthesis (vancomycin, **5**) and membrane recognition (amphotericin, **6**), and also target specific proteins (staurosporine, **7**) and/or protein complexes (cardiac glycosides, e.g., digitoxin **8**). The functional contribution of carbohydrates to biologically active natural products has been reviewed in a variety of outstanding compilations.[2–8] A clear picture is emerging from the exciting work highlighted in these reviews of how sugar ligands can mediate drug targeting, biological activity, and pharmacology, which suggests that altering glycosylation patterns on secondary metabolites has high potential for the generation of novel therapeutics.

There are a number of routes for altering the glycosylation of complex metabolites. 1) synthesis/semisynthesis relies upon the total synthesis of analogues or synthetic modification of intermediates usually produced by fermentation or hydrolysis of the natural product itself. An advantage of this approach is that the variants produced are only limited by the available chemistry and synthetic expertise. However, a significant disadvantage is the enormous structural complexity of many glycosylated natural products (e.g., **1**). In vivo methods for altering glycosylation include 2) genetic engineering/combinatorial biosynthesis and 3) bioconversion. The first of these includes disruption of natural sugar ligand biosynthetic pathways (SLBPs) in a producing host to provide essentially shunt metabolites and/or the heterologous expression of SLBP components to present hybrid glycosylation patterns (Scheme 2 A).[9–12] The second relies upon the feeding of aglycons or aglycon analogues to strains containing SLBPs (Scheme 2 B).[13, 14] A significant advantage of these routes is the ability to access new compounds by fermentation. Yet the price of in vivo methods may be twofold: first, SLBPs significantly bias the available chemistry and clearly limit the extent of accessible sugar variation in the final product;[15] second, in vivo approaches are likely to be limited by toxicity. For example, novel variants with high antibacterial properties might kill the producing bacterium long before the active variants can be observed. Thus, in the case of anti-infectives, in vivo methods may actually favor the isolation of unwanted, biologically inactive derivatives.

A final route for altering glycosylation is through biocatalysis or in vitro chemoenzymatic strategies. This so-called in vitro glycorandomization (IVG) approach takes advantage of combining the limitless flexibility of the chemical synthesis of unique sugar precursors with the inherent or engineered substrate promiscuity of enzymes to activate (nucleotidylyltransferases) and attach (glycosyltransferases) these carbohydrates to various natural product aglycons (Scheme 3).[4, 8, 16–20] Specifically for IVG, natural and "unnatural" sugar-1-phosphate precursors are chemically synthesized and attached to various aglycons by a one-pot, two-enzyme (nucleotidylyltransferase/glycosyltransferase) process. This methodology is advantageous in that it combines the strength of chemical synthesis with the ease of regio- and stereospecific enzymatic coupling of sugars to extremely complex aglycon structures. An early example of this process as applied to the nonribosomal peptides,[21–24] reveals that the first glycosyltransferase in vancomycin biosynthesis (GtfE) is capable of accepting many "unnatural" activated NDP-sugars, and that the products of the first reaction are also accepted by the second glycosyltransferase (GtfD) in the pathway (Scheme 4).[23, 24] Assuming that GtfD holds similar promiscuity toward the NDP-sugar; this suggests the potential for an exponential library growth, that is, true combinatorial biocatalysis. Furthermore, this methodology allows for the efficient incorporation of sugars bearing "reactive handles" (e.g., azides,[25] thiols,[25] ketones,[25] and aminooxy[26] substituents) that can then be specifically modified, in the context of a very

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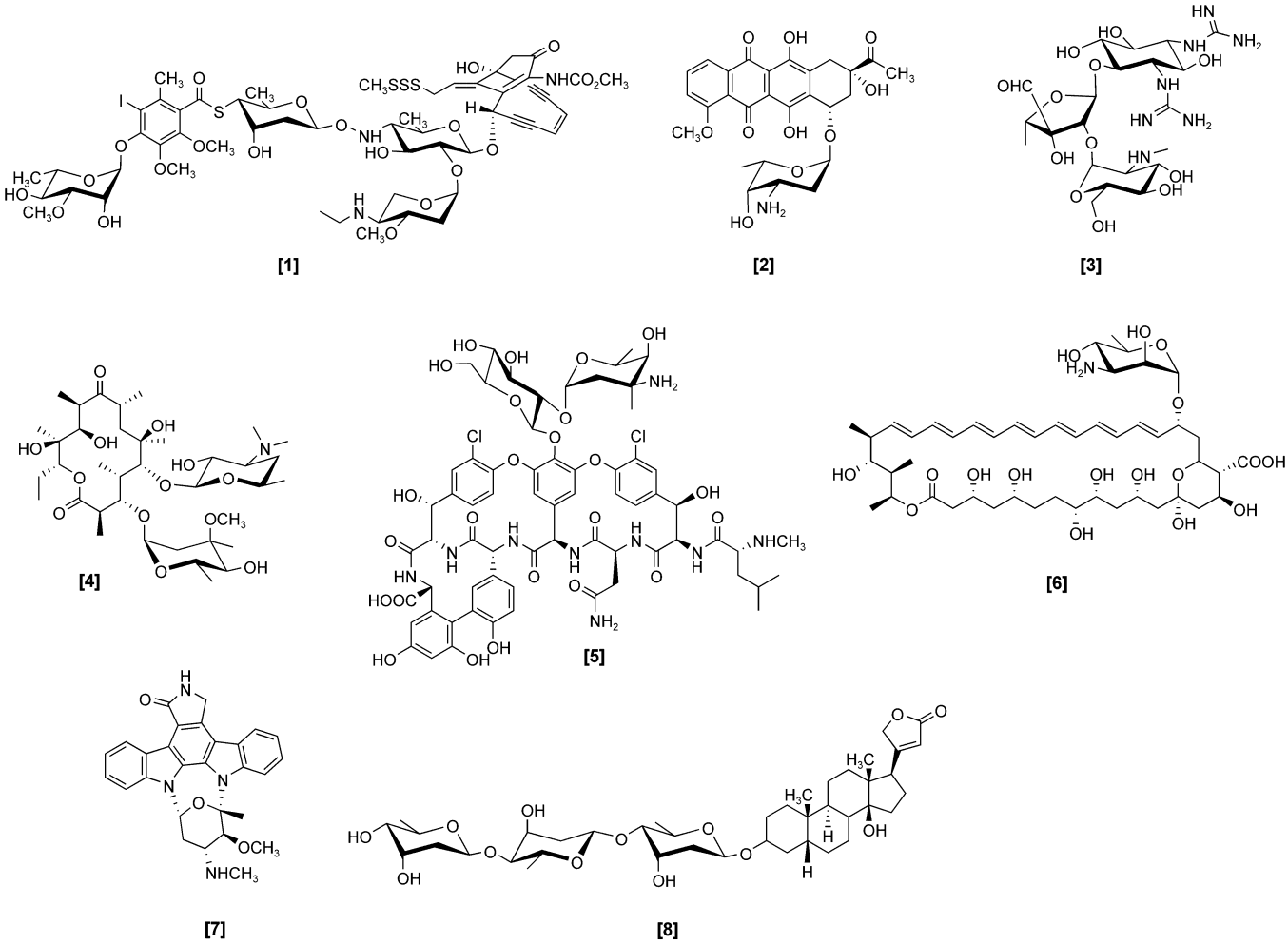


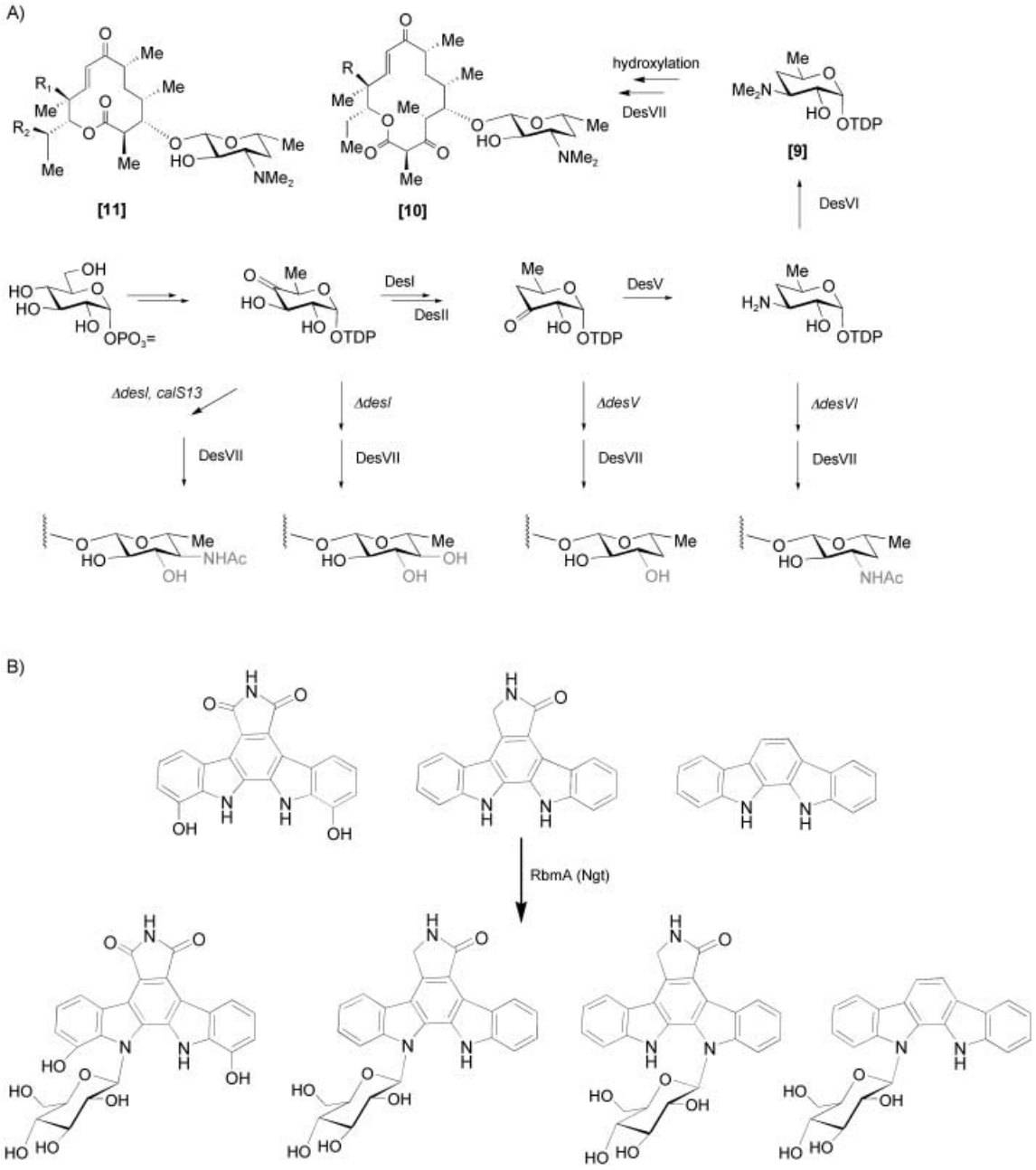
Scheme 1. *Representative examples of therapeutically relevant glycosylated secondary metabolites.*

complex natural product architecture, to enhance the diversity of the final glycorandomized natural product library.

A critical component to this approach includes the ability to enhance the promiscuity of the two enzyme families essential to the glycorandomization strategy, namely, nucleotidylyltransferases and glycosyltransferases. One approach might be the application of evolutionary methods in conjunction with a screen or selection for enzymes with desired properties.[27, 28] Given the nature of the reactions involved and the desire to develop enzymes capable of accepting large libraries of substrates, an evolutionary approach might prove challenging. Alternatively, rational design based upon structure might also provide mutants with the desired properties. This review highlights the application of structure-based engineering toward enhancing the promiscuity of these two enzyme families essential to the glycorandomization strategy.

2. E_p, A Model Nucleotidylyltransferase for Glycorandomization

Out of the vast number of available nucleotidylyltransferases, structure-based engineering work began with the uniquely promiscuous *rml*A-encoded α-D-glucopyranosyl phosphate thymidylyltransferase (E_p) from *Salmonella enterica* LT2.[29] E_p catalyzes the conversion of α-D-glucopyranosyl phosphate (Glc-1-P) and dTTP to dTDP-α-D-glucose (dTDP-Glc) and pyrophosphate (PP_i), through a single sequential displacement mechanism,[18] and is unique among nucleotidylyltransferases in that it displays unusual promiscuity toward both its nucleotide triphosphate (NTP) and the sugar phosphate substrates.[16, 17, 20] Specifically, E_p was demonstrated to convert a wide array of derivatized α-D-hexopyranosyl and α-D-pentopyranosyl phosphates to their corresponding dTDP *and* UDP nucleotide sugars (Scheme 5 A). However, limitations, as a function of sterics, ring conformation, and/or electrostatics, prohibit the use of E_p in a true combinatorial sense. Thus, a program was initiated to rationally engineer E_p variants capable of utilizing any sugar phosphate imaginable. The implication of a set of such E_p variants is the subsequent ability to generate, in a simple one-pot reaction, diverse libraries of NDP-sugars. These libraries, in conjunction with downstream glycosyltransferases and further chemical modification, form the basis for IVG.



Scheme 2. *A) An example of the application of genetic engineering toward changing macrolide glycosylation. Disruption (as indicated by "Δ") of genes leading to the biosynthesis of dTDP-desosamine (**9**), a precursor to pikromycin (**10**)/methymycin (**11**) and related macrolides in* Streptomyces (S.) venezuluae, *leads to macrolides bearing novel sugars. In addition, introduction of biosynthetic genes from other pathways (ΔdesI,* calS13*—which incorporates a sugar 4-aminotransferase from the calicheamicin pathway in* Micromonospora echinospora*) can lead to further diversity in glycosylation. B) An example of the application of bioconversion toward altering indolocarbazole glycosylation. In this example, the* N*-glycosyltransferase gene from rebeccamycin biosynthesis in* S. aerocolonigenes *was expressed in the heterologous host* S. lividans. *Unnatural aglycons were then fed to the Ngt-*S. lividans *recombinant strain fermentation leading to the novel indolocarbazole analogues.*

3. Three-Dimensional Structure of E_p

Several recent X-ray crystallographic studies of E_p[18] and its homologues in *Pseudomonas aeruginosa* (RmlA)[30] and *Escherichia coli* (G1p-TT)[31] have elucidated the structures of these enzymes and revealed that they function as symmetrical tetramers (Figure 1 A). The interactions between the subunits are dominated by helix – helix packing of four large helices in the center of the tetrameric assembly. The four active site pockets—one on each monomer—are located close to, but not overlapping with, the subunit interfaces. The E_p monomer is a two-domain molecule. The domain containing the active site has overall resemblance, including the location of the active site to other nucleotide binding proteins containing the common α/β "Rossmann" fold. The second E_p domain, which packs tightly to the side of the active site domain, is involved in the intermonomer interactions generating the E_p tetramer. The closest E_p structural homologues include other nucleotidylyltransferases

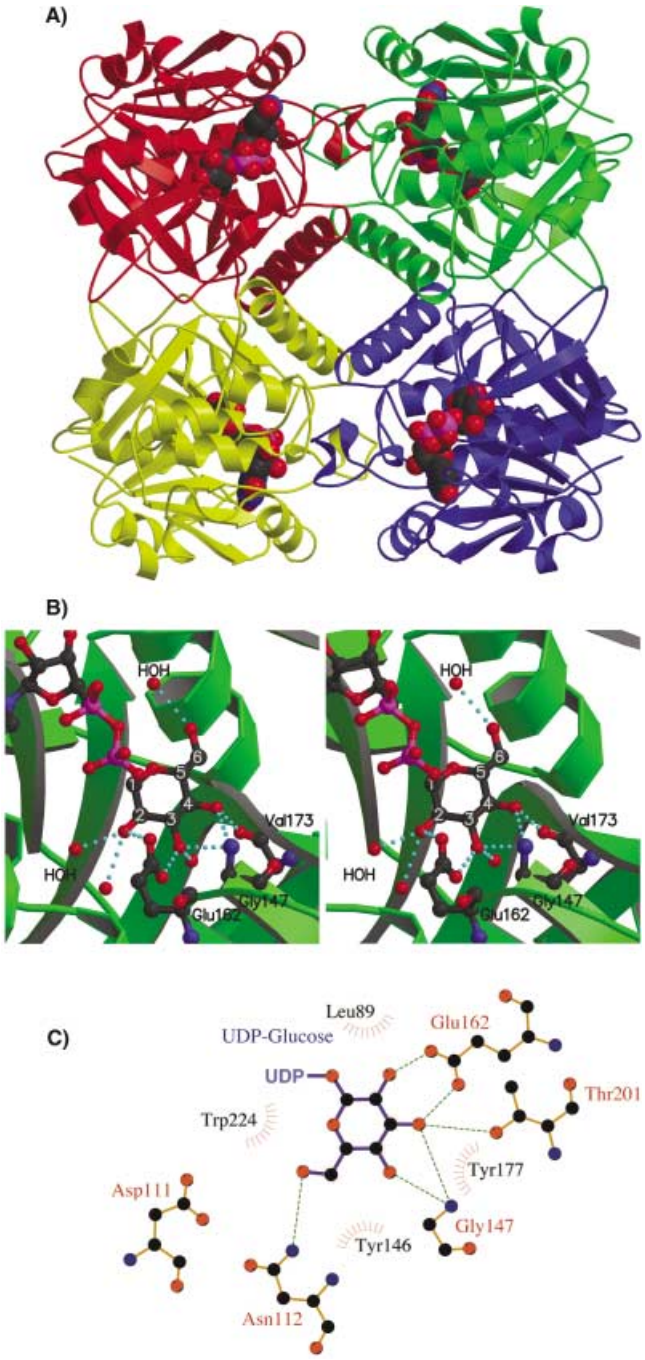


Figure 1. *Structure of E_p. A) structure of the E_p tetramer (PDB ID: 1IIN); B) Stereoview of the E_p active site with bound substrate (reproduced with permission from ref. [18]); C) Schematic representation of the E_p – substrate contacts (reproduced with permission from ref. [19]]).*

and glycosyltransferases, such as the *E. coli* GlmU[32] and the *Bacillus subtilis* SpsA.[33]

The structural studies of E_p and its homologues were complemented by kinetic data to characterize their catalytic mechanism, specifically supporting a sequential ordered S_N2-type single-displacement mechanism and ruling out the alternative ping-pong double-displacement mechanism.[18, 31] The location of the Mg^{2+} cofactor, which is strictly required for enzyme activity, was also identified; this revealed that the metal ion functions by electrostatic stabilization of the leaving group, while also playing a structural role in folding the substrate-binding region of E_p around itself to fix the NTP at an optimal position for the catalytic event.[18]

4. Structure-Based Engineering of E_p and New Substrates Accepted by Mutants

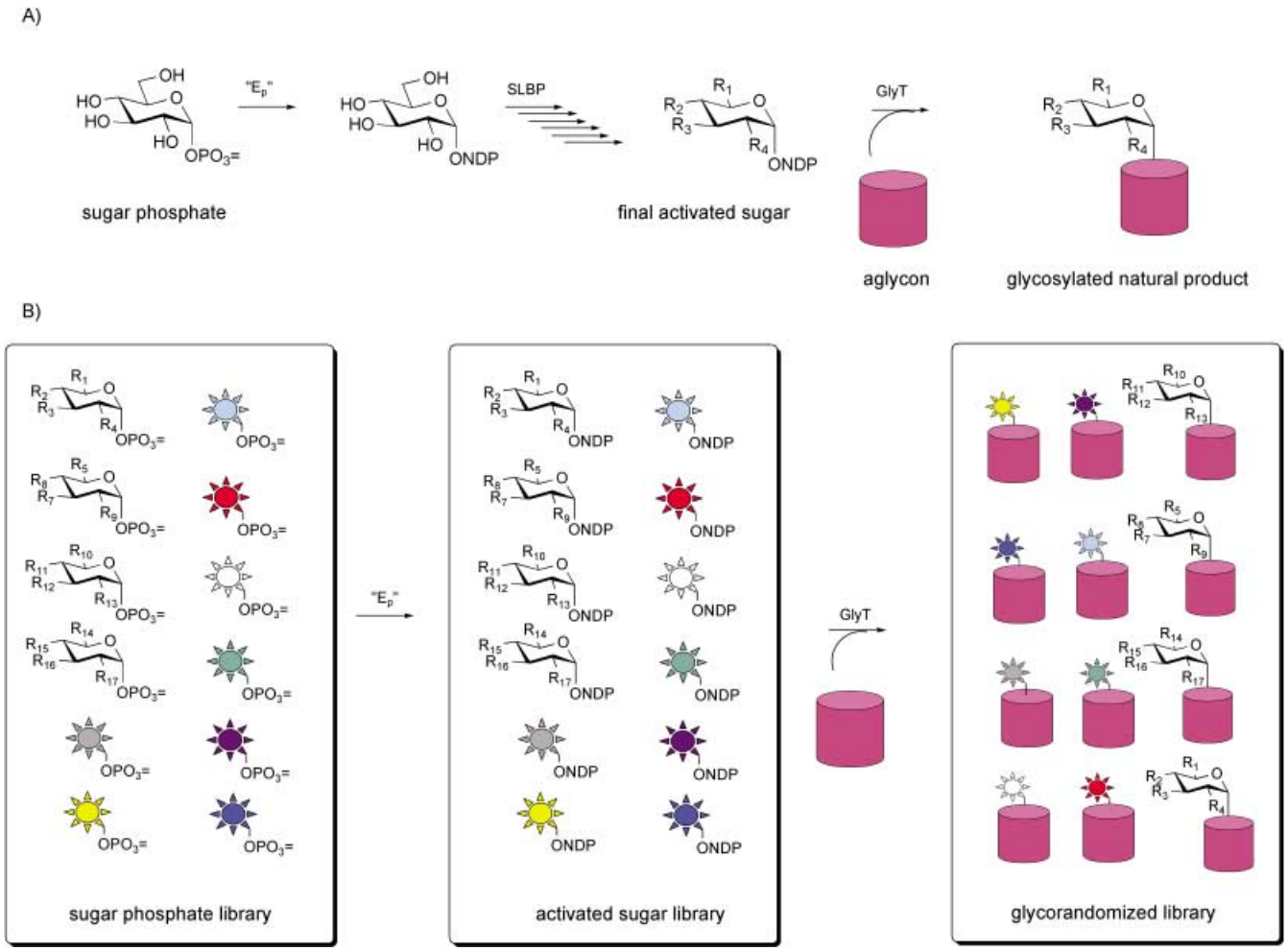
Most importantly, the various reported enzyme – product and enzyme – substrate crystal structures unraveled the precise molecular details of substrate recognition and substrate specificity of E_p (Figure 1 B), providing the information necessary for the enzyme-engineering experiments required for the generation of E_p variants as IVG components. Figure 1 C summarizes the observed contacts between the enzyme and substrate in the active-site pocket. The sugar moiety sits on a hydrophobic bed composed of leucine and isoleucine residues and is positioned by its interaction with several side chains through hydrogen bonding with the sugar hydroxyl groups. By using the structural information as a guide, a systematic effort was initiated to alter residues within the sugar-binding pocket that might hinder the binding of the "unnatural" sugar phosphates that failed as substrates of wild-type E_p. Modeling such substrates into the active site revealed that both steric and electrostatic constraints preclude their binding. In addition to constraints imposed by side-chain atoms, main-chain atoms also prevented access to some sugars; this creates additional challenges to engineering efforts.

C6 substitutions

The initial attempt at rational engineering of E_p substrate promiscuity included two "unnatural" sugar phosphates (Scheme 5 B), acetamido-6-deoxy-α-D-glucopyranosyl phosphate (**12**) and α-D-glucopyranuronic acid 1-(dihydrogen phosphate) (**13**), which have bulky substitutions at the sugar C6 position and are not accepted by the wild-type enzyme.[18] Based upon the determined E_p structure, a W224H mutation was designed to decrease steric constraints imposed at C6 of the substrate while providing a partial positive charge to assist in binding of **13**. Functional analysis of this mutant revealed that it was indeed capable of converting both of the targeted compounds. The structural elucidation of W224H revealed an astonishing and unexpected active site side-chain rearrangement that creates a large gap surrounding C6, clearly consistent with its impressive substrate promiscuity.[19] As an illuminating example of the unexpected in "rational" design, this case is one in which the design expectations and the catalytic outcome are consistent although the precise structural basis is distinct from the predicted structural consequences of the given mutation.

C2/C3 substitutions

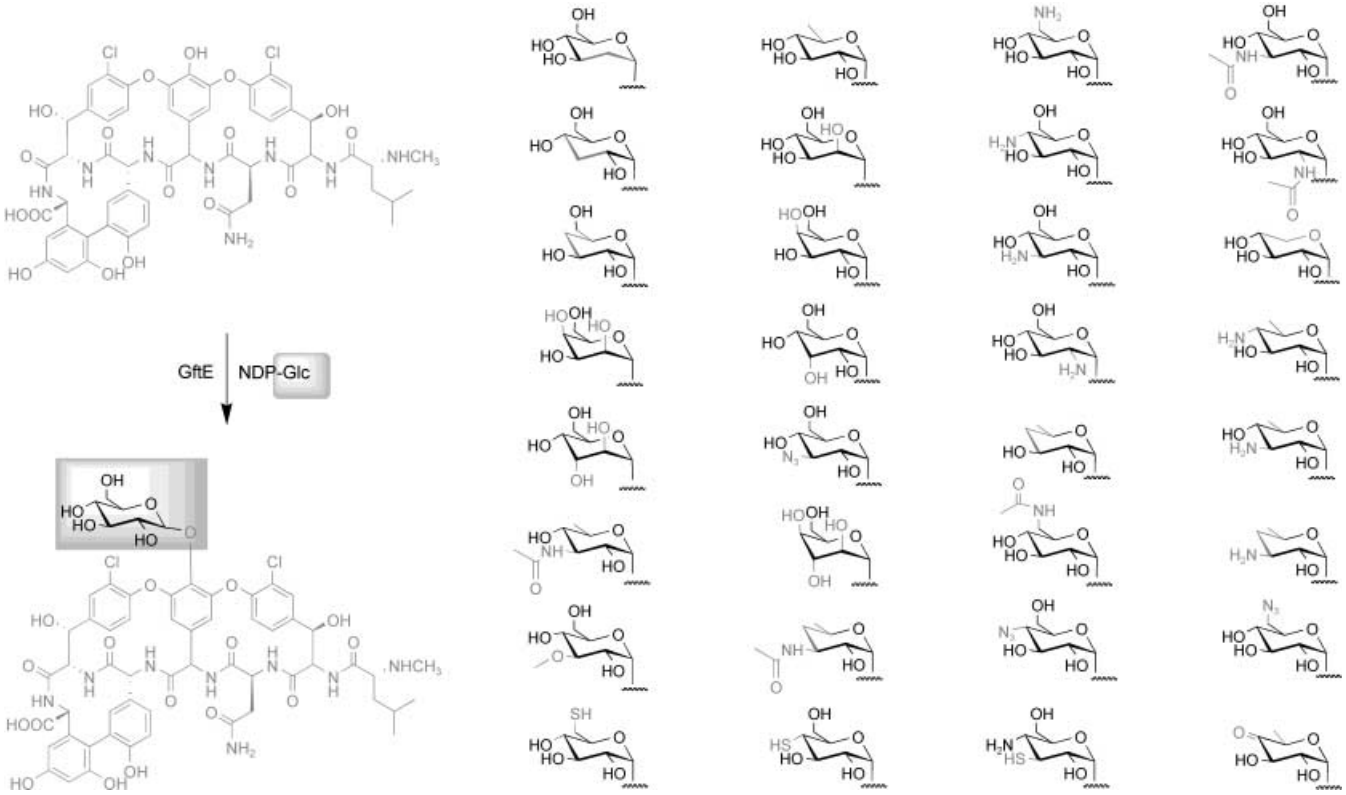
Another engineering challenge involved increasing the promiscuity of E_p toward substrates with unique C2, C3, and C4 substitutions. One such substrate set, the α-D-hexose series,



Scheme 3. *A) The standard route for secondary metabolite glycosylation in vivo. Generation of the fully functionalized activated NDP-sugar can require up to ten distinct transformations by unique enzymes prior to the culminating glycosyltransferase (GlyT)-catalyzed attachment to the aglycon. B) Schematic for natural product in vitro glycorandomization illustrating the great potential for enhanced diversity through the simplistic bypass of the many specific SLBP enzymes.*

contains a number of representatives poorly utilized by the wild-type enzyme.[19] Modeling of failed substrates bearing a C2-epimeric configuration of glucose (altrosyl- (**14**), talosyl- (**15**), and idosyl-phosphate (**16**)) revealed that the main steric infringement upon "unnatural" substrate binding was imposed by the Leu89 side chain. These studies also suggested that a Leu89 – Thr substitution would relieve steric constraints while simultaneously supplying a potential hydrogen-bonding partner. Remarkably, the designed L89T mutant resulted in the production of six new nucleotide sugars.[19] This is especially encouraging since the enhanced promiscuity of L89T did not affect its natural-substrate turnover rate. Furthermore, the conversion of **16**, which is predicted to adopt predominately the $_4C^1$ conformer, suggests that L89T may accept substrates that adopt alternative chair conformations; this adds to the potential utility of this designed mutant. Structural elucidation of the L89T E_p variant revealed that the γ oxygen in Thr89 is approximately 4 Å away from the sugar C2 hydroxyl and that this gain of >1 Å (relative to wild-type E_p) may account for this mutant's ability to accept the C2 epimers of glucose. Furthermore, the L89T structure suggests that, in addition to relieving C2 steric constraints, this mutation also alleviates infringements at C3 and C4 through an adjustment or "slipping" of the sugar base in the enlarged active site pocket. Such "slipping" could also explain its ability to accept alternative chair conformations. Cumulatively, the success of this designed mutation was exceptionally high in that three of the four targeted compounds became successful substrates. In this particular example, the design, anticipated result, and experimental determinations were consistent and highlight the potential of structure-based enzyme engineering.

In a similar fashion, modeling of the poorly utilized substrates containing a C3-epimeric configuration of glucose (altrosyl- (**14**), idosyl- (**16**), allosyl- (**17**), and gulosylphosphate (**18**)) revealed that while main-chain atoms were preventing access, a Tyr177 – Phe substitution (which supports the hexopyranoside ring in the active site pocket) could potentially lower the position of the sugar phosphates and provide the additional space needed by the axial C3 and C2 hydroxyl groups in this epimeric series. Yet, the Y177F mutant was relatively unsuccessful in that a twofold enhancement of the conversion of **17** was the only observable biocatalytic benefit. The Y177F mutant structure revealed that while, as designed, there was adequate room for movement of



Scheme 4. *Representative vancomycin analogues generated by IVG. The typical reaction catalyzed by GtfE is the attachment of α-D-Glc (as illustrated on the left). By using IVG, Glc has been replaced by a number of "unnatural" and uniquely functionalized sugars presenting a library of novel "glycorandomized" vancomycin analogues. Certain members of this library can be further elaborated through specific chemical modifications owing to the incorporation of sugars bearing "reactive handles" (e.g., thiols, azides, amines, and ketones).*

the sugar base lower into the binding pocket, it had actually moved only very slightly.[19] In this example, the structure-based design and anticipated structural consequences were consistent, yet the determined catalytic consequences were not predicted.

In addition to the C6 acetamido derivative discussed in the previous section, attention was also directed to 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate (**19**).[18] A T201A substitution was designed to decrease the steric interference at the sugar positions C2 and/or C3 for compounds and, as expected, was found to substantially increase the conversion of **19**. The structure of the T201A mutant has yet to be determined.

5. Glycosyltransferase Structure and Structure-Based Engineering

Although the structure-based engineering of a glycosyltransferase has yet to be reported, the past few years have seen encouraging progress in this field, and the necessary foundations have clearly been set for this approach. A number of high-resolution glycosyltransferase structures have been reported over the past decade and these structures have been reviewed in a number of compilations.[34, 35] The structure of GtfB (Figure 2 A),[36] which is a glycopeptide glucosyltransferase (GT) decorating the 4-hydroxyphenylglycine of vancomycin's ana-

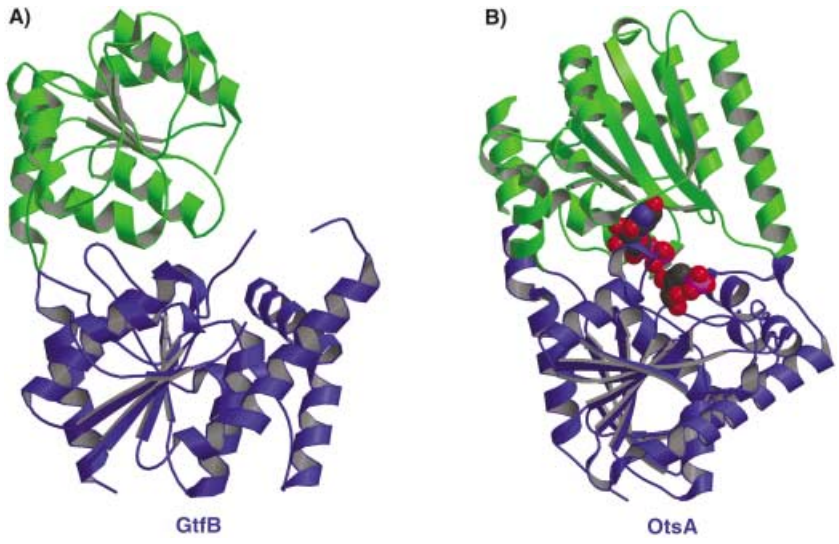
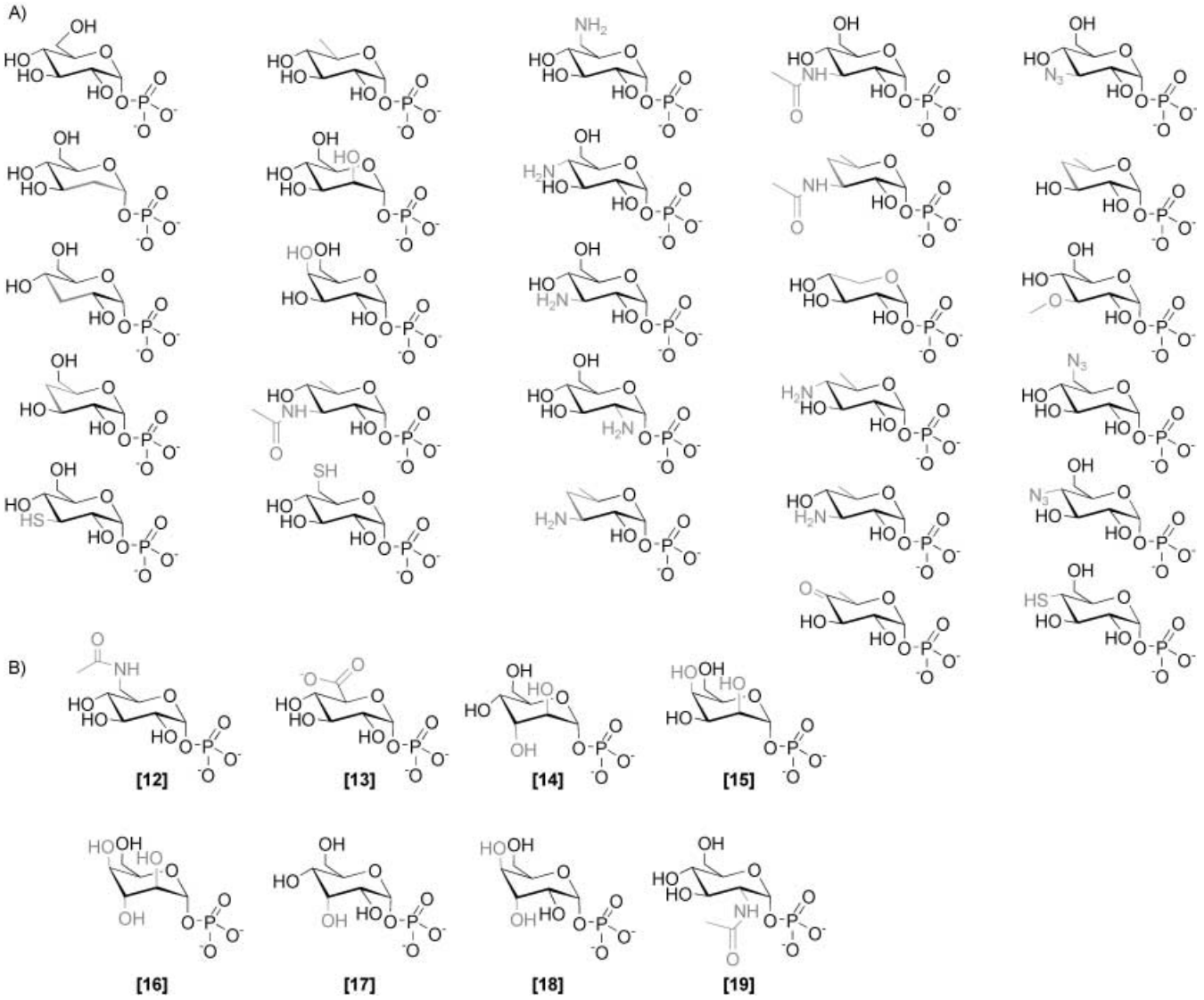


Figure 2. *Structure of related glycosyltransferases A) GtfB (PDB ID: 1IIR); B) OtsA (PDB ID: 1GZS). The N-terminal domain is in green, the C-terminal domain is in blue, the bound UDP and α-D-glucose-6-phosphate substrates are in red/black.*

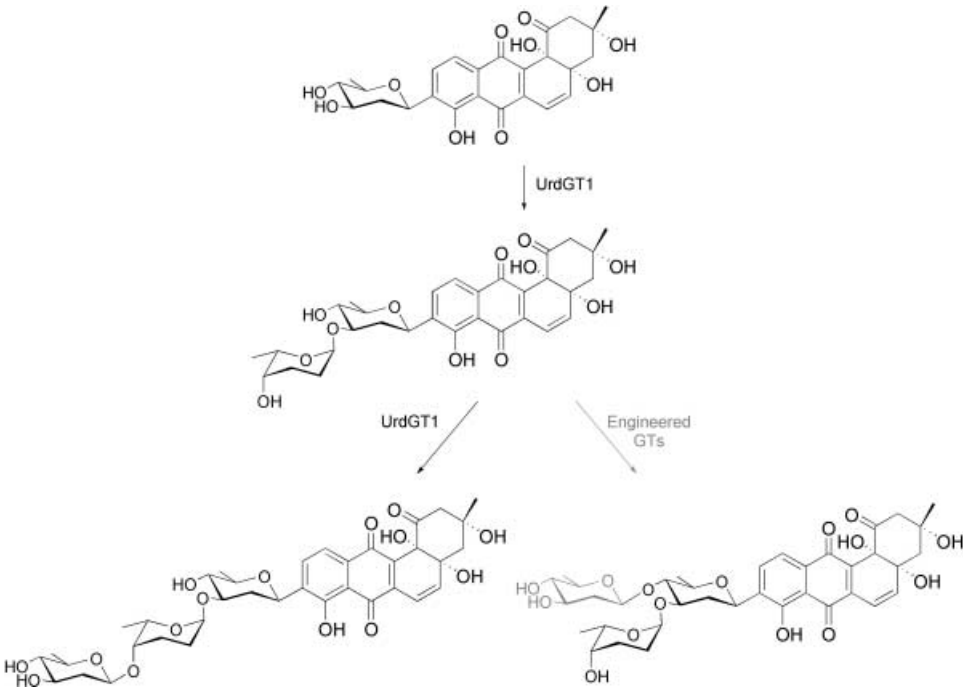


Scheme 5. *A) "Unnatural" substrates of wild-type E_p; B) "Unnatural" substrates of structure-based engineered E_p variants which completely failed (or were very poor) as substrates of the wild-type enzyme.*

logue chloroeremomycin, and the related structure of OtsA (Figure 2B) are particularly appealing for the biosynthetic chemist.[39] The results illustrate that this first natural-product GT structure follows the two-domain design now common to these catalysts. Although attempts to cocrystallize the enzyme with its UDP-Glc donor substrate failed, the interdomain gap could be recognized as a plausible site to accommodate the nucleotide sugar. From sequence homologies it is also reasonable to suggest that the C-terminal domain of GTs harbors the nucleotide-binding apparatus. The acceptor-binding site of GtfB was proposed to reside within the N-terminal domain where a hydrophobic patch was found. While these specific designations have not been tested experimentally, the two-domain blueprint opens up intriguing possibilities for hybrid transferases composed of domains of different origins. Future work must explore whether this approach is suitable to elegantly blend sugar donor and acceptor selectivities of given parental enzymes, thus expanding the possibilities of a glycosyltransfer by design.

6. Current Progress in Glycosyltransferase Engineering

While structure-based engineering of glycosyltransferases is still in the planning phase, designed glycosyltransferases based upon sequence alignment strategies have recently been reported. The biosynthetic route to the angucyclic glycosides urdamycin A and B is unique in that it features two highly homologous glycosyltransferases (UrdGT1b and UrdGT1c, 90% identical), which display different substrate selectivities for both the nucleotide sugar donor and acceptor.[37] In the biosynthesis of urdamycin, UrdGT1c transfers L-rhodinose to an equatorial 3-OH group of the preceding D-sugar, closing an α-(1$\rightarrow$3) glycosidic bond, while UrdGT1b accounts for the attachment of D-olivose through a β-(1$\rightarrow$4) glycosidic bond to the axial 4-OH of L-rhodinose (Scheme 6). The most significant sequence differences between UrdGT1b and UrdGT1c are found in the N-terminal section, particularly between positions 52–82 which



Scheme 6. *Schematic of urdamycin glycosyltransferase engineering.*

are located within a hypervariable section among many natural product GTs. In comparison to the GtfB structure,[36] this region corresponds to the region bridging β3 and β4 in the N-terminal domain.

Recently, a set of elegant random mutagenic and domain-swapping experiments revealed this hypervariable region to dictate both donor and acceptor selectivity in UrdGT1b and UrdGT1c and distinguished ten amino acids as vital for substrate specificity.[38] By randomizing these amino acids in a GT-gene library, the parental enzyme selectivities could be fused. In addition, a new breed of glycosyltransferase was generated in this work which was capable of modifying a unique glycosylation target site. Surprisingly, none of the ten key amino acids identified was able to dictate selectivity independently but instead multiple amino acid alterations within these ten were required to produce the novel catalytic activities in comparison to the wild-type. Future work is expected to focus on harnessing the potential of this region to design multipurpose glycosyltransferases to support both IVG and in vivo engineering of novel natural products.

7. Conclusions and Prospects

In the light of both pure and applied pharmaceutical sciences, enzyme engineering has already greatly supported the development and diversification of bioactive natural products. Researchers dealing with pure science appreciate the detailed insight into enzymatic mechanisms underlying the biosynthesis of natural products and their building blocks (e.g., modified and functionalized sugars). At the applied end, these insights have led, in part, to the IVG approach.

Although IVG is a recent strategy, its versatility has been impressively demonstrated by a library of diversified vancomycins, antibiotics of last resort against multiresistant pathogens. Compared to solely in vivo-based strategies, IVG appears superior in that it smartly integrates the potential of traditional synthetic chemistry and does not select against the most potent metabolites that might kill the microbial host strain. Furthermore, it relies on only a small set of enzymes, thereby eliminating the need for large, multienzymatic cascades. IVG is also advantageous over the pure synthetic approach owing to the complexity of many natural product variants (e.g., nonribosomal peptides like vancomycin).[40]

Future work, including further synthetic or enzymatic sugar processing to feed the IVG process in conjunction with investigations on glycosyltransferases, promises to enhance the diversification of novel or known drug leads.

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Keywords: antibiotics · enzyme catalysis · glycosyl phosphate · natural products · transferases

[1] P. Potier, *Actual. Chim.* **1999**, *11*, 9.
[2] A. C. Weymouth-Wilson, *Nat. Prod. Rep.* **1997**, *14*, 99.
[3] T. Vogt, P. Jones, *Trends Plant Sci.* **2000**, *5*, 380 – 386.
[4] J. S. Thorson, T. J. Hosted, Jr., J. Jiang, J. B. Biggins, J. Ahlert, *Curr. Org. Chem.* **2001**, *5*, 139 – 167.
[5] V. Kren, L. Martinkova, *Curr. Med. Chem.* **2001**, *8*, 1303 – 1328.
[6] P. Jones, T. Vogt, *Planta* **2001**, *213*, 164 – 174.
[7] K. Keegstra, N. Raikhel, *Curr. Opin. Plant Biol.* **2001**, *4*, 219 – 224.
[8] "Glycosylated Natural Products" J. S. Thorson, T. Vogt in *Carbohydrate-Based Drug Discovery* (Ed.: C.-W. Wong), Wiley-VCH, Weinheim, **2002**.
[9] L. S. Zhao, N. L. S. Que, Y. Xue, D. H. Sherman, H.-w. Liu, *J. Am. Chem. Soc.* **1998**, *120*, 12 159 – 12 160.
[10] L. S. Zhao, D. H. Sherman, H.-w. Liu, *J. Am. Chem. Soc.* **1998**, *120*, 10 256 – 10 257.
[11] S. A. Borisova, L. S. Zhao, D. H. Sherman, H.-w Liu, *Org. Lett.* **1999**, *1*, 133 – 136.
[12] L. S. Zhao, J. Ahlert, Y. Q. Xue, J. S. Thorson, D. H. Sherman, H.-w. Liu, *J. Am. Chem. Soc.* **1999**, *121*, 9881 – 9882.

[13] a) T. Ohuchi, T. A. Ikeda-Araki, A. Watanabe-Sakamoto, K. Kojiri, M. Nagashima, M. Okanishi, H. Suda, *J. Antibiot*. **2000**, *53*, 393; b) C. Sanchez, I. A. Butovich, A. F. Brana, J. Rohr, C. Mendez, J. A. Salas, *Chem. Biol*. **2002**, *9*, 519 – 531; c) C.-G. Hyun, T. Bililign, J. Liao, J. S. Thorson, *ChemBioChem* **2003**, *1*, 114 – 117.
[14] C. Albermann, C.-G. Hyun, J. S. Thorson, unpublished results.
[15] X. M. He, H.-w. Liu, *Annu. Rev. Biochem*. **2002**, *71*, 701 – 754.
[16] J. Jiang, J. B. Biggins, J. S. Thorson, *J. Am. Chem. Soc*. **2000**, *122*, 6803 – 6804.
[17] J. Jiang, J. B. Biggins, J. S. Thorson, *Angew. Chem*. **2001**, *113*, 1550 – 1553; *Angew. Chem. Int. Ed*. **2001**, *40*, 1502 – 1505.
[18] W. A. Barton, J. Lesniak, J. B. Biggins, P. D. Jeffrey, J. Jiang, K. R. Rajashankar, J. S. Thorson, D. B. Nikolov, *Nat. Struct. Biol*. **2001**, *8*, 545 – 551.
[19] W. A. Barton, J. B. Biggins, J. Jiang, J. S. Thorson, D. B. Nikolov, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 13 397 – 13 402.
[20] J. Jiang, C. Albermann, J. S. Thorson, *ChemBioChem* **2003**, *4*, 443 – 446.
[21] P. J. Solenberg, P. Matsushima, D. R. Stack, S. C. Wilkie, R. C. Thompson, R. H. Baltz, *Chem. Biol*. **1997**, *4*, 195 – 202.
[22] H. C. Losey, M. W. Peczuh, Z. Chen, U. S. Eggert, S. D. Dong, I. Pelczer, D. Kahne, C. T. Walsh, *Biochemistry* **2001**, *40*, 4745 – 4755.
[23] H. C. Losey, J. Jiang, J. B. Biggins, M. Oberthur, X.-Y. Ye, S. D. Dong, J. S. Thorson, D. Kahne, C. T. Walsh, *Chem. Biol*. **2002**, *9*, 1305 – 1314.
[24] X. Fu, C. Albermann, J. Jiang, J. Liao, C. Zhang, J. S. Thorson, *Nat. Biotechnol*. **2003**, in press.
[25] C. Albermann, J. Jiang, J. S. Thorson, unpublished results.
[26] J. M. Langenhan, J. Liao, J. S. Thorson, unpublished results.
[27] E. T. Farinas, T. Bulter, F. H. Arnold, *Curr. Opin. Biotech*. **2001**, *12*, 545 – 551.
[28] H. Y. Tao, V. W. Cornish, *Curr. Opin. Chem. Biol*. **2002**, *6*, 858 – 864.
[29] L. Lindquist, R. Kaiser, P. R. Reeves, A. A. Lindberg, *Eur. J. Biochem*. **1993**, *211*, 763 – 770.
[30] W. Blankenfeldt, M. Asuncion, J. S. Lam, J. H. Naismith, *EMBO J*. **2000**, *19*, 6652 – 6663.
[31] S. Zuccotti, D. Zanardi, C. Rosario, L. Sturla, M. Tonetti, M. Bolognesi, *J. Mol. Biol*. **2001**, *313*, 831 – 843.
[32] K. Brown, F. Pompeo, S. Dixon, D. Mengin-Lecreulx, C. Cambillau, Y. Bourne, *EMBO J*. **1999**, *18*, 4096 – 4107.
[33] S. J. Charnock, G. J. Davies, *Biochem*. **1999**, *38*, 6380 – 6385.
[34] U. M. Ünligil, J. M. Rini, *Curr. Opin. Struc. Biol*. **2000**, *10*, 510 – 517.
[35] Y. Hu, S. Walker, *Chem. Biol*. **2002**, *9*, 1287 – 1296.
[36] A. M. Mulichak, H. C. Losey, C. T. Walsh, R. M. Garavito, *Structure* **2001**, *9*, 547 – 557.
[37] D. Hoffmeister, K. Ichinose, A. Bechthold, *Chem. Biol*. **2001**, *8*, 557 – 567.
[38] D. Hoffmeister, B. Wilkinson, G. Foster, P. J. Sidebottom, K. Ichinose, A. Bechthold, *Chem. Biol*. **2002**, *9*, 287 – 295.
[39] R. P. Gibson, J. P. Turkenburg, S. J. Chamock, R. Lloyd, G. J. Davis, *Chem. Biol*. **2002**, *9*, 1337 – 13 346.
[40] D. L. Boger, *Med. Res. Rev*. **2001**, *21*, 356 – 381.

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