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Learning Nature's Strategies for Making Deoxy Sugars: Pathways, Mechanisms, and Combinatorial Applications

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I. Introduction

Carbohydrates have been the focus of growing attention among biological molecules in recent years due to the increased recognition of their vital roles in many physiological processes. Areas in which their significance has been well-established include cellular adhesion and cell−cell recognition, fertilization, protein folding, neurobiology, xenotransplantation, and target recognition in the immune response. Apart from the expanding appreciation of carbohydrates as a whole, there exists a distinct class of carbohydrates that has received special attention—the deoxysugars. These sugars are derived from common sugars by the replacement of at least one hydroxyl group with a hydrogen or a non-*O*-linked functional group.[1] Such a substitution generally induces a fundamental change in the chemical properties of the resulting sugar, such as enhancement of its thermal stability and hydrophobicity. These properties contribute to the wide range of activities exhibited by deoxysugars as a class, including their role in lipopolysaccharides, glycoproteins, and glycolipids, where they serve as ligands for cell−cell interactions or as targets for toxins, antibodies, and microorganisms.[2] An increasing number of medical disorders have been ascribed to anomalies in the sugar composition of various glycoconjugates. In addition to their involvement in human physiology, the deoxysugars also serve as important constituents of numerous secondary metabolites in bacteria, including cardiac glycosides and macrolide antibiotics. Removal of deoxysugars from these clinically relevant molecules often abolishes their efficacy or greatly decreases their specificity.[2] Considering these reasons, the possibility of altering or exchanging the crucial deoxysugar structure(s) to enhance or vary the physiological characteristics of the parent molecules is appealing. Achievement of such a goal, however, relies on a thorough understanding of the deoxysugar biosynthetic pathway of each target molecule, including genetic, enzymic, and mechanistic information.

Inspired by the intriguing biological activities of this class of unusual sugars and the potentially perplexing multistep conversions required for their formation, we initiated a research program to study the pathways and mechanisms of deoxysugar biosynthesis more than a decade ago. Our main efforts have focused on the 3,6-dideoxyhexoses found almost exclusively in the lipopolysaccharide of Gram-negative bacteria.[1] These deoxysugars are well-known antigenic determinants that contribute to the serological specificity of many immunologically active polysaccharides. Mechanistic and genetic information gleaned from our studies of 3,6-dideoxyhexoses have facilitated our later investigations into the biosynthesis of 2,6- and 4,6-dideoxyhexoses, which are commonly found in cardiac glycosides and antibiotics. More recently, we have also started to explore the feasibility of altering nature's biosynthetic machinery to make modified deoxysugars. There is little doubt that the progress made in understanding deoxysugar biosynthesis is due to joint contributions from many research teams working on different fronts. However, this Account is not intended to be an update of the entire field; instead, this is a highlight of what has been learned thus far from our own work, focusing on the highly studied 3,6-dideoxyhexoses and how these insights have allowed the postulation of biosynthetic pathways for other deoxysugars found in macrolide antibiotics. In addition, the impact of this knowledge on the emerging field of combinatorial biosynthesis is also discussed.

II. Biosynthesis of 3,6-Dideoxyhexoses

Among the vast number of monosaccharides identified as components of *O*-specific polysaccharides, the 3,6-dideoxyhexoses have attracted particular attention due to their established roles in defining microbial pathogenicity and virulence.[1] The biosynthesis of 3,6-dideoxyhexoses, represented by the formation of ascarylose (**8**, 3,6-dideoxy-L-*arabino*-hexose) found in the *O*-antigen of *Yersinia pseudotuberculosis* VA, is shown in Figure 1.[3] Conversion of D-glucose-1-phosphate (**1**) and cytidine triphosphate (CTP) to the nucleotide sugar cytidine 5′-diphospho-D-glucose (CDP-D-glucose) (**2**) by α-D-glucose-1-phosphate cytidylyltransferase (E_p) initiates the pathway.[4] This is followed by an irreversible intramolecular oxidation−reduction catalyzed by the NAD^+-dependent CDP-D-glucose 4,6-dehydratase (E_{od}).[5] The nascent product, CDP-

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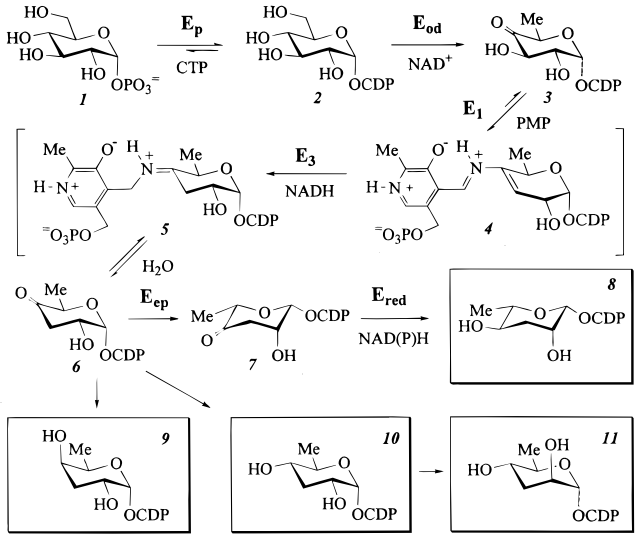


FIGURE 1.

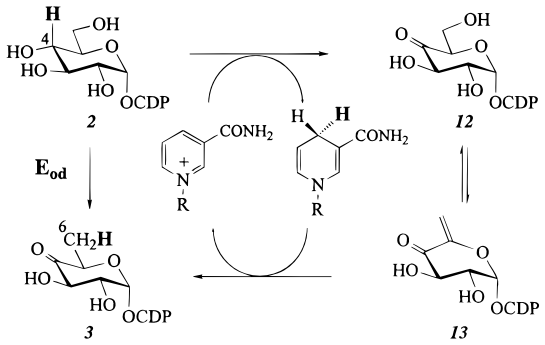


FIGURE 2.

6-deoxy-D-*glycero*-L-*threo*-4-hexulose (**3**), is then transformed into CDP-3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose (**6**) in two consecutive enzymatic reactions mediated by CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose-3-dehydrase (E_1)[6] and CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose-3-dehydrase reductase (E_3).[7] The final conversions are carried out by CDP-3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose-5-epimerase (E_{ep}), which inverts the configuration of **6** at C-5, and CDP-3,6-dideoxy-D-*glycero*-L-*glycero*-4-hexulose-4-reductase (E_{red}), which catalyzes the reduction of **7** at C-4 to give the desired product, CDP-L-ascarylose (**8**).[8]

As illustrated in Figure 1, the product of E_1/E_3 catalysis also serves as the immediate precursor for CDP-D-abequose (**9**), CDP-D-paratose (**10**), and CDP-D-tyvelose (**11**).[3] Clearly, the subsequent actions of a stereospecific reductase and/or epimerase in each case determine the final outcome for this common intermediate, providing the structural diversity in this class of unusual sugars. To study the overall pathway of ascarylose formation and to elucidate the mechanism of each enzymatic transformation, we have cloned, sequenced, and expressed most of the genes in the *asc* (ascarylose) gene cluster.[9] The pioneering work by Strominger and co-workers, combined with our own efforts in this area, has demonstrated that the two key deoxygenation events at C-6 and C-3 are the most interesting steps in the ascarylose biosynthetic sequence.

II.A. Mechanism of C-6 Deoxygenation. The C-6 deoxygenation catalyzed by E_{od}, a homodimeric enzyme isolated from *Y. pseudotuberculosis*, is a prototypical reaction necessary for the biosynthesis of all 6-deoxyhexoses.[5] Various experiments have shown that E_{od} catalysis consists of three discrete steps as illustrated in Figure 2: oxidation of CDP-D-glucose (**2**) to the corresponding 4-ketohexose **12**, C5/C6 dehydration to a 4-keto-$\Delta^{5,6}$-glucoseen intermediate (**13**), and reduction at C-6 to give the 4-keto-6-deoxyhexose product **3**.[5] Generation of the 4-keto group lowers the pK_a of the 5-H and, thus, facilitates the subsequent C5/C6 dehydration. Since the 4-keto group remains intact in the E_{od} product, it also serves as an activating group promoting transformations beyond the E_{od} reaction to the final products (see Figure 1). As depicted in Figure 2, the overall transformation catalyzed by E_{od} is in fact an intramolecular oxidation–reduction where an enzyme-bound NAD^+ receives the 4-H as a hydride in the oxidative half-reaction and passes the reducing equivalents to C-6 of the dehydration product **13** in the reductive half-reaction. While most other nicotinamide-dependent enzymes utilize $NAD(P)^+$ as a cosubstrate, E_{od} clearly belongs to a select group of enzymes in which the pyridine nucleotide coenzyme is regenerated after each catalytic cycle and is in essence a catalytic prosthetic group.

To develop a means to control and/or regulate this important enzymatic catalysis, we have synthesized CDP-6-deoxy-6,6-difluoro-α-D-glucose (**14**) and have determined that it is a mechanism-based inactivator for E_{od}.[10] The inactivation is active-site directed and irreversible, with a k_{inact} of 2.4×10^{-2} min^{-1} and a K_I of 0.94 mM. Further analysis indicated that the inactivation is likely the result of the covalent trapping of an active site nucleophile by the 4-keto-$\Delta^{5,6}$-glucoseen (**13**), which is the expected turnover product of the inhibitor **14** after being processed by E_{od} (Figure 3). Detection of **12** as the actual turnover product lent further credence to the proposed mechanism. This difluoroglucose derivative is the first mechanism-based inactivator known for E_{od} and has the potential to serve as a general inhibitor for this class of enzymes. Since C-6 deoxygenation is the common entry to the formation of all 6-deoxyhexoses,[1] many of which play key roles as cellular components or as an indispensable part of secondary metabolites, the development of effective inhibitors for E_{od} and perhaps other enzymes of this class may afford better control and regulation of the biosynthesis of these unusual sugars.

II.B. Mechanism of C-3 Deoxygenation. C-3 deoxygenation, on the other hand, is a more difficult process that requires two enzymes: E_1, a pyridoxamine 5′-phosphate (PMP)/[2Fe-2S]-containing homodimeric enzyme,[6] and E_3, a NADH-dependent [2Fe-2S]-containing flavoenzyme.[7] While E_3 closely resembles many electron-transfer iron–sulfur flavoproteins in the ferredoxin-$NADP^+$ reductase (FNR) family,[7c] E_1 stands alone as a coenzyme B_6-dependent catalyst that recognizes PMP rather than PLP (pyridoxal 5′-phosphate), and also con-

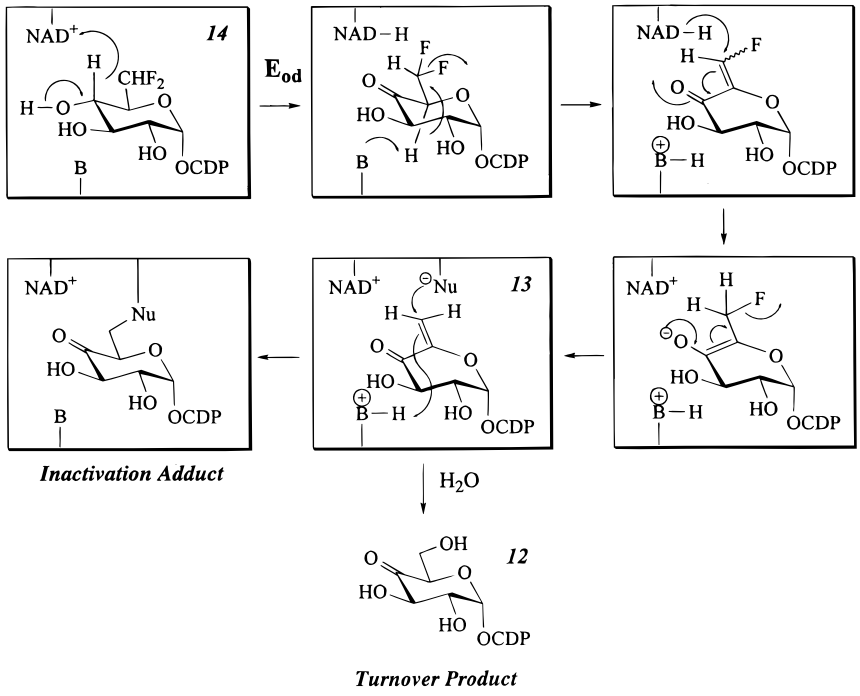


FIGURE 3.

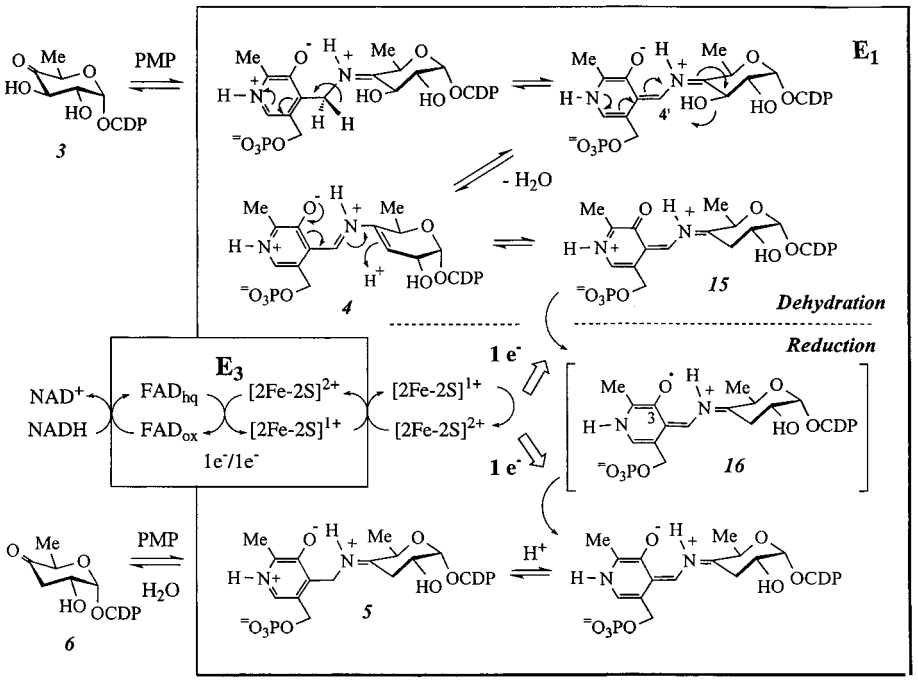


FIGURE 4.

tains an essential iron−sulfur cluster.[6c,d] A recent study revealed that a complex between E_1 and E_3 is a prerequisite for activity, and the apparent dissociation constant for the complex was estimated to be 288 nM.[11]

On the basis of chemical and spectroscopic evidence, the overall reaction consists of two events—dehydration and reduction (Figure 4).[3] The E_1 reaction is initiated by Schiff base formation between **3** and enzyme-bound PMP, followed by abstraction of the C-4′ *pro-S* hydrogen by an active site base, His220, which triggers expulsion of the 3-OH group to give a $\Delta^{3,4}$-glucoseen intermediate (**4**).[6b,d] While the intermediate PMP−$\Delta^{3,4}$-glucoseen complex (**4**) has never been isolated, reversible hydration at the *si* face of **4** has been demonstrated by isotopic incorporation of $[^{18}O]H_2O$.[6b] It has also been shown that the replacement of the 3-OH group with a solvent hydrogen in the E_1−E_3 coupled reaction proceeds with retention of configuration.[12] Together, these observations imply that the catalysis in the active site of E_1 is likely a suprafacial process occurring at the *si* face of the PMP−substrate complex, as is the case with most other coenzyme B_6-dependent enzymes.[13]

A clear relationship between E_1 and other coenzyme B_6-dependent enzymes is revealed by sequence align-

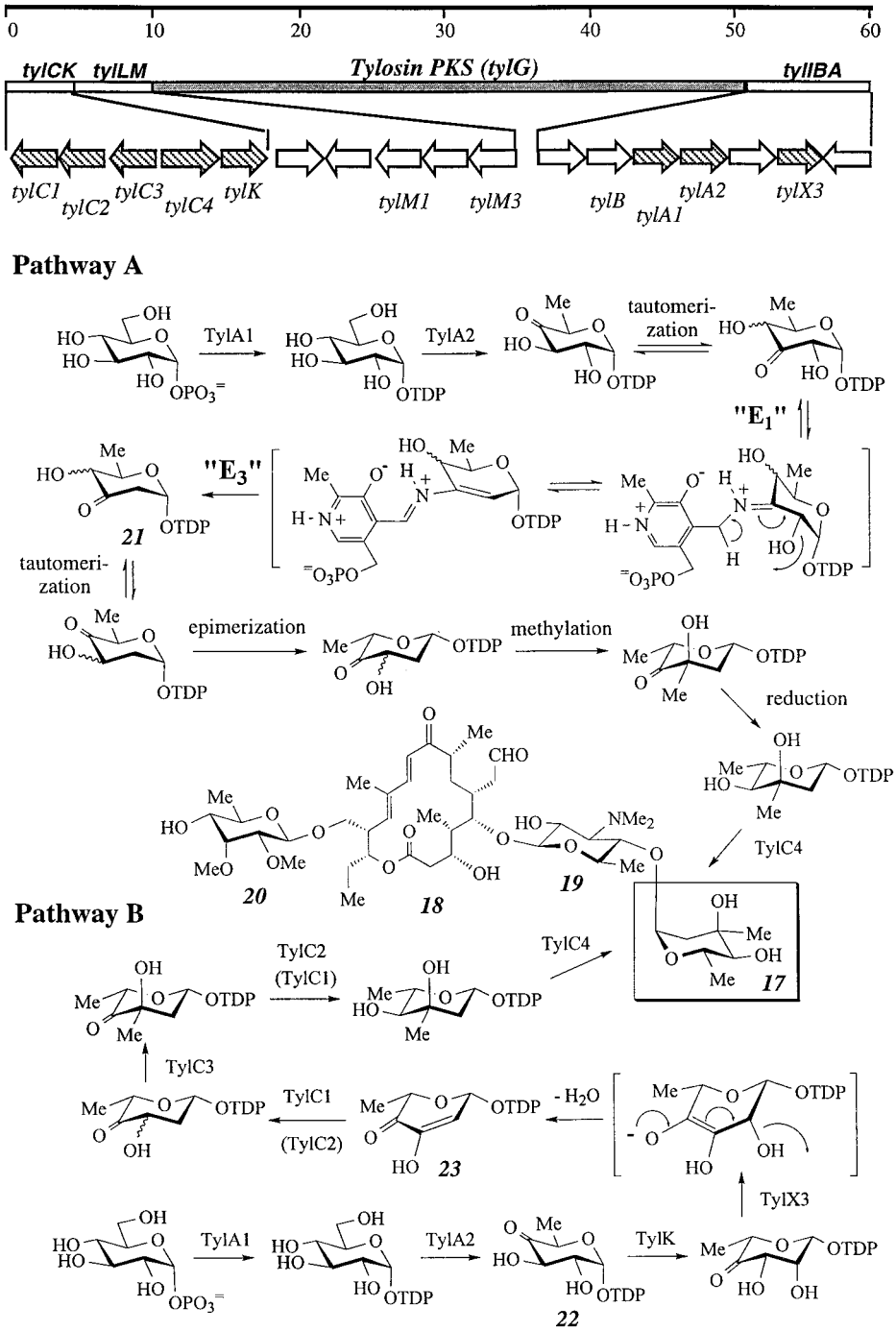


FIGURE 5.

ments.[6d] One significant difference, though, is that the highly conserved lysine in a typical PLP-dependent catalyst has been replaced with a histidine at position 220 in E_1. This lysine normally forms a Schiff base with PLP and also functions as the base catalyzing the deprotonation of the substrate−coenzyme aldimine adduct to initiate the catalysis. Since the substitution with histidine is unique to E_1, which only recognizes PMP, it is conceivable that this single point substitution (Lys → His) may be responsible for converting a normal PLP-dependent enzyme to a PMP-dependent dehydrase.

The subsequent reduction of the glucoseen intermediate by E_3 begins with a hydride transfer from NADH to FAD, followed by sequential one-electron transfers from the reduced flavin through the iron−sulfur centers of E_3 and E_1 to give **5**.[7a] Since these iron−sulfur centers are one-electron carriers and are essential for catalysis, the reduction of the PMP−glucoseen intermediate (**4**) must occur via a radical process.[13] The presence of two radical intermediates during turnover was detected by stopped-flow spectrophotometry and rapid freeze-quench EPR under both aerobic and hypoxic conditions.[14] While the first radical is clearly the flavin semiquinone, the nature of the second radical, which has a *g* value of 2.003 and shows no apparent hyperfine splitting, is less well-characterized. Of the few conceivable routes to form such

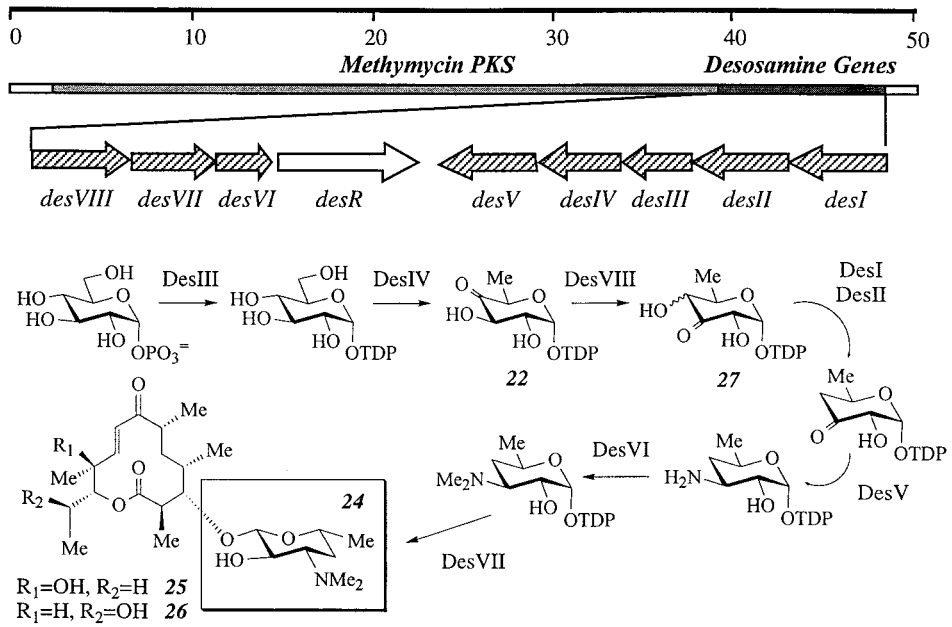


FIGURE 6.

an organic radical intermediate, that in which the unpaired spin develops primarily on the C-3 oxygen of PMP (**16**) is considered the most reasonable and most consistent with the observed data.

Formation of the proposed phenoxyl radical **16** has been hypothesized to involve a tautomerization of the glucoseen−PMP intermediate **4** to a PMP−quinone methide species (**15**), which can then serve as the electron acceptor during the reduction.[13,14] Thus, PMP in this catalysis appears to have a dual function of being responsible for the anion-induced dehydration and also of being an integral part of the subsequent redox process. This combined capacity to effect both dehydration and one-electron redox reactivity places E_1 in its own class, with the C-3 deoxygenation a unique example of a C−O bond cleavage event. Moreover, the PMP−glucoseen radical can now be added to the growing list of mechanistically important bioorganic radical intermediates.[15]

III. Biosynthesis of Other Deoxysugars

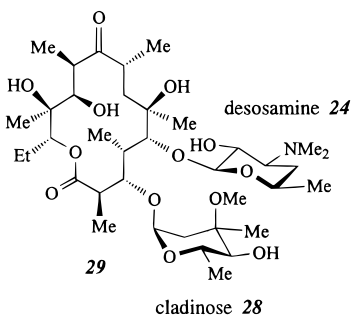
The insights gained from our studies of the biosynthesis of 3,6-dideoxyhexoses have made us more appreciative of the catalytic divergence of the modes of deoxygenation and the mechanistic intricacies of the enzymes involved. More importantly, this knowledge has laid the foundation for exploring the biosynthesis of other unusual deoxysugars present in secondary metabolites, especially those found in various polyketide and aminoglycoside antibiotics. Early biosynthetic work on polyketide antibiotics has focused on the genetics of polyketide synthases (PKSs), which catalyze the aglycon skeleton assembly.[16] Recent sequencing results beyond the PKS region, as well as correlation of blocked mutants with phenotypes, have provided critical evidence revealing that the sugar biosynthetic genes are also part of the overall antibiotic biosynthetic gene cluster.[3] However, unlike the PKS gene cluster that is composed of highly organized modules, the sugar biosynthetic genes are scattered at both ends of the PKS cluster, making it a challenge to distinguish them from those for regulation or aglycon modification that are also interspersed in the same region. Furthermore, in compounds with more than one attached sugar moiety, the assignment of these genes to the appropriate sugar biosynthetic pathway can be quite difficult. Despite these obstacles, a number of sugar biosynthetic genes from various antibiotic biosynthetic pathways have been sequenced. Comparisons of these gene sequences have enabled the initial proposals for the biosynthesis of these sugars. Summarized below is the current knowledge on the biosynthesis of 2,6- and 4,6-dideoxyhexoses as exemplified by our studies on the formation of L-mycarose (**17**) from tylosin (**18**; see Figure 5) and D-desosamine (**24**) from methymycin/neomethymycin (**25/26**; see Figure 6). It should be noted that both D-desosamine and an L-mycarose derivative (L-cladinose, **28**) are also found in erythromycin (**29**) of *Saccharopolyspora erythraea*, whose sugar biosynthetic gene clusters have recently been sequenced.[17] The availability of the gene information of the *ery* cluster has allowed us to confirm the postulated assignment of the biosynthetic genes of these two unusual sugars in different organisms.

III.A. Biosynthesis of Mycarose: Possible Mechanism of C-2 Deoxygenation. Mycarose (**17**) is a 2,6-dideoxy-D-*threo*-4-hexose found in many antibiotics, including tylosin (**18**) and erythromycin (**29).** To study the biosynthesis of this unusual sugar, we chose tylosin as our working system. Tylosin is a macrolide antibiotic isolated from *Streptomyces fradiae* and is composed of a polyketide aglycon and three unusual sugars—mycarose (**17**), mycaminose (**19**), and mycinose (**20**). Early studies on the tylosin biosynthetic gene cluster (*tyl*) have shown that the *tylG* region harbors the PKS genes, while the *tylIBA*, *tylLM*, and *tylCK* regions contain genes for mycaminose and mycarose formation (Figure 5).[18] Recently, we have se-

quenced the extended segments flanking the PKS genes and have identified 17 open reading frames (ORFs) in the *tylCK*, *tylLM*, and *tylIBA* regions. Further analysis based on sequence similarities to other sugar biosynthetic genes, especially those reported by Cundliffe and co-workers, who have also sequenced the *tylIBA* and *tylLM* segments of the *tyl* cluster,[19] has allowed most of the ORFs in these regions to be tentatively assigned.

As highlighted in Figure 5, two genes in the *tylIBA* region are believed to be involved in the biosynthesis of all three deoxysugars: *tylA1* encodes α-D-glucose-1-phosphate thymidylyltransferase (E_p equivalent), and *tylA2* encodes TDP-D-glucose 4,6-dehydratase (E_{od} equivalent). Other genes that are assigned to enzymes involved in the mycarose pathway reside in the *tylCK* region: *tylC1* and *tylC2* are both homologous to sugar oxidoreductase genes, *tylC3* is the putative *S*-adenosylmethionine-dependent *C*-methyltransferase gene, and *tylK* and *tylC4* are the epimerase and glycosyltransferase genes, respectively. Initially, it was presumed that formation of mycarose (**17**) would resemble that of 3,6-dideoxysugars since the only required modification would be tautomerization of the C-4 keto group to C-3 before dehydration and reduction by an E_1/E_3 equivalent (Figure 5, pathway A). However, examination of the *tylCK* genes revealed that both the E_1 and E_3 equivalents are missing in this region. This result indicated that *S. fradiae* must rely on alternative chemistry to accomplish the task of C-2 deoxygenation.

More insight into the mechanism of C-2 deoxygenation was gained upon comparison of the mycarose genes from the tylosin cluster with genes that have been identified for mycarose biosynthesis in the *ery* cluster. As expected, a similar set of genes encoding two reductases (*eryBII* and *eryBIV*), an epimerase (*eryBVII*), a methyltransferase (*eryBI*), and a glycosyltransferase (*eryBV*)[17] were also found in the *ery* cluster. However, an additional gene in the *ery* cluster, *eryBVI*, has been postulated to be involved in mycarose biosynthesis and is a likely candidate for the dehydrase gene needed for C-2 deoxygenation. This gene is highly homologous to the unassigned gene *tylX3* in the tylosin cluster that is present downstream in the *tylIBA* region (Figure 5). Initially, *tylX3* was surmised to be part of the amination machinery in the biosynthesis of mycaminose (**19**), which is the aminodeoxysugar of tylosin. If this is the case, its equivalent, *eryBVI*, in the *ery* cluster must also play a similar role in the biosynthesis of desosamine (**24**), the aminosugar component of erythromycin (**29**). However, since no such gene (*tylX3/eryBVI*) was found in the methymycin/neomethymycin gene cluster (see section III.B), which also contains desosamine as the only sugar component, the involvement of *eryBVI* and *tylX3* in the formation of aminosugars can be excluded. Instead, they are more likely the long-sought dehydrase genes whose translated products catalyze the C-2 deoxygenation.



Therefore, sequence comparison of these genes has led to an alternative pathway for the biosynthesis of mycarose, which includes dehydration, reduction, methylation, and a second reduction (Figure 5, pathway B). Although the identities of TylA1 and TylA2 are unequivocal, the remaining events leading to mycarose formation are as yet only tentatively established. For example, the assigned roles of the TylC1 and TylC2 proteins may be reversed, and the epimerization catalyzed by TylK may occur at a later stage. In addition, the catalytic mechanism of TylX3 remains unresolved. However, nature has clearly devised a different approach to accomplish C-2 deoxygenation in the biosynthesis of mycarose via a reductive cleavage distinct from both C-6 and C-3 deoxygenations found in the biosynthesis of 3,6-dideoxyhexoses.

III.B. Biosynthesis of Desosamine: Possible Mechanism of C-4 Deoxygenation. Desosamine (**24**) is a 3-amino-3,4,6-trideoxyhexose found in several macrolide antibiotics such as methymycin (**25**), neomethymycin (**26**), narbomycin, picromycin, and erythromycin (**29**). To investigate the biosynthesis of this prototypical aminodeoxy sugar, we chose to study methymycin and neomethymycin produced in *Streptomyces venezuelae*.[20] A 13 kilobase (kb) stretch of DNA downstream from the PKS genes of the methymycin/neomethymycin gene cluster was found to harbor the entire desosamine biosynthetic gene cluster.[20,21] Among the nine ORFs mapped in this segment, eight are believed to be involved in desosamine formation. The ORF assignments are based on sequence similarities to other sugar biosynthetic genes, especially those derived from the erythromycin cluster that has been independently characterized by Gaisser et al.[17a] and Summers et al.[17c]

The translated amino acid sequences of *desIII* and *desIV* are very similar to those of *tylA1* and *tylA2* found in the tylosin cluster,[19] and thus, can be assigned to code for α-D-glucose-1-phosphate thymidylyltransferase and TDP-D-glucose 4,6-dehydratase, respectively, as depicted in Figure 6. Sequence alignments have also allowed the *desVI* and *desVII* genes to be designated as an *N*,*N*-dimethyltransferase[22] and a glycosyltransferase,[20] respectively. Both *desI* and *desV* gene products exhibit modest sequence homology to E_1 (25% and 23% identity, respectively) in the 3,6-dideoxysugar pathway. However, DesI and DesV both show stronger similarity to TylB (36% and 60% identity, respectively),[23] a putative PLP-dependent aminotransferase gene from the tylosin cluster (Figure 5).[19a] Since DesV more closely resembles TylB, it is conceivable that the DesV protein is a PLP-dependent aminotransferase that effects C-3 amination, while the DesI enzyme acts like an actual E_1 mimic whose function is to catalyze C-4 deoxygenation in desosamine formation. However, it

should be pointed out that E_1 not only is a PMP enzyme, but also contains a [2Fe-2S] cluster in the active site.[6c,d] The lack of a presumed iron−sulfur binding motif in DesI clearly reflects the distinction between DesI and E_1. The only significant homologue of *desII* in the database is *eryCV* from the erythromycin cluster, which has been postulated to be a reductase that participates in C-4 deoxygenation to make desosamine.[17a,c] Thus, *desII* may also be assigned a similar role in the formation of desosamine in the methymycin/neomethymycin pathway. The remaining gene, *desVIII*, displays protein sequence similarity to a number of P-450 enzymes, but lacks the highly conserved cysteine residue that normally coordinates the heme iron. Its tentative assignment as a tautomerase is based on an analogous role proposed for *eryCII*, a *desVIII* equivalent in the erythromycin pathway.[17b,c] The DesVIII and EryCII proteins may catalyze the conversion of the 4-ketosugar to the corresponding 3-ketosugar intermediate, a common prerequisite for the transamination at C-3.

On the basis of these gene assignments and mechanistic intuition for the construction of aminodeoxy sugars, a plausible pathway has been proposed (Figure 6). This route is identical to that postulated by Summers et al. according to their study of the *ery* cluster.[17c] An alternative route has been suggested by Leadlay and co-workers in which the order of C-4 deoxygenation and C-3 transamination is reversed.[17a,b] However, our gene deletion results, as discussed in section IV, are more consistent with the reaction sequence shown in Figure 6. Clearly, genetic studies alone are not sufficient to piece together the complete biosynthetic pathway, and more vigorous biochemical investigations are required. Nevertheless, the influence of earlier work, especially that from the 3,6-dideoxyhexoses, can be appreciated. The availability of a database of well-characterized enzyme sequences is also critical in ascribing functions to these newly identified genes. Even though several of these gene assignments are still tentative at this stage, they have provided the means to genetically alter the structures of the corresponding natural products, as discussed below.

IV. Construction of New Sugars by Manipulating Nature's Biosynthetic Machinery

The emergence of pathogenic bacteria that are resistant to many commonly used antibiotics poses a serious threat to human health and has been the impetus of the present resurgent search for new antimicrobial agents. Recently, a genetic approach that "reprograms" the PKS genes involved in the biosynthesis of macrolide antibiotics has shown great promise for creating novel compounds that may exhibit new or enhanced biological activities. So far, such a combinatorial biosynthetic methodology has led to an impressive array of polyketide structures; however, without the sugar components that are essential for target recognition and binding, these new compounds are ineffective. While synthesizing the activated sugar components and performing the subsequent coupling to the aglycons by chemical means is a conceivable alternative, a complete "assembly line" production by nature's own biosynthetic machinery to directly give the final product would be more desirable. Hence, future success in making new macrolide antibiotics by the combinatorial biosynthetic strategy is contingent upon having the capability of assembling a repertoire of novel sugar structures and having the ability to couple these sugars to the structurally diverse macrolide aglycons.

In an initial attempt toward achieving the aforementioned goals, we have tested whether new methymycin/neomethymycin analogues carrying modified sugars could be generated by altering the desosamine biosynthetic genes. The *desVI* and *desV* genes, predicted to encode the *N,N*-dimethyltransferase and aminotransferase, respectively, were chosen as our initial targets. Interestingly, fermentation of the *desVI* deletion mutant strain, which no longer produced methymycin and neomethymycin, resulted in the accumulation of two new macrolides, **30** and **31**, carrying an *N*-acetylated aminodeoxyhexose (**32**).[22] Similarly, analysis of the *desV* deletion mutant led to the isolation of two new macrolides, **33** and **34**, carrying a 4,6-dideoxysugar (**35**) (Figure 7).[23] Since deletion of the *desV* gene leads to the incorporation of a 4-deoxysugar in the final products, it can be surmised that deoxygenation at C-4 most likely occurs prior to the transamination at C-3 as shown in Figure 6. Thus, these results not only confirm the assigned functions of the DesVI and DesV proteins, but also provide significant insights into the reaction sequence of desosamine biosynthesis. These findings also indicate that the glycosyltransferase (DesVII) of this pathway is capable of recognizing and processing sugar substrates other than TDP-desosamine. However, it is interesting that the sugar structures predicted on the basis of the proposed pathway seem to have undergone further modification in both cases, i.e., acetylation of the C-3 amine and reduction of the C-3 keto group. Whether the observed modification is a necessary step for self-protection must await further examination.

The fact that new macrolides bearing modified sugars can be produced by the deletion mutants is an exciting result that demonstrates not only a relaxed substrate specificity of the glycosyltransferase, but also the feasibility of preparing novel sugars via a genetic engineering approach. It should be pointed out that a few glycosyltransferases involved in the biosynthesis of polyketide and peptide antibiotics have been shown to exhibit flexible specificity toward modified aglycons.[24,25] In fact, glycosyltransferases of the daunorubicin[26] and erythromycin[17b,27] pathways are able to recognize modified sugars and catalyze their coupling to the respective aglycons. Since the methymycin/neomethymycin glycosyltransferase can also tolerate structural variants of its sugar substrate, a relaxed substrate specificity may be a general characteristic of the glycosyltransferases involved in the biosynthesis of secondary metabolites.

Another area where we have applied genetic engineering is in the exchange of related genes from different sugar pathways in an attempt to expand the dimensions of

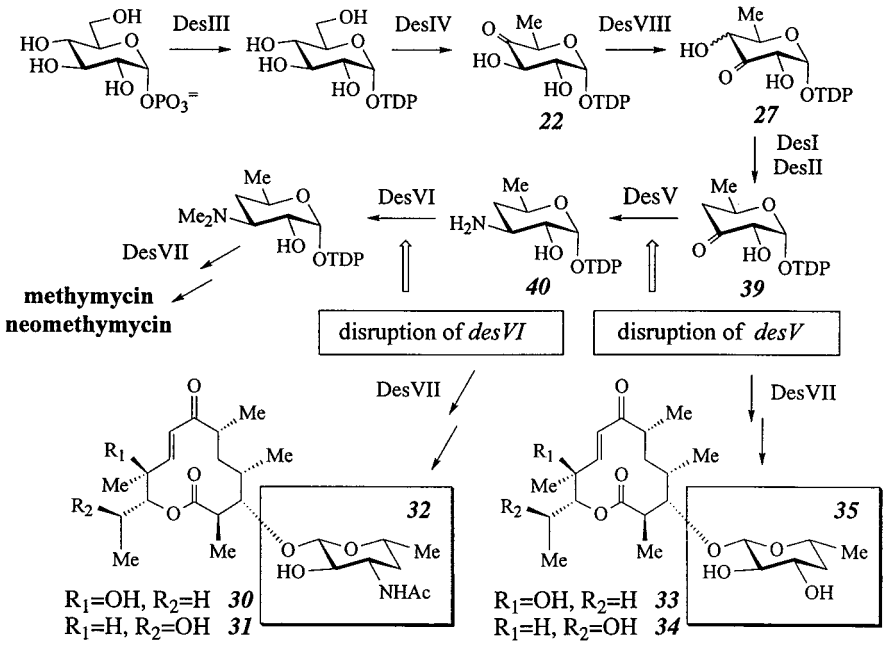


FIGURE 7.

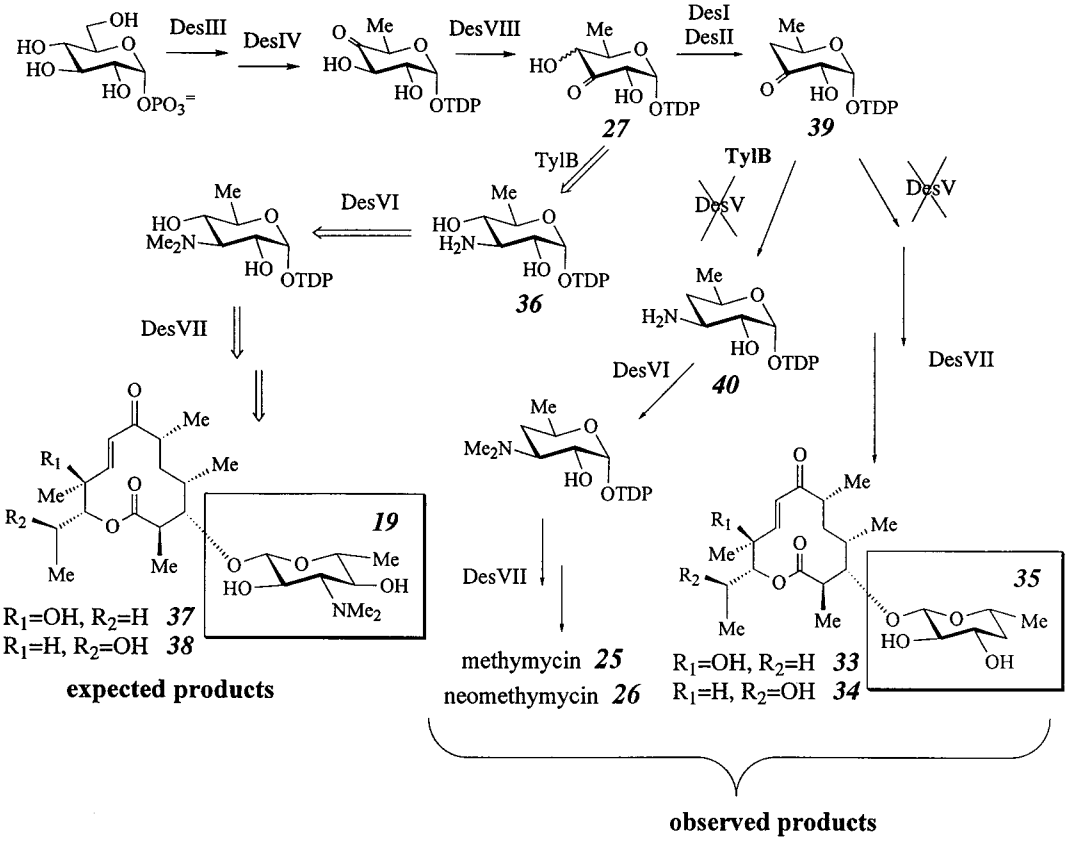


FIGURE 8.

structural diversity. For example, we have found that the *desVI* gene, which encodes an *N*,*N*-dimethyltransferase in the desosamine pathway, can be effectively replaced by the analogous gene *tylM1*[28] from the mycaminose (**19**) pathway of the tylosin cluster (Figures 5 and 8). Another gene exchange we have examined is the substitution of *desV* in the desosamine pathway with *tylB*, which encodes a similar aminotransferase in the mycaminose pathway (Figures 5 and 8). This resulted in the production of **33** and **34** along with small amounts of methymycin (**25**) and neomethymycin (**26**).[23] Since the real substrate of TylB is **27**, which is also an intermediate in the desosamine pathway, it was our prediction that the TylB enzyme in this mutant strain would partially intercept this intermediate and convert it to the corresponding 3-amino-3,6-dideoxyhexose **36** (see Figure 8). The previous experiment

already demonstrated that DesVI is a competent surrogate of TylM1, and therefore, the resulting aminosugar was expected to be readily *N*,*N*-dimethylated by DesVI to mycaminose (**19**) and incorportated into the final products. However, the fact that no mycaminose-containing products were observed suggested that TylB cannot compete with DesI for **27** in the mutant strain. Nonetheless, formation of methymycin and neomethymycin revealed that TylB can convert an unnatural substrate **39** to the immediate precursor of desosamine **40**, and thereby demonstrated a relaxed substrate specificity for TylB.

The results from these and other genetic manipulations attest to the potential for future production of customized glycosylated natural products. Continuing ventures into this burgeoning field of combinatorial biosynthesis hold great promise for the discovery of "hybrid natural products" with new and exciting biological activities.

V. Conclusion

In the past 10 years we have witnessed an explosion in our understanding of the pathways and mechanisms of deoxysugar biosynthesis. Yet, despite tremendous progress in this area, important questions continue to emerge. We have discovered how the C-3 deoxygenation is accomplished by the E_1/E_3 complex. However, this enzyme pair is not observed in the 2,4- and 4,6-dideoxyhexose pathways, and we have yet to determine nature's strategy for carrying out C−O bond cleavage in these cases. In addition to studying the mechanisms of sugar biosynthetic enzymes, efforts in determining the functions of the individual genes in the deoxysugar pathways have intensified as interest in combinatorial biosynthesis has gained momentum. The concept of genetically engineering biosynthetic pathways to produce new compounds with clinically useful activities is appealing. However, combinatorial biosynthesis using deoxysugar pathways is only in the early stages of development, and requires more in-depth investigations in order to increase our understanding of the pathways, improve our ability to accurately predict the biosynthetic routes of deoxysugars, and gain knowledge for manipulating the genes involved. What we have learned from previous studies on the enzymes in the 3,6-dideoxysugar pathway has proven the feasibility of this process and laid the foundation for future work needed to accomplish these goals. Further promise for success in this endeavor has been provided by recent findings of enzymes with diminished substrate specificity. Altogether, the efforts to increase our knowledge of deoxysugar biosynthesis have highlighted the elegance and complexity of nature's strategies for producing these unusual sugars, and shown potential for the development of novel and useful compounds by exploiting the deoxysugar biosynthetic machinery in a combinatorial biosynthetic approach.

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