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Pathway engineering of anthracyclines: Blazing trails in natural product glycodiversification

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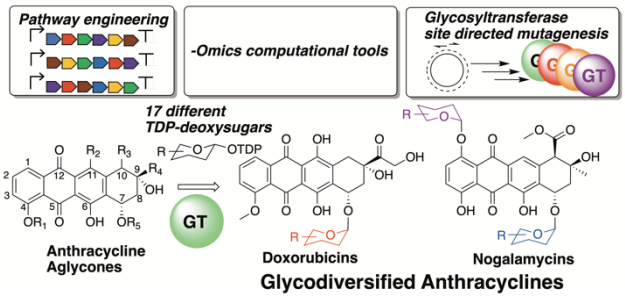


ABSTRACT

The anthracyclines are structurally diverse anticancer natural products that bind to DNA and poison the topoisomerase II – DNA complex in cancer cells. Rational modifications in the deoxysugar functionality are especially advantageous for synthesizing drugs with improved potency. Combinatorial biosynthesis of glycosyltransferases and deoxysugar synthesis enzymes is indispensable for the generation of glycodiversified anthracyclines. This Synopsis considers recent advances in glycosyltransferase

structural biology and site-directed mutagenesis, pathway engineering, and deoxysugar combinatorial biosynthesis with a focus on the generation of “new-to-nature" anthracycline analogs.

The actinomycetes are an order of gram-positive bacteria that occupy a wide range of terrestrial and marine habitats [1]. Actinomycetes produce a plethora of bioactive substances notable for their antibacterial, anticancer, and immunosuppressive activities [2]. Among these bioactive substances, the anthracyclines are some of the most structurally diverse and important chemicals for their potent anticancer and antibacterial properties [3]. The anthracyclines exhibit a characteristic anticancer mechanism of action involving intercalation into DNA and poisoning of topoisomerase II-mediated unwinding of DNA supercoils [4]. The anthraquinone moiety of the anthracyclines intercalates between DNA bases, while the carbohydrate unit at 7-position enhances binding through interactions with the minor groove of DNA (Figure 1A). Anthracyclines can undergo redox activation by chelating cellular Fe^{2+}, which can lead to the formation of hydroxyl free radicals that cause additional macromolecular damage to DNA and RNA [5]. The aminosugar moiety can form methylene adducts with guanine in the minor groove of DNA (Figure 1B) [6]. The aminosugar also enhances water solubility, plays a role in substrate recognition by *p*-glycoprotein (i.e. mutations in *p*-glycoprotein are responsible for cellular resistance), and impacts clearance of the drug from the body [7–9]**.** The anthracyclines suffer from dose-limiting cardiotoxicity [10], which has motivated efforts to study the biosynthesis of the anthracyclines and to generate new drug analogs with improved clinical properties. The combination of DNA and chromatin damage has been shown to be the main cause of cardiotoxicity of doxorubicin [11]. The modification of doxorubicin to *N*,*N*-dimethyl-doxorubicin abrogated double-stranded break activity, while retaining the ability to induce chromatin damage. Mouse models showed no cardiotoxicity after treatment with *N*,*N*-dimethyl doxorubicin [11]. This suggests that the

development of next-generation clinical anthracyclines might be achieved through modification of the carbohydrate units.

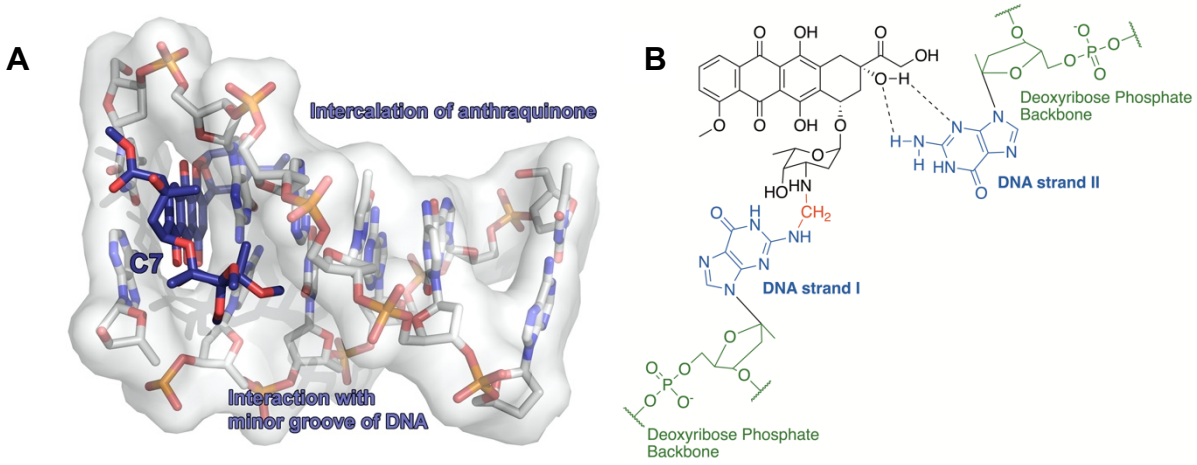


Figure 1 The binding of anthracyclines to DNA. A) Structure of the nogalamycin-DNA complex (PDB: 182D) reveals the intercalation of the anthraquinone aglycone between DNA bases and positioning of the carbohydrate unit at 7-position in the minor groove of DNA, B) Formation of methylene adducts of doxorubicin to double-stranded DNA.

The anthracyclines are aromatic polyketides with a 7,8,9,10-tetrahydro-tetracene-5,12-quinone scaffold and between one to eight saccharide moieties attached [12,13]**.** At least 500 naturally occurring anthracyclines have been isolated to date [14]. The polyketide scaffold exhibits great structural diversity in nature, however, only six of the fifteen possible types of *peri*-hydroxyanthraquinone chromophores have been described to date. In addition, anthracyclines feature *O*-glycosides primarily at phenolic oxygens at 7-position and/or 10-position, although in a few cases *O*-glycosylation is observed at 1-position and 4-position [14]. Examples of well-characterized anthracyclines include daunorubicin (**1**), doxorubicin (**2**), nogalamycin (**3**), aclacinomycin (**4**), elloramycin (**5**), 8-demethyl-tetracenomycin C (8-DMTC, **6**), and keyicin (**7**) (Figure 2).

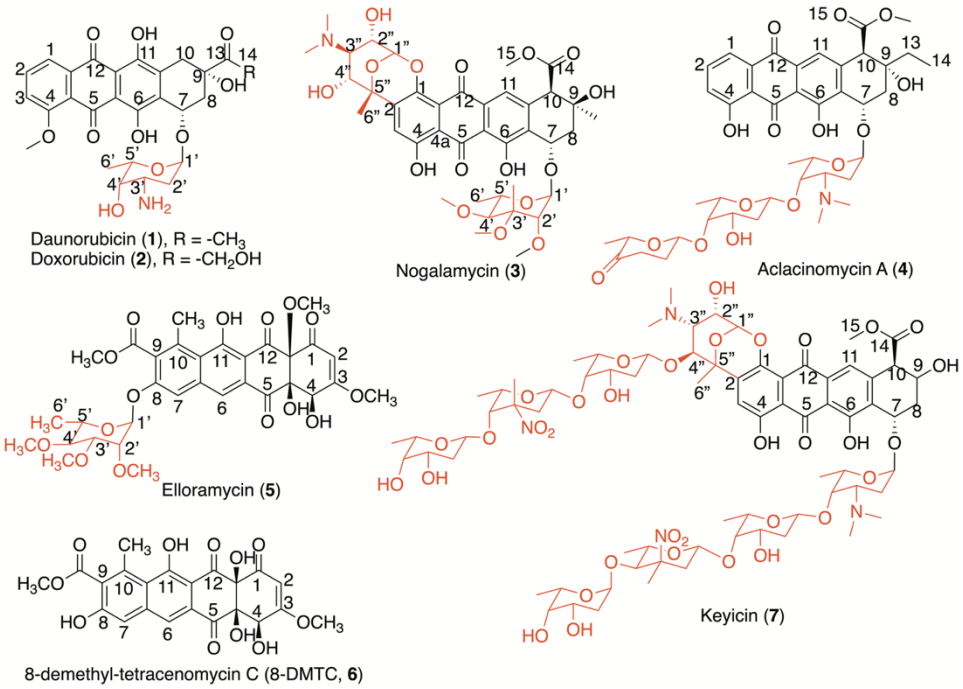


Figure 2 Structures of anthracycline natural products. Sugar moieties are indicated in red.

Anthracyclines undergo similar biosynthetic routes via a type II polyketide synthase (PKS), though their structural diversity derives in large part from the complement of post-PKS tailoring enzymes [15]. The biosynthesis of doxorubicin involves the iterative condensation of an acetyl-CoA or propionyl-CoA starter unit (e.g. synthesized and transferred by acyltransferase DpsC and propionyl-CoA synthetase DpsD) with nine malonyl-CoA extender units by a minimal PKS composed of ketoacyl synthase alpha (KSα, DpsA), ketoacyl synthase beta (KSβ, DpsB), acyl carrier protein (ACP, DpsG) (Figure 3) [16,17]. The minimal PKS forms an extended complex with a 9-ketoreductase (DpsE), aromatase (DpsF), and second/third-ring cyclase (DpsY) before undergoing C-12 anthraquinol oxygenation (DnrG) and release of the first isolable tricyclic intermediate, aklanonic acid, that is common to most anthracycline biosynthetic pathways [18]. Additional *O*-methylation (DnrC), fourth ring cyclization (DnrD), and 7-

ketoreduction (DnrE) yields aklavinone (**8**) [19]. C-11 oxygenation gives rise to the aglycone ε-rhodomycinone (**9**) [20]. TDP-L-daunosamine (**10**) and TDP-L-rhodosamine (**11**) are the most common aminosugars appended to the 7-position for most of the anthracyclines [14]. The biosynthesis of **10** starts from D-glucose-6-phosphate, which is interconverted to D-glucose-1-phosphate by phosphoglucomutase (Pgm) (Figure 3). TDP-glucose-synthase (GS, DnmL/RfbA) condenses thymidine monophosphate with D-glucose-1-phosphate to form TDP-D-glucose, which is then 6-deoxygenated by NDP-D-glucose-4,6-dehydratase (4,6-DH, DnmM/RfbB) to form TDP-4-keto-6-deoxy-D-glucose [21]. TDP-4-keto-6-deoxy-D-glucose then undergoes 2-deoxygenation via 2,3-dehydratase (2,3-DH, DnmT), 3-aminotransfer (AT, DnmJ), 3,5-epimerization (EPI, DnmU), and 4-ketoreduction (4-KR, DnmV) to afford **10** (Figure 3) [22–25]. **10** undergoes dual *N*-methylations to form **11**.

Glycosylation at the 7-position of aglycone **9** to form rhodomycin D (**12**) requires the action of two enzymes to function properly, one *O*-glycosyltransferase (e.g. DnrS) and a P450-auxiliary protein (e.g. DnrQ) that is hypothesized to function as an allosteric activator [26,27]. Inactivation of either DnrQ or DnrS in the daunorubicin producer *Streptomyces peucetius* resulted in the accumulation of the aglycone **9**, demonstrating the essentiality of both gene products for glycosylation of **12.** The structure of the erythromycin desosaminyltransferase EryCIII in complex with its activating P450 homolog, EryCII, demonstrated that these enzymes form an α2β2 heterodimer [28]. EryCII forms tight non-covalent interactions with EryCIII that putatively causes a conformational shift in the *N*-terminus aglycone acceptor and *C*-terminus sugar donor domains that results in the transfer of TDP-D-desosamine to 3-α-mycarosylerythronolide

B [28]. The co-expression of the P450 homolog DesVIII along with desosaminyltransferase DesVII enhances glycosylation efficiency by 10^3-fold as compared to DesVII alone [29]. The glycosyltransferases of anthracycline pathways (e.g. DnrQS, AknST) are thought to function similarly and could be worthy targets for future structural biology studies.

Compound **12** undergoes additional methyl esterase (DnrP) and 4-*O*-methyltransferase (DnrK) activity to afford 13-deoxy-daunorubicin (**13**) [30]. **13** is oxygenated at 13-position by CYP450 oxygenase DoxA to form **1** and is then hydroxylated at 14-position to afford **2** [31]. The characterization of the doxorubicin biosynthetic pathway provides a fertile substratum for metabolic engineering of the pathway to improve production titers and to generate new anthracyclines via combinatorial biosynthesis. In this Synopsis, we first consider recent advances in pathway engineering of deoxysugar metabolism and polyketide biosynthesis, including substrate precursor engineering and overexpression of rate-limiting structural genes (Figure 4). Pathway engineering is a powerful technique for redirecting carbon flux from primary cellular metabolism towards the production of anthracyclines. Secondly, we discuss the recent discovery of the **7** biosynthetic pathway, which employed state-of-the-art techniques, such as deep transcriptomic sequencing and co-culturing to elicit expression of a cryptic pathway. Thirdly, we describe initial endeavors to elucidate the structural biology of anthracycline glycosyltransferase SnogD and the rational site-directed mutagenesis of substrate-flexible ElmGT. Fourthly, we examine recent efforts to exchange the appended deoxysugar moiety with non-natural deoxysugar donors via the combinatorial biosynthesis of deoxysugar biosynthesis genes. These recent

advances have expanded the toolbox for combinatorial biosynthesis of these impressive biosynthetic pathways, which could usher in the next generation of anthracyclines with

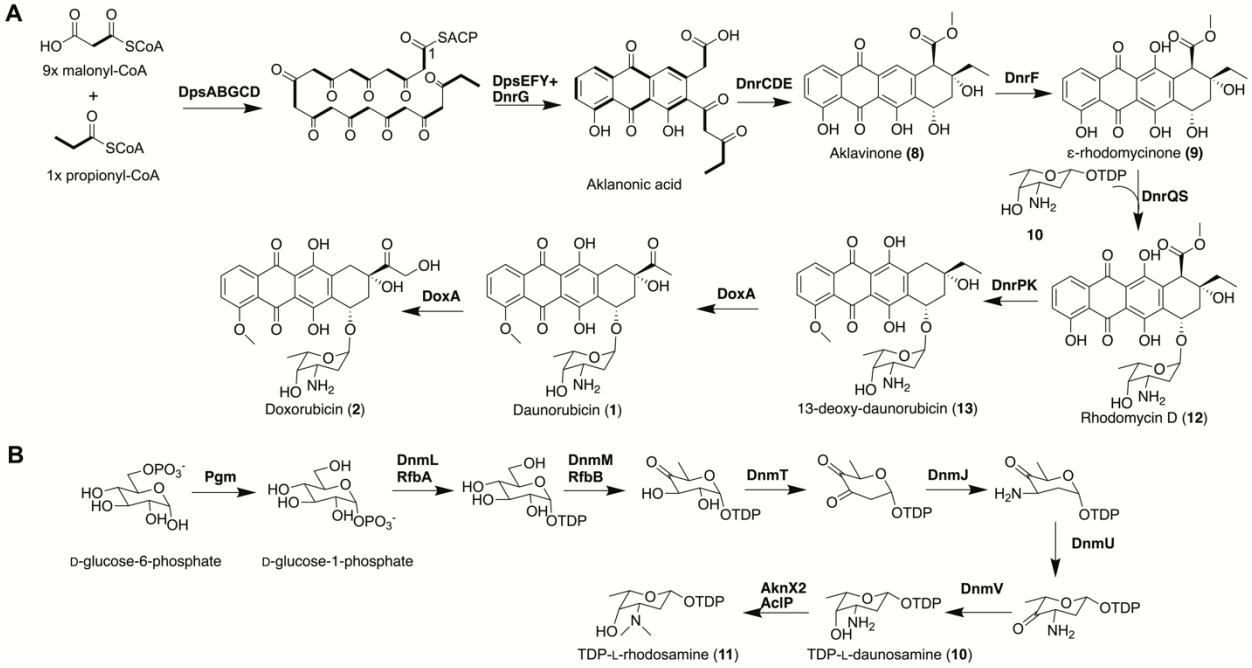


Figure 3 Scheme for polyketide biosynthesis of daunorubicin (**1)** and doxorubicin (**2**) (panel A) and deoxysugar biosynthesis of TDP-L-daunosamine (**10**) and TDP-L-rhodosamine (**11**) (panel B).

improved biological activities.

1. Pathway Engineering

1.1. Overexpression of structural biosynthetic genes

Streptomyces peucetius exhibits notable limitations concerning doxorubicin production that have engendered pathway engineering efforts to overcome rate-limiting blocks in the production [32]. Some of these limitations include poor glycosylation efficiency, low expression of deoxysugar biosynthetic genes, low conversion of **1** to **2** by DoxA, and feedback regulation [24–26,31]. Consequently, pathway engineering provides an attractive solution for overcoming transcriptional imbalances via overexpression of

structural biosynthetic genes. The overexpression of the glycosyltransferase DnrS alone or together with the DnrQ auxiliary protein resulted in a 1.2 or 2.8-fold, respectively, increase in **2** production titers [33]. The overexpression of the deoxysugar biosynthesis enzymes TDP-D-glucose synthase (e.g. DesIII) and TDP-D-glucose-4,6-dehydratase (e.g. DesIV) increased production by 2.6-fold. The combined overexpression of *desIII/desIV* and *dnrQ/dnrS* demonstrated a synergistic 5.6-fold increase in **2** production titers.

1.2. Substrate precursor engineering

Overexpression of structural biosynthetic genes is one successful approach towards improving anthracycline production titers, but substrate precursor engineering of biochemical building blocks (e.g. acetyl-CoA, D-glucose-6-phosphate) is a promising technique to funnel carbon from central metabolism towards polyketide biosynthesis. Overexpression of phosphoglucomutase (Pgm, *sco7443*) and the acetyl-CoA carboxylase complex (ACCase, *ovmGIH*) increased precursor substrate levels of glucose-1-phosphate and malonyl-CoA, respectively, which ubiquitously enhanced production titers of **2**, **3**, **5**, **6**, and steffimycin by 20-60% (Figure 4) [34]. The same technique has also been utilized in *Streptomyces argillaceus* to increase the production of the anticancer polyketide, mithramycin by overexpressing Pgm and ACCase [35]. Overexpression of *sco7443* resulted in higher titers of mithramycin early in the logarithmic growth phase of the engineered strain (e.g. 180% after 3 days and 62% after 4 days) before leveling off to a 9% increase as compared to *S. argillaceus* harboring an empty plasmid vector. This result was further supported by a decrease in D-glucose-6-phosphate concentrations (e.g. 6% - 61%) and an increase in D-glucose-1-

phosphate concentrations (e.g. 15% to 34%) in the engineered strain. Overexpression of *ovmGIH* increased malonyl-CoA titers in the engineered strain by 3-fold as compared to wildtype *S. argillaceus*. Also, overexpression of *ovmGIH* in *S. argillaceus* resulted in an increase in mithramycin production by 21%. Most importantly, overexpression of both *ovmGIH* and *sco7443* in the *S. argillaceus* M7W1 (*mtmW*$^-$) mutant strain resulted in a 2-fold increase in the production of mithramycin SK and a 5-fold increase in the production of mithramycin SDK. Both of these derivatives have improved anticancer activity as compared to the parent compound mithramycin [36]. Remarkably, this work demonstrated that pathway engineering can be used to increase production titers of valuable polyketide analogs with improved anticancer properties. Similar techniques could be utilized to enhance production titers of clinically important anthracycline analogs.

1.3. Harnessing β-oxidation for polyketide biosynthesis

Recent studies have identified new metabolic targets for overexpression to augment carbon flux towards polyketide biosynthesis, a critical development that could increase the yields of analogs that were previously unisolable. *Streptomyces coelicolor*, a model actinomycete for studying actinorhodin biosynthesis, stores much of its carbon as triacylglycerols following the logarithmic phase growth [37]. Deep sequencing of the transcriptome of wildtype actinorhodin producer *S. coelicolor* M145 and an industrial actinorhodin overproducer, *S. coelicolor* HY01 revealed a significant increase of β-oxidation degradation products in the industrial producer strain. The transcripts corresponding to genes involved in β-oxidation were significantly upregulated during the stationary phase in *S. coelicolor* M145 and *S. coelicolor* HY01. Specifically,

acyltransferase *sco6196* was significantly upregulated during the stationary phase in the HY01 strain as compared to the M145 wildtype, which provided a clue as to the enzyme which might be responsible for higher carbon flux towards actinorhodin. The identification of acyltransferase *sco6196* as a potential "metabolic switch" bridging β-oxidation to polyketide synthesis is potentially a universal strategy for enhancing production titers of anthracyclines in most actinomycetes (Figure 4) [37].

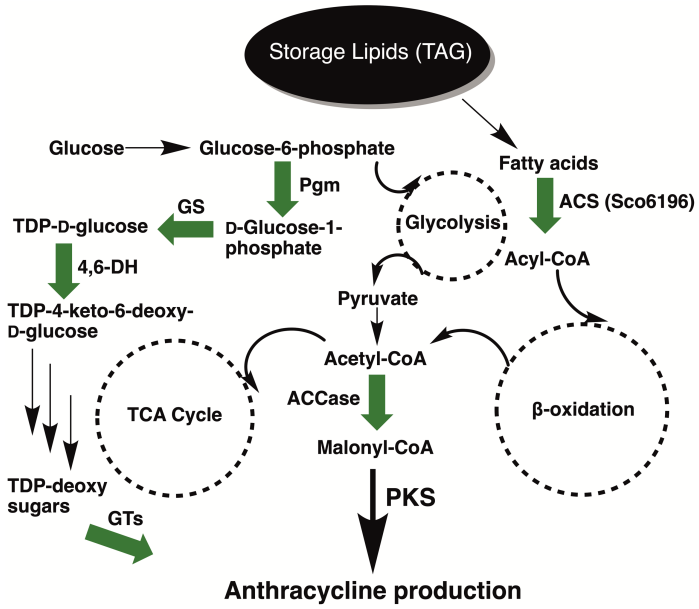


Figure 4 Biochemical scheme for anthracycline pathway engineering. Enzymes that have been overexpressed are indicated by green arrows. Pgm = phosphoglucomutase, GS = NDP-D-glucose synthase, 4,6-DH = NDP-D-glucose-4,6-dehydratase, GT = glycosyltransferase, ACS = acyltransferase, *sco6196* = acyltransferase from *Streptomyces coelicolor* M145, ACCase = acetyl-CoA carboxylase complex, TAG = triacylglycerols, TDP = thymidine diphosphate.

2. Advances in glycosylation of anthracyclines

Glycosylation is an indispensable biosynthetic modification that influences the antitumoral activity of the anthracyclines [4]. Glycosyltransferases (GTs) are enzymes that catalyze the attachment of deoxysugar moieties to anthracyclinone scaffolds, which

recognize a deoxysugar donor substrate and an anthracyclinone acceptor substrate [38]. An exhaustive description of deoxysugar biosynthesis is beyond the scope of this review, though several comprehensive reviews have previously been published on this subject [39–41]. In this section, we describe novel advances in glycodiversification of anthracyclines due to deoxysugar pathway engineering and site-directed mutagenesis of glycosyltransferases. These findings set the stage for future combinatorial biosynthesis of rationally designed anthracyclines with substitutions in the glycosylation pattern.

2.1. Elloramycin

Compounds **5** and **6** are anthracycline antibiotics produced by *Streptomyces olivaceus* strain Tü 2353 (Figure 2) [42]**.** Recently, the mechanism of action for the tetracenomycins was shown to occur through inhibition of peptide translation via binding to the large ribosomal subunit of prokaryotes and humans, which accounts for their dual antibacterial and anticancer activities [43]. **5** features an 8-*O*-glycosidically linked 2, 3, 4-tri-*O*-methyl-α-L-rhamnose sugar appendage. The biosynthetic gene cluster for production of **5** has been cloned on cosmid cos16F4 and encodes the genes necessary for the biosynthesis of polyketides **5** and **6**, in addition to the gene for the sugar-flexible glycosyltransferase, *elmGT*. [44]. The *Streptomyces lividans* (cos16F4) heterologous expression system is an ideal platform for assessing the combinatorial biosynthesis of TDP-deoxysugar pathways. ElmGT exhibits remarkable flexibility towards the transfer of many TDP-deoxysugar substrates to **6**, including > 20 different deoxysugars in both D- and L-configurations [45–49]. Salas and coworkers generated a series of “sugar plasmids” directing biosynthesis of TDP-deoxysugar pathways using the multicopy plasmid pEM4

and expression of biosynthetic genes from the strong *ermE*p* erythromycin resistance up-promoter [50]. In one study, Nybo et al. generated a construct, pKOL, expressing the *oleSEVWL* genes from the TDP-L-oleandrose pathway for the formation of TDP-4-keto-L-olivose. Heterologous expression of plasmid pKOL in the *Streptomyces lividans* (cos16F4) host resulted in the successful generation of two derivatives, including the known analog, 8-demethyl-8-α-L-olivosyl-tetracenomycin C and the new analog 8-demethyl-8-(4'-keto)-α-L-olivosyl-tetracenomycin C [51]. This report revealed additional substrate flexibility by ElmGT towards a TDP-4-keto-L-sugar, which is unusual among natural product GTs.

2.2. Nogalamycin

The **3** biosynthetic gene cluster (*snog*) from *Streptomyces nogalater* ATCC 27451 has been cloned in *Streptomyces albus* J1074 [52]. Heterologous expression of cosmid pSnogaori containing the majority of the gene cluster resulted in the production of nogalamycinone, nogalamycin R (**14**), nogalamycin F (**15**), and 3',4'-demethoxy-nogalose-1-hydroxynogalamycinone (**16**) (Figure 5). Expression of the entire metabolic pathway using a two-plasmid system led to formation of **3** and confirmed that all biosynthetic genes were present [52].

The production of **15** by pSnogaori was surprising since an L-olivose sugar was featured at the 1-position instead of the expected L-nogalamine, which hinted at relaxed substrate flexibility on the part of glycosyltransferase SnogD. This result was explained due to the endogenous TDP-L-olivose biosynthesis in *S. albus* J1074, which effectively competed with TDP-L-rhodosamine for binding by SnogD. The functions of the two glycosyltransferases SnogD and SnogE were studied with gene inactivation

experiments. The strain lacking *snogD* produced primarily **16** and whereas, in the absence of *snogE*, *S. albus* produced primarily nogalamycinone, which established SnogE as the 7-*O*-L-nogalose glycosyltransferase and SnogD as the 1-*O*-L-rhodosaminyl glycosyltransferase, respectively.

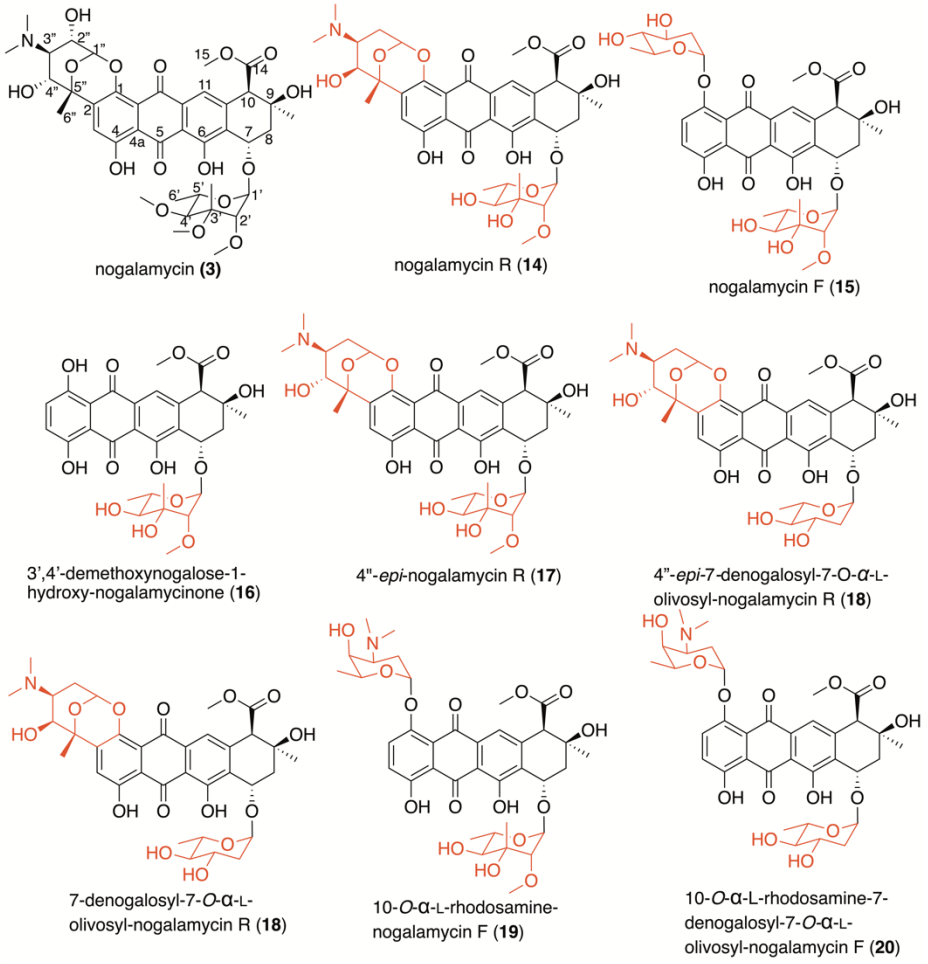


Figure 5 Glycosylated analogs of nogalamycin reported from gene inactivation experiments.

Structure determination of the glycosyltransferase SnogD from the nogalamycin biosynthetic pathway to 2.6 Å resolution to gain insight into the mechanism of carbohydrate transfer in anthracycline biosynthesis [53]. The authors determined the structure of the *apo*-enzyme and the enzyme with a bound nucleotide, 2-deoxyuridine-5'-diphosphate. SnogD consists of one Rossman fold in the *N*-terminal aglycone

acceptor domain (residues 1-209) and one Rossman fold in the *C*-terminal sugar donor domain (residues 228-390). This framework is characteristic of the glycosyltransferase B family (GT-B). The authors inactivated residues His25 and His301, which resulted in the abrogated function of the resulting SnogD mutants when expressed *in vitro* and *in vivo*. This is the first structural report of a crystallized anthracycline glycosyltransferase, which opens the door for future attempts at glycosyltransferase engineering to expand the substrate flexibility of these utilitarian enzymes towards accepting alternative aglycones and TDP-deoxysugar donors. Engineering of SnogD, for example, via the introduction of single or multiple point mutations or via rational domain swapping could afford new glycosyltransferase catalysts useful for deoxysugar interchange experiments (Figure 6).

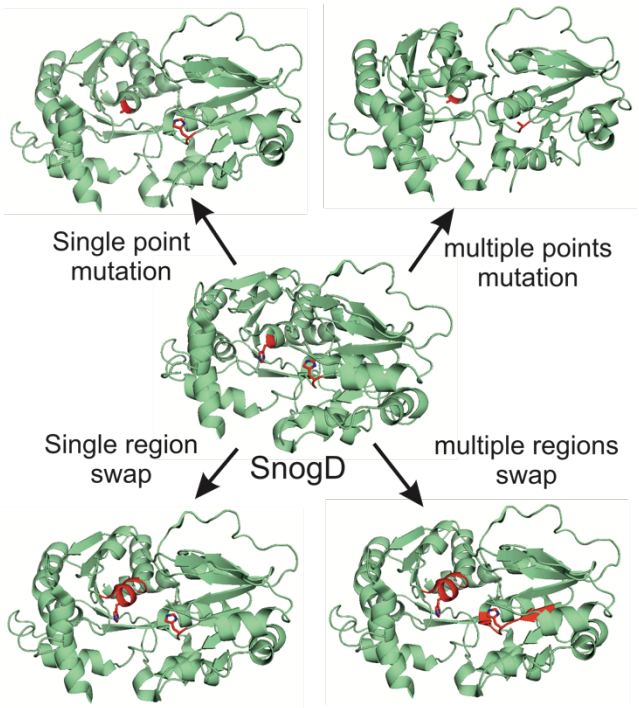


Figure 6 Structural biology and site-directed mutagenesis of SnogD as strategies for developing new glycosyltransferase catalysts. Mutations can focus on single or multiple points mutations or even swapping entire regions or domains between glycosyltransferases.

The key structural feature of **3** is the attachment of L-nogalamine with an additional C5''-C2 bond, which positions the carbohydrate unit perpendicular to the aglycone that allows interactions with the major groove of DNA [54]. In addition, both C2'' and C4'' hydroxyl groups of L-nogalamine are important for hydrogen bonding interactions of **3** with DNA [54]. Nonetheless, the pathway intermediates isolated from *S. albus* typically contained L-rhodosamine (2''-deoxy-4''-*epi*-L-nogalamine) as the carbohydrate unit. Recently, the Rieske enzyme SnoT and two non-heme Fe(II) and α-ketoglutarate dependent enzymes SnoK and SnoN are responsible for late-stage modifications in the biosynthesis of L-nogalamine [55,56]. Complementation experiments with *snoN* in the **14**-producing strain resulted in new 4"-epimerized nogalamycins: 4"-*epi*-nogalamycin R (**17**) and 4"-*epi*-7-denogalosyl-7-*O*-α-L-olivosyl-nogalamycin R (**18**) (Figure 5). The deletion of *snoK* from pSnogaORI resulted in the production of new nogalamycins lacking the C5"-2 carbocycle: 10-*O*-α-L-rhodosamine-nogalamycin F (**19**) and 10-*O*-α-L-rhodosamine-7-denogalosyl-7-*O*-α-L-olivosyl-nogalamycin F (Figure 5) (**20**)**.** The sequence of events leading to the formation of **3** includes 2''-hydroxylation of 1-*O*-L-rhodosamine by SnoT, followed by C5''-C2 carbocyclization by SnoK and 4" epimerization by SnoN (Figure 7). Non-heme Fe(II) and α-ketoglutarate dependent enzymes can perform demanding oxidative transformations using a reactive Fe(IV)=O center. Despite the drastically different chemistry catalyzed by SnoK and SnoN, the catalytic difference appears to depend largely on the positioning of the substrates in front of the activated iron-oxo center. This was demonstrated in a recent study where SnoN was engineered to catalyze carbocyclization after insertion of just three residues from SnoK [57].

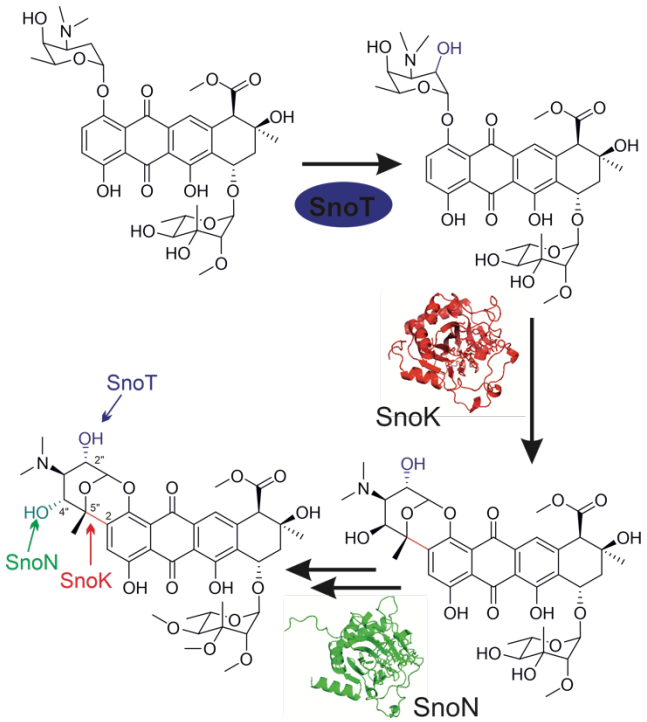


Figure 7 Late-stage biosynthesis of nogalamycin via SnoT, SnoK, and SnoN. SnoT is a Rieske oxygenase that carries out 2”-hydroxylation of 1-*O*-L-rhodosamine. SnoK and SnoN are non-heme iron enzymes that form the C5”-C2 carbon-carbon bond and catalyze 4”-epimerization of **3**, respectively.

2.3. Keyicin

Compound **7** was discovered using an innovative co-culturing technique between *Rhodococcus sp.* WMMA185 and *Micromonospora sp*. WMMB235 that elicited the expression of the cryptic **7** biosynthetic gene cluster (*kyc*) from the latter host [58]. The **7** biosynthetic pathway contains a non-heme iron enzyme ortholog, *kyc54*, of *snoK* from nogalamycin biosynthesis that putatively catalyzes the formation of the C2-C5" bond in the epoxyoxocin ring system [55]. The epoxyoxocin ring system of **7** is inverted at C4’ relative to nogalamycin, which the authors hypothesized is due to the presence of the C4’-*O*-glycosidically linked trisaccharide containing 2-deoxy-L-fucose, a novel L-configured nitrosugar (e.g. 3”-demethoxy-L-evernitrose), and 2-deoxy-L-fucose (Figure

2). **7** also contains a tetrasaccharide appended at the 7-*O*-position composed of L-rhodosamine, 2-deoxy-L-fucose, a second novel nitrosugar (e.g. 3"-demethoxy-L-rubranitrose), and 2-deoxy-L-fucose (Figure 2). In total, there are seven putative GTs (e.g. *kyc12, kyc20, kyc24, kyc25, kyc29, kyc32,* and *kyc52*) responsible for the transfer of the seven deoxysugar moieties. Supporting evidence for participation of the encoded GT gene products in glycosylation of the **7** polyketide scaffold was provided by proteomics studies of 8 day-grown cultures of *Micromonospora sp.* WMMB235 in which four of the GT proteins were detected at the time of greatest production of glycosylated metabolites [59]. These studies have opened the door for heterologous expression of these newly identified GT catalysts and deoxysugar biosynthetic enzymes in other anthracycline producing organisms, which could result in the production of anthracyclines decorated with novel nitrosugars.

2.4. Doxorubicin

1 and **2** are the best-characterized anthracyclines that are currently used in the clinic, and they have broad-spectrum activity against a variety of human cancers [60]. Like the other anthracyclines, daunorubicin and doxorubicin inhibit the growth of cancer by poisoning the action of topoisomerase II on supercoiled DNA [61]. The polyketide functionality intercalates into the GC-rich DNA and the indispensable 7-*O*-L-daunosamine moiety anchors the drug inside the DNA double helix [8]. The carbohydrate moiety is an attractive functionality for the alteration of structure-activity-relationships (SAR) since this position influences the potency and efficacy of the anthracycline (Figure 1) [8]. In a pioneering study, *S. peucetius* was engineered for the production of 4'-

epi-doxorubicin (e.g. epirubicin) via knockout of the endogenous TDP-4'-keto- 2,3,6-trideoxyhexulose reductase gene *dnmV* and expression of alternative 4-ketoreductase genes *eryBIV* and *avrE* [62]. Epirubicin is a clinically advantageous derivative of doxorubicin since it exhibits a two-fold higher maximum lifetime dose as compared to doxorubicin [63].

1 and **2** are produced by the actinomycete *Streptomyces peucetius* [64,65], yet the native production host exhibits several transcriptional and metabolic limitations that diminish production titers of **2** and the most biologically active analogs [32]. Therefore, several groups have developed heterologous expression hosts for the production of glycosylated analogs of daunorubicin. The heterologous host, *Streptomyces venezuelae*, has been developed for combinatorial biosynthetic expression of anthracycline glycosyltransferase and deoxysugar biosynthetic genes to produce glycosylated derivatives of doxorubicin [66]. The strain was engineered to confer self-resistance to **2** by incorporation of the doxorubicin resistance genes *drrABC*. The genetic expression of "deoxysugar plasmids" in actinomycete hosts results in flooding the biosynthetic pathway with deoxysugar donors, which can outcompete endogenous deoxysugars for binding inside the active site of glycosyltransferases [67,68]. The authors hypothesized that if the glycosyltransferase active site exhibits some flexibility towards alternative deoxysugar donors, then the foreign sugar can be appended to the anthracycline aglycone. The authors generated constructs encoding several heterologous TDP-deoxysugar biosynthetic pathways: TDP-L-daunosamine (**10**), TDP-L-rhodosamine (**11**), TDP-3-*N*-methyl-L-daunosamine (**21**), TDP-4-*epi*-L-daunosamine (**22**), TDP-3-*N*-methyl-4-*epi*-L-daunosamine (**23**), TDP-L-nogalamine (**24**), TDP-L-

ristosamine (**25**), TDP-3-*N*-methyl-L-ristosamine (**26**), TDP-L-megosamine (**27**), TDP-4-*epi*-L-vancosamine (**28**), TDP-L-rhamnose (**29**), TDP-D-olivose (**30**), TDP-L-olivose (**31**), TDP-D-digitoxose (**32**), and TDP-L-digitoxose (**33**) (Figure 8).

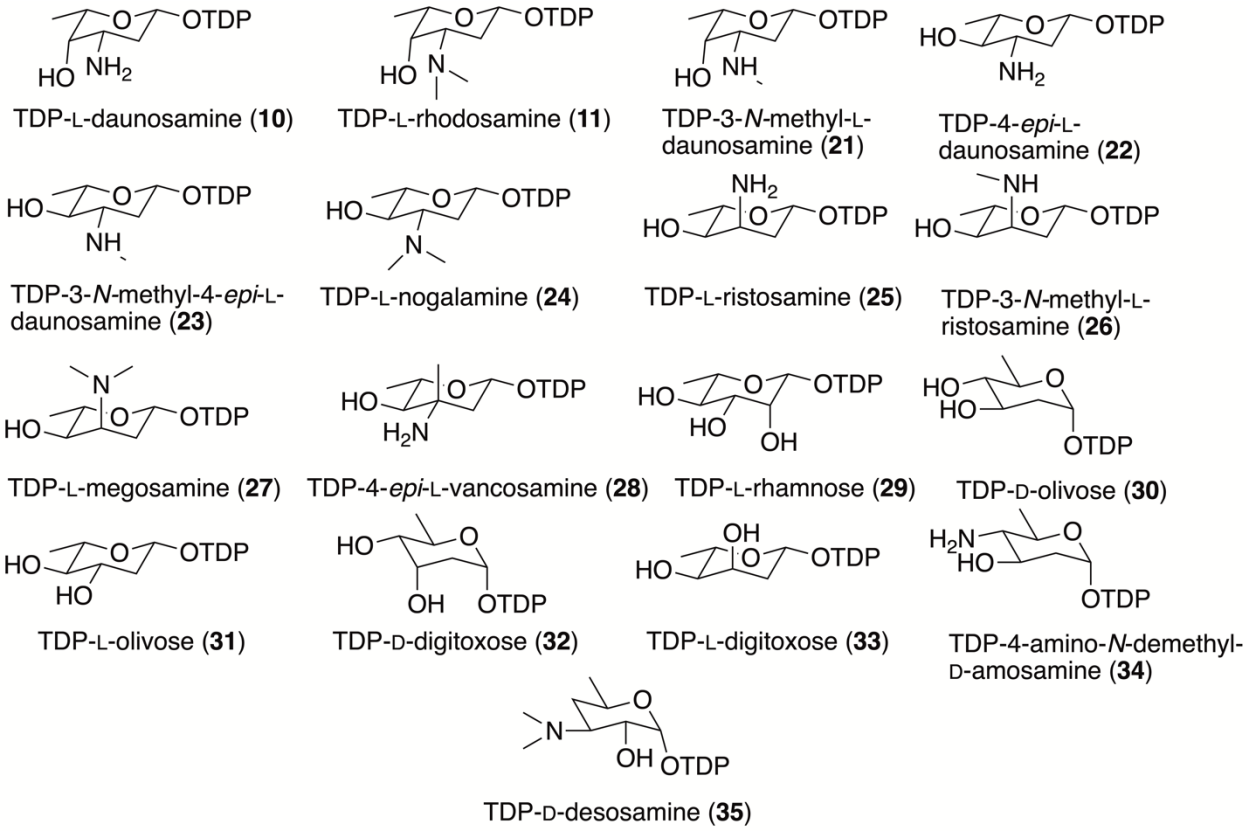
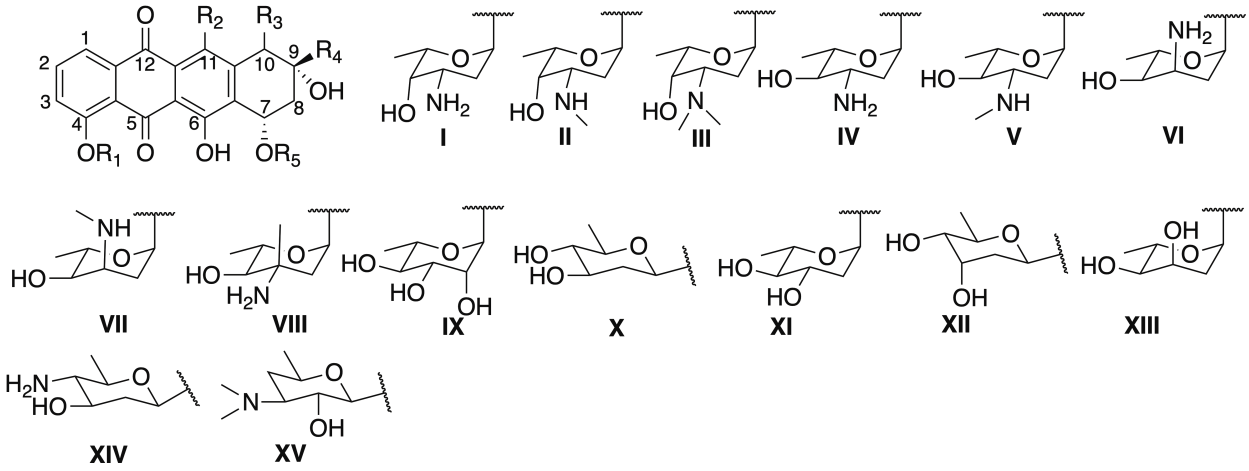


Figure 8 TDP-deoxysugar donors used for glycodiversification of anthracyclines.

The authors conducted a biotransformation experiment in which they fed in **9** into the strain co-expressing the resistance genes, glycosyltransferase, and heterologous deoxysugar pathways. In this work, the authors were able to reconstitute the late doxorubicin biosynthetic steps and produce 2.8 ± 0.5 μM **12**, 0.9 ± 0.04 μM **1**, and 1.1 ± 0.13 μM **2**. The glycosyltransferase AknS and its CYP450-auxiliary protein AknT exhibited surprising donor substrate flexibility towards non-canonical TDP-deoxysugars. AknST demonstrated turnover of TDP-L-daunosamine, TDP-L-rhodosamine, TDP-L-

ristosamine, TDP-L-vancosamine, TDP-D-digitoxose, TDP-L-digitoxose, and TDP-L-rhamnose. In total, the authors generated twenty anthracyclines, including seven new rhodomycin D derivatives that were characterized by HPLC-ESI-MS/MS analysis (**1**, **2**, **12**, **36 – 52**) (Figure 9). However, production titers of the new derivatives were too low to enable NMR spectroscopic characterization, which indicates further metabolic engineering efforts are required to harness the full potential of the method.

A one-pot combinatorial biosynthesis system was established in *Streptomyces venezuelae* for the production of aglycones and TDP-deoxysugars [69]. The PKS genes (*dpsABGCDEFY*+*dnrGCDEF*) required for the production of **8** and **9** were integrated into the ϕC31 *attB* site of the *S. venezuelae* chromosome. The authors were able to achieve production and export of 3.78 mg/L **8** production in strain YJ183/pAKV and 0.68 mg/L **9** production in strain YJ183/pRHO. Co-cultivation of these strains with strains engineered to express glycosyltransferases and TDP-deoxysugar biosynthetic pathways led to the production of glycosylated anthracyclines. The authors interrogated the donor substrate flexibility of AknST towards **10**, **11**, **21**, **22**, **25**, and novel deoxysugars TDP-L-vancosamine (**28**), TDP-4-amino-*N*-demethyl-D-amosamine (**34**), and TDP-D-desosamine (**35**) (Figure 8). The authors generated 16 glycosylated analogs of doxorubicin, including 7 new compounds characterized by HPLC-ESI-MS/MS (**56** – **62**), though yields were too low for NMR spectroscopic structure elucidation (Figure 9). This work demonstrates that the substrate-flexible AknST and combinatorial biosynthesis of heterologous TDP-deoxysugar biosynthetic pathways are powerful tools for glycodiversification of anthracyclines.



Number	Compound	R_1	R_2	R_3	R_4	R_5
12	Rhodomycin D	H	OH	CO_2CH_3	CH_2CH_3	I
36	3'-*N*-methyl-rhodomycin D	H	OH	CO_2CH_3	CH_2CH_3	II
37	L-rhodosaminyl-rhodomycin D	H	OH	CO_2CH_3	CH_2CH_3	III
38	4'-*epi*-rhodomycin D	H	OH	CO_2CH_3	CH_2CH_3	IV
39	3'-*N*-methyl-4'-*epi*-rhodomycin D	H	OH	CO_2CH_3	CH_2CH_3	V
40	L-ristosaminyl-rhodomycin D	H	OH	CO_2CH_3	CH_2CH_3	VI
41	3'-*N*-methyl-L-ristosaminyl-rhodomycin D	H	OH	CO_2CH_3	CH_2CH_3	VII
42	4'-*epi*-L-vancosaminyl-rhodomycin D	H	OH	CO_2CH_3	CH_2CH_3	VIII
43	L-rhamnosyl-rhodomycin D	H	OH	CO_2CH_3	CH_2CH_3	IX
44	D-olivosyl-rhodomycin D	H	OH	CO_2CH_3	CH_2CH_3	X
45	L-olivosyl-rhodomycin D	H	OH	CO_2CH_3	CH_2CH_3	XI
46	D-digitoxosyl-rhodomycin D	H	OH	CO_2CH_3	CH_2CH_3	XII
47	L-digitoxosyl-rhodomycin D	H	OH	CO_2CH_3	CH_2CH_3	XIII
1	Daunorubicin	CH_3	OH	H	$COCH_3$	I
48	3'-*N*-methyl-daunorubicin	CH_3	OH	H	$COCH_3$	II
49	4'-*epi*-daunorubicin	CH_3	OH	H	$COCH_3$	IV
50	4'-*epi*-L-vancosaminyl-daunorubicin	CH_3	OH	H	$COCH_3$	VIII
2	Doxorubicin	CH_3	OH	H	$COCH_2OH$	I
51	3'-*N*-methyl-doxorubicin	CH_3	OH	H	$COCH_2OH$	II
52	Epirubicin	CH_3	OH	H	$COCH_2OH$	IV
53	L-daunosaminyl-aklavinone	H	H	CO_2CH_3	CH_2CH_3	I
54	3'-*N*-methyl-L-daunosaminyl-aklavinone	H	H	CO_2CH_3	CH_2CH_3	II
55	L-rhodosaminyl-aklavinone	H	H	CO_2CH_3	CH_2CH_3	III
56	4'-*epi*-L-daunosaminyl-aklavinone	H	H	CO_2CH_3	CH_2CH_3	IV
57	L-ristosaminyl-aklavinone	H	H	CO_2CH_3	CH_2CH_3	VI
58	4'-*epi*-L-vancosaminyl-aklavinone	H	H	CO_2CH_3	CH_2CH_3	VIII
59	4'-*N*-demethyl-D-amosaminyl-aklavinone	H	H	CO_2CH_3	CH_2CH_3	XIV
60	4'-*N*-demethyl-D-amosaminyl-rhodomycin D	H	OH	CO_2CH_3	CH_2CH_3	XIV
61	D-desosaminyl-aklavinone	H	H	CO_2CH_3	CH_2CH_3	XV
62	D-desosaminyl-rhodomycin D	H	OH	CO_2CH_3	CH_2CH_3	XV

Figure 9 Glycodiversified anthracycline analogs produced via combinatorial biosynthesis. New analogs produced in studies by Han et al.[66] and Ryu et al.[74] are indicated in red typeface.

3. Conclusion

The past two decades have witnessed a revitalization of the anthracyclines with respect to glycodiversification and pathway engineering of these molecules. Most anthracyclines in clinical use, such as epirubicin, pirarubicin, and idarubicin, are semi-synthetic derivatives of natural products [70]. However, it is important to note that the chemical space available for modification by pathway engineering or organic synthesis differs significantly [71]. Of particular interest is the ability of *Actinomycetes* to generate hundreds of diverse carbohydrates through dedicated biosynthetic pathways that have now been characterized for the most part [72]. Glycosylation has a significant impact on the biological activity of anthracyclines, and therefore, glycodiversification of naturally occurring anthracyclines would allow access to previous underexplored chemical space for biological testing [73].

The development of *Streptomyces venezuelae* as chasses for combinatorial biosynthesis of novel glycosylated analogs offers an excellent starting point [66]. These studies revealed, for the first time, the moderate substrate promiscuity of glycosyltransferases AknS, SnogE, and StfG towards non-canonical TDP-deoxysugar donor substrates. The approach is enhanced by a novel one-pot co-culturing system for synthesis of aklavinone, ε-rhodomycinone, and TDP-amino-trideoxysugars separately to generate new analogs [74]. The major drawback of the methodology is that the production titers for most of these compounds were <10 mg/L due to insufficient catalytic efficiency of the glycosyltransferases towards non-cognate substrates. In future efforts, these foundational discoveries can be improved via the incorporation of pathway engineering and glycosyltransferase engineering.

The crystallization of SnogD provided the first insight into the structure of an anthracycline glycosyltransferase. The identification of discrete *N*-terminal aglycone acceptor and *C*-terminal deoxysugar donor typical of glycosyltransferases sets the stage for future efforts to engineer these enzymes to perform an expanded repertoire of transfer reactions. Other glycosyltransferases, such as ElmGT, feature unusually relaxed donor-substrate specificity. For example, wildtype ElmGT efficiently transferred TDP-4'-keto-L-olivose to 8-DMTC, which was surprising, given that ElmGT was not able to previously turnover TDP-4'-keto-L-rhamnose, which more closely resembles its natural substrate [51]. Using a different approach, site-directed mutagenesis of the loosely conserved α/β/α motif of the nucleoside-diphosphate binding region of ElmGT was engineered to modulate the deoxysugar transfer to 8-DMTC. Altogether, these discoveries signify that engineering of glycosyltransferases could provide additional enzymatic catalysts for transferring exotic deoxysugar donor substrates.

The last twenty years have seen the synthesis of new anthracycline analogs and the development of a deeper understanding of the systems by which these analogs can be created. Once the remaining issues regarding the specificity of carbohydrate transfer can be solved, hundreds of the next generation of anthracycline analogs can be developed in a rapid manner using synthetic biology to expand the chemical space of this important class of pharmaceuticals.

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