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MINI-REVIEW

Achievements and impacts of glycosylation reactions involved in natural product biosynthesis in prokaryotes

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Abstract Bioactive natural products, such as polyketides, flavonoids, glycopeptides, and aminoglycosides, have been used as therapeutic agents. Many of them contain structurally diverse sugar moieties attached to the aglycone core structures. Glycosyltransferases (GTs) catalyze the attachment of nucleotide-activated sugar substrates to acceptor aglycones. Because these sugar moieties are usually essential for biological activity, in vivo pathway engineering in prokaryotic hosts and in vitro enzymatic approaches coupled with GT engineering are currently being used to synthesize novel glycosylated derivatives, and some of them exhibited improved biological activities compared to the parent molecules. Therefore, harnessing the potential of diverse glycosylation reactions in prokaryotes will increase the structural diversity of natural products and the possibility to generate new bioactive products.

Keywords Glycosylation · Glycosyltransferase · Natural products · Prokaryotes

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Introduction

A diverse range of bioactive natural products, such as polyketides, flavonoids, glycopeptides, and aminoglycosides, are often glycosylated with mono-, di-, tri-, or oligosaccharides. They have useful biological activities for clinical applications, including antibiotics (e.g., pikromycin, kanamycin, vancomycin), antitumors (kaempferol, apigenin), anti-inflammatories (quercetin), and antioxidants (naringenin). Glycosyltransferases (GTs) catalyze the glycosylation of acceptor molecules with the activated nucleotide sugar, resulting in the formation of *O*-, *N*-, *S*-, and *C*-glycosidic bonds (Gantt et al. 2011). A range of highly modified unusual sugar structures are generated through multiple enzymatic modifications of common sugar precursors and transferred to the natural product aglycones in prokaryotes (Thibodeaux et al. 2007). The sugar moiety usually affects their biological activity and is responsible for the specific contacts with molecular targets (Gantt et al. 2011). Therefore, significant efforts have gone into the biological generation of novel natural product derivatives by modifying their glycosylation patterns due to the difficulties in chemical modification of the sugar moieties of the structurally complex natural products (Gantt et al. 2011).

The common biological strategies used for the generation of novel glycosylated natural products are illustrated in Fig. 1. Inactivation of the gene(s) involved in specific sugar biosynthesis, expression of heterologous sugar biosynthesis genes or the substrate-flexible GT, or combination of both strategies have been used to generate novel glycosylated natural products in producer strains in vivo. Nonproducer strains, in which the genes for the biosynthesis of the native aglycone and sugar are deleted and recombinant sugar biosynthesis pathway and GT are expressed, can be used as the bioconversion hosts for the glycosylation reaction of

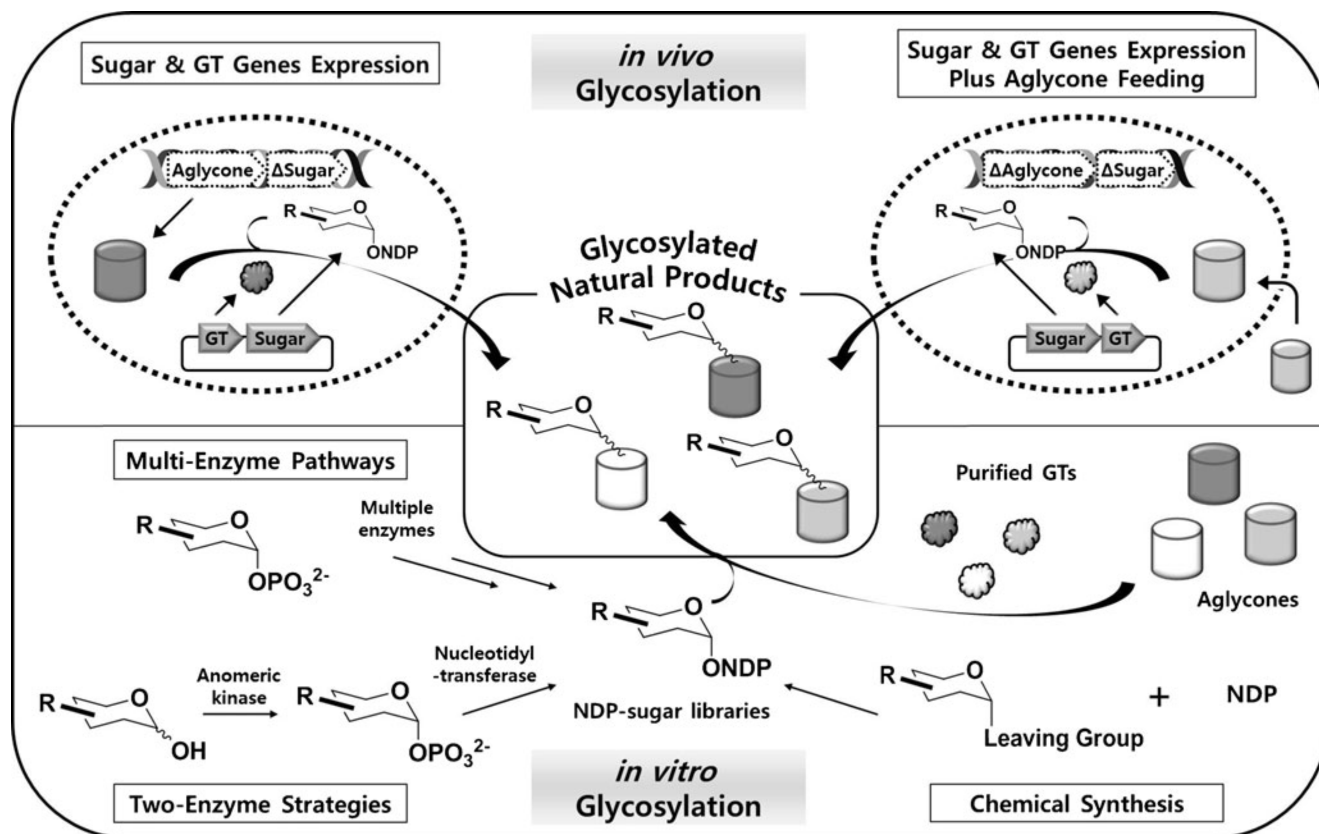


Fig. 1 Various approaches for the development of novel glycosylated natural products

exogenously fed aglycone (Salas and Méndez 2007; Thibodeaux et al. 2007; Han et al. 2012). For decades, it has been shown that GTs involved in the biosynthesis of natural products in prokaryotes have substrate flexibility toward both sugar donor and aglycone acceptor and these findings have facilitated the development of in vitro approaches to generate structurally diverse natural products with altered glycosylation patterns (Gantt et al. 2011). Nucleoside diphosphate (NDP) sugars can be generated through conventional multiple enzyme reactions or glycorandomization strategy, which takes advantage of substrate-flexible anomeric sugar kinases and nucleotidyltransferases (Fu et al. 2003) that are transferred onto aglycone by flexible GTs to create structurally diverse natural products.

Since there are several reviews covering the biosynthesis of unusual nucleotide sugars in microbial natural products (Thibodeaux et al. 2007, 2008), herein we highlight some of the examples of in vivo pathway engineering in prokaryotic hosts and in vitro enzymatic synthesis for the generation of new glycosylated natural products with an emphasis on glycosylation reactions catalyzed by the substrate-flexible prokaryotic GTs, which is crucial to the success of biological glycodiversification of natural products. We also describe recent advances in the engineering of GTs to alter or expand their substrate specificity.

Glycosylation reactions in polyketide biosynthesis

Polyketides are a structurally diverse class of natural products found in bacteria, fungi, and plants and exhibit a variety of biological activities including antibacterial, antifungal, immunosuppressive, anticancer, anticholesterol, and antiparasitic properties (Hertweck 2009). They are biosynthesized by polyketide synthases (PKSs) and broadly divided into three classes: the "complex" or "reduced" polyketides synthesized by type I PKSs such as macrolide, polyether, and polyene; the aromatic polyketides generally synthesized by type II PKSs such as anthracycline and angucycline; and the small aromatic polyketides synthesized by chalcone synthase-like type III PKSs such as flavonoids, stilbene, and flavolin (Shen 2003). The deoxysugar moiety of polyketides has been shown to be essential for their biological activity, and various GTs involved in the biosynthesis of polyketide have been found to exhibit substrate flexibility toward their sugar donor or aglycone acceptor and, sometimes, to both (Gantt et al. 2011). Here, we will summarize some glycosylation reactions catalyzed by the representative flexible prokaryotic GTs involved in polyketide biosynthesis and their use for glycodiversification of polyketides.

DesVII GT in *Streptomyces venezuelae*, a notable example of substrate-flexible macrolide GTs, catalyzes the attachment

of thymidine-5′-diphospho (TDP)-D-desosamine onto 12- and 14-membered macrolactone rings, 10-deoxymethynolide (1) and narbonolide (2), to make YC-17 (3) and narbomycin (4), respectively (Borisova et al. 2004, 2006). Several new glycosylated derivatives of 1 and 2 were generated by attachment of various unnatural sugars. A mutant strain of *S. venezuelae* was engineered by deletion of TDP-D-desosamine biosynthetic gene cluster and heterologous expression of unnatural sugar biosynthetic gene cassettes for the biosynthesis and attachment of various unnatural sugars to 1 and 2. The sugar flexible GT, DesVII, was able to attach the various sugars (Yamase et al. 2000; Hong et al. 2004; Han et al. 2012). Especially 3'-*O*-demethyl-D-chalcosyl narbonolide (5) and L-rhamnosyl narbonolide (6) (Fig. 2a) were shown to exhibit greater antibacterial activity than 4 and the clinically relevant erythromycin (Han et al. 2012). In addition, in *S. venezuelae*, which was genetically manipulated to be deficient in the production of its macrolide antibiotics by deletion of the gene cluster encoding the pikromycin PKS and desosamine biosynthetic enzymes, DesVII was also shown to attach these unnatural sugars to the exogenously supplemented nonnative aglycone, 16-membered tylactone (Jung et al. 2007; Han et al. 2011b). In an in vitro system, DesVII was also shown to attach native or various unnatural sugars to acyclic and cyclic aglycones (Kao et al. 2006; Borisova et al. 2008). For the glycosylation reactions to proceed efficiently, some GTs in polyketide biosynthesis, including DesVII, require an auxiliary protein, such as DesVIII, although its actual role in catalysis is unclear (Lu et al. 2005; Yuan et al. 2005; Hong et al. 2007).

Anthracyclines are a member of drugs used mainly in cancer chemotherapy. A range of GTs involved in the biosynthesis anthracyclines has been studied. Elloramycin (7) is an anthracycline-type antitumor drug that is related to tetracenomycin C, which is produced by *Streptomyces olivaceus* Tü2353 (Drautz et al. 1985). ElmGT in the elloramycin biosynthetic gene cluster normally transfers an L-rhamnose to 8-demethyltetracenomycin C (8-DMTC, 8) (Blanco et al. 2001), and the resulting sugar moiety is further modified by trimethylation generating 7 (Fig. 2b). Heterologous expression of cosmid 16F4, which contains the elloramycin biosynthesis cluster of *S. olivaceus* Tü2353 in the PKS-deleted mutant of the urdamycin producer *Streptomyces fradiae* Tü2717/ΔPKS and in the mithramycin producer *Streptomyces argillaceus* ATCC 12956, resulted in the production of several novel glycosylated derivatives of 8. ElmGT was able to transfer various sugars including a disaccharide onto 8. These transfers were not catalyzed by GT of the *S. fradiae* or *S. argillaceus* strains, only ElmGT can catalyze the glycosylation of 8 using various sugars (Wohlert et al. 1998). It was found that two mithramycin GTs in *S. argillaceus* (MtmGI and MtmGII) were responsible for the formation of the diolivosyl disaccharide, by currently unknown mechanisms, which was then transferred by ElmGT to 8 generating 8-demethyl-8-*O*-β-D-olivosyl-(1″→3′)-*O*-β-D-olivosyl tetracenomycin C (9, Fig. 2b) (Blanco et al. 2001). Co-expression of cosmid 16F4 and various sugar biosynthesis cassette genes in *Streptomyces lividans* resulted in the glycosylation of 8 with various NDP sugars (Fischer et al. 2002; Lombó et al. 2004; Pérez et al. 2005; Nybo et al. 2012).

Aranciamycin (10), which belongs to anthracycline group, is produced from *Streptomyces echinatus* (Keller-Schierlein and Müller 1970) and AraGT in the aranciamycin biosynthetic gene cluster normally transfers an L-rhamnose onto aranciamycinone aglycone (Sianidis et al. 2006). Expression of the aranciamycin biosynthetic genes including AraGT in heterologous hosts *Streptomyces diastatochromogenes* Tü6028 and *S. fradiae* A0 resulted in the production of several novel aranciamycins (Luzhetskyy et al. 2007, 2008). One of them, aranciamycin E (11), which is hydroxylated at C1 position and lacks any deoxysugar at C7 position (Fig. 2b), showed the best inhibitory activity against human tumor cell lines (Luzhetskyy et al. 2008), indicating that the hydroxylation pattern and glycosylation pattern of aglycone play critical roles in biological activities.

Another anthracycline steffimycin (12), which resembles aranciamycin, is produced by *Streptomyces steffisburgensis* and StfG GT involved in steffimycin biosynthesis transfers an L-rhamnose moiety to C7 position of aglycone (Gullón et al. 2006). Several novel glycosylated steffimycins were produced in *Streptomyces albus* through the heterologous expression of the steffimycin biosynthetic genes along with various kinds of deoxysugar biosynthetic gene cassettes or flexible 3′-*O*-methyltransferase OleY (Olano et al. 2008). Two of novel steffimycin derivatives, D-digitoxosyl-8-demethoxy-10-deoxysteffimycinone (13) and 3'-*O*-methylsteffimycin (14) (Fig. 2b), showed improved antitumor activities.

The anthracycline antitumor agent aclacinomycin A (15) produced by *Streptomyces galilaeus* is composed of an aklavinone aglycone and a trisaccharide moiety, which is catalyzed by two GTs, AknS and AknK (Räty et al. 2000, 2002). AknS transfers the first sugar TDP-L-rhodosamine to the aglycone, and then AknK transfers the second sugar TDP-2-deoxy-L-fucose and the third sugar TDP-L-rhodinose consecutively (Lu et al. 2004a, 2005; Leimkuhler et al. 2007). AknK attached the various unnatural second or third sugars to aklavinone aglycone and also to other monoglycosylated aglycones (Lu et al. 2004a). Combination of AknS/AknT (AknT is an auxiliary protein required for efficient glycosylation reaction) and AknK generated the novel glycosylated aclacinomycins, including 2-deoxy-1-fucosyl-2-deoxy-1-fucosyl-rhodosaminyl-aklavinone (16) which has a trisaccharide moiety (Fig. 2b) (Lu et al. 2005; Leimkuhler et al. 2007). Additionally, glycosylated derivatives of doxorubicin were produced in *S. venezuelae*-based combinatorial biosynthetic system by using AknS/AknT pair. AknS efficiently transferred TDP-L-daunosamine or TDP-L-rhodosamine to ε-rhodomycinone generating rhodomycin D (17) and L-

rhodosaminyl-rhodomycin D (18), respectively (Fig. 2b) (Han et al. 2011a). Therefore, AknS/AknT pair and AknK can be a useful glycosylation tool to generate the diverse anthracycline variants.

Nogalamycin (19), produced by *Streptomyces nogalater*, is also an anthracycline antibiotic (Torkkell et al. 2001) and contains two deoxysugars: one is a nogalose, which is attached to nogalamycinone at C7 position by SnogE GT, and the other deoxysugar is a nogalamine, which is existed as an unusual epoxyoxocin moiety connected through C1 and C2 positions, transferred by SnogD GT (Siitonen et al. 2012). The heterologous expression of the nogalamycin gene cluster in *S. albus* led to the production of three novel compounds such as nogalamycin R (20), 3′,4′-demethoxynogalose-1-hydroxynogalamycinone (21), and nogalamycin F (21) (Fig. 2b). Only 20 showed weak activity against topoisomerase II (Siitonen et al. 2012). In addition, SnogD was able to remove the deoxysugar from 22, but not from 21, which suggests that SnogD catalyzes both the *O*-glycosylation reaction at C1 position of aglycone and the reverse glycosylation reaction (Siitonen et al. 2012). There are several studies regarding reversibility in the glycosyltranfer reaction, which could be a useful tool for rare NDP-sugar synthesis and sugar/aglycone exchange (Zhang et al. 2006a, b).

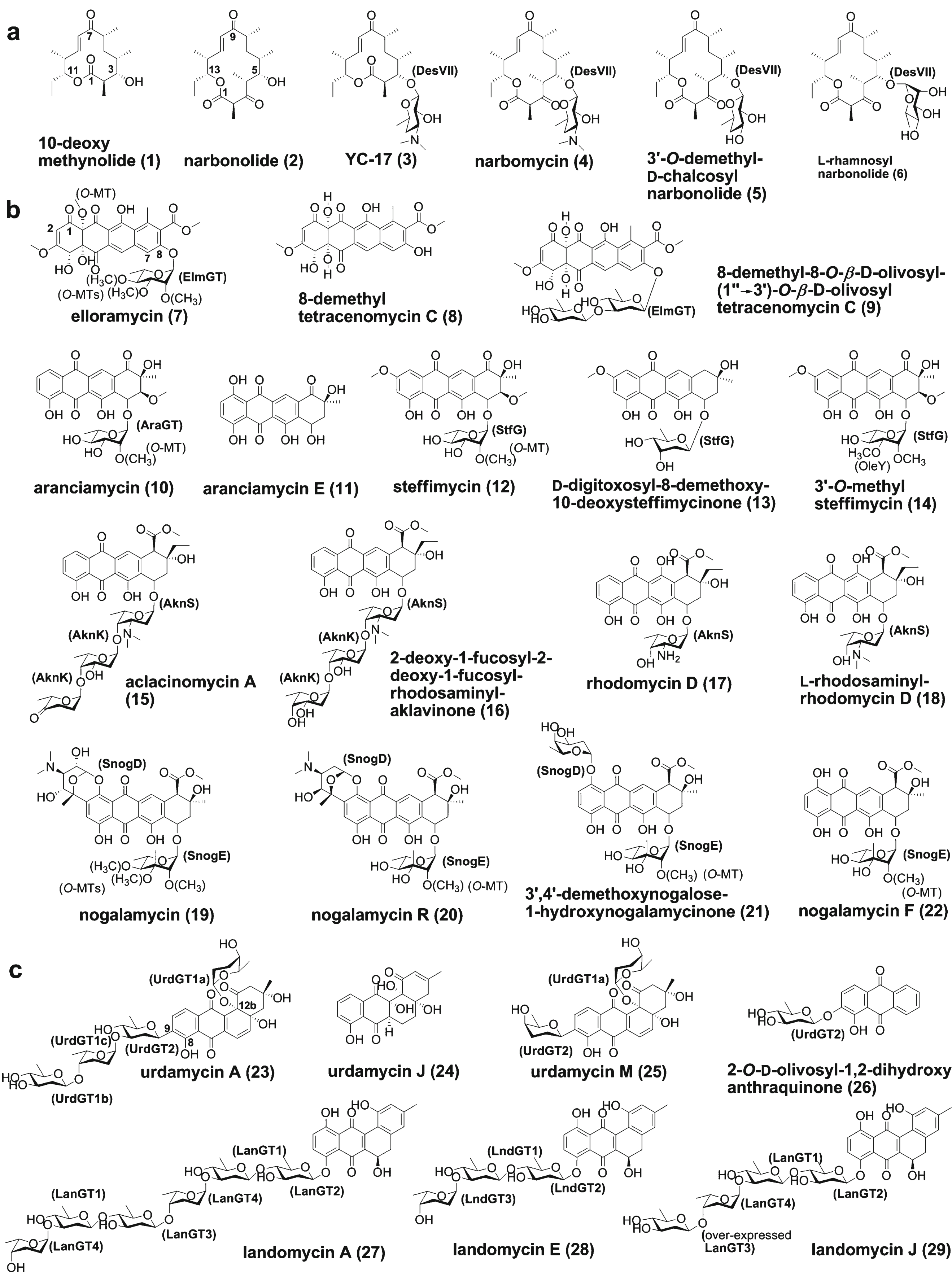
Another group of polyketide, angucyclines, exhibits mainly anticancer and antibacterial activities. Urdamycin A (23) is an angucycline-type antibiotic and anticancer agent produced by *S. fradiae* Tü2717 (Drautz et al. 1986). This agent is composed of a tetrasaccharide moiety, *C*-glycosidically linked D-olivose, and three additional *O*-glycosidically linked deoxysugars (two L-rhodinoses and one D-olivose). Four GTs (three *O*-GTs, UrdGT1a, UrdGT1b, and UrdGT1c, and one *C*-GT, UrdGT2) are involved in the glycosylation of 23 (Künzel et al. 1999; Trefzer et al. 2000). UrdGT2 catalyzes the formation of a glycosidic C-C bond between aglycone and sugar moiety (Vogt and Jones 2000). Disruption of the *urdGT2* gene in *S. fradiae* Tü2717 resulted in the formation of novel urdamycins. Interestingly, urdamycin J (24; Fig. 2c) was shown to display good anticancer activity in in vitro tests (Künzel et al. 1999). Genetic manipulation of deoxysugar biosynthetic genes of urdamycin biosynthesis in *S. fradiae* Tü2717 showed that UrdGT2 was able to transfer NDP-D-rhodinose instead of NDP-D-olivose to C9 position, generating urdamycin M (25; Fig. 2c) (Hoffmeister et al. 2000). Further in vivo work showed that UrdGT2 could also attach a D-olivose to the *C*-position of various aglycones (Trefzer et al. 2002; Dürr et al. 2004; Luzhetskyy et al. 2005a, b; Baig et al. 2006). These results demonstrate the potential of using UrdGT2 for the generation of novel *C*-glycosylated polyketide compounds. Interestingly, UrdGT2 showed the unusual *O*-glycosylating ability. In urdamycin PKS-deleted strain *S. fradiae*, UrdGT2 transferred NDP-D-olivose onto the exogenously supplemented aglycone, 1,2-dihydroxyanthraquinone, generating *O*-glycosylated compound, 2-*O*-β-D-olivosyl-1,2-dihydroxyanthraquinone (26; Fig. 2c). This result suggests that UrdGT2 has the potential ability for both *C*- and *O*-glycosylation (Dürr et al. 2004).

Fig. 2 Structures of polyketides and novel glycosylated derivatives. **a** Macrolide YC-17 (3)/narbomycin (4) and their glycosylated derivatives. **b** Anthracycline-type polyketides andtheir glycosylated derivatives. **c** Angucycline-type polyketides and their glycosylated derivatives. GTs responsible for the attachment of the corresponding sugar moieties are indicated in parenthesis

Landomycin A (27), which also belongs to the angucycline group, is produced by *Streptomyces cyanogenus* S136 and consists of a landomycinone aglycone and an *O*-glycosidically linked hexasaccharide moiety comprised of two repeating trisaccharide units of D-olivose-D-olivose-L-rhodinose (Henkel et al. 1990). Only four GTs participate in the glycosylation of hexasaccharide moiety of landomycin A (von Mulert et al. 2004). LanGT1 and LanGT4 catalyze the transfer of two sugars, the second and the fifth D-olivose moieties and the third and the sixth L-rhodinose moieties, respectively (Trefzer et al. 2001; Luzhetskyy et al. 2005a), while LanGT2 and LanGT3 transfer one sugar, the first and the fourth D-olivose moiety, respectively (Luzhetskyy et al. 2004, 2005b). Twenty-seven shows better antitumor activity than landomycin E (28; Fig. 2c), which contains the shorter sugar moiety catalyzed by three GTs (LndGT1, LndGT2, and LndGT3) (Ostash et al. 2004). Expression of lanGTs in urdamycin GT genes-defective mutant of *S. fradiae* Tü2717, except for *urdGT2* gene, resulted in the formation of novel angucyclines that contain the second sugar D-olivose attached to the *C*-glycosidically linked D-olivose (Trefzer et al. 2001). In addition, expression of *lanGT4* gene in the urdamycin producer *S. fradiae* Tü2717 resulted in the production of two novel urdamycins, which has an extra L-rhodinose moiety each. As a result, LanGT4 seems to be a useful tool for saccharide extensions (Hoffmeister et al. 2004). In addition, the overexpression of *lanGT3* gene in *S. cyanogenus* S136 resulted in the production of novel landomycin J containing a tetrasaccharide moiety (29; Fig. 2c). This showed that the unbalancing of the GTs by overexpressing one of the GT genes involved in multiple glycosylation reactions can lead to the formation of novel compounds (Zhu et al. 2007).

Glycosylation reactions in flavonoid biosynthesis

Flavonoids are a class of plant secondary metabolites with diverse biological functions that are frequently found in



a
10-deoxy methynolide (1)
narbonolide (2)
YC-17 (3)
narbomycin (4)
3'-O-demethyl-D-chalcosyl narbonolide (5)
L-rhamnosyl narbonolide (6)
(DesVII)
b
elloramycin (7)
(O-MT)
(O-MTs)
(ElmGT)
8-demethyl tetracenomycin C (8)
8-demethyl-8-O-β-D-olivosyl-(1''→3')-O-β-D-olivosyl tetracenomycin C (9)
aranciamycin (10)
(AraGT)
aranciamycin E (11)
steffimycin (12)
(StfG)
D-digitoxosyl-8-demethoxy-10-deoxysteffimycinone (13)
3'-O-methyl steffimycin (14)
(OleY)
aclacinomycin A (15)
(AknS)
(AknK)
2-deoxy-1-fucosyl-2-deoxy-1-fucosyl-rhodosaminyl-aklavinone (16)
rhodomycin D (17)
L-rhodosaminyl-rhodomycin D (18)
nogalamycin (19)
(SnogD)
(SnogE)
nogalamycin R (20)
3',4'-demethoxynogalose-1-hydroxynogalamycinone (21)
nogalamycin F (22)
c
urdamycin A (23)
(UrdGT1a)
(UrdGT1b)
(UrdGT1c)
(UrdGT2)
urdamycin J (24)
urdamycin M (25)
2-O-D-olivosyl-1,2-dihydroxy anthraquinone (26)
landomycin A (27)
(LanGT1)
(LanGT2)
(LanGT3)
(LanGT4)
landomycin E (28)
(LndGT1)
(LndGT2)
(LndGT3)
landomycin J (29)
(over-expressed LanGT3)

fruits, vegetables, and cereals and thus are common components of our daily diet (Gattuso et al. 2007). In humans, they are most commonly known for their antioxidant, antibacterial, and antiviral activities; ability to protect against cardiovascular disease and cancer; and display of antiallergic, hepatoprotective, cytostatic, apoptotic, estrogenic, or antiestrogenic properties (Harborne and Williams 2000; Middleton et al. 2000). 3-*O*-rhamnosyl quercetin (30) and 3-*O*-rhamnosyl kaempferol (31) (Fig. 3) isolated from plants have been shown to possess better antiviral and antibacterial activities compared to flavonol aglycones (Choi et al. 2009; Tatsimo et al. 2012), demonstrating the impact of glycosylation on the biological activity of flavonoids. Moreover, the low solubility of flavonoids, which limits the application of this class of natural products, can be overcome by glycosylation (Li et al. 2004a).

Some plants GTs are exploited for the glycosylation of flavonoid in a bioconversion system using *Escherichia coli*.

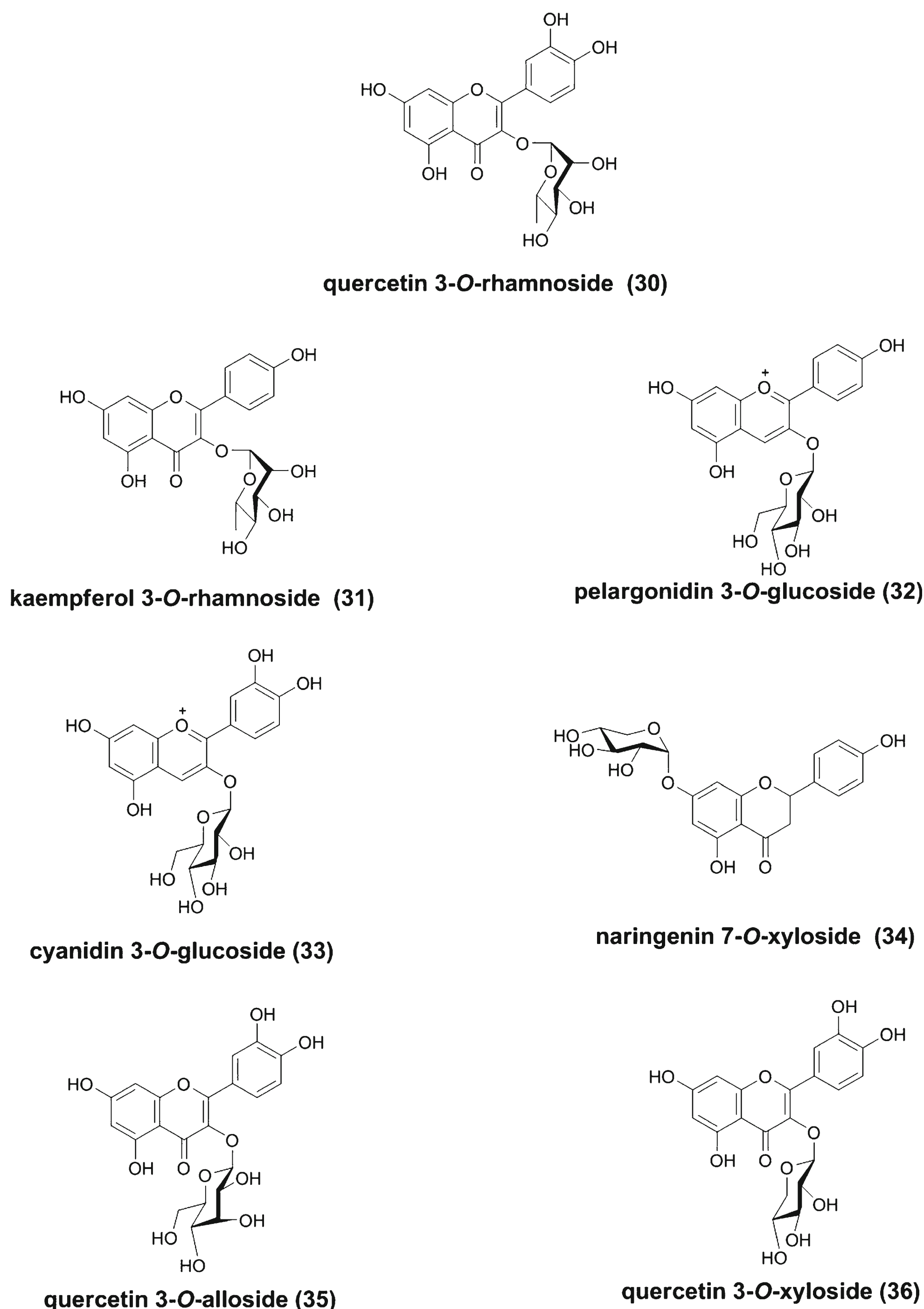


Fig. 3 Structures of novel glycosylated flavonoids

Expression of an artificial gene cluster that contains the four plant-derived genes, flavanone 3β-hydroxylase from *Malus domestica*, dihydroflavonol 4-reductase from *Anthurium andraeanum*, anthocyanidin synthase from *M. domestica*, and uracil-5′-diphospho (UDP)-glucose:flavonoid 3-*O*-glucosyltransferase from *Petunia hybrida* converted the flavanones, naringenin and eriodictyol, into colored anthocyanins, 3-*O*-glucosyl pelargonidin (32) and 3-*O*-glucosyl cyanidin (33) (Fig. 3) (Yan et al. 2005). A combination of the prokaryotic sugar biosynthetic pathway and plant GT have been used to create more structurally diverse glycosylation patterns in flavonoids. UDP-glucose dehydrogenase gene (*calS8*) and UDP-glucuronic acid decarboxylase gene (*calS9*) from *Micromonospora echinospora* spp. *calichensis*, and 7-*O*-GT gene (*arGt*-4) from *Arabidopsis thaliana* were expressed together with an integrated copy of glucose-1-phosphate-1 uridylyltransferase gene (*gal*U) from *E. coli* K12 in *E. coli* BL21 (DE3)/Δ*pgi*, in which the glucose phosphate isomerase gene (*pgi*) was deleted to increase the intracellular pool of UDP-glucose, a precursor of UDP-xylose. The engineered *E. coli* was fed with naringenin to produce 7-*O*-xylosyl naringenin (34; Fig. 3) (Simkhada et al. 2009). Similarly, the pools of TDP-L-rhamnose and TDP-6-deoxy-D-allose sugars were developed separately by combining the respective sugar biosynthetic genes from different bacterial sources in *E. coli* BL21 (DE3)/Δ*pgi* along with the GT gene (*arGt*-3) from *A. thaliana.* When the recombinants were supplemented with extracellular quercetin and kaempferol, the respective strains produced 30, 31, and 3-*O*-allosyl quercetin (35; Fig. 3) (Simkhada et al. 2010). Recently, co-expression of the genes involved in the biosynthesis of UDP-glucose from glucose-6-phosphate (phosphoglucomutase gene (*nfa44530*) from *Nocardia farcinica* and *galU* from *E. coli* K12) and the genes for the conversion of UDP-glucose to UDP-xylose (*calS8* and *calS9* from *M. echinospora* spp. *calichensis*) resulted in the high-yield bioconversion of quercetin to 3-*O*-xylosyl quercetin (36; Fig. 3) by ArGt-3 in an engineered *E. coli* BL21 (DE3)/Δ*pgi*Δ*zwf*Δ*ushA*. This strain was engineered by knocking out the three genes, *pgi*, *zwf* (encoding D-glucose-6-phosphate dehydrogenase), and *ushA* (UDP-glucose hydrolase), to increase the conversion of glucose-6-phosphate to UDP-xylose (Pandey et al. 2012).

Microbial GTs have also been used for the biosynthesis of flavonoid glycosides. GT from *Bacillus cereus*, BcGT-1, could use apigenin, genistein, kaempferol, luteolin, naringenin, and quercetin as sugar acceptors, resulting in the production of 3-*O*-glucosyl kaempferol, 7-*O*-glucosyl kaempferol, 7-*O*-glucosyl naringenin, 3-*O*-glucosyl quercetin, 7-*O*-glucosyl quercetin, 7-*O*-glucosyl apigenin, 4′-*O*-glucosyl apigenin, and 4′-*O*-glucosyl luteolin in an *E. coli* bioconversion system expressing the *BcGT-1* (Ko et al. 2006). In an in vitro system, the purified recombinant OleD GT from *Streptomyces antibioticus*, which transfers UDP-glucose to the macrolide oleandomycin aglycone, was able to catalyze glycosylation of various flavonoids with UDP-glucose: flavone (apigenin, luteolin), flavonol (kaempferol), flavanone (naringenin), and isoflavone (daidzein, genistein) (Choi et al. 2012).

Glycosylation reactions in nonribosomal glycopeptide biosynthesis

Glycopeptide antibiotics, such as vancomycin and teicoplanin, which consist of a heptapeptide moiety synthesized by nonribosomal peptide synthetase, are clinically important for the treatment of life-threatening infections caused by Gram-positive bacteria (Donadio and Sosio 2008). The heptapeptide skeleton is generally further modified by chlorination, methylation, sulfation, or glycosylation. Due to its pharmacological importance, efforts to create novel glycosylated derivatives of glycopeptide have been made over the past decades.

Vancomycin (37) biosynthesis cluster has two GTs, GtfE attaches D-glucose onto 4-OH of 4-hydroxyphenylglycine at C4 position of vancomycin alycone scaffold and GtfD transfers L-vancosamine to the 2-OH group of the glucosyl peptide (Losey et al. 2001, 2002). Similarly, chloroeremomycin (38), which is a member of the vancomycin family of antibiotics, has three sugars including one D-glucose and two L-4-*epi*-vancosamines attached by GtfA, GtfB, and GtfC. GtfA transfers L-4-*epi*-vancosamine to the β-OH of β-hydroxytyrosine at C6 position of vancomycin aglycone. GtfB and GtfC, which exhibit significant homology to GtfE and GtfD, respectively, attach a D-glucose and an L-4-*epi*-vancosamine at C4 position of vancomycin aglycone, respectively (Losey et al. 2001; Lu et al. 2004b). Likewise, balhimycin (39) GT, bGtfB, which shows high similarity to GtfB and GtfE, is mostly supposed to have same function. Another balhimycin GT, bGtfA, shows high similarity to GtfA and may be involved in the attachment of L-dehydrovancosamine at C6 position of aglycone (Pelzer et al. 1999). In the teicoplanin (40) biosynthesis, three GTs are contained, tGtfA and tGtfB add an *N*-acetylglucosamine at C6 and C4 positions of teicoplanin aglycone, respectively, and it is supposed that the attachment of D-mannose at C7 position is catalyzed by the putative mannosyltransferase Orf3* (Li et al. 2004b; Sosio et al. 2004; Howard-Jones et al. 2007).

Hybrid glycopeptide antibiotics, xylosyl vancomycin aglycone (41), glucosyl A41030A (42), and glucosyl A47934 (43) (Fig. 4), were produced by glycosylation reaction of GtfE from the vancomycin producer, both in vitro and in vivo. Forty-one, 42, and 43 were generated by purified GtfE expressed in *E. coli*, and expression of the *gtfE* gene in the A47934-producing *S. toyocanesis* also generated 43 (Solenberg et al. 1997). In addition, the substrate flexibility

of the GtfE enzyme was examined with a number of novel sugar donors prepared by chemoenzymatic synthesis and both vancomycin and teicoplanin aglycones. As a result, small libraries of novel glycopeptides were generated, such as vancomycin and teicoplanin aglycones possessing deoxyglucose or analogues with amino groups at the 2-, 3-, 4-, and/or 6 position of the glucose ring (Losey et al. 2001, 2002; Fu et al. 2003). These novel glycopeptide derivatives generated by GtfE might be used as substrates for GtfD, which can transfer a 4-*epi*-vancosamine moiety to each of the analogues generated by GtfE, resulting in the production of compounds 4-*epi*-vancosaminyl-(1″→2′)-4-aminoglucosyl vancomycin aglycone (44) and 4-*epi*-vancosaminyl-(1″→2′)-4-deoxyglucosyl teicoplanin aglycone (45) (Fig. 4) (Losey et al. 2002). Therefore, the combined actions of GtfE and GtfD have been successfully used to create novel glycopeptides. Also, using vancomycin aglycone, the GtfB and GtfC attached UDP-glucose and UDP-L-4-*epi*-vancosamine in tandem, generating novel glycopeptide epivancomycin (46; Fig. 4) (Walsh et al. 2001). Interestingly, 46 showed nearly the same activity as vancomycin, although other vancomycin/teicoplanin derivatives showed significantly reduced activity compared to the parent glycopeptide. Furthermore, using two flexible GTs, galactosyltransferase from *Helicobacter pylori* and sialyltransferase from *Pasteurella multocida*, novel glycosylated vancomycins were enzymatically synthesized. The biological activity of galactoxyl (1‴→4″) vancomycin (47; Fig. 4) was improved against several MSSA and MRSA strains (Oh et al. 2011).

Glycosylation reactions in aminoglycoside biosynthesis

Aminoglycoside antibiotics have been widely used clinically since the first use of streptomycin as an effective antibiotic in the treatment of tuberculosis (Schatz et al. 1944; Rybak and Whitworth 2005). These antibiotics consist of a central aminocyclitol ring, such as streptamine, streptidine, or 2-deoxystreptamine (2-DOS). Structurally, the clinically important 2-DOS-containing aminoglycosides can be divided into the 4,5-disubstituted aminoglycosides (neomycin (48) and butirosin (49)) and 4,6-disubstituted aminoglycosides (gentamicin (50), kanamycin (51), and tobramycin (52)) based on the position of their glycosidic linkages (Fig. 5).

The first glycosylation step of aminoglycosides involved in the formation of pseudodisaccharides is the addition of *N*-acetylglucosamine to 2-DOS. NeoM from the neomycin gene cluster was first characterized as the UDP-*N*-acetylglucosamine GT (Fan et al. 2008; Yokoyama et al. 2008). GenM1 from the gentamicin gene cluster, BtrM in the butirosin cluster, and other homologs in the aminoglycoside biosynthetic gene clusters also catalyze the same glycosylation step to give 2′-*N*-acetylparomamine, which is further deacetylated to paromamine by the 2′-*N*-acetylparomamine deacetylases, such as BtrM and its homologs (Park et al. 2012).

A second glycosylation is required for the formation of the pseudotrisaccharides. In 4,5-disubstituted aminoglycosides, *O*-ribosylation for the generation of ribostamycin, a key intermediate for neomycin and butirosin, is catalyzed by BtrL and BtrP in the butirosin biosynthetic gene cluster (Kudo et al. 2007). BtrL catalyzes the phosphoribosylation of neamine (6′-aminated paromamine) to form 5″-phosphoribostamycin using 5-phosphoribosyl-1-pyrophosphate as a ribosyl donor and BtrP dephosphorylates 5″-phosphoribostamycin to generate ribostamycin. In 4,6-disubstituted aminoglycosides, GenM2 in the gentamicin cluster and KanM2 in the kanamycin cluster were also found to be responsible for the attachment of UDP-xylose and UDP-kanosamine (3-glycosamine) to paromamine, respectively (Park et al. 2008a; Park et al. 2011).

Interestingly, several GTs of aminoglycosides are flexible toward NDP-sugar donors and/or acceptors. KanM1 in the kanamycin cluster of *Streptomyces kanamyceticus* was found to accept both UDP-glucose and UDP-*N*-acetylglucosamine as glycosyl donors but preferentially transfers glucose to 2-DOS, while the NemD, TobM1 from the tobramycin gene cluster, and GenM1 had a preference for the UDP-*N*-acetylglucosamine as a cosubstrate for attachment to 2-DOS (Park et al. 2011). The second GT in kanamycin biosynthesis, KanM2, was found to be able to transfer two different sugar donors, UDP-glucose and UDP-kanosamine, to four different pseudodisaccharides (paromamine, neamine, 2′-deamino-2′-hydroxyparomamine, and 2′-deamino-2′-hydroxyneamine). KanM2 has also been shown to transfer UDP-kanosamine preferentially to sugar acceptor compared with UDP-glucose (Kudo et al. 2009; Park et al. 2011). BtrL and RacK of ribostamycin cluster catalyzes ribosylation of paromamine as well as neamine to produce 6′-deamino-6′-hydroxyribostamycin (pseudoribostamycin) and ribostamycin, respectively (Kudo et al. 2007; Kurumbang et al. 2011). The remarkable substrate specificities of GTs involved in the biosynthesis of aminoglycosides provided the potential for the biosynthesis of a variety of aminoglycoside derivatives.

Engineering of glycosyltransferases

Most of the GTs that catalyze the attachment of NDP sugars to their acceptor fall into two structurally different types of folds, GT-A and GT-B, and the prokaryotic GTs associated with secondary metabolite biosynthesis belong to the GT-B family. Members of the GT-B family consist of two Rossmann-type domains separated by a deep cleft. The N-terminal domain provides the acceptor-binding site, whereas the C-terminal domain is responsible for binding the sugar donor (Rix et al. 2002; Breton et al. 2006; Thibodeaux et al. 2007).

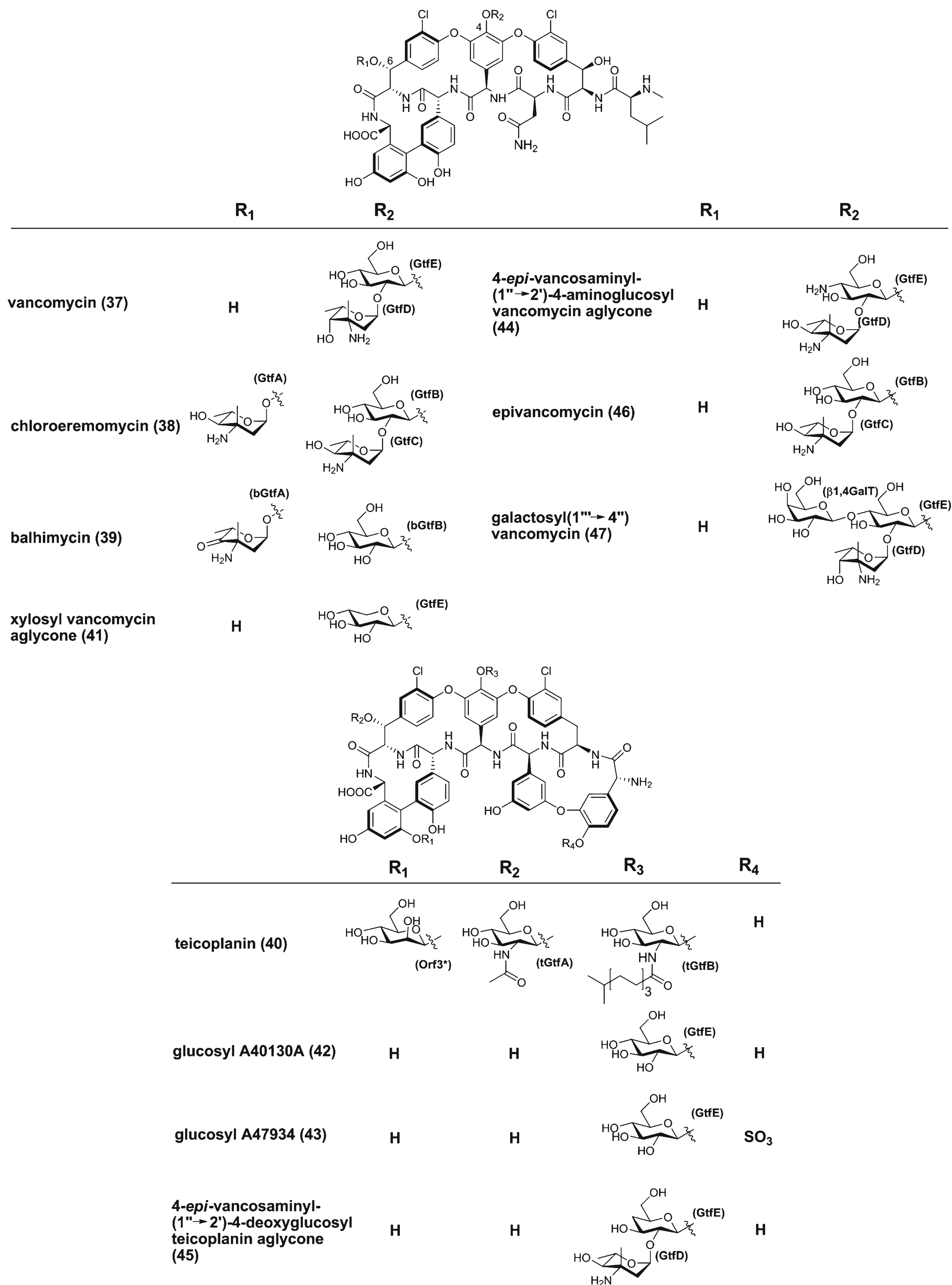
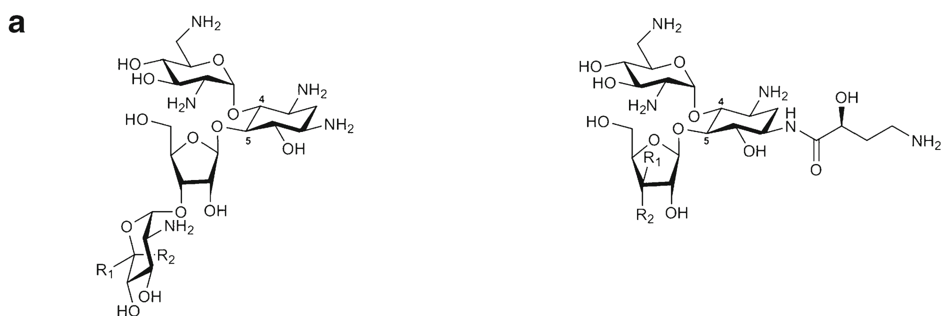


Fig. 4 Structures of glycopeptides and novel glycosylated derivatives. GTs responsible for the attachment of the corresponding sugar moieties are indicated in parenthesis

UrdGT1b and UrdGT1c involved in urdamycin biosynthesis from *S. fradiae* Tü2717 show different specificities for both nucleotide sugar and acceptor substrates but share a surprisingly high number (91 %) of identical amino acids. A

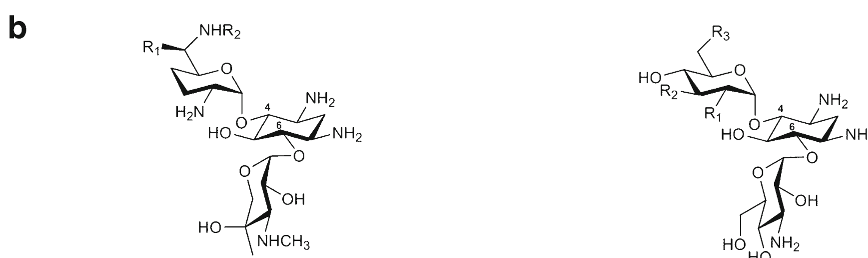
a



48	R_1	R_2
neomycin B	H	CH_2NH_2
neomycin C	CH_2NH_2	H

49	R_1	R_2
butirosin A	OH	H
butirosin B	H	OH

b



50	R_1	R_2
gentamicin C1	CH_3	CH_3
gentamicin C2	CH_3	H
gentamicin C1a	H	H

51	R_1	R_2	R_3
kanamycin A	OH	OH	NH_2
kanamycin B	NH_2	OH	NH_2
kanamycin C	NH_2	OH	OH
tobramycin (52)	NH_2	H	NH_2

Fig. 5 Structures of aminoglycosides. **a** Aminoglycosides which contain 4,5-disubstituted 2-deoxystreptamine. **b** Aminoglycosides which contain 4,6-disubstituted 2-deoxystreptamine

region of 31 amino acids within the N-terminal part of UrdGT1b and UrdGT1c differing in 18 positions was identified to control the substrate specificity of both enzymes. It was shown to be possible to alter the selectivity for both the donor and the acceptor by mutating this region. Consequently, a novel compound carrying an unusual branched saccharide chain was generated, demonstrating that the NDP-sugar donor substrate specificity is determined by the C-terminal domain and the N-terminal domain (Hoffmeister et al. 2001, 2002).

A simple high-throughput screening method using a fluorescent surrogate acceptor, 4-methylumbelliferone, has successfully been applied to expand the substrate specificity of oleandomycin GT (OleD) from *S. antibioticus* by directed evolution. The triple mutant OleD (P67T/S132F/A22V) with considerable expansion of both donor and acceptor specificities was generated by error-prone PCR and site-directed mutagenesis. The evolved triple mutant accepted 15 of the 22 sugar nucleotide donors examined, 12 of which were not accepted by wild-type OleD, with catalytic efficiency improvements ranging from 7- to 300-fold. Furthermore, the activity of mutant OleD toward the small phenolic acceptors, including flavonoid and isoflavonoid, was improved significantly. These results demonstrate the ability to change the GT specificity and proficiency via a few mutations and the potential to create promiscuous GT variants by screening for efficiency only toward a single acceptor–donor pair (Williams et al. 2007).

A domain swapping method to expand the substrate specificity of aminoglycoside GT revealed its potential for use in the synthesis of new aminoglycoside derivatives. A chimeric gene library encoding a random length of the N terminus of KanM1 from kanamycin cluster and C terminus of GtfE from vancomycin cluster was constructed by the

time-dependent incremental truncation method and screened by the plate-based whole cell color assay, which detects pH changes according to the proton release during the glycosylation reaction. The most active mutant displayed significantly improved activity for the transfer of several NDP sugars onto 2-DOS and acquired new donor specificities (Park et al. 2008b). Similarly, by swapping the N- and C-terminal domains of glycopeptide GTs, GtfB and GtfA from chloroeremomycin cluster and Orf10* (tGtfB) and Orf1 (tGtfA) from teicoplanin cluster, hybrid vancomycins were generated by GtfB-Orf10* and GtfA-Orf1 chimeras (Truman et al. 2009).

Landomycin GTs were engineered in order to introduce a new substrate specificity. Two landomycin GTs, LanGT1 catalyzing the transfer of the second and the fifth D-olivose in landomycin A biosynthesis from *S. cyanogenus* S136 and LndGT1 catalyzing the transfer of the second D-olivose in landomycin E from *Streptomyces globisporus* 1912 share 74.8 % identical amino acids. Chimeric genes were made by swapping that focused on exchanging the N-terminal region, in which most of the differences are placed, and expressed in *lanGT1*-deleted mutant of *S. cyanogenus* S136, resulting in the production of various landomycin derivatives (Krauth et al. 2009). Moreover, it has been shown that the hybrid LanGT2 generated by amino acids substitution of the *O*-GT LanGT2 with the *C*-GT UrdGT2 can catalyze *C*-glycosylation reactions (Härle et al. 2011).

Prospects

For years, in vivo and in vitro combinatorial biosynthesis technologies using substrate-flexible GTs have been used for the synthesis of unnatural natural products with altered glycosylation patterns that may have efficacious bioactivities. Understanding the biochemical processes that are involved in the biosynthesis of sugar moieties of natural products and the glycosylation reactions coupled with attempts to engineer GTs will play a key role in the biological synthesis of novel glycosylated products. Recently, the structures of four GTs (CalG1, CalG2, CalG3, and CalG4) in calicheamicin biosynthetic pathway have been reported and provided a valuable resource for understanding the relationship between structure and function (Chang et al. 2011). Advances in the knowledge of GTs structure can provide valuable information for the engineering of these enzymes to create more structural glycodiverse natural products.

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