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Review Article

Digitoxose as powerful glycosyls for building multifarious glycoconjugates of natural products and un-natural products

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ABSTRACT

Digitoxose, a significant 2,6-dideoxyhexose found in nature, exists in many small-molecule natural products. These digitoxose-containing natural products can be divided into steroids, macrolides, macrolactams, anthracyclines, quinones, enediynes, acyclic polyene, indoles and oligosaccharides, that exhibit antibacterial, anti-viral, antiarrhythmic, and antitumor activities respectively. As most of digitoxose-containing natural products for clinical application or preclinical tests, this review also summarizes the biosynthesis of digitoxose, and application of compound diversification by introducing sugar plasmids. It may provide a practical approach to expanding the diversity of digitoxose-containing products.

1. Introduction

Glycosylated natural products are widely distributed in nature and serve as reliable scaffolds for drug discovery and development due to their diverse structures. Over the past decades, a variety of sugars have been discovered in naturally occurring glycosylated products. Specifically, 2,6-dideoxyhexoses, the hydroxyl group at C-2 and C-6 substituted by a hydrogen atom, are crucial for the bioactivity of the active natural compounds.

Digitoxose is a group of 2,6-dideoxyhexoses that is unusual observed in natural products. It exists in ten groups in the natural compounds (Fig. 1), named L-digitoxose (**I**), 4-*O*-methyl-L-digitoxose (**II**), 4-*O*-isopropyl ketone-L-digitoxose (**III**), 4-*O*-methyl ketone-5-*C*-acetic acid-L-digitoxose (**IV**), 4-*O*-methyl ketone-L-digitoxose (**V**), 3-*O*-methyl-L-digitoxose (**VI**), D-digitoxose (**VII**), 4-*O*-methyl-D-digitoxose (**VIII**), 3-*O*-methyl-D-digitoxose (**IX**), 4-deoxy-thio-D-digitoxose (**X**), 4-keto-D-digitoxose (**XI**) respectively. There are almost 50 small natural products with digitoxose moiety have been discovered and studied. The digitoxose attached to secondary metabolites is essential for various bioactivities, including antibacterial, antitumor and antifungal. Therefore, digitoxose shows it irreplaceable position in natural products as a part of structures.

Most of glycosyl modifications can improve the draggability of natural products, such as the increase of water solubility and the decrease of toxicity. In recent years, heterologous expression of sugar plasmids based on digitoxose biosynthesis is a common strategy to add digitoxose to compounds. Since there is no comprehensive review about digitoxose biological activities and biosynthesis, we summarize the structures and bioactivities of 50 digitoxose containing glycosides while discussing their biosynthetic pathway and application, to highlight significance for future endeavors in glycosylated secondary metabolites discovery.

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2. Structures and bioactivities

2.1. Macrolides and macrolactams

The first group of compounds with digitoxose moiety are macrolides and macrolactams, mainly includes macrolides, polyene macrolides, polyene macrolactams, and spirotetronates (Fig. 2). As a class of antimicrobial drugs with carbon lactone rings, most of them are effective against bacteria or fungi, some for mycoplasma, chlamydia, legionella, spirochetes and rickettsia [1,2].

Tylosin IIIC (**1**), a derivative of tylosin, is a 16-membered macrolide produced by the mutagenic mutant *Streptomyces fradiae* NRRL2702 [3]. The C-5 of tylosin lactone skeleton is glucosylated with *β*-L-mycosamine and *β*-D-mycinose. The other sugar group of tylosins IIIC, *β*-D-digitoxose, is attached to C-23 via engineering. Tylosin IIIC shows an inhibitory effect on a variety of Gram-positive pathogenic bacteria including *Bacillus circulans* ATCC 9966, *Corynebacterium equi* IAM 1038, and *Staphylococcus aureus* FDA 209P, and its biological activity in these strains is better promotion than that of tylosin.

Another of macrolides is ammocidins (A-D), which was discovered from *Saccharothrix* sp. AJ9571 by natural screening [4,5]. All of the ammocidin comprised of 6-deoxy-*α*-L-glucose at C-9, and the major differences between ammocidins are the number of olivomycose attached to *O* at C-24. Ammocidin A (**2**), with two deoxy sugars attach to C-24 (*β*-D-digitoxose and *β*-D-olivomycose), was able to cause apoptosis of Ras-dependent Ba/F3–V12 cells (IC_{50} = 66 ng/ml) by reducing the phosphorylation level. There are two olivomycoses linked in ammocidin B, which is less potent than ammocidins A, but more effective than ammocidins C and D (with no glycosyl modification). This also established that deoxysugar, in particular digitoxose, plays a key role in the cytotoxic activity of ammocidins.

Polyene macrolide is a macrolactone ring with 25–37 carbon atoms, which has conjugated polyene structure and corresponding hydroxyl groups, and one or more sugars are connected by glycoside bonds [6]. *O*-glycosides loaded by polyene macrolides are predominately *β*-D-mycosamine, and some are carried additionally to further improve pharmacological properties. Typical polyene macrolide nystatin has a broad-spectrum antifungal effect, including the inhibition of *Cryptococcus*, *Candida albicans*, *Coccidioides*, and has therapeutic functions on infectious diseases caused by protozoa and parasites. It is mainly used for the treatment of fungal infections in the digestive tract and superficial skin fungal infections such as candidal vaginitis and vulvitis in clinic [7–9]. At present, the recognized antibacterial mechanism of nystatin is that it forms a complex with sterols in fungal cell membrane, resulting in a large loss of intracellular small molecular substances and ions [10]. In recent years, nystatin has also been used in combination with metronidazole and nifuratel to treat trichomonas vaginitis [11,12]. There are three components of nystatin from *S. noursei* A94, including nystatin A1, nystatin A3 (**3**), and polyfungin B (**4**) [13,14]. Differ from nystatin A1, the OH at C-24 of nystatin A3 and polyfungin B was modified with *α*-L-digitoxose. showing the higher stability than that of nystatin A1. In addition, the titer of polymycin B was 10879.0 u/mg, which was much higher than the titer of nystatin A1 (3956.0 u/mg) [15]. Our research group tested the toxicity of the three components of nystatin. The lethal dose of L-digitoxose-modified polyfungin B and nystatin A3 to wild zebrafish embryo were 8.92 ng/embryo and 3.49 ng/embryo respectively, while nystatin A1 was highly toxic with 2.56 ng/embryo.

Polyene macrolide selvamicin (**5**) was isolated from *Actinobacteria pseudonocardia* HH130629-09 symbiotic with ants in ant nests [16]. Similar to the clinical anti-infective drug nystatin, selvamicin has macrolide skeleton and additional two sugars at the canonical glycosylation site, including atypical 6-deoxymannose at C-15 and 4-*O*-methyl-digitoxose sugar at C-27. Selvamicin showed milder antifungal activity against human pathogen *C. albicans* (MIC = 23.0 μM) and improved water solubility (2.3 μM) compared to nystatin A1 (MIC = 1.0 μM, water solubility = 0.3 μM). Besides, a representative member of polyene macrolactams with five-membered ring, cremimycin (**6**), contains methyl-*β*-D-digitoxose at C-10 and was demonstrated to have inhibitory activities against Gram-positive bacteria [17,18].

PA-46101 A (**7**) belongs to the family of spirotetronates, containing 5′-*O*-methyl-*β*-D-digitoxose at C-7, were demonstrated to have antibiotic properties involving the activities against Gram-negative bacteria, anaerobic Gram-positive and a small part of aerobic Gram-positive bacteria [19]. Unusual 17-membered macrocyclic polyketides, versipelostatins A-F (**8**–**13**), were extracted from the *S. versipellis* 4083-SVS6, containing a spirotetronate skeleton and two or three sugar moieties (at least one *β*-D-Digitoxose) at C-19 [20,21]. Studies reported that versipelostatins A-F were able to reduce the expression of molecular chaperone GRP78 through inhibiting ER stress, which suggested that oligosaccharide chains played a vital role in regulation [22]. In particular, the inhibitory activity against GRP78 of versipelostatin A, versipelostatin D, and versipelostatin F was 3.5 μg/mL, 4.3 μg/mL, and 0.3 μg/mL respectively. These findings indicate that the deoxysugar component of versipelostatins, particularly *α*-L-oleandrose-*β*-D-digitoxose, may plays a significant role in GRP78 inhibition.

Some of the spirotetraenoic acid families are linked to two sugar side chains, one of which is N-desmethylcarbamate-*β*-D-tetronitrose at C-17 and the other at C-9 is consisting of three 2,6-dideoxyhexoses. Lobophorins A, B, E and F (**14**–**17**), were produced by a South China deep sea *S. sp.* SCSIO 01127, all of which are typically C9–*O*-glycosides bearing L-digitoxoses [23,24]. In comparison with the lobophorins A and B, lobophorin E and F are more potent against *S. aureus* ATCC 29213 because of the absence of hydroxyl group at C-32. Particularly

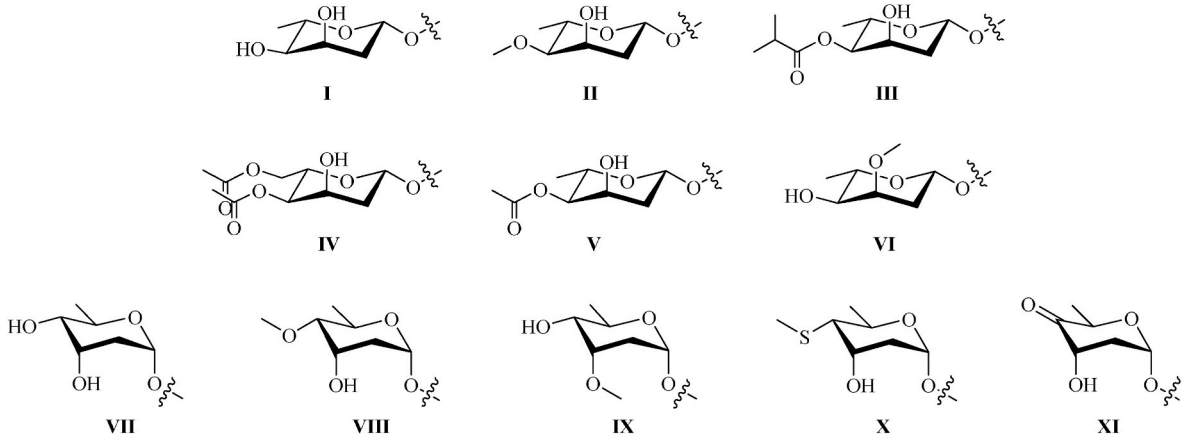


Fig. 1. Ten types of digitoxose presented in natural products (**I-XI**).

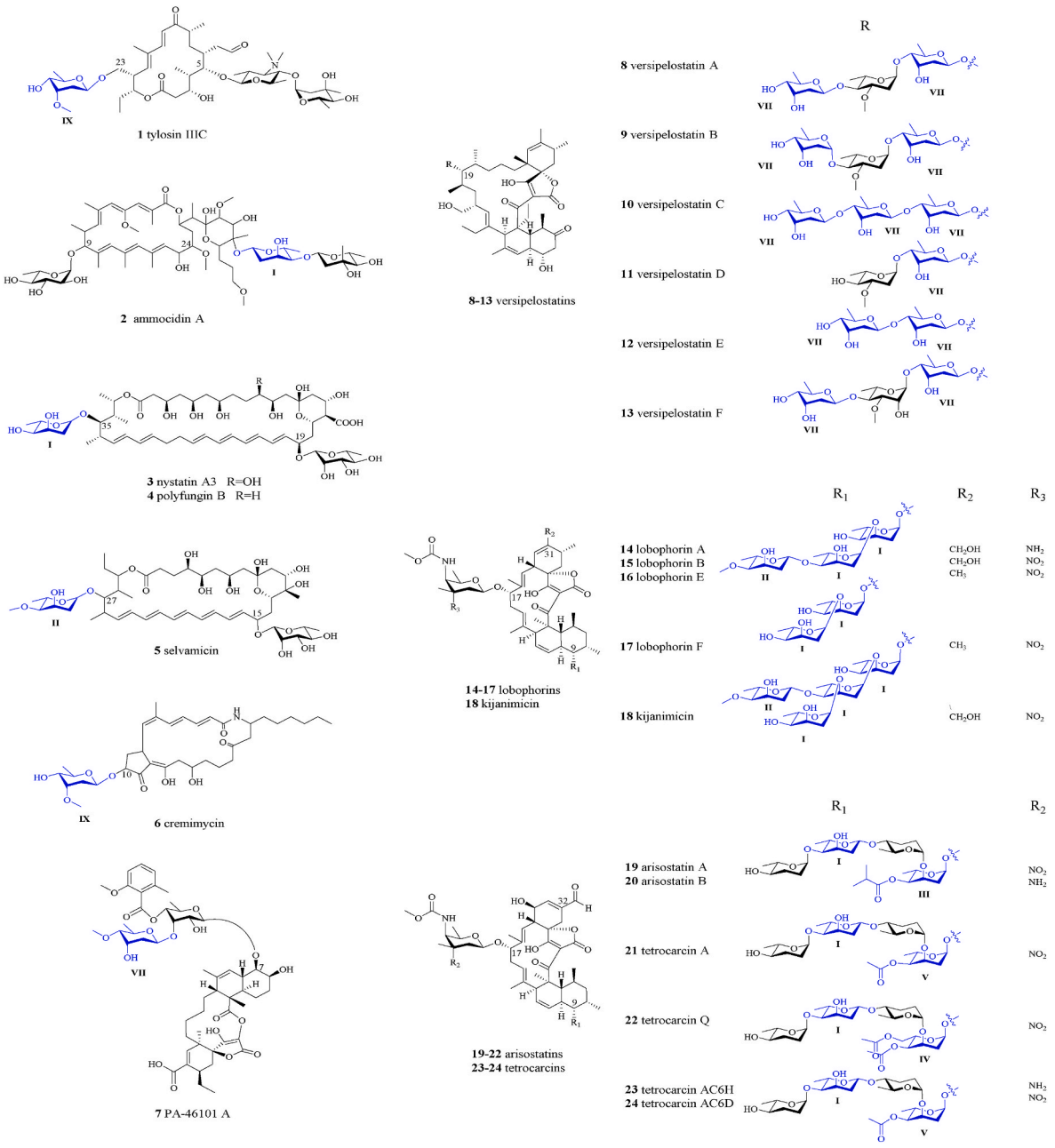


Fig. 2. Structures of digitoxose containing macrolides and macrolactams (**1**–**24**).

lobophorin F has efficient antibacterial activities with MIC values of 8 μg/mL as well as inhibitory activities against human tumor cell SF-268 (IC_{50} = 6.82 μM), NCI–H460 (IC_{50} = 3.16 μM) and MCF-7 (IC_{50} = 2.93 μM). It also shows that, although digitoxose is essential, the activity is independent of the longer the sugar chain. A lobophorin derivative, kijanimicin (**18**) was isolated from *Actinomadura kijaniata* SCC 1256, containing a pentacyclic core, one rare nitro-containing tetraoxosugar D-kijanose at C-17, and four L-digitoxoses at C-9, consisting of three *α*-L-digitoxoses and one *β*-L-digitoxose, which play vital roles in bioactivity [25]. Kijanimicin shows the versatile activities against Gram-positive bacteria, anaerobic bacteria and malaria, it also shows antitumor activity [26].

Micromonospora sp. TP-A0316 has been reported to produce arisostatins A-B (**19**–**20**) along with tetrocarcin A (**21**) (Fig. 2) [27,28]. They were demonstrated to inhibit the growth of the Gram-positive bacteria, such as *Bacillus subtilis* and *Staphylococcus aureus*. Arisostatin A also showed notable *in vitro* cytotoxicity against cancer cell lines in various organs such as lung, brain and breast. In addition, tetrocarcins AC6D and AC6H (**23**–**24**) are also spirotetronate glycosides having the above similar structures [29]. At concentrations of 0.78 and 0.39 μg/mL, respectively, tetrocarcin AC6D had the cytotoxicity against B16 melanoma cells and P388 leukemia. It is worth mentioning that tetrocarcin Q (**22**), isolated from *M. carbonacea* LS276, has a different sugar type (6-*O*-acetylated) at the C-9 position [30]. With MIC value of 12.5 μg/mL, tetrocarcin Q exhibited antibacterial activity against *Bacillus subtilis* ATCC 63501. Despite the lack of a clear exposition on the role of digitoxose, the removal of deoxysugar chain results in the completely loss of inhibitory activity of aglycone (tetrocarcin F1) against *B. subtilis* ATCC 63501. This observation indirectly underscores the significant influence of the sugar chain on the activity [30].

2.2. Anthracyclines and quinones

Jadomycin B (**25**) (Fig. 3) is a unique member of the angucycline

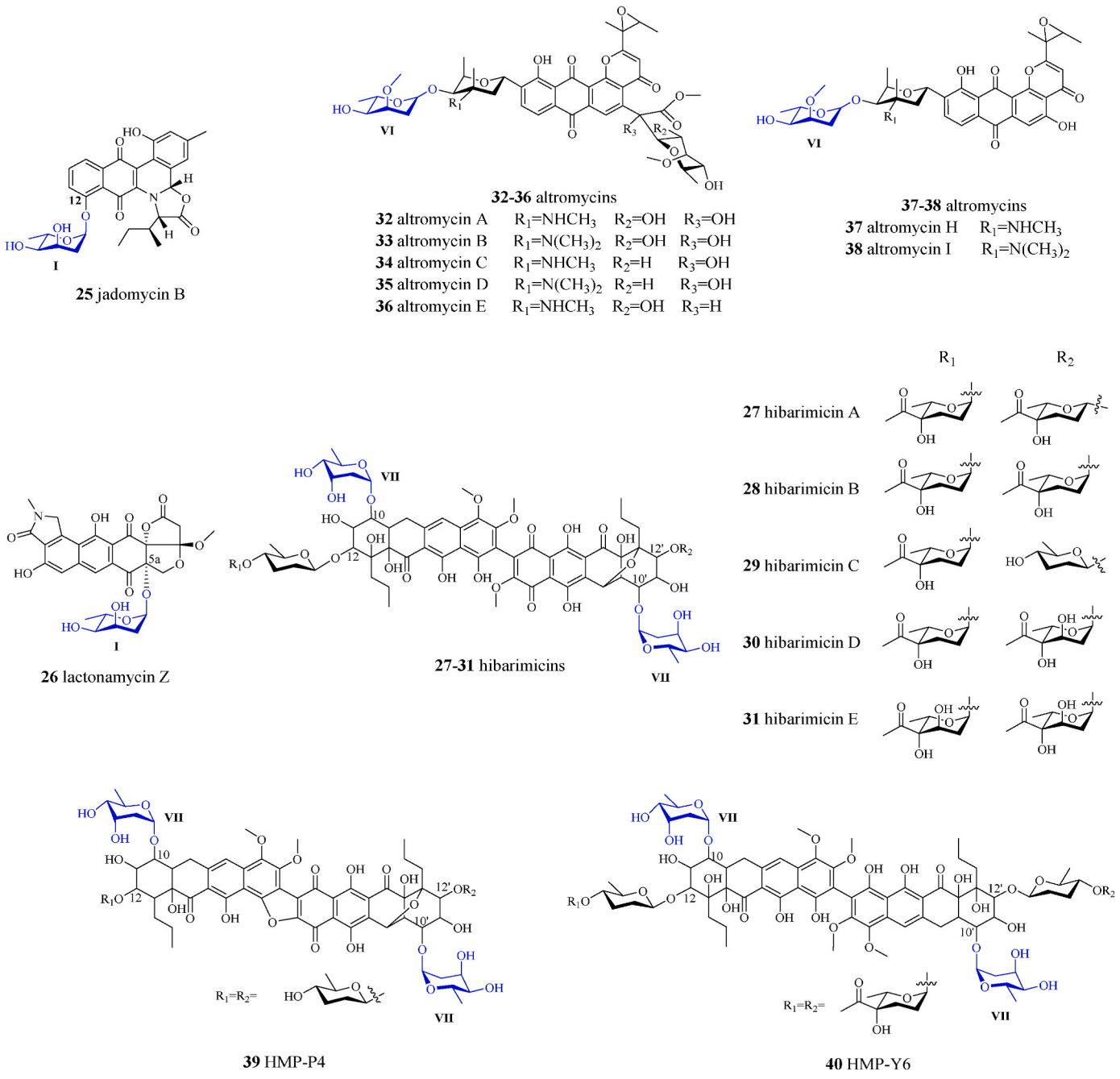


Fig. 3. Structures of anthracyclines and quinones with digitoxose moiety (**25**–**40**).

family, the structural signature of which is an 8*H*-benz [*b*] oxazolo [3,2-*f*] phenanthridine [31]. As a product of the C-8-*O*-glycosylation of jadomycin A, jadomycin B contains *α*-L-digitoxose, produced by *S. venezuelae* ISP5230 when cultured at 37 °C with nutritional pressure. Determination of biological activity indicates that jadomycin B displays much of functions, including anti-viral, antimicrobial, as well as inhibiting aurora-B activity [32–34]. It also an effective agent for inhibiting the cytotoxicity of tumor cells, like eradicating grown of MCF7 breast cancer cells in culture [35]. Surprisingly, jadomycin B has an anti-yeast activity that jadomycin A does not, implying that digitoxose plays a vital role in antimicrobial activity. Lactonamycin Z (**26**), one lactonamycinone-containing natural product, is C-5a-*O*-glycosides with *α*-L-digitoxose isolated from *S. sanglieri* Strain AK 623. The metabolite **26** inhibits gastric adenocarcinoma effectively, and displays weak activity against Gram-positive bacteria.

Majority of the antibiotics produced by *Microsporum* have antitumor activity, such as hibarimicins (A-E, HMP-P4, and HMP-Y6) [36–38]. The structures of hibarimicins (**27**–**31, 39**–**40**) consist of a highly oxidized naphthoquinone and six distinct sugars (Fig. 3). In all cases, C-10 and C-10′ are both modified by *α*-L-digitoxose. Studies on the effects of hibarimicins on human myeloid leukemia HL-60 cells and cell membrane v-Src tyrosine kinase had shown that hibarimicin B was a strong and selective inhibitor for src tyrosine kinase with differentiation-inducing activities of HL-60 cells [39]. Hibarimicin E similarly induced HL-60 cells differentiation but had no v-Src kinase inhibitory activity. Additionally, all the components of hibarimicins also showed *in vitro* antitumor activities and moderate inhibitory activity against Gram-positive bacteria.

Altromycins A-I (**32**–**38**) (Fig. 3), were isolated from *Actinomyces* AB1246E-26 and the mixture of them has good activities against Gram

positive bacteria [40–42]. What should be of concern is that altromycin B was found to bind to the to N7 guanidine of guanine in major groove, forming a drug-DNA complex as a member of the anti-tumor antibiotic pluramycin family.

2.3. Steroids and their glycosides

Some glycoside steroid products containing digitoxose are found in plants. For example, a new 8,14-cyclopropyl glycoside with a tetrasaccharide modification (*β*-D-oleandrose-*β*-D-digitoxose- *β*-D-oleandrose-*β*-D-oleandrose), tuberoside A_1 (**41**) (Fig. 4), was found in the aerials part of the plant *Asclepias tuberosa* L [43]. *Sphaeranthus indicus* Linn, a plant found all over the Indian plains, produced eudesmanolide glycoside with a *β*-D-digitoxose [44]. Compound **42**, a new eudesmanolide glycoside, showed antifungal activity toward *Aspergillus fumigatus*, *A. flavus* and *Saccharomyces cerevisiae*. Although the functions of digitoxose in **41** and **42** are unknown, it plays a crucial role as a C-3 modified sugar group in the core structure of cardiac glycosides and steroidal glycosides.

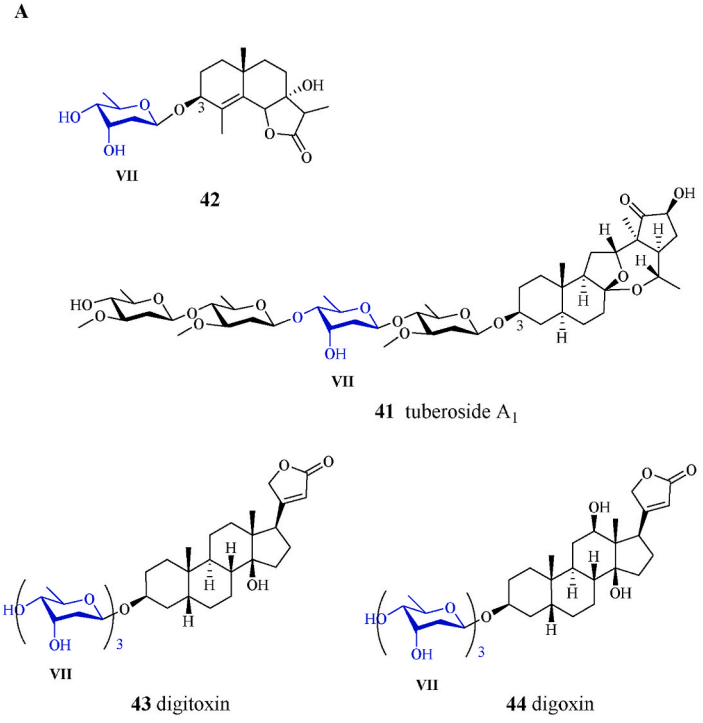
Cardiac glycoside usually takes steroid mother nucleus as aglycone, with glycosyl at C-3 position and its C-17 position is connected with unsaturated lactone ring [45]. Digitoxigenin- and digoxigenin-based digitoxin (**43**) and digoxin (**44**), isolated from *Digitalis purpurea*, are cardiac glycosides containing three *β*-D-digitoxoses commonly used in clinical for the treatment of heart failure and supraventricular arrhythmias at present [46]. They played a positive inotropic role by inhibiting Na^+/K^+-ATPase activity of cell membrane and increasing Ca^{2+} level in myocardial cells [47]. A study of binding affinities for ATPase showed that there was a positive correlation between affinity and inhibitory efficacy in most cardiac glycosides [48]. Among them, the relative binding affinities (RBA) of digitoxigenin monodigitoxoside containing one-glycosyl group was 24.7 times that of digitoxigenin without sugar group modification. With the prolongation of the sugar chain, the RBA is weakened, but also higher than that of digitoxigenin. The same results were obtained for digoxigenin and its digitoxose-modified compounds (Fig. 4 B).

Various studies reported that both digitoxin and digoxin had a wide range of antitumor activities, including prostatic carcinoma, breast carcinoma as well as adenocarcinoma [49]. In recent years, digitoxin has also been found to have a certain therapeutic effect on glioblastoma, renal-cell carcinoma and lung cancer [50,51]. The structure-activity relationship of digitoxin indicates that mono-digitoxoside derivative exhibit higher antitumor activity than di- and tri-*O*-digitoxoside derivatives of digitoxigenin, but is accompanied by higher cytotoxicity.

2.4. Others

The enediyne antitumor antibiotics are produced by actinomyces, and have a pharmacodynamic core structure of enediyne consisting of two alkynyl groups coupled by double bonds [52]. Due to their unique molecular structure, novel mechanism of action and strong biological activity, enediynes are the most active antitumor substances found so far [53]. Since calicheamicin (**45**) (Fig. 5) was first discovered by Lederle Laboratories in 1987, this complex secondary metabolite has attracted the attention of scientists in different professional fields [54]. Digitoxose, as the intermediary linkage part, is responsible for connecting the structural regions, the warhead and enediyne aglycon of calicheamicin. Here, digitoxose is not only a sugar chain but also serves as a key structure of calicheamicin.

Typically, the ten-membered enediyne antitumor antibiotics calicheamicin (**45**) and esperamicin (**46**) (Fig. 5) have strong cytotoxic effect on some mouse xenografts such as leukemia P388 and melanoma B16, and have good efficacy in the dose range of 0.5–1.5 μg/kg and 0.1 μg/kg, respectively [55]. Since the calicheamicin monoclonal antibody conjugate, mylotarg, had an excellent therapeutic effect on recurrent acute myeloid leukemia, it was approved for marketing by the US FDA in 2000. Studies on antitumor mechanisms have shown that DNA fragmentation caused by enediynes was not direct, but enediynes was activated as the active free radical intermediate, inducing DNA dehydro fragmentation of DNA chain [56]. Besides, the acyclic polyene metabolites α-lipomycin (comprise of *β*-D-digitoxose, **47**) and its aglycon



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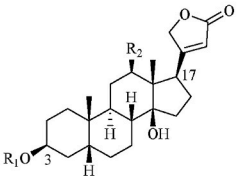

	R_1	R_2	RBA
digitoxigenin	H	H	11.10±6.70
digitoxigenin monodigitoxoside	monodigitoxose	H	0.36±0.17
digitoxigenin bisdigitoxoside	bisdigitoxose	H	0.45±0.13
digitoxin (**43**)	trisdigitoxose	H	0.51±0.26
digoxigenin	H	OH	2.19±0.70
digoxigenin monodigitoxoside	monodigitoxose	OH	0.83±0.15
digoxigenin bisdigitoxoside	bisdigitoxose	OH	1.25±0.84
digoxin (**44**)	trisdigitoxose	OH	2.13±1.02

Fig. 4. Digitoxose containing steroids and their glycosides (**41**–**44**)
A Structures of compound **41**–**44**. **B** Relative binding affinities of **43**, **44,** and their derivatives.

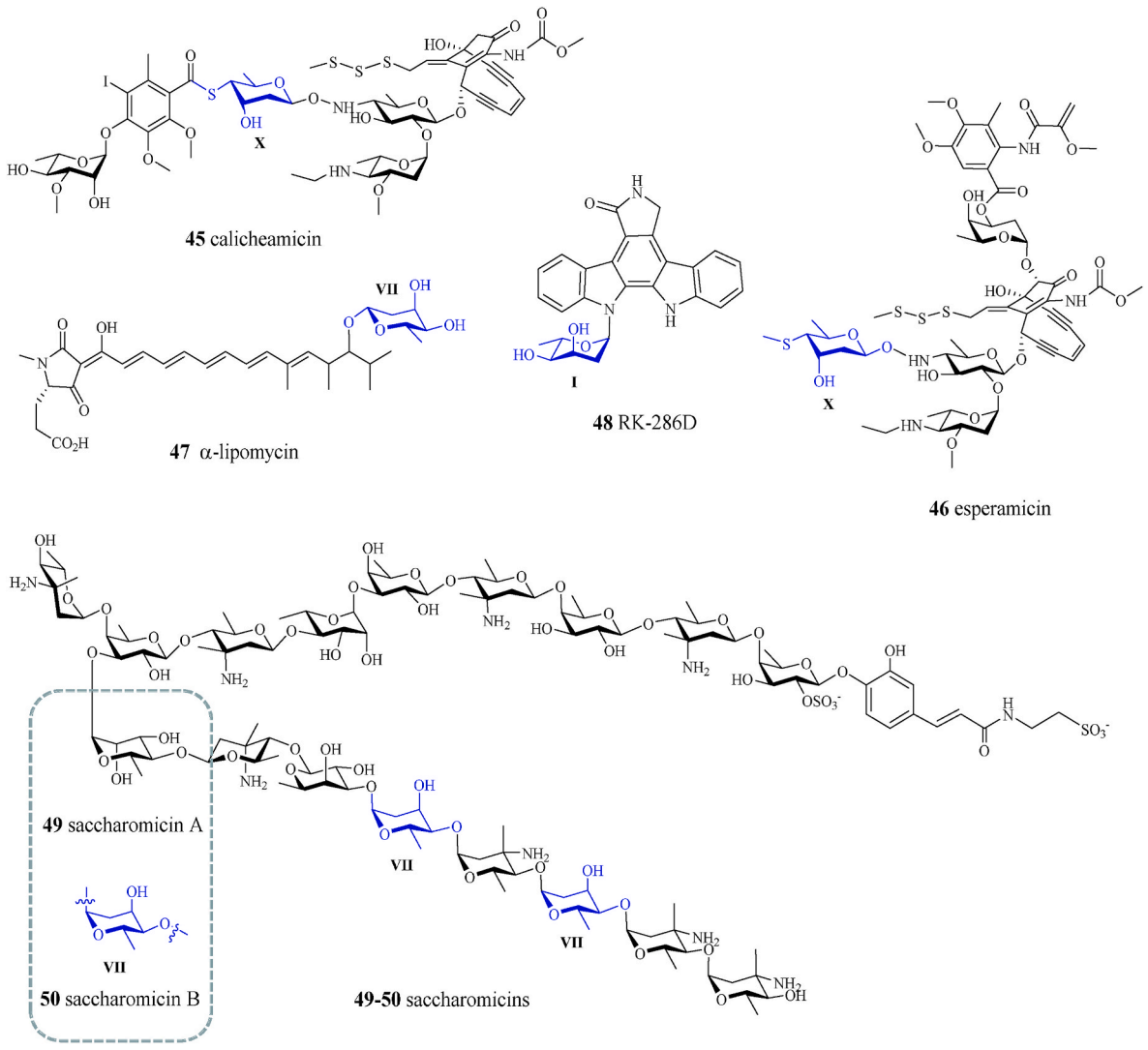

Fig. 5. Structures of digitoxose containing natural products (**45**–**50**).

β-lipomycin were isolated from *S. aureofaciens* Tu 117. It was established that 3-polyenoyltetramic acid α-lipomycin against Gram-positive bacteria [57].

RK-286D (**48**) (Fig. 5) was found as a novel indolocarbazole antibiotic with sugar *α*-L-digitoxose from *S. sp.* RK-286. RK286D had inhibitory activity on protein kinase C and blister formation in K-562 cells, but no antibacterial activity [58]. Saccharomicins were a rare member of oligosaccharide with 17 sugar moieties isolated from a rare actinomycete *Saccharothrix espanaensis* [59]. Complex of saccharomicin A and saccharomicin B acts on bacterial membrane and had the activity against Gram-positive bacteria. The difference between saccharomicin A and saccharomicin B (**49**–**50**) in mono-saccharide units is in tenth, *α*-L-rhamnose or *α*-L-digitoxose respectively (Fig. 5). Among them, saccharomicin B with *α*-L-digitoxose was more efficient in killing *E. faecalis* and *S. pneumoniae in vitro* than that of saccharomicin A.

3. Biosynthesis of TDP-digitoxose in bacteria

The biosynthesis of digitoxoses has been inferred in various strains (Fig. 6), such as *M. chalcea* NRRL 11289 [60], *P.* HH130629-09 [16], *S. versipellis* 4083-SVS6 [61], *S. sp.* MJ635-86F5 [62], and *S. sanglieri* AK 623 [63]. Notably, it seems that digitoxoses are activated as TDP-digitoxoses before it can be used as donors. Therefore, the purpose of this section is to introduce the synthetic mechanism of TDP-digitoxoses. The TDP-digitoxose includes two configurations: TDP-L-digitoxose and TDP-D-digitoxose, and their synthetic pathways comprise a common pathway and two branches (Fig. 6).

The formation of TDP-L-digitoxose was first discovered in jadomycin B from strain *S. venezuelae* ISP5230 at 2002 [31]. The molecules lack of digitoxose were produced by the knocking out the genes *jadO*, *jadP*, *jadQ*, *jadS*, *jadT*, *jadU* and *jadV*, meaning that these genes are responsible for the digitoxose biosynthesis. And in 2007, synthetases KijD5, KijD4, KijB1, KijD10, KijD11 and KijC2 from in kijanimicin producing strain *A. kijaniata* have realized the enzymatic synthesis of TDP-L-digitoxose *in vitro* [64]. However, due to the instability of TDP-L-digitoxose, high-resolution MS data and ^{1}H NMR spectrum were not obtained.

In general, there are six steps for the biosynthesis of TDP-L-digitoxose, corresponding to six enzymes. The synthesis of digitoxose begins with the activated monosaccharide **51**, which is the first metabolite in the gluconeogenic pathway. Subsequently, the C-4 and C-6 positions of the **52**-sugar ring were dehydrated to synthesize important intermediate **53**, this is an irreversible process. The steps from **51** to **53** are commonly found in biosynthetic pathways of unusual sugars. The generation of keto group at the C-4 position provides a suitable cyclic deoxygenation environment for C-2 and C-3. The dehydration reaction at C-2 and C-3 is subsequently triggered by 2,3-dehydratase KijB1, obtaining intermediate **54**. Then catalyzed by ketoreductase KijD10, with NADPH as a cofactor, C-3 keto group was reduced to hydroxyl group, in preparation

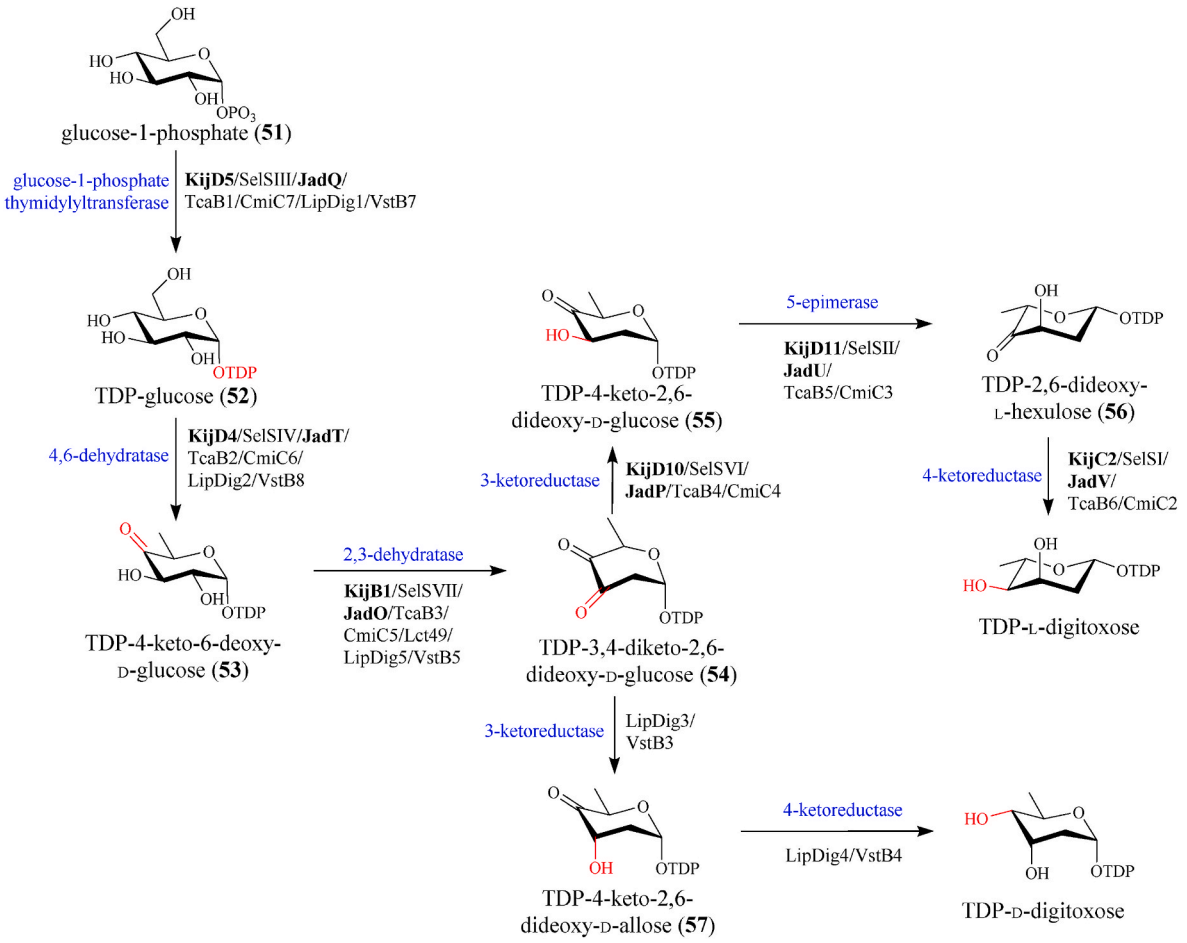

Fig. 6. Biosyntheses of TDP-digitoxose.

for the subsequent epimerization. As shown in Fig. 6, the intermediate **55** occurs conformational change and obtains the intermediate **56**. Finally, taking **56** as a substrate and catalyzing C-4 keto group protonation by reductase to obtain TDP-L-digitoxose. What is noteworthy is that L-digitoxose of some compounds is modified with methyl group at C-4, such as lobophorin [65] and selvamycin [16]. This process is catalyzed by methyltransferase, but the sequence of this process and aglycone connecting glycosyl is not clear. After the synthesis of TDP-L-digitoxose, it was transferred and linked to the aglycones by glycosyltransferase to generate TDP and the corresponding glycosides.

It is noteworthy that the biosynthetic routes of TDP-D-digitoxose are five steps [57,61], differs from that of TDP-L-digitoxose is from the fourth step (Fig. 6): The product catalyzed by C-3-ketoreductase is **57** because the keto group at C-3 position is reduced to a hydroxyl group in the axial configuration (not equatorial configuration). Afterwards, the product **57** is catalyze by reductase of C-4 to obtain TDP-D-digitoxose.

To date, it is remain difficult to reconstruct its biosynthesis pathway of TDP-digitoxose *in vitro*. This may be related to the catalytic efficiency of enzymes in the natural pathway and the stability of intermediates. Only the structures and mechanisms of enzyme KijD10 in the *A. kijaniata* has been explored. KijD10 is classified into the superfamily of glucose/fructose oxidoreductase enzymes, catalyzing C-3 reduction during the reduction of the intermediate TDP-3,4-diketo-2,6-dideoxy-D-Glucose [66]. Its active site, Lys 102, contributes one proton to the reaction, promoting the elimination of C-3 keto. But thankfully, in the biosynthetic pathway of TDP-digitoxose, the amino acid sequences used for the same routes have high similarity, which facilitates the functional analysis of genes related to the synthesis of digitoxose-containing natural products *in vitro*.

4. Relaxed substrate specificity for digitoxose glycosyltransferase

Glycosyltransferases (GTs) are a class of important catalytic enzymes that can transfer glycosyl groups to and form a target substrate via glycosidic bonds [67]. GTs is divided into two structural superfamilies, GT A and GT B. Majority of GTs from natural products belong to GT B family, and its N-terminal and C-terminal bind aglycone and sugar donor respectively [68]. In here, GTs is divided into aglycone-targeting GT and glycosyl-linking GT (Table 1), in order to analyze their function and specificity.

Selvamicin, cremimycin are macrolides, they are catalyzed by SelSV and CmiM5 respectively, to complete the final loading of digitoxose. Anthraquinones jadomycin B and lactonamycin Z contain the digitoxose, which are attached by the GTs JadS and Lct36. In addition, LipGtf is responsible for lipomycin biosynthesis, thus it is a GT targeting acyclic polyene. However, only JadS among them has been characterized with highly flexible substrate specificity. Therefore, this part first focuses on the GT in jadomycin B biosynthesis.

The ligation of the L-digitoxose in jadomycin B producing strain *S. venezuelae* ISP5230 is performed after the formation of the oxazolone ring, a process that is catalyzed by GT JadS [69]. In the detection of metabolites accumulated in mutant Δ*jadO* (*jadO* was identified as a gene encoding 2,3-dehydratase), a new glycosylated modifier ILEVS1080 (**58**) was unexpectedly found [70] Fig. 7. ILEVS1080 was 6-deoxy-L-altrose-modified jadomycin, and the sugar was synthesized under the catalysis of epimerase JadU and ketoreductase JadV, and then recognized and loaded by glycosyltransferase JadS. This result indicates that JadS can accept C-2 hydroxylated L-digitoxose as substrate, and is

Table 1
GTs from natural products with digitoxose moiety.

Glycosyltransferase	Strain	Function	Literature
Aglycone-targeting GTs			
SelSV	*Pseudonocardia*	GT involved in the biosynthesis	[16]
CmiM5	*Streptomyces* sp. MJ63586F5	GT involved in the cremimycin biosynthesis	[62]
LobG3	*Streptomyces* sp. SCSIO 01127	GT involved in the lobophorin biosynthesis	[65]
JadS	*Streptomyces* sp. ISP5230	GT involved in the jadomycin B biosynthesis	[69]
Lct36	*Streptomyces sanglieri* AK 623	GT involved in the lactonamycin Z biosynthesis	[63]
LipGtf	*Streptomyces aureofaciens* Tü117	GT involved in the lipomycin biosynthesis	[57]
Glycosyl-linking GTs			
LobG2	*Streptomyces* sp. SCSIO 01127	GT involved in the biosynthesis of saccharide chains of lobophorin	[65]
Unknow			
KijA4, C1, C3, C4	*Actinomadura kijaniata*	GTs involved in the kijanimicin biosynthesis	[64]
TcaT1 T2, T3, T4, M	*Micromonospora chalcea* NRRL 11289	GTs involved in the tetrocarcin A biosynthesis	[60]

worthy of further exploit to generate novel glycosylated derivatives. The tolerance of glycosyltransferases to sugar substrates is also reported in urdamycin and methymycin [71,72].

In addition, GTs in biosynthesis of spirocyclic tetracarboxylic acid antibiotics lobophorin A and B has been elaborated recently. Lobophorins A and B (**14**–**15**) have a pentacyclic aglycone and two sugar chains, including three L-digitoxose moieties linked at C-9 position and one D-kijanose (or 3-amino-D-kijanose) linked at C-17 position. The lobophorin biosynthesis gene cluster was identified from *S.* sp. SCSIO 01127, and three GT-encoding genes, lobG1-G3, were knocked out, yielding five new compounds (**59**–**65**) Fig. 7 [65]. **59** was isolated from Δ*lobG1* mutant, and it was confirmed that LobG1 was responsible for adding D-kijanose or 3-amino-D-kijanose at C-17. **60** and **61** without digitoxose, suggesting that LobG3 may be to attach two L-digitoxoses at C-9 in lobophorins. **62** and **63** only contain two digitoxoses at C-9, suggesting that LobG2 is responsible for loading the terminal L-digitoxose moiety. The BAC plasmid pCSG5560 containing the lobophorin gene cluster was heterologously expressed in the model strain *S. coelicolor* M1154. Two of lobophorin analogs (**64**–**65**) were isolated in strain M1154-pCSG5560 after amplification fermentation, with D-kijanose and 3-amino-D-kijanose at the C-9 position respectively [73]. To identify the GT responsible for the C-9 kijanose glycosyl group, LobG3 was further knocked out in this strain and molecules **60** and **61** with no glycosyl modification at the C-9 position were re-accumulated [73]. The result demonstrates that the substrate flexibility of LobG3 can also accept D-kijanose (or its variants with a 3-amino or hydroxyl group)) as substrates in addition to iteratively loaded two L-digitoxoses. However, kijanimicin and tetrocarcin A are also loaded with sugar chains composed of several digitoxoses, but it is not clear which GTs catalyze the connection and installation has not been clarified.

5. Introduction of digitoxose biosynthetic genes to generate more glycosylated products

In recent years, with the analysis of sugar biosynthesis gene clusters, the use of sugar plasmids to incorporate different sugars has attracted great attention. By introducing "Sugar Cassette Plasmids", the subsidiary sugars of compounds can be changed, and even produce novel compounds having better pharmacological properties. As shown in Fig. 8, plasmids pMP3*BII and pLNBIV contain genes involved in biosynthesis of digitoxose [74]. Among them, deoxysugar plasmid pLNBIV contained genes *oleV*, *oleW*, *eryBIV*, *oleY*, *oleL*, *oleS* and *oleE*, which were used to synthesize L-digitoxose. OleS, OLE, OleV, OleW, OleL and EryBIV correspond to glucose-1-phosphate thymidylyltransferase, 4,6-dehydratase, 2,3-dehydratase, 3-ketoreductase, epimerase, 4-ketoreductase in sequence, respectively. Then, the digitoxose was modified by methylation by OleY. In addition, the genes of the biosynthetic

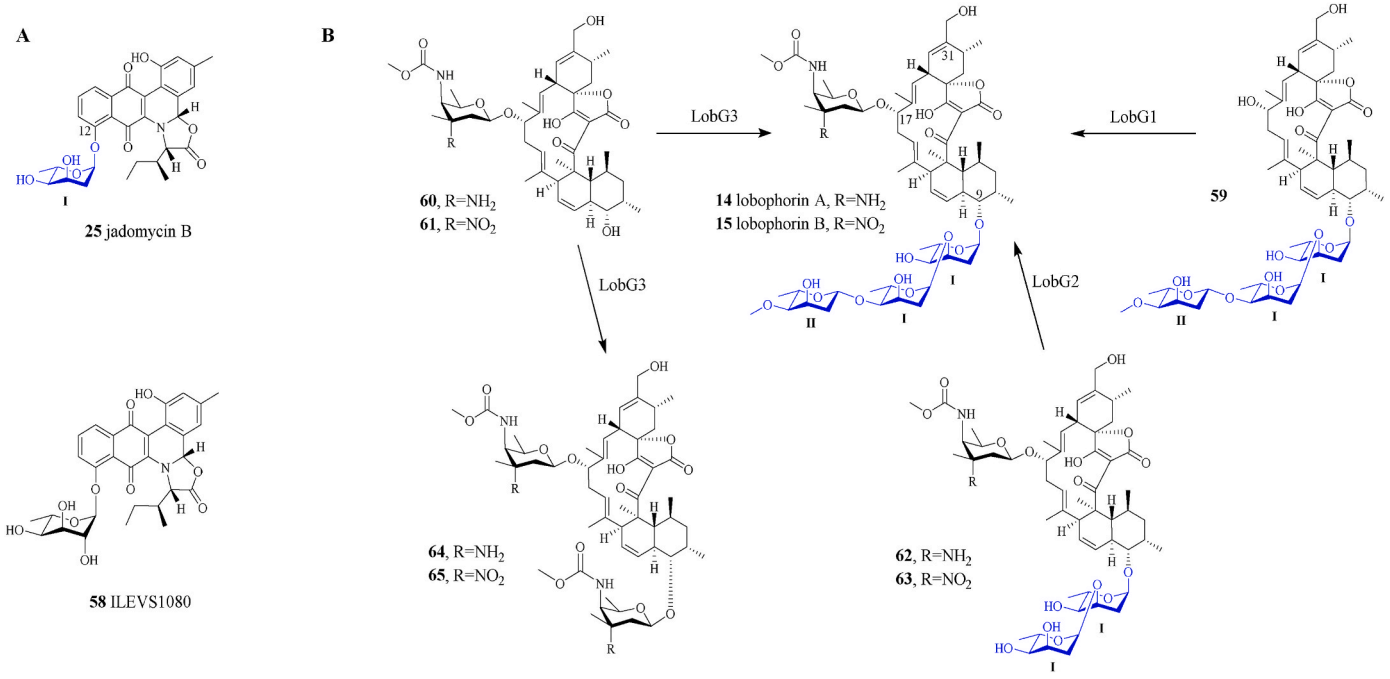

Fig. 7. Modification of glycosyl side chain by blocking digitoxose synthetic pathway.
A jadomycin B. **B** lobophorins A and B.

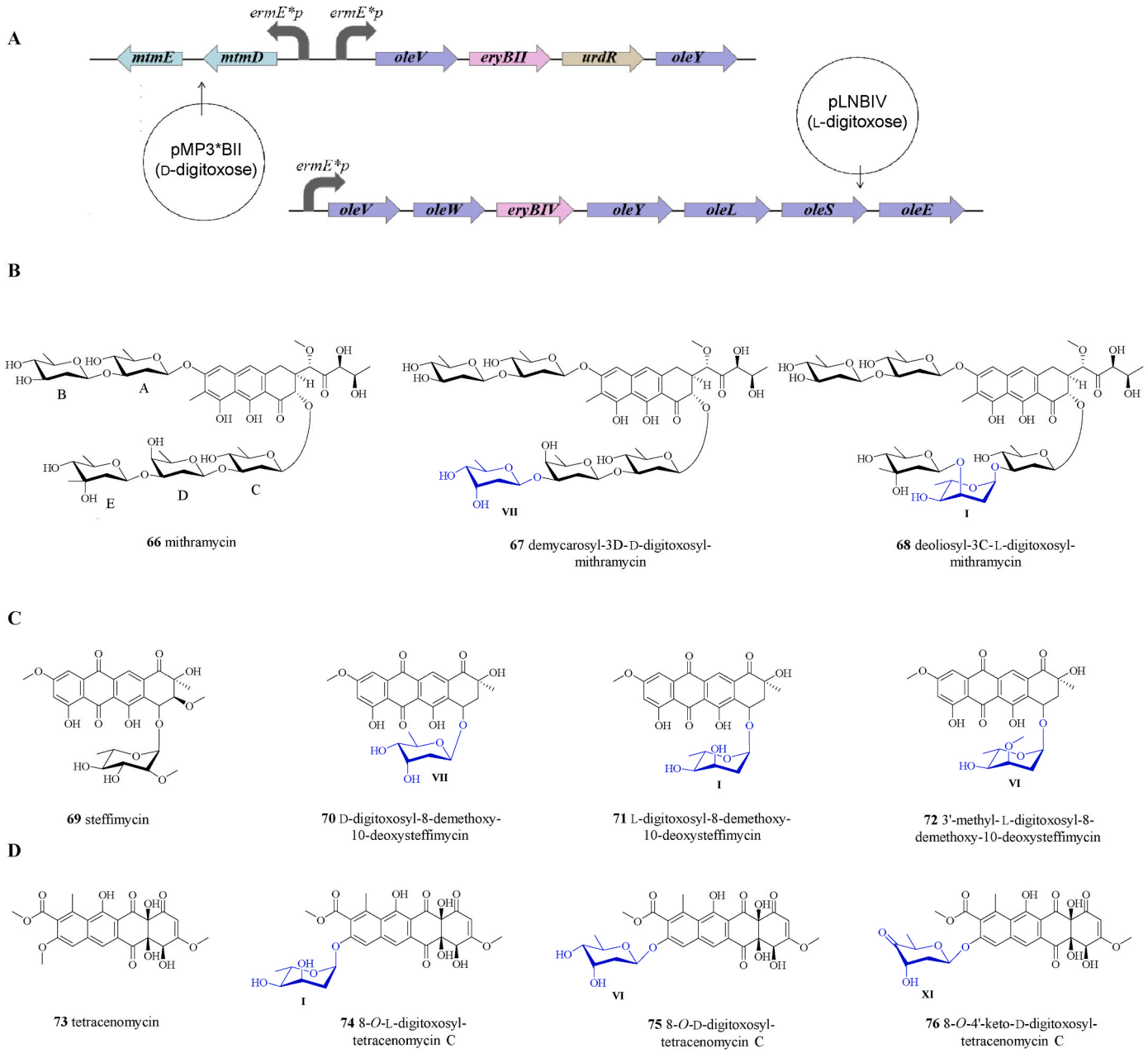

Fig. 8. Engineer glycosylation of bioactive compounds by sugar plasmids encode the biosynthesis of digitoxoses. **A** Schematic of the formation of digitoxose by plasmids PLNBIV and pMP3*BII. **B** Chemical structures of MTM and its derivatives. **C** Chemical structures of steffimycin and its derivatives. **D** Chemical structures of tetracenomycin and its derivative.

pathway of deoxyglucose D-digitoxose (*mtmE*, *mtmD*, *oleV*, *eryBII*, *urdR*, *oleY*) were cloned into plasmids pMP3*BII, and their expression were controlled by a highly expressed promoter *ermEp** [74].

Natural product drugs have some limitations, especially toxicity to organs, which usually limits their clinical use. This had prompted people to develop new drug derivatives with good therapeutic effects and few side effects through combinatorial biosynthesis or chemical synthesis. Like mithramycin (**66**), a clinical drug used to treat hypercalcemia and several types of cancer, including myeloid leukemia and testicular carcinoma [75–77]. In recent years, mithramycin has also been demonstrated to protect neuronal cells and inhibit a variety of parasites which cause dangerous infections of the central nervous system. It may be a potential drug for the treatment of neurodegenerative diseases [78]. However, due to the serious side effects of mithramycin, including toxicities of liver, kidney and bone marrow, the treatment window of mithramycin in humans is very narrow [79].

With a tricyclic chromophore as the core, mithramycin contains two aliphatic side chains at C-3 and C-7, as well as a trisaccharide chain (D-olivose, D-oliose and D-mycarose) and a disaccharide chain (two D-olivoses) at C-2 and C-6 respectively. In order to solve the limitation of mithramycin, studies have used sugar plasmids to replace the deoxyhexose moieties to obtain new modified molecules. Introduction of plasmid pMP3*BII into the mutant strain M3W1 producing mithramycin with C-3 side chain modification resulted in the production of a new derivatives containing D-digitoxose (**67**) with higher antitumor activity and lower toxicity than that of mithramycin [80]. It is more suitable for development into antitumor agent. And the biosynthesis genes of L-digitoxose were overexpressed in mithramycin producing strain *S. argillaceus* ATCC 12956 through the plasmid pLNBIV [81]. As a result, a new compound with L-digitoxose modification (**68**) could better

induce apoptosis of human breast cancer cell line MCF-7 (estrogen receptor (ER)-positive) and had a significant effect on MDA-231 (ER-negative), a human breast cancer cell line not inhibited by MTM. Although the other three positions in the five deoxysugars of mithramycin are hardly affected, heterologous expression of digitoxose biosynthesis pathway genes is an effective method to change the constitution of mithramycin's glycosyl groups.

Steffimycin (**69**) and tetracenomycin (**73**) are drugs of anthracycline used to treat bacterial infection and cancer. Both of them have an oxidatively modified linear decaketide as the core and different in O-methylation at C-9 and glycosyl modification at C-4. However, the resistance and cardiotoxicity of steffimycin and tetracenomycin may contraindicate them use. Take steffimycin as an example: the antitumor activity assay results showed that the GI_{50} of steffimycin against colon adeno carcinoma, non-small cell lung cancer, and breast adeno carcinoma ranged from 2.61 to 6.79 μM. To develope new derivatives by modifying the sugar moiety [82–84], plasmids pMP3*BII and pLNBIV were introduced respectively into *S. albus*, a strain used to heterologous expression the gene cluster of steffimycin. There are three new digitoxose containing derivatives (**70**–**72**) produced [74], indicating that the glycosyltransferase StfG of steffimycin has substrate promiscuity and was capable of recognizing a variety of D- and L-deoxyhexoses. It is noteworthy that compound **70** with D-digitoxos moiety shows significantly increased antitumor activities (GI_{50} less than 1.0 μm) compared to steffimycin.

Coincidentally, pLNBIV was introduced into tetracenomycin producing strain *S. species*, producing the 8-demethyl-8-L-digitoxosyl-tetracenomycin C (**74**) [85]. In addition, a plasmid named pDDIG, similar to pMP3*BII, directing the biosynthesis of D-digitoxose. By engineer biosynthesis of D-digitoxose in tetracenomycin producing strain *S. coelicolor* M1146:cos16F4iE, two new tetracenomycin derivants, 8-*O*-D-digitoxosyl-tetracenomycin C (**75**) and 8-O-4-keto-D-digitoxosyl-tetracenomycin C (**76**), were generated [86]. These products once again confirmed the feasibility of using biosynthesis plasmids of digitoxose to produce new glycosylated bioactive compounds by altering saccharide patterns.

6. Conclusions

Digitoxose is an important 2,6-dideoxyhexose in nature. Here, we summarize the structures and activities of natural small molecules with digitoxose modification. These compounds with complex structures are mainly macrolides and anthraquinones, and also involve some steroids, enediynes and oligosaccharide, that exhibit antibacterial, anti-viral, antiarrhythmic, and antitumor activities. Among these limited numbers of digitoxose-containing secondary metabolites, like digitoxigenin, digoxigenin, nystatin and calicheamicin have been practically used clinically. In general, digitoxose-containing compounds are an important source of drugs discovery and development and the glycosylation modified portion of digitoxose plays an irreplaceable role in the biological activity of these compounds.

Speculation and generalization of the biosynthesis of TDP-digitoxose in various strains made it possible to reconstruct its biosynthesis pathway *in vitro*. However, due to the low catalytic efficiency of enzymes in the natural pathway and the low stability of intermediates, this goal still needs more refined technologies to achieve. Although there have been a number of studies reporting methods for the total synthesis of L-digitoxose, natural product analogs will be expensive or difficult to obtain by chemical methods alone [87,88]. Studies on the function of various glycosyl synthetic genes currently mainly depend on gene inactivation and amino acid sequence alignment of gene-encoded products. Fortunately, the synthesis of rare sugars can be replaced by metabolic engineering, in which sugar modifications are added to compounds *in vivo* by combinatorial biosynthesis in the form of sugar plasmids. This may be a practical method to enlarge the diversity of digitoxose containing small molecules.

Author contribution

LK and BL conceived the review. LK and GZ analyzed data. LK and BL wrote the manuscript. All authors read and approved the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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