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Biosynthesis of Glycosylated Derivatives of Tylosin in *Streptomyces venezuelae*

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***Streptomyces venezuelae* YJ028, bearing a deletion of the entire biosynthetic gene cluster encoding the pikromycin polyketide synthases and desosamine biosynthetic enzymes, was used as a bioconversion system for combinatorial biosynthesis of glycosylated derivatives of tylosin. Two engineered deoxysugar biosynthetic pathways for the biosynthesis of TDP-3-*O*-demethyl-D-chalcose or TDP-L-rhamnose in conjunction with the glycosyltransferase-auxiliary protein pair DesVII/DesVIII were expressed in a *S. venezuelae* YJ028 mutant strain. Supplementation of each mutant strain capable of producing TDP-3-*O*-demethyl-D-chalcose or TDP-L-rhamnose with tylosin aglycone tylactone resulted in the production of the 3-*O*-demethyl-D-chalcose, D-quinovose, or L-rhamnose-glycosylated tylactone.**

Keywords: Tylosin, combinatorial biosynthesis, *Streptomyces venezuelae*

Polyketides are clinically important natural products that include antibacterial, anticancer, immunosuppressive, and antifungal compounds [9]. Their activities stem from the presence of a polyketide macrolactone ring (aglycone) with one or more deoxysugars attached. The deoxysugar moiety often greatly affects their biological activity, being responsible for specific contacts with molecular targets [12]. Tylosin, a polyketide antibiotic produced from *Streptomyces fradiae*, is composed of a 16-membered ring and three sugars, D-mycaminose, D-mycinose, and L-mycarose. It binds to ribosomes and thereby inhibits bacterial growth by preventing protein synthesis [4]. The growing number of antibiotic-resistant bacteria has heightened interest towards generation of novel glycosylated antibiotics [3]. To accomplish this, significant efforts have gone into chemical and enzymatic syntheses of structurally diverse glycosylated polyketide derivatives [7, 10, 13]. However, because of the troubles in the chemical modification of the deoxysugar moieties and *in vitro* enzymatic synthesis of nucleotide-activated deoxysugar, *in vivo* combinatorial biosynthesis could be a useful approach in the preparation of structurally diverse glycosylated derivatives in quantities adequate to allow determination of their bioactivity. *Streptomyces venezuelae* has been developed as a host for combinatorial biosynthesis of macrolide antibiotics [5, 6]. In addition to being amenable to genetic manipulation, *S. venezuelae* has a relatively fast growth rate and requires a short culture period for metabolite production. *S. venezuelae* also has the glycosyltransferase-auxiliary protein DesVII/DesVIII capable of using unnatural deoxysugars and unnatural aglycones as substrates, thereby being an important tool for combinatorial biosynthesis [2]. These characteristics make *S. venezuelae* an efficient combinatorial biosynthesis system for producing novel macrolide antibiotics. We previously developed a combinatorial biosynthesis system based on *S. venezuelae*, which is able to attach D-quinovose and D-olivose to 10-deoxymethynolide, narbonolide, and tylactone [6].

In this study, we constructed a *S. venezuelae* system expressing engineered deoxysugar biosynthetic pathways for the biosynthesis of TDP-3-demethyl-D-chalcose or TDP-L-rhamnose together with the glycosyltransferase-auxiliary protein pair DesVII/DesVIII (Fig. 1). The 16-membered ring macrolactone tylactone was fed to this engineered host, which in turn successfully produced 3-*O*-demethyl-D-chalcosyl, L-rhamnosyl, and D-quinovosyl derivatives by the action of the substrate-flexible DesVII/DesVIII (Fig. 1).

In order to biosynthesize and attach deoxysugars to exogenously fed tylactone in *S. venezuelae* YJ028, in which the entire biosynthetic gene cluster encoding the pikromycin polyketide synthases and desosamine biosynthetic enzymes was deleted [6], pODDC2 [15] and pLRHM3 [11] were

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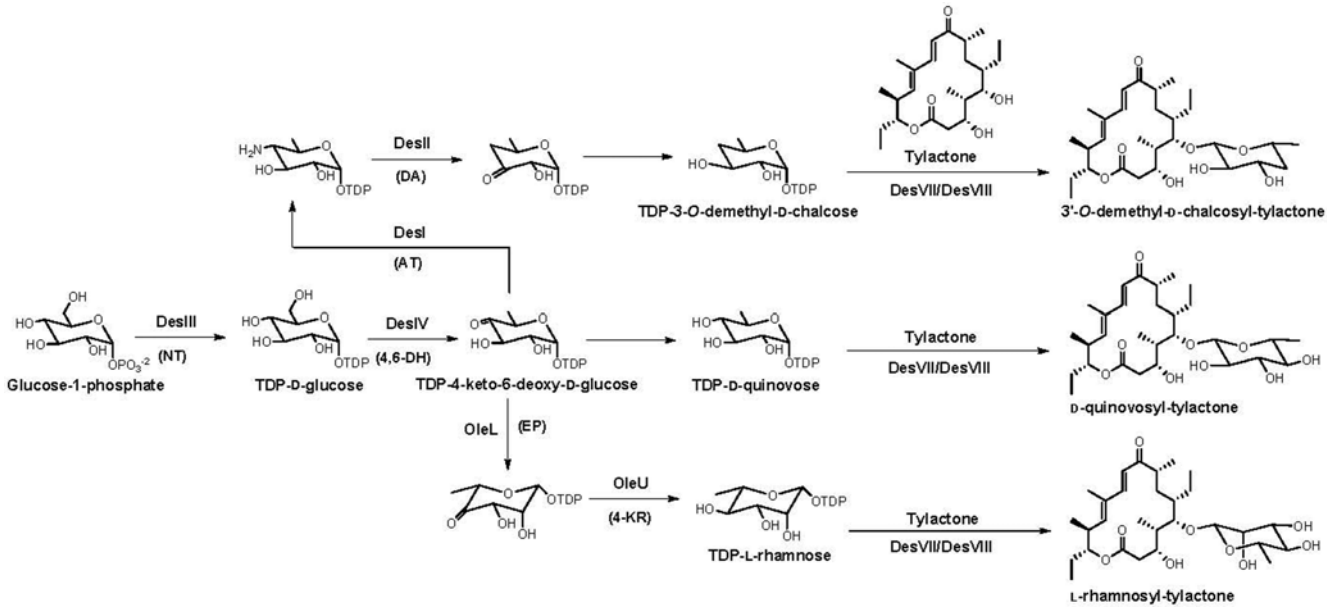

Fig. 1. Proposed biosynthetic pathway of 3'-*O*-demethyl-D-chalcose, D-quinovose, and L-rhamnose directed by the plasmids described in this work and proposed structures of 3'-*O*-demethyl-D-chalcosyl, D-quinovosyl, and L-rhamnosyl-tylactone produced in *S. venezuelae* mutant strain.
The functions of the proteins carrying out each step are shown in brackets: NT, Nucleotidylyl transferase; 4,6-DH, 4,6-dehydratase; AT, aminotransferase; DA, deaminase; EP, epimerase; 4-KR, 4-ketoreductase.

constructed (Fig. 2). The high-copy number *Escherichia coli–Streptomyces* shuttle vector pSE34 containing a strong *ermE** promoter (P_{ermE*}) plus a thiostrepton resistance marker was used as an expression plasmid [14]. DNA fragments containing several deoxysugar biosynthetic genes were amplified by PCR with specific deoxyoligonucleotide primers and template DNAs (Table 1). PCR was performed using Pfu polymerase (Fermentas) under the manufacturer's recommended conditions. Each DNA fragment contained a *Pac*I–*Spe*I site upstream of the ribosome binding site and a *Xba*I site downstream of the stop codon to facilitate subcloning. To construct pODDC2 carrying *desVIII*, *desVII*, *desIII*, *desIV*, *desI*, and *desII*, and pLRHM3 containing *desVIII*, *desVII*, *desIII*, *desIV*, *oleL*, and *oleU*, the following general method was employed: the *Pac*I–*Xba*I fragment containing one gene was cloned to *Pac*I–*Spe*I-digested Litmus28 carrying other genes. This process was repeated until all the genes involved in the biosynthesis of deoxysugars and their transfer were combined in Litmus28 and then transferred into pSE34 digested with *Pac*I–*Xba*I. The function of each gene product is shown in Fig. 1. These plasmids were introduced into the *S. venezuelae* YJ028 [6]. For production of glycosylated tylactone derivatives, a *S. venezuelae* YJ028 mutant strain carrying pODDC2 (YJ028/pODDC2) or pLRHM3 (YJ028/pLRHM3) was cultivated at 30°C for 72 h in 50 ml of SCM liquid medium

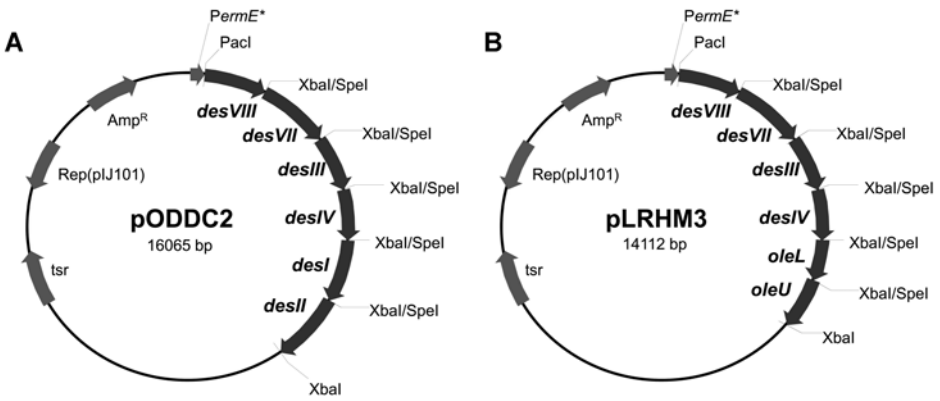

Fig. 2. Expression plasmids carrying genes involved in sugar biosynthesis and its transfer.
A. pODDC2 containing *desVIII, desVII, desIII, desIV, desI,* and *desII.* **B**. pLRHM3 containing *desVIII, desVII, desIII, desIV, oleL,* and *oleU.*

Table 1. Deoxyoligonucleotide primers used in this study.

Primer[a]	Sequence (5′–3′)[b]	Restriction enzyme	Source of DNA (Gene Accession No.)
desVIII-VII F	TTAATTAAACTAGTACCGGCAAGGAAGGACACGACGCC	*Pac*I–*Spe*I	*Streptomyces venezuelae* ATCC 15439
desVIII-VII R	TCTAGAGCGCAGATACAGGGGTGAGGCCTG	*Xba*I	(GenBank AF079762.1)
desI-II F	TTAATTAAACTAGTGACGGTGGCCCGGAGGG	*Pac*I–*Spe*I	*Streptomyces venezuelae* ATCC 15439
desI-II R	TCTAGATGCGGGTCAGCGCAGGAAGCCGCG	*Xba*I	(GenBank AF079762.1)
desIII-IV F	TTAATTAAACTAGTTAACTCGCCACGCCGACCGTT	*Pac*I–*Spe*I	*Streptomyces venezuelae* ATCC 15439
desIII-IV R	TCTAGAGAGCTCCTCGTAGGCGGCCTT	*Xba*I	(GenBank AF079762.1)
oleL F	TTAATTAAACTAGTATCGCTCCGAGCCCGAAGGGA	*Pac*I–*Spe*I	*Streptomyces antibioticus* ATCC 11891
oleL R	TCTAGAGCCGGCCAGTACGAGGGCCTT	*Xba*I	(GenBank AF055579.2)
oleU F	TTAATTAAACTAGTGTACCGCGACAACCGC	*Pac*I–*Spe*I	*S. antibioticus* ATCC 11891
oleU R	TCTAGAGAAGAGGGCCAGGTCGTGCACGC	*Xba*I	(GenBank AF055579.2)

[a]F and R represent forward and reverse direction of corresponding primers, respectively.
[b]Restriction sites are underlined.

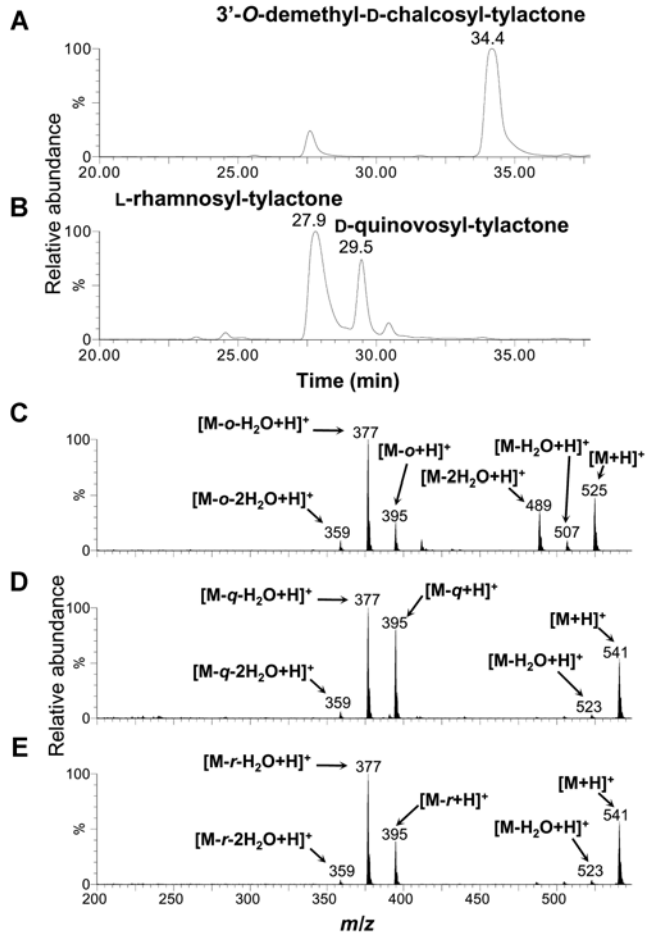

Fig. 3. LC–ESI–MS and ESI–MS/MS analyses of glycosylated derivatives of tylactone obtained from *S. venezuelae* YJ028/pODDC and YJ028/pLRHM2 supplemented with tylactone.
LC–ESI–MS chromatograms of (**A**) extract obtained from *S. venezuelae* YJ028/pODDC2 supplemented with tylactone and (**B**) extract obtained from *S. venezuelae* YJ028/pLRHM3 supplemented with tylactone. ESI–MS/MS spectra of (**C**) 3'-*O*-demethyl-D-chalcose (*O*)-glycosylated tylactone produced by *S. venezuelae* YJ028/pODDC2, and (**D** and **E**) D-quinovose (*q*), and L-rhamnose (*r*)-glycosylated tylactone produced by *S. venezuelae* YJ028/pLRHM3, respectively.

[14] supplemented with 5 μg/ml tylactone. The purified tylactone was obtained from Professor Eric Cundliffe (University of Leicester, UK). The cultures of *S. venezuelae* YJ028/pODDC2 and YJ028/pLRHM3 were extracted with a solid-phase extraction (SPE) procedure using an OASIS HLB cartridge (Waters), as previously described [8]. Liquid chromatography–electrospray ionization–mass spectrometry (LC–ESI–MS) was performed in a Waters/Micromass Quattro micro/MS interface using a Waters Nova-Pak C18 column (150×3.9 mm, 5 μm) in the positive-ion mode. The relative amount of each compound produced was compared using peak intensity obtained from the LC–ESI–MS chromatogram. The production level of each glycosylated compound was calculated by averaging the yield from three separate cultivations and extractions.

LC–ESI–MS analysis of the SPE extract of YJ028/pODDC2 fed with tylactone showed that a peak with *m/z*=525 was detected at a retention time of 34.4 min, corresponding to 3'-*O*-demethyl-D-chalcosyl-tylactone (Fig. 3A). The SPE extract of YJ028/pLRHM3 fed with tylactone was analyzed by LC–ESI–MS, and two peaks with *m/z*=541 were observed at a retention time of 27.9 and 29.5 min, corresponding to L-rhamnosyl-tylactone and D-quinovosyl-tylactone, respectively (Fig. 3B). The co-production of D-quinovosyl-tylactone in this strain can be explained as that 4-keto-6-deoxy-D-glucose, an intermediate of L-rhamnose, was reduced by a *S. venezuelae* pathway-independent reductase, synthesizing D-quinovose [1]. 3'-*O*-Demethyl-D-chalcosyl-tylactone with *m/z*=525 fragmented into characteristic ions at *m/z*=507 corresponding to the dehydrated form of 3'-*O*-demethyl-D-chalcosyl-tylactone, at *m/z*=489 corresponding to the sequentially dehydrated form of 3'-*O*-demethyl-D-chalcosyl-tylactone, at *m/z*=395 for the loss of 3-*O*-demethyl-D-chalcose, at *m/z*=377 corresponding to the dehydrated form of tylactone, and *m/z*=359 corresponding to the sequentially dehydrated form of tylactone from the parent ion upon MS/MS spectrometry (Fig. 3C). D-Quinovosyl-tylactone with *m/z* =541 also resulted in characteristic ions

at *m/z*=523 corresponding to the dehydrated form of D-quinovosyl-tylactone, at *m/z*=395 for the loss of D-quinovose, at *m/z*=377 corresponding to the dehydrated form of tylactone, and at *m/z*=359 corresponding to the dehydrated form of tylactone in ESI−MS/MS analysis (Fig. 3D). L-Rhamnosyl-tylactone with *m/z*=541 fragmented into characteristic ions at *m/z*=523 corresponding to the dehydrated form of L-rhamnosyl-tylactone, at *m/z*=395 for the loss of L-rhamnose, at *m/z*=377 corresponding to the dehydrated form of tylactone, and at *m/z*=359 corresponding to the sequentially dehydrated form of tylactone from the parent ion upon MS/MS spectrometry (Fig. 3E). Approximately 25%, 14%, and 31% of tylactone were converted to 3'-*O*-demethyl-D-chalcosyl, D-quinovosyl, and L-rhamnosyl-tylactone, respectively.

Here, we converted tylactone to 3'-*O*-demethyl-D-chalcosyl, D-quinovosyl, and L-rhamnosyl-tylactone by a *S. venezuelae*-derived combinatorial biosynthesis system carrying 3-*O*-demethyl-D-chalcose or L-rhamnose biosynthetic genes in conjunction with the glycosyltransferase genes *desVII*/*desVIII*. These glycosylated analogs of tylactone were previously produced *in vitro* [2]. Purified enzyme pair DesVII/DesVIII is capable of recognizing TDP-3-*O*-demethyl-D-chalcose, TDP-D-quinovose, and TDP-L-rhamnose as sugar donors and tylactone as a sugar acceptor, synthesizing 3'-*O*-demethyl-D-chalcosyl, D-quinovosyl, and L-rhamnosyl-tylactone, respectively. However, the percentage conversion into glycosylated compounds was relatively low with 10−20% conversion for 3'-*O*-demethyl-D-chalcosyl-tylactone, <5% conversion for D-quinovosyl-tylactone, and 10−20% conversion for L-rhamnosyl-tylactone [2]. The improved conversion yields of glycosylated tylactone analogs and the efficient *in vivo* synthesis of TDP-sugars that does not require multistep enzymatic or chemical synthesis using the *S. venezuelae* system clearly shows that *in vivo* combinatorial biosynthesis is a useful tool in the generation of structurally diverse glycosylated macrolides and development of novel drugs against bacterial infections.

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