Bioconversion of Tetracycline Antibiotics to Novel Glucoside Derivatives by Single-Vessel Multienzymatic Glycosylation

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Tetracyclines are broad-spectrum antibiotics clinically prescribed for treating a variety of bacterial infections [1]. Several analogs of tetracyclines, such as chlorotetracycline, oxytetracycline, tetracycline, and dimethylchlorotetracycline, are naturally produced from different \textit{Streptomyces} species, including \textit{S. aureofaciens} [2], \textit{S. rimosus}, and \textit{S. viridofaciens} [1]. However, some tetracycline analogs, such as methacycline, rolitetracycline, doxycycline, and minocycline, are produced by semisynthetic approaches [3–5]. Tigecycline (Tygacil) is one of the glyclyclcline group of semisynthetic compounds lately approved by the United States Food and Drug Administration (USFDA) as a third-generation tetracycline antibiotic drug. Although several tetracycline analogs are in clinical use to treat various diseases, many commensal and pathogenic strains are recently becoming resistant to tetracycline antibiotics because of the spread of the tetracycline resistance gene [6, 7]. Thus, the search for a novel analog of the tetracycline antibiotic to overcome resistant strains is a current priority in the scientific community for treating different diseases.

The single-vessel multienzyme UDP-\alpha-D-glucose recycling system was coupled with a forward glucosylation reaction to produce novel glucose moiety-conjugated derivatives of different tetracycline antibiotic analogs. Among five tetracycline analogs used for the reaction, four molecules (chlorotetracycline, doxycycline, meclotetracycline, and minotetracycline) were accepted by a glycosyltransferase enzyme, YjiC, from \textit{Bacillus licheniformis} to produce glucoside derivatives. However, the enzyme was unable to conjugate sugar units to rolitetracycline. All glucosides of tetracycline derivatives were characterized by ultraviolet absorbance maxima, ultra-pressure liquid chromatography coupled with photodiode array, and high-resolution quadruple time-of-flight electrospray mass spectrometry analyses. These synthesized glucosides are novel tetracycline derivatives.

**Keywords:** Novel tetracycline derivatives, glycosylation, single-vessel multienzyme reaction, UDP-\alpha-D-glucose recycling system
produce different analogs [8]. Recently, Professor Thorson’s group has synthesized 37 different analogs of doxycycline glycoside analogs using the neoglycosylation approach [9]. Among the new analogs, a 2'-amino-α-D-glucoside-conjugated derivative exhibited activity comparable to doxycycline pharmacophore [9]. Because of the potential effect of sugar conjugation on doxycycline, we applied an enzymatic glycosylation approach to generate analogs of different tetracycline derivatives in this study. Moreover, the USFDA recently approved oritavancin, a semisynthetic lipoglycopeptide antibiotic, to treat adults with skin infections. The molecule possesses two 4-epi-vancosamines and a glucose monosaccharide in its structure. The 4-epi-vancosamine is believed to play a crucial role in exerting activity by interacting with peptides nearby, but distinct from the terminal D-alanyl-D-alanine (or D-lactate) in both S. aureus and enterococci [10–12]. Thus, oritavancin is active against vancomycin-resistant organisms. Dalbavancin and telavancin are other second-generation semisynthetic glycolipopeptides approved by the USFDA for treatment of skin infections [13]. Thus, glycosylation is attracting attention to conjugate diverse sugars to different lead molecules.

Most glycosylation post-modification reactions of natural products are performed by glycosyltransferase (GT) enzymes that can transfer the sugar moiety from an activated sugar donor to acceptor molecules [14, 15]. Several GTs that can conjugate sugars to small molecules have been identified from different sources, such as plants and microbes [16]. However, only a few sugar-conjugated tetracyclines are reported from natural sources. For example, dactylocycline derivatives [17–19], TAN-1518 [20], and SF2575 [21] are produced by different organisms (Fig. 1A).

**Fig. 1.** (A) Structures of naturally occurring tetracycline glycosides and their producer organisms, and (B) various tetracycline derivatives used for glucosylation reaction in this study.
In the last few years, we have found that a GT from *Bacillus licheniformis* DSM-13 (YjiC) has broad substrate flexibility. It is able to accept a wide range of small molecules as acceptors and different NDP-sugars as sugar donors \[22-26\]. We applied the same YjiC for in vitro glucosylation of selected natural and synthetic tetracycline analogs (Fig. 1B) using a single-vessel UDP-\(\alpha\)-\(\delta\)-glucose recycling system (Fig. 2). This is the first report of the production of tetracycline glucosides by an enzymatic glucosylation reaction. The generated molecules are most likely to be novel compounds.

Acetyl phosphate kinase (ACK) from *Escherichia coli* K-12 uses acetyl-Pi, and phosphorylates UDP to generate uridine triphosphate (UTP). Another enzyme, glucose 1-phosphate uridylyltransferase (GalU), also from the same species, transfers UDP from UTP to glucose 1-phosphate and produces UDP-\(\alpha\)-\(\delta\)-glucose, which is the donor substrate for YjiC GT. Both glucose 1-phosphate and acetyl phosphate are commercially available at low cost.

Soluble fractions of the three proteins (ACK, GalU, and YjiC) were produced in the *E. coli* BL21(DE3) cytosol. The molecular mass was approximately 38 kDa for ACK and 45 kDa for YjiC. The respective bands of these soluble proteins were clearly observed by SDS-PAGE analysis (Fig. 3). Detail methods for the protein expression and preparation are explained in the supporting materials.

Five different tetracycline derivatives (chlorotetracycline, doxycycline, meclotenacycline, minotetracycline, and rolitetracycline) purchased from Tokyo Chemical Industry...
(Japan) were used for the in vitro single-vessel glucosylation reactions. The single-vessel reaction was carried out by putting all enzymes (ACK, GalU, YjiC) along with their required components for the reaction in 1 ml of final volume. For this reaction, each crude soluble protein fraction (~30 μg/ml of final concentration) was used. Other ingredients of the reaction mixture were 100 mM Tris-HCl buffer (pH 7.5) with 2 mM UDP-α-D-glucose, 2 mM each of tetracycline derivatives, 10 mM MgCl₂, 50 mM α-D-glucose 1-phosphate, and 200 mM acetyl phosphate. The final reaction mixture was incubated at 37°C for 3 h. After the 3 h of incubation, the reaction was quenched by adding a double volume of chilled nearly 100% methanol. The control reaction had no UDP-α-D-glucose.

UPLC-PDA analysis showed novel peaks in four tetracycline analogs (chlorotetracycline, doxytetracycline, meclotetracycline, and minotetracycline) (Fig. 1B). However, no novel peak was observed in the rolitetracycline derivative. Since novel peaks appeared in four different tetracyclines, the same reaction mixture was further analyzed by HR-QTOF ESI/MS to determine whether these new peaks might be possible glucoside derivatives of the respective tetracycline analog.

The standard chlorotetracycline appeared at the retention time (Rt) of 3.21 min. However, the novel peak supposed to be a glucoside derivative appeared at the lower Rt of 2.35 min (Fig. 4(i)). The maximum UV absorbance of each peak was determined. The λ_{max} of standard chlorotetracycline was at 230, 268, and 371 nm (Fig. 4A(ii)). However, it was shifted to 241 and 270 nm for the peak with Rt of 2.35 min (Fig. 4A(iii)). HR-QTOF ESI/MS analysis of the standard peak (Rt 3.21 min) showed a prominent spectrum of m/z 479.1218 with a calculated mass of 479.1221 Da for the molecular formula C_{22}H_{28}ClN_{2}O_{8} in the protonated state. The novel peak in the reaction mixture with Rt 2.35 min showed a prominent spectrum at m/z 641.1753 with a calculated mass of 641.1749 Da for the molecular formula C_{35}H_{32}ClN_{2}O_{13} (Fig. 4A(iv-v)). This mass corresponded to the molecular mass of the glucose-conjugated derivative. The relative conversion percentage of chlorotetracycline to its glucoside was approximately 6%.

In the doxytetracycline reaction mixture, the doxytetracycline standard appeared at Rt 3.30 min. However, a new peak assumed to be a glucoside derivative of doxytetracycline appeared at Rt 2.32 min (Fig. 4B(ii)). Both peaks were further analyzed by spectroscopic and spectrometric analyses. Spectroscopic analysis revealed a similar pattern of UV absorbance maxima. The λ_{max} for the standard doxytetracycline was at 269 and 348 nm. For the presumed glucoside derivative, it was at 268 and 339 nm (Fig. 4B(ii-iii)). This analysis clearly showed that the new observed peak was a derivative of standard doxytetracycline. These peaks were further analyzed for their exact molecular masses. The standard peak showed a prominent spectrum of m/z 445.1613 for which the calculated mass was 445.1611 Da for the molecular formula C_{22}H_{27}N_{2}O_{13} in the protonated state (Fig. 4B(iv)). The new peak at Rt 2.32 min showed a spectrum of m/z 607.2132 (Fig. 4B(v)) for which the calculated mass was 607.2139 Da for the molecular formula C_{39}H_{32}N_{10}O_{15}. This exact mass resembles the mass of the monoglucosylated derivative of doxytetracycline. The conversion percentage of doxytetracycline was relatively small (below 3%).

The meclotetracycline reaction mixture showed the standard molecule at Rt 3.54 min with a new peak at Rt 2.51 min (Fig. 4C(i)). The maxima UV absorbance of the standard molecule was at 243 and 346 nm for standard meclotetracycline. It was subtly changed to new peaks (at 240 and 339 nm) (Fig. 4C(ii-iii)). These same peaks were further analyzed by HR-QTOF ESI/MS analysis to determine their exact molecular masses. As a result, the standard meclotetracycline at Rt 3.54 min showed a distinct spectrum of m/z 477.1063 for which the calculated exact mass was 477.1065 Da for the molecular formula C_{22}H_{25}ClN_{2}O_{8} in protonated form (Fig. 4C(iv)). The new peak in the UPLC at Rt 2.51 min showed a spectrum at m/z 639.1589. It resembled the protonated monoglucosyl-conjugated derivative of meclotetracycline with a molecular formula of C_{35}H_{32}ClN_{2}O_{13} for which the calculated exact mass was 639.1593 Da (Fig. 4C(v)).

Similar to the aforementioned tetracycline analogs, the reaction mixture of minotetracycline was also analyzed. In UPLC-PDA, the standard minotetracycline appeared at Rt 2.59 min while a new peak appeared at Rt 2.19 min (Fig. 4D(i)). Both peaks had a similar pattern of UV absorbance maxima. The standard showed prominent λ_{max} at 221, 266, and 354 nm. The new peak had slight-shifted λ_{max} (at 260 and 339 nm) (Fig. 4D(ii-iii)). Mass spectrometry analysis of the standard showed the prominent spectrum of m/z 458.1941, for which the molecular formula C_{25}H_{27}N_{2}O_{13} had a calculated mass of 458.1927 Da in protonated form. However, the new peak showed the increased mass of m/z 620.2452, which resembled the single sugar-conjugated minotetracycline, for which the calculated exact mass was 620.2455 Da with a molecular formula of C_{38}H_{29}N_{10}O_{12} in protonated mode (Fig. 4D(iv-v)). UPLC-PDA analysis of the rolitetracycline reaction mixture did not show any new peaks (data not shown). Thus, the reaction mixture was not further analyzed by HR-QTOF ESI/MS.
Fig. 4. UPLC-PDA coupled with HR-QTOF ESI/MS analyses of reaction mixtures of four different tetracycline glucosides. 
(A) Chlorotetracycline: (i) UPLC-PDA chromatogram of chlorotetracycline glucosylation reaction mixture; (ii) $\lambda_{\text{max}}$ of standard chlorotetracycline; (iii) $\lambda_{\text{max}}$ of glucoside of chlorotetracycline; (iv) ESI/MS spectrum of chlorotetracycline standard; and (v) ESI/MS spectrum of glucoside of chlorotetracycline. (B) Doxytetracycline: (i) UPLC-PDA chromatogram of doxytetracycline glucosylation reaction mixture; (ii) $\lambda_{\text{max}}$ of standard doxytetracycline; (iii) $\lambda_{\text{max}}$ of glucoside of doxytetracycline; (iv) ESI/MS spectrum of doxytetracycline standard; and (v) ESI/MS spectrum of glucoside of doxytetracycline. (C) Meclotetracycline: (i) UPLC-PDA chromatogram of meclotetracycline glucosylation reaction mixture; (ii) $\lambda_{\text{max}}$ of standard meclotetracycline; (iii) $\lambda_{\text{max}}$ of glucoside of meclotetracycline; (iv) ESI/MS spectrum of meclotetracycline standard; and (v) ESI/MS spectrum of glucoside of meclotetracycline. (D) Minotetracycline: (i) UPLC-PDA chromatogram of minotetracycline glucosylation reaction mixture; (ii) $\lambda_{\text{max}}$ of standard minotetracycline; (iii) $\lambda_{\text{max}}$ of glucoside of minotetracycline; (iv) ESI/MS spectrum of minotetracycline standard; and (v) ESI/MS spectrum of glucoside of minotetracycline.
The conversion of substrates to products was relatively very small. Glucoside derivatives of doxycycline and mectetetracycline were produced in trace amount. In comparison with the other substrates, chlorotetracycline conversion to its glucoside was slightly higher. However, such quantity was not enough for structural analysis or biological activity determination. Thus, we did not scale up the reaction mixture of the tetracycline reactions for purification of the glucoside derivatives. Hence, further experiments for the complete structure elucidation and biological activities of the modified compounds were not performed.

Since the YjiC enzyme has been found to be very non-regiospecific for glucosylation reaction with small molecules [23, 27], the prediction for the exact position of glucose conjugation to tetracycline analogs is difficult. However, in the case of tetracycline analogs, YjiC produced a single product for all molecules used in this study except for rolitetracycline, which was not accepted any longer for the reaction. The possible positions of glucose conjugation in tetracycline might be the OH-groups of all four rings (A-D). However, OH-groups of the B and C rings could be engaged in intramolecular H-bonding with adjacent keto functional groups. In the A-ring of tetracyclines, the OH-group at the third position could be possibly hindered for glucosylation because of bulky methyl groups attached at the C-4 position and conceivable engagement of 3-OH to form H-bonding with neighboring amide functional groups present at the C-2 position. Thus, the glucose is most likely to be conjugated at the 10β-OH group in the D-ring of the tetracycline analogs used in this study.

In the in vitro glucosylation reaction, several problems have been reported for low bioconversion reaction, including the following: (i) competitive inhibition of binding of UDP-α-D-glucose to the donor substrate binding site by side products of the forward glucosylation reaction (i.e., UDP); (ii) reversible nature of the GT enzyme, which can hydrolyze the glucoside product to aglycone and UDP-α-D-glucose; (iii) low enzyme catalytic properties; and (iv) low enzyme preference toward acceptor substrates. It has been found that YjiC has high catalytic conversion efficiency in the forward direction with different acceptor substrates [23, 27]. However, it also exhibits a reversible glucosylation nature [24]. Thus, we applied the UDP-α-D-glucose recycling system for two main purposes: (i) to prevent competitive inhibition of binding of UDP-α-D-glucose by recycling UDP, and (ii) to prevent a reverse reaction. However, the conversion of tetracycline analogs to their respective glucosides has been found to be very low. This gives the clue that the enzyme has low preferences toward tetracycline molecules. Attachment of a bulky group in rolitetracycline might have prevented the molecule from reaching the catalytic cleft of GT. The results of this research could become a basis to explore the possibility of synthesizing glucosylated derivatives of different tetracyclines using the GT enzyme.

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**Conflict of Interest**

The authors have no financial conflicts of interest to declare.

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