Biosynthesis of Rhamnosylated Anthraquinones in *Escherichia coli*

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Rhamnose is a naturally occurring deoxysugar present as a glycogenic component of plant and microbial natural products. A recombinant mutant *Escherichia coli* strain was developed by overexpressing genes involved in the TDP-L-rhamnose biosynthesis pathway of different bacterial strains and *Saccharothrix espanaensis* rhamnosyl transferase to conjugate intrinsic cytosolic TDP-L-rhamnose with anthraquinones supplemented exogenously. Among the five anthraquinones (alizarin, emodin, chrysazin, anthrarufin, and quinizarin) tested, quinizarin was biotransformed into a rhamoside derivative with the highest conversion ratio by whole cells of engineered *E. coli*. The quinizarin glycoside was identified by various chromatographic and spectroscopic analyses. The anti-proliferative property of the newly synthesized rhamnoside, quinizarin-4-O-α-L-rhamnoside, was assayed in various cancer cells.

**Keywords:** Rhamnosyltransferase, quinizarin, *Saccharothrix espanaensis*

Anthraquinones are naturally occurring aromatic organic compounds found in plants, fungi and actinomycetes [1]. The dihydroxyanthraquinones constitute the most important group and are largely used as dyes and in the manufacture of dye intermediates [2, 3]. In addition, anthraquinone derivatives exert a wide range of biological activities [4–10]. They have also been used as anticancer agents to treat breast cancer and acute leukemia [11, 12].

Emodin (1,3,8-trihydroxy-6-methylanthraquinone), which is an active compound isolated from several Chinese herbs, is traditionally used as a laxative agent. Treatment with emodin has been shown to result in body weight reduction, lipid-lowering, blood glucose control, and anti-inflammatory effects [13]. Danthron (1,8-dihydroxyanthraquinone) was isolated from the root and the rhizome of *Rheum palmatum L.*, used in traditional medicine [14]. Alizarin red S is used for histological characterization of calcium deposits [15]. Quinizarin occurs as a glycoside in small amounts in the root of the madder plant, *Rubia tinctorum* [16], and is used as a fungicide and pesticide [17] as well as an inhibitor of tumor cell growth [18]. It is an inexpensive dye used to color gasoline and heating oil; it also acts as an intermediate for the synthesis of indanthrene- and alizarin-derived dyes [2]. Further, anthraquinone glycosides exhibit stronger activity than free aglycones [19].

*Saccharothrix*, a member of the order Actinomycetales [20], generates glycosylated natural products. The *S. espanaensis* genome carries 106 glycosyltransferase (GT) genes [21]. One of the GTs has been recently characterized as a promiscuous rhamnosyl transferase (7665) [21], which glycosylates anthraquinones using thymidine diphosphate (TDP)-L-rhamnose as a sugar donor. With the aim of producing different rhamnoside derivatives of anthraquinone, we used an *Escherichia coli* BL21 (DE3) Δ*pgi* Δ*zwf* Δ*galU* strain [22] developed by blocking glucose phosphate isomerase (*pgi*), glucose-6-phosphate dehydrogenase (*zdh*), and uridylyltransferase (*galU*) genes to divert carbon flow from glucose to TDP-L-rhamnose via G-1-P and dTDP-glucose. *E. coli* BL21 (DE3) Δ*pgi*Δ*zwf*Δ*galU* was further engineered by introducing TDP-L-rhamnose biosynthesizing genes harboring plasmids pCDFDuet-TGSDH and pACYCDuet-EPKR, carrying genes for biosynthesis of...
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TDP-L-rhamnose (tgs: TDP-glucose synthase from Thermus caldophilus GK24; dl: dTDP-D-glucose 4,6-dehydratase from Salmonella typhimurium LT2; epi: TDP-4-keto-6-deoxyglucose 3, 5-epimerase from Streptomyces antibioticus Tü99; and kr: TDP-glucose 4-ketoreductase from S. antibioticus Tü99) [23, 24] and pET28a (+)-7665 carrying a rhamnosyltransferase from S. espanaensis (7665) [21] (Fig. S1). The fully grown cells of engineered E. coli were used to biotransform five anthraquinones into respective O-rhamnosides. Five different anthraquinones were added for biotransformation into respective O-rhamnosides. After 20 h of isopropyl β-D-1-thiogalactopyranoside (IPTG)-induced culture, we supplemented anthraquinone exogenously at a final concentration of 0.2 mM. The biotransformation reaction was continued for the next 28 h at 20°C, followed by extraction using a double volume of ethyl acetate and analysis via high-performance liquid chromatography (HPLC-PDA).

The HPLC-PDA analysis of each sample yielded product peaks at shorter retention times (tR) than the substrate peak in each reaction mixture, as expected. New peaks appearing at tR ~ 19.6 min in alizarin; tR ~ 21.06 min in anthrarufin; tR ~ 21.24 min in chrysazin; tR ~ 23.53 min in emodin; and tR ~ 21.2 min in quinizarin were suspected to be rhamnose-conjugated derivatives (Fig. 2). These samples were further analyzed.

Fig. 1. Schematic diagram representing the metabolic engineering of E. coli BL21(DE3) for the biosynthesis of rhamnose-conjugated anthraquinones.

The genes (pgi, zaf, and galU) were knocked out of the genome. The dTDP-L-rhamnose was generated in the cytosol of engineered E. coli by overexpressing the respective genes in the sugar pathway. Rhamnosyl transferase (7665) from S. espanaensis was used for the conjugation of sugar to the exogenously supplemented anthraquinones (emodin, chrysazin, alizarin, anthrarufin, and quinizarin).

TDP-L-rhamnose (tgs: TDP-glucose synthase from Thermus caldophilus GK24; dl: dTDP-D-glucose 4,6-dehydratase from Salmonella typhimurium LT2; epi: TDP-4-keto-6-deoxyglucose 3, 5-epimerase from Streptomyces antibioticus Tü99; and kr: TDP-glucose 4-ketoreductase from S. antibioticus Tü99) [23, 24] and pET28a (+)-7665 carrying a rhamnosyltransferase from S. espanaensis (7665) [21] (Fig. S1). The fully grown cells of engineered E. coli were used to biotransform five anthraquinones into their respective rhamnosides (Fig. 1). The anti-proliferative activity of quinizarin rhamnoside was assessed and the results were significant compared with those of the corresponding aglycone.

First, we employed a versatile post-biosynthesis modifying enzyme, O-rhamnosyltransferase (7665), derived from S. espanaensis for the biosynthesis of anthraquinone rhamnosides in the engineered E. coli mutant strain overexpressing genes for TDP-L-rhamnose. Five different anthraquinones were added for biotransformation into respective O-rhamnosides. After 20 h of isopropyl β-D-1-thiogalactopyranoside (IPTG)-induced culture, we supplemented anthraquinone exogenously at a final concentration of 0.2 mM. The biotransformation reaction was continued for the next 28 h at 20°C, followed by extraction using a double volume of ethyl acetate and analysis via high-performance liquid chromatography (HPLC-PDA).

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by high-resolution quadruple time-of-flight electrospray ionization (HR-QTOF ESI/MS) to confirm the conjugation of rhamnose moiety with each anthraquinone substrate added exogenously. The mass spectra displayed an exact mass of emodin m/z 271.06 [M+H]+, while the mass spectrum of m/z 417.11 [M+H]+ resembled the rhamnose-conjugated derivative of emodin. Similarly, chrysazin, quinizarin, anthrarufin and alizarin conjugated to rhamnose m/z 409.08 [M+H]+ were established based on the mass analysis of respective product peaks. The mass spectra were obtained along with their sister fragments of chrysazin, quinizarin, anthrarufin and alizarin m/z 241.05 [M+H]+ (Fig. S2). The biotransformation reaction analysis by HPLC-PDA and ESI/MS revealed that the engineered strain converted all exogenously supplemented substrates to products. The conversion percentage of emodin, chrysazin, quinizarin, anthrarufin and alizarin were 2.4%, 2.5%, 17%, 10.7%, and 3%, respectively. Based on the highest conversion, a further study of quinizarin alone was carried out.

We increased the bioconversion of quinizarin via supplementation of different concentrations (0%, 2%, 4%, 6%, and 8%) of glucose in cultures grown under identical conditions during biotransformation. The change in conversion percentage of quinizarin to product was monitored at different time intervals (from 0 to 60 h). The result showed that supplementation of 2% additional glucose improved the conversion from 22% (36 h, without additional glucose) to 75% (48 h) (Figs. S3 and S4). The addition of glucose facilitated cell growth and product yield.

The product was purified by using prep-HPLC and then subjected to nuclear magnetic resonance (NMR) analyses. While comparing the 1H NMR spectra of standard quinizarin and the reaction product, signals from the parent compound containing 2-hydroxyl groups in the symmetrical position were detected at δ 12.71 (1H, s) while in the reaction compound hydroxyl group, the signals were detected at δ 12.88 (1H, s) (Table 1, Figs. S5a and S5b). The anomeric proton (1'-H) was consistent with δ 5.48 (d, J = 1.7 Hz, 1H); however, the anomeric proton coupling constant (J = 1.7 Hz) confirmed that the conjugation of rhamnose moiety was in α-configuration. In addition, based on the 13C NMR analysis of the reaction product, the new peak appeared at δ 100.01 ppm for anomeric carbon and other carbon peaks between 70 and 80 ppm along with a CH3 peak at 18.3 ppm. All the peaks were assigned to their respective carbon as shown in Figs. S6a and S6b. To confirm the position of sugar conjugation, we analyzed 1H-13C correlation using heteronuclear single quantum
coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) spectroscopy. The result supported the correlation between the observed anomeric carbon and anomeric proton revealed by HSQC (Fig. S7). Similarly, the carbon C-4 of the quinizarin signal appearing at $\delta$ 150.48 ppm showed a direct correlation with the observed anomeric proton at $\delta$ 5.48 ppm in HMBC (Fig. S8). Based on these results, the product was established as quinizarin 4-$\alpha$-L-rhamnoside.

Previous studies showed that the anticancer effects of anthraquinones were associated with the suppression of cancer cell proliferation [25]. We thus evaluated the effects of quinizarin and its derivative on the proliferation of A375SM melanoma, AGS gastric cancer, MCF-7 breast cancer, and U87MG brain cancer. The inhibitory effect of quinizarin rhamnoside was greater than that of aglycone in all cancer cell lines tested. This result showed that approximately 70% of AGS gastric cancer cells failed to grow in the presence of 50 $\mu$M concentration of quinizarin rhamnoside while the suppression of cell growth was only 20% under the same concentration of quinizarin. Although subtle growth reduction was observed with rhamnoside derivative, the decrease in cell proliferation of MCF-7 breast cancer cells and U87MG brain cancer was not significantly different with quinizarin and its rhamnoside derivative (Fig. 3).

Chemical synthesis of anthraquinone glycosides requires multiple steps, uses hazardous chemicals, and is therefore an environmentally unfriendly approach [26]. Moreover, production of anthraquinone rhamnosides in practical quantities from plant sources has been tedious and impractical as biosynthesis in large quantity from these sources is difficult to achieve while purification and extraction are more challenging because of the presence of a large number of other metabolites [27]. Therefore, regiospecific biosynthesis using engineered recombinant microbial cells is superior in terms of sustainability while being eco-friendly and enabling easy fermentation and scale-up for industrial biosynthesis [28]. This study provides a broad overview of the modification of anthraquinones by rhamnosylation using an engineered E. coli strain in a sustainable way. The antiproliferative activities

| Table 1. Comparison of $^1$H- and $^{13}$C-NMR spectra of quinizarin and quinizarin-4-$\alpha$-L-rhamnoside measured in DMSO-d$_6$. |
|---|---|---|---|
| Position | $^1$H-NMR | $^{13}$C-NMR | $^1$H-NMR | $^{13}$C-NMR |
| 1-OH | 12.71 ($s$, 1H) | 12.88 ($s$, 1H) | 158.57 | 157.89 |
| 2 | 7.45 ($s$, 1H) | 7.38 ($d$, $J = 9.3$ Hz, 1H) | 131.24 | 129.48 |
| 3 | 7.45 ($s$, 1H) | 7.38 ($d$, $J = 9.3$ Hz, 1H) | 131.24 | 127.20 |
| 4-OH | 12.71 ($s$, 1H) | - | 158.57 | 150.48 |
| 5 | 7.98 ($dd$, $J = 5.8$, 3.3 Hz, 1H) | 7.90 ($d$, $J = 32.7$ Hz, 1H) | 128.57 | 126.54 |
| 6 | 8.27 ($dd$, $J = 5.8$, 3.3 Hz, 1H) | 8.16 ($dd$, $J = 34.9$, 7.6 Hz, 2H) | 134.78 | 134.24 |
| 7 | 8.27 ($dd$, $J = 5.8$, 3.3 Hz, 1H) | 8.16 ($dd$, $J = 34.9$, 7.6 Hz, 2H) | 134.78 | 132.31 |
| 8 | 7.98 ($dd$, $J = 5.8$, 3.3 Hz, 1H) | 7.90 ($d$, $J = 32.7$ Hz, 1H) | 128.57 | 126.20 |
| 9 | | | 188.59 | 188.59 |
| 10 | | | 188.59 | 180.95 |
| 11 | | | 114.58 | 116.01 |
| 12 | | | 114.58 | 120.59 |
| 13 | | | 136.99 | 135.60 |
| 14 | | | 136.99 | 134.87 |
| 1' | 5.48 ($d$, $J = 1.7$ Hz, 1H) | | 100.01 |
| 2' | 3.96 ($dd$, $J = 4.4$ Hz, 1H) | | 72.82 |
| 3' | 3.36 ($dd$, $J = 9.1$, 4.5 Hz, 1H) | | 75.05 |
| 4' | 4.81 ($d$, $J = 5.8$ Hz, 1H) | | 70.59 |
| 5' | 4.06 ($s$, 1H) | | 72.17 |
| 6'-CH$_3$ | 1.12 ($s$, 1H) | | 18.31 |

Multiplicities are indicated by $s$ (singlet), $d$ (doublet), $t$ (triplet), $q$ (quartet), and $m$ (multiplet), including coupling constant $J$. 

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of the newly synthesized molecule prompted further investigations into the search for novel molecules in medicinal chemistry.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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Fig. 3. Inhibitory effects of quinizarin and quinizarin-4-O-α-L-rhamnoside derivative (denoted as quinizarin-R) on cancer cells including AGS gastric cancer, A375SM skin cancer, MCF-7 breast cancer and U87MG brain cancer.
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