Journal of Molecular Catalysis B: Enzymatic 124 (2016) 1–10

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Synthetic analog of anticancer drug daunorubicin from daunorubicinone using one-pot enzymatic UDP-recycling glycosylation

Luan Luong Chu[a,1], Ramesh Prasad Pandey[a,1], Ju Yong Shin[a], Hye Jin Jung[a], Jae Kyung Sohng[a,*]

[a] Institute of Biomolecule Reconstruction, Department of BT-Convergent Pharmaceutical Engineering, Sun Moon University, 70 Sunmoon-ro 221, Tangjeong-myeon, Asan-si, Chungnam 31460, Republic of Korea

ARTICLE INFO

Article history:
Received 12 August 2015
Received in revised form
10 November 2015
Accepted 18 November 2015
Available online 24 November 2015

Keywords:
Daunorubicinone
Glucosylation
One-pot reaction
Byproduct recycling
Biocatalysis

ABSTRACT

Doxorubicin and daunorubicin are two widely used anticancer drugs containing deoxyaminosugar unit in their structure. Altering the sugar moiety could generate novel drugs, which could have different biological potency than daunorubicin and doxorubicin. In this study, daunorubicinone was used as a substrate for the production of two different novel glucoside derivatives via enzymatic glycosylation, and their physical and biological activities were assessed and compared with standard compounds. A one-pot enzymatic glycosylation system was designed *in vitro* to maintain continuous generation of UDP-α-D-glucose, which is used as the donor substrate by the glycosyltransferase enzyme, YjiC, for the glycosylation of acceptor molecule, daunorubicinone, aglycone of daunorubicin. The result indicates an increased conversion (~74.8%) of daunorubicinone 7-*O*-α-D-glucoside and ~22.2% of daunorubicinone-7,9-di-*O*-α-D-glucoside in one-pot UDP-α-D-glucose recycling system in comparison to regular reaction at 90 min incubation period. Both the glucosylated daunorubicinone exhibited improved water solubility, and stability over a wide range of pH (4.5–8.5) and high temperatures. However, anticancer activity assay against four different cancer cell lines showed complete loss of anticancer property upon glucosylation of daunorubicinone.

1. Introduction

Daunorubicin is an anthracycline antibiotic produced by *Streptomyces peucetius* [1] and is known as one of the most effective chemotherapeutic agents widely used for the treatment of acute lymphoblastic or myeloblastic leukemias [2]. Similar to doxorubicin, the mode of actions of daunorubicin is mainly owing to its ability to intercalate with DNA, leading to inhibition of DNA replication and transcription or break down of double-strand DNA and inhibiting topoisomerase II [3,4]. Despite daunorubicin's extensive use for cancer treatment, its clinical effectiveness is limited, because of serious side effects such as cardiomyopathy [5], skin ulcerations [6], hematologic toxicity and infections, immunosuppressive and teratogenic activities [7], congestive heart failure [8], as well as the appearance of multidrug resistance in tumor cells [9,10]. To address these disadvantages, the biosynthetic modifications and the development of novel analogs of daunorubicin as potential antitumor agents are essential.

The chemical structure of daunorubicin consists of daunorubicinone aglycone and daunosamine sugar moiety (Fig. 1). Daunorubicinone is structurally characterized as a tetracyclic ring with adjacent quinone-hydroquinone groups in rings C-B, a methoxy substituent at C-4 in ring D, and a short side chain at C-14 in ring A carrying a methyl group. Daunosamine is attached by a glycosidic linkage at the C-7 of ring A [11]. The sugar moiety has significant role in affecting the pharmacological activity and direct interacting with DNA to exhibit antitumor efficacy [12]. The amino sugar is a hydrophilic structural component of daunorubicin, enhancing its solubility and pharmacokinetics [13]. The change in the chemical structure of the amino sugar affects the affinity of daunorubicin to the cell membrane and DNA by changing the polarity and charge [14]. The modification of the sugar moiety could reduce toxicities [12] and overcome multidrug resistance [15].

Although aglycones of many natural products exhibit biological activity when liberated intracellularly, daunorubicinone has

* Corresponding author. Fax: +82 41 544 2919.
E-mail address: sohng@sunmoon.ac.kr (J.K. Sohng).
[1] Authors contributed equally to this work.

http://dx.doi.org/10.1016/j.molcatb.2015.11.020
1381-1177/

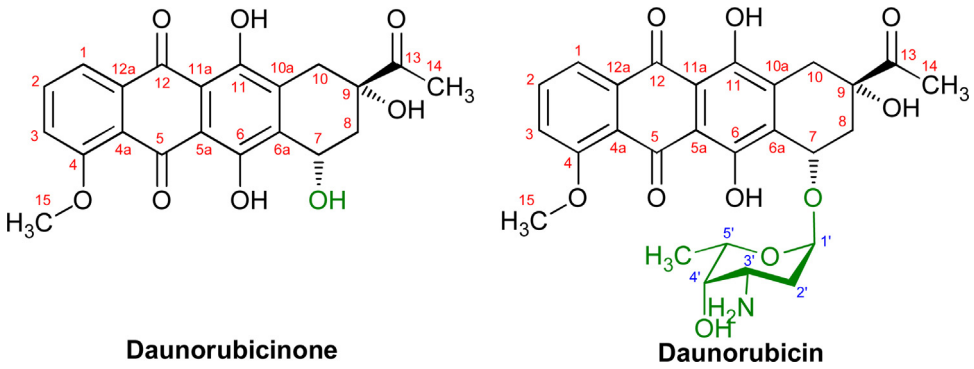


Fig. 1. Structures of daunorubicinone and daunorubicin.

not been reported to have any such activities. Therefore, synthesis of a new daunorubicin analog from daunorubicinone is expected to have potent biological activities for drug production. Several derivatives of daunorubicinone have been prepared by chemical synthesis. Chemically synthesized 7-*O*-epoxyalkyl daunorubicinone was reported to have antimicrobial activities and inhibited the nucleic acid and protein synthesis in P388 cells [16]. Using alkaline deacetylation method, daunorubicinone-7-D-glucuronide has been synthesized; however, the chemical and biological properties of the daunorubicinone-7-D-glucuronide as a potential prodrug has not been reported [17]. Another analog of daunorubicin with halogen (Cl, Br, or I) in the place of amino group at C-3′ position of the sugar moiety showed activities against resistant tumor cells [18].

Glycosylation is one of the most important and promising post-biosynthesis modifications of natural products in nature, enhancing solubility, stability or bioactivity of the parent compounds [19]. Enzymatic glycosylation has been developed as one of the most prominent techniques to generate novel compounds [20]. A large number of natural glucosides were synthesized by using nucleotide diphosphate (NDP)-sugars by *in vitro* glycosylation reactions [21–26]. The enzymatic and microbial synthesis of natural product glycosides have overcome costly, tedious and time-consuming multistep chemical synthesis processes [27–29]. However, one of the major problems of *in vitro* enzymatic glycosylation is the use of expensive NDP-sugar such as uridine diphosphate (UDP)-α-D-glucose as the sugar donor, which hinders easy industrial scale-up of the process. Therefore, to increase the productivity and decrease the cost of the reaction and biosynthesis of compounds, one-pot enzymatic glycosylation system has been developed by recycling the by-product of the glycosylation reaction such as UDP and generating UDP-α-D-glucose from additional supply of glucose-L-phosphate [30–32].

In this study, a one-pot enzymatic system with UDP-α-D-glucose regeneration was developed for efficient glycosylation of daunorubicinone by using a uridine diphosphate glycosyltransferases (UGT), YjiC from *Bacillus licheniformis* DSM 13 (Scheme 1). Two novel glucoside derivatives of daunorubicinone were successfully produced, and their water solubility and stability were also studied. Moreover, the anticancer properties of newly synthesized derivatives were compared to daunorubicinone and daunorubicin standards against AGS, B16F10, HepG2, and Hela cells.

2. Materials and methods

2.1. Chemicals and reagents

Standard daunorubicinone, α-D-glucose-1-phosphate, acetyl phosphate, UDP-α-D-glucose and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Genechem (Deajeon, Korea). Deuterium oxide (D_2O) and dimethyl sulfoxide-d_6 (DMSO-d_6) were obtained from Sigma–Aldrich (St. Louis, MO, USA). High-performance liquid chromatography (HPLC) grade acetonitrile and water purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). All other chemicals were purchased from standard commercial sources and were of the highest quality available.

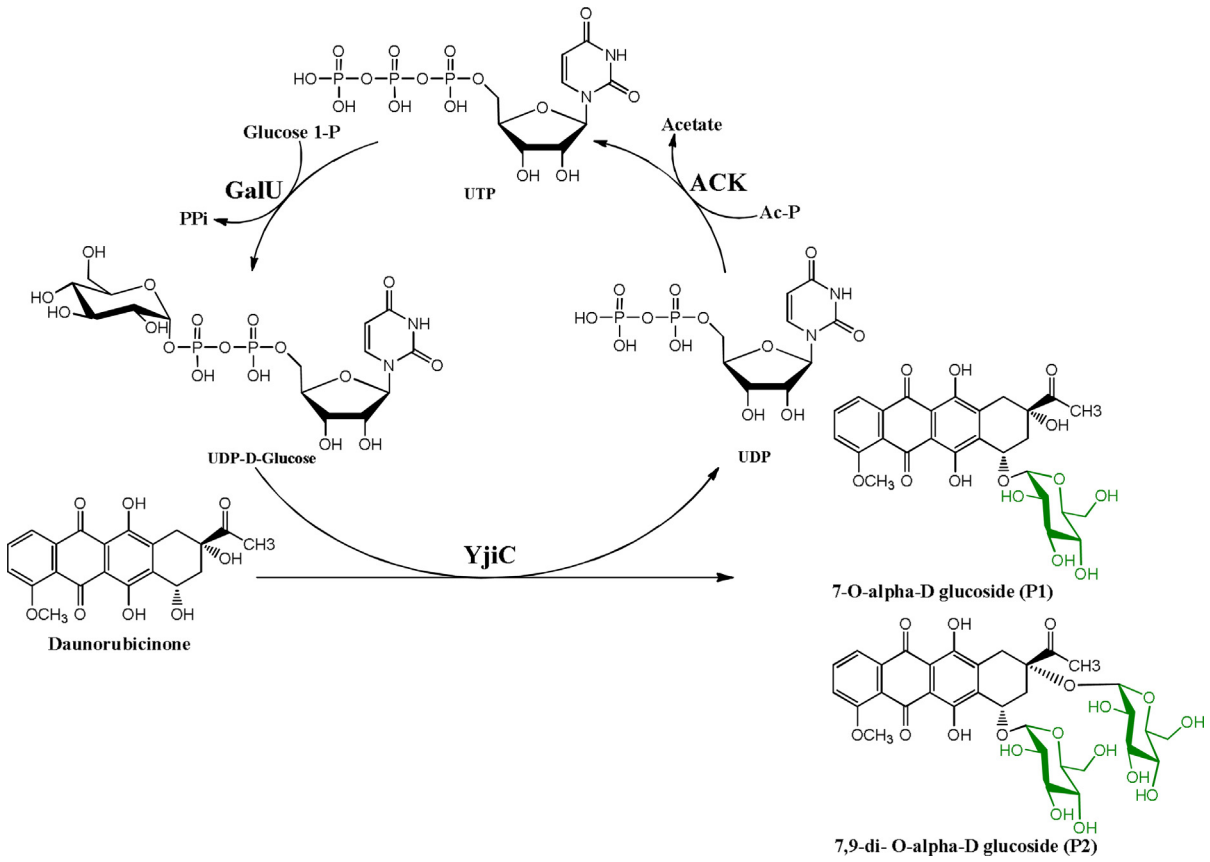
2.2. Plasmids, microorganisms and culture conditions

Escherichia coli BL21 (DE3) (Stratagene, USA) was used as the host for the protein expression and production. Recombinant plasmids for acetate kinase (ACK-pET24ma), α-D-glucose-1-phosphate uridilyltransderase (GalU-pET24ma) [33] and glycosyltransferases (YjiC-pET28a) [23] were used. All *E. coli* strains were cultured in Luria Bertani (LB) liquid medium at 37 °C under shaking at 180 rpm. An appropriate amount of antibiotics (ampicillin up to 100 μg/mL and kanamycin up to 50 μg/mL) was used for the selection and maintenance of the recombinant strains.

2.3. Protein expression, purification and quantification

The recombinant strains were cultured in 5 mL LB medium supplemented with appropriate antibiotics at 37 °C, 180 rpm for 6 h. For protein production, the pre-inoculum (200 μL) was transferred in a 250 mL sharking flask containing 50 mL LB medium supplemented with appropriate antibiotics and then incubated at 37 °C, 180 rpm. When the optical density at 600 nm (OD_{600}) reached 0.6, the protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration at 0.5 mM, and the growth was continued at 20 °C for 20 h. The cells were harvested by centrifugation at 842 × *g* rpm for 15 min and suspended twice in 50 mM Tris–HCl (pH 7.5) buffer containing 10% glycerol. The cells were suspended in 1 mL of the same buffer containing 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were lysed by sonication using a Sonosmasher (2 × 1 min, output control 5, 50% duty cycle; Sonicator, Heat Systems, Ultrasonic, Inc.). Following centrifugation at 13,475 × *g* for 30 min at 4 °C, the resulting soluble and insoluble protein fractions were analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The crude enzymes were used for enzymatic glycosylation.

For the regular reactions, the crude YjiC was applied to a TALON metal nickel affinity resin (Takara Bio, Shiga, Japan) equilibrated with buffer containing 300 mM NaCl and 50 mM Tris–HCl (pH 7.5). The resin-bound protein was eluted using discontinuous imidazole gradient (10, 50, 100, 200, and 500 mM) prepared in the equilibration buffer. The fraction containing purified protein were analyzed by 12% SDS–PAGE and further concentrated using Amicon® Ultra 15 mL filters (Millipore, 30 K NMWL device; Milford, MA, USA). Protein quantitation was performed by Bradford protein assay [34].



Scheme 1. One-pot enzymatic glucosylation system for the synthesis of daunorubicinone glucosides with regeneration of UDP-α-D-glucose. Abbreviations: ACK: acetate kinase; GalU: UDP-α-D-glucose synthase; UDP: uridine-5′-diphosphate; UTP: uridine-5′-triphosphate; Ac-P: acetylphosphate; Glucose-1-P: glucose-1-phosphate; PPi: inorganic pyrophosphate.

2.4. Enzymatic reaction conditions

A 200 μL regular reactions were carried out using 50 μgm L^{-1} purified YjiC and 2 mM daunorubicinone and other substrates in 50 mM Tris–HCl buffer (pH 7.5) with 2 mM UDP-α-D-glucose and 10 mM $MgCl_2$. Subsequently, the 200 μL one-pot reaction mixture for the synthesis of daunorubicinone glucopyranosides was prepared consisted of ~50 μgm L^{-1} each crude enzyme (ACK, GalU and YjiC), 2 mM daunorubicinone in 50 mM Tris–HCl buffer (pH 7.5) with 2 mM UDP-α-D-glucose, 10 mM $MgCl_2$, 50 mM α-D-glucose-1-phosphate, and 200 mM acetyl phosphate. The mixture reaction was incubated at 37 °C, and 20 μL of the sample was removed at different intervals and quenched by adding 40 μL of methanol. The samples were analyzed by high-performance liquid chromatography-photodiode array (HPLC-PDA).

To study the effects of concentration of UDP-α-D-glucose, the one-pot reaction was carried out as aforementioned condition adding various concentrations of cofactor. For example, 0.5 mM, 1 mM, 1.5 mM, 2 mM and 3 mM of UDP-α-D-glucose was used in five different reaction mixtures and products were analyzed at different time points (5; 15; 30; 60; 90; 120 min). Similarly, the optimal pH of the one-pot reaction was also determined by using the optimal UDP-α-D-glucose concentration. Similar reaction mixture was prepared varying the pH of the buffer (pH 4.5; 5.5, 6.5; 7.5; 8.5; 10; 11) and reaction was proceed at 37 °C. The outcome of the reaction was monitored at different time points.

For preparative-scale glycosylation, the reaction was carried out in 5 mL volume with optimized cofactor concentration and pH of the buffer in a shaking incubator at 37 °C. The reaction was stopped by adding 5 mL cold methanol. Protein residue was removed by centrifugation at 13,475 × *g* for 30 min at 4 °C. The supernatant was concentrated by evaporation and dissolved in 2.5 mL methanol before purification by preparative-HPLC.

2.5. Analytical procedures

Thin layer chromatography (TLC) and HPLC-PDA were carried out to detect the glycosylation products. TLC was performed using Silica gel 60 F_{254} plates (Merck, Darmstadt, Germany). An aliquot ~2 μL of the reaction mixture was loaded onto a normal phase silica plate and developed in a solvent system of 10:1.5:1.0:0.2 (v/v/v/v) ethyl acetate: methanol:water:toluene in a closed TLC chamber. The plate was allowed to air-dry in a hood and then soaked rapidly in 5% H_2SO_4 in methanol. Finally, the plate was dried in an oven at 90 °C until visible spots were clearly observed.

For the HPLC-PDA analysis, the reaction mixture was analyzed by reversed-phase column (Mightysil RP-18 GP 250–4.6 (5 μm), Kanto Chemical, Japan) at a UV absorbance of 254 nm. The binary mobile phases comprised solvent A (0.05% trifluroacetic acid (TFA) in HPLC-grade water) and solvent B (100% acetonitrile, CH_3CN). Total flow rate was maintained at 1 mL min^{-1} for 30 min program. The percentage of solvent B used is as follows: 0–20% (0–5 min), 50% (5–10 min), 70% (10–15 min), 90% (15–20 min), 10% (20–25 min), 10% (25–30 min).

The preparative purification of the enzymatic reaction was achieved by preparative HPLC (Shimazu, Tokyo, Japan) with C_{18} column (YMC-Pack ODS-AQ) (150 × 20 mm I.D., 10 μm) connected to a UV detector at a UV absorbance of 254 nm using a 36-min binary program with CH_3CN 20% (0–5 min), 40% (5–10 min),

40% (10–15 min), 90% (15–25 min), 90% (25–30 min) and 10% (30–36 min) at a flow rate of 10 mL min^{-1}.

High-resolution quadruple time-of-flight electrospray ionization-mass spectrometry (HR-QTOF ESI/MS) analysis was performed in the positive ion mode using an ACQUITY (UPLC, Waters Corp., Billerica, MA, USA) column coupled with a SYNAPT G2-S (Water Corp.). The purified compounds were dried, lyophilized, and dissolved in DMSO-d_6 and subjected to 800 MHz Bruker, Biospin NMR for ^{1}H NMR and ^{13}C NMR analyses.

2.6. Water solubility determination

For determination of water solubility, daunorubicinone, daunorubicin, daunorubicinone 7-*O*-α-D-glucoside, and daunorubicinone 7,9-di-*O*-α-D glucoside were dissolved in phosphate-buffered saline (PBS) at pH 7.4 to a final concentration at 1 mM. The mixtures were vortexed for 30 min and centrifuged at 13,475 × *g* for 10 min. The supernatant was collected, filtered through a 0.45-μm syrige filter and diluted ten times in methanol. The samples (20 μL) were then analyzed directly via HPLC-PDA at 254 nm. The concentrations of daunorubicinone, daunorubicin, daunorubicinone 7-*O*-α-D-glucoside, and daunorubicinone 7,9-di-*O*-α-D glucoside dissolved in PBS buffer were calculated by the regression equations.

2.7. Thermal and pH stability of daunorubicinone glucoside

To determine the pH stability, the dried products were dissolved to a final concentration at 0.15 mM in 200 μL of Tris–HCl buffer at varying pH (4.5–12) and left for 30 min at 25 °C before HPLC analysis. To determine the thermal stability of the daunorubicinone glucosides, each compound was incubated at 40, 50, 60, 70, 80, 90 and 100 °C at pH 7.5 for 30 min. Aliquots (20 μL) were analyzed directly via HPLC. Stability of the products was calculated by the regression equations.

2.8. Anti-cancer activity test

To evaluate the effects of the different novel analogs of daunorubicinone glucosides on the proliferation and viability of AGS (gastric carcinoma), B16F10 (skin melanoma), HepG2 (Hepatic carcinoma), and Hela cervical cancer cells, cells were grown in RPMI 1640 medium (Invitrogen, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Invitrogen). All the cells were maintained at 37 °C in a humidified 5% CO_2 incubator. For the cell growth assay, cells seeded at 2×10^3 cell/well in 96-well plates (SPL Life Sciences, Gyeonggi, Korea) were treated with each compound in a serial dilution (10, 5, 2.5, 1.25, 0.625, 0.3125 μM) for 72 h. Cell growth was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay.

3. Results and discussion

3.1. Protein expression and purification

The recombinant proteins, ACK (38 kDa), GalU (38 kDa), and YjiC (45 kDa) were overexpressed in *E. coli* BL21 (DE3) and obtained in soluble fraction (Supplementary Fig. S1). The crude enzymes were used for one-pot reactions. For testing the enzyme activity, YjiC was purified and used for regular reactions. SDS–PAGE analysis of purified YjiC is shown in Supplementary Fig. S1.

3.2. Enzymatic synthesis of the daunorubicinone glucoside

In regular glycosylation reactions, YjiC was used to modify daunorubicinone to its glucoside using UDP-α-D-glucose as the sugar donor. The reaction mixture was incubated at 37 °C for 90 min and quenched with methanol and applied to silica F_{254} plates for TLC analysis along with standard daunorubicinone. The TLC analysis shows a distinct new spot at a retention factor (R_f) of ~2.9 (Supplementary Fig. S2). The new spot with a lesser R_f value (higher polarity) than daunorubicinone (R_f ~6.0) might be the target monoglucosylated daunorubicinone. The same reaction mixture was also injected for HPLC analysis. The HPLC chromatogram reveals the appearance of new peak in regular glucosylation reaction (Fig. 2). The retention time (t_R) of standard daunorubicinone was observed at 18.28 min in the same HPLC condition at 254 nm UV absorbance. The exact mass of daunorubicinone $[M+H]^+$ m/z^+ was observed at ~399.1082 corresponding to molecular formula $C_{21}H_{18}O_8$, for which the exact calculated mass was ~399.1080 (Supplementary Fig. S3A). A new peak (t_R) at 15.1 min (P_1) could be the probable target product. P_1 was further analyzed by HR-QTOF ESI/MS. The mass spectrum shows the peak at an exact mass of ~583.1457 for $[M+Na]^+$ m/z^+ resembling the mass of the monoglucoside derivative of daunorubicinone with a molecular formula ($C_{27}H_{28}NaO_{13}$) for which the calculated mass for $[M+Na]^+$ m/z^+ was ~583.1428 (Supplementary Fig. S3B). The HPLC-PDA analysis also showed ~26.20% conversion of daunorubicinone to its glucoside within 90 min in regular reaction. The results indicate that the substrate-versatile glycosyltransferase, YjiC, which has been characterized for its promiscuous activity toward various small molecules such as flavonoids [21,23,28] and macrolides [25], could be used for the synthesis of daunorubicinone glucoside derivatives, which structurally mimic daunorubicin. The *in vitro* enzymatic synthesis using YjiC opened up prospects for the synthesis of the compound within the tetracyclic ring containing natural products. The similar strategy could be applied for other NDP-sugar to generate the libraries of compound with different glycosylation approaches.

3.3. Production of glycosylated daunorubicinone by one-pot systems

The production of daunorubicinone glucoside using YjiC and the corresponding sugar nucleotide, UDP-α-D-glucose, was achieved by the regular glycosylation reaction. However, this process is expensive to carry out in a large-scale reaction, because of the high cost of UDP-α-D-glucose. Furthermore, UDP, one of the products of the enzymatic glycosylation reaction, is responsible for deglycosylation activity of YjiC [23]. Consequently, the glycosylation reaction rate gradually decreases as UDP accumulates in the reaction mixture. Beside, in many cases, UDP acts an inhibitor of glycosyltransferase activity by competing at the donor substrate binding site with UDP-sugar [30]. Thus, the conversion of substrate to its glucoside is limited unless a higher concentration of UDP-sugar is present in the regular glycosylation reaction mixture. To overcome such drawbacks, one-pot enzymatic system was developed with *in situ* UDP-α-D-glucose regeneration. As shown in Scheme 1, UDP produced from UDP-α-D-glucose by YjiC was used for UDP-α-D-glucose regeneration and continued to the reaction system by acetate kinase (ACK). The ACK catalyzes the phosphorylation of UDP to form UTP with the consumption of acetyl-phosphate. Then, glucose-1-phosphate uridylyltransferase (GalU) carried out a key step in the generation of UDP-α-D-glucose and catalyzed the transfer of UDP to α-D-glucose-1-phosphate to yield UDP-α-D-glucose, which is the sugar donor molecule in the glycosylation reaction. Both α-D-glucose-1-phosphate and acetyl-phosphate are required in this system. The reaction components are inexpensive and easily available, thus eventually lowering the cost of the reactions.

The TLC analysis of one-pot reaction mixture showed two new spots at 15 and 90 min under the same reaction conditions (temperature, pH, buffer, and incubation time) with regular glycosylation

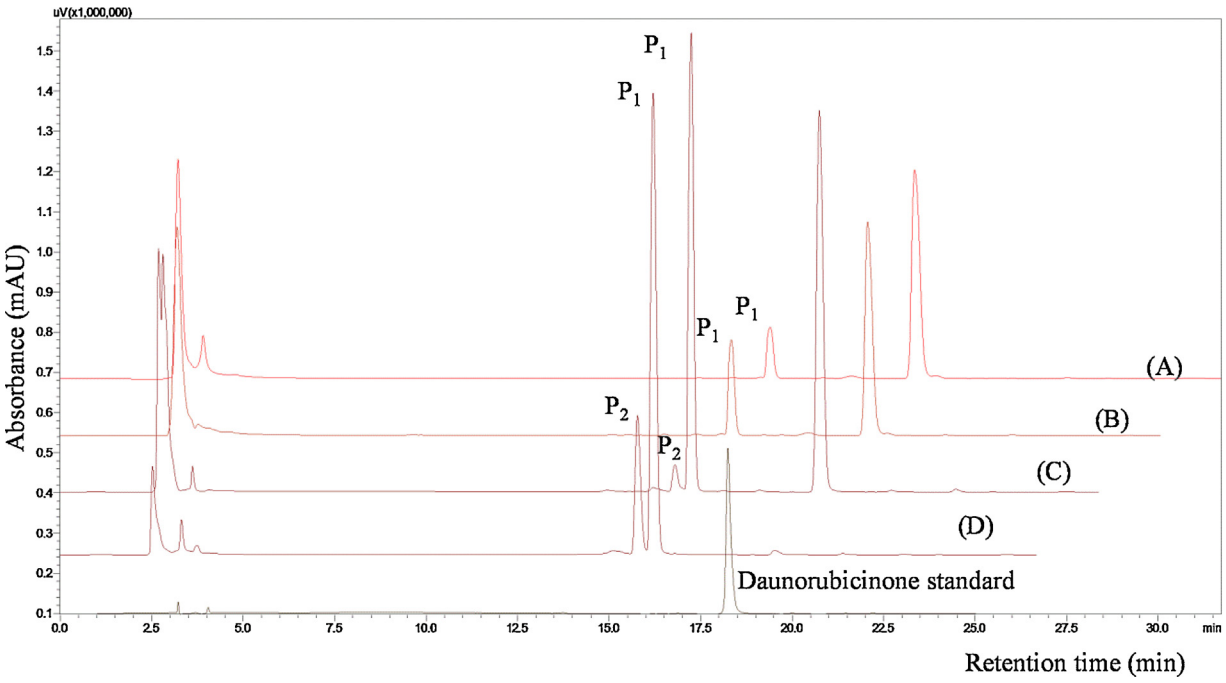


Fig. 2. The HPLC-PDA analyses of glycosylation reaction of daunorubicinone with UDP-α-D-glucose. P_1 and P_2 have been identified as mono-glucoside (t_R ~15.178) and di-glucoside (t_R ~14.776) of daunorubicinone (t_R ~ 18.28): (A) and (B) general glycosylation reaction for 15 min and 90 min incubation; (C) and (D) one-pot glycosylation reaction for 15 min and 90 min incubation.

reaction (Supplementary Fig. S2). The spot with a higher R_f value (~2.9) is similar to the product of regular reaction; however, a novel spot with a lesser R_f value (~1.4) than the monoglucosylated product might be the diglucosylated daunorubicinone derivative. The HPLC-PDA analysis of the same one-pot reaction mixture showed two distinct peaks at t_R of 15.17 min (P_1) and 14.77 min (P_2). P_1 had exactly similar t_R with the single product of the regular reaction (Fig. 2). Novel product P_2 showed the exact mass $[M+Na]^+$ m/z^+ at ~745.1948 corresponding to the calculated mass of the diglucoside derivative of daunorubicinone with molecular formula ($C_{33}H_{38}NaO_{18}$) for $[M+Na]^+$ m/z^+ was ~583.1956 (Supplementary Fig. S3C). In the one-pot enzymatic reaction, daunorubicinone was more efficiently converted to respective glucosides. The result indicates an increased conversion of ~74.8% in the one-pot system compared to 26.2% in the regular glycosylation reaction. In addition, novel product P_2 was formed within 15 min, which was not seen in the regular glycosylation reaction. Thus, the designed one-pot glycosylation system was applied for the preparative-scale production of daunorubicinone glucosides.

3.4. Optimization of cofactor concentration, pH and scale-up of one-pot system

To study the effects of concentration of UDP-α-D-glucose on the one-pot reaction, several reactions were performed with 2 mM daunorubicinone and varied concentrations of cofactor. The total conversion of daunorubicinone to daunorubicinone glucosides was determined. Analysis of reaction mixtures at different time intervals showed favored biosynthesis of daunorubicinone glucosides under higher UDP-α-D-glucose concentration than lower concentration of UDP-α-D-glucose (Fig. 3A). While using 0.5 mM of UDP-α-D-glucose in the reaction, the total conversion of daunorubicinone to glucoside derivative was ~63.64% in 2 h incubation. But, upon increasing the concentration of UDP-α-D-glucose to 1.0 mM, the conversion was elevated to 93.45% in 2 h. Similarly, further increase in UDP-α-D-glucose concentration to 1.5 mM, the conversion of dounorubicinone was 98% at the same duration of reaction

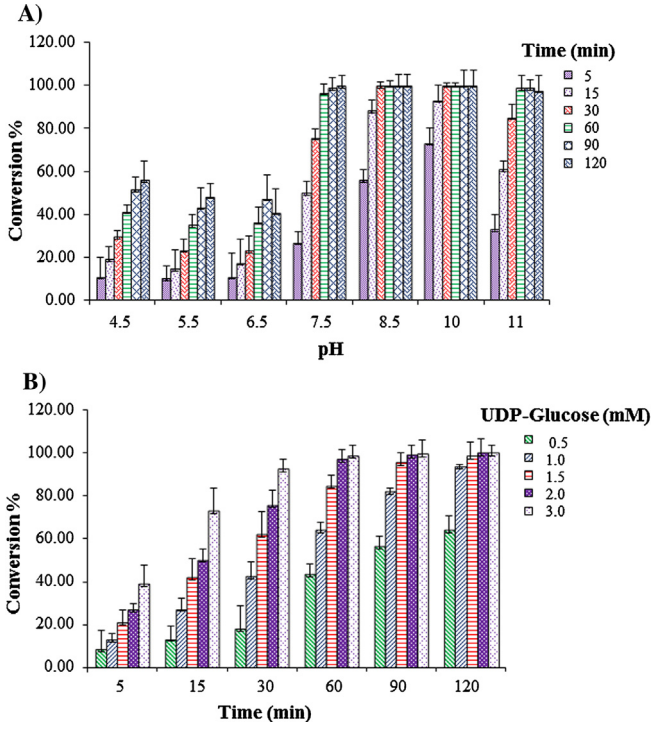


Fig. 3. (A) Effects of UDP-α-D-glucose concentration on the one-pot reaction. (B) The optimization of pH on the one-pot glycosylation reaction. The bar diagrams are marked with error bars with standard deviation.

incubation. When 2.0 mM of UDP-α-D-glucose was used, approximately 98.8% conversion of daunorubicinone was observed within 90 min. Similarly, upon use of 3.0 mM of UDP-α-D-glucose, 98.4% total conversion was achieved within an hour of reaction incubation. The result also showed that the proportion of production of P_1 was always higher in comparison to P_2 at different concentration of UDP-α-D-glucose. After complete conversion of daunorubicinone,

the production of P_1 decreased slowly while increasing the concentration of P_2 (Supplementary Table 1). There are two possible reasons behind increased concentration of P_2 upon longer incubation of reaction mixture. The first reason could be because of higher stability of P_2 than P_1 in the reaction mixture. Secondly, P_1 could be used as substrate by the enzyme to produce P_2 as in our previous report [23]. These results indicated that one-pot glycosylation reactions can be controlled to produce desired product in higher concentration by varying the concentration of cofactor and incubation time of the reaction mixture.

The optimal pH for the one-pot reaction was also determined by performing reactions at different pH (4.5–11.0) of the Tris–HCl buffer. The results showed that the conversion of substrate was increasing above pH 7.5 in the one-pot reaction (Fig. 3B). When the reaction was carried out using low pH of the buffer such as 4.5, 5.5 and 6.5, the total conversion of daunorubicinone to its glucoside derivatives was limited to below 50% until 2 h of reaction incubation. However, when the higher pH of the buffer (7.5, 8.5, 10 and 11) was used, drastic difference in substrate conversion was observed. In pH 7.5, 100% substrate conversion was achieved in 90 min. But, the same conversion percentage was noticed within 30 min when 8.5 and 10.0 pH of buffer was used for the reaction. However, when buffer with pH 11.0 was used for the reaction, the conversion of substrate was slightly slower than in buffer with pH of 8.5 and 10.0 (Fig. 3B). The maximum conversion was achieved after 90 min of incubation. The reason behind favorable glycosylation activity at comparatively higher pH could be because of neutralization of proton (H^+) released during glycosylation reaction by alkaline buffer.

To study the formation of the glucosylated products, 5 mL of the one-pot glycosylation reaction was started at pH 10.0 with 2 mM of daunorubicinone and the same amount was added at an interval of 30 min with the progress of the reaction, and the conversion of each product was calculated at different time points. The result showed that the maximum conversion of P_1 (66.96%) and P_2 (17.14%) were obtained at 6 h after addition of total 12 mM of daunorubicinone (Fig. 4A). The results demonstrated the capability of the one-pot reactions for high-conversion production of the glucosylated products.

One of the purposes of this experiment was to produce the analog of daunorubicin more economically and efficiently based on the one-pot enzyme glycosylation reaction from inexpensive material. The one-pot reaction approach showed that the catalysis of many enzymes could be performed in a single reaction vessel with high stereo- and regioselectivity with many advantages of mild and eco-friendly reaction conditions [35]. In our previous report [32], UDP and ATP were generated and recycled for the glycosylation of xanthonoid derivative. In this system, solely UDP was recycled after the glucosylation. Boycotting the ATP utilization step for the production of UDP, UDP produced after the glycosylation was directly recycled using initial UDP-α-D-glucose, which contributed to reduce cost of the reaction. Moreover, the one-pot UDP-recycling system was found to operate efficiently to convert daunorubicinone to its glucosides. Several previous studies [30–32] also showed enhanced glycosylation upon immediate removal of UDP, a product inhibitor of UGTs. A similar phenomenon was also observed in our one-pot glucosylation. An important feature of this method is the easy biosynthesis and purification of glycosylated products rather than multistep biosynthesis and tedious purification steps of NDP-sugars [36].

3.5. Structural elucidation of daunorubicinone glycosides

For determination of the structure of daunorubicinone glucoside derivatives, each product was isolated by preparative HPLC and analyzed by one-dimensional NMR (^{1}H and ^{13}C NMR). The results are listed in Table 1. The ^{1}H NMR spectrum of P_1 shows the presence of an anomeric proton signal at a chemical shift δ = 5.25 ppm (d, J = 3.9 Hz, 1H), confirming the attachment of one glucose molecule to the aglycone with an alpha (α) configuration. The proton signal of the hydroxyl group of C-7 at δ = 6.11 ppm (s, 1H) was absent in the ^{1}H NMR spectrum of P_1. Moreover, the proton signal at C-7 showed the same signal as daunorubicin (Table 1, Supplementary Figs. S4A–S6A). A total of 27 carbon peaks were observed in the ^{13}C NMR spectra, confirming to a molecular formula of $C_{27}H_{28}O_{13}$. The signal at δ = 103.82 ppm was assigned to the anomeric carbon (Table 1, Supplementary Figs. S4B–S6B), confirming compound P_1 as daunorubicinone 7-*O*-α-D-glucoside. Similarly, the ^{1}H NMR spectrum of P_2 (Table 1, Supplementary Fig. S7A) showed two anomeric protons at δ = 5.00 ppm (d, J = 5.0 Hz, 1H) and δ = 4.98 ppm (d, J = 5.4 Hz, 1H), representing alpha (α) configuration of the two sugar moieties, whereas other proton signals for sugar moieties were observed in the range δ 3.16–5.33 ppm (Table 1, Fig. S3). The proton signals of the two hydroxyl groups of C-7 and C-9 at δ 6.11 and 7.91 ppm were absent in the ^{1}H NMR spectrum of P_2. Other protons of the aglycone showed the same signals as that of daunorubicinone, indicating that the positions for two sugar moieties were confirmed to be at C-7 and C-9 positions. From the ^{13}C NMR analysis (Table 1, Supplementary Fig. S7B), beside 21 carbons of daunorubicinone, two anomeric carbons C-1′ and C-1″ of the two sugar moieties appeared at δ values 104.48 and 102.89 ppm. Moreover, the signals observed in the region δ 61.28–77.37 ppm were assigned to the sugar carbons. Therefore, P_2 was identified as daunorubicinone 7,9-di-*O*-α-D-glucoside.

Most of anthracyclines such as doxorubicin, daunorubicin, epirubicin, and idarubicin consists of tetracyclic aromatic polyketide planar scaffold coupled to L-deoxysugars at the 7th hydroxyl position. We successfully produced two glucoside derivatives of daunorubicinone, one of them containing a glucose moiety at 7th hydroxyl position of ring A and the other containing glucose at both 7th and 9th hydroxyl position by *in vitro* enzymatic reaction. A possible reasons for the formation of these structures may be attributed to the molecular shape of anthracyclines, which are characterized by the presence of an electron deficient planar chromophore (ring B, C, and D) and a sugar moiety oriented almost perpendicularly to the plane of the chromophore and linked to the alicylic ring A, the latter being in the half-chair conformation. Another possible reason could be the hydroxyl group at 7th and 9th of ring A of daunorubicinone does not form hydroxyl bonds with adjacent bases [37]. Thus, the attached sugar moiety and further modification in ring A of anthracycline molecule is essential for the intercalation activity of daunorubicin with DNA minor groove and thus exerting the bioactivity. Applying various approaches that could be either total chemical synthesis or combinatorial biosynthesis approaches, more than 2000 different derivatives of anthracyclines have been developed [37]. Our approach of *in vitro* glycosylation of daunorubicinone also contribute to produce novel glucosylated derivatives.

3.6. Solubility, and thermal and pH stability of daunorubicinone glycosides

For the determination of water solubility, daunorubicinone, daunorubicin, daunorubicinone-7-*O*-α-D-glucoside, and daunorubicinone-7,9-di-*O*-α-D-glucoside were dissolved in PBS buffer and analyzed by HPLC-PDA. The results show improved water solubility of daunorubicinone after glucose conjugation (Fig. 4B). Although being 5.25 times higher than daunorubicinone (0.064 mM), the solubility of daunorubicinone 7-*O*-α-D-glucoside (0.336 mM) in water is lower than daunorubicin (0.567 mM). As expected, the daunorubicinone 7,9-di-*O*-α-D-glucoside has the highest water solubility than other derivatives with 14.36 and 1.62 times higher than the daunorubicinone aglycone and daunorubicin, respectively. Our data were in accordance with the results

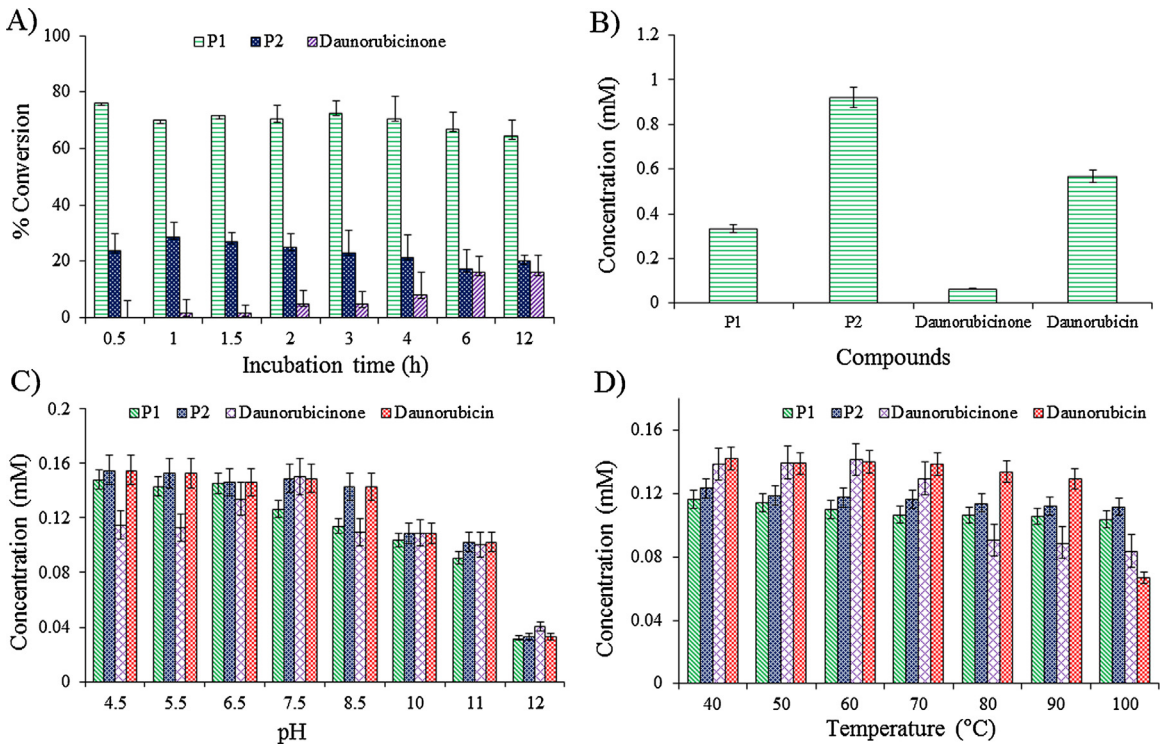


Fig. 4. (A) The production of P_1 and P_2 from 5 mL one-pot reaction at different time intervals. (B) Solubility of daunorubicinone, daunorubicin, P_1 and P_2. (C) Effect of temperature on the thermostability of P_1 and P_2. (D) Effect of pH on the stability of P_1 and P_2.

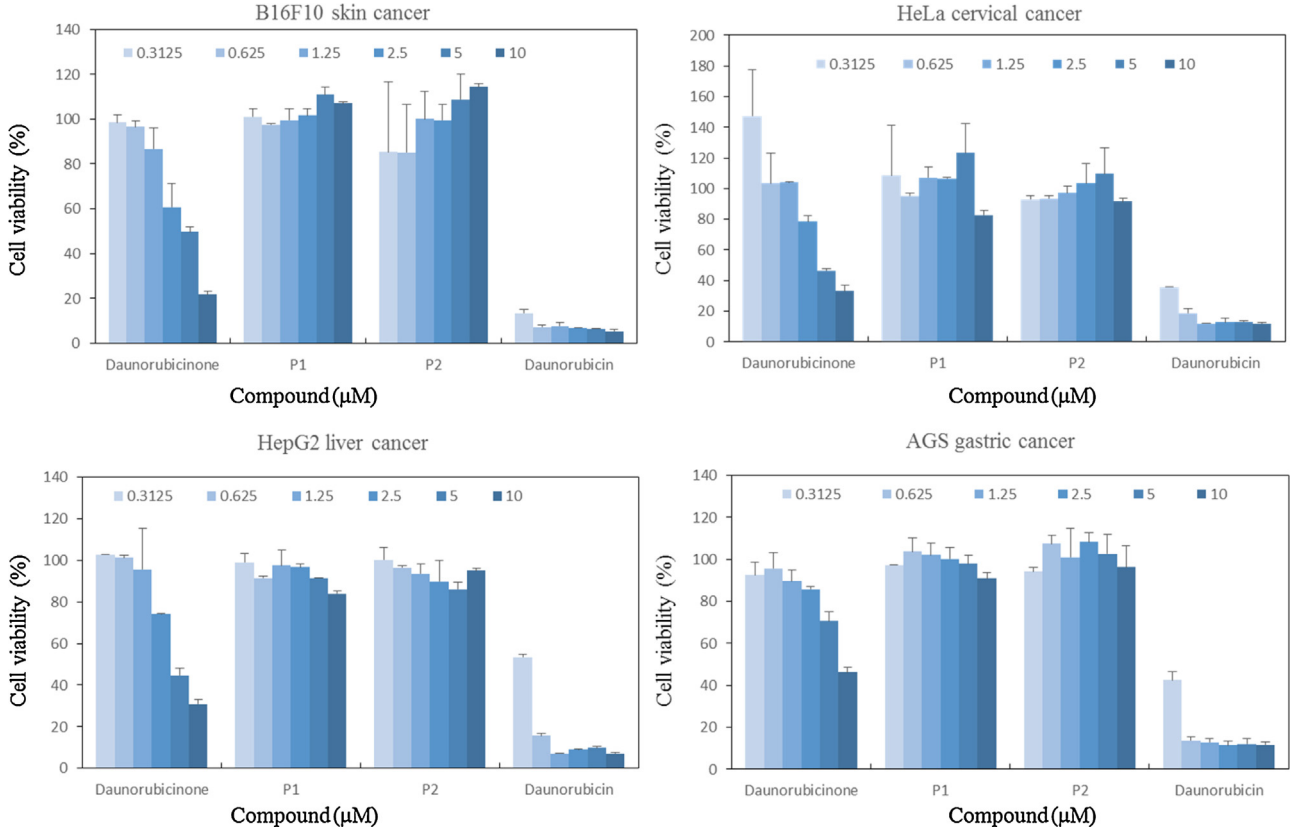


Fig. 5. Anticancer activities of compounds. The cells were treated with various concentrations (0.3125 ~ 10 μM) of each compound.

Table 1
Comparison of ^{1}H NMR of daunorubicinone, daunorubicin with two glycosylated daunorubicione. ^{1}H NMR of daunorubicin and daunorubicione was performed at 700 MHz in DMSO-d_6 whereas the ^{1}H NMR of daunorubicinone glucosides was determined at 800 MHz. Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), qn (quintet), m (multiplet) and br (broad) if necessary. Chemical shift are reported in parts per million (ppm) and coupling constants *J* are given in Hz when appropriate.

No.	Daunorubicinone		Daunorubicin		P_1		P_2	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1	7.72–7.58 (m, 1H)	119.48	7.64 (dd, *J* = 8.1, 1.6 Hz, 1H)	120.20	7.65 (d, *J* = 7.6 Hz, 1H)	119.5	7.67 (p, *J* = 3.8 Hz, 1H)	120.29
2	7.91 (d, *J* = 5.3 Hz, 2H)	137.89	7.90–7.86 (m, 1H)	136.74	7.96–7.84 (m, 2H)	136.73	7.93 (d, *J* = 4.7 Hz, 2H)	136.72
3		118.57		119.47		118.60		119.53
4		161.28		161.26		161.24		161.28
4-OCH_3	4.00 (s, 3H)	63.54	3.98 (s, 3H)	57.07	3.99 (s, 4H)	57.07	4.00 (s, 3H)	57.10
4a		120.49		120.40		120.46		120.53
5		186.98		186.98		187.10		187.20
5a		111.26		111.15		111.48		111.61
6-OH	13.97 (s, 1H)	155.25	14.01 (s, 1H)	154.93	13.99 (s, 1H)	156.31	13.98 (s, 1H)	154.66
6a		135.2		135.82		135.70		135.29
7	5.33 (d, J = 7.3 Hz, 1H)	72.95	4.92 (dd, *J* = 5.6, 3.3 Hz, 1H)	70.47	4.94 (t, *J* = 4.9 Hz, 1H)	70.61	5.31 (dd, *J* = 5.0, 3.2 Hz, 1H)	70.55
7-OH	6.11 (s, 1H)							
8-Ha	2.17 (dt, *J* = 14.0, 2.3 Hz, 1H)	36.4	2.17 (ddd, *J* = 14.4, 3.1, 1.4 Hz, 1H)	36.37	2.46–2.38 (m, 1H)	36.67	2.43–2.35 (m, 1H)	36.91
8-Hb	2.02–1.92 (m, 1H)	76.81	2.09 (dd, *J* = 14.3, 5.8 Hz, 1H)	75.46	2.14 (dd, *J* = 14.7, 5.1 Hz, 1H)	76.29	2.18 (dd, *J* = 14.6, 5.1 Hz, 1H)	88.08
9-OH	5.06 (s, 1H),		7.90 (s, 1H)		7.96–7.84 (m, 1H)			
9-CO-CH_3	2.31 (s, 3H)		2.28 (s, 3H)		2.29 (s, 3H)		2.28 (s, 3H)	
10-Ha	3.05–2.95 (m, 1H)	32.55	2.99–2.84 (m, 2H)	31.98	3.02–2.94 (m, 2H)	32.53	3.09–2.97 (m, 3H)	32.51
10-Hb	2.89 (d, *J* = 18.2 Hz, 1H)			135.03		135.16		135.20
10a		134.02						
11-OH	13.27 (s, 1H)	156.27	13.22 (s, 1H)	156.53	13.21 (s, 1H)	158.27	13.22 (s, 1H)	156.28
11a		111.03		111.08		111.33		111.36
12		186.88		186.89		186.84		186.92
12a		136.7		135.16		135.26		135.80
13		212.33		212.36		212.73		212.65
14		24.90		24.63		24.94		24.91
5′-ChH_3			1.17 (d, *J* = 6.5 Hz, 3H)	17.19				
1′			5.29 (dd, *J* = 3.8, 1.5 Hz, 1H)	99.64	5.25 (d, *J* = 3.9 Hz, 1H)	103.82	5.00 (d, *J* = 5.0 Hz, 1H)	104.48
1″							4.98 (d, *J* = 5.4 Hz, 1H)	102.89
2′a			1.89 (td, *J* = 12.8, 3.8 Hz, 1H)	28.66	4.63–4.46 (m, 3H); 3.75 (ddd,	74.21	5.33 (s, 1H), 4.93 (d, J = 3.5 Hz, 1H);	74.28
2′b			1.69 (dd, *J* = 12.6, 4.3 Hz, 1H)		*J* = 11.8, 5.4, 2.2 Hz, 1H); 3.50 (dt,		4.89 (d, *J* = 4.0 Hz, 1H); 4.68–4.53	
3′			5.47 (d, *J* = 6.1 Hz, 1H)	47.04	*J* = 11.8, 6.0 Hz, 1H); 3.24–3.19 (m,	77.08	(m, 4H); 4.32 (d, *J* = 7.9 Hz, 1H); 3.75	76.58
4′			3.60 (dd, *J* = 6.3, 2.5 Hz, 1H)	66.60	1H); 3.17 (dd, *J* = 8.9, 5.1 Hz, 1H);	69.24	(ddd, *J* = 11.9, 5.3, 1.9 Hz, 1H); 3.69	68.96
5′			4.21 (dt, *J* = 7.4, 6.0 Hz, 1H)	66.54	3.09 (td, *J* = 9.3, 5.3 Hz, 1H); 2.92 (t,	77.52	(ddd, *J* = 11.7, 5.4, 2.2 Hz, 1H); 3.53	77.37
6′					J = 17.5 Hz, 1H)	61.60	(dt, *J* = 11.6, 5.8 Hz, 1H); 3.45–3.34	61.54
2″							(m, 8H); 3.30–3.22 (m, 2H); 3.16	72.88
3″							(dtd, *J* = 11.6, 8.7, 4.4 Hz, 3H).	76.32
4″								68.84
5″								77.02
6″								61.28

reported previously [26,38]. The results indicate that the glucosylation of daunorubicinone aglycone enhanced water solubility by the attachment of bulky hydrophilic glucose moiety.

The study of thermal and pH stability of novel glucosylated derivatives of daunorubicinone was carried out at various pH (4.5–12) and temperatures (40, 50, 60, 70, 80, 90, and 100 °C). The results show that daunorubicinone 7-*O*-α-D-glucoside exhibited the highest stability in the pH range 4.5–6.5, whereas daunorubicinone 7,9-di-*O*-α-D-glucoside was most stable in the pH range 4.5–8.5 (Fig. 4C). Similarly, daunorubicin is unstable at pH > 8.5, whereas daunorubicinone is stable in the pH range 6.5–7.5. The results indicate that glucosylation of daunorubicinone increased the pH stability range in comparison to daunorubicinone and daunorubincin. Similarly, the results also show that the two glucosylated daunorubicinone derivatives were more stable at higher temperatures than their parent compound. Daunorubicinone and daunorubicin showed stability in the temperature up to 70 and 90 °C respectively; however, daunorubicinone 7-*O*-α-D-glucoside and daunorubicinone 7,9-di-*O*-α-D-glucoside showed thermal stability up to 100 °C (Fig. 4D). These results suggest that the daunorubicinone glucosides are relatively stable at high-temperature conditions.

3.7. Anticancer activities of compounds

To evaluate the pharmaceutical potential of glucosylated daunorubicinone as a chemotherapeutic agent for cancer treatment, daunorubicin, daunorubicinone standard, and two glucosylated daunorubicinone derivatives (daunorubicinone 7-*O*-α-D-glucoside and daunorubicinone 7,9-di-*O*-α-D-glucoside) were assayed against various cancer cell lines using the MTT colorimetric assay. Daunorubicin and daunorubicinone showed anticancer activities against various cell lines at the concentrations of 0.625 and 10 μM, respectively (Fig. 5). This is the first report of the activities of daunorubicinone against metastatic melanoma cell as B16F10, AGS, Hela, and HepG2. However, two glucosylated daunorubicinone derivatives did not exhibit inhibitory activities. The results confirm that daunorubicinone 7-*O*-α-D-glucoside and daunorubicinone 7,9-*O*-α-D-diglucoside lost anticancer properties of daunorubicinone upon glucosylation. A reason could be the absence of the amino group at C-3′ position of the sugar moiety of the glycosylated daunorubicinone, which showed the important role for drug DNA binding affinity and antitumor activity of anthracyclines [39]. Moreover, in the case of novobiocin, the glucopyranoside was less toxic than the parent compound as their antibacterial activity [40]. In another instance, quercetin aglycone is more potent than the glucoside [41]. Thus, generating the new derivatives provides avenues for further discourse on the biological activities and other pharmacokinetic or pharmacodynamic property of daunorubicinone.

4. Conclusions

In this study, the glucosylated derivatives of daunorubicinone were synthesized by one-pot enzymatic glycosylation. Daunorubicinone 7-*O*-α-D-glucoside and daunorubicinone 7,9-di-*O*-α-D-glucoside were obtained at 66.96% and 17.14% conversion at 6 h after addition of 12 mM of the substrate in 5 mL volume reaction. The conjugation of the glucose moiety to daunorubicinone aglycone increases the apparent water solubility and thermal and pH stability. The enzymatic system presented here provide a useful method for glycodiversification of various natural products by using inexpensive starting material, thus lowering the cost of the production of target natural products glycosides. We believe that this method is a promising approach for the production of important glycosylated compounds using inexpensive materials.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

This work was carried out the support of "Leaders in INdustry-university Cooperation" Project, supported by the Ministry of Education (MOE).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2015.11.020.

References

[1] A. Rabbani, R.M. Finn, J. Ausió Bioessays 27 (2005) 50–56.
[2] J.M. Lubieniecka, J. Graham, D. Heffner, R. Mottus, R. Reid, D. Hogge, T.A. Grigliatti, W.K. Riggs, Front. Genet. 4 (2013) 231.
[3] F. Zunino, G. Capranico, Anticancer Drug Des. 5 (1990) 307–317.
[4] A.R. Han, J.W. Park, M.K. Lee, Y.H. Ban, Y.J. Yoo, E.J. Kim, E. Kim, B.G. Kim, J.K. Sohng, Y.J. Yoon, Appl. Environ. Microbiol. 77 (2011) 4912–4923.
[5] D.T. Harrison, L.A. Sanders, Ann. Intern. Med. 85 (1976) 339–341.
[6] J.J. Reilly, J.P. Neifeld, S.A. Rosenberg, Cancer 40 (1977) 2053–2056.
[7] R.J. Maral, M. Jouanne, Cancer Treat. Rep. 65 (1981) 9–18.
[8] P.K. Singal, T. Li, D. Kumar, I. Danelisen, N. Iliskovic, Mol. Cell. Biochem. 207 (2000) 77–86.
[9] T. Tsuruo, H. Iida, S. Tsukagoshi, Y. Sakurai, Cancer Res. 42 (1982) 4730–4733.
[10] J. Czepas, K. Gwoździński, Biomed. Pharmacother. 68 (2014) 1149–1159.
[11] G. Minotti, P. Menna, E. Salvatorelli, G. Cairo, L. Gianni, Pharmacol. Rev. 56 (2004) 185–229.
[12] F. Zunino, G. Pratesi, P. Perego, Biochem. Pharmacol. 61 (2001) 933–938.
[13] D.W. Henry, Cancer Treat. Rep. 63 (1979) 845–854.
[14] N.R. Bachur, M. Steele, W.D. Meriwether, R.C. Hildebrand, J. Med. Chem. 19 (1976) 651–654.
[15] G. Capranico, R. Supino, M. Binaschi, L. Capolongo, M. Grandi, A. Suarato, F. Zunino, Mol. Pharmacol. 455 (1994) 908–915.
[16] V. Prikrylová, H. Lipavská, J.V. Jizba, J. Fuska, M. Podojil, Z. Vaněk, J. Antibiot. (Tokyo) 38 (1985) 1714–1718.
[17] Y.S. Rho, S.Y. Kim, W.J. Kim, Y.K. Yun, H.S. Sin, D.J. Yoo, Synth. Commun. 34 (2004) 3497–3511.
[18] N. Aligiannis, N. Pouli, P. Marakos, A.L. Skaltsounis, H. Pratsinis, Bioorg. Med. Chem. Lett. 12 (2002) 3505–3507.
[19] F.D. Bruyn, J. Maertens, J. Beauprez, W. Soetaert, M.D. Mey, Biotechnol. Adv. 33 (2015) 288–302.
[20] F.D. Bruyn, B.D. Paepe, J. Maertens, J. Beauprez, P.D. Cocker, S. Mincke, C. Stevens, M.D. Mey, Biotechnol. Bioeng. 112 (2015) 1594–1603.
[21] R.B. Gurung, E.H. Kim, T.J. Oh, J.K. Sohng, Mol. Cells 36 (2013) 355–361.
[22] R.P. Pandey, S. Malla, D. Simkhada, B.G. Kim, J.K. Sohng, Appl. Microbiol. Biotechnol. 97 (2013) 1889–1901.
[23] R.P. Pandey, T.F. Li, E.H. Kim, T. Yamaguchi, Y.I. Park, J.S. Kim, J.K. Sohng, Appl. Environ. Microbiol. 79 (2013) 3516–3521.
[24] R.P. Pandey, P. Parajuli, N. Koirala, J.W. Park, J.K. Sohng, Appl. Environ. Microbiol. 79 (2013) 6833–6888.
[25] P. Parajuli, R.P. Pandey, N. Koirala, Y.J. Yoon, B.G. Kim, J.K. Sohng, AMB Express 4 (2014) 31.
[26] N. Koirala, R.P. Pandey, D.V. Thang, H.J. Jung, J.K. Sohng, J. Ind. Microbiol. Biotechnol. 41 (2014) 1647–1658.
[27] S. Malla, R.P. Pandey, B.G. Kim, J.K. Sohng, Biotechnol. Bioeng. 110 (2013) 2525–2535.
[28] R.P. Pandey, P. Parajuli, N. Koirala, J.H. Lee, Y.I. Park, J.K. Sohng, Mol. Cells 37 (2014) 172–177.
[29] G.P. Ghimire, N. Koirala, R.P. Pandey, H.J. Jung, J.K. Sohng, World J. Microbiol. Biotechnol. 31 (2015) 611–619.
[30] S. Masada, Y. Kawase, M. Nagatoshi, Y. Oguchi, Y.K. Terasaka, H. Mizukami, FEBS Lett. 581 (2007) 2562–2566.
[31] K. Terasaka, Y. Mizutani, A. Nagatsu, H. Mizukami, FEBS Lett. 586 (2012) 4344–4350.
[32] T.T. Le, R.P. Pandey, R.B. Gurung, D. Dhakal, J.K. Sohng, Appl. Microbiol. Biotechnol. 98 (2014) 8527–8538.
[33] H.C. Lee, S.D. Lee, J.K. Sohng, K. Liou, J. Biochem. Mol. Biol. 37 (2004) 503–506.
[34] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[35] A.F. Taboada, I.D.L. Mata, M. Arroyo, J.F. Lucas, Appl. Microbiol. Biotechnol. 97 (2013) 3773–3785.

[36] J.W. Phillip, C.J. Thibodeaux, H.W. Liu, Methods Enzymol. 459 (2009) 521–544.
[37] V. Kren, T. Rezanka, FEMS Microbiol. Rev. 32 (2008) 858–889.
[38] C.Z. Wu, J.H. Jang, M. Woo, J.S. Ahn, J.S. Kim, Y.S. Hong, Appl. Environ. Microbiol. 4 (2012) 7680–7686.
[39] C. Temperini, L. Messori, P. Orioli, C.D. Bugno, F. Animati, G. Ughetto, Nucleic Acids Res. 31 (2003) 1464–1469.
[40] L. Heide, Biotechnol. Adv. 27 (2009) 1006–1014.
[41] G. Williamson, G.W. Plumb, Y. Uda, K.R. Price, M.J.C. Rhodes, Carcinogenesis 17 (1996) 2385–2387.