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NOTE

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Production of a new triterpenoid disaccharide saponin from sequential glycosylation of ganoderic acid A by 2 *Bacillus* glycosyltransferases

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ABSTRACT

Ganoderic acid A (GAA) is a lanostane-type triterpenoid, isolated from medicinal fungus *Ganoderma lucidum*, and possesses multiple bioactivities. In the present study, GAA was sequentially biotransformed by 2 recently discovered *Bacillus* glycosyltransferases (GT), BtGT_16345 and BsGT110, and the final product was purified and identified as a new compound, GAA-15,26-O-β-diglucoside, which showed 1024-fold aqueous solubility than GAA.

Keywords: ganoderic acid A, glycosyltransferase, triterpenoid, glucosides, saponin

Triterpenoids are composed of 6 isoprenoid subunits and wildly exist in nature (Muffler *et al.* 2011). Many triterpenoids possess potent bioactivities and the bioactive triterpenoids usually comprise penta- or tetracyclic structures. In plants, many triterpenoids are naturally modified with sugar linkages to form triterpenoid saponins, which contain more diversified bioactivities. For example, triterpenoids isolated from Ginseng are almost present as saponin forms, which possess more bioactivities than their aglycon forms (Shi *et al.* 2019). In addition, some studies have also demonstrated that different number and different position of sugar linkages would greatly affect the bioactivities of the saponins (Shi *et al.* 2019). Thus, finding new triterpenoid saponins becomes an attractive issue in the field of new drug discovery.

Ganoderma lucidum is a medicinal fungus with many pharmacological functions and *Ganoderma* triterpenoids are the key constituents performing these bioactivities (Yang *et al.* 2019). Ganoderic acid A (GAA) is the first identified *Ganoderma* triterpenoid with multiple bioactivities validated, and the first bioactive ganoderic acid undergoing phase I trial (Liang *et al.* 2019). Although several hundreds of triterpenoids have been isolated and identified from *Ganoderma* fungus, there is no natural *Ganoderma* triterpenoid saponins found yet (Xia *et al.* 2014; Yang *et al.* 2019). Therefore, modification (glycosylation) of natural *Ganoderma* triterpenoids to triterpenoid saponins is a promising strategy both to create new compounds and to expand the bioactivities of *Ganoderma* triterpenoid.

In contrast to chemical modification, enzymatic modification (biotransformation) has more advantages, such as mild reaction conditions, high regio/stereoselection, and less side reactions (Muffler *et al.* 2011; Sultana and Saify 2013). Especially, specific glycosylation cannot be chemically conducted in a

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single step due to the multiple hydroxyl groups in both the sugar donor and acceptor molecules. Thus, regioselective glycosylation of molecules is usually achieved through biotransformation by whole cells or purified enzymes (Hofer 2016). In nature, glycosylation is usually catalyzed by glycosyltransferase (GT), which transfers the sugar moiety of a donor molecule, such as uridine-diphosphate glucose (UDP-G), to an acceptor molecule, including large molecules like proteins or polysaccharides and small molecules like flavonoids or triterpenoids (Tiwari *et al.* 2016). According to a carbohydrate-activating enzyme (CAZy) database, GTs are classified into 107 families, where GTs catalyzes small molecules are classified into GT1 family (Cantarel *et al.* 2009). Although over 500 000 GTs have been discovered, only 7 bacterial GTs are identified to possess glycosylation activity towards triterpenoids (Chang *et al.* 2020, in press). Among the 7 triterpenoid-catalyzing GTs, 2 GTs, BtGT_16345 from *Bacillus thuringiensis* GA A07 (Chang *et al.* 2019) and BsGT110 from *Bacillus subtilis* American Type Culture Collection 6633 (Chang *et al.* 2019), are unique that they share low homology in amino acid sequences with the others (Chang *et al.* 2020, in press). In addition, BtGT_16345 is the only GT28 with triterpenoid-catalyzing activity, while the others belonged to GT1. Most triterpenoid-catalyzing GTs catalyze glycosylation at multiple positions toward triterpenoids, while BtGT_16345 regioselectively catalyzes C-15 hydroxyl glycosylation toward GAA (Chang *et al.* 2019). On the other hand, most GT1 has optimal reaction rate under neutral or alkaline conditions, while BsGT110 is an unique GT that catalyzes the glycosylation of GAA at the C-26 carboxyl position under acidic conditions, but loses most of this activity under alkaline ones (Chang *et al.* 2019). Taken the above together, it is highly plausible to create a new *Ganoderma* triterpenoid saponins through sequential biotransformation of GAA using the 2 recently discovered *Bacillus* GTs.

Our previous studies showed that BtGT_16345 had 3-fold higher catalytic efficiency (k_{cat}/k_M) than that of BsGT110 (Chang *et al.* 2019; Chang *et al.* 2019). Moreover, the equilibrium constant (K_{eq}) for the glycosylation of hydroxyl group of a sugar acceptor ($K_{eq} > 10$) is much higher than that of carboxyl group ($K_{eq} < 1$) when using UDP-G as a sugar donor (Mestrom *et al.* 2019). Therefore, to obtain the maximal conversion of GAA at the first step, BtGT_16345 was used as the first enzyme to conduct the biotransformation of GAA. Twenty μg/mL of BtBT_16345 obtained from the recombinant *Escherichia coli* BL-21 (DE3) in our previous study (Chang *et al.* 2019) was added into 1 mg/mL of GAA obtained from Baoji Herbest Bio-Tech (Xi-An, Shaanxi, China) in the presence of 10 mM of phosphate buffer (PB) pH 7.4, 10 mM of $MgCl_2$, and 10 mM of UDP-G purchased from Cayman Chemical (Ann Arbor, MI, USA). The reaction was conducted at 30 °C for 1 h. After reaction, samples from the reaction mixture were taken out and analyzed with an ultra-performance liquid chromatography (UPLC), where the stationary phase was a C18 column (Acquity UPLC BEH C18, 1.7 μm, 2.1 i.d. × 100 mm, Waters, MA, USA), and the mobile phase was 1% acetic acid in water (A) and methanol (B); the linear gradient elution condition was 0 min with 36% B to 7 min with 81% B at a flow rate of 0.2 mL/min; the detection condition was set at 254 nm (Chang *et al.* 2019). The results showed that almost all GAA (retention time; $t_R = 9.07$ in Figure 1a) was converted to GAA-15-*O*-β-glucoside ($t_R = 7.26$ in Figure 1b) at the tested condition and this result was consistent with our previous study (Chang *et al.* 2019). Then, to generate a novel GAA saponin, BsGT110 obtained from the recombinant *E. coli* BL-21 (DE3) in the previous study (Chang *et al.* 2019) was used as a second biocatalyst to perform the biotransformation. Equal volume of 50 mM PB, pH 6.0, containing

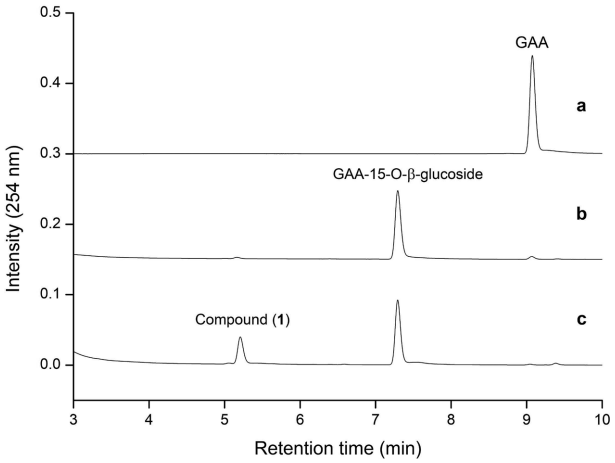

Figure 1. Ultra-performance liquid chromatography (UPLC) analysis of the biotransformation product of GAA by 2 *Bacillus* glycosyltransferases (GT), BtGT_16345 and BsGT110. (a) GAA only. (b) Biotransformation product of GAA by 20 μg/mL of BtGT_16345 at 30 °C for 1 h in the presence of 10 mM of phosphate buffer (PB) pH 7.4; 1 mg/mL of GAA; and 10 mM of uridine-diphosphate glucose (UDP-G). (c) Biotransformation product of the reaction product in (b) by 20 μg/mL of BsGT110 at 40 °C for 1 h in the presence of 50 mM of PB pH 6.0; and another 10 mM of UDP-G.

20 μg/mL of BsGT110 and 10 mM of UDP-G was added into the reaction mixture from the first biotransformation conducted by BtGT_16 345, and the reaction mixture was incubated at 40 °C for another 1 h. At the end of the reaction, the reaction mixture was analyzed by UPLC. The result showed that, in addition to GAA-15-*O*-β-glucoside, a new peak, compound (**1**), appeared at RT of 5.15 min (Figure 1c). The yield of compound (**1**) in the reaction was 27%, which was similar with that in the biotransformation of GAA by BsGT110 (Chang *et al.* 2019).

To resolve the chemical structure of compound (**1**), the biotransformation was scaled-up to 25 mL, where 25 mg of GAA was used. After sequential biotransformation by the 2 GTs, the reaction mixture was mixed with equal volume of methanol and applied into a preparative high-performance liquid chromatography (HPLC) system. The preparative HPLC was equipped with an Inertsil ODS 3 column (10 μm, 20 i.d. × 250 mm, GL Sciences, Eindhoven, The Netherlands), and the mobile phase was the same as that in the UPLC system, but with a flow rate of 15 mL/min (Chang *et al.* 2019). From the 25 mL of reaction, 9.6 mg of the product was purified. The chemical structure of the purified compound (**1**) was resolved with mass and nuclear magnetic resonance (NMR) spectral analysis. The mass analysis of the compound showed an $[M\text{-}H]^-$ ion peak at *m/z*: 839.2 in the electrospray ionization mass (ESI-MS) spectrum corresponding to the molecular formula $C_{42}H_{63}O_{17}$. Then ^1H and ^{13}C NMR spectra, including distortionless enhancement by polarization transfer (DEPT), heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond connectivity (HMBC), correlation spectroscopy (COSY), and nuclear Overhauser effect spectroscopy (NOESY) spectra, were obtained, and the ^1H- and ^{13}C-NMR signal assignments were conducted accordingly (Figures S1-S7). In addition to the signals of GAA, 14 proton signals (from 3.05 to 5.31 ppm) and 12 carbon signals (from 60.5 to 103.6 ppm) indicated the presence of 2 glucose moieties. The attachment of glucose moiety to C-15 of GAA was confirmed by the HMBC correlation from the anomeric proton of Glc H-1′ (δ_H 4.24, d, $J = 7.9$ Hz) to C-15 (δ_C 81.0) and from H-15 (δ_H 4.82, dd, $J = 9.3, 6.3$ Hz) to Glc C-1′ (δ_C 103.6), and the H-H NOESY correlation between Glc H-1′ and H-15. The attachment of glucose

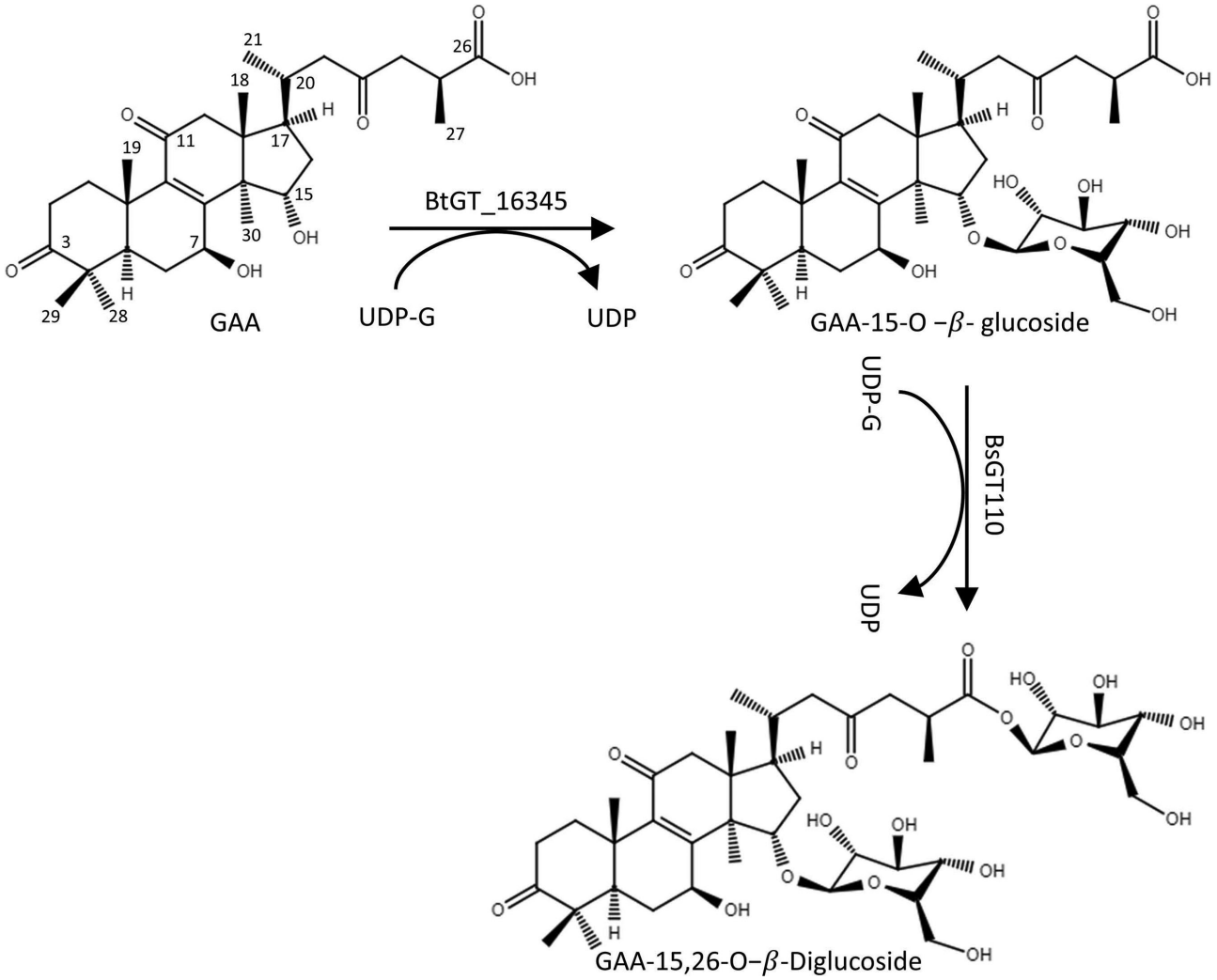

Figure 2. Sequential biotransformation process of GAA by BtGT_16345 and BsGT110.

moiety to C-26 of GAA was confirmed by the HMBC correlation from the anomeric proton of Glc H-1″ (δ_H 5.27, d, J = 8.2 Hz) to C-26 (δ_C 173.9). The observation of the large coupling constants (J = 7.9-8.2 Hz) of the anomeric protons confirmed that the 2 glycosidic bonds of compound (**1**) were in the β-configuration, which was consistent with the inverting mechanism of GTs. Thus compound (**1**) was demonstrated to be GAA-15,26-O-β-diglucoside. The key HMBC and NOESY correlations of compound (**1**) are shown in Figure S8, and the spectroscopic data are listed in Table S1. Figure 2 illustrates the sequential biotransformation process of GAA by the 2 *Bacillus* GTs.

Previous studies have proven that aqueous solubility of flavonoids can be improved through glycosylation (Huang *et al.* 2016), which would advance the oral bioavailability of the original molecules (Zhao *et al.* 2019; Fu *et al.* 2019). Thus, the aqueous solubility of GAA and its glycosylated derivatives, GAA-15-O-β-glucoside and GAA-15,26-O-β-diglucoside, were determined with the methods we used in a previous study (Chang *et al.* 2020, in press). In brief, the tested compounds were vortexed in distilled deionized (d.d.) H_2O for 1 h at 25 °C and then centrifuged at 14 000 × *g* and filtrated through a 0.2 μm nylon membrane. The filtrate was mixed with equal volume of methanol and analyzed with UPLC. The concentrations of the tested compounds were calculated based on their peak areas using calibration curves prepared with UPLC analyses of authentic samples. The results showed that the aqueous solubility of GAA-15,26-O-β-diglucoside was the highest in all tested compounds with 1024.4-fold of GAA, while the aqueous solubility of GAA-15-O-β-glucoside was 59.9-fold of GAA (Table 1).

Table 1. Aqueous solubility of GAA and its derivatives at 25°C

Compound	Aqueous solubility (mg/L)[a]	Fold[b]
GAA	15.37 ± 0.36	1
GAA-15-O-β-glucoside	920.17 ± 44.97	59.9
GAA-15,26-O-β-diglucoside	15 745.36 ± 1270.39	1024.4

[a]The mean solubility ± standard deviations was calculated from results of 3 independent experiments.
[b]The fold of aqueous solubility of GAA glucoside derivatives is expressed as relative to that of GAA, normalized to 1.

In addition to improving aqueous solubility, glycosylation might also improve the bioactivities of the original molecules. For example, quercetin glycoside exerts the antimicrobial effect via inhibition of DNA gyrase and topoisomerase IV, while its aglycone by targeting D-alanine: D-alanine ligase (Azizah *et al.*

2020). Ginseng saponins have been demonstrated to possess more diversified bioactivities, involving the central nervous system, cardiovascular system, immune system, anticarcinogenicity, and diabetes mellitus, than ginseng triterpenoid aglycones (Shi *et al.* 2019). Although no *Ganoderma* triterpenoid saponin discovered in nature, the results of the present study and the previous ones highlight a promising strategy to create multiple, complex, and more bioactive *Ganoderma* triterpenoid saponins. In particular, the sequential biotransformation using 2 recently discovered *Bacillus* GTs, BtGT_16345 and BsGT110, warrants the creation of new triterpenoid saponins with diverse bioactivity from the hundreds of natural *Ganoderma* triterpenoids.

Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Author contribution

T.-S.C., J.-Y.W., C.-M.C., and H.-J.T. designed the study. Y.-L.T. performed all experiments. T.-S.C. wrote the initial draft of the manuscript and J.-Y.W., C.-M.C., and H.-J.T. revised the manuscript. All authors contributed to analysis and interpretation of the data.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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