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# Enzymatic Synthesis of β-Glucosylglycerol and Its Unnatural Glycosides Via β-Glycosidase and Amylosucrase S

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β-Glucosylglycerol (β-GG) and their derivatives have potential applications in food, cosmetics and the healthcare industry, including antitumor medications. In this study, β-GG and its unnatural glycosides were synthesized through the transglycosylation of two enzymes, *Sulfolobus shibatae* β-glycosidase (SSG) and *Deinococcus geothermalis* amylosucrase (DGAS). SSG catalyzed a transglycosylation reaction with glycerol as an acceptor and cellobiose as a donor to produce 56% of β-GGs [β-D-glucopyranosyl-(1→1/3)-D-glycerol and β-D-glucopyranosyl-(1→2)-D-glycerol]. In the second transglycosylation reaction, β-D-glucopyranosyl-(1 → 1/3)-D-glycerol was used as acceptor molecules of the DGAS reaction. As a result, 61% of α-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1/3)-D-glycerol and 28% of α-D-maltopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1/3)-D-glycerol were synthesized as unnatural glucosylglycerols. In conclusion, the combined enzymatic synthesis of the unnatural glycosides of β-GG was established. The synthesis of these unnatural glycosides may provide an opportunity to discover new applications in the biotechnological industry.

**Keywords:** Glycerol glycosides, β-glycosidase, amylosucrase, transglycosylation

## Introduction

β-Glucosylglycerol (β-GG) is found in some higher species of plants [1–3] and is also known as the structural unit of cell membrane components (non-bilayer glycolipids) in microorganisms, algae, plants, and animal tissues [4]. β-GG can exist in two stereochemical forms, β-D-glucopyranosyl-(1 → 1/3)-D-glycerol [two products of β-D-glucopyranosyl-(1→1)-D-glycerol and β-D-glucopyranosyl-(1→3)-D-glycerol] and β-D-glucopyranosyl-(1→2)-D-glycerol [5]. The free hydroxyl groups positioned in the glycerol of β-GG typically are in esterified forms with one or two fatty acid molecules [6]. β-GG and their derivatives could be used in food, cosmetics, and in the healthcare industry as biodegradable and food-grade, non-ionic surfactants and moisturizing agents [5, 7].

In addition, β-GG has been shown to exhibit inhibitory effects on Epstein-Barr virus activation. Colombo *et al.* (1996) assessed a series of glycosylglycerols without fatty acyl residues in glycerol subunits to ascertain the structural features responsible for the cancer chemopreventing activity. Their study discovered several bioactive glycosylglycerols including β-GG which exhibited a strong inhibitory effect on Epstein-Barr virus activation [5]. Colombo *et al*. (1998) also screened for cancer chemopreventing agents and found several glycosylglycerols to be active. Anti-tumor promoting effects were optimized by controlling the acyl chain length of β-GG [6]. Several studies of the anti-tumor promoting activity of glycosylglycerols obtained from natural sources have been reported. Several monoglycosyl- and diglycosyl glycerols obtained from the cyanobacterium *Phormidium tenue* from *Citrus hystrix* and *Chlorella vulgaris* exhibited anti-tumor promoting activity [8–11]. These results indicated that additional sugars can be attached to

the glucosyl moiety of glycosylglycerol to diversify their biological activities.

Recently, even though oligosaccharides and glycoconjugates are synthesized through a rather complex enzymatic biosynthetic pathway in which several specific glycosyltansferases are involved in biological systems, the demand for oligosaccharides and the synthesis of glycoconjugates has risen since they are used as probes for biological research and as lead compounds for drug and vaccine discovery [12]. Glycoconjugates can be synthesized through chemical and biological (enzymatic) methods. The chemical methods are not suitable for use in the food and cosmetic industry due to the use of toxic reagents and solvents and the large number of tedious protection and deprotection steps [13, 14]. Otherwise, enzymatic biosynthesis has several advantages over chemical methods such as its high regioselectivity, mild reaction condition, and non-toxic process [15, 16]. Therefore, an enzymatic synthesis is adequate for the synthesis of glycoconjugates including β-GG.

β-Glycosidase (E.C. 3.2.1.21) has gained wide acceptance in the area of biosynthesis due to its broad substrate specificity and high stereo-selectivity. It normally catalyzes the hydrolysis of β-glycosidic linkage in the substrate while retaining an anomeric configuration through a double-displacement mechanism [17]. In the presence of appropriate nucleophiles other than water, the biosynthesis of glycoconjugates can occur instead of the hydrolysis reaction [17]. β-Glycosidase driven biosynthesis has been employed in the synthesis of a variety of biologically important compounds [18, 19]. In addition, amylosucrase (ASase, E.C. 2.4.1.4) is a versatile enzyme that hydrolyzes sucrose to equal amounts of glucose and fructose [20]. Simultaneously, it can transfer a released glucose to the 4-positions of other glucose molecules to form α-1,4 glucan as well as to other acceptor molecules containing -OH groups to synthesize new glycoconjugates. ASase is particularly useful for the glycosylation of bio-materials due to its ability to utilize sucrose, a relatively inexpensive donor molecule [21–23].

In this study, we described the biosynthesis of natural and unnatural GGs through enzymatic methods using two enzymes – β-glycosidase from *Sulfolobus shibatae* (SSG) and amylosucrase from *Deinococcus geotharmalis* (DGAS). The synthesis of unnatural glycerol glycosides was successfully accomplished through the transfer of additional sugars using DGAS to β-GG synthesized by SSG. The enzymatic biosynthesis of these compounds may broaden the opportunities of novel enzyme-driven biosynthesis applicable to the production of valuable glycoconjugates.

## Materials and Methods

### Chemicals and Enzymes

Cellobiose and glycerol were obtained from Sigma–Aldrich (USA). Sucrose was purchased from Duchefa Biochemistry (Haarlem, Netherlands). Two glycoside hydrolases, α-glucosidase from yeast (OYC Americas, USA) and β-amylase from *Bacillus cereus* [24], were employed to determine the glycosidic bonds of the transglycosylation products. All other chemicals used in this study were of analytical grade and obtained either from Sigma–Aldrich or Junsei Chemical Co. Ltd. (Japan).

### Enzyme Preparation

*Escherichia coli* MC1061 strain harboring the cloned DGAS gene (*dgas*) (*Deinococcus geothermalis* ASase) in the pHCXHD vector (pHC-*dgas*) and *E. coli* BL21(DE3) strain harboring the cloned *ssg* (*Sulfolobus shibatae* β-glycosidase) in the pRSET-B vector (pRB-*ssg*) were utilized for the production of DGAS and SSG, respectively. Purified enzymes were prepared according to the method described in the previous reports [19, 25]. In final, DGAS and SSG were then concentrated to 1 and 5 mg/ml, respectively, in the buffer [100 mM sodium citrate buffer (pH 5) and 50 mM Tris-HCl buffer (pH 7) for SSG and DGAS, respectively] using ultrafiltration (30,000 MW cut-off membrane; Amicon, USA). Protein concentrations were determined by the bicinchoninic acid (BCA) method using a BCA protein assay kit (Thermo Fisher Scientific, USA) with bovine serum albumin as a standard. Enzyme purities were checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis and each enzyme was diluted to an appropriate concentration before being used for transglycosylation.

### Enzyme Assay

DGAS activity was measured by determining hydrolysis activity using the dinitrosalicylic acid (DNS) method. The substrate solution contained a final 150 mM sucrose in 50 mM Tris-HCl buffer (pH 7). After pre-incubating at 45°C for 5 min, the reaction was initiated by adding 10 µl of the enzyme solution to the 90 µl substrate solution and then continued for 10 min. The presence of the reducing sugar in the reaction mixture was determined by adding 300 µl DNS solution, followed by boiling for 5 min. The absorbance of the final reaction solution was measured at 550 nm using a microplate reader (Tecan Infinite M200, USA). Reducing sugar concentrations were calculated using fructose as a standard. One unit of DGAS was defined as the amount of enzyme that produced 1 µmole of fructose per min under assay conditions.

SSG activity was determined using *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPβG) at a 1 mM final concentration in a 30 min assay. Assays were performed in 1 ml of 100 mM sodium citrate buffer (pH 5) containing *p*-NPβG and enzyme solution. After pre-incubation of the substrate solution at 75°C for 5 min, the reaction was initiated with 10 µl of the diluted enzymes solution and continued for 30 min before being terminated by the addition of 1 ml of 100 mM NaOH solution. Absorbance at 410 nm

was measured by a spectrophotometer (Beckman DU 730, Fullerton, CA, USA) and expressed in μmol of *p*-nitrophenol (*p*-NP) using a standard graph prepared under the same conditions. One unit of SSG activity was defined as the amount of enzyme required to release 1 μmol of *p*-NP per min at 75°C under the assay conditions described above.

**Biosynthesis of Glycerol Glycosides by Enzymatic Reactions**

The basic reaction mixture for transglycosylation consisted of 250 mM of cellobiose as a donor, 1 M of glycerol as an acceptor, and 30 unit/ml of SSG in 5 ml of 100 mM sodium citrate buffer (pH 5). Afterwards, the reaction was continued up to 15 h at 75°C and kept in 4°C for termination. To enhance the production yield of β-glucosylglyerol, different concentrations of cellobiose (16, 63, 125, 250, and 500 mM) with 1 M glycerol were tested to obtain the saturation point. The glycerol concentration was then controlled to be between 0.5 to 1.75 M with 250 mM of cellobiose.

To synthesize second glycosylated products, the reaction was performed with 150 mM of β-D-glucopyranosyl-(1→1/3)-D-glycerol as an acceptor molecule and 150, 300, and 450 mM of sucrose as a donor compound in 50 mM Tris-HCl buffer (pH 7). The reaction was initiated by adding DGAS (3 unit/ml) and continued for 15 h at 30°C. The enzyme reaction was stopped by heating in boiling water for 5 min.

**Thin Layer Chromatography (TLC) and High Performance Anion Exchange Chromatography (HPAEC) Analyses**

The detection and identification of products in the reaction were achieved by TLC and HPAEC analyses. TLC analysis was performed with Whatman K5F silica gel plates (Whatman, UK) after activating at 110°C for 30 min. An aliquot (2 μl) of the reaction mixture was loaded onto a plate and developed with a solvent system of isopropyl alcohol-ethyl acetate-water (3:1:1, v/v/v) in a TLC chamber. Ascending development was performed at room temperature. The plate was allowed to dry in a hood and then developed by rapidly soaking in 0.3% (w/v) *N*-(1-naphthyl)-ethylenediamine and 5% (v/v) $H_2SO_4$ in methanol. The plate was dried and placed in an oven for 10 min at 110°C to visualize the reaction spots.

HPAEC analysis was performed to measure the amount of glycerol glycosides. Samples were analyzed using a CarboPac MA1 column (0.4 × 25 cm; Dionex Co., USA) connected to a Thermo Fischer Scientific Dionex ICS-5000 system with an electrochemical detector. Analysis of the glycerol glycosides was achieved with an isocratic buffer (614 mM NaOH) over 60 min.

**Recycling-HPLC for the Separation of Transglycosylated Products**

A recycling preparative HPLC system (LC-9104, JAI, Japan) was used for the isolation of glycerol glycosides. Three milliliters of the sample were applied to a JAIGEL-W252 (2 cm × 50 cm, JAI) column connected in tandem with a JAIGEL-W251 (2 cm × 50 cm, JAI) and guard columns. The sample was eluted with deionized water at a flow rate of 3 ml/min. The results were recorded by an RI detector and the fractions corresponding to the detected peaks were collected and freeze-dried. The purity of each sample was confirmed through TLC and HPAEC analysis.

**Fast Atom Bombardment Mass Spectrometry (FAB-MS) and Nuclear Magnetic Resonance (NMR) Analyses**

Both negative and positive fast atom bombardment mass spectrometry (FAB-MS) were recorded on a JEOL JMS 700 (JEOL, Japan). Twenty milligrams of purified enzyme reaction product was exchanged with $D_2O$ and dissolved in 0.5 ml pure $D_2O$. The prepared sample was then loaded into 5 mm NMR tubes. $^{13}C$ and $^{1}H$ NMR spectra of the purified product were obtained from a Varian Inova AS 400 MHz BMR spectrometer (USA). As an internal chemical shift reference, tetramethylsilane was used by dissolution in $CD_3OD$ at 23°C.

## Results and Discussion

**Biosynthesis of β-Glucosylglycerol Products Using SSG**

β-Glucosylglycerols (β-GG) were enzymatically synthesized from glycerol through the intermolecular transglycosylation activity of SSG. Fig. 1 summarizes the results of the intermolecular bioconversion of 250 mM cellobiose by SSG in the presence of 1 M glycerol. In TLC analysis, a distinct new spot (spot T) appeared in the reaction with glycerol compared to the reaction without glycerol (lane 3). This result revealed that glycerol acted as an acceptor for the

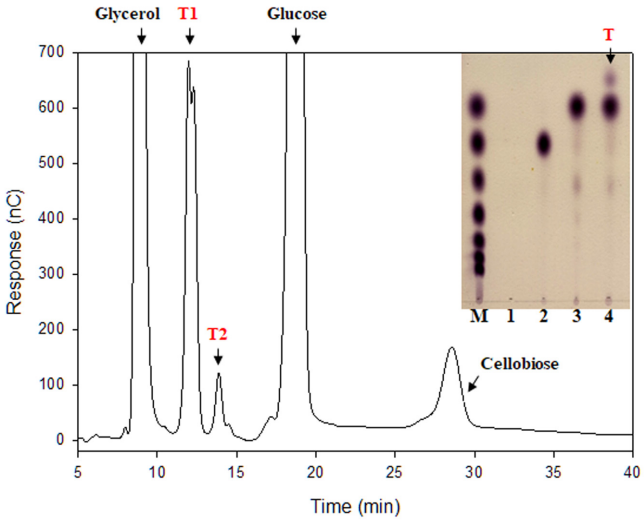


**Fig. 1.** TLC and HPAEC analysis of transglycosylated products synthesized by β-glycosidase from *Sulfolobus shibatae*. In the TLC result, Lane M, standard makers for glucose to maltoheptaose. Lane 1, glycerol, Lane 2, cellobiose, Lane 3, 0.25 M cellobiose reacted with SSG. Lane 4, 0.25 M celloiose and 1 M glycerol mixed with SSG. In the HPAEC result, 0.25 M cellobiose reacted with SSG in the presence of 1 M glycerol.

SSG transglycosylation reaction and converted to the transglycosylation product. Different from TLC analysis, however, two new apparent peaks (T1 and T2) were observed in the SSG transglycosylation reaction in the HPAEC analysis. The compounds in these peaks were obviously different from glycerol, glucose, and cellobiose, implying that they were transglycosylation products of SSG. Previously, de Roode M *et al*. performed the biosynthetic production of glycerol glucoside from glycerol and glucose with almond β-glucosidase [26] and three compounds [β-D-glucopyranosyl-(1→1)-D-glycerol, β-D-glucopyranosyl-(1→3)-D-glycerol, and β-D-glucopyranosyl-(1→2)-D-glycerol] were identified from the products. Among those, β-D-glucopyranosyl-(1→1)-D-glycerol and β-D-glucopyranosyl-(1→3)-D-glycerol were stereoisomer forms and not easily distinguishable in TLC and HPAEC analyses. Therefore, it was assumed that the two new peaks in the HPAEC chromatogram were possibly three β-GG. To determine the structure of T1 and T2, recycling preparative HPLC was performed and the transglycosylation products were successfully separated from each other as shown in Fig. S1. Even in TLC analysis, the two compounds were split into two distinct spots (lanes 3 and 4). The isolated T1 and T2 were matched on the peaks designated as T1 and T2 in HPAEC analysis (12 and 14 min), respectively. These analytical results reinforced that at least two unidentified glycerol transglycosylation products (T1 and T2) were synthesized by SSG. More careful examination revealed that the isolated T1 was detected as a double peak on HPAEC analysis whereas the isolated T2 was observed as a single peak (Fig. 1). Previous HPAEC analysis supported the fact that stereoisomers of any compound could appear as a double peak because they are not completely separated by HPAEC [27, 28]. When glycosyl transfer products were synthesized using sucrose as a donor and glycerol as an acceptor by ASase of *Methylobacillus flagellatus*, two peaks were observed in HPAEC analysis. Among them, one peak was a double peak, and the structural determination of the sample in the double peak revealed the presence of two isomer compounds [α-D-glucopyranosyl-(1→1)-D-glycerol and α-D-glucopyranosyl-(1→3)-D-glycerol]. This result suggested that T1 may be a mixture of stereoisomers of glycerol transglycosylation products [β-D-glucopyranosyl-(1→1)-D-glycerol and β-D-glucopyranosyl-(1→3)-D-glycerol].

**Structural Determination of Transglycosylated Products**

The purified T1 and T2 were analyzed using MASS and NMR spectroscopy. FAB/MS analysis of T1 and T2 revealed that the molecular ion peak was observed at *m/z* 277.2 $(M+Na)^+$ (data not shown), which exactly matched the calculated molecular mass of glucosylglycerol. $^1$H NMR and $^{13}$C NMR results are shown in Table 1. In $^{13}$C and $^1$H NMR analyses, T1 was determined to be the mixture of β-D-glucopyranosyl-(1→1)-D-glycerol and β-D-glucopyranosyl-(1→3)-D-glycerol. This mixture of compounds was designated as βg1,3gly or β-D-glucopyranosyl-(1→1/3)-D-glycerol hereafter. An oxygenated methylene carbon signal (C-1) was observed at $\delta_C$ 72.3/72.4 due to the glycosylation shift, which was observed at $\delta_C$ 62. It confirmed that the transferred glucose molecule was connected to C1 in the glycerol molecule. The carbon signals of the sugar moieties were observed at $\delta_C$ 104.5/104.7, 75.0/75.1, 77.8, 71.5/71.6, 77.9, and 62.6, suggesting the presence of a D-glucosyl group. The bond between glucose and glycerol was determined to be a β-glycosidic linkage according to two coupling constants (*J*=8.0/8.0 Hz) at 4.287/4.294 ppm. $^{13}$C NMR results revealed 14 signals (the signals of C1, C2, C1′, C2′, and C4′ were observed as double peaks), which is more than the 9 glucosylglycerol signals. $^1$H NMR results were shown as 2 signals. These results indicated that T1 was truly a mixture of β-D-glucopyranosyl-(1→1)-D-glycerol and β-D-glucopyranosyl-(1→3)-D-glycerol.

In the $^{13}$C and $^1$H NMR analyses, T2 was determined to be β-D-glucopyranosyl-(1→2)-D-glycerol (βg2gly). The chemical shift of C2 in the glycerol molecule changed significantly from 72 to 83.1. It confirmed that the transferred glucose molecule was connected to C2 in the glycerol molecule. In addition, $^1$H NMR analysis revealed that the glucose molecule was transferred to C2 in the glycerol molecule with a β-anomeric configuration based on the coupling constant (*J*=7.6 Hz) of the glucose anomeric proton signal observed at 4.423 ppm.

**Biosynthesis of the Second Transglycosylated Products Using DGAS**

Glucose was transferred from cellobiose to glycerol and the synthetic reaction was optimized. The concentration of glycerol and cellobiose was controlled to obtain the maximum production of βg1,3gly because a small amount of βg2gly can be negligible (ca 5%). Fig. 2 shows the conversion yield of cellobiose to β-GG when mixed with 30 unit/ml of SSG. The SSG transglycosylation reaction on 1 M glycerol was carried out with six different concentrations of cellobiose (15, 62, 125, 250, 375, and 500 mM). At concentrations lower than 250 mM of cellobiose, the production of β-GG is not saturated. The conversion was decreased in concentrations higher than 250 mM cellobiose,

**Table 1.** $^{13}C$ (100 MHz) and $^{1}H$ (400 MHz) NMR data of glucosylglycerols (unit : ppm, in $CD_3OD$)

| | | $^{13}C$ NMR | | | | | | | $^{1}H$ NMR | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | References Glycerol [29] Glucose [30] | Reference βg2gly [31] | Reference βg1,3gly [26] | βg2gly | βg1,3gly | αgβg1,3gly | αmβg1,3gly | Reference βg2gly [26] | Reference βg1,3gly [26] | βg2gly | βg1,3gly | αgβg1,3gly | αmβg1,3gly |
| Glycerol | 1 | 62.0 | 62.0 | **71.3, 71.6** | 63.3 | **72.3, 72.4** | **72.3, 72.4** | **72.3, 72.4** | | | | | | |
| | 2 | 72.0 | **82.7** | 70.8, 71.0 | **83.1** | 72.1, 71.9 | 72.7, 72.9 | 72.7, 72.9 | | | | | | |
| | 3 | 62.0 | 61.8 | 62.9 | 62.6 | 64.0 | 64.3 | 64.3 | | | | | | |
| β-D-glucopyranose | 1′ | 105.4 | 103.9 | 102.9, 103.1 | 104.3 | 104.5, 104.7 | 104.2, 104.5 | 104.2, 104.5 | 4.45 (d, ***J=7.9 Hz***) | 4.32, 4.33 (d, ***J=7.9, 7.9 Hz***) | 4.423 (d, ***J=7.6 Hz)*** | 4.287, 4.294 (d, ***J=8.0 Hz)*** | 4.369, 4.372 (d, ***J=8.0, 8.4 Hz***) | 4.370, 4.373 (d, ***J=8.0, 8.0 Hz***) |
| | 2′ | 74.8 | 74.5 | 73.5 | 75.2 | 75.0, 75.1 | 74.9, 75.0 | 74.9, 75.0 | | | | | | |
| | 3′ | 78.1 | 77.7 | 76.0 | 78.0 | 77.8 | 78.0 | 78.0 | | | | | | |
| | 4′ | 71.4 | 71.0 | 70.0 | 71.8 | 71.5, 71.6 | **78.7** | **78.7** | | | | | | |
| | 5′ | 78.1 | 77.3 | 76.3 | 77.8 | 77.9 | 76.5 | 76.4 | | | | | | |
| | 6′ | 62.5 | 62.3 | 61.1 | 62.7 | 62.6 | 62.6 | 62.6 | | | | | | |
| α-D-glucopyranose | 1″ | | | | | | 101.5 | 101.4 | | | | | 5.264, 5.274 (d, ***J=4.0, 4.0 Hz)*** | 5.264, 5.274 (d, ***J=2.8, 2.8 Hz)*** |
| | 2″ | | | | | | 73.6 | 73.7 | | | | | | |
| | 3″ | | | | | | 74.8 | 74.8 | | | | | | |
| | 4″ | | | | | | 71.3 | **79.0** | | | | | | |
| | 5″ | | | | | | 74.6 | 74.6 | | | | | | |
| | 6″ | | | | | | 62.4 | 62.4 | | | | | | |
| α-D-glucopyranose | 1‴ | | | | | | | 101.7 | | | | | | 5.256, 5.257 (d, ***J=2.8, 2.8 Hz)*** |
| | 2‴ | | | | | | | 73.1 | | | | | | |
| | 3‴ | | | | | | | 75.2 | | | | | | |
| | 4‴ | | | | | | | 71.3 | | | | | | |
| | 5‴ | | | | | | | 73.4 | | | | | | |
| | 6‴ | | | | | | | 62.4 | | | | | | |

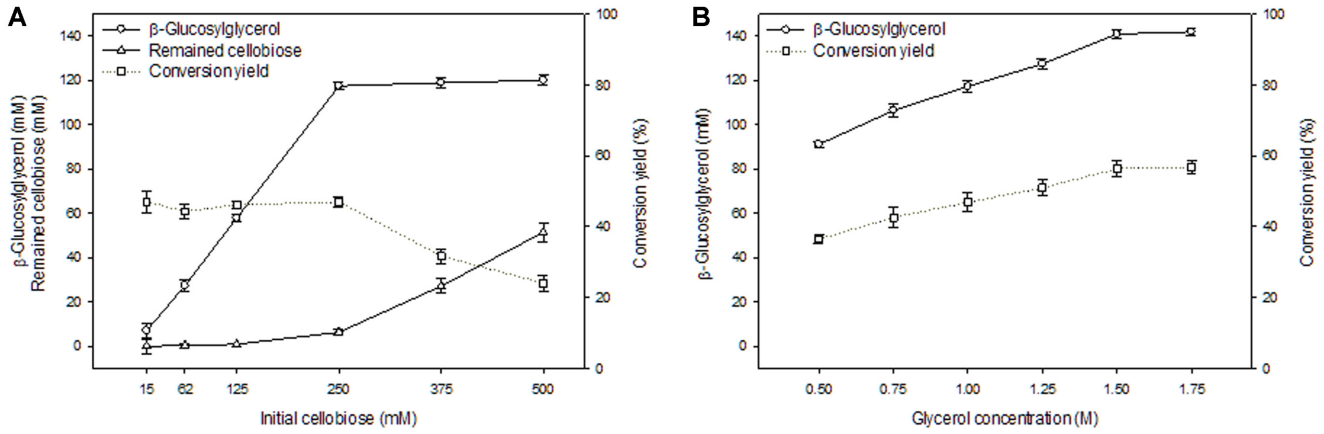


**Fig. 2.** The amount of β-glucosylglycerol synthesized with different molar ratios of cellobiose (donor) and glycerol (acceptor).
The ratios of cellobiose and glycerol in the reactions were (**A**) 15 to 500 mM cellobiose with 1 M glycerol and (**B**) 0.5 to 1.75 M glycerol with 250 mM cellobiose.

and a significant amount of cellobiose still remained (Fig. 2A). This result revealed that more than 250 mM of cellobiose concentration was saturated against enzyme concentrations. Therefore, glycerol concentrations should be controlled. Fig. 2B shows the results of optimum concentrations of glycerol in which 250 mM cellobiose was present as a donor and the concentration of glycerol was varied at six different concentrations (0.5, 0.75, 1, 1.25, 1.50, and 1.75 M). The more glycerol added to produce β-GG, the more increased conversion yield was observed up to 1.5 M.

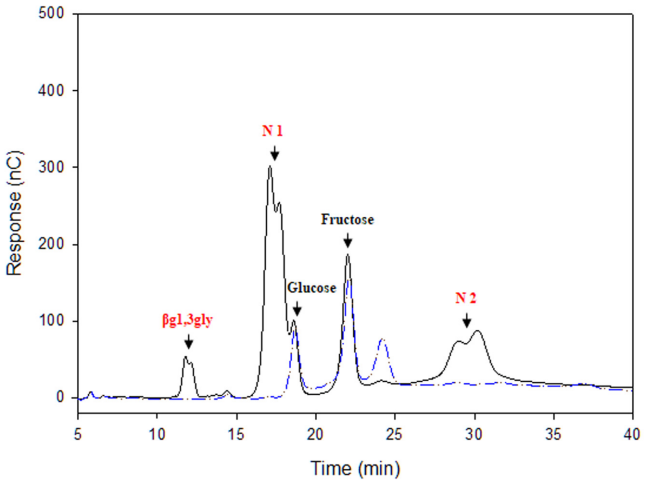


**Fig. 3.** HPAEC analysis of second transglycosylated products synthesized by amylosucrase from *Deinococcus geothermalis*.
The chromatogram represents the reaction products of 300 mM of sucrose and DGAS (black line) in the presence or (blue line) absence of 150 mM of βg1,3gly.

Therefore, 1.5 M of glycerol was suitable for the first transglycosylation. This result suggested that the cellobiose concentration was fixed at 250 mM as a final concentration, whereas the glycerol concentration was kept at 1.5 M in the reaction mixture. Finally, the maximum conversion yield from cellobiose to β-GG was determined to be 56%.

The second transglycosylation was performed to confirm that βg1,3gly could act as an acceptor molecule. DGAS has a high conversion yield and produced fewer intermediate products in the presence of acceptor molecules [21–23]. DGAS was employed to elongate β-GG to its glycosides using sucrose as the sole donor molecule. As a result of HPAEC analysis in Fig. 3, new peaks (N1 and N2) appeared at 17 and 30 min in the reaction of sucrose and β-GG by DGAS, respectively. In addition, N1 and N2 were observed as double peaks. Therefore, the possible structures of the two glycosylated products of N1 and N2 were expected to be α-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1/3)-D-glycerol (αgβg1,3gly) and α-D-maltopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1/3)-D-glycerol (αmβg1,3gly), respectively. When 150 mM of βg1,3gly reacted with 150, 300, and 450 mM of sucrose with 3 unit/ml of DGAS, βg1,3gly was converted to 61% of αgβg1,3gly and 28% of αmβg1,3gly.

## Structural Determination of the Second Transglycosylated Products

To determine structural diversity, purified N1 and N2 were analyzed using enzymatic hydrolysis, MASS, and NMR spectroscopy. The FAB/MS analysis of N1 and N2 revealed that the molecular ion peak was observed at *m/z* 439.0 and 601.0 $(M+Na)^+$ (data not shown), which exactly

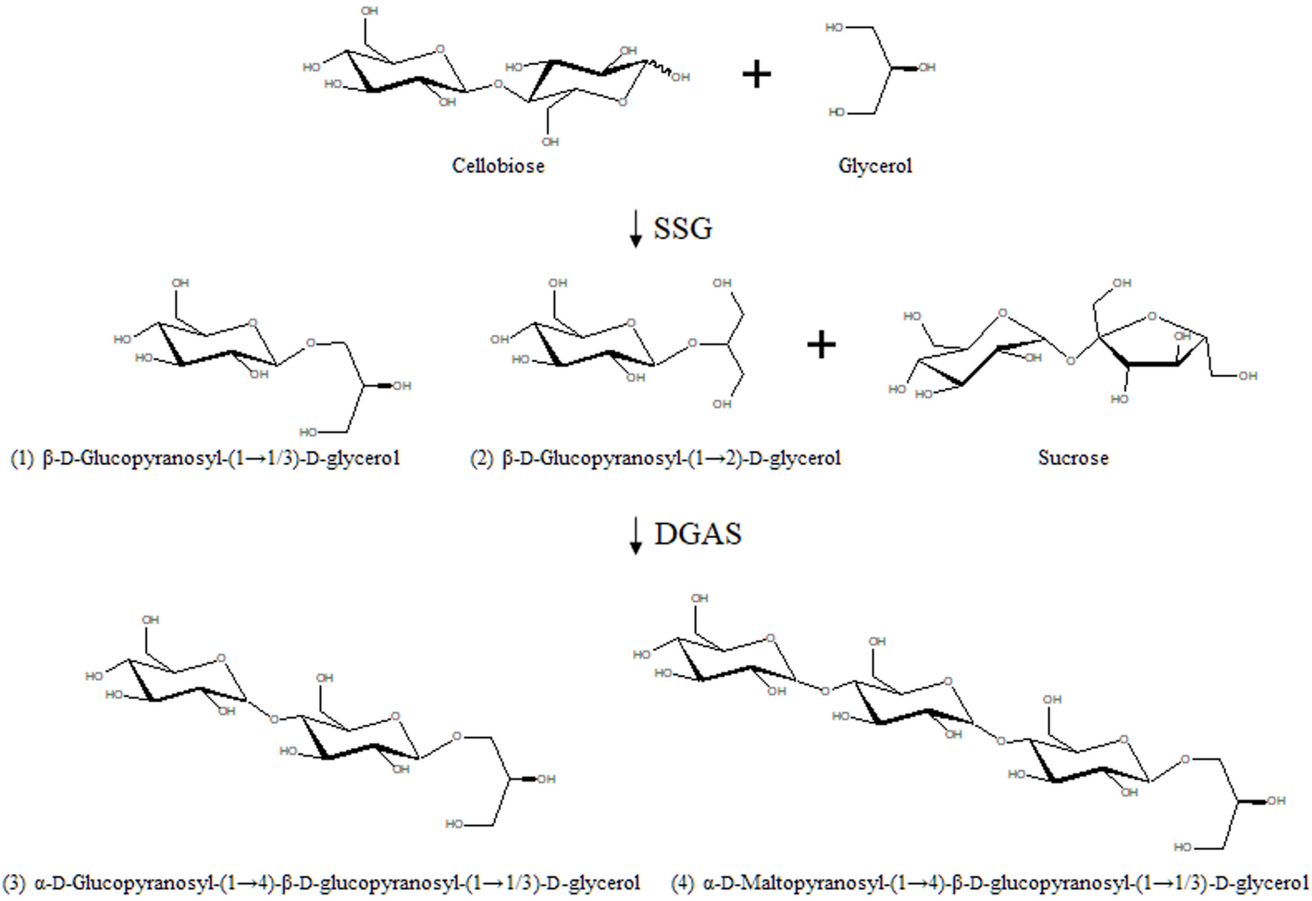


**Fig. 4.** Structure and synthesis scheme of glucosylglycerols.
The biosynthesis of natural (1 and 2) and unnatural glucosylglycerol (3 and 4) was successfully accomplished using SSG and DGAS.

matched the calculated molecular mass of diglucosylglycerol and triglucosylglycerol, respectively.

$^{1}$H NMR and $^{13}$C NMR results are shown in Table 1. N1 was determined as α-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1/3)-D-glycerol (αgβg1,3gly) by $^{1}$H NMR and $^{13}$C NMR analysis. An oxygenated methine carbon signal (C-4′) was observed at $\delta_C$ 78.7 due to the glycosidation shift, which is observed at $\delta_C$ 71.4. In addition, αgβg1,3gly was almost hydrolyzed as β-GG and glucose through treatment with α-glucosidase (data not shown). These results confirmed the connection between the glycosyl unit (C-1′′) and the C-4′ of the glucose. The bonds of diglucosylglyerol were determined to be β and α glycosidic cross-linkages from glycerol according to four coupling constants ($J$=8.0/8.4 Hz at 4.369/4.372 and $J$=4.0/4.0 Hz at 5.264/5.274). The $^{13}$C NMR spectrum showed 19 carbon signals (the signals of C1, C2, C1′, and C2′ were observed as double peaks), which is more than the 15 of diglucosylglycerol. The $^{1}$H NMR results are shown as 4 signals. These results confirm that N1 is α-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 1/3)-D-glycerol.

Similarly, the structure of N2 was determined by $^{1}$H NMR and $^{13}$C NMR. An oxygenated methine carbon signal (C-4′′) was observed at $\delta_C$ 79.0 due to the glycosidation shift, which is observed at $\delta_C$ 71.3. In enzymatic hydrolysis reaction, αmβg1,3gly was degraded to β-GG and glucose through treatment with α-glucosidase, as well as β-GG and maltose were observed by the treatment of β-amylase. These results confirmed the connection between the glycosyl unit (C-1′′′) and the C-4′′ of the glucose. The bonds of triglucosylglycerol were determined to be β, α, and α-glycosidic cross-linkages from glycerol according to six coupling constants ($J$=8.0/8.0 Hz at 4.370/4.373, $J$=2.8/2.8 Hz at 5.264/5.274, and $J$=2.8/2.8 Hz, 5.256/5.257). $^{13}$C NMR results are shown as 25 signals, which is more than the 21 of triglucosylglycerol. The $^{1}$H NMR results are shown

as 6 signals. These results indicate thatα-D-maltopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1/3)-D-glycerol is the structural conformation of N2.

In this study, the biosynthesis of natural and unnatural glucosylglycerol was successfully established using two enzymes – SSG and DGAS. β-D-Glucopyranosyl-(1 → 1/3)-D-glycerol and β-D-glucopyranosyl-(1 → 2)-D-glycerol were synthesized from glycerol and cellobiose using SSG, and additional sugars were transferred from sucrose to β-D-glucopyranosyl-(1 → 1/3)-D-glycerol using DGAS. Finally, unnatural glucosylglycerols were synthesized and their structures were determined to be α-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1/3)-D-glycerol and α-D-maltopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 1/3)-D-glycerol by enzymatic digestion, MASS, and NMR. The structure and overall synthesis scheme is described in Fig. 4.

The use of combinatorial enzymes for the synthesis of glycoconjugates is still in the initial stages of research. The possibility of the combinatorial enzymatic biosynthesis of biological molecules was shown in this study. β-GG glycosides synthesized by the combination of two enzymes could exhibit different biological properties depending on the modification of their additional sugar residues. Expanding the combination of enzymes and substrates to synthesize unnatural glycosides will broaden the opportunity to find new biomolecules which may possess novel biological activities.

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## Conflict of Interest

The authors have no financial conflicts of interest to declare.

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