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# Enzymatic synthesis of salicin glycosides through transglycosylation catalyzed by amylosucrases from *Deinococcus geothermalis* and *Neisseria polysaccharea*

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ABSTRACT

Amylosucrase (ASase, EC 2.4.1.4) is a member of family 13 of the glycoside hydrolases that catalyze the synthesis of an α-(1→4)-linked glucan polymer from sucrose instead of an expensive activated sugar, such as ADP- or UDP-glucose. Transglycosylation reactions mediated by the ASases of *Deinococcus geothermalis* (DGAS) and *Neisseria polysaccharea* (NPAS) were applied to the synthesis of salicin glycosides with sucrose serving as the glucopyranosyl donor and salicin as the acceptor molecule. Two salicin glycoside transfer products were detected by TLC and HPLC analyses. The synthesis of salicin glycosides was very efficient with NPAS with a yield of over 90%. In contrast, DGAS specifically synthesized only one salicin transglycosylation product. The transglycosylation products were identified as α-D-glucopyranosyl-(1→4)-salicin (glucosyl salicin) and α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-salicin (maltosyl salicin) by NMR analysis. The ratio between donor and acceptor had a significant effect on the type of product that resulted from the transglycosylation reaction. With more acceptors present in the reaction, more glucosyl salicin and less maltosyl salicin were synthesized.



## 1. Introduction

Transglycosylation is a mechanism for glycosidic bond formation in which a glycosyl molecule in a donor compound is transferred to a hydroxyl group of an acceptor molecule.[1] Recently, the essential roles of many glycosyl-linked biomolecules (glycoconjugates) and oligosaccharides in biological systems have been revealed. They play important roles in host defense, cell–cell recognition, ligand–receptor binding, and cell signaling.[2–6] The therapeutic use of oligosaccharides or glycoconjugates as analogs of biologically important molecules to eliminate or diminish the symptoms of various diseases has been considered.[6,7] Currently, chemical methods of glycoconjugate (or oligosaccharide) synthesis are often slow and regiospecifically nonspecific. These syntheses also generally require multi-step processes under harsh conditions. On the contrary, enzymatic biotransformation has been employed as an alternative method for manufacturing pharmaceuticals, fine chemicals, and food ingredients, due to the highly selective catalytic reactions and the use of environmentally friendly, mild reaction conditions.[8–10]

Many enzymes that catalyze transglycosylation reactions have been employed to synthesize oligosaccharides or glycoconjugate molecules.[8,9] The resulting glycoside-transfer products exhibited improved pharmacological and physicochemical properties such as sweetness, water solubility, stability, and transportation through membrane barriers or by body fluids, as well as having better biological activities.[11–14] For example, maltogenic amylase from *Bacillus stearothermophilus* (BSMA) and *Thermus* sp. (ThMA) has been used to synthesize various glycosyl-transfer products such as naringin glycosides, glucosyl ascorbic acid, and puerarin glycosides.[15–17] Recently, a novel α-glucosidase-selective inhibitor with 10-fold enhanced selectivity, as compared to acarbose, toward α-glucosidase over α-amylase activity was synthesized by ThMA using acarviosine glucose as a donor and 3-α-D-glucopyranosylpropen (αGP) as an acceptor.[11,15,16] This final product, α-acarviosinyl-(1→9)-3-α-D-glucopyranosylpropen, was a potent hypoglycemic agent. Cyclodextrin glycosyltransferase (CGTase) favorably catalyzes transglycosylation reactions, whereas many other related α-amylase family enzymes are hydrolases. Therefore, the intermolecular transglycosylation activity of CGTase was successfully accepted to produce a variety of glycosyl-transfer products.[18,19]

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Amylosucrase (ASase, EC. 2.4.1.4) is a member of family 13 of the glycoside hydrolases (GH13, α-amylase family). Among the GH13 family, ASase has the remarkable enzymatic property of not only having an unusual specificity for sucrose hydrolysis, but also synthesizing an amylose-like polymer without using an expensive activated sugar, such as ADP- or UDP-glucose as a donor molecule.[20–24] Until now, this interesting enzyme has only been reported in bacteria of the *Neisseria* and *Deinococcus* genera, and has been studied exclusively in *Neisseria polysaccharea*.[20,25,26] ASase from *N. polysaccharea* (NPAS) is secreted to produce the polysaccharide outside of cells, while ASase of other bacteria is found inside cells.[20] Recently, a hypothetical protein from *Deinococcus geothermalis* (DGAS), which is highly homologous to NPAS, was found, and the corresponding gene was cloned and expressed in *Escherichia coli*.[27] The recombinant DGAS synthesized an insoluble amylose polymer accompanied by side reactions (sucrose hydrolysis, sucrose isomer, and soluble maltooligosaccharide formation). Most interestingly, DGAS has exceptionally thermostable characteristics, as demonstrated by a half-life of 6.8 h at 55 °C, whereas the half-life of NPAS is only 21 h at 30 °C. Although it is obvious that AS performs a transglycosylation reaction when sucrose is used as the sole substrate, the transglycosylation activity toward acceptor molecules other than sucrose has not been investigated. In this study, the transglycosylation activities of recombinant DGAS and NPAS were employed to synthesize salicin glycosides using sucrose as a glucose donor and salicin as an acceptor. The high yield synthesis of two salicin derivatives by ASases was also confirmed. Finally, the molecular structures of salicin transfer products and optimal transglycosylation reaction conditions of ASases are discussed.

## 2. Results and discussion

### 2.1. Expression and purification of rDGAS and rNPAS

The genes corresponding to DGAS and NPAS from *D. geothermalis* DSM 11300 and *N. polysaccharea* ATCC 43768 were amplified by PCR. The absence of possible error in the nucleotide sequence of the PCR-generated product was confirmed by DNA sequencing analysis. The resulting PCR fragment corresponding to *npas* was inserted into the *E. coli* expression vector, pRSET-B vector, to generate pRSET-NPAS, whereas the fragment coding for *dgas* was added into the pGEX-4T-1 vector, making pGEX-DGAS. Therefore, the final recombinant NPAS (rNPAS) was tagged by six histidines, while rDGAS was fused to the glutathione S-transferase (GST). An attempt to use the pRSET-B vector to express rDGAS was not successful. The recombinant *E. coli* cells transformed with either pRSET-NPAS or pGEX-DGAS showed a blue-staining halo around the colony when the cells were grown in sucrose-containing medium and were stained with iodine vapor, indicating that an amylose-like polymer was produced by these cells (data not shown). In the pRSET-NPAS vector, there were an extra 33 amino acids attached to the N-terminus of the matured NPAS since the expression cassette contained six-histidine tags for simple purification, T7 gene 10 leader amino acids to provide protein stability, and the Xpress™ epitope for easy detection of the fusion protein. These extra 33 amino acids did not eliminate or impair the enzymatic activity of rNPAS. Similarly, the GST-fused DGAS showed apparent ASase activity with its tagged GST protein on the N-terminus. Both rDGAS and rNPAS were efficiently purified using a Ni-NTA affinity column and a Glutathione–Sepharose™ High-Performance affinity column, respectively, and then used for further experiments.

### 2.2. Comparison of enzymatic properties of rDGAS and rNPAS

rNPAS showed its highest sucrose hydrolysis activity at 35 °C, and over 80% of its activity was retained between 30 and 40 °C

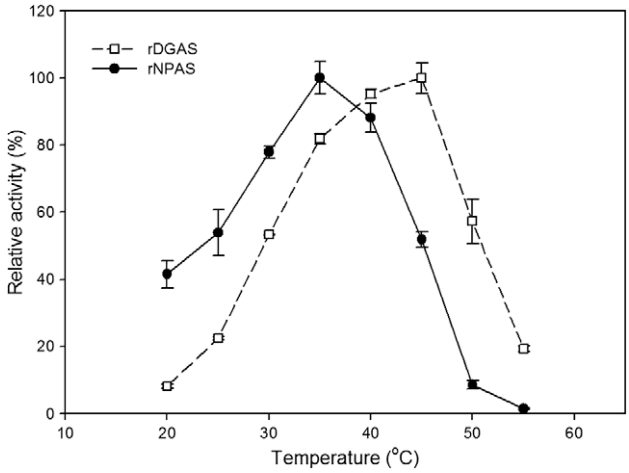


**Figure 1.** Effect of temperature on the hydrolysis activities of rNPAS and rDGAS. rNPAS is described by closed circles, while rDGAS is shown as open circles.

(Fig. 1). However, its activity was significantly reduced at temperatures over 50 °C. In rDGAS, the most favorable hydrolysis reaction temperature was 45 °C, and its enzymatic activity was maintained at more than 80% of its optimal activity in the range of 35–45 °C. Furthermore, the hydrolysis activity of rDGAS was maintained at about 60% of its maximum activity, even at 50 °C. The half-life of rNPAS was less than 1 min at 50 °C, whereas that of rDGAS was 28.1 h at the same temperature, indicating that rDGAS is much more thermostable than rNPAS.[27] The optimal pHs for rNPAS and rDGAS were pH 6 and pH 8, respectively. When the polymerization activity of rDGAS was investigated by high-performance anion-exchange chromatography (HPAEC), more oligosaccharides were detected at lower temperatures (25 °C and 30 °C) than at its optimal hydrolysis temperature (45 °C).[27] Since polymerization activity is associated with transglycosylation activity, the transglycosylation reaction of rDGAS was performed at 30 °C. Due to the heat-labile properties of rNPAS, its polymerization activity could not be analyzed at higher temperatures (>40 °C). Previously, various synthetic reactions of amylose-like polymers by rNPAS were performed at 30 °C.[28] Therefore, the transglycosylation reaction of rNPAS was also performed at 30 °C.

### 2.3. Production of salicin transfer product

Salicin is a naturally occurring plant glycoside found in the bark of poplar and willow trees. This compound was used as an analgesic and antipyretic.[29] It has a D-glucopyranose unit attached by a β-linkage to the phenolic hydroxyl group of salicyl alcohol. When salicin is employed as an acceptor of transglycosylation reactions of ASase, transglycosylation products can be easily detected using a UV detector due to the hydroxy group on the phenyl group of the compound. In this study, salicin transfer products were enzymatically synthesized by employing the transglycosylation activity of rNPAS and rDGAS. TLC analysis of the salicin transglycosylation reaction showed that at least one salicin transfer product was synthesized by both enzymes (Fig. 2). However, HPLC analysis revealed that there were two distinct salicin transfer products (designated as compounds **1** and **2**) in the rNPAS reaction, but only one product (**1**) in the rDGAS reaction (Fig. 3).

The yield of transfer products was incredibly high, reaching over 80% in the rNPAS reaction. The total yields of salicin transfer products (**1** and **2**) from 0.5%, 1%, and 2% acceptor (salicin) with 4% donor (sucrose) were 99%, 99%, and 97%, respectively, implying that all acceptors were glycosylated by rNPAS (Table 1). Even when higher concentrations of acceptor (3% and 4%) were present in the

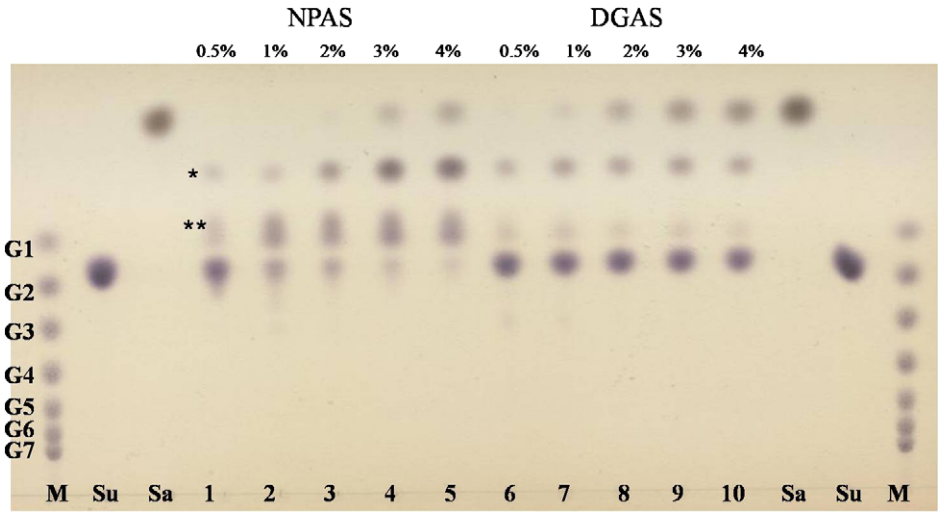


**Figure 2.** TLC analysis of salicin transglycosylation reactions of rNPAS and rDGAS. Lane M, G1(glucose)–G7(maltoheptaose) standard; lane Su, 0.05 M sucrose; lane Sa, 2% (0.069 M) salicin; lanes 1–10 are the transglycosylation reaction mixtures with various concentrations of salicin as acceptors by rNPAS (lanes 1–5) and rDGAS (lanes 6–10). Lanes 1 and 6, 0.5% (0.017 M) salicin; lanes 2 and 7, 1% (0.03 M) salicin; lanes 3 and 8, 2% (0.069 M) salicin; lanes 4 and 9, 3% (0.1 M) salicin; lanes 5 and 10, 4% (0.14 M) salicin. All reactions contained 4% sucrose as a donor. The spots representing compounds **1** and **2** are designated by * and **, respectively.

reaction, the yields of transfer products were still close to 85%. Interestingly, the portion of **1** increased as the concentration of acceptor increased. The yields of the rDGAS reaction were a little lower than those of the rNPAS reaction. The yield achieved with rDGAS was over 70% when less than 1% of acceptor existed in the reaction. However, the yield decreased to 43%, 38%, and 29% when the amounts of acceptor in the reaction mixture increased to 2%, 3%, and 4%, respectively (Table 1). Interestingly, only a very small quantity of **2**, if any, was synthesized by rDGAS, as compared to the rNPAS reaction.

Usually, the synthesis of glycosidic linkages in nature is carried out by glycosyltransferases (EC 2.4) that use activated glycosides as the glycosyl donors. Typical glycoside donors are expensive nucleotide sugars such as ADP-glucose, UDP-glucose, and UDP-galactose.[30,31] In contrast, ASase employs a considerably inexpensive substrate (sucrose) as a glycoside donor molecule, leading to large

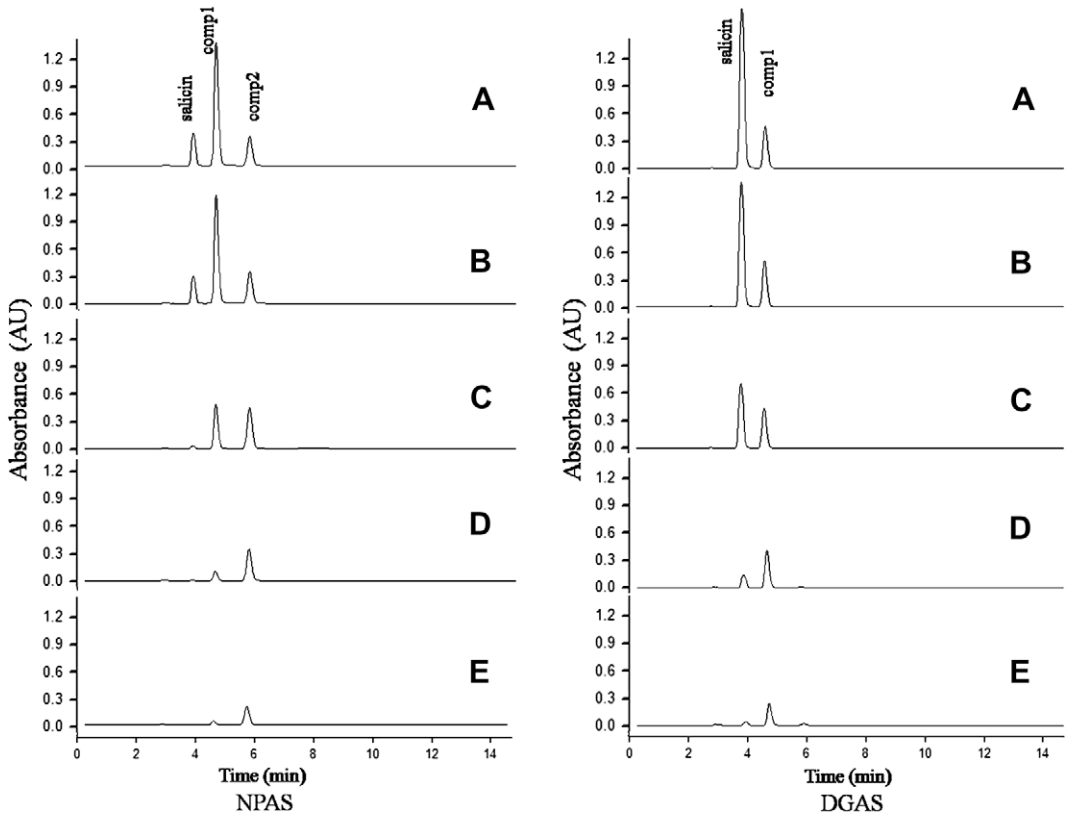


**Figure 3.** HPLC analysis of salicin transglycosylation reactions of rNPAS and rDGAS. (A) reaction with 4% (0.14 M) salicin; (B) reaction with 3% (0.1 M) salicin; (C) reaction with 2% (0.069 M) salicin; (D) reaction with 1% (0.03 M) salicin; (E) transglycosylation reaction with 0.5% (0.017 M) salicin.

**Table 1**
Yield of transglycosylation products by rNPAS and rDGAS[a]

| Salicin concentration (%) | Yield (%) | | | | | |
|---|---|---|---|---|---|---|
| | NPAS | | | DGAS | | |
| | Compound 1 | Compound 2 | Total | Compound 1 | Compound 2 | Total |
| 0.5 | 15 | 84 | 99 | 79 | 5 | 83 |
| 1 | 20 | 79 | 99 | 68 | 3 | 70 |
| 2 | 46 | 50 | 97 | 43 | ND | 43 |
| 3 | 62 | 22 | 86 | 37 | ND | 37 |
| 4 | 65 | 18 | 84 | 29 | ND | 29 |

[a] All reactions contained 4% sucrose as a donor.

industrial interest in employing this enzyme for biotechnological synthesis of glycoconjugates or polysaccharides.[21,32] Our results showed that both rNPAS and rDGAS can efficiently utilize the energy generated from the cleavage of the glycosidic bond between glucose and fructose in sucrose to synthesize other glycosidic linkages in transglycosylation products, such as salicin transfer products.

Interestingly, the ratio of transfer products, **1** and **2**, varied as the concentration of acceptor molecule was changed in the rNPAS reaction. At higher salicin concentrations (3% and 4%), **1** was the predominant transfer product over **2**, while at lower acceptor concentrations (0.5%, 1%, and 2%), significant quantities of **2** were generated. This result indicates that the transfer product could be controlled by adjusting the concentration of the acceptor molecule in the rNPAS transglycosylation reaction. In the rDGAS reaction, **1** was the predominant transglycosylation product with only insignificant or minimal amounts of **2** detected, although the yield achieved with rDGAS was lower than that of the rNPAS transglycosylation reaction. Therefore, rDGAS could be employed in a specific reaction to make a certain single transglycosylation product.

### 2.4. Effect of temperature and the concentration of donor molecules in the transglycosylation reaction by rDGAS

rDGAS was known to have a maximum hydrolysis activity at 45 °C, while its highest transglycosylation activity was observed at lower temperatures (25–30 °C).[27] Since quite a few donor molecules of sucrose remained in the reaction mixture (Fig. 4), the salicin transglycosylation reaction was performed at 45 °C to monitor whether the hydrolysis reaction was a rate-limiting step in the transglycosylation reaction. The result showed that the salicin transglycosylation product was not increased at 45 °C compared to 30 °C, implying that the hydrolysis reaction was not a rate-limiting step to perform transglycosylation in rDGAS (Fig. 4). The effect of the concentration of donor molecule on the production of salicin transglycosylation product was examined (Table 2). As the ratio of donor concentration over acceptor concentration (D/A) was increased, the yield of salicin transglycosylation product increased. At a D/A of 0.5, the yield was only 23%, but was approximately 87% at a D/A of 12.

### 2.5. Purification and structural determination of salicin transfer products

Two salicin transglycosylation products in the rNPAS reaction were purified by a preparative recycling HPLC system. Both products

**Table 2**
The effect of donor/acceptor ratio (D/A) on the yield of salicin transglycosylation products

| Donor concn (M) | Acceptor concn | D/A | Yield (%) |
|---|---|---|---|
| 0.05 | 3% (0.1 M) | 0.5 | 23 |
| 0.1 | | 1 | 30 |
| 0.3 | | 3 | 35 |
| 0.6 | | 6 | 73 |
| 0.8 | | 8 | 80 |
| 1.2 | | 12 | 87 |

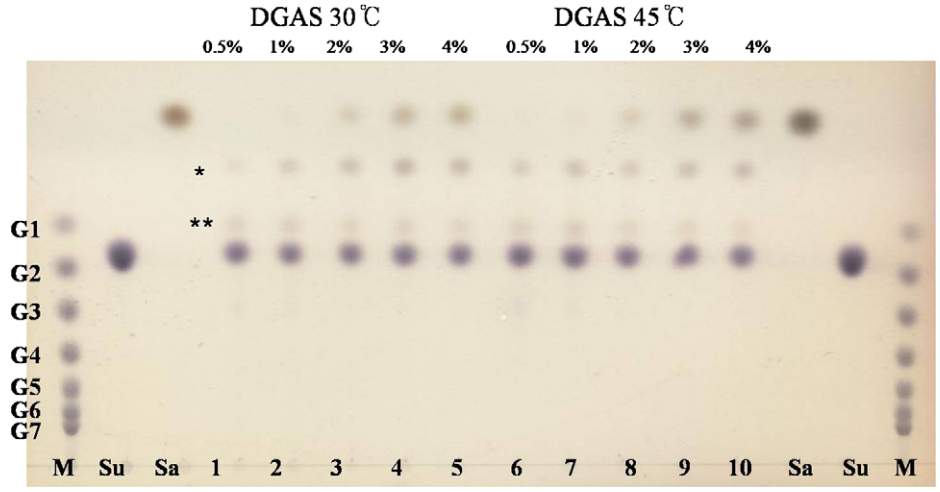


**Figure 4.** TLC analysis of salicin transglycosylation reaction of rDGAS performed at two different temperatures (30 °C and 45 °C). Lane M, G1(glucose)–G7(maltoheptaose) standard; lane Su, 0.05 M sucrose; lane Sa, 2% (0.069 M) salicin; lanes 1–10 are the transglycosylation reaction mixtures with various concentrations of salicin as acceptors by DGAS at 30 °C (lanes 1–5) and DGAS at 45 °C (lanes 6–10). Lanes 1 and 6, 0.5% (0.017 M) salicin; lanes 2 and 7, 1% (0.03 M) salicin; lanes 3 and 8, 2% (0.069 M) salicin; lanes 4 and 9, 3% (0.1 M) salicin; lanes 5 and 10, 4% (0.14 M) salicin. The spots representing compounds **1** and **2** are designated by * and **, respectively.

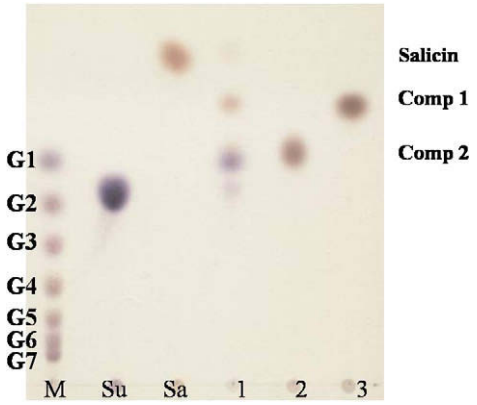


**Figure 5.** TLC analysis of the purified salicin transfer products. Lane M, G1(glucose)–G7(maltoheptaose) standard; lane Su, 0.05 M sucrose (0.5 μL); lane Sa, 0.069 M salicin (0.5 μL); lane 1, transglycosylation reaction mixture prepared with rNPAS; lane 2, purified compound 2; lane 3, purified compound 1.

were successfully isolated as single compounds, and confirmed as individual clear spots on TLC (Fig. 5). The molecular masses of the purified salicin transfer products, **1** and **2**, were determined to be 448 Da and 610 Da, respectively, by matrix-assisted laser-desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometric (MS) analysis. These molecular masses corresponded to the calculated molecular masses of glucosyl salicin and maltosyl salicin. When various signals of **1** and **2** in their $^{1}H$ and $^{13}C$ nuclear magnetic resonance (NMR) spectra were compared with those of salicin, seven of them (group 1 in Table 3) were assigned to the (hydroxymethyl)phenyl group, and six (group 2 in Table 3) were assigned as glucose units of salicin. Compound **1** showed 19 carbon signals, including those of the salicin molecule, and **2** had 25 signals containing those of salicin and **1**. This result, combined with MALDI-TOFMS analysis, implies that **1** contains a glucose molecule connected to salicin, whereas two glucose units are attached to the salicin molecule in **2**.

Analysis of $^{1}H$ and $^{13}C$ NMR data was used to determine the type of glycosidic linkage in two compounds (**1** and **2**) between the glycosyl (or two glucosyl groups) and D-glucopyranosyl group of salicin. Chemical shifts of **1** in the $^{13}C$ NMR spectra were compared with those of salicin (Table 3). The position for the terminal glucose linked to the salicin was determined through the glycosylation shift of the C-4′ signal of the glucose moiety in salicin. Compound **1** showed 19 carbon signals including those of a terminal glucose molecule. The chemical shift of C-4′ in the glucose unit of salicin changed greatly from 71.34 to 80.85 ppm (+9.51 ppm) in **1**, confirming that the transferred glucosyl group was connected to C-4′ in the glucose unit of salicin. Similarly, the chemical shift of C-4′ in the glucose unit of salicin was principally altered from 71.34 to 80.86 ppm (+9.52 ppm) in **2**. In addition, the chemical shift of C-4″ in the transferred glucosyl unit of **1** was distinctly changed from 71.48 to 81.32 ppm (+9.84 ppm) in **2**. In addition, $^{1}H$ NMR analysis revealed that the glucosyl (or maltosyl) residue was transferred to C-4′ in the glucose unit of salicin to give the α-anomeric configuration based on the coupling constant ($J$ = 4.0 Hz) of the glucose anomeric proton signal observed at 5.18 ppm. Two-dimensional HMBC spectra of **1** and **2** confirmed the molecular structures of both compounds (data not shown). The structure of **1**was identified as α-D-glucopyranosyl-(1→4)-salicin, in which the transferred D-glucose was attached to C-4′ of the glucose moiety of salicin by an α-(1→4)-linkage. Likewise, **2** was determined to be α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-salicin (Fig. 6). The transglycosylation product observed in the rDGAS reaction was confirmed to be the same as **1**. These results demonstrate that the type of glycosidic bond formed in the transglycosylation products was exclusively the α-(1→4)-glycosidic linkage, which must be a specific characteristic of ASases (rNPAS and rDGAS).

**Table 3**
$^{13}C$ and $^{1}H$ NMR analyses of salicin transglycosylation products

| Carbon atom | | $^{13}C$ NMR | | | $^{1}H$ NMR | | |
|---|---|---|---|---|---|---|---|
| | | Salicin | 1 | 2 | Salicin | 1 | 2 |
| Group 1 salicyl alcohol | 1 | 156.99 | 156.99 | 156.99 | | | |
| | 2 | 132.03 | 132.03 | 132.03 | | | |
| | 3 | 129.75 | 129.75 | 129.75 | 7.31 (dd, $J$ = 7.6, 1.2 Hz) | 7.31 (br d, $J$ = 7.6, 1.2 Hz) | 7.31 (br d, $J$ = 7.6, 1.2 Hz) |
| | 4 | 123.62 | 123.62 | 123.62 | 7.00 (ddd, $J$ = 7.6, 7.6, 1.2 Hz) | 7.00 (ddd, $J$ = 7.6, 7.6, 1.2 Hz) | 7.00 (ddd, $J$ = 7.6, 7.6, 1.2 Hz) |
| | 5 | 129.85 | 129.85 | 129.85 | 7.23 (ddd, $J$ = 7.6, 7.6, 1.2 Hz) | 7.21 (ddd, $J$ = 7.6, 7.6, 1.2 Hz) | 7.21 (ddd, $J$ = 7.6, 7.6, 1.2 Hz) |
| | 6 | 116.96 | 116.96 | 116.96 | 7.18 (dd, $J$ = 7.6, 1.2 Hz) | 7.18 (br d, $J$ = 7.6 Hz) | 7.18 (br d, $J$ = 7.6 Hz) |
| | 7 | 60.98 | 60.98 | 60.93 | 4.75 (d, $J$ = 13.2 Hz) | 4.75 (d, $J$ = 13.2 Hz) | 4.75 (d, $J$ = 13.2 Hz) |
| | | | | | 4.55 (d, $J$ = 13.2 Hz) | 4.55 (d, $J$ = 13.2 Hz) | 4.55 (d, $J$ = 13.2 Hz) |
| Group 2 Glc (I) | 1′ | 103.29 | 103.29 | 103.07 | 4.86 (d, $J$ = 8.0 Hz) | 4.88 (d, $J$ = 8.0 Hz) | 4.88 (d, $J$ = 8.0 Hz) |
| | 2′ | 75.05 | 74.64 | 74.74 | 3.48 (dd, $J$ = 8.0, 7.6 Hz) | 3.54 (dd, $J$ = 9.2, 8.0 Hz) | 3.54 (dd, $J$ = 9.2, 8.0 Hz) |
| | 3′ | 77.96 | 77.69 | 77.67 | 3.43 (dd, $J$ = 8.0, 7.6 Hz) | 3.71 (dd, $J$ = 9.2, 9.2 Hz) | 3.71 (dd, $J$ = 9.2, 9.2 Hz) |
| | 4′ | 71.34 | 80.85 | 80.86 | 3.38 (dd, $J$ = 8.0, 8.0 Hz) | 3.62 (dd, $J$ = 9.2, 9.2 Hz) | 3.62 (dd, $J$ = 9.2, 9.2 Hz) |
| | 5′ | 78.21 | 76.79 | 76.78 | 3.40 (ddd, $J$ = 8.0, 5.2, 2.0 Hz) | 3.53 (ddd, $J$ = 9.2, 4.4, 2.0 Hz) | 3.53 (ddd, $J$ = 9.2, 4.4, 2.0 Hz) |
| | 6′ | 62.53 | 61.99 | 62.02 | 3.87 (dd, $J$ = 12.0, 2.0 Hz) | 3.87 (dd, $J$ = 12.0, 2.0 Hz) | 3.87 (dd, $J$ = 12.0, 2.0 Hz) |
| | | | | | 3.68 (dd, $J$ = 12.0, 5.2 Hz) | 3.83 (dd, $J$ = 12.0, 4.4 Hz) | 3.83 (dd, $J$ = 12.0, 4.4 Hz) |
| Group 3 Glc (II) | 1″ | | 102.85 | 102.85 | | 5.18 (d, $J$ = 4.0 Hz) | 5.18 (d, $J$ = 4.0 Hz) |
| | 2″ | | 74.14 | 73.75 | | 3.43 (dd, $J$ = 9.6, 4.0 Hz) | 3.43 (dd, $J$ = 9.6, 4.0 Hz) |
| | 3″ | | 75.05 | 74.90 | | 3.60 (dd, $J$ = 9.6, 9.2 Hz) | 3.83 (dd, $J$ = 9.2, 9.2 Hz) |
| | 4″ | | 71.48 | 81.32 | | 3.25 (dd, $J$ = 9.2, 9.2 Hz) | 3.51 |
| | 5″ | | 74.79 | 73.35 | | 3.68 (dd, $J$ = 9.2, 5.2, 2.0 Hz) | 3.73 |
| | 6″ | | 62.53 | 62.13 | | 3.82 (dd, $J$ = 12.0, 2.0 Hz) | 3.87 (dd, $J$ = 12.0, 2.0 Hz) |
| | | | | | | 3.63 (dd, $J$ = 12.0, 5.2 Hz) | 3.83 (dd, $J$ = 12.0, 4.4 Hz) |
| Group 4 Glc (III) | 1‴ | | | 102.62 | | | 5.2 (d, $J$ = 4.0 Hz) |
| | 2‴ | | | 74.23 | | | 3.43 (dd, $J$ = 9.6, 4.0 Hz) |
| | 3‴ | | | 75.06 | | | 3.6 (dd, $J$ = 9.6, 9.2 Hz) |
| | 4‴ | | | 71.46 | | | 3.25 (dd, $J$ = 9.2, 9.2 Hz) |
| | 5‴ | | | 74.62 | | | 3.68 (ddd, $J$ = 9.2, 5.2, 2.0 Hz) |
| | 6‴ | | | 62.72 | | | 3.82 (dd, $J$ = 12.0, 2.0 Hz) |
| | | | | | | | 3.63 (dd, $J$ = 12.0, 5.2 Hz) |

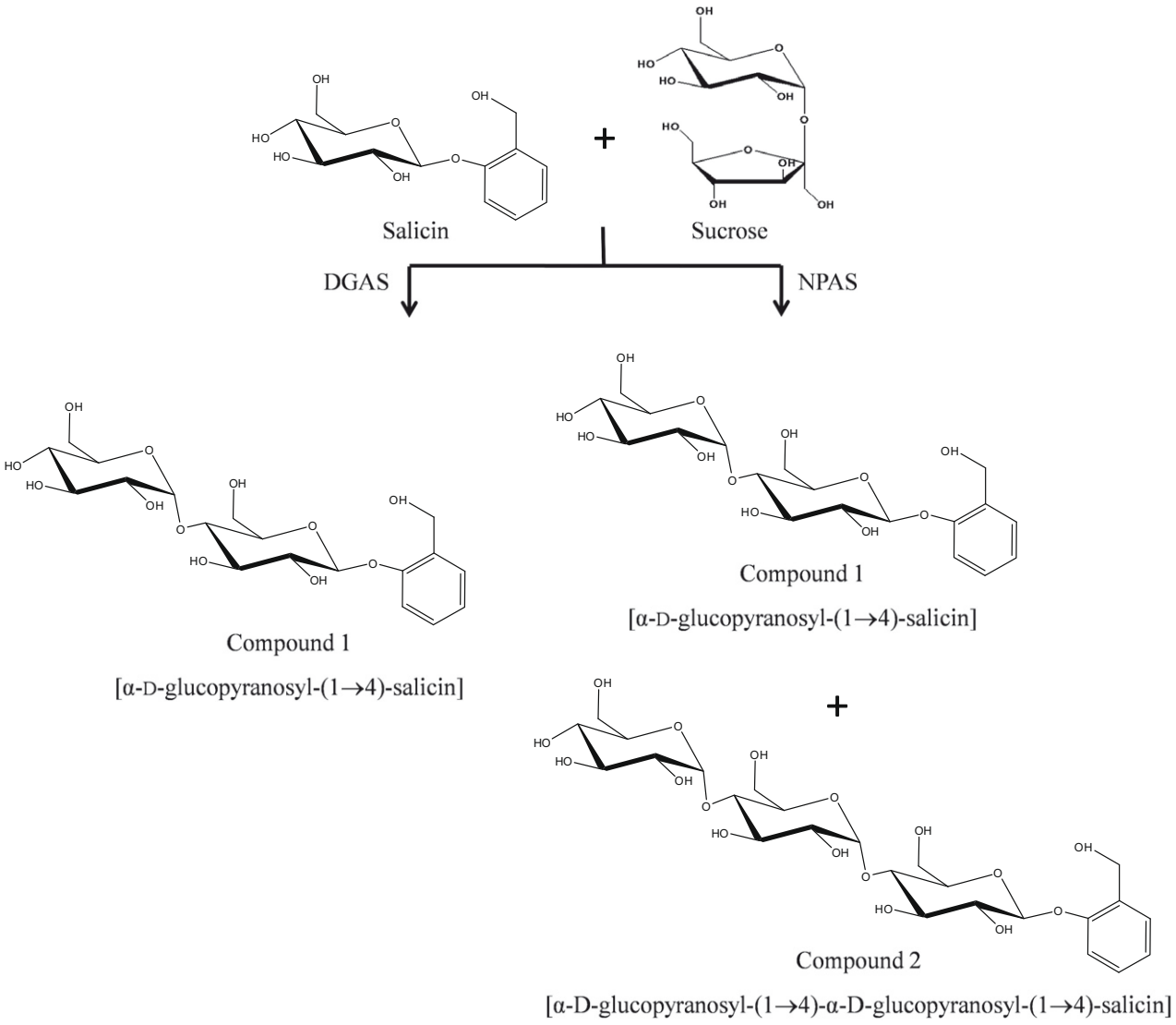


**Figure 6.** Structures of salicin and transglycosylated salicin derivatives [α-D-glucopyranosyl-(1→4)-salicin and α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-salicin].

The transglycosylation activity of rNPAS has been employed to modify glycogen and starch-related α-glucans.[28,33] When rNPAS was employed to glucosylate glycogen particles using sucrose as the donor molecule, the initial sucrose/glycogen (donor/acceptor) ratio strongly affected the morphology and structure of the resulting insoluble glucans. As this ratio was increased, the external glycogen chains were extended by the enzyme, resulting in dendritic nanoparticles with diameters 4–5 times those of the initial particles.[28] Until now, the only report about small molecules as possible acceptors of ASases was that of our previous research on monosaccharides, such as galactose, xylose, methyl α-D-glucopyranoside, and methyl β-D-glucopyranoside as acceptors for the DGAS transglycosylation reaction.[27] In this study, we have shown for the first time that a molecule other than a monosaccharide or α-glucans can be efficiently employed as an acceptor in the transglycosylation reaction of ASases.

## 3. Experimental

### 3.1. Bacterial strains and plasmids

*N. polysaccharea* (ATCC 43768) was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA), while *D. geothermalis* DSM 11300 was obtained from Dr. Michael Daly at the Uniformed Services University of the Health Sciences. *E. coli* DH10B [$F^-$ *ara*D139 Δ(*ara leu*)7697 Δ*lac*X74 *gal*U *gal*K *rps*L *deo*R Φ80*lac*ZΔM15 *end*A1 *nup*G *rec*A1 *mcr*A Δ(*mrr hsdRMS mcrBC*)] and BL21 [$F^-$, *omp*T, *hsd*$S_B$($r_B-$, $m_B-$), *dcm*, *gal*, λ(DE3)] used as hosts for cloning and expression studies were grown in Luria–Bertani (LB) medium containing 1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) NaCl, supplemented with ampicillin (100 μg/mL). pGEM T-easy vector (Promega, Madison, WI, USA), pRSET-B vector (Invitrogen, Carlsbad, CA, USA), and pGEX-4T-1 vector (Amersham Biosciences, Buckinghamshire, UK) were used for PCR-cloning and expression.

### 3.2. Enzymes and chemicals

Restriction endonucleases and modifying enzymes, such as T4 DNA ligase and *Pfu* DNA polymerase, were purchased from New England Biolabs (Beverly, MA, USA), Solgent (Seoul, Korea), or Promega (Madison, WI, USA). The PCR products and DNA restriction fragments were purified using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA, USA). All chemicals used in this study, including D-(−)-salicin (2-(hydroxymethyl)phenyl-D-glucopyranoside), were of reagent grade and obtained from Sigma Chemical Co. (St Louis, MO, USA).

### 3.3. Construction of NPAS and DGAS expression plasmids

The gene corresponding to NPAS was amplified by PCR using two primers designed based on the *N. polysaccharea* (ATCC 43768) ASase nucleotide sequence (Genbank accession number, AJ011781). The two primers matched with the 5′ and 3′ ends of the *npas* gene were NPAS1 (5′-GGA TCC GAT GTT GAC CCC CAC GCA GCA A-3′) and NPAS2 (5′-GGC AAG CTT CAG GCG ATT TCG AGC CAC AT-3′), containing BamHI and HindIII recognition sites (underlined), respectively. The standard conditions for PCR were as follows: one cycle of denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, extension at 72 °C for 2 min, and extra extension at 72 °C for 10 min. The PCR product of the *npas* gene made with *Pfu* DNA polymerase was cloned into the pGEM T-easy vector after an A-tailing reaction, and the nucleotide sequence of the PCR-generated insert was then determined by using the BigDye Terminator Cycle Sequencing Kit for ABI377 PRISM (Perkin–Elmer Inc., Boston, MA, USA). The insert was excised from pGEM T-easy vector using BamHI and HindIII and ligated into pRSET-B vector treated with the same restriction enzymes to create pRSET-NPAS. The *npas* gene in the pRSET-NPAS was controlled by a T7 promoter. Therefore, *E. coli* BL21 was transformed with the resulting recombinant plasmids for efficient expression. The amplification and cloning of the *dgas* gene were described previously.[27] The final expression vector in which the DNA fragment corresponding to the *dgas* gene was inserted into pGEX-4T-1 vector was designated as pGEX-DGAS.

### 3.4. Purification of recombinant NPAS

Ni-NTA affinity column chromatography was used to purify recombinant $His_6$-tagged NPAS. Recombinant *E. coli* BL21 cells harboring pRSET-NPAS were grown in 500 mL of LB containing 100 μg/mL ampicillin at 37 °C. When the optical density of the cells reached approximately 0.6, the cells were induced by adding 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown for 3 h. The cells were harvested by centrifugation at 10,000 *g* for 20 min at 4 °C. The cell pellet was thoroughly suspended in lysis buffer [50 mM $NaH_2PO_4$, 300 mM NaCl, and 10 mM imidazole (pH 8)] and disrupted by sonication (Sonifier 450, Branson, Danbury, CT, USA; output 4, 10 s × 6, constant duty) in an ice bath. The cellular debris was spun down by centrifugation at 10,000 *g* for 30 min at 4 °C. The filtered supernatant was passed through a Ni-NTA affinity column (Qiagen). Before eluting $His_6$-tagged NPAS, the Ni-NTA affinity column was washed with washing buffer [50 mM $NaH_2PO_4$, 300 mM NaCl, and 20 mM imidazole (pH 8.0)] and finally rNPAS protein was eluted with elution buffer [50 mM $NaH_2PO_4$, 300 mM NaCl, and 250 mM imidazole (pH 8.0)]. The eluted fraction was dialyzed to remove the excess imidazole.

The expression of GST-tagged DGAS was performed as described above. The purification steps of rDGAS were almost the same as those of rNPAS, except for the lysis buffer used. The lysis buffer used to resuspend the pellet for the GST-tagged DGAS was phosphate-buffered saline (PBS) buffer (140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, and 1.8 mM $KH_2PO_4$, pH7.3). The supernatant was applied to a Glutathione–Sepharose™ High-Performance affinity column (Amersham Biosciences) pre-equilibrated with PBS buffer. The column was washed twice with PBS buffer. The bound recombinant DGAS was eluted with a 50-mM Tris–HCl buffer (pH 8.0) containing a 30 mM concentration of reduced glutathione. The elimination of fused glutathione S-transferase protein from the purified recombinant DGAS was carried out using thrombin treatment when necessary. The purity of rNPAS and rDGAS was checked by SDS–PAGE using 10% (w/v) polyacrylamide gels and Coomassie brilliant blue R-250 for staining. Protein concentrations were determined using the Bradford reagents kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard.

### 3.5. Enzyme assay

The hydrolysis activities of the ASases were determined by the dinitrosalicylic acid (DNS) method. The reaction mixture included 100 μL of 4% sucrose, 100 μL of deionized water, and 250 μL of 100 mM sodium citrate buffer (pH 6.0) for rNPAS and 100 mM Tris–HCl buffer (pH 8.0) for rDGAS. After the substrate solution was preincubated at 30 °C for 5 min, the reaction was initiated by the addition of 50 μL of the enzyme solution, and then continued for 10 min before being terminated by the addition of 500 μL of DNS solution, followed by inactivation by boiling. The absorbance of the reducing sugar at 545 nm was measured by a spectrophotometer (Beckman DU 730, Fullerton, CA, USA). Reducing sugar concentration was calculated using fructose as the standard. One unit of AS was defined as the amount of enzyme that catalyzes the production of 1 μmol fructose/min in the assay conditions.

### 3.6. Transglycosylation reactions of rNPAS and rDGAS

Transglycosylation reactions of rNPAS and rDGAS were performed with 0.5 mL of substrate solution, containing 4% (0.11 M) sucrose as a substrate and 0.5% (0.017 M), 1% (0.03 M), 2% (0.07 M), 3% (0.1 M), and 4% (0.14 M) salicin as the acceptor or containing 0.05 M, 0.1 M, 0.3 M, 0.6 M, 0.8 M, and 1.2 M sucrose as a substrate with 3% (0.1 M) salicin as an acceptor in 100 mM sodium citrate buffer (pH 6.0) for rNPAS reactions and 100 mM Tris–HCl buffer (pH 8.0) for rDGAS. The reactions were carried out at 30 °C for 14 h with 60 U/mL of each enzyme, followed by heating in boiling water for 30 min. The transglycosylated products in the reactions were analyzed by TLC and HPLC. The transglycosylation yield was defined as the ratio of synthesized salicin transglycosylated products to the initial salicin added.

### 3.7. Analytical methods

The detection and identification of transglycosylation products in the reaction were carried out with HPLC and TLC analyses. HPLC analysis was performed as follows. The reaction mixtures were boiled for 30 min to inactivate the enzyme and were centrifuged at 10,000 *g* for 5 min. The supernatants were filtered using a 0.45-μm filter and analyzed with a SUPELCO™ LC-$NH_2$ column (25 cm × 4.6 mm, 5 μm) connected to a Shimadzu LC-10AD*vp* system with SPD-10A UV–vis detector at 265 nm. The mobile phase (75:25 acetonitrile–deionized water) was delivered at a flow rate of 1.00 mL/min. All solvents were filtered, degassed, and kept under pressure. TLC analysis was performed with Whatman K5F silica gel plates (Whatman, Kent, 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 3:1:1 (v/v/v) *n*-BuOH–HOAc–$H_2O$ in a TLC tank. Ascending development was repeated once at room temperature. The plate was allowed to air-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 MeOH. The plate was dried and placed in an oven for 10 min at 110 °C to visualize the reaction spots.

### 3.8. Preparative recycling HPLC analysis

Each transfer product was fractionated and purified by a preparative recycling HPLC system (LC-9104, JAI, Tokyo, Japan) equipped with refractive index detection. Three milliliters of the transglycosylation reaction mixture were loaded onto a JAIGEL-W252 column (2 × 50 cm), which was connected with

two JAIGEL-W251 columns (2 × 50 cm) and an RI detector RI-50 (JAI, Tokyo, Japan). Deionized water was used for the elution of the sample at a flow rate of 3 mL/min. The purified products were lyophilized and used for NMR analysis.

### 3.9. NMR analysis

Salicin and salicin transfer products were dissolved in 0.5 mL of pure $CD_3OD$, and then placed into 5-mm NMR tubes. NMR spectra were obtained on a Varian Inova AS 400 NMR spectrometer (Varian, Palo Alto, CA, USA). COSY (homonuclear correlation spectroscopy), HMBC (heteronuclear multiple bond correlation spectroscopy), HSQC (heteronuclear single quantum correlation), and $^{13}C$ and $^{1}H$ NMR spectra were recorded, following standard experimental protocol. Tetramethylsilane (TMS) in $CD_3OD$ at 23 °C was used as the internal chemical shift reference.

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