J. Microbiol. Biotechnol. (2018), 28(11), 1859–1864
https://doi.org/10.4014/jmb.1807.07043

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# Stepwise Synthesis of Quercetin Bisglycosides Using Engineered *Escherichia coli* S

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*Received: July 24, 2018*
*Revised: September 10, 2018*
*Accepted: September 13, 2018*

First published online
September 20, 2018

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S upplementary data for this paper are available on-line only at http://jmb.or.kr.

pISSN 1017-7825, eISSN 1738-8872

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Synthesis of flavonoid glycoside is difficult due to diverse hydroxy groups in flavonoids and sugars. As such, enzymatic synthesis or biotransformation is an approach to solve this problem. In this report, we used stepwise biotransformation to synthesize two quercetin bisglycosides (quercetin 3-*O*-glucuronic acid 7-*O*-rhamnoside [Q-GR] and quercetin 3-*O*-arabinose 7-*O*-rhamnoside [Q-AR]) because quercetin *O*-rhamnosides contain antiviral activity. Two sequential enzymatic reactions were required to synthesize these flavonoid glycosides. We first synthesized quercetin 3-*O*-glucuronic acid [Q-G], and quercetin 3-*O*-arabinose [Q-A] from quercetin using *E. coli* harboring specific uridine diphopsphate glycosyltransferase (UGT) and genes for UDP-glucuronic acid and UDP-arabinose, respectively. With each quercetin 3-*O*-glycoside, rhamnosylation using *E. coli* harboring UGT and the gene for UDP-rhamnose was conducted. This approach resulted in the production of 44.8 mg/l Q-GR and 45.1 mg/l Q-AR. This stepwise synthesis could be applicable to synthesize various natural product derivatives in case that the final yield of product was low due to the multistep reaction in one cell or when sequential synthesis is necessary in order to reduce the synthesis of byproducts.

**Keywords:** Bioconversion, flavonoid glycosides, glycosyltransferase

## Introduction

Synthesis of glycones is a difficult task due to regioselectivity, and enzymatic synthesis could facilitate it. Glycosylation is carried out by uridine diphosphate-dependent glycosyltransferases (UGTs) [1]. Most UGTs show substrate and regioselectivity, although some display promiscuity [2]. Flavonoids are one of the most abundant natural compounds [3], and flavonoid glycosides are common forms found in plants [4]. Thus, this is a good model compound to study regioselective synthesis using UGTs. In addition, bioconversion of flavonoids into flavonoid glycosides using *Escherichia coli* harboring UGT could be possible [5, 6], and the three-dimensional structure of flavonoid UGTs are available [2]. UGTs use nucleotide sugars (mostly UDP-sugar) as sugar donors and, therefore, have specificity for flavonoids and nucleotide sugar. Glucose, galactose, glucuronic acid, xylose, arabinose, and rhamnose are common sugars that are attached to flavonoids [7]. UGTs have been used as biocatalysts for the glycosylation of flavonoids, and various flavonoid glycosides, including flavonoid *O*-monoglycosides, flavonoid *O*-diglycosides, and flavonoid *O*-bisglycosides, have been synthesized in engineered *E. coli* [5, 8]. For the synthesis of diverse flavonoid glycosides using *E. coli*, UGTs and nucleotide sugar biosynthetic genes were introduced. This approach achieved what could not be achieved through chemical synthesis.

*E. coli* synthesizes various nucleotide sugars, including UDP-glucoside, UDP-galactoside, UDP-glucuronic acid, UDP-*N*-acetylglucosamine, and TDP-glucoside [9]. These could serve as sugar donors for UGTs to synthesize flavonoid glycones. In addition, these nucleotide sugars can serve as substrates for the synthesis of other nucleotide sugars, such as UDP-arabinoside, UDP-xyloside, and UDP-rhamnoside [10, 11]. Therefore, *E. coli* is a good system to synthesize flavonoid glycosides. In addition, quercetin rhamnosides contain antiviral activity [12, 13]. In this

**Table 1.** Plasmids and *E. coli* strains used in this study.

| Plasmids or *E. coli* strain | Relevant properties or genetic marker | Source or reference |
|---|---|---|
| Plasmids | | |
| pACYCDuet | P15A ori, $Cm^r$ | Novagen |
| pCDFDuet | CDF ori, $Str^r$ | Novagen |
| pETDuet | f1 ori, $Amp^r$ | Novagen |
| pGEX 5X-2 | pBR322 ori, $Amp^r$ | GE Healthcare |
| pE-AmUGT-Ecugd | pET + *UGT* from *A. majus* + *ugd* from *E. coli* | [14] |
| pG-D3 | pGEX 5X-2 + *AUGT78D3* from *A. thaliana* | [10] |
| pA-AtUXS-Ecugd | pACYC + *UXS* from *A. thaliana* + *ugd* from *E. coli* | [10] |
| pC-C1-RHM | pACYCD + *AtUGT89C1* + *RHM2* from *A. thaliana* | [15] |
| pC-OsUXE | pCDF + *UGE* from *Oryza sativa* | [10] |
| Strains | | |
| BL21 (DE3) | $F^-$ *ompT hsdS*$_B$($r_B^-$ $m_B^-$) *gal dcm lon* (DE3) | Novagen |
| B407 | BarnA harboring pG-D3, pA-AtUXS-Ecugd, and pC-OsUXE | [10] |
| B509 | BarnA harboring pG-VvUGT and pA-Ecugd | [14] |
| B-R | BL21 harboring pC-C1-RHM | [14] |

report, we synthesized quercetin bisglycosides, Q-GR, and Q-AR. We used a stepwise synthesis method. First, quercetin 3-*O*-monoglycosides (quercetin 3-*O*-glucuronic acid [Q-G], and quercetin 3-*O*-arabinoside [Q-A]) were synthesized in *E. coli.* Subsequently, using this as a substrate, rhamnosylation was carried out in the second *E. coli*. Throughout this approach, 77.7 μM Q-AR and 71.8 μM Q-GR from 100 μM quercetin were synthesized, respectively.

## Materials and Methods

### Synthesis and Analysis of Metabolite

Genes involved in Q-A synthesis (*AtUGT78D3*, *OsUXE*, *AtUXS*, and *Ecugd*) were cloned previously [10]. Genes for the synthesis of Q-G were cloned previously [14]. *AtUGT89C1* and *AtRHM2* were cloned and named pC-C1-Rham [15]. The *E. coli* strains used in this study are listed in Table 1.

The synthesis of Q-A and Q-G was carried out as described before [10, 14]. M9 medium containing antibiotics and 100 μM quercetin was used for the synthesis of Q-A and Q-G. Proteins in the *E. coli* strain B-R grown in LB medium containing spectinomycin were induced by adding IPTG at the concentration of 1 mM and incubating cells at 18°C for 30 h. The cells were harvested and resuspended in the culture filtrate containing either Q-A or Q-G, which was further incubated at 30°C with shaking. Optimal cell concentration of *E. coli* strain B-R was determined by adjusting the cell density to $OD_{600}$ = 1, 2, 3, 4, 5, and 6.

For the purification of the reaction product, the culture filtrate was passed through a column (20 × 3 cm) packed with a non-polar copolymer styrene–divinylbenzene adsorbent resin (HP20, Samyang, Korea). The binding reaction product was eluted with methanol and was evaporated to dryness. The sample was further purified using HPLC [16]. The structure of the product was determined using nuclear resonance spectroscopy (NMR) [15]. Complete assignments of the Q-GR and the Q-AR were in the supplementary data.

### Molecular Modeling of AtUGT89C1

AtUGT89C1 was used to convert quercetin 3-*O*-glucoside and quercetin 3-O-rhamnoside into quercetin 3-*O*-glucoside 7-*O*-rhamnoside and quercetin 3,7-*O*-dirhamnoside [15]. In order to test whether AtUGT89C1 could use Q-A and Q-R as a substrate, we carried out the molecular modeling. The model structure of AtUGT89C1 was obtained from the SWISS-MODEL server (https://swissmodel.expasy.org) by using the crystal structure of hydroquinone glucosyltransferase (PDB ID: 2vg8) as a template (sequence identity = 27.98%). Uridine-5’-diphosphate (UDP) in the template was merged into the model structure, and the UDP was modified into UDP-rhamnoside using the BUILD module of the MAESTRO program (Schrödinger Inc.). The resulting AtUGT89C1-UDP-rhamnoside complex was subjected to energy minimization to obtain a stable, low-energy conformation. Energy minimization was performed using the MacroModel module incorporated in the Maestro (Schrödinger Inc) (conjugate gradient minimization with 0.05 convergence criteria, the OPLS-AA force field, and GB/SA continuum water model). The ligand structures (Q-G and Q-A) were prepared using the BUILD module, which were energy-minimized through the MacroModel module incorporated in the Maestro (Schrödinger Inc). The energy-minimized structure of AtUGT89C1-UDP-rhamnoside complex was used for ligand-docking in order to determine the binding

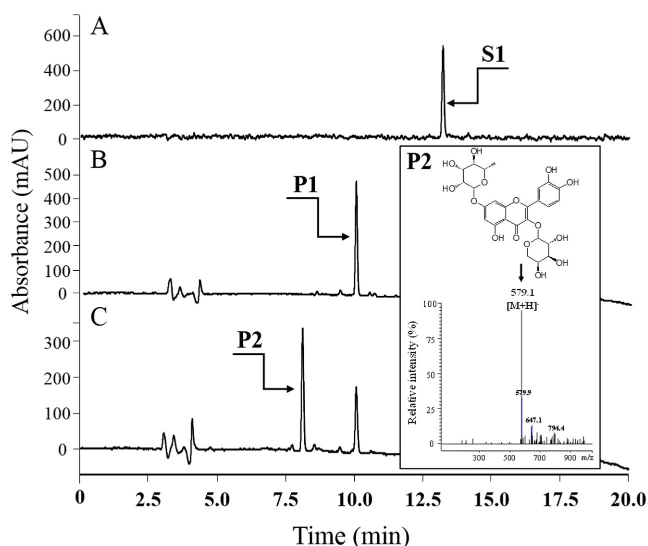


**Fig. 1.** Stepwise synthesis of quercetin 3-*O*-arabinoside-7-*O*-rhamnoside.

A, standard quercetin; B, reaction product from strain B407 (P1); C, reaction product from strain B-R fed the reaction product from strain B407. P1 is quercetin 3-*O*-arabinoside, and P2 is quercetin 3-*O*-arabinoside-7-*O*-rhamnoside. Inset is the mass spectra of the reaction product (P2).

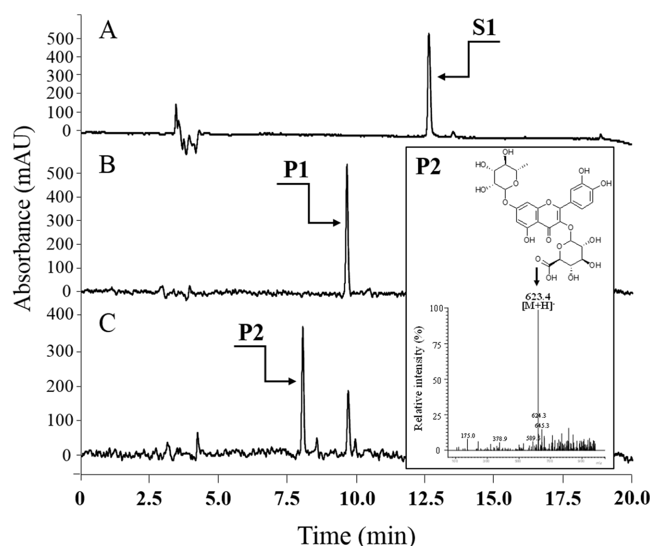


**Fig. 2.** Synthesis of quercetin 3-*O*-glucuronic acid 7-*O*-rhamnoside.

A, standard quercetin (S1); B, reaction product from strain B509; C, reaction product from strain B-R fed the reaction product from strain B509. P1 is quercetin 3-*O*-glucuronic acid, and P2 is quercetin 3-*O*-glucuronic acid 7-*O*-rhamnoside. Inset is the mass spectra of the reaction product.

mode of the flavonoid conjugates. We used the protein preparation utilities in Maestro to assign the charge state of ionizable residues, add hydrogens, and carry out energy minimization. The ligands were docked into the modeled AtUGT89C1-UDP-rhamnoside complex using GLIDE (http://www.schrodinger.com). The default setting of the extreme precision mode of GLIDE was employed for the docking, and the top scored pose was chosen for the binding mode analysis.

## Results and Discussion

### Synthesis of Quercetin *O*-Bisglycosides Using Engineered *E. coli*

Quercetin is one of the abundant flavonoids in plants containing five hydroxy groups. This is a good substrate for the synthesis of quercetin *O*-bisglycosides. Quercetin *O*-bisglycosides were synthesized by two sequential glycosylation reactions. The first reaction was the attachment of sugar from UDP-glucuronic acid and UDP-arabinoside to the 3-OH group of quercetin. UGTs for each reaction were cloned from plants, and *E. coli* harboring each UGT and nucleotide sugar biosynthesis gene(s) were used to synthesize these quercetin 3-*O*-glycosides. For the synthesis of Q-A, the strain B407 [10] was used, and this strain harbored genes for the biosynthesis of UDP-arabinose from UDP-glucuronic acid and UGT by having a high specificity for quercetin and UDP-arabinose. The B407 converted 100 μM of quercetin into Q-A (Fig. 1B). The strain B509 [14], which harbored UGTs specific for quercetin and UDP-glucuronic acid and the *ugd* encoding UDP-glucose dehydrogenase that converts UDP-glucose into UDP-glucuronic acid, was used (Table 1). This strain also converted 100 μM of quercetin into Q-G (Fig. 2B).

The second reaction used the Q-G or the Q-A as a sugar acceptor, and UDP-rhamnose as a sugar donor. AtUGT89C1 can use quercetin 3-*O*-glucoside and quercetin 3-*O*-rhamnoside as a substrate and UDP-rhamnoside as a sugar donor [15]. However, it is unknown whether AtUGT89C1 could use Q-G or Q-A. In order to examine this, we conducted molecular docking using the modeled structure of AtUGT89C1. Each quercetin 3-*O*-glycoside along with UDP-rhamnose could fit into the active site of AtUGT89C1 (Fig. 3). The binding modes of Q-G or Q-A into AtUGT89C1 were very similar. Three amino acid residues (Ser152, Gln159, and Arg162) were key for binding to the sugar acceptor. Among them, Arg162 and Gln159 formed a hydrogen bond with hydroxy groups of sugar in Q-A or Q-G. Ser152 formed two hydrogen bonds with 3′ and 4′ hydroxy groups of quercetin. These results indicated that AtUGT89C1 can be used for the second glycosylation reaction.

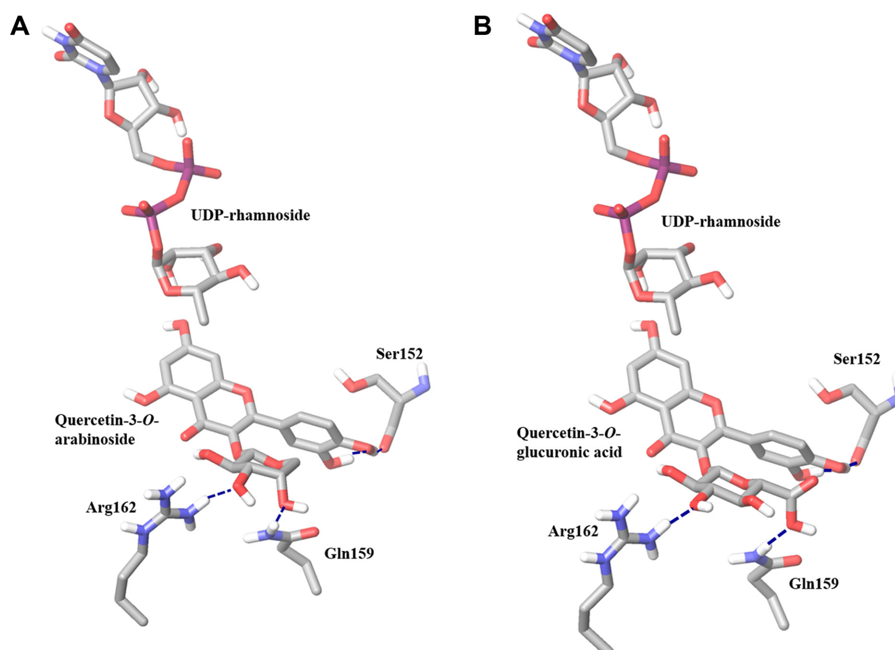


**Fig. 3.** Molecular docking of quercetin 3-*O*-arabinoside and UDP-rhamnoside (**A**) or quercetin 3-*O*-glucuronide and UDP-rhamnoside (**B**) into AtUGT89C1.

Based on the molecular modeling result, we carried out the second reaction to synthesize quercetin *O*-bisglycosides. For the second glycosylation reaction, we used the strain B-R, which contained AtUGT89C1 and RHM encoding rhamnose synthase, which converts UDP-glucose into UDP-rhamnose. In order to optimize the conversion of Q-A and Q-G into the corresponding 7-*O*-rhamnoside, the optimal initial cell concentration was examined. After protein induction in strain B-R by adding IPTG, the cells were harvested. The cells were resuspended with M9 medium at the concentration of 1, 2, 3, 4, 5, and 6 with 100 µM of substrate. The conversion of each quercetin 3-*O*-glycoside into the corresponding quercetin *O*-bisglycoside increased until $OD_{600} = 5$. However, at $OD_{600} = 6$, the conversion decreased. Therefore, we used the cell concentration at $OD_{600} = 5$.

Culture filtrate of each quercetin 3-*O*-glycoside was mixed with the strain B-R at $OD_{600} = 5$, and the production of quercetin *O*-bisglycoside was monitored. Production of Q-AR was increased and reached to maximum at 36 h, at which approximately 45.1 mg/l (77.7 µM) Q-AR was synthesized, and 6.7 mg/l (15.4 µM) Q-A remained. The synthesis of Q-GR reached to the maximum at 24 h, at which 44.8 mg/l (71.8 µM) was synthesized while 12.1 mg/l (25.3 µM) of Q-G remained. Starting from quercetin, Q-AR, and Q-GR showed 77.7% and 71.8% conversion rates, respectively.

**Structural Determination of Reaction Products**

The structures of quercetin *O*-bisglycosides were determined by NMR experiments. The purified Q-GR dissolved in DMSO-$d_6$, and $^1$H, $^{13}$C, $^1$H-$^1$H COSY, $^1$H-$^1$H NOESY, $^1$H-$^{13}$C HMQC, and $^1$H-$^{13}$C HMBC NMR experiments were performed. There were five aromatic signals in the $^1$H NMR spectrum. They were easily assigned as protons of quercetin. Two $^1$H signals at 5.55 ppm and 5.47 ppm were identified as H-R2 and H-G6, respectively. The chemical shifts of these two protons were distinguishable from other sugar protons because they were positioned between two oxygen atoms. Four NOE correlations, H-R2/H-6, H-R2/H-8, H-G6/H-2′, and H-G6/H-6′ in the NOESY spectrum identified the sugar positions (Fig. 5A). The glucuronic acid was attached to 3-OH while the rhamnose was attached to 7-OH via O-linked glycosylation. The $^1$H of methyl from rhamnose showed at 1.12 ppm, and the other protons from glucuronic acid and rhamnose were dispersed from 3.28 ppm to 3.85 ppm. From these, the structure of this compound was determined to be Q-GR.

$^1$H, $^{13}$C, $^1$H-$^1$H NOESY, $^1$H-$^{13}$C HMQC, and $^1$H-$^{13}$C HMBC NMR experiments were also carried out with the purified

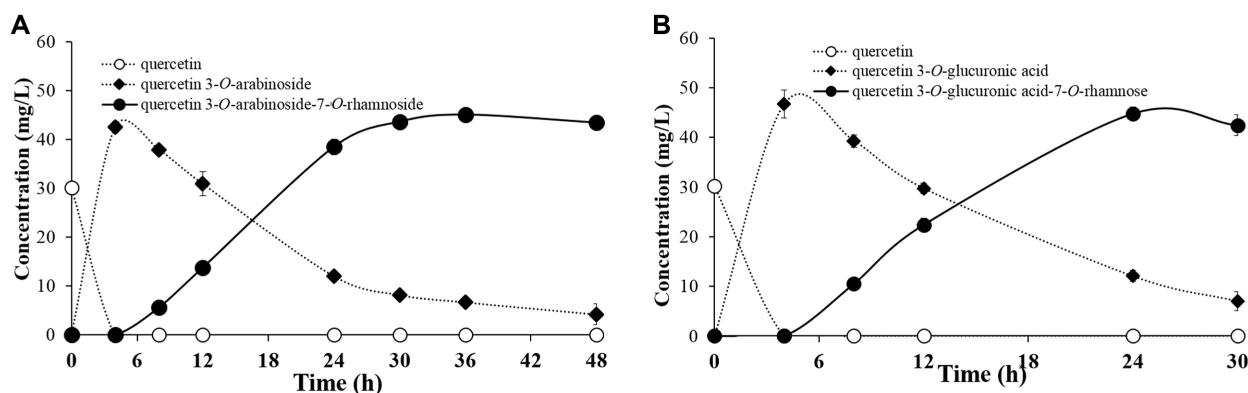


**Fig. 4.** Sequential synthesis of quercetin 3-*O*-arabinoside 7-*O*-rhamnoside (**A**) and quercetin 3-*O*-glucuronic acid 7-*O*-rhamnoside (**B**).

Quercetin 3-*O*-arabinoside and quercetin 3-*O*-glucuronic acid were synthesized using strain B407 and B509, respectively. Subsequently, the culture filtrate was mixed with strain B-R, and the resulting mixture was incubated for 48 h.

product of putative Q-AR. All $^{1}$H and partial $^{13}$C peaks were assigned as mentioned above. Several important NOE and HMBC cross peaks were observed to confirm the structure (Fig. 5B). In the NOESY spectrum, H-2′ and H-6′ correlated with H-A2. H-A2 was also long-range coupled to the C-3 of quercetin in the HMBC spectrum. The H-R2 showed NOEs with H-6 and H-8 and long-range coupling with C-7 in the HMBC spectrum. These data supported that the synthesized compound was Q-AR.

We successfully synthesized Q-AR and Q-GR. Chemical synthesis of flavonoid *O*-bisglycosides has been carried out throughout multiple steps [17, 18]. However, these two compounds have not been synthesized previously. Stepwise synthesis using two kinds of cells has an advantage over synthesis using one cell containing all genes (called a monoculture system). Synthesis using a monoculture system often produced byproduct(s). AtUGT89C1 showed less regioselectivity and substrate specificity. It could transfer a rhamnose group from UDP-rhamnose to 3-OH of quercetin to form quercetin 3-*O*-rhamnoside. Subsequently, the quercetin 3-*O*-rhamnoside could be used for the second round rhamnosylation of AtUGT89C1 to form quercetin 3,7-*O*-bisrhamnoside [15]. However, the synthesis of quercetin 3-*O*-glycosides in one cell using regioselective UGT could prevent the formation of the byproducts as shown in this study. Recently, modulating cell ratios to consist of more than three different cells to synthesize anthocyanin has been attempted to maximize productivity [18]. This approach is called a polyculture system, which could reduce the byproduct formation and maximize the synthesis of the main product.

Due to the promiscuity of AtUGT89C1, it could be possible to synthesize other quercetin 3-*O*-glycoside 7-*O*-rhamnoside. Previously, we showed the biosynthesis of

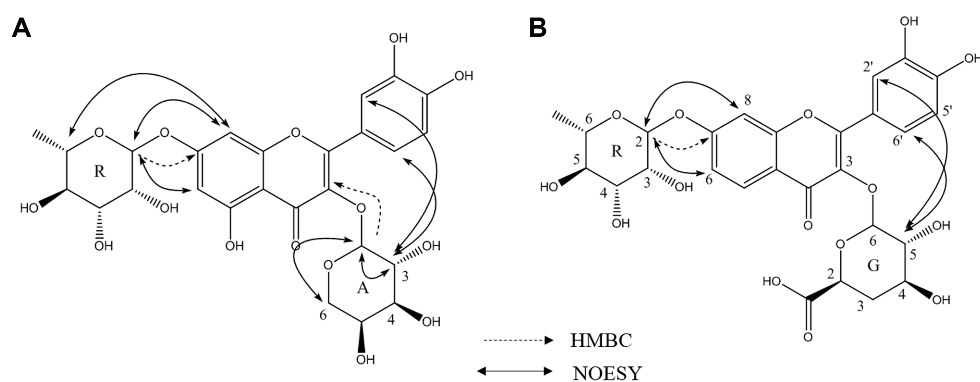


**Fig. 5.** Important long-range couplings and NOE correlations observed in the HMBC and NOESY spectra for the structure identifications of quercetin 3-*O*-arabinose 7-*O*-rahmnoside (**A**) and quercetin 3-*O*-glucuronic acid 7-*O*-rhamnoside (**B**).

quercetin 3-*O*-glucoside 7-*O*-rhamnoside and quercetin *O*-3,7-bisrhamnoside [15]. Quercetin 3-O-*N*-acetylglucosimine [20], quercetin 3-*O*-galactoside [14], and quercetin 3-*O*-xyloside [10] were synthesized previously. Molecular docking study of these three quercetin 3-*O*-glycosides showed a similar binding mode to quercetin 3-*O*-arabinose and quercetin 3-*O*-glucuronic acid (data not shown). Therefore, other flavonoid glycosides using AtUGT89C1 could be achievable.

## Acknowledgments

This paper was supported by Konkuk University in 2016.

## Conflict of Interest

The authors have no financial conflicts of interest to declare.

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