Specific hydroxylation and glucuronidation of 2'-hydroxyflavanone by *Streptomyces coeruleorubidus* NRRL B-2569

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Short title: Hydroxylation and glycosylation of 2'-hydroxyflavanone

ABSTRACT

Flavonoids constitute a class of natural compounds with varied bioactivities. Nevertheless, the potential health benefits of flavonoids for humans are often compromised by their low water solubility and limited bioavailability. In this study, four derivatives, namely 2',5'-dihydroxyflavanone (**2**), 5'-dihydroxyflavone-2'-*O*-*β*-D-glucuronide (**3**), and two isomers of hydroxyflavanone-2'-*O*-*β*-D-glucuronide (**4** and **5**), were biosynthesized from substrate 2'-hydroxyflavanone (**1**) through the specific hydroxylation and glucuronidation using *Streptomyces coeruleorubidus* NRRL B-2569. Product **2** was identified as a known compound while **3**-**5** were structurally characterized as new structures through extensive 1D and 2D NMR analysis. The water solubility of obtained products **3**-**5** were enhanced by 36 to 340 times compared to the substrate. Moreover, the antioxidant assay revealed that **3** exhibited improved 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity compared to the substrate, decreasing the logIC_{50} from 10.77 ± 0.05 μM to 9.55 ± 0.05 μM. Compound **3** also displayed significantly higher anticancer activity than the substrate 2'-hydroxyflavanone against Glioblastoma 33 cancer stem cells (GSC33), decreasing the IC_{50} from 25.05 μM to 7.07 μM. Thus, *S. coeruleorubidus* NRRL B-2569 stands out as an effective tool for modifying flavonoids, thereby enhancing their water solubility and bioactivities.

Key words: 2'-Hydroxyflavanone; Antioxidant; Anticancer; Hydroxylation; Glucuronidation

Dietary flavonoids showcase a wide array of biological activities, offering diverse benefits to human health, including anti-inflammatory, anticancer, anti-obesity, antioxidant, and antimicrobial effects (1). Nonetheless, the low water solubility of flavonoids impedes their potential health benefits for humanity (2). In recent years, researchers have been dedicating their efforts to exploring effective methods for producing natural product glycosides for a variety of reasons (3, 4). Chief among these reasons is the fact that glycosylation can diversify the chemical pool of natural products, thereby offering numerous potential candidates for new drug development (5). Sugar moieties generally improve water solubility of dietary flavonoids, with some glycosides exhibiting improved bioavailability, stability, and bioactivity or reduced toxicity relative to their aglycones (6, 7).

The extraction of flavonoid glycosides from plants is time-consuming and often suffers from low yields, such as the isolation of quercetin-3-rutinoside from the fruits of *Ficus sycomorus* (8). This is not only due to their naturally low abundance but also because the yield is heavily influenced by various factors, including seasonal and vegetation conditions (9-11). Similarly, the organic synthesis of target glycosides presents challenges, as it necessitates the protection and deprotection of reactive groups in substrates, exemplified by the chemical synthesis of quercetin-3-*O*-glucuronide (12). Additionally, the utilization of costly catalysts and the generation of toxic wastes make chemical synthesis an economically inefficient and environmentally unfriendly approach for large-scale production of bioactive glycosides (13, 14).

Compared to the aforementioned methods, biotransformation is emerging as an

appealing alternative with significant potential to produce bioactive and novel flavonoid derivatives. This process can enhance water solubility through various reactions such as glycosylation and hydroxylation (15). Microbial biotransformation can facilitate the biosynthesis of glycosides through an affordable, straightforward process, conducted under mild conditions (16). *Streptomyces* are renowned for their capacity to produce various bioactive molecules through diverse biosynthetic pathways (17). In this work, we employed 2'-hydroxyflavanone, a natural compound abundant in fruits and vegetables, known for its remarkable anticancer effects (18), as the substrate to biosynthesize novel glycosides through microbial biotransformation using *Streptomyces coeruleorubidus* NRRL B-2569. Four derivatives, namely 2',5'-dihydroxyflavanone (**2**), 5'-dihydroxyflavone-2'-*O*-*β*-D-glucuronide (**3**), and two isomers of hydroxyflavanone-2'-*O*-*β*-D-glucuronide (**4** and **5**) were synthesized, all exhibiting varying degrees of enhanced water solubility. Additionally, compound **3** demonstrated stronger radical scavenging and anticancer activities compared to the substrate. The biotransformation products could serve as promising candidates for further investigations as potentially active compounds, offering valuable insights into structure-bioactivity relationships. *S. coeruleorubidus* NRRL B-2569 could be used as an effective biocatalyst to structurally modify other flavonoids through specific hydroxylation and glucuronidation.

MATERIALS AND METHODS

General experimental procedures The analysis and purification of the

products were performed using an Agilent 1200 HPLC instrument equipped with an Agilent Eclipse Plus-C_{18} column (5 µm, 250 mm × 4.6 mm). The samples were eluted with a methanol-water gradient (35:65 to 95:5, v/v) over 30 minutes, containing 0.1% formic acid, at a flow rate of 1 mL/min. Molecular weights of the compounds were confirmed using an Agilent 6130 single quadrupole LC-MS.

NMR spectra were collected by dissolving the purified compounds in deuterated dimethyl sulfoxide (DMSO-d_6) and analyzing them with a Bruker Avance III HD Ascend-500 NMR instrument (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) at the Department of Chemistry and Biochemistry, Utah State University. Chemical shift (δ) values are reported in parts per million (ppm), and coupling constants (J values) are reported in hertz (Hz).

Antioxidant assays were conducted using a SpectraMax® 190 microplate reader (Molecular Devices, USA). Ascorbic acid (vitamin C) and 2'-hydroxyflavanone were purchased from Sigma-Aldrich (USA) and Tokyo Chemical Industry (USA), respectively. Compounds were purified by chromatography using Sephadex™ LH-20 (Cytiva, USA) and normal-phase silica gel (40-60 µm, VWR® Agela Technologies, USA). All solvents were obtained from Fisher Scientific, and Milli-Q water was used throughout the study.

S. coeruleorubidus NRRL B-2569 was provided by the United States Department of Agriculture-ARS Culture Collection (NRRL) and cultured in Yeast-Malt (YM) medium, consisting of yeast extract (4 g/L), malt extract (10 g/L), and glucose (4 g/L) at 28 °C. Glucose and yeast extract were obtained from Thermo Fisher Scientific, while

malt extract was acquired from Gibco™. For mammalian cell culture maintenance, Dulbecco's Modified Eagle Medium (DMEM), Ham's F12 media, fetal bovine serum (FBS), trypsin-EDTA (0.25%) (all from Gibco, MA, USA), and Accutase Cell Dissociation Reagent (Millipore, MA, USA) were used. Tetrazolium MTT and 96-well plates were purchased from Fisher Scientific. Absorbance in the cell viability assay was measured using a BioTek Synergy HTX Multimode Reader. Additionally, 99.7% DMSO was obtained from Sigma-Aldrich.

A Waters Acquity UPLC system equipped with an I-Class Binary Solvent Manager and an H-Class Flow Through Needle autosampler hyphenated to a Waters Xevo-G2-XS Quadrupole Time-of-Flight (QToF) mass spectrometer was used to acquire high resolution mass spectra (HRMS). The UPLC was performed using a Waters Acquity Premier HSS T3 C18 column (1.8 µM, 2.1 mm × 150 mm). The samples were eluted with acetonitrile-water (2:98 to 95:5, v/v, over 12 minutes, containing 0.1% formic acid) at a flow rate of 0.4 mL/min. The mass analysis was carried out in ESI negative mode with the capillary voltage (1.0 kV), cone voltage (50 V), source temperature (100 °C), desolvation gas temperature (450 °C), cone gas flow rate (50 L/h), and desolvation gas flow rate (750 L/h) as the major parameters. The collision cell energy was set to 6V for acquisition of MS1 spectra, and to 30 V for acquisition of MS2 spectra using Argon as the collision gas.

Cultivation of *S. coeruleorubidus* NRRL B-2569 for the biotransformation of 2'-hydroxyflavanone and its time-course analysis To assess the biotransformation capability of *S. coeruleorubidus* NRRL B-2569 for 2'-

hydroxyflavanone, the strain was cultured in 50 mL of YM medium on a rotary shaker at 250 rpm and 28 °C for 3 days. Afterward, 4 mg of 2'-hydroxyflavanone, dissolved in 100 μL of DMSO, was introduced into the culture medium, which was then incubated under the same conditions for an additional 3 days. Subsequently, 1 mL samples of the cultures were collected and centrifuged at 15,000 ×g for 10 minutes. The supernatant was analyzed using HPLC at 300 nm. For time-course analysis, the procedure was repeated with 1 mL of cultivation broth sampled every 24 hours for 6 days post-substrate addition, with 100 μL of the supernatant injected into the HPLC after centrifugation.

Product extraction and isolation To isolate the biotransformation products of 2'-hydroxyflavanone for structural elucidation, *S. coeruleorubidus* NRRL B-2569 was cultured in a 2-L Erlenmeyer flask containing 500 mL of YM medium. A total of 50 mg of 2'-hydroxyflavanone was utilized for the biotransformation process. Following 6 days of cultivation at 250 rpm and 28 ℃, the *S. coeruleorubidus* NRRL B-2569 culture was centrifuged at 4,000 ×g for 20 minutes to collect the supernatant. The harvested cultivation broth was then evaporated under reduced pressure at 38 ℃, and the resulting residue was dissolved in 15 mL of 50% methanol-water (v/v). Subsequently, the dissolved residue was mixed with 20 g of silica gel. After drying, the crude sample was subjected to normal phase silica gel column chromatography, using a chloroform-methanol (5:2, v/v) elution to eliminate most of the endogenous metabolites of *S. coeruleorubidus* NRRL B-2569. The samples were then filtered and subsequently passed through a Sephadex LH-20 column chromatography, eluting with methanol-

160 water (1:1, v/v). Products-enriched fractions were pooled and subjected to further
161 separation via reverse-phase HPLC, employing a methanol-water gradient elution
162 system (35-95%, 0-15 min) containing 0.1% formic acid (v/v) to isolate the target
163 products. Subsequently, further purification was conducted using a gradient elution
164 method (32-32%, 0-20 min; 35-95%, 20-30 min), resulting in the isolation of product
165 **3** (2.6 mg). Similarly, reverse-phase HPLC employing an isocratic elution of methanol-
166 water (50-50%, 0-30 min) was utilized to isolate products **4** (2.9 mg) and **5** (2.4 mg).
167 To isolate compound **2** (1.5 mg), the cultivation process was terminated on day 3, and
168 a 150 mL of cultivation broth sample was subjected to the same isolation procedures as
169 mentioned earlier, excluding the HPLC purification steps. All purified products were
170 dissolved in DMSO-d_6 and subjected to NMR analysis, and their chemical structures
171 were characterized based on the NMR spectra. The co-injection sample of products **4**
172 and **5** were eluted with a methanol-water gradient (35:65 to 95:5, v/v) over 30 minutes,
173 containing 0.1% formic acid, at a flow rate of 1 mL/min.

174 **Determination of the water-solubility of products** The purified products were
175 evaluated for water solubility using a previously described method (19). Purified 2'-
176 hydroxyflavanone biotransformation products were used to create standard curves for
177 determining their water solubility. Briefly, the purified compounds and the substrate
178 were each mixed with 600 µL of distilled water in Eppendorf tubes at 25 °C. Ultrasonic
179 agitation was applied to aid dissolution. After 60 minutes of sonication, the samples
180 were centrifuged at 13,000 × *g* for 10 minutes. The supernatant from each sample was
181 analyzed using reverse-phase HPLC to determine compound concentrations, with a

methanol-water gradient elution system (35-95%, 0-15 min) containing 0.1% formic acid (v/v). The substrate, 2'-hydroxyflavanone, was analyzed using a different elution gradient (50-95%, 0-15 min). Each test was performed in triplicate, and the water solubility of each sample is reported as the mean ± standard deviation (SD).

Antioxidant assay Antioxidant assays were conducted following the methodology outlined in our previous work (19). Due to the substrate's sub-detectable DPPH radical scavenging activity, we adjusted the treatment concentration range to 8,000-500 μM for the substrate and compounds **3**, **4**, and **5**. Conversely, the concentration range for vitamin C was set from 1,000-62.5 μM to remain within the scavenging assay's dynamic range. To account for the significant intra-treatment variance in IC_{50} values caused by the substrate's low activity, the data were logarithmically transformed for more accurate comparisons between the substrate and its derivatives. Log IC_{50} values were calculated in triplicate where applicable and analyzed using one-way ANOVA. Pairwise comparisons among treatments were conducted with the Tukey-Kramer adjustment to control the family-wise error rate.

Procedures for cell culture maintenance Glioblastoma 33 cells were cultured as spheroids in T25 flasks using serum-free media composed of 70% DMEM and 30% Ham's F12. The media was supplemented with B-27 (1:50), epidermal growth factor (EGF) (1:5000), and fibroblast growth factor (bFGF) (1:5000). Spheroids were dissociated and passaged with Accutase Cell Dissociation Reagent and were passaged every 5-6 days to maintain their size at less than 1 mm. The media was refreshed every 48 hours.

Seeded cell monolayers were cultured in media containing 90% Ham's F12 and 10% fetal bovine serum. The mouse fibroblast cell line L929 (ATCC, VA, US) was maintained in media with 90% DMEM and 10% fetal bovine serum. Cells were passaged using trypsin-EDTA (0.25%) and cultured at 37 °C with 5% CO_2 until reaching 80% confluency. L929 cells were passaged at a seeding density of 1.5×10^4 cells/cm^2.

Cell viability assay The effect of each compound on cell viability was assessed using the MTT Assay (20). Glioblastoma 33 cancer stem cells (GSC33) and L929 fibroblast cells (L929) were seeded at a density of 10^4 cells per well in clear, flat-bottomed 96-well plates. Cells were allowed to adhere overnight for 12 hours at 37 °C in a CO_2 incubator. After attachment, 200 µL of each treatment was added to the wells, with each treatment group consisting of three wells. The compounds were initially dissolved in DMSO and then diluted in cell culture media, resulting in final concentrations ranging from 0 to 100 µM, with 1% DMSO as the delivery vehicle. Cells were incubated with the treatments for 24 hours at 37 °C in a CO_2-buffered cell culture incubator. Following incubation, the media was replaced with fresh media without treatment. Next, 10 µL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 4 hours at 37 °C. The media was then aspirated, leaving the formazan crystals. To dissolve the crystals, 50 µL of DMSO was added to each well and incubated for 30 minutes at 37 °C. Absorbance at 540 nm was measured using a spectrophotometer to determine cell viability with the following equation:

$$Viability\ \% = \frac{OD540_{sample} - OD540_{blank}}{OD540_{control} - OD540_{blank}} * 100$$

The control wells, assumed to have 100% viability, contained cells incubated with only 1% DMSO. Blank wells contained only 50 µL of DMSO. IC_{50} values were calculated using GraphPad Prism 9 with a nonlinear regression curve fit [inhibitor] *vs.* normalized response, and the data were plotted using the same software.

RESULTS

Biotransformation of 2'-hydroxyflavanone by *S. coeruleorubidus* NRRL B-2569 In our prior study, *S. coeruleorubidus* NRRL B-2569 was identified as a viable biocatalyst capable of catalyzing the biotransformation of 2'-hydroxyflavone into its glucuronidated derivative (19). We posited that this strain could potentially catalyze the introduction of a glucuronic acid moiety to other bioactive flavonoids, such as 2'-hydroxyflavanone. To test our hypothesis, 2'-hydroxyflavanone underwent incubation with *S. coeruleorubidus* NRRL B-2569. Subsequent HPLC analysis unveiled the biosynthesis of four additional polar metabolites, namely, product **2** (14.8 min), product **3** (10.5 min), product **4** (18.6 min), and product **5** (19.0 min) from the incubation of 2'-hydroxyflavanone (23.3 min) with *S. coeruleorubidus* NRRL B-2569 (Fig. 1A). The UV absorption spectra of products **2**-**5** closely resembled that of 2'-hydroxyflavanone, indicating that these four polar products are derivatives of the substrate (Figs. 1B-E). Among them, the UV spectra of compounds **4** and **5** exhibit remarkable similarity, implying a high degree of structural resemblance between products **4** and **5**. The ESI-

MS spectra of compounds **2-5** (Figs. 1F-I) display corresponding quasimolecular ions [M-H]- at *m/z* 254.9, 431.1, 414.9, and 415.1, respectively, indicating that the molecular weights of these products are 256, 432, 416, and 416. These weights are 16, 192, 176, and 176 mass units greater than that of the substrate (*m/z* 240), suggesting the potential introduction of hydroxyl and/or glucuronic acid moieties to the substrate, resulting in the formation of these four products.

Structural characterization of the biotransformed products of 2'-hydroxyflavanone by *S. coeruleorubidus* NRRL B-2569 Product **2** was isolated as a brown, amorphous powder. Its molecular weight of 256 corresponds to a molecular formula of $C_{15}H_{12}O_4$. Compound **2** harbors a hydroxyl group at B ring, which was proved by the observation of an ABX spin system determined in the tri-substituted aromatic ring B [δ_H/δ_C 6.60 (dd, J = 8.6, 3.0 Hz)/115.7 (CH-4'), 6.69 (d, J = 8.6 Hz)/116.2 (CH-3'), and 6.89 (d, J = 2.9 Hz)/113.1 (CH-6')]. The HMBC spectrum revealed a correlation between H-2 at δ_H 5.70 and C-6' at δ_C 113.1, confirming that the hydroxyl group is attached at C-5'. This was further supported by the ^{13}C NMR, which showed a downfield carbon signal at 149.9 (C-5'). Based on the NMR analysis (Figs. S1-S5), compound **2** was characterized as 2',5'-dihydroxyflavanone by comparing with the NMR data from reported literature (21).

Product **3** was isolated as an orange, amorphous powder. The molecular formula of product **3** was determined to be $C_{21}H_{20}O_{10}$, based on the $[M-H]^-$ peak at *m/z* 431.0984 (calculated: 431.0984 for $C_{21}H_{19}O_{10}$) in the high-resolution electrospray ionization mass spectrometry (HRESIMS) spectrum (Fig. S6), indicating the possible addition of

a hydroxyl group and a sugar moiety to the substrate. The NMR analysis (Figs. S7-S11) was performed to further elucidate the chemical structure of **3**. Compound **3** is similar to **2** except that an additional sugar moiety was observed. In addition to the 15 signals belonging to the substrate, six extra carbon signals at δ_C 170.1, 102.4, 75.4, 75.4, 73.0, and 71.3 were found in the spectra (Fig. S8), together with the additional proton signals at δ_H 3.20-4.76 in the ^{1}H NMR spectrum (Fig. S7), suggesting that a sugar moiety has been added to 2',5'-dihydroxyflavanone (product **2**). Unlike the common sugar glucose, this sugar moiety has a quaternary carbon signal at δ_C 170.1, indicating the presence of a carboxyl group in the sugar moiety. Therefore, both the ^{1}H and ^{13}C signals of this sugar moiety are consistent with a glucuronic acid moiety (3). Moreover, the ^{1}H NMR spectrum showed a doublet at δ_H 4.76, indicative of an anomeric proton with a coupling constant of 7.6 Hz, and the chemical shift along with the *J*-coupling value were consistent with that of *β*-D-glucuronic acid. The correlation of the anomeric H-1" signal at δ_H 4.76 to C-2' signal at δ_C 146.2 in the HMBC spectrum (Fig. S11) revealed that the glucuronic acid moiety was located at C-2'. Based on the above spectral evidence, compound **3** was identified to be a new compound, namely, 5'-dihydroxyflavone-2'-*O*-*β*-D-glucuronide and all signals were assigned accordingly (Fig. 2A and Table 1).

Product **4** was isolated as a white, amorphous powder. It has a molecular formula of $C_{21}H_{20}O_9$, as indicated by the $[M-H]^-$ peak at *m/z* 415.1041 (calculated: 415.1035 for $C_{21}H_{19}O_9$) in the HRESIMS spectrum (Fig. S12), indicating that it also contains a glucuronic acid moiety. The NMR analysis (Figs. S13-S17) was performed to further elucidate the chemical structure of **4**. The NMR spectra of compounds **4** and **3** are very

similar, except that two sets of ABCD coupled methines were observed in the di-substituted aromatic A and B rings by analyzing ^{1}H-^{1}H COSY spectrum (Fig. S15). Moreover, the glucuronic acid moiety was determined to be attached at C-2' by observing the correlation between H-1" at δ_H 4.98 and C-2' at δ_C 153.4. Based on the above spectral evidence, the compound was identified to be a new compound, namely hydroxyflavanone-2'-*O*-*β*-D-glucuronide (Fig. 2A and Table 1).

Product **5** was also isolated as a white, amorphous powder. The $[M-H]^-$ peak at *m/z* 415.1042 (calculated: 415.1035 for $C_{21}H_{19}O_9$) in the HRESIMS spectrum (Fig .4D and Fig. S18) confirms that product **5** (yellow, amorphous powder) also has the same formula of $C_{21}H_{20}O_9$ as product **4** (Fig .4C and Fig. S12), indicating that they are two isomers. The chemical shift values (Figs. S13, 14, 19, and 20) of carbons and protons in products **4** and **5** align closely, with the exception of a few signals near the bond between C-2 and C-1' and signals on the glucuronic acid moiety: [δ_C 74.0 for **4**/73.2 for **5** (C-2), 43.2 for **4**/42.8 for **5** (C-3), 128.3 for **4**/127.9 for **5** (C-1'), 153.4 for **4**/153.8 for **5** (C-2'), 114.8 for **4**/115.2 for **5** (C-3'), 129.4 for **4**/129.7 for **5** (C-4'), 126.6 for **4**/127.3 for **5** (C-5'), and δ_H 3.09 and 2.74 for **4**/3.18 and 2.81 for **5** (H-3)]. Therefore, the different configurations (*R* and *S*) at the bond between C-2 and C-1' result in the observed variations in chemical shifts for these signals. NMR data of **5** (Figs. S19-S23) were assigned accordingly (Fig. 2A and Table 1). To further confirm that the isolated products **4** and **5** are distinct compounds, we conducted a co-injection analysis of both products on HPLC. The methanol-water gradient (35:65 to 95:5, v/v, 0-35 min) method instead of the isocratic elution of methanol-water (50-50%, v/v, 0-30 min) was used to

obtain better HPLC profiles of products **4** and **5**. HPLC analysis unequivocally revealed that product **4** elutes at 16.9 minutes, while product **5** elutes at 17.5 minutes, confirming their differentiation as distinct biotransformed products with unique retention times (Fig. 4A). Notably, both products exhibited nearly identical UV absorption spectra (Fig. 4B).

Proposed biosynthetic pathways for biotransformed products Based on the chemical structures of these isolated products, we proposed potential biosynthetic pathways for products **2**-**5**, which were biotransformed from 2'-hydroxyflavanone by *S. coeruleorubidus* NRRL B-2569 (Fig. 2B). A dedicated hydroxylase or monooxygenase from *S. coeruleorubidus* NRRL B-2569 can catalyze the hydroxylation of the substrate 2'-hydroxyflavanone to produce 2',5'-dihydroxyflavanone (**2**). This hydroxylation is regio-specific and only occurs at C-5', indicating that the corresponding enzyme is highly selective. In the meantime, the substrate 2'-hydroxyflavanone can undergo direct glycosylation to form hydroxyflavanone-2'-*O*-*β*-D-glucuronide (**4** and/or **5**) facilitated by a dedicated glucuronyltransferase from *S. coeruleorubidus* NRRL B-2569. Regarding the biosynthesis of 5'-dihydroxyflavone-2'-*O*-*β*-D-glucuronide (**2**), two potential pathways exist: either the hydroxylase of this strain utilizes hydroxyflavanone-2'-*O*-*β*-D-glucuronide (**4** and/or **5**) as a substrate for hydroxylation; or the intermediate **2** needs to be synthesized first and followed by the action of glucuronyltransferase for the final production of **3**. Similarly, the glucuronyltransferase is also regio-selective and only glycosylate the flavonoid at 2'-OH.

Time-course analysis of the bioconversion of 2'-hydroxyflavanone into

products by *S. coeruleorubidus* NRRL B-2569 Despite the successful isolation of all four products, we were surprised to observe that the color of compound **2** dissolved in DMSO-d_6 turned pink during the recovery process using Sephadex LH-20. Compound **2** is a hydroxylated product, unlike the other three products, which contain a sugar moiety. Therefore, to test the hypothesis that compound **2** is an intermediate, we conducted a time-course study on the bioconversion process of 2'-hydroxyflavanone by *S. coeruleorubidus* NRRL B-2569 over 6 days. As depicted in Fig. 2C, this strain initiated the biotransformation of 2'-hydroxyflavanone on the second day, achieving near-complete conversion of the substrate into products after 5 days of cultivation. This highlights the efficiency of *S. coeruleorubidus* NRRL B-2569 in catalyzing the biotransformation of 2'-hydroxyflavanone. Regarding all the biotransformation products, the content of each product varied daily. Products **4** and **5** steadily increased from day 2 to day 4 and remained at a stable production level from day 4 to day 6. Compound **3** was detected in the HPLC trace on day 3 and showed an increasing trend through day 6. Instead, compound **2** initially increased from day 2 to day 3, followed by a decrease from day 3 to day 4, and completely disappeared after day 5. This pattern underscores the likelihood that compound **2** plays a role as an intermediate in the biosynthesis of compound **3**.

Water solubility of 2'-hydroxyflavanone and its biotransformed products Water solubility plays a crucial role in enhancing the beneficial effects of bioactive compounds in the human body. Limited water solubility hinders the utility of numerous natural products, including flavonoids (22). Thus, we determined the water solubility

of the three products with higher polarity using HPLC (The water solubility of substrate **1** and product **2** were determined in previous study) (23). The water solubility of compounds **3**-**5** were computed in triplicate (n = 3). The resulting pairwise comparison analysis demonstrated that glucuronidation can significantly improve the water solubility of natural product, increasing the water solubility of 2'-hydroxyflavanone (substrate **1**) from 11.11 ± 0.45 μM to 3.73 ± 0.05 g/L (product **3**), 436.52 ± 3.16 mg/L (product **4**), and 394.31 ± 4.02 mg/L (product **5**), respectively ($p < 0.001$) (Fig. 3A). Moreover, the two-tailed *t* test analysis demonstrated that the water solubility between products **4** and **5** are significantly different (Fig. 3B), indicating that different spatial arrangement of atoms (such as *R*/*S* isomers in this study) may also show differences in solubility due to their different interactions with water molecules. While isomers share the same chemical structure, they often exhibit significant differences in pharmacology and pharmacokinetics, particularly regarding solubility (24-26). These results suggested that this microbial glycosylation process can convert 2'-hydroxyflavanone into more water-soluble products. The identified glycosylating strain in this study could serve as a valuable tool for glycosylating other bioactive flavonoids, potentially yielding new derivatives with enhanced water solubility.

Antioxidant activity of biotransformed products of 2'-hydroxyflavanone

Recently, we demonstrated that hydroxylated and/or glycosylated 2'-hydroxyflavanone can markedly enhance the radical scavenging activity of the non-scavenging substrate 2'-hydroxyflavone (23). These findings motivated us to investigate the scavenging abilities of the 2'-hydroxyflavanone glycosides obtained in this study. Antioxidant

assays were performed on 2'-hydroxyflavanone, ascorbic acid, together with **3**, **4**, and **5,** as the antioxidant activity of **2** was determined in the previous study (23). The results were analyzed by fitting the data with one-way ANOVA, and the $\log IC_{50}$ value for each compound was computed in triplicate (n=3). Furthermore, 2'-hydroxyflavanone exhibited relatively low DPPH radical scavenging activity, leading to a weak correlation and notable variability among the IC_{50} replicate measurements. To compare 2'-hydroxyflavanone with its glycosidic derivatives, the data were logarithmically transformed. Consistent with findings from our previous research, compounds **4** and **5** showed no detectable radical scavenging activity, making it impossible to determine $\log IC_{50}$ values for these two compounds. The loss of scavenging activity is likely attributed to the removal of the free hydroxyl group through *O*-glycosylation. In contrast, compound **3** demonstrated significantly enhanced radical scavenging activity compared to the substrate, reducing the $\log IC_{50}$ value from 10.77 ± 0.05 μM to 9.55 ± 0.05 μM (Fig. 3B).

Anticancer activity of biotransformed products of 2'-hydroxyflavanone

Based on our prior research, glycosylated 2'-hydroxyflavanone demonstrates enhanced anticancer activity (23). This finding prompted us to investigate the anticancer activities of the novel products obtained in this study. Anticancer activity assays were performed on GSC 33 cells, and the data were analyzed using one-way ANOVA to compare the unmodified substrate with its glycosides (Figs. 3C and 3D). Pairwise comparison analysis indicated that compound **3** possesses significantly enhanced anticancer activity, reducing the mean IC_{50} from 25.05 μM to 7.07 μM ($p < 0.05$). Conversely, compounds

4 and **5** either showed reduced or comparable activity to the substrate. Due to the increased impact of compound **3** on the cell viability of GSC33, we further examined its selectivity effects on a normalcell line. The L929 mouse fibroblast cell line is commonly used in cytotoxicity studies and serves as a valuable comparison to GSC33. Compound **3** exhibited its greatest effect at a concentration of 50 μM, with minimal impact on cell viability at higher concentrations. Interestingly, the viability of the L929 cell line at this concentration was higher compared to GSC33. Specifically, the mean viability of the L929 cell line was 57.8%, whereas it was 30.2% for GSC33 (Fig. 3E).

DISCUSSION

Biotransformation proves effective in structurally modifying bioactive natural products to enhance their physicochemical properties and biological activities. Alongside the structurally diverse biotransformed products, there is potential to generate isomeric compounds as well. In this study, following six days of incubation of 2'-hydroxyflavanone with *S. coeruleorubidus* NRRL B-2569, we identified two biotransformed products with nearly identical retention times on HPLC (compound **4** at 18.6 min and compound **5** at 19 min). However, through comprehensive 1D and 2D NMR analysis (Table 1), we determined that these products are actually two isomeric compounds. Both UV and ESI-MS spectra further confirmed the high structural similarity of compounds **4** and **5**. We hypothesized that this is due to the unspecified *R* or *S* stereochemistry of 2'-hydroxyflavanone (CAS number: 17348-76-4) purchased from Tokyo Chemical Industry. Despite the similar polarity of these two products, we

successfully isolated them using only the HPLC isocratic elution method, which offers a viable approach for isolating similar compounds following biotransformation in future studies. However, both antioxidant and anticancer bioactivities have been shown to be either similar to or decreased compared to the substrate 2'-hydroxyflavanone. Therefore, we believe it is unnecessary to further confirm the absolute structure of compounds **4** and **5** obtained in this study.

Impurities are often inevitable during product isolation, particularly when isolating isomers. In this study, the singlet at a chemical shift of approximately 4 ppm in the ^{1}H NMR spectrum (Fig. S19) of product **5** correlates with a carbon signal at around 56 ppm in the HSQC spectrum (Fig. S22). However, both signals correspond to impurities. A zoomed-in HMBC analysis reveals no correlation between the impurity proton at 4 ppm and any of the carbons associated with product **5** (Fig. S23). This impurity peak was also observed in product **3**, indicating that these additional signals are indeed due to impurities. We also obtained the MS2 spectra (Figs. S12 and S18) for both products **4** and **5**. The butterfly comparison plot of the MS2 spectra (Fig. S24) shows that they are indistinguishable in the MS2 space. Therefore, both the MS1 and MS2 high-resolution mass spectra suggest that products **4** and **5** are isomers. The chemical structure of biotransformed product **3** closely resembles that of 2'-dihydroxyflavanone-5'-*O*-4''-*O*-methyl-*β*-D-glucoside from a previous study (23), both being hydroxylated and glycosylated versions of the substrate 2'-hydroxyflavanone. However, product **3** exhibited enhanced anticancer activity compared to 2'-hydroxyflavanone against Glioblastoma 33 cancer stem cells, whereas 2'-dihydroxyflavanone-5'-*O*-4''-*O*-methyl-

β-D-glucoside from the previous study showed reduced anticancer activity. We hypothesize that attaching the sugar moiety to the 2'-OH position of the substrate and/or using glucuronic acid moiety instead of the 4"-*O*-*β*-D-methyl-glucose moiety may enhance the drug's interaction with its cellular receptor. This modification likely promotes a more favorable alignment between the modified 2'-hydroxyflavanone derivative and the active site of the target receptor or enzyme, thereby increasing the drug's binding capacity and inhibitory activity. Further supporting this hypothesis is the widely recognized phenomenon known as the Warburg effect, which suggests that cancer cells have an increased number of glucose transporters on their surface due to their elevated rate of glycolysis. Therefore, glycosylation of natural products presents a promising strategy for targeting cancer-specific cells in potential therapies. This approach enhances the selectivity of cytotoxic compounds for cancer cells, which, due to their higher expression of glucose transport proteins, are more likely to absorb the glycosylated compounds. In contrast, normal cells, with fewer glucose transport proteins, are less likely to take up these compounds. Regarding antioxidant bioactivity, this study reaffirms that glycosylation typically reduces the antioxidant activity of flavonoids but suggests that hydroxylation may enhance activity by introducing additional phenolic groups. Further investigation is needed to explore the detailed mechanisms of action.

In conclusion, diverse 2'-hydroxyflavanone derivatives were biosynthesized by *S. coeruleorubidus* NRRL B-2569 with enhanced water solubility or biological activities. All products have enhanced water solubility, and **3** possess stronger antioxidant activity

and improved anticancer activity than the substrate. **3** showed a selectivity towards cancer cells. This study introduces an environmentally-friendly bioprocess as an effective method for discovering medically relevant compounds (novel flavonoid derivatives). The various derivatives of 2'-hydroxyflavanone produced could be utilized for investigating structure-bioactivity relationships in both *in vitro* and *in vivo* research, with potential applications in dietary supplement and pharmaceutical industries.

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TABLE 1. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data for compounds **2-5.**

Position	Compound **2**		Compound **3**		Compound **4**		Compound **5**	
	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
2	74.4, CH	5.70 (1H, dd, *J*=13.1, 2.7 Hz)	73.8, CH	5.99 (1H, dd, *J*=13.3, 2.6 Hz)	74.0, CH	6.06 (1H, dd, *J*=13.4, 2.6 Hz)	73.2, CH	6.05 (1H, dd, *J*=13.4, 2.8
3	42.6, CH_2	3.11 (1H, dd, *J*=16.8, 13.2 Hz)	43.2, CH_2	3.01 (1H, dd, *J*=16.8, 13.4 Hz)	43.2, CH_2	3.09 (1H, dd, *J*=16.7, 13.5 Hz)	42.8, CH_2	3.18 (1H, dd, *J*=16.8, 13.
		2.77 (1H, dd, *J*=16.8, 2.8 Hz)		2.74 (1H, dd, *J*=16.8, 2.7 Hz)		2.74 (1H, dd, *J*=16.8, 2.7 Hz)		2.81 (1H, dd, *J*=16.8, 2.9
4	192.0, C		191.6, C		191.7, C		191.8, C	
5	126.4, CH	7.79 (1H, dd, *J*=8.1, 1.7 Hz)	126.3, CH	7.81 (1H, dd, *J*=8.0, 1.6 Hz)	126.3, CH	7.82 (1H, dd, *J*=7.8, 1.6 Hz)	126.3, CH	7.82 (1H, dd, *J*=7.8, 1.5
6	121.4, CH	7.08-7.11 (2H, m, overlapped)	121.4, CH	7.10-7.13 (2H, m, overlapped)	121.4, CH	7.10-7.15 (4H, m, overlapped)	121.4, CH	7.09-7.13 (3H, m, overla
7	136.2, CH	7.60 (1H, m)	136.1, CH	7.60 (1H, m)	136.2, CH	7.60-7.63 (2H, m, overlapped)	136.2, CH	7.59 (1H, m)
8	118.0, CH	7.08-7.11 (2H, m, overlapped)	118.0, CH	7.10-7.13 (2H, m, overlapped)	118.1, CH	7.10-7.15 (4H, m, overlapped)	118.1, CH	7.09-7.13 (3H, m, overla
9	161.5, C		161.3, C		161.4, C		161.4, C	
10	120.6, C		120.7, C		120.7, C		120.7, C	
1'	125.6, C		129.7, C		128.3, C		127.9, C	
2'	146.3, C		146.2, C		153.4, C		153.8, C	
3'	116.2, CH	6.69 (1H, d, *J*=8.6 Hz)	117.1, CH	6.95 (1H, d, *J*=8.9 Hz)	114.8, CH	7.10-7.15 (4H, m, overlapped)	115.2, CH	7.16 (1H, d, *J*=8.7 Hz)
4'	115.7, CH	6.60 (1H, dd, *J*=8.6, 3.0 Hz)	115.4, CH	6.72 (1H, dd, *J*=8.8, 2.9 Hz)	129.4, CH	7.36 (1H, m)	129.7, CH	7.38 (1H, m)
5'	149.9, C		152.7, C		122.4, CH	7.10-7.15 (4H, m, overlapped)	122.4, CH	7.09-7.13 (3H, m, overla
6'	113.1, CH	6.89 (1H, d, *J*=2.9 Hz)	112.8, CH	7.01 (1H, d, *J*=2.9 Hz)	126.6, CH	7.60-7.63 (2H, m, overlapped)	127.3, CH	7.63 (1H, dd, *J*=7.6, 1.5
1"			102.4, CH	4.76 (1H, d, *J*=7.6 Hz)	101.0, CH	4.98 (1H, d, *J*=7.4 Hz)	100.9, CH	5.00 (1H, d, *J*=7.6 Hz)
2"			73.0, CH	3.20 (1H, d, *J*=7.7 Hz)	72.9, CH	3.22-3.29 (2H, m, overlapped)	73.0, CH	3.25 (1H, t, *J*=9.0 Hz)
3"			75.4, CH	3.24 (1H, t, *J*=8.9 Hz)	75.3, CH	3.22-3.29 (2H, m, overlapped)	75.4, CH	3.30 (1H, t, *J*=8.9 Hz)
4"			71.3, CH	3.39 (1H, t, *J*=9.2 Hz)	71.3, CH	3.41 (1H, t, *J*=9.1 Hz)	71.3, CH	3.39 (1H, t, *J*=9.2 Hz)
5"			75.4, CH	3.82 (1H, d, *J*=9.7 Hz)	75.4, CH	3.93 (1H, t, *J*=9.7 Hz)	75.4, CH	3.92 (1H, d, *J*=9.7 Hz)
6"			170.1, C		170.1, C		170.1, C	

627 **Figure legends**

628 **FIG. 1.** Biotransformation of 2'-hydroxyflavanone into products **1**-**4** by *S.*
629 *coeruleorubidus* NRRL B-2569. (A) HPLC analysis (300 nm) of the biotransformation
630 of 2'-hydroxyflavanone by *S. coeruleorubidus* NRRL B-2569 after 3 days of
631 fermentation. (B) UV spectra comparison of 2'-hydroxyflavanone and compound **1**; (C)
632 UV spectra comparison of 2'-hydroxyflavanone and compound **2**; (D) UV spectra
633 comparison of 2'-hydroxyflavanone and compound **3**; (E) UV spectra comparison of 2'-
634 hydroxyflavanone and compound **4**; (F) ESI-MS (-) spectrum of compound **1**; (G) ESI-
635 MS (-) spectrum of compound **2**; (H) ESI-MS (-) spectrum of compound **3**; (I) ESI-MS
636 (-) spectrum of compound **4**.

637 **FIG. 2.** Chemical structures of **1**-**4** and their proposed biosynthetic pathways and the
638 time-course analysis. (A) Chemical structures of 2'-hydroxyflavanone and products **1**-
639 **4** with selected HMBC and ^{1}H-^{1}H COSY correlations. (B) Proposed biosynthetic
640 pathways of **1**-**4**. Substrate (2'-hydroxyflavanone) is circled in red, and hydroxylated
641 and glycosylated derivatives of 2'-hydroxyflavanone are outlined with a dashed and
642 solid blue line, respectively. (C) Time-course analysis of the bioconversion of 2'-
643 hydroxyflavanone into products by *S. coeruleorubidus* NRRL B-2569. Samples were
644 taken from the biotransformation broth every 24 hours and analyzed by HPLC at 300
645 nm.

646 **FIG. 3.** Water solubility, antioxidant activity, and anticancer activity of biotransformed
647 products. All data are presented as mean ± SD from three independent experiments. (A)
648 Water solubility of 2'-hydroxyflavanone and its selected biotransformed products. a)

One-way analysis of variance (ANOVA) results of the water solubility with pairwise comparisons between 2'-hydroxyflavanone (substrate **1**) and glycosylated products **3**-**5**, where *** indicates p-value < 0.001. b) Statistical analysis of the water solubility between products **4** and **5** was performed by using two-tailed t test, where *** indicates p-value < 0.001. Data are presented as the mean ± SD from three independent experiments. (B) One-way analysis of variance (ANOVA) results of the antioxidant activity with pairwise comparisons between 2'-hydroxyflavanone (substrate) and its slected biotransformed products. ***$p < 0.001$. (C) MTT assay of GSC33 viability after compound treatment. (D) IC_{50} values of the tested compounds toward GSC33 cells. *$p < 0.05$; n.s., no significante difference ($p > 0.05$). (E) Comparison of the viability of L929 and GSC33 at 50 μM of product **5**. *$p < 0.05$.

FIG. 4. Comparison of products **4** and **5**. (A) HPLC traces of product **4** (top), product 5 (middle), and co-injection of products **4** and **5** (bottom). (B) UV spectra comparison of products **4** and **5**. (C) HRMS spectrum of product **4** (Top) and HRMS spectrum of product **5** (bottom).

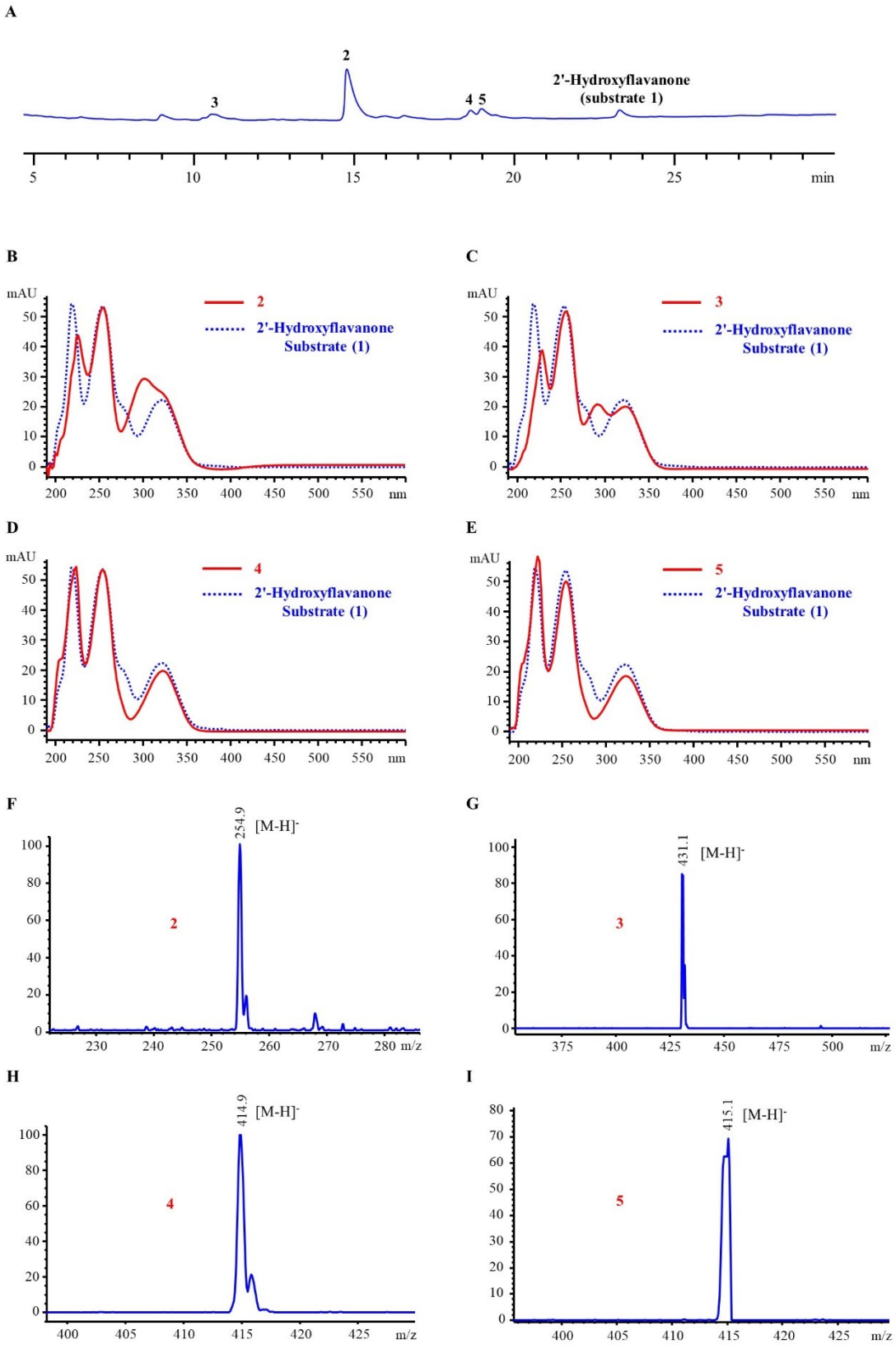
A
2
3
4 5
2'-Hydroxyflavanone
(substrate 1)
5
10
15
20
25
min
B
mAU
2
2'-Hydroxyflavanone
Substrate (1)
nm
C
3
2'-Hydroxyflavanone
Substrate (1)
D
4
2'-Hydroxyflavanone
Substrate (1)
E
5
2'-Hydroxyflavanone
Substrate (1)
F
254.9
[M-H]-
2
m/z
G
431.1
[M-H]-
3
H
414.9
[M-H]-
4
I
415.1
[M-H]-
5

Fig. 1

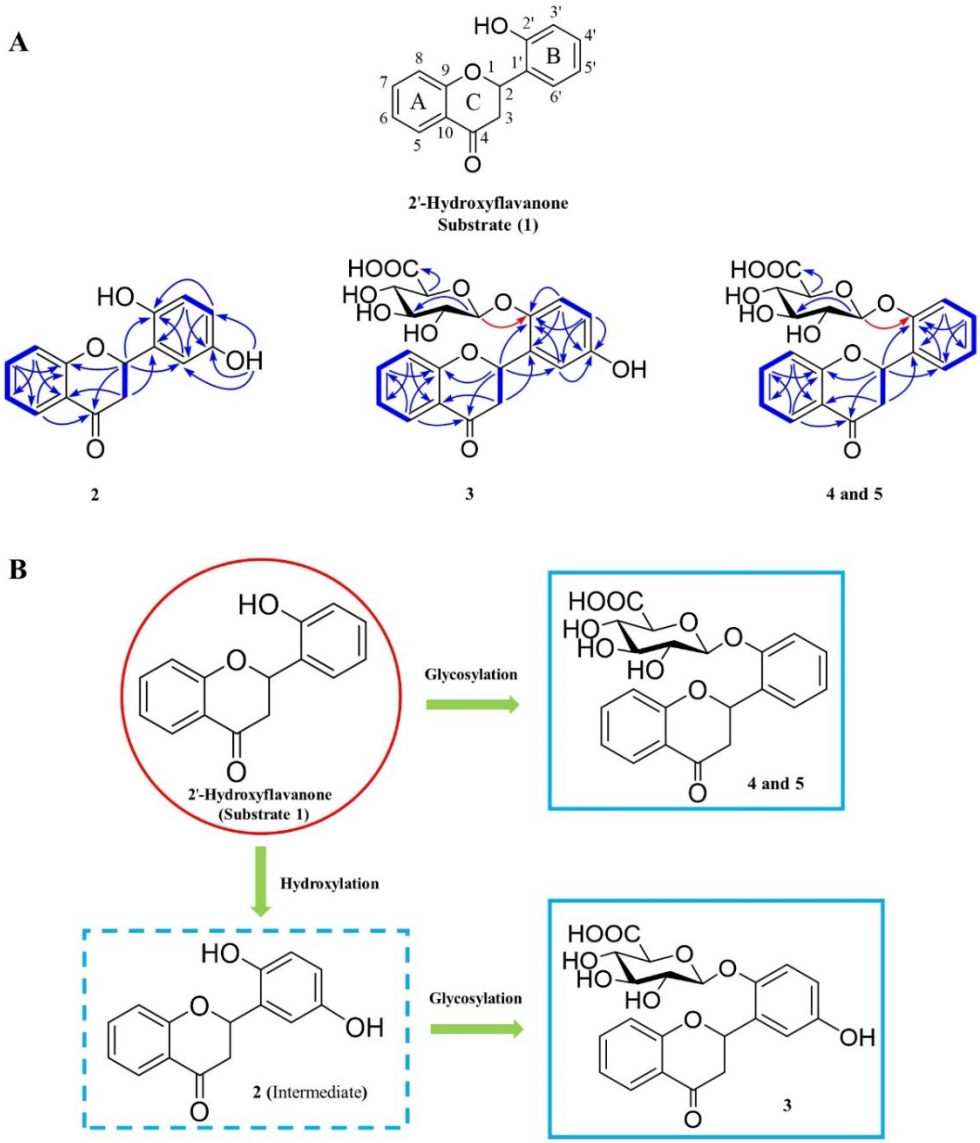
A
2'-Hydroxyflavanone
Substrate (1)
2
3
4 and 5
B
2'-Hydroxyflavanone
(Substrate 1)
Glycosylation
4 and 5
Hydroxylation
2 (Intermediate)
Glycosylation
3
C
Glycosylated products
2 (Intermediate)
Hydroxylated product
2'-Hydroxyflavanone
(Substrate 1)
1 d
2 d
3 d
4 d
5 d
6 d
min

Fig. 2

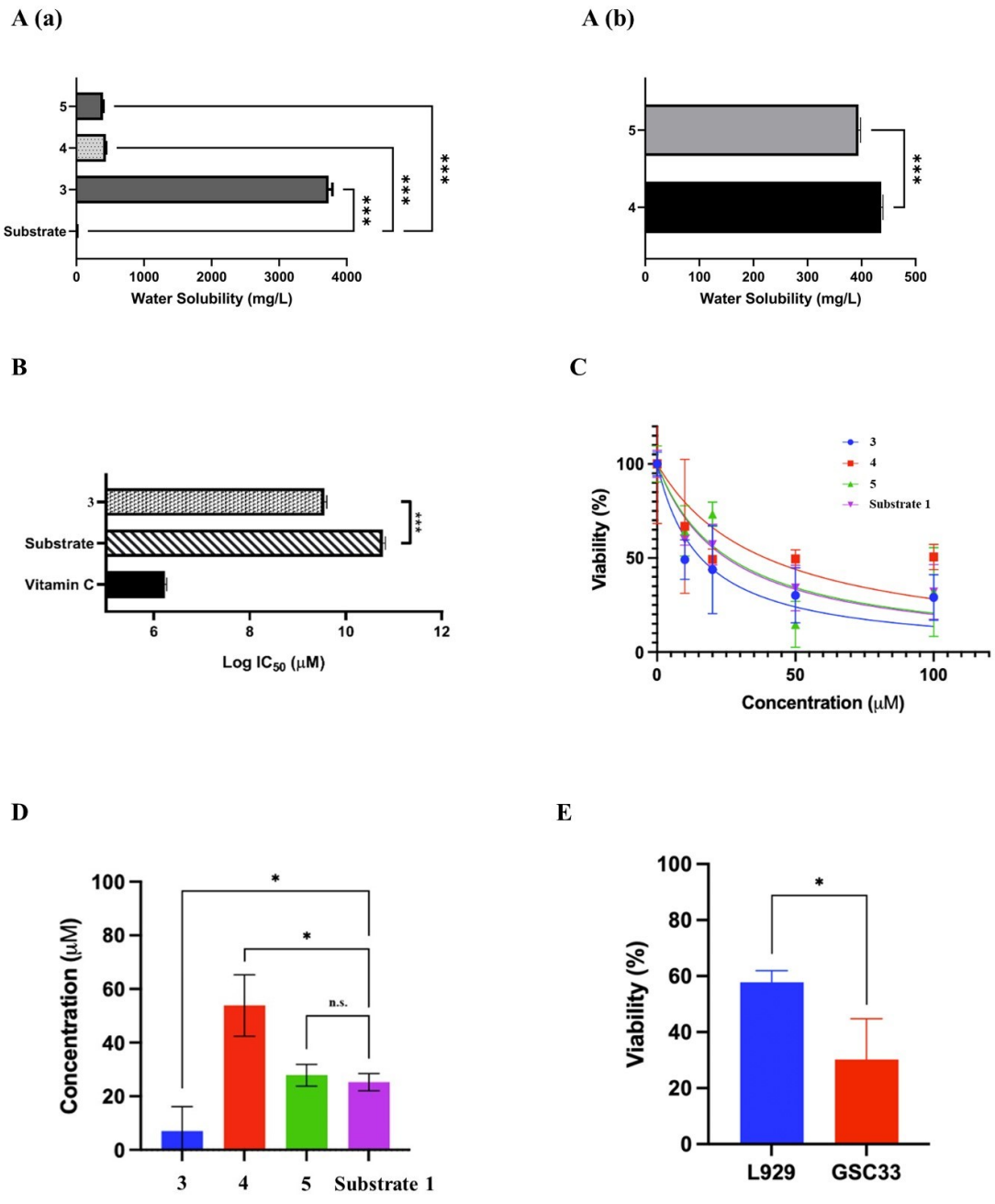
A (a)
5
4
3
Substrate
***
***
***
0
1000
2000
3000
4000
Water Solubility (mg/L)
A (b)
5
4
***
0
100
200
300
400
500
Water Solubility (mg/L)
B
3
Substrate
Vitamin C
***
6
8
10
12
Log IC50 (μM)
C
3
4
5
Substrate 1
Viability (%)
100
50
0
0
50
100
Concentration (μM)
D
Concentration (μM)
100
80
60
40
20
0
*
*
n.s.
3
4
5
Substrate 1
E
Viability (%)
100
80
60
40
20
0
*
L929
GSC33

Fig. 3

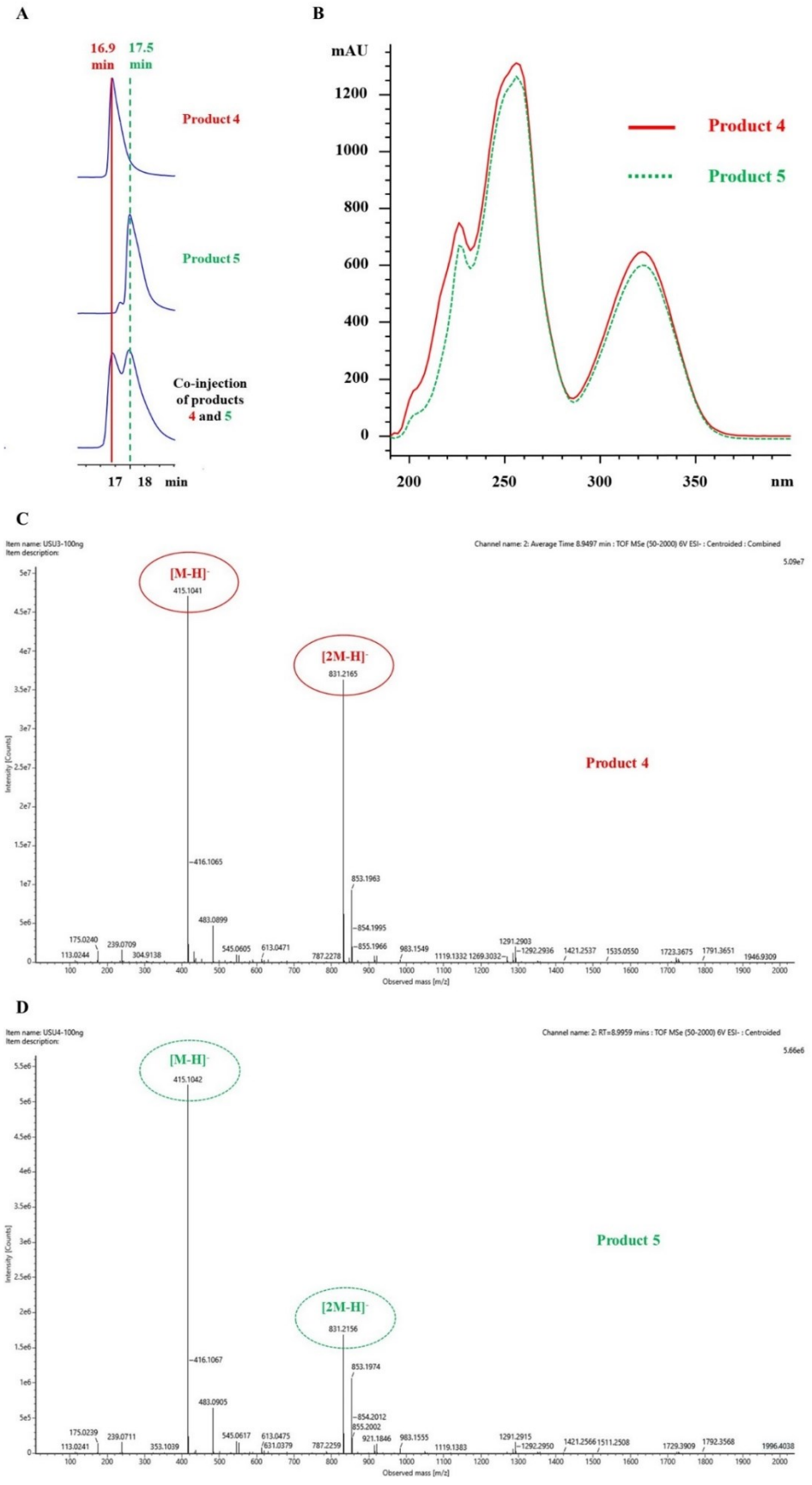
A
16.9 min
17.5 min
Product 4
Product 5
Co-injection of products 4 and 5
17
18
min
B
mAU
1200
1000
800
600
400
200
0
200
250
300
350
nm
Product 4
Product 5
C
Item name: USU3-100ng
Item description:
Channel name: 2: Average Time 8.9497 min : TOF MSe (50-2000) 6V ESI- : Centroided : Combined
5.09e7
[M-H]-
415.1041
[2M-H]-
831.2165
Product 4
Intensity [Counts]
Observed mass [m/z]
D
Item name: USU4-100ng
Item description:
Channel name: 2: RT=8.9959 mins : TOF MSe (50-2000) 6V ESI- : Centroided
5.66e6
[M-H]-
415.1042
[2M-H]-
831.2156
Product 5
Intensity [Counts]
Observed mass [m/z]

Fig. 4