Creating diverse glycosides of 2'-hydroxyflavone through microbial glycosylation

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Abstract

Four new 2'-hydroxyflavone glycosides, namely hydroxyflavone-2'-*O*-*β*-D-glucuronide (**1**), hydroxyflavone-2'-*O*-*α*-L-rhamnoside (**2**), hydroxyflavone-2'-*O*-*β*-D-glucoside (**3**), and hydroxyflavone-2'-*O*-4''-*O*-methyl-*β*-D-glucoside (**4**), were biosynthesized through microbial glycosylation using *Streptomyces coeruleorubidus* NRRL B-2569, *Streptomyces toxytricini* NRRL 15443, *Escherichia coli* BL21(DE3)/pWZ8, and *Beauveria bassiana* ATCC 7159, respectively. Compounds **1**-**4** were structurally characterized through extensive analysis of 1D and 2D NMR spectroscopic data. The water solubility of glycosylated products **1**-**4** were enhanced by 7 to 15 times compared to the substrate 2'-hydroxyflavone. Moreover, antioxidant assays revealed that compounds **1** and **2** exhibited stronger 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity than the substrate, decreasing the logIC_{50} by 68.7% and 80.7%, respectively. Therefore, this research provides several effective biocatalysts that can be used for structural modification of flavonoids for enhanced water solubility and biological activities.

Keywords:

Glycosylation

2'-hydroxyflavone

Microorganisms

Antioxidant

1. Introduction

Flavonoids are a large group of phenolic natural products with more than 8,000 members [1]. They are abundant in foods and have diverse health-promoting activities such as antioxidant, antibacterial, antifungal, anti-inflammatory, and anti-angiogenic activities [2]. However, many flavonoids show low water solubility, which limits their bioavailability and health benefits in the human body. As an effective method for improving the bioavailability of flavonoids, glycosylation can potentially enhance the water solubility, stability, and other biological activities of plant flavonoids [3] and control the absorption, distribution, metabolism, and excretion of secondary metabolites [4]. For example, the absorption of quercetin glycosides in onions was higher (52%) than quercetin (24%) [5]. In addition, most flavonoid drugs with clinical applications are glycosides, such as rutin (quercetin-3-*O*-rutinoside) and puerarin (daidzein-8-*C*-glucoside) [3].

Due to the importance of the sugar moiety, many studies have focused on discovering effective biocatalysts for glycosylation of flavonoids, such as synthesizing quercetin glucosides [6] and its glucuronides using engineered strains [7]. While some glycosylated products exhibit improved water solubility and biological activities over their aglycons, most nucleotide sugars (sugar donors) are costly and difficult to acquire, which makes the *in vitro* enzymatic reaction approach uneconomical for large scale production of desired products [8]. Although there were some reports on preparation of sugar nucleotides, it is still not economically viable considering the labor, effort, and time required [9]. In addition, chemical methods for the synthesis of flavonoid

glycosides are sometimes difficult due to their structural complexity and tedious protection and de-protection steps of the hydroxyl groups [10]. Other methods to obtain flavonoid glycosides such as sequential extraction of quercetrin (quercetin glycoside) from plants are time-consuming and environmentally unfriendly [11]. Bioconversion represents an alternative approach for generating flavonoid glycosides in an more effective and economical way, because microbes themselves can synthesize various nucleotide sugars, including UDP-D-glucose, UDP-D-glucuronic acid and dTDP-L-rhamnose [12]. Moreover, microbial biotransformation is cost-effective, environmentally-friendly, regio- and stereoselective, and can proceed under mild conditions [13]. Based on these advantages, in this work we chose to explore microbes for their ability to synthesize various flavonoid glycosides.

The hydroxyl groups of flavonoids can interact with DNA and proteins and thus affect the biological activities [14]. Even though both flavonoids and their glycosides are investigated intensively, one of the subgroups, 2'-hydroxyflavones, are much less studied due to lower abundance compared with 3'/4'/5'-hydroxyl-containing flavones. However, some 2'-hydroxyflavones have potent bioactivities. For examples, morin has marked anticancer activity against leukemia and melanoma [15], and oxyanin A showed an inhibitory effect on the transcription factor NFκB in normal human embryonic kidney HEK293 cells [16]. Thus, 2'-hydroxyflavonoids represent a group of bioactive molecules with promising therapeutic potential, such as cancer treatments. Synthesis of new derivatives would expand the chemical diversity in this group and provide new entities for bioactivity screening. In this work, we used the rare natural product 2'-

hydroxyflavone, first isolated in the early 1970s from *Primula florindea* [17], as the parent compound to synthesize four new glycosides **1**-**4** using various microbial strains. All these products have enhanced water solubility and **1** and **2** showed stronger radical scavenging activity than the substrate.

2. Experimental section

2.1. General experimental procedures

Agilent 1200 HPLC instrument with an Agilent Eclipse Plus-C_{18} column (5 μm, 250 mm × 4.6 mm) was used to analyze and purify the products. The samples were eluted with methanol-water (35:65 to 95:5, v/v, over 30 minutes, containing 0.1 % formic acid) at a flow rate of 1 mL/min. Low-resolution ESI-MS spectra were obtained on an Agilent 6130 single quadrupole LC-MS to confirm the molecular weights of glycosides. All purified compounds were dissolved in deuterated dimethyl sulfoxide (DMSO-d_6) to collect the NMR spectra on a Bruker Avance III HD Ascend-500 NMR instrument (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) located in the Department of Chemistry and Biochemistry, Utah State University. The chemical shift (δ) values are given in parts per million (ppm). The coupling constants (J values) are reported in hertz (Hz). IR spectra were recorded via VARIAN 640 FT-IR (USA). Antioxidant assays were carried out on a SpectraMax® 190 microplate reader (Molecular Devices Co., USA). Ascorbic acid was purchased from SIGMA-Aldrich (USA). 2'-Hydroxyflavone was purchased from INDOFINE Chemical Company Inc (USA). Compounds were purified through chromatography using Sephadex™ LH-20 (Cytiva, USA), and normal phase silica gel (40-60 μm, VWR® Agela Technologies,

USA). All solvents were purchased from Fisher Scientific. Milli-Q water was used throughout this study.

2.2. Strains, media, and culture conditions

Streptomyces sp. NRRL S-1521, *Streptomyces chromofuscus* NRRL B-12175, *Streptomyces pulveraceus* ATCC 31906, *Streptomyces* sp. NRRL B-1140, *Streptomyces* sp. NRRL B-1677, *Streptomyces coeruleorubidus* NRRL B-2569, and *Streptomyces toxytricini* NRRL 15443 were kindly provided by the United States Department of Agriculture ARS Collection (NRRL). *Streptomyces pulveraceus* ATCC 31906 was purchased from the American Type Culture Collection (ATCC). The strains were routinely grown 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. Engineered strain *Escherichia coli* BL21(DE3)/pWZ8 [6] with glucosyltransferase gene was routinely grown at 37°C on LB liquid medium supplemented with kanamycin (50 μg/mL). *Beauveria bassiana* ATCC 7159 was obtained from ATCC and grown in Potato Dextrose Broth (PDB) at 28°C.

2.3. Screening of different actinomycete strains for 2'-hydroxyflavone glycosylation

To test the ability of actinomycetes to glycosylate 2'-hydroxyflavone, we screened seven strains, namely, *Streptomyces* sp. NRRL S-1521, *S. chromofuscus* NRRL B-12175, *S. pulveraceus* ATCC 31906, *Streptomyces* sp. NRRL B-1140, *Streptomyces* sp. NRRL B-1677, *S. coeruleorubidus* NRRL B-2569, and *S. toxytricini* NRRL 15443. These bacteria were grown in 50 mL of YM medium in a rotary shaker at 250 rpm and 28°C for 3 days. 2'-Hydroxyflavone (4 mg) was dissolved in DMSO and added into

each culture. The cultures were incubated under the same conditions for an additional 3 days. After that, 1 mL of fermentation broth was sampled and centrifuged at 15,000 ×g for 10 min. The supernatant was analyzed by HPLC at 300 nm. *E. coli* BL21(DE3)/pWZ8 was grown in 50 mL of LB medium and induced as reported previously [6] and tested for the ability to glycosylate 2'-hydroxyflavone. Similarly, *B. bassiana* ATCC 7159 was grown in 50 mL of PDB medium and incubated with the substrate for HPLC analysis [18].

2.4. Product extraction and isolation

To isolate the biotransformation products of 2'-hydroxyflavone for structure elucidation, *S. coeruleorubidus* NRRL B-2569 and *S. toxytricini* NRRL 15443 were cultivated in 2-L Erlenmeyer flasks, containing 500 mL of YM medium. *E. coli* BL21(DE3)/pWZ8 and *B. bassiana* ATCC 7159 were also cultivated in 2-L Erlenmeyer flasks, but with 500 mL of LB and PDB medium, respectively. In total 20 mg of 2'-hydroxyflavone was used for each biotransformation. After 2 days, both *S. coeruleorubidus* NRRL B-2569 and *S. toxytricini* NRRL 15443 cultures were centrifuged at 4,000 ×g for 10 min to collect the supernatant. The collected fermentation broths were dried under reduced pressure at 38°C, and the residue was recovered in 3 mL of 67% methanol-water (v/v). The samples were filtered and subjected to fractionation using a Sephadex LH-20 column, eluted with methanol-water (1:1, v/v). The product-containing fractions were combined and further separated by reverse phase HPLC, and eluted with methanol-water (40-95%, 0-20 min) containing 0.1% formic acid (v/v) to yield products **1** (13.0 mg) and **2** (11.5 mg). Similarly, *E. coli*

BL21(DE3)/pWZ8 culture was centrifuged after 3 days, and the supernatant fraction was successively extracted with 500 mL of n-butyl alcohol three times. The extract was dried under reduced pressure, and the residue was dissolved in 2 mL of methanol and 1 mL of distilled water. After the Sephadex LH-20 column chromatography, product-containing fractions were combined and the solvent was removed *in vacuo*. Different amounts of methanol (1 mL, 0.5 mL and 0.2 mL) were used to wash the residue three times, and the sample was centrifuged at 15,000 ×g for 10 min to collect product **3** (2.0 mg) for NMR. The biotransformation broth of *B. bassiana* ATCC 7159 was centrifuged after 3 days' incubation and the supernatant fraction was dried and treated with the same method for Sephadex LH-20 column chromatography. Product-containing fractions were combined and dried for normal phase silica gel column chromatography, eluted with chloroform-methanol (10:1, v/v). Finally, product-containing fractions were combined and purified to yield product **4** (3.0 mg) by reverse phase HPLC with isocratic elution of methanol-water (52 %, v/v, 0-20 min). All purified products were dissolved in DMSO-d_6 and subjected to NMR analysis, and their chemical structures were characterized by NMR spectra.

Hydroxyflavone-2'-*O*-β-D-glucuronide (**1**): White, amorphous powder; UV_{max} 247, 308 nm; IR ν_{max} (film) 3242.9, 2985.9, 1614.8, 1556.0, 1468.1, 1379.7, 1216.3, 1088.8 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; negative ESIMS *m/z* 412.8 $[M-H]^-$; positive HRESIMS *m/z* 415.1013 $[M+H]^+$ (415.1029 calcd for $C_{21}H_{19}O_9$).

Hydroxyflavone-2'-*O*-α-L-rhamnoside (**2**): White, amorphous powder; UV_{max} 248, 307 nm; IR ν_{max} (film) 3408.9, 2932.2, 1621.8, 1661.7, 1480.8, 1383.6, 1236.2, 1057.8

cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; negative ESIMS *m/z* 419.0 $[M+Cl]^-$, 429.0 $[M+HCOO]^-$; positive HRESIMS *m/z* 385.1273 $[M+H]^+$ (385.1287 calcd for $C_{21}H_{21}O_7$).

Hydroxyflavone-2'-*O*-β-D-Glucoside (**3**): White, amorphous powder; UV_{max} 250, 310 nm; IR ν_{max} (film) 3436.8, 3261.6, 2920.4, 1620.8, 1561.9, 1468.1, 1381.2, 1210.6, 1063.4 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; negative ESIMS *m/z* 435.0 $[M+Cl]^-$, 445.0 $[M+HCOO]^-$; positive HRESIMS *m/z* 401.1218 $[M+H]^+$ (401.1236 calcd for $C_{21}H_{21}O_8$).

Hydroxyflavone-2'-*O*-4''-*O*-methyl-β-D-glucoside (**4**): White, amorphous powder; UV_{max} 244, 308 nm; IR ν_{max} (film) 3351.1, 2970.5, 1627.3, 1466.3, 1375.1, 1241.9, 1096.0 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; negative ESIMS *m/z* 449.0 $[M+Cl]^-$, 458.9 $[M+HCOO]^-$; positive HRESIMS *m/z* 415.1371 $[M+H]^+$ (415.1393 calcd for $C_{22}H_{23}O_8$).

2.5. Determination of the water-solubility of products

The purified products were tested for their water solubility as previously described [19]. Purified 2'-hydroxyflavone glycosides were used to establish standard curves for quantifying water solubility of each sample. Briefly, the purified compounds and the substrate were each mixed with 300 μL of distilled water in an Eppendorf tube at 25°C. An ultrasonic cleaner was used to facilitate the dissolution. After 30 min of sonication and centrifugation at 13,000 × g for 10 min, supernatant of each sample was analyzed by HPLC to determine the compound concentrations in the solution. All samples were performed in triplicate and water solubility of each sample are expressed as the mean ± standard deviation (SD).

2.6. Antioxidant assay

The DPPH radical scavenging assay was performed to evaluate the antioxidant activity of all four new compounds using a reported method with minor modifications [20]. Briefly, 180 μL of 150 μM fresh DPPH in MeOH were mixed with 20 μL of samples at different concentrations (62.5, 125, 250, 500, and 1000 μM in DMSO) in the wells of 96-well plates. The natural antioxidant ascorbic acid and the substrate 2'-hydroxyflavone were used as the positive and negative controls, respectively. After shaking in the dark for 30 min at room temperature, the reaction was detected by measuring the absorbance $A_{sample+DPPH}$ at 517 nm using a microplate reader. A_{sample} means the blank measurement for each tested compound; specifically, 20 μL sample was mixed with 180 μL of MeOH. A_{DPPH} is the absorbance of the mixture of 20 μL of distilled water and 180 μL of DPPH. A_{blank} indicates the absorbance of the mixture of 20 μL of distilled water and 180 μL of MeOH. All tests were performed in triplicate. The formula DPPH scavenging activity (%) = $[1-(A_{sample+DPPH} - A_{sample})/(A_{DPPH} - A_{blank})] \times 100\%$. The log[$IC_{50}$ values (the concentration required to scavenge 50% of radicals)] of tested compounds were calculated using the GraphPad Prism software. The results are expressed as the mean ± SD. Significant differences were determined by one-way analysis of variance (ANOVA) using the SAS Studio web-based environment, and $p < 0.05$ was considered significant. All samples were performed in triplicate and antioxidant activity of each sample is expressed as the mean ± SD.

3. Results and discussion

Actinomycetes, such as *Streptomyces*, are known to produce structurally and

functionally diverse natural products and contain abundant biosynthetic enzymes. We hypothesized that some of these strains may have versatile glycosyltransferases that can introduce particular sugar moieties to bioactive flavonoids such as 2'-hydroxyflavone. To this end, 2'-hydroxyflavone was incubated with seven different streptomycete strains, including *Streptomyces* sp. NRRL S-1521, *S. chromofuscus* NRRL B-12175, *S. pulveraceus* ATCC 31906, *Streptomyces* sp. NRRL B-1140, *Streptomyces* sp. NRRL B-1677, *S. coeruleorubidus* NRRL B-2569, and *S. toxytricini* NRRL 15443. HPLC analysis revealed that two more polar metabolites, at 17.0 min for product **1** and 21.0 min for product **2** respectively, were biosynthesized from 2'-hydroxyflavone by *S. coeruleorubidus* NRRL B-2569 and *S. toxytricini* NRRL 15443 (Fig. 1a), while the other five strains could not metabolize the substrate. The UV absorption spectra of the products were both similar to that of 2'-hydroxyflavone, suggesting that these two polar products are derivatives of the substrate (Figs. 1b and c). ESI-MS spectra of **1** and **2** (Figs. 1d and 1e) showed the corresponding quasimolecular ions $[M-H]^-$ at *m/z* 412.8 and $[M+Cl]^-$ at *m/z* 419.0, respectively, suggesting that their molecular weights are 414 and 384, which are 176 and 146 mass units larger than the substrate. These data indicated that a glucuronic acid and a rhamnose might have been introduced to the substrate to yield the two products.

Compound **1** was isolated as a white, amorphous powder. Its molecular formula was deduced to be $C_{21}H_{18}O_9$ based on the $[M+H]^+$ peak at *m/z* 415.1013 (415.1029 calcd for $C_{21}H_{19}O_9$) in high-resolution electrospray ionization mass spectrometry (HRESIMS) spectrum (Fig. S1), indicating that this compound has thirteen degrees of

unsaturation. To further elucidate the chemical structure, **1** was analyzed by NMR (Figs. S2-S6). The ^{13}C NMR analysis (Fig. S3) presented 21 peaks in the spectrum. In addition to the 15 signals belonging to the substrate, six additional carbon signals at δ_C 170.0, 99.6, 76.0, 75.5, 73.0, and 71.3 were found in the spectra, together with the additional proton signals at δ_H 3.34-3.98 in the ^1H NMR spectrum (Fig. S2), suggesting that a sugar moiety has been added to 2'-hydroxyflavone. Unlike the common sugar glucose, this sugar moiety has a quaternary carbon signal at δ_C 170.0, indicating the presence of a carboxyl group in the sugar moiety. Therefore, both the ^1H and ^{13}C signals of this sugar moiety are consistent with a glucuronic acid moiety [7]. Moreover, the ^1H NMR spectrum showed a doublet at δ_H 5.32, indicative of an anomeric proton with a coupling constant of 7.2 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 5.55 to C-2' signal at δ_C 154.0 in the HMBC spectrum (Fig. 2 and Fig. S6) revealed that the glucuronic acid moiety was located at C-2'. The above data together with the detailed analysis of its 2D NMR spectra confirmed its structure as hydroxyflavone-2'-*O*-*β*-D-glucuronide and all signals were assigned accordingly (Table 1).

Compound **2** was isolated as a white, amorphous powder. It has twelve degrees of unsaturation, according to its molecular formula $C_{21}H_{20}O_7$ that was determined based on the $[M+H]^+$ peak at *m/z* 385.1273 (385.1287 calcd for $C_{21}H_{21}O_7$) in the HRESIMS spectrum (Fig. S7). The NMR spectra of **2** were collected (Figs. S8-S12). The ^1H (Fig. S8) and ^{13}C NMR (Fig. S9) spectra were similar to those of **1** except the sugar moiety signals. Unlike glucuronic acid in **1**, this sugar moiety has a carbon signal at δ_C 17.9

instead of δ_C 170.0, together with a doublet proton signal at δ_H 1.12 (d, J = 6.2 Hz), indicating the presence of a methyl group on the sugar moiety. Both the ^1H and ^{13}C signals of this sugar unit supported the presence of a rhamnose moiety. The ^{13}C-undecoupled-NMR analysis presented that the $^1J_{C1'',H1''}$ value of this compound is 170.5 Hz (Fig. 3), which matched well with the typical value of α-glycosyl anomer (170 Hz for α-configuration and 160 Hz for β-configuration) [21]. Moreover, the ^1H NMR spectrum showed a doublet at δ_H 5.56 indicative of an anomeric proton with a coupling constant of 1.2 Hz, and the chemical shift along with the J value were consistent with those of α-L-rhamnose [22]. HMBC spectrum of **2** revealed the correlation of H-1'' at δ_H 5.55 to C-2' at δ_C 154.0 (Fig. 2 and Fig. S12), which confirmed that **2** has a rhamnose moiety at C-2'. Based on the above spectral evidence, the compound was identified as a new compound, namely hydroxyflavone-2'-*O*-α-L-rhamnoside. Its ^1H and ^{13}C NMR data were assigned and are shown in Table 1.

Both **1** and **2** are new compounds generated through microbial glycosylation. This encouraged us to further explore other microbes to create additional new glycosides of 2'-hydroxyflavone. *B. bassiana* ATCC 7159 is well known for its microbial glycosylation capability [23]. We recently identified a versatile glucosyltransferase from this strain and engineered it into *Escherichia coli* BL21(DE3) to make a glycosylating strain, *E. coli* BL21(DE3)/pWZ8 [6]. We next tested *E. coli* BL21(DE3)/pWZ8 and *B. bassiana* ATCC 7159 for their ability to glycosylate 2'-hydroxyflavone. HPLC analysis revealed that two polar metabolites, at 16.5 min for product **3** and 17.5 min for product **4** respectively, were biosynthesized from 2'-

hydroxyflavone by *E. coli* BL21(DE3)/pWZ8 and *B. bassiana* ATCC 7159 (Fig. 4a). The UV absorption patterns of the products were similar to that of the substrate, suggesting that these two polar products are also derivatives of 2'-hydroxyflavone (Figs. 4b and 4c). ESI-MS analysis of **3** revealed that the corresponding quasimolecular ions $[M+Cl]^-$ at *m/z* 435.0 and $[M+HCOO]^-$ at *m/z* 445.0, indicating that its molecular weight is 400. Similarly, based on the ion peaks $[M+Cl]^-$ at *m/z* 449.0 and $[M+HCOO]^-$ at *m/z* 458.9, the molecular weight of **4** was deduced to be 414. The molecular weights of **3** and **4** are 162 and 176 mass units larger than the substrate, based on which we proposed that a glucose moiety and a methylated glucose moiety have been added to 2'-hydroxyflavone, respectively.

Compound **3** was isolated as a white, amorphous powder and has a molecular formula of $C_{21}H_{20}O_8$, determined based on the $[M+H]^+$ peak at *m/z* 401.1218 (401.1236 calcd for $C_{21}H_{21}O_8$) in the HRESIMS spectrum (Fig. S13), indicating twelve degrees of unsaturation. To further elucidate the chemical structure, the NMR spectra of **3** were collected (Figs. S14-S18). Analysis of its ^{13}C NMR (Fig. S15) and HSQC (Fig. S17) spectra indicated additional signals for five CH at δ_C 100.1, 77.2, 76.8, 73.3, and 69.6 as well as one CH_2 at δ_C 60.6, which is a typical diastereotopic carbon signal from glucose. The presence of proton signals δ_H 3.19-3.70 in the ^1H NMR spectrum (Fig. S14) further suggested that a glucose moiety has been added to 2'-hydroxyflavone, and both the ^1H and ^{13}C signals of the glucose moiety are consistent with the published data [6]. Moreover, the ^1H NMR spectrum showed a doublet at δ_H 5.14, indicating an anomeric proton with a coupling constant of 7.3 Hz, and the chemical shift along with

the *J* value were consistent with those of *β*-D-glucose. HMBC spectrum of **3** revealed the correlation of H-1" at δ_H 5.55 to C-2' at δ_C 154.0 (Fig. 2 and Fig. S18), which confirmed that **3** has a glucose moiety at C-2'. On the basis of the above evidence and analysis of COSY, HSQC and HMBC spectra, compound **3** was identified as hydroxyflavone-2'-*O*-*β*-D-glucoside and its proton and carbon signals were assigned accordingly (Table 1).

Finally, **4** was isolated as a white, amorphous powder. The $[M+H]^+$ peak at *m/z* 415.1371 (415.1393 calcd for $C_{22}H_{23}O_8$) in the HRESIMS spectrum (Fig. S19) revealed that **4** has a molecular formula of $C_{22}H_{22}O_8$, suggesting that this contains an additional CH_2 compared with that of **3** but with the same degrees of unsaturation. The NMR analysis (Figs. S20-S24) was performed to further elucidate the chemical structure of **4**. Among the 22 carbon signals in the ^{13}C NMR spectrum (Fig. S21), 15 signals belonging to the skeleton of 2'-hydroxyflavone were determined to be similar to those present in **1**-**3**. The seven remaining carbon signals at δ_C 99.7, 78.9, 76.5, 75.7, 73.5, 60.1, and 59.7 in the ^{13}C NMR spectrum, together with the proton signals in the range of δ_H 3.07-3.64 in the ^{1}HNMR spectrum (Fig. S20), further suggested that a typical methyl glucose was added to 2'-hydroxyflavone [18]. Compared to the glucose moiety in **3**, one additional methoxy carbon signal was observed at δ_C 59.7, and the HMBC spectrum of **4** revealed the correlation of methoxy group at δ_H 3.46 to C-4" at δ_C 78.9 (Fig. 2 and Fig. S24). Therefore, both the ^1H and ^{13}C signals of this sugar moiety supported the presence of a 4"-*O*-methyl-glucose moiety. Moreover, the ^1H NMR spectrum showed a doublet at δ_H 5.16, corresponding to the anomeric proton of this

sugar moiety with a coupling constant of 7.7 Hz, which was consistent with that of 4"-*O*-*β*-D-methyl-glucose [23]. The HMBC spectrum of **4** revealed the correlation of H-1" at δ_H 5.16 to C-2' at δ_C 155.3 (Fig. 2 and Fig. S24), which confirmed that **4** has a 4"-*O*-*β*-D-methyl-glucose moiety at C-2'. Based on the above spectral evidence, the compound was identified to be a new compound, namely hydroxyflavone-2'-*O*-4"-*O*-methyl-*β*-D-glucoside (Table 1).

We have previously discovered and identified the dedicated glucosyltransferase, named BbGT, from this fungal strain. Incorporation of this enzyme into *E. coli* led to the construction of *E. coli* BL21(DE3)/pWZ8, which specifically produced the glucoside **3** from 2'-hydroxyflavone in this work. Interestingly, as seen in Fig. 4a, both **3** and **4** are synthesized by *B. bassiana* ATCC 7159. There is an additional methyltransferase in *B. bassiana* ATCC 7159, which explains the synthesis of the two glycosylated products in this fungal culture.

Water solubility is critical for the beneficial effects of bioactive compounds in the human body. Poor water solubility hinders the use of many natural products including flavonoids [24]. Therefore, we measured the water solubility of the four new glycosides using HPLC. The water solubility of compounds **1**-**4** were computed in triplicate (n = 3) and determined to be 34.8 ± 0.9 mg/L, 17.3 ± 0.6 mg/L, 36.1 ± 1.3 mg/L, 33.2 ± 0.7 mg/L, respectively, which are around 7 to 15 times than that of the substrate 2'-hydroxyflavone (2.5 ± 0.7 mg/L) (Fig. 5a), indicating that microbial glycosylation is an effective tool to convert 2'-hydroxyflavone into more water-soluble products. The glycosylating strains identified in this work could be used as a potential tool to

glycosylate other bioactive flavonoids to yield new derivatives with improved water solubility.

Many plant flavonoids have antioxidant activity. A previous study showed that the *O*-glycoside of chrysoeriol is a more efficient scavenger of DPPH radical [25]. This finding encouraged us to further discover how glycosylation affects the antioxidant activity of 2'-hydroxyflavone. Antioxidant assays were conducted and the results were analyzed by fitting the data with one-way ANOVA. The $\log IC_{50}$ value for each compound was computed in triplicate (n = 3) and utilized as the response variable in the statistical model. The substrate exhibited a relatively low antioxidant activity, with scavenging activities ranging between 0.4% to 8.0%. In the interest of making pairwise comparisons on antioxidant activity between the unmodified substrate and its glycosides, we transformed the data logarithmically. The resulting pairwise comparison analysis demonstrated that glucuronidation (for **1**) and rhamnosylation (for **2**) significantly improve radical scavenging activity, decreasing the mean $\log IC_{50}$ from 65.2 ± 8.6 μM to 20.4 ± 0.7 μM and 12.6 ± 0.8 μM, respectively ($p < 0.001$) (Fig. 5b). The $\log IC_{50}$ values of the glucosylated products (**3** and **4**) were not significantly different from that of the substrate. Though the antioxidant activities of **1** and **2** are not as high as the positive control (ascorbic acid), these findings indicate that glucuronidation and rhamnosylation may be effective tools for improving the radical scavenging activity of otherwise inert substrates, providing a simple method for future discoveries of medically-relevant new compounds.

The arrangement of functional groups of flavonoids can affect their antioxidant

activity, therefore, the number, position, and structure of the sugars can affect the antioxidant properties [26]. For example, the antioxidant effect of 7-*O*-glycosylated flavonoids was weakened in rat mitochondria compared to flavonoids glycosylated at C-3 [27]. This is due to the fact that glycosylation results in charge changes on the oxygen atoms of the hydroxyl groups, which further affects their antioxidant abilities. Specifically, 7-*O*-glycosylation decreases the negative charge on the oxygen atom at position 3 [28]. The general trend is that aglycones exhibit higher antioxidant activity than their relevant glycosides [29], even though sugar moieties generally enhance the bioavailability [30]. In this work, we found that **3** and **4** showed reduced antioxidant activity after glycosylation compared to the substrate. On one hand, some researchers have suggested that lack of free hydroxyl groups important for hydrogen abstraction and radical scavenging may lead to the low antioxidant activity [26]. On the other hand, some others proposed that the decreased antioxidant activity was due to the properties of the sugar itself, rather than displacement of a free OH [27]. However, rutin, with a rhamnose sugar, does not show apparently reduced antioxidant activity relative to its aglycone [27]. Researchers also found that glucuronidated resveratrol has comparable antioxidant activity with the resveratrol [31]. Therefore, glycosylation could have very different effects on the antioxidant activity of flavonoids. While **3** and **4** showed lower antioxidant activity than 2'-hdyroxyflavone, **1** and **2** have enhanced activity than the substrate. Since all the four glycosylation reactions occur at the same position, we propose that the introduced sugar moiety plays a role in the antioxidant activity. The detailed mechanisms remain to be investigated in future work.

4. Conclusion

In conclusion, four new glycosides were prepared from 2'-hydroxyflavone using different microbial strains. All products have enhanced water solubility and compounds **1** and **2** possess stronger antioxidant activity than the substrate. Overall this research provides effective methods of biosynthesizing sufficient quantity of novel flavonoid derivatives in a green and efficient way, which paves the way for the further *in vivo* research on the function of sugar moieties on physicochemical and biological properties. Meanwhile, as the European Union Law mentions [32], the products obtained by biotransformation of natural compounds are still considered natural; therefore, this research can be used as the reference for further application of glycosylated compounds as ingredients of dietary supplements and pharmaceuticals. This work thus adds new strains into the biocatalytic glycosylation toolbox that can be used for creating diverse glycosides of flavonoids for enhanced waster solubility and biological activities.

Credit authorship contribution statement

Jie Ren: Biotransformation, product analysis purification, structure elucidation, measurement of water solubility, antioxidant assay, and manuscript preparation. Caleb Don Barton: Antioxidant assay, statistical analysis, and manuscript preparation. Jixun Zhan: Funding acquisition, project design and administration, supervision, methodology, and manuscript preparation.

Declaration of competing interest

The authors declare no conflicts of financial interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at

Figures S1-S24, 1D (^{1}H and ^{13}C) and 2D NMR (HSQC, COSY, HMBC) and HRESIMS spectra for compounds **1-4**

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Table 1

^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data for compounds **1-4** in DMSO-d_6.

Position	**1**		**2**		**3**		**4**	
	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	160.7, C		161.9, C		160.3, C		160.3, C	
3	112.2, CH	7.04 (1H, s)	111.6, CH	6.74 (1H, s)	112.2, CH	7.09 (1H, s)	112.2, CH	7.08 (1H, s)
4	177.2, C		176.9, C		177.2, C		177.2, C	
5	124.7, CH	8.06 (1H, dd, *J*=7.9, 1.5 Hz)	124.8, CH	8.08 (1H, dd, *J*=7.9, 1.6 Hz)	124.7, CH	8.06 (1H, dd, *J*=7.9, 1.5 Hz)	124.7, CH	8.06 (1H, d, *J*=7.8 Hz)
6	125.4, CH	7.50 (1H, t, *J*=7.5 Hz)	125.5, CH	7.52 (1H, t, *J*=7.5 Hz)	125.3, CH	7.50 (1H, t, *J*=7.5 Hz)	125.3, CH	7.50 (1H, t, *J*=7.5 Hz)
7	134.2, CH	7.82 (1H, m)	134.4, CH	7.83 (2H, m, overlapped)	134.2, CH	7.83 (1H, m)	134.2, CH	7.83 (1H, t, *J*=7.7 Hz)
8	118.6, CH	7.74 (1H, d, *J*=8.3 Hz)	118.6, CH	7.75 (1H, d, *J*=8.3 Hz)	118.6, CH	7.74 (1H, d, *J*=8.3 Hz)	118.6, CH	7.74 (1H, d, *J*=8.4 Hz)
9	156.0, C		156.0, C		156.0, C		156.0, C	
10	123.2, C		123.1, C		123.2, C		123.2, C	
1'	121.0, C		121.6, C		120.7, C		120.7, C	
2'	154.9, C		154.0, C		155.4, C		155.3, C	
3'	115.3, CH	7.37 (1H, d, *J*=8.5 Hz)	115.7, CH	7.37 (1H, d, *J*=8.4 Hz)	115.4, CH	7.36 (1H, d, *J*=8.5 Hz)	115.3, CH	7.35 (1H, d, *J*=8.5 Hz)
4'	132.7, CH	7.57 (1H, m)	132.7, CH	7.57 (1H, m)	132.7, CH	7.56 (1H, m)	132.7, CH	7.55 (1H, t, *J*=7.6 Hz)
5'	122.2, CH	7.23 (1H, t, *J*=7.6 Hz)	122.0, CH	7.21 (1H, t, *J*=7.5 Hz)	121.9, CH	7.22 (1H, t, *J*=7.6 Hz)	122.0, CH	7.22 (1H, t, *J*=7.6 Hz)
6'	129.4, CH	7.93 (1H, dd, *J*=7.8, 1.5 Hz)	130.0, CH	7.83 (2H, m, overlapped)	129.2, CH	7.93 (1H, dd, *J*=7.8, 1.5 Hz)	129.2, CH	7.93 (1H, d, *J*=7.6 Hz)
1"	99.6, CH	5.32 (1H, d, *J*=7.2 Hz)	98.7, CH	5.56 (1H, d, *J*=1.2 Hz)	100.1, CH	5.14 (1H, d, *J*=7.3 Hz)	99.7, CH	5.16 (1H, d, *J*=7.7 Hz)
2"	73.0, CH	3.34 (2H, m, overlapped)	70.0, CH	3.86 (1H, dd, *J*=3.2, 1.7 Hz)	73.3, CH	3.34 (2H, m, overlapped)	73.5, CH	3.32 (1H, t, *J*=8.4 Hz)
3"	76.0, CH	3.34 (2H, m, overlapped)	70.5, CH	3.56 (1H, dd, *J*=9.3, 3.4 Hz)	76.8, CH	3.34 (2H, m, overlapped)	76.5, CH	3.44 (2H, m, overlapped)
4"	71.3, CH	3.42 (1H, m)	71.7, CH	3.29 (1H, t, *J*=9.3 Hz)	69.6, CH	3.19 (1H, m)	78.9, CH	3.07 (1H, t, *J*=9.3 Hz)
5"	75.5, CH	3.98 (1H, d, *J*=9.6 Hz)	69.9, CH	3.50 (1H, dd, *J*=9.4, 6.2 Hz)	77.2, CH	3.40 (1H, m)	75.7, CH	3.44 (2H, m, overlapped)
6"	170.0, C		17.9, CH_3	1.12 (1H, d, *J*=6.2 Hz)	60.6, CH_2	3.70 (1H, m), 3.48 (1H, m)	60.1, CH_2	3.64 (1H, m), 3.51 (1H, m)
4"-OCH_3							59.7, CH_3	3.46 (1H, s)

Fig. 1. Screening of actinomycetes for the ability to glycosylate 2'-hydroxyflavone. (a) HPLC analysis (300 nm) of biotransformation of 2'-hydroxyflavone by seven actinomycete strains. (i) 2'-hydroxyflavone + YM medium; (ii) 2'-hydroxyflavone + *Streptomtces* sp. NRRL S-1521; (iii) 2'-hydroxyflavone + *S. chromofuscus* NRRL B-12175; (iv) 2'-hydroxyflavone + *S. pulveraceus* ATCC 31906; (v) 2'-hydroxyflavone + *Streptomtces* sp. NRRL B-1140; (vi) 2'-hydroxyflavone + *Streptomtces* sp. NRRL B-1677; (vii) 2'-hydroxyflavone + *S. coeruleorubidus* NRRL B-2569; (viii) 2'-hydroxyflavone + *S. toxytricini* NRRL 15443. (b) UV spectra comparison of the substrate and **1**. (c) UV spectra comparison of the substrate and **2**. (d) ESI-MS (-) spectrum of **1**. (e) ESI-MS (-) spectrum of **2**.

Fig. 2. Selected HMBC correlations for **1**-**4**.

Fig. 3. 1D ^{13}C-undecoupled NMR spectrum analysis of **2** for determing the configuration of rhamnoside (circled by the red line).

Fig. 4. Biotransformation of 2'-hydroxyflavone into various glycosides by an engineered *E. coi* and a fungal strain. (a) HPLC analysis (300 nm) of biotransformation of 2'-hydroxyflavone by the two strains. (i) 2'-hydroxyflavone + YM medium; (ii) 2'-hydroxyflavone + *E. coli* BL21(DE3)/pWZ8; (iii) 2'-hydroxyflavone + *B. bassiana* ATCC 7159. (b) UV spectra comparison of the substrate and **3**. (c) UV spectra comparison of the substrate and **4**. (d) ESI-MS (-) spectrum of **3**. (e) ESI-MS (-) spectrum of **4**.

Fig. 5. Water solubility and antioxidant activity of glycosylated products **1**-**4** compared to the substrate (2'-hydroxyflavone). (a) Water solubility of 2'-hydroxyflavone and its

glycosides. 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'-hydroxyflavone (substrate) and glycosylated compounds **1**-**4**, where *** indicates *p*-value < 0.001, n.s. indicates no significante difference ($p > 0.05$). Data are presented as the mean ± SD from three independent experiment.

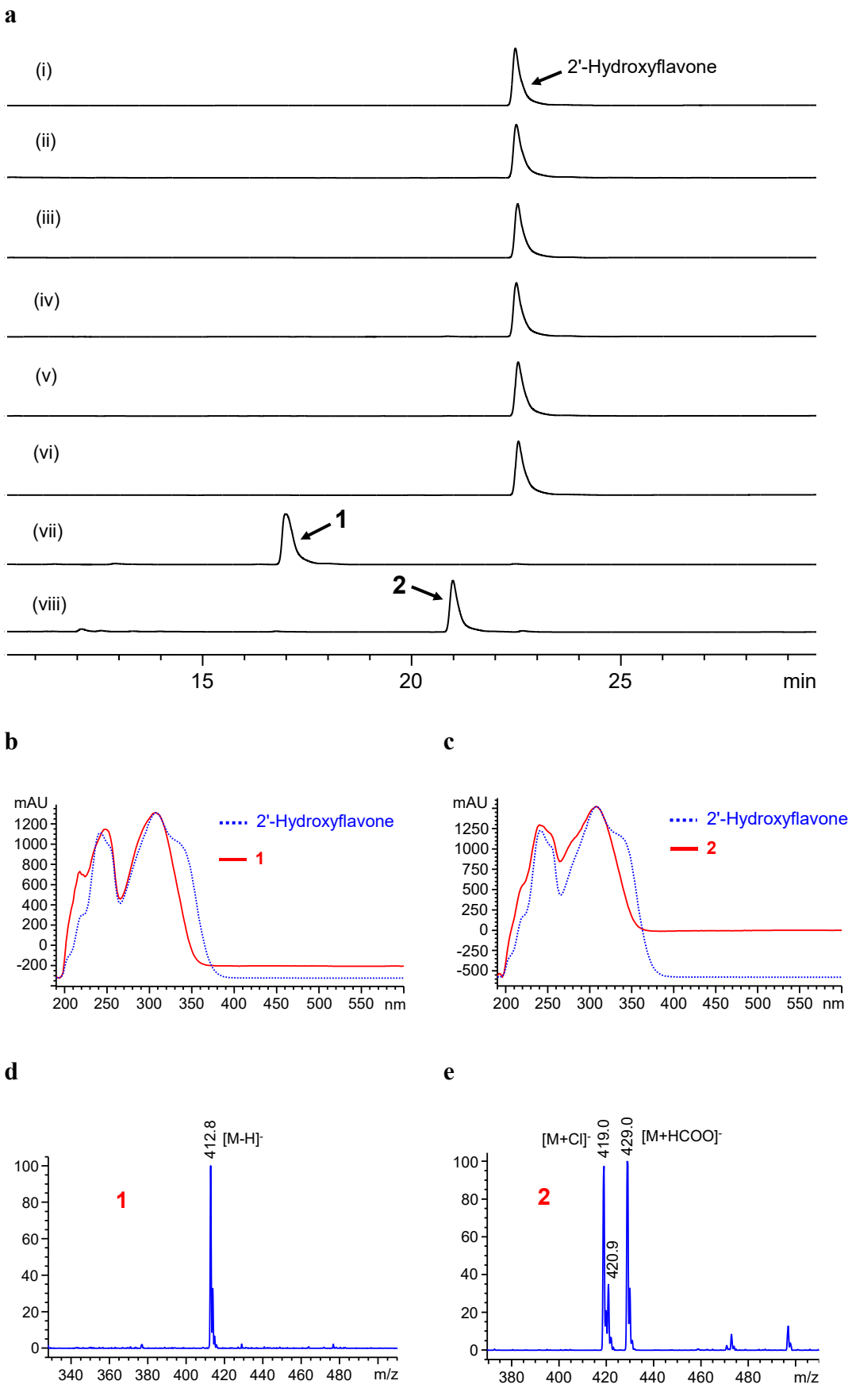
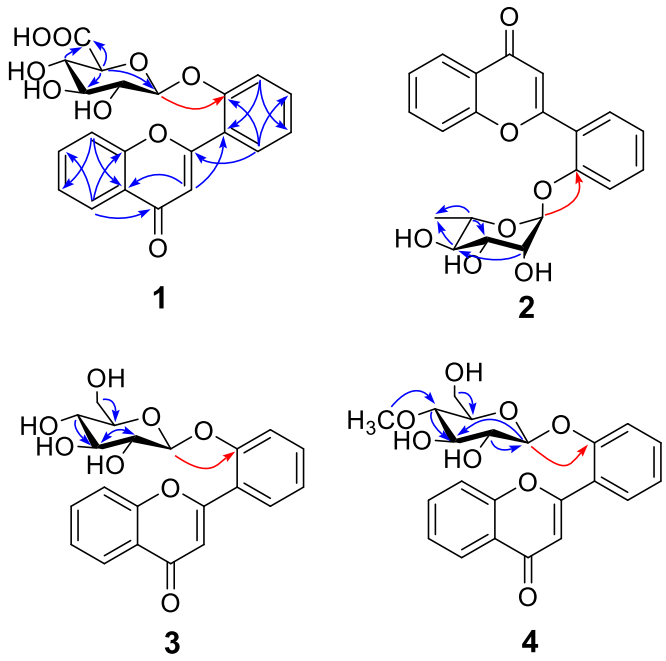

a
(i)
2'-Hydroxyflavone
(ii)
(iii)
(iv)
(v)
(vi)
(vii)
1
(viii)
2
15
20
25
min
b
mAU
2'-Hydroxyflavone
1
nm
c
mAU
2'-Hydroxyflavone
2
nm
d
412.8
[M-H]-
1
m/z
e
[M+Cl]-
419.0
429.0
[M+HCOO]-
2
420.9
m/z

Figure 1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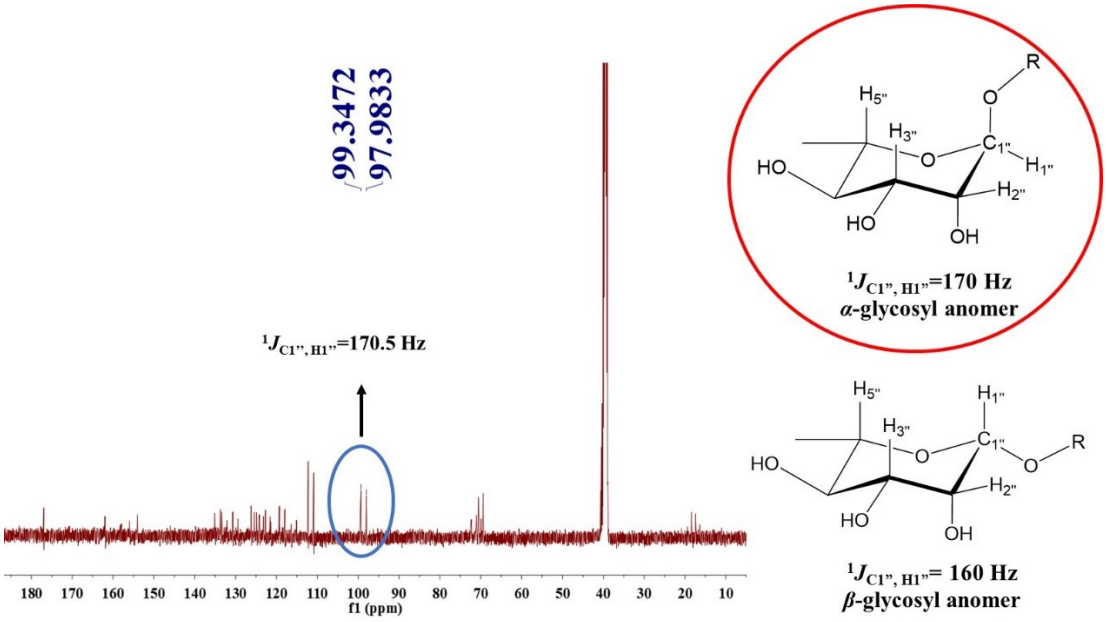
O
O
4

Figure 2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97.9833
$^1J_{C1'',H1''}$=170.5 Hz
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
f1 (ppm)
$^1J_{C1'',H1''}$=170 Hz
α-glycosyl anomer
$^1J_{C1'',H1''}$= 160 Hz
β-glycosyl anomer

Figure 3

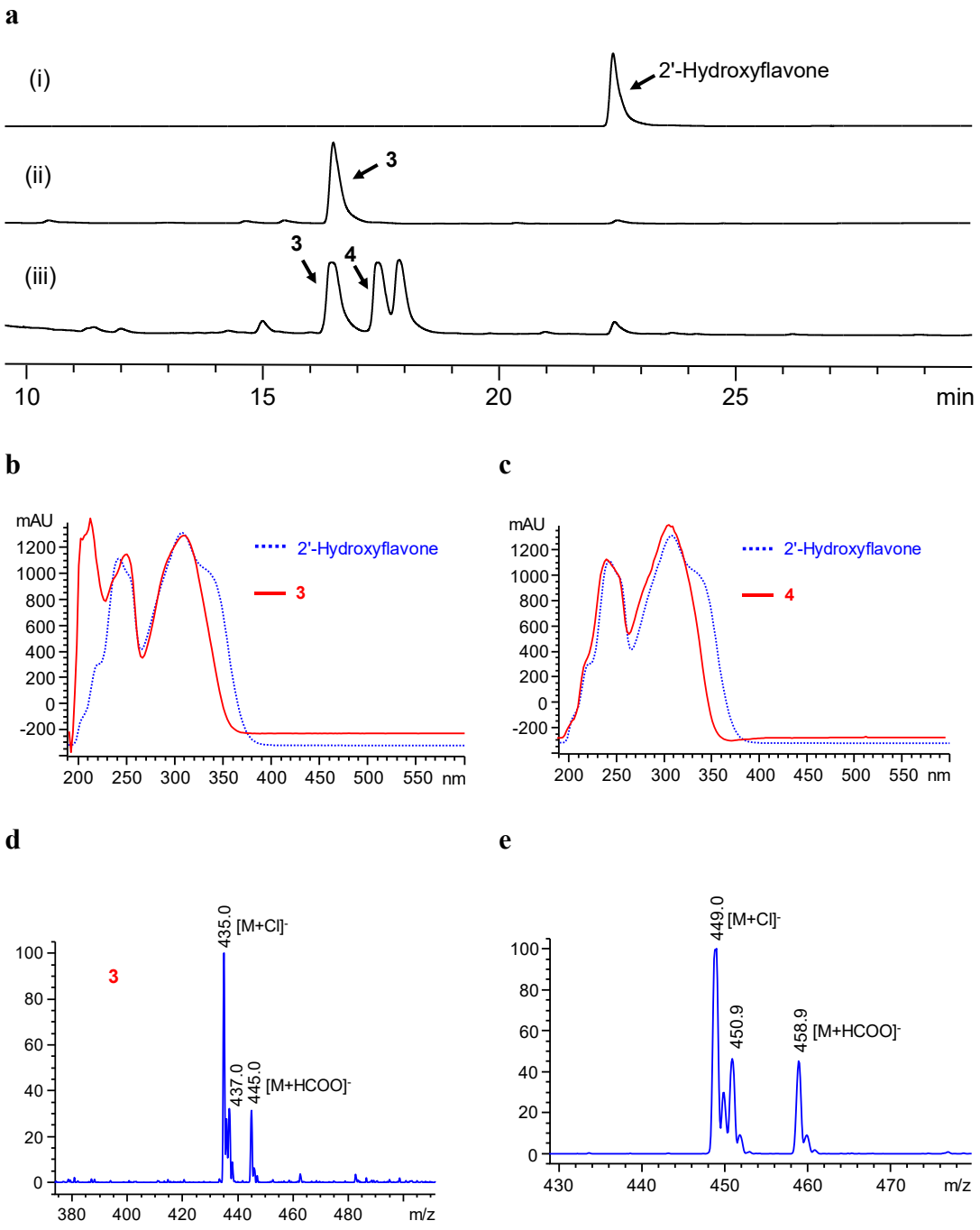
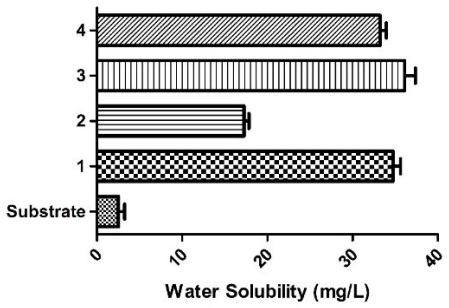

a
(i)
2'-Hydroxyflavone
(ii)
3
(iii)
3
4
10
15
20
25
min
b
mAU
2'-Hydroxyflavone
3
200 250 300 350 400 450 500 550 nm
c
mAU
2'-Hydroxyflavone
4
200 250 300 350 400 450 500 550 nm
d
3
435.0 [M+Cl]-
437.0
445.0 [M+HCOO]-
380 400 420 440 460 480 m/z
e
449.0 [M+Cl]-
450.9
458.9 [M+HCOO]-
430 440 450 460 470 m/z

Figure 4

a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3
2
1
Substrate
0
10
20
30
40
Water Solubility (mg/L)

b

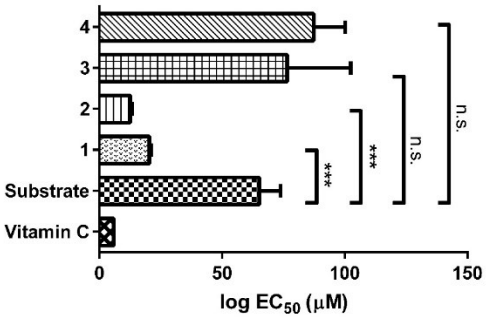
4
3
2
1
Substrate
Vitamin C
***
***
n.s.
n.s.
0
50
100
150
log EC50 (μM)

Figure 5