Enzyme-mediated transglycosylation of rutinose (6-*O*-α-L-rhamnosyl-D-glucose) to phenolic compounds by a diglycosidase from *Acremonium* sp. DSM 24697

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Abstract

The structure of the carbohydrate moiety of a natural phenolic glycoside can have a significant effect on the molecular interactions and physicochemical and pharmacokinetic properties of the entire compound, which may include anti-inflammatory and anticancer activities. The enzyme 6-*O*-α-rhamnosyl-β-glucosidase (EC 3.2.1.168) has the capacity to transfer the rutinosyl moiety (6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranose) from 7-*O*-rutinosylated flavonoids to hydroxylated organic compounds. This transglycosylation reaction was optimized using hydroquinone (HQ) and hesperidin as rutinose acceptor and donor, respectively. Since HQ undergoes oxidation in a neutral to alkaline aqueous environment, the transglycosylation process was carried out at pH values ≤6.0. The structure of 4-hydroxyphenyl-β-rutinoside was confirmed by NMR, that is, a single glycosylated product with a free hydroxyl group was formed. The highest yield of 4-hydroxyphenyl-β-rutinoside (38%, regarding hesperidin) was achieved in a 2-h process at pH 5.0 and 30 °C, with 36 mM OH-acceptor and 5% (v/v) cosolvent. Under the same conditions, the enzyme synthesized glycoconjugates of various phenolic compounds (phloroglucinol, resorcinol, pyrogallol, catechol), with yields between 12% and 28% and an apparent direct linear relationship between the yield and the pK_a value of the aglycon. This work is a contribution to the development of convenient and sustainable processes for the glycosylation of small phenolic compounds. Volume 66, Number 1, Pages 53–59, 2019

Keywords: *α-rhamnosyl-β-glucosidase, hesperidin, hydroquinone*

1. Introduction

Phenolic metabolites are widely distributed in nature, especially in plants, and have gained attention because of their pharmacological functions as chemoprotectants, and antitumor, antioxidant, antibacterial, and anti-inflammatory compounds [1, 2]. The tailoring of these phenolic compounds with different carbohydrates can lead to modifications in the bioavailability and the pharmacokinetic and physicochemical properties of the resulting glycoconjugates compared with the respective aglycons [3, 4]. The synthesis of new glycoconjugates is highly relevant to the pharmaceutical and food industry. An interesting and often convenient approach is to obtain these compounds through transglycosylation reactions based on retaining glycoside hydrolases. Numerous enzymatic glycosylations for primary and secondary OH- groups have been reported [5–8]; but the glycosylation of phenolic hydroxyl groups is less common [9, 10]. In many cases, the advantage of the enzymatic glycosylation dwells in its high stereo- and regioselectivity. The yield, however, can be low, especially with

Abbreviations: *HQ, hydroquinone; HQ-rutinoside, 4-hydroxyphenyl-β-rutinoside.*

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phenolic acceptor molecules, in view of their low nucleophilicity [11]. On the other hand, aromatic alcohols (e.g., benzyl alcohol) were reported to give better glycosylation yields compared with primary alcohols with enzymatic fructosylations using a levansucrase, resulting in an inverse correlation between yield and pK_a value of the acceptor alcohols [9].

Hydroquinone (HQ) is an effective inhibitor of melanogenesis because of its tyrosinase inhibition activity [12]. However, it has a low chemical stability in aqueous environments and this limits its practical and technological applications [13]. Some glycosylated derivatives of HQ have been approved for cosmetic applications and treatment of hyperpigmentation issues caused by excessive synthesis of melanin [14]. As an example, HQ-β-glucoside (β-arbutin), which naturally occurs in a variety of plants such as bearberry, exhibits low toxicity and has already been used as a skin-whitening agent [15]. This glycoconjugate showed higher chemical stability, skin-whitening activity, and a more pronounced tyrosinase inhibition than the unmodified aglycon. Interestingly, the glycosylated HQ variants reported to date showed tyrosinase inhibition regardless of the bound sugar moiety, thus presumably any glycosylated form of HQ could lead to potential cosmetic applications [14, 16–20]. The disaccharide rutinose (6-*O*-L-rhamnosyl-D-glucose) is a constituent of some flavonoids, terpenoids, anthocyanins, and other secondary plant metabolites [16]. Rutinosylated glycoconjugates have potential medicinal applications ascribed to the presence of the terminal L-rhamnopyranose moiety [21]. It appears that rutinose-containing glycoconjugates are resistant to hydrolysis in human tissues because of the absence of rutinoside-attacking glycosidases such as rhamnosidases or α-L-rhamnosyl-β-D-glucosidases [22]. Thus, rutinose-capped HQ may offer an additional advantage over other HQ-containing glycoconjugates in that it may be less prone to enzymatic degradation than, for example, glucosylated HQ when applied to human skin. Moreover, rutinosides have potential applications as food additives and antiviral agents [23, 24]. Transglycosylation is the method of choice for the synthesis of numerous rutinosides. Two main approaches using α-L-rhamnosyl-β-D-glucosidases, which are diglycosidases, have been pursued from either rutin [25, 26] or hesperidin as a rutinose donor [5]. In this work, we explored the transglycosylation specificity of the α-rhamnosyl-β-glucosidase from the fungus *Acremonium* sp. DMS 24697 for the synthesis of rutinose-based conjugates containing phenolic aglycons.

2. Materials and Methods

2.1. Chemicals and stock solutions

HQ (hydroquinone = benzene-1,4-diol), hesperidin (3′,5,7-trihydroxy-4′-methoxyflavanone 7-rhamnoglucoside), and hesperetin (3′,5,7-trihydroxy-4′-methoxyflavanone) were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade methanol (LiChrosolv®) was obtained from Merck (Darmstadt, Germany). The other chemicals were from standard sources. The pK_a values for phloroglucinol, resorcinol, pyrogallol, catechol, HQ, and phenol were obtained from PubChem. To prepare stock solutions for the enzymatic assays, the flavonoids (180 mM) were solubilized in dimethylsulfoxide and suspended in water. The buffers used were 50 mM sodium citrate (pH 3.0–5.0), sodium phosphate (pH 6.0–8.0), and Tris–HCl (pH 6.8–8.8). Standards for the HPLC analysis were prepared by diluting the stock solutions in the mobile phase.

2.2. Enzyme source

Acremonium sp. DSM 24697 was cultured in the presence of hesperidin, which acts as both a carbon source and an inducer of the α-rhamnosyl-β-glucosidase [27]. The enzyme was purified from the culture supernatant to homogeneity by hydrophobic interaction and anion-exchange chromatography, as described previously [27]. To quantify the enzymatic activity, the hydrolysis of the substrate hesperidin was measured (see below).

2.3. Enzymatic reactions

For quantification of the hydrolytic activity of the α-rhamnosyl-β-glucosidase, the reaction mixtures, which contained 450 μL of substrate (0.11%$_{w/v}$ hesperidin in 50 mM sodium citrate buffer, pH 5.0) and 50 μL of suitably diluted enzyme solution, were incubated at 60 °C for 1 h. The reactions were stopped by adding 500 μL of 3,5-dinitrosalicylic acid [27]. Then, the reaction mixtures were incubated at 100 °C for 10 min and subsequently cooled before measuring the absorbance at 540 nm. One unit of α-rhamnosyl-β-glucosidase activity was defined as the amount of enzyme required to release 1 μmol of rutinose per min. The transglycosylation reactions were performed at 30 °C for 24 h in 50 mM sodium phosphate buffer (pH 6.0), containing 1.8 mM hesperidin (donor), 1.8 mM OH-acceptor (as indicated below), 10%$_{v/v}$ dimethylformamide (DMF), and 0.02 U mL^{-1} of α-rhamnosyl-β-glucosidase (unless stated otherwise). The enzymatic reactions were stopped at 100 °C (10 min).

2.4. Analytical assays

The residual substrates and the reaction products were analyzed by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) using a KONIK-500-A series HPLC system attached to a KONIK Uvis 200 detector. The TLC analysis (Silicagel 60 W) was performed using ethyl-acetate/2-propanol/water (3:2:2) as the mobile phase and the anthrone reagent for staining. The total activity (hydrolysis plus transglycosylation) was determined by quantifying the released hesperetin at 320 nm [28]. The TLC images were analyzed using the software ImageJ 1.38x (National Institutes of Health, United States; http://rsb.info.nih.gov/ij/). The 32-bit color images were split into red, green, and blue (RGB) components. Images corresponding to the red component were selected for their highest signal-to-noise ratio, and integrated optical density units were used for relative quantification of

rutinose and rutinosylated compounds. The reaction samples were extracted with ethyl acetate and the water phase deproteinized according to the procedure of Contin et al. [29] previous to HPLC injection. The column was a reversed-phase LiChroCART® 125-4 (12.5 cm length, 4 mm internal diameter) containing a LiChrospher® 5-μm RP-18 sorbent (pore size 100 Å). Isocratic elution was performed with 40%$_{v/v}$ methanol in water at 40 °C at a flow rate of 1.0 mL min^{-1}. The assays were performed in triplicate, and peak areas and extinction coefficients were calculated from the chromatograms of authentic standards recorded at 285 nm.

2.5. HQ stability

The chemical stability of HQ was assessed by measuring the UV-spectra at different pH values using 50 mM sodium citrate (pH 4.0 and 5.0) or sodium phosphate buffer (pH 6.0) at 30 °C with a USB4000 UV-vis spectrophotometer (Tecnocientifica, Ocean Optics, Buenos Aires, Argentina).

2.6. Synthesis of rutinosides

HQ-rutinoside was enzymatically synthesized in a reaction volume of 1.0 mL at 30 °C using buffers of 50 °mM (as specified below). The reaction mixtures were incubated for up to 24 h and contained 1.8 mM hesperidin, 1.8 mM HQ, 0.02 U mL^{-1} of 6-*O*-α-rhamnosyl-β-glucosidase, and 2%$_{v/v}$ of cosolvent. The concentration of cosolvent (DMF or DMSO) was optimized in the range of 0%–30%$_{v/v}$, performing 2-h reactions in 50 mM sodium citrate buffer (pH 5.0) at 30 °C. The acceptor (HQ) concentration was optimized in the presence of 5%$_{v/v}$ of cosolvent. The same reaction conditions were applied for the synthesis of other aromatic rutinosides. The reaction products were purified using a LH 20 Sephadex column (1.5 × 150 cm; flow rate of 0.1 mL min^{-1}) with methanol/water (4:1) as a mobile phase. All fractions containing the transglycosylation products were pooled and dried by evaporation and subsequent lyophilization.

2.7. Spectral characterization

NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer (600.23 MHz for ^{1}H, 150.94 MHz for ^{13}C) in CD_3OD at 25 °C (see the Supplementary Tables S1–S5). The residual solvent peak was used as an internal standard (δ_H 3.330 ppm, δ_C 49.60 ppm). The NMR experiments—^{1}H, ^{13}C, COSY, HSQC, and HMBC—were performed using the manufacturer's software. The assignment of individual proton spin systems was achieved by COSY experiments and transferred to carbons by HSQC. The positions of the aglycons and glycosidic linkages were deduced from heteronuclear correlations extracted from HMBC experiments.

3. Results and Discussion

3.1. Hesperidin hydrolysis and HQ stability

Hesperidin is generated in the agro- and fruit-processing industry on a large scale as a byproduct, representing an inexpensive and renewable starting material for specific bio-

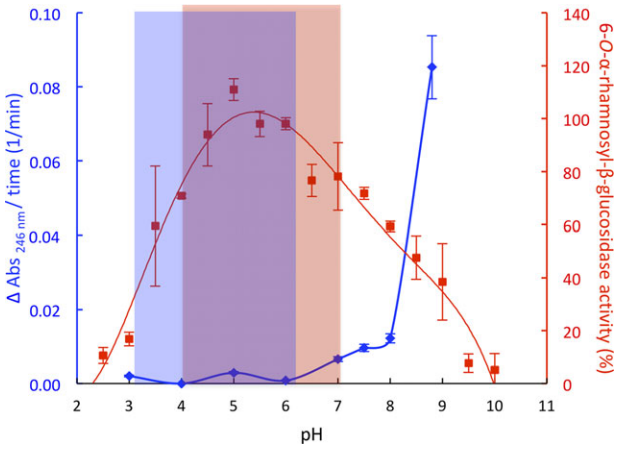
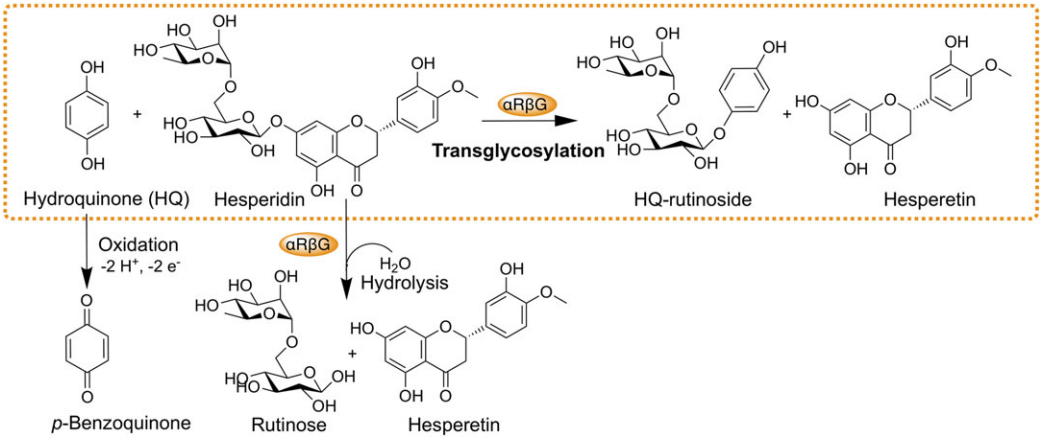


FIG. 1 *Effect of pH on the oxidation of hydroquinone to p-benzoquinone (●), and 6-O-α-rhamnosyl-β-glucosidase activity (■) [27]. The red area highlights the pH range of enzymatic activity higher than 80%, whereas the blue area shows the pH range where hydroquinone is stable. The overlapping area indicates the pH range that was used in the optimization of the transglycosylation reactions.*

catalytic processes. The enzyme α-rhamnosyl-β-glucosidase from *Acremonium* sp. DSM 24697 has been shown to efficiently catalyze both hesperidin hydrolysis and transglycosylation reactions with hesperidin as a rutinose donor [27]. However, the use of HQ as an aglycon called for a thorough optimization of the transglycosylation reactions because of its instability under oxygen-containing conditions [13]. The chemical oxidation of HQ to *p*-benzoquinone (λ_{max} at 246 nm) at room temperature was evaluated at different pH values (Fig. 1). HQ was found to be unstable at alkaline conditions, whereas in acidic environments, the oxidation reaction proceeded slowly with negligible reaction rates at pH ≤ 6.0. In this context, a low HQ stability was encountered at pH 7.0 in the amylosucrase-catalyzed production of α-arbutin, which resulted in a low conversion yield of 1.3%. Interestingly, the stability of HQ was considerably improved by adding the antioxidant ascorbic acid to the reaction mixture, which markedly augmented the conversion yield [30]. Considering that the α-rhamnosyl-β-glucosidase exhibits a high activity (>75% relative activity) between pH 4.0 and 7.0, and HQ has been found to be stable at a pH ≤ 6.0, a pH range of 4.0–6.0 was selected to further assess the transglycosylation reactions (Fig. 1).

3.2. Transglycosylation of HQ

The rutinosylation of HQ renders the potentially bioactive compound HQ-rutinoside, which can be viewed as rhamnosylated arbutin (Scheme 1). To synthesize HQ-rutinoside in a single step, transglycosylation reactions were performed with the α-rhamnosyl-β-glucosidase from *Acremonium* sp. DSM 24697 in the presence of HQ and hesperidin as the sugar acceptor and donor, respectively. The rutinosylation of HQ was successfully



SCHEME 1 *Enzymatic synthesis of HQ-rutinoside using the 6-O-α-rhamnosyl-β-glucosidase from Acremonium sp. DSM 24697.*

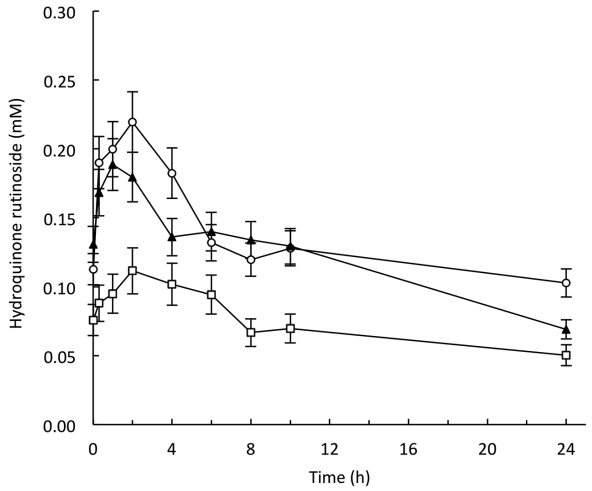


FIG. 2 *Time course of the production of HQ–rutinoside at different pH values using 0.02 U mL^{-1} of 6-O-α-rhamnosyl-β-glucosidase; pH 4.0 (□), pH 5.0 (○), and pH 6.0 (▲).*

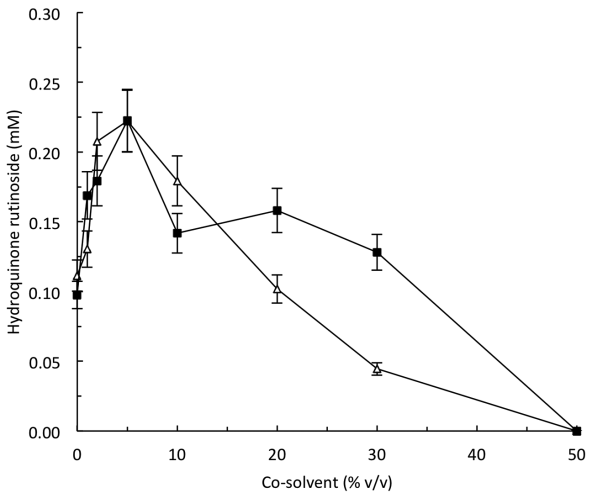


FIG. 3 *Effect of cosolvent concentration on the transrutinosylation of HQ in the presence of DMF (△) and DMSO (■).*

achieved at pH 6.0 with 4 U L^{-1} of α-rhamnosyl-β-glucosidase, with the highest product concentration of 0.18 mM after 25 h of reaction time with a 10% yield regarding the acceptor concentration. As the next step, three reactions were performed at pH 4.0, 5.0, and 6.0 with a five times higher enzyme concentration (Fig. 2). The highest transglycosylation activity was found at pH 5.0 as for the hydrolytic reaction (Figs. 1 and 2), while the maximum yield was observed after 1 or 2 h of reaction time, regardless of the pH. The time course indicated that the formed rutinosyl-HQ was rehydrolyzed by the enzyme, resulting in low product concentrations of 0.05–0.12 mM rutinosyl-HQ when approaching the thermodynamic equilibrium. In a previous report, we have shown that this enzyme efficiently rutinosylates specific OH-acceptor compounds such as 2-phenylethanol, geraniol, and nerol without displaying significant hydrolysis activities [5]. However, in the case of 4-methylumbelliferone [10] or HQ, which both are good leaving groups, a kinetic control of the enzymatic process has to be applied for optimal yields.

3.3. Effect of cosolvents and OH-acceptor concentrations

An approach to enhance the yield of enzymatic processes that involve hydrophobic substrates in aqueous media is the use of organic co-solvents as a means to increase the availability of the substrate in the reaction medium [31]. In this work, the cosolvents DMF and DMSO were assessed in concentrations of up to 50% (v/v) for the transglycosylation-based synthesis of HQ-rutinoside. Both solvents improved the transglycosylation yields in concentrations of 2%–10% (v/v), whereas at higher concentrations (>20%$_{v/v}$ DMF and >30%$_{v/v}$ DMSO), they turned out to be deleterious for the process (Fig. 3). The increased

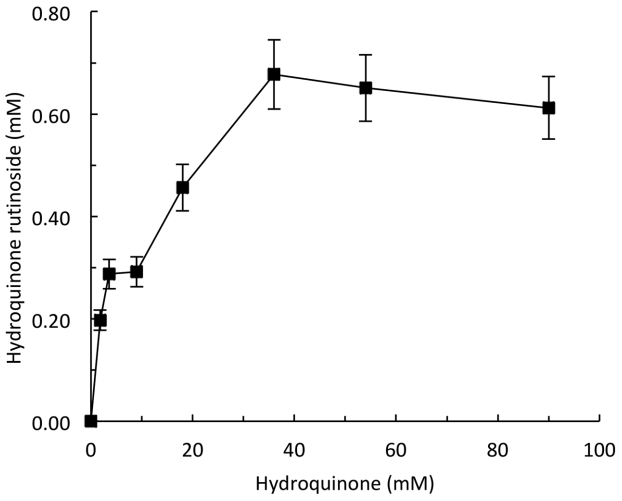


FIG. 4 *Effect of OH-acceptor (HQ) concentration on the enzymatic synthesis of HQ-rutinoside.*

yields can be explained by a better solubility and availability of the hydrophobic substrate in the presence of the co-solvent and/or a change in the reaction equilibrium [32]. On the other hand, the reduced transglycosylation yield at higher co-solvent concentrations is probably attributed to enzyme instability issues. Another explanation for reduced yields may be found in a decreased activity coefficient for the acceptor, as reported for a levansucrase-catalyzed transglycosylation reaction in the presence of various concentrations of the co-solvent 2-methyl-2-propanol [9].

The optimum OH-acceptor concentration for the transglycosylation reaction was determined to be 36 mM of HQ with 38% of hesperidin being converted to the transglycosylation product (Fig. 4). The reduced enzyme activity at higher HQ concentrations may be explained by enzyme inactivation processes in the presence of this compound [16]. On the other hand, the robust dextransucrase, a glucosyltransferase from *Leuconostoc mesenteroides*, displayed its optimal activity at 450 mM HQ, however, the product yield was low (0.44%) with respect to HQ [14]. An approach to overcome the enzyme inactivation or inhibition caused by high HQ concentrations may be the use of an enzymatic reactor with a feeding strategy that avoids high acceptor concentrations [33].

3.4. Substrate specificity

The transglycosylation product of the α-rhamnosyl-β-glucosidase-catalyzed reaction with HQ as acceptor was identified as 4-hydroxyphenyl β-rutinoside (HQ–rutinoside) through NMR analysis (Supplementary Table S1). It is noteworthy that only mono-rutinosylated HQ was detected, suggesting that the enzyme exhibits a negligible substrate affinity towards the generated glycoconjugate. In contrast, the retaining endo-acting glycoside hydrolases which were shown to glycosylate HQ produced several products. Specifically, the amylosucrase from *Deinococcus geothermalis* was shown to transfer glucoside residues from sucrose to (+)-catechin, resulting in two major transglycosylation products, a monoglucopyranoside and a maltoside, together with several (+)-catechin maltooligosaccharides in lower amounts [34]. Similarly, cyclodextrin glucanotransferase-catalyzed reactions with maltodextrin and HQ as donor and acceptor compounds, respectively, led to the formation of HQ-glycosides containing up to seven glucose units [20]. Furthermore, byproducts were also found with exo-acting glycosidases, that is, transglucosylation of HQ using maltose as a glucosyl donor and the α-glucosidase from baker's yeast yielded two products, HQ-α-glucoside and HQ-α-isomaltoside [16]. Although, the transglycosylation reactions rendered more than one reaction product, in none of the cases the second OH-group of the HQ was glycosylated, the OH-groups of the sugar moiety being more prone to glycosylation. This fact explains the inhibitory effect of these glycoconjugates on tyrosinases, since in all cases one OH-aromatic group remains exposed. Concerning the bioprocess, a reduced amount of byproducts is advantageous, therefore, the above-mentioned endo-acting enzymes with polysaccharides as donor substrates produced several oligosaccharide-aglycon derivatives, which substantially complicates the downstream process.

Optimization of the reaction conditions resulted in an improved productivity for the production of HQ rutinoside by a factor of 53; the established conditions were used for the glycosylation of phenol and additional phenol derivatives such as phloroglucinol, resorcinol, pyrogallol, and catechol (Fig. 5). These are interesting monohydric or dihydric compounds with a rather wide distribution in nature, mainly as secondary plant metabolites [1]. In addition, phloroglucinol was reported to be produced by *Pseudomonas fluorescens* [35]. The isolated yields of the transrutinosylation reactions ranged from 13 to 34% for all above-mentioned phenol derivatives (Fig. 6). The conversion of phenol, however, was determined to be very low with a yield of 0.3%, probably because of the instability of the biocatalyst in the presence of this compound. The NMR data of the isolated transglycosylation products are shown in the Supplementary Tables S1–S4.

Considering the classical two-step displacement mechanism for the hydrolysis reaction in family GH5 glycosidases, an inverse relationship between the pK_a value of the acceptor and the transglycosylation yield would be expected, as reported for the fructosylation reactions catalyzed by a levansucrase [9]. Generally, a higher tendency to be deprotonated (as manifested by a lower pK_a value) should lead to greater nucleophilicity. Thus, hydroxyls with lower pK_a values should more easily become activated (deprotonated) nucleophiles, which should facilitate their attack on the glycosyl–enzyme intermediate. However, the transglycosylation yields with acceptors **1–5** showed a positive relationship regarding the pK_a values (Fig. 6). For this reason, we suggest that the binding or entrance to the active site is mainly responsible for the differences in the observed transglycosylation yields. In other words, the different acceptor accommodations in the active site determine the outcome of the competition between water and

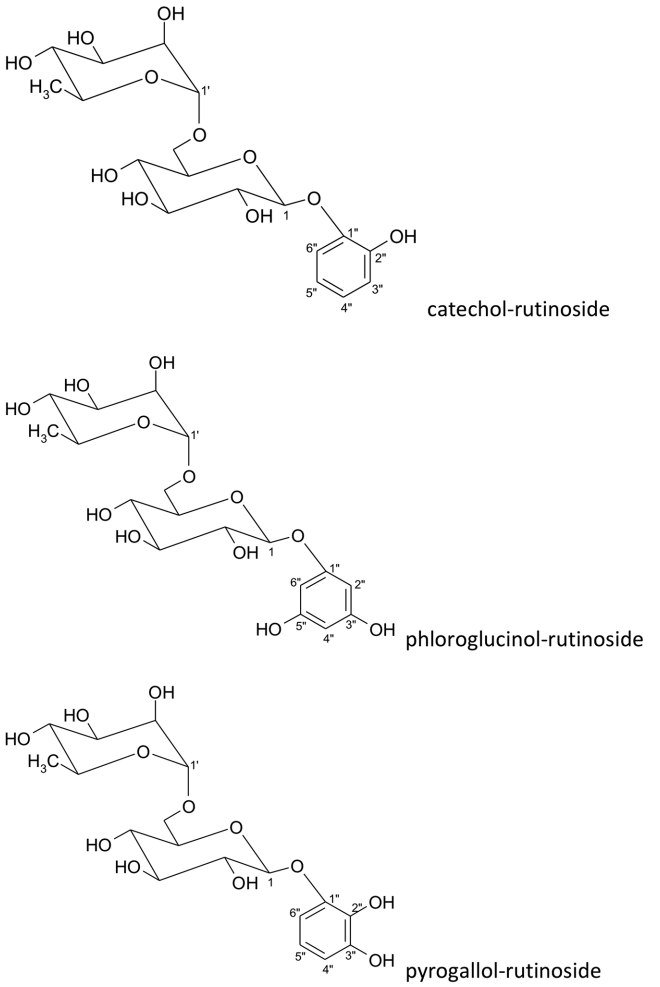


FIG. 5 *Structures of synthesized compounds.*

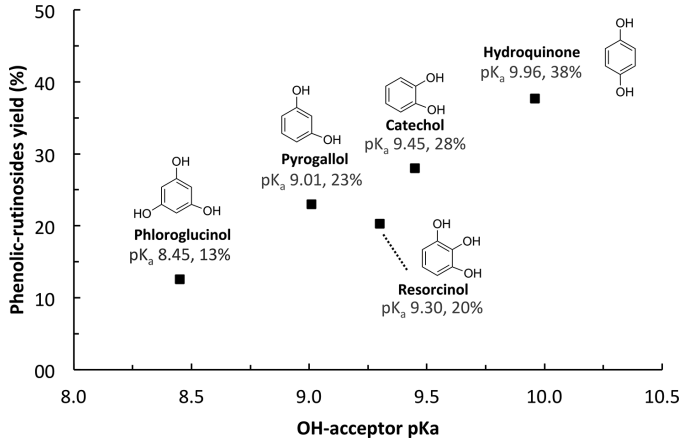


FIG. 6 *Apparent dependence of transglycosylation yield on the acceptor pK_a value using the 6-O-α-rhamnosyl-β-glucosidase from Acremonium sp. DSM 24697. Phenol (pK_a 9.99) is not represented in the graph due to its low yield (0.3%).*

the phenolic acceptor molecule in the attack on the covalent glycosyl–enzyme intermediate. A similar relationship was observed for the rutinosidase from tartary buckwheat where the transglycosylation yields decreased in the order vanillic acid (pK_a 8.81), ferulic acid (pK_a 8.65), caffeic acid (pK_a 8.69), and sinapic acid (pK_a 9.21) [24, 36]. With the exception of sinapic acid, for which a high steric hindrance is expected, a direct correlation between yield and pK_a was observed.

Rutinosides can have interesting medicinal activities. For instance, the reported glycosylated HQ compounds have been shown to inhibit human tyrosinase [16–18]. In addition, phenolic acid rutinosides were produced in transglycosylation reactions using a rutinosidase from tartary buckwheat (*Fagopyrum tataricum*) seeds; these compounds exhibited enhanced antiviral activities against feline calicivirus [24]. The α-rhamnosyl-β-glucosidase from *Acremonium* sp. DSM 24697 is a versatile biocatalyst that accepts various aliphatic and aromatic compounds as sugar acceptors [5, 10]. In this work, we have shown that this enzyme is an interesting biocatalyst for the rutinosylation of various phenolic compounds with acceptable yields. Only monorutinosylated products were generated, and a single transglycosylation product was formed with pyrogallol as an acceptor, which underlines the selectivity of the reaction. As shown recently, transrutinosylating enzymes can be used in bi-enzymatic reactions, enlarging their application potential. Transrutinosylation by diglycosidases in conjunction with the subsequent decapping of the formed glycoconjugates by rhamnosidases opens a route to the high-yield production of various glucosides [26]. This work is a contribution to the development of convenient and sustainable processes with synthetic enzymes for the glycosylation of small phenolic compounds, which may have their applications in medicine, health care or cosmetics.

4. Acknowledgements

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The authors declare that they have no conflict of interest.

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