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α-L-Rhamnosyl-β-D-glucosidase (Rutinosidase) from *Aspergillus niger*: Characterization and Synthetic Potential of a Novel Diglycosidase

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Abstract: We report the first heterologous production of a fungal rutinosidase (6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosidase) in *Pichia pastoris*. The recombinant rutinosidase was purified from the culture medium to apparent homogeneity and biochemically characterized. The enzyme reacts with rutin and cleaves the glycosidic linkage between the disaccharide rutinose and the aglycone. Furthermore, it exhibits high transglycosylation activity, transferring rutinose from rutin as a glycosyl donor onto various alcohols and phenols. The utility of the recombinant rutinosidase was demonstrated by its use for the synthesis of a broad spectrum of rutinosides of primary (saturated and unsaturated), secondary, acyclic and phenolic alcohols as well as for the preparation of free rutinose. Moreover, the α-L-rhamnosidase-catalyzed synthesis of a chromogenic substrate for a rutinosidase assay – *para*-nitrophenyl β-rutinoside – is described.

Keywords: diglycosidase; enzyme catalysis; glycosides; rutinosidase; transglycosylation

Introduction

α-L-Rhamnosyl-β-D-glucosidases (rutinosidases) constitute an important, albeit poorly characterized class of glycosidases. They catalyze the hydrolysis of rutin and/or hesperidin, cleaving the glycosidic linkage between the disaccharide rutinose and the aglycone, leaving the rutinose unit intact. These enzymes (EC 3.2.1.168) belong to the enzyme family of diglycosidases. Structural information on rutinosidases, which are typically microbial or plant in origin, is very limited: neither primary nor tertiary structures are known. On the other hand, the application of and screening for novel rutinosidases have been described in a number of publications in the last five years.[1–5] This highlights the potential of these enzymes, which can often catalyze both hydrolytic and transglycosylation reactions, for agricultural, biomedical and food chemistry applications.[6]

Rutin (**1**, quercetin 3-*O*-β-rutinoside), hesperidin (**2**, hesperetin 7-*O*-β-rutinoside) and naringin (**3**) (Figure 1) are major flavonoid glycosides of some

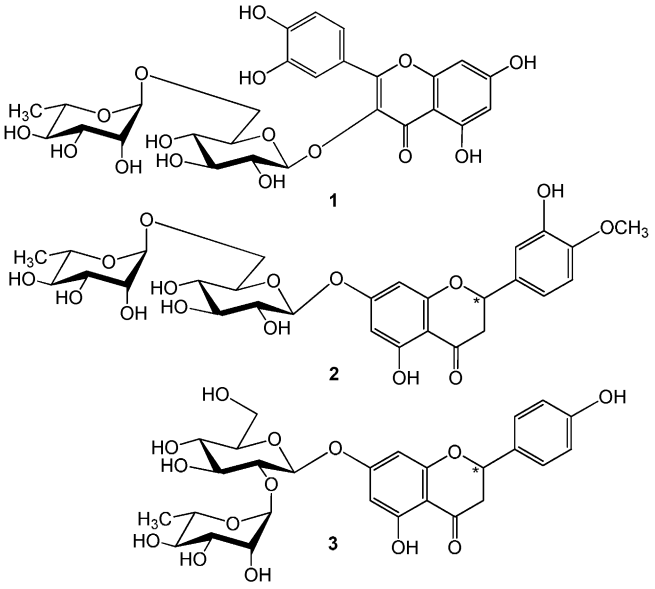


Figure 1. Potential substrates for rutinosidase.

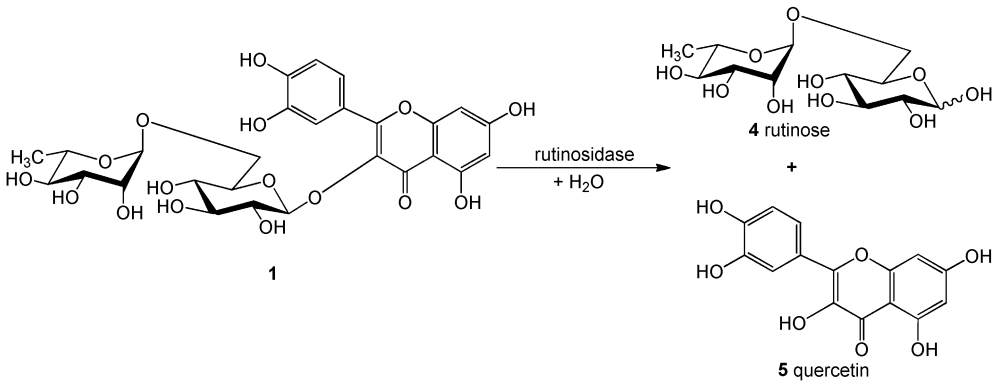


Figure 2. Rutinosidase-catalyzed hydrolysis of rutin (**1**).

plants (mainly buckwheat and common rue, and also tobacco) and fruits, such as apple, tomato, grape and citrus.[6] Waste products of the agro- and fruit juice-producing industry contain large amounts of rutin and hesperidin, which represent inexpensive starting materials for the synthesis of rutinosylated glycoconjugates with potentially interesting biological and pharmacological activities and for the preparation of rutinose.

Rutinose (**4**, 6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranose) (Figure 2) is a disaccharide with potential medical applications attributed to the L-rhamnopyranose moiety.[7] Due to the limited availability of rutinosidases, this disaccharide has so far been unavailable for research and practical exploitation.

Rhamnosidases, which cleave rhamnose from rutin and hesperidin and other rutinose-containing glycoconjugates, are non-mammalian glycosidases. Thus it appears that rutinose-capped compounds are resistant to hydrolysis in human tissues, which lack α-L-rhamnosidases.[7,8] As a consequence, rutinosylated glycoconjugates could provide novel biological activities and/or altered pharmacokinetic properties; for example, resorption of the rutinosides can only occur in the lower intestines (caecum) after cleavage of the glycosidic bond by rhamnosidase-producing human gut microflora. Rutinosylated compounds can also be used as food additives, ingredients in cosmetics and antiviral agents.[5,9] Furthermore, rutinose-containing glycopolymers may have entrapment abilities for fibroblasts due to the presence of rhamnose-recognizing receptors on their surface.[10]

We present here the first heterologous production of a rutinosidase from *Aspergillus niger* K2 in *Pichia pastoris*. The enzyme exhibited high-yielding transglycosylation activities, which is especially interesting and advantageous for synthetic applications. The recombinant enzyme was used for the production of various rutinosides, some of which were synthesized for the first time as well as for the preparation of the disaccharide rutinose (Figure 2).

Results and Discussion

Screening for Rutinosidase Activity

Since rutin (**1**) occurs in many plants it is not surprising that a number of microorganisms have developed metabolic routes for its degradation. The initial step in this catabolic pathway is the cleavage of the glycosidic bond, catalyzed by rutin-specific rutinosidases, also called rutinases.[11]

A collection of 17 fungal strains was screened for extracellular rutinosidase activity using the transglycosylation assay with rutin as a donor and butanol as an acceptor (Table 1). Because the strains exhibited also α-rhamnosidase and β-glucosidase activities (releasing *para*-nitrophenol by the sequential hydrolysis of **8**), the use of a transglycosylation assay was essential for the unambiguous detection of rutinosidase activity. Enzymes with high transglycosylation activities were therefore screened for. Six strains were found to be active generating *n*-butyl rutinoside in the assay in less than 2 h (Table 1). Interestingly, only rutin but not hesperidin served as an inducer of rutinosidase activity.

We have tried to use the highly active enzyme from *Chaetomium globosum* for the sequencing taking into account that its genome was available (http://www.broadinstitute.org/annotation/genome/chaetomium_globosum/Home.html), but we were not able to purify the active protein to the quality suitable for MS. We have obtained some sequences but no fit was found. Then we have decided for *Aspergillus niger*, which is generally accepted as a fungal model organism and its genome was available.[12]

Cloning of Rutinosidase from *Aspergillus niger*

The availability of the genome sequence of *Aspergillus niger* CBS 513.88[12] prompted us to opt for the *A. niger* K2 strain as a source of the rutinosidase-encod-

Table 1. Fungal strains screened for the rutinosidase activity.[a]

Strain	Activity[b]
Acremonium persicinum 1850 CCF	–
Aspergillus niger K2 CCIM	+
Aspergillus oryzae 1066 CCF	+
Aspergillus terreus TK CCF	–
Circinella muscae 1568 CCF	–
Fusarium culmorum 2680 CCF	–
Fusarium oxysporum 377 CCF	–
Chaetomium globosum	+++
Micromucor ramannianus 1022 CCF	–
Mucor circinelloides 2598 CCF	++
Mucor dimorphosporus 2576 CCF	–
Mucor wosnessenskii 2606 CCF	–
Penicillium chrysogenum 1269 CCF	+
Penicillium oxalicum 2430 CCF	++
Talaromyces flavus 2686 CCF	–
Trichoderma polysporum 1555 CCF	–
Umbelopsis vinacea 1097 CCF	–

[a] Screening for rutinosidase was performed in a total volume of 300 μL (100 μL of 150 mM rutin in 50% DMSO as a donor; 80 μL of *n*-butanol; 70 μL of 50 mM citrate-phosphate buffer, pH 5.0; 50 μL of culture medium). Products of the reaction (*n*-butyl β-rutinoside) were monitored by TLC (acetone/formic acid/water = 30:1:1, v/v/v).

[b] Transglycosylation product formation in: +++ 30 min; ++ 60 min + 120 min; – no activity.

ing gene even though this strain did not have the highest activity. To identify the gene in the genomic DNA sequence, peptide sequences of the purified enzyme had to be determined. Consequently, the extracellular rutinosidase from *A. niger* K2 was purified to apparent homogeneity from the medium using three chromatographic steps, including cation exchange, size-exclusion chromatography, and chromatofocusing, but the overall yield of the pure enzyme was only 0.04%.

The resulting enzyme was analyzed by SDS-PAGE and the band corresponding to a protein of a molecular mass of ~75 kDa was excised from the gel and subjected to trypsin digestion followed by MALDI-TOF mass spectrometry. The obtained mass spectrometric data (Supporting Information, Table S29) allowed an unambiguous identification of the putative gene with the accession numbers CAK39791 and XP 001392228.1, which was tentatively annotated in the GenBank as a β-1,3-exoglucosidase-encoding open reading frame.

Expression and Purification of Recombinant Rutinosidase

Amplification of the corresponding gene was accomplished by PCR using chromosomal DNA from *A. niger* K2 as the template and specific primers, which were derived from the gene segments of CAK39791 encoding the N- and C-termini of the putative mature enzyme. Cloning the PCR product downstream of the α-factor-encoding DNA segment in pPICZαA enabled the production of the fungal rutinosidase in *P. pastoris* and its secretion into the medium. A two-step purification (cation exchange and size-exclusion chromatography) gave the recombinant rutinosidase in apparent homogeneity and 30% yield (Figure 3).

The purified rutinosidase had a specific hydrolytic activity of 0.6 U mg^{-1} in the spectrophotometric enzyme assay with *para*-nitrophenyl β-rutinoside (**8**) as the substrate. Seven cycles of automated Edman degradation revealed EFRAPLA as the N-terminal sequence of the recombinant rutinosidase, confirming the correct N-terminus with the APLA sequence obtained from the mature enzyme lacking its fungal signal sequence of 19 amino acids. The expression of the correct protein product was verified by MALDI-TOF analysis of peptide fragments generated by trypsin and endoproteinase Asp-N digestion of the recombinant rutinosidase (Supporting Information, Figure S30).

Deglycosylation of the purified rutinosidase (both wild-type and recombinant) with Endo H_f did not affect the enzyme activity and resulted in a pronounced shift of the protein band on SDS-PAGE compared to the native recombinant enzyme (Figure 3). The molecular mass of the deglycosylated protein was determined to be ~45 kDa, which is in good agreement with the calculated molecular mass of 41.3 kDa for the mature protein of 367 amino acids. An isoelectric point of 5.0 (recombinant rutinosidase) was determined using chromatofocusing.

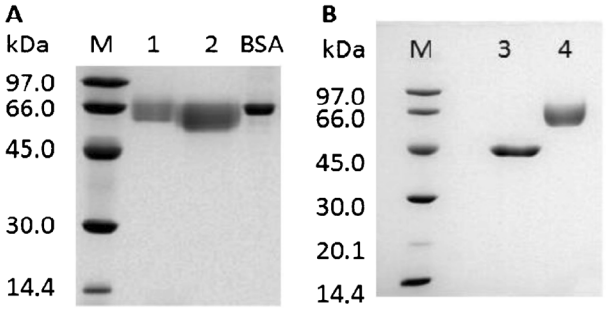


Figure 3. SDS-PAGE, 10% gel **A:** 1 – crude secreted recombinant rutinosidase (after dialysis), 2 – purified recombinant rutinosidase; **B:** 3 – Endo H_f-deglycosylated purified recombinant rutinosidase; 4 – untreated purified recombinant rutinosidase.

Purified rutinosidase (recombinant) displayed traces of β-D-glucopyranosidase (~1%) activity and absolutely no α-L-rhamnopyranosidase activity.

Several fungal and plant-derived rutinosidases were described in the past, although no sequence information was available. Three were shown to be exclusively specific for 3-*O*-linked rutinosides.[13–15] In contrast to these, the extracellular α-L-rhamnosyl-β-D-glucosidase from *Acremonium* sp. was solely active on 7-*O*-linked rutinosides such as hesperidin, and did not accept rutin as a substrate.[3] Although the rutinosidase described in this report exhibited hydrolytic activity towards both rutin and hesperidin, rutin is a *ca.* ten times better substrate (Supporting Information, Figures S24 and S25). On the other hand, no activity was found with naringin (naringenin 7-*O*-neohesperidoside), another major flavonoid diglycoside in grapefruit. Thus it appears that the rutinosidase from *A. niger* only recognizes rutinose as the glycone moiety, whereas its aglycone specificity appears to be relaxed.

Optimum reaction conditions for the recombinant rutinosidase were determined with **8** as the colorimetric substrate. The highest enzyme activity was observed at 50 °C at a pH of 3.0 (Figure 4 and Figure 5). Temperatures exceeding 55 °C resulted in major loss of activity. The dependence of the enzyme activity on pH was characterized by a sharp decrease below pH 2.5 with a gradual loss of activity above pH 3.0, the enzyme being completely inactive at pH 2.0 and 7.0. The influence of DMSO and ethanol on the activity of the enzyme was determined. A substantial loss in activity was observed in the presence of DMSO or ethanol (10%) which caused a more than 60% loss of enzyme activity (Figure 6). DMSO is important for this transglycosylation because of the insufficient solubility of rutin in water.

The kinetic parameters of the rutinosidase with *para*-nitrophenyl rutinoside as the substrate have been determined to be (pH 5.0; 25 °C): $K_M = 0.598 \pm 0.113$ mM, $V_{max} = 0.154 \pm 0.0186$ min^{-1} (see Figure S31 in the Supporting Information).

The relatively low pH optimum of this extracellular enzyme correlates well with the habitat of *A. niger*, which often generates organic acids (e.g., citric acid) or grows on fruit substrates in an acidic environment in the presence of glycosylated flavonoids.

Interestingly, the cloned rutinosidase gene with its sequence accession number CAK39791 has been assigned in the CAZy database to the class 5–subfamily 23 of glycoside hydrolases,[16] together with the characterized β-primeverosidase from *Penicillium multicolor* TS-5 (EC 3.2.1.149; BAG70961)[17] and 7 more fungal enzymes. Significantly more information, including one tertiary[18] and several primary structures, is available on primeverosidases. These enzymes constitute a different class of diglycosidases, cleaving the glycosidic linkage between the aglycone and β-primeverose (6-*O*-β-D-xylopyranosyl-β-D-glucopyranose).[6,19] In order to gain some insight into the structural diversity of diglycosidases, we performed a phy-

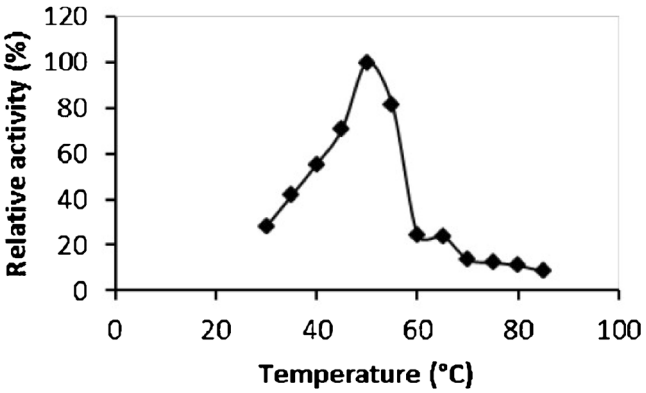


Figure 4. Temperature profile of the recombinant rutinosidase. The effect of temperature on the activity of the enzymes was determined in 50 mM citrate-phosphate buffer (pH 3.5).

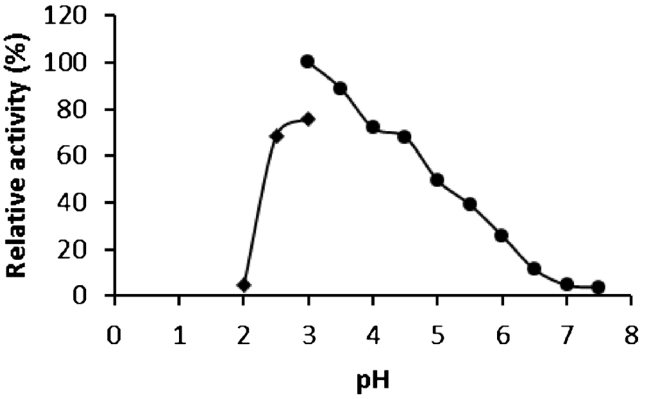


Figure 5. pH profile of recombinant rutinosidase at 35 °C; glycine-HCl buffer (–◆–) and Britton–Robinson buffer (–●–).

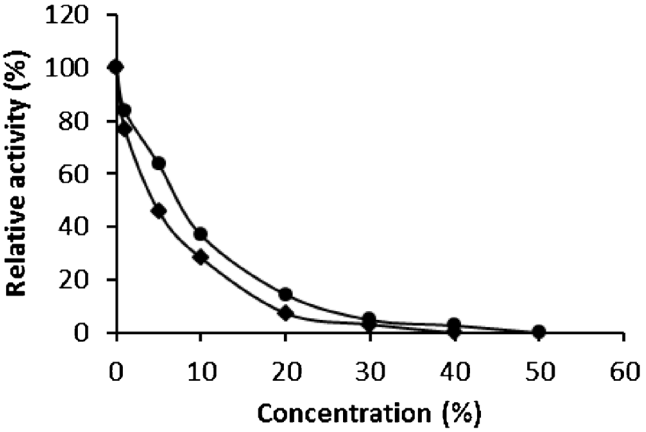


Figure 6. The influence of ethanol (–●–) and DMSO (–◆–) on the activity of the recombinant rutinosidase.

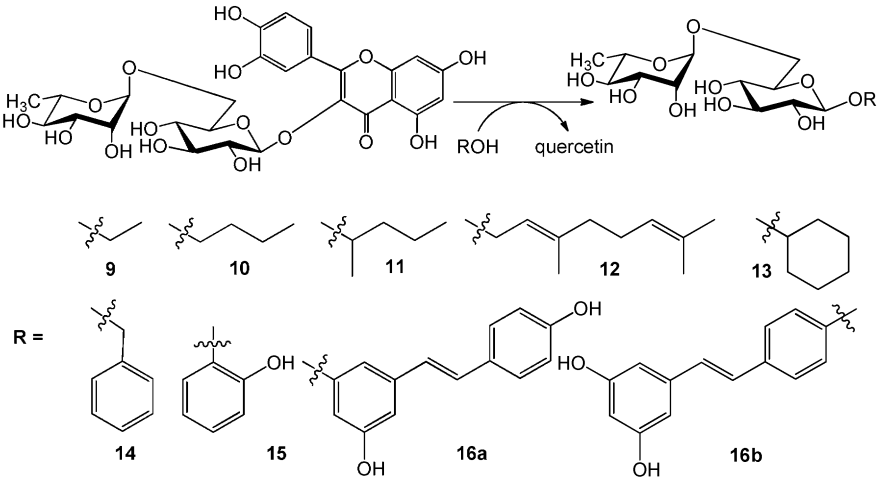


Figure 7. Rutinosidase-catalyzed synthesis of various types of β-rutinosides.

logenetic analysis of the rutinosidase with 6 known diglycosidases from plants and fungi, 9 GH-5–subfamily-23 sequences in the CAZy database, and the 15 putative fungal glycosidases, which best matched to the rutinosidase-encoding sequence in a BLASTP search (Supporting Information, Figure S26). The search revealed that the cloned rutinosidase most closely resembled 4 glycosidases from *Aspergillus* strains with protein sequence identities of 69–99%. The diglycosidases from plants clustered in a separate clade whereas the putative fungal diglycosidases together with the GH-5–subfamily-23 sequences and the cloned rutinosidase grouped in a different clade.

This suggests that the putative and characterized fungal diglycosidases within clade B can be considered structurally similar. The alignment of these protein sequences with the sequence of the primeverosidase whose structure has been determined (BAC78656)[17] revealed 9 conserved residues (composed of F, G, H, S, T, V, W and Y). However, no clear indication of catalytically important residues could be found. It appears that the diglycosidases in clade B are phylogenetically too distant from the primeverosidase-encoding sequence BAC78656 to extract structural data.

Enzymatic Synthesis of β-Rutinosides

The strong transglycosylation activity of the rutinosidase enabled the facile synthesis of various rutinosides using rutin as the disaccharide donor and a broad range of primary, secondary, saturated or unsaturated alcohols as acceptors with linear or cyclic carbon chains, including phenolic compounds (Figure 7). The transglycosylation reactions were monitored by TLC and the reactions were stopped when the product concentration reached its highest level. The molecular mass of each synthesized rutinoside was determined by mass spectrometry, and the structures of the transglycosylation products were confirmed by NMR analysis.

The highest yield of the investigated transglycosylation reactions was determined to be 46% with ethanol as an acceptor (Table 2). The transglycosylation product yields decreased with increasing chain length and bulkiness of the acceptor compounds. Interestingly, phenolic acceptors such as catechol (**15**) and resveratrol (**16**) were also glycosylated, although in lower yields. To the best of our knowledge, the enzymatic glycosylation of these phenolic compounds (**15** and **16**) with retaining glycosidases has not been described yet. It is worthwhile to note in this context that the E197S mutant of the *Humicola insolens* Cel7B glycosidase catalyzed the synthesis of flavonoid glycosides, transferring the disaccharide donor lactosyl fluoride to several flavonoids.[20]

The enzymatic transglycosylation of compound **16**, which contains three phenolic groups, yielded a mixture of two regioisomeric glycosides in the ratio of 2:1 **16a**:**16b** (C-3 and C-4′ glycosides). Preparation of rutinosides using transglycosylations of aliphatic alcohols

Table 2. Transglycosylation yields of rutinosidase from *A. niger* (recombinant) with various acceptors.

Acceptor	Product	Yield [%]
ethanol	**9**	46.0
n-butanol	**10**	44.6
pentan-2-ol	**11**	37.9
geraniol	**12**	4.1
cyclohexanol	**13**	22.2
benzyl alcohol	**14**	18.2
catechol	**15**	27.1
resveratrol	**16**	8.0

have been described in the past with a crude extract of *Fagopyrum tataricum* seeds;[1] here, methyl-, ethyl-, propyl-, isopropyl- and benzyl-β-rutinosides were obtained in relatively high yields (84–94%).

The synthesis of rutinose-containing glycoconjugates has many potential applications. The glycosylation of several flavonoids has been shown to increase their aqueous solubility and bioavailability. For instance, quercetin 7-rhamnoside exhibited a more potent antiviral activity against porcine epidemic diarrhea virus than the flavonoid aglycone.[21] Recently, Katayama and co-workers reported on the increased antiviral activity of various rutinosylated phenolic acids against feline calicivirus, which is a surrogate of gastroenteritis-causing noroviruses, compared to the respective aglycone.[5] Rotavirus infections were inhibited *in vitro* by several flavonoids, including hesperidin, whereas no inhibition was detected for the mere aglycones.[22] Rutinose-containing compounds are expected to have interesting pharmacological and medicinal applications.[7,8,23] It has been shown that rutinose-containing flavonoids are only absorbed in the intestines after hydrolysis of the rhamnose catalyzed by human gut microflora.[24]

Rutinosidases that have been used so far were isolated from various microbial sources, often not properly determined taxonomically and/or not available from public collections. This often resulted in the generation of irreproducible results. Although the gene (accession numbers CAK39791 and XP 001392228.1) has been ascribed to a putative β-1,3-exoglucosidase (*A. niger*), we have assigned it to a rutinosidase activity. The first known sequence of a rutinosidase will thus allow for further data-mining for other rutinosidase sequences and also for a detailed structural study (crystallization) of this enzyme. Thanks to the commercially available preparation of the synthetic genes, this enzyme is now available to the whole scientific community.

Conclusions

In conclusion, we present here the first complete sequence of a functional rutinosidase (*A. niger* K2, Supporting Information, Tables S27 and S28), its recombinant expression, full biochemical characterization and its application in the synthesis of various rutinosides in good yields. The recombinant production of rutinosidase will allow the widespread and universal application of this enzyme, since to date this enzyme has been prepared from various complex natural sources. Both the native and recombinant enzymes cleaved rutinose both from rutin and hesperidin. Rutin, which is a commodity chemical, can serve as a rutinosyl donor in the tranglycosylation of various primary (saturated and unsaturated), secondary, alicyclic and phenolic alcohols, thus demonstrating the universal application of this enzyme in glycosylation. The selective cleavage of rutin by rutinosidase provides (after respective purification) a source of rutinose.

This method opens also an access to the respective β-glucopyranosides as it is easy to trim respective rutinosides by removing selectively α-L-rhamnose. For this purpose recombinant α-L-rhamnosidase from *A. terreus* void of β-glucosidase activity (expressed in *P. pastoris*) can be used.[25] We have exemplified this selective enzymatic trimming of rutinosides in the preparation of quercetin 3-β-glucopyranoside from rutin.[26]

We have thus provided an affordable tool for the preparation of rutinosides of various aglycones at a preparative scale, which will enable further detailed study of these compounds.

Experimental Section

Materials

A DNeasy Mini Plant Kit from Qiagen was used for DNA isolation. A High Pure Plasmid Isolation Kit and Genopur Plasmid Midi Kit (Roche, DE) were used for plasmid isolation. An EasySelect *Pichia* Expression Kit was purchased from Invitrogen (US). Oligonucleotide primers were obtained from Generi Biotech (CZ). The pGEM.T Easy Vector System was purchased from Promega (US). The Advantage® 2 PCR Kit was purchased from Clontech (US). The enzymes and buffers for DNA manipulations were obtained from New England Biolabs (US). Media components were from Oxoid (UK). All other chemicals, including *para*-nitrophenyl β-D-glucopyranoside, were purchased from Sigma–Aldrich.

Strains, Media and Cultivation Conditions

The fungal strains used for activity screening are deposited in the Culture Collection of the Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague (CCIM) or in the Czech Collection of Fungi, Charles University in Prague (CCF). The cultures were maintained on slants using the following medium [$g\,L^{-1}$]: agar-agar, 20; bacto-peptone, 5; and malt extract, 35. The medium (200 mL) was inoculated with a suspension of spores in a 0.1% Tween 80 solution. The fungal strains were cultivated in 500-mL round-bottomed flasks in a rotary shaker at 28 °C, 200 rpm, and 50 mm eccentricity. The production medium consisted of [$g\,L^{-1}$]: rutin or hesperidin, 5.0; KH_2PO_4, 15.0; NH_4Cl, 4.0; KCl, 0.5; yeast extract, 5.0; casein hydrolysate, 1.0, and 1.0 mL of trace element Vishniac solution at pH 5.0.[27] After sterilization, each flask was supplemented with 1.0 mL of sterile 10% $MgSO_4 \cdot 7\,H_2O$ (v/w).

The bacterial strain *Escherichia coli* JM109 (Promega, USA) was cultivated in Luria-Bertani (LB) medium composed of [$g\,L^{-1}$]: tryptone, 10.0; yeast extract, 5.0 and NaCl, 5.0, pH 7.0 at 37 °C for 24 h. The plates for the selection of

transformants contained [g L^{-1}]: tryptone, 10.0; yeast extract 5.0; NaCl 5.0; agar 15.0; cultivation at 37 °C for 24 h.

Pichia pastoris KM71H (Invitrogen) was grown in YPD, BMGY and BMG media at 28 °C. After electroporation, the *P. pastoris* transformants were grown under selective conditions in YPD medium containing Zeocin (100 mg L^{-1}). All *P. pastoris* media were prepared according to the EasySelect *Pichia* Expression Kit manual (Invitrogen).

Purification of Wild-Type Rutinosidase from *A. niger*

Aspergillus niger K2 CCIM was cultivated for 6 days at 28 °C and 250 rpm in shaking flasks in the presence of 0.5% rutin as an inducer of rutinosidase. The mycelium was subsequently removed by filtration through an asbestos-cellulose filter (C10, Vertex, CZ). The filtrate (2 L, total 70 U) was dialyzed overnight against a 10 mM sodium acetate buffer (pH 3.6) and then loaded onto a Fractogel EMD SO_3^- column (1.5 × 10.0 cm, Merck), which was equilibrated with 10 mM sodium acetate (pH 3.6). The enzyme was eluted with a linear gradient of NaCl (0 to 0.5 M) at a flow rate of 5 mL min^{-1}.

The active fractions were pooled and subsequently concentrated by ultrafiltration to a final volume of 2 mL using cellulose membranes with a 10 kDa cut-off (Millipore). The concentrated protein sample was then subjected to size-exclusion chromatography using a Superdex 200 10/300 GL column (1.0 × 30 cm, GE Healthcare Life Sciences) equilibrated with 25 mM piperazine, 150 mM NaCl, pH 5.5. A chromatofocusing column (Mono P 5/200 GL, Amersham, Biosciences, SE), which was equilibrated with 25 mM piperazine (pH 6.3), was used as the final purification step. After the protein sample was injected onto the column, a polyampholyte elution buffer (3.0 mL, Polybuffer 74; GE Healthcare Life Sciences) was applied yielding in total 0.03 U (0.04%). The purified enzyme was stored for several months at 4 °C with no significant loss of activity.

In-Gel Digestion and MALDI-TOF Mass Spectrometry

Coomassie Brilliant Blue-stained protein bands were excised from the gel, cut into small pieces and destained using 50 mM 4-ethylmorpholine acetate (pH 8.1) in 50% MeCN. After complete destaining, the gel was washed with water, shrunk by dehydration in MeCN and rehydrated again in water. The supernatant was removed and the gel was partly dried in a SpeedVac concentrator. The gel pieces were then incubated overnight at 37 °C in a cleavage buffer containing 25 mM 4-ethylmorpholine acetate, 5% MeCN and sequencing grade trypsin (100 ng; Promega, US) or Asp-N protease (10 ng; Roche, DE). The resulting peptides were extracted with 40% MeCN/0.1% TFA.

An aqueous 50% MeCN/0.1% TFA solution of α-cyano-4-hydroxycinnamic acid (5 mg mL^{-1}; Sigma–Aldrich, USA) was used as a MALDI matrix. One μL of the peptide mixture was deposited on the MALDI plate, allowed to air-dry at room temperature and was overlaid with 0.4 μL of the matrix. MALDI mass spectra were measured on an Ultraflex III instrument (Bruker Daltonics, Bremen, DE) equipped with LIFT technology for MS/MS analysis. The peptide mass spectra were acquired in the mass range of 700–4000 Da and calibrated externally using a mixture of peptide standard PepMix (Bruker Daltonics, DE). Both MS and MS/MS data were searched against the NCBInr20130120 database subset of fungal proteins using the in-house MASCOT search engine (http://www.matrixscience.com).

Cloning

The primers were designed based on the sequence of the putative β-1,3-exoglucosidase-encoding gene (accession numbers CAK39791 and XP 001392228) of *A. niger* CBS 513.88.[12] The forward primer FWRUT, which contained an *Eco*RI restriction site (italics), was designed to comprise the N-terminus of the mature protein, which lacks the fungal N-terminal signal sequence as determined by Signal IP 4.0:[28] 5′-CC*GAATTC*CGGGCCCCCCTAGCCAGCCCCCCAAAC-3′. The sequence of the reverse primer RERUT, which contained a *Kpn*I restriction site (italics), was derived from the C-terminus of the putative enzyme: 5′-CGG*GGTACC*CCGTCAATTGAAATACCCCTCGTCAATAAACG-3′. PCR reactions were performed using a GeneE Thermal Cycler (Techne) with an optimized amount of isolated DNA as a template in a 50 μL reaction volume, containing 1 μL of Advantage 2 Polymerase Mix, 1 μL of dNTPs (10 mM each), 2 μL of 10 μM FWRUT and RERUT, and 2 μL of DMSO.

The PCR conditions were as follows: initial denaturation at 95 °C for 1 min, followed by 35 cycles of denaturation at 95 °C for 30 sec, primer annealing at 50 °C for 30 sec, extension at 68 °C for 3 min, with a final extension of 10 min at 70 °C. After ligation of the PCR product (amplified with primers without restriction sites) into pGEM-T Easy and the subsequent transformation of the ligation mix into *E. coli* JM109, the resulting plasmid pGEM-RUT was isolated and subsequently analyzed by restriction digestion and sequencing using an ABI PRISM 3130xl sequencer (Applied Biosystems). The full-length gene was re-amplified using the primers mentioned above and pGEM-RUT as a template. The resulting PCR product was spin-column purified, digested with *Eco*RI and *Kpn*I and inserted into the ligation-ready pPICZα A expression vector. The correct sequence of the resulting plasmid, which was designated pPICZαA-RUT, was confirmed by DNA sequencing.

Phylogenetic Analysis

Multiple alignments of diglycosidase-encoding protein sequences were generated using Clustal W[29] within the software package MEGA version 4,[30] which was used for the construction of a phylogenetic tree. An unrooted phylogenetic tree was constructed using the Neighbor-Joining method with 1000 bootstrap resamplings. The evolutionary distances were computed using the Poisson correction method. All positions containing alignment gaps were only eliminated in pairwise sequence comparisons.

Expression of Rutinosidase in *Pichia pastoris*

The recombinant plasmid pPICZαA-RUT was linearized with *Pme*I, purified by phenol/chloroform extraction and subsequently electroporated into electro-competent *P. pastoris* KM71H cells (*Pichia* program, Electroporator, Bio-Rad, DE). The transformants were screened on plates with YPDS medium [g L^{-1}]: yeast extract, 10; peptone, 20; glu-

cose, 20; sorbitol, 182.2; agar-agar, 15; Zeocin at a final concentration of 1 mg mL^{-1}. The presence of the rutinosidase gene in the transformants was confirmed by PCR with 5′AOX and 3′AOX universal primers using yeast genomic DNA as a template and using the procedures from the EasySelect *Pichia* Expression Kit manual. 100 mL BMGH (buffered minimal glycerol) medium in 1-L baffled conical flasks was inoculated with positive single colonies and grown at 28 °C in a shaking incubator (250 rpm) until the culture reached an OD_{600} = 2–6. Afterwards, the cells were harvested by centrifuging at 3000 *g* for 10 min at room temperature. The supernatant was removed and the cell pellet resuspended in 30 mL BMMH (buffered minimal methanol) medium, containing 0.5% methanol (v/v) instead of 1% glycerol (v/v) to induce gene expression (media described in the "EasySelect *Pichia* Expression Kit", Invitrogen, US). The induction was maintained for 4 days by adding methanol to a final concentration of 0.5% (v/v) every 24 h. The 1 mL culture aliquot was centrifuged at 17,000 *g* in a table-top microcentrifuge for 3 min at room temperature at 24 h intervals to analyze the enzyme expression to the medium by SDS-PAGE and enzyme assay.

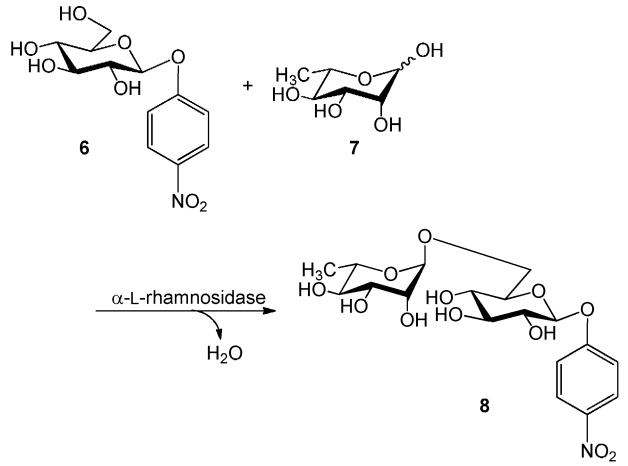
The clones with the highest production of recombinant rutinosidase were used for protein production.

Purification of Recombinant Rutinosidase

After 6 days of cultivation the cells were harvested by centrifugation (3,000 *g*, 10 min at 4 °C). The supernatant (600 mL, total 72 U) was dialyzed against 6 L of 10 mM sodium acetate buffer for 2 h and then diluted with an equal amount of deionized water and the pH was adjusted to 3.6 with 10% acetic acid. This solution was loaded onto a Fractogel EMD SO_3^- column (1.5 × 10.0 cm, Merck, DE) equilibrated with 10 mM sodium acetate buffer (pH 3.6) and eluted with a linear gradient of NaCl from 0 M to 1.0 M at a flow rate of 5 mL min^{-1}. Fractions were analyzed by enzyme assay and those containing active rutinosidase were pooled and concentrated by ultrafiltration using cellulose membranes with a 10 kDa cut-off (Millipore, USA). The concentrated protein was finally purified by gel filtration on a Superdex 200 10/300 GL column (1.0 × 30 cm, 50 mM citrate-phosphate buffer, 150 mM NaCl, pH 5.0, Amersham Bioscience, SE) to yield 21.6 U (30%).

Enzymatic Synthesis of *para*-Nitrophenyl β-Rutinoside (8)

The title compound **8** was prepared from *para*-nitrophenyl β-D-glucopyranoside (**6**) and L-rhamnose (**7**) (Scheme 1) by reverse hydrolysis catalyzed by the recombinant α-L-rhamnosidase from *A. terreus*.[26] Substrate **6** (150 mg, 0.498 mmol) and rhamnose (**7**, 410 mg, 2.498 mmol) were dissolved in citrate-phosphate buffer (4.0 mL, 50 mM, pH 5.0), α-L-rhamnosidase (7 U) was added and the mixture was incubated at 35 °C (Thermomixer, Eppendorf, DE). The reaction was monitored by TLC (*n*-propanol/H_2O/NH_4OH = 7:2:1, v/v/v; Kieselgel 60 F_{254}, Merck, DE) and stopped by heating (100 °C, 10 min), typically after 72 h. Spots on TLC were visualized by charring with 5% H_2SO_4 in EtOH. The reaction mixture was loaded onto a BioGel P-2 column (BioRad, USA, 1.5 × 150 cm, eluent water), fractions containing the product were pooled and evaporated, and re-purified in a silica gel column (1.5 × 50 cm, eluent *n*-propanol/H_2O/NH_4OH = 7:2:1, v/v/v). Unreacted substrate **6** could be almost completely recuperated. The title compound **8** was isolated as a white powder (TLC R_f = 0.57); yield: 6.2 mg (0.014 mmol, 2.8% related to the substrate **6**). MS (MALDI-TOF): *m/z* = 469.1 $[M+Na-H]^+$, calcd. mass for $C_{18}H_{24}NO_{12}Na$: 469.12 (^{13}C NMR Table S2, Figure S22, ^1H NMR Table S4, Figure S23 in the Supporting Information).



Scheme 1. Enzymatic preparation of *para*-nitrophenyl β-rutinoside (**8**).

Enzyme Assay

Rutinosidase activity was determined spectrophotometrically using **8** as a substrate. The reaction mixture contained **8** (10 μL of 10 mM), 10 μL of 50 mM citrate-phosphate buffer pH 5.0 and 30 μL of the enzyme solution. The reaction was incubated at 35 °C for 10 min and stopped by the addition of 1 mL of 0.1 M Na_2CO_3. The released *para*-nitrophenol was determined spectrophotometrically at 420 nm. One unit of enzyme activity was defined as the amount of enzyme releasing 1.0 μmol of *para*-nitrophenol per minute under the assay conditions.

Transglycosylation Activity

Transglycosylation activity was assayed in a total volume of 300 μL (100 μL of 150 mM rutin in 50% DMSO as the donor; 80 μL of *n*-butanol; 70 μL of 50 mM citrate-phosphate buffer, pH 5.0; 50 μL of enzyme solution) (culture medium). *n*-Butanol was selected as an optimal screening acceptor for tranglycosylation mainly due to high yields compared to other alcohols. The formation of the transglycosylation product (*n*-butyl β-rutinoside) was monitored by TLC (acetone/formic acid/water = 30:1:1, v/v/v).

Protein concentration was determined by Bradford assay (Bio-Rad) using bovine serum albumin as a standard.[31]

Substrate Specificity

Rutin (**1**, 10 mg, 0.016 mmol), hesperidin (**2**, 10 mg, 0.016 mmol) or naringin (**3**, 10 mg, 0.017 mmol) were suspended in 0.9 mL of 50 mM citrate-phosphate buffer,

pH 7.0; 0.1 mL of rutinosidase (0.1 mg of protein) was added to the suspension and the mixture was incubated at 35 °C (Thermomixer, Eppendorf, DE). The reaction was monitored by TLC (acetone/H_2O/CH_3COOH = 15:1:1, v/v/v) and by HPLC.

Analytical HPLC

The deglycosylations of **1** and **2** were monitored with Shimadzu Prominence UFLC system(Kyoto, JP) consisting of a DGU-20A mobile phase degasser, two LC-20AD solvent delivery units, a SIL-20ACHT cooling autosampler, a CTO-10AS column oven and SPD-M20A diode array detector. The column was a Chromolith Performance RP-18e (100 × 3 mm i.d., Merck, Darmstadt, DE). The PDA data were acquired in the 200–450 nm range and the signals corresponding to 360 nm (for **1** and **5**) or 282 nm (for **2** and **17**) were extracted. Gradient elution: mobile phase A (CH_3CN/H_2O/HCO_2H (5/95/0.1; v/v/v); mobile phase B (CH_3CN/H_2O/HCO_2H (80/20//0.1; v/v/v); gradient I (deglycosylation of **1**) 0–4 min, 7–30% B; 4–5 min, 30% B; 5–6 min, 30–7% B; gradient II (deglycosylation of **2**) 0–4 min 30% B; 4–6 min 30% B; 6–7 min 30–7% B. Flow rate was 1.5 mL min^{-1} at 25 °C. All samples were dissolved in methanol.

SDS-PAGE

Proteins were analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Brilliant Blue R-250 staining.

pI Determination

Extracellular rutinosidase was loaded onto a chromatofocusing column (Mono P 5/200 GL, Amersham Bioscience, SE) equilibrated with 25 mM piperazine buffer (pH 5.5) with pre-gradient of 3.0 mL Polybuffer 74 (pH 4.0).

Effect of pH and Temperature on the Activity of Recombinant Rutinosidase

The effect of pH and temperature on rutinosidase activity was determined using **8** as a substrate. The enzyme activity was measured at 35 °C in Britton–Robinson buffer (40 mM H_3PO_4, 40 mM CH_3COOH, 40 mM H_3BO_3 – pH was adusted with 200 mM NaOH) within the pH range 3.0–7.5 and in glycine-HCl buffer at pH 2.0–3.0. The temperature optimum was measured in 50 mM citrate-phosphate buffer (pH 3.5) in the temperature range 30–85 °C. Thermostability was measured at 50 °C with 50 mM citrate-phosphate buffer (pH 3.5) at 10 min or 30 min intervals. The enzyme activities were measured under standard assay conditions as described above. All assays were done in triplicate.

N-Terminal Sequencing

For N-terminal sequencing, the rutinosidase was electroblotted from 10% SDS-PAGE gel onto a PVDF membrane and analyzed by automated Edman degradation using a Procise 491 Protein Sequencer (Applied Biosystems, USA).

Deglycosylation of Native Wild-Type and Native Recombinant Rutinosidase

The rutinosidase was deglycosylated at 37 °C for 24 h with an ENDO H_f Kit (New England BioLabs, USA). Both reducing and non-reducing conditions for deglycosylation were employed.

Spectral Characterization

NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer (400.13 MHz for ^1H, 100.61 MHz for ^{13}C - compounds **9**, **10**, **11**, **14**, **15**) and a Bruker Avance III 600 MHz spectrometer (600.23 MHz for ^1H, 150.94 MHz for ^{13}C - compounds **12**, **13**, **16**) in CD_3OD at 25 °C (data and the spectra *in extenso* in the Supporting information). The assignment of individual proton spin systems was achieved by COSY experiments, and then transferred to carbons by HSQC. The position of an aglycone and glycosidic linkage was deduced using heteronuclear correlations in HMBC experiments.

Mass spectra were measured on Platform LC mass spectrometer (Waters, USA) in ESI mode, with Masslynx software. Source temperature was 80 °C, ionization energy was 0.2–0.4 keV. Positive-ion spectra in reflectron mode were also measured on Ultraflex III MALDI-TOF mass spectrometer (Bruker-Daltonics, Bremen, DE). A 50 mg mL^{-1} solution of 2,5-dihydrobenzoic acid in 50% MeCN/0.1% TFA was used as the MALDI matrix. The sample (0.4 μL) dissolved in water was allowed to dry at ambient temperature and overlaid with 0.3 μL of the matrix solution directly on the MALDI target.

6-*O*-α-L-Rhamnopyranosyl-β-D-glucopyranose (rutinose, 4)

Rutin (**1**, 200 mg, 0.328 mmol) was dissolved in DMSO (250 μL), diluted with citrate phosphate buffer (50 mM, pH 5.0) to make up 2.5 mL and the rutinosidase (0.5 U) was added. The mixture was incubated at 35 °C with shaking. The reaction was monitored by TLC (acetone/water/formic acid = 30/1/1, v/v/v; Kieselgel 60 F_{254}, Merck) and stopped by heating (100 °C, 10 min), typically after 24 h. The reaction mixture was diluted with water (5 mL), the pH was adjusted to pH 3 and precipitated quercetin was filtered off. The filtrate was evaporated and purified in a silica gel column (1.5 × 50 cm, mobile phase: acetone/water/formic acid = 30:1:1, v/v/v). Fractions containing the product were pooled and concentrated to dryness. The title compound (**4**) was isolated as a colorless amorphous solid (TLC R_f = 0.20); yield: 38.5 mg (0.118 mmol, 63.0%). ^{13}C NMR and ^1H NMR data are listed in Table S1 in the Supporting Information.

Transglycosylation Reactions: General Procedure

Rutin (**1**, 130 mg, 0.213 mmol) and the respective acceptor were dissolved in DMSO (250 μL), diluted with citrate phosphate buffer (50 mM, pH 5.0) to make up 2.5 mL, rutinosidase (0.5 U) was added and the mixture was incubated at 35 °C. All reactions were monitored by TLC (acetone/water/formic acid = 30/1/1, v/v/v; Kieselgel 60 F_{254}, Merck) and stopped by heating (100 °C, 10 min), typically after 24 h. The reaction mixture was purified in a silica gel column (1.5 ×

50 cm, mobile phase: acetone/water/formic acid = 30:1:1, v/v/v). The fractions containing the product were pooled and concentrated to dryness.

Ethyl α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (ethyl rutinoside, 9): The reaction was performed as described in the general procedure, ethanol (3 mL, 51.3 mmol) was used as the acceptor. **9** was isolated as a colorless amorphous solid (TLC $R_f = 0.37$); yield: 34.7 mg (0.098 mmol, 46.0% relative to the donor). MS (ESI): $m/z = 377.0$ $[M+Na]^+$, calcd. mass for $C_{14}H_{26}O_{10}Na$: 377.1 (^{13}C NMR in Table S2, Figure S6; ^1H NMR in Table S3, Figure S7 in the Supporting Information).

***n*-Butyl α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (*n*-butyl rutinoside, 10):** The reaction was performed as described in the general procedure, *n*-butanol (750 μL, 8.16 mmol) was used as an acceptor. **10** was isolated as a colorless amorphous solid (TLC $R_f = 0.56$); yield: 37.7 mg (0.095 mmol, 44.6% relative to the donor). MS (MALDI): $m/z = 405.1$ $[M+Na]^+$, calcd. mass for $C_{16}H_{30}O_{10}Na$: 405.2 (^{13}C NMR in Table S2, Figure S8; ^1H NMR in Table S3, Figure S9 in the Supporting Information).

Pentan-2-yl α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (pentan-2-yl rutinoside, 11): The reaction was performed as described in the general procedure, pentan-2-ol (750 μL, 6.91 mmol) was used as an acceptor. **11** was isolated as a colorless amorphous solid (TLC $R_f = 0.60$); yield: 32 mg (0.081 mmol, 37.9% relative to the donor). MS (MALDI): $m/z = 419.1$ $[M+Na]^+$, calcd. mass for $C_{17}H_{32}O_{10}Na$: 419.2 (^{13}C NMR in Table S2, Figure S10; ^1H NMR in Table S3, Figure S11 in the Supporting Information).

(*E*)-3,7-Dimethylocta-2,6-dien-1-yl [α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside] (geranyl rutinoside, 12): The reaction was performed as described in the general procedure: rutin was used as the donor (**1**, 130 mg, 0.213 mmol) and geraniol (700 μL, 4.03 mmol) was used as the acceptor. The title compound **12** was isolated as a colorless amorphous solid (TLC $R_f = 0.63$); yield: 4.0 mg (9 μmol, 4.1% relative to the donor). MS (ESI): $m/z = 485.3$ $[M+Na]^+$, calcd. mass for $C_{22}H_{38}O_{10}Na$: 485.2 (^{13}C NMR in Table S2, Figure S12; ^1H NMR in Table S3, Figure S13 in the Supporting Information).

Cyclohexyl α-L-Rhamnopyranosyl-(1→6)-β-D-glucopyranoside (cyclohexyl rutinoside, 13): The reaction was performed as described in the general procedure: rutin was used as a donor (**1**, 130 mg, 0.213 mmol), cyclohexanol (700 μL, 6.73 mmol) as the acceptor. The title compound **13** was isolated as a colorless amorphous solid (TLC $R_f = 0.27$); yield: 19.3 mg (0.047 mmol, 22.2% relative to the donor). MS (ESI): $m/z = 431.1$ $[M+Na]^+$, calcd. mass for $C_{18}H_{32}O_{10}Na$: 431.2 (^{13}C NMR in Table S2, Figure S14; ^1H NMR in Table S4, Figure S15 in the Supporting Information).

Benzyl α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (benzyl rutinoside, 14): The reaction was performed as described in the general procedure, benzylalcohol (450 μL, 4.32 mmol) was used as the acceptor. The title compound **14** was isolated as a colorless amorphous solid (TLC $R_f = 0.55$); yield: 16.1 mg (0.039 mmol, 18.2% relative to the donor). MS (MALDI): $m/z = 439.1$ $[M+Na]^+$, calcd. mass for $C_{19}H_{28}O_{10}Na$: 439.1 (^{13}C NMR in Table S2, Figure S16; ^1H NMR in Table S4, Figure S17 in the Supporting Information).

2-Hydroxyphenyl α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (catechol rutinoside, 15): The reaction was performed as described in the general procedure: rutin was used as a glycosyl donor (**1**, 75 mg, 0.122 mmol), catechol (80 mg, 0.727 mmol) was used as the acceptor. The title compound **15** was isolated as a colorless amorphous solid (TLC $R_f = 0.50$); yield: 14.0 mg (0.033 mmol, 27.1% relative to the donor). MS (MALDI): $m/z = 441.1$ $[M+Na]^+$, calcd. mass for $C_{18}H_{26}O_{11}Na$: 441.1 (^{13}C NMR in Table S2, Figure S18; ^1H NMR in Table S4, Figure S19 in the Supporting Information).

5,4′-Trihydroxy-*trans*-stilbene-3-yl-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside] 16a and 3,5-trihydroxy-*trans*-stilbene-4′-yl-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside] 16b (resveratrol rutinoside): The reaction was performed as described in the general procedure: rutin was used as a donor (1, 130 mg, 0.213 mmol), resveratrol (60 mg, 0.263 mmol) as an acceptor. Product **16** was isolated as a colorless amorphous solid (TLC $R_f = 0.45$); yield: 9.3 mg (0.017 mmol, 8.0% relative to the donor). NMR showed that the product consists of an (inseparable) mixture of two compounds **16a** and **16b** in the ratio 2:1 (C-3 and C-4′ glycosides). MS (ESI): $m/z = 535.2$ $[M-H]^-$, calcd. mass for $C_{26}H_{31}O_{12}$: 535.2 (^{13}C NMR in Table S5, Figure S20; ^1H NMR in Table S5, Figure S21 in the Supporting Information).

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12 α-L-Rhamnosyl-β-D-glucosidase (Rutinosidase) from *Aspergillus niger*: Characterization and Synthetic Potential of a Novel Diglycosidase

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