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A sustainable one-pot two-enzymes synthesis of naturally occurring arylalkyl glucosides

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Abstract: A sustainable, convenient and scalable one-pot two-enzymes method for the glucosylation of arylalkyl alcohols has been developed. The reaction scheme is based on a transrutinosylation catalyzed by a rutinosidase from *A. niger* using the cheap commercially available natural flavonoid rutin as glycosyl donor, followed by a selective 'trimming' of the rutinoside unit catalyzed by a rhamnosidase from *A. terreus*. The process has been validated with the syntheses of several natural bioactive glucosides, that could be isolated in up to 75 % yield avoiding silica gel chromatography.

Introduction

Substituted benzyl, phenylethyl and phenylpropenyl glucosides, such as **1a-8a** (**Figure 1**), are bioactive natural compounds largely occurring in plants.[1] For example, salidroside (**3a**) is associated with a wide number of pharmacological effects, including antioxidant, anti-inflammatory and anticancer activities,[2-4] whereas trandirin (**5a**) and coniferin (also named citrusin, **7a**) are studied for their potential antidepressant[5] and hypotensive[6] effects, respectively. Those biological effects are often related to the structures of the aglycones but, importantly, the physicochemical, pharmacological, and toxicity profiles of these substances are strongly influenced by the conjugation with the sugar.[7]

These compounds can be isolated in minor amounts from natural sources or can be synthesized chemically. For instance, in a recent work we synthesized the glucoside coniferin (**7a**).[8] The synthetic pathway was straightforward, based on well-defined and reliable protocols, nevertheless the sequence of protective and deprotective reactions consisted of seven synthetic steps. Moreover, two chromatographic purifications were required

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to obtain the product from glucose and the respective primary alcohol as starting materials. Not only such procedures are time-consuming and result in overall mediocre yields, but they use large amounts of solvents, silica gel and various toxic chemicals.

Therefore, alternative and greener approaches able to improve selectivity and efficacy under mild reaction conditions would offer viable solutions.
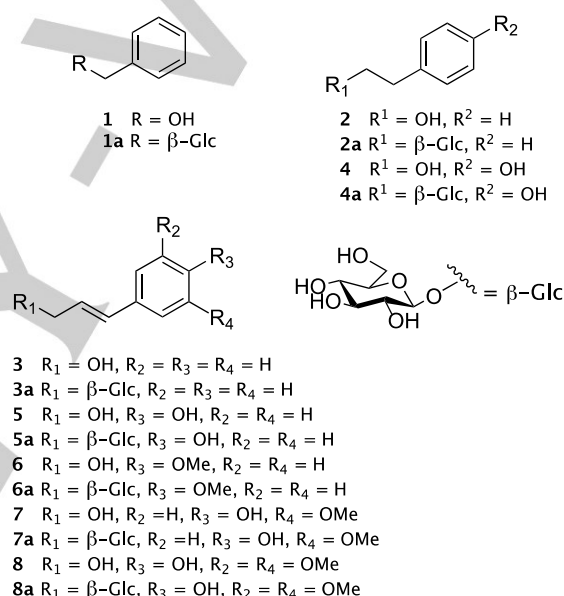

1   R = OH
1a R = β–Glc

2   R¹ = OH, R² = H
2a R¹ = β–Glc, R² = H
4   R¹ = OH, R² = OH
4a R¹ = β–Glc, R² = OH

3   R_1 = OH, R_2 = R_3 = R_4 = H
3a R_1 = β–Glc, R_2 = R_3 = R_4 = H
5   R_1 = OH, R_3 = OH, R_2 = R_4 = H
5a R_1 = β–Glc, R_3 = OH, R_2 = R_4 = H
6   R_1 = OH, R_3 = OMe, R_2 = R_4 = H
6a R_1 = β–Glc, R_3 = OMe, R_2 = R_4 = H
7   R_1 = OH, R_2 =H, R_3 = OH, R_4 = OMe
7a R_1 = β–Glc, R_2 =H, R_3 = OH, R_4 = OMe
8   R_1 = OH, R_3 = OH, R_2 = R_4 = OMe
8a R_1 = β–Glc, R_3 = OH, R_2 = R_4 = OMe

= β–Glc

Figure 1. Compounds **1-8** and natural products **1a-8a**.

In general, the structural complexity and the 'fragility' of natural bioactive compounds makes them an ideal target for biocatalysis which, by exploiting the inherent properties of enzymes in terms of selectivity and efficacy under mild reaction conditions, offers interesting solutions.
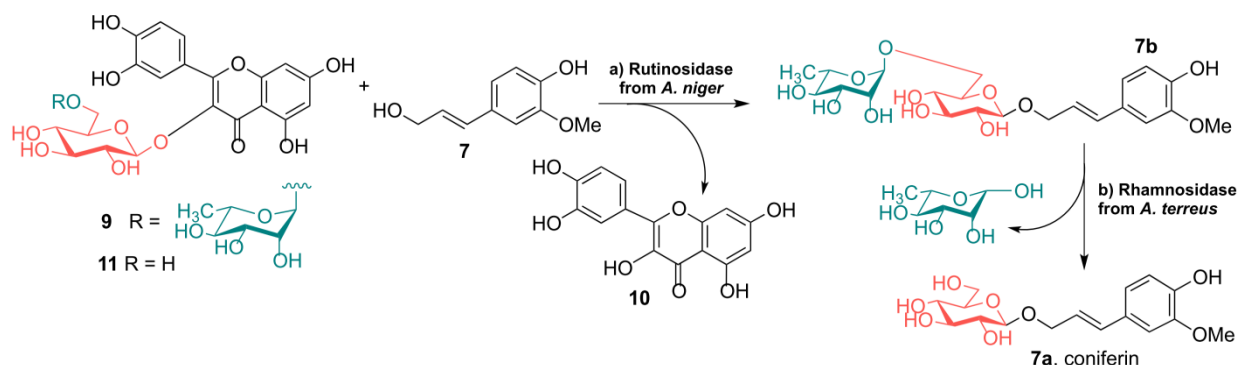
The formation of stereoselective glycosidic bonds in nature is mainly catalyzed by glycosyl transferases, a group of enzymes that however need activated sugars (UDP-glucose or other nucleosides, glucosyl phosphate). [8-11] Alternatively, it may be accomplished via the transglycosylation action of glycosidases, the enzymes that in nature hydrolyze glycosidic bonds,[12,13] or by their artificial mutants, the so-called glycosynthases.[12] The latter enzymes are generated by site directed mutagenesis, depleting natural glycosidases from their hydrolytic attitude. Due to the special configuration of their active sites, they can catalyze the formation of a glycosidic bond, but they are not able to hydrolyze it anymore. Moreover, they also usually need activated sugar

acceptors, such as fluorides or azides and requires considerable

agents. [18,19] Besides this, rutinose-containing glycopolymers may

Scheme 1. Two-enzymatic synthesis of coniferin (**7a**).

Among the number of papers published on this topic, a few years ago Akita *et al.* described the synthesis of a series of glucosides of primary alcohols, including compounds **3a-7a**, exploiting a cheap and available β-glucosidase from almonds.[14] Products were obtained from the unprotected glucose and respective alcohol in a single step.

However, isolated yields were moderate to low and purification steps by silica gel chromatography (where typically losses of polar compounds occur due to partial irreversible adsorption) were always required.

Rutin (**9**) is a glycosyl derivative of the flavonoid quercetin (**10**; glycon: rutinose, α-L-rhamnopyranosyl-β-D-glucopyranoside). [15] It is an important phytochemical contained in various plants and fruits (buckwheat, tobacco, apples, tomatoes, citrus and grapes), which was named after one of its primary natural sources, the common rue *Ruta graveolens*. [16] Rutin is now produced as a multi-ton commodity chemical from the Brasilian tree Fava d'anta (*Dimorphandra mollis*); it is also accumulated as a waste byproduct in different manufactory processes (e.g., the production of fruit juices). Rutin has a GRAS status (cf. www.hc-sc.gc.ca) and it is used in plethora of nutraceuticals and pharmaceutical preparation as a capillary/blood vessel protectant and as an antiviral agent. Therefore, rutin is a highly suitable, inexpensive (presently it costs less than 50 €/Kg) and non-toxic starting material for the synthesis of rutinosylated conjugates. All these properties make rutin superior to the typically used glycosyl donors for glycosidase-catalyzed synthesis, e.g. expensive and toxic nitrophenyl glycosides.

We have recently shown that the diglycosidase rutinosidase from *Aspergillus niger* (cloned and expressed in *Pichia pastoris*[17]) has very strong transglycosylation activity both for aliphatic alcohols and phenols. This diglycosidase is much more efficient in transglycosylation than other commonly used (mono)glycosidases. This reaction has another advantage: all the reactants and products are non-toxic (natural) flavonoids and thus they do not compromise potential pharmaceutical and/or nutraceutical applications. Rutinosylated substances can be used also as food additives, ingredients in cosmetics and antiviral

have entrapment abilities for fibroblasts due to the presence of rhamnose-recognizing receptors on their surface. [20]

Often, however, the corresponding β-glucosides are required. For this purpose, α-L-rhamnosidases are perfectly suitable to catalyze the selective de-rhamnosylation. We have in our hands a highly efficient thermo- and alkali-stable α-L-rhamnosidase from *Aspergillus terreus*, which is now available as a heterologous protein produced by a recombinant strain of *P. pastoris*.[21] This enzyme preparation, devoid of β-glucosidase activity, has been shown to efficiently and selectively cleave the α(6→1) rhamnosyl unit of rutin to yield isoquercitrin (**11**). [15,22]

We present here a sustainable, convenient and scalable telescoping two-enzymes glucosylation of arylalkyl alcohols, exploiting rutin as a glycosyl donor.

Results and Discussion

Transglycosylations are kinetically controlled reactions where the product may become a substrate for a subsequent hydrolysis catalyzed by the same enzyme, lowering thus the yields. Therefore, the first critical point of our investigation was the thorough optimization of the rutinosidase-catalyzed reaction. As shown in **Scheme 1**, coniferyl alcohol (**7**), the aglycone of the natural hypotensive agent coniferin (**7a**), was chosen as a model glycosyl acceptor. When **7** was subjected to the rutinosidase action following the published transglycosylation protocol (a 50 mM 1:1 mixture of rutin and glycosyl acceptor dissolved in a 50 % v/v DMSO solution in citrate-phosphate buffer at pH 5, incubated at 35 °C for 7 h with 0.026 U mmol$^{-1}_{substrate}$ of rutinosidase), only 10 % isolated yield of the desired disaccharide derivative **7b** was obtained since the major rutinosidase-catalyzed reaction was rutin hydrolysis.

To optimize the reaction, we have focused on three key parameters: the enzyme amount, the glycosyl donor/acceptor stoichiometric ratio and the amount of cosolvent added.

After a whole set of experiments (for details see Supplementary Material, sections S1 and S2), we found that the use of a lower amount of rutinosidase (optimum value ca 0.013 U mmol$^{-1}_{alcohol}$) and a reaction time of 7-8 h strongly improved transglycosylation over hydrolysis. Moreover, an initial excess of alcohol acceptor over the glycosyl donor (a starting mixture of

FULL PAPER

rutin/alcohol 0.5 : 1 eq) gave better results. To limit a significant substrate inhibition caused by rutin, a lower amount of the cosolvent DMSO (15 % v/v instead of 50 %) was used to decrease the active concentration of rutin in the reaction. In this system, the donor was just suspended and only partially dissolved.

To make this procedure fully scalable and green, a purification strategy avoiding the use of silica gel chromatography was developed. By fully exploiting the chemo-physical differences between the starting alcohol **7**, the product **7b**, the residual rutin **9** (if any), the produced quercetin **10** and the free disaccharide rutinose always formed as a byproduct, it was possible to purify the rutinoside **7b** by means of i) centrifugation, to recover the produced quercetin (**10**); ii) extraction with organic solvents, to recover the unreacted alcohol and; iii) solid phase extraction on Amberlite®XAD4 non-ionic resin. [23] Accordingly, the produced quercetin (**10**) was removed by centrifugation, the unreacted coniferyl alcohol was extracted with AcOEt, the hydrolyzed rutinose, the buffer salts and the residual co-solvent DMSO were easily removed in the eluate from the product **7b** that was adsorbed to the XAD4 resin. The DMSO used in the procedure does not compromise the green concept: its toxicity is very low (this compound is currently used in cosmetics and medications to increase the skin permeability) and its residual presence could not be detected – by NMR – in the isolated products.

The preparative scale transrutinosylation of **7** was conducted with 1 g of alcohol. Rutin (a total of 1.5 eq) was portion-wise added (0.5 equivalent each time) during 8 h (the disappearance of rutin was monitored by TLC).

The product **7b** was obtained in 70 % isolated yield and fully characterized by MS ([M + Na$^+$] *m/z* 511.3) and NMR. In addition to the expected signals due to the coniferyl moiety, the presence of a disaccharide unit was clearly demonstrated by the respective signals in the ^{1}H-NMR spectrum. Specifically, the anomeric protons of the rhamnopyranose and glucopyranose units were doublets (*J* = 1.6 Hz and *J* = 7.8 Hz, respectively) centered at 4.80 and 4.37 ppm. The presence of the rhamnopyranose unit was further confirmed by the signal of its methyl group (δ_H 1.30, d, *J* = 6.3 Hz).

Thus, the initial transglycosylation reaction was also highly regioselective, as no formation of phenolic glycoside at C-1 OH was observed.

After the optimization of the first step, the consecutive step, i.e., the enzymatic trimming of the rhamnose unit, was straightforward. The citrate-phosphate solution (50 mM, pH 5) of rutinoside **7b** (200 mg, 100 mM) incubated at 35 °C for 3 h with 2 U mmol^{-1}$_{rutinoside}$ of enzyme, followed by XAD4 solid phase extraction, gave the target coniferin (**7a**) in 90 % isolated yield. **7a** was characterized by MS ([M + Na$^+$] *m/z* 365.2 Da) and ^{1}H- and ^{13}C-NMR, which showed the presence of both the coniferyl aglycone and the glucopyranosyl moiety. These data agreed with literature data. [24]

As a further improvement of the efficiency of the synthetic process, we investigated the possibility of a one-pot approach by merging the two enzymatic reactions. We used the so-called '*telescoping synthesis*': the reactants were added sequentially to the reactor without any intermediate purification or work-up and the reaction ran in one pot. Two-enzymes glucosylation of coniferyl alcohol was achieved by sequentially combining the transglycosylation grafting and the rhamnose trimming in the same reactor. Only, rutinosidase had to be thermally inactivated prior adding the rhamnosidase (0.5 U mmol^{-1}$_{substrate}$) and stirring the final reaction mixture overnight. Following the previously described purification procedure using XAD4 resin, the target coniferin (**7a**) was obtained in 75 % isolated yield. In comparison, the chemical synthesis of **7a** required seven reactions (protection of carbohydrate hydroxyl groups, deprotection of the anomeric OH, activation of the anomeric OH, protection of the phenolic hydroxyl group, condensation between activated sugar and the aglycone, deprotection of the phenolic moiety, deprotection of the sugar hydroxyl groups) and two silica gel chromatography. Therefore, this enzymatic approach is superior to the chemical one in terms of better atom economy. [8]

Both enzymes used in this work, i.e., rutinosidase and α-L-rhamnosidase, do not need to be recycled. In fact, they are both produced heterologously by *P. pastoris* and their production costs are very low. Typical activity is ca 0.15 U/mL cultivation broth. This means that less than 1 mL of the medium needs to be used for 1 g of coniferyl alcohol. The previously described experiments were run in the presence of purified rutinosidase, but we have verified that even crude enzyme (just fermentation medium) can be used equivalently. By rough estimation the substrate used for our optimization study (coniferyl alcohol) is significantly (ca 100 – 1000 times) more expensive than the enzymes used, thus making non-economic the additional work required to regenerate the biocatalysts. Moreover, the use of immobilized enzymes is not compatible with the reaction protocol, as we work with a suspension of substrates and therefore we need soluble enzymes.

On the contrary, polystyrene microporous resin Amberlite XAD4 [23] can be well reused in tens of cycles and refreshed by simple washing with MeOH or acetone. When the resin starts to lose capacity, well assessed regeneration protocols are available (see Supplementary Information, **S6**). This means that the resin can be technically used for tens to hundreds of cycles.

The same protocol as above was then applied to the alcohols **1-8** and the results are summarized in Table 1. All the compounds were accepted by the rutinosidase, except for sinapyl alcohol (**8**). Compounds **4-6** behaved analogously to **7** and with coumaryl alcohol (**5**) the transglycosylation reaction was also fully regioselective. Compounds **1-3** were also glycosylated, but apparently, they were less reactive, as the isolated yield were lower. A more careful investigation of the reaction kinetics by HPLC (see Supplementary Information, sections **S1** and **S3**) showed that the rutinosides **1b-3b** were formed, but they were hydrolyzed much faster than the rutinosides of the phenylpropenyl alcohols **4-7**. Therefore, the reactions needed to be carefully monitored and optimized.

Table 1. Telescoping two-enzymes synthesis of glucosides.

| Substrate	Product	Isolated Yield [%]
1[a]	1a	12
2[a]	2a	24
3[a]	3a 3c	44.5[b] 4.5[b]
4	4a	70
5	5a	68
6	6a	58
7	7a	75
8	NO	--

[a] Reactions with substrates 1, 2 and 3 were run for 24 h instead of 8 h; [b] Relative ratio estimated by ^{1}H NMR.

Tyrosol (**3**) behaved differently in terms of regioselectivity (Scheme 2). The MS spectra of the isolated product ([M + Na+] *m/z* 232.2) confirmed the introduction of a glucose moiety. However, the ^{1}H-NMR spectrum clearly showed the presence of two products in a 10 : 1 ratio. The signal due to the α-anomeric proton (H-10) of the major one, a doublet with *J* = 7.8 Hz resonating at 4.29 ppm could be overlapped to the corresponding signal of the glucoside of phenylethanol (**2a**), therefore it could be possible to assign the structure **3a** to this compound.

The significant downfield shift of the aromatic protons of the minor one (the two coupled doublets (*J* = 8.4 Hz) due to the AA'BB' aromatic system resonating at 7.18 and 7.03 ppm, whereas they were recorded at 7.08 and 6.71 ppm in **3a**), indicated the glucosylation of the phenolic OH (**3c**).

To further investigate the glycosylation of this interesting compound, we decided to isolate the intermediate tyrosol rutinosides **3b** and **3d**. Following the previously described protocol, a mixture of the expected regioisomers **3b** and **3d** (MS and ^{1}H-NMR in accordance with the proposed structures), was again obtained, this time in 3 : 1 ratio. The different observed regioisomeric ratio between **3a** and **3c** vs. **3b** and **3d** could be explained taking in account the different reaction time allowed for the transrutinosylation step in the two protocols, that was longer in the telescopic synthesis. A detailed kinetic study performed by monitoring the reactions by HPLC (see Supplementary Material, sections **SI, Figure S2**), clearly showed that the phenolic rutinoside **3d** could be hydrolyzed faster than the alkyl one **3b**.

The separation of the two regioisomeric glycosides (**3a** from **3c**, **3b** from **3d**) proved to be quite troublesome and several techniques failed, i.a. silica gel flash chromatography and reverse phase preparative HPLC. Separation of the regioisomeric rutinosides **3b** and **3d** could be achieved by gel-filtration using a Biogel P2 (BioRad), and the major product **3b** could be obtained in a 94% purity, as determined by ^{1}H-NMR.
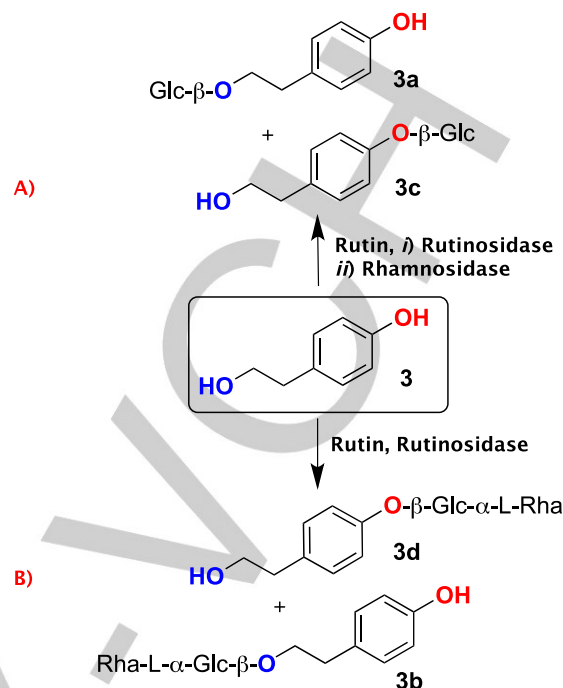
Scheme 2. Glycosides of tyrosol (**3**). A) Two-enzymatic synthesis of tyrosol glucosides **3a** and **3c**; B) Rutinosidase-catalyzed synthesis of tyrosol rutinosides **3b** and **3d**.

Conclusions

The proposed synthetic protocols allowed the preparation, in good isolated yields, of two types of glycosylated products, rutinosides and β-glucopyranosides, and specifically of valuable natural glucosides. This goal was achieved using the glycosyl donor rutin, presently a byproduct in various manufactory processes and a cheap and biocompatible food-grade commodity chemical, and avoiding the use of protective groups, toxic activating agents, expensive reagents, environment-unfriendly experimental procedures requiring dry solvents and harsh conditions and, last but not least, avoiding chromatographic purifications. Moreover, the flavonoid quercetin (**10**), the main by-product of our synthesis has a high value per se and is easily recovered (centrifugation) during our established purification method.

All these aspects (the use of a renewable feedstock, the use of biocatalysts, the use of less hazardous chemical synthesis, the use of safer solvents and chemicals, the reduced number of synthetic derivatives) perfectly fit with most of the so-called '12 principles of Green Chemistry' proposed by Anastas and Warner a few years ago. [25]

Experimental Section

3.1. Materials, chemicals and equipment

NMR spectra were recorded on Bruker AC400 spectrometer (400 or 500 MHz) in MeOH-d_4, DMSO-d_6 or D_2O, MS spectra were recorded on Bruker Esquire 3000 Plus spectrometer. NMR and the MS spectra *in extenso* are available in the Supplementary Material, sect. SI. Biotranformations were carried out using a G24 Environmental Incubator New Brunswick Scientific Shaker (Edison, USA) or a Thermomixer Comfort (Eppendorf, DE).

Phenylpropanoid alcohols were prepared from their corresponding acids, following standard procedures (Supplementary Material S1).

The enzymatic glucosylations were monitored by TLC [precoated silica gel 60 F$_{254}$ plates (Merck, DE); mobile phase = AcOEt : MeOH : HCOOH , 4 : 1 : 0.5; development = UV lamp, Komarovsky reagent (1 mL of 50 % ethanolic H_2SO_4 with 10 mL of 2% methanolic 4-hydroxybenzaldehyde)].

HPLC analysis were conducted using a Jasco 880-PU pump equipped with a Jasco 875-UV/Vis detector Kinetex 5µm EVO C18 100Å 150 × 4.6 mm column, gradient of A = aqueous TFA (1 ppm, pH 2.5-3) and B = CH_3CN as a mobile phase (0 min 80% A; 1-15 min 70 % A; 15-20 min 20 % A; 20-25 min 80 % A), flow rate 0.8 mL/min at 25 °C, detection at 270 nm. α-L-Rhamnosyl-β-D-glucosidase (rutinosidase) from *Aspergillus niger* and α-L-rhamnosidase from *Aspergillus terreus* were prepared according to the literature and were used based on their respective activities determined by the previously published assays. [14,15]

All other reagents were of the highest purity grade from commercial suppliers.

3.2. Two-step two-enzymatic synthesis of coniferin (7a)

a) A suspension of rutin (1.70 g, 2.78 mmol) in a 15 % solution of DMSO in citrate-phosphate buffer (50 mM pH 5) containing coniferyl alcohol (1.00 g, 5.56 mmol) and rutinosidase from *A. niger* (0.073 U) were prepared and incubated at 35 °C and 750 rpm. The consumption of the glycosyl donor was checked by TLC analysis and 1.70 g of rutin was added again to the reacting mixture after 3 and 5 h. After 8 h the obtained yellow suspension was heated to 100 °C for 10 minutes and then cooled again to room temperature, diluted with ten 400 mL of citrate-phosphate buffer and centrifuged, recovering only the supernatant. The pH was changed from 5 to 7.5 – 7.7 and the water layer was extracted with AcOEt (2 × 200 mL) and then, after removing the residual AcOEt via concentration in vacuo, it was loaded loaded to a column (5 × 30 cm) with Amberlite XAD4 resin (300 g) in water (elution with slow gradient H_2O to MeOH), affording the pure rutinoside **7b** (yellow solid, 755 mg, 70 %). The resin can be simply reused after washing with pure MeOH followed by extensive washing with water.

¹H-NMR (400 MHz; MeOD, 25 °C): δ 7.04 (d, *J* = 1.9 Hz, 1H: H-5), 6.89 (dd, *J* = 8.2, 1.9 Hz, 1H: H-3), 6.76 (d, *J* = 8.1 Hz, 1H: H-3), 6.60 (d, *J* = 15.9 Hz, 1H: H-7), 6.21 (ddd, *J* = 15.8, 6.8, 5.9 Hz, 1H: H-8), 4.80 (d, *J* = 1.6 Hz, 1H: H-16), 4.48 (ddd, *J* = 12.3, 5.8, 1.3 Hz, 1H: H-9$_A$), 4.37 (d, *J* = 7.8 Hz, 1H: H-10), 4.28 (ddd, *J* = 12.3, 7.0, 1.1 Hz, 1H: H-9$_B$), 4.01 (dd, *J* = 11.3, 1.7 Hz, 1H: H-15$_A$), 3.88 (s, 3H: OCH$_3$), 3.73-3.64 (m, 3H: H-15$_B$, H-18, H-20), 3.44-3.21 (m, 6H: H-11, H-12, H-13, H-14, H-17, H-19), 1.30 (d, *J* = 6.3 Hz, 3H: H-21).

¹³C-NMR (101 MHz; MeOD, 25 °C): δ 147.7, 146.3, 133.1, 128.9, 122.1, 119.7, 114.8, 109.2, 101.6, 100.9, 76.7, 75.5, 73.7, 72.6, 70.97, 70.83, 70.3, 69.5, 68.4, 66.8, 54.9, 29.3, 16.7

EI-MS, *m/z* 511.3 [M+Na]⁺.

b) Rhamnosidase from *A. terreus* (0.82 U) was dissolved in citrate-phosphate buffer (50 mM, pH 5) solution of **7a** (200 mg, 0.41 mmol, 2.5 mL). The resulting mixture was incubated at 35 °C and 750 rpm for 3 h. After checking the complete conversion of the starting rutinoside into the target glucoside by TLC analysis, heated to 100 °C for 10 minutes and then cooled again to room temperature to be subjected to an Amberlite XAD4 solid phase extraction (loaded and washed with H_2O; eluted with MeOH), affording pure coniferin, **7a** (yellow solid, 126 mg, 90%).

¹H-NMR (400 MHz; MeOD, 25 °C): δ 7.03 (d, *J* = 1.9 Hz, 1H: H-5), 6.87 (dd, *J* = 8.2, 1.9 Hz, 1H: H-3), 6.76 (d, *J* = 8.1 Hz, 1H: H-2), 6.59 (d, *J* = 15.9 Hz, 1H: H-7), 6.21 (ddd, *J* = 15.9, 6.7, 6.0 Hz, 1H: H-8), 4.51 (ddd, *J* = 12.4, 5.9, 1.4 Hz, 1H: H-9$_A$), 4.39 (d, *J* = 7.8 Hz, 1H: H-10), 4.32 (ddd, *J* = 12.4, 6.8, 1.2 Hz, 1H: H-9$_B$), 3.90 (dd, *J* = 11.9, 2.0 Hz, 1H: H-15$_A$), 3.88 (s, 3H: OCH$_3$), 3.70 (dd, *J* = 11.9, 5.4 Hz, 1H: H-15$_B$), 3.41-3.23 (m, 4H: H-11, H-12, H-13, H-14).

¹³C-NMR (101 MHz; MeOD, 25 °C): δ 147.7, 146.3, 132.9, 129.0, 122.4, 119.8, 114.8, 109.3, 101.8, 76.74, 76.57, 73.7, 70.3, 69.6, 61.4, 55.0.

EI-MS, *m/z* 365.2 [M+Na]⁺.

3.3. Telescoping two-enzymatic glucosylation

General procedure: arylalkyl alcohol (1 eq, 128 mM) and rutinosidase from *A. niger* (0.013 U mmol^{-1}$_{substrate}$) were dissolved in a 15 % solution of DMSO in citrate-phosphate buffer (50 mM pH 5) and incubated at 35 °C and 750 rpm. The glycosylation reaction was started by adding 0.5 eq of rutin. Monitoring the consumption of the glycosyl donor by TLC analysis, two portions of rutin, 0.5 eq each, were added. The transglycosylations were conducted for 8 or 16 h, depending on the substrate used. When all the rutin was consumed the suspension was heated to 100 °C for 10 minutes and then cooled again to 35 °C and rhamnosidase from *A. terreus* (0.5 U mmol^{-1}$_{substrate}$) was added to the mixture, which was incubated overnight at 35 °C and 750 rpm. The complete conversion of the intermediate disaccharide to the target β-glucopyranoside was checked by TLC analysis and then the resulting yellow suspension was cooled to room temperature, diluted with ten volumes of citrate-phosphate buffer and centrifuged. The solid proved to be the expected quercetin (**10**) containing ca 20-25 % of unreacted rutin. The supernatant water layer, after shifting the pH from 5 to 7.5 – 7.7, was extracted twice with half the volume of AcOEt and then, after removing the residual AcOEt in vacuo, subjected to an Amberlite XAD4 solid phase extraction (mobile phase H_2O : MeOH, from 100 : 0 to 0 : 100), affording the pure arylalkylglucoside.

Compound 1a: According to the General Procedure, compound **1a** (30 mg, 0.11 mmol, isolated yield: 12 %, yellow oil, 24 h) was obtained from 100 mg of benzyl alcohol (**1**) and 846 mg of rutin (**9**).

¹H-NMR (500 MHz; MeOD, 25 °C): δ 7.44 (d, *J* = 7.3 Hz, 2H: H-2, H-6), 7.35 (t, *J* = 7.4 Hz, 2H: H-3, H-5), 7.29 (t, *J* = 7.3 Hz, 1H: H-1), 4.95 (d, *J* = 11.8 Hz, 1H: H-7$_A$), 4.69 (d, *J* = 11.8 Hz, 1H: H-7$_B$), 4.38 (d, *J* = 7.7 Hz,

FULL PAPER

1H: H-10), 3.92 (d, J = 10.2 Hz, 1H: H-15$_A$), 3.72 (dd, J = 11.8, 5.5 Hz, 1H: H-15$_B$), 3.39-3.26 (m, 6H: H-11, H-12, H-13, H-14).

^{13}C-NMR (101 MHz; MeOD, 25 °C): δ 132.4, 122.55, 122.48, 122.0, 96.6, 71.41, 71.36, 71.33, 71.32, 68.49, 68.44, 65.04, 65.03, 56.1, 33.7.

EI-MS, *m/z* 293.1 [M+Na]$^+$.

Compound 2a: According to the General Procedure, compound **2a** (36 mg, 0.20 mmol, isolated yield: 24 %, yellow solid, 24 h) was obtained from 100 mg of 1-phenyl ethanol (**2**) and 750mg of rutin (**9**).

^{1}H-NMR (500 MHz; MeOD, 25 °C): δ 7.27 (d, J = 4.5 Hz, 4H: H-2, H-3 H-5, H-6), 7.19 (q, J = 4.4 Hz, 1H: H-1), 4.32 (d, J = 7.8 Hz, 1H: H-10), 4.11 (dt, J = 9.5, 7.5 Hz, 1H: H-8$_A$), 3.88 (dd, J = 11.7, 1.5 Hz, 1H: H-15$_A$), 3.81-3.76 (m, 1H: H-8$_B$), 3.69 (dd, J = 11.8, 5.2 Hz, 1H: H-15$_B$), 3.39-3.19(m, 4H: H-11, H-12, H-13, H-14), 2.96 (td, J = 7.3, 1.6 Hz, 2H: H-7).

^{13}C-NMR (101 MHz; MeOD, 25 °C): δ 133.4, 123.3, 122.67, 122.64, 120.53, 120.50, 97.7, 71.43, 71.29, 68.4, 65.0, 56.1, 33.7, 30.6.

EI-MS, *m/z* 307.0 [M+Na]$^+$.

Compound 3a, *salidroside*: According to the General Procedure, compound **3a** (106 mg, 0.35 mmol, isolated yield: 49 %, yellow solid, 24 h) was obtained from 100 mg of tyrosol (**3**) and 663 mg of rutin (**9**).

^{1}H-NMR (500 MHz; D$_2$O, 25 °C): δ 7.05 (d, J = 8.3 Hz, 2H: H-3, H-5), 6.69 (d, J = 8.3 Hz, 2H: H-2, H-6), 4.29 (d, J = 7.8 Hz, 1H: H-10), 4.04-3.99 (m, 1H: H-8$_A$), 3.85 (d, J = 10.7 Hz, 1H: H-15$_A$), 3.68 (td, J = 13.2, 5.8 Hz, 3H: H-7$_A$, H-8$_B$, H-15$_B$), 3.36 (t, J = 8.7 Hz, 1H: H-11), 3.31-3.24 (m, 3H: H-7$_A$, H-12, H-14), 3.18 (t, J = 8.4 Hz, 1H: H-13).

^{13}C-NMR (101 MHz; D$_2$O, 25 °C): δ154.3, 128.45, 128.26, 113.7, 101.8, 75.59, 75.41, 72.6, 69.6, 69.1, 60.3, 38.0, 33.9.

EI-MS, *m/z* 323.2 [M+Na]$^+$.

Compound 4a, *rosin*: According to the General Procedure, compound **4a** (140 mg, 0.47 mmol, isolated yield: 70 %, yellow solid, 8 h) was obtained from 100 mg of cinnamyl alcohol (**4**) and 684 mg of rutin (**9**).

^{1}H-NMR (400 MHz; MeOD, 25 °C): δ 7.44-7.42 (m, 2H: H-2, H-6), 7.31 (dd, J = 8.2, 6.7 Hz, 2H: H-3, H-5), 7.23 (t, J = 7.3 Hz, 1H: 1), 6.70 (d, J = 16.0 Hz, 1H: H-7), 6.39 (ddd, J = 16.0, 6.4, 5.8 Hz, 1H: H-8), 4.55 (ddd, J = 12.8, 5.7, 1.5 Hz, 1H: H-9$_A$), 4.39 (d, J = 7.8 Hz, 1H: H-10), 4.35 (ddd, J = 12.8, 6.5, 1.4 Hz, 1H: H-9$_B$), 3.91 (dd, J = 11.9, 2.0 Hz, 1H: H-15$_A$), 3.71 (dd, J = 11.9, 5.3 Hz, 1H: H-15$_B$), 3.42-3.24 (m, 4H:H-11, H-13, H-14).

^{13}C-NMR (101 MHz; MeOD, 25 °C): δ 136.8, 132.4, 128.2, 127.3, 126.1, 125.3, 102.0, 76.72, 76.59, 73.7, 70.3, 69.4, 61.4.

EI-MS, *m/z* 319.2 [M+Na]$^+$.

Compound 5a, *triandrin*: According to the General Procedure, compound **5a** (141 mg, 0.45 mmol, isolated yield: 68 %, yellow solid, 8 h) was obtained from 100 mg of 4-hydroxycinnamyl alcohol (**5**) and 603 mg of rutin (**9**).

^{1}H-NMR (400 MHz; D$_2$O, 25 °C): δ 7.32 (d, J = 8.0 Hz, 2H: H-3, H-5), 6.83 (d, J = 7.8 Hz, 2H: H-2, H-6), 6.58 (d, J = 15.9 Hz, 1H: H-7), 6.16 (dt, J = 15.0, 7.1 Hz, 1H: H-7), 4.47-4.40 (m, 2H: H-9$_A$, H-10), 4.31-4.27 (m, 1H: H-9$_B$), 3.86 (d, J = 12.6 Hz, 1H: H-15$_A$), 3.69-3.66 (m, 1H: H-15$_B$), 3.44-3.24 (m, 4H: H-11, H-12, H-13, H-14).

^{13}C-NMR (101 MHz; D$_2$O, 25 °C): δ 215.4, 155.6, 133.6, 128.9, 128.2, 122.3, 115.7, 101.1, 75.92, 75.90, 73.2, 70.5, 69.7, 60.8, 38.8, 30.3.

EI-MS, *m/z* 335.1 [M+Na]$^+$.

Compound 6a, *vimalin*: According to the General Procedure, compound **6a** (115 mg, 0.35 mmol, isolated yield: 58 %, yellow solid, 8 h) was obtained from 100 mg of 4-methoxycinnamyl alcohol (**6**) and 558 mg of rutin (**9**).

^{1}H-NMR (500 MHz; MeOD, 25 °C): δ 7.35 (d, J = 8.7 Hz, 2H: H-3, H-5), 6.87 (d, J = 8.8 Hz, 2H: H-2, H-6), 6.61 (d, J = 16.0 Hz, 1H: H-7), 6.22 (dt, J = 15.9, 6.3 Hz, 1H: : H-8), 4.51 (ddd, J = 12.5, 5.8, 1.3 Hz, 1H: H-9$_A$), 4.38 (d, J = 7.8 Hz, 1H: H-10), 4.30 (ddd, J = 12.4, 6.8, 1.1 Hz, 1H: H-9$_B$), 3.90 (dd, J = 11.9, 2.2 Hz, 1H: H-15$_A$), 3.78 (s, 3H: OCH$_3$), 3.71 (dd, J = 11.9, 5.5 Hz, 1H: H-15$_B$), 3.41-3.24 (m, 4H: H-11, H-12, H-13, H-14).

^{13}C-NMR (101 MHz; MeOD,25 °C): δ 154.1, 127.0, 124.1, 122.0, 117.5, 108.3, 96.5, 71.36, 71.20, 68.4, 64.9, 64.3, 56.1, 49.0, 33.7.

EI-MS, *m/z* 349.2 [M+Na]$^+$.

Compound 7a, *coniferin*: According to the General Procedure, compound **7a** (508 mg, isolated yield: 75 %, yellow solid, 8 h) was obtained from 250 mg of coniferyl alcohol (**7**) and 1.30 g of rutin. The NMR and mass spectra were in accordance with those described above.

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Entry for the Table of Contents

FULL PAPER

A one-pot two-enzymes synthesis of arylalkyl glucosides exploiting the activity of a rutinosidase and a rhamnosidase is here proposed. The cheap, non-toxic, natural flavonoid rutin was selected as a convenient glycosyl-donor and the target glucosides were obtained in isolated yield up to the 75%, avoiding the use of PGs, activating agents and flash column chromatography on silica gel, producing the valuable quercetin as the only recovered by-product.
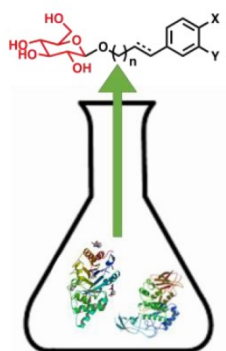
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Page No. – Page No.

A sustainable one-pot two-enzymes synthesis of naturally occurring arylalkyl glucosides.