Robinobiosylation of tyrosol by seed meal from Rhamnus cathartica.

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Short Report

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Abstract

Tyrosol robinobioside was prepared under catalysis of robinobiosidase-containing seed meal from common buckthorn *Rhamnus cathartica*. Robinin, a flavonoid isolated from the flowers of black locust (*Robinia pseudoacacia*) served as the donor of robinobiose. The glycosylation proceeded predominantly on the primary hydroxyl of tyrosol, typically yielding mixtures of isomeric glycosides in ratios of 5:1 to 8:1 with overall yields of robinobiosides higher than 20%. This is the first robinobiosylation promoted under enzymatic catalysis.

Short Communication

Diglycosidases, a group of glycoside hydrolases that hydrolyze the glycosidic bonds between disaccharides and aglycones, are an interesting group of enzymes with expected industrial applications (Koseki et al. 2018). Four types of β-endoglucosidases, namely rutinosidases/hesperidinases, primeverosidases, acuminosidases and vicianosidases are usually referred to as diglycosidases, which are specifically hydrolyzing diglycosides possessing β-glucopyranoside core bonded to an aglycon and substituted at position 6-*O*- by α-L-rhamnopyranose, β-D-xylopyranose, β-D-apiofuranose or α-L-arabinopyranose, respectively. On the other hand, almost 90 years ago, Zemplén and Gerecs reported that an enzyme from the seeds of *Rhamnus utilis* (Rhamnaceae) catalyzed the release of intact disaccharide robinobiose (6-*O*-α-L-rhamnopyranosyl-α,β-D-galactose) from the robinin, the flavonoid from flowers of black locust (*Robinia pseudoacacia*) (Zemplén and Gerecs 1935a) and rutinose from rutin (Zemplén and Gerecs 1935b). This enzyme is somehow distinct from the four mentioned diglycosidases, since it is not a strict endoglucosidase and deglycosylates also disaccharides bearing rhamnose at the nonreducing end in position 6-*O*- of the core monosaccharide (glucose or galactose). Shimokoriyama (1949) and later Suzuki (1962) reported the presence of similar enzyme in seeds of *Rhamnus japonica* and *R. dahurica*, var. *nipponica* and named it rhamnodiastase. The term rhamnodiastase is however somehow misleading since it is used also for mixtures of α-L-rhamnosidase and β-D-glucosidase completely hydrolyze rutinosides and other diglycosides to monosaccharides (Suzuki 1962). It is therefore more correct to refer to the catalytic ability to release intact robinobiose from its glycoside as the robinobiosidase activity despite of the wider specificity of the enzyme. The occurrence of the enzyme seems to be rather general across the genera *Rhamnus*.

Diglycosidases have high application potential in biocatalysis thanks to their ability to catalyze the synthesis of oligosaccharides or anomerically pure tailored diglycosides without the risk of the formation of product regioisomers in the sugar fragment of the product (Mazzaferro et al. 2012, 2019; Kotik et al. 2021). They were used in the synthesis of pure standards of natural diglycosidic aroma precursors (Tsuruhami et al. 2005) or building blocks for structured phenylethanoid glycosides (Bassanini et al. 2017).

The main drawback of preparative applications of diglycosidases remains in the limited availability of appropriate substrates. Although preparation of synthetic substrates has been reported (Bourbouze et al.

1972; Mazzaferro et al. 2012), the diglycosidase-driven reactions usually rely on plant-derived flavonoids and glycosides such as rutin, hesperidin, furcatin or vicianín (Imaseki and Yamamoto 1961; Ahn et al. 2007; Karnišová Potocká et al. 2021). As an analogy, enzymatic robinobiosylations may be performed with flavonoid robinin as the natural robinobiose donor for transglycosylation.

Phenylethanoid glycosides (PEGs) are the group of plant secondary metabolites with wide scale of pharmacological activities (Xue and Yang 2016; Wu et al. 2020). Structurally, the PEGs are β-D-glucosides of tyrosol or hydroxytyrosol substituted on their glucopyranoside ring by other monosaccharides (mainly α-L-rhamnopyranose, β-D-apiofuranose, α-L-arabinopyranose or β-D-xylopyranose) and/or hydroxycinnamic acids such as ferulic, coumaric, caffeic or sinapic acid. Due to the nature of their glycon part, the disaccharidic PEGs are ideal models for study of synthetic applications of diglycosidases.

Non-natural PEGs have been also prepared by replacing β-D-glucose in the core structure by other saccharides such as α-D-galactose (Potocká et al. 2015), β-D-galactose (Qi et al. 2017), β-D-fructose (Hollá et al. 2019) or β-D-xylose (Nieto-Domínguez et al. 2017) and tested for their biological activities. Disaccharidic variants of these non-glucoside analogues of PEGs are highly demanded to widen the library of candidates available for pharmacoactive substances. In this Short Communication we report our experience with the preparation of a new diglycoside – tyrosol robinobioside by transglycosylation from robinin catalyzed by seed meal of common buckthorn *Rhamnus cathartica*.

Flowers of black locust were dried at room temperature. In a typical preparation of robinin concentrate, 162 grams of dry flowers were extracted three times for 1 hour with hot water. The combined extracts (3 L) were filtered and applied on column of Amberlite XAD-4 (25 x 4.5 cm) and eluted with water and 20 % ethanol to remove free sugars. Robinin (**1**) together with other aromatic substances were eluted with neat ethanol. The ethanolic solution was concentrated, the solid residue was dissolved in boiling water and left overnight in refrigerator. The precipitated **1** was separated by filtration, dissolved in methanol, mixed with Celite and the solvent was evaporated to dryness. The solid material was applied on the top of a column of silica gel equilibrated in chloroform. Robinin **1** was eluted by a gradient of methanol in chloroform. Fractions comprising **1** were combined and evaporated. To improve the overall yield, the supernatant after precipitation of **1** was evaporated and chromatographed the same way to provide a less pure fraction. The overall yield of **1** was 1.64 g. Its identity was confirmed by HPLC against the commercial robinin standard (Biosynth, Bratislava, Slovakia) and by techniques of ^{1}H and ^{13}C NMR and was found to comprise as an impurity up to 10 % of kaempferol-3-*O*-rutinoside **2**. The observable signals of **2** were compliant with data reported by Leong et al. (2008).

Robinin (1)

^{1}H NMR (600 MHz, DMSO-d_6 + 10% CD_3OD) δ 8.10 (d, *J* = 8.5 Hz, 2H, H-3', H-5'), 6.81 (d, *J* = 2.1 Hz, 1H, H-8), 6.45 (d, *J* = 2.2 Hz, 1H, H-6), 6.88 (d, *J* = 8.6 Hz, 2H, H-2', H-6'), 5.55 (d, *J* = 1.9 Hz, 1H, H-1''''), 5.36 (d, *J* = 7.7 Hz, 1H, H-1"), 4.40 (d, *J* = 1.5 Hz, 1H, H-1'''), 3.86 (dd, *J* = 3.4, 1.8 Hz, 1H, H-2''''), 3.64 (dd, *J* = 9.4, 3.3 Hz, 1H, H-3''''), 3.62 (d, *J* = 2.9 Hz, 1H, H-4"), 3.60 (dd, *J* = 10.4, 4.3 Hz, 1H, H-6"a), 3.57 (dd, *J* = 9.6, 7.6 Hz, 1H,

H-2"), 3.60 – 3.55 (m, 1H, H-5"), 3.48 – 3.44 (m, 1H, H-5""), 3.41 (dd, *J* = 9.8, 3.5 Hz, H-3"), 3.40 – 3.36 (m, 1H, H-5'"), 3.39 (dd, *J* = 3.5, 1.7 Hz, 1H, H-2'"), 3.31 (dd, *J* = 9.4, 3.4 Hz, H-3'"), 3.31(t, *J* = 9.4 Hz, 1H, H-4""), 3.28 (dd, *J* = 10.0, 6.1 Hz, H-6"b), 3.09 (t, *J* = 9.4 Hz, H-4'"), 1.13 (d, *J* = 6.1 Hz, 3H, CH_3""), 1.06 (d, *J* = 6.3 Hz, 3H, CH_3'").

^{13}C NMR (151 MHz, DMSO-d_6) δ 177.6 (C-4), 161.6 (C-5), 160.9 (C-7), 160.2 (C-4'), 157.1 (C-8a), 156.0 (C-2), 133.6 (C-3), 131.1 (C-2', C-6'), 120.7 (C-1'), 115.1 (C-3', C-5'), 105.6 (C-4a), 101.9 (C-1"), 100.0 (C-1'"), 99.4 (C-6), 98.4 (C-1""), 94.7 (C-8), 73.6 (C-5"), 73.0 (C-3"), 71.9 (C-4'"), 71.6 (C-4""), 71.1 (C-2"), 70.6 (C-3'"), 70.4 (C-2'"), 70.3 (C-3""), 70.1 (C-5'"), 69.8 (C-2""), 68.3(C-5""), 68.0 (C-4"), 65.2 (C-6"), 17.9 (C-6'", C-6""").

Kaempferol-3-*O*-rutinoside (2)

^{1}H NMR (600 MHz, DMSO-d_6 + 10% CD_3OD), detectable signals δ 8.02 (d, *J* = 8.9 Hz, 2H, H-3', H-5'), 6.86 (d, *J* = 8.8 Hz, 2H, H-2', H-6'), 6.73 (d, *J* = 2.1 Hz, 1H, H-8), 6.43 (bs, 1H, H-6), 5.33 (d, *J* = 7.6 Hz, 1H, H-1"), 4.40 (bs, 1H, H-1'"), 3.69 (m, 1H, H-6"a), 3.31 (m, 1H, H-6"b), 3.23 (dd, *J* = 9.8, 7.6 Hz, 1H, H-2"), 1.02 (d, *J* = 6.3 Hz, 3H, CH_3"").

In a typical preparative reaction, the seeds of *Rhamnus cathartica* were ground with a coffee mill and sieved. 200 milligrams of this fine powder were added together with 300 mg of **1** to 10 mL of 0.2 M tyrosol (**3**) solution in water. Based on pre-optimized conditions, the reaction mixture was left on magnetic stirrer at 37 °C for one hour. The reaction was quenched by boiling in a water bath for 10 minutes and after cooling, the reaction mixture was filtered by suction and centrifuged. The supernatant was concentrated under vacuum and centrifuged to separate from the newly formed precipitate. All precipitates were extracted three times with water and decanted. The supernatants were combined, concentrated and applied to a column of Diaion HP-20 (8 x 3.8 cm) equilibrated in water and eluted with gradient of ethanol in water. The fractions comprising the products were concentrated and separated by chromatography on silica gel eluted with a gradient of methanol in chloroform to give 41 mg (23 %) of the mixture of tyrosol robinobioside isomers **4a** and **4b** with observable amounts of tyrosol rutinosides **5a** and **5b**. The detectable NMR signals from the mixture for **5b** were consistent with the literature (Karnišová Potocká et al. 2021).

4-Hydroxyphenetyl robinoside (4a)

^{1}H NMR (600 MHz, CD_3OD) δ 7.07 (d, *J* = 8.4 Hz, 2H, Ph), 6.69 (d, *J* = 8.5 Hz, 2H, Ph), 4.75 (d, *J* = 1.7 Hz, 1H, H-1"), 4.26 (d, *J* = 7.5 Hz, 1H, H-1'), 4.02 – 3.95 (m, 1H, OCH_2aa), 3.84 (dd, *J* = 11.0, 4.4 Hz, 1H, H-6'a), 3.80 (dd, *J* = 3.6, 1.5 Hz, H-2"), 3.79 (d, *J* = 2.9 Hz, 1H, H-4'), 3.73 - 3.66 (m, 1H, CH_2ab), 3.69 - 3.62 (m, 3H, H-5', H-6'b, H-5"), 3.63 (dd, *J* = 9.5, 3.4 Hz, 1H, H-3"), 3.51 (dd, *J* = 9.7, 7.6 Hz, 1H, H-2'), 3.46 (dd, *J* = 9.7, 3.3 Hz, 1H, H-3'), 3.37 (t, *J* = 9.5 Hz, 1H, H-4"), 2.84 (m, 2H, CH_2b), 1.26 (d, *J* = 6.2 Hz, 3H, CH_3).

^{13}C NMR (151 MHz, CD_3OD) δ 156.8 (C-Ph), 130.9 (2xCH-Ph), 130.9 (C-Ph), 116.1 (2xCH-Ph), 105.0 (C-1'), 102.1 (C-1"), 75.0 (C-5'), 74.9 (C-3'), 74.0 (C-4"), 72.5 (C-2'), 72.4 (C-3"), 72.2 (C-2"), 72.2 (CH_2a), 70.4 (C-4'),

69.8 (C-5''), 67.7 (C-6'), 36.4 (CH_2b), 18.1 (CH_3).

4-(2-Hydroxyethyl)phenyl robinoside (4b)

^{1}H NMR (600 MHz, CD_3OD), detectable signals δ: 7.15 (d, *J* = 8.5 Hz, 2H, Ph), 7.03 (d, *J* = 8.1 Hz, 2H, Ph), 4.80 (d, *J* = 7.7 Hz, 1H, H-1'), 4.72 (d, *J* = 1.7 Hz, H-1''), 3.73 – 3.70 (overlapped with multiplet, 2H, OCH_2a), 2.76 (t, *J* = 7.1, Hz, 2H, CH_2b).

^{13}C NMR (151 MHz, CD_3OD), detectable signals δ: 130.8 (2xCH-Ph), 117.8 (2xCH-Ph), 103.0 (C-1'), 102.1 (C-1''), 64.3 (CH_2a), 39.4 (CH_2b).

4-Hydroxyphenetyl rutinoside (5a)

^{1}H NMR (600 MHz, CD_3OD), detectable signals δ: 7.07 (d, *J* = 8.4 Hz, 2H, Ph), 6.71 (d, *J* = 8.3 Hz, 2H, Ph), 4.75 (d, *J* = 1.9 Hz, 1H, H-1''), 4.27 (d, *J* = 7.8 Hz, 1H, H-1'), 4.02 – 3.95 (m, OCH_2aa), 3.98 (dd, 1H, H-6'a), 3.83 (bd, *J* = 2.8 Hz, 1H, H-2''), 3.74 – 3.65 (m, 3H, CH_2ab, H-5'', H-3''), 3.61 (dd, *J* = 11.1, 6.1 Hz, 1H, H-6'b), 3.42 – 3.27 (overlapped with CD_3OD, H-5', H-3', H-4', H-4''), 3.17 (dd, *J* = 9.2, 7.8 Hz, 1H, H-2'), 2.84 (m (overlapped), 2H, CH_2b), 1.26 (d, *J* = 6.3 Hz, 3H, CH_3).

^{13}C NMR (151 MHz, CD_3OD), detectable signals δ: 130.9 (2xCH-Ph), 116.2 (2xCH-Ph), 104.5 (C-1'), 102.3 (C-1''), 78.1 (C-3'), 76.9 (C-5'), 75.1 (C-2'), 74.0 (C-4''), 72.4 (C-3''), 72.3, 72.2 (C-2'', CH_2a), 71.7 (C-4'), 69.8 (C-5''), 68.1 (C-6'), 39.4 (CH_2b), 18.1 (CH_3).

4-(2-Hydroxyethyl)phenyl rutinoside (5b)

^{1}H NMR (600 MHz, CD_3OD), detectable signals δ: 7.16 (d, *J* = 8.8 Hz, 2H, Ph), 7.01 (d, *J* = 8.6 Hz, 2H, Ph), 4.83 (overlapped with HDO, H-1'), 4.70 (d, *J* = 1.7 Hz, H-1''), 3.71– 3.66 (overlapped with multiplet, 2H, OCH_2a), 2.71 (t, *J* = 7.2, Hz, 2H, CH_2b).

^{13}C NMR (151 MHz, CD_3OD), detectable signals δ: 130.9 (2xCH-Ph), 117.7 (2xCH-Ph), 103.0 (C-1'), 102.1 (C-1''), 64.7 (CH_2a), 39.6 (CH_2b).

Although being expensive compound, **1** is relatively easy to prepare by extraction from flowers of black locust (Sando 1932). We routinely prepare it in approximately 1 % yield and purity above 90 % (HPLC). The impurity **3** occurs also in the commercial standard of robinin in even higher content. Since the plant material – dried black locust flowers – is also available commercially, it is possible to prepare **1** at the multigram level in any biochemical laboratory without deep experience with procedures of organic chemistry and independently on the year season. Therefore, this flavonoid is a good candidate for use as substrate in routine enzymatic robinobiosylations.

Plant seeds comprising glycosidase activities are quite commonly used, for example, in enzymatic synthesis of β-D-glucosides, including β-D-glucosylation of tyrosol. Reversed hydrolysis catalyzed by

various seeds from various plants from *Rosaceae* proceeds on the primary hydroxyl of tyrosol (Lu et al. 2007), as does transrutinosylation catalyzed by the flower buds of *Sophora japonica* (Karnišová Potocká et al. 2021). In the presented experiment, the transrobinosylation of tyrosol by seed meal from *R. cathartica* proceeded smoothly within 1 hour according to the Scheme 1. Contrary to the mentioned glycosylations with plant materials, the reaction catalyzed by seeds of *R. cathartica* was not chemoselective and provided in two separate reactions mixtures of isomers of tyrosol β-robinobioside **4a** and **4b** in ratios 5:1 and 8:1. The b-anomeric configuration for the galactopyranose (d 4.26 (d, 1H, H-1')) was determined from a $^{3}J_{H1,H2}$ coupling constants values (7.5 Hz). The aglycon of **4a** showed A_2B_2-type aromatic protons (δ 7.07 (d, *J* = 8.4 Hz, 2H, Ph) and 6.69 (d, *J* = 8.5 Hz, 2H, Ph)) and two methylenes of the 4-hydroxyphenylethyl alcohol part, CH_2b display one signal (δ 2.84 (m, 2H, CH_2b) and CH_2a show separated proton signals (δ 4.02 – 3.95 (m, 1H, OCH_2aa) and 3.73 - 3.66 (m, 1H, CH_2ab)). The ^{1}H NMR spectrum of **4b** shows a set of proton signals for the 1,4-disubstituted phenyl ring at δ 7.15 (d, *J* = 8.5 Hz, 2H, Ph) and 7.03 (d, *J* = 8.1 Hz, 2H, Ph) as well as a benzylic methylene at δ 2.76 (t, J = 7.1, Hz, 2H, CH_2b) and a hydroxymethyl at 3.73 – 3.70 (overlapped with multiplet, 2H, OCH_2a), indicating the presence of a 2-(4-hydroxyphenyl)ethanol moiety and the galactosylation of the phenolic OH.

The low ability to distinguish between the primary and phenolic hydroxyls of aglycon is more typical for microbial glycosidases (Bassanini et al. 2017; Haluz et al. 2023) and represents a complication in hydroxylation of tyrosol. We were not able to separate **4a** and **4b** each from other by standard column chromatography. The reaction therefore deserves further study with special focus on product separation and study of reaction conditions to either suppress formation of one of the products or its removal by secondary hydrolysis during longer reaction times and at the risk of lower chemical yields. On the other hand, the enzyme glycosylating phenols may be employed in synthesis of pharmacoactive substances with enhanced bioavailability (Šimčíková et al. 2014; Mazzaferro et al. 2019).

The final product comprised also observable amounts of tyrosol rutinosides **5a** and **5b** formed by transrutinosylation from contaminating kaempferol-3-rutinoside **2** since the *Rhamnus cathartica* seed meal displays also rutinosidase activity (Scheme 2). For the future, it is necessary to find a method of selective removal of **2** from robinin, for example, by selective hydrolysis with pure rutinosidases.

In conclusion, here we report the first ever example of enzymatic robinobiosylation. The reaction, catalyzed by the seeds of *Rhamnus cathartica*, extends the range of diglycosidase-catalyzed syntheses of structured oligoglycosides known to date. Moreover, despite its lower chemoselectivity, the seed preparation was found to catalyze the glycosylation of phenolic hydroxyls, a useful property in biocatalytic applications.

Declarations

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Conflict of interest The authors declare no conflict of interest

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schemes

schemes 1 and 2 are available in the Supplementary Files section.

Supplementary Files

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- Scheme2.tif