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Leuconostoc mesenteroides glucansucrase synthesis of flavonoid glucosides by acceptor reactions in aqueous-organic solvents

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Abstract—The enzymatic glucosylation of luteolin was attempted using two glucansucrases: the dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F and the alternansucrase from *L. mesenteroides* NRRL B-23192. Reactions were carried out in aqueous-organic solvents to improve luteolin solubility. A molar conversion of 44% was achieved after 24 h of reaction catalysed by dextransucrase from *L. mesenteroides* NRRL B-512F in a mixture of acetate buffer (70%)/bis(2-methoxyethyl) ether (30%). Two products were characterised by nuclear magnetic resonance (NMR) spectroscopy: luteolin-3′-O-α-D-glucopyranoside and luteolin-4′-O-α-D-glucopyranoside. In the presence of alternansucrase from *L. mesenteroides* NRRL B-23192, three additional products were obtained with a luteolin conversion of 8%. Both enzymes were also able to glucosylate quercetin and myricetin with conversion of 4% and 49%, respectively.
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Keywords: Flavonoid; Glucosylation; Luteolin; Glucansucrase; *Leuconostoc mesenteroides*; Acceptor reaction

1. Introduction

Flavonoids are polyphenolic compounds existing widely throughout the plant kingdom. These natural compounds are used in the food, cosmetic and pharmaceutical industries and have recently been shown to possess potential pharmacological properties. They were first described to play a role in the maintenance of blood capillary wall integrity and capillary resistance.[1,2] A number of studies have demonstrated that, depending on their structure, flavonic compounds may also exhibit anti-inflammatory, anti-allergic, anti-oxidant or anti-tumoural activities, conferring to these polyphenols a potential protective function against cardiovascular and coronary heart diseases and against certain forms of cancer.[1–7]

One major drawback of flavonoids is their poor water solubility, which limits their practical applications. Thus, in order to improve the flavonoid hydrophilicity, enzymatic glucosylation has been reported on various phenolic compounds: (+)-catechin,[8–14] hesperidin,[15,16] neohesperidin,[16] naringin[16,17] and rutin.[18,19] The glucosylated catechin has been shown to be 100-fold more soluble than catechin,[13] while the glucosylated naringin is 1000-fold more soluble than naringin.[16] An example is the capillary protectant activity of rutin (quercetin-3-O-rutinoside). Enzymatic glucosylation produced 4^G-α-D-glucopyranosyl-rutin, which is 30,000-fold more soluble than rutin and thus leading to an improved biological activity.[18,19]

Glucansucrases (GS, EC 2.4.1) are a family of enzymes, which catalyse two kinds of reaction: (1) the synthesis of glucan from sucrose and (2) the acceptor reaction. The acceptor reaction corresponds to the transfer of an α-D-glucopyranosyl unit from the donor

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(sucrose) onto either a mono or disaccharide to form low molecular weight oligosaccharides, or onto a non-carbohydrate hydroxylated compound, which leads to the formation of a glycoconjugate.

Dextransucrase (E.C. 2.4.1.5) from *Leuconostoc mesenteroides* NRRL B-512F produces a glucan called dextran, which consists of 95% of α-(1→6) osidic linkages and 5% of α-(1→3) branched linkages.[20–22] Another enzyme of particular interest is the alternansucrase (E.C. 2.4.1.140) from *L. mesenteroides* NRRL B-23192. This enzyme synthesises from sucrose an alternating α-(1→6) and α-(1→3) linked D-glucan, called alternan.[23,24]

Glucansucrases have been widely used for the glucosylation of saccharides[25] used as acceptor molecules, primarily resulting in the formation of oligosaccharides[26–28] and for the glucosylation of unusual saccharides like cellobiose,[29] acarbose,[30] methyl hexopyranosides,[31] alkylglucosides,[32] alditols, aldosulose, sugar acids[33] and salicin.[34] Due to the enzymatic nature of these reactions and the solubility properties of these acceptors, the reaction medium of choice was water.

However, only three studies have focused on the glucosylation of non-saccharide molecules: phenol acting as acceptor for B-1299CB-BF563 dextransucrase;[34] salicyl alcohol as acceptor for B-742CB dextransucrase;[35] catechol and its derivatives acting as acceptors for *Streptococcus mutans* GS-5 glucosyltransferase-D.[11,12] Various water-miscible organic solvents were used to facilitate the transglucosylation of catechol derivatives. These results have shown that the synthetic potential use of these enzymes is not restricted to conventional saccharides. Only one study has reported the activity and the stability of glucansucrase in the presence of water-miscible organic solvents.[36]

The aim of our work was to investigate the ability of glucansucrases to glucosylate non-water soluble flavonoids and to determine the effect of glucosylation on the water solubility of the resulting glucosylated flavonoid. Conditions enabling the enzymatic glucosylation of one particular flavonoid, namely luteolin were first established. Products were characterised and subsequently tested for their water solubility. This approach was then applied to five other flavonoids in order to develop the potential use of glucansucrases to glucosylate flavonoids.

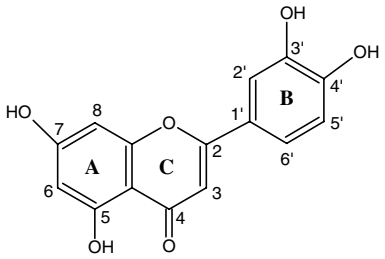


Figure 1. Structure and chemical numbering of luteolin (3′,4′,5,7-tetrahydroxy-flavone or 2-(3′,4′-dihydroxyphenyl)-5,7-dihydroxy-4*H*-1-benzopyran-4-one).

2. Results and discussion

Glucosylation of luteolin (Fig. 1) was chosen as the model reaction with glucansucrases.

2.1. Influence of organic solvent on substrate solubility and glucansucrase activity

Luteolin acceptor was slightly soluble (14 μM) in the buffered medium usually employed for dextransucrase or alternansucrase (sodium acetate buffer 20 mM, pH 5.2). As luteolin was poorly soluble in the reaction medium, various organic solvents were tested to find a solvent able to solubilise both luteolin and sucrose. As shown in Table 1, bis(2-methoxyethyl) ether (MEE) and dimethyl sulfoxide were the only solvents in which both acceptor and donor could be solubilised.

The effect of these solvents on the glucansucrase activity was then investigated (see Table 2). It appears that

Table 1. Solubility of luteolin and sucrose in sodium acetate buffer and in organic solvents

	$[Luteolin]_{max}$ (mM)[a]	$[Sucrose]_{max}$ (mM)[a]
NaOAc buffer (20 mM, pH 5.2)	0.014	2000.0
Acetonitrile	1.5	—
1,4-Dioxane	8.9	0
Methanol	10.6	6.4
Ethyl acetate	12.7	—
MEE (bis[2-methoxyethyl] ether)	456.5	0
Dimethyl sulfoxide	560.8	120.0

[a] Luteolin and sucrose concentrations were calculated from HPLC peaks area after calibration with standards.

Table 2. Substrate solubility and glucansucrase activity measured in a mixture of AcONa buffer and organic solvents

		Substrate solubility (mM)		Glucansucrase activity (%)	
		Sucrose	Luteolin	Dextransucrase B-512F	Alternansucrase B-23192
AcONa Buffer (20 mM, pH = 5,2)		2000	0.014	100	100
AcONa Buffer (70)	**MEE (30)**	**1316**	**11**	**55**	**70**
AcONa Buffer (70)	**ME_2SO (30)**	**1297**	**10**	**59**	**58**
AcONa Buffer (60)	MEE (40)	1186	15	8	50
AcONa Buffer (60)	Me_2SO (40)	1114	13	27	32
AcONa Buffer (50)	MEE (50)	977	90	5	28
AcONa Buffer (50)	Me_2SO (50)	902	15	3	15

alternansucrase is less inactivated than dextransucrase in the presence of an organic solvent. Both enzymes retained more than 50% activity in the presence of dimethyl sulfoxide or MEE when their concentration did not exceed 30% of acetate buffer (v/v). This percentage corresponds to the best compromise between substrate solubility and enzyme activity and thus was selected for the glucosylation reaction.

2.2. Glucosylation reaction

After 24 h of reaction catalysed by dextransucrase B-512F in acetate buffer (70)/MEE (30), the luteolin conversion was 44% (see Table 3). Two new more polar compounds were observed by HPLC (Fig. 2).

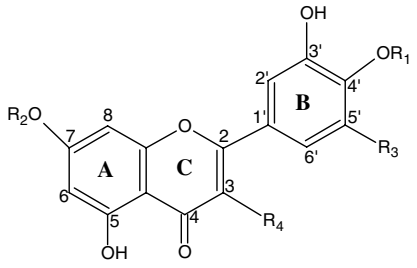
LC–MS analysis showed that they correspond to luteolin derivatives with a molecular mass of 448 g mol^{-1} ($m/z = 449$ $[MH]^+$), confirming the transfer of one glucose unit onto luteolin. Proton NMR analysis of P1 and P2 was then used to deduce the position of glucosylation. For free luteolin, signals at 7.34 and 6.88 ppm were assigned to the H-2′ and H-5′ of the luteolin moiety (B-ring), respectively. However, in the ^1H NMR spectra of P1 the proton signal of H-2′ showed a downfield shift of 0.40 ppm, in comparison to that of luteolin. This indicated the presence of a glycosidic bond at the 3′-hydroxyl group (H-2′ shifts at 7.74 ppm). In the case of P2, the proton signal of H-5′ shifted by 0.40 ppm at 7.28 ppm, indicating the presence of a glycosidic bond on the 4′-hydroxyl group. The α-configuration of the anomeric carbon in glucose was deduced, for both P1 and P2, from chemical shifts of the H-1″ at 5.43 and 5.50 ppm, respectively. The corresponding $J_{1'',2''}$ coupling constant of 3.7 Hz was also characteristic of an α-linkage.

From these results, we concluded that the structure of compound P1 was luteolin-4′-O-α-D-glucopyranoside and P2 was luteolin-3′-O-α-D-glucopyranoside (Fig. 2). The major glucosylated product P1 was further analysed and the total assignment of ^1H and ^{13}C resonances was made through COSY, HMQC and HMBC experiments (see Table 4).

The water solubility of luteolin-4′-O-α-D-glucopyranoside (P1) and luteolin-3′-O-α-D-glucopyranoside (P2) was compared to the parent luteolin (6 μM, 1.7 mg L^{-1}) and was found to be 8-fold more soluble. The linking position of the glucosyl residue onto the aromatic B-ring did not appear to influence the solubility of the glucosylated luteolins.

Next, glucosylation reaction was carried out with alternansucrase B-23192, using the experimental conditions previously established. After 24 h of reaction, we observed five products (called P3–P7) on the HPLC chromatogram (Fig. 3). Luteolin conversion was lower (8%) than that previously obtained with the dextransucrase B-512F.

Table 3. Transglucosylation of diverse flavonoids using either dextransucrase B-512F or alternansucrase B-23192 and molar ratio of the various flavonoid glucosides



Flavonoid	Luteolin[a]		Quercetin[b]	Myricetin[c]	Diosmetin[d]	β-D-Glucopyranosyl-7-diosmetin[e]	Diosmin[f]
Enzyme	DS*	ASR*	ASR*	DS*	DS*	DS*	DS*
Conversion (%)	44%	8%	4%	49%	0	0	0
Molar ratio of flavone monoglucoside	P1: 17% P2: 27%	P6: 2.5% P7: 1.5%	P9: 2.5% P10: 1%	P11: 27% P12: 22%	—	—	—
Molar ratio of flavone di-glucoside	—	P4: 2% P5: 1.5%	P8: 0.5%	—	—	—	—
Molar ratio of flavone tri-glucoside	—	P3: 0.5%	—	—	—	—	—

DS*: Dextransucrase, ASR*: alternansucrase.
[a] $R_1 = H$, $R_2 = H$, $R_3 = H$, $R_4 = H$.
[b] $R_1 = H$, $R_2 = H$, $R_3 = H$, $R_4 = OH$.
[c] $R_1 = H$, $R_2 = H$, $R_3 = OH$, $R_4 = OH$.
[d] $R_1 = CH_3$, $R_2 = H$, $R_3 = H$, $R_4 = H$.
[e] $R_1 = CH_3$, $R_2 = glucose$, $R_3 = H$, $R_4 = H$.
[f] $R_1 = CH_3$, $R_2 = rutinose$, $R_3 = H$, $R_4 = H$.

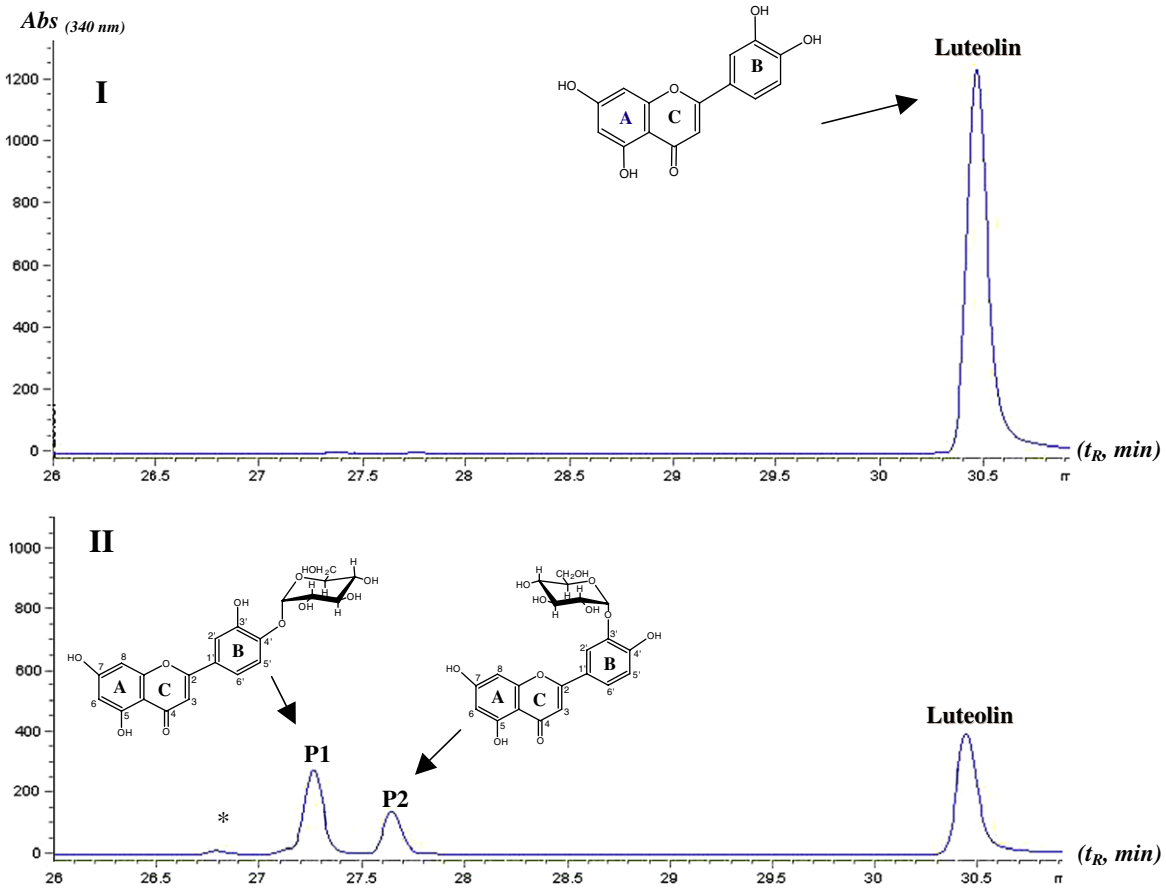


Figure 2. HPLC analysis of reaction mixtures after incubation of luteolin (9 mM) in the presence of dextransucrase B-512F (3 U mL^{-1}), sucrose (120 mM) and MEE 30% (v/v). I: at initial reaction time, II: after 24 h incubation, with luteolin conversion of 44%; t_R = retention time. Peak identification: P1: luteolin-4′-O-α-D-glucopyranoside and P2: luteolin-3′-O-α-D-glucopyranoside. ∗: not determined.

LC/MS coupling revealed that P6 and P7 are mono-glucosides (molecular mass of 448 g mol^{-1}), P4 and P5 are di-glucosides (molecular mass of 611 g mol^{-1}) and P3, a tri-glucoside (molecular mass of 773 g mol^{-1}).

Using LC–NMR, it was confirmed that P6 and P7 are identical to the luteolin mono-glucoside synthesised with the dextransucrase B-512F (P1 and P2, respectively), P4 and P5 are di-glucosides at 4′-position and P3 is a tri-glucoside at the same 4′-position. It was not possible to determine the nature of the linkages between the glucosyl residues. However, the specificity of alternansucrase and its ability to synthesise both α-(1→3) and α-(1→6) linkages suggest that the diglucosyl may be isomaltosyl or nigerosyl moieties. From our results, it appears that only two sites of glucosylation are recognised by glucansucrases, showing that both enzymes are selective towards the hydroxyl groups of B ring. This is in agreement with the observation made by Meulenbeld and Hartmans[12] who proposed that two vicinal hydroxyl groups are necessary for glucosylation of catechol.

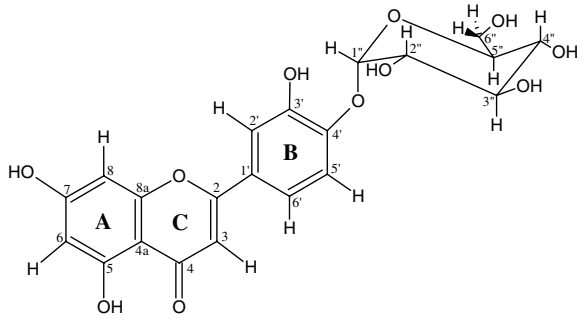
We should point out that it was also possible to detect several luteolin glucosylation products containing up to eight glucosyl residues by LC–MS, but unfortunately the yields were too low for the NMR structural characterisation.

2.3. Glucosylation of other flavonoids catalysed by dextransucrase B-512F or alternansucrase B-23192

After the successful luteolin conversion, acceptor reaction conditions of luteolin were subsequently applied to the glucosylation of five other flavonic acceptors, which are almost insoluble in conventional aqueous systems (see Table 3). Using these conditions, quercetin and myricetin were successfully glucosylated.

Quercetin differs from luteolin by the presence of an additional hydroxyl group at 3-position of the C-ring (Fig. 4) and its solubility in the reaction medium is 8-fold lower. After 24 h of glucosylation reaction catalysed by alternansucrase B-23192, we observed three products (conversion ratio of 4%, see Fig. 4), which were further characterised by LC/MS and LC/NMR analysis as two mono-glucosides at 4′- or 3′-position (P9 and P10 respectively, molecular mass of 464 g mol^{-1}) and one di-glucoside at 4′-position (P8, molecular mass of

Table 4. ^{13}C NMR and ^{1}H NMR chemical shifts for luteolin-4′-O-α-D-glucopyranoside



	δ ^{13}C (ppm, in Me_2SO-d_6)	δ ^{1}H (ppm, in Me_2SO-d_6)
Aglycone		
2	163.7	
3	104.5	6.82 (s)
4	182.2	
4a	104.3	
5	161.9	
6	99.4	6.21 (s)
7	164.7	
8	94.5	6.51 (s)
8a	157.8	
1′	125.3	
2′	114.0	7.50 (s)
3′	147.9	
4′	148.7	
5′	117.2	7.35 (d, $J = 8.3$ Hz)
6′	119.0	7.52 (d, $J = 8.3$ Hz)
4′-Glucoside		
1″	99.7	5.43 (d, $J = 3.7$ Hz)
2″	72.4	3.39 (m)
3″	73.6	3.73 (m)
4″	70.4	3.20 (m)
5″	74.4	3.51 (m)
6″	61.2	3.61 (m)
6″		3.48 (m)

626 g mol^{-1}). Hence, it was confirmed that the hydroxyl group at 3-position was not glucosylated. The presence of the 3′,4′-catechol structure in the B-ring is mainly responsible for the transglucosylating activity of these glucansucrase in the presence of flavonoids.

Regarding myricetin, this flavonoid bears two additional hydroxyl groups compared to luteolin: one at 3-position of the C-ring (like quercetin) and one at 5′-position of the B-ring (Fig. 5). Myricetin solubility in the reaction medium is the same for luteolin, and in this case, two glucosylation products (P11 and P12) were observed after 24 h of reaction catalysed by dextransucrase B-512F (conversion ratio of 49%, see Fig. 5). The LC/MS and LC/NMR data showed that they were two mono-glucosides (molecular mass of 480 g mol^{-1}) at 3′- and 4′-positions of the B-ring.

In conclusion, the ability of glucansucrases to catalyse the glucosylation in aqueous-organic solvents of non-water soluble flavonoid has been demonstrated for the first time in this study. The best flavonoid conversion was achieved for luteolin (44%) and myricetin (49%) using dextransucrase. The glucosylation of such flavonoids clearly puts in evidence the ability of this enzyme to specifically glucosylate non-natural acceptor substrates in a regioselective and stereoselective manner.

3. Experimental

3.1. Materials

L. mesenteroides NRRL B-512F (EC 2.4.1.5) and B-23192 (EC 2.4.1.140) were supplied by the National Research Regional Laboratories NRRL, Peoria (IL, USA). Luteolin, diosmetin, diosmin, quercetin, β-D-glucopyranosyl-7-diosmetin and myricetin were supplied by SERVIER Laboratories (Orléans, France). Bis(2-methoxyethyl) ether (MEE) was purchased from Aldrich. All other chemicals used were commercially available and of chemically pure grade.

3.2. Methods

3.2.1. Analytical methods

3.2.1.1. High performance liquid chromatography (HPLC). The efficiency of the glucosylation reaction was evaluated by HPLC using a Hewlett Packard 1050 series pump, autosampler, UV detector and HP 1047A series refractometer. To study flavonoid consumption and flavonoid–glucoside formation, HPLC analyses were performed on an analytical C18 column (Prontosil Eurobond, 5.0 μm, 250 × 4.0 mm; Bischoff Chromatography, Germany) thermostated at 30 °C, by using a gradient of water/trifluoroacetic acid (99.90:0.10, v/v) and acetonitrile/trifluoroacetic acid (99.90:0.10, v/v) at a flow rate of 1 mL min^{-1}: 0 min (100/0), 10 min (100/0), 40 min (40/60), 45 min (0/100). The different compounds (flavonoids and glucosylated flavonoids) were quantified by UV detection at 255 and 340 nm. The reaction progress was also evaluated by measuring sucrose consumption (fructose formation) through an analytical NH_2 column (Waters-Spherisorb Amino 5.0 μm, 250 × 4.0 mm; Bischoff Chromatography, Germany) thermostated at 30 °C, with a mixture of water/acetonitrile (20:80, v/v) as eluent and a linear elution at a flow rate of 0.5 mL min^{-1}. The different compounds were quantified by refractometry.

3.2.1.2. Liquid chromatography/mass spectrometry (LC/MS). The molecular mass of the luteolin glucosylation products was determined by LC/MS coupling using a Micromass LC/TOF (Alliance Waters). The compounds were ionised by electrospray ionisation on the positive mode with a source at 100 °C. LC was performed with a C18 column as previously described.

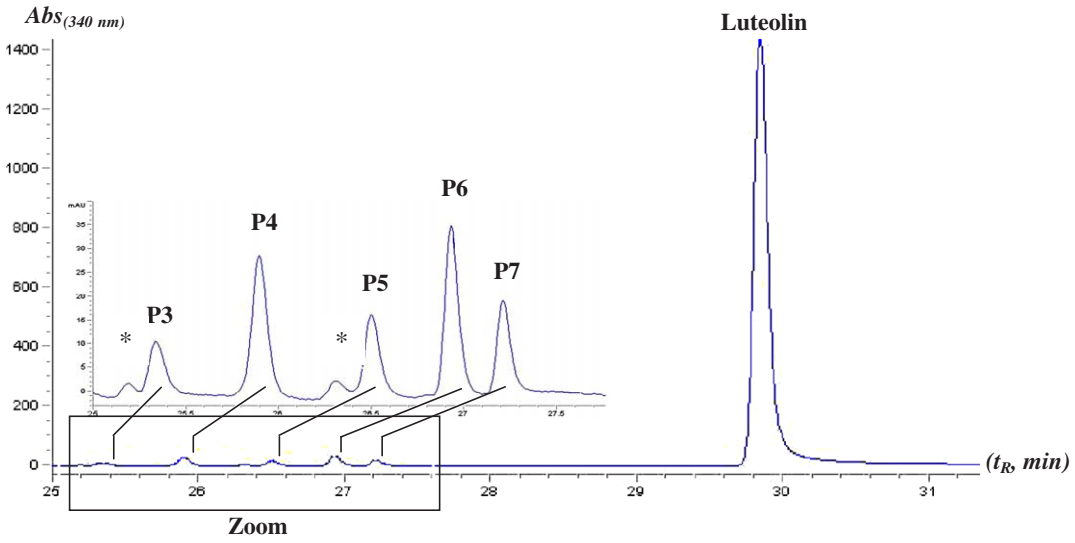


Figure 3. HPLC analysis of reaction mixtures after incubation of luteolin (9 mM) in the presence of alternansucrase B-23192 (3 U mL^{-1}), sucrose (120 mM) and MEE 30% (v/v). After 24 h reaction, luteolin conversion of 8%. The insert shows an enlargement of the reaction product chromatogram. Peaks: P3: luteolin-4′-O-α-D-tri-glucoside, P4 and P5: two forms of luteolin-4′-O-α-di-glucosides, P6 (=P1): luteolin-4′-O-α-D-glucopyranoside and P7 (=P2): luteolin-3′-O-α-D-glucopyranoside. ∗: not determined.

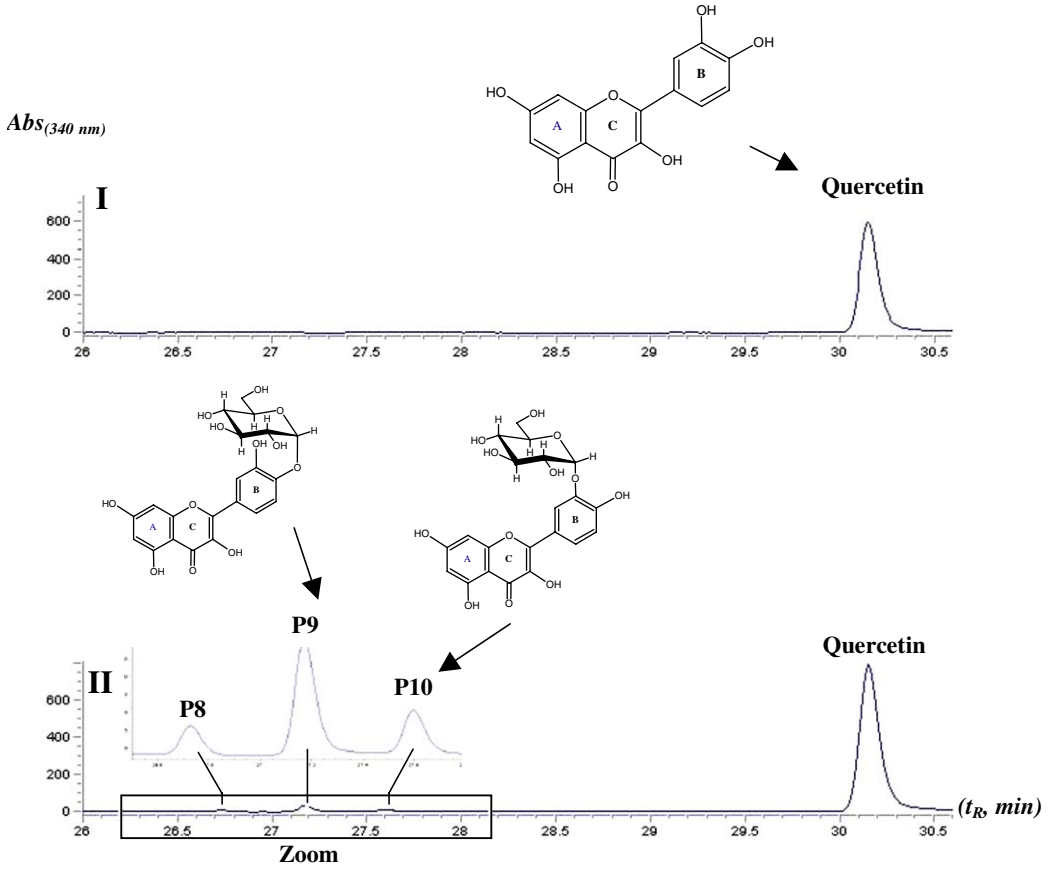


Figure 4. HPLC analysis on C18 column of reaction mixtures after incubation of quercetin (9 mM) in the presence of alternansucrase B-23192 (3 U mL^{-1}), sucrose (120 mM) and MEE 30% (v/v). I: at initial time of reaction, II: after 24 h of reaction, with a transfer ratio of 4%. The insert shows an enlargement of the reaction product chromatogram retention times. Peaks: P8: quercetin-4′-O-α-D-di-glucopyranoside, P9: quercetin-4′-O-α-D-glucopyranoside and P10: quercetin-3′-O-α-D-glucopyranoside.

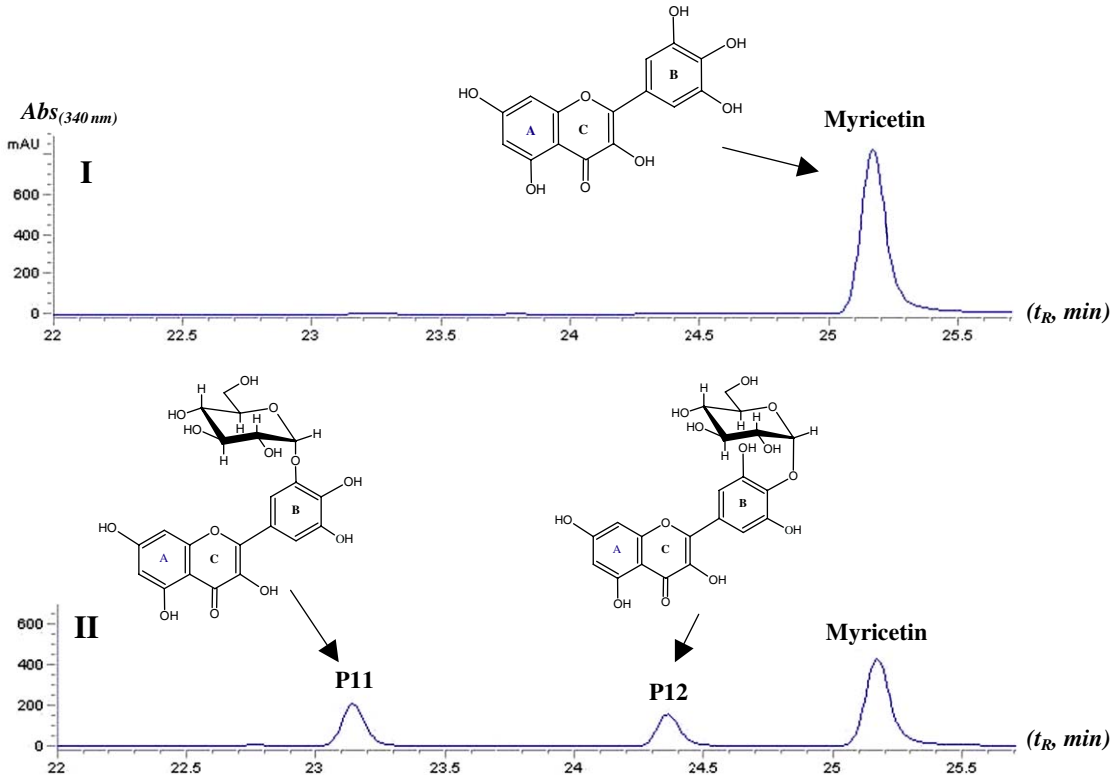


Figure 5. HPLC analysis on C18 column of the reaction mixture after incubation of myricetin (9 mM) in the presence of dextransucrase B-512F (3 U mL^{-1}), sucrose (120 mM) and MEE 30% (v/v). I: at initial reaction time, II: after 24 h reaction and with a conversion of 49%. Peaks: P11: myricetin-3′-O-α-D-glucopyranoside and P12: myricetin-4′-O-α-D-glucopyranoside.

The different compounds (flavonoids and glucosylated flavonoids) were quantified by UV detection at 255 nm.

3.2.1.3. Liquid chromatography/nuclear magnetic resonance (LC/NMR). ^{1}H NMR spectra (400.13 MHz) were recorded in Me_2SO-d_6 on a BRUKER AVANCE 400 spectrometer. LC was conducted as previously described by using deuterium oxide instead of water. The different compounds (flavonoids and glucosylated flavonoids) were quantified at 340 nm.

3.2.1.4. Nuclear magnetic resonance (NMR). ^{1}H NMR data (500.13 MHz), ^{13}C NMR data (125.76 MHz) and 2D NMR data (COSY, HMQC and HMBC) were recorded in Me_2SO-d_6 on a BRUKER DRX 500 spectrometer.

3.2.2. Determination of substrate solubility in hydro-organic solvents

3.2.2.1. Luteolin acceptor. Luteolin solutions were prepared by dissolving the flavone in MEE or Me_2SO until they reached saturation (457 and 561 mM, respectively). Then, acetate buffer (20 mM, pH 5.2) was gradually added into these organic solutions to determine the solubility at various co-solvent concentrations. The samples were centrifuged at 10,000 rpm for 10 min, and an aliquot of supernatant was diluted to adequate concentration with Me_2SO in order to ensure a concentration lower than 0.5 g L^{-1}. The luteolin concentration was estimated by HPLC using the same conditions as those described above.

3.2.2.2. Sucrose donor. Sucrose solutions were prepared by dissolving the sugar in acetate buffer (20 mM, pH 5.2) until it reached saturation (2 M). Then, MEE (or Me_2SO) was gradually added into this aqueous buffer to determine its solubility at various co-solvent concentrations.

The samples were centrifuged (10 min, 10,000 rpm). An aliquot of supernatant was diluted with the mixture of water/acetonitrile (20:80, v/v) used as HPLC mobile phase and analysed using the same conditions as those described above by HPLC with an analytical NH_2 column.

3.2.3. Water solubility of glucosylated compounds. Luteolin, luteolin-3′-O-α-D-glucopyranoside and luteolin-4′-O-α-D-glucopyranoside were tested for their water solubility. Each compound was dissolved at 2.7 mg mL^{-1} with distilled water. The mixtures were vortexed for 2 min, kept overnight at room temperature, then centrifuged at 10,000 rpm for 10 min. The concentration of each sample was estimated by HPLC (conditions as

described above for luteolin solubility in different hydro-organic solvents).

3.2.4. Glucansucrase activity. GS activity was determined by measuring the initial rate of fructose production by using the 3,5-dinitrosalicylic acid assay.[37,38] Thus, one unit of glucansucrase activity was defined as the amount of enzyme that caused the consumption of 1 μmol of sucrose per minute at 30 °C and, as a consequence, the release of 1 μmol of fructose per minute. Reactions were carried out at 30 °C in sodium acetate buffer (20 mM, pH 5.2), sucrose 100 g L^{-1} and various solvent concentrations.

3.2.5. Enzymatic transglucosylation. Transglucosylation reaction was carried out at 30 °C in acetate buffer (20 mM, pH 5.2) containing different concentrations of water-miscible organic solvents, sucrose (120 mM), flavonic acceptor (9 mM) and glucansucrase (3 U mL^{-1}). At fixed times an aliquot of the reaction mixture was heated at 95 °C for 5 min to stop the reaction. Then, the samples were centrifuged at 10,000 rpm for 10 min, and an aliquot of the supernatant was diluted to the adequate concentration with Me_2SO or a mixture of water/acetonitrile (20:80, v/v). HPLC analysis using a C18 column or a NH_2 column, respectively, allowed to determine the amounts of substrates and products.

3.2.6. Preparation of luteolin glucosides. A reaction mixture (388 mL) containing luteolin (1 g, 3.5 mmol), sucrose (16.1 g, 47 mmol), MEE (30%, v/v), dextransucrase B-512F (3 U mL^{-1}) and sodium acetate buffer (20 mM, pH 5.2) was incubated at 30 °C until total sucrose consumption. Then, the reaction was stopped after 7 h of reaction by heating the mixture at 95 °C for 5 min. The reaction mixture was extracted with 100 mL (10 * 10 ml) of ethyl acetate and dried on $MgSO_4$. The organic phase layer was evaporated until a yellow powder was obtained, which was dried in a desiccator. The mixture was fractionated by preparative chromatography with a C18 column (PROCHROM LC-50 Phase SYMMETRY C18, 7.0 μm) using a gradient of water/trifluoroacetic acid (99.90:0.10, v/v) and acetonitrile/trifluoroacetic acid (99.90:0.10, v/v) at a flow rate of 60 mL min^{-1}: 0 min (85/15), 45 min (60/40). The fractions containing the products were collected and lyophilised. The structure of pure luteolin mono-glucoside P1 was confirmed by ^1H and ^{13}C NMR analyses.

3.2.7. Structural characterisation of luteolin glucosides. Luteolin-4′-O-α-D-glucopyranoside (P1): LC/MS: Anal. Calcd for $C_{21}H_{20}O_{11}$: 449.2 $[MH]^+$. Found: 449.3; LC/NMR: ^1H and ^{13}C NMR data given in Table 3.

Luteolin-3′-O-α-D-glucopyranoside (P2): LC/MS: Anal. Calcd for $C_{21}H_{20}O_{11}$: 449.2 $[MH]^+$. Found: 449.3; LC/^1H NMR (Me_2SO-d_6): δ 7.74 (s, 1H, H-2′), 7.56 (d, 1H, $J_{6',5'} = 8.3$ Hz, H-6′), 6.97 (d, 1H, H-5′), 6.60 (s, 1H, H-3), 6.50 (s, 1H, H-8), 6.21 (s, 1H, H-6) and 5.50 (d, 1H, $J_{1'',2''} = 3.7$ Hz, H-1″).

3.2.8. Structural characterisation of quercetin glucosides. Quercetin-4′-O-α-D-di-glucopyranoside (P8): LC/MS: Anal. Calcd for $C_{27}H_{30}O_{17}$: 627.2 $[MH]^+$. Found: 627.3; LC/^1H NMR (Me_2SO-d_6): δ 7.63 (s, 1H, H-2′), 7.61 (s, 1H, H-6′), 7.29 (s, 1H, H-5′), 6.46 (s, 1H, H-8), 6.21 (s, 1H, H-6), 5.53 (s, 1H, Glc-1, H-1″) and 5.21 (s, 1H, Glc-2, H-1″).

Quercetin-4′-O-α-D-glucopyranoside (P9): LC/MS: Anal. Calcd for $C_{21}H_{20}O_{12}$: 465.2 $[MH]^+$. Found: 465.3; LC/^1H NMR (Me_2SO-d_6): δ 7.65 (s, 1H, H-2′), 7.63 (s, 1H, H-6′), 7.25 (s, 1H, H-5′), 6.45 (s, 1H, H-8), 6.20 (s, 1H, H-6) and 5.50 (s, 1H, H-1″).

Quercetin-3′-O-α-D-glucopyranoside (P10): LC/MS: Anal. Calcd for $C_{21}H_{20}O_{12}$: 465.2 $[MH]^+$. Found: 465.3; LC/^1H NMR (Me_2SO-d_6): δ 8.00 (s, 1H, H-2′), 7.65 (s, 1H, H-6′), 6.97 (s, 1H, H-5′), 6.47 (s, 1H, H-8), 6.20 (s, 1H, H-6) and 5.45 (s, 1H, H-1″).

3.2.9. Structural characterisation of myricetin glucosides. Myricetin-3′-O-α-D-glucopyranoside (P11): LC/MS: Anal. Calcd for $C_{21}H_{20}O_{13}$: 481.2 $[MH]^+$. Found: 481.3; LC/^1H NMR (Me_2SO-d_6): δ 7.58 (s, 1H, H-2′), 7.37 (s, 1H, H-6′), 6.45 (s, 1H, H-8), 6.20 (s, 1H, H-6) and 5.42 (s, 1H, H-1″).

Myricetin-4′-O-α-D-glucopyranoside (P12): LC/MS: Anal. Calcd for $C_{21}H_{20}O_{13}$: 481.2 $[MH]^+$. Found: 481.3; LC/^1H NMR (Me_2SO-d_6): δ 7.20 (s, 1H, H-2′), 7.20 (s, 1H, H-6′), 6.45 (s, 1H, H-8), 6.20 (s, 1H, H-6) and 5.20 (s, 1H, H-1″).

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