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Microbial glucuronidation of polyphenols

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ABSTRACT

In a typical series of experiments, several polyphenol *O*-β-D-glucuronides (including human metabolites) deriving from two flavonoids (naringenin, quercetin) and several stilbenoids (*trans*-resveratrol, rhapontigenin, deoxyrhapontigenin) have been obtained from the aglycones or their natural glycosides (Naringenin-7-O-glucoside, rutin, piceid, rhapontin or deoxyrhapontin), in a one step biotransformation and in moderate to high yields by incubation with a *Streptomyces* sp. strain M52104. Regioselectively glucuronidated products have been separated by chromatographic methods, then extensively characterized by MS and NMR. In all cases glucuronidation is β-stereospecific, but not completely regioselective. When present, the 4′(para)-hydroxyl group is generally favoured, then the 7-OH-group of flavonoids (corresponding to the 3-OH of stilbenoids). Several pure O-β-D-glucuronidated derivatives, commercially not available, have been prepared in high purity by this method.

1. Introduction

Polyphenols are a group of natural chemical substances (over 8000 distinct compounds) characterized by the presence of more than one phenol group per molecule, with a widespread occurrence in commonly consumed fruits and vegetables [1]. Among them, stilbenoids and flavonoids seem to have biological activities and especially antioxidant characteristics with potential health benefits [2]. Polyphenol antioxidants scavenge free radicals (singlet oxygen, peroxynitrite and hydrogen peroxide) [3,4] and thus may contribute to continually remove various reactive oxygen species from cells to maintain healthy metabolic function and reduce the risk of oxidative stress, neurodegenerative or cardiovascular diseases [5], inflammation [6] and cancer [7]. In addition, there is increasing evidence that reactive oxygen species play a pivotal role in the process of ageing [8].

Most *in vitro* biological activities have been assessed by experimentation using the aglycones. However, in nature, flavonoids and stilbenoids are mainly found as glycosylated conjugates, which may greatly affect their antioxidant properties [9,10]. Morover recent studies have shown the limited bioavailability of most polyphenols and the role of conjugated species such as glucosides or glucuronides as absorption and long-lived circulating forms [11–15].

Although deglycosylation is likely to occur either pre- or postabsorption [16,17], metabolism of these compounds *in vivo* leads to a neo-conjugation of one or more hydroxyl groups with sulfate and D-glucuronic acid. As the antioxidant properties of polyphenols are generally associated to the presence of *ortho*-phenolic groups, the nature and position of these substitutions will affect subsequent biological activities, possibly reducing or abolishing the activities seen with the aglycone [18–21].

Conjugation of xenobiotics with glucuronic acid or sulfate are known as common detoxification pathways that lead to an increased solubility and a higher molecular weight (necessary for excretion in the bile). The ingested glycosides undergo hydrolysis in the gut by intestinal microflora and endogenous enzymes to their aglycones. Within the enterocyte, aglycones are then glucuronidated [22–24] and can pass through the enterocyte basolateral membrane and hence into the vascular system and circulating blood, or are transferred back into the luminal compartment by P-glycoprotein and multi-drug resistance proteins [18,25]. In addition untransformed aglycones may be glucuronidated by hepatic microsomes. Overall uptake of an individual polyphenol is thus a complex interplay between the biochemistry of polyphenol glycosides, metabolism of their aglycones and the rate of transport of each form. However it is recognized that the major (if not exclusive) part of flavonoids and stilbenoids circulates in blood as their conjugated forms, glucuronides or sulfates [26].

More work is thus necessary to elucidate the potential role and intrinsic activity of different conjugated forms of polyphenols, provided regiospecific synthetic conjugates will be available. *In vitro* experiments using flavonoids and stilbenoids aglycones or glycoside conjugates with cultured cells require careful consideration

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of absorption and bioavailability for their appropriate interpretation [14,15]. To further investigate the glucuronidation of several flavonoid or stilbenoid substrates, detailed kinetic analyses are required using sensitive analytical measurements. The most accurate approach for such determinations is to monitor the formation of glucuronides using LC/MS methods complemented by calibration with authentic metabolites. However, the number of readily available glucuronide standards required for method development is limited.

The preparation of glucuronides presents significant challenges for existing methods of glucuronidation. Various methods have been described to obtain such conjugates, involving chemical and chemoenzymatic methods, extraction from plants, isolation from blood or urine after consumption of the flavonoid, or enzymic synthesis with crude microsomal preparations or purified glucuronyltransferases. Chemical methods of glucuronidation are based on the Koenigs–Knorr reaction or related procedures but require one or more deprotection steps to liberate free glucuronide; in addition they often suffer from poor yields and side reactions due in part to the low reactivity of glucuronic acid derived glycosyl donors or unexpected drawbacks in the deprotection reactions [27–34]. An efficient chemoenzymatic method of oxidation of the corresponding glucosides (frequently found as natural products in plants) by the laccase-TEMPO system [35] cannot be used because of the presence of free phenolic groups, rendering the substrate and products very sensitive to a laccase-catalyzed polymerisation. Enzymatic methods of synthesis employ either crude (permeabilized) liver microsomal preparations [36–38], S9 intestinal fractions [19], or purified uridine 5′-diphosphoglucuronosyl transferases (UGTs), a superfamily of enzymes responsible for glucuronidation reactions, with uridine-5′-diphosphoglucuronic acid (UDPGA) as glycosyl donor [39,40]. These procedures provide a mild and stereospecific synthesis in a single step. However, UGTs are substrate-specific to the acceptor alcohol and practical considerations often limit this procedure to very small scale syntheses. Given the limitations associated with existing methods, the development of improved glucuronidation protocols for the production in significant amounts of regio- and stereoselectively conjugated derivatives is an important challenge.

Microbial transformation methods are known to produce, in addition to oxidized metabolites of xenobiotics, some glycosylated metabolites of flavonoids or stilbenoids aglycones, mainly D-glucosylated conjugates [41,42]. However, a few strains have already demonstrated their ability to produce O-β-D-glucuronides of drugs and drug metabolites [43,44]. We have investigated the use of one of these strains, a *Streptomyces* sp. from the Bertin Pharma collection, to prepare in multimilligram amounts various glucuronides of typical dietary flavonoids, such as naringenin or quercetin and some stilbenoids, such as resveratrol, rhapontigenin or deoxyrhapontigenin, starting either from the free aglycones or from their naturally found glycosides.

2. Experimental

2.1. Chemicals

Naringenin, rutin and deoxyrhapontin were purchased from Sigma–Aldrich France, naringenin-7-O-glucoside and quercetin from Extrasynthese (Genay, France). Resveratrol and piceid (resveratrol 3-β-D-glucoside) from Bertin Pharma (Montigny-le-Bretonneux, France). Rhapontin was purchased from Carl Roth (Lauterbourg, France). Deionised water was obtained from a Milli-Q Gradient water system. HPLC-grade acetonitrile, formic acid (98%), Amberlite XAD-16 and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich France. All other products and solvents were of the highest purity available.

2.2. Analytical methods

The separation and quantification of glucuronide metabolites was carried out using an Agilent model 1100 liquid chromatograph interfaced with UV and MS detection. Separations were performed at 40 °C on Nucleodur (Macherey-Nagel) C18 columns (5 μ, 4 mm × 70 mm or 3 mm × 250 mm) using water–acetonitrile gradients, containing 0.1% formic acid. Detection was carried out with an Agilent DAD detector (200–400 nm) and a Quatro Micromass spectrometer (Waters) operated in negative mode from 150 to 900 D.

^{1}H- and ^{13}C-NMR spectra (500.13 MHz and 125.77 MHz, respectively) were recorded on a Brucker Avance-500 spectrometer in $^{2}H_2O$, methanol-d_4 or DMSO-d_6 solvents. All NMR spectra were measured at 20 °C, and proton and carbon chemical shifts were referenced to the solvent signals: 2.50 ppm for proton and 39.52 ppm for carbon in the case of DMSO-d_6, and 3.31 ppm for proton and 49.00 ppm for carbon in the case of CD_3OD. Two-dimensional correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY), rotating frame nuclear Overhauser effect spectroscopy (ROESY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) experiments were performed using standard pulse sequences. Proton chemical shift assignments were obtained by the analysis of COSY and TOCSY spectra and carbon chemical shift assignments were made by the analysis of HSQC and HMBC spectra. For reference, a complete proton and carbon peak assignment of the NMR spectra was made on the parent compounds.

2.3. Cultivation of microorganism

The strain M52104, isolated from soil, was maintained on ISP medium 2 agar (Difco) slants at 25–28 °C. Liquid medium for cultures contained 2.0% D-(+)-glucose, 0.5% yeast extract (Difco), 0.5% soytone (Difco), 0.5% NaCl, 0.5% K_2HPO_4 in deionised water, adjusted to pH 7 with HCl solution. Media were sterilised by autoclaving at 121 °C during 20 min. Strain M52104 has been identified by 16S rDNA sequencing at the DSMZ collection as a member of the *Streptomyces* genus, closely related (99.8%) to *S. tubercidicus* or a subspecies of *S. libani*. It is freely available from the authors through a usual Material Transfer Agreement procedure.

2.4. Preparative syntheses and isolation of products

Streptomyces sp. M52104 was grown at 28 °C with orbital shaking in 3 L-conical flasks containing 1 L-volumes of liquid medium during 72 h. Flavonoid and stilbenoid substrates, solubilized in DMSO (2% final concentration) when not sufficiently soluble in water, were added at 0.1–0.6 g/L final concentration and incubations were continued for 48–192 h and monitored by LC/UV/MS. When bioconversion was completed, the cells were removed by centrifugation (13,000 × *g*, 20 min), the supernatant was filtered on GF/A filter (Whatman), and the aqueous phase treated with Amberlite XAD-16; the resin was repeatedly washed with water, then the glucuronides were eluted with ethanol. After evaporation to a small volume, the ethanol eluate residue was solubilized in a minimal volume of DMSO and submitted to successive injections (0.5 ml) on a semi-preparative Nucleodur (Macherey–Nagel) C18 column (10 μ, 32 mm × 250 mm) using a water–acetonitrile gradient, containing 0.1% formic acid. Collected fractions were evaluated for purity by LC/UV/MS and those fractions that contained sufficient quantity and purity of the glucuronide of interest were pooled, evaporated and lyophilized. In some cases a complementary

separation was carried out on the same column using a gradient of water/acetonitrile, containing10 mM ammonium acetate. Final purity (in the 90–98% range) was estimated from analytical HPLC and UV absorption between 230 and 320 nm using the aglycone molecular absorption as a reference.

3. Results and discussion

A summary of isolated yields of the various polyphenol glucuronides obtained is given in Table 1.

3.1. Quercetin glucuronidation

Quercetin **1a** (Scheme 1) is the main flavonol in the human diet, and one of the most commonly used in studies of biological activity of flavonoids. Much attention has been paid to its antioxidant and free-radical scavenging properties [45] and its biological activities, such as antithrombotic and anticarcinogenic activities [46]. Quercetin is an effective inhibitor of xanthine oxidase and lipoxygenase, enzymes involved in processes such as inflammation, atherosclerosis, cancer and ageing. The major circulating forms of quercetin found in human plasma after consumption of food containing quercetin and its glycosides are glucuronides and sulfates [11,38].

A number of different quercetin-conjugated metabolites, including 3′-O-methyl substituted (isorhamnetin) derivatives, have been described in animals and humans. However, in the absence of easily available or extensively characterized authentic standard compounds, their identification and quantitation was essentially based on HPLC/MS and HPLC/UV data or comparison with minute amounts of mixtures of enzymatically produced derivatives. At least four of them were detected as the major circulating forms: quercetin-3-glucuronide, quercetin-3′-sulfate, isorhamnetin-3-glucuronide (the 3′-O-methylated product of quercetin-3-glucuronide) and an uncharacterised quercetin-diglucuronide, accompanied by minor levels of the 4′-glucuronides of quercetin and isorhamnetin [21,37,38]. On the other hand, *in vitro* studies have shown that the apparent affinity of UDP-glucuronosyltransferases from human liver cell-free extracts follows the order 4′- > 3′- > 7- > 3-OH, although the apparent maximum rate of formation is for the 7-position. The 5-position does not appear to be a site for conjugation.

Quercetin-3-O-β-D-glucuronide (**4**) has been precedently obtained by extraction from green beans (7–15 mg per kilogram of plant material) [37], and chemically synthesised in poor yields (5–25%), as quercetin-3′-O-β-D-glucuronide (**5**) and isorhamnetin-3-O-β-D-glucuronide, after due selective protection of 3′-, 4′- and 7-hydroxyl groups, then extensively characterized by MS and NMR spectroscopy [27,47,48]. Other glucuronides such as quercetin-7-O-β-D-glucuronide **(3)** and quercetin-4′-O-β-D-glucuronide (**2**) have been produced in milligram amounts by chemical multistep synthesis [48] or sub-milligram amounts by enzymatic methods and mainly characterized by MS spectroscopy [37,48–51].

When quercetin (**1a**, 0.1 g/L) was incubated with grown cultures of strain M52104 during 65 h at 28 °C, it disappeared completely from the culture medium, and several products identified as glucuronidated compounds by HPLC/MS (*m*/*z* 477 and *m*/*z* 301, corresponding to the loss of 176 mass units, as the glucuronide moiety) were detected in the centrifuged supernatant, some of them in large amounts (Fig. 1A). After adsorption on XAD-16, elution with ethanol and semi preparative HPLC purification, it was possible to recover the major 4′-O-β-D-glucuronide **2** (*Rt* = 19.21 min, 50%), an unseparated mixture of 3- and 7-O-β-D-glucuronides **4** and **3** (*Rt* = 14.27 min, 38%) in a respective 3:1 ratio, and a minor undetermined glucuronide conjugate (*Rt* = 20.39 min, 5%). Similar results were obtained when the initial concentration of quercetin was increased up to 0.6 g/L and incubation was prolonged.

A very similar pattern was observed when rutin **1b** (quercetin 3-O-rhamnoglucoside, 0.1 g/L) was submitted to the same incubation conditions (90 h, 28 °C), with a complete disappearance of rutin. Moreover an additional peak with *m*/*z* 785 corresponding to an undetermined quercetin 3-O-glucoside glucuronide, possibly resulting from partial hydrolysis and glucuronidation, was detected. A change in HPLC solvent to an ammonium acetate buffered-acetonitrile gradient allowed an easy separation of glucuronides **3** and **4** and preparation of pure samples.

Identification of the glucuronidation positions was secured on the purified products by mass spectrometry 1D ^{1}H and ^{13}C NMR (see Supplementary material Figs. S1, S6, S7 and Tables S1, S2) and 2D NMR, using the usual COSY, TOCSY, HSQC and HMBC sequences. The major quercetin-4′-O-β-D-glucuronide (**2**) showed, in addition to the proton resonance pattern of the aglycone, the presence of the anomeric hydrogen as a doublet at 5.00 ppm, with a coupling constant *J* = 7.6 Hz, indicating a β-configuration, associated with well separated H-5″ as a doublet at 4.08 ppm (*J* = 9.6 Hz) and 2″-, 3″- and 4″-hydrogens as triplets at 3.5–3.7 ppm. 2D-COSY was used for the respective identification of these protons and glucuronidation position was determined by HMBC, with a clear ^{3}J correlation of the anomeric hydrogen with C-4′ (at 147.5 ppm), itself identified by its ^{3}J cross correlations with H-2′ and H-6′.

The 7-O-β-D-glucuronide **(3)** [48] was similarly identified, with an anomeric hydrogen at 5.17 ppm (*J* = 7.4 Hz) associated with H-5″ at 4.16 ppm (d, *J* = 9.6 Hz) and 2″–4″ hydrogens as overlapping signals at 3.5–3.6 ppm. Identification of the glucuronidation position followed from HMBC experiments with a ^{3}J cross-correlation of the anomeric hydrogen with C-7 (at 164.2 ppm), itself identified by cross correlations with H-6 and H-8.

The 3-O-β-D-glucuronide (**4**) was similarly identified [33,52,53], with an anomeric hydrogen at 5.22 ppm (*J* = 7.7 Hz) associated with a highly shielded H-5″ at 3.75 ppm (d, *J* = 9.7 Hz) and 2″–4″ hydrogens as overlapping signals at 3.5–3.6 ppm. Identification of the glucuronidation position followed from HMBC experiments with a ^{3}J cross-correlation of the anomeric hydrogen with C-3 (at 135.3 ppm).

The last undetermined glucuronide metabolite (*m*/*z* 477 and *m*/*z* 301) was tentatively identified as the 3′-O-β-D-glucuronide (**5**), but the amount recovered was too small and contaminated with other products so that its NMR spectra could not be securely interpretated.

The similarity of chromatographic profiles of quercetin and rutin metabolites by strain M52104 indicates that hydrolysis by glycosidase(s) is a predominant preliminary reaction, but the observed presence in rutin derivatives of a minor glucuronide–glucoside (*m*/*z* = 785) shows that glucuronidation of only partially hydrolysed rutin is possible. Contrarily to what is found for total human liver extracts, where the 7-O-glucuronidation is predominant [50], or with recombinant UGTs 1A3 and 1A9, where the predominant glucuronidation positions are respectively 3′ and 3 [54], the preferred glucuronidation position of our *Streptomyces* strain is the 4′ position. As expected, the 5-hydroxy position of quercetin does remain unglucuronidated.

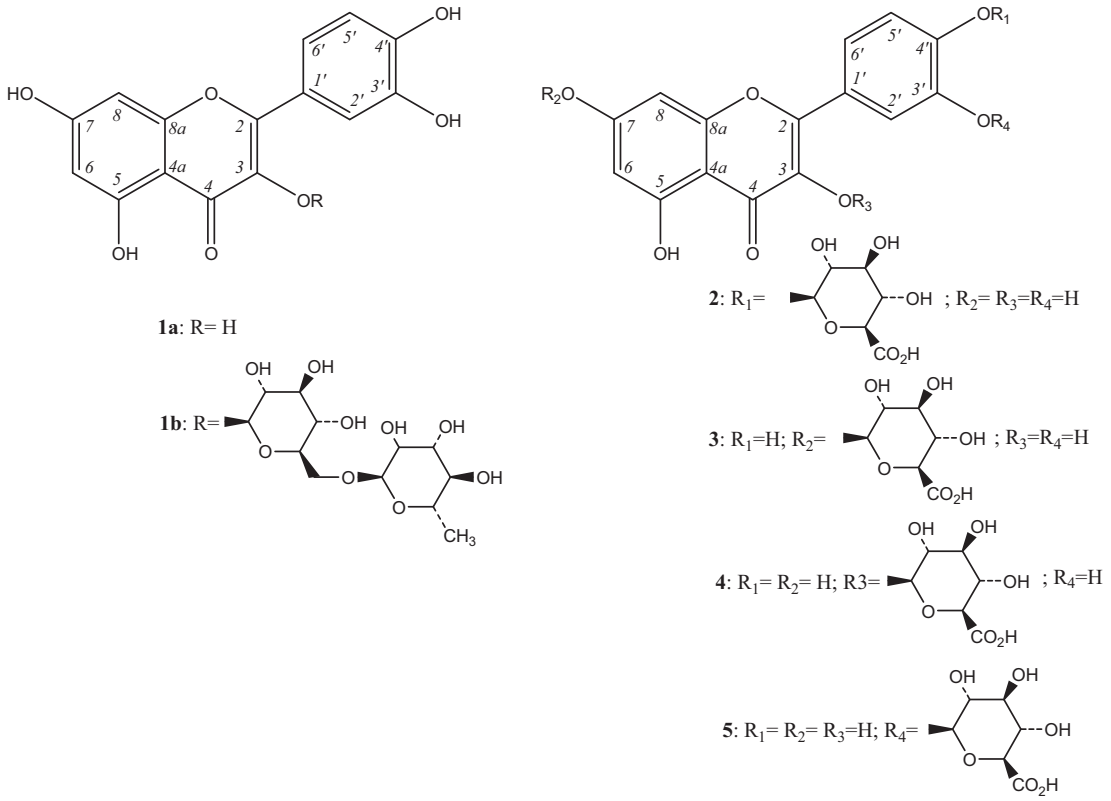
3.2. Naringenin glucuronidation

Naringenin **6a** (Scheme 2), in the form of its 7-O-rhamnoglucoside derivative, naringin, is one of the major flavanone compounds of citrus species such as grapefruit, and as such is consumed either in the fresh fruit or its juice. The C-2 chiral center of naringenin is majorily, if not exclusively *S*, by virtue of the stereospecificity of the cyclizing enzyme responsible for the formation of ring B [55,56]. How-

Table 1
Isolated yields of glucuronidated metabolites obtained by incubation of flavonoids or stilbenoids with *Streptomyces* sp. M52104.

Substrate	Substrate concentration (g/L)	Incubation time (h)	Conversion (%)	Glucuronidated metabolites	Yield (%)
Quercetin (**1a**)	0.1–0.6	65	100	**2**	50
				3	9
				4	19
				5	5
Rutin (**1b**)	0.1	90	100	**2**	40
				3	11
				4	20
				ND*	–
(2S)-Naringenin (**6a**)	0.1	65	100	**7**	25
				8	5
(2S)-Naringenin-7-O-β-D-glucopyranoside (**6b**)	0.1	65	100	**7**	–
				8	–
trans-Resveratrol (**9a**)	0.1	48	100	**11**	90
				12	10
trans-Resveratrol-7-O-β-D-glucopyranoside (**9b**)	0.1–0.3	48–72	100	**11**	68
				12	27
trans-Deoxyrhapontin (**10**)	0.12	72	100	**13**	60
				14	10
trans-Rhapontin (**15**)	0.3	48	100	**16**	60
				17	<5
				ND*	–

* ND = undetermined minor metabolite.



Scheme 1.

ever NMR studies have shown some epimerization at C-2 during maturation of the fruits or even in purified glycosides, resulting in the formation of different ratios of diastereomeric glycoside conjugates. Naringenin and its glycosides have been extensively studied in terms of their anticarcinogenic, antiviral and anti-inflammatory activities. As for other flavonoids, after absorption and hydrolysis of the glycoside, the aglycone enters blood circulation as conjugates, mainly sulfates and glucuronides, also formed in hepatic cells, particularly as naringenin 4′ and 7-O-β-D-glucuronides. Both glucuronides have been chemically synthesized from the aglycone, involving delicate protection-deprotection steps, in 12–30% overall yields and only in diastereoisomeric forms [34]. Naringenin 4′ and 7-O-β-D-glucuronides have been also prepared in very low amounts from urine after consumption of grapefruit juice or pure naringin [57].

When (2*S*)-Naringenin-7-O-glucoside (**6b**, 0.1 g/L) or its aglycone, (2*S*)-naringenin (**6a**, 0.1 g/L) were incubated with grown cultures of strain M52104 during 65 h at 28 °C, both substrates disappeared completely from the culture medium, and two products (**7**) and (**8**), identified as glucuronidated compounds by HPLC/MS

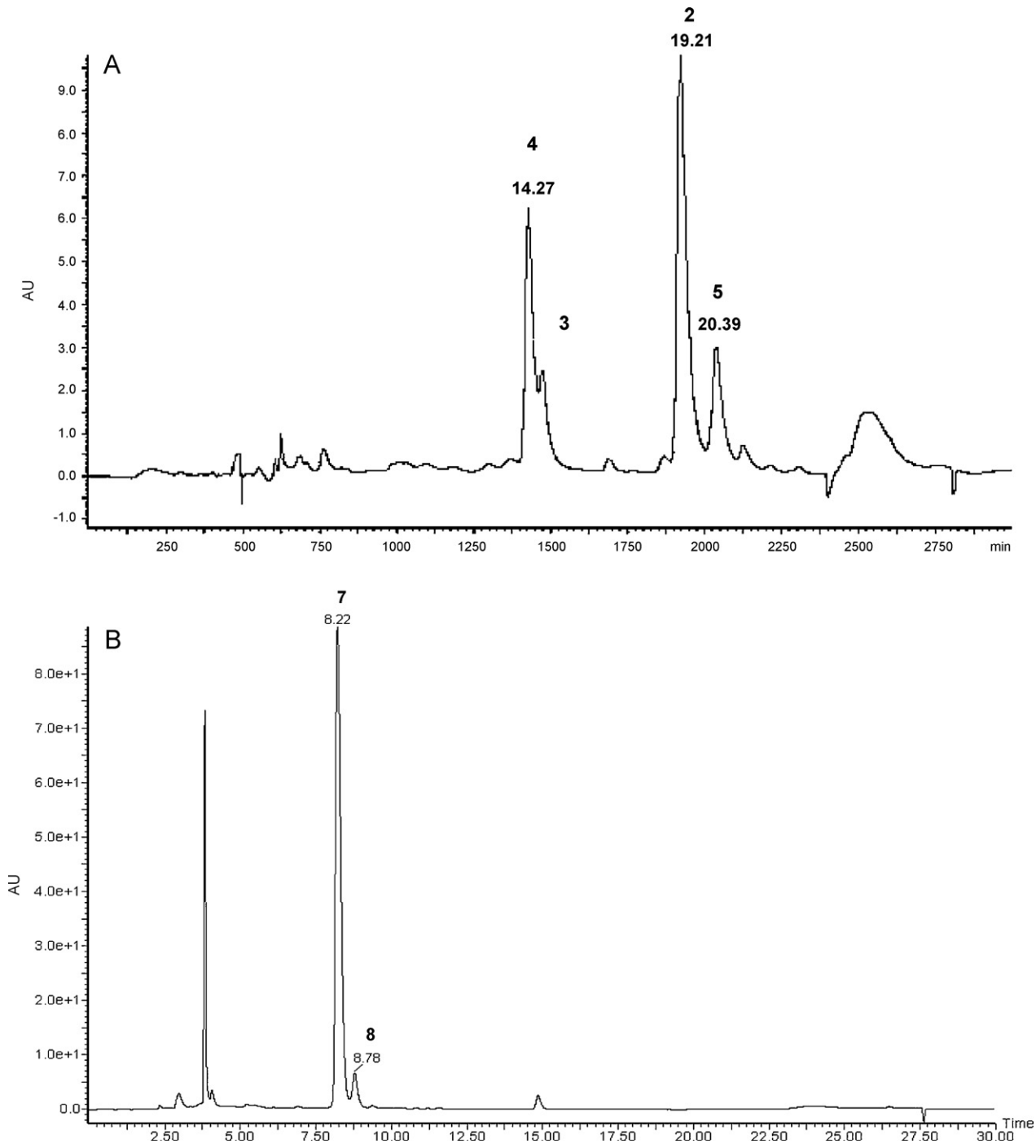
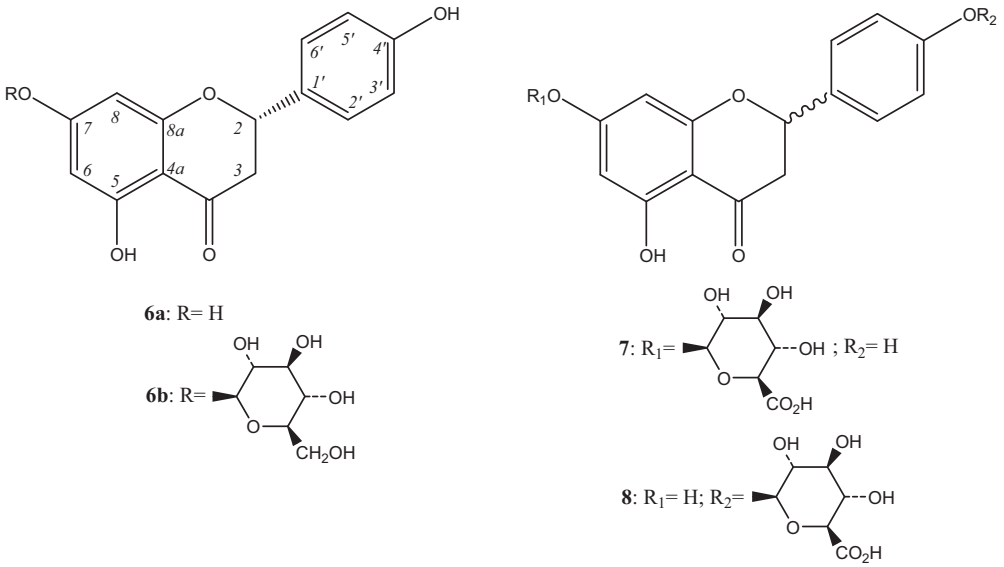


Fig. 1. HPLC/UV chromatographic profiles (assays as described under Section 2.2) of the conversion of flavonoids by strain M52104. (A) Conversion of quercetin (**1a**) (0.1 g/L, 65 h). Eluted peaks **2–5** all correspond to glucuronidated metabolites with *m*/*z* 477. (B) Conversion of naringenin (**6a**) (0.1 g/L, 65 h). Eluted peaks **7** and **8** correspond to metabolites with *m*/*z* 447.

(*m*/*z* 447 and *m*/*z* 271, corresponding to the loss of 176 mass units) were detected in the centrifuged supernatant (Fig. 1B). After adsorption on XAD-16 resin, elution with ethanol then semipreparative HPLC on a C18 column, two main glucuronidated conjugates were isolated as pure compounds and identified by comparison with published data [34,58], mass spectrometry and detailed ^{1}H and ^{13}C NMR studies (see Supplementary material Figs. S2, S8, S9 and Table S3), using 2D-COSY, TOCSY, NOESY, HSQC and HMBC sequences.

The major naringenin-7-O-β-D-glucuronide (**7**), obtained in 25% yield and >98% purity, showed in ^{1}H NMR a rather complex spectrum due to the presence of two sets of proton signals corresponding to the presence of two epimers in about 1:1 ratio: the aglycone signals showed overlapping split doublets for H-6 and H-8 (Table S3), associated with 1″-anomeric hydrogen signals at 5.05 and 5.04 ppm (two doublets with J = 7.7 Hz), H-5″ at 4.07 and 4.06 ppm (two overlapping doublets, J = 9.7 Hz) and 2″–4″ hydrogens as overlapping signals at 3.48–3.60 ppm. Most characteristic of the presence of (2*S*) and (2*R*) epimeric compounds were the overlapping duplicated signals of H-2 (2 dd) at 5.36 and 5.35 ppm and H-3a /H-3b (2 dd), at 3.16, 3.13/2.74, 2.71 ppm, with characteristic coupling constants of H-2 with H-3a, H3b for axial and equatorial



Scheme 2.

hydrogens ($J_{2,3}$ = about 7 Hz and 2.8 Hz, respectively). The ^{13}C NMR spectrum exhibited similarly splitted signals for most carbons of the A and B rings of the aglycone (Table S3). Identification of the glucuronidation position was deduced from HMBC experiments with a ^{3}J cross-correlation of the anomeric hydrogens with each respective C-7 (at 167.0 and 164.7 ppm), itself identified by their cross correlations with respective H-6 and H-8.

In the literature, the presence of two sets of ^{1}H NMR signals in flavanone glycosides has been often observed and was successively attributed to conformers of the optically pure glycoside derivative [59,60], or more frequently to the presence of diastereomeric conjugates resulting from the racemization of the aglycone part of the glycosides by ring-opening under basic conditions [55,56,61]. This reaction would lead to the formation of the corresponding unstable chalcones, which rapidly recyclize to flavanones in a non-stereospecific manner [62], affording epimeric compounds. Nonenzymatic chalcone formation is known to occur very readily for flavanones such as naringin which contains a blocked 7-hydroxyl group and a free 4′-hydroxyl group [63]. Such a stereomerization has also been already observed in natural fruits or fruit extracts on ageing [55,56], or by solubilisation of pure glycosides in various solvents, particularly in DMSO [61].

The minor naringenin-4′-O-β-D-glucuronide (**8**) was obtained in about 5% yield and 95% purity, and showed in ^{1}H NMR a simpler spectrum (see Supplementary material Fig. S9 and Table S3) due to the presence of only one set of proton signals corresponding apparently to the presence of a single epimer at C-2 position: H-6 and H-8 were seen as singlets at 5.91 and 5.89 ppm, as the B-ring signals, but integrated only for less than one hydrogen each, as a result of hydrogen to deuterium exchange with the NMR solvent and as previously described in the literature for several dihydroxylated A-ring flavonoids [56]. H-2 was at 5.42 ppm, coupled with both H-3 (2 dd, $J_{3ax\text{-}3eq}$ = 17.0 Hz, $J_{3ax\text{-}2}$ = 12.6 Hz, $J_{3eq\text{-}2}$ = 2.8 Hz). The glucuronide moiety was characterized by the 1″ anomeric hydrogen resonance at 5.04 ppm (J = 7.3 Hz), H-5″ as a doublet (J = 9.8 Hz) at 3.98 ppm, H-2″(m) at 3.62 ppm and H-3″ and H-4″ as overlapping signals at 3.51 ppm. All data from 2D ^{1}H and ^{13}C NMR confirmed the proposed structure and HMBC clearly indicated the position of glucuronidation by a ^{3}J cross-correlation of the 1″-anomeric hydrogen with C-4′ (at 158.9 ppm), itself correlated with H-2′ and H-6′. However a splitting of some signals could be observed in ^{13}C 1D NMR (Table S3), attesting the presence of diastereomeric forms of this 4′-O-glucuronide derivative. The apparent absence of a clear splitting of ^{1}H signals (as precedently described for the 7-O-glucuronide) is possibly due to its relatively symmetrical structure.

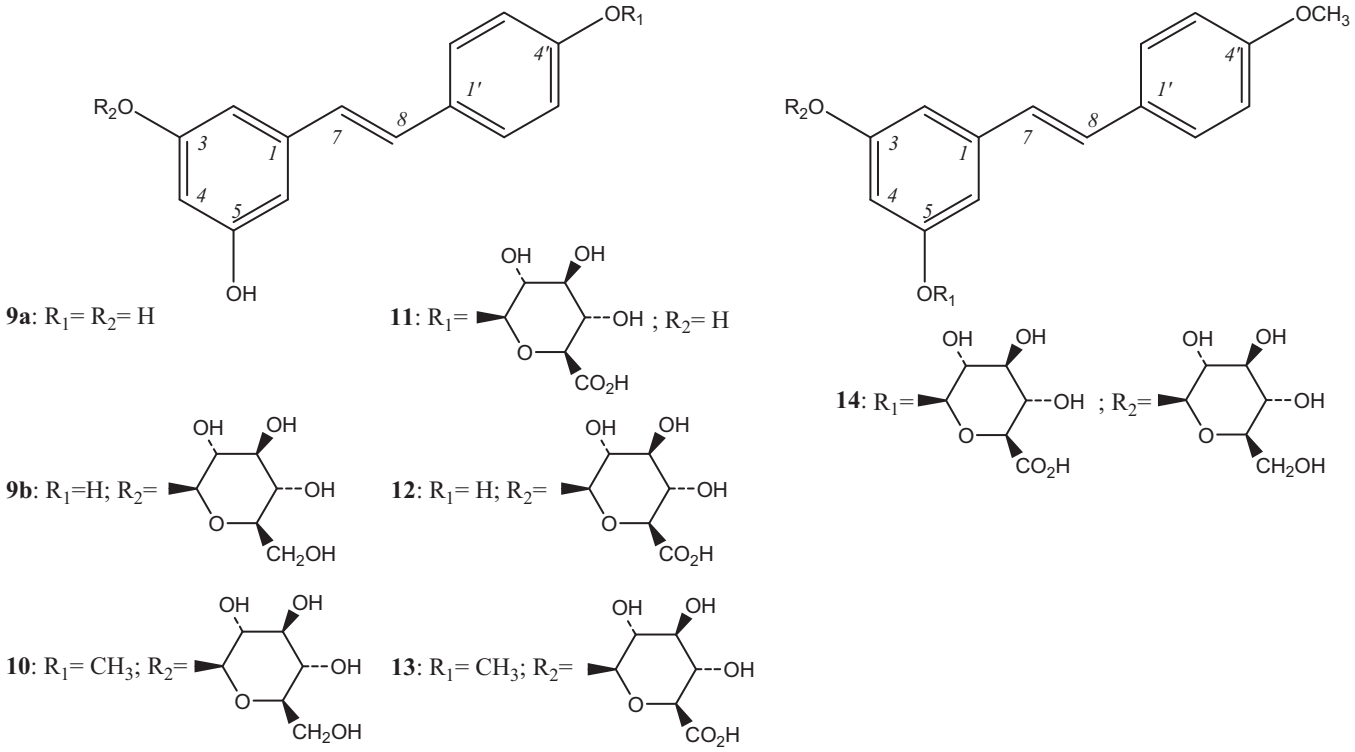
Unexpectedly, the regioselectivity of the glucuronidations observed with naringenin is opposite to that observed with quercetin, the 7-O-glucuronidation being largely favoured over the 4′-O-glucuronidation.

3.3. Resveratrol and related stilbenoids glucuronidation

Resveratrol **9a** (Scheme 3) is a constituent of red wine polyphenols and has been also isolated from different plant sources, frequently as piceid (or polydatin) **9b**, its chemically stable [10] and water-soluble 3-O-β-D-glucopyranoside derivative. Considered as the responsible agent for the “french paradox”, *trans*-resveratrol exhibits numerous beneficial activities [64]: as general antioxidant [65], modulator of lipoproteins metabolism [66], inhibitor of platelets aggregation [67], cardioprotective agent [67,68] anticancer agent [7,69], antifongic [70], phytoestrogen [71], antiHIV [72], etc. Its metabolism in human involves hydroxylation to piceatannol (3,5,3′,4′-tetrahydroxy *trans*-stilbene) [73], conjugation to 3- or 4′-O-monosulfate and 3- and 4′-O-β-D-glucuronopyranosides [13]. As a consequence of its poor bioavailability [12] *trans*-resveratrol is absorbed in the small intestine as glucuronidated conjugates, and circulates in blood mainly as glucuronides [12,16,23,24,74]. It is also actively glucuronidated in liver by several UGT isoforms (UGTA1, UGTA9 and UGTA10) with a preference for 3-glucuronidation [22,74,75].

The isomeric *cis*-resveratrol, present in minor amounts in natural products may be formed from *trans*-resveratrol, and is also active as antioxidant. The specific activities of *cis*-resveratrol have not been specifically documented. As *trans*-resveratrol, the *cis*-isomer is metabolized by liver or intestinal microsomes [22] as well as recombinant UGTs [76] to glucuronide conjugates.

Separate chemical syntheses of 3- and 4′-O-β-D-glucuronopyranosides (**12**, **11**) of *trans*-resveratrol have been described by Learmonth [28] in 3 or 4 steps and overall yields of 27% and 30%, respectively. Higher yields were obtained in another separate synthesis using silyl-protected derivatives [75]. An alternative one-pot synthesis involving a semi-preparative HPLC separation of the regioisomers [75] afforded 18% and 13% yields, respectively. In a modified method, the



Scheme 3.

3-O-β-D-glucuronopyranoside of *trans*-resveratrol (**12**) was obtained in an overall yield of 10% [31]. Analytical samples were generally obtained from blood or urine of animals treated with resveratrol or piceid, or by transglycosylation of resveratrol catalyzed by specific UGT isoforms [22].

When *trans*-resveratrol (**9a**, 0.1 g/L) solubilized in DMSO was incubated at 28 °C with a culture of M52104, grown for 72 h as described in the experimental part, resveratrol completely disappeared after 48 h (Fig. 2A), corresponding to the formation of two major glucuronidated products in a 9:1 ratio, as estimated by HPLC/UV and MS.

After the usual treatment (centrifugation, filtration of the supernatant and adsorption–elution from XAD-16), the crude product was submitted to semi-preparative HPLC to give >95% pure *trans*-resveratrol 4′-O-β-D-glucuronide **11** (60%) and *trans*-resveratrol 3-O-β-D-glucuronide **12** (5%), identified by LC/MS [22] and ^{1}H ^{13}C NMR [28,75]. 1D and 2D NMR measurements were carried out in $^{2}H_2O$ and DMSO-d_6, for comparison with the literature data [22,28,75]. The major glucuronide **11** presented perfectly symmetrical resonances for protons and carbons at C-2 and C-6, as well as C-2′, C-3′ and C-6′, C-5′, as expected for a 4′-O-glucuronide (see Supplementary material Fig. S11 and Table S4). The anomeric hydrogen at 5.04 ppm (J = 7.5 Hz) was characteristic of a β-glycosidic bond and presented in HMBC a clear cross-correlation signal with C-4′ and reciprocally. In addition NOESY experiments showed the expected correlation peaks between H-1″ and H-3′, H-5′ at 7.01 ppm.

The minor glucuronide presented an anomeric hydrogen signal at 4.97 ppm as a doublet (J = 7.7 Hz) characteristic of a β-glycoside conjugate and all 1D and 2D NMR data were consistent with an unsymmetrical 3-O-β-D-glucuronide derivative **12** with a slight difference in the H-2 (6.66 ppm) and H-6 (6.58 ppm) resonances (see Supplementary material, Table S4). In addition, HMBC experiments showed a clear cross-peak between H-3 and the anomeric 1″-carbon and reciprocally (H-1″/C-3), and ROESY experiments clearly showed cross-correlation peaks between H-1″ and H-2, H-4.

The concentration of added resveratrol in the incubation with M52104 cultures had to be strictly limited to 0.1 g/L, as the glucuronidation was completely inhibited at higher concentrations (Table 2).

Similar results were obtained when piceid, the *trans*-resveratrol 3-*O*-β-D-glucoside (**9b**, 0.2 g/L) was used as substrate in the same conditions, affording the same glucuronide products in similar yields. Again, an increase of the piceid concentration above 0.3 g/l resulted in a complete inhibition of the glucuronidation reaction, due to the fast formation by hydrolysis of the corresponding amount of *trans*-resveratrol (Table 2).

In all experiments, *cis*-resveratrol was not formed in significant amounts, excepted when incubations were prolonged after the disappearance of the substrate. In such a case, even the *cis*-resveratrol formed appeared to be transformed into two glucuronides (data not shown), probably similar to the corresponding 3′ and 4-glucuronidated metabolites formed by human liver and intestinal microsomes, as well as recombinant human UGTs [22,76]

When deoxyrhapontin (**10**, 0.12 g/l), the 3-O-β-D-glucoside of deoxyrhapontigenin, solubilized in DMSO, was incubated at 28 °C with a culture of M52104, grown for 72 h as described in the experimental part, the substrate completely disappeared after 72 h (Fig. 2B), with the formation of two products in a 85:15 ratio, separated by HPLC/MS with M/z at 417 and 579, and corresponding to monoglucuronides of deoxyrhapontigenin and deoxyrhapontin, respectively After the usual treatment (centrifugation, filtration of the supernatant and adsorption–elution from XAD-16), the crude product was submitted to semi-preparative HPLC to give pure *trans*-deoxyrhapontigenin-3-O-β-D-glucuronide **13** (60%) and *trans*-deoxyrhapontin-5-O-β-D-glucuronide **14** (10%), identified by LC/MS [22] and ^{1}H, ^{13}C 1D and 2D NMR measurements (see

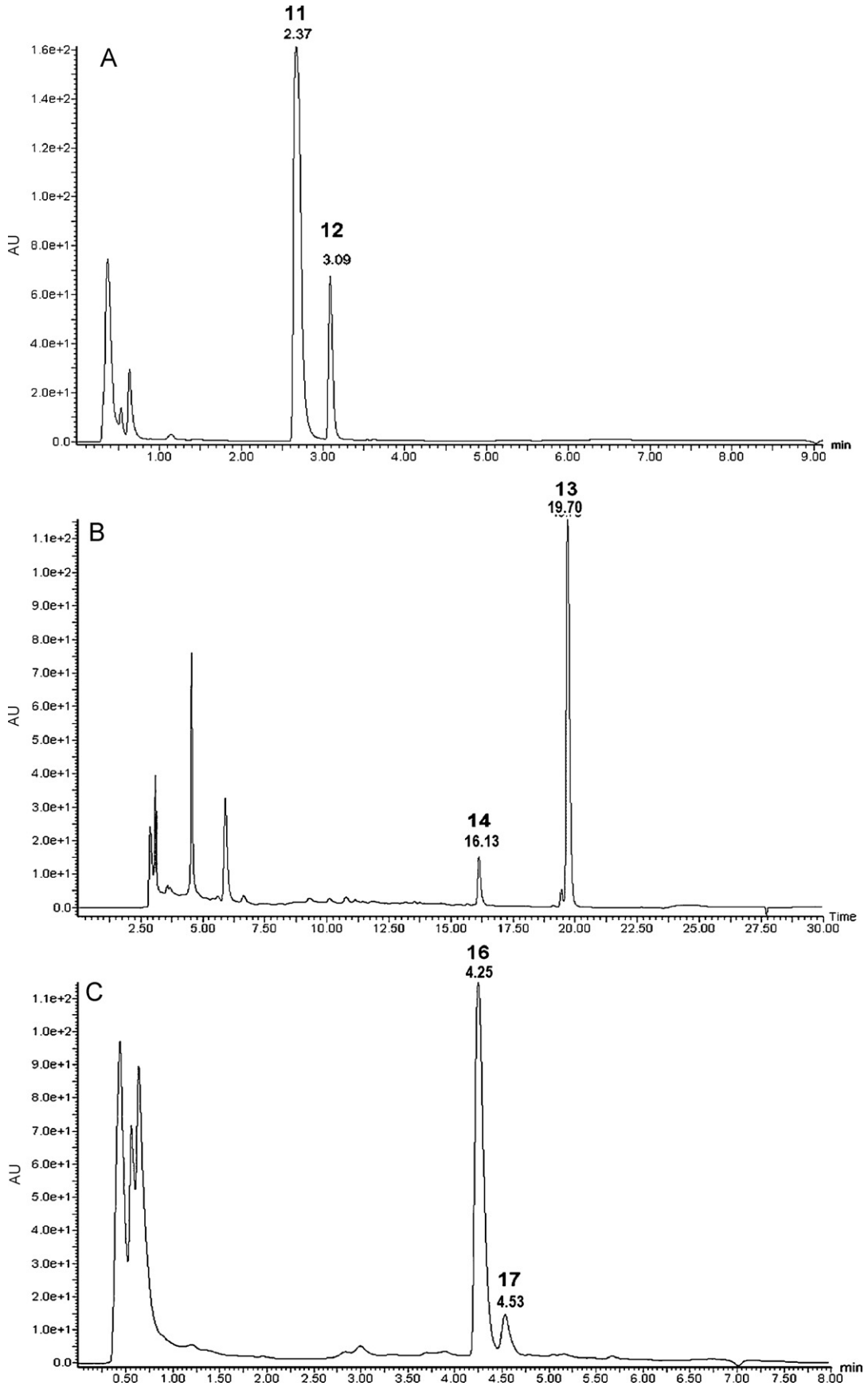
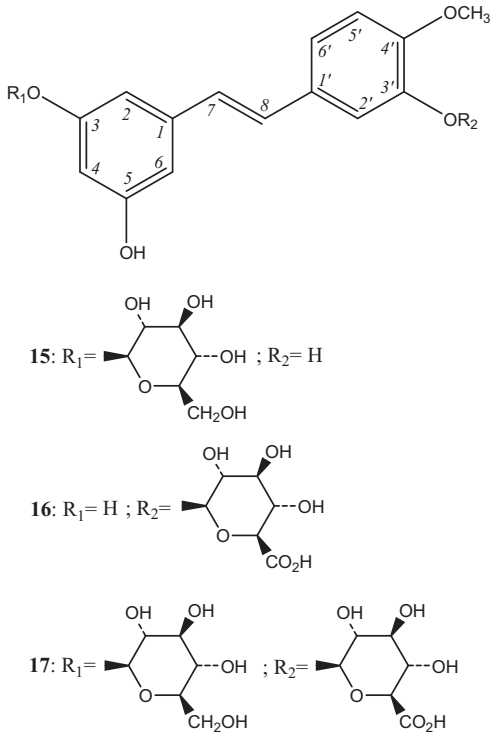


Fig. 2. HPLC/UV chromatographic profiles (assays as described under Section 2.2) of the conversion of stilbenoids by strain M52104. (A) Conversion of resveratrol (**9a**) (0.1 g/L, 29 h). (B) Conversion of deoxyrhapontin (**10**) (0.12 g/L, 24 h). (C) Conversion of rhapontin (**15**) (0.2 g/L, 48 h).

Table 2
Products of *trans*-resveratrol or piceid transformation by strain M52104. Incubations were carried out at 27 °C during 48 h after addition of various concentrations of substrates in the culture medium. Percent of products was determined from areas of peaks in the HPLC/UV profile of the crude supernatant.

Substrate	g/L	4′-O-glucuronide (**11**) (%)	3-O-glucuronide (**12**) (%)	*trans*-Resveratrol (%)	*cis*-Resveratrol[a] (%)
trans-Resveratrol (**9a**)	0.1	90	10	0	0
	0.2	<1	<1	98	–
Piceid (**9b**)	0.1	68	27	0	<1
	0.2	68	28	0	<1
	0.3	66	29	0	<1
	0.4	12	4	82	~2
	0.5	10	2	86	~2
	0.6	12	2	83	~3

[a] Including the corresponding 3- and 4′-O-glucuronides.



Scheme 4.

Supplementary material, Figs. S4, S12 and Table S5) [28,75] (see Scheme 4).

When rhapontin (scheme 4), the 3-O-β-D-glucoside of rhapontigenin (**15**, 0.3 g/l), solubilized in DMSO, was incubated at 28 °C with a culture of M52104, grown for 72 h as described in the experimental part, the substrate completely disappeared after 48 h (Fig. 2C), with the formation of two products characterized in HPLC–MS by a molecular peak at m/z = 433: a major glucuronidated product (85%), and another minor glucuronidated product (about 10%). In addition a small amount of a product with m/z = 595 was detected, which was not further identified by NMR, but probably corresponds to a monoglucuronide of rhapontin (**17**, <5%)

After centrifugation, filtration of the supernatant and adsorption-elution from XAD-16, the crude product was submitted to semi-preparative HPLC to yield pure *trans*-rhapontigenin-3′-O-β-D-glucuronide **16** (60%), identified by MS and ^{1}H, ^{13}C 1D and 2D NMR measurements [22] (see Supplementary material Figs. S5, S13 and Table S5).

4. Conclusion

In the present study, we have described a simple and productive microbial method for the easy preparation in a 10–200 mg scale of some typical flavonoid or stilbenoid glucuronides, starting either from the aglycones or from the corresponding glucosides or rhamnoglucosides, their usual derivatives found and extracted from plants. This indicates in the microorganism used, beside a high glycosidase activity, the absence of active glucuronidase(s), allowing the final formation in quantitative yields of O-glucuronide derivatives. The polyphenol glucuronides obtained constitute bioavailable, stabilized and soluble forms of powerful antioxidant molecules. The regioselectivity of the glucuronide transfer, probably using a UDPGA internal pool, is essentially directed, as previously shown [44] to phenolic hydroxylic groups, with yield variations depending on the reactivity or exposition of these groups, and necessitating in most cases chromatographic separations. On the other hand, the glucuronic acid transfer stereoselectively affords β-glucuronides.

However the major glucuronidated metabolites found do not always correspond to the ones majorily formed in animal systems and which constitute the circulating forms of the aglycones. The easy access to such diverse glucuronides will certainly help for the *in vivo* and *in vitro* elucidation of polyphenol pharmacology and metabolism. Moreover, polyphenol glucuronides may act not only as systemic transporters of non specific antioxidant activities, scavenging free radicals in tissues and cells after deglucuronidation, but also as potential therapeutic agents when transporting polyphenols able to scavenge free radicals into β-glucuronidase-enriched cells, as for example some cancer tumors, or as glucuronidase-targetted conjugated monoclonal antibodies. The extension of the glucuronidation reactions described in this paper to other active polyphenols is currently under study in our laboratory.

Conflict of interest

The authors report no conflict of interest. All of the studies reported herein were supported by Bertin-Pharma. One of the authors (C. Marvalin) was employee of Bertin-Pharma. Part of this work was presented as a poster at the 2009 AAPS Annual Meeting and Exposition, November 2009, Los Angeles, http://abstracts.aapspharmaceutica.com/ExpoAAPS09/CC/forms/attendee/index.aspx?content=sessionInfo&sessionId=2336.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2011.07.015.

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