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Biocatalyzed Synthesis and Structural Characterization of Monoglucuronides of Hydroxytyrosol, Tyrosol, Homovanillic Alcohol, and 3-(4′-Hydroxyphenyl)propanol

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Abstract: The biocatalytic synthesis and purification of *O*-β-D-monoglucuronide conjugates of hydroxytyrosol, tyrosol, homovanillic alcohol, and 3-(4′-hydroxyphenyl)propanol, using porcine liver microsomes, are described here. The glucuronides were synthesized, analyzed and separated by HPLC-UV, identified by HPLC-MS, and their structures unequivocally established by NMR techniques. The outcome of the glucuronidation reaction depends on the structure of the phenolic compounds. Thus, the glucuronidation of hydroxytyrosol, biocatalyzed with porcine liver microsomes, proceeded exclusively on the phenolic hydroxy groups. The regioselectivity was similar to that observed for human and rat liver microsomes, the 4′-hydroxy position being more favorable than the 3′-hydroxy one. In the case of tyrosol, homovanillic alcohol, and hydroxyphenylpropanol, two products were formed during microsomal glucuronidation: a major one, the phenolic *O*-β-D-glucuronidated derivative and, a minor one, the *O*-β-D-glucuronidated aliphatic alcohol.

Keywords: biocatalytic synthesis; glucuronidation; liquid chromatography; metabolism; NMR spectroscopy; olive oil antioxidants

Introduction

The discovery of antioxidant activities, *in vitro*, *ex vivo*, and in animal models, of the most important olive oil phenols, hydroxytyrosol (HOTYR) and tyrosol (TYR), has stimulated an intense research on their bioavailability, metabolism, and disposition in humans. The foundation for these studies is that the debate about the potential benefits for human health of olive oil phenolic compounds requires the support of clinical studies on their bioavailability.[1,2] Initial investigations performed both in humans[3,4] and animals[5] have shown that olive oil phenolic compounds are absorbed from the intestinal tract. They are also subjected to an extensive first pass metabolism, both in the intestinal epithelium and in the liver, leading to the formation of phase II metabolites, mainly methyl, sulfate, and glucuronide conjugates. HOTYR and TYR are nearly undetectable in their free form both in plasma and urine. Therefore, the potential health benefits of olive oil phenolic compounds should be attributed to phase II metabolites or to their enzymatic hydrolysis at the cellular level, freeing the phenol moiety. Preliminary results for some metabolites isolated from rat urine seem to support these views, since glucuronides of HOTYR are more active scavengers than HOTYR, while partially purified sulfated conjugates do not exhibit scavenging behavior.[5] Thus, the investigation of the biological activity of phase II metabolites of olive oil phenolic compounds, presumably inactive in the context of the general rules applied for the metabolism of most xenobiotics, becomes particularly relevant in the present case.

Following reports on HOTYR metabolic disposition in humans,[3,6] two of the main competing pathways consist of *O*-glucuronide conjugation by uridine 5′-diphosphoglucuronyl transferases (UDPGTs), and *O*-methylation by catecholmethyltransferase (COMT) leading to the formation of homovanillic alcohol (HVAlc).[4,7] This last compound is further *O*-glucuronoconjugated at the free phenolic position after *O*-methylation. TYR, as a monohydroxylated phenylethanol, cannot be methylated by COMT and undergoes direct *O*-glucuronidation. Therefore, the synthesis of glucuronoconjugates of HVAlc, TYR, and especially of HOTYR (i.e., the phenolic compound with the highest antioxidant activity) is of paramount importance for their identification, quantification, and determination of their biological and antioxidant properties in biological samples. Moreover, the properties of these conjugates could be compared with those of their metabolic precursors, which are well established, characterized and used so far in research.[8]

A variety of methods for the preparation of glucuronide conjugates of phenolic compounds has been reported. The chemical approach could be considered the cheapest one, but its main drawback is that most chemical procedures yield mixtures of α- and β-anomers along with side-products.[9] In addition, the distinct reactivity of the OH groups, when more than one hydroxy function is present in the phenolic compound, may lead to selective glucuronidation that has to be adjusted by the reagent and/or reaction conditions. Biocatalysis-assisted synthesis using tissue homogenates,[10] subcellular fractions[11,12] or recombinant UDPGT enzymes[13] as a source of UDPGT activity is an alternative strategy for the straightforward preparation of regioisomeric pure *O*-glucuronides. One important advantage of this approach is the exclusive formation of the β-D-glucuronoconjugates due to the stereo- and regioselectivity of enzymes. Recently the formation of both phenolic β-D-regioisomers in the glucuronidation reaction of HOTYR, which have also been observed in our preliminary experiments, has been reported.[14]

Several studies on the substrate specificities of different isoforms of UDPGTs in humans report that the aglycone specificities are determined by the N-terminal structure isoforms[15,16] and large overlaps among the acceptor specificity is well documented.[17–19] Therefore, each UDPGT may glucuronidate more than one substrate and it could have tissue-specific expression and also activity. The combination of substrate specificity overlapping and substrate promiscuity makes it difficult to assess the active UDPGT isoform on a specific substrate.

Other alternatives could be the use of immobilized enzymes[10,20] or recombinant UDPGTs[13] but both of these methods are very laborious and/or expensive approaches. Moreover, it has been reported that the degree of conversion for the immobilized enzymes was lower than that in the reaction with the enzyme in solution and also the raw material cost would rise due to both a high uridine 5′-diphosphoglucuronic acid (UDPGA) consumption[10] and additional preparation of immobilized enzymes.[20] UDPGT activities are totally dependent on the phospholipid content of the membrane and, as a result, are usually inactive when isolated from the endoplasmic reticulum. This makes their isolation, purification and eventually the overexpression in its active form very difficult.[21]

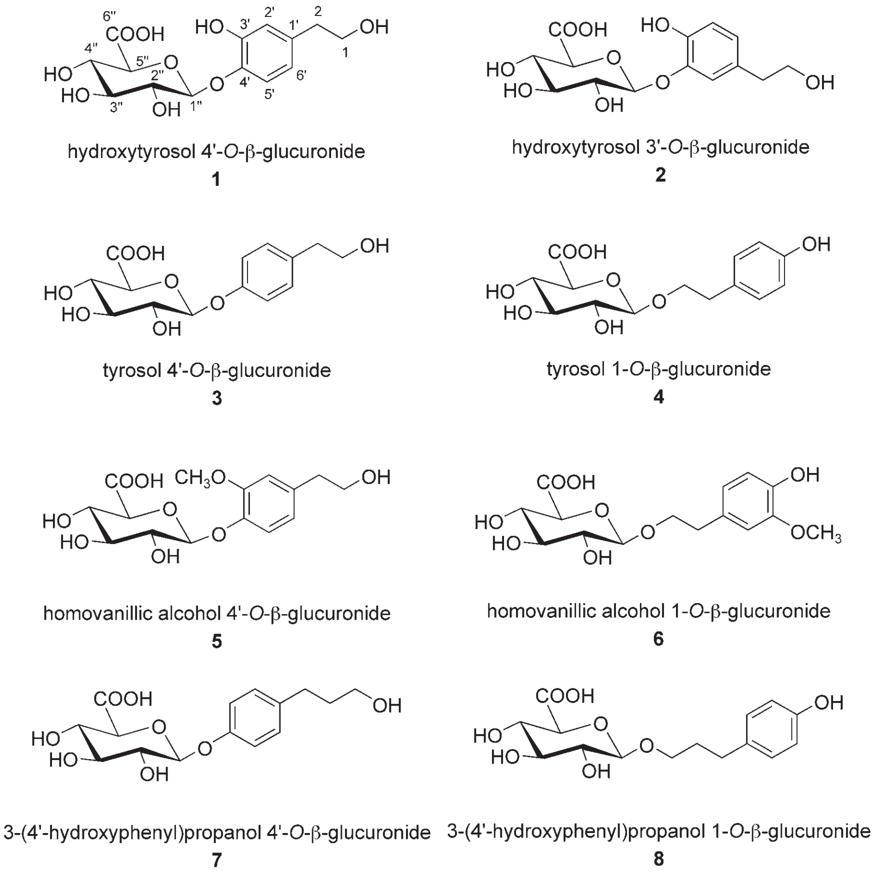
We describe here, for the first time, the preparative synthesis and characterization of *O*-β-D-glucuronides of hydroxytyrosol, tyrosol, homovanillic alcohol and 3-(4′-hydroxyphenyl)propanol (HOPhPr) (see Figure 1) using porcine liver microsomes (PLM) as a catalyst. In addition, we report on conditions for the HPLC separation, purification, monitoring, and quantification of the glucuronoconjugates. Thus, the complex UDPGTs pool present in the liver microsomal fraction is a favorable combination of the different natural isoforms with broad activities that could be considered a cheap and useful source of UDPGT for synthetic purposes.

Results and Discussion

Glucuronidation Reactions using Liver Microsomes

To establish the best reaction conditions, HOTYR was chosen as a model and its glucuronidation with PLM was monitored by HPLC-UV (see Experimental Section for a description of the HPLC method). The reaction was carried out by adding the substrate dissolved in DMSO to a pre-activated mixture containing liver microsomes and uridine 5′-diphosphoglucuronic acid (UDPGA) and the incubation mixture was stirred at 35 °C for 10 h. After 2 h, the chromatogram showed several new peaks. Two of them [Figure 2 a) Pr1 and Pr2] were identified as monoglucuronoconjugates of hydroxytyrosol by HPLC-MS, and their structures were unequivocally assigned by NMR as the two regioisomers: 4′-*O*-β-D-glucuronide (**1**) and 3′-*O*-β-D-glucuronide (**2**), respectively (see below). The third one corresponds to the UDP from the activated glucuronic acid. None of these main peaks were observed when the reaction was carried out in the absence of either UDPGA or microsomal fraction (data not shown).[22]

Liver microsomes from humans (HLM) and rats (RLM) were also used under the same reaction conditions to compare their *in vitro* capacity for HOTYR conjugation with that of PLM [plots of hydroxytyrosol glucuronides formation *versus* substrate concentration for porcine (PLM), human (HLM) and rat (RLM) liver microsomes are shown in the Supporting Infor-



hydroxytyrosol 4'-*O*-β-glucuronide
1

hydroxytyrosol 3'-*O*-β-glucuronide
2

tyrosol 4'-*O*-β-glucuronide
3

tyrosol 1-*O*-β-glucuronide
4

homovanillic alcohol 4'-*O*-β-glucuronide
5

homovanillic alcohol 1-*O*-β-glucuronide
6

3-(4'-hydroxyphenyl)propanol 4'-*O*-β-glucuronide
7

3-(4'-hydroxyphenyl)propanol 1-*O*-β-glucuronide
8

Figure 1. Structures of the olive oil phenol monoglucuronides synthesized.

mation; see Figure S1]. All three types of microsomes afforded approximately the same degree of glucuronidation conversion and were able to introduce the glucuronide residue into both catechol hydroxy groups. In all cases, the reactions were regioselective towards one of the hydroxy groups of HOTYR resulting in the formation of Pr1 as the major product. Hence, the 4′-hydroxy was more favorable to enzymatic glucuroconjugation than the 3′-hydroxy of HOTYR. These findings are in agreement with the previous reported glucuronidation regioselectivity of nitrocatechols.[11] Slight differences were observed regarding the regioselectivities between glucuronides among the species tested. Thus, the regioisomers ratio, expressed as a proportion of Pr1 to Pr2 peak areas, was around 1.5, 1.2, and 3.0 for HLM, RLM, and PLM respectively (see Figure S1 in the Supporting Information).

Consequently, PLM appears to be an appropriate source of highly active UDPGTs for use in *O*-β-D-glucuronide preparative biosynthesis. In addition, PLM is inexpensive, easily available and a suitable model for *in vitro* studies. The regioselectivity of PLM towards HOTYR was of the same order as that observed for HLM and RLM, which are widely used in drug metabolism research. On the basis of data obtained for HOTYR it was assumed that PLM should display similar glucuronidation conversions towards the other substrates: TYR, HVAlc, and HOPhPR.

Figure 2 shows the chromatographic profiles of small-scale test reactions for TYR, HVAlc, and HOPhPr after 2 h of incubation. The newly formed peaks were identified as UDP and the two monoglucuronoconjugated products, Pr1 and Pr2, whose molecular weights were confirmed by mass spectrometric analysis. Furthermore, an NMR structural analysis (see below) showed that the major product was the 4′-*O*-β-D-glucuronide (**3**, **5**, and **7**) and the minor one was the 1-*O*-β-D-glucuronide (**4**, **6**, and **8**) with a glucuronidation ratio around 9:1 (see Figure 1 and Table 1).

After product identification and separation, the synthesis of the selected glucuronides on a preparative scale was performed. The experiments carried out on an analytical scale revealed that the substrates, especially HOTYR, underwent some degradation during the reaction. Thus, the preparative method was adjusted to 8 h reaction time on the basis of stability tests and reaction conversion to products (see Figures S2 and S3 in the Supporting Information). Moreover, to minimize side reactions, probably due to oxidation in the presence of oxygen mediated by the basic pH of the reaction mixture, the enzymatic glu-

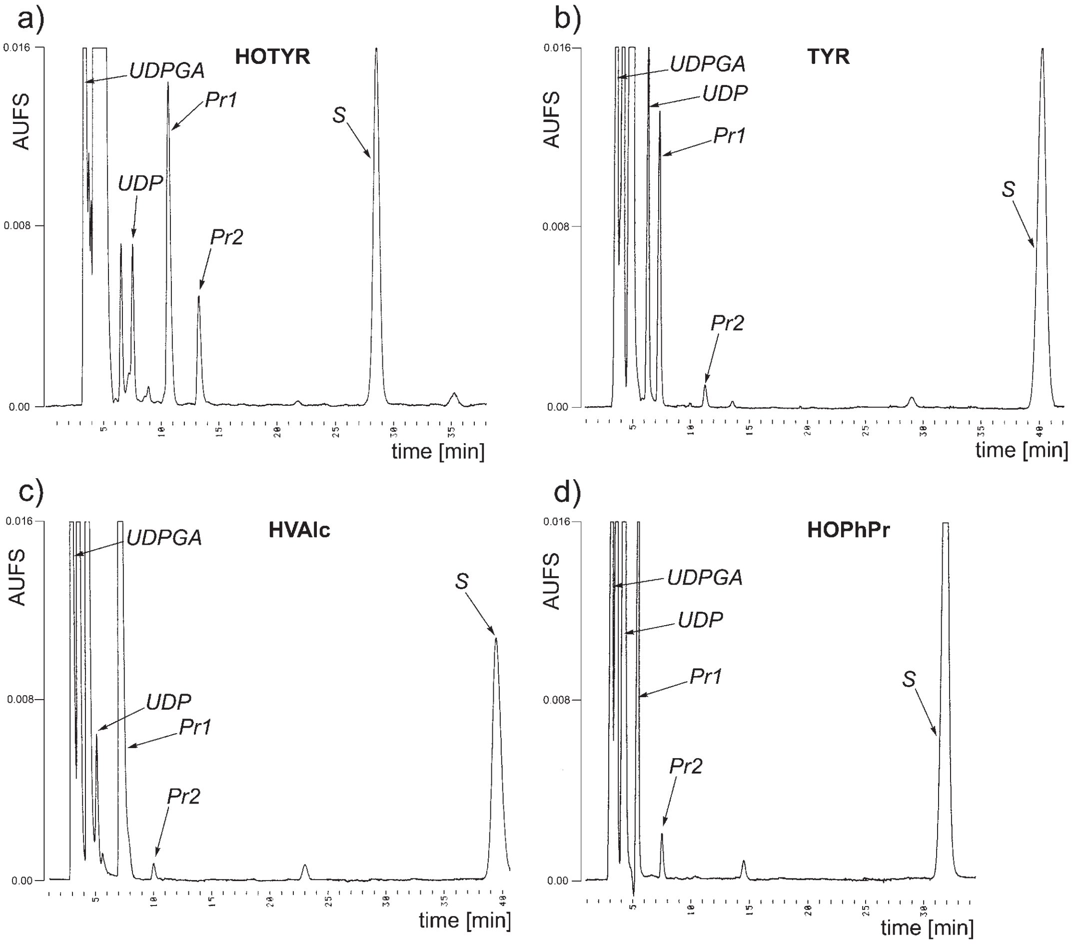


Figure 2. HPLC-UV chromatographic analysis after 2 h of incubation using porcine liver microsomes (PLM) with 20 mM UDPGA and 10 mM substrates. **a)** HOTYR, 5% acetonitrile in 5 mM ammonium acetate pH 5.0; **b)** TYR, 7% acetonitrile in 5 mM ammonium acetate pH 5.0; **c)** HVAlc, 8% acetonitrile in 5 mM ammonium acetate pH 5.0; **d)** HOPhPr, 10% acetonitrile in 5 mM ammonium acetate pH 5. UDPGA: uridine 5′-diphosphoglucuronic acid (cofactor); UDP: uridine 5′-diphosphate; Pr1: product of glucuronidation 1; Pr2: product of glucuronidation 2.

Table 1. Syntheses of monoglucuronides of HOTYR, TYR, HVAlc and HOPhPr.

Substrate [mg]	Product	Conversion to product [%][a]	Isolated yield [mg, %]
HOTYR (15.4)		74	
	1		11.9 (36.1)
	2		5.1 (15.4)
TYR (13.8)		100	
	3		13.1 (41.7)
	4		1.7 (5.4)
HVAlc (16.8)		95	
	5		30.3 (88.0)
	6		2.1 (6.1)
HOPhPr (15.2)		100	
	7		18.4 (56.0)
	8		5.6 (5.5)

[a] After 8 h incubation for HOTYR, TYR and HOPhPr and 6 h for HVAlc.

curonidation was carried out under a nitrogen atmosphere. Furthermore, the HPLC conditions found at the analytical scale were also scaled up to purify the corresponding glucuronides.

Table 1 summarizes the results obtained after the preparative synthesis of glucuronides. The conversion to glucuronides during the preparative synthesis was lower than that in the small-scale reactions, except for HVAlc, which was completely glucuronidated in 6 h. Although the reaction mixtures were protected from oxygen, there were some losses of substrate during the syntheses (i.e., about 25% for HOTYR and 5% for HVAlc, results not shown). As already mentioned, all substrates contained at least two functional hydroxy groups and the reactions provided a mixture of two different monoglucuronides. Using HPLC-UV as a purification method we were able to isolate both products of glucuronidation for each substrate. The yields for each monoglucuronide are listed in Table 1. Again, as in the case of HOTYR, their structure identification was carried out by ESI-MS/MS and NMR spectroscopy.

Mass Spectrometry

The abundance of deprotonated molecules $[M-H]^-$ recorded in negative ESI-MS indicated the molecular weight of the synthesized glucuronides. The ion spectra of $[M-H]^-$ showed at least two diagnostic product ions $[M-H-Gluc]^-$ and $[Gluc-H]^-$ (175.0^-) for the main synthesised compounds (**1**, **2**, **3**, **5**, and **7**), clearly indicating the presence of the glucuronide moiety. The position of the glucuronic acid moiety could not be determined from these data and no MS/MS differences were observed for the regioisomers of HOTYR glucuronoconjugates (see Experimental Section).

NMR Spectroscopy

All compounds **1–8** were completely characterized by the use of 1D and 2D NMR methods (see Supporting Information). As a general trend, a chemical shift between $\delta = 4.25–4.30$ was observed for the anomeric H-1″ proton of the glucuronic acid residue for the 1-*O*-β-D-glucuronides **4**, **6**, and **8**, whereas a considerable downfield shift was observed in the 3′- or 4′-*O*-β-D-glucuronides **1**, **2**, **3**, **5**, and **7** ($\delta =$ *ca.* 4.8–4.9) due to the anisotropic effect of the aromatic ring. The β-configuration of the glucuronic acid moiety was determined in all derivatives by the large doublet splitting of about 7 Hz observed in the anomeric H-1″ proton resonance. This confirmed the axial-axial *J* interaction between the H-1″ and H-2″ protons, and the downfield chemical shift of the anomeric C-1″ carbon (about $\delta = 101–104$). Remarkably, some dynamic aspects in the glucuronic acid ring concerning intra-hydrogen bonding between OH-4″ and OH-6″ hydroxy groups could be deduced from the analysis of 1D ^1H and ^{13}C spectra. This was experimentally evidenced by the presence of broad signals for the H-4″/H-5″/H-6″ protons and C-4″/C-5″/C-6″ carbons in some ^1H and ^{13}C spectra, respectively.

The position of the glucuronic acid residue was fully confirmed by NOE enhancements observed in selective 1D NOESY spectra, and by the intense cross-peaks observed in the HMBC spectra due to inter-glycosidic three-bond proton-carbon correlations (see Supporting Information). Thus, the anomeric H-1″ proton showed clear homonuclear NOEs with their neighboring aromatic protons and HMBC cross-peaks with the quaternary aromatic carbons in **1**, **2**, **3**, **5**, and **7**. In particular, the regioisomers **1** and **2** were unequivocally differentiated from these NOESY spectra. Moreover, a definitive NOE was observed between the anomeric H-1″ proton and the aliphatic CH_2 systems in **4**, **6**, and **8**. HMBC correlation peaks were also detected between the anomeric H-1″ proton and the aliphatic CH_2 carbon and between the CH_2 protons and the anomeric glucuronic acid carbons. It is noteworthy that in 1-*O*-β-D-glucuronides **4**, **6**, and **8**, the overall flexibility of the aliphatic side-chain was shown to be partially restricted due to *O*-substitution, and two well-differentiated resonances were visible for all diastereotopic RCH_2OGluc spin systems.

Conclusions

A methodology has been developed for the biocatalyzed syntheses of glucuronides with a single-step product isolation and in high yield. This method has provided, for the first time, glucuronide metabolites of the antioxidant phenolic compounds present in olive oil in a ratio close to that observed during the *in vivo* phase II metabolism in human hepatoma cells.[14]

The described methodology and the experimental data are of great practical use in developing analytical protocols to quantify olive oil antioxidants in biological fluids. The preparative HPLC-UV method has allowed us to perform a one-step separation and isolation of different monoglucuronidated products in high purity as required for reference materials in analytical studies, or standards for *in vivo* and *in vitro* experiments.

The methodology developed can be successfully applied to other situations where the synthesis and isolation of regioisomerically glucuronoconjugated substrates is of relevance.

Experimental Section

Chemicals and Reagents

Tyrosol [2-(4′-hydroxyphenyl)ethanol] (TYR) and hydroxytyrosol [2-(3′,4′-dihydroxyphenyl)ethanol] (HOTYR) were purchased from Extrasynthesis (France). Homovanillic alcohol [2-(4′-hydroxy-3′-methoxyphenyl)ethanol] (HVAlc), 3-(4′-hydroxyphenyl)propanol (HOPhPr), and UDP-glucuronic acid (UDPGA) were from Aldrich (Steinheim, Germany). Solvents were of HPLC grade and all other reagents used in this work were of analytical grade. Human liver microsomes (HLM), $20\ mg \cdot mL^{-1}$ of microsomal protein, were purchased from CellzDirect (USA).

Preparation of Microsomes

Tissue samples were obtained by dissection of a porcine liver within the two hours following animal sacrifice. Porcine liver was treated with sucrose solution as previously described[23] and cut into small 25–30 g pieces which were immediately snap-frozen in liquid nitrogen and kept at −80 °C prior to microsomes isolation.

Porcine liver microsomes were prepared by differential centrifugation as described before[24] and stored at −80 °C. They were used in the synthesis as a source of uridine 5′-diphosphoglucuronyl transferases. Protein concentration of microsomes samples was determined by the method of Bradford[25] using bovine serum albumin as standard.

Analytical HPLC-UV and HPLC-MS

Analytical chromatographic separations and analyses of microsomal reaction mixtures were done on an Atlantis C_{18} 5 μm column (4.6×150 mm) (Waters). The HPLC-UV instrument consisted of a LaChrom Pump L-7100 (Merck, Hitachi) equipped with a manual injector coupled to a UV LC-75 Spectrophotometric Detector (Perkin–Elmer). The HPLC-MS instrument consisted of an Agilent HPLC pump (Agilent, 1100 Series) with an automatic injector, coupled to a mass spectrometer equipped with an electrospray ion source (ESI) (Bruker, Daltonics GmbH). UV and MS of eluting peaks were recorded. As mobile phase 5–10 % acetonitrile in 5 mM ammonium acetate pH 5.0 (see below) was used. The elution of compounds was detected by UV absorbance at 215 nm at a flow rate of 0.5 $mL·min^{-1}$ or by MS detection at flow rate of 0.35 $mL·min^{-1}$ in the negative ionization mode. The injection volume was 10 μL. Retention factors (k') for the glucuronides are given below.

Mass Spectrometric Characterization

The mass spectra were obtained in a Bruker Esquire 3000 instrument equipped with an electrospray ion source (ESI). Samples, at concentrations from 10 to 100 $μg·mL^{-1}$, were introduced by direct injection, and negative ion MS and MS/MS were obtained.

NMR Spectroscopy

All 1D and 2D ^{1}H NMR spectra were recorded at 310 K in a Bruker AVANCE spectrometer operating at 500.13 MHz for ^{1}H and equipped with a 5 mm TCI cryogenically cooled probe. 1D ^{13}C spectra were obtained in a Varian Mercury 400 instrument operating at 100.62 MHz for ^{13}C. The solvent was CD_3OD. ^{1}H and ^{13}C chemical shifts assignments were carried out using 2D COSY and 2D HSQC experiments when appropriate and full structural characterization was confirmed by selective 1D NOESY and 2D HMBC experiments (see Supporting Information).

Small-Scale *O*-Glucuronides Synthesis

Reaction conditions were based on those earlier described for benzyl alcohol[26] with slight modifications. Thus, an aliquot (20 μL) of a stock solution of the substrate (100 mM freshly prepared stock solutions in 20 % DMSO) was added to the Tris-HCl buffer (180 μL, 100 mM, pH 8.0) containing $CaCl_2$ (6 mM), UDPGA (20 mM), dithiothreitol (1 mM), BSA [3 % (w/v)], and UDPGT (2 mg microsomal protein). The reaction was carried out in an Eppendorf tube placed on a reciprocal shaker at 35 °C. Aliquots (25 μL) were withdrawn at 0, 30, 120, 300 and 600 min of incubation and quenched in ice-cold, acidified methanol (75 μL, 2.9 % acetic acid in 100 % methanol, pH 3.3). The proteinaceous precipitate was removed by centrifugation at 10,000 *g* for 10 min, the supernatant was placed in a new tube and the pellet was washed and re-precipitated two times using a 1:3 mixture of water/methanol. The double treatment of the proteinaceous pellet was introduced because in this way we found an additional recovery of products and of substrates up to 5 % (results not shown). The supernatants were collected together, evaporated under vacuum and dissolved in mobile phase (125 μL, 5–10 % v/v acetonitrile in 5 mM ammonium acetate pH 5). The samples were assayed by HPLC-UV (215 nm) to monitor both the consumption of substrates and formation of products. Identification of glucuronides was performed by HPLC-MS analysis.

Preparative Synthesis and Purification of *O*-Glucuronides

The analytical-scale reaction was scaled up to a volume of 10 mL and the reaction was performed in a 25-mL glass tube (previously purged with nitrogen) with 10 mM substrate per batch. The rest of the conditions were identical to those described for the small-scale reaction. After 8 h of incubation, the synthesis was stopped, the supernatants were collected, and the solvent removed under vacuum. The residue was dissolved in 25 mL of the corresponding mobile phase, filtered through 0.2 μm filter (MilliQ), and applied to a column (Atlantis C_{18}, 5 μm, 19×150 mm). The column was eluted at a rate of 10 $mL·min^{-1}$ in isocratic conditions based on 4–8 % of acetonitrile in 5 mM ammonium acetate pH 5.0 mobile phase (see below). Fractions were collected and pooled as indicated for the analytical HPLC-UV detection, and the mobile phase was removed by lyophilization. The obtained residues were weighed, reconstituted in either CD_3OD or 5 mM aqueous ammonium acetate pH 5.0, and subjected to NMR or MS/MS analyses, respectively.

Synthesis of Hydroxytyrosol 4′-*O*-β-D-Glucuronide (1) and Hydroxytyrosol 3′-*O*-β-D-Glucuronide (2)

The title compounds were synthesized following the above described procedure for the synthesis and purification of *O*-β-D-glucuronides. Reaction of hydroxytyrosol (15.4 mg, 0.1 mmol) yielded 11.9 mg (0.036 mmol, 36.1 %) of **1** and 5.1 mg (0.015 mmol, 15.4 %) of **2**.

Hydroxytyrosol 4′-*O*-β-D-Glucuronide (1): ^{1}H NMR (500.13 MHz, CD_3OD): δ=7.13 (d, J=8.15 Hz, H-5′), 6.72 (d, J=1.87 Hz, H-2′), 6.63 (dd, J=8.15, 1.73 Hz, H-6′), 4.68 (d, J=6.98 Hz, H-1″), 3.68 (t, J=7.16 Hz, H-1), 3.58–3.43 (m, H-5″, H-2″, H-3″ and H-4″), 2.69 (t, J=7.12 Hz, H-2); ^{13}C NMR (100.62 MHz, CD_3OD): δ=173.0 (C-6″), 149.5 (C-3′), 145.6 (C-4′), 136.2 (C-1′), 121.4 (C-6′), 117.8 (C-5′), 116.4 (C-2′), 102.8 (C-1″), 77.2 (C-3″), 73.1 (C-5″), 72.8 (C-2″), 72.7 (C-4″), 64.3 (C-1), 39.7 (C-2); purity by HPLC-UV: 99.8 % (k'=1.1); HPLC-MS (4 % acetonitrile in 5 mM ammonium acetate, pH 5.0): m/z=328.0 $[M-H]^-$, 153.0 $[M-H-Gluc]^-$.

Hydroxytyrosol 3′-*O*-β-D-Glucuronide (2): ^{1}H NMR (500.13 MHz, CD_3OD): δ=7.09 (d, J=0.78 Hz, H-2′), 6.79 (dd, J=8.19, 1.51 Hz, H-6′), 6.76 (d, J=8.14 Hz, H-5′), 4.76 (d, J=6.56 Hz, H-1″), 3.77 (bs, H-5″), 3.69 (t, J=7.09 Hz, H-1), 3.58–3.48 (m, H-2″, H-3″ and H-4″), 2.71 (t, J=7.07 Hz, H-2); ^{13}C NMR (100.62 MHz, CD_3OD): δ=175.1 (C-6″), 147.2 (C-3′), 146.8 (C-4′), 132.0 (C-1′), 125.4 (C-6′), 120.3 (C-

5′), 117.2 (C-2′), 102.7 (C-1″), 77.4 (C-3″), 73.1 (C-5″), 72.8 (C-2″), 72.7 (C-4″), 64.5 (C-1), 39.8 (C-2); purity by HPLC-UV: 97.8% (k' = 1.6); HPLC-MS (4% acetonitrile in 5 mM ammonium acetate, pH 5.0): m/z = 328.0 $[M-H]^-$, 153.0 $[M-H-Gluc]^-$.

Synthesis of Tyrosol 4′-*O*-β-D-Glucuronide (3) and Tyrosol 1-*O*-β-D-glucuronide (4)

The title compounds were synthesized following the above described procedure. Reaction of tyrosol (13.8 mg, 0.1 mmol) yielded 13.1 mg (0.042 mmol, 41.7%) of **3** and 1.7 mg (0.005 mmol, 5.4%) of **4**.

Tyrosol 4′-*O*-β-D-Glucuronide (3): ^{1}H NMR (500.13 MHz, CD_3OD): δ = 7.13 (d, J = 8.36 Hz, H-2′ and H-6′), 7.04 (d, J = 8.43 Hz, H-3′ and H-5′), 4.87 (d, J = 7.10 Hz, H-1″), 3.76 (bs, H-5″), 3.70 (t, J = 7.12 Hz, H-1), 3.57–3.45 (m, H-2″, H-3″ and H-4″), 2.76 (t, J = 7.12 Hz, H-2); ^{13}C NMR (100.62 MHz, CD_3OD): δ = 176.6 (C-6″), 157.8 (C-4′), 134.3 (C-1′), 130.9 (C-2′ and C-6′), 118.0 (C-3′ and C-5′), 102.7 (C-1″), 77.8 (C-3″), 76.7 (C-5″), 74.8 (C-2″), 73.7 (C-4″), 64.4 (C-1), 39.5 (C-2); purity by HPLC-UV: 98.8% (k' = 1.0); HPLC-MS (4% acetonitrile in 5 mM ammonium acetate, pH 5.0): m/z = 313.0 $[M-H]^-$, 137.0 $[M-H-Gluc]^-$.

Tyrosol 1-*O*-β-D-Glucuronide (4): ^{1}H NMR (500.13 MHz, CD_3OD): δ = 7.07 (d, J = 8.46 Hz, H-2′ and H-6′), 6.69 (d, J = 8.49 Hz, H-3′ and H-5′), 4.30 (d, J = 7.78 Hz, H-1″), 4.08 (m, H-1), 3.69 (m, H-1), 3.56 (d, J = 9.0 Hz, H-5″), 3.45 (t, J = 9.12 Hz, H-4″), 3.40 (t, J = 8.86 Hz, H-3″), 3.22 (dd, J = 8.83, 8.00 Hz, H-2″), 2.84 (t, J = 7.49 Hz, H-2); ^{13}C NMR (100.62 MHz, CD_3OD): δ = 177.2 (C-6″), 156.8 (C-4′), 131.0 (C-1′), 130.8 (C-2′ and C-6′), 116.1 (C-3′ and C-5′), 104.3 (C-1″), 77.9 (C-3″), 76.1 (C-5″), 75.0 (C-2″), 73.8 (C-4″), 72.2 (C-1), 36.3 (C-2); purity by HPLC-UV: 98.5% (k' = 2.16); HPLC-MS (4% acetonitrile in 5 mM ammonium acetate, pH 5.0): m/z = 313.0 $[M-H]^-$, 137.0 $[M-H-Gluc]^-$.

Synthesis of Homovanillic Alcohol 4′-*O*-β-D-Glucuronide (5) and Homovanillic Alcohol 1-*O*-β-D-Glucuronide (6)

The title compounds were synthesized following the above described procedure. Reaction of homovanillic alcohol (16.8 mg, 0.1 mmol) yielded 30.3 mg (0.088 mmol, 88.0%) of **5** and 2.1 mg (0.006 mmol, 6.1%) of **6**.

Homovanillic Alcohol 4′-*O*-β-D-Glucuronide (5): ^{1}H NMR (500.13 MHz, CD_3OD): δ = 7.12 (d, J = 8.25 Hz, H-5′), 6.91 (s, H-2′), 6.79 (d, J = 8.23 Hz, H-6′), 4.89 (d, J = 7.31 Hz, H-1″), 3.86 (s, -OMe), 3.74 (t, J = 7.03 Hz, H-1), 3.72 (t, J = 4.63 Hz, H-5″), 3.60–3.51 (m, H-2″, H-3″ and H-4″), 2.78 (t, J = 7.02 Hz, H-2); ^{13}C NMR (100.62 MHz; CD_3OD): δ = 176.3 (C-6″), 150.3 (C-3′), 146.1 (C-4′), 135.6 (C-1′), 122.7 (C-6′), 118.1 (C-5′), 114.6 (C-2′), 102.8 (C-1″), 77.2 (C-3″), 77.0 (C-5″), 74.5 (C-2″), 73.4 (C-4″), 64.2 (C-1), 56.7 (-OMe), 39.6 (C-2); purity by HPLC-UV: 99.78% (k' = 2.0); HPLC-MS (6% acetonitrile in 5 mM ammonium acetate, pH 5.0): m/z = 343.0 $[M-H]^-$, 167.0 $[M-H-Gluc]^-$.

Homovanillic Alcohol 1-*O*-β-D-Glucuronide (6): ^{1}H NMR (500.13 MHz, CD_3OD): δ = 6.81 (s, H-2′), 6.66–6.60 (m, H-5′ and H-6′), 4.25 (d, J = 7.79 Hz, H-1″), 4.06 (m, H-1), 3.77 (s, -OMe), 3.66 (m, H-1), 3.50 (d, J = 9.6 Hz, H-5″), 3.38 (t, J = 9.6 Hz, H-4″), 3.34 (t, J = 8.7 Hz, H-3″), 3.18 (t, J = 8.9, 7.8 Hz, H-2″), 2.81–2.76 (m, H-2); ^{13}C NMR (100.62 MHz; CD_3OD): δ = 174.9 (C-6″), 147.4 (C-3′), 144.5 (C-4′), 130.1 (C-1′), 122.4 (C-6′), 116.1 (C-5′), 113.7 (C-2′), 104.1 (C-1″), 77.7 (C-3″), 76.2 (C-5″), 74.8 (C-2″), 73.6 (C-4″), 72.0 (C-1), 56.4 -OMe), 36.6 (C-2); purity by HPLC-UV: 96.4% (k' = 3.4); HPLC-MS (6% acetonitrile in 5 mM ammonium acetate, pH 5.0): m/z = 343.0 $[M-H]^-$, 167.0 $[M-H-Gluc]^-$.

Synthesis of 3-(4′-Hydroxyphenyl)propanol 4′-*O*-β-D-Glucuronide (7) and 3-(4′-Hydroxyphenyl)propanol 1-*O*-β-D-Glucuronide (8)

The title compounds were synthesized following the above described procedure. Reaction of 3-(4′-hydroxyphenyl)propanol (15.2 mg, 0.1 mmol) yielded 18.4 mg (0.056 mmol, 56.0%) of **7** and 1.8 mg (0.0055 mmol, 5.5%) of **8**.

3-(4′-Hydroxyphenyl)propanol 4′-*O*-β-D-Glucuronide (7): ^{1}H NMR (500.13 MHz, CD_3OD): δ = 7.05 (d, J = 8.56 Hz, H-2′ and H-6′), 6.98 (d, J = 8.62 Hz, H-3′ and H-5′), 4.81 (d, J = 7.25 Hz, H-1″), 3.67 (d, J = 8.97 Hz, H-5″), 3.49 (t, J = 6.8 Hz, H-1), 3.46–3.41 (m, H-2″, H-3″ and H-4″), 2.56 (t, J = 7.5 Hz, H-3), 1.74 (q, J = 6 Hz, H-3); ^{13}C NMR (100.62 MHz, CD_3OD): δ = 176.5 (C-6″), 157.5 (C-4′), 137.4 (C-1′), 130.3 (C-2′ and C-6′), 118.0 (C-3′ and C-5′), 102.8 (C-1″), 77.8 (C-3″), 76.7 (C-5″), 74.8 (C-2″), 73.7 (C-4″), 62.2 (C-1), 35.7 (C-3), 32.3 (C-2); purity by HPLC-UV: 99.8% (k' = 2.3); HPLC-MS (8% acetonitrile in 5 mM ammonium acetate, pH 5.0): m/z = 327.0 $[M-H]^-$, 151.0 $[M-H-Gluc]^-$.

3-(4′-Hydroxyphenyl)propanol 1-*O*-β-D-Glucuronide (8): ^{1}H NMR (500.13 MHz, CD_3OD): δ = 7.02 (d, J = 8.10 Hz, H-2′ and H-6′), 6.68 (d, J = 8.07 Hz, H-3′ and H-5′), 4.26 (d, J = 7.75 Hz, H-1″), 3.98 (dd, J = 15.62, 6.46 Hz, H-1), 3.60–3.48 (m, H-1 and H-5″), 3.45 (t, J = 8.80 Hz, H-4″), 3.40 (t, J = 8.90 Hz, H-3″), 3.23 (t, J = 8.27 Hz, H-2″), 2.62 (t, J = 7.54 Hz, H-3), 1.90–1.83 (m, H-2); ^{13}C NMR (100.62 MHz, CD_3OD): δ = 173.1 (C-6″), 154.9 (C-4′), 132.8 (C-1′), 128.9 (C-2′ and C-6′), 114.5 (C-3′ and C-5′), 103.3 (C-1″), 76.5 (C-3″), 73.5 (C-5″), 72.8 (C-2″), 72.3 (C-4″), 69.2 (C-1), 33.2 (C-3), 31.5 (C-2); purity by HPLC-UV: 99.0% (k' = 4.9); HPLC-MS (8% acetonitrile in 5 mM ammonium acetate, pH 5.0): m/z = 327.0 $[M-H]^-$, 151.0 $[M-H-Gluc]^-$.

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