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Abstract

Resorcinol bis-(diphenylphosphate) (RDP) is widely used as flame retardant in electrical/electronic products and constitutes a suitable alternative to decabrominated diphenyl ether. Due to its toxicity and its recently reported ubiquity in electronics and house dust, there are increasing concerns about human exposure to this emerging contaminant. With the aim of identifying human-specific biomarkers, the *in vitro* metabolism of RDP and its oligomers was investigated using human liver microsomes and human liver cytosol. Mono- and di-hydroxy-metabolites, together with glucuronidated and sulfated metabolites were detected. Regarding RDP oligomers, only a hydroxy-metabolite of the dimer could be detected. RDP and its oligomers were also readily hydrolyzed, giving rise to a variety of compounds, such as diphenyl phosphate, *para*-hydroxy-triphenyl phosphate and *para*-hydroxy RDP, which were further metabolized. These degradation products or impurities are possibly of environmental importance in future studies.

Keywords: resorcinol bis-(diphenylphosphate); human liver microsomes; metabolism; *in vitro;* mass spectrometry

Introduction

After the phase-out of polybrominated diphenyl ethers (PBDEs), organophosphorus flame retardants (PFRs) have been increasingly used to comply with flammability standards in consumer products (plastics, textile, paints, etc.).[1] Resorcinol bis-(diphenylphosphate) (RDP or PBDPP; CAS no. 125997-21-9) is a PFR used mainly in polycarbonate/acrylonitrile butadiene styrene and of poly (p-phenylene) oxide/high impact polystyrene polymers in electronics.[2] It is considered as a primary substitute for Deca-BDE in electronic enclosures, such as television sets.[2,3] In addition to its major use in thermoplastics/styrene polymers, other uses reported in the EU are in polyvinylchloride plastics, polyurethanes, paints and coatings and pigment dispersions.[4] In 2006, United States production/import volume for RDP was reported as up to 250,000 kg.[5]

There is little toxicity and environmental data for RDP in literature yet. A recent report of EPA shows low to moderate toxicity of RDP to humans, high to very high aquatic toxicity *(Daphnia magna* 48-hour EC_{50} = 0.7 mg/L), moderate persistence and high bioaccumulation potential.[2] The presence of toxic impurities (triphenyl phosphate, TPHP; typically 1-5% w/w) and breakdown products (phenol, resorcinol) may also influence the toxicity of the technical RDP.[6]

RDP has been found in a variety of electronics that are usually present in homes (TV sets, printers, powerboards, etc.)[7,8] and it migrates easily from these products into the house dust.[9] RDP has been recently reported in indoor dust samples from the Netherlands, Greece and Sweden.[9] High concentrations (<0.04-520 µg/g) were found in dust collected on electronic equipment. Levels decreased in dust collected further away from the electronics, suggesting that electronic products at home are a contamination source. In analogy with other FRs, human

exposure to RDP is probably due to inhalation and/or ingestion of house dust.[10] To the best of our knowledge, the presence of RDP in biological samples has not yet been reported.

There are only few studies which investigated the *in vivo* or *in vitro* biotransformation of PFRs in the literature. We have reported the *in vitro* metabolism of TPHP, tris (2-butoxyethyl) phosphate (TBOEP), tris (2-chloroisopropyl) phosphate (TCIPP), TDCIPP and tris(chloroethyl) phosphate (TCEP) using HLM and human liver S9 fraction[11] and of 2-ethylhexyldiphenyl phosphate (EHDPHP) by HLM.[12] All PFRs showed a wide array of oxidative metabolites next to the corresponding diesters. Regarding RDP, there is only one study in literature that investigated the *in vivo* metabolism and disposition of ^{14}C-RDP in rats, mice, and monkeys.[13] The major fecal metabolites were resorcinol, diphenyl phosphate (DPHP), hydroxy-DPHP, hydroxy-RDP and dihydroxy-RDP. Major urinary metabolites were resorcinol, resorcinyl glucuronide and resorcinyl sulfate. There were no differences in the metabolism of RDP between species.

In this study, we aimed at investigating the *in vitro* metabolism of RDP with human liver preparations and the hydrolysis under physiological conditions (pH 7.4, 37°C). According to technical reports[14], RDP suffers from hydrolytic instability and this could lead to more bioavailable and toxic breakdown products that could be also further metabolized in the human body. *In vitro* experiments were carried out with HLM and human liver cytosol (HLCYT) to determine the role of human cytochrome P450 (CYP), uridine glucuronic acid transferase (UGT) and sulfotransferase (SULT) enzymes in the metabolism of RDP. Liquid chromatography (LC) coupled with quadrupole-high-resolution mass spectrometry (Q-TOFMS) was employed for the identification of possible metabolite classes by a combination of a targeted screening approach,

based on an *in silico* generated database, and an untargeted screening analysis. As a result, we proposed a possible metabolism pathway of RDP and suitable candidate biomarkers for the human exposure to this ubiquitous contaminant.

Experimental section

Chemicals and reagents

Pooled human liver microsomes (HLM; *n*=50, mixed gender) and pooled human liver cytosol (HLCYT, mix gender) were purchased from Tebu Bio (Boechout, Belgium) and Sigma-Aldrich (Bornem, Belgium), respectively. RDP standard (98% purity) was obtained from TRC (Toronto, Canada); reduced β-nicotinamide adenine dinucleotide 2'-phosphate (NADPH; purity>95%), uridine 5'-diphosphoglucuronic acid (UDPGA; purity>98%), adenosine 3'-phosphate 5'-phosphosulfate (PAPS; purity>96%), DPHP and TPHP-d_{15} (used as internal standard, IS) were purchased from Sigma–Aldrich (Bornem, Belgium). The standard TPHP was purchased from Chiron AS (Trondheim, Norway). The DPHP-d_{10} standard (also used as IS) was custom synthesized by Dr. Vladimir Belov (Max Planck Institute, Göttingen, Germany). Acetonitrile, methanol and ammonium acetate were purchased from Merck KgA Chemicals (Darmstadt, Germany). *para*-HO-TPHP was synthesized by the Max Planck Institute (Göttingen, Germany) with a purity of 98%. *meta*-HO-TPHP was synthesized by the Organic Chemistry Synthesis Group from Duke University (NC, USA) and kindly donated by prof. Heather Stapleton (Duke University, NC, USA). Ultrapure water (18.2 MΩ) was obtained using an Elga LabWater water purification instrument (Saint Maurice, France). Oasis WAX cartridges (3 cc, 60 mg, 30 µm) were obtained from Waters (Milford, Massachusetts, USA).

Database of RDP metabolites

As previously reported,[11,12] our approach was to prepare a database of metabolites for QTOF-based targeted screening that can be produced from RDP by specific enzymes present in the used human liver preparations. The use of specific software for the prediction of metabolites greatly simplified this task, providing a list of possible candidates and also estimated values for physico-chemical properties of the generated structures, such as calculated hydrophobicity (calculated log K_{ow}), that are useful for confirmation purposes in chromatography.

The structures of the metabolites of RDP were predicted by the Meteor Nexus program (Lhasa limited, UK). Since RDP contain a number of oligomers (*n*=1-7), prediction experiments for RDP oligomers (*n*=1-3) were run separately. According to the literature, the major component of RDP polymer is the oligomer where *n*=1, which typically accounts for 95-99% of the mixture.[2] The balance is made up of higher oligomers (*n*=2, 3, etc.) and a TPHP impurity (1-5% w/w). The following prediction parameters were used: metabolism was outlined to include the transformations by CYP, phosphatases, alcohol dehydrogenases or ADH, SULT and UGT enzymes, the maximum number of metabolic steps was set at three, and the maximum number of predicted metabolites was set at 200. The degree of likelihood included probable, plausible and equivocal in decreasing rank of likelihood. Relative reasoning level was set at two, and human was selected as the only species of interest. By using these parameters, Meteor Nexus predicted a list of 17 metabolites for RDP, 31 for RDPn2 (nº oligomers =2) and 38 for RDPn3 (nº oligomers =3).

123

124 ***In vitro biotransformation and chemical hydrolysis assays***

125 **1) Chemical hydrolysis experiments:** TRIS buffer (pH 7.4 at 37°C) solutions of RDP (0.5-50 µM at
126 30 min incubation) were prepared. The incubations were stopped in ice and by adding
127 methanol (50% v/v in final extract) in order to minimize further chemical hydrolysis.

128 **2) HLM metabolism by CYP enzymes:** A reaction mixture containing 100 mM TRIS buffer
129 (containing 5 mM $MgCl_2$; pH 7.4 at 37°C), HLM (0.5 mg/mL, final protein concentration) and
130 RDP (20 µM, final concentration, 1% methanol v/v) in a total volume of 1 mL was prepared in 2
131 mL Eppendorf tubes. The mixture was pre-incubated in a shaking water bath at 37°C for 5 min.
132 The reaction was started by adding NADPH (1 mM, final concentration) dissolved in buffer. A
133 new aliquot of NADPH was added after 1 h incubation to ensure sufficient cofactor amount
134 throughout the experiment.

135 **3) HLM metabolism by UGT enzymes:** To investigate the formation of the glucuronidated
136 metabolites derived from the hydroxy metabolites of RDP, RDP (20 µM, final concentration)
137 mixtures were incubated with HLM as described above. So, after 2 h incubation, the reaction
138 was stopped by placing the tubes on ice for 1 min. Subsequently, the whole content of each
139 tube was transferred to a 2 mL Eppendorf vial and centrifuged at 8,000 rpm for 5 min. After
140 centrifugation, the supernatant (buffer containing the un-metabolized EHDPHP and the CYP-
141 generated metabolites) was transferred into a new set of glass tubes and mixed with freshly
142 prepared pooled HLM (0.5 mg/mL, final concentration). Alamethicin (10 µg/mL, final
143 concentration, 1% v/v in dimethyl sulfoxide, DMSO) was added to the reaction mixture to
144 increase membrane porosity and therefore facilitate diffusion of the substrate to the

membrane-bound UGTs present inside the HLM phospholipid bilayer.[15] The reaction mixtures were pre-incubated for 5 min in a shaking water bath at 37°C. The glucuronidation reaction was started by adding UDPGA (1 mM, final concentration). A new aliquot of UDPGA was added after 1 h incubation to ensure sufficient cofactor amount throughout the experiment.

4) HLCYT metabolism by SULT enzymes: Samples were prepared as described above for UGT experiments omitting the addition of alamethicin, using HLCYT (0.5 mg/mL, final concentration) instead of HLM and starting the reaction by addition of PAPS (0.1 mM, final concentration) instead of UDPGA.

All CYP, UGT and SULT reactions were quenched after 2 h by adding 1 mL of ice-cold acetonitrile and placing the tubes on ice. Ammonium acetate (155 mg) was added to each tube (~2 M in water). The solution was vortex-mixed for 1 min and then ultracentrifuged (8,000 rpm, 30s). After the phase-separation by salting-out with ammonium acetate, the acetonitrile layer was separated and transferred into a new glass tube. The liquid-liquid extraction was done 3 times and all the supernatants (3 mL acetonitrile) combined and evaporated to dryness (N_2, 40°C). The extracts were reconstituted in 150 µL of methanol containing the internal standards DPHP-d_{10} and TPHP-d_{15} to a final concentration of 100 ng/mL. The extracts were vortexed for 20 s and transferred into a vial for analysis.

Cofactor and enzyme negative control samples were routinely prepared for every batch of experiments concerning CYP, UGT and SULT reactions as described above, but devoided of the specific cofactor or enzyme of interest in each case. Within each batch of experiments, blanks containing only the buffer underwent the whole procedure (incubation+liquid-liquid extraction). Background contamination was not found in any of the blanks.

After the screening phase, and to further confirm the formation profiles of the hydroxy metabolites of RDP, a second set of experiments (profiling) was conducted over a range of incubation time values (0 to 60 min) at 0.1 mg/mL HLM; HLM concentration (0.025 to 0.5 mg/mL) for 15 min and RDP concentration (0.5-50 µM) at 0.1 mg/mL HLM and 15 min incubation time.

LC-QTOF MS analysis method

RDP metabolites produced by CYP, UGT and SULT enzymes and hydrolysis products were identified by LC–HRMS using a 1290 series LC system coupled to a 6530 Q-TOF-HRMS (Agilent Technologies) equipped with an ESI Jet Stream ion source. The chromatographic separation of the hydroxy metabolites was achieved by using an Agilent Extend Zorbax C18 column (50 x 2.1 mm, 3.5 µm) and the following mobile phase composition: 2 mM ammonium acetate in water (A) and acetonitrile (B). The gradient elution for the hydroxy metabolites and the hydrolysis products was 5% B during 1 min, linear gradient from 5 to 45% B from 1 to 3 min, linear gradient from 45 to 55% B from 3 to 5 min, isocratic conditions at 55% B from 5 to 5.8 min, linear gradient from 55 to 80% B from 5.8 to 8.5 min and linear gradient from 80 to 90% B from 8.5 to 12 min. Different stationary and mobile phases were necessary for achieving good separation of the glucuronidated and sulfated metabolites. The column employed was a Agilent Eclipse Plus C8 column (150 x 2.1 mm, 3.5 µm) and the mobile phase was 2 mM ammonium acetate in water (A) and methanol (B). The gradient elution was as follows: isocratic conditions at 5% B during 5 min, linear gradient from 5 to 80% B from 0 to 20 min, then linear gradient from 80% B to 90% B from 20 to 25 min. In both gradient programs, the flow was 0.3

189 mL/min, the column oven temperature was set at 30°C, the injection volume was 3 µL and the
190 re-equilibration time after injection was 10 min.
191 The Q-TOF-MS was run in ESI positive and negative mode scanning *m/z* from 30 to 1,500 amu at
192 a scan rate of 2.5 spectra/s. The following MS parameters were used: gas temperature 300°C,
193 gas flow 8 L/min, nebulizer pressure 20 psi, sheath gas temperature 275°C, sheath gas flow 12
194 L/min. Nozzle, capillary, fragmentor, and skimmer voltages were set to 500, 3000, 100, and 65
195 V, respectively. The instrument was calibrated during run times by monitoring positive ions
196 with reference masses *m/z* 59.0604 and 922.0098 and negative ions with *m/z* 112.9856 and
197 980.01528
198 For quantitation or semi-quantitation of hydroxy metabolites (profiling experiments), hydrolysis
199 products and impurities, the samples were analyzed using an Agilent 6460 triple quadrupole MS
200 with a standard ESI source. This detection system provides a higher linearity range, thus
201 facilitating quantitation. Table S-1 shows the main transitions and MS parameters used in this
202 method. Calibration curves for DPHP, *para*-HO-TPHP and *meta*-HO-TPHP (0.05-2 µg/mL) were
203 prepared in methanol or in water:methanol 50:50 v/v for hydrolysis experiments.
204
205 ***Data analysis***
206 In a first stage, the metabolites and hydrolysis products of RDP were identified by LC-
207 QTOF-MS analysis. **1) Targeted approach:** the list of predicted metabolites generated by
208 Meteor was imported (in the *.csv* format) in the Mass Hunter Qualitative analysis software
209 (Agilent) by using the "Identify compounds" tool for databases in order to verify the presence
210 of the predicted metabolites. **2) Untargeted approach:** RDP metabolites were screened by

211 using the Mass Hunter Qualitative Analysis software and the "Find compound by molecular
212 feature" tool. In both targeted and untargeted screening, $[M+H]^+$ and $[M-H]^-$ were selected as
213 major ions in positive and negative mode, respectively. Ions coming from the formation of
214 adducts (e.g. $[M+NH_4]^+$, $[M+Na]^+$, etc.), neutral loss ($[M-H_2O-H]^-$) or dimers were also included
215 for formulae generation since they are commonly formed in the ESI Jet Stream ion source
216 employed in this study.
217 In a second stage, MS/MS experiments were carried out using different collision induction
218 dissociation (CID) energy values (5 to 30 eV) for further confirmation of the previously identified
219 metabolites (by targeted or untargeted screening) trough fragmentation patterns. As suggested
220 by other authors,[16] the presence of false positive peaks due to in-source fragmentation of other
221 (related) compounds was investigated.
222 The main criteria for identification of metabolites in both targeted and untargeted approaches
223 were as follows: (a) the measured *m/z* value should be within 10 ppm of its calculated value; (b)
224 the isotope pattern should be matched within 7.5% of the predicted abundances; (c) if the
225 precursor *m/z* was fragmented, its fragmentation pattern should be explainable and showing at
226 least one structure specific fragment and d) the calculated log K_{ow} of the metabolite should be
227 in accordance with its reverse-phase LC retention time in relation with the calculated log K_{ow}
228 and retention times of DPHP, TPHP, RDP and the identified metabolite classes. Further
229 quantitative or semi-quantitative experiments were done by LC-QQQ-MS, according to the
230 conditions specified in Table S-1.
231
232

Results and discussion

Sample preparation

Different sample preparation procedures were studied for the clean-up/concentration of the aqueous incubation samples. The introduction of a sample preparation step aimed at minimizing matrix effects which could hamper the detection of metabolites. It could also increase the lifetime and good performance of the LC column and the stability of the metabolites in the final organic extracts. The following procedures were investigated: a) common solvents for liquid-liquid extraction after HLM incubations reported for other FRs (ethyl acetate, methyl-tert-butylether, MTBE:hexane 1:1 v/v); b) anion exchange solid-phase extraction (SPE) as previously reported for the analysis of PFR metabolites in urine[17] and c) salting-out extraction in mixtures acetonitrile:water with ammonium acetate. The salting-out procedure was selected as optimal due to the higher apparent recoveries, lower consumption of organic solvent and materials and the simplicity of the procedure. Detailed information about the sample preparation optimization is given in SI.

Chemical Hydrolysis of RDP

The hydrolysis half-life of RDP in water at pH 7 has been calculated as 17 days[4], while slow mineralization rates have been recently reported with a value of 18% after 28 days.[18] In this study, the chemical hydrolysis of RDP was investigated in the buffer (100 mM TRIS buffer containing 5 mM $MgCl_2$; pH 7.4 at 37 ºC) to simulate general physiological conditions as those found in serum and tissues. The identification of RDP degradation products is also relevant for future environmental studies, which up to now only focused on the formation of DPHP.[19]

A variety of hydrolysis products of RDP and its oligomers were identified, including DPHP, RDP-[Phe] (loss of a phenyl ring from RDP), *meta*-hydroxy-TPHP, *meta*-hydroxy-RDP and *meta*-hydroxy-RDPn2. They were all present as impurities in the RDP standard, probably due to its hydrolytic instability, and increased after the incubation at 37°C in the buffer. The *meta*-position of the hydroxy group is the only possible considering the structure of RDP and the hydrolytic breakdown of the molecule as shown in Figure 1. Although not specified in the Figure, each hydrolysis product may breakdown in the lower mass products, e.g., *meta*-hydroxy-RDPn2 could be hydrolyzed further into *meta*-hydroxy-RDP and then into *meta*-hydroxy-TPHP and successively until DPHP. The compound RDPn2-[Phe] was only minor.

All hydrolysis products were identified with a mass accuracy <10 ppm. Further MS/MS experiments for structure confirmation by specific fragments, showed DPHP as the main fragment in *meta*-hydroxy-TPHP and in RDP-[Phe] and the fragment corresponding to a loss of a phenyl ring (or RDP-[Phe]) as the main fragment for *meta*-hydroxy-RDP and for RDPn2-[Phe]. The identities of *meta*-hydroxy-TPHP and DPHP were also confirmed by the injection of authentic standards and by matching MS spectra and retention times. Figure 1 shows all identified hydrolysis products of RDP.

The hydrolysis products or impurities were present in the same amounts by incubations containing HLM and by those containing only buffer, so that they were mainly produced by chemical hydrolysis and not by enzymatic reactions (see Figure S-1). *Meta*-hydroxy-TPHP and of DPHP was calculated with their authentic standard as 135±10 and 36±5 pmol per nmol of RDP for a hydrolysis time of 30 min at concentrations of 1-20 µM RDP.

Hydroxy metabolites of RDP

In the HLM incubations, we observed a number of hydroxy metabolites that were not present in the cofactor, neither in the enzyme negative controls. This confirms the formation of these metabolites by NADPH-consuming enzymes, most probably CYP. A hydroxy-metabolite [HO-RDP (1)], a di-hydroxy-metabolite [di-HO-RDP] and a hydroxy- and a di-hydroxy-triphenyl phosphate [HO-TPHP(1), di-HO-TPHP] were identified. Figure 2 shows all the identified metabolites of RDP.

All identified metabolites complied with the rule of mass accuracy, meaning that the parent compound was identified with a mass error below 10 ppm, as shown in Table S-2. MS/MS experiments confirmed the structure of the identified metabolites. Fragmentation spectra showed the structure-specific fragment diphenyl phosphate (DPHP) as the main ion in hydroxy- and dihydroxy-TPHP and another specific fragment ([$C_{12}H_{11}O_4P$] - the hydrolysis product named as RDP-[Phe]) corresponding to a loss of a phenyl ring from hydroxy-RDP and dihydroxy-RDP.

Regarding the oligomers of RDP, only the hydroxy-metabolite of RDPn2 could be detected as a minor metabolite, not being present in the cofactor neither in the enzyme negative controls. This is in agreement with the fact that the oligomers are less abundant that the monomer in the RDP formulation[2] as explained in the experimental section. Differently from TPHP and RDP, we did not detect a dihydroxy metabolite derived from the RDP dimer. This could be just due to the limited sensitivity of the method that was lower for the dihydroxy metabolites in comparison with the hydroxy metabolites.

299 Hydroxy-TPHP was most probably oxidized in the *para* position as predicted by the Meteor
300 software and as described previously by our research group.[12] This was confirmed by the
301 injection of the authentic standard of *para*-hydroxy-TPHP (for matching both spectra and
302 retention times). The formation of *para*-hydroxy-TPHP was formed in the range 19-45 pmol per
303 nanomol of RDP (at 3-10 µM of RDP, 30 min incubations, 0.2 mg/mL HLM). The most probable
304 oxidation position for the hydroxy-RDP (with one or two oligomers) predicted by Meteor was
305 also *para*, but this could not be confirmed due to the unavailability of standards. Minor peaks
306 corresponding to hydroxy-DPHP and hydroxy-(RDP-[Phe]) were identified at long incubations
307 times (3 h), but due to their low abundance, they were not considered for further study.
308 Since TPHP is present in the RDP formulations (around 2% w/w), the contribution of the
309 impurity in the formation of the observed metabolites was studied. For this purpose, HLM
310 incubations containing only TPHP were prepared at the calculated level that is present as
311 impurity in the RDP solution used for the biotransformation experiments. *Para*-hydroxy-TPHP
312 was exclusively formed from the TPHP impurity. However, only around 15% of the dihydroxy-
313 TPHP was derived from TPHP. The remaining 85% of dihydroxy-TPHP probably originates from a
314 second oxidation of *meta*-hydroxy-TPHP.
315 A profile study was done for further confirmation of the hydroxy metabolites. For this purpose,
316 the formation of the hydroxy metabolites was monitored under different incubation times (5-
317 60 min); HLM concentrations (0.05-0.5 mg/mL) and RDP concentrations (0.5-50 µM). As can be
318 seen in Figure S-2, the concentration (or MS signal intensity) of the metabolites increased as
319 these influential factors also increased.

DPHP was also formed as O-dearylation metabolite at concentrations significantly higher than those produced by chemical hydrolysis at long incubation times (>2 h), both in HLM and in cofactor negative controls. Although no differences in the DPHP formation were observed at incubation times <60 min (see Figure S-1), the formation of DPHP was higher with HLMs and cofactor negative controls than by chemical hydrolysis at longer times (e.g. around 65, 52 and 35 pmol of DPHP per nmol of RDP were formed in 0.2mg/mL HLM, cofactor negative controls and in only buffer incubations of 3 h, values are an average of three independent experiments; relative standard deviations below 10%; 10 µM of RDP). This suggests that DPHP is also a metabolite and not only a hydrolysis product of RDP as occurring for other PFRs, namely TPHP[11] and EHDPHP.[12] The presence of DPHP in cofactor negative controls suggests that not only CYP enzymes (HLM), but also NADPH-independent liver microsomal phosphatase enzymes, such as paraoxonases and aryl esterases may be involved.[19]

Glucuronidated and sulfated metabolites

By incubating the buffer containing the hydroxy metabolites of RDP and/or only the hydrolysis products with pooled HLM or HLCYT, alamethicin and UDPGA or PAPS, several new chromatographic peaks appeared. None of these peaks were detected in HLM/HLCYT or cofactor negative controls. We could thus conclude that several sulfated and glucuronidated metabolites were formed (Figure 2). These conjugates arose from the metabolism of hydroxy- and dihydroxy-TPHP and RDP metabolites by UGT and SULT enzymes and also from the hydrolysis products.

341 The main identified metabolites were phenyl sulfate, TPHP sulfate, RDP sulfate, TPHP
342 glucuronide and RDP glucuronide and glucuronidated and sulfated metabolites with an hydroxy
343 group [resorcinyl sulfate, hydroxy-TPHP sulfate (two isomers), hydroxy-RDP sulfate, hydroxy-
344 TPHP glucuronide (two isomers) and hydroxy-RDP glucuronide (two isomers)]. All these
345 compounds were identified on the basis of mass accuracy (mass error below 10 ppm for parent
346 compound) and showed structure-specific fragments in MS/MS experiments, i.e. DPHP for all
347 TPHP derivatives and for the hydroxy-RDP glucuronide and RDP-[Phe] for all other RDP
348 derivatives. For the lower mass metabolites, the main fragments were the phenoxy ion $[C_6H_5O]^-$
349 for phenyl sulfate and its double oxidized form $[C_6H_5O_2]^-$ for the resorcinyl sulfate.
350 As a result of these experiments, we observed that the glucuronidated and sulfated metabolites
351 with an additional unconjugated –OH group were formed exclusively through the sulfation or
352 glucuronidation of the hydroxy metabolites of RDP. On the other hand, the glucuronidated and
353 sulfated metabolites not having an additional unconjugated –OH group, were formed mainly
354 from the hydrolysis products of RDP. This fact could be explained by the higher abundance of
355 the hydrolysis products compared with their isomers produced by CYP (e.g. about ten times
356 more *meta*-HO-TPHP was produced by chemical hydrolysis than *para*-HO-TPHP was formed by
357 CYP under same conditions). RDP-glucuronide was an exception and was formed both from
358 *meta*-hydroxy-RDP (hydrolysis product) and from the *para*-hydroxy-RDP generated by CYP
359 enzymes. So, SULT and UGT enzymes reacted also with the compounds having hydroxy groups
360 in *meta*-position. Although *meta*-hydroxy metabolites were not produced by CYP enzymes,
361 these compounds could be present in the human body due to hydrolysis at physiological
362 conditions.

363 Figure 3 shows the relative abundance of the identified metabolites according with the MS
364 signal of their main MRM transitions in QQQ (average of three independent experiments). In
365 terms of MS peak area the dihydroxy metabolites were more abundant than the monohydroxy
366 metabolites and sulfated metabolites were formed with 2 to 5 times higher than glucuronide
367 conjugates. However, these are only relative values that can be influenced by the unknown
368 formation rate of the hydroxy metabolites used as substrate to generate the glucuronidated
369 and sulfated metabolites, the limited solubility of RDP and the differences between *in vitro* and
370 *in vivo* experiments, which can be substantial.
371
372 ***Comparison with the in vitro metabolism of other PFRs***
373 The number of studies investigating the metabolism of PFRs is still very limited in the
374 literature. The reported metabolites of TPHP, a structural analogous or building block of RDP
375 (and also an impurity) were a hydroxy- metabolite (two isomers), a di-hydroxy- metabolite (two
376 isomers), DPHP and a metabolite resulting from oxidation and *O*-dearylation of TPHP.[11] Only
377 the more abundant metabolites of TPHP, the hydroxy- and one dihydroxy-TPHP, could be
378 detected in RDP experiments since they were formed from the TPHP impurity an not from RDP
379 itself. RDP was similarly metabolized by CYP enzymes, producing also hydroxy-RDP and
380 dihydroxy-RDP, glucuronide and sulfate conjugates. The formation of DPHP from RDP by
381 chemical hydrolysis and enzymatic reactions is relevant for future exposure studies. Up to now,
382 the urinary levels of DPHP have been correlated mainly with the levels of TPHP in house dust,
383 since it was considered the main parent compound forming this metabolite. [20,21] In future

investigations, it would be necessary to include also the contributions of RDP and the recently reported EHDPHP[12], both ubiquitous in house dust.

The results are also consistent with the reported study about the *in vivo* metabolism and disposition of RDP (rat, monkey and mice) that was published in 2000.[13] The major fecal metabolites were dihydroxy-TPHP, hydroxy-TPHP, dihydroxy-RDP, and hydroxy-RDP. Major urinary metabolites were identified as resorcinol, resorcinyl glucuronide, and resorcinyl sulfate. So, specific sulfated or glucuronidated metabolites of RDP were not reported. These low molecular weight compounds could come also from a variety of other compounds, such as TPHP, DPHP or phenol. The specific sulfated and glucuronides metabolites of RDP identified in our study provide a better understanding of the metabolic pathway of RDP. Besides, a hydroxy metabolite for the RDP dimer and a variety of hydrolysis products or impurities are also reported in this study for the first time.

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406 **Supporting Information Available**

407 The Supporting Information provides detailed information about 1) the detection and
408 quantification of metabolites and hydrolysis products by QQQ-MS and QTOF-MS, 2) the profile
409 formation of hydrolysis products and metabolites, 3) the efficiency of the different sample
410 preparation methods (in terms of relative MS signal intensity of the reported compounds) and
411 4) LC-MS chromatograms of the identified metabolites and compounds. This information is
412 available free of charge via the Internet at http://pubs.acs.org/

413

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Figure 1. *Hydrolysis products of RDP and RDP oligomers*

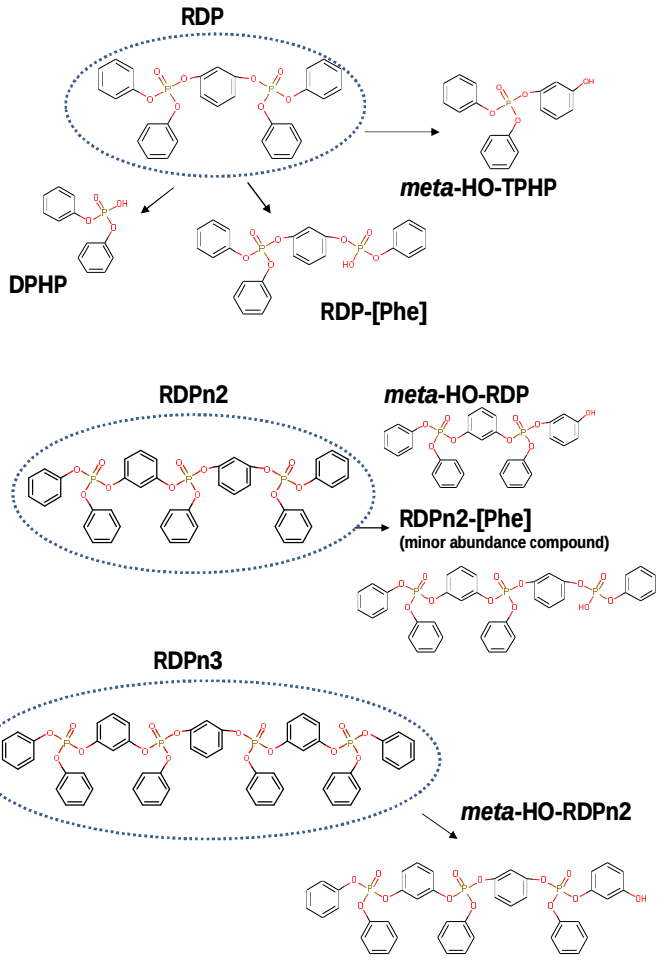


***Figure 2**. Metabolites of A) RDP and the B) dimer RDPn2 identified in this study. Those derived from TPHP (RDP impurity) and meta-HO-TPHP (RDP hydrolysis product) are also shown in A), since they are present in RDP solutions. (Abbreviations: HYD, chemical hydrolysis; PHO?, phosphatases, possible pathway but not confirmed)*

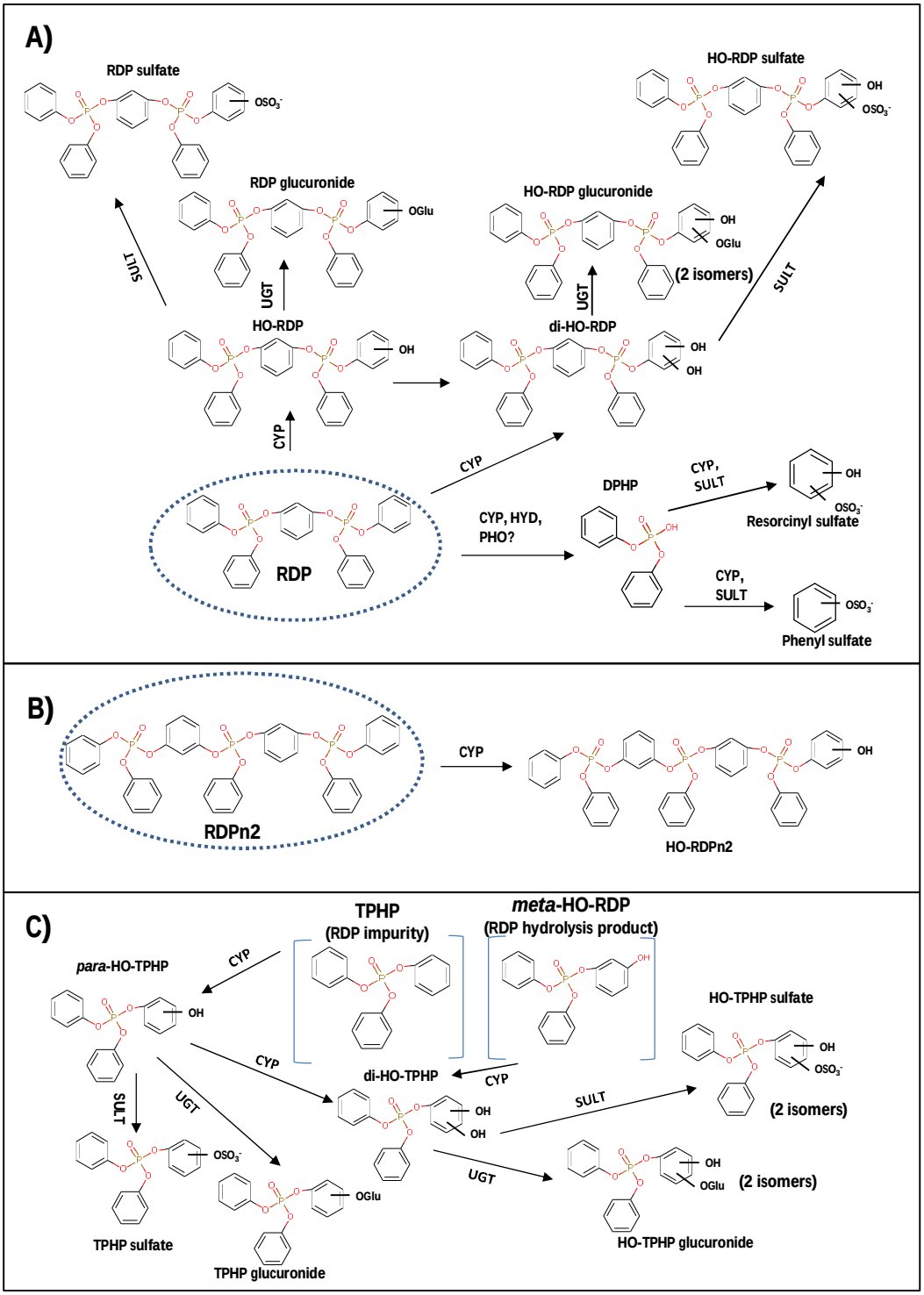
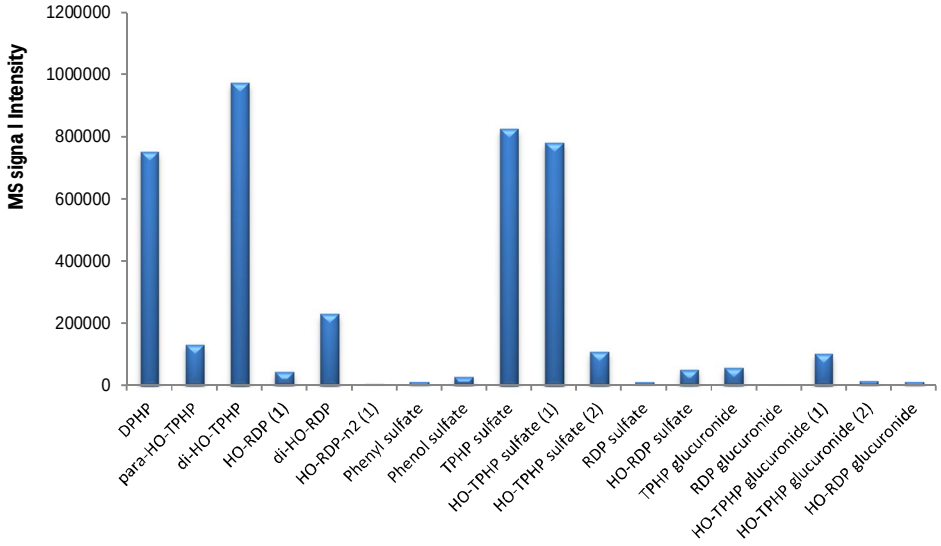
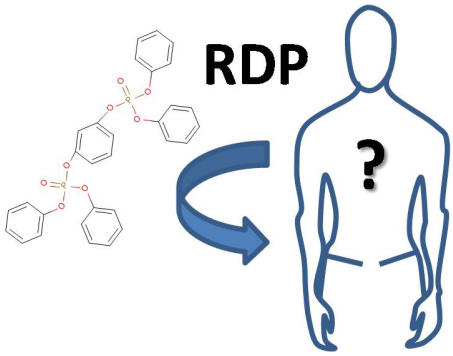


Figure 3. Relative abundance expressed as MS signal (main transition, QQQMS) of RDP metabolites. Conditions: 3 h incubation for CYP-mediated reaction and 3 h incubation for UGT- or SULT-mediated reactions, 0.3 mg/mL HLM, 20 µM RDP (average of three independent experiments).



495 **TOC/Abstract Art**



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