In vitro human metabolism of the flame retardant resorcinol bis-(diphenylphosphate) (RDP)

Ana Ballesteros-Gómez[†,$,*], Nele Van den Eede[†], Adrian Covaci[†*]

[†]Toxicological Center, University of Antwerp, Universiteitsplein 1, 2610 Antwerp, Wilrijk, Belgium

* Corresponding authors:

E-mail: a.m.ballesterosgomez@gmail.com and adrian.covaci@uantwerpen.be

Toxicological Center, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium

$ - present address: Institute for Environmental Studies (IVM), VU University, De Boelelaan 1087, 1081 HV Amsterdam, the Netherlands

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.

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

57 exposure to RDP is probably due to inhalation and/or ingestion of house dust.[10] To the best of

58 our knowledge, the presence of RDP in biological samples has not yet been reported.

59 There are only few studies which investigated the *in vivo* or *in vitro* biotransformation of PFRs in

60 the literature. We have reported the *in vitro* metabolism of TPHP, tris (2-butoxyethyl)

61 phosphate (TBOEP), tris (2-chloroisopropyl) phosphate (TCIPP), TDCIPP and tris(chloroethyl)

62 phosphate (TCEP) using HLM and human liver S9 fraction[11] and of 2-ethylhexyldiphenyl

63 phosphate (EHDPHP) by HLM.[12] All PFRs showed a wide array of oxidative metabolites next to

64 the corresponding diesters. Regarding RDP, there is only one study in literature that

65 investigated the *in vivo* metabolism and disposition of ^{14}C-RDP in rats, mice, and monkeys.[13]

66 The major fecal metabolites were resorcinol, diphenyl phosphate (DPHP), hydroxy-DPHP,

67 hydroxy-RDP and dihydroxy-RDP. Major urinary metabolites were resorcinol, resorcinyl

68 glucuronide and resorcinyl sulfate. There were no differences in the metabolism of RDP

69 between species.

70 In this study, we aimed at investigating the *in vitro* metabolism of RDP with human liver

71 preparations and the hydrolysis under physiological conditions (pH 7.4, 37°C). According to

72 technical reports[14], RDP suffers from hydrolytic instability and this could lead to more

73 bioavailable and toxic breakdown products that could be also further metabolized in the human

74 body. *In vitro* experiments were carried out with HLM and human liver cytosol (HLCYT) to

75 determine the role of human cytochrome P450 (CYP), uridine glucuronic acid transferase (UGT)

76 and sulfotransferase (SULT) enzymes in the metabolism of RDP. Liquid chromatography (LC)

77 coupled with quadrupole-high-resolution mass spectrometry (Q-TOFMS) was employed for the

78 identification of possible metabolite classes by a combination of a targeted screening approach,

79 based on an *in silico* generated database, and an untargeted screening analysis. As a result, we

80 proposed a possible metabolism pathway of RDP and suitable candidate biomarkers for the

81 human exposure to this ubiquitous contaminant.

82

83 **Experimental section**

84 ***Chemicals and reagents***

85   Pooled human liver microsomes (HLM; n=50, mixed gender) and pooled human liver

86 cytosol (HLCYT, mix gender) were purchased from Tebu Bio (Boechout, Belgium) and Sigma-

87 Aldrich (Bornem, Belgium), respectively. RDP standard (98% purity) was obtained from TRC

88 (Toronto, Canada); reduced β-nicotinamide adenine dinucleotide 2'-phosphate (NADPH;

89 purity>95%), uridine 5'-diphosphoglucuronic acid (UDPGA; purity>98%), adenosine 3'-

90 phosphate 5'-phosphosulfate (PAPS; purity>96%), DPHP and TPHP-d_{15} (used as internal

91 standard, IS) were purchased from Sigma–Aldrich (Bornem, Belgium). The standard TPHP was

92 purchased from Chiron AS (Trondheim, Norway). The DPHP-d_{10} standard (also used as IS) was

93 custom synthesized by Dr. Vladimir Belov (Max Planck Institute, Göttingen, Germany).

94 Acetonitrile, methanol and ammonium acetate were purchased from Merck KgA Chemicals

95 (Darmstadt, Germany). *para*-HO-TPHP was synthesized by the Max Planck Institute (Göttingen,

96 Germany) with a purity of 98%. *meta*-HO-TPHP was synthesized by the Organic Chemistry

97 Synthesis Group from Duke University (NC, USA) and kindly donated by prof. Heather Stapleton

98 (Duke University, NC, USA). Ultrapure water (18.2 MΩ) was obtained using an Elga LabWater

99 water purification instrument (Saint Maurice, France). Oasis WAX cartridges (3 cc, 60 mg, 30

100 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 (n° oligomers =2) and 38 for RDPn3 (n° oligomers =3).

123

In vitro biotransformation and chemical hydrolysis assays

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

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

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

145   membrane-bound UGTs present inside the HLM phospholipid bilayer.[15] The reaction mixtures

146   were pre-incubated for 5 min in a shaking water bath at 37°C. The glucuronidation reaction was

147   started by adding UDPGA (1 mM, final concentration). A new aliquot of UDPGA was added after

148   1 h incubation to ensure sufficient cofactor amount throughout the experiment.

149   **4) HLCYT metabolism by SULT enzymes**: Samples were prepared as described above for UGT

150   experiments omitting the addition of alamethicin, using HLCYT (0.5 mg/mL, final concentration)

151   instead of HLM and starting the reaction by addition of PAPS (0.1 mM, final concentration)

152   instead of UDPGA.

153   All CYP, UGT and SULT reactions were quenched after 2 h by adding 1 mL of ice-cold acetonitrile

154   and placing the tubes on ice. Ammonium acetate (155 mg) was added to each tube (~2 M in

155   water). The solution was vortex-mixed for 1 min and then ultracentrifuged (8,000 rpm, 30s).

156   After the phase-separation by salting-out with ammonium acetate, the acetonitrile layer was

157   separated and transferred into a new glass tube. The liquid-liquid extraction was done 3 times

158   and all the supernatants (3 mL acetonitrile) combined and evaporated to dryness (N_2, 40°C).

159   The extracts were reconstituted in 150 µL of methanol containing the internal standards DPHP-

160   d_{10} and TPHP-d_{15} to a final concentration of 100 ng/mL. The extracts were vortexed for 20 s and

161   transferred into a vial for analysis.

162   Cofactor and enzyme negative control samples were routinely prepared for every batch of

163   experiments concerning CYP, UGT and SULT reactions as described above, but devoided of the

164   specific cofactor or enzyme of interest in each case. Within each batch of experiments, blanks

165   containing only the buffer underwent the whole procedure (incubation+liquid-liquid

166   extraction). Background contamination was not found in any of the blanks.

167   After the screening phase, and to further confirm the formation profiles of the hydroxy

168   metabolites of RDP, a second set of experiments (profiling) was conducted over a range of

169   incubation time values (0 to 60 min) at 0.1 mg/mL HLM; HLM concentration (0.025 to 0.5

170   mg/mL) for 15 min and RDP concentration (0.5-50 µM) at 0.1 mg/mL HLM and 15 min

171   incubation time.

172

173   ***LC-QTOF MS analysis method***

174   RDP metabolites produced by CYP, UGT and SULT enzymes and hydrolysis products

175   were identified by LC–HRMS using a 1290 series LC system coupled to a 6530 Q-TOF-HRMS

176   (Agilent Technologies) equipped with an ESI Jet Stream ion source. The chromatographic

177   separation of the hydroxy metabolites was achieved by using an Agilent Extend Zorbax C18

178   column (50 x 2.1 mm, 3.5 µm) and the following mobile phase composition: 2 mM ammonium

179   acetate in water (A) and acetonitrile (B). The gradient elution for the hydroxy metabolites and

180   the hydrolysis products was 5% B during 1 min, linear gradient from 5 to 45% B from 1 to 3 min,

181   linear gradient from 45 to 55% B from 3 to 5 min, isocratic conditions at 55% B from 5 to 5.8

182   min, linear gradient from 55 to 80% B from 5.8 to 8.5 min and linear gradient from 80 to 90% B

183   from 8.5 to 12 min. Different stationary and mobile phases were necessary for achieving good

184   separation of the glucuronidated and sulfated metabolites. The column employed was a

185   Agilent Eclipse Plus C8 column (150 x 2.1 mm, 3.5 µm) and the mobile phase was 2 mM

186   ammonium acetate in water (A) and methanol (B). The gradient elution was as follows: isocratic

187   conditions at 5% B during 5 min, linear gradient from 5 to 80% B from 0 to 20 min, then linear

188   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.

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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 °C) 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]

255 A variety of hydrolysis products of RDP and its oligomers were identified, including DPHP, RDP-

256 [Phe] (loss of a phenyl ring from RDP), *meta*-hydroxy-TPHP, *meta*-hydroxy-RDP and *meta*-

257 hydroxy-RDPn2. They were all present as impurities in the RDP standard, probably due to its

258 hydrolytic instability, and increased after the incubation at 37°C in the buffer. The *meta*-

259 position of the hydroxy group is the only possible considering the structure of RDP and the

260 hydrolytic breakdown of the molecule as shown in Figure 1. Although not specified in the

261 Figure, each hydrolysis product may breakdown in the lower mass products, e.g., *meta*-

262 hydroxy-RDPn2 could be hydrolyzed further into *meta*-hydroxy-RDP and then into *meta*-

263 hydroxy-TPHP and successively until DPHP. The compound RDPn2-[Phe] was only minor.

264 All hydrolysis products were identified with a mass accuracy <10 ppm. Further MS/MS

265 experiments for structure confirmation by specific fragments, showed DPHP as the main

266 fragment in *meta*-hydroxy-TPHP and in RDP-[Phe] and the fragment corresponding to a loss of a

267 phenyl ring (or RDP-[Phe]) as the main fragment for *meta*-hydroxy-RDP and for RDPn2-[Phe].

268 The identities of *meta*-hydroxy-TPHP and DPHP were also confirmed by the injection of

269 authentic standards and by matching MS spectra and retention times. Figure 1 shows all

270 identified hydrolysis products of RDP.

271 The hydrolysis products or impurities were present in the same amounts by incubations

272 containing HLM and by those containing only buffer, so that they were mainly produced by

273 chemical hydrolysis and not by enzymatic reactions (see Figure S-1). *Meta*-hydroxy-TPHP and of

274 DPHP was calculated with their authentic standard as 135±10 and 36±5 pmol per nmol of RDP

275 for a hydrolysis time of 30 min at concentrations of 1-20 µM RDP.

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Hydroxy metabolites of RDP

280   In the HLM incubations, we observed a number of hydroxy metabolites that were not

281 present in the cofactor, neither in the enzyme negative controls. This confirms the formation of

282 these metabolites by NADPH-consuming enzymes, most probably CYP. A hydroxy-metabolite

283 [HO-RDP (1)], a di-hydroxy-metabolite [di-HO-RDP] and a hydroxy- and a di-hydroxy-triphenyl

284 phosphate [HO-TPHP(1), di-HO-TPHP] were identified. Figure 2 shows all the identified

285 metabolites of RDP.

286 All identified metabolites complied with the rule of mass accuracy, meaning that the parent

287 compound was identified with a mass error below 10 ppm, as shown in Table S-2. MS/MS

288 experiments confirmed the structure of the identified metabolites. Fragmentation spectra

289 showed the structure-specific fragment diphenyl phosphate (DPHP) as the main ion in hydroxy-

290 and dihydroxy-TPHP and another specific fragment ($[C_{12}H_{11}O_4P]$ - the hydrolysis product named

291 as RDP-[Phe]) corresponding to a loss of a phenyl ring from hydroxy-RDP and dihydroxy-RDP.

292 Regarding the oligomers of RDP, only the hydroxy-metabolite of RDPn2 could be detected as a

293 minor metabolite, not being present in the cofactor neither in the enzyme negative controls.

294 This is in agreement with the fact that the oligomers are less abundant that the monomer in the

295 RDP formulation[2] as explained in the experimental section. Differently from TPHP and RDP, we

296 did not detect a dihydroxy metabolite derived from the RDP dimer. This could be just due to the

297 limited sensitivity of the method that was lower for the dihydroxy metabolites in comparison

298 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.

320   DPHP was also formed as O-dearylation metabolite at concentrations significantly higher than

321   those produced by chemical hydrolysis at long incubation times (>2 h), both in HLM and in

322   cofactor negative controls. Although no differences in the DPHP formation were observed at

323   incubation times <60 min (see Figure S-1), the formation of DPHP was higher with HLMs and

324   cofactor negative controls than by chemical hydrolysis at longer times (e.g. around 65, 52 and

325   35 pmol of DPHP per nmol of RDP were formed in 0.2mg/mL HLM, cofactor negative controls

326   and in only buffer incubations of 3 h, values are an average of three independent experiments;

327   relative standard deviations below 10%; 10 µM of RDP). This suggests that DPHP is also a

328   metabolite and not only a hydrolysis product of RDP as occurring for other PFRs, namely TPHP[11]

329   and EHDPHP.[12] The presence of DPHP in cofactor negative controls suggests that not only CYP

330   enzymes (HLM), but also NADPH-independent liver microsomal phosphatase enzymes, such as

331   paraoxonases and aryl esterases may be involved.[19]

332

333   ***Glucuronidated and sulfated metabolites***

334   By incubating the buffer containing the hydroxy metabolites of RDP and/or only the

335   hydrolysis products with pooled HLM or HLCYT, alamethicin and UDPGA or PAPS, several new

336   chromatographic peaks appeared. None of these peaks were detected in HLM/HLCYT or

337   cofactor negative controls. We could thus conclude that several sulfated and glucuronidated

338   metabolites were formed (Figure 2). These conjugates arose from the metabolism of hydroxy-

339   and dihydroxy-TPHP and RDP metabolites by UGT and SULT enzymes and also from the

340   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

384   investigations, it would be necessary to include also the contributions of RDP and the recently

385   reported EHDPHP[12], both ubiquitous in house dust.

386   The results are also consistent with the reported study about the *in vivo* metabolism and

387   disposition of RDP (rat, monkey and mice) that was published in 2000.[13] The major fecal

388   metabolites were dihydroxy-TPHP, hydroxy-TPHP, dihydroxy-RDP, and hydroxy-RDP. Major

389   urinary metabolites were identified as resorcinol, resorcinyl glucuronide, and resorcinyl sulfate.

390   So, specific sulfated or glucuronidated metabolites of RDP were not reported. These low

391   molecular weight compounds could come also from a variety of other compounds, such as

392   TPHP, DPHP or phenol. The specific sulfated and glucuronides metabolites of RDP identified in

393   our study provide a better understanding of the metabolic pathway of RDP. Besides, a hydroxy

394   metabolite for the RDP dimer and a variety of hydrolysis products or impurities are also

395   reported in this study for the first time.

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Supporting Information Available

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

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**Figure 1.** *Hydrolysis products of RDP and RDP oligomers*
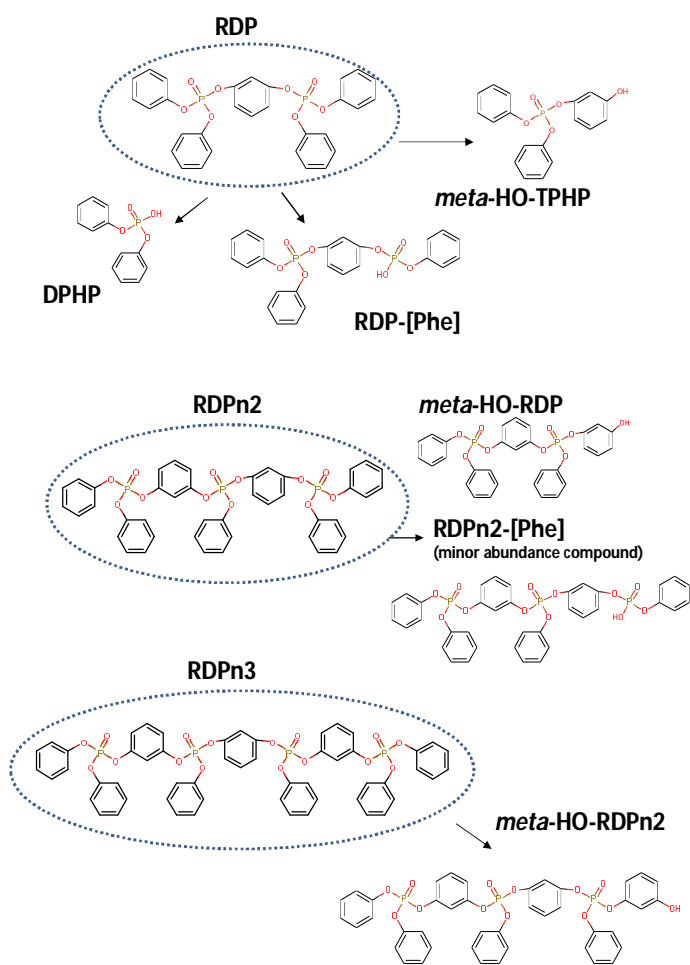
484 **Figure 2**. *Metabolites of A) RDP and the B) dimer RDPn2 identified in this study. Those derived from TPHP*
485 *(RDP impurity) and meta-HO-TPHP (RDP hydrolysis product) are also shown in A), since they are present*
486 *in RDP solutions. (Abbreviations: HYD, chemical hydrolysis; PHO?, phosphatases, possible pathway but*
487 *not confirmed)*

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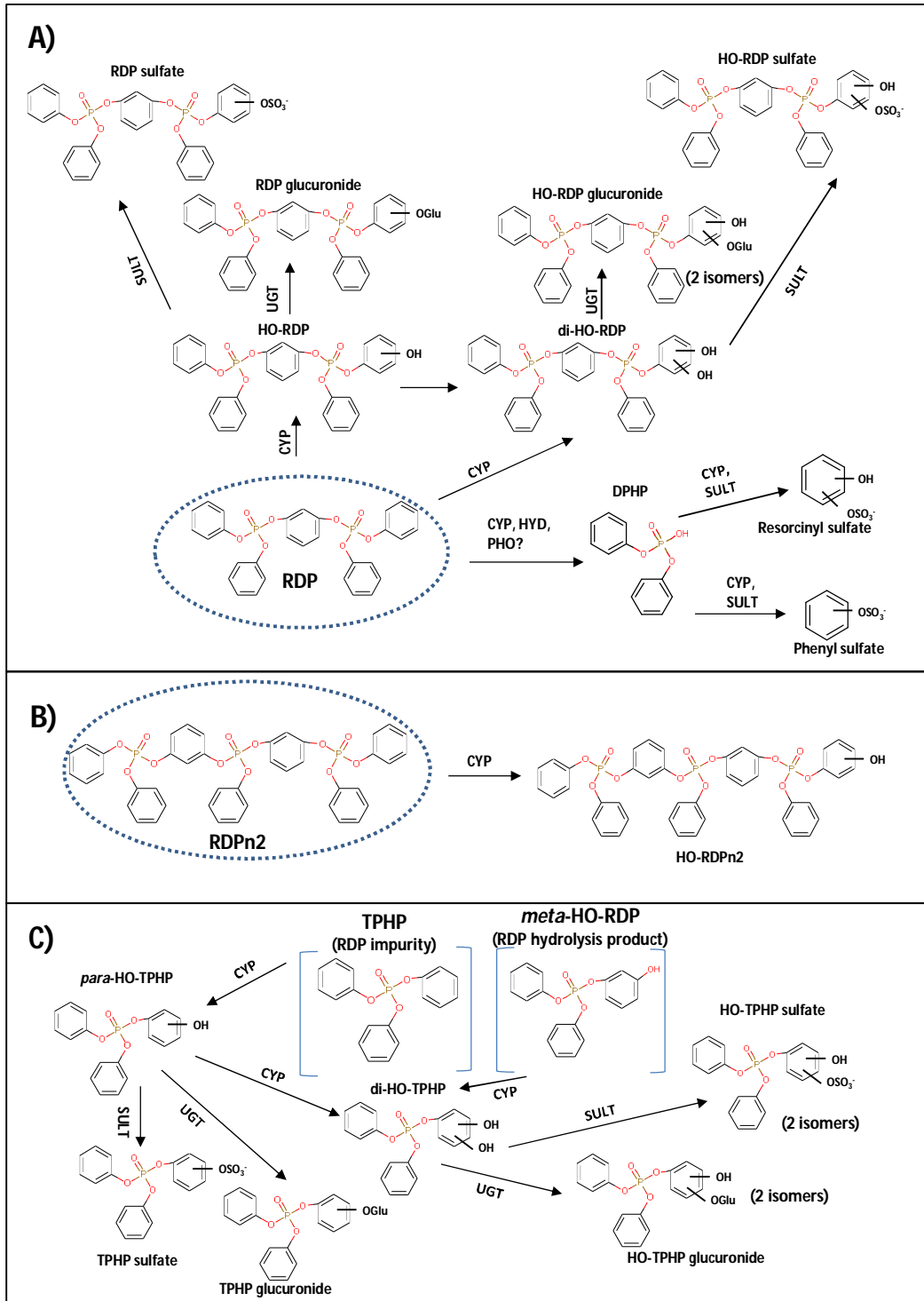
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490   *Figure 3. Relative abundance expressed as MS signal (main transition, QQQMS) of RDP metabolites.*
491   *Conditions: 3 h incubation for CYP-mediated reaction and 3 h incubation for UGT- or SULT-mediated*
492   *reactions, 0.3 mg/mL HLM, 20 µM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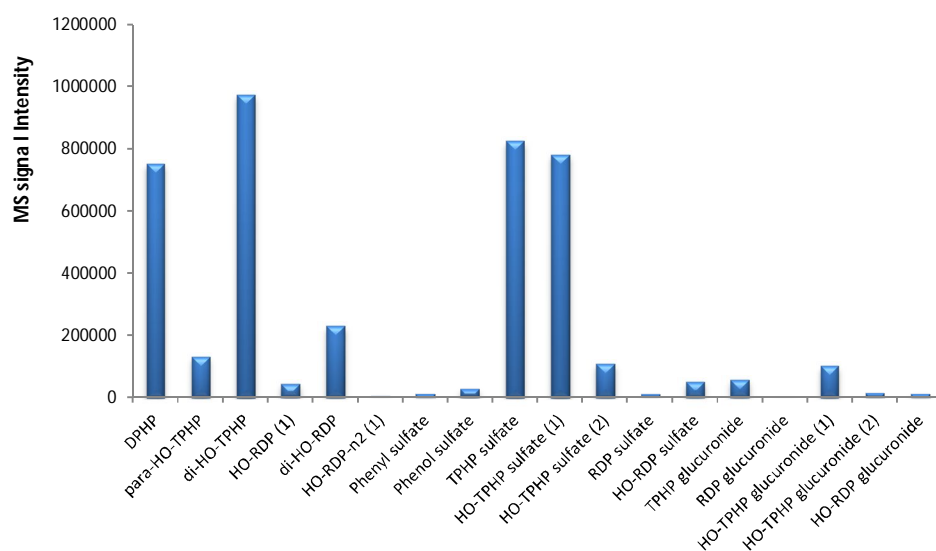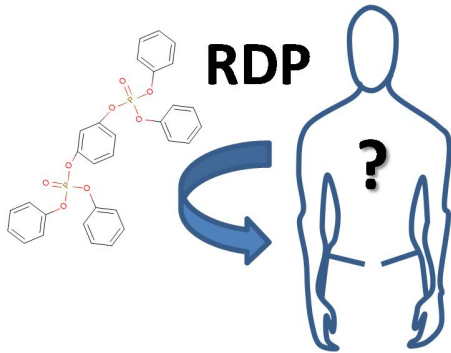

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495 **TOC/Abstract Art**

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