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CASE STUDY ON THE USE OF INTEGRATED APPROACHES FOR TESTING AND ASSESSMENT TO INFORM READ-ACROSS OF p-ALKYLPHENOLS: REPEATED-DOSE TOXICITY

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CASE STUDY ON THE USE OF INTEGRATED APPROACHES FOR TESTING AND ASSESSMENT FOR 90-DAY RAT ORAL REPEATED-DOSE TOXICITY OF CHLOROBENZENE-RELATED CHEMICALS

IOMC INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

Environment Directorate

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

Paris 2020

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OECD Environment Directorate,
Environment, Health and Safety Division
2, rue André-Pascal
75775 Paris cedex 16
France
Fax : (33-1) 44 30 61 80
E-mail : ehscont@oecd.org

Forward

OECD member countries have been making efforts to expand the use of alternative methods in assessing chemicals. The OECD has been developing guidance documents and tools for the use of alternative methods such as (Q)SAR, chemical categories and Adverse Outcome Pathways (AOPs) as a part of Integrated Approaches for Testing and Assessment (IATA). There is a need for the investigation of the practical applicability of these methods/tools for different aspects of regulatory decision-making, and to build upon case studies and assessment experience across jurisdictions.

The objective of the IATA Case Studies Project is to increase experience with the use of IATA by developing case studies, which constitute examples of predictions that are fit for regulatory use. The aim is to create common understanding of using novel methodologies and the generation of considerations/guidance stemming from these case studies.

This case study was developed by Kao Corporation (BIAC) for illustrating practical use of IATA and submitted to the 2019 review cycle of the IATA Case Studies Project. This case study was reviewed by the project team. The document was endorsed at the 4th meeting of the Working Party on Hazard Assessment in June 2020.

The following case study was also reviewed in the project in 2019:

1. CASE STUDY ON USE OF AN INTEGRATED APPROACH TO TESTING AND ASSESSMENT (IATA) AND NEW APPROACH METHODS TO INFORM A THEORETICAL READ-ACROSS FOR DERMAL EXPOSURE TO PROPYLPARABEN FROM COSMETICS, ENV/JM/MONO(2020)16.
2. CASE STUDY ON THE USE OF INTEGRATED APPROACHES FOR TESTING AND ASSESSMENT FOR SYSTEMIC TOXICITY ARISING FROM COSMETIC EXPOSURE TO CAFFEINE, ENV/JM/MONO(2020)17.
3. CASE STUDY ON THE USE OF INTEGRATED APPROACHES FOR TESTING AND ASSESSMENT FOR 90-DAY RAT ORAL REPEATED-DOSE TOXICITY OF CHLOROBENZENE-RELATED CHEMICALS, ENV/JM/MONO(2020)18.
4. CASE STUDY ON THE USE OF INTEGRATED APPROACHES TO TESTING AND ASSESSMENT FOR PREDICTION OF A 90 DAY REPEATED DOSE TOXICITY STUDY (OECD 408) FOR 2-ETHYLBUTYRIC ACID USING A READ-ACROSS APPROACH FROM OTHER BRANCHED CARBOXYLIC ACIDS, ENV/JM/MONO(2020)20.
5. CASE STUDY ON THE USE OF INTEGRATED APPROACHES TO TESTING AND ASSESSMENT FOR READ-ACROSS BASED FILLING OF DEVELOPMENTAL TOXICITY DATA GAP FOR METHYL HEXANOIC ACID, ENV/JM/MONO(2020)21.
6. CASE STUDY ON THE USE OF INTEGRATED APPROACHES TO TESTING AND ASSESSMENT FOR IDENTIFICATION AND CHARACTERISATION OF PARKINSONIAN HAZARD LIABILITY OF DEGUELIN BY AN AOP-BASED TESTING AND READ ACROSS APPROACH, ENV/JM/MONO(2020)22.

7. CASE STUDY ON THE USE OF INTEGRATED APPROACHES TO TESTING AND ASSESSMENT FOR MITOCHONDRIAL COMPLEX-III-MEDIATED NEUROTOXICITY OF AZOXYSTROBIN - READ-ACROSS TO OTHER STROBILURINS, ENV/JM/MONO(2020)23.

These case studies are illustrative examples, and their publication as OECD monographs does not translate into direct acceptance of the methodologies for regulatory purposes across OECD countries. In addition, these cases studies should not be interpreted as official regulatory decisions made by the authoring member countries.

In addition, a considerations document summarising the learnings and lessons of the review experience of the case studies is published with the case studies:

REPORT ON CONSIDERATIONS FROM CASE STUDIES ON INTEGRATED APPROACHES FOR TESTING AND ASSESSMENT (IATA) -Fifth Review Cycle (2019) -, ENV/JM/MONO(2020)24.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology.

Abstract

The general purpose of this case study is to develop the Integrated Approaches to Testing and Assessment (IATA) for repeated dose toxicity (RDT) and suggest key elements for the read-across that may be used for regulatory purposes in member countries. Furthermore, the specific purpose is to fill the existing gap in available data of the sub-chronic rat oral RDT for para-alkylphenols (p-alkylphenols) based on the category approach combined with the use of mode of action (MoA)/adverse outcome pathway (AOP)-based *in vitro* methods. The liver was selected as the main target organ.

In this case study, p-alkylphenols were selected as category members to predict No-Observed-Adverse-Effect Level (NOAEL) values using the read-across approach. It is well known that p-Alkylphenols could induce hepatotoxicity by forming the reactive metabolite quinone methide (QM) and its binding to nucleophiles such as glutathione (GSH). To assess the toxicity of p-alkylphenols based on the similarity of metabolism, category members were sub-grouped based on common alkyl substituents, and their toxicity was assessed by integrating *in silico*, *in vitro*, and *in vivo* data. First, various *in silico* tools were used to simulate structural alerts, intestinal absorption, and metabolism. Next, dansylated GSH (dGSH) binding and cell viability assays were performed to investigate how much reactive metabolites including QM bound with the parent compound and to study the effect on cytotoxicity *in vitro*. As a result, structural alerts for hepatotoxicity and the rate of intestinal absorption were very similar among all category members, whereas metabolism and biological responses were different. The amount of dGSH adducts and cytotoxicity decreased according to the elongation or structural hindrance at the 4-position. These data suggest that QM-induced hepatotoxicity would be prevented by structurally hindered substituents at the 4-position. Considering the chemical reactivity of substituents, the lowest NOAEL values of source members were used to target other members with more complex structures.

Overall, the results of this case study suggest that the read-across under the category approach, considering the similarity of MoA/AOP, focused mainly on metabolism could support to predict NOAEL values for a specific target organ, considering the data gaps between *in vitro* and *in vivo*. However, *in vitro* methods were not enough to evaluate toxicokinetics/dynamics quantitively and fill gaps between *in silico*, *in vitro*, and *in vivo* data. In the future, additional *in vitro* or new approach methodologies (NAMs) will need to be combined to improve IATA-based read-across.

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1. Introduction

On March 11, 2013, the European Union (EU) completed the institution of the ban on the sale of animal-tested cosmetics after that date. Subsequently, over 40 countries worldwide have banned or restricted animal testing of cosmetics and cosmetic ingredients. Consequently, alternative assessment methods to animal testing have been developed with various endpoints. For example, the European Chemicals Agency (ECHA) recommends the use of alternative methods under the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation, especially having endpoints such as skin corrosion/irritation, serious eye damage/irritation, and skin sensitisation. Therefore, animal testing should be avoided in the development of cosmetics and chemical registration processes.

Systemic toxicity refers to systemic adverse effects (AEs) that occur in one or more organs (e.g., liver, kidney, etc.) or systems (such as the nervous, immune, and circulatory systems) by single (acute toxicity) or repetitive exposure (repeated dose toxicity, RDT). This toxicity is one of the most challenging endpoints to evaluate without animal testing because of the complicated underlying mechanism. Under such conditions, Integrated Approaches to Testing and Assessment (IATA) contribute to achieving a conclusion with high scientific validity on non-animal risk assessment. More specifically, read-across is an alternative method for filling data gaps based on an analogue or categorical approach (ECHA, 2017), which allows quantitative (e.g., No-Observed-Adverse-Effect Level [NOAEL]) and qualitative (e.g., target organ and absorption, distribution, metabolism, and excretion [ADME]) evaluation. The purpose of this case study was to verify the usefulness of read-across using a MoA/AOP-based IATA to establish a specific read-across strategy that would be acceptable for chemical substance registration.

p-Alkylphenols are compounds in which benzene is substituted with a hydroxyl (OH) and an alkyl (CnH_2n+_1) group, and they have various 4-alkyl substituents (e.g., butylated hydroxytoluene [BHT] has tert-butyl at the 2, 6-positions and a methyl group at the 4-position). The main industrial applications of alkylphenols are as raw materials for nonionic surfactants, plastics, resins, pesticides, perfumes, and photoresistant substances (photographic colour forming agents) and as antioxidants.

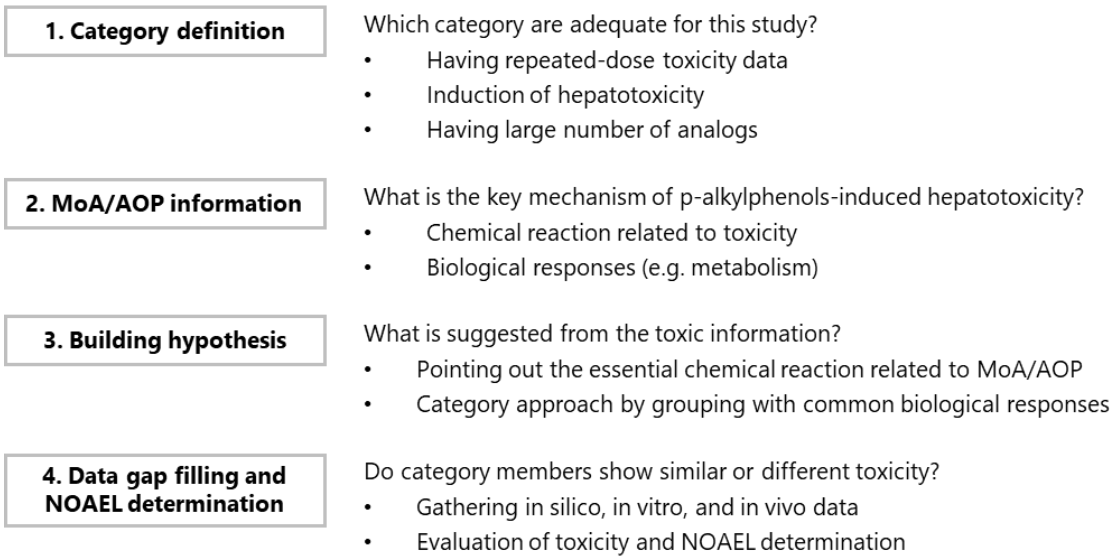
In this case study, the 28-day rat oral repeated dose endpoint of p-alkylphenols was predicted for a specific target organ using MoA/AOP-related *in silico* and *in vitro* data. This case study can contribute to refine read-across approach for the risk assessment of systemic toxicity without animal testing under the REACH regulation.

2. Purpose

2.1. Purpose of use

The general purpose of drafting this case study is to provide further insight into the Organisation for Economic Co-operation and Development (OECD) guidance on IATA. Furthermore, the specific purpose is to verify the usefulness of the read-across approach using MoA/AOP-based IATA to establish a specific and acceptable read-across strategy for chemical substance registration. To develop alternative methods for systemic toxicity, this case study is drafted with the aim to establish an IATA-based read-across approach for 28-day repeated dose toxicity with a focus on hepatotoxicity as the target organ. The target chemicals are p-alkylphenols with a wide industrial application and much information on MoA/AOP. The read-across workflow is summarised in Figure 1.

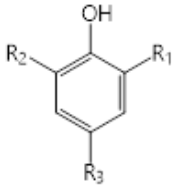
Figure 1. Overview on the four main assessment steps of read- across workflow



2.2. Target chemical(s)/category definition

The target chemicals in this case study are 2,4,6-tri-alkylphenols, which are classified as p-alkylphenols, and their identified structures are presented in Figure 2. Substituents at the 2,6-positions (R_1 and R_2) are defined as straight or structurally hindered alkyl groups or both (C_nH_{2n+1}, $1 \leq n \leq 4$), and R_3 is defined as a straight alkyl chain (C_nH_{2n+1}, $1 \leq n \leq 8$). The category members that meet the endpoints (28-day rat oral repeated dose) and had enough analogues are identified by searching HESS v.3.8, ECHA, COSMOS, AMBIT, and ToxCast library databases. In addition, category members with the same substituents at the 2,6-positions are sub-grouped to allow application of the category approach to read-across. The final category members and grouping definition are listed in Table 1.

Figure 2. Defined structure of 2,4,6-tri-alkylphenols in this study



R_1, R_2, R_3 = alkyl group (C_nH_{2n+1})

(carbon number at R_1 and R_2 $1 \leq n \leq 4$; at R_3 $1 \leq n \leq 8$)

2.3. Endpoint(s)

The target endpoint is sub-chronic repeated dose toxicity (28-day exposure period) via the oral route in rats. In addition, the liver was chosen as the target endpoint because several studies suggest that quinone methide formation via oxidisation of p-alkylphenols induces oxidative stress induced hepatotoxicity and the primary target organ of p-alkylphenols is the liver. Therefore, in this study, the NOAEL values of the target chemicals are predicted based on hepatotoxicity, which are determined by histopathological changes such as hypertrophy/swelling, hyperplasia, degeneration, necrosis of hepatocytes, and proliferation of bile-duct cells. The liver is a major target organ in systemic toxicity and is susceptible to toxic effects at minimally administered concentrations. On the other hand, toxic effects in multiple organs and other tissues often appear at high concentrations, such as the maximum dose concentration. In this case study, common toxicological effects other than hepatotoxicity are not observed between the p-alkylphenols and information regarding the toxicological effects other than hepatotoxicity is limited. Therefore, the NOAEL values of hepatotoxicity of 2,4,6-tri-alkylphenols defined as “predicted NOAEL (liver) values” are predicted in this case study.

3. Hypothesis for category approach

p-Alkylphenols with at least one benzylic hydrogen may be oxidised to para-quinone methides (p-QMs) by cytochrome P450 (Tajima *et al.,* 1985; Thompson D., *et al.* 1990). The oxidation could occur by two successive one-electron transfers from the aromatic π system via phenoxy radical intermediate. QMs are electrophilic with a positive charge density centred mainly on the exocyclic methylene carbon (Turner, 1964). QMs are formed from the two-electron oxidation of phenols with ortho or para methyl or alkyl groups. This metabolite has tendency to undergo Michael addictions with nucleophiles such as glutathione (GSH) to form benzylic adducts (Bolton *et al.,* 1992; Monks and Jones, 2002; Thompson and Moldéus, 1991).

GSH, a major antioxidant and cell signal regulator, is generally maintained at a relatively constant concentration through a balance between its synthesis and turnover. However, GSH depletion in the hepatocytes could cause oxidative stress through tumour necrosis factor (TNF)-α and FasL killing, nuclear factor (NF)-κB, c-Jun N-terminal kinase (JNK), and mitochondrial cell death pathways. These effects could lead to various types of liver injury such as steatosis, hypertrophy, and necrosis *in vivo* (Yuan and Kaplowitz, 2009). These QM-induced hepatotoxic mechanisms are summarised as MoA/AOP of p-alkylphenols in Figure 3.

In case of p-alkylphenols, a variety of alkyl substituents could affect the formation and reactivity of QMs (Bolton *et al.,* 1995). As for the reactivity of compounds with substituents at the 4-positions, one study revealed that 4-sec-butylphenol was more toxic than 4-tert-butylphenol because of its ability to form QM. Another study showed that the reactivity of a series of QM compounds was BHT-QM > 4-ethyl-BHT-QM (E-BHT-QM) >4-isopropyl-BHT-QM (I-BHT-QM), and that toxicity was also observed following covalent binding to rat liver microsomal and tissue slide proteins (Reed *et al.,* 2001). On the other hand, one study reported that quinone methides were generated by disproportionation of phenoxy radicals generated by the ferricyanide oxidation of 2,6-di-tert-butyl-4-isopropylphenol, 2,6-di-tert-butyl-4-sec-butylphenol, 4-(butan-2-yl)-2-tert-butyl-6-methylphenol (Toteva and Richard, 2011). Therefore, it can be hypothesised that more complicated 4-alkyl substituent could reduce the potency of QM formation and related hepatotoxicity such as GSH depletion and hepatocellular toxicity.

The 2,6-Alkyl substituents may also affect the reactivity of QM. Large, hydrophobic alkyl substituents adjacent to the oxo group provide no stabilising influence and effectively prevent solvent interactions with the carbonyl oxygen. Therefore, 2,6-di-tert-butyl-4-methylphenol (BHT; member No. 1, CAS No. 128-37-0) with QM (BHT-QM) should exist mainly in the uncharged form and undergo normal Michael additions of nucleophiles. In addition to structural factors responsible for altering QM reactivity, data on the enzymatic conversion of phenolic compounds to these electrophilic metabolites have been obtained using rat liver microsomes and isolated rat hepatocytes (Bolton *et al.,* 1992). 2,4-Dimethyl-6-tert-butylphenol (BDMP; member No. 2, CAS No. 1879-09-0) destroyed hepatocyte viability within 1–2 h, as measured by the loss of plasma membrane integrity, but BHT (member No. 1, CAS No. 128-47-0) and 2,4,6-trimethylphenol (TMP; member No. 3, CAS No. 527-60-6) had little or no effect at a similar concentration range. In this context, variety of 2,6-alky substituents has to be considered for the read-across in this case study.

Taken above toxicological information, the following two points are considered as important hypothesis.

Hypothesis 1: Category members with QM formation could promote hepatotoxicity.

Hypothesis 2: The 4-alkyl structure could affect the strength of hepatotoxicity of 2,4,6-tri-alkylphenols. Especially, more complicated 4-alkyl substituent could reduce the potency of QM formation and related hepatotoxicity such as GSH depletion and hepatocellular toxicity.

In this case study, therefore, category approach is applied for the read-across by classifying the category members with 4-alkyl structure; "QM formation group" (member No. 1–14) and "QM not formation group" (member No. 15–20). As for metabolism, it is expected that chemicals with longer, branched alkyl chain, or a tert-butyl group at the 4-position could have low potency of QM formation and GSH depletion whereas QM formation is no longer possible with a tert-butyl group at the 4-position. This implies that these category members with hindered structure at the 4-poisiton would show different MoA/AOP shown in Figure 3.

4. Category members

4.1. Identification and selection of category members

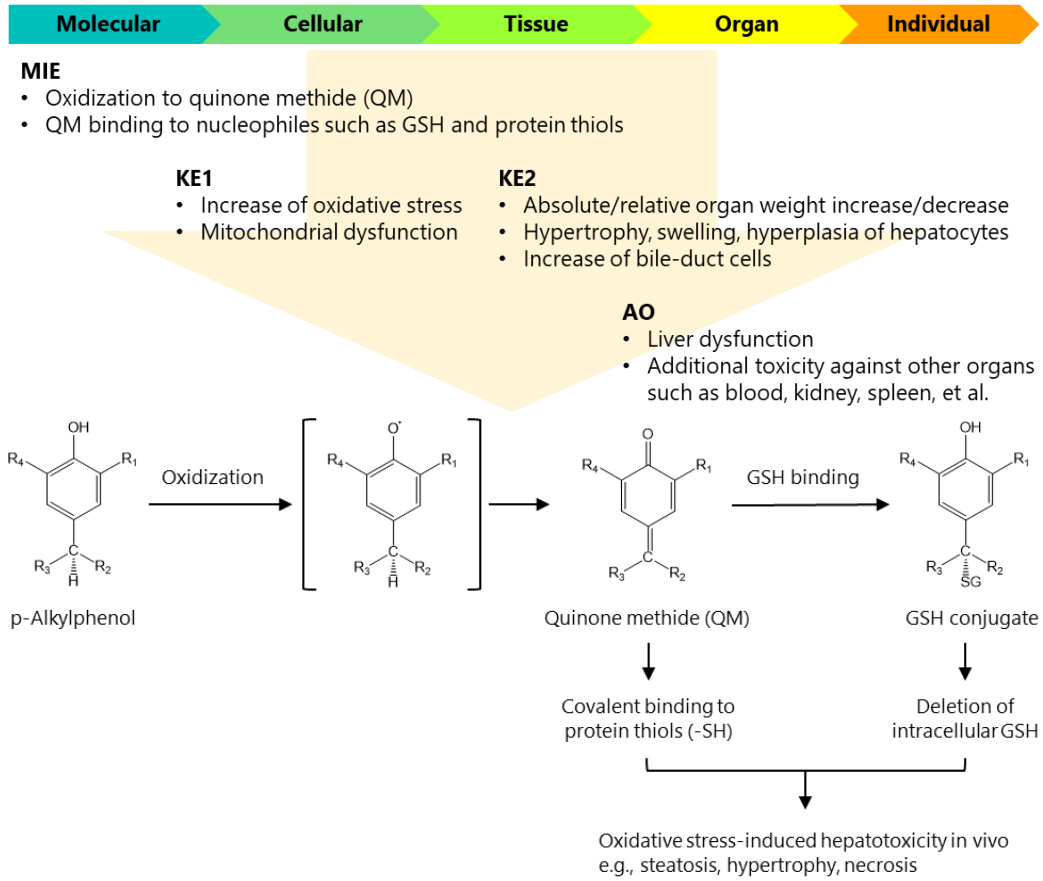
First, the Hazard Evaluation Support System Integrated Platform (HESS) v.3.8[1]. was used to select target category referring to chemical boundaries with endpoints (e.g., hepatotoxicity, nephrotoxicity, and neurotoxicity). In this study, the category with hepatotoxicity and enough information on MoA/AOP was selected, and p-alkylphenols were identified as target category members. Then, additional analogues were identified by searching HESS v.3.8, ECHA[2], Integrated *In silico* Models for the Prediction of Human Repeated Dose Toxicity of COSMetics to Optimise Safety[3] (COSMOS), AMBIT[4], PubMed[5], and Environmental Protection Agency (EPA) ToxCast Screening Library[6] databases.

Finally, 2,4,6-tri-alkylphenols with straight or structurally hindered substituents at the 2,4,6-positions or both are identified as category members. Carbon numbers at the 2,6- and 4-positions are defined as $1 \leq n \leq 4$ and $1 \leq n \leq 8$, respectively (Figure 2). Total 20 chemicals are defined as category members (listed in Table 1), and their MoA/AOP is summarised in Figure 3.

[1] Hazard Evaluation Support System Integrated Platform (HESS): https://www.nite.go.jp/en/chem/qsar/hess-e.html
[2] ECHA: https://echa.europa.eu/search-for-chemicals
[3] Integrated *in silico* Models for the Prediction of Human Repeated Dose Toxicity of COSMetics to Optimise Safety (COSMOS): http://www.cosmostox.eu/what/COSMOSdb/
[4] AMBIT: https://ambitlri.ideaconsult.net/tool2/ui
[5] PubMed: http://www.ncbi.nlm.nih.gov/pubmed
[6] Environmental Protection Agency (EPA) ToxCast Screening Library: https://comptox.epa.gov/dashboard/chemical_lists/toxcast

Figure 3. Overview of mode of action (MoA)/adverse outcome pathway (AOP) of p-alkylphenols

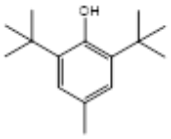
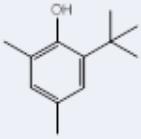
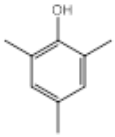
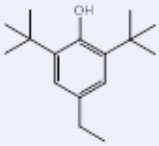
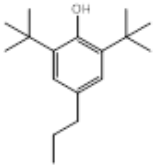
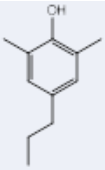
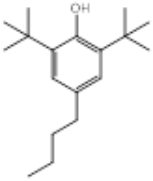
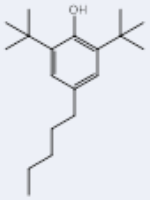
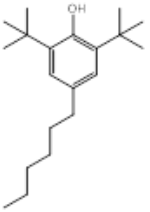
MoA/AOP contain a molecular initiating event (MIE), some key events (KEs), and an adverse outcome (AO). p-Alkylphenols are absorbed through the gastrointestinal tract. They then reach the liver and are oxidised to the intermediate quinone methide (QM). QM attacks nucleophiles such as glutathione (GSH) and binds to protein thiols. As a result, oxidative stress is caused by depletion of intracellular GSH and dysfunction of normal proteins. Then, various oxidative stress and hepatotoxicity could occur *in vivo*.

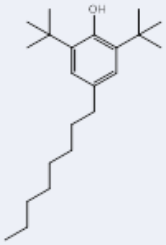
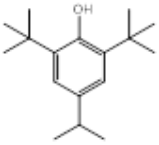
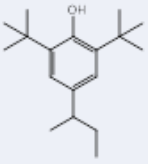
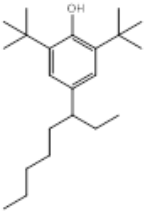
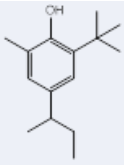
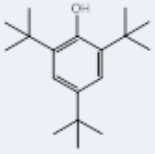
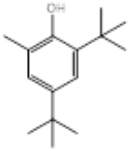
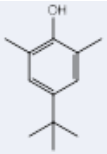
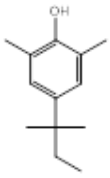


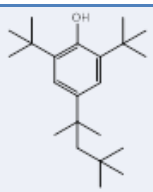
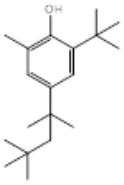
4.2. List of category members

All category members and the definition of grouping are shown in Table 1.

Table 1. 2,4,6-tri-Alkylphenol category members in this case study

Member No.	Name	CAS No.	Chemical structure	Endpoint data*
QM formation group				
1	2,6-Di-tert-butyl-4-methylphenol, BHT	128-37-0		Yes (source)
2	2,4-Dimethyl-6-tert-butylphenol, BDMP	1879-09-0		Yes (source)
3	2,4,6-Trimethylphenol, TMP	527-60-6		Yes (source)
4	2,6-Di-tert-butyl-4-ethylphenol	4130-42-1		Yes (source)
5	2,6-Di-tert-butyl-4-propylphenol	4973-24-4		No (target)
6	2,6-Dimethyl-4-propyl-phenol	13037-82-6		No (target)
7	2,6-Di-tert-butyl-4-butylphenol	5530-30-3		No (target)
8	2,6-Di-tert-butyl-4-pentylphenol	4973-26-6		No (target)
9	2,6-Di-tert-butyl-4-hexylphenol	56280-62-7		No (target)

10	2,6-Di-tert-butyl-4-octylphenol	35309-87-6		No (target)
11	2,6-Di-tert-butyl-4-isopropylphenol	5427-03-2		No (target)
12	2,6-Di-tert-butyl-4-sec-butylphenol	17540-75-9		Yes (source)
13	2,6-Di-tert-butyl-4-(2-ethylhexyl)phenol	816462-78-9		No (target)
14	4-(Butan-2-yl)-2-tert-butyl-6-methylphenol	51067-63-1		No (target)
QM not formation group				
15	2,4,6-Tri-tert-butylphenol	732-26-3		Yes (source)
16	2,4-Di-tert-butyl-6-methylphenol	616-55-7		No (target)
17	2,6-Dimethyl-4-tert-butylphenol	879-97-0		No (target)
18	2,6-Dimethyl-4-(2-methylbutan-2-yl)phenol	91798-63-9		No (target)

19	2,6-Di-tert-butyl-4-(1,1,3,3-tetramethylbutyl)phenol	65796-87-4		No (target)
20	4-(1,1,3,3-tetramethylbutyl)-2-(1,1-dimethylethyl)-6-methylphenol	104066-40-2		No (target)

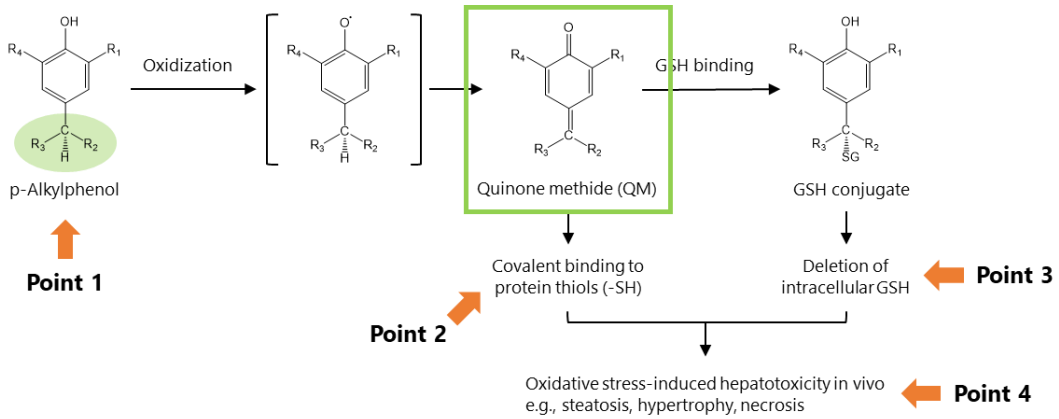
* Sub-chronic repeated dose toxicity (90 > exposure period ≥ 28 days) via the oral route in rats

5. Justification of data gap filling

5.1. Data gathering

In this case study, mainly four points are pointed out by construction of MoA/AOP-based read-across (shown in Table 4). Each *in silico* tool and *in vitro* method are shown in Table 2 and Annex III, respectively.

Figure 4. Summary of IATA-based read-across design



Point 1: Category members were classified into two groups; QM formation and not formation groups.

Point 2: QM formation was investigated by using simulated and published data.

Point 3: MoA/AOP-based biological responses were investigated by GSH trapping test and cell viability assay (detailed protocol is addressed in Annex III).

Point 4: The predicted NOAEL (liver) values of target members were determined by integrated data gap filling including published *in vivo* data.

Simulation

Table 2. Tools used for prediction and justification in this read-across

Name of tools	Reference	Description of use
ChemTunes•ToxGPS v.3	MN-AM (Database and Knowledgebase for Safety Evaluation and Risk Assessment, Altamira LLC and Molecular Networks GmbH) (https://www.chemtunes.com/)	Calculation of structural similarities using MACCS and RDkits fingerprint.
Hazard Evaluation Support System (HESS) Integrated Platform v.3.8	National Institute of Technology and Evaluation (https://www.nite.go.jp/en/chem/qsar/hess-e.html) (March 2019)	1. Definition of category boundary and members: categories with hepatotoxic information (Rank A) were identified by searching relevant databases. "Rank A" is defined as follows: categories containing well-known toxicity information (e.g., AOP, chemical reaction) and enough data on repeated-dose studies. 2. Metabolism simulation using the following profilers - Observed rat Liver metabolism - Liver Metabolism Simulator
OECD QSAR Toolbox v.4.4	OECD (http://www.oecd.org/chemicalsafety/risk-assessment/oecd-qsar-toolbox.htm) (February 2018)	1. Examination of physical/chemical properties, ADME and MoA/AOP-related structural alerts (e.g., GSH binding, protein binding *et al.*). 2. Metabolism simulation using the following profilers - Observed rat *in vivo* metabolism - Observed rat liver S9 metabolism - Observed rat liver metabolism with quantitative data - *In vivo* rat metabolism simulator - Rat liver S9 metabolism simulator 3. Simulation of bioavailability by "Lipinski rules Oasis" profiler: Lipinski's Rule of Five is a rule of thumb to evaluate drug likeness, or determine if a chemical compound with a certain pharmacological or biological activity has properties that would make it a likely orally active drug in humans.
Derek Nexus v.6.0.1	Lhasa Ltd. (http://www.lhasalimited.org/products/dereknexus.htm)	Examination of hepatotoxic alerts using following modules - Hepatotoxicity in mammal - Mitochondrial dysfunction in mammal
CASE Ultra v.1.7.0.5	MultiCASE Inc. (http://www.multicase.com/products/prod01.htm)	1. Examination of hepatotoxic alerts using five modules below - LIVER_BDUCT (Human bile duct disorders) - LIVER_CHOLEST (Human holestasis) - LIVER_DAMAGE (Human acute liver damage) - LIVER_FTEST (Human liver function test (blood test on liver enzymes release)) - LIVER_GALL) (Human gall bladder disorders) 2. Simulation of human intestinal absorption (%)

Experiments

Dansylated GSH (dGSH) trapping test and cell viability assay were performed for available compounds (limited to category member No. 1, 2, 4, 9, 10, 12, 15, and 16). The obtained compounds and detailed protocol are shown in Annex III.

5.2. Data and methods

5.2.1. In silico alerts

The OECD QSAR Toolbox v.4.3, Derek Nexus v.6.0.1, and CASE Ultra v.1.6.2.1 were used to simulate structural alerts related to hepatotoxicity. The simulated data using OECD QSAR Toolbox showed "negative" or "out of rules", whereas data using Derek Nexus and CASE Ultra showed something "positive" except for member No. 11 (Table 3 and Appendix. Data matrix, IATA for "Case Study on the Use of Integrated Approaches for Testing and Assessment to Inform Read-Across of p-Alkylphenols: Repeated-Dose Toxicity"). These data suggested that source and target members would cause hepatotoxicity.

Table 3. *In silico* profilers related to hepatotoxicity

Member No.	Hepatotoxicity in mammal [1)]	Mitochondrial dysfunction in mammal [1)]	LIVER_BDUCT (Human bile duct disorders) [2)]	LIVER_CHOLEST (Human cholestasis) [2)]	LIVER_DAMAGE (Human acute liver damage) [2)]	LIVER_FTEST (Human liver function test (blood test on liver enzymes release)) [2)]
1	Probable	Equivocal	Negative	Inconclusive	Negative	Positive
2	Plausible	Equivocal	Negative	Inconclusive	Negative	Positive
3	Plausible	No prediction	Negative	Inconclusive	Negative	Positive
4	Plausible	Equivocal	Negative	Inconclusive	Negative	Positive
5	Plausible	Equivocal	Negative	Inconclusive	Negative	Positive
6	Plausible	No prediction	Negative	Inconclusive	Negative	Positive
7	Plausible	Equivocal	Negative	Inconclusive	Negative	Positive
8	Plausible	Equivocal	Negative	Inconclusive	Negative	Positive
9	Plausible	Equivocal	Negative	Inconclusive	Negative	Positive
10	Plausible	Equivocal	Negative	Inconclusive	Negative	Positive
11	No prediction	No prediction	Negative	Negative	Negative	Negative
12	No prediction	Equivocal	Negative	Inconclusive	Negative	Positive
13	Plausible	Equivocal	Negative	Inconclusive	Negative	Positive
14	No prediction	Equivocal	Negative	Inconclusive	Negative	Positive
15	No prediction	Equivocal	Negative	Inconclusive	Negative	Positive
16	No prediction	Equivocal	Negative	Inconclusive	Negative	Positive
17	No prediction	No prediction	Negative	Inconclusive	Negative	Positive
18	No prediction	No prediction	Negative	Inconclusive	Negative	Positive
19	No prediction	Equivocal	Negative	Inconclusive	Negative	Positive
20	No prediction	Equivocal	Negative	Inconclusive	Negative	Positive

1) Derek Nexus v.6.0.1; 2) CASE Ultra v.1.7.0.5
Glossary in Derek Nexus prediction are following. “Probable” means that there is proof that the proposition is true. “Plausible” means that there is at least one strong argument that the proposition is true and there are no arguments against it. “Equivocal” means that there is an equal weight of evidence (WoE) for and against the proposition. “No prediction” in DEREK means no alert found for this endpoint in some category members.

5.2.2. ADME and QM formation

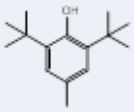
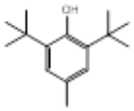
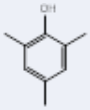
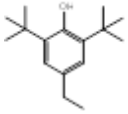
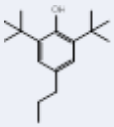
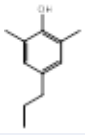
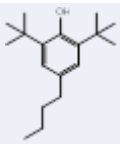
First, the ECHA database, Cosmetic Ingredient Review (CIR), and PubMed were searched to find basic ADME information. The available data are summarised in the Appendix. Data matrix, IATA for "Case Study on the Use of Integrated Approaches for Testing and Assessment to Inform Read-Across of p-Alkylphenols: Repeated-Dose Toxicity" (limited to category member No. 1, 2, 3, and 15). Category member No. 1 (2,6-di-tert-butyl-4-methylphenol; BHT, CAS No. 128-37-0), 2 (2,4-dimethyl-6-tert-butylphenol; BDMP, CAS No. 1879-09-0), and 15 (2,4,6-tri-tert-butylphenol, CAS No. 732-26-3) are rapidly absorbed through the gastrointestinal tract.

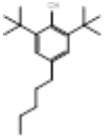
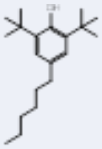
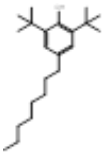
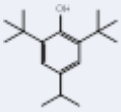
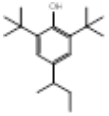
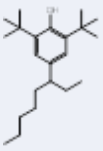
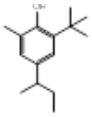
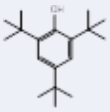
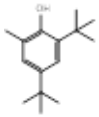
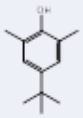
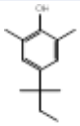
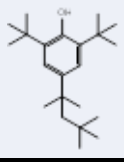
As for QM formation, member No. 1 (2,6-di-tert-butyl-4-methylphenol; BHT, CAS No. 128-37-0), 2 (2,4-dimethyl-6-tert-butylphenol; BDMP, CAS No. 1879-09-0), and 3 (2,4,6-trimethylphenol; TMP, CAS No. 527-60-6) are oxidised to para-QM (BHT-QM, BDMP-QM and TMP-QM, respectively) and could conjugate with GSH. However, member No. 15 (2,4,6-tri-tert-butylphenol, CAS No. 732-26-3) does not form QM since there is no H atom present. On the other hand, one study reported that quinone methides were generated by disproportionation of phenoxy radicals generated by the ferricyanide oxidation of 2,6-di-tert-butyl-4-isopropylphenol (member No. 11), 2,6-di-tert-butyl-4-sec-butylphenol (member No. 12), 2,6-di-tert-butyl-4-(2-ethylhexyl)phenol (member No. 13), and 4-(butan-2-yl)-2-tert-butyl-6-methylphenol (member No. 14) (Toteva and Richard, 2011).

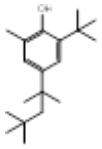
Next, bioavailability, absorption, and metabolism were simulated by OECD QSAR Toolbox v.4.4 and/or CASE Ultra v.1.6.2.1. The rate of intestinal absorption was largely similar (Table 4 and Appendix. Data matrix, IATA for "Case Study on the Use of Integrated Approaches for Testing and Assessment to Inform Read-Across of p-Alkylphenols: Repeated-Dose Toxicity"). Especially, elongation of the 4-alkyl chain increased the intestinal absorption. As for metabolism, QM formation was found in category members with linear alkyl at the 4-position, but not those with branched alkyl except for member No. 11 (2,6-di-tert-butyl-4-isopropylphenol, CAS No. 5427-03-2), 12 (2,6-di-tert-butyl-4-sec-butylphenol, CAS No. 17540-75-9), 13 (2,6-di-tert-butyl-4-(2-ethylhexyl)phenol, CAS No. 816462-78-9) (Table 5 and Annex II), and 14 (4-(butan-2-yl)-2-tert-butyl-6-methylphenol, CAS No. 51067-63-1).

The above data suggest that 2,4,6-tri-alkylphenols would be absorbed rapidly through the gastrointestinal tract and metabolised via a different pathways since not all members are able to form a QM. Additionally, QM formation rather than logK_{ow} or the rate of intestinal absorption, could play more essential role in the hepatotoxicity of 2,4,6-tri-alkylphenols.

Table 4. Physical/chemical properties and the rate of intestinal absorption

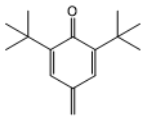
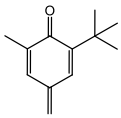
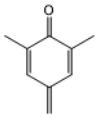
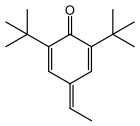
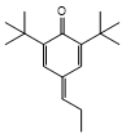
Member No.	Chemical structure	Molecular weight [1)]	logK_{ow} [1)]	Lipinski Rule Oasis [2)]	Human intestinal absorption [3)]
1		220.36	5.03	Less bioavailable	93
2		178.28	4.52	Bioavailable	97
3		136.20	3.15	Bioavailable	95
4		234.38	5.52	Less bioavailable	93
5		248.41	6.01	Less bioavailable	94
6		192.30	5.01	Bioavailable	98
7		262.44	6.50	Less bioavailable	95

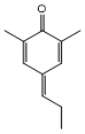
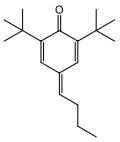
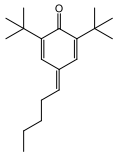
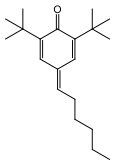
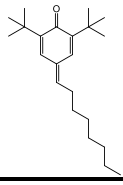
8		276.47	6.99	Less bioavailable	95
9		290.49	7.48	Less bioavailable	95
10		318.55	8.47	Less bioavailable	97
11		248.41	5.94	Less bioavailable	94
12		262.44	6.43	Less bioavailable	94
13		318.55	8.39	Less bioavailable	97
14		220.36	5.92	Less bioavailable	97
15		262.44	6.39	Less bioavailable	94
16		220.36	5.88	Less bioavailable	92
17		178.28	4.52	Bioavailable	97
18		164.25	4.14	Bioavailable	97
19		318.55	8.24	Less bioavailable	97

20		276.47	7.73	Less bioavailable	95

1) EPI Suite v.4.11; 2) OECD QSAR Toolbox v.4.4 3) CASE Ultra v.1.6.2.1

Table 5. Summarisation of simulated and/or published data on QM formation (Yes/No)

Member No.	1	2	3	4	5
CAS No.	128-37-0	1879-09-0	527-60-6	4130-42-1	4973-24-4
Formation of QM[1, 2)]	Yes 	Yes 	Yes 	Yes 	Yes 

Member No.	6	7	8	9	10
CAS No.	13037-82-6	5530-30-3	4973-26-6	56280-62-7	35309-87-6
Formation of QM[1, 2)]	Yes 	Yes 	Yes 	Yes 	Yes 

Member No.	11	12	13	14	15
CAS No.	5427-03-2	17540-75-9	816462-78-9	51067-63-1	732-26-3
Formation of QM[1, 2)]	Yes (Toteva and Richard, 2011)	Yes (Toteva and Richard, 2011)	Yes (Toteva and Richard, 2011)	Yes (Toteva and Richard, 2011)	No

Member No.	16	17	18	19	20
CAS No.	616-55-7	879-97-0	91798-63-9	65796-87-4	104066-40-2
Formation of QM[1, 2)]	No	No	No	No	No

1) HESS v.3.8 and 2) OECD QSAR Toolbox v.4.4 were utilised to simulate metabolism, especially QM formation.

5.2.3. In vitro responses

First, a dansylated glutathione (dGSH) trapping assay was performed to investigate the rate of GSH binding with reactive metabolites (Figure 4). The amount of dGSH adducts with member No. 1, 2, 4, and 12 in QM formation group were much more than member No. 15 and 16 in QM not formation group. However, the GHS amount of No. 10 is almost equal to the level of member No. 15 and 16. This data implied that the steric hindrance at the 4-position could reduce the rate of QM formation, and diminish the cytotoxicity because QM is known to be a major toxic metabolite of p-alkylphenols. To prove this hypothesis, a cell viability assay was performed on parent compounds. The results showed that cell viability

tended to decrease at higher concentration, but there was no clear difference among the tested compounds except for member No. 2 (2,4-dimethyl-6-tert-butylphenol; BDMP, CAS No. 1879-09-0) (data shown in Figure 5). Referring to the endpoint data, source member No. 2 showed more severe hepatotoxicity and lower NOAEL than No. 1, 4, and 12.

In published studies, member No. 1 (BHT, CAS No. 128-37-0) caused GSH depletion (Bolton *et al.,* 1992) and mitochondrial swelling in rat hepatocytes (Fusi *et al.,* 1991), whereas the decrease in cytotoxicity was moderate compared to that of BDMP (member No. 2: 2,4-dimethyl-6-tert-butylphenol, CAS No. 1879-09-0) even after 120 min (Bolton *et al.,* 1992). Additionally, BHT-QM induced higher formation of GSH conjugates and covalent binding to cellular proteins in rat liver microsomes and tissue slides than its congeners (4-ethyl-BHT [E-BHT] (No. 4) and 4-isopropyl-BHT [I-BHT]) (No. 11) (Reed *et al.,* 2001).

Taken above data, it was suggested that the steric hindrance such as elongation or branching of the 4-alkyl substituents could reduce the rate of QM formation and cytotoxicity. This also implied these 2,4,6-tri-alkylphenol would show low hepatotoxicity *in vivo*.

Figure 5. Amount of dansylated glutathione (dGSH) adducts formed with tested compounds

Rat liver microsomes were incubated with each compound (limited in member No. 1, 2, 4, 10, 12, 15, and 16) at 0.1, 0.5, and 1 mM for 60 min, and the supernatants were analysed using ultra-high-performance liquid chromatography (UPLC).

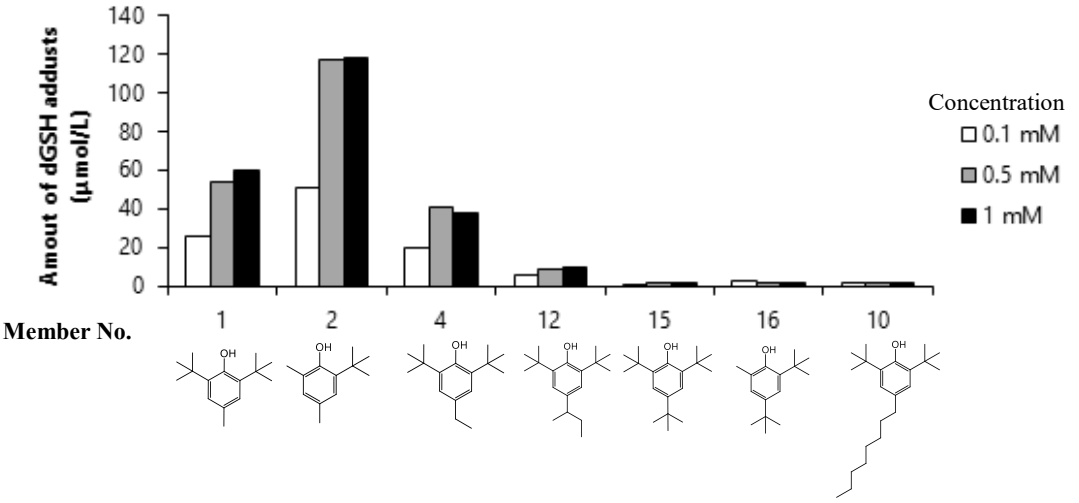
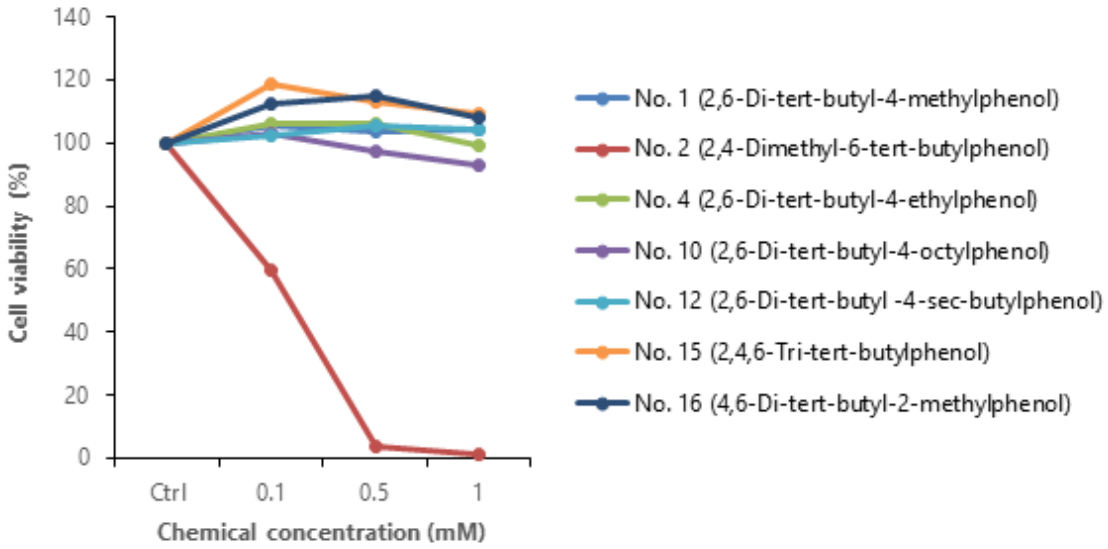


Figure 6. Effects of test compounds on viability of primary rat hepatocytes

Rat hepatocytes were incubated with each compound (limited in member No. 1, 2, 4, 10, 12, 15, and 16) at 0.1, 0.5, and 1 mM for 72 h, and the viability was measured using luminescence.



5.2.4. In vivo toxicity

Available data for source compounds (member No. 1−4, 12, and 15) are summarised in Annex IV. All source compounds except for member No. 3 (TMP; 2,4,6-trimethylphenol, CAS No. 527-60-6) induced hepatotoxicity, as evidenced by alterations in histopathological parameters and weight changes (e.g., hypertrophy of hepatocytes, organ weight increase). As for QM formation group, member No. 1 (BHT; 2,6-di-tert-4-methylphenol, CAS No. 128-37-0) caused the most severe hepatotoxicity at the highest dose (e.g., hepatocyte necrosis, fibrosis, bile duct cell proliferation, hepatocyte hyperplasia, pigment-laden macrophages, and glycogen depletion/accumulation). In a 28-day rat repeated-dose study of member No. 1, periportal hepatocyte necrosis was observed at 500 mg/kg/day BHT and centrilobular liver necrosis at 1000 mg/kg/day BHT exposure for up to 4 days (Powell *et al.* 1986). The persistent and active nature of the lesions in rats dosed with 500 mg BHT/kg for 28 days, combined with evidence of cell damage at doses equivalent to those associated with hepatic tumours (250 mg BHT/kg), suggests that chronic liver cell damage may be involved in their aetiology (Powell *et al.* 1986). Therefore, liver tumour can be considered an AO for this case study as one of the source compounds can induce this neoplasia at 250 mg/kg bw/day after 2,5 years (Olsen *et al.* 1986 *apud* Powell *et al.* 1986). In contrast, member No. 4 (2,6-di-tert-butyl-4-ethylphenol, CAS No. 4130-42-1), 12 (2,6-di-tert-butyl-4-sec-butylphenol), and 15 (2,4,6-tri-tert-butylphenol) only caused hepatocyte hypertrophy.

5.3. Justification

In this case study, the read-across hypothesis suggested that the potency of QM formation could affect hepatotoxicity of 2,4,6-tri-alkylphenols. Then, category members were categorised into two groups; One was “QM formation group” and another was “QM not formation group”. The *in silico* analysis revealed that ADME-related data, especially

intestinal absorption, was not largely different among all category members. This implied that the difference of physical-chemical properties did not affect the level of liver exposure. Therefore, it was necessary to consider the hepatotoxicity in the description of hazard assessment.

QM formation group

dGSH assay was performed for a part of category members in QM formation group. As expected, member No. 1 (BHT, CAS No. 128-37-0) and 2 (BDMP, CAS No. 1879-09-0) showed clear formation of dGSH adducts, while member No. 10 (2,6-di-tert-butyl-4-octylphenol, CAS No. 35309-87-6) did not (data shown in Figure 4). It is known that metabolic enzyme cytochrome (CYP) act in QM formation. It meant that the length at 4-alykl chain could change the rate of QM formation. Considering the results of dGSH assay, it was suggested that the hepatotoxicity was as follows: member No. 1 (BHT, CAS No. 128-37-0) > 4 (2,6-Di-tert-butyl-4-ethylphenol, CAS No.4130-42-1) > 10 (2,6-di-tert-butyl-4-octylphenol, CAS No. 35309-87-6) due to its ability to form QM. *In vitro* cell viability assay showed that except for member No. 2, which had the highest amount of dGSH adducts, the viability did not decrease (data shown in Figure 5). These indicated that the ability of dGSH binding was mostly within the acceptable range of detoxification, and, therefore, the cell viability did not decrease. As for *in vivo* information in QM formation group, member No. 2 (BDMP, CAS No. 1879-09-0) showed most severe pathological changes including hepatocellular necrosis, and followed by member No. 1 (BHT, CAS No. 128-37-0) > 4 (2,6-di-tert-butyl-4-ethylphenol, CAS No. 4130-42-1). Member No. 12 (2,6-di-tert-butyl-4-isopropylphenol, CAS No. 17540-75-9) showed only hypertrophy of hepatocytes. This toxic potential was correlated with the ability of dGSH binding *in vitro*. Then, it was suggested that the reactivity of parent chemicals with GSH was important to estimate hepatotoxicity. On the other hand, member No. 3 (TMP, 527-60-6), which had potency of QM formation, did not show hepatotoxicity *in vivo*.

QM not formation group

The group members had no difference in ADME properties, especially intestinal absorption. As for *in vitro* reactivity, member No. 15 (2,4,6-tri-tert-butylphenol, CAS No. 732-26-3) and 16 (2,4-di-tert-butyl-6-methylphenol, CAS No. 616-55-7) showed almost no amount of dGSH adducts and cell viability did not decrease. The *in vivo* data of member No. 15, however, showed hepatocellular necrosis. This suggested that these group members could not form QM, and the hepatotoxicity could be caused by its parent and other metabolites. It indicated that they would show different MoA/AOP in Figure 3.

Strategy for predicted NOAEL (liver) values determination

In this case study, the structure at the 4-position was simpler in all source members than it was in target compounds. This implied that the target members would cause lower hepatotoxicity than source members. On the other hand, the MoA/AOP among category members in QM not formation group was not revealed clearly. Then, target members in QM not formation group were (member No. 15–20) defined as “NOAEL not determined”. And the NOAEL values were applied for target members in QM formation group (member No. 1–14). Additionally, considering the similarity of chemical reactivity, these category members were sub-grouped with common 4, 6-substituents to conduct NOAEL (liver) determination more severely. Each result of read-across is addressed below and summarised in Table 6.

6. Strategy for integrated conclusion of data gap filling

6.1. Uncertainty

Factor	Uncertainty	Comment
Hypothesis used for the read-across	Low	The category members shared the basic structure of p-alkylphenols with alkyl substituents of variable structure at the 2, 4, 6-positions. Some studies on the toxicity of p-alkylphenols have suggested that structures at the 4-position could affect metabolism and strength of hepatotoxicity. Therefore, the read-across under category approach was applied for category members by grouping them according to the same alkyl substituents at the 2, 6-positions. For data gap filling, MoA/AOP-based *in vitro* methods such as dGSH trapping test and cell viability assay were performed.
Structural similarity	Low	The basic structure of category members looks like very similar because they have commonly alkyl substituents at the 2, 4, 6-positions. First, category members were categorised into two groups based on the similarity of metabolism. One was "QM formation group" (member No. 1–14) which could show QM-induced hepatotoxicity considering published and simulated data. Another was "QM not formation group" (member No. 15–20). Additionally, they were sub-grouped by the same alkyl substituents at the 2, 6-positions–i.e., Group 1 (2,6-di-tert-butylphenol), Group 2 (2-methyl-6-tert-butyl or 6-methyl-2-butylphenol), and Group 3 (2,6-mi-methyl). The structural similarity which was calculated by MACCS and RDkits fingerprint was very high (shown in Annex I and Appendix. Data matrix, IATA for "Case Study on the Use of Integrated Approaches for Testing and Assessment to Inform Read-Across of p-Alkylphenols: Repeated-Dose Toxicity").
Similarity of physical/chemical properties	Medium	The $\log K_{ow}$ for all category members ranged from 3 to 10, and molecular weight ranged from 136 to 374. In contrast, the rate of intestinal absorption differed only slightly. In fact, intestinal absorption calculated using CASE Ultra were quite similar among all category members. This suggest that the difference of physical/chemical properties such as $\log K_{ow}$ and molecular weight do not necessarily affect the biological properties.
Similarity of toxicokinetic data	Medium	*In silico* analysis were performed to predict ADME such as intestinal absorption (%) and Rule of Lipinski. Published information on ADME and toxicokinetics was available for some category members. The simulated data showed that the rate of intestinal absorption were almost common among all category members; whereas QM formation was different depending on the alkyl structure at the 4-position and its reactivity.
Similarity of other supportive data (e.g. data related to key event)	Medium	An examination of structural alerts revealed similar hepatotoxicity among all category compounds. Data from the dGSH trapping assay and metabolism simulation by *in silico* tools indicated that compounds with simple alkyl structure at the 4-position showed high amount of dGSH adducts and had potency of QM formation. However, compounds with longer alkyl chain or branched alkyl decreased the amount of dGSH adducts. In addition, compounds with steric hindrance at the 4-position did not show QM formation in metabolism simulation. These data suggested that the alkyl structure at the 4-position could affect the rate of QM formation, GSH depletion, and related hepatotoxicity.
Number of analogues used for the read-across	Medium	The number of analogues used for the category approach was almost enough. HESS, COSMOS, AMBIT, PubMed, and EPA ToxCast Screening Library were used to identify category members. In this study, various 2,4,6-tri-alkylphenols with different substituents at the 4-position were selected as category members. However, source members were not enough because sub-group 2 and 3 had only one compound. It was considered that at least two or more compounds were needed to brush up the uncertainty of NOAEL determination.
Quality of the endpoint data used for the read-across	Low	Quality of *in vivo* tests was high using two reliable methods: the GLP study for member No. 1 and 12, and GLP-compliant OECD TG 407 or 422 studies for member No. 2–4, and 15. These studies describe the test conditions and toxicity findings after dosing and, therefore, the quality of endpoint data was high.
Similarity of the endpoint data (among source and target chemicals)	Medium	All source members except for member No. 3 (TMP; 2,4,6-trimethylphenol, CAS No. 527-60-6) showed hepatotoxicity-related histopathological changes such as liver weight changes and hepatocellular hypertrophy, with more severe effects observed at high doses. However, there was no clear correlation between structure of substituents at the 4-position and NOAEL value. Consequently, *in vivo* studies were not performed under the same conditions such as the exposure period (28, 45, or 90 days) and doses (lowest and highest NOAEL values).
Concordance and weight of evidence of all data used for justifying the hypothesis	Medium	Category members with same substituents at 2,6-positions were grouped for the category approach. *In vitro* data were combined with physical/chemical information, *in silico* data, and reported repeated-dose toxicity data. Particularly, simulation of metabolism and examination of potency of binding to QM reactive metabolites are important points related to MoA/AOP of 2,4,6-tri-alkylphenols. This multifactorial analysis using the read-across approach could provide insight into mechanisms of toxicity based on chemical and biological properties. In contrast, it was difficult to determine how each metabolite influenced toxicity because of the lack of quantitative data in this study. Moreover, the molecular basis for the toxicity of p-alkylphenols has not been fully elucidated. The predicted NOAEL (liver) values of target compounds were ultimately determined considering the worst-case scenario.
Overall uncertainty of the read-across	Medium	Uncertainty associated with the read-across approach for the repeated-dose toxicity of 2,4,6-tri-alkylphenols was determined to be medium. Category compounds were divided into two groups (with or without QM formation). Metabolism and hepatotoxicity data suggested that structure of alkyl substituents at 4-position influenced toxicity. In contrast, there was little variation in predicted NOAEL (liver) values among group members. Results of AOP-based *in vitro* assays indicated that toxicity trend could be predicted using biological responses to simulated or published data. Finally, suitable NOAEL (liver) values of target compounds were determined considering the worst-case scenario across the source members in each group. These results indicate that both chemical and biological data were essential for IATA-based read-across.

6.2. Integrated conclusion

In this case study, the structure at the 4-position was simpler in all source members than it was in target compounds. On the other hand, the MoA/AOP among category members in QM not formation group was not revealed clearly. Therefore, only the predicted NOAEL (liver) values of target members in QM formation group (member No. 5-11, 13+14) were finally determined. Additionally, these category members were sub-grouped with common 4, 6-substituents to conduct NOAEL (liver) determination more severely considering the similarity of chemical reactivity. Each result of read-across is addressed below and summarised in Table 6.

Sub-group 1: 2,6-Di-tert-butylphenol (member No. 1, 4, 5, 7–13, 15, 19)

In the metabolism simulation, QMs were not detected in category chemicals with steric hindrance (member No. 10, 15, and 19). Member 10, however, is structurally able to form a QM. Additionally, the amount of dGSH adducts reduced in following order: member No. 1 (4-methyl) > 4 (4-ethyl) > 12 (4-sec-butyl) > 15 (4-tert-butyl) ≒ 10 (4-octyl) (data shown in Figure 4). And several published studies on the correlation between the QM reactivity and alkylphenol-induced hepatotoxicity suggested that the elongation or hindered structure at the 4-position could reduce QM formation, GSH depletion, covalent binding to proteins and related cytotoxicity (Bolton *et al.,* 1992; Reed *et al.,* 2001). Furthermore, member No. 1 exhibited the most severe histopathological changes in the endpoint data. Accordingly, member No. 1 was first expected to be the most hepatotoxic among Group 1 members. On the other hand, the NOAEL value of source member No. 1 (25 mg/kg/day) was higher than No. 4 (15 mg/kg/day). Member No. 1 exhibited greater histopathological changes (e.g., hepatocyte necrosis, fibrosis, bile duct cell proliferation, hepatocyte hyperplasia, pigment-laden macrophages, and glycogen depletion/accumulation) at the maximum dose (500 mg/kg/day) than No. 4, which just induced hepatocyte hypertrophy at 250 mg/kg/day. It is difficult to directly compare the NOAEL values between source members in terms of Point of Departure (PoD), because it depends on not only the potential of toxicity but also test conditions. Considering the hepatotoxicity *in vivo*, member No.1 was more potent than No. 4 according to the severity of toxicological effect. However, the NOAEL of member No. 4 (15 mg/kg/day) was applied for target members No. 5, 7–13 as the worst-case scenario (lowest tested NOAEL in the group). The predicted NOAEL (liver) values of target member No. 16 and 20 were not determined since they could not form QM referring to the metabolism simulation (data shown in Annex II and Appendix. Data matrix, IATA for "Case Study on the Use of Integrated Approaches for Testing and Assessment to Inform Read-Across of p-Alkylphenols: Repeated-Dose Toxicity").

Sub-group 2: 2-Methyl-6-tert-butylphenol or 6-Methyl-2-butylphenol (member No. 2, 14, 16, 20)

Group 2 could be assessed by applying the same hypothesis as Group 1. In the metabolism simulation, QMs were detected only in member No. 2 (2,4-dimethyl-6-tert-butylphenol, CAS No. 1879-09-0) and its amount of dGSH adducts was higher than target No. 16 (2,4-di-tert-butyl-6-methylphenol, CAS No. 616-55-7) (data shown in Figure 4). As for member No. 2, hepatotoxic effects such as swelling and necrosis of hepatocytes were detected *in vivo* at 30 and 150 mg/kg/day. As for target members, one study reported on the potency of QM formation of member No. 14 (Toteva and Richard, 2011). On the other hand, *in silico* data revealed that target member No. 16 and 20 did not form QM (data shown in Annex II and Appendix. Data matrix, IATA for "Case Study on the Use of Integrated Approaches for Testing and Assessment to Inform Read-Across of p-Alkylphenols: Repeated-Dose Toxicity"). This suggested that toxicity could occur via another MoA/AOP

than the one shown in Figure 3. Thus, the predicted NOAEL (liver) values of target member No. 16 and 20 were not determined. Therefore, the predicted NOAEL (liver) values of member No. 2 (6 mg/kg/day) was just applied for target member No. 14. in group 2 as the worst-case scenario.

Sub-group 3: 2,6-Di-methylphenol (member No. 3, 6, 17, 18)

In the metabolism simulation, QMs were detected in member No. 3 (TMP; 2,4,6-trimethylphenol, CAS No. 527-60-6) and 6 (2,6-Dimethyl-4-propyl-phenol), which had linear alkyl chains at the 4-position, whereas was not found in No. 17 (2,6-Dimethyl-4-tert-butylphenol, CAS No. 879-97-0) and 18 (2,6-Dimethyl-4-(2-methylbutan-2-yl)phenol, CAS No. 91798-63-9) (data shown in Figure 4). Although member No. 3 had potency of QM formation, it just showed squamous hyperplasia in the forestomach but not hepatotoxicity *in vivo* (data summarised in Annex I). Another study revealed that TMP (member No. 3) formed GSH conjugates like BHT (member No. 1), but the viability of primary rat hepatocytes was in the range of 80–90% even at the longest exposure time (120 min) (Bolton *et al.* 1992) which is also similar to member No. 1. Considering metabolism and chemical reactivity, it was speculated that member No. 3 could have low toxicity due to oxidisation and binding to GSH, and that the methyl group at the 2, 6-positions would allow a more rapid reaction with intracellular GSH and other nucleophiles than a tert-butyl group, with QM rapidly cleared from the liver. Thus, the QM-related toxicity of member No. 3 could not be greater than that of BHT (member No. 1). In addition, the structural hindrance of target member No. 6 could induce less QM formation and alleviation of intracellular GSH depletion. Taken all these data into account, we concluded that Group 3 category members do not fit in MoA/AOP shown in Figure 3, and may have different toxicity pattern than Group 1 and 2 category members. Therefore, no predicted NOAEL (liver) values for target member No. 6, 17, and 18 were proposed. To address the potential influence of a methyl group at the 2 and/or 6 position further data are needed since member No. 2 (2,4-Dimethyl-6-tert-butylphenol) shows higher toxicity than No. 1 and No. 3.

Table 6. Summary of integrated conclusion

	Sub-group	Number of category member	Predicted NOAEL (liver) values determination
QM formation group (linear 4-alkyl)	1	No. 1, 4, 5, 7–13	All target members in this sub-group had longer alkyl chain at the 4-position than source members. This implied that target members could show lower hepatotoxicity than source ones. Therefore, a worst-case scenario was applied for NOAEL determination. At last, the NOAEL of member No. 4 (15 mg/kg/day) was applied for category members No. 5, 7–13.
	2	No. 2, 14	The NOAEL for No. 2 (6 mg/kg/day) was applied for member No. 14
	3	No. 3, 6	As for *in vivo* data, source member No. 3 did not induce hepatotoxicity. Thus, the NOAEL value of target member No. 6 was not proposed.
QM not formation group (branched 4-alkyl)	1	No. 15, 19	NOAEL not determined
	2	No. 16, 20	NOAEL not determined
	3	No. 17, 18	NOAEL not determined

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Annex I. Structural similarities

Similarities with MACCS and RDkits fingerprint calculated by ChemTunes•ToxGPS v.3

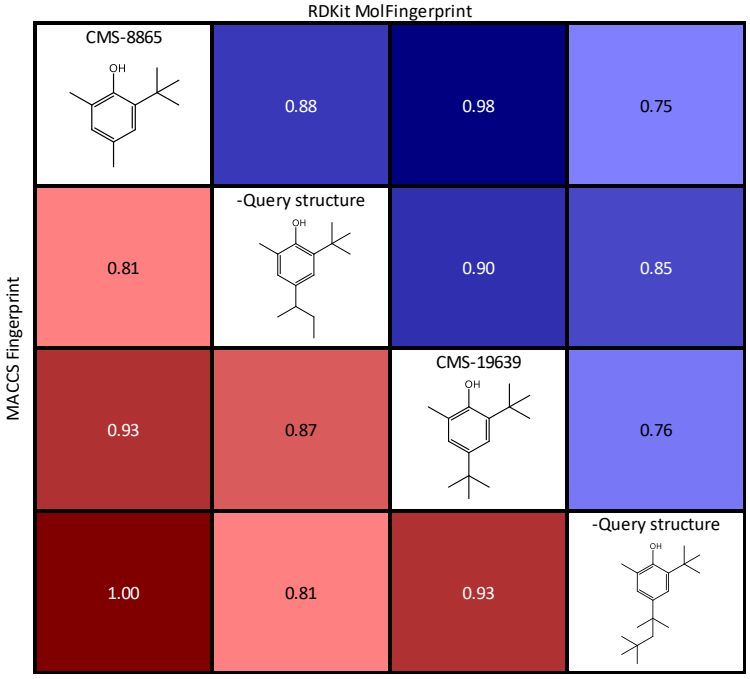
Sub-group 1: 2,6-Di-tert-butylphenol (member No. 1, 4, 5, 7–13, 15, 19)

RDKit MolFingerprint

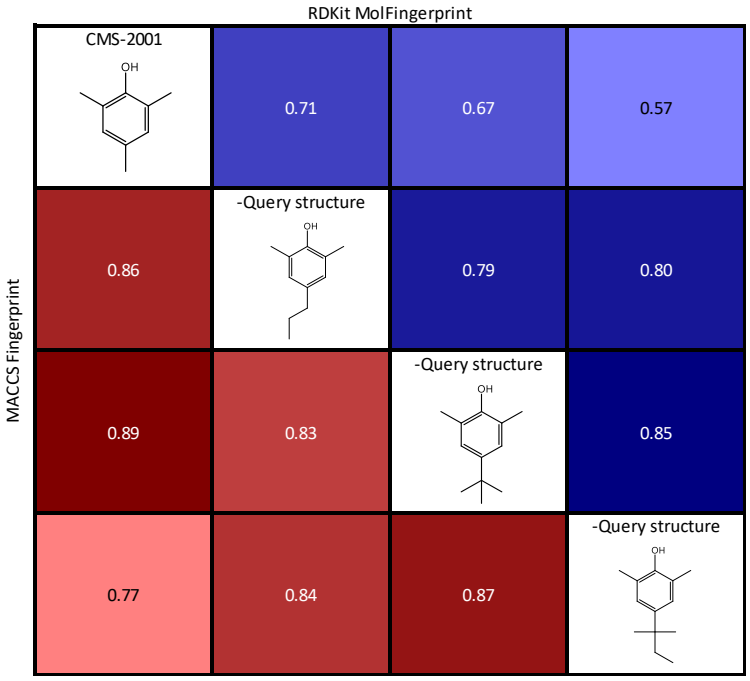
MACCS Fingerprint

CMS-224	0.99	0.89	0.99	0.99	0.93	0.90	0.88	0.86	0.76	0.85	0.79
0.93	CMS-6177	0.90	1.00	1.00	0.94	0.91	0.88	0.86	0.76	0.85	0.79
0.86	0.93	CMS-6218	0.90	0.90	0.96	0.95	0.92	0.90	0.85	0.89	0.89
1.00	0.93	0.86	CMS-1326	1.00	0.94	0.91	0.88	0.86	0.76	0.85	0.79
1.00	0.93	0.86	1.00	CMS-21499	0.94	0.91	0.88	0.86	0.76	0.85	0.79
0.89	0.96	0.90	0.89	0.89	-Query structure	0.97	0.94	0.92	0.81	0.91	0.85
0.83	0.90	0.97	0.83	0.83	0.93	-Query structure	0.97	0.95	0.84	0.94	0.88
0.78	0.84	0.91	0.78	0.78	0.88	0.94	-Query structure	0.98	0.84	0.97	0.90
0.78	0.84	0.91	0.78	0.78	0.88	0.94	1.00	-Query structure	0.83	0.99	0.92
0.93	0.86	0.81	0.93	0.93	0.83	0.78	0.84	0.84	-Query structure	0.82	0.84
0.78	0.84	0.91	0.78	0.78	0.88	0.94	1.00	1.00	0.84	CMS-24676	0.93
0.78	0.84	0.91	0.78	0.78	0.88	0.94	1.00	1.00	0.84	1.00	-Query structure

Sub-group 2: 2-Methyl-6-tert-butylphenol or 6-Methyl-2-butylphenol (member No. 2, 14, 16, 20)



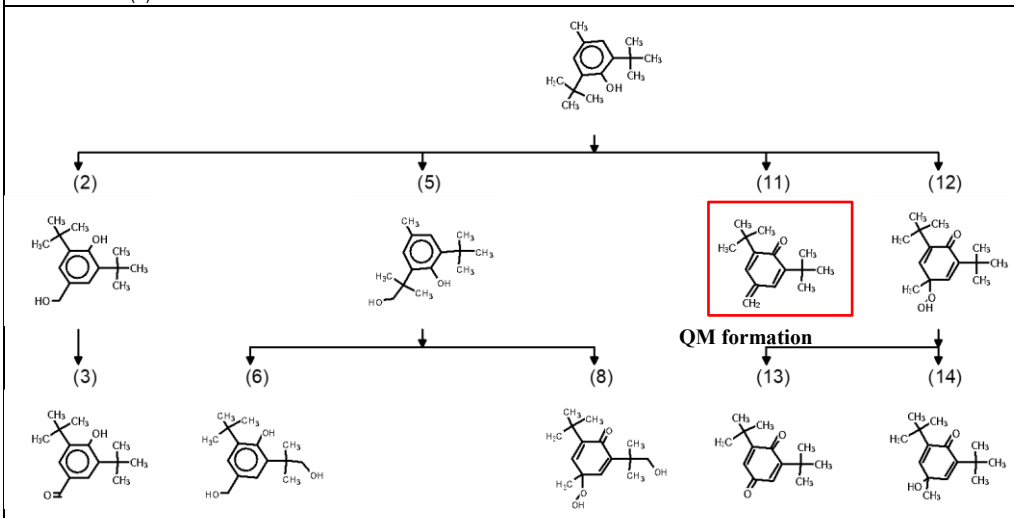
Sub-group 3: 2,6-Di-methylphenol (member No. 3, 6, 17, 18)



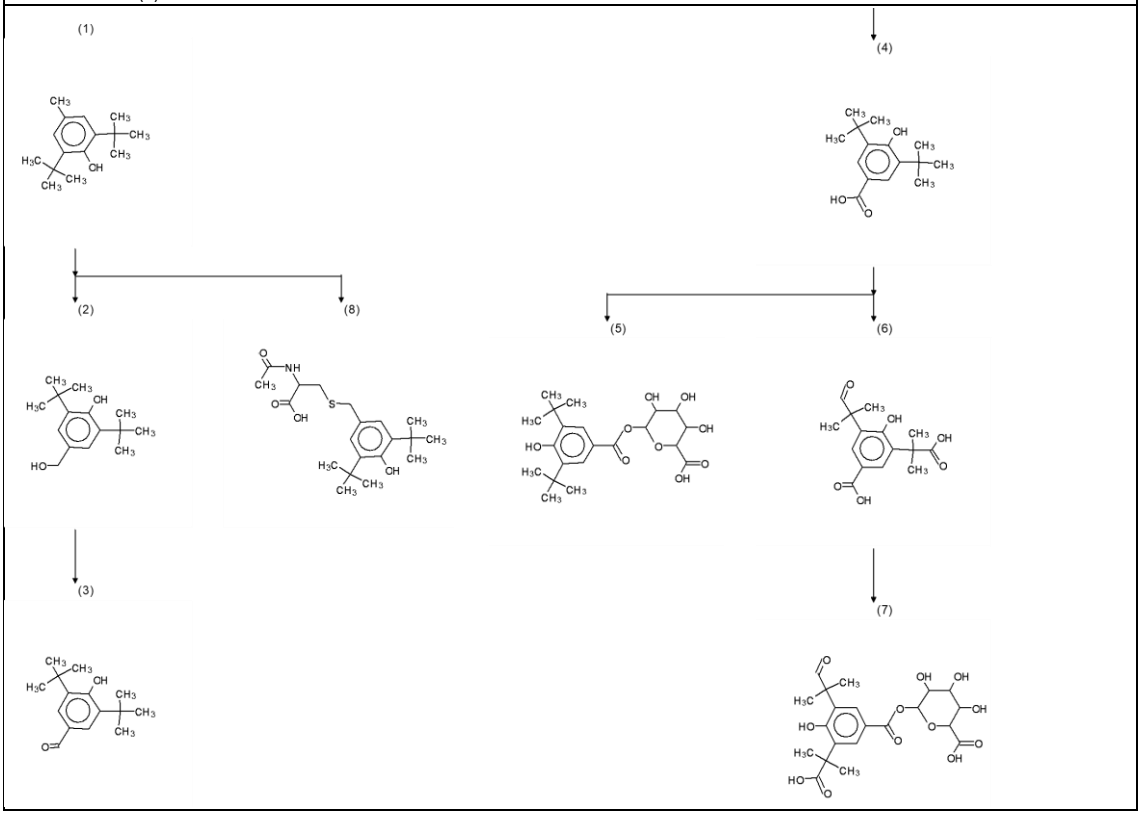
Annex II. Summary data of observed or simulated metabolites

Member No. 1: 2,6-Di-tert-butyl-4-methylphenol, BHT (CAS No. 128-37-0)

Reference (in vitro): Ladomery LG, Ryan AJ, Wright SE. (1967a). The excretion of [^{14}C] butylated hydroxytoluene in the rat. J Pharm Pharmacol. 19 (6): 383–7.

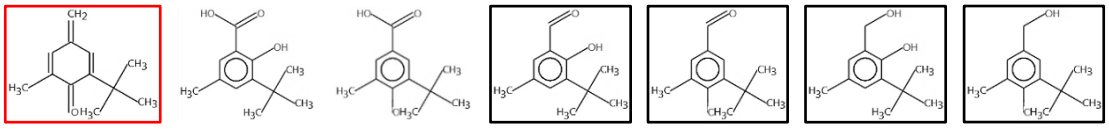


Reference (in vivo): Daniel JW, Gage JC, Jones DI. (1968). The metabolism of 3,5-di-tert-butyl-4-hydroxytoluene in the rat and in man. Biochem J. 106 (4): 783–90.



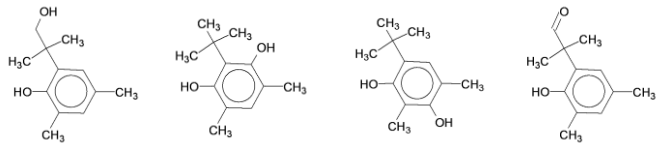
Member No. 2: 2,4-Dimethyl-6-tert-butylphenol (CAS No. 1879-09-0)

QSAR Toolbox: Total 7 metabolites including QM (shown in red frame) were found.



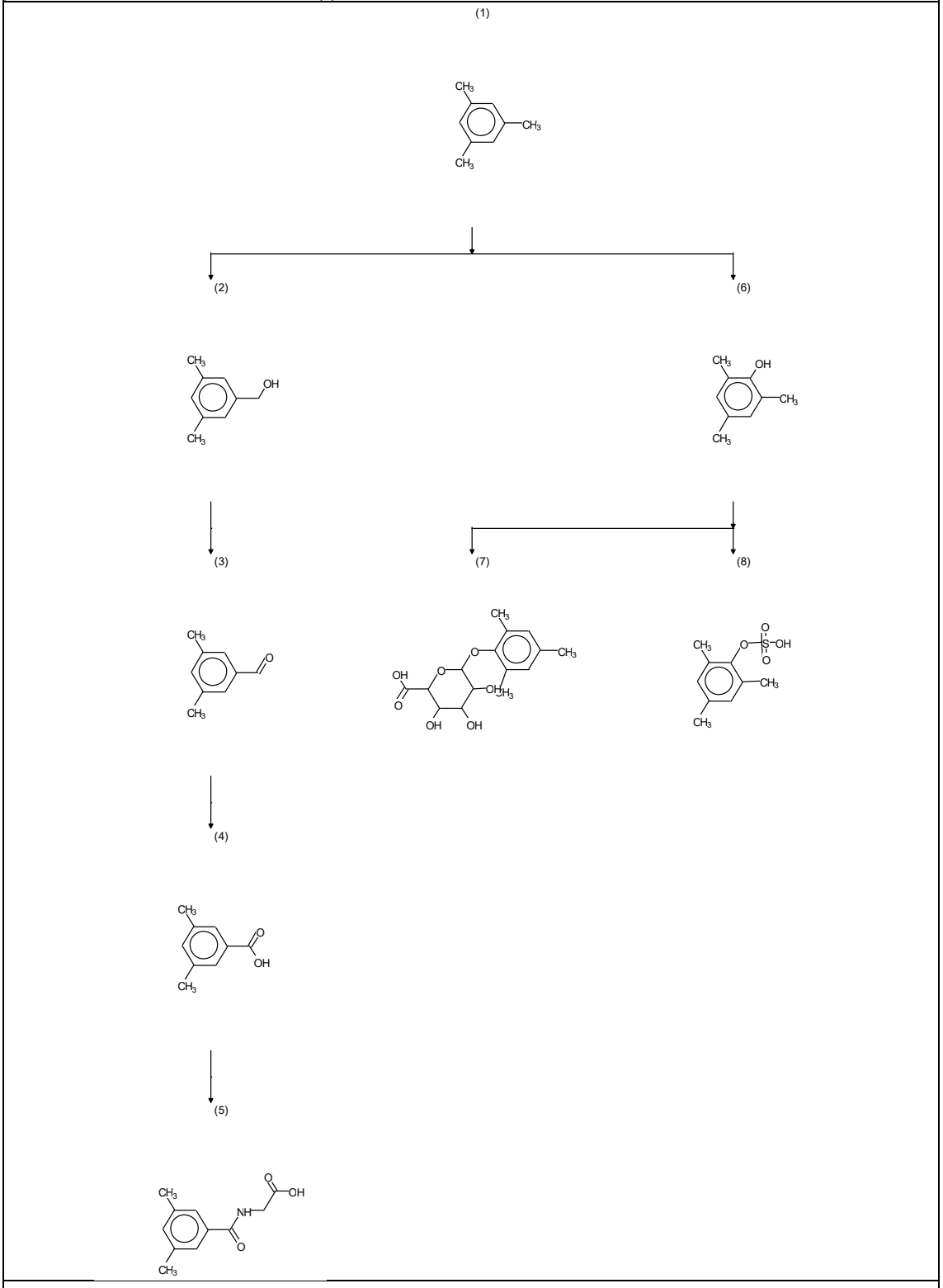
QM formation

HESS: 4 metabolites in QSAR Toolbox (shown in black frame above) and 4 metabolites below were found.

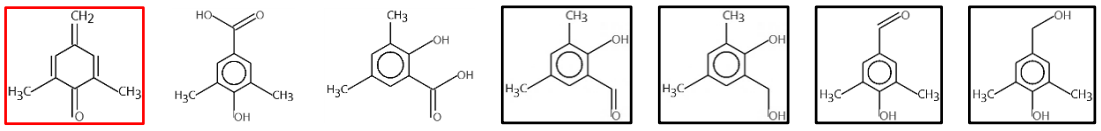


Member No. 3: 2,4,6-Tri-methylphenol, TMP (CAS No. 527-60-6)

Reference (in vivo): Mikulski PI, Wiglusz R. (1975). Toxicol Appl Pharmacol. The comparative metabolism of mesitylene, pseudocumene, and hemimellitene in rats. 31 (1): 21–31.

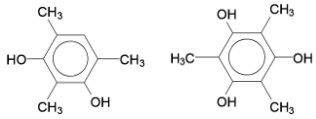


QSAR Toolbox: Total 16 metabolites including QM (shown in red frame) were found.



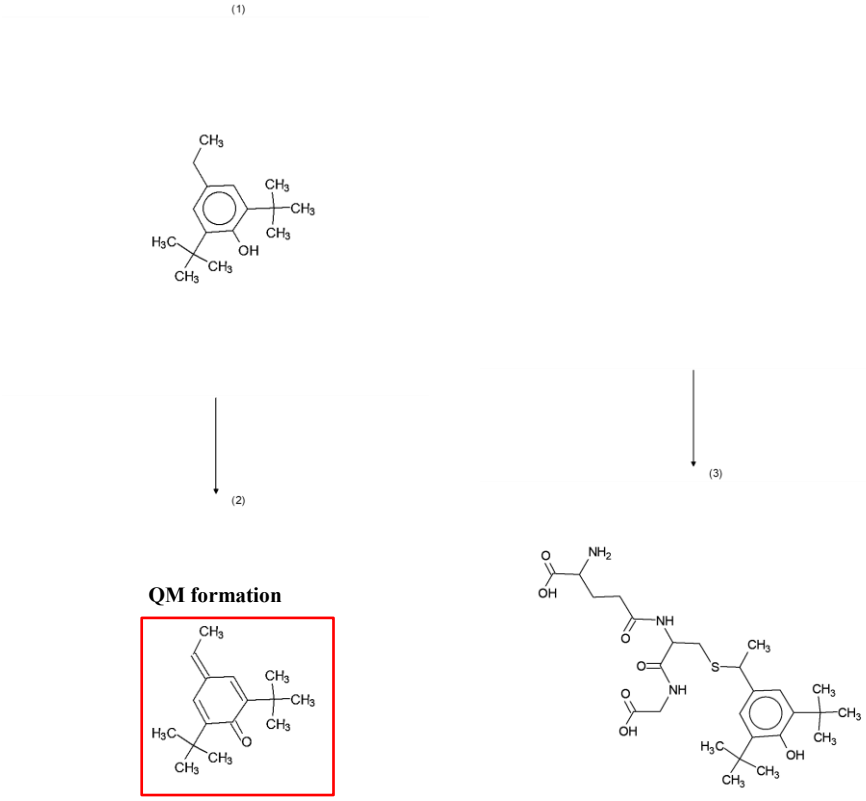
QM formation

HESS: 4 metabolites in QSAR Toolbox (shown in black frame above and QM) and 2 metabolites below were found.



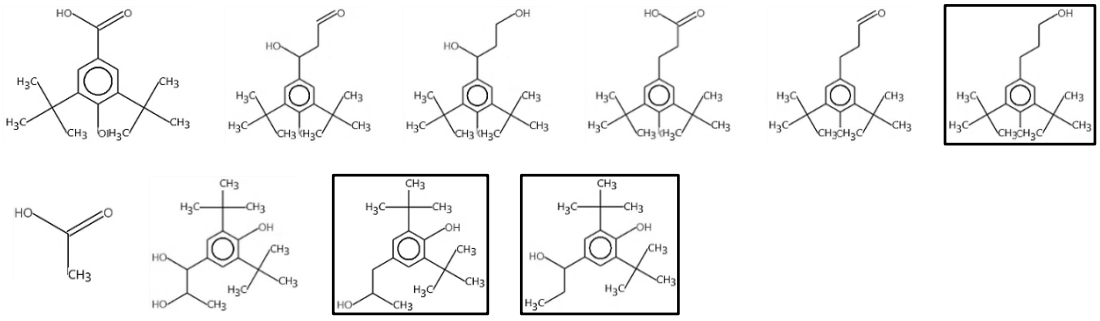
Member No. 4: 2,6-Di-tert-butyl-4-ethylphenol (CAS No. 4130-42-1)

Reference (in vitro): Reed M, Fujiwara H, Thompson DC. (2001). Chem Biol Interact. 28; 138(2): 155–170.

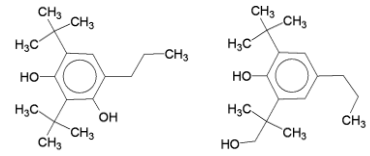


Member No. 5: 2,6-Di-tert-butyl-4-propylphenol (CAS No. 4973-24-4)

QSAR Toolbox: Total 10 metabolites were found.

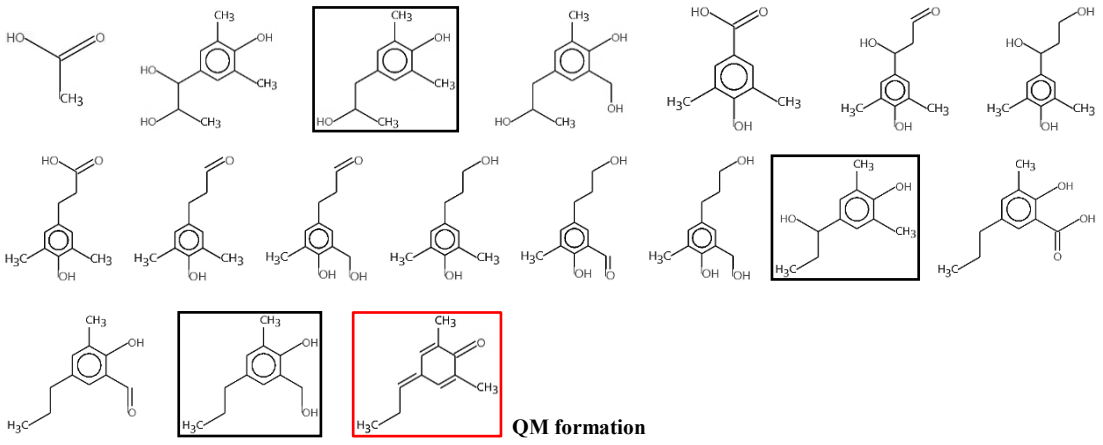


HESS: 3 metabolites in QSAR Toolbox (shown in black frame above) and additional 2 metabolites below were found.

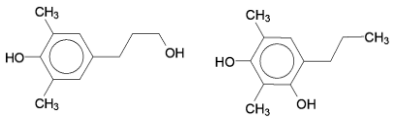


Member No. 6: 2,6-Dimethyl-4-propylphenol (CAS No. 13037-82-6)

QSAR Toolbox: Total 18 metabolites including QM (shown in red frame) were found.

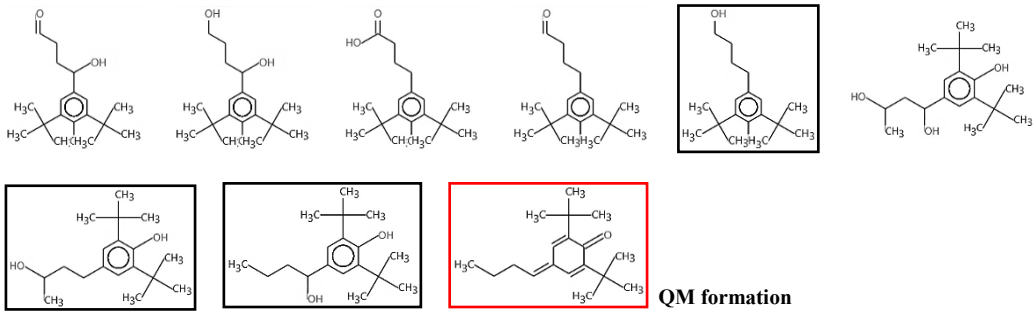


HESS: 3 metabolites in QSAR Toolbox (shown in black frame above) and 2 metabolites below were found.



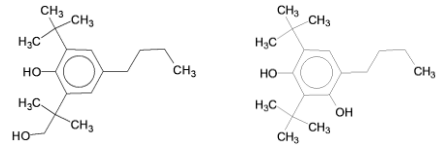
Member No. 7: 2,6-Di-tert-butyl-4-butylphenol (CAS No. 5530-30-3)

QSAR Toolbox: Total 9 metabolites including QM (shown in red frame) were found.



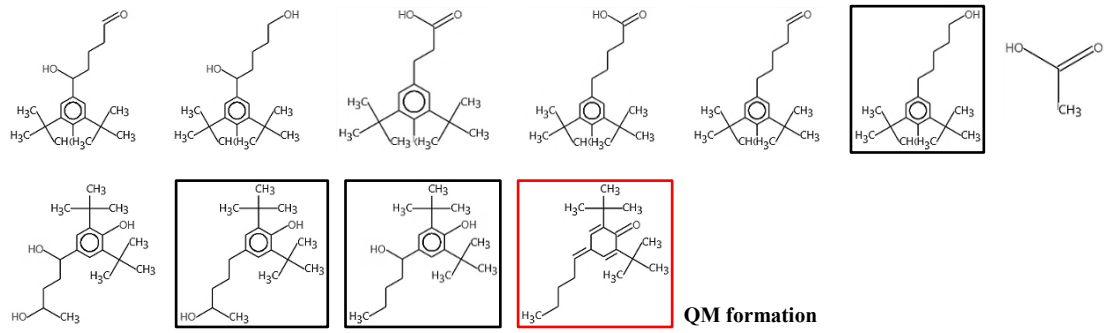
QM formation

HESS: 3 metabolites in QSAR Toolbox (shown in black frame above) and 2 metabolites below were found.



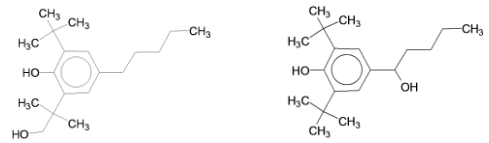
Member No. 8: 2,6-Di-tert-butyl-4-pentylphenol (CAS No. 4973-26-6)

QSAR Toolbox: Total 11 metabolites including QM (shown in red frame) were found.



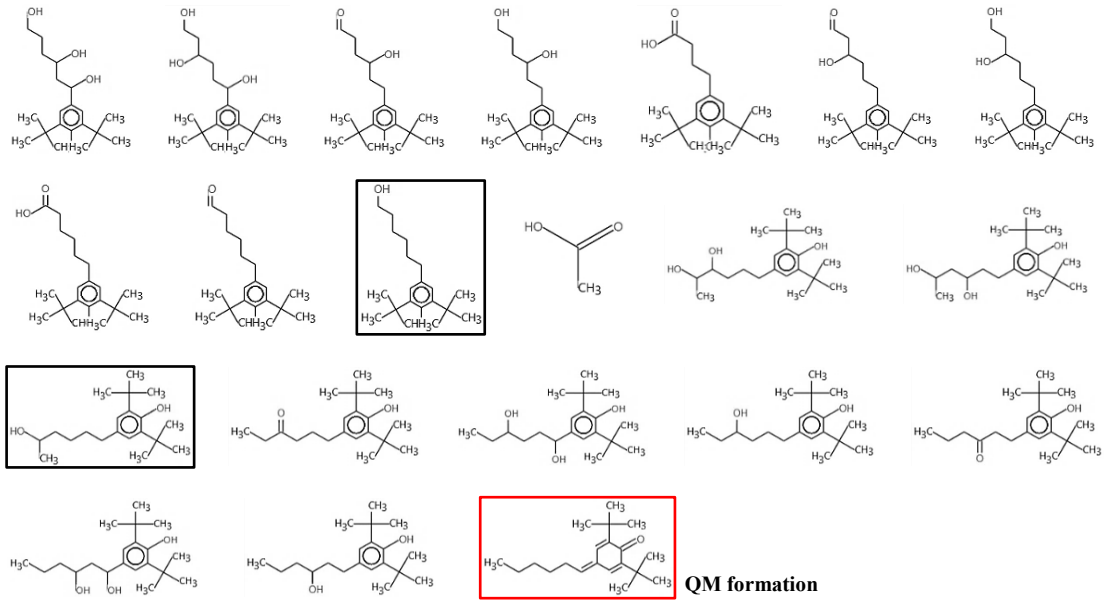
QM formation

HESS: 3 metabolites in QSAR Toolbox (shown in black frame above) and 2 metabolites below were found.

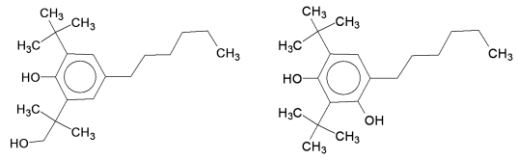


Member No. 9: 2,6-Di-tert-butyl-4-hexylphenol (CAS No. 56280-62-7)

QSAR Toolbox: Total 21 metabolites including QM (shown in red frame) were found.

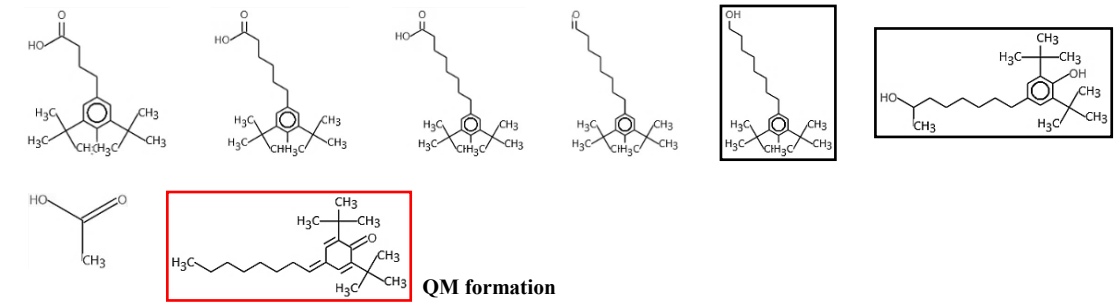


HESS: 2 metabolites in QSAR Toolbox (shown in black frame above) and 2 metabolites below were found.

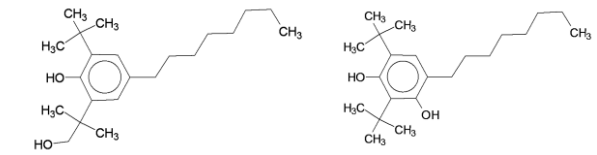


Member No. 10: 2,6-Di-tert-butyl-4-octylphenol (CAS No. 35309-87-6)

QSAR Toolbox: Total 8 metabolites including QM (shown in red frame) were found.

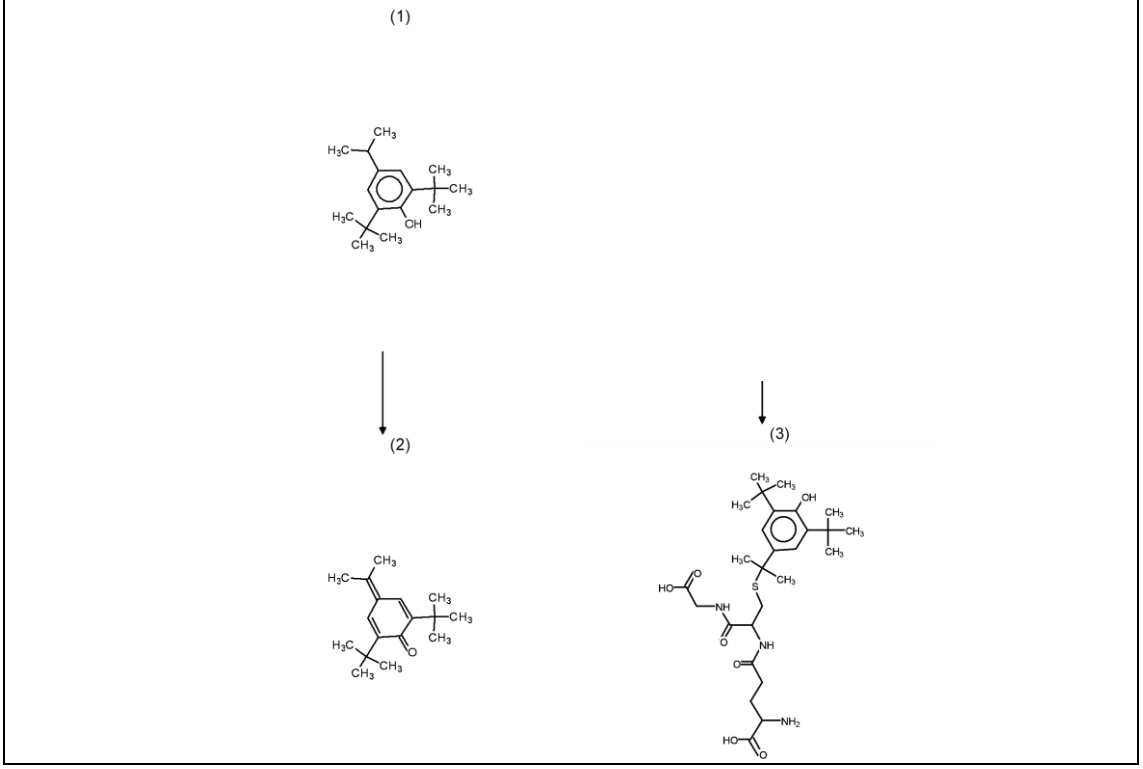


HESS: 2 metabolites in QSAR Toolbox (shown in black frame above) and 2 metabolites below were found.



Member No. 11: 2,6-Di-tert-butyl-4-isopropylphenol (CAS No. 5427-03-2)

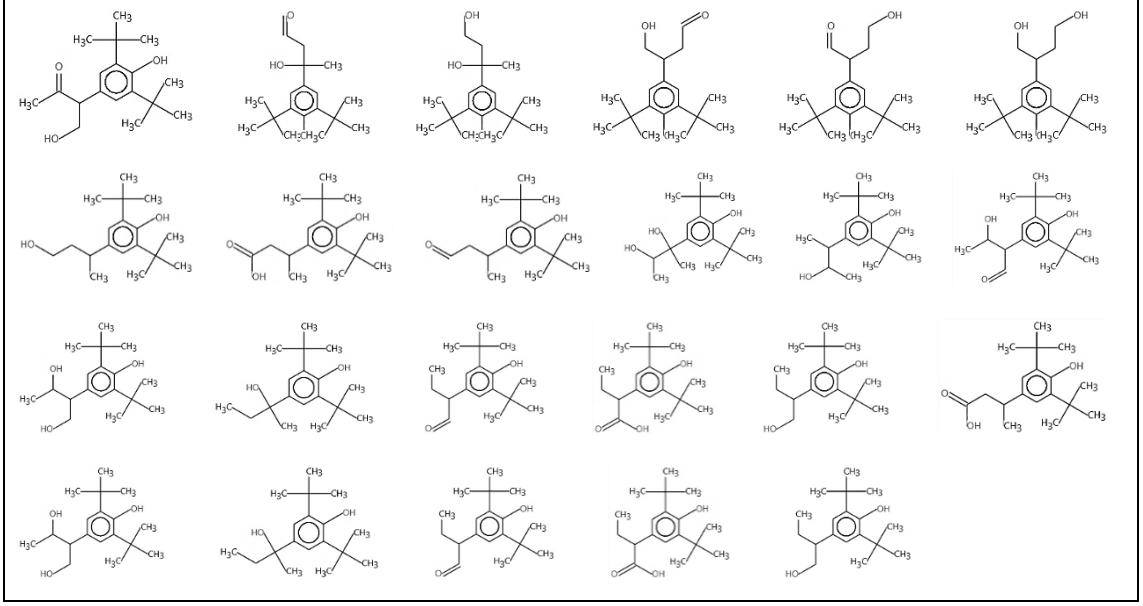
Reference (in chemico): Toteva MM, Richard JP. (2011). The Generation and Reactions of Quinone Methides. Adv Phys Org Chem. 1; 45: 39–91.



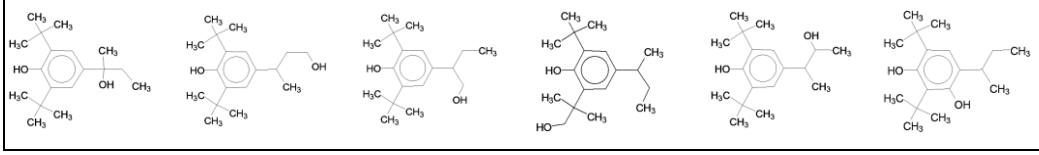
Member No. 12: 2,6-Di-tert-butyl-4-sec-butylphenol (CAS No. 17540-75-9)

Reference (in chemico): Toteva MM, Richard JP. (2011). The Generation and Reactions of Quinone Methides. Adv Phys Org Chem. 1; 45: 39–91.

QSAR Toolbox: Total 23 metabolites were found.



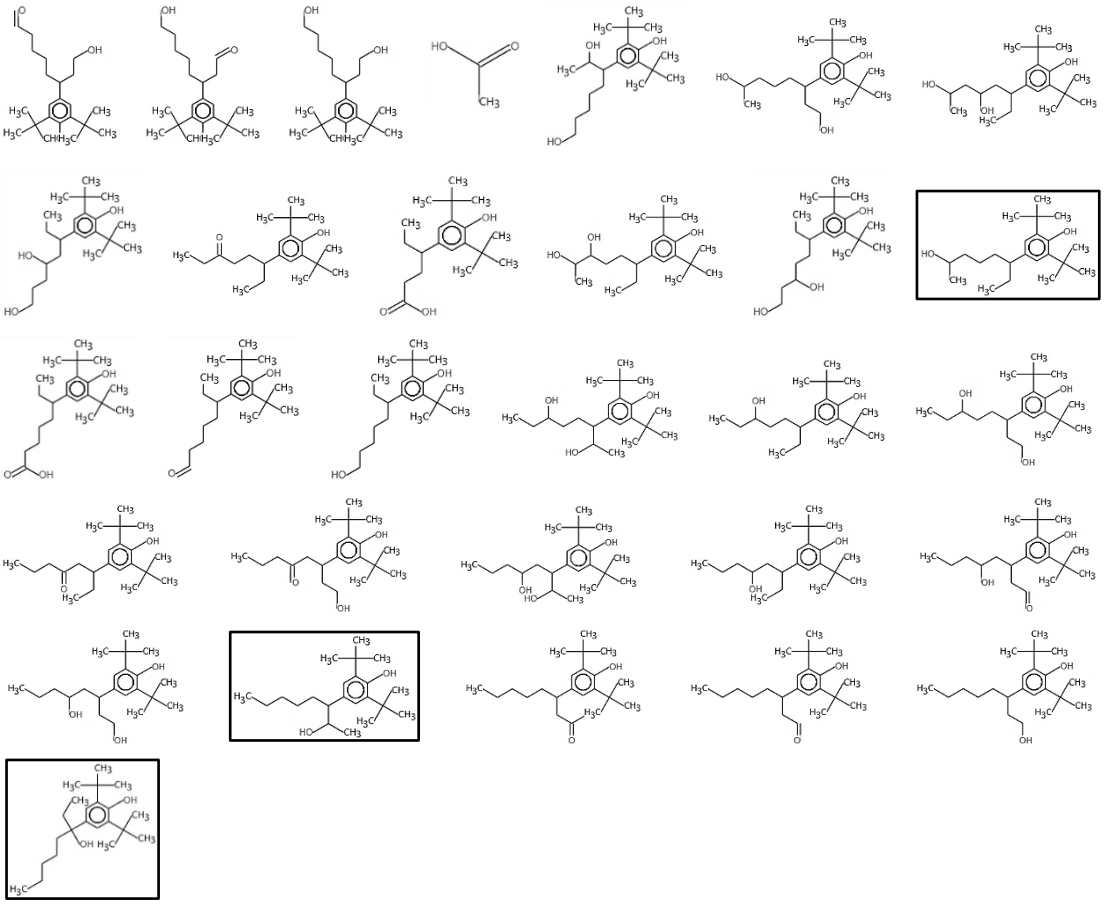
HESS: Total 6 metabolites were found.



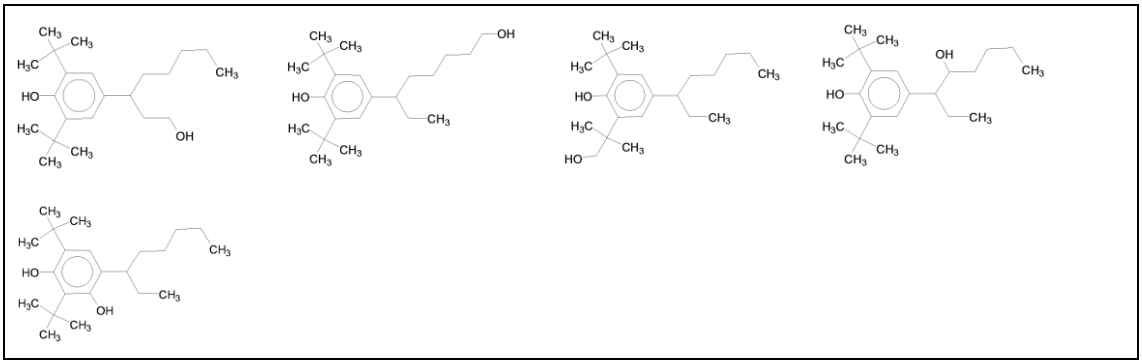
Member No. 13: 2,6-Di-tert-butyl-4-(2-ethylhexyl)phenol (CAS No. 816462-78-9)

Reference (in chemico): Toteva MM, Richard JP. (2011). The Generation and Reactions of Quinone Methides. Adv Phys Org Chem. 1; 45: 39–91.

QSAR Toolbox: Total 30 metabolites were found.



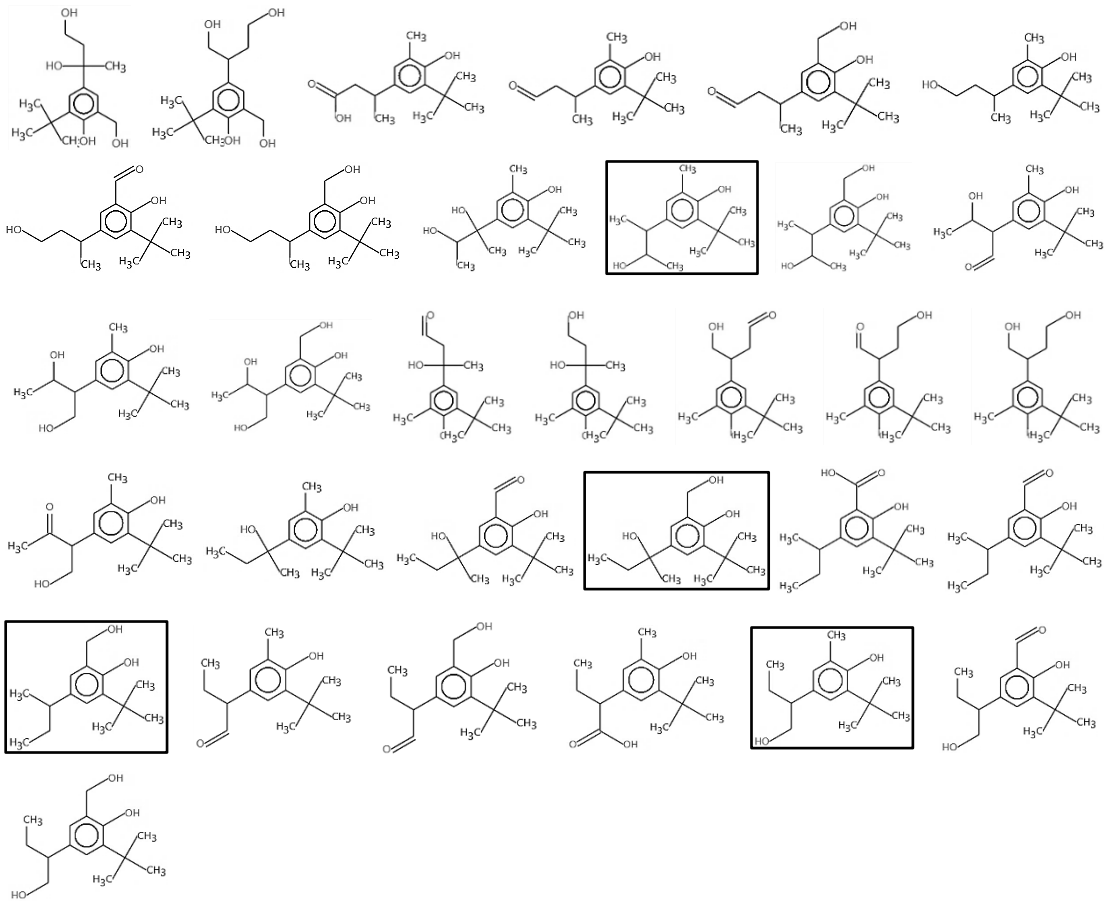
HESS: 3 metabolites in QSAR Toolbox (shown in black frame above) and 5 metabolites below were found.



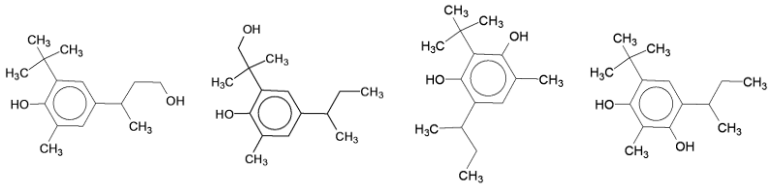
Member No. 14: 4-(Butan-2-yl)-2-tert-butyl-6-methylphenol (CAS No. 51067-63-1)

Reference (in chemico): Toteva MM, Richard JP. (2011). The Generation and Reactions of Quinone Methides. Adv Phys Org Chem. 1; 45: 39–91.

QSAR Toolbox: Total 32 metabolites were found.

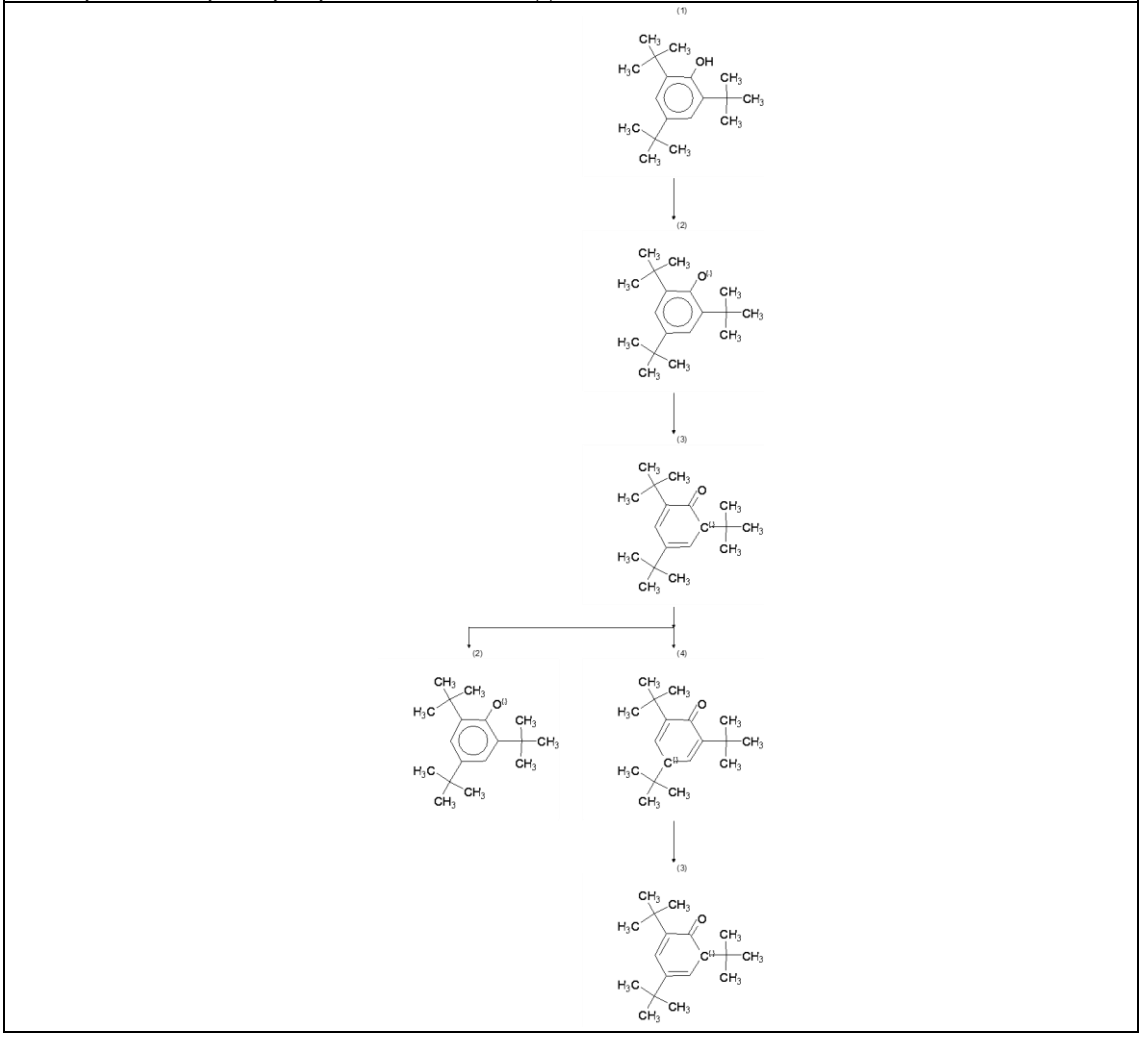


HESS: 4 metabolites in QSAR Toolbox (shown in black frame above) and 4 metabolites below were found.



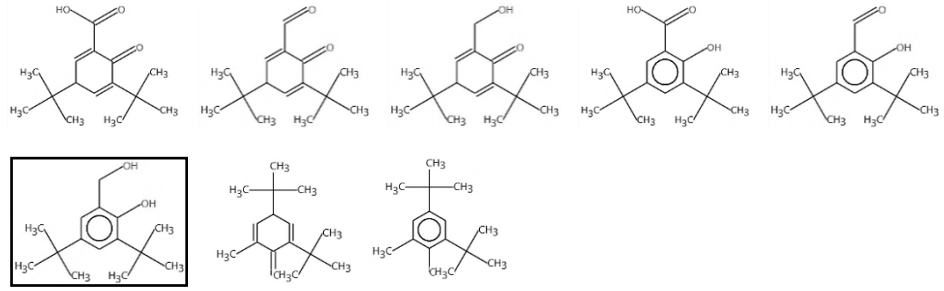
Member No. 15: 2,4,6-Tri-tert-butylphenol (CAS No. 732-26-3)

Reference (in vivo): Takahashi O, Hiraga K. (1983). Metabolic studies in the rat with 2,4,6-tri-t-butylphenol: a haemorrhagic antioxidant structurally related to butylated hydroxytoluene. Xenobiotica. 13 (5): 319–26.

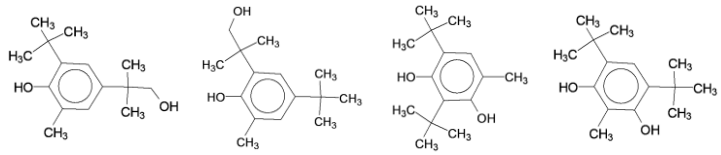


Member No. 16: 2,4-Di-tert-butyl-6-methylphenol (CAS No. 616-55-7)

QSAR Toolbox: Total 8 metabolites were found.

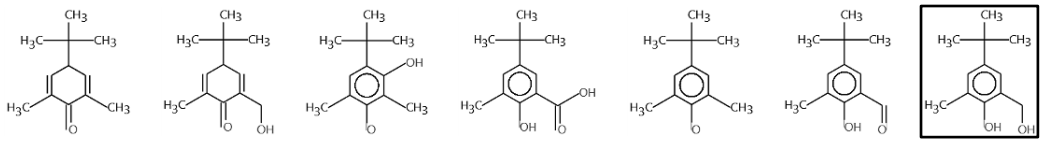


HESS: 1 metabolite in QSAR Toolbox (shown in black frame above) and 4 metabolites below were found.

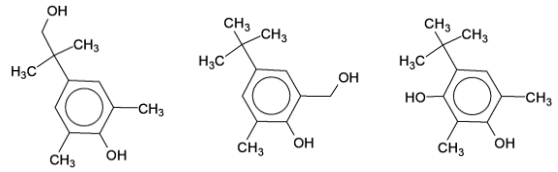


Member No. 17: 2,6-Dimethyl-4-tert-butylphenol (CAS No. 879-97-0)

QSAR Toolbox: Total 7 metabolites were found.



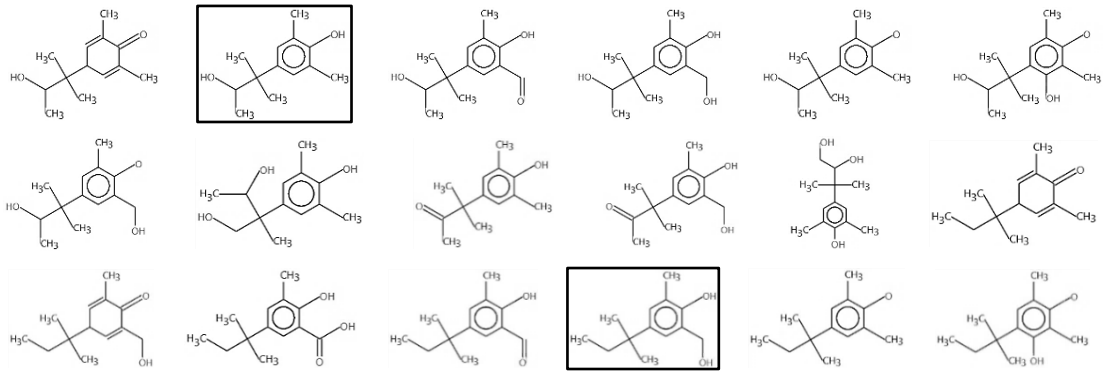
HESS: 1 metabolite in QSAR Toolbox (shown in black frame above) and 2 metabolites below were found.



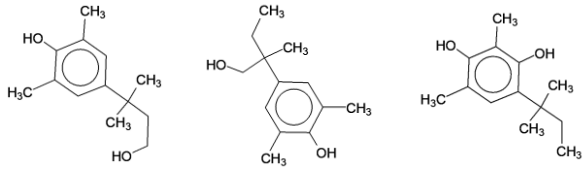
Member No. 18: 2,6-Dimethyl-4-(2-methylbutan-2-yl)phenol (CAS No. 91798-63-9)

Reference (in chemico): Toteva MM, Richard JP. (2011). The Generation and Reactions of Quinone Methides. Adv Phys Org Chem. 1; 45: 39–91.

QSAR Toolbox: Total 18 metabolites were found.

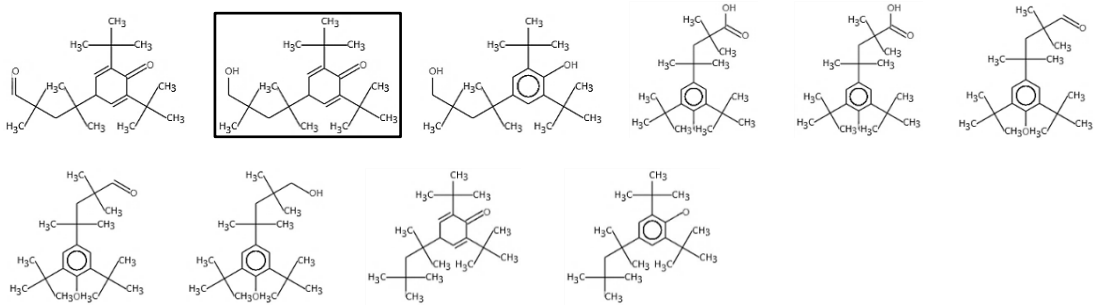


HESS: 2 metabolites in QSAR Toolbox (shown in black frame above) and 3 metabolites below were found.

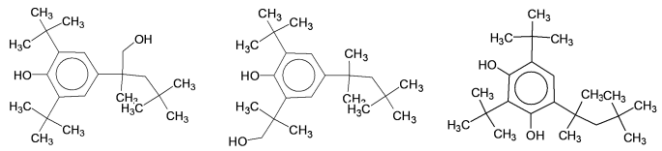


Member No. 19: 2,6-Di-tert-butyl-4-(1,1,3,3-tetramethylbutyl)phenol (CAS No. 65796-87-4)

QSAR Toolbox: Total 10 metabolites were found.



HESS: 1 metabolite in QSAR Toolbox (shown in black frame above) and 3 metabolites below were found.



Annex III. Detailed protocols of *in vitro* assay

Compounds

The compounds used in this study were follows: member No. 1 (2,6-di-tert-butyl-4-methylphenol, BHT; #D0228), 2 (2,4-dimethyl-6-tert-butylphenol, BDMP; #B0903), 3 (2,4,6-tri-methylphenol, TMP; #T0486), 4 (2,6-di-tert-butyl-4-ethylphenol; #D1667), 12 (2,6-di-tert-butyl-4-sec-butylphenol; #B2774), and 15 (2,4,6-tri-tert-butylphenol, #T0359) were purchased from Tokyo Chemical Industry (Tokyo, Japan); No. 10 (2,6-di-tert-butyl-4-octylphenol; #PBCM1284666) was from Chemieliva Pharmaceutical (Shanghai, China); and No. 16 (2,4-di-tert-butyl-6-methylphenol; #S879916-50MG) was from Sigma-Aldrich (St. Louis, MO, USA).

dGSH trapping assay [non-GLP-compliant]

dGSH trapping assay is widely used to screen reactive metabolites of drugs based on chromatographic detection of fluorescence following metabolic reactions with microsomes. International Genetic Standard Sprague-Dawley rat liver microsomes (#1610290) were purchased from XenoTech (Tokai, Japan). Incubations were carried out at 37°C for 60 min. Each reaction included the test compounds (0.1, 0.5, or 1 mM), microsomal proteins (3 mg/ml microsome), dGSH (1 mM), and β-nicotinamide adenine dinucleotide phosphate (1.1 mM). The reaction was terminated by adding 1 mM/l Tris (2-carboxyethyl) phosphine methanol solution. Samples were maintained for about 60 min in a −20°C freezer, then centrifuged at 3000 rpm at 4°C for 10 min to pellet the proteins. The amount of dGSH adduct in the supernatant was determined by ultra-high performance liquid chromatography (UPLC) with a fluorescence detector (Nihon Waters K.K., Tokyo, Japan). Aliquots of each sample were injected into an ACQUITY UPLC ethylene bridged hybrid C18 column (1.7 µm, 2.1 × 100mm) (Nihon Waters K.K.). The mobile phase (0.2% acetonitrile water, 0.2% formic acid) was linearly programmed from 20% formic acid/acetonitrile to 70% in 9.33 min, held at 90% for 1.3 min, then returned to 20% in 0.37 min and maintained for 3 min at a flow rate of 0.5 ml/min. The excitation and emission wavelengths were set to 340 and 525 nm, respectively. The UPLC auto-sampler and column were set to 4°C and 40°C, respectively. Empower software (Nihon Waters K.K.) was used to calculate the amount of dGSH adducts from the calibration curve peak area value. The basic technical information on this test can be found elsewhere (Dieckhaus *et al.,* 2005; Gan *et al.,* 2005).

Cell viability assay [non-GLP-compliant]

Cryopreserved primary hepatocytes from male Sprague-Dawley rats (#R16E31; KaLy-Cell, Plobsheim, France) were obtained from Kurabo (Osaka, Japan). The cells were cultured according to the manufacturer's protocol on a 96-well plate coated with collagen at 37°C and % CO_2. On the day before treatment with test compounds, the cells were seeded at 2 × 10^4 cells/well in culture medium for 24 h. 1 M stock solution of test compounds were prepared in dimethyl sulfoxide (DMSO) and the cells were treated with them at a concentration of 0.1, 0.5, or 1 mM. Control cells were treated with conditioned medium containing 0.1% DMSO. After incubation for 72 h, the number of viable cells was determined with CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI,

USA); the optical density was measured on a microplate reader (SparkControl; Tecan Japan, Kawasaki, Japan). The percent cell viability was calculated from emission values relative to that of the control at each concentration. The assay was performed with samples prepared in triplicate and was independently performed at least three times.

Annex IV. Summary of rat oral repeated-dose toxicity data

Member No. 1: 2,6-Di-tert-butyl-4-methylphenol, BHT (CAS No. 128-37-0)

References	Powell CJ, Connelly JC, Jones SM, Grasso P, Bridges JW. (1986). Hepatic responses to the administration of high doses of BHT to the rat: their relevance to hepatocarcinogenicity. Food Chem Toxicol. 24 (10-11): 1131–43.
Species/strain	Rats/Wistar
Sex	Male
Route of admin.	Oral: gavage
Exposure period	28 days (daily exposure)
Doses	0, 25, 250, 500 mg/kg/day
GLP	Yes (Klimisch score 1)
Test substance	Purity: 99.9 %; vehicle: arachis oil
NOAEL	= 25 mg/kg/day (male)
Result	General signs: Marginally lower than that of the control group (500 mg/kg/day). No deaths occurred during the study period. Liver biochemistry: Increase of proteins, glucose 6-phosphatase activity, ethoxycoumarin-O-deethylase, and epoxide hydrolase activity (250 and 500 mg/kg/day) Liver weight: Slight (25 mg/kg/day), moderate (250 mg/kg/day) and marked increase (500 mg/kg/day) Histopathological examination: Necrosis of hepatocyte in periportal region, fibrosis, proliferation of bile-duct cell, hepatocyte hypertrophy, hepatocyte hyperplasia, pigment-laden macrophages, glycogen depletion (500 mg/kg/day), glycogen accumulation (250 and 500 mg/kg/day) Immunochemistry: Moderately-increased staining intensity in the hypertrophied viable hepatocytes adjacent to the areas of damage (500 mg/kg/day)
Other findings	Tissue levels: Higher in fat tissue than liver after the treatment for 28 days

Member No. 2: 2,4-Dimethyl-6-tert-butylphenol (CAS No. 1879-09-0)

References	MHW (1994). Unpublished Report on Combined Repeat Dose and Reproductive/Developmental Toxicity Screening Test of 6-tert-butyl-2,4-xylenol. (HPV/SIDS Test conducted by MHW, Japan)
Species/strain	Rat/CD(SD), SPF
Sex	Male/female
Route of admin.	Oral: gavage
Exposure period	Males, 45 days/females, from 14 days before mating to day 3 of lactation
Doses	0, 6, 30, 150 mg/kg/day
GLP	Yes (OECD TG 422) (Klimisch score 1)
Test substance	Purity: 98.5 %; vehicle: corn oil
NOEL	6 mg/kg/day (male), 30 mg/kg/day (female)
Result	General signs: No clinical signs related to the treatments were noted. However, two dead animals (one of them during the delivery) were observed in female rats (150 mg/kg/day) at the end of the gestation period. Haematology: Decrease of haematocrit, haemoglobin and red blood cells, increase in reticulocytes (slight trend of anaemia) in males (150 mg/kg/day). Clinical biochemistry: Decrease of GOT, and increase of γ-GTP in males (30 and 150 mg/kg/day) Liver weight: Weight increase and enlargement of the liver in males (30 and 150 mg/kg/day) and females (150 mg/kg/day) Histopathological examination: Swelling of centrilobular hepatocytes in males (150 mg/kg/day); swelling and necrosis of centrilobular hepatocytes, and single cell necrosis in females (150 mg/kg/day)
Other findings	Kidney: Weight increase and enlargement in males (30 and 150 mg/kg/day) and females (150 mg/kg/day). Histopathological examination showed swelling of liver cells in the centrilobules in both males and females (150 mg/kg/day), and showed degeneration and protein cast of the proximal renal tubules, PAS positive granules deposited at renalpapilla in females (150 mg/kg/day). General signs: The dead females and females with pups which all died showed increased incidences of parakeratosis of the tongue, oesophageal swelling and necrosis of centrilobular hepatocytes, as well as a variety of degenerative charges, single cell necrosis and mitosis in the liver.

Member No. 3: 2,4,6-trimethylphenol, TMP (CAS No. 527-60-6)

References	Safety Institute of Science of Mitsubishi Chemical (2007)
Species/strain	Rats/CD(SD), SPF
Sex	Male/female
Route of admin.	Oral: gavage
Exposure period	Males, 42 days; females, from 14 days before mating to day 4 of lactation; females (satellite), 42 days
Doses	0, 10, 60, 300 mg/kg/day
GLP	Yes (OECD TG 422) (Klimisch score 1)
Test substance	Purity: 99.8 %; vehicle: 0.1w/v% Tween 80+ 0.5w/v% Sodium carboxymethylcellulose solution
NOAEL	10 mg/kg/day (squamous hyperplasia in the forestomach) (male/female)
Result	General signs: No clinical signs related to the treatments were noted. No deaths occurred during the study period. Liver: No other changes considered to represent adverse effects in the liver
Other findings	Stomach: Squamous hyperplasia of forestomach in both sex (60 and 300 mg/kg/day), oedema in one male, foveola hyperplasia and erosion in the glandular stomach in males (300 mg/kg/day)

Member No. 4: 2,6-Di-tert-butyl-4-ethylphenol (CAS No. 4130-42-1)

References	Ministry of Health, Labour and Welfare (Japan Existing Chemical Database (JECDB; https://dra4.nihs.go.jp/mhlw_data/jsp/SearchPageENG.jsp), Access on Jan. 2011)
Species/strain	Rats/Sprague Dawley
Sex	Male/female
Route of admin.	Oral: gavage
Exposure period	28 days (daily exposure)
Doses	0, 15, 60, 250 mg/kg/day
GLP	Yes (OECD TG 407) (Klimisch score 1)
Test substance	Vehicle control: 0.5% methylcellulose solution
NOAEL	= 15 mg/kg/day (male/female)
Result	General signs: No clinical signs related to the treatments were noted. No deaths occurred during the study period. Haematology: Level increase of platelet, fibrinogen and APTT in both sex, and PT in males (250 mg/kg/day) Clinical biochemistry: Increase of total cholesterol and total protein in both sex, and phospholipid in males (250 mg/kg/day); decrease of chlorine in males (250 mg/kg/day) Liver weight: Absolute/relative weights increase in both sex (60 and 250 mg/kg/day) Histopathological examination: Hypertrophy of centrilobular hepatocytes (60 and 250 mg/kg/day)
Other findings	Thyroid: Hypertrophy of follicular cells in males (60 mg/kg/day) and in both sex (250 mg/kg/day)

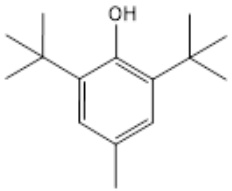
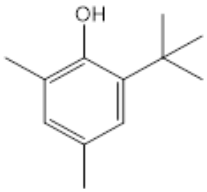
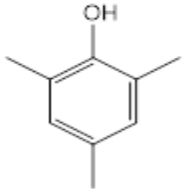
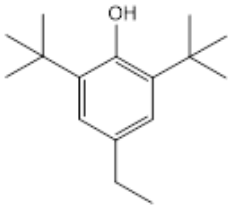
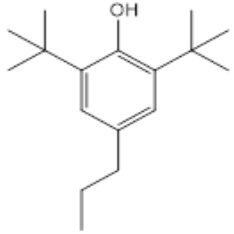
Member No. 12: 2,6-Di-tert-butyl-4-sec-butyl-phenol (CAS No. 17540-75-9)

References	Safety Research Institute for Chemical Compounds Co., Ltd.
Species/strain	Rats/CD(SD), SPF
Sex	Male/female
Route of admin.	Oral: gavage
Exposure period	28 days (daily exposure)
Doses	0, 15, 60, 250 mg/kg/day
GLP	Yes (Klimisch score 1)
Test substance	Purity: 99.0 %; vehicle: corn oil
NOAEL	15 mg/kg/day (male), < 15 mg/kg/day (female)
Result	General signs: Loose stools in both sex (250 mg/kg/day). No deaths occurred during the study period. Haematology: Increase of APTT and PT in males (60 mg/kg/day) and females (250 mg/kg/day); decrease of lymphocyte and eosinophil in males (60 mg/kg/day) and in females (15 mg/kg/day) and WBC in females (60 mg/kg/day) Clinical biochemistry: Increase of total cholesterol in males (60 and 250 mg/kg/day) and in females (15, 60 and 250 mg/kg/day); increase of AST, ALT, ALP and decrease of total bilirubin in both sex (250 mg/kg/day); decrease of potassium and IP in males (250 mg/kg/day); increase of ureain females (250 mg/kg/day) Liver weight: Absolute (250 mg/kg/day) and relative weight increase in females (15, 60 and 250 mg/kg/day) Histopathological examination: Hypertrophy of centrilobular hepatocytes, small granuloma and fatty degeneration in females (250 mg/kg/day)
Other findings	Spleen: Absolute weight increase in females (250 mg/kg/day) Uterus: Relative weight increase in females (250 mg/kg/day) Kidney: Increase of UN in males (250 mg/kg/day); decrease of pH in females (250 mg/kg/day)

Member No. 15: 2,4,6-tri-tert-butylphenol (CAS No. 732-26-3)

References	ECHA registration dossier (2019a). US EPA OPPTS 870.3650
Species/strain	Rats/Wistar, SPF
Sex	Male/female
Route of admin.	Oral: gavage
Exposure period	Males were exposed for 29 days, i.e. 2 weeks prior to mating, during mating, and up to the day prior to scheduled necropsy. Females were exposed for 41 to 56 days, i.e. during 2 weeks prior to mating, during mating, during post-coitum, and during at least 4 days of lactation (up to the day prior to scheduled necropsy).
Doses	0, 3, 10, 30 mg/kg/day
GLP	Yes (OECD TG 422; combined repeated-dose toxicity study with the reproduction / developmental toxicity screening test)
Test substance	Vehicle: corn oil
NOAEL	3 mg/kg/day (based on higher liver weights at 10 and 30 mg/kg/day, with hepatocellular hypertrophy and necrosis at 30 mg/kg/day)
Result	General signs: No clinical signs related to the treatments were noted. No deaths occurred during the study period. Haematology: Decrease of neutrophil counts, and increase of lymphocyte counts in females (30 mg/kg/day); increase of APTT and PT in males (60 mg/kg/day) and females (250 mg/kg/day); decrease of WBC in females (60 mg/kg/day) Clinical biochemistry: Increase of total protein, albumin, calcium, cholesterol, glucose, and decrease of urea in females (30 mg/kg/day); increase of Potassium and decrease of total bilirubin in both sex (30 mg/kg/day); increase of total cholesterol Liver weight: Relative weight increase in males (30 mg/kg/day) and females (10 and 30 mg/kg/day) Histopathological examination: Hypertrophy of hepatocytes in both sex (10 and 30 mg/kg/day), necrosis of hepatocytes in single male and female (30 mg/kg/day)
Other findings	No other changes considered to represent adverse effects

Appendix. Data matrix, IATA for "Case Study on the Use of Integrated Approaches for Testing and Assessment to Inform Read-Across of p-Alkylphenols: Repeated-Dose Toxicity"

Chemical ID						
Number of category member		**Member 1**	**Member 2**	**Member 3**	**Member 4**	**Member 5**
CAS		128-37-0	1879-09-0	527-60-6	4130-42-1	2,6-Di-tert-butyl-4-propylphenol
Name		2,6-Di-tert-butyl-4-methylphenol, BHT	2,4-Dimethyl-6-tert-butylphenol, BDMP	2,4,6-Trimethylphenol, TMP	2,6-Di-tert-butyl-4-ethylphenol	4973-24-4
SMILES		CC1=CC(=C(C(=C1)C(C)(C)C)O)C(C)(C)C	CC1=CC(=C(C(=C1)C(C)(C)C)O)C	CC1=CC(=C(C(=C1)C)O)C	CCC1=CC(=C(C(=C1)C(C)(C)C)O)C(C)(C)C	CCCC1=CC(=C(C(=C1)C(C)(C)C)O)C(C)(C)C
Structure						
Summary of data gap filling						
Sub-chronic repeated-dose toxicity (oral route, rat)	Experimental result	**Wistar rats (male), oral (gavage), 28-days, 0, 25, 250, 500 mg/kg/day [GLP data]** **Result** General signs: Marginally lower than that of the control group (500 mg/kg/day). No deaths occurred during the study period. Liver biochemistry: Increase of proteins, glucose 6-phosphatase	**Sprague Dawley rats (male), oral (gavage), males, 45 days/females, from 14 days before mating to day 3 of lactation, 0, 6, 30, 150 mg/kg/day [GLP data, OECD TG 422]** **Result** General signs: No clinical signs related to the treatments were noted. However, two dead	**Sprague Dawley rats (male), oral (gavage), males, 42 days/females, from 14 days before mating to day 3 of lactation, 0, 10, 60, 300 mg/kg/day [GLP data, OECD TG 422]** **Result** General signs: No clinical signs related to the treatments were noted. No deaths occurred	**Sprague Dawley rats (male), oral (gavage), 28-days, 0, 15, 60, 250 mg/kg/day [GLP data, OECD TG 407]** **Result** General signs: No clinical signs related to the treatments were noted. No deaths occurred during the study period. Haematology: Level increase of platelet, fibrinogen and APTT	N/A

		activity, ethoxycoumarin-O-deethylase, and epoxide hydrolase activity (250 and 500 mg/kg/day) Liver weight: Slight (25 mg/kg/day), moderate (250 mg/kg/day) and marked increase (500 mg/kg/day) Histopathological examination: Necrosis of hepatocyte in periportal region, fibrosis, proliferation of bile-duct cell, hepatocyte hypertrophy, hepatocyte hyperplasia, pigment-laden macrophages, glycogen depletion (500 mg/kg/day), glycogen accumulation (250 and 500 mg/kg/day) Immunochemistry: Moderately-increased staining intensity in the hypertrophied viable hepatocytes adjacent to the areas of damage (500 mg/kg/day) Tissue levels: Higher in fat tissue than liver after the treatment for 28 days **Data source: Powell *et al.* (1986)**	animals (one of them during the delivery) were observed in female rats (150 mg/kg/day) at the end of the gestation period. Haematology: Decrease of haematocrit, haemoglobin and red blood cells, increase in reticulocytes (slight trend of anaemia) in males (150 mg/kg/day). Clinical biochemistry: Decrease of GOT, and increase of γ-GTP in males (30 and 150 mg/kg/day) Liver weight: Weight increase and enlargement of the liver in males (30 and 150 mg/kg/day) and females (150 mg/kg/day) Histopathological examination: Swelling of centrilobular hepatocytes in males (150 mg/kg/day); swelling and necrosis Kidney: Weight increase and enlargement in males (30 and 150 mg/kg/day) and females (150 mg/kg/day). Histopathological examination showed swelling of liver cells in the centrilobules in both males and females (150 mg/kg/day), and showed degeneration and protein cast of the proximal renal tubules, PAS positive granules deposited at renalpapilla in females (150	during the study period. Liver: No other changes considered to represent adverse effects in the liver Stomach: Squamous hyperplasia of forestomach in both sex (60 and 300 mg/kg/day), oedema in one male, foveola hyperplasia and erosion in the glandular stomach in males (300 mg/kg/day) **Data source: Safety Institute of Science of Mitsubishi Chemical (2007)**	in both sex, and PT in males (250 mg/kg/day) Clinical biochemistry: Increase of total cholesterol and total protein in both sex, and phospholipid in males (250 mg/kg/day); decrease of chlorine in males (250 mg/kg/day) Liver weight: Absolute/relative weights increase in both sex (60 and 250 mg/kg/day) Histopathological examination: Hypertrophy of centrilobular hepatocytes (60 and 250 mg/kg/day) Thyroid: Hypertrophy of follicular cells in males (60 mg/kg/day) and in both sex (250 mg/kg/day) **Data source: Ministry of Health, Labour and Welfare (Japan Existing Chemical Database (JECDB) Access on Jan. 2011)**	

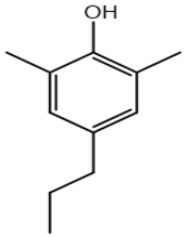
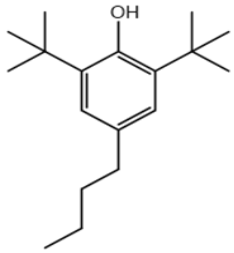
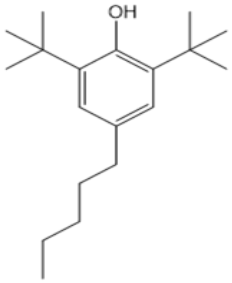
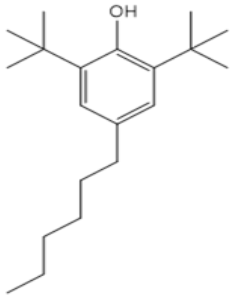
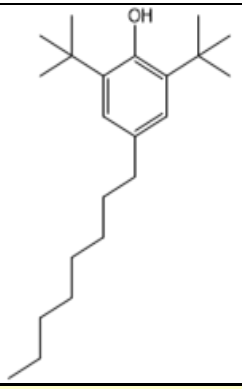
			mg/kg/day). General signs: The dead females and females with pups which all died showed increased incidences of parakeratosis of the tongue, oesophageal swelling and necrosis of centrilobular hepatocytes, as well as a variety of degenerative charges, single cell necrosis and mitosis in the liver. **Data source: MHW (1994)**			
	Integrated conclusion	NOAEL = 25 mg/kg/day	NOAEL = 6 mg/kg/day (male), 30 mg/kg/day (female)	NOAEL = 10 mg/kg/day (squamous hyperplasia in the forestomach) (male/female)	NOAEL = 15 mg/kg/day	derived result
Molecular profiling related to the category hypothesis						
Metabolite*	Observed Rat *In vivo* metabolism [2)]	5 metabolites	Not tested	Not tested	Not tested	Not tested
	Observed Rat Liver S9 metabolism [2)]	Not tesed	Not tested	Not tested	Not tested	Not tested
	Observed rat liver metabolism with quantitative data [2)]	Not tested	Not tested	Not tested	Not tested	Not tested
	in vivo Rat metabolism simulator [2)]	4 metabolites	7 metabolites	7 metabolites	4 metabolites	13 metabolites
	Rat liver S9 metabolism simulator [2)]	4 metabolites	7 metabolites	7 metabolites	5 metabolites	6 metabolites
	Observed rat Liver metabolism [5)]	20 metabolites	N/A	N/A	2 metabolites	N/A

	Liver Metabolism Simulator [5]	4 metabolites	9 metabolites	7 metabolites	3 metabolites	5 metabolites
Physical-chemical data						
Molecular weight (EPI Suite™ v.4.11) [1]		220.36	178.28	136.20	234.38	248.41
Boiling point (deg C) (MPBPVP estimate) [1]		296.49	264.87	229.63	310.94	324.48
Melting point (deg C) (MPBPVP estimate) [1]		83.01	66.48	40.68	92.16	100.91
Vapour pressure (mm Hg 25 deg C) (MPBPVP estimate) [1]		0.00177	0.0121	0.0204	0.00217	2.88E-05
Water solubility (mg/L) (WSKOW v.1.41) [1]		5.748	29.64	1539	2.115	0.6747
$logP_{ow}$ (measured value) (TSCATS experimental database match) [1]		5.10	N/A	2.73	N/A	N/A
$logP_{ow}$ (calculated value) (KOWWIN v.1.68 estimate) [1]		5.03	4.52	3.15	5.52	6.01
…						
Kinetics**						
Absorption		Rapidly absorbed through the gastrointestinal tract (OECD, 2002)	Rapidly absorbed from the gastrointestinal tract (ECHA, 2019b)	N/A	N/A	N/A
Distribution		30 and 45 ppm BHT in fat (male and female rats, respectively); lower levels in the liver (Daniel and Gage, 1965)	-	N/A	N/A	N/A
Metabolism		Oxidation of the 4-methyl group and one or both tert-butyl substituents (Daniel 1986); Enterohepatic dissemination of BHT-COOH and its glucuronide derivative (Ladomery *et al.* 1967b; Holder *et al.* 1970); $t_{1/2}$ =	Alkyl-, alkenyl-, and aryl-substituted phenols and their corresponding esters is conjugated with sulphate and glucuronic acid after hydrolysis of the esters (ECHA, 2019b)	Oxidation to p-quinone methides (TMP-QM) by cytochrome P450 and its conjugate with glutathione GSH (TMP-SG) (ECHA, 2019c)	N/A	N/A

		7–10 days in fat and liver (Daniel and Gage, 1965)				
Excretion		A parent in urine and to a lesser extent in feces (Verhagen *et al.* 1989); 10% BHT in faeces and less than 1% BHT-COOH in urine after 24 h (ECHA, 2019d)	Primary in the urine (ECHA, 2019b)	N/A	N/A	N/A
Supporting data related to the target endpoint(s)						
In vivo	Toxicogenomics	N/A	N/A	N/A	N/A	N/A
	…	N/A	N/A	N/A	N/A	N/A
In vitro	Alternative method A	N/A	N/A	N/A	N/A	N/A
In silico	Protein binding potency [2)]	Not found	Not found	Not found	Not found	Not found
	Protein binding potency GSH [2)]	Not possible to classify according to these rules (GSH)	Not possible to classify according to these rules (GSH)	Not possible to classify according to these rules (GSH)	Not possible to classify according to these rules (GSH)	Not possible to classify according to these rules (GSH)
	GSH reactivity [2)]	Not calculated	Not calculated	Not calculated	Not calculated	Not calculated
	Hepatotoxicity in mammal [3)]	Probable	Plausible	Plausible	Plausible	Plausible
	Mitochondrial dysfunction in mammal [3)]	Equivocal	Equivocal	Not detected	Equivocal	Equivocal
	LIVER_BDUCT (Human bile duct disorders) [4)]	Negative	Negative	Negative	Negative	Negative
	LIVER_CHOLEST (Human holestasis) [4)]	Inconclusive	Inconclusive	Inconclusive	Inconclusive	Inconclusive
	LIVER_DAMAGE (Human acute liver damage) [4)]	Negative	Negative	Negative	Negative	Negative

	LIVER_FTEST (Human liver function test (blood test on liver enzymes release)) [4)]	Positive	Positive	Positive	Positive	Positive
	LIVER_GALL (Human gall bladder disorders) [4)]	Negative	Negative	Inconclusive	Negative	Negative
Other data	Battery approach	N/A	N/A	N/A	N/A	N/A
	Defined approach of IATA	N/A	N/A	N/A	N/A	N/A
	Formation of quinone methide (QM)	Yes	Yes	Yes	Yes	Yes

* More relevant metabolite such as toxicant
**General outline of relative comparative kinetics
reference
1) EPI SuiteTM v.4.11
2) OECD QSAR Toolbox v.4.3
3) Derek Nexus v.6.0.1
4) CASE Ultra v.1.7.0.5
5) HESS v.3.8

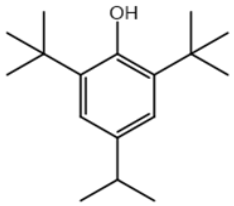
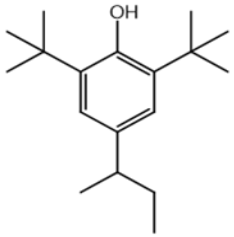
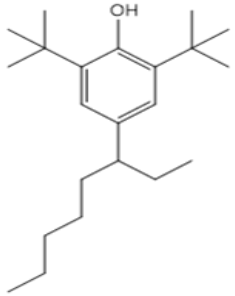
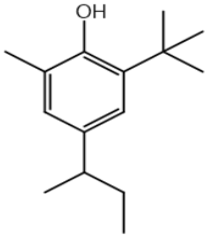
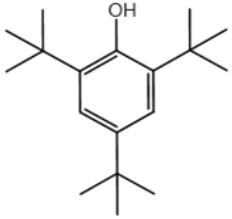
Chemical ID						
Number of category member		**Member 6**	**Member 7**	**Member 8**	**Member 9**	**Member 10**
CAS		2,6-Dimethyl-4-propyl-phenol	2,6-Di-tert-butyl-4-butylphenol	2,6-Di-tert-butyl-4-pentylphenol	2,6-Di-tert-butyl-4-hexylphenol	2,6-Di-tert-butyl-4-octylphenol
Name		13037-82-6	5530-30-3	4973-26-6	56280-62-7	35309-87-6
SMILES		CCCC1=CC(=C(C(=C1)C)O)C	CCCCC1=CC(=C(C(=C1)C(C)(C)C)O)C(C)(C)C	CCCCCC1=CC(=C(C(=C1)C(C)(C)C)O)C(C)(C)C	CCCCCCC1=CC(=C(C(=C1)C(C)(C)C)O)C(C)(C)C	CCCCCCCCC1=CC(=C(C(=C1)C(C)(C)C)O)C(C)(C)C
Structure						
Summary of data gap filling						
Sub-chronic epeated-dose toxicity (oral route, rat)	Experimental result	N/A	N/A	N/A	N/A	N/A
	Integrated conclusion	derived result	derived result	derived result	derived result	derived result
Molecular profiling related to the category hypothesis						
Metabolite*	Observed Rat *In vivo* metabolism [2)]	Not tested	Not tested	Not tested	Not tested	Not tested
	Observed Rat Liver S9 metabolism [2)]	Not tested	Not tested	Not tested	Not tested	Not tested
	Observed rat liver metabolism with	Not tested	Not tested	Not tested	Not tested	Not tested

	quantitative data [2)]					
	in vivo Rat metabolism simulator [2)]	21 metabolites	8 metabolites	10 metabolites	20 metabolites	7 metabolites
	Rat liver S9 metabolism simulator [2)]	11 metabolites	9 metabolites	9 metabolites	5 metabolites	5 metabolites
	Observed rat Liver metabolism [5)]	N/A	N/A	N/A	N/A	N/A
	Liver Metabolism Simulator [5)]	5 metabolites	5 metabolites	5 metabolites	4 metabolites	4 metabolites
Physical-chemical data						
Molecular weight (EPI Suite™ v.4.11) [1)]		164.25	262.44	276.47	290.49	318.55
Boiling point (deg C) (MPBPVP estimate) [1)]		264.86	337.12	348.98	360.69	383.79
Melting point (deg C) (MPBPVP estimate) [1)]		61.91	109.26	117.27	125.17	140.97
Vapour pressure (mm Hg 25 deg C) (MPBPVP estimate) [1)]		0.00219	1.12E-05	4.57E-06	1.88E-06	3.15E-07
Water solubility (mg/L) (WSKOW v.1.41) [1)]		72.92	0.2145	0.068	0.0215	0.002136
$logP_{ow}$ (measured value) (TSCATS experimental database match) [1)]		N/A	N/A	N/A	N/A	N/A
$logP_{ow}$ (calculated value) (KOWWIN v.1.68 estimate) [1)]		4.14	6.50	6.99	7.48	8.47
...						
Kinetics**						
Absorption		N/A	N/A	N/A	N/A	N/A
Distribution		N/A	N/A	N/A	N/A	N/A
Metabolism		N/A	N/A	N/A	N/A	N/A
Excretion		N/A	N/A	N/A	N/A	N/A

Supporting data related to the target endpoint(s)						
In vivo	Toxicogenomics	N/A	N/A	N/A	N/A	N/A
	…	N/A	N/A	N/A	N/A	N/A
In vitro	Alternative method A	N/A	N/A	N/A	N/A	N/A
In silico	Protein binding potency [2)]	Not found	Not found	Not found	Not found	Not found
	Protein binding potency GSH [2)]	Not possible to classify according to these rules (GSH)	Not possible to classify according to these rules (GSH)	Not possible to classify according to these rules (GSH)	Not possible to classify according to these rules (GSH)	Not possible to classify according to these rules (GSH)
	GSH reactivity [2)]	Not calculated	Not calculated	Not calculated	Not calculated	Not calculated
	Hepatotoxicity in mammal [3)]	Plausible	Plausible	Plausible	Plausible	Plausible
	Mitochondrial dysfunction in mammal [3)]	Not detected	Equivocal	Equivocal	Equivocal	Equivocal
	LIVER_BDUCT (Human bile duct disorders) [4)]	Negative	Negative	Negative	Negative	Negative
	LIVER_CHOLEST (Human holestasis) [4)]	Inconclusive	Inconclusive	Inconclusive	Inconclusive	Inconclusive
	LIVER_DAMAGE (Human acute liver damage) [4)]	Negative	Negative	Negative	Negative	Negative
	LIVER_FTEST (Human liver function test (blood test on liver enzymes release)) [4)]	Positive	Positive	Positive	Positive	Positive
	LIVER_GALL (Human gall bladder disorders) [4)]	Inconclusive	Negative	Negative	Negative	Negative

Other data	Battery approach	N/A	N/A	N/A	N/A	N/A
	Defined approach of IATA	N/A	N/A	N/A	N/A	N/A
	Formation of quinone methide (QM)	Yes	Yes	Yes	Yes	Yes

* More relevant metabolite such as toxicant
**General outline of relative comparative kinetics
reference
1) EPI SuiteTM v.4.11
2) OECD QSAR Toolbox v.4.3
3) Derek Nexus v.6.0.1
4) CASE Ultra v.1.7.0.5
5) HESS v.3.8

Chemical ID						
Number of category member		**Member 11**	**Member 12**	**Member 13**	**Member 14**	**Member 15**
CAS		5427-30-2	17540-75-9	816462-78-9	51067-63-1	732-26-3
Name		2,6-Di-tert-butyl-4-isopropylphenol	2,6-Di-tert-butyl-4-sec-butylphenol	2,6-Di-tert-butyl-4-(2-ethylhexyl)phenol	4-(Butan-2-yl)-2-tert-butyl-6-methylphenol	2,4,6-Tri-tert-butylphenol
SMILES		CC(C)C1CC(C(O)C(C1)C(C)(C)C)C(C)(C)C	CCC(C)C1=CC(=C(C(=C1)C(C)(C)C)O)C(C)(C)C	CCCCC(CC)CC1=CC(=C(C(=C1)C(C)(C)C)O)C(C)(C)C	CCC(C)C1=CC(=C(C(=C1)C)O)C(C)(C)C	CC(C)(C)C1=CC(=C(C(=C1)C(C)(C)C)O)C(C)(C)C
Structure						
Summary of data gap filling						
Sub-chronic repeated-dose toxicity (oral route, rat)	Experimental result	N/A	**Sprague Dawley rats, oral (gavage), 28-days, 0, 15, 60, 250 mg/kg/day [GLP data]** **Result** General signs: Loose stools in both sex (250 mg/kg/day). No deaths occurred during the study period. Haematology: Increase of APTT and PT in males (60 mg/kg/day) and females (250 mg/kg/day); decrease of lymphocyte and eosinophil in males (60 mg/kg/day) and in females (15 mg/kg/day) and WBC in females	N/A	N/A	**Sprague Dawley rats (male), oral (gavage), 28-days, 0, 3, 10, 30 mg/kg/day [GLP data, OECD TG 422; combined repeated-dose toxicity study with the reproduction / developmental toxicity screening test]** **Result** General signs: No clinical signs related to the treatments were noted. No deaths occurred during the study period. Haematology: Decrease of neutrophil counts, and increase of lymphocyte counts in females

			(60 mg/kg/day) Clinical biochemistry: Increase of total cholesterol in males (60 and 250 mg/kg/day) and in females (15, 60 and 250 mg/kg/day); increase of AST, ALT, ALP and decrease of total bilirubin in both sex (250 mg/kg/day); decrease of potassium and IP in males (250 mg/kg/day); increase of ureain females (250 mg/kg/day) Liver weight: Absolute (250 mg/kg/day) and relative weight increase in females (15, 60 and 250 mg/kg/day) Histopathological examination: Hypertrophy of centrilobular hepatocytes, small granuloma and fatty degeneration in females (250 mg/kg/day) Thyroid: Hypertrophy of follicular cells in males (60 mg/kg/day) and in both sex (250 mg/kg/day) Spleen: Absolute weight increase in females (250 mg/kg/day) Uterus: Relative weight increase in females (250 mg/kg/day) Kidney: Increase of UN in males (250 mg/kg/day); decrease of pH in females (250 mg/kg/day) **Data source: Safety Research**			(30 mg/kg/day); increase of APTT and PT in males (60 mg/kg/day) and females (250 mg/kg/day); decrease of WBC in females (60 mg/kg/day) Clinical biochemistry: Increase of total protein, albumin, calcium, cholesterol, glucose, and decrease of urea in females (30 mg/kg/day); increase of Potassium and decrease of total bilirubin in both sex (30 mg/kg/day); increase of total cholesterol Liver weight: Relative weight increase in males (30 mg/kg/day) and females (10 and 30 mg/kg/day) Histopathological examination: Hypertrophy of hepatocytes in both sex (10 and 30 mg/kg/day), necrosis of hepatocytes in single male and female (30 mg/kg/day) **Data source: ECHA registration dossier (ECHA, 2019a). US EPA OPPTS 870.3650**

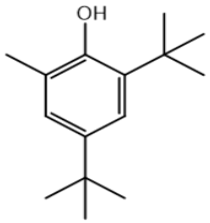
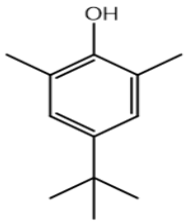
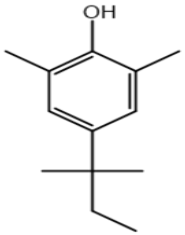
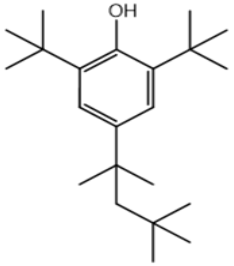
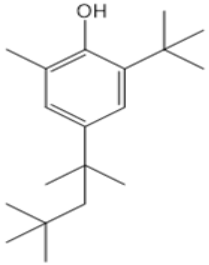
			Institute for Chemical Compounds Co., Ltd.			
	Integrated conclusion	derived result	NOAEL = 15 mg/kg/day (male), < 15 mg/kg/day (female)	derived result	derived result	NOAEL = 3 mg/kg/day (based on higher liver weights at 10 and 30 mg/kg/day, with hepatocellular hypertrophy and necrosis at 30 mg/kg/day)
Molecular profiling related to the category hypothesis						
Metabolite	Observed Rat *In vivo* metabolism [2]	Not tested	Not tested	Not tested	Not tested	3 metabolites
	Observed Rat Liver S9 metabolism [2]	Not tested	Not tested	Not tested	Not tested	Not tested
	Observed rat liver metabolism with quantitative data [2]	Not tested	Not tested	Not tested	Not tested	Not tested
	in vivo Rat metabolism simulator [2]	5 metabolites	20 metabolites	16 metabolites	39 metabolites	2 metabolites
	Rat liver S9 metabolism simulator [2]	4 metabolites	8 metabolites	10 metabolites	13 metabolites	0 metabolites
	Observed rat Liver metabolism [5]	2 metabolites	N/A	N/A	N/A	3 metabolites
	Liver Metabolism Simulator [5]	4 metabolites	6 metabolites	7 metabolites	8 metabolites	3 metabolites
Physical-chemical data						
Molecular weight (EPI Suite™ v.4.11) [1]		254.46	262.44	318.55	220.36	262.44
Boiling point (deg C) (MPBPVP estimate) [1]		298.58	329.61	376.80	302.33	324.50
Melting point (deg C) (MPBPVP estimate) [1]		57.09	102.22	134.15	87.33	104.33

Vapour pressure (mm Hg 25 deg C) (MPBPVP estimate) [1)]		7.45E-05	2.07E-05	5.7E-07	0.000144	0.0002
Water solubility (mg/L) (WSKOW v.1.41) [1)]		0.1942	0.2479	0.002468	1.154	0.512
logP_{ow} (measured value) (TSCATS experimental database match) [1)]		N/A	N/A	N/A	N/A	6.06
logP_{ow} (calculated value) (KOWWIN v.1.68 estimate) [1)]		6.52	6.43	8.39	5.92	6.39
…						
Kinetics						
Absorption		N/A	N/A	N/A	N/A	Rapidly absorbed through the gastrointestinal tract (Takahashi and Hiraga, 1983)
Distribution		N/A	N/A	N/A	N/A	C_{max} = 100 µg/g in blood, 10 µg/g in liver (2–3 h), 5 µg/g in spleen (1.5–2.5 h), 200 µg/g in fat (over 24 h) (ECHA, 2019a)
Metabolism		N/A	N/A	N/A	N/A	Not metabolised to less lipophilic compounds such as BHT; oxidised to the 2,4,6-tri-tert-butylphenoxy radical (ECHA registration dossier); $t_{1/2}$ = 18.2 min for the α phase and 11.8 h for the slower β phase (ECHA, 2019a)
Excretion		N/A	N/A	N/A	N/A	Metabolites but not a parent are detected in faeces and bile (ECHA, 2019a); 2,4,6-tri-tert-butylphenoxy radical in faeces via bile
Supporting data related to the target endpoint(s)						
In vivo	Toxicogenomics	N/A	N/A	N/A	N/A	N/A
	…	N/A	N/A	N/A	N/A	N/A

In vitro	Alternative method A	N/A	N/A	N/A	N/A	N/A
In silico	Protein binding potency [2)]	Not found	Not found	Not found	Not found	Not found
	Protein binding potency GSH [2)]	Not possible to classify according to these rules (GSH)	Not possible to classify according to these rules (GSH)	Not possible to classify according to these rules (GSH)	Not possible to classify according to these rules (GSH)	Not possible to classify according to these rules (GSH)
	GSH reactivity [2)]	Not calculated	Not calculated	Not calculated	Not calculated	Not calculated
	Hepatotoxicity in mammal [3)]	Not detected	Not detected	Plausible	Not detected	Not detected
	Mitochondrial dysfunction in mammal [3)]	Not detected	Equivocal	Equivocal	Equivocal	Equivocal
	LIVER_BDUCT (Human bile duct disorders) [4)]	Negative	Negative	Negative	Negative	Negative
	LIVER_CHOLEST (Human holestasis) [4)]	Negative	Inconclusive	Inconclusive	Inconclusive	Inconclusive
	LIVER_DAMAGE (Human acute liver damage) [4)]	Negative	Negative	Negative	Negative	Negative
	LIVER_FTEST (Human liver function test (blood test on liver enzymes release)) [4)]	Negative	Positive	Positive	Positive	Positive
	LIVER_GALL (Human gall bladder disorders) [4)]	Negative	Negative	Negative	Inconclusive	Negative
Other data	Battery approach	N/A	N/A	N/A	N/A	N/A
	Defined approach of IATA	N/A	N/A	N/A	N/A	N/A

	Formation of quinone methide (QM)	Yes	Yes	Yes	Yes	Not detected

* More relevant metabolite such as toxicant
**General outline of relative comparative kinetics
reference
1) EPI SuiteTM v.4.11
2) OECD QSAR Toolbox v.4.3
3) Derek Nexus v.6.0.1
4) CASE Ultra v.1.7.0.5
5) HESS v.3.8

Chemical ID						
Number of category member		**Member 16**	**Member 17**	**Member 18**	**Member 19**	**Member 20**
Name		616-55-7	879-97-0	91798-63-9	65796-87-4	104066-40-2
CAS		2,4-Di-tert-butyl-6-methylphenol	2,6-Dimethyl-4-tert-butylphenol	2,6-Dimethyl-4-(2-methylbutan-2-yl)phenol	2,6-Di-tert-butyl-4-(1,1,3,3-tetramethylbutyl)phenol	4-(1,1,3,3-tetramethylbutyl)-2-(1,1-dimethylethyl)-6-methylphenol
SMILES		CC1=CC(=CC(=C1O)C(C)(C)C)C(C)(C)C	CC1=CC(=CC(=C1O)C)C(C)(C)C	CCC(C)(C)C1=CC(=C(C(=C1)C)O)C	CC(C)(C)CC(C)(C)C1=CC(=C(C(=C1)C(C)(C)C)O)C(C)(C)C	CC1=CC(=CC(=C1O)C(C)(C)C)C(C)(C)CC(C)(C)C
Structure						
Summary of data gap filling						
Sub-chronic repeated-dose toxicity (oral route, rat)	Experimental result	N/A	N/A	N/A	N/A	N/A
	Integrated conclusion	derived result	derived result	derived result	derived result	derived result
Molecular profiling related to the category hypothesis						
Metabolite	Observed Rat *In vivo* metabolism [2)]	Not tested	Not tested	Not tested	Not tested	Not tested
	Observed Rat Liver S9 metabolism [2)]	Not tested	Not tested	Not tested	Not tested	Not tested
	Observed rat liver metabolism with	Not tested	Not tested	Not tested	Not tested	Not tested

	quantitative data [2)]					
	in vivo Rat metabolism simulator [2)]	8 metabolites	9 metabolites	22 metabolites	10 metabolites	16 metabolites
	Rat liver S9 metabolism simulator [2)]	3 metabolites	3 metabolites	8 metabolites	3 metabolites	6 metabolites
	Observed rat Liver metabolism [5)]	N/A	N/A	N/A	N/A	N/A
	Liver Metabolism Simulator [5)]	5 metabolites	3 metabolites	5 metabolites	4 metabolites	6 metabolites
Physical-chemical data						
Molecular weight (EPI Suite™ v.4.11) [1)]		220.36	178.28	192.30	318.55	276.47
Boiling point (deg C) (MPBPVP estimate) [1)]		296.49	264.87	281.13	360.61	337.13
Melting point (deg C) (MPBPVP estimate) [1)]		83.01	66.48	76.56	143.02	112.69
Vapour pressure (mm Hg 25 deg C) (MPBPVP estimate) [1)]		0.00221	0.00352	0.000625	1.58E-06	1.03E-05
Water solubility (mg/L) (WSKOW v.1.41) [1)]		1.242	29.64	9.62	0.003306	0.00159
$logP_{ow}$ (measured value) (TSCATS experimental database match) [1)]		N/A	N/A	N/A	N/A	N/A
$logP_{ow}$ (calculated value) (KOWWIN v.1.68 estimate) [1)]		5.88	4.52	5.01	8.24	7.73
…						
Kinetics						
Absorption		N/A	N/A	N/A	N/A	N/A
Distribution		N/A	N/A	N/A	N/A	N/A
Metabolism		N/A	N/A	N/A	N/A	N/A
Excretion		N/A	N/A	N/A	N/A	N/A
Supporting data related to the target endpoint(s)						

In vivo	Toxicogenomics	N/A	N/A	N/A	N/A	N/A
	…	N/A	N/A	N/A	N/A	N/A
In vitro	Alternative method A	N/A	N/A	N/A	N/A	N/A
In silico	Protein binding potency [2)]	Not found	Not found	Not found	Not found	Not found
	Protein binding potency GSH [2)]	Not possible to classify according to these rules (GSH)	Not possible to classify according to these rules (GSH)	Not possible to classify according to these rules (GSH)	Not possible to classify according to these rules (GSH)	Not possible to classify according to these rules (GSH)
	GSH reactivity [2)]	Not calculated	Not calculated	Not calculated	Not calculated	Not calculated
	Hepatotoxicity in mammal [3)]	Not detected	Not detected	Not detected	Plausible	Not detected
	Mitochondrial dysfunction in mammal [3)]	Equivocal	Not detected	Not detected	Equivocal	Equivocal
	LIVER_BDUCT (Human bile duct disorders) [4)]	Negative	Negative	Negative	Negative	Negative
	LIVER_CHOLEST (Human holestasis) [4)]	Inconclusive	Inconclusive	Inconclusive	Inconclusive	Inconclusive
	LIVER_DAMAGE (Human acute liver damage) [4)]	Negative	Negative	Negative	Negative	Negative
	LIVER_FTEST (Human liver function test (blood test on liver enzymes release)) [4)]	Positive	Positive	Positive	Positive	Positive
	LIVER_GALL (Human gall bladder disorders) [4)]	Inconclusive	Inconclusive	Positive	Inconclusive	Positive
Other data	Battery approach	N/A	N/A	N/A	N/A	N/A

	Defined approach of IATA	N/A	N/A	N/A	N/A	N/A
	Formation of quinone methide (QM)	Not detected	Not detected	Not detected	Not detected	Not detected

* More relevant metabolite such as toxicant
**General outline of relative comparative kinetics reference
1) EPI SuiteTM v.4.11
2) OECD QSAR Toolbox v.4.3
3) Derek Nexus v.6.0.1
4) CASE Ultra v.1.7.0.5
5) HESS v.3.8