Agency technical report on the classification and labelling of:

2-(4-*tert*-butylbenzyl) propionaldehyde and 4-*tert*-butylbenzoic acid and 3-(4-*tert*-butylphenyl)propionaldehyde [1] 4-*tert*-butyltoluene [2] 4-*tert*-butylbenzaldehyde [3] methyl 4-*tert*-butylbenzoate [4]

EC Number: 201-289-8 and 202-696-3 and 242-016-2 [1]; 202-675-9 [2]; 213-367-9 [3]; 247-768-5 [4]
CAS Number: 80-54-6 and 98-73-7 and 18127-01-0 [1]; 98-51-1 [2]; 939-97-9 [3]; 26537-19-9 [4]

March 2026

Contents

Brief summary

The conclusion of the Agency technical report is that 2-(4-*tert*-butylbenzyl) propionaldehyde, 4-*tert*-butylbenzoic acid, 3-(4-*tert*-butylphenyl)propionaldehyde [1], 4-*tert*-butyltoluene [2], 4-*tert*-butylbenzaldehyde [3] and methyl 4-*tert*-butylbenzoate [4] meet the classification criteria for:

Repr. 1B; H360Fd (May damage fertility. Suspected of damaging the unborn child)

Is this in agreement with the RAC opinion? YES

RAC proposed the inclusion of a new note to accompany these entries in Annex VI of EU CLP:

“The classification of mixtures as reproductive toxicant is necessary if the sum of the concentrations of individual substances covered by this entry, forming the same metabolite, in the mixture as placed on the market is equal to, or above, the applicable generic concentration limit for the assigned category, or a specific concentration limit given in this entry.”

The Agency appreciates the intention of the note and can support it in principle, however the wording of the note will be given further consideration in the Agency Opinion.

At the time of publication, this mandatory classification and labelling (MCL) has not been agreed and/or adopted in Great Britain.

This is a targeted technical report which only considers Reproductive Toxicity. This was the only hazard class considered in the EU Committee for Risk Assessment (RAC) Opinion.

4-*tert*-butylbenzoic acid has an existing MCL which includes Acute Tox. 4 (H302) and STOT RE 1 (H372). Acute toxicity and STOT RE are not assessed in this technical report, therefore Acute Tox. 4 (H302) and STOT RE 1 (H372) should be retained in the GB MCL of this substance.

Introduction

Under Article 37 of the GB CLP Regulation[1], the Agency[2] is required to produce a technical report for each substance on which the Committee for Risk Assessment (RAC) of the European Chemicals Agency produces an opinion[3].

This technical report documents an independent scientific assessment, conducted by HSE technical specialists, of the classification and labelling of 2-(4-*tert*-butylbenzyl) propionaldehyde and 4-*tert*-butylbenzoic acid and 3-(4-*tert*-butylphenyl)propionaldehyde [1], 4-*tert*-butyltoluene [2], 4-*tert*-butylbenzaldehyde [3], methyl 4-*tert*-butylbenzoate [4].

Table 1. Information considered in the scientific assessment

Document	**Included in assessment**
EU CLH report	Yes
Annexes to the EU CLH report	Yes
RAC opinion	Yes
Background document	Yes
Information submitted during the EU public consultation process (RCOM table, including attachments)	Yes
RAC minority opinion(s)	Not applicable
Other information:	Not applicable

This information has been evaluated against the classification and labelling criteria set out in the GB CLP Regulation.

[1]The retained CLP Regulation (EU) No. 1272/2008 as amended for Great Britain

[2] HSE acting in its capacity as the GB CLP Agency

[3] Under Article 37(4) of Regulation (EU) No 1272/2008 on classification, labelling and packaging of substances and mixtures

Overview of current and proposed classification and labelling

Table 2a. Current and proposed classification and labelling of 2-(4-*tert*-butylbenzyl) propionaldehyde

	Index No.	International Chemical Identification	EC No.	CAS No.	Classification		Labelling			Specific Concentration Limits, M-factors	Notes
					Hazard Class and Category Code(s)	Hazard Statement Code(s)	Pictogram, Signal Word Code(s)	Hazard Statement Code(s)	Suppl. Hazard Statement Code(s)		
GB MCL List entry	605-041-00-3	2-(4-*tert*-butylbenzyl) propionaldehyde	201-289-8	80-54-6	Repr. 1B	H360Fd	GHS08 Dgr	H360Fd			
EU dossier submitter's proposal	605-041-00-3	2-(4-*tert*-butylbenzyl) propionaldehyde	201-289-8	80-54-6	**Retain** Repr. 1B	**Retain** H360Fd	**Retain** GHS08 Dgr	**Retain** H360Fd			**Add** Note *
EU RAC opinion	605-041-00-3	2-(4-*tert*-butylbenzyl) propionaldehyde	201-289-8	80-54-6	**Retain** Repr. 1B	**Retain** H360Fd	**Retain** GHS08 Dgr	**Retain** H360Fd			**Add** Note *
Agency technical report conclusion	605-041-00-3	2-(4-*tert*-butylbenzyl) propionaldehyde	201-289-8	80-54-6	**Retain** Repr. 1B	**Retain** H360Fd	**Retain** GHS08 Dgr	**Retain** H360Fd			**Add** Note *
Resulting MCL entry on GB MCL list	605-041-00-3	2-(4-*tert*-butylbenzyl) propionaldehyde	201-289-8	80-54-6	Repr. 1B	H360Fd	GHS08 Dgr	H360Fd			Note *

* New note: The classification of mixtures as reproductive toxicant is necessary if the sum of the concentrations of individual substances covered by this entry, forming the same metabolite, in the mixture as placed on the market is equal to, or above, the applicable generic concentration limit for the assigned category, or a specific concentration limit given in this entry.

Table 2b. Current and proposed classification and labelling of 4-*tert*-butylbenzoic acid

	Index No.	International Chemical Identification	EC No.	CAS No.	Classification		Labelling			Specific Concentration Limits, M-factors	Notes
					Hazard Class and Category Code(s)	Hazard Statement Code(s)	Pictogram, Signal Word Code(s)	Hazard Statement Code(s)	Suppl. Hazard Statement Code(s)		
GB MCL List entry	607-698-00-1	4-*tert*-butylbenzoic acid	202-696-3	98-73-7	Repr. 1B STOT RE 1 Acute Tox. 4	H360F H372 H302	GHS07 GHS08 Dgr	H360F H372 H302			
EU dossier submitter's proposal	607-698-00-1	4-*tert*-butylbenzoic acid	202-696-3	98-73-7	**Retain** STOT RE 1 Acute Tox. 4 **Modify** Repr. 1B	**Retain** H372 H302 **Modify** H360Fd	**Retain** GHS07 GHS08 Dgr	**Retain** H372 H302 **Modify** H360Fd			**Add** Note *
EU RAC opinion	607-698-00-1	4-*tert*-butylbenzoic acid	202-696-3	98-73-7	**Retain** STOT RE 1 Acute Tox. 4 **Modify** Repr. 1B	**Retain** H372 H302 **Modify** H360Fd	**Retain** GHS07 GHS08 Dgr	**Retain** H372 H302 **Modify** H360Fd			**Add** Note *
Agency technical report conclusion	607-698-00-1	4-*tert*-butylbenzoic acid	202-696-3	98-73-7	**Retain** STOT RE 1 Acute Tox. 4 **Modify** Repr. 1B	**Retain** H372 H302 **Modify** H360Fd	**Retain** GHS07 GHS08 Dgr	**Retain** H372 H302 **Modify** H360Fd			**Add** Note *
Resulting MCL entry on GB MCL list	607-698-00-1	4-*tert*-butylbenzoic acid	202-696-3	98-73-7	Repr. 1B STOT RE 1 Acute Tox. 4	H360Fd H372 H302	GHS07 GHS08 Dgr	H360Fd H372 H302			Note *

* New note: The classification of mixtures as reproductive toxicant is necessary if the sum of the concentrations of individual substances covered by this entry, forming the same metabolite, in the mixture as placed on the market is equal to, or above, the applicable generic concentration limit for the assigned category, or a specific concentration limit given in this entry.

Table 2c. Current and proposed classification and labelling of 3-(4-*tert*-butylphenyl)propionaldehyde [1], 4-*tert*-butyltoluene [2], 4-*tert*-butylbenzaldehyde [3], methyl 4-*tert*-butylbenzoate [4]

	Index No.	International Chemical Identification	EC No.	CAS No.	Classification		Labelling			Specific Concentration Limits, M-factors	Notes
					Hazard Class and Category Code(s)	Hazard Statement Code(s)	Pictogram, Signal Word Code(s)	Hazard Statement Code(s)	Suppl. Hazard Statement Code(s)		
GB MCL List entry	No existing entry										
EU dossier submitter's proposal	TBD	3-(4-*tert*-butylphenyl)propionaldehyde [1] 4-*tert*-butyltoluene [2] 4-*tert*-butylbenzaldehyde [3] methyl 4-*tert*-butylbenzoate [4]	242-016-2 [1] 202-675-9 [2] 213-367-9 [3] 247-768-5 [4]	18127-01-0 [1] 98-51-1 [2] 939-97-9 [3] 26537-19-9 [4]	Repr. 1B	H360Fd	GHS08 Dgr	H360Fd			Note *

	Index No.	International Chemical Identification	EC No.	CAS No.	Classification		Labelling			Specific Concentration Limits, M-factors	Notes
					Hazard Class and Category Code(s)	Hazard Statement Code(s)	Pictogram, Signal Word Code(s)	Hazard Statement Code(s)	Suppl. Hazard Statement Code(s)		
EU RAC opinion	TBD	3-(4-*tert*-butylphenyl)propionaldehyde [1] 4-*tert*-butyltoluene [2] 4-*tert*-butylbenzaldehyde [3] methyl 4-*tert*-butylbenzoate [4]	242-016-2 [1] 202-675-9 [2] 213-367-9 [3] 247- 768-5 [4]	18127-01- 0 [1] 98-51-1 [2] 939-97-9 [3] 26537-19-9 [4]	Repr. 1B	H360Fd	GHS08 Dgr	H360Fd			Note *
Agency technical report conclusion	TBD	3-(4-*tert*-butylphenyl)propionaldehyde [1] 4-*tert*-butyltoluene [2] 4-*tert*-butylbenzaldehyde [3] methyl 4-*tert*-butylbenzoate [4]	242-016-2 [1] 202-675-9 [2] 213-367-9 [3] 247- 768-5 [4]	18127-01- 0 [1] 98-51-1 [2] 939-97-9 [3] 26537-19-9 [4]	Repr. 1B	H360Fd	GHS08 Dgr	H360Fd			Note *

	Index No.	International Chemical Identification	EC No.	CAS No.	Classification		Labelling			Specific Concentration Limits, M-factors	Notes
					Hazard Class and Category Code(s)	Hazard Statement Code(s)	Pictogram, Signal Word Code(s)	Hazard Statement Code(s)	Suppl. Hazard Statement Code(s)		
Resulting MCL entry on GB MCL list	TBD	3-(4-*tert*-butylphenyl)propionaldehyde [1] 4-*tert*-butyltoluene [2] 4-*tert*-butylbenzaldehyde [3] methyl 4-*tert*-butylbenzoate [4]	242-016-2 [1] 202-675-9 [2] 213-367-9 [3] 247- 768-5 [4]	18127-01- 0 [1] 98-51-1 [2] 939-97-9 [3] 26537-19-9 [4]	Repr. 1B	H360Fd	GHS08 Dgr	H360Fd			Note *

TBD: To be determined

* New note: The classification of mixtures as reproductive toxicant is necessary if the sum of the concentrations of individual substances covered by this entry, forming the same metabolite, in the mixture as placed on the market is equal to, or above, the applicable generic concentration limit for the assigned category, or a specific concentration limit given in this entry

Background

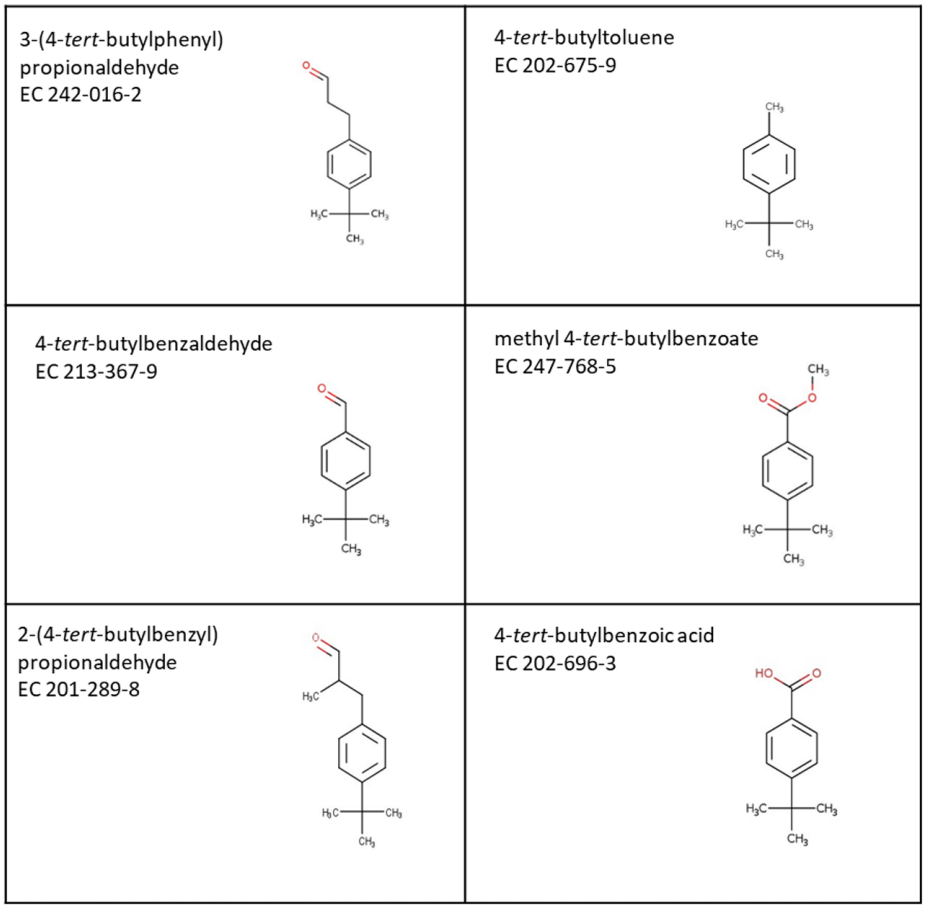
Active substance in Plant Protection Products: ☐

Active substance in Biocidal Products: ☐

Chemical registered under REACH: ☒

Six substances, referred to as the bourgeonal group, were assessed in the relevant 2025 RAC Opinion (ECHA, 2025). The substances included 2-(4-*tert*-butylbenzyl) propionaldehyde (bourgeonal), 4-*tert*-butylbenzoic acid (TBBA), 3-(4-*tert* butylphenyl)propionaldehyde (lysmeral), 4-*tert*-butyltoluene, 4-*tert*-butylbenzaldehyde and methyl 4-*tert*-butylbenzoate (see Figure 1).

Figure 1. The bourgeonal group members and their chemical structures (taken from p. 20 of the CLH report; CLH, 2023)



According to the CLH report (2023), these substances are used as fragrances/perfumes and/or for masking in products such as cosmetics, cleaning and washing agents, polishes and wax blends, biocides, air care products etc. Some of these substances are also used as intermediates, while TBBA is also used as a binding agent in paints and coatings. According to the Swedish Products Register, the concentration of some of these fragrances in products ranges between 0.0001–1% with a median range of 0.1–0.2%.

These substances were grouped in a category by the DS, Sweden, according to Article 13 of REACH, which complies with the OECD principles for the validation of chemical grouping, for read-across purposes. The category included TBBA and substances which are predicted/proven to metabolise into TBBA. The latter substances contain a benzene ring with a substituent that can degrade to a carboxylic acid group and a *tert*-butyl group in para position. Experiments with bourgeonal, lysmeral, 4-*tert*-butyltoluene and 4-*tert*-butylbenzaldehyde have demonstrated formation of TBBA in vivo, while methyl 4-*tert*-butylbenzoate has been predicted to metabolise into TBBA based on its chemical structure (a methylester that can be hydrolysed to a carboxylic acid) and a profiling scheme built in the OECD QSARs Toolbox for the identification of TBBA precursors. The described structural similarity and evidence that these substances metabolise into TBBA formed the basis for grouping TBBA and precursor substances into the bourgeonal group. Except for the structural similarity and the predicted/proven formation of common metabolite TBBA, the proposed read-across of these substances was also supported by similar physicochemical properties, toxicokinetics (see ADME section of this report) and toxicological datasets (see Reproductive toxicity section of this report). While for some members of the bourgeonal group, such as bourgeonal and 4-*tert*-butyltoluene, available studies were deemed of limited value for classification purposes, the observed effects were consistent with other members of the bourgeonal group, such as lysmeral and TBBA, and were considered supportive of the proposed grouping and read-across approach.

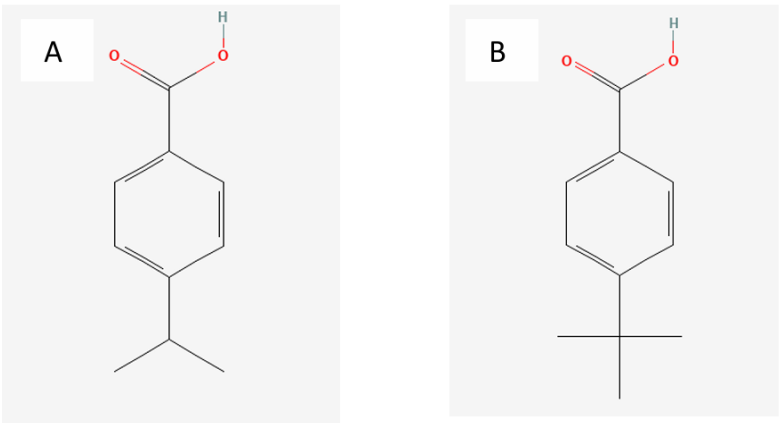
The only hazard class assessed in this report and the relevant RAC Opinion is reproductive toxicity. TBBA has a harmonised and mandatory classification in the EU and GB, respectively, as Repr. 1B (H360F). Adverse effects were described on the male reproductive system in the relevant RAC Opinion (ECHA, 2011), which were characterised by testicular lesions, spermatotoxic effects and infertility. There were no data on the developmental toxicity potential of TBBA (ECHA, 2011). Lysmeral also has a harmonised and mandatory classification in the EU and GB, respectively, as Repr. 1B (H360Fd). Regarding its classification for adverse effects on sexual function and fertility, reported findings were of similar nature to TBBA. Data on TBBA were considered as supportive evidence in the relevant RAC Opinion on lysmeral, as the metabolite was considered responsible for the effects of lysmeral on the male reproductive system and fertility (ECHA, 2019).

In terms of the scope of the current CLH proposal on the bourgeonal group, the DS did not aim to revise the classifications of TBBA for effects on sexual function and fertility or the overall classification of lysmeral for reproductive toxicity. New studies have become

available for both substance since the publication of the relevant RAC opinions (ECHA, 2011; ECHA, 2019); however, these included in vitro and ex vivo studies, which the DS considered would not have an effect on the conclusions already drawn by RAC. Instead, both substances were considered reference substances for the proposed read-across, and the DS proposed that a note is added to their harmonised classifications to account for additive reproductive effects in mixtures resulting from the common metabolite TBBA. Additionally, a Repr. 2 (H361d) classification was proposed to be added to the harmonised classification of TBBA based on the proposed read-across approach. Data were available for all of the other substances in the bourgeonal group, except for methyl 4-*tert*-butylbenzonate. The available data on bourgeonal and 4-*tert*-butylbenzaldehyde were considered supportive of the read-across in terms of effects on the male reproductive system; however, the studies were considered of limited value for classification as stand-alone.

The bourgeonal group bares structural similarity to another group of substances, known as the cyclamal group. The cyclamal group includes p-cymene, 3-p-cumenyl-2-methylpropion aldehyde, 3-(p-cumenyl) propion aldehyde, 4-isopropyl benzaldehyde and their common metabolite, 4-isopropylbenzoic acid (4-iPBA). 4-iPBA is structurally similar to TBBA, differing only by a methyl group at the benzylic carbon (see Figure 2). Both groups present with similar reproductive toxicity properties; however, the DS prepared a separate CLH proposal for the cyclamal group due to the different common main metabolite between the cyclamal and bourgeonal group and due to a difference in the strength of data for developmental toxicity. Additionally, the systemic toxicity profile of the bourgeonal group appeared to be slightly more marked. RAC agreed with the DS's approach, but noted that evidence from both groups was considered relevant in the assessment of the other in a weight of evidence of approach.

Figure 2. Structures of the metabolites 4-iPBA (A) and TBBA (B) (taken from the CLH report; CL, 2023)



Scientific assessment of the physical, human health and environmental hazard classes

Physical Hazards

Not assessed in the CLH report or RAC opinion.

Health Hazards

ADME

3-(4-tert-butylphenyl)propionaldehyde (bourgeonal)

Two *in vivo* studies were available with bourgeonal.

The first one was a 14-day dose-range finding (DRF) study (Study report, 2019) for a combined repeated-dose toxicity (RDT) study with reproduction/developmental toxicity screening test, which investigated TBBA levels in the plasma. The study was not conducted according to a test guideline (TG) or GLP. Five Crl:CD Sprague Dawley (SD) rats/sex/dose were exposed to 0, 5, 25 and 50 mg/kg bw/d bourgeonal (purity: unknown) in corn oil via oral gavage 3 times per day for 14 consecutive days.

Bourgeonal and TBBA concentrations were below the detection limit in all animals before the first dose. TBBA was detected in plasma samples from 25 mg/kg bw/d on study day 1. On day 14, TBBA plasma concentrations quickly increased and maintained steady state from 0.5 to 24 hr in both sexes and at all dose levels. In females at ≥ 25 mg/kg bw/d, TBBA levels were almost twice as high and increased in a nearly dose-proportional manner. The parent substance was either detected below the concentration limit or at much lower levels than TBBA, while concentrations of day 14 were many times lower than on day 1.

In the second study (Givaudan, 2009), sexually mature male CD rats were administered 25, 100 and 250 mg/kg bw/d bourgeonal for 5 days. After the 5th dose, urine samples were analysed for the presence of TBBA, 4-iso-butylbenzoic acid and 4-iPBA. All animals at the top dose were killed in extremis by day 2 of the study. One mortality was also noted at 100 mg/kg bw/d. In the remaining animals, unconjugated TBBA was detected in urine in a dose-dependent manner (mean levels: 35.75 µg/mL and 274.8 µg/mL at 25 and 100 mg/kg bw/d, respectively).

Overall, oral administration of bourgeonal in rats resulted into increases in plasma TBBA levels, which were more pronounced and with dose-dependency in females. The parent substance was either present at very low concentrations in plasma or below the detection limit (Study report, 2019). Oral administration of bourgeonal also lead to urinary excretion of TBBA in rats with dose-dependency (Givaudan, 2009).

There was also an *in vitro* metabolism study with bourgeonal (Laue *et al.,* 2017). See 4-*tert*-butylbenzoic acid (TBBA) under the ADME section of this report.

4-tert-butyltoluene

Five *in vivo* studies were available with 4-*tert*-butyltoluene.

The first study (Ingebrigtsen and Walde, 1982; Wald and Scheline, 1983; GLP status: unspecified) examined the distribution of p-*tert*-butyltoluene in the rat and guinea pig. The test substance was radiolabelled with ^{14}C and administered to male Wistar rats and male Dunkin Hartley guinea pigs via oral gavage and inhalation at doses of 100 mg/kg bw.

The test substance was well-absorbed via both routes. It distributed quickly and was eliminated after a few days. In the rat, the oral dose was mainly eliminated via the urine, but also through faeces. Elimination was generally biphasic with slower elimination observed from 6 days post-treatment. Following oral administration, 73% of the radioactivity was eliminated in the urine and faeces of the rat by day 3, and 83% by day 10.

The second study (Study report, 1982; GLP status: unspecified) aimed to investigate the metabolism of p-*tert*-butyltoluene following oral administration in the rat. Eight male albino SPF rats/dose were administered graduated doses of the test substance in rape oil for 5 consecutive days via oral gavage. Dose levels were reported as 25 and 100 mg/kg bw/d p-*tert*-butyltoluene (purity: unknown). The study also included a group of 4 control males. After the final dose, urine was collected for 24 hours and samples were analysed for metabolite using gas chromatography-mass spectrometry (GC-MS).

TBBA was detected in the urine of all treated rats in a dose-dependent manner. Analysis indicated additional metabolites were also present, but the secondary metabolite, p-*tert*-butylhippuric acid (TBHA; the glycine conjugate of TBBA), was not detected. The authors considered that TBBA made up a considerable amount of the excreted metabolites. They also hypothesised that TBBA was likely eliminated conjugated to glucuronide, but were unable to confirm this due to the analytical procedure used for the study.

In the third study (Gerarde, 1960; non-GLP), rats were exposed (route: unspecified) to p-*tert*-butyltoluene (purity: unknown). Based on a lack of change in the urinary sulfate ratio (inorganic/total), the author considered that the p-methyl group or one of the methyl groups of the tertiary butyl moiety of the test substance was oxidized in the liver to hydroxy- and

carboxyl derivatives, rather than *in situ* oxidation of the aromatic ring system. The author presumed metabolites were eliminated as glucuronide or glycine conjugates.

In the fourth study (Rasmussen *et al.,* 1980; GLP status: unspecified), male mice were exposed to 1000 ppm p-*tert*-butyltoluene via inhalation for up to 8 hours for up to 5 days. The study investigated the uptake and elimination of the test substance from mesenterial fat and the brain. It was concluded that p-*tert*-butyltoluene does not tend to accumulate in fat or nervous tissue based on complete elimination of the test substance 24 hours after a single 4-hour exposure and barely detectable amounts after 5 days of 4-hour exposures.

The final study (Study report, 1985; GLP status: unspecified) investigated the presence of different metabolites in the urine of mice, guinea pigs and dogs using GC. Six male SPF albino mice and five male Himalayan guinea pigs were administered 100 mg/kg bw/d 4-*tert*-butyltoluene (purity: unknown) in rape oil via oral gavage for 5 days. Two dogs were also treated with 100 mg/kg bw/d test substance (vehicle: unspecified) for the same amount of time via oral capsule. Urine was collected for 24 hours after the final dose. In both mice and guinea pigs, the main metabolite in urine was TBHA (25.2% in mice, 53.9% in guinea pigs), whereas TBBA levels were either below the detection limit or very low. In dogs, TBBA was the main metabolite (3.9%), whereas the concentration of TBHA was < 1%.

Overall, 4-*tert*-butyltoluene was readily absorbed and rapidly distributed in rats and guinea pigs following oral and inhalation exposure. The majority of the test substance was excreted within 3 days of treatment via urine and faeces following oral exposure, and there was no evidence of accumulation (Ingebrigsten & Walde, 1982; Wald & Scheline, 1983). Accumulation was also not evident in the nervous system and fat of mice following repeated inhalation exposure (Rasmussen et al., 1980). TBBA was identified as the main metabolite in rat urine, where levels increased with dose-dependency. The glycine conjugate of TBBA, TBHA, was not detected in rat urine (Study report, 1982). Similar findings were reported in dogs, where TBBA was the major metabolite and TBHA levels were minor. In contrast, TBHA was the major metabolite in the urine of mice and guinea pigs (Study report, 1985). Regarding metabolism in the rat, Gerarde (1960) offered a proposed mechanism described as oxidation of 4-*tert*-butyltoluene in the liver to hydroxyl- and carboxyl derivatives, which were then assumed to be eliminated as glucuronide or glycine conjugates.

There was also an *in vitro* metabolism study with 4-*tert*-butyltoluene (Laue *et al.,* 2017). See 4-*tert*-butylbenzoic acid (TBBA) under the ADME section of this report.

4-tert-butylbenzaldehyde

Two *in vivo* studies were available on 4-*tert*-butylbenzaldehyde.

In the first study (Study report, 1982; GLP status: unspecified), 8 male SPF albino rats/group were treated with 12.5 or 50 mg/kg bw test substance in rape oil via oral gavage

for 5 days. A control group of 4 males was also included. Urine was collected for 24 hours after the final doses and analysed using GC. TBBA was identified as a metabolite, but not TBHA.

The second study (Study report, 1985; GLP status: unspecified) investigated the presence of different metabolites in the urine of mice, guinea pigs and dogs using GC. Six male SPF albino mice and five male Himalayan guinea pigs were administered 100 mg/kg bw/d 4-*tert*-butyltoluene (purity: unknown) in rape oil via oral gavage for 5 days. Two dogs were also treated with 100 mg/kg bw/d test substance (vehicle: unspecified) for the same amount of time via oral capsule. Urine was collected for 24 hours after the final dose. In the mice and guinea pigs, TBHA was identified as the main metabolite, while TBBA was detected in very low concentrations. In dogs, the opposite applied.

Overall, metabolism studies with 4-*tert*-butylbenzaldehyde reported similar findings to studies with 4-*tert*-butyltoluene — TBBA was identified as the main metabolite in the urine of rats and dogs, while TBHA was identified as the main metabolite in the urine of mice and guinea pigs.

2-(4-tert-butylbenzyl)propionaldehyde (lysmeral)

Ten studies were available on lysmeral — six *in vivo* and four *in vitro* studies.

The first *in vivo* study (Study report, 1995) was conducted according to GLP and the protocol used was equivalent to OECD TG 417. Four Wistar-derived RORO (Ibm:RORO (SPF)) albino male rats/group were administered a single dose of 25 or 100 mg/kg bw ^{14}C-lysmeral in rapeseed oil via oral gavage. Blood and plasma were collected at various timepoints post-dosing. The study showed that lysmeral absorbed quickly following oral administration (T_{max} < 4hours). The elimination half-life was between 8–9.8 hours.

The second study (Study report, 2006) examined toxicokinetics in five male Wistar rats/group following single administration of 50 mg/kg bw lysmeral (purity: 99.1%) or lysmerylic acid in olive oil via oral gavage. The study was not GLP, but was conducted in accordance with the OECD principles for GLP. Blood was sampled before and at the various timepoints following treatment. Lysmeral was not detected in any plasma samples in either group, while lysmerylic acid was detected in all plasma samples in both groups. Concentrations were highest directly after and 4 hours after administration for the lysmerylic acid and lysmeral groups, respectively, with higher C_{max} and $AUC_{(0–24h)}$ for the lysmerylic acid group.

A similar study was performed in male mice (Study report, 2006; non-GLP) with similar findings — lysmeral was not detected in any plasma sample in either group, while lysmerylic acid was detected in all plasma samples in both groups. Concentrations were highest directly after administration with both substances, with higher C_{max} and $AUC_{(0–24h)}$ for the lysmerylic acid group.

In the fourth study (Study report, 1982; GLP status: unspecified), 8 male Albino (SPF) rats/group were administered 100 or 400 mg/kg bw/d lysmeral (purity: unspecified) in rapeseed oil via oral gavage for 5 days. A control group of 4 male rats was also included. Urine was collected for 24 hours after the last dose and analysed using GC-MS. TBBA was detected in the urine of treated males in a dose-related manner. The authors hypothesised that TBBA was eliminated as a glucuronide conjugate; however, they were unable to detect such conjugates due to the analysis method used. TBHA was not detected in any sample.

A similar study (Study report, 1985; GLP status: unspecified) was performed in rats, mice, guinea pigs, monkeys and dogs. TBBA was the main metabolite in the urine samples of treated rats (males and females), monkeys (males only) and dogs (males only). In the urine samples of guinea pigs (males only) and mice (males only), TBHA was the main metabolite. The concentration of the minor metabolite in all species was very low.

There was also one human study (Scherer *et al*., 2017; non-GLP), where the metabolism and excretion kinetics of lysmeral were investigated following a single oral administration in human volunteers. The test substance was dissolved in ethanol and applied as a chocolate coated eatable waffle cup containing approximately 20 mL coffee, milk or water. Each volunteer ingested 5.26 mg lysmeral. The highest concentrations of the main metabolites, lysmerol, lysmerylic acid, TBBA and TBHA, were excreted between 3–6 h after ingestion. Lysmerol and lysmerylic acid appeared slightly earlier in the urine in comparison to secondary metabolites, such as hydroxyl-lysmerylic acid and TBBA. Urinary excretion was rapid with more than 90% of all measured metabolites being excreted within 12 hours, and complete elimination within 48 hours. TBBA, lysmerol, hydroxy-lysmerylic acid and lysmerylic acid made up 14.3%, 1.82%, 0.20% and 0.16% of the applied dose, respectively.

The study also included a preliminary test, where a single 65-year-old non-smoking male was dermally exposed to a single application of lysmeral-containing sunscreen which had been fortified to a lysmeral content of 6.5 mg/g. His urine was analysed up to 48 hours after administration. The same main metabolites were present in this urine as in the main oral study. Peak concentration of lysmerol and lysmerylic acid were excreted 3–6 hours after exposure, similarly to the oral study. In contrast, TBBA and TBHA were not detected in the urine until 12 hours after exposure. TBBA was the most abundant metabolite, making up 0.67% of the applied dose, followed by TBHA (0.04%), lysmerol (0.02%), and lysmerylic acid (0.012%).

Overall, the *in vivo* animal studies showed that lysmeral is absorbed quickly following single oral administration in rats (Study report, 1995). The substance metabolises quickly, and lysmerylic acid was detected in blood samples, whereas the parent substance was not detected in blood samples of rats and mice (Study report, 2006). Following repeated oral administrations in rats, dose-dependent increases were noted in TBBA in the urine (Study

report, 1982), which was identified as the main urinary metabolite in this species, as well as in monkeys and dogs. Similarly to the previously described substances of the bourgeonal group, TBHA was the main urinary metabolite in guinea pigs and mice (Study report, 1985). In humans, TBBA was among the most major metabolites in urine following oral and dermal exposure. TBBA appeared slightly later than other major metabolites following oral exposure (up to 6 hours), and much later following dermal exposure (from 12 hours). Excretion of lysmeral was completed within 48 hours after ingestion (Scherer *et al*., 2017).

Four *in vitro* studies with lysmeral were also available.

The first study (Study report, 1982; GLP status: unspecified) investigated the metabolism of lysmeral in rat hepatocytes. Lysmeral was metabolised to TBBA, which made up 50% of the applied radioactivity, and to another unidentified metabolite, which made up 7% of the applied radioactivity.

The second study (Study report, 2010; GLP compliant) was a comparative metabolism assay which investigated qualitative and quantitative differences in lysmeral metabolism in the hepatocytes and microsomes of rats, mice, rabbits, and humans. Hepatocytes and microsomes from male Han-Wistar rats, CD1-mice, New Zealand White rabbits and humans were exposed to various concentrations of ^{14}C-lysmeral (purity: 97.8%) in DMSO. Testosterone was used as the positive control, and behaved as expected in both assays.

In the microsomal assay, metabolism of lysmeral was extensive with up to 9 metabolites detected using Radio-High-Performance Liquid Chromatography. All metabolites were more polar than the parent substance. Lysmerylic acid, lysmerol and hydroxy-lysmerol were detected in samples from all investigated species; however, there were some quantitative differences (higher levels of hydroxy-lysmerol in rats and rabbits than in mice and humans; higher levels of lysmerylic acid in rats and mice than in rabbits and humans; higher levels of lysmerol in humans than in rabbits, mice or rats). In hepatocytes, metabolism was also extensive with up to 8 metabolites detected. Metabolites were more polar than lysmeral. Lysmerylic acid was the main metabolite in all species. Qualitative differences included the detection of TBHA in rodents, but not in rabbits or humans. The most notable quantitative difference was in the detection of TBBA across species. TBBA was detected in higher levels in rat hepatocytes (8.3–29.3% of ROI) and in higher concentrations at lower lysmeral doses. In mice, rabbits and humans, TBBA levels made up to 0.5%, 2.0% and 7.5% of the ROI, respectively. The quantitative difference in TBBA levels between rats and humans was statistically significant.

Overall, the *in vitro* studies demonstrated that lysmeral is extensively metabolised in the hepatocytes of different species, where lysmerylic acid was the main metabolite. TBBA was a common metabolite among species; however, quantitative differences were noted

— specifically, TBBA formation was much more extensive in rat hepatocytes than in the hepatocytes of humans, mice or rabbits.

For the third and fourth *in vitro* studies, see Laue *et al*. (2017) and Laue *et al.* (2020) under 4-*tert*-butylbenzoic acid (TBBA) of the ADME section of this report.

A number of dermal ADME studies with lysmeral were available in the CLH report and its Annex. These were not evaluated in depth in the RAC Opinion; however, RAC noted that dermal uptake was generally lower than uptake through the oral route.

4-tert-butylbenzoic acid (TBBA)

Only *in vitro* metabolism and human biomonitoring studies were available with TBBA.

One study by McCune *et al.* (1982) (non-GLP) investigated the effect of para-4-*tert*-butylbenzoic acid (p-TBBA) (purity: 99%) and other substances on hepatic gluconeogenesis and lipogenesis using hepatocytes from Wistar rats. According to the study, p-TBBA inhibited fatty acid synthesis in rat hepatocytes. An increase was noted in long-chain acyl coenzyme A (CoA) levels, which were potentially considered to be p-TBBA-CoA conjugates, and a decrease was noted in CoA and acetyl-CoA levels. Exposure to p-TBBA also inhibited gluconeogenesis from lactate, but not proline or glycerol, and lead to an increase in lactate levels.

Laue *et al*. (2017) investigated the *in vitro* metabolism of TBBA, bourgeonal, 4-*tert*-butyltoluene and lysmeral, as well as two members of the cyclamal group, cyclamal and 3-(p-cumenyl)propion aldehyde (cyclemax) in suspended and plated rat hepatocytes. TBBA or 4-iPBA were noted among other metabolites. In suspended hepatocytes, these metabolites were detected unconjugated, while in plated hepatocytes, which were considered a better test model, TBBA and 4-iPBA formed high levels of stable conjugates with CoA. Treatment with various concentrations of TBBA and lysmeral led to the formation of high levels of TBBA-CoA conjugates, which were mostly stable over 22 hours. For bourgeonal, 4-*tert*-butyltoluene and cyclemax, data on TBBA-CoA levels was reported as relative to levels formed after treatment with 50 µM lysmeral, and therefore stability over time was difficult to assess; however, the relative levels of 4-*tert*-butyltoluene and cyclemax decreased over 22 hours. The study also investigated the formation of CoA-conjugates following incubation of substances in rat liver S9 fraction. The only substance tested that was relevant to the bourgeonal group was lysmerylic acid. Formation of TBBA-CoA was minimal (approx. 1% relative to treatment with TBBA).

Laue *et al.* (2020) also conducted a comparative *in vitro* metabolism study with TBBA, lysmeral, bourgeonal, 4-*tert*-butyltoluene and members of the cyclamal group (cyclamal, cyclemax and 4-iPBA) in plated rat, rabbit and human hepatocytes. CoA conjugates formed and accumulated following incubation with substances from both the bourgeonal and cyclamal group in rat hepatocytes, and these were considered to be TBBA-CoA and 4-iPBA-CoA, respectively. In rabbit and human hepatocytes, low levels of TBBA-CoA

formed following treatment with lysmeral without accumulation — TBBA-CoA levels decreased over time. The same applied to human and rabbit hepatocytes treated with members of the cyclamal group. In rabbit cultures, CoA conjugates did not accumulate despite higher levels being detected than in rat hepatocytes.

The study also compared the levels of metabolites formed in rat and human hepatocytes following treatment with lysmeral or cyclamal. The main metabolite in human hepatocytes was lysmerylic acid, while levels of TBBA showed a very slight increase over 22 hours. In rats, a sharp increase in TBBA levels was noted over 22 hours, which was inverse to the decrease in lysmerylic acid levels. The same pattern of formation was noted with 4-iPBA following cyclamal treatment (see Figure 3). RAC noted that the slower rate of increase of the relevant metabolites in human cells might be an indication that TBBA/4-iPBA-CoA levels in human cells may increase over time and *in vitro* testing might require a longer incubation period than 22 hours. Additionally, supplemental information showed variations in TBBA levels over time between different and within the same cell batches of rat hepatocytes following lysmeral treatment.

Figure 3. The levels of the different metabolites in rat (a) and human (b) hepatocytes following treatment with lysmeral (top graphs) or cyclamal (bottom graphs) in the Laue et al. (2020) study (taken from p. 42 of the RAC opinion; ECHA, 2025)

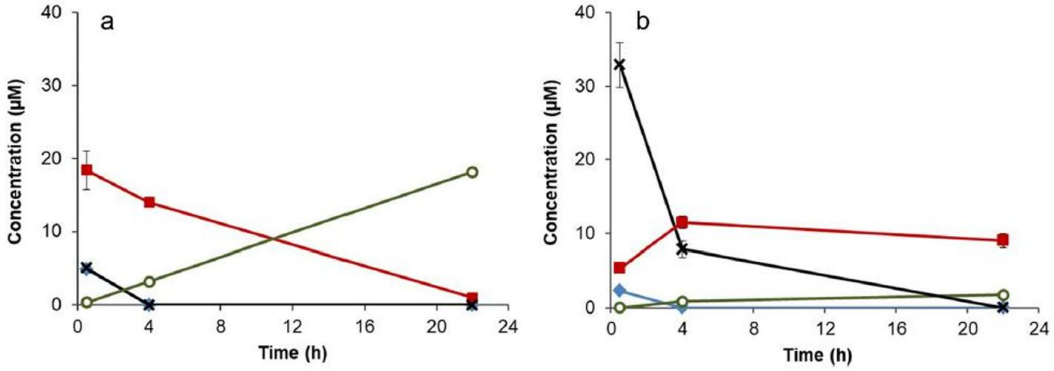


Fig. 4 Decrease of parent and formation of phase I metabolites in plated rat and human hepatocytes exposed to 3-(4-*tert*-butylphenyl)-2-methylpropanal (BMHCA). Plated primary rat (**a**) and human (**b**) hepatocytes were exposed to 50 µM BMHCA for 0.5, 4 and 22 h in triplicate and parent (closed diamonds) and selected phase I metabolites (closed squares, BMHCA acid; crosses, BMHCA alcohol; open circles, *p*-*t*BBA) analysed by GC–MS

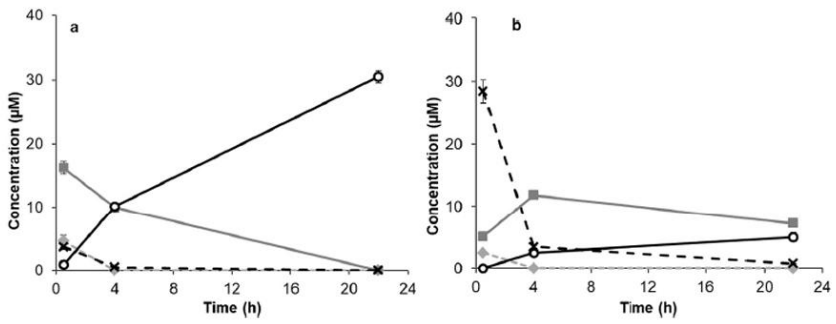


Fig. 4. Decrease of parent and formation of key phase I metabolites in plated rat and human hepatocytes exposed to CA. Plated rat (a) and human (b) hepatocytes were exposed to 50 µM CA for 0.5, 4 and 22 h in triplicate. Parent (closed diamonds) and selected phase I metabolites (closed squares, CA acid (M2); crosses, CA alcohol (M1); open circles, *p*-iPBA (M3) were analyzed by GC-MS. Samples were derivatised with (trimethylsilyl)diazomethane in methanol prior to analysis and M2 and M3 detected as methyl ester. Data from supplementary information of (Laue et al., 2020).

The authors also investigated the levels of two endogenous C8-CoA conjugates following treatment with lysmeral. In rat hepatocytes, lysmeral treatment inhibited the formation of these conjugates, while levels remained stable or increased over 22 hours in controls. This effect was not observed in rabbit or human hepatocytes.

The authors considered that the accumulation of metabolite-CoA conjugates was responsible for the adverse effects of the tested substances on reproduction and on kidney and liver function. They interpreted the results of this experiment as evidence that the proposed mode of action (MoA) of CoA conjugate accumulation was rat-specific.

CoA conjugates were also investigated in a study by Givaudan (2017) following treatment of rat hepatocytes with various substances, including substances related/belonging to the bourgeonal and cyclamal groups. The study attempted to correlate the formation of benzoic acid-CoA conjugates formed in rat hepatocytes following exposure to members of the bourgeonal and cyclamal groups with LOAEL values for adverse effects on the male reproductive system in *in vivo* rat studies. This study was not analysed in the RAC opinion.

Two biomonitoring studies were also available.

The first one (Murawski *et al.,* 2020) investigated lysmeral metabolites in the urine of children and adolescents in Germany. First-morning void urine samples (N = 2133) were collected from German residents between the ages of 3–17 years. TBBA, lysmerol, lysmerylic aid and hydroxy-lysmerylic acid were the main metabolites and were identified in 100%, 99%, 40% and 23% of the samples, respectively. Lysmerylic acid and its hydroxy- form were below the level of detection, and TBBA had the highest mean concentration (10.21 µg/L) followed by lysmerol (1.528 µg/L). Urinary metabolite concentrations were generally higher in girls, and usage of lysmeral-containing products, such as fragrances, personal care products etc., was positively associated with urinary concentrations.

The second study (Scherer et al., 2021) investigated 329 urine samples from the Environmental Specimen Bank for lysmeral metabolites. Samples were collected between 2000–2018. TBBA and lysmerol were detected in almost all of the samples in quantifiable amounts. The study identified a decreasing trend in the level of these metabolites in urine over time.

Methyl 4-tert-butylbenzoate

There were no toxicokinetic studies with methyl 4-*tert*-butylbenzoate. This member of the bourgeonal group was predicted to metabolise to TBBA based on its chemical structure (a methyl ester that can be hydrolysed to a carboxylic acid) and a profiling scheme built in the OECD QSARs Toolbox for the identification of TBBA precursors.

Acute Toxicity

Not assessed in the CLH report or RAC opinion.

Specific target organ toxicity – single exposure (STOT SE)

Not assessed in the CLH report or RAC opinion.

Skin corrosion/irritation

Not assessed in the CLH report or RAC opinion.

Serious eye damage/irritation

Not assessed in the CLH report or RAC opinion.

Respiratory sensitisation

Not assessed in the CLH report or RAC opinion.

Skin sensitisation

Not assessed in the CLH report or RAC opinion.

Specific target organ toxicity – repeated exposure (STOT RE)

Not assessed in the CLH report or RAC opinion.

Germ cell mutagenicity

Not assessed in the CLH report or RAC opinion.

Carcinogenicity

Not assessed in the CLH report or RAC opinion.

Reproductive toxicity

Classification agreed by RAC:

Effects on sexual function and fertility

3-(4-tert-butylphenyl)propionaldehyde (bourgeonal)

Three studies in rats were available for the assessment of effects on sexual function and fertility with bourgeonal — one combined repeated dose toxicity (RDT) study with reproduction/developmental toxicity screening test which also included a dose-range finding (DRF) study, and a toxicity screening test.

The dose selection for the main OECD TG 422 study was decided according to a non-GLP, non-guideline 14-day DRF study (Study report, 2019). Five Crl:CD(SD) rats/sex/dose were exposed to 0, 5, 25 and 50 mg/kg bw/d bourgeonal (purity: unknown) in corn oil via oral gavage 3 times per day for 14 consecutive days.

Mortalities in this study included two males at 50 mg/kg bw/d. The first one occurred on day 1 and was considered unrelated to treatment. The second mortality occurred on the last day and was considered likely treatment-related despite the lack of clinical signs prior to death or the lack of findings upon necropsy. There were no mortalities in the rest of the groups. Clinical signs were only observed in females from 25 mg/kg bw/d — these included suspected dehydration (based on skin turgor) and a low incidence of hunched posture and thin appearance. Marked decreases in overall body weight gain were observed in females from 25 mg/kg bw/d (-116% and -248% at 25 and 50 mg/kg bw/d, respectively), and mean body weight was decreased at 25 mg/kg bw/d (-4–9%) and at 50 mg/kg bw/d (-5–18%) with associated decreases in food consumption. There were no such effects in males. Other systemic toxicity indications included changes in mean absolute and relative liver weight in males from 5 mg/kg bw/d with histopathological findings of periportal to midzonal hepatocellular vacuolation from 5 mg/kg bw/d and centrilobular hepatocellular hypertrophy from 25 mg/kg bw/d. The latter effect was also observed in top-dose females.

In terms of sexual function and fertility, macroscopic examinations revealed one top-dose male with decreased testicular size. Changes in mean absolute and relative testes weight were observed at 50 mg/kg bw/d. Histopathological examinations revealed vacuolation of Sertoli cells and of the seminiferous tubular epithelium in the testes from 5 mg/kg bw/day, the latter of which was characterised by fine microvesicular vacuolation within the cytoplasm of seminiferous tubule epithelium and uniformly affected all stages of spermatogenesis (spermatogonia, spermatocyte, and spermatid). Degeneration of the seminiferous tubular epithelium in the testes was also observed from 5 mg/kg bw/d. The

effects on the testes were accompanied by secondary findings of cribriform change, cellular debris and hypospermia in the epididymides from 25 mg/kg/day.

Sperm assessments revealed decreases in motility at 5 mg/kg bw/d (77% vs 84% in controls) and little to no sperm at 25 and 50 mg/kg bw/d (18% and 3%, respectively, vs 84% in controls). It was considered that the infrequent increased spermatid head retention by the Sertoli cells, degeneration of maturing spermatids, round spermatids and/or elongating spermatids, exfoliation/degeneration of germ cells, increased cellular debris, and moderate to marked hypospermia noted during the histopathological assessments may have contributed to the overall decrease in sperm motility. In terms of morphology, all sperm samples from 25 mg/kg bw/d except for one contained headless and detached sperm.

In females, changes in mean absolute and relative weight were noted in the ovaries from 25 mg/kg bw/d and in the uterus at 50 mg/kg bw/d accompanied by uterine atrophy. Based on the marked decreases in body weight gain in females in these groups, these changes were considered of uncertain toxicological significance by the study authors.

The DS considered that the single instance of decreased testes size, the statistically significant differences in testes weight with histopathological findings in males, and the statistically significant changes in uterine weight and uterine atrophy in females were not relevant for classification due to excessive toxicity at the top-dose level. Effects in males in the lower dose levels, including histopathological findings in the testes at 5 and 25 mg/kg bw/d and in the epididymides at 25 mg/kg bw/d, and spermatotoxic effects at 25 mg/kg bw/d, were considered evidence of an adverse effect on the male reproductive system by the DS.

In consideration of the longer exposure period in the main combined RDT study with reproduction/developmental toxicity screening test (OECD TG 422 study; GLP), 5 mg/kg bw/d was determined to be the maximum tolerated dose (MTD), and the dose levels in the main study were set at 0, 0.5, 1 and 5 mg/kg bw/d bourgeonal (purity: 99%; vehicle: corn oil). Animals were treated via oral gavage once daily. The parental generation (P0) consisted of 10 Crl:CD(SD) rats/sex/dose. Treatment commenced 14 days before cohabitation. F0 males were exposed for 42–45 days and females were treated until lactation day (LD) 12 if they delivered a litter, or until gestation day (GD) 25 if they did not. F1 pups were not directly exposed to the test substance. The DS and REACH Registrant assigned the main study a Klimisch score of 1. The full study report was available to the DS.

In males, there were no mortalities, clinical signs or effects on body weight, body weight gain, food consumption, functional observational battery and motor activity assessments, haematology and clinical chemistry assessments or coagulation. Upon necropsy, there were no effects reported on organ weights or following macroscopic and microscopic

examinations. The only effect noted was a decrease in mean serum T4 levels from 1 mg/kg bw/d (-13% and -10% at 1 and 5 mg/kg bw/d, respectively) which lacked dose dependency or statistical significance and was considered incidental by the study authors. In terms of effects on sexual function and fertility, sperm assessments were not performed in the main study and there was no effect on mating and fertility indices or cohabitation time.

Similarly to the males, there were no systemic toxicity indications in females. Two mortalities, one in the low-dose and one in the middle-group, were considered unrelated to treatment. In terms of effects on sexual function and fertility, there were no effects on the oestrus cycle, mating and fertility indices or cohabitation time, parturition, the mean number of implantations, or any other reproductive parameters.

There were no effects on sexual function and fertility in this study. Based on the lack of systemic toxicity indications, the DS concluded that the top-dose level was too low.

The non-guideline 5-day toxicity screening test (Study report, 2009) was conducted according to GLP. Six Crl:CD® (SD)IGS BR male rats/dose were exposed to 0, 25, 100 and 250 mg/kg bw/d bourgeonal (purity: 98.4%) in corn oil via oral gavage for 5 days. The study also included a positive control group which was administered 250 mg/kg bw/d lilial (lysmeral). Animals were deprived of food on day 5 and sacrificed on day 6. The study was assigned a Klimisch score of 2 by the Registrant.

Mortalities included all top-dose males, 3 of which were killed in extremis after the first treatment due to poor clinical condition. The rest of the top-dose males were sacrificed on day 2 prior to dosing due to the high mortality rate in this group. The cause of death for the males killed in extremis could not be identified. Clinical signs prior to death included underactivity, prostrate or hunched posture, reduced body temperature, partially closed eyelids, irregular breathing and piloerection. Upon necropsy, 2/3 males presented with dark liver and 2/3 with distended stomach. One male at 100 mg/kg bw/d was also killed in extremis on day 1 due to poor clinical condition. This animal presented similar clinical signs to the top-dose group and also had a distended stomach upon necropsy. Similar clinical signs to the decedent animals were also observed in the rest of the males at 100 mg/kg bw/d in addition to loose faeces and yellow faecal staining on the body. Piloerection and loose faeces were also noted at the low-dose level.

Body weight loss was reported in the remaining top-dose males prior to sacrifice on day 2 (15–30 g), in middle-dose males on day 1 (14 g) and days 3–5, and in low-dose males on day 1 (10 g). At the low and middle dose, body weight stasis or lack of effects on body weight were reported in the rest of the study days. Additionally, mean food consumption was not decreased in these groups. Other indications of systemic toxicity included kidney depressions (2/6), thickened forestomach (2/6) and pale livers (5/6) at 100 mg/kg bw/d. The latter effect was also observed at 25 mg/kg bw/d (6/6). Histopathological findings

included generalised hepatocyte vacuolation from 25 mg/kg bw/d (low dose: 2/6; middle dose: 5/6) and cortical tubular vacuolation in the kidneys at 100 mg/kg bw/d (5/6).

Effects on the male reproductive system included enlarged epididymides at 100 mg/kg bw/d (3/6) and histopathological findings of seminiferous tubular degeneration/atrophy, Sertoli cell vacuolation and inflammation in the epididymides. Evaluations on spermatogenesis and the integrity of various cell types in the seminiferous tubules revealed multinucleate giant cells and luminal sloughing of spermatogenic cells in the testes, and reduced numbers of spermatozoa and sloughed germ cells in the lumen. Overall, 5/6 animals at 100 mg/kg bw/d showed treatment-related effects in the testes and epididymides. Additionally, the metabolite, TBBA, was detected in the urine of animals at 25 and 100 mg/kg bw/d (mean concentration: 35.8 and 275 µg/ml, respectively).

Overall, RAC considered that the macroscopic and microscopic findings in the epididymides and testes at 100 mg/kg bw/d were indicative of adverse effects on the male reproductive system, which were relevant for classification. However, they noted that these findings were observed in the presence of systemic toxicity, evident by one mortality at 100 mg/kg bw/d, clinical signs from 25 mg/kg bw/d, and macroscopic and histopathological findings in the kidneys and liver.

4-tert-butyltoluene

Eight studies were available for the assessment of effects on sexual function and fertility with 4-*tert*-butyltoluene in various species.

Four of the available studies were conducted in rats.

The first study was a reproduction/developmental toxicity screening test (Study report, 2007a), conducted according to GLP and OECD TG 421. The study was assigned a Klimisch score of 1 by the Registrant. The full report was not available to the DS, and the reported differences in the body weight of parental animals were estimated from graphs by the DS and are therefore associated with uncertainty. The parental generation (P0) consisted of 12 SD (Crj:CD(SD)IGS, SPF) rats/sex/dose, which were treated with 0, 1.5, 5, 15 and 50 mg/kg bw/d p-*tert*-butyltoluene (purity: 96.94%) in corn oil via oral gavage. Treatment started 14 days prior to mating, with males treated for 50–52 days and females treated until postnatal day (PND) 3 (between 41–45 days).

In males, one mortality was noted at 50 mg/kg bw/d. Prior to death, the animal exhibited transient salivation, decreased locomotor activity, soiled fur, reddish urine and hypothermia. Macroscopic examinations also showed atrophy of the thymus and ulcer on the anterior gastric mucosa in the decedent animal. Clinical signs in the rest of the dose groups were limited to transient salivation at the low dose and soiled fur in one top-dose male. Statistically significant decreases in body weight were noted between days 18–49 at 15 mg/kg bw/d and between days 4–49 at 50 mg/kg bw/d. According to the CLH report, these were estimated to be -13% on day 49 at 15 mg/kg bw/d and -6% and -19% on days

4 and 49, respectively, at 50 mg/kg bw/d. There were generally no effects on food consumption with the exception of statistically significant decreases on days 13 and 48 in the top-dose group.

In terms of effects on sperm, assessments revealed decreases in sperm motility ratio, path velocity, straight line velocity, curvilinear velocity, sperm viability, sperm survivability, sperm count, and sperm count per one gram of the left cauda epididymis at 15 mg/kg bw/d, with an increase in beat cross frequency and ratios of morphological abnormality of sperms (ratios of abnormality in the head, tail and the total of those). In the top-dose group, all sperm parameters, including sperm count, were decreased, and only samples from one animal among those with low number of motile sperm could be used to assess velocity and sperm viability parameters. Similarly, only five males could be used for morphology assessments, which revealed increases in the ratios of abnormality in the head, tail and the total of those.

In terms of effects on male reproductive organs, decreased absolute weights of the testis and epididymis were observed at 15 and 50 mg/kg bw/d and were statistically significant in all cases except for testis weight at 15 mg/kg bw/d (testis: -8% and -82% at 15 and 50 mg/kg bw/d, respectively; epididymis: -12% and -34% at 15 and 50 mg/kg bw/d, respectively). Non-statistically significant decreases were also observed in the relative weight of these organs at 50 mg/kg bw/d (testis: -44%; epididymis: -20%). Macroscopic examinations revealed atrophy of the testes and epididymides in one male at 15 mg/kg bw/d and in 11/11 males at 50 mg/kg bw/d. In the decedent top-dose male, there was atrophy of the testes, epididymides, seminal vesicles and prostate. Microscopic examinations of the testes revealed atrophy of the seminiferous tubules and hyperplasia of Leydig cells in some males at 15 mg/kg bw/d (4/12 and 2/12, respectively) and in all males at 50 mg/kg bw/d. In the epididymides, there was a decrease in sperm count in 4/12 males at 15 mg/kg bw/d and in all males at 50 mg/kg bw/d.

In females, mortalities included one female at 15 mg/kg bw/d and 6/12 animals at 50 mg/kg bw/d. Clinical signs prior to death included hypothermia, decreased locomotor activity and transient salivation from 15 mg/kg bw/d and prone position, piloerection, soiled fur and bradypnea at 50 mg/kg bw/d. Upon necropsy, there was a dark red spot in the glandular stomach of the decedent animal at 15 mg/kg bw/d and pale liver in 1/6 and atrophy of the thymus in 1/6 animals at 50 mg/kg bw/d.

In surviving animals, clinical signs included transient salivation from 15 mg/kg bw/d and hypothermia, decreased locomotor activity, staggering gait, lacrimation, diarrhoea and muscle relaxation at 50 mg/kg bw/d. In terms of body weight, statistically significant decreases prior to mating were noted only in the 50 mg/kg bw/d group between study days 4–15 (est. -8%). During gestation, statistically significant decreases were noted on GD 7 and 14 at 5 mg/kg bw/d (est. -8% and -10%, respectively), and between GD 7–21 at 15 mg/kg bw/d (est. -9%) — there was no information on the 50 mg/kg bw/d group in the CLH

report or its Annex. During lactation, a statistically significant decrease in body weight was noted on LD 4 at 5 mg/kg bw/d (est. -13%), which was correlated with a statistically significant decrease in food consumption. On the same day, a statistically significant decrease was observed in one animal at 15 mg/kg bw/d (est. -11%) — there was no information on the 50 mg/kg bw/d group in the CLH report or its Annex. Terminal body weights were statistically significantly decreased from 5 mg/kg bw/d (est. -13%, -18% and -29% at 5, 15 and 50 mg/kg bw/d, respectively).

In terms of effects on the female reproductive organs, there was no effect on the weight of the ovaries in any groups and there were no histopathological findings in the 50 mg/kg bw/d group. There were no effects on the oestrus cycle.

In terms of effects on reproduction, there were no effects on time to mating. One pair failed to mate at 50 mg/kg bw/d, but there was no statistically significant effect on the copulation index. There were statistically significant decreases in the fertility index from 15 mg/kg bw/d, with 8 non-pregnant females at 15 mg/kg bw/d (fertility index: 33.3%) and no pregnant females at the top dose (fertility index: 0%). There were no effects in the duration of gestation or parturition in females that achieved pregnancy. However, one dam at 15 mg/kg bw/d suffered total litter loss on day 1, resulting in a decreased gestation index at this dose level (66.7%). There were no statistically significant differences in number of pregnant corpora lutea, number of implantations and implantation index up to 15 mg/kg bw/d. Statistically significant decreases were observed in the number of pups born (-26%) at 15 mg/kg bw/d. The number of live pups on LD 0 was also statistically significantly decreased (-58%) and there was also an increase in the mean number of stillborn pups (4.7 vs 0.1 in controls), which was not statistically significant, at 15 mg/kg bw/d. At this dose level, decreases were reported in the delivery index (82.7% vs 94.1% in controls), birth index (49.3% vs 93.6% in controls) and live birth index (63% vs 99.5% in controls).

In this study, several effects were reported on the male reproductive system, including decreases in sperm motility and increased abnormal sperm morphology, decreased testes and epididymides weight and atrophy in these organs in combination with histopathological findings and decreases in the sperm count. While these effects were most severe at the top-dose level (50 mg/kg bw/d) where systemic toxicity was marked (mortality, clinical signs, decreased body weight), adverse effects were also observed at 15 mg/kg bw/d. At this dose level, systemic toxicity indications in males were limited to decreases in body weight, which were estimated not to exceed 13%. Effects at 15 mg/kg bw/d in males were therefore considered relevant for classification by RAC. Decreases in the fertility index were also reported from 15 mg/kg bw/d with 0 pregnancies achieved at the top-dose level. However, in females systemic toxicity was evident from 5 mg/kg bw/d in the form of statistically significant decreases in body weight, while clinical signs and mortality were reported from 15 mg/kg bw/d. There were no effects on the female reproductive organs.

The second study in rats was a 28-day RDT study (Study report, 2007b), which was conducted according to GLP and OECD TG 407. The study was assigned a Klimisch score of 1 by the Registrant. Twelve SD (Crj:CD(SD)IGS SPF) rats/sex/dose were administered 0, 1.5, 5, 15 and 50 mg/kg bw/d p-*tert*-butyltoluene (purity: 95.93%) in corn oil via oral gavage for 28 days. Half of the animals were necropsied upon cessation of treatment, and half of them were observed for a recovery period of 14 days before sacrifice.

There were no mortalities or effects on body weight during the treatment period, except for a statistically significant decrease in the body weight of top-dose females at termination at the end of the treatment period (-13%). Clinical signs included transient salivation in both sexes from 15 mg/kg bw/d. There were some statistically significant changes in food consumption throughout the dosing period in both sexes from 15 mg/kg bw/d; however, these were transient and were not considered toxicologically significant. Transient statistically significant increases in water consumption were noted in top-dose females throughout the study and in middle-dose males throughout the treatment period, while increases in water consumption in top-dose males were observed for the majority of the treatment period (days 3–24).

Haematological examinations revealed dose-dependent and statistically significant decreases in activated partial thromboplastin time (APTT) and fibrinogen in males from 5 mg/kg bw/d. In females, fibrinogen levels were statistically significantly increased at 15 mg/kg bw/d and decreased at 50 mg/kg bw/d. There was no effect on APTT in females, while statistically significant changes in platelet counts included a decrease at 15 mg/kg bw/d and an increase at 50 mg/kg bw/d. There were other changes in individual haematological parameters during treatment, which were slight and did not indicate toxicologically significant effects based on the lack of effects on other parameters.

Upon necropsy at the end of the treatment period, there were increases in relative liver weight in males at 15 mg/kg bw/d (+20%) and in absolute (+39%) and relative (+55%) weight at 50 mg/kg bw/d. At the top dose, these effects were accompanied by periportal hepatocyte hypertrophy in 4/6 males. None of these effects were observed upon recovery. In females, increases in both absolute and relative weight were noted from 15 mg/kg bw/d (15 mg/kg bw/d: +37% and +48%, respectively; 50 mg/kg bw/d: +60% and +83%, respectively) at the end of the treatment period. Histopathological findings included periportal hepatocyte hypertrophy in 1/6 females. Upon recovery, the effects on liver weight persisted (relative weight only at 15 mg/kg bw/d; absolute and relative at 50 mg/kg bw/d — no quantitative data available) without treatment-related histopathological findings.

Findings in biochemical chemistry assessments at the end of the treatment period were supportive of toxic effects on the liver. These included statistically significant decreases in total protein and triglycerides (dose-dependent) and increases in AST from 5 mg/kg bw/d, decreases in albumin (dose-dependent) and increases in the albumin to globulin ratio and

total bilirubin (dose-dependent) from 15 mg/kg bw/d, and increases in total cholesterol at 50 mg/kg bw/d in males. In females, there were statistically significant decreases in total protein, albumin, triglycerides and total cholesterol (non-significant) and an increase in gamma-glutamyl transferase (gGT) from 15 mg/kg bw/d, and an increase in total bilirubin at 50 mg/kg bw/d at the end of the treatment period. These effects did not persist upon recovery in either sex.

Urinalysis assessments revealed a statistically significant increase in urine volume from 15 mg/kg bw/d and decreases in urine specific gravity and pH (non-significant) at 50 mg/kg bw/d in males. In females, significantly increased urine volume and decreased pH (non-significant) were noted at the top dose.

In terms of effects on the reproductive system, in males there were significant decreases in absolute and relative weight of the testis (-65% and -61%, respectively) and in the absolute weight of the epididymis (-23%) at the top dose. Histopathological findings included atrophy of the seminiferous tubules, hyperplasia of Leydig cells in the testes and a decrease in sperm count in the lumen of the ductus epididymis in 6/6 top-dose males, with statistical significance. Upon recovery, effects on the weight of reproductive organs persisted, with additional decreases in the relative weight of the epididymis, but without statistical significance for the effects on absolute and relative testis weight. Histopathological findings persisted in the top-dose recovery group with the same incidence and statistical significance. In females, the only effect noted was a statistically significant decrease in absolute ovary weight (-28%) at the top dose, which was not apparent after recovery. There were no histopathological findings in the female reproductive organs.

In this study, the most notable effects included statistically significant decreases in testes and epididymides weight in top-dose males, which were accompanied by histopathological findings in these organs, including atrophy and decreased sperm counts. Systemic toxicity indications in males were limited to mild liver toxicity. The DS considered that adverse effects on the male reproductive system were observed in the absence of marked systemic toxicity.

The rest of the available studies consisted of four non-guideline 5-day toxicity screening test with 4-*tert*-butyltoluene, two of which were conducted in rats. All studies were assigned a Klimisch score of 2.

In the first test (Study report, 1982a; non-GLP), seven male albino SPF rats/group were administered 0 or 200 mg/kg bw/d 4-*tert*-butyltoluene (unknown purity) in rape oil via oral gavage for 5 days. There were no mortalities or clinical signs. Body weight loss was observed between treatment days 1–3 in the treated rats, which improved by the end of the treatment period (no statistical analysis, no quantitative data). One of the treated rats presented with liver inflammation upon necropsy, and histopathological findings of acute

hepatitis and acute interstitial nephritis were evident in all treated animals; although, the study authors noted that this was potentially due to a parasitic infestation.

A treatment-related decrease in testes weight was observed in the treated rats with histopathological changes in the seminiferous tubules of all dosed rats and lesions in the epithelium comprised degeneration of spermatocytes and spermatids, reduction of spermatozoa as well as appearance of giant cells. There were no effects on Sertoli cells and interstitial Leydig cells.

In the second test (Study report, 1982b; non-GLP), eight male albino SPF rats/group were administered 12.5, 25, 50 or 100 mg/kg bw/d p-*tert*-butyltoluene (unknown purity) in rape oil via oral gavage for 5 days. A vehicle control group of 4 males was also included. There were no mortalities. Clinical signs included loss of hair, shaggy fur, hunched posture, lethargy and diarrhoea from 50 mg/kg bw/d. Transient body weight loss was observed at 25 and 50 mg/kg bw/d between days 2–4. By day 5, the body weight of the males on 25 mg/kg bw/d was only slightly decreased in comparison to the control (-4%). In the 50 mg/kg bw/d group, the body weight decrease was more pronounced (-12%), while in the 100 mg/kg bw/d body weight loss was recorded throughout the treatment period which resulted in a marked decrease of 24% on day 5. Other indications of systemic toxicity included decreases in the absolute weight of the kidneys from 50 mg/kg bw/d and in the absolute weight of the liver at 100 mg/kg bw/d. Gross pathology examinations revealed pale kidneys in 3/8 top-dose males, pale liver at 50 and 100 mg/kg bw/d (1/8 and 3/8, respectively) and delineation of the hepatic lobules from 50 mg/kg bw/d with increased severity at the top dose (slight in 6/8 males at 50 mg/kg bw/d; slight in 2/8 and severe in 4/8 at 100 mg/kg bw/d).

Regarding effects on the male reproductive system, there was a marked decrease in absolute testis weight at the top dose (-59%). Histopathological findings in the seminiferous epithelium were noted from 50 mg/kg bw/d and included severe cell deformations in the germinal epithelium — mainly degenerated spermatids and spermatocytes, reduced spermatozoa and sporadic giant cells. The severity of such findings was much higher in the top-dose group (grade 3 in 86.3% and grade 2 in 12.4% of samples at 100 mg/kg bw/d vs. grade 3 in 20.2% and grade 2 in 29.1% of samples at 50 mg/kg bw/d).

Overall, effects on the male reproductive system were observed in both of these rat studies. These included decreases in testes weight accompanied by degeneration of the germinal epithelium in the absence of systemic toxicity at 200 mg/kg bw/d in 1982a study. In the 1982b study, there were similar adverse findings on the male reproductive system from 50 mg/kg bw/d; however, significant systemic toxicity was observed from this dose level in the form of clinical signs, body weight decreases and liver toxicity.

The third 5-day toxicity screening test (Study report, 1984a; non-GLP) was conducted in male Himalayan spotted guinea pigs (SPF). Two groups of 5 males each were administered 0 or 100 mg/kg bw/d p-*tert*-butyltoluene (unknown purity) in rape oil via oral gavage for five days. There were no mortalities, clinical signs or effects on body weight. Absolute and adjusted (per 100g of body weight) testes weights were comparable between the control and treated groups. Histopathological examinations revealed slight damage to the germinal epithelium of the seminiferous tubules in 2/5 control males and 1/5 treated males, and moderate damage in 1/5 treated males. Histological assessment of cross sections of the seminiferous tubules showed a higher incidence of findings in treated males (91% unaffected vs. 97.5% in controls) and findings of higher severity (grade 1: 4.5% vs. 2.3% in controls; grade 2: 1.8% vs. 0% in controls; grade 3: 2.7% vs. 0.2% in controls). The study authors concluded that in this study p-*tert*-butyltoluene had a harmful effect on the germinal epithelium of male guinea pigs.

The fourth 5-day toxicity screening study (Study report, 1984b; non-GLP) was conducted in male Beagle dogs. Two males were administered 100 mg/kg bw/d p-*tert*-butyltoluene (unknown purity; no vehicle) via oral capsule for 5 days. One control male (sham-exposed) was also included. There were no mortalities, clinical signs or effects on body weight. There were no effects in the epididymides in any of the dogs. There was a small quantity of randomly disseminated seminiferous tubules with nearly total depopulation of germinal epithelium in both testes in one of the treated dogs. The concerned tubules showed early signs of spermatogenesis and Sertoli cells. The control dog presented with few multinucleated giant cells in the lumen of the seminiferous epithelium in the testes.

The fifth 5-day toxicity screening study (Study report, 1984c; non-GLP) was conducted in male albino SPF mice. Two groups of 6 males each were administered 0 or 100 mg/kg bw/d p-*tert*-butyltoluene (unknown purity) in rape oil via oral gavage for five days. There were no mortalities or clinical signs. The body weight of the treated males was slightly higher than the controls throughout the study (+4–9%). There was an increase in absolute testes weight in the treated mice (+17%) and a decrease in adjusted testes weight (per 100 g body weight) (-12%). Histopathological assessments revealed a slight increase in the incidence of slight damage of the germinal epithelium (3/6 vs. 1/6 in controls). Histological assessment of cross sections of the seminiferous tubules showed a slightly higher incidence of findings in treated males (94.83% unaffected vs. 95.75% in controls) and findings of higher severity (grade 1: 4.25% vs. 4.08% in controls; grade 2: 0.25% vs. 0% in controls; grade 3: 0.67% vs. 0.17% in controls). There were no other effects on the testes or epididymides.

4-tert-butylbenzaldehyde

Five non-guideline 5-day toxicity screening tests were available for the assessment of 4-*tert*-butylbenzaldehyde on sexual function and fertility — two in rats, one in guinea pigs, one in dogs and one in mice. All studies were assigned a Klimisch score of 2.

The first study (Study report, 1981; GLP status unknown) was conducted on male SPF albino rats. Eight males/dose were administered 6.5, 12.5, 25 and 50 mg/kg bw/d p-*tert*-butylbenzaldehyde (purity: unknown) in rape oil via oral gavage for 5 days. A control group of 4 males was also included. There were no mortalities. Clinical signs included slight aggressiveness in 3/8 rats at 12.5 mg/kg bw/d and slight hair loss in 1/8 rats at 50 mg/kg bw/d. Body weight loss was noted throughout the study period at the top dose, and transiently at 25 mg/kg bw/d. Body weight gain was decreased from 12.5 mg/kg bw/d (-27%, -60%, -160% at 12.5, 25 and 50 mg/kg bw/d, respectively). There were no statistically significant effects on liver or kidney weights. At necropsy, 2/8 top-dose males presented with marbled liver and 1/8 males at 25 mg/kg bw/d had a small dell on the right kidney.

In terms of effects on the male reproductive organs, there was a slight decrease in absolute testis weight at 50 mg/kg bw/d (-14%). Histopathological assessments revealed a higher percentage of convoluted tubules with degenerated cells or detritus in the lumina of the seminiferous epithelium from 25 mg/kg bw/d. Injuries at the top-dose level were moderate to severe. Assessments of cross sections of seminiferous tubules revealed a higher incidence of findings from 25 mg/kg bw/d (unaffected samples: 53.6% and 1.5% at 25 and 50 mg/kg bw/d, respectively vs. 78.1% in controls) and a higher severity of findings from 25 mg/kg bw/d (25 mg/kg bw/d: grade 2: 5.7%, grade 3: 6.1%; 50 mg/kg bw/d: grade 2: 43.8%, grade 3: 27.1%; controls: 0% for both gradings). Interstitial and Sertoli cells were unaffected.

In the second study (TSCATS, 1982; GLP status unknown), 0 or 100 mg/kg bw/d p-*tert*-butylbenzaldehyde (purity: unknown) in rape oil were administered to 7 male SPF albino rats/group via oral gavage for 5 days. There were no mortalities or clinical signs. Body weight loss was recorded on day 2 for the treated group, but weight gain was observed throughout the rest of the study. In the treated group, terminal body weight and overall body weight gain were 8% and 53% lower, respectively, in comparison to the control. There were no effects on liver or kidney weights. In the liver, the incidence of histopathological findings was higher and more severe in the control (inflammatory infiltration of the portal tract by mainly mononuclear cells associated with alterated bile ducts, multifocal necrosis in the parenchyma and accumulation of lymphocytes in the parenchyma). In the kidneys, there was an increased incidence of lymphocytic interstitial inflammation in control animals and a single incidence of subcapsular lymphocytic infiltration. All males, treated or controls, presented with acute hepatitis and acute interstitial nephritis. This was potentially caused by a parasitic infection according to the authors.

In terms of effects on reproductive organs, there was a slight decrease in absolute testes weights in the treated group (-16%). Histopathological assessments revealed minimal to moderate degeneration of spermatids and spermatocytes (5/7), minimal reduction of

spermatozoa (1/7) and minimal to moderate appearance of multinucleate giant cells (7/7). Sertoli cells and Leydig cells were unaffected.

Overall, effects on the male reproductive system were similar in nature in both studies. These included slight decreases in absolute testes weight at 50 mg/kg bw/d in the 1981 study and at 100 mg/kg bw/d in the 1982 study, and histopathological findings from 25 mg/kg bw/d and at 100 mg/kg bw/d, respectively. These effects were noted in the presence of systemic toxicity, indicated by decreased body weight gain from 12.5 mg/kg bw/d in the 1981 study, while systemic toxicity was also evident in controls in the 1982 study potentially due to the presence of a parasitic infection.

In the third study (Study report, 1984e; GLP status: unknown), five male Himalayan guinea pigs/group were administered 0 or 100 mg/kg bw/d p-*tert*-butylbenzaldehyde (purity: unknown) in rape oil via oral gavage for 5 days. There were no mortalities, clinical signs or effects on body weight. There were no statistically significant differences in absolute or relative testes weight. Histopathological assessments revealed slight damage of the germinal epithelium in 2/5 control and 1/5 treated animals, but higher levels of detritus in the lumen of the seminiferous tubules in treated males. Histological assessments of cross section of the seminiferous tubules showed a higher incidence of findings in treated animals (unaffected samples: 87.9% vs. 97.5% in controls). There were no effects on the epididymides.

In the fourth study (Study report, 1984f; GLP status: unknown), two male Beagle dogs were treated with 100 mg/kg bw/d p-*tert*-butylbenzaldehyde (purity: unknown) via oral capsule for 5 days. One control dog was also included in the study. There were no mortalities or clinical signs. Body weight loss was reported in both treated animals (-1.1 and -0.8 kg each). There was no information on the body weight of the control dog. In one of the treated dogs, there was slight damage of the germinal epithelium with nearly total depopulation in both testes and detection of only early spermatogenesis and Sertoli cells in the affected seminiferous tubules. The second dog presented with multinucleated giant cells in the testes, which was also observed in the control dog. There were no effects on the epididymides.

In the fifth study (Study report, 1984d; GLP status: unknown), six male albino SPF mice/group were administered 0 or 100 mg/kg bw/d p-*tert*-butylbenzaldehyde (purity: unknown) in rape oil via oral gavage for 5 days. There were no mortalities, clinical signs or effects on body weight. There were no effects on absolute or relative testes weight. Histopathological assessments revealed slight damage of the germinal epithelium in the testes of 1/6 controls and 4/6 treated males. Histological assessments of cross sections of the seminiferous tubules did not report a difference in the incidence of findings between control and treated animals; however, the severity of findings was higher in treated males (grade 2: 0.33% vs. 0% in controls; grade 3: 1.17% vs. 0.17% in controls).

2-(4-tert-butylbenzyl)propionaldehyde (lysmeral)

Twenty studies were available with lysmeral, nineteen of which had been previously assessed by RAC in their opinion on lysmeral in 2019 (ECHA, 2019). The findings of these studies have been summarised in Table 3.

Lysmeral has a harmonised and mandatory classification as Repr. 1B (H360Fd) under EU and GB CLP, respectively. Adverse effects on sexual function and fertility were characterised by decreased male reproductive organ weights, histopathological findings of degeneration in the testes, decreased sperm counts and increases in abnormal sperm morphology in rats. These effects were also observed in dogs. Decreased fertility was also observed in two DRF rat studies for one-generation reproductive toxicity studies (BASF SE, 2006; BASF SE, 2017B). While this effect was not observed in the modified extended one-generation reproductive toxicity study (EOGRTS) (BASF SE, 2017), RAC had considered that the dose levels in this study were too low (ECHA, 2019).

In other species, such as mice, guinea pigs and monkeys, effects on the male reproductive system were not evident or not as pronounced as in the rat and dog studies. However, RAC at the time had considered that the lack of positive findings was likely caused by doses that were too low or exposure periods that were too short.

The 2019 RAC opinion on lysmeral also discussed a potential MoA behind the lack of effects in some species. The harmonised classification of metabolite TBBA (Repr. 1B (H360F) was considered as supportive evidence in their opinion on lysmeral, as this metabolite was considered responsible for the adverse effects on the testes and sperm. Toxicokinetic studies indicated differences between species, particularly in the formation of TBBA-CoA conjugates, which was much more pronounced in rat hepatocytes. Based on these differences, the potential of the adverse effects being specific to certain species was discussed. RAC had noted that these differences revolved around the level of TBBA-CoA conjugate formation in different species and were not qualitative in nature. RAC additionally had considered that mechanistic data was not available in dogs, where adverse effects on the male reproductive system had also been observed. They concluded that there was no evidence that adverse effects were mediated only through this particular MoA, and noted that a direct effect of lysmeral on target tissues could not be excluded.

Only one animal study with lysmeral (Study report, 1987) has been reported in the current CLH report on the bourgeonal group which was not considered in the 2019 RAC opinion on lysmeral. This was a non-guideline, non-GLP RDT study in rats with limited reporting. The observed effects on the male reproductive system were considered supportive of lysmeral's current classification by RAC. Additionally, any other new studies or papers revolved around potential MoAs and the relevance of effects to humans (see ADME section of this report).

Table 3. Available studies on lysmeral

Study protocol	General Toxicity	Effects on reproduction
Oral studies in rats		
One-generation DRF study (BASF SE, 2006c) Non-guideline, non-GLP 10 F0 Wistar rats/sex/dose Oral administration (dietary) for 12 weeks, mated after 6 weeks of treatment, F0 females sacrificed after PND 21 Lysmeral in sunflower oil microencapsulated in gelatine (a.i. purity: 30.7%) Doses: 0, 400, 800, 1700 and 3400 ppm a.i. doses: Males: 0, 14, 28, 62.6, 116.8 mg/kg bw/d Females: 0, 10–15, 18.3–29.4, 62.7, 123.2 mg/kg bw/d (ranges reported for the two lower dose levels due to dose adjustments during gestation and lactation; no adjustment for the two highest doses due to the lack of offspring)	**F0 males:** <u>From 800 ppm:</u> Increased relative liver weight (+10–20%) Increased ALT (+20–45%), ALP (+30–55%) and glutamate dehydrogenase (4–5-fold) <u>At 3400 ppm:</u> Dose-dependent decreases in body weight (-5–30%), body weight gain (-10–40%) and food consumption (-15%) Increased relative kidney weight (+15%) Increased gGT (2-fold) **F0 females:** Decreased body weight and body weight gain: • during/after premating (-5–10% and -10–30%, respectively) from 800 ppm • during gestation and lactation (approx. -10% for both) at 800 ppm Decreased food consumption during lactation (-20%) (no data on dose levels) No effects on liver or kidneys weights Increased gGT (2–8-fold) at all dose levels Decreased serum cholinesterase (-50–65%) at all dose levels	**F0 males:** <u>From 1700 ppm:</u> Decreased male fertility index (1700 ppm: 10%; 3400 ppm: 0%; controls and lower dose groups: 100%) Decreased mating index (1700 ppm: 80%; 3400 ppm: 50%; controls and lower dose groups: 100%) Decreased relative testes weight (-30–45%) and cauda epididymis (-30–40%) Diffuse testes degeneration (moderate: 8/10; moderate to severe: 2/10) and aspermia of the epididymis (10/10) Decreased testicular spermatic heads (6 mio vs 121 mio in controls) Decreased epididymal sperm heads (2 mio vs 591 in controls) Motile sperm 0% <u>At 3400 ppm:</u> Decreased seminal vesicle weight (-10%) and prostate (-20%) Hyperplasia of Leydig cells (9/10) **F0 females:** No data on corpora lutea <u>From 400 ppm:</u> Decreased mean implantation sites (9.9, 8.5, 8.8, 1 and 0 at 0, 400, 800, 1700 and 3400 ppm, respectively) Increased mean post-implantation loss (5.1%, 16.2%, 11.1%, 100% at 0, 400, 800 and 1700 ppm, respectively — no data at 3400 ppm due to no implantations)

	Increased glutamate dehydrogenase from 800 ppm (5–75%)	From 1700 ppm: Decreased female fertility index (1700 ppm: 13%; 3400 ppm: 0%; controls and lower dose groups: 100%) Only 1/8 mated females was pregnant at 1700 ppm, effect associated to adverse effects on male reproductive organs — only one implantation in this female, which was resorbed **F1 pups:** Up to 800 ppm: No effect on gestation and live birth index — no stillborn pups Decreased number of pups delivered (9.4, 8.7, 7.9, 0 and 0 at 0, 400, 800, 1700 and 3400 ppm, respectively) No effect on the number of litters or pup survival between PND 0–4 and PND 4–21 Decreased pup body weight on PND 0 (400 ppm: -19%; 800 ppm: -22%) and on PND 21 (400 ppm: -17%; 800 ppm: -21%) Decreased pup body weight gain (400 ppm: -16%; 800 ppm: -21%) From 1700 ppm: No litters from 1700 ppm
One-generation DRF study (BASF SE, 2017b) Non-guideline, GLP 10 F0 Wistar rats/sex/dose Oral administration (dietary) for 8 weeks, mated after 2 weeks of treatment, F0 females sacrificed after PND 21 Lysmeral in sunflower microencapsulated in alginate (a.i. purity: 17.7%) Doses: 0, 230, 750 and 2300 ppm a.i. doses:	**F0 males:** From 230 ppm: Increased absolute and relative liver weight (230 and 750 ppm: <10%; 2300 ppm: +14–30%) From 750 ppm: Decreased food consumption from week 1 (750 ppm: -7%; 2300 ppm: -9%) Decreased total protein At 2300 ppm: Decreased body weight starting from week 2 (-5–11%) and body weight gain (-45–84%)	**F0 males:** At 2300 ppm: Decreased relative cauda epididymis (-19%), epididymis (-16%) and seminal vesicles (-19%) weight Minimal to moderate tubular degeneration in testis (3/10 vs. 1/10 in controls), and minimal to moderate ductal atrophy in epididymis (8/10), slight to moderate oligospermia (6/10) and slight to moderate cellular debris (2/10) vs. 0 in controls Decreased epididymal sperm heads (469 mio vs 674 mio in controls) Decreased motile sperm (25% vs. 85% in controls) Increased abnormal sperm (72% vs 6.2% in controls)

Males: 0, 2.3–2.8, 7.4–9.1 and 25.1–27.5 mg/kg bw/d Females: 0, 3.3–3.7, 10.6–11.9 and 21–34.7 mg/kg bw/d (ranges reported due to differences in lysmeral uptake in males pre- and post-mating, and due to differences in uptake during premating and gestation and a 50% dose reduction in females during lactation)	Increased incidence of liver discolouration Decreased albumin, globulin, cholesterol, triglycerides, sodium and calcium Increased AST (+26%) **F0 females:** <u>From 750 ppm:</u> Decreased body weight during gestation with recovery of body weight at the end of lactation period (2300 ppm: GD 14: -10–16%; lactation: -10%; with less severity at 750 ppm) Decreased food consumption during premating and lactation (2300 ppm: premating week 1: -14%; lactation: -44–48%; with less severity during the first two weeks of lactation at 750 ppm) Decreased total protein, albumin, globulin, triglycerides, sodium and calcium Increased AST (+23–47%) and gGT (9–24-fold) <u>At 2300 ppm:</u> Body weight loss during pre-mating (week 1) Decreased body weight gain during premating and gestation (-32–59%) Decreased cholesterol Increased creatinine, total bilirubin, chloride and inorganic phosphate	No effect on testicular spermatid head counts Decreased male fertility index (40% vs. 90–100% in controls and lower dose groups) **F0 females:** <u>At 2300 ppm:</u> Decreased female fertility index (44% vs 90–100% in controls and other treated groups) Statistically significantly decreased mean implantation sites (4.5 vs 10.1–11.5 in controls and other treated groups) Increased post-implantation loss (16.7% vs 3.7–3.9% in controls and other treated groups) Increased gestation duration (23 days vs 22.2 days in controls) — considered secondary to the lower number of pups per litter, leading to a lower stimulus in starting the process of parturition **F1 pups:** No effects on live birth index due to no increases in stillborn rate in treated groups in comparison to the control <u>From 750 ppm:</u> Decreased pup survival between PND 0–4 (95%, 99%, 86% and 75% at 0, 230, 750 and 2300 ppm, respectively) No effect on pup mortality between PND 4–21 at any dose level Decreased pup body weight on PND 0 and PND 21 (750 ppm: PND 0: -17%, PND 21: -13–21%; 2300 ppm: PND 0: -18%, PND 21: -30–32%) Decreased pup body weight gain (750 ppm: -13%; 2300 ppm: -33%) <u>At 2300 ppm:</u> Statistically significantly decreased mean number of pups delivered (4.0 vs 9.7–11.3 in controls and other treated groups)

<table>
<tr>
<td></td>
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<td>Decreased number of litters (4 vs. 9–10 in controls and other treated groups)

Effect on sex ratio (67–69% females vs. 53% in controls) —considered secondary to low pup numbers</td>
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<tr>
<td>Modified EOGRTS
(BASF SE, 2017)

OECD TG 443, GLP

35 F0 Wistar rats/sex/dose for controls to middle-dose groups; 40 F0 Wistar rats/sex for the top dose group; Two control groups (plain diet and capsules without lysmeral)

Oral administration (dietary), F0 from 13 days before mating, F1 pups continued in the same fashion as the F0 parental animals.

F1 pups assigned to 7 cohorts:
<table>
<tr><th>Cohort</th><th>Designation</th><th>Animals/ Cohort</th><th>Puberty</th><th>Approx. age at necropsy</th></tr>
<tr><td>1A</td><td>Reproductive PND 90</td><td>20 M + 20 F</td><td>Yes</td><td>13 weeks</td></tr>
<tr><td>1B</td><td>Reproductive (= F1 parental animals)</td><td>25 M + 25 F</td><td>Yes</td><td>19–25 weeks</td></tr>
<tr><td>2A</td><td>Neurotoxicity PND 75–90</td><td>10 M + 10 F*</td><td>Yes</td><td>11 weeks</td></tr>
<tr><td>2B</td><td>Neurotoxicity PND 22</td><td>10 M + 10 F*</td><td>No</td><td>3 weeks</td></tr>
<tr><td>3</td><td>Immunotoxicity</td><td>10 M + 10 F*</td><td>Yes</td><td>8–9 weeks</td></tr>
</table>
</td>
<td>F0 males:
None

F0 females:
<u>from 4.5 mg/kg bw/d:</u>
Decreased mean serum acetylcholinesterase activity (4.5 mg/kg bw/d: -16%; 15.1 mg/kg bw/d: -21%; in comparison to placebo controls)

Significant absolute and relative weight increase of the liver weight (4.5 mg/kg bw/d: abs: 112%, rel: 110% — within HCD; 15.1 mg/kg bw/d: abs: 119%; rel: 120%)

<u>at 15.1 mg/kg bw/d:</u>
Decreased food consumption during lactation (-5%)

Decreased body weight during gestation (GD 20: ss -5%) and the first two weeks into lactation (LD 14: ss -4%)

Decreased body weight gain during premating (-14%) and gestation (ss -12%)

Higher red blood cell counts, haemoglobin and haematocrit values

Prolonged prothrombin time (i.e. reduced synthesis of coagulation factors), increased gGT activity and reduced albumin levels

Minimal to slight centrilobular hypertrophy accompanied by minimal to slight apoptosis/single cell necrosis of hepatocytes</td>
<td>F0 males:
No effect on male fertility or mating index

<u>At 15.1 mg/kg bw/d:</u>
No findings in sperm motility (86% vs. 88% in placebo control) or sperm head counts in testes (108 mio/g vs. 102 mio/g in placebo control) and epididymides (717 mio/g vs. 723 mio/g in placebo control)

Slightly higher (not statistically significant) mean percentage of abnormal sperms (9.8%) above the placebo controls (6.3%) and HCD (6.0–6.6 %)

F0 females:
No effect on female fertility or mating index, gestation index, gestation duration, oestrous cycle duration, mean number of implantation sites, post-implantation loss, mean number of pups delivered, live birth index, total litter numbers, sexual organ weights and gross and histopathological findings

F1 pups:
<u>At 15.1 mg/kg bw/d:</u>
Decreased pup body weight on PND 1 (-15%) and PND 21 (-10%)

Slight decrease in viability index between PND 0–4 (93% vs. 97% in controls)

No effect on viability between LD 4–21

No effects on anogenital distance or index</td>
</tr>
</table>

4A	Cholinesterase PND 22	10 M + 10 F*	No	3 weeks
4B	Cholinesterase adult	10 M + 10 F*	Yes	11–12 weeks

F1B cohort mated to produce the F2 generation
F1B cohort and F2 pups sacrificed at weaning

Lysmeral encapsulated (a.i. purity: 17.7%)

Doses:
Nominal: 0, 75, 230 and 750 ppm;
a.i.: 0, 13, 41 and 133 ppm
Achieved a.i. doses: 0, 1.4, 4.5 and 15.1 mg/kg bw/d

Periportal vacuolation and multinucleated hepatocytes in few animals

F1 males:
<u>At 15.1 mg/kg bw/d:</u>
Decreased body weight after weaning (up to -11%)

Higher red blood cell counts and haemoglobin values and decreased mean corpuscular volumes

F1 females:
<u>from 4.5 mg/kg bw/d:</u>
Significant absolute and relative liver weight increases (4.5 mg/kg bw/d: abs: 116%, rel: 109% — above HCD; 15.1 mg/kg bw/d: abs: 126%, rel: 128%)

<u>At 15.1 mg/kg bw/d:</u>
Decreased food consumption during lactation (-5%)

Decreased body weight during gestation (GD 20: ss -8%) and lactation (LD 14: ss -4%)

Decreased body weight gain during premating (-4%) and gestation (ss -11%)

Higher red blood cell counts and haemoglobin values and decreased mean corpuscular volumes

Prolonged prothrombin time (i.e. reduced synthesis of coagulation factors), increased gGT activity and reduced albumin levels.

Minimal to slight centrilobular hypertrophy and minimal to slight apoptosis/single cell necrosis of hepatocytes.

Higher number of cannibalized pups around PND 1 (20 vs. 1 in placebo controls) — most cannibalizations (16) were clustered in 2 litters (total of 5 litters affected with 9, 7, 2, 1 and 1 cannibalized pup(s), respectively)

F1B males:

No effect on male fertility or mating index, or sperm parameters

<u>At 15.1 mg/kg bw/d:</u>
Ss delay in time to preputial separation (42.3 days vs. 41.6 days in controls — within HCD (40.5–45.2 days)

F1B females:

No effect on time to vaginal opening, female fertility, mating or gestation index, gestation duration, oestrous cycle duration, differential ovarian follicle count, post-implantation loss, live birth index, total litter numbers, sexual organ weights and gross and histopathological findings

<u>At 15.1 mg/kg bw/d:</u>
Ss decrease in mean number of implantation sites (10.5 vs 12.3 in controls) within the historical control range (9.4-13.9 implants/dam)

Ss decrease in mean number of pups delivered (10.1 vs 12 in controls) — associated to low number of implantation sites

F2 pups:

No effects on anogenital distance or index, cannibalised pups or sex ratio on PND 1 or 21

<u>At 15.1 mg/kg bw/d:</u>
Decreased pup body weight on PND 1 (-13%) and PND 21 (-10%)

Slight decrease in viability index between PND 0–4 (95% vs. 99% in controls)

No effect on viability between LD 4–21

<table>
<tr><td>Time course study
(BASF SE, 2006A)
Non-guideline, non-GLP
5 male rats/time point
Time points: 1, 2, 3, 4 or 14 days
Oral administration (gavage)
Lysmeral (purity: 99.1%) or Lysmerylic acid
Dose: 50 mg/kg bw/d</td><td>Males:
<u>At 14-day timepoint:</u>
Non-significant decrease in body weight gain (lysmeral: -25%; lysmerylic acid: -20%)</td><td>Males:
<u>From days 1 to 2:</u>
Slight to severe testicular atrophy on day 1 (lysmeral: 2/5; lysmerylic acid: 3/5) and from day 2 (5/5 in both groups),
Described as diffuse tubular testicular degeneration and fine vacuolar change of pachytene spermatocytes up to apoptotic cell death.
<u>At 14-day timepoint:</u>
Decreased sperm motility, spermatid count in testes and cauda epididymal sperm count with both substances
Altered sperm morphology</td></tr>
<tr><td rowspan="3">5-day toxicity screening test
(Givaudan, 1986B)
Non-guideline, GLP
8 male rats/group
Oral administration (gavage), 5 days
Lysmeral (purity: unknown)
Doses: 0, 25, 50, 100, 200 and 400 mg/kg bw/d</td><td colspan="2">Summarised together in the RAC Opinion and CLH report (CLH, 2017; ECHA 2019)</td></tr>
<tr><td rowspan="3"><u>from 50 mg/kg bw/d:</u>
Clinical signs
Initial body weight loss/decreased body weight
Macroscopic liver changes
<u>from 100 mg/kg bw/d:</u>
Decreased kidney weight</td><td rowspan="3"><u>from 50 mg/kg bw/d:</u>
Degeneration and loss of seminiferous/ testicular tubule epithelium
Minimal to marked atrophy of testes
Degenerated germ cells
Decreased sperm counts
<u>from 100 mg/kg bw/d:</u>
Decreased testes weight
Decreased prostate and seminal vesicles size</td></tr>
<tr><td>5-day toxicity screening test
(Givaudan, 1991A)
Non-guideline, GLP
5 male rats/group
Oral administration (gavage), 5 days
Lysmeral (purity: 99.1%)
Doses: 0, 25, 50 and 100 mg/kg bw/d</td></tr>
<tr><td>5-day toxicity screening test
(Newberne, 1990A)</td></tr>
</table>

Non-guideline, GLP status unknown 8 male rats/group Oral administration (gavage), 5 days Lysmeral (purity: unknown) Doses: 0, 50, 100, 200 and 400 mg/kg bw/d		
90-day RDT study **(Givaudan, 1986A)** OECD TG 408 with deviations, GLP 14 rats/sex/group, one additional satellite top-dose group of 14 rats/sex/dose with a 4-week recovery period Oral administration (gavage), 5 days/week Lysmeral (purity: 97.8%) Doses: 0, 2, 5, 25 and 50 mg/kg bw/d Effects on females not reported due to lack of reported findings in the female reproductive organs	**Males:** <u>From 25 mg/kg bw/d:</u> Increased absolute (+24–45%) and relative (+21–45%) liver weight, reversible in recovery decreased plasma cholinesterase activity (-30–70%) and plasma cholesterol levels (-40–70%) from 25 mg/kg bw/day <u>At 50 mg/kg bw/d:</u> hepatic lipid droplet content, reversible in recovery slight increase in AST, reversible in recovery	<u>At 50 mg/kg bw/d:</u> Spermatoceles in the epididymides (67%), deterioration upon recovery (79%) Testicular atrophy Histopathological findings in the testes and epididymides in 14/14 males vs. 2/14 in controls, reversible in recovery: • Disturbances of spermatogenesis (29% of organs affected vs. 11% in controls) • Disturbed spermiogenesis (21% of organs affected vs 0% in controls) • Increased Sertoli cell-only tubules (29% of organs affected vs. 11% in controls) • Increased surface density in Leydig cells (21% of organs affected vs. 11% in controls) • Spermatoceles in the epididymides (67% of organs affected vs. 0% in controls) Evaluated in epididymides without spermatocele, reversible in recovery: • Decreased density of spermatozoa (80% of organs affected vs. 11% in controls) • Nucleated cells (100% of organs affected vs. 11% in controls)
24- or 52-day RDT study **(Study report, 1987)** **Not assessed in the RAC Opinion (2019) on Lysmeral**		<u>From 24 days:</u> Decreased absolute testes weight (24 days: -25%; 52 days: -40%) Decreased absolute seminal vesicles weight (24 days: -12%; 52 days: -9%)

Non-guideline, non-GLP 20 Fuellingsdorft albino rats/sex/group Oral administration (gavage), exposure for 24 or 52 days Lysmeral (purity: 97.4%) Doses: 0 or 50 mg/kg bw/d Limited information available		At 52 days: Decreased absolute prostate weight (-12%)
Oral studies in dogs		
90-day RDT study (Givaudan, 1990B) comparable to OECD TG 409, GLP 3 dogs/sex/group Oral administration (gelatine capsule), 90 days Lysmeral (purity: 97.6%) Doses: 0, 4.4, 22.3 and 44.6 mg/kg bw/d	From 22.3 mg/kg bw/d: Occasional diarrhoea At 44.6 mg/kg bw/d: Vomiting	No effects
9-week pilot RDT study (Givaudan, 1990A) Non-guideline, GLP status unknown 2 male Beagle dogs Oral administration (gelatine capsule), 9 weeks Lysmeral (purity: 95%) Increasing doses of 47–564 mg/kg bw/d	Occasional vomiting (2/2) and diarrhoea (1/2) Decreased body weight Increased glutamate dehydrogenase and ALT Multifocal liver inflammation (2/2)	Mild atrophy in seminiferous tubules (necrosis of germ cells, multinucleated giant cells in tubular lumen) (2/2)
14-day screening RDT study (BASF SE, 2008A) Non-guideline, GLP	From 200 mg/kg bw/d: Vomitus, diarrhoea, decreased body weight gain and food efficiency in all animals	At 200 mg/kg bw/d: 1/10 dogs: massive diffuse degeneration of seminiferous tubules, hyperplasia of Leydig cells in the testes, aspermia and epithelial

10 male Beagle dogs/group Oral administration (gelatine capsule), 14 days Lysmeral (purity: 99.1%) Doses: 0, 20, 200 and 1000 mg/kg bw/d decreased to 500 mg/kg bw/d due to vomitus and diarrhoea	Body weight loss in some animals Increased absolute and relative liver weight (+30–40%) and centrilobular hypertrophy of hepatocytes Prolongation of APPT, increased serum magnesium, potassium and inorganic phosphate Decreased AST and ALT <u>At 500/1000 mg/kg bw/d:</u> Decreased glucose levels	vacuolation in the epididymides, decreased testes and epididymides size 1/10 dogs: slight, one-sided and focal degeneration of seminiferous tubules – also observed in HCD and potentially incidental <u>At 20 and 500/1000 mg/kg bw/d:</u> Decreased prostate size — not considered treatment-related due to lack of dose-response and histopathological findings
14-day screening RDT study (BASF SE, 2008B) Non-guideline, GLP 10 male Beagle dogs/group Oral administration (gelatine capsule), 14 days Lysmeral (purity: 99.1%) Doses: 0 or 200 mg/kg bw/d	Vomitus (7/10) and diarrhoea (4/10) Mean body weight loss (-0.2 kg vs. +0.1 kg in controls) – mainly due to 2/10 dogs with massive body weight loss Decreased food efficiency (up to -25%) from day 3 Increased absolute (+14%) and relative (+17%) liver weight Centrilobular hepatocyte hypertrophy Increased ALT (+80%), AST (+310%), APPT (+10%) and decreased serum triglycerides (-35%) Decreased RBC count (-5%), Hb (-5%), Ht (-10%), reticulocyte count (-60%) Increased serum urea (+45%), creatinine (+25%), calcium (+5%), magnesium (+20%)	Decreased absolute and relative testes weight (approx. -25% for both) and prostate weight (slight) Slight to severe degeneration of seminiferous tubules (9/10 dogs) Unilateral decrease in testicular length or width of ≥ 3 mm (6/10 dogs) Decreased progressively motile spermatozoa and/or morphological alterations (9/10 dogs) Increased spermatozoa with damaged plasma membrane (3/10 dogs) Morphological sperm alterations consisted mainly of mid-piece anomalies (cytoplasmatic droplets) and less frequently sperm neck anomalies (paraxial tail attachment, cytoplasmatic droplets) Minimal to moderate multifocal prostate atrophies (3/10 dogs)
Oral studies in mice		

Time course study **(BASF SE, 2006B)** Non-guideline, non-GLP 5 male C57BL/6NCrl mice/time point Time points: 1, 2, 3, 4 or 14 days Oral administration (gavage) Lysmeral (purity: 99.1%) or Lysmerylic acid Doses: 50 mg/kg bw/d	Lysmerylic acid: Non-significant decreases in body weight gain (-33%) at day 14	No effects upon macroscopic or microscopic examinations of the testes Lysmeral: Decreased ratio of normal to abnormal sperm only at the 3- and 4-day timepoints No effects on other sperm parameters (sperm motility, spermatid count in testes or cauda epididymis) Lysmerylic acid: Significantly decreased ratio of normal to abnormal sperm at 1-day timepoint Decreased total sperm numbers in cauda epididymis at 2-day timepoint
Oral studies in guinea pigs		
5-day toxicity screening test **(Givaudan, 1983)** Non-guideline, non-GLP 5 male guinea pigs/group Oral administration (gavage), 5 days Lysmeral (purity: unknown) Doses: 0 or 100 mg/kg bw/d	No effects	No effects
Oral studies in monkeys		
5-day toxicity screening test **(Givaudan, 1984G)** Non-guideline, non-GLP 2 male Rhesus monkeys/group Oral administration (dietary), 5 days	No effects	No effects

Lysmeral (purity: unknown) Doses: 0 or 100 mg/kg bw/d		
Oral studies in rabbits		
15-day RDT study (BASF SE, 2008C) Non-guideline, non-GLP 5 male rabbits/group Oral administration (gavage), 15 days Lysmeral (purity: 99.1%) Doses: 0, 30, 100, 300 mg/kg bw/d	No clinical signs or effects on body weight and food consumption	No effects on the testes or cauda epididymis, or sperm parameters 1/5 males at 30 mg/kg bw/d: moderate diffuse degeneration of the seminiferous tubules, moderate oligospermia, moderate mixed inflammation in the epididymides 1/5 males at 100 mg/kg bw/d: decreased testes and epididymides size, severe diffuse degeneration of seminiferous tubules in the examined left testis, severe atrophy and aspermia in the left epididymides The above isolated histopathological findings were not considered treatment-related due to a lack of dose response
Dermal studies in rats		
5-day dermal toxicity screening test (Givaudan, 1991A) Non-guideline, GLP 5 male rats/group Dermal administration, 6 h/d for 5 days Lysmeral (purity: 99.1%) Doses: 0, 250, 500, 1000 and 2000 mg/kg bw/d	<u>At 2000 mg/kg bw/d:</u> Very slight decrease in body weights (-2%) No clinical signs No relevant findings upon necropsy	<u>At 2000 mg/kg bw/d:</u> Marked testicular atrophy Marked disorganization of the epithelial structure of the seminiferous tubules with Decreased number of germ cells and increased number of degenerating germ cells (inclusive giant cells) (5/5 males) Slight to moderate immature/degenerating germ cells (5/5 males) and spermatocele (1/5 males) in the epididymides
In vitro studies		
Oestrogenic activity of benzyl salicylate, benzyl benzoate and butylphenylmethylpropional (Lilial/lysmeral) in oestrogen-responsive MCF7 human breast cancer cells using human recombinant ER	N/A	Lysmeral inhibited the binding of [^{3}H]oestradiol to oestrogen receptors in MCF7 cells up to a maximal inhibition of 47%. It also induced CAT gene expression, increased the growth rate of MCF7 cells and increased the expression of the oestrogen-regulated

alpha and ER beta **(Charles and Darbre, 2009)** **Not assessed in the RAC Opinion (2019) on Lysmeral** Non-guideline, non-GLP Lysmeral (purity: 95%)		gene pS2 mRNA following a period of oestrogen deprivation, but not to the same extent as 17β-oestradiol.

4-tert-butylbenzoic acid

Ten studies were available with 4-*tert*-butylbenzoic acid (TBBA), which included five *in vivo* studies conducted in rats via different routes of exposure and one human study, summarised in Table 4, and four *ex vivo* studies. All studies except for the *ex vivo* studies had been previously assessed in the CLH report and RAC Opinion on TBBA (CLH, 2010; ECHA, 2011).

Table 4. Available studies with TBBA

Study protocol	General Toxicity	Effects on reproduction
Oral studies in rats		
Mating trial focused on male fertility (Hoechst, 1987) Non-guideline, GLP status: unknown 10 male Wistar rats/group Oral administration (dietary) 1st trial: exposure for 70 days prior to mating, then each male was mated with 2 untreated females 2nd trial (recovery group): males that were not fertile in the 1st trial (successful impregnation of at least 1/2 females) were remated following a 70-day period without dietary exposure to TBBA TBBA (purity: unknown) Doses: 0, 20, 100, or 500 ppm a.i doses: 0, 1.6, 7.9 and 41 mg/kg bw/d	Decreased body weight gain and body weight (-14%) at 500 ppm, reversible upon recovery No effects on organ weights upon necropsy	At 1st trial: 1/10 males at 100 ppm did not prove their fertility — 2/20 females: sperm positive but not pregnant; 2/20 females: sperm negative 10/10 males at 500 ppm did not prove their fertility — 3/20 females: sperm positive but not pregnant (sired by 3/10 males); 17/20 females: sperm negative 9, 8 and 7 males at 0, 20 and 100 ppm, respectively, impregnated both females At 2nd trial: All remaining males at 100 ppm (1/10) and 500 ppm (10/10) proved their fertility — 8/10 top-dose males and the one male at 100 ppm impregnated both females. At necropsy: Decreased absolute testes weight upon recovery at 500 ppm (-12% in comparison to controls) Minor lesions at the germinative epithelium, confined to few tubules only, at 500 ppm. No histopathological changes on the prostate, seminal vesicles, epididymides or sperm.
90-day RDT study (Hunter et al., 1965) Non-guideline, non-GLP 10 Carworth Farm rats/sex/group Oral administration (dietary), 90 days TBBA (purity: unknown)	Absolute and relative weight impairment of the liver and the kidney Renal tubular and papillary necrosis from 100 ppm Statistically significantly decreased body weight from 316 ppm	Testes atrophy caused by destruction of the epithelium of the seminiferous tubules (no detailed data provided) from 100 ppm Statistically significantly decreased absolute and relative testes weight at 316 ppm (abs: -23%) and at 1000 ppm (abs: -65%)

Doses: 0, 100, 316, 1000, 3160 and 10000 ppm a.i. doses: Males: 0, 6, 21 and 75 mg/kg bw/d Females: 0, 8, 27 and 89 mg/kg bw/d (up to 1000 ppm, as the top two doses levels resulted in mortality and excessive toxicity)		
Dermal studies in rats		
7- or 13-week RDT dermal study (Cagen et al., 1989) Non-guideline, non-GLP 7-week administration: 7 Fischer 344 rats/sex/group 13-week administration: 13 Fischer 344 rats/sex/group TBBA and diethanolamine salt prepared in deionized water Dermal application (topical on clipped skin) for 5 days per week Doses: 0, 17.5, 35, 70 and 140 mg/kg bw/d	No clinical signs or mortalities Decreased body weight (-10%) and body weight gain in males at 140 mg/kg bw/d Absolute and relative organ weight impairment of the liver and the kidney from 17.5 mg/kg bw/d in both exposure groups Microscopic lesions in the liver and kidneys	Statistically significant decreased absolute and relative testes at 70 mg/kg bw/d in both exposure groups <u>7 weeks:</u> 70 mg/kg bw/d: absolute: left: -41%; right: -43%; relative: -38% 140 mg/kg bw/d: absolute: left: -67%; right: -58%; relative: -59% <u>13 weeks:</u> 70 mg/kg bw/d: absolute: left: -45%; right: -45%; relative: -41% 140 mg/kg bw/d: absolute: left: -60%; right: -57%; relative: -50% Decreased sperm head counts and LDH-X enzyme activity from 70 mg/kg bw/d in both exposure groups Testicular lesions: moderate to severe diffuse seminiferous tubular degeneration, with most affected tubules containing spermatogonia, primary and secondary spermatocytes, early spermatids and Sertoli cells, but being devoid of late spermatids, and few seminiferous tubules containing only Sertoli cells and a few spermatogonia. Degenerative tubules and epididymal tubular lumina also contained numerous testicular giant cells in both exposure groups.
28-day RDT dermal study (Shell, 1975) Non-guideline, non-GLP 8 male Carworth Farm E rats/group Dermal administration (topical on shaved skin) TBBA (purity: unknown) in DMSO	Decreased body weight gain and body weights from 30 mg/kg bw/d	Decreased relative and absolute testes weights and degeneration of germinal epithelium at 60 mg/kg bw/d

Doses: 0, 7.5, 15, 30 and 60 mg/kg bw/d		
Inhalation studies in rats		
Acute inhalation study (Shell, 1982a) Non-guideline, non-GLP 6 male Fischer 344 rats/group Inhalation (dust) (no data on MMAD), 4 hours, observation period: 14 days TBBA (purity: unknown) Doses: 0, 495, 668, 958, 1802 mg/m^3 Controls were exposed to air only, not dust	No information on general toxicity	Statistically significant decreases in mean testis weight from 495 mg/m^3 (-47%, -46% and -54% in all exposed rats in comparison to the control (data missing for one dose level)) Decreased mean testicular sperm count (-85%, -84%, -91% and -99% at 495, 668, 958, 1802 mg/m^3, respectively) Absence of late spermatids in the seminiferous tubules at 495 mg/m^3 All stages of differentiating spermatids were absent at 1802 mg/m^3 Tubules containing Sertoli cells only and tubules with multinucleated giant cells were also prevalent (no data on dose level)
4-day RDT inhalation study (Shell, 1982b) Non-guideline, non-GLP 8 male Fischer 344 rats/group Inhalation (in air) (no data on MMAD), 6h/d for 4 days, then 3 days recovery followed by 3 more days of exposure, no observation period TBBA (purity: unknown) Doses: 0, 12.5, 106 and 525 mg/m^3	Mortalities: 2/8 males at 106 mg/m^3 7/8 males at 525 mg/m^3	Decreased testis weight in surviving males from 106 mg/m^3 Decreased testicular sperm counts from 12.5 mg/m^3 (-21%, -61% and -96% at 12.5, 106 and 525 mg/m^3, respectively) Absence of late spermatids, presence of multinucleated giant cells, and reduction in spermatogenic cell types in surviving animals from 106 mg/m^3
Human studies		
Human observational study (Whorton et al., 1981) Non-GLP	N/A	33/90 men underwent a vasectomy and did not contribute to semen examinations 51/57 remaining men provided at least 1 semen sample and 39/57 provided 2 semen samples

90 exposed male workers, within 5 different jobs, 103 unexposed males who did not work at the facility without previous exposure to testicular toxicants Control group was subsequently expanded to a total of 335 individuals, including control men from similar studies Occupational exposure of TBBA		Semen samples from exposed mean had a mean count of 72 mil sperm/ml sperm vs. 78 mil in controls 8/51 exposed individuals (15.7%) had a sperm count of less than 20 mil sperm/ml sperm vs. 7/103 individuals in the initial control group. When the control group was expanded, 25 individuals in total (7.5%) had such sperm counts. The difference was statistically significant or non-significant depending on the analysis method used. Oligospermia in exposed individuals was influenced by multiple other factors, such as orchitis after mumps, testicular hernias, sclerosis of the penis, as evident by urological-clinical data. There was no such information available for the control group. It was concluded that based on the small sample size of exposed individual and confounding factors, the increased incidence of oligospermia in exposed workers was of questionable significance.

Four *ex vivo* studies were available, one of which was submitted by Industry during the public consultation.

The first study (Study report, 2019a) was conducted using primary seminiferous tubules from 9 male juvenile SD rats, which were cultured according to Bio-AlteR® technology. The study was not conducted according to a test guideline or GLP. Two structural isomers of TBBA were used in this study, p-TBBA and meta-3-*tert*-butylbenzoic acid (m-TBBA). Methoxyacetic acid (MAA) was used as positive control. Cultures were initially exposed to the test substances, vehicle or positive control from day 2, and every 2 ± 1 days onwards. On days 7 and 14, cells were detached, trypsinised and pooled, and an aliquot was used for flow cytometric analysis and to determine cell numbers and viability. On days 8 and 15, cells were detached and pooled for CoA-TBBA conjugate determination (3 replicates per treatment condition). Samples for metabolome analyses were also obtained on the same days. The integrity of the blood-testis barrier was determined by trans-epithelial electrical resistance (TEER) measurements before treatment (day 2) and after treatment (days 5 to 21).

The positive control acted as expected by strongly decreasing the number of studied germ cell population, lowering TEER on all assessed days and increasing the number of somatic cells, validating the sensitivity and responsiveness of the cell cultures. Treatment with p-TBBA only caused a slight and transient decrease in TEER measurements, indicating a low effect on the blood-testis barrier. It did not have any effect on viability and caused a dose-dependent increase in the number of somatic cells on day 7 but not day 14. It also caused an increase in the number of spermatogonia on both days. It did, however, have an adverse effect on the meiotic process of germ cells, leading to: dose-dependent decreases in the number of middle to late pachytene spermatocytes, secondary spermatocytes and round spermatids on day 7; decreases in the number of middle to late pachytene spermatocytes at the top concentration only; decreases in the number of secondary spermatocytes at the middle and top concentrations; and decreases in the number of round spermatids at all 3 tested concentrations. Conjugates of p-TBBA and CoA were detected at all concentration levels on both days, with trace amounts around the detection limit at the low concentration. Treatment with m-TBBA had similar effects on the blood-testis barrier and viability. In contrast, it did not have an effect on the number of somatic cells and it only had a slight effect on the meiotic process of the germ cells. On day 7, it led to decreases in the number of middle to late pachytene spermatocytes and of secondary spermatocytes at the low and middle concentration and in the number of round spermatids at the low concentration only. On day 14, it led to a decrease in the number of round spermatids at the top dose, and to an increase in the number of middle to late pachytene spermatocytes in all 3 tested concentrations without dose-dependency. Conjugates of m-TBBA and CoA were not detected at any concentration level. Additionally, metabolome analysis showed that samples treated with m-TBBA were in close proximity to the control on both days, while treatment with p-TBBA led to a clear and

concentration-dependent separation from control clusters at both timepoints with lower levels of several lipids and some amino acids (glutamate, glutamine, 5-oxoproline).

The protocol of the second *ex vivo* study (Study report, 2020) was similar to the 2019a study. The only notable differences were that primary seminiferous tubules were derived from 3 instead of 9 rats, the number of cells, viability determinations and flow cytometry analyses were performed on days 8 and 15, and there was no determination of CoA-TBBA conjugation or metabolome analyses.

The positive control (MAA) acted as expected by decreasing the number of studied germ cell population and increasing the number of somatic cells, validating the sensitivity and responsiveness of the cell cultures. Neither p-TBBA or m-TBBA had an effect on cell viability. Treatment with p-TBBA led to increases in the number of somatic cells (dose-dependent) and in the number of spermatogonia (middle and top concentrations only) on day 8 only. It also had an adverse effect on the meiotic process of germ cells. On day 8 decreases were noted in the number of middle to late pachytene spermatocytes at the top concentration and in the number of secondary spermatocytes and round spermatids at all 3 tested concentrations with dose-dependency. On day 15, dose-dependent decreases were noted in the number of the same cells at all 3 tested concentrations. Treatment with m-TBBA only slightly increased the number of somatic cells at the 3 tested concentrations on day 8. The significance of its effect on the meiotic process of germ cells was also questionable. On day 8, there were increases in the number of spermatogonia, young and middle to late pachytene spermatocytes and secondary spermatocytes. On day 15, the only decrease noted was in the number of middle to late pachytene spermatocytes and of secondary spermatocytes at the low concentration, while increases in the number of these cell types were noted at the top concentration.

The protocol of the third *ex vivo* study (Study report, 2019b) was similar to the other studies. The most notable difference was that this study was conducted with primary seminiferous tubules derived from the pooled testes of a young male human, who had undergone castration and previous treatment with an antiandrogenic compound. The individual's treatment had been replaced by Finasteride (inhibitor of 5-alpha-reductase) and Provames (oestradiol) several months prior. Additionally, the number of cells, viability determinations and flow cytometry analyses were performed on days 14 and 21, and CoA-TBBA conjugate determination was performed on days 15 and 22. There was no metabolome analysis in this study.

The positive control decreased the number of somatic cells on day 14 only and decreased the number of spermatogonia and pachytene spermatocytes on day 21 only. However, the numbers of secondary spermatocytes and round spermatids were increased on day 14 and days 14 and 21, respectively. It was noted that the presence of cellular debris may have distorted the flow cytometry analysis resulting in an artificial increase in the number

of round spermatocytes. Based on these findings, it was considered that the positive control demonstrated an effect only on the early stages of spermatogenesis only.

Treatment with p-TBBA had no effect on cell viability. Decreases in the number of somatic cells were noted at the middle and top concentrations on day 14, but without dose-dependency and with no effects on the number of somatic cells on day 21. Treatment with p-TBBA resulted in an increase in the total number of germ cells and the number of spermatogonia on D14 and D21 at the top concentration. There were no clear dose-related effects on the number of pachytene spermatocytes, secondary spermatocytes and round spermatids. Treatment with m-TBBA did not affect cell viability. The number of somatic cells was slightly decreased from the middle concentration on day 14, and a slight decrease was also noted on day 21 at the top concentration with dose-dependency. The number of germ cells and spermatogonia was also not affected by treatment, while a slight decrease in pachytene spermatocytes was only observed on day 21. At the same timepoint, a dose-dependent increase was reported in the number of secondary spermatocytes, while the number of round spermatids was not clearly affected. Similarly to the positive control, it was noted that the presence of cellular debris may have influenced the flow cytometry analysis for both p-TBBA and m-TBBA.

p-TBBA-CoA conjugates were only detected in trace amounts around the detection limit in 2/6 replicates at the top concentration at the 22-day timepoint. Following treatment with m-TBBA, no relevant conjugates were detected in any of the replicates at any timepoint. Negative and positive control samples were also negative for any relevant conjugates.

The fourth *in vivo* study was submitted by Industry during the EU public consultation process. This study investigated the effect on TBBA on rat 3D seminiferous tubule cultures with a focus on the inhibition of late-stage ex-vivo spermatogenesis by p-TBBA (Hareng *et al*., 2023). For this purpose, ex-vivo 3D cells cultures of seminiferous tubules derived by SD rats were exposed to p-TBBA and m-TBBA. Cells were harvested after 1 (experiment 1) or 2 weeks (experiment 2). The study protocol was similar to the other ex-vivo studies. MAA and 1,3-dinitrobenzene were used as positive controls and DMSO was used the vehicle control.

Treatment with positive controls resulted in expected decreases in TEER measurements. Treatment with p-TBBA or m-TBBA did not have an effect on the blood-testis barrier. Treatment with positive controls resulted in marked decreases in the percentage of total germ cells in both experiments, as expected. Treatment with p-TBBA led to dose-dependent decreases in the percentage of mid/late pachytene spermatocytes (statistically significant), secondary spermatocytes and round spermatids (statistically significant) in both experiments, while treatment with m-TBBA did not have an effect on spermatocyte populations. Treatment with TBBA also led to dose- and time-dependent increases in the levels of TBBA-CoA conjugates, which was not observed with m-TBBA or positive controls. Treatment with p-TBBA also led to changes in the level of metabolites associated

with lipid and energy metabolism, which was not apparent upon treatment with m-TBBA. Haren et al. (2023) also referenced in vivo studies with m-TBBA, where there was no testicular toxicity; however, RAC (and the Agency) did not have access to these studies and were therefore unable to assess them.

Methyl 4-tert-butylbenzoate

There are no available studies with methyl 4-*tert*-butylbenzoate.

Summary, mode of action and conclusions

A summary of all relevant effects on sexual function and fertility was provided by RAC:

Text copied from pages 29-31 of the RAC opinion:

The majority of studies were carried out in **rats** which consistently demonstrated adverse effects on male sexual function and fertility for all tested members of the bourgeonal group. The effects on male reproductive organs included **small testis** (14-d study, bourgeonal, Study report, 2019) and **decrease in testis weight** in several studies (14-d study, bourgeonal, Study report, 2019; two 5-d studies, 4-tert-butyltoluene, Study report, 1982a and 1982b; two 5-d studies, 4-tert butylbenzaldehyde, Study report, Study report, 1981 and TSCATS study, 1982; mating trial, TBBA, Hoechst, 1987; 90-d study, TBBA, Hunter et al., 1965), both, **testis and epididymis weight**, were reduced in two studies (OECD TG 421, 4-tert-butyltoluene, Study report, 2007a; OECD TG 407, 4-tert-butyltoluene, Study report, 2007b). In studies with lysmeral, weight of testis, epididymis, seminal vesicle and prostate was reduced (12-week study, lysmeral, BASF SE, 2006C; 8-week study, lysmeral, BASF SE, 2017B) and in the 24-/52-d study with lysmeral (Study report, 1987) **weight of testis, seminal vesicle and prostate** was reduced after 24 and 52 days.

Degeneration and atrophy of seminiferous tubules was observed in several studies (14 day study, bourgeonal, Study report, 2019; 5 day, bourgeonal, Study report, 2009; two 5 day studies, 4-tert-butyltoluene, Study report, 1982a and 1982b; two 5 day studies, 4-tert butylbenzaldehyde, Study report, Study report, 1981 and TSCATS study, 1982; 28-d study, 4 tert-butyl toluene, Study report, 2007b; OECD TG 421, 4-tert-butyltoluene, Study report, 2007a; 1-14-d study, Lysmeral, BASF 2006a; three 5-d studies with lysmeral (5-d study, lysmeral, Givaudan, 1986b; 5-d study, lysmeral, Givaudan, 1991a; 5-d study, lysmeral, Newberne, 1990a); 90-d study, TBBA, Hunter et al., 1965), in the OECD TG 421 study with 4-tert-butyltoluene (Study report, 2007a) **atrophy was also seen in testis, epididymis, seminal vesicle and prostate**. Sertoli cell vacuolation and Leydig cell hyperplasia was observed in several studies. Presence of luminal cell debris in seminiferous tubules and cribriform change in epididymis was described for bourgeonal (14-d, DRF for OECD TG 422, Study report, 2019). For most of these effects a dose- and time-dependent increase in incidence and severity could be observed.

Sperm parameters were also affected in several studies and included lower sperm numbers, reduced sperm motility and changes in sperm morphology (14-d study, bourgeonal, Study report, 2019; 5 day, bourgeonal, Study report, 2009; 28-d study; OECD TG 421, 4-tert-butyltoluene, Study report, 2007a; OECD TG 407, 4-tert-butyltoluene, Study report, 2007b, 5-d study, 4-tert butylbenzaldehyde, TSCATS study, 1982; 12-week study, lysmeral, BASF SE, 2006C; 8-week study, lysmeral, BASF SE, 2017B; OECD TG 408, lysmeral, Givaudan, 1986a) and in several studies depletion of germinal epithelium was observed (two 5-d studies, 4-tert-butyltoluene, Study report, 1982a and b; 5-d study, 4-tert-butylbenzaldhyde, Study report, 1981; 5-d study, 4-tert-butylbenzaldhyde, TSCATS study, 1982; mating trial, TBBA, Hoechst, 1987). These effects mostly showed a dose-dependent increase in incidence and severity.

Fertility was affected in the OECD TG 421 study with 4-tert-butyltoluene (OECD TG 421, 4-tert butyltoluene, Study report, 2007a). One pair did not copulate and no pregnancies was obtained in the top dose group. Fertility index was also lower in the next lower dose group.

No effects on reproductive function or fertility or general toxicity were seen in the OECD TG 422 study with bourgeonal (Study report, 2019), but dosing in this study is not considered adequate (i.e. doses too low).

Fertility of lysmeral was investigated in several studies and reduced fertility was seen in two DRF studies for a one generation study (BASF SE, 2006c and BASF SE, 2017B). No effects were seen in the OECD TG 443 study (OECD TG 443, modif., lysmeral, BASF SE, 2027B), which can be explained by the low doses applied.

TBBA was tested in a mating trial where males were exposed to different concentrations of TBBA for 70 days prior mating. At the top dose no pregnancies were obtained when these treated males were mated with untreated females. The effect was reversible after 70 days, when a second mating trial was conducted (Hoechst, 1987).

In some studies **female reproductive organs** were also affected, including change in ovary and uterus weights at mid- and top dose and uterus atrophy at top dose (bourgeonal, 14-d study, Study report, 2019), ovary weights were also reduced in an OECD TG 407 study with 4-tert butyltoluene (Study report, 2007b). No other relevant findings were observed in females.

For TBBA and lysmeral there are also rat studies via **dermal** route and for TBBA also for the inhalation route. Results from these studies also demonstrated comparable effects as for the oral route, including reduced testis weight, reduced numbers of sperm/spermatids and depletion of germinal epithelium.

Some members of the bourgeonal group were also tested in other species. Short term studies investigating lysmeral exposure in **dogs** revealed clear effects in two of three studies. In a 14-d study (BASF SE, 2008a) reduced prostate weight was seen at 40 mg/kg

bw/d (though not at higher doses) and at 200 mg/kg bw/d reduced testis / epididymis size, reduced testis weight, degeneration of seminiferous tubules, slight hyperplasia of Leydig cells, aspermia and epithelial vacuolation in epididymis were observed, which were not reported in the top dose (top dose was reduced from 1000 to 500 mg/kg bw/d due to excessive toxicity). In a second 14-d study (BASF SE, 2008b) effects included reduced testis and prostate (slight) weight, reduced size of testis (6/10), slight to severe degeneration of seminiferous tubules (9/10) minimal to moderate multi focal prostate atrophy 3/10), reduced progressive motility of spermatozoa (8/10), spermatozoa with damaged plasma membrane (3/10) and increase in morphologically altered spermatozoa (9/10) at the single dose of 100 mg/kg bw/d tested (not observed in controls). No effects were seen in the third 14-d study at lower concentrations (Givaudan, 1990b). There are two further 5-d toxicity studies available in dogs, one for 4-tert butyltoluene (Study report, 1984b) and the other for 4-tert butylbenzaldehyde (Study report, 1984f). At 100 mg/kg bw/d 4-tert butyltoluene (Study report, 1984b) resulted in a small quantity of seminiferous tubules with nearly total depopulation of germinal epithelium in both testes in 1/2 treated dogs (no effect in the single control animal) and treatment with 100 mg/kg bw/d 4-tert butylbenzaldehyde (Study report, 1984f) induced 60 cross sections of seminiferous tubules with nearly total depopulation of germinal epithelium in testis of one treated dog, no effects in the second treated and the single control animal.

No adverse effects on male reproductive organs or spermatotoxicity were observed in the single **rabbit** study (14-d, lysmeral, BASF SE, 2008c) or in the 14-d study in **mice** (1-14-d study, lysmeral, BASF SE, 2006b), though RAC is of the view that the doses tested in both studies were too low (in line with the RAC opinion on lysmeral from 2019, RAC, 2019). There were another three 5-d mouse studies available, two of which were positive (4-tert butyltoluene, Study report, 1984c: effects on testis weight, slight damage of germinal epithelium in testes: 1/6 control animals, 3/6 treated animals, very slight increase in injured testis tissue in treated animals compared to controls; 4-tert butylbenzaldehyde, Study report, 1984d: Slight damage of germinal 30 epithelium in testes: in 4/6 treated animals, 1/6 in controls), while one was negative (lysmeral, Givaudan, 1990b, single dose of 100 mg/kg bw/d).

Two of the three 5-d studies in **Guinea pigs** were positive (4-tert butyltoluene, Study report, 1984a and 4-tert-butylbenzaldehyde, Study report, 1984e: in both studies a dose of 100 mg/kg bw/d resulted in damage of germinal epithelium, which was more severe and involved more cross sections of seminiferous tubules compared to controls), while the study with lysmeral (Givaudan, 1984g, lysmeral at 100 mg/kg bw/d) was negative.

No effects were seen in a single study with **rhesus monkeys** (Givaudan, 1984g) using two males at the single dose of 100 mg/kg bw/d. A minor finding was decreased numbers of spermatozoa per tubule.

In most studies the above-described effects on sexual function and fertility were seen at doses without general toxicity or without marked toxicity. However, in some studies there

was considerable general toxicity at higher doses (including lethality, considerable weight loss, adverse effects on liver and kidneys).

End of RAC text

RAC additionally performed an in-depth analysis of the effects observed in the available studies with the bourgeonal group and the presence/absence of systemic toxicity at the dose levels where adverse effects were observed (see Table 4 of the RAC opinion; ECHA, 2025). They concluded that effects were generally observed in the absence of severe systemic toxicity. They additionally noted that in studies of longer exposure, adverse effects were observed at lower doses, while rats were evidently more sensitive than other species in relation to the observed adverse effects.

RAC generally considered that effects on the male reproductive system were evident following exposure to several members of the bourgeonal group, with the exception of methyl 4-*tert*-butylbenzoate, where read-across was supported based on *in silico* evidence. Overall, the observed effects on the male reproductive system were considered as clear evidence of adverse effects, which formed a comprehensive toxicological database of similar effects across the bourgeonal group that supported the proposed read-across, while effects on the female reproductive system were less consistent and were considered supportive for classification.

Comments by Industry during the EU public consultation disputed the relevance of the observed effects on the male reproductive system in rats to humans. In response, RAC performed a comprehensive analysis of the potential MoAs behind the observed toxicity, and additionally considered data on the structurally-similar cyclamal group.

The reproductive toxicity associated with the bourgeonal and cyclamal groups has been associated with the formation of their respective common metabolites, TBBA and 4-iPBA, which has been shown using *in vitro, in vivo* or *in silico* methods and is also supported by the structural similarity of the members of each group and their similar toxicological effects on the male reproductive system. For both groups, the rat was found to be the most susceptible species to these effects, while RAC noted that the presentation of milder effects in other species, such as the guinea pig, mouse or rhesus monkey, may be explained by lower sensitivity, the use of doses that were too low, or the assessment of effects in studies of shorter length. In any case, the presentation of effects in other species, even if mild, supported that the testes and sperm were sites of target toxicity for these groups. As the effects were not specific to the rat only, RAC concluded that human relevance could not be excluded.

Industry supported their argument that effects on the male reproductive system were rat-specific by proposing an MoA of accumulation of TBBA/4-iPBA-CoA conjugates and subsequent decreases in endogenous CoA conjugates that in their view was responsible for the observed adverse effects following exposure with members of the bourgeonal or cyclamal groups. The non-relevance to humans was supported based on *in vitro*

comparative metabolism studies where accumulation of these conjugates was demonstrated in rat hepatocytes, but not in human or rabbit hepatocytes. It was proposed that accumulation of these conjugates led to sequestration of endogenous CoA reserves and subsequent disruption of fatty acid synthesis or direct effects of these conjugates on fatty acid synthesis, leading to a lack of lipids during spermatogenesis.

An in-depth analysis of the MoA data can be found in the supplemental information section of the RAC opinion (ECHA, 2025), and a summary of RAC's analysis can be found below.

Overall, RAC considered that accumulation of 4-iPBA/TBBA-CoA was not consistently shown in the *in vitro* studies with rat hepatocytes, with increases and decreases reported in levels of 22 hours, while variability was also noted between the different but also the same batches of cells. Based on this, they considered that comparisons between rat and human hepatocytes were not meaningful. They also noted that the number of experiments with rabbit and human hepatocytes was low and therefore their reproducibility was questionable, while noting that incubation periods in the relevant studies may have been too short to assess CoA conjugate accumulation in hepatocytes from these species.

Despite the accumulation of p-TBBA-CoA conjugates in *ex vivo* studies, RAC considered that there was no effect on the blood-testis barrier in these studies and that germ cell parameters were not meaningfully impacted in comparison to the positive controls. In terms of effects on metabolites involved in lipid and energy metabolism, where changes were observed following treatment with p-TBBA but not with m-TBBA, RAC noted that these assessments were only conducted in rat cells, not in other species, and therefore did not allow for any conclusions to be drawn on the underlying MoA or its relevance to other species.

They also noted that *in vivo* formation of 4-iPBA-Coa conjugates in the liver and testes has only been demonstrated in the rat (Natsch *et al.,* 2021), and while TBBA has been detected in human urine, it is not possible to conclude on 4-iPBA-CoA/TBBA-CoA levels in testis or liver in species other than the rat.

They overall concluded that the available literature did not allow for firm conclusions regarding the MoA behind the testicular and sperm toxicity observed in the available studies, while the link between CoA conjugate accumulation and the observed adverse effects has not been demonstrated in the available *in vitro, ex vivo* and *in vivo* investigations. In any case, other potential MoAs were also discussed that could potentially be behind the observed adverse effects, such as endocrine disruption and oxidative stress, while RAC considered that the MoA industry proposed has not been conclusively demonstrated not to be relevant to humans.

Overall, RAC considered that based on the adverse effects observed in available studies with substances from the bourgeonal group, as summarised above, and the lack of conclusive evidence that these effects are not relevant to humans, the bourgeonal group warranted classification with Repr. 1B (H360F).

The DS additionally considered the application of specific concentration limits (SCLs) for adverse effects on sexual function and fertility, but concluded that this was not necessary based on calculated ED_{10} values for lysmeral and TBBA which indicated medium potency.

Additionally, the DS proposed that a note is added to the classification of the bourgeonal group substances to account for additive reproductive effects in mixtures resulting from the common metabolite TBBA. RAC agreed with the DS and considered the addition of the below note on additivity was supported by the formation of the common metabolite, TBBA, for all group members of the bourgeonal group.

* New note: The classification of mixtures as reproductive toxicant is necessary if the sum of the concentrations of individual substances covered by this entry, forming the same metabolite, in the mixture as placed on the market is equal to, or above, the applicable generic concentration limit for the assigned category, or a specific concentration limit given in this entry.

Effects on development

3-(4-tert-butylphenyl)propionaldehyde (bourgeonal)

There was only one study available.

The combined RDT study with reproduction/developmental toxicity screening test (Anonymous, 2019; GLP; OECD TG 422) has been described in detail in the sexual function and fertility section of this report. In summary, 10 Crl:CD(SD) rats/sex/dose were treated with 0, 0.5, 1 and 5 mg/kg bw/d bourgeonal (purity: 99%; vehicle: corn oil) from 14 days prior to mating until LD 21 for females. F1 pups were not directly exposed to bourgeonal.

The top-dose level was chosen based on the findings of a DRF study. However, there were no indications of systemic toxicity in either sex, and RAC considered that the MTD had not been reached in this study.

In terms of effects on development, there was a statistically significant decrease in the number of pups found dead between PND 1–3 at 0.5 and 1 mg/kg bw/d, which was caused by an increase in pup mortality in the control group. RAC noted that since there was no decrease in pup mortality at the top dose, it is likely that pup survival was decreased at the top dose; however, in the absence of historical control data (HCD), it was not possible to draw reliable conclusions. Other effects on F1 pups included a statistically significant decrease in mean body weight on PND 9 and 12 at the top dose (-11–12%), which was reported to be within the HCD range of the testing facility, and decreases in the mean concentration of serum T4 on LD 12 in both sexes from the low dose (males: -2%, -18% and -22% at 0.5, 1 and 5 mg/kg bw/d, respectively; females: -18%, -26% and -26% at 0.5, 1 and 5 mg/kg bw/d, respectively). Only the decreases in females at 1 and 5 mg/kg bw/d were statistically significant. One F1 pup/sex/litter was microscopically examined with

no effects reported in the thyroid or parathyroid glands. There were no effects on anogenital distance or nipple retention.

4-tert-butyltoluene

Two studies were available with 4-*tert*-butyltoluene.

The first study was a reproduction/developmental toxicity screening test (Study report, 2007a; GLP; OECD TG 421) and has been described in detail in the sexual function and fertility section of this report. In summary, 12 SD (Crj:CD(SD)IGS, SPF) rats/sex/dose, were treated with 0, 1.5, 5, 15 and 50 mg/kg bw/d p-*tert*-butyltoluene (purity: 96.94%) in corn oil via oral gavage from 14 days prior to mating until PND 3 for females.

Systemic toxicity indications in males included one top-dose male dying with poor clinical condition prior to death, limited clinical signs only in one other top-dose male and statistically significant decreases in body weight (at 15 mg/kg bw/d: est. -13% on day 49; at 50 mg/kg bw/d: est. -6% and -19% on days 4 and 49, respectively. In females, mortalities were more extensive (1/12 at 15 mg/kg bw/d; 6/12 animals at 50 mg/kg bw/d; poor clinical condition prior to death). Surviving top-dose females presented with clinical signs. Statistically significant decreases in body weight were noted during the premating period for top-dose females (est. -8%), and during gestation and lactation for females from 5 mg/kg bw/d (est. up to -13% at 5 mg/kg bw/d; est. up to -11% at 15 mg/kg bw/d). Terminal body weights were statistically significantly decreased from 5 mg/kg bw/d (est. -13%, -18% and -29% at 5, 15 and 50 mg/kg bw/d, respectively).

In terms of effects on development, one dam at 15 mg/kg bw/d suffered total litter loss on day 1, resulting in a decreased gestation index at this dose level (66.7% vs. 100% in the lower dose groups — no data on controls). There were no statistically significant differences in the number of pregnant corpora lutea, number of implantations and implantation index up to 15 mg/kg bw/d. However, there was a decrease in fertility at this dose level (8/12 non-pregnant females) and statistically significant decreases in the number of pups born (-26%). The number of live pups on LD 0 was also statistically significantly decreased (-58%) and there was also an increase in the mean number of stillborn pups (4.7 vs 0.1 in controls), which was not statistically significant. At this dose level, decreases were reported in the delivery index (82.7% vs 94.1% in controls), birth index (49.3% vs 93.6% in controls) and live birth index (63% vs 99.5% in controls). Pup viability was also decreased during the lactation period in the 15 mg/kg bw/d group, with decreases in the mean number of live pups on LD 4 (4.5 vs 14.3 in controls) and in the viability index on LD 4 (45% vs 98.8% in controls). Mean pup body weight was decreased on LD 0 and 4 at 5 mg/kg bw/d (LD 0: males: -11%, females: -8%; LD 4: males: -16%, females: -15%) and at 15 mg/kg bw/d (LD 0: -32% for both sexes; LD 4 (based on pups from one dam only): males: -13%, females: -10%). There were no treatment-related external abnormalities, clinical signs or findings upon necropsy in any of the groups.

Overall, RAC considered that in this study maternal toxicity was excessive at the top-dose level. Maternal toxicity was also evident at 15 mg/kg bw/d, based on one mortality and decreases in body weight during the gestation and lactation period and at termination. At this dose level, adverse effects on development were evident by increased pup mortality (decreased delivery, birth and live birth index) and decreases in pup body weight at birth and on LD 4 (up to -32%). At 5 mg/kg bw/d, maternal toxicity indications included decreases in body weight from the gestation period and at termination (-13%). Effects in pups at this dose level included decreases in pup body weight at birth (up to -11%) which further progressed by LD 4 (up to -16%). RAC considered that the decrease in pup viability and pup body weight at 15 mg/kg bw/d were evidence of an adverse effect on development, albeit in the presence of significant maternal toxicity, while at 5 mg/kg bw/d pups presented with decreases in body weight in the presence of mild maternal toxicity.

The second study was a non-guideline prenatal developmental toxicity (PNDT) study (Hass et al., 1996), which investigated long-lasting learning and memory impairments induced by prenatal exposure to 4-*tert*-butyltoluene in rats. The GLP status of the study was unspecified. Limited information was available on this study. Female Mol:Wistar rats (number: unspecified) were exposed to 0 or 0.12 mg/l (20 ppm) 4-*tert*-butyltoluene (purity: unknown; vehicle: unspecified) by inhalation (type: unspecified) for 6h/d between GD 7–20. Effects on the pups were monitored for 22 months after delivery. This study was assigned a Klimisch score of 4.

According to the study report, there was no maternal toxicity (no further data). There was no effect on pup viability. Pup body weight was decreased until LD 10 and there was a delay in the ontogeny of reflexes, even when the parameter was adjusted for body weight. At 3 months of age, treated female pups presented with non-statistically significant increases in latencies and swim length (p = 0.6%). Some indications of impaired memory were also observed three weeks later, but without statistical significance (p = 8.7%). These effects were not observed at 17 months of age, but increases in latencies and swim length indicating memory impairments recurred at the 22-month timepoint in the first 3 trials and after a 4-day break in testing. These effects were also not statistically significant (p = 5.5%). Overall, RAC considered that the information provided on the study design as insufficient to draw any firm conclusion for classification purposes.

4-tert-butylbenzaldehyde

There were no studies available on 4-*tert*-butylbenzaldehyde.

2-(4-tert-butylbenzyl)propionaldehyde (lysmeral)

There were four studies available on lysmeral, all of which had been previously assessed in the relevant CLH report and RAC Opinion (CLH, 2017; ECHA, 2019). Three of the studies have already been described in detail in the sexual function and fertility section in this report (see Table 3).

In summary, the first study was a DRF study for a one-generation study (BASF SE, 2006c; non-guideline; non-GLP). Ten F0 Wistar rats/sex/dose were treated with 0, 400, 800, 1700 and 3400 ppm lysmeral in sunflower oil microencapsulated in gelatine (a.i. purity: 30.7%) via the diet from 6 weeks before mating for a total of 12 weeks. F0 females sacrificed after PND 21. The dietary doses corresponded to 0, 14, 28, 62.6, 116.8 mg/kg bw/d in males, and 0, 10–15, 18.3–29.4, 62.7, 123.2 mg/kg bw/d in females.

Maternal toxicity indications included decreased body weight and body weight gain during/after premating from 800 ppm (-5–10% and -10–30% at 800 and 3400 ppm, respectively) and during gestation and lactation (approx. -10% for both periods) at 800 ppm. Decreased food consumption was noted during lactation (-20%) (no data on dose levels). There was also a decrease in choline esterase levels (-50–60%) and an increase in gGT (2–8-fold; without increase in liver weights) at all dose levels.

In this study, there was an increase in the rate of post-implantation loss from the low dose, but without dose dependency (5.1%, 16.2%, 11.1%, 100% at 0, 400, 800 and 1700 ppm, respectively). There were no implantations at the top-dose level. At 1700 ppm was 1/8 females was pregnant with one implantation, which was resorbed. The decreased fertility index at this dose level (13%) was associated with the adverse effects on the male reproductive organs (see Table 3). These effects resulted in no pups being born from 1700 ppm. Slight decreases in the mean number of pups were noted up to 800 ppm (9.4, 8.7, 7.9 at 0, 400 and 800 ppm, respectively). There were no stillborn pups or effects on pup survival between PND 0–4 and PND 4–21 up to 800 ppm. The only effects noted were decreases in pup body weight and body weight gain from 400 ppm (body weight: on PND 0: 400 ppm: -19%; 800 ppm: -22%; on PND 21: 400 ppm: -17%; 800 ppm: -21%; body weight gain: 400 ppm: -16%; 800 ppm: -21%).

RAC considered that maternal toxicity in this study was evident from the low-dose level in the form of clinical chemistry perturbations. They noted that the increased post-implantation loss at the low dose and the decreased pup body weights at birth and weaning at the same dose level were relevant for classification, due to lack of evidence that these effects were secondary to the observed maternal toxicity. They did however note the high variability in the post-implantation rate in this study and the lack of dose response. Additionally, they considered that there was also no evidence that effects observed at 800 ppm were secondary to maternal toxicity, while noting that maternal toxicity at this dose level was more pronounced in the form of body weight and body weight gain decreases in addition to clinical chemistry perturbations.

The second study was also a DRF study for a one-generation study (BASF SE, 2017B; non-guideline; GLP). Ten F0 Wistar rats/sex/dose were treated with 0, 230, 750 and 2300 ppm lysmeral in sunflower microencapsulated in alginate (a.i. purity: 17.7%) via the diet from 2 weeks before mating for 8 weeks. F0 females sacrificed after PND 21. Dietary

doses corresponded to 0, 2.3–2.8, 7.4–9.1 and 25.1–27.5 mg/kg bw/d in males, and 0, 3.3–3.7, 10.6–11.9 and 21–34.7 mg/kg bw/d in females.

Maternal toxicity in this study was evident from 750 ppm in the form of transient body weight decreases which recovered towards the end of the study, decreases in food consumption which were particularly pronounced during the lactation period for top-dose females, and clinical chemistry changes which were potentially indicative of liver toxicity. Decreases in body weight did not exceed 16% at the top-dose level and the degree of decrease at 750 ppm was not specified but described as less severe. However, at the top dose decreases in body weight gain were marked (> 30%).

Decreases in fertility were noted at the top-dose, which were correlated to statistically significantly decreases in mean implantation sites (4.5 vs 10.1–11.5 in controls and other treated groups) and increased post-implantation loss (16.7% vs 3.7–3.9% in controls and other treated groups). This resulted in a statistically significantly decrease in the mean number of pups delivered (4.0 vs 9.7–11.3 in controls and other treated groups) and in the number of litters (4 vs. 9–10 in controls and other treated groups) at this dose group, and a secondary effect on the sex ratio. There were no stillborn pups at any dose level. However, pup survival was decreased between PND 0–4 from 750 ppm (95%, 99%, 86% and 75% at 0, 230, 750 and 2300 ppm, respectively). This effect did not persist between PND 4–21. Decreases were also noted in mean pup body weight at birth (750 ppm: -17%; 2300 ppm: -18%) and at weaning (750 ppm: -13–21%; 2300 ppm: -30–32%) and in pup body weight gain (750 ppm: -13%; 2300 ppm: -33%) from 750 ppm.

RAC considered that the decreases in pup survival and in pup body weight at 750 ppm were relevant for classification due to lack of evidence that these effects were secondary to the observed maternal toxicity.

In the modified EOGRTS (BASF SE, 2017; OECD TG 443; GLP), 35–40 F0 Wistar rats/sex/dose were treated with 0, 75, 230 and 750 ppm encapsulated lysmeral (a.i. purity: 17.7%) via the diet from 13 days before mating. Dietary doses corresponded to achieved a.i. doses of 0, 1.4, 4.5 and 15.1 mg/kg bw/d. F1 pups were assigned to 7 cohorts, including reproductive toxicity, neurotoxicity, immunotoxicity and cholinesterase cohorts, and continued in the same fashion as the F0 parental animals. The F1B cohort was mated to produce the F2 generation, and the study was concluded with the termination of the F1B cohort and their F2 pups at weaning.

In F0 females, maternal toxicity indications included decreased body weight during gestation (GD 20: ss -5%) and the first two weeks into lactation (LD 14: ss -4%), decreased body weight gain during premating (-14%) and gestation (ss -12%) and decreased food consumption during lactation (-5%) at 15.1 mg/kg bw/d. Liver toxicity was also evident by significant increases in absolute and relative liver from 4.5 mg/kg bw/d (4.5 mg/kg bw/d: abs: 112%, rel: 110% — within HCD; 15.1 mg/kg bw/d: abs: 119%; rel: 120%) and histopathological findings of minimal to slight centrilobular hypertrophy accompanied by minimal to slight apoptosis/single cell necrosis of hepatocytes, periportal vacuolation

and multinucleated hepatocytes at the top dose. Some haematological and clinical chemistry parameters and coagulation were also affected.

There were no effects on the fertility of F0 females, mean number of F1 pups delivered, live birth index, total litter numbers, anogenital distance/index or sex ratio. An increase in post-implantation loss was noted from the low dose; however, this was not statistically significant or dose-dependent (4.8%, 7.6%, 5.4% and 7.4% at 0, 1.4, 4.5 and 15.1 mg/kg bw/d, respectively). Effects in pups included decreased pup body weight on PND 1 (-15%) and PND 21 (-10%) and a slight decrease in viability index between PND 0–4 (93% vs. 97% in controls), which was not evident between LD 4–21, at 15.1 mg/kg bw/d. There was also a higher number of cannibalized pups around PND 1 (20 vs. 1 in placebo controls) at the top dose, with most cannibalizations (16) clustered in 2 litters (total of 5 litters affected with 9, 7, 2, 1 and 1 cannibalized pup(s), respectively). There were no signs of developmental neurotoxicity or immunotoxicity in the relevant F1 cohorts. In top-dose male pups, there was a delay in preputial separation, which was within HCD and was attributed to a general developmental delay of male pups at this dose level. There were no effects on the time to vaginal opening in female F1 pups.

Maternal toxicity indications in F1B females included decreases in body weight during gestation (GD 20: ss -8%) and lactation (LD 14: ss -4%) and in body weight gain during premating (-4%) and gestation (ss -11%) at the top dose. Food consumption was also decreased during lactation (-5%) at this dose level. Liver toxicity was also evident in F1 dams, based on increases in absolute and relative liver weight from 4.5 mg/kg bw/d (4.5 mg/kg bw/d: abs: 116%, rel: 109% — above HCD; 15.1 mg/kg bw/d: abs: 126%, rel: 128%) and histopathological findings of minimal to slight centrilobular hypertrophy and minimal to slight apoptosis/single cell necrosis of hepatocytes at the top dose. Changes were also noted in some haematological and clinical chemistry parameters and coagulation at 15.1 mg/kg bw/d.

There were no effects on the fertility of F1B females including no effects on post-implantation loss and no effects on live birth index or total litter numbers at any dose level. There was a statistically significant decrease in the mean number of pups delivered at 15.1 mg/kg bw/d (10.1 vs 12 in controls), which was associated to low number of implantation sites at this dose level (10.5 vs 12.3 in controls; within HCD range (9.4–13.9 implants/dam). In the F2 pups, there were no effects on anogenital distance or index or sex ratio on PND 1 or 21. There was also no effect on the number of cannibalised pups in this generation. Effects were limited to decreased pup body weight on PND 1 (-13%) and PND 21 (-10%) at 15.1 mg/kg bw/d and a slight decrease in viability index between PND 0–4 (95% vs. 99% in controls), which was not evident between LD 4–21.

RAC considered that the top-dose level of this study was too low to induce increases in post-implantation loss similar to the DRF studies, as the study design aims to produce enough viable pups for the multiple cohorts in the F1 generation. Overall, some maternal

toxicity was evident in both generations at the top dose in the form of liver toxicity and mild but statistically significant decreases in body weight throughout the study. The most notable effect on development was a decrease in pup body weight at birth and weaning in both generations (> 10%) at the top dose. RAC considered that this effect was relevant for classification due to no correlation between dam body weight loss and pup body weights according to their analysis of the full study report and due to persistence of this effect until weaning.

The final study was a PNDT study (BASF SE, 2004), conducted according to OECD TG 414 and GLP. Twenty-five female Wistar rats/group were administered nominal doses of 0, 5, 15 and 45 mg/kg bw/d lysmeral (purity: 98.1%) in olive oil via oral gavage between GD 6–20. The actual intake was 0, 4.1, 12.7 and 40.7 mg/kg bw/d.

Clinical signs included transient salivation at the top dose. Statistically significant decreases in body weight were noted between GD 13–20 at the top dose (approx. -7% at study termination). The body weight gain of this group was also statistically significantly decreased, with body weight loss between GD 6–8 (-110%), which was accompanied by a statistically significant decrease in food consumption (-18%), and an overall decrease in food consumption of 25% in comparison to the control. When mean body weights were adjusted for gravid uterine weight, the decrease at the top dose was even more pronounced (-32%). The only effect noted at the middle dose was a statistically significant decrease in body weight gain between GD 6–8 (-56%), which did not persist — overall body weight gain and mean body weight on GD 20 were comparable to the control. Other indications of maternal toxicity included increases in relative liver weight from the low dose (9%, 11% and 19% at 4.1, 12.7 and 40.7 mg/kg bw/d, respectively. These increases were accompanied by elevated ALT levels (+20–30%) from the middle dose and elevated glutamate dehydrogenase levels (+79%) at the top dose. Other effects in clinical chemistry assessments included decreased serum and erythrocyte cholinesterase activity from the middle dose.

In terms of effects on gestational and litter parameters, a non-statistically significant decrease was observed in gravid uterine weight at the top dose (-20%). While there was no effect on the mean number of corpora lutea or implantation sites, the rate of post-implantation loss was increased at the top dose with statistical significance and high variations (15.1 ± 20.25% vs 4.4 ± 7.35% in controls). This effect was outside of the reported HCD range (3.4–1.3%). The mean number of foetuses/live foetuses per dam was decreased at the top dose (7.4 vs 8.1 foetuses in the control) outside of HCD ranges (7.6–9.8 foetuses/dam). Mean foetal weight per litter was statistically significantly decreased from the middle dose (-8% and -19% at 12.7 and 40.7 mg/kg bw/d, respectively). There were no effects on pre-implantation loss, sex ratio or placental weight, while there were no dead foetuses, abortions or premature births in any of the groups.

Observed malformations at the top dose, such as anasarca with a small spleen, polydactyly due to a supernumerary phalanx, and cervical hemivertebra, were considered sporadic, and the total foetal (1.8%), litter (13%) and foetal/litter (2.4%) incidence was within HCD ranges (foetal: 0–2.7%; litter: 0–25%; foetal/litter: 0–2.79%). There was no effect on the mean percentage of affected foetuses with soft tissue variations per litter. In terms of skeletal variations, the litter incidence was 100% in all groups, while the foetal incidence and the number of affected foetuses per litter was increased from the middle dose with statistical significance for the latter (foetal: 91%, 91%, 99% and 98% at 0, 4.1, 12.7 and 40.7 mg/kg bw/d, respectively; mean number of affected foetuses/litter: 98.1%, 92%, 99.1% and 98.3% at 0, 4.1, 12.7 and 40.7 mg/kg bw/d, respectively). The incidence of some parameters was above HCD ranges at the top dose. The DS of the 2017 CLH proposal on lysmeral considered that these increases were secondary to the decreased foetal weights and maternal toxicity in these groups. The only other statistically significant effect was the presentation of liver discolouration in foetuses from the middle dose, which was not evident in the control or low-dose groups (1.7% and 15.5% at 12.7 and 40.7 mg/kg bw/d, respectively). RAC noted at the time that these effects were in line with the observed liver changes in dams of the same dose groups.

Considering that maternal toxicity was marked at the top-dose level in this study, RAC concluded that the effects on foetal body weight and the increased incidence of some skeletal variations over HCD ranges were secondary effects. They concluded that effects in this study were not relevant for classification.

Overall, RAC considered that the increased post-implantation loss in the DRF studies in the presence of some maternal toxicity and the presentation of decreased pup body weights across studies provided some evidence of developmental toxicity. Based on these effects they concluded that lysmeral warranted classification with Repr. 2 (H361d) for effects on development in the 2019 RAC Opinion (ECHA, 2019).

4-tert-butylbenzoic acid

There were no studies available on 4-*tert*-butylbenzoic acid.

Methyl 4-tert-butylbenzoate

There were no studies available on methyl 4-*tert*-butylbenzoate.

Summary and conclusions

Relevant effects for classification from the studies with bourgeonal and 4-*tert*-butyltoluene included:

- Statistically significant decreases in mean body weight in the post-natal period at 5 mg/kg bw/d in the OECD TG 422 study with bourgeonal. These decreases were noted to be within the HCD range and were observed in the absence of maternal toxicity. RAC considered that the top-dose level of this study was too low.

- Decreases in pup body weight at birth and on LD 4 (up to -16% in male pups and up to -15% in females pups) at 5 mg/kg bw/d in the absence of significant maternal toxicity in the OECD TG 421 study with 4-*tert*-butyltoluene.
- Decreases in pup viability, based on a decrease in the number of live pups (-58%) and an increase in the number of stillborn pups (4.7 vs. 0.1 in controls), and indications of increased post-implantation loss, based on the decreased number of live pups at birth (-26%) at 15 mg/kg bw/d in the OECD TG 421 study with 4-*tert*-butyltoluene, albeit in the presence of significant maternal toxicity.

RAC considered that these effects were in line with the observed effects on development with lysmeral that had supported classification with Repr. 2 (H361). They considered that overall the data on the bourgeonal group supported that there was some evidence of developmental toxicity, and that classification with Repr. 2 was warranted based on the read-across approach.

Comments during the EU public consultation, including a comment by the Agency, raised questions towards the validity of the read-across for developmental effects, due to the absence of data on the common metabolite, TBBA, and proposed that a weight of evidence approach might be more appropriate for this endpoint. The DS acknowledged that fewer studies on the bourgeonal group substances were generally available for this endpoint. However, they considered that evidence of developmental toxicity was apparent in the available studies. They additionally noted similar effects on development with the structurally-related cyclamal group. While this group was not included in the proposed read across in the CLH proposal, both groups are structurally similar and present with similar toxicokinetics and target organ toxicity. While it was deemed preferable to prepare separate CLH proposals and read-across approaches for the two groups, RAC considered that the data on the cyclamal group was also relevant for this assessment in a weight of evidence approach.

Overall, based on some evidence of developmental toxicity, the lack of evidence that these effects were not relevant to humans and the read-across approach, RAC concluded that the bourgeonal group warranted classification with Repr. 2 (H361).

In terms of lysmeral and TBBA, whose classification had already been assessed in previous RAC opinions, there were no changes proposed to the classification of lysmeral, while the classification of TBBA was proposed to be modified to add Repr. 2 (H361d) based on read-across. The DS additionally considered proposing a SCL for developmental toxicity, based on calculated ED_{10} values of 4.1 mg/kg bw/d for bourgeonal and 3.9 mg/kg bw/d for 4-*tert*-butyltoluene. These values were at the lower end of the medium potency range; however, the DS concluded that the SCL for Category 2 developmental effects would be similar to the SCL for Category 1 fertility effects, and concluded that an SCL is not necessary due to the Repr. 1B (H360F) classification of substances in the bourgeonal group.

Effects on or via lactation

There was no human evidence regarding the effect of the bourgeonal group on or via lactation.

Additionally, there were no studies regarding the presence of bourgeonal or relevant metabolites in milk.

Two rat studies were available for the assessment of this endpoint. The OECD TG 422 study with bourgeonal (Study report, 2019) and the OECD TG 421 study with 4-*tert*-butyltoluene (Study report, 2007a). Both studies have been described in detail under the Sexual function and fertility and Developmental toxicity sections of this report. Neither of these studies included any investigations on the quantity, quality or composition of milk.

Pups in the OECD TG 422 study with bourgeonal were potentially exposed to the test substance via lactation until LD 21, while the lactation period in the OECD TG 421 study with 4-*tert*-butyltoluene was only up until LD 4. While decreases in pup body weight were observed in both of these studies and there were also some indications of decreased pup survival, there was no clear evidence that these effects were caused by adverse effects of the test substances on or via the milk.

RAC concluded that the bourgeonal group does not warrant classification for adverse effects on or via lactation.

Classification proposed by the Agency:

The Agency agrees with RAC's conclusion on classification. **The bourgeonal group warrants classification with Repr 1B; H360Fd (May damage fertility. Suspected of damaging the unborn child).**

The Agency also agrees with the inclusion of the below note to account for additive effects due to the demonstrated/predicted formation of common metabolite, TBBA, by members of the bourgeonal group.

* New note: The classification of mixtures as reproductive toxicant is necessary if the sum of the concentrations of individual substances covered by this entry, forming the same metabolite, in the mixture as placed on the market is equal to, or above, the applicable generic concentration limit for the assigned category, or a specific concentration limit given in this entry.

Aspiration hazard

Not assessed in the CLH report or RAC opinion.

Environmental hazards

Hazardous to the aquatic environment

Not assessed in the CLH report or RAC opinion.

Other hazards

Hazardous to the ozone layer

Not assessed in the CLH report or RAC opinion.

Overall conclusion

The Agency has evaluated the RAC Opinion, its rationale and any additional scientific evidence that may have been made available to HSE against the criteria for classification and labelling in the GB CLP Regulation and technical guidance.

The Agency technical report **agrees** with the classification proposed by RAC for the following hazards:

Repr. 1B; H360Fd (May damage fertility. Suspected of damaging the unborn child)

Overall, the conclusion is to **agree** with the RAC opinion.

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ECHA (2024c) Guidance on the Application of the CLP Criteria, Part 4: Environmental Hazards; and Part 5: Additional Hazards. Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, version 4.0, ref: ECHA-24-G-05-EN. Available at https://www.echa.europa.eu/

For all other references, please see the EU CLH report and the EU RAC opinion (available at: https://echa.europa.eu/registry-of-clh-intentions-until-outcome)

CLH (2010) CLH report (including Annexes): Proposal for Harmonised Classification and Labelling Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2. Substance Name: 4-tert-butylbenzoic acid; Date: 2010; Written by: Germany; Accessed date: 03/2026

CLH (2017) CLH report (including Annexes): Proposal for Harmonised Classification and Labelling Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2. Substance Name: 2-(4-tert-butylbenzyl) propionaldehyde; Date: 2017; Written by: Germany; Accessed date: 03/2026

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ECHA (2019) Committee for Risk Assessment (RAC) Opinion (including Annexes) proposing harmonised classification and labelling at EU level of 2-(4-tert-butylbenzyl) propionaldehyde; Reference CLH-O-0000001412-86-259/F; Date: 28/01/2019, Accessed date: 03/2026

ECHA (2025) Committee for Risk Assessment (RAC) Opinion (including Annexes) proposing harmonised classification and labelling at EU level of 2-(4-tert-butylbenzyl) propionaldehyde and 4-tert-butylbenzoic acid and 3-(4-tert-butylphenyl)propionaldehyde [1], 4-tert-butyltoluene [2], 4-tert-butylbenzaldehyde [3], methyl 4-tert-butylbenzoate [4]; Reference CLH-O-0000007586-63-01/F; Date: 27/10/2025, Accessed date: 03/2026

Documents published as part of the EU CLH process: Source: European Chemicals Agency, http://echa.europa.eu/

Glossary of terms used in Agency technical reports

4-iPBA	4-isopropylbenzoic acid
µg	microgram
ADME	Absorption, distribution, metabolism, excretion
Agency, the	HSE, acting in its capacity as the GB CLP Agency
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
APTT	Activated partial thromboplastin time
AR	Applied radioactivity
AST	Aspartate aminotransferase
ATE	Acute toxicity estimate
AUC_x	Area under the concentration–time curve up to time point x
BCF	Bioconcentration factor
BOD	Biological Oxygen Demand
BR	Barrier-raised
bw	Body weight
CAR	Competent Authority Report
CAS	Chemical Abstracts Service
CD	Caesarean-derived
CI	Confidence interval
CL	Confidence limits
CLH	Harmonised Classification and Labelling
CLP	Classification, labelling and packaging (of substances and mixtures)
C_{max}	Maximum concentration
CO_2	Carbon dioxide
CoA	Coenzyme A
COD	Chemical Oxygen Demand
Crj	Charles River Japan
Crl	Charles River Laboratories
CV	Coefficient of Variation
d	Day
DAR	Draft Assessment Report
DMSO	Dimethyl sulfoxide
DOC	Dissolved Organic Carbon
DRF	Dose-range finding
DS	Dossier Submitter
DT	Dissipation time OR degradation time (also DissT or DegT where apparent)

DT_{50}	Dissipation half-life OR degradation half-life (hours or days), see also above
dw	Dry weight
ECHA	European Chemicals Agency
ECx	x% effect concentration
EFSA	European Food Safety Authority
EOGRTS	Extended one-generation reproductive toxicity study
ER	Oestrogen receptor
E_rCx	x% effect concentration based on growth rate
EU	European Union
g	gram
GB	Great Britain
GC-MS	Gas chromatography-mass spectrometry
GD	Gestation day
gGT	γ-glutamyl transferase
GLP	Good Laboratory Practice
h	Hours
Hb	Haemoglobin
HCD	Historical control data
HSE	Health and Safety Executive
Ht	Haematocrit
IGS	International Genetic Standardized
kg	kilogram
K_{OC}	Organic carbon-water partition coefficient
K_{OW}	Octanol-water partition coefficient
LC_x	x% lethal effect concentration
LD	Lactation day
LHD-X	Lactate dehydrogenase-X
LOAEL	Lowest Observed Adverse Effect Level
MAA	Methoxyacetic acid
MCL	Mandatory Classification and Labelling
M-factor	Multiplying factor
mg	milligram
mio	million
mL	millilitre
MMAD	Median mass aerodynamic diameter
MoA	Mode of action
m-TBBA	meta-3-*tert*-butylbenzoic acid
MW	Molecular weight
N	Number
NOEC	No-observed effect concentration
OECD	Organisation for Economic Co-operation and Development

QSAR	Quantitative structure-activity relationship
PND	Postnatal day
PNDT	Prenatal developmental toxicity
ppm	Parts per million
p-TBBA	para-4-*tert*-butylbenzoic acid
RAC	Risk Assessment Committee
RAR	Renewal Assessment Report
RBC	Red blood cell
RCOM	Response to comments document
RDT	Repeated-dose toxicity
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals regulation
ROI	Region of Interest
SD	Sprague-Dawley
SPF	Specific Pathogen-Free
STOT-RE	Specific target organ toxicity – repeated exposure
STOT-SE	Specific target organ toxicity – single exposure
TBBA	4-*tert*-butylbenzoic acid
TBD	To be determined
TBHA	p-*tert*-butylhippuric acid
TEER	Trans-epithelial electrical resistance
TG	Test Guideline
T_{max}	Time to maximum concentration
TSCATS	Toxic Substances Control Act Test Submissions
US EPA	United States Environmental Protection Agency
wt	Weight
wwt	Wet weight

Further information

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