Substance Name:

(±)-1,7,7-trimethyl-3-[(4-methylphenyl)methylene]bicyclo[2.2.1]heptan-2-one covering any of the individual isomers and/or combinations thereof (4-MBC)

EC Number: -

CAS Number: -

MEMBER STATE COMMITTEE SUPPORT DOCUMENT FOR IDENTIFICATION OF

(±)-1,7,7-TRIMETHYL-3-[(4-METHYLPHENYL)METHYLENE]BICYCLO[2.2.1]HEPTAN-2-ONE COVERING ANY OF THE INDIVIDUAL ISOMERS AND/OR COMBINATIONS THEREOF (4-MBC)

AS A SUBSTANCE OF VERY HIGH CONCERN BECAUSE OF ITS ENDOCRINE DISRUPTING PROPERTIES (ARTICLE 57(f) - HUMAN HEALTH)

Adopted on 29 November 2021

This document has been prepared according to template: TEM-0049.03

CONTENTS

TABLES

ABBREVIATIONS

3-BC	3-benzylidene camphor
4-MBC	4-methylbenzylidene camphor
A	Androgen
Abs	Absolute
AC50	Concentration at 50% of maximum activity
AD	Androstenedione
ADME	Absorption, distribution, metabolism and excretion
AGD	Anogenital distance
AO	Adverse outcome
AOP	Adverse outcome pathway
AP	Alkaline phosphatase
AR	Androgen receptor
AUC	Area under curve
BW	Body weight
CF	Conceptual Framework
CLP	Classification, Labelling and Packaging
C_{max}	Maximum concentration
DES	Diethylstilbestrol
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
DNT	Developmental neurotoxicity
E	Estrogen
E2	17-beta-estradiol
EATS	Estrogen/Androgen/Thyroid/ Steroidogenesis
EC_{50}	50 % effective concentration
EC	Effect concentration
ECHA	European Chemicals Agency
ED	Endocrine disrupter
ED_{50}	50 % effective dose
EDSP	Endocrine Disruptor Screening Program
EFSA	European Food Safety Authority
ELISA	Enzyme linked immunosorbent assay
EOGRTS	Extended One-Generation Reproductive Toxicity Study
ER	Estrogen receptor
E-Screen	Cell proliferation assay in MCF-7 cells
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
hER	Human estrogen receptor
HPG axis	Hypothalamic/pituitary/ gonadal axis
hPR	Human progesterone receptor
HPT axis	Hypothalamic/pituitary/ thyroid axis
HTS	High throughput screening
IC_{50}	50 % inhibitory concentration
IPCS	International Programme on Chemical Safety
K1-4	Klimisch categories
KE	Key event
KER	Key event relationship
LABC	Levator ani-bulbocavernosus muscle complex
LD_{50}	Median (50%) lethal dose
LH	Luteinising hormone
LOD	Limit of detection
LoE	Line of Evidence
LOEC	Lowest-observed-effect -concentration
µM	Micromolar
MCF-7	Breast cancer cell line. Michigan Cancer Foundation-7
MIE	molecular initiating event
mg/kg	Milligram per kilograms
MoA	Mode of action
MOS	Margin of Safety
MPO	Medial preoptic area
NOAEL	No observed adverse effect level
NOEC	No-observed-effect-concentration
NS	Non-statistically significant
OECD	Organisation for Economic Co-operation and Development
OMC	Octyl-methoxycinnamate
OVX	Ovariectomised
PE	Proliferative effect
PND	Postnatal day
PR	Progesterone receptor
PTU	Propylthiouracil
REACH	Registration, evaluation, authorisation and restriction of chemicals
Rel	Relative
RT-PCR	real-time Polymerase Chain Reaction
S	Steroidogenesis
S.C.	Subcutaneous
SCCP	Scientific committee on Consumer Products
SD	Standard deviation
SHGB	Sex hormone-binding globulin
SPF	Specific-Pathogen Free
SVHC	Substance of very high concern
T	Thyroid
T3	Tri-iodothyronine (thyroid hormone)
T4	Thyroxine (thyroid hormone)
TG	Test guideline

TH(s)	Thyroid hormone(s)
TPO	Thyroperoxidase
TR	Thyroid hormone receptor
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone
US EPA	United States Environmental Protection Agency
VO	Vaginal opening (or patency)
VMH	Ventromedial Hypothalamic nucleus
VTG	Vitellogenin
WHO	World Health Organisation
WoE	Weight of Evidence
YES	Yeast estrogen screen

IDENTIFICATION OF SUBSTANCES OF VERY HIGH CONCERN ON THE BASIS OF THE CRITERIA SET OUT IN REACH ARTICLE 57

Substances Name: (±)-1,7,7-trimethyl-3-[(4-methylphenyl)methylene]bicyclo[2.2.1]heptan-2-one covering any of the individual isomers and/or combinations thereof (4-methylbenzylidene camphor, 4-MBC)

- The substances are identified as substances of equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of Regulation (EC) No 1907/2006 (REACH) according to Article 57(f) of REACH Regulation.

(±)-1,7,7-trimethyl-3-[(4-methylphenyl)methylene]bicyclo[2.2.1]heptan-2-one covering any of the individual isomers and/or combinations thereof (commonly referred to as 4-methylbenzylidene camphor or 4-MBC) are identified as substances of very high concern in accordance with Article 57(f) of Regulation (EC) 1907/2006 (REACH) because of their endocrine disrupting properties for which there is scientific evidence of probable serious effects to human health which gives rise to an equivalent level of concern to those substances listed in points (a) to (e) of Article 57 REACH. This identification is made based on the available evidence for 4-MBC. Due to the structural similarity between the different isomers and in the absence of evidence on the individual isomers, it has to be assumed that all individual isomers and/or combinations thereof have endocrine disrupting properties.

Endocrine disrupting (ED) properties of 4-MBC relevant for human health:

Thyroid mode of action

There is some evidence of thyroid-related (T) endocrine activity based on the few available *in vitro* studies and strong evidence in a larger number of *in vivo* studies showing a consistent pattern of increased thyroid stimulating hormone (TSH) and tri-iodothyronine (T3). There is also strong evidence for adverse effect on the thyroid gland in several *in vivo* studies (e.g. increased weight and altered histopathological findings). According to the Mode of Action analysis (MoA), there is strong evidence that the adverse effects on the thyroid gland are plausibly linked to the thyroid disrupting endocrine activity seen *in vitro* and *in vivo*. According to the ECHA/EFSA ED guidance (ECHA/EFSA 2018), such effects are considered relevant for human health and could pose a hazard to humans, in particular if alterations of thyroid hormones should occur during the critical windows of pre- and postnatal neurological development. Such adverse health effects on offspring neurodevelopment are often irreversible and can have consequences later in life. Neurodevelopment has not been sufficiently investigated with 4-MBC, and therefore such effects cannot be excluded. Irrespective of the identified knowledge gaps related to neurodevelopment, the consistently seen adverse effects on thyroid gland weight and histopathology clearly show that 4-MBC is a thyroid hormone system disrupting chemical.

Estrogenic mode of action

There is strong evidence for endocrine activity related to estrogen receptor activation. The available *in vitro* assays provide strong evidence for induction of estrogenic response in the E-

screen and for ER agonism. *In vivo*, several mechanistic studies show altered growth of estrogen sensitive tissues, including increased uterus weight in uterotrophic assays and altered expression of estrogen-regulated genes in several target tissues, confirming the strong evidence of an estrogenic activity.
In female rodents, there is moderate to strong scientific evidence that combined perinatal and adult exposure to 4-MBC can lead to adverse effects on sexual behaviour (reduced proceptive and receptive behaviour, and increased rejection behaviour towards the male) as well as a moderate degree of evidence for other adverse effects on female reproductive development (changes in ovary weight, uterine weight and ano-genital distance (AGD), vaginal opening (VO)). In addition, there is weak-moderate evidence of alterations in circulating follicle stimulating hormone (FSH), luteinising hormone (LH) and gonadotropin releasing hormone (GnRH) levels *in vivo*. The performed MoA analysis shows a biologically plausible link beween the estrogenic endocrine mechanism and the reported adverse effects. The molecular initiating event is activation of the ER(s), which can result in increased ER activity in specific tissues, including specific areas of the brain. If such changes occur during the first two weeks of postnatal life, the female brain is not organised properly. This can lead to disrupted regulation of LH and FSH in adulthood and may as a consequence adversely affect sexual behaviour. Additionally, altered ER signalling has been shown in some studies to alter female AGD, ovary and uterus development and timing of sexual maturation.

In male rodents, there is moderate to strong scientific evidence of persistent reductions in prostate weight in several studies in adult animals, whereas the results are less clear from the available developmental toxicity studies. The Mode of action analysis shows that estrogens are important regulators of adult prostate growth and function and that increased ER signalling may affect prostate growth during early prostate development. Although patterns of effects of estrogenic substances may vary, it is biologically plausible that the observed effects of 4-MBC are related to increase in estrogen signalling. In addition to the role of estrogens, it has been shown that dysregulation of the FSH system plays a significant role in prostate growth. Although the evidence is currently limited, it is biologically plausible that altered gonadotropin secretion may contribute to the observed changes in adult prostate growth.

Other potential modes of action
In addition, there is some supportive *in vitro* evidence showing androgen receptor (AR) antagonistic activity. This endocrine activity could also plausibly contribute to the adverse effects on both the male and female reproductive system in rodents.

Summary of the ED assessment
Therefore, there is scientific evidence to conclude that 4-MBC are endocrine disruptors via T and E modalities, according to a mode of action analysis including an evaluation of biological plausibility.

Equivalent level of concern

- 4-MBC exposure has been shown to consistently disrupt the thyroid hormone system and consequently cause adverse effects on the thyroid gland. Thyroid hormone system disruption can have potentially serious and irreversible effects on humans, in particular on neurodevelopment. This can impact the quality of life and raises societal concern of a

high and increasing burden. A number of vulnerable populations may be particularly susceptible to thyroid hormone (TH) disruption induced by 4-MBC. Pregnancy is likely to be a period of sensitivity to the alteration of TH regulation, with potential consequences for neurodevelopment of the offspring.

- The observed adverse reproductive effects in male and female rodents have been plausibly linked to the estrogenic mode of action, shown both *in vitro* and *in vivo* after 4-MBC exposure. These, and potentially other adverse effects in humans, caused by endocrine disruption via the E modality are considered serious, as similar effects in humans could cause sub- and infertility. For humans, sub- and infertility is not only detrimental to the propagation of the species, but it also has a major impact on quality of life. Additionally, fertility treatment and counselling carry high societal costs.

Based on the available studies, it may be difficult to establish a safe level of 4-MBC. Mixture effects, where substances act additively or with synergistic effects, cannot be excluded and this might impact the threshold of toxicity. Moreover, the difficulty to establish a safe level with sufficient certainty raises concern particularly on the capacity to manage safe use of the substances for sensitive populations. Establishing safe levels for these particularly sensitive populations is surrounded with large uncertainties, and alterations of thyroid hormones during the critical windows of pre- and post-natal neurological development may have consequences later in life. The complexity of the response in reaction to thyroid disturbance is not fully characterised and understood, and considering the range of functions influenced by THs, it is also highly challenging to fully characterise these effects in experimental studies.

Altogether, this gives rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of REACH.

Conclusion

Overall, it is concluded that the substances *(±)-1,7,7-trimethyl-3-[(4-methylphenyl) methylene]bicyclo[2.2.1]heptan-2-one* covering any of the individual isomers and/or combinations thereof *(4-methylbenzylidene camphor, 4-MBC)* meet the criteria of Article 57(f) of REACH, due to their endocrine disrupting properties for which there is scientific evidence of probable serious effects to human health which give rise to an equivalent level of concern to those for other substances listed in paragraphs (a) to (e) of Article 57 of REACH Regulation.

Registration dossiers submitted for the substances? Yes

PART I

Justification

1 Identity of the substances and physical and chemical properties

1.1 Name and other identifiers of the substances

This proposal addresses (±)-1,7,7-trimethyl-3-[(4-methylphenyl)methylene]bicyclo[2.2.1] heptan-2-one, covering any of the individual isomers and/or combinations thereof (referred to hereafter as "4-MBC" or "the substances"). This SVHC identification is based on the available evidence for 4-MBC. Due to the structural similarity between the different isomers and in the absence of evidence on the individual isomers, it has to be assumed that all individual isomers and/or combinations thereof have endocrine disrupting properties.

Structural formulae of the possible isomers:

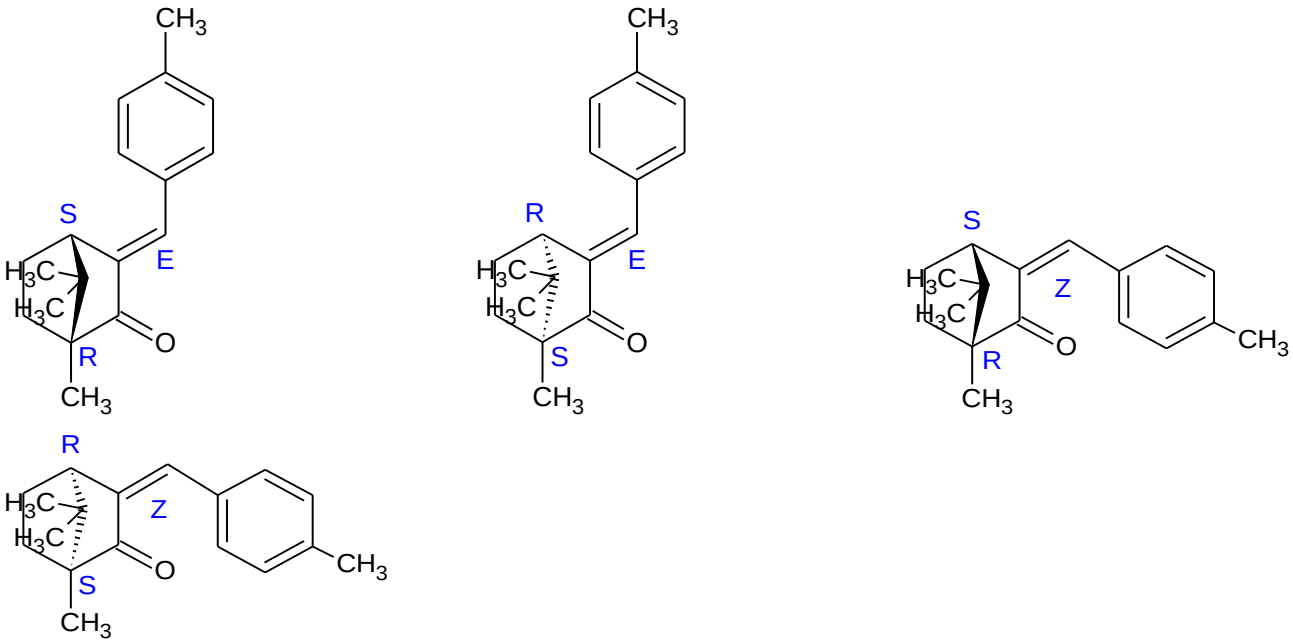
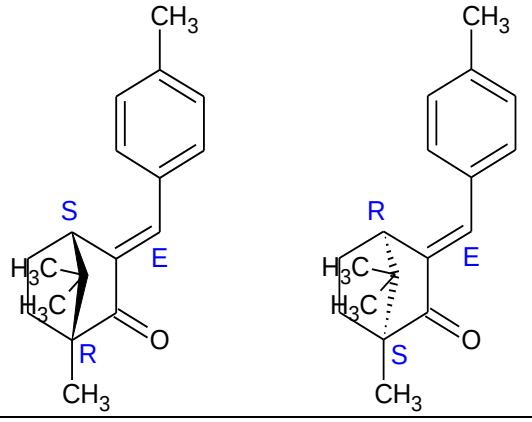


Table 1.1 reports the identifiers associated to the registration dossier submitted under REACH for the substance currently on the market. Table 1.2 reports the identifiers previously associated to the registration dossier.

Table 1.1: Substance identity (3*E*)-1,7,7-trimethyl-3-[(4-methylphenyl)methylene]bicyclo[2.2.1]heptan-2-one[1]

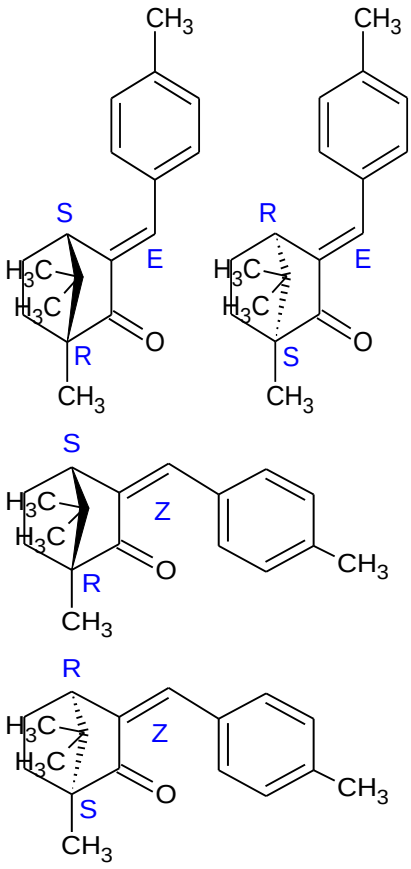
EC number:	-
EC name:	-
CAS number (in the EC inventory):	1782069-81-1
IUPAC name:	(3*E*)-1,7,7-trimethyl-3-[(4-methylphenyl)methylene]bicyclo[2.2.1]heptan-2-one
Molecular formula:	C18H22O
Molecular weight:	254.4 g/mol
Structural formula	

Substance type: ☐ Mono-constituent ☒ Multi-constituent ☐ UVCB

Table 1.2: Substance identity (±)-1,7,7-trimethyl-3-[(4-methylphenyl)methylene]bicyclo[2.2.1]heptan-2-one

EC number:	253-242-6
EC name:	(±)-1,7,7-trimethyl-3-[(4-methylphenyl)methylene]bicyclo[2.2.1]heptan-2-one
CAS number:	36861-47-9
IUPAC name:	1,7,7-trimethyl-3-[(4-methylphenyl)methylene]bicyclo[2.2.1]heptan-2-one
Molecular formula:	C18H22O
Molecular weight:	254.4 g/mol

[1] Registration dossier: https://echa.europa.eu/da/registration-dossier/-/registered-dossier/25426 (July 2021)

Synonyms:	4-MBC 4-Methylbenzylidenecamphor 1,7,7-Trimethyl-3-(4-methylbenzylidene)bicyclo(2.2.1)heptan-2-one 3-(4-Methylbenzylidene)-dl camphor 3-(4-Methylbenzylidene)-DL-camphor 3-(4-Methylbenzylidene)camphor 3-(*p*-Methylbenzylidene)-DL-camphor 3-(*p*-Methylbenzylidene)bornan-2-one 3-(*p*-Methylbenzylidene)camphor Bicyclo(2.2.1)heptan-2-one, 1,7,7-trimethyl-3((4-methylphenyl)methylene) *p*-Methylbenzylidenecamphor
Structural formula	

Substance type: ☐ Mono-constituent ☒ Multi-constituent ☐ UVCB

Table 1.3: Non-exhaustive list of substances covered by the proposed entry:

Name	EC Number	CAS Number
(1*S*,3*E*,4*R*)-1,7,7-trimethyl-3-(4-methylbenzylidene)bicyclo[2.2.1]heptan-2-one	-	852541-30-1
(1*R*,3*E*,4*S*)-1,7,7-trimethyl-3-(4-methylbenzylidene)bicyclo[2.2.1]heptan-2-one	-	95342-41-9

(1*R*,3*Z*,4*S*)-1,7,7-trimethyl-3-(4-methylbenzylidene)bicyclo[2.2.1]heptan-2-one	-	852541-21-0
(1*S*,3*Z*,4*R*)-1,7,7-trimethyl-3-(4-methylbenzylidene)bicyclo[2.2.1]heptan-2-one	-	852541-25-4
(3*E*)-1,7,7-trimethyl-3-(4-methylbenzylidene)bicyclo[2.2.1]heptan-2-one	-	1782069-81-1
(1*R*,4*S*)-1,7,7-trimethyl-3-(4-methylbenzylidene)bicyclo[2.2.1]heptan-2-one	-	741687-98-9
(±)-1,7,7-trimethyl-3-[(4-methylphenyl)methylene]bicyclo[2.2.1]heptan-2-one	253-242-6	36861-47-9

1.2 Physicochemical properties

Table 1.4: Overview of physicochemical properties (based on the registration information[1])

Property	Description of key information	Value	Reference/source of information
Physical state at 20°C and 101.3 kPa	Information reported from the test item Certificate of Analysis from a GLP toxicology study.	Pale white to white crystalline solid	ECHA dissemination site
Melting/freezing point	Differential scanning calorimetry	70.4 °C at atmospheric pressure (1009 hPa)	OECD Guideline 102, 1995 EU Method A.1, 2008
Boiling point	Differential scanning calorimetry	357-358 °C corrected to normal atmospheric pressure (1013 hPa)	OECD Guideline 103, 1995 EU Method A.2, 2008
Vapour pressure	Effusion method: vapour pressure balance	$4.9 \cdot 10^{-4}$ Pa at 20°C, $1.0 \cdot 10^{-3}$ Pa at 25°C, $2.9 \cdot 10^{-2}$ Pa at 50°C	OECD Guideline 104, 2006 EU Method A.4
Density	Air comparison pycnometer (for solids)	1.108 g/cm^3 at 20 °C ± 0.01 °C (compared to water at 4 °C)	OECD Guideline 109, 2012 EU Method A.3
Water solubility	Column elution method	1.08 ± 0.15 mg/L at 20 °C (pH 5-6)	OECD Guideline 105, 1995
Partition coefficient n-octanol/water (log value)	Octanol-water. Analytical method: HPLC	log Pow 5.1 at 23°C.	OECD Guideline 117, 1989

2 Harmonised classification and labelling

There is no harmonised classification for 4-MBC and the substances are therefore not covered in Annex VI to the CLP Regulation.

According to the classifications provided by the company in the REACH registration, 4-MBC is very toxic to aquatic life (Aquatic Acute 1, H400, M=1), very toxic to aquatic life with long lasting effects (Aquatic Chronic 1, H410, M=1) and may cause damage to organs through prolonged or repeated exposure (STOT RE 2, H373, Thyroid, oral, inhalation). In addition, the classification provided by the company to ECHA in CLP notifications identifies that the substances are also suspected of damaging fertility or the unborn child (Repr. 2, H361), causes skin irritation (Skin Irrit. 2, H315) and causes serious eye irritation (Eye Irrit. 2, H319)[2].

The registrant of the substance (CAS No 1782069-81-1) provides the following justification for the classification of the substance as STOT RE 2, which is available in the public registration dossier[1] under the endpoint summary for repeated dose toxicity:

Justification for classification or non-classification:
The stimulatory response shown in the thyroid in this study was not typical of the consequences of the feedback mechanism resulting from enhanced hepatic clearance of circulating thyroxine following hepatic enzyme induction. The evidence of an enzyme-inducing effect in the liver in this study was weak in comparison with the signs of thyroid stimulation, suggesting the potential for some direct thyroid effect as well as an adaptive response to possible hepatic enzyme induction. Consequently, the oral NOEL in this rat study was determined to be 25 mg/kg bw/day and the NOAEL to be >50 but <125 mg/kg bw/day (GHS Category 2 classification range is 10-100 mg/kg bw/day). In order to confirm the potential for STOT-RE classification GHS Category 2, it is important to establish whether the effect on the thyroid seen in the key study is adaptive or otherwise, is toxicologically relevant and is demonstrative of organ dysfunction. The authors state that the changes seen in liver weight and blood chemistry are not indicative of a hypermetabolic state hence the pronounced, threshold response demonstrated in the thyroid could be a direct effect of treatment. Therefore, it is concluded that the test item should be classified as Category 2 for STOT-RE.

3 Environmental fate properties

Not relevant for the identification of the substances as SVHC in accordance with Article 57(f) of the REACH Regulation.

[2] C&L Inventory database: http://echa.europa.eu/web/guest/information-on-chemicals/cl-inventory-database (July 2021)

4 Human health hazard assessment

4.1 Toxicokinetics

4.1.1 Non-human information

In two non-guideline studies by Schauer *et al.* (2006) and by Völkel *et al.* (2006), plasma levels and toxicokinetics were investigated in rats following dermal and oral exposure of 4-MBC. In Schauer *et al.* 2006 Wistar rats (3 males and 3 females) were exposed to dermal 4-MBC doses of 400 and 2000 mg/kg body weight (bw) applied in a formulation using an occlusive patch for 24 hours. Concentrations of 4-MBC and its metabolites were monitored over 96 hours in plasma. Peak plasma levels of 4-MBC were 200 (dose of 400 mg/kg bw) and 1200 pmol/mL (dose of 2000 mg/kg bw) in male and female rats. These levels remained constant for up to 24–48 hours after dermal application. Two major metabolites were identified in the plasma of rats after dermal application: 3-(4-carboxybenzylidene)-6-hydroxycamphor and 3-(4-carboxybenzylidene) camphor[1].

In Völkel *et al.* (2006), male and female Sprague–Dawley rats (n = 3 per group) were administered single oral doses of 25 or 250 mg/kg bw of 4-MBC in corn oil. The metabolites formed were characterized and the kinetics of elimination for 4-MBC and its metabolites from blood and urine were determined. Metabolites of 4-MBC were characterized as 3-(4-carboxybenzylidene) camphor and as four isomers of 3-(4-carboxybenzylidene)-hydroxycamphor. After oral administration only very low concentrations of 4-MBC were present in blood and the peak concentrations of 3-(4-carboxybenzylidene)camphor were approximately 500-fold above those of 4-MBC; blood concentrations of 3-(4-carboxybenzylidene)-6-hydroxycamphor were below the limit of detection. Blood concentration of 4-MBC and 3-(4-carboxybenzylidene)camphor peaked within 10 hours after 4-MBC administration and then decreased with half-lives of approximately 15 hours. No major differences in peak blood levels between male and female rats were seen. In urine, one isomer of 3-(4-carboxybenzylidene) hydroxycamphor was the predominant metabolite [3-(4-carboxybenzylidene)-6-hydroxycamphor], the other isomers and 3-(4-carboxybenzylidene)camphor were only minor metabolites excreted with urine. However, urinary excretion of 4-MBC-metabolites represents only a minor pathway of elimination for 4-MBC, since most of the applied dose was recovered in feces as 3-(4-carboxybenzylidene)camphor and, to a smaller extent, as 3-(4-carboxybenzylidene)-6-hydroxycamphor. The results show that absorbed 4-MBC undergoes extensive first-pass biotransformation in rat liver resulting in very low blood levels of the parent 4-MBC. Enterohepatic circulation of glucuronides derived from the two major 4-MBC metabolites may explain the slow excretion of 4-MBC metabolites with urine and the small percentage of the administered doses recovered in urine (Völkel et al 2006).

Schlumpf *et al.* 2008a

See section 4.10.3.2 for study description. The paper describes measurements of 4-MBC in milk from rat offspring (doses of 7 and 0.7 mg/kg 4-MBC) and in human breast milk samples. A comparison showed that at the dose of 7 mg/kg, the 4-MBC concentration in rat milk was only 11 times higher than the highest value found so far in human milk. Hence, the study provided good evidence that humans (in 2008) had measurable levels of 4-MBC in the breast milk and that these levels were not very different from those measured in rats exposed to 4-MBC doses of 0.7 and 7 mg/kg per day.

4.1.2 Human information

In a study by Schauer *et al.* (2006), plasma levels and toxicokinetics were also investigated in human subjects following dermal exposure[1]. Six humans (3 male and 3 female subjects) were exposed to 4-MBC by topical application of a commercial sunscreen formulation containing 4% 4-MBC (w/w), covering 90% of the body surface and resulting in a mean dermal 4-MBC dose of 22 mg/kg bw. Concentrations of 4-MBC and its metabolites were monitored over 96 hours in plasma and urine. Peak plasma levels (C_{max}) of 4-MBC were reached 6 hours after dermal application (200 pmol/mL in males and 100 pmol/mL in females). After the 6-hour sampling point, 4-MBC concentrations decreased following 1st order kinetics with a half-life of 9 hours to reach the lower limit of quantitation at 48 hours (males) or 36 hours (females) after the application (1-20 pmol/mL). The area under the curve (AUC) values were 3884 and 1909 pmol/mL hours in males and females, respectively. In plasma, 3-(4-carboxybenzylidene) camphor was the major metabolic product, whereas, in urine, 3-(4-carboxybenzylidene)-6-hydroxycamphor and a glucuronide of 3-(4-carboxybenzylidene)camphor were the main metabolites. Less than 1% of the applied dose of 4-MBC was recovered in urine as metabolites.

In an opinion document on 4-MBC by the Scientific Committee on Consumer Products (SCCP, 2008) a toxicokinetic approach was used to derive a Margin of Safety (MoS) for 4-MBC. A mean dermal absorption value of 1.96 µg/cm² from an applied substance amount of 178 µg/cm² yielded a calculated dermal absorption value of 1.10%[1].

Schlumpf *et al.* 2008a
The paper describes measurements of 4-MBC in milk from rat offspring (doses of 7 and 0.7 mg/kg 4-MBC) and in human breast milk samples. See above in non-human information and in section 4.10.3.2.

4.1.3 Summary of toxicokinetics.

Differences in toxicokinetics between dermal and oral exposure to 4-MBC have been investigated in studies by Schauer *et al*. (2006) and Völkel *et al.* (2006). From these studies it is apparent that the kinetics of 4-MBC do differ in relation to route of exposure, causing different absorption patterns, and different internal levels of 4-MBC and its metabolites. Two major metabolites were identified in the plasma of rats and humans after dermal application: 3-(4-carboxybenzylidene)-6-hydroxycamphor and 3-(4-carboxybenzylidene)camphor. These metabolites were previously identified in rats after oral administration of 4-MBC; however, the pattern of metabolites in plasma differs depending on the route of exposure: the plasma concentration of the parent compound 4-MBC is much higher after topical application as compared to after oral administration. In addition, the plasma levels of 3-(4-carboxybenzylidene)-6-hydroxycamphor were below the LOD (limit of detection) after oral administration to rats, whereas this metabolite was clearly detected in the plasma of humans and rats after dermal application. As observed after oral administration of 4-MBC, peak plasma levels of both metabolites were much higher than plasma levels of 4-MBC, suggesting intensive biotransformation of 4-MBC. The AUC of 3-(4-carboxybenzylidene) camphor was much higher after oral application of 250 mg/kg bw as compared to dermal application of 400 mg/kg bw. This suggests major differences in the relative contribution of biotransformation pathways to the bioavailability of 4-MBC after different routes

of application (Schauer *et al*., 2006), as plasma concentrations of the metabolites diverge after oral and dermal exposures. Schlumpf *et al.* 2008a provided evidence of 4-MBC excretion to rat and human breast milk.

Additionally, a recent human biomonitoring report has measured concentrations of 4-MBC metabolites in urine samples from German children and adolescents (Murawski et al. 2021). This study showed that 4-MBC metabolites were only found in quantifiable amounts in single cases and that in the period 2014-17, exposures were generally very low. This is also the conclusion from two Danish biomonitoring studies in children and adolescents, as these did not find 4-MBC metabolites in any of the investigated samples (Frederiksen et al., 2017; Krause et al., 2017). As these publications only reported on biomonitoring results and did not attempt to link the chemical exposure data to any physiological changes in the test subjects, these data have not been included in the ED assessment of 4-MBC.

4.2 Acute toxicity

4.2.1 Non-human information

4.2.1.1 Acute toxicity: oral

The acute oral toxicity of 4-MBC was determined in a 16-day limit test conducted according to OECD 401. Male and female Fü-albino SPF rats, 6 to 7 weeks old, were dosed with 4-MBC at a nominal concentration of 2000 mg/kg bw in Standard Suspending Vehicle by oral gavage. The dose volume was 10 mL/kg. Rats were observed for mortality, clinical signs and body weight. The lethal dosage of 50 % (LD_{50}) was determined to be > 2000 mg/kg bw. There were no effects on body weight development and no clinical signs or autopsy findings were recorded[1].

4.2.1.2 Acute toxicity: dermal

The acute dermal toxicity of 4-MBC was determined in a 14-day limit test conducted similar to the OECD Guideline 402. A nominal concentration of 10 g/kg bw 4-MBC in peanut oil DAB 7 (1:1) was applied to a 6 x 6 cm area of shaved skin of male and female Wistar-AF/HAN-EMD-SPF rats. A vehicle control was included in the study. The treated area was occluded for 24 hours, after which substance residues were washed off with water. Rats were observed for mortality, intoxication, local changes and body weight. The LD50 was determined to be > 10 g/kg bw. There were no signs of absorptive intoxication, symptoms of irritation or pathoanatomical abnormalities. All rats lost weight in the first two days after application, however weight gain was continuous thereafter[1].

4.2.2 Summary of acute toxicity

One key study is available for the acute oral toxicity endpoint and one key study is available for acute dermal toxicity. As the acute oral and dermal studies resulted in predicted lethal dose LD50 levels in excess of the 2000 mg/kg limit dose, it was concluded by the registrant that 4-MBC does not fulfil the criteria for classification for acute toxicity according to the CLP Regulation (EC) No 1272/2008.

4.3 Irritation

Not relevant for the identification of the substances as SVHC in accordance with Article 57(f) of the REACH Regulation.

4.4 Corrosivity

Not relevant for the identification of the substances as SVHC in accordance with Article 57(f) of the REACH Regulation.

4.5 Sensitisation

Not relevant for the identification of the substances as SVHC in accordance with Article 57(f) of the REACH Regulation.

4.6 Repeated dose toxicity

Repeated dose toxicity studies from the registration dossier were included in the assessment. See study descriptions and discussion in section 4.10.

4.7 Mutagenicity

Not relevant for the identification of the substance as SVHC in accordance with Article 57(f) of the REACH Regulation.

4.8 Carcinogenicity

No carcinogenicity studies have been performed. Studies might have been relevant for the identification of the substance as SVHC in accordance with Article 57(f) of the REACH Regulation, as endocrine mediated cancers (for instance thyroid neoplasia) might have been identified.

4.9 Toxicity for reproduction

4.9.1 Effects on fertility

Reproductive toxicity studies from the registration dossier were included in the assessment. See study description and discussion in section 4.10.

4.9.2 Developmental toxicity

Apart from the studies included in the assessment of Endocrine disruption (See study descriptions and discussion in section 4.10.), a prenatal developmental toxicity study was

conducted according to TG 414 (Unpublished, 1988). This test was not specifically designed to detect endocrine disruptors, but may provide parameters that are sensitive, but not diagnostic of EATS modalities according to the ECHA/EFSA ED guidance (ECHA/EFSA 2018).

Prenatal Developmental Toxicity Study – OECD TG 414 (Unpublished, 1988)

Groups of female rats were dosed with 4-MBC at dose levels of 0, 10, 30 and 100 mg/kg bw/day by oral gavage between days 6 to 15 of gestation. The females (n=25) were killed on day 20 of gestation and the uterine contents examined in detail to evaluate any potential effects on reproduction and the embryo. The skeletal and soft tissue were examined only in the females. All litters were examined externally.

From this treatment regime, the dose levels of 10 and 30 mg/kg bw/day proved to be non-toxic to the pregnant female rat. However, the dose level of 100 mg/kg bw/day was shown to be minimally toxic to the dams as demonstrated by a slightly lower body weight gain by these females. There was no evidence of an effect of treatment on maternal reproductive parameters (number of resorptions, corpora lutea, implantations) at any of the dose levels examined.

A small but statistically significant reduction in body weight was recorded for fetuses from Group 4 (100 mg/kg bw/day). Corresponding to these lighter fetal weights in Group 4, there was a lower degree of ossification of the sternum and the extremities seen in fetuses from this treatment group. Since a level of maternal toxicity was seen in this treatment group (slightly reduced weight gain), this incidence of reduced ossification was considered to be secondary to this effect on the dams. The dose-dependent increase of rudimentary lumbar ribs in both sexes of fetuses from Groups 3 and 4 (30 and 100 mg/kg bw/day) was also attributed to stress in the dams being sufficient to express the developmental instability inherent in this species.

Consequently, the NOAEL for both maternal and fetal toxicity in this study was determined to be 30 mg/kg bw/day. There was no effect on sex ratio.

Overall, no endocrine-related endpoints were investigated in the offspring. If adverse effects on female fertility had been seen, for instance an increase in pre- or post-implantation loss, this could have been an indication of effects sensitive to but not diagnostic of EATS. However, no such effects were seen in the present nor in the only other study where this endpoint was assessed, the TG421 reproductive toxicity screening study (Unpublished 2004).

Study assessment: This was a guideline study and the quality and reliability of the study was high. A large group size was used (n=25). The study was conducted before the assay update in 2018, which means additional endpoints that would have been relevant for an ED assessment were not investigated in this study (1, reliable without restriction).

4.10 Endocrine Disruption – Human health

4.10.1 General approach

The subsequent sections of the report are organized in the following way:

Section 4.10.2 presents the literature search that was employed for assessment of endocrine disrupting properties.

Section 4.10.3 provides study summaries. Section 4.10.3.1 provides summaries of all identified *in vitro* studies. These summaries are presented according to year of publication and include descriptions of methods, results and an assessment of study quality using the Klimisch score criteria adapted to *in vitro* methodology. Section 4.10.3.2 provides summaries of all *in vivo* studies from the open literature, organised according to investigated endpoints and chronology, while section 4.10.3.3 summaries results from the studies in the registration dossier (based on the provided study reports).
All the *in vivo* summaries include information regarding study design, results and assessment of study reliability, using Klimisch score (Klimisch *et al.* 1997) combined with an expert judgement statement.
In section 4.10.3.4, the human information is presented.

In section 4.10.4, all of the available data is organized into Lines of Evidence (LoE) for T-modality. Table 4.3 shows *in vitro* mechanistic data, Table 4.4 shows *in vivo* mechanistic data and Table 4.5 shows *in vivo* adversity.

In section 4.10.5, LoE for EAS modalities are presented: Table 4.8 shows *in vitro* mechanistic data via EAS modalities, Table 4.9 show *in vivo* mechanistic data via E modality, Table 4.10 show *in vivo* mechanistic data (hormone measurements) via EAS modalities and table 4.11 shows *in vivo* adversity (female and male reproductive system, nervous system) via EAS modalities.

In section 4.10.6, Mode of Action (MoA) analyses are performed for relevant adverse outcomes (T and EAS mediated).

Finally, in section 4.10.7 an overall weight of evidence analysis is performed in order to assess whether 4-MBC fulfils the WHO/IPCS definition for identification as an endocrine disrupter (WHO/IPCS, 2002) as interpreted by the JRC Endocrine Advisory Group (2013). These conclusions are carried forward to section 6.1 "Equivalent level of concern assessment", where it is evaluated whether 4-MBC can be identified as substances of equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of Regulation (EC) No 1907/2006 (REACH) according to Article 57(f) of REACH Regulation.

Annex 1 summarises information on systemic toxicity in the performed animal studies.

4.10.2 Literature search

A literature search was performed, in order to identify all studies from the open literature relevant for assessment of endocrine disrupting properties of 4-MBC in relation to human health. Additionally, an assessment of the results presented in study reports from the registration dossier was performed.
To ensure inclusion of all possibly relevant literature, a single concept search strategy was used, as suggested in the ECHA/EFSA guidance (ECHA/EFSA 2018). This type of search was conducted in PubMed. Search strings and number of hits are presented in Table 4.1 below.

Table 4.1: Date, database, search string and number of articles.

Date of search	Database	Search string	Number of articles	Comment
October 2019, and again on May 5th 2021. The last search did not result in any additional relevant publications.	PubMed	((4-MBC) OR (4-methylbenzylidene camphor) OR (36861-47-9)) AND ((rats OR mice OR human OR toxicity) OR (endocrin* OR hormon* OR androgen* OR estrogen* OR thyroid* OR steroid*))	173 publications Manual screening narrowed the result to 34 potentially relevant publications. 9 additional studies were identified through these publications.	The title and abstracts of the identified articles were screened manually. Expert judgement was also used to determine whether a study was likely to provide information of relevance for the ED assessment.

The last PubMed search performed on May 5th 2021. This resulted in 173 hits. Of these 34 were relevant for the ED assessment of 4-MBC. In addition, 8 publications and a poster abstract were included in the present assessment. The studies by Gomez *et al.* 2005, Morohoshi *et al.* 2005, Schreurs *et al.* 2005, Kunz *et al.* 2006, Kunz & Fent 2006, Schmutzler *et al*. 2007, Hofmann *et al.* 2009, Schiffer *et al.* 2014 and Rehfeld *et al.* 2016 were identified through references in other publications or identified through information on the ECHA dissemination site. Several of these publications were *in vitro* screening studies testing a wide range of chemicals. These types of studies are often difficult to identify using the presented search strategy, as the names of all tested chemicals are not always included as search terms.
It should be noted that environmental data for 4-MBC were not reviewed and thus not considered for the assessment.

The quality and reliability of all *in vitro* and *in vivo* studies were assessed, and each study was assigned a reliability score based on the Klimisch categories 1, 2, 3 or 4 (Klimisch *et al*., 1997) combined with an expert judgement statement. • 1: reliable without restriction • 2: reliable with restriction • 3: not reliable or • 4: not assignable

4.10.3 Study summaries

In this section, an overview of the identified *in vitro* and *in vivo* studies are presented. Table 4.2 shows a study overview, describing model, effect modality and conceptual framework (CF) level (OECD Guidance Document 150). Hereafter study summaries are presented, in the same order. These include descriptions of methods, results and assessment of study quality and reliability. The CF level is in accordance with ECHA/EFSA guidance (ECHA/EFSA 2018).
Toxcast data is briefly presented below the table.

Table 4.2: Study overview, sorted by model and modalities.

Reference	Model	EATS or Other	CF level	Klimisch Score	Note
Schlumpf *et al.* 2001	*In vitro* *In vivo*	E	2, 3	2	*The same reliability score is given for both in vivo and in vitro results presented in this publication*
Schreurs *et al.* 2002	*In vitro*	E	2	2	
Tinwell *et al.* 2002	*In vitro* *In vivo*	E	2, 3	2	*The same reliability score is given for both in vivo and in vitro results presented in this publication*
Mueller *et al.* 2003	*In vitro*	E	2	2	
Ma *et al*. 2003	*In vitro*	A	2	2	
Schlumpf *et al.* 2004a	*In vitro*	E	2	2	
Schmutzler *et al.* 2004	*In vitro* *In vivo*	T	2, 3	2 & 3	*The in vivo part of this study was assigned a reliability score of 3*
Gomez *et al.* 2005	*In vitro*	E	2	2	
Morohoshi *et al.* 2005	*In vitro*	E	2	2	
Heneweer *et al.* 2005	*In vitro*	E	2	2	
Klann *et al.* 2005	*In vitro*	E	2	2	
Matsumoto *et al.* 2005	*In vitro*	E	2	4	*Publication is in Japanese*
Schreurs *et al.* 2005	*In vitro*	E, A, other (progesto-genic)	2	2	
Kunz *et al.* 2006	*In vitro*	E	2	2	
Kunz & Fent 2006	*In vitro*	E, A	2	2	
Schmutzler *et al*. 2007	*In vitro*	T	2	4	*Refers to new data in poster abstract (Schmutzler et al. 2006)*
Minh *et al.* 2008	*In vitro*	E	2	2	
Schmitt *et al.* 2008	*In vitro*	E	2	2	
Hofmann *et al.* 2009	*In vitro*	T	2	2	
Nashev *et al.* 2010	*In vitro*	A, S	2	2	
Song *et al.* 2013	*In vitro*	T	2	2	

Jiménez-Díaz *et al.* 2013	*In vitro*	E, A	2	2	
Schiffer *et al.* 2014	*In vitro*	Other (CatSper)	2	2	
Yin *et al.* 2015	*In vitro*	Other (progesto-genic)	2	2	
Jocsak *et al.* 2016	*In vitro*	E	2	2	
Rehfeld *et al.* 2016	*In vitro*	Other (CatSper)	2	2	
Rehfeld *et al.* 2018	*In vitro*	Other (CatSper)	2	2	
Ashby *et al.* 2004	*In vivo*	E	3	2	
Seidlova-Wuttke *et al.* 2006a	*In vivo*	E, T	3	3	
Seidlova-Wuttke *et al.* 2006b	*In vivo*	E	3	3	
Carou *et al.* 2008	*In vivo*	E, A, S	3	2	
Carou *et al.* 2009a	*In vivo*	E, A, S	3	3	
Carou *et al.* 2009b	*In vivo*	E, A, S	3	3	
Schlumpf *et al.* 2004b	*In vivo*	Other	-	4	*Only qualitative reporting (arrows indicating increase/ decrease) but no actual results.*
Durrer *et al.* 2005	*In vivo*	E, A, S	3	2	
Durrer *et al.* 2007	*In vivo*	E, A, S	4	2	
Maerkel *et al.* 2005	*In vivo*	E	4	3	
Maerkel *et al.* 2007	*In vivo*	E, T	4	2	
Hofkamp *et al.* 2008	*In vivo*	E, A, S	4	3	
Schlumpf *et al.* 2008a	*In vivo*	Other	1	4	*Summary of existing data. New data on 4-MBC in rat- and human breast milk*
Schlumpf *et al.* 2008b	*In vivo*	Other	-	4	*Only qualitative reporting (arrows indicating increase/ decrease) but no actual results.*
Faass *et al.* 2009	*In vivo*	E, S	4	2	
Unpublished, 2004	*In vivo*	E, A, T, S	4	2	*OECD TG 421, rat*
Unpublished, 1984a	*In vivo*	E, A, T, S	4	1	*OECD TG 408, oral rat*
Unpublished, 1983a	*In vivo*	E, A, T, S	4	1	*17-day oral study, rat*
Unpublished, 1983b	*In vivo*	E, A, T, S	4	2	*28-day oral study, rat*
Unpublished, 2003	*In vivo*	T	4	3	*14-day study conducted in dogs*
Unnamed, 2003	*In vivo*	T	4	3	*21-day study conducted in dogs*
Unpublished, 2005	*In vivo*	E, A, T, S	4	1	*OECD TG 411, dermal rat*

Unpublished, 1995	*In vivo*	T	3	2	*Conducted in humans*
Janjua *et al*. 2004	*In vivo*	E, A, S	3	2	*Conducted in humans*

A search in **ToxCast Dashboard**[3] was conducted on May 6th 2021, using the search term '4-MBC' and Cas# '36861-47-9'. The search was performed in both the TOXCAST/TOX21 database, and using the EDSP21 (Endocrine Disruption Screening Program for the 21st Century) Dashboard. This dashboard was developed by the US EPA to help evaluate chemicals for endocrine-related activity, and reports results from the ED relevant assays performed in ToxCast. It was therefore chosen here to report results from the EDSP21 dashboard only.
For all assays reporting activity of 4-MBC in the EDSP21 database, a cytotoxicity limit was set to a value of 12.82 µM, based on mean the cytotoxicity caused by 4-MBC in 15 *in vitro* systems with different approaches. In the ED relevant assays, all reported AC50 values were above this cytotoxicity limit. Even though this cytotoxicity limit was not specific of the cell lines where the endocrine activity was measured, the validity of the obtained results may be questioned. It was therefore chosen not to include results from the EDSP21 assays in the lines of evidence and ED assessment of 4-MBC, but just briefly report the EDSP21 findings below:

4-MBC showed estrogen receptor agonism in 1 out of 6 assays with an AC50 of 13.18 µM (TOX21_ERa_LUC_VM7_Agonist). No ER antagonism was reported. Androgen receptor (AR) antagonism was seen in 2 out of 8 assays, with the following AC50 values; 36.31µM (TOX21_AR_BLA_Antagonist_ratio) and 44.56 µM (TOX21_AR_LUC_MDAKB2_Antagonist _0.5nM_R1881). No AR agonism was reported. 4-MBC showed no effect on steroidogenesis in the two performed aromatase assays (CYP19A1 assay). The thyroid hormone data for 4-MBC showed TR antagonism in one out of two assays, with an AC50 of 54.79 µM (TOX21_TR_LUC_GH3_Antagonist), and TSHR agonist activity in 2 out of 9 assays, with the following AC50 values; 51.59 µM (TOX21_TSHR_HTRF_Agonist_ch1) and 44.11 µM (TOX21_TSHR_Agonist_ratio).

[3] CompTox Chemicals Dashboard | TOXCAST Chemicals (epa.gov)

4.10.3.1 Study summaries; *in vitro* studies

Schlumpf *et al.* 2001

The estrogenic effects of 4-MBC were investigated in two *in vitro* assays, the E-Screen based on cell proliferation activity in human mammary tumor cell line MCF-7 at six concentrations of 4-MBC ranging from 10^{-7} to 5 x 10^{-5} M (0.025-12.7 mg/L) and induction of estrogen-regulated pS2 protein levels in MCF-7 cells at 5, 10 and 50 µM 4-MBC. Treated cells were incubated in test medium, and 17β-estradiol (E2) was used as a positive control. Cell proliferation was measured after six days incubation and optical density measured by spectrophotometry as a count of cell numbers. pS2 protein levels were determined by radioimmunoassay. 4-MBC acted as partial estrogenic agonist, with a proliferative effect (PE) of 13.49 and a relative PE compared to E2, of 79.54, reaching around 88% of maximal E2 activity. Statistically significant effects on MCF-7 cell proliferation occurred at 4-MBC concentrations of 5 x 10^{-6} (1.27 mg/L) and above. A 50 % effective concentration (EC50) of 3.02 µM (0.77 mg/L) was determined. The PE was completely blocked by the pure estrogen receptor (ER) antagonist ICI 182780, indicating an ER-mediated effect. In terms of pS2 protein expression 4-MBC produced a statistically significant increase at 10 µM, which also indicates an ER-mediated effect. 4-MBC was 5 to 6 orders of magnitude less potent than E2.

Study assessment: This study is allocated a reliability rating of 2, reliable with restrictions. The study used a standard study design which was reported in sufficient detail, and was scientifically acceptable.

Schreurs *et al.* 2002

The *in vitro* estrogenic and anti-estrogenic activity of 4-MBC was investigated in a HEK293 reporter gene assay. The authors did not report whether cytotoxicity was measured in the study design. 4-MBC was found to activate the human estrogen receptor (hER)α at 10 µM and 100 µM, which resulted in luciferase activity of approximately 65% and 55%, respectively, of maximal E2 induction. 4-MBC was also found to activate the hERβ receptor at 10 µM and 100 µM, which resulted in luciferase activity of approximately 10% and 75%, respectively, of maximal E2 induction. The study reported activation of the hERα and β receptors by 4-MBC. No clear dose-dependent antagonistic effect was seen for either ERα or ER β.

Study assessment: This study was assigned a reliability score of 2, reliable with restrictions for determination of estrogenic activity in vitro. This experiment used a well-established approach. The study was reported in sufficient detail, and even though there was no reporting of cytotoxicity it was scientifically acceptable.

Tinwell *et al.* 2002

Three *in vitro* assays were reported in this publication in order to assess the estrogenic effects of 4-MBC. An *ER competitive binding assay* was conducted using the cytosol isolated from uteri of 25-day-old female Alpk:APfSD rats. The uterine cytosol was incubated with 4-MBC and 3H-E2. 10-fold dilutions of E2, Diethylstilbestrol (DES) and 4-MBC (5 x10^{-10} to 5 x10^{-4} M) were tested. The authors conducted two experiments, each with duplicate dose levels of 4-MBC and of vehicle alone (Dimethylsulfoxid, DMSO) to ascertain a 100% binding value. The top dose of 4-MBC tested (5 x10^{-4} M) was the apparent limit of its solubility in the assay medium. 4-MBC reproducibly displaced E2 from ER at the highest tested dose (5 x10^{-4} M), but no more than 20% displacement could be achieved. Since the test substance was approaching its aqueous solubility

limits the authors questioned whether the apparent competitive binding of the substrates to ER was artifactual, and concluded that the data provided equivocal evidence that 4-MBC binds competitively to ER.
In a *yeast ER transactivation assay* (yeast estrogen screen – YES), a DNA sequence encoding the human ER was integrated into the genome of the *Saccharomyces cerevisiae* yeast strain that also contained an expression vector in which an ER response element was cloned upstream of the reporter gene *LacZ* (encoding the enzyme β-galactosidase). The top doses used in the assay were 10^{-3} M for 4-MBC and 10^{-8} M for E2. 4-MBC reproducibly, but only marginally, increased optical absorbance, and the authors also classified this response as equivocal, but concluded that toxicity had not been a limiting factor. The plateau nature of the assay response to 4-MBC made the authors conclude that re-solution of the agent into the medium may have become the limiting factor in the assay response.
In an E-Screen *cell proliferation assay,* MCF-7cells were cultured for 5 days, then incubated with the appropriate concentration of 4-MBC for 6 days and counted visually using a hemocytometer. Proliferation of MCF-7 cells was increased by incubation with 1-10 µM 4-MBC for 6 days in culture. The results were representative of several experiments. At concentrations above $2x10^{-5}$ M 4-MBC, a statistically significant decrease in the number of viable cells was observed, indicating that 4-MBC may be cytotoxic to these cells at high concentrations. Maximal effects on MCF-7 cell proliferation was observed at 10 µM 4-MBC in this system; these levels of proliferation were similar to those reported for 4-MBC by Schlumpf *et al.* 2001. In summary, 4-MBC showed clear estrogenic activity in MCF-7 cells but only equivocal evidence of estrogenicity *in vitro* in ER binding and yeast transactivation assays.
Study assessment: This study was assigned a reliability score of 2, reliable with restrictions. The study used standard and well documented assays which were all reported in sufficient detail and scientifically acceptable.

Mueller *et al.* 2003
The estrogenicity of 4-MBC was evaluated in a series of assays using Ishikawa cells, as well as primary rat and human hepatocytes. First the authors examined the ability of E2 and 4-MBC to activate alkaline phosphatase (AP) in human endometrial Ishikawa cells, a well-characterized surrogate marker for estrogenic activity. There was statistically significant induction of AP activity (2.8 ± 0.5 fold) compared with the vehicle control at the highest dose of 10 µM (2.5 mg/L), indicating weak estrogenic activity. The activity was inhibited by the anti-estrogen ICI 182780 confirming that the observed induction of AP was mediated by ER. Secondly the binding affinity of 4-MBC to endogenous ER in Ishikawa cells was analysed. In this assay there was no binding affinity of 4-MBC to endogenous ER, i.e. there was no inhibition of specific binding of labelled E2 to ER (IC50 > 8.2 µM (2.1 mg/L). This result was consistent with the findings from a commercially available ER binding kit used by the authors, in which no binding affinity for either estrogen receptor α (ERα) nor estrogen receptor β (ERβ) was observed up to the highest dose tested (IC50 > 3mM).
Subsequently, the authors analysed the ability of 4-MBC and the potent estrogen DES to not only bind to, but also activate ERα or ERβ in Ishikawa cells. Transactivation studies were performed using mammalian expression plasmids for human ERα or human ERβ and the 3xERE-Luc reporter construct. No activity was observed up to a dose of 1 µM 4-MBC; however, in the dose range of 10 µM up to 150 µM (highest dose was limited due to cytotoxicity) a concentration-dependent increase in transactivation in parental Ishikawa cells as well as in Ishikawa cells with

additional transfected ERα or ERβ was observed. Interestingly, 4-MBC showed a higher potency to activate ERβ compared to ERα. Overall, these results showed that 4-MBC had a weak potency to activate ERα and to a higher extent ERβ in Ishikawa cells. Presence of the anti-estrogen ICI 182780 resulted in an abrogation of activity, thereby providing evidence that the measured transactivation was mediated by ER. In contrast to its weak agonistic activity on ERα and ERβ, 4-MBC effectively inhibited the activity of DES to induce ERα or ERβ in a dose-dependent fashion. These results indicate that 4-MBC possesses marked antagonistic properties on the ER. 4-MBC showed no increased estrogenic potency in transactivation studies with rat or human hepatocytes compared to Ishikawa cells, indicating that estrogenic metabolites were not formed.
Study assessment: This study was assigned a reliability score of 2, reliable with restrictions. This experiment used well-established approaches and the study was reported in sufficient detail, and was scientifically acceptable.

Ma *et al*. 2003

The potential androgenic and anti-androgenic activities of 4-MBC were investigated in an *in vitro* MDA-kb2 assay. MDA-kb2 cells are human breast cancer cells stably transfected with a luciferase reporter gene that is driven by an androgen response-element containing promoter. This cell line expresses high levels of functional endogenous androgen receptor (AR) and also glucocorticoid receptor. ERα and the progesterone receptor (PR) are not detectable at the RNA level and ERβ is only expressed at low levels. Cells were incubated overnight in the presence or absence of dihydrotestosterone (DHT; 0.1 or 0.5 nM) to distinguish androgenic and anti-androgenic activity at test concentrations of 1×10^{-7} to 1×10^{-4} M (0.025-25 mg/L). Cytotoxicity was monitored using the MTT dye reduction assay. 4-MBC did not result in any androgenic or anti-androgenic effects in this assay at the tested concentrations.
Study assessment: This study was assigned a reliability score of 2, reliable with restrictions. The study used a well-established and well documented assay. The study was reported in sufficient detail, and was scientifically acceptable.

Schlumpf *et al.* 2004a (data also shown in Seidlova-Wuttke *et al.* 2006a)

The potential estrogenic activity of 4-MBC was investigated in three different assays. The E-Screen using a 96 well plate version of the assay and cell proliferation was measured by cell counts derived from optical density readings. The EC50 value for cell proliferative activity was reported as the mean from 5 independent experiments. ER binding affinity was investigated in recombinant ERα and ERβ ligand-binding assays, and additionally binding of 4-MBC to proteins in cytosolic porcine extracts of uteri was also tested, as the authors considered that there may be unidentified E2 binding proteins present within the uterine cytosol. In the E-Screen, an EC50 of 3.99 µM (1.01 mg/L) was determined for 4-MBC with the maximum increase as a percentage of E2 being 58.7%.
4-MBC bound to the recombinant ERβ preparation with an EC50 of 35.3 µM (8.9 mg/L) as well as to the cytosolic preparation of the uterus with an EC50 of 112 µM (25.4 mg/L), but not to the ERα protein (EC50 > 10^{-3}or 254 mg/L). 4-MBC was 4 orders of magnitude less potent than E2 to displace the radioactive tracer from the recombinant ERβ and 6 orders of magnitude less potent in the cytosolic-binding assays.
Study assessment: This study is allocated a reliability rating of 2, reliable with restrictions. The E-Screen is well-established in the literature. The assay was reported in sufficient detail and even though there was no reporting of cytotoxicity it was scientifically acceptable.

Schmutzler *et al.* 2004

This manuscript primarily reported *in vivo* findings but also reported the results of an *in vitro* thyroid peroxidase (TPO) assay. Human FTC-133 thyroid carcinoma cells were stably transfected with a plasmid coding for human TPO. The assay was performed three times in duplicates. 4-MBC was added to TPO-containing protein extracts at concentrations ranging from 1 nM to 100µM, but did not inhibit TPO activity.

Study assessment: The in vitro part of the study was assigned a reliability score of 2, reliable with restrictions. The study used well-established assay and was reported in sufficient detail.

Gomez *et al.* 2005

The estrogenic activity of 4-MBC was investigated in a reporter gene assay, using ERα and ERβ. The human HeLa cell line was used. The luciferase assays were conducted at 4-MBC concentrations between 1 x 10^{-7}M to 1 x 10^{-5}M (0.0254-2.54 mg/L). Cytotoxicity was not monitored. 4-MBC caused an estrogenic response as it resulted in activation of the ERα cell line, with approximately 40% transactivation at concentrations of 3 x 10^{-6}M and above, relative to treatment with 1 x 10^{-8}M E2. A slight (<30%) activation of the ERβ receptor was observed in the same concentration range.

Study assessment: This study was assigned a reliability score of 2, reliable with restrictions. It used a well-established and well documented assay. Even though there was no reporting of cytotoxicity, the study was well reported, and scientifically acceptable.

Morohoshi *et al.* 2005

The estrogenic activity of 4-MBC was evaluated in two *in vitro* assays: An enzyme linked immunosorbent assay (ELISA)-based estrogen competitive receptor binding assay (ER-ELISA) and a modified yeast two-hybrid estrogen assay with and without addition of a rat liver preparation, S9 mix. The ER-ELISA assay is a colourimetric assay that measures the binding affinity of chemicals to the hERα. From the graphs, 4-MBC seemed to exhibit an estrogenic response, but the authors stated that 4-MBC caused no estrogenic activity because it impaired the antibody-antigen reaction in the ELISA assay indicating a toxic effect. 4-MBC also caused no agonist activity with or without S9 mix in the yeast assay and there was no antagonist activity with or without out S9 mix. No cytotoxic effect was observed. However, the highest concentration in the present study was only 37.5 µM for ER-ELISA and 10 µM for the yeast two-hybrid assay.

Study assessment: The study is allocated a reliability rating of 2, reliable with restrictions. The methods used, whilst non-standard are well documented and scientifically acceptable.

Heneweer *et al.* 2005

The effects of 4-MBC were investigated on estrogen-regulated pS2-gene transcription in MCF-7 cells (E-Screen). The test concentrations were not specified in the text but there were six doses with a maximum concentration of 10 µM (2.5 mg/L). E2 was used as positive control. There was a concentration-dependent increase in pS2-gene transcription with 4-MBC, showing an estrogenic response. The maximum increase in pS2 gene transcription was approximately 3- to 4-fold above control levels, which was similar to that of E2. A full concentration–response curve was not obtained for 4-MBC and an EC_{50} could not be determined. 4-MBC caused a 50% increase in basal pS2 gene transcription at a concentration of 1.9 µM (0.48 mg/L).

Study assessment: This study was assigned a reliability score of 2, reliable with restrictions. It used a well-established approach, and even though there was no reporting of cytotoxicity, the study was well reported, and scientifically acceptable.

Klann *et al.* 2005

The study aim was to clarify estrogenicity of 4-MBC by three experimental approaches: (1) by investigating the effects on cell proliferation in MCF-7 cells (E-Screen), (2) by testing ER binding in cytosolic preparations of amphibian liver cells, and (3) by testing its ability to induce an ER-dependent gene in isolated amphibian hepatocytes. MCF-7 breast cancer cells in 24-well plates were treated with 17*β*-E2 (1 µM), 4-MBC (10 µM), or vehicle (ethanol). After 18 h, the cell proliferation index was determined. When grown in phenol-red-free medium containing charcoal/dextrane-treated serum, ethanol-treated control cells showed a proliferation index of 4.5 +0.9%. In cells cultured in the presence of E2, proliferation index was significantly elevated to 13.8±3.1%. Exposure to 4-MBC resulted in a similar increase in proliferation rate as compared to the control, and reached a level of 11.5±4.1%. In MCF-7 cells grown in full RPMI medium (Roswell Park Memorial Institute – growth medium) and treated with vehicle (ethanol) for 18 h, proliferation index was 15.2±2.1% (n=6). In MCF-7 cells cultured in the presence of 17*β*-E2, the proliferation index was significantly elevated to 21.0±1.1% (n=6). Addition of 4-MBC resulted in an increase in the portion of DNA-synthetizing cells to a level of 22.2±3.8% which was significantly different from the control. These results indicate that 4-MBC in µM concentrations accelerated cell proliferation rate in MCF-7 human breast cancer cells.

In the ER binding assay, hepatocytes of the South African clawed frog *X. laevis* were used to determine 4-MBC's potential to bind to the cytosolic ER. These cells express ERs that bind E2 with high affinity. Competitive binding experiments using [^{3}H]-E2 as a tracer and unlabeled E2 as the competitor showed that displacement of the radiotracer by 10 µM of E2 was complete. When 4-MBC was used as the competitor, displacement of tracer was observed at concentrations higher than 10 µM. However, the tracer could not be entirely displaced by 4-MBC, even at very high concentrations up to 1 mM. This indicates that 4-MBC does bind specifically to cytosolic E2 binding sites, but binding affinity is moderate to low compared with that of the endogenous agonist.

A gene induction assay was used to determine whether 4-MBC acts as an agonist to the ER. Hepatocytes of male *Xenopus* frogs were isolated and cultured in the presence of E2 (positive control, 1 nM–10 µM), 4-MBC (1 nM–1 mM), or ethanol as solvent control and measured induction of an estrogen-responsive gene in comparison with a gene product of an estrogen-insensitive gene, elongation factor 1α. Treatment of cultured hepatocytes with E2 significantly induced the ER gene by more than 1.5-fold in the concentration range 1 nM–10 µM in comparison with the vehicle-treated control cells. In contrast, 4-MBC at 1 nM was ineffective in inducing the ER gene. At concentrations of 0.1 and 10 µM, however, 4-MBC induced the ER gene almost as strongly as E2 at the respective concentrations (n=5 experiments). The cells did not tolerate 4-MBC well in the culture medium at a concentration of 1 mM, since many cells detached from the bottom of the culture dish and were lost during the culture period, which may explain why there was no significant ER gene induction observed in these cells. The authors conclude that 4-MBC has the potential to weakly bind to endogenous ER of vertebrates and to activate ER-dependent signaling mechanisms leading to altered gene expression.

Study assessment: This study was assigned a reliability score of 2, reliable with restrictions. The study used a non-standard, but well-established and well documented assay. The study was well

reported, and scientifically acceptable.

Matsumoto *et al.* 2005

This publication is in Japanese, but does show tables and figures in English. Some information on the study methods can be derived from these tables, but no details are available. 4-MBC was investigated in a MCF-7 cell proliferation assay (E-Screen), where it was shown to act as an ER agonist by causing a significant increase in the three highest tested concentrations. Cell growth of the MCF-7 cells was reduced when ER antagonist ICI182780 was added. 4-MBC was also weakly positive using the CHOOSER assay, measuring alkaline phosphatase activity by Chooser cells, although the increase was only significant in the highest tested concentration. The study also confirmed binding of 4-MBC to ERα and ERβ using a human ER competitive binding assay, but with 10-30 times lower potency than Bisphenol A and 30-100 times lower potency than Benzophenone-2.

Study assessment: This study was assigned a reliability score of 4, not assignable, because apart from figures and tables the study was reported in Japanese. The study however seemed to use well-established assays.

Schreurs *et al.* 2005

The *in vitro* (anti-)androgenic and (anti-)progestogenic activity of 4-MBC, was investigated in the AR- Chemical Activated Luciferase gene Expression Assay (CALUX) and progesterone receptor (PR) CALUX assays. The cell line employed consisted of U2-OS cells, which were stably transfected with either the human androgen receptor (hAR) or the human PR (hPR). Both assays were based on the transactivation of the hAR or the hPR, and the subsequent induction of luciferase. Antagonistic activity was determined by measuring the inhibitory effect of 4-MBC following co-incubation of the cells with 0.1 nM (1×10^{-10} M) DHT (for anti-androgenic activity) and 30 pM (3×10^{-11} M) of the progestin ORG2058 (for anti-progestogenic activity). The methods do not specifically state the tested concentration range, but the figures indicate that the substances were tested in the range of 10^{-9} to 10^{-5} M. Each assay was run as three separate experiments with each concentration tested in triplicate. The paper does not mention whether cytotoxicity was monitored. 4-MBC was found to have an antagonistic effect on the AR with and 50% inhibitory concentration IC_{50} of 7.1 µM and an antagonistic effect on the PR, with an IC50 of 0.9 µM**.** 4-MBC did not show any agonistic androgenic or progestogenic activity. The paper also includes method for (anti-)estrogenicity, as measured in HEK293 reporter cell lines stably transfected with ERα and ERβ. To measure anti-estrogenicity, cells were incubated with both 4-MBC and E2 concentrations of 3 and 100 pM for hERα and hERβ, respectively. As positive controls for ER antagonism, 4-hydroxytamoxifen and ICI 182,780 were used. Data on 4-MBC from the assay with HEK93 cells have been published previously (Schreurs *et al*. 2002), but in the present paper IC50 values were provided. 4-MBC showed agonism toward hERα (IC50 of 6.2 uM), and toward hERβ (IC50 of 14 uM) whereas no anti-estrogenic effects were seen.

Study assessment: This study was assigned a reliability score of 2, reliable with restrictions. The study was not conducted according to an international guideline, but used well-established transactivational based assay. The study failed to report cytotoxicity of dose levels tested.

Kunz *et al.* 2006

This study investigated the *in vitro* estrogenic activity of 4-MBC in a yeast transactivational assay. A recombinant yeast (Saccharomyces cerevisiae) was stably transfected with the human

ERα. The tested concentration range was not specifically stated, but the figures indicate that 4-MBC was tested in the range of 10^{-9} to 10^{-2} M (0.00025-2543 mg/L). Cytotoxicity, manifested by significantly reduced yeast growth or cell lysis, was monitored. The results are expressed as a percentage of the maximal effect produced by the dose–response curve of E2. 4-MBC did not result in any effects on hERα activation, indicating that it did not have any agonistic estrogenic activity in this yeast transactivational assay.
Study assessment: This study was assigned a reliability score of 2, reliable with restrictions. The study used a well-established yeast based assay. The study was well-documented and scientifically acceptable.

Kunz & Fent 2006
The *in vitro* (anti-)estrogenic and (anti-)androgenic activity of 4-MBC was investigated in transactivational assays. The recombinant yeast (S. cerevisiae) was employed, which was stably transfected with either the hERα or the hAR. Transactivation led to induction of β-galactosidase, causing a colour change of the substrate. The antagonistic activity was measured in the assay by the addition of the natural ligands E2 or DHT, respectively, at a concentration that produced 65% of the maximal response, followed by the addition of 4-MBC or the antagonistic standards 4-hydroxytamoxifen or flutamide, respectively. The tested concentration range was 10^{-7} to 10^{-2} M (0.0025 - 25437 mg/L). Cytotoxicity was controlled for in this study by measurement of yeast cell growth (turbidity measurements). 4-MBC did not cause any cytotoxicity in any assay up to the highest tested concentration of 10^{-2} M. No estrogenic transactivation was measurable in the estrogen agonism assay, but clear anti-estrogenic activity was observed. Complete inhibition of E2-induced activity was measured at the highest concentration tested and a full dose-response curve was obtained. The IC_{50} was determined as 8.73×10^{-5} M (22 mg/L), which represented a potency 190 times lower than the known anti-estrogen 4-hydroxytamoxifen. No androgenic transactivation was measured, but clear anti-androgenic activity was observed. A full dose-response curve with complete inhibition of DHT was observed in the anti-androgenic assay. The IC50 was determined as 1.18×10^{-5} M (3 mg/L), which represents a potency 3 times less than the anti-androgen reference standard flutamide.
Study assessment: This study was assigned a reliability score of 2, reliable with restrictions. The study used a well-established and well documented assay. The study was well reported, and scientifically acceptable.

Schmutzler *et al.* 2007
This publication is mainly a review paper. The only new information it provides is the following: After 5 days of incubation with 4-MBC, iodide uptake into FTRL-5 cells was inhibited at concentrations of 0.1 and 1.0 µM.
Study assessment: Unfortunately, the Schmutzler et al. 2007 manuscript only refers to a poster abstract (Schmutzler et al. 2006) for the original description of methods and results, as the data from this in vitro assay have not been published elsewhere. Since the poster abstract has not been subject to peer review, this study was assigned a reliability score of 4, not assignable.

Minh *et al.* 2008
The authors constructed a vector system containing the promoter region of the Xenopus laevis vitellogenin A2 gene including estrogen response elements, and the genes for firefly luciferase. This vector was transfected into other cell lines. Cells which had stably integrated the vector-

DNA into the genomic DNA were selected. SiG12 cells responded to treatment with ER ligands, including 4-MBC, which in this study was chosen as a positive control for estrogenic activity. Relative luciferase-mRNA abundance was determined by RT-PCR after 24 h treatment with ER-ligands or ethanol. 4-MBC significantly induced luciferase gene transcription, as a consequence of ER-activation in the SiG12 cells. Means of the data obtained with E2, Bisphenol A and 4-MBC were significantly different from the mean of the vehicle-treated control but not significantly different among each other. Hence all tested ER ligands activated luciferase expression and activity in SiG12 cells in a concentration range between 1 x 10^{-11} and 1x10^{-6} M. The EC50 value of 4-MBC was 6 x10^{-9}.
Study assessment: This study was assigned a reliability score of 2, reliable with restrictions. The study used a non-standard, but well documented assay. Even though there was no reporting of cytotoxicity, the study was well reported, and scientifically acceptable.

Schmitt *et al.* 2008
The estrogenic activity of 4-MBC was investigated in a yeast transactivation assay (YES assay). A recombinant yeast (Saccharomyces cerevisiae) was used. The assay used a stably transfected hERα, and is based on the induction of β-galactosidase, following activation of the receptor. Eight replicates per concentration were tested, but cytotoxicity was not reported. The positive control was E2. 4-MBC attained only 8% of maximal E2 response. However, the two highest concentrations of 4-MBC (100 and 300 µM) showed a significant higher response in β-galactosidase activity compared to the negative control. The EC_{10} was reported as 25.9 µM (6.59 mg/L) and the EC_{50} was as 44.3 µM (11.3 mg/L). The EC_{50} value was much higher than for E2, but the results did indicate an intrinsic activity of the hERα in the yeast cells.
Study assessment: This study was assigned a reliability score of 2, reliable with restrictions. The study used a well-established yeast-based assay. Even though there was no reporting of cytotoxicity, the study was well reported, and scientifically acceptable.

Hofmann *et al.* 2009
The effects of 4-MBC on the thyroid system were investigated in two mechanistic assays, 1) a TRα luciferase-based reporter assay and 2) expression of deiodinase type I (DI01) as an endogenous triiodothyronine (T3) target gene, both using the human hepatocarcinoma cell line HepG2. The assays were used to assess both the agonistic and antagonistic (i.e. by co-incubation T3) effects. There was significant activation of TRα1-cotransfected cells by 4-MBC with a lowest observed effect concentration (LOEC) of 1 µM (0.25 mg/L). The increase was 1.3-fold compared with the solvent control. The reference substance T3 resulted in a 122-fold induction. 4-MBC acted antagonistically in the competitive activation assay with a LOEC of 10 µM (2.5 mg/L) and with a 5-fold change reported. 4-MBC alone significantly increased DI01 expression but DI01 was not affected by co-exposure with 1 nM T3. These assays suggest some agonist and antagonist activity of the thyroid system.
Study assessment: The study is allocated a reliability score of 2, reliable with restrictions. The non-standard methodology used while new is well described in sufficient detail and scientifically acceptable.

Nashev *et al.* 2010

The enzyme 17β-hydroxysteroid dehydrogenase type 3 (17β-HSD3) catalyses the last step of testosterone synthesis in testicular Leydig cells and plays an essential role during male sexual development. The enzyme 17β-HSD5 catalyses the conversion of androstenedione (AD) to testosterone in testis and prostate. 17β-HSD2 converts testosterone to AD and E2 to estrone (E1) and is expressed in uterus, liver, kidney, intestine and adipose tissue, and 17β-HSD1 is important for the formation of E2 from estrone but can also convert AD to testosterone and is expressed in placenta, uterus, breast and adipose tissue. These enzyme activities were measured in Human Embryonic Kidney HEK-293 cells transfected with plasmids expressing human 17β-HSD1, 2, 3 and 5. For assessment of 17β-HSD3 & 5 activity the HEK-293 cells were incubated with 20 µM of 4-MBC and 200 nM AD for 45 min, followed by determination of testosterone formation. To assure that the observed inhibition was not caused by cell death, cytotoxicity was assessed. HEK-293 cells were also transfected with plasmids for human 17β-HSD1 and 17β-HSD2. Here the cells were incubated with 200 nM radiolabeled E2.
These experiments showed that 4-MBC concentration-dependently inhibited 17β-HSD3 mediated conversion of AD to testosterone, with estimated IC50 of 10.7 µM. 4-MBC did not inhibit 17β-HSD5, but did inhibit17β-HSD1 and 17β-HSD2 activity. 17β-HSD2 was inhibited with an IC_{50} of 5.9 µM. 17β-HSD1 was weakly inhibited by 4-MBC (IC_{50} of 70 µM)
Additionally, a gene transactivation assay for the human androgen receptor was performed. Here HEK-293 cells were transfected with plasmid for human AR, MMTV-lacZ β-galactosidase reporter and pCMV-LUC luciferase transfection control. After 24 h, cells were incubated with 0.2 nM testosterone and 4-MBC for 24 h, and analysed for luciferase and β-galactosidase activities. 4-MBC did not activate the AR at concentrations up to 20 µM, but did act in an anti-androgenic manner by inhibiting testosterone-dependent AR activation (84 % AR activation compared to testosterone).
Study assessment: This study was assigned a reliability score of 2, reliable with restrictions. This experiment used well-documented approaches and was scientifically acceptable.

Song *et al.* 2013

The aim of this study was to identify biomarkers that could be used for detecting deiodinase deficiency status on the cellular level. Gene expression profiles were compared between deiodinase knockdown (DKD) human neuroblastoma (SH-SY5Y) cells and T3-treated SH-SY5Y cells. Using qRT-PCR, the expression of the selected candidate biomarkers was tested after the exposure of cells to well-known deiodinase-disrupting chemicals for 48h, including 4-MBC. After cells were exposed to deiodinase-disrupting chemicals, no T3 or thyroxine (T4) was detectable in cell lysates or culture supernatants. Furthermore, the expression of the potential biomarker genes was upregulated or downregulated. 10 genes (ID2, ID3, CCL2, TBX3, TGOLN2, C1orf71, ZNF676, GULP1, KLF9, and ITGB5) were identified as useful biomarkers to identify the disruption of deiodinase activity upon exposure to environmental chemicals. Four of these (CCL2, TBX3, TGOLN2, C1orf71) showed 4-8 times induction after incubation with 60 µM 4-MBC, indicating that 4-MBC may affect deiodinase activity.
Study assessment: The study is allocated a reliability rating of 2, reliable with restrictions. The non-standard methodology used while new is well described in sufficient detail and scientifically acceptable.

Jiménez-Díaz *et al.* 2013

Interactions of 4-MBC with the hERα and hAR were investigated using two *in vitro* bioassays based on reporter gene expression and cell proliferation assessment. For the E-Screen bioassay, MCF-7 cells were used. In each experiment, a dose–response curve for E2 and a negative control were included. Agonistic assays were performed in the presence of increasing concentrations (0.01–10 μM) of 4-MBC. Tests were done in triplicate for each concentration. Results were expressed as proliferative effect. The antagonistic activities were determined by co-incubation with the agonist E2 at 100 pM.
Agonistic activity of hAR in PALM cells was tested in the presence of increasing concentrations (0.01–10 μM) of the tested chemicals. Tests were performed in quadruplicate for each concentration. Results were expressed as a percentage of maximal luciferase activity. Maximal luciferase activity (100%) was obtained in the presence of 10 nM R1881. The antagonistic activities of these chemicals were determined by co-incubation with R1881 (0.3 nM). In all performed assays, 4-MBC did not show cytotoxic activity, and the percentage of viable cells ranged from 95% to 100%, in the concentration range tested (0.01–10 μM).
In the E-screen, 4-MBC (10 μM) increased the number of cells by approximately 2.8-fold (EC_{50} = 24.14 μM). 4-MBC did not antagonize E2-induced proliferation in MCF-7 cells. 4-MBC did not show agonistic AR activity (tested in the concentration range of 0.01–10 μM). When the AR antagonistic activity was tested, 4-MBC proved to be potent hAR antagonist at 10 μM concentration (IC_{50} = 9.12 μM, respectively), strongly inhibiting the luciferase activity induced R1881. In conclusion 4-MBC showed estrogenic activity in the E-Screen bioassay and potent hAR antagonism.
Study assessment: This study was assigned a reliability score of 2, reliable with restrictions. The study used a well-established and well documented assay. The study was well reported, and scientifically acceptable

Schiffer *et al.* 2014
The effects of 4-MBC on activation of the sperm-specific CatSper channel, which is usually controlled by progesterone and prostaglandins, was investigated. Activation of the CatSper channel induces Ca^{2+} influx which is believed to control sperm motility and acrosomal exocytosis (transport of a secretory granule which is a physiological requirement for fertilization). The study was conducted with human and mouse sperm. The study measured changes in Ca^{2+} flux, sperm motility based on the flagellar beat (asymmetry and frequency) of head-tethered sperm and acrosomal exocytosis. Progesterone (2 μM) was used as a positive control. Inhibition of the Ca^{2+} signalling was also assessed using the known inhibitor MDL12330A (100 μM). Concentrations of 0.1, 1 and 10 μM (0.025, 0.25 and 2.5 mg/L) 4-MBC were used for Ca^{2+} flux assays, whereas 6.8 and 30 μM (1.7 and 7.6 mg/L) were used for the other assays (sperm motility and acrosomal exocytosis). The exposure duration for these tests is short (seconds). The results were reported based on up to four separate experiments. 4-MBC had no effect on Ca^{2+} flux in mouse sperm. In human sperm, at 0.1 and 1μM, 4-MBC evoked a biphasic increase in Ca^{2+} flux similar to that observed with progesterone whereas at 10 μM the response was more sustained. The EC_{50} for inducing Ca^{2+} flux was 6.83 ± 2.26 μM (1.73 mg/L). The response was inhibited 96.64% by the CatSper inhibitor MDL12330A. The results suggest that 4-MBC competes with progesterone for CatSper activation. 4-MBC was found to lower the frequency and enhance the asymmetry of the beat of human sperm flagella at concentrations of 6.8 and 30 μM. Acrosomal exocytosis was found in 25–40% of sperm exposed to 4-MBC at 6.8 μM, which was similar to the response with progesterone.

Study assessment: The study is allocated a reliability rating of 2, reliable with restrictions. The non-standard methodology used while new is well described in sufficient detail and scientifically acceptable.

Yin *et al.* 2015

This *in vitro* study used human endometrial epithelial adenocarcinoma Ishikawa cells to investigate anti-progestogenic activity of 4-MBC (test concentrations from 10^{-8} to 10^{-5} M). This was compared with the effect of three selective PR modulators. Effects of 4-MBC in combination with progesterone on the progesterone-sensitive target gene estrogen sulfotransferase (SULT1E1) were obtained by RT-qPCR. The SULT1E1, which plays a critical role in the inactivation of estrogens and in the pathogenesis of estrogen dependent tumors, was identified as the most responsive marker in Ishikawa cells to anti-progestogenic effects by gene expression profiling in the research group's previous work. The induction of progesterone on SULT1E1 mRNA levels by progesterone was concentration-dependently antagonized by RU486, UPA and ZK137316, whereas 4-MBC had no effect on SULT1E1 mRNA levels, indicating no anti-progestogenic effect in this test system.

Study assessment: This study was assigned a reliability score of 2, reliable with restrictions. The study used a non-standard, but well documented assay. The study was reported in sufficient detail, and is scientifically acceptable.

Jocsak *et al.* 2016

This study investigated if 4-MBC affects ERβ mRNA expression in primary cerebellar cell cultures. Primary rat cerebellar cell cultures were divided into two main groups: "glia containing" (Glia+) and "glia reduced" (Glia−). In Glia+, both 4-MBC and 4-MBC + E2 + T3 treatment resulted in an increase in ERβ transcription, with a two-fold increase after 4-MBC (not significant), and a three-fold increase after 4-MBC + E2 + T3 treatment. In Glia−, there was a two-fold decrease in ERβ mRNA levels after 4-MBC- and a seven-fold decrease after 4-MBC + E2 + T3 administration. The study showed that 4-MBC affected ERβ mRNA expression in these cell cultures.

Study assessment: This study was assigned a reliability score of 2, reliable with restrictions. The study used a non-standard, but well documented assay. The study was reported in sufficient detail, and scientifically acceptable.

Rehfeld *et al.* 2016

In this study 4-MBC was investigated for its ability to induce Ca^{2+} signals in human sperm cells. Suboptimal Ca^{2+} influx is associated with reduced male fertility, and correct CatSper function is absolutely essential for fertilization. This study used human non-capacitated sperm cells exposed to between 10^{-3} and 10^{3} µM (0.00025 and 254 mg/L) 4-MBC for up to 232 seconds. The results were expressed based on three separate experiments. 4-MBC was among the most potent of all 29 tested UV filters, and at 10 µM the mean relative maximal induction of Ca^{2+} signal was 97% of progesterone response at 5 µM. The EC_{50} for 4-MBC was 0.52 ± 0.31 µM. The Ca^{2+} signals induced by 4-MBC was fast and transient and resembled the Ca^{2+} signal induced by progesterone, suggesting a similar mode of action between this UV filter and progesterone.

Study assessment: The study is allocated a reliability rating of 2, reliable with restrictions. The non-standard methodology used, whilst new, is well described in sufficient detail and scientifically acceptable.

Rehfeld *et al.* 2018

Progesterone released by the cumulus cells surrounding the egg induces a Ca^{2+} influx into human sperm cells, via the CatSper Ca^{2+} channel, and thereby controls sperm function. 4-MBC (and other chemical UV filters) have been shown to induce a Ca^{2+} influx through CatSper, thus mimicking the effect of progesterone on Ca^{2+} signalling. This study hypothesised that 4-MBC could also mimic the effect of progesterone on sperm function. The study was conducted using a single concentration of 10 µM (2.5 mg/L) 4-MBC along with a positive control (progesterone at 5 µM) and a negative control (0.2% DMSO, maximum). The endpoints were sperm viability, acrosome reaction, sperm penetration with methylcellulose and hyperactivation measured using computer assisted semen analysis (CASA).

There was a significant increase in viable acrosome reacted sperm cells following exposure to 10 µM 4-MBC, similar to the effect observed with progesterone. The observed increase in sperm penetration in 4-MBC exposed sperm was not statistically significant. There were no significant effects of 4-MBC on hyperactivation or sperm viability. In conclusion, chemical UV filters that mimic the effect of progesterone on Ca^{2+} signalling in human sperm cells can similarly mimic the effect of progesterone on acrosome reaction and sperm penetration.

Study assessment: The study is allocated a reliability rating of 2, reliable with restrictions. The non-standard methodology used, whilst new, is well described in sufficient detail and scientifically acceptable. There was only a single test concentration.

4.10.3.2 Study summaries; *in vivo* studies from open literature

Study summaries are organized according to year of publication, and for each study, the quality is assessed.

Schlumpf *et al.* 2001

An uterotrophic assay was performed using immature Long-Evans rats. Beginning on postnatal day (PND) 21, female pups (n=9-19) in 4-MBC dosed groups and 28 controls, received chow containing several concentrations of 4-MBC (0, 66, 119, 211, 337, 402, 1980 mg/kg bw/day) for 4 days, until PND 25. Test chemical (including 4-MBC) were dissolved either in acetone or in 99% ethanol and added to powdered chow. Body weight was recorded at the beginning and at the end of the treatment period. Mean body weights of experimental groups were in the same range as that of the controls (initial weight 38.0 ± 4.5 g, final weight 48.8 ± 3.8 g). At the end of the treatment period, the animals were decapitated under light ether anesthesia. The uterus was excised and weighed. 4-MBC treated animals exhibited a dose-dependent increase in uterine weight which was statistically significant from at a dose of 119 mg/kg bw/day and had an effective dose $(ED)_{50}$ of 309 mg/kg bw/day.

In the same publication, results from another uterotrophic study were obtained using dermal application of 4-MBC in immature females (n=4-10) of the Rat Nu (hairless) strain (Ico: OFA hr/hr). Female rat pups were treated on 6 consecutive days. From PND 21 to PND 26, 4-MBC (2.5%, 5.0%, 7.5% (w/w) in olive oil) or olive oil (vehicle control) was applied twice daily at an interval of 3–4 hr. On PND 27, the animals were weighed and decapitated under light ether anesthesia. The uterus was removed and weighed. The treatment group was unknown to the person dissecting the uterus. Total amounts of 4-MBC applied to the skin per day were calculated to be 137.5, 275, and 412.5 mg for 2.5, 5.0, and 7.5% 4-MBC concentrations, respectively. The dose absorbed from the 5% solution in oil was by the study authors tentatively calculated as 37 mg/kg bw/day. Following dermal application of 4-MBC in olive oil twice daily for 6 days, immature rats of the hr/hr (hairless) strain exhibited an increase in uterine weight, with a significant increase induced by 5% and 7.5% 4-MBC. The mean uterine weight of the 5% 4-MBC group was also significantly higher than that of the 2.5 or 7.5% groups, yielding a bell-shaped dose–response curve. The control uterine weight of this strain appeared to be slightly lower than that of Long-Evans rats, even though the animals were 2 days older.

Study assessment: The study is generally well described, but no CAS-number or purity of the chemical is given. In spite of this the study is assessed to be of high quality (2, reliable with restrictions).

Tinwell *et al.* 2002

19 to 20 days old (38–50 g) female Alpk:APfSD (Wistar-derived) rats (n=12) were allowed to acclimatize for 24 hours and hereafter either dosed orally with 4-MBC doses of 0, 500 or 800 mg/kg bw/day for three days or dosed subcutaneously (s.c.) using doses of 0, 500 or 1000 mg/kg bw/day for three days. Each female received a single oral or s.c. injections on each of 3 days (PND 20–21 to 23–24). DES was used as a positive control agent for the uterotrophic assays (5 µg/kg). All animals were terminated 24 hours after the last of three daily administrations. The uterus was trimmed, blotted and weighed. Then it was dried overnight (24 hours) at 70°C, and reweighed.

DES exposure resulted in marked increases in uterine weight (300-400%) in the absence of

evident toxicity. 4-MBC was also clearly active in the immature rat uterotrophic assay by both routes of exposure. Both blotted and dry uterine weights were increased in all test groups ($p < 0.01$). Clinical signs of toxicity were observed following the third oral administration of 800 mg/kg 4-MBC. At termination, a statistically significant reduction in body weight gain was seen in both groups exposed to 4-MBC by oral gavage. No indications of toxicity or adverse effects on body weight were seen in females exposed to 4-MBC by s.c. injections. In the orally dosed animals the uterine weight increases were 50 and 90 % in the two 4-MBC dose groups respectively, compared to controls, whereas no dose response pattern was seen in the two s.c. dosed groups. Here both 4-MBC dose groups had increases in uterine weight of approx. 30%.
Study assessment: The study is generally well described, uses a relatively high group size for an uterotrophic assay (n=12), is well reported and is assessed to be of high quality (2, reliable with restrictions).

Ashby *et al.* 2004

This publication shows results from several uterotrophic tests with 4-MBC, along with results from other test chemicals which were tested for ED properties using different test methods. For the uterotrophic studies with 4-MBC, female Alpk:APfSD (Wistar derived) rats received an s.c. injection on 3 consecutive days and were killed 24 hours after the last dose. Unfortunately, the group size used in the different experiments was not mentioned, making the reliability of the results harder to assess. 4-MBC was given at 0 or 1000 mg/kg; 5 µg/kg DES was used as a positive control. Some of the studies also included additional groups of animals exposed to 4-MBC together with the gonadotropin-releasing hormone (GnRH) antagonist ANT. For the different experiments, animals of different ages at the start of the uterotrophic assays, were used. All animals were killed 24 hours after the last dose. The uterus was removed, trimmed free of fat, blotted and weighed. It was then dried overnight and reweighed.
In the first experiment 4-MBC failed to induce a significant increase in uterine weight, which was in contrast to the uterotrophic results that had previously been reported from the same laboratory (Tinwell *et al.* 2002). In the Tinwell *et al.* 2002 study, the authors used a group size of 12, so it is plausible that a comparable group size was used in the new experiments, but it cannot be known with certainty. The first experiment showed no increase in uterine weight after exposure to 4-MBC, but the control uterine weights were markedly heavier than usual: 35.1 ± 5.9 compared with 23.3 ± 4.3 in the previous study. Thus, a repeat of this study was undertaken: Here, control uterine weights were within normal range, and 4-MBC induced a significant (33%), increase in uterine weight. This activity was abolished by the GnRH inhibitor ANT, suggesting that the uterotrophic effects were mediated centrally, possibly via a temporal advance in puberty rather than via a direct effect of 4-MBC on the uterus. Two further experiments were performed in which the effect of the initial age of the rat was investigated. In both studies, the response to 4-MBC was highly significant when using rats that were 19-20 days old at the beginning of the study (26-29% increase in uterine weight); the increase in uterine weight after 4-MBC exposure was still significant but smaller with 20-21 days old animals (18-19% increase), and the response was not significant when the older animals (21-22 and 22-23 days old) were used. In conclusion, in all but one experiment (which had an abnormally high uterine weight in control animals) statistically significant increases in uterine weight were seen in 4-MBC treated animals compared to the controls, as long as the initial age of the test animals was 19-21 days old (the age recommended in the test guideline for the uterotrophic assay). Hence, in spite of the message the authors try to convey in the abstract and discussion, this study actually corroborates the

findings of an uterotrophic effect of 4-MBC.
Study assessment: A large drawback in the description of these studies is that the number of animals used in the different experiments is not provided anywhere in the publication. However, the same authors have previously published an uterotrophic study using a group size of 12, and compare the present results to those, so it is likely that a similar group size was used here. And even if a smaller group size was used in the present studies (the OECD test guideline for the uterotrophic assay only mandates a group size of 6), most of the experiments performed in this publication shows statistically significant increases in uterine weight after exposure to 4-MBC. Therefore, the overall conclusion that can be drawn based on these results is that in immature female rats exposed to 4-MBC an uterotrophic response is induced. Inclusion of more animals that those already used in these studies, would probably only make the statistical significance larger. Apart from lacking animal number, the remaining details regarding the study design are relatively well reported and therefore the study is assessed to be of median quality (2, reliable with restrictions).

Schmutzler *et al.* 2004
Adult ovariectomized Sprague Dawley (SD) rats (n=8-11) were treated with 4-MBC (or other suspected endocrine disrupters nonylphenol and Octyl-methoxycinnamate (OMC) in the feed for 12 weeks, on the background of either a soy-free or soy-containing diet. Dose levels were listed as 2.5 & 12.5 g/kg 4-MBC, and calculated to 66 and 310 mg/animal/day, but no animal body weight was provided. In the present publication endpoints relevant for regulation via the thyroid axis were measured. At both doses of 4-MBC thyrotropin (TSH) levels were statistically significantly elevated (both with and without soy in the feed). T4 serum levels were statistically significantly decreased whereas T3 levels were increased without soy but decreased using feed with soy. In the liver, type I 5-deiodinase was decreased at both doses, but the changes were not statistically significant.
Study assessment: The description of the study design is relatively poor. No CAS-number or purity of the chemical is given, it is not clear exactly how many animals are included in each dose group, and even more importantly no information on systemic toxicity or animal body weights during the study or calculation of dose in mg/kg bw/day is provided. The variability in the hormonal measurements seems large and hormonal effects are not very consistent using the two different types of feed. Reporting of thyroid gland weight and histopathology would have markedly improved the publication. The study is assessed to be of low-median quality (3, not reliable).

Seidlova-Wuttke *et al.* 2006a
Adult ovariectomized SD rats (n=12) were treated with 4-MBC in the feed for 12 weeks. A soy-free diet was used. Dose levels of 4-MBC were only stated as 57.5 and 250 mg/animal /day. Animal weight at the beginning of the study were 250 g and the animals gained between 60-100 gram during the experiment, meaning that the doses can be calculated to be approximately, 180-230 mg/kg bw/day in the low dose group and 750-1000 mg/kg bw/day in the high dose group. This calculation was not performed in the publication. Other dose groups in the study were exposed to either OMC or E2.
4-MBC caused a small but significant reduction in animal weight gain, whereas the positive control E2, caused a very marked decrease in body weight gain. Both doses of 4-MBC increased uterine weights almost equally (by about 50%), but according to this publication the effect was

only significant in the low dose group. For comparison, E2 increased uterine weight by about 700%. Both doses of 4-MBC decreased T4 levels, and increased TSH, while non-significant increases were seen for T3. No dose-response relationship was observed for any of the thyroid parameters, and on these parameters E2 had no effect. Opposite the effect of E2, both doses of 4-MBC caused significantly increased serum LH levels. 4-MBC did not affect serum cholesterol levels or low-density-lipo protein levels, but both doses caused reductions in the size of fat depots and reductions in serum leptin levels.
The authors concluded that 4-MBC exposure had profound effects in the hypothalamo–pituitary–thyroid axis, in fat tissue and on serum lipids, but many of the effects were not similar to those exerted by E2.
Study assessment: The description of the study design has some serious inadequacies, especially when analysed together with the information from the Seidlova-Wuttke 2006b publication. One of the authors has confirmed that the data in these two publications stem from the same animal study. There are however some marked differences in the study description, especially related to the chemical exposure. In addition, some of the same results (uterine weights) are presented twice in the two publication but with different conclusions regarding statistical significance, even though the same statistical methods are described in both publications. No CAS-number or purity of the chemical is given, no calculation of dose in mg/kg bw/day is provided (and hence needs to be calculated by the reader). The study is therefore assessed as being not reliable (score 3).

Seidlova-Wuttke *et al.* 2006b
Adult ovariectomized SD rats (n=12) were treated with 4-MBC in the feed for 12 weeks. Doses were shown as 57.5 and 250 mg/animal/day, but animal weights were not provided in the publication. It is not stated anywhere in the publication, but based on correspondence with Professor Körhle, one of the study's authors, it has been confirmed that the data provided in this publication is generated in the same animal study as the one reported in the Seidlova-Wuttke et al. 2006a publication. Based on this information it can be derived that the low and high dose levels of 4-MBC were in the range of 230 and 1000 mg/kg bw/day respectively. In the present publication, both doses of 4-MBC were also shown to cause increased uterine weights, but now only significantly so in the high dose group. The graph showing this data was almost identical to the one in the 2006a publication, except that the only significant effect on uterine weight occurred in the low dose group. Nowhere in the publications do the authors refer to the other publication or mention that results from the same animal experiment were reported twice, but with different conclusions regarding the statistics. This publication specifies that the high dose of 4-MBC resulted in reduced gain of body weight during the first 6 weeks of treatment. Therefore, these animals were set to the low dose of 4-MBC. They experienced a catch-up in weight gain such that, at the end of the experiments, the weights of the animals did not differ from those of the controls. This information is not included in the 2006a publication, even though the results presented in the two publications stem from the same animal study.
In the uterus and vagina, both doses of 4-MBC affected histopathology (increased epithelium thickness), but markedly less than E2. In the bone, 4-MBC shared the anti-osteoporotic effects of E2 but the mechanism of action of 4-MBC appeared to be different than of E2. Slight effects on three estrogen-regulated genes in the uterus (PR, Insulin-like growth factor-1 and ERβ) were observed under 4-MBC, while E2 had profound stimulatory (PR and IGF) or inhibitory (ERβ) effects on these parameters.
Study assessment: The description of the study design is poor. No CAS-number or purity of the

chemical is given, no information on systemic toxicity and animal body weights during the study are provided, and no calculation of dose in mg/kg bw/day is present. Most importantly it is not stated anywhere in the publication that the data have been generated in the same in vivo study as reported in the 2006a-publication, even though some of the same data (uterine weights) seem to be reported twice. The study is assessed to be of low-median quality (3, not reliable).

Carou *et al.* 2008

Wistar male adult rats (n=10-12) were either injected s.c. with 4-MBC (purity 99.9%) for 5 days (at doses of 2 or 10 mg/kg) or for 2 days (at doses of 2 and 20 mg/kg). In all rats serum prolactin, LH and FSH concentration were measured. The hypothalamus of rats injected during 2 days were also dissected to study GnRH release. Rats that received 2 and 10 mg/kg of 4-MBC for 5 days showed a decrease in the LH and FSH serum concentration. In rats injected for 2 days, serum LH decreased with 2 and 20 mg/kg and FSH decreased with 2 mg/kg. Ex vivo hypothalamic GnRH release also decreased in these animals. Prolactin levels were unaffected. These results show that low doses of 4-MBC may inhibit the reproductive axis in adult male rats. A MoA suggested by the authors is facilitation of the negative feedback mechanism produced by estrogens in the hypothalamus, with the following decrease of gonadotropin concentration. In addition, 4-MBC may act as an estrogen agonist by changing the expression of estrogen receptors in the hypothalamus.

Study assessment: The study is well described and assessed to be of high quality (2, reliable with restrictions).

Carou *et al.* 2009a

4-MBC was administered s.c. to female rats from pregnancy onset to the day of birth in doses of 20, 100 and 500 mg/kg. The injections were given every second day. One experiment was run with the low dose exposure, and one experiment with the two high doses. It is stated that 15 dams were treated with 4-MBC, but not how many controls were included in the studies. Elsewhere in the manuscript the group sizes are reported to be between 8-13. The litter is most likely not viewed as the statistical unit. The male offspring were sacrificed at postnatal days 15 or 30 to determine testicular weight, gonadotropin and prolactin serum levels and also GnRH and amino acids release from the hypothalamus (*ex vivo*). The exposure to 20 mg/kg bw/day did not affect hormone levels in the male offspring, apart from a significant increase in LH in 30-day old male offspring. The variation in this group was very high, making it impossible to assess whether this change was caused by a single or at few very high outlier values, or if the LH concentrations in this group were generally higher. Doses of 100 and 500 mg/kg bw/day were stated to cause a decrease in absolute testicular weight on PND 15. Unfortunately, no information regarding offspring body weights (neither on PND 15 nor 30) nor any information on systemic toxicity were provided in the manuscript, and the unit provided when stating that absolute testes weights were reduced was 'mg/100 g bw'. The two high doses caused decreased LH, GnRH and glutamate levels, in prepubertal rats (15-day-old specimens), but increased gonadotropin (LH and FSH) concentration and aspartate levels in peripubertal rats (30-day-old specimens), without changes in testicular weight. Prolactin levels were unaltered in all dose groups. The results indicate that administration of 4-MBC during development may affect the endocrine system in male rats during the prepubertal and pubertal stages.

Study assessment: This study has substantial shortcomings. The number of animals used is stated as n= 8-13, but it is impossible to evaluate how many litters these offspring came from,

but probably no more than 5 in each dose group - and litter effects do not seem to have been included in the statistical analysis of the data. The authors state that the doses used correspond to those previously tested (Tinwell et al. 2002), but in the present study s.c. injections were only given every other day and not each day like in the Tinwell study. Most of the hormonal results and the results on testes weight results are written in the main manuscript text rather than in tables or figures, making it very difficult to get an overview of the results, and generally the reporting of the study is rather poor. The study is poorly described and assessed to be of low quality (3, not reliable)

Carou *et al.* 2009b

The 4-MBC was administered s.c. to female Wistar rats (n=10-13) during pregnancy, in a dose of 100 mg/kg every second day. Offspring were weaned on PND21 and timing of vaginal opening (VO) and estrous cyclicity (results not reported) were investigated in the female offspring. The litters were sacrificed at 70 days to determine gonadotrophin serum levels, uterine weight and ex vivo GnRH release from the hypothalamus.

The male rats showed a marked (40-50%) decrease in serum LH and FSH concentrations in adulthood, and an 80% decrease in GnRH secretion. The 4-MBC exposed female rats were reported to have a 3 days advance on the VO, but no change in adult uterine weight. There was a very marked increase in serum LH (400%) and FSH (90%) concentrations, whereas hypothalamic GnRH release was not modified. The authors concluded that prenatal administration of 4-MBC disrupted the gonadal axis in a sexual dimorphic mode that could be connected with the physiological sexual differences in the development of gonadotrophin secretion hypothalamic control mechanisms.

Study assessment: In the present study a group size of 10-13 dams per group was used, and only one male and female per litter were assessed. Unfortunately, only one dose group was included, and therefore no dose-response relationships can be made. The authors state that the dose used corresponds to one previously tested (Tinwell et al. 2002), but in the present study s.c. injections were only given every other day and not each day like in the Tinwell study. Very little data on maternal and developmental toxicity is provided, and unfortunately the results on VO are written in the main manuscript text rather than shown in tables or figures, making it very difficult to get an overview of the results. Also, body weights of the offspring should have been provided when reporting results related to VO. 3 days early sounds like a rather substantial effect, but it is difficult to assess it properly. Generally, the study is poorly described and is assessed to be of low-median quality (3, not reliable).

One research group has published 6 publications on the endocrine disrupting effects of developmental 4-MBC exposure in rats (Durrer *et al.* 2005; Durrer *et al.* 2007; Maerkel *et al.* 2005; Maerkel *et al.* 2007; Hofkamp *et al.* 2008; Faass *et al.* 2009), and additional three review articles (Schlumpf *et al.* 2004b; Schlumpf *et al.* 2008a, 2008b). The three reviews only summarize results in a qualitative way (arrows indicating direction of the observed effects) and the result provided in these publications have therefore not been used in the lines of evidence. The results provided in the 6 publications with original data seem to stem from the same series of developmental toxicity studies with 4-MBC. The studies have been performed in several blocks, and some doses have been repeated and the results combined with previously obtained data, in order to obtain a larger group sizes. Some of the tested doses also seem to have been

tested separately. Unfortunately, often it is difficult to assess exactly which of the results are from the same animal study, because it has not been reported very clearly in the different publications exactly how many developmental toxicity studies with 4-MBC have been performed. The overall study design used in these studies is described in detail here, and therefore not repeated under the description of each of the publications from this research group.
The studies investigated effects of pre- and postnatal exposure of Long Evans rats to 4-MBC (7, 24, 47 mg/kg bw/day) (0.1, 0.33, 0.66 g/kg chow) administered in chow to the parental generation 10 weeks before mating, during gestation and lactation. Exposure of the offspring continued until adulthood (12 weeks of age). An original dose of 70 mg/kg bw/day was discontinued because of markedly reduced postnatal survival of F1 (reported in Schlumpf *et al.* 2004b).
The original study included a control group (n=8 litters) and 3 treatment groups (n=5-6). An additional study investigating a lower dose of 0.7 mg/kg (0.01 g/kg chow) (n=4), used a concurrent control group (n=5). This was presented in the Durrer *et al.* 2005 paper and these animals were not included in the results on gene expression in the brain (Maerkel *et al.* 2005, 2007). Offspring were weighed on PND 2, 4, 6, 9, 12, 13, 14, at puberty onset and in adulthood. For this endpoint (and possibly also some other endpoints) additional litters were included into the study (reported in Durrer *et al.* 2007). Hence the group size for this endpoint was n=17, 12, 13 and 12 litters in the 4 dose groups respectively. Approximately 3 offspring/sex were examined from each litter. The offspring were examined in the perinatal period for reproductive organ weight changes on PND 14 (results not shown in any publication apart from qualitatively in males in Schlumpf *et al.* 2004b paper). The offspring were investigated for timing of sexual maturation (females from day 30, males from day 41) (Durrer *et al.* 2007) and for reproductive organ weight at the age of 12 weeks (females in diestrus). Offspring survival rate, righting reflex, anogenital distance (AGD) and eye opening are reported in a PhD thesis (Durrer 2004), but have not been published in a manuscript and are unfortunately not publically available. An additional estrogen challenge was performed in the adult offspring. In order to assess possible changes in sensitivity to estrogens, a cohort of adult offspring (both male and female) were gonadectomized on day 70, had a single injection with E2 (at 10 or 50 µg/kg) or vehicle on day 84, and sacrificed 6 hours later.

Schlumpf *et al.* 2004b

This review paper reported the first results from this study. Unfortunately, results were only reported qualitatively, i.e. stated if endpoints were increased/decreased or unaltered in exposed offspring compared to controls, but did not show any actual measurements/values. Neither did the authors inform on group sizes used or the fact that the 0.7 mg/kg dose group was run in a separate experiment than the other dose groups. Based on the results provided in this publication it is not possible to assess properly the data. Fortunately, the results are properly reported in later publications.
Study assessment: In the present publication no actual values are shown, and a lot of information on study design, group sizes and methods is missing. The quality of the underlying animal study itself is assessed to be moderate but the presentation of the results in the present manuscript is poor. This study was assigned a reliability score of 4, Not assignable.

Durrer *et al.* 2005

This publication reported how 4-MBC exposure (0.7, 7, 24 and 47 mg/kg bw/day), affected estrogen regulated gene expression in the uteri of adult female offspring. The study showed that 4-MBC altered mRNA levels encoding for ERα, ERβ, PR and the androgen receptor, determined by RT-PCR in the uterus. Western-blot analyses of the same tissue homogenates indicated changes in ERα but not ERβ and only PR proteins in the 0.7 mg/kg dose group. In adult ovariectomized (OVX) and E2 treated offspring, acute up-regulation of PR and down-regulation of ERα and AR by E2 were reduced in 4-MBC-exposed rats. The reduced response to E2 was accompanied by reduced coactivator SRC-1 mRNA and protein levels. The data indicated that developmental exposure to 4-MBC affected the regulation of estrogen target genes and the expression of nuclear receptor co-regulators in uterus at mRNA and protein levels, but also showed that different genes were differently affected at different doses, and generally no clear dose-response relationships were observed. Absolute and relative uterine weights and body weights of 4-MBC exposed rat offspring at 12 weeks of age did not differ significantly from untreated controls, except for the 24 mg/kg dosage group, which showed a slight increase in absolute and relative uterine weights.

Study assessment: The original developmental study is not very well described, e.g. no information on toxicity was provided, whereas the endpoints investigated in this report seem to be reported thoroughly. Regarding group size for the investigated gene expression endpoints, it is stated to be between 6-9, and for body and uterus weight n=10-25, but it is not stated how many litters these offspring originate from, and litter effect is not included in the statistical analysis of the data. Overall, the study is assessed to be of median quality (2, reliable with restrictions).

Durrer *et al.* 2007

This paper reports effects of 4-MBC exposure on sexual maturation of male and female offspring and subsequent effects on male reproductive development. 4-MBC delayed onset of male puberty (preputial separation) in a dose-related manner. The effect was marked and statistically significant in the 7, 24 and 47 mg/kg groups, with delays of around 3 days in the high dose group. The male body weights of 4-MBC exposed offspring's at preputial separation were at control levels. This could indicate a general developmental delay caused by exposure of 4-MBC, since exposed males became sexually mature 2-3 days later than control males, when they reached a weight of around 180 grams. In females timing of VO was not statistically significantly affected, i.e. VO occurred at a mean age of 38-39 days in all dose groups. Yet, the female body weight at this age was statistically significantly smaller in all 4-MBC exposed groups compared to controls. This indicated that in females, the age of the animals and not their weight seemed to be determining for when they reached sexual maturation.

In this publication, additional litters were included for assessment of reproductive organ weights (reaching an n= 21-34, from 7-11 litters per group). Unlike many of the other publications on developmental toxicity, it was in this paper specified for each investigated endpoint how many individual animals had been assessed and from how many litters. 4-MBC caused dose-dependent decreases in absolute and relative prostate weights from a dose of 7 mg/kg and above (both when analysed for individual animals and for litter means). The two higher doses of 4-MBC also statistically significantly increased testis weight. In the high dose group a non-statistically significant decrease in epididymis weight was observed and the same was true for seminal vesicle weight. At these doses liver weight were unaffected (data not shown).

AR, insulin-like growth factor-1 (IGF-1), ERα and ERβ expression in prostate were altered at mRNA and protein levels, with stronger effects in dorsolateral than ventral prostate. To assess sensitivity of target genes to estrogens, offspring were castrated on postnatal day 70, injected with E2 (10 or 50 µg/kg, s.c.) or vehicle on postnatal day 84, and sacrificed 6 hr later. Acute repression of AR and IGF-1 mRNAs by E2, studied in ventral prostate, was reduced by 4-MBC exposure. This was accompanied by reduced co-repressor N-CoR (nuclear receptor co-repressor) protein in ventral and dorsolateral prostate, whereas steroid receptor coactivator-1 (SRC-1) protein levels were unaffected. The data indicate that 4-MBC affects development of male reproductive functions and organs, with a lowest observed effect on gene expression changes seen at a level of 0.7 mg/kg. Nuclear receptor co-regulators were revealed as targets for endocrine disruptors, as shown for N-CoR in prostate and SRC-1 in uterus. This may have widespread effects on gene regulation.
Study assessment: This publication and its results are assessed to be of median quality, because relatively high group and litter sizes were used for all assessed endpoints, and it was properly reported. It is not possible to determine whether these effects occur due to perinatal exposure to 4-MBC, adult exposure or a combination of both (2, reliable with restrictions).

Maerkel *et al.* 2005
This paper describes results from the same series of developmental toxicity studies with 4-MBC, and focuses on how the developmental and continued exposure to 4-MBC (7, 24 and 47 mg/kg) affects gene expression in the brain. mRNA expression of PR, an estrogen-regulated gene, is investigated by real-time RT-PCR in two brain areas; the medial preoptic area and ventromedial hypothalamic nucleus (VMH). Additionally, the response to an acute E2 challenge in adult gonadectomized offspring is investigated. The study analysed intact 12-week-old male and female offspring under steady state conditions and adult gonadectomized offspring 6 h after a single s.c. injection of E2 (10 or 50mg/kg), in order to assess estrogen sensitivity. At steady state conditions, statistically significantly higher PR mRNA expression in VMH of control females was seen compared to control males. 4-MBC exposed females exhibited a decrease in PR mRNA to levels of control males. The increase in PR mRNA in response to E2 was higher in VMH of males of all 4-MBC groups as compared to control males. PR mRNA levels were similar in MPO of control males and females. Developmental 4-MBC exposure increased PR mRNA levels in male MPO, but did not statistically significantly change female levels. The acute response to the lower E2 dose was decreased in MPO of 4-MBC exposed males, whereas females of the 7 mg/kg dose group exhibited an increased reaction to 50 mg/kg of E2. The data indicate that developmental exposure to 4-MBC can interfere with sexually dimorphic gene expression in brain in a sex- and region-specific manner. Stated by the authors, the induction of the PR mRNA by estrogens in VMH is closely linked with the induction of female sexual behaviour.
Study assessment: The study is assessed to be of low-median quality. The original developmental study is not very well described, e.g. no information on group size or toxicity is provided. Regarding group size for the investigated gene expression endpoints it is stated to be between 6-8/sex, but there is no information on how many litters these animals originally are derived from (3, not reliable).

Maerkel *et al.* 2007
This paper reports effects of 4-MBC exposure on thyroid endpoints in the offspring and mRNA of estrogen target genes involved in control of sexual behaviour, measured in brains of adult

offspring. Gene expression was investigated by real-time RT-PCR in VMH and MPO of the brain. The treatment up to a dose of 47 mg/kg did not affect maternal body weight gain during pregnancy or number of pups. Body weight was slightly reduced in offspring of both sexes at 47 mg/kg on PND 14 but no longer at 12 weeks of age. On thyroid endpoints, the dose of 7 mg/kg had no effect. At higher doses absolute and relative thyroid gland weights were increased in adult male and female offspring by 25-32% in the 24 mg/kg dose and by 40-53% in the high dose group. Serum TSH levels showed increases, but these were only statistically significant in high dose females (51%). Serum T4 levels were unaffected in both sexes at all tested dose levels, whereas T3 levels showed slight but statistically significant increases in female from the two highest dose groups.
4-MBC exposure affected mRNA levels of ER alpha and PR in a sex- and region-specific manner. In order to assess possible changes in sensitivity of target genes to estrogens, offspring were gonadectomized on day 70, injected with E2 or vehicle on day 84, and sacrificed 6 h later. The acute induction of PR mRNA by E2 was enhanced by 4-MBC in male and female VMH and female MPO, whereas male MPO exhibited reduced responsiveness of both genes. Steroid receptor coactivator SRC-1 mRNA levels were increased in female VMH and MPO. The data indicate profound sex- and region-specific alterations in the regulation of estrogen target genes at brain level. Effect patterns in baseline and E2-induced gene expression in the brain differed from those in uterus and prostate reported previously.
Study assessment: The study is relatively well described and assessed to be of median quality. It looks like all of the results related to the PR mRNA have already been published in the Maerkel et al. 2005 paper, yet this is not mentioned in the present publication.
The group sizes are reported for all assessed endpoints, and are between 6-9 samples for the gene expression results and between 5-21 for the thyroid endpoint. However, the number of examined litters in this study is not included, making difficult to establish how robust the study design is (2, reliable with restrictions).

Hofkamp *et al.* 2008
In a block of animals receiving chow containing low doses of 4-MBC (0.7 or 7 mg/kg bw/day) (n=4), 1-day old male offspring were examined using digital photographs of serial sections. The authors identified, contoured, and aligned the epithelial ducts from specific regions of the developing prostate, plus the accessory sex glands and calculated the total volume for each region from three-dimensional, surface-rendered models. At birth, one male pup from each litter was taken out of the cage, anesthetized and decapitated. The lower part of the body was sectioned, immediately fixed and transferred to the University of South Dakota for final tissue preparation, three-dimensional (3-D) reconstruction, and morphometric analysis. Fetal exposure to 4-MBC (7.0 mg/kg bw/day) resulted in a statistically significant and marked (60-70%) increase in tissue volume in the prostate and accessory sex glands. Treated males exhibited a 62% increase in the number of ducts in the caudal dorsal prostate. Increased distal branching morphogenesis appears to be a consequence of exposure in the ventral region, resulting in a 106% increase in ductal volume. Generally, quite marked increases in size were seen at a dose of 7 mg/kg bw/day. Similar investigations at higher doses would have provided very useful knowledge as to the dose-response relationship of such changes in the male offspring exposed prenatally to 4-MBC.
Study assessment: The study is well described, but due to the low group size (n=4) it is assessed to be of low-median quality (3, not reliable).

Schlumpf *et al.* 2008a

This paper is mainly a review of the effects of 4-MBC. It summarizes knowledge on the ED properties of 4-MBC obtained *in vitro*, in 90-day studies by academia and industry, and in the developmental studies the research group has performed itself. New results provided in the paper included measurements of 4-MBC in rat milk on PND 6 from offspring exposed to 4-MBC doses of 7 and 0.7 mg/kg. The study also presented measurements of 4-MBC in human breast milk samples and comparison of the two. This comparison showed that at the dose of 7 mg/kg, the 4-MBC concentration in rat milk was only 11 times higher than the highest value found so far in human milk, indicating a potential risk caused by this UV filters. Hence, the study provided good evidence that humans (in 2008) had measurable levels of 4-MBC in the breast milk and that these levels were not very different from those measured in rats exposed to 4-MBC doses of 0.7 and 7 mg/kg per day.

Study assessment: Like the Schlumpf et al. 2004b review, the present mix of review and reporting of new data is rather uninformative and its reliability is therefore assessed to be category 4 " not assignable". However, the review did provide clear evidence on human relevance and this part was assessed to be of median quality (2, reliable with restriction).

Schlumpf *et al.* 2008b

This paper is another review paper, summarizing the *in vivo* effects of 4-MBC after developmental exposure. Also, here the results are only shown qualitatively, and the provided information has therefore not been included further in the present assessment.

Study assessment: This publication was assigned a reliability score of 4, Not assignable

Faass *et al.* 2009

This paper reports effects of 4-MBC exposure on estrous cyclicity and sexual behaviour in adult female offspring. Vaginal smears of female offspring (7, 24 and 47 mg/kg) were collected at 3-4 month of age for 21 days. A different batch of animals was used for investigation of female sexual behaviour (in groups exposed to 7 & 24 mg/kg) aged 11-13 weeks. Here estrous cycles were studied for 10-14 days before performing behavioural tests, in order to correctly identify proestrus. Female sexual behaviour (when placed with untreated males) was recorded on videotape in adult female offspring on proestrus evening at the beginning of the dark phase.

Even though more females from the 47 mg/kg group were irregular in their estrous cyclicity compared to control (25% vs. 5%), the difference was not statistically significant. The 4-MBC exposure did, on the other hand, markedly alter the behaviour of these females in a dose-related manner, seen as reduced proceptive behaviour (jump and ear wiggling), reduced receptive behaviour (lordosis quotient), and increased rejection behaviour towards the male. The effects were statistically significant both when analysed on individual basis (n=12-14) and on litter basis (n=6-7) and were statistically significant in both examined dose groups. This behavioural change resulted in only half as many mounts during the 15-minute test in the high dose group compared to controls (22.6 in controls vs. 10.6 in the 24 mg/kg group). The authors conclude that the data demonstrates that female sexual behaviour represents a sensitive target of endocrine disrupters and point to an involvement of PR in VMH. Overall, the study suggests possible alterations in estrous cyclicity, which might have been statistically significant if a larger group size had been used, but strong evidence of adverse effects in terms of altered sexual behaviour in females at doses of 7 and 24 mg/kg. These correlated well with the changes in

gene expression in the brain which the authors state are thought to be linked to sexual behaviour (reported in the Maerkel *et al*. 2007 paper).
Study assessment: The study is well described and assessed to be of median-high quality. The group sizes are reported for all assessed endpoints, both in terms of individual animals and litters (2, reliable with restrictions).

4.10.3.3 Studies in the registration dossier

The following studies from the registration dossier[1] have been included in the performed ED assessment (table 4.3). Each study is described in detail below, and summary results have been included in the assembled lines of evidence.

Table 4.3: Overview of studies from the registration dossier

Study	**Klimisch score**
Enhanced reproductive toxicity screening study in rats, OECD TG 421 (Unpublished, 2004)	2
90-day oral repeated dose toxicity study in rats, OECD TG 408 (Unpublished, 1984a)	1
17-day oral repeated dose toxicity in rats (Unpublished, 1983a)	1
28-day oral repeated dose toxicity study in rats (Unpublished, 1983b)	2
14-day oral tolerability study in dogs (Unpublished, 2003)	3
21-day oral tolerability study in dogs (Unnamed, 2003)	3
Dermal 90-day repeated dose toxicity study in rats, OECD TG 411 (Unpublished, 2005)	1

Additionally, a prenatal developmental toxicity study has been performed (Unpublished, 1988). This study is described in section 4.9.2.

Enhanced reproductive toxicity screening study in rats, OECD TG 421 (Unpublished, 2004)
An 'enhanced' screening reproductive/developmental toxicity study, based on TG421 has been performed. As a deviation from the test guideline, no exposure of parental males was performed. Instead, additional hormonal measurements were included in the study design.
Groups of 10 female Wistar rats (HanBrl:WIST SPF) were dosed with 4-MBC at dose levels of 12.5, 25, or 50 mg/kg bw/day at a standard dose volume of 10 mL/kg of vehicle (hydroxypropyl methylcellulose) by gavage for 28 days prior to mating, during pairing, gestation and lactation periods, amounting to a total of 84 days. Parental males remained untreated (used for pairing only) and F1 offspring were not directly treated with 4-MBC.

Before mating the estrous cycles of the parental females was monitored. Parental females were sacrificed at postnatal day 21. Blood samples were analysed for hormone levels and a range of organs were weighed and prepared for microscopic examination, including uterus, ovaries and thyroid. At birth, offspring body weights and AGD was measured, and on PND 12 nipple retention was registered. During the postnatal period, developmental indices like pinna unfolding, incisor eruption, eye opening and coat development were registered. Also timing of testes descent and preputial separation in the male offspring and VO in the female offspring was registered.

Assessment of neuro-behaviour was performed in male and female offspring using a water maze, testing learning, memory and re-learning. Half the F1 offspring were sacrificed at weaning (PND21), the other half on PND 55. At necropsy special attention was directed to organs of the reproductive system and the thyroid gland. The following organs were weighted and prepared for microscopic examination: uterus, cervix, ovaries, testes, epididymis, seminal vesicle, prostate, pituitary, and thyroid.

In parental females no clinical signs or adverse reactions to treatment were noted, and there were no effects on mortality, body weight gain or food consumption in any dose group. A statistically significant increase in water consumption was recorded for females from all treatment groups throughout the various phases of the study with the highest intake recorded for those treated at 50 mg/kg bw/day.

On day 21 of the pre-paring period, a range of hormonal measurements were performed in parental females. It is worth noting that the group size for these investigations was small (n=3-5), adding a lot of variation to the results. No exposure related differences in T4 levels were seen. TSH concentrations were increased by 15-25 % in the mid and high dose groups, but this was not statistically significant, due to the very large variation in the data. T3 levels showed a dose-dependent increase, but the 25% increase in the high dose was not statistically significant. FSH levels were unaffected in the parental females after 21 days of exposure. LH levels showed a dose dependent decrease, with the high dose group showing a 62% lower concentration than controls. Due to large variation in the data this was not statistically significant. Prolactin levels were elevated in mid and high dose females (100% increases in both groups), and E2 levels showed a 45% increase in the high dose group. None of these effects were statistically significant. Testosterone levels were below level of detection.

There were no effects on any of the reproductive parameters included in the scope of investigations (estrous cyclicity, fertility indices, mating performance, duration of gestation, implantation rate, post-implantation loss, litter size and postnatal pup loss).

After 84 days of exposure, dams (n=10) were necropsied on PND 22 (time of weaning of the pups). No statistically significant effects on the weight or histopathology of reproductive or endocrine organs were noted. It is however worth noting, that the mean thyroid gland weight in the high dose dams was 19% increased, compared to control dams (both for absolute and relative weights). This effect was not statistically significant, most likely due to the relatively large variation observed for this endpoint. This large nominal increase in thyroid gland weight, at a dose which showed no effect on body weight (nominal increase of 1%), indicates that like in many other oral toxicity studies with 4-MBC, the thyroid gland was very likely also a target organ in this study. No exposure-related effects were noted on ovary weights. Uterus weights showed a dose-dependent increase, which reached 8 % in the high dose animals, but was not statistically significant.

On PND 22 hormone levels were investigated on 10 dams per group. T4 and TSH levels were not affected by the treatment at this age. T3 concentrations showed an increase of between 8-11% in all three dose groups compared to controls, but the effect was not statistically significant. At this age testosterone levels were still below the level of detection, while E2 and prolactin

levels were not affected by the exposure. In the high dose group, FSH levels were increased by 260% (i.e. 3.6 times higher than in control animals) whereas LH levels were increased 16 and 34 times (by 1500% and by 3300%) in the mid and high dose groups. Yet the variation in the exposed animals was so high that these differences did not reach statistical significance.

In F1 offspring, no statistically significant effects on body weight were observed during the course of the study. Developmental indices (pinna unfolding, incisor eruption, onset of coat development and eye opening) did not differ from controls. AGD showed statistically significant increases in both male and female offspring exposed to higher doses of 4-MBC. In males the effect was statistically significant in the high dose group (9 % increase, 2.75 mm compared to 2.52 mm in control) and in the females an increase between 9-19 % was seen in all three dose groups. The effect was statistically significant in the low (11% increase compared to control) and high dose (19% increase compared to control) group. Whether or not body weights at the time of AGD assessment were statistically significantly affected by 4-MBC exposure cannot be determined, based on the information provided in the study report. When initially reporting on pup body weights throughout the study, no statistically significant effects were noted on PND 1, in either males or females. In these analyses the litter was used as the statistical unit, as an n=10 is provided in the tables. This is the correct method of analysing this type of data. As specified in Guidance Document 43, on mammalian reproductive toxicity testing and assessment (OECD 2008), statistical analysis of fetal and neonatal data should always be conducted with careful consideration of the influence of litter on analytical outcome. It is critical that littermates are not treated as independent observations in the statistical analysis. For instance, in the analysis of neonatal body weight data, treating as few as 2 litter mates per litter as independent observations, can increase the nominal alpha by a factor of almost 3 (OECD 2008).
In the tables reporting AGD, pup body weights on PND 1 are reported again, but slightly differently than before. These tables show that males from the mid and high dose groups had significantly increased body weight compared to control males and the same pattern is seen for the female pups. Based on these data the relative AGD (i.e. AGD compared to body weight) were no longer statistically significantly affected. The validity of this calculation method, using an n=56-60 as provided in the AGD tables, is questionable. In the analysis performed for AGD assessment, litter was most likely not used as the statistical unit, skewing the results of the statistical analysis of both AGD and body weight. It is unclear why different methodologies were used in different parts of the report, and how inclusion of litter in the statistical analysis of the AGD data would have affected the results.

There were no signs of nipple retention in the males, but the full study report did not report any actual nipple retention results, only a text stating that no males with nipple retention were seen. In the females from the low dose group, VO was seen 1.6 day later than in the control group. This effect was statistically significant. In the mid and high dose groups VO was seen 0.4 and 1.0 days later than in controls. The effects in these group were not statistically significant, and therefore the significant effect in the low dose group was not viewed as a treatment related finding. However, it is possible that 4-MBC dosed female offspring from the low and high dose groups had marginally delayed sexual maturation.
In the males, no treatment-related differences were seen in the timing of testes descent or on preputial separation. These data were correctly analysed using litter as the statistical unit.

In the neurobehavioural test in the water maze, there was no indication of adverse effects on learning or memory function in male or female offspring. This endpoint was correctly assessed using litter as the statistical unit. Unfortunately, the results from the water maze tasks were only assessed in a binary manner, so that each animal was registered as either completing or failing the task. This reduced the sensitivity of the test, compared to e.g. registering continuous endpoints like the time needed to complete the task. Also, it was not assessed whether 4-MBC exposure affected the two sexes differently. As spatial learning is sexually dimorphic (Vorhees and Williams, 2014), sex differences in relation to the effects of 4-MBC remain unknown.

In male offspring on PND 22, no statistically significant effects on organ weights were seen. However, thyroid weights showed a non-statistically significant increase in a dose related manner and in the high dose males it was 33% increased, while body weighs in this group were only 1 % increased. Testes, prostate and epididymis weights were unaffected, whereas seminal vesicle weights showed a 12 % non-statistically significant decrease in the high dose group. The decrease in relative weight was 16%.
In female offspring at the PND 22 thyroid gland weights were unaffected by the exposure. Mid and high dose females showed an increase in ovary weights (25 % in both dose groups), an effect that was statistically significant in the high dose group. Uterus weight showed a dose-dependent decrease that was statistically significant in the high dose groups (39%). However, since histopathological investigation of ovaries and uterus did not show any treatment related effects, the significantly altered ovary and uterus weights were in the study report considered not to be treatment related. A similar pattern was seen for the relative organ weights, since the female body weights on PND 22 were unaffected by treatment. Unfortunately, serum hormone concentrations were no assessed at this age.

Necropsy results from PND 55 males showed no statistically significant effects on the thyroid glands. The majority of the male reproductive organs were unaffected by treatment. The prostate weights showed a 10% non-statistically significant decrease in the high dose animals compared to controls and seminal vesicle weights showed a non-statistically significant 10% increase in low and high dose animals and a statistically significant 20% increase in the mid dose group.

In the PND 55 females no treatment–related effects were observed on the reproductive organ weights nor on the thyroid gland weight. Histological examination revealed no treatment-related findings on ovaries, uterus, testes prostate or thyroid gland.

Hormonal measurements were performed in male and female offspring on day 55. The tables showing the results, report an n=30 for males and females, indicating that litter effects were most likely not taken into account in the statistical analysis of these data. In both male and female offspring, levels of T4 were not affected by the exposure. In high dose males and females T3 levels were decreased (16 and 11% respectively), reaching statistical significance in the males. Also, TSH concentrations were decreased (16%) in high dose males, reaching statistical significance. TSH levels were unaffected in the females.
In the females, no treatment related effects were seen on E2, LH or prolactin levels.
FSH levels were decreased by 61% in the high dose group, but the large variation in the data prevented this decrease from reaching statistical significance.

FSH levels also showed a dose-dependent decrease in males and the reductions of 27% and 56% in the mid and high dose groups were statistically significant, but were for some reason considered incidental and not treatment related in the study report. LH concentrations in the males showed a dose-dependent decrease of 20% and 34 % on the mid and high dose groups, but these reductions did not reach statistical significance. In the males E2 and prolactin concentration were unaffected by treatment. Testosterone concentrations were dose-dependently decreased in all three dose groups, but the reductions of 26%, 33% and 60% in the three groups respectively were not statistically significant, due to very large variation, especially in the control group. The large variation in this group was primarily caused by two very high outlier values, which were 5-7 times higher than the group mean, and one could argue that they should have been excluded from the analysis. That would very likely have caused the observed testosterone decreases to be statistically significant.

Study assessment: The study was only assessed to be of median quality, because even though it was a guideline study, with a robust study design, the study itself had several shortcomings (as discussed in more details above). The fact that very few adverse systemic toxicity effects were seen (no effect on body weights, food consumption, liver weights) indicates that the chosen high dose was too low - as some signs of systemic toxicity should be apparent in the high dose group in order to properly assess reproductive toxicity. Thus, including a higher dose in this study would likely have strengthened the conclusions regarding effects on endocrine sensitive endpoints.

The group size was 10 dams per group, which is comparable to the group size used in several of the developmental toxicity studies from the open literature. The statistical analysis was often inconsistently reported and most likely not always correctly performed. The variation in many of the assessed endpoints (especially some of the hormonal measurements) was often extremely large, which resulted in lack of statistical significance on endpoints which seemed to show a biologically relevant effect. And most importantly, all of the statically significant effects that were seen in the study report were concluded not to be treatment related and were therefore not included in the study summary and conclusions. As a consequence, the study summary provided in the ECHA dissemination site stated that no endocrine related endpoints in dams or offspring were affected by the exposure. However clearly a somewhat different conclusion has emerged from the present evaluation of the data which is provided in the original study report (2, reliable with restrictions).

<u>90-day oral repeated dose toxicity study in rats, OECD TG 408 (Unpublished, 1984a)</u>

Groups of male and female Wistar rats (n=20/sex) were fed 4-MBC in the diet for a 3-month period at varying concentrations in order to maintain a daily intake of 0, 50, 125 or 312 mg/kg bw/day. At the end of the 3-month treatment period, some animals from each treatment group were maintained off-treatment for a period of 1 month to assess the progression or regression of any treatment-related changes. Since no no-effect-level was established in this initial study, a separate study was commenced in male and female rats with a new control group and a new low dietary inclusion concentration equivalent to an achieved dose level of 25 mg/kg bw/day. Thyroid hormone status was examined after 7 weeks of exposure, at study termination and again off in off-treatment period.

The bw of males in all dose groups and females treated with 25 and 50 mg/kg corresponded to the controls. A transiently reduced bw gain was seen in females treated with 125 mg/kg and from the start of the study in females treated with 312 mg/kg bw/day, compared with the

controls. The statistically significant differences were evened out in the off-treatment period. Food consumption was also statistically significantly reduced in females treated with 312 mg/kg. At the dose level of 50 mg/kg bw/day and above there was evidence of a stimulatory effect on the thyroid gland in males and females comprising increases in circulating T3 and TSH levels, increased thyroid weights and histopathological changes of the thyroid. Females were more sensitive to the hormonal effects than the males, whereas thyroid weight changes were seen at lower doses in males than in females. Statistically significant elevated mean TSH concentrations were seen in females at 50 mg/kg bw/day and above (termination) and at 312 mg/kg bw/day (at week 7) and in males at 312 mg/kg bw/day (off-treatment period). Statistically significant elevated mean T3 concentrations were seen in females at doses of 50 mg/kg bw/day and above (week 7 + termination) and in males at 50 mg/kg bw/day and above (off treatment period) and at 312 mg/kg bw/day at termination. Statistically significant increases in mean absolute and relative thyroid weights were clearly observed in males and females treated at 125 and 312 mg/kg bw/day and slightly in males treated at 50 mg/kg bw/day. By end of the off-treatment period, the increased absolute and relative thyroid weights were irreversible in males and females at 312 mg/kg bw/day. The pathological lesions of the thyroid (follicular hyperplasia and hypertrophy, presence of mitosis, loss of follicle roundness and lumen, epithelium stratification etc.) showed some dose dependency and graded as slight to moderate in the groups treated at 50 and 125 mg/kg bw/day and as strongly pronounced at 312 mg/kg bw/day. At the end of the follow-up recovery period the changes in the thyroid were still apparent albeit less pronounced. The increase of the thyroid weight associated with thyroid alterations, were considered as morphologic evidence of thyroid stimulation and varied from mild, moderate to intense. All males and females treated 50 mg/kg and above had histopathological activation of the thyroid by end of the treatment period.

Liver weights were statistically significantly increased in females at 125 mg/kg bw/day and in males at 312 mg/kg bw/day. In high dose males, statistically significant reductions in prostate weight were observed, but prostate histopathological examination were reported as being 'not remarkable.

Apart from a few isolated cases, in which statistically significant deviations were found on T4 levels in rats, the T4 values were normal at the dose level of 25 mg/kg bw/day and above. The rats showed no treatment-related effects at the dose level of 25 mg/kg bw/day and this dose is, therefore, designated the NOAEL.

Overall, the study provides strong evidence of adverse effects on thyroid hormone signalling as well as on prostate weight, after prolonged adult exposure to 4-MBC (hormonal effects seen after 7 weeks and histopathological effects on the thyroid gland seen after 13 weeks).

Study assessment: This was a guideline study and the quality and reliability of the study was high. A large group size was used (n=20/sex) and there was good consistency between the effects on thyroid hormone measurements, thyroid gland weights and thyroid histopathology in both examined sexes, and in a clear dose-related manner (1, reliable without restriction).

<u>17-day repeated dose oral toxicity in rats (Unpublished, 1983a)</u>

Male and female Wi-AF/Han (SPF) albino rats were administered 4-MBC by oral gavage in a 17-day study. Ten rats of each sex were administered 30 or 300 mg/kg bw/day 4-MBC in peanut oil or as control (vehicle only) at a volume of 5 mL/kg/day, once daily on 17 consecutive days. On the final day of treatment (Day 17), a blood sample was taken to determine TSH levels. 24 hours after the final treatment (Day 18), all animals were killed and underwent necropsy. 4-MBC

was clinically tolerated at 30 mg/kg bw/day and could be considered as the NOAEL. The only statistically significant finding in the rats treated at this dose level was the reduced weight of the prostate. At 300 mg/kg bw/day, statistically significant effects included reduced prostate weight, increased thyroid gland weight (140% in males and 160% in females), increased serum TSH (1.9 fold in males and 7.5 fold in females), increased kidney weights (males only), increased adrenal gland weights (males only) and reduced thymus weight (females only). Hypertrophy of the follicular epithelium with occasional incisures of the thyroid gland was also observed pathologically (4/20 in controls, 8/20 at 30 mg/kg and 16/20 at 300 mg/kg). Therefore, 300 mg/kg bw/day is the dose considered to be an adverse effect level, and it can be concluded that the study provides strong evidence of adverse effects on thyroid hormone signalling, at doses of 300 mg/kg after only 17 days exposure in adult animals. Histopathological investigation was only performed on the thyroid gland.
Study assessment: This was not a guideline study but the quality and reliability of the study was high. An adequate group size for a repeated dose toxicity was used (n=10/sex) and there was good consistency between the effects on TSH, thyroid gland weights and thyroid histopathology in both sexes (1, reliable without restrictions).

28-day oral repeated dose toxicity study in rats (Unpublished, 1983b)
The experiment was performed to investigate an excessive and toxic dose for side effects particularly on the thyroid, with the thyroid depressant propylthiouracil (PTU) as a reference substance. Male and female Wi-AF/HanEMD-SPF albino rats were administered 4 -MBC by oral gavage in a 4-week study. Ten rats of each sex were administered 1000 mg/kg bw/day 4-MBC in peanut oil or a control (vehicle only) at a volume of 5 mL/kg/day, once daily for 4 weeks. The reference group was administered approximately 20 mg/kg bw/day PTU in drinking water. 4-MBC was toxic at 1000 mg/kg bw/day and affected several endpoints including; animal behaviour, appearance (grooming, hypersalivation, dishevelled fur), and statistically significant reductions in body weight gain (20% lower body weight in males and 10% lower in females), food consumption and statistically significant increased water consumption.
Animals in the 4-MBC treatment group showed dilated, excessively full stomachs, a reduction in size of the thymus, a slight enlargement of the thyroids (less apparent in females than in males, 10/10 males, (8/10 females). In males, the atrophy of the accessory sex glands was observed and in the female animals enlargement of the liver and a reduction in the size of the adrenals. 4-MBC exposure increased absolute thyroid weights by approximately 1.9-fold for both sexes (absolute and relative weight), along with increased liver weight (males 37%, females 65%, absolute and relative), reduced thymus weight (males 36%, females 52%, absolute and relative), increased adrenal weight (males 30%, females 26%, relative), reduced ovary weight (21%, absolute), and prostate weights (35%, absolute and relative). No effects observed on the testes and uterus. All the results reported above reached statistical significance. In the histopathological examination, males displayed mild (1/10 animals), moderate (5/10) to marked (4/10) stimulation of the thyroid, whereas mainly mild stimulation was observed in the females (7/10), while 2/10 were moderately to greatly stimulated.
T3 serum levels were statistically significant increased by 96% in males and 28% in females, and T4 serum levels were statistically significant decreased by 30% in males and 23% in females, compared to the control. The mechanism of 4-MBC was different to that of the reference substance PTU, as clearly indicated by the difference in thyroid effects. In conclusion, this study provided moderate evidence of adverse effects on thyroid hormone signalling and reproductive

toxicity effects, at doses of 1000 mg/kg after 28 days exposure in adult animals. Effects on the thyroid were clearly occurring and effects on the reproductive organs were seen, but their interpretation was made difficult by marked systemic toxicity.
Study assessment: This was not a guideline study and the quality and reliability of the study was moderate. An adequate group size was used (n=10/sex) but only one dose of 4-MBC was tested, and it was so high that it induced marked systemic toxicity, complicating the interpretation of the effects on the thyroid gland and reproductive organs. There was however consistency between the effects on thyroid hormones, thyroid gland weights and thyroid histopathology in both sexes (2, reliable with restrictions).

14-day oral tolerability study in dogs (Unpublished, 2003)
4-MBC was administered by oral gavage to 1 male and 1 female beagle dog over a period of 14 days in an oral tolerability study. The two dogs were dosed with 20 mg/kg on day 1, 100 mg/kg on day 2, 500 mg/kg on day 3, 2500 mg/kg on day 4 and 500 mg/kg on days 5 - 14. Parameters analysed were food consumption, body weight, clinical signs, electrocardiogram and blood pressure, haematology, clinical chemistry, gross pathology and histopathology of a long list of organs, including thyroids. The male dog showed minimal activation of the thyroids. This finding was characterised by predominantly small and middle-sized follicles filled with colloid and lined regularly by iso and high prismatic epithelium. The variation of the normal height of the follicular epithelium in this strain of dog is wide, although in this dog the high-prismatic appearance was slightly more pronounced. The toxicological relevance of the thyroid finding was in the study report described as questionable, due to its "minimal degree" and minimal activation of the thyroids in the male dog, characterised by predominantly small and middle-sized follicles filled with colloid and lined regularly by iso and high prismatic epithelium. All other findings were sporadic and of spontaneous origin. In conclusion, the study provides inconclusive evidence of adverse effects on thyroid hormone signalling in dogs.
Study assessment: The use of differing doses throughout the study makes it difficult to determine exactly which dose levels lead to adverse effects in the animals. This was not a guideline study and the quality and reliability of the study was low due to the use of only 2 animals (3, not reliable).

21-day oral tolerability study in dogs (Unnamed, 2003)
4-MBC was orally administered by gavage to 4 beagle dogs (2 males, 2 females) over a period of 3 weeks. The four dogs were dosed with 0 mg/kg on day 1, 20 mg/kg on day 4, 100 mg/kg on day 8 and 500 mg/kg on days 11 - 21. Each dose escalation step was followed by a wash-out phase of 2 or 3 days.
The analysed parameters included food consumption, body weight, clinical signs, electrocardiogram, blood pressure, haematology, clinical chemistry, gross pathology and histopathology. T3, T4 and TSH concentrations were measured in blood samples at the following time points; one week before the start of treatment, on week 3 of treatment and on Days 1, 4, 8, 11 and 18 (both before and 2, 4, 6 and 24 hours after treatment). No changes in mortality, clinical signs or body weight were observed. Dose levels up to 500 mg/kg did not lead to any toxicological relevant alterations in thyroid hormone levels or thyroid gland histopathology. It is worth noting that during the first 10 days of the study the animals only received 2 doses of 4-MBC. Thus, in reality, the study was an 11-day exposure study and not a 21 day- study. The employed exposure (11 days exposure to 500 mg/kg bw/day) showed no evidence of adverse

effects on thyroid hormone signalling in dogs.
Study assessment: This was not a guideline study and the quality and reliability of the study was low. Only 4 dogs were used in the study (3, not reliable). This study summary was solely based on the information available on the ECHA dissemination site[1].

Dermal 90-day repeated dose toxicity study in rats, OECD TG 411 (Unpublished, 2005)
Topical administration of 4-MBC at a dose of 100, 400 or 2000 mg/kg bw/day by a daily 6 -hour semi-occlusive application during 90 or 91 days (100 and 400 mg/kg bw/day) or 11 days (2000 mg/kg bw/day), resulted in no systemic clinical signs of toxicity. At study termination organs were weighted and analysed histopathologically and serum was analysed for total T3, T4 and TSH. A slight decrease in bodyweight was observed in the high dose group at week 2 and this group had to be terminated on test day 15 due to severe skin reactions. Signs of local dermal reaction were recorded for many of the animals treated with 4-MBC, comprising transient signs of irritation such as erythema, scab formation, scaling and epidermal lesions. The formation of wounds, general edema, necrosis fissures and watery discharge were observed in the group treated at 2000 mg/kg bw/day. The severity as well as the incidence of general erythema, scaling, scabs and epidermal lesions aggravated with increasing dosage and, in general, the males appeared to be less sensitive than the females. The daily administration of 400 mg/kg bw/day produced no treatment-related pathological findings. Histopathology of tissues from the high dose group was confined to the thyroid glands and there were no treatment-related changes seen in this organ. No differences in serum levels of TSH, total T3 and total T4 were observed at Day 90 in males and females treated at 100 or 400 mg/kg bw/day when compared with the controls. 4-MBC was found to be absorbed in a dose-dependent manner and metabolized into its two major metabolites after dermal application. Based on the results of this study, 400 mg/kg bw/day of the test material was established as the NOAEL.
In conclusion, no reproductive or thyroid effects were found in this study. This lack of any effects differs from the results from the repeated dose toxicity studies, especially since high doses (400 mg/kg bw/day) and long-term exposure (90 days) was used in the present study. The most likely explanation is that 4-MBC acts differently after dermal than after oral exposure. Possibly the metabolism of 4-MBC is different, but still uterotrophic effects have been observed after dermal applications (though less marked than after oral exposure), so this does not fully explain the lack of positive results.
Study assessment: The quality and reliability of the study was high as several doses and an adequate group size was used (n=10/sex) (1, reliable without restrictions).

4.10.3.4 Study summaries - Human information

Few non-guideline absorption studies have been conducted in humans. One study investigated the effect of a 6% formulation over 14 days and report no effects on thyroid hormone levels (Unpublished, 1995). The second study looked at the effect of a 10% formulation over 4 days and report no effect on reproductive hormone levels (Janjua *et al.* 2004). A third study provided results on 4-MBC in human breast milk samples and indicated that humans (in 2008) had measurable levels of 4-MBC in the breast milk and that these levels were not very different from those measured in rats exposed to 4-MBC doses of 0.7 and 7 mg/kg per day (Schlumpf *et al.* 2008a – see section 4.10.3.3).

Unpublished, 1995

In a human dermal exposure study, 4-MBC was applied twice daily to healthy male and female volunteers for 14 days to determine the effects on the human thyroid hormone system. A 6% 4-MBC (maximum allowable concentration in the EU at the time of study) or placebo water in oil emulsion were applied on 1200 cm2 total surface area of the body and left uncovered for 4 hours after every dose. Blood samples were taken for analysis of thyroid function (TBG, TSH, FT4, FT3, T4 and T3) on days 1, 4, 7, 12, 14, 16 and 21 and thyroid volume was estimated pre-study and post-study using ultrasound data. 4-MBC was well-tolerated in the dose and application regime. There were no statistically significant differences between the exposed and placebo for any of the thyroid function measurements. Gender, smoking and the use of hormonal contraceptives had no effect on any of the thyroid function measurements. The total thyroid volume for the 4-MBC treatment group was statistically lower compared to the placebo, however post-study volumes were not statistically significant from pre-test volumes for both treatment group, indicating that measurement of thyroid volume has some limitations regarding the accuracy of the method. Overall, the application of 4-MBC did not seem to affect the thyroid hormone status of the participants. It is possible that like in the rodent model, there is a difference in adverse outcome depending on the route of exposure, and that in human dermal application also does not cause as marked effects on the thyroid hormone system as oral exposure would have done.
Study assessment: The quality and reliability of the study was moderate. It was well-designed and adequately reported but only included a relatively small number of test subjects (n=24/sex) (2, reliable with restrictions).

Janjua *et al*. 2004

The systemic uptake of 4-MBC and the effect on reproductive hormones were investigated in humans. 15 young males and 17 postmenopausal females were assigned to daily whole-body topical application of 2 mg/cm2 of basic cream formulation without (control) and with a 10% (wt/wt) formulation of 4-MBC for 4 consecutive days. Levels of FSH, Sex hormone-binding globulin (SHBG), LH, inhibin B, testosterone and estradiol were assessed. No biologically significant effects on hormone levels (FSH, LH, SHBG and estradiol) were observed. Minor but statistically significant differences in hormone levels between the control and treatment groups were observed for testosterone, estradiol, and inhibin B at some time points, however these did not seem to be related to the exposure to 4-MBC and at least some of these statistically significant differences may be chance findings.
Study assessment: The quality and reliability of the study was moderate. It was well-designed and adequately reported but only included a relatively small number of test subjects (2, reliable with restrictions).

4.10.4 Lines of evidence – T modality

The LoE for the T modality are assembled below. Table 4.4 shows evidence from *in vitro* studies regarding endocrine activity via T modality. Table 4.5 shows the evidence for *in vivo* endocrine activity via T modality and table 4.6 shows the evidence for adverse effects via T modality *in vivo.*

Table 4.4 Lines of evidence for **endocrine activity** via **T modality** (*in vitro* mechanistic). All reported changes are statistically significant with $p<0.05$, unless specified otherwise.

Reference	Modality	Effect classification	Effect target	Observed effect	Assessment of each line of evidence
Hofmann *et al.* 2009 (Klimisch 2)	T	*In vitro* mechanistic	Disruption of the Thyroid Hormone system	Both agonist & antagonist activity on the TRa	**Some evidence for thyroid hormone (TH) disrupting properties** Few studies have investigated the same endpoints. However, the data indicates that 4-MBC can act as both an agonist and antagonist on the thyroid receptor and increase deiodinase gene expression. K3/K4 studies: Thyroid peroxidase inhibition was not observed. One study showed decrease iodine uptake.
Hofmann *et al.* 2009 (Klimisch 2)	T	*In vitro* mechanistic	Disruption of the Thyroid Hormone system	↑ DIO1 expression	
Song M *et al.* 2013 (Klimisch 2)	T	*In vitro* mechanistic	Disruption of the Thyroid Hormone system	Upregulated expression of genes associated with deiodinase activity	
Schmutzler *et al.* 2004 (Klimisch 3)	T	*In vitro* mechanistic	Disruption of the Thyroid Hormone system	No TPO inhibition	
Schmutzler *et al.* 2007 (Klimisch 4)	T	*In vitro* mechanistic	Disruption of the Thyroid Hormone system	Decreased iodine uptake into FTRL-5 cells	

Table 4.5: Lines of evidence for **endocrine activity** via **T modality** (*in vivo* mechanistic). All reported changes are statistically significant with p<0.05, unless specified otherwise. NS: Non-statistically significant

Reference	Modality	Effect classifi-cation	Effect target	Species	Exposure	Route	Dose (mg/kg bw/day)	General toxicity	Observed effect on thyroid hormone level	Assessment of each line of evidence
OECD TG 408 Unpublished, 1984a (Klimisch 1)	T	*In vivo* mecha-nistic	Thyroid hormon e (TH) levels	Rat	90 day study (n=20/ sex)	Feed	50, 125, 312. A dose of 25 was included with its own control group.	Decreased bw gain in females at 312 mg/kg (transient in 125 mg/kg). No effect on male bw. Relative liver weights were increased in females at 125 and males at 312 mg/kg bw/day.	All doses from 50 mg/kg and above caused stimulatory effect on the thyroid gland in males and females, including increases in circulating T3 and TSH levels. T4 levels were normal except from a few isolated cases.	**Moderate-Strong evidence** of effect on TH levels. All rodent studies using oral exposure report effects on THs after exposure. There is a very consistent pattern of increased TSH and T3. In studies examining higher doses, T4 was typically decreased, whereas studies investigating lower doses showed unaltered T4 levels. A dermal study in rats showed no effects on TH levels, and also no effects were seen in a dermal study in humans.
Unpublished, 1983a (Klimisch 1)	T	*In vivo* mecha-nistic	TH levels	Rat	17 day repeated dose (n=10/ sex)	Oral	30, 300	No effect on bw or food consumption	Increased serum TSH (1.9 fold in males and 7.5 fold in females) at 300 mg/kg bw/day	
Unpublished, 1983b (Klimisch 2)	T	*In vivo* mecha-nistic	TH levels	Rat	28 day repeated dose (n=10/ sex)	Oral	1000	Marked systemic toxicity, seen as clinical signs and markedly reduced bw.	T3 serum levels were increased by 96% in males and 28% in females, and T4 serum levels were decreased by 30% in males and 23% in females, compared to the controls.	
OECD TG 421 Unpublished, 2004 (Klimisch 2)	T	*In vivo* mecha-nistic	TH levels	Rat	21 days exposure in the pre-mating period (n =3-5)	Oral	12.5, 25, 50	In parental females there were no treatment-related clinical signs, no mortality and no effects on bw gain or food consumption in any dose. Water consumption was increased in the high dose group.	Females: T4 levels unaffected. TSH marginally increased by 15-25% in mid and high dose group (NS. Likely due to high variation). T3 dose-dependently increased with a 25% increase in the high dose group (NS. Likely due to high variation).	
OECD TG 421 Unpublished, 2004 (Klimisch 2)	T	*In vivo* mecha-nistic	TH levels	Rat	Premating, gestation, lactation (84 days exposure. n=10 parental females/ exposure group)	Oral	12.5, 25, 50	4-MBC exposure caused no effect on dam bw after 84 days of exposure, but water consumption was increased in the high dose group.	Dams on PND22 (after 84 days of exposure): No effect on T4 and TSH. Small (8-11%), increase in T3 in all dose groups (8-11%) (NS. Likely due to high variation.	

OECD TG 421 Unpublished, 2004 (Klimisch 2)	T	*In vivo* mecha-nistic	TH levels	Rat	Gestation, lactation until PND 22 (n=30)	Oral	12.5, 25, 50	No decreases in offspring bw were observed during the course of the study (including on PND 55) in 4-MBC exposed animals. Liver weights were not assessed.	Offspring PND55 Males: No effects on T4. T3 (16%) and TSH (16%) levels were decreased in high dose group. Females: No effects on T4 and TSH. T3 levels were NS decreased in high dose group (11%).	K3/K4 studies: Two studies in rats point in the same direction as the other rats studies whereas one study in dogs showed no effects on TH levels
Maerkel *et al*. 2007 (Klimisch 2)	T	*In vivo* mecha-nistic	TH levels	Rat	Pre-mating to PND 12.	Feed	7, 24, 47	Bw were unaffected at 12 weeks of age (PN 84). Liver weights were not reported here but were reported as unaltered in adult male offspring in the Durrer *et al.* 2007.	Adult offspring: Increased levels of TSH in females in the high dose group and a slight increase in T3 (middle and high dose group). No effects on male TSH and T3. Serum T4 levels were unaffected in both sexes.	
OECD 411 Unpublished, 2005 (Klimisch 1)	T	*In vivo* mecha-nistic	TH levels	Rat	90 days (n=20/sex)	Derma l	100, 400, 2000	No effect on body or liver weight seen at 100 and 400 mg/kg. Highest dose terminated after 11 days due to severe local effects.	No treatment-related changes on T3, T4 or TSH levels were observed.	
Unpublished, 1995 (Klimisch 2)	T	*In vivo* mecha-nistic	TH levels	Human (male and female)	14 days, twice daily	Derma l	6% on 1200 cm2 body surface	No systemic toxicity observed	There was no effect on thyroid hormone levels (T3, T4, TSH).	
Schmutzler *et al.* 2004 (Klimisch 3)	T	*In vivo* mecha-nistic	TH levels	Rat	12 weeks (adult, ovariecto mized rats) (n=8-11)	Feed	66, 310 mg/ animal/day	Clinical symptoms, body and liver weight were not reported in this publication	At both doses TSH and T3 levels were elevated, and T4 serum levels were decreased. In the liver, type I 5-deiodinase was decreased in both treatment groups (NS)	
Seidlova-Wuttke *et al*. 2006a (Klimisch 3)	T	*In vivo* mecha-nistic	TH levels	Rat	12 weeks (adult ovariecto mized rats) (n=12)	Feed	230, 1000	Animals exposed the to both doses of 4-MBC gained less bw than controls	Both doses caused decreased T4, increased TSH and NS increase in T3 levels.	
Unnamed, 2003 (Klimisch 3)	T	*In vivo* mecha-nistic	TH levels	Dog	21 day study (n = 2/sex)	Oral	0 mg/kg on day 1, 20 mg/kg on day 4, 100 mg/kg on day 8, 500 mg/kg on days 11 - 21.	No general toxicity was observed	No effect on thyroid hormone levels (T3, T4, TSH).	

Table 4.6: Lines of evidence for **adverse effects** *in vivo* via **T modality**. All reported changes are statistically significant with p<0.05, unless specified otherwise. NS: Non-statistically significant.

Reference	Modality	Effect target	Species	Exposure	Route	Dose (mg/kg bw/day)	General toxicity	Observed effects	Assessment of each line of evidence
OECD TG 408, Unpublished 1984a (Klimisch 1)	T	Thyroid gland	Rat	90 days (n=20 animals /sex)	Feed	0, 50, 125, 312. A dose of 25 was included with its own control group.	Decreased bw gain in females at 312 mg/kg (transient at 125 mg/kg). No effect on male bw. Relative liver weights were increased in females at 125 and males at 312 mg/kg bw/day.	Doses of 50, 125 & 312 mg/kg bw/day induced increases in thyroid weights and follicular hyperplasia, hypertrophy etc. in both sexes in a dose related manner. The changes in the thyroid were still apparent at the end of the follow-up recovery period albeit less pronounced. No effects seen at 25 mg/kg bw/day.	**Strong evidence for effect on the thyroid gland** All oral rat studies investigating thyroid weight, report an increase, and almost all report adverse histopathology findings on the thyroid gland. A rat study using dermal exposure route did not see effects on thyroid gland histopathology. K3/K4 studies: Two studies were performed in dog. In one of them indications of adverse effects on histopathology were seen, in the other one there were no effects.
Unpublished, 1983a (Klimisch 1)	T	Thyroid gland	Rat	17-day study (n=10/sex)	Oral	0, 30, 300	No effect on bw or food consumption	At 300 mg/kg bw/day the thyroid gland weight was increased (140% in males and 160% in females). Hypertrophy of the follicular epithelium with occasional incisures of the thyroid gland was also observed pathologically (4/20 in controls, 8/20 at 30 mg/kg and 16/20 at 300 mg/kg).	
Unpublished, 1983b (Klimisch 2)	T	Thyroid gland	Rat	28-day study (n=10/sex)	Oral	0, 1000	Marked systemic toxicity, seen as clinical signs and markedly reduced bw (20% lower in males and 10 % lower in females). Liver weights were increased and thymus and adrenal weights reduced.	Thyroid weights increased approximately 1.9 fold in both sexes. Males displayed mild (1/10 animals), moderate (5/10) to marked (4/10) stimulation of the thyroid, whereas mainly mild stimulation (7/19 animals. 2 animals were moderately-marked) were observed in the females.	
OECD TG 421 Unpublished, 2004 (Klimisch 2)	T	Thyroid gland	Rat	Pre-mating, mating and lactation (n=10)	Oral	12.5, 25, 50	No effect on dam bw after 84 days of exposure, i.e. at the time of weaning, but water consumption was increased in the high dose group. Liver weight was not assessed.	Dams on PND22 (after 84 days of exposure): Marked increase of 19% in thyroid weight in the high dose group (NS. Likely due to high variation)	
OECD TG 421 Unpublished, 2004 (Klimisch 2)	T	Thyroid gland	Rat	Gestation, lactation until PND 22 (n=10)	Oral	12.5, 25, 50	No effect on offspring bw on PND 22. Liver weights not assessed.	Offspring PND22: Marked increase (33%) in thyroid gland weights in male offspring at weaning (NS. Likely due to high variation). No effect in females.	

OECD TG 421 Unpublished, 2004 (Klimisch 2)	T	Thyroid gland	Rat	Gestation, lactation until PND 22 (n=10)	Oral	12.5, 25, 50	No effect on offspring bw on PND 55. Liver weights not assessed.	Offspring PND55: No effect on male or female thyroid gland weight or histology.	
Maerkel *et al.* 2007 (Klimisch 2)	T	Thyroid gland	Rat	Fetal, postnatal, pubertal and adult exposure	Feed	7, 24, 47	Offspring bw were unaffected at 12 weeks of age (PN 84). Liver weights were not reported here but were reported as unaltered in the Durrer *et al.* 2007	Adult offspring: 24 and 72 mg/kg bw/day markedly increased thyroid weights (abs. and rel.) in a dose related manner in males and females (24 mg/kg: 40-53 %; 72 mg/kg: 25-32 %). No effect was seen at 7 mg/kg bw/day.	
OECD TG 411 Unpublished, 2005 (Klimisch 1)	T	Thyroid gland	Rat	90-day study (n=20/sex). Only 11 days in 2000 mg/kg bw/day dose.	Dermal	100, 400, 2000	No effect on body or liver weight seen at 100 and 400 mg/kg. Highest dose terminated after 11 days due to severe local effects.	No effect on thyroid gland was seen at any dose level.	
Unpublished, 2003 (Klimisch 3)	T	Thyroid gland	Dog	14-day study (n=1/sex)	Oral	20 mg/kg on day 1, 100 mg/kg on day 2, 500 mg/kg on day 3, 2500 mg/kg on day 4 and 500 mg/kg on days 5–14	No effect on food consumption, bw, clinical chemistry or gross pathology. Vomiting seen after treatment with 2500 mg/kg in males.	The male dog showed minimal activation of the thyroid gland, characterized by small and middle-sized follicles. No effects were seen in the female dog.	
Unnamed, 2003 (Klimisch 3)	T	Thyroid gland	Dog	21 day study (n=2/sex)	Oral	0 mg/kg on day 1, 20 mg/kg on day 4, 100 mg/kg on day 8 and 500 mg/kg on days 11-21.	No general toxicity was observed	There was no effect on thyroid gland histopathology. No general toxicity was observed	
TG 421 Unpublished, 2004 (Klimisch 2)	Sensitive to, but not diagnostic of T	Nervous system	Rat	Gestation, lactation until PND 22 (n=10)	Oral	12.5, 25, 50	No effect in offspring bw were observed. Offspring liver weights were not assessed.	Offspring: No effect on learning and memory in male or female offspring.	**Not sufficient evidence to determine whether learning and memory are affected by developmental exposure to 4-MBC** Only investigated in one study using too low top dose , a group size of 10 and a rather insensitive scoring method for the behavioural results, is not in itself robust enough to exclude effects on neurodevelopment.

4.10.4.1 Summary of the lines of evidence – T mediated effects

The lines of evidence presented in Table 4.4-4.6 can be summarised as follows:

Table 4.7: WoE for T-mediated endocrine activity and adverse effects

T-mediated endocrine activity *in vitro*:

- There is some *in vitro* evidence for thyroid disrupting effects, supporting conclusions on endocrine activity seen *in vivo*. Few studies have investigated the same endpoints, but the overall data indicate that 4-MBC can act as both thyroid receptor agonist and antagonist (Hofmann *et al.* 2009), and upregulate deiodinase gene expression (Hofmann *et al.* 2009; Song M *et al.* 2013).
  K3/K4 studies: One study investigated thyroid peroxidase, but did not see any effect (Schmutzler *et al.* 2004), and the other indicated a decrease in iodine uptake (Schmutzler *et al*. 2007).

T-mediated endocrine activity *in vivo*:

- There is moderate-strong evidence of endocrine activity due to effects on thyroid hormone levels in rats exposed during adulthood. A consistent pattern of increased TSH and T3 and in some studies decreased or unaffected T4 levels are seen (Unpublished, 1983a; Unpublished 1983b; Unpublished, 1984a; Unpublished, 2004). For perinatally exposed rats, where exposure stopped at weaning, decreased TSH and T3 was seen in 55 day old offspring (Unpublished, 2004) whereas in a study where exposure continued after weaning, increased TSH and T3 in adult offspring were found (Maerkel *et al*. 2007). None of the perinatal studies showed effects on T4. A dermal study in rats (Unpublished, 2005) and a dermal experimental study in humans (Unpublished, 1995) reported no effects on thyroid hormone levels.
  K3/K4 studies: Two studies of lower reliability found decreased T4 levels in rats exposed during adulthood (Schmutzler *et al*. 2004; Siedlova-Wuttke *et al*. 2006a). Another study (n=2/sex) reported no effects on thyroid hormone levels in an oral dog study (unpublished 2003).

T-mediated *in vivo* adverse effects:

- There is strong evidence of adverse effects on the thyroid gland, as several studies report increased thyroid weight and histopathology after oral exposure (Unpublished, 1984a; Unpublished, 1983a; Unpublished 1983b; Unpublished, 2004; Maerkel *et al*. 2007). Only an oral study investigating adult (PND55) rats exposed perinatally (Unpublished, 2004), and a dermal study in rats (Unpublished, 2005) report no effects.
  In a developmental toxicity study (Unpublished, 2004) no exposure-related effects on learning and memory were observed.
  K3/K4 studies: No clear results on the thyroid in two oral studies conducted in dogs (n=1-2/sex) (Unnamed 2003, unpublished 2003).

4.10.4.2 A discussion of the overall evidence and relation to possible other toxicity is presented below

Endocrine activity via T modality:

Integrated lines of evidence led to the conclusion that there is **strong evidence of endocrine activity via T modality** (Table 4.4-4.5)**.** Few studies investigated the same endpoints in relation to thyroid disruption, but the collected **mechanistic data show some evidence for thyroid disrupting properties *in vitro*** (Hofmann *et al*. 2009; Song M *et al*. 2013; Schmutzler *et al*. 2004). The *in vivo* mechanistic data (Table 4.5) show **moderate-strong evidence of effect on thyroid hormone levels** in rats exposed during adulthood with an overall pattern of increased TSH and T3 and, in some studies, decreased or unaffected T4 levels (Unpublished, 1984a; Unpublished, 2004; Unpublished, 1983a; Unpublished, 1983b). One study investigating perinatal exposure found decreased TSH and T3 in 55 day old offspring that were no longer exposed after PND 22 (Unpublished, 2004), where increased TSH and T3 levels were seen in adult offspring after continued exposure after weaning (Maerkel *et al*. 2007). The perinatal studies did not see effects on T4. Two studies reported no effects on thyroid hormone levels – a dermal study in rats (Unpublished, 2005), a dermal experimental study in humans (Unpublished, 1995).

Two published studies of lower reliability found evidence of altered TSH, T4 (Seidlova-Wuttke *et al.* 2006a) and T3 levels (Schmutzler *et al.* 2004) and another study found no evidence of such effects in dogs (n=2/sex) exposed orally (Unpublished, 2003).

Adverse effect related to T modality:

The lines of evidence showed **strong evidence to conclude that 4-MBC has adverse effects via T modality *in vivo*** (Table 4.5). There is strong evidence for adverse effect on the thyroid system with several studies reporting increased thyroid weight after oral exposure (Unpublished, 1984a; Unpublished, 1983a; Unpublished, 1983b; Unpublished, 2004; Unpublished, 2003; Maerkel *et al*. 2007). In the oral 90-day study (Unpublished, 1984a) thyroid gland weights were elevated and histopathology was adversely affected in both male and female rats (n=20/sex) in a dose-dependent manner. The effects were statistically significant at doses of 50, 125 and 312 mg/kg. In a developmental toxicity screening study using only gestational and lactational exposure to 4-MBC (Unpublished, 2004) thyroid gland weight in the dams at weaning (PND 22) showed a (non-statistically significant) 20% increase, and in the male offspring a non-statistically significant 33% increase. No adverse thyroid effects were seen in the offspring on PND 55, suggesting that the adverse effects on the gland were transient if exposure was discontinued. Doses of 24 and 72 mg/kg bw/day markedly and dose-dependently increased thyroid weights (absolute and relative) in adult male and female offspring exposed during development, when exposure continued into adulthood (Maerkel *et al.* 2007).

A dermal study in rats, showed no effects on the thyroid glands, at doses which in an oral study would likely have affected the animals (Unpublished, 2005). It is possible that the lack of toxicological effects was related to differences in toxicokinetics, due to the different route of exposure. In two oral studies in dogs, which due to their low number of animals wereassigned a lower reliability score (K3), no clear thyroid effects were seen (Unpublished, 2003; Unnamed, 2003).

In an oral developmental toxicity study (Unpublished, 2004) no exposure-related effects on learning and memory were observed. Since only one study was performed and the employed

methods for the assessment of learning behaviour had some important limitations (as discussed in 4.10.3.3), there is not sufficient evidence to determine whether learning and memory are affected by developmental exposure to 4-MBC.

There are no indications of increased thyroid activity mediated through increased liver enzyme induction. Specific investigations have been made to assess liver enzyme induction in male rats administered 312 mg/kg bw/day 4-MBC in the feed for 28 days. In this repeated dose toxicity study no mortalities were observed, 4-MBC was clinically tolerated, and no significant differences were observed in body weight, feed consumption or liver weight (absolute and relative), compared to the controls. It was concluded that the thyroid effects caused by 4-MBC exposure are most likely not mediated through liver enzyme induction. However, the study did not examine any other organs than the liver and T hormones were not measured (Unpublished, 1984b).

Assessment whether T mediated endocrine activity and adverse effects been sufficiently investigated.

The ECHA/EFSA ED guidance (ECHA/EFSA, 2018) states that when adversity is observed based on 'EATS-mediated' parameters, the biological plausibility of the link between the 'EATS-mediated' adversity and endocrine activity should be documented through a MoA analysis. In the guidance, this is presented in table 5 "High level summary of the scenarios, including the next steps in the assessment" (ECHA/EFSA 2018).
For 4-MBC both T adversity and activity were observed. There would be a sufficient data set for T-mediated adversity to support a conclusion on *absence* of T-mediated adversity. Hence, a MoA analysis should be performed (Scenario 2b).

Table 4.8: Selection of relevant scenario (T-modality)

Adversity based on T-mediated parameters	**Positive mechanistic OECD CF level 2/3 Test**	**Scenario**	**Next step of the assessment**	**Scenario selected**
No (sufficiently investigated)	Yes/No	1a	Conclude: ED criteria not met because there is not "**EAS-mediated**" adversity	
Yes (sufficiently investigated)	Yes/~~No~~	1b	Perform MoA analysis	x
No (not sufficiently investigated)	Yes	2a (i)	Perform MoA analysis (additional information may be needed for the analysis)	
No (not sufficiently investigated)	No (sufficiently investigated)	2a (ii)	Conclude: ED criteria not met because no **EAS-mediated endocrine activity** observed	
No (not sufficiently investigated)	No (not sufficiently investigated)	2a (iii)	Generate missing level 2 and 3 information. Alternatively, generate missing "EATS-mediated" parameters. Depending on the outcome move to corresponding scenario	
Yes (not sufficiently investigated)	Yes/No	2b	Perform MoA analysis	

4.10.5 Lines of evidence – EAS modalities

The LoE for the modalities EAS and other modalities sensitive to, but not diagnostic of EAS are assembled below. Table 4.9 shows evidence from *in vitro* studies regarding endocrine activity by the modalities via EAS and other. Table 4.10-4.11 show the evidence for *in vivo* endocrine activity via the EAS modalities. Table 4.12 shows the evidence for adverse effects via the EAS modalities.

Table 4.9: Lines of evidence **endocrine activity** via **EAS and other modalities** (*in vitro* mechanistic). All reported changes are statistically significant with p<0.05, unless specified otherwise. NS: Non-statistically significant.

Reference	Modality	Effect classification	Effect target	Observed effect	Assessment of each line of evidence
Schlumpf *et al*. 2001 (Klimisch 2)	E	*In vitro* mechanistic	E-screen	↑ in MCF-7 cell proliferation and in pS2 protein expression	**Strong evidence for endocrine activity *in vitro* in studies on induction of estrogenic response** All 6 studies show a response in the E-screen measured as proliferation, gene or protein expression. K3/K4 studies: One study shows increased proliferation in the E-screen
Tinwell *et al*. 2002 (Klimisch 2)	E	*In vitro* mechanistic	E-screen	Clear ↑ MCF-7 proliferation	
Schlumpf *et al.* 2004a (Klimisch 2)	E	*In vitro* mechanistic	E-screen	↑ in MCF-7 cell proliferation.	
Klann *et al* 2005 (Klimisch 2)	E	*In vitro* mechanistic	E-screen	↑ MCF-7 proliferation	
Heneweer *et al.* 2005 (Klimisch 2)	E	*In vitro* mechanistic	E-screen	↑pS2 gene transcription	
Jiménez-Díaz *et al.* 2013 (Klimisch 2)	E	*In vitro* mechanistic	E-screen	↑ MCF-7 proliferation, no antagonistic effect	
Matsumoto *et al.* 2005 (Klimisch 4)	E	*In vitro* mechanistic	E-screen	↑ MCF-7 proliferation	
Klann *et al*. 2005 (Klimisch 2)	E	*In vitro* mechanistic	Estrogen Receptor (ER) agonism	4-MBC bound to endogenous ER in frogs and activated ER-dependent gene expression.	**Strong evidence for endocrine activity *in vitro* in studies on ER agonistic properties** Different *in vitro* test systems have been employed to investigate activation of the estrogen receptor. The majority of the studies (7/11) did conclude that 4-MBC acts as an ER agonist, some however conclude that the potency is weak.
Gomez *et al.* 2005 (Klimisch 2)	E	*In vitro* mechanistic	ER agonism	Activation of ER a&b receptor	
Schreurs *et al*. 2002 (Klimisch 2)	E	*In vitro* mechanistic	ER agonism	Activation of hERa&b in HEK293 reporter gene assay	
Minh *et al.* 2008 (Klimisch 2)	E	*In vitro* mechanistic	ER agonism	ER activation seen as activated luciferase expression in SiG12 cells	
Schmitt *et al.* 2008 (Klimisch 2)	E	*In vitro* mechanistic	ER agonism	hERα activation in YES assay	
Jocsak *et al.* 2016 (Klimisch 2)	E	*In vitro* mechanistic	ER agonism	Affected ERb mRNA expr. in cerebellar cells	
Mueller *et al.* 2003 (Klimisch 2)	E	*In vitro* mechanistic	ER agonism	Weak potency to activate ERα and to a higher extent ERb in transfected human endometrial Ishikawa cells	
Tinwell *et al.* 2002 (Klimisch 2)	E	*In vitro* mechanistic	ER agonism	Equivocal evidence of estrogenicity in YES assay	
Morohoshi *et al.* 2005 (Klimisch 2)	E	*In vitro* mechanistic	ER agonism	No agonist activity in YES assay	
Kunz & Fent 2006 (Klimisch 2)	E	*In vitro* mechanistic	ER agonism	No agonistic effect on hERα in YES assay	
Kunz *et al.* 2006 (Klimisch 2)	E	*In vitro* mechanistic	ER agonism	No agonistic effect on hERα in YES assay	

Kunz & Fent 2006 (Klimisch 2)	E	*In vitro* mechanistic	ER antagonism	Clear antagonistic effect on hERα in YES assay	**Equivocal evidence for ER antagonism** The evidence for ER antagonism is equivocal. Of the four studies investigating this, two found clear antagonistic effects whereas the other two studies showed no antagonistic effects on the ER.
Mueller *et al.* 2003 (Klimisch 2)	E	*In vitro* mechanistic	ER antagonism	Marked antagonistic properties on the ERα & β in human endometrial Ishikawa cells transfected with ERα or ERβ	
Schreurs *et al.* 2005 (Klimisch 2)	E	*In vitro* mechanistic	ER antagonism	No antagonism on ERα or ERβ in stably transfected HEK293 reporter cell lines	
Morohoshi *et al*. 2005 (Klimisch 2)	E	*In vitro* mechanistic	ER antagonism	No antagonist activity in YES assay	
Schlumpf *et al.* 2004a (Klimisch 2)	E	*In vitro* mechanistic	ER binding	Binding to recombinant ERb (but not a) in ligand-binding assay	**Equivocal evidence for ER binding.** One study concludes evidence of ER binding while another study found equivocal evidence. One study showed no binding. K3/K4 studies: In one study weak binding was seen.
Morohoshi *et al*. 2005 (Klimisch 2)	E	*In vitro* mechanistic	ER binding	No binding to hERA in an ER-ELISA assay	
Tinwell *et al.* 2002 (Klimisch 2)	E	*In vitro* mechanistic	ER binding	Equivocal evidence of ER binding in competitive binding assay	
Matsumoto *et al.* 2005 (Klimisch 4)	E	*In vitro* mechanistic	ER binding	Weak binding to ERα and ERb	
Ma *et al.* 2003 (Klimisch 2)	A	*In vitro* mechanistic	AR agonism	No agonist activity on AR in MDA-kb2 assay	**No evidence of AR Agonism** None of the five studies report an agonistic response on the AR.
Schreurs *et al.* 2005 (Klimisch 2)	A	*In vitro* mechanistic	AR agonism	No agonist activity in AR callux	
Kunz & Fent 2006 (Klimisch 2)	A	*In vitro* mechanistic	AR agonism	No agonistic effect on hAR in YES assay	
Nashev *et al.* 2010 (Klimisch 2)	A	*In vitro* mechanistic	AR agonism	No activation of the AR	
Jiménez-Díaz *et al.* 2013 (Klimisch 2)	A	*In vitro* mechanistic	AR agonism	No agonistic effect	
Ma *et al* 2003 (Klimisch 2)	A	*In vitro* mechanistic	AR antagonism	No antagonist activity on AR in MDA-kb2 assay	**Moderate-strong evidence for endocrine activity in studies on AR antagonism** 4/5 studies did report a marked antagonistic effect on the AR. None of the five studies report an agonistic response.
Schreurs *et al.* 2005 (Klimisch 2)	A	*In vitro* mechanistic	AR antagonism	Marked antagonistic activity in AR callux	
Kunz & Fent 2006 (Klimisch 2)	A	*In vitro* mechanistic	AR antagonism	Clear antagonistic effect on hAR in YES assay	
Nashev *et al.* 2010 (Klimisch 2)	A	*In vitro* mechanistic	AR antagonism	Weak antagonistic effect of the AR	
Jiménez-Díaz *et al.* 2013 (Klimisch 2)	A	*In vitro* mechanistic	AR antagonism	Potent AR antagonism	
Nashev *et al.* 2010 (Klimisch 2)	S	*In vitro* mechanistic	Steroidogenesis	Inhibition of steroid hormone synthesis enzymes 17b-HSD3, 17b-HSD1 & 2	**Some evidence for endocrine activity in studies on steroidogenesis.** Only investigated in one study.

Schreurs *et al.* 2005 (Klimisch 2)	Other (progesterone)	*In vitro* mechanistic	Progesterone receptor activity	No agonist but marked antagonistic activity in PR callux	**Equivocal evidence for effects on activation of the progesterone receptor** The results in the two studies differ.
Yin *et al.* 2015 (Klimisch 2)	Other (progesterone)	*In vitro* mechanistic	Progesterone receptor activity	No anti-progestogenic effect in Ishikawa cells assessed by SULT1E1 mRNA levels	
Schiffer *et al.* 2014 (Klimisch 2)	Other (progesterone)	*In vitro* mechanistic	Mimicking effects of progesterone in sperm cell	Biphasic increase in Ca2+ flux similar to that observed with progesterone. 4-MBC also lowed the frequency and enhanced the asymmetry of the beat of sperm flagella. Acrosomal exocytosis was found in 25–40% of exposed sperm exposed, which was like the response with progesterone	**Moderate evidence for endocrine activity in studies on progesterone mimicking in sperm cells** 4-MBC exposure mimics the effects of progesterone in human sperm cells, causing a Ca2+ influx into the sperm cells through the CatSper channel. The studies show a clear pattern of effects, but it is not clear if this is sign of endocrine activity.
Rehfeld *et al.* 2016 (Klimisch 2)	Other (progesterone)	*In vitro* mechanistic	Mimicking effects of progesterone in sperm cell	The Ca2+ signals induced by 4-MBC in human sperm cells resembled the one induced by progesterone, suggesting a similar MoA	
Rehfeld *et al.* 2018 (Klimisch 2)	Other (progesterone)	*In vitro* mechanistic	Mimicking effects of progesterone in sperm cell	Increase in viable acrosome reacted sperm cells, similar to the effect of progesterone. Increase in sperm penetration was NS, and no effects on hyperactivation or sperm viability.	

Table 4.10: Lines of evidence for **endocrine activity** via **E modality** (*in vivo* mechanistic). All reported changes are statistically significant with p<0.05, unless specified otherwise.

Reference	Moda-lity	Effect classifi-cation	Effect target	Species	Expo-sure	Route	Dose (mg/kg bw/day)	General toxicity	Observed effect related to E-modality	Assessment of each line of evidence
Schlumpf *et al.* 2001 (Klimisch 2)	E	*In vivo* mechanistic	Utero-trophic assay	Rat	6 days, PND 21-26 (n=4-10)	Dermal	Total amount applied each day: 137.5, 275, 412.5 mg	Mean bw on PND 26 were unaffected by treatment, liver weight were not assessed	Increased uterine weight at 275 and 412.5 mg doses (The 275 mg dose was calculated/ estimated to 37 mg/kg bw/day).	**Strong evidence of endocrine activity *in vivo* seen as effect in uterotrophic assay** All 5 studies report an increase in uterine weight after exposure. One study (Ashby *et al*. 2004) reports a lack of effect in animals older than 21 days, but do see the effect on PND 19-21.
Schlumpf *et al.* 2001 (Klimisch 2)	E	*In vivo* mechanistic	Utero-trophic assay	Rat	4 days, PND 21-24 (n=9-19)	Feed	66, 119, 211, 337, 402, 1980	Mean bw on PND 24 were unaffected by treatment, liver weight were not assessed	Increase in uterine weight from 119 mg/kg bw/day and above and had an ED_{50} of 309 mg/kg bw/day. Bw was not affected.	
Tinwell *et al.* 2002 (Klimisch 2)	E	*In vivo* mechanistic	Utero-trophic assay	Rat	For three days from PND 19-20 (n=12)	Oral	500, 800	Clinical signs of toxicity after the third oral administration of 800 mg/kg. At termination, reduced bw was seen in both exposed groups. Liver weights were not assessed.	Increased uterine weight at both dose levels (50 and 90% in a dose related manner).	
Tinwell *et al.* 2002 (Klimisch 2)	E	*In vivo* mechanistic	Utero-trophic assay	Rat	For three days from PND 19-20 (n=12)	s.c.	500, 1000	No signs of general toxicity were seen, liver weights were not assessed.	Increased uterine weight at both dose levels (approximately 30% in both groups, no dose response pattern).	
Ashby *et al.* 2004 (Klimisch 2)	E	*In vivo* mechanistic	Utero-trophic assay	Rat	Three days from PND 19-21 or older animals	s.c.	1000	No bw reductions were seen in any of the performed studies. Liver weights were not assessed.	Uterotrophic effects were seen when using animals on PND 19-21. No effect on uterine weight was seen in older animals (PND 21-23).	
Maerkel *et al.* 2007 (Klimisch 2)	E	*In vivo* mechanistic	Gene and protein expression	Rat	Fetal, postnatal, pubertal and adult exposure	Feed	7, 24, 47	4-MBC treatment did not affect bw at 12 weeks of age (PN 84). Liver weights were not reported here but were reported as unaltered in adult male offspring in the Durrer *et al.* 2007	Adult offspring: alterations in gene expression of ERα and progesterone receptor in the brain indicate profound sex- and region-specific alterations in the regulation of estrogen target genes at brain level.	**Moderate-strong evidence for altered gene and protein expression on estrogen sensitive genes in the brain, uteri and prostate** in offspring from all dose groups, exposed during development and
Durrer *et al.* 2005 (Klimisch 2)	E	*In vivo* mechanistic	Gene and protein expression	Rat	Fetal, postnatal, pubertal and adult exposure	Feed	0.7, 7, 24, 47	Female offspring bw at 12 weeks of age were not affected by treatment, liver weights were not reported.	Adult offspring: altered expression of estrogen regulated genes in the uteri at all examined dose levels (0.7, 7, 24 and 47 mg/kg bw/day). Different genes were affected at different doses, and no	

									clear dose-response relationship was observed.	with continued exposure in adulthood. K3/K4 studies: Two studies with lower reliability provided supporting evidence.
Durrer *et al.* 2007 (Klimisch 2)	E	*In vivo* mechanistic	Gene and protein expression	Rat	Fetal, postnatal, pubertal and adult exposure	Feed	7, 24, 47	Body weight at onset of puberty was at control level in males On PND 55 when reproductive organs weights were assessed there were no effects on body weight.	Adult offspring: Expression of estrogen regulated genes in the prostate was affected.	
Maerkel *et al.* 2005 (Klimisch 3)	E	*In vivo* mechanistic	Gene and protein expression	Rat	Fetal, postnatal, pubertal and adult exposure	Feed	7, 24, 47	Offspring bw were not reported, but we know from other publications that they were unaffected by treatment.	12 week old offspring: alterations in gene expression of progesterone receptor in sexually dimorphic areas of the brain at all 4-MBC doses tested. The responsiveness of estrogen target genes was changed after acute injection of E2 in adult gonadectomised offspring.	
Seidlova-Wuttke *et al.* 2006b (Klimisch 3)	E	*In vivo* mechanistic	Gene and protein expression	Rat	12 weeks (adult) (n=12)	Feed	230, 1000	Animals exposed to both doses of 4-MBC gained less bw than control animals. Liver weights were not reported.	Changes in expression of estrogen regulated genes in the uterus.	
Seidlova-Wuttke *et al.* 2006a (Klimisch 3)	E	*In vivo* mechanistic	Uterine weight changes in OVX females	Rat	12 weeks (adult) (n=12)	Feed	230, 1000	Animals exposed to both doses of 4-MBC gained less bw than controls Liver weights were not reported	Both doses caused an increase in uterine weight of approximately 50%. This publication states that the increase was only statistically significant in the low dose group.	**Evidence of endocrine activity *in vivo* seen as uterine weight changes in OVX females in two unreliable studies.** The two publications report results from the same animal study. Increased uterine weights were seen in both dose groups but based on the provided data it is not possible to determine if the effect was statistically significant in the low dose, the high, both or none of them.
Seidlova-Wuttke *et al.* 2006b (Klimisch 3)	E	*In vivo* mechanistic	Uterine weight changes in OVX females	Rat	12 weeks (adult) (n=12)	Feed	230, 1000	The high dose of 4-MBC resulted in reduced gain of bw during the first 6 weeks of treatment. Therefore these animals were set to the low dose of 4-MBC. They experienced a catch-up in weight gain such that, at the end of the experiments, the weights of the animals did not differ from those of the controls.	Both doses caused an increase in uterine weight of about 50%. Lack of dose-response is not surprising given that the animals in the two groups dosed with 4-MBC received the same dose during the second half of the study. The statistical results presented in this publication states that effects in the high dose were statistically significant, but not in the low dose. Increased epithelium thickness in uterus and vagina.	

Table 4.11: Lines of evidence for **endocrine activity** via **EAS modalities** (*in vivo* mechanistic). Effect target: Hormone measurements. All listed endpoints relate to the EAS modalities. All reported changes are statistically significant with p<0.05, unless specified otherwise. NS: Non-statistically significant.

Reference	Moda-lities	Effect classifi-cation	Effect target	Species	Exposure	Route	Dose (mg/kg bw/day)	General toxicity	Observed effect	Assessment of each line of evidence
OECD TG 421 Unpublished, 2004 (Klimisch 2)	EAS	*In vivo* mechanistic	Luteinising Hormone (LH) level	Rat	21 days exposure in the pre-mating period (n =3-5 parental females)	Oral	12.5, 25, 50	In maternal animals there were no clinical signs, and no effects on bw gain or food consumption	LH levels dose-dependently decreased, being 62% lower in high dose parental female group (NS – likely due to high variation).	**Weak-moderate evidence of endocrine activity *in vivo* in studies showing effect on LH levels in males and females** **Females:** Two robust studies in rats report NS effects on LH levels after exposure. One study reports no effects. One dermal study conducted in humans reported no effects K3/K4 studies: Two studies with lower reliability support effects on LH levels after exposure. **Males:** One study in adult rats reported effects on LH levels after short term exposure. The effect was supported by a robust study, showing dose-related but NS effects on LH levels in developmentally exposed males. One dermal study conducted in humans reported no effects
OECD TG 421 Unpublished, 2004 (Klimisch 2)	EAS	*In vivo* mechanistic	LH level	Rat	Premating, gestation, lactation (84 days exposure). (n=10 parental females/exposure group)	Oral	12.5, 25, 50	No effect on dam bw after 84 days of exposure, i.e. at the time of weaning. Liver weight was not assessed.	Dams on PND22: In high dose group, LH increased by 1500% and 3300% in mid and high dose groups, respectively (corresponding to 16 and 34 times higher than control group). Effects were NS, likely due to high variation in the groups.	
OECD TG 421 Unpublished, 2004 (klimisch 2)	EAS	*In vivo* mechanistic	LH level	Rat	Gestation, lactation until PND 22 (n=30)	Oral	12.5, 25, 50	No decreases in offspring bw were observed on PND 55. Offspring liver weights were not assessed.	Offspring PND55 Males: LH was dose-dependently decreased in mid (20%) and high (34%) dose groups (NS). Females: No effects on LH	
Carou *et al.* 2008 (Klimisch 2)	EAS	*In vivo* mechanistic	LH level	Rat	2 days (adult males) (n=10-12)	s.c.	2, 20	Body and liver weights were not reported, but the low doses and short exposure period probably did not affect either	LH was decreased in both groups (males).	
Carou *et al.* 2008 (Klimisch 2)	EAS	*In vivo* mechanistic	LH level	Rat	5 days (adult males) (n=10-12)	s.c.	2, 10	Body and liver weights were not reported, but the low doses and short exposure period probably did not affect either	LH was decreased in both groups (males).	
Janjua *et al* (2004) (Klimisch 2)	EAS	*In vivo* mechanistic	LH level	Human	4 consecutive days (15 young males, 17 postmenopausal females)	Dermal	2 mg/cm2 with 10% (wt/wt) formulation	Not assessed	There were no biological effects on LH.	
Carou *et al.* 2009b (Klimisch 3).	EAS	*In vivo* mechanistic	LH level	Rat	Every second day during	s.c.	100	Dam bw were not reported. Offspring	PND 70 male offspring: 40-50% decrease in serum LH levels.	

					pregnancy (n=10-13)			bw on PND 70 were unaffected	PND 70 female offspring: 400% increase in serum LH levels	K3/K4 studies: Two studies with lower reliability support effects on LH levels after exposure.
Carou *et al.* 2009a (Klimisch 3)	EAS	*In vivo* mechanistic	LH level	Rat	Every second day from pregnancy onset to day of delivery	s.c.	20, 100, 500	Body and liver weights were not reported in neither dams or offspring	In offspring males, exposure to 20 mg/kg bw/day increased LH in 30-day-old males. Doses of 100 and 500 mg/kg bw/ day caused a decrease in LH level in prepubertal rats (15-day-old males), and an increase in LH concentrations in peripubertal rats (30-day-old males).	
Seidlova-Wuttke *et al.* 2006a (Klimisch 3)	EAS	*In vivo* mechanistic	LH level	Rat	12 weeks (adult) (n=12)	Feed	230, 1000	Animals exposed to both doses of 4-MBC gained less bw than control animals. Liver weights were not reported.	Both doses caused increased serum LH levels (opposite the effect of E2) in ovariectomised female rats.	
OECD TG 421 Unpublished, 2004 (Klimisch 2)	EAS	*In vivo mechanistic*	Follicle stimu-lating hormone (FSH) level	Rat	21 days exposure in the pre-mating period (n =3-5 females)	Oral	12.5, 25, 50	In maternal animals there were no clinical signs and no effects on bw gain or food consumption.	FSH not affected in parental females.	**Weak-moderate evidence of endocrine activity *in vivo* in studies showing effect on FSH levels in males and females** **Females:** One robust study in rats reports effects on FSH levels, however the effects were NS. One dermal study conducted in humans reported no effects. K3/K4 studies: One study with lower reliability support effects on FSH levels after exposure. **Males:** Two robust studies in rats report decreased FSH levels after exposure.
OECD TG 421 Unpublished, 2004 (Klimisch 2)	EAS	*In vivo mechanistic*	FSH level	Rat	Premating, gestation, lactation (84 days exposure). (n=10 parental females/exposure group)	Oral	12.5, 25, 50	No effect on dam bw after 84 days of exposure. Liver weight was not assessed.	Dams, PND22: In high dose group, FSH increased by 260% (3.6 times higher than control). Effects were NS, likely due to high variation.	
OECD TG 421 Unpublished, 2004 (Klimisch 2)	EAS	*In vivo mechanistic*	FSH level	Rat	Gestation, lactation until PND 22 (n=30)	Oral	12.5, 25, 50	No decreases in offspring bw were observed on PND 55. Offspring liver weights were not assessed.	Offspring PND55 Males: Decreases in FSH levels was seen in mid (27%) and high (56%) dose groups. Females: A decrease (61%) in FSH level was seen in high dose group (NS, likely due to high variation).	
Carou *et al*. 2008 (Klimisch 2)	EAS	*In vivo mechanistic*	FSH level	Rat	5 days (adult) (n=10-12)	s.c.	2, 10	Body and liver weights were not reported, but were probably not affected	FSH was decreased in both groups (males).	
Carou *et al*. 2008 (Klimisch 2)	EAS	*In vivo mechanistic*	FSH level	Rat	2 days (adult) (n=10-12)	s.c.	2, 20	Body and liver weights were not	FSH was decreased the 2 mg/kg bw/day group (males).	

								reported, but were probably not affected		One dermal study conducted in humans reported no effects. K3/K4 studies: Two studies with lower reliability support effects on FSH levels after exposure.
Janjua *et al.* (2004) (Klimisch 2)	EAS	*In vivo mechanistic*	FSH level	Human	4 consecutive days (15 young males, 17 postmenopausal females)	Dermal	2 mg/cm2 with 10% (wt/wt) formulation	Not assessed	There were no biological effects on FSH.	
Carou *et al*. 2009b (Klimisch 3).	EAS	*In vivo mechanistic*	FSH level	Rat	Every second day during pregnancy (n=10-13)	s.c.	100	Offspring bw on PND 70 were unaffected	PND 70 male offspring: 40-50% decrease in FSH level. Female offspring: 90% increase in FSH levels.	
Carou *et al*. 2009a (Klimisch 3)	EAS	*In vivo mechanistic*	FSH level	Rat	Every second day from pregnancy onset to day of delivery	s.c.	20, 100, 500	Body and liver weights were not reported	In male offspring doses of 100 and 500 mg/kg bw/ day caused an increase in FSH level in peripubertal rats (30-day-old males).	
Carou *et al.* 2008 (Klimisch 2)	EAS	*In vivo* mechanistic	Gonadotropin releasing hormone (GnRH)	Rat	2 days (adult males) (n=10-12)	s.c.	2, 20	Body and liver weights were not reported, but were probably not affected	Ex-vivo hypothalamic GnRH release was decreased in both doses.	**Some evidence of decreased GnRH secretion** (ex-vivo measurements) **Females:** K3/K4: One study with lower reliability showed unaffected GnRH release in adult prenatally exposed offspring. **Males:** In one robust study a decrease was seen after 2-day exposure in adult rats. K3/K4: Two studies with lower reliability showed decreased GnRH release in adult prenatally exposed offspring.
Carou *et al.* 2009b (Klimisch 3)	EAS	*In vivo* mechanistic	GnRH	Rat	Every second day during pregnancy (n=10-13)	s.c.	100	Offspring bw on PND 70 were unaffected	PND 70 male offspring 80% decrease in GnRH secretion. PND 70 female offspring: GnRH release not affected.	
Carou *et al.* 2009a (Klimisch3)	EAS	*In vivo* mechanistic	GnRH level	Rat	Every second day from pregnancy onset to day of delivery	s.c.	20, 100, 500	Body and liver weights were not reported	Doses of 100 and 500 mg/kg bw/ day caused a decrease in GnRH level in prepubertal rats (15-day-old males).	
OECD TG 421 Unpublished, 2004 (Klimisch 2)	EAS	*In vivo* mechanistic	Testosterone level	Rat	Gestation, lactation until PND 22 (n=30)	Oral	12.5, 25, 50	No decreases in offspring bw were observed on PND 55. Offspring liver weights were not assessed.	Offspring PND55 Males: Testosterone was decreased in all three groups in a dose related manner (26%, 33%, 60%	**Weak-moderate evidence of decreased testosterone** One robust animal study found dose-

									respectively. NS, likely due to large variation and outliers).	dependent but NS decreases in male offspring. One human study found no effects related to 4-MBC exposure (males and females).
Janjua *et al.* (2004) (Klimisch 2)	EAS	*In vivo* mechanistic	Testosterone	Human	4 consecutive days (15 young males, 17 postmenopausal females)	Dermal	2 mg/cm2 with 10% (wt/wt) formulation	Not assessed	Effects on testosterone levels were seen at some time points, but these seemed unrelated to exposure and were considered chance findings.	
OECD TG 421 Unpublished, 2004 (Klimisch 2)	EAS	*In vivo* mechanistic	Prolactin level	Rat	21 days exposure in the pre-mating period (n =3-5)	Oral	12.5, 25, 50	There were no clinical signs and no effects on bw gain or food consumption	Prolactin levels were elevated in mid and high dose parental females (100% in both groups), but the increase was NS.	**Evidence of no effect on prolactin levels in males and females**
OECD TG 421 Unpublished, 2004 (klimisch 2)	EAS	*In vivo* mechanistic	Prolactin level	Rat	Gestation, lactation until PND 22 (n=30)	Oral	12.5, 25, 50	No decreases in offspring bw were observed on PND 55. Offspring liver weights were not assessed.	Offspring PND55 Males and females: No effects on prolactin.	
Carou *et al.* 2008 (Klimisch 2)	EAS	*In vivo* mechanistic	Prolactin level	Rat	2 days (adult males) (n=10-12)	s.c.	2, 20	Body and liver weights were not reported, but were probably not affected	Prolactin levels were unaffected (males).	
Carou *et al.* 2009a (Klimisch 3)	EAS	*In vivo* mechanistic	Prolactin level	Rat	Every second day from pregnancy onset to day of delivery	s.c.	20, 100, 500	Body and liver weights were not reported	Prolactin levels were unaffected (males)	
OECD TG 421 Unpublished, 2004 (Klimisch 2)	EAS	*In vivo* mechanistic	Estradiol (E2) level	Rat	21 days exposure in the pre-mating period (n =3-5)	Oral	12.5, 25, 50	There were no clinical signs and no effects on bw gain or food consumption	Females: E2 levels increased 45% in high-dose group (NS).	**Equivocal evidence for effect on serum E2 levels in both males and females** No consistent effects on E2 levels were seen in the TG 421 study or in the human study in both males and females, K3/K4 studies: A study of lower reliability did found decreased serum E2 in 4-MBC exposed OXV female rats treated for 12 weeks.
OECD TG 421 Unpublished, 2004 (Klimisch 2)	EAS	*In vivo* mechanistic	E2 level	Rat	Premating, gestation, lactation (84 days exposure). (n=10 parental females/exposure group)	Oral	12.5, 25, 50	No effect on dam bw after 84 days of exposure. Liver weight was not assessed.	Dams on PND22 No effect on E2 levels.	
OECD TG 421 Unpublished, 2004 (Klimisch 2)	EAS	*In vivo* mechanistic	E2 level	Rat	Gestation, lactation until PND 22 (n=30)	Oral	12.5, 25, 50	No decreases in offspring bw were observed on PND 55. Offspring liver weights were not assessed.	Offspring PND55 Males and females: No effects on E2	

Janjua *et al.* (2004) (Klimisch 2)	EAS	*In vivo* mechanistic	E2 level	Human	4 consecutive days (15 young males, 17 postmenopausal females)	Dermal	2 mg/cm2 with 10% (wt/wt) formulation	Not assessed	There were no biological effects on estradiol. Some effects were seen on estradiol levels, but these seemed unrelated to exposure and were considered chance findings.	
Seidlova-Wuttke *et al.* 2006a (Klimisch 3)	EAS	*In vivo* mechanistic	E2 level	Rat	12 weeks (adult) (n=12)	Feed	230, 1000	Animals exposed to both doses of 4-MBC gained less bw than control animals. Liver weights were not reported.	Both doses caused decreased serum E2 levels in ovariectomised female rats.	

Table 4.12: Lines of evidence for **adverse effects** *in vivo* via **EAS modalities.** Effect target: Female and male reproductive system. Nervous system. All reported changes are statistically significant with p<0.05, unless specified otherwise. NS: Non-statistically significant.

Reference	Effect Classify-cation	Effect target	Species	Exposure	Route	Dose (mg/kg bw/day)	General toxicity	Observed effect	Assessment of each line of evidence
Faass *et al.* 2009 (Klimisch 2)	EAS	Fem. Reprod. system	Rat	Fetal, postnatal, and adult exposure	Feed	7, 24, 47	Bw of adult female 4-MBC-exposed offspring was unchanged	Sexual behaviour in adult female offspring was altered in a dose-related manner. The effects were seen as reduced proceptive and receptive behaviour, and increased rejection behaviour towards the male. The effects were seen in both examined dose groups (7 & 24 mg/kg), on individual bases (n=12-14) and on litter bases (n=6-7).	**Moderate-strong evidence for adverse effects on female sexual behaviour** Sexual behaviour was altered in adult females on both individual and litter basis.
Faass *et al.* 2009 (Klimisch 2)	EAS	Fem. Reprod. system	Rat	Fetal, postnatal, pubertal and adult exposure	Feed	7, 24, 47	Bw of adult female 4-MBC-exposed offspring was unchanged	25% of the female offspring in high dose group had irregular estrous cycling compared to 5% in the control group, indicating an increase that was, however, NS.	**Moderate evidence for adverse effects on female reproductive organ weight, AGD, pubertal timing and estrous cycling.** **Ovary weight:** 1 out of 2 studies in adult females report reduced ovary weight. In this high-dose study, bw was also reduced. In perinatally exposed offspring increased ovary weight was reported at weaning, whereas no effects were seen on PND55.
Durrer *et al.* 2007 (Klimisch 2)	EAS	Fem. Reprod. system	Rat	Fetal, postnatal, and adult exposure	Feed	7, 24, 47	Bw of adult male 4-MBC–exposed offspring were in the control range, and their liver weight were unaltered. Bw at onset of puberty was at control level in males, but slightly reduced in females.	Timing of vaginal opening (VO) was not affected.	
OECD TG 421 Unpublished, 2004	EAS	Fem. Reprod. system	Rat	21 days exposure in the pre-mating (n	Oral	12.5, 25, 50	There were no clinical signs and no effects on bw gain or food consumption	Maternal estrous cycling not affected (adult non-pregnant females)	

(Klimisch 2)				=3-5)					
OECD TG 421 Unpublished, 2004 (Klimisch 2)	EAS	Fem. Reprod. system	Rat	Gestation, lactation until PND 22 (n=10). Parental females exposure group	Oral	12.5, 25, 50	No effect in offspring bw were observed. Offspring liver weights were not assessed.	Offspring: female AGD was increased in low and high dose groups (between 9-19% increases compared to control). See study description for details. VO was delayed in the low dose group.	**Uterine weight:** No effects were seen in two studies with adult exposure. In perinatally exposed offspring one study showed reduced uterus weight at PND 22 but not at PND 55. Another study showed no effect in adult females exposed during development. No studies found any effect on histology. **AGD:** The one study investigating this endpoint found significantly increased AGD in exposed animals. **VO:** Indications of delayed VO were seen in one study where exposure stopped at weaning. In another developmental study, where exposure continued, no effect on sexual maturation was seen. K3/K4 studies: In a less robust developmental study using a markedly higher doses, VO was shown to be 3 days advanced. **Estrous cyclicity**: Was not affected in females exposed during adulthood whereas a NS increase in irregular estrous cycling was observed in a study with developmental and continued exposure.
OECD TG 421 Unpublished, 2004 (Klimisch 2)	EAS	Fem. Reprod. system	Rat	Gestation, lactation until PND 22 (n=10). Parental females exposure group	Oral	12.5, 25, 50	No effect on offspring bw on PND 22. Liver weights not assessed	Offspring PND22: 25% increase in ovary weight in mid and high dose group (NS in mid dose). Uterus weight was decreased in mid and high dose (39%) (NS in mid dose). No effects on ovary and uterus histology.	
OECD TG 421 Unpublished, 2004 (Klimisch 2)	EAS	Fem. Reprod. system	Rat	Gestation, lactation until PND 22 (n=10). Parental females exposure group	Oral	12.5, 25, 50	No decreases in offspring bw were observed on PND 55. Offspring liver weights were not assessed.	Offspring PND55: No effect on reproductive organ weights or histology. Exposure stopped at weaning.	
OECD TG 421 Unpublished, 2004 (Klimisch 2)	EAS	Fem. Reprod. system	Rat	Premating, gestation, lactation (84 days exposure). (n=10)	Oral	12.5, 25, 50	No effect on dam bw after 84 days of exposure. Liver weight was not assessed.	Dams on PND22 (after 84 days of exposure): No effect on ovary or uterus weight.	
28-day oral, Unpublished, 1983b (Klimisch 2)	EAS	Fem. Reprod. system	Rat	28-day study (n=10/sex)	Oral	0, 1000	4-MBC induced clinical signs and markedly reduced bw (20% lower in males and 10 % lower in females). Liver weights were increased and thymus and adrenal weights reduced.	Reduced ovary weight. No effect on uterus weight	
Carou *et al.* 2009b (Klimisch 3)	EAS	Fem. Reprod. system	Rat	Dams exposed during pregnancy (n=10-13)	s.c.	100	Offspring bw on PND 70 were unaffected	No effect on adult uterine weight in developmentally exposed female offspring	
Carou *et al.* 2009b (Klimisch 3).	EAS	Fem. Reprod. system	Rat	Every second day during pregnancy (n=10-13)	s.c.	100	Offspring bw at the time of VO were not assessed. Body weighs on PND 70 were unaffected	VO in offspring was 3 days advanced.	
OECD TG 414 Unpublished 1988 (Klimisch 1)	Sensitive to, but not diagnostic of, EAS	Fem. Reprod. system	Rat	Dams exposed during pregnancy (gestation day 6-15 (n=25 females)	Oral	0, 10, 30, 100	Dose level of 100 mg/kg was shown to be minimally toxic to the dams as demonstrated by a slightly lower body weight gain by these females	Dams: Number of implantation, corpora lutea, post-implantations loss were unaffected by the treatment.	**Evidence for no adverse effects on reproductive parameters sensitive but not diagnostic of EATS**

OECD TG 421 Unpublished, 2004 (Klimisch 2)	Sensitive to, but not diagnostic of, EAS	Fem. Reprod. system	Rat	Premating, gestation, lactation (84 days exposure). (n=10)	Oral	12.5, 25, 50	No effect on dam bw after 84 days of exposure. Liver weight was not assessed.	Dams: no effects on implantation rate, post-implantation loss	
17-day oral, Unpublished, 1983a (Klimisch 1)	EAS	Male reprod. system	Rat	17-day study (n=10/sex per dose group)	Oral	0, 30, 300	No effect on bw or food consumption, but non-specific clinical signs (salivation, excessive grooming) were observed at both doses	Prostate weight was reduced at both doses.	**Moderate-strong evidence for effect on the male reproductive system** 7 studies reported weight of different male sex organs and all studies saw effects. Among these 6 studies looked at prostate weight and 4 studies reported reduced adult prostate weight and one study a tendency towards reduced weight. One K3 study looked at volume and reported increased volume of neonatal prostate. One study investigated AGD and nipple retention. AGD was increased. Two studies investigated sexual maturation, 1 study reported no effect and 1 study reported delayed sexual maturation.
28-day oral, Unpublished, 1983b (Klimisch 2)	EAS	Male reprod. system	Rat	28-day study (n=10/sex)	Oral	0, 1000	Marked systemic toxicity, seen as clinical signs and markedly reduced bw (20% lower in males and 10 % lower in females). Liver weights were increased	Atrophy of the accessory sex glands was observed and prostate weight was reduced. No effect on testes.	
OECD TG 408, Unpublished 1984a (Klimisch 1)	EAS	Male reprod. system	Rat	90 day study (n=20 animals /sex)	Feed	0, 50, 125, 312. A dose of 25 was included with its own control group.	Decreased bw gain in females at 312 mg/kg (transient at 125 mg/kg). No effect on male bw. Relative liver weights were increased in females at 125 and males at 312 mg/kg bw/day.	In high dose males, reduced prostate weight was observed.	
OECD TG 421 Unpublished, 2004 (Klimisch 2)	EAS	Male reprod. system	Rat	Gestation, lactation until PND 22 (n=10). Parental females exposure group	Oral	12.5, 25, 50	No effect on offspring bw on PND 22. Liver weights were not assessed.	Offspring PND22: 12% decrease in seminal vesicle weight in high dose group (NS). No effect on testes, prostate and epididymis weights.	
TG 421 Unpublished, 2004 (Klimisch 2)	EAS	Male reprod. system	Rat	Gestation, lactation until PND 22 (n=10). Parental females exposure group	Gestation, lactation until PND 22 (n=10). Parental females exposure group	12.5, 25, 50	No effect on offspring bw on PND 55. Liver weights were not assessed.	Offspring PND55: Prostate weight was 10% decreased in high dose group (NS). Seminal vesicle weight was 10% increased in low and high dose (NS), 20% increase in the mid dose group. No effect on other male reproductive organ weight or histology.	
OECD TG 421 Unpublished, 2004 (Klimisch 2)	EAS	Male reprod. system	Rat	Gestation, lactation until PND 22 (n=10). Parental females exposure group	Oral	12.5, 25, 50	Offspring bw on day 1 were increased in the mid and high dose groups, but it is unclear if the effect was statistically significant	Offspring: AGD was increased in the high dose group (9%). No effect on timing of testes descent or sexual maturation. No effect on nipple retention, however the reporting of the results was limited.	
Durrer *et al.* 2007	EAS	Male reprod.	Rat	Fetal, postnatal, pubertal and	Feed	7, 24, 47	Body weight at onset of puberty was at control	Delayed male puberty in all exposure groups, in a dose-	

(Klimisch 2)		system		adult exposure			level in males, but slightly reduced in females. On PND 55 when reproductive organs weights were assessed there were no effects on body weight.	related manner, with a delay of preputial separation of approximately 3 days in the high dose group. In adult male offspring, absolute and relative prostate weights were decreased in all dose groups. 24 and 47 mg/kg bw/day increased testis weight. Epididymis and seminal vesicle weight were reduced in the high dose group, however, NS.	
Carou *et al.* 2009a (Klimisch 3)	EAS	Male reprod. system	Rat	Dams exposed from pregnancy onset. Litters killed on PND 13 or 30	s.c.	20, 100, 500	Body and liver weights were not reported	Testicular weight (absolute) was decreased in 100 and 500 mg/kg bw/ day groups on PND 15. No effect was seen on testes weight on PND 30.	
Hofkamp *et al.* 2008 (Klimisch 3)	EAS	Male reprod. system	Rat	Dams exposed during gestation (n=4)	Feed	0.7, 7	Offspring bw were not reported, but we know from other publications that doses of 0.7 and 7 mg/kg does not affect offspring bw in the early postnatal period.	PND 1: 4-MBC caused proliferative effect on prostate growth. An increase of 60-70% in accessory sex glands and prostate volume was seen, caused by increasing number and volume of ducts in the prostate.	
OECD TG 421 Unpublished, 2004 (Klimisch 2)	Sensitive to, but not diagnostic of, EAS	Nervous system	Rat	Gestation, lactation until PND 22 (n=10). Parental females exposure group	Oral	12.5, 25, 50	No effects on bw. Liver weights were not assessed.	Offspring: No effect on learning and memory in male or female offspring.	**Not sufficient evidence to determine whether learning and memory are affected by developmental exposure to 4-MBC** Only investigated in one study using relatively low doses, a group size of 10 and a rather insensitive scoring method for the behavioural results, is not in itself robust enough to exclude effects on neurodevelopment by 4-MBC exposure.

4.10.5.1 Summary of the line of evidence - EAS mediated effects

The lines of evidence presented in Table 4.9-4.12 can be summarised as follows:

Table 4.13: WoE for EAS-mediated endocrine activity and adverse effects

EAS mediated endocrine activity *in vitro*:

- Several *in vitro* assays from good quality studies provide strong evidence for estrogenic activity, as induction of estrogenic response is seen in the E-screen measured as proliferation, gene or protein expression (Schlumpf *et al*. 2001; Tinwell *et al.* 2002; Schlumpf *et al.* 2004a; Klann *et al.* 2005; Matsumoto *et al.* 2005 (K4); Heneweer *et al.* 2005; Jiménez-Díaz *et al.* 2013) and (weak) ER agonistic properties is seen in various studies (Klann *et al.* 2005; Gomez *et al.* 2005; Schreurs *et al.* 2002; Minh *et al.* 2008; Schlumpf *et al.* 2004a; Schmitt *et al.* 2008; Jocsak *et al.* 2016; Matsumoto *et al.* 2005 (K4); Mueller *et al.* 2003). Some studies reported no ER agonistic effect or equivocal evidence on ER binding (Tinwell *et. al* 2002; Schlumpf et al. 2004a, Matsumoto *et al.* 2005 (K4); Morohoshi *et al.* 2005; Kunz & Fent 2006; Kunz *et al.* 2006).
- Several *in vitro* studies (4/5) provide moderate-strong evidence for androgen receptor antagonism, but no effect on androgen receptor agonism (Ma *et al.* 2003; Schreurs *et al.* 2005; Kunz & Fent 2006; Nashev *et al.* 2010; Jiménez-Díaz *et al.* 2013).
- One study provides *in vitro* evidence for effects on steroidogenesis (Nashev *et al.* 2010) while there is moderate-strong evidence for progesterone mimicking in sperm cells (Schiffer *et al.* 2014; Rehfeld *et al.* 2016; Rehfeld *et al.* 2018).

E mediated endocrine activity *in vivo*:

- Uterotrophic assays in immature females show strong evidence of estrogenic activity *in vivo*, seen as increased uterine weight after exposure (Schlumpf *et al*. 2001; Tinwell *et al*. 2002; Ashby *et al*. 2004).
  K3/K4 studies: Additional evidence is present from a study showing increased uterine weights in OXV females after 3-months exposure to 4-MBC (Seidlova-Wuttke *et al*. 2006a, Seidlova-Wuttke *et al*. 2006b)
- In rats exposed during fetal, postnatal, pubertal and adult period, there is moderate-strong evidence that estrogen sensitive genes in the brain were affected in a sexually dimorphic pattern after exposure (Maerkel *et al*. 2007) and estrogen regulated genes in the uteri (Durrer *et al*. 2005) and prostate (Durrer *et al.* 2007) were altered
  K3/K4 studies: Two studies also found alterations in gene expression in the brain (Maerkel *et al.* 2005) or uteri (Seidlova-Wuttke *et al.* 2006b).
- *In vivo* studies in rats exposed in adulthood, show weak-moderate evidence of endocrine activity *in vivo*, as effects were seen on gonadotropin levels, primarily reduced levels of both LH and FSH (Unpublished, 2004; Carou *et al*. 2008). The same pattern is seen in perinatally exposed offspring (Unpublished, 2004).
  K3/K4 studies: A few studies of lower reliability, also support that changes in LH and FSH levels occur in 4-MBC exposed animals (Seidlova-Wuttke *et al.* 2006a; Carou *et al.* 2009a; Carou *et al.* 2009b).
  An experimental study in humans using dermal exposure report no effects on gonadotropin levels (Janjua *et al*. 2004).

EAS mediated *in vivo* adverse effects:

- There is moderate-strong evidence of adverse effect related to EAS modalities, as altered female sexual behaviour was seen in offspring exposed during fetal, postnatal, pubertal and adult life (Faass *et al*. 2009). There is also moderate evidence for effects

on ovary weight, uterine weight, AGD, VO and estrous cycling (Unpublished, 1983b; Unpublished, 2004; Carou *et al*. 2009b, (K3)).
No effect was reported on other female reproductive parameters sensitive to, but not diagnostic of EAS (Unpublished, 1988; Unpublished, 2004)

- There is moderate-strong evidence for adverse effects on the male reproductive system, with effects seen in prostate and other male reproductive organs (Unpublished, 1983a,b; Unpublished, 1984a; Unpublished, 2004;). Decreased prostate weight was seen in several of these good quality/reliable studies (Unpublished, 1983a, 1983b; 1984a; Unpublished, 2004; Durrer *et al*. 2007). Effect on AGD (increase) and sexual maturation (delay in preputial separation) were also reported (Unpublished, 2004; Durrer *et al*. 2007).
  K3/K4: Studies with lower reliability also indicate adverse effects on the male reproductive system (Carou *et al*. 2009a; Hofkamp *et al*. 2008)

4.10.5.2 A discussion of the overall evidence and relation to possible other toxicity is presented below:

Endocrine activity related to EAS modalities:

The lines of evidence for *in vitro* studies on endocrine activity (Table 4.8) led to the conclusion that there is **strong evidence of endocrine activity seen as an estrogenic response in the E-screen**. All 6 reliable studies and one study of lower reliability found a clear estrogenic response, typically at concentrations around 10µM or lower (Schlumpf *et al.* 2001; Tinwell *et al.* 2002; Schlumpf *et al.* 2004a; Klann et al. 2005; Matsumoto *et al.* 2005 (K4); Heneweer *et al.* 2005; Jiménez-Díaz *et al.* 2013). In most of the studies, MCF-7 cell proliferation was increased and some studies also measured pS2-protein expression or pS2 gene transcription, and here an increase was seen (Schlumpf *et al.* 2001, Heneweer *et al.* 2005). **Strong evidence for ED activity on ER agonistic properties** was found, as the majority of the published studies (7/11) did find 4-MBC to transactivate the ER (Klann *et al.* 2005; Gomez *et al.* 2005; Schreurs *et al.* 2002; Minh *et al.* 2008; Schmitt *et al.* 2008; Jocsak *et al.* 2016; Mueller *et al.* 2003). The specific ER activation to either the ERα or ERβ was investigated. Some studies found clear activation of the ERα and ERβ receptors (Gomez *et al.* 2005; Schreurs *et al.* 2002), some found potent activation or binding to ERβ but weak activation or lack of binding toERα (Mueller *et al.* 2003; Schlumpf *et al.* 2004a). A study with lower reliability found weak binding to both receptor subtypes (Matsumoto *et al.* 2005 (K4)). Differences in study design and investigated concentrations may explain some of the observed differences. Jocsak *et al.* 2016 showed that 4-MBC exposure could also affect mRNA expression of the ERβ. Other studies did not specify the receptor subtype they investigated (Klann *et al.* 2005, Minh *et al.* 2008), but did find an estrogenic response in the employed assays. One study concluded that the evidence of ER binding was equivocal (Tinwell *et al.* 2002), whereas three other studies performed in yeast cells transfected with hER (YES assay) concluded no agonistic effects of 4-MBC treatment (Morohoshi *et al.* 2005; Kunz & Fent 2006; Kunz *et al.* 2006). In contrast, a study by Schmitt *et al.* (2008) did show ERα binding in a YES assay, and they proposed that the apparent difference in results could be due to a different design of their YES assay, since theirs included an additional enzymatic digestion of the yeast cells which may have increased its sensitivity. Furthermore, the negative study by

Morohoshi *et al.* (2005) only tested relatively low concentrations of 4-MBC which could also explain some of the discrepancies with the previous studies showing estrogenic responses in vitro, though often at higher test concentrations.

The evidence for ER antagonism was equivocal. Of the four studies investigating this, two found clear antagonistic effects whereas the other two studies showed no antagonism on ERα or ERβ. The clear antagonistic effects were seen on hERα in a YES assay (Kunz & Fent 2006) and in human endometrial Ishikawa cells transfected with ERα or ERβ (Mueller *et al.* 2003), whereas the two studies which showed no antagonism investigated ERα or ERβ activity in stably transfected HEK293 cell (Schreurs *et al.* 2005) and in a YES assay (Morohoshi *et al.* 2005). The employed test systems could therefore not explain the differences in results.

None of the five studies investigating effects of 4-MBC on the AR, showed an agonistic response (Ma *et al.* 2003; Schreurs *et al.* 2005; Kunz & Fent 2006; Nashev *et al.* 2010; Jimenez-Diaz *et al.* 2013). However, four of these studies did find a weak (Nashev *et al.* 2010) or even marked antagonistic effects on the AR (Schreurs *et al.* 2005; Kunz & Fent 2006; Jiménez-Díaz *et al.* 2013), hence there was **moderate-strong evidence for endocrine activity seen as AR antagonism.** The effect could not be explained by cytotoxicity (Nashev *et al.* 2010; Kunz & Fent 2006; Jiménez-Díaz *et al.* 2013. Not reported in Schreurs *et al.* 2005).

Supportive evidence for endocrine activity seen as effects on steroidogenesis was present as inhibition of steroid hormone synthesis enzymes 17β-HSD3, 17β-HSD1 & 17β-HSD2 was reported by (Nashev *et al.* 2010). However, this was only investigated in one study.

Studies investigating PR agonism/antagonism are few and equivocal. Schreurs *et al.* 2005 found no agonistic but marked antagonistic activity in PR callux reporter gene assay, whereas Yin *et al.* (2015) showed no anti-progestogenic effect in Ishikawa cells, assessed by SULT1E1 mRNA levels. The different findings between transactivation assays and tissue-specific cell models (e.g. Ishikawa cells) could be due to different sensitivities of the assays. Yin *et al.* (2015) discussed in their paper that the PR CALUX assay displays high hPR expression, resulting in a high sensitivity towards PR ligand and that the Ishikawa assay might therefore be less sensitive than the PR-CALUX transactivation assay.

While effects of 4-MBC on the PR are unresolved, it is clear that 4-MBC exposure **mimics the effects of progesterone in human sperm cells**, by causing an Ca^{2+} influx into the sperm cells through the CatSper channel. This mechanism has been shown in three separate publications, though all performed by the same research group. Schiffer *et al.* (2014) found 4-MBC to cause an increase in Ca^{2+} flux, similar to that observed with progesterone. Rehfeld *et al. (*2016; 2018) also showed that the Ca^{2+} signals induced by 4-MBC in human sperm cells resembled the ones induced by progesterone and that this exposure caused a statistically significant increase in viable acrosome reacted sperm cells. It is not clear if this response can be evaluated as an indication of endocrine activity.

The lines of evidence on endocrine activity *in vivo* (Table 4.9) led to the conclusion that there is **strong evidence for endocrine activity *in vivo***, as 4-MBC induces an **estrogenic response in target tissue**, as uterine weight changes in both immature and OVX females. Increased uterine weights were reported in uterotrophic assays using both oral, dermal and s.c. exposure (Schlumpf *et al.* 2001; Tinwell *et al.* 2002). When Ashby and co-workers (2004) tried to repeat their own findings of estrogenic activity in the

uterotrophic assay (reported in Tinwell *et al.* 2002), their new results indicated that uterotrophic effect were most consistently seen when using females aged 19-21 days at the beginning of the study. Studies with lower reliability also show increases in uterine weights in longer exposure scenarios, as three months of dosing of adult female ovariectomized rats caused a 50% increases in uterine weights (Seidlova-Wuttke *et al.* 2006 a; Seidlova-Wuttke *et al.* 2006; Seidlova-Wuttke *et al.* 2006b). The statistical significance of the effects was not properly disseminated in the two publications, and the effects on uterus weight did not seem to be dose-related, but apparently only the high dose of 1000 mg/kg bw/day was used in the first half of the study. In addition, changes in estrogen-regulated gene expression in the uterus were also observed in this study (Seidlova-Wuttke *et al.* 2006b). The conclusion on moderate-strong evidence for an estrogenic response in target tissue is also clear from findings of **altered gene and protein expression on estrogen sensitive genes in the brain** (Maerkel *et al*. 2007), **uteri** (Durrer *et al*. 2005) and **prostate** (Durrer *et al.* 2007) in offspring from all dose groups, exposed during development and with continued exposure in adulthood.

There is **weak-moderate evidence of endocrine activity *in vivo*** from effects on **gonadotropin levels;** reliable studies measuring these hormones found changes in LH and FSH levels (Unpublished, 2004; Carou *et al*. 2008) and these results are corroborated by results from studies with lower reliability pointing in the same direction (Seidlova-Wuttke *et al*. 2006a; Carou *et al*. 2009a; Carou *et al*. 2009b). In adult females exposed prior to mating, quite low doses of 4-MBC caused LH levels to decrease by 60% (non-statistically significant effect), while FSH levels were unaffected. In adult male rats receiving low dose s.c. injections for 2 or 5 days, statistical significant decreased serum concentrations of LH and FSH and decreases hypothalamic GnRH release were seen (Carou *et al.* 2008).
GnRH levels were also affected in dams and offspring in the extended reproductive toxicity screening study (Unpublished, 2004). Here 4-MBC exposure stopped at weaning on PND 22 and the highest investigated dose was 50 mg/kg bw/day. In dams on PND22, FSH concentrations were increased by 260% in high dose group (3.6 times higher than control) and LH was increased by 1500% and 3300% in mid and high dose groups, respectively (corresponding to 16 and 34 times higher than control group). The effects were non-statistically significant, likely due to high variation in the data. In male offspring on PND55, a statistically significant decrease in FSH levels was seen in mid (27%) and high (56%) dose groups. LH was dose-dependently decreased in mid (20%) and high (34%) dose groups (non-statistically significant). In the female PND 55 offspring, a non-statistically significant decrease of 61% in FSH was seen, whereas LH was unaffected. In a developmental study with lower reliability, using a dose of 100 mg/kg every other day during pregnancy, 40-80% decreases in LH, FSH and GnRH levels were seen in the adult male offspring and 90-400% increases in FSH and LH concentrations were seen in the adult female offspring (Carou *et al.* 2009b). In another developmental study with lower reliability, doses of 100 & 500 mg/kg during pregnancy altered LH and FSH concentrations in pre- and peripubertal male offspring, but in opposite directions on PND 15 (decrease) and 30 (increase) (Carou *et al.* 2009a). An unreliable study in adult OXV females exposed for 12 weeks, showed statistically significant increases in the circulating levels of LH (Seidlova-Wuttke *et al.* 2006a).

One experimental dermal study conducted in humans reported no effects on gonadotropins, which may be due to the exposure route as well as the relative short exposure period of 4 days (Janjua *et al*. 2004).

Adverse effect related to EAS modalities:

There is **moderate-strong evidence for adverse effect related to EAS modalities,** as **altered female sexual behaviour was seen in offspring exposed during fetal, postnatal, pubertal and adult life** (Faass *et al*. 2009). The exposures very markedly and in a dose-related-manner altered the sexual behaviour of adult female offspring. The effects were reduced proceptive and receptive behaviour, and increased rejection behaviour towards the male. The effects were statistically significant both when analysed on individual basis (n=12-14) and on litter basis (n=6-7) and were statistically significant in both examined dose groups (7 & 24 mg/kg). The behavioural results were closely correlated to findings of altered gene expression in sexually dimorphic areas of the brain in two studies with lower reliability (Maerkel *et al.* 2005, Maerkel *et al.* 2007).

There is **moderate evidence for other adverse effects on female reproduction, including changes in reproductive organ weights, AGD, VO and estrous cycling** (Unpublished, 1983b; Unpublished, 2004; Faass *et al*. 2009). In one study using a high dose that also induced systemic toxicity, resulted in reduced ovary weights in adult females, (Unpublished, 1983b). In the TG 421 reproductive toxicity screening study, where exposure stopped at weaning, no effects on ovary or uterus weight were seen in dams on PND 22 (after 84 days of exposure) (Unpublished, 2004). 4-MBC did however statistically significant increase ovary weights in the high dose offspring, when measured at weaning. The effects were no longer seen on PND55. This study also found statistically significant reduced uterus weights in offspring's at PND 22, but not at PND 55. None of the studies found any effects on ovary or uterus histology.

AGD was statistically significant increased in the female offspring from low and high dose groups in the TG421 reproductive screening study (Unpublished, 2004). No consistent pattern was seen for VO. Indications of delayed VO was seen in the TG421 study. A statistically significant effect was seen in the low dose group, but not at higher exposures. As exposure in this study stopped at weaning, the females were no longer exposed to 4-MBC at the time of the assessment. In another developmental study, where exposure continued throughout puberty, no effect on female sexual maturation was seen (Durrer *et al.* 2007). Estrous cyclicity was not affected in females exposed during adulthood (Unpublished, 2004) whereas a non-statistically significant increase in irregular estrous cycling was seen in a study with developmental and continued exposure (Faass *et al.* 2009). A developmental study with lower reliability, showed no effect on uterus weight in adult females exposed during fetal development while VO was shown to be 3 days advanced (Carou *et al.* 2009b). The different exposure period and markedly higher doses used in this study may have affected the hormonal system differently that the lower perinatal exposure employed in the TG421 study and the study by Durrer *et al*. 2007. However, the study is assessed to be not reliable, and therefore relatively little emphasis is put on its findings.

No effects of 4-MBC were reported on other female reproductive parameters (number of resorptions, implantations, corpora lutea) (Unpublished 1988; unpublished 2004). Absence of effect one these parameters alone cannot lead to a conclusion that 4-MBC have

no endocrine disrupting effects, as the endpoints investigated are only sensitive but not diagnostic to EATS.

There is **moderate-strong evidence for adverse effect on the male reproductive system** (reproductive organ weight and histopathology, AGD and sexual maturation) (Unpublished, 1983a; Unpublished, 1983b; Unpublished, 1984a; Unpublished, 2004; Durrer *et al*. 2007) with decreased prostate weight seen in several studies (Unpublished, 1983a,b; 1984a; Unpublished, 2004; Durrer *et al*. 2007). In the 90-day oral study (Unpublished, 1984a), reduced prostate weights were apparent in the high dose group of 312 mg/kg and in the 17-day expose study, prostate weights were reduced in both the low (30 mg/kg) and the high dose groups (300 mg/kg) (Unpublished, 1983a). At the systemic toxic dose of 1000 mg/kg, 28-day exposure caused atrophy of the accessory sex glands in the males, including statistically significant weight reductions of the prostate (Unpublished, 1983b). The prostate was also a target in the developmental studies from the Schlumpf group. In adult male offspring, 4-MBC caused dose-dependent decreases in absolute and relative prostate weights from a dose of 7 mg/kg and above (Durrer *et al.* 2007). Expression of estrogen regulated genes in the prostate was also affected (Durrer *et al.* 2007).
No effects on the prostate weight were observed in the developmentally exposed male offspring in the TG421 guideline study (Unpublished, 2004) on PND 22, but on PND 55 a non-statistically significant 10% decrease in prostate weight was seen. In the dermal repeated-dose toxicity study, no adverse effects on the prostate were observed at doses of 100 and 400 mg/kg (Unpublished, 2005). Histological examination of prostate was carried out only in the 90-day study, showing no differences between dose groups. In a study of lower reliability, a dose of 7 mg/kg bw/day was shown to cause proliferative effect on prostate growth, when assessed on PND 1. A very marked increase (60-70%) in prostate volume was seen at this age, caused by increasing number and volume of ducts in the prostate (Hofkamp *et al.* 2008). Unfortunately, the study only used a group size of 4, did not look at doses higher than 7 mg/kg, and did not assess the same endpoints at a later age.
Overall, the consistency findings in the repeated dose studies lead to the conclusion of **strong evidence for adverse effect on prostate growth.**
Other adverse effects on the male reproductive system have only been sporadically found after 4-MBC exposure in developmental studies, and a consistent pattern of effects is not seen. In adult males dosed for 17 or 90 days, reproductive organs other than the prostate gland were not adversely affected (Unpublished 1983a; Unpublished 1984a). Effects on accessory sex glands and prostate were found in male rats in a 28-day study, but no effects on testes (Unpublished 1983b). In a robust developmental studies, 4-MBC doses of 24 and 47 mg/kg were reported to cause statistically significant increases in testis weights (Durrer *et al.* 2007) and a non-statistically significant decrease in epididymis and seminal vesicle weights were also observed (Durrer *et al.* 2007). In the TG421 guideline study, a 12% (non-statistically significant) decrease in seminal vesicle weight was seen in high dose group at weaning. At this age, no effects on testes or epididymis weights were seen. On PND55, seminal vesicle weights showed a 10% increase in low and high dose males (non-statistically significant), and a 20% increase in the mid dose group (statistically significant). No effect on other male reproductive organ weight or histology

were noted (Unpublished, 2004). In the TG 421 guideline study, AGD was statistically significant increased in the high dose males. No effect on nipple retention was seen (Unpublished, 2004), however the reporting of this endpoint was limited. Results related to timing of male puberty differed between studies and are difficult to conclude on. Statistically significant delay in puberty (preputial separation) was seen in male offspring exposed perinatally and during post-weaning to doses of 7, 24 and 47 mg/kg, with delays of around 3 days in the high dose group (Durrer *et al.* 2007). This delay could be mediated by an endocrine MoA, but it is also possible that the effects were caused by a general delay in postnatal development. In the TG421 study also effects on sexual maturation of the male offspring were seen (Unpublished, 2004). This difference could possibly be explained by the fact that in the TG421 study offspring exposure stopped at weaning.
In developmental studies with lower reliability s.c. doses of 100 and 500 mg/kg bw/ day were reported to cause a decrease in testicular weight in prepubertal rats, but no change in testes weight in peripubertal rats (Carou *et al.* 2009a) and no effects on timing of the preputial separation in male offspring (Carou *et al.* 2009b).
Despite the lack of clear patterns of effect on other male reproductive endpoints, the findings for prostate are considered sufficient evidence for adverse effect related to EAS modalities. It is possible that the prostate is more sensitive to the estrogenic properties of 4-MBC than the other male reproductive endpoints, which may be more likely to be adverse affected by exposure to anti-androgenic chemicals. Hence, lack of clear effects on other male reproductive endpoints does not negate the consistent effects seen on the prostate gland. The available data are sufficient for that conclusion, but the lack of an extended one- or a two-generation study on 4-MBC implies that there would not be a sufficient data-set to conclude on *absence* of EAS-mediated adversity.

Assessment of whether EAS mediated endocrine activity and adverse effects have been sufficiently investigated. The ECHA/EFSA ED guidance (2018) states that when adversity is observed based on 'EATS-mediated' parameters, the biological plausibility of the link between the 'EATS-mediated' adversity and endocrine activity should be documented through a MoA analysis. In the guidance, this is presented in table 5 (ECHA/EFSA 2018).
For 4-MBC both adversity and activity related to EAS modalities was observed. There would however not be a sufficient data set for EAS-mediated adversity to support a conclusion on *absence* of EAS-mediated adversity. Hence, a MoA analysis should be performed (Scenario. 2b).

Table 4.14: Selection of relevant scenario (EAS-mediated parameters)

Adversity based on EAS-mediated parameters	**Positive mechanistic OECD CF level 2/3 Test**	**Scenario**	**Next step of the assessment**	**Scenario selected**
No (sufficiently investigated)	Yes/No	1a	Conclude: ED criteria not met because there is not "**EAS-mediated**" adversity	
Yes (sufficiently investigated)	Yes/No	1b	Perform MoA analysis	
No (not sufficiently investigated)	Yes/No	2a (i)	Perform MoA analysis (additional information may be needed for the analysis)	
No (not sufficiently investigated)	No (sufficiently investigated)	2a (ii)	Conclude: ED criteria not met because no **EAS-mediated endocrine activity** observed	
No (not sufficiently investigated)	No (not sufficiently investigated)	2a (iii)	Generate missing level 2 and 3 information. Alternatively, generate missing "EATS-mediated" parameters. Depending on the outcome move to corresponding scenario	
Yes (not sufficiently investigated)	Yes~~/No~~	2b	Perform MoA analysis	X

4.10.6 Mode of action (MoA) analysis

To evaluate the plausible link between adverse effects and endocrine activity, a MoA was performed taking into account *inter alia* the ECHA/EFSA guidance (ECHA/EFSA 2018). ECHA/EFSA guidance highlights that both biological plausibility and empirical support are weighted, however biological plausibility is the most influential consideration.
For 4-MBC the MoA analysis is carried out for three separate hypotheses:

- Interference with the thyroid hormone system
- ER activation causing altered sexual behaviour in females
- ER activation causing male reproductive toxicity

For simplicity and consistency with LoE reporting, we here present the MoA analysis for T modality first and for E modality next.

4.10.6.1 Postulated MoA for interference with the thyroid hormone system

Table 4.15: Summary table on Key Events for Mode of Action analysis (Thyroid)

Title	**Interference with thyroid hormone system**	
Hypothesis	The molecular initiating event (MIE) is not fully characterised, and several possible MIEs could cause the observed changes in circulating T3 and T4 concentrations. Altered thyroid hormone concentrations led to an increase in TSH, which in turn causes thyroid gland toxicity with increased weight and hypertrophy of the thyroid gland. This is an adverse effect which indicates clearly that 4-MBC may also affect human thyroid function. Consequently, 4-MBC exposure could lead to thyroid hormone-mediated altered neurodevelopment.	
	Brief description of event	**Supporting evidence**
MIE	Molecular: Binding to TR, and/or altered iodine uptake, and/or altered deiodinase activity and /or a yet unidentified MIE	Moderate evidence Only few studies have investigated the thyroid disrupting properties of 4-MBC *in vitro*. However, the data indicate that 4-MBC is capable of acting as both an agonist and antagonist on the thyroid hormone receptor (Hofmann *et al.* 2009), may decrease iodine uptake (Schmutzler *et al*. 2007 (K4)), and upregulate deiodinase gene expression (Hofmann *et al* 2009; Song M *et al.* 2013). Notably, 4-MBC does not appear to inhibit TPO *in vitro* (Schmutzler *et al.* 2004), nor do the observed changes in thyroid hormone levels *in vivo* seem to be mediated through changes in liver catabolism (Unpublished, 1984b)
KE1	Organ: Altered T3 and T4 concentrations in serum	Moderate evidence T3 concentrations were consistently increased in oral toxicity studies in rats (Unpublished, 1984a; Unpublished, 2004; Unpublished, 1983b, Maerkel *et al.* 2007). In some of the oral rat studies, 4-MBC exposure caused decreases in circulating T4 concentrations (Unpublished, 1983b). Two studies with lower reliability also saw decreased T4 concentrations (Schmutzler *et al.* 2004; Seidlova-Wuttke *et al.* 2006a) In some reliable studies, T4 levels were not statistically significant affected (Unpublished, 2004; Maerkel *et al.* 2007). Overall, there were consistent effects on serum T3 concentrations. The studies investigating higher doses of 4-MBC often found T4 levels to be decreased, whereas studies investigating lower doses (up to 50 mg/kg bw/day) typically did not see a decrease in serum T4.
KE 2	Organ/tissue: Increased TSH concentration	Strong evidence Studies consistently show increased levels of circulating TSH in orally exposed rats (Unpublished, 1984a; Unpublished, 2004; Unpublished, 1983a; Maerkel *et al.* 2007) and the same effects were seen in two studies with lower reliability (Schmutzler *et al*. 2004; Seidlova-Wuttke *et al*. 2006a).
AO1	Organism: Thyroid gland toxicity	Strong evidence Increased weight and altered histopathology of the thyroid gland is seen in the majority of studies investigating these endpoints (Unpublished, 1984a; Unpublished, 1983a; Unpublished, 1983b; Unpublished, 2004; Maerkel *et al*. 2007), as long as exposure is still ongoing at the time of assessment. In a dermal study in rats (Unpublished, 2005) no adverse

		effects were noted. In humans there is equivocal information regarding changes in thyroid gland size after 4-MBC exposure for 14 days. In a less reliable oral repeated dose study in dogs, 4-MBC exposure caused no adverse effects on the thyroid gland (Unpublished, 2003)
A02	Organism: Altered neurodevelopment	Insufficient evidence There is one study investigating neurobehavioural changes after perinatal exposure to 4-MBC (Unpublished, 2004). This study did not show any adverse effects on learning or memory, but these results are not robust enough to conclude whether or not adverse effects on neurodevelopment will occur after 4-MBC-mediated thyroid hormone disruption.

4.10.6.2 Assessment of biological plausibility of link between endocrine activity and adverse effect – T modality

The biological plausibility of key event relationships was analysed as presented in Table 4.16. The evidence for this analysis is not limited to 4-MBC, but is strengthened by evidence from other models and studies on other endocrine disrupters. This analysis of biological plausibility thus includes understanding of physiology, endocrinology and toxicology, and information from studies on other chemicals or knockout models.

Table 4.16: Analysis of biological plausibility of Key Event Relationships (Thyroid)

Title	**<u>Interference with thyroid hormone system</u>**	
	Brief description of key event relationship (KER)	**Supporting evidence**
MIE to KE1	MIE to Altered T3 and T4 concentrations in serum	Strong A number of different MIEs could be responsible for the observed effects, either singly or in combinations. A recent synthesis of AOPs relevant for thyroid disruption revealed a convergence of KEs at the point of altered circulatory thyroid hormone levels (Foster *et al.* 2021). Hence, it is not imperative to pin down a specific MIE to perform the MoA analysis. 4-MBC has been shown to act as both an agonist and antagonist towards the TR. Only a limited number of chemicals can bind the TR (Freitas *et al*. 2014), but when xenobiotics bind directly to thyroid receptors, allosteric effects on TRs may alter their ability to mediate thyroid hormone action, which could alter TH concentrations in serum (Yamauchi & Ishihara, 2006; Zoeller & Tan, 2007, Crofton 2008). Additionally, altered iodide uptake or altered deiodinase activity could be partially or fully responsible for the observed changes in circulating thyroid hormone concentrations, as many chemicals possessing these modes of action have been shown to adversely affect the thyroid hormone system (Crofton 2008, Noyes *et al.* 2019, Foster *et al.* 2021).
KE1	Altered thyroid	Moderate

to KE2	hormone concentrations to increased TSH levels	Generally, when circulating levels of thyroid hormones are low, this stimulates TRH release from the hypothalamus, and increases TSH release from the pituitary (Hill *et al.* 1989). The pattern of thyroid hormone perturbations observed for 4-MBC could indicate a moderate degree of inhibition of T4 formation. This inhibition leads to a compensatory increase in TSH production, which would maintain T4 concentrations within an (almost) normal range, whereas increases in T3 levels would be seen. Complicating the interpretation of the response is the fact that 4-MBC may also affect the deiodinases. 4-MBC exposure could therefore increase the conversion of T4 to T3, resulting in further increase of T3. This process however most likely occurs primarily in the peripheral tissues, rather than at serum level.
KE2 to AO1	Increased TSH to increased follicular growth and higher thyroid weight	Strong The follicular cells in the thyroid respond to increased TSH stimulation by increasing in number (hyperplasia) and size (hypertrophy), in order to increase thyroid hormone output. In the presence of continued chemical exposure and a consequent chronic TSH stimulation, there is an increase in the thyroid weight (Hill *et al*. 1998; Hurley *et al*. 1998; Capen 1999; Smith *et al*. 1991). In rodents, continued thyroid stimulation by TSH eventually leads to neoplasia of the thyroid follicular cells (Hill *et al*. 1998; Hurley *et al*. 1998; Capen 1999; Smith *et al*. 1991).
KE1 to AO2	Altered TH concentrations to altered neurodevelopment	Strong This key event relationship has been described in detail in AOP 42 as well as in AOP 54 (AOP 42 & 54 in AOP wiki[4]). All relevant references are provided in these two OECD endorsed AOPs. In short, marked perturbations in T4 during fetal and neonatal development will result in impaired neurodevelopment. Such adverse neurological effects can be seen as changes in a number of molecular and morphological endpoints in the brain, as well as in behavioural changes. However, typically the employed behavioural tests are not very sensitive to thyroid hormone disruption (Ramhøj *et al*. 2020). The fact that no adverse behavioural effects on learning and memory were seen in a developmental toxicity screening study (Unpublished, 2004) using relatively low doses, a group size of 10 rats and a rather insensitive scoring method for the behavioural results, is not in itself robust enough to exclude effects on neurodevelopment by 4-MBC exposure.

4.10.6.3 Human relevance of MoA – T modality

Even though thyroid hormone disruption may be quantitatively more sensitive in rats than in humans, human relevance should be assumed (ECHA/EFSA 2018). See further discussion of this point in conclusion table 4.17.

[4] Aop:42 - AOP-Wiki (aopwiki.org) https://aopwiki.org/wiki/index.php/Aop:42.
Aop:54 - AOP-Wiki (aopwiki.org) https://aopwiki.org/wiki/index.php/Aop:54

4.10.6.4 Conclusion on the Mode of Action analysis – T modality

Table 4.17: Conclusions on Mode of Action analysis – T modality

Mode of action analysis	There is strong evidence of endocrine activity (Tables 4.3, 4.4, 4.14). There is strong evidence for adverse effects of 4-MBC (Tables 4.5, 4.14)
Biological plausibility	It is biologically plausible that adverse effects are due to the endocrine activity of 4-MBC (Table 4.15).
Dose and temporal concordance	The effects on the thyroid hormone system occur both with exposure during adulthood, and in developmental toxicity studies, but generally, increasing doses of 4-MBC and exposures over a longer period of time, have been shown to cause more severe effects than lower doses and shorter exposure periods.
Essentiality, consistency, analogy and specificity	For determining essentiality, it should be demonstrated whether or not downstream KEs and/or the adverse effect is prevented/decreased if an upstream event is experimentally blocked. No such studies have been provided for 4-MBC. The data on endocrine activity and adverse effects are consistent, specifically for changes in TSH levels and thyroid gland weight and histology. The effects on thyroid gland are considered specific and not resulting from non-endocrine modes of action. No alternative non-endocrine mode of action is demonstrated.
Human relevance	Even though thyroid hormone disruption may be quantitatively more sensitive in rats than in humans, human relevance should be assumed (ECHA/EFSA 2018). This is especially in the case of 4-MBC, as these substances have been shown not to exert their thyroid disrupting effects through liver enzyme induction - a MoA which by some is viewed as not being relevant for humans. Disruption of thyroid hormone signalling during fetal and neonatal development is plausibly linked to adverse neurodevelopmental effects (OECD endorsed AOPs 42 & 54 -AOPwiki). Lack of effects on learning and memory in one study investigating this endpoint after developmental 4-MBC exposure is not in itself robust enough to exclude risk of thyroid-mediated effects on neurodevelopment by 4-MBC exposure. One human study examined the relationship between 4-MBC exposure and effect on T-relevant parameters. The lack of effects in this dermal study does not negate all of the findings showing thyroid effects in experimental animals receiving oral 4-MBC exposure. Furthermore, one study showed that human exposure to 4-MBC was potentially problematic, as concentrations is some breast milk samples were comparable to those measured in rats exposed orally to 4-MBC doses of 0.7 and 7 mg/kg per day (Schlumpf *et al.* 2008a).
Identified uncertainties	
No one- or two-generation studies have been performed	The lack of an extended one- or a two-generation guideline study is not an uncertainty in relation to identification of effects on T3, T4 and TSH concentrations, or on adverse effects on the thyroid gland itself. The available repeated dose – and developmental studies provide strong evidence of this. However, the top dose used in the TG421 study was too low, as no signs of systemic toxicity were observed. Including a higher dose in this study would likely have strengthened the conclusions regarding effects on the thyroid hormone system. In terms of identification of any additional adverse effects related to the observed developmental thyroid hormone disruption, lack of more thorough investigations of neurodevelopmental effects is an identified uncertainty. Presently however, even the EOGRTS study (OECD TG 443) performed with a DNT cohort does not include investigation of sensitive and specific endpoints to sufficiently address potential adverse neurodevelopmental effects caused by thyroid hormone disruption. Therefore, performance of additional developmental guideline studies would probably not add much relevant information to the assessment of thyroid-mediated adversity on developmental neurotoxicity.

Different effect levels/no effect levels observed in different *in vivo* studies.	A consistent pattern of effects was observed in all oral studies in rats. In contrast, a dermal study in rats did not show any thyroid related effects and only very weak indications of thyroid effects were seen in a dermal study in humans. It is possible that the dermal route led to a potentially lower systemic dose than oral exposure. Furthermore, differences in toxicokinetics between dermal and oral exposure to 4-MBC may explain these differences. Toxicokinetic studies investigating this problem have been performed (Schauer *et al.* 2006 and Völkel *et al.* 2006). From these studies it is apparent that the kinetics of 4-MBC do differ in relation to route of exposure, causing different absorption patterns, and different internal levels of 4-MBC and its metabolites. This could in turn affect the endocrine disrupting effects. As far as we know, it has not been tested whether this difference is solely accountable for the differences in toxicological outcome in oral and dermal studies in rats.
Lack of a clearly identified MIE for 4-MBC	The lack of an MIE is not an issue for the performed MoA analysis because several review papers on thyroid hormone disruption, including a recent synthesis of AOPs (Foster *et al.* 2021), have described a convergence of KEs at the point of altered circulatory thyroid hormone levels. Hence, it is not imperative to pin down a specific MIE in order to perform the MoA analysis.
Conclusion: The analysis leads to the conclusion that it is biologically plausible that oral exposure of 4-MBC disrupts thyroid hormone production. This causes increased TSH secretion, which leads to adverse effects on the thyroid gland, seen as increased thyroid gland weight and altered histopathology. It is plausible that through its thyroid hormone system disruptive effects, 4-MBC could also adversely affect neural development. The mode of action of 4-MBC is based on "EATS-mediated adversity", and the substances is considered to be an endocrine disrupter. No alternative non-endocrine mode of action is demonstrated.	

4.10.6.5 Postulated MoAs for interference with female and male reproductive system – E modality

The hypothesised MoA for effects of 4-MBC on **female reproductive function** is presented in Table 4.18. Specifically, the molecular initiating event (MIE) is activation of the estrogen receptor(s). In females, altered estrogen signalling causes changes to the organisation of brain areas which regulate gonadotropin release in adulthood and control female sexual behaviour. These neurodevelopmental changes on sexual behaviour are biologically plausibly linked to the endocrine disrupting effects of 4-MBC and can be viewed as sufficient evidence of female reproductive toxicity. Additionally, other indications of disrupted female reproductive development are seen, as altered ER signalling has also been shown to increase female AGD, alter ovary and uterus weights at the time of weaning, and possibly affect the timing of sexual maturation and estrous cyclicity. These results support the conclusion that female reproductive development is adversely affected by 4-MBC.

Table 4.18: Summary table on Key Events for Mode of Action analysis (Female reproduction)

Title	**Activation of ER to female reproductive toxicity**	
Hypothesis	The molecular initiating event is activation of the ER(s). In females, increased ER signalling results in increased ER activity in tissues, including the brain. If changes occur during the first two postnatal weeks, the female brain is not organised properly. This leads to disrupted regulation of LH and FSH in adulthood and adversely affected sexual behaviour.	
	Brief description of event	**Supporting evidence**
MIE	Molecular: Activation of ER	Strong Lines of evidence show strong evidence for endocrine activity related to estrogen receptor activation. Several *in vitro* assays provide evidence for induction of estrogenic response in the E-screen measured as proliferation, gene or protein expression (Schlumpf *et al.* 2001; Tinwell *et al.* 2002; Schlumpf *et al.* 2004a; Klann *et al.* 2005; Heneweer *et al.* 2005; Jiménez-Díaz *et al.* 2013) and strong evidence for ER agonistic properties (Klann *et al.* 2005; Gomez *et al.* 2005; Schreurs *et al.* 2002; Minh *et al.* 2008; Schlumpf *et al.* 2004a; Schmitt *et al.* 2008; Jocsak *et al.* 2016; Mueller *et al.* 2003).
KE1	Cell: Increased ER signalling in target tissue	Strong Lines of evidence show clear signs of altered ER signalling in target tissues. *In vivo*, several studies show altered growth of estrogen sensitive tissues, such as increased uterus weight in uterotrophic assays in immature females (Schlumpf *et al.* 2001; Tinwell *et al.* 2002; Ashby *et al.* 2004). Altered expression of estrogen regulated genes in the uteri of adult female offspring exposed during development (Durrer *et al.* 2005) was also seen. Additionally, indications of increased uterine weight, combined with increased epithelium thickness and altered expression of estrogen regulated genes was seen in uteri and vaginas of adult OXV females in studies with lower reliability (Seidlova-Wuttke *et al.* 2006a; Seidlova-Wuttke *et al.* 2006b).
KE2	Organ/tissue: Altered organisation	Weak No studies have investigated the female brains during the first

	of sexually dimorphic areas of the brain	postnatal weeks. Alterations to gene expression (progesterone receptor, ERalpha) have been seen in sexually dimorphic areas of the brain (VMH) in adult offspring exposed perinatally and continued to adulthood (Maerkel *et al.* 2007), which supports that gene expression changes may also have occurred in the female offspring during early postnatal life. A recent review by Ruszkiewicz et al. 2017 also concluded that 4-MBC has shown region- and sex-dependent alteration in the oestrogenic genes in the brain. The structurally similar 3-BC substance also caused alterations in gene expression in the uterus and sexually dimorphic areas of the brain in offsprings (Faass et al. 2009).
KE 3	Organ/tissue: Altered regulation of gonadotropin secretion in females	Weak-moderate In the extended reproductive toxicity screening study (Unpublished, 2004) a dose of 50 mg/kg resulted in a non-statistically significant decrease of 61% in FSH, in the 55-day old female offspring, whereas LH was unaffected. In a developmental study with lower reliability, a higher dose (100 mg/kg every other day) given during pregnancy only, increased FSH and LH levels by 90-400% in adult female offspring (Carou *et al.* 2009b). Together, these results indicate persistent effects on programming of the hypothalamo-pituitary-gonadal axis and altered gonadotrophin secretion in perinatally exposed female offspring.
AO1	Organism: Altered function of sexually dimorphic areas of the brain	Moderate-strong Altered sexual behaviour of adult female offspring (reduced proceptive and receptive behaviour, and increased rejection towards the male) in a robust study, using perinatal exposure continued into adulthood (Faass *et al.* 2009).
AO2	Organism: Altered female reproductive development	Moderate Statistically significant increased AGD (a finding also observed with other estrogens) was seen in neonatal female offspring from low and high dose groups (Unpublished, 2004). In high dose offspring from the same study, increased uterus and ovary weights were observed without changes in histopathology at PND 22. These changes were no longer evident on PND 55 (Unpublished, 2004). Delayed VO was seen in low and high dose female offspring, only statistically significant in low dose (Unpublished, 2004). A developmental study using similar dose levels showed no effect on sexual maturation in the female offspring (Durrer *et al.* 2007), Indications of irregular estrous cycling was seen in females exposed during development and adulthood (though not statistically significant) (Faass *et al.* 2009). Additionally, a developmental study with lower reliability, showed VO to occur 3 days earlier than in control animals (Carou *et al.* 2009b).

The biological plausibility of key event relationships was analysed as presented in Table 4.19. The evidence for this analysis is not limited to 4-MBC, but is strengthened by evidence from other models and studies on other endocrine disrupters. This analysis of biological plausibility thus includes understanding of physiology, endocrinology and toxicology, and information from studies on other chemicals or knockout models.

Table 4.19: Analysis of biological plausibility of Key Event Relationships (Female reproduction)

Title	**Activation of ER to female reproductive toxicity**	
	Brief description of key event relationship (KER)	**Supporting evidence**
MIE to KE1	Activation of ER to Increased ER signalling in target tissue	Strong Estrogen receptor activation leads to increased estrogen receptor signalling.
KE1 to KE2	Increased ER signalling in target tissues to Altered organisation of sexually dimorphic areas of the brain	Strong In rodents, organisation of the sexually dimorphic areas of the brain, takes place during the early postnatal life, due to absence of gonadal steroids [Arnold & Gorski 1984; Gorski *et al.* 1977]. If female rats are treated with estrogen or testosterone in the early postnatal period, correct organisation of the female brain will be disrupted (Arnold & Gorski 1984; Gorski *et al.* 1977). The preoptic area and the VMH serve as major sites that accumulate estrogen receptors in the brain (Pfaff *et al.* 1973). These regions of the female brain are therefore most affected by exposure to steroid hormones during early postnatal development (Herath *et al.* 2001).
KE 2 to KE 3	Altered organisation of sexually dimorphic areas of the brain to Altered regulation of gonadotropin secretion	Moderate One of the consequences of a disrupted (de-feminised) brain organisation during the first two postnatal weeks, is lack of cyclic release of pituitary gonadotropins in adulthood (Harlan & Gorski 1977a, Gorski 1977b; Mennin & Gorski 1975).
KE3 to AO1	Altered regulation of gonadotropin secretion to Altered function of sexually dimorphic areas of the brain	Strong Correct cyclic release of gonadotropins (especially afternoon surges of LH on the day of proestrous) is needed for correct performance of sexual behaviour. Females treated neonatally with steroid hormones will fail to exhibit lordosis behaviour (Barraclough & Gorski 1962, Harris *et al.* 1965). This fits well with the fact that the VMH is the estrogen and progesterone feedback centre in the brain, which regulates female sexual behaviour (Davis *et al.* 1979, Davis *et al.* 1982, Rubin *et al.* 1980). Exposure to potent steroid hormones such as testosterone and estradiol are not the only exposures, which can elicit this response. A study using the estrogenic substance octylphenol (OP) has shown that when given to female rats in the neonatal period, this estrogenic compound can affect the secretion of LH, and FSH, and adversely affect the display of sexual receptive behaviour in adulthood. These results indicate that early-life exposure to endocrine disrupting chemicals with an estrogenic mechanism of action, can interfere with sexual differentiation of the brain and cause adverse effect on sexual behaviour through misregulation of gonadotropin secretion in adulthood (Herath *et al.* 2001).
KE1 to AO2	Increased ER signalling in target tissues to Altered female reproductive development	Moderate-strong It is well established that an increased estrogenic response can cause altered growth of estrogen-regulated female reproductive organs and is reflected by increases in uterus and ovary weights in developing females (OECD Guidance Document 150). Studies on ovariectomised mice have established that ER (especially alpha) signalling impacts uterine growth (weight) (Lindberg *et*

		al. 2002). Female AGD can be influenced by altered androgen/estrogen balance. No clear signalling pathways have yet been established, but the increased female AGD seen for 4-MBC has been seen for several other estrogenic compounds, including ethinyl estradiol (Schwartz *et al*. 2019) DES (Johansson *et al.* 2021) as well as phytoestrogens (Mandrup *et al.* 2013). Increased ER signalling may delay or accelerate the timing of VO, depending on dose and exposure period. For 4-MBC and DES lower doses given during development caused a small delay in sexual maturation, whereas higher doses given prenatally advanced the time of sexual maturation. Lower doses of DES given to rats perinatally resulted in slight delay in VO (Johansson *et al.* 2021). Another rat study on DES found delayed VO at low neonatal s.c. exposures (1 ug/kg/day) but saw earlier VO at a high exposure dose (10 ug/kg/day) (Franssen *et al*. 2014). In humans, estrogenic chemical DES is associated with early menarche (D'Aloisio *et al.* 2013), highlighting the human relevance of the association between estrogen activity and altered puberty onset. Increased ER signalling may cause irregular cycling in exposed offspring, as seen for other estrogens that 4-MBC**.** Kwon *et al*. 2000 found that DES exposure in utero and during lactation lead to irregular estrous cyclicity in 4-month old offspring. Ohmukai *et al*. 2017 showed earlier onset of age-related irregular cycling after neonatal exposure to DES.

The hypothesised MoA for 4-MBC effects on **male reproductive function** is presented in Table 4.20. Specifically, the molecular initiating event (MIE) is activation of the estrogen receptor(s). In males, increased estrogen receptor signalling can affect adult prostate function. The influence of estrogens on early prostate development is well established, but has only been studied to limited degree for 4-MBC. Therefore, the data on adult prostate weight is central to the mode of action evaluation.

Table 4.20: Summary table on Key Events for Mode of Action analysis (Male reproduction)

Title	**Activation of ER to male reproductive toxicity**	
Hypothesis	The molecular initiating event is activation of the ER(s). In developing males, increased ER signalling can alter prostate development and prostate function in adulthood.	
	Brief description of event	**Supporting evidence**
MIE	Molecular: Activation of ER	Strong Lines of evidence show sufficient evidence for endocrine activity related to estrogen receptor activation. Several *in vitro* assays provide evidence for induction of estrogenic response in the E-Screen measured as proliferation, gene or protein expression (Schlumpf *et al*. 2001; Tinwell *et al.* 2002; Schlumpf *et al.* 2004a; Klann *et al.* 2005; Matsumoto *et al.* 2005 (K4); Heneweer *et al.* 2005; Jiménez-Díaz *et al.* 2013) and some evidence for (weak) ER agonistic properties (Klann *et al.* 2005; Gomez *et al.* 2005; Schreurs *et al.* 2002; Minh *et al.* 2008; Schlumpf *et al.* 2004a; Schmitt *et al.* 2008; Jocsak *et al.* 2016; Matsumoto *et al.* 2005 (K4); Mueller *et al.* 2003).
KE1	Organ: Increased ER signalling in target tissues	Strong Lines of evidence show clear signs of altered ER signalling in target tissues. *In vivo* data on ER signalling in males is absent, but in females several studies showed altered growth of estrogen sensitive tissues, such as increased uterus weight in uterotrophic assays in immature females (Schlumpf *et al*. 2001; Tinwell *et al*. 2002; Ashby *et al*. 2004) *In vitro*, MCF-7 cells show increased expression of estrogen-regulated genepS2 (Heneweer *et al.* 2005). A study with lower reliability indicates increased uterine weight, combined with increased epithelium thickness in uterus and vagina of adult OXV females (Seidlova-Wuttke *et al.* 2006b).
KE 2	Organ/tissue: Altered gonadotropin secretion	Weak-Moderate Lines of evidence show evidence of altered gonadotropin levels in male and female rats exposed perinatally or in adulthood, primarily reduced levels of both LH and FSH (Unpublished, 2004; Carou *et al*. 2008). More specifically, with adult male exposure serum LH and FSH levels were statistically significantl decreased after 2 and 5 days of s.c. exposure (Carou *et al*. 2008). In perinatally exposed male offspring, LH was dose-dependently decreased (20-34%, non-statistically significant), and FSH statistically significantly decreased (27-56%) at PND 55 (Unpublished, 2004). In studies with lower reliability, results showed that in male offspring exposed s.c. during pregnancy, a decrease in LH was seen on PND 15, whereas an increase in both LH and FSH levels was seen on PND 30 (Carou *et al*. 2009a). On PND 70 decrease in serum LH and FSH levels was seen (Carou *et al*. 2009b), and altered LH eves were seen in adult OVX females after 12 weeks

		of exposure to 4-MBC (Seidlova-Wuttke *et al*. 2006a)
AO1	Organism: Male reproductive toxicity	Moderate-strong Clear evidence of persistent changes was seen for adult prostate. Reduced prostate weight was reported in a 17-day study (Unpublished, 1983a) and in a 90-day study (Unpublished, 1984a). Reduced prostate weight was also reported in a 28-day study, but the dose was very high, which induced systemic toxicity (Unpublished, 1983b). The 90-day study reported no histological changes in prostate, whereas histopathological changes in the prostate were not investigated in the other repeated dose toxicity studies. Effects on prostate weight at various ages are also reported after developmental exposure. In offspring exposed perinatally no effect was seen on PND22, but on PND55 a 10% reduction, not statistically significant, was reported. In this study, no effects were seen on histology (Unpublished, 2004). In a study where exposure continued during fetal, postnatal, pubertal and adult life a decreased absolute and relative prostate weight was reported in adult offspring. Estrogen regulated genes in the prostate were also affected (Durrer *et al*. 2007). In a study with low reliability (K3), exposure during gestation had a proliferative effect on prostate growth, causing an increase of 60-70% in prostate volume on PND 1. The increased volume was due to increased number and volume of ducts in the prostate (Hofkamp *et al*. 2008). Some evidence of effect on androgen-sensitive endpoints: Anogenital distance and nipple retention were investigated in one study using perinatal exposure. Anogenital distance was statistically significantly longer in exposed male offspring. No effect was seen on timing of sexual maturation (Unpublished, 2004). In a study where exposure continued during fetal, postnatal, pubertal and adult life a delay in male sexual maturation was seen. The preputial separation was dose-related and was approximately 3 days delayed in the high dose group (Durrer *et al*. 2007).

4.10.6.6 Assessment of biological plausibility of link between estrogenic endocrine activity and adverse effect

The biological plausibility of key event relationships was analysed as presented in Table 4.21. The evidence for this analysis is not limited to 4-MBC, but is strengthened by evidence from other models and studies on other endocrine disrupters. This analysis of biological plausibility thus includes understanding of physiology, endocrinology and toxicology, and information from studies on other chemicals or knockout models.

Table 4.21: Analysis of biological plausibility of Key Event Relationships (Male reproduction)

Title	**Activation of ER to male reproductive toxicity**	
	Brief description of key event relationship (KER)	**Supporting evidence**
MIE to KE1	Estrogen receptor activation To Increased ER signalling in target tissues	Strong Estrogen receptor activation leads to increased estrogen receptor signalling.
KE1 to KE2	Increased ER signalling in target tissues To Altered gonadotropin secretion	Strong Hypothalamic GnRH neurons control secretion of gonadotropins FSH and LH. In adults, these neurons are regulated by negative feedback both at the pituitary and the hypothalamic level. Estrogen receptors repress GnRH secretion directly and via adjacent Kiss1 neurons, and in male humans and experimental animals estradiol exposure leads to decreases in serum gonadotropin secretion (reviewed by Guercio *et al*. 2020).
KE1 and KE 2 to AO1	Increased ER signalling in target tissues and Altered gonadotropin secretion to Male reproductive toxicity	Moderate - strong Estrogens are important regulators of adult prostate growth and function. In adult prostate, ERα is expressed in stromal cells, while ERβ is primarily present in luminal epithelial cells, and these receptors may play opposing roles with proliferative and anti-proliferative effects on prostate tissue (Prins and Korach 2008). Stromal proliferation in response to estrogen treatment may be mediated through stromal ERα. In the prostate epithelium, ERβ may be the key mediator of estrogen-induced events, and knock-out models indicate that ERβ has an anti-proliferative role (Prins and Korach 2008). In line with this, certain phytoestrogens have been suggested as beneficial to (adult) prostate health due to anti-proliferative actions. During early prostate development, increased ER signalling may enhance prostate growth. In fetal organ culture, DES increased the outgrowth of prostate dose-dependently, and the effect was counteracted through the co-administration of anti-estrogenic ICI168, 387 (Gupta 2000). Although patterns of effects of estrogenic substances may vary, it is biologically plausible that the observed effects of 4-MBC enhancing outgrowth of neonatal prostate epithelium and reducing adult prostate growth are related to increase in estrogen signalling. In addition to the role of estrogens, it has been shown that dysregulation of the FSH system plays a significant role in prostate growth (Porter *et al.* 2001, Crawford *et al.* 2014). Although the evidence is currently limited, it is biologically plausible that altered gonadotropin secretion may contribute to the observed changes in adult prostate growth. Male anogenital distance (AGD) is programmed by steroid hormones during late fetal life (Schwartz *et al.* 2019). Altered estrogen signalling and gonadotropin secretion during the male programming window (from gestation day 14-18) is likely to cause hormonal imbalances which can alter the development of the AGD. Too low concentrations of androgens typically cause a reduction in male AGD, wheras the specific mechanisms behind increases in male AGD have not yet been characterized. However, imbalance (at tissue level) in the ratio

		between androgens and estrogen could be part of the mechanism behind increased male AGD.

4.10.6.7 Human relevance of MoA – E modality

Relevance to humans is assumed by default in the absence of appropriate scientific data demonstrating non-relevance.

4.10.6.8 Conclusion on the Mode of Action analysis – E modality

Table 4.22: Conclusions on Mode of action analysis – E modality

Mode of action analysis	There is strong evidence of endocrine activity (estrogen receptor activation) (Table 4.8-4.10, 4.17, 4.19). There is strong evidence for adverse effects of 4-MBC (Table 4.11).
Biological plausibility	It is biologically plausible that adverse effects are due to the endocrine activity of 4-MBC (Table 4.18, 4.20).
Dose and temporal concordance	Females: Dose-dependent effects on female sexual behaviour were seen in a study with perinatal and continued pubertal and adult exposure, as the effects were more marked and statistically significant in the highest tested dose (24 mg/kg bw/day) compared to the lower dose (7 mg/kg bw/day). Females exposed to 47 mg/kg were not investigated. Males: Effects on prostate weight after adult and perinatal exposure (exposure ending at weaning) showed effects down to 30 mg/kg, whereas a study continuing exposure during fetal, postnatal, pubertal and adult life report effects down to 7 mg/kg (lowest dose tested) indicating that continued exposure over several life stages may cause a more pronounced effect. In this study the effect was dose-dependent.
Essentiality, consistency, analogy and specificity	For determining essentiality it should be demonstrated whether or not downstream KEs and/or the adverse effect is prevented/decreased if an upstream event is experimentally blocked. Such studies were not performed for 4-MBC. The data on endocrine activity and adverse effects are consistent, specifically for estrogenic effects *in vitro* and for changes in prostate weight. Only one study examined adverse effect on female behaviour and consistency thus cannot be evaluated. The observed adverse effects on female behaviour and prostate weights are considered specific and not resulting from non-endocrine modes of action. No alternative non-endocrine mode of action is demonstrated.
Human relevance	There are no data indicating that these endocrine modes of action are not relevant to humans. Thus, human relevance is assumed by default. No epidemiological studies examined the relationship between 4-MBC exposure and effect on EAS relevant adverse effects. In young men and postmenopausal women, effects on endocrine activity were examined, but no clear changes in gonadotropin levels were observed after 4 days of dermal exposure to 4-MBC. Minor but statistically significant differences in hormone levels between the control and treatment groups were observed for testosterone, estradiol, and inhibin B at some time points, however these did not seem to be related to the exposure to 4-MBC and at least some of these statistically significant differences may be chance findings.
Identified uncertainties	

No extended one- or two-generation studies have been performed	An enhanced OECD TG421 screenings study was performed – with focus on endocrine measurements and endocrine endpoints. Compared to a 2-generation study, several more endocrine endpoints were included in the TG421 study, hence more endpoints relevant to assessing ED are available that would have been in a TG416 study. Compared to a TG 443 EOGRTS the group size in the performed study was smaller (10 litters per group, and not 20), which may have decreased the sensitivity of some of the findings. Furthermore the top dose used in this study was too low, as no signs of systemic toxicity were observed. Including a higher dose in this study would likely have strengthened the conclusions regarding effects on endocrine sensitive endpoints. In the developmental toxicity studies which were available in the open literature exposure continued after weaning; it is therefore not possible to discern which of the observed effects in these studies were caused solely by the developmental exposure and which ones were caused by a combination of fetal, postnatal, pubertal and adult exposure to 4-MBC. Only the 90-day study reported investigations of prostate histopathology and showed no effects.
Different effect levels/no effect levels observed in different *in vivo* studies.	Female sexual behaviour was only investigated in one study and therefore consistency of this adverse reproductive effect cannot be examined. For males there was a consistent pattern of effect on prostate gland weight in all the studies, but the effect level/no effect level varies. This difference could be explained by differences in study design including the life stages covered by exposure. However, there is a rather large difference in effect level/no effect level between the adult 17- and 90-day study, with the lowest effect dose being observed in the shorter 17-day study. This may be a chance finding or reflect an increased sensitivity with short-term exposure in young adults when prostate growth is assumed to be more pronounced than in older adults.
Lack of a clear description of biological pathways leading from estrogen receptor activation to adverse reproductive effects	In females, the reported effect patterns on changes in estrogen sensitive adverse outcomes was mixed. Notably, this type of mixed patterns of different direction of effects depending on age and dose in rat models is also seen for other estrogenic compounds. An example of this is DES, which is well known to have endocrine disrupting properties in the human. For DES, differing effects are also seen on puberty timing, estrous cycling. In males, despite some uncertainties in the MoA description for the relationship between estrogen receptor activation and adverse effects on prostate, the hypothesis is considered relevant, as evidence of biological plausibility and analogous effects for other estrogenic substances is provided.
Conclusion: The analysis leads to the conclusion that it is biologically plausible that estrogen receptor activation leads to adverse effects on the reproductive system, specifically altered female sexual behaviour and altered prostate weight in males. The mode of action of 4-MBC is based on "EATS-mediated adversities", and the substances is considered to be an endocrine disrupter. No alternative non-endocrine mode of action is demonstrated.	

4.10.7 Overall conclusion on endocrine disruption with regards to human health

The overall conclusion is that 4-MBC can be considered endocrine disruptor for human

health. This is based on sufficient information on an endocrine mode of action and the adverse effects following exposure of 4-MBC.

5 Environmental hazard assessment

Environmental data for 4-MBC was not reviewed and thus not included in this document

6 Conclusions on the SVHC Properties

6.1 Assessment under Article 57(f)

6.1.1 Summary of the data on the intrinsic/hazardous properties

There is strong evidence for endocrine activity and adverse effect related to the T and E modalities.

Thyroid mode of action
For the T-modality, there is some evidence of T-related endocrine activity based on the few available *in vitro* studies and strong evidence in a larger number of *in vivo* studies showing a consistent pattern of increased TSH and T3. The exact MIE responsible for the disruption in circulating thyroid hormones cannot with certainly be determined, because of the relatively limited number of available *in vitro* data. In spite of this data gap, it is concluded that there is sufficient evidence of endocrine activity based on the few available *in vitro* studies and a larger number of *in vivo* studies, showing changes in circulating THs. There is also strong evidence for adverse effect on thyroid gland in several *in vivo* studies (e.g., increased weight and altered histopathological findings). According to the MoA analysis, there is strong evidence that the adverse effects on the thyroid gland are plausibly linked to the thyroid disrupting endocrine activity seen *in vitro* and *in vivo*. Such effects are considered relevant for human health and could pose a hazard to humans, in particular if alterations of thyroid hormones should occur during the critical windows of pre- and postnatal neurological development. Adverse health effects on offspring neurodevelopment are often irreversible and can have consequences later in life. Neurodevelopment has not been sufficiently investigated with 4-MBC, and therefore such effects cannot be excluded. Irrespective of the identified knowledge gaps related to neurodevelopment, the consistently seen adverse effects on thyroid gland weight and histopathology clearly show that 4-MBC is a thyroid hormone system disrupting chemical.

Estrogenic mode of action
There is strong evidence for endocrine activity related to estrogen receptor activation. The available *in vitro* assays provide strong evidence for induction of estrogenic response in the E-screen and for ER agonism. *In vivo*, several mechanistic studies show altered growth of estrogen sensitive tissues, including increased uterus weight in uterotrophic assays and altered expression of estrogen-regulated genes in several target tissues, confirming the strong evidence of an estrogenic activity.
In females, there is moderate to strong scientific evidence that combined perinatal and

adult exposure to 4-MBC can lead to adverse effects on sexual behaviour (reduced proceptive and receptive behaviour, and increased rejection behaviour towards the male) as well as a moderate degree of evidence for other adverse effects on female reproductive development (changes in ovary weight, uterine weight, ano-genital distance (AGD) and vaginal opening (VO). In addition, there is weak-moderate evidence of alterations in circulating follicle stimulating hormone (FSH), luteinising hormone (LH) and gonadotropin releasing hormone (GnRH) levels *in vivo*. The performed MoA analysis shows a biologically plausible link beween the estrogenic endocrine mechanism and the reported adverse effects. The molecular initiating event is activation of the ER(s), which can result in increased ER activity in specific tissues, including specific areas of the brain. If such changes occur during the first two weeks of postnatal life, the female brain is not organised properly. This can lead to disrupted regulation of LH and FSH in adulthood and may as a consequence adversely affect sexual behaviour. Additionally, altered ER signalling has been shown in some studies to alter female AGD, ovary and uterus development and timing of sexual maturation.

In males, there is moderate to strong scientific evidence of persistent reductions in prostate weight in several studies in adult animals, whereas the results are less clear from the available developmental toxicity studies. The Mode of action analysis shows that estrogens are important regulators of adult prostate growth and function and that increased ER signalling may affect prostate growth during early prostate development. Although patterns of effects of estrogenic substances may vary, it is biologically plausible that the observed effects of 4-MBC are related to increase in estrogen signalling. In addition to the role of estrogens, it has been shown that dysregulation of the FSH system plays a significant role in prostate growth. Although the evidence is currently limited, it is biologically plausible that altered gonadotropin secretion may contribute to the observed changes in adult prostate growth.

In conclusion, based on a weight of evidence evaluation, 4-MBC is assessed to meet the WHO WHO/IPCS definition of an endocrine disruptor (WHO/IPCS, 2002) as interpreted by the JRC Endocrine Advisory Group (2013), with both estrogenic and thyroid disrupting MoA, leading to adverse reproductive effects in both males and females and adverse effect on the thyroid gland.

6.1.2 Equivalent level of concern assessment

6.1.2.1 Human health

4-MBC exposure gives rise to an equivalent level of concern to substances listed in Article 57 points (a) to (e) due to its endocrine disrupting properties for human health.

4-MBC exposure has been shown to consistently disrupt the thyroid hormone system and consequently cause adverse effects on the thyroid gland. Thyroid hormone system disruption can have potentially serious and irreversible effects on humans, in particular on neurodevelopment. This can impact the quality of life and raises societal concern of a high and increasing burden. A number of vulnerable populations may be particularly susceptible to thyroid hormone (TH) disruption induced by 4-MBC. Pregnancy is likely to be a period of sensitivity to the alteration of TH regulation, with potential consequences for neurodevelopment of the offspring.

The observed adverse reproductive effects in males and females have been plausibly linked to the estrogenic mode of action, shown both *in vitro* and *in vivo* after 4-MBC exposure. These, and potentially other adverse effects in humans, caused by endocrine disruption via the E modality are considered serious, as similar effects in humans could cause sub- and infertility. For humans, sub- and infertility is not only detrimental to the propagation of the species, but it also has a major impact on quality of life. Additionally, fertility treatment and counselling carry high societal costs.

Based on the available studies, it may be difficult to establish a safe level of 4-MBC. Mixture effects, where substances act additively or with synergistic effects, cannot be excluded and this might impact the threshold of toxicity. Moreover, the difficulty to establish a safe level with sufficient certainty raises concern particularly on the capacity to manage safe use of the substances for sensitive populations. Establishing safe levels for these particularly sensitive populations is surrounded with large uncertainties, and alterations of thyroid hormones during the critical windows of pre- and post-natal neurological development may have consequences later in life. The complexity of the response in reaction to thyroid disturbance is not fully characterised and understood, and considering the range of functions influenced by THs, it is also highly challenging to fully characterise these effects in experimental studies.

6.1.2.2 Summary of the ELoC assessment

Altogether, this gives rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 REACH.

6.1.3 Conclusion on the Article 57(f) assessment

(±)-1,7,7-trimethyl-3-[(4-methylphenyl)methylene]bicyclo[2.2.1]heptan-2-one covering any of the individual isomers and/or combinations thereof (commonly referred to as 4-methylbenzylidene camphor or 4-MBC) are identified as substances of very high

concern in accordance with Article 57(f) of Regulation (EC) 1907/2006 (REACH) because of their endocrine disrupting properties for which there is scientific evidence of probable serious effects to human health which gives rise to an equivalent level of concern to those substances listed in points (a) to (e) of Article 57 REACH. This proposal is made based on the available evidence for 4-MBC. Due to the structural similarity between the different isomers and in the absence of evidence on the individual isomers, it has to be assumed that all individual isomers and/or combinations thereof have endocrine disrupting properties.

Endocrine disrupting (ED) properties of 4-MBC relevant for human health:

Thyroid mode of action

There is some evidence of thyroid-related (T) endocrine activity based on the few available *in vitro* studies and strong evidence in a larger number of *in vivo* studies showing a consistent pattern of increased thyroid stimulating hormone (TSH) and tri-iodothyronine (T3). There is also strong evidence for adverse effect on the thyroid gland in several *in vivo* studies (e.g. increased weight and altered histopathological findings). According to the Mode of Action analysis (MoA), there is strong evidence that the adverse effects on the thyroid gland are plausibly linked to the thyroid disrupting endocrine activity seen *in vitro* and *in vivo*. According to the ECHA/EFSA ED guidance (ECHA/EFSA 2018), such effects are considered relevant for human health and could pose a hazard to humans, in particular if alterations of thyroid hormones should occur during the critical windows of pre- and postnatal neurological development. Such adverse health effects on offspring neurodevelopment are often irreversible and can have consequences later in life. Neurodevelopment has not been sufficiently investigated with 4-MBC, and therefore such effects cannot be excluded. Irrespective of the identified knowledge gaps related to neurodevelopment, the consistently seen adverse effects on thyroid gland weight and histopathology clearly show that 4-MBC is a thyroid hormone system disrupting chemical.

Estrogenic mode of action

There is strong evidence for endocrine activity related to estrogen receptor activation. The available *in vitro* assays provide strong evidence for induction of estrogenic response in the E-screen and for ER agonism. *In vivo*, several mechanistic studies show altered growth of estrogen sensitive tissues, including increased uterus weight in uterotrophic assays and altered expression of estrogen-regulated genes in several target tissues, confirming the strong evidence of an estrogenic activity.

In female rodents, there is moderate to strong scientific evidence that combined perinatal and adult exposure to 4-MBC can lead to adverse effects on sexual behaviour (reduced proceptive and receptive behaviour, and increased rejection behaviour towards the male) as well as a moderate degree of evidence for other adverse effects on female reproductive development (changes in ovary weight, uterine weight, ano-genital distance (AGD) and vaginal opening (VO). In addition, there is weak-moderate evidence of alterations in circulating follicle stimulating hormone (FSH), luteinising hormone (LH) and gonadotropin releasing hormone (GnRH) levels *in vivo*. The performed MoA analysis shows a biologically plausible link beween the estrogenic endocrine mechanism and the reported adverse effects. The molecular initiating event is activation of the ER(s), which can result in increased ER activity in specific tissues, including specific areas of the brain. If such

changes occur during the first two weeks of postnatal life, the female brain is not organised properly. This can lead to disrupted regulation of LH and FSH in adulthood and may as a consequence adversely affect sexual behaviour. Additionally, altered ER signalling has in some studies been shown to alter female AGD, ovary and uterus development and timing of sexual maturation.

In male rodents, there is moderate to strong scientific evidence of persistent reductions in prostate weight in several studies in adult animals, whereas the results are less clear from the available developmental toxicity studies. The Mode of action analysis shows that estrogens are important regulators of adult prostate growth and function and that increased ER signalling may affect prostate growth during early prostate development. Although patterns of effects of estrogenic substances may vary, it is biologically plausible that the observed effects of 4-MBC are related to increase in estrogen signalling. In addition to the role of estrogens, it has been shown that dysregulation of the FSH system plays a significant role in prostate growth. Although the evidence is currently limited, it is biologically plausible that altered gonadotropin secretion may contribute to the observed changes in adult prostate growth.

Other potential modes of action
In addition, there is some supportive *in vitro* evidence showing androgen receptor (AR) antagonistic activity. This endocrine activity could also plausibly contribute to the adverse effects on both the male and female reproductive system in rodents.

Summary of the ED assessment
Therefore, there is scientific evidence to conclude that 4-MBC are endocrine disruptors via T and E modalities, according to a mode of action analysis including an evaluation of biological plausibility.

Equivalent level of concern

- 4-MBC exposure has been shown to consistently disrupt the thyroid hormone system and consequently cause adverse effects on the thyroid gland. Thyroid hormone system disruption can have potentially serious and irreversible effects on humans, in particular on neurodevelopment. This can impact the quality of life and raises societal concern of a high and increasing burden. A number of vulnerable populations may be particularly susceptible to thyroid hormone (TH) disruption induced by 4-MBC. Pregnancy is likely to be a period of sensitivity to the alteration of TH regulation, with potential consequences for neurodevelopment of the offspring.

- The observed adverse reproductive effects in male and female rodents have been plausibly linked to the estrogenic mode of action, shown both *in vitro* and *in vivo* after 4-MBC exposure. These, and potentially other adverse effects in humans, caused by endocrine disruption via the E modality are considered serious, as similar effects in humans could cause sub- and infertility. For humans, sub- and infertility is not only detrimental to the propagation of the species, but it also has a major impact on quality of life. Additionally, fertility treatment and counselling carry high societal costs.

Based on the available studies, it may be difficult to establish a safe level of 4-MBC. Mixture effects, where substances act additively or with synergistic effects, cannot be excluded and this might impact the threshold of toxicity. Moreover, the difficulty to establish a safe level with sufficient certainty raises concern particularly on the capacity to manage safe use of the substances for sensitive populations. Establishing safe levels for these particularly sensitive populations is surrounded with large uncertainties, and alterations of thyroid hormones during the critical windows of pre- and post-natal neurological development may have consequences later in life. The complexity of the response in reaction to thyroid disturbance is not fully characterised and understood, and considering the range of functions influenced by THs, it is also highly challenging to fully characterise these effects in experimental studies.

Altogether, this gives rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 REACH.

Conclusion

Overall, it is concluded that the substances *(±)-1,7,7-trimethyl-3-[(4-methylphenyl) methylene]bicyclo[2.2.1]heptan-2-one* covering any of the individual isomers and/or combinations thereof *(4-methylbenzylidene camphor, 4-MBC)* meet the criteria of Article 57(f) of REACH, due to their endocrine disrupting properties for which there is scientific evidence of probable serious effects to human health which give rise to an equivalent level of concern to those for other substances listed in paragraphs (a) to (e) of Article 57 of REACH Regulation.

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Annex 1: Table with summarised information on systemic toxicity in the performed animal studies

Study	Experimental design	Route	Doses	Systemic toxicity, body and liver weights
Schlumpf *et al.* 2001	6 days, PND 21-26 (n=4-10)	Dermal	Total amount applied each day: 137.5, 275, 412.5 mg	Mean body weights on PND 26 were unaffected by treatment, liver weight were not assessed
Schlumpf *et al.* 2001	4 days, PND 21-24 (n=9-19)	Feed	66, 119, 211, 337, 402, 1980	Mean body weights on PND 24 were unaffected by treatment, liver weight were not assessed
Tinwell *et al.* 2002	For three days , starting on PND 19-20 (n=12)	Oral	500, 800	Clinical signs of toxicity after the third oral administration of 800 mg/kg. At termination, reduced body weight was seen in both exposed groups. Liver weights were not assessed.
Tinwell *et al.* 2002	For three days, starting on PND 19-20 (n=12)	s.c.	500, 1000	No signs of general toxicity were seen, liver weights were not assessed.
Ashby *et al.* 2004	Three days, starting on PND 19-21 or older animals	s.c.	1000	No body weight reductions were seen, liver weights were not assessed.
Carou *et al.* 2008	2 days (adult) (n=10-12)	s.c.	2, 20	Body and liver weights were not reported, but the low doses and short exposure period probably did not affect either
Carou *et al.* 2008	5 days (adult) (n=10-12)	s.c.	2, 10	Body and liver weights were not reported, but the low doses and short exposure period probably did not affect either
Durrer *et al.* 2005	Fetal, postnatal, pubertal and adult exposure	Feed	0.7, 7, 24, 47	Female offspring body weights at 12 weeks of age were not affected by treatment, liver weights were not reported.
Durrer *et al.* 2007	Fetal, postnatal, and adult exposure	Feed	7, 24, 47	Body weights of adult male 4-MBC–exposed offspring were in the control range, and their liver weight were unaltered. Body weight at onset of puberty was at control level in males, but slightly reduced in females.
Maerkel *et al.* 2007	Fetal, postnatal, pubertal and adult exposure	Feed	7, 24, 47	4-MBC treatment did not affect maternal body weight gain during pregnancy, or the number of pups per litter. Body weight was reduced by 15-20% in offspring of both sexes at the highest dose level at PN 2 and PND 14 (only statistically significant on day 14), but was at control levels at 12 weeks of age (PN 84). Liver weights were not reported here but were reported as unaltered in adult male offspring in the Durrer *et al.* 2007 publication.
Faass *et al.* 2009	Fetal, postnatal, and adult exposure	Feed	7, 24, 47	Body weight of adult female 4-MBC-exposed offspring was unchanged

Study	Experimental design	Route	Doses	Systemic toxicity, body and liver weights
Unpublished, 2004	21 days exposure in the pre-mating period (n =3-5))	Oral	12.5, 25, 50	In parental females there were no treatment-related clinical signs, no mortality and no effects on body weight gain or food consumption in any dose, but water consumption was increased in the high dose group
Unpublished, 2004	Premating, gestation, lactation (84 days exposure). n=10 parental females/exposure group)	Oral	12.5, 25, 50	4-MBC exposure caused no effect on dam body weights after 84 days of exposure, i.e. at the time of weaning, but water consumption was increased in the high dose group. Liver weight was not assessed.
Unpublished, 2004	Gestation, lactation until PND 22 (n=30)	Oral	12.5, 25, 50	No statistically significant decreases in offspring body weights were observed during the course of the study in 4-MBC exposed animals. If anything offspring body weights on day 1 were increased in the mid and high dose groups, whereas no effects were seen on PND 22 and PND 55. Offspring liver weights were not assessed.
Unpublished, 1984a	TG408 (n=20 animals /sex)	Feed	0, 50, 125, 312. A dose of 25 was included with its own control group.	Decreased body weight gain in females at 312 mg/kg. No effect on male bodyweight. Relative liver weights were increased in females at 125 and males at 312 mg/kg bw/day. No effects were seen at 25 mg/kg bw/day.
Unpublished, 1984b	28 day repeated dose (only focus on liver enzymes)	Oral	312	No changes in body or liver weight were observed
Unpublished, 1983a	17-day study (n=10/sex per dose group)	Oral	0, 30, 300	No effect on body weight or food consumption, but non-specific clinical signs (salivation, excessive grooming) were observed at both doses. At 300 mg/kg/day statistically significant increased kidney weight (males), adrenal gland weight (males) and reduced thymus weight (females) was seen.
Unpublished, 1983b	28-day study (n=10/sex)	Oral	0, 1000	Marked systemic toxicity, seen as clinical signs and markedly reduced body weights (20% lower in males and 10 % lower in females). Liver weights were increased and thymus and adrenal weights reduced.
Unpublished, 1988	TG414 (n=25) Gestation day 6-15	Oral	0, 10, 30, 100	The dose levels of 10 and 30 mg/kg bw/day proved to be non-toxic to the pregnant female rat. However, the dose level of 100 mg/kg bw/day was shown to be minimally toxic to the dams as demonstrated by a slightly lower body weight gain by these females
Unpublished, 2005	90 days (n=20/sex)	Dermal	100, 400, 2000	No effect on body or liver weight seen at 100 and 400 mg/kg. Highest dose terminated after 11 days due to severe local effects.

Unpublished, 1995	Humans; 14 days, twice daily	Dermal	6% on 1200 cm2 body surface	No systemic toxicity observed
Janjua *et al*. 2004	Humans; 4 consecutive days (15 young males, 17 postmenopausal females)	Dermal	2 mg/cm2 with 10% (wt/wt) formulation	Not assessed
Studies with lower reliability				
Seidlova-Wuttke *et al.* 2006a	12 weeks (adult) (n=12)	Feed	60 mg/day and 285 mg/day, corresponding to approximately 230 and 1000 mg/kg bw/day	During the study control animals gained around 95 grams. Exposure to the positive control E2 caused a very marked decrease in the same period, body weight in these animals was only around 15 grams. Animals exposed to the low dose of 4-MBC gained around 65 grams. Animals exposed to the high dose of 4-MBC showed a reduction in body weight gain during the first weeks of treatment, and the dose was therefore reduced to the low dose level. At the end of the study weight gain was around 60 grams. The information on dose reduction was not provided in this publication, only in the Seidlova-Wuttke et al 2006 publication.
Seidlova-Wuttke *et al.* 2006b	12 weeks (adult ovariectomized, n=12)	Feed	230, 1000	The authors reported that no signs of toxicity were observed under application of 0.6 mg E2, 57.5 or 250 mg of 4-MBC; However, both the tested E2 dose and the high dose of 4-MBC resulted in reduced body weight gain during the first 6 weeks of treatment. The high dose 4-MBC animals were therefore changed to the low dose of 4-MBC. They experienced a catch-up in weight gain such that, at the end of the experiment, the weights of the animals did not differ from those of the controls.
Carou *et al.* 2009a	Every second day from pregnancy onset to day of delivery	s.c.	20, 100, 500	Body and liver weights were not reported in either dams or offspring
Carou *et al.* 2009b	Every other day during pregnancy (n=10-13)	s.c.	100	Dam body weights were not reported. Offspring body weights on PND 70 were unaffected
Maerkel *et al.* 2005	Fetal, postnatal, pubertal and adult exposure	Feed	7, 24, 47	Offspring body weight were not reported, but we know from other publications that they were unaffected by treatment.
Hofkamp *et al.* 2008	Dams exposed during gestation (n=4)	Feed	0.7, 7	Offspring body weights were not reported, but we know from other publications that doses of o.7 and 7 mg/kg does not affect offspring body weight in the early postnatal period.
Schmutzler *et al.* 2004	12 weeks (adult, ovariectomized rats) (n=8-11)	Feed	66, 310 mg/ animal/day	Clinical symptoms, body and liver weight were not reported in this publication

Unpublished, 2003	14-day study in dogs (1 female and 1 male)	Oral	20 mg/kg on day 1, 100 mg/kg on day 2, 500 mg/kg on day 3, 2500 mg/kg on day 4 and 500 mg/kg on days 5 – 14	No effect on food consumption, body weight, clinical chemistry or gross pathology. Vomiting seen after treatment with 2500 mg/kg in males.
Unnamed, 2003	21 day study in dogs (n = 4, 2 males and 2 females)	oral	0 mg/kg on day 1, 20 mg/kg on day 4, 100 mg/kg on day 8 and 500 mg/kg on days 11 - 21.	No general toxicity was observed