Additional information report

For a Substance under targeted consultation Harmonised Classification and Labelling Process

Substance Name: Hexyl Salicylate

EC Number: 228-408-6

CAS Number: 6259-76-3

Contact details for Additional information report submitter:

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CONTENTS

1 INTRODUCTION

France, as dossier submitter, has elaborated a CLH report for hexyl salicylate (HS) on 2 endpoints, including reproductive toxicity. The present document is specifically related to reproductive toxicity, and in particular to developmental toxicity.

As no developmental data is available on HS in animals or humans, assessment has been based on read-across from data on structural analogues: methyl salicylate (MeS), sodium salicylate (NaS) and salicylic acid (SA). This read-across was based on the hypothesis that HS, MeS and NaS hydrolyse into a common product, SA. Therefore, based on the known toxicity of SA on development, it is expected that these substances should have similar toxicological effects.

Before the elaboration of the CLH report, read-across was already proposed for reproductive toxicity by the registrants in the HS registration dossier based on data about several salicylates including MeS. Besides, as detailed later, HS has been evaluated in 2012 by NL and in the conclusion document published in 2018, read-across for HS from other salicylates was accepted regarding reproductive toxicity. It means that it was judged unnecessary to request further testing.

The only available study giving evidence of the metabolisation of HS to SA was an *in vitro* dermal absorption test with freshly isolated human excised skin (OECD Test Guideline 428) requested during the Substance Evaluation process.

During the RAC-59 Working Group (WG) on CLH for HS, the RAC rapporteur and some RAC members have expressed some doubts regarding the adequacy of this read-across:

- the *in vitro* dermal absorption test was poorly described in the CLH report;
- it refers exclusively to metabolism of HS after dermal application whereas the studies investigating reproductive toxicity with MeS, NaS and SA and showing effects were conducted after oral or subcutaneous administration;
- no quantitative *in vivo* data has been provided to support the alleged common metabolism of methyl salicylate and hexyl salicylate;
- the paper from Belsito *et al.* (2007) (Research Institute for Fragrance Materials) indicating that 17 salicylates including HS are expected to undergo extensive hydrolysis, primarily in the liver, to SA, was only mentioned in the CLH report without any details. RAC members highlighted that metabolism of HS to SA is only possible but without a quantitative indication of probability;
- two metabolic pathways (glucuronidation and hydrolysis) were identified by the ECHA team and RAC wanted further information on the proportion to which each contributed to its metabolism/degradation.

Overall, even if it is acknowledged that a part of HS would be metabolized into SA, the kinetics of this hydrolysis is unknown and therefore, it was questioned if the quantity of SA produced would be sufficient to reach a concentration that could cause developmental toxicity.

No consensus on classification of HS for developmental toxicity was reached during this WG.

As ECHA provided additional data during the RAC-59 WG (e.g. QSAR estimation), it was agreed to conduct a targeted consultation.
The present document aims at describing new data presented during RAC WG and also identified after this meeting in order to strengthen the justification of the read-across.

2 DATA SOURCES

During RAC WG, it was asked from the dossier submiter (FR) to:

- contact the registrant in order to have access to the study report of the *in vitro* dermal absorption test,
- contact the RIFM for potential unpublished data on toxicokinetics of salicylates.

Moreover, France has looked in the litterature for additional data to clarify the concerns expressed during the RAC-59 WG and investigated the QSAR report from ECHA.

3 DATA DISCUSSED DURING RAC WG

This chapter aims at describing more in depth data broached or discussed during the RAC WG.

3.1 TOXICOKINETIC DATA

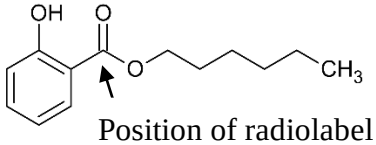
3.1.1 Data from the RIFM

The RIFM (Research Institute for Fragrance Materials) was contacted and does not have unpublished data on toxicokinetics on salicylates.

3.1.2 Study report of the in vitro dermal absorption test on HS

France had access to the study report of the *in vitro* dermal absorption test as requested by the RAC WG. A study summary is provided below and a detailed summary is available in a confidential Annex (separate document).

The *in vitro* dermal absorption test was performed by the registrant according to OECD Test Guideline 428 in order to investigate the dermal absorption of ^{14}C-radiolabelled hexyl salicylate in human skin membranes using flow-through diffusion cells and physiological saline containing 6% PEG 20 as the receptor fluid.



Position of radiolabel

Human skin membranes were prepared from frozen skin samples derived from breast and abdomen obtained from 4 female donors. Hexyl salicylate, undiluted or as 0.1 and 20% in dipropylene glycol, was applied to split-thickness skin membranes (n = 8) for 8h. Exposure was terminated by washing with a 3% soap solution and the skin

membranes were tape-stripped at termination of the study 24h after exposure. Adequate solubility of hexyl salicylate in the receptor fluid was demonstrated. Skin preparation integrity was also assessed. Detailed information on sampling times, procedures and analysis of skin was provided.
At each concentration of hexyl salicylate, the majority of the applied radioactivity was removed by washing at 8h (97.6%, 87.9% and 93.5% at concentrations of 100%, 20% and 0.1%, respectively). Only relatively small amounts of radioactivity (0.15%, 0.64% and 1.00%, respectively) were detected in the receptor fluid.

In a separate metabolism phase, 0.1% of ^{14}C-radiolabelled hexyl salicylate in dipropylene glycol was applied to breast or abdomen skin membranes (n=3) obtained from fresh human skin tissue from two female donors using static diffusion cells and tissue culture medium as receptor fluid.
Analysis of the receptor fluid showed almost no hexyl salicylate, but **identified salicylic acid as the major component, indicating metabolism of hexyl salicylate by dermal esterases**. Hexyl salicylate and salicylic acid were identified in the skin extracts. Figures are available in the confidential annex.
The authors indicated that calculation of dermal absorption for hexyl salicylate should take into account the potential for metabolism to salicylic acid in the skin. As non-viable skin membranes were used in the first phase of the study (diffusion cells), little or no metabolism would have occurred. Thus, the dermal absorption values in this first phase might underestimate the total level of absorption. The authors assumed that all the hexyl salicylate present in the skin was potentially metabolised and absorbed as salicylic acid. Therefore, the calculated dermal absorption values were 0.8%, 7.8% and 2.7% for hexyl salicylate concentrations of 100, 20 and 0.1% respectively. France could not explain why the dermal absorption values were not linear with dilution.

A limitation of the dermal absorption test was identified in view of the requirements of the OECD 428 guideline. The results for relevant reference chemicals were not made available to demonstrate the performance and reliability of the test system in the performing laboratory.

3.1.3 QSAR report

ECHA has presented a modelling of phase I metabolism of MS and HS during the RAC WG. In order to support the metabolic similarity between these two substances, the QSAR modelling has been further investigated and resulting data are described in the chapter 4.1.3.

4 OTHER RELEVANT DATA SUPPORTING READ-ACROSS

This chapter aims at describing additional relevant data in order to strengthening read-across.

4.1.1 Other assessments

4.1.1.1 Substance Evaluation by the Netherlands

The Netherlands performed a Substance Evaluation on HS, published in 2018. The conclusion of this evaluation is that there is a need for follow-up regulatory action at EU level, namely, an Harmonised Classification and Labelling process ("*In view of the eMSCA, the registration dossier contains information on the main metabolite of hexyl salicylate, salicylic acid, that would prompt a classification Repr. 1B. However, in 2016 RAC has issued an opinion on salicylic acid proposing a harmonised classification for the endpoint reproductive toxicity, Repr. 2; H361d. RAC stated the following: "Taking into account the available data, including pharmacokinetics, in vitro tests with ASA and salicylic acid, developmental studies in animals (positive findings in rat and monkey studies and a negative rabbit study), human epidemiology and medical experience, the RAC considered classification of salicylic acid as Repr. 2; H361d (Suspected of damaging the unborn child) to be justified". Although it is the registrants' responsibility to consider the consequence of the classification of salicylic acid,* ***a harmonised classification for reproduction toxicity is proposed, that should follow the RAC opinion and thus read: Repr. 2; H361d (Suspected of damaging the unborn child). The harmonised classification could be extended including a group of salicylates, as the read across by the Registrants, indicate a common mechanism, where salicylic acid is the main metabolite of the salicylate group. Hexyl salicylate is rapidly and almost completely metabolized to salicylic acid via all routes of exposure (based on toxicokinetics data in the Registration dossier, a supporting read across document in IUCLID, and following the results from the requested in vitro dermal absorption study).*** *At this moment, no notifications of classification and labelling of hexyl salicylate are made for reproductive toxicity, while the substance is wide dispersively used. Following the opinion of RAC for salicylic acid, the classification and labelling proposal for hexyl salicylate should read: Repr. 2; H361d (Suspected of damaging the unborn child)*").

As seen, the **proposed read across here in the framework of the CLH report is fully in line with the conclusion of NL in the Substance Evaluation process**.

4.1.1.2 Group regulatory management options of ECHA

ECHA performed in 2021 a group regulatory management options (RMOA) for various salicylate esters including HS.

According to ECHA: *All substances have a* ***suspected health hazard for developmental toxicity derived from Salicylic acid, which is the common metabolite for all salicylates****. It has a harmonised classification as Repr. 2 […]. The suspected hazard for the other substances is based on read-across from methyl salicylate and salicylic acid (metabolite of all). There is no detailed knowledge on the toxicokinetics for all the different esters, however it is assumed that esterases can hydrolyse the esters of salicylic acid quickly (rates unknown) in vivo and therefore resulting in the same active metabolite for all of them.*

The conclusion was as follow: "*Need for (further) EU regulatory risk management – harmonised classification for reprotoxicity […]*
Developmental toxicity is a known or likely hazard for most group members; for hexyl salicylate (EC 228-408-6) there is a CLH proposal for Repr. 2 based on methyl salicylate data (developmental effects) for which a RAC opinion for Repr. 2 has also been issued*. A third substance (phenyl salicylate, EC 204-259-2) has a self-*

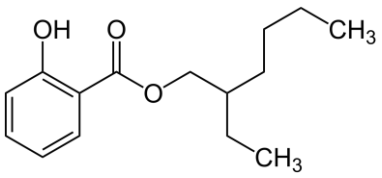
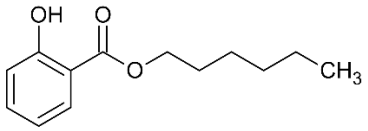
classification of Repr. 2 (fd). Although several group members are lacking data, a group-wide pattern emerges around the formation of the same metabolite, salicylic acid, thus classification as Repr. 2 is appropriate; due to the widespread uses of the group members and the commonality in their functions and uses, which would indicate a potential for substitutability, harmonised classification would add value by ensuring proper classification in the context of the use of these substances by workers and proper description of RMMs in exposure scenarios and supply chain communication. Moreover, harmonised classification would facilitate regulatory risk management under other legislation."

The group RMOA of ECHA for salicylate esters especially included ethylhexyl salicylate (EHS) which is an additional structural analogue particularly close to HS. Thus, based on their structural similarities, EHS may be another relevant candidate for read-across in addition to MeS, NaS and SA for the assessment of HS, that was not initially identified in the CLH report. Data about physicochemical properties, toxicokinetics and developmental toxicity of EHS is provided below in order to justify the relevance of this candidate for read-across.

4.1.2 Other relevant data on an additional structural analogue: ethylhexyl salicylate (EHS)

4.1.2.1 Identity and physicochemical properties of HS and EHS

Table 1: Identity and physicochemical properties of ethylhexyl salicylate

Substance name	2-ethylhexyl salicylate	Hexyl salicylate
EC number	204-263-4	228-408-6
CAS number	118-60-5	6259-76-3
Molecular formula	$C_{15}H_{22}O_3$	$C_{13}H_{18}O_3$
Structural formula		
Molecular weight or molecular weight range	250.33 g/mol	222.28 g/mol
Water solubility	0.074 mg/L at 20°C (registration dossier)	2 mg/L at 23°C (NL Sev 2018)
Log Pow	5.94 (registration dossier)	5.5 (NL Sev 2018)
Vapour pressure	1.8 x 10^{-2} Pa at 20°C (registration dossier)	7.7 x 10^{-2} Pa at 23°C (NL Sev 2018)

EHS is a structural analogue particularly close to HS. Indeed, both substances share similar alkyl chain length. Besides, these 2 molecules also share a same steric hindrance. Additionally, HS and EHS share similar physicochemical properties (see Table 2).

4.1.2.2 Toxicokinetic data on EHS

Bury *et al.* (2019) studied urinary metabolites of the UV filter 2-ethylhexyl salicylate as biomarkers of exposure in humans. A study summary including only relevant results for the purpose of the present document is provided below and a detailed summary is available in Annexes. This paper was considered relevant by France to justify that EHS is a potential relevant candidate for read-across for the assessment of HS as it shows metabolism of EHS to SA in humans after oral exposure.

In order to provide human toxicokinetic data on EHS as a tool for risk assessment, Bury *et al.* (2019) studied metabolism and urinary metabolite excretion of EHS after oral exposure (single dose: 57.4-75.5 µg/kg bw) in three male volunteers. Seven EHS metabolites were identified, including three EHS specific metabolites that were quantitatively investigated. Additionally, salicylic acid (SA), as ester cleavage product, and its follow-up metabolite salicyluric acid (SUA) (Figure 1) were found at the retention times t_R = 9.6 and 8.4 min, respectively, with rather high peak heights at least for SUA, indicating its presence at rather high concentrations.

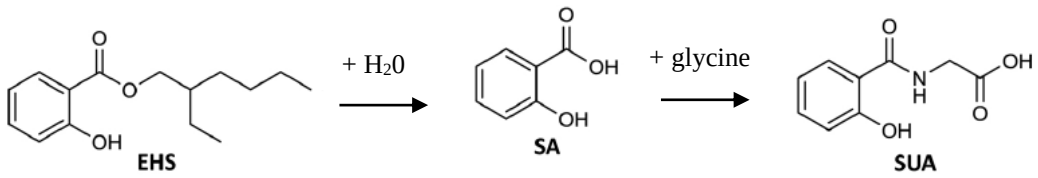


Figure 1: Human EHS metabolism to SA and SUA

The identities of SA and SUA were confirmed using analytical standards. However, SA and SUA are not specific biomarkers of exposure to EHS and are therefore not interesting regarding the initial purpose of the study. The extent of formation of SA and SUA in urine related to metabolism of EHS has thus not be quantified by the authors. Moreover, due to the different possible sources of SA and SUA, including nutritional sources, there were significant background levels of these two metabolites already in the pre-dose samples. This would have made the exact quantification of dose-related SA and SUA rather inaccurate, the authors having not dosed isotope-labeled EHS. The fact remains that data unambiguously identified SA and SUA as metabolites of EHS in the study.
Besides, as the 3 specific EHS metabolites analysed in urine accounted for less than 1% of the orally applied EHS dose, the authors indicated that it could be expected that the major share of EHS dose was eliminated via urine as the non-specific metabolites SA and SUA, which is likely the predominant metabolic pathway.

This study, as well as the physicochemical properties of EHS, are considered sufficient to the DS to consider EHS as a relevant substance for read across with HS.

4.1.2.3 Reproduction/Developmental Toxicity Screening Test on EHS (OECD 421)

A reproduction/developmental toxicity screening test on EHS was performed by the registrant according to OECD Test Guideline 421. This study was considered relevant by France as EHS is a relevant substance for read across with HS. Moreover, the effects reported are similar to those found with other salicylates (as methyl salicylate or salicylic acid).
The study summary provided below is based on ECHA Dissemination website (France did not have access to the study report). A detailed summary is also available in Annexes

EHS was administered once daily by gavage in corn oil as vehicle at dosages of 25, 80, and 250 mg/kg body weight/day in male and female rats. Control animals received the vehicle only. Male rats were exposed for 28 days and female rats for approximately 7 weeks, i.e. 14 days prior to pairing, through the pairing and gestation periods until the F1 generation reached day 4 post partum.
At the high dose level, one female was found dead on day 23 of the gestation period which was considered to be a result of birth complications. Slight but non-significant changes on body weight gain in female rats were also observed at this dose.
Reduction in gestation index (number of females with living pups as a percentage of females pregnant), increase in incidence of post-implantation loss resulting in a lower litter size and prolonged gestation period were observed at 80 and 250 mg/kg bw/d. Reduction in gestation index and increase in incidence of post-implantation loss were statistically significant and dose dependent effects, so these findings were considered to be test item-related adverse effects. Based on the individual data, increased post-implantation loss occurred predominantly in females with prolonged gestation.
Reduction in absolute body weights of pups was observed at 250 mg/kg bw/d and was considered to be test item-related adverse effect.

Based on the observation of increased post-implantation loss, reduction in gestation index and lower litter size, the LOAEL for developmental toxicity is 80 mg/kg bw/d and the NOAEL is 25 mg/kg bw/d. The LOAEL for maternal toxicity is 250 mg/kg bw/d.
This study shows indubitably developmental toxicity of EHS, and the effects reported are similar to those found with other salicylates (as MeS, NaS or SA).

4.1.3 QSAR report

In order to support the metabolic similarity between methyl salicylate and hexyl salicylate that was questioned during RAC WG, two *in silico* software tools have been used: Meteor and TIMES. Moreover, as EHS has been identified by FR as another relevant candidate for read-across in addition to MeS, NaS and SA for the assessment of HS, QSAR modelling was also performed on this substance.

Meteor

Meteor Nexus v.3.1.0 (Lhasa Ltd., Leeds, UK) was used with the default settings.

Meteor is a rule-based software tool that contains a knowledge-based of reactions and rules relating structure and biotransformation to predict both phase I and II xenobiotic metabolites of a query chemical structure.

The Site of Metabolism (SOM) Scoring with molecular mass variance is the default setting in Meteor Nexus for assessing the likelihood of observed metabolites. The SOM methodology builds on the Static Scoring methodology by tailoring the score using experimental data for compounds (from the metabolism database) that match the same biotransformation, have similar molecular weights and are chemically similar around the site of metabolism. The methodology is complex and users can control how many metabolites they are presented with by modifying the processing constraints associated with the methodology. It must be noted that Site of Metabolism Scores do not represent quantitative information.

Results:

Table 2: Biotransformation predicted for each compounds

Biotransformation	Phase (enzyme)	Methyl salicylate score	Hexyl salicylate score	Ethylhexyl salicylate
144: Hydrolysis of acyclic carboxylic Esters	Phase I (hydrolase)	831	887, 541, 297, 463, 359, 284, 393, 300, 345	904
225: 4-Hydroxylation of 1,2-Disubstituted Benzenes	Phase I (CYP450)	294	314, 295, 505	320
072: Hydroxylation of Penultimate Alkyl Methylene[1]	Phase I (CYP 450)	/	591, 389	653, 296
073: Hydroxylation of terminal methyl[1]	Phase I (CYP 450)	/	324, 310, 380	389
074: Hydroxylation of Antepenultimate Alkyl Methylene[1]	Phase I (CYP 450)	/	429	291
075 Hydroxylation of Alkyl Methine[2]	Phase I (CYP 450)	/	/	351
241: Oxidation of primary alcohols[3]	Phase I (ADH)	/	485, 313	571
036: Conjugation of Aryl and Alkyl Carboxylic Acids with Glycine	Phase II (ACS, AANAT)	407	434	443
027: Glucuronidation of Aromatic Alcohols	Phase II (UGT)	340, 382	363, 342, 383, 273	370, 364, 502, 282, 272
019: O-sulphonation of aliphatic alcohols[3]	Phase II (SULT)	/	315	/
023: Glucuronidation of Primary and Secondary Aliphatic and Benzylic Alcohols[3]	Phase II (UGT)	/	315	345, 279

acyl-coenzyme A synthetase (ACS), amino acid N-acyltransferase (AANAT), glucuronyltransferase (UGT), sulfotransferase (SULT), cytochrome P450 (CYP 450)

[1] These biotransformations (072, 073 and 074) do not apply to MS. The biotransformation 019 does not apply to MS and EHS.
[2] The biotransformation (075) does not apply to MS and HS.
[3] These biotransformations (241 and 023) do not apply to MS. The biotransformation 019 does not apply to MS and EHS.

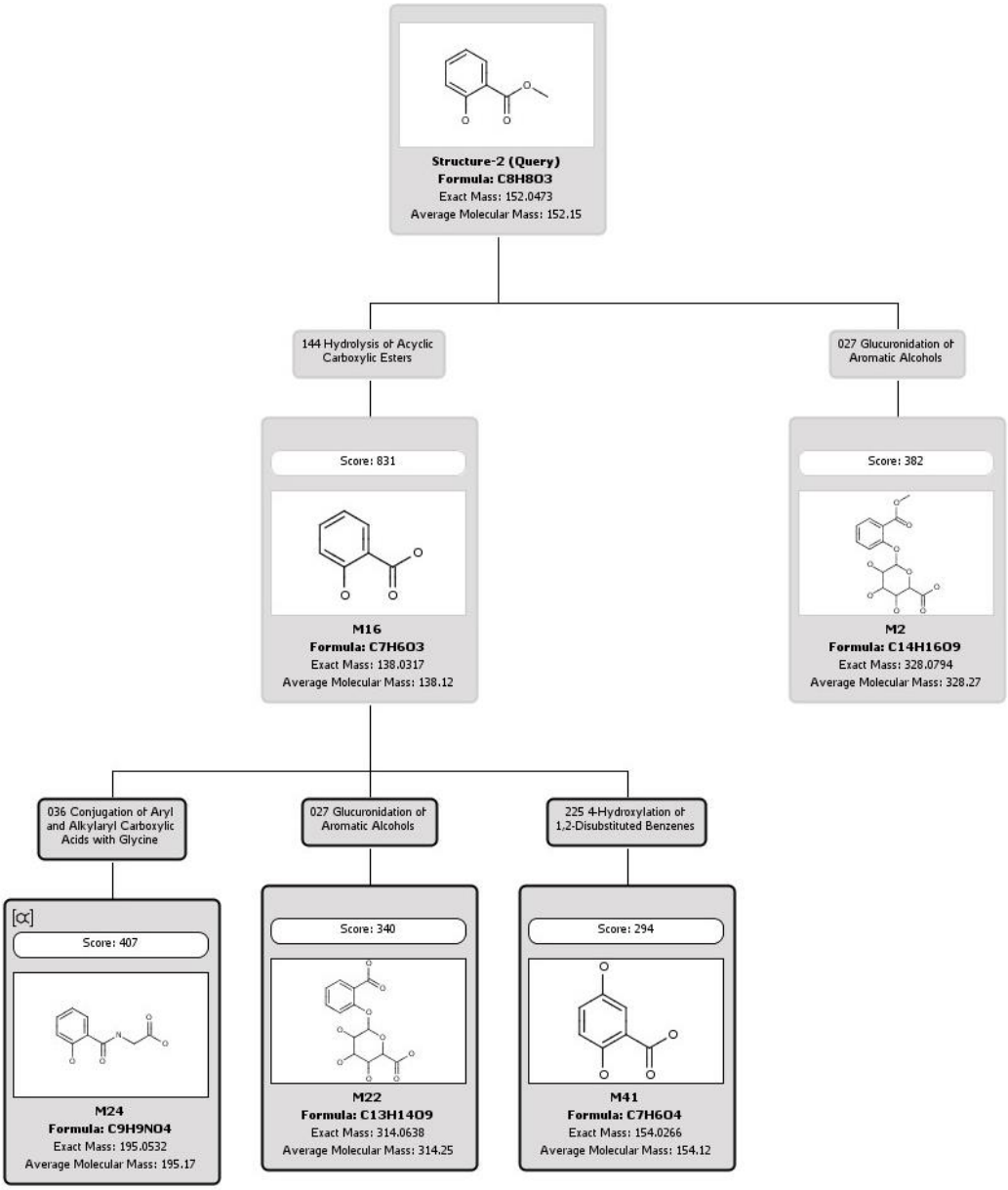
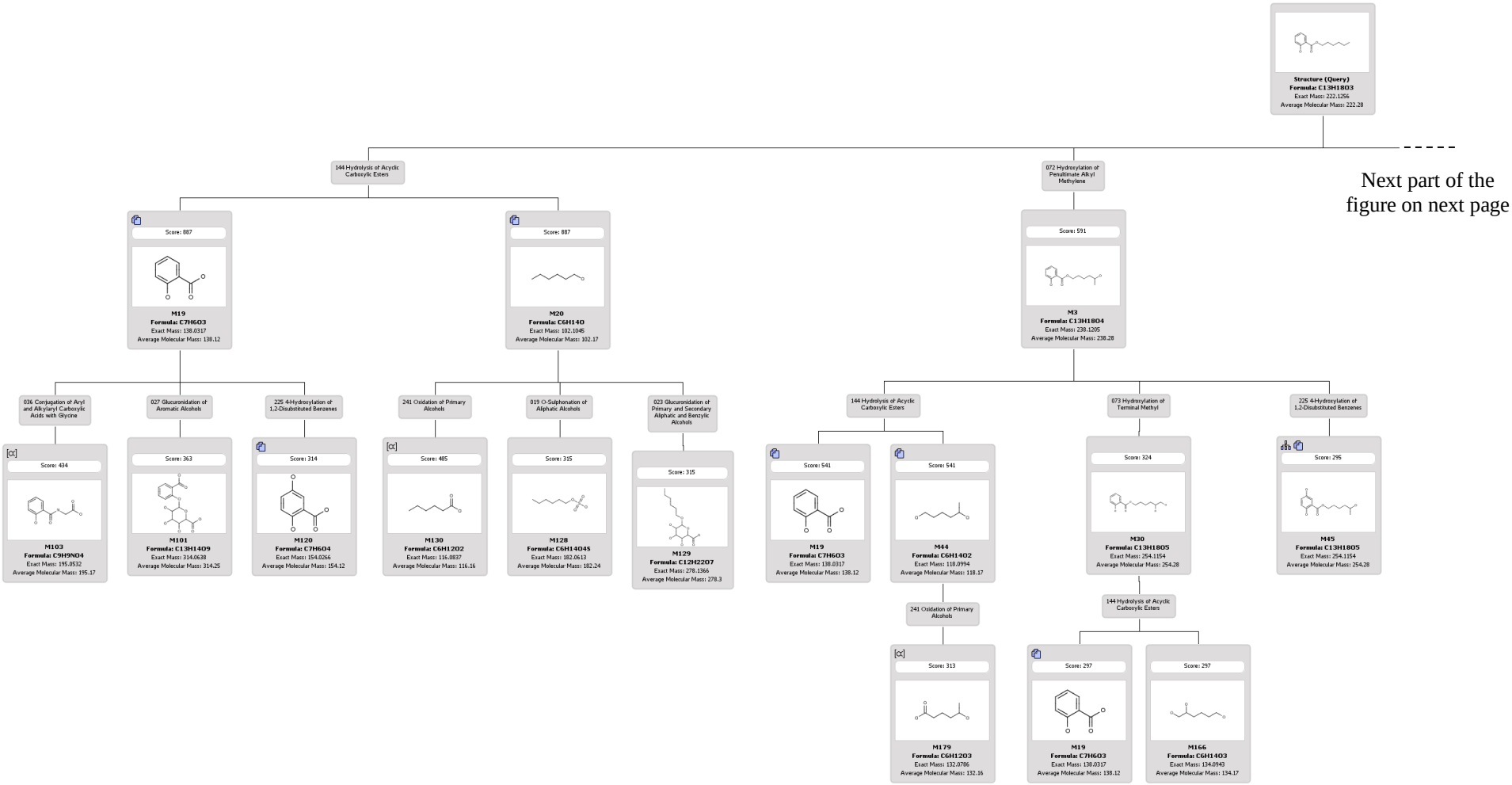


Figure 1: Metabolic trees (maps) for methyl salicylate (Meteor)



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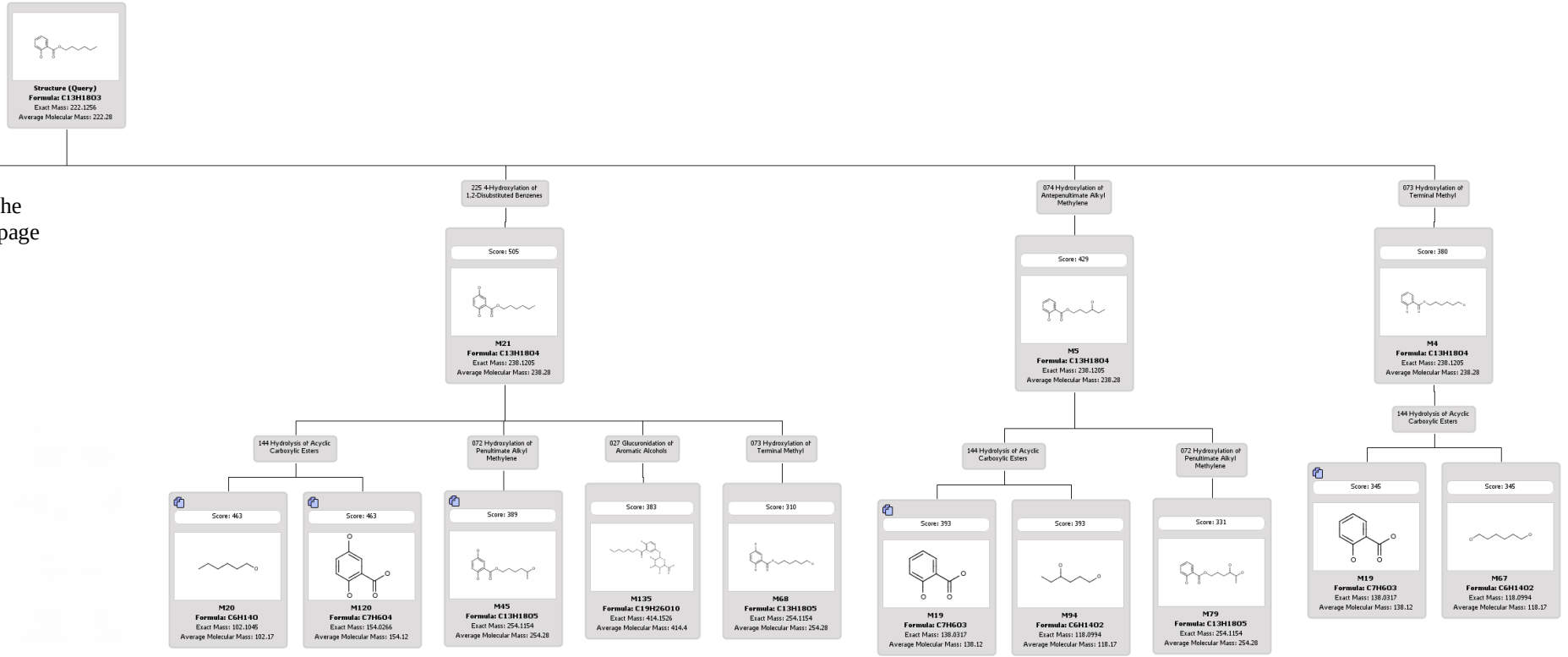

Figure 2: Metabolic trees (maps) for Hexyl salicylate (Meteor)

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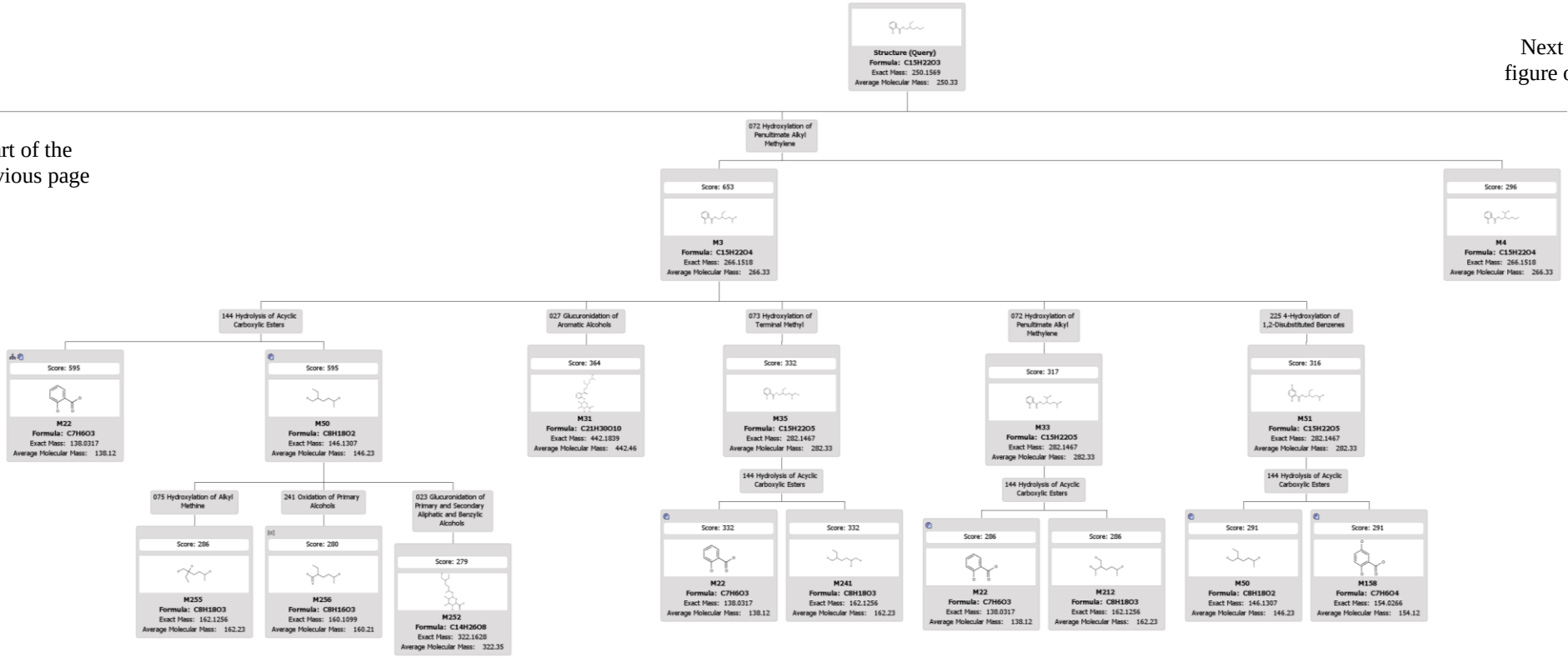

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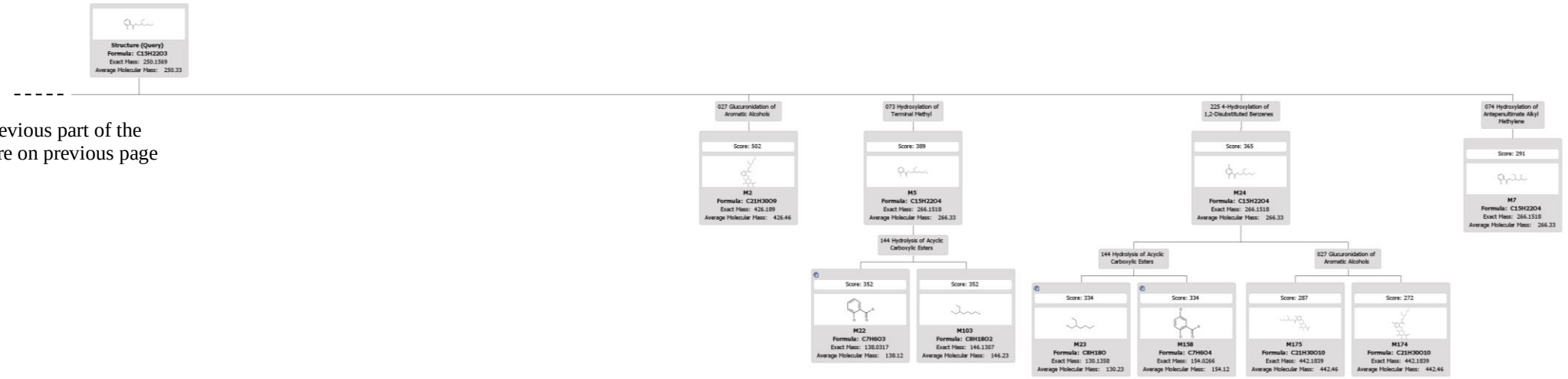

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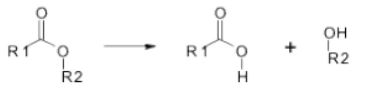
Figure 3: Metabolic trees (maps) for Ethylhexyl salicylate (Meteor)

Discussion:
For the three compounds, the highest score is obtained for biotransformation 144 "Hydrolysis of Acyclic Carboxylic Esters (Score 887 for hexyl salicylate, 831 for methyl salicylate and 904 for ethylhexyl salicylate)." This biotransformation corresponds to the hydrolysis of MS, HS or EHS to salicylic acid and the respective alcohol. The following information are available on this biotransformation in the METEOR report:



The acyl bond cannot be in a ring
R1, R2 = carbon excluding:

(a) either R1 or R2 as a saturated aliphatic carbon with no attached hydrogens or

(b) R1 and R2 are both, or a combination of:
(i) a saturated aliphatic carbon with one attached hydrogen,
(ii) a fully carbon-substituted sp2 (e.g. alkene) carbon or
(iii) an aromatic carbon.

"This biotransformation describes the hydrolysis of acyclic carboxylic esters, excluding only those with groups considered to offer steric hindrance to hydrolysis based on simple structural criteria. Esters such as vinyl acetate with an unsubstituted alkene group on the ester oxygen atom give the aldehyde by tautomerism of the enol product. Ester hydrolysis is frequently a very facile reaction, which may occur additionally by chemical (non-enzymatic) means. QSAR studies on *in vitro* hydrolysis rates of over 80 esters in human blood have indicated that the ease of enzymatic hydrolysis decreases with increasing steric shielding of the carbonyl group (the most significant factor) and increases if electron withdrawing groups increase the positive charge and electrophilicity of the carbonyl carbon. A positive correlation with lipophilicity was also indicated (Buchwald and Bodor 1999, Buchwald 2001). In general, esters of phenols are more easily hydrolysed than esters of alcohols. The presence of alkyl amino groups a few bonds from the ester function is also associated with an increase in enzymatic and non-enzymatic hydrolysis rates (Testa and Mayer 2003a). For esters with chiral centres close to the ester function, such as cocaine, and prodrugs of oxazepam, propranolol or ibuprofen, a high degree of enantioselectivity may be exhibited (Testa and Mayer 2003b). Ester hydrolase enzyme systems display significant activity *in vivo* at sites other than the liver such as the kidney, intestine, plasma and others. It has been suggested that, often, many different enzymes are active on the same substrate. The biotransformation is characterised by wide and unpredictable interspecies differences although generally rodents metabolise esters faster than humans (Buchwald 2001)."

On the basis of this information, although a higher lipophilicity is expected for HS and EHS compared to MS, there is no ground to consider that hydrolysis would be less likely. It may also be of interest to note that the nearest neighbours contributing to the prediction in Meteor for the biotransformation prediction of hydrolysis (144 in Meteor) of HS, EHS and MS into salicylic acid were not fully the same substances, maybe due to differences in molecular weight. Nevertheless, for the 3 compounds, MS, HS and EHS, a similar biotransformation prediction to salicylic acid (144) was proposed with the highest score.

Regarding the other predicted biotransformations 019, 023 and 241 of HS, which are not predicted for MS, they are related to the transformation of the alcohol. The

biotransformation 019 is also not predicted for EHS. This metabolite is not expected to have an effect on the reproductive toxicity and may thus be disregarded for the comparative profile.
Additional biotransformations predicted for HS and EHS, and not predicted for MS, is the hydroxylation of the alkyl chain at different sites (biotransformation 072, 073 and 074). The resulting metabolites may be further biotransformed to salicylic acid and the corresponding alcohol (biotransformation 144 predicted by Meteor). Therefore, although the biotransformation such as hydrolysis is predicted with more occurrence for HS and EHS, the nature of the biotransformation between the three compounds can be considered similar. There were no biotransformation predicted for MS that was not predicted to occur also for HS and EHS.

Times

There are two metabolism simulators in TIMES: *in vitro* rat S9 metabolism and *in vivo* rat metabolism. The following version of the software was used: OASIS TIMES ver. 2.29.1.88.

About the in vitro rat S9 model: "The simulation of metabolism is focused on the correct reproduction of experimentally observed metabolites. The current *in vitro* rat liver metabolic simulator (transformation table) represents electronically designed set of 517 structurally generalized, hierarchically arranged biotransformation reactions. These molecular transformations are characteristic for the metabolism in the presence of *in vitro* experimental systems such as rodent liver microsomes and S9 fraction. Each transformation in the simulator consists of source and product structural fragments and inhibiting "masks". A probability of occurrence is ascribed to each transformation, which determines its hierarchy in the transformation list. Thus, the modelling is based on the set of principal molecular transformations, and the *in vitro* "logic" of the commonly observed metabolism with the corresponding experimental systems."

About the in vivo rat model: "The simulation of metabolism is focused on the correct reproduction of experimentally observed metabolites. The current *in vivo* rat metabolic simulator (transformation table) represents electronically designed set of 622 structurally generalized, hierarchically arranged biotransformation reactions. These molecular transformations are characteristic for the *in vivo* metabolism in rats. Each transformation in the simulator consists of source and product structural fragments, and inhibiting "masks". A probability of occurrence is ascribed to each transformation, which determines its hierarchy in the transformation list. Thus the modelling is based on the set of principal molecular transformations, and the *in vivo* "logic" of the commonly observed xenobiotics metabolism in living rats."
The following types of molecular transformations are included in the *in vivo* simulator:

- 26 abiotic (non-enzymatic) reactions. The highest priority (probability of occurrence) is assigned to these reactions. This subset of reactions includes also transformations of highly-reactive functional groups and intermediates, such as tautomerizations, arene epoxide rearrangements to phenols, etc. which occur spontaneously.
- 479 enzymatic phase I transformations such as aliphatic C-oxidation, aromatic C hydroxylation, oxidative N- and O-dealkylation, epoxidation, ester and

amide hydrolysis, carbonyl group reduction, nitro and azo group reduction, N-hydroxylation, oxidative deamination, beta-oxidation, ring cleavage, hydrolytic cleavage, aromatization, decarboxylation, dehalogenation, etc.

- 104 enzymatic phase II transformations, such as glucuronidation, sulfation, glutathione and mercapturic acid conjugation, N-acetylation, etc., which, unlike the *in vitro* systems, are believed to occur with high priority *in vivo*.

The derivation of the structural domain of simulator is based on atom-centred fragments

Results:

Metabolic trees were obtained with default setting in TIMES. Two models were used as described above (*in vitro* rat S9 model, and *in vivo* rat). The transformation maps were obtained in two modes: considering Phase I metabolism reactions only, or, considering both Phase I and Phase II reactions.

In the metabolic trees, two types of numbers are shown (P and Q)

- **P (Prob., intrinsic)** is the probability of the current transformation from transformation table.
- **Quantity of metabolite** depends on both – probability to be obtained and probability to metabolize:

Q = <probability to obtain> x (1 - <probability to metabolize>)

Quantity of parent is calculated under the assumption that the probability to obtain is equal to 1: <probability to obtain> = 1

Q(parent) = 1 - <probability to metabolize>

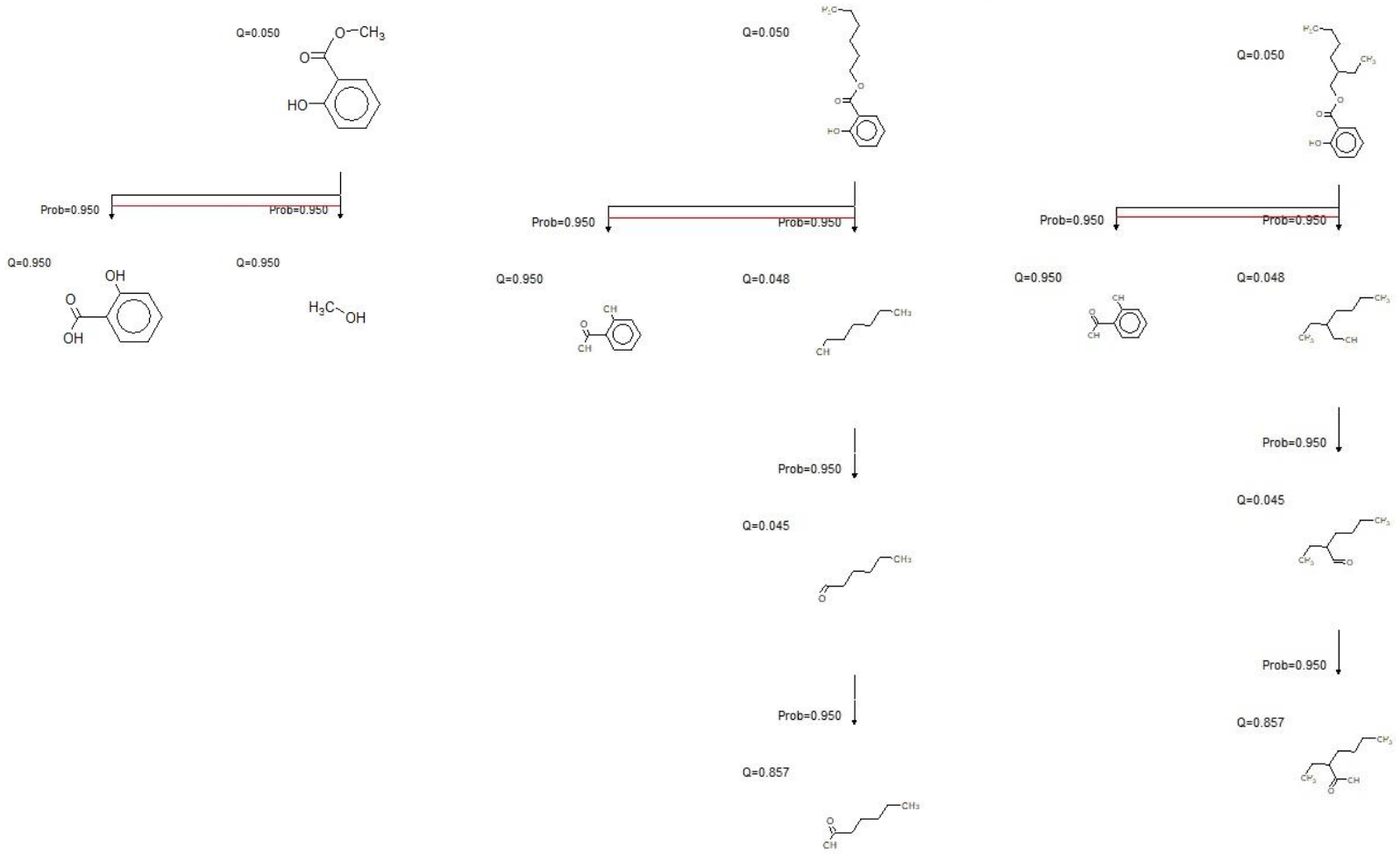


Figure 4: TIMES_In vitro S9_Phase I only_MS (left part), HS (in the middle) and EHS (right part)

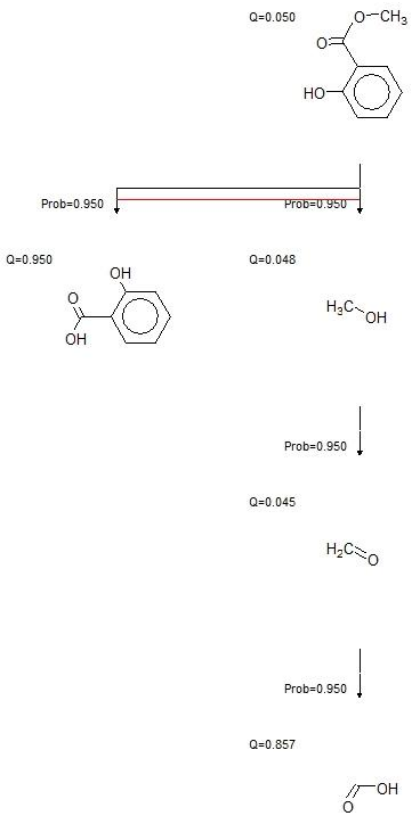


Figure 5: TIMES_In vivo rat_Phase I only_MS

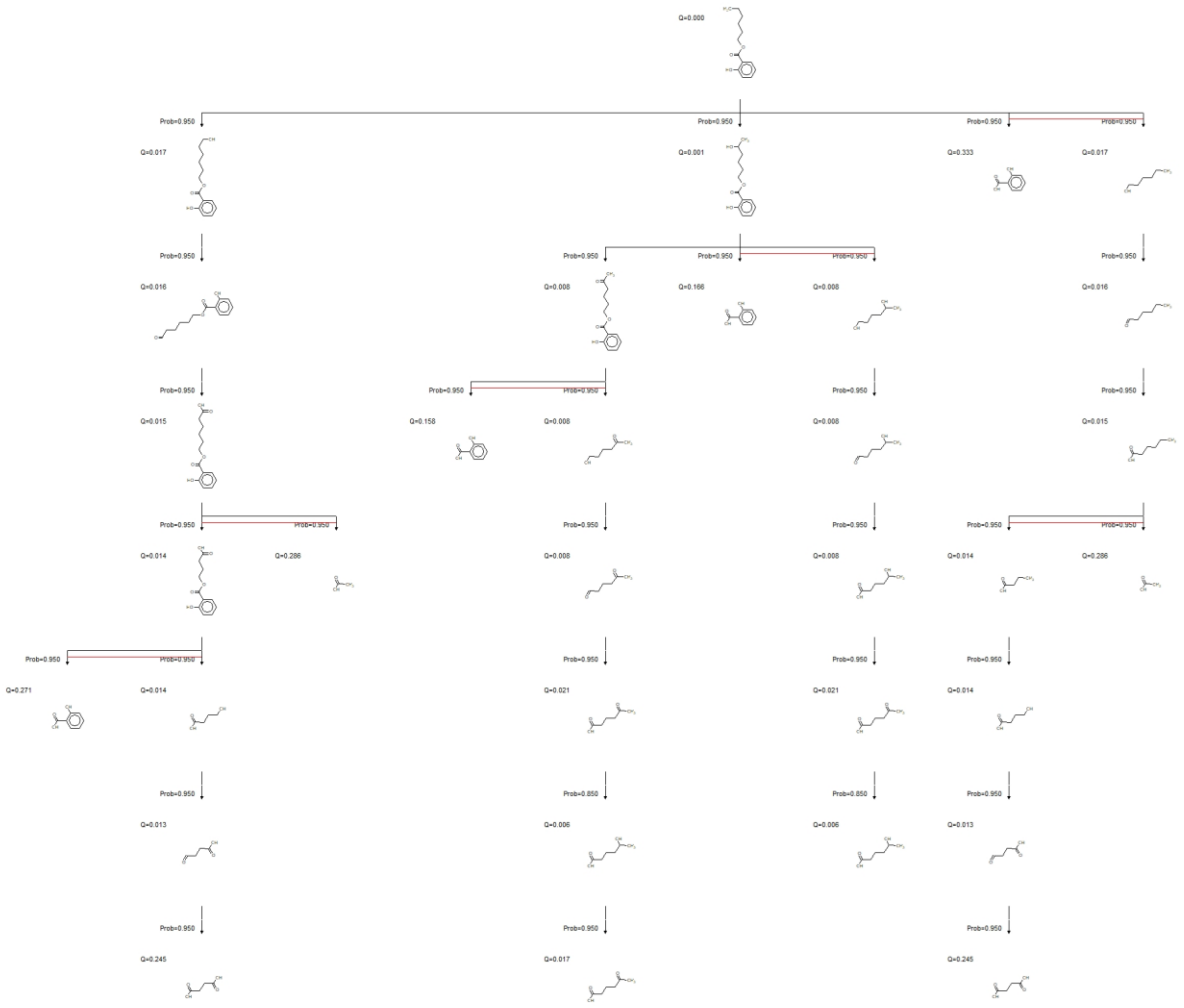
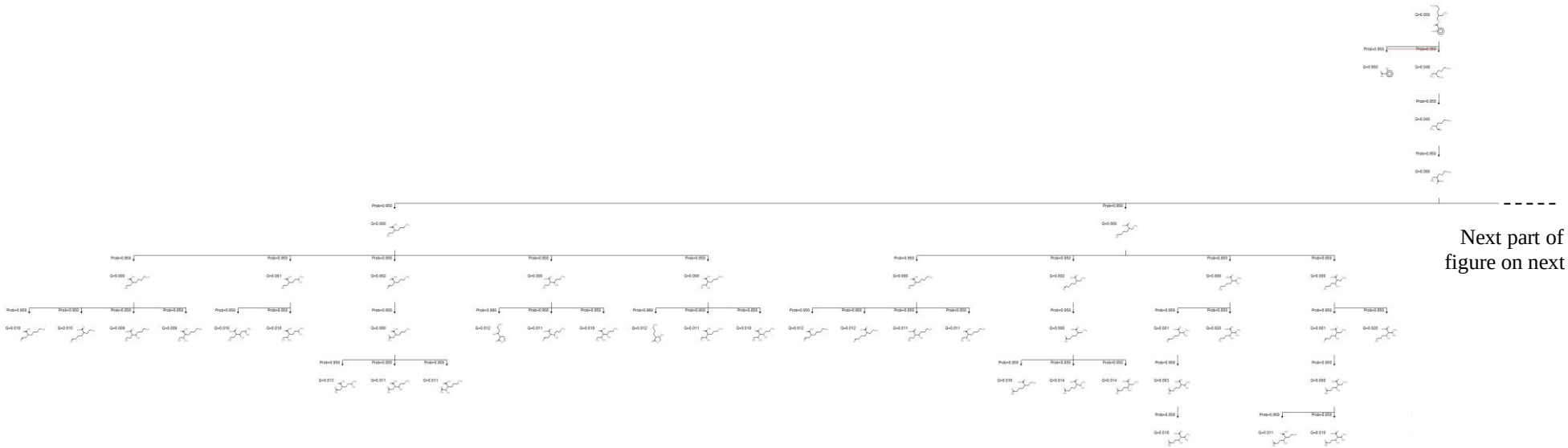


Figure 6: TIMES_In vivo rat_Phase I only_HS



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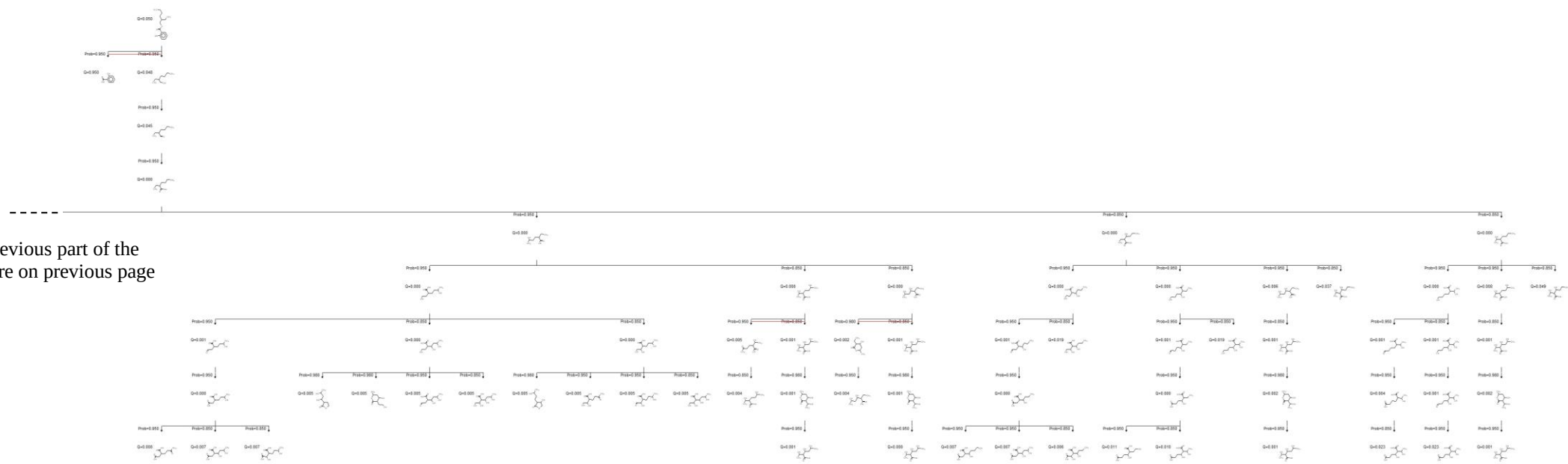

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Figure 7: TIMES_In vivo rat_Phase I only_EHS

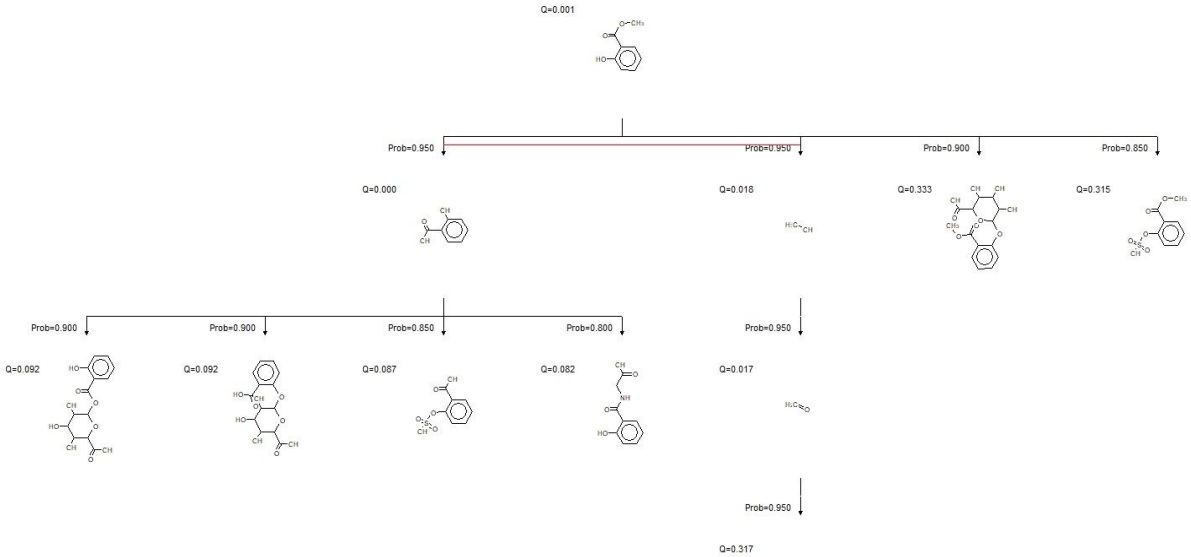


Figure 8: TIMES_In vivo rat_Phase I and Phase II_MS

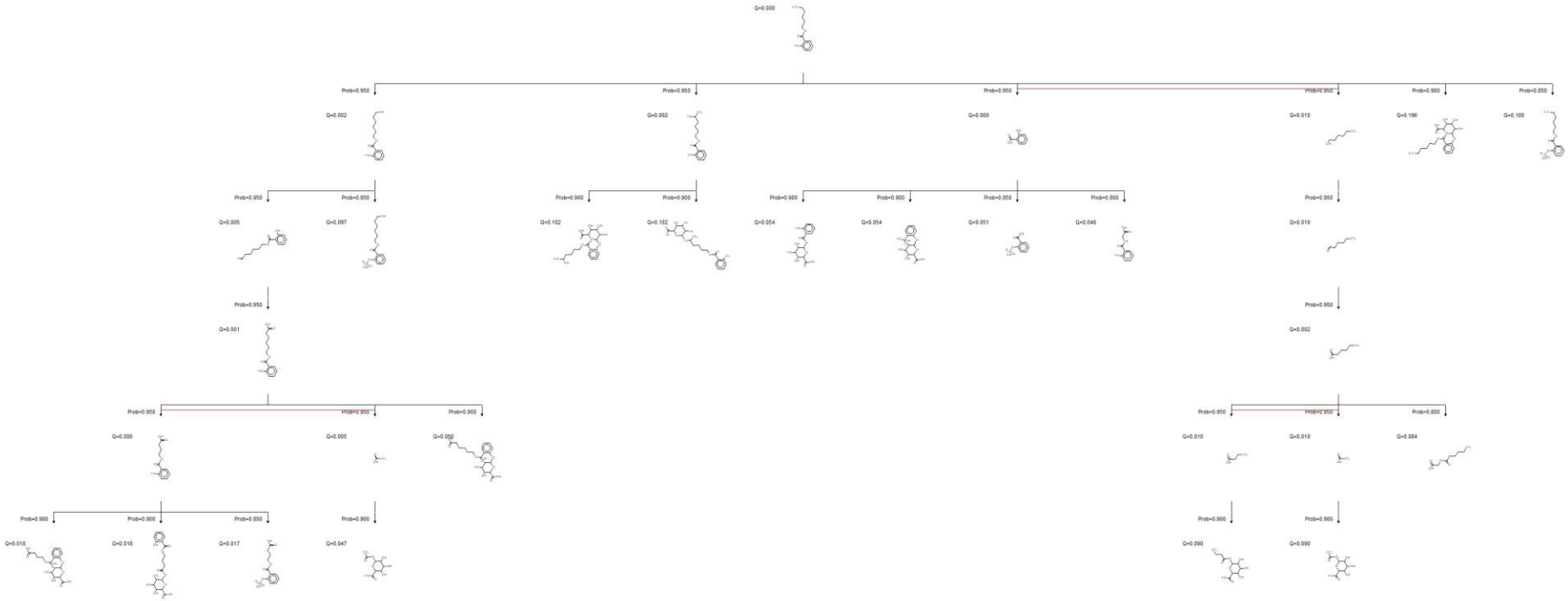


Figure 9: TIMES_In vivo rat_Phase I and Phase II_HS

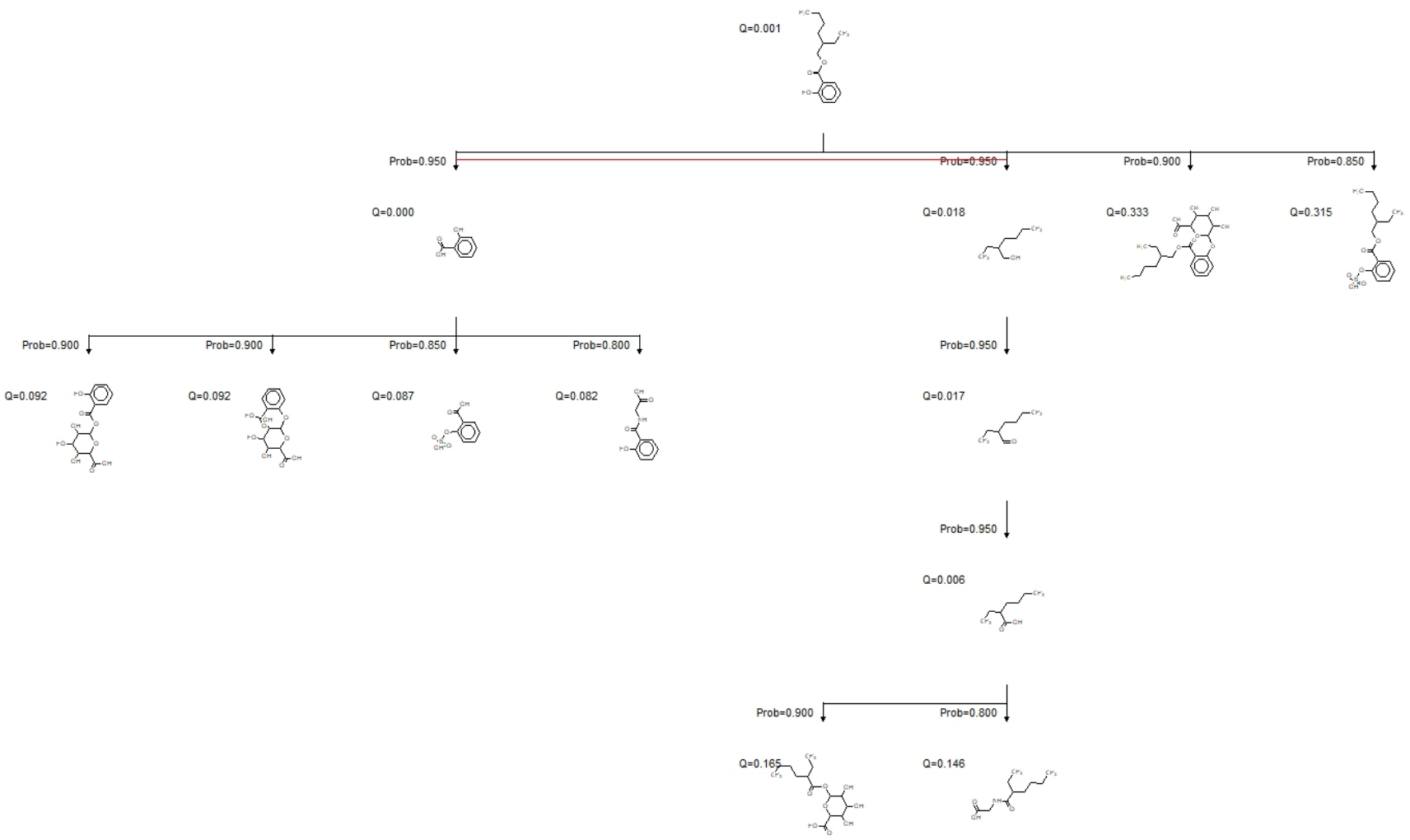


Figure 10: TIMES_In vivo rat_Phase I and Phase II_EHS

Discussion:

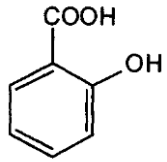
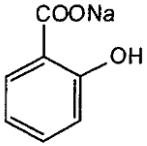
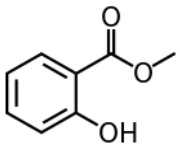
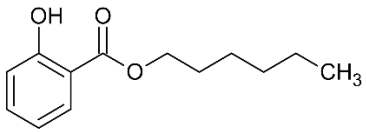
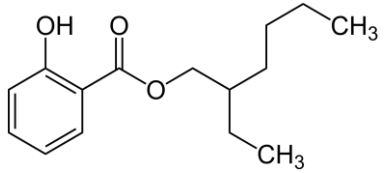
As in Meteor, TIMES predicts with high probability **the phase I hydrolysis of MS, HS and EHS to salicylic acid**. Quantitative information predicts similar rate of hydrolysis (considering phase I). A similar profile is obtained *in vitro* for the three compounds with the hydrolysis to salicylic acid and respective alcohol with approximately 50% hydrolysis to salicylic acid.

As predicted by Meteor, *in vivo*, hydroxylation of the alkylated chain leads to additional metabolites not observed with MS. In contrast to Meteor, TIMES does not predict further hydrolysis of the hydroxylated metabolites of HS and EHS to salicylic acid.
Nevertheless, considering quantitative prediction in the prediction of both phase I and phase II metabolism, salicylic acid is still the major metabolite for MS, HS and EHS.

5 COMPARISON OF PHYSICOCHEMICAL AND TOXICOLOGICAL DATA OF HS, MeS, SA, NaS and EHS

In order to assess the relevance of this read-across, physicochemical and toxicological data of HS, MeS, SA, NaS and EHS, as additional structural analogue of HS, were compared in the table below.

Table 3: Comparative data on physicochemical parameters and human health endpoints (issued from the CLH report on hexyl salicylate with added information on ethylhexyl salicylate)

Endpoints	Salicylic acid	Sodium salicylate	Methyl salicylate	Hexyl salicylate	Ethylhexyl salicylate
CAS number	69-72-7	54-21-7	119-36-8	6259-76-3	118-60-5
Structure					
Classification	Acute Tox 4 – H302 Eye Dam. 1 – H318 Repr. 2 – H361d (ATP13)	No harmonized classification	Acute Tox 4 – H302 Repr. 2 – H361d Skin Sens. 1B – H317 Aquatic Chronic 3 – H312 (ATP17)	No harmonized classification	No harmonized classification
Water solubility	Slightly soluble in water (2.17 x 10^3 mg/L at 20°C) (Merck 2006)	Soluble in water 1.25 x 10^6 mg/L in water (Merck 2006)	0.67 x 10^3 mg/L in water at ambient T (FR Sev 2021)	2 mg/L at 23°C (NL Sev 2018)	0.074 mg/L at 20°C (registration dossier)
Log P_{ow}	2.26 (Hansch, Leo 1995)	No data	2.55 (FR Sev 2021)	5.5 (NL Sev 2018)	5.94 (registration dossier)
Vapour pressure	8.2.10^{-5} mmHg at 25°C (Daubert, Danner 1989) soit 1.1 x 10^{-2} Pa at 25°C	No data	10 Pa at 22°C 100 Pa at 51°C (FR Sev 2021)	7.7 x 10^{-2} Pa at 23°C (NL Sev 2018)	0.018 Pa at 20°C
ADME	- Absorption: rapid by oral route - Distribution: distributed to several organs - Metabolism: 2 major urinary metabolites, salicyluric acid and salicyl-glucuronic acid found in rats; also metabolism in a small proportion to oxidative metabolites (2,3- and 2,5-dihydroxybenzoic acid) found in	- Absorption: rapid by oral route in rats. - Distribution: data from structurally-related salicylates (MeS) indicate wide distribution via blood and no bioaccumulation is expected after oral and dermal exposure. - Metabolism: rapid hydrolysis to free salicylate in rats.	- Absorption: well absorbed by oral route; oral bioavailability of 100% is assumed; very different values from 1 to 93% for dermal route; no data for inhalation exposure. - Distribution: widely distributed via blood and no bioaccumulation expected	- Absorption: no data for oral and inhalation route; expected to be poorly absorbed by inhalation route based on Log P and water solubility; data are contradictory for oral route; absorption varied from 0.8% to 7.8% for dermal route for concentrations between 100 and 0.1% HS. - Distribution: data from	- Absorption: well absorbed via the oral route (100% absorption assumed), low absorption via the dermal route in an *in vitro* study (3%); inhalation exposure is not relevant due to low vapour pressure. (registration dossier) - Distribution: data from structurally-related salicylates (MeS) indicate wide distribution via blood and no

	rats. - Elimination: these metabolites and free unchanged SA are almost exclusively excreted in the urine. (CLH report on salicylic acid 2014)	- Elimination: data from structurally-related salicylates (MeS) indicate main and rapid excretion in the urine. (CLP report on salicylic acid 2014)	after oral and dermal administrations. - Metabolism: rapid and extensive hydrolysis to SA and methanol. After oral administration, 80% of MeS were hydrolysed in 90 minutes in humans; in dogs, hydrolysis is 95% complete in 1h and in rats, MeS is completely hydrolysed to free salicylate within 20 min. After dermal administration, free salicylate rapidly appears in blood and level of unhydrolysed MeS is low. SA obtained is then conjugated with either glycine or glucuronide and excreted in the urine as salicyluric acid and acyl and phenolic glucuronides. Methanol is metabolized to corresponding aldehyde and acid and ultimately to CO_2. (CLH report on methyl salicylate 2018) QSAR modelling with Meteor and TIMES predicted hydrolysis of MeS (50% in vitro) to SA and methanol (ECHA 2021) - Elimination: mainly and rapidly in the urine after oral and dermal administration; low level in the faeces. (CLH report on methyl salicylate	structurally-related salicylates (MeS) indicate wide distribution via blood and no bioaccumulation is expected after oral and dermal exposure. - Metabolism: metabolism to SA by human skin esterases in an *in vitro* dermal absorption test; the QSAR Toolbox predicted the metabolites SA, hexanol, hexanal and hexanoic acid. (CLH report on hexyl salicylate 2020) QSAR modelling with Meteor and TIMES predicted hydrolysis of HS (50% in vitro) to SA and hexanol, hydroxylation of the alkyl chain at different sites leading to different metabolites that may be further biotransformed to SA and the corresponding alcohol (ECHA 2021). - Elimination: data from structurally-related salicylates (MeS) indicate main and rapid excretion in the urine. (CLH report on hexyl salicylate 2020)	bioaccumulation is expected after oral and dermal exposure. - Metabolism: unchanged EHS in traces (t_R = 16.6 min) and metabolism to hydroxyl-EHS (5OH-EHS) (t_R = 12.5 min), 5oxo-EHS (t_R = 12.9 min), carboxylheptyl salicylate (cx-EHS) (t_R = 12.1 min), dinor EHS carboxylic acid metabolite, SA (t_R = 9.6 min), salicyluric acid (t_R = 8.4 min) in humans after oral exposure (Bury et al. 2019); also metabolism to 2-ethylhexanol (registration dossier). QSAR modelling with Meteor and TIMES predicted hydrolysis of EHS (50% in vitro) to SA and 2-ethyl-1-hexanol, hydroxylation of the alkyl chain at different sites leading to different metabolites that may be further biotransformed to SA and the corresponding alcohol (ECHA 2021). - Elimination: fast excretion in the urine (peak urinary concentrations of 5OH-EHS, 5oxo-EHS and cx-EHS were found 1.6-2.6h after dose and >95% of the total amounts were excreted within 24h); it is expected that the major share of EHS dose was eliminated via urine as SA and SUA. (Bury et al. 2019)

			2018)		
Acute toxicity	Classified as Acute Tox 4 - H302 LD_{50} oral = 400-3700 mg/kg LD_{50} dermal > 2000 mg/kg bw	LD_{50} oral = 930-1200 mg/kg LD_{50} dermal > 2000 mg/kg bw	Classified as Acute Tox 4 – H302 ATE = 580 mg/kg bw LD_{50} dermal > 2000 mg/kg bw	LD_{50} oral and dermal > 5000 mg/kg bw	LD_{50} oral and dermal (rat) > 5000 mg/kg bw
	Acute oral toxicity of salicylates is moderate, with toxicity generally decreasing with increasing size of the ester R-group. Likely related to the relative proportion of the molecular weight release as SA followed hydrolysis. Methanol is of higher toxicity than the other alcohol metabolites and this is likely to explain the higher acute toxicity of methyl salicylate compared to the other salicylates.				
Irritation	Moderately to minimally irritant in solutions to animal skin. Mild transient irritant to human skin in formulation. Dermal irritation in repeated-dose toxicity studies (CIR, 2003). No dermal irritation (registration data)	No dermal irritation (registration data)	No dermal irritation (FR SeV 2021)	The mean scores for skin irritation do not trigger classification as a skin irritant (registration data)	No dermal irritation (registration data)
	Classified as Eye Dam. 1	Mildly irritation to eye (registration data)	Eye Dam 1 required based on *in vitro* study (FR SeV 2021)	No eye irritation (registration data)	Slightly irritant to eye (registration data)
Sensitisation	One LLNA positive (CIR, 2003). Contradictory results in LLNA, negative in a MEST and QSAR model predict no sensitisation potential of SA (registration data)	QSAR model predict no sensitisation potential of NaS. Only 1 positive reaction among 31 patients (registration data)	Classified as Skin Sens. 1B	One LLNA positive (registration data) Modified Draize test positive (Lapczynski *et al*. (2007)) Maximisation assay negative (Lapczynski *et al*. (2007)) Low incidence of reactions in humans. Classification proposed by France	Not sensitizing (registration data)
Repeated-dose toxicity	No target organ reported (registration data), bones (Abbott, 1978)	Target organs: kidney and liver (registration data); bones (Abbott, 1978)	Target organs: bone and liver NOAELs of 50 mg/kg bw/day based on 2-year studies in rats and dogs (FR SeV 2021)	No data available for oral route	No particular target organ reported in a OECD 421 study at doses up to 250 mg/kg bw/day (registration data)
Mutagenicity	Not mutagenic in bacteria and in	Negative in Ames test and DNA	Negative in bacteria.	Negative in Ames test	Negative in bacteria and mammalian

	mammalian cells. Contradictory data for chromosome aberrations (SCCNFP, 2002).	cell-binding assay using Ehrlich ascites cells (CIR, 2003)	Contradictory results for chromosomal aberrations (FDA, 2006; FR SeV 2021).	(registration data)	cells (registration data)
	Negative *in vivo* in sister chromatid exchange assay and in chromosome aberration assay (Giri, 1996)	Contradictory data for clastogenic effects *in vivo* (Giri, 1996)	Negative in a micronucleus assay (FDA, 2006; FR SeV 2021)	No data available	No data available
Carcinogenicity	Not carcinogenic in rats by oral route (CIR, 2003).	No *in vivo* data (CIR, 2003)	Even if not fully adequate carcinogenicity study conducted according to current test guidelines is available, no carcinogenic concern has been raised for methyl salicylate (FR SeV 2021)	No data available	Based on negative genotoxicity data derived for EHS and supportive information from a 2-year carcinogenicity study with MeS as read-across substance, carcinogenic properties are not expected (registration data).
Toxicity on reproduction	No adequate study on fertility. Inhibition of human sperm mobility *in vitro* (CIR, 2003). Increased mean gestation period after treatment on GD20 & 21 in rodents (CIR, 2003).	No adequate study on fertility. Increased duration of gestation (CIR, 2003)	No effect on fertility (FDA, 2006; FR SeV 2021)	No data available	No effect on fertility (registration data)
Toxicity for the development	Foetal death, growth retardation and malformations (kidney and skeletal) in rats. Classified as Repr. 2 – H361 based on experimental studies with salicylic acid, methyl salicylate, sodium salicylate and acetylsalicylic acid and on human data with acetylsalicylic acid.	Foetal death, growth retardation and malformations (mainly skeletal) in prenatal toxicity study in rats.	Lethality, external malformations, visceral/skeletal anomalies and growth retardation in rats (registration data; FDA (2006); FR SeV 2021). The lowest NOAEL for developmental toxicity can be set at < 60 mg/kg bw/day (but > 20 mg/kg bw/day) based on skeletal variations.	No data available	Increased post-implantation loss, reduction in gestation index and lower litter size in an OECD 421 study (registration data). LOAEL set by the registrants: 80 mg/kg bw/day and NOAEL: 25 mg/kg bw/day.

			Classified as Repr. 2 based on a read-across with SA.		
	When various salicylates were administered to rats twice on GD9 (subcutaneous route) (Koshakji, 1973), different developmental toxicity profiles were observed depending on the salicylate tested. When comparing molecular structure and developmental findings, it appears that COOH and OH must be adjacent for inducing teratogenic effects. It also appears that substitution of OH group by SH or NH_2, substitution of COOH for $CONH_2$ or addition of OH groups to SA eliminates teratogenic properties.				

6 OVERALL RELEVANCE OF THE PROVIDED INFORMATION

Taking into account the overall information provided above, some arguments are given below in order to answer the different doubts expressed by the RAC rapporteur and members about the adequacy of the read-across during the RAC-59 Working Group (WG) on CLH for HS.

- **Argument 1: The *in vitro* dermal absorption test was poorly described.**

France examined the study report of the *in vitro* dermal absorption test as requested during the RAC WG. Details are provided in Annexes. No deviations were noticed compared to the OECD guideline 428.

- **Argument 2: The *in vitro* dermal absorption test refers exclusively to metabolism of HS after dermal application whereas the studies investigating reproductive toxicity with MeS, NaS and SA and showing developmental effects were conducted after oral or subcutaneous administration. Besides, no quantitative data *in vivo* has been provided to support the alleged common metabolism of MeS/NaS and HS.**

France acknowledges that there is no toxicokinetics data with HS by oral route. It should be noted that this is not a REACH requirement. Therefore, this type of information is rarely available in Reach registration dossier.

In the framework of Substance Evaluation, the NL requested the *in vitro* dermal absorption assay examined in the CLH report and the present document. No additional information was considered necessary by NL regarding toxicokinetics of HS.

- **Argument 3: The paper from Belsito *et al.* (2007) (RIFM) indicating that 17 salicylates including HS are expected to undergo extensive hydrolysis, primarily in the liver, to SA, was only mentioned in the CLH report without any details. RAC members highlighted that metabolism of HS to SA is only possible but without a quantitative indication of probability.**

When contacted by France, the RIFM informed that they do not have unpublished data on toxicokinetics on salicylates.

- **Argument 4: Two metabolic pathways (glucuronidation and hydrolysis) were identified by the ECHA team and the proportion of each was questioned by the RAC.**

The Meteor report from the ECHA confirmed that a similar profile is obtained *in vitro* for MS and HS for the hydrolysis to SA and respective alcohol with approximately 50% hydrolysis to SA. *In vivo*, SA is still the major metabolite for both MS and HS but with different rates of hydrolysis.

Thus, there is no ground to consider that hydrolysis of HS would be less likely than hydrolysis of MS. Moreover, it can be expected that kinetics of hydrolysis would be

faster than the one of glucuronidation due to steric hindrance. Indeed, the alkyl chain of HS implies a steric hindrance of the molecule that does not facilitate glucuronic acid, which is a voluminous molecule, to react on the reaction site on HS. Thus, this may not be in favour of glucuronidation. On the contrary, H_20 is a small molecule that can easily reach the reaction site on HS to hydrolyse it. Besides, it can be anticipated that commercial aqueous solution of HS could already contains SA and thus humans could also be exposed to SA when handled HS products.

- **Argument 5: Overall, even if it can be concluded that a part of HS would be metabolized into SA, it is unknown if the quantity of SA produced will be sufficient to cause developmental toxicity.**

Some difference in toxicokinetics (in particular in terms of absorption) may be anticipated between HS and MeS based on physico-chemical properties (i.e. HS is less soluble than MeS and more hydrophobic). However, it is very difficult to predict the metabolic changes a substance may undergo on the basis of physico-chemical information alone.

Even if the quantity of SA that will be formed *in vivo* from HS is unknown, there is evidence that SA will be formed when exposed to HS. As the CLP regulation is based on the assessment of hazards and not risks, the notion of quantity is not the major issue here because it refers to the notion of exposure. Moreover, developmental effects caused by MeS and EHS appear at relatively low doses. Thus, even if SA might be generated at lower concentrations after HS metabolism, effects at relevant and not too high doses can be expected.

The relevance of read-across for HS with MeS, NaS and SA was also supported by previous assessments: one made by the registrant in its registration dossier, one made by the NL in the context of SeV process and one made by ECHA in the context of a group RMOA for various salicylate esters. Both have concluded that read-across is appropriate and that HS should be classified Repr. 2 H361d (Suspected of damaging the unborn child) based on the common metabolite, SA, which is responsible for developmental toxicity.

Finally, a literature search was also performed to identify data with another analogous substance, EHS, that can support the read-across. This analogue is particularly structurally close to HS because it also contains a long alkyl chain. Besides, these 2 molecules share a same steric hindrance. Additionally, HS and EHS share similar physicochemical properties: log Pow of 5.5 and 5.94, water solubility of 2 mg/L and 0.074 mg/L, respectively for HS and EHS, and vapour pressure of the same order of magnitude for both molecules (10^{-2} Pa). The information below can be taken into account in a weight of evidence to conclude on the relevance of the read-across:

- Metabolism of EHS to SA was shown in humans after oral exposure (*Bury et al.* 2019).

- Increased post-implantation loss, reduction in gestation index and lower litter size are reported from the tested dose of 80 mg/kg bw/day EHS in an OECD 421 study.

These effects are consistent with those found with MeS. According to the CLH report on MeS:
Developmental effects, characterized by lethality, are also consistently reported in fertility studies in both mice and rats:

- *decreases in litter size, number of liveborn progeny per female, viability (liveborn), survival (survivors on day 4) and weaning survival at 150 mg/kg bw/day in the Collins et al. (1971) study in rats;*
- *higher number of deaths between birth and day 5 at 250 mg/kg bw/day in the Anonymous (1978a) study in rats;*
- *"slightly smaller litter size" from 375 mg/kg bw/day at birth in the Anonymous (1978b) study in rats;*
- *reduced pup viability, decrease in the mean number of litter, in the average of pups per litter and the proportion of pups born alive at 500 mg/kg bw/day in the NTP (1984b) study in mice.*

Finally, a QSAR modelling was also performed on EHS in addition to the ones performed on MS and HS. It confirmed that a similar profile is obtained *in vitro* for MS, HS and EHS for the hydrolysis to SA and respective alcohol with approximately 50% hydrolysis to SA. *In vivo*, SA is still the major metabolite for the three compounds but with different rates of hydrolysis.

7 CONCLUSION ON CLASSIFICATION AND LABELLING FOR REPRODUCTIVE TOXICITY

Based on all data described in the present document, France confirms its position and considers that the read-across proposed for HS is adequate:

- Physical-chemical parameters are coherent to consider these salicylates into a family approach.
- The *in vitro* dermal absorption study and the QSAR estimations all predict that HS is metabolised into SA.
- Developmental toxicity data point into a similar toxicity among the substances of this family.
- The read-across has been acknowledged by all the entities (ECHA, NL, FR, registrant) working on the substance(s).

Therefore, considering the RAC opinions for MeS and SA as Repr. 2 for development, HS should also be classified as Repr. 2 – H361d.

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9 ANNEXES

9.1 DETAILED SUMMARY ON THE IN VITRO DERMAL ABSORPTION TEST ON HS

Confidential annex to this document (separate document)

9.2 DETAILED STUDY SUMMARY ON TOXICOKINETICS OF EHS

Study reference:

Bury D., Griem P., Wildemann T., Brüning T., Koch H. Urinary metabolites of the UV filter 2-ethylhexyl salicylate as biomarkers of exposure in humans. Toxicology Letters. 2019

- Test Substance: 2-ethylhexyl salicylate (EHS)
  - EC number: 204-263-4
  - CAS number: 118-60-5
  - Degree of purity: 99.7%
  - Impurities: not indicated

- Method
  - Three healthy male volunteers (age: 28–32 years; body weight: 75–93 kg) each received a single oral dose of approximately 5 mg EHS (weighed exactly), corresponding to 57.4–75.5 µg/(kg body weight). The EHS dose was provided dissolved in 1 mL ethanol diluted with 9 mL water in a chocolate coated waffle cup. The applied doses were more than a factor of 1000 below the no observed adverse effect level (NOAEL) of a short-term (male rats: 28 days, female rats: approx. 7 weeks) repeated dose toxicity study performed in rats (NOAEL: 250 mg/(kg body weight * d) and 80 mg/(kg body weight * d) for male and female rats, respectively).
    For one week prior to the study, the volunteers abstained from using any products containing EHS to avoid interfering exposures. Urine samples were collected in 250 mL polyethylene (PE) containers immediately before dose (t_0) and consecutively and completely for 48 h after the dose, and stored at -20°C until analysis. Times of urine collection were recorded by the volunteers. Urine volumes were determined via the weight difference between filled and empty PE containers and urinary creatinine was determined according to Jaffe (1886) by contract analysis (L.u.P. GmbH Labor und Praxisservice; Bochum, Germany).
    The identification of EHS metabolites was performed using a triplequadrupole-MS suspect screening approach previously

applied to the UV filter octocrylene by the same group (Bury et al., 2019b). All urine samples from one volunteer were prepared by enzymatic deglucuronidation as described in Bury et al. (2019a), however with omission of internal standards (which were not yet available at that time). These processed urine samples were then analyzed with online-SPE-LC–MS/MS.

5OH-EHS, 5oxo-EHS, and 5cx-EPS were quantified by stable isotope dilution analysis using an online-SPE-LC–MS/MS method, recently published by the group. In short, after addition of a pure β-glucuronidase from E. coli K12, buffer, and stable isotope labelled internal standards, urine samples were incubated at 37 °C for 3 h for the cleavage of glucuronic acid conjugates. Then, samples were frozen overnight, thawed, equilibrated to ambient temperature and centrifuged at 1900 g for 10 min. 100 µL of the supernatant were analyzed. The limits of quantification (LOQ) were 0.05 µg/L (5OH-EHS), 0.15 µg/L (5oxo-EHS), and 0.01 µg/L (5cx-EPS).

Elimination half-lives were calculated using the equation $t_{1/2}$ = ln(2)/k, with k being the kinetic constant of the exponential decline in excreted metabolite amounts. k was obtained by exponential regression of the metabolite excretion rates (ER in µg/h) vs. the midpoint of each time interval (t in h) (described by the mathematical expression $ER(t) = ER_{max} * e^{-kt}$, with ER_{max} the peak metabolite excretion rate) using Microsoft Excel 2010. The calculation of urinary excretion fractions (including background correction) and daily intakes is described in the Supplementary Material of the paper.

- Test Type
  - In vivo human data

- Results

Only relevant results for the purpose of the present document are described below.

  - In addition to the 3 EHS specific metabolites (5OH-EHS, 5oxo-EHS, and 5cx-EPS) that were identified and quantitatively investigated, salicylic acid (SA), as ester cleavage product, and its follow-up metabolite salicyluric acid (SUA) were found at t_R = 9.6 and 8.4 min, respectively, with rather high peak heights at least for SUA, indicating its presence at rather high concentrations.

    The elution order of the (in part tentatively) identified EHS metabolites was plausible with the most polar metabolite SUA eluting first, followed by SA, 5cx-EPS, tentative 2cx-MHS, 5OH-EHS, the second tentative OH-EHS isomer, and 5oxo-EHS. The identities of 5OH-EHS, 5oxo-EHS, 5cx-EPS, SA, and SUA were confirmed using analytical standards.

    The authors indicated that it could be expected that the major share of EHS dose was eliminated via urine as the non-specific

metabolites SA and SUA. Moreover, they concluded that, as urinary excretion fractions were rather low for 5OH-EHS, 5oxo-EHS, and 5cx-EPS, the total breakdown to SA was likely the predominant metabolic pathway.

9.3 DETAILED STUDY SUMMARY ON TOXICITY TO REPRODUCTION (DEVELOPMENT) OF EHS

Study reference:
Study report, 2012. Registration dossier of EHS (ECHA 2021).

- Materials and methods
    - 3.2.1 Reproduction/Developmental Toxicity Screening Test (OECD 421)

- Test substance: 2-ethylhexyl salicylate
    - EC number: 204-263-4
    - CAS number: 118-60-5
    - Degree of purity: not indicated
    - Impurities: not indicated

- Test animals
    - Species/strain/sex: rat/RccHanTM: WIST(SPF)/male and female
    - Number of animals per sex per dose: 11
    - Age and weight of animals at the study initiation: 11 weeks at start of treatment; Males: 312 to 351 g Females: 208 to 244 g at start of treatment

- Administration/exposure
    - Route of administration – oral (gavage)
    - Doses/concentration levels, rationale for dose level selection: 0, 25, 80 and 250 mg/kg/day, the dose levels were selected based on a previous dose range-finding toxicity study in Han Wistar rats, Harlan Laboratories Study D54872, using dose levels of 0, 100, 300 and 1000 mg/kg/day, resulting in mortality and adverse toxic effects at the dose level of 1000 mg/kg bw/day and adverse toxic effects at the dose level of 300 mg/kg bw/day.
    - Duration and frequency of test/exposure period: 28 days for males and approximately 7 weeks for females; treatment was administered once daily.
    - Control group and treatment : yes with concurrent vehicle (corn oil)
    - Post exposure observation period: not indicated
    - Vehicle: identification, concentration and volume used, justification of choice of vehicle (if other than water): corn oil from Carl Roth GmbH; batch number: 292189296; expiry date (retest date): 02-Aug-2017; storage conditions: room

temperature (20 ± 5 °C); dose volume: 4 mL/kg bw; dose concentrations: 0.00, 6.25, 20.00 and 62.50 mg/mL; no justification of choice of vehicle

- Test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation: Animals were treated with Neo Heliopan® OS which is an oil-soluble UVB absorber containing EHS. Diet: Pelleted standard Harlan Teklad 2018C (batch no. 43/12) rodent maintenance diet (Provimi Kliba SA, 4303 Kaiseraugst / Switzerland) was available ad libitum. Water: Community tap-water from Füllinsdorf was available ad libitum in water bottles. On the first treatment day, samples from the control group as well as three samples (top, middle and bottom) of about 1 g of each concentration were taken prior to dosing for analysis of concentration and homogeneity. To confirm the stability (8 days), samples of about 1 g of each concentration were taken from the middle of each aliquot used on day 7 of the treatment. During the last week of the treatment, samples were taken from the middle to confirm concentration. The aliquots for analysis of dose formulations were frozen (-20 ± 5 °C) and delivered on dry ice to B. Bürkle (Harlan Laboratories Ltd., Zelgliweg 1, 4452 Itingen / Switzerland) and stored there at -20 ± 5 °C until analysis.

  The samples were analyzed by GC coupled to an FID detector following an analytical procedure provided by the Sponsor and adapted at Harlan Laboratories. The test item was used as the analytical standard. Analyzed samples were not discarded without written consent from the study director.

  In conclusion, the results indicate the accurate use of the test item and corn oil as vehicle during this study. Application formulations were found to be homogeneously prepared and sufficient formulation stability under storage conditions was approved.
- Actual doses (mg/kg bw/day) and conversion factor from diet/drinking water test substance concentration (ppm) to the actual dose, if applicable: not indicated

- Statistical methods: The following statistical methods were used to analyze food consumption, body weights and reproduction data:
  - Means and standard deviations of various data were calculated.
  - The Dunnett-test (many to one t-test) based on a pooled variance estimate was applied if the variables could be assumed to follow a normal distribution for the comparison of the treated groups and the control groups for each sex.
  - The Steel-test (many-one rank test) was applied instead of the Dunnett-test when the data could not be assumed to follow a normal distribution.
  - Fisher's exact-test was applied if the variables could be dichotomized without loss of information.

- Results
  - Maternal toxic effects: slight but non-significant changes on weight gain at 250 mg/kg bw/d; at this dose, one female was found dead on day 23 of the gestation period which was considered to be a result of birth complications
    LOAEL (maternal toxicity) = 250 mg/kg bw/d
  - Embryonic/teratogenic effects: reduction in gestation index (number of females with living pups as a percentage of females pregnant), increase in incidence of post-implantation loss resulting in a lower litter size and prolonged gestation period at 80 and 250 mg/kg bw/d. These findings were considered to be test item-related adverse effects and reduction in gestation index and increase in incidence of post-implantation loss were statistically significant and dose dependent effects. Based on the individual data, increased post-implantation loss occurred predominantly in females with prolonged gestation. Reduction in absolute body weights of pups was observed at 250 mg/kg bw/d and was considered to be test item-related adverse effect.
    Based on the observation of increased post-implantation loss, reduction in gestation index and lower litter size, the LOAEL for developmental toxicity is 80 mg/kg bw/d and the NOAEL is 25 mg/kg bw/d.