Comparative Effects of Di-(2-ethylhexyl)phthalate and Di-(2-ethylhexyl)terephthalate Metabolites on Thyroid Receptors: In Vitro and In Silico Studies

Nicolas Kambia, Isabelle Séverin, Amaury Farce, Laurence Dahbi, Thierry Dine, Emmanuel Moreau, Valérie Sautou, Marie-Christine Chagnon

Genomic effects on thyroid receptors of di-(2-ethylhexyl)terephthalate metabolites, a di-(2-ethylhexyl)phthalate substitute in medical devices: *in silico and in vitro* studies

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ABSTRACT

Background: Plasticizers added to polyvinylchloride used in medical devices can be released into patients' biological fluids. Di-(2-ethylhexyl)phthalate (**DEHP**), a well-known reprotoxic and endocrine disruptor, must be replaced by alternative compounds. Di-(2-ethylhexyl) terephthalate (**DEHT**) is an interesting alternative candidate due to its lower migration from PVC and its lack of reprotoxicity. However, there is still a lack of data to support the safety of its human metabolites with regard to their hormonal properties on the thyroid system.

Objectives: in order to evaluate the effects of **DEHT** metabolites on thyroid/hormone receptors, they were first synthesized and then compared *in vitro* and *in silico* to the effects of **DEHP** metabolites, as both plasticizers are highly metabolized.

Methods: **DEHP** and **DEHT** monoesters and oxidized compounds were first synthesized and then investigated using T-screen assays to assess interference with triiodothyronine (T3). Docking studies were also performed to determine the potential interactions of the different metabolites with the TRα and TRβ receptors.

Results: The oxidized metabolites of **DEHT** have no effect on T3 receptors whereas 5-hydroxy-mono-(ethylhexyl)phthalate (5-OH **MEHP**) appears to be primarily an agonist for TRβ at a concentration above 0.2 µg/mL. A synergistic effect with T3 was also observed. Mono-(ethylhexyl) phthalate (**MEHP**) and mono-(ethylhexyl) terephthalate (**MEHT**) were also active on T3 receptors. *In vitro,* **MEHP** was a partial agonist at concentrations between 10 µg/mL and 20 µg/mL. In contrast, **MEHT** was an antagonistic at non-cytotoxic concentrations (2-5 µg/mL) in a concentration-dependent manner. The results obtained with docking are consistent with those of the T-screen and provide additional information on the preferential affinity of monoesters and 5-OH-**MEHP** for TRβ.
Conclusion: This study highlights a lack of interactions by oxidized metabolites on thyroid receptors, confirming the interest of **DEHT** as an alternative to **DEHP**.

KEYWORDS: **DEHT**, *in silico*, T-screen assay, hormonal activities, thyroid receptors,

INTRODUCTION

Polyvinyl chloride (PVC) is a material widely used in medical devices (MDs), including infusion sets or lines, feeding tubes and tubing, umbilical catheters, oxygen masks, endotracheal tubes, blood transfusors and bags or extracorporeal circuits. Plasticizers are added to the polymer to improve the flexibility and softness of the PVC. However, since they are not covalently bonded to the PVC matrix, they can easily migrate from the MD and come into contact with patients during medical procedures (*SCENIHR, 2016, Bernard 2015*). Neonates in intensive care units are known to be exposed to one of these plasticizers, di-(2-ethylhexyl) phthalate (**DEHP**) present in many MDs (*Mallow et al., 2014; Fischer et al., 2013; Stroustrup et al., 2020*). This phthalate is now classified as a CMR1B (Cancerigen Mutagen or Reprotoxic) substance under the Classification Labeling and Packaging (CLP) Regulation *(Regulation EU, 1278/2002)* due to its effects on reproduction and fertility. The use of **DEHP** in PVC MDs has been called into question by the European authorities and has been restricted for several years. It must now not exceed 0.1% by mass of the plasticized material, as defined by European regulation n°2017/745 on MDs (*Regulation EU 2017/745).* Other plasticizers, such as di-(2-ethylhexyl)terephthalate (**DEHT**), were proposed to replace **DEHP** to soften the PVC in MDs *(SCENIHR, 2016).* This additive is particularly interesting because less is released from the PVC MDs than **DEHP** (*Bernard et al. 2015*) and therefore

there is less exposure. **DEHT** is less active than **DEHP** in inducing peroxisome proliferation in rats, which can be explained by the small amount of monoester produced during **DEHT** metabolism (*Barber et al., 1994*). **DEHT** is principally hydrolyzed to both terephthalic acid (TPA) and 2-ethylhexanol (EH), with both metabolites being rapidly removed *in vivo*. This extensive hydrolysis of **DEHT** to TPA and EH allows only a small fraction to be converted into the monoester and then ultimately to the corresponding oxidized metabolites *(CPSC, 2018)*. **MEHT** exhibits a lower cytotoxicity than **MEHP** and its cytotoxicity occurs (0.05 mg/mL) at a much higher concentration than those measured in body fluids (*Eljezi et al., 2017*). In animal studies, **DEHT** has shown no reprotoxic effects, a low developmental toxicity and no genotoxicity (*Faber et al., 2007*; *CPSC, 2018*). However, toxicity data are not complete as there is a lack of information regarding the hormonal activities of **DEHT** and/or its metabolites resulting from *in vivo* hydrolysis and oxidation. It is, however, very important to assess these activities on hormones since they can occur at very low doses and can have a significant impact on the development of children when they are exposed during critical periods of their development. In a previous study, we performed an *in vitro* investigation on the potential endocrine-disrupting effects of **DEHT** and its metabolites on estrogen and androgen receptors and on steroid synthesis. This study demonstrated that **DEHT** and its metabolites exhibit much weaker effects on hormonal activities than **DEHP**. However, special attention must be paid to the 5-hydroxy metabolite of mono-(ethylhexyl)terephthalate (5-OH-**MEHT**) due to co-stimulation of estrogen alpha and human androgen receptors and an increase in estrogen synthesis (*Kambia et al., 2019*). To date, data are lacking the effects of **DEHT** and its ultimate metabolites on thyroid hormonal activity. Many studies have demonstrated a correlation between the exposure to **DEHP** metabolites and thyroid function (*Kim et al., 2019; Villanger et al., 2020, Ghisari et al., 2019; Huang et al., 2017),* effects which may have an impact on neurodevelopment in children. Indeed, the development of the nervous system is extremely dependent on thyroid hormones during the *in utero* period and the first two years of life, with critical windows of vulnerability. (*Zoeller et al., 2004; Vulsma et al., 2000; De Cock et al., 2012; Qian et al., 2019*). Newborns and premature neonates hospitalized in intensive care units are particularly vulnerable. It is therefore very important to assess whether plasticizers from MDs, and the corresponding metabolites found in the body, affect the thyroid and the active concentrations.

The objective of this study was to use *in silico* and *in vitro* methodologies to assess the effects of **DEHT** metabolites on thyroid hormonal activities and to compare them with those of **DEHP,** as both plasticizers are highly metabolized.

MATERIALS AND METHODS

Metabolites of DEHP and DEHT

Primary and secondary metabolites of **DEHP** and **DEHT** were synthesized and characterized by the IMOST team (UMR 1240, INSERM) in Clermont-Ferrand, France. The compounds tested are shown Table 1. The purity of all our synthesized metabolites and their corresponding intermediates exceeded 95% (HPLC/MS and RMN). **MEHT** was synthesized according to the method described par Eljezi *et al.* (*Eljezi et al. 2017*). All synthesis processes are described in the supplementary data (appendix 1).

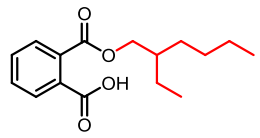
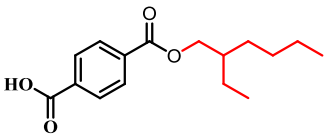
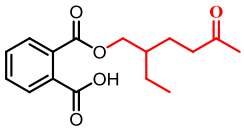
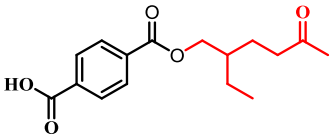
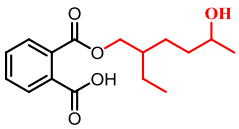
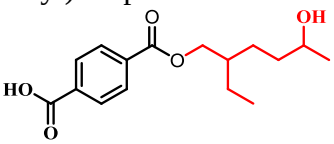
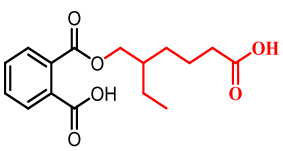
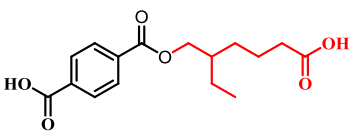
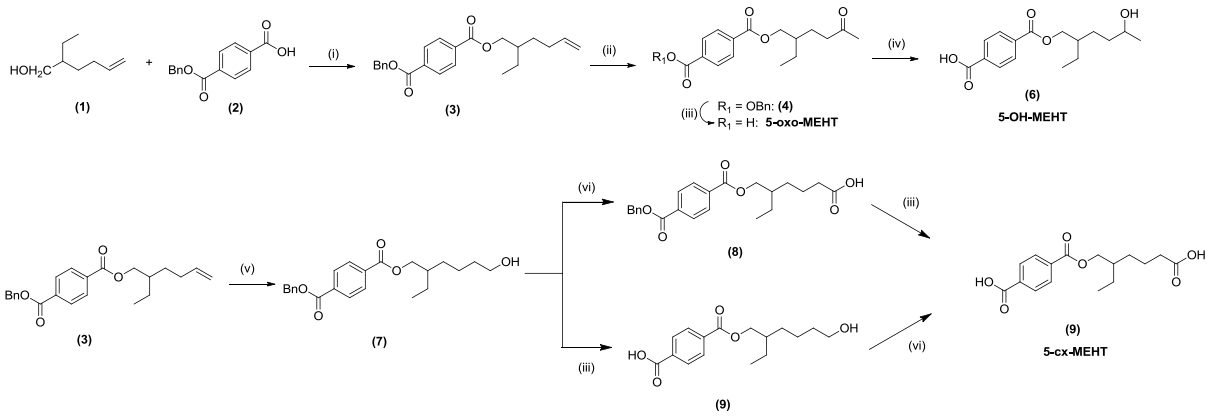
DEHP Metabolites	**DEHT Metabolites**
MEHP: mono-(2-ethylhexyl)phthalate 	**MEHT** or **MEHTP**: mono-(2-ethylhexyl)terephthalate 
5-oxo-MEHP or **MEOHP**: mono-(2-ethyl-5-oxohexyl)phthalate 	**5-oxo-MEHT** or **MEOHTP**: mono-(2-ethyl-5-oxohexyl)terephthalate 
5-OH-**MEHP** or **MEHHP**: mono-(2-ethyl-5-hydroxyhexyl)phthalate 	**5-OH-MEHT** or **MEHHTP**: mono-(2-ethyl-5-hydroxyhexyl)terephthalate 
5-cx-MEHP or **MECPP**: mono-(2-ethyl-5-carboxypentyl)phthalate 	**5-cx-MEHT** or **MECPTP**: mono-(2-ethyl-5-carboxypentyl)terephthalate 

Table 1: Structures and denominations of **DEHP** and **DEHT** metabolites

Access to the secondary metabolites 5-OH-MEHT, 5-oxo-MEHT and 5-cx-MEHT.

Metabolites **5-OH-MEHT, 5-oxo-MEHT** and **5-cx-MEHT** were synthesized from 2-ethylhex-5-en-1-ol **(1)** and 4-((benzyloxy)carbonyl)benzoic acid **(2)** previously synthesized and characterized (^{1}H, ^{13}C NMR and HRMS) by Nüti *et al*. and our lab INSERM U1240 *(Nüti et al., 2005; Eljezi et al., 2017*), respectively (Scheme 1). Derivatives **(4)** and **(7)** were obtained by Wacker oxidation of the vinylic group at ω-position of compound **(3)** in a mixture of $PdCl_2$ and parabenzoquinone or a hydroboration reaction, respectively. Compound **(4)** was then converted into **5-oxo-MEHT (5)** by hydrogenation, with the ketone group then being reduced with $NaBH_4$ to form **5-OH-MEHT (6)**. Finally, compound **(7)** was successively oxidized (Jones reagent) and reduced (Method A; or inverse for Method B) to give **5-cx-MEHT (9)**. The purity of all synthesized metabolites was over 95% (HPLC/MS).



Conditions and reactants: (i) DMAP, DCC, dichloromenthane, rt, overnight; (ii) $PdCl_2$; DMF/H_2O (17/3, v/v), rt, overnight; (iii) H_2, Pd/C 10%,absolute ethanol, 4 hours; (iv) $NaBH_4$, rt, overnight; (v) (a) B_2H_6, anhydrous THF, rt, 1 h; (b) NaOH (3M), then H_2O_2 30%, 50°C, 2 h; (vi) CrO_3/H_2SO_4, acetone; rt; 30 min

<u>Scheme 1:</u> Synthesis pathways of **5-oxo-MEHT**, **5-OH-MEHT** and **5-cx-MEHT**.

Preparation of samples

All compounds were dissolved in 100% ethanol and tested in a concentration range of 0.2 µg/mL to 20 µg/mL. The maximum concentration of ethanol in the culture medium was 0.1% in order to avoid any cytotoxic effect of the vehicle.

T-screen assay.

<u>Cell culture and treatment</u>

The assay is based on thyroid hormone dependent cell growth of the rat pituitary tumor cell line GH3 (ATCC, CCL-82.1). The GH3 cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 in DMEM/F12 (Gibco) supplemented with 10% fetal bovine serum

(PAN, Biotech). Passaging was carried out in 75 cm^2 tissue culture flasks every four days by releasing the cells from the substrate using 0.25% (w/v) trypsin. The T-screen was performed as previously described (Schriks *et al*., 2006). GH3 cells at 80% confluence were incubated for 48 h in serum-free PCM medium (which was changed once after 24h), as originally described by Sirbasku *et al.* (*Sirbasku et al.,* 1991). PCM consists of phenol red-free DMEM/F12 with 15 mM HEPES, 10 µg/ml bovine insulin, 10 µM ethanolamine, 10 ng/ml sodium selenite, 10 µg/ml apo-transferrin, and 500 µg/mL bovine serum albumin. The cells were then harvested in PCM medium using a cell scraper and plated at a density of 2500 cells/well (100 µl) on a 96-well plate. Following an attachment period of 2 h to 3 h, the cells were exposed in triplicate and for 96 h to various concentrations of the chemicals to be tested (100 µL, 2x dosing exposure concentration in PCM medium) either alone or in combination with 0.25 nM T3. Control wells contained cells and test medium with the same amount of ethanol (0.1%) as the exposed cells.

Cytotoxicity/viability

GH3 cells kept a basal activity but were unable to divide in PCM medium without T3. Subsequently, and following a 4 h incubation period with 10 µl/well of 0.1 mg/mL resazurin (Sigma-Aldrich) in PBS, cell proliferation was measured as relative fluorescence units (RFUs) resulting from the reduction of non-fluorescent resazurin to the fluorescent product resorufin. Fluorescence was measured at λex = 530 nm and λem = 590 nm on a microplate reader. A chemical was considered cytotoxic if the fluorescence was less than the fluorescence of the vehicle control minus 3 fold the standard deviation.

Data analysis

Cell proliferation was expressed as a function of the maximum response observed at 10 nM T3 (in agonist mode) or 0.25 nM T3 (in antagonist mode). The response for the solvent control was set at 0%.

Statistical analysis

Obtained data were statistically analyzed using the PC program GraphPad Prism 6.00 (GraphPad Software Incorporated, San Diego, CA, USA). Descriptive statistical characteristics (arithmetic mean, minimum, maximum, standard deviation and coefficient of variation) were evaluated. One-way analysis of variance (ANOVA) and the Dunnett´s

multiple comparison test were used for statistical evaluations. The level of significance was set at ***P < 0.001; **P < 0.01 and *P < 0.05.

Docking

Metabolites of **DEHP** and **DEHT** were docked into TRα1, the only TRα subtype to bind T3, and TRβ1, the only β subtype crystallized. The coordinates of the receptor subtypes were taken from the RCSB ProteinDatabank under the entry 4lnw (*Souza et al., 2014*) crystallized with T3, and 1n46 (*Dow et al., 2003*), bound to an agonist, respectively. The ligands were extracted manually and both molecules were assigned using the Gasteiger-Hückel method. The ligands were subjected to an energy minimization using the maximin2 protocol of the Sybyl 6.9.1 molecular modeling package. The co-crystallized T3 was cross-docked with very good precision, indicating that the docking protocol was sound. It consisted of a 30-solution GOLD run in a binding site defined as a sphere of 10 Å around the co-crystallized ligand with 100,000 operations and the ChemPLP scoring function. The 30 poses were manually inspected to define the most representative conformations, chosen as the best scored solution from the largest cluster of poses. In a few cases, a high score, but not the best, was selected as it was more representative of all the poses. The two receptor subtypes only differed in 15 residues, all of which were remote from the binding site. The superimposition of the structures is also fairly good, with a Root Mean Square over the heavy atoms of 2.42 Å, which is only very slightly higher than the worst resolution (2.2 Å). The docking results were therefore fairly comparable, the discussion of the docking will focus on TRα, which benefits from a better resolution.

RESULTS

Impact on thyroid dependent cell growth

The T-screen assay was used as a fast and functional assay to assess interference with T3 receptors (agonistic or antagonistic potency of xenobiotics at cellular level (Figure 1)).

A concentration dependent antagonist effect starting at 2 µg/mL was observed, becoming significant at 5 µg/mL with **MEHT**. Above 5 µg/mL, **MEHT** was cytotoxic for the cell line. **MEHT** metabolites had no effect.

In contrast, **MEHP** was a partial agonist (around tenfold) between 10 µg/mL and 20 µg/mL. Derived oxidative metabolites, such as OH metabolite, were 2 fold more active, with a

significant effect at lower concentrations (0.2 µg/mL, 2 µg/mL and 5 µg/mL) and with a concentration dependency. A synergistic response was also observed when cells were co-treated with T3 up to a concentration of 10 µg/mL. 5-oxo-MEHP significantly inhibited cell growth at 10 µg/mL in the presence of T3 and at non-cytotoxic concentrations. Higher concentrations of **5-oxo-MEHP** were very cytotoxic for the cells. The CX metabolite had no effect.

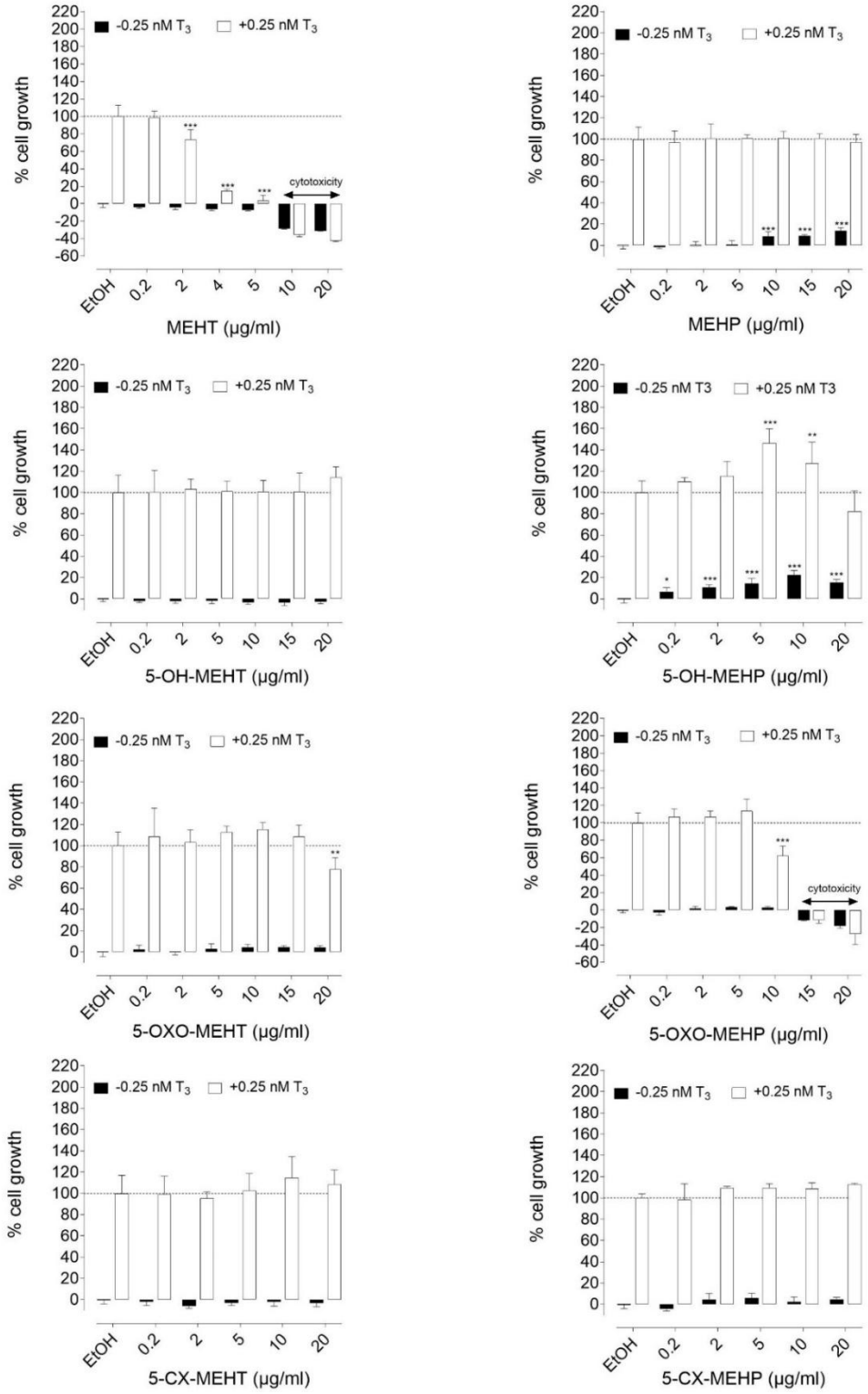


Figure 1: Effects of plasticizer metabolites on the T-Screen assay. GH3 cells were exposed for 96h to increasing concentrations of the chemicals either alone or in the presence of T3. Cell proliferation was expressed relative to the maximum response observed at 10 nM T3 (in agonist mode) or 0.25 nM T3 (in antagonist mode). The response for the solvent control was set at 0%. The values are mean ± SD of at least two independent experiments performed in triplicate. * Significantly different from control (*$p<0.05$, **$p<0.01$ and ***$p<0.001$).

Docking

T3 was docked into the two subtypes to verify the docking protocol. A single conformation was achieved which was almost superimposable with the co-crystallized conformation. It was tightly bound by a strong ionic interaction between the acid and arginine 228 and 262 at the base of the pocket. At the other extremity, the phenol formed a hydrogen bond with His381. The only difference was a slight twist of the acid chain to better interact with the arginines. (Figure 2)

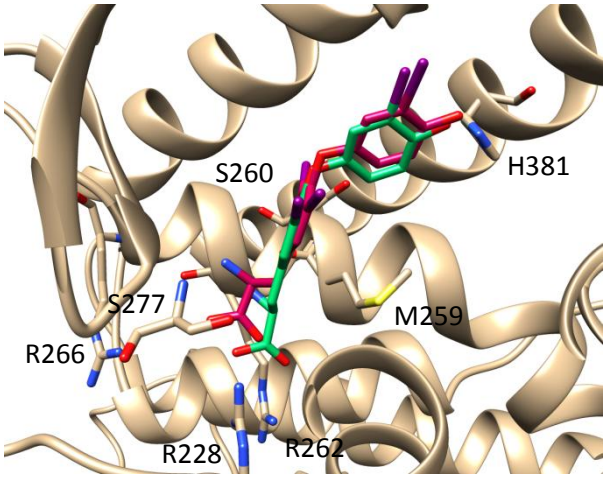


Figure 2: Docking of T3 (green) Vs its crystallographic position (red) in 4lnw

The monoesters fared well. **MEHT** showed a single conformation, with an excellent conservation of the position of the aromatic ring close to arginines 228, 262 and 266, which interacted with its free acid *via* strong salt bridges. The remaining ester side chain occupied the other side of the pocket and, with only a small amount of fluctuation, was positioned above His 381. Compared to T3, the aromatic group of **MEHT** was at the opposite end of the pocket. **MEHP**, in contrast, fitted into the binding site with its aromatic group very close to the position of the distal phenyl of the hormone. The free acid was also able to bind to His 381, although the orientation of the interaction was not perfect in the crystallographic conformation of the histidine side chain. The ester occupied the part of the pocket close to the arginines. (Figure 3)

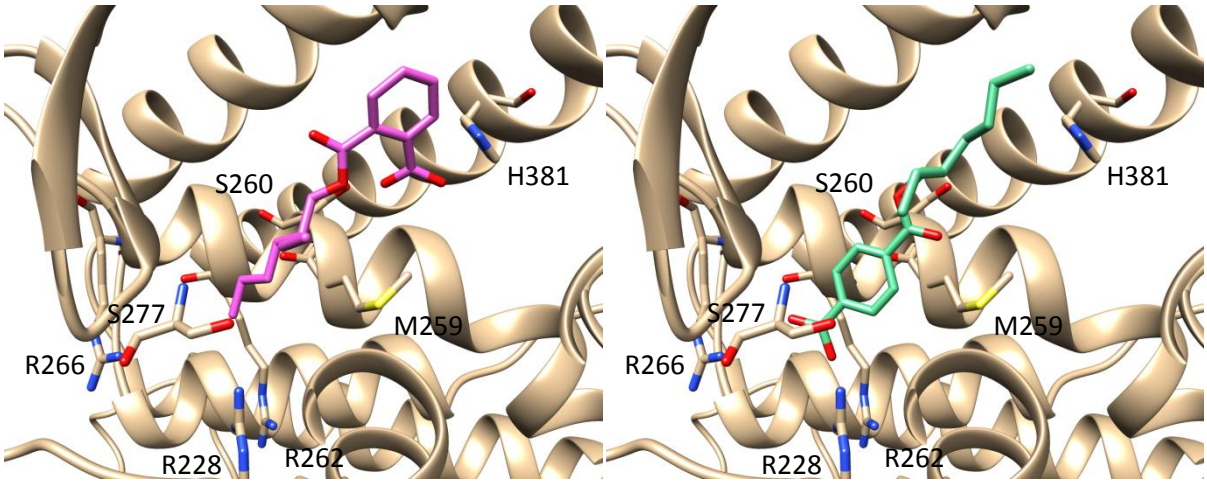


Figure 3: Docking of **MEHP** (left) and **MEHT** (right) in TRα_1 (4lnw)

The hydroxylation of the monoester metabolite in **5-OH-MEHT** did not modify its placement in the pocket. However, the hydroxyl at the end of the ester chain only formed inconsistent hydrogen bonds with the skeleton of Gly290, which may be deleterious due to the rather hydrophobic nature of the pocket around this residue. **5-OH-MEHP** behaved in much the same way, keeping the same position as its parent but with the acid binding to His381 and the hydroxyl group at the other end of the molecule forming hydrogen bonds with the skeleton of Met259 and, occasionally, with that of Ala283 (Figure 4)

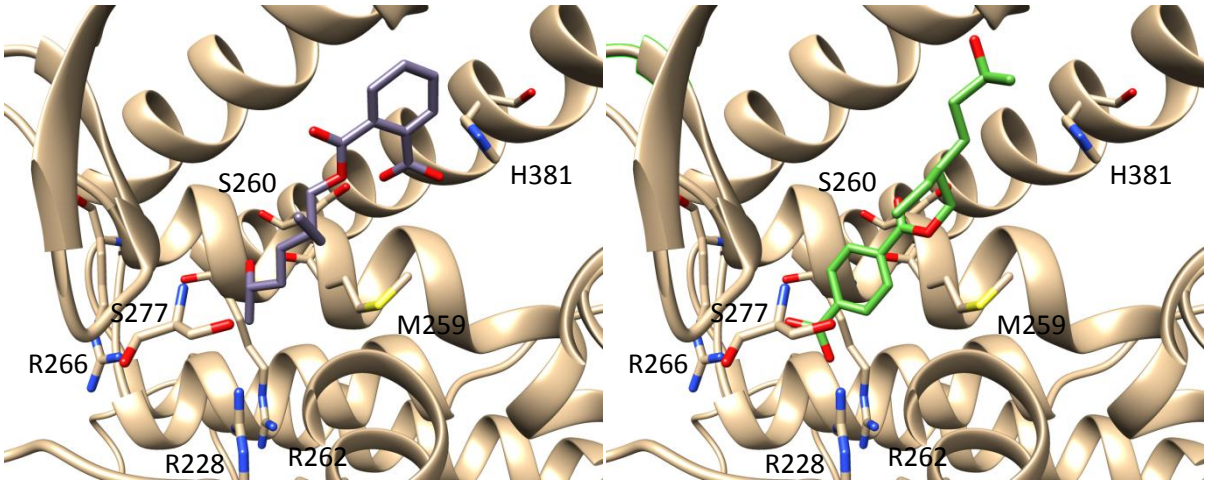


Figure 4: Docking of 5-OH-**MEHP** (left) and 5-OH-**MEHT** (right) in TRα_1 (4lnw)

DISCUSSION

*Impact on thyroid hormones

Thyroid hormones have a wide range of biological effects in vertebrates, both during fetal and prenatal development and with regard to the development of sex organs and the central nervous system in mammals (*Portefield and Henrich, 1993; Bernal and Nunez, 1995*). The T screen is based on thyroid hormone dependent cell growth of a rat pituitary tumor cell line and is used as a model to study basic thyroid hormone dependent cell physiology, and to study the interference of compounds with thyroid hormones at a cellular level (*Hohenwarter et al, 1996, Gutleb et al., 2005*).

For the **DEHT** metabolites, only **MEHT** was an antagonist for cell line proliferation at very low concentrations. In contrast, **DEHP** metabolites were agonists, particularly the hydroxylated metabolite (**5-OH-MEHP**), demonstrating a synergism when cells were co-treated with T3 up to a concentration of 10 µg/mL. **5-Oxo-MEHP** only inhibits cell growth at 10 µg/mL and was very cytotoxic for the cells above this concentration. Our data agree with data published by Ghisari that demonstrated a low dependent potency activation of **DEHP** between 10^{-6} M and 10^{-5} M. However, Ghisari *et al.* did not examine **DEHP** metabolites (*Ghisari et al., 2009*). In a TR reporter gene assay using a recombinant *Xenopus laevis* cell line, BBP, DBP and **DEHP** were reported to exhibit a T3-antagonistic activity, and to inhibit the expression of the endogenous TRβ gene (*Sugiyama et al., 2005).* **DEHP** was also shown to interfere with the binding of T3 to TRβ (*Ishihara et al., 2003*), suggesting that the compound may bind to the receptor. Rodent studies have also confirmed the effects of **DEHP** on the thyroid (*Dong et al., 2017; Liu et al., 2015*), where significant influences on thyroid hormones and metabolism were observed. Specifically, proliferative changes were noted in the thyroid *in vivo*, raising the concern of a potential thyroid carcinogenicity of **DEHP**. Recently, Kim *et al.* investigated whether **DEHP** could induce proliferative changes and DNA damage in 8505C thyroid carcinoma cell lines both in vitro and in the thyroid tissue of rats treated orally with **DEHP** for 90 days from juvenile to full maturation in vivo (*Kim et al., 2019*). They showed that **DEHP** can stimulate thyroid cell proliferation and DNA damage through the activation of the TSHR pathway, as TSHR plays a key role in the proliferation and differentiation of thyroid cells (*De Felice et al., 2004*). All *in vitro* and *in vivo* data suggested that **DEHP** is able to influence thyroid tissues at low doses.

Though, studies on the *in vitro* effects of plasticizer metabolites on the TH system are scarce, it is well known that diesters are highly metabolized *in vivo.* Reduced serum thyroid hormone levels have been well documented in human populations, with higher urine phthalate metabolites in various regions around the globe (*Boas et al., 2006; Gao et al., 2017; Meeker et al., 2007; Wu et al., 2013*).

In this study, we observed that **5-OH-MEHP,** in contrast to **MEHP,** also induced a decrease in the presence of T3 at the highest non-cytotoxic concentration, whereas below this concentration we saw a synergism at 5 µg/mL. In this assay, cell proliferation was measured as a consequence of T3 activation but the mechanism of cell proliferation is a complex biological phenomenon. For example, GH3 cells also express PPARs, which exhibit anti-proliferative activity upon ligand binding (*Chen et al., 2008*). In this study, we confirmed that it is linked to T3 as induction by T3 was modulated in the presence of **5-OH-MEHP** with a synergistic response when the cells were coexposed. Interestingly, human studies have found an inverse association between **MEHP** metabolites in the urine and concentrations of free T4 and total T3 levels in adult men (*Meeker et al., 2007*), which suggests that **DEHP** can disrupt the homeostasis of the thyroid-pituitary axis. Ghisary identified a potential antagonistic effect of a mixture of plasticizers, including 6 phtalates, with regard to T screen assays, confirming that it is important to consider a combined effect (*Ghisary et al., 2009*).

* **Docking**

The monoesters **MEHP** and **MEHT** displayed a rather strong binding mode in the pocket, with a single coherent conformation found for each. It is noteworthy that **MEHP**, with its aromatic group in close proximity to His381, closely imitates the binding mode of the natural T3 agonist despite lacking any strong interaction with the arginines. On the other hand, **MEHT**, presenting its acid at the entry of the pocket in front of the arginines, has a different binding mode in which the aromatic is at the opposite end to His 381 and has no interactions with this part of the binding site.

The presence of a hydroxyl group on the monoester metabolite did not alter its placement in the pocket, with **5-OH-MEHP** once again being closer to the natural agonist and **5-OH-MEHT** positioning its aromatic group the other way around, far from His381. This conformation is also somewhat destabilized by the poor hydrophobic fit between the added hydroxyl group and the rather hydrophobic area around His381.

Further oxidation of the hydroxyl to a carbonyl group led to the same positioning of the derivatives. In the case of **5-oxo-MEHP**, the same position close to His381 was maintained, and the carbonyl formed some hydrogen bonds, although it did not exhibit a clear preference, binding to Arg228, the Ser277 skeleton or side chain or to nothing, with roughly the same propensity. It may therefore be a less than perfect fit for the TR binding site and clearly inferior to the hydroxylated metabolite. The carbonyl group of **5-oxo-MEHT** formed no interactions and lay in the middle of the pocket with a poor fit to its surroundings.

Interestingly, of the 30 solutions determined for **5-cx-MEHP**, no correctly superimposable conformation was identified. Although all were placed in the same area, there was a good deal of fuzziness in their position, most probably due to the different relative positions of the acids in T3 and in **5-cx-MEHP** hindering a perfect fit to the binding site. The addition of a second acid at the end of the remaining ester on **5-cx-MEHT** gave the same position as the other compounds derived from **DEHT**. The acid of the ester side chain lay squarely in the middle of the pocket and was unable to form any interactions. We can assume it is worse than its congeners.

Table 2 summarizes the theoretical and experimental affinities toward TRα1 obtained *in silico* and *in vitro.*

Table 2: Theoretical and experimental affinities towards TRα1 of **DEHP** and **DEHT** metabolites derived from the *in silico* and *in vitro* bioassays.

Compounds studied	***In silico* studies [1] Affinity towards TRα1**	***In vitro* studies Agonist/antagonist activities on cell proliferation**
MEHT	**++**	+++ antagonist or cytotoxic
MEHP	**+++**	++ agonist
5-OH-MEHT	**+/-**	0
5-OH-MEHP	**++++**	++++ agonist and synergic activities
5-oxo-MEHT	**0**	+ antagonist
5-oxo-MEHP	**+**	++ antagonist or cytotoxic
5-cx-MEHT	**0**	0
5-cx-MEHP	**0**	0

0: no affinity or no activity
+/-: inconclusive affinity or activity
+: low to very low affinity or activity at concentration ≥ 20 µg/ml
++: low to medium affinity or activity at concentration ≥ 10 µg/ml
+++: medium to strong affinity or activity at concentration ≥ 5 µg/ml
++++: very strong affinity or activity at concentration ≥ 2 µg/ml
NT: not tested

[1] *Classification is based on three criteria:*
- The superposition of the conformations (n = 30) of the chemical structures in the TRα1 receptor,
- The number of bindings between groupings in the structure and the TRα1 receptor,
- The type of bond (hydrogen or ionic).
This makes it possible to estimate a relative potential for interaction between the chemical structure and the target (TRα1).

* **In vitro data versus biomonitoring values**

In neonatal intensive care units (NICU), the use of many plasticized PVC medical devices overexpose neonates to phthalates (*Stroustrup et al., 2020; Mallow et al., 2014; Fischer Fumeaux et al., 2015).* Biomonitoring studies have shown high concentrations of oxidized metabolites of **DEHP** in the urine of neonates. Urinary levels of **5-OH-MEHP** may exceed 0.2 µg/mL, a concentration at which we have demonstrated a TRβ agonist effect that was confirmed *in vitro* as being partial and which has a synergistic effect with T3. The maximum values of **5-OH-MEHP** measured in the urine of newborns hospitalized in NICU range from 0.43 µg/mL to 13.1 µg/mL, i.e. 2 to 65 times higher than the concentration activating thyroid receptors (*Demirel et al., 2016; Strommen et al., 2016; Calafat et al., 2004; Green et al., 2005; Pinguet et al., 2019*). Certain medical procedures, such as extracorporeal circulation (Extracorporeal membrane oxygenation, cardiopulmonary bypass), respiratory assistance, and intravenous nutrition are recognized as situations with a high risk of exposure, which can explain the high values in multi-exposed patients. In these studies, the median values of urinary **5-OH-MEHT** concentrations are generally less than 0.2 µg/mL. *Calafat et al.* (2004) and *Green et al.* (2005) have shown higher urinary levels of 2.22 µg/mL and 0.26 µg/mL, respectively. However, these studies were carried out in 2004 and 2005 and it can be assumed that exposure to **DEHP** *via* MDs has decreased in the 15 years following the recommendations of the current SCENIHR and the European regulations. However, a recent study carried out specifically in newborns in cardiac surgery showed that CEC MDs remain highly exposed to **DEHP** (*Gaynor et al.*, 2019). Indeed, Gaynor *et al.* (2019) demonstrated

that the level of **5-OH-MEHP** passes from 0.01 µg/mL to 0.229 µg/mL after cardiopulmonary bypass. Our study showed that **MEHP** had partial agonist effects on TR receptors at concentrations of 10 µg/mL to 20 µg/mL. The data in the literature show that the concentrations of **MEHP** in biological media are significantly lower than these values, including the study by Eckert et al. who directly measured **MEHP** in blood in contact with extracorporeal circulation lines during heart surgery in newborns. The maximum concentration of **MEHP** found in the blood of these patients was 0.56 µg/mL *(Eckert et al., 2020)*

With regard to **DEHT** metabolites, only **MEHT** showed an effect on cellular growth with an antagonistic effect on T3 at non cytotoxic concentrations of 2 µg/mL to 5 µg/mL using the T-screen assay. There is little biomonitoring data for **DEHT** in the literature, even less measuring the exposure of hospitalized newborns. Lessmann et al. studied the exposure of non-hospitalized children over 4 years of age to **DEHT** *(Lessmann et al., 2017*). However, only the oxidized metabolites of **MEHT** were measured in the urine. Pinguet *et al.* presented results of the exposure of patients hospitalized in NICU to certain plasticizers, including **DEHT** (Pinguet *et al., 2019*). The median of the urinary concentrations of **MEHT** in this cohort of patients was lower than the limit of quantification (0.018 ng/mL) and the maximum concentration measured was 9.90 ng/mL, 200 times less than the concentration showing an antagonistic effect on T3 identified using the T screen assay in this study (Pinguet *et al.*, 2019). In the Armed Neo clinical trial, **MEHT** levels found in the urine of premature babies hospitalized in NICU were also much lower than this value, with a maximum of 1.32 ng/mL (unpublished data). Therefore, it would appear unlikely that **MEHT** levels of 2 µg/mL would be reached in the biological media of hospitalized newborns. **DEHT** is a plasticizer which has a low migration from PVC medical devices (*Bernard et al., 2015; Bernard et al., 2018*). In addition, **MEHT**, a metabolite resulting from the enzymatic hydrolysis of **DEHT**, is very rapidly transformed into oxidized derivatives, which have no *in vitro* effect on T3 dependent cell proliferation. The urinary excretion factor is 0.02% and 6% for **DEHT** and **MEHP** respectively (*Lessmann et al, 2016*).

CONCLUSION

We can conclude that **under these experimental conditions**, and with regard to the use of alternative methods, that **DEHT** and its metabolites (except for **MEHT)** have no effect on T3

hormonal activities when compared to **DEHP**. Taking all these data into account, along with human biological enzymatic data, there appears to be no safety concerns with **DEHT** compared to **DEHP**

Declaration of interest

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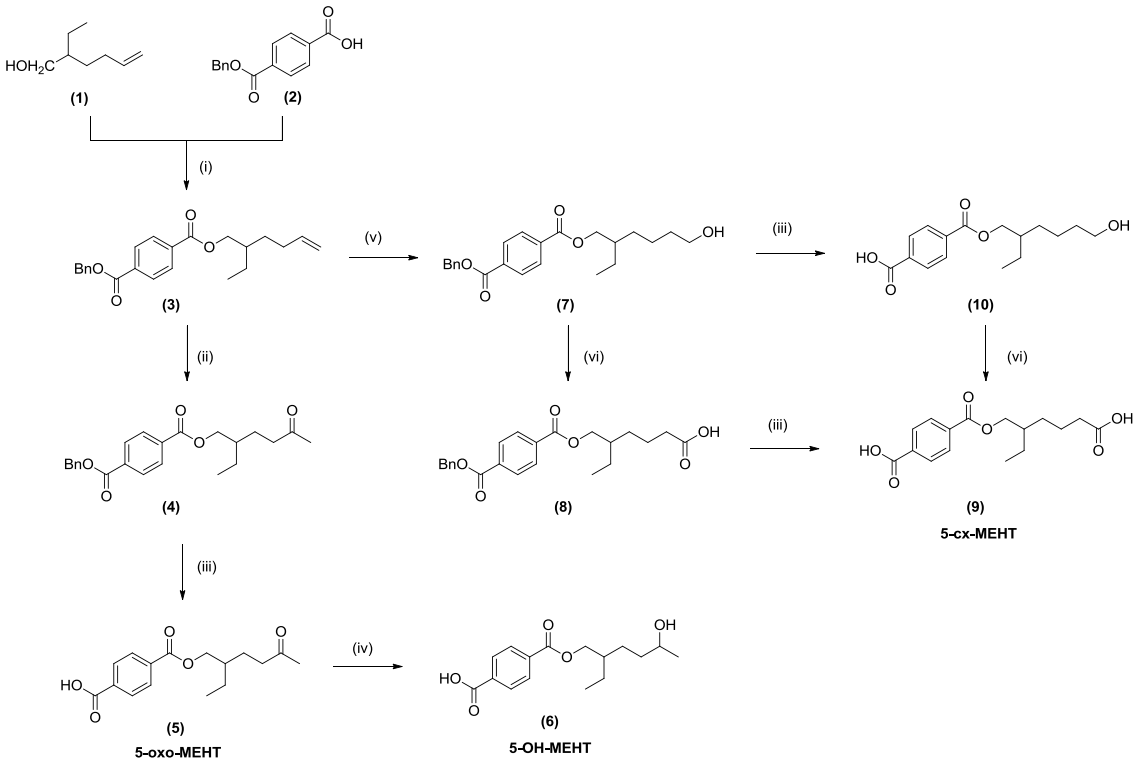
Appendix 1: supplementary data - Synthesis of DEHP and DEHT metabolites

Materials for Chemical Syntheses

Unless otherwise mentioned, all manipulations were performed under argon; all reagents were purchased from the following commercial suppliers: Sigma-Aldrich, Acros Organics, Carlo Erba, TCI Europa, Alpha Aesar. Anhydrous DMF, anhydrous trimethylamine, anhydrous pyridine were purchased from Acros Organics. THF was dried over a Pure Solv™ Micro Solvent Purification System (Sigma-Aldrich) with an alumina column. Dichloromethane was distilled over hydride calcium. Nuclear magnetic resonance (NMR) spectra were acquired on a Bruker AC-200 or 500 operating at 200 or 500 for ^{1}H NMR and 50 or 125 MHz for ^{13}C NMR, respectively. All ^{1}H and ^{13}C NMR spectra are reported in δ units, parts per million (ppm). Coupling constants are indicated in Hertz (Hz). The following abbreviations are used for spin multiplicity: s = singlet, d = doublet, t = triplet, q = quadruplet, m =multiplet, and brs = broad singlet. TLC was performed on pre-coated silica gel sheets (POLYGRAM® 60F254 plates) and visualized under UV light (254 nm). Revelators used were KMnO4 (1.5 g $KMnO_4$, 10 g K_2CO_3, and 1.25 mL 10% NaOH in 200 mL water) and ninhydrin (1.5 g ninhydrin in 100 mL of n-butanol with 3 mL AcOH). Column chromatography was performed using silica gel normal phase (35-70 µm). Uncorrected melting points (Mp) were measured on an IA9100 Digital Melting Point Apparatus. Infrared spectra (IR) were recorded on a Bruker FT Vector 22. The HRMS analysis was performed using a Thermo Exactive benchtop Orbitrap®

instrument (UCA PARTNER, Clermont-Ferrand, France). DEHP, MEHP, 5-OH-MEHP, 5-oxo-MEHP and 5-cx-MEHT were synthesized using the procedures previously described by Nüti (Nüti et al., 2005)



Conditions and reactants: (i) DMAP, DCC, dichloromenthane, rt, overnight; (ii) $PdCl_2$; DMF/H_2O (17/3, v/v), rt, overnight; (iii) H_2, Pd/C 10%,absolute ethanol, 4 hours; (iv) $NaBH_4$, rt, overnight; (v) (a) B_2H_6, anhydrous THF, rt, 1 h; (b) NaOH (3M), then H_2O_2 30%, 50°C, 2 h; (vi) CrO_3/H_2SO_4, acetone; rt; 30 min

Benzyl (2-ethylhex-5-en-1-yl)terephthalate (3)

4-((benzyloxy)carbonyl)benzoic acid **(2)**[2] (2 g, 7.80 mmoles), DMAP (1.37 g, 11.24 mmoles) dissolved in anhydrous dichloromethane (75 mL), cooled to 0°C, under argon atmosphere, DCC (2.32 g, 11.24 mmoles) was added to a solution of 2-ethylhex-5-en-1-ol **(1)**[1] (1.2 g, 9.30 mmoles). The reaction mixture was allowed to warm to room temperature and was then stirred overnight. The reaction mixture was filtered through Celite® 545 and washed with dichloromethhane (2 * 20 mL). The filtrate was evaporated under reduced pressure and the resulting crude product was purified on silica gel eluted with dichloromethane/cyclohexane (9/1, v/v) to produce the expected compound **(3)** (1.91 g, 66%). IR (cm^{-1}) ν 1246, 1263, 1716, 2117, 2929; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.95 (t, J = 7.5 Hz, 3H), 1.17-1.51 (m, 4H, 1.71—1.76 (m, 1H), 2.10-2.15 (m, 2H), 4.27 (d, J = 5.6 Hz, 4.94-4.96 (m, 1H), 5.00-5.04 (m, 1H), 5.38 (s, 2H), 5.76- 5.94 (m, 1H), 7.33-7.39 (m, 3H), 7.43-7.45 (m, 2H), 8.08 (m, 2H), (8.12 (m, 2H); ^{13}CNMR (125 MHz, $CDCl_3$) δ 11.0, 23.9, 30.2, 31.0, 38.4, 67.1, 67.5, 114.7, 128.2, 128.3, 128.4, 128.7, 129.5, 129.7, 133.9, 134.4, 135.7, 138.6, 165.6, 165.6; HRMS for $C_{23}H_{26}O_4$ m/z $[M+H]^+$ calc.: 367.18311; found: 367.19023.

Benzyl (2-ethyl-5-oxohexyl)terephthalate (4)

A solution of compound **(3)** (0.39 g, 1.06 mmoles) dissolved in 2 mL of DMF/H_2O (17/3, v/v) was added dropwise to a solution of $PdCl_2$ (0.197g, 1.11 moles) and *para*-benzoquinone (0.132 g, 1.22 mmoles) dissolved in 10 mL of DMF/H_2O (17/3, v/v). The resulting mixture was stirred overnight in the dark. A solution of HCl (3N) (20 mL) was added dropwise to the

reaction mixture. The reaction solution was extracted with diethyl ether (3 * 30 mL). The combined organic layers were washed with brine (30 mL), dried over $MgSO_4$, filtered and evaporated under reduced pressure. The crude product was purified on silica gel eluted with a gradient dichloromethane/methanol (100 to 98/2) to produce the expected compound **(4)** (0.247 g, 61%) as an oil. IR (cm^{-1}) ν 1246, 1263, 1716, 2931; ^1H NMR (200 MHz, $CDCl_3$) δ 0.99 (t, J = 7.4 Hz), 1.45-1.51 (m, 2H), 1.70-1.76 (m, 3H), 2.17 (s, 3H), 2.51-2.56 (m, 2H), 4.28 (d, J = 4.4 Hz, 2H), 5.40 (s, 2H), 7.38-7.49 (m, 5H), 8.09 (d, J = 8.6 Hz, 2H), -8.17 (d, J = 8.6 Hz, 2H);^{13}C NMR (125 MHz, $CDCl_3$) δ 11.1, 23.9, 30.2, 31.0, 38.4, 67.1, 67.5, 114.8, 128.3, 128.4, 128.7, 129.5, 129.7, 133.9, 134.7, 135.7, 138.6, 165.6, 165.9; HRMS for $C_{23}H_{26}O_5$ m/z $[M+ H]^+$ calc.: 383.17802; found: 383.18549.

4-(((2-Ethyl-5-oxohexyl)oxy)carbonyl)benzoic acid (5) (5-oxo-MEHT)

Pd/C 10% (24.7 mg) was added to a solution of compound **(4)** (0.247 g, 0.64 mmoles) dissolved in absolute ethanol (30 mL). The resulting mixture was degassed three times and stirred under hydrogen atmosphere for 4 h. The reaction solvent was filtered on a Celite® 545 pad and rinsed with 50 mL of absolute ethanol. The filtrate was evaporated under reduced pressure. The crude product was purified on silica gel with ethyl acetate to produce the expected compound **(5)** (0.148 g, 79%) as a solid. Mp: 87-89°C; IR (cm^{-1}) ν 1259, 1313, 1678, 1708, 2552, 2960; ^1H NMR (200 MHz, $CDCl_3$) δ 1.00 (t, J = 7.4 Hz, 3H), 1.39-1.60 (m, 2H), 1.72-1.82 (m, 3H), 2.20 (s, 3H), 2.58 (t, J = 7.1 Hz, 2H), 4.30 (d, J = 4.1 Hz, 2H), 8.14 (d, J = 8.6 Hz, 2H), -8.19 (d, J = 8.6 Hz, 2H), 10.2 (brs, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 11.0, 24.0, 24.9, 38.5, 40.6, 67.6, 129.5, 130.2, 132.9, 135.1, 165.9, 170.6, 209.0; HRMS for $C_{16}H_{20}O_5$ m/z $[M+ H]^+$ calc.: 292.13107; found: 291.12415.

4-(((2-Ethyl-5-hydroxyhexyl)oxy)carbonyl)benzoic acid (6) (5-OH-MEHT)

$NaBH_4$ (0.031 g, 0.06 mmoles) was added portion wise (very exothermic reaction) to a solution of ketone compound **(5)** (0.079 g, 0.02 mmoles) dissolved in absolute ethanol, cooled to 0°C with an ice-bath. After stirring overnight at room temperature, water (20 mL) was added with caution, followed by a solution of HCl (1N) (pH = 1). The aqueous solution was extracted with ethyl acetate (3*20 mL). The combined organic layers were dried over $MgSO_4$, filtered and evaporated under reduced pressure. The crude product was purified on silica gel eluted with ethyl acetate/ethanol (75/25; v/v) to provide the expected compound **(6)** (0.069 g, 87%) as a solid. Mp: 74-76°C; IR (cm^{-1}) ν 1271, 1311, 1686, 1709, 2550, 2851, 2929, 2961, 3211; ^1H NMR (500 MHz, $CDCl_3$) δ 0.95 (t, J = 7.4 Hz, 3H), 1.22 (d, J = 6.1 Hz, 3H), 1.45-1.54 (m, 4H), 1.73-1.76 (m, 2H), 3.82-3.86 (m, 1H), 4.28 (d, J = 5.6 Hz, 2H), 8.07-8.13 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 10.8, 23.4, 23.6, 27.2, 33.8, 39.5, 67.2, 68.4, 129.2, 130.1, 135.5, 134.9, 165.7, 169.6; HRMS for $C_{16}H_{22}O_5$ m/z $[M+ H]^+$ calc.: 294.14672; found: 293.13979.

Benzyl (2-ethyl-6-hydroxyhexyl)terephthalate (7)

B_2H_6 (1M) (1.96 mL, 1.96 mmoles) was added dropwise to a solution of compound **(3)** (0.54 g, 1.47 mmoles) dissolved in anhydrous THF (10 mL), cooled to 0°C. The resulting mixture was stirred to room temperature for 1 hour. NaOH (3M) (182 µL), followed by 33%

H_2O_2 (182 µL) were then added dropwise. The resulting mixture was heated to 50°C for 2 h. After cooling to room temperature, water (10 mL) was added. The resulting mixture was extracted with diethyl ether (3 * 10 mL). The combined organic layers were washed with brine (30 mL), dried over $MgSO_4$, filtered and evaporated under reduced pressure. The crude product was purified on silica gel and eluted with dichloromethane/cyclohexane (8/2; v/v) to produce the expected compound **(7)** (0.262 g, 79%) as an oil. IR (cm^{-1}) ν 1264, 1716, 2932; ^1H NMR (200 MHz, $CDCl_3$) δ 0.98 (t, J = 7.4 Hz), 1.46-1.58 (m, 7H), 1.63-1.80 (m, 2H), 3.67 (d, J = 6.3 Hz, 2H), 4.29 (d, J = 5.6 Hz, 2H), 5.41 (s, 2H), 7.39-748 (m, 5H), 8.09-8.19 (m, 4H); ^{13}C NMR δ (125 MHz, $CDCl_3$) 11.1, 23.0, 23.9, 33.0, 39.0, 62.8, 67.1, 67.6, 128.3, 128.3, 128.4, 128.7, 129.5, 129.7, 129.7, 133.9, 134.4, 135.7, 165.7, 165.9; HRMS for $C_{23}H_{28}O_5$ m/z $[M+ H]^+$ calc.: 384.19367; found: 385.20062.

5-(((4-((benzyloxy)carbonyl)benzoyl)oxy)methyl)heptanoic acid (8)

11.31 mL of Jones' reagent (prepared from 670 mg CrO_3, 600 µL H_2SO_4 and 5 mL of water) was added dropwise to a solution of compound **(7)** (0.43 g, 1.12 mmoles) dissolved in acetone (3 mL), cooled to 0°C. After stirring for 30 min at room temperature, water (10 mL) was carefully added. The mixture was then extracted with diethyl ether (3*30 mL). The combined organic layers were dried over $MgSO_4$, filtered and evaporated under reduced pressure to produce an oil. The crude product was purified on silica gel and eluted with ethyl acetate/cyclohexane (2/8, v/v) to produce the expected compound **(8)** (0.113 g, 26%) as an oil. IR (cm^{-1}) ν 1264, 1715, 2959; ^1H NMR (500 MHz, $CDCl_3$) δ 0.95 (t, J = 7.5 Hz, 3H), 1.45-1.48 (m, 4H), 1.70-1.77 (m, 3H), 2.37 (t, J = 7.3 Hz, 2H), 2.27 (d, J= 5.4 Hz, 2H), 5.38 (s, 2H), 7.25-7.38 (m, 3H), 7.40-7.45 (m, 2H), 8.07-8.09 (m, 2H), 8.12-8.14 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ11.0, 21.5, 23.8, 30.3, 34.1, 38.8, 67.0, 67.4, 127.9, 128.4, 128.8, 129.5, 129.7, 134.0, 134.4, 136.0, 165.5, 166.0, 179.3; HRMS for $C_{23}H_{26}O_6$ m/z $[M- H]^+$ calc.: 397.17294; found: 397.16547.

4-(((2-Ethyl-6-hydroxyhexyl)oxy)carbonyl)benzoic acid (10)

10% Pd/C (0.02 mg) was added to a solution of compound **(8)** (0.2 g, 0.52 mmoles) dissolved in absolute ethanol (20 mL). The resulting mixture was degassed three times and stirred under a hydrogen atmosphere for 4 h. The reaction solvent was filtered on a Celite® 545 pad and rinsed with 20 mL of absolute ethanol. The filtrate was evaporated under reduced pressure to produce the expected compound **(10)** (0.138 g, 90%). The product was used for the next step without purification. Mp: 64-66°C; IR (cm^{-1}) ν 1261, 1427, 1506, 1680, 1708, 2856, 2932; ^1H NMR (500 MHz, MeOD) δ 0.94 (t, J = 7.5 Hz, 3H), 1.42-1.53 (m, 9H), 1.70-1.73 (m, 1H), 3.54 (t, J = 6.5 Hz, 2H), 4.25 (d, J = 5.4 Hz, 2H), 8.03 (d, J = 8.2 Hz, 2H), 8.07 (d, J = 8.2 Hz, 2H); ^{13}C NMR (125 MHz, MeOD) δ 10.2, 22.4, 23.8, 30.1, 32.1, 39.0, 61.5, 66.8, 129.4, 129.7, 133.3, 136.0, 165.9, 168.2; HRMS for $C_{16}H_{22}O_5$ m/z $[M+ H]^+$ calc.: 295.1539; found: 295.1540.

4-(((5-Carboxy-2-ethylpentyl)oxy)carbonyl)benzoic acid (9) (5-cx-MEHT)

Method A: 10% Pd/C (0.02 g) was added to compound **(8)** (0.2 g, 0.054 mmoles) dissolved in absolute ethanol (20 mL). The reaction mixture was degassed three times and stirred under a hydrogen atmosphere for 7 h. The reaction mixture was filtered on a pad of Celite® 545. The filtrate was evaporated under reduced pressure. The crude product was purified on silica gel and eluted with ethyl acetate to produce the expected compound **(9)** (0.017 g, 8%) as a solid.

Method B: 107 µL of Jones' reagent (prepared from 670 mg CrO_3, 600 µL H_2SO_4 and 5 mL of water) was added dropwise to a solution of compound **(10)** (0.1 g, 0.34 mmoles) dissolved in acetone (1.5 mL), cooled to 0°C. After stirring for 30 min at room temperature, water (10 mL) was carefully added. The mixture was extracted with diethyl ether (3*15 mL). The combined organic layers were dried over $MgSO_4$, filtered and evaporated under reduced pressure. The crude product was purified on silica gel and eluted with ethyl acetate to produce the expected compound **(9)** (0.130 g, 96%) in solid form.

Mp: 65-67°C; IR (cm^{-1}) ν 1266, 1685, 1712, 2560, 2875, 2924, 2957; ^{1}H NMR (200 MHz, MeOD) δ 0.99 (t, J = 7.4 Hz, 3H), 1.38-1.75 (m, 9H), 4.32 (d, J = 5.5 Hz, 2H), 8.08 (m, 4H); ^{13}C NMR (125 MHz, MeOD) δ 10.6, 23.9, 23.8, 30.3, 32.5, 39.0, 61.2, 67.3, 129.0, 129.4, 133.5, 135.8, 165.7, 165.7; HRMS for $C_{16}H_{20}O_6$ m/z $[M- H]^+$ calc.: 307.12599; found: 307.11904.

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