Toxicologically important DDT metabolites
Synthesis, enantioselective analysis and kinetics

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Abstract

DDT was extensively and globally used as a pesticide in agriculture and for malaria vector control from the 1940's until the 1970's. Due to its heavy use, DDT became ubiquitously distributed throughout the environment. DDT and several DDT metabolites are persistent organic pollutants. Two DDT metabolites, 3-MeSO$_2$-DDE and o,p'-DDD have been proved to be tissue specific toxicants in the adrenal cortex. They are bioactivated to reactive intermediates which bind covalently to the adrenal cortex causing cell death. Due to its tissue specific toxicity o,p'-DDD has been used as a chemotherapy drug for adrenal cancer in humans. The efficacy and potency is however low and o,p'-DDD treatment is associated with serious side effects. 3-MeSO$_2$-DDE has been suggested as a potential alternative therapeutic agent.

A key aim of this thesis has been to improve the understanding of the kinetics of the two adrenocorticolytic compounds o,p'-DDD, its two enantiomers and 3-MeSO$_2$-DDE. To meet this objective chemical synthesis and enantioselective analysis were required. Furthermore, *in vitro* toxicity of o,p'-DDD enantiomers and diastereomers were performed.

An 11 step synthesis of 3-SH-DDE has been developed to promote both labelled and unlabelled synthesis of 3-alkylsulfonyl-DDE. Toxicokinetic studies showed that 3-MeSO$_2$-DDE and o,p'-DDD were accumulated in tissues and retained in adipose tissue in minipigs. 3-MeSO$_2$-DDE however had a twice as long biological $t_{1/2}$ and a considerably lower V$_d$ compared to o,p'-DDD. Suckling offspring were more exposed to 3-MeSO$_2$-DDE than their mothers who were given 3-MeSO$_2$-DDE orally. Interindividual differences in enantiomer kinetics in minipigs were observed suggesting polymorphism among the minipigs. Preparative isolation of the o,p'-DDD enantiomers is presented allowing determination of the absolute structures of the o,p'-DDD enantiomers by X-ray. The pure enantiomer of o,p'-DDD showed significant differences in toxicity in human adrenocortical cells.

In memory of my father/ En memoria de mi padre

Luis Aurelio Cantillana Perez

Por haber buscado un mejor futuro para sus hijos

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Abbreviations

o,p'-DDD	1,1-dichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane
p,p'-DDE	1,1-dichloro-2,2-bis(4-chlorophenyl)ethene
o,p'-DDE	1,1-dichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethene
o,p'-DDT	1,1,1-trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane
p,p'-DDT	1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane
3-MeSO$_2$-DDE	1,1-dichloro-2-(3-methylsulfonyl-4-chlorophenyl)-2-(4-chlorophenyl)ethene
ACC	Adrenocortical carcinoma
CD	Cyclodextrin
Cl	Clearance
CYP	Cytochrome P450
ECD	Electron capture detection
EF	Enantiomer fraction
ER	Enantiomeric ratio
EI	Electron ionisation
F	Bioavailability
GC	Gas chromatography
HPLC	High performance liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
PCB	Polychlorinated biphenyl
POP	Persistent organic pollutants
QA	Quality assurance
QC	Quality control
S/N	Signal to noise ratio
$t_{1/2}$	Half-life
V$_d$	Volume of distribution

List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals I-VI. The published articles are reproduced here with the permission of the publisher.

I   Synthesis of 2-(4-chlorophenyl)-2-(4-chloro-3-thiophenol)-1,1-dichloroethene (3-SH-DDE) via Newman-Kwart rearrangement - A precursor for synthesis of radiolabeled and unlabeled alkylsulfonyl-DDEs.

Cantillana T., Sundström M., Bergman Å.

In press. *Chemosphere* **2009**

II   Pharmacokinetics of the adrenocorticolytic compounds 3-methylsulphonyl-DDE and *o,p'*-DDD (mitotane) in minipigs.

Hermanson V., **Cantillana T**., Hovander L., Bergman Å., Ljungvall K., Magnusson U., Törneke K., Brandt I.

Cancer Chemotherapy and Pharmacology **2008**, 61, 267-274.

III Toxicokinetics of the CYP11B1-activated adrenal toxicant 3-MeSO$_2$-DDE in mother and offspring following oral administration to lactating minipigs

Cantillana T., Kismul H., Aleksandersen M., Tanum M., Sörvik I., Verhaegen S., Hovander L., Bergman Å., Ropstad E., Brandt I. Manuscript.

IV   Interindividual differences in *o,p'*-DDD enantiomer kinetics examined in Göttingen minipigs.

Cantillana T., Lindström V., Eriksson L., Brandt I., Bergman Å.

In press. *Chemosphere* **2009**.

V   Chiral effects in adrenocorticolytic action of *o,p'*-DDD (mitotane) in human adrenal cells.

Asp V., **Cantillana T**., Bergman Å., Brandt I.
Submitted.

VI   (2S)-1,1-dichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane.
Cantillana T., Eriksson L.
Acta Cryst. **2009**, E65, o297.

1 Introduction

The word malaria comes from Italian and means bad air (mal=bad, aria=air), which stems from the belief that foul smelling air or miasmas were the cause of the disease. This confusion made people combat malaria by planting water loving and aromatic eucalyptus trees in swamps. It was first in 1898 that the link between the *plasmodium* parasite, the anopheles mosquito and man was made by R. Ross, a military doctor working in India. Malaria prevention was then focused on killing the mosquito larvae, this was done by filling the breeding sites with petroleum. Efforts were also made to stop the spreading by educating people about the disease and infected people were treated with quinine. Despite all the efforts the war against malaria was not won. It was not until the use of chemical vector control was implemented that the fight against malaria showed dramatic positive results. The use of the larvicide Paris Green (copper aceto arsenite, $Cu(C_2H_3O_2)2.3Cu(AsO_2)$) [1] in the 1930's made an important contribution to vector control as it was effective, cheap and easy to apply. Pyrethrum insecticide (a natural insecticide derived from chrysanthemum) [2] was also introduced in the 1930's and was used with great success as it was more effective in killing the mosquitoes and cost only a third of the larval control programme. A disadvantage with the pyrethrum spraying was that it had to be repeated weekly during the peak seasons and its use was therefore labour intensive [3].

A major advance in vector control came in the form of dichlorodiphenyltrichloroethane (DDT). This compound had been synthesised for the first time in 1874 [4] but it was in the late 1930's [5] that its insecticidal properties were discovered. DDT was used in agriculture before it was introduced as an anti-malaria agent by the US army in the Second World War and after the war it was in use worldwide. The use of DDT in malaria prevention led to enormous optimism as the pesticide proved to be highly effective in killing the malaria vector, interrupting the transfer of the malaria parasite thanks to its spatial repellence and irritant effect on malaria vectors. DDT is also cheap and easy to use and has long residual efficacy when sprayed on walls and ceilings (6-12 months). In Europe and North America DDT was widely used and within a few years malaria was eradicated from both continents [3].

Due to its extensive use as a pesticide in agriculture and in malaria vector control, DDT soon became ubiquitously distributed throughout the environment. In the environment, *p,p'*-DDT is degraded to *p,p'*-DDE (1,1-dichloro-2,2-bis(4-chlorophenyl)ethene) and *p,p'*-DDD (1,1-dichloro-2,2-bis(4-chlorophenyl)ethane), *p,p'*-DDE being more persistent than the parent

compound. This persistence, induced by its high lipophilicity and low reactivity, provides the necessary conditions to bioaccumulate in organisms and to biomagnify in food webs.

DDT is highly toxic to aquatic organisms, fish and to some species of amphibians. The major DDT metabolite, DDE causes eggshell thinning leading to embryo deaths in predatory birds [6]. Lifetime treatment with DDT induced liver tumour in mice in a dose related manner. In another mice study DDT also increased incidences of lung tumour. DDE and DDD are also carcinogenic in mice [7]. IARC concluded that there is sufficient evidence for the carcinogenicity of DDT in experimental animals and has classified it as a possible human carcinogen. Many epidemiologic studies have been conducted to see if there is evidence for DDT carcinogenicity in humans. The authors have reported both positive and negative associations between exposure to DDT and the development of tumours in humans [7]. Several *in vitro* studies have shown DDT and its metabolites to have estrogenic activity [8] and DDE has been shown to act as an androgen antagonist [9].

$3\text{-MeSO}_2\text{-DDE}$ is a metabolite of *p,p'*-DDE formed by cytochrome P450, through the mercapturic acid pathway and intestinal microfloral activity. $3\text{-MeSO}_2\text{-DDE}$ is found in wildlife and humans and it has been proved to be a very potent toxicant in the adrenal cortex in mice. In the late 1950's *o,p'*-DDD was found to cause cell death in the adrenal gland in dog and has since the 1960's been used as a drug for adrenal cancer in humans with the aim to decrease cortisol hypersecretion and inhibit tumour growth.

Growing concern about DDT's adverse effects in the environment led to restrictions and bans in many countries in the early 1970's. Surprisingly, 70 years after its introduction, the available data on DDT's safety is somewhat limited. No living organism is DDT-free and the possible contribution of DDT to increase cancer risk and its potential role as an endocrine disruptor deserves further investigations.

There were an estimated 247 million malaria cases among 3.3 billion people at risk in 2006, causing nearly a million deaths, a majority of which were children under 5 years of age. It has been estimated that 109 countries were plagued with malaria epidemics in 2008, 45 within the WHO African region [10]. There is still a need for DDT and it is used for disease vector control simply because there is no alternative of equivalent efficacy and operational feasibility, especially for high transmission areas. There is an urgent need to develop alternative products and methods not only to reduce reliance on DDT and achieve its ultimate elimination but also to sustain effective malaria vector control.

To estimate the human health risk of exposure to DDT additional studies are required that also take the more persistent metabolites into consideration because our lack of knowledge today makes it impossible to accurately estimate the risks. Most of the human epidemiological studies have been conducted in developed countries where the population were exposed more than 30 years ago and now have a very low exposure to DDT. This could explain the inconclusive evidence of possible harmful effects of DDT in humans. More research should be directed towards the developing countries that still use DDT. The effects of DDT could be studied in high-risk population such as occupationally exposed people or individuals living in the malaria infested areas. Furthermore, regarding infants' health and DDT exposure, information is even scarcer. Taking into account the possible cancer risks mentioned above and that infants are still exposed to high levels of DDTs in several countries, more both pre- and postnatal studies should be done. These studies should increase our knowledge and enable more accurate risk assessments for infants. There is also a need to balance the enormous benefits for individuals at risk for malaria, and the negative environmental consequences of uncontrolled DDT use.

1.1   Aims

The *p,p'*-DDT metabolite, 3-MeSO$_2$-DDE has been proved to be a highly tissue specific toxicant in the adrenal cortex in mice. It is activated by CYP11B1 into a reactive intermediate of unknown structure which binds covalently to the adrenal cortex causing cell death. The lack of knowledge about the reactive intermediate structure made it urgent to synthesise this compound and other similar alkyl DDE sulfones in their radiolabelled and unlabelled forms for structure-reactivity relationship studies. The ability of 3-MeSO$_2$-DDE to interact with CYP11B1 makes it suitable as a PET (Positron Emission Tomography) tracer if carbon-11 is introduced into the molecule. Throughout my thesis work it was an important goal to find methods for synthesis of 3-SH-DDE to be used as a precursor for the DDE-methyl sulfones and related compounds.

o,p'-DDD has been used as a chemotherapeutic drug for adrenocortical cancer in humans since the 1960's. The efficacy and potency is however low and *o,p'*-DDD treatment is frequently associated with severe side effects. 3-MeSO$_2$-DDE has been suggested as a lead compound for an improved therapeutic agent for adrenal cancer, due to its specific toxicity in the adrenal cortex. To address this issue one objective of the research leading up to my thesis was to assess and compare pharmacokinetics of *o,p'*-DDD and 3-MeSO$_2$-DDE. Since *o,p'*-DDD is chiral it was also of interest to study the influence of the enantiomers on toxicity and distribution, as enantiomers have been known to present different pharmacodynamics and pharmacokinetics.

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2 DDT and related compounds

2.1 DDT

DDT was first synthesised in 1874 by Zeidler but its insecticidal properties were discovered by Müller in the late 1930's. Technical DDT contains 65-80% *p,p'*-DDT, 15-21% *o,p'*-DDT and up to 4% *p,p'*-DDD (Figure 2.1) [11]. DDT is an odourless colourless crystalline solid, it is lipophilic (log K_{ow} = 5.9, technical DDT log K_{ow} = 4.9-6.9), semi-volatile and its presence is ubiquitous in the environment. In the environment, *p,p'*-DDT is degraded to *p,p'*-DDE and *p,p'*-DDD. *p,p'*-DDE is more persistent than the parent compound. The physicochemical properties of DDT and its major metabolites enable these compounds to accumulate readily in organisms via the surrounding medium and food [12]. On the basis of the ecotoxicity of *p,p'*-DDT and/or its metabolites, Sweden was the first country to ban the use of DDT in the early 1970's and shortly after most developed countries followed.
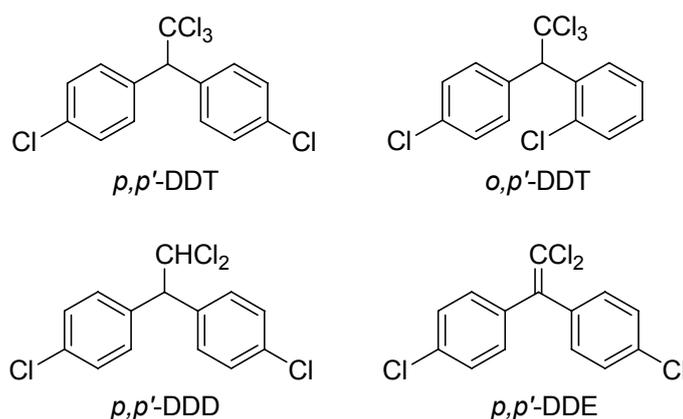

Figure 2.1. Structure of the compounds in technical DDT and one of DDT's major metabolite *p,p'*-DDE.

2.1.1 Use

DDT was widely used during the Second World War to protect military areas and civilians from the spread of malaria, typhus and other vector borne diseases [13]. It was commercialised in 1945 and was widely used in agriculture to control pest insects such as the pink boll worm on cotton, codling moth, Colorado potato beetle and the European corn borer [14]. In the early 1960's about 400,000 tonnes of DDT was used annually worldwide, of which 70-80% was used in agriculture [7]. By this time it had been credited for the eradication of malaria from the United States and Europe [15]. As a result of the environmental damage caused by DDT, its use was restricted or banned in most developed countries after 1970.

In 2001, at the Stockholm Convention on Persistent Organic Pollutants, a legally binding treaty was adopted and entered into force in 2004. In total, 162 nations have ratified the Stockholm convention document where governments will take measures to minimize and eliminate the use of 12 persistent organic pollutants (POPs). Nine of the 12 POPs are organochlorine pesticides including DDT. DDT was granted a health exemption for use in countries where malaria is still a major public health concern. However, the use is strictly regulated by the introduction of a DDT register. Presently sixteen countries have exemption to use DDT for vector control and three of them (China, India and Ethiopia) for production as well. China and India also have an exemption for DDT use as an intermediate in the production of dicofol (2,2,2-trichloro-1,1-bis(4-chlorophenyl)ethanol). Dicofol is used as a miticide for a wide variety of fruits, vegetables and crops. In contrast to DDT, dicofol does not possess any persistent characteristic but is classified as a class III "slightly hazardous" pesticide by WHO and as a possible human carcinogen by the US EPA [16].

2.1.2 Health risks

An early human study in 1956 where 51 volunteers from correctional institutions were administered low (2-3 µg/kg/day) and high (0.4-0.6 mg/kg/day) doses of DDT combined with food for 18 months showed no sign of illness or other symptoms [17]. This study also gave valuable information on DDT's absorption and storage in lipophilic tissues. DDT has low acute toxicity in humans and doses as high as 285 mg DDT/kg body weight have been accidentally ingested by humans with no fatal results [18]. DDT poisoning usually results in dizziness, headache, tremor, confusion and fatigue. Occupational exposure to DDT in retired workers from Costa Rica was associated with neurobehavioral symptoms in a dose-response pattern [19]. It is only in the last decades that more rigorous epidemiological research has focused to reveal any possible adverse effect of DDT exposure in humans. Positive associations between DDT and pancreatic-, liver- and biliary tract-cancer, multiple myeloma, cardiovascular disease and possibly diabetes have been found in different cohort studies [20]. A large study of DDT and adverse reproductive outcomes was done by Longnecker and co-workers in 2001, where 44,000 children born between 1959 and 1966 were included. The study showed a significant statistical increase in preterm births and low birth weight of this children with increasing DDE concentrations in serum [21]. Further, other studies have found increased risk of prostate cancer among farmers and pesticide applicators and increased risk for pancreatic cancer in chemical manufacturing workers and insecticide applicators exposed to DDT [20]. Other recent case studies suggest that DDT may be related to neurological impairment and that various neurobehavioral functions deteriorated

significantly with increasing years of DDT application in retired malaria-control workers [20]. Although many epidemiological studies associate different types of cancer and other adverse outcomes with DDT as described above, there are as many that fail to find any association making it very difficult for authorities to set policies about DDT's future use.

2.1.3   Effects in biota - mechanism of action

DDT is toxic to freshwater and marine organisms, fishes, amphibians and birds. Numerous studies have shown a link between DDE and eggshell thinning in predatory and fish-eating birds. Possible mechanisms of this effect have been studied and the leading hypothesis involves an inhibition by *p,p'*-DDE of prostaglandin synthesis in the shell gland mucosa [22]. Prostaglandins are lipid compounds derived from fatty acids. They are hormones with a wide variety of actions such as regulation of the calcium movement. Calcium carbonate from the shell gland is important for the formation of the egg shell. Other DDT-induced hormonal imbalances are associated with e.g. embryo lethality, decreased egg size and weight and reduced post-hatch survival in avian wildlife. The estrogenicity of DDT also induces hormonal imbalances in alligators affecting reproduction. *p,p'*-DDE in particular but organochlorines in general also influence sexual dimorphism in turtles. Reviews have suggested that during periods of energy stress (starvation, nesting, migration or thermal stress) DDT is mobilized from the fat deposits and is redistributed to the brain where it induces neurological effects in wildlife [11].

2.2   DDD

Technical DDT contains about 15-21% *o,p'*-DDT and 4% *p,p'*-DDD. *p,p'*-DDD by itself was used as an insecticide but is no longer commercially produced. There are no production figures available for *p,p'*-DDD but the production is believed to have been small. *o,p'*-DDT is degraded to *o,p'*-DDD in the environment, to a large extent by abiotic reductive dechlorination. However, low levels are usually found in the environment. *o,p'*-DDD under the brand name Lysodren (Bristol, Meyer) [11] is used as a chemotherapeutic drug for adrenal cancer due to its selective toxicity to the adrenal cortex. Lysodren was approved by the Food and drug administration (FDA, USA) in 1970 but is not available in all countries and was approved by the European Medicines Agency as late as in 2004 [23].

2.2.1   *o,p'*-DDD toxicity - mechanism of action

p,p'-DDD was found in the late 1940's to induce cytotoxic atrophy in the adrenal gland in dogs [24]. Some years later other scientists came to the conclusion that the atrophy studied was caused by the contaminant *o,p'*-DDD

and not *p,p'*-DDD [25,26]. The suggested mechanism of action causing the cell death is a CYP-catalysed hydroxylation of the side chain β-carbon and a subsequent dehydrochlorination resulting in a reactive acylchloride. This reactive intermediate binds covalently to mitochondrial proteins or is transformed to *o,p'*-DDA by addition of water [27,28] (Figure 2.2). Another proposed mechanism contributing to the cytotoxicity is oxidative damage through production of free radicals [29]. Dogs are sensitive to *o,p'*-DDD toxicity, but it has also been proven a selective toxicant in human [30], birds [31] and mink [32].
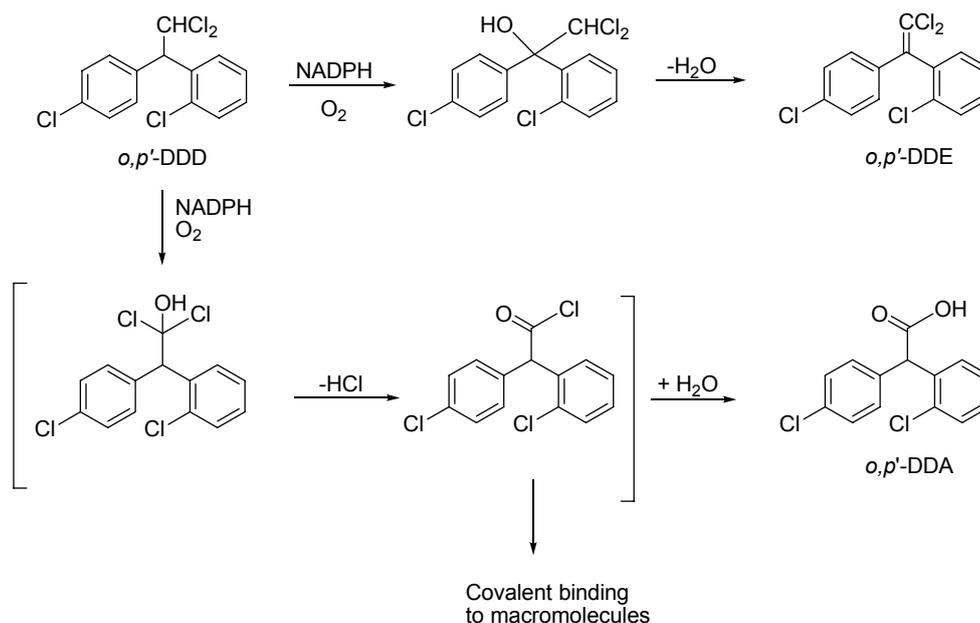

Figure 2.2. Proposed pathways for the metabolism of *o,p'*-DDD through aliphatic oxidation in mammals.

2.2.2 Adrenal glands

The adrenal glands are located in the thoracic abdomen situated atop the kidneys. They are surrounded by the adipose capsule and the renal fascia. The adrenal gland consists of two parts, an inner medulla and an outer cortex (Figure 2.3). The medulla produces mainly adrenaline, noradrenaline and dopamine. The cortex is divided into three functional layers; *zona glomerulosa* which is the main site for production of mineralocorticoids such as aldosterone, *zona fasciculata* which is responsible for producing glucocorticoids (cortisol/corticosterone) and *zona reticularis*, producing androgens. All adrenocortical hormones are synthesised from cholesterol. Cholesterol is transported to the inner mitochondrial membrane where it is converted into pregnenolone. Accordingly, production of hormones in the adrenal cortex is limited by transport and conversion of cholesterol. Damages to the adrenal gland may have serious consequences for the steroid hormone synthesis. Aldosterone disorder may affect the regulation of extracellular

potassium or sodium levels and blood volume. Cortisol derangement could affect among other things lipolysis as well as production and levels of glucose. High blood supply and high lipid content makes the adrenal gland a target organ for many xenobiotics [11].
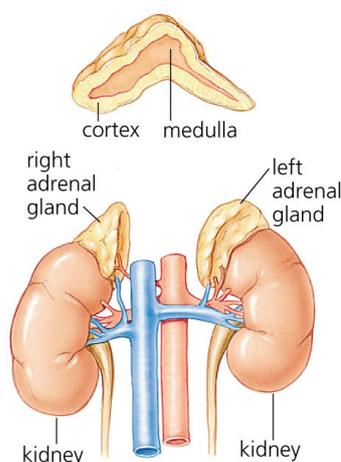

Figure 2.3. The adrenal glands are located above the kidneys and divided in an inner medulla and outer cortex.

2.2.3 Adrenal disorders

Adrenal cortical carcinoma (ACC) is a rare aggressive cancer form with an incidence rate of 0-2 cases per million persons per year and can develop at any age in both women and men. The incidence peaks of ACC seem to occur in the first and fourth decades of life. Several studies have shown that children have better prognosis after tumour removal than adults. ACC carries a poor prognosis due to the difficulty to diagnose in an early phase and the poor response of chemotherapeutic agents, only 20-25% of the patients survive more than 5 years after the diagnosis [33]. ACC's main symptoms are Cushing's syndrome (cortisol excess), Conn syndrome (aldosterone excess) and feminization/virilism (estrogen/androgen excess) [34]. The best curative treatment is complete surgical excision of the tumour but when the tumour is inoperable or recurrent, *o,p'*-DDD (Lysodren) is used as an adjuvant drug. *o,p'*-DDD appears to be the only pharmacological agent that both inhibits corticoid biosynthesis and destroys adrenocortical cells. Despite its effectiveness as a cytotoxic drug the overall results of *o,p'*-DDD therapy have not been uniform from one group to the next and the treatment has been limited by serious side effects (nausea, vomiting, anorexia, diarrhoea, lethargy and somnolence). Several reports indicate that only one third of the treated patients respond to *o,p'*-DDD [34].

2.3 3-MeSO₂-DDE

3-MeSO$_2$-DDE is a metabolite of *p,p'*-DDE, formed by several steps involving activation and conjugation. *p,p'*-DDE is oxidized to an arene oxide by cytochrome P450, then reacts with glutathione (GSH) and after dehydration the conjugate is transformed via the mercapturic acid pathway (MAP) to a cysteine conjugate. The cysteine conjugate is then excreted with the bile to the gastrointestinal tract where the conjugate is cleaved by C-S lyase, also called β-lyase, to SH-DDE. SH-DDE is methylated by S-adenosylmethionine (SAM) and oxidized in a two step oxidation by CYP-450 in the liver to 3-MeSO$_2$-DDE [35-37] (Figure 2.4).
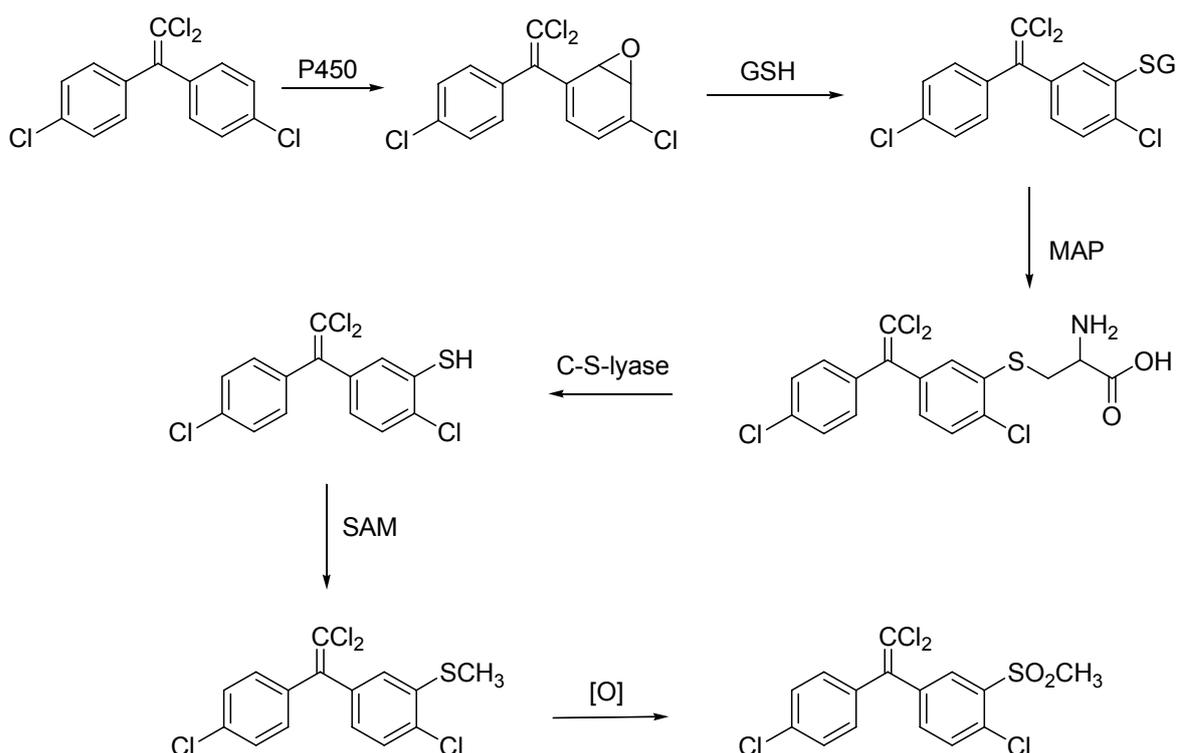

Figure 2.4. Schematic pathway for biotransformation of *p,p'*-DDE to 3-MeSO$_2$-DDE.

3-MeSO$_2$-DDE was first detected in seal blubber from the Baltic sea in 1976 [38] and nowadays it is frequently reported in wildlife [39-41] and humans; in human milk [42], human tissues [43] and plasma [44]. 3-MeSO$_2$-DDE is also detected in high concentrations in humans from countries where people are still exposed to DDT, e.g. in Mexico [45] and Slovakia [46] but is decreasing with time in countries where there is no recent use of DDT, as shown in human milk from Sweden [47].

2.3.1 3-MeSO₂-DDE toxicity - mechanism of action

3-MeSO$_2$-DDE is also a very potent toxicant to the adrenal cortex causing cell death particularly in mice [48]. 3-MeSO$_2$-DDE is activated by CYP11B1 into a reactive intermediate which binds covalently to adrenocortical proteins causing cell death [49]. The structure of the reactive intermediate that

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generates the toxicity is still unknown. The endogenous function of CYP11B1 is to catalyse the formation of adrenal glucocorticoids and decreased corticosterone plasma levels have been observed both in suckling mouse pups and in the lactating dam following a single oral dose to the dam. This finding indicates a reduced CYP11B1 activity [50]. There are known species differences regarding the adrenocorticolytic activity of 3-MeSO$_2$-DDE, it binds irreversible in mice [51] and ex-vivo in hamster, rat and guinea pig tissue slice [52] but not in mink [32]. The metabolic activation and covalent binding of 3-MeSO$_2$-DDE has also been studied *ex-vivo* in normal and cancerous human adrenal tissue slice culture. 3-MeSO$_2$-DDE was selectively bound to the *zona fasciculata* and *reticularis* where CYP11B1 is expressed but no binding was observed in the *zona glomerulosa* and the adrenal medulla [53]. Studies in pregnant and lactating mice showed high and tissue specific irreversible binding of 3-MeSO$_2$-DDE in the adrenal cortex of the foetus and pups. 3-MeSO$_2$-DDE was transferred to the foetus and to the pups via the placenta and milk. These findings indicated that the mechanism of metabolic activation of 3-MeSO$_2$-DDE is functional from foetal life to adulthood [54].

2.4   DDTs in human milk

The relatively rich lipid content of human milk (2-5%) compared to plasma and the non invasive sampling method makes milk an ideal matrix for monitoring lipophilic pollutants. It provides a measure of maternal body burden and an opportunity to estimate the intake levels by infants during breastfeeding. Human milk fat is composed of 98% triglycerides, 0.7% phospholipids and 0.5% cholesterol [55]. The fat composition in human milk can however vary according to maternal BMI (body mass index), dietary intake, smoking habits and weight loss during lactation. Twenty five percent of the lipids in human milk comes from diet and the remaining, 75% is mobilized from the stored fat in adipose tissue [56], making human milk a good elimination pathway for exogenous lipophilic compounds. There are several factors that can affect the levels of exogenous lipophilic compounds in human milk e.g. number of births, maternal age, diet and timing of sampling [57].

There are numerous studies reporting DDT and DDE concentrations in human milk from various countries (Table 2.1). It seems clear that DDT and DDE levels are in general decreasing worldwide, more pronounced in countries where DDT has been banned for many years [58-61]. In these countries the detected levels of DDTs in human milk and plasma are a reflection of food exposure. In contrast to the decreasing levels in developed countries, countries in Latin America, Africa and Asia still use DDT or have used until recently for vector control, leading to high human exposure of DDT (see Table 2.1).

Very little information is available on adverse effects of DDT/DDE exposure in infants exposed via breastfeeding. However, much more has been done concerning adverse effects on infant development after pre- or postnatal PCB exposure. Negative association between postnatal exposure to environmental levels of PCBs and infants' mental and motor development has been reported [62,63].

Several laboratory animal studies showed that *p,p'*-DDE exhibited prenatal antiandrogen activity and delayed the onset of puberty in rats [9]. Perez (2003) has shown that DDE induces apoptosis in human mononuclear cells *in vitro* and DDE exposure is associated with increased apoptosis in Mexican children. This finding could implicate a health risk, considering the chronic exposure to DDE and the potential effects of apoptosis in cells of the immune system [64].

Unfortunately there are only a few studies that have looked at $3\text{-MeSO}_2\text{-DDE}$ levels in human milk. Mother's milk may be an important route of exposure of $3\text{-MeSO}_2\text{-DDE}$ for breastfed children. Children differ from adults in their susceptibility to hazardous chemicals due to the fact that many physiological systems are not fully developed. To my knowledge there are no studies of adverse effects in children exposed to $3\text{-MeSO}_2\text{-DDE}$ via milk or the placenta. Studies in animals have shown that the amounts of DDT/DDE transferred via mother's milk are much greater than the amounts transferred to the foetus via the placenta [65].

Taken into consideration the risk for adrenal damages caused by $3\text{-MeSO}_2\text{-}$DDE it is important to study the levels of this metabolite in highly exposed populations and especially in the more vulnerable infants. WHO has established an acceptable daily intake (ADI) value for ∑DDTs of 20 µg/kg/day [66] but in many countries breastfed infants have daily intakes above the recommended ADI. In a Mexican study [67] the estimated daily intake (EDI) via maternal milk ranged from 1 to 414 µg/kg/day. The EDI also exceeded the recommended ADI in breastfed children in Thailand, Tunisia and Brazil with a mean value of 51 [68], 24 [69] and 33 µg/kg/day [70], respectively. In South Africa the EDI for DDE significantly exceeded the WHO guideline in almost all the milk samples ranging from 260-4,700 µg/kg/day [71]. These results indicate that many of the infants are exposed to higher levels than recommended and the possible health implications cannot be ignored. Ironically, it has been suggested that increased levels of DDE are associated with a reduced period of lactation [72].

Table 2.1. Median (min-max) concentrations (ng/g lipid weight) of p,p'-DDT, p,p'-DDE and 3-MeSO$_2$-DDE in human milk in various countries. (References to the authentic scientific reports or reviews are given in the table).

Country	year	n	p,p'-DDT	p,p'-DDE	3-MeSO$_2$-DDE	Ref
Australia	1995	60	225 (6-960)	960 (150-3900)	n.r.	[73]
Australia	2002-2003	157	6.96	279	n.r.	[74]
Belgium[1]	2006	197	n.d.	211	n.r.	[75]
Brazil	1992	40 (1p)	180	1520	n.r.	[76]
Brazil[2]	2001-2002	69	72	343	n.r.	[70]
Canada	1992	497	18.7	169	n.r.	[59]
Canada	1992	50			0.26	[77]
China (Beijing)	1983	50	1630	5890	n.r.	[78]
China (Beijing)	2005-2006	40	3.9 (1.2-17)	112 (30-1010)	n.r.	[79]
China (Shenyang)[1]	2002	20	40	830	n.r.	[80]
China (Shenyang)	2006-2007	36	3.5 (n.d-14.6)	117 (15.7-763)	n.r.	[79]
China (Hong Kong)[1]	1999	132	390	2480	n.r.	[81]
China (Hong Kong)[1]	2001-2002	238 (10p)	99 (71-166)	1380 (810-1910)	n.r.	[82]
China (Tianjin)[2]		50		10.4 (5.3-20.2)	n.r.	[82]
Czechoslovakia[1]	1993	26	716	1129	n.r.	[83]
Denmark	1997-2001	65	6 (2-38)	134 (25-428)	n.r.	[84]
Egypt[1]	1996	60	2.9	21.5	n.r.	[83]
Finland	1997-2001	65	3 (1-13)	59 (19-331)	n.r.	[84]
Germany[1]	1991	113	30	500	n.r.	[81]
Germany	2005	39	4 (LOQ-60)	87 (20-1070)	n.r.	[85]
Greece[1]	1995-1997	112	66	721	n.r.	[83]
Indonesia (Purwakarta)	2002	18	17 (2.2-2400)	430 (25-12000)	n.r.	[86]
Italy[1]	1987	64	150	2200	n.r.	[81]
Italy	1998-2000	29	(9.4-44)	(210-510)	n.r.	[87]
Japan[1]	1989	6			0.1	[88]
Japan[3]	1972	12	538 (130-1380)	1686 (640-2630)	n.r.	[58]

n.d. = not detected, n.r. = not reported, p = pooled samples, [1] mean, [2] geo mean, [3] average

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Table 2.1 (cont.). Median (min-max) concentrations (ng/g lipid weight) of p,p'-DDT, p,p'-DDE and 3-MeSO$_2$-DDE in human milk in various countries. (References to the authentic scientific reports or reviews are given in the table).

Country	year	n	p,p'-DDT	p,p'-DDE	3-MeSO$_2$-DDE	Ref
Japan[3]	1998	49	18 (43-1227)	270 (77-997)	n.r.	[58]
Jordan[1]	1992	59	2522	5680	n.r.	[83]
Kuwait[1]	2000	32	12	833	n.r.	[83]
Mexico[1]	1997-1998	60	650 (nd-4270)	4000 (180-34280)	n.r.	[89]
Mexico (Huasteca Potosina)[2]	2006	32	126 (19-5661)	503 (37-4423)	7 (0.15-176)	[67]
Mexico (San Luis Potosi)[2]	2006	20	28 (13-121)	54 (20-312)	0.1 (0.1-0.-8)	[67]
Mexico (El Ramonal)	2004	7	911 (323-2071)	3100 (1153-15875)	2.8 (0.2-9)	[67]
Norway	2000-2002	29	8 (2.8-15)	99 (34-278)	n.r.	[61]
Poland	1989-1992	277	537	5745	n.r.	[90]
Russia[1]	1996-1997	140	133 (3-691)	900 (70-3824)	n.r.	[91]
Saudi Arabia[1]	1998	115	65	183	n.r.	[83]
South Africa[1]	2004	30	(n.d-1880)	(560-2570)	n.r.	[71]
Sweden	1972	75	630	2300	5	[60]
Sweden	1992	20	32	251	0.5	[60]
Thailand	1998	25	2630	6540	n.r.	[68]
Tanzania	1992	9	3034	2547	n.r.	[92]
Tunisia[1]	2003-2005	237	256 (1-2499)	676 (3-6800)	n.r.	[69]
UK[1]	1997-1998	168	40	430	n.r.	[83]

n.d. = not detected, n.r. = not reported, p = pooled samples, [1] mean, [2] geo mean, [3] average

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3 Synthesis of alkyl aryl sulfones

Sulfones are useful in a wide range of fields such as agrochemicals, pharmaceuticals and polymers [93,94]. The main method of preparing alkyl aryl sulfones is sulfonylation via the Friedel-Craft (FC) reaction between alkylsulfonyl halides and aromatic compounds catalysed by a suitable Lewis acid (e.g., $AlCl_3$, $FeCl_3$, $ZnCl_2$, $SbCl_5$, CF_3SO_3H) [95](Figure 3.1).
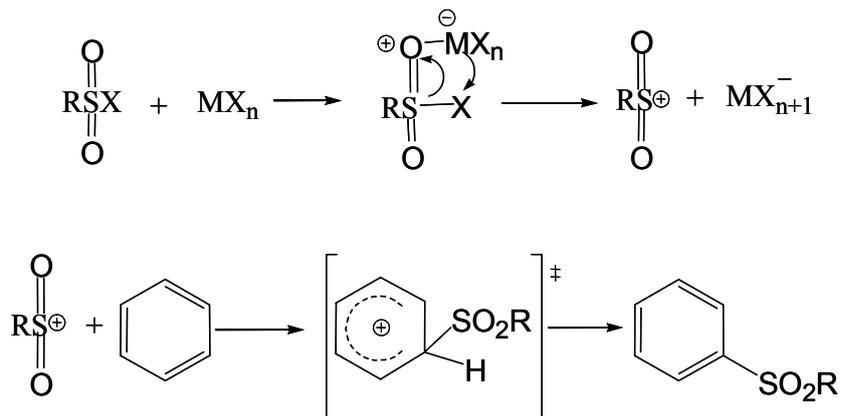

Figure 3.1. Mechanism of Friedel-Craft sulfonylation catalysed by a Lewis acid.

The disadvantages of this method have been low selectivity of sulfonylated isomers, the use of hazardous and moisture-sensitive Lewis acids and the highly corrosive conditions of the reagents. A more eco-friendly method for FC sulfonylation has been suggested by Choudary (2000) where Lewis acids are replaced by solid acids. Solid acids such as Fe^{+3}-montmorillonite and zeolite beta showed higher *para* selectivities compared to the conventional method [96]. In FC sulfonylation the reaction kinetic depends on the catalytic strength of the Lewis acid, the electrophilicity of the sulfonyl reagent and the activity of the aromatic compound. Many researchers have tried to increase the reactivity of the catalysts used in FC sulfonylation and by this method increase the yield [97-99]. The activity of triflic acid (CF_3SO_3H), has been dramatically increased by the addition of a catalytic amount of bismuth(III) chloride by forming bismuth triflate ($Bi(OSO_2CF_3)_3$) [100].

The Newman-Kwart rearrangement (NKR) is a well studied and valuable method for converting phenols to thiophenols [101,102] via O-thiocarbamates (Figure 3.2). This approach can be used to access other sulfur-containing functional groups such as sulfones. The advantages of this method are the use of cheap phenols with a wide variety of substitution patterns and the selectivity of the thiol-position. The disadvantage is the high temperature required for the rearrangement (200-300°C) which is necessary since the

migration of the O- to S-aryl requires high activation energy. Electron withdrawing groups are known to aid the rearrangement, either reducing the reaction time or lowering the required temperature. Electron donating groups and sterically hindered compounds on the other hand have shown to slow down the migration or to inhibit it [103]. The rearrangement is proposed to proceed via a four-centre transition state and should be stabilised by polar solvents but only very little sensitivity to solvents has been demonstrated. However, a significant reaction rate increase is observed when formic acid is used as a solvent [104].
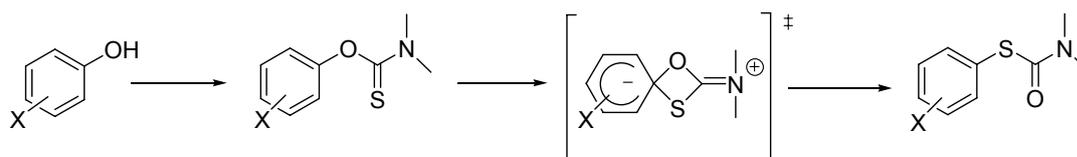
Figure 3.2. Proposed Newman-Kwart rearrangement via a four centre transition state.

3.1 Background to Paper I

3-MeSO$_2$-DDE is a metabolite of *p,p'*-DDE and is nowadays detected in wildlife [39-41] and in humans [42-44]. 3-MeSO$_2$-DDE is activated in the adrenal cortex by CYP11B1 to form a reactive intermediate which binds covalently to adrenocortical proteins causing cell death [49] in specific species. Accordingly there have been several reasons to synthesise both un-labelled and radiolabelled 3-MeSO$_2$-DDE to promote toxicological studies. One of the aims in **Paper I** was to improve the poor yield of the established sulfonylation of *p,p'*-DDE [105]. Furthermore, since the structure of the reactive metabolic intermediate is not yet known, it was also an aim to synthesise 3-SH-DDE as a precursor to be used for structure-reactivity related studies of different alkyl DDE sulfones.

Positron emission tomography (PET) is a nuclear medicine imaging technique used for example in imaging tumours, in the search of metastases and in the diagnosis of certain diffuse brain diseases. PET produces a three-dimensional image or picture of functional processes in the body by detecting gamma rays emitted indirectly by a positron-emitting radionuclide (tracer). Radionucleides used in PET scanning are typically isotopes with short half lives such as [11]C (~20 min), [13]N (~10 min), [15]O (~2 min), and [18]F (~110 min). PET technology can be used to trace the biological pathway of any compound in living humans (and many other species as well), provided it can be radiolabelled with a PET isotope. The ability of 3-MeSO$_2$-DDE to interact with CYP11B1 makes it suitable as a PET tracer if carbon-11 is introduced into the 3-SH-DDE compound.

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At the department we have some experience in NKR, e.g. it is applied to convert 2,6-dichlorophenol to 2,6-dichlorothiophenol, a conversion that was afforded in high yields. 2,6-dichlorothiophenol was further methylated with [14C]-methyl iodide and oxidized to 2,6-dichloro-[14C]methylsulfonyl-bensen (unpublished). Labelled 2,6-dichloro-methylsulfonyl-bensen was required for toxicological studies since it has been found to be a tissue selective toxicant in the olfactory mucosa in rodents. The toxicity is manifested as necrosis in the Bowman's gland followed by degeneration and shedding of the neuroepithelium [106]. The satisfactory results obtained in the conversion of phenol to thiophenol made us expect positive results in the conversion of 3-OH-DDE to 3-SH-DDE.

3.2 Synthesis of 3-SH-DDE

Repeated attempts to synthesise 3-MeSO$_2$-DDE via sulfonylation of p,p'-DDE with methanesulfonic anhydride and aluminium chloride were made (Scheme 3.1) [105]. Unfortunately, formation of undesired by-products and low yield made me look for new synthetic methods.
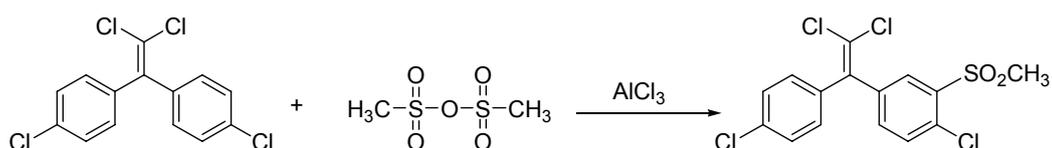
Scheme 3.1

Bismuth triflate (Bi(OSO$_2$CF$_3$)$_3$) has been shown to catalyse FC sulfonylation of several aromatic compounds, including non-activated ones [107], and was tested in the sulfonylation of p,p'-DDE (Scheme 3.2).
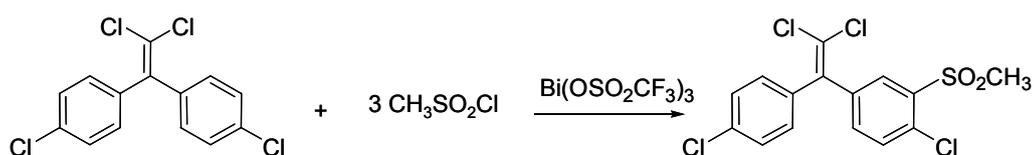
Scheme 3.2

The proposed mechanism was a ligand exchange between the catalyst (Bi(OSO$_2$CF$_3$)$_3$) and sulfonylchlorides leading to the thermally unstable trifluoromethanesulfonic alkylsulfonyl anhydride (RSO$_2$OSO$_2$CF$_3$). In a second step, RSO$_2$OSO$_2$CF$_3$ reacts with an arene and gives the desired ArSO$_2$R and triflic acid. In a third step the triflic acid reacts with a new sulfonyl chloride and the RSO$_2$OSO$_2$CF$_3$ is re-formed *in situ* [107]. Unfortunately, this attempt failed and no sulfonylation was achieved. One reason could be heterolytic dissociation of RSO$_2$OSO$_2$CF$_3$ to RSO$_2^+$

$OSO_2CF_3^-$ and a subsequent loss of SO_2 yielding inactive alkyltriflate R^+O $SO_2CF_3^-$ as has been suggested for alkylsulfonyl chlorides[108] (Figure 3.3).

$$R\text{-}SO_2\text{-}O\text{-}SO_2CF_3 \xrightarrow{\text{Ar-H}} R\text{-}SO_2\text{-}Ar$$

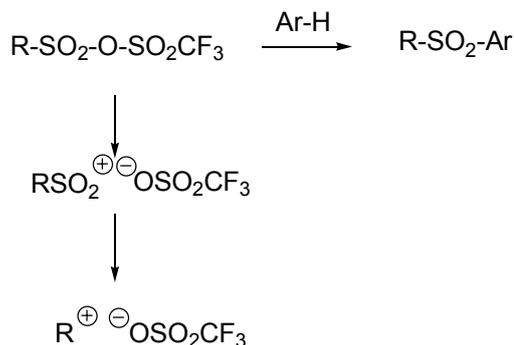$$RSO_2^{\oplus}\,{}^{\ominus}OSO_2CF_3$$

$$R^{\oplus}\,{}^{\ominus}OSO_2CF_3$$

Figure 3.3. Proposed dissociation of trifluoromethanesulfonic alkylsulfonyl anhydride in the sulfonylation of arenes.

NKR was used in the conversion of 3-OH-DDE to 3-SH-DDE but first the appropriate starting material, 3-OH-DDE, had to be synthesised. The requirement of having the OH-group in the *meta*-position of the DDE, resulted in that 2,2,2-trichloro-1-(4-chloro-3-methoxyphenyl)ethanol (**5**) was chosen as a reagent in the DDT synthesis. In order to prepare **5** a multi-step procedure was developed as described in **Paper I** and shown in Scheme 3.3.
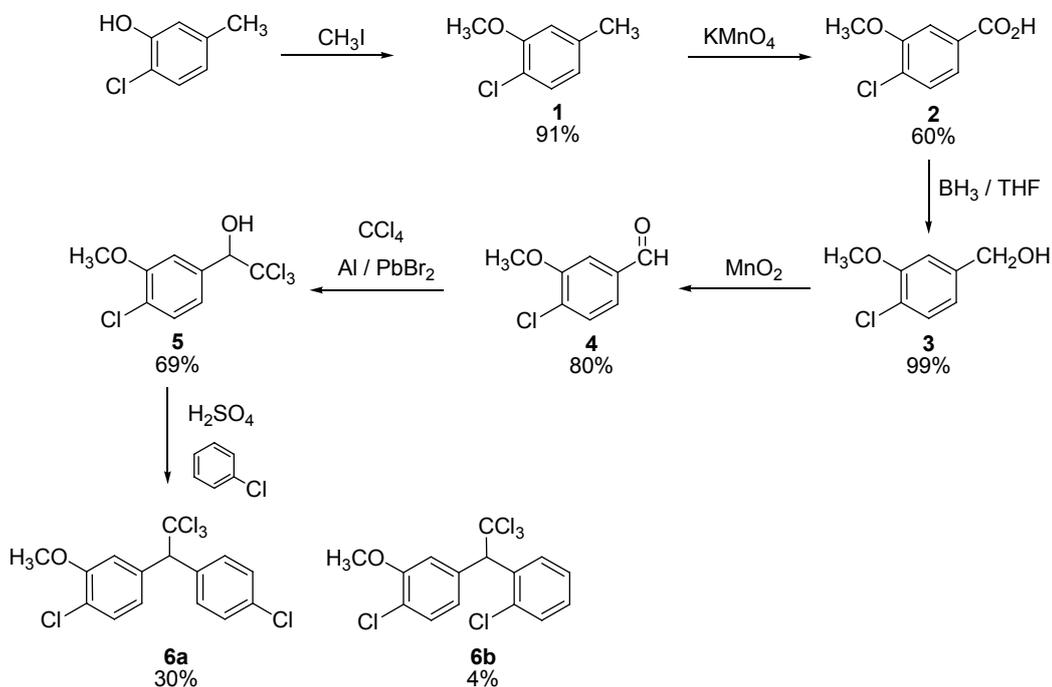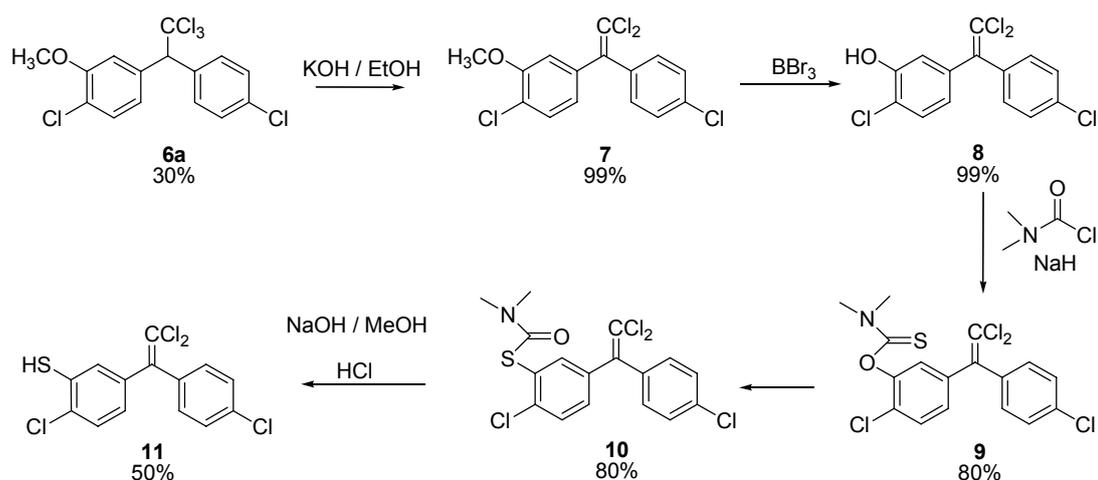

Scheme 3.3. Synthesis of 2-(4-chlorophenyl)-2-(3-methoxy-4-chlorophenyl)-1,1,1-trichloroethane, 3-MeO-DDT and overall yield.

Briefly, 2-chloro-5-methylphenol was first methylated and further oxidized to the corresponding benzoic acid (**2**) by potassium permanganate. The acid was

reduced to 4-chloro-3-methoxy-benzaldehyde (**4**) via 4-chloro-3-methoxy-benzene methanol (**3**) by boron hydride and manganese oxide, respectively. A lead/aluminium bimetal system was used to carry out the reductive addition of tetrachloromethane to **4** to obtain 2,2,2-trichloro-1-(4-chloro-3-methoxy-phenyl)ethanol (**5**), the starting material needed for the synthesis of the DDT-analogue. 3-MeO-DDT (**6a**) was synthesised as described by Bailes, 1945 (Scheme 3.3) [109].

Further, the desired 3-SH-DDE (**11**) was afforded after five steps as shown in Scheme 3.4. 3-MeO-DDT was reduced to 3-MeO-DDE (**7**) with potassium hydroxide in ethanol and demethylated with boron tribromide in dichloromethane to 3-OH-DDE (**8**). 3-OH-DDE was deprotonated by sodium hydride and formed O-DDE dimethylthiocarbamate (**9**) after a nuchleophilic substitution attack on dimethyl thiocarbamoyl chloride. The desired product, S-DDE dimethylthiocarbamate (**10**) was obtained by heating **9** in a sealed glass vial for the NKR as described in **Paper I**. Small sample amounts were taken from the reaction vial at different times and the conversion was followed by GC/MS. When the conversion was successful the product was hydrolysed with sodium hydroxide in methanol to afford **11**.

Scheme 3.4. Synthesis of 2-(4-chlorophenyl)-2-(4-chloro-3-thiophenol)-1,1-dichloroethene, 3-SH-DDE , via NKR and overall yield.

Since the synthesis of the starting material, 2,2,2-trichloro-1-(4-chloro-3-methoxyphenyl)ethanol (**5**) required several steps, one attempt to reduce the steps and in that way improve the synthetic method was made. Iodine(V) reagents are used in organic synthesis as a tool for single electron transfer-based oxidation of several compounds [110-112]. *o*-iodylbenzoic acid (IBX) has been used in benzylic oxidation to aldehydes in high yields [113]. IBX was synthesised as described earlier [114] using oxone (potassium peroxymonosulfate sulfate) and tested in the oxidation of 1-chloro-2-methoxy-4-methyl-benzene (**1**) to the corresponding aldehyde (**4**) (Scheme

3.5). Nicolaou has suggested a single electron transfer (SET) mediated reaction forming a benzylic radical intermediate which undergoes a second IBX facilitated SET to give a benzylic carbocation.
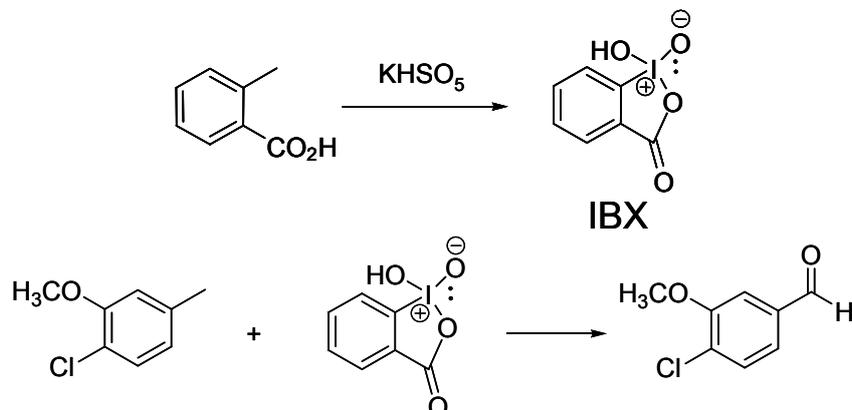
Scheme 3.5

All the reactions in **Paper I** were followed by TLC when possible and the products were purified by crystallisation or silica gel columns. All products were identified by GC/MS analysis on an ion trap GCQ Finnigan MAT instrument, operated in electron ionization (EI) mode. The GC was equipped with a DB-5HT (30m × 0.25mm × 10μm) column from J&W Scinetific (Folsom, USA). ^{1}H-NMR and ^{13}C-NMR spectra were recorded in CDCl$_3$ on Bruker Avance II spectrometer at 400 MHz with CDCl$_3$ as internal standard.

3.3 Results and discussion

Unfortunately, IBX did not oxidise 1-chloro-2-methoxy-4-methyl-benzene but the reaction was only tested once. Hence, it may still be worth trying with other reaction times and temperatures.

The method used in **Paper I** to synthesise 4-chloro-3-methoxy-benzaldehyde (**4**) has been described before [115] but some minor modifications were made therein and the overall yield after 4 steps was 43%.

DDT can be synthesised in various ways by using different reaction temperatures and reaction times [109,116]. In **Paper I** the reaction mixture was heated after adding the reactants and the reaction was stopped after 6 hours. A rather poor yield of 30% was obtained compared to other studies where pure 4,4'-DDT has been afforded in 45-60% yield [117]. The 4,4'-DDT synthesis in general seems to be a reaction with poor yield, this may be explained by competing reactions giving undesired by-products. In this work two isomers are formed, 3-MeO-4,4'-DDT (**6a**) and 3-MeO-2',4-DDT (**6b**) in a 7:1 ratio. According to Mosher (1946) the sulfonation of the reagents is another competing reaction which could be the main limiting factor in the condensation of DDT [116].

The conversion of 3-OH-DDE to 3-SH-DDE was followed by GC/MS since the aryl dimethylthiocarbamate compound are known to give specific fragments that are characteristic for the S- and O-aryl demethylthiocarbamate compound, respectively [118]. The principal fragmentation by EI is the cleavage of the bond between oxygen or sulfur and an adjacent carbon resulting in the formation of four possible types of ions (Figure 3.4).
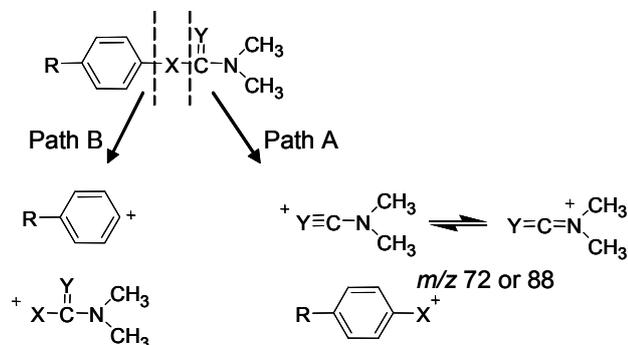

Figure 3.4. Proposed ions formed in GC/MS (EI), where Y and X could be S or O depending on the aryl dimethylthiocarbamate analysed.

Prabhakar found that the predominant ion formed in the S-aryl dimethylthiocarbamates is m/z 72 corresponding to $[OCN(CH_3)_2]^+$. Whereas, O-aryl dimethylthiocarbamates showed two ions; m/z 88 $[SCN(CH_3)_2]^+$ and m/z 72 $[OCN(CH_3)_2]^+$. O-aryl dimethylthiocarbamate was suggested to undergo a NKR rearrangement at the ion source in the mass spectrometer. S-aryl dimethylthiocarbamates are more thermally stable and do not undergo rearrangement in the ion source which only gives the characteristic fragment m/z 72 as the predominant ion. Using this theory we followed the conversion of O-DDE-dimethylthiocarbamate to S-DDE-dimethylthiocarbamate by GC/MS (EI) and the optimal reaction conditions were determined. The only ions observed for O-DDE dimethylthiocarbamate were m/z 88 (100), 72 (90) and also the ion corresponding to $[M-Cl]^+$ m/z 384 (75). For S-DDE dimethylthiocarbamate ions at m/z 72 (100) and 384 (20) were observed. $[M-Cl]^+$ is usually only observed for O-aryl dimethylthiocarbamates if the aryl has an electron withdrawing group and has not been observed for the S-aryl dimethylthiocarbamates, which is not the case here though the intensity of $[M-Cl]^+$ is less for S-DDE dimethylthiocarbamate.

The ideal condition for the NKR was at 250°C for 30 minutes. Below this temperature no rearrangement was observed and above 250°C the O-dimethylthiocarbamate bond was cleaved and the starting material, 3-OH-DDE was recovered. Longer reaction times resulted in decreasing yields, DDE-dimethylthiocarbamate has one chlorine atom in the *ortho*-position which seemed to enhance the reaction rate [103], as shown by the relatively short reaction time. The NKR gave a satisfactory overall yield of 64% and the hydrolysis of S-DDE dimethylthiocarbamate gave a lower yield of 50%

something which could be improved since the reaction was incomplete after recovering non-hydrolysed starting material.

The overall yield after 11 synthetic steps is low (~3%) and evidently reflects the low yield (30%) obtained in the DDT synthesis. Taken into consideration that almost all the step (8 of 11) gave yields above 70% and that the low yield of the hydrolysis step and the oxidation of 1-chloro-2-methoxy-4-methyl-benzene to 4-Chloro-3-methoxy-benzaldehyde could be improved, this can be considered a good way to synthesise 3-SH-DDE. The DDT synthesis could possibly also be improved by testing the effect of different catalysts on reaction yield.

4 Toxicokinetics in general

Toxicokinetics is often studied in conjunction with toxicodynamics. Toxicodynamics explores the interaction of potentially toxic substances with target sites and the biochemical and physiological consequences. Toxicokinetics includes the study of the process of **a**bsorption and **d**istribution of a potentially toxic substance and the rate at which it is eliminated from the body due to **m**etabolism and/or **e**xcretion (ADME).

The absorption of a substance into an organism is dependent on the administration/exposure route (via the respiratory tract, gastrointestinal tract or skin) and involves processes such as active and passive transport. After entering the blood, the distribution of the substance is dependent on the blood flow and on the affinity of a substance for various tissues. Substances absorbed and distributed in the body are biotransformed by metabolic enzymes mainly in the liver to more water soluble substances to enhance their elimination, of course biotransforming enzymes are widely distributed throughout the whole body. In humans, the main routes of elimination of an absorbed substance or its metabolites are excretion via urine and/or feces. Kidney excretion is one of the most important routes as a lot of substances are eliminated from the body via this organ, even though substances have to be biotransformed to more water–soluble products prior to excretion in urine. The other major elimination pathway is fecal excretion of non-absorbed substances, via bile and intestinal excretion. Other routes of elimination can be of some importance such as exhalation of volatile substances/metabolites and via mother's milk for lipid-soluble compounds. Human milk has a lipid content of 3-4% and lipid-soluble substances diffuse along with fats from plasma into the mammary gland and are excreted with milk during lactation. The secretion of toxic compounds into milk may be of particular importance as it can expose the infant to potentially high concentrations of the pollutant.

The simplest method to gather information on the ADME of a compound is by sampling blood/plasma over time. Changes in plasma concentrations of the substance are then considered to reflect the changes in tissue concentrations. Four different parameters are important in toxicokinetic studies. 1) Apparent volume of distribution (V_d) is a hypothetical volume of fluid required to contain the total amount of a substance in the body at the same concentration as that present in plasma. The magnitude of V_d is substance-specific and represents the extent of distribution of a substance out of plasma into other tissues. Thus, substances with high affinity for tissues will have V_d values that exceed the actual body volume and a substance that predominantly remains in the plasma will have a low V_d that approximates the actual volume of plasma.

2) Clearance (Cl) describes the rate of elimination (biotransformation and excretion) from the body for a substance. Cl is described in terms of volume of fluid containing substance that is cleared per unit of time (e.g. mL/min). High Cl values indicate an efficient and rapid removal of the substance whereas low Cl values show a slow and less efficient removal from the body. 3) The biological half life ($t_{1/2}$) describes the time required for the amount of a substance in a biological system to be reduced to one half of its primary value. The biological half life is dependent of the biotransformation rate, excretion and storage. Binding to or dissolving in tissues such as plasma proteins, liver, kidney and fat can result in longer half-lives. 4) The extent of absorption of a substance by a living organism can be determined in toxicokinetic studies and is called bioavailability (F). F values ranges between 0 and 1, complete absorption gives an $F = 1$ and F values < 1 indicates incomplete absorption due to limited absorption after oral dosing, intestinal or hepatic first-pass effect [119,120].

4.1   Background to Paper II and III

o,p'-DDD has been used since the 1960's as a cytotoxic drug in the treatment of ACC when the tumour is inoperable or as an adjuvant drug after surgery. *o,p'*-DDD is activated by a CYP catalyzed reaction to a reactive intermediate that binds covalently to the adrenal cortex leading to necrosis both in the tumour and in metastases (Chapter 2, 2.2.1). In addition, oxidative damage through production of free radicals contributes to the adrenocorticolytic effect [29]. *o,p'*-DDD also exerts a direct effect on glucocorticoid secretion by inhibiting the steroidogenic enzymes CYP11A1 and CYP11B1 leading to inhibition of the intramitochondrial conversion of cholesterol to pregnenolone and the conversion of 11-deoxycortisol to cortisol [121]. The efficacy and potency of *o,p'*-DDD is however low and the treatment is frequently associated with side effects but since the frequency of ACC is very low, little research has focused on developing new therapeutic alternatives. Since 3-MeSO$_2$-DDE is a highly potent adrenal toxicant in mice and is also bioactivated *ex-vivo* in human adrenal tissue (see Chapter 2.3), it has been proposed as a lead compound for an improved drug for ACC in humans replacing *o,p'*-DDD [122]. The pharmacokinetics of both compounds are investigated and compared in **Paper II**.

3-MeSO$_2$-DDE is present in humans [43,44] and is also known to be excreted in human milk [42]. Studies in pregnant and lactating mice showed that 3-MeSO$_2$-DDE was transferred via the placenta and milk. Both the lactating mice and the foetus showed specific irreversible binding in the adrenal cortex indicating the mechanism of metabolic activation to be present from foetal

life to adulthood [54]. No risk assessment of 3-MeSO$_2$-DDE in highly DDE-exposed human has to our knowledge been carried out and given the evidence of its specific adrenal toxicity; a risk assessment of this environmental pollutant should be directed towards early life-stage exposure. The toxicokinetics of 3-MeSO$_2$-DDE in mother and offspring following a single oral dose to lactating minipigs was studied in **Paper III.**

4.2 Toxicokinetics of 3-MeSO$_2$-DDE and *o,p'*-DDD in minipigs

In **Paper II** the toxicokinetics of 3-MeSO$_2$-DDE and *o,p'*-DDD was studied and compared in 10 female Göttingen minipigs (Ellegaard, Dalmose, Denmark). The minipigs were divided into two groups of five and administrated with a single oral dose of 30 mg 3-MeSO$_2$-DDE or *o,p'*-DDD/kg b.w. with the test compounds dissolved in corn oil. Blood and adipose tissue samples were continuously collected over a period of 180 days. Samples from the adrenals, liver, kidneys and brain were taken at the end of the study. Only plasma, adipose tissue and liver levels were presented in **Paper II**. Kinetic parameters were calculated from plasma and adipose tissue analysis as described in **Paper II**.

In **Paper III** the excretion of 3-MeSO$_2$-DDE via milk to suckling offspring following a single oral dose of 3-MeSO$_2$-DDE to lactating minipigs was studied. Five sows were given a single oral dose of 3-MeSO$_2$-DDE (15 mg/kg b.w) two days *post partum*. Milk, maternal adipose tissue and plasma samples from both the sows and piglets were collected at different times during a period of 28 days. Adrenal, adipose tissue and liver samples were taken from all the animals at the end of the experiment.

The Göttingen minipig was selected as a non-rodent animal model in **Paper II** and **III** because they have similar physiology to humans. Their metabolic activity and enzymatic processes have close parallels with human systems. This species is often used in pharmacological and toxicological studies [123].

4.2.1 Sample extraction and preparation
The blood samples were extracted as described by Hovander (2000) [124]. In short, the internal standard (IS) was added to the plasma samples (1 g) and the samples were denaturized with hydrochloric acid (6 M) and 2-propanol, thereafter extracted with *n*-hexane/metyl-*tert*-butyl ether (*n*-Hx/MTBE). Further, a washing step with a potassium chloride solution was included in order to wash out any co-extractable compounds. The method used for the extraction of the milk samples (1g) was similar to the one described above. Instead of hydrochloric acid and MTBE the milk samples were denaturized with formic acid and extracted with diethyl ether [125].

Adipose tissue was extracted according to a method originally developed by Jensen (1983) [126]. The method was scaled down and adjusted for the sample size applied (1 g). This allowed the extraction to be performed in test tubes instead of glass funnels, shortening time consumption and limiting the amount of solvent used. The adipose tissue was mixed with acetone:*n*-hexane and homogenized. The lipids were extracted twice with *n*-Hx:MTBE. The samples were then spiked with the IS. The other tissue samples were extracted with a modified Jensen method that has been shown to be more efficient for leaner tissues [127]. The tissue samples were mixed with 2-propanol and diethyl ether (DEE), homogenized and extracted with 2-propanol and *n*-Hx:DEE.

Lipid content in plasma could be determined gravimetrically or enzymatically and studies have shown that both methods are in good agreement [128]. Enzymatic lipid determination is preferable for plasma samples due to low lipid levels and the small sample volumes required for measurement. The lipid amounts in **Paper II** and **III** were determined gravimetrically (see Table 4.1). The lipid content in plasma was low, 0.2-0.6% (<10 mg per sample) which resulted in difficulties obtaining reliable lipid amounts. The concentrations in all the tissues are accordingly presented on a fresh weight basis to avoid uncertainty when comparisons to plasma concentrations are made. From a toxicokinetic point of view concentrations on fresh weight basis are preferred as it is important to know the dose at the target tissue. The lipid content in the tissues and plasma was still determined and reported to allow comparisons between studies. As shown in Table 4.1 the lipid content was a bit higher in the piglets compared to the sows, which is not surprising since the piglets were gaining weight during the study.

Table 4.1. Mean lipid content (%) ±SD determined for all the tissues analyzed in Paper II and III.

	Plasma	Adipose	Liver	Adrenal	Kidney	Brain	Milk
Sow	0.2 ±0.03	70 ±13	4 ±0.8	6 ±1	4 ±1	9 ±1	10 ±2
Piglet	0.6 ±0.02	78 ±5	5 ±1	9 ±2	n.c.	n.c.	n.c.

n.c. = not collected

It is important to remove all lipids in a sample since even traces of lipid may disturb the gas chromatographic measurements. Lipid removal and clean up consisted in several steps and are illustrated in Figure 4.1. Two different methods were used depending upon the analyte in question, 3-MeSO$_2$-DDE or *o,p'*-DDD. In **Paper II**, possible phenolic compounds/metabolites were separated from neutrals with a 0.5 M potassium hydroxide partitioning step prior to lipid removal.
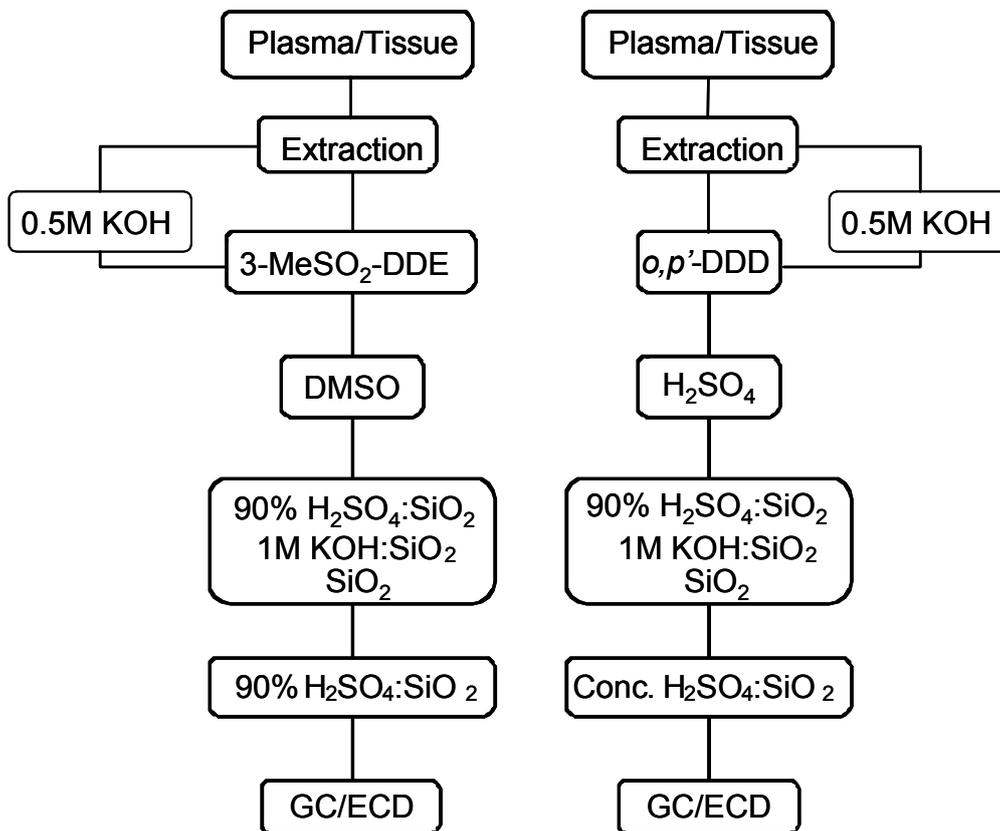
Figure 4.1. General scheme for extraction and clean up used in Paper II and III for plasma, milk and tissues. KOH partition step was only done in Paper II.

The samples containing 3-MeSO$_2$-DDE were treated with dimethyl sulfoxide (DMSO) to separate 3-MeSO$_2$-DDE from compounds without a sulfone functional group. This is a non-destructive method used to separate lipids from analytes [129,130]. The DMSO partitioning step used for 3-MeSO$_2$-DDE failed for the samples containing *o,p'*-DDD since the analyte was partitioned in both the hexane and DMSO phases. The *o,p'*-DDD samples were treated with conc. sulfuric acid instead, a destructive method that is not recommended when unknown analytes are analyzed. Sulfuric acid destroys the polyunsaturated fats by oxidation of the double bonds. Sulfuric acid has also been used to separate sulfone containing compounds from non-sulfone compounds since sulfone containing compounds partition to the acid due Lewis acid/base pair formation [43]. Any remaining lipids were removed by silica gel columns. A multilayer column packed with silica, silica treated with potassium hydroxide and silica treated with sulfuric acid was applied as described elsewhere [131]. 3-MeSO$_2$-DDE was eluted with dichloromethane (DCM) and *o,p'*-DDD with *n*-Hx:DCM (1:1). Further sample clean up was necessary and a smaller column with silica gel impregnated with sulfuric acid was used to remove the final traces of extracted matter.

4.2.2   GC analysis and QA/QC

The samples in **Paper II** and **III** were quantified by gas chromatography (GC) with electron capture detection (ECD). GC/ECD is useful when the

analytes are known, standards are available and when there are no interfering co-elutions. In **Paper II** and **III** the analytical issue only concerned two analytes and their surrogate standards which facilitated the use of GC/ECD. The linearity of the ECD was controlled by running series of dilution standard mixtures in parallel to the samples. The quantification was made by using the standard surrogate method and a single point of the external standard within the range of linearity. The limit of detection (LOD) was set to a signal to noise (S/N) ratio of above three (0.1 pg) and the limit of quantification (LOQ) was defined as S/N ratio above ten.

Solvent blank and samples taken at time zero were analyzed in parallel with all samples to detect any potential contamination. No co-eluting contamination was neither detected in the solvent blanks nor in the samples taken prior to administration of the minipigs (time zero). The quality of the analytical method was tested by calculating recoveries analysing reference material spiked with the IS and the two analytes.

In **Paper II**, recovery studies were performed at one concentration (20 ng) in human plasma and bovine liver. In **Paper III**, two concentration levels were used in human milk (10 and 100 ng). Results from the recovery studies are shown in Table 4.2. The recoveries were considered to be acceptable.

Table 4.2. Mean recoveries ±SD of the analytes and the IS, 3-CB-141 and CB189 at low concentration (10-20 ng).

		3-MeSO$_2$-DDE	3-CB-141	*o,p'*-DDD	CB189
Paper II					
	plasma	80 ±10	89 ±15	90 ±9	91 ±11
	adipose	88 ±5	84 ±11	60 ±16	89 ±10
Paper III					
	milk	60 ±5	70 ±2	-	-

4.3 Results and discussion

In **Paper II** maximal plasma concentrations of 3-MeSO$_2$-DDE (1,980 ±830 ng/g f.w.) and *o,p'*-DDD (790 ±390 ng/g f.w) were reached at eight hours after exposure. Plasma concentrations showed individual differences indicated by large standard deviations. The compounds were rapidly distributed to other tissues and/or eliminated. At C$_{max}$, the plasma concentrations of 3-MeSO$_2$-DDE were about two times higher than *o,p'*-DDD (Figure 4.2). After 4 days only very low concentrations of *o,p'*-DDD remained in plasma (14 ±7 ng/g f.w.) compared to 3-MeSO$_2$-DDE (620 ±98 ng/g f.w.). Unexpectedly, a second concentration peak at day 60 was observed in the 3-MeSO$_2$-DDE plasma samples (Figure 4.2). Upon further investigation this appeared to be due to a weight loss in four of the minipigs.
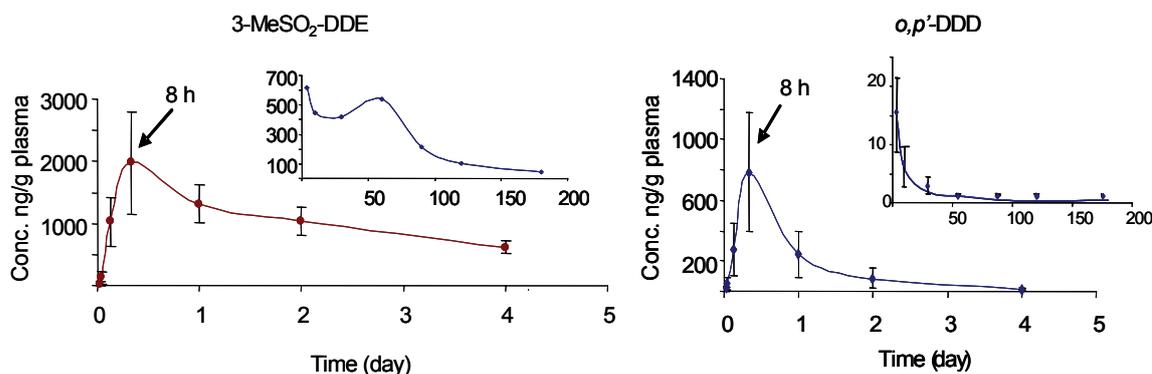
Figure 4.2. Mean plasma concentrations of 3-MeSO$_2$-DDE and *o,p'*-DDD (ng/g f.w.) in minipigs over 180 days after a single oral dose of 30 mg/kg b.w.

$t_{1/2}$ in plasma was calculated to 50 and 28 days for 3-MeSO$_2$-DDE and *o,p'*-DDD, respectively. There is a risk to overestimate the determined $t_{1/2}$ when the detected concentrations are low, as in the case for *o,p'*-DDD after 180 days. The calculated mean residence time (MRT) is less affected by this factor and was considered to be a more appropriate parameter to describe the substances biological half lives. MRT for 3-MeSO$_2$-DDE and *o,p'*-DDD was 56 and 12 days, respectively.
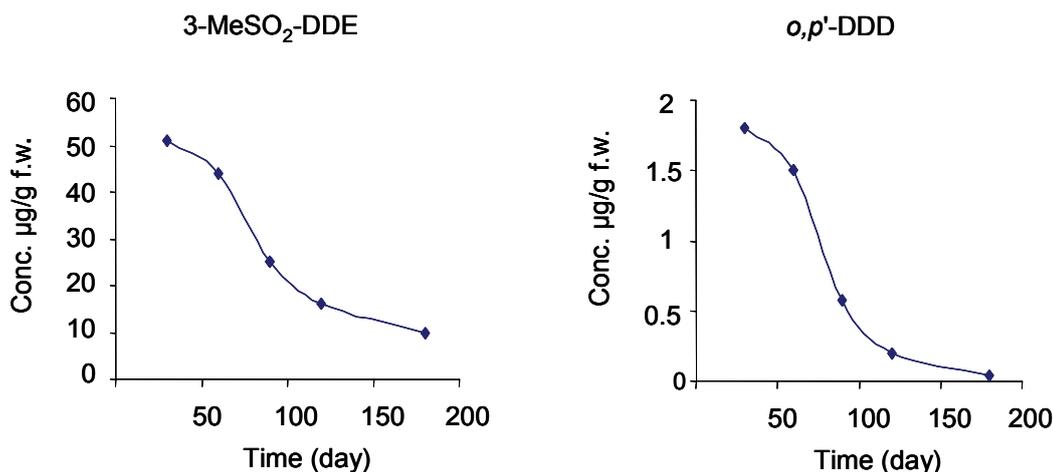
Figure 4.3. Mean concentrations in minipig adipose tissue of 3-MeSO$_2$-DDE and *o,p'*-DDD (µg/g f.w.) over 180 days after a single oral dose (30 mg/kg b.w.).

In adipose tissue, the concentrations were high which is not surprising considering the fact that both compounds are lipophilic with log K$_{ow}$ of 5.4 and 4.7 for *o,p'*-DDD and 3-MeSO$_2$-DDE, respectively. Also here the concentrations of 3-MeSO$_2$-DDE are higher (25-100 times) than *o,p'*-DDD (see Figure 4.3). Although both substances are lipophilic the partitioning between fat and plasma was different for the two compounds. The fat/plasma ratio at day 30 was 15 times higher for *o,p'*-DDD than 3-MeSO$_2$-DDE which confirms the higher lipophilicity of *o,p'*-DDD. The $t_{1/2}$ in fat was similar of those in plasma for both compounds, 22 and 52 days for *o,p'*-DDD and 3-MeSO$_2$-DDE, respectively.

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Since the bioavailability (F) of the compounds studied was not known for minipigs the volume of distribution (V_d) and clearance (Cl) was calculated as V_d/F and Cl/F and are shown in Table 4.3. The much faster elimination of o,p'-DDD from plasma compared to that of 3-MeSO$_2$-DDE was also confirmed by the larger clearance (Cl/F) of o,p'-DDD. The differences in clearance could be explained by o,p'-DDD being faster biotransformed than 3-MeSO$_2$-DDE. There are known metabolic pathways for o,p'-DDD in humans as well as in other species but no metabolic pathway has yet been shown for 3-MeSO$_2$-DDE. Both test compounds had a large V_d/F but the much larger V_d/F value for o,p'-DDD confirms the greater lipophilicity of this compound compared to 3-MeSO$_2$-DDE.

Table 4.3. Kinetic parameters calculated for 3-MeSO$_2$-DDE and o,p'-DDD in plasma and adipose tissue after a single oral dose to minipigs (median values).

	Kinetic parameter	3-MeSO$_2$-DDE	o,p'-DDD
Plasma	C_{max} (ng/g f.w.)	1,700	800
	T_{max} (h)	8	8
	$t_{1/2}$ (days)	50	28
	MRT (days)	56	12
	V_d/F (L)	0.8×10^3	20×10^3
	Cl/F (L/h)	0.5	22
Adipose tissue	$t_{1/2}$ (days)	52	22

The tissue concentrations at day 180 are shown in Table 4.4, both on fresh weight and lipid weight basis. In liver, the mean concentration of o,p'-DDD (0.5 ±0.3 ng/g f.w.) was of the same magnitude as the plasma concentration at day 180. However the mean concentration of 3-MeSO$_2$-DDE (800 ±500 ng/g f.w.) was 20 times higher than the plasma concentration at 180 days indicating a potential non-specific protein binding. 3-MeSO$_2$-DDE levels have been reported to be higher in liver than in fat in humans and seals [40,43,132].

The brain samples were divided into two different types; one from the cerebellum and four from the cerebrum from each group. No o,p'-DDD was detected, but in contrast 3-MeSO$_2$-DDE was found in both the cerebellum and the cerebrum, 600 ng/g f.w and mean 350 ±80 ng/g f.w., respectively. In kidney the mean concentration of o,p'-DDD and 3-MeSO$_2$-DDE were 0.1 ±0.05 ng/g f.w. and 700 ±300 ng/g f.w., respectively.

Only few adrenal samples were collected but surprisingly the concentration of 3-MeSO$_2$-DDE (7,400 ng/g f.w.) was extremely high compared to o,p'-DDD (0.7 ng/g f.w.). The concentrations of 3-MeSO$_2$-DDE in the adrenal were of the same magnitude as the concentrations in adipose tissue at day 180 when compared on a fresh weight basis, but four times higher if based on lipid weights, indicating a possible storage or retention in the adrenal. Unfortunately, only one adrenal sample was collected and more tissue should

be analyzed before a retention/storage of 3-MeSO$_2$-DDE in the adrenal gland can be verified. In general the concentrations of 3-MeSO$_2$-DDE were higher than *o,p'*-DDD during the whole studied period due to longer $t_{1/2}$.

Table 4.4. Mean tissue concentrations ±SD of 3-MeSO$_2$-DDE and *o,p'*-DDD on fresh weight and lipid weight basis 180 days after a single oral dose (30 mg/kg b.w.) to five minipigs.

Tissue (d 180)	n	3-MeSO$_2$-DDE ng/g f.w.	*o,p'*-DDD ng/g f.w.	3-MeSO$_2$-DDE ng/g l.w.	*o,p'*-DDD ng/g l.w.
Plasma	5	41 ±13	0.5 ±0.3	21,000 ±6,000	250 ±140
Adipose	5	9,000 ±4,000	40 ±41	12,000 ±2,000	50 ±50
Abd. adipose	5	7,800 ±3,000	9 ±6	11,000 ±3,000	13 ±10
Liver	5	800 ±500	0.5 ±0.3	21,000 ±10,000	15 ±9
Kidney	4[a]	700 ±300	0.09 ±0.05	19,000 ±600	3 ±2
Adrenal	3[b]	7,400	0.7 ±1	46,000	10 ±15
Cerebrum	4	350 ±80	<LOD	4,000 ±1,000	<LOD
Cerebellum	1	600	<LOD	6,200	<LOD

[a] for 3-MeSO$_2$-DDE n = 3, [b] for 3-MeSO$_2$-DDE n = 1

In conclusion, the results from **Paper II** show that 3-MeSO$_2$-DDE is efficiently retained in the liver of minipigs and a possible retention in the adrenal is also observed. 3-MeSO$_2$-DDE is eliminated slower than *o,p'*-DDD and the slower elimination might make it challenging to design appropriate dosage regimes in ACC treatment.

In **Paper III** the plasma concentrations in the lactating sows reached a C$_{max}$ at eight hours after administration (mean 998 ±605 ng/g f.w.). 3-MeSO$_2$-DDE was rapidly distributed, and after 10 days *post partum*, distribution equilibrium appeared. Mean plasma concentrations of 3-MeSO$_2$-DDE on fresh weight basis for the sows and the piglets during the whole experimental period are given in Figure 4.4. The plasma levels of 3-MeSO$_2$-DDE in the piglets (ranging from 200 to 2,700 ng/g f.w.) were about five times higher than the levels in the mothers (ranging from 50 to 1,900 ng/g f.w.) throughout the whole studied period. The milk samples (ranging from 1,500 to 79,000 ng/g f.w.) showed much higher levels of 3-MeSO$_2$-DDE than the plasma samples, this is at least partly due to much more lipids in milk than in plasma. The lipid content varied a lot between milk samples ranging from 6-19%.
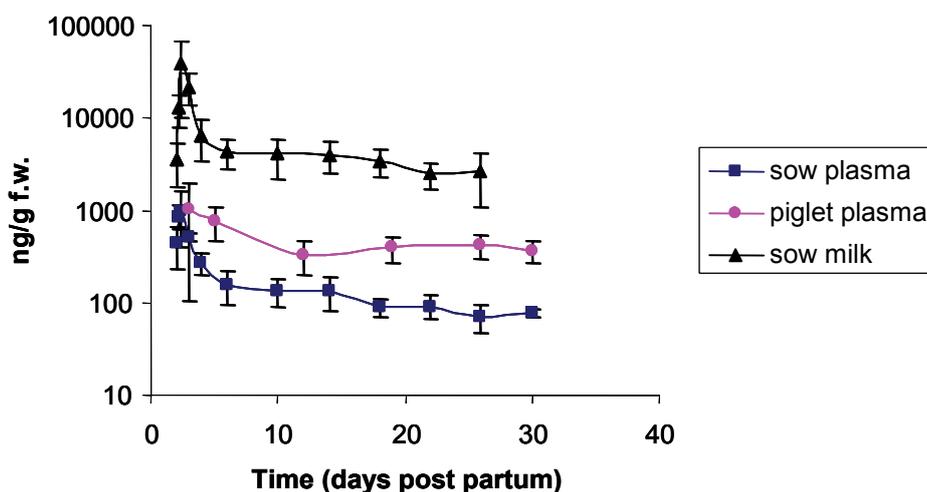
Figure 4.4. Mean concentrations ±SD (ng/g f.w., Logarithmic scale) of 3-MeSO$_2$-DDE in plasma and milk from lactating sows and in plasma from suckling piglets. The sows were administrated one single oral dose of 15 mg 3-MeSO$_2$-DDE/kg b.w. two days *post partum*.

Tissue concentrations are shown in Table 4.5 on both lipid weight basis and fresh weight basis for comparison. The 3-MeSO$_2$-DDE concentrations in adipose tissue taken at different times from the lactating sows showed great individual differences indicated by large standard deviations (see Figure 2 in Paper III). The mean levels of 3-MeSO$_2$-DDE in adipose tissue of the piglets were at day 30 *post partum* higher than in their mothers. A strong accumulation of 3-MeSO$_2$-DDE in fat and a pronounced accumulation in adrenals and liver were observed in both the mothers and offspring confirming the observations made in **Paper II**. The retained tissue levels in the piglets were consistently higher than in the mothers

Table 4.5. Mean concentrations ±SD of 3-MeSO$_2$-DDE in tissues of lactating sows (SA-SE) and their litter (LA-LE) 28 days after administration of a single oral dose (15 mg/kg b.w.) given 2 days *post partum*.

	n	Adipose µg/g f.w.	Liver µg/g f.w.	Adrenal µg/g f.w.	Adipose µg/g l.w.	Liver µg/g l.w.	Adrenal µg/g l.w.
SA-SE	5	24 ±14	1.6 ±0.4	0.7 ±0.3	20 ±15	47 ±18	11 ±4
LA-LE	20	54 ±21	3.5 ±0.8	2 ±0.6	68 ±25	75 ±20	25 ±8

The results from **Paper III** revealed a pronounced elimination of 3-MeSO$_2$-DDE in milk exposing suckling offspring for high levels. Despite a rapid weight gain in the piglets during the course of the study the accumulated levels of 3-MeSO$_2$-DDE in the piglets were higher than in the sows. These observations and the possible risk for adrenal damages in children should be taken into account in human risk assessments, focusing on breastfed infants rather than adults.

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5 Chirality in general

Louis Pasteur was the first to successfully separate a chiral compound into its enantiomers, separating the two sodium ammonium tartrate enantiomers, an achievement made in 1848 [133]. He showed that solutions of the two crystal types he had isolated rotated plane polarised light in opposite directions, right (*d*) and left (*l*). Stereoisomers are compounds made up of the same atoms, bonded by the same sequence of chemical bonds but possessing different three dimensional structures which are not interchangeable. Stereoisomers are divided into enantiomers which are mirror images of each other and diastereomers which are molecules without mirror images. A molecule that is not identical with its mirror image and possesses no plane of symmetry is chiral. A 1:1 mixture of an enantiomeric pair forms a racemate. The atom that carries four different substituents is called the asymmetric or stereogenic center and the individual enantiomers are characterized by different absolute configurations around this centre, *R* and *S*, respectively. Enantiomers physical properties, such as melting point, boiling point, refractive index and solubility are identical, making abiotic environmental processes like air-water exchange, sorption and abiotic transformation non-enantioselective. The only major physico-chemical difference between the enantiomers lies in the way they interact with plane polarized light, levorotatory rotation (−) and dextrorotatory rotation (+) also expressed as *l* and *d*, respectively [134].

However, the pharmacokinetic and pharmacodynamic properties may differ depending on the absolute structure of the enantiomer. Pharmacokinetic processes like absorption, distribution, biotransformation and excretion can thus be enantioselective. This is caused by individual enantiomer interactions with biogenic chiral molecules such as carrier proteins, enzymes and receptors. On the other hand, passive processes such as diffusion of lipid-soluble substances across gastrointestinal- and other membranes are not enantioselective. Pharmacodynamics may differ between enantiomers, each of them expressing different biological effects (qualitatively) or with different potency (quantitatively) [135]. The most potent enantiomer is called *eutomer* and the one with less potency, *distomer*. The thalidomide tragedy is often quoted as an example of a qualitatively acting compound, based on the fact that the *R*-enantiomer is sedative and the *S*-enantiomer is teratogenic. Many thought that the thalidomide incident could have been avoided if the drug had been enantiopure, only containing the *R*-enantiomer but this is not entirely correct since the pure isomers racemise through opening of the phthalimide ring [135]. Nowadays, chiral drugs are prepared with high enantiomeric purity due to potential side effects of the other enantiomer.

Chiral chemicals used for technical purposes and as pesticides are released into the environment as racemic mixtures [136]. The relative abundances of

enantiomers can however change in the environment due to microbiological degradation or after uptake and retention in biota due to e.g. enantioselective metabolism and/or accumulation. Changes in the 1:1 ratio of enantiomers are reported as enantiomeric ratio (ER) or enantiomer fraction (EF) in environmental chemistry while as enantiomeric excess (EE) in organic chemistry. ER has been defined by Faller (1991) [137] as:

$$ER = \frac{E_+}{E_-}$$

where E_+ and E_- represent the peak area, peak height or concentration of the (+)-enantiomer and (−)-enantiomer, respectively. A racemic mixture has an ER value of 1 and a range from 0 to infinity (∞). The ER may be perceived in a misleading manner since a one unit change below 1 and above one has entirely different meanings. In my mind, a better representation of the chiral signature has been introduced by Harner and co-workers as the enantiomer fraction (EF) [138]. EF is defined as:

$$EF = \frac{E_+}{(E_+ + E_-)} \quad EF_x = \frac{E_1}{(E_1 + E_2)}$$

where E_1 and E_2 are the first and second eluting enantiomer, if the enantiomer specific rotation of plane-polarized light is unknown. The EF can only range from 0 to 1, with EF = 0.5 for a racemic mixture. It is based on a bounded additive scale that is linear, finite and symmetric in sample distribution around the racemic value. Harner also suggested the equation EF = ER/(ER + 1) for the conversion of ER values to EF. Conversion between ER and EF statistics values can however be problematic as discussed by Ulrich (2003) [139]. Substantial discrepancies are observed converting statistically data (e.g. median, mean, standard deviation), especially when the ER is greater than 1 as it has large variability due to asymmetrical data around 1.

The relative abundance of enantiomers in the environment has been taken as an indicator of biological degradation since abiotic transformations are non-enantioselective. Atmospheric transport processes are also non-enantioselective and there is a constant input to the atmosphere from racemic and non-racemic sources by volatilization and transport. Thus, enantiomeric composition in the atmosphere might serve as a tracer of vapour exchange with water and soil and for atmospheric long range transport [140].

Enantioselective separations by gas chromatography can be achieved by applying columns with chiral stationary phases. The enantiomer separation is achieved by the formation of diastereomeric, energetically unequal association complexes between the enantiomers and the chiral stationary phase (CSP) [134]. The most commonly used CSP in enantiospecific analysis by GC are cyclodextrins (CD). Cyclodextrins are cyclic glucose oligomers with 6 (α), 7 (β) or 8 (γ) glucose units that have a hydrophilic outer surface

with its hydroxyl groups and an interior cavity that is hydrophobic (Figure 5.1). The interior cavity favours the selective inclusion and trapping of the analyte. The hydroxyl groups can be derivatised to modify the CD and enhance the selectivity and the stability of the column. Commercially available CD columns are often derivatised to various degrees. Small changes in the CD composition can significantly influence the enantioselective properties which might lead to reproducibility problems between columns manufactured to be identical. Chiral compounds may be sensitive to composition changes of CD and even reversed enantiomer elution order has been observed as a consequence thereof [141]. To stabilize the column even further, the CD is dissolved in or bound to an achiral stationary phase, e.g. polysiloxane [142]. Although several modified CD-columns are commercially available it is impossible to resolve "all" chiral analytes using one single CD-column. Overlap of different isomers is a problem, as for other GC separations, and tandem-columns consisting of an achiral column connected to a chiral column have been used to enhance the resolution of the analytes [143]. An even more sophisticated technique to overcome problems of co-elution may be multidimensional (MD) GC, which involves separation on two serial columns with different selectivity [144,145].
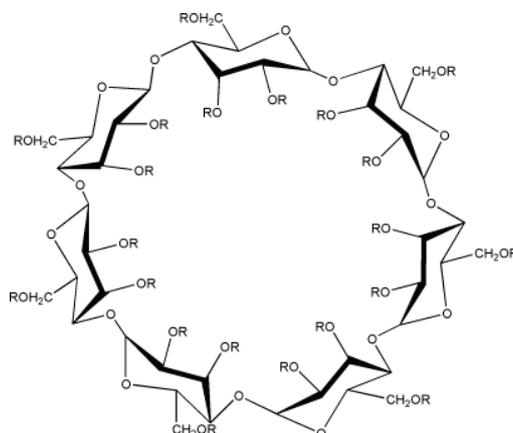
Figure 5.1. Chemical structure of β-cyclodextrin (7 glucose units), R = alkyl group.

5.1   Background to Paper IV, V and VI

o,p'-DDT and its metabolite *o,p'*-DDD are both chiral. Usually the concentrations of these compounds are too low in biota to enable assessment of their concentrations and to determine enantiomeric ratios. Despite the low levels there are some studies evaluating the enantiomeric composition of *o,p'*-DDT and *o,p'*-DDD in the environment and in biota [143,146-148]. There is concern that chiral pollutants could affect human and wildlife health by disrupting normal hormone interactions. It has been demonstrated that (–)-*o,p'*-DDT is a more oestrogenic than the (+)-enantiomer [149]. Unfortunately,

not much is known about enantiospecific toxicological properties of *o,p'*-DDT and *o,p'*-DDD.

To be able to compare enantiomeric compositions from different studies it is important to establish a rule where the enantiomers in the future should be identified by at least their (+) and (−) form before calculations of EF are made. There are studies presenting EFs where the enantiomeric absolute configuration or optical rotation is not known which can create problems when comparing data, especially if different chiral columns are used as reversed elution order is not uncommon. Isolation of pure enantiomer followed by x-ray analysis or NMR are methods required for structure elucidations.

As a registered drug for ACC treatment, *o,p′*-DDD (Lysodren) is to our knowledge administered as a racemic mixture. Due to possible enantiospecific interactions of chiral exogenous compounds with endogenous enzymes it is of interest to study possible differences in the *o,p'*-DDD enantiomers kinetics. In **Paper II**, plasma and tissue samples were collected from minipigs administrated one single oral dose of a racemic mixture of *o,p'*-DDD which gave an ideal base to study the enantiomeric distribution over time, both between tissues and individuals. To our knowledge, the individual contribution of each *o,p'*-DDD enantiomer to the intended pharmacological effects and the toxic side effects has never been studied. However, it is not rare for enantiomers to possess different pharmacodynamic properties, as is the case with e.g. thalidomide [150]. To be able to identify and perform enantiospecific toxicological tests it was necessary to isolate and determine the absolute configuration of the *o,p'*-DDD enantiomers.

5.2 Enantioselective analysis

In **Paper IV**, the two enantiomers of *o,p'*-DDD were isolated by HPLC. Pure enantiomers (~30 mg) were obtained after fractionation of the two enantiomers applying a semi-preparative permethylated γ-CD column. Base line separation was obtained when a mobile phase of methanol:water (80:20) and 1% triethylamine:acetic acid (1:2 v/v) was used. Each pure enantiomer was recrystallized in methanol. The absolute configuration of the enantiomers was determined by X-ray crystallography and the X-ray data of the first eluting enantiomer (E1), from the separation system described above, is reported in **Paper VI**. The optical characteristics of both enantiomers were measured with a polarimeter. The separation achieved by HPLC of the two enantiomers and their absolute configuration is shown in Figure 5.2.
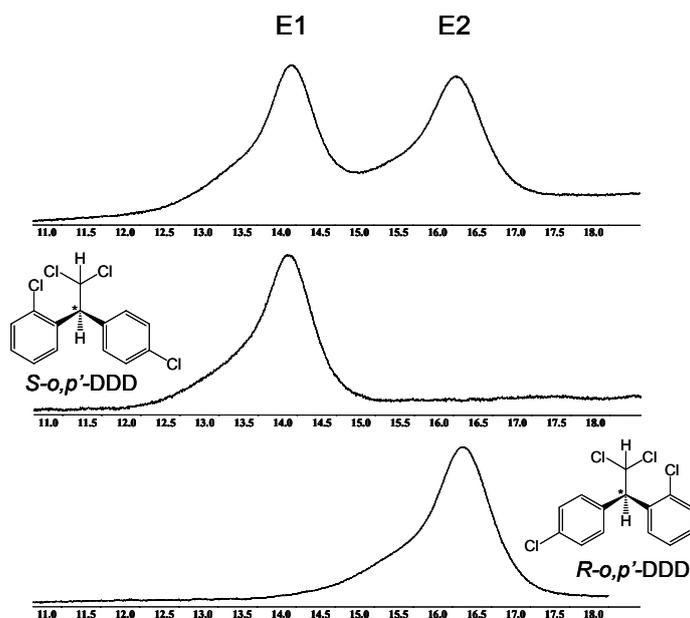
Figure 5.2. HPLC chromatogram of racemic *o,p'*-DDD and the two isolated enantiomers. The absolute configuration of the enantiomers was determined by X-ray crystallography and is shown under the structures.

In **Paper IV**, plasma and tissue samples that were collected from minipigs administrated one single oral dose of a racemic mixture of *o,p'*-DDD (**Paper II**) were analysed by GC/ECD. High enantiospecific resolution of the two *o,p'*-DDD enantiomers was achieved using a 20% *tert*-butyldimethylsilyl-β-CD column dissolved in 15% phenyl- and 80% methylpolysiloxane (Figure 5.3). The GC temperature programme was set to 10°C/min up to 230°C. Isothermal oven temperature is commonly used for the best possible resolution and to minimize the bleeding of the column, but this usually increases the retention time of the analytes.
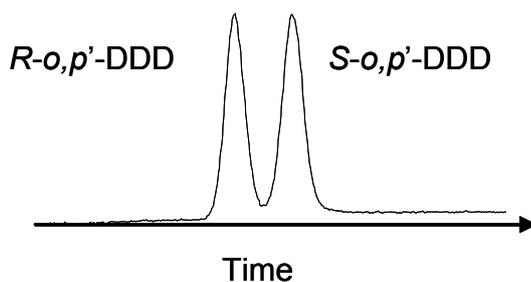
Figure 5.3. GC separation of racemic *o,p'*-DDD standard achieved by a PM-β-CD column.

The pure *o,p'*-DDD enantiomers when analysed by GC/ECD, using a PM-β-CD column, showed reversed elution order compared to HPLC analysis (compare Figure 5.2 and 5.3). Here the first eluting enantiomer from the gas chromatographic separation was the (*R*)-(+)-*o,p'*-DDD with (*S*)-(–)-*o,p'*-DDD as the second eluting enantiomer. The result of reversed eluting order is not unusual as observed when different CD columns and systems are used [151].

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5.3 Enantioselective effects in adrenocortycolytic action

In **Paper V**, the isolated and pure (*R*)-(+)-*o,p'*-DDD and (*S*)-(–)-*o,p'*-DDD were tested for effects on cell viability and cortisol formation in the human adrenocortical cell line H295R. The enantiospecific effects were compared to those of racemic *o,p'*-DDD and the isomers; *m,p'*- and *p,p'*-DDD. The method used to assess respective effects is described in **Paper V**. In short, all the studied compounds were dissolved in dimethyl sulfoxide (DMSO) and added to an assay medium obtaining different total concentrations (ranging from 1.25 to 20 μM). The cells were incubated at 37˚C for 72 hours and the cell viability was determined during the last two hours of incubation by the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Formed formazan, a reduction product of MTT, was dissolved in sodium dodecyl sulfate, hydrochloric acid and incubated overnight at 37˚C. Cell viability was expressed as percent viability of DMSO control cells. For measurement of cortisol levels all the DDD isomers and *R*-, *S*-*o,p'*-DDD were dissolved in DMSO and added to assay medium yielding concentrations ranging from 0.625 to 12.5 μM. The cells were incubated at 37˚C for 24 hours. Cortisol levels were measured using an enzyme immunoassay kit and expressed as percent of DMSO control levels.

5.4 Results and discussion

In **Paper IV,** the isolation of the *o,p'*-DDD enantiomers (E1 and E2) by HPLC fitted with a PM-γ-CD column was carried out successfully. Only E1 was re-crystallized to thin plate-like crystals suitable for X-ray analysis at the first attempt. E1 was determined to be (*S*)-(–)-*o,p'*-DDD by X-ray crystallography (**Paper VI**) and polarimetry (Figure. 5.4). Thus, the second eluting enantiomer was (*R*)-(+)-*o,p'*-DDD, which has been confirmed by X-ray crystallography (unpublished, Eriksson pers. commun.).
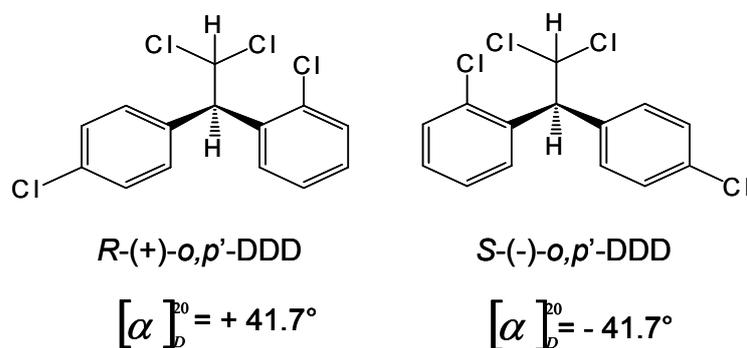

$$R\text{-}(+)\text{-}o,p'\text{-DDD} \qquad S\text{-}(-)\text{-}o,p'\text{-DDD}$$

$$\left[\alpha\right]_D^{20} = +41.7° \qquad \left[\alpha\right]_D^{20} = -41.7°$$

Figure 5.4. Absolute configurations as determined by X-ray crystallography and specific rotation by polarimetry.

Further, in **Paper IV**, the EF in plasma and tissues from 5 minipigs (M1-M5) exposed to one single oral dose of a racemic mixture of *o,p'*-DDD (**Paper II**) was determined by GC/ECD. Plasma and adipose tissues had been collected at different times over a period of 180 days. Up to three hours after

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administration of racemic *o,p'*-DDD, the EFs in plasma were close to 0.5 (0.4-0.5) for all the five minipigs indicating a non-enantioselective uptake of the racemic mixture. With time, three of the minipigs (M1, M4-5) showed a slight increase in EF indicating a possible faster elimination of (*S*)-(−)-*o,p'*-DDD but the EF were back to racemic value 30 days after administration. In contrast, two of the minipigs (M2-3) showed a significant decrease in EF with time indicating a faster elimination of (*R*)-(+)-*o,p'*-DDD (Figure 5.5 a). Thirty days after administration only the (*S*)-(−)-*o,p'*-DDD peak was detected in these two minipig plasma samples.
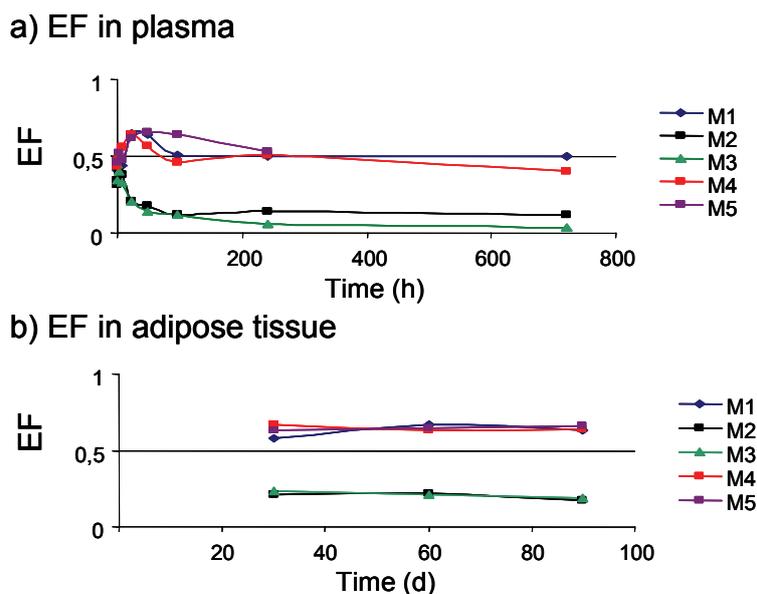
Figure 5.5. EF in minipig M1-M5 plasma (a) and adipose tissue (b) after a single oral dose of racemic *o,p'*-DDD over time.

Adipose tissue was first taken 30 days after administration and thereafter every thirty days up to 180 days. The EFs showed the same pattern as in plasma. Three of the minipigs (M1, M4-5) had EFs ranging from 0.63 to 0.65 and the other two (M2-3) had mean EF of 0.19 and 0.21, over a period of three months (Figure 5.5 b).

Kidney and liver samples taken at the termination of the study (**Paper II**), 180 days after administration of *o,p'*-DDD were also enantioselective analysed. Surprisingly the EFs in the kidney samples were racemic for all the five minipigs. The reason behind this observation is not yet understood but possibly non-enantioselective metabolism/elimination in kidney may be involved. Unfortunately, the determination of EF in the liver samples was impossible due to matrix disturbances during quantification.

The interindividual differences in minipigs observed in **Paper IV** imply that either the metabolism or the distribution of *o,p'*-DDD was affected by polymorphism. This observation may be of particular importance with regard to the pharmacological use of *o,p'*-DDD. The question of whether ACC

patients will show similar interindividual differences in enantiomer disposition remains to be demonstrated.

In **Paper V**, cell viability was measured and a concentration-dependent cytotoxicity in H295R cells was observed for all three DDD isomers and both *o,p'*-DDD enantiomers. The shapes of the concentration-response curves for the (*R*)-(+)-*o,p'*-DDD and (*S*)-(−)-*o,p'*-DDD enantiomers showed different slopes/kinetics. (*S*)-(−)-*o,p'*-DDD being somewhat more cytotoxic than the (*R*)-enantiomer (see Figure 2a in Paper V). The differences in slopes were less pronounce at 20 μM which was the highest concentration tested. The racemic mixture showed a concentration-response curve below the pure enantiomers curve (see Figure 2b in Paper V). Consequently, the racemic mixture produced more cytotoxicity than the sum of the two pure enantiomers at corresponding concentrations. The *m,p'*- and *p,p'*-DDD isomers displayed identical concentration-response curves and cytotoxic potency as racemic *o,p'*-DDD.

Cortisol secretion was also measured in H295R cells at non-cytotoxic conditions. All three DDD isomers and both enantiopure (*R*)- and (*S*)-*o,p'*-DDD decreased cortisol secretion in a concentration-dependent way. (*R*)-(+)-*o,p'*-DDD initially decreased cortisol secretion more potently than (*S*)-(−)-*o,p'*-DDD, although at 12.5 μM concentration, the two curves seem to converge (see Figure 3a in Paper V). In contrast to cell viability, the concentration-response curve of the racemic *o,p'*-DDD on cortisol secretion lay between the two enantiomer curves (see Figure 3b in Paper V). Similarly to the cytotoxic results, the three DDD isomers displayed equal potency in cortisol secretion.

In conclusion, the *in vitro* results in adrenocortical H295R cells do not strongly support single enantiomer *o,p'*-DDD therapy for ACC patients. On the other hand the present study did not address the possibility that the toxic side effects on the central nervous system and gastrointestinal tract could vary between the enantiomers. Administration of a single enantiomer could possibly reduce the frequency and/or severity of these adverse effects in ACC patients.

6 Concluding remarks

Novel data on the toxicokinetics of 3-MeSO$_2$-DDE and *o,p'*-DDD in minipigs has resulted from this work. Both compounds are distributed and retained in adipose tissue in the minipigs. Unlike *o,p'*-DDD, 3-MeSO$_2$-DDE was found in high concentrations in liver and the adrenal. 3-MeSO$_2$-DDE was eliminated much slower than *o,p'*-DDD from the pigs indicating a potential problem using this compound as an improved drug for ACC, instead of *o,p'*-DDD.

Unfortunately very little is known about the biotransformation of 3-MeSO$_2$-DDE while the pathways for *o,p'*-DDD biotransformation are well understood. In the future, identification and quantification of 3-MeSO$_2$-DDE metabolites should be carried out. Further work is required, due to known species differences in the adrenocorticolytic effect of 3-MeSO$_2$-DDE culminating in the importance for studies of the effects of this compound in humans.

The toxicokinetics of 3-MeSO$_2$-DDE in mother and offspring following a single oral dose to lactating minipigs was studied. 3-MeSO$_2$-DDE was eliminated via mother's milk exposing suckling offspring to high concentrations of 3-MeSO$_2$-DDE. Despite a rapid weight gain of the piglets the accumulated levels of 3-MeSO$_2$-DDE in plasma, liver and adrenal were twice as high as in the sows. These observations support the contention that risk assessment of 3-MeSO$_2$-DDE should be focussed on exposure and adrenal toxicity during the neonatal/postnatal period.

The two *o,p'*-DDD enantiomers were isolated by HPLC, a requirement for further structure elucidations by X-ray crystallography. The absolute configuration of the pure enantiomers of *o,p'*-DDD were determined for the first time. Also, the optical rotation of plane polarised light was measured. Hence any future studies of *o,p'*-DDD can now be done enantioselective and discussed in relation to the absolute structures of the compounds.

Significant interindividual differences in enantioselective kinetics of *o,p'*-DDD were observed in the minipigs. Two of the five minipigs administrated a single oral dose of racemic *o,p'*-DDD showed a significant faster elimination of (*R*)-(+)-*o,p'*-DDD. This may be an effect of polymorphism in the minipigs. Further, pharmacokinetic studies in humans are warranted to see if similar interindividual enantioselective differences are present also in humans in general but in ACC patients, in particular.

The pure enantiomers were assessed for enantiospecific toxicity in the human cell line H295R. The difference in enantiospecific toxicity was small but

significant, although too small to suggest single enantiomer therapy of ACC. Still we do not know whether the severe side effects of Lysodren are caused by one or both of the enantiomers and whether these side effects could be reduce by single enantiomer therapy.

A pathway has for the first time ever been developed for the synthesis of 3-SH-DDE. This compound makes it possible to perform structure-reactivity studies to find compounds more potent than 3-MeSO$_2$-DDE. 3-SH-DDE synthesis creates also opportunities for PET-studies. The new synthetic pathway could be further improved to minimize the steps required for the synthesis of 3-OH-DDE.

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