Partition, sorption & biodegradation of dialkoxybenzenes that modulate insect behavior

by

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B.Sc., Sharif University of Technology, 2007

Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of
Master of Science

in the
Department of Chemistry
Faculty of Science

SIMON FRASER UNIVERSITY

Summer 2012

Approval

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Degree: **Master of Science**

Title of Thesis: ***Partition, sorption & biodegradation of dialkoxybenzenes that modulate insect behavior***

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Date Defended/Approved: July 20, 2012

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Abstract

Plant protection is an important part of modern agriculture, in which high-yielding crop varieties are at risk of diseases and insect pest attacks. Dialkoxybenzenes are promising new insect control agents. Some of these compounds mimic naturally occurring odorants that modulate insect behavior. Before applying these compounds, however, their persistence and biodegradability at the application site and in the environment must be understood. In this study, the octanol-water partition coefficient, volatility and sorption on soil components (sand, clay and organic matter) of selected dialkoxybenzenes were investigated. In general, these compounds showed acceptable physical-chemical properties. Biodegradation experiments of 1-allyloxy-4-propoxybenzene were done with three strains of *Pseudomonas putida*. Two of the three strains of *P. putida* tested were able to metabolize 1-allyloxy-4-propoxybenzene. The first step in metabolizing by strain ATCC 17453 was dealkylation. *In vitro* tests with CYP101A1 (cytochrome $P450_{cam}$, a camphor hydroxylase), revealed that the dealkylation is catalyzed by this enzyme.

Keywords: Biodegradation; Octanol-water partition coefficient; Sorption; Insecticides; *Trichoplusia ni*; Cytochrome P450; *Pseudomonas putida*

I dedicate my dissertation work to my lovely parents Hossein and Safura, for their love, endless support and encouragement.

Acknowledgements

This thesis would not have been completed without substantial supports from many individuals at SFU. First, I would like to sincerely thank my supervisor, Dr. Erika Plettner for giving me the opportunity to join her group and for her continual support during my work at SFU. I would also like to thank my supervisory committee members, Dr. Margo Moore and Dr. Charles Walsby, for their great comments and input about my research. I would like to thank Dr. Noham Weinberg and Mr. Jacob Spooner for their help in “calculation of partial molar volumes”.

Brinda Prasad, Jason Nardella, Dr. Yang Yu, Dr. Hao Chen and Dr. Carlos Castillo, Linda Cameron, Yulia Rozen, Govardhana Pinnelli are also acknowledged for their helpful discussions.

Last, but not least, I would like to thank my husband, Ahmad, for his understanding and love during the past few years. His support and encouragement was in the end what made this dissertation possible. My parents, Hossein and Safura, receive my deepest gratitude and love for their dedication and the many years of support during my undergraduate studies that provided the foundation for this work.

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Abbreviations

CYP	Cytochrome P40
DDT	Dichlorodiphenyltrichloroethane
DEET	N,N-Diethyl-meta-toluamide
DMSO	Dimethyl sulfoxide
E.coli	*Escherichia coli*
GC	Gas Chromatography
*m*CPBA	*meta*-Chloroperoxybenzoic acid
NADP	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear Magnetic resonance
P.putida	*Pseudomonas putida*
T.ni	*Trichoplusia ni*

1. Introduction

For millennia, people have used different types of poisons to kill insect pests. In the past, the only compounds available were those obtained from nature. For example, nicotine was among the first widely used insecticides (Soloway, 1976). Other examples of insecticides from natural sources include rotenone and chrysanthemic acid, the active component of pyrethrum (Soloway, 1976). (***Fig1.1***)

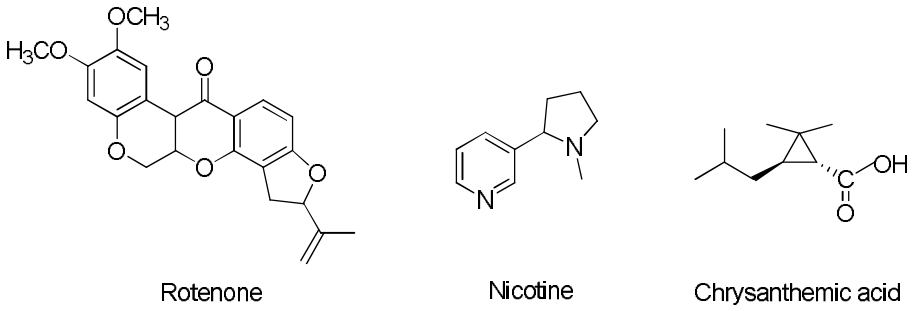

Fig. 1.1

Structure of some naturally occurring insecticides

With the advent of modern organic chemical synthesis, people have learned to make new compounds, not previously found in nature. This has led to the synthesis of a variety of insecticides and many other substances targeted to specific species of pests. Some examples of synthetic insecticides are, DDT (1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane), endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepine-3-oxide), Malathion (diethyl 2-

[(dimethoxyphosphorothioyl)sulfanyl]butanedioate) and carbaryl (1-naphthyl methylcarbamate). (***Fig 1.2***)

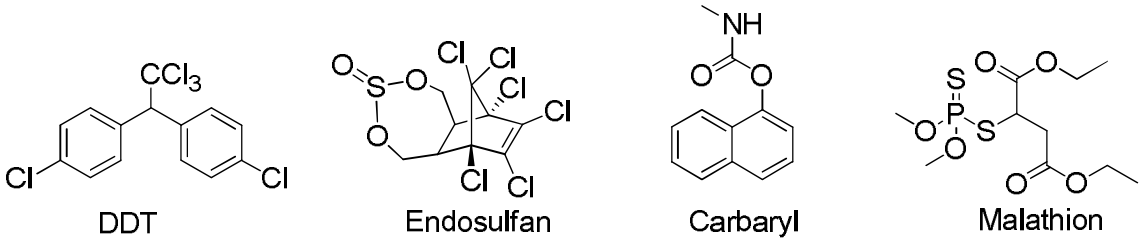

Fig. 1.2

Structures of selected synthetic insecticides

The discovery of the insecticidal properties of DDT in the 1940's marked the beginning of production of a large variety of synthetic pesticides. Many of these compounds are resistant to degradation either by physical, chemical or biological agents in the environment and have become major pollutants (Horrigan *et al.*, 2002). Another concern about pesticides is their effect on non-target organisms. It has been estimated that only 0.1% of a pesticide applied to the environment actually reaches the target pest; the excess pesticide contaminates air, water and soil (Pimentel and Levitan, 1986).

As mentioned, many pesticides can persist for a long time in the ecosystem. For example, hydrophobic pesticides like endosulfan or DDT are very persistent in the environment because the compound itself and the partial degradation products are hydrophobic, and soil microorganisms are not able to fully metabolize them (Bhalerao and Puranik, 2007) (Dimond *et al.*, 1971). The fate of organic compounds is controlled by diverse processes such as sorption to soil components, uptake by plants, biodegradation, volatilization, photo-degradation and chemical degradation. These

processes direct the transportation and transformation of chemicals in the environment (Cavoski *et al.*, 2008).

1.1. Deterrent insect control

Plants release organic compounds into the environment to protect themselves against pathogens and pests. For example, plant leaves make particular waxes and proteins on their surface to protect them against pathogens (Shepherd and Wagner, 2007). Some leaves have sticky coating hairs that can make the movement of small insects on their surfaces very difficult. (Wagner, 1991). In addition, some plant compounds such as monoterpenes, low molecular weight alcohols, aldehydes and esters, are volatile and spread fast through air or soil (Reddy and Guerrero, 2004) (Landolt and Phillips, 1997). Generally, plants use these volatile compounds as messages to communicate with the neighbouring environment (Knudsen *et al.*, 2006). For example, volatile compounds such as hexenal and methyl salicylate help plants in their defence against herbivores and pathogens (Goff and Klee, 2006). They can also attract beneficial insects, e.g. pollinators that help in plant reproductive development or parasitoids that attack herbivorous insects (see below). Plant defensive approaches *via* volatile compounds can be classified into direct and indirect defence categories. Direct defences have an immediate negative effect on the herbivore, while the indirect defences include higher trophic levels (Price *et al.*, 1980). In the indirect defence, volatile compounds attract natural enemies of the herbivore, i.e. parasitoids or predators, which can dynamically reduce the density of herbivore (Pare and Tumlinson, 1999). Both strategies can be either constitutive (always expressed) or inducible (appear only when needed) (Dicke, 1994). Plant secondary chemical defences, such as phytoecdysteroids

(Dinan, 2001), also discourage insect herbivory, either as feeding or oviposition deterrents or by interrupting larval growth, rather than by killing insects directly (Isman, 2006).

Antifeedants are substances that inhibit normal feeding behaviour of an insect and are found among all of the major classes of secondary metabolites: alkaloids, phenolics and terpenoids. Mainly triterpenes and their derivatives, such as phytoecdysteroids (Rharrabe *et al.*, 2011) or the limonoids (Mordue and Blackwell, 1993) are well known in this group. In addition, another important class of compounds involved in the defence of plants are phenolics (Bhattacharya *et al.*, 2010).

Plants require phenolic compounds for many essential development processes such as growth, defence, reproduction and many other functions. There are several examples of phenolic compounds that are active as feeding deterrents against bacteria and fungi (Wallsgrove, 1994). These compounds have been identified in various structures such as monomeric, dimeric and polymeric phenolics. It also has been found that phenolic compounds are biosynthesized either via the shikimic acid or malonic acid pathways in plants (Kefeli *et al.*, 2003).The most frequent phenolic compounds found in plants are flavonoids which form the backbone of the polyphenolic class of proanthocyanidins (tannins). (***Fig1.3***)

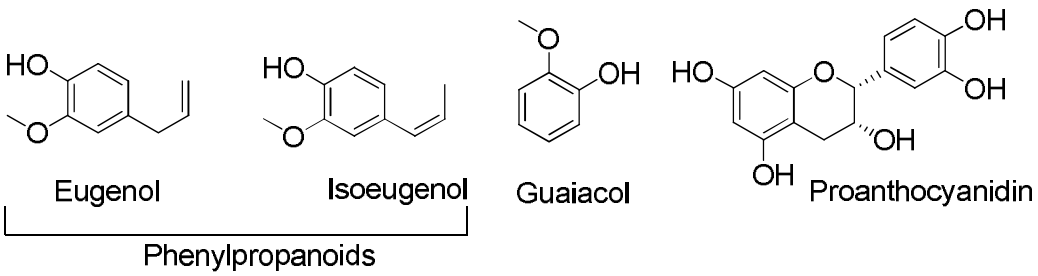

Fig. 1.3

Structure of some naturally occurring phenolic compounds

Low molecular weight phenol derivatives are also present in-wood smoke, which has insect repellent and insecticidal properties. Some of the compounds found in wood smoke are phenol derivatives such as guaiacol (1-hydroxy-2-methoxybenzene), 1,2-dimethoxybenzene, 1-ethoxy-2-methoxybenzene, 1-propoxy- 2-methoxybenzene, eugenol, and isoeugenol (**Fig 1.3**), which are known to have insect-repellent and insecticidal activities (Bortolomeazzi *et al.*, 2007).

1.2. Dialkoxybenzenes

Dialkoxybenzenes are a group of low molecular weight, dihyroquinone, resorcinol or catechol synthetic derivatives that mimic some naturally occurring compounds, such as phenylpropanoids (**Fig 1.3**). It has been found that these compounds have anti-feeding and anti-oviposition effects on *T.ni* (Akhtar *et al.*, 2007; Akhtar *et al.*, 2010), feeding deterrence against gypsy moth larvae (Graham, Forbes, Plettner, unpublished) and modulation of the responses of the pheromone olfactory system in the gypsy moth (Plettner and Gries, 2010a). The compounds can be synthesized in a straightforward procedure from commercially available chemicals (Paduraru *et al.*, 2008).

In previous research, the antifeedant, oviposition deterrent, and toxic effects of individual dialkoxybenzene compounds were assessed against the cabbage looper, *T.ni*, in laboratory bioassays. The research results showed that p-dialkoxybenzene libraries were the most active, with 65% of the members exhibiting feeding deterrence in the range of 53–100% in leaf disc choice bioassays. It was also found that the best oviposition and feeding deterrent compounds from this group are compounds with 2-3 carbon substituents (i.e., propyl, ethyl, allyl) (Akhtar *et al.*, 2007; Akhtar *et al.*, 2010).

The highest feeding deterrence was seen for 1-propoxy-4-allyloxybenzene. This compound also exhibited low toxicity to the larvae and low oviposition deterrence against female moths. In laboratory tests this compound has comparable efficacy to commercial feeding deterrents such as neem oil (Cameron and Plettner, unpublished), a widely used botanical insect control agent (Isman, 2006). Also 1-propoxy-4-allyloxybenzene is more deterrent than DEET (Akhtar *et al.*, 2010), an insect repellent originally developed against mosquitoes (Brown and Hebert, 1997). (***Fig1.4***) Therefore, some dialkoxybenzenes have potential for development as commercial insect control agents with selectivity towards Lepidoptera (Akhtar *et al.*, 2010).

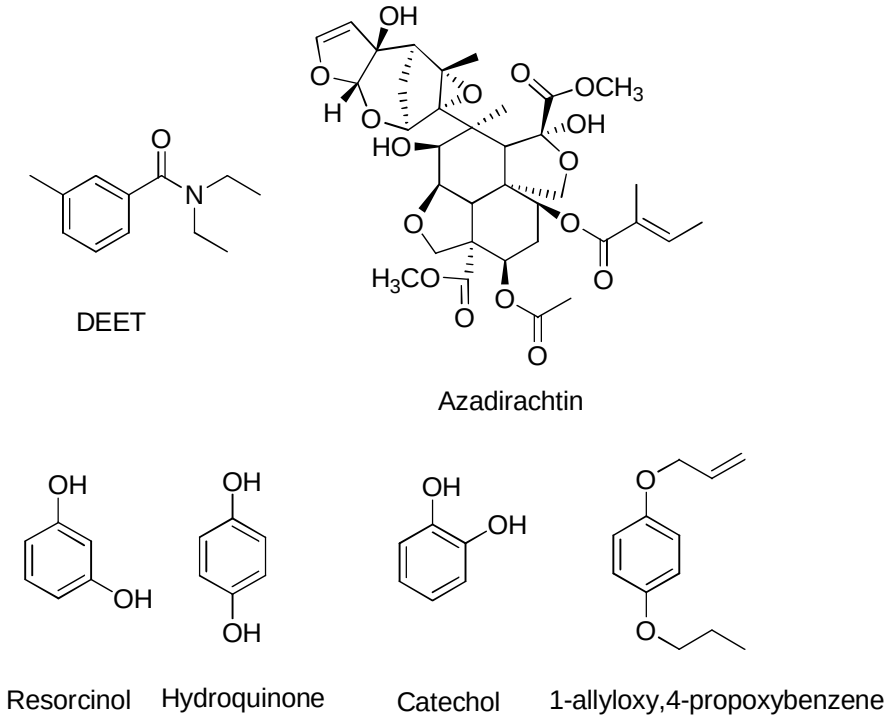

Fig. 1.4

Structure of dialkoxybenzenes and other insect repellent compounds

1.3. Cabbage looper

The cabbage looper, *Trichoplusia ni* (Lepidoptera: *Noctuidae*) is as an important field and greenhouse pest in North America. It is a pest of cruciferous plants but also attacks several other crops including lettuce, beets, peas, celery, tomatoes, certain ornamentals and weeds (Akhtar *et al.*, 2010). The cabbage looper larvae consume three times their weight in plant material daily, so they can cause a lot of damage (Antonious *et al.*, 2007).

Considering that *T. ni* has evolved resistance against many synthetic insecticides (Jiang *et al.*, 2009) and against the microbial insecticide *Bacillus thuringiensis* (Wang et al., 2007*)* there is a need to develop new insect control tools for this pest.

1.4. Physicochemical Properties of Pesticides

The prediction of the transport and fate of chemicals in the environment requires knowledge of their physical and chemical properties. Therefore the physicochemical properties of candidate compounds play important roles in the design of new pesticides. When a pesticide is used in the environment, it will partition between water, air, soil, and living organisms. Pesticides are distributed in the environment by physical processes such as sorption and volatilization, and they can then be degraded by chemical and biological processes.

In the following sections, some of the physicochemical properties of pesticides that affect activity and environmental fate will be discussed.

1.4.1. Water solubility

Water solubility is a fundamental property defined as the concentration of a pesticide dissolved in water when that water is at equilibrium with the pure chemical. In practical terms, water solubility indicates the tendency of a pesticide to be removed from soil by runoff (if the compound is hydrophilic) or its persistence in the soil (if the compound is hydrophobic). The most important cause of aquatic pollution by pesticides is leaching of surface-applied pesticides through the soil and into the ground water (Gustafson, 1989).

Water solubility alone, however, cannot be used for predicting the leaching of pesticides through soil, because the pollutants will distribute themselves between water and other phases: 1) hydrophobic phases (modeled by the octanol-water partition coefficient), 2) air and 3) the surface of solid materials.

1.4.2. Water- Octanol partition coefficient

Lipophilicity of a pesticide is an important physicochemical property that determines the fate of a pesticide in the environment. Lipophilicity of a compound is usually expressed as its partition coefficient between 1-octanol and water (K_{ow}) (Leo *et al.*, 1971). (***Eq.1***) K_{ow} values are predictive of uptake and partitioning of a compound in plants and animals, as well as sorption to soils. Consequently, K_{ow} also reflects the potential of the compound to leach from plants, animals or soil: the higher the K_{ow}, the less the compound will leach from solid and/or biological phases (see Eq. 1).

Partitioning is the process occurring when a solute is added to two liquids, i.e. octanol and water, contacting each other and forming two phases. The solute distributes between the two phases until equilibrium is reached at concentrations dependent upon molecular interactions between the solute and the two liquids. The K_{ow} is defined by the following equation:

$$K_{ow} = \frac{C_o}{C_w}$$ Equation 1.

Where C_o is concentration of the compound in the octanol phase after equilibrium and C_w is concentration of compound in the water phase after equilibrium. Octanol water partitioning is usually reported as Log P values, where P = K_{ow}. This coefficient reflects

the hydrophobicity of a compound: the larger K_{ow} (or log P), the more hydrophobic the compound.

The octanol–water system is a commonly used reference for modeling the partition between water and a biophase (Jaworska *et al.*, 1995). This physicochemical parameter is used for prediction of distribution of chemicals in the environment, estimating bioaccumulation in animals and plants and also in predicting the toxic effects of a substance in QSAR (Quantitative structure activity relationship) studies (Dimitrov *et al.*, 2002). This is because of the amphiphilicity of 1-octanol which makes it a good model of the phospholipids and proteins found in biological systems (Amellal *et al.*, 2001).

1.4.3. Volatility

Compounds used for pest control contaminate the atmosphere by evaporating. Volatilization and air transport are major pathways of pesticide movement (Dorfler *et al.*, 1991). Therefore, the investigation of pesticide evaporation is important for environmental research. It is also important to understand the evaporation process of pesticide droplets on targets to increase the control efficiency of applied pesticide (Samsonov *et al.*, 1998). Volatilization of a compound is dependent on three main factors:

1- The physicochemical properties of compound: vapour pressure, water solubility, partition and adsorption constants.

2- The type of the target surface: for example, evaporation is different from soil than from a plant surface. (Rudel, 1997)

3- Environmental factors: for example, air and surface temperatures, soil moisture and soil components affect the partitioning of the compound and, hence, its volatility. (Burgoyne and Hites, 1993)

Volatilization of pesticides has been studied comprehensively, and results of this research shows that this is the major elimination mechanism for many pesticides. For instance, 90 percent of applied trifluralin (2,6-Dinitro-N,N-dipropyl-4-(trifluoromethyl)aniline), used as a pre-emergence herbicide, evaporated in a week (White *et al.*, 1977).

1.4.4. Interaction of pesticides with soil

A considerable portion of the pesticides applied in agriculture remain associated with the soil for long periods of time. Therefore, it is important to study the toxicity and stability of these chemicals in the soil environment. Soil plays a major role in determining the fate of chemical pollutants (Bollag *et al.*, 1992). In the soil, pesticides can be changed by biotic or abiotic processes giving rise to new products. It is believed that the soil properties have an important influence on the persistence of pesticides and their transformation products (Walker *et al.*, 1992).

Soil is a mixture of organic matter, minerals, and water. The solid part of a typical soil is approximately 5% organic matter and 95% inorganic matter. The majority of inorganic components are primary and secondary minerals; the primary minerals include quartz and micas and the secondary compounds are phyllosilicates (clay minerals),

allophanes, and metal oxides (Buytaert *et al.*, 2005). Quartz and micas are simple SiO_2 minerals but clay minerals are mainly aluminosilicates. Clay minerals have layered structures consisting of silica tetrahedral and alumina octahedral sheets with a ratio of 1: 1 or 2: 1 (H. Bohn, 1985). Soil organic matter contains non-decayed plant and animal tissues, their partial decomposition products, and the soil biomass. For example, the organic matter includes high molecular weight organic materials such as polysaccharides, proteins and mostly (85-90%) humic acids. Soil also contains simpler substances such as sugars, amino acids, and other small molecules (Dube *et al.*, 2001).

The largest quantities of applied pesticides are deposited on the soil, even though the application target may be a crop and volatilization can also happen. In the soil, pesticide molecules can adsorb on the surface of solid particles or partition between aqueous and hydrophobic phases. This distribution will affect the behaviour of a pesticide in the soil. Sorption will determine whether the pesticide will persist in the soil or will be washed out to the ground water and become a pollutant (Wauchope *et al.*, 2002).

Pesticides with low polarity are mainly adsorbed by organic matter of soil because of hydrophobic interactions. However, for more polar molecules, other soil components (minerals) are major sorbents. These sorption processes are all happening in the presence of water as solvent. When soil is dry, it can be sorptive for both polar and non-polar compounds (Delle Site, 2001).

1.5. Degradation of pesticides

1.5.1. Biodegradation

Microorganisms are able to degrade many pesticides by utilizing enzyme catalyzed processes that result in reduced complexity of the chemical. The degradation is called mineralization if the pesticide is degraded to CO_2 and inorganic products. Degradation can either induce growth if the compound is used as C- and energy source or non-proliferation (non-growth) as the microorganisms degrade without gaining energy. Mineralization is often growth-linked so the number of degrading bacteria increases as the compound concentration decreases, until the compound has been completely metabolized (Aislabie and Lloydjones, 1995).

1.5.1.1. Biodegradation of aromatic compounds

Aromatic compounds, such as phenylalanine or tyrosine, are found in all organisms. Beside the natural occurrence, some aromatic compounds such as benzene, toluene, xylene, etc. have been added to the environment (Jindrova *et al.*, 2002). However, plants and animals lack a degradation pathway for recycling carbon from benzenes, and only bacteria and fungi are able to degrade these compounds either by an aerobic or an anaerobic pathway (Fuchs *et al.*, 2011). Non-polar aromatic compounds such as benzene or toluene are first oxidized by oxygenases. These are enzymes that reduce O_2, and insert oxygen atom(s) into the organic substrate. If both oxygens are inserted, the enzyme is a dioxygenase and if only one oxygen is inserted the enzyme is a monooxygenase. This degradation pathway is then followed by other reactions that eventually lead to ring cleavage. (***Fig 1.5***)

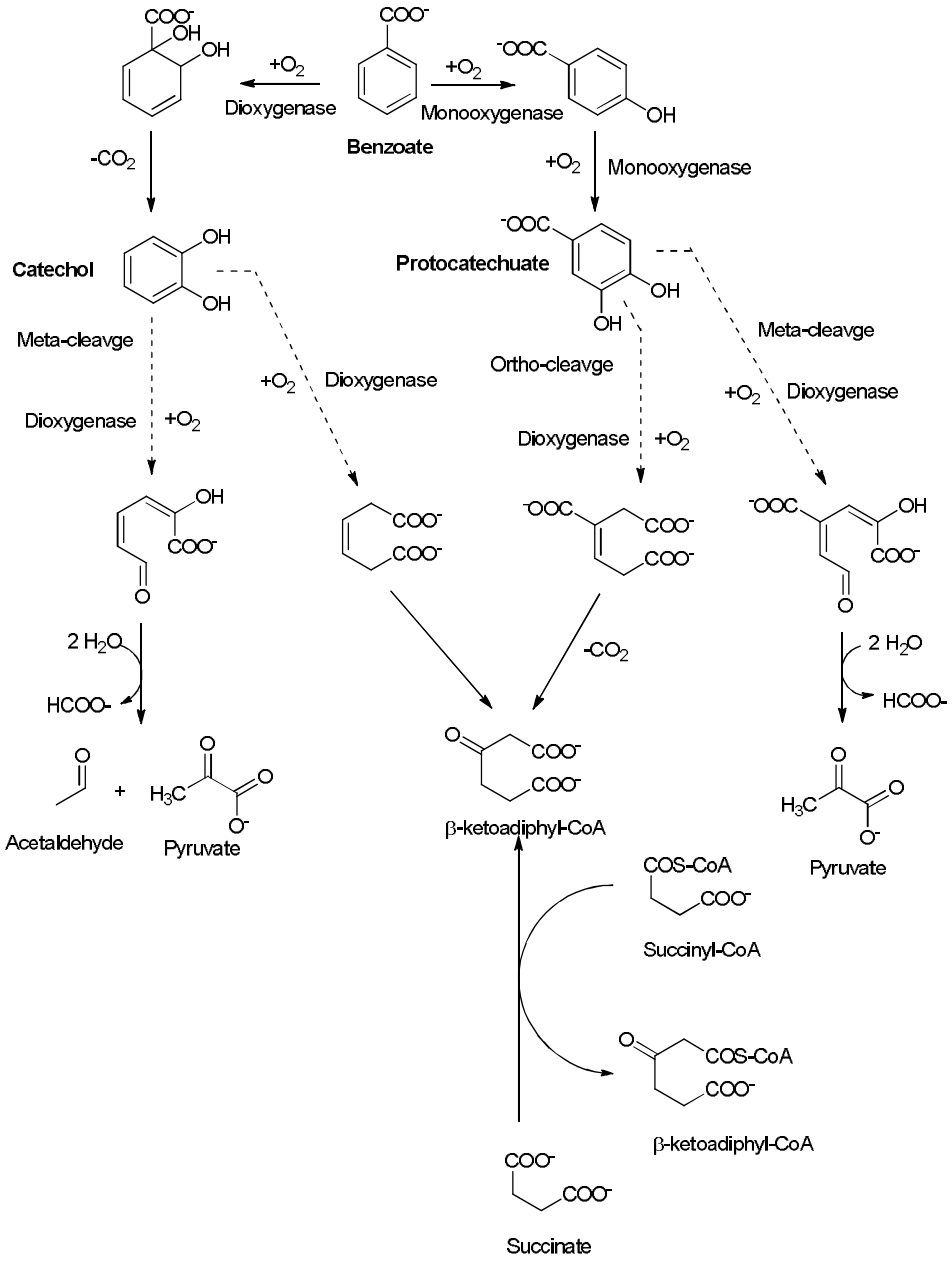

Fig. 1.5

Aerobic degradation of benzoate in bacteria and fungi

(Fuchs *et al.*, 2011)

Species of the genus *Pseudomonas* are able to metabolize a large number of organic compounds including aromatic hydrocarbons which gives them a high potential for biodegradation of pollutants (Rossellomora *et al.*, 1994). Within this genus *Pseudomonas putida* is found widely distributed in soil and water and well known for metabolizing environmental pollutants especially aromatic compounds (Seo *et al.*, 2009). For example *P.putida* is able to mineralize benzene and toluene via the toluene dioxygenase (TOD) pathway. In these cases the genes for the catabolic enzymes are located on the TOL plasmid. (Mosqueda *et al.*, 1999).

Cytochrome P450 is a key monooxygenase in *P. putida* which functionalizes nonpolar aromatic compounds for further degradation. Cytochrome P450 enzymes are heme-containing monooxygenases involved in a number of vital processes including degradation of xenobiotics. One important reaction catalyzed by these enzymes is hydroxylation of hydrocarbons through the following scheme (Schlichting *et al.*, 2000):

$$NADPH + H^+ + O_2 + RH \rightarrow NADP^+ + H_2O + ROH$$

This reaction occurs through a seven-step catalytic cycle depicted in ***Fig 1.6.*** The resting enzyme [1] has water bound to the iron at the axial coordination site of the iron porphyrin. When the organic substrate binds to the active site, the axial water molecule is displaced, giving rise to square-pyramidal complex [2], with the substrate pointed above the iron. This complex is reduced to [3]; O_2 binds, giving [4]. This complex is reduced again and protonated, giving [5]. Protonation of the distal OH group of the hyropreroxo moiety of [5] causes water to split off and compound [6] to form. This is the active Fe-oxo species that then hydroxylates the hydrocarbon in a two-stage mechanism. First, [6] abstracts a hydrogen atom from C-H bond, giving complex [7] and

a carbon radical. Recombination of the OH radical from [7] and the carbon radical gives the alcohol products. Finally, displacement of the alcohol product by water restores the resting state of the enzyme (Montellano, 1995; Schlichting *et al.*, 2000).

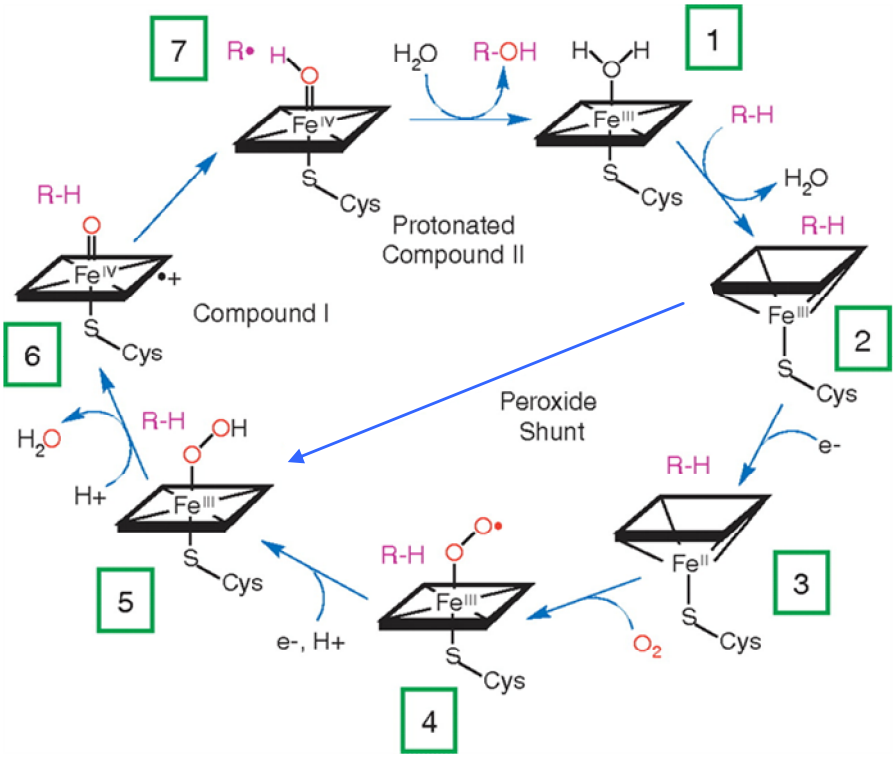

Fig. 1.6

Schematic representation of the P450 catalytic cycle and peroxide shunt pathway

(Ener *et al*., 2010)

P450 enzymes are also well known for the oxidation of carbon atoms located next to a heteroatom (O, S, N), which leads to heteroatom dealkylation (Cryle *et al.*, 2003). (***Fig 1.7***)

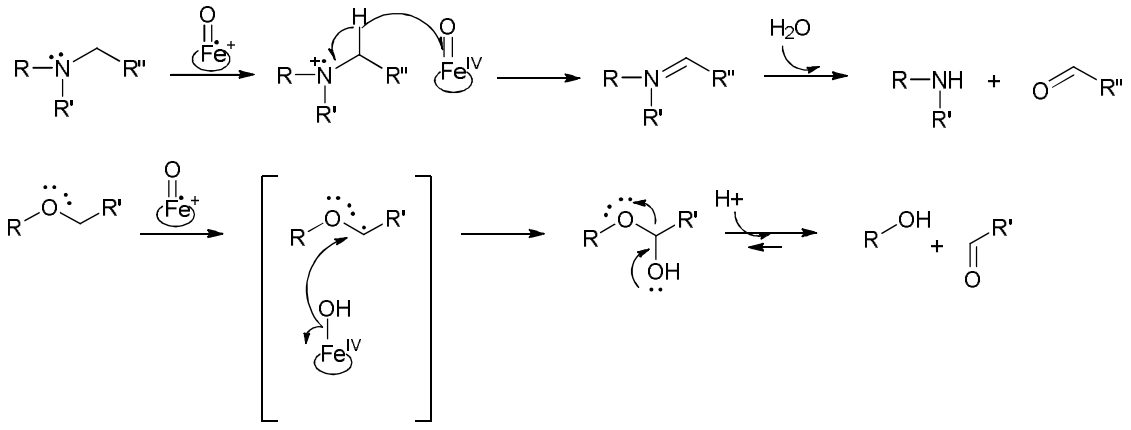

Fig. 1.7

Mechanism of heteroatom oxidation and dealkylation by P450
(Cryle *et al.*, 2003)

Cytochromes P450 catalyze an important first step in the functionalization of hydrophobic compounds. Other enzymes such as dioxygenases can then degrade the structure further, as shown, for example, in ***Fig 1.5***.

1.5.2. Photo-degradation

Pesticides are usually sprayed onto soil or plants, so they are exposed to UV light and atmospheric radicals. In fact, photolysis on the surface of leaves and of the soil can be an important pathway for degradation of pesticides, after their release into the environment. For example photodegradation is the major degradation pathway for Imidacloprid (1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylidene-amine) with a photodegradation half life of 45 minutes (Wamhoff and Schneider, 1999). (***Fig 1.8***)

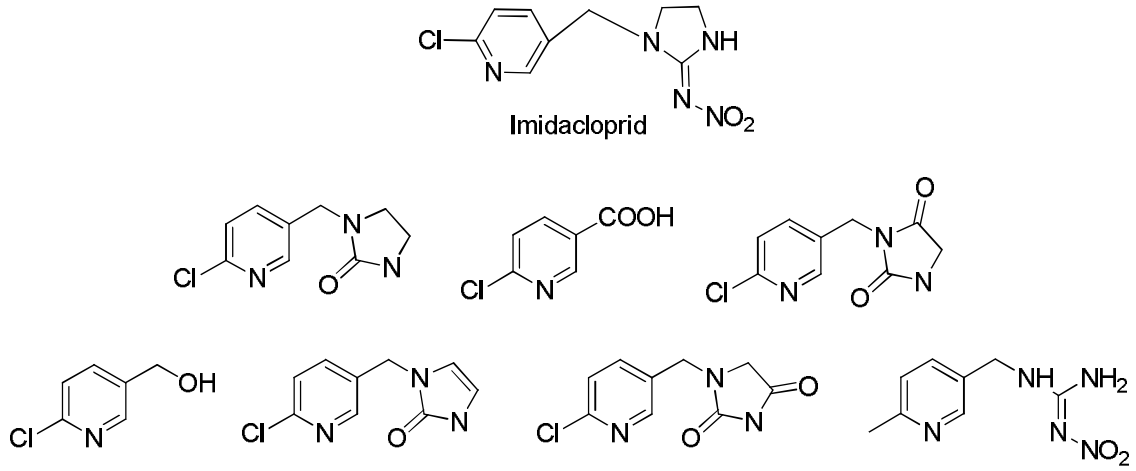

Fig. 1.8

Photo-products of imidacloprid
(Wamhoff and Schneider, 1999)

Photodegradation happens by direct and indirect photolysis. In direct photolysis, the pesticide absorbs UV light and then decomposes either by reacting with other compounds in the environment or with itself. Indirect photolysis occurs when natural

substances in the environment absorb sunlight and form radicals which then react with pesticides (Franko *et al.*, 2005). The factors that determine whether a pollutant will be photo-degraded are: 1) whether the compound has a chromophore that absorbs in the UV or visible range, 2) how light-exposed the compound is on the surface of leaves or soil particles and 3) the presence of other substances (e.g. moisture, pH or formulation of compounds) (Katagi, 2004).

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2. Partition, sorption and structure activity relation study of dialkoxybenzenes that modulate insect behavior

This chapter comprises the manuscript "**Partition, sorption and structure activity relation study of dialkoxybenzenes that modulate insect behavior**" which was prepared for the journal *Environmental Science and Technology*.

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Preface

I have conducted all the experiments for this work. I also wrote the manuscript. Jacob Spooner helped with section 2.4 (Partial molar volume) and 2.5 (Molar surfaces). Table 2.2 and Figure 2.3 on this chapter were prepared by Jacob Spooner.

2.1. Abstract

Some dialkoxybenzenes are promising new insect control agents. These compounds mimic naturally occurring odorants that modulate insect behavior. Before applying these compounds, however, their persistence and biodegradability at the application site and in the environment should be understood. The fate of organic compounds in the environment is a complex phenomenon which is influenced by many processes such as sorption to soil components, volatilization, and uptake by plants, as well as biotic and abiotic chemical degradation. In this study, the octanol-water partition coefficient, volatility and sorption on soil components (sand, clay and organic matter) of selected dialkoxybenzenes as well as structure-activity relationships with regard to partition, volatility and sorption were investigated.

2.2. Introduction

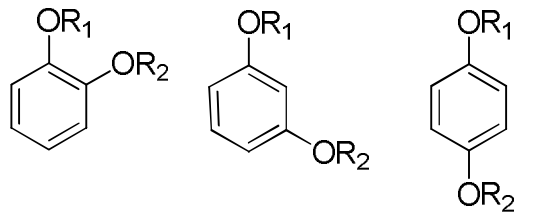
The fate of organic compounds in the environment is a complex phenomenon which is influenced by many processes such as sorption to soil components, uptake by plants, biodegradation, volatilization, photodegradation and chemical degradation. (Kah and Brown, 2007). These processes control the transport, transformation and biological impact of chemical substances in the environment. For example, rotenone, a natural product sold for insect control, can accumulate in soils, if the degradation capacity of those soils is exceeded by frequent applications.(Cavoski *et al.*, 2008)

After application, a significant portion of the pesticides applied in agriculture remains associated with the soil over long periods of time. Sorption of organic compounds to soil is the most important factor that will determine whether compounds will persist or be transported and become a water pollutant (Wauchope *et al.*, 2002). Strongly soil-adsorbed compounds will result in low contamination of water (in the ground or at the surface),(Duran-Alvarez *et al.*, 2012) whereas compounds of high water solubility will dissolve and be washed away by rain and surface water (Reid *et al.*, 2000). Furthermore, other investigations have concluded that the biodegradation rate of adsorbed compounds is dependent on the particle size and type of the sediment, for example, bacterial density has been shown to be lower on sand particles than on silt or clay (Amellal *et al.*, 2001). Therefore, it is important to understand the partition of new pest control agents between soil components, biophases, water and air.

The octanol–water system is a widely used reference for modeling the partition between water and a biophase such as soil or the fat tissue of a living organism (Jaworska *et al.*, 1995). The reason is that 1-octanol is amphiphilic; it has a polar, hydrogen bond donating and accepting hydroxyl group and a non-polar alkyl chain that is sufficiently long to make 1-octanol a separate phase from water. This amphiphilicity of 1-octanol makes it a good model of the phospholipids and proteins found in biological systems (Amellal *et al.*, 2001). The partition properties of an organic compound in biological systems are highly influenced by its hydrophobicity which is quantified as the octanol-water coefficient (K_{ow}). Partition of compounds in soil systems, in turn, affects their sorption to the soil components and, consequently, their biodegradation by soil-dwelling organisms (Duran-Alvarez *et al.*, 2012). This article presents an investigation of the partition, volatility and sorption of low-molecular weight dialkoxybenzenes, which are

being developed as new insect behavioral control agents for agricultural applications (Plettner, 2009). Dialkoxybenzenes have been chosen because they mimic naturally occurring plant odorants, have been shown to affect olfactory responses (Plettner and Gries, 2010b) and behaviors of Lepidoptera (moths) (Akhtar *et al.*, 2007)'(Akhtar *et al.*, 2010) and can easily be prepared from commodity chemicals (Paduraru *et al.*, 2008). For instance, 1,4-diethoxybenzene and 1-methoxy-4-propoxybenzene are among the strongest agonists of gypsy moth (*Lymantria dispar*) pheromone responses.(Plettner and Gries, 2010b; Gong and Plettner, 2011) Furthermore, 1-allyloxy-4-propoxybenzene is a feeding deterrent against cabbage looper (*Trichoplusia ni*) with relatively low toxicity, whereas 1,4-diethoxybenzene is a feeding and oviposition deterrent but also a toxicant against *T. ni*. (Akhtar *et al.*, 2010) Since *T. ni* has evolved resistance against many insecticides, it is essential to develop new methods, that do not involve killing the insects directly, to protect crops from leaf damage by this insect.

In this study, octanol-water partitioning, volatility and sorption to models of three soil components (clay, sand and organic matter) have been measured for *ortho- meta* and *para*, diethoxy, dipropoxy and diallyloxybenzene, as well as 1-allyloxy-4-propoxybenzene (**Fig 2.1**).



$R_1 = R_2$ = ethyl, propyl or allyl

R_1= allyl and R_2= propyl (para congener only)

Fig. 2.1

Dialkoxybenzenes characterized in this study

We discuss our results in terms of structure-activity relationships and compare the partitioning, sorption and volatility of the dialkoxybenzenes to the properties of commonly used insecticides.

2.3. Experimental

Test Compounds. Dialkoxybenzenes were synthesized from the corresponding dihydroxybenzenes by stepwise monoalkylations, as described elsewhere (Paduraru *et al.*, 2008). Dimethyl sulfoxide (DMSO, 99.9% chemical purity) from Caledon Laboratories Ltd (Georgetown, ON, Canada). All solvents were distilled prior to use. Sand (washed and ignited) was purchased from EMD chemicals. 1-Octanol (99% chemical purity), Kaolinite ($Al_2O_3 \cdot 2SiO_2 \cdot 2H_2O$), humic acid sodium salt (technical grade) and DEET (N,

N-diethyl-3-methylbenzamide) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Water was distilled and filtered (18 MΩ) on a Barnstead Nanopure water system. Cat litter (Safeway™) was used as a model of clay in some sorption tests. Kaolinite was used as a clay model in other tests. Cellulose was used as a model of the organic component of soil in most tests with the exception that humic acid was tested with 1-allyloxy-4-propoxybenzene.

2.3.1. Octanol /water partition coefficient

To determine the octanol-water partition coefficient for dialkoxybenzes, a defined volume of water (1 mL) was added to an identical volume of octanol with defined compound concentration (0.7 mg/ml). Vials were incubated at 25±1 ºC overnight (16 h). Four replicates were completed for each compound.

The next day, the water was separated from the octanol phase. Hexane: EtOAc (5:1) was used for the extraction of compounds from water (2×1 mL). The separated octanol phase was diluted with hexane: EtOAc (5:1). Both extracts were dried over sodium sulphate. Aliquots of the sample extracts (1µL) were analyzed by gas chromatography (GC), on a Perkin Elmer Clarus 500 instrument (Avondale, PA, USA) equipped with a flame ionization detector and a 30 m×0.25 mm i.d.×0.25 µm film thickness SPB-5 column (Supelco, Bellefonte, PA, USA). The temperature program used was as follows: initial temperature 100°C, rate 10°C per minute, final temperature 250°C (10min). Retention Indices were determined for the compounds by comparison of their retention times to the retention times of straight-chain alkanes (Freeman, 1981).

Octanol /water partition coefficient validation. In order to confirm the accuracy of the method used for finding K_{ow}, the octanol-water partition coefficient of

DEET was determined by two experiments: one experiment was done as above, and the other followed the same procedure except that *ortho*-dipropoxybenzene (100 ng/mL) was used as internal standard in the extracting solvent.

2.3.2. Volatility Experiments

To measure gas-liquid equilibrium constants, a known concentration of each compound was prepared in hexane. A defined volume of each solution (2 mL) was transferred to a vial (4 mL), fitted with a Teflon-lined septum, and incubated at 25±1 ºC overnight. Full equilibrium of the chemical solute between liquid and gas phases is required for the determination of volatility. The next day, 1 mL of the headspace gas was taken by a gas-tight syringe and directly injected into the GC for analysis.

2.3.3. Sorption Experiments

Since soil constituents differ in various forms, in this experiment the focus was on the following soil components: sand, clay and cellulose; the latter was a model of the more complex and variable organic matter found in soil (Wauchope *et al.*, 2002). Clay, sand and organic matter are common components in soils in all parts of the world,(Randall J Schaetzl, 2005) which make these data applicable various geographic regions. Sorption of the dialkoxybenzenes to the solid phase was determined by processing organic matter (cellulose or humic acid), sand and clay (Kaolinite or cat litter) in batch equilibrium experiments at room temperature (25±1 º C). The solid phase (100 mg) and water containing 1% (w/v) of the compound were transferred to glass vials (4 mL), fitted with Teflon-lined septa. Clay was pre-wetted in 1 mL distilled water and then transferred to the vials, because it absorbed a lot of water. An equilibration time of 24 hours was chosen for the sorption experiments. For analysis, 1 mL of head space gas of

each vial was taken by a gas-tight syringe and injected to the GC. The mixed samples were centrifuged for 5 minutes and the supernatant (water phase) was removed and extracted with hexane: EtOAc (5:1). The same solvent was used for extraction of compounds from the solid phase. Both extracted solutions were dried over sodium sulphate and analyzed by GC.

Sorption Experiment with Kaolinite Sorption of 1-allyloxy-4-propoxybenzene onto kaolinite (Aluminum silicate, $Al_2O_3 \cdot 2SiO_2 \cdot 2H_2O$) was determined by the same method as outlined above for the other batch equilibration experiments.

Sorption Experiment with humic acid Sorption of 1-allyloxy-4-propoxybenzene on humic acid sodium salt was determined as described above; with the exception that humic acid was precipitated, at the end of the incubation time, by adding 1 mL of saturated $CaCl_2$ then by centrifuging.

2.4. Partial molar volumes

In an attempt to interpret the physical properties of the dialkoxybenzenes, their partial molar volumes in water and octanol were calculated using molecular dynamics simulations. Previous research has shown that there is a correlation between molecular volumes, logP and soil sorption of some pesticides, such as phenylureas (Reddy and Locke, 1994).

Volumes were calculated using the displacement volume model which uses constant pressure MD (Molecular Dynamic) simulations to obtain the partial molar volume of the solute **X**, in a system of N solvent particles **S**, $N \cdot$**S**, as an Archimedean displacement volume: $V(\mathbf{X}) = V(\mathbf{X}+N\mathbf{S}) - V(N\mathbf{S})$.

The calculations were performed using the GROMACS package (Berendsen *et al.*, 1995; Lindahl *et al.*, 2001; Van der Spoel *et al.*, 2005) for a system of 1000 water molecules in a cubic box with periodic boundary conditions. The system was maintained at a constant pressure and a constant temperature matching experimental conditions using Berendsen temperature and pressure coupling (Berendsen *et al.*, 1984). The MD trajectories were obtained using leap-frog integration with 1 fs time step with an interaction cut off radius of 0.9 nm. The solutes and the octane solvent were described using the OPLS force field (Jorgensen *et al.*, 1996) and the flexible SPC model was used for the water solvent (Toukan and Rahman, 1985).

2.5. Molecular surfaces

Molecular surfaces were calculated as solvent-excluded surfaces (Richards, 1977) using van der Waals radii augmented by addition of an increment to account for the effects of thermal expansion and specific solvent-solute interactions. The augmenting increments were determined by adjusting the values of molecular volumes confined by the surfaces to the volumes obtained in molecular dynamics simulations.

2.6. Results

2.6.1. Volatility

The compound concentration after partitioning in the liquid phase (C_l), and the concentration of the substance in the gas phase (C_g) were determined by gas chromatography. The volatility was calculated by $K_{l/g} = C_l / C_g$. The measured log $K_{l/g}$ values of different dialkoxybenzenes are listed in **Table 2.1**. The larger $K_{l/g}$ the less volatile the compound. The most volatile compound is 1,2-diethoxybenzene with a $K_{l/g}$

value of 1.18. This result is not surprising, as this compound had the smallest volume and molecular weight. As expected, the larger propyl homologs had higher masses and volumes and, therefore, lower volatilities. Interestingly, the diallyl compounds were least volatile (had the largest log $K_{l/g}$). It is possible that the π bonds of the diallyl compounds mediate more extensive intermolecular Van der Waals interactions than the alkyl groups of the other compounds. Among the diallyl compounds, the *ortho* and *meta* compounds were more volatile than the *para* congener. The minimized structure of the *meta* and *para* diallyloxybenzenes were planar whereas the structure of the *ortho* congener had one C=C bond perpendicular to the benzene ring and the other at 45°, and these protruding allyl groups may weaken intermolecular van der Waals interactions in this case. Conversely, the *para* diallyloxybenzene was the most elongated, planar structure that could have the most extensive stacking interactions, thereby giving the lowest volatility of all the compounds studied. The 1-allyloxy-4-propoxybenzene had a log $K_{l/g}$ intermediate between that of the *para*-dipropoxy- and *para*-diallyloxybenzenes. The 1-allyloxy-4-propoxybenzene minimized structure had all carbons of both side chains in the plane of the benzene and the side chains in the *anti* conformation. Because more hydrogens protrude above and below the carbon framework on the side with the alkyl substituent, stacking interactions between the molecules should be less extensive than in *para*-diallyloxybenzene. The allyl compounds were generally more compact than the propyl compounds (**Table 2.1**), and there was an inverse linear relationship between $K_{l/g}$ and the calculated molar volumes, with exception of the diethoxybenzenes (**Fig 2. 2A**).

Table 2.1 Octanol-water partition coefficients (log K_{ow}) and liquid-gas partition coefficients (log $K_{l/g}$) for dialkoxybenzenes

	Log $K_{l/g}$ [1]	**Log K_{ow} [1]**	**Volume [2] (cm^3/mol)**
1,2-diethoxybenzene	1.18 ± 0.06	1.58 ± 0.20	163.18
1,2-dipropoxybenzene	2.77 ± 0.20	0.30 ± 0.12	195.11
1,2-diallyloxybenzene	6.13 ± 0.21	2.70 ± 0.18	183.17
1,3-diethoxybenzene	3.37 ± 0.50	1.02 ± 0.14	165.13
1,3-dipropoxybenzene	3.54 ± 0.03	0.98 ± 0.23	197.63
1,3-diallyloxybenzene	5.95 ± 0.20	3.80 ± 0.12	185.41
1,4-diethoxybenzene	3.21 ± 0.08	4.32 ± 0.09	164.97
1,4-dipropoxybenzene	3.22 ± 0.10	1.46 ± 0.06	197.50
1,4-diallyloxybenzene	9.25 ± 0.42	3.50 ±0.14	187.31
1-allyloxy-4-propoxybenzene	4.33 ± 0.12	2.40 ± 0.17	191.40

[1] Mean ± S. E. of 4 replicates

[2] Calculated using the GROMACS package, as described in the methods.

2.6.2. Octanol/water partition coefficient (K_{ow})

The compound concentration after partitioning in the water phase (C_w), and the concentration of the substance in the octanol phase (C_o) were determined by GC. The partition coefficient was calculated as $K_{ow} = C_o/C_w$.

The accuracy of the K_{ow} value was verified by checking the mass balance of the starting amount of the compound compared to the total amount of the compound partitioned between the two phases. The measured log K_{ow} values of different dialkoxybenzenes are listed in **Table2.1**.

The K_{ow} is representative of hydrophobicity, because the K_{ow} of compounds is inversely proportional to their water solubility.(Barnsley, 1982) Among the measured alkoxybenzenes, *para*-diethoxybenzene, had the largest log K_{ow} value of 4.32 at 25°C, a surprising result, given that this compound had the shortest alkyl chains. *Para*-dipropoxybenzene had a surprisingly low log K_{ow} value compared to the diethoxy homolog, and the diallyl congener was intermediate. Interestingly, the log K_{ow} value for 1-allyloxy-4-propoxybenzene was 2.4, intermediate between the values obtained for the *para*-dipropoxy and *para*-diallyloxy compounds.

There seem to be two trends among these compounds with regard to correlation between logK_{ow} and the molar volume. For p-diethoxybenzene, the diallyloxybenzene set, 1-allyloxy-4-propoxybenzene and the dipropoxybenzene set, there was an unexpected inverse correlation **(Figure 2.2B**). This correlation suggests that p-diethoxybenzene was more hydrophobic than the dipropoxy set, even though it has shorter hydrocarbon chains. For o- and m-diethoxybenzene and the diallyloxy set there was a positive linear correlation between logK_{ow} and molar volume. This trend is expected, because the larger hydrophobic substituent in the diallyl congeners gave the overall compounds higher hydrophobicity than the o- or m-diethyl versions.

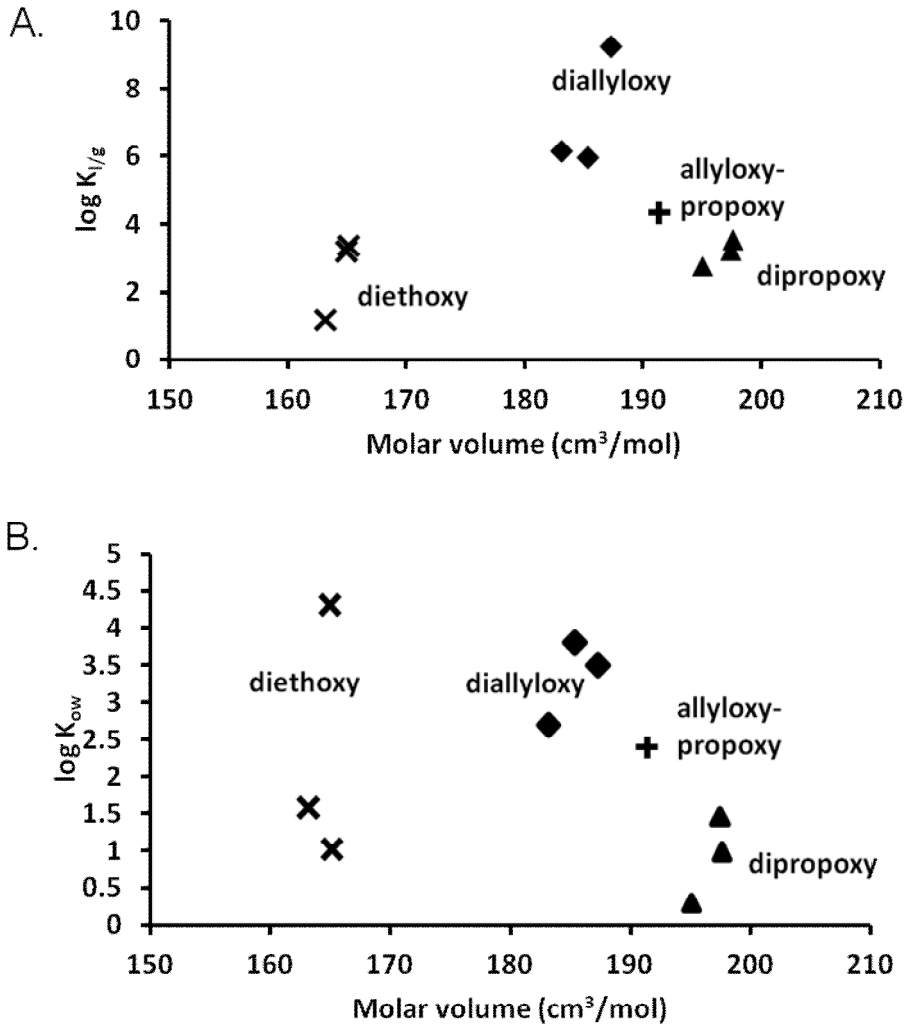


Fig. 2.2

A. Correlation between volatility constants and molar volumes of dialkoxybenzenes. B. Correlation between octanol-water partition values and molar volumes of dialkoxybenzenes

2.6.3. Correlations with hydrophobic/hydrophilic surface areas

Since molecular surface area can be subdivided into its hydrophilic and hydrophobic sections, it seems to offer a better descriptor of molecular hydrophobicity

than molecular volume. We therefore attempted to correlate the experimental values of $\log K_{ow}$ to the calculated molecular surface areas (**Table 2.2**) according to linear equation

$$\log K_{ow} = (G_w - G_o)/(RT) = g_{lw}S_{lw} + g_{bw}S_{bw} - g_{lo}S_{lo} - g_{bo}S_{bo} + g_{ow}$$

Where G, R, T, and S denote Gibbs energy, gas constant, temperature, and area, respectively. Subscripts l, b, o, and w abbreviate hydrophi***l***ic, hydropho***b***ic, ***o***ctanol, and ***w***ater. Coefficients g found by linear regression were (in the order of their appearance in the above equation): 63.16 nm^{-2}, -21.76 nm^{-2}, 63.39 nm^{-2}, -19.06 nm^{-2}, and 8.65 . The values of $\log K_{ow}$ estimated from the equation correlated well with the experimental data (**Fig. 2.3**). Still, 1, 4-diethoxybenzene produced a significant outlier.

Table 2.2 Volumes, surface areas, and estimated octanol-water partion coefficients (log K_{ow}) for dialkoxybenzenes

	Volume[1] (cm^3/mol)		Augmenting radii increments[2] (nm)		Hydrophobic Surface Area[3] (nm^2)		Hydrophilic Surface Area[3] (nm^2)		log K_{ow}[4]
	octanol	water	octanol	water	octanol	water	octanol	water	
1,2-diethoxybenzene	163.4	165.1	0.084	0.085	2.35	2.36	0.15	0.15	2.06
1,2-dipropoxybenzene	198.3	197.6	0.088	0.088	2.81	2.81	0.12	0.12	1.05
1,2-diallyloxybenzene	182.2	185.4	0.079	0.081	1.86	1.87	0.96	0.98	3.86
1,3-diethoxybenzene	165.1	163.2	0.085	0.084	2.36	2.35	0.12	0.12	2.53
1,3-dipropoxybenzene	194.5	195.1	0.085	0.086	2.78	2.80	0.11	0.11	0.80
1,3-diallyloxybenzene	191.0	183.2	0.084	0.080	1.92	1.88	0.93	0.91	2.75
1,4-diethoxybenzene	164.0	165.0	0.085	0.085	2.36	2.36	0.14	0.14	2.26
1,4-dipropoxybenzene	197.1	197.5	0.086	0.087	2.76	2.77	0.14	0.14	0.92
1,4-diallyloxybenzene	187.1	187.3	0.083	0.083	1.86	1.86	0.98	0.98	3.43

This table was prepared by Jacob Spooner.

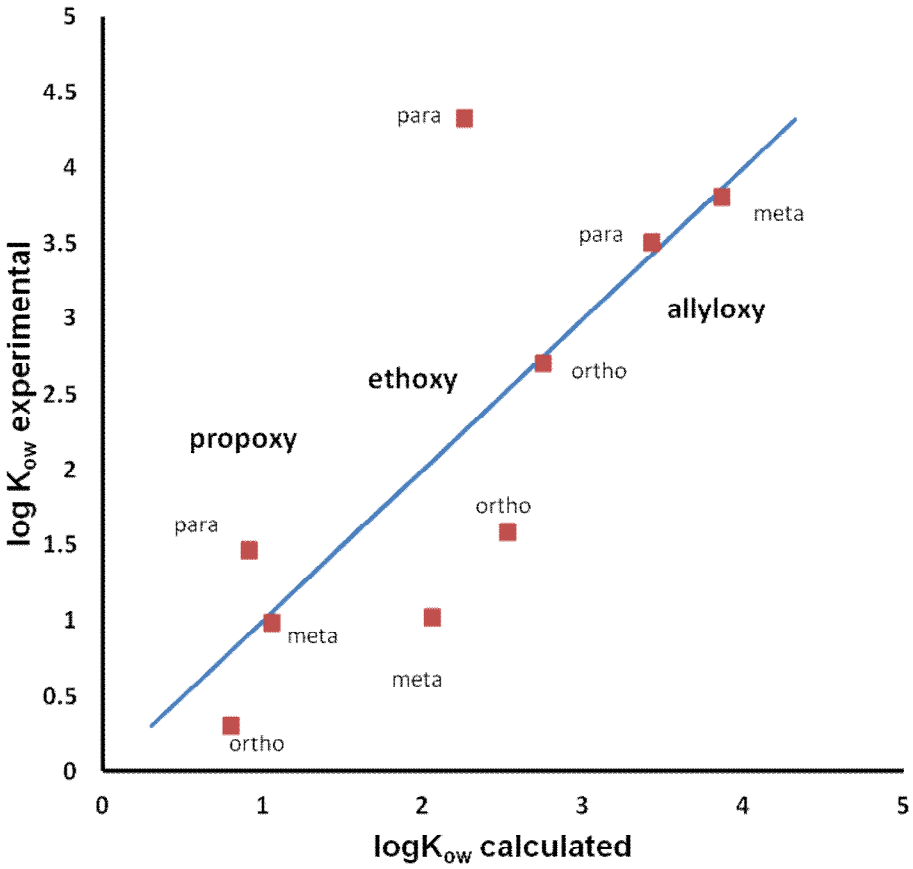


Fig. 2.3

Correlation between experimental octanol-water partition coefficients and those estimated from the hydrophobic/hydrophilic molecular surface areas

This figure has been prepared by Jacob Spooner.

2.6.4. Octanol /water partition coefficient validation

The octanol water partition coefficient of DEET was determined to be 2.97±0.41 in the usual experiment and 2.80 ±0.37 in the experiment with internal standard. These two values do not differ significantly (Anova, $P > 0.05$), confirming that our quantization method does not require addition of an internal standard to the extraction solvent.

Nonetheless, these values differ from the literature values of 2.02,(Noble, 1993)2.58(Jahn *et al.*, 2010)and 2.00 (Fediuk *et al.*, 2010) which have been obtained by different methods.

2.6.5. Sorption

Sorption of compounds to three different soil components was examined. Results are presented in **Fig 2.4**. In all cases, the significant majority of compound was adsorbed by the solid phase in comparison to the amount of compound in the liquid and gas phases. (Anova, $P < 0.05$) In all three experiments (sand, clay, cellulose) *meta*-dipropoxy benzene was more adsorbed by the solid phase than other tested compounds. *Para* diethoxybenzene exhibited the lowest adsorption on clay and sand (Kruskal–Wallis, $P < 0.05$) while *ortho* dipropoxybenzene had the lowest adsorption on cellulose in comparison to other dialkoxybenzenes (Kruskal–Wallis, $P < 0.05$). **Figure 2.5** shows sorption of 1-allyloxy-4-propoxybenzene on different solid substrates. There were no significant differences in the sorption of this compound on cellulose vs. humic acid or on cat litter vs. Kaolinite, thus validating our use of cellulose as a model for organic soil matter and of cat litter for clay components in other tests.

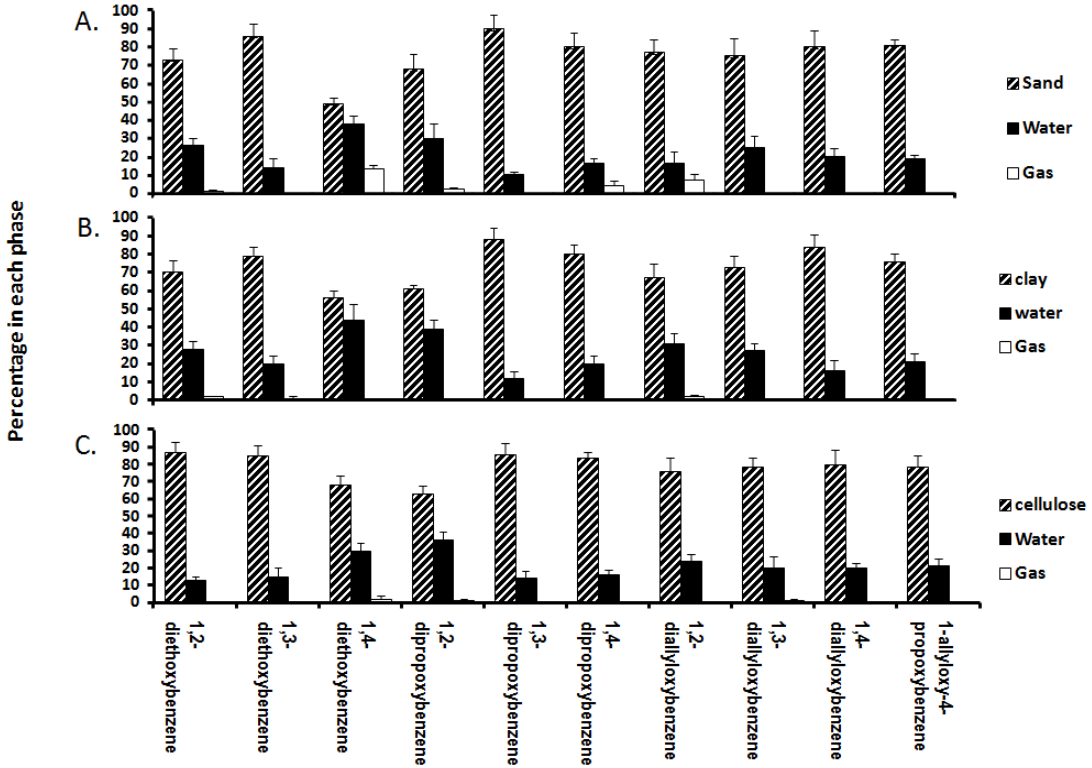


Fig. 2.4 Sorption of dialkoxybenzenes: A. on sand; B. on clay; and C. on cellulose

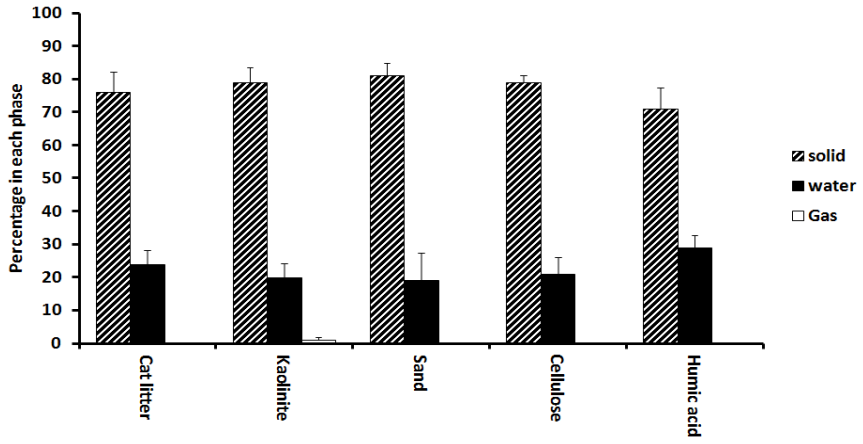


Fig. 2.5

Sorption of 1-allyloxy-4-propoxybenzene on different solid substrates

2.6.6. Volatility

Generally, volatility of these compounds was not significant, with one exception: para diethoxybenzene. This compound showed some volatility when partitioned between sand, air and water. This result is consistent with the result obtained in the volatility tests, namely, that diethoxybenzene was the most volatile compound.

2.7. Discussion

In general, these compounds showed acceptable physical-chemical properties in comparison to other pesticides that are currently used or have been used in the past. For example, 1-allyloxy-4-propoxybenzene has a logP value of 2.4, which is lower than logP for DDT (6.19), carbaryl (2.76), endosulfan (3.62) or malathion (2.89). Higher logP values mean that the compounds are more hydrophobic than 1-allyloxy-4-propoxybenzene, and as a result there is a higher likelihood of bioaccumulation in the ecosystem (Streit, 1992). In contrast, a negative logP value is also not favorable, since such a value means that the compound readily dissolves in water and can be moved by water through soil. For example, solubility of dicamba (3, 6-dichloro-2-methoxybenzoic acid) (logP= -1.69)(Gerstl and Helling, 1987) in water is 8310 mg/L(Armbrust, 2000) and that solubility may lead to the contamination of groundwater.

Sorption is a process that governs and controls pesticide degradation in soil. However, the effect of sorption on biodegradation is complicated and depends on many factors, such as microorganisms, soil properties and characteristics of a chemical itself. In some cases, pesticides (e. g., paraquat) are irreversibly bound to soil components which make them isolated from degrading organisms (Burns and Audus, 1970). However, most of the pesticides used currently (i.e. carbamates, organophosphates and etc.) reversibly partition between a solid soil component and water (Karickhoff, 1981). In some cases sorbed compound is available for further microbial or chemical degradation (Katayama *et al.*, 2010). For example, bacteria themselves may also be sorbed, which can make biodegradation possible, even for sorbed compounds (Ogram *et al.*, 1985). In this study, we have found that dialkoxybenzenes adsorb more onto solid phases (soil components) than they partition into the aqueous or gas phase. However, these results

were obtained in a closed system. Under environmental conditions, where there is running water, rain, etc., results might be different.

It is interesting to note that 1, 4-diethoxybenzene and 1, 2-dipropoxybenzene were both significant outliers in terms of their correlation of partition properties and molar volume. In particular, 1, 4-diethoxybenzene behaved more hydrophobically in the octanol-water partition test than expected, and 1,2-dipropoxybenzene was opposite. Both of these compounds were adsorbed less than all the other compounds on the solid phases we tested, and they both showed higher proportion in the water. Thus, when partitioned between water and 1-octanol, 1,4-diethoxybenzene appears much more hydrophobic than when partitioned between water, air and a solid phase. Consistent with the high K_{ow}, this compound was the most toxic of all the compounds we tested against *T. ni*. (Akhtar *et al.*, 2007)

The 1,4-diethoxybenzene was also the most volatile compound, and this was also apparent in the solid/water/air partitioning tests.

The unusual K_{ow} values of 1,4-diethoxybenzene and 1,2-dipropoxybenzene might be explained by their intermolecular interactions with each other and with water. Gaussian calculations show that 1,4-diethoxybenzne molecules are flatter in organic solvent compared to water, whereas 1,2-dipropoxybenzene or 1-allyloxy-4-propoxybenzene molecules have almost the same conformation in organic solvent and in water (**Fig 2. 6**). This difference in conformational partitioning may help to explain the unexpectedly high hydrophobicity of 1,4-diethoxybenzene, compared to the other two compounds. In the organic phase, the 1,2-diethoxybenzene molecules may pack better against the solvation sphere than in the aqueous phase. In addition, moving from the

organic to the aqueous phase may carry an entropic penalty, due to the conformational change.

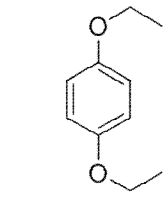
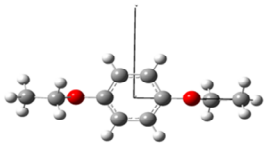
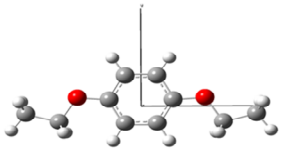
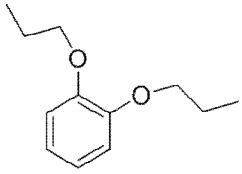
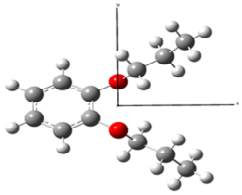
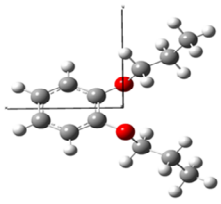
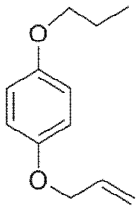
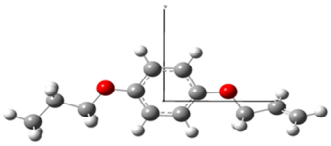
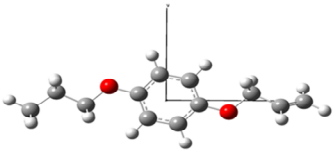
Solvent → Compound ↓	Heptane	Water
		
		
		

Fig 2.6

Computed conformations of 1,4-diethoxybenzene and 1,2-dipropoxybenzene in an organic solvent (heptane) and in water

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3. Biodegradation of 1-allyloxy- 4-propoxybenzene by selected strains of *Pseudomonas Putida*.

This chapter comprises the manuscript "**Partition, sorption and structure activity relation study of dialkoxybenzenes that modulate insect behavior**" which was prepared for the journal *Environmental Science and Technology*.

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Preface

I have conducted all the experiments for this work. I also wrote the manuscript.

3.1. Abstract

Dialkoxybenzenes constitute a class of organic compounds with anti feeding and oviposition effects on the cabbage looper, *Trichoplusia ni.* Among them, 1-allyloxy-4-propoxybenzene has the highest feeding deterrence activity and potential for development as commercial insect control agent. To develop this compound, its fate in the environment needs to be studied. The fate of organic compounds in the environment depends on their biodegradability in the soil. We present results of laboratory biodegradation experiments of 1-allyloxy-4-propoxybenzene with three strains of *Pseudomonas putida*. Two of the three strains of *P. putida* tested were able to metabolize 1-allyloxy-4-propoxybenzene. Both strains required induction of the catabolic pathway. Specially, strain ATCC 17453 (which contains the CAM plasmid) metabolized 1-allyloxy-4-propoxybenzene by first dealkylating. This gave both possible monoalkoxy phenols after five days, followed by dihydroquinone after 8 days. *In vitro* tests with CYP101A1 (cytochrome $P450_{cam,}$ a camphor hydroxylase), revealed that the dealkylation is catalyzed by this enzyme.

3.2. Introduction

The compound 1-allyloxy-4-propoxybenzene is a prospective pest control agent with high feeding deterrence activity against cabbage looper, *Trichoplusia ni*. It also shows low contact toxicity and oviposition deterrence towards the cabbage looper larvae and female moths, respectively.(Paduraru *et al.*, 2008) The physicochemical

properties of 1-allyloxy-4-propoxybenzene and related compounds have been tested and described elsewhere (Ebrahimi et al, chapter 2).

In order to develop this compound further as a potential control agent against cabbage looper, we have tested its bacterial degradation. Here we present the toxicity of this compound against laboratory strains of *Escherichia coli* and *P. putida,* as well as biodegradation by these strains of *P. putida*. The biological degradation of toxic compounds has been recognized for several decades, and the study of biological degradation processes in polluted soil and water has increased in recent years.(Providenti *et al.*, 1993) Many studies have shown that microbial degradation of organic compounds and contaminants can be a possible way to remove contaminants from the environment (Leahy and Colwell, 1990) (Juhasz and Naidu, 2000) (Samanta *et al.*, 2002).

Compounds with aromatic moieties are found in all organisms. For example, phenylalanine, tryptophan, tyrosine and their derivatives such as dopamine or the phenylpropanoids are widely distributed in nature. Besides the naturally occurring aromatic compounds, others, such as benzene, toluene and xylene have been added to the environment by humans (Jindrova *et al.*, 2002). These compounds eventually need to be degraded, to prevent their accumulation, and this degradation is carried out by soil microorganisms. Among soil microorganisms, species of the genus *Pseudomonas* are able to metabolize a large number of organic compounds, including aromatic hydrocarbons, which gives them a high potential for biodegradation of pollutants.(Rossellomora *et al.*, 1994) Within this genus, *Pseudomonas putida* are found widely distributed in soil and water and are well known for metabolizing environmental pollutants, especially aromatic compounds.(Mosqueda *et al.*, 1999)

Aromatic compounds are usually metabolized by oxygenases, and most of the time the product is catechol (1, 2-dihydroxybenzene). This degradation pathway is then followed by ring cleavage catalysed by ring-cleaving dioxygenases.(Fuchs *et al.*, 2011) For instance, *P. putida* (Trevisan) Migula (ATCC 17483) is a strain which contains the naphthalene plasmid. This plasmid encodes naphthalene metabolism enzymes and also the “meta pathway” enzymes for catechol metabolism. The ortho and meta-pathway enzymes of catechol metabolism are induced in ATCC 17483 during growth on naphthalene or salicylate or during growth in the presence of 2-aminobenzoate.(Connors and Barnsley, 1982) Some strains of *P. putida* are also capable of growth on toluene and xylene (i.e. ATCC 33015); in these strains the genes for the catabolic enzymes are located on a TOL plasmid.(Worsey and Williams, 1975) The TOL plasmid of *P. putida* encodes enzymes for the oxidation of toluene to benzoate and xylenes to toluates, and for the further oxidation of benzoate and toluates (Ramos *et al.*, 1987).

Cytochrome $P450_{cam}$ (CYP101A1), which is found in another strain of *P. putida* (Trevisan) Migula (ATCC17453), is a key enzyme which functionalizes monoterpenes (such as D(+)-camphor) and aromatic compounds for further degradation. Cytochromes P450 are monooxygenases involved in the oxidation of many compounds, including degradation of xenobiotics (Schlichting *et al.*, 2000). One reaction catalyzed by CYP101A1 is hydroxylation of D(+)-camphor.(Bradshaw *et al.*, 1959) However, cytochromes P450 are also well known for oxidation of substrates containing carbon next to a heteroatom (O, S, N). This reaction leads to heteroatom dealkylation,(Cryle *et al.*, 2003) or heterocycle ring opening (Sielaff *et al.*, 2001).

In this work, toxicity assays and biodegradation experiments were done with three strains of *P. putida*: ATCC 17453 (camphor metabolizing), ATCC 17484

(naphthalene metabolizing), and ATCC 33015 (toluene/xylene metabolizing*),* to better understand biodegradation of 1-allyloxy-4-propoxybenzene*.* We have found that the induced $P450_{cam}$-containing strain can dealkylate 1-allyloxy-4-propoxybenzene, and *in vitro* assays with recombinant $P450_{cam}$ confirmed that this enzyme is responsible for dealkylation.

3.3. Materials and methods

All the bacterial strains were purchased from American Type Culture Collection (ATCC). Dialkoxybenzenes were synthesized from the corresponding dihydroxybenzenes by stepwise monoalkylations, as described elsewhere.(Paduraru *et al.*, 2008) Dimethyl sulfoxide (DMSO, 99.9% chemical purity) was from Caledon Laboratories Ltd (Georgetown, ON, Canada). All solvents were distilled prior to use. m-Chloro perbenzoic acid (m-CPBA) (≤77% c.p) was purchased from Sigma-Aldrich and purified by a known method.(Aggarwal *et al.*, 1998)

3.3.1. Statistical Analysis

ANOVA (student t test) was performed within each series of the three replicates of treatments (indicated with capital letters), and for comparison between each treatment and its corresponding control (indicated with small letters). Kruskal–Wallis two way comparison tests were chosen to compare data between series and, if significantly different, are indicated with a star (*). Both tests were done on raw data (amount in ng) of degradation time courses. Significance level was $P < 0.05$. These tests were done using JMP, Version 8. SAS Institute Inc., Cary, NC and GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California USA.

3.3.2. Toxicity assays

The IC_{50} values of dialkoxybenzenes were obtained with three strains of soil bacteria (*P. putida* ATCC17484, *P. putida* ATCC17453 and *P. putida* ATCC33015) and one enteric species of bacterium (*E. coli* XL-1-BL), with the following compounds: 1-allyloxy-4-propoxybenzene, *para*- diethoxybenzene, *para*- dipropoxybenzene and *meta* – diallyloxybenzene.

LB media (250 mL) were inoculated with a culture of each bacterial strain that had been grown overnight. These cultures were grown at 300 rpm with good aeration until an O.D. of 0.1 was reached. Aliquots (5 mL) of this culture were transferred to a series of Falcon tubes, to which each compound was added in varying concentrations. The final concentrations of these compounds ranged from 1µM to 700 µM. Dimethyl sulfoxide (DMSO), the solvent used for preparing the stock solutions, was used as one of the controls. The aliquots were grown for 1 hr at 27^{o}C and incubated overnight at 4°C. They were diluted 10^4× and 10^6 × in three replicates, then plated on non-antibiotic LB-agar plates in two replicates (25 and 50 µL) and incubated overnight at 37°C. The next day, colonies were counted and the concentration of colony forming units (CFU/mL) was calculated. The IC_{50} was calculated by graphing CFU/mL *vs*. the logarithm of concentration of the compounds.

3.3.3. Biodegradation Experiments

Degradation experiments were carried out with 1-allyloxy-4-propoxybenzene. The same method was used for all strains of *P. putida*, with minor modifications in the media used for culture. For example, for *P. putida* strain (ATCC 17453), bacteria were inoculated into regular beef broth (Difco™ Nutrient Broth) and incubated over night. The grown culture acted as an inoculum for fresh regular beef broth, and this culture was

grown with shaking (250 rpm) until an optical density (OD) of 0.7-0.9 was reached. The culture was then centrifuged at 6000 rpm, in a Beckmann Avanti centrifuge, using rotor JLA 8.100 (4355 $\times$ g) at 4 ºC for 30 min. The pellets were suspended into M_9 minimal media: $Na_2HPO_4.7H_2O$, KH_2PO_4, NaCl, $MgSO_4$, $CaCl_2$, and NH_4Cl. To make the solution completely homogenous, the culture was shaken for a few minutes (250 rpm) and was then distributed into four different Erlenmeyer flasks (three *treatments*, one *control*). One additional control was prepared using minimal medium without inoculation. One mL of 1-allyloxy-4-propoxybenzene solution in DMSO (10 mg/mL) was added to 99 mL of bacterial culture in the treatments and also the control without bacteria to get the final concentration of 100 ppm. One control flask was left containing bacteria but no compound. Zero time samples were taken from each flask. In order to monitor the evaporation of the compound from the system, the flasks were covered by a cork that held a Porapak® (Waters Corporation, MA, USA) column in the middle. Samples were collected as described below. The treatments and controls were incubated for two weeks. Samples were taken every 24 hours. Porapak® columns were exchanged every 24 h and were rinsed with 500 µL Hexane: EtOAc (5:1) twice, and the eluent was stored in vials and refrigerated until analysis by GC-mass spectrometry (GC-MS).

Two experiments were done with this strain: one without induction by D(+)-camphor, the other with induction. In the latter case, 6 mL of 10 mM D-(+)-camphor in DMSO was added to the nutrient broth during the overnight culture.

3.3.3.1. Sampling and Extraction procedure:

Samples taken from the culture (1 mL) were centrifuged at 10,000 $\times$ g for 5 min and the supernatants were transferred to the vials (14$\times$ 45 mm, 1D) containing NaCl (100 mg). The pellets were washed with sterilized H_2O, centrifuged at 10000 $\times$ g for 5

min and were transferred to fresh vials containing NaCl (100 mg). Both supernatant and pellet solutions were acidified to the pH < 7. Then each pellet and supernatant was extracted with hexane: EtOAc (5:1) dried using Na_2SO_4 and stored in vials. The supernatants were also extracted with hexane: EtOAc (5:1) dried using Na_2SO_4 and transferred to fresh vials. The volume of dried solution was recorded for each extract. Vials were queued at 4°C for GC injection.

A second degradation experiment was carried out using the same procedure as above with another *P. putida* strain from the American Type Culture Collection (ATCC 17484). This strain has the "Naphthalene plasmids" responsible for the *ortho* and *meta* pathways of catechol and resorcinol metabolism in *pseudomonads* that are capable of metabolizing naphthalene (Connors and Barnsley, 1982).

A third degradation experiment was carried out using the same procedure as above with *P. putida* strain from the American Type Culture Collection (ATCC 33015). This strain has the TOL plasmid, responsible for metabolism of toluene and xylene (Worsey and Williams, 1975).

The experiment was carried out with 1-allyloxy-4-propoxybenzene as a carbon source. Strain ATCC 33015 was inoculated into benzoate medium (ATCC MEDIUM #1271), which consists of: $(NH_4)_2HPO_4$ (3.0 g), KH_2PO_4 (1.2 g), NaCl (5.0 g), $MgSO_4.7H_2O$ (0.2g), yeast extract (0.5g), distilled water (1.0 L). After sterilization, sodium benzoate (3.0g) was filter-sterilized and added to the medium. The culture grown acted as an inoculum for fresh benzoate medium, and the growth of this culture was monitored until an optical density (OD) of 0.7-0.9 was reached. The culture was then centrifuged at 4355 $\times$ g at 4 ºC for 30 min and the pellets were suspended into M9

minimal medium as described above. Gas phase and culture monitoring was done as described above.

3.3.4. In vitro Assays with Isolated P450$_{cam}$

In vitro enzymatic assays were performed in 5 ml of 50 mM phosphate buffer (100 mM K^+) (pH 7.4), and recombinant P450cam that had been expressed in *E. coli* and purified as described previously (Rojubally *et al.*, 2007) (Prasad *et al.*, 2011) m-CPBA was used as a shunt agent for the catalytic cycle; the reaction mixture contained 1.5 µM P450cam, m-CPBA (1 mM) and the substrate 1-allyloxy-4-propoxybenzene (5 mM). The experiment was performed in two replicates, and three controls were run in parallel with the treatments: 1) Blank: without enzyme but all other reactants added, 2) Enzyme and the substrate added to the buffer without m-CPBA, 3) m-CPBA and enzyme added without substrate.

The reaction mixture was incubated for 24 hours at 22°C. Samples were taken after 2, 4, 6, 24 hours and $CHCl_3$ were added to extract product(s) and the substrate. A second extraction of the aqueous phase by $CHCl_3$ followed. The combined organic layers were dried over $MgSO_4$ and analyzed by GC-MS.

3.4. Results and Discussion

3.4.1. Toxicity

IC_{50} values were determined for 1-allyloxy-4-propoxybenzene, *para*-diethoxybenzene, *para*- dipropoxybenzene, *meta* –diallyloxybenzene against *P. putida* ATCC17484, *P. putida* ATCC17453, *P. putida* ATCC33015, *E. coli* XL-1-BL. Results (***Table 3.1***) show that *para*- diethoxybenzene was most toxic against *E. coli* and *P.*

putida ATCC33015. *E. coli* XL-1-BL in total seemed to be the most sensitive bacterial species and strain among the ones tested. The 1-allyloxy-4-propoxybenzene was the least toxic compound against three *P. putida* strains among the compounds tested.

Table 3.1 IC_{50} values (µM) for four dialkoxybenzenes with four bacterial strains

Bacteria species → / **Compounds ↓**	*P. putida* ATCC17484 (NAPH)	*P. putida* ATCC17453 (CAM)	*P. putida* ATCC33015 (TOL)	*E. coli* XL-1-BL
1-allyloxy-4-propoxybenzene	454	365	280	45.5
Para- diethoxybenzene	233	32.4	20.3	8.96
Para- dipropoxybenzene	54.6	25.7	110	25.0
para -diallyloxybenzene	53.0	78.4	32.0	36.3

3.4.2. Biodegradation

The concentration of 1-allyloxy-4-propoxybenzene in the degradation experiment with *P. putida* (ATCC 17484) (***Fig. 3.1A***) decreased by approximately 40 percent more than in the control with substrate in the medium but no bacteria (Anova, $P < 0.05$). The control provides an indication of how much the compound evaporates during the experiment, and evaporation was the same in all three experiments (there was ~ 15% less total compound on the last day relative to the first day). It is important to note that this strain did not require any additives that induce the catabolic pathway.

The experiment with *P. putida* ATCC 33015 revealed that there was only a small change in the concentration of 1-allyloxy-4-propoxybenzene in comparison to the control (***Fig. 3.1B***), which suggests that the TOL pathway, even when induced with benzoic acid, is not optimal for the biodegradation of 1-allyloxy-4-propoxybenzene.

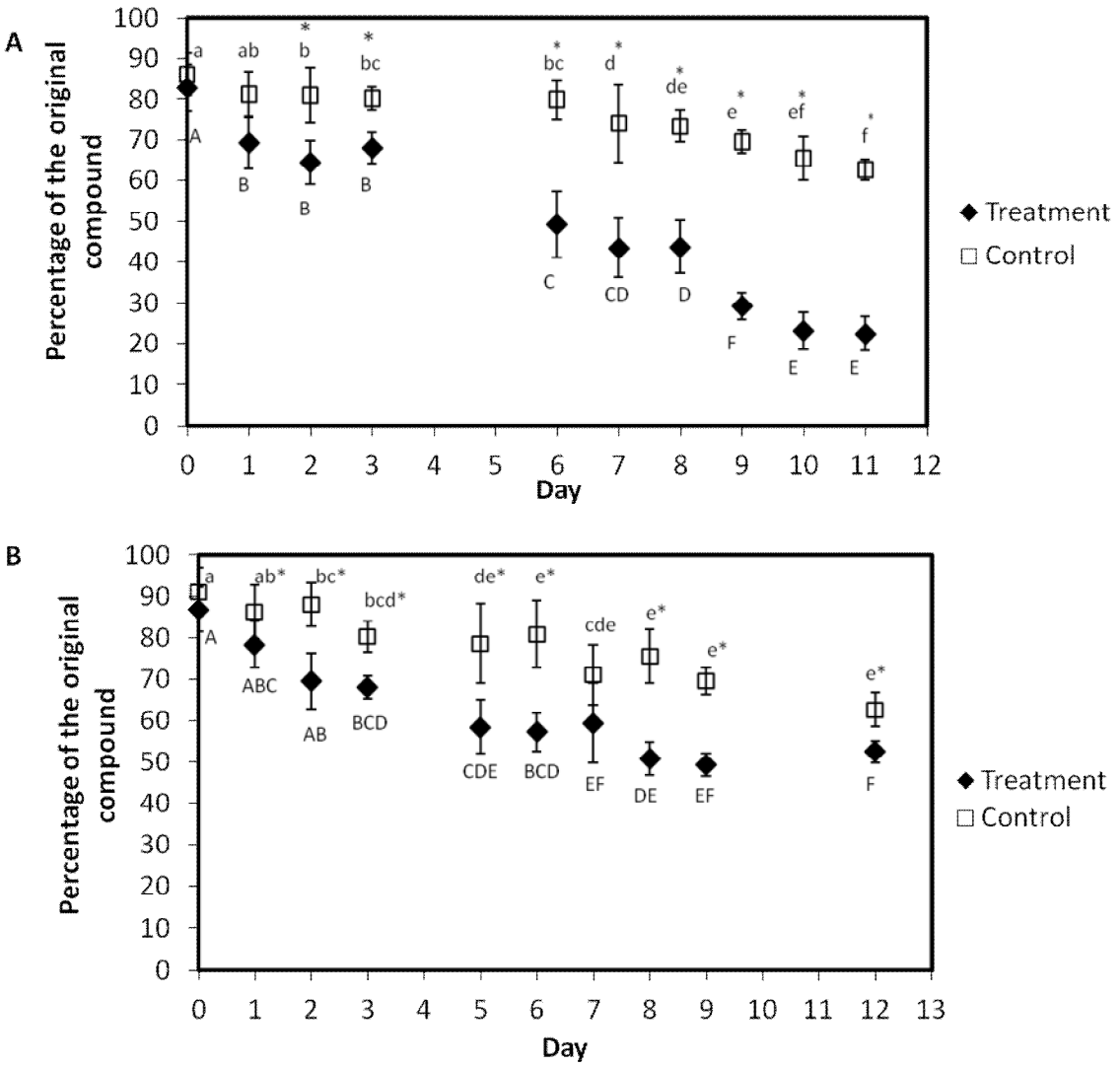

Fig. 3.1

Degradation of 1-allyloxy-4-propoxybenzene by A. P.putida (ATCC 17484), and B. by P.putida(ATCC 33015).

The percentage of starting material left, after adding the totals found in the pellet, solution and gas phases. Letters refer to comparisons (ANOVA) within a series (treatment with capital letters; control with lower case letters), and * refer to pairwise comparison between a treatment and its respective control (Kruskal-Wallis). All comparisons were done on the raw data (total amounts in µg, see supplemental information).

The concentration of 1-allyloxy-4-propoxybenzene in the degradation curve obtained with *P. putida* (ATCC 17453) (***Fig. 3.2A***) decreased with time. In the final day

of this experiment, almost 75% of the compound was either metabolized or had evaporated. The control (from which the compound can only evaporate) contained significantly more compound than the treatment samples (Kruskal–Wallis, $P < 0.05$); therefore, it can be concluded that this bacterial species and strain has the ability to metabolize 1-allyloxy-4-propoxybenzene. GC-MS analysis of products confirms that p-allyloxyphenol and dihydroxybenzene (= dihyroquinone) are two main metabolites. It is important to note that, in this case, camphor had to be included in the medium to induce the camphor catabolism pathway. Without camphor, this strain did not degrade 1-allyloxy-4-propoxybenzene (***Fig. 3.2B***). The two points in the treatment with low percentages of substrate are probably due to experimental error, as this was the first time course done in this series of experiments.

To assess how much of the compound irreversibly sorbs on the bacterial pellets, we performed an experiment with a boiled culture of *P. putida* (ATCC 17453). Results are shown in supplemental information and confirm that decreasing in concentration of substrate is comparable to the control experiment without bacteria culture.

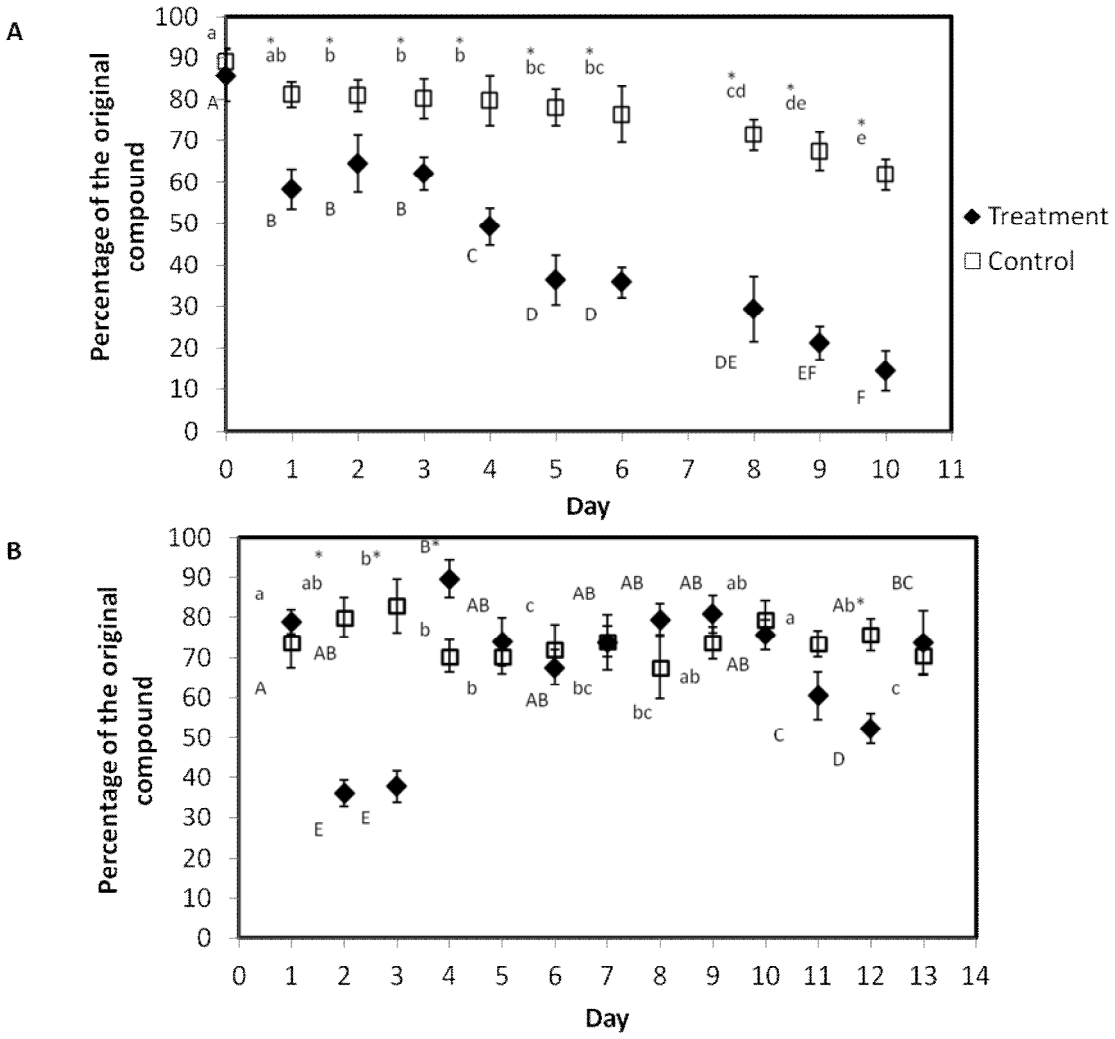

Fig. 3.2

Degradation of 1-allyloxy-4-propoxybenzene by Pseudomonas Putida (ATCC 17453) A. with camphor induction. B. without camphor induction.

The percentage of starting material left, after adding the totals found in the pellet, solution and gas phases .See Fig. 1 for an explanation of the statistical tests done.

3.4.3. In vitro Assays with Isolated P450$_{cam}$

To determine whether P450$_{cam}$ is involved in the formation of dealkylated products, an experiment was performed with purified, isolated P450$_{cam}$. Production of the metabolites from the P450$_{cam}$ assay was monitored by extracting samples with chloroform and analyzing them by GC-MS. Identification and quantification of intermediates was performed based on their MS spectra and by chromatographic comparison with standards. The analyses yielded three distinct peaks (p-propoxyphenol and p-allyloxyphenol have the same retention time of 17.0 min on the program as mentioned above). The peaks with retention times of 15.5 min and 19.0 min in samples drawn at 2-24 h intervals corresponded with those of the standard compounds p-dihydroxybenzene and 1-allyloxy-4-propoxybenzene, respectively (Supplemental information). The time course of relative quantities of metabolites measured is depicted in ***Fig. 3.3***.

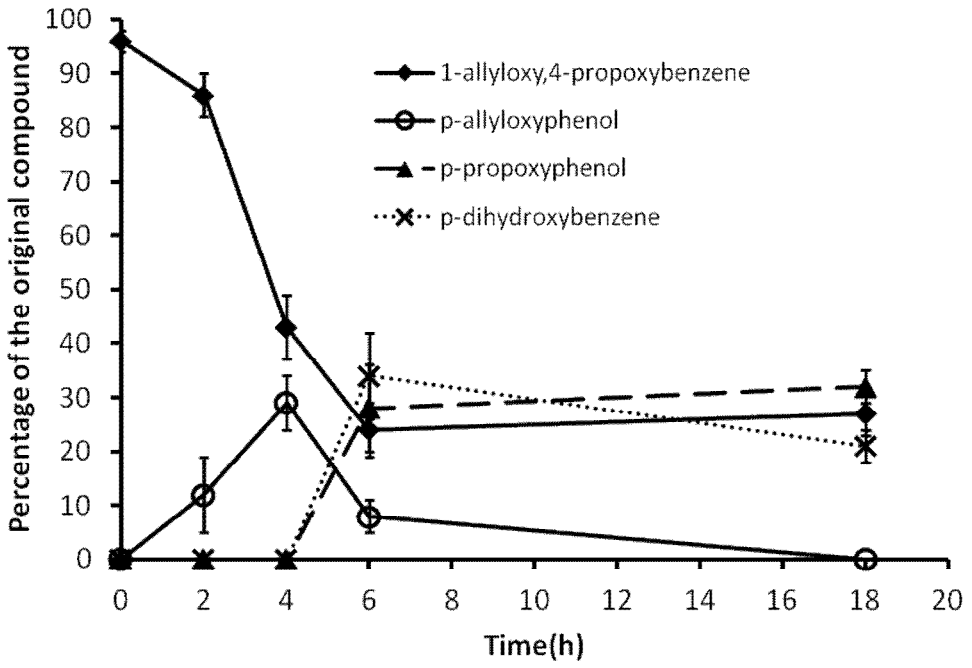

Fig. 3.3

Time course of relative quantities of metabolites

Measured in an *in vitro* experiment with expressed, purified cytochrome $P450_{cam}$ (CYP101A1), shunted with m-CPBA

Overall, the objective of this study was to determine whether 3 strains of *P. putida* with the naphthalene, camphor and TOL plasmids, are able to metabolize 1-allyloxy-4-propoxybenzene, as well as the biodegradation rate.

Results of biodegradation experiments demonstrate that two of the three strains were able to catabolize 1-allyloxy-4-prpoxybenzene. It seems that among the species that have been tested *P. putida* (ATCC 17453) is the best candidate for degrading this compound, provided it is induced with D-(+)-camphor. Using GC-MS, we demonstrated that strain ATCC 17453 degraded 1-allyloxy-4-propoxybenzene through dealkylation and

production of p-allyloxyphenol, p-propoxyphenol and p-dihydroxybenzene. The degradation time course with this bacterial strain also suggests that the p-dihydroxybenzene levels increase over the first few days and then start decreasing after 9 days. We additionally provide evidence for the involvement of cytochrome $P450_{cam}$ (CYP101A1) in dealkylation of 1-allyloxy-4-prpoxybenzene as the initial step of biodegradation. A degradation pathway for 1-allyloxy- 4-prpoxybenzene has been proposed, based on these results and on literature about the degradation of aromatic compounds. First, the P450 dealkylates the dialkoxybenzene stepwise, to yield 1,4-dihydroxybenzene (= dihydroquinone) (**Fig. 3.4,** enzyme 1). This compound can be oxidized to 1,2,4-trihydroxybenzene which is immediately oxidized further by the same enzyme (hydroquinone 1,2-dioxygenase, **Fig. 3.4**, enzyme 2) to 4-hydroxymuconic semialdehyde.(Sielaff *et al.*, 2001) This intermediate is oxidized by 4-hydroxymuconic acid semialdehyde dehydrogenase (**Fig. 3.4**, enzyme 3) to the corresponding diacid.(Sielaff *et al.*, 2001) Tautomerism to the keto form gives 4-keto hex-2-enedioic acid (= maleyl acetic acid), which is reduced by maleyl acetate reductase (Aggarwal *et al.*, 1998) to 3-keto-hexanedioic acid (= 3-oxoadipic acid). This compound can then be further degaded, for example, to succinate (Mucha *et al.*, 2010).

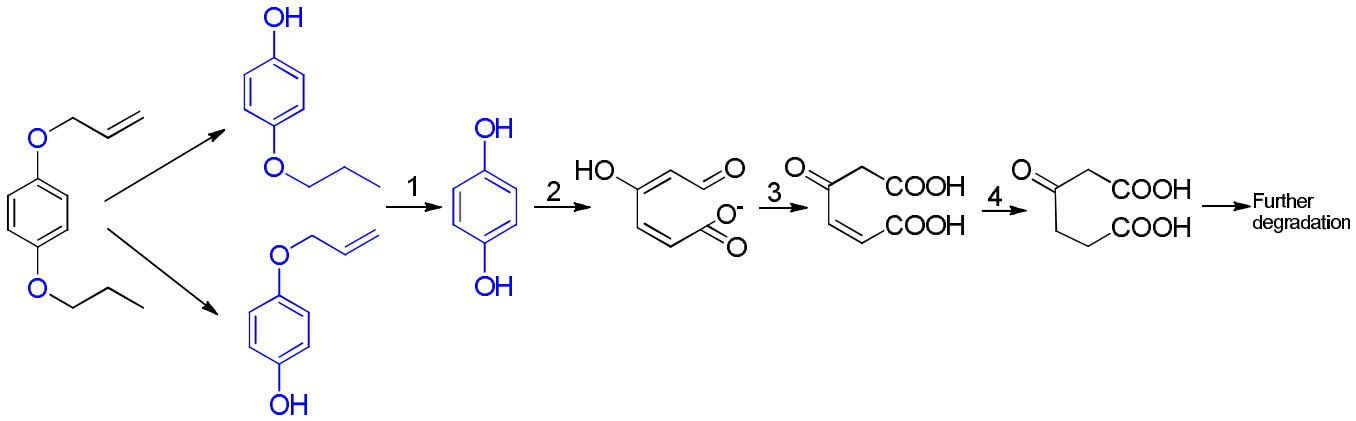

Fig. 3.4

Proposed pathway for the biodegradation of 1-allyloxy-4-propoxybenzene in P. putida ATCC 17453, a D-(+)-camphor metabolizing strain, that is induced by camphor

1: cytochrome $P450_{cam}$ (CYP101A1) (this work), 2: hydroquinone 1,2-dioxygenase,(Spain and Gibson, 1991) 3: 4-hydroxymuconic semialdehyde dehydrogenase,(Spain and Gibson, 1991) 4: maleylacetate reductase.(Kaschabek and Reineke, 1995)

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3.5. Supporting Information

Table S3.1 Biodegradation Experiment details: ATCC 17453 with camphor induction

Day	Gas-Treatment (µg)			Supernatant-Treatment(µg)			Pellet-Treatment(µg)			TOTAL(µg)			TOTAL %			
	Rep.1	*Rep.2*	*Rep.3*	*Rep.1*	*Rep.2*	*Rep.3*	*Rep.1*	*Rep.2*	*Rep.3*	*Rep.1*	*Rep.2*	*Rep.3*	*Rep.1*	*Rep.2*	*Rep.3*	**Avg.**
0	11.30	9.48	11.00	49.05	55.84	45.80	24.65	26.68	23.20	85.00	92.00	80.00	85.00	92.00	80.00	**85.67**
1	12.54	10.30	14.61	27.93	28.18	30.48	16.53	15.72	18.42	57.00	54.20	63.50	57.00	54.20	63.50	**58.23**
2	13.30	12.03	14.12	34.58	29.22	36.71	18.62	16.04	19.77	66.50	57.30	70.60	66.50	57.30	70.60	**64.80**
3	15.12	15.60	14.02	30.24	31.20	28.03	17.64	18.20	16.35	63.00	65.00	58.40	63.00	65.00	58.40	**62.13**
4	9.70	11.52	9.20	29.11	23.04	23.92	15.09	13.44	12.88	53.90	48.00	46.00	53.90	48.00	46.00	**49.30**
5	9.84	7.02	9.60	20.91	15.86	20.40	10.25	7.63	10.00	41.00	30.50	40.00	41.00	30.50	40.00	**37.17**
6	8.80	8.50	7.70	20.80	15.70	18.20	10.40	8.50	9.10	40.00	32.70	35.00	40.00	32.70	35.00	**35.90**
8	8.36	6.38	4.97	19.76	15.08	11.75	9.88	7.54	5.88	38.00	29.00	22.60	38.00	29.00	22.60	**29.87**
9	6.24	5.06	4.12	13.00	11.22	9.13	6.76	5.72	4.65	26.00	22.00	17.90	26.00	22.00	17.90	**21.97**
10	3.50	4.37	2.81	6.86	9.69	5.18	3.64	4.94	2.81	14.00	19.00	10.80	14.00	19.00	10.80	**14.60**
Day	**Control(µg)**			**Control(µg)**			**Control(µg)**			**TOTAL(µg)**			**TOTAL %**			
	Rep.1	*Rep.2*	*Rep.3*	*Rep.1*	*Rep.2*	*Rep.3*	*Rep.1*	*Rep.2*	*Rep.3*	*Rep.1*	*Rep.2*	*Rep.3*	*Rep.1*	*Rep.2*	*Rep.3*	**Avg.**
0	12.00	16.70	19.78	49.07	48.72	43.86	31.23	23.58	22.36	92.30	89.00	86.00	92.30	89.00	86.00	**89.10**
1	18.81	22.06	22.12	43.61	41.08	40.29	23.09	18.86	16.59	85.50	82.00	79.00	85.50	82.00	79.00	**82.17**
2	16.84	18.42	17.78	48.15	41.74	39.42	19.61	21.14	20.10	84.60	81.30	77.30	84.60	81.30	77.30	**81.07**

3	16.67	16.63	15.88	40.95	48.82	38.56	22.68	19.55	21.17	80.30	85.00	75.60	80.30	85.00	75.60	**80.30**
4	16.39	7.50	19.04	44.64	48.71	42.23	22.57	16.79	21.53	83.60	73.00	82.80	83.60	73.00	82.80	**79.80**
5	23.68	15.84	20.02	40.23	40.04	36.96	19.09	18.63	20.02	83.00	74.50	77.00	83.00	74.50	77.00	**78.17**
6	11.39	27.51	20.30	44.82	36.40	33.60	20.79	19.09	16.10	77.00	83.00	70.00	77.00	83.00	70.00	**76.67**
8	11.40	22.12	18.20	41.04	36.40	33.60	16.56	17.48	18.20	69.00	76.00	70.00	69.00	76.00	70.00	**71.67**
9	15.18	21.73	16.51	35.19	33.02	30.48	18.63	18.25	16.51	69.00	73.00	63.50	69.00	73.00	63.50	**68.50**
10	15.74	18.58	15.50	29.73	32.24	31.62	12.83	15.18	14.88	58.30	66.00	62.00	58.30	66.00	62.00	**62.10**

Table S3.2 Biodegradation Experiment details: ATCC 17453 without camphor induction-Treatment.

Day	**Gas-Treatment (µg)**			**Supernatant-Treatment(µg)**			**Pellet-Trearment(µg)**			**TOTAL(µg)**			**TOTAL %**			
	Rep.1	*Rep.2*	*Rep.3*	*Rep.1*	*Rep.2*	*Rep.3*	*Rep.1*	*Rep.2*	*Rep.3*	*Rep.1*	*Rep.2*	*Rep.3*	*Rep.1*	*Rep.2*	*Rep.3*	**Avg.**
1	17.16	12.92	13.94	44.46	43.32	46.74	16.38	19.76	21.32	78.00	76.00	82.00	78.00	76.00	82.00	**78.67**
2	5.94	6.63	7.98	18.81	22.23	20.90	8.25	10.14	9.12	33.00	39.00	38.00	33.00	39.00	38.00	**36.67**
3	6.46	7.98	10.66	19.04	20.90	20.50	8.50	9.12	9.84	34.00	38.00	41.00	34.00	38.00	41.00	**37.67**
4	16.38	21.84	20.46	51.87	42.00	53.01	22.75	20.16	19.53	91.00	84.00	93.00	91.00	84.00	93.00	**89.33**
5	16.17	17.60	15.18	41.90	45.60	39.33	15.44	16.80	14.49	73.50	80.00	69.00	73.50	80.00	69.00	**74.17**
6	15.12	15.62	15.09	34.65	40.47	39.10	13.23	14.91	14.41	63.00	71.00	68.60	63.00	71.00	68.60	**67.53**
7	18.96	14.52	16.72	39.50	37.62	43.32	20.54	13.86	15.96	79.00	66.00	76.00	79.00	66.00	76.00	**73.67**
8	13.60	14.25	14.04	45.60	42.00	47.08	20.80	18.75	21.48	80.00	75.00	82.60	80.00	75.00	82.60	**79.20**
9	15.77	14.04	13.77	47.31	44.46	46.17	19.92	19.50	21.06	83.00	78.00	81.00	83.00	78.00	81.00	**80.67**
10	14.44	15.84	15.01	43.32	41.04	45.03	18.24	15.12	18.96	76.00	72.00	79.00	76.00	72.00	79.00	**75.67**
11	11.88	14.30	11.97	30.78	37.05	35.91	11.34	13.65	15.12	54.00	65.00	63.00	54.00	65.00	63.00	**60.67**
12	13.52	14.56	9.50	27.56	29.68	28.50	10.92	11.76	12.00	52.00	56.00	50.00	52.00	56.00	50.00	**52.67**
13	22.12	21.28	12.35	40.29	38.76	37.05	16.59	15.96	15.60	79.00	76.00	65.00	79.00	76.00	65.00	**73.33**

Table S3.3 Biodegradation Experiment details: ATCC 33015

Day	Gas-Treatment (µg)			Supernatant-Treatment(µg)			Pellet-Treatment(µg)			TOTAL(µg)			TOTAL %			
	Rep.1	*Rep.2*	*Rep.3*	*Rep.1*	*Rep.2*	*Rep.3*	*Rep.1*	*Rep.2*	*Rep.3*	*Rep.1*	*Rep.2*	*Rep.3*	*Rep.1*	*Rep.2*	*Rep.3*	**Avg.**
0	24.96	18.40	12.87	49.92	52.44	50.88	21.12	21.16	21.25	96.00	92.00	85.00	96.00	92.00	85.00	**91.00**
1	16.15	17.67	13.93	49.30	53.01	46.75	19.55	22.32	21.32	85.00	93.00	82.00	85.00	93.00	82.00	**86.67**
2	22.14	20.90	25.94	42.64	52.25	41.82	17.22	21.85	20.24	82.00	95.00	88.00	82.00	95.00	88.00	**88.33**
3	20.54	16.80	17.38	41.08	47.88	41.87	17.38	19.32	19.75	79.00	84.00	79.00	79.00	84.00	79.00	**80.67**
5	17.71	20.64	16.25	42.35	43.86	38.50	16.94	21.50	18.25	77.00	86.00	73.00	77.00	86.00	73.00	**78.67**
6	22.10	21.06	11.95	44.20	41.31	45.05	18.70	18.63	19.00	85.00	81.00	76.00	85.00	81.00	76.00	**80.67**
7	14.64	21.60	21.78	31.72	40.80	32.94	14.64	17.60	17.28	61.00	80.00	72.00	61.00	80.00	72.00	**71.00**
8	18.72	19.24	23.44	37.44	37.74	38.16	15.84	17.02	18.40	72.00	74.00	80.00	72.00	74.00	80.00	**75.33**
9	15.41	15.62	19.16	36.85	40.47	34.84	14.74	14.91	18.00	67.00	71.00	72.00	67.00	71.00	72.00	**70.00**
12	12.60	13.65	15.52	34.20	37.05	31.20	13.20	14.30	17.28	60.00	65.00	64.00	60.00	65.00	64.00	**63.00**
Day	**Cotrol(µg)**			**Control(µg)**			**Control(µg)**			**TOTAL(µg)**			**TOTAL %**			
	Rep.1	*Rep.2*	*Rep.3*	*Rep.1*	*Rep.2*	*Rep.3*	*Rep.1*	*Rep.2*	*Rep.3*	*Rep.1*	*Rep.2*	*Rep.3*	*Rep.1*	*Rep.2*	*Rep.3*	**Avg.**
0	20.02	19.20	17.80	50.96	42.40	48.95	20.02	18.40	22.25	91.00	80.00	89.00	91.00	80.00	89.00	**86.67**
1	18.25	17.71	17.20	37.96	40.81	46.44	16.79	18.48	22.36	73.00	77.00	86.00	73.00	77.00	86.00	**78.67**
2	20.25	15.60	15.87	39.00	34.45	37.26	15.75	14.95	15.87	75.00	65.00	69.00	75.00	65.00	69.00	**69.67**
3	17.68	16.25	15.12	35.36	33.80	38.88	14.96	14.95	18.00	68.00	65.00	72.00	68.00	65.00	72.00	**68.33**
5	17.94	11.50	10.64	35.88	26.00	31.36	15.18	12.50	14.00	69.00	50.00	56.00	69.00	50.00	56.00	**58.33**

6	14.08	12.00	11.40	35.84	24.96	33.60	14.08	11.04	15.00	64.00	48.00	60.00	64.00	48.00	60.00	**57.33**
7	15.18	11.44	12.20	34.98	29.12	34.16	15.84	11.44	14.64	66.00	52.00	61.00	66.00	52.00	61.00	**59.67**
8	10.12	10.08	13.92	25.76	26.88	30.74	10.12	11.04	13.34	46.00	48.00	58.00	46.00	48.00	58.00	**50.67**
9	10.78	12.88	10.07	27.44	23.46	29.68	10.78	9.66	13.25	49.00	46.00	53.00	49.00	46.00	53.00	**49.33**
12	10.56	11.66	11.97	26.88	29.68	29.64	10.56	11.66	15.39	48.00	53.00	57.00	48.00	53.00	57.00	**52.67**

Table S3.4 Biodegradation Experiment details: ATCC17484

Day	Gas-Treatment (µg)			Supernatant-Treatment(µg)			Pellet-Trearment(µg)			TOTAL(µg)			TOTAL %			
	Rep.1	Rep.2	Rep.3	Rep.1	Rep.2	Rep.3	Rep.1	Rep.2	Rep.3	Rep.1	Rep.2	Rep.3	Rep.1	Rep.2	Rep.3	Avg.
0	18.04	13.60	14.62	46.74	45.60	49.02	17.22	20.80	22.36	82.00	80.00	86.00	82.00	80.00	86.00	82.67
1	11.88	12.34	14.49	37.62	41.38	37.95	16.50	18.88	16.56	66.00	72.60	69.00	66.00	72.60	69.00	69.20
2	11.69	13.65	17.94	34.44	35.75	34.50	15.38	15.60	16.56	61.50	65.00	69.00	61.50	65.00	69.00	65.17
3	12.78	16.38	15.84	40.47	31.50	41.04	17.75	15.12	15.12	71.00	63.00	72.00	71.00	63.00	72.00	68.67
6	12.10	10.78	9.68	31.35	27.93	25.08	11.55	10.29	9.24	55.00	49.00	44.00	55.00	49.00	44.00	49.33
7	9.84	9.68	10.12	22.55	25.08	26.22	8.61	9.24	9.66	41.00	44.00	46.00	41.00	44.00	46.00	43.67
8	11.52	8.14	10.12	24.00	21.09	26.22	12.48	7.77	9.66	48.00	37.00	46.00	48.00	37.00	46.00	43.67
9	5.61	5.89	4.25	18.81	17.36	14.25	8.58	7.75	6.50	33.00	31.00	25.00	33.00	31.00	25.00	29.67
10	3.61	5.04	4.08	10.83	15.96	13.68	4.56	7.00	6.24	19.00	28.00	24.00	19.00	28.00	24.00	23.67
11	3.42	5.94	4.29	10.26	15.39	12.88	4.32	5.67	5.42	18.00	27.00	22.60	18.00	27.00	22.60	22.53
Day	**Control(µg)**			**Control(µg)**			**Control(µg)**			**TOTAL(µg)**			**TOTAL %**			
	Rep.1	Rep.2	Rep.3	Rep.1	Rep.2	Rep.3	Rep.1	Rep.2	Rep.3	Rep.1	Rep.2	Rep.3	Rep.1	Rep.2	Rep.3	Avg.
0	18.92	14.96	14.62	49.02	50.16	49.02	18.06	22.88	22.36	86.00	88.00	86.00	86.00	88.00	86.00	86.67
1	15.48	15.13	14.49	49.02	50.73	37.95	21.50	23.14	16.56	86.00	89.00	69.00	86.00	89.00	69.00	81.33
2	16.53	14.43	22.88	48.72	37.79	44.00	21.75	16.49	21.12	87.00	68.70	88.00	87.00	68.70	88.00	81.23
3	14.40	20.54	18.26	45.60	39.50	47.31	20.00	18.96	17.43	80.00	79.00	83.00	80.00	79.00	83.00	80.67
6	19.36	16.72	16.28	50.16	43.32	42.18	18.48	15.96	15.54	88.00	76.00	74.00	88.00	76.00	74.00	79.33

7	17.18	16.50	16.72	39.38	42.75	43.32	15.04	15.75	15.96	71.60	75.00	76.00	71.60	75.00	76.00	74.20
8	18.82	13.64	17.82	39.20	35.34	46.17	20.38	13.02	17.01	78.40	62.00	81.00	78.40	62.00	81.00	73.80
9	10.71	13.49	12.58	35.91	39.76	42.18	16.38	17.75	19.24	63.00	71.00	74.00	63.00	71.00	74.00	69.33
10	12.22	12.29	10.91	36.65	38.93	36.59	15.43	17.08	16.69	64.30	68.30	64.20	64.30	68.30	64.20	65.60
11	10.81	15.40	11.59	32.43	39.90	34.77	13.66	14.70	14.64	56.90	70.00	61.00	56.90	70.00	61.00	62.63

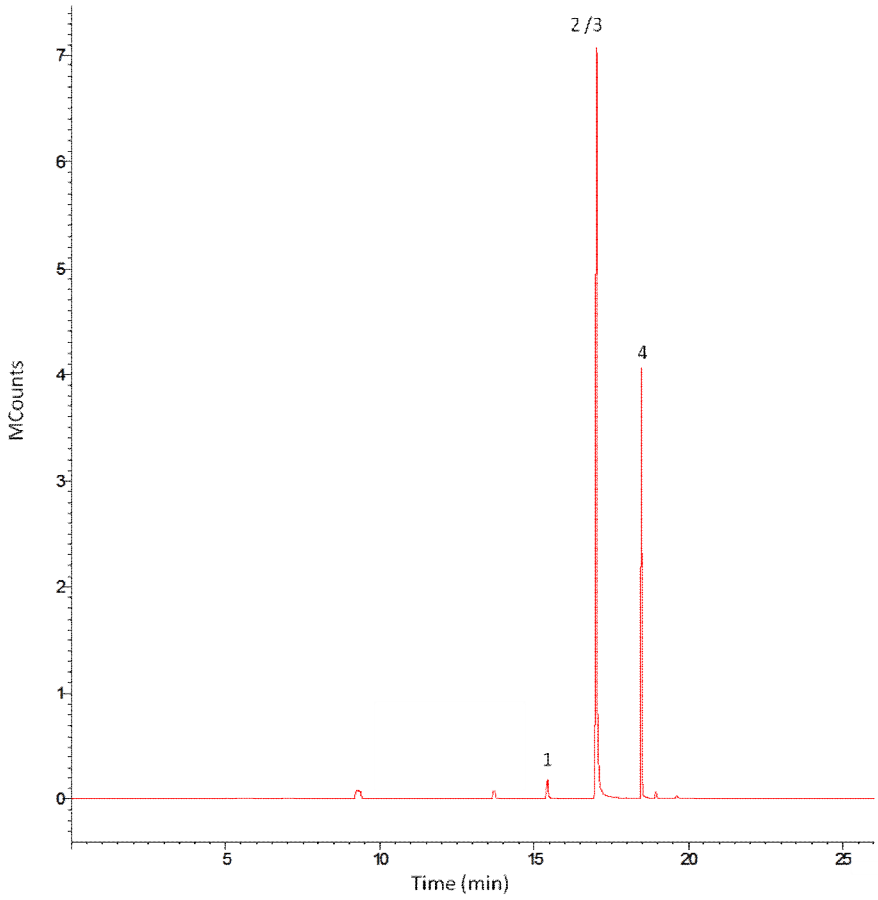

Fig. S3.1

GC chromatograph of the metabolites from the P450$_{cam}$ assay

Peaks 1, 2, 3 and 4 represent P-dihydroxybenzene, P-allyloxyphenol, P-pripoxyphenol and 1-allyloxy-4-propoxybenzene respectively.

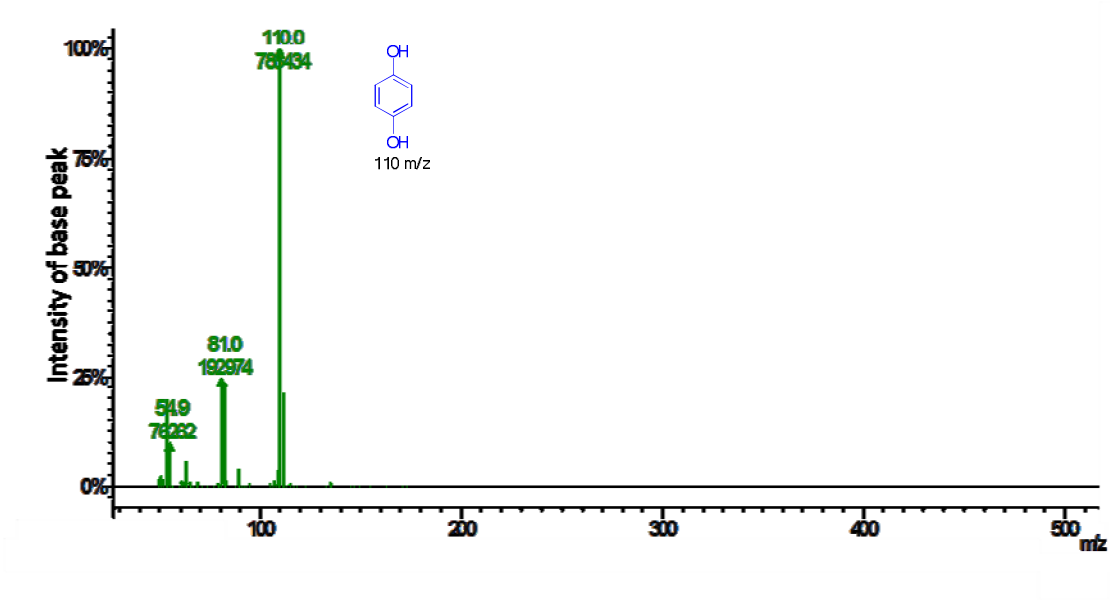

Fig. S3.2

GC-MS analysis of compound1: P-dihydroxybenzene.

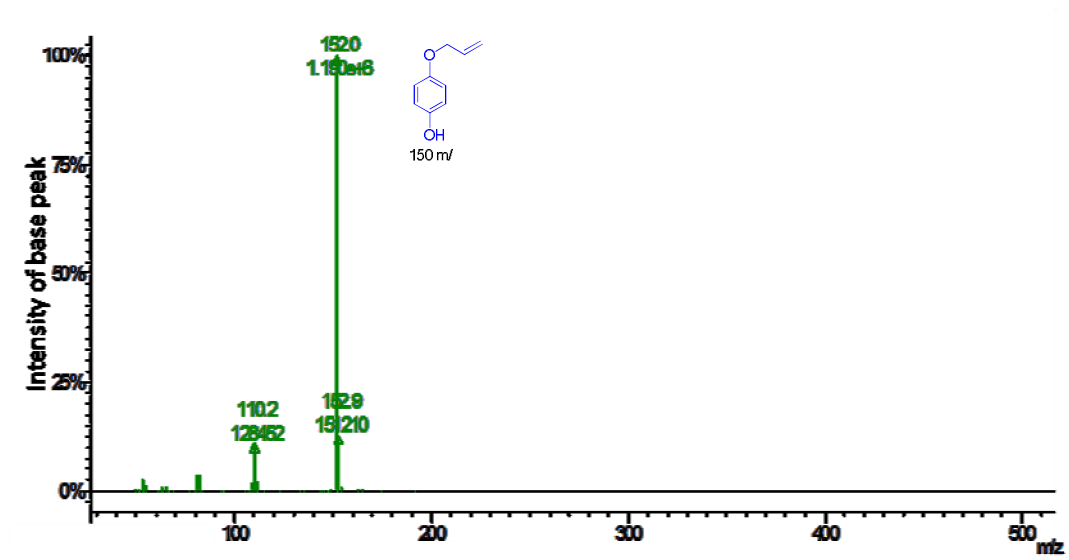

Fig. S3.3

GC-MS analysis of compound 2: P-allyloxyphenol.

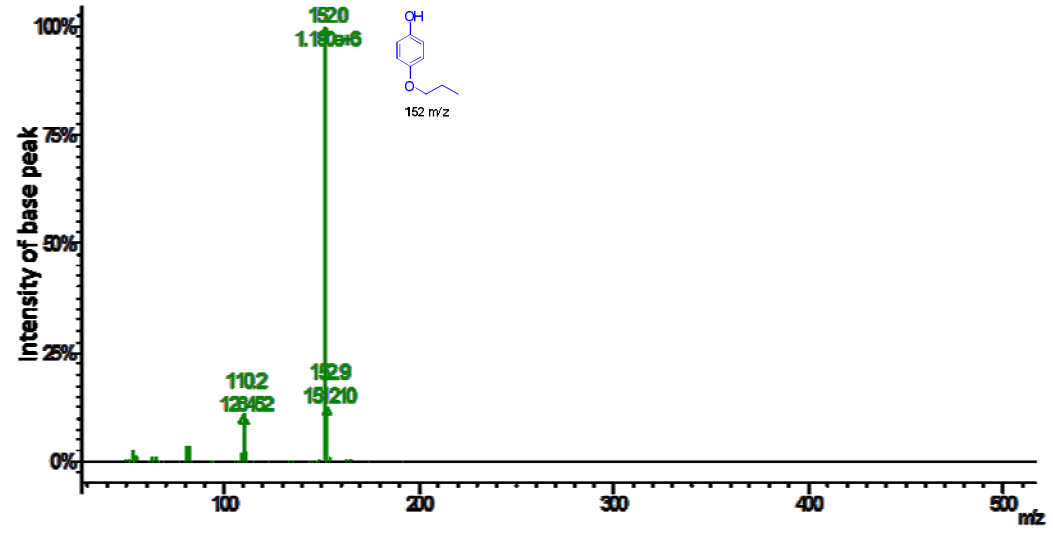

Fig. S3.4

GC-MS analysis of compound 3: P-propoxyphenol

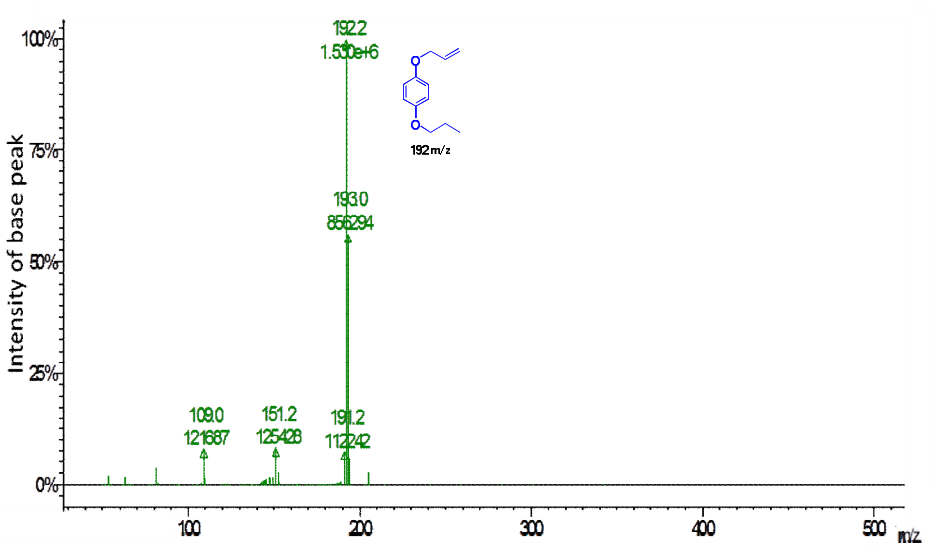

Fig. S3.5

GC-MS analysis of compound 4: 1-allyloxy-4-propoxybenzene

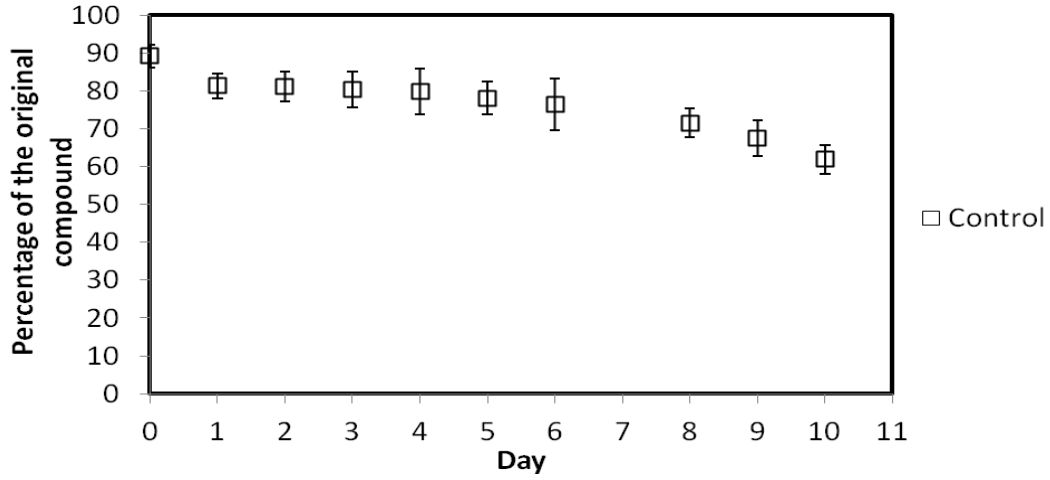

Fig S3.6

Degradation of 1-allyloxy-4-propoxybenzene by boiled Pseudomonas Putida (ATCC 17453)

4. Summary and Future work

Dialkoxybenzenes are new promising insect control compounds. It has been found that these compounds have anti feeding and anti-oviposition effects against *T.ni* and feeding deterrence against gypsy moth larvae. Among various isomers and homologs, the one with the highest feeding deterrence against *T. ni* was 1-allyloxy-4-propoxybenzene. This compound has potential for development as commercial insect control agent with selectivity towards Lepidoptera.

In this study, the octanol-water partition coefficient, volatility and sorption on soil components (sand, clay, cellulose and humic acid) of selected dialkoxybenzenes were investigated. It has been found that these compounds have acceptable physical-chemical properties in comparison to other pesticides. Toxicity of these compounds against laboratory strains of *E.coli* and *P.putida,* as well as biodegradation by different strains of *P.putida* were also investigated. Two of the species have the ability to degrade 1-allyloxy-4-propoxybenzene. In one of these strains that harbours a D-(+)-camphor degradation pathway, the first step was dealkylation of the compound by cyctochrome $P450_{cam}$ (CYP101A1) at either end, which gave both possible monoalkoxy phenols. A second dealkylation gave dihydroquinone. *In vitro* tests with CYP101A also confirmed the biodegradation experiment results and revealed that the dealkylation is catalyzed by this P450 enzyme.

At present, the biodegradation experiments were done with three bacterial species. However, in order to find more bacterial species with the ability to metabolize dialkoxybenzenes, similar experiments should be done on soil samples (Santoshkumar *et al*., 2010). This could help us to find out if different microorganism communities in the soil cooperate to mineralize the compound (Ghazali *et al.,* 2004). It would also be interesting to compare the biodegradation rates in soil samples with the rates seen here in the single strain experiments.

The future endeavours in this project would also include further experiments in order to find how much 1-allyloxy-4 propoxybenzene is absorbed and degraded by the leaves of treated plants. (Ryan *et al*., 1988) (Dust *et al*., 2011) This is a very important experiment because the compound may eventually be used in horticulture.

We would also need to perform more studies to find the best formulation and delivery method of the compound. For example, we need to find a solvent that is less toxic than ethanol (which I used here), yet miscible with water.

Furthermore, in order to ascertain the safety of compound for human and other non-target living organisms more toxicology studies need to be done, for example, on vertebrates and on beneficial insects such as honey bees.

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