CHAPTER 1

INTRODUCTION

1.1 Statement of problem

1.1.1 Dichlorodiphenyl trichloroethane (DDT)

DDT is an organochlorine pesticide that creates most concern for problems of translocation and biomagnifications. Technical DDT is composed of an approximately 3:1 mixture of 1,1,1-trichloro-2,2-di(4-chlorophynyl) ethane (*p,p'*-DDT) and 1, 1,1-trichloro-2(2-chlorophynyl)-2-(4-chlorophenyl) ethane (*o,p'*-DDT) (Burow et. al., 1999). It has been strongly connected to endocrine disruption activity (Sharpe and Skakkebaek, 1993), resulting in adverse human and wildlife developmental defects, and potentially causing cancer. During the early 1940s, DDT was commercially manufactured in the United States and heavily used in agriculture for the control of pest insects. Widespread distribution of these pesticides peaked in the early 1960s because of extensive use in agricultural, industrial, and domestic applications (Huff, 1980).

DDT was a widely used chemical to control insects on agricultural crops and insects that carry diseases like malaria and typhus. The United States Environmental Protection Agency (U.S. EPA) banned the use of DDT in the United States in 1973, and it was no longer being produced in this country. However, DDT still is being used and produced in limited quantities in other countries. Food is the primary pathway of DDT exposure for the general population. However, food imported from other countries that still use DDT might have DDT contamination (Wandiga, 2001). Food

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from tropical regions may contain more DDT because of its greater use in these regions. A major metabolite of *p,p'* DDT is 1, 1'-(2, 2-dichloroethenylidene) - bis [4-chlorobenzene] (*p,p'*-DDE). It can be formed in human or in the environment. DDE is more persistent than DDT in the environment and in human. The presence of DDT in the human body reflects either a relatively recent exposure or cumulative past exposure. High DDT to DDE ratio may indicate recent exposure, and low DDT to DDE ratio may indicate an exposure in the more distant past (Walter and Aimin, 2005). The health effects associated with DDT after large accidental exposures or workplace exposures have been described (Hayes, 1976). Elevations of liver enzymes in serum have been observed in exposed workers. The toxic effects of DDT demonstrated in experimental animals include infertility (Jonsson *et al*., 1975), a decrease in the number of implanted ova (Lundberg, 1974), intrauterine growth retardation (Fabro *et al*., 1984), cancer (Cabral *et al.,* 1982), neurological developmental disorders (Eriksson *et al.,* 1990) and fetus death (Clement and Okey, 1974). The association of DDT exposure and breast cancer has been studied but inconclusive (Helzlsouer *et al.,* 1999; Hoyer *et al*., 2000; Hunter *et al.,* 1997; Williams *et al.,* 1988). International agency for research on cancer (IARC) (1987; 1991) classifies DDT (*p,p'*-DDT) as a possible human carcinogen; National toxicology program (NTP) considers that DDT is reasonably anticipated to be a human carcinogen; and the U.S. EPA (Besbelli, 1999) has classified DDT as a probable human carcinogen.

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1.1.2 Metabolism and tissue distribution of DDT

The metabolism and tissue distribution of DDT has been studied in several species (Hayes *et al.,* 1958; Hayes *et al.,* 1965). In man, *p,p'*-DDT is readily absorbed from the intestinal tract and converted mainly into DDE and 1,1-dichloro-2,2-di(4-chlorophenyl) ethane (*p,p'*-DDD) which is further converted to bis (*p*-chlorophenyl) acetic acid (DDA) (Morgan *et al.,* 1971; Hayes *et al*., 1971). DDA is excreted in the urine and the total amount of urinary DDA is less than one – third of total excreted DDT and its metabolites (Morgan and Roan, 1971; Hayes *et al.,* 1971). DDT and its metabolites are mainly excreted via the bile into the feces. DDT and DDE were shown to be highly persistent in the adipose tissue and elimination of DDT from the fat was very slow with a half–life of about 2 years (Morgan and Roan, 1971). The depletion rate of DDT and its metabolites from the body lay in the order: *p,p'*-DDA > *p,p'*-DDD > *o,p'*-DDT > *p,p'*-DDT > *p,p'*-DDE (Fawcett et al., 1981). There are marked species differences in the rate of disappearance of DDT from adipose tissue. Man exhibited extraordinary slowness in the depletion as compared with monkey, dog, and rat (Fawcett et al., 1981). The rates appear to reflect the activity and efficiency of the enzymes, possibly including cytochrome P–450 which are responsible for the dehydrochlorination and dechlorination of DDT (Jenson *et. al.*, 1957) (Figure 1.1).

The metabolism of *o,p'*-DDT (Figure 1.2) has not been so extensively studied, as compared with that of *p,p'*-DDT. Rats treated with *o,p'*-DDT or *o,p'*-DDD extensively metabolized these compounds to ring-hydroxylated products (Feil *et al.*, 1973). Several mono- and dihydroxylated metabolites were formed with aromatic

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hydroxylation occurring primarily at the 3 and/or 4 position of the ring. Conjugation reactions of *o,p'*-DDA with glycine, serine, and aspartic acid occurred. Human metabolism of *o,p'*-DDD was very similar to that of rats, as revealed by the nature of urinary products (Reif *et al*., 1974). Thus, the metabolism of DDT in man probably occurs via the same major pathways as found in the laboratory rats. The striking feature is the slow rate of metabolism in man. Since DDT as well as dieldrin is metabolized by reactions involving cytochrome P-450, human cytochrome P-450 might not be as effective as that of rats and mice.

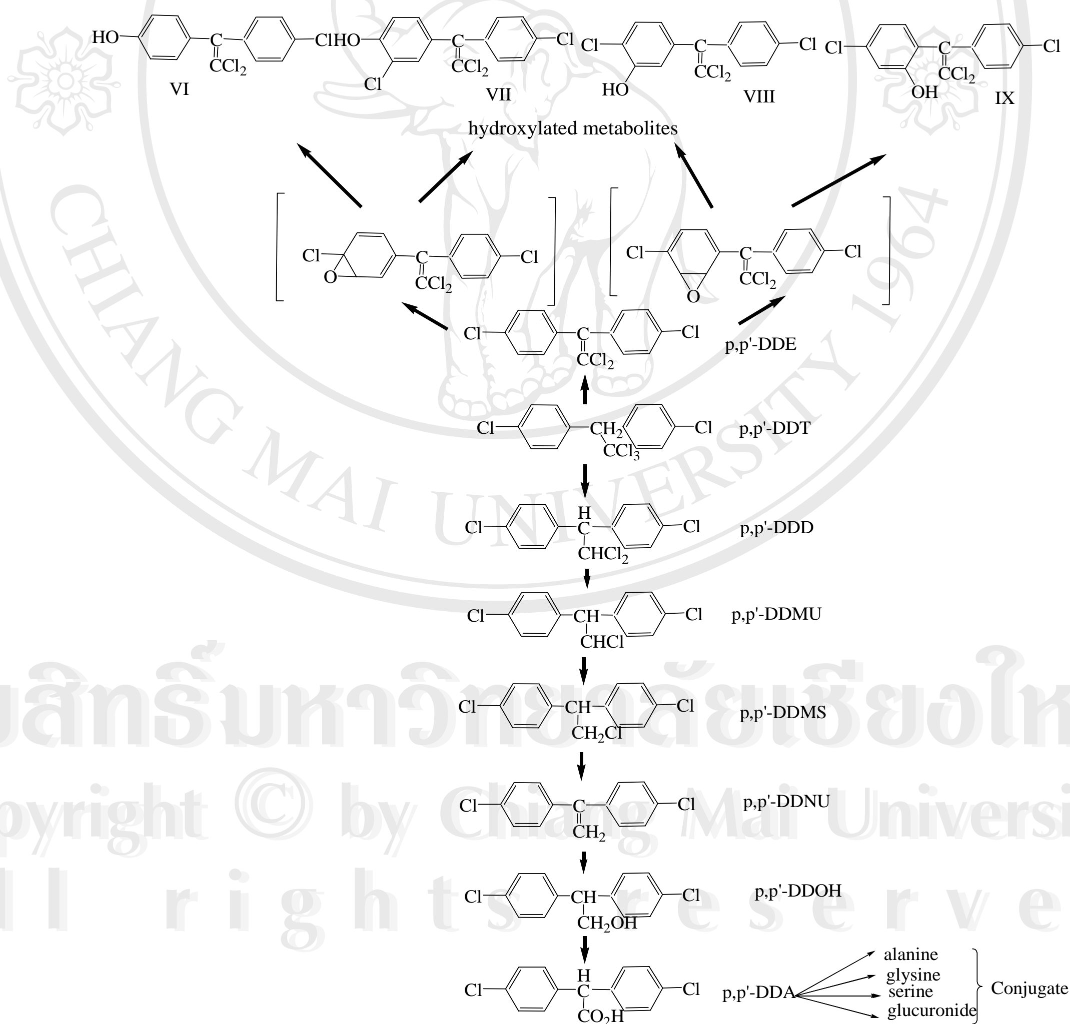

Figure1.1 Metabolic pathways of *p,p'*-DDT in mammals. (Miyamoto *et al*., 1988)

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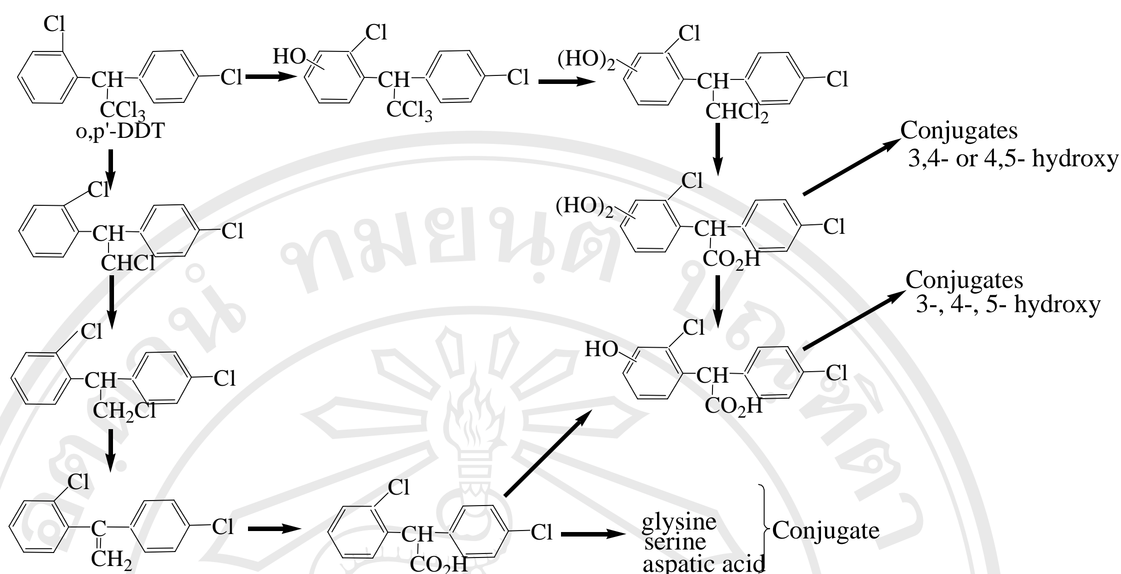

Figure 1.2 Metabolic pathways of *o, p'*-DDT in mammals. (Miyamoto *et al.,* 1988)

1.2.3 Standard techniques for detection of DDT and its derivatives

Standard techniques for detection of DDT and its derivatives are gas chromatography – electron capture detection (GC-ECD) and mass spectrometry (MS) detector are widely used methods for identification and quantification of DDT and other chlorinated pesticides. They can detect low concentration down to ng/mL level (ppb) (White *et al*., 1970). However, this technique has disadvantages, such as required clean sample preparation required, high solvent consumption, expensive equipment, and skill person for operation. Therefore, a new method that will enable a rapid and inexpensive determination of pesticides and metabolites i.e. DDT and its metabolite in a large number of samples is needed. Accordingly, scientists have developed a simple and inexpensive technique for massive sample load called immunoassay (IA) such as Enzyme-linked immunosorbent assay (ELISA) that developed from immunochemistry technique.

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1.1.4 Immunoassay

An immunoassay is a biochemical test that measures the level of a substance in a biological liquid, typically serum or urine, using the reaction of an antibody or antibodies to its antigen. The assay takes advantage of the specific binding of an antibody to its antigen. Detecting the quantity of antibody or antigen can be achieved by a variety of methods. One of the most common is to label either the antigen or antibody. The label may consist of an enzyme (enzyme immunoassay, EIA), radioisotopes such as I-125 (radioimmunoassay, RIA) or fluorescence (immunofluorescence assay, IFA). Immunoassays can be competitive or noncompetitive. In a competitive immunoassay, the antigen in the unknown sample competes with labeled antigen to bind with antibodies. The amount of labeled antigen bound to the antibody site is then measured. In noncompetitive immunoassays, antigen in the unknown is bound to the antibody site, then labeled antibody is bound to the antigen. The amount of labeled antibody on the site is then measured.

1.1.4.1 Monoclonal antibody

Monoclonal antibodies (mAb) are antibodies that are identical because they were produced by one type of immune cell, all clones of a single parent cell. Given (almost) any substance, it is possible to create monoclonal antibodies that specifically bind to that substance; they can then serve to detect or purify that substance. This has become an important tool in biochemistry, molecular biology and medicine. When it has been established by serological screening that the immunized animal produces antibodies with the correct specificity, the spleen, which contains many antibody-producing cells, is removed and grown in culture. Clones producing antibodies with

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the desired properties are then selected. Single hybridoma cell derived from the fusion of B-lymphocytes with myeloma cells (Kohler *et. al*., 1975). The hybridoma cells can then be propagated almost indefinitely in culture and will continue to produce the Ab of the lymphocyte parent. Since an individual lymphocyte produces only a single Ab type, all of the Ab molecules were produced by a hybridoma cell line derived from a single hybrid cell are identical and have the same binding properties the process was shown in figure 1.3. It is possible to select antibodies that react with chemical structures specific for a single pesticide molecule, or alternatively a structure shared by a group of pesticides. In addition to their high specificity, monoclonal antibodies also have the advantage of consistency. It is always possible to reculture the hybridomas and produce more antibodies with exactly the same characteristics (Aamand *et al*., 2004). Therefore, the hybridoma technology guarantees the unlimited production of mAbs with constant characteristics (Hock *et. al*., 1995). Owing to the great effort involved in mAb production many IAs still employ pAb. Here, Ig genes can be cloned, introduced and expressed in inexpensive and relatively simple host systems (Ward *et al*., 1995; Choudary *et. al.,* 1995; Kramer *et al.,* 2004). The main properties of pAbs, mAbs are listed in Table 1.1

1.1.4.2 Antibody Structure

Antibodies are glycoproteins of the immunoglobulin class produced by the immune system against foreign material such as pathogens or xenobiotics, and bind the target substance with high selectivity and affinity. Although there are five distinct classes of Ab in higher mammals (IgA, IgD, IgE, IgG, IgM) IgG makes up approximately 80% of the total Ig in human serum. IgG, figure 1.4, consists of two

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identical heavy (H) chains and two identical light (L) chains stabilized and linked by inter- and intrachain disulfide bonds. The H- and L-chains are organized into variable and constant regions. The Ag binding site (combining site) is formed by the association of parts of the variable regions of the hand L-chains, located at the amino terminal end. The variable regions of both chains are organized into three hypervariable or complementarity determining regions (CDRs) separated by four framework regions. The greatest amino acid sequence variation occurs within the CDRs whereas the framework regions are more conserved. It is assumed that the association of the CDR regions forms the combining site. The lower part of the molecule, the Fc (antibody fragment containing the crystallizable fragment) is responsible for some important biological effecter functions such as complement fixation and is not necessary for Ag or hapten binding. It contains the last heavy chain domains. The whole of the Ig molecule or Ab fragments, F(ab)2 and Fab (antibody fragments containing the antigen binding site(s)) can be used in IAs. A substance that after injection into the body of a vertebrate induces a specific Ab synthesis is called Ag. Ags are principally macromolecules, for instance proteins, polysaccharides or nucleic acids. Small molecules (haptens) such as pesticides have to be coupled to a macromolecular carrier to elicit an Ab response. The ability of an Ab molecule to bind an Ag or a hapten specifically is controlled by structural and chemical interactions between the ligand and the Ab at the combining site. The Ag–Ab interaction is reversible and does not involve formation of covalent bonds (Roitt and Cooke, 1987). The binding is a result of a variety of interactions such as hydrophobic, ionic, H-bonding, and van der Waals forces. The relative affinity of the Ab increases

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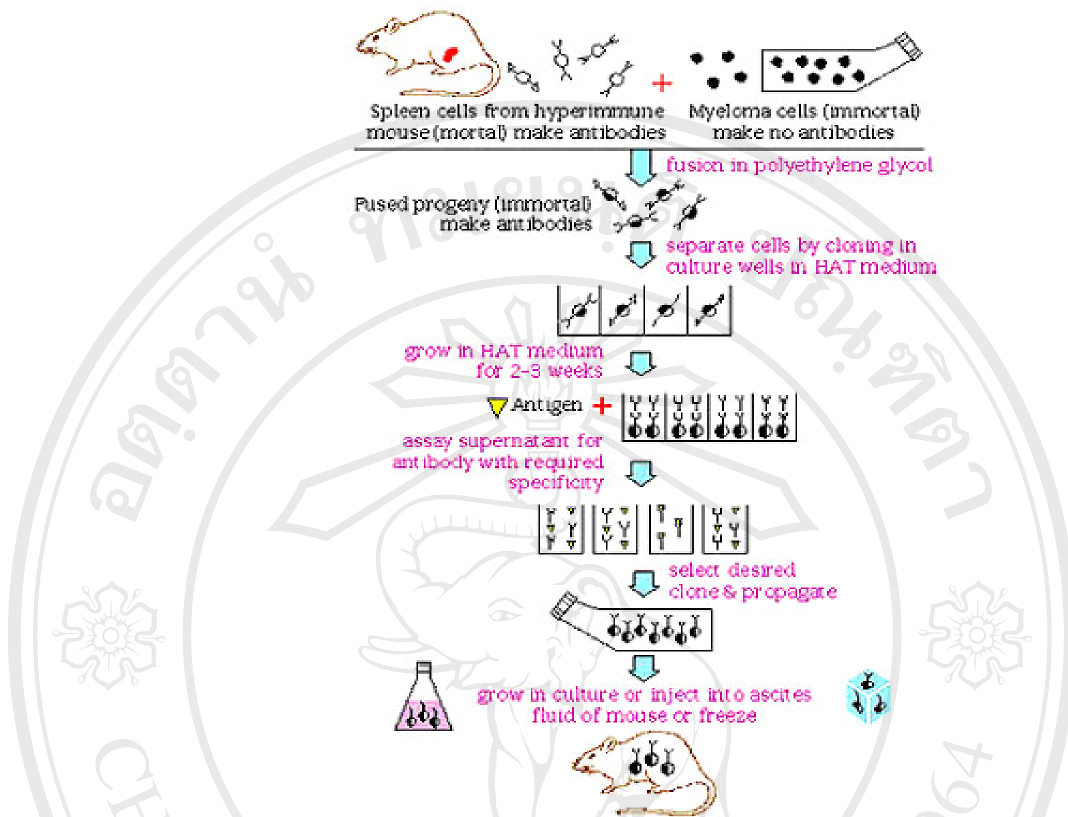

Figure 1.3 Monoclonal antibody production. (www.biotech.uiuc.edu_fusion)

Table 1.1 Properties of polyclonal and monoclonal antibody. (Andrea, 2000)

Properties	pAb (Ab from serum)	mAb (Ab from hybridoma cells)
Supply	Limited and variable	Unlimited production possible
Uniformity	Changing properties with different sera and bleedings	Constant properties of a mAb
Affinity	Mixture of Ab with different affinity often higher with pAb	Uniformly high or low, can be selected by testing
Cross-reactivity	Results from different selectivity and low affinity interactions	Different, dependent upon the individual Ab
Classes and subclass	Typical spectrum	One defined isotype
Demands on Ag	High purity required for specific antisera	Impure Ags or mixture of Ags can be used for immunization pure Ags necessary for screening
Cost	Low	High

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with the number of specific chemical interactions between the analyte and the amino acid residues in the Ab combining site. Therefore, the selectivity and sensitivity of an immunoassay is controlled by the nature of the Ag–Ab binding process.

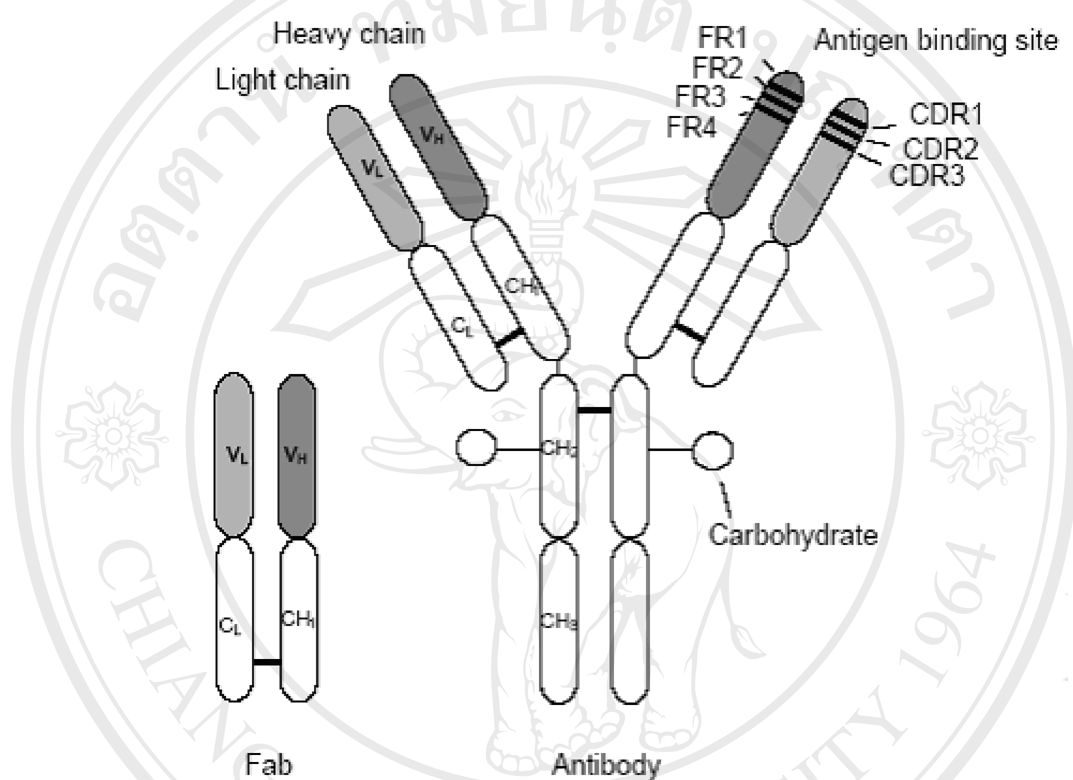

Figure 1.4 Structure of IgG Ab and its fragments (modified from Hock *et al,.* 1995). VL is the variable region of light chain, VH is the variable region of heavy chain, CL is the constant region on the light chain, CH1, CH2, CH3 is a constant region on the heavy chain.

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1.2 Literature review of antibody to DDT and its derivatives production

Immunochemical techniques are alternative and complementary methods for the analysis of pesticides for their sensitivity, simplicity, and cost-effectiveness. These methods do not require expensive, highly-sophisticated instrumentation and it is possible to adapt them for field measurement. The major component of all

immunoassays is the antibody that is responsible for specific and sensitive recognition of an analyte. First, several research groups attempted to develop antibodies to DDT and related compounds using protein conjugates of DDA as an immunogen (Haas and Guardia, 1968; Banerjee, 1987). The antibodies obtained exhibited high affinity to DDA. On the other hand, these antibodies poorly recognized DDT. Burgisser *et al.* (1990) designed a hapten based on dicofol (Figure 1.5). A radioimmunoassay using monoclonal IgM developed against *p,p*'- DDT conjugated with kelthane- BSA has shown sufficient sensitivity, with a detection limit for *p,p*'-DDT of about 3.5 µg/mL. However, this antibody lacked of selectivity and could not distinguish that between *p,p*'-DDT and *p,p*'-DDE.

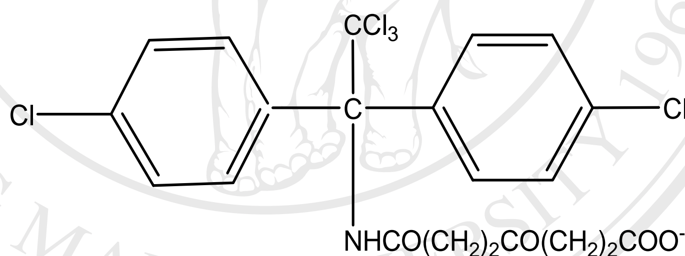

Figure 1.5 The structure of hapten reported by Burgisser *et al.* in 1990.

Banerjee et al. (1996) utilized diamino derivatives of DDT, DDE, and DDA as haptens and developed several competitive ELISAs with IC_{50} values in the range of 180-360 µg/L for standards of each of these pesticides. In 1997, Abad *et al.* described the preparation of several DDT derivatives (Figure 1.6) applied as immunogens for preparation of sensitive mAbs. No application to samples was reported. They developed mAbs to immunogen (dicofol conjugated with KLH) with IC_{50} values in the range of 0.74-4.08 µg/mL as determined by homologous conjugate-coated assays.

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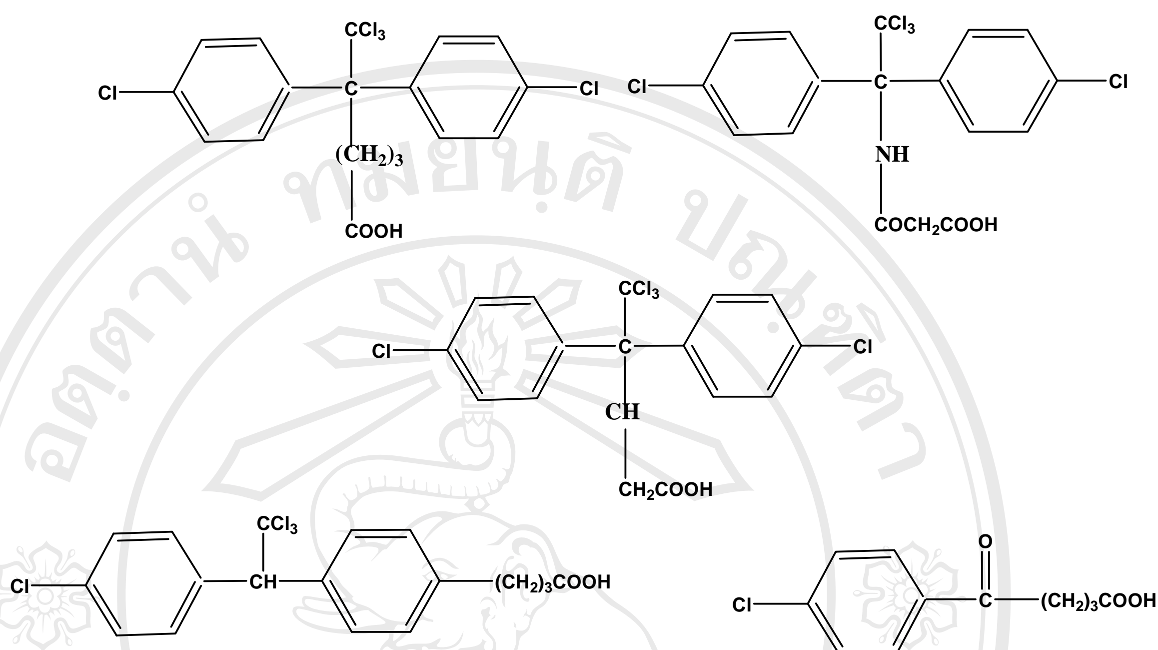

Figure 1.6 The structure of haptens reported by Abad *et al.* in 1997.

In 1998, Beasley et al. described preparation of haptens based on different amides of DDA, dicofol and DDE derivatives, and an analogue of DDT bearing a substitution at one of *p*-chloro atoms (Figure 1.7). The immunoassays based on these haptens, were applied to the detection of DDT and DDE in water, soil, and selected foods. They found altering the IC_{50} from 0.013–0.015 μg/mL in soil and 0.020 μg/mL in custard DDT for the 1 h extracts and to 0.020 μg/mL in soil and 0.050 μg/mL in custard DDT for the 16 h extracts.

Haptens were produced by Hong *et al*. (2002) (Figure 1.8). The result found DDA conjugated with KLH and 5,5-Bis(4-chlorophenyl)-5-chloropentanoic acid (DDCP) conjugated with KLH could be used as immunogens to produce antibodies to

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DDT and that 3-[6,6-Bis(4-chlorophenyl)-6-hydroxyhexanoylamino]propanoic acid (DDHHAP) conjugated with OVA was the best capture Ag for the generation of

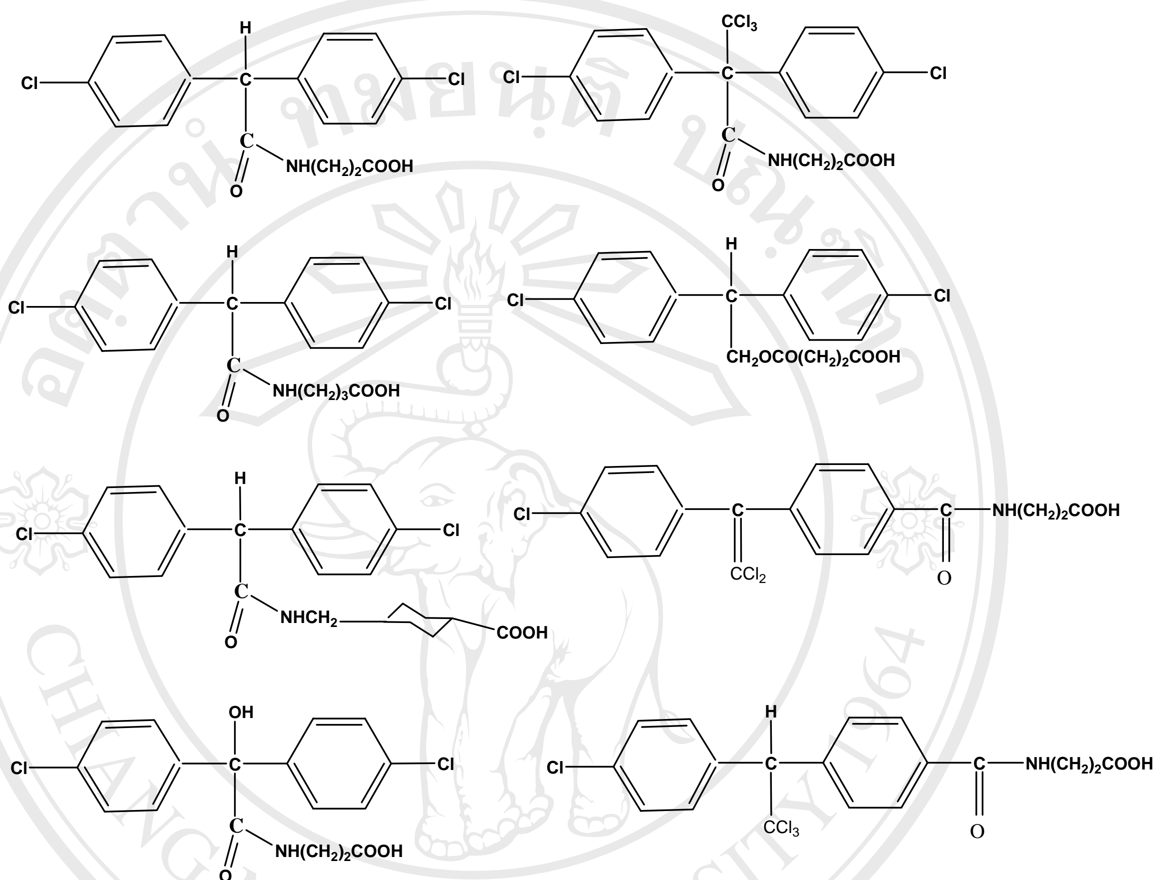

Figure 1.7 The structure of haptens reported by Beasley *et al.,* 1998.

dose-response curve, using the selected pair of PAb and DDHHAP-OVA coating ligand. DDT analysts could be detected simultaneously with the detection limit of 0.0003 μg/mL for DDT and 0.0034 μg/mL for DDA and DDE by an indirect enzyme immunoassay. In 2003, Hong *et al.* found DDA-KLH immunogen and DDA-OVA coating ligand were the best combination for detection of *p,p*'- DDT and a wide range of DDT analytes (Hong *et al*., 2003).

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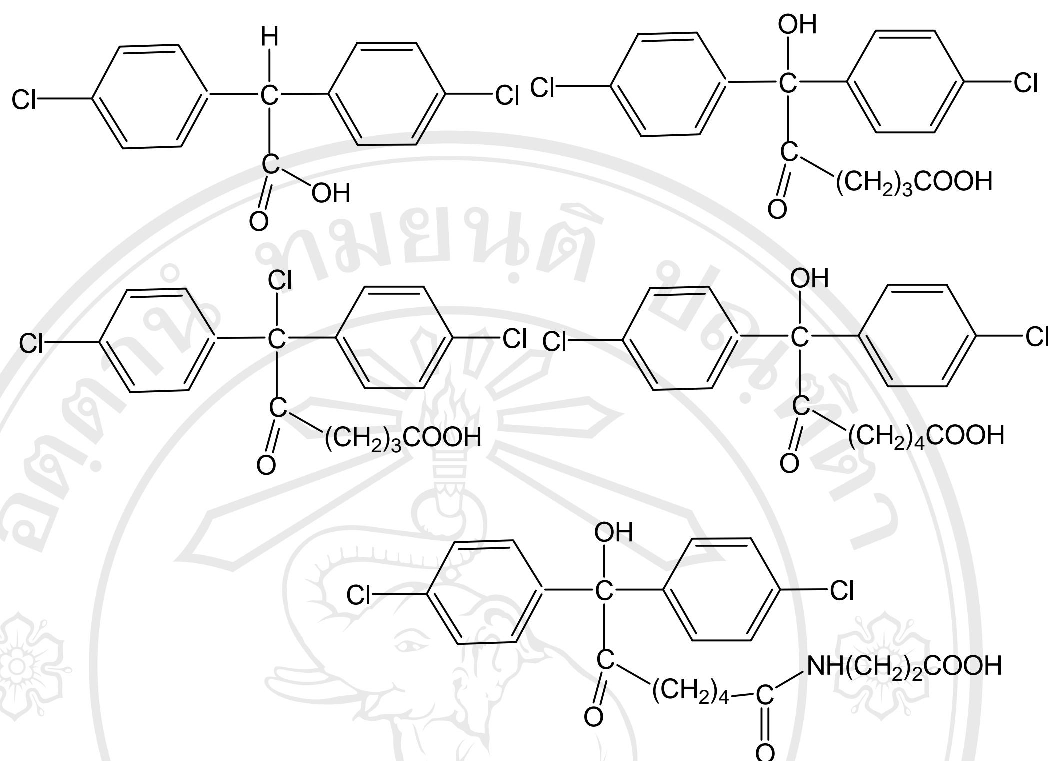

Figure 1.8 The structure of haptens reported by Hong *et al*., 2002.

1.3 DDT and its derivatives of the present study

Chemical structures of DDT and its derivatives proposed in the present study are shown in Table 1.2. The table is confined to compounds that have the same major structure of DDT.

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Table 1.2 Common name, chemical name, and structure of DDT and its derivatives.

Common name	Chemical name	Structure
p,p'-DDT	1,1'-(2,2,2-trichloroethylidene)- bis[4-chlorobenzene]	
o,p'-DDT	1, 1,1-trichloro-2(2-chlorophynyl)-2-(4-chlorophenyl) ethane	
p,p'-DDE	1,1'-(2,2-dichloroethenylidene)- bis[4-chlorobenzene]	
o,p'-DDE	1, 1-dichloro-2(2-chlorophynyl)-2-(4-chlorophynyl)ethane	
p,p'-DDD	1,1'-(2,2-dichloroethylidene)-bis[4-chlorobenzene]	
p,p'-DDA	2,2-bis(4-chlorophenyl)- acetic acid	
Dicofol(Kelthane)	4-chloro-alpha-(4-chlorophenyl)-alpha-(trichloromethyl) benzenemethanol	
DCBH	4,4-dichlorobenzhydrol	

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1.4 Hypothesis of the present study

The basis for the development of new immunological analyses is the antibody that reacts with DDT and its derivatives, the so-called antigens. DDT and its derivatives are too small to induce an immunological response. Linking the hapten to larger carrier molecules it is possible to deceive the immune system into starting the production of antibodies against the DDT and its derivatives. To initiate antibody production, the hapten-carrier complex is injected into animals, e.g. mice, thus inducing an immunological response resulting in the production of antibodies against the hapten-carrier complex. Antibodies are produced by B-cells, each producing a single antibody species, which recognize a specific structure on DDT and its derivatives. As the animal contains many B-cells which all produce antibodies, a range of antibodies reacting with different structures on them and with different affinity are generated. By purification of the antibodies from the blood serum, a polyclonal antibody serum is obtained, containing antibodies from different B-cells. However, it is more appropriate to produce mAb, specific antibodies all arising from the one B-cell clone.

1.5 Purposes of the study

The general purpose of the study was to produce and characterize anti-DDT antibody. The specific purposes are to:

1. To prepare haptens based on chlorinated aromatic rings by chemical synthesis.
2. To prepare immunogen by conjugation method.

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3. To produce antibody to DDT using animal immunization and hybridoma technique.
4. To characterize produced antibody for their analytical sensitivity and specificity.

The present study was done following to conceptual frame work that showed in figure 1.9**.**

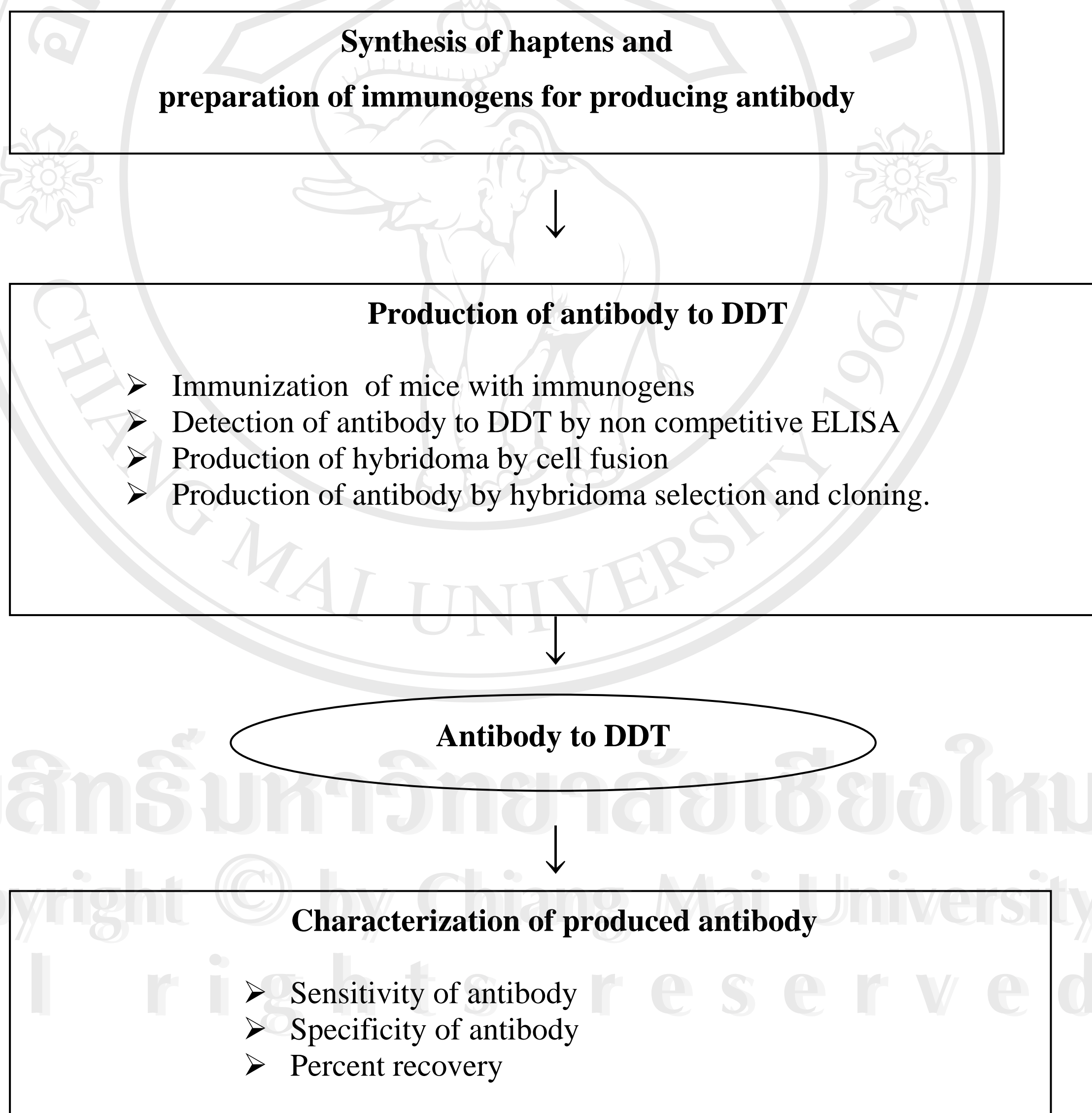

Figure 1.9 Conceptual framework of the present study.

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1.6 Benefits of the study outcome

The antibodies can be used to develop test kit for detecting DDT and its derivatives in biological and environmental matrices.

1.7 Key words and definition

Antibody (Ab) is a type of glycol-protein molecule produced by cells of the immune system, in response to the presence of a foreign substance, known as antigen. Antibodies bind to antigens in a precise physico/chemical manner and thus neutralize their activity. Over a lifetime, the body will produce thousands of different antibodies to the range of antigens it encounters. Antibody molecules consist of two "light" and two "heavy" protein chains, only part of which actually come into contact with the antigen. The area of the antibody known as the variable region is different and specific for each type of antibody.

Antigen is any agent or substance that stimulates an immune response. Antigens are often foreign microorganisms such as bacteria or viruses, or the substances they produce.

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Clone is a term applied to genes, cells, or entire organisms which are produced from - and are genetically identical to - a single common ancestor gene, cell or organism (i.e. an identical copy). Cloning of genes and cells to create many copies in the lab is a common, essential procedure for biomedical research. Note that several processes commonly described as cell "cloning" that produce cells that are almost but not

completely genetically identical to the ancestor cell. "Cloning" of organisms from embryonic cells occurs in nature (e.g. identical twins).

DDT and its derivatives are chemicals having the structure like the DDT such as dichlorodiphenyl dichloroethylene (DDE), dichlorodiphenyl dichloroethane (DDD), and dicofol.

Enzyme-Linked Immunosorbent assay (ELISA) is an assay that relies on an enzymatic conversion reaction and is used to detect the presence of specific substances.

Hapten is a small molecule which can elicit an immune response only when attached to a large carrier such as a protein; the carrier may be one which also does not elicit an immune response by itself.

Hybridoma is a type of hybrid cell produced by fusing a normal cell with a tumor cell. When lymphocytes (antibody-producing cells) are fused to the tumor cells, the resulting hybridomas produce antibodies and maintain rapid, sustained growth, producing large amounts of an antibody. Hybridomas are the source of monoclonal antibodies.

Immunogen is any substance that provokes the immune response when introduced into the body. An immunogen is always a macromolecule (protein, polysaccharide). Its ability to provoke the immune response depends on its foreignness to the host, molecular size, chemical composition and heterogeneity (e.g. different amino acids in a protein).

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Monoclonal antibody is antibody that recognizes a specific type of antigen and that can therefore be used as a tool for diagnosing a particular disease or highly specific, purified antibody that is derived from only one clone of cells and recognizes only one antigen.

Polyclonal antibody - Ab that derived from multiple clones, each recognizes a active against a specific antigen, each recognizing a different epitope or region of the antigen.

Sensitivity is the ability of an antibody to be detected molecules of chemical. Also refers to a statistical measure of the accuracy of a screening test.

Specificity is determined by the steric (three-dimensional) match of antigen and antibody as well as by the number of molecular interactions taking place between both molecules.

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