

## Curious about Pesticide Action<sup>†</sup>

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**S** Supporting Information

**ABSTRACT:** The safe and effective use of pesticides requires knowledge of their mode of action in pests and adverse effects in nontarget organisms coupled with an understanding of their metabolic activation and detoxification. The author and his laboratory colleagues were privileged to observe, participate in, and sometimes influence these developments for the past six decades. This review considers contributions of the Berkeley and Madison laboratories to understanding insecticides acting at voltage-gated sodium and GABA-gated chloride channels and the nicotinic receptor and at serine hydrolases and other targets as well as the action of insecticide synergists and selected herbicides and fungicides. Some of the discoveries gave new probes, radioligands, photoaffinity labeling reagents, and understanding of reactive intermediates that changed the course of pesticide investigations and related areas of science. The importance of coupling mode of action with metabolism and design with serendipity is illustrated with a wide variety of chemotypes.

**KEYWORDS:** binding site, fungicide, herbicide, insecticide, metabolism, mode of action, photoaffinity ligand, radioligand

### ■ EARLY CURIOSITY

I had the privilege to observe, participate in, and sometimes influence the golden age of pesticide research<sup>1</sup> during the past 60 years and hopefully continuing into the future. As a teenager, my fascination with collecting insects and field biology shifted to pesticide effects as DDT, 2,4-D, and a multitude of agrochemicals became available in the 1940s. The constant question of “how does it work” was soon focused on organophosphorus (OP) insecticide mode of action and metabolism and over the years shifted to the novel effects of many chemotypes on organisms and vice versa. My graduate studies combined biochemistry, entomology, and plant physiology consistent with my broad curiosity about pesticide action. Graduate school was interrupted by service in the U.S. Air Force Atomic, Biological and Chemical Warfare program but still pursuing problems on toxicant mode of action. Following my Ph.D. degree in 1954, I set up the Pesticide Chemistry and Toxicology Laboratory of the University of Wisconsin at Madison, a group of chemists and biologists that moved in 1964 to the Environmental Chemistry and Toxicology Laboratory (ECTL) of the University of California at Berkeley. This brief chronicle of the Berkeley and Madison explorations illustrates an era of learning about pesticide mode of action and metabolism with particular reference to mechanisms of selective toxicity.<sup>2</sup>

### ■ VOLTAGE-GATED SODIUM CHANNEL (FIGURE 1)

**DDT and Pyrethroid Action.** DDT (**1**) in the 1940s started a new era in pest insect control dependent primarily on neurotoxins. It was soon recognized that **1** and the pyrethrins such as pyrethrin I (**2**) (Figure 1) and the synthetic pyrethroids including permethrin (**3**) and deltamethrin (**4**) shared a common nerve

target such that knockdown resistance and other nerve insensitivity factors to **1** generally conferred cross-resistance to **2–4**. All of these compounds act in insects on the voltage-gated sodium channel by prolonging the inactivation current. ECTL research on pyrethroid neurotoxicity contributed to recognizing differences by compound type in their effects in mammals and insects referred to as type I actions for **2** and **3** and type II actions for **4**. A target site model proposed by others is now available for **4** in the housefly sodium channel on the basis of studies defining mutations conferring reduced insect sensitivity and residues differing between insects and mammals contributing to selective action.

**Photostabilized Yet Biodegradable.** Toxicological and environmental restrictions on the use of the unacceptably stable **1** helped pyrethroids such as **3** and **4** fill much of the gap in controlling crop-chewing pests and disease-transmitting mosquitoes. The pyrethrins including **2** were found to be very rapidly metabolized, requiring a synergist such as piperonyl butoxide (PBO) (considered later) acting as a cytochrome P450 (CYP450) inhibitor for effective and economical use. The photostabilized synthetic pyrethroids (**3**, **4**, and others) were metabolically labile from CYP450 oxidase and “pyrethroid esterase” action, thereby avoiding most of the persistence problems of DDT. These developments were facilitated by ECTL structure–biodegradability findings on a large series of pyrethroids with insects and mammals In Vivo and in vitro by CYP450 and esterase action. The metabolic reactions are generally detoxifications except the glutathione (GSH)-catalyzed

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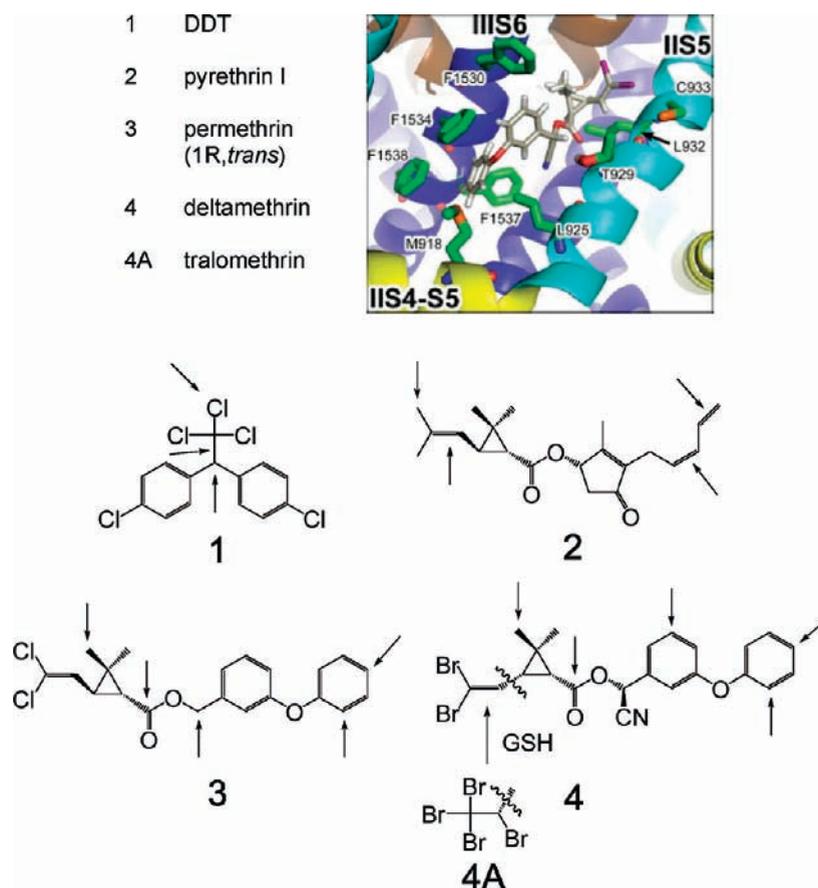
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**Figure 1.** DDT and pyrethroids acting on the voltage-gated sodium channel showing sites of metabolism (†) and docking prediction for deltamethrin (4) in the housefly binding site. Reproduced with permission from Davies and Williamson.<sup>3</sup> Copyright 2009 Bayer AG.

formation of 4 from the tetrabromo derivative and proinsecticide tralomethrin (4A).

## ■ GABA-GATED CHLORIDE CHANNEL (FIGURE 2)

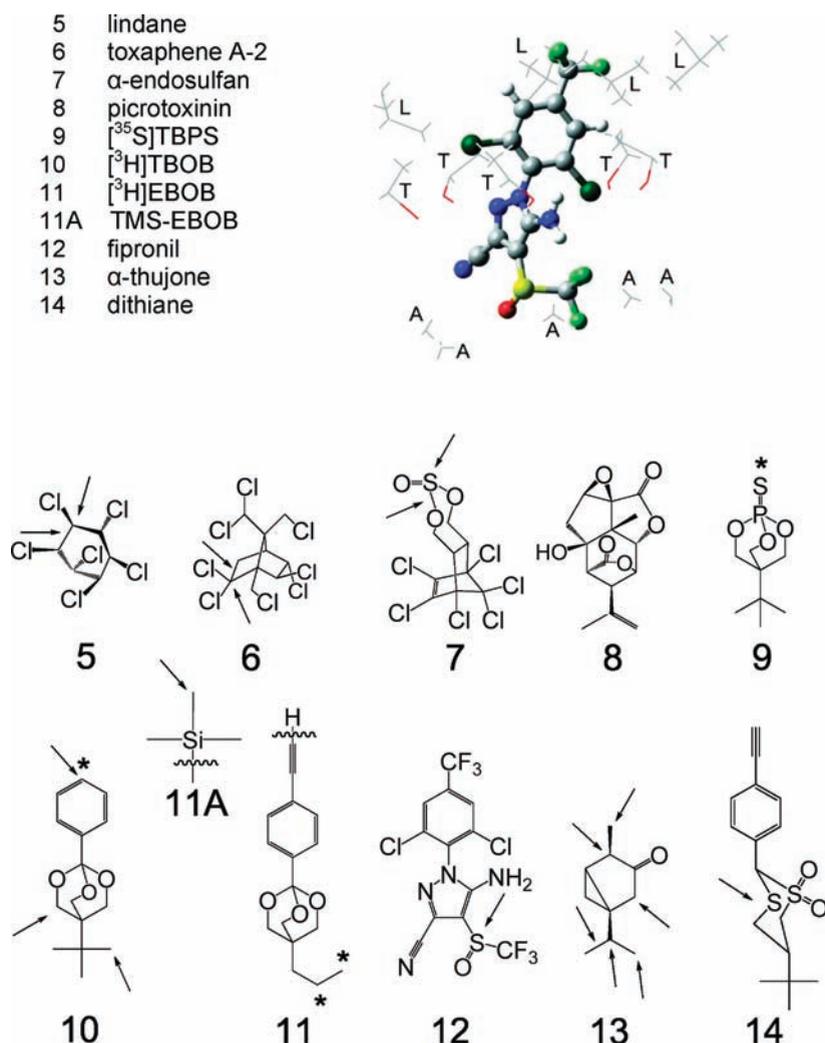
**Noncompetitive Blocker Site.** The unprecedented success of DDT led quickly to a multitude of other readily accepted and broadly used chlorinated hydrocarbons including lindane (5, Figure 2) and octachlorobornane A-2 (6) (the principal active ingredients of hexachlorocyclohexane and toxaphene, both mixed with many isomers and related compounds) and the cyclodienes [e.g.,  $\alpha$ -endosulfan (7)]. These polychlorocycloalkanes were known to be neurotoxicants with a different target and no cross-resistance to 1. In the early 1980s the target was finally defined as the heteropentameric  $\gamma$ -aminobutyric acid (GABA)-gated chloride channel and, more specifically, the noncompetitive blocker or picrotoxinin (8) site readily assayed with the radioligands [<sup>35</sup>S]TBPS (9), [<sup>3</sup>H]TBOB (10), and [<sup>3</sup>H]EBOB (11) from ECTL research. This is also the target of the major insecticide fipronil (12) (discovered in 1987 and introduced in 1993) and  $\alpha$ -thujone (13) (a mildly active botanical insecticide and the main active ingredient other than ethanol of the liquor absinthe). There is considerable specificity for some compounds in blocking the insect versus the mammalian brain GABA receptor/chloride channel, and in the most simplified case (human receptor  $\beta$ 3 homopentamer as a model for the receptor of *Drosophila melanogaster* or *Musca domestica*) there appears to

be a common binding site for compounds 5–14. Structurally optimized trioxabicyclooctanes (11) and dithianes (14) are highly potent on both insect and mammalian GABA receptors. The binding site of the avermectin-type compounds (channel openers) in housefly brain membranes was found to be closely coupled to the [<sup>3</sup>H]EBOB site (channel blocker) because the same structure–activity relationships were observed for avermectin analogues in inhibiting [<sup>3</sup>H]avermectin and [<sup>3</sup>H]EBOB binding.

**Metabolism and Photochemistry.** Contact insecticides 5–7 are highly lipophilic and slowly metabolized by CYP450 oxidation (5–7) and reductive dechlorination (6). Biooxidation reactions convert 7 and 12 to the insecticidal sulfate and sulfone, respectively. Photochemical bridging of cyclodienes other than 7 to form cage compounds and photodesulfonylation of 12 yield potent channel blockers. The selectively toxic trimethylsilylethynyl derivative 11A is more effectively oxidatively bioactivated to 11 in houseflies than in mice with even higher selectivity for the *n*-butyl analogue. The dithiane series (e.g., 14) is readily biooxidized to various combinations of sulfoxide, sulfone, sulfoxide–sulfone, and disulfone derivatives of widely varying biological activity.

## ■ SERINE HYDROLASE INHIBITORS (FIGURE 3)

**Secondary Targets.** Physostigmine (15) [a methylcarbamate (MC) from calabar beans], the MC insecticide carbaryl (16), and OP chemical warfare agents such as sarin (17) (Figure 3) led to the discovery of the critical importance of

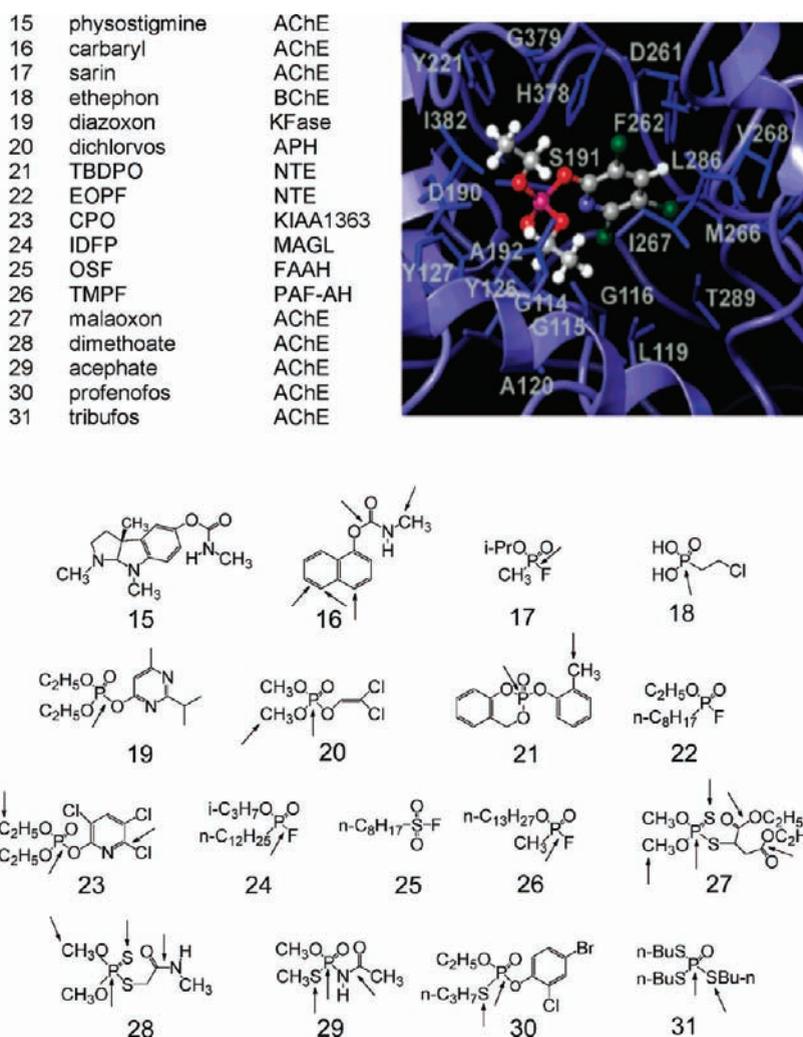


**Figure 2.** Insecticides acting on the GABA receptor showing sites of metabolism (†) (6 extrapolated from a heptachlorobornane analogue) and docking prediction for fipronil in the human recombinant  $\beta 3$  homopentamer. Reproduced with permission from Chen et al.<sup>4</sup> Copyright 2006 National Academy of Sciences of the United States of America.

acetylcholinesterase (AChE) as the MC and OP target. Secondary lesions and sites of action were recognized in increasing numbers from the 1930s to date on exposure to OP industrial chemicals and pesticides inhibiting serine hydrolases other than AChE. The plant growth regulator and ethylene generator ethephon (**18**) is selective for butyrylcholinesterase (BChE). Teratogenesis induced in avian embryos by diazoxon (**19**) and other OPs and MCs such as **16** results from kynurenine formamidase (also known as arylformamidase) inhibition, blocking NAD synthesis important in developmental programming. Acyl-peptide hydrolase, a possible target of cognition-enhancing drugs, is selectively inhibited by dichlorvos (**20**). There are also several important OP targets in lipid metabolism. OP-induced delayed neuropathy with more than 50000 human cases is now attributed to inhibition of neuropathy target esterase (NTE), identified as a lysophosphatidylcholine hydrolase, by OPs such as TBDPO (**21**) (most important) and EOPF (**22**) (model compound). OPs such as CPO (bioactivated chlorpyrifos) (**23**) and IDFP (**24**) (model compound) inhibit endocannabinoid metabolism by monoacylglycerol lipase and fatty acid amide hydrolase (FAAH), resulting in elevated endocannabinoid levels of 2-arachidonoylglycerol and anandamide, respectively, leading to OP-induced cannabinoid

effects. Organosulfonate **25** gives selective FAAH inhibition. The platelet-activating factor (PAF) acetylhydrolase (AH) is very sensitive to TMPF (**26**). The application of affinity-based protein profiling to serine hydrolases starting about 2000 was important in recognizing and characterizing OP targets more sensitive and relevant than AChE dependent on the specificity pattern.

**Bioactivation and Detoxification.** The CYP450-catalyzed methyl oxidation of tri-*o*-cresyl phosphate and albumin-catalyzed cyclization to yield TBDPO (**21**) (a potent delayed neurotoxicant) is of particular interest. OP and MC AChE inhibitors are also substrates for other hydrolases; for example, KIAA1363 hydrolyzes **23** and albumin hydrolyzes **16** with serine and tyrosine, respectively, at their reactive sites. Detoxifying enzymes of unusually high activity are potential prophylactic or antidotal agents. GSH *S*-transferases *O*-dealkylate the *O*-methyl and *O*-ethyl OPs. CYP450s bioactivate all phosphorothionates (P=S  $\rightarrow$  P=O) and many phosphorothiolates [RSP  $\rightarrow$  RS(O)P]. OPs of low acute toxicity to mammals such as malathion (**27**) and dimethoate (**28**) undergo facile enzymatic hydrolysis at the carboxyester or carboxamide substituents. The selective toxicity of acephate (**29**) involves a two-step bioactivation by acetamide



**Figure 3.** Insecticides and other serine hydrolase inhibitors showing sites of metabolism (†) and docking prediction for chlorpyrifos oxon in the KIAA 1363 active site. Reproduced from Nomura et al.<sup>5</sup> Copyright 2006 American Chemical Society.

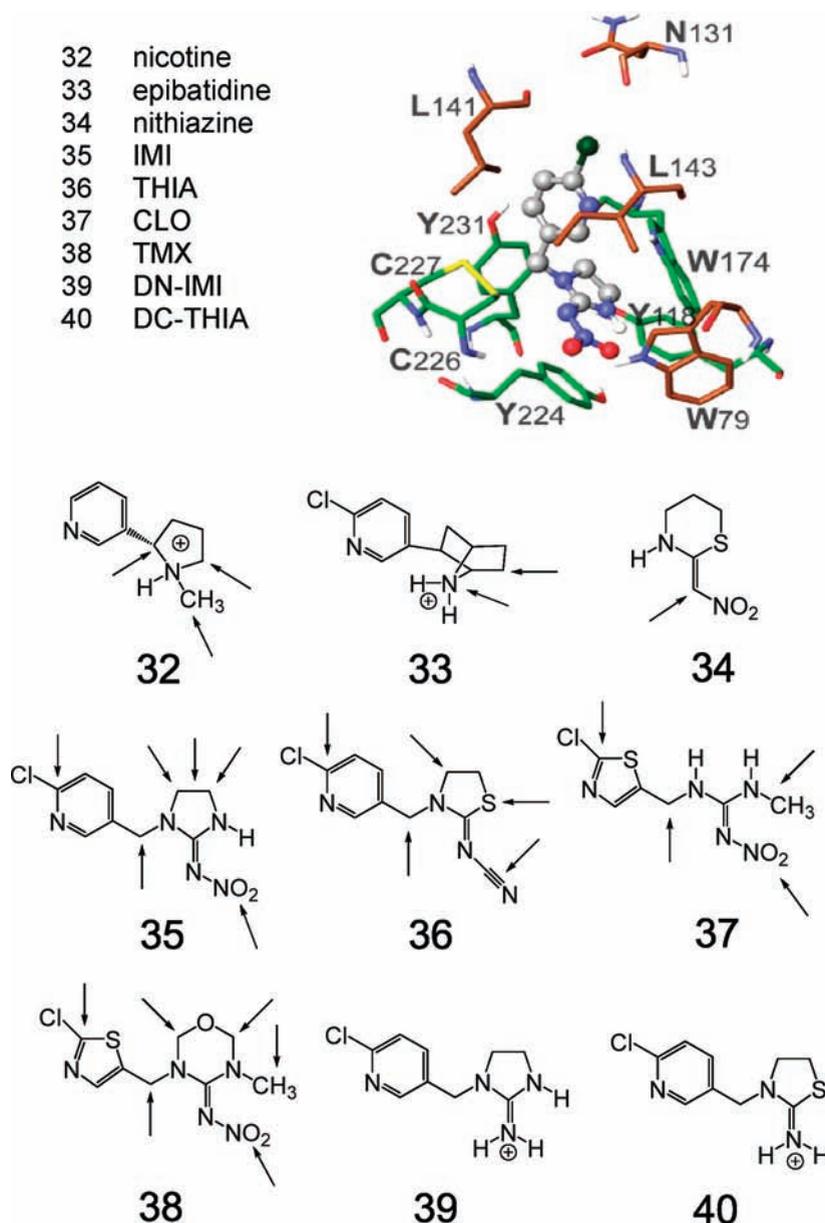
hydrolysis and then P–S–CH<sub>3</sub> sulfoxidation, which is more efficient in insects than in mammals. The chiral specificity of the profenofos (30) enantiomers for AChE inhibition is reversed on enzymatic sulfoxidation. CYP450-catalyzed sulfoxidation converts tribufos (31) to a broad-spectrum serine hydrolase inhibitor.

### ■ NICOTINIC RECEPTOR (FIGURE 4)

**Selective for Insect nAChR.** Nicotine (32, Figure 4) for more than 200 years was the principal botanical insecticide for control of sucking insect pests on plants. It acts in mammals on the  $\alpha 4\beta 2$  nicotinic acetylcholine receptor (nAChR) and in insects on an analogous receptor. Structural modifications of 32 were not successful in improving insecticidal effectiveness and safety. Epibatidine (33) from the skin of an Ecuadorian frog is much more potent than 32 on the mammalian  $\alpha 4\beta 2$  nAChR. Screening new types of compounds for insecticidal effectiveness revealed an active dibromonitroalkane optimized to the nitromethylene derivative nithiazine (34). Further optimization yielded imidacloprid (35), thiacloprid (36), and clothianidin (37) with an electronegative nitroimine or cyanoimine pharmacophore, which were much more selective than 32 or 33 with a

cationic moiety for insects versus mammals. Preferred radioligands to examine these sites are [<sup>3</sup>H]35 for the insect nAChR and [<sup>3</sup>H]32 or [<sup>3</sup>H]33 for the mammalian nAChR. The higher toxicity of neonicotinoids to insects than mammals is attributable largely to the corresponding difference in sensitivity of their nAChRs. The molecular basis for their selectivity became evident from photoaffinity labeling and X-ray crystallography of the ACh binding proteins from *Aplysia californica* and *Lymnaea stagnalis* docked with neonicotinoids and nicotinoids in their active sites and then extrapolation of the findings to insect and mammalian nAChRs.

**Facile Metabolism.** Neonicotinoid detoxification is largely attributed to CYP450 oxidations at the indicated carbon or sulfur sites or aldehyde oxidase reduction of the nitro to nitroso and amino substituents. Thiamethoxan (38) is bioactivated to 37 and *N*-desmethyl-38. Potency similar to 32 on the  $\alpha 4\beta 2$  receptor is observed for desnitroimidacloprid (39) and descyanothiacloprid (40), which are protonated metabolites of 35 and 36, respectively. Other metabolic reactions are imidazolidine hydroxylation or desaturation (35), *N*-demethylation of 37, and sulfoxidation and hydrolysis of the cyano substituent of 36. IMI (35) is persistent enough in mammals to be an excellent flea control agent for cats and dogs.

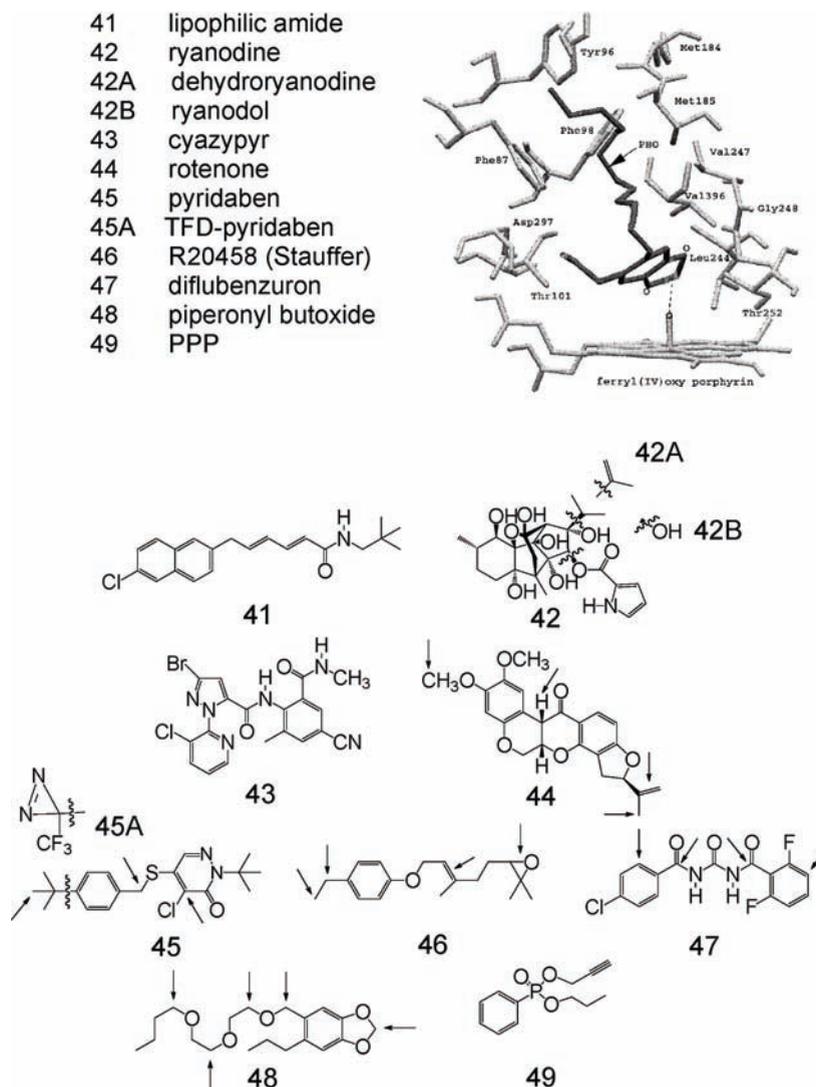


**Figure 4.** Nicotinoids and neonicotinoids acting on the nicotinic receptor showing sites of metabolism (†) and docking prediction for imidacloprid in *Myzus*  $\alpha 2/\beta 1$  binding site. Reproduced with permission from Tomizawa et al. <sup>6</sup> Copyright 2008 National Academy of Sciences of the United States of America.

### ■ DIVERSE INSECTICIDE MECHANISMS (FIGURE 5)

**Other Targets.** Lipophilic amide or isobutyramide insecticides originated from pellitorine and dihydropiperidine as botanical and plant-derived prototypes and progressed to considerably more potent analogues (41, Figure 5) never commercialized, presumably because of inadequate potency–selectivity–stability features for effective and safe use. They act at a sodium channel site different from the pyrethrins with no target site cross resistance. The botanical insecticide Ryania contains ryanodine (42), structurally identified in 1968 as an active ingredient, and 16 years later the equally active dehydroryanodine (42A) was found in even larger amounts, allowing the preparation of [<sup>3</sup>H]42 and starting a new era in ryanodine receptor and insecticide research. The hydrolysis product ryanodol (42B) was also found to be a potent insecticide with low mammalian toxicity

acting at a target different from 42, possibly a potassium channel. Most derivatives of 42 and 42B are of reduced potency and insecticidal activity, but the focus on this target led eventually but not directly to phthalamic and anthranilic acid amide insecticides such as cyazypyr (43), activating the calcium channel at very low levels. Rotenone (44) has been used as an insecticide and piscicide for more than 150 years. It is a respiratory poison inhibiting NADH-ubiquinone oxidoreductase of complex I. Several insecticides including pyridaben (45) act at the same site as rotenone, evident from their use as radioligands in competitive binding assays. Photoaffinity labeling with trifluoromethyl diazirinyl analogue 45A revealed complex I derivatization at the PSST subunit, which couples electron transfer from iron–sulfur cluster N2 to quinone. Action in nerve is more critical in lethality than that in muscle. Toxicity to fish and mammals has restricted interest in this oxidoreductase target site. Juvenoids



**Figure 5.** Other insecticides and related compounds considered in ECTL mode of action and detoxification research showing a proposed model of piperonyl butoxide (PBO) in the active site of CYP450<sub>cam</sub>. Reproduced from Bertini et al.<sup>7</sup> Copyright 2009 American Chemical Society.

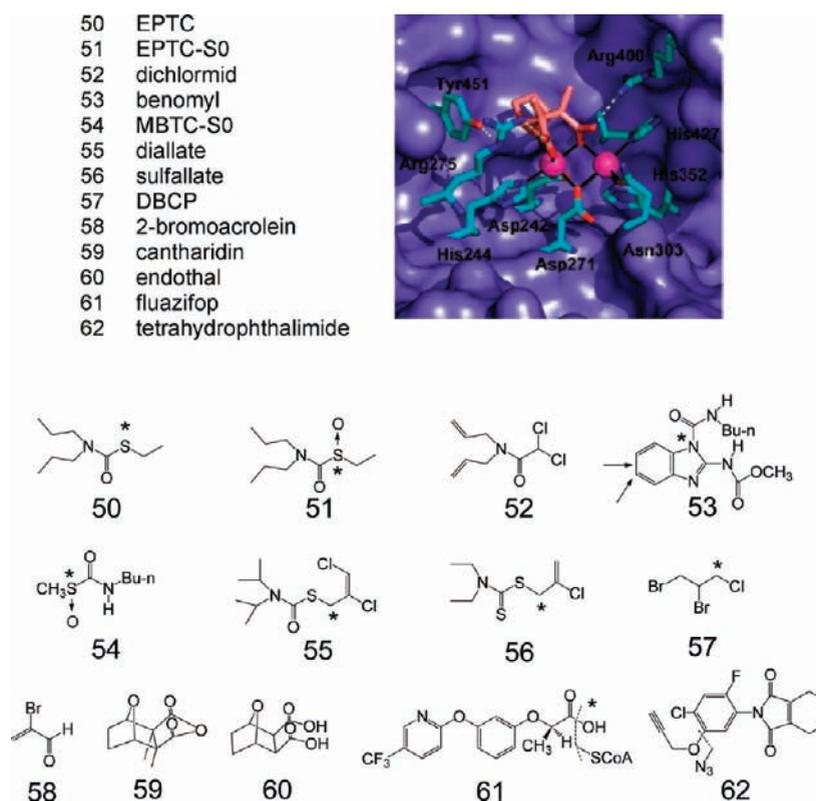
(46) and chitin synthesis inhibitors (47), also studied at Berkeley, are other very effective non-neurotoxic insecticides. The fungal antibiotic nikkomycin was radiosynthesized and shown to inhibit chitin synthetase, whereas 47 and its analogues were found to block the final polymerization step in chitin synthesis but not chitin synthetase itself.

**Metabolic Lability and Synergist Action.** The lipophilic amides are readily biodegraded (e.g., by amide hydrolysis and aryl, arylmethylene, and *N*-methylene hydroxylation for the phenyl isobutyl analogue of 41) and photolyzed, requiring stabilization for practical use. The sites of CYP450 metabolism of juvenoid 46 are well-defined, and almost all metabolites are detoxification products. Interestingly, studies on the metabolism of 46 led to the discovery of cytosolic epoxide hydrolase. Most insecticides are metabolized by CYP450s, and their potency on insects is greatly increased by CYP450 inhibitors such as PBO (48) or *O*-propyl *O*-2-propynyl phenylphosphonate (49). Compound 48 is the most important synergist because of its use with pyrethrins and the pyrethroids. Expanded use of these CYP450 inhibitors raises toxicological questions for mammals and challenges in delivering the insecticide and synergist for maximum

effectiveness. The same relationships apply to ester insecticides detoxified by OP-sensitive hydrolases such as “pyrethroid esterases” sensitive to inhibitors such as 31.

## HERBICIDES, FUNGICIDES, AND MUTAGENESIS (FIGURE 6)

ECTL herbicide research established that the metabolic activation of important thiocarbamates (e.g., EPTC, 50) involves previously unrecognized thiocarbamate sulfoxides (51), and the action of dichloroacetamides (e.g., 52) as safeners or “antidotes”, lowering the phytotoxicity of thiocarbamates to corn but not to weeds, results from inducing in the crop only the synthesis of GSH and GSH *S*-transferase that rapidly detoxify the metabolically activated herbicide. The safener binding protein, isolated and identified in corn, may contribute to herbicide safety, but the mechanism is not completely defined. Important thiocarbamate herbicides (e.g., 50) and the fungicide benomyl (53) via thiocarbamate sulfoxide metabolites (51 and 54) are potent mitochondrial aldehyde dehydrogenase inhibitors and may therefore sensitize agricultural workers to ethanol intoxication.



**Figure 6.** Herbicides, fungicides, and mutagens examined for mode of action in ECTL research showing sites of oxidative metabolic activation (\*) and active site structure of the catalytic domain of protein phosphatase 5  $Mn_2PP_5$  complexed with cantharidin (59). Reproduced with permission from Keserü et al.<sup>8</sup> Copyright 1999 Wiley.

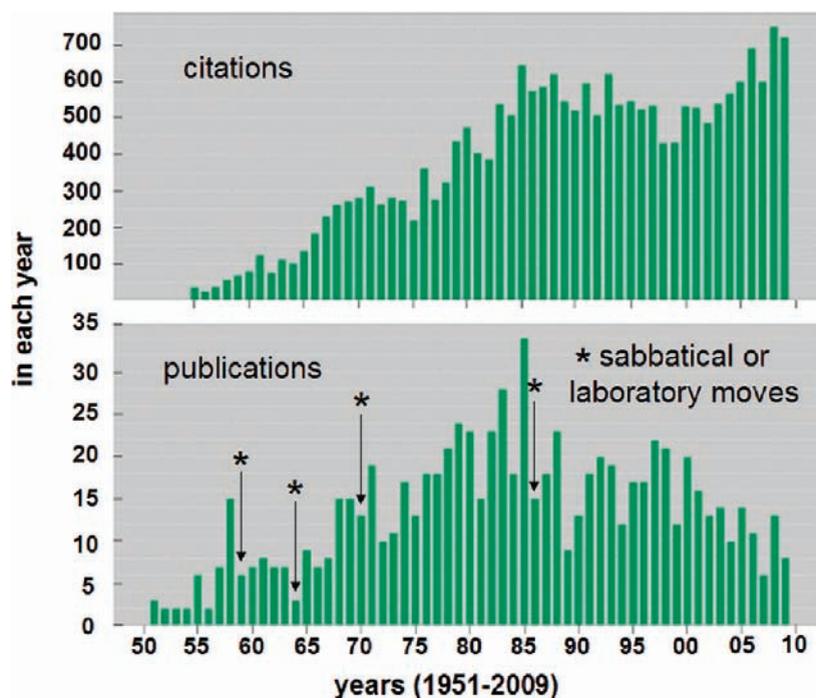
The mutagenic and/or carcinogenic activity of the chloroallyl thiocarbamates diallate (55) and sulfallate (56) and dibromochloropropane (DBCP) (57) are attributable to 2-haloacrolein metabolites such as 58. Cantharidin (59), the active ingredient of blister beetles and the purported aphrodisiac in Spanish fly, acts by inhibiting protein phosphatase 2A. The structurally related herbicide endothal (60) acts the same way in mammals, and its phytotoxicity is probably due to a similar mechanism in plants. The stereospecific metabolic conversion of aryloxypropionates (e.g., 61) to their coenzyme A esters greatly enhances their potency as inhibitors of acetylcoenzyme A carboxylase. A  $^3H$ -labeled *N*-aryltetrahydrophthalimide developed as a radioligand (62) effectively quantitates the protoporphyrinogen oxidase target, which is very similar in plants and mammals.

## ■ STILL CURIOUS

**Coupling Mode of Action and Metabolism.** Understanding and optimizing pesticidal activity requires knowledge of structure–activity relationships at the primary target site coupled with structure–biodegradability relationships at the organismal level. Substituents are introduced or replaced to enhance target fit and control rates of bioactivation or detoxification. As a result, pesticides are becoming more potent and selective and generally more complex in structure. Three approaches have been used in studies to couple pesticide mode of action with metabolism so the ultimate active agent is examined. The first or traditional approach was to separately define the chemistry and biology by metabolite recognition, isolation, identification, and synthesis. The authentic standard was then bioassayed in appropriate systems or, less reliably, its biological properties were predicted

from related compounds. The second approach was to coin-cubate metabolite-generating and -assaying systems. The generating system could be a cell, a cell fraction, or an enzyme such as the S9 fraction or CYP450 with or without NADPH. The assay system might involve AChE (cholinergic) or membrane receptors with [ $^{35}S$ ]TBPS (GABAergic) or *Salmonella typhimurium* (mutagenesis). Useful input comes from computational models. A third and very informative approach involves comparing the structure–activity relationships in vitro at a primary target with the organismal effect. Does the in vitro activity faithfully predict the In Vivo action? This experiment is then repeated with an inhibitor or synergist such as 48 or 49 to determine if the resulting block of metabolism improves the correlation, thereby defining which compounds are detoxified or activated by CYP450. As an alternative, important information is obtained with gene knockouts of either the target or the metabolism system.

**Impact of Discoveries.** Research discoveries are sometimes evaluated by their impact reduced to numbers as a convenient basis for publication analysis. Major advances are cited many times, whereas minor adaptations are largely ignored. The ISI Web of Knowledge provides a convenient source for publication analysis (Figure 7). The author has about 800 publications and 31 patents from 1951 through 2009 (see the Supporting Information). This averages one paper per month or one paper per researcher per year. There were about 22000 citations averaging 27 per paper and an H-index of 71. Sabbaticals abroad (1958–1959, 1971–1972, and 1985–1986) in England, France, Germany, Greece, Japan, Sweden, and Switzerland and a laboratory move from Madison to Berkeley (1964) were times of decreased publication rate, but usually involved a change in the topics considered.



**Figure 7.** Publications and citations in each year 1951–2009 for John E. Casida (or John Casida) based on ISI Web of Knowledge. Asterisks designate sabbaticals or laboratory move.

**Curiosity Is the Key.** Scientific advances are dependent on asking the right questions at the right time and having the intellectual and institutional environments to pursue the answers. Unique discoveries require the freedom to explore pesticide action in whatever direction our curiosity and serendipity might lead us. Only then can we continue to devise and use safe and effective pesticides to maintain food resources and health for an expanding human population.

## ■ ASSOCIATED CONTENT

**Supporting Information.** List of publications and patents of the author for the period 1951–2009 (similar but not identical to compilation in ISI Web of Science). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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## ■ NOTE ADDED AFTER ASAP PUBLICATION

Figure 6 was corrected after original ASAP publication of August 11, 2010. The corrected version is found with the publication of October 22, 2010.