# THE MICROBIAL TOXICITY ASSESSMENT AND BIODEGRADATION OF TRICLOPYR USING *Arthrobacter globiformis* and *Pseudomonas pictorum*

A thesis submitted in partial fulfillment of the

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## ABSTRACT

Triclopyr (3,5,6-trichloro-2-pyridinyloxyacetic acid) was investigated for biodegradation using *Arthrobacter* and *Pseudomonas sp.* bacteria. No evidence of the proposed major metabolite, 3,5,6-trichloro-2-pyridinol was found in sole carbon source or enriched growth medium experiments. Triclopyr, and various salts of triclopyr were also tested for toxicity using a modified INT reduction method which relied upon the enzymes from dead, lysed cells in the mature culture. The $EC_{50}$ values for *Arthrobacter* and *Pseudomonas sp.* bacteria were found to be 46 and 470 ppm respectively for triclopyr. The proposed major metabolite 3,5,6-trichloro-2-pyridinol was found to be considerably more toxic to both bacteria than triclopyr. The $EC_{50}$ results were compared to those for 2,4-D and picloram using the same method. Analysis of triclopyr by gas chromatography and $^{13}C$ nuclear magnetic resonance was investigated in order to simultaneously quantitate the herbicide and its proposed major metabolite. Synthesis of triclopyr using various starting materials was also investigated to achieve an efficient laboratory scale synthesis. Different synthetic routes will be useful for further studies which may make use of radiolabeled triclopyr since selective inclusion of the radiolabel depends on the synthesis used.

## PURPOSE OF THIS STUDY

The purpose of this study was to investigate the biodegradation of triclopyr by two bacterial species: *Pseudomonas pictorum* Gray and Thornton ATCC No. 22284 and *Arthrobacter globiformis* Conn National Research Council No. 32001. In order to study the biodegradation of triclopyr by these two bacteria, a toxicity test was developed to determine the appropriate concentration of triclopyr required so as to not hinder the replication of the bacteria. A tetrazolium indicator: 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) was used in a 48 hour exposure test. In order to complete this study several synthetic routes were developed for the convenient laboratory scale production of triclopyr. Analytical methods for the quantitative determination of triclopyr [VII], possible metabolites and synthetic intermediates were also developed. These included traditional methods such as gas chromatography and a less traditional method for this kind of analysis, $^{13}C$ nuclear magnetic relaxation (NMR), which is gaining popularity for biological uses.

# CHAPTER 1 INTRODUCTION

## 1.1 History

The discovery of plant growth regulators by Kogl in 1934 (1) has led to the development of synthetic compounds (herbicides) which can selectively control plant growth, allowing for the elimination of weeds and brush from croplands and right of ways, economically and with minimum harm to the cultivated plants.

The chemical structure and form (i.e. free acid, salt form, or ester) of the herbicide can be manipulated to facilitate the uptake of the compound selectively at the root or in the post emergent cuticle region (2). The review of plant uptake, transport and metabolism of xenobiotic compounds by Morrison and Cohen (2) emphasizes the importance of assessing the toxicity of these growth regulators in the target environment, as well as in areas that may be indirectly affected by movement of the herbicides. Equally important is the toxicity assessment of metabolites of the xenobiotic compound. Transformation can lead to other biologically active compounds (1,2) which may persist and disrupt the ecological balance of the environment. Persistence can lead to resistance in target organisms. DDT is a good example of this phenomenon (3).

Chlorinated phenols have been known to have herbicidal properties for over four decades (4). Chlorination of the ring structure is used to increase the dissociation of the hydroxyl group (4), thereby increasing the physiological activity of the compound. Blackman, Parke and Garton (4)

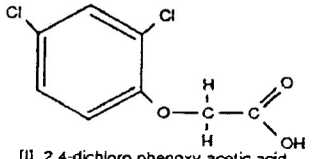
found that the biological activity of chlorinated phenols increases by one order of magnitude with each additional chlorine atom on the ring although positional isomers will show different activities. Other research at this time indicated there is a relationship between the stereochemistry of 2-aryloxybutyric acids and their biological activity. The D-isomers were found to be many times more biologically active than the L-isomers (5). Herbicide absorption has also been correlated with structural properties, and the degree of chlorination was found to affect the solubility of the compound. Decreased solubility leads to less movement in the soil thereby allowing greater contact with the target organism (6).

Further research into the physical and chemical properties of herbicidal compounds included the elucidation of the crystal structure of 2,4-dichlorophenoxy- acetic acid [I] and 2,4,5-trichlorophenoxyacetic acid [II] (7-10). The degree of auxin-like activity was found to correlate with the proximity of the acetate group and a non chlorinated carbon ($C_6$) of the aryl ring which carries a partial positive charge, a situation similar to that found for 3-indoyl acetic acid [III] (7,9). Foy and Change (11), however, did not find a correlation between the auxin behaviour of picloram [IV] and triclopyr [VII] and heavy metal chelation as was proposed by previous authors (12). EDTA and other metal chelators, however, do exhibit auxin activity (11). It was also reported that the degree of lipid solubility has an effect on the activity of the herbicide (9).

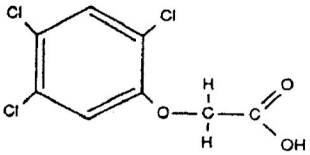
## 1.2 Tolerance Levels

As analytical techniques have improved in the past decades and the reliance on commercially produced herbicides has also increased, more attention has been focused on the persistence and degradation of these compounds. Acceptable tolerance levels of herbicides and other organochlorine compounds for humans and livestock are published by governments and academic institutions to minimize the exposure to these compounds. Triclopyr [VII], a relatively new herbicide, was listed in the 1985 U. S. Federal register (13). Levels of tolerance are 500 parts per million (ppm) in forage grasses and hay (livestock), with 0.1 ppm listed for milk, 0.05 ppm for meat and 0.5 ppm for organ meat (human consumption). This is a combined listing which includes a proposed metabolite, 3,5,6-trichloro-2-pyridinol [VIII]. Triclopyr [VII] equine toxicity has been investigated (14), with acute clinical symptoms found when four oral doses of 300 mg/kg body weight were given daily. This heavy dose schedule was five times the estimated maximal intake at the highest recommended application rate for this herbicide (14). The $LD_{50}$ (rat) is listed at 713 mg/kg (15) or 1675 to 2165 mg/kg (16) for the free acid, apparently varying with the source of the information.The commercially available preparations, Garlon 3A and Garlon 4 (the triethylamine salt and the butoxyethyl ester respectively) are reported to have an $LD_{50}$ (rat) of 2500 mg/kg (17). This compares to the reported $LD_{50}$ (rat) of 2,4-D [I] (300 to 1000 mg/kg). Both triclopyr [VII] and 2,4-D [I] are somewhat more toxic than picloram [IV] ($LD_{50}$ (rat) 8200 mg/kg) (17, 18). A recent review (19) of 2,4- D [I], picloram [IV] and triclopyr [VII]

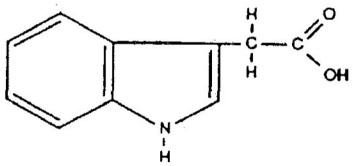
indicated that each of these herbicides is environmentally safe when used at the recommended application rate and showed that there is little evidence of contamination of consumer products. Mullison (19) claimed that triclopyr [VII] is rapidly degraded although there was no mention of the formation of degradation products in the report. This author also states that the half life of triclopyr [VII] in aerobic soil is 30 days (19).



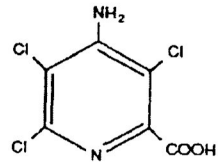
[I] 2,4-dichloro phenoxy acetic acid 2,4-D



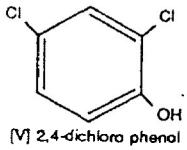
[II] 2,4,5-trichloro phenoxy acetic acid 2,4,5-T



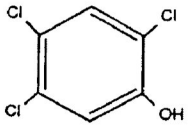
[III] Indole-3-acetic acid IAA



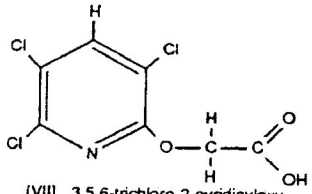
[IV] 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid Picloram



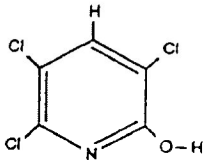
[V] 2,4-dichloro phenol



[VI] 2,4,5-trichlorophenol



[VII] 3,5,6-trichloro-2-pyridinyloxy acetic acid Triclopyr



[VIII] 3,5,6-trichloro-2-pyridinol

## 1.3 Persistence in Soils

Bailey and White (20) and Olloffs (21) reviewed the persistence of xenobiotics in soils citing seven controlling factors, chemical decomposition, photochemical decomposition, microbial decomposition, volatilization, movement, uptake by plants and organisms, and adsorption. These seven parameters must be investigated before it can be safely said that the herbicide will be useful for its purpose yet not be disruptive to non target plants or organisms.

The persistence and movement of triclopyr [VII] have been studied by several groups (22-27). Field studies comparing triclopyr [VII] to numerous other established herbicides (22,23) indicate that the persistence of triclopyr [VII] in soils is moderate in most normal situations (1-2 months at 0-5 cm) while downward movement was quite low with only a few plots showing residues of triclopyr at the 5-15 cm level. In some of the test plots in Sweden (23) the acid preparation and the butoxy ethyl ester preparation of triclopyr [VII] persisted up to two years but the climate of the test area was noted to bear an effect on the degradation of the two compounds. Other influences on persistence in soils are pH, oxygen availability, the amount of organic material in the soil, water content and clay content (23). In both of these studies (22, 23), the herbicide remaining in the soil was measured using bioassay techniques. This type of measurement can furnish only semiquantitative estimates of the herbicide remaining in soils since bioassays have a high degree of uncertainty. Chemical analysis of the herbicide residues provides a more accurate value. Also bioassays cannot

distinguish between the biological activity of the herbicide and that of possible metabolites.

Radosevich and Bayer (24) determined that triclopyr [VII], picloram [IV] and 2,4,5-T [II] all showed optimum root and leaf uptake in warm climates and long daylight conditions. Triclopyr [VII] was found to have the fastest uptake of the three herbicides tested. The method of analysis used by Radosevich and Bayer was not able to distinguish between the herbicide and possible metabolites. The authors used radioactive herbicide preparations and photographic plates to locate the herbicide uptake.

An aerial application of the triethylamine salt preparation of triclopyr [VII] was monitored for persistence (25). Gas chromatographic analysis of soil and water extracts indicated that the residues of triclopyr [VII] decreased from 4.4-18.0 ppm to less than 0.1 ppm in 166 days. There was no detection of triclopyr [VII] or its proposed metabolite 3,5,6-trichloro-2-pyridinol [VIII] in two streams running through the test area. Only one soil sample contained 3,5,6-trichloro-2-pyridinol [VIII] in a detectable quantity (0.28 ppm at 28 days from application). Choon, Oloffs, and Szeto (26) approximated the environment of a British Columbia rain forest in a laboratory experiment to determine the persistence and movement of triclopyr [VII] and Garlon 4 in soil. After 54 days the herbicide had migrated only marginally (less than 10 cm) down a soil column and most of the triclopyr [VII] was converted into 3,5,6-trichloro-2-pyridinol [VIII] (85 % conversion). These authors also reported that 10% of the triclopyr [VII] was converted into 3,5,6-trichloro-2-methoxy pyridine [IX] via a suggested microbial mechanism.

Siltanen *et al* . (27) used gas chromatography to analyze residual triclopyr [VII] levels in wild berries obtained from sprayed conifer plots to determine the extent of contamination in fruits. At an application rate of 2.25 kg/ha, the residual triclopyr [VII] in cowberries was 1.9 ppm and 4.0 ppm in bilberries.

### 1.4 Applications

Much has been written on the effectiveness of triclopyr [VII] as a herbicide for brush control (28-38), conifer release (39-43) and as an aquatic herbicide in rice production (44). The application of triclopyr [VII] in a mixture with 2,4-D [I] and picloram [IV] was found to be more effective in producing leaf brownout and defoliation than triclopyr [VII] alone. It was reported, though, that the overall performance of triclopyr [VII] is suitable for killing mixed hardwoods (28,29). Triclopyr [VII] control of weeds which are hardy, resistant to above ground destruction and produce abundant fruit such as autumn olive (*Eleagnus umbellata*) (30), blackcurrants (32), matagouri (*Discaria toumatou*) (33), gorse (*Ulex europaeus*) (34), honey mesquite (*Prosopis glandulosa*) (35,36), chamise (*Adenostoma fasciculatum*) (37) and maypop passion flower (*Passiflora incarnata*) (38) gave mixed results. All were controlled except the chamise which responded best to glyphosate. Hardwood destruction with triclopyr [VII] is safe to conifers at 2.2-4.4 kg/ha (39-41). King, 1985 (43) indicated that minimal conifer injury is noted when the herbicide is applied in early spring and fall. Aquatic applications of triclopyr [VII] for the control of aquatic weeds to

increase rice crops were proven effective (44). No study of the persistence or degradation of triclopyr [VII] was undertaken by these authors.

## 1.5 Mechanisms of Herbicide Degradation

### 1.5.1 Introduction

Research into the fate of xenobiotic compounds can be separated into two general categories: the field studies which can show the results of herbicide degradation in distinct geographic areas and the laboratory studies which elucidate the mechanisms of degradation. The former branch of study is limited in its approach as the soil microcosm prevents the elucidation of degradative mechanisms (45). The latter technique can provide insights into degradative processes but invariably does not approximate the heterogeneous environment (45) with respect to movement and other soil processes.

Some of these processes consist of degradation through hydrolysis, soil-microbial transformation, photolysis and oxidation (45). The herbicides may also be repartitioned through soil absorption, leaching, bioconcentration, volatilization, ligand exchange and ionization .

### 1.5.2 Soil and Microbial Enzymes

The most important factors in the removal of herbicides from the environment are the soil-microbial degradation mechanisms. In general,

biotransformation tends to make compounds more water soluble by increasing their polarity (46). This, in turn, can allow for stronger adsorption on inorganic soils by ion exchange, if the polar form is able to be ionized (47) rather than simple partitioning of the material into the organic component of soils, as occurs with hydrophobic organic compounds (48). If one considers the enzyme activity of soil to include the native microbial, flora and fauna components, then the activity can be classified as having extracellular and endocellular components (49,50).

The extracellular component can be further separated into the activity of accumulated and continuously released extracellular enzymes (49,50). The accumulated enzyme fraction can be bound to nonproliferating microbial cells, intact dead microbial cells, and microbial cellular fragments or they may be free enzymes from microbes, soil fauna and plant roots (49,50). The continuously released fraction can originate from active, viable microorganisms or from plant roots (49,50). The free enzyme fraction in soils tends to become interspersed in the colloidal soil (51). Endocellular enzymes rely on the uptake of substrates by the host organism for biotransformation to take place. Enzyme types in soil include oxidoreductase, hydrolase, transferase, and lyase (50). Over fifty different soil enzymes had been studied by 1978 (50) but no work on the isomerase and ligase groups had been reported.

### 1.5.3 Microbial Degradation

Bacterial species variation in soils has been studied since the late nineteenth century (52), and correlations with various soil types have been made. Soil microorganisms seem to be of an opportunistic nature with species survival in a given area requiring metabolic adjustments to the environmental fluctuations of organic content, water availability and temperature (52). In general, most extracellular enzyme activity is considered to originate from enzymes released by the cell which are specifically designed to withstand the external environment and are sometimes activated by the external conditions (53).

The uptake of xenobiotic compounds by microorganisms is dependent on the size, charge, and structural configuration of the molecule (51,54,55). Compounds which are lipophilic in nature, tend to be absorbed more readily into the cell membrane (54,55). The effect a herbicide can exert on the environment is influenced by its fate (53). Biodegradation can lead to increased nutrients and a subsequent increase in cell numbers. If the compound exerts a physiological action the microorganism growth can be modified positively, causing proliferation. Alternatively, a negative effect leads to eventual cell death (53). The three types of microbial metabolism recognized as distinct activities are fermentation, aerobic and anaerobic respiration (56). Fermentation occurs independently of oxygen with organic compounds acting as electron acceptors (56). In aerobic respiration the final receptor of electrons is oxygen while in anaerobic respiration the electron

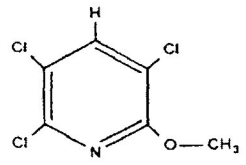
acceptors are nitrates and sulfates (56). In each case the products of the biotransformation of a given compound will be different.

As previously mentioned, the limitations of laboratory degradation studies are caused by the lack of interaction that is present in the heterogeneous system. Some degradative processes lead to toxic metabolites which limit the growth of specific microorganisms, while other microbial species may be able to utilize these metabolites thus detoxifying the environment for the inhibited species. This can lead to an equilibrium between the species which has been proven to be established in some experiments with up to four different microorganisms involved (56). Audus (57) indicated that the equillibrium may occur with 2,4-D [I] by noting a long lag phase in his soil percolation experiments: 10-14 days elapsed before the compound disappeared. Also, the induction of enzyme systems required for the removal of the substrate should be considered. The difference between enzyme induction and microbial equilibrium in a heterogeneous system would be difficult to distinguish as the cause of the degradation process. In fact, both processes are likely to occur simultaneously. Audus did manage to isolate a soil bacterium which was responsible for disappearance of the 2,4-D [I] and he was able to grow it on a 2,4-D [I] basal medium (57). Identification of the bacterium proved to be elusive to Audus, although he found it had characteristics similar to the *Bacterium globiforme* group (57).

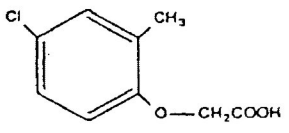
As well as the research by Audus into 2,4-D [I] and 2-methyl-4-chlorophenoxyacetic acid [X] (MCPA) (57-59), numerous other investigations into the degradation of these compounds have been published (60-72). Other work was published on the fates of phenols and

phenolic compounds, which are primary metabolites of the phenoxy herbicides and are also important industrial chemicals (73-81). A summary of the reviewed work on the fates of phenoxy herbicides and phenols is presented (Table 1.1 and 1.2). The proposed pathway for degradation of 2,4-D [I] by two *Pseudomonas sp.* (67) is illustrated in Figure 1.2.

The early published data on the metabolism of phenoxy herbicides relied (to some extent) on indirect analysis, such as: bioassay techniques (57, 58, 64), thin layer chromatography (57-59), ultraviolet absorption spectroscopy (59) and oxygen consumption (65). Bioassay measurements such as root growth stimulation (57,58,64) do not account for the auxin behaviour of accumulated metabolites. Thin layer chromatography and gas chromatography are not conclusive unless the results are verified using different stationary phases since the basis for identification relies on similar behaviour (interaction with a stationary phase) between the test compound and a known compound and not on structural elucidation (NMR, mass spectroscopy *etcetera*). Ultraviolet absorption spectroscopy is subject to interfering absorption spectra when samples are impure. Manometric measurements (for the consumption of oxygen) are at best crude, especially if the mass of substrate to be oxidized by the microorganism is small.




[IX] 3,5,6-trichloro-2-methoxypyridine




[X] 2-metyl-4-chlorophenoxy acetic acid
MCPA

| Author | Date | Ref. | Compound(s) | Microorganism | Metabolite(s) |
|---|---|---|---|---|---|
| Audus | 1951 | 57 | 2,4-D/MCPA | Bacterium globiforme group | None identified |
| Audus | 1952 | 58 | 2,4-D/MCPA | Bacterium globiforme group | 2,4-D/MCPA-Phenols |
| Audus | 1955 | 59 | 2,4-D | Bacterium globiforme group | Two not identified |
| Gaunt | 1971 | 60 | MCPA | Group III Pseudomonas sp. | 5-chloro-o-cresol<br>5-chloro-3-methylcatechol |
| Gaunt | 1971 | 61 | MCPA | Group III Pseudomonas sp. | 5-chloro-o-cresol<br>glyoxylate |
| Gaunt | 1971 | 62 | MCPA | Pseudomonas NCIB 9340 as a cell free extract | 5-chloro-3-methylcatechol<br>cis-cis-gamma-chloro-alpha-methylmuconate<br>gamma-carboxymethylene-alpha-methyl-delta-butenolide |
| Gaunt | 1961 | 63 | MCPA | Group III Pseudomonas sp. | 5-chloro-o-cresol<br>gamma-carboxymethylene-alpha-methyl-delta-butenolide |
| Jensen | 1952 | 64 | 2,4-D/MCPA | Flavobacterium aquatile<br>Possible Corynebacterium sp.<br>Nocardia coeliaca | None identified<br>None identified<br>None formed |
| Loos | 1967 | 65 | 2,4-D | Arthrobacter sp. | 2,4-dichlorophenol |
| Steenson | 1957 | 66 | 2,4-D/MCPA | Flavobacterium peregrinum<br>Achromobacter sp. | 2,4-dichlorophenol<br>2,4-dichlorophenol |
| Evans | 1971 | 67 | 2,4-D | Two Pseudomonas sp. | 2,4-dichlorophenol, 2-chlorophenol, 3,5-dichlorocatechol, alpha-chloromuconate |
| | | | 2,4-D | Pseudomonas NCIB 9340 | 2,4-dichloro-6-hydroxyphenoxyacetate, 2,4-dichlorophenol |
| | | | | Pseudomonas NCIB 9340 as a cell free extract | 3,5-dichlorolcatechol, alpha-gamma-dichloromuconate, gamma-carboxymethylene-alpha-chloro-delta-butenolide |
| Evans | 1957 | 68 | p-chlorophenoxyacetate | Pseudomonas sp. | ß-chloromuconic acid |
| Loos | 1967 | 69 | 2,4-D/MCPA | Arthrobacter sp. | 2,4-dichlorophenol, 2-methyl-4-chlorophenol |
| Osman | 1964 | 70 | 2,4-D (and esters) | Aquatic mixed cultures (lake water) | 2,4-dichlorophenol (esters were hydrolysed) |
| Smith | 1972 | 71 | 2,4-D (and esters) | Mixed cultures (prairie soil) | esters were hydrolysed |
| Faulkner | 1965 | 72 | 2,4-D/MCPA | Aspergillus niger | 2,4-dichloro-4-hydroxyphenoxyacetic acid, 4-chloro-5-hydroxy-2-methylphenoxyacetic acid |

**Table 2.1 Microbial Degradation of 2,4-D, MCPA, and Related Compounds**

| Author | Date | Ref. | Compound(s) | Microorganism | Metabolite(s) |
|---|---|---|---|---|---|
| Evans | 1947 | 73 | Phenol | Vibrio 01 | Catechol |
| | | | Benzoic acid | Vibrio 01 | o-benzoquinone |
| Chambers | 1964 | 74 | Various substituted phenols | Pseudomonas sp.<br>Flavobacterium sp.<br>Achromobacter sp.<br>Xanthomonas sp. | None investigated. The benzoic acids, benzaldehydes, cresols, and methylphenols were easily degraded. The nitro compounds, benzene, and halogenated aromatics were most resistant. |
| Cartwright | 1966 | 75 | Vanillic acid | Pseudomonas fluorescens | β-oxoadipate |
| Neujahr | 1970 | 76 | Phenol | Trichosporon cutaneum | Catechol |
| Yang | 1975 | 77 | Phenol | Pseudomonas putida | Catechol |
| | | | | Trichosporon cutaneum | Catechol |
| Buswell | 1975 | 78 | Phenol | Bacillus stearothermophilus | catechol |
| | | | o-cresol | Bacillus stearothermophilus | 3-methylcatechol |
| | | | m-cresol | Bacillus stearothermophilus | 4-methylcatechol |
| | | | p-cresol | Bacillus stearothermophilus | 4-methylcatechol |
| Hill | 1975 | 79 | Phenol | Pseudomonas putida | None investigated. P putida was found to have a high rate of dissapearance of phenol. |
| Neufeld | 1980 | 80 | Phenol | Activated sludge (anaerobic degradation) | Various volatile organic acids, not identified. |
| Portier | 1983 | 81 | Phenol, 4-nitrophenol 2,4,6-tri-chlorophenol, 2-chloro-phenol. | Mississippi River sediment containing mixed flora | None identified. The chlorinated phenols were most resistant to degradation. |

**Table 2.2 Microbial Degradation of Phenols Related to 2,4-D**

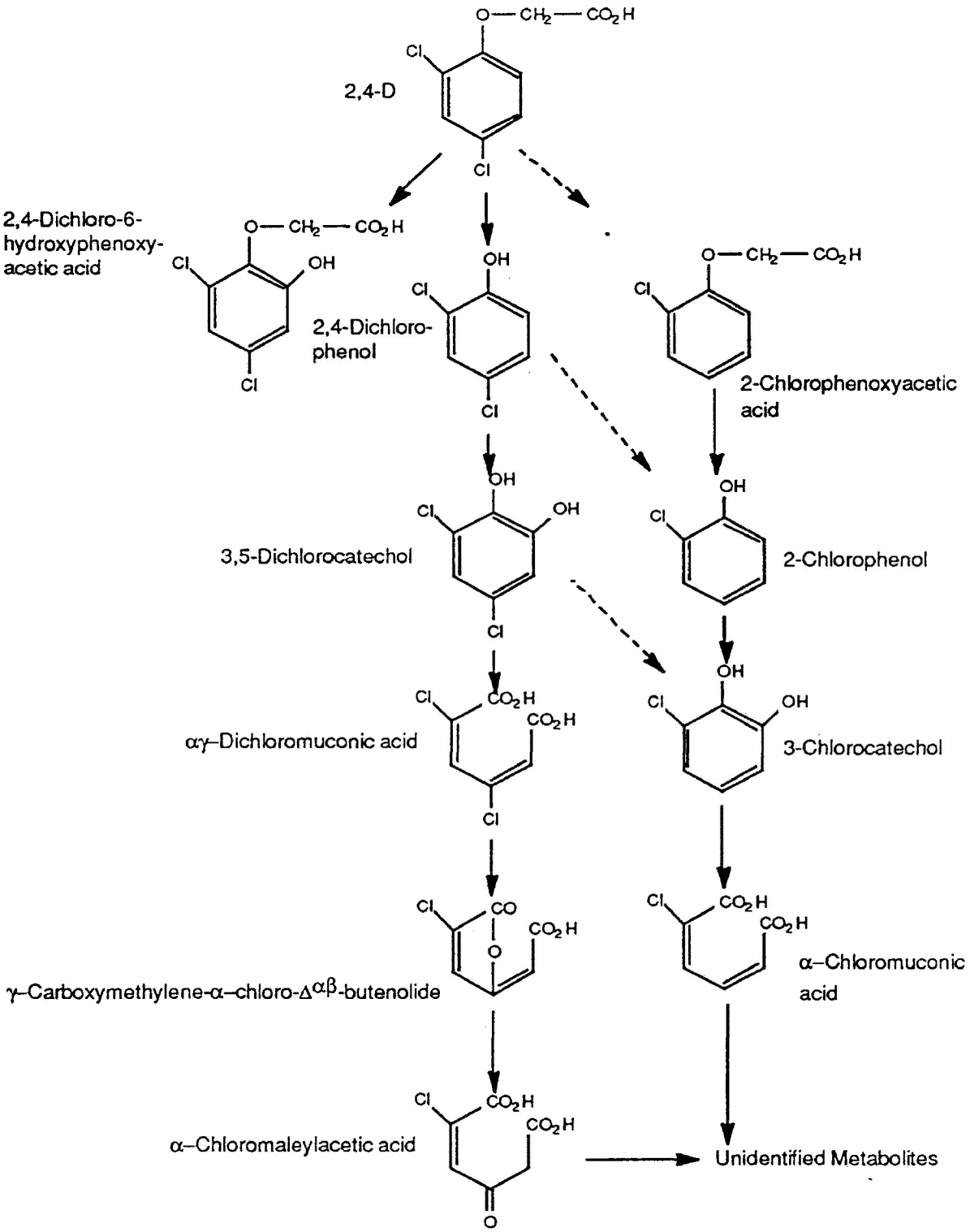


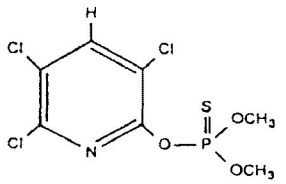

**Fig. 2.1 Pathway for 2,4-D Degradation Proposed by Evans *et al*. (67)**

### 1.5.4 Tryclopyr [VII] Degradation

Very little data has been reported on the degradation of triclopyr [VII] other than results from field studies which have indicated that the herbicide dissappears from the soil over time (22-27). These studies do not account for the irreversible binding of the parent compound in soils nor do they report the formation of metabolic products by soil enzymes as the result of microbial metabolism. Choon, Oloffs and Szeto (26) reported that triclopyr [VII] is extensively converted to 3,5,6-trichloro-2-pyridinol [VIII] and 3,5,6-trichloro-2-methoxypyridine [IX]. There was no mention of specific microorganisms in their study and the conversion of triclopyr [VII] to 3,5,6-trichloro-2-methoxypyridine [IX] is not encountered in the analogous 2,4-D [I] degradation studies. The proposed metabolite for triclopyr [VII] (3,5,6-trichloro-2-pyridinol [VIII]) is also encountered in the soil and microbial degradation (82-84) and also the higher organism metabolism (85-87) of chlorpyrifos [XI] (o,o-dimethyl-o-(3,5,6-trichloro-2-pyridyl)phosphorothionate), which is a broad spectrum insecticide. Getzin (82) found that chlorpyrifos [XI] degraded from dry soil surfaces as well as moist soils indicating a clay activated degradation of the insecticide can take place. Getzin (83) reported that the half life for chlorpyrifos [XI] was significantly longer in autoclaved soils. In view of the fact that the sterilization of soil would result in the destruction of the exogenous enzyme fractions as well as those endogenous to the microbial population, the results point to a degradative process that may be inorganicaly mediated. Szeto and Sundaram also describe 3,5,6-trichloro-2-pyridinol [VIII] as the toxic

breakdown product of chlorpyrifos [XI]. They do not indicate whether the metabolite is toxic to insects or non target organisms (84). Rats and sheep fed doses of chlorpyrifos [XI] were found to excrete 3,5,6-trichloro-2-pyridinol [VIII] in their feces while the glucoronide conjugate of 3,5,6-trichloro-2-pyridinol [VIII] was found in the urine. Plants (86) and insects (87) were also found to produce the metabolite.

Smith (88) investigated the ultraviolet decomposition of 3,5,6-trichloro-2-pyridinol [VIII]. Smith found that in water the 3,5,6-trichloro-2-pyridinol [VIII] structure readily loses the chlorine atoms via light induced mechanisms producing HCl and various di, tri and tetrols. Unfortunately he does not give analytical proof of the formation of these di, tri, and tetrols apart from mentioning the formation of the HCl and colored compounds. Furthermore, one of the structures proposed in his photodecomposition scheme has two pentavalent carbons (two aromatic ring carbons with ketone groups attached). It is physically impossible for a carbon atom to bear five formal bonds. Aromaticity must be lost before a hydroxy group can be oxidized to an aldehyde in this type of structure. In view of the above it is important to investigate the environmental degradation of triclopyr [VII] with respect to the potential toxicity of metabolites such as 3,5,6-trichloropyridinol [VIII]. To be concerned only with the degradation of the parent compound would not present the true picture of its enviromental fate.




[XI] Phosphorothioic acid O,O-dimethyl O-(3,5,6-trichloro-2-pyridinyl) ester
Chlorpyrifos-methyl

## 1.6 Methodology for the Assessment of Herbicide Toxicity

### 1.6.1 Applications of Toxicity Assesment Tests

As well as investigating the fate of xenobiotic compounds in the target flora and fauna, the effects they can exert on non target organisms must be investigated. Since the rate of disappearance of a given compound relies on the ability of the microorganisms in the local environment to assimilate it, a great deal of toxicity research is focused on bacteria and fungi. Also, microorganisms are used as models for other species since they replicate at very high rates. As well, this makes them useful for studying mutagenicity.

The release of effluents by the pulp and paper industry is presently regulated by the Canadian federal government while other industries (oil refineries, chloralkali plants, base metal mining, meat and poultry processing) follow suggested guidelines (89). In the near future all industries and municipalities will become responsible for their own effluent control and analysis (90). For obvious reasons, it is not feasible for complete chemical analysis to be performed on effluent wastes on a regular basis. As an alternative, toxicity assessment provides a relatively inexpensive means of controlling the release of toxic materials. One such test which is currently accepted is the 96 hour acute trout toxicity test as this provides information related to the mortality of a fish species (89). Such a result is considered evidence and can be used in legal situations (89).

### 1.6.2 General Types of Toxicity Tests

Buikema *et al.* (91) and Bitton (92) emphasised the importance of using bacteria, algae, macroinvertabrae and fish in toxicity tests as these groups of organisms make up a considerable component in the biomass and are major contributors to the food chain. Buikema *et al.* (91) also defined the main types of tests. Acute toxicity tests are short term studies which indicate a response of the organism exposed to a logarithmic series of dilutions of a toxin over a set amount of time. The final result is usually reported as the concentration which causes a 50 % mortality ($LC_{50}$) in that time. The term $EC_{50}$ (effective concentration) can be used when the mortality of an organism is difficult to establish and a different response is required to measure the effect of the toxin (91). The sublethal chronic test is used to reflect the effects of various concentrations of a toxin on the reproduction, growth and survival of an organism (91). The parameter reported for such a test is known as the Maximum Acceptable Toxic Concentration (MATC), which is the highest possible concentration that does not exert an effect on the test organism (91). This type of test is usually designed so as to include an entire life cycle of a species and is subject to much variability due to the time span involved. Bioaccumulation tests are used to determine the accumulation of a given compound in a test organism. The value is sometimes reported for aquatic species as a ratio of the final tissue concentration over the water concentration (91).

Babich and Stotzky, (93) recommended that microbial toxicity assays which reflect a disruption in an ecological process be used in water quality

criteria. This response would be reported as $EcD_{50}$ (Ecological Dose 50 %) and is thought to represent the influence of abiotic (environmental) factors on a compounds toxicity. The physical and chemical factors of the environment cited by Babich and Stotzky (93,94) include: pH, redox potential, aeration status, buffering capacity, anion-cation composition, water content, clay mineralogy, hydrous metal oxides, organic content, ion exchange capacity, temperature, solar radiation, hydrostatic pressure and osmotic pressure. These factors influence the bioavailability of a toxin through ligand exchange, irreversible absorption, ionization, solubility and chemical transformation. Bird *et al* . (95) echo these points by cautioning against the use of growth media in tests for metal species. as chelation of the metal species is possible. Hartman and Martin (96) reported on the effects of bentonite clay in the toxicity of glyphosate. A decreased bioavailability of the herbicide was found upon the addition of suspended clay particles. Also, Herzel and Murty (97), studied the effects of carrier solvents used to increase the water solubility of hydrophobic compounds. Their results indicated that the use of acetone does not enhance the solubility of hydrophobic compounds, so that the actual concentration is lower than the apparent concentaration in many acute toxicity tests. Ideally, toxicity tests should be designed to simulate the environment in which the test compound is released.

### 1.6.3 Methods Which Utilize Enzymes

In microbial toxicity assays, the most common parameter measured is enzyme inhibition (92). Bitton (92) listed the common biological activities which can be easily measured with simple apperatus: dehydrogenase, ATPase, bioluminescence, and the biological indicators of biomass such as ATP, lipopolysaccharides (gram negative bacterial cell wall), muramic acid (bacteria and blue green algae cell wall) and chlorophyll *a* content (blue green algae).

Genotoxicity can be measured with the Ames® test, which relies on the frequency of mutations in the histidine operon of *Salmonella typhimurium*. Also available is an assay which reflects the ability of prophage *Mu* to utilize *Escherichia coli* for replication (98). If the lytic cycle of the prophage is disrupted by a genotoxin then the decrease in plaque formation will indicate toxicity (98).

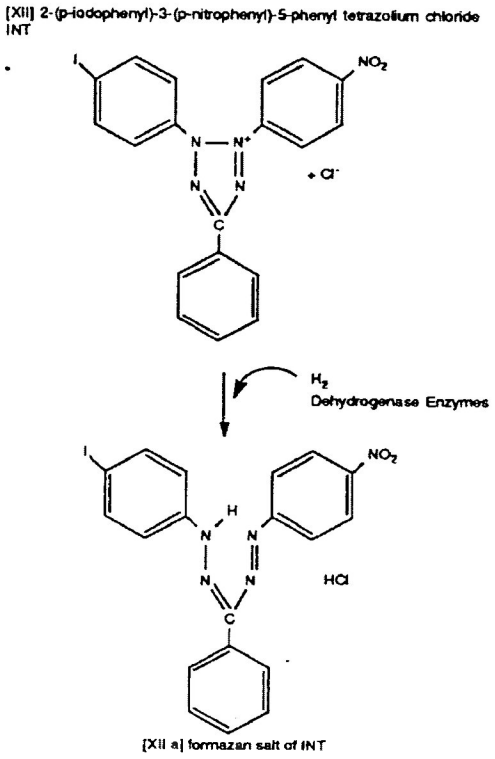
The Microtox® assay, which utilizes bacterial bioluminescence (a part of the electron transport chain in some species) has been evaluated and compared to other established toxicity tests by several investigators (99-103). Dutka and Kwan (100) compared the Microtox® system with the inhibition of flagella motility of *Spirillum volutans* and with the growth rate of *Pseudomonas fluorescens* . The rapid nature of the Microtox® test makes it particularly useful for on the spot effluent testing (99,100). The results from a comparison of the Microtox® test with *Spirillum volutans* motility, the inhibition of respiration in activated sludge and the inhibition of activated sludge TTC-dehydrogenase activity (triphenyltetrazoliumchloride reduction),

led Dutka *et al* . (101) to recommend that more than one toxicity test be used to asses the potential toxicity of a given compound. Dutka reiterated this point in another comparison study (102), also stating that standardization is required so that interlaboratory results will be comparable. McFeters *et al.* (103) compared the Microtox® test to another bioluminescent assay (developed by Tchan) which uses *Photobacterium phosporium*, NZ11D and a luminescent algae *Dunaliella tertiolecta* . The sensitivities of the two tests were different depending on the type of compounds tested. Photosynthesis inhibiting herbicides gave a more toxic response in the test utilizing the algae. Most other chemicals tested were found to be more toxic by the Microtox® test.

Other methods which reflect changes in the dehydrogenase activity of bacteria employ dyes such as resazurin (104), or tetrazolium salts (105-111) (commonly; triphenyltetrazolium chloride and 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride [XII], known as TTC and INT respectively which upon reduction form colored formazans, for example XIIa). With the resazurin method unreduced dye is measured at 610 nm (104). The amount of unreduced resazurin is inversely proportional to dehydrogenase activity in the test culture. Liu and Thomson (104) demonstrated that the method is useful for relatively insoluble compounds. Cenci and Morozzi (106) compared TTC-dehydrogenase activity to methods which measure the oxygen respiration rate of activated sludge. These authors found that the two different methods will allow for the classification of some metal toxins as respiratory enzyme inhibitors or inhibitors of dehydrogenase enzyme activity, depending on the enzyme method they

inhibit most. Some other toxicity assesment assays utilizing tetrazolium salt reduction require the visual inspection of the intracellular crystals of INT-formazan against a counter stain of malachite green (107,111), while other methods have been reported which require extraction of the INT-formazan before it is quantified (108,109) or employ direct measurement without extraction (110).

INT is unique when compared to other dye substances since it is the reduced form which is highly coloured. Thus, measurement of the colour intensity can be directly related to the reducing properties of the medium (enzyme constituent), rather than measuring a decrease in colour intensity (105).



### 1.6.4 Measurements of ATP Production

The ATP content of bacterial (112) and algal cultures (113) is useful for measuring the percentage of a given population of cells that is killed by a toxin, as ATP is converted to ADP when a cell dies (92). After the ATP is extracted from the cells with boiling tris buffer, a luciferin-luciferase bioluminescent reaction is performed utilizing the ATP which was extracted. The light generated by the reaction is measured with a photometer, and the intensity is a reciprocal function of mortality. As a routine method for measuring the toxic effects on microorganisms, ATP extractions and then bioluminescent reactions would appear to be time consuming and expensive.

### 1.6.5 Algae and Miscelaneous Methods

The measurement of the response of algae to toxic compounds is commonly done by cell counts performed manually or with particle counting devices (113-116). A recent development is the miniaturization of the cultures with microplates (113-115). Some other microbial responses to toxic substances are the generation of carbon dioxide and inorganic nitrogen from soil microorganisms (117) and activated sludge (118,119). The oxidation of nitrite to nitrate by a pure culture of *Nitrobacter sp.* has also been used to measure the toxicity of test compounds (120).

Freshwater macroinvertebrae organisms are used in toxicity tests because of their biomass contribution to the food chain and the ease of maintaining cultures (121-123). These organisms can also be easily monitored throughout their life cycle (121,123). Gerisch *et al.* (121,122) used *Daphnia magna* Straus to study the acute and chronic toxic effect of triclopyr [VII] (as the triethyl amine salt) on invertebrae species. The acute toxicity test result ($LC_{50}$) was reported to be 1170 ppm for triclopyr [VII] (as the triethyl amine salt), based on a 48 hour exposure with no aeration of the tanks and no feeding (121). The chronic endpoint test result (MATC) was reported to be between 80.7 and 149.0 ppm (121,122). The chronic endpoint test used neonate *Daphnia magna* Straus which matured and replicated over the 21 day test. The literature published on toxic assessment tests of triclopyr [VII] is quite scarce. No attention has been focused on the effects of triclopyr [VII] on microbial populations and algae.

### 1.7 Synthesis of Triclopyr [VII]

Triclopyr [VII] is not commercially available in pure form. To complete this study, a suitable laboratory scale preparation of triclopyr [VII], had to be found. A selection of laboratory scale preparations is theoretically available and some of these were investigated with two objectives in mind: first to find the least time consuming synthesis and second to allow for selective labeling of the compound if studies requiring radiolabeled compounds are to be subsequently undertaken.

### 1.7.1 Methods Analogous to 2,4-D [I] Preparations

Triclopyr [VII] is a relatively new herbicide discovery (13) when compared to 2,4,5-T [II], picloram [IV] and 2,4-D [I]. Consequently, there is a scarcity of data on the synthesis of triclopyr [VII]. Since triclopyr [VII] is a pyridine analogue of 2,4,5-T [II] and 2,4-D [I], synthetic methods used to produce these herbicides may be useful in the synthesis of triclopyr [VII].

The synthesis of 2,4,5-T [II] and 2,4-D [I] was first reported by Porknoy (123). He mixed equimolar quantities of 2,4-dichlorophenol [V] or 2,4,5-trichlorophenol [VI] with monochloroacetic acid and a slight excess of sodium hydroxide. The aqueous solutions were evaporated by heating until almost dry. After redissolving in water and acidifying with hydrochloric acid, 85 % yields of the chlorophenoxy acids were obtained. Dawson, Pycock and Smith (124) attempted the elimination of the chloride ion from monochloroacetate with a large number of other bases (34 in total). The hydroxyl anion and the carbonate anion were found to be most efficient in the removal of chlorine. This experiment indicated that anymore than a slight excess of base would lead to diminished yields of the chlorophenoxyacetic acids. To be technically correct, the removal of the chlorine by base is not an elimination as described by Dawson, Pycock and Smith (124). The removal of chlorine is in fact part of a bimolecular nucleophillic reaction ($S_N2$) which is initiated by a more basic ion than the chloride ion. This was proposed by Smith (125) and indirectly confirmed by Synerholm and Zimmerman (126). Their experiments involved the production of ß-(2,4-Dichlorophenoxy)propionic acid and other phenoxy acids with longer side

chains (butyric, valeric, and caproic acids). In these reactions no rearrangement of the side chain occurred indicating the removal of the chloride ion takes place by bimolecular substitution and not via the formation of a cation intermediate.

Eckstein *et al.* (127) attempted to improve the condensation of the phenolate ion with sodium chloroacetate by adding various salts (sodium chloride, magnesium chloride, or sodium sulphate) to the reaction mixture in an effort to prevent the hydrolysis of the chloroacetate. Yields were not improved by the addition of the inorganic salts. Also, Eckstein *et al.* erroneously explained the reaction by proposing the elimination of the chloride ion as a separate step, followed by the formation of a carbonium ion from the sodium acetate molecule. This would indicate the reaction proceeds via a unimolecular elimination ($S_N1$) reaction mechanism rather than a $S_N2$ mechanism.

Synerholm and Zimmerman (128) reported that the rates and yields of these reactions were increased when the sodium acetate was replaced with an ester. This is likely due to the removal of the repulsion between the two anions in Porknoy's reaction. Markin (129) exemplified the increased reactivity of the esters by using the diethyl ester of dibromo succinate to produce meso-a,a'-diphenoxysuccinic acid. Morris and Bohnert (130) reported that the formation of the methyl ester of triclopyr [VII] was possible with the use of methyl chloroacetate and the sodium or potassium salt of 3,5,6-trichloro-2-pyridinol [VIII]. This reported method (130) is a patented procedure (1978, Germ. Offen. 2,756,056.) and, is used by the Dow Chemical Company to produce triclopyr [VII].

An understanding of the differences between the structure of the ring in triclopyr [VII] and 2,4-D [I] is necessary in order to predict the differences in the reactivities of the pyridinol, and phenol structures. Since the ring structure of triclopyr [VII] is a pyridine ring, the geometric and electronic nature is different from the phenyl ring of 2,4-D [I]. The inclusion of a nitrogen into the ring of pyridine causes a perturbation in the pi orbital overlap ($sp^2$) therefore, the aromatic stabilization energy is lower than that of benzene (131). The heteroatom has slightly shorter bond lengths to the neighboring carbons relative to the carbon-carbon bonds of pyridine (132,133). Also, the heteroatom is not located exactly in the plane of the ring (132,133). These factors contribute to the resonance energy difference between benzene (36 kcal./mole) and pyridine (31.9 kcal./mole) (131). The geometric perturbation coupled with the increased electronegativity of nitrogen, relative to carbon, causes the ortho and para carbons in the ring to be slightly electron deficient. This electron deficiency affects the ortho and para carbons' capabilities to participate in reactions which require electrophillic attack upon the ring. This deactivation is felt less at the meta position so that electrophillic attack is meta directed.

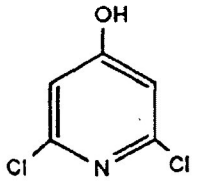
The reactivity of 3,5,6-trichloro-2-pyridinol [VIII], in preparations similar to Porknoy's synthesis of 2,4-D [I] (123), is affected by the electronic and geometric character of the pyridinol structure as mentioned in the previous paragraph. Since the heteroatom is electron withdrawing, a tautomeric equilibrium occurs between the pyridinol and pyridone structures (tautomeric equilibrium structures can be found at the end of 1.7.1). In

solution this tautomerism is affected by the nature of the solvent (134,135). Also the position and electronic nature of ring substituents will affect the tautomerism (135-141). Another important contributing factor to the tautomerism of the pyridinol molecule is the location of the oxygen on the ring (135,141). The 2-hydroxypyridine isomer is less affected by ring substituents than the 4-hydroxypyridine isomer (141). The keto form of 2 or 4-hydroxy pyridine tends to be favored in solvents that are non polar, while the hydroxy form is more favoured in polar solvents (134,135). The ring substituents can destabilize certain contributing structures to the overall resonance hybrid structure, thereby increasing the contributions of other structures (141). In general, evidence suggests that $C_3$ or $C_5$ chlorine substituents on the 2 pyridinol ring tend to drive the equilibrium towards the pyridone form (136, structures can be found at the end of 1.7.1). However, the 4-pyridinol ring structure favours the hydroxy form when there is a chlorine atom a to the nitrogen (141). This is due to the electron withdrawing capability of the chlorine atom, which decreases the acidity of the proton attached nitrogen in the pyridone form (141). In the 4-hydroxy form, the chlorine is also capable of reducing the basicity of the nitrogen (141). Therefore appreciable amounts of the hydroxy form can be present in this equilibrium. Similar to the 4-pyridinol case, chlorine in the 6-position of 2-pyridinol should favour the hydroxy tautomer over the keto form. Specifically, for 3,5,6-trichloro-2-pyridinol [VIII] no clear cut assumptions can be made as to which form predominates in solution. Although the substituent effects have not been fully elucidated (141), there may very well be conflicting forces between the chlorine atoms in the 3,5-positions and the chlorine atom in the

6- position. Whatever tautomeric form is favoured, the nucleophilic character of the oxygen atom will be somewhat decreased by the nitrogen in the ring. It may also be deactivated by the chlorine atoms in the 3,5-positions. Therefore, relative to phenolate, the pyridinolate ion will be less reactive in $S_N2$ reactions such as the displacement of chlorine from chloroacetic acid.

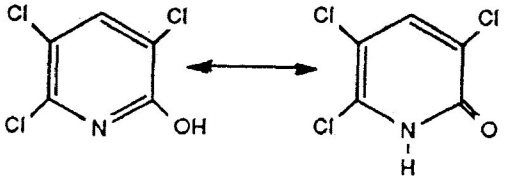
Another method of interest to produce triclopyr [VII], which is based on a 2,4-D [I] preparation, is the reaction of ethyl diazoacetate with 3,5,6-trichloro-2-pyridinol [VIII] (142,143). Cava and Bhattacharyya (142) reported that the yield of triclopyr [VII] by this preparation of its ethyl ester and subsequent hydrolysis was only 17 %. The highest yields obtained when various halogenated 2-pyridinols react with ethyl diazoacetate are found when the pyridine ring has only one chlorine atom or when the ring has chlorine atoms in the 3,5-position (142). The authors gave no reason for the difference in reactivities of the various substituted pyridinols but it is likely due to the degree of halogenation. If there is a high degree of electron density at the nitrogen with respect to the oxygen, as may be the case with 3,5,6-trichloro-2-pyridinol [VIII], then carboxymethylene may bond to the nitrogen forming the N-alkyl product (143). Oddly, Maas *et al.* have illustrated their N-alkyl isomer with two pentavalent carbons in the side chain. This is likely a typographical error though, and is of no importance to the chemistry they have proposed, unlike the previously mentioned pentavalent structures proposed by Smith (88).


3,5-dichloro-2-pyridone




2,6-dichloro-4-hydroxypyridine

From discussion: Chlorine in the meta position with respect to Nitrogen (3,5-positions) tends to favour the ketone tautomer while ortho chlorine groups will decrease the acidity of the hydrogen on the nitrogen atom in the pyridone form. This in turn will favour the pyridinol tautomer.




3,5,6-trichloro-2-pyridinol 3,5,6-trichloro-2-pyridone

The Keto-Enol Tautomerism of 3,5,6-trichloro-2-pyridinol

### 1.7.2 Methods Requiring 2,3,5,6-tetrachloropyridine [XIII]

Apart from the previously mentioned preparations which are based on methods used to prepare 2,4-D [I] and 2,4,5-T [II], triclopyr [VII] has been synthesized by two other methods (144,145). Both of these preparations require 2,3,5,6-tetrachloropyridine [XIII] as a starting material. This is a problem since 2,3,5,6-tetrachloropyridine [XIII] is not commercially available. A laboratory method for the syntheis of triclopyr [VII] via these routes must therefore include the preparation of this starting material.

Sell and Dootson (146) first reported the preparation of 2,3,5,6-tetrachloropyridine [XIII]. By heating tetrachloroisonicotinic acid [XXIII] in sealed tubes with water, 2,3,5,6-tetrachloropyridine [XIII] and carbon dioxide is formed. They obtained similar results when the acid was distilled with glycerol (146). No yields were reported for either of the forementioned reactions.

Catalytic cycloaddition can be used to prepare 2,3,5,6-tetrachloropyridine [XIII] (147). By autoclaving (180° C.) the acid chloride of trichloroacetic acid with acrylonitrile and copper(I)chloride in acetonitrile as the solvent, 2,3,5,6-tetrachloropyridine [XIII] was produced along with 3,5,6-trichloro-2-pyridinol [VIII] (147). The patent for this reaction (European Patent Application 30,214 November 10, 1979) did not contain information on the yield of this reaction.

The sealed tube reaction of glutarimide with phosphorus pentachloride produces a mixture of halogenated pyridines (148-151).

Phosphorus pentachloride is a good donor of chlorine in this situation as the byproduct of the reaction is phosphorus oxychloride which has an especially stable phosphorus-oxygen double bond. This byproduct, phosphorus oxycloride, in the presence of chlorine gas also appears to increase the yield of chlorinated pyridines. Careful control of the temperature of this preparation is necessary for good yields of the product, 2,3,5,6-tetrachloropyridine [XIII]. If the reaction is kept at 270° C. for 24 hours the yield of 2,3,5,6-tetrachloropyridine [XIII] is 61 %. When the mixture is allowed to react for more than 24 hours, additional chlorination of the pyridine ring occurs leading to the formation of pentachloropyridine in a 25 % yield.

Pentachloropyridine can be converted to 2,3,5,6-tetrachloropyridine [XIII] by reductive dechlorination (149,152,153). This is facilitated by zinc metal in an aqueous HCl solution. At 110° C. 95 % of the pentachloropyridine is converted to 2,3,5,6-tetrachloropyridine [XIII] (153).

Similar to the reaction of glutarimide with phosphorus pentachloride, 2,6-dichloropyridine can be reacted with a Lewis acid ($Al_2Cl_3$, $SnCl_2$, or $FeCl_3$) and chlorine gas at high temperatures in a melt, to produce 2,3,5,6-tetrachloropyridine [XIII] (154-157). The small amount of the Lewis acid catalyst required for the conversion of 2,6-dichloropyridine is contradictory to the so called swamping catalyst effect that was reported by Pearson *et al.* (158). In the presence of a Lewis acid, the lone pair of electrons on the nitrogen heteroatom is presumed to associate with the Lewis acid (158). Then upon chlorination, and the release of a proton from the pyridine ring a second pyridine ring is protonated at the nitrogen and $AlCl_4^-$ associates with

it as a conjugate base. Since this salt complex is not capable of chlorination, low yields can only be obtained. It appears that the catalyst need not be present in high concentrations (154-158) and perhaps this is what diminished the yields obtained by Pearson *et al.* (158).

Another preparative route for the formation of 2,3,5,6-tetrachloropyridine [XIII] is the chlorination of 2,6-diaminopyridine at the 3 and 5-positions followed by the bis-diazotation of the structure with sodium nitrite and HCl (159). However, Chen and Flowers (159) did not furnish a specific procedure for this preparation. Yields are reported to be around 45-65 % of tetrahalopyridine by this method.

The compound 3,5,6-trichloro-2-pyridinol [VIII], which can be obtained by chlorination of the 3,5-positions of 6-chloro-2-pyridinol ($H_2O_2$ 30%, HCl conc.) (160) can be converted to 2,3,5,6-tetrachloropyridine [XIII]. This is accomplished by treatment of 3,5,6-trichloro-2-pyridinol [VIII] with phosphorus pentachloride in a phosphorus oxychloride solution, under pressure (160). Once again, the phosphorus pentachloride will provide chlorine atoms as it forms the especially stable phosphorus-oxygen bond.

The previously mentioned syntheses (144,145), which require 2,3,5,6-tetrachloropyridine [XIII] to produce triclopyr [VII], are described in patents held by the Dow Chemical Company (144,145). One method is a two step procedure which requires the mixing of 2,3,5,6-tetrachloropyridine [XIII] with paraformaldehyde and an alkali-metal cyanide. This reaction (144) must initially proceed in an aprotic environment, to prevent the formation of HCN. The second step is the hydrolysis of the resulting 3,5,6-trichloro-2-pyridyloxyacetonitrile [XIV] to triclopyr [VII].

The other preparation (145) is the reaction of methyl hydroxyacetate with 2,3,5,6-tetrachloropyridine [XIII] to form the methyl ester of triclopyr [XV] which can then be hydrolyzed with dilute base or acid. Both of these forementioned methods are reported to give excellent yields. But both methods are, however, dependent on the formation of 2,3,5,6-tetrachloropyridine [XIII], which in the laboratory at least appeared to be expensive in terms of time and labour.

## 1.8 Analysis of Triclopyr [VII]

### 1.8.1 Extraction and Cleanup

It was previously mentioned that triclopyr [VII] is structurally similar to 2,4-D [I]. Consequently, the extraction and analysis of triclopyr [VII] can be based on methods used in the analysis of 2,4-D [I]. The proposed metabolite of triclopyr [VII], 3,5,6-trichloro-2-pyridinol [VIII], is also a metabolite of chlorpyrifos [XI]. Therefore, methods for the extraction and analysis of 3,5,6-trichloro-2-pyridinol [VIII] can be based on procedures for chlorpyrifos [XI] investigations.

The nature of the medium in which the herbicide is found, influences the cleanup and extraction technique. Therefore, available literature methods for these analysis must be modified to accommodate the uniqueness of the sample. Some factors that can influence cleanup and extraction procedures include the pH, and matrix of the sample and also the chemical characteristics of other compounds that require extraction. Sample

pH may require adjustment to ensure the optimum extraction of compounds that may be present in the sample in their ionic form (*i.e.* as salts). The composition of the sample will govern whether cleanup is required, since the coextraction of interfering substances is often a problem with soil and biological samples. Also, some sample components (for example soils) can strongly bind polar and ionic compounds, making pH adjustment and solvent selection very critical to ensure good recovery of the test compound. If more than one compound is to be extracted from a sample, then the characteristics of all molecular species must also be considered to ensure good recovery.

The extraction of 2,4-D [I] has often been performed with diethyl ether (161-163). Other extraction methods use saturated calcium hydroxide solutions for the removal of 2,4-D [I] adsorbed on soils (164) and acidified acetone solutions for the removal of 2,4-D [I] from citrus fruits (162).

An effective cleanup procedure for 2,4-D [I] is the use of macroreticular adsorption resins (164-167) or gel permeation techniques (168). Adsorption resins such as the Amberlite XAD series are small beads which swell in the presence of various solvents. The cavities can then selectively retain molecules of appropriate size until a suitable solvent is used to elute them. Success with this system seems variable. Good recoveries are found mainly with samples that contain high concentrations of 2,4-D [I] (164). Florisil, a combination of silicagel and alumina, is used for gel permeation cleanup procedures (168). This method is particularly useful for the separation of herbicides (and other organochlorine compounds) from lipids. The basis of the separation is the difference in molecular size of the

compared to reference chromatograms. Also the separated compounds can be removed from the chromatogram and redissolved for further analysis. By adjusting the polarity of the mobile phase (solvent mixtures) separation of species can be easily achieved.

Thin layer chromatography has also been used to measure concentrations of chlorpyrifos [XI] and 3,5,6-trichloro-2-pyridinol [VIII] in tap water and bananas (176). Quantitation of the two compounds was performed with a densitometer after development with ammoniacal silver nitrate solution.

A considerable amount of literature has been published on the application of gas chromatographic techniques for herbicide analysis. Since resolution and detection of complex mixtures is achievable, it has been applied to synthetic work and metabolism studies. In order to accurately quantitfy compounds by gas chromatography, resolution of the peaks is critical. Also few columns available for gas chromatography are capable of allowing the elution of organic acids of high molecular weight. Both of the forementioned problems are alleviated by derivatization of the polar structures to produce an alkyl or silylester in the case of organic acids and ethers in the case of organic hydroxyl groups (170-173, 177-186). The resulting derivatives of the organic acids and phenols are more volatile and are less strongly retained in the liquid (stationary) phase, thereby allowing for greater resolution of the compounds. Derivatization techniques can also be used to increase the sensitivity of the method. When electron capture is used as the detection system it is sometimes beneficial to esterify the acid with a halogenated alcohol to raise the detection sensitivity (183-185). Many

alkyl and silyl derivatization techniques have been applied to the analysis of 2,4-D [I] and other phenoxy herbicides. Khan (183), Cochrane (184) and Siltanen and Mutanen (185) have all written extensive reviews of gas chromatographic applications in herbicide analysis.

An analytical tool which may have a major impact on future studies of biological compounds is $^{13}C$ NMR. Historicaly, the early development of nuclear magnetic resonace spectroscopy was limited to proton spectra. The development of more sensitive machines and the manipulation of various NMR techniques has allowed for the elucidation of spectra from other elements. $^{31}P$ NMR is now used on a wide spread basis to study intracellular metabolic processes *in vitro* and *in vivo* This is possible through the use of surface coils and the recent improvements in magnets used to produce the field. The ability of $^{31}P$ NMR to distinguish inorganic phosphorus from adenosine triphosphate and adenosine diphosphate makes it particularly useful for the study of intracellular energy transfer processes.

$^{13}C$ NMR has been limited somewhat by the low natural abundance (*Ca.* 1%) of the isotope. Although this low isotopic abundance indicates that sample concentrations must be high for analysis, it is also useful since the signals from proton-carbon coupling are almost negligible relative to the $^{13}C$ signal. The lack of proton-carbon signals and the intentional proton decoupling makes $^{13}C$ NMR spectrum very simple to interprete.

Two routes can be used for the study of metabolic products with $^{13}C$ NMR. The first is based on the natural abundance of the $^{13}C$ isotope, which may require a fairly high concentration of the material to be studied and can

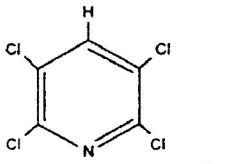
be subject to some interference. The second route is based on enrichment with labeled compounds which require parent compounds (starting materials) that are enriched with $^{13}C$. This latter route is more useful for non invasive metabolic studies as the signal generated by the enriched materials is considerably stronger than the non enriched background. The cost of $^{13}C$ enriched reagents is a major drawback in these experiments.

Some recent applications of $^{13}C$ NMR are the pharmaceutical quality control of the antibiotic fungal metabolite gentamicin (187,188), glycosylated protein structure studies (189), and *in vivo* and *in vitro* cellular biosynthetic pathways (190-198). Some metabolism studies pursued by investigators include tryptophan biosynthesis by a genetically modified *E.coli.* (190), yeast chemotaxonomy (192), glycogen synthesis in rat liver (193), glucose metabolism in bacterium (196) and guinea pig brain slices (197), and liver regeneration in rats after injury (198).

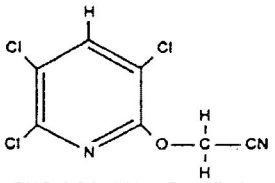
Other recent work with $^{13}C$ NMR has included the correlation of the chemical shift parameter to carcinogenicity for chlorinated aromatic compounds (199). This is a novel method for the prediction of carcinogenicity. Average chemical shifts for the entire ring structure are used to predict whether a compound is a carcinogen or not. Isomer elucidation for polychlorinated pyridines has also been reported (200). Substituted pyridines have been studied to predict chemical shift patterns (201-204). These chemical shift experiments are providing information which will allow better mathematical predictions of chemical shifts in aromatic ring systems.

Chemical shift comparisons, of both literature data and experimental data, are quite difficult. Caution must be observed to ensure that all pertinent

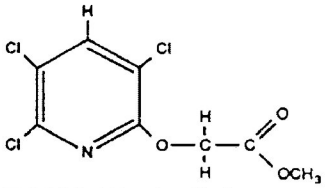
operating parameters are equal, otherwise the chemical shift data will not be reproducible. Bremser and Fachinger (205) have presented a formula which determines the uncertainty factor as an arbitrary number. Some of the parameters which these authors (205) consider important are: solvents, instruments, relaxation times, proton couplings and heterocouplings.



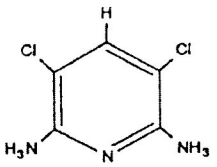
[XIII] 2,3,5,6-tetrachloropyridine



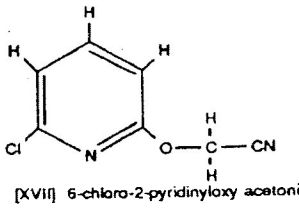
[XIV] 3,5,6-trichloro-2-pyridinyloxy acetonitrile



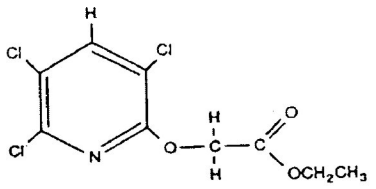
[XV] 3,5,6-trichloro-2-pyridinyloxy methyl acetate Triclopyr-methyl ester



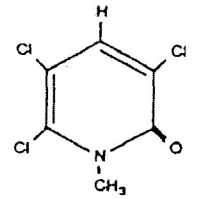
[XVI] 2,6-diamino-3,5-dichloropyridine



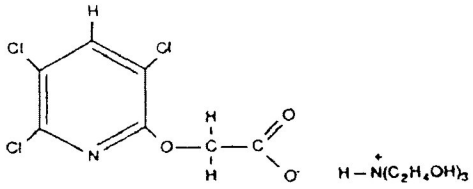
[XVII] 6-chloro-2-pyridinyloxy acetonitrile



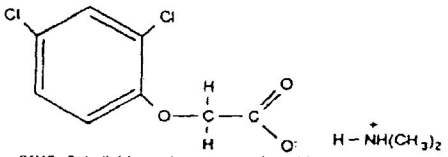
[XVIII] 2,5,6-trichloro-2-pyridinyloxy ethyl acetate



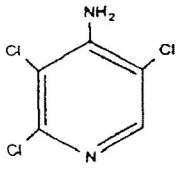
[XIX] N-methyl-3,5,6-trichloro-2-pyridone



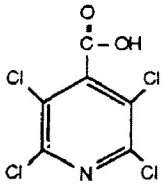
[XX] Triclopyr-triethanolamine Salt



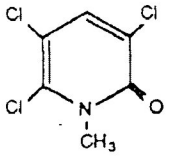
[XXI] 2,4-dichloro phenoxy acetic acid
2,4-D Dimethylamine salt



[XXII] 4-amino-3,5,6-trichloro pyridine
Decarboxy Picloram



**[XXIII] Tetrachloroisonicotinic acid**



**[XXIV] N-methyl-3,5,6-trichloro-2-pyridone**

# CHAPTER 2 SYNTHETIC CHEMISTRY

## 2.1 Introduction

The synthetic routes used in the production of 2,4-D [I] have been discussed in Section 1.7.1. This section deals with the adaptation of some of these syntheses for the laboratory preparation of triclopyr [VII]. Also, 2,3,5,6-tetrachloropyridine [XIII] was prepared to investigate the synthetic routes which make use of this precursor to produce triclopyr [VII] (refer to page 33).

## 2.2 Synthesis of 3,5,6-trichloro-2-pyridinol [VIII]

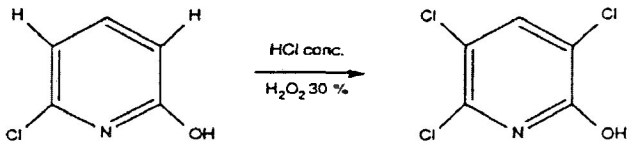
In order to follow the synthetic methods developed by Porknoy (123), which utilize 2,4-dichlorophenol [V] and 2,4,5-trichlorophenol [VI], 3,5,6-trichloro-2-pyridinol [VIII], which is not commercially available, had to be synthesized.

The method used to produce 3,5,6-trichloro-2-pyridinol [VIII] was similar to that reported by den Hertog (160 Scheme 2.1). 6-chloro-2-pyridinol is chlorinated with concentrated HCl and 30 % $H_2O_2$. Yields for this reaction varied from 11 % to 65 %. Since the reaction medium is strongly oxidizing, higher yields were obtained when the product was separated from the mixture without delay. In several attempts at this synthesis, glacial acetic acid was used to aid in the dissolution of the starting material, 6-chloro-2-pyridinol. The use of glacial acetic acid as a solvent was not worthwhile and

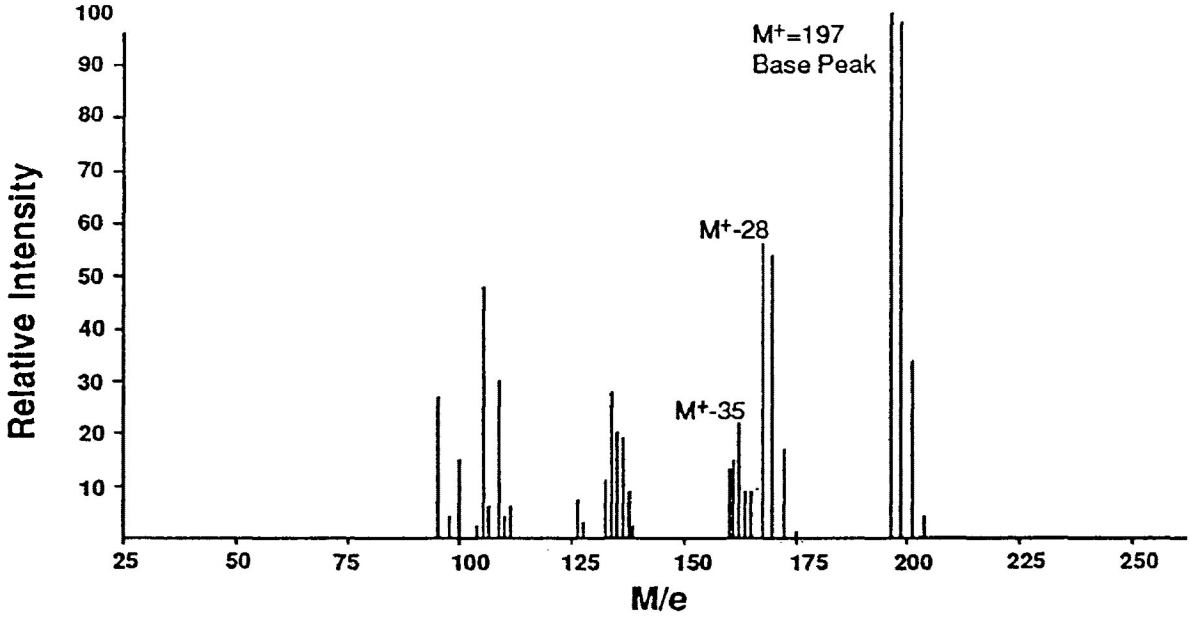
should be avoided, since pyridines can be converted to pyridine N-oxides in the presence of glacial acetic acid and hydrogen peroxide (138). Another point which may be important for obtaining high yields, is the sequence in which HCl and hydrogen peroxide are added. If the acid is kept in slight excess in the mixture then chlorine is formed spontaneously upon the addition of the hydrogen peroxide. Therefore, the oxidizing nature of the medium is minimized and side reactions are also controlled. Cooling the mixture below ambient temperature early in the reaction is also to be avoided. The formation of chlorine is inhibited by cooling, thus, when the mixture is brought to ambient temperature, the formation of chlorine proceeds vigorously, and is difficult to control.

The infrared spectrum of the product obtained with the above methods is identical to that of an authentic sample of 3,5,6-trichloro-2-pyridinol [VIII] supplied by the Dow Chemical Company, Midland Michigan. The proton NMR spectrum contains a single signal in the aromatic region at d = 7.8 ppm. The mass spectrum of the product (Figure 2.1) contains the molecular ion, which is also the base peak at M/e 197. This peak is clustered in a series of peaks which are spaced two atomic mass units apart, indicating the chlorine isotope pattern for three chlorine atoms is present. The probable loss of carbon monoxide yields another cluster of peaks centered at M/e 169 (Figure 2.1 [i]). This ion is likely a five membered heterocyclic ring with three chlorine atoms still present, as indicated by the isotope pattern. Another strong set of peaks is encountered at M/e 162 (Figure 2.1 [ii]) which indicates the probable loss of one chlorine radical.

Since the yield by this method was adequate, and required no labour intensive work up to isolate the product, there was no need to look for other methods for the pyridinol.




Scheme 2.1



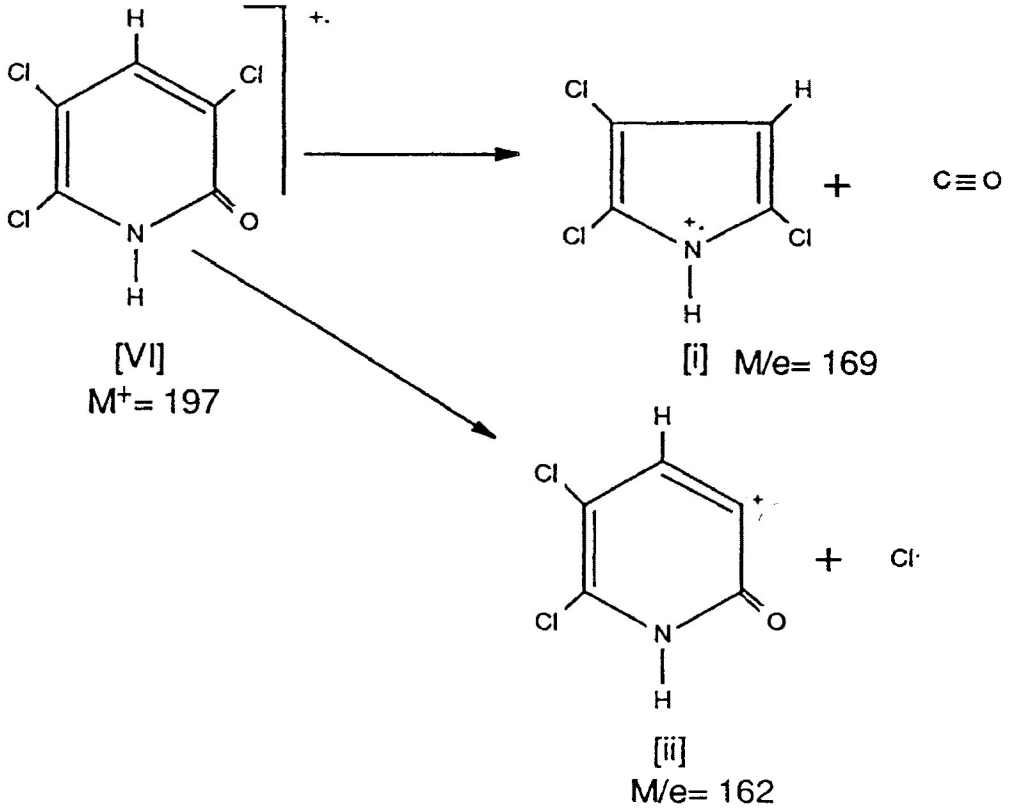




**Fig. 2.1 3,5,6-trichloro-2-pyridinol [VI] Mass Spectrum and Fragmentation Pattern**

## 2.3 Synthesis of Triclopyr [VII] from 3,5,6-trichloro-2-pyridinol [VIII]

Initial attempts at the preparation of triclopyr [VII] from 3,5,6-trichloro-2-pyridinol [VIII] and a-chloroacetic acid, via the $S_N2$ reaction mechanism analogous to Porknoy's synthesis of 2,4-D [I] (123), were unsuccessful (Scheme 2.2). When 3,5,6-trichloro-2-pyridinol [VIII] was mixed with an aqueous sodium hydroxide solution; dissolution was difficult, thereby decreasing the concentration of the reacting species in solution. No product was formed when either a slight excess or a ten times molar excess of a-chloroacetic acid was used. Refluxing in either case had no effect. It was suspected that the pyridone tautomer of 3,5,6-trichloro-2-pyridinol [VIII] was predominant. If this is the case, then the electron density at the oxygen on the ring is decreased. This decreased electron density lowers the ability of 3,5,6-trichloro-2-pyridinol [VIII] to attack the a-carbon on a-chloroacetic acid. Another problem may be the deactivation by the chlorine in position 3. Also, this chlorine may be hydrolyzed in the presence of base.

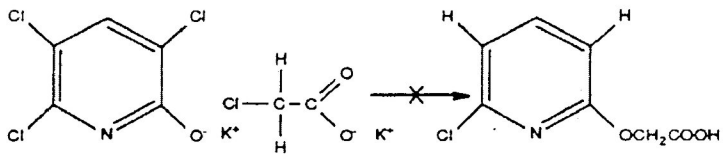
The reaction was then performed using di-n-butyl ether as a solvent. An attempt with this solvent was undertaken since $S_N2$ reactions proceed best in non polar medium. This reaction yielded no product.

Since the 3,5,6-trichloro-2-pyridinol [VIII] structure is almost certainly deactivated with respect to the proposed $S_N2$ reaction, 6-chloro-2-pyridinol was used as a starting material. As discussed in the introduction, the chlorine in the 6-position may decrease the influence the heteroatom has on forming the pyridone tautomer. In three attempts, using di-n-butyl ether,

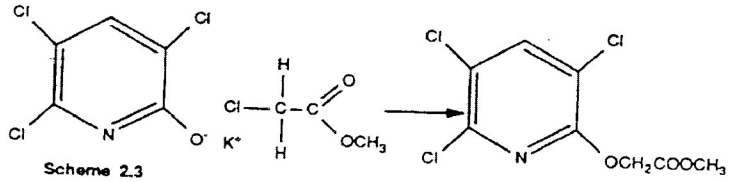
methanol, and dimethyl sulphoxide (DMSO) respectively as the solvent, trace amounts of the product were formed as determined by gas chromatography. Potassium hydroxide was used as the base for these preparations. The reaction of chloroacetic acid with 6-chloro-2-pyridinol was then attempted, using sodium bicarbonate as the base and acetone as the solvent. Again, the yields were poor. It was then suspected that the reacting species, chloroacetic acid (as a salt), is too electronically repulsive to react with 3,5,6-trichloro-2-pyridinol [VIII] or 6-chloro-2-pyridinol.

To alleviate the electronic repulsion caused by the reacting anions, methyl monochloroacetate was used instead of chloroacetic acid. This preparation (Scheme 2.3) was similar to that of Morris and Bonhert (130). Using dipotassium carbonate as the base, a yield of 53 % was achieved. Proton NMR indicated the product was methyl-3,5,6-trichloro-2-pyridinyloxyacetate [XV]. In hexadeuteroacetone a single signal at d = 3.75 ppm (integrating as 3 protons) accounted for the methyl ester. At d = 5.05 ppm the methylene protons (two protons) were found as a single signal . The single aromatic proton gave a signal at d = 8.15 ppm . The mass spectrum of the product also confirmed its structure as that of methyl-3,5,6-trichloro-2-pyridinyloxyacetate (XV, Figure 2.2) and gave the molecular ion at 269 (isotope pattern for three chlorine atoms present). A signal at M/e 210 (ion i in Figure 2.2, $M^+$ -59) indicated the loss of the carboxymethyl group. The base peak at $M^+$ -210 indicates this fragment is favored when the carboxy methyl ester is the positively charged ion and the ring structure with the methylene ether side group is the radical (*i . e .* structure i in Figure 2.2 but

with the charge on the $CO_2CH_3$ fragment). The second major fragmentation is the loss of the ether group from the pyridine ring (M/e=180, ii, Figure 2.2).



Scheme 2.2



Scheme 2.3

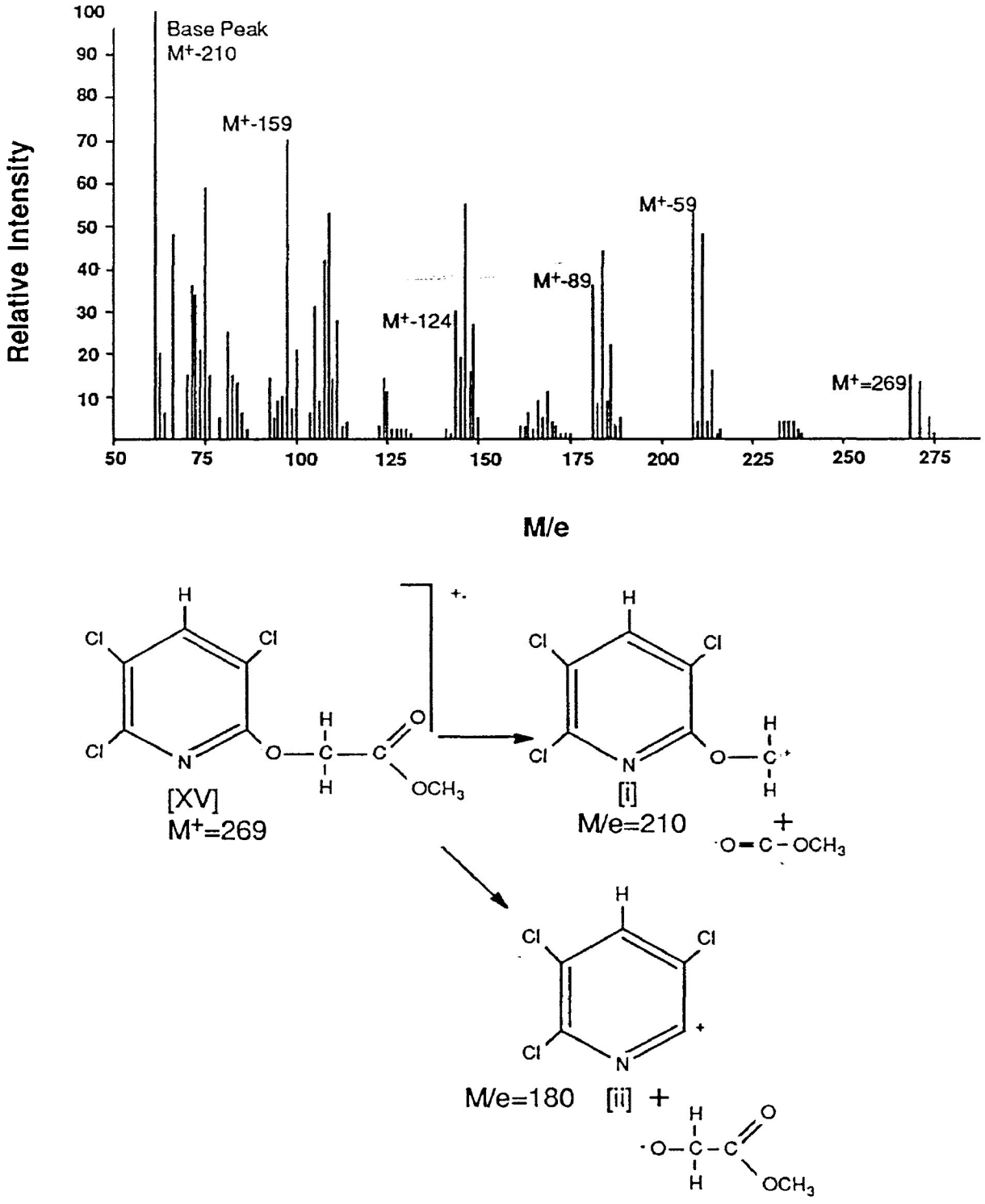



**Fig. 2.2 Triclopyr-methyl ester [XV] Mass Spectrum and Fragmentation Pattern**

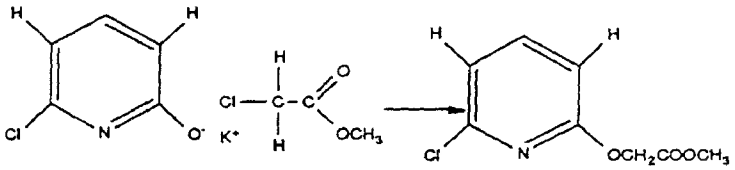
## 2.4 Synthesis of Triclopyr [VII] from 6-chloro-2-pyridinol [XV]

The monochloro analogue of methyl-3,5,6-trichloro-2-pyridinyloxyacetate [XV], methyl-6-chloro-2-pyridinyloxyacetate [XVI] was synthesized in an attempt to improve the yield of the the coupling reaction (Scheme 2.4). If the yield of the coupling reaction was significantly higher using a monochloro substituted ring, then chlorination afterwards could yield more triclopyr ester. Following the same procedure used to produce methyl-3,5,6-trichloro-2-pyridinyloxyacetate [XV], a 55 % yield of methyl-6-chloro-2-pyridinyloxyacetate [XVI] was obtained. This indicated that the reaction conditions and the degree of ring chlorination had no significant effect. Indeed, the reason for the inhibition of the reactions using chloroacetic acid were probably due to the electronic repulsion of the two reacting anions.

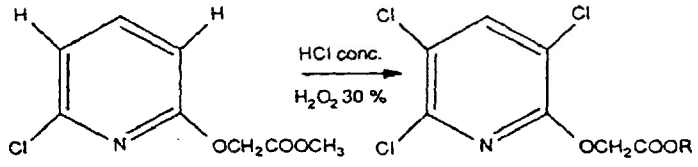
Proton NMR confirmed the product as methyl-6-chloro-2-pyridinyloxyacetate [XVI], with the methoxy signal at 3.75 ppm and the methylene signal at 4.8 ppm. The three adjacent aromatic protons gave three distinct doublets (all three protons are in different environments) $\delta$ = 7.7, 7.6, and 7.5. The mass spectrum (Figure 2.3) contained the molecular ion at M/e = 201, with the isotope pattern expected for one chlorine atom. Fragmentations followed patterns similar to those encountered for methyl-3,5,6-trichloro-2-pyridinyloxyacetate [XV] (See Figure 2.2). One fragmentation which was not observed in the mass spectrum of methyl-3,5,6-trichloro-2-pyridinyloxyacetate [XV] (Figure 2.2) was the four center

rearrangement to produce ion [ii] in Figure 2.3. Also, ion [i] in Figure 2.3 (which is a particularly stable cation that is encountered frequently in mass spectroscopy) was observed at M/e = 170.

As previously mentioned, the monochloro substituted pyridinol was used in an attempt to improve the yield of methyl-3,5,6-trichloro-2-pyridinyloxyacetate [XV] by chlorinating after the ether was formed. Therefore, chlorination of the compound methyl-6-chloro-2-pyridinyloxyacetate [XVI] was attempted (Scheme 2.5). By using the same procedure used to chlorinate 6-chloro-2-pyridinol an unsatisfactory yield of 25 % was obtained. The crude product was as a mixture containing triclopyr [VII] and methyl-3,5,6-trichloro-2-pyridinyloxyacetate [XV] along with unreacted methyl-6-chloro-2-pyridinyloxyacetate [XVI] and 6-chloro-2-pyridinlyoxyacetic acid. Silica gel column chromatography allowed separation of the mixture. The triclopyr [VII] fraction was esterified with boron trichloride and methanol, then combined with the methyl-3,5,6-trichloro-2-pyridinyloxyacetate [XV] fraction to give the 25 % yield. The proton NMR spectrum of this material was identical to that of methyl-3,5,6-trichloro-2-pyridinyloxyacetate [XV]. Clearly, chlorination after the formation of the ether side group is not a useful procedure.



Scheme 2.4




Scheme 2.5

R = H, $CH_3$

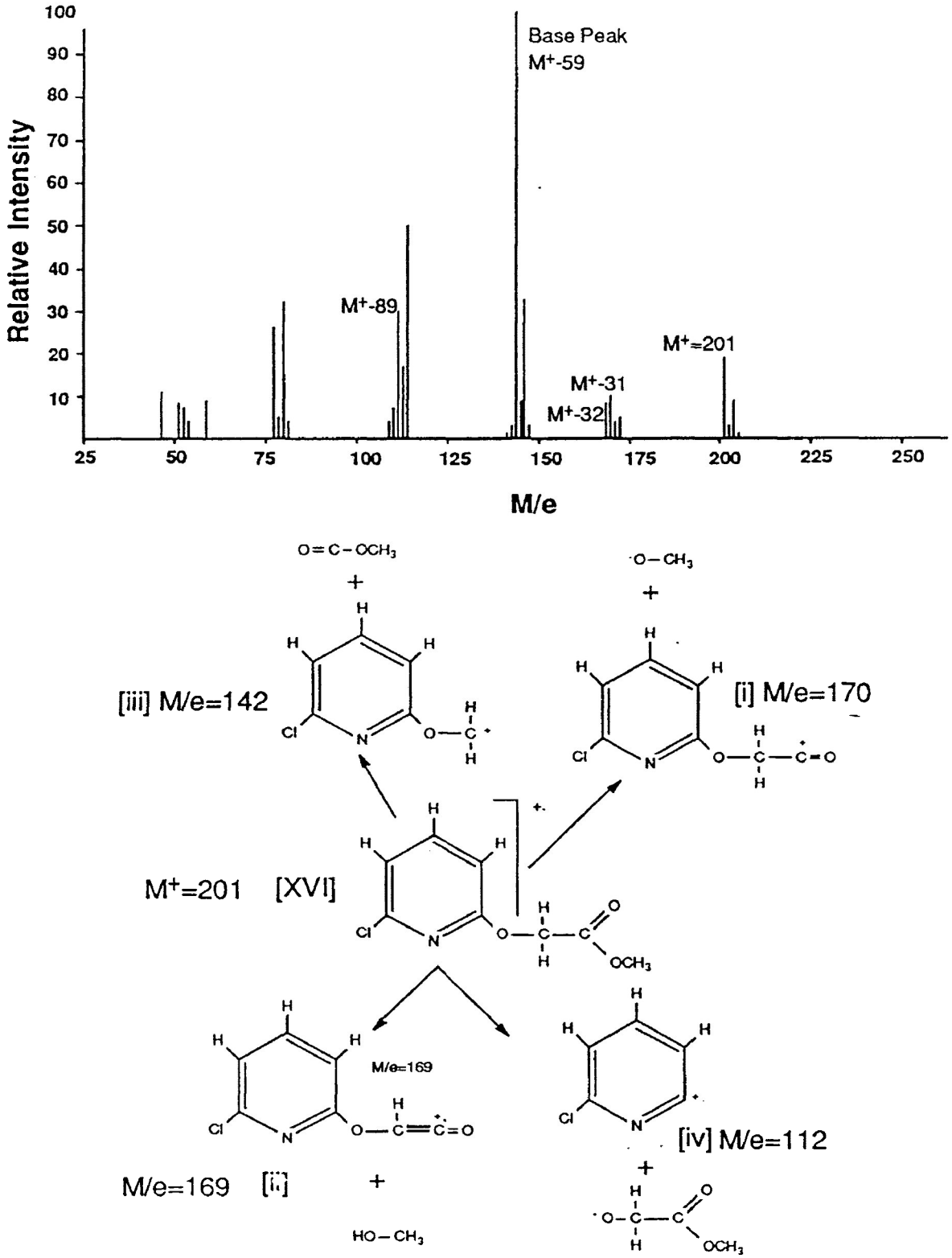



Fig. 2.3 Methyl-6-chloro-2-pyridinyloxyacetate [IX] Mass Spectrum and Fragmentation Pattern

## 2.5 Synthesis of 2,3,5,6-tetrachloropyridine [XIII]

Synthesis of triclopyr [VII] via the Dow Chemical Company patent method which requires 2,3,5,6-tetrachloropyridine [XIII] (144) was attempted. In order to prepare triclopyr [VII] by this method a supply of 2,3,5,6-tetrachloropyridine [XIII], which is not commercially available, was required. The various synthetic methods currently available for the preparation of 2,3,5,6-tetrachloropyridine [XIII] are disscussed in Section 1.7.2.

Various sources of chlorine were used in the first attempts at synthesizing 2,3,5,6-tetrachloropyridine [XIII]. 2,6-dichloropyridine was mixed with hydrogen peroxide (30 %) and concentrated HCl (Scheme 2.6). Only starting material was recovered after heating the mixture. N-chlorosuccinamimide and benzoyl peroxide were also used as a source of chlorine (Scheme 2.6). Refluxing 2,6-dichloropyridine with n-chlorosuccinamimide and benzoyl peroxide in carbon tetrachloride yielded trace amounts of a compound presumed to be 2,3,6-trichloropyridine as determined by gas-liquid chromatography. Since only trace amounts were formed after refluxing the mixture in carbon tetrachloride for 48 hours, gas chromatographic parameters were the only indication of the increased substitution of chlorine in the ring.

In a next attempt at producing 2,3,5,6-tetrachloropyridine [XIII], 3,5,6-trichloro-2-pyridinol [VIII] was refluxed in carbon tetrachloride with phosphorus pentachloride as the source of chlorine (Scheme 2.6). Once again only starting material was recovered. These three reactions were

carried out in an attempt to avoid the use of pressurized vessels such as the sealed tube reactions mentioned in Section 1.7.2 (146-151).

Since the three previous reactions were unsuccesful in the production of 2,3,5,6-tetrachloropyridine [XIII], the method initially reported by den Hertog (160) was attempted. 3,5,6-trichloro-2-pyridinol [VIII] was allowed to react with phosphorus pentachloride in a Carius tube which contained phosphorus oxychloride (Scheme 2.6). In contrast to Den Hertog's report (160), the desired product was not obtained.

One preparation which appeared to be convenient, for the synthesis of 2,3,5,6-tetrachloropyridine, is the chlorination of 2,6-diaminopyridine followed by the bis-diazotization of the 2,6-amino groups (159). This method (159) was attempted several times (Scheme 2.7). The preparation led to the formation of 3,5-dichloro-2,6-diaminopyridine [XVI] in a 24 % yield. The product was analyzed by proton NMR and mass spectroscopy. The proton NMR spectrum indicated that there was only one aromatic proton remaining. The mass spectrum contained the molecular ion at M/e = 177 (Figure 2.4). The molecular ion was, in fact, a group of three peaks as would be expected from two chlorine atoms. A second peak is observed at M/e= 141 (ion i, Figure 2.4). This is probably due to the formation of HCl. The bis-diazotization step of the reaction was then carried out on a portion of the isolated material (Scheme 2.7). The Sandmeyer reaction yielded 11 % tetrachloropyridine.

Chen and Flowers (159) reported that yields in this reaction are improved when the two steps are carried out in the same vessel without isolation of the intermediate3,5-dichloro-2,6-diaminopyridine. The reported

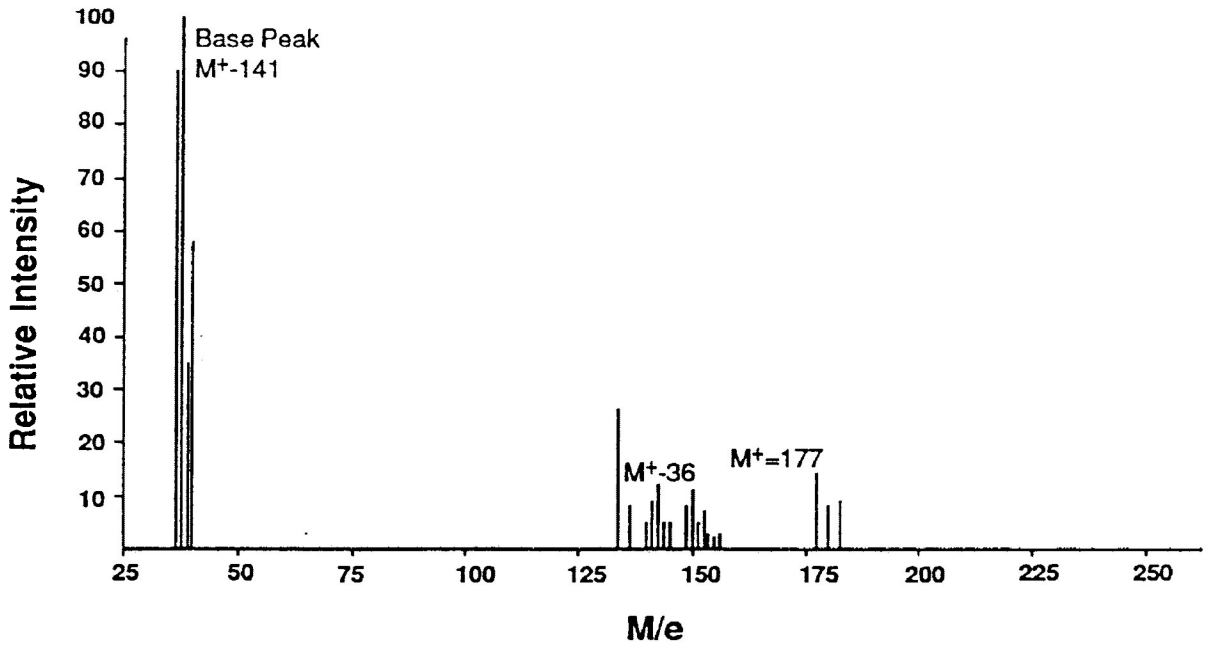
yield (159) for 2,3,5,6-tetrachloropyridine [XIII] by this preparation is 45 % but in preparations attempted in this way only tars were produced. After the reaction of the starting material 2,6-diaminopyridine with chlorine (produced from HCl and $H_2O_2$, or chlorine gas bubbled into the solution), and the neutralization of the acid with base (NaOH or $NaHCO_3$) a tarry mass precipitated. This may be due to the loss of chlorine from the ring caused by the base. Chen and Flowers (159) did not elaborate on the method, nor did they mention possible byproducts. This procedure was therefore considered unsuitable and the method was not further pursued.

The reaction of 2,6-dichloropyridine with chlorine gas and a Lewis acid (154-157) was also attempted (Scheme 2.8).The starting material was melted by heating to 180 ° and after the addition of the Lewis acid ($AlCl_3$) and a crystal of iodine the mixture was stirred vigorously in an atmosphere of chlorine gas. After 22 hours, gas chromatography of an aliquot of the mixture indicated that the dichloropyridine was slowly converted into a higher molecular weight compound. After 58 hours a significant portion of the dichloro compound had been converted to what was suspected to be trichloropyridine. After 72 hours gas chromatography and thin layer chromatography (silica gel) indicated that three compounds were present. This reaction was repeated several times with the same results. Yields are dependent on the length of time the reaction is allowed to proceed. Almost all of the material is recovered and easily separated using silica gel columns with hexane as the solvent. The material which has not reacted, or only partially reacted (trichloropyridine), can then be reused in subsequent reactions.

The 2,3,5,6-tetrachloropyridine [XIII] was analyzed by mass spectroscopy and $^{13}C$ NMR. The mass spectrum had a molecular ion at M/e=215 (Figure 2.5). The ion (i, Figure 2.5), at M/e=180 is thought to be due to the loss of one chlorine radical from the ion [XIII]. A second fragmentation is the suspected loss of two chlorine atoms to give ion ii at M/e=145 this cannot be proven since a metastable peak was not found. The isotope pattern for a structure with four chlorine atoms is found around the molecular ion, the pattern for three chlorine atoms is found at the first fragment (M/e = 180, 182, 184, 186) and the pattern for two chlorine atoms is found at the second fragment (M/e = 145, 147, 149).

$^{13}C$ NMR is useful for the elucidation of isomers of halogenated pyridines. By comparing the experimental chemical shifts to literature values (200) the isomers present can be recognized. For symmetrical tetrachloropyridine the chemical shifts in the $^{13}C$ NMR spectrum are expected to be 145.9, 129.6, 140.0, 129.6 and 145.9 ppm for C-2 through C-6 respectively (200). The observed spectrum of the obtained product contained signals 146.2, 129.9, 140.4, 129.9 and 145.9 ppm for C-2 through C-6 respectively indicating that the compound obtained was of high purity with no other isomers present.

The results of the above attempts show that 2,3,5,6-tetrachloropyridine [XIII] is difficult to obtain. The most succesfull route *i . e.* the chlorination of 2,6-dichloropyridine (154-157) is also the longest in terms of reaction time and it requires the handling of chlorine gas with careful monitoring to control the gas flow. In terms of separation and purification the most succesfull method furnishes a crude product which is easily purified.



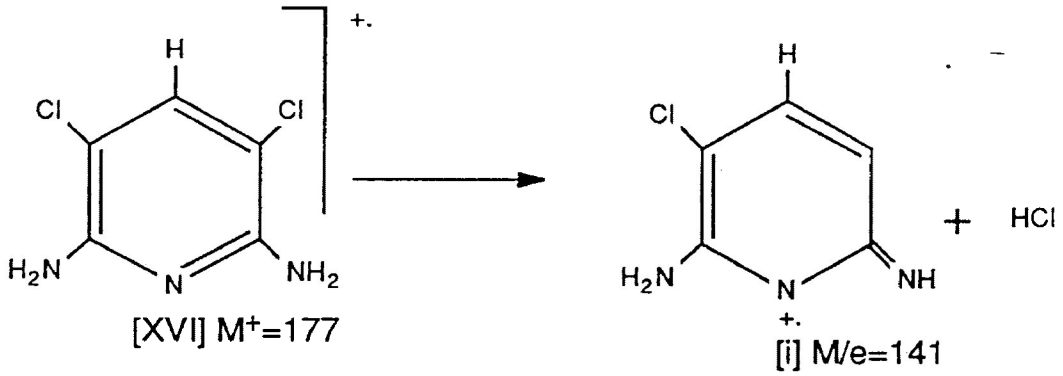
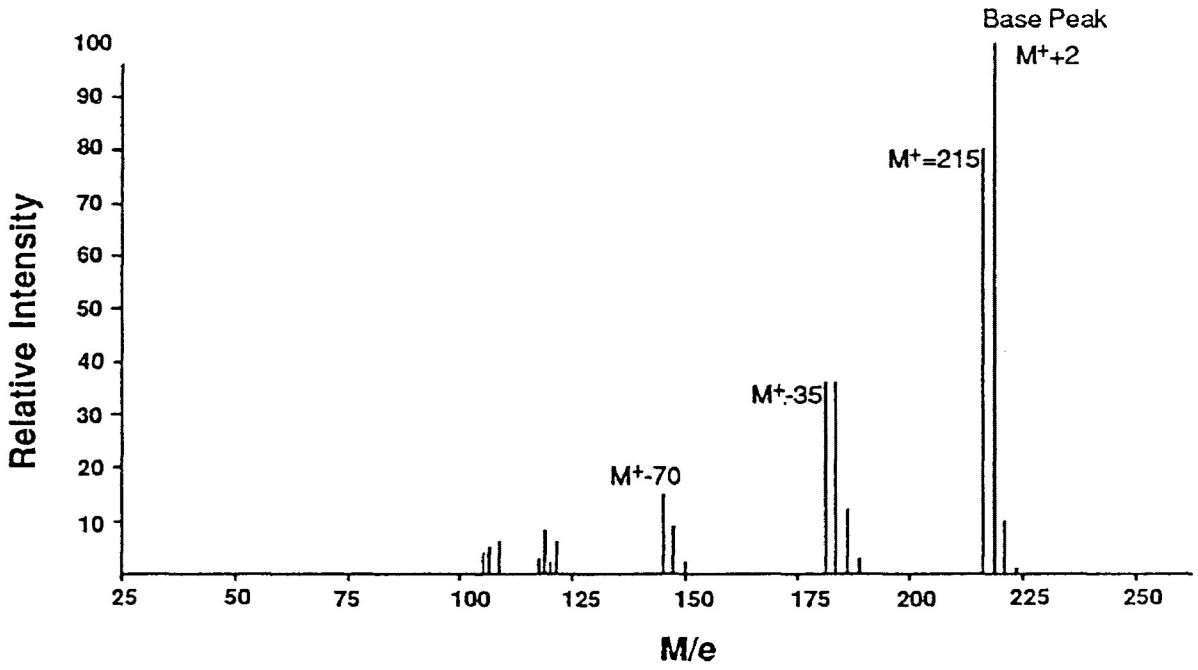




Fig. 2.4 2,6-diamino-3,5-dichloropyridine [XVI] Mass Spectrum and Fragmentation Pattern



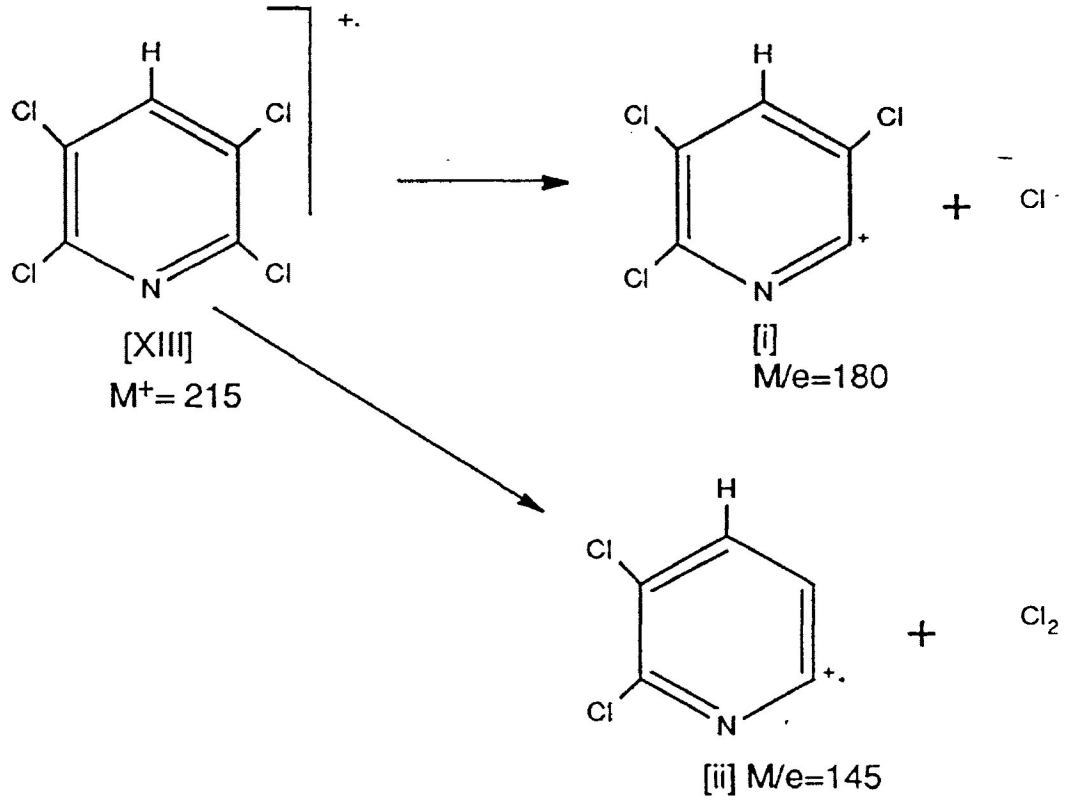
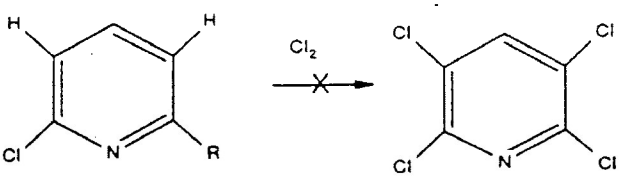



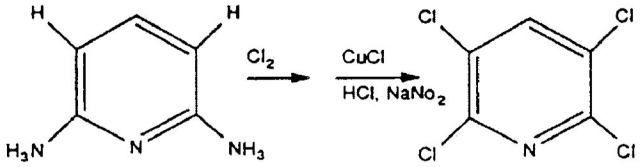

**Fig. 2.5 2,3,5,6-tetrachloropyridine [XIII] Mass Spectrum and Fragmentation Pattern**




R = Cl :
$Cl_2$ from: HCl (conc.), $H_2O_2$
N-chlorosuccinamide, benzoyl peroxide

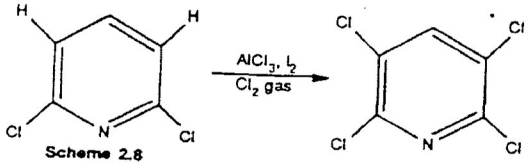
R = OH :
Chlorine from: $PCl_5$ (reflux)
$PCl_5$ (sealed tube)

**Scheme 2.6**




$Cl_2$ from: HCl (conc.) $H_2O_2$
$Cl_2$ gas

**Scheme 2.7**




**Scheme 2.8**

## 2.6 Synthesis of Triclopyr [VII] from 2,3,5,6-tetrachloropyridine [XIII]

2,3,5,6-tetrachloropyridine [XIII] is required for the synthesis of triclopyr [VII] via the Dow patent method (United States Patent 3,862,952 January 28, 1975) (144). This method which is designed for industrial

purposes, was pursued to determine if it was suitable for laboratory scale preparations and to compare the yield obtained in the laboratory with the reported yield (144).

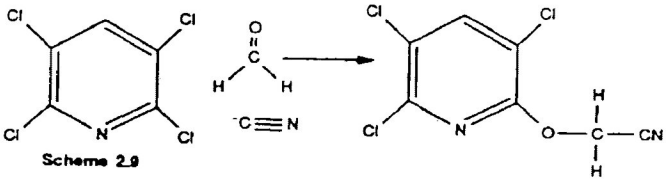
From 2,3,5,6-tetrachloropyridine [XIII], triclopyr [VII] is prepared by first forming 3,5,6-trichloro-2-pyridinlyoxyacetonitrile [XIV] (144) (Scheme 2.9). This is easily acomplished by stirring 2,3,5,6-tetrachloropyridine [XIII] with paraformaldehyde and then slowly adding sodium cyanide. The solvent used must be aprotic (DMSO). When the reaction was carried out on a lab scale the yield of 3,5,6-trichloro-2-pyridinlyoxyacetonitrile [XIV] was found to be 70 %.

Analysis of the product by proton NMR and mass spectroscopy confirmed the identity of the product. The proton NMR spectrum had a single signal at $\delta$ = 8.23 ppm from the aromatic proton, along with a signal at $\delta$ = 5.33 ppm (two protons), which can be assigned to the methylene group adjacent to the nitrile. The mass spectrum of the product (Figure 2.6) confirmed the molecular ion ($M^+$=236) and a fragment at M/e=210 (ion i, Figure 2.6) indicating the loss of a cyanide radical. A second fragment at M/e=180 (ion ii, Figure 2.6) is likely due to the loss of the entire side chain including the ether oxygen or the los of formaldehyde from (i). Infrared spectroscopy proved to be not convincing as a method for determining of the presence of the nitrile group, since the C-N stretching vibration is not strongly absorbing when this group is is ß to an ether group (209).

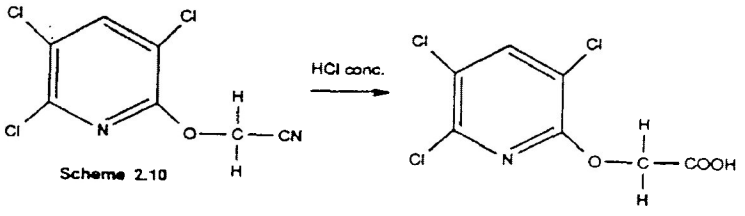
From the nitrile, triclopyr [VII] is prepared by hydrolyzing the nitrile group with concentrated HCl (Scheme 2.10). After five hours of stirring 3,5,6-trichloro-2-pyridinlyoxyacetonitrile [XIV] with concentrated HCl a yield of 81

% of triclopyr [VII] was obtained. Thus an overall yield of about 58 % was obtained calculated on the basis of 2,3,5,6-tetrachloropyridine [XIII].

The resulting triclopyr [VII] was analysed by proton NMR and mass spectroscopy. The proton signals are $\delta$ = 8.1 ppm for the aromatic proton, $\delta$ = 5.0 ppm for the two equal methylene protons and $\delta$ = 2.75 ppm for the broad signal caused by the acidic proton. The mass spectrum contained an observable molecular ion at M/e = 255 (Figure 2.7). The first fragment [I] at M/e=237 (Figure 2.7 ) indicates the loss of a water molecule from the carboxyl and methylene groups. The second fragment [ii] is at M/e = 209 (Figure 2.7) and is likely due to the loss of carbon monoxide and water. No metastable peak was found to confirm this mechanism. The third fragmentation is the loss of the side chain to give the ion [iii] at M/e=180 (Figure 2.7). The isotope pattern required for three chlorine atoms was present for all three fragments.



Scheme 2.9




Scheme 2.10

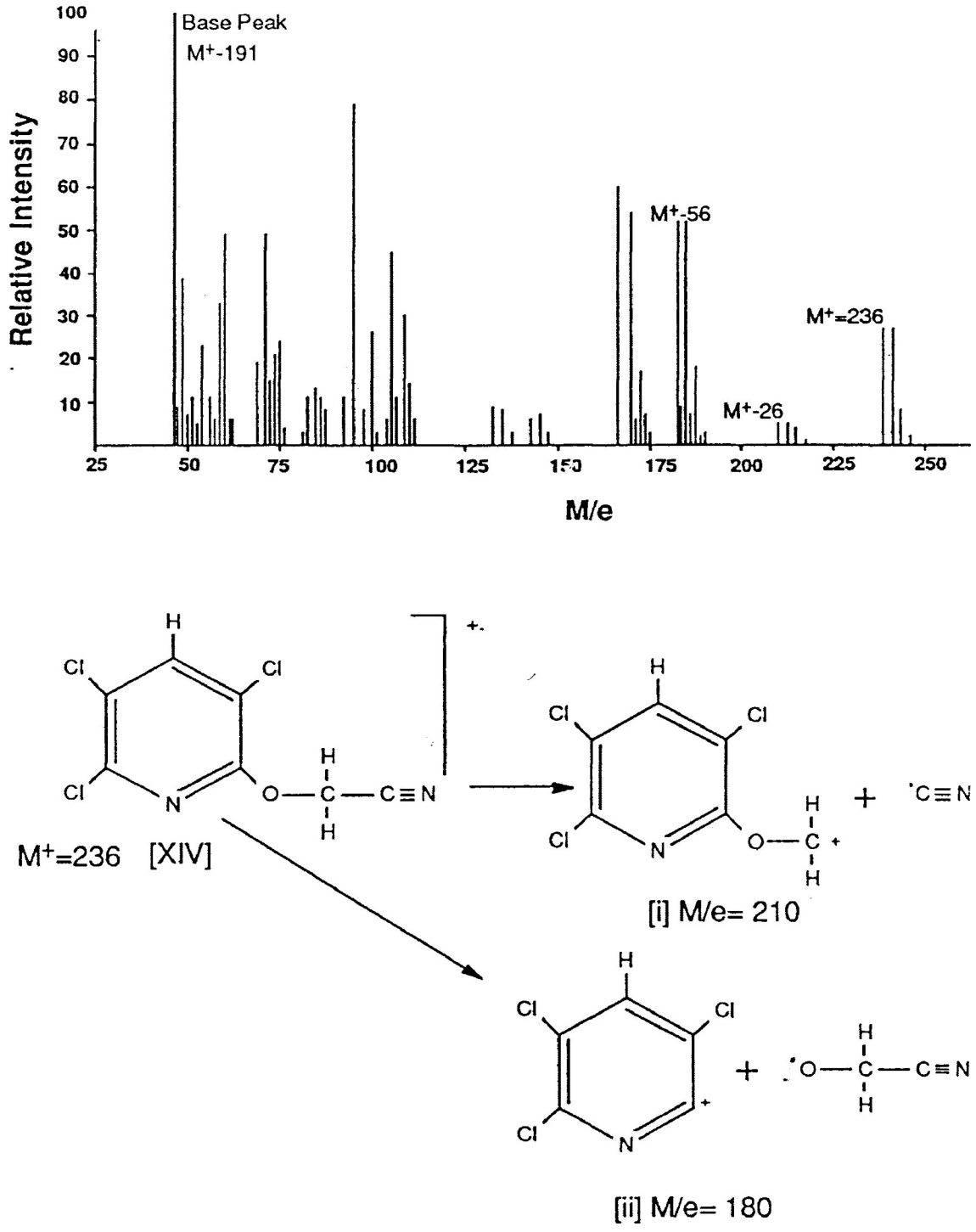
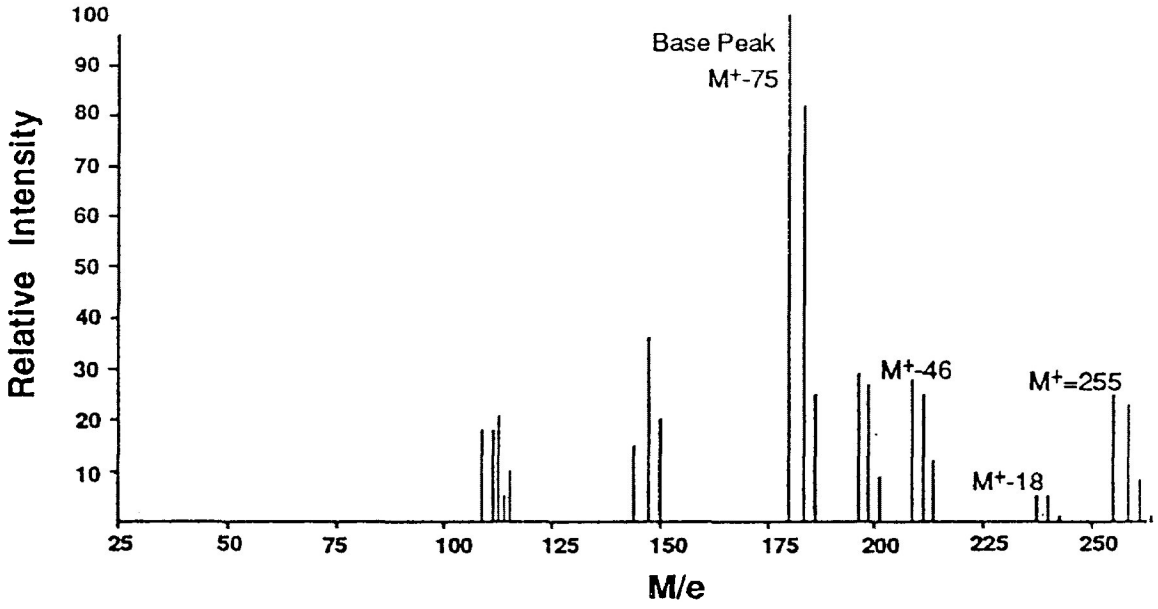



**Fig. 2.6 3,5,6-trichloro-2-pyridinyloxyacetonitrile [XIV] Mass Spectrum and Fragmentation Pattern**



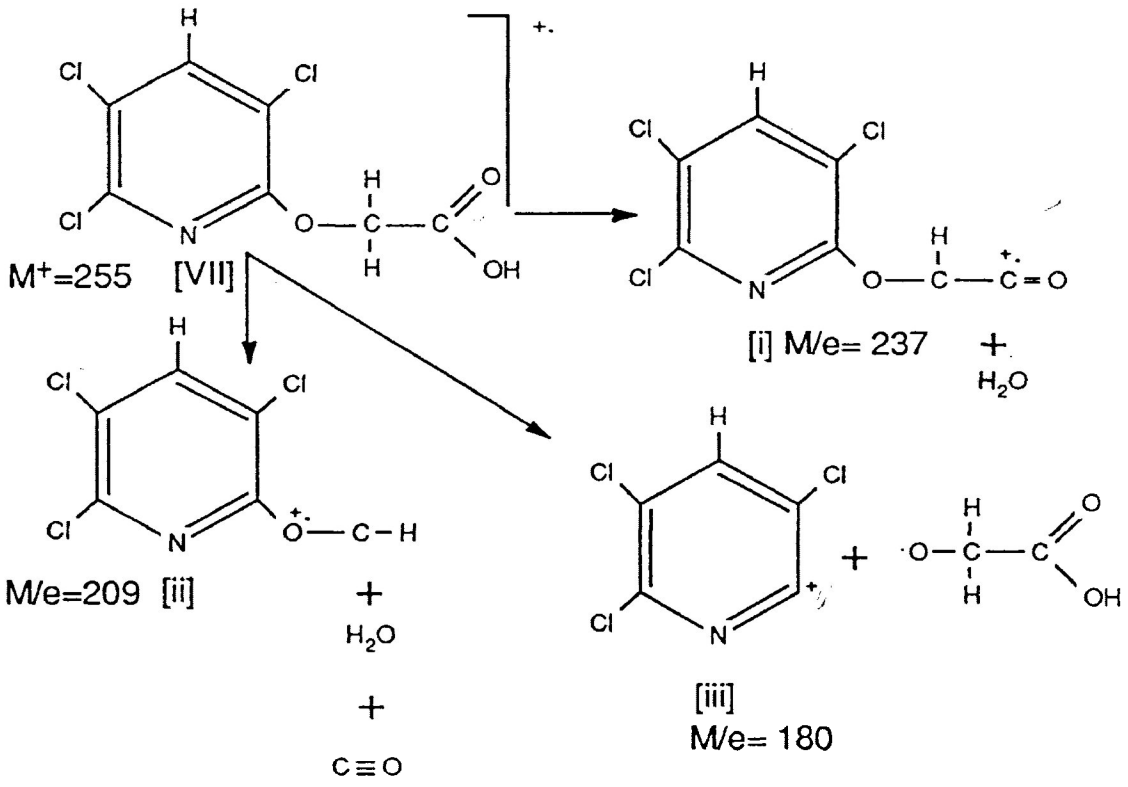




**Fig. 2.7 Triclopyr [VII] Mass Spectrum and Fragmentation Pattern**

## 2.7 Synthesis of Triclopyr [VII] from 2,6-dichloropyridine

The preparation of 2,3,5,6-tetrachloropyridine [XIII] is time consuming. In order to avoid the synthesis of 2,3,5,6-tetrachloropyridine [XIII] the following preparation was attempted.

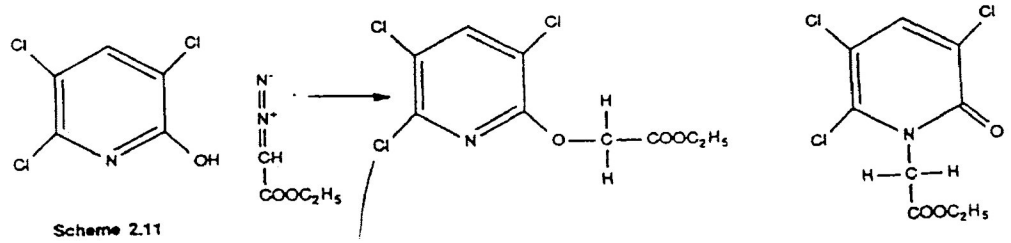
Since 2,6-dichloropyridine is commercially available, the formation of a monochloro analogue to 3,5,6-trichloro-2-pyridinlyoxyacetonitrile [XIV] was attempted (analogous Scheme 2.9). If yields were reasonable by this route, then the laborious preparation of 2,3,5,6-tetrachloropyridine [XIII] could perhaps be avoided. However the preparation of 6-chloro-2-pyridinyloxyacetonitrile gave a yield of only 25 %. It appears that for this reaction to proceed, there must be sufficient electron withdrawl at the 2-position on the pyridine ring. Without decreased electron density at the 2-position on the ring, the attacking species cannot displace the chloride ion. Since the yield of this preparation was so low, the hydrolysis of the nitrile was not performed. Moreover, the earlier preparation of monochloro ester (Section 2.4) indicated that subsequent chlorination could not be used to obtain the desired product.

## 2.8 Synthesis of Triclopyr [VII] using Ethyl Diazoacetate and 3,5,6-trichloro-2-pyridinol [VIII]

Another attempt to synthesize triclopyr [VII] was the reaction of 3,5,6-trichloro-2-pyridinol [VIII] with ethyl diazoacetate (142,143) (Scheme 2.11). As previously mentioned in Section 1.7.1, the reported yield of this

preparation is only 17 % (142). The possibility that this reported yield could be improved was the reason for attempting this reaction. Since the preparation requires the addition of the ethyl diazoacetate to melted 3,5,6-trichloro-2-pyridinol [VIII], precautions must be taken to guard against explosion. The reaction mixture was examined by gas chromatography and thin layer chromatography (silica gel). Along with a considerable amount of unreacted 3,5,6-trichloro-2-pyridinol [VIII] and product, there were unidentified materials present. The yield as estimated by gas chromatography only was 24 % of the O-alkyl product [XVIII], ethyl-3,5,6-trichloro-2-pyridinyloxyacetate, and the yield of N-alkyl product [XIX], 3,5,6-trichloro-1-ethylacetate-2-pyridone could not be estimated since no reference sample was available.

The estimated gas chromatographic yield was determined by comparing the signal response of an authentic sample of triclopyr-ethyl ester [XVIII] and an internal standard. From the known mass of the compounds used, the yield can be estimated. An authentic sample of triclopyr-ethyl ester [XVIII] was prepared by acid catalysed esterification of triclopyr [VII] with ethanol.

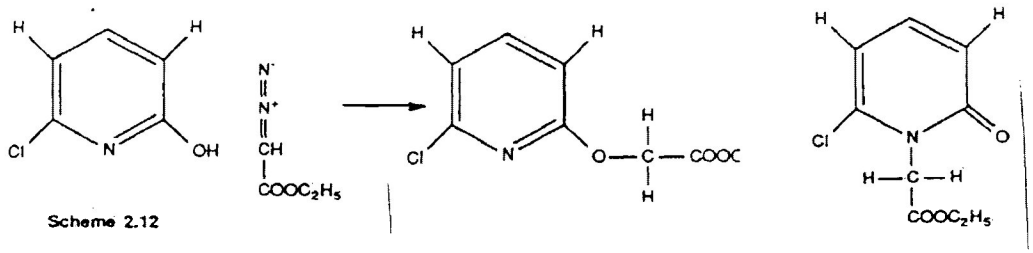


Scheme 2.11

## 2.9 Synthesis of Triclopyr [VII] using Ethyl Diazoacetate and 6-chloro-2-pyridinol

The ethyl diazoacetate reaction was also attempted with 6-chloro-2-pyridinol (Scheme 2.12). Similar to the forementioned reaction in Section 2.8, the results were observed by gas chromatography, and the desired product was not isolated. Although a yield was not estimated for this reaction, gas chromatography and thin layer chromatography observations indicated that the yield was very low. Also, the inherent risk of explosion (refluxing ethyl diazoacetate) does not make this procedure very attractive.

Even if this reaction had been successful in yielding substantial quantities of ethyl-6-chloro-2-pyridinyloxyacetate, the subsequent chlorination of the 3, and 5-positions (see Section 2.4) would not be efficient enough to make this a suitable method of producing triclopyr [VII].



Scheme 2.12

## 2.10 Miscelaneous Compounds

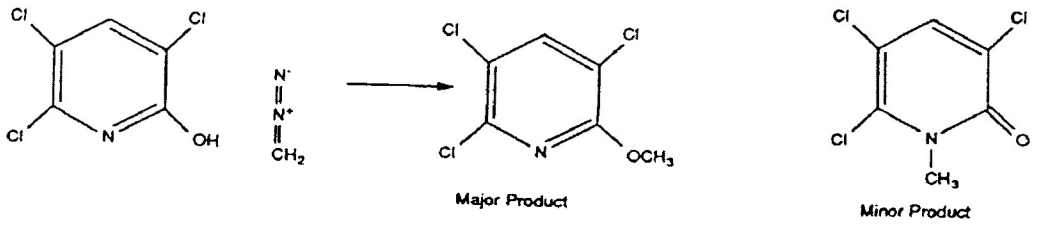
Several other compounds were required as reference compounds to complete this study. Some of these compounds were commercially available or obtained as gifts, but several others had to be prepared.

The triethanolamine salt of triclopyr [XX] was prepared by stirring triethanolamine with triclopyr [VII]. The dimethyl amine salt of 2,4-D [XXI] was prepared by bubbling dimethyl amine gas into a solution of 2,4-D [I] in acetone. Decarboxypicloram [XXII] was prepared previously in this laboratory by Baarschers *et al.* (206).

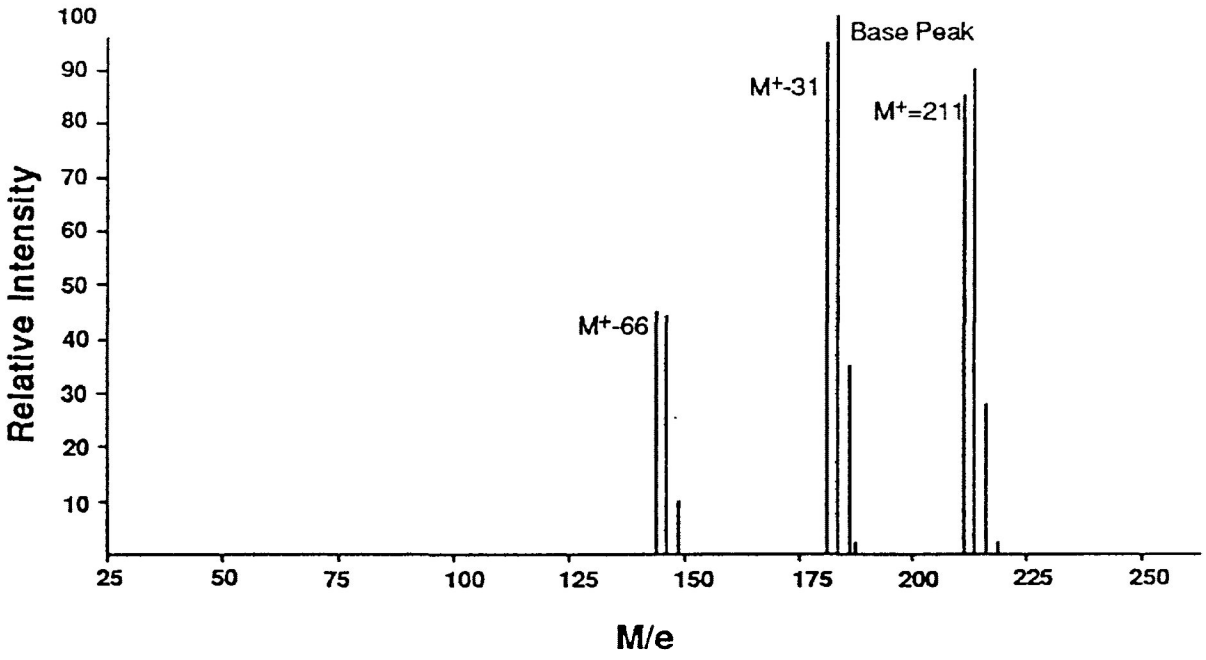
In order to develop a suitable gas chromatographic analytical method for quantifying triclopyr [VII] (Section 3.2), and it's possible metabolite 3,5,6-trichloro-2-pyridinol, 3,5,6-trichloro-2-methoxypyridine [IX] was prepared by reacting 3,5,6-trichloro-2-pyridinol [VIII] with diazomethane (Scheme 2.13). The mass spectrum and proton NMR of the purified product (a small

quantity of N-methyl product is formed) indicated that the compound formed was indeed 3,5,6-trichloro-2-methoxypyridine [XXIII] and not the N-alkyl structural isomer [XXIV]. The proton NMR had two signals, one corresponded to an aromatic proton at 7.70 ppm, and the other to the three protons on the methoxy carbon at 4.0 ppm. The mass spectrum fragmentation pattern contained the molecular ion at M/e=211 (Figure 2.8). The first fragmentation (M/e=180) (Figure 2.8 [i]) was the probable loss of the methoxy group as a radical. The second fragmentation may have been the loss of HCl and formaldehyde from the ring (M/e=145) (Figure 2.8 [ii]).

Authentic triclopyr [VII], authentic 3,5,6-trichloro-2-pyridinol [VIII], and 2,4-D [I] were obtained as a gift from the Dow Chemical Company, Midland Michigan. Picloram [IV] was purchased from the Aldrich Chemical Company, Milwaukee Wisconson. 2,4-dichlorophenol [V] was purchased from the J.T. Baker Chemical Company, Phillipsburg New Jersey.




Scheme 2.13



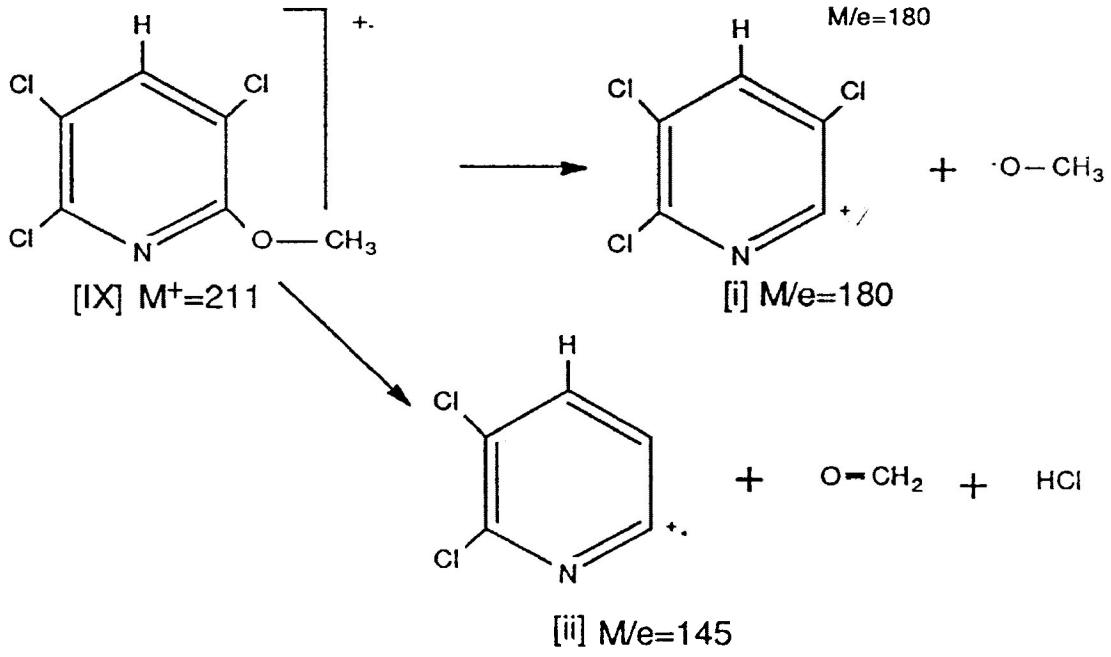




**Fig. 2.8 3,5,6-trichloro-2-methoxypyridine [IX] Mass Spectrum and Fragmentation Pattern**

# CHAPTER 3 ANALYTICAL METHODS

## 3.1 Introduction

In order to study the effect of microorganisms on the environmental fate of triclopyr [VII], suitable analytical techniques were required. Also, convenient and simple analytical techniques were required for monitoring of the syntheses previously discussed. Thin layer chromatography and gas liquid chromatography are both well suited for these purposes, provided the selection and preparation of suitable derivatives can be achieved. Two other analytical techniques which were used in the present study are ultraviolet spectroscopy and $^{13}C$ NMR. Both of these methods were found to be useful in degradation studies.

## 3.2 Gas Chromatography

Gas chromatographic analysis of triclopyr [VII] and its proposed major metabolite, 3,5,6-trichloro-2-pyridinol [VIII] required derivatisation on the packed columns selcted for this study. Consequently, the two tautomeric forms of the major metabolite (see Section 1.7.1) are capable of being methylated in varying proportions, depending on the choice of methylating reagent. The formation of N- and O-methyl derivatives can lead to discrepancies in the quantitation of the metabolite. This problem has been

ignored in the literature on triclopyr degradation (26), and chlorpyrifos degradation (82-87).

Two packed columns and one megabore (530 µm) column were tested for the separation of triclopyr [VII] and 3,5,6-trichloro-2-pyridinol [VIII]. The two packed columns were 5 % SE 30 and the mixed phase 3% OV 101/3 % OV 17 both on Gas Chrom Q (80-100 mesh) support. The commercially prepared 530 µm megabore column was a DB-5 (5 % phenyl and 95 % methyl silicone) column, J. and W. Scientific Company).

Initial work on the SE 30 column indicated derivatisation of both compounds was necessary. The free acid of triclopyr [VII] was retained on the column, as expected, and the compound 3,5,6-trichloro-2-pyridinol [VIII] tailed significantly. The commercially available derivatisation reagent methelute (trimethylanilinium hydroxide in methanol, Pierce Chemical Co.) was found to be unsuitable for the methylation of triclopyr [VII] because it gave two peaks on the chromatograph. The first peak had the same retention time recorded for an authentic sample of the methyl ester of triclopyr [XV] (210), which was prepared using dimethylsulphate. The second peak immediatly following the first, may have indicated the substitution of a methoxy group for a chlorine on the ring. The structure of the second derivative could not be elucidated since methelute is an on column derivatisation reagent.

Methelute was also unsuited for the derivatisation of 3,5,6-trichloro-2-pyridinol [VIII]. This compound contains a heterocyclic nitrogen atom which is capable of being methylated when the molecule is in the pyridone form (see tautomeric structures, Section 1.7.1). Therefore, two methylated

derivatives can be formed, and on methylation by this method two peaks were indeed formed on the gas chromatograph. One of these is the O-methyl derivative [IX], which was verified by the coincidental GC retention time with authentic 3,5,6-trichloro-2-methoxypyridine [IX] (Scheme 2.13). The other compound was found to be the N-methyl isomer. That the second peak was the N-methyl isomer (3,5,6-trichloro-1-methyl-2-pyridone) was established by the distinct fragmentation pattern contained in the mass spectrum analysis of an isolated sample of this material (210). Similar to the spectrum 3,5,6-trichloro-2-pyridinol [VI], the mass spectrum contains the loss of carbon monoxide from the ring resulting in the subsequent formation of a 5-membered heterocyclic ring (Figure 2.1, ion i is representative of this characteristic fragmentation).

An alternate approach to the derivatisation problem was required. It was found that triclopyr [VII] could be readily methylated using boron trichloride (10 % w/v) in methanol. This methylation process occurs when the reaction mixture is heated at 60° C. for 20 minutes in a sand bath. It was found that 3,5,6-trichloro-2-pyridinol [VIII] was not methylated by this technique. Also this methylating reagent was incapable of substituting a methyl group for a chlorine on the ring of triclopyr [VII].

The excess methanol and $BCl_3$ are then removed with a gentle stream of nitrogen. After all of the methanol is removed, the residue is dissolved in dichloromethane and 0.1 ml of BSA (N-O-bis (trimethylsilyl) acetamide) is used to silylate the 3,5,6-trichloro-2-pyridinol [VIII] in the mixture. The volume of BSA required can be decreased to 0.02 ml but if any water or methanol is present this reagent would be consumed. The

stationary phase chosen for this technique was a mixture of 3% OV 101 and 3 % OV 17 as it provided a reasonably sharp peak for the methylated triclopyr [VII]. Temperature programming was useful for decreasing the analysis time. This analysis is therefore suitable for the simultaneous analysis of triclopyr [VII] and the suspected metabolite, 3,5,6-trichloro-2-pyridinol [VIII]. Also, the proposed metabolite 3,5,6-trichloro-2-methoxypyridine [IX] (26) would be detected by this method without any artifact formation.

An internal standard is required for the quantitation of the derivatives,and dimethyl sebacate was found to be the compound of choice. The retention time of dimethyl sebacate was longer than that of 3,5,6-trichloro-2-pyridinol [VIII] and shorter than that of triclopyr [VII]. The peak shape was also suitable for comparison with both compounds. Figure 3.1 is a representative GC trace of triclopyr [VII] and 3,5,6-trichloro-2-pyridinol [VIII] with dimethyl sebacate internal standard. Figure 3.2 is a GC trace of a culture extract of triclopyr [VII] with derivatisation. Dichloromethane proved to be a good solvent for most gas chromatographic applications.

This procedure was found to be adequate for samples containing 0.2 mg/ml of triclopyr [VII] and 3,5,6-trichloro-2-pyridinol [VIII] (by flame ionization detection) based on the linearity of the calibration curves and comparisons with calibration standards containing authentic triclopyr methyl ester.

Preliminary studies with the megabore column, previously described, indicated that underivatised triclopyr [VII] was retained on the column when the flow rate was set for "capillary column" mode (2 ml/min). Derivatised

triclopyr had a similar retention time to the packed column results. When the flow rate for the megabore column was increased to 20 ml/min underivatised triclopyr [VII] eluted from the column without tailing.

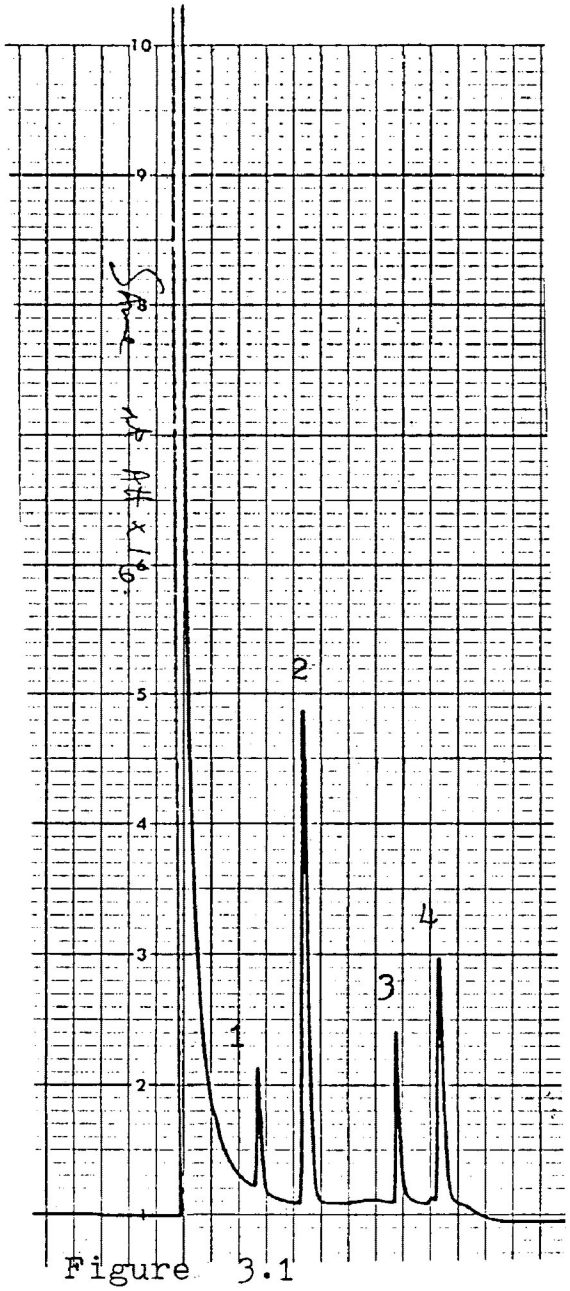



Figure 3.1

1,2:0, and N-silyl-derivatives of 3,5,6-trichloro-2-pyridinol.
3; Dimethyl sebacate, Internal Standard.
4: Triclopyr-methyl ester

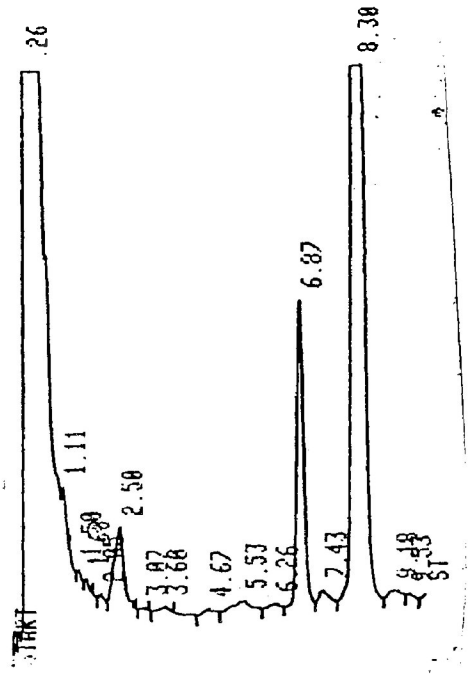



Figure 3.2
6.87 minutes- Dimethyl Sebacate
8.38 minutes- Triclopyr-methyl ester

### 3.3 $^{13}C$ NMR

When using gas chromatography or ultraviolet spectroscopy, waste products of the test organisms unrelated to the test compounds can interfere with the analysis. Consequently, in recovery and metabolism experiments it is necessary to clean-up extracts which are to be analyzed. Loss of material can occur through the introduction of a clean-up step. In developing such a procedure these losses must be accounted for by performing the procedure on a known quantity of the material. The use of $^{13}C$ NMR for the analysis of degradation products reduces the need for these clean up procedures.

Some of the metabolic experiments undertaken in this study utilized trypticase soy broth (Difco Laboratories, Detroit, Michigan), a general purpose growth medium for non fastidious bacteria. This medium will allow for relatively rapid growth of the bacterial population. Upon extraction of the herbicide a great deal of lipid material is also partitioned into the organic phase. This is especially so at the low pH required for the successful extraction of triclopyr [VII]. Consequently, gas chromatographic analysis of the material is subject to interference from extraneous peaks which are sometimes coincidental with peaks representing the compounds of interest. Furthermore the analysis must be performed at high operating temperatures for some time, to remove materials with long retention times. This slows the analysis considerably.

It was thought that in $^{13}C$ NMR analysis there would be less cleanup requirement, providing the analyte is concentrated enough to produce sufficiently strong signals in the recorded spectrum. Also the non destructive

nature of $^{13}C$ NMR makes it attractive, since the sample can be analyzed afterwards by other methods .

It was found that in the series of experiments by which $^{13}C$ NMR was used to investigate the possible breakdown of triclopyr [VII], the presence of 50 to 100 mg of herbicide in the sample was required to provide adequate signals. To ensure that the concentration of triclopyr [VII] used did not inhibit the growth of the bacterium, 1 L cultures were used. After the culture was extracted and the extract was dried, reconstitution to a standard volume was performed with deuterochloroform containing tetramethylsilane which is required as an internal standard for spectrometer calibration. Non deuterated chloroform can be used, but some deuterated species must be present to allow the sample signals to be locked. The solvent used must also contain a reasonably non-volatile internal standard for comparing signal strengths (tetramethyl silane is too volatile for quantitative work). Ideally the internal standard used should provide a single signal to avoid the complication of the spectrum and should provide a strong signal which does not interfere with signals for the test compound. For these particular experiments, tert butyl bromide was used because it met all the requirements by providing a strong uncomplicated signal. The predominant signal of tert butyl bromine is in the low ppm region far from the aromatic region. The tertiary carbon of tert butyl bromine provides a strong reference signal which is enhanced by the Overhauser effect due to the three methyl groups attached to it (211).

Signal noise encountered in these metabolic studies is relatively large due to the heterogeneous nature of the sample. Although this does

impart some uncertainty in quantitative studies, the error involved is not significant enough to require spectral comparisons with blank culture extracts. It is the heterogeneous nature of the impurities which allow for the success of $^{13}C$ NMR over other techniques. Impurities which interfere with gas chromatographic analysis become background noise in $^{13}C$ NMR and do not normally interfere with the spectrum of interest. Figure 3.3 is a representative spectrum of triclopyr [VII] extracted from a culture of *Arthrobacter globiformis* in trypticase soy broth at pH 2. Similarly Figure 3.4 is a representative spectrum of 3,5,6-trichloro-2-pyridinol added to a culture extract obtained from *Arthrobacter globiformis* in trypticase soy broth at pH 2.

To measure the quantity of herbicide remaining after the incubation period, ratios between assigned signal intensities are measured. The signals which are assigned to carbons from the herbicide are compared to the predominant signal from the internal standard. Known concentrations of the herbicide are measured under the same conditions as the test samples. From the signal intensity ratios of the test compound and the internal standard at various concentrations of the former, a calibration curve can be made. The lower limit of detection in culture media extracts is is determined by the signal to noise ratio. The noise, as perceived by the instrument detector, is mostly the lipid material and other waste products in the extract. In these experiments the lower limit of detection (of the analyte) was found to be 25 mg when the extract was reconstituted in 3 mL of solvent.

Triclopyr [VII] and the proposed metabolite, 3,5,6-trichloro-2-pyridinol [VIII] can be distinguished by the distint chemical shifts in the respective

spectrums (Figure 3.3 and 3.4). The metabolism of triclopyr by the bacteria can be determined by the decrease in intensity in any one of the signals in the recorded spectrum, when compared to the internal standard signal intensity. The signal which arises from the methylene carbon on the side chain of triclopyr is best suited for this as there can be no question that the 3,5,6-trichloro-2-pyridinol signals would interfere in the rare instance that the signal lock is unstable. For 3,5,6-trichloro-2-pyridinol [VIII] the best signal for quantitation is arises from $C_3$.

This procedure is limited by two factors: The sample must be concentrated enough to produce a signal, also, analysis time is long (the samples in this study required 4000 scans which takes over 8 hours due to the long relaxation times of $^{13}C$ NMR).

Natural abundance studies are severely limited by the equipment used, and the concentration of the test compound, this is not the case in studies which use $^{13}C$ enriched material. Specifically, for triclopyr [VII], if one carbon in the ether side chain and another in the ring, were enriched with $^{13}C$ then the signal to noise ratio would be improved. The synthesis of $^{13}C$ enriched triclopyr [VII] is possible, especially in the ether side chain. The reaction of 2,3,5,6-tetrachloropyridine [XIII] and either $^{13}C$ enriched KCN or paraformaldehyde may furnish a reasonable yield. The production of triclopyr [VII] with a ring position enriched with $^{13}C$ would be more difficult (150). The cost of $^{13}C$ enriched materials is still prohibitive for most experiments, but as the demand increases for $^{13}C$ enriched materials, this may change. The results of the recovery studies are discussed in Section 4.3.

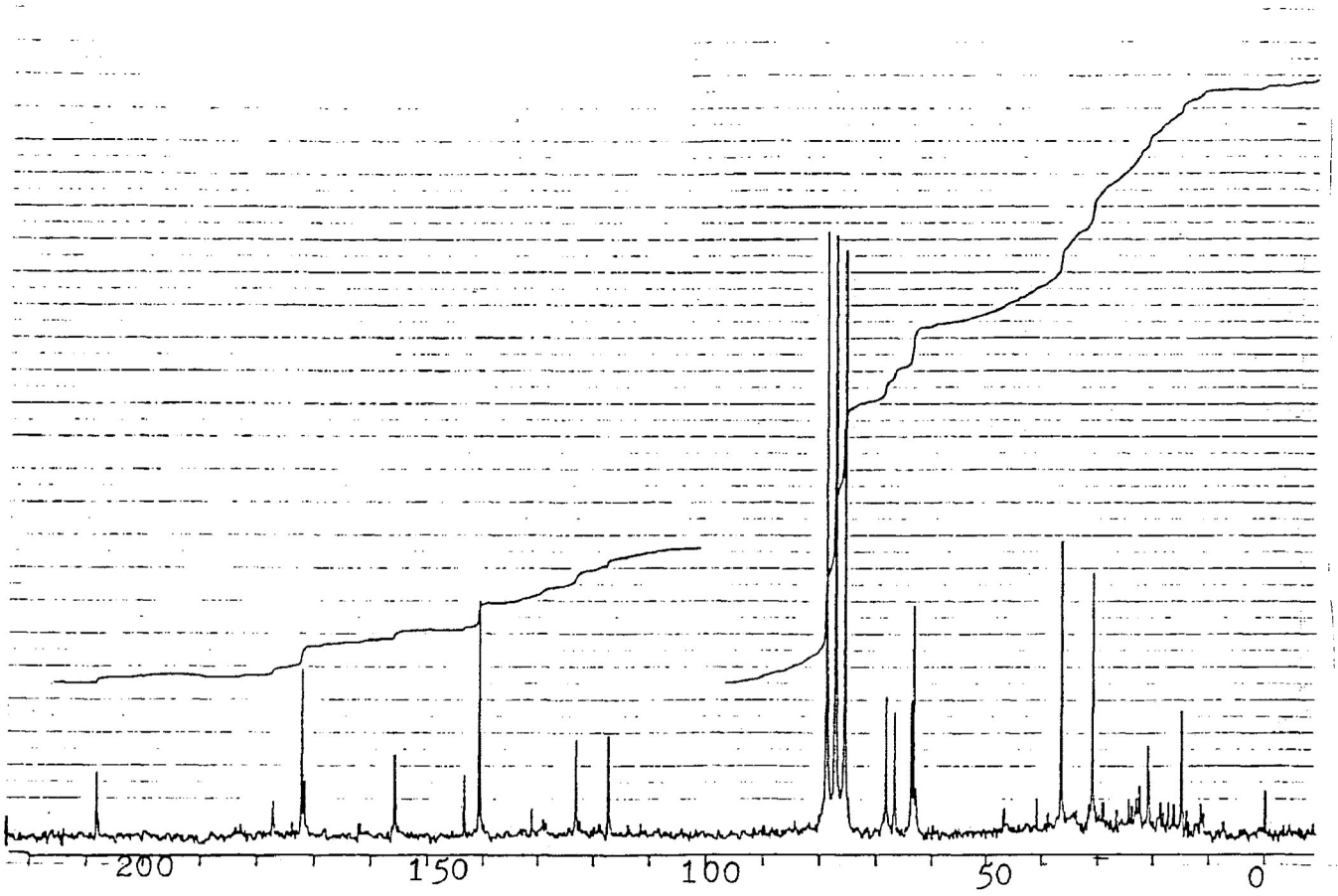



Figure 3.3 C-13 NMR Spectrum of Triclopyr

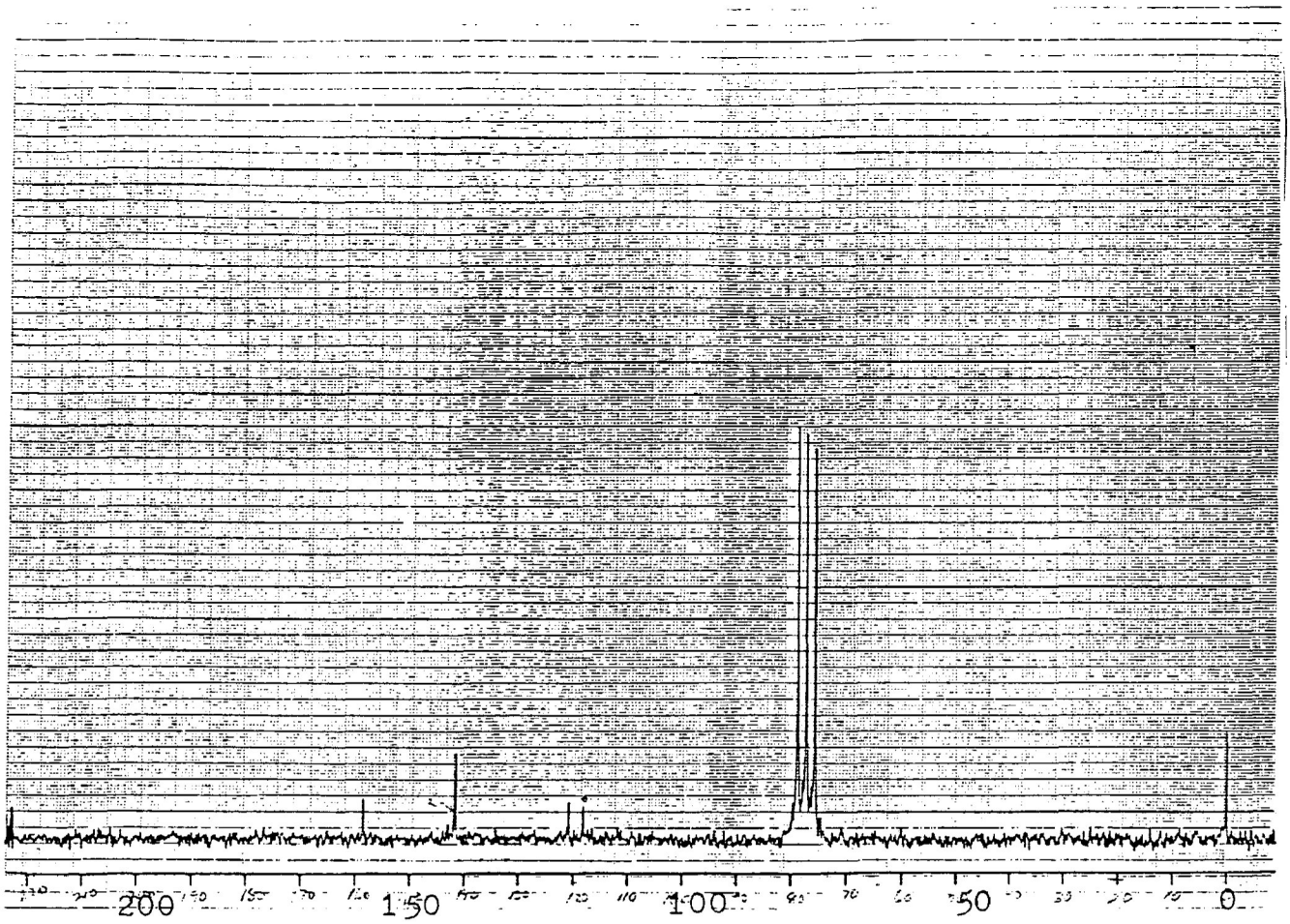



Figure 3.4 C-13 NMR Spectrum of 3,5,6-trichloro-2-pyridinol

# CHAPTER 4 THE TOXICITY, UPTAKE, AND DEGRADATION OF TRICLOPYR [VII] USING TWO SOIL BACTERIA

## 4.1 Introduction

In order to assess the effects of triclopyr [VII] on soil microorganisms, two representative species were selected as test organisms. *Pseudomonas pictorum* Gray and Thornton ATC No. 22284, and *Arthrobacter globiformis* Conn NRC No. 32001 were selected on the basis of the ability of other species within these two bacterial genera to cleave the side chain of 2,4-D [I] at the ether group, to produce the compound 2,4-dichlorophenol [V] (57-59, 65-69). The purpose of this part of the study was to determine the dose related response of these two microorganisms to triclopyr [VII] and other structurally related herbicides (2,4-D [I], and picloram [IV]). Also, the dose related response of the proposed metabolite of triclopyr [VI], 3,5,6-trichloro-2-pyridinol was compared with the dose related response of the metabolite of 2,4-D [I], 2,4-dichlorophenol [V] and the metabolite of picloram, decarboxypicloram [XXII]. The results obtained by this method were compared to those of a fungal dose related response method previously used by Baarschers *et al* (208) and an algae microplate toxicity test also used by Baarschers *et al* (208).

The metabolism of triclopyr [VII] in pure cultures of the bacterium was also investigated. Bioacccumulation experiments, using trypticase broth enriched with triclopyr [VII] and a culture medium containing triclopyr [VII] as a sole carbon source, were performed.

To asses the potential effects of dose related toxicity in a bacterium, a suitable indicator of toxic response is required. Although bacteria would seem well suited for population counts using automated particle counters, this is not the case. Liquid cultures of bacteria tend to form microcolonies, this is especially true with mucoid, filamentous or flagellar species. Colony counting procedures which require serial dilutions are cumbersome, time consuming and mainly suited for non enriched culture mediums (such as water samples). Consequently, an indicator of population size and viability is required to asses the response of a bacterial population to a specific toxin. A brief review of microbial toxicity assessment methodology is presented in chapter 1.

## 4.2 Microbial Toxicity Assessment of Triclopyr [VII]

The method which has been developed for this part of the study is a modification of the method developed by Bitton *et al*. (110) which measures dehydrogenase activity through the reduction of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) without an extraction step.

The initial intention was to use the direct assay INT method (110), in this laboratory without any modifications. The reduction of INT is thought to be caused by the electron transport chain (dehydrogenase enzymes specifically) of respiring bacteria (107-111). Assays have been developed in which the degree of INT reduction is measuraed by visualization of the intracellular INT-formazan salts using a microscope (107,111). But bacterial cultures in liquid medium contain a great deal of extracellular enzymes

which originate from dead lysed cells. Consequently, the measurement of INT-formazan salts by direct spectroscopy, or after the separation of the insoluble salt by extraction cannot be considered to be solely due to intracellular dehydrogenase activity. Since the reductive capability of the culture and media reflects the sum of all the bacteria both viable and lysed cells alike, it was thought that the test compound should be present in the culture from the time it is inoculated. Bitton *et al*. (110) added the potential toxin to the culture 30 minutes prior to the reaction with INT. Therefore, the test compound must directly inhibit reductive enzymes to be considered inhibitory by this method. In the modified method used in this study, the toxin is added to the culture media prior to the inoculation with the bacterium. This allows the toxin to affect the bacteria in terms of growth inhibition and allows the toxic assessment of compounds which do not exert an effect on the dehydrogenase system.

Some preliminary experiments proved that the two methods were different. If Bitton's reported procedure (110) is a measure of intracellular dehydrogenase activity, the culture medium with the bacterial cells removed should not reduce INT to the formazan form. In order to investigate this aspect the cells were removed from the culture by two methods; centrifugation for 15 minutes at 6,000 g, and filtration through 0.2 μm membrane filters. The cells were washed with pH 7.6 phosphate buffer and recentrifuged. The cells were then separated from the supernatant and resuspended in fresh buffer. The cell free culture medium and the buffered cell suspensions were then tested by Bitton's method (incubation with the toxin for 30 minutes followed by measurement the reduction of INT

spectrophotometrically). The increase in absorbance of the solutions over time is due to INT reduction by dehydrogenase enzymes. It was found that the cell free extracts exhibited the same absorbance change that was obtained the intact bacterial culture. The buffered cell suspension had very little absorbance increase, hence the bulk of INT reduction is occurring in the culture medium and not within the cells. Other experiments involved measuring the effects of a metal salt ($ZnSO_4$) and an organic acid (triclopyr [VII]) by both methods. Using Bitton's method (110) the metal salt inhibited the reduction of INT but triclopyr [VII] (6.3 - 800 ppm concentration range) did not. When the organic acid was present through the growth phase of the bacterium, a decrease of INT activity related to dosage is found. Clearly, it can be seen that the direct INT method (110) is useful only for the inhibition of the dehydrogenase enzymes in bacteria, as the test compound is in contact with the culture medium for only 30 minutes. Commercially available dehydrogenase enzyme such as the ethanol and lactate analysis systems available from Boeringer Manheim (Manheim W. Germany) likely could be substituted for the bacteria in Bitton's proceedure (110), thus measurement of the toxic effects of various compounds with a standardized amount of dehydrogenase enzyme each time would be possible. INT could also be replaced as the indicator, by measuring NADH conversion at 340 nm. This would be an improvement since INT formazan is an insoluble salt that eventually settles to the bottom of the cuvette limiting the time available for accurate photometric measurements.These could be adapted to measure the enzyme inhibition by compounds similar to the procedure used by Baarschers (~~211~~ 208) for thiophosphate pesticides.

The modified method is useful for the study of toxic effects on the bacterium but the method does not allow for the elucidation of the toxic mechanism. Indeed the measurements of dehydrogenase activity in the culture are simply a reflection of the cumulative growth of the bacterium over a period of time in contact with a toxic substance. This makes the method useful for measuring other effects including growth enhancement. In experiments with $ZnSO_4$, low concentrations actually increased growth, which is illustrated by the increase in INT reduction relative to a reference culture, that contains no added zinc, other than what is present in the culture medium. The growth enhancement effect is also noticed with some of the herbicides tested. At low concentrations the influence of the herbicides may not be so severe as to negatively influence the normal physiology of the cell but may actually increase the reproduction of bacteria.

Probit-log curves were plotted of the average slope of the change in absorbance over time at the test concentration ranges (see Section 5.5 for the formula). The results of the modified INT assays are summarized in Table 4.1.The $EC_{50}$ results indicate that more than one species must be tested as the inhibitory concentrations were significantly different for the two species used. In general, *Arthrobacter globiformis* is more susceptible to the herbicides than *Pseudomonas pictorum* . This difference was noticed in all the tests except in the case of 2,4-dichlorophenol [V]. The low concentrations of 2,4-dichlorophenol [V] required for inhibition may increase the errors which are inherent in bioassays of this nature. Therefore this method should serve as a guide and $EC_{50}$ results should be considered as an indication of the concentration range required to inhibit the microorganisms growth. The

potential metabolite of triclopyr [VII], 3,5,6-trichloro-2-pyridinol [VIII] , which is analogous to the metabolite of 2,4-D [I], 2,4-dichlorophenol [V], is also considerably more toxic than the parent compound. This illustrates the importance of assessing the toxicity of potential metabolites as well as the parent compound. The $EC_{50}$ results obtained from the tests using the salts of the herbicides are all higher than the parent compounds $EC_{50}$ results. This indicates that the compounds used to form the salts do not adversely contribute to the inhibition of growth of the microorganism.

The errors of probit-log models, as described by Buikema *et al* (91) for toxicity assessment did not occur in any of the analyses. Some errors described in the paper (91) are: slope values of zero, which would be encountered if the compound was not toxic to the test organism, two $EC_{50}$ values on one curve which could be encountered with a dilution error, or with the test compound exerting more than one dose related effect on the test organism. Figure 4.1 through Figure 4.6 illustrate some curve shapes derived from the probit-log plots. Ideally, the line should be straight at the 50 % inhibition area as deviations in this area will affect the derived $EC_{50}$ result. At the extreme ends of the test plot the slope will decrease. This is because low concentrations will have no effect and high concentrations will have exhibited the maximum possible effect on the culture.

The fungal plate and algalmicroplate toxicity tests used by Baarschers (208) were compared to the INT assay. The algae microplate growth assay and fungal plate method results are presented in Tables 4.2 and 4.3. In general, the algae (*Chlorella vulgaris* and *Chlorella pyrenoidosa*) are more susceptible to the herbicide's influence than the

fungi (*Trichoderma viride, Mortierella isabellina* and *Saprolegnia parasitica*) and bacteria. This is likely due to the close relationship between algae and plants (chlorophyll production) in contrast to plants and bacteria or fungus. The success of the INT method, as a toxicity assessment procedure, is confirmed by the results obtained between the different species tested by the three methods and also by the results obtained when using different herbicides in the same method. If the $EC_{50}$ results obtained with different types of microorganisms were spread over several orders of magnitude (i.e. fungal and bacterial $EC_{50}$ results) then one or two of the methods may be providing doubtful results. Also, if the results obtained by the same microorganism with different herbicides, that are structurally analogous to each other (specifically triclopyr [VII] and 2,4-D [I]), are spread over several orders of magnitude then there may be problems with the method providing the two compounds exert the same physiological effect. These two forementioned methods of verifying the test are not absolute proof of its validity especially if the test organisms have very different sensitivities to the herbicides tested, or if the herbicides have different effects in the same microorganism, but it does give some confidence in the method.

Some limitations of the three different toxicity methods used in this study are the use of culture media and specific pH ranges. These two parameters can greatly affect the available concentration of metals in solution thereby making them not suitable for the toxicity assessment of metals (95). Also the interaction of some test compounds and INT has been noticed. 2,4-dichlorophenol [V] and 3,5,6-trichloro-2-pyridinol [VIII] cause a precipitate at the higher dose levels. Since both of these compounds have

low $EC_{50}$ values it has little effect on the results providing a reagent blank is used to compensate for any turbidity that may occur.

**Table 4.1 Bacterial Toxicity of Triclopyr [VII], 2,4-D, Picloram and Some of Their Salts and Possible Metabolites as $EC_{50}$ Values in ppm.**

| Compound | *A. globiformis* | *P. pictorum* |
|---|---|---|
| Triclopyr[a] | 46 | 470 |
| Triclopyr Salt[b,c] | 490 | >800 |
| Trichloropyridinol | 12 | 32 |
| 2,4-D[a] | 270 | 490 |
| 2,4-D Salt[d] | 750 | 770 |
| 2,4-dichlorophenol | 4 | 0.7 |
| Picloram[a] | 60 | 380 |
| Decarboxy Picloram | 190 | 90 |

a: Free Acid b:Triethanolamine Salt c: Values for Amine Salts as Free Acid Equivalent d: Dimethylamine Salt

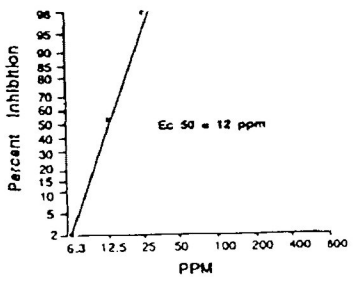



Fig.4.1 INT Test of 3,5,6-trichloropyridin-2-ol Toxicity with *Arthrobacter globiformis*

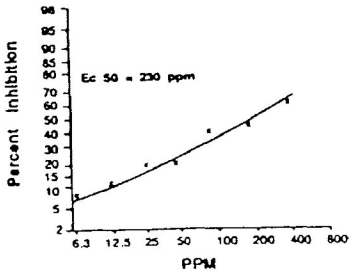



Fig. 4.2 INT Test of 2,4-D Toxicity with *Arthrobacter globiformis*

**Table 4.2 Algal Toxicity of Triclopyr, 2,4-D, Picloram and Some of Their Salts and Possible Metabolites as $EC_{50}$ Values in ppm.** (208)

| Compound | *Chlorella vulgaris* | *Chlorella pyrenoidosa* |
|---|---|---|
| Triclopyr[a] | 11 | 80 |
| Triclopyr Salt[b,c] | 8 | 8 |
| Trichloropyridinol | 3 | 2 |
| 2,4-D[a] | 19 | 56 |
| 2,4-D Salt[d] | 15 | 34 |
| 2,4-dichlorophenol | 9 | 4 |
| Picloram[a] | >160 | >160 |
| Decarboxy Picloram | 49 | 8 |

Notes a-d as for Table 4.1

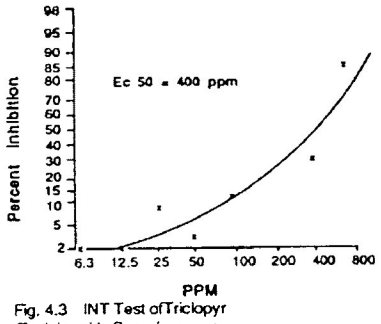



Fig. 4.3 INT Test of Triclopyr Toxicity with *Pseudomonas pictorum*

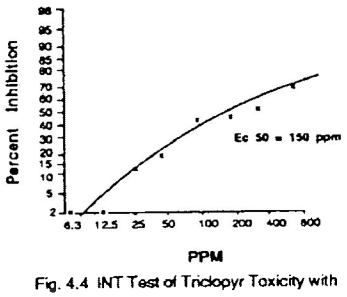



Fig. 4.4 INT Test of Triclopyr Toxicity with *Arthrobacter globiformis*

**Table 4.3 Fungitoxicity of Triclopyr, 2,4-D, Picloram and Some of Their Salts and Possible Metabolites as $EC_{50}$ Values in ppm.**(208)

| Compound | *T. viride* | *S. parisitica* | *M. isabellina* |
|---|---|---|---|
| Triclopyr[a] | 220 | 250 | 250 |
| Triclopyr Salt[b,c] | >1600 | 700 | 750 |
| Trichloropyridinol | 13 | 11 | 15 |
| 2,4-D[a] | 350 | 220 | 400 |
| 2,4-D Salt[d] | >1600 | 1000 | 800 |
| 2,4-dichlorophenol | 10 | 23 | 50 |
| Picloram[a] | >400 | -[e] | -[e] |
| Decarboxy Picloram | 50 | 80 | 60 |
| Picloram Salt | >1600 | >1600 | >1600 |

Notes a-e as for Table 4.1 f:Triisopropylamine Salt

## 4.3 Recovery Studies

Two different types of experiments were undertaken to determine if triclopyr [VII] is utilized by *Arthrobacter globiformis* and *Pseudomonas pictorum*. Experiments using established cultures grown in trypticase soy broth and inoculated with the herbicide during the exponential growth phase were attempted and also sole carbon source experiments using triclopyr [VII] in a trace element solution were tried. The results of the sole carbon

source, and enriched culture medium experiments are summarized in table 4.4.

The two bacterial species (*Arthrobacter globiformis* and *Pseudomonas pictorum* ) used for the recovery study did not utilize the herbicide and no metabolites were found in any of the experiments. Unlike the experiments performed by Adus (57-59), in which the bacteria were selected by survival in soil with 2,4-D added, the bacteria for the experiments described in this paper were not known to metabolize triclopyr and were not selected from soil. Since each species from a given genus has many variants, it is not possible to state that *Pseudomonas pictorum* and *Arthrobacter globiformis* do not utilize triclopyr but the two variants used did not do so in these experiments.

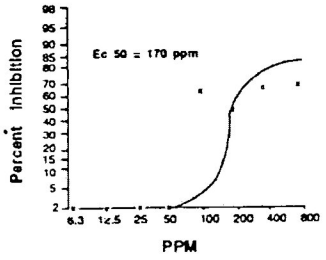



Fig. 4.5 INT Test of Triclopyr Toxicity with *Arthrobacter globiformis*

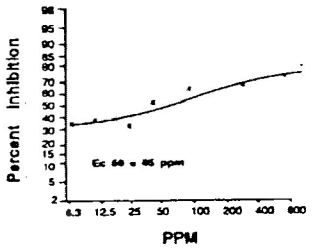



Fig. 4.6 INT Test of 2,4-D Toxicity with *Arthrobacter globiformis*

**Table 4.4 Percent Recovery of Triclopyr [VII] from Trypticase Soy Broth and Trace Element Liquid Cultures of *A. globiformis* and *P. pictorum***

| Concentration of triclopyr [VII] | $^{13}C$ NMR | G.C. | UV |
|---|---|---|---|
| 75[abi] | 100[c] | 96[cd] | |
| 75[aei] | 100[c] | 94[cd] | |
| 75[abj] | -[f] | 100[g] | 100[h] |
| 75[aej] | -[f] | 100[g] | 100[h] |

a: ppm. b: *A. globiformis* . c: corrected for 88 % recovery efficiency of control. d: OV 101 (3 %) / OV 17 (3 %) packed column, and derivatisation. e: *P. pictorum* f: not performed g: without extraction or derivatisation using $DB_5$ (5 % phenyl and 95 % methyl silicone) megabore column. h: culture solution used directly. i: trypticase soy broth cultures. j: trace element cultures (triclopyr as a sole carbon source).

# CHAPTER 5 EXPERIMENTAL

## 5.1 Instrumentation

Proton nuclear magnetic resonance ($^1$HNMR) and carbon-13 nuclear magnetic resonance spectra ($^{13}$C NMR) spectra were measured with a Bruker WP-80 Spectrometer, using hexadeuteroacetone or deuterochloroform solutions. Tetramethylsilane was used as an internal standard (d=0 ppm).

Mass spectra were measured with a Hitachi-Perkin-Elmer RMU-7 double focusing mass spectrometer. The samples were introduced *via* the direct or liquid inlet depending on the melting point of the sample.

Infrared spectra were recorded with a Beckman IR-2 spectrophotometer and a Perkin-Elmer 1320 IR spectrophotometer. All spectra were run in chloroform using NaCl sample cells.

Combustion analyses were performed with a Perkin-Elmer model 240 elemental analyser and a Perkin-Elmer model 260 elemental analyser equipped with a Hewlett-Packard Touch Screen microcomputer controller.

Melting points are uncorrected.

All gas chromatography was performed using a Perkin-Elmer model 3920B gas chromatograph equipped with a flame ionization detector and glass columns (1.8 X 6.4 mm OD, 3 % OV-101 and 3 % OV-17 stationary phase on Gaschrom Q (80-100 mesh support) or a 530 μm column coated with 5 % phenyl and 95 % methyl silicone stationary phase. GC traces were recorded using a Fisher Recordall Series 5000 strip chart recorder or a Hewlett-Packard Model 3390 A integrator.The carrier gas used was nitrogen at 30 mL/min. Isothermal methods were used at temperatures between 100-195°, depending on the sample to be analysed. The solvent used for the injection of samples was dichloromethane.

Triclopyr [VII] was converted to its methyl ester derivative by heating the sealed sample with 1.0 mL of $BCl_3$ in methanol (10 % W/V) obtained from Milton Roy Co., State College, PA. Heating the sample at 60° for 20 minutes was sufficient for the complete derivatisation of the sample. The methanol and residual $BCl_3$ are then removed with a gentle stream of nitrogen gas and then silylation of the 3,5,6-trichloro-2-pyridinol [VIII] was performed by adding 0.2 mL of BSA reagent to the vial. The internal standard used for metabolism studies was dimethyl sebacate.

Thin layer plates were prepared from glass slides using either silica gel or alumina. The mobile phase was benzene or benzene/methanol (95:5) depending on the polarity of the samples. Since permanent color development was not required, iodine vapor was used. Silver nitrate and ammonia were used to develop some plates but iodine was found to give a better reaction with most compounds.

## 5.2 Synthetic Chemistry

### 5.2.1 3,5,6-trichloro-2-pyridinol [VIII]

To two g of 6-chloro-2-pyridinol dissolved in concentrated HCl (12 mL). $H_2O_2$ (30 %, 8 mL) was added dropwise (20 min.) with vigorous mixing. After the addition of the $H_2O_2$ was complete the mixture was diluted with 75 mL of distilled water, and extracted with toluene (2 X 15 mL). The combined toluene extracts were washed with sodium metabisulphite solution to remove the excess chlorine. After flash evaporating the solution the solid residue was crystallized from toluene. 2.1 g were obtained by decanting. After the balance of solvent was removed the crystals were dried *in vacuo*. M. p. 176 °, Lit. m. p. 174-175 ° (110) Mixed m. p. with Dow authentic 3,5,6-trichloro-2-pyridinol [VIII] 176 °. Calculated for $C_5H_2ONCl_3$: C, 30.27 ; H, 1.06 ; N, 7.06. Found : C, 30.65 ; H, 1.11 ; N, 7.19 %. $M^+$=197, 199, 201, 203 (100, 98, 35, and 2 % respectively). Major fragments at M/e 169, 171, 173, 175 (55, 53, 18, and 1 % respectively) and 162, 165, 167 (23, 10, and 10 % respectively).

### 5.2.2 Triclopyr [VII] (as the Methyl Ester)

One gram of 3,5,6-trichloro-2-pyridinol [VIII] was dissolved in methylmonochloro acetate (10.0 g). Dipotassium carbonate (0.9 g) was

added with vigorous mixing. The mixture was refluxed for 60 minutes, diluted with cold distilled water (100 mL) and extracted with toluene (2 X 25 mL ). The extract was washed with water (3 X 20 mL). The dried residue from the extraction was dissolved in benzene (2 mL) and added to the top of a silica gel column (7.5 cm long and 2.25 cm in diameter) which had previously been washed with 50 mL of benzene. After 100 mL of benzene was passed through the column the solvent was removed from the eluate under a gentle stream of nitrogen at 60 °. When crystals formed the sample was removed from the heat and allowed to cool slowly. The remaining solvent was decanted upon cooling and the sample was dried *in vacuo* . G.C. analysis produced a single peak. Yield from starting material was 0.71 g (53 %). M.P. 107 °. Calculated for $C_8H_6O_3NCl_3$ : C, 35.52 ; H, 2.24 ; N, 5.18. Found : C, 35.56 H, 2.23 ; N, 5.20. $M^+$= 269, 271, 273, 275 (15, 14, 5, and 1 % respectively). Major fragments at M/e 210, 212, 214, 216 (52, 50, 15, and 2 % respectively), 180, 182, 184, 186 (37, 44, 22, and 2 % respectively), and M/e 59 (100 %).

### 5.2.3 Methyl 6-chloro-2-pyridinyloxy acetate

One gram of 6-chloro-2-pyridinol was dissolved in methyl monochloroacetate (15.0 g). Dipotassium carbonate (1.35 g) was added and the solution was refluxed for 2 hours. GC analysis indicated the reaction was not complete, but it was stopped at this time. The mixture was added to cold distilled water (50 mL) and then extracted into toluene (3 X 20 mL). The extract was washed 3 times with distilled water and dried with anhydrous

sodium sulphate. Removal of the solvent by flash evaporation, after removing the sodium sulphate by filtration left an oily residue which was light green in color. This oil was dissolved in 2 mL of benzene and placed on a 10 X 2.25 cm column of silicagel which had previously been washed with 50 mL of benzene. A further 100 mL of benzene was passed through the column and the product was dried *in vacuo.* The residue was crystallized from benzene (3 mL). Colour plate crystals were formed. Yield from starting material is 55 % (0.85 g). M.p. 62-63 ° . Calculated for $C_8H_8O_3NCl$ : C, 47.66 ; H, 4.00 ; N 6.95. Found : C, 47.47 ; H , 4.03 ; N, 6.83. $M^+$ = 201, and 203 (20 and 10 % respectively). Major fragments at M/e = 170, 172 (10 and 5 % respectively), 169, 171 (8 and 4 % respectively), 112 (32 %) and M/e =142 (100%).

5.2.4 Triclopyr [VII]

(as the Methyl Ester from 6-chloro-2-pyridinyloxymethyl acetate)

0.25 g of 6-chloro-2-pyridinyloxymethyl acetate was dissolved in 2.5 mL of glacial acetic acid with mixing. Concentrated HCl (6 mL) and $H_2O_2$ (1 mL, 30 %) was added dropwise to the solution. GC analysis indicated several compounds were present in the solution. Silicagel thin layer plates (benzene mobile phase) indicated that triclopyr [VII] as the methyl ester, starting material, triclopyr [VII] and another unidentified compound were present. The chlorination was repeated on the product. The material was then extracted into benzene (3 X 15 mL) and the extract was washed with dilute sodium thiosulphate solution and then distilled water. The solvent was

removed by flash evaporation and the residue was redissolved in 2 mL of benzene. A 10 cm column of silicagel which had previously been washed with benzene (100 mL) was used to separate the components of the mixture. 100 mL of benzene was passed through the column eluting the starting material. A furthur 100 mL of benzene eluted triclopyr [VII] as the methyl ester. The third fraction was also eluted with 100 mL of benzene. GC analysis of this fraction gave one peak upon derivatization with 10 % $BCl_3$ in methanol (w/v) indicating it was likely triclopyr [VII] as the free acid. The two forms of triclopyr [VII] were combined after the free acid fraction was methylated with 10 % boron trichloride in methanol (w/v). Crystalization from 0.3 mL of benzene yielded 25 % (0.073 g) triclopyr [VII] as the methyl ester. M.p. 105 °. Calculated for $C_8H_6O_3NCl_3$ : C, 35.52 ; H, 2.24 ; N, 5.18 ; Found : C, 35.77; H, 2.22; N, 5.03. $M^+$ at M/e= 269, 271, 273 (12, 10, and 4 % respectively). Major fragments at M/e= 210, 212, 214, 216 (51, 46, 15, and 1 % respectively), M/e= 180, 182, 184 (33, 42, and 21 % respectively), and M/e = 59 (100 %).

5.2.5 2,3,5,6-tetrachloropyridine [XIII]

(from 2,6-dichloropyridine)

Three g of 2,6-dichloropyridine was mixed with 0.09 g of $AlCl_3$ and a crystal of $I_2$. The mixture was heated to 180 ° and stirred. Chlorine gas was introduced through the top of the vessel which had a vent leading into a 25 cm column of carbon tetrachloride. This column of liquid allowed for a slight pressurization of the gas in the reaction vessel and also allowed the gas

flow to be monitored. GC analysis performed on an aliquot after 24 hours indicated the conversion of the starting material into a compound with a higher molecular weight which was presumed to be 2,3,6-trichloropyridine. GC analysis after 58 hours indicated that most of the starting material was converted into this intermediate species. A significant amount of the reaction mixture was also found in a third fraction indicated by gas chromatogaraphy after 58 hours. This was presumed to be 2,3,5,6-tetrachloropyridine [XIII]. After 72 hours the reaction was stopped and the mixture was dissolved in 50 mL of dichloromethane. This mixture was washed with 50 mL of thiosulphate solution (to remove excess chlorine) and then with 50 mL of distilled water. Silicagel thin layer chromatography with hexane resolved three compounds in the mixture. Separation of the three compounds was performed using a 20 cm column of silicagel which was previously washed with hexane. The material was dissolved in 3 mL of benzene and added to the top of the column. The first fraction was eluted with hexane (100 mL) . The hexane was removed by flash evaporation and the residue was dried *in vacuo* . M. p. 92 °, Lit. m. p. 91 ° (144). Calculated for $C_5HNCl_4$ : , C 27.69 ; H, 0.47 ; N, 6.46 ; Found : C, 27.59 ; H, 0.52 ; N, 6.42. $M^+$ at M/e= 215, 217, 219, 221 (85, 100, 10, and 2 % respectively). Major fragments at 180, 182, 184, 186 (35, 35, 12, and 3 % respectively), M/e= 145, 147, 149 (15, 10, and 2 % respectively).

5.2.6 2,3,5,6-tetrachloropyridine [XIII]

(from 2,6-diaminopyridine)

Two g of 2,6-diaminopyridine was dissolved in 12 mL of concentrated HCl with vigorous mixing. A further 12 mL of concentrated HCl and 2 mL of 30 % $H_2O_2$ were added dropwise over 15 minutes. After two hours the excess acid was neutralized with NaOH. Extraction was performed using ethyl acetate (3 X 25 mL). The solution was dried with anhydrous sodium sulphate and then filtered. The solvent was then removed by flash evaporation and the residue was crystallized twice from a minimum amount of benzene. Yield of 2,6-diamino-3,5-dichloropyridine 23.6 % (0.80 g). M. p. 215 °. $M^+$ at M/e= 177, 179, 181 (15, 10, and 10 % respectively). Major fragments at M/e= 141, 143, 145 (12, 5, and 5 % respectively), and M/e= 36 (100 %).

O.64 g of 2,6-diamino-3,5-dichloropyridine was dissolved in 8.5 mL of concentrated HCl and 8.5 mL of $H_2O$. The mixture was cooled to 0 ° and 2.4 g of $NaNO_2$ dissolved in 5 mL of distilled water was added slowly. This was followed by the addition of 1.05 g of CuCl which was dissolved in 15 mL of 1 M HCl . The mixture was gradually allowed to warm to room temperature and was then heated to 45 ° for 30 minutes. The mixture was then allowed to cool to room temperature and was extracted with dichloromethane (3 X 25 mL). The combined extracts were washed with dilute sodium bicarbonate solution (25 mL) and with distilled water (3 X 25 mL). The solution was dried

with anhydrous sodium sulphate and filtered. The solvent was removed *in vacuo* and the residue was purified by filtering through a 5 cm silicagel column which was previously washed with hexane. The material eluted by 50 mL of hexane was isolated by the removal of the solvent by flash evaporation and dried *in vacuo.* GC analysis confirmed the product as 2,3,5,6-tetrachloropyridine [XIII], as compared to the previous preparation (5.2.5). The m. p. of the compound produced by this synthesis was 92-93 ° . Lit. m.p. 91 °(144). Yield of 2,3,5,6-tetrachloropyridine 11 % (0.087 g).

5.2.7 Triclopyr [VII] (from 2,3,5,6-tetrachloropyridine [XIII])

1.06 g of 2,3,5,6-tetrachloropyridine [XIII] was dissolved in 10 mL of dimethylsulphoxide. With vigorous stirring, 0.17 g of paraformaldehyde was added. The mixture formed a slurry. O.25 g of NaCN was added and the temperature was allowed rise from 20 ° to 45 °. After 90 minutes the temperature had dropped to 30 °. The mixture was then heated to 100 ° for 10 minutes. Distilled water (15 mL) was added and the mixture was extracted with dichloromethane (2 X 20 mL). The combined extracts were washed with distilled water (10 mL), dried with anhydrous sodium sulpate, and filtered. Flash evaporation of the solvent left a semisolid residue. A Kugelrohr apparatus was used to separate the unreacted 2,3,5,6-tetrachloropyridine [XIII] from the product by distillation below 100 °. The product was distilled off above 120 ° leaving a high boiling residue. The material that distilled between 120 and 132 ° was a white solid upon cooling. Recrystalization using ethanol formed needles which were

separated from the solvent by decanting. The remaining solvent was removed *in vacuo.* The m. p. of the product was 88 °. Yield of 3,5,6-trichloro-2-pyridinyloxy acetonitrile [XIV] was 70 % (0.81 g). Calculated for $C_7H_3N_2OCl_3$ : C, 35.41 ; H, 1.27 ; N, 11.79 ; Found : C, 35.16 ; H, 1.19 ; N, 11.63. $M^+$ at M/e= 236, 238, 240, 242 (25, 25, 10, and 2 % respectively). Major fragments at M/e= 210, 212, 214, 216 (5,5,4, and 1 % respectively), M/e= 180, 182, 184, 186 (52,52, 20 and 3 % respectively), and M/e= 45 (100 %).

0.4 g of 3,5,6-trichloro-2-pyridinyloxyacetonitrile [XIV] was stirred with 6.0 mL of concentrated HCl at 70 ° for 5 hours. The resulting slurry was filtered through scintered glass and washed with 10 mL of distilled water. Benzene dissolved the product from the filter. The solvent was then removed by flash evaporation. The residue was dissolved in a dilute sodium hydroxide solution (50 mL, which forms the sodium salt of the product) and washed with benzene (2 X 25 mL, to remove unreacted nitrile). After the benzene extractions, the aqueous phase was reacidified and reextracted with benzene (2 X 25 mL). The combined benzene extracts were then washed with dilute sodium bicarbonate solution (1 X 10 mL) and water (2 X 25 mL). The benzene solution was dried with anhydrous sodium sulphate and filtered. The solvent was removed by flash evaporation and the product was dried *in vacuo.* The residue was then crystallized from warm chloroform. White needles were obtained. M.p. 150 ° *versus* authentic triclopyr [VII] (Dow) 151 °. Yield of triclopyr [VII] 81 % (0.35 g , from nitrile). Therefore the yield based on 2,3,5,6-tetrachloropyridine [XIII] is 58 %.

Calculated for $C_7H_4NO_3Cl_3$ : C, 33.01 ; H, 1.58 ; N, 5.50 ; Found : C, 33.25 ; H, 1.41 ; N, 4.98. $M^+$ at M/e= 255, 257, 259 (28, 26 and 12 % respectively). Major fragments at M/e= 237, 239, 241 (8, 8, and 2 % respectively), M/e= 209, 211, 213 (32, 28, and 14 % respectively), M/e= 209, 211, 213 (100, 83, and 23 % respectively).

5.2.8 Triclopyr [VII] from 2,6-dichloropyridine

2.86 g of 2,6-dichloropyridine was mixed with 15 mL of dimethyl sulphoxide and 0.68 g of paraformaldehyde. 1.0 g of NaCN was added. Treatment of the mixture was the same as in Section 5.27. The final yield of product after separation from unreacted material was 25 %. MS, GC analysis confirmed the identity of the material as 2,6-dichloro-2-pyridinyloxyacetonitrile.

5.2.9 Triclopyr [VII] from 3,5,6-trichloro-2-pyridinol [VIII] and Ethyl Diazoacetate

2.51 g of 3,5,6-trichloro-2-pyridinol [VIII] was heated to 180 °. The melt was vigorously stirred while 0.6 g of ethyl diazoacetate was added dropwise during 45 minutes. Precautions must be taken since ethyl diazoacetate is explosive. After the addition of the ethyl diazoacetate, the mixture was cooled and dissolved in benzene. Silicagel thin layer chromatography (benzene / ethanol, 8:1 mobile phase) indicated that there was unreacted 3,5,6-trichloro-2-pyridinol [VIII] and a small amount of an unidentified

material which was considerably less polar than 3,5,6-trichloro-2-pyridinol [VIII]. Since the amount of material converted by this reaction appeared small, GC analysis was used to estimate the yield of material. From the comparison with authentic triclopyr [VII] as ~~an~~ the ethyl ester and an internal standard, the yield of O-alkyl product was 24 % and N-alkyl product was presumed to be the other major fraction noted on the gas chromatograph.

5.2.10 Ethyl 6-chloro-2-pyridinyloxyacetic ester (from 6-chloro-2-pyridinol and Ethyl Diazoacetate)

1.63 g of 6-chloro-2-pyridinol was treated as in Section 5.2.9. Analysis using silicagel thin layer plates and GC indicated the yield was similar to that obtained in Section 5.2.9. No further purification was initiated since the yield of triclopyr [VII] as the ethyl ester was not high enough to make this synthesis feasible.

5.2.11 Triclopyr [VII] triethanolamine Salt.

1.0 g of triclopyr [VII], dissolved in 50 mL of benzene, was mixed with 1.38 g of triethanolamine in 15 mL of benzene. After stirring for 1 hour the solvent was removed by flash evaporation. The residue was crystallized from a minimum amount of acetone. The product was separated from the acetone by decanting and the residual acetone was removed *in vacuo*. The yield of the product was 79 %. The m. p. was 125-127 °. Calculated for

$C_{13}H_{19}N_2O_6Cl_3$ : C, 38.47 ; H, 4.72 ; N, 6.90 ; Found : C, 39.11 ; H, 4.75 ; N, 6.76.

5.2.12 3,5,6-Trichloro-2-methoxypyridine

0.30 g of 3,5,6-trichloro-2-pyridinol [VIII] was dissolved in 150 mL of diethyl ether. 2.2 g of Diazald (N-methyl-N-nitroso-p-toluene sulphonamide) was dissolved in 30 mL of ether in another flask which was connected by a hollow tubing to the flask containing the 3,5,6-trichloro-2-pyridinol [VIII] and ether solution. 0.4 g of KOH was dissolved in 10 mL of 96 % ethanol. This solution was added to the Diazald solution with great care in a fume hood. The liberated diazomethane was bubbled into the etherel solution of 3,5,6-trichloro-2-pyridinol [VIII]. After the evolution of diazomethane was complete, the ether and excess diazomethane was removed with a gentle stream of nitrogen. GC analysis indicated that there were three compounds present. One compound had a retention time identical to that of unreacted 3,5,6-trichloro-2-pyridinol [VIII]. The two other compounds had retention times indicating the O and N-methyl derivatives of 3,5,6-trichloro-2-pyridinol [VIII] as compared with specimens obtained by methylating with methelute. A 30 cm column of silica gel which had been previously washed with benzene was used to separate the various components of the residue. Increasing concentrations of ether and then chloroform in benzene (starting with pure benzene) were used to elute the fractions. The O-methyl species was isolated and crystallised from ethanol: distilled water 99:1. The solvent was removed by decanting and the product was dried *in vacuo.* M. p. 61 °. $M^+$ at

M/e= 211, 213, 215, 217 (85, 93, 29 and 2 % respectively). Major fragments at M/e= 180, 182, 184, 186 (90, 100, 34, and 2 % respectively), M/e= 145, 147, 149 (43, 42, and 10 % respectively).

5.2.13 Triclopyr [VII] as ~~an~~ the Ethyl Ester from Authentic Triclopyr [VII]

0.11 g of triclopyr [VII] was dissolved in 5 mL of ethanol and 5 mL of hexane. 0.25 mL of concentrated HCl was added and the mixture was refluxed for 1 hour. The solvent was distilled off and the residue was redissolved in 20 mL of hexane. The hexane solution was washed with distilled water (2 X 10 mL). The hexane solution was dried with anhydrous sodium sulphate and then filtered. After removing the solvent by flash evaporation the product was dried *in vacuo.* This material was used to estimate the yield of triclopyr [VII] ethyl ester obtained in section 5.2.9 as it had an identical retention time with one of the products in 5.2.9.

**5.3 Bacterial Culture Maintenance**

The freeze dried bacterial cultures of *Pseudomonas pictorum* Gray and Thornton ATC No. 22284 and *Arthrobacter globiformis* Conn NRC No. 32001 were rehydrated from their lyophylized form using trypticase soy broth. Stock cultures were maintained at 28 °C on trypticase soy agar plates for the entire length of this study.

## 5.4 INT Toxicity Assesments

The stock bacterial cultures were used to inoculate liquid trypticase cultures 24 hours before the test was initiated. Aseptic techniques were practised throughout the toxicity tests to maintain pure cultures. The cultures for the toxicity assessment tests were trypticase soy broth (8 mL) in glass vials (12 mL capacity). Bacterial cultures in the exponential growth phase (24 hours old) were used to inoculate these vials (0.1 mL inoculum). The test compound which had been dissolved in ethanol was then added to the vials. The blank culture received ethanol only. The concentration ranges of the test compound used were between 0.63 and 800 ppm with a final ethanol concentration of 3 %. The cultures were incubated for 48 hours at 28 ° C with the caps left loose to ensure oxygen exchange. A single mixing of the vial contents was performed after 24 hours to distribute the cells. After 48 hours, triplicate samples of each culture were tested.

To 0.8 mL of phosphate buffer (pH 7.6), 1.6 mL of the culture broth was added and mixed. This was placed in a quartz cuvette and 0.8 g % of INT (distilled water and ethanol 1:1) was quickly added and mixed. The change in absorbance was measured for three minutes at 490 nm at ambient temperature (21-25 ° ). The percentage of growth inhibition of the culture was determined from the decrease in slope between the blank culture without added toxicant and the test cultures using the formula :

$$\%\text{inhibition} = \{ (\Delta \text{ abs } / t)_T / (\Delta \text{ abs } / t)_B \} \text{ X } 100 \% - 100 \%.$$

Where :

$(\Delta \text{ abs } / t)_T$ = The average slope of the triplicate analysis of each test concentration.

$(\Delta \text{ abs } / t)_B$ = The average slope of the triplicate analysis of the blank culture.

The $Ec_{50}$ of each test was derived by plotting the percent inhibition of each test against the concentration of the toxic compound. A probit scale was used for percent inhibition and a log scale was used for concentration. The results of these tests are presented in Table 4.1.

### 5.5 Metabolism Studies

The metabolic studies used either established cultures or newly inoculated cultures which were in contact with the herbicide from the start. Two types of medium were used: trypticase soy broth to which the herbicide was added after autoclaving, or trace element and vitamin enriched distilled water to which the herbicide had been added after autoclaving as the sole carbon source. In either culture medium the herbicide was added with an ethanol carrier. The final ethanol concentration was adjusted to 0.2 %.

One liter cultures of the two bacteria were grown in trypticase soy broth which contained 75 ppm triclopyr [VII]. After 72 hours of incubation on a shaker table (75 rpm at 20° C) the cultures were adjusted to pH 2 from pH 7.6 with concentrated HCl. The free acid form of triclopyr [VII] was then

extracted with 300 mL of ethyl acetate (three 100 mL portions). Emulsions were eliminated by passing the mixtures through filter paper. The filter paper was then rinsed with 25 mL of ethyl acetate which was pooled with the extracts. After drying the extracts with anhydrous sodium sulphate, the solvent was removed by flash evaporation. The residues were then analytically transfered to $^{13}C$ NMR tubes using 3 ml of deuterated chloroform which contained the internal standard (tert butyl bromide). After the $^{13}C$ NMR spectrums were recorded the residues were transferred to septum capped vials, the solvent was removed under a gentle stream of nitrogen and the procedure for derivitization was followed (Section 3.2). Analysis by gas chromatography was performed and the results were compared to the results obtained from the $^{13}C$ NMR spectrums (Table 4.4).

For the sole carbon source experiments the cultures were inoculated into a trace element medium which contained 75 ppm of triclopyr. The cultures were incubated on a shaker table (75 rpm at 20 °) for two weeks. An aliquot of the test cultures was removed and analyzed at day 3, 8, and 14 by UV spectroscopy. At day 14 gas chromatography was also performed to verify the UV analysis and to determine if any metabolic products had been produced which may have escaped detection in the UV analysis. The results of these experiments are described in Section 4.3.

### 5.6 Culture Media

Trypticase soy broth was purchased in powder form from Difco Laboratories Detroit Mi. To 1 L of distilled water, 30 grams of media was added stirred and autoclaved at 121 ° (15 psi steam) for 15 minutes. Agar (15 grams) was added prior to autoclaving to make plates.

For the sole carbon source experiments a mineral medium consisting of $FeCl_3.6H_2O$, 78.5 mg $CuSO_4.5H_2O$, 40.5 mg $MnSO_4.4H_2O$ and 88 mg $ZnSO_4.7H_2O$ made up to 250 mL with distilled water), vitamin stock (25 µg biotin, 500 µg thiamine, 500 µg pyridoxine and 25 µg inositol made up to 100 mL with 40 % ethanol and diluted 100 fold with distilled water) and trace element stock $Ca(NO_3)_2.4H_2O$ (100 mg), $NH_4NO_3$ (100 mg), $KH_2PO_4$ (50 mg), $MgSO_4.7H_2O$ (50 mg) $KNO_3$ (50 mg), $MnSO_4.4H_2O$ (0.5 mg), $H_3BO_3$ (0.5 mg), $ZnSO_4.7H_2O$ (0.5 mg), $CuSO_4.5H_2O$ (0.5 mg), $Na_2MoO_4.2H_2O$ (0.2 mg), $Co(NO_3)2.6H_2O$ (0.25 mg), and $NH_4$Fe-citrate (25 mg) per litre of distilled water was used.

# CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS

The toxicities of the herbicide triclopyr and its potential metabolite 3,5,6-trichloro-2-pyridinol towards *Pseudomonas pictorum* Gray and Thornton ATC No. 22284 and *Arthrobacter globiformis* Conn NRC No. 32001 are comparable to those of 2,4-D and its metabolite 2,4-dichlorophenol as well as to those of picloram and its metabolite decarboxy picloram. When compared to the results of the toxicity tests using algae, the bacteria were less succeptible to the herbicide's influence. The fungi were generally as succeptible to the toxic effects of the herbicide as the bacteria.

*Pseudomonas pictorum* Gray and Thornton ATC No. 22284 and *Arthrobacter globiformis* Conn NRC No. 32001 did not convert triclopyr to 3,5,6-trichloro-2-pyridinol under the experimental conditions described in this thesis. As these bacteria are each strains of quite a large genus and each contains many species types it cannot be concluded that some *Pseudomonas or Arthrobacter* species are not capable of degrading triclopyr. To improve the chances of finding bacteria which can utilize triclopyr, enrichment culturing from soil isolates should be attempted.

The patent method described by Markley (144) is suitable for providing reasonable yields of triclopyr on a laboratory scale, although the starting material for this synthesis (2,3,5,6-tetrachloropyridine) is difficult to obtain. The procedure described by Morris and Bonhert (130) proved to be the fastest and most economical for producing triclopyr on a laboratory scale requiring only minor modifications.

From an analytical viewpoint $^{13}C$ NMR was useful in determining the recovery of triclopyr in the metabolism studies without the necessity of separating the compound from the media. The sensitivity of $^{13}C$ NMR would be improved if $^{13}C$ labeled triclopyr was used in future metabolic studies. This may also make it no longer necessary to concentrate the analytes thereby eliminating several steps and decreasing handling error.

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