STUDIES RELATED TO THE CHEMISTRY AND ENVIRONMENTAL PERSISTENCE OF METHOXYCHLOR AND ITS DERIVATIVES

BY

© JAMES PAUL VUKMANICH

A THESIS SUBMITTED TO THE DEPARTMENT OF CHEMISTRY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

Lakehead University
Thunder Bay, Ontario, Canada

OCTOBER, 1983

ABSTRACT

An investigation of some of the chemical and biological factors influencing the environmental fate of methoxychlor (1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane) has been conducted.

Methoxychlor is an organochlorine insecticide which is used extensively in Canada and has been proposed as a potential DDT replacement. It has been reported in the liter ature that methoxychlor exhibits a low toxicity towards most mammals and is also partially degraded by these organisms. Although much information exists on the interaction of this insecticide with higher animals, less information is available on the interaction between this compound and organisms at lower tropic levels, especially the microorganisms. Methoxy chlor, because of its use as a larvicide in and around natural water systems, has the potential to interact directly with non-target aquatic biota. Therefore an investigation concerning the interaction between methoxychlor and aquatic microorganisms was initiated. The microorganisms chosen for this study were common species of green algae, *Chlorella pyrenoidosa* and *Chlorella vulgaris*. The biological effects of methoxychlor have been studied *in vitro* using axenic cultures of the test algae.

Known metabolites and derivatives of methoxychlor required for this study were synthesized. The extensive modi fication of the literature methods for the preparation of these compounds has resulted in improved yields and purer

products for virtually all compounds synthesized. The synthesis of 1-monochloro-2,2-bis (*p*-hydroxyphenyl) ethylene, a known methoxychlor metabolite, has been developed and almost completed. The synthesis of this compound has not been reported in the literature before. The product of the previously reported base-promoted rearrangement of 1,1-dichloro-2,2-bis (*p*-hydroxyphenyl) ethane has been identified and a mechanism for its formation postulated.

Also, to aid in the selection of solvents used for the extraction of the polar metabolites of methoxychlor from aqueous samples, the *p*-values (ie. extractability) and solubilities (in water and organic solvents) of these compounds have been determined.

The toxicity of methoxychlor and hydroxychlor (1,1,1-trichloro-2,2-bis(*p*-hydroxyphenyl)ethane) towards both species of algae was assessed. Although both species are tolerant of relatively high concentrations of these compounds, the hydroxy analogue was much more toxic than the parent insecticide. Growth rate studies revealed that both methoxychlor and hydroxychlor had little effect on the overall growth of *Chlorella pyrenoidosa*. The growth of *Chlorella vulgaris* was however, significantly depressed by methoxychlor, while on the other hand hydroxychlor stimulated the growth of this alga. Recovery experiments indicated that the uptake of methoxychlor and hydroxychlor by *Chlorella pyrenoidosa* is mainly due to physical adsorption. In the case of *Chlorella vulgaris* very little of the added methoxychlor could be

recovered. Recovery experiments conducted with *Chlorella vulgaris* and hydroxychlor suggest that at least some of this compound is reductively dechlorinated by this organism.

ACKNOWLEDGEMENTS

I wish to thank my research supervisor, Dr. W. H. Baarschers for his guidance and encouragement during the time this study was in progress.

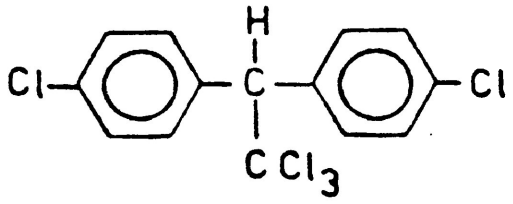
I also wish to thank Dr. T. J. Griffith, Mr. R. Chuchman and Mr. K. Pringnitz of the Lakehead University Science Instrumentation Laboratory for their help with the various spectroscopic measurements.

Finally, I must convey my deepest appreciation to my wife, Rebecca, for her moral support and invaluable assistance with the illustrations.

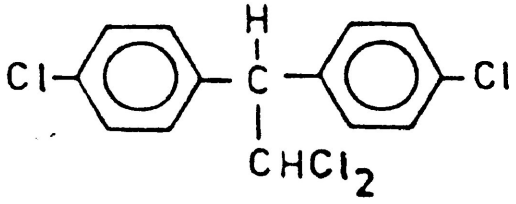
TABLE OF CONTENTS

CHAPTER I: INTRODUCTION

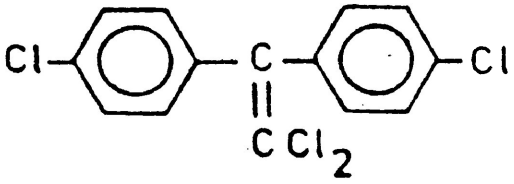
The development and widespread use of the organochlorine pesticides during and after the Second World War has made possible tremendous advances in the production of food and fiber, and has also contributed significantly to public health on a worldwide basis. The insecticide DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane, I, figure 1-1) is a primary example of this group of pesticides and is probably best known for its outstanding success in antimalarial campaigns. Its simplicity of manufacture, low acute mammalian toxicity (LD_{50}, 200mg/Kg, male rat), and effectiveness against a broad range of insect pests have, in the past, contributed to its wide spread use and acceptance. The chemical stability, low vapour pressure (1.5×10^{-7}mm at 20°C), high solubility in lipids (ca. 100,000ppm), and low solubility in water (0.002ppm) of this compound also add to its effectiveness as an insecticide (1). However, DDT and its major metabolites, DDD (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane, II), and DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene, III), because of the above chemical properties are among the most persistent compounds known and are classic examples of environmental contaminants. Perhaps the most serious aspect of environmental pollution by DDT is its almost universal distribution in fresh and sea waters, and the ability of aquatic animals to concentrate it in their body lipids to levels thousands of times higher through direct absorption and storage. Predatory birds and



DDT(I), 1,1,1-trichloro-2,2-bis (*p*-chlorophenyl) ethane



DDD(II), 1,1-dichloro-2,2-bis (*p*-chlorophenyl) ethane



DDE(III), 1,1-dichloro-2,2-bis (*p*-chlorophenyl) ethylene

FIG. 1-1. DDT and its major metabolites.

terrestrial animals also accumulate DDT and its metabolic products. The environmental impact of DDT has been well documented in numerous review articles (2,3,4) and in several books (5,6). As a result of the increasing concern regarding the longterm bioactivity and persistence of DDT, its use in Canada was restricted in 1969 (7).

The problems associated with the persistence and bioaccumulation of organochlorine pesticides have, to a certain extent, been answered by the use of "softer" or more biodegradable compounds. These replacement compounds of

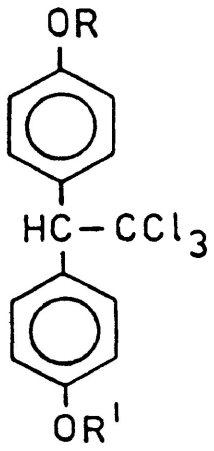
necessity must be effective against target pests. However, at the same time the alteration products formed through degradation by biochemical, photochemical and other chemical pathways should also be non-persistent in the environment.

Methoxychlor (1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl) ethane, IV, see figure 1-2) is an organochlorine pesticide related to DDT and is one of the earliest compounds to receive attention as a potential DDT replacement (8).

Gardner and Bailey (7) have reviewed much of the early literature on methoxychlor. This early work included methoxy chlor degradation, bioaccumulation and bioassy, but the major ity of the work reviewed dealt with the toxicity of methoxy chlor. The toxicity studies were largely confined to mammals, birds, and fish. In general methoxychlor displays a lower acute toxicity towards mammals and birds, than does DDT. Reported lethal doses (LD_{50}) of methoxychlor for mammals range from 800mg/Kg for mice to 10,000mg/Kg for dogs. These authors also reported that methoxychlor was quite toxic to fish (LC_{50} figures range from 14ug/L to 2000ug/L), although massive fish kills have never been reported when methoxychlor was used at the recommended rates for insect control. It was also report ed that unlike DDT, the cumulative storage of relatively high levels of methoxychlor by most terrestrial animals does not generally occur.

One of the most comprehensive studies on the metabo lism and bioaccumulation of methoxychlor was reported by

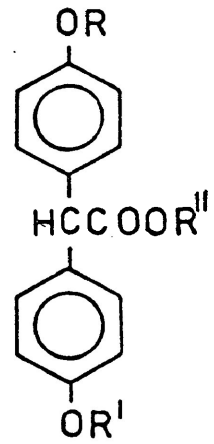
Kapoor *et. al.* (8). Radiotracer studies by these authors demonstrated clearly that methoxychlor was biodegraded in mammals by a stepwise O-dealkylation of the methoxy substi tuents. It was observed that 98.3% of the methoxychlor administered to mice was eliminated within 24 hours. These animals degraded methoxychlor to phenolic products, the major metabolites being, 1,1,1-trichloro-2-(*p*-methoxyphenyl)-2-(*p*-hydroxyphenyl)ethane(methoxyhydroxychlor, V) and 1,1,1-trich loro-2,2-bis(*p*-hydroxyphenyl)ethane(hydroxychlor, VI). Minor degradation products also found were, 1,1-dichloro-2,2-bis (*p*-hydroxyphenyl)ethylene(hydroxychlor olefin, XIV), 2,2-bis (*p*-hydroxyphenyl)acetic acid(dihydroxy acid, VIII), and 4,4'-dihydroxybenzophenone (XIX), (see figure 1-2 for structures). These authors also incubated labelled methoxychlor with mouse liver microsomes and found some of the above metabolites present, suggesting that O-dealkylation in the mammalian liver is the major pathway for methoxychlor metabolism. Al though methoxychlor is structurally related to DDT it is potentially more biodegradable by virtue of the alkoxy substi tuents on the aromatic rings of this molecule. It was suggested (1) that such sites provide the necessary "handle" for the attack of hepatic multifunction oxidase enzymes found in a wide variety of organisms. The DDT molecule on the other hand, because of the stability of the aryl chlorine bonds, is not susceptable to attack at these sites and is therefore not readily degraded. Kapoor and co-workers also developed a "model ecosystem" in which the bioaccumulation and ecological



IV methoxychlor, R = R' = CH_3

V methoxyhydroxychlor, 1,1,1-trichloro-2-(*p*-methoxyphenyl) -2- (*p*-hydroxyphenyl) ethane, R = H, R' = CH_3

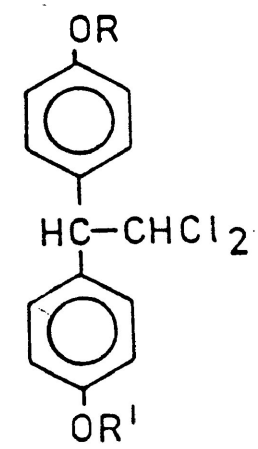
VI hydroxychlor, 1,1,1-trichloro-2,2-bis (*p*-hydroxyphenyl) ethane, R = R' = H



VII dimethoxy acid, 2,2-bis (*p*-methoxyphenyl) acetic acid, R = R' = CH_3, R" = H

VIII dihydroxy acid, 2,2-bis (*p*-hydroxyphenyl) acetic acid, R = R' = R" = H

IX dimethoxy acid methyl ester, 2,2-bis (*p*-methoxyphenyl) acetic acid methyl ester, R = R' = R" = CH_3

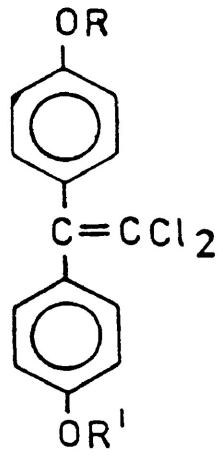


X dimethoxydichloro derivative, 1,1-dichloro-2,2-bis (*p*-methoxyphenyl) ethane, R = R' = CH_3

XI dihydroxydichloro derivative, 1,1-dichloro-2,2-bis (*p*-hydroxyphenyl) ethane, R = R' = H

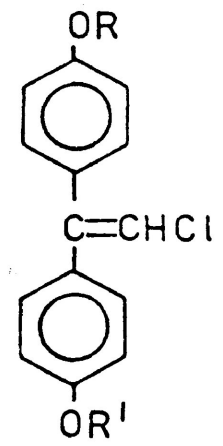
XII dibenzyldichloro derivative, 1,1-dichloro-2,2-bis (*p*-benzyloxyphenyl) ethane, R = R' = C_7H_7

FIG. 1-2. Methoxychlor and its metabolites and chemical derivatives.



XIII methoxychlor olefin, 1,1-dichloro-2,2-bis (*p*-methoxyphenyl) ethylene, R = R' = CH_3

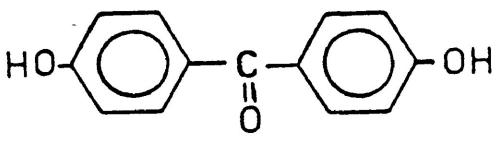
XIV hydroxychlor olefin, 1,1-dichloro-2,2-bis (*p*-hydroxyphenyl) ethylene, R = R' = H



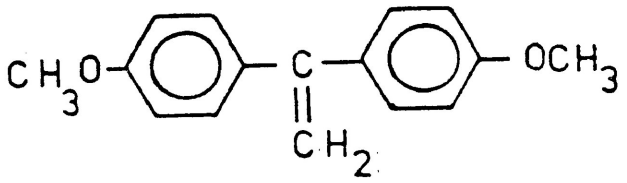
XV dimethoxymonochloro olefin, 1-chloro-2,2-bis((*p*-methoxyphenyl) ethylene, R = R' = CH_3

XVI dibenzylmonochloro olefin, 1-chloro-2,2-bis (*p*-benzyloxyphenyl) ethylene, R = R' = C_7H_7

XVII dihydroxymonochloro olefin, 1-chloro-2,2-bis (*p*-hydroxyphenyl) ethylene, R = R' = H



'XIX 4,4'-dihydroxybenzophenone



XVIII methoxy olefin, 2,2-bis (*p*-methoxyphenyl) ethylene

FIG. 1-2. cont'd

magnification of methoxychlor could be assessed. Using such a system methoxychlor was found in fish, at the top of the food chain, in concentrations 1500 times that found in water. Comparative studies with DDT showed magnification factors of 90,000 times. Methoxychlor appeared to be in dynamic equili brium with the ecosystem while DDT and its metabolites were stored in organisms at the highest tropic levels. Thus Kapoor's group was able to demonstrate clearly the biodegrada bility of methoxychlor through metabolism and "model ecosystem" studies and suggested the compound as a replacement for DDT. These authors concluded by stressing the need for further research concerning the metabolism and characterization of metabolites in organisms and their fate in the ecosystem.

Of course the fate of pesticides in the environment is not exclusively determined by biological factors. Thus, depend ing on the chemical reactivity of a pesticide, the action of sunlight, and the pH and mineral content of water and soil may also play a role in the degradation of these compounds. The photodecomposition of methoxychlor has been examined in water and as a thin film. Zepp *et. al.* (9) found that the major photolysis product in water was 1,1-dichloro-2,2-bis (*p*-methoxy phenyl) ethylene (XIII, see figure 1-2). MacNeil *et. al.* (10) investigated the photolysis of methoxychlor as a thin film sprayed on pyrex glass and in addition to the above olefin (XIII), another product 1,1-dichloro-2,2-bis (*p*-methoxyphenyl) ethane (X, see figure 1-2) was also formed. The degradation of methoxychlor in water has been studied by Wolfe *et. al.*(11).

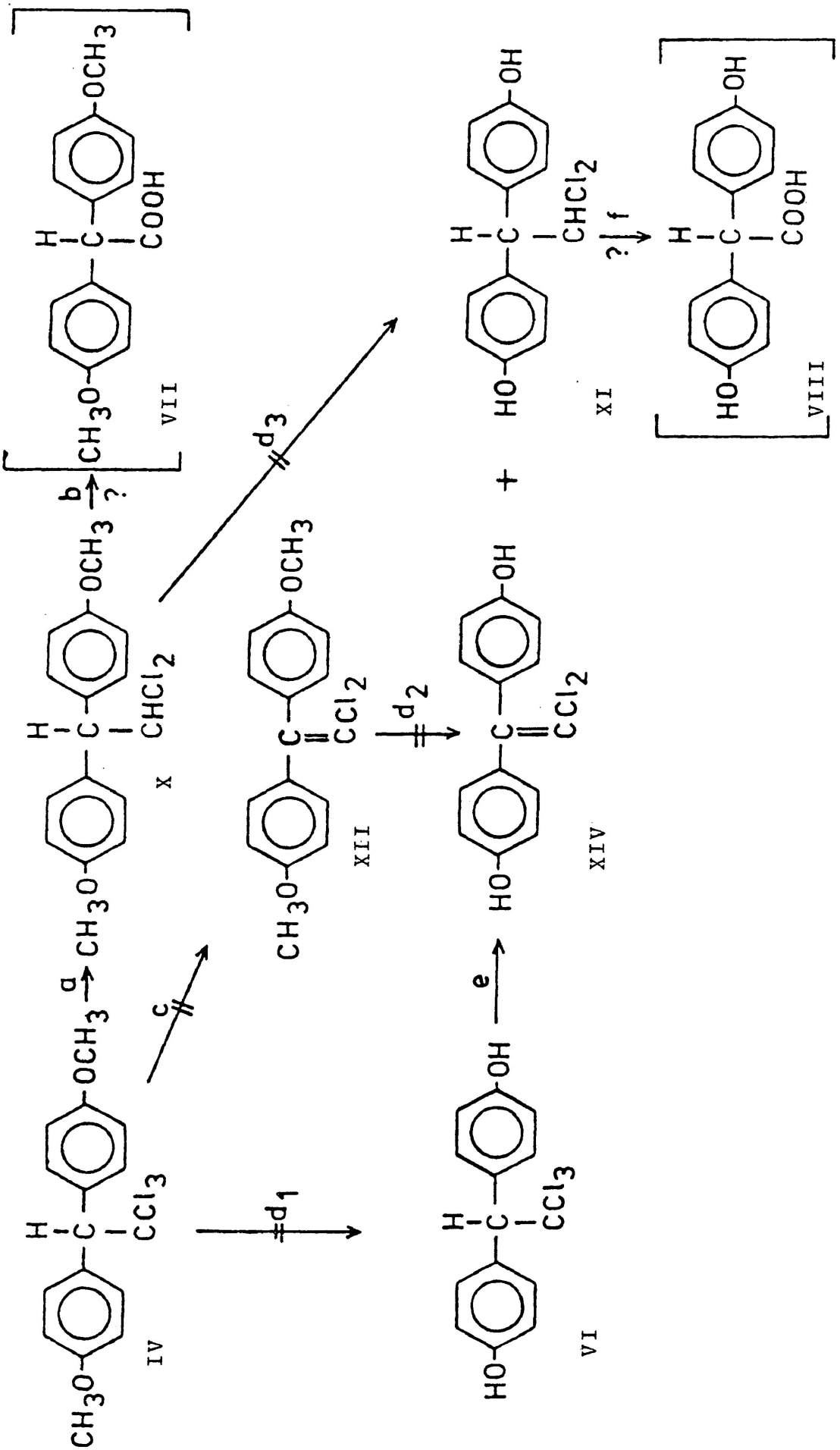
These authors reported that the degradation rate of methoxy chlor at 27°C and pH 5 was about twelve times faster than the rate of DDT under the same conditions. At pH 12 and 27°C Wolfe and co-workers found that the major degradation product was 1,1-dichloro-2,2-bis (*p*-methoxyphenyl) ethylene (XIII). Walker (12) on the other hand, reported that surface water samples with salinities up to 28ppt amended with methoxychlor and incubated in the dark at 30°C caused no change in methoxy chlor concentration even after 84 days.

The usage patterns of methoxychlor in Canada have also been reviewed. Gardner and Bailey (7) reported that methoxy chlor is mainly used for biting fly control. Methoxychlor has been used in aerial spray programs against mosquitoes and blackflies in Northern Canada. In Western Canada it has been used to control heavy infestations of blackflies on rangeland. In some of these areas methoxychlor is introduced directly into the surrounding water systems (calculated maximum concentration 0.31ppm) to kill larvae before they emerge as adults. These authors also pointed out that methoxychlor is strongly absorbed on suspended solids and sediment of lakes and streams during larviciding operations. It was suggested that more research in this area is needed in order to assess the behaviour of methoxychlor on soil and in benthic sediments, and particularly the interaction of this insecticide with microorganisms which inhabit these ecological niches.

Recently this area of study has come under closer

scrutiny by several authors. Baarschers *et. al.* (13) and Bharath (14) have studied the toxicity of methoxychlor and many of its degradation products which may be present in the envir onment (see figure 1-2, structures IV to VI and X and XI) towards several species of soil and aquatic fungi. These authors reported that the phenolic derivatives of methoxychlor were significantly more toxic to the test fungi than the parent insecticide. It was observed that the aquatic fungus *Saprolegnia parasitica* was most sensitive, being totally inhibited by hydroxychlor (VI) at concentrations as low as 3.1ppm. The above authors concluded their report by noting the significance of the degradation products, and stated that current scientific opinions concerning the use of "soft" pesticides, be tempered by the observation that the degradation products may also have the potential for environmental damage.

In a subsequent paper Baarschers *et. al.* (15) described the partial biodegradation of methoxychlor by the bacterium *Klebsiella pneumoniae*. These researchers demonstrated clearly that this bacterium was capable of degrading methoxychlor and the phenolic analogue hydroxychlor (VI) via reductive dechlor ination to the dimethoxydichloro derivative (X) and the dihydroxydichloro derivative (XI) respectively (see scheme 1-1, steps a and e). Incubation of the dimethoxydichloro derivative (X) with this bacterium also showed that this organism was incapable of cleaving the methoxy substituents. These authors also proposed a metabolic pathway (see scheme 1-1)



Scheme 1-1. Pathway suggested by Baarschers *et. al.* (15) for the degradation of methoxychlor by *K. pneumoniae*.

in which some of the known biodegradation steps of methoxychlor are formulated, others are potential routes formulated by analogy with known pathways for DDT (16). The above authors suggested that the diphenyl acetic acid derivatives (VII) and (VIII) may be the end products in the biodegradation of the corresponding dichloro derivatives (X) and (XI) respectively but this was not experimentally confirmed.

In another recent study Fogel *et. al.* (17) reported the biodegradation of methoxychlor in natural soil samples could be enhanced by exposing an initial anaerobic culture to aerobic conditions after three months. These authors reported that ring-U-^{14}C-labelled methoxychlor undergoes initial degradation to methoxychlor olefin (XIII, see figure 1-2) in anaerobic conditions but not in aerobic conditions. They stated that dehydrochlorination must be biologically mediated since samples poisoned with mercuric chloride did not degrade methoxychlor. Under sequential conditions (ie. anaerobic then aerobic) a 70 fold increase in $^{14}CO_2$ evolution with respect to strictly anaerobic conditions, was observed. Although the microorganism(s) responsible for the degradation were not identified, the formation of carbon dioxide from the aromatic ring carbon atoms under these conditions has not been previously reported. Incubation periods of three months under each condition were used and over this period these authors recovered only 10% of the initially added methoxychlor. It should be pointed out however that conversion to the olefin (XIII) and especially the

amount of carbon dioxide reported to be evolved represented only a small fraction of the methoxychlor that could not be recovered. It is unfortunate that these authors have used hexane as an extraction solvent in the recovery of methoxy chlor from soil samples. It has already been pointed out that methoxychlor can potentially undergo O-dealkylation to form other phenolic alteration products. These products due to their polarity, may not be extracted efficiently with a non-polar solvent such as hexane and thus would not be recov ered completely. Consideration of these polar metabolites could well have resolved mass balance discrepencies noted in the above report.

Bharath (14) in addition to the previously described toxicity study also investigated the metabolism of methoxychlor by various species of fungi. This author reported that three species, *Mortierella isabellina*, *Mortierella pusilla*, and *Saprolegnia parasitica* could, to some extent, metabolize methoxychlor via O-dealkylation. The main products observed were, methoxyhydroxychlor (V), and hydroxychlor (VI, see figure 1-2 for structures). He also reported that methoxychlor appeared to be strongly adsorbed by these fungi and because of this phenomenon the work was confined to qualitative measure ments of the metabolites. Bharath recognized that freshwater algae may also contribute to the degradation of methoxychlor and recommended that these organisms be studied.

The algae are a very important group of non-target

organisms which can also be exposed to pesticides. This diverse class of microorganisms can be found in both aquatic and terrestrial environments. In their role as primary producers the aquatic algae not only form the basis of most aquatic food chains but are also the primary oxygen producers in these ecosystems. The soil algae are probably the major contributor of photosynthesized organic material to the carbon cycle, inhibit soil erosion and increase the moisture-holding capacity of the soil. Some members principally the blue-green algae are able to fix atmospheric nitrogen thereby increasing the nitrogen content of the soil (18). Interference with the normal activities of algae may, therefore, be expected to have potentially serious consequences on the overall productivity of land-based and aquatic ecosystems.

The use of methoxychlor as a larvacide in water prompted several researchers to investigate the effects of this insecticide on the growth, photosynthetic ability, and nitrogen fixing ability of aquatic algae. For example, Poorman (19) conducted growth experiments with the aquatic species *Euglena gracilis* in pure cultures exposed to methoxy chlor. This author found that methoxychlor at concentrations of 100ppm depressed the growth of this alga by 17% for a 24 hour exposure period, while lower concentrations (ie. 10ppm or less) stimulated growth. Seven day exposure experiments with methoxychlor (100, 50, and 10ppm) all stimulated growth. How ever this author did not comment on the observed "lag" period

(ie. 24 hour experiment) nor its significance. Furthermore no attempt was made to investigate possible metabolic products from the stimulated cultures. Poorman did however point out that no morphological changes in the test alga exposed to methoxychlor were observed.

Butler *et. al.* (20) stressed the need for studying natural phytoplankton communities. Therefore these authors studied the effects of methoxychlor on the growth (in terms of biomass present) of algal isolates from 36 field samples and found that no significant change in growth occurred in these populations exposed to 0.001 and 0.01ppm methoxychlor respectively. In a subsequent paper, Butler *et. al.* (21) analyzed, by gas chromatography, extracts of the above 36 algal isolates after incubation with methoxychlor, to collect evidence for metabolism. The recoveries of methoxychlor for all 36 isolates ranged from 34 to 79% suggesting to these authors that metabolism may be responsible for the loss, although they did point out that disappearance of a pesticide from an actively growing algal culture may not conclusively demonstrate metabolism. It should also be pointed out that the above authors used a mixture of benzene and hexane to extract the algal cultures. In view of the polar nature of some potential metabolites this extraction method is subject to the limitations mentioned before (p. 12).

Page and Coleman (22) studied the effects of methoxy chlor (100ppb) on the photosynthetic ability of natural popu lations of freshwater algae *in situ*. Monitoring experiments,

conducted at 19 regular intervals over a period of several months on a succession of algal populations, showed that in only a few cases did methoxychlor inhibit photosynthesis. In these cases inhibition ranged from 9.7 to 64%. These authors also stated that it was difficult to correlate the inhibitory response with any one type of algae present. However, in one experiment a *Dinobryon sp.* and *Ankistrodesmus sp.* appeared most sensitive to this insecticide. It is unfortunate that the above authors did not include any of the known alteration products of methoxychlor in their study.

Kricher *et. al.* (23) observed the effects of methoxy chlor on the growth (biomass present) and productivity (photosynthetic carbon assimilation) of pure cultures of *Chlorella pyrenoidosa.* In this study the inhibition of algal growth after 7 days exposure to methoxychlor at a concentra tion of 100ppb was found to be 17%. The productivity of this species in the presence of 1ppm methoxychlor was also lowered to the same extent. In reviewing the above report an impor tant consideration concerning the results and experimental method has come to mind. In most liquid culture experiments, and as noted by the above authors, the insecticide is usually added to the culture as a solution in an organic solvent (in this case acetone). In the Kricher study (23) acetone was present to the extent of 1%, and these authors noted that 1% acetone caused extensive lysis of the *Chlorella* cells. Certainly the control cultures also contained acetone, however in view of the present results (see page 95) it could be

argued that Kricher *et. al.* may have unknowingly observed the synergistic effect of methoxychlor and acetone on the test alga.

The degradation and accumulation of methoxychlor by microorganisms was also studied by Paris *et. al.* (24). These authors, in addition to studying algae, included fungi and bacteria. Of the four bacterial populations screened for the interaction of this insecticide with pure cultures, only one *Flavobacterium harrisonii*, caused a decrease in methoxychlor concentration after 216 hours of incubation. The second order rate constant for the disappearance of methoxychlor was computed to be 1.1×10^{-13} hr.$^{-1}$. These authors noted that a 72 hour acclimation period was required by this bacterium before degradation commenced and that degradation ceased after 30% of the added methoxychlor was consumed. The main product of bacterial degradation was reported to be methoxychlor olefin (XIII). Experiments on fungal degradation were attempt ed by these authors, but the *Aspergillis sp.* used did not effect biodegradative changes.

Sorption studies on algae, fungi and bacteria for methoxychlor were also reported by the above group. Paris and co-workers found that all three types of organisms adsorbed methoxychlor, both in live cultures as well as the autoclaved cells. Equilibration times were 30 minutes for bacteria and algae and 16 hours for fungi. Comparative sorption studies on two species of bacteria revealed that the number of polar

groups on the cell wall may determine sorption rather than total lipid content of the cell. This group of researchers did not attempt to carry out degradation studies with methoxy chlor and algae. The physical adsorption of methoxychlor by freshwater algae has also been studied by O'Kelly and Deason (25), these authors report similar findings to those of Paris' group.

It is clear that some of the apparent inconsistencies in the reported sensitivity of algal growth and photosynthesis in the presence of methoxychlor may be due to species and strain differences. However many short term photosynthesis studies do not always reflect the results of long term growth studies. Perhaps future algal studies in pure culture concern ing organic pollutants can be modelled after protocols out lined by the U.S. Environmental Protection Agency proposed for inorganic pollutants (26). All too frequently samples were taken once after a set time period, rather than monitor ing algal growth effects regularly over a longer period of time. Also many studies restrict concentrations to one or two values within the solubility limits of the pesticide. Considering the adsorption of methoxychlor on organisms (24), soil and sediments (7) algae may very well be exposed to concentrations much higher than would be possible in aqueous solution. Reports on the metabolism of methoxychlor by algae appear to be non-existent. This cannot be due to a lack of an intrinsic ability of these organisms for degrading

organochlorine pesticides. For example, Alexander (27) reported that DDT was degraded by a *Cylindrospermum sp.* and Sweeny (28) has observed the metabolism of lindane by a *Chlorella sp.*

It is clear from the foregoing discussion that bacteria and fungi play an important role in the degradation of methoxy chlor. The contribution of algae is uncertain and has received relatively little attention. Also the elucidation of the effects of metabolites and other environmental alteration products of methoxychlor is extremely important because these substances may be more toxic or may have different mobilities than the parent insecticide especially in aquatic microbial environments.

In view of the above statements, the purpose of this thesis then, is twofold:

(i) To observe the effects of methoxychlor and its alteration products on the growth of green algae and to investigate the possibility that these photosynthesizing microorganisms may also be involved in the degradation of this insecticide.

(ii) To expand, in anticipation of further degradative work, the study of the synthetic chemistry and physical properties of methoxy chlor metabolites and derivatives.

For the first of these two objectives two species of green algae, *Chlorella pyrenoidosa* and *Chlorella vulgaris* were

selected.

Chlorella pyrenoidosa was chosen primarily because it is a common species found in both terrestrial and aquatic environments. It is also particularly sensitive to photo synthetic and respiratory inhibitors and as such would make an ideal test organism (29). *Chlorella vulgaris*, a common soil alga, was chosen mainly for its reported ability to metabolize other organochlorine compounds (28).

While the biological work described in this thesis is of primary importance from an environmental viewpoint, another aspect of methoxychlor study became evident during the course of this work. Many synthetic procedures, cited in the envir onmental literature for the preparation of methoxychlor derivatives, have been derived from earlier unrelated synthetic studies. Consequently some of the methods reported are inefficient, lack full experimental details or result in prod ucts with questionable purities. Therefore it was found necessary to devote considerable effort to reinvestigate several synthetic procedures in order to improve yields in the preparation of several known methoxychlor derivatives. In addition, some methoxychlor alteration products were prepared which have not been described in the literature before.

For example, in the previously described degradation study (p. 9) a few gaps in the degradation pathway (scheme 1-1) still exist. In response to this Bharath (14) attempted the synthesis of 1-monochloro-2,2-bis(p-hydroxyphenyl)ethylene

(dihydroxymonochloro olefin, XVII, see figure 1-2) which was considered by him to be a potential metabolite and required for further study. Unfortunately this synthesis resulted in an anomalous product which was only partially characterized. Thus, considerable effort has now been made not only to prepare this compound (XVII) but also to investigate Bharaths original synthesis. At the present time the multistep synthe sis of the dihydroxymonochloro olefin (XVII) has been partially completed. It should also be pointed out that during the course of the present study Davison *et. al.* (30) identified this olefin as one of many metabolites isolated from goats fed methoxychlor, without however, describing a chemical synthesis.

Another serious problem concerning the environmental chemistry of methoxychlor relates to the lack of reliable information on the extractability and solubility of the various alteration products (metabolic or photochemical) of methoxychlor. If the recovery and detection of these compounds from biological and environmental samples are to be accomplished with efficiency and confidence then quantitative measurements must be made regarding these properties. This lack of physical data, not only for methoxychlor but also many other pesticides reported in the environmental literature, has been criticised recently. For example, Gunther *et. al.* (31) found that out of 738 reports dealing with pesticide solubility, 297 papers contained "useless" data (ie. reported

solubility in descriptive terms like, soluble, not soluble etc.). In another review by Dao *et. al.* (32) on pesticide extraction, it was noted that no obvious consideration for the solubility of the residual pesticide in the organic solvent used for extraction was reported. Clearly the need to do further research in this area is indicated.

CHAPTER II: CHEMICAL AND PHYSICAL PROPERTIES OF METHOXYCHLOR AND ITS DERIVATIVES

Introduction

Various derivatives of methoxychlor are known from photodegradation (9) and biodegradation studies (8). Others, not yet isolated in such studies, may be anticipated to be of importance based on analogies with the biodegradation of DDT. Such derivatives (V to XIX, p. 5 and 6) are not commercially available, however methods for their preparation have been published and several of these methods have been used in the present investigation. In many instances these published procedures give little in the way of experimental details or suffer from less than satisfactory yields. Thus, the major emphasis of the present synthetic work was to prepare and characterize some of the anticipated metabolites which will no doubt be isolated, as studies in this area progress. Probably the most important of these metabolites are the diphenyl acetic acids (VII and VIII), and the dihydroxymono chloro olefin (XVII).

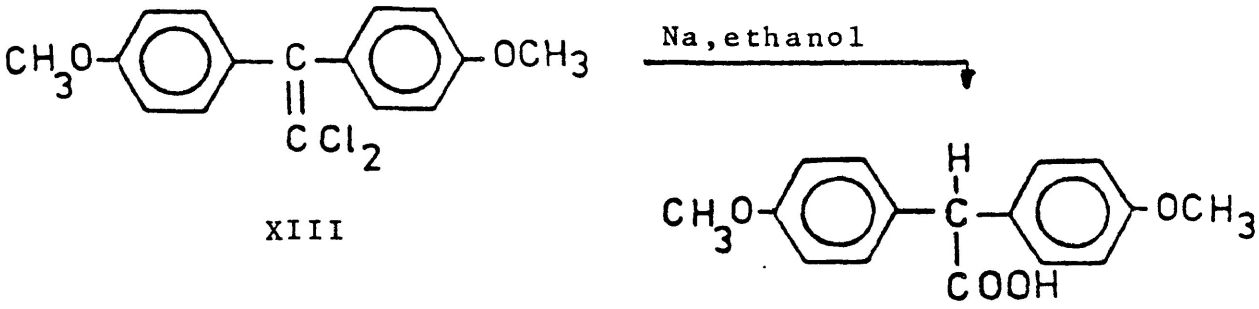
In addition to this synthetic work some of the existing preparative methods have been modified so as to provide better yields and purer products. In view of the above mentioned scarcity of factual information on solubilities and extracta bility of some of these compounds, several such measurements are included in this study (p.80).

Preparations

The preparation of 2,2-bis(*p*-methoxyphenyl) acetic acid (VII) and 2,2-bis(*p*-hydroxyphenyl) acetic acid (VIII) has been described by a number of authors.

Hubacher (33) prepared the dimethoxy acid (VII) by heating 1,1-dichloro-2,2-bis(*p*-methoxyphenyl)ethylene (XIII) with metallic sodium and ethanol in a nickel autoclave (see page 24, equation 2-1). Although the acid was characterized by elemental analysis and melting point (110°), the method is somewhat inconvenient and the major disadvantage is the poor yield reported (18%). In the same paper, Hubacher has also described two methods for preparing the dihydroxy acid (VIII). The first method (equation 2-2), involved baking (210°) a dry mixture of the dimethoxy acid (VII) with pyridine hydrochlor ide. Apparently this was the only demethylating procedure which yielded the desired product, whereas conventional methods were stated to give tars. This author recrystallized the crude dihydroxy acid from benzene and reported the melting point as 146.5-151°, but the yield was not stated. In the second method described (equation 2-3), the dihydroxy acid (VIII) was prepared via the condensation of phenol with glyoxylic acid in aqueous sulphuric acid. The method is very convenient and a fair yield (45 to 58%) was reported. Hubacher noted that a by-product of this reaction, 3-(*p*-hydroxy phenyl)-2-coumaranone (XXIV) was also isolated and probably formed by the *ortho-para* condensation and subsequent lactone

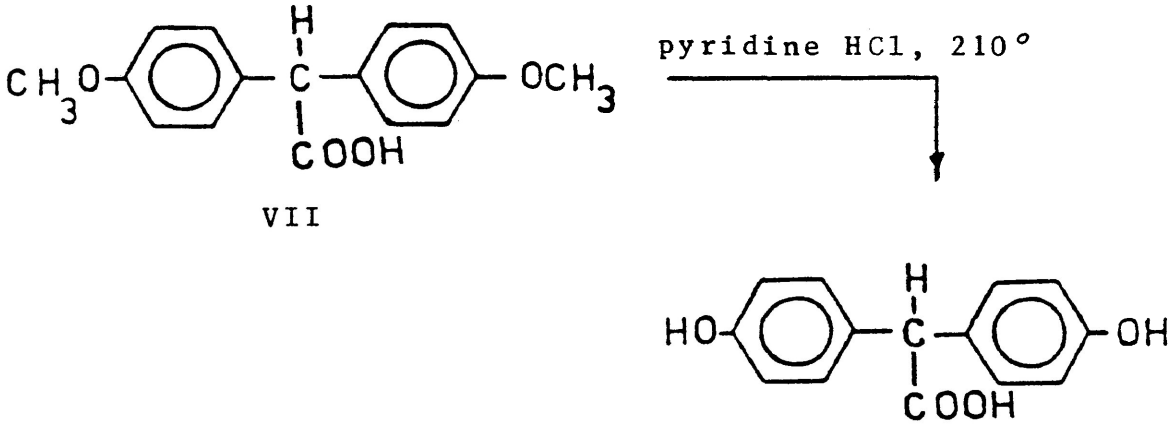
Eq. 2-1



XIII

VII

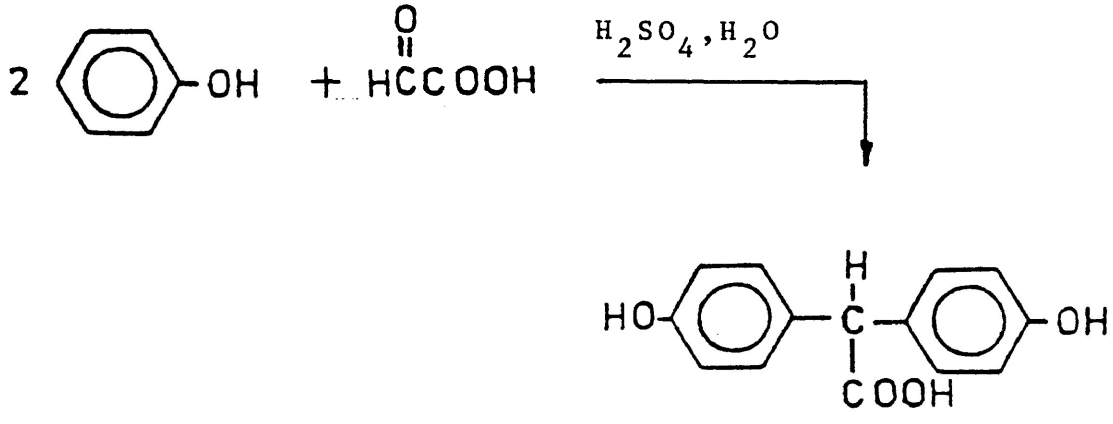
Eq. 2-2



VII

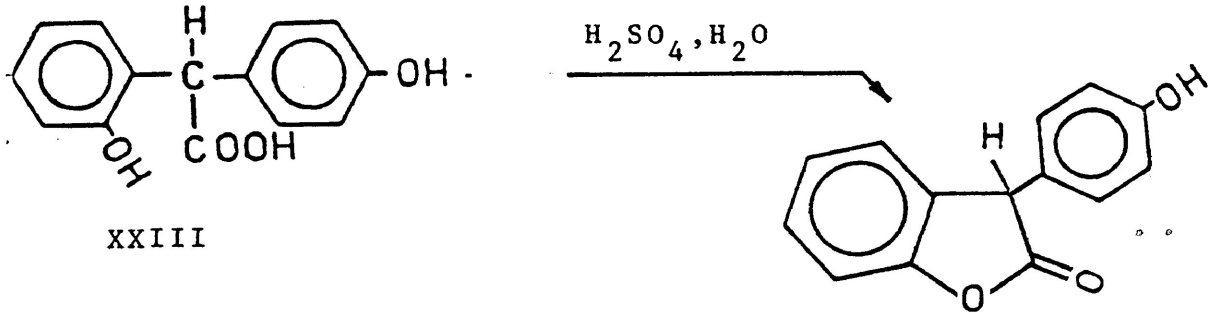
VIII

Eq. 2-3



VIII

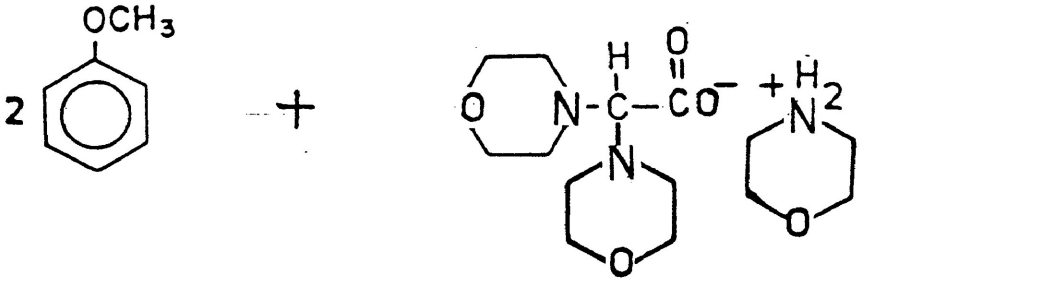
Eq. 2-4



XXIII

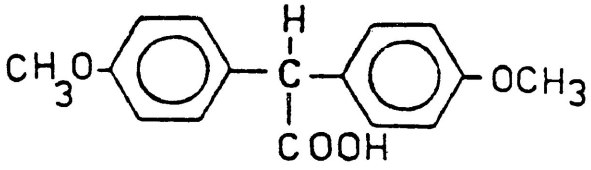
XXIV

Eq. 2-5



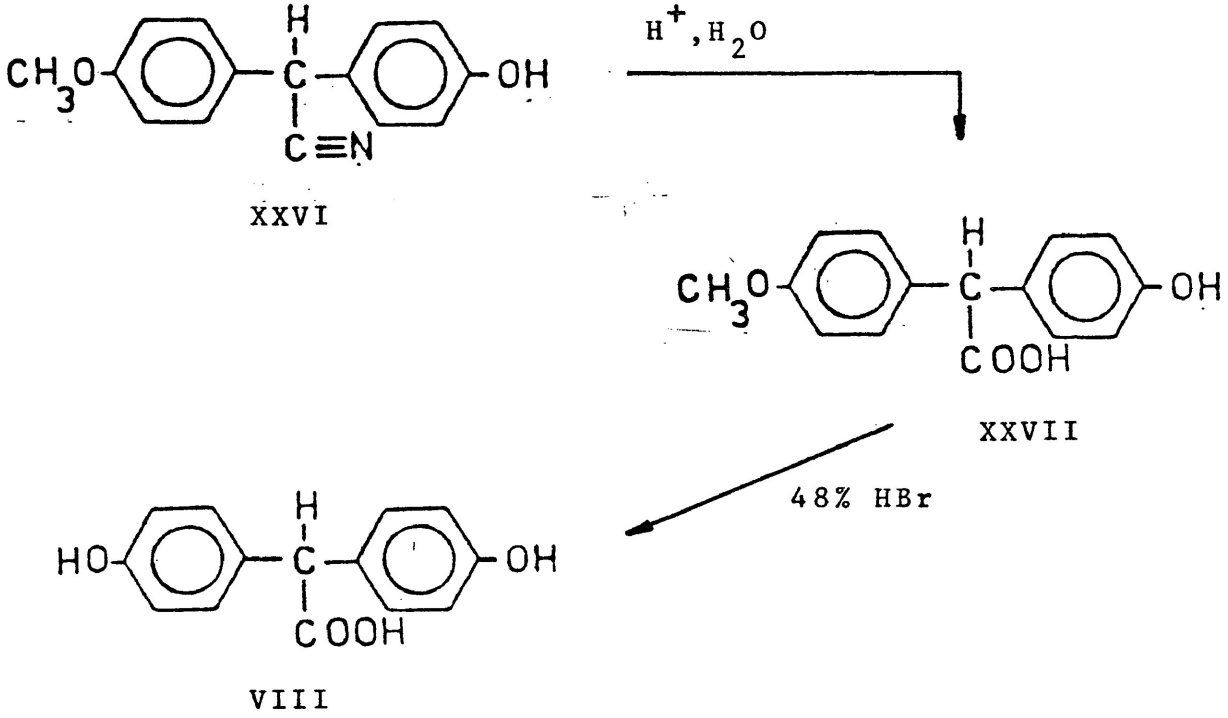
XXV

CH_3COOH, H_2SO_4, H_2O



VII

Eq. 2-6



formation of the initially formed 2-(*o*-hydroxyphenyl)-2-(*p*-hydroxyphenyl)acetic acid (XXIII) (see equation 2-4).

Brault and Kerfanto (34) developed a general procedure for the preparation of various diaryl acetic acids employing the morpholine salt of α,α-di(N-morpholino)acetic acid (XXV) prepared from dichloroacetic acid (see equation 2-5). These authors prepared the dimethoxy acid (VII) by condensing anisole with the above salt in an aqueous mixture of acetic and sulphuric acids. The reported yield of this reaction, based on the morpholine salt precursor dichloroacetic acid, was 74%. It was suggested that the morpholine salt acts as

a potential source of glyoxylic acid for the reaction. It is not known whether the dihydroxy acid (VIII) could be prepared in this way.

Nathanson (35) has reported the preparation of the dihydroxy acid (VIII) without the use of glyoxylic acid (see page 26, equation 2-6). This author first prepared 4-hydroxy-4'-methoxydiphenylacetonitrile (XXVI) by the condensation of phenol with the cyanohydrin of anisaldehyde in the presence of sulphuric acid. The acetonitrile was then hydrolyzed to the corresponding acid (XXVII), which was subsequently demethylated with 48% HBr in glacial acetic acid to yield the dihydroxy acid (VIII). There is little advantage of using this method as compared to Hubacher's one step synthesis. However, it may be argued that Nathanson's reaction is of greater synthetic utility for it appears that the method could be expanded as a potential route to the less accessible unsymmetrically substituted acids, such as 4-hydroxy-4'-methoxy diphenylacetic acid (XXVII).

In the present work a modified synthesis for the preparation of the two acid derivatives (VII and VIII) of methoxychlor was developed. This method has three distinct advantages as compared to the previous methods: Firstly, it avoids the use of an autoclave and both acids may be prepared at room temperature; secondly by-products are not formed in any appreciable amounts which would lower the yield of the desired product, and thirdly the method can be applied to the

preparation of both the dimethoxy acid (VII) and the dihydroxy acid (VIII). Thus, 2,2-bis(*p*-methoxyphenyl)acetic acid (VII) was prepared by condensing anisole and glyoxylic acid in the presence of concentrated sulphuric acid with glacial acetic acid as the solvent. It was found that temperature control of the reaction mixture is important and during the addition of the sulphuric acid the temperature should not rise above 30°, otherwise an appreciable amount of polymeric material is formed. The acid (VII) was obtained as a colourless syrup which crystallized on standing. The yield of the reaction based on glyoxylic acid was 80%. Several attempts to recry stallize this acid according to details in the literature were unsuccessful. The purity of this compound was therefore determined indirectly by gas chromatography, combustion analysis and determination of the neutralization equivalent. In addition, the methyl ester (IX) of this acid was prepared using boron trifluoride-methanol reagent. The methyl ester was a crystalline compound (m.p. 63.5-64°) which was fully characterized by combustion analysis.

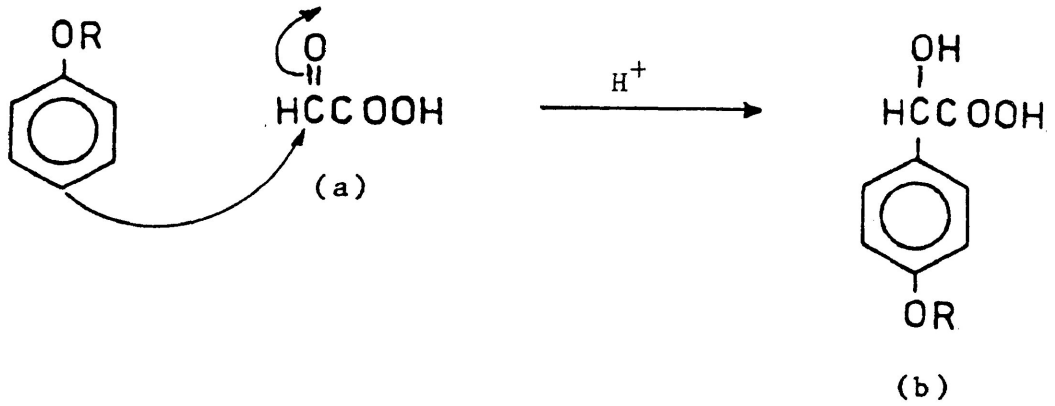
The synthesis of 2,2-bis(*p*-hydroxyphenyl)acetic acid (VIII) was accomplished similarly, using phenol instead of anisole. The yield of this reaction was 42% and comparable to that reported in the literature (33). The dihydroxy acid (VIII) was readily recrystallized from a mixture of ether and benzene, and the solvent of crystallization could be removed by heating the material in a high vacuum. The acid (VIII)

thus obtained could be characterized by combustion analysis. The attempted esterification of the dihydroxy acid (VIII) with boron trifluoride-methanol reagent led to the esterification of the carboxyl group as well as the methylation of the phenolic hydroxy groups, resulting in the formation of the methyl ester of the dimethoxy acid (IX) (see above). No further attempts to prepare the methyl ester of the dihydroxy acid (VIII) were made. Gas chromatographic analysis of silylated samples of the dimethoxy (VII) and dihydroxy (VIII) acids on an SE-30 (5%) column (see p.108) proceded smoothly and the respective retention times relative to DDT were 0.84 and 1.25.

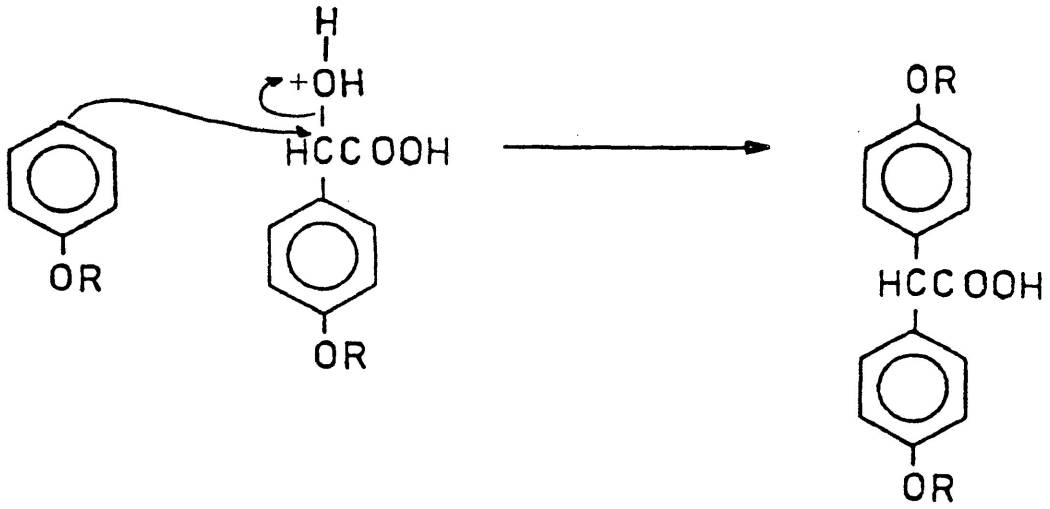
The mechanism for the formation of these acids is analoguous to that of the Baeyer condensation reaction (36). This general reaction mechanism is presented in scheme 2-1 on page 30. The first step is the nucleophilic addition of a substituted benzene to the glyoxylic acid (a) molecule resulting in the formation of a secondary alcohol intermed iate (b). The second step involves the acid catalyzed substitution of the hydroxyl group of this intermediate with a second molecule of the substituted benzene, yielding the diaryl acetic acid with the loss of a molecule of water.

The NMR spectra (figures 2-1 and 2-2) of the dimeth oxy (VII) and dihydroxy (VIII) acids respectively, which have not been described in the literature before are very distinc tive. Common to both spectra is the characteristic "quartet"

Step one:



Step two:



VII or VIII

R= H, CH_3

Scheme 2-1. Reaction mechanism for the formation of the diphenyl acetic acids, (VII) and (VIII).

in the aromatic region indicative of a para-substituted benzene ring moiety. The additional fine structure surrounding the main signals, further confirms this structural feature (37). This "quartet" corresponding to eight aromatic protons is located between 6.75-7.25ppm for the dimethoxy acid and 6.65-7.18ppm for the dihydroxy acid. Another common feature is the signal arising from the benzylic proton. This signal, a singlet, appears at 4.91ppm and 4.8ppm for the dimethoxy and dihydroxy acids respectively. The slight upfield shift of this signal for the dihydroxy acid can be attributed to the greater electron donating effect of the hydroxy substituents (38). This effect and its influence, on the acidity of the benzylic hydrogen in other methoxychlor derivatives, also plays a role in the dehydrohalogenation reactions discussed below (p.74). The signal corresponding to the six methoxy protons of the dimethoxy acid occurs as a well defined singlet at 3.75ppm and is characteristic of this acid. A broad signal at 10.75ppm was attributed to the carboxyl group proton of this acid. The proton signals arising from the phenolic hydroxy groups and the carboxyl group of the dihydroxy acid (VIII) were not observed (see figure 2-2). This is a well known phenomenon in NMR spectrometry and is due to the dependence of these "active hydrogen" signals on solvent and concentration effects (39). However a comparison of the integrals of the aromatic and benzylic proton signals revealed a ratio of 8:1 respectively, which is in agreement with the

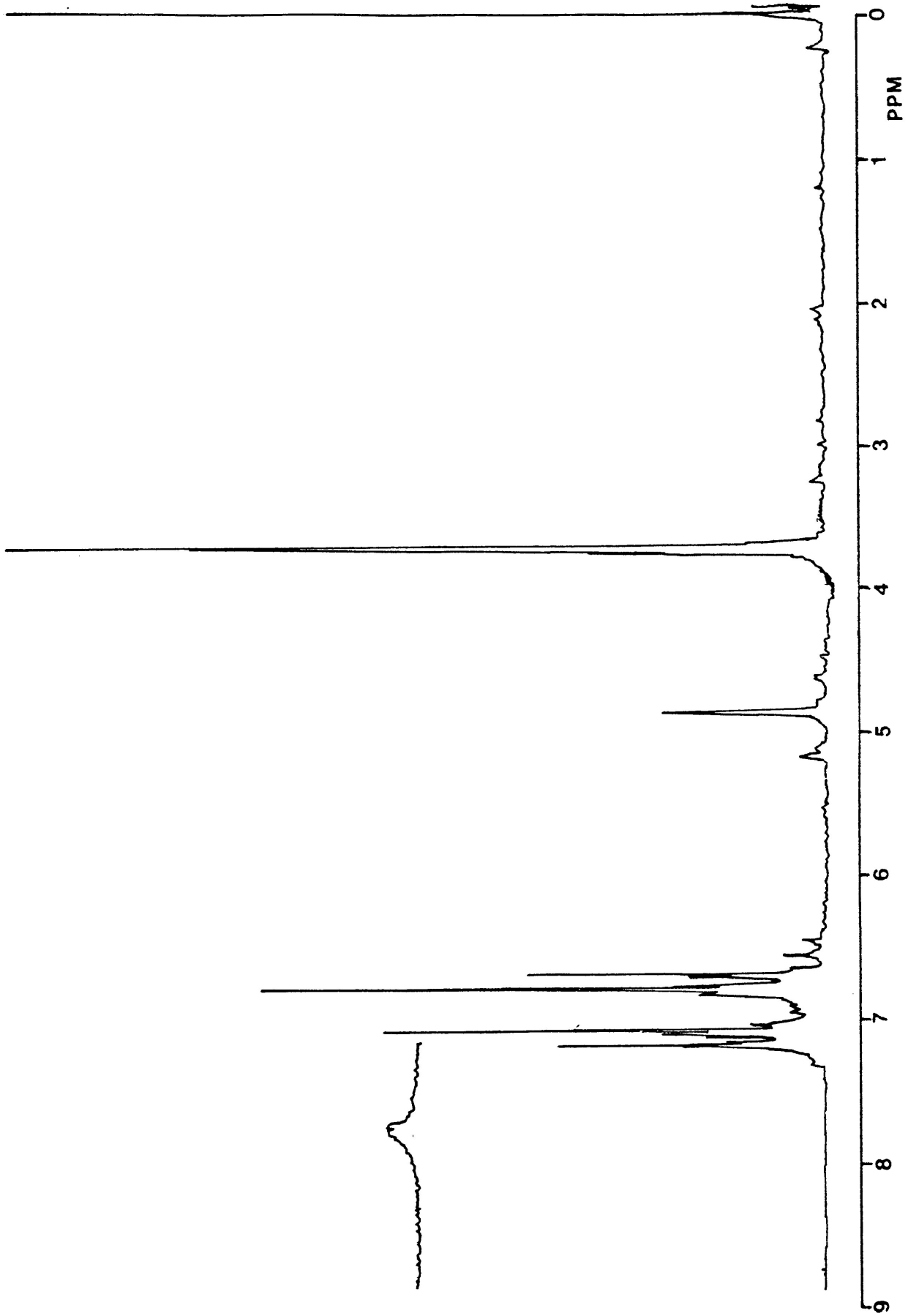


FIG. 2-1. NMR spectrum 2,2-bis(*p*-methoxyphenyl)acetic acid, VII.

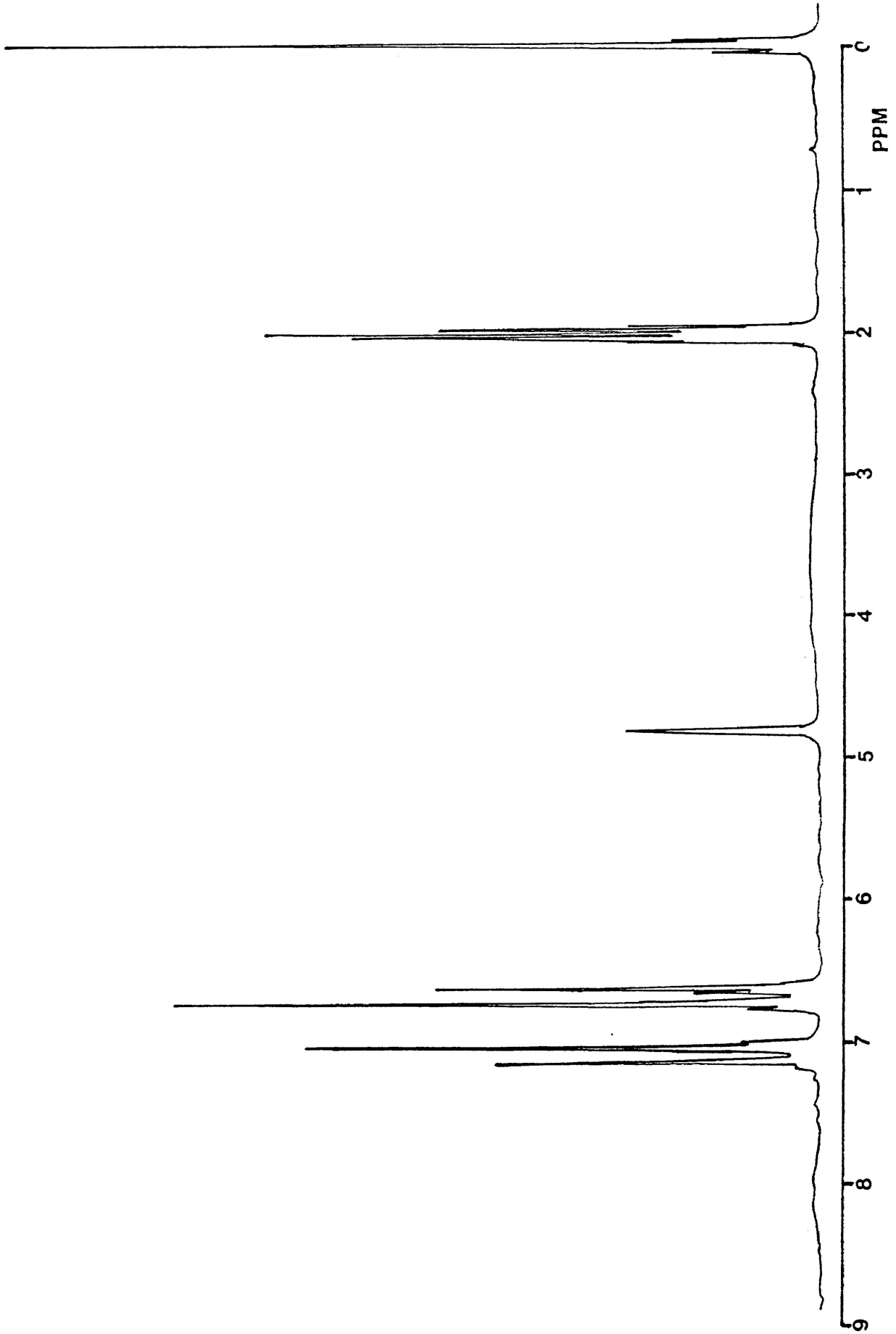


FIG. 2-2. NMR spectrum 2,2-bis(*p*-hydroxyphenyl)acetic acid, VIII.

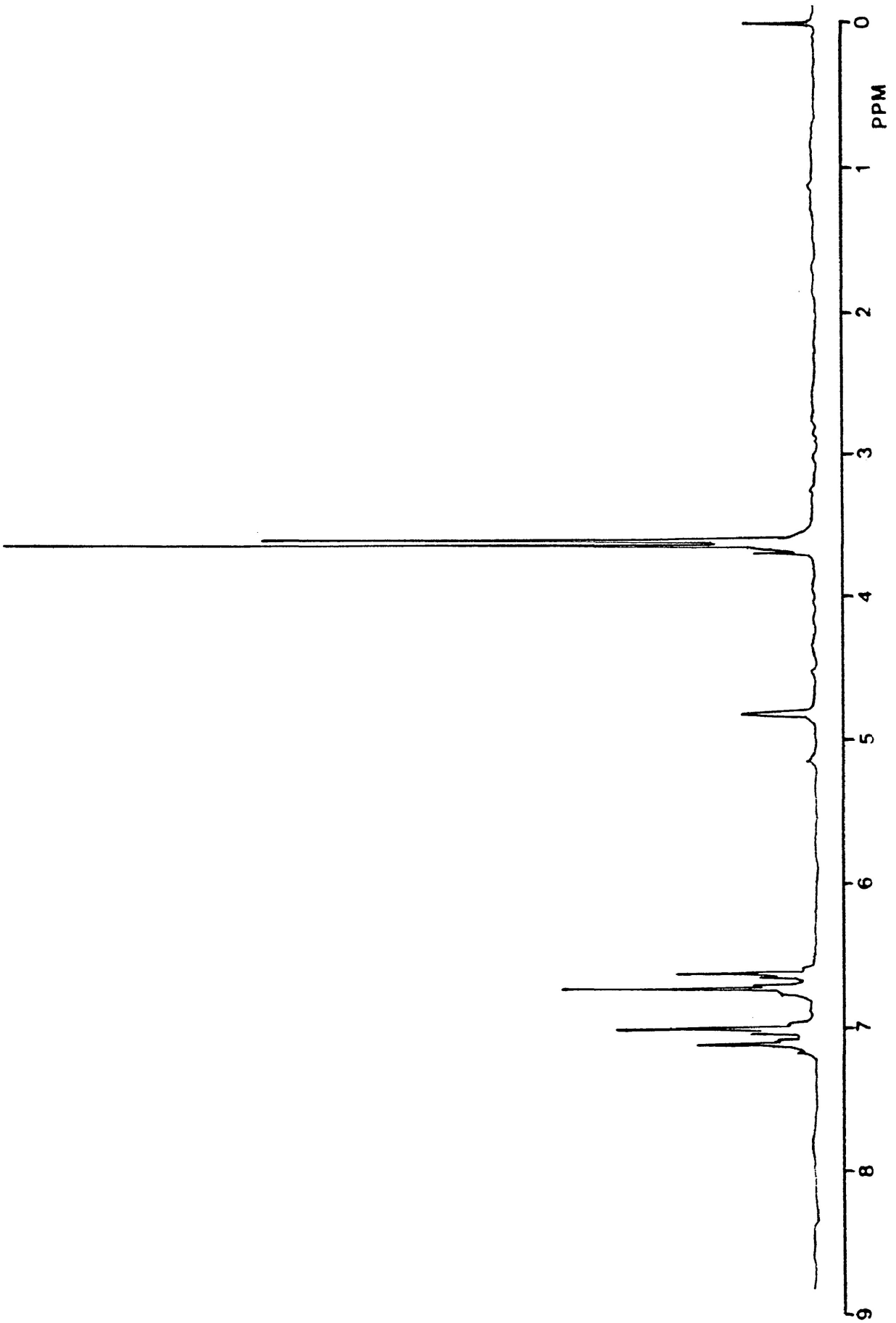


FIG. 2-3. NMR spectrum 2,2-bis(*p*-methoxyphenyl)acetic acid methyl ester, IX.

structure of this acid.

The NMR spectrum (figure 2-3) of the methyl ester of the dimethoxy acid (IX) is in agreement with its structure. The only new feature of their spectrum is the signal at 3.65 ppm corresponding to the three methoxy protons of the ester group. In all other respects the spectrum is similar to that of the parent acid.

The mass spectrum (figure 2-4) of the methyl ester (IX) contained a clearly observable molecular ion peak at m/e 286. The base peak at m/e 227 results from the concerted loss of carbon dioxide and a methyl radical (see scheme 2-2).

The identity and purity of the dihydroxy acid (VIII) was similarly confirmed by the mass spectrum, which contained the molecular ion peak at m/e 244.

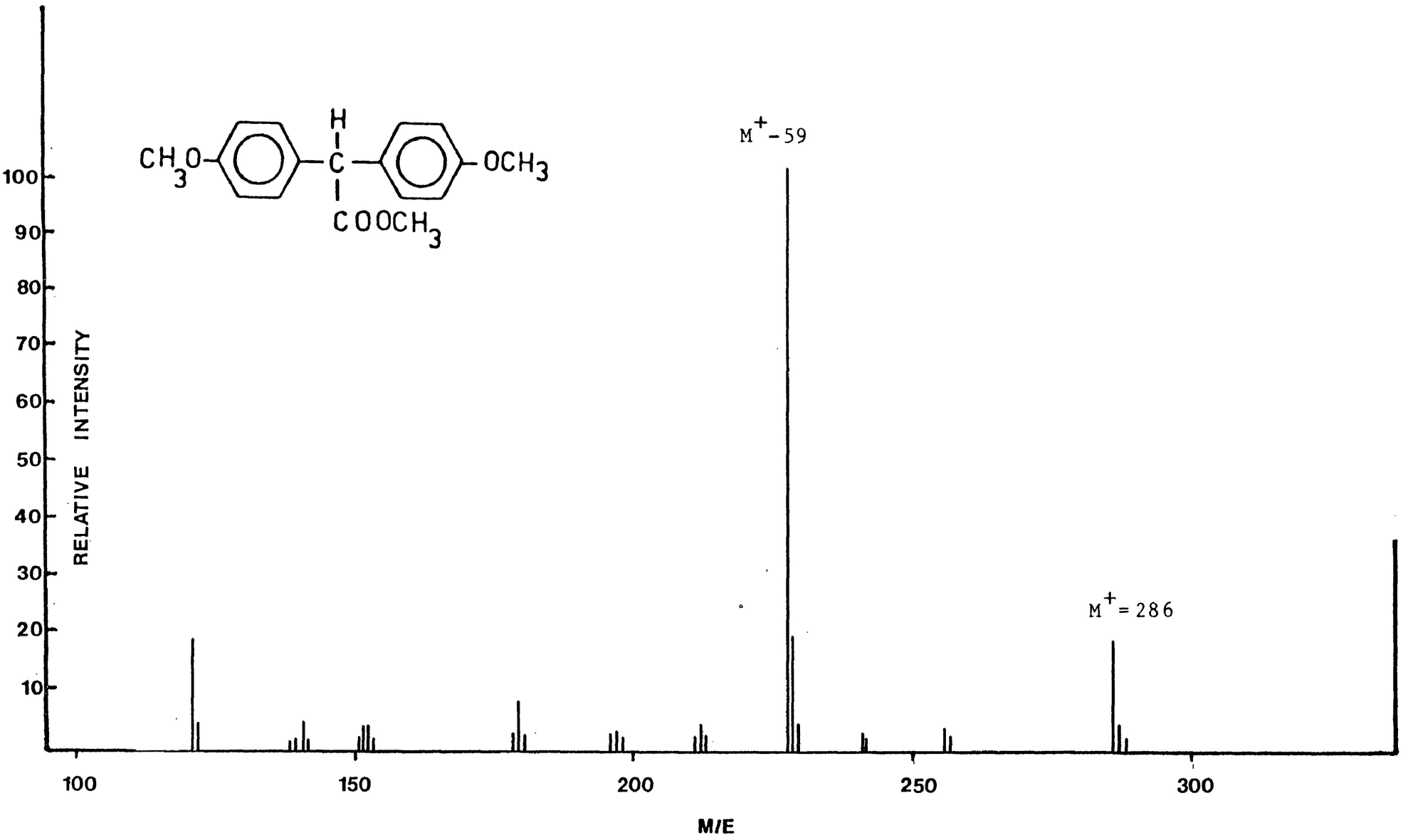
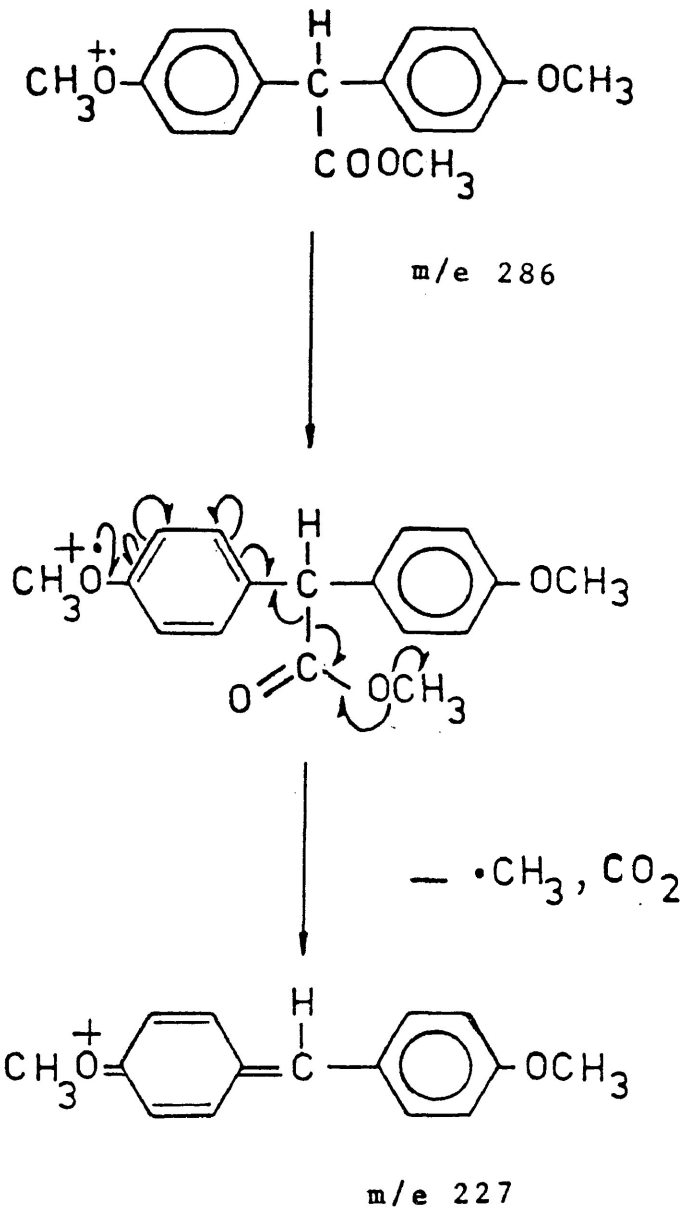


FIG. 2-4. Mass spectrum of 2,2-bis(*p*-methoxyphenyl)acetic acid methyl ester, IX.

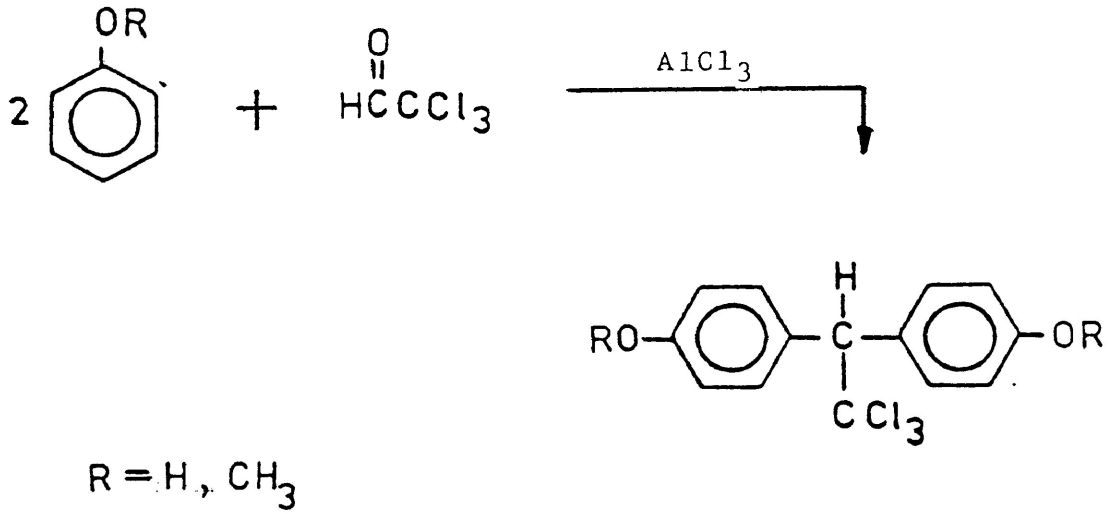


Scheme 2-2. Mass spectral fragmentation pattern for 2,2-bis (*p*-methoxyphenyl)acetic acid methyl ester, IX.

Bharath (14) prepared a number of symmetrical and unsymmetrical derivatives of methoxychlor, using literature methods, and noted that the yields of some of these proced ures are disappointingly low. The syntheses of several of these compounds were re-investigated and improved methods for their preparation have been developed.

One general modification involved the sequence of addition of the various reagents. The condensation of a substituted aromatic compound with a chlorinated acetaldehyde according to equation 2-7 is usually catalyzed by aluminum trichloride, added as a dry powder to the reaction mixture. It was found that better results could be obtained by prepar ing a slurry of aluminum trichloride in the reaction solvent, to which the other reagents are then added subsequently. This allowed for better protection of the moisture sensitive Lewis acid catalyst and greater ease of manipulation.

Eq. 2-7.



The preparation of 1,1,1-trichloro-2,2-bis(*p*-hydroxy phenyl)ethane (VI, hydroxychlor) was the one exception where the above general modification did not lead to an improvement in yield. In fact, only 8% of the desired compound was obtained, which is similar to the results obtained by Bharath (14).

However, hydroxychlor (VI) could be prepared in much shorter time and significantly better yield by using concen trated sulphuric acid as the catalyst and carbon tetrachloride as the solvent (method (b), see page115). Thus, when phenol was dissolved in a chilled, heterogeneous mixture of carbon tetrachloride and concentrated sulphuric acid, and a solution of chloral was added dropwise to the vigourously stirred mixture, hydroxychlor (VI) was obtained in 21% yield in one hour.

The synthesis of 1,1-dichloro-2,2-bis(*p*-methoxyphenyl) ethane (X) was carried out according to the procedure reported by Zepp *et.al.* (9). The yield of this reaction was not reported by these authors. When this method was modified in terms of the addition sequence, as described above, it was found that the dimethoxydichloro derivative (X) could be obtained in 22% yield.

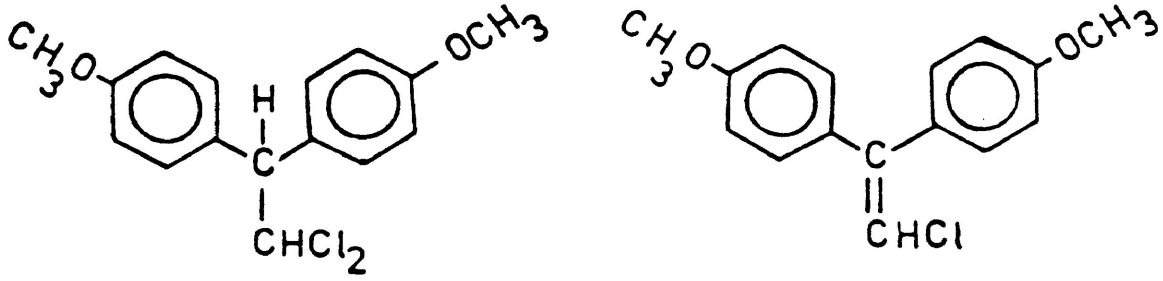
An improved yield was also achieved for the synthesis of 1,1-dichloro-2,2-bis(*p*-hydroxyphenyl)ethane (XI). This compound was obtained in 20% yield by condensing phenol and dichloroacetal in the presence of anhydrous aluminum trich

loride using carbon disulphide as the solvent. This modified procedure using carbon disulphide rather than chloroform (as reported in the literature (14)) resulted in a 53% increase in yield as compared to the literature preparation. This improvement in yield is particularly significant in view of the fact that appreciable quantities of this compound were required for the study of the unusual rearrangement reaction reported by Bharath (see page 60).

The 1-chloro-2,2-bis(*p*-methoxyphenyl)ethylene (XV) derivative of methoxychlor, which was not prepared by Bharath, was also prepared in the present study by modifying a method reported in the literature. Mendel *et. al.* (16) prepared this compound by refluxing the dimethoxydichloro derivative (X) in a 2% solution of sodium hydroxide in ethanol. In the present study, various dehydrohalogenation experiments (see page 74) indicated that the dimethoxymonochloro olefin (XV) could be prepared under a number of reaction conditions. From these experiments one method was adopted as a preparative procedure. Thus, this dimethoxymonochloro olefin (XV) was obtained in 73% yield by refluxing the dimethoxydichloro derivative (X) in a 1N methanolic potassium hydroxide solu tion. The pure product (m.p. 79°) was obtained after recrystallization from ethanol.

The NMR spectrum (figure 2-5) of 1-chloro-2,2-bis(*p*-methoxyphenyl)ethylene (XV), which has not been described in the literature before, is a good example of how NMR spectrom etry can be used for confirming structural changes as the

result of a given reaction. It is evident from the structures (shown in diagram, 2-1), that the starting material (X) is a symmetrical molecule with its plane of symmetry lying between the two aromatic rings. No such plane can be assigned to the structure of the product molecule (XV) and therefore it is unsymmetrical.



X XV

Dia. 2-1. Symmetry differences between compounds X and XV.

A comparison of the NMR spectra (figure 2-5 and 2-6) of these compounds clearly shows this difference in symmetry in terms of the methoxy group signals in the region from 3.5 to 4.0ppm. In the symmetrical dimethoxydichloro derivative (X) the two methoxy groups are in an identical chemical environ ment and therefore give rise to a single six proton signal. In the unsymmetrical dimethoxymonochloro olefin (XV), however the two methoxy groups are in slightly different chemical

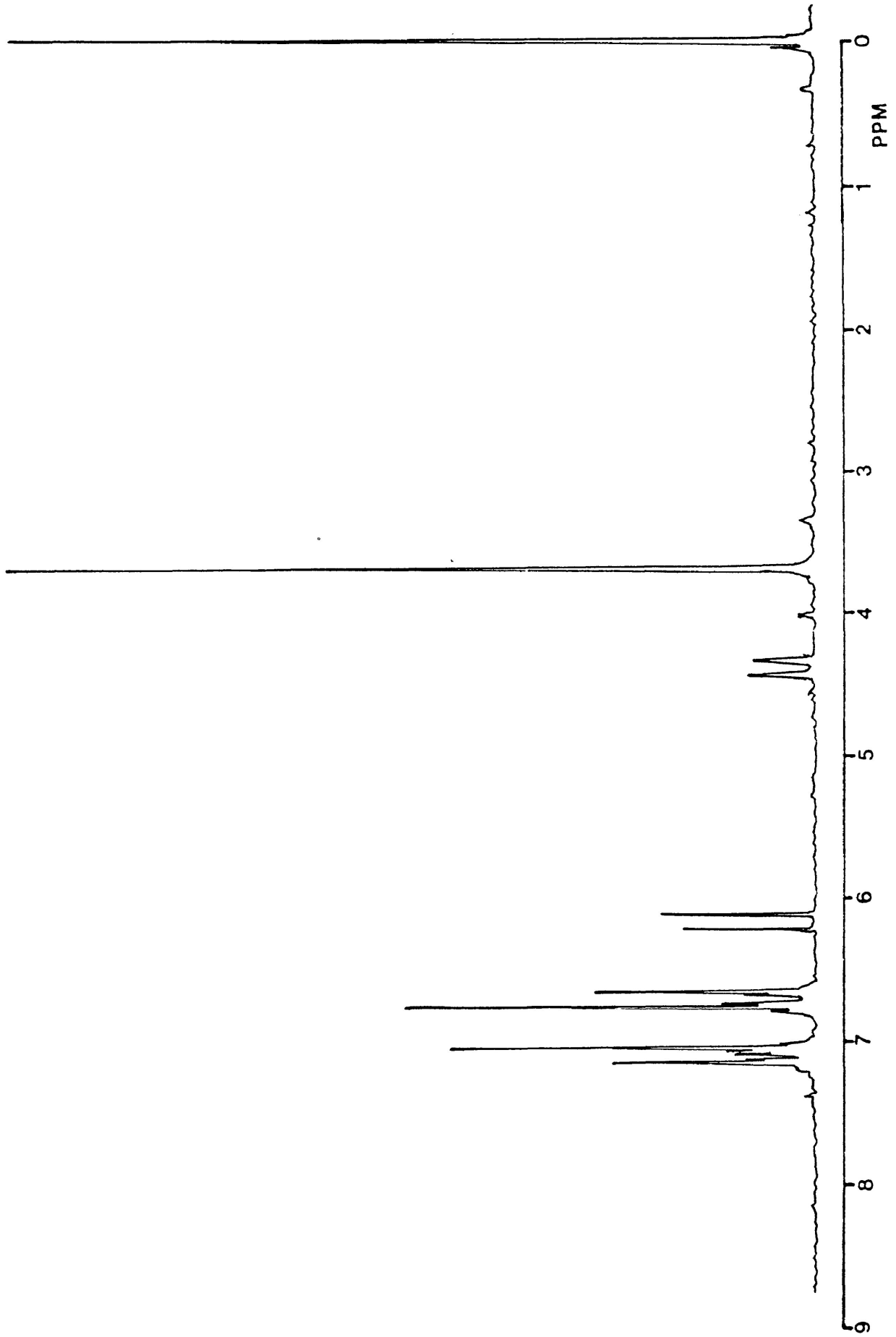


FIG. 2-5. NMR spectrum 1,1-dichloro-2,2-bis(*p*-methoxyphenyl)ethane, X.

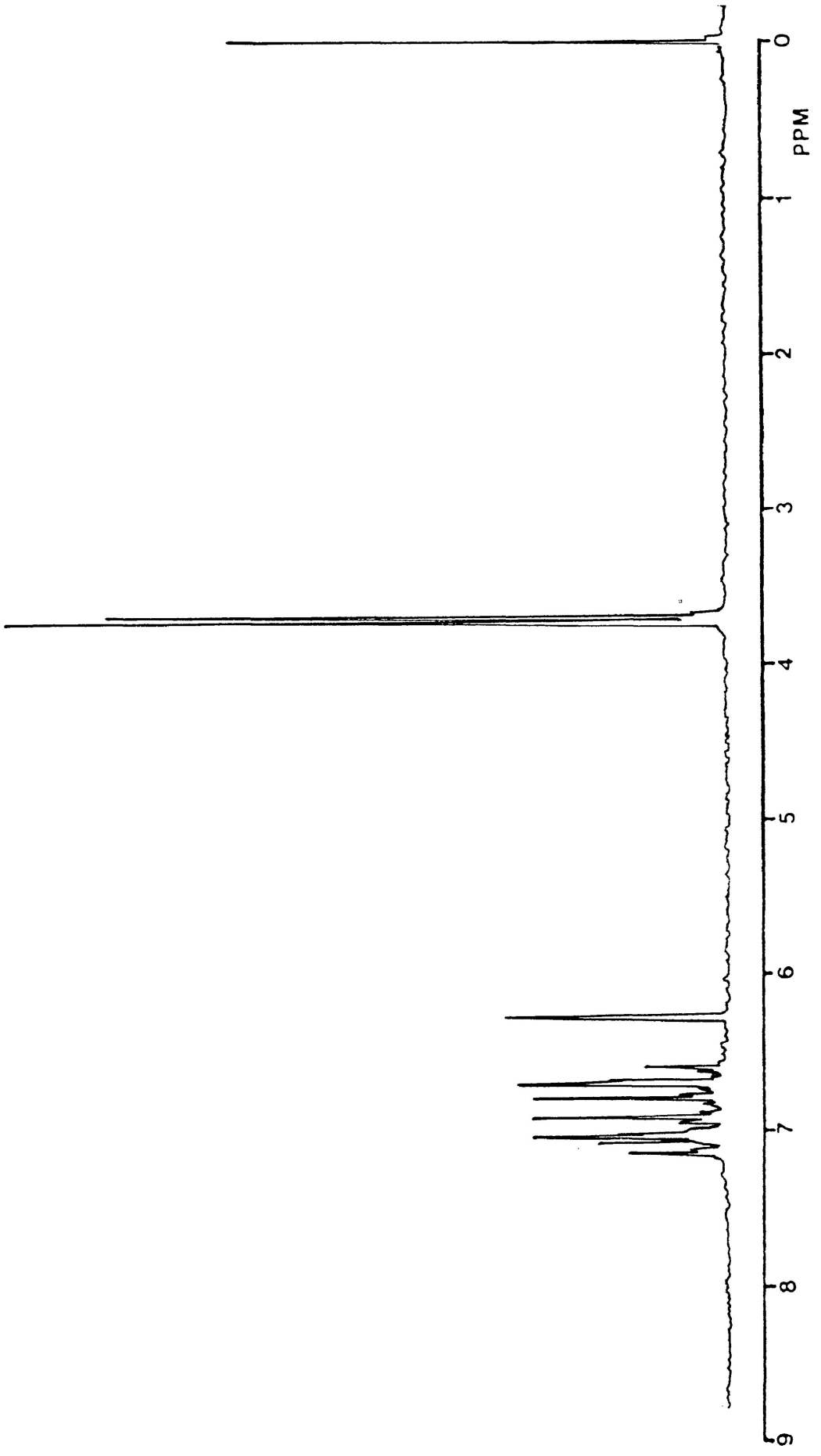


FIG. 2-6. NMR spectrum 1-chloro-2,2-bis(*p*-methoxyphenyl)ethylene, XV.

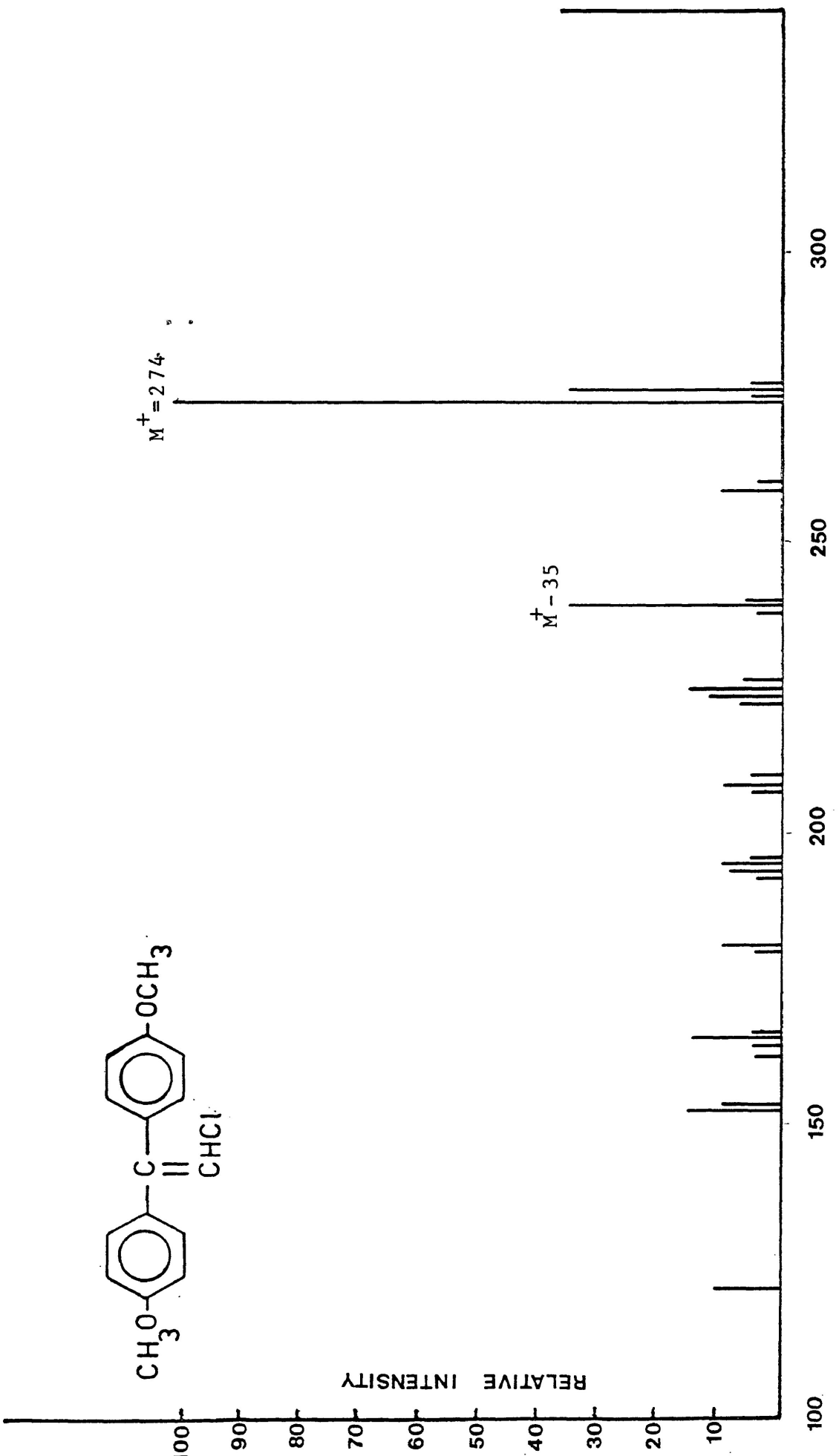
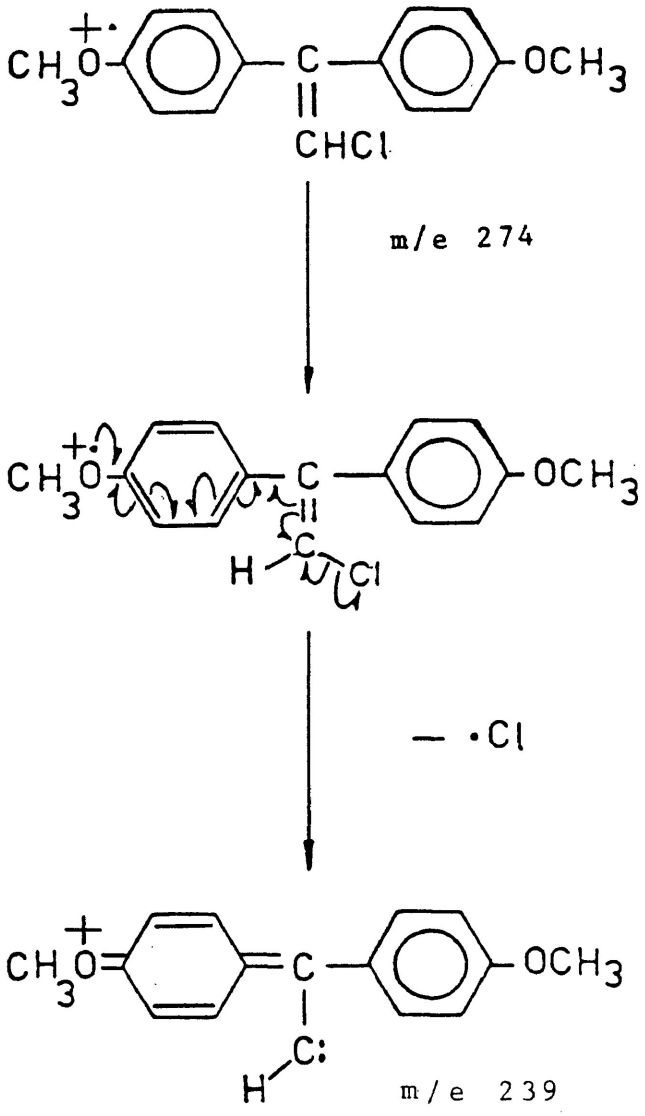


FIG. 2-7. Mass spectrum of 1-chloro-2,2-bis(*p*-methoxyphenyl)ethylene, XV.



Scheme 2-3. Mass spectral fragmentation pattern for 1-chloro-2,2-bis(*p*-methoxyphenyl)ethylene, XV.

environments and hence, two closely spaced, three proton signals are observed.

The mass spectrum (figure 2-7) of the dimethoxymono chloro olefin (XV) contains a strong molecular ion peak, which is also the base peak at m/e 274. A major fragment corresponding to the loss of chlorine (see scheme 2-3) is also evident in the spectrum, (see p. 44 and 45).

In view of the ready dehydrohalogenation of the dimethoxydichloro derivative (X), it is tempting to assume that the corresponding dihydroxydichloro derivative (XI) can be dehydrohalogenated in the same way. Bharath (14) attempted this reaction and failed to isolate any of the desired olefin, but instead, obtained a rearrangement product which was partially characterized (see page 60). With this in mind two attempts were made to prepare this olefin by methods which did not involve the dehydrohalogenation of the dihydroxy dichloro derivative (XI). These attempts are outlined in scheme 2-4.

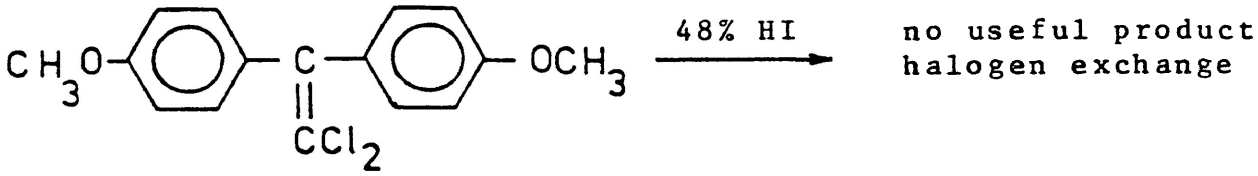
The cleavage of an ether linkage by halogen acids, especially hydriodic acid, is a common and widely used method in organic synthesis. Route (a) was based upon this method. The 1-chloro-2,2-bis(*p*-methoxyphenyl)ethylene (XV) was warmed in a solution of 48% HI however, analysis results of the isolated product indicated that ether cleavage had not been effected and that halogen exchange took place instead. An attempt (route b) was then made to cleave the methoxy groups in the corresponding non-chlorine containing olefin (XVIII) in

the hope that the resulting dihydroxydiphenyl ethylene (XXVIII) could be monochlorinated subsequently.

The starting material, olefin(XVIII), for route (b) was prepared via the Grignard reaction of *p*-methoxyphenyl magnesium bromide with *p*-methoxyacetophenone, and the dehydration of the resulting tertiary alcohol to give 2,2-bis(*p*-methoxyphenyl)ethylene(XVIII)in an overall yield of 65% (equation 2-8). It was clear that the difficult step in this reaction sequence would be the monochlorination of the olefinic bond. Usually halogenation of a double bond results in addition, while in this reaction substitution was required. Bothner-By (40) reported the successful monobromination of an olefin of similar structure (equation 2-9) by using free bromine in carbon disulphide. Using similar reaction conditions a chlorination, as opposed to a bromination, was attempted using the dimethoxy olefin(XVIII)as a model compound. This reaction led to a mixture of chlorinated products which contained the desired monochlorinated product in very low yield. Since there was no reason to assume that the dihydroxy olefin (aa, route (b)) would give any better results this method was not further pursued.

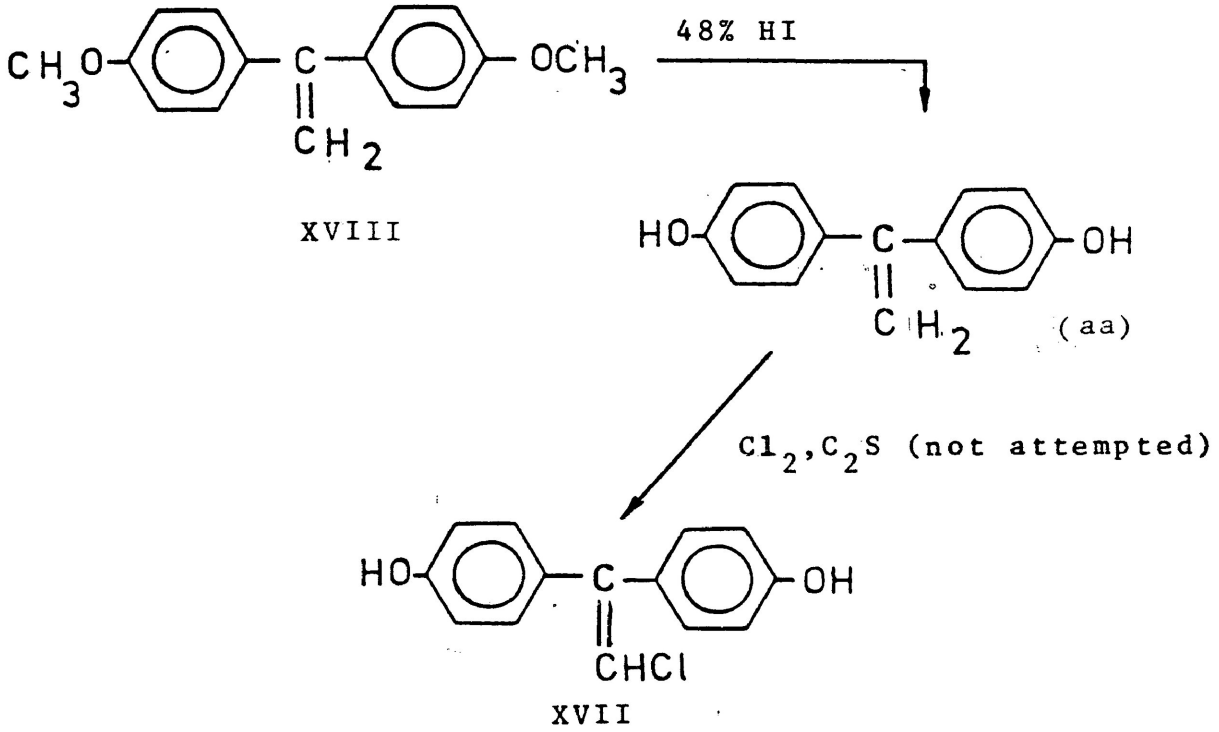
The preparation of the 1-chloro-2,2-bis(*p*-hydroxyphenyl)ethylene (XVII) was then attempted by a third route (scheme 2-5, route c). Unlike the previous attempts this route was based on the dihydroxydichloro derivative (XI) as the starting material. It was noted earlier that the

Route (a)



XV

Route (b)

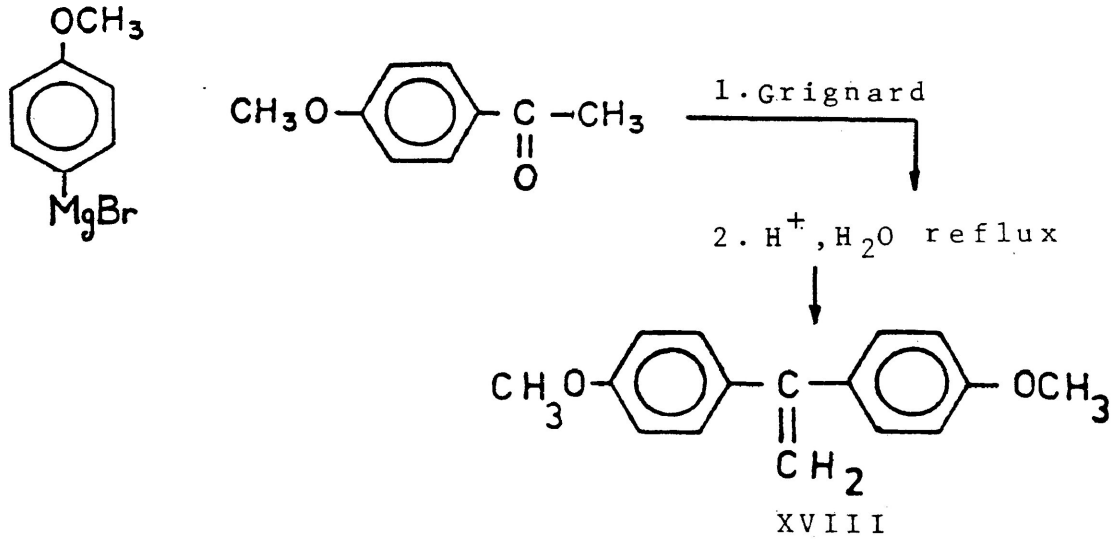


XVIII

XVII

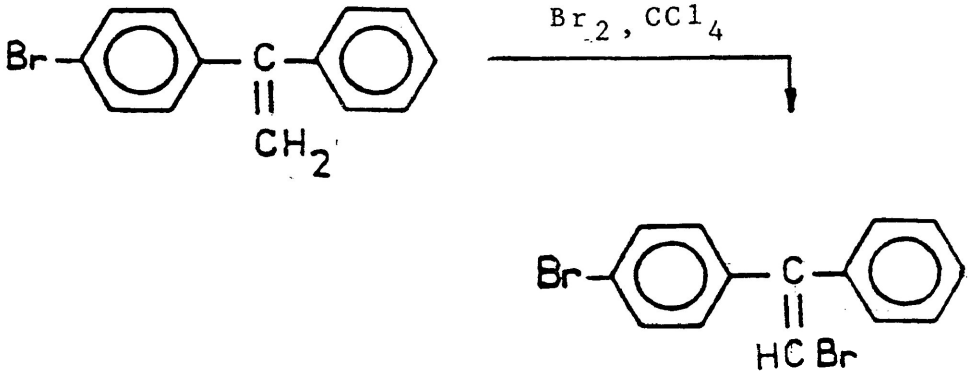
Scheme 2-4. Attempted routes to the dihydroxymonochloro olefin, XVII.

Eq. 2-8

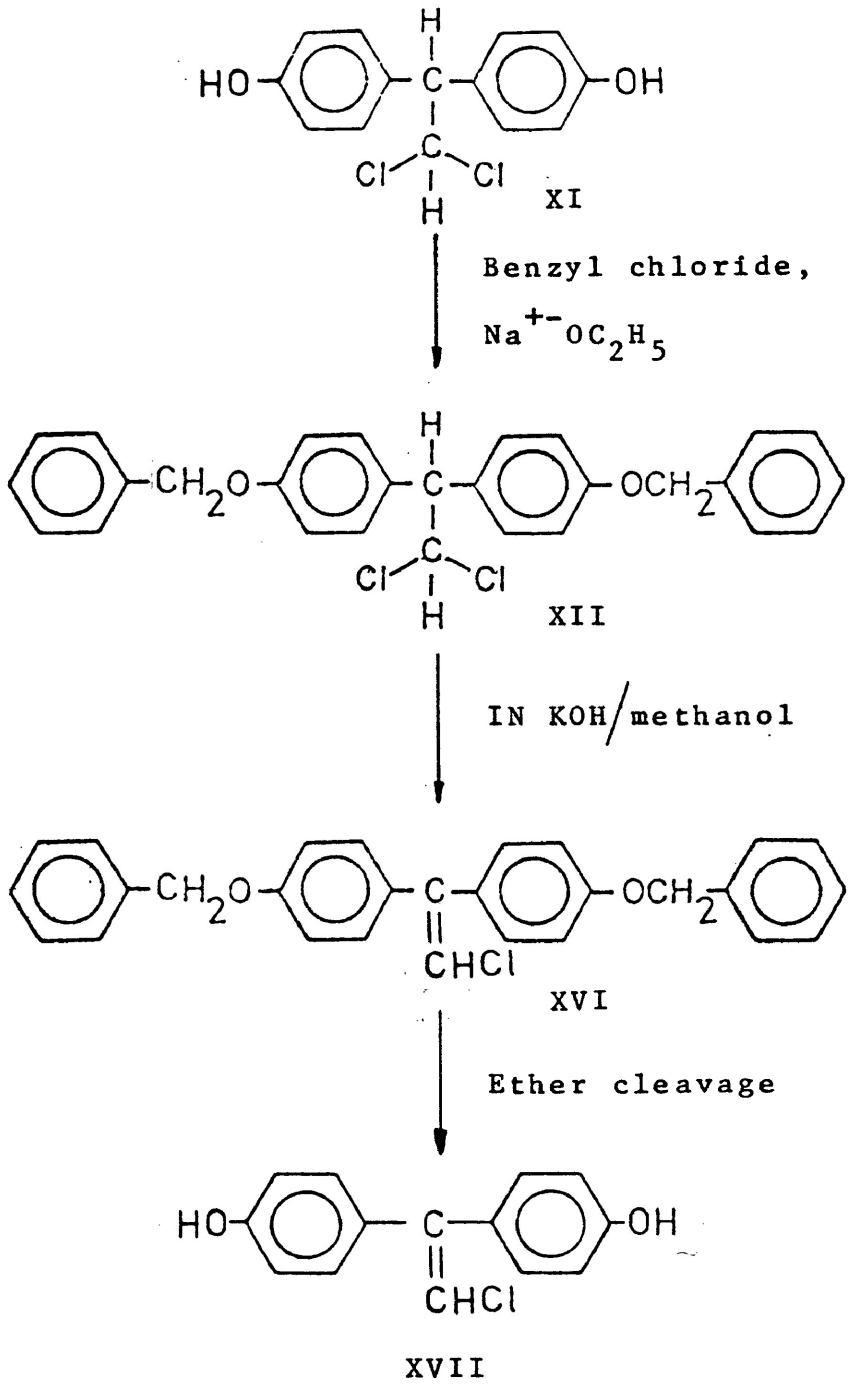


XVIII

Eq. 2-9



Route (c)



Scheme 2-5. Potential route to the dihydroxymonochloro olefin, XVII.

rearrangement reaction was facilitated by the presence of the para hydroxy substituent groups on the aromatic rings of compound (XI), and that the corresponding methoxy ether analogue (X) successfully underwent dehydrohalogenation to yield the corresponding monochloro olefin (XV). It was also shown (see route a) that these methoxy groups could not be cleaved successfully. The above results then, suggest a need for a readily cleavable protective group which, once in place would allow for the smooth elimination of hydrogen chloride from the molecule and yet inhibit the rearrangement reaction. The benzyloxy group proved to be a successful choice. As a protective group it is stable toward the basic conditions of the elimination reaction, yet the benzyloxy moiety is easily cleaved by a number of reagents (41).

The dibenzyldichloro derivative (XII), which has not been reported in the literature before, was obtained in low yield (22%) by refluxing the dihydroxydichloro derivative (XI) with an ethanolic solution of benzyl chloride in the presence of sodium ethoxide. The pure product, after recry stallization from ethanol, had a m.p. 76-79°. The NMR spectrum (figure 2-8) of the dibenzyldichloro derivative (XII) contains a number of new signals not found in the spectrum of the starting material. Two new singlets are present: one at 7.36ppm corresponding to the ten aromatic protons of the benzyloxy groups, and the other at 5.13ppm corresponding to four protons of the benzylic methylene moieties. These

signals provide good evidence that the desired compound had been formed. The mass spectrum (figure 2-9 of the compound (XII) did not contain the molecular ion peak. However a peak at m/e 372 corresponding to the loss of a benzyl radical from the parent ion is present in the spectrum. The base peak at m/e 289 corresponds to the loss of a dichloromethyl radical from the m/e 372 ion, and this fragmentation step can be verified by the presence of a metastable peak at m/e 224 (see scheme 2-6).

The next step in the synthetic sequence was to remove the elements of hydrogen chloride from the dibenzyldichloro derivative (XII) by reaction with alkali. The elimination reaction was effected by gently refluxing this compound (XII) in 1N methanolic potassium hydroxide solution. In this way a good yield (57%) of the desired 1-chloro-2,2-bis(*p*-benzyl oxyphenyl)ethylene (XVI) was obtained. The NMR spectrum (figure 2-10) is consistent with the expected structure and is characterized by the existence of a singlet at 6.43ppm corresponding to a single olefinic proton on the chlorine bearing carbon. The mass spectrum (figure 2-11) of the dibenzylmonochloro olefin (XVI) contained a weak but observable molecular ion peak at m/e 426. The base peak at m/e 91 is the result of heterolysis of the C-O bond of the benzyl ether moiety. The initially formed ion probably rearranges to form the stable tropylium ion (a). Another fairly abundent ion, at m/e 390, is formed as a result of the loss of chlorine from the parent ion (see scheme 2-7).

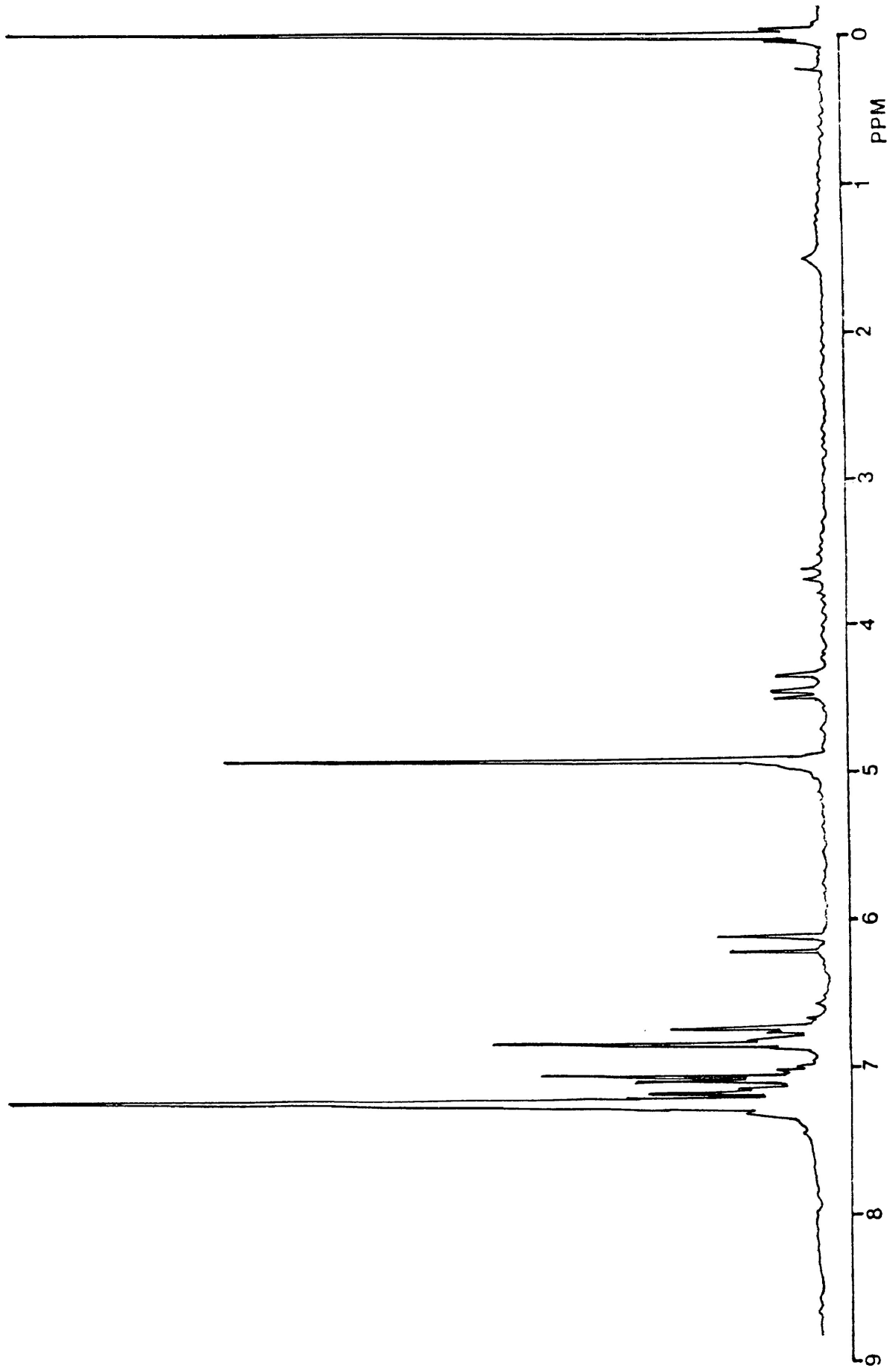


FIG. 2-8. NMR spectrum 1,1-dichloro-2,2-bis(*p*-benzyloxyphenyl)ethane, XII.

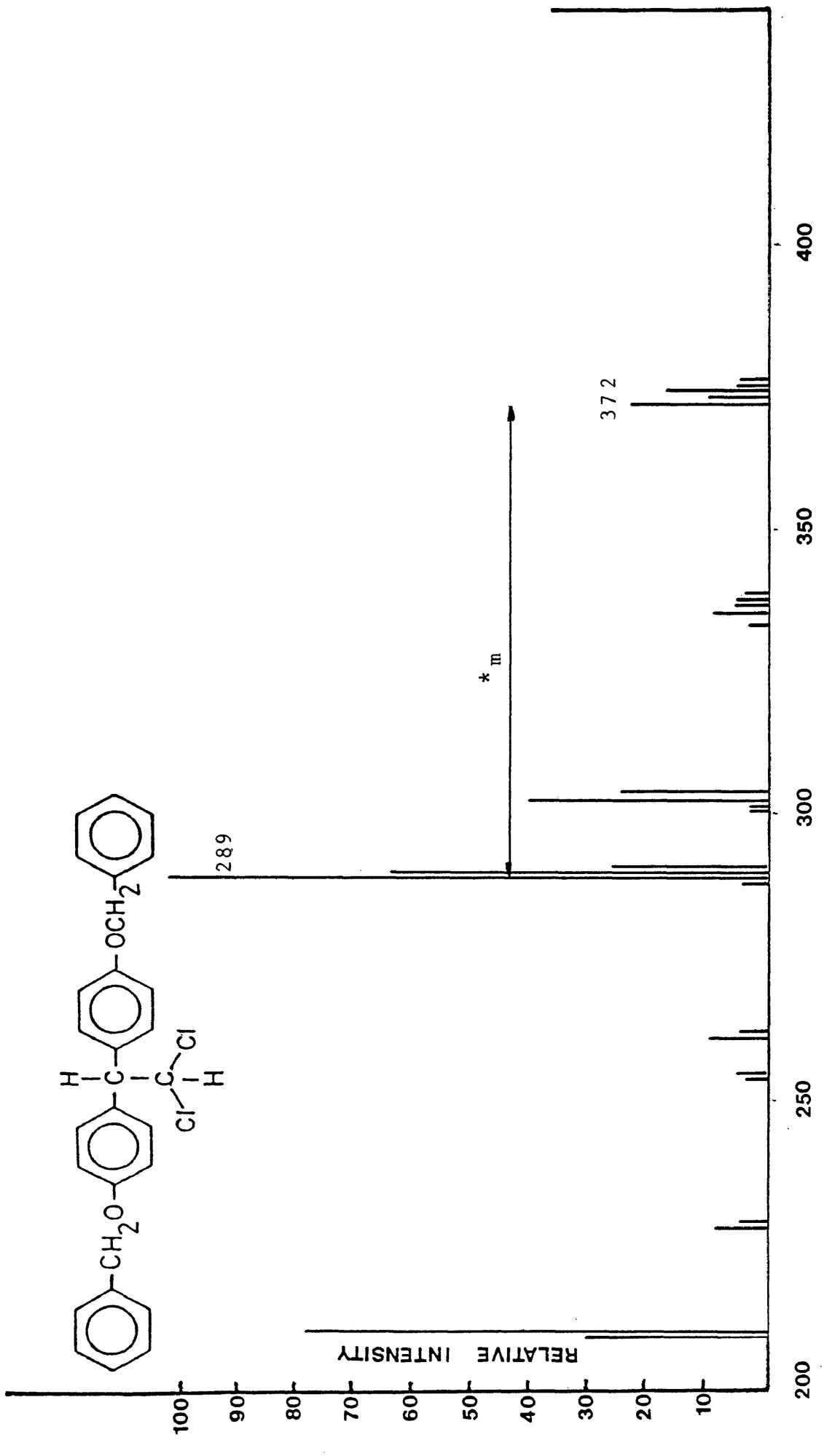
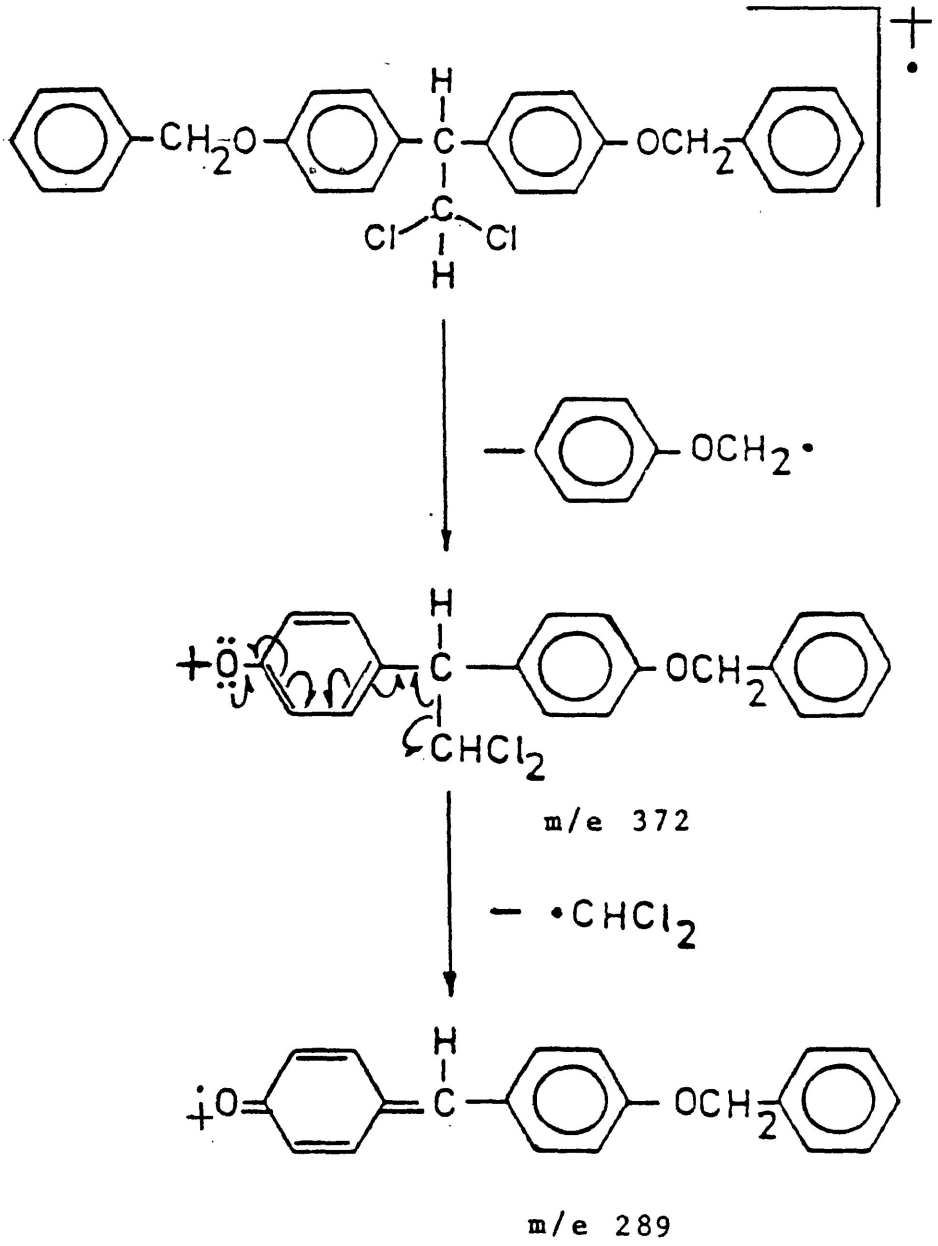


FIG. 2-9. Mass spectrum 1,1-dichloro-2,2-bis(*p*-benzyloxyphenyl)ethane, XII.



Scheme 2-6. Mass spectral fragmentation pattern for 1,1-dichloro-2,2-bis(*p*-benzyloxyphenyl)ethane, XII.

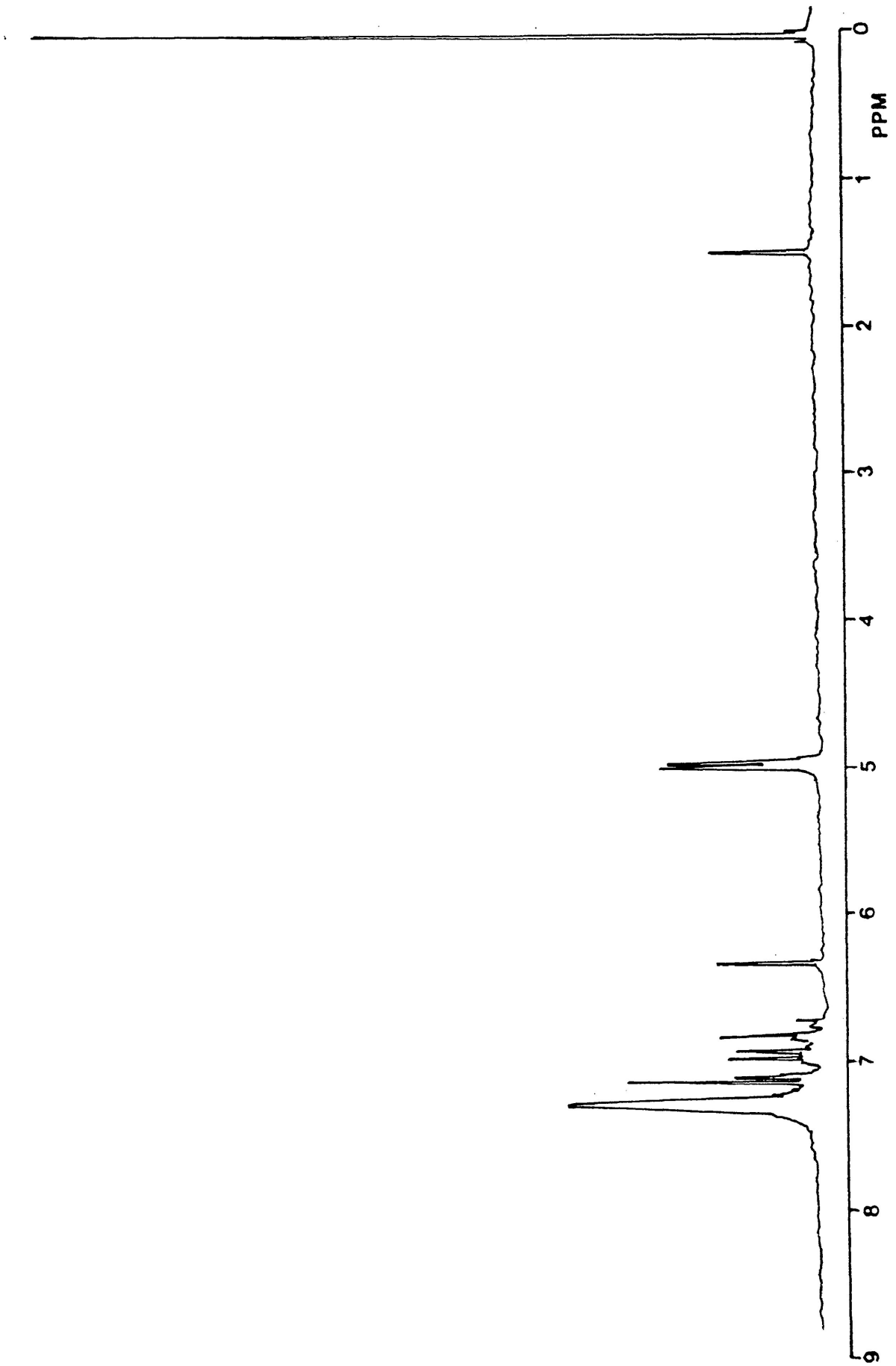


FIG. 2-10. NMR spectrum 1-chloro-2,2-bis(*p*-benzyloxyphenyl)ethylene, XVI.

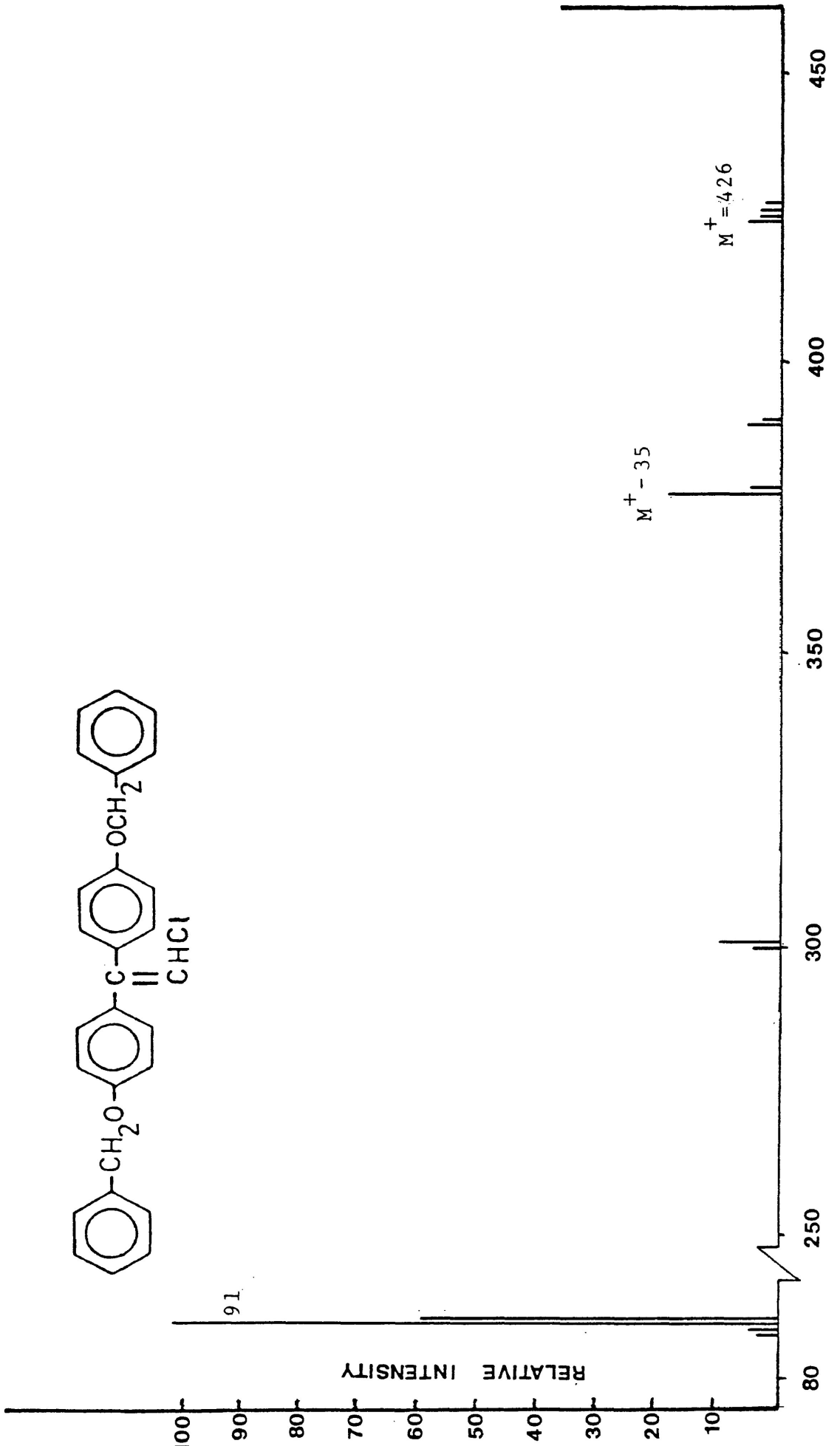
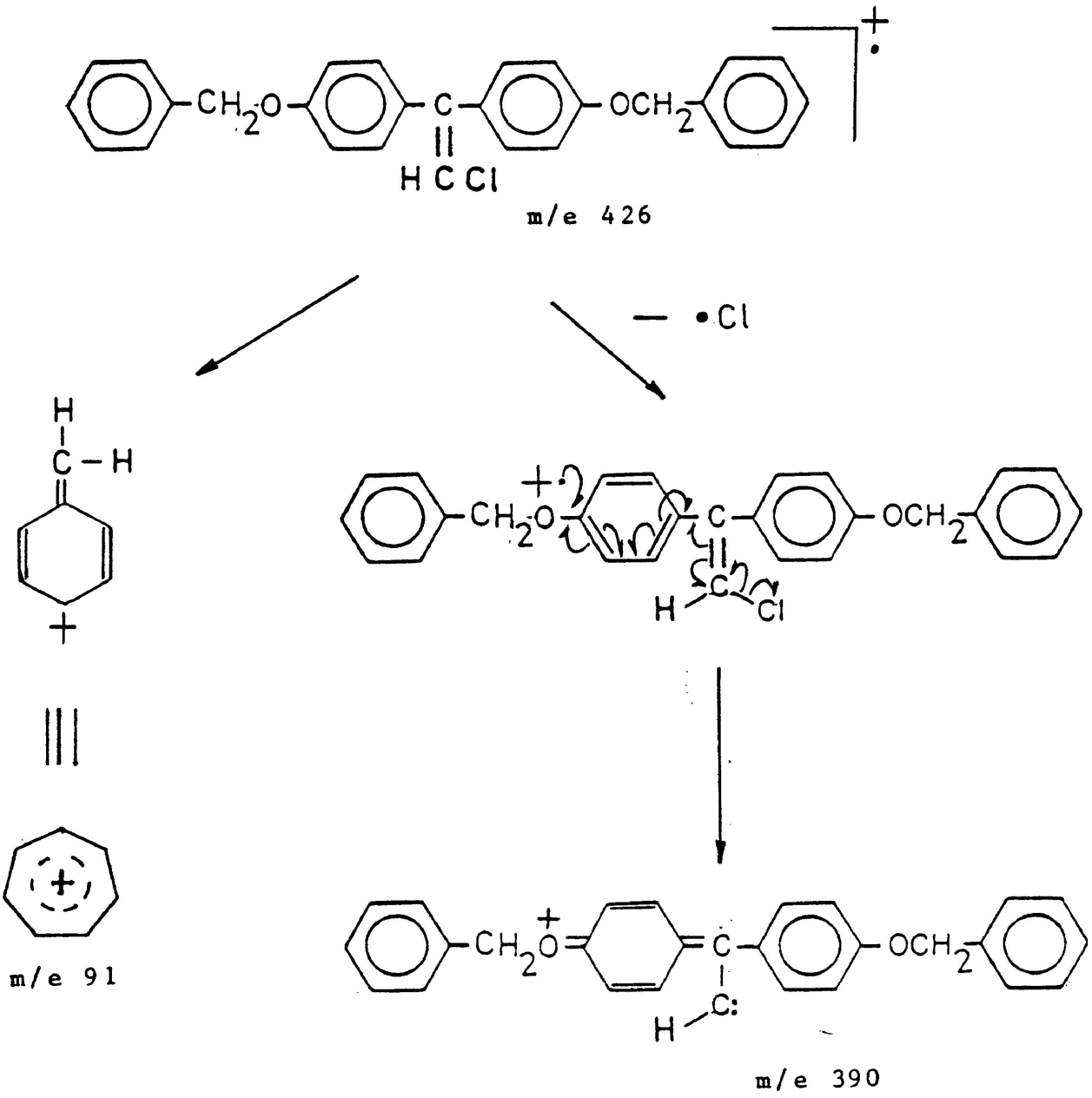


FIG. 2-11. Mass spectrum 1-chloro-2,2-bis(*p*-benzyloxyphenyl)ethylene, XVI.



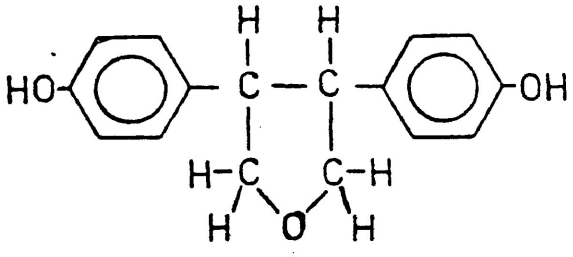
Scheme 2-7. Mass spectral fragmentation of 1-chloro-2,2-bis(*p*-benzyloxyphenyl)ethylene, XVI.

As depicted in scheme 2-5 (route c) the dibenzyl monochloro olefin (XVI) upon acid hydrolysis, would be expected to yield the desired dihydroxymonochloro olefin (XVII). At the present time this step has not been completed. However, in view of the ease with which benzyl ethers cleave under these conditions, it is anticipated that little problem would be encountered in the last step of this synthesis. Recently, Davison *et. al.* (30) have identified the dihydroxymonochloro olefin (XVII) as one of the metabolites of goats fed methoxy chlor. Clearly, the chemical synthesis of this compound is important and necessary so that larger quantities of this material be available for study of its physical and toxicolo gical properties. In addition, this olefin (XVII) has been implicated as an intermediate in the possible biodegradation of methoxychlor to the corresponding diphenyl acetic acid (VIII) (14). Therefore, if the synthesis of the dihydroxy monochloro olefin (XVII) can be achieved, then the complete general biodegradative pathway for methoxychlor may evolve, similar to and analoguous with the metabolic pathway now known for DDT.

Rearrangement Reaction

The only question that remains concerning the synthetic chemistry of these compounds is the unknown rearrangement product obtained in low yield by Bharath, from the alkali promoted dehydrohalogenation of the dihydroxy dichloro derivative (XI). It was considered to be of scientific significance to attempt an elucidation of the structure of this compound. Such attempts were carried out in parallel with the above described synthesis of the dihydroxymonochloro olefin (XVII) since an understanding of this rearrangement might be of value in developing a synthetic route to the olefin.

Bharath (14) isolated a compound which, according to the mass spectroscopic data, contained no chlorine and had a molecular weight of 256. On the basis of this and elemental analysis data, he concluded that a rearrangement had taken place and the product was tentatively assigned molecular formula $C_{16}H_{16}O_3$ and a plausible structure (XXII, diagram 2-2) was suggested.

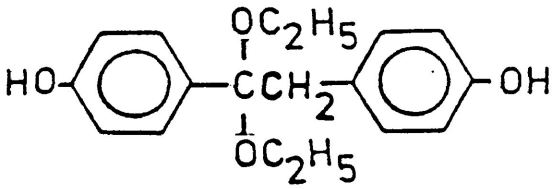


XXII

DIA. 2-2. Structure suggested by Bharath (14)

Using the method described by Bharath, the synthesis of this compound was repeated. The expected rearrangement product was obtained and was found identical to Bharath's product by the usual comparison of physical properties. The reaction conditions were then modified so as to improve the yield of this product. Thus, when the dihydroxydichloro derivative (XI) was refluxed in an ethanolic potassium hydroxide solution for one hour, the rearrangement product could be obtained in 93% yield. The crude product was recrystallized from a mixture of chloroform, benzene and ethyl acetate. The pure crystalline material (m.p. 189-193°) was homogeneous by thin layer chromatography and the combustion analysis suggested that this compound had a molecular formula $C_{18}H_{22}O_4$.

The most useful information concerning the structure of this rearrangement product was obtained by the interpreta tion of the proton-NMR spectrum and the mass spectrum. On the basis of evidence obtained by these techniques, as discussed below, structure (XX, diagram 2-3) was proposed.



4,4'-dihydroxydesoxybenzoin diethyl Ketal (XX)

DIA. 2-3. Proposed structure of the rearrangement product.

The NMR spectrum (figure 2-12) of this compound is fairly complex, but is consistent with the proposed structure. The methyl triplet at 0.9ppm and the complex multiplet centered at 3.15ppm corresponding to two methylene groups are indicative of the ethoxy moieties of the ketal. Bharath had originally assumed these ethyl signals to result from solvent of crystallization.

However, when the material was sublimed before analysis, it could be confirmed that the ethyl groups are indeed a part of the molecule. Double resonance experiments suggested that these two signals are coupled to one another although the results are not conclusive. The singlet peak at 4.14ppm was assigned to the methylene group protons adjacent to the aromatic ring (ie. benzyl protons) and are in a region where the signals due to such protons are known to occur. The aromatic signal in the region between 7.05 - 6.68ppm has the characteristic "quartet" pattern indicative of a para-substituted benzene ring. The phenolic hydroxyl protons are quite acidic and give rise to a singlet located at 8.09ppm.

The mass spectrum (figure 2-13) of this compound is very characteristic and contained many peaks which were helpful in confirming the proposed structure. The spectrum contained a very weak peak at m/e 302 which corresponds to the molecular ion of the proposed structure (XX). The low intensity of the molecular ion is a general characteristic which is common to both ketals and acetals, and is due to the presence

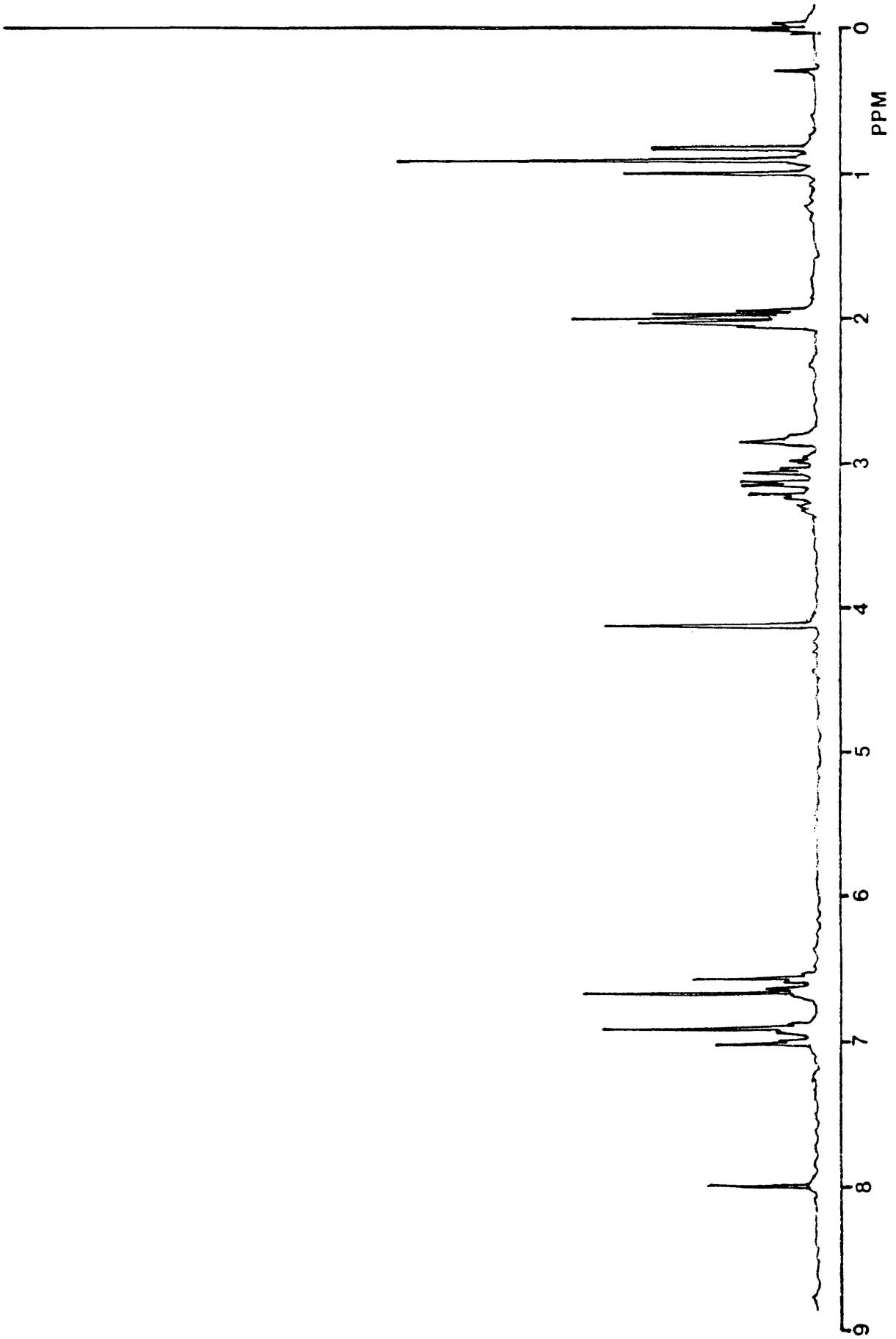


FIG. 2-12. NMR spectrum 4,4'-dihydroxydesoxybenzoin diethyl Ketal, XX

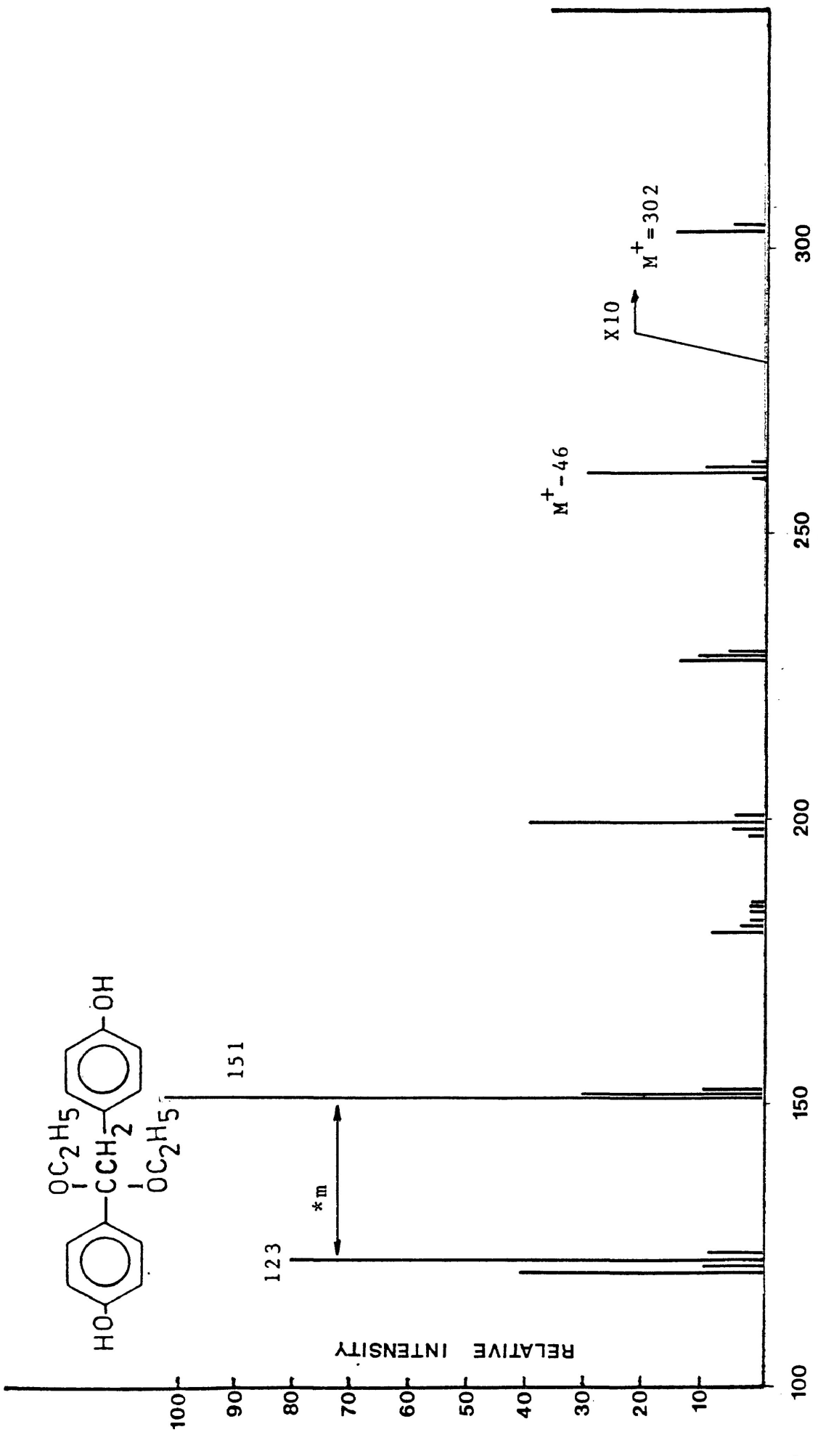
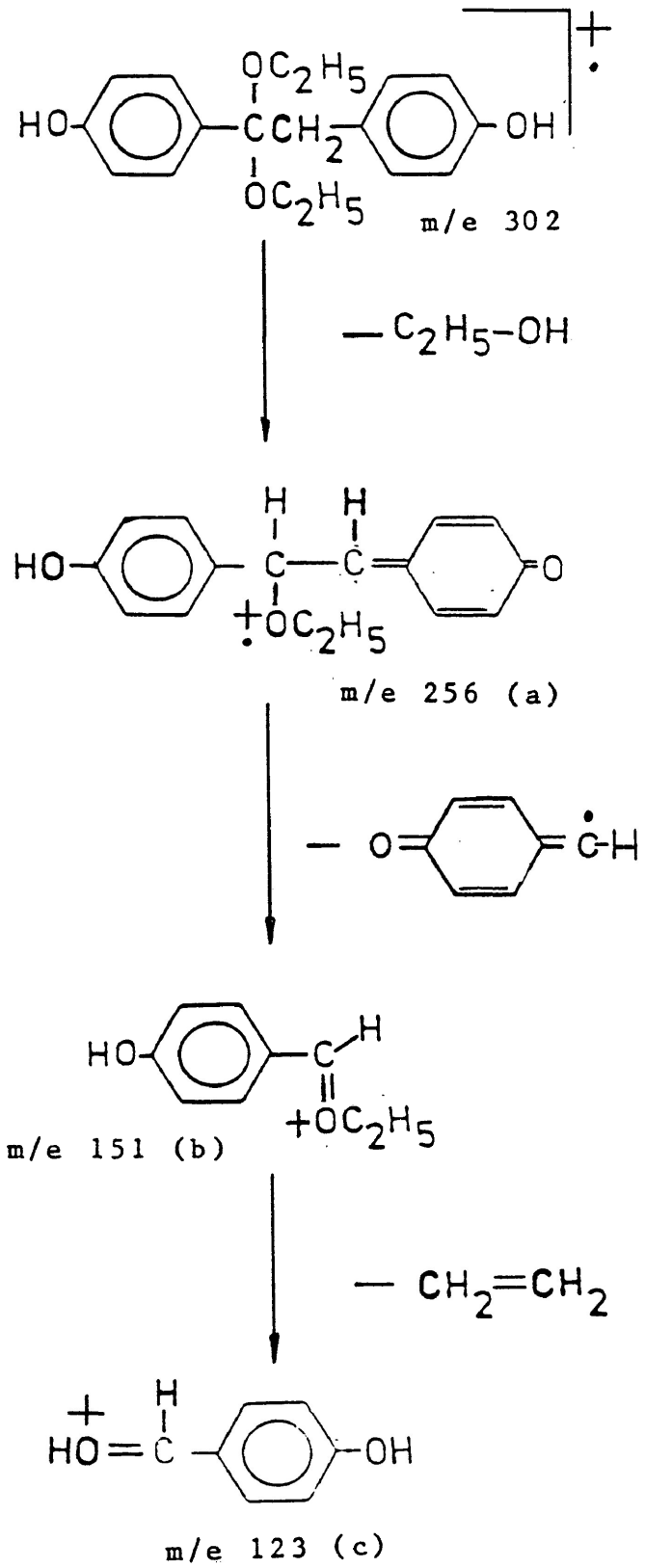


FIG. 2-13. Mass spectrum 4,4'-dihydroxydesoxybenzoin diethyl Ketal, XX

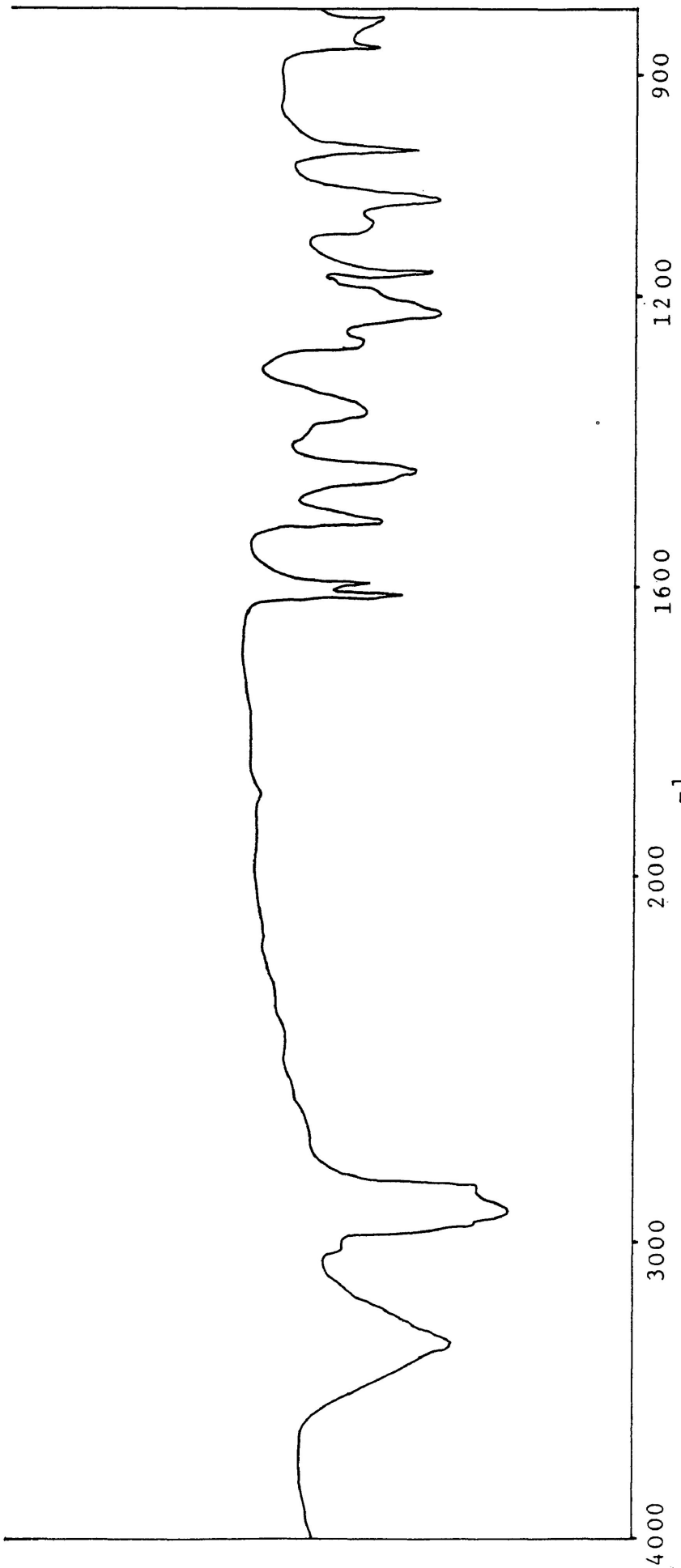


Scheme 2-8. Mass Spectral fragmentation of 4,4'-dihydroxy-desoxybenzoin diethyl ketal, XX.

of a number of bonds which upon cleavage give rise to some very stable positive ions (41) (see scheme 2-8). In many cases the molecular ion of such compounds cannot be determined directly. The peak at m/e 256 would correspond to the loss of ethanol from the parent compound and such a loss is not unusual for diethyl ketals (42). It is then reasonable to assume that this new species (a) becomes the parent ion for subsequent fragmentations. The base peak is found at m/e 151 (b) and corresponds to the loss of radical C_7H_5O from ion m/e 256 (a). The fragmentation of ion m/e 151 to ion m/e 123 (c) occurs in one step by the expulsion of ethylene and is verified by the presence of a metastable peak at m/e 100.

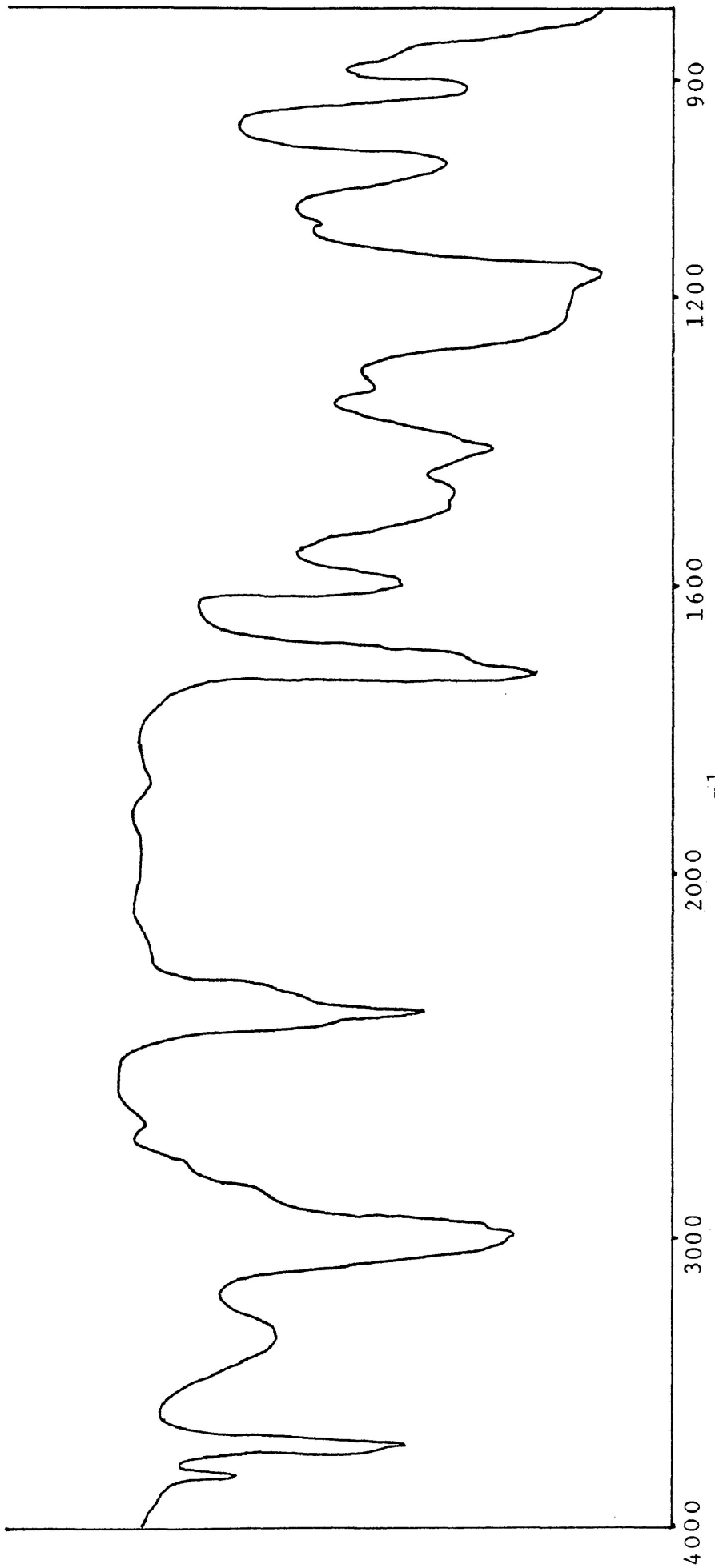
The infrared spectrum (figure 2-14) of the rearrangement product (XX) also provided some evidence of a ketal structure. The presence of three bands at 1070, 1100 and 1170cm^{-1} are very characteristic of the coupled vibration (C-O-C) in simple ketals (43).

Further evidence confirming the proposed structure of the rearrangement product (XX) was obtained by hydrolysis of this compound to the corresponding ketone (XXI). This hydrolysis was effected by warming compound (XX) in dilute aqueous acid for 12 hours. The product was recrystallized from chloroform. The infrared spectrum (figure 2-15) of the hydrolysis product (XXI) contained a very strong absorption at 1719cm^{-1} (C=O, stretch) indicating that a ketone was isolated. This absorption band was not present in the infrared spectrum



*nujol mull

FIG. 2-14. Infrared spectrum* of 4,4'-dihydroxydesoxybenzoin diethyl Ketal, XXI.

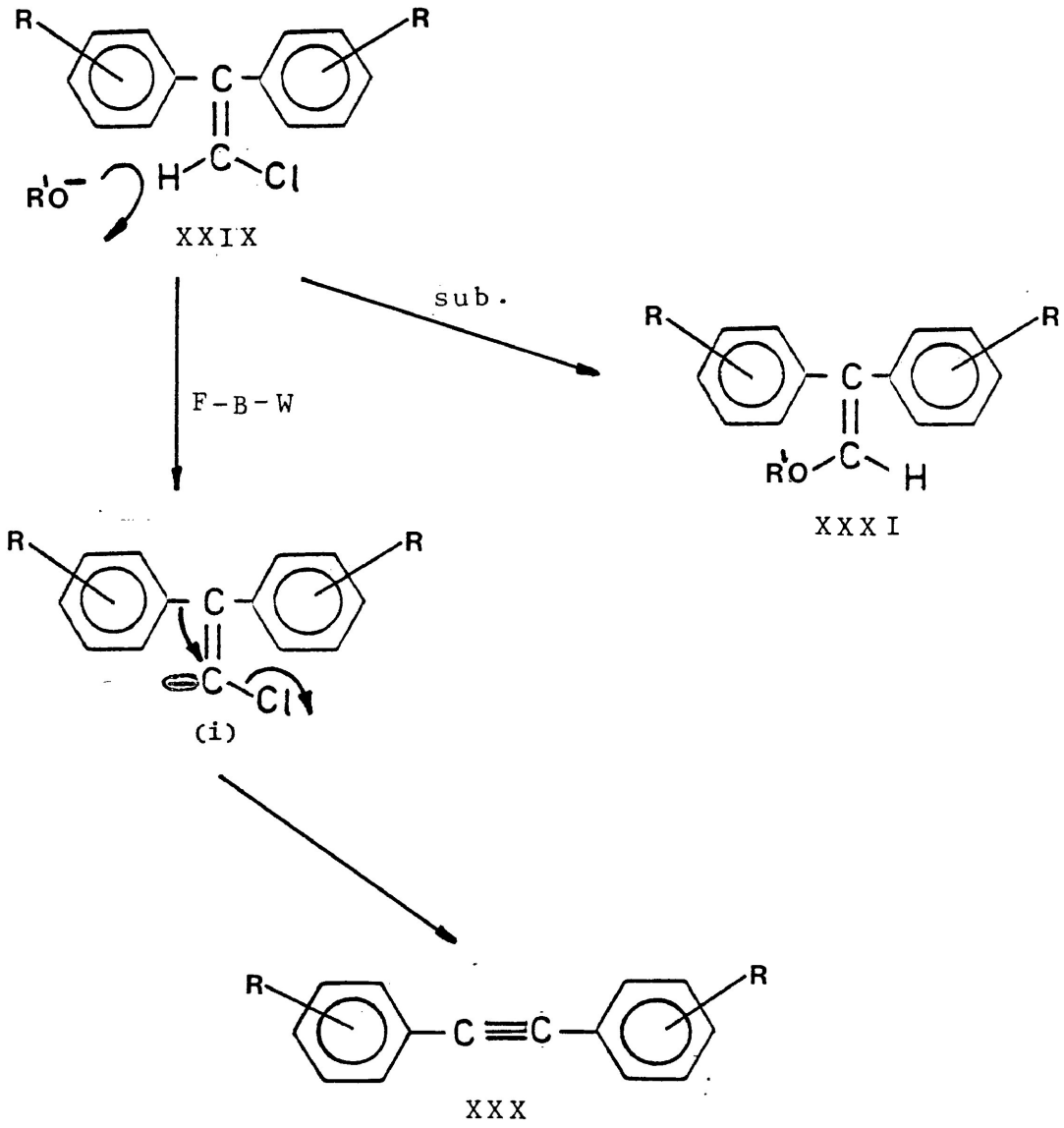


* in $CHCl_3$

FIG. 2-15. Infrared spectrum* of hydrolysis product from compound, XX.

(figure 2-14) of the ketal (XX). The carbonyl stretching band for this ketone (XXI) is in agreement was observations made by Jones *et. al.* (44) for aromatic substituent effects on the carbonyl stretching absorption in various acetophenones. Attempts to record the mass spectrum of this compound were unsuccessful.

In many ways the observed rearrangement reaction bears a striking resemblance to the base promoted elimination - rearrangement reaction reported by Fritsch (45) and others (46, 47). The so called Fritsch - Buttenburg - Wiechell rearrangement (referred to below as the F-B-W rearrangement) involves the rearrangement of various diarylmonohaloethylenes (XXIX) to the corresponding diarylacetylenes or tolans (XXX, see scheme 2-9). As a minor product the vinyl ether (XXXI) is sometimes isolated in various amounts depending on the reaction conditions and substituents on the aromatic rings. The stereochemistry and mechanism of this reaction has been investigated by Bothner - By (40) and by Pritchard and Bothner - By (48) respectively. There is general agreement that the reaction procedes through a configurationally stable vinyl carbanion (i, scheme 2-9), however whether aryl ring migration occurs concomitant with halogen expulsion is still debated and appears to be dependent upon the aromatic ring substituents (49). The stereochemistry of the reaction has been determined and it appears that the aryl ring trans to the halogen migrates. Presumably the vinyl ether by-product is



$R\dot{O}^-$ = alkoxide

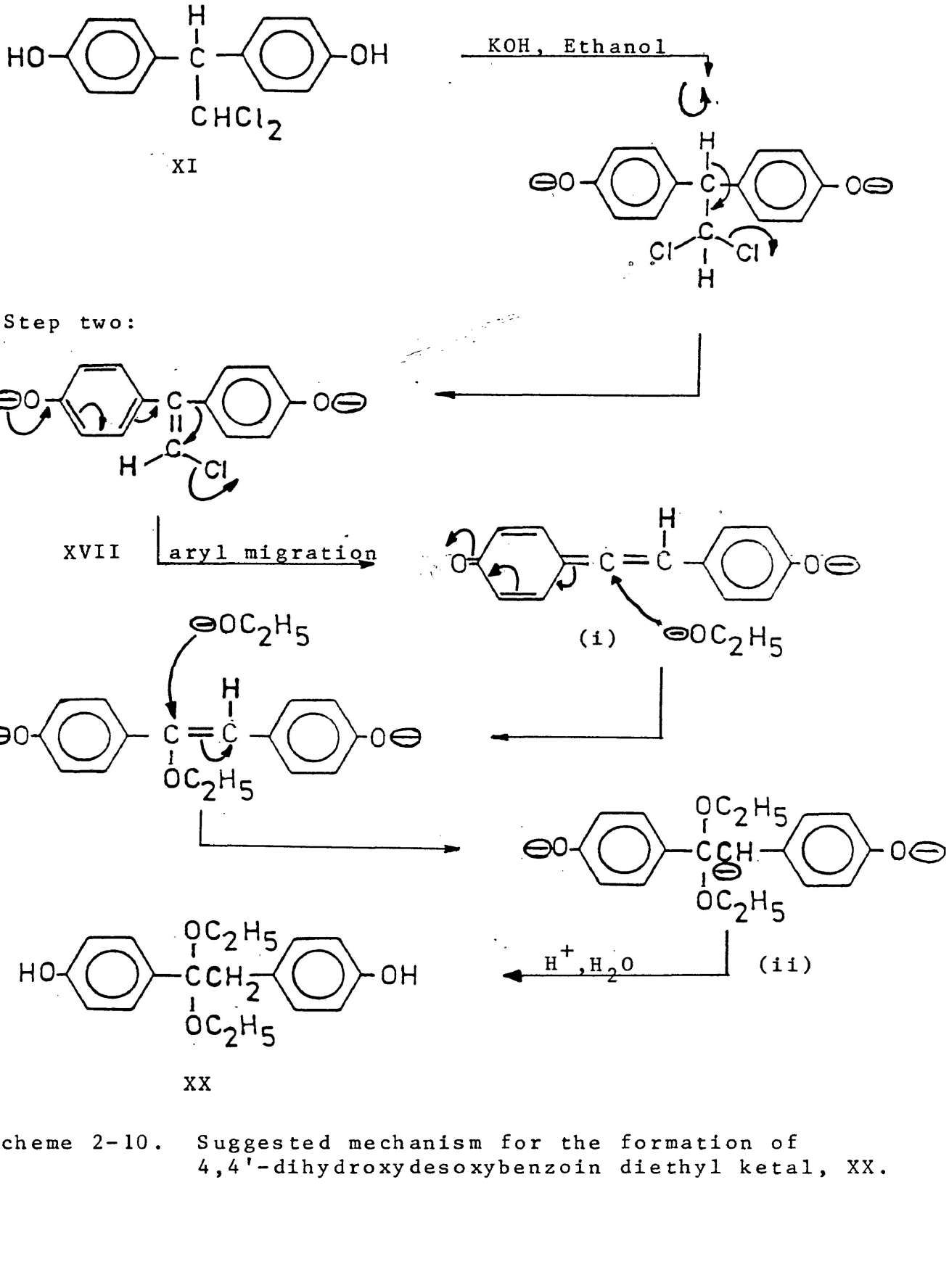
Scheme 2-9. Mechanism of the Fritsch-Buttenburg-Wiechell rearrangement.

formed by substitution.

Considering the above facts it is apparent that the present rearrangement is different from the classical F-B-W rearrangement. Two differences are clearly evident. Firstly, the reaction conditions of the present reaction, as far as temperature and reflux time are concerned, differ substantially from the general reaction conditions used for alkoxide induced F-B-W rearrangement. Consider the following; the present rearrangement occurs at a temperature of 100° in one hour, the F-B-W rearrangement reaction conditions are far more drastic, and reaction times from 8 hours to 30 days at temperatures between 150° to 200° have been reported. Thus, the reaction conditions for alkoxide induced rearrangements play an important role in determining the final outcome of the reaction. Secondly, no systematic study of this reaction, reported in the literature, has included a phenolic precusor molecule. It is believed that this special case led to an anomalous Fritsch - Buttenburg - Wiechell rearrangement where tolan formation is suppressed. In the present work the rearrangement of the dihydroxydichloro derivative (XI) resulted in the formation of a ketal (XX). A possible mechanism for its formation is outlined in scheme 2-10.

It is suggested that the formation of the ketal (XX) occurs in two distinct steps. Step one is a base promoted elimination of hydrogen chloride from the starting material (XI) to form the intermediate monochloro olefin (XVII). In

Step one:



Scheme 2-10. Suggested mechanism for the formation of 4,4'-dihydroxydesoxybenzoin diethyl ketal, XX.

step two, this olefin then undergoes a rearrangement via aryl ring migration, assisted by the electronic shift through the other aryl group, with expulsion of chloride ion. The resulting quinone structure (i) would then be susceptible to vinyl addition of the incoming ethoxide ion. A second ethoxide ion could conceivably add to the alpha-carbon resulting in the formation of carbanion (ii), and acid work-up would then yield the final product (XX).

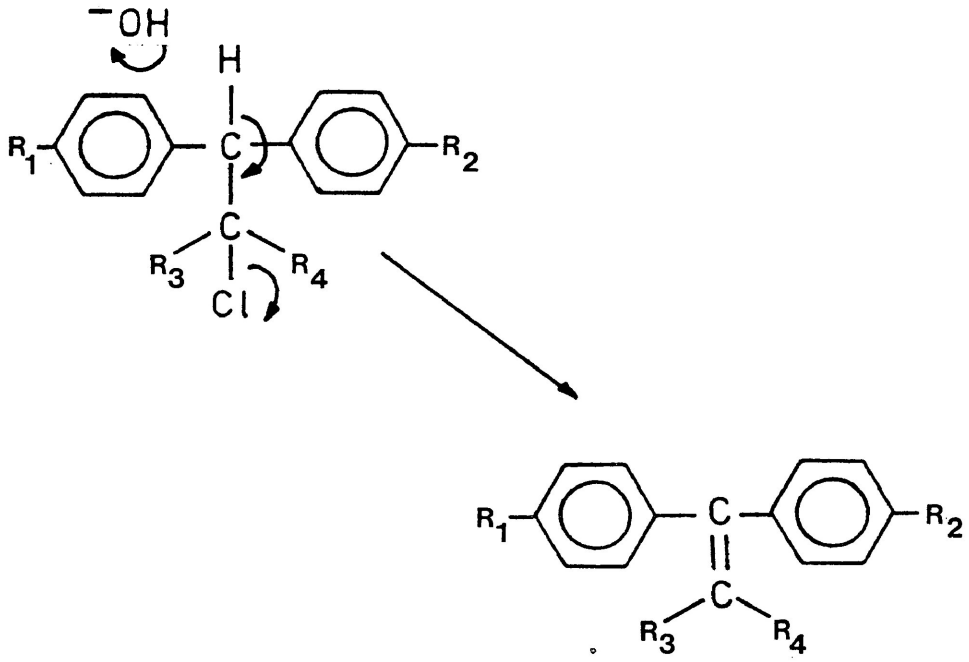
The suggestion that this rearrangement proceeds via the intermediate olefin (XVII), may be confirmed once that olefin is obtained via the alternate synthesis discussed above. It should be pointed out that the proposed mechanism is not intended to refute or support the Fritsch - Buttenburg - Wiechell rearrangement mechanism even though similarities exist. It has been advanced merely to suggest that ketal formation is possible under the conditions of the reaction (ie. ketal formation usually results by reaction of an alcohol with a ketone under acidic conditions). Clearly the mechanism proposed for the formation of the ketal (XX) does not involve an intermediate ketone.

Dehydrohalogenation Studies

The purpose of these experiments was to establish the extent of dehydrohalogenation of methoxychlor (IV), hydroxychlor (VI), dimethoxydichloro derivative (X) and the dihydroxydichloro derivative (XI) in aqueous and non-aqueous media. This is an important aspect of the chemistry of these compounds with respect to biodegradation studies, since it was found (15) that hydroxychlor olefin (XIV) can be formed merely by autoclaving a neutral culture containing hydroxychlor (VI). The effects brought about by varying: (i) the reaction time, (ii) solvent medium and (iii) pH level in buffered and unbuffered media were studied. The results of these experiments are summarized in tables 2-1 to 2-4. Some of the information obtained from these studies was applied to practical synthetic problems encountered in the preparative work and also to the biological work. To a limited extent these results could also be related to the environmental aspects of the chemical degradation of the above compounds. However, it is recognized that in addition to this data only kinetic studies can provide meaningful information on the persistence of these compounds in the field from a viewpoint of chemical degradation.

One such study (11) was reported but limited to methoxychlor itself, and did not address other metabolites. Cristol *et. al.* (50, 51), however, studied the mechanism and kinetics of the alkaline dehydrohalogenation of many substituted 2,2-diarylchloroethanes including DDT and methoxychlor.

According to these authors the reaction mechanism is formally an E2 type elimination (see scheme 2-11).



Scheme 2-11. Mechanism of dehydrohalogenation of the 2,2-diaryldichloroethanes.

The rate of the reaction is dependent upon the acidity of the benzylic hydrogen. This in turn is dependent upon the electron-attracting or electron-repelling properties of the para substituents on the benzene rings (ie. R_1 and R_2) and the effects of increasing alpha chlorine substitution (ie. R_3 and R_4). Cristol reported that electron-withdrawing groups at R_1 and R_2 will increase the rate of the reaction, while electron-donating groups have the opposite effect. It was

reported that increasing the number of alpha chlorine substituents (maximum of three) also increased the rate of the reaction.

In the present study all test compounds gave the corresponding olefins, with the exception of the dihydroxy dichloro derivative (XI) which undergoes rearrangement as discussed above. The results (table 2-1) indicated that quantitative conversion from methoxychlor to methoxychlor olefin (XIII) could be achieved by refluxing the pesticide in 3N methanolic potassium hydroxide solution for two hours. This reaction is an ideal preparative method for this olefin. It was also noted that methoxychlor will, to a small extent (ca. 1% yield) undergo dehydrochlorination in boiling water. The dehydrochlorination of hydroxychlor (VI) was found to occur under a wide variety of reaction conditions. The highest yield of hydroxychlor olefin (XIV) was attained using 3N methanolic potassium hydroxide. A significant amount of hydroxychlor olefin (XIV) was produced in boiling water, dilute aqueous acid and in various buffered solutions, which reflects the ease with which hydroxychlor undergoes dehydro chlorination in these media. This finding is of practical importance when considering a design protocol of metabolic experiments with hydroxychlor. The fact that dehydrohalo genation of some methoxychlor derivatives can occur by heating with water means that hydroxychlor olefin (XIV), for example, could be formed as an artifact when cultures

TABLE 2-1. Results of the dehydrohalogenation reactions with methoxychlor, IV.

Expt. no.	Reaction Conditions Medium	Time(hr)	pH	% Olefin formed	% Unreacted methoxychlor
M1	dilute HCl	0.5	2	0	90.4
M2	dilute HCl	0.5	5	0.2	94.4
M3	water	0.5	7	0	100
M4	3N KOH/methanol	0.5	12	89.6	5.6
M5	dilute HCl	2	2	0	93.3
M6	dilute HCl	2	5	0.5	85.3
M7	water	2	7	1.3	95.6
M8	3N KOH/methanol	2	12	100	0

TABLE 2-2. Results of the dehydrohalogenation reactions with hydroxychlor, VI.

EXPT. no.	Reaction Conditions Medium	Time(hr)	pH	% Olefin formed	% Unreacted hydroxychlor	*Other products
H1	water	0.5	7	3.5	84.1	1
H2	water	2	7	3.8	88.1	1
H3	dilute HCl	0.5	5	16.3	78.8	1
H4	dilute HCl	2	5	17.3	75.2	1
H5	3N KOH/methanol	0.5	12	91.1	1.2	2
H6	3N KOH/water	0.5	12	78.8	0	3
H7	N/2 KOH/methanol	0.5	12	78.9	16.1	2
H8	N/2 KOH/methanol	2	12	76.3	6.5	2
H9	phthalate buffer	0.5	2	11.6	0	4
H10	phthalate buffer	0.5	6	3.5	91.2	4
H11	phthalate buffer	0.5	8	2.3	88.7	4
H12	bicarb. buffer	0.5	10	22.4	5.7	5
H13	phosphate buffer	0.5	12	17.7	27.8	5

* unidentified by-products, numbers 1 to 5 represent by-products differentiated on the basis of retention times from gas chromatographic analysis.

TABLE 2-3. Results of the dehydrohalogenation reactions with the dimethoxydichloro derivative, X.

Expt. no.	Reaction Conditions Medium	Time(hr)	pH	% Olefin formed	% Unreacted dimethoxydichloro
DM1	water	2	7	0	90.5
DM2	IN KOH/methanol	2	10	93.9	0
DM3	3N KOH/water	0.5	10	0	94.8
DM4	IN KOH/methanol	0.5	10	94.3	0
DM5	triethylamine	0.5		96.1	0
DM6	pyridine	2		0	91.5
DM7	3N KOH/ethanol	0.5	10	0	69.2
DM8	methanolic KI	2		0	98.9

TABLE 2-4. Results of the dehydrohalogenation reactions with the dihydroxydichloro derivative, XI.

Expt. no.	Reaction Conditions Medium(20mL)	Time(hr)	% Product[1] formed	% Unreacted dihydroxydichloro
DH1	0.2g KOH/ethanol	0.5	89.7	9.1
DH2	0.2g KOH/ethanol	1.0	96.2	2.4
DH3	0.2g KOH/ethanol	1.5	80.8	14.7

1 compound undergoes rearrangement. experiments conducted to assess optimal conditions for reaction.

containing hydroxychlor are sterilized by autoclaving before extraction. Particularly metabolic studies using pathogenic organisms (eg. *Klebsiella pneumoniae*, 15) would require such sterilization after the experiment to reduce risk during work up, therefore other sterilization techniques, without the use of heat, are necessary (eg. addition of alcohol). Similar arguments apply to methoxychlor as well. It was also observed that a number of unidentified by-products were formed during the reaction which appear to be dependent upon medium used. The largest amounts of by-products were produced when hydroxychlor (VI) was dehydrochlorinated in aqueous buffered media (see table 2-2, experiments H9-H13).

The dehydrochlorination of the dimethoxydichloro derivative (X) was also examined. This compound yielded the corresponding monochloro olefin (XV) in non-aqueous basic medium. A preparative method for the synthesis of the monochloro olefin (XV) was developed from these experiments and was discussed earlier. It should be mentioned that strong organic bases such as triethylamine may also be used to dehydrochlorinate this compound (see table 2-3, experiment DM5).

Solubility and *p*-value study

The polarity of the phenolic metabolites of methoxychlor (compounds, VI, VIII, XI.) has led Baarschers *et. al.* (15) to question the use of non-polar extraction solvents for bacterial and fungal degradation studies on methoxychlor, because of the possibility that these metabolites, if present, may not

be extracted. In the present study the solubility of hydroxychlor (VI, a major metabolite of methoxychlor) in benzene (commonly used as an extraction solvent in many reports) was determined. The water solubilities of the diphenyl acetic acid derivatives (VII) and (VIII) have also been determined. The results of these measurements are presented in table 2-5.

In addition to the solubility data the distribution behaviour of the above derivatives has been studied in a few solvent/water systems (see table 2-6). The partition coefficient K, (see below),

$$K = \frac{p}{q}$$

K = partition coefficient

p = fractional amount in non-polar phase

q = fractional amount in polar phase

where, $p + q = 1$.

has generally been used to express the partition of a solute between two immiscible phases. However, the distribution behaviour is more conveniently described by its p - value (52).

$$p = \frac{A_n}{A_s}$$

A_n = analysis of solvent phase (non-polar) before distribution.

A_s = analysis of non-polar phase after distribution.

when, $V_n = V_p$ = volumes of non-polar and polar phases respectively and are equivolume.

TABLE 2-5. Solubilities of selected derivatives in various solvents.

Compound	Solvent	Solubility (ppm)[1]
hydroxychlor	benzene	194
dimethoxy acid	water	199
dihydroxy acid	water	17,656

1 based on triplicate analyses.

TABLE 2-6. *p*-values of selected derivatives in various solvent systems.

Compound	Concentration in organic phase (ppm)	System	*p*-value[2]
hydroxychlor	62	benzene/water	0.82
hydroxychlor	118	benzene/water	0.82
hydroxychlor	84	ethyl acetate/water	0.96
hydroxychlor	106	ethyl acetate/water	0.97
dimethoxy acid	454	ethyl acetate/water	0.79
dihydroxy acid	248	ethyl acetate/water	0.82

2 based on triplicate analyses.

The *p*-value (or extraction *p*-value) is readily calculated and experimentally very easy to determine (see page127) when equal volumes of each phase are used. The *p*-value is defined as the fraction of the total solute that distributes itself in the non-polar phase of an equivolume solvent pair. For example, the *p*-value for hydroxychlor (VI) in an ethyl acetate/water system is 0.97. This means that 97% of the solute is in the non-aqueous phase (ie. ethyl acetate) and 3% in the aqueous phase. The same partition expressed as a K is 32.2; obviously the partition coefficient does not convey as clear a picture of distribution as does the *p*-value. The *p*-value experiment results clearly show that ethyl acetate is an efficient solvent for the extraction of the phenolic derivatives of methoxychlor. Therefore this solvent was used throughout the metabolic experiments described in this thesis.

CHAPTER III : THE EFFECTS OF METHOXYCHLOR AND ITS DERIVATIVES ON GREEN ALGAE

Introduction.

Two common species of green algae, *Chlorella pyrenoidosa*, and *Chlorella vulgaris* were chosen as test organisms. *Chlorella* are single celled algae that have been widely used in the study of respiration and photosynthesis. The cells are small and generally spherical with a single parietal chloroplast. Asexual reproduction is by autospores (ie. having the same distinctive shape as the parent cell) and is the only means of reproduction (53).

As part of the present investigation several attempts were made to assess the interaction of two compounds, methoxy chlor (IV) and hydroxychlor (VI) with these two algal species. These attempts may be described in terms of three distinct stages:

(i) The measurement of toxicity effects in terms of dosage-response relationships similar to those obtained for fungi (13).

(ii) The study of effects of the pesticide on the growth of algal cultures at constant concentration levels.

(iii) The study of bioaccumulation and/or metabolism, by extraction of cultures and analysis of extracts.

The results of a study of these interactions with algae

form an important part of the overall assessment of the environmental impact of this insecticide.

There are several methods available for the measure ment of growth, in terms of the biomass present in the algal cultures. The following methods are listed in order of preference according to the U.S. Environmental Protection Agency's protocol on algal growth measurement (26):

(i) Electronic cell counting.

(ii) Gravimetric methods based on dry weight.

(iii) Fluorescence measurements of algal chlorophyll.

(iv) Direct microscopic enumeration - Hemacytometer.

(v) Absorbance/turbidity measurements at specified wavelength.

The first method although generally assumed to be the most accurate could not be used in the present study because the equipment was not readily available.

Several investigators have used the turbidity method and the closely related absorbance method for algal growth assessment. The U.S. Environmental Protection Agency (EPA) criticises the use of the absorbance method when low cell densities lead to the observation of 0.06 absorbance units or less (26). In the above report it was noted that no distinction could be made between 5000 and 115,000 cells/mL when culture

densities were measured as an absorbance using a spectrophoto meter at 750nm. The same report does however, describe a good correlation between electronic cell counts and absorbance measurements in high density cultures (ie. above 0.06 abosrbance units). Also, Meyers (54) found a good correlation between absorbance and population density of *Chlorella pyrenoidosa* when measured using a spectrophotometer at 600nm. In the present study initial experiments with the absorbance method (see page110.) showed a linear relationship between absorbance and population density by serial dilution of a given algal culture, which is in agreement with the above observation. Since in the present study absorbance was generally recorded above 0.06 absorbance units, this method was considered adequate for measuring algal population densities.

Toxicity Studies

A logical first approach to a systematic study of the interactions between pesticides and microorganisms is the assessment of the toxic effects the pesticide may have on the organism(s). The accurate measurement of dosage-response relationships is the most complete and most informative method of studying such toxic effects.

The bioassay procedure used for this study of two algal species is similar to that used previously for an assess ment of the fungal toxicity (14) of the compounds discussed presently. Thus, a series of stagnant liquid cultures containing logarithmically increasing concentrations of test

compound were allowed to grow (light exposure 16 hours per 24 hour period, for further experimental details see page110.) for various time periods, and the cell densities of all cultures measured at the end of that period.

Toxicity measurements for *Chlorella pyrenoidosa* at methoxychlor concentrations below 25ppm indicated an average inhibition of 27% (see figure 3-1.). This species appears to be more sensitive to hydroxychlor (VI) and suffers an average inhibition of approximately 25% at concentrations below 12.5ppm (figure 3-2). The dosage-response curve (figure 3-3) for *Chlorella vulgaris* in the presence of methoxychlor revealed an average inhibition of less than 5% at concentrations below 12.5ppm. *Chlorella vulgaris* in the presence of hydroxychlor (figure 3-4) did not appear to be inhibited to any extent at concentrations of less than 12.5ppm. However complete inhibi tion of growth was observed for hydroxychlor concentrations above 12.5ppm.

The most characteristic feature of the algal dosage -response curves is the abrupt and complete inhibition of growth between concentrations from 12.5ppm to 25ppm of test compound. This phenomenon was not observed in the previous fungal toxicity study. The fungal dosage-response curves (figure 3-5, reproduced here from Bharath's thesis, 14) show, in general, a much more gradual dosage-response function.

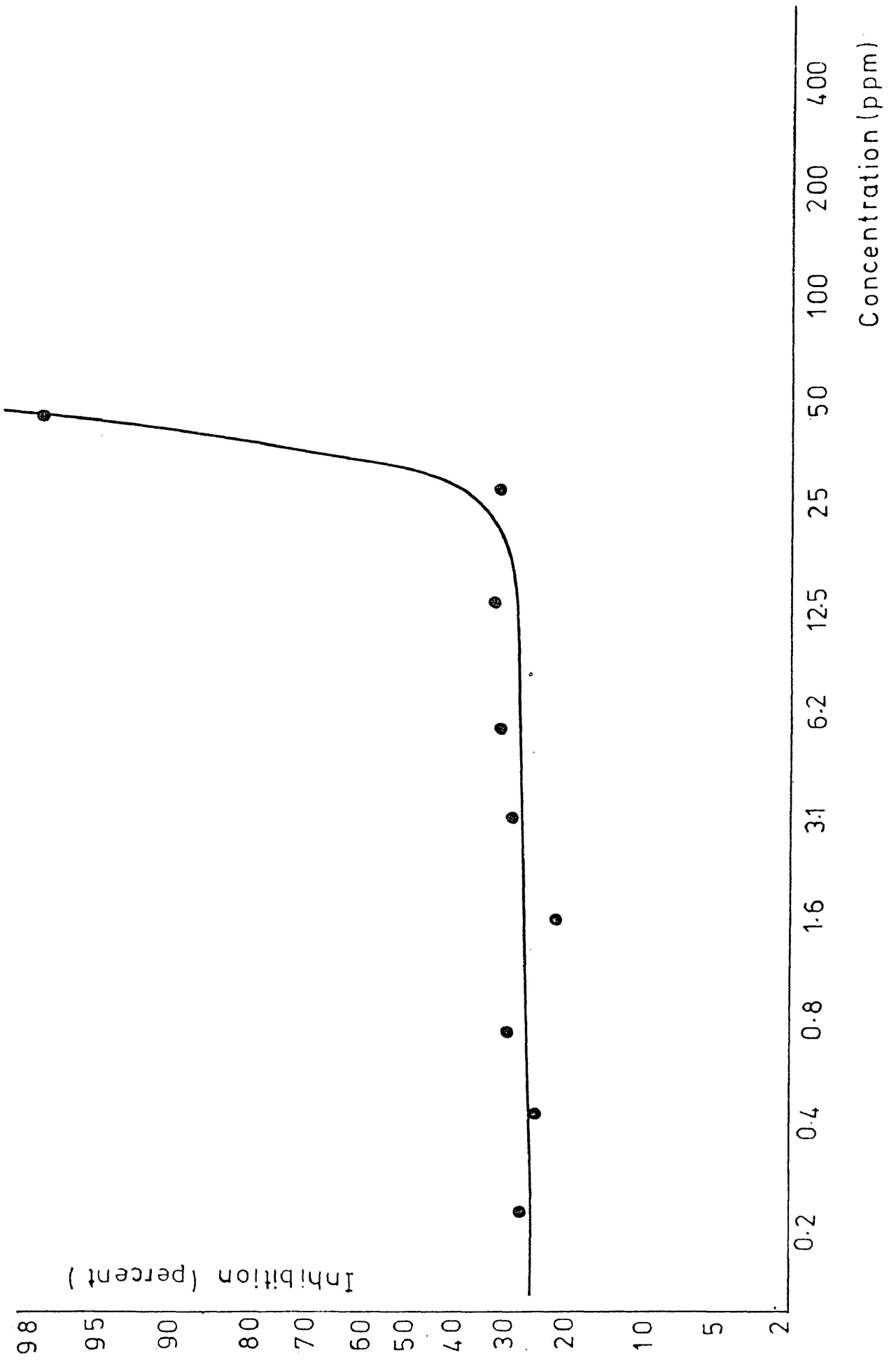


FIG. 3-1. Dosage-response curve of *Chlorella pyrenoidosa* assayed with methoxychlor, IV.

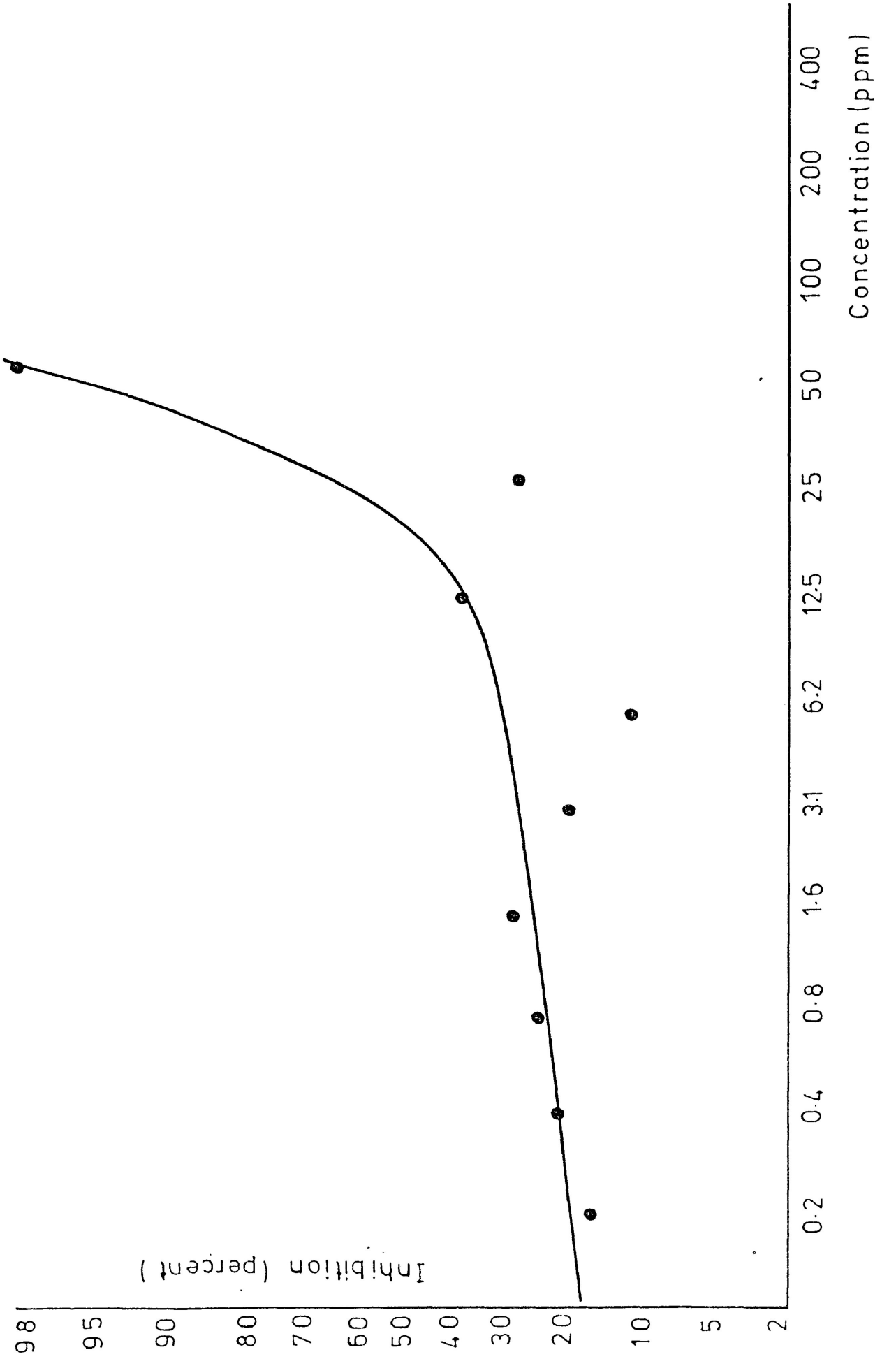


FIG. 3-2. Dosage-response curve of *Chlorella pyrenoidosa* assayed with hydroxychlor, VI.

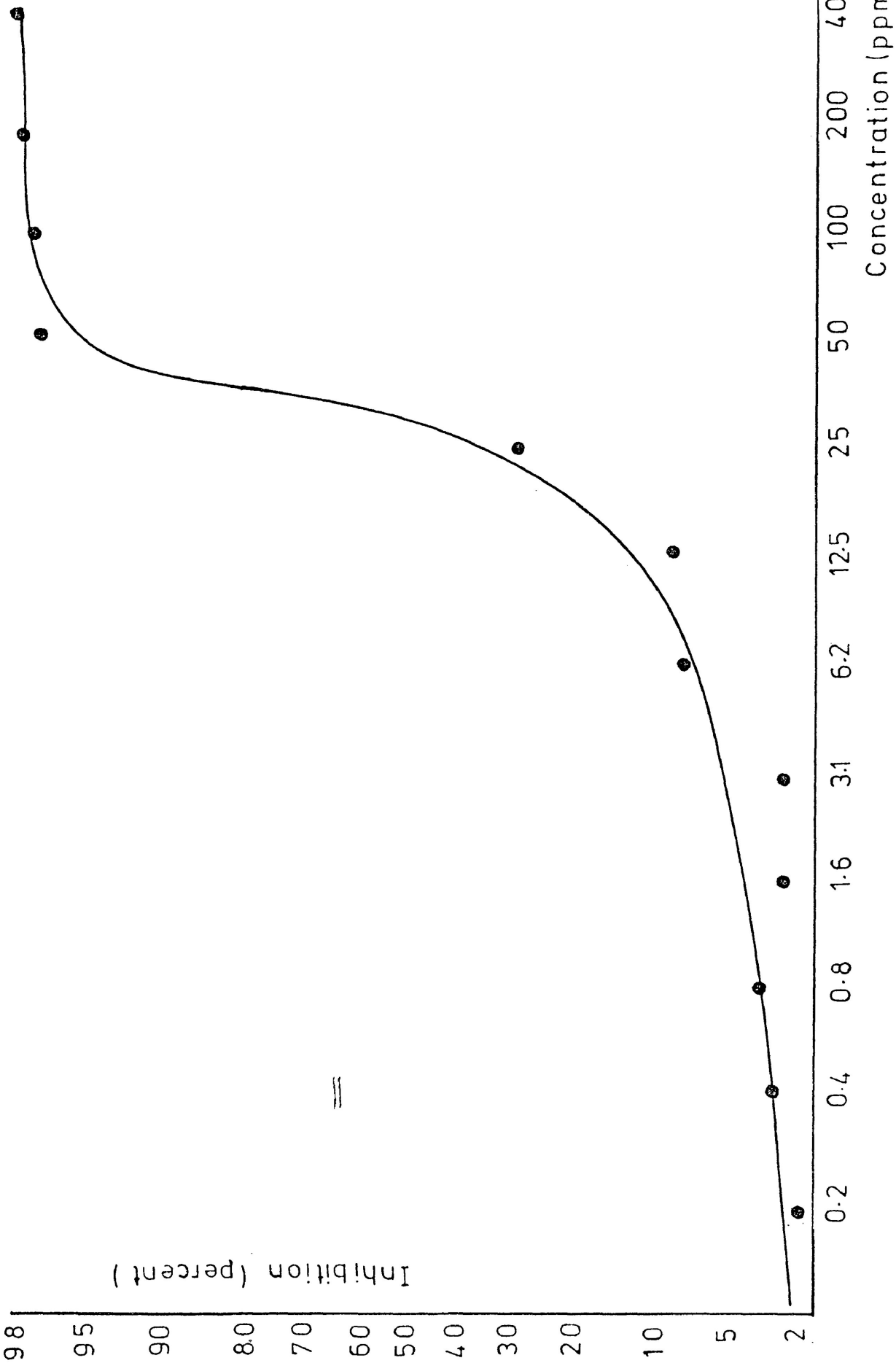


FIG. 3-3. Dosage-response curve of *Chlorella vulgaris* assayed with methoxychlor, IV.

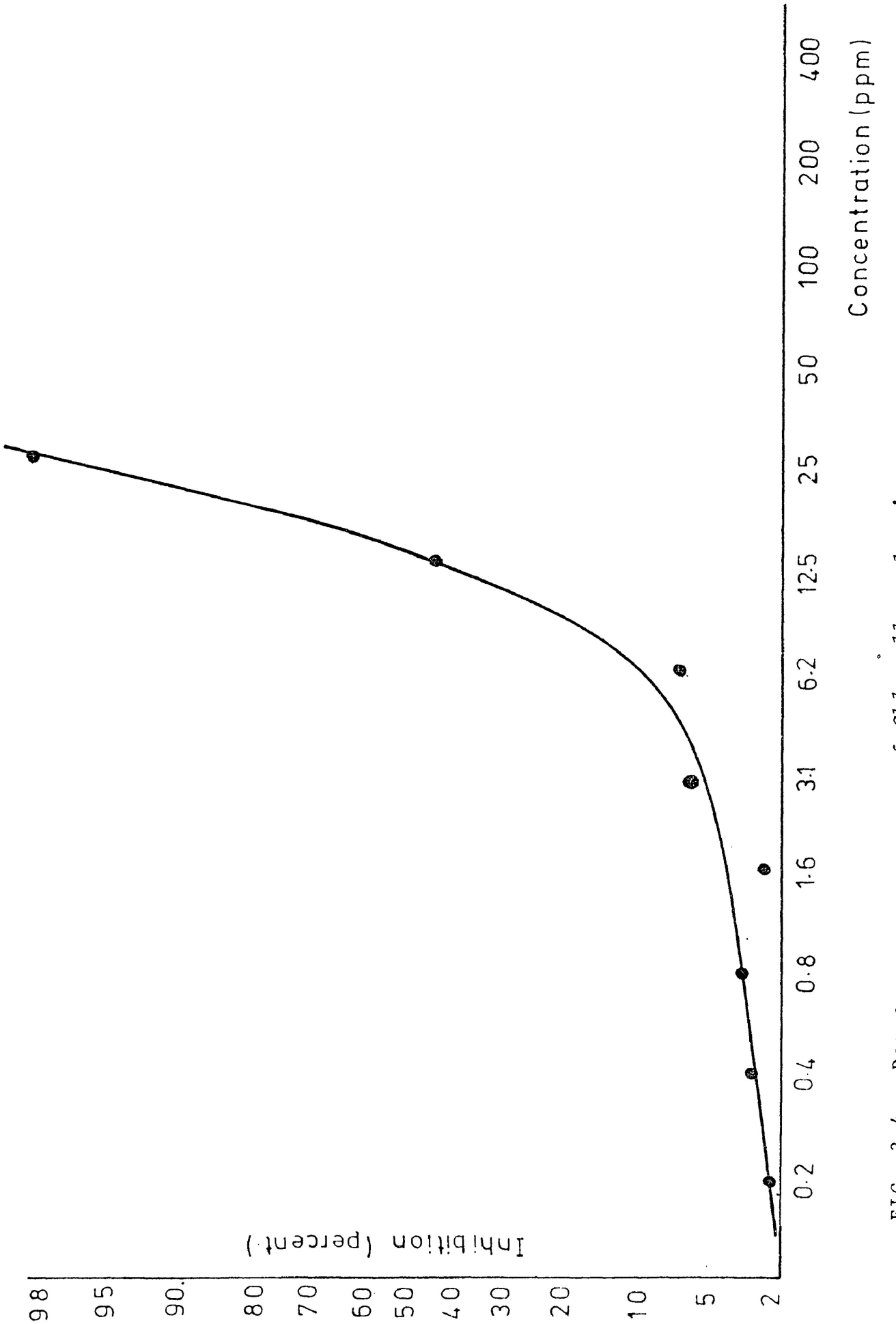


FIG. 3-4. Dosage-response curve of *Chlorella vulgaris* assayed with hydroxychlor, VI.

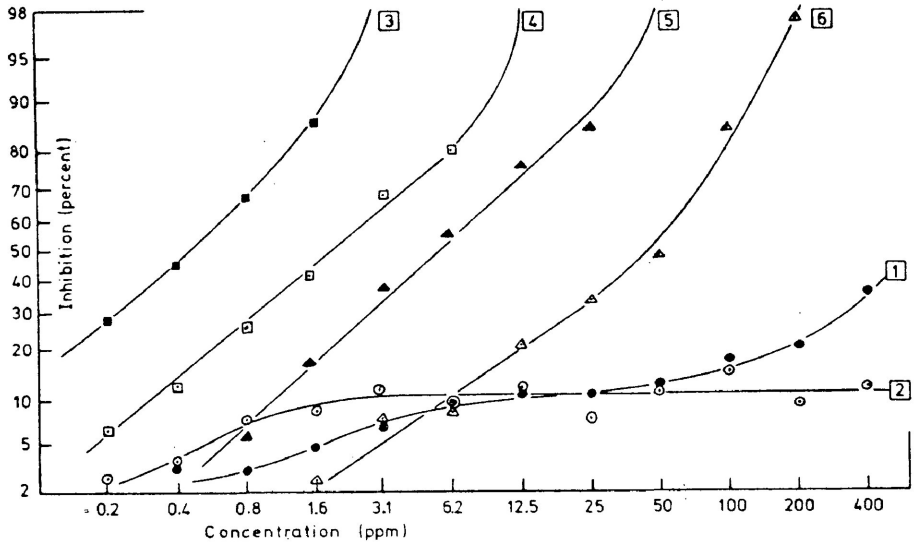


FIG. 3-5. Typical fungal dosage-response curves obtained by Bharath (14), (reproduced by permission of the author.)

The dosage-response curves obtained for *Chlorella pyrenoidosa* and *Chlorella vulgaris* show that these algae are subject to a drastic algicidal effect at a specific concentration level for each compound. This "cut-off" concentration was about 25ppm for methoxychlor and 12.5ppm hydroxychlor. It was also determined experimentally, that the size of the inoculum did not alter these "cut-off" concentrations. It may also be concluded that both algal species are more tolerant of methoxychlor than hydroxychlor, at least in terms of the above

"cut-off" concentrations. This conclusion must be considered with caution in view of the solubility differences between the two compounds. At 12.5ppm hydroxychlor is well within its solubility limit for aqueous media (76ppm, 8), on the other hand, at 25ppm the solubility limit of methoxychlor (0.1ppm, 56) is greatly exceeded. Therefore, the real concentration of methoxychlor in solution may be much less than indicated.

Growth Rate Studies

In order to determine the nature of the inhibiting effects of methoxychlor and hydroxychlor on the test algae, a series of growth rate experiments were carried out. The basic difference between the toxicity measurements (ie. dosage -response curves) and the growth rate experiments was in the way growth was monitored. In the toxicity study inhibition of algal growth was measured for each concentration level of a series of *increasing concentrations* of the test compound. All absorbance measurements were then made at the *end of the growth period*, which was determined by periodically measuring the growth of a set of control cultures. In the growth rate study a *fixed concentration* of test compound (chosen on the basis of the inhibition study) was used. Algal growth, of both test and control cultures was measured by absorbance *throughout the growth period* until the control cultures showed signs of decay. Thus in the present study the algae were grown in a stagnant mineral medium (25mL) containing one of the test compounds in concentrations determined by the "cut-off" points of the

toxicity curves (p.87) determined earlier and exposed to "cool white" fluorescent lights (400 ft.-candles, light exposure 16 hours per 24 hour period.) The correct amounts of methoxychlor or hydroxychlor (VI) were administered as solutions in 0.1mL acetone to each culture. Growth was monitored throughout the growth period by absorbance measure ments and compared to a set of control cultures to which only acetone (0.1mL) was added. The growth curves for both species in the presence of test compound and the corresponding control are presented in figures 3-6 to 3-9.

There are a number of general observations that may be discussed before analyzing the individual growth curves. The shapes of both test and control cultures resemble the exponential growth function which is characteristic of all biological growth patterns. The curves, with one exception (discussed later) level off after 10 to 12 days probably because at that point either the nutrient medium becomes exhausted or self-inhibition occurs due to overcrowding of the population. All test cultures exhibited a lag period of 12 to 48 hours when compared to the control cultures. This observation has led to two possible explanations. Either the added chemical inhibits cell division (algistatic effect) during this time period, thus depreseing growth rate, or the population suffers an initial toxic response (algicidal effect) whereby a certain percentage of the inoculum population is killed. In the second case it is assumed that the survivors

re-establish a normal growth pattern similar to the control cultures. It should be noted that for a given experiment (ie. one series of algal cultures for one test compound) the inoculum used was of uniform size for both control and test cultures, however the size of inocula may not necessarily be identical to that used for another experiment.

The growth curves (figure 3-6) *Chlorella pyrenoidosa* with methoxychlor show that this organism experiences a lag period of about 12 hours when compared to the control cultures. However, after this period the progress of the two cultures is essentially the same over the entire test period indicating similar growth rates.

Another observation is that the total population of the test cultures usually does not exceed that of the controls. When growth levels off, the total population difference between test and control cultures is about 5%. This difference may be accounted for in part, if it is assumed that the cause of the lag period is indeed a partial destruction of the inoculum (see above), by the initial damage to the population during the lag period. It thus appears that methoxychlor at 25ppm level only marginally affects the growth of *Chlorella pyrenoidosa*. This is in agreement with the observations of other workers who have commented on the apparent insensitivity of this species toward methoxychlor and other organo-chlorine pesticides. For example, Kricher *et. al.* (23) found that neither methoxychlor nor mirex significantly affects the

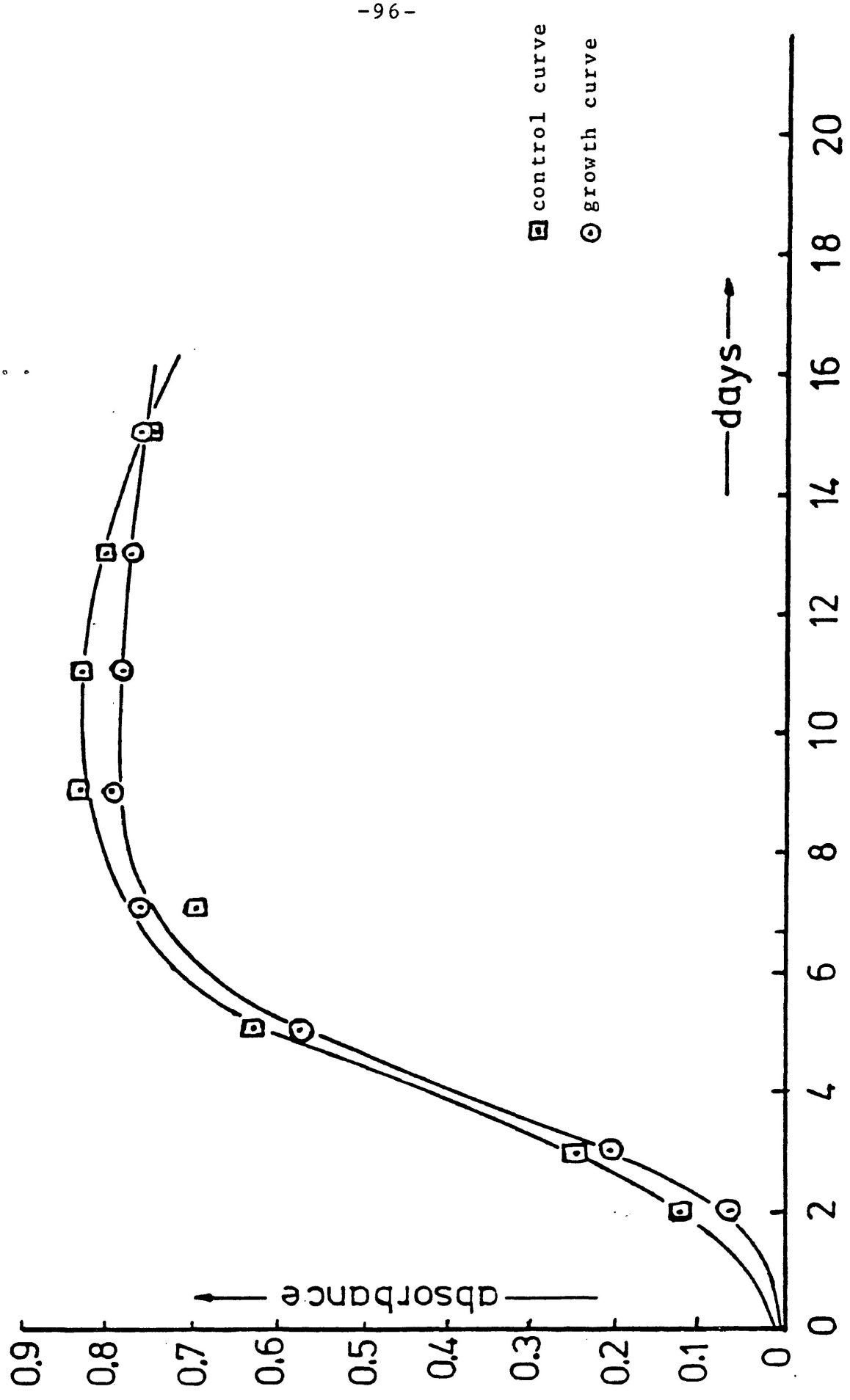


FIG. 3-6. Growth curve of *Chlorella pyrenoidosa* in the presence of methoxychlor, IV, (25ppm).

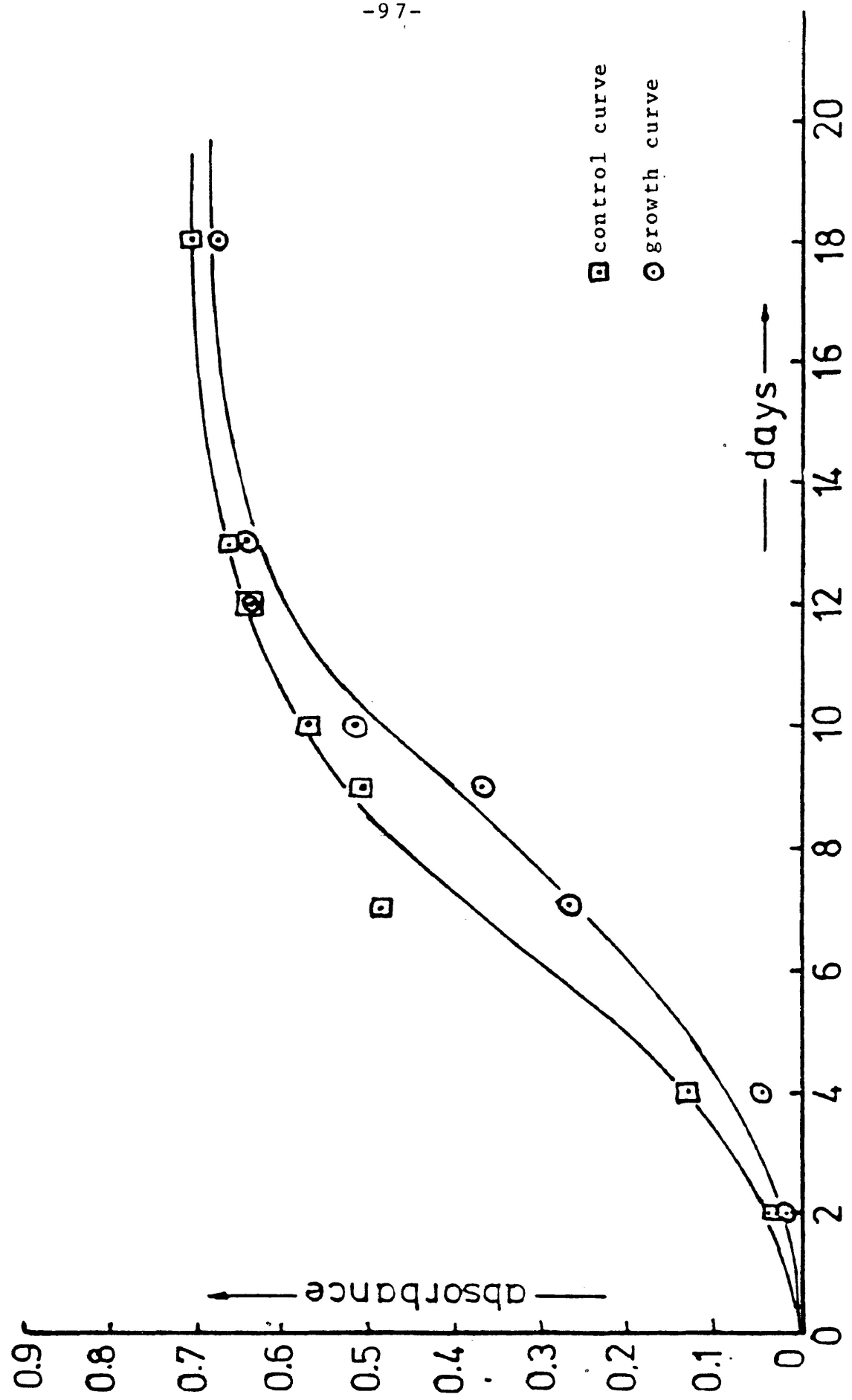


FIG. 3-7. Growth curve of *Chlorella pyrenoidosa* in the presence of hydroxychlor, VI, (12.5ppm).

growth or productivity of *Chlorella pyrenoidosa*. Other researchers (57, 58) have found similar effects with DDT on this species and have attributed the results to the low water solubility of this pesticide.

The growth curves (figure 3-7) for *Chlorella pyrenoidosa* with hydroxychlor show a lag period of about 24 hours in relation to the control cultures. In this case this lag is maintained during a major portion of the growth curve, indicating a modest degree of growth inhibition. The fact that the final population density of the test cultures is within 5% of the controls may be indicative of adaptation or metabolism.

In the case of *Chlorella vulgaris* in the presence of methoxychlor (figure 3-8) the inhibition of growth is manifested not merely as an initial lag period, but rather as a clear inhibition of growth of approximately 70% as compared to the controls during the entire growth period, with a final reduction in population density by about 60%, which is in agreement with the results of the toxicity study (see dosage-response curve, figure 3-3 at 25ppm). It is clear that *Chlorella vulgaris* is considerably more sensitive towards methoxychlor than *Chlorella pyrenoidosa*. The shape of the test culture curve also suggests the absence of any adaptation.

Certainly the most interesting growth curve obtained was that for *Chlorella vulgaris* with hydroxychlor (figure 3-9). In this case a lag period of nearly two days is observed,

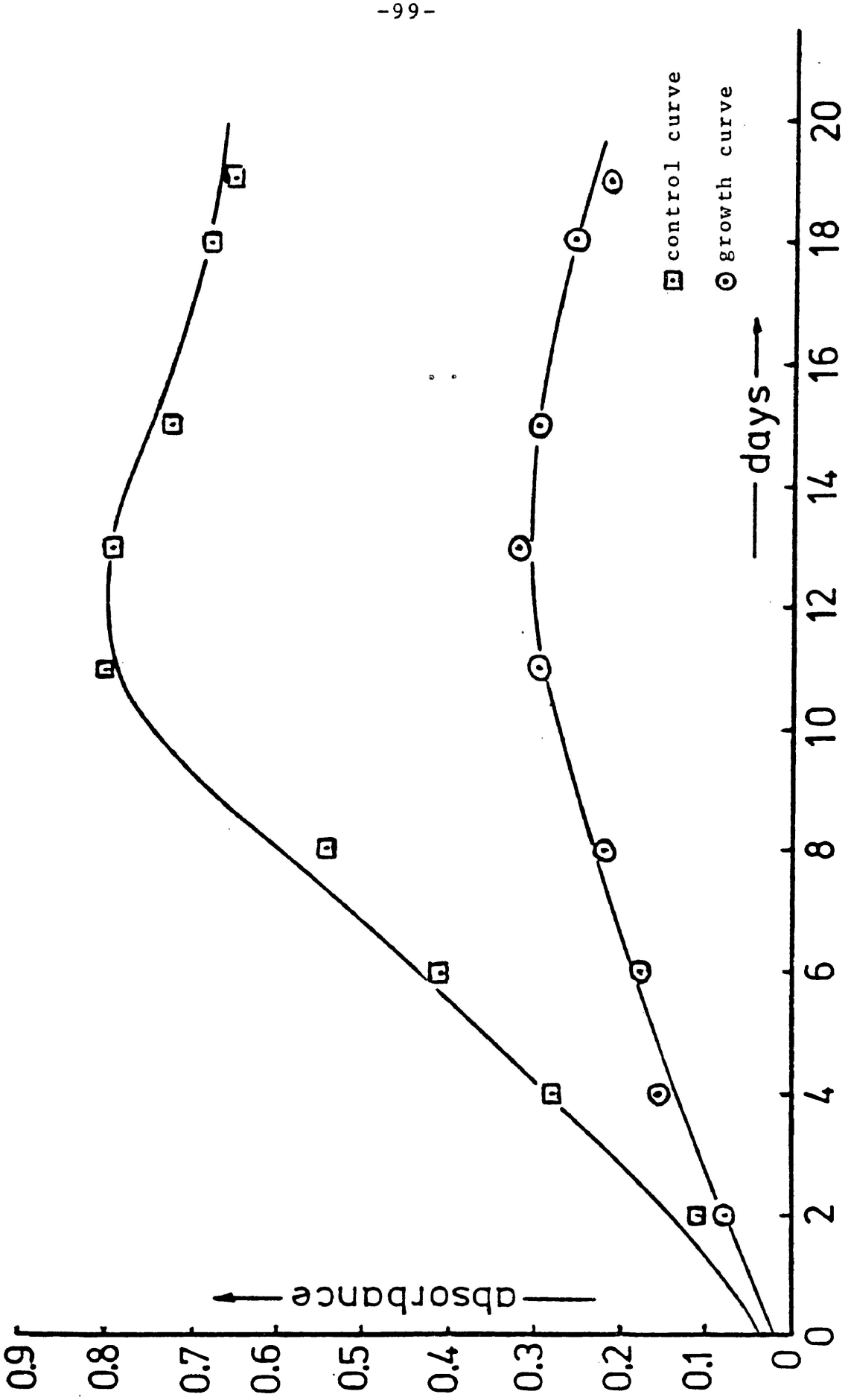


FIG. 3-8. Growth curve of *Chlorella vulgaris* in the presence of methoxychlor, IV, (25ppm).

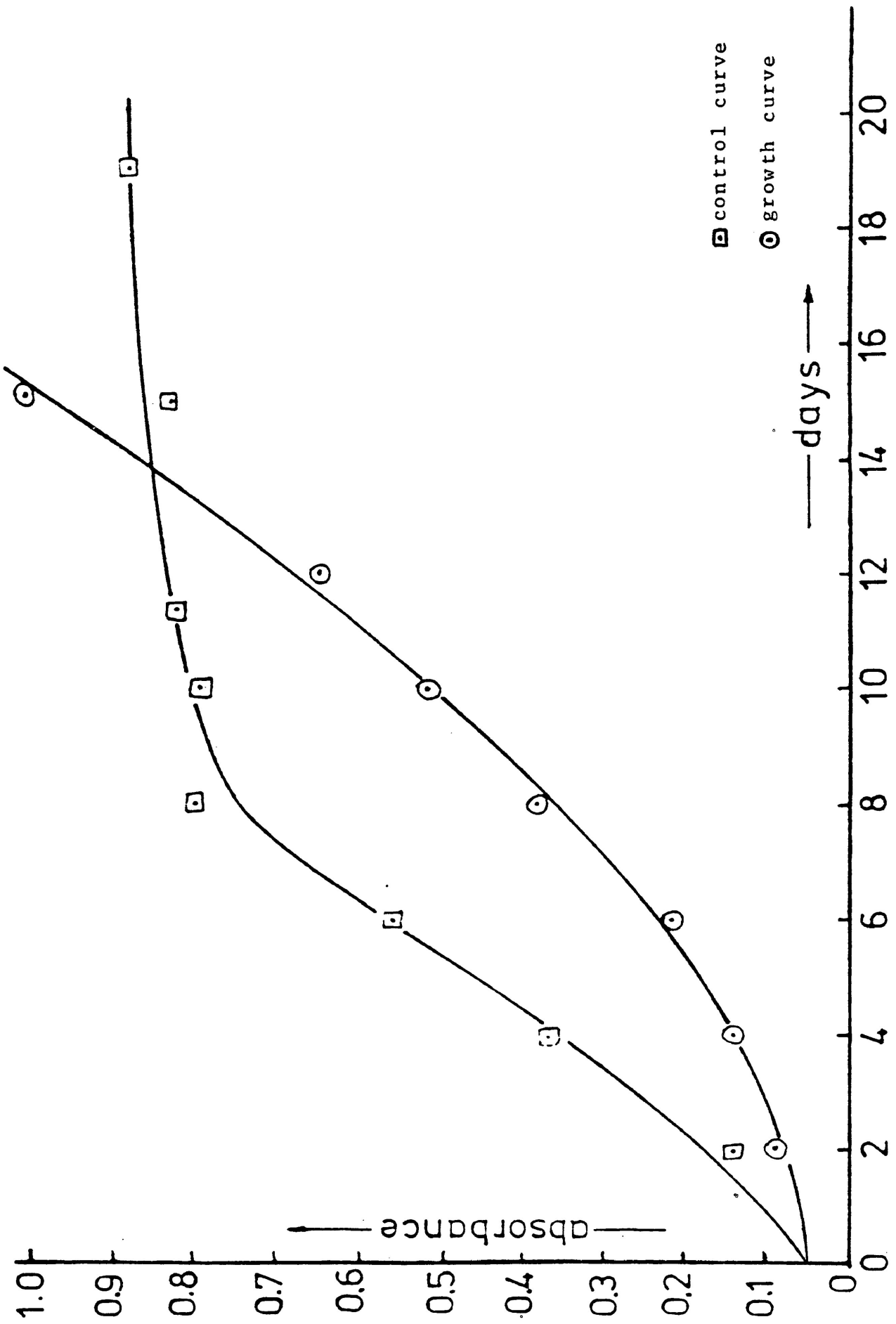


FIG. 3-9. Growth curve of *Chlorella vulgaris* in the presence of hydroxychlor, VI, (12.5ppm).

followed by approximately ten days of inhibited growth. The shape of the growth curve for the control cultures is as expected, similar to that observed earlier (see figure 3-8), however, the growth of the test cultures appears to continue for some time, so that the final population density is well beyond that of the control cultures. A possible explanation for this behaviour is that in the early stages of growth, cell division is inhibited (algistatic effect). Following this period the organism may adapt to hydroxychlor (VI) and eventually use it as an energy source. Further evidence in support of a suggested metabolic breakdown of hydroxychlor (VI) by *Chlorella vulgaris* was obtained from the recovery experiments discussed below (p.106). Analyses of extracts of this species grown in the presence of hydroxychlor (VI) indicated the presence of the dihydroxydichloro derivative (XI) which is a known reductive dechlorination product of hydroxychlor.

In conclusion it may be noted that these two closely related algal species react quite differently towards these compounds. In the natural environment such an effect could alter the natural species composition, placing a stress on the delicate balance established between various other organisms. The observation that hydroxychlor which is a known degradation product of the parent pesticide, is more toxic to both *Chlorella* species is in agreement with earlier observations concerning the toxicity of these compounds towards fungi (13).

It is reasonable to assume that this effect is related to the difference in water solubility of these two compounds. Thus, once more it becomes evident from these experiments that the assessment of the environmental impact of any pesticide is not truly complete without some knowledge of the effects of its biochemical and photochemical alteration products.

In addition, some of the inconsistencies between the toxicity data (previously discussed) and the results of the growth rate studies may be due to factors outlined by Bailey (55). Since the toxicity study was based on a bioassay procedure similar to the method for fungi using agar plates, then the validity of such results must be questioned with the following arguements in mind. Bailey stated that dosage-response data are only valid if: (i) there is no lag period due to an initial dying of cells in the inoculum, (ii) there is no localized depletion of the inhibiting substance at the growth front due to metabolism and (iii) the growth rate must remain constant during the period of the assay. It is unlikely that in liquid media where rapid diffusion is possible, localized depletion occurs. The other two criteria are not satisfied because the growth rate studies did verify that a lag period is experienced by the algae and that growth rate varied over the test period.

Recovery Experiments

In view of the relatively mild toxic effects methoxy chlor and hydroxychlor (VI) have on the test algae at lower

concentrations, the purpose of this part of the study was to collect evidence for the uptake, by adsorption or metabolism, of the test compounds by these organisms. Experiments were therefore designed to quantitatively measure the recovery of added methoxychlor or hydroxychlor from actively growing algal cultures after various time periods. The recovery experiments were conducted by adding the appropriate test compound (10ppm) to actively growing four day old liquid cultures (100mL), which were then grown for an additional 3, 48, and 96 hour time periods on a rotatory shaker table. At the end of the appropriate time period the whole cultures were extracted with ethyl acetate and the extracts analyzed by gas chromatography and also by thin layer chromatography. Although the recovered amounts (see table 3-1.) varied considerably, some general trends could be observed.

In the case of *Chlorella pyrenoidosa* the recovery figures suggest that both methoxychlor and hydroxychlor are physically adsorbed by this alga. This conclusion is based on the observation that no further decrease in recovery occurs after 48 hours contact with either chemical. If the loss of pesticide in these two cases were due to metabolism it would be expected that a continuous decrease would take place, since at this point (4 days plus 48 hrs) the cultures have reached an equilibrium growth stage but are not as yet dying (see growth curve, figure 3-6). It is thus likely that a fairly rapid equilibrium is established which remains

TABLE 3-1. Quantitative analyses of algal cultures from recovery experiments.

Species	Contact time(hr)	[1]Compound	[2]Recovery(%)	Metabolites
Chlorella pyrenoidosa	3	methoxychlor	47.5	
	48		34.3	
	120		38.9	
Chlorella pyrenoidosa	3	hydroxychlor	85.6	
	48		76.3	
	96		68.1	
Chlorella vulgaris	3	methoxychlor	60.0	
	48		8.3	
	120		1.0	
Chlorella vulgaris	3	hydroxychlor	33.7	
	48		15.7	
	120		0	dihydroxy dichloro,XI.

1 culture grown in presence of 10ppm of respective compound.

2 average value based on analyses of triplicate cultures.

constant. This suggestion is further supported by the fact that recovery of the less polar methoxychlor from *Chlorella pyrenoidosa* cultures is considerably lower than the more water soluble hydroxychlor. The lower value for methoxychlor suggests that this compound is more strongly adsorbed by the algal cells than hydroxychlor. Methoxychlor is a relatively non-polar molecule and may be readily adsorbed by the lipid rich algal cell. Hydroxychlor, on the other hand is a more polar molecule and as such is more lipophobic and is expected to partition itself in favour of the polar aqueous medium. This could account for its decreased adsorption and so larger amounts can be recovered.

A similar adsorption phenomenon was observed by Paris *et. al.* (24). These researchers found that the organo-chlorine pesticides methoxychlor and toxaphene were adsorbed by *Chlorella pyrenoidosa* and other microorganisms. They also noted that equilibrium was reached in 0.5 hours and the process was reversible. Moss *et. al.* (59) using ^{14}C-methoxychlor observed that sorption of methoxychlor appears to be mainly physical, since much of the labelled compound sorbed by algae was exchangeable with unlabelled material.

The chromatograms (both G.C. and T.L.C.) of the above extracts of *Chlorella pyrenoidosa* failed to reveal any break-down products of either test compound.

Extraction of *Chlorella vulgaris* cultures inoculated with the two test compounds gave quite different results.

The recovery after three hours contact time is now greater (60%) for the non-polar methoxychlor than for the polar hydroxychlor (33.7%), and in both cases no pesticide could be recovered after 120 hours incubation. These findings suggest that in the case of *Chlorella vulgaris* adsorption is not the only mechanism for uptake of these compounds. It is suggested that at least some of the hydroxychlor (VI) is metabolized. This suggestion is reinforced by the possible presence of the dihydroxydichloro derivative (XI) in the extracts of cultures containing hydroxychlor (VI) after 120 hours. Analysis of the extracts of these cultures by thin layer chromatography (silicagel, 1:10 v/v methanol/chloroform), after treatment with the spray reagent produced a blue colouration characteristic of the dihydroxydichloro derivative (XI). This very tentative result could not as yet be confirmed by gas chromatography. The above evidence is in agreement with the results obtained from the growth rate studies which indicated that the growth of *Chlorella vulgaris* was stimulated in the presence of hydroxychlor (VI) and may be using this compound as an energy source. The results of the growth rate experiments and the recovery experiments compliment each other and these results do indeed form a clear picture of the possible metabolism of hydroxychlor.

The recovery experiments conducted with *Chlorella vulgaris* and methoxychlor are somewhat inconclusive. No evidence of breakdown products could be found and in view of

the recovery of methoxychlor from the three hour cultures (60%), the recovery from the 120 hour cultures (less than 1%) is low.

An attempt was made to monitor the formation of the diphenyl acetic acid derivatives (VII and VIII) from these cultures, but analysis by thin layer chromatography failed to reveal the presence of either acid. The extraction solvent could not be at fault here since *p*-value measurements (p. 80) revealed that ethyl acetate is an efficient extraction solvent for these acids.

Furthermore, in view of the results from the metabolism work with *Chlorella vulgaris* and hydroxychlor (VI), future studies should be advanced with this combination in order to confirm the somewhat tentative results obtained. Perhaps a longer incubation period is necessary, in order to fully acclimatize this species to the presence of hydroxychlor (VI).

CHAPTER IV: EXPERIMENTAL METHODS

(i) Analysis Techniques

Nuclear magnetic resonance instrumentation details; - the nuclear magnetic resonance (NMR) spectra of samples in either deuterochloroform or hexadeuteroacetone were recorded using a Bruker WP-80 spectrometer with tetramethylsilane as an internal standard at δ = 0ppm.

Mass spectral instrumentation details; - the mass spectra were recorded using a Hitachi-Perkin-Elmer RMU-7 double focusing mass spectrometer. All samples were introduced via the direct inlet.

Spectrophotometric instrumentation details; - the infrared spectra were recorded on a Beckmann IR-12 spectrophotometer. Samples were prepared as either nujol mulls or in chloroform solution; ultraviolet spectra were recorded using a Cary model 14 spectrophotometer and the samples were prepared in ethanol solution.

Elemental analysis instrumentation details; - the combustion analyses were carried out with a Perkin-Elmer model 240 elemental analyser.

Gas-liquid chromatography method; - a Perkin-Elmer model 3920B gas chromatograph equipped with dual flame ionization detectors and dual glass columns (1.8m x 6.4mm i.d.) was used at temperatures from 230° to 250°C, with nitrogen as the carrier gas at 25mL/min. Liquid phases, on Gaschrome Q (80-100 mesh) were silicone gum SE-30 (5%) and Dexsil 300 (3%).

Qualitative identifications were made by determining the retention times relative to ethoxychlor for the dimethoxy derivatives (IV, IX, X, XIII, IV), retention times for the dihydroxy derivatives (VI, XI, XIV) and acid derivatives (VII, VIII) after silylation were measured relative to DDT. Quanti tative measurements were determined by measuring the ratios of the heights of the peak of the compound in question and the peak heights of an internal standard compound (60). Thus, extracts or compounds were made up to 1mL with an internal standard solution (either ethoxychlor or DDT in benzene). Dihydroxy derivatives were treated with Tri-sil reagent (0.2mL, Pierce Chemical Co.) prior to dilution with the internal standard solution containing DDT. Calibration curves derived from the analyses of solutions of the internal standard containing a range of known concentrations of the test-compound, then allowed for the quantitative analysis of all samples. New calibration curves were prepared for each experiment.

Thin-layer chromatography method; - the method employed was that described by Kapoor *et. al.* (8). Silicagel plates were eluted with one of three solvent systems: ether/pet. ether(30-60°)(1:9), ether/hexane(3:1), and pet. ether(30-60°)/ chloroform/methanol(3:2:1) depending on sample polarity. Characteristic colours for the various compounds were produced after spraying the plates with an acetone solution of 0.5% diphenylamine and 0.5% zinc chloride, heating the plates at

110°C for 10 minutes, and exposing them to ultraviolet radiation (254nm). Grey-black colours were obtained for the diaryltrichloroethanes (IV) and (VI), blue colours were observed for the diaryldichloroethanes (compounds, X, XI and XII) and a yellow colour was produced for the diarylmonochloro ethylenes (XV and XVI, see pages 5 and 6 for structures).

Algal culture and growth measurement method; - algal studies were conducted with cultures of *Chlorella pyrenoidosa* (ATCC no. 7516) and *Chlorella vulgaris* (ATCC no. 9765) using a stagnant liquid culture technique (20). The growth vessels were large pyrex test tubes (25 x 200mm) capped with a layer of standard thickness of absorbent cotton sandwiched between cheesecloth and secured over the tube opening with a rubber band. The growth medium (25mL) consisted of dextrose (10g), cassamino acids (10g), mineral stock solutions (10mL each, see appendix I), and microelement stock (1mL, see appendix I) per litre of distilled water. After autoclaving (15 minutes at 15p.s.i.) the tubes were cooled and inoculated with inocula (0.5mL) withdrawn from a four day old actively growing liquid culture (50 mL). The tube cultures were then placed at an angle of 30 degrees to the horizontal in a growth chamber and exposed to "cool white" fluorescent lights at 400ft-candles for 16 hours per 24 hour period at an ambient temperature of 23°C. The growth was monitored by periodically measuring the absorbance of the cultures (diluted 1:9 with distilled water) using a Fisher electrophotometer II, model 81 equipped with a

red filter.

(ii) Materials Used

All solvents and chemicals obtained for the synthetic work were reagent grade and were used without further purification unless stated otherwise. Melting points were determined with an electrothermal capillary tube melting point apparatus and are uncorrected.

(iii) Synthetic Chemistry

2,2-bis(p-methoxyphenyl)acetic acid; - the dimethoxy acid (VII) was prepared using a method similar to that of White and Sweeney (61). Anisole (2.92g) was mixed with glyoxylic acid (1.0g) in glacial acetic acid (10mL). Concentrated sulphuric acid (2mL) was added dropwise to the stirred mixture while the temperature was maintained below 30°. The resulting solution was stirred at room temperature for forty-one hours. The solvent was removed *in vacuo* and the residue dissolved in a saturated aqueous sodium bicarbonate solution (50mL). This solution was extracted with methylene chloride (50mL) and the extract discarded. The aqueous layer was acidified (10% H_2SO_4) and extracted with methylene chloride (2 x 50mL). The combined extracts were washed with water, dried (Na_2SO_4), and the solvent removed *in vacuo*. The product (2.96g, 80% yield) was a clear syrup, which crystallized on standing. Recrystallization attempts were unsuccessful. Titration of 0.2331g of this product with 0.1016N NaOH (8.19mL) indicated a molecular weight of 280 (calcd. M.W. = 272). The NMR spectrum ($CDCl_3$) contained signals at: 10.75(-COOH,1H), 7.25-6.75(aromatic,8H), 4.91(benzylic proton,1H), 3.75($-OCH_3$,6H)ppm.

Analysis: Calcd. for $C_{16}H_{16}O_4$: C,70.84; H,5.57; Found: C,70.50; H,5.86%.

The methyl ester was prepared by treating the acid (0.336g) with boron trifluoride - methanol reagent (2mL) at

room temperature for 4 hours. Purification of the residue by column chromatography (silicagel, 2% ether/hexane) yielded the desired ester (IX, 0.108g) which was recrystallized from chloroform, m.p. 63.5-64°, Lit. 65° (33). The NMR spectrum ($CDCl_3$) contained signals at: 7.23-6.73(aromatic,8H), 4.8 (benzylic proton,1H), 3.69($-OCH_3$,6H), 3.65(methyl ester,3H) ppm. The mass spectrum contained M^+ at m/e 286(19%, $C_{17}H_{18}O_4^+$) and the base peak at m/e 227(100%, $C_{15}H_{15}O_2^+$).

Analysis: Calcd. for $C_{17}H_{18}O_4$: C,71.33; H,6.33; Found: C,71.01; H,6.39%

2,2-bis(p-hydroxyphenyl)acetic acid; - the dihydroxy acid (VIII), was prepared by the same method used above for the dimethoxy acid (VI). Phenol (10.2g) was mixed with glyoxylic acid hydrate (4.8g) in glacial acetic acid (32mL). Concentrated sulphuric acid (6mL) was added dropwise to the stirred mixture and the temperature was kept below 30°. The resulting solution was stirred for forty-one hours at room temperature. The solvent was removed *in vacuo* and the residue dissolved in water (50mL). The solution was acidified (20% HCl) and extracted with ether (3 x 50mL). The combined extracts were washed with water, dried (Na_2SO_4), and the solvent removed *in vacuo*. The crude product was recrystal lized from a mixture of ether and benzene to give the pure acid (VIII, 5.35g, 42% yield), which after drying at 100° *in vacuo* had m.p. 145-147°, Lit. 145-151° (33). The acid (VIII), after treatment with Tri-sil reagent was homogeneous

by gas chromatography (see method p.108) and had RRT(DDT) = 1.25. The NMR spectrum (acetone-D6) contained signals at: 7.18-6.65(aromatic,8H), 4.81(benzylic proton,1H)ppm. The mass spectrum contained M^+ at m/e 244(100%,$CC_{14}H_{12}O_4^+$).

Analysis: Calcd. for $C_{14}H_{12}O_4$: C,68.85; H,4.95; Found: C,68.67; H,5.02%.

1,1,1-trichloro-2,2-bis(p-hydroxyphenyl)ethane; - hydroxychlor (VI) was synthesized by two methods.

Method (a) (8): Phenol (9.41g) was added to a slurry of anhydrous aluminum trichloride (8.0g) in chloroform (150mL, dry, ethanol free) in a nitrogen atmosphere. A solution of chloral (7.4g, freshly distilled from the hydrate with conc. H_2SO_4) in dry chloroform (10mL) was added dropwise to the stirred slurry while the temperature was kept below 0°. After the addition was complete the mixture was stirred for an additional half hour at 0°, and for a further twenty hours at room temperature. The reaction mixture was poured into cold water (300mL) and acidified (20% HCl). The organic layer was separated and the aqueous layer extracted with ethyl acetate (3 x 50mL). The combined extracts and organic layer were washed with water, dried (Na_2SO_4) and the solvent evaporated. The crude product was recrystallized from a mixture of ethanol and benzene to yield pure hydroxychlor (VI, 1.17g, 7.3% yield), m.p. 199-201°, Lit. 202-204° (8). The NMR spectrum (acetone-D6) contained signals at: 7.53-6.7(aromatic,8H), 5.03(benzylic proton,1H), 2.95(phenolic-OH,2H; D_2O exchangable)ppm. The

mass spectrum contained M^+ at m/e 316(9%,$C_{14}H_{11}Cl_3O^+$) and the base peak at m/e 199(100%, $C_{13}H_{11}O_2^+$).

Analysis: Calcd. for $C_{14}H_{11}Cl_3O_2$: C,52.95; H,3.49; Found: C,52.74; H,3.45%.

Method (b): Phenol (23.5g) was mixed with a cold solution (0°) of concentrated sulphuric acid (25mL) in carbon tetrachloride (100mL). Chloral (17.69g, freshly distilled) in carbon tetrachloride (10mL) was added dropwise to the vigourously stirred heterogeneous mixture while the reaction temperature was kept below 35°. After the addition was complete, the mixture was stirred for one hour at room temperature. After the reaction period, the solvent layer was separated from the insoluble product residue and the solvent was retained. The product residue, a mauve coloured syrup, was poured onto chipped ice (100g) and the mixture neutralized with 2N NaOH. The aqueous mixture was extracted with ethyl acetate (3 x 75mL). The extracts and the solvent layer were combined and washed with water, dried (Na_2SO_4) and the solvent removed *in vacuo*. The crude product was recrystallized from a mixture of ethanol and benzene to yield pure hydroxychlor (VI, 7.9g, 21% yield), m.p. 204-205°. The product was identical in all respects to that obtained by method (a) above.

1,1-dichloro-2,2-bis(p-methoxyphenyl)ethane; - the dimethoxydichloro derivative (X) was prepared by an improved method as discussed on page 38 .

Anisole (10.8g) was added to a cooled (0°) suspension of anhydrous aluminum trichloride (7.4g) in chloroform (80mL, dry, ethanol free) in nitrogen. Dichloroacetal (4.4g) in chloroform (10mL) was added dropwise to the stirred solution and the temperature was kept below 0°. The resulting mixture was stirred for half an hour at 0°, and a further 4.5 hours at room temperature. The reaction mixture was poured into ice water (300mL). The chloroform layer was separated and retained. The aqueous layer was extracted with ethyl acetate (2 x 50mL). The combined extracts and chloroform layer were washed in water, dried (Na_2SO_4), and solvent removed *in vacuo*. The crude product was a colourless oil which was crystallized from ethanol to yield pure dimethoxydichloro.derivative (X, 1.63g, 23% yield), m.p. 113-114°, Lit. 116° (9). The NMR spectrum ($CDCl_3$) contained signals at: 7.23-6.73(aromatic, 8H), 6.23(-$CHCl_2$, doublet J=4Hz,1H), 4.43(benzylic proton, doublet J=4Hz,1H), 3.74(-OCH_3,6H)ppm. The mass spectrum contained M^+ at m/e 310(16%,$C_{16}H_{16}Cl_2O_2^+$) and the base peak at m/e 227(100%,$C_{15}H_{15}O_2^+$).

Analysis: Calcd. for $C_{16}H_{16}Cl_2O_2$: C,61.75; H,5.18; Found: C,61.88; H,5.23%.

1,1-dichloro-2,2-bis(p-hydroxyphenyl)ethane; - the dihydroxydichloro derivative (XI) was prepared by an improved method as discussed on page 39 .

Phenol (18.4g) was added to a cooled (0°) slurry of anhydrous aluminum trichloride (20g) in dry carbon disulphide

(125mL) in nitrogen. A solution of dichloroacetal (18.7g) in carbon disulphide (20mL) was added dropwise to the stirred mixture while the temperature was maintained below 0°. After the addition the mixture was stirred for an additional 16 hours at room temperature. The reaction mixture was poured onto chipped ice and acidified (20% HCl). The mixture was extracted with ethyl acetate (3 x 50mL). The combined extracts were washed with water (800mL), dried (Na_2SO_4) and the solvent removed *in vacuo*. The crude product was recrystallized from a mixture of ethanol and benzene to yield the pure dihydroxydichloro derivative (XI, 5.16g, 18% yield), m.p. 178-179°, Lit. 178-179°,(14). The NMR spectrum (acetone -D6) contained signals at: 7.31-6.69(aromatic,8H), 4.43(benzylic proton, doublet J=4Hz,1H), 2.90(-OH,2H)ppm. The dichloromethyl proton signal was masked by the signal of the aromatic protons. Its presence at 6.75ppm was confirmed by spin decoupling. The mass spectrum contained M^+ at m/e 282(10%,$C_{14}H_{12}Cl_2O_2^+$) with the base peak at m/e 199(100%, $C_{13}H_{11}O_2^+$).

Analysis: Calcd. for $C_{14}H_{12}Cl_2O_2$: C,59.59; H,4.25; Found: C,59.31; H,4.21%.

1-chloro-2,2-bis(p-methoxyphenyl)ethylene; - the dimethoxymonochloro olefin (XV) was prepared by an improved synthetic method developed from the dehydrohalogenation studies (see also page 80).

The dimethoxydichloro derivative (X, 811mg) was refluxed (oil bath 100°) with a IN methanolic potassium hydroxide solution (50mL) for 45 minutes. The solvent was removed *in vacuo* and the residue dissolved in ice water (50mL). The aqueous mixture was acidified (20% HCl) and extracted with methylene chloride (2 x 50mL). The combined extracts were washed with water, dried (Na_2SO_4), and the solvent removed *in vacuo*. Recrystallization of the crude product from ethanol afforded the pure dimethoxymonochloro olefin (XV, 0.522g, 73% yield), m.p. 78-79°, Lit. 80.5-81°C (16). The NMR spectrum ($CDCl_3$) contained signals at: 7.30-6.75 (aromatic,8H), 6.40(-C=CHCl,1H), 3.83($-OCH_3$,3H), 3.79($-OCH_3$,3H) ppm. The mass spectrum contained M^+ at m/e 274(100%,$C_{16}H_{15}O_2^+$).

Analysis: Calcd. for $C_{16}H_{15}ClO_2$: C,69.95; H,5.50; Found: C,69.66; H,5.40%.

1,1-bis(p-methoxyphenyl)ethylene; - 4-Methoxybromo benzene (1.87g) was refluxed with dry magnesium turnings (0.243g) in dry ether (80mL) in nitrogen. 4-Methoxyaceto phenone (1.5g) in dry ether (20mL) was added dropwise to the above Grignard reagent. The mixture was refluxed for 16 hours in nitrogen. Aqueous sulphuric acid (10mL, 10% H_2SO_4) was added and the mixture refluxed for an additional 4 hours. The reaction mixture was extracted with ether (2 x 50mL) and the combined extracts were washed with water, dried (Na_2SO_4) and the solvent removed *in vacuo*. The residue was recrystallized from ethanol yielding the pure dimethoxy olefin (XVIII, 1.5g,

65% yield), m.p. 143°. The NMR spectrum ($CDCl_3$) contained signals at: 7.25-6.75(aromatic,8H), 5.25($C=CH_2$,2H), 3.76($-OCH_3$,6H)ppm.

Analysis: Calcd. for $C_{16}H_{16}O_2$: C,79.98; H,6.71; Found: C,80.04; H,6.70%.

Attempts to convert this olefin into 1-chloro-2,2-bis(*p*-methoxyphenyl)ethylene (XV) by direct substitution with gaseous chlorine in carbon disulphide were unsuccessful.

1,1-dichloro-2,2-bis(p-benzyloxyphenyl)ethane; - the dibenzyldichloro derivative (XII) was prepared by a modified literature method (62).

The dihydroxydichloro derivative (XI, 2.68g) was refluxed in a mixture of benzyl chloride (10mL), sodium ethoxide (1.3g, freshly prepared) in absolute ethanol (60mL) for 2.3 hours (oil bath 90°, N_2atmosphere). The solvent was removed *in vacuo*. The residue was dissolved in water (100mL) and extracted with methylene chloride (2 x 50mL). The combined extracts were washed with aqueous base (10% NaOH), water, dried (Na_2SO_4) and the solvent removed by evaporation. The crude product was heated (60°) *in vacuo* to remove residual benzyl chloride. The crude residue was recrystallized from ethanol to yield the pure product (XII, 0.95g, 22% yield), m.p. 76-79°. The NMR spectrum ($CDCl_3$) contained signals at: 7.36(benzyl aromatic,10H), 7.30-6.83(aromatic,8H), 6.28(dichloromethyl, doublet J=8Hz,1H), 5.13(benzylic ether protons, 4H), 4.45(benzylic proton, doublet J-8Hz,1H)ppm. The mass

spectrum did not contain the molecular ion peak. Major peaks were found at m/e 372(23%,$C_{21}H_{17}Cl_2O_2^+$) and m/e 289(100%, $C_{20}H_{16}O_2^+$).

Analysis: Calcd. for $C_{28}H_{24}Cl_2O_2$: C,72.63; H,5.22; Found: C,73.01; H,5.28%.

1-chloro-2,2-bis(p-benzyloxyphenyl)ethylene; - the dibenzylmonochloro olefin (XVI) was prepared by the same method developed for compound (XV).

The dibenzyldichloro derivative (XII, 0.95g) was refluxed with IN methanolic potassium hydroxide (60mL) for 1.5 hours. The solvent was removed *in vacuo* and the residue dissolved in water (75mL). The aqueous mixture was extracted with ether (3 x 50mL). The combined extracts were washed with water, dried (Na_2SO_4) and the solvent removed *in vacuo*. The crude product was recrystallized from ethanol and yielded pure dibenzylmonochloro olefin (XVI, 0.497g, 57% yield), m.p. 95-97°. The NMR spectrum contained signals at: 7.40(benzyl aromatic,10H), 7.23-6.85(aromatic,8H), 6.43(chloro olefinic proton, 1H), 5.09(benzylic ether proton,2H), 5.07(benzylic ether proton,2H)ppm. The mass spectrum contained M^+ at m/e 426(7%,$C_{28}H_{23}O_2Cl^+$) and base peak at m/e 91(100%, $C_7H_7^+$).

Analysis: Calcd. for $C_{28}H_{23}O_2Cl$: C,78.77; H,5.43; Found: C,78.47; H,5.57%.

4,4'-dihydroxydesoxybenzoin diethyl ketal; - this ketal (XX) was prepared by a method developed from the

dehydrohalogenation studies on compound (XI) (see also page 79).

The dihydroxydichloro derivative (XI, 0.402g) was mixed with a solution of potassium hydroxide (0.4g) in ethanol (40mL). The mixture was refluxed in nitrogen for one hour. The solvent was removed *in vacuo* and the residue dissolved in ice water (50mL). The aqueous mixture was acidified (20% HCl) and extracted with ether (3 x 50mL). The combined extracts were washed with water, dried (Na_2SO_4) and the solvent removed *in vacuo*. Recrystallization of the crude product from a mixture of chloroform, benzene, and ethyl acetate yielded pure ketal (XX, 0.3994g, 93% yield), m.p. 189-193°. The product was homogeneous on T.L.C.(silicagel, ether:benzene/1:4) and produced a yellow spot with the spray reagent. The NMR spectrum (acetone-D6) contained signals at: 8.09(-OH,2H), 7.05-6.68(aromatic,8H), 4.14(benzylic proton,2H), 3.15(methy lene protons, multiplet,4H), 0.9(methyl protons, triplet,6H) ppm. The infrared spectrum contained absorptions at 1070, 1100, and 1170cm^{-1}(C-O-C). The mass spectrum contained M^+ at m/e 302(1.5%,$C_{18}H_{22}O_4^+$) and the base peak at m/e 151(100%, $C_9H_{11}O_2^+$).

Analysis: Calcd. for $C_{18}H_{22}O_4$: C,71.5; H,7.33; Found: C,71.44; H,7.27%.

4,4'-dihydroxydesoxybenzoin (XXI); - the ketal (XX, 0.0091g) was dissolved in water (10mL) containing concentrated HCl (5 drops) and the mixture was heated (oil bath, 50°) for

12 hours. The reaction mixture was diluted with water (50mL) and extracted with ether (2 x 50mL). The combined extracts were washed with water, dried (Na_2SO_4), and the solvent removed *in vacuo*. The crude product (90mg) was recrystal lized from chloroform to give the ketone (XXI), which had m.p. 174-179° and was homogeneous on T.L.C.(silicagel, pet. ether:chloroform:methanol/3:2:1). The infrared spectrum contained a very strong absorption at 1719cm^{-1}(C=O). The ultraviolet spectrum (ethanol) contained λ (max) 287nm (ε=10,567), which shifted to λ (max) 344nm (ε=13,373) upon the addition of NaOH.

(iv) Dehydrohalogenation Reactions

hydroxychlor; - ten samples of hydroxychlor (VI, 100mg each) were refluxed (oil bath, 100°) in the following solutions (20mL): water, aqueous HCl(pH=5), 3N methanolic potassium hydroxide, 3N aqueous potassium hydroxide, 0.5N methanolic potassium hydroxide, three aqueous phthalate buffer solutions at pH=2.2, 6, and 8 respectively, aqueous bicarbonate buffer solution(pH=10), and an aqueous phosphate buffer solution(pH=12) for 0.5 hours in nitrogen. Three additional samples of hydroxychlor (100 mg each) were refluxed (oil bath, 100°) in solutions (20mL) of: water, aqueous HCl(pH=5), and 0.5N methanolic potassium hydroxide for 2.0 hours in nitrogen.

Each of the samples was treated separately. Organic solvents when present were removed *in vacuo*. The residues were dissolved in water (50mL), acidified (20% HCl), and the aqueous mixtures extracted with ethyl acetate (50mL). The respective extracts were washed with water, dried (Na_2SO_4), and evaporated to dryness.

The dry residues were weighed, and dissolved in ethyl acetate (10mL). A 0.5mL aliquot was transferred to a 1mL serum cap vial and the solvent removed in a stream of dry nitrogen. Each residue was treated with Tri-sil (0.2mL) and the internal standard solution (0.8mL, DDT solution) was added. The samples were analyzed by gas chromatography as described (see page108). The results are summarized in

Table 2-2 (see page 78).

methoxychlor; - four samples of methoxychlor (IV, 100mg each) were refluxed (oil bath, 100°) in the following solutions (20mL): water, aqueous HCl(pH=2), aqueous HCl (pH=5), and 3N methanolic potassium hydroxide for 0.5 hours. An additional four samples were refluxed under the same conditions for a period of 2 hours.

The aqueous reaction mixtures were extracted separately with ether (50mL). The methanol from the basic reaction mixture was removed *in vacuo* and the residue was dissolved in water (20mL) acidified (20% HCl), and extracted with ether (50mL). The extracts were dried (Na_2SO_4) and evaporated to dryness.

The dry residues were weighed, dissolved in 10mL ethyl acetate and a 0.5mL aliquot from each sample transferred to a 1mL serum cap vial and the solvent removed in a stream of dry nitrogen. To the residues ethoxychlor internal standard solution (1.0mL) was added and the samples analyzed by gas chromatography (see above). The results are summarized in Table 2-1 (see page 77).

dimethoxydichloro derivative; - four samples of the dimethoxydichloro derivative (X, 100mg each) were refluxed (oil bath, 100°C) in the following solutions (20mL): 3N aqueous potassium hydroxide, IN methanolic potassium hydr oxide, 3N ethanolic potassium hydroxide, and triethylamine for 0.5 hours. An additional four samples (100mg each) were

refluxed (oil bath, 100°) in solutions (20mL) of: water, IN methanolic potassium hydroxide, a methanolic potassium iodide solution (500 mg KI in 20mL methanol) and pyridine for 2 hours. The cooled reaction mixtures were acidified (20% HCl) and each was extracted with ethyl acetate (50mL). The extracts were washed with water, dried (Na_2SO_4) and solvent removed *in vacuo*. The dry residues were weighed, and each was dissolved in 10mL ethyl acetate. A 0.5mL aliquot from each sample was transferred to a 1mL serum cap vial and solvent removed in a stream of dry nitrogen. To the residues ethoxychlor internal standard solution (1.0mL) was added and the resulting mixtures analyzed by gas chroma tography. The results are tabulated in Table 2-3, (see page 79).

dihydroxydichloro derivative; - the dihydroxydichloro derivative (XI, 200mg) was refluxed (oil bath, 100°) in a solution of ethanol (20mL) containing potassium hydroxide (0.2g). Two 5.00mL aliquots were withdrawn after 0.5 and 1.0 hour reflux time respectively. The remaining reaction mixture was allowed to reflux for a further 0.5 hours. The aliquots and remaining reaction mixture were quenched with aqueous acid (20% HCl) and each was extracted with ether (20mL). The extracts were washed with water, dried (Na_2SO_4) and solvent removed *in vacuo*.

The dry residues were weighed, then each dissolved in ethyl acetate (10.0, 10.0, and 20.0mL respectively). A

1.00mL aliquot of each solution was transferred to a 1mL serum cap vial and the solvent removed in a stream of dry nitrogen. Each residue was prepared for gas chromatographic analysis as above. The results of subsequent gas chromato graphic analysis are given in Table 2-4 (see page 79).

(v) Solubility and *p*-value Measurements

Solubility of hydroxychlor in benzene; - twenty-five milligrams of hydroxychlor (VI) was added to benzene (50mL) and the mixture stirred in a tightly capped volumetric flask at room temperature for 48 hours. The resulting mixture was filtered through a plug of glass wool. The first 20mL of filtrate was rejected and the next 20mL was retained for quantitative gas chromatographic analysis as described on page 108.

Results: The solubility of hydroxychlor (VI) in benzene was 194ppm (based on triplicate analyses, see also table 2-5 page 82 .)

Solubilities of the dimethoxy and dihydroxy acids in water; - twenty-five milligrams of the dimethoxy acid (VII) was added to distilled water (50mL) and the mixture stirred in a tightly capped volumetric flask at room temperature for 48 hours. The resulting mixture was filtered through a plug of glass wool. The first 20mL of filtrate was rejected and the next 20mL was retained. Triplicate 1.00mL aliquots of the retained filtrate were transferred to 1mL serum cap vials and the water removed under a stream of dry nitrogen. Each sample was treated with Tri-sil (0.2mL) and diluted with DDT internal standard solution (0.8mL). The samples were then analyzed by gas chromatography (p.108).

The dihydroxy acid (VIII) water solubility was determined by titration using standard sodium hydroxide solution.

Thus, two grams of the dihydroxy acid was added to distilled water (50mL) and the mixture stirred in a tightly capped volumetric flask at room temperature for 48 hours. The resulting mixture was filtered in the same manner as described above. Triplicate 5.00mL aliquots of the filtrate were titrated with 0.0908N NaOH solution using a pH meter. The average titer was 11.94mL.

Results: The water solubility of the dimethoxy acid (VII) was found to be 199ppm. The water solubility of the dihydroxy acid (VIII) was found to be 17,656ppm (see also table 2-5 page 82.)

p-value of hydroxychlor; - two stock solutions of hydroxychlor (VI) in benzene (Omni Solv, BDH Chemicals Ltd.) were prepared by weighing 0.00615g and 0.01182g hydroxychlor into two 100mL volumetric flasks respectively and diluting with benzene to the mark. Two additional stock solutions of hydroxychlor (VI) in ethyl acetate (glass distilled) were prepared by the same technique using 0.00839g and 0.01056g hydroxychlor respectively. A 5.0 mL aliquot of each of the above stock solutions was transferred to separate 10mL screw cap vials containing 5.0mL of distilled water which had previously been equilibrated with the appropriate solvent (benzene or ethyl acetate). The vials were hand shaken for one minute and allowed to settle. A 2.0mL aliquot of the organic phase from each vial was withdrawn, dried (Na_2SO_4), transferred to separate 5mL vials and solvent removed under

a stream of dry nitrogen. Each dry residue was treated with 0.2mL Tri-sil reagent and diluted with 0.8mL DDT internal standard solution. The samples were analyzed by gas chromatography using an SE-30 (5%) column as described (see page 108). A 2.0mL aliquot of each original stock solution was also prepared for gas chromatographic analysis by the same technique and analyzed subsequently. The *p*-values were calculated as the ratio of the material in the organic phase to that originally present in the stock solution.

Results: The *p*-value for hydroxychlor (VI) was 0.82 (62 ppm in benzene/water system), 0.82 (118 ppm in benzene/water system), 0.96 (84 ppm in ethyl acetate/water system) and 0.97 (106 ppm in ethyl acetate/water system), see also table 2-6 page 82.

Dimethoxy acid and dihydroxy acid p-value measurements; - a stock solution of the dimethoxy acid (VII) in ethyl acetate was prepared by weighing 0.04541g of the acid into a 100mL volumetric flask and diluting to the mark with ethyl acetate. A stock solution of the dihydroxy acid (VIII) in ethyl acetate was also prepared by the same technique using 0.02481g of the acid. The *p*-values for the acid derivatives (VII) and (VIII) were determined by gas chromatography using the same technique as described for hydroxychlor (VI) above.

Results: The *p*-value for the dimethoxy acid (VII) was 0.79 (454 ppm in ethyl acetate/water system). The *p*-

value for the dihydroxy acid (VIII) was 0.82(248 ppm in ethyl acetate/water system), see also table 2-6 page 82.

(vi) Experiments with Algae

Dosage-response curves; - triplicate cultures (25mL) were prepared for each concentration level at logarithmically increasing concentrations of each test compound (methoxychlor, IV, hydroxychlor, VI.). The correct amounts were added to the media in acetone (0.1mL) immediately after autoclaving. A series of control cultures were also prepared to which only acetone (0.1mL) had been added. The cooled tubes were inoculated with inocula (0.5mL) withdrawn from a four day old actively growing liquid culture (50mL) and test cultures grown as described on page 110. The growth of the control cultures was monitored by absorbance measurements (see p. 110 for details) until a maximum was reached, signifying the end of the growth period (usually between 10 and 14 days). At this time the absorbance of the test cultures was measured. The degree of inhibition of algal growth was calculated from the mean differences between the controls and the test cultures, and expressed as a percentage of the former. Dosage-response curves were obtained by plotting percentage inhibition on a probit scale (63) against pesticide concentration on a log. scale. The resulting dosage-response curves are given on pages 88 to 91.

Growth rate measurements; - the growth rate study was conducted using the stagnant liquid culture method as previously described. Test cultures (25mL) were prepared in the same manner as described above for the toxicity measurements. The media for the test cultures contained 12.5ppm hydroxychlor

(VI) or 25ppm methoxychlor (IV) for both species. A series of control cultures were also prepared. All cultures were inoculated and grown under the same conditions as described before. The initial absorbance of the cultures (triplicate samples of both test and control cultures) was measured as above and every two days following inoculation until the cultures began to decay. Growth curves were obtained by plotting the absorbance verses the time on linear graph paper. These curves are presented on pages 96 to100.

Recovery measurements; - liquid algal cultures (100mL) were prepared as before (p.110.) and grown for four days on a rotatory shaker table (stroke 12cm, 90r.p.m.) under fluorescent lights (400ft.-candles, see page 110 for additional details.) To triplicate cultures was added the appropriate test compound (1mg) in acetone (0.1mL), and these cultures were allowed to grow for additional time periods varying from 3 to 120 hours. At the end of the respective time periods the cultures were extracted with ethyl acetate (3 x 50mL). The combined extracts from each culture were dried (Na_2SO_4) and solvent removed *in vacuo*. The dry residues were dissolved in the appropriate internal standard solution and analyzed by gas chromatography (see page108). The results are recorded in Table 3-1 on page 104.

CHAPTER V: CONCLUSIONS

From the experiments discussed in chapters II and III a few general conclusions may be made.

The synthetic chemistry of many of the derivatives of methoxychlor have been re-investigated primarily due to the low yields reported in some of the earlier literature. It was necessary to improve on these procedures so that they be available for other chemical and biological studies. For example, the synthesis of hydroxychlor (VI), was improved because it was needed in quantity for many chemical and biological studies. The synthesis of the dihydroxydichloro derivative (XI) was also improved as this compound was also needed in quantity for many chemical studies.

Along with the synthetic work some of the important physical properties of these derivatives, such as solvent and water solubility and extractability have been investigated primarily because of the lack of reliable data in the literature. These properties are especially important if environmental studies on these compounds are undertaken. In view of the results it has been found that ethyl acetate is an efficient extraction solvent for methoxychlor metabolites including the more polar hydroxy derivatives and the carboxylic acid derivatives.

The preparative synthesis of the 1-chloro-2,2-bis(p-hydroxyphenyl)ethylene (XVII) derivative, once completed, is important for two reasons. Firstly, it has been mentioned

that this compound has been identified in goats (30)as a methoxychlor metabolite. If quantities of this material were available then further biodegradation work with *Klebsiella pneumoniae* may proceed, as this metabolite has been suggested as a possible biointermediate in the formation of the methoxychlor acid derivatives (14).

Secondly, from a more chemical point of view with regards to the observed rearrangement reaction, the dihydroxymonochloro olefin (XVII) may provide a chemical tool in elucidating the mechanism of the formation of ketal (XX) from the dihydroxydichloro derivative (XI).

The toxicological experiments with green algae show that although the test species are tolerant of relatively high concentrations of methoxychlor and hydroxychlor (ie. 25ppm and 12.5ppm respectively), the hydroxy analogue was much more toxic than the parent insecticide. It has also been observed that one of the algal species, *Chlorella vulgaris*, is to some extent capable of reductive dechlorination of hydroxychlor (VI) to the dihydroxydichloro derivative (XI). It is recommended that this somewhat tentative result, be confirmed by additional metabolism experiments in the future.

APPENDIX I

Mineral stock solutions

Ten millilitres of each of the following stock solutions were added to 938 millilitres of algal growth medium.

KCl	1.5g/L
KH_2PO_4	5.0g/L
KNO_3	5.0g/L
$MgSO_4 \cdot 7H_2O$	10.0g/L
$Ca(NO_3)_2 \cdot 4H_2O$	10.0g/L

Microelement stock solution

One millilitre of the following stock solution was added to 938 millilitres of algal growth medium.

$MnSO_4 \cdot 4H_2O$	125mg/L
H_3BO_3	125mg/L
$ZnSO_4 \cdot 7H_2O$	125mg/L
$CuSO_4 \cdot 5H_2O$	125mg/L
$Na_2MoO_4 \cdot 2H_2O$	5mg/L
$Co(NO_3)_2 \cdot 6H_2O$	6.3mg/L
ferric ammonium citrate	6.3 g/L

REFERENCES

1. Metcalf, R.L., I.P. Kapoor, and A.S. Hirwe. Development of biodegradable analogues of DDT. Chemtech. Feb. 105-109. 1972.

2. Metcalf, R.L.. A Century of DDT. J. Agric Food Chem. 21: 511-519. 1973.

3. Kenaga, E.E.. Guidelines for environmental study of pesticides: Determination of bioconcentration potential. Res. Rev. 44: 73-113. 1972.

4. Bevenue, A.. The bioconcentration aspects of DDT in the environment. Res. Rev. 61: 37-112. 1976.

5. Brooks, C.T.. "Chlorinated Insecticides, Volume II, Biological and Environmental Aspects." CRC Press, Ohio. 1974.

6. Edwards, C.A.. "Persistent Pesticides in the Environment." CRC Press, Ohio. 1975.

7. Gardner, D.R., and J.R. Bailey. Methoxychlor, its effects on environmental quality. National Research Council of Canada, Publication No. 14102. 1975.

8. Kapoor, I.P., R.L. Metcalf, R.F. Nystrom, and G.K. Sanga. Comparative metabolism of methoxychlor, methiochlor and DDT in mice, insects, and in a model ecosystem. J. Agric. Food Chem. 18: 1145-1152. 1970.

9. Zepp, R.G., N.L. Wolfe, J.A. Gordon, and R.C. Fincher. Light-induced transformations of methoxychlor in aquatic systems. J. Agric. Food Chem. 24: 727-733. 1976.

10. MacNeil, J.D., R.W. Frei, S. Safe, and O. Hutzinger. Electron-Donor-Acceptor complexing reagents in the analysis of pesticides. V. The analysis of pesticide decomposition products via electron-donor-acceptor complexes; Photolysis of Methoxychlor. J. Assoc. Off. Anal. Chem. 55(6): 1270-1275. 1972.

11. Wolfe, N.L., R.G. Zepp, D.F. Paris, G.L. Baughman, and R.C. Hollis. Methoxychlor and DDT Degradation in Water: Rates and Products. Environ. Sci. Technol. 11: 1077-1081. 1977.

12. Walker, W.W.. Insecticide persistence in natural seawater as affected by salinity, temperature, and sterility. U.S. Environmental Protection Agency. Ocean Springs MS. EPA600/3-78-044. 1978.

13. Baarschers, W.H., A.I. Bharath, M. Hazenberg, and J.E. Todd. Fungitoxicity of methoxychlor and fenitrothion and the environmental impact of their metabolites. Can. J. Bot. 58(4): 426-431. 1980.

14. Bharath, A.I.. M.Sc. Thesis, Lakehead University. 1980.

15. Baarschers, W.H., A.I. Bharath, J. Elvish, and M. Davies. The biodegradation of methoxychlor by *Klebsiella pneumoniae*. Can. J. Microbiol. 28: 176-179. 1982.

16. Mendel, J.L., A.K. Klein, J.T. Chen, and M.S. Walton. Metabolism of DDT and some other chlorinated organic compounds by *Aerobacter aerogenes*. J. Assoc. Off. Anal. Chem. 50: 897-903. 1967.

17. Fogel, S., R.L. Lancione, and A.E. Sewall. Enhanced biodegradation of methoxychlor in soil under sequential environmental conditions. Appl. and Environ. Microbiol. 44: 113-120. 1982.

18. McCann, A.E., and D.R. Cullimore. Influence of pesticides on soil algal flora. Res. Rev. 72: 1-31. 1979.

19. Poorman, A.E.. Effects of pesticides on *Euglena gracilis*. I. Growth studies. Bull. Environ. Contam. Toxicol. 10: 25-28. 1973.

20. Butler, G.L., T.R. Deason, and J.C. O'Kelley. Effect of atrazine, 2,4-D,methoxychlor, carbaryl and diazinon on the growth of planktonic algae. British Phycol. Jour. 10: 371-376. 1975.

21. Butler, G.L., T.R. Deason, and J.C. O'Kelley. Loss of five pesticides from cultures of 21 planktonic algae. Bull. Environ. Contam. Toxicology. 13: 149-152. 1975.

22. Page, D.L. and B.R. Colman. The effect of methoxychlor on carbon assimilation by freshwater phytoplankton. Can. J. Bot. 54: 2848-2851. 1976.

23. Kricher, J.G., J.C. Urey, and M.L. Hawes. The effects of mirex and methoxychlor on the growth and productivity of *Chorella pyrenoidosa*. Bull. Environ. Contam. Toxicol. 14: 617-620. 1975.

24. Paris, D.F., D.L. Lewis, J.T. Barnett, Jr., and G.L. Baughman. Microbial degradation and accumulation of pesticides in aquatic systems. U.S. Environmental Protection Agency. Athens, GA. EPA-660/3-75-007. 1975.

25. O'Kelley, J.C. and T.R. Deason. Degradation of pesticides by algae. U.S. Environmental Protection Agency. EPA 600/3-76-002. 1976.

26. Anonymous. The *Selenastrum capricornutum* Printz Algal Assay Bottle Test: Experimental design, application and data interpretation. U.S. Environmental Protection Agency. Corvallis OR. EPA-600/9-78-018. 1978.

27. Alexander, M.. Microbial degradation of pesticides. Office of Naval Research, Department of the Navy. N00014-76-C-0019. 1977.

28. Sweeney, R.A.. Metabolism of lindane by unicellular algae. Proc. 12th Conf. Great Lakes Res. Internat. Assoc. Great Lakes Res. 98-102. 1969.

29. Kratky, B.A. and G.F. Warren. The use of three simple, rapid bioassays on 42 herbicides. Weed Res. 11: 257-259. 1971.

30. Davison, K.L., C.J.H. Lamoureux, and V.J. Feil. Methoxy chlor metabolism in goats. 2. Metabolites in bile and movement through skin. J. Agric. Food Chem. 31: 166-168. 1983.

31. Gunther, F.A., W.E. Westlake, and P.S. Jaglan. Reported solubilities of 738 pesticide chemicals in water. Res. Rev. 20: 1-148. 1968.

32. Dao, T.H., T.L. Lavy, and J. Dragun. Rationale of the solvent selection for soil extraction of pesticide residues. Res. Rev. 87: 91-104. 1983.

33. Hubacher, M.H.. Bis(*p*-hydroxyphenyl)acetic acid. J. Org. Chem. 24: 1949-1951. 1959.

34. Brault, A. and M. Kerfanto. Nouvelle method de preparation des acides diarylacetiques. Compt. Rend. Acad. Sci. 258: 5465-5466. 1964.

35. Nathanson, G.. Bis(4-hydroxyphenyl)acetic acid derivatives. J. Org. Chem. 26: 1420-1422. 1961.

36. Schneller, G.H. and G.B.L. Smith. Unsymmetrical Analogues of DDT. J. Am. Chem. Soc. 70: 4057-4059. 1948.

37. Williams, D.H. and M.A. Fleming. Spectroscopic methods in organic chemistry. McGraw-Hill Book Co. (U.K.) Ltd., Maidenhead, England. p. 118. 1973.

38. Morrison, R.T. and R.N. Boyd. Organic Chemistry. Allyn and Bacon, Inc. Boston. p. 342. (3rd. edition). 1973.

39. See reference 37. p. 84.

40. Bothner-By, A.A. Stereochemistry of the Rearrangement Involving Migration between Multiply-bonded Carbons. J. Am. Chem. Soc. 77: 3293-3296. 1955.

41. Biemann, K. Mass Spectrometry, Organic chemical applications. McGraw-Hill Book Co. New York. p. 101. 1962.

42. Budzikiewicz, H., C. Djerassi and D. H. Williams. Mass Spectrometry of Organic Compounds. Holden-Day, Inc. San Francisco. p. 270. 1967.

43. Conley, R.T.. Infrared Spectroscopy. Allyn and Bacon, Inc. Boston. p. 128-130. 1966.

44. Jones, N., W.F. Forbes and W.H. Mueller. The infrared carbonyl stretching bonds of ring substituted acetophen ones. Can. J. Chem. 35: 504-510. 1957.

45. Fritsch, P.. Ann. 279: 319. 1894.

46. Buttenburg, W.P.. *ibid* 279: 327. 1894.

47. Wiechell, H.. *ibid* 279: 337. 1894.

48. Pritchard, J.G. and A.A. Bothner-By. Base-initiated dehydrohalogenation and rearrangement of 1-halo-2,2-diphenylethylenes in *t*-butyl alcohol. The effect of deuterated solvent. J. Physical Chem. 64: 1271-1277. 1960.

49. Kobrich, G.. Eliminations from olefins. Angew. Chem. internat. Edit. 4: 49-68. 1965.

50. Cristol, S.J.. A kinetic study of the dehydrochlorination of substituted 2,2-diphenylchloroethanes related to DDT. J. Am. Chem. Soc. 67: 1494-1498. 1945.

51. Cristol, S.J., N.L. Hause, A.J. Quant, H.W. Miller, K.R. Eilar, and J.S. Meek. Mechanisms of elimination reactions. VI. The kinetics of dehydrochlorination of various 2,2-diarylchloroethanes. J. Am. Chem. Soc. 74: 3333-3337. 1952.

52. Beroza, M., M.N. Inscoe and M.O. Bowman. Distribution of pesticides in immiscible binary solvent systems for cleanup and identification and its application in the extraction of pesticides from milk. Res. Rev. 30: 1-61. 1969.

53. Hill, J.B., H.W. Popp and A.R. Grove, Jr.. Botany. McGraw-Hill Book Co. New York. p. 369. (4th edition). 1967.

54. Meyers, J.. The culture of algae for physiological research. In: The culturing of algae. J. Brunel, G.W. Prescott, and L.H. Tiffany, editors. The Charles F. Kettering Foundation. Yellow Springs, Ohio. 1950.

55. Bailey, J.A., G.A. Carter and R.A. Skipp. The use and interpretation of bioassays for fungitoxicity of phytoalexins in agar media. Physiol. Plant Pathol. 8: 189-194. 1976.

56. Richardson, L.T. and D.M. Miller. Fungitoxicity of chlorinated hydrocarbon insecticides in relation to water solubility and vapour pressure. Can. J. Bot. 38: 161-175. 1960.

57. Palmer, C.M. and T.E. Maloney. Preliminary screening for algicides. Ohio Jour. Sci. 55: 1. 1955.

58. Ukeles, R.. Growth of pure cultures of marine phyto plankton in the presence of toxicants. Appl. Microbiol. 10: 532. 1962.

59. Moss, S.W., G.L. Blume, J.C. O'Kelley and T.R. Deason. Sorption and degradation of methoxychlor by planktonic algae. In preparation. 1976. Cited in: Algae and pesticides, G.L. Butler. Res. Rev. 66: 19-62. 1977.

60. Takimoto, Y., A. Murano, and J. Miyamoto. Analytical methods for Sumithion in technical products and formulated materials. Res. Rev. 60: 22-24. 1976.

61. White, W.C. and T.R. Sweeney. The metabolism of 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloro ethane (DDT). I. A metabolite from rabbit urine, di(*p*-chlorophenyl) acetic acid; its isolation, identification and synthesis. Public Health Reports. 60: 66-71. 1945.

62. Stephenson, O. and W.A. Waters. Chemical investigations of the insecticide DDT and its analogues. Part II. Symmetrical analogues. J. Chem. Soc.. 339-343. 1946.

63. Dimond, A.E., J.G. Horsfall, J.W. Heuberger, and E.M. Stoddard. Role of the dosage-response curve in the evaluation of fungicides. Conn. Agr. Expt. Sta. Bull. no. 451: 635-667. 1941.