Part I : Phosphorus Analogs of Arylsulfonylhydrazones

Part II : Aldophosphamide Substrate-like Inhibitors

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A Thesis

Presented to

The Faculty of the College of Pharmacy

University of Houston

---

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Daniel James Good

May, 1978

## Dedication

This thesis is dedicated to my mother; for without her devotion, intercession, and prayers, I never would have persevered in this educational achievement.

Acknowledgments

Sincere appreciation is expressed to Dr. L. A. Cates for his guidance, patience, and concern throughout the course of the research and the preparation of this thesis.

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Finally, I wish to thank Ms. Deborah Jones for her immense patience in the various stages in the preparation of this manuscript.

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## Abstract

A new class of anticancer agents, arylsulfonylhydrazones of 2-formyl pyridine N-oxide, has previously been reported. Trace metal chelation by these hydrazones had been proposed as the mode of action in their antitumor activity. Four new phosphorus analogs of these hydrazones, two of which were cupric chelates, were synthesized and tested against Ehrlich carcinoma, Sarcoma 180, and P388 lymphocytic leukemia. Additionally, the concurrent administration of these compounds with a solution of cupric chloride was investigated to determine the possibility of a potentiating effect of cupric chloride.

A chelation study of these phosphorus analogs was also undertaken.

Part I : Phosphorus Analogs of Arylsulfonylhydrazones

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Abstract

A key metabolite in the activation pathway of cyclophosphamide, a potent oncolytic agent, has been identified to be aldophosphamide [2-formyl ethyl-N,N-bis-(2-chloroethyl)phosphorodiamidate]. Through hepatic enzymatic action, aldophosphamide is rendered inactive by being oxidized to carboxyphosphamide, a non-cytotoxic metabolite.

To act as potential substrates for the enzymes responsible for the degradation of aldophosphamide, several structural modifications of the basic nucleus were attempted. One potential substrate-like inhibitor was isolated as a 2,4-dinitrophenyl hydrazone. The precursor to this potential inhibitor was also isolated.

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# Part I : Phosphorus Analogs of Arylsulfonylhydrazones

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# CHAPTER I

# INTRODUCTION

# CHAPTER I

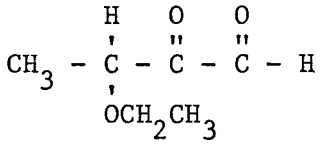
## INTRODUCTION

The search for an effective chemotherapeutic agent in the treatment of cancer has, for several decades, been the primary objective of much research into the eradication of this most dreaded of all human disease states. During phases of this research many theories have been proposed attempting to rationalize various methods of chemical attack upon neoplastic cells. One method, first proposed by Furst[1,2] as early as 1960, that has shown particular promise and which is beginning to gain acceptance as an effective oncolytic procedure, is trace metal chelation.

As a general class, thiosemicarbazones were shown by Domagk[3] in 1946 to have antitubercular activity; and by Johnson[4] in 1952 and Gausman[5] in 1953 to have anti-fungal activity. Erlenmeyer[6] in 1953 demonstrated that these activities were due to the active metal chelates of these thiosemicarbazones.

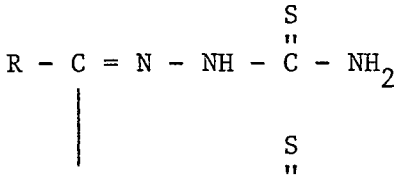
In 1956, Underwood[7] reported that several ketoaldehydes were active against certain strains of Newcastle disease and influenza viruses in chick embryos. Underwood and Weed[8] later found that glyoxal and methyl glyoxal were active against various viremias.

Furst[9] in 1957 demonstrated that 3-ethoxy-2-ketobutyraldehyde (I, Kethoxal) was active against Ehrlich Hanschka ES ascites tumor. Subsequently, French[10] in 1958, showed that various ketoaldehydes, namely kethoxal, and several dialdehydes were active against several leukemic and ascites tumors.



I

As a result of the preceeding studies, several bis-thiosemicarbazone derivatives of these ketoaldehydes were prepared to evaluate their oncolytic potential. Among these were the bis-thiosemicarbazones of glyoxal (II), methyl glyoxal (III), and kethoxal (IV, KTS).



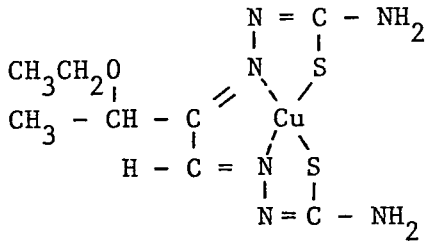
II: R = H

III: R = $CH_3$

IV: R = $CH(CH_3)-OCH_2CH_3$

French in 1958 reported that KTS and several related compounds, when administered in the diet, were active against Sarcoma 180. In the same year they postulated that these bis-thiocarbazones might act as metal chelators.[12]

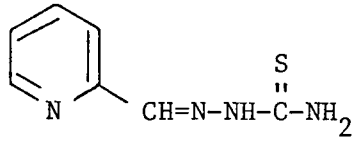
In order to investigate the most readily isolable of the metal chelates, Gingras[13] in 1962 prepared a number of bis-thiosemicarbazones to use in an antifungal study. They reported that most of the bis-thiosemicarbazones did form isolable 1:1 chelates with $Cu^{2+}$; but that with $Cu^{+}$, $Fe^{2+}$, $Fe^{3+}$, $Zn^{2+}$, $Sn^{2+}$, $Ag^{+}$ no such products were isolated. This copper:ligand complex (V), using KTS as the ligand, was postulated by Bahr[14] in 1952 to have the following structure:



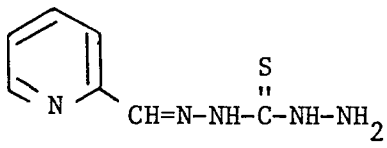
V

The bis-thiosemicarbazones of ketoaldehydes were investigated for antitumor activity, again by Petering[15] in 1962. He showed that, against certain tumors in mice, these derivatives were able to retard the tumor's growth. Subsequently, in 1964,[16,17] it was demonstrated that several bis-thiocarbazones of ketoaldehydes, of which KTS was found to be the most active, did retard the growth of a broad spectrum of rodent tumors including Sarcoma 180, Ehrlich carcinoma, Walker 256 carcinoma, Gurrin carcinoma, Jensen sarcoma, and Murphy-Sturn lymphoma. A number of KTS complexes were studied[18,19] and the most active was found to be a $Cu^{2+}$ chelate.

Related to the ketoaldehydes in their antitumor properties are the α-N-heterocyclic carboxaldehydes.[20,21] As early as 1956, Brockman[22] reported that pyridine-2-carboxaldehyde thiosemicarbazone (VI) and pyridine-2-carboxaldehyde thiosemicarbohydrazone (VII) showed activity against several types of experimental leukemia.



VI



VII

In 1965, French[20] postulated a hypothesis involving the chelating ability of these thiosemicarbazones. In it he suggested that pyridine-2-carboxaldehyde thiosemicarbazone, while acting as a tridentate ligand, preferred to form octahedral coordination complexes with divalent metals of the upper right-hand side of the transition elements in the periodic table. This hypothesis is shown graphically in Figure 1.

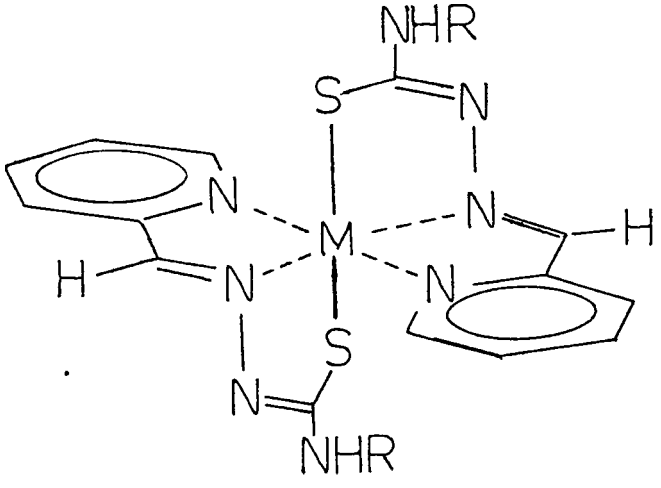
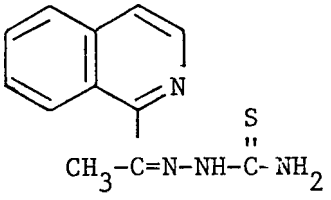


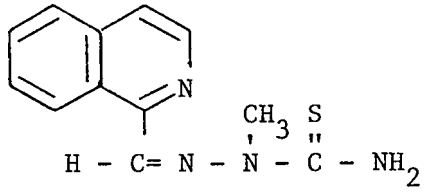
Figure 1
Hypothetical chelation sites of
formyl heterocyclic thiosemicarbazazones

The structure activity relationships in the formyl thiosemicarbazone side chain ($-CH=NNHC(S)NH_2$) were investigated by Agrawal and Sartorelli[23] in 1969 to determine both the oncolytic and toxic effects. Since the tridentate ligand system had been postulated earlier,[20] it was believed that substitution on this side chain would alter the activity relationships of these compounds in mice bearing Sarcoma 180 ascites cells. This

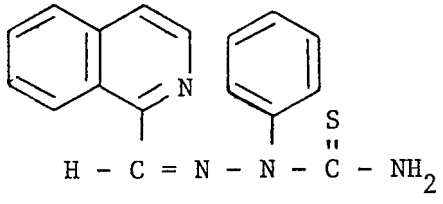
was indeed found to be the case in that substitution of the hydrogen atom with methyl (VIII) in the formyl group of the side chain resulted in a compound of reduced tumor-inhibitory activity as compared with the unsubstituted 1-formylisoquinoline thiosemicarbazone, an active carcinostatic compound. Likewise, substitution of the N-2' with either methyl (IX)



VIII



IX



X

or phenyl (X) produced inactive compounds. This would seem to be in agreement with the tridentate theory in that N-2' substitution would prevent lactim formation and subsequent prevention of chelation by the necessary resonance hydrid anion: = N - N = C ($S^-$) - (Figure 2).

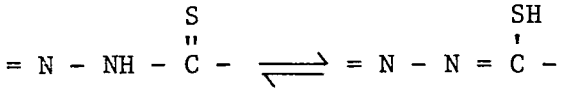
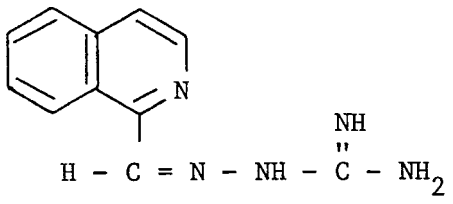


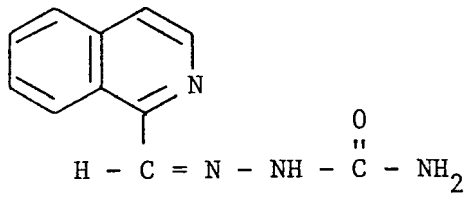
Figure 2 - Lactim Formation

In the same study it was determined that not only did substitution on the formyl group and the N-2' position produce inactive compounds, but that the terminal amide group, either intact or substituted with small substituents, appeared to be critical for activity.

Substitution of the sulfur by an imine group (XI) resulted in an active tumor inhibitor. However, substitution of the sulfur with an oxygen (XII) produced an inactive compound due to the fact that semicarbazones are less active than thiosemicarbazones as metal chelators.



XI

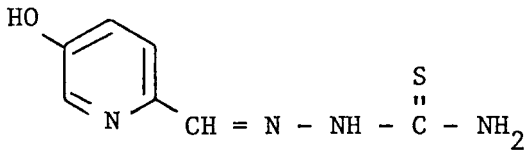


XII

In an effort to determine the mechanism of action of both the bis-thiosemicarbazones and the thiosemicarbazones, several studies were undertaken by Sartorelli.[24-28] For the bis-thiosemicarbazones it was determined that the mechanism of kethoxal bis-thiosemicarbazone (KTS) differed from that of $Cu^{2+}$ KTS.[24,25] Using the ligand KTS alone, it was reported that the mechanism of action was the retardation of DNA synthesis resulting from inhibition of the enzyme systems involved in the incorporation of formate into the thymidine residues of DNA.[25] The enzymes involved were dihydrofolate reductase, 5,10-methylene tetrahydrofolate dehydrogenase, and, the one most sensitive to KTS, thymidylate synthetase. The mechanism for $Cu^{2+}$ KTS differed in that the particular enzyme system that was inhibited in DNA synthesis was thymidine kinase, the catalyst for direct

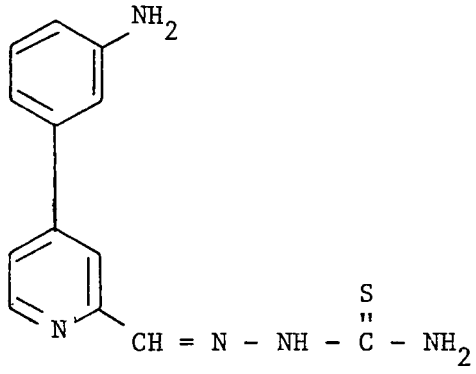
phosphorylation of thymidine and deoxyuridine.[24] It was also reported that the cytotoxicity of this complex was due to the dissociation of $Cu^{2+}$ from KTS and the subsequent accumulation of copper within the cell resulting in the inhibition of thymidine kinase. For the thiosemicarbazones, it was reported that formyl heterocyclic-thiosemicarbazone (FTS) caused the retardation of DNA synthesis by inhibiting the enzyme ribonucleotide diphosphate reductase (RDR) needed for the reduction of ribonucleotides to deoxyribonucleotides.[26-28] Moore[29-30] suggested in 1970 and 1971 that the inhibitory action of FTS was due to its coordination with a ferrous ion in RDR or that the iron chelate of FTS inhibited RDR directly. French[31] reported, also in 1970, that an intact formyl-thiosemicarbazone side chain *alpha* to the heterocyclic ring nitrogen was necessary for inhibitory action, a conclusion agreeing with the hypothetical structure needed for activity postulated in 1965 (see Figure 1).

To determine the effects of ring substitution of FTS on tumor inhibition, a series of 2-formyl pyridine thiosemicarbazones were synthesized by Blanz and Coworkers[32] in 1970. They reported that the substitution of a hydroxyl in place of a hydrogen at the 5-position (XIII) showed the greatest increase in inhibitory activity of all analogs tested. French[31] in 1970 reported that these thiosemicarbazones were of low water solubility; therefore, the increase in activity of XIII may be due to the increase in solubility afforded by the introduction of the hydroxyl group in the heterocyclic ring.



XIII

Agrawal in 1974[33] and again in 1975[34] reported upon the synthesis and oncolytic activity of 2-formyl-4-(m-aminophenyl)pyridine thiosemicarbazone (XIV). They reported that, against the murine ascitic neoplasms Sarcoma

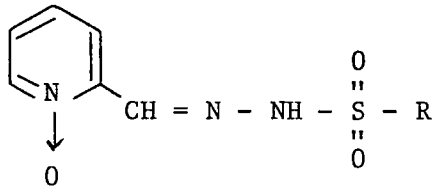


XIV

180, Ehrlich Carcinoma, and Hepatoma 129, XIV showed strong inhibitory activity and was more potent than XIII in the inhibition of RDR.[35]

In 1976, Sartorelli and coworkers[36] investigated the potential activity of a new class of oncolytic agents: arylsulfonylhydrazones of 2-formyl pyridine N-oxide (XV). They reported that these hydrazones exhibited antineoplastic activity against a broad spectrum of transplanted tumors including Sarcoma 180, Hepatoma 129, Ehrlich carcinoma, leukemia

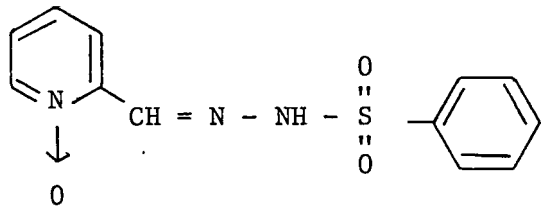
L1210, and a subline of Sarcoma 180 resistant to -N-heterocyclic carboxaldehyde thiosemicarbazones. They found that replacement of the pyridine



XV

ring with benzene, quinoline, or isoquinoline resulted in loss of activity and that movement of the formyl hydrazone side chain from the 2 to the 3 or 4-position of the pyridine N-oxide ring produced inactive agents. They also reported that the pyridine N-oxide was necessary for anticancer activity in compounds containing the formyl hydrazone side chain in the 2 position, since an analog containing only the pyridine function was essentially inactive. An interesting finding reported in this same study was the fact that contrary to the thiosemicarbazones where a bulky group in the terminal R-position decreased activity,[23] bulky groups in this position on XV increased activity. The mode of antitumor action was found to be similiar to that of $Cu^{2+}$ KTS, that is, by inhibition of thymidine and uridine incorporation in DNA and RNA, respectively.

Recently Agrawal and Sartorelli[37] studied the various structural modifications on the antineoplastic activity of the benzenesulfonylhydrazone of 2-formyl pyridine N-oxide (XVI) towards mice bearing either Sarcoma 180 or leukemia L1210. It was determined that substitution on the



XVI

pyridine ring did not cause a loss in activity of all analogs tested except one: a chlorine in the 4-position did lead to a pronounced decrease in biological activity. They also reported that the aldehydic proton was essential for tumor inhibitory activity as that substitution of this proton with either a methyl group or an oxygen atom resulted in inactive derivatives. This parallels their earlier findings[23] with the formyl heterocyclic thiosemicarbazones.

# CHAPTER II

# DISCUSSION

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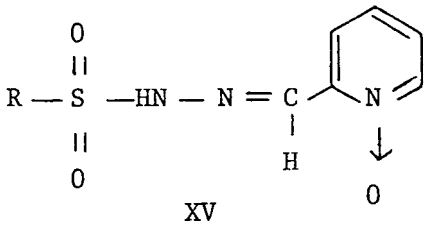
# DISCUSSION

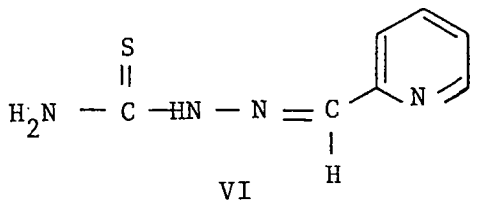
## A. Rationale

It was reported by Sartorelli and coworkers[36] that a new class of anticancer agents, aryl sulfonylhydrazones of 2-formylpyridine N-oxide (XV) exhibited activity in mice bearing Sarcoma 180 ascites cells.



XV

The precursors to this new class of oncolytic agents were the α-N-heterocyclic carboxaldehyde thiosemicarbazones of which pyridine-2-carboxaldehyde thiosemicarbazone (VI) is a representative example.



VI

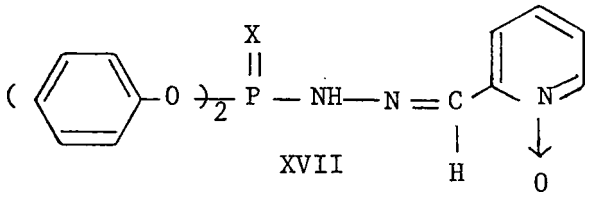
These thiosemicarbazones, although of confirmed potent antitumor activity, were difficult to administer due to their low water solubilities.[31] As a consequence, various structural modifications were attempted to circumvent this problem.

Compounds of type XV above were synthesized and were shown to have antitumor activity against a broad spectrum of transplanted tumors including a subline of Sarcoma 180 resistant to α-N-heterocyclic carboxaldehyde thiosemicarbazones.[36]

Therefore, in this study, and in earlier work by Cates,[38] the substitution of a P(O) and P(S) in lieu of a $SO_2$ or C(O), the introduction of various substituents off the phosphorus moiety, and the modification to include two hydrazone groups were undertaken to determine the oncolytic, solubility, and chelative characteristics of this potentially new class of anticancer agents.

B. Methods

It was of interest to synthesize compounds such as XVII to determine their potential oncolytic nature, their solubilities, and their chelating abilities. The proposed synthesis of the phosphorous analog of XVII where

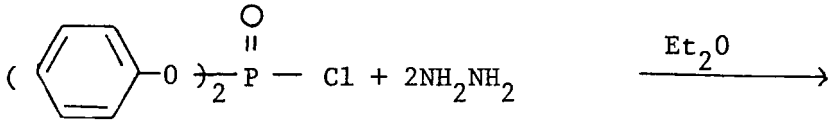


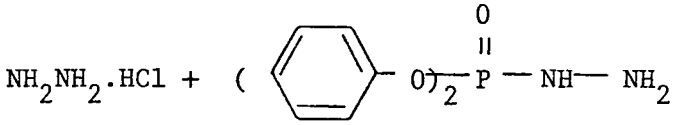
XVII

where X = O, S

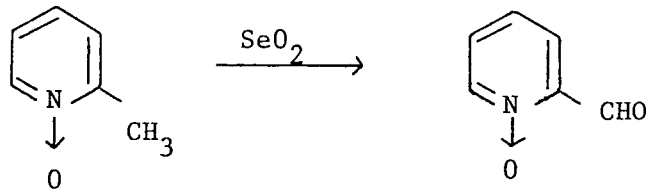
X = oxygen involves the preparation of diphenyl phosphoric hydrazide (XVIII),[49] selenium oxide oxidation of 2-picoline-N-oxide to yield (XIX),[51] and then, following a method suggested by Agrawal,[39] the condensation of XVIII with XIX to obtain the diphenyl phosphoric hydrazone of pyridine-2-carboxaldehyde N-oxide (XX) (Scheme I).

Scheme I

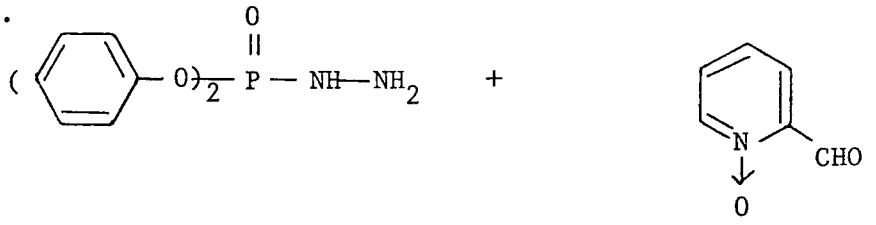


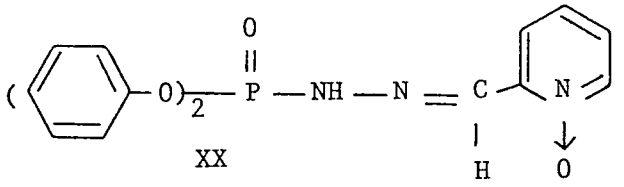


XVIII



XIX

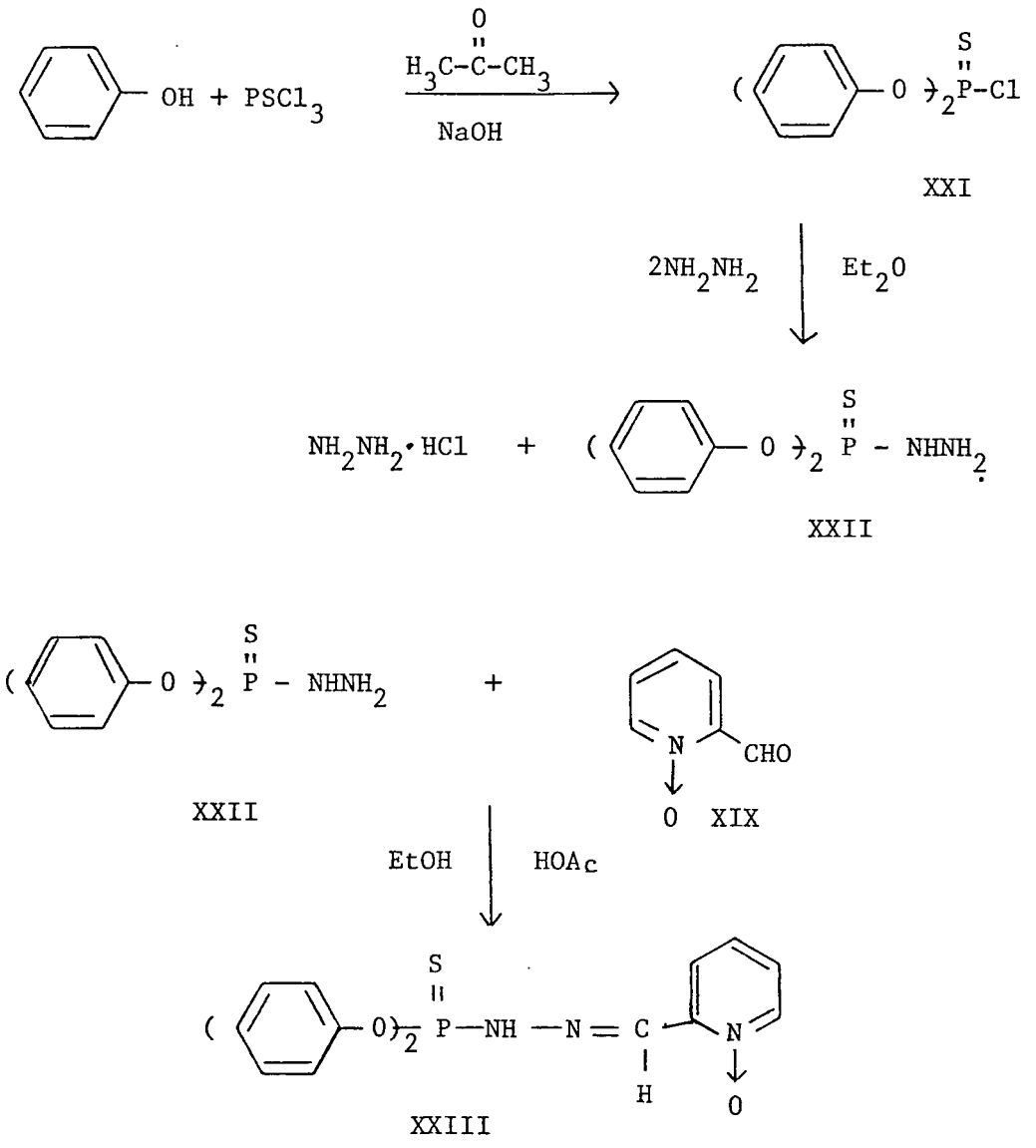




XX

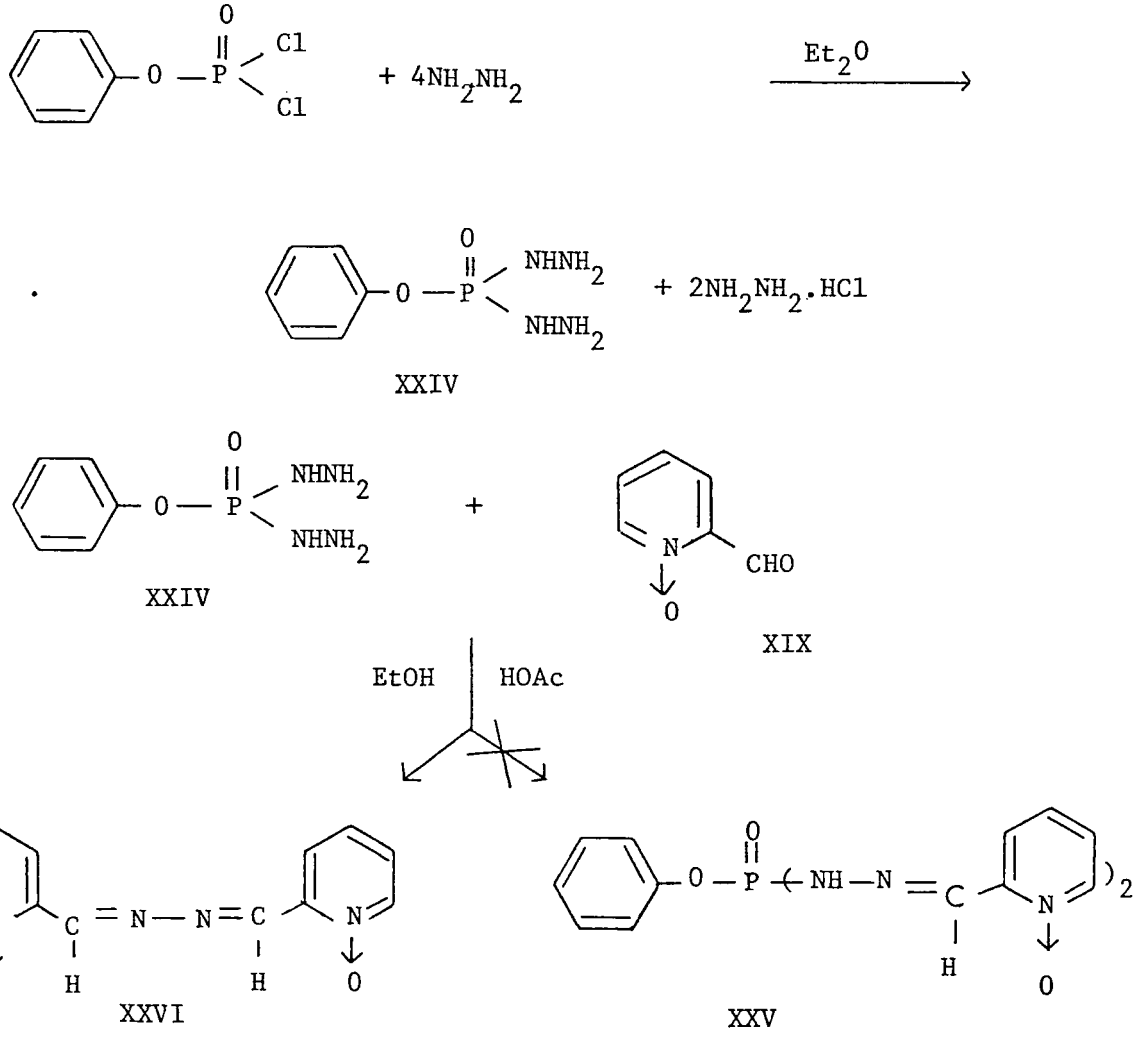
The synthesis of the thiophosphoric analog where X = sulfur involved the preparation of diphenyl chlorothiophosphate (XXI),[53] the introduction of a hydrazine side chain onto XXI to obtain diphenyl thiophosphoric hydrazide (XXII),[54] and then, following the preceeding method,[39] the condensation of XXII with XVIII to obtain the diphenyl thiophosphoric hydrazone of pyridine-2-carboxaldehyde N-oxide (XXIII) (Scheme II).

Scheme II



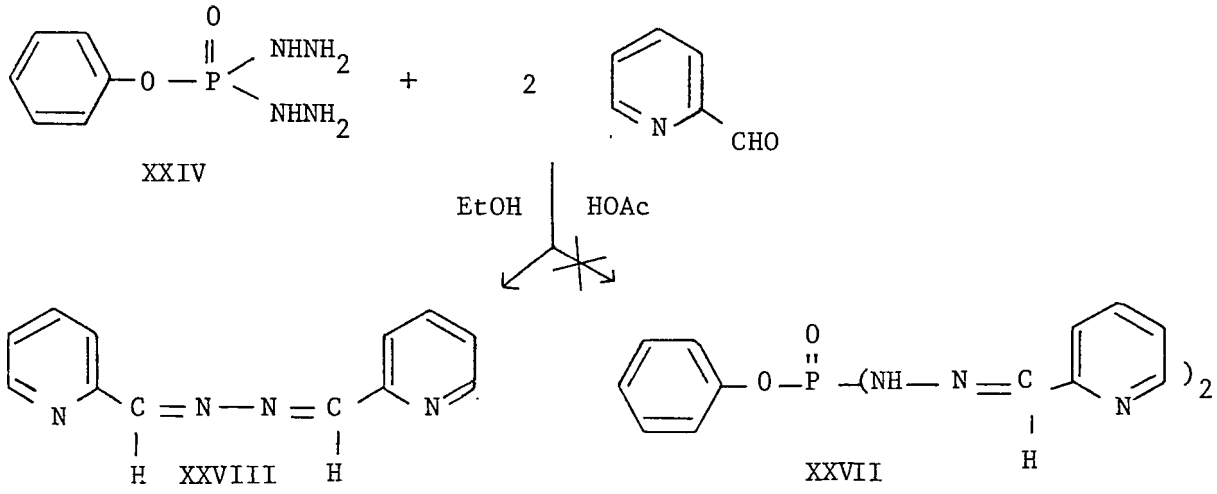
The synthesis of the phenyl phosphoric dihydrazone of pyridine-2-carboxaldehyde N-oxide was attempted to determine if this dihydrazone had greater antitumor activity by vitrue of a greater ability to chelate trace metals (cf. work by Petering[18,19]). This synthesis involved the preparation of phenyl phosphoric dihydrazide (XXIV),[49] and then following a method reported by Audrieth,[40] the condensation of XXIV with XIX to obtain the phenyl phosphoric dihydrazone of pyridine-2-carboxaldehyde N-oxide (XXV) (Scheme III). Unfortunately, the only product isolated was 2-formyl pyridine N-oxide bis-hydrazone (XXVI).

Scheme III



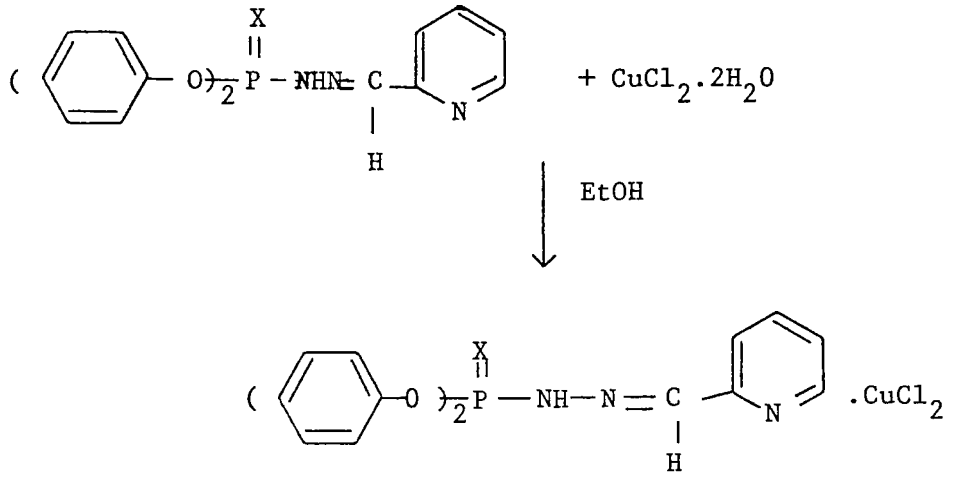
Since the above synthesis failed to produce XXV, the pyridine N-oxide was thought to be the cause; therefore, pyridine-2-carboxaldehyde[41] was used to determine if the N-oxide function was indeed preventing the desired product from forming. This synthesis involved the condensation of XXIV with pyridine-2-carboxaldehyde to obtain the phenyl phosphoric dihydrazone of pyridine-2-carboxaldehyde (XXVII) (Scheme IV). Again, unfortunately, the only product isolated was 2-formyl pyridine bis-hydrazone (XXVIII).

Scheme IV




In order to investigate the potential oncolytic activity of a preformed chelate, two copper chelates were synthesized following the method described by Gingras.[42] The synthesis of the cupric chloride chelates of the diphenyl phosphoric and thiophosphoric hydrazones of pyridine-2-carboxaldehyde involved the addition of a cupric chloride solution to the appropriate hydrazone[43,44] to yield XXIX and XXX, respectively. (Equation 1).

Equation 1




X = O XXIX

X = S XXX

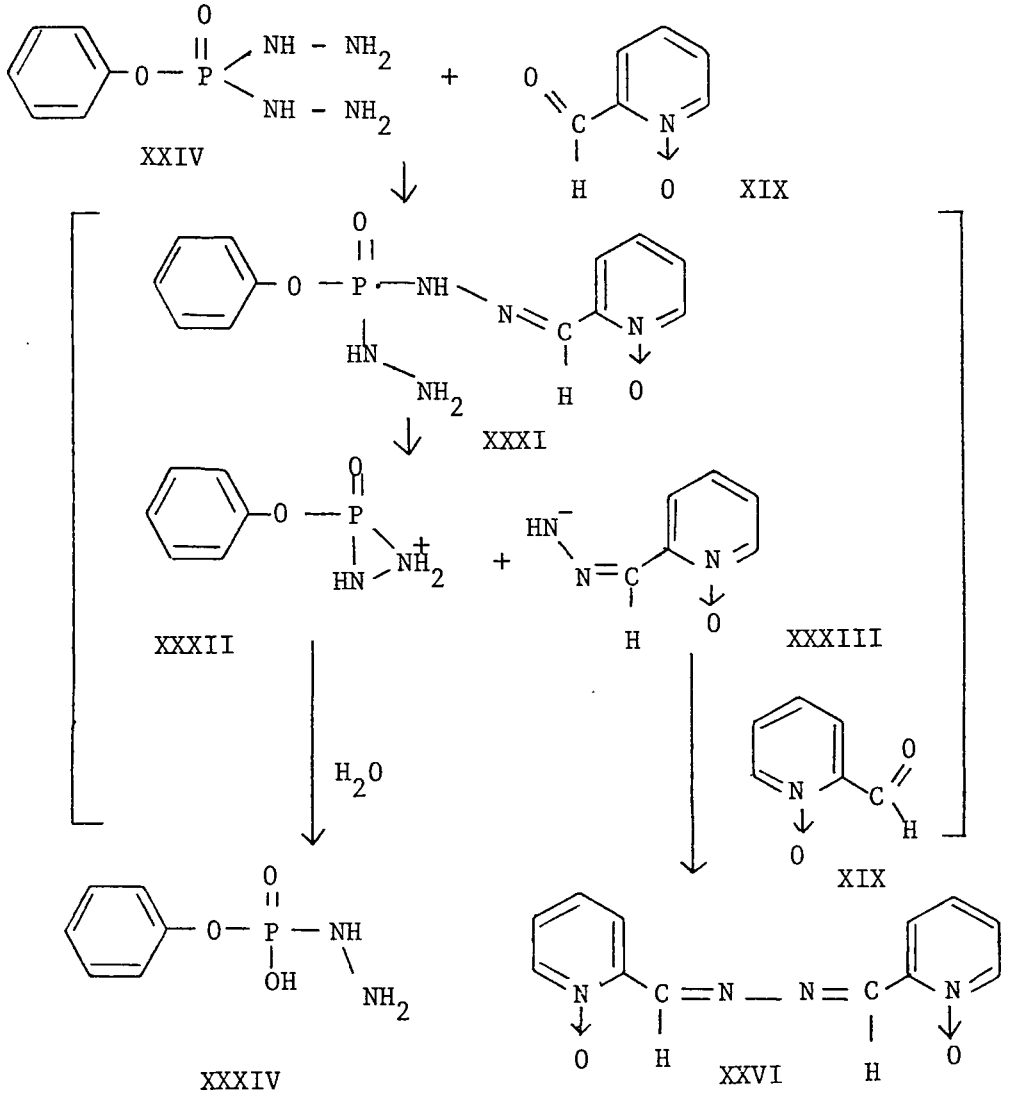
The chelation of trace metals had been found to be important in the anticancer activity of 2-formyl heterocyclic thiosemicarbazones[23] and in the phosphorus analogs of these compounds.[38] Since the former preferred to form octahederal coordination complexes with divalent metals of the right-hand side of the first row transition elements,[20] a 1 X $10^{-4}$ M alcoholic solution of XX and XXIII, along with their respective non-N-oxide forms,[43,44] were each individually added to a 1 X $10^{-4}$ M alcoholic solution of the following metals in a 1:1 ratio: $CuCl_2 \cdot 2H_2O$, $FeCl_2 \cdot 4H_2O$, $CoCl_2 \cdot 6H_2O$, and $H_2SeO_3$. Due to a hyperchromic effect observed *via* UV absorption for the cupric ion-ligand complex alone, this metal was used to determine the chelation ratios of each ligand. Initially both the metal and ligand concentrations were 1.67 X $10^{-5}$M. Then in increments of 1.67 X $10^{-5}$M, the ligand concentration was increased causing a hyperchromic effect in the region of 300 - 400 nm until further addition of the ligand caused no increase in the absorption intensity.

The concentration at which this occured, 5.01 X $10^{-5}$ M for the non-N-oxide form of XX, 6.68 X $10^{-5}$ M for both XXIII and its non-N-oxide form, was used to calculate the chelation ratio.

C. Results

The failure to isolate compounds XXV and XXVI is unfortunate for these compounds would have provided valuable information concerning both the oncolytic and chelative properties of this type of dihydrazone. It was of interest to examine the reasons for the formation of the unexpected products from the reactions depicted in Schemes III and IV. A plausible mechanism to account for the observed products is shown in Scheme V,

Scheme V



whereby, following the formation of the mono-hydrazone, intramolecular nucleophilic attack of the free hydrazino-amino results in the expulsion of XXXII. At this point, XXXIII can be easily envisioned, following a proton transfer, to react with the second mole of XIX to give the observed 2-formyl pyridine N-oxide bis-hydrazone (XXVI).

While the elemental analysis of XXIX was found to be within $\pm$ 0.4% for carbon, hydrogen and nitrogen, the copper and chlorine concentrations were within $\pm$ 1.6%. Similarly, the concentrations of carbon, hydrogen and copper were within $\pm$ 0.4% for XXX, but the concentrations of nitrogen and chlorine fell within $\pm$ 1.7%. Gingras[45] in 1960 and Van Giessen[46] in 1968 reported that with copper chelates of low solubility the elemental analyses were often unsatisfactory. Van Giessen suggested that this might be due to the presence of solvent ligands which were included in coordination complex making the analyses difficult to interpret. Nevertheless, the analysis results for the other elements present: carbon, hydrogen and nitrogen, preclude, more so in XXIX than in XXX, the chelation ratio of anything else but 1:1, with XXX having water incorporated into the copper complex.

In keeping with the hypothetical structure necessary for chelation, as proposed by French[20] in 1965 (see Figure 1), and since this chelation was found to be important to antitumor activity of 2-formyl heterocyclic thiosemicarbazones[23] and their phosphorus analogs,[38] the ability of XX and XXIII, along with their non-N-oxide forms,[43,44] to chelate cupric ion was investigated.

As was expected XX did not chelate cupric ion (Figure 4), whereas its non-N-oxide form did (Figure 5). This can be explained by the fact that, as in the suggested model (Figure 1), the nitrogen atom of the aromatic heterocyclic ring needs to have its unshared pair of electrons unencumbered so as to participate in chelation. In XX this pair of electrons form a coordinate covalent bond with oxygen, so its ability to chelate at this point is inhibited.

On the other hand, both XXIII and its non-N-oxide form did chelate cupric ion (Figures 6 and 7, respectively). This seems to suggest that the mechanism of complexation of the P(S) analog is completely different from that of the P(O).

The chelation ratios of each complex suggests the same conclusion. In a study of 2-formyl heterocyclic thiosemicarbazones,[47] it was determined that the ligand-copper chelate ratio was 1:1. Further, in a study of benzaldehyde 4',4'-dimethyl thiosemicarbazone with cupric chloride,[48] it was found that two complexes were formed - one that was soluble in acetone and another that was insoluble in acetone, acid, base, ether, or alcohol. The former complex was assigned a ligand-metal chelation ratio of 2:1, while the latter a ratio of 1:1. From figures 5 through 7 (shown in spectral data section), it can be shown that the ligand-metal chelation ratios of the non-N-oxide form of XXIX, XXX, and the non-N-oxide form of XXX are 3:1, 4:1, and 4:1, respectively. Since the isolated complexes of these compounds had a ratio of 1:1, these results concur with the above in that a different chelation ratio applies for a complex in solution as compared to the same complex in solid form. This is in contrast, however, to the earlier work in this study,[38] for there it was

shown that, while the isolated complexes did form 1:1 ligand metal ratios, the complexes in solution formed 2:1 ratios. A possible answer to this dilemma was proposed in this same study. It is possible that the chelate may exist in two isomers - the E and Z conformations (Figure 3 ).

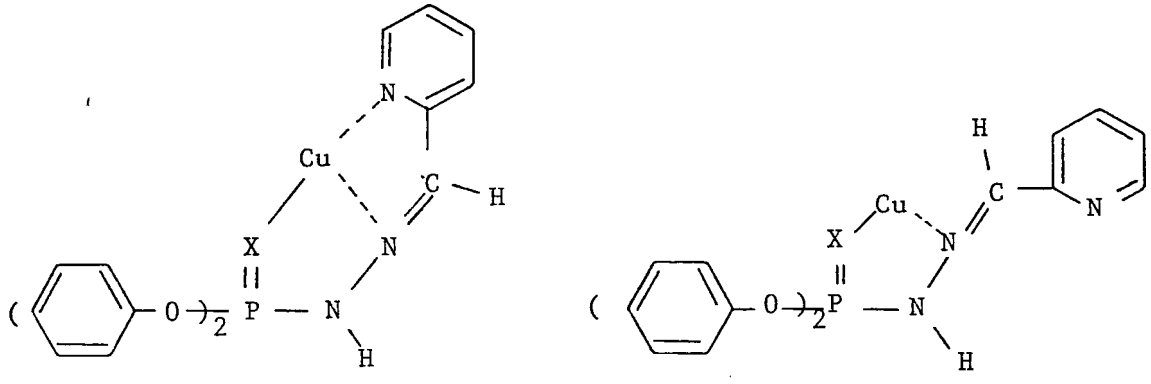



Figure 3
Conformational isomers of 2-formyl heterocyclic phosphoric hydrazones

The copper chelate of XXIX in solution as well as the soluble chelates of the earlier study may exist in the E-conformation where all three binding sites may be used. On the other hand, since the coordinate covalent bond with oxygen to the heterocyclic N atom did not hinder chelation of XXX, it is possible that the copper complex of both XXVIII and its non-N-oxide form may exist preferentially in the Z-conformation. Although the above would explain the different chelation ratios of the chelates in this study, it does not explain satisfactorily

the difference in chelation ratios between the complex of the non-N-oxide form of XXX and the complexes investigated in the earlier study. Therefore, it would seem appropriate to state that further work is warranted to determine just what is the correct mechanism of chelation of these 2-formyl heterocyclic phosphoric and thiophosphoric hydrazones in their role as potential oncolytic agents.

The biological activities of XX, XXIII, their non-N-oxide forms: XX-N and XXIII-N, respectively, XXIX and XXX were determined by measuring the increase in survival times after administration of these compounds to mice bearing Ehrlich carcinoma, Sarcoma 180, and P388 lymphocytic leukemia (see Appendix). All compounds, except the two copper chelates - XXIX and XXX, were administered first by themselves and then concurrently with a copper solution to ascertain whether a potentiating effect resulted with added copper.

The effects of phosphoric and thiophosphoric hydrazones, concurrently administered copper-ligand complexes, and two cupric chelates against P388 lymphocytic leukemia are shown in Table I (all tables appear in the Appendix). The administration of XX, XX-N, XXIII and XXIII-N at 100 mg/kg doses did not seem to significantly increase the survival times against P388. Likewise, the concurrent administration of copper with each ligand showed no increase in survival time, but actually a decrease. This might be due to systemic toxicity of $CuCl_2$ in mice for even the administration of $CuCl_2$ alone caused a decrease in survival time. The administration of XXIX and XXX at a dose of 6.1 mg/kg also showed a decrease in survival time. Interestingly, the administration of XXX at a dose of 12.1 mg/kg resulted in a substantial increase in survival time.

The survival study of XXIII, XXX, and concurrently administered copper-ligand complex of XXIII against Sarcoma 180 is shown in Table II. A decrease in survival time occured for all ligands. A similar decrease occured for the $CuCl_2$ alone. However, the concurrent administration of copper with XXIII. in contrast to the P388 study above, did increase survival time, although of little consequence.

The survival times of XXIII, XXIX, and the concurrent administration of a copper-ligand complex of XXIII against Ehrlich carcinoma are shown in Table III. A significant increase in survival is noted for both ligands with a substantial increase in the concurrent administration of copper with XXIII.

Therefore, it appears from this rather limited study that bulky groups off the phosphorus atom are favorable to antitumor activity as opposed to less bulky groups such as ethoxy.[38,55] Unfortunately only the P(S) analog was tested against Sarcoma 180 and Ehrlich Carcinoma so a true comparison from the data presented here is not possible at this time. However, the P(S) analog has shown itself in more recent work[55] to be the more active analog, a fact reflected in the Ehrlich carcinoma study.

A conclusion can be drawn, none the less, that a concurrent administration of cupric chloride does indeed enhance the activity of these hydrazones, at least in the Ehrlich carcinoma tumor system. This fact is in agreement with the earlier studies that trace metal chelation does play a role in the oncolytic activities of these 2-formyl heterocyclic phosphoric and thiophosphoric hydrazones.

# CHAPTER III

# EXPERIMENTAL

# CHAPTER III

# EXPERIMENTAL

## A. Chemicals and Reagents

All chemicals and reagents were obtained either from the various listed chemical suppliers or were prepared using the procedures given.

1. Aldrich Chemical Co., Inc., Milwaukee, Wisconsin
   (a) Diphenyl chlorophosphate
   (b) Phenyl dichlorophosphate

2. J. T. Baker Chemical Co., Phillipsburg, New Jersey
   (a) Ferrous chloride
   (b) Phenol

3. Eastman Kodak Co., Rochester, New York
   (a) Hydrazine
   (b) Thiophosphoryl chloride

4. Fisher Scientific Co., Fair Lawn, New Jersey
   (a) Cobaltous chloride
   (b) Selenous Acid
   (c) Sodium metal

5. Mallinckrodt Chemical Works, St. Lewis, New York
   (a) Ammonium molybdate

6. Matheson, Coleman and Bell Manufacturing Chemists, Norwood, Ohio
   (a) Cupric chloride

7. Ammonium molybdate test reagent. The reagent consists of a two per cent ammonium molybdate solution in water added to a five per cent nitric acid solution in water.[56]

B. Instrumentation

The melting points were taken on a Thomas-Hoover Unimelt capillary melting point apparatus, Arthur H. Thomas Co., Philadelphia, Pa., and are uncorrected.

U. V. Absorbance spectra were recorded on a Beckman grating spectrophotometer, Model DB-GT, Beckman Instruments, Inc., Fullerton, California.

Proton nuclear magnetic resonance spectra were recorded on a Varian Model EM-360 nuclear magnetic resonance spectrometer, Varian Associates, Inc., Walnut Creek, California, using tetramethylsilane as an internal standard.

Fourier transform proton nuclear magnetic resonance spectra were obtained on a Varian Associates Model XL-100 Fourier transform spectrometer operating at 100.06 MHz and equipped with a Nicolet Model TT-100 data system and a NT-440 frequency synthesizer. All $^{1}$H-NMR shifts are reported in parts per million ($\delta$) downfield from an internal tetramethylsilane reference. Typical instrument parameters were: pulse width, 6 μsec.; pulse delay, 2 sec.; data table size, 8K, acquisition time, 2.728 sec.; sweep width, 1502 Hz; and exponential line broadening, 0.20 Hz.

CI mass spectra were obtained on a Varian mass spectrometer, model MAT 112S, Varian Associates, Inc., Walnut Creek, California. Samples were introduced by direct probe using methane as the ionization gas.

Infrared spectra were recorded on a Perkin-Elmer infrared spectrophotometer, Model 283, Perkin-Elmer Co., Norwalk, Connecticut. All samples were prepared as KBr pellets at a concentration of 1 mg/300 mg KBr.

Elemental analyses were preformed by Atlantic Microlab, Inc., Atlanta, Georgia, and by Galbraith Laboratories, Inc., Knoxville, Tennessee.

C. Syntheses

1. Synthesis of the diphenyl phosphoric hydrazone of pyridine-2-carboxaldehyde N-oxide (XX)

a. Synthesis of diphenoxyphosphoric hydrazide (XXVIII)

This procedure follows the method described by Klement and Knollmuller.[49] To a 500 ml three neck reaction flask, equipped with a thermometer and a reflux condenser, containing hydrazine (12.8 g, 0.40 mol) in 250 ml anhydrous diethyl ether, was added, dropwise with stirring, diphenyl chlorophosphate (26.8 g, 0.10 mol) in 50 ml of anhydrous diethyl ether. After complete addition the solution was refluxed for one hour. The reaction mixture was filtered and the precipitate washed with water several times to remove any residual hydrazine hydrochloride. The precipitate was air dried to give 21.93 g (83.0%) of the product melting at 112-114° (lit. 112-116°).[49] The reaction product was characterized by NMR[50] ($CDCl_3$): δ 3.4 (broad s,2),δ5.1 (broad d, J = 34Hz,1), δ7.2 (s,10).

b. Synthesis of 2-pyridine carboxaldehyde-N-oxide (XIX)

This procedure follows the method of Jerchel and co-workers.[51] Selenous Acid (50 g, 0.39 mol) was reduced to selenium oxide by heating the former in a covered crucible and collecting the sublimate. In a 250 ml three neck reaction flask, equipped with a reflux condenser, selenium oxide (10.2 g, 0.092 mol) and distilled 2-picoline-N-oxide (10.0 g, 0.092 mol)

was added to 60.0 ml of distilled pyridine. The resulting solution was heated at reflux for four hours. The solution was allowed to cool, then, was spin evaporated to yield a yellowish, waxy residue. This residue was resuspended in benzene to remove the last traces of pyridine as an azeotrope. The benzene solution was evaporated and the residue allowed to remain under 4.0 mm Hg vacuum for two hours to yield 3.7 g (32%) of the yellowish, waxy product.

NMR (DMSO-$d_6$): δ6.85 (d, J = 6.0Hz,1), δ7.45 (m, 2), 8.20 (m, 1), 10.3 (s, 1).

c. Synthesis of the diphenyl phosphoric hydrazone of pyridine-2-carboxaldehyde N-oxide (XX)

To a 250 ml round bottom reaction flask, equipped with a reflux condenser and thermometer, containing diphenoxy phosphoric hydrazide (7.92 g, 0.03 mol) in approximately 50 ml of absolute ethanol, was added one ml of glacial acetic acid. Then, 2-pyridine carboxaldehyde-N-oxide (3.7 g, 0.03 mol) was added with stirring. The solution was heated at 75° for one and one-half hours. It was allowed to cool and then stand overnight. The precipitate was collected by filtration. Distilled water was slowly added to the filtrate until faintly cloudy. The resulting precipitate was recrystallized from benzene/petroleum ether (bp 40-60°) to yield 6.12 g (55.2%) of the product melting at 134-135°.

NMR ($CDCl_3$) (Figure 8): δ 7.15 (s,10). δ 7.2 (m, 2), δ 7.8 (m, 1), δ 8.15 (m, 1) δ 8.75 (s, 1), δ 10.15 (d, J = 33.0 Hz, 1, exchangable).

Elemental analysis of $C_{18}H_{16}N_3O_4P$, M.W.: 369.305

Calculated: C 58.53, H 5.47, N 11.38

Found: C 58.66, H 5.47, N 11.38

IR[52] (KBr) (Figure 14): 3440 (w), 3140 (m), 1590 (m), 1495 (s), 1460(s), 1220-1190 (three bands, s). 950 $cm^{-1}$ (s).

2. Synthesis of the diphenyl thiophosphoric hydrazone of pyridine-2-carboxaldehyde N-oxide (XXIII)

a. Synthesis of diphenyl chlorothiophosphate (XXI)

This procedure follows the method of Authenrieth and Hildlebrand.[53] To a solution of phenol (20.6 g, 0.22 mol) in 140 ml of 10% NaOH and 60 ml of acetone in a 250 ml reaction flask, kept at 0° *via* an ice bath, was added dropwise a solution of thiophosphoryl chloride (18.5 g, 0.11 mol) in 10 ml acetone. The reaction was stirred for five hours and then allowed to stand overnight. A white crystalline material had formed along with a darker material. The darker material was carefully removed and washed with 95% ethanol to yield 5.0 g (15.6%) of the product melting at 63-66° (lit. 67-68°).[53]

NMR ($CDCl_3$): 7.30 (s).

IR (KBr): 1590 (m), 1480 (s), 1160 (s), and 950 $cm^{-1}$ (s).

b. Synthesis of Diphenyl thiophosphoric hydrazide (XXII)

This procedure follows the method described by Tolksmith.[54] To a solution of hydrazine (4.62 g, 0.144 mol) in 65 ml anhydrous diethyl ether in a 250 ml reaction flask at 20° was added diphenyl chlorothiophosphate (9.9 g, 0.034 mol) in 100 ml anhydrous ether. The temperature rose to 25° during the addition. After complete addition, the mixture was refluxed for three hours. After cooling, the ether layer was decanted, washed twice with water, then spin evaporated. To the resulting oil was added 100 ml of petroleum ether (bp 40-60°). Upon adding a few milliliters of chloroform a white precipitate immediately formed. This was filtered and air dried to yield 9.05 g (92.9%) of the product melting at 64-66° (lit. 64°).[54]

NMR ($CDCl_3$): δ 7.3 (s, 10), δ4.6 (m, 1) δ3.5 (s, 2)

IR (KBr): 3240 (broad, s), 1590 (s), 1490 (s), 1400 (m), 1210 (s), 1190 (s), 1160 (s), and 920 $cm^{-1}$ (s).

c. Synthesis of the diphenyl thiophosphoric hydrazone of pyridine-2 carboxaldehyde N-oxide (XXIII)

To a solution of 2-pyridine carboxaldehyde-N-oxide (0.89g, 7.2 mol) in 10 ml of absolute ethanol and 1.0 ml acetic acid in a 100 ml beaker was added XXII (2.0 g, 7.2 mmol) in 10 ml absolute ethanol. The reaction mixture was heated in a water bath at 60-80°. Within 15 minutes, while stirring, a white precipitate began to form along the sides and on the bottom of the beaker. The reaction mixture was removed from the water bath and allowed to cool slowly. The white precipitate was filtered and air dried to yield 2.13 g (76.8%) of the product melting at 158-160°.

NMR ($CDCl_3$) (Figure 9): δ 7.1 (m, 12), δ 7.9 (m, 1), δ 8.15 (m, 1), δ9.05 (s, 1), δ 10.6 (d, J = 39.0 Hz, 1, exchangable).

IR (KBr) (Figure 15): 3450 (broad, m), 1190-1230 (triplet, s), 940 (s), 1590 (s), 3100 (m), and 1490 $cm^{-1}$ (s)

Elemental analysis: $C_{18}H_{16}N_3O_3SP$, M.W.: 385.371

Calculated: C 56.10, H 4.18, N 10.90

Found: C 55.98, H 4.22, N 10.87

3. Attempted Synthesis of the phenyl phosphoric dihydrazone of pyridine-2-carboxaldehyde N-oxide (XXV)

a. Attempted Synthesis of Phenyl phosphoric dihydrazide (XXIV)

To a solution of hydrazine (40.4g, 1.26 mol) in 250 ml anhydrous diethyl ether in a 500 ml three neck reaction flask, equipped with a reflux condenser and a thermometer, was added, dropwise with stirring, a solution of phenyl dichlorophosphate (50.5 g, 0.26 mol) in 50 ml of

anhydrous diethyl ether. After complete addition, the mixture was heated at reflux for five hours. An oily precipitate was collected and put into a Soxhlet extraction apparatus. After ten hours of refluxing with ether, the only compounds isolated were the starting materials.

b. Synthesis of Phenyl phosphoric dihydrazide (XXIV)

This procedure follows the method described by Klement and Knollmuller.[49] To a solution of hydrazine (76.92 g, 2.4 mol) in 350 ml of anhydrous diethyl ether in a 1000 ml three neck reaction flask, equipped with a reflux condenser and a thermometer, was added, dropwise with stirring, a solution of phenyl dichlorophosphate (77.99 g, 0.4 mol) in 100 ml anhydrous diethyl ether. After complete addition, the mixture was refluxed for one hour. The ether layer was decanted from the reaction mixture; the white solid residue was then extracted with 500 ml of warm absolute ethanol. After several recrystallizations from absolute ethanol, 1.26 g (1.56%)[57] of the reaction product was isolated which melted at 101-103° (lit. 103°).[49]

NMR (DMSO-$d_6$): $\delta$ 7.3 (s, 5), $\delta$ 5.7 (d, J = 27Hz, 2), $\delta$ 3.5 (broad s, 4).

IR (KBr): 3300 (broad, s), 1590 (s), 1480 (s), 1260 (s), and 750 $cm^{-1}$ (s).

c. Attempted Synthesis of the phenyl phosphoric dihydrazone of pyridine-2-carboxaldehyde N-oxide (XXV)

To a 100 ml beaker containing a solution of 2-pyridine carboxaldehyde N-oxide (0.74g, 6.0 mmol) in 20 ml absolute ethanol and 1.0 ml acetic acid was added, with stirring, a solution of XXIV (0.5g, 2.5 mmol) in 20 ml absolute ethanol. The mixture was heated in a water bath at 60 - 70° for 45 minutes. A yellowish, fluffy precipitate was collected by filtration and upon air drying yielded 0.268 g of a yellow crystalline material which decomposed at 250 - 280°. Recrystallization from ethanol

yielded 0.237 g of a compound decomposing at 267-268°.

100 MHz Fourier transform NMR ($CDCl_3$) (Figures 10 and 11): δ 9.2 (s, 2), δ 8.3 - 8.1 (m, 4),δ 7.36 - 7.25 (m, 4).

CI mass spectrum: Molecular ion peak occured at m/e = 242

IR (KBr): 3390 (broad,m), 3050 (m), 1610 (s), 1430 (s), 1240 (s), and 770 $cm^{-1}$ (s).

Elemental analysis: $C_{12}H_{10}N_4O_2$, M.W.: 242.23

Calculated: C 59.50, H 4.13, N 23.14

Found: C 59.34, H 4.19, N 23.03

Sodium Fusion Test using Ammonium Molybdate Test Reagent: Several determinations did show the presence of nitrogen.

From the above data, it was determined that the only product isolated was 2-formyl pyridine N-oxide bis-hydrazone (XXVI).

4. Attempted Synthesis of the phenyl phosphoric dihydrazone of pyridine-2-carboxaldehyde (XXVII)

To a 100 ml beaker containing a solution of 2-pyridine carboxaldehyde[41] (0.64 g, 6.0 mmol) in 20 ml absolute ethanol and 1.0 ml acetic acid was added, with stirring, a solution of XXIV (0.5 g, 2.5 mmol) in 20 ml absolute ethanol. The mixture was heated for one hour on a water bath at 60-70°. A yellowish precipitate was collected by filtration and upon air drying yielded 0.23 g of a yellowish crystalline material melting at 145 - 147° (lit. 148-149° for XXVIII).[58]

100 MHz Fourier transform NMR ($CDCl_3$) (Figures 12 and 13): δ 8.76 - 8.68 (m,2), δ 8.18 - 8.09 (m, 2), δ 7.88 - 7.12 (m, 2), δ 7.43 - 7.29 (m, 2).

CI mass spectrum: molecular ion peak occured at m/e = 210

IR (KBr): 3460 (broad, m), 3050 (m), 3000 (m), 1660 (s), 1570 (s),

1465 (s), 1430 (s), 980 (s), and 775 $cm^{-1}$ (s).

Elemental analysis: $C_{12}H_{10}N_4$, M.W.: 210.23

Calculated: C 68.55, H 4.80, N 26.65

Found: C 68.57, H 5.13, N 26.41

From the above data, it was determined that the only product isolated was 2-formyl pyridine bis-hydrazone (XXVIII).

5. Synthesis of the cupric chloride chelate of the diphenyl phosphoric hydrazone of pyridine-2-carboxaldehyde (XXIX)

To a 100 ml beaker containing the diphenyl phosphoric hydrazone of pyridine-2-carboxaldehyde (1.5 g, 4.2 mmol)[43] in 20 ml absolute ethanol was added, slowly, a saturated solution of cupric chloride dihydrate in absolute ethanol until no additional precipitate formed. The reaction mixture was stirred for 15 minutes; the precipitate was then filtered, washed several times with triple-distilled water, and dried five hours *in vacuo* to yield 1.96 g (94.6%) of the desired product melting at 205-207°.

Elemental Analysis: $C_{18}H_{16}N_3O_3P.CuCl_2$, M.W.: 487.751

Calculated: C 44.32, H 3.31, N 8.61, Cu 13.03, Cl 14.54

Found: C 44.18, H 3.45, N 8.48, Cu 14.23, Cl 12.85

6. Synthesis of the cupric chloride chelate of the diphenyl thiophosphoric hydrazone of pyridine-2-carboxaldehyde (XXX)

To a 100 ml beaker containing the diphenyl thiophosphoric hydrazone of pyridine-2-carboxaldehyde (1.0 g, 2.7 mmol)[44] in 20 ml absolute ethanol was added, slowly, a saturated solution of cupric chloride dihydrate in absolute ethanol until no additional precipitate formed. After stirring the reaction mixture for 15 minutes, the precipitate was filtered, washed several times with triple-distilled water, then dried five hours *in vacuo* to yield 0.70 g (47.9%) of the desired product decomposing at 175-177°.

Elemental analysis: $C_{18}H_{16}N_3O_2PS.CuCl_2.2\ 1/2\ H_2O$, M.W.: 548.861

Calculated: C 39.39, H 3.85, N, 7.65, Cu 11.58, Cl 12.92

Found: C 39.06, H 3.26, N 9.40, Cu 11.53, Cl 13.70

D. Method of Chelation Studies

A 1.0 X $10^{-4}$ M alcoholic solution of each ligand was individually combined with a 1 X $10^{-4}$ M alcoholic solution of cupric chloride. The volumes used were calculated to produce ligand:metal ratios of from 1:2 to 5:1. To keep the total volume constant at 30 ml for each determination, 95% ethanol was added in decreasing amounts to each ligand: metal ratio determination. Representative examples for ligand:metal ratios of 3:1 and 4:1 are illustrated below:

| | Ligand | Metal | 95% Ethanol | L:M Ratio |
|---|---|---|---|---|
| Volumes used | 15 ml | 5 ml | 10 ml | 3:1 |
| | 20 ml | 5 ml | 5 ml | 4:1 |

# CHAPTER IV

## Spectral Data

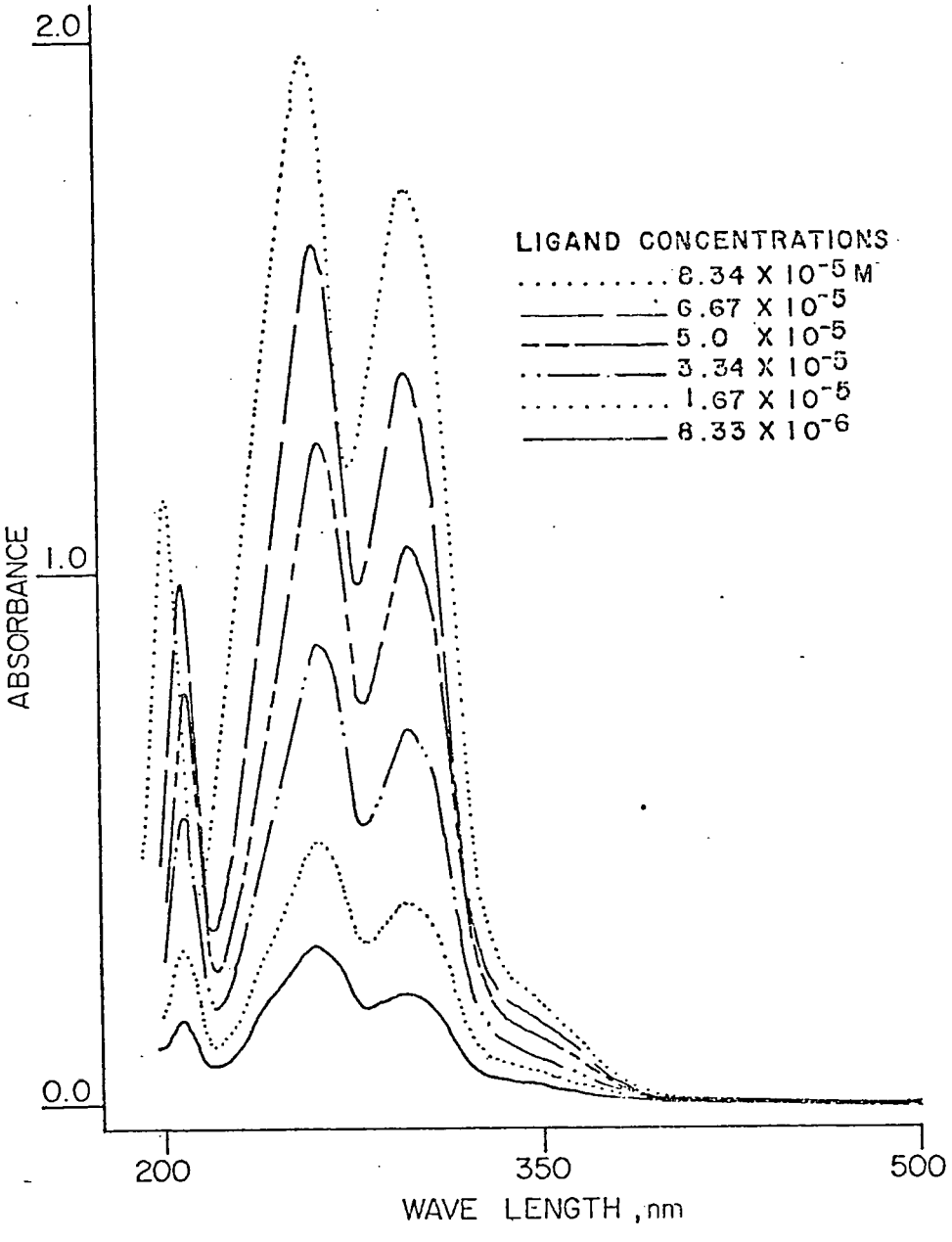



Figure 4. UV spectrum of the diphenyl phosphoric hydrazone of pyridine-2-carboxaldehyde N-oxide (XX) in the presence of 1 X $10^{-4}$ M alcoholic solution of cupric chloride.

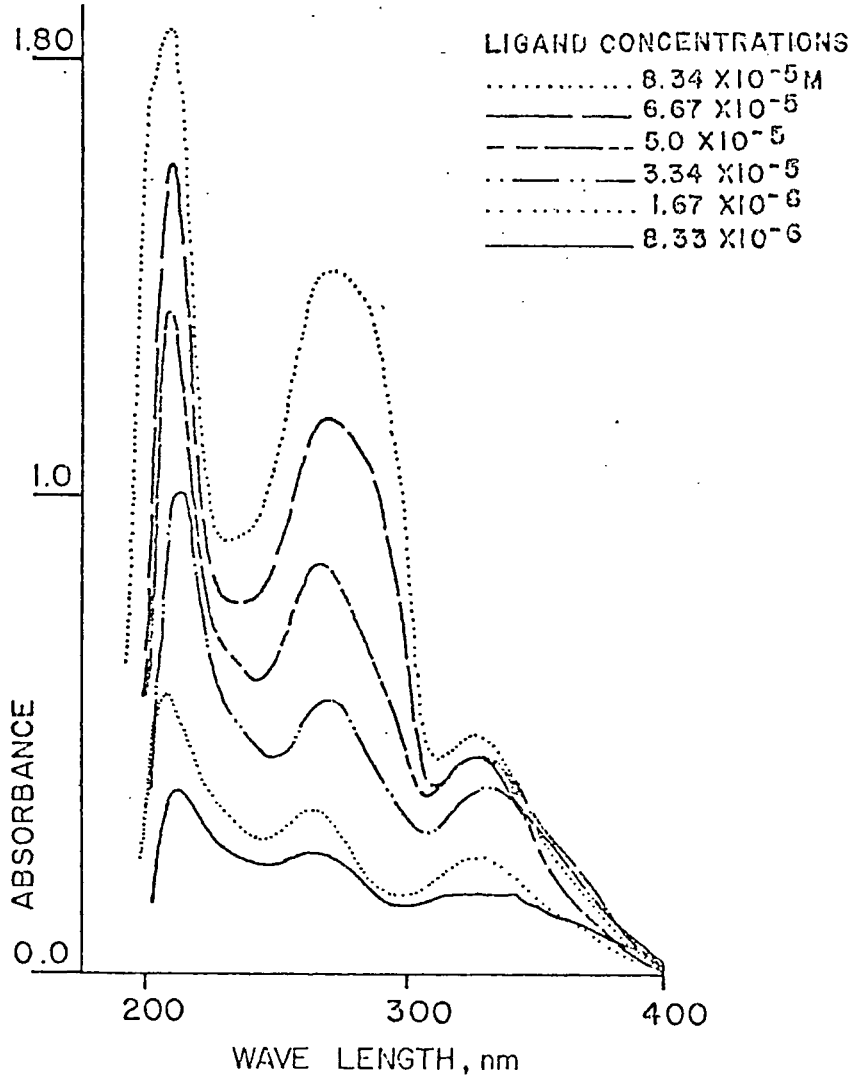



Figure 5. UV spectrum of the diphenyl phosphoric hydrazone of pyridine-2-carboxaldehyde in the presence of a $1 \times 10^{-4}$ M alcoholic solution of cupric chloride.

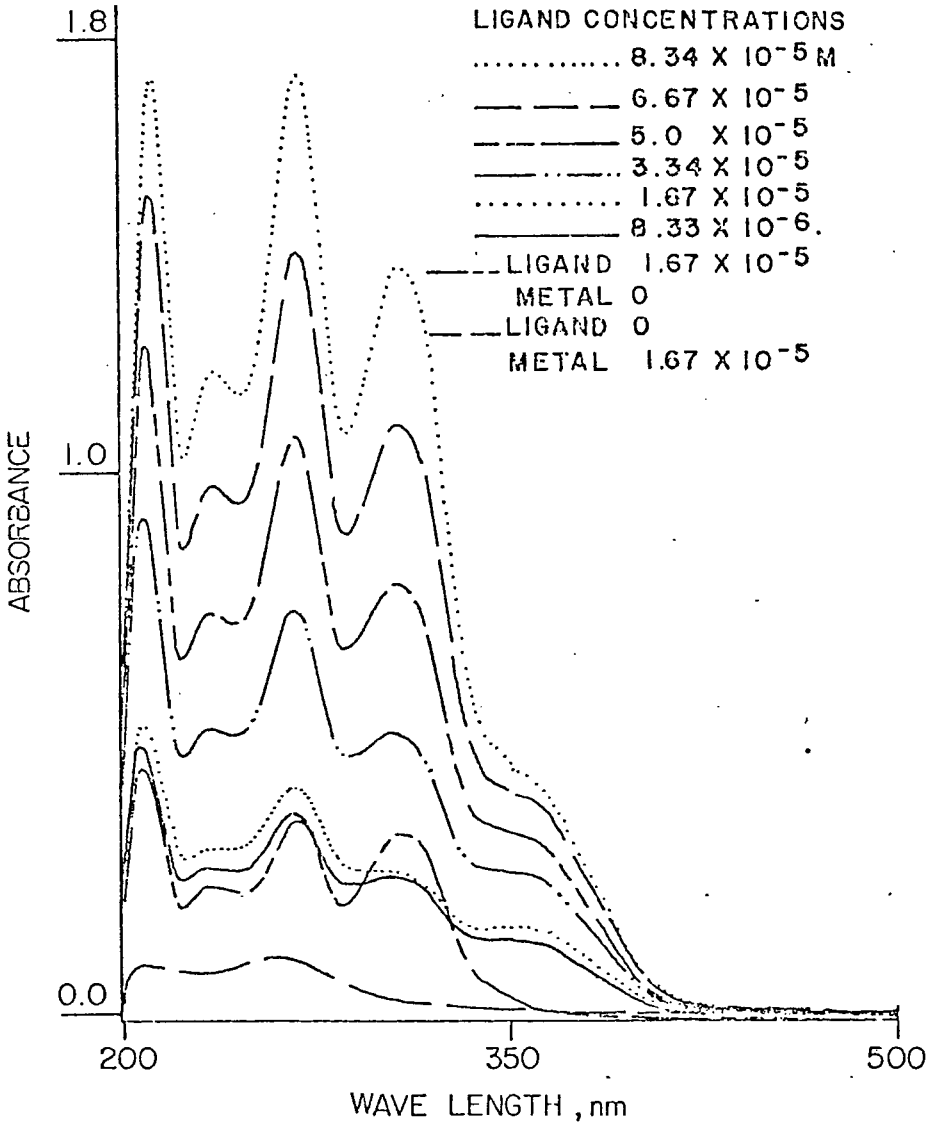



Figure 6. UV spectrum of the diphenyl thio-phosphoric hydrazone of pyridine-2-carboxaldehyde N-oxide (XXIII) in the presence of a 1 X $10^{-4}$ M alcoholic solution of cupric chloride.

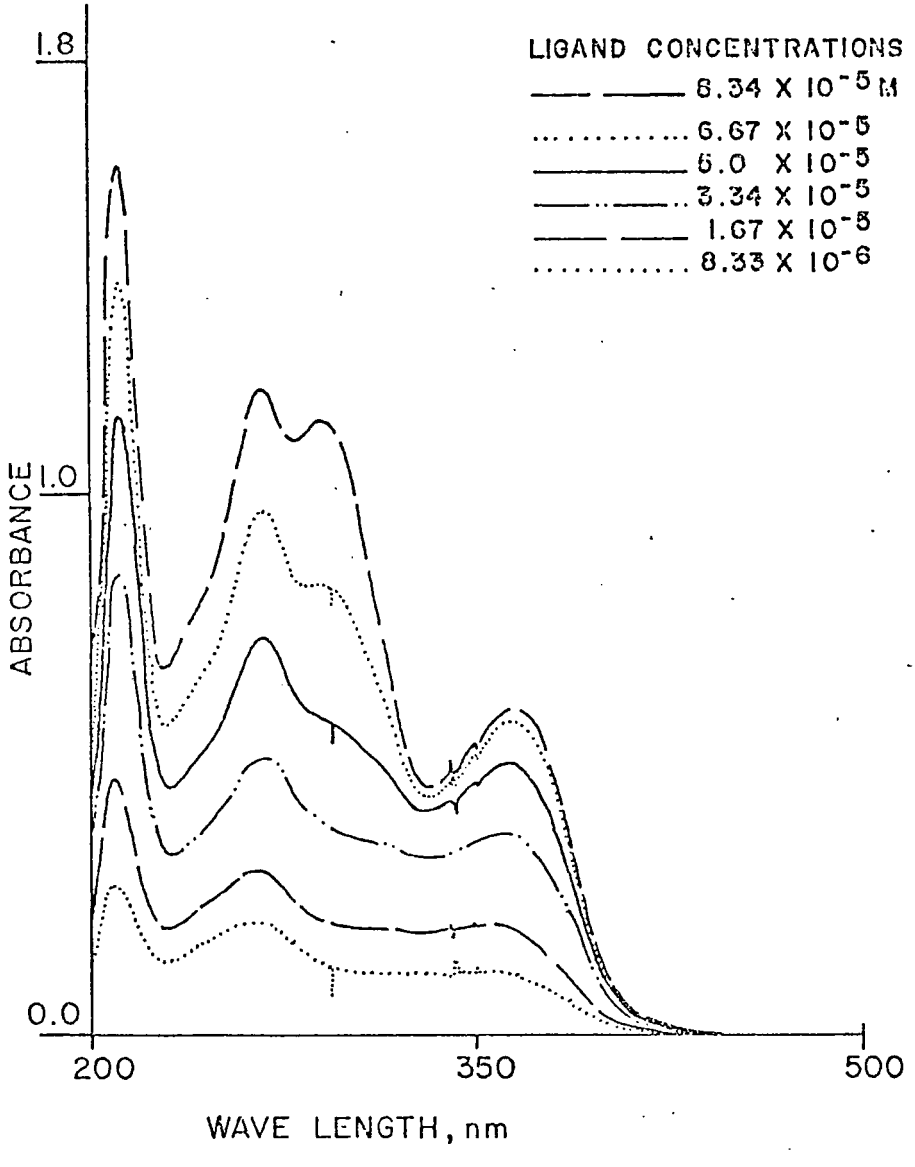



Figure 7. UV spectrum of the diphenyl thiophosphoric hydrazone of pyridine-2-carboxaldehyde in the presence of a 1 X $10^{-4}$M alcoholic solution of cupric chloride.

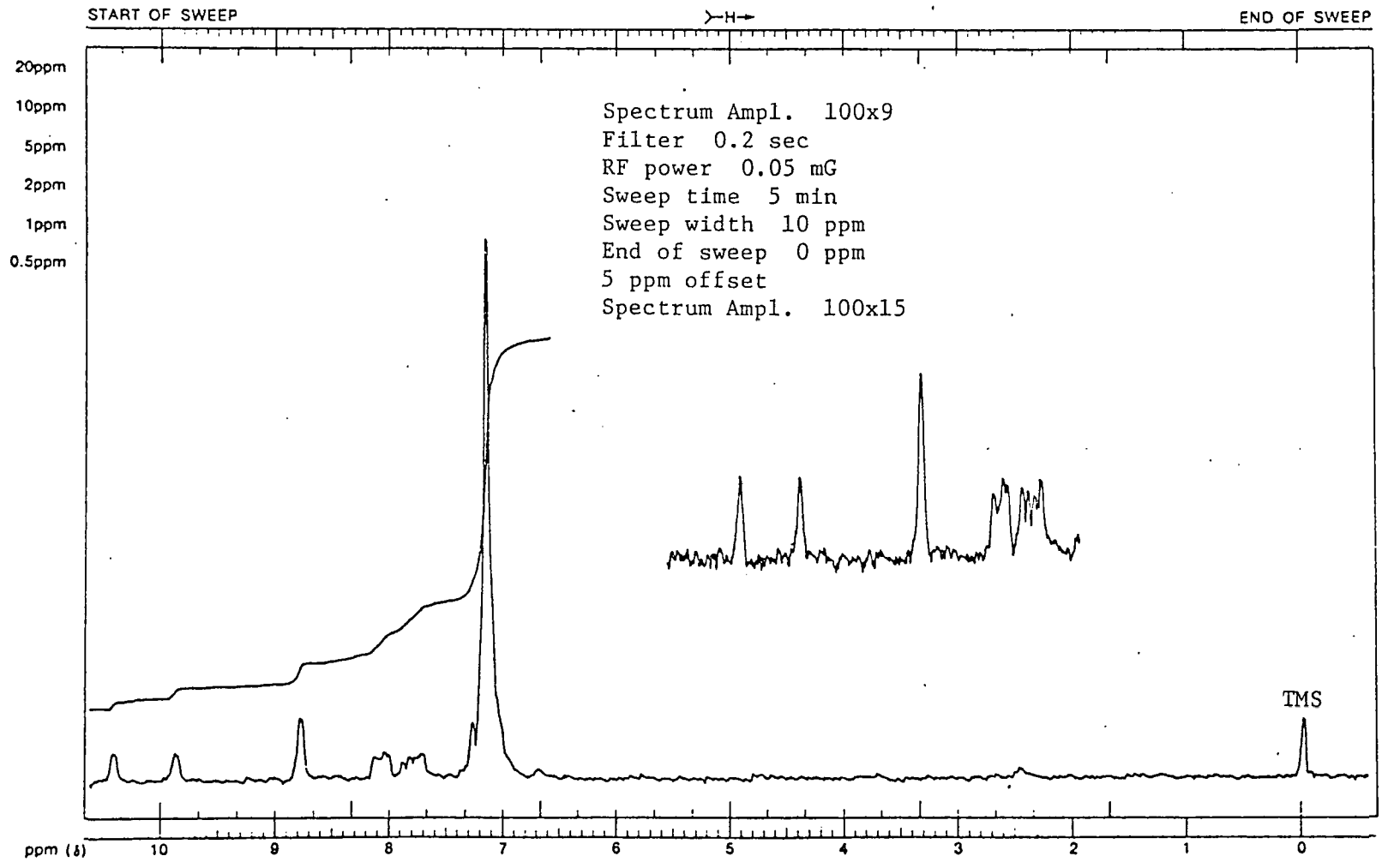



Figure 8. 60 MHz NMR spectrum of the diphenyl phosphoric hydrazone of pyridine-2-carboxaldehyde N-oxide (XX)

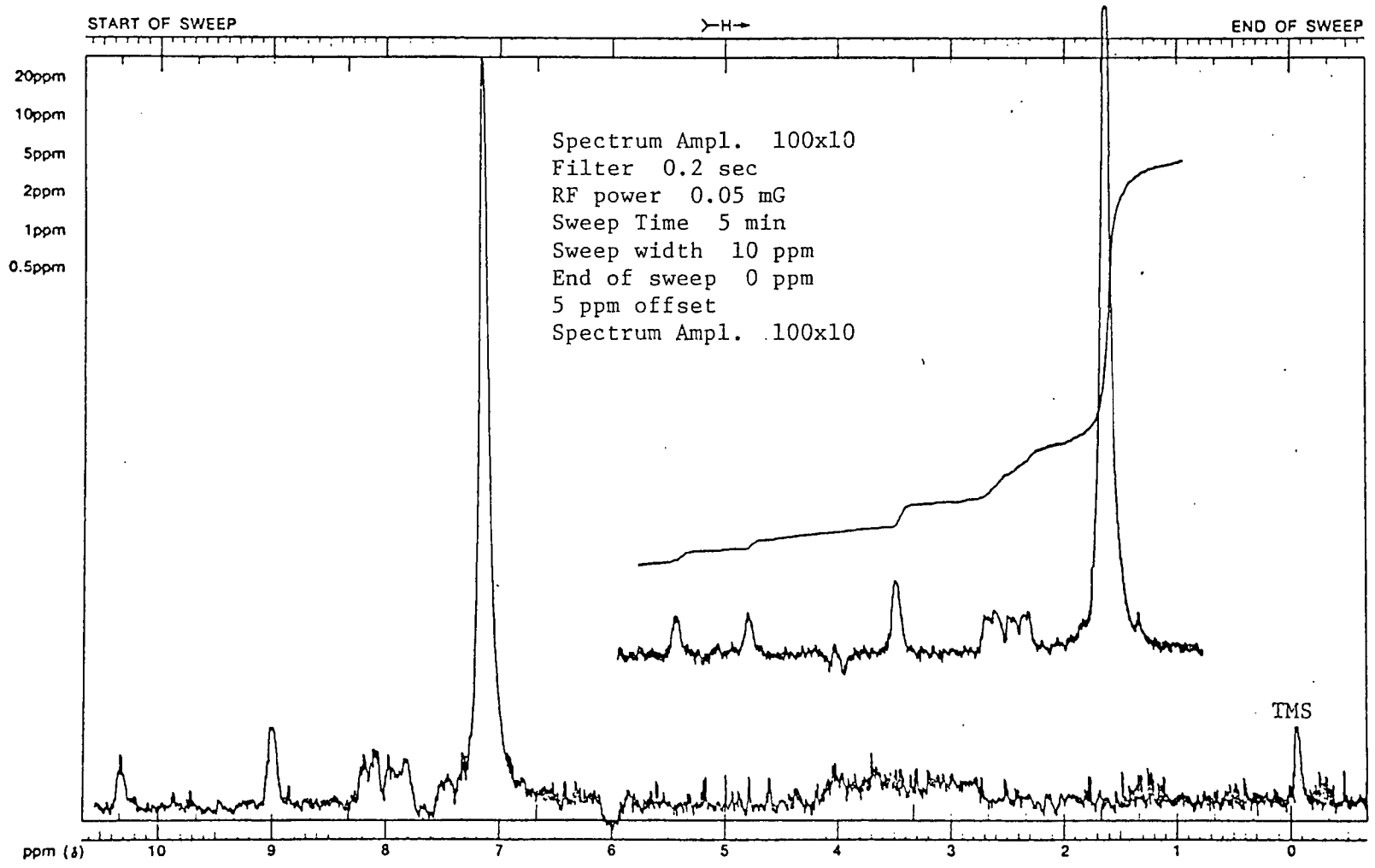



Figure 9. 60 MHz NMR spectrum of the diphenyl thiophosphoric hydrazone of pyridine-2-carboxaldehyde N-oxide (XXIII)

100 MHz NMR spectrum of 2-formyl pyridine
N-oxide bis-hydrazone (XXVI)

| | | | |
|---|---|---|---|
| EX = | 25APR78 | AT = | 2.72793 SEC |
| P2 = | 6.00000 USEC | SW = | 1501.50 |
| D5 = | 2.0000 SEC | OF = | 1240.11 |
| ACQ = | 16 | SF = | 100.060 |
| SIZE = | 16384 | EM = | .200000 |

| LINE # | HEIGHT | FREQ(HZ) | PPM |
|---|---|---|---|
| 1 | 631.656 | 924.150 | 9.23596 |
| 2 | 123.106 | 828.124 | 8.27627 |
| 3 | 97.215 | 825.821 | 8.25326 |
| 4 | 170.004 | 824.761 | 8.24266 |
| 5 | 89.399 | 822.285 | 8.21792 |
| 6 | 247.191 | 820.675 | 8.20183 |
| 7 | 56.668 | 817.775 | 8.17285 |
| 8 | 138.739 | 815.537 | 8.15048 |
| 9 | 106.008 | 814.569 | 8.14081 |
| 10 | 130.923 | 810.766 | 8.10280 |
| 11 | 270.639 | 736.714 | 7.36272 |
| 12 | 315.583 | 732.734 | 7.32295 |
| 13 | 148.510 | 729.061 | 7.28623 |
| 14 | 254.030 | 727.913 | 7.27476 |
| 15 | 255.984 | 727.352 | 7.26916 |
| 16 | 1034.68 | 726.107 | 7.25672 |

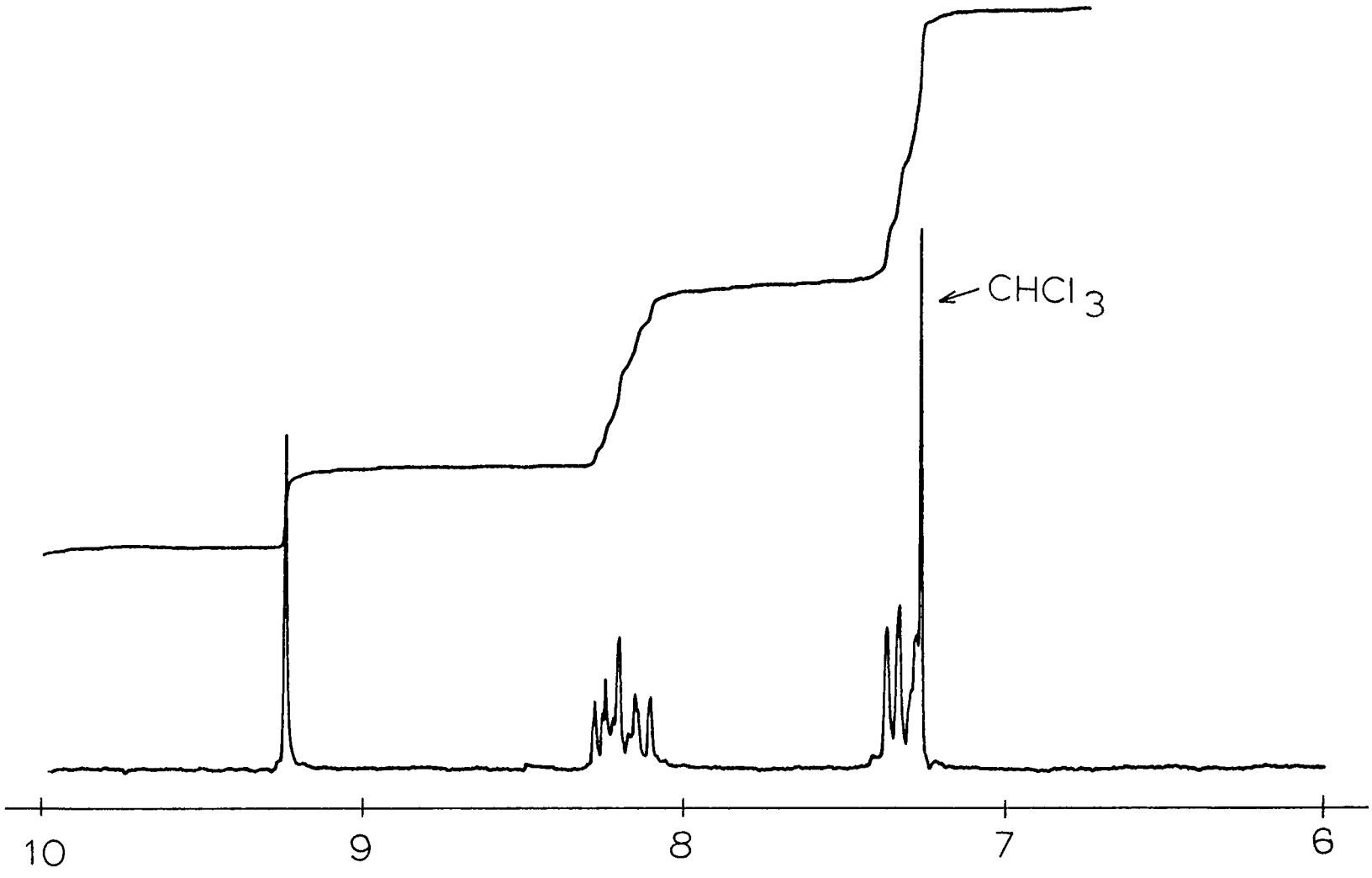



Figure 10. 100 MHz NMR spectrum of 2-formyl pyridine N-oxide bis-hydrazone (XXVI)

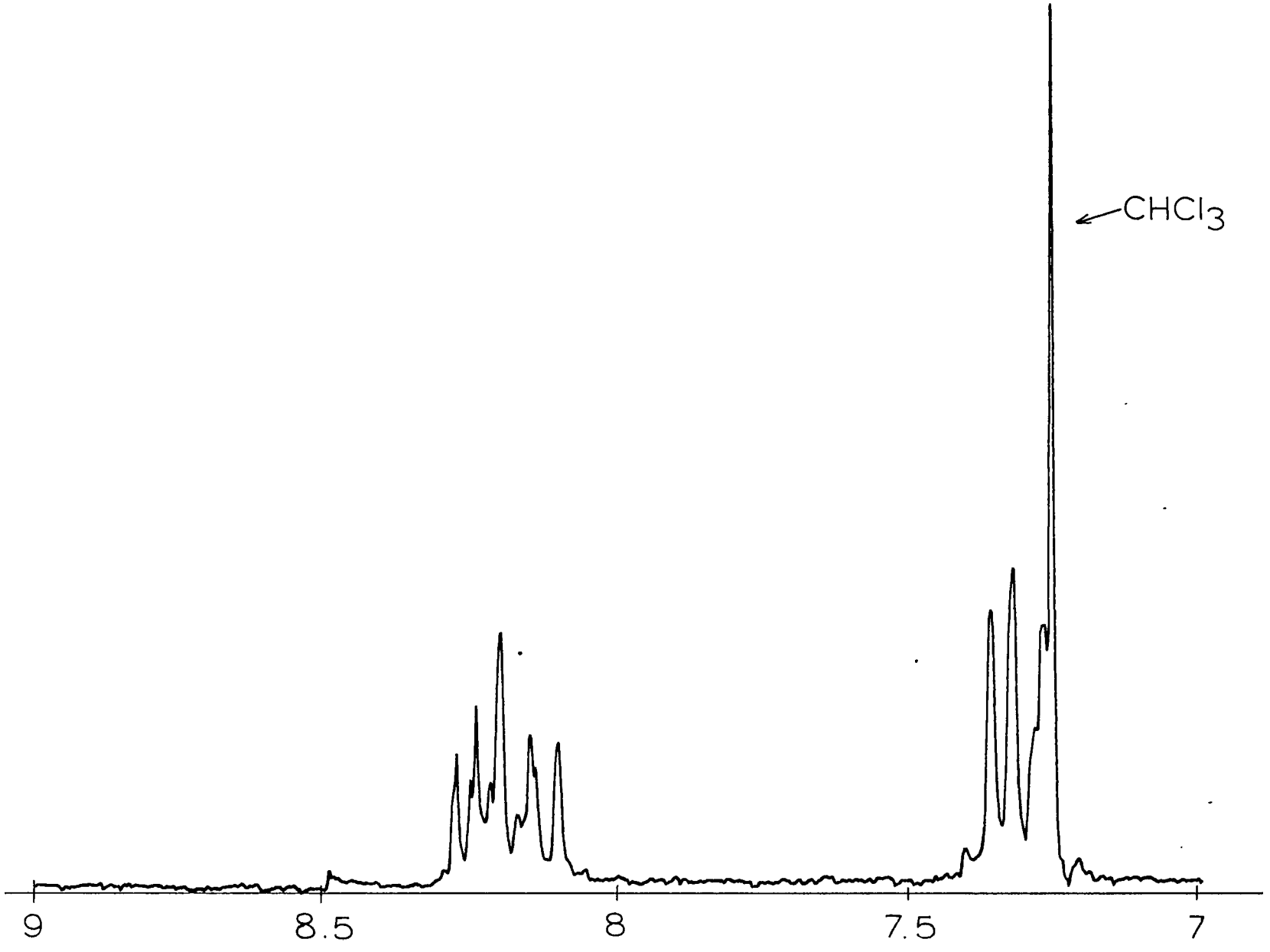



Figure 11. 100 MHz NMR spectrum of 2-formyl pyridine N-oxide bis-hydrazone (XXVI) (expanded scale)

100 MHz NMR spectrum of 2-formyl pyridine
bis-hydrazone (XXVIII)

| | | | |
|---|---|---|---|
| EX = | 25APR78 | AT = | 2.72793 SEC |
| P2 = | 6.00000 USEC | SW = | 1501.50 |
| D5 = | 2.00000 SEC | OF = | 1241.16 |
| ACQ = | 16 | SF = | 100.060 |
| SIZE = | 16384 | EM = | .200000 |

| LINE # | HEIGHT | FREQ(HZ) | PPM |
|---|---|---|---|
| 1 | 125.352 | 876.731 | 8.76205 |
| 2 | 159.807 | 875.015 | 8.74491 |
| 3 | 146.623 | 874.259 | 8.73735 |
| 4 | 176.699 | 871.933 | 8.71411 |
| 5 | 189.852 | 871.196 | 8.70673 |
| 6 | 766.765 | 868.414 | 8.67893 |
| 7 | 186.022 | 818.416 | 8.17925 |
| 8 | 195.620 | 811.467 | 8.10981 |
| 9 | 311.177 | 810.438 | 8.09952 |
| 10 | 195.132 | 809.415 | 8.08929 |
| 11 | 106.553 | 788.787 | 7.88314 |
| 12 | 112.489 | 787.036 | 7.86565 |
| 13 | 195.819 | 781.145 | 7.80677 |
| 14 | 192.141 | 779.564 | 7.79097 |
| 15 | 98.7106 | 773.413 | 7.72949 |
| 16 | 96.6964 | 771.658 | 7.71195 |
| 17 | 149.629 | 743.271 | 7.42825 |
| 18 | 165.056 | 741.902 | 7.41457 |
| 19 | 142.534 | 738.281 | 7.37838 |
| 20 | 161.120 | 737.108 | 7.36666 |
| 21 | 127.718 | 735.699 | 7.35257 |
| 22 | 116.456 | 734.497 | 7.34057 |
| 23 | 109.193 | 730.852 | 7.30413 |
| 24 | 105.516 | 729.736 | 7.29298 |
| 25 | 236.499 | 726.939 | 7.26503 |

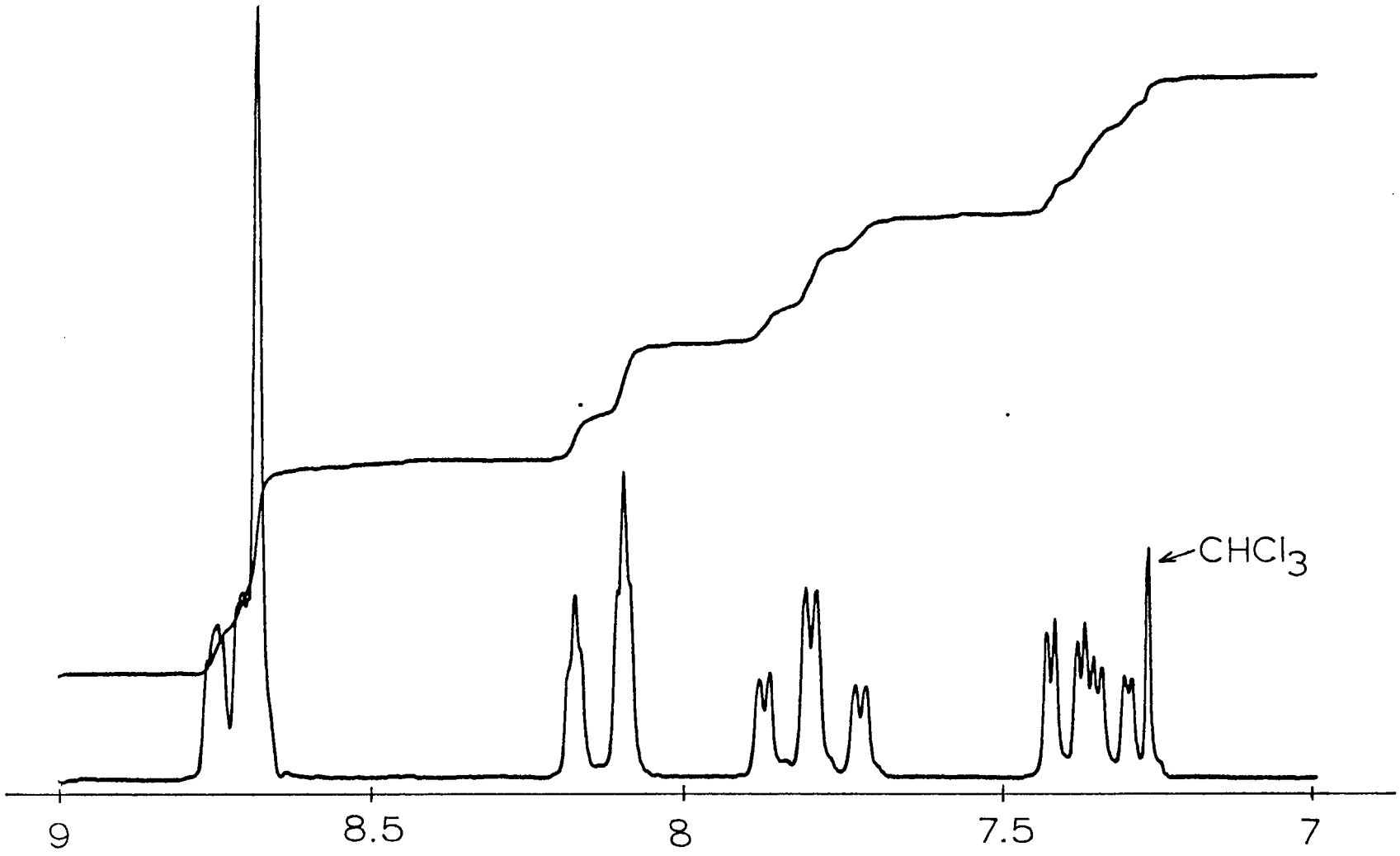



Figure 12. 100MHz NMR spectrum of 2-formyl pyridine bis-hydrazone (XXVIII)

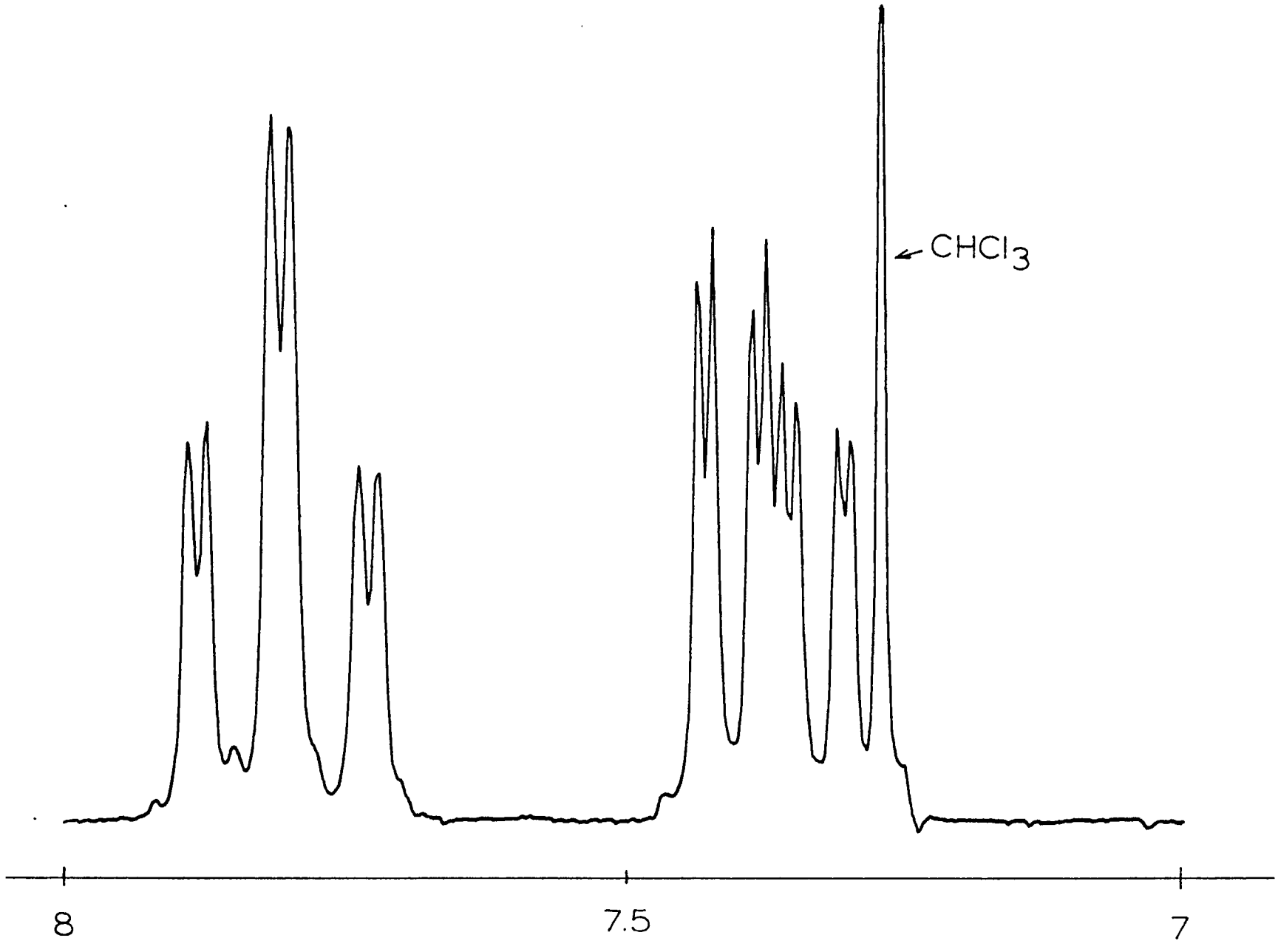



Figure 13. 100 MHz NMR spectrum of 2-formyl pyridine bis-hydrazone (XXVIII) (expanded scale)

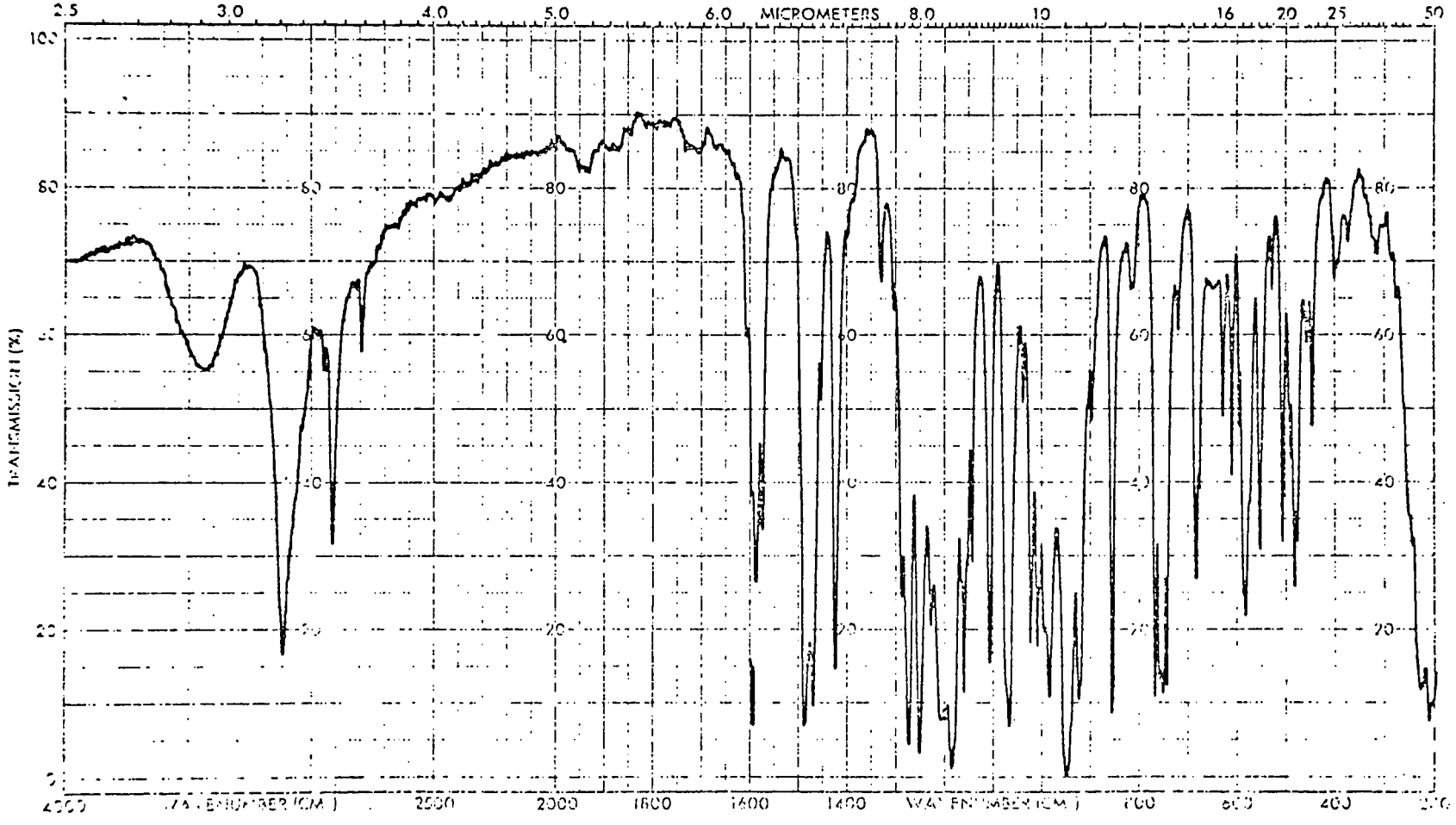



Figure 14. IR spectrum of the diphenyl phosphoric hydrazone of pyridine-2-carboxaldehyde N-oxide (XX)

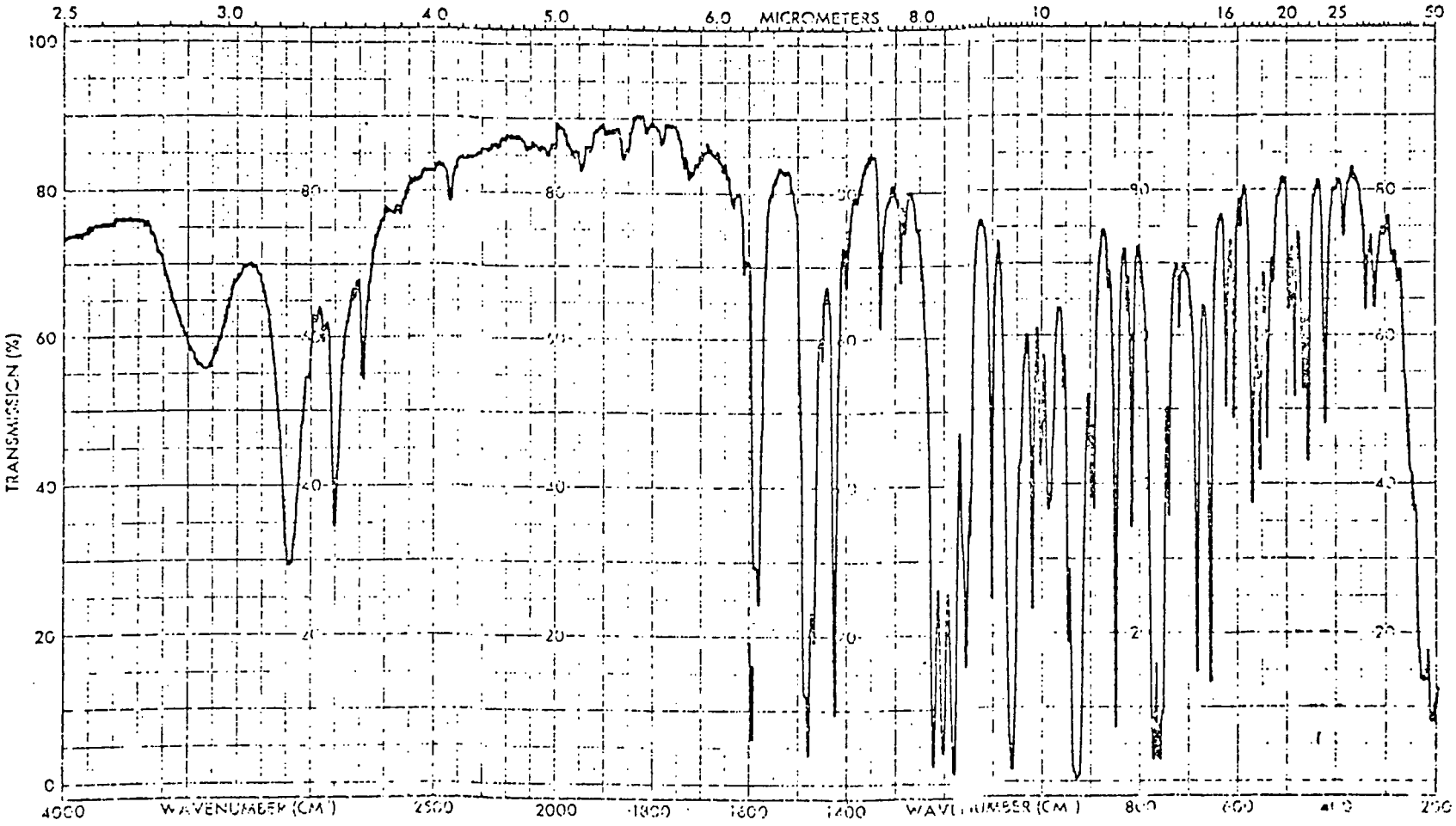



Figure 15. IR spectrum of the diphenyl thiophosphoric hydrazone of pyridine-2-carboxaldehyde N-oxide (XXIII)

# CHAPTER V

# REFERENCES

## CHAPTER V

## REFERENCES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# Appendix

Biological Evaluations

Preliminary in vivo oncolytic activities of XX, XXIII, their non-N-oxide forms: XX-N and XXIII-N, respectively, XXVII and XXVIII against Ehrlich carcinoma, Sarcoma 180, and P388 lymphocytic leukemia were conducted under the supervision of Dr. A. P. Kimball by Mr. Norman Caron, both of the Department of Biophysical Sciences, University of Houston.

1. Transplantation

The Ehrlich carcinoma ascites cells were grown in female Swiss albino mice, while the Sarcoma 180 Carcinoma and the P388 lymphocytic leukemia cells were grown in female CD-1 mice. Using donor mice bearing a seven-day tumor growth, the intraperitoneal ascitic fluid was withdrawn and centrifuged at approximately 1,500 rpm for 30 seconds. The supernatant was then decanted. If red blood cells were present, the packed cells were washed with normal saline (0.9% sodium chloride solution) and centrifuged for 30 seconds two times. After decanting the supernatants, the packed cells were then resuspended in distilled water. Within 30 seconds, two volumes of 1.8% normal saline was added to make the resulting solution isotonic. After centrifuging for 30 sec., the supernatant was decanted off. The packed cells were resuspended in normal saline and diluted approximately 500 fold. In the case of Ehrlich and Sarcoma 180 carcinomas, $4 \times 10^6$ cells, and for P388, $1 \times 10^5$ cells in 0.1 ml suspensions were administered to groups of six female mice, Swiss albino in the case of Ehrlich carcinoma and $BDF_1$ in the cases of Sarcoma 180 and P388.

2. Methods

The survival studies were carried out using the above tumor cells in the appropriate mice by administering, intraperitoneally, suspensions

of all compounds to be tested in normal saline with a few drops of Tween 80 if difficulties in solubility arose. Therapy began 24 hours after transplantation and was continued once daily for six consecutive days. The single compounds were arbitrarily administered at a previously determined non-toxic dose of 100 mg/kg while the concurrent administration of some of the compounds with copper using female Swiss albino mice required reduction to 40 + 8 mg/kg due to toxicity of these combinations. The copper chelates were likewise administered at maximum non-toxic doses. All compounds were administered in volumes of 0.2 - 0.4 ml with controls given injections of comparable volumes of vehicle. When the compound and copper were administered concurrently, solutions of the two were mixed in the syringe prior to injection. The mice were observed each day until death or sacrifice and the average life span was calculated.

Table I

Survival Study of Phosphoric and Thiophosphoric Hydrazones, Concurrently Administered Copper-Ligand Complexes; and Cupric Chelates Against P388 Lymphocytic Leukemia

| | Compound | Dosage[a] mg/kg | %T/C[b] |
|---|---|---|---|
| group 1 | Cell Control | | 10.3 ± 0.2[c] |
| group 2 | $CuCl_2$ Control | 8 | 94 |
| group 3 | XX | 100 | 111 |
| group 4 | XX + Cu | 50+8 | 95 |
| group 5 | XX - N | 100 | 117 |
| group 6 | XX - N + Cu | 40+8 | 92 |
| group 7 | XXIII | 100 | 113 |
| group 8 | XXIII + Cu | 40+8 | 94 |
| group 9 | XXIII - N | 100 | 102 |
| group 10 | XXIII - N + Cu | 40+8 | 84 |
| group 11 | XXIX | 6.1 | 89 |
| group 12 | XXX | 6.1 | 92 |
| group 13 | XXX | 12.2[d] | 133 |

[a]Administered once a day for six consecutive days beginning 24 hours after tumor implantation.
[b]%T/C = treated/control X 100
[c]Average survival time in days including Standard Error.
[d]Dosage of XXX was doubled to investigate effect on survival time.

Table II

Survival Study of XXIII, XXIX, and Concurrently Administered Copper-Ligand Complex of XXIII Against Sarcoma 180

| | Compound | Dosage[a] mg/kg | %T/C[b] |
|---|---|---|---|
| group 1 | Cell Control | | 18.2 ± 0.3[c] |
| group 2 | $CuCl_2$ Control | 8 | 98 |
| group 3 | XXIII | 100 | 93 |
| group 4 | XXIII + Cu | 40 + 8 | 106 |
| group 5 | XXIX | 6.1 | 99 |

[a,b,c]Refer to Table I.

Table III

Survival Study of XXIII, XXIX, and Concurrently Administered Copper-Ligand Complex of XXIII Against Ehrlich Carcinoma

| | Compound | Dosage[a] mg/kg | %T/C[b] |
|---|---|---|---|
| group 1 | Cell Control | | 16.3 ± 0.6[c] |
| group 2 | $CuCl_2$ Control | 8 | 98 |
| group 3 | XXIII | 100 | 144 |
| group 4 | XXIII + Cu | 40 + 8 | 204 |
| group 5 | XXIX | 6.1 | 160 |

[a,b,c]Refer to Table I

# Part II : Aldophosphamide Substrate-like Inhibitors

LIST OF EQUATIONS

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LIST OF SCHEMES

LIST OF FIGURES

# CHAPTER I

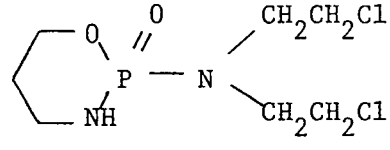
# INTRODUCTION

# CHAPTER I

## INTRODUCTION

Much research has been undertaken to date to utilize the reportedly greater occurrence of phosphoramidases in neoplastic cells than in normal cells.[1,2] Consequently, many types of phosphorus-nitrogen compounds to be used as potential substrates for these enzymes have been studied, with emphasis quickly going to phosphorylated nitrogen mustards due to their ability to alkylate both DNA and RNA.

The most effective, and therefore the most studied, nitrogen-mustard derivative has been 2-[bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide (cyclophosphamide, Cytoxan, I).

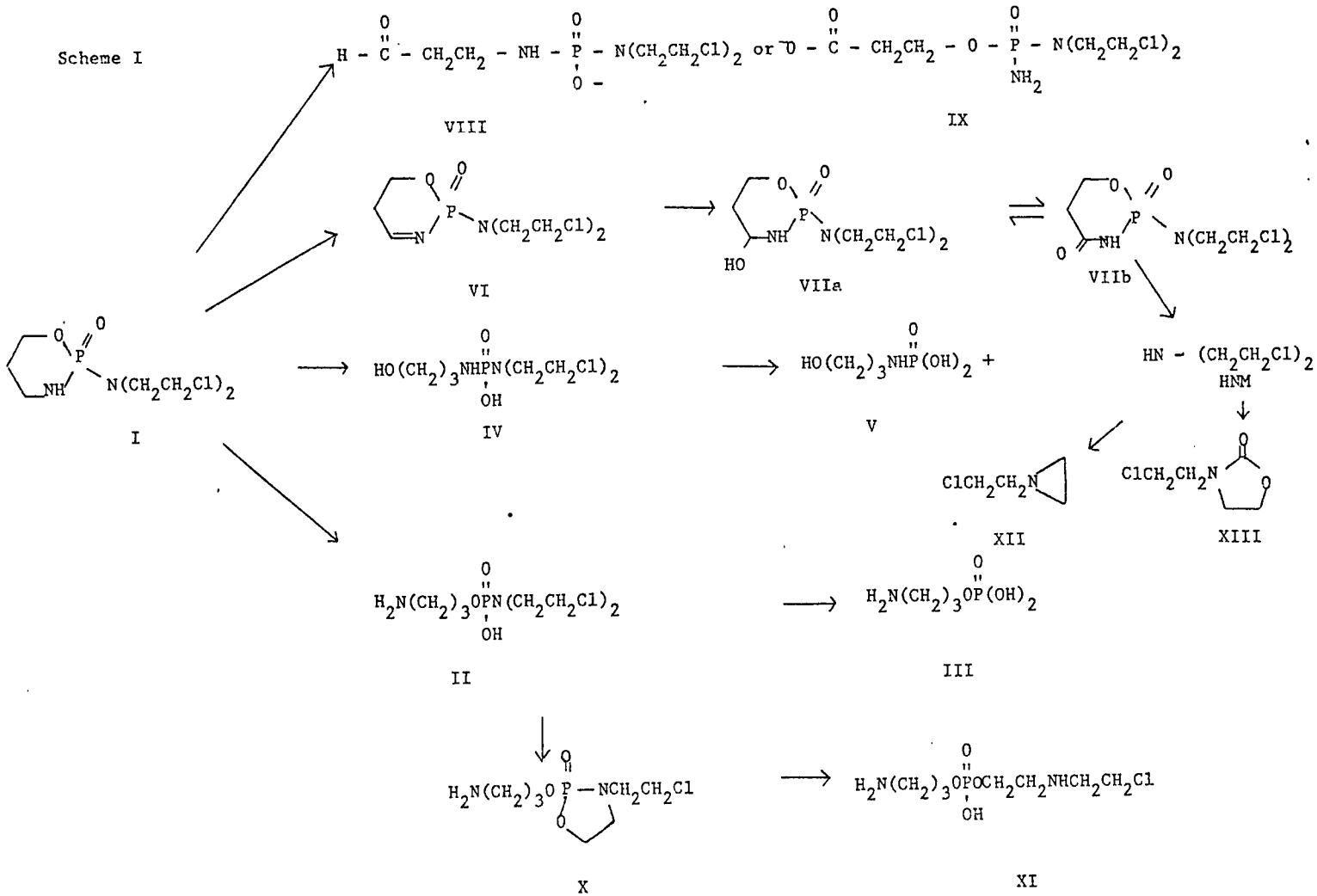


I

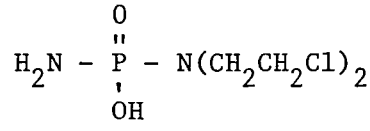
It was suggested by Foley[3] in 1961 that the role of cyclophosphamide was that of a transport structure which needed activation to become biologically effective. He also suggested that this activation process occurred primarily in the liver. Although still not completely understood, cyclophosphamide is believed to undergo metabolic activation involving the release of the nitrogen mustard, 2,2'-dichloro-N-diethyl amine (HNM).

The first in a series of studies to determine the metabolites of cyclophosphamide (Scheme I) was performed by Arnold[4,5] in 1958. He suggested that I was activated by a two step hydrolysis to HNM involving

Scheme I



the sequence I $\longrightarrow$ IV (or II) $\longrightarrow$ V (or III) + HNM. Both cytoxol alcohol (IV, 2,2'-dichloro-N-diethyl-N'-3-hydroxypropyl phosphorodiamidic acid) and cytoxol amine (II) were perceived as metabolites and potential active forms of cyclophosphamide. The latter was reported by Grunicke[6] in 1965 to be a product of the hydrolytic cleavage of I; and that, in contrast to I itself, II showed marked antitumor activity *in vitro*. Bunnett,[7] in 1967, proposed that II could be involved in a cyclization process with further activation in the sequence II $\longrightarrow$ X $\longrightarrow$ XI. Friedman[8] in 1963 and Maddock[9] in 1966 reported that IV and the related compound, phosphoramide mustard (XIV), were comparable in inhibitory activity to cyclophosphamide without prior activation toward several mouse tumors.



XIV

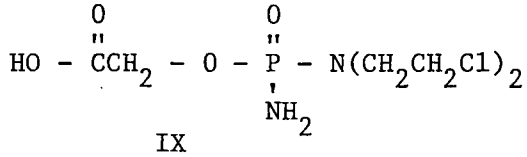
Brock[10] in 1963 cited evidence for the involvement of oxidation in the activation of I, and that microsomes found in the liver were responsible for this oxidative activation utilizing triphosphopyridine nucleotide and molecular oxygen. It was also reported in the same study that *in vivo* activation of I resulted from a metabolic process requiring the presence of oxygen and that a simple hydrolysis was not correct.

Likewise, through further studies, it was determined that other activated metabolites were also formed in the liver. Among these were VIII, *via* an O-dealkylase, and IX, *via* an N-dealkylase. Although the latter showed little, if any, cytotoxicity,[11,12] the former was designated

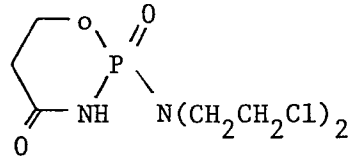
as a highly hydrophilic anion which may permeate cancer cells more readily than normal cells.[13]

The end product of the microsomal activation of I, 1-(2-chloroethyl) aziridine (XII), was reported by Rauen[14] in 1964 to be from the oxidation of the metabolic intermediates VI, VII, and HNM.

Struck[15] in 1971 reported the isolation and identification of carboxyphosphamide [IX, 2-carboxyethyl-N,N-bis(2-chloroethyl) phosphorodiamidate], as the major urinary metabolite when I was administered *iv.* to dogs and, then in the same study, to humans. They also reported that 4-ketocyclophosphamide ($VII_b$) was one of the minor isolated metabolites (see Scheme I). In this same study, it also was reported that



IX

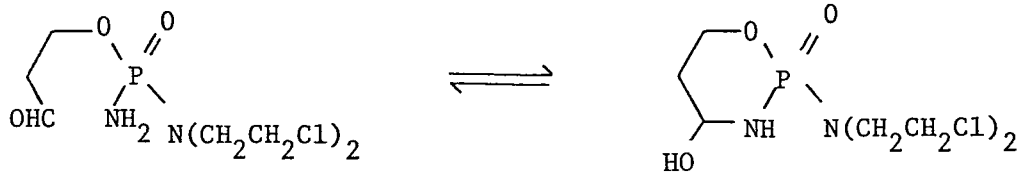


VIIb

neither of these two metabolites exhibited much cytotoxicity toward human epidermoid cancer *in vitro* or to leukemia L1210 cells *in vivo*. The same investigation was repeated recently by Schaumloeffel[16] with concurring results.

In 1972, Hill[17,18] discovered that the initial metabolite of I, after hepatic enzyme action, could be oxidized to IX by various cellular enzymes as well as by purified aldehyde oxidase and a commercial preparation of NAD - linked aldehyde dehydrogenase. This initial metabolite was

reported to be the open-ring aldehyde, 2-formylethyl-N,N-bis(2-chloroethyl) phosphorodiamidate (XV) which was thought to exist in tautomeric



XV VIIa

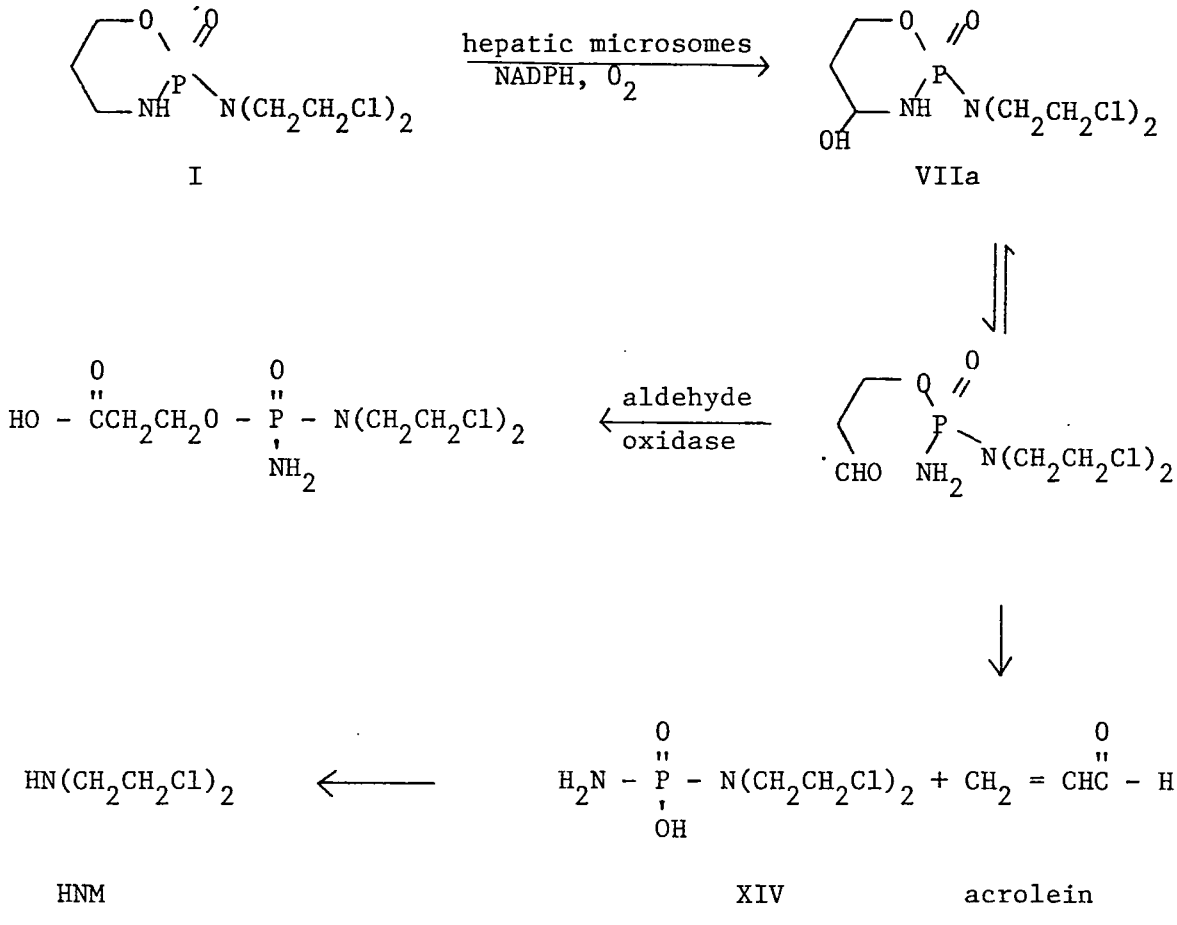
equilibrium with 4-hydroxycyclophosphamide (VIIa) (see above and Scheme I). The proposed trivial name for XV was "aldophosphamide". In these same studies the following were also reported: (a) that XV was highly toxic to human epidermoid carcinoma number two and leukemic L1210 cells, (b) that the oxidation of XV to IX could be inhibited by substrates or inhibitors of aldehyde oxidase, and (c) that the combination of I with aldehyde oxidase substrates was more toxic to leukemia L1210 cells than was I alone.

Sladek[19] in 1973 described the generation of an alkylating aldehyde from cyclophosphamide *in vivo* and *in vitro*; but, due to its instability, this aldehyde was isolated only as a semicarbazone derivative.

Then in 1974 Connors[20] reported the isolation of VIIa, the initial major metabolite of I. Although it was also too unstable to isolate in pure form, it was trapped by reaction with ethanol.

The currently accepted metabolic pathway of cyclophosphamide (I), first proposed by Norpoth[19] in 1973, is illustrated in Scheme II. As can be seen from this scheme, the activation of cyclophosphamide (I) to

Scheme II




aldophosphamide (XV), *via* the proper enzymes in the liver, produces in turn the probable cytotoxic agent, phosphoramide mustard (XIV).[21] However, without suitable substrates, the aldehyde oxidase would prefererentially oxidize XV to the corresponding inactive carboxyphosphamide (IX), and thus prevent much of XV from ever becoming activated.

In a continuing study, Struck[22] in 1974 managed to synthesize aldophosphamide semicarbazone XVI from VIIa. He found that XVI was identical

by TLC in four solvent systems to an alkylating semicarbazone derivative of an aldehyde produced in a model oxygenase system.

In more recent work, Myles[23] was able to synthesize XV, but agreeing with the above results, found it to be very unstable at room temperature, decomposing to give acrolein.

Similarly, Fenselau[24] in 1977, isolated XV as a cyanohydrin derivative from an incubation mixture of I with mouse liver microsomes *in vitro* and from the plasma of a cyclophosphamide(I)-treated patient.

Therefore, from the evidence stated above, it would seem that aldophosphamide is indeed a key metabolite in the metabolic pathway of this increasingly prescribed oncolytic agent, cyclophosphamide (I).

# CHAPTER II

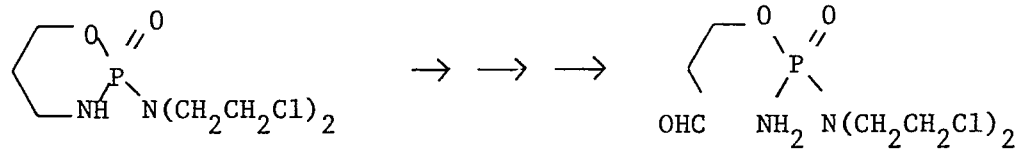
# DISCUSSION

# CHAPTER II

# DISCUSSION

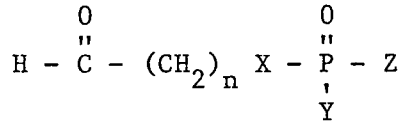
## A. Rationale

Due to the fact that aldophosphamide (XV) had been identified as a key metabolite in the activation pathway of cyclophosphamide (I)[22],



I XV

compounds of type XVII were to be synthesized to act as potential substrates



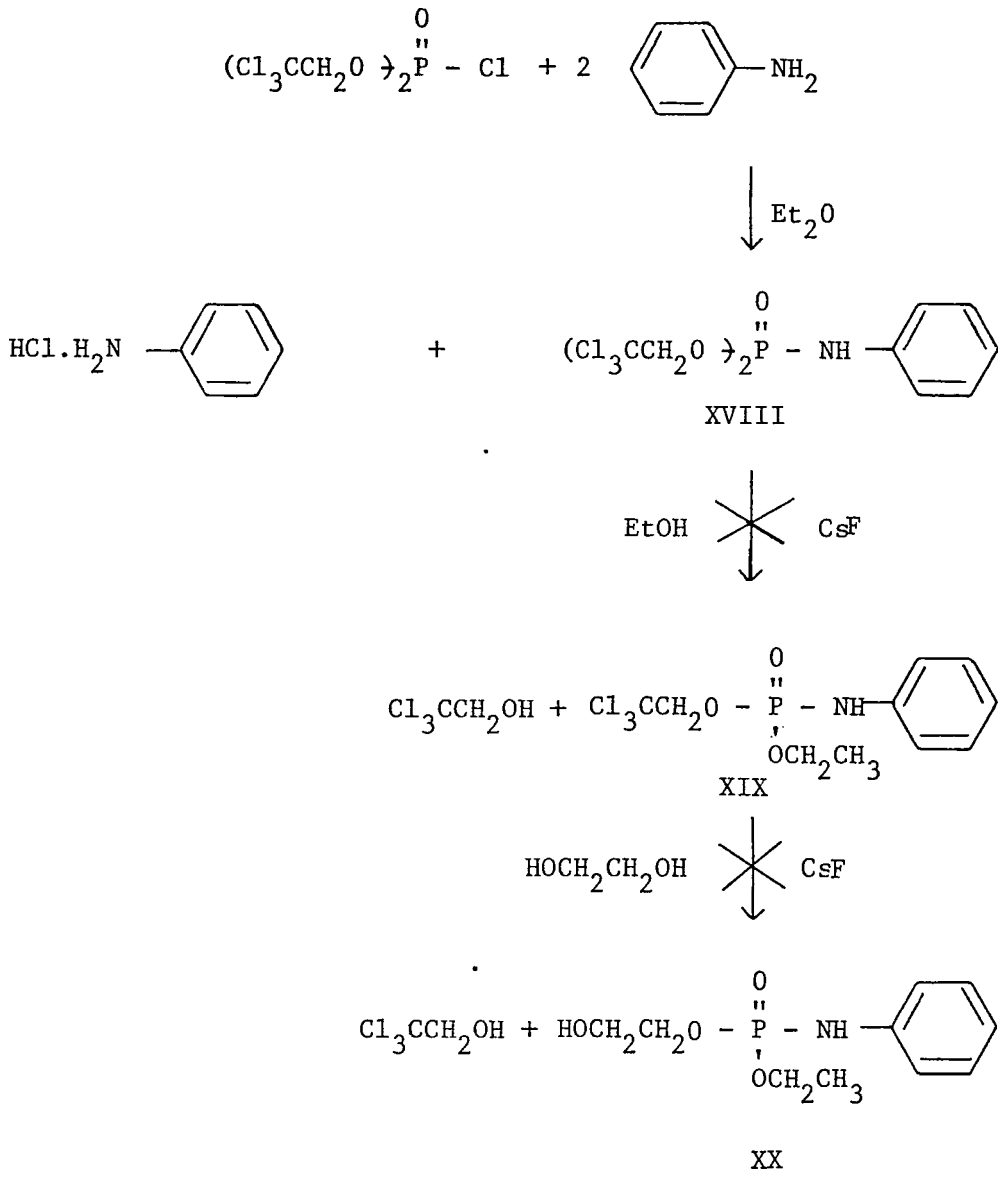
XVII

of aldehyde oxidase responsible for the degradation of XV. This would allow accumulation of XV and other cytotoxic metabolites by reducing the extent of conversion to inactive carboxyphosphamide (IX) and, possibly, increase duration of action and reduce toxicity.

## B. Methods

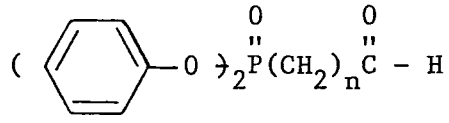
The proposed syntheses of a compound of type XVII involved the synthesis of 0,0'-di-(2,2,2-trichloroethyl)-N-phenyl phosphoramidate (XVIII),[25] the transesterification of XVIII with ethanol in the presence of cessium floride to obtain 0-(2,2,2-trichloroethyl)-0-ethyl-N-phenyl phosphoramidate (XIX),[26] and then, following the same method as described above,[26] the further transesterification with ethylene glycol to yield

Scheme III



XX (Scheme III). It was hoped that XX could be oxidized to its corresponding aldehyde, but since XIX was not isolated, XX could not be synthesized.

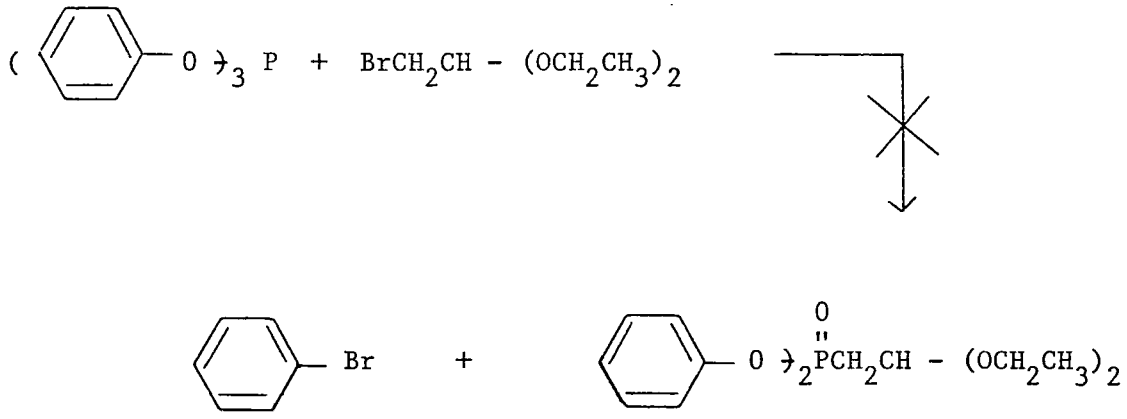
Due to the failure in the isolation of either XIX or XX from the above synthesis, a compound of type XXI below was synthesized in the hope of decreasing water solubility and increasing stability in order to facilitate isolation and purification.



XXI, where n = 1

The synthesis of diphenyl formyl methyl phosphonate (XXI) involved first the synthesis of 0,0'-diphenyl formyl methyl phosphonate diethyl acetal (XXII) using triphenyl phosphite[27] (Equation 1). When this procedure

Equation 1

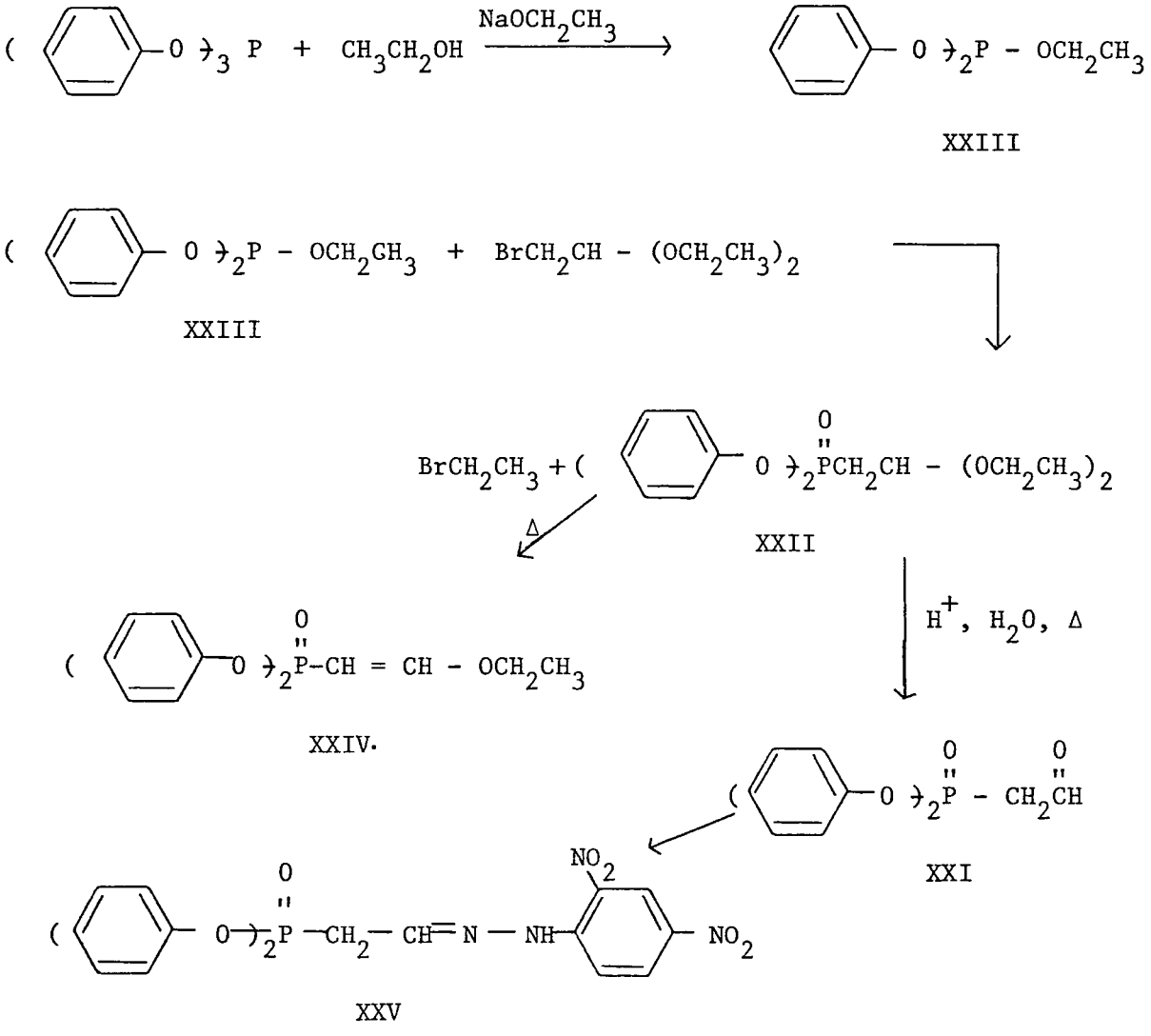


XXII

was used, only the starting materials were isolated. So an alternate method was employed. This initially involved the preparation of diphenyl ethyl phosphite (XXIII),[28] then the addition of this to bromoacetaldehyde diethyl acetal to yield XXII.[27] The first attempt at the hydrolysis of XXII gave 0,0'-diphenyl (2-ethoxyvinyl) phosphonate (XXIV) as the only

product. The second attempt did give XXI, but could only be isolated as the 2,4-dinitrophenyl hydrazone (XXV, Scheme IV)

Scheme IV

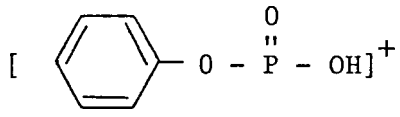
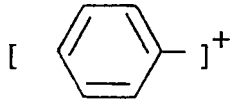


C. Results

The difficulties that arose in the preparation of these compounds are those found in any investigation and synthesis of organophosphorus compounds: their isolation and purification.

In the synthesis of XXI, an unexpected event occured. Because an insufficient quantity of water was present in the first hydrolysis of XXII, the attempt at the fractional distillation of the hydrolysis product resulted in the formation of XXIV. This loss of only one ethoxy group was caused, not from the hydrolysis by the acid/water present, but from the applied heat needed in the distillation process. Because this was an unknown compound, it was of interest to characterize it. The NMR, IR, and elemental analysis confirmed the proposed structure. The EI-Mass spectrum likewise showed that XXIV had been formed. The molecular weight of this compound was calculated to be 304.285. The major peaks are listed below:

| m/e | Fragment |
|---|---|
| 305 | $[(C_6H_5-O)_2P(O)CH=CH-OCH_2CH_3+H]^+$ |
| 304 | $[(C_6H_5-O)_2P(O)CH=CH-OCH_2CH_3]^+$ |
| 289 | $[(C_6H_5-O)_2P(O)CH=CH-OCH_2]^+$ |
| 275 | $[(C_6H_5-O)_2P(O)CH=CH-O]^+$ |
| 259 | $[(C_6H_5-O)_2P(O)CH=CH]^+$ |
| 258 | $[(C_6H_5-O)_2P(O)CH=CH-H]^+$ |
| 211 | $[C_6H_5-O-P(O)CH=CHOCH_2CH_3]^+$ |
| 165 | $[C_6H_5-O-P(O)CH=CH-H]^+$ |

| m/e | Fragment |
|---|---|
| 157 |  |
| 77 |  |

A second attempt at the hydrolysis of XXII using sufficient water did produce the corresponding aldehyde XXI. However, after repeatedly trying to fractionally distill the reaction product from the crude mixture, and having no desired compounds collected, it was thought too unstable to isolate in this manner. Therefore it was isolated as the 2,4-dinitrophenyl hydrazone (XXV).

I feel that a suggestion concerning the possible future isolation of XXI is warranted. It might be possible to separate this compound either by column chromatography or by high pressure liquid chromatography. For, to satisfactorily interpret the results of the aldehyde oxidase inhibition study that will subsequently be performed, an analytically pure sample of XXI must first be obtained.

# CHAPTER III

# EXPERIMENTAL

# CHAPTER III

# EXPERIMENTAL

## A. Chemicals and Reagents

All chemicals and reagents were obtained either from the various listed chemical suppliers or were prepared using the procedures given.

1. Aldrich Chemical Co., Inc., Milwaukee, Wisconsin
   - (a) bis-(2,2,2-trichloroethyl)phosphoro chloridate
   - (b) Bromoacetaldehyde diethyl acetal
   - (c) Triphenyl phosphite

2. Alfa Products, Danvers, Massachusetts
   - (a) Cesium fluoride

3. J. T. Baker Chemical Co., Phillipsburg, New Jersey
   - (a) Aniline

4. Eastman Kodak Co., Rochester, New York
   - (a) Hydroquinone

5. Fisher Scientific Co., Fair Lawn, New Jersey
   - (a) Ethylene glycol
   - (b) Sodium metal

6. 2,4-dinitrophenyl hydrazine test solution[29]

The test solution was prepared by adding 0.4 g of 2,4-dinitrophenyl hydrazine to 2.0 ml concentrated sulfuric acid in a 25 ml Erlenmeyer flask. Water (3 ml) was added dropwise, with swirling, until solution was complete. To this warm solution was added 10.0 ml of 95% ethanol.

B. Instrumentation

The melting points were taken on a Thomas-Hoover Unimelt capillary melting point apparatus, Arthur H. Thomas Co., Philadelphia, Pa., and are uncorrected.

Proton nuclear magnetic resonance spectra were recorded on both a Varian Model EM-360 and T-60 nuclear magnetic resonance spectrometers, Varian Associates, Inc., Walnut Creek, California, using tetramethylsilane as an internal standard.

Fourier transform proton nuclear magnetic resonance spectra were obtained on a Varian Associates Model XL-100 Fourier transform spectrometer operating at 100.06 MHz and equipped with a Nicolet Model TT-100 data system and a NT-440 frequency synthesizer. All $^{1}$H-nmr shifts are reported in parts per million ($\delta$) downfield from an internal tetramethylsilane reference. Typical instrument parameters were: pulse width, 8 μsec; pluse delay, 3 sec.; data table size, 8K; acquisition time, 4.546 sec.; sweep width, 900 Hz; and exponential line broadening, 0.10 Hz.

Infrared spectra were recorded on a Perkin-Elmer infrared spectrophotometer, Model 283, Perkin-Elmer Co., Norwalk, Connecticut. Liquid samples were recorded neat using two sodium chloride salt plates. Solid samples were recorded by preparing a KBr pellet at a concentration of 1 mg/300 mg KBr.

Mass spectra were obtained on a Hewlett-Packard mass spectrometer, Model 5941A, coupled with a model 5710 A gas chromatograph and a Model 5933A recording system for data collection, Hewlett-Packard, Avondale, Pennsylvania.

Elemental analyses were performed by Atlantic Microlab, Inc., Atlanta, Georgia.

C. Syntheses

1. Attempted Synthesis of O-(2-hydroxyethyl)-O'-ethyl-N-phenyl phosphoramidate (XX)

a. Synthesis of O,O'-di-(2,2,2-trichloroethyl)-N-phenyl phosphoramidate (XVIII). This procedure follows a method described by Klement and Knollmuller.[25] To a solution of distilled aniline (24.6 g, 0.26 mol) in 150 ml anhydrous diethyl ether in a 1000 ml reaction flask was added, dropwise with stirring, a solution of bis-(2,2,2-trichloroethyl) phosphoro chloridate (50.0 g, 0.13 mol) in 250 ml anhydrous diethyl ether. A white, cloudy precipitate formed during the addition. After 1.5 hours, the precipitate was filtered, washed several times with distilled water, and recrystallized from hot alcohol to yield 30.7 g (53.4%) of the reaction product melting at 138-140°.

NMR (DMSO-$d_6$): $\delta$ 8.55 (d, J = 10.2 Hz, 1), $\delta$ 7.15 (s,5), $\delta$ 4.70 (d, J = 7.2Hz, 4).

IR (KBr): 3430 (broad, w), 3160 (m), 2980 (w), 2900 (w), 1600 (m), 1500 (s), 1250 (s), 1120 (s), and 740 $cm^{-1}$ (s).

Elemental analysis of $C_{10}H_{10}Cl_6NO_3P$, M.W.: 435.905

Calculated: C 27.55, H 2.31, N 3.21

Found: C 27.62, H * , N 3.67 (*Machine malfunction)

b. Attempted Synthesis of O-(2,2,2-trichloroethyl)-O'-ethyl-N-phenyl phosphormamidate (XIX).

This procedure follows the method of Ogilvie, et al.[26] To a suspension of O,O-di-(2,2,2-trichloroethyl)-N-phenyl phosphoramidate (21.8 g, 0.05 mol) in 500 ml absolute ethanol in a 1000 ml reaction flask

was added, with stirring, cesium fluoride (75.9 g, 0.5 mol). During a period of 94 hours, at various intervals, a TLC in chloroform was performed. Evidence of a new component was visible after reaction was allowed to progress for this period of time. Reaction mixture was filtered and the filtrate evaporated to yield a waxy residue weighing 105.4 g. Dissolving this residue in chloroform, filtering the insoluble precipitate, and evaporating the filtrate yielded 42.2 g of a white, solid mass. The reaction product (21.1 g) was chromatographed on silica gel (70-230 mesh ASTM). Elution with first 100% chloroform and then increased to 3% methanol/chloroform gave the starting materials as the only isolated compounds.

c. Attempted Synthesis of O-(2,2,2-trichloroethyl)-O'-ethyl-N-phenyl phosphoramidate (XIX)

The procedure was the same as described above except the reaction was allowed to proceed for one week. Again the only compounds isolated were starting materials.

2. Synthesis of diphenyl formyl methyl phosphonate (XXI).

a. Attempted Synthesis of diphenyl formyl methyl phosphonate diethyl acetal (XXII).

This procedure follows the method described by Razumov and Moskva.[27] To a 50 ml reaction flask, equipped with a reflux condenser, thermometer, and nitrogen inlet stream, containing triphenyl phosphite (10.04 g, 0.032 mol) was added bromoacetaldehyde diethyl acetal (15% excess, 7.94 g, 0.038 mol). The resulting solution was heated at reflux for six hrs. Upon fractional distillation, the only compounds isolated were the starting materials.

b. Synthesis of diphenyl ethyl phosphite (XXIII).

This procedure follows the method described by Shakirova and Imaev.[28] Sodium ethoxide was prepared by adding clean sodium metal (2.39 g, 0.1 mol), portion wise, to absolute ethanol (69.11 g, 1.5 mol). The excess ethanol was removed under reduced pressure; and the sodium ethoxide was stored in a vacuum oven at 120° until used. To a 100 ml reaction flask, equipped with a reflux condenser, thermometer, and nitrogen inlet stream, containing triphenyl phosphite (31.03 g, 0.1 mol) and sodium ethoxide (0.68 g, 0.01 mole) was added absolute ethanol (4.61 g, 0.1 mol). The resulting solution was heated at reflux for one hour. Fractional distillation of the reaction mixture gave 17.54 g (33.4%) of the product boiling at 105-115 at 0.6 mm Hg. (lit. 150-155 at 4 mm Hg).[28]

NMR ($CDCl_3$) (Figure 1): δ 7.1 (m, 10), δ 4.2 (m, J = 6.0 Hz, 2), δ 1.3 (t, J = 6.0 Hz, 3)

IR (Neat) (Figure 7): 3040 (m), 2980 (m), 1580 (s), 1470 (s), 1200 (s), 1040 (s), 850 (s), and 760 $cm^{-1}$ (s).

c. Synthesis of diphenyl formyl methyl phosphonate diethyl acetal (XXII)

This procedure follows the method described by Razumov and Moskva.[27] To a 100 ml reaction flask, equipped with a reflux condenser, thermometer, and a nitrogen inlet stream, containing diphenyl ethyl phosphite (13.11 gm, 0.05 mol) was added bromoacetaldehyde diethyl acetal (15% excess, 11.33 gm, 0.058 mol). The resulting solution was heated at 185-195° for three hours. Let cool and stand sealed under nitrogen in the refrigerator overnight. Fractional distillation of the reaction mixture gave 8.59 g of product boiling at 170-190° at 0.10 mm Hg. A second fractional distillation

gave 2.30 g (13.1%) of the reaction product boiling at 197-199° at 0.15 mm Hg.

NMR ($CDCl_3$) (Figure 2): δ 7.2 (s, 10), δ 5.2-4.8 (m,1), δ 4.0-3.35 (m, 4) δ 2.8-2.25 (doublet of doublets, J = 6.0 and 18.0 Hz, 2), δ 1.2 (t, J = 6.0, 6).

IR (Neat) (Figure 8): 3065 (w), 2970 (m), 1590 (s), 1490 (s), 1190 (s), and 960 $cm^{-1}$ (s).

Elemental analysis of $C_{18}H_{23}O_5P$, M.W.: 350.335

Calculated: C 61.71, H 6.62

Found: C 61.64, H 6.64

d. Attempted synthesis of diphenyl formyl methyl phosphonate (XXI)

This procedure follows the method described by Razumov and Moskva.[27] To a 100 ml reaction flask, equipped with a reflux condenser, thermometer, and nitrogen inlet stream, containing diphenyl formyl methyl phosphonate diethyl acetal (3.86 g, 0.011 mol) was added 0.1 ml of an eight percent solution of hydrochloric acid along with a few crystals of hydroquinone. The resulting suspension was heated at 70-75° for five hours. Fractional distillation at 195-205° at 0.3 mm Hg gave 1.1 g of a solid material. Recrystallization from anhydrous diethyl ether gave 0.27 g (8.1%) of a solid compound melting at 79-81°.

60 MHz NMR ($CDCl_3$) (Figure 3): δ 7.26 (m, 11), δ 5.02-4.78 (doublet of doublets, J = 13.8 and 11.0 Hz, 1),δ 4.0-3.8 (q, J = 7.1 Hz, 2), δ 1.38-1.23 (t, J = 7.1 Hz, 3)

From the NMR spectrum it was determined that the only reaction product isolated was 0,0-diphenyl (2-ethoxyvinyl) phosphonate (XXV). Additional spectral data were gathered to confirm structure.

EI mass spectrum (Figure 11): Molecular ion peak occured at m/e 304.

IR (KBr) (Figure 9): 3050 (m), 2990 (m). 1610 (s), 1580 (s), 1470 (s), 1250 (s), 1220 (s), 1170 (s), 950 (s), 760 (s), 680 (s), and 640 $cm^{-1}$ (s).

Elemental Analysis of $C_{16}H_{17}O_4P$, M.W.: 304.285

Calculated: C 63.15, H 5.64

Found: C 63.14, H 5.65

e. Synthesis of diphenyl formyl methyl phosphonate (XXI)

The procedure is the same as in the preceeding attempted synthesis. To a 100 ml reaction flask containing diphenyl formyl methyl phosphonate diethyl acetal (9.42 g, 0.027 mol) was added 1.0 ml of an eight percent solution of hydrochloric acid along with a few crystals of hydroquinone. The resulting suspension was heated at 85-90° for four hours. Let reaction mixture stand sealed under nitrogen overnight. Attempts at fractional distillation failed to produce the desired reaction product, therefore, the 2,4-dinitrophenyl hydrazone was attempted.

1. 2,4-dinitrophenyl hydrazone: positive test for aldehyde

To a 50 ml reaction flask containing 1.0 g of the above reaction mixture in 20.0 ml of ethanol (95%) was added 15.0 ml of the 2,4-dinitrophenyl hydrazine test reagent. The resulting mixture was allowed to stand overnight. The fluffy, yellow precipitate was filtered, washed several times with water, and then recrystallized from ethanol (95%) to give 0.51 g of reaction product melting at 147-149°.

NMR (DMSO-$d_6$) (Figure 6): $\delta$ 11.75 (s,1), $\delta$ 8.95 (split s, 1), $\delta$ 8.6-7.8 (m, 3), $\delta$ 7.7-7.0 (m, 10), $\delta$ 3.9-3.25 (doublet of doublets, J = 6.0 and 24.0 Hz, 2).

IR (KBr) (Figure 10): 3450 (broad, w), 3285 (s), 3120 (w), 2960 (w), 1620 (s), 1575 (s), 1520 (s), 1480 (s), 1360 (s), 1320 (s), 1260 (s), and 960 $cm^{-1}$ (s).

Elemental analysis of $C_{20}H_{17}N_4O_7P$, M.W.: 456.345

Calculated: C 52.63, H 3.76, N 12.28

Found: C 52.66, H 3.76, N 12.29

# CHAPTER IV

## Spectral Data

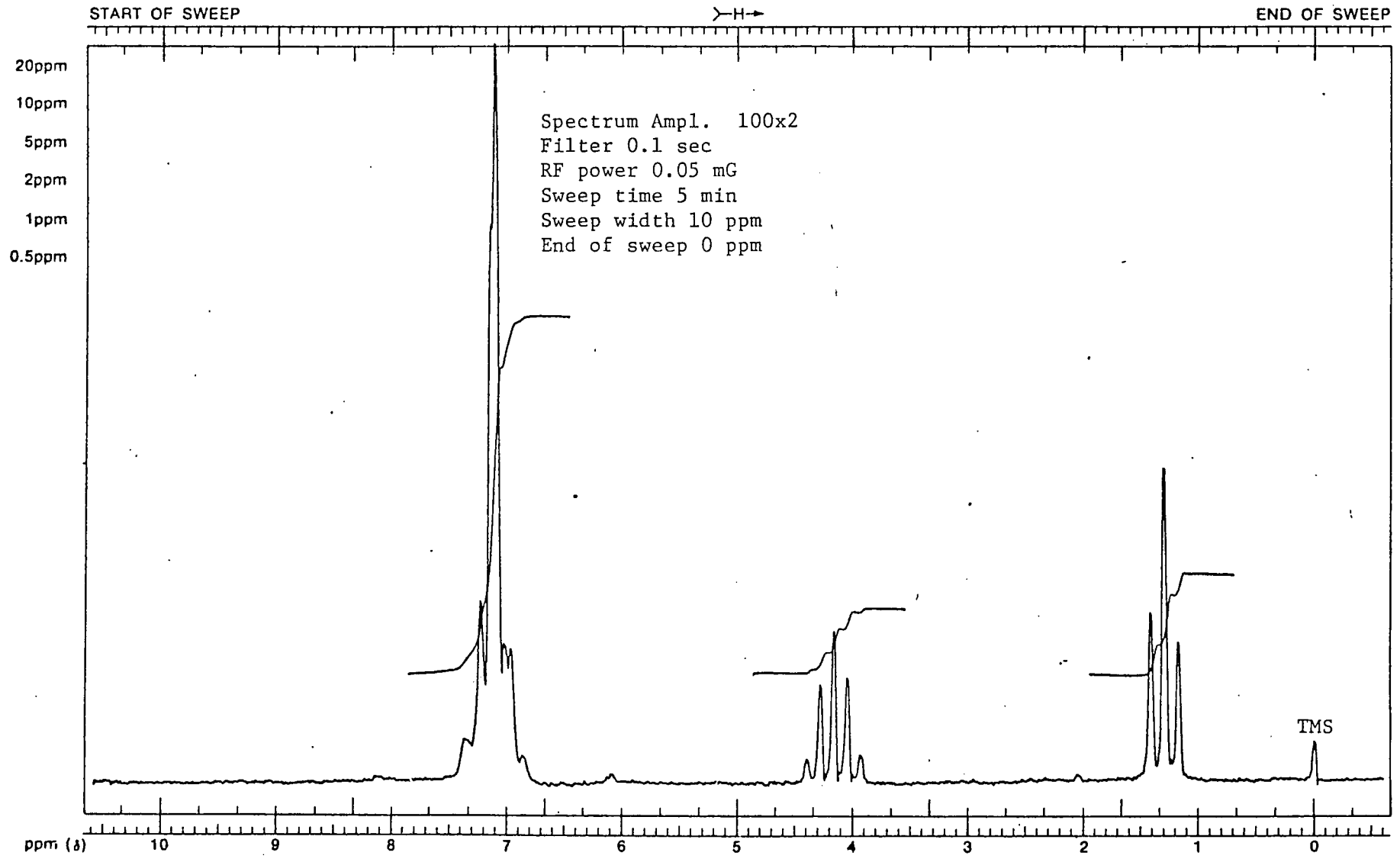



Figure 1. 60 MHz NMR spectrum of diphenyl ethyl phosphite (XXIII)

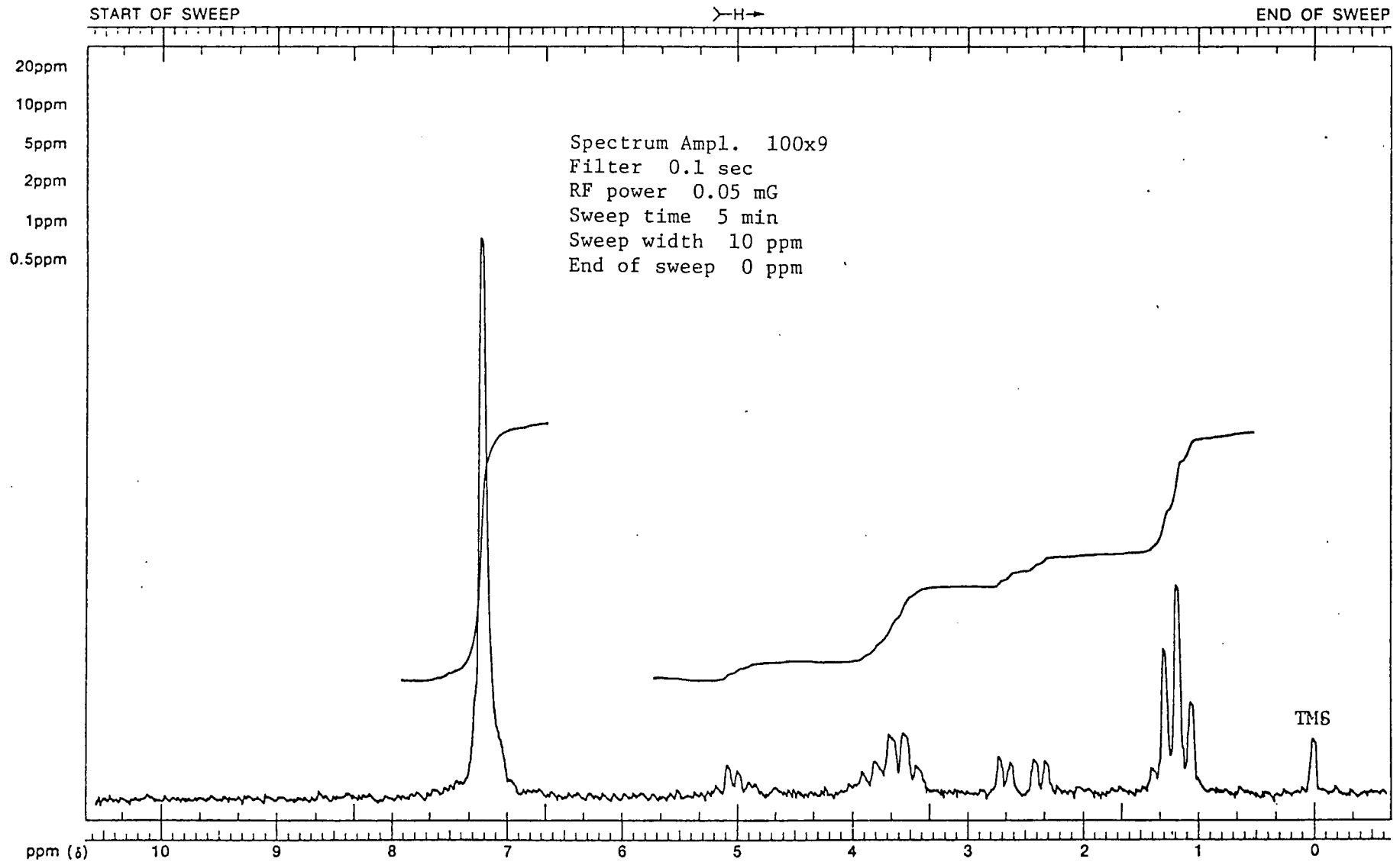



Figure 2. 60 MHz NMR spectrum of diphenyl formyl methyl phosphonate diethyl acetal (XXII)

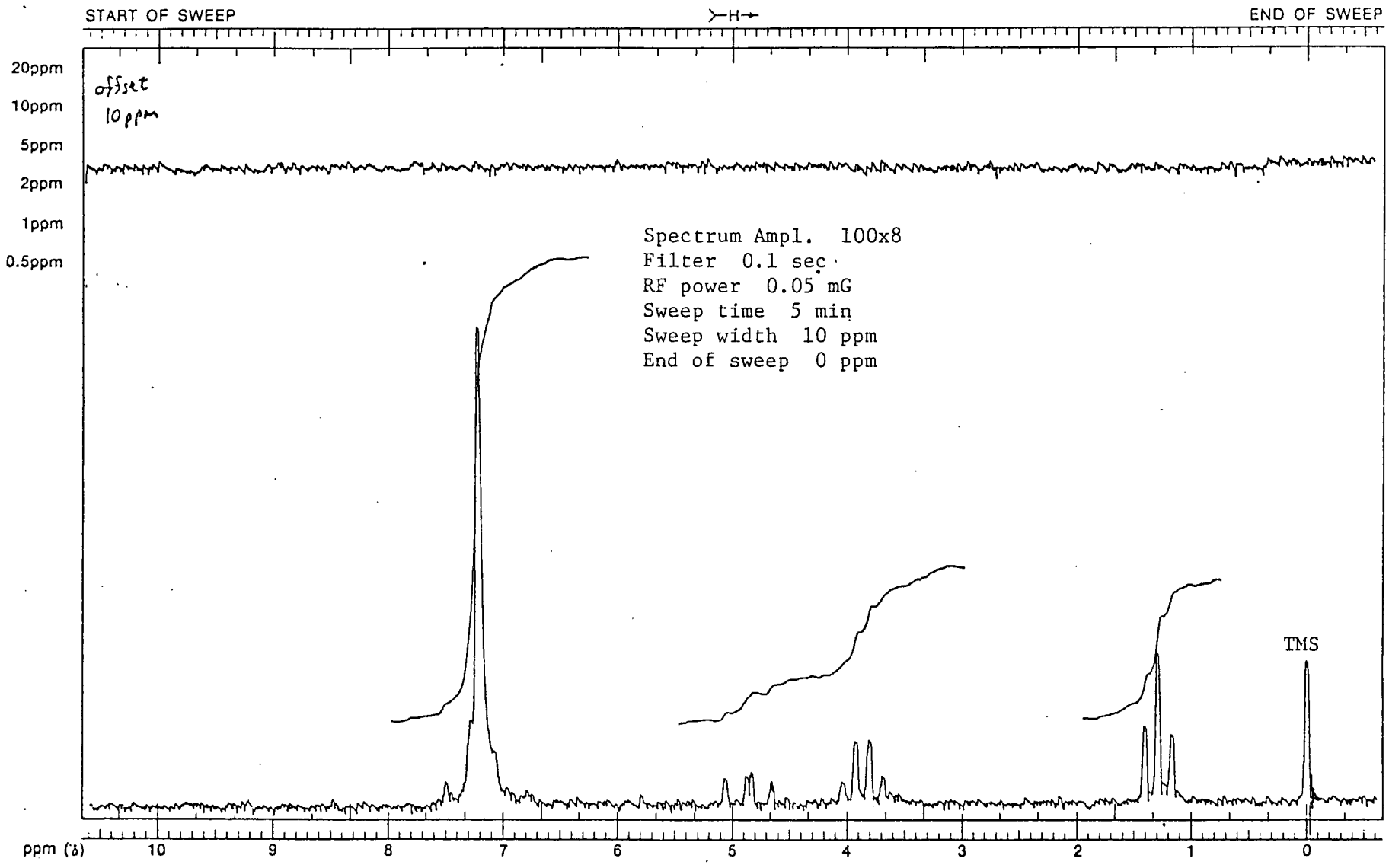



Figure 3. 60 MHz NMR spectrum of 0,0-diphenyl (2-ethoxy vinyl)phosphonate (XXIV)

100 MHz NMR spectrum of 0,0-diphenyl
(2-ethoxy vinyl)phosphonate (XXXI)

| | | | |
|---|---|---|---|
| EX = | 1PLS | AT = | 4.54656 SEC |
| P2 = | 8.00000 USEC | SW = | 900.900 |
| D5 = | 3.00000 SEC | OF = | 260.654 |
| ACQ = | 16 | SF = | 100.060 |
| SIZE = | 16384 | EM = | .100000 |

| LINE# | HEIGHT | FREQ(HZ) | PPM |
|---|---|---|---|
| 1 | 29.3275 | 746.315 | 7.45868 |
| 2 | 43.6174 | 735.963 | 7.35521 |
| 3 | 79.8342 | 734.413 | 7.33973 |
| 4 | 73.1890 | 733.544 | 7.33105 |
| 5 | 83.8931 | 732.855 | 7.32415 |
| 6 | 90.9503 | 731.828 | 7.31389 |
| 7 | 82.5122 | 731.331 | 7.30893 |
| 8 | 57.0108 | 729.588 | 7.29121 |
| 9 | 302.602 | 726.523 | 7.26087 |
| 10 | 288.861 | 726.085 | 7.25650 |
| 11 | 267.243 | 724.761 | 7.24326 |
| 12 | 108.269 | 722.328 | 7.21895 |
| 13 | 115.547 | 721.429 | 7.20996 |
| 14 | 68.7525 | 718.563 | 7.18132 |
| 15 | 67.7416 | 716.809 | 7.16379 |
| 16 | 44.3078 | 715.923 | 7.15503 |
| 17 | 49.9155 | 714.410 | 7.13982 |
| 18 | 50.2321 | 713.723 | 7.13295 |
| 19 | 40.2528 | 502.999 | 5.02698 |
| 20 | 41.7787 | 491.987 | 4.91692 |
| 21 | 38.1471 | 489.400 | 4.89106 |
| 22 | 36.6746 | 478.386 | 4.78100 |
| 23 | 27.0043 | 401.200 | 4.00959 |
| 24 | 81.3334 | 394.142 | 3.93906 |
| 25 | 84.3127 | 387.096 | 3.86864 |
| 26 | 27.7291 | 380.068 | 3.79840 |
| 27 | 103.588 | 137.662 | 1.37580 |
| 28 | 199.333 | 130.613 | 1.30535 |
| 29 | 91.1372 | 123.456 | 1.23382 |
| 30 | 47.5618 | 3.31946 | .033174 |
| 31 | 60.4364 | 2.23036 | .022290 |
| 32 | 91.282 | 1.29255 | .012917 |
| 33 | 859.233 | .000000 | .000000 |

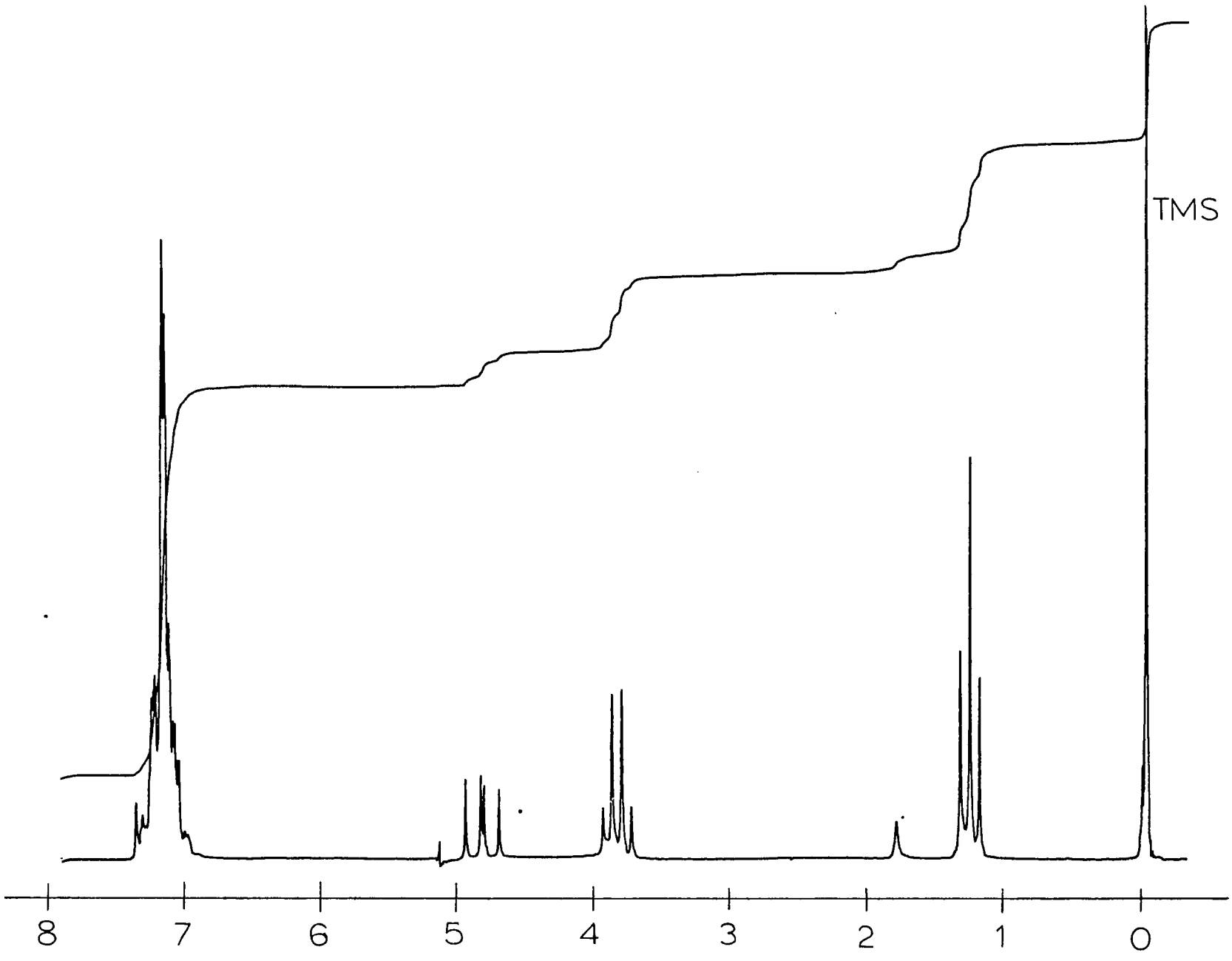



Figure 4. 100 MHz NMR spectrum of 0,0-diphenyl (2-ethoxy vinyl)phosphonate (XXIV)

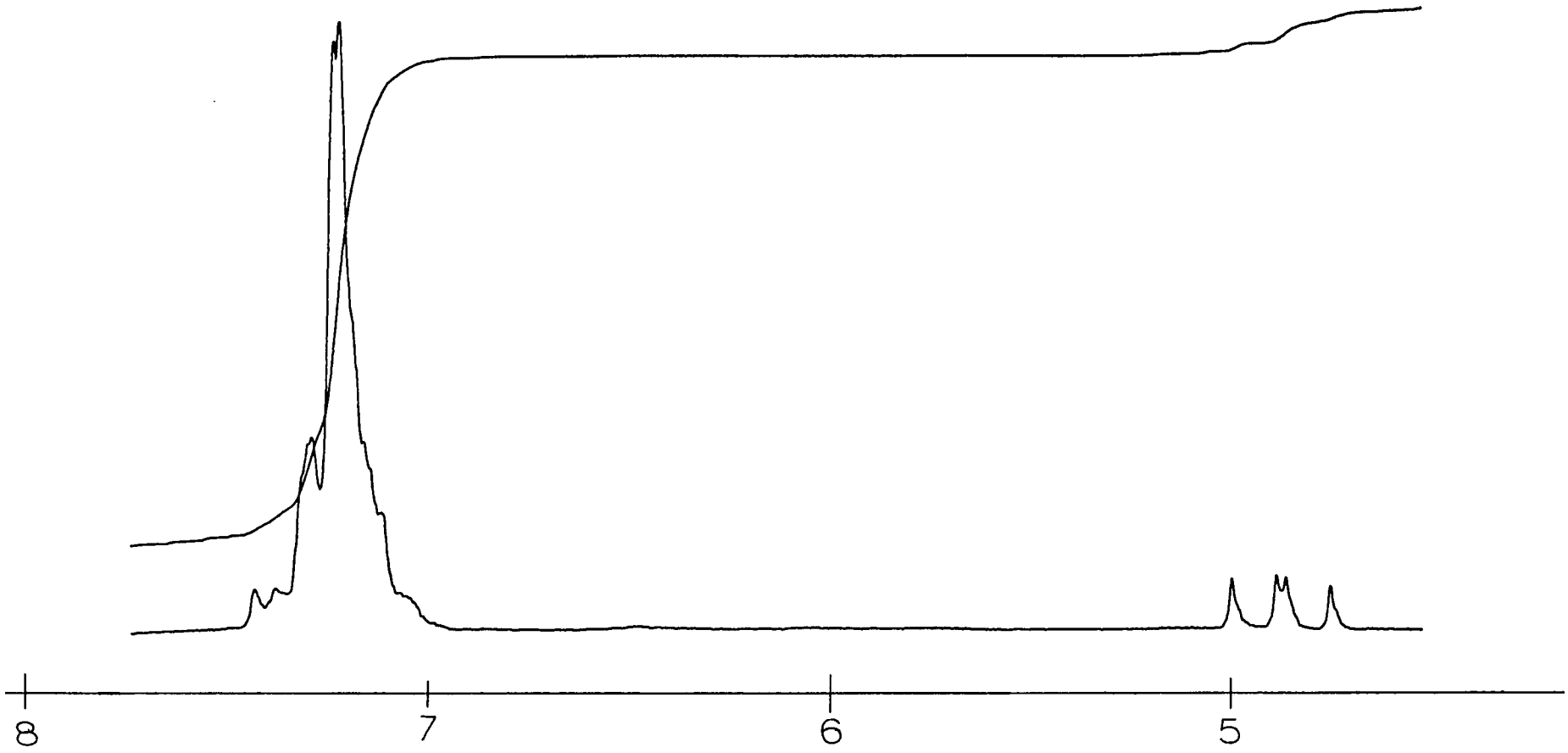



Figure 5. 100 MHz NMR spectrum of 0,0-diphenyl (2-ethoxy vinyl)phosphonate (XXIV) (expanded scale)

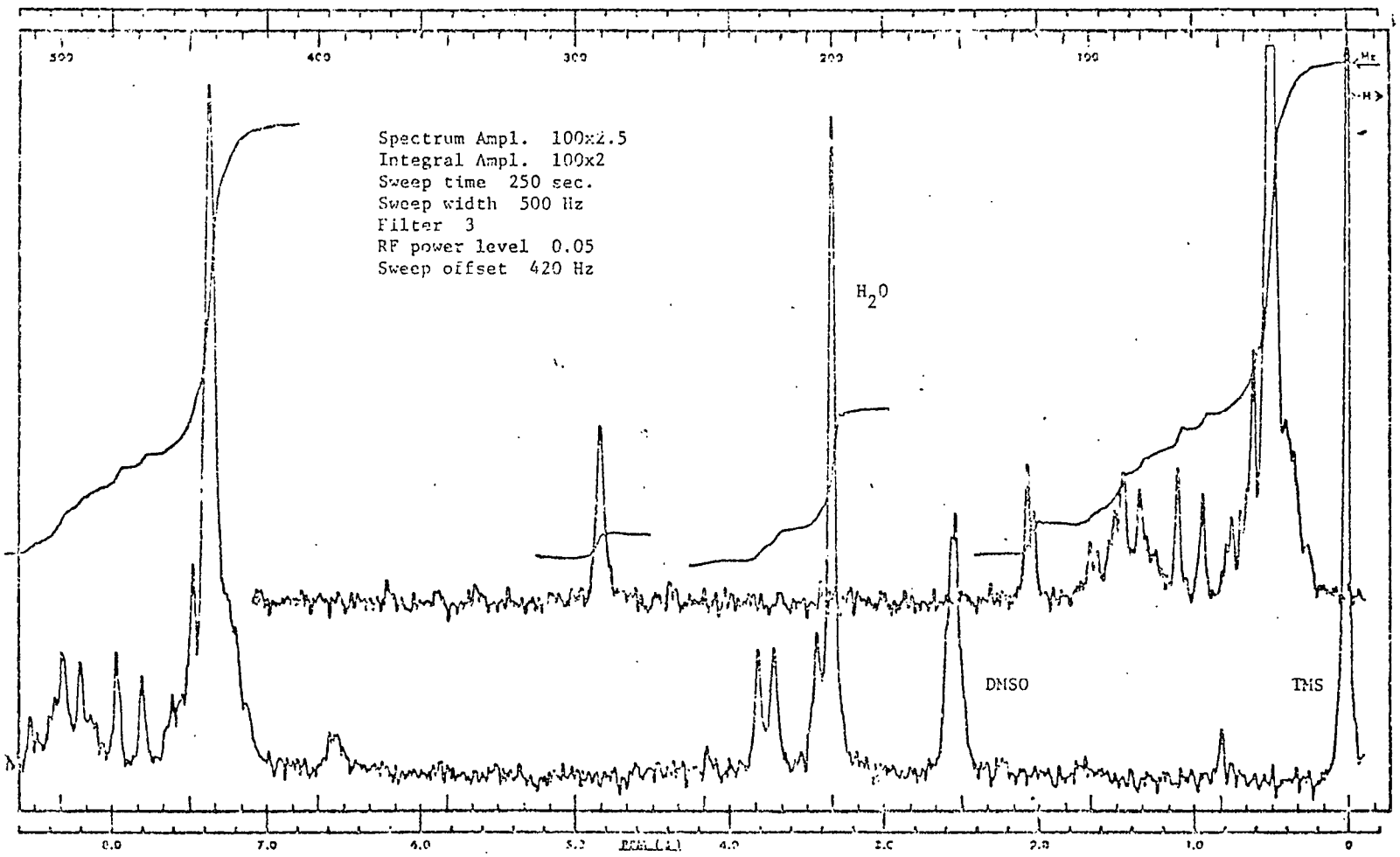



Figure 6. 60 MHz NMR spectrum of the 2,4-dinitro phenyl hydrazone of diphenyl formyl methyl phosphonate (XXV)

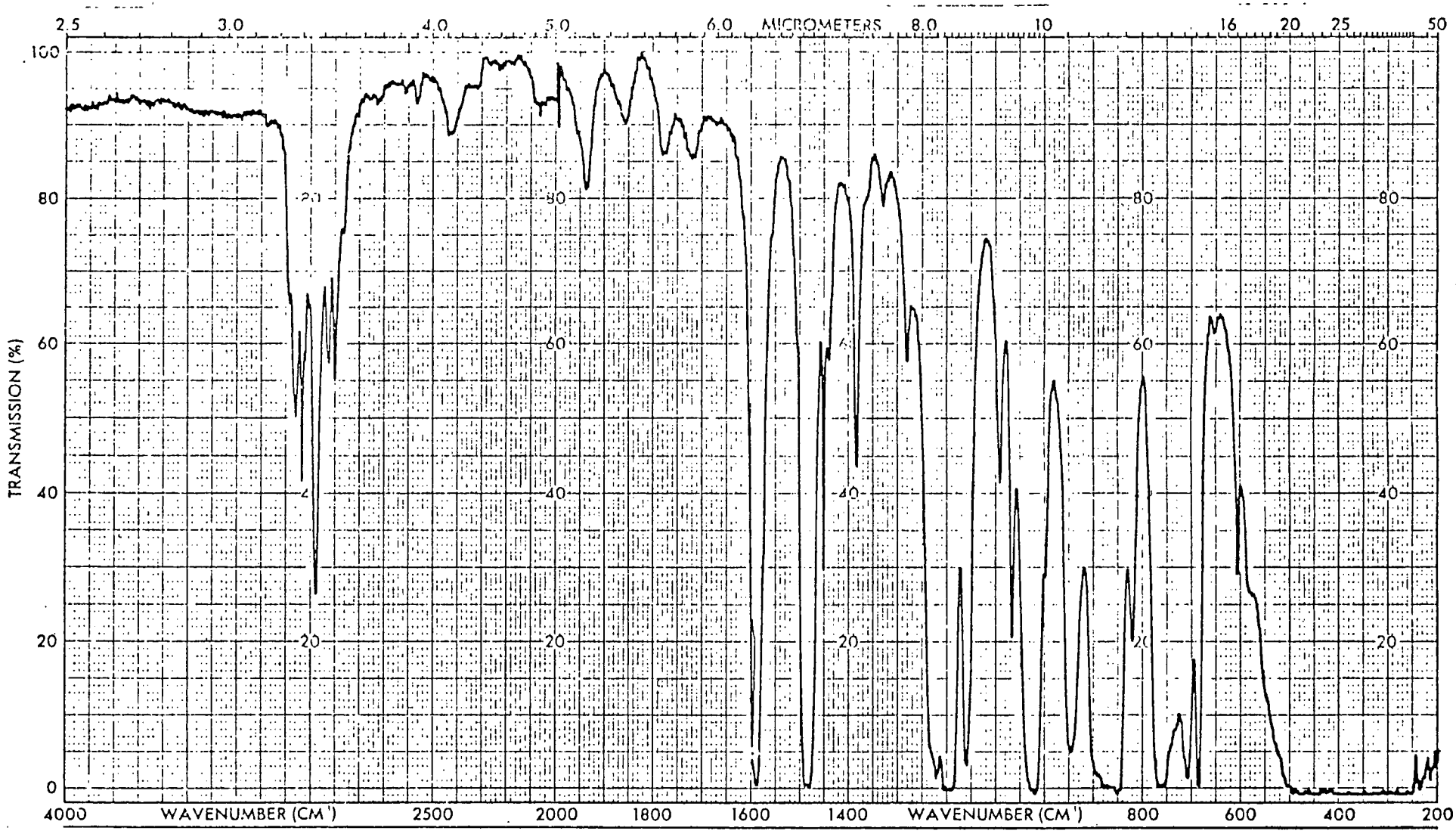



Figure 7. IR spectrum of diphenyl ethyl phosphite (XXIII)

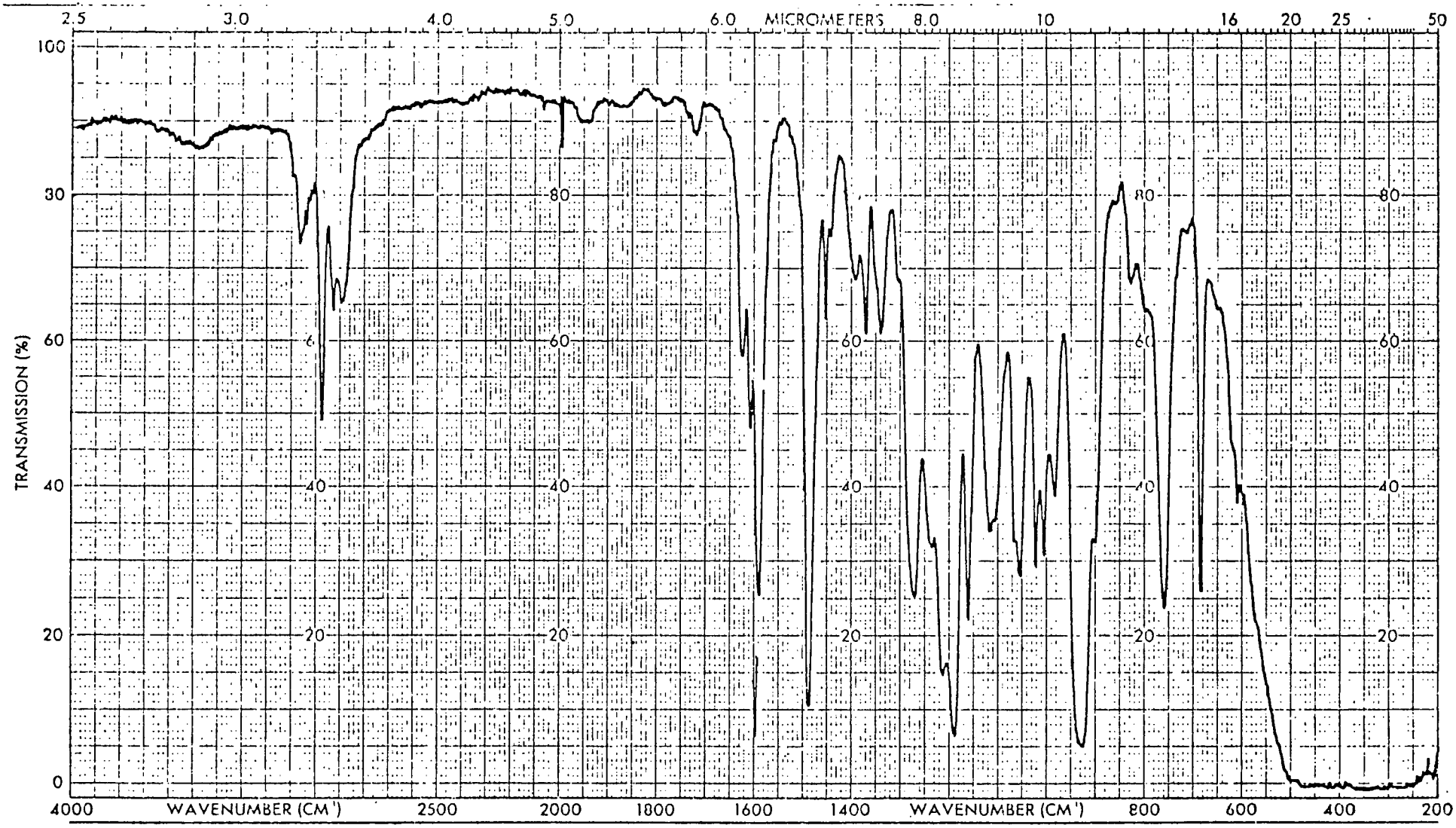



Figure 8. IR spectrum of diphenyl formyl methyl phosphonate diethyl acetal (XXII)

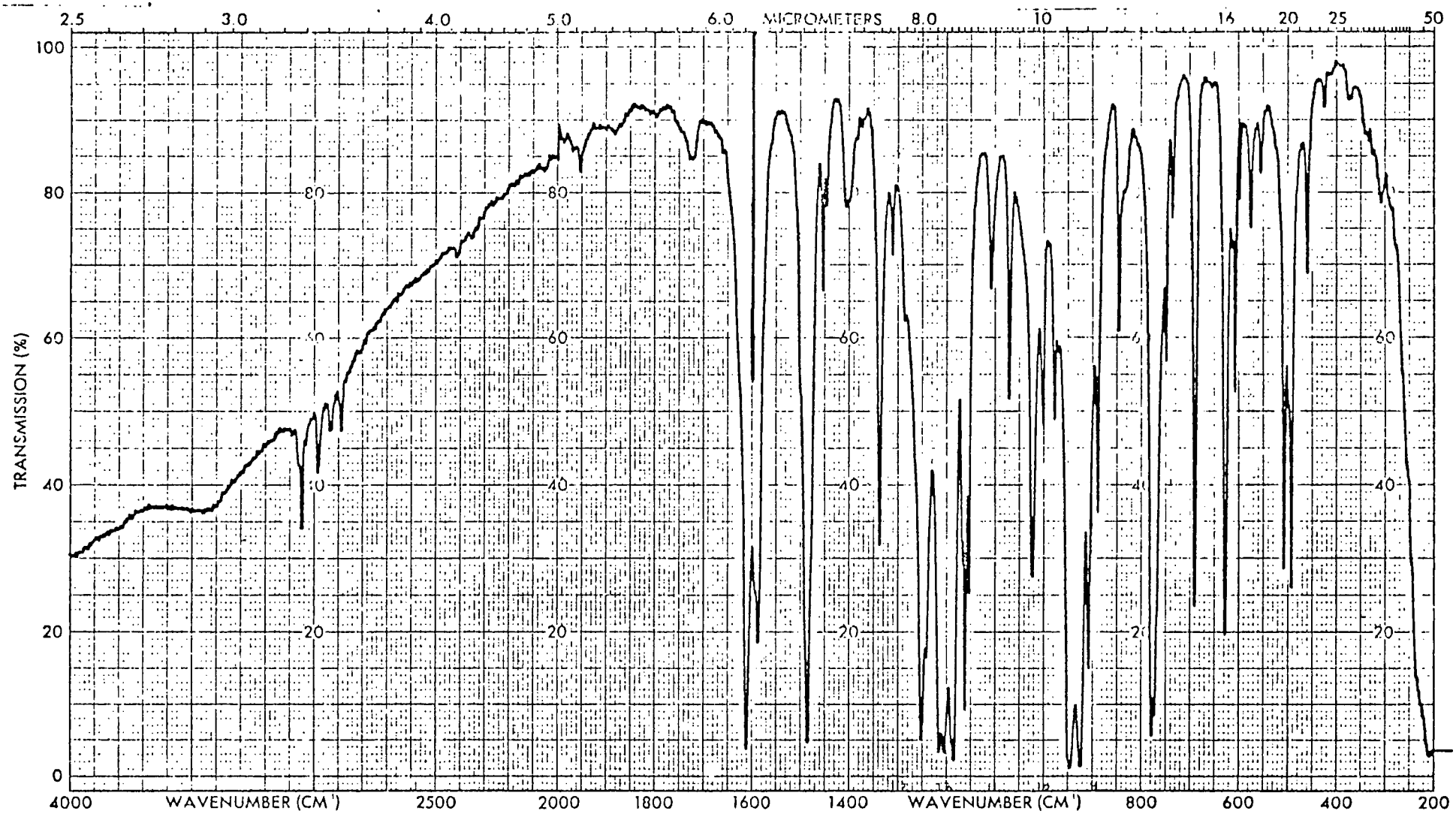



Figure 9. IR spectrum of 0,0-diphenyl (2-ethoxy vinyl)phosphonate (XXIV)

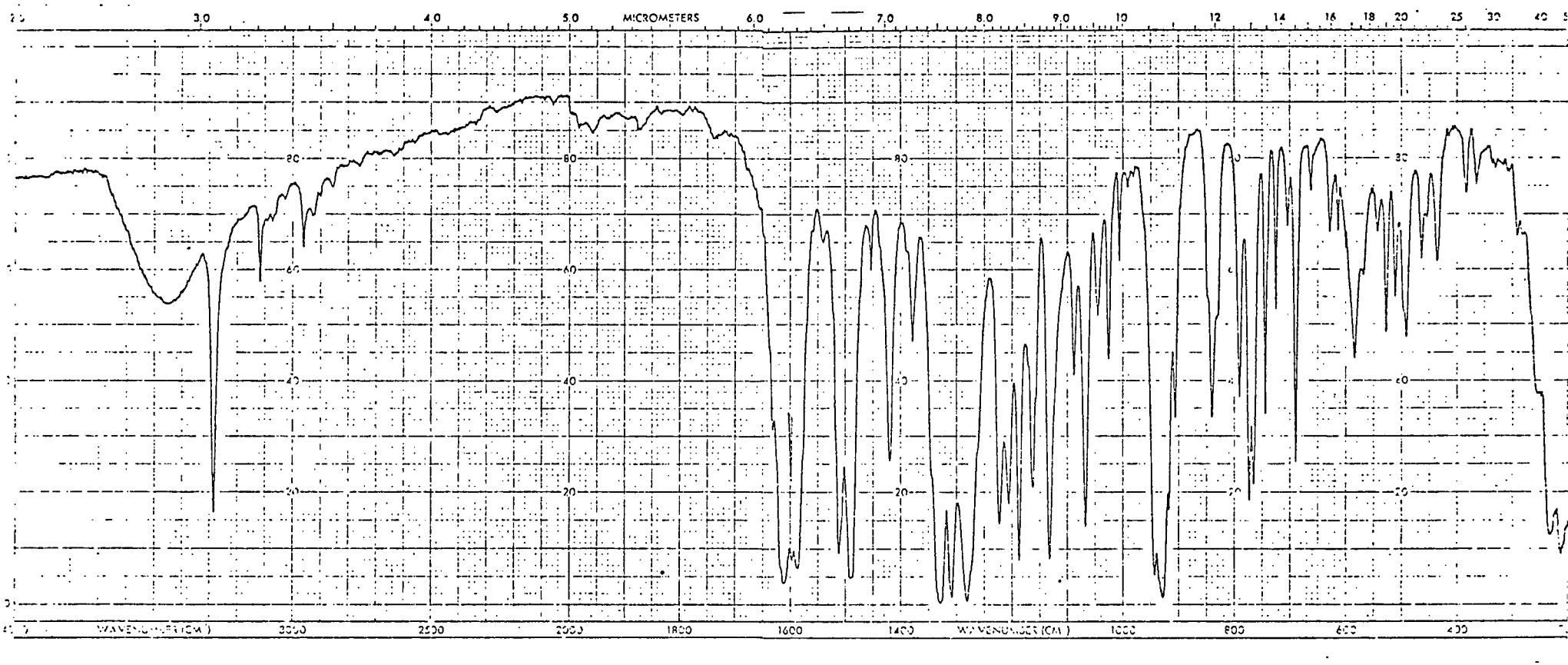



Figure 10. IR spectrum of the 2,4-dinitrophenyl hydrazone of diphenyl formyl methyl phosphonate (XXV)

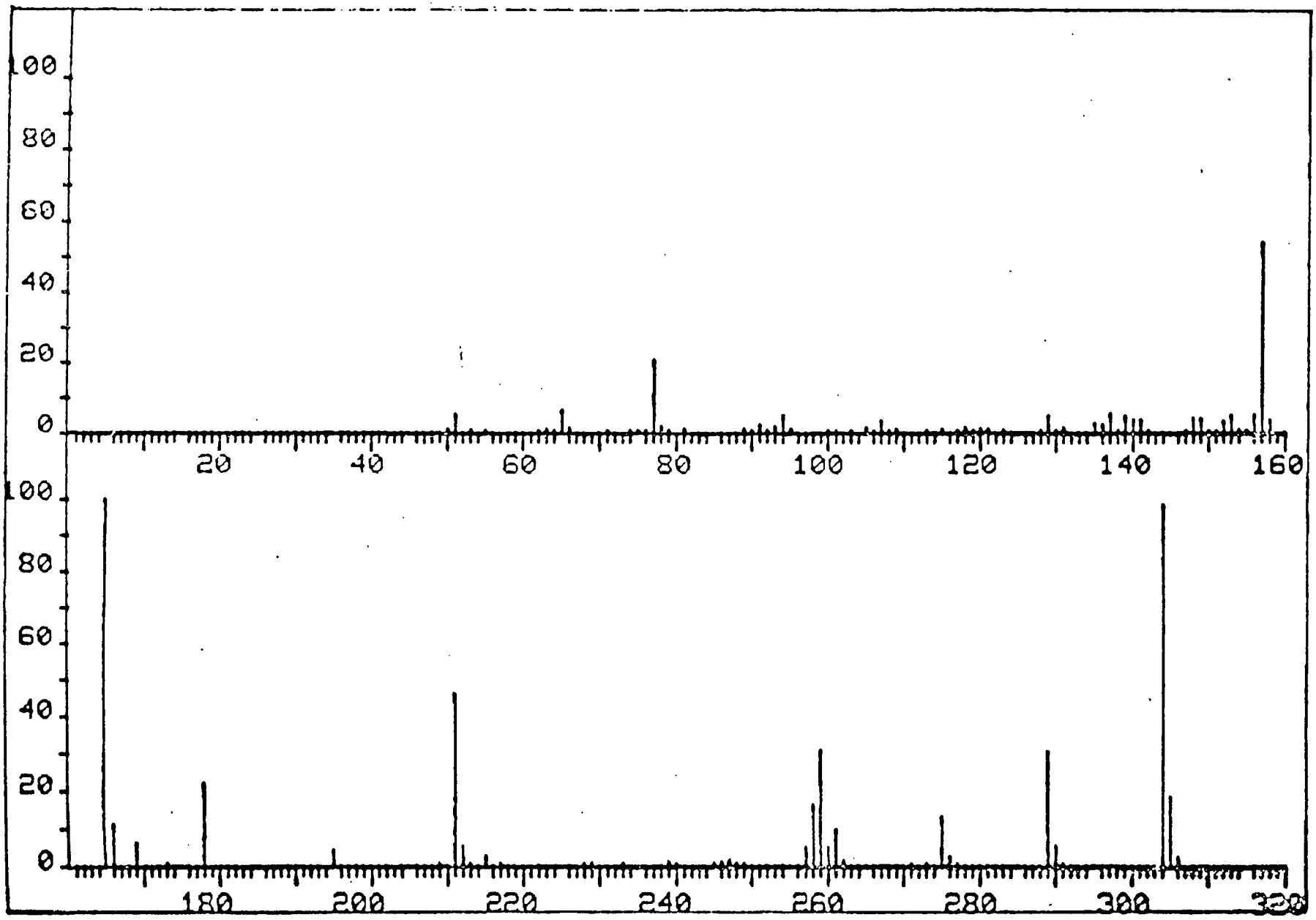



Figure 11. EI mass spectrum of 0,0-diphenyl (2-ethoxy vinyl)phosphonate (XXIV)

# CHAPTER V

# REFERENCES

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