INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

STRUCTURAL DETERMINATIONS BY ANALYTICAL ANALYSIS OF 7-PHOSPHANORBORNADIENE DERIVATIVES AND AMINO ACID ENANTIOMERS

A Thesis

Presented to

The Faculty of Graduate Studies

of

Lakehead University

by

STEPHEN W. FRATPIETRO

In partial fulfilment of requirements

for the degree of

Master of Science

September 3, 2000

ABSTRACT

STRUCTURAL DETERMINATION BY ANALYTICAL ANALYSIS OF 7-PHOSPHANORBORNADIENE DERIVATIVES AND AMINO ACID ENANTIOMERS

Stephen Fratpietro
Lakehead University

Supervisors:
Dr.C. Gottardo/ Dr.A.N. Hughes

The purpose of Chapter 1 is to create a bicyclic, two-coordinate phosphorus derivative in the form of a 2,3-benzo-1,4,5,6-tetraphenyl-7-phosphanorbornadiene anion and measure its experimental ^{31}P NMR chemical shift. This value is compared to a theoretical NMR shift of δ +1085 ppm calculated by D.B. Chesnut at Duke University using *ab initio quantum mechanics*. This is the largest downfield chemical shift ever predicted for an organophosphorus compound. Diphenylacetylene was reacted with solid Li to produce 1,4-dilithio-1,2,3,4-tetraphenylbutadiene (LTPB). Then, LTPB was reacted with dichlorophenylphosphine to produce pentaphenyl-phosphole, which was oxidized with 30% hydrogen peroxide to pentaphenylphosphole oxide. Next, this oxide was reacted with benzyne to synthesize 2,3-benzo-1,4,5,6,7- pentaphenyl-7-phosphabicyclo[2.2.1]hept-5-ene oxide which has a ^{31}P NMR chemical shift of δ + 96 ppm. Reduction of this product using trichlorosilane afforded products with ^{31}P NMR chemical shifts of δ +57 and +58 ppm which could possibly be the two isomers of 2,3-benzo-1,4,5,6,7-pentaphenyl-7- phosphabicyclo[2.2.1]hept-5-ene. Further reduction of these crude products was attempted using elemental sodium but with no success. Because of a time restraint and the difficulty in obtaining a stable 7-phosphanorbornadiene, the bicyclic, two- coordinate anionic phosphorus derivative could not be synthesized and proven to have a ^{31}P NMR chemical shift of δ +1085 ppm.

Amino acid racemization occurs according to the environment to which the amino acids are exposed. Racemization occurs when laevorotary-forms of amino acids are converted to dextrorotary-forms of amino acids by exposure to weak acids or bases, over time. This conversion in ancient samples was found to take place at the same rate as degradation of DNA. It was found [1] that if the D/L ratio of aspartic acid was lower than 0.08 in a bone or tissue sample, viable DNA could be extracted from it. In Chapter 2 this research attempted to derivatize enantiomeric amino acids with L-Marfey's Reagent to produce diastereomers that could be separated using capillary electrophoresis, gas chromatography, and high pressure liquid chromatography. Problems with the equipment, contamination, unrepeatable results and a variety of unknown factors plagued this portion of the research. This mode of testing for the presence of viable DNA was deemed a non-viable process.

ACKNOWLEDGEMENTS

I would like to thank a number of people that came to my aid in many aspects of this research. Thanks goes out to Dr. Christine Gottardo and Dr. A.N. Hughes for allowing me to work on the phosphorus project and Dr. El Molto, Dr. Ryan Parr and Christine, once again, for the idea of the amino acid project. Christine was a tremendous help when many problems arose in both aspects. Thanks to Dave Corbett and Ainsley Bharath for acquiring equipment and chemicals needed for this research. Thanks to Bert Harding in Chem. Stores for all of the exercise he allowed me to have as I spent countless hours going up and down stairs and wandering the halls of the campus looking for him. Thanks to Keith Pringnitz for the valuable NMR instument training and troubleshooting as well as running C:H:N analysis for this research. Thanks to Ain Raitsakas for running GC/MS samples for me as well as Al MacKenzie for his expertise and help in allowing me access to the equipment used. Thanks to George (Jerzy) Arct for running UV spectra of the derivatizing agents used for the amino acid project. Thanks to Dr. Greg Spivak for ideas involving Titanocene derivatives that would cleave off oxygen ligands from phosphorus atoms. Thanks greatly to Eleanor Jensen for running the HPLC (the working one) and gathering the last bits of information for this research. As well, thanks to Dr. Jeff Banks and Dr. Robert Omeljaniuk who valiantly fought with their own HPLC systems to get them operational but failed miserably. Finally, I appreciated everyone who visited me in the dungeon while I was doing my time.

TABLES

ABBREVIATIONS

aDNA	ancient deoxyribonucleic acid	PPPO	pentaphenylphosphole-oxide
Ala	Alanine	psi	pounds per square inch
Asn	Asparagine	δ	chemical shift
BSTFA	bis(trimethylsilyl)trifluoroacetamide	Thr	Threonine
CD	cyclodextrin		
CE	capillary electrophoresis		
CHDA	cyclohexyldiethylamine		
D-/L-Asp	dextrorotary-/ laevorotary-Asparagine		
DMAP	(4-dimethylamino)pyridine		
DNA	deoxyribonucleic acid		
equiv	equivalents		
GC	gas chromatography		
GC/MS	gas chromatography/ mass spectrometry		
HPLC	high pressure liquid chromatography		
Leu	Leucine		
mol.wt.	molecular weight		
NMR	nuclear magnetic resonance		
Phe	Phenylalanine		
7-PNBD	7-phosphanorbornadiene		
7-PNBDO	7-phosphanorbornadiene-oxide		
PPP	pentaphenylphosphole		

TABLE OF CONTENTS

THESIS OVERVIEW:

All compounds are molecules that are unique in their internal structure. Variations in bond length, bond angle, ligands and stability of the molecule play an important role in how the structure of the compound can be analyzed. Chemical structures are studied through various techniques based on specific properties of the molecule.

One analytical technique may be more suited to analyzing a sample than another. In the following thesis, ^{31}P NMR spectroscopy will be used to monitor the transformation of a simple phosphorus molecule into a complex structure based solely on the orientation and types of ligands attached to the phosphorus atom. Various arrangements of ligands around the phosphorus atom will produce different chemical shifts which help identify each molecule that incorporates phosphorus atoms.

Capillary electrophoresis is another technique that will be used to differentiate between amino acid enantiomers based on mirror-image orientations about a chiral centre. Because it is difficult to distinguish between amino acid enantiomers due to their similar properties, each enantiomer will be derivatized with a chiral molecule {Nα-(2,4-dinitro-5- fluorophenyl)-L-alaninamide} that will produce diastereomers, providing two molecules with different properties. These molecules will then be separated using capillary electrophoresis. The use of NMR spectroscopy may distinguish between amino acid enantiomers, when run in the presence of a chiral shift reagent, however, it does not provide an effective analysis. Electrophoresis may work on phosphorus compounds but most comparative data exists in the form of ^{31}P NMR chemical shifts. Every molecule has specific properties that one can manipulate in order to acquire analytical data. This research focuses on two specific examples.

CHAPTER 1:

^{31}P NMR STUDIES OF 7-PHOSPHANORBORNADIENE ANIONS

INTRODUCTION:

Every molecule is characterized by the number, nature, and bonding of atoms incorporated into its structure. Various techniques can be used to acquire important data relating to a specific molecule. Among the most common techniques is the use of Nuclear Magnetic Resonance (NMR) spectroscopy by which chemical shifts are measured relative to a specific reference compound. Each nucleus in a molecule will have a distinct chemical shift depending on the characteristics of the nucleus being measured (^{1}H, ^{13}C, ^{31}P) and onany additional interactions with other nuclei that affect its orientation. Each chemical shift is measured relative to a reference shift, so as to assign it a numerical value. Similar structures will have approximately similar chemical shifts within a specific range, so one is able to predict where a specific chemical shift may appear. By performing NMR spectroscopy on the compounds in a sequence of reactions, it is possible to observe the success of each reaction by analyzing each product on the NMR spectrometer and observing changes in the various chemical shifts. These shifts should be compared to literature values and may vary depending on the presence of electron donating or electron withdrawing groups present. The following research utilizes ^{31}P NMR spectroscopy for the majority of the data obtained because molecules that contain phosphorus atoms display distinct trends which allow their ^{31}P chemical shifts to be predicted.

Phosphorus atoms have the ability to have one of six possible coordination states. Phosphorus compounds that have a coordination number of one or two are not as abundant as phosphorus-containing molecules with coordination states of three to five. Nevertheless, with respect to ^{31}P NMR spectral data, a trend exists in the number and

nature of ligands attached to a phosphorus atom. The higher the coordination number of the phosphorus atom, the further upfield its ^{31}P NMR chemical shift will be found. The lower its coordination number, the further downfield its ^{31}P NMR chemical shift will be found. Using 85% phosphoric acid as a reference (δ 0 ppm), any ^{31}P peak found at a higher field strength to this reference is considered positive and downfield, while ^{31}P peaks found at a lower field strength to the reference are considered negative and upfield.

Compounds containing phosphorus with a coordination of one are few in number. The ^{31}P NMR chemical shifts of these phosphaalkynes may be affected by the electro-negativity of the R group (R-C≡P) [2]. The phosphorus atom, itself, uses a hybrid orbital with increasing p-character for the σ bond to carbon. The unshared lone pair of electrons occupies an orbital with increasing s-character [2]. This increase in the shielding of a phosphorus nucleus produces a ^{31}P NMR shift at a higher field strength relative to the shift of a two-coordinate phosphorus compound. One-coordinate phosphorus species span across a range of δ +96 ppm to δ -207 ppm [2].

Two coordinate phosphorus species have ^{31}P NMR chemical shifts which span across the spectrum, although no theory correctly explains this large diversity. Cyclic two-coordinate phosphorus species do exist as pure anions (Figure 1-1).

Figure 1-1. Anionic forms of cyclic two-coordinate phosphorus species [2].

In a related trend, changing the oxidation state of phosphorus also affects its ^{31}P NMR

chemical shift. A three-coordinate phosphorus atom in a +3 oxidation state normally uses p-orbitals for binding to the ligands. A strong screening of the phosphorus nucleus occurs as the high s-character of the phosphorus lone pair of electrons [2] is diminished. This causes the ^{31}P signal to shift downfield (Figure 1-2).

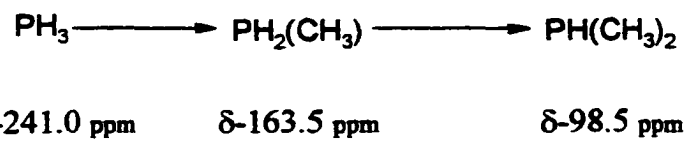


Figure 1-2. Decreasing s-character of the phosphorus lone pair leads to downfield shifts [2].

A three coordinate phosphorus atom in a +5 oxidation state usually involves an sp^2 hybridized framework [2]. These $p\pi$-$p\pi$ stabilized compounds are influenced in shift by the double bonds present (Figure 1-3) [2]. Complex A in its +3 state has a ^{31}P NMR chemical shift of δ -237.4 ppm [2] while complex B in a +5 state has a ^{31}P NMR chemical shift found more downfield at δ +174.0 ppm [2].

Figure 1-3. Comparison of phosphorus in a P(III) +3 state and P(III) +5 state [2].

In a ring system, ^{31}P NMR chemical shifts depend on ring size [2,3] and angle increments [3] produced from the presence of double bonds and the nature of ligands attached (Figure 1-4).

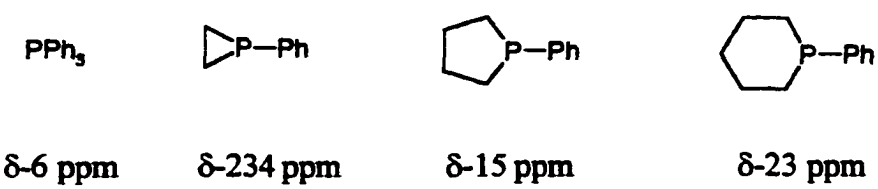
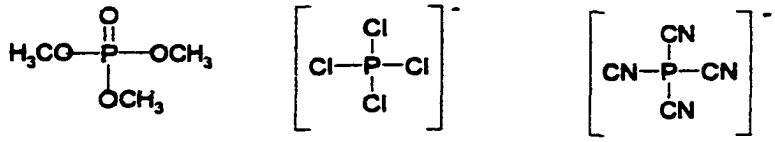


Figure 1-4. Comparison of three-coordinate phosphorus species in ring systems [4].

As the phosphorus atom coordination number increases to four, it uses sp^3 hybridized orbitals for sigma-bonding and includes d-orbital participation for only those ligands considered to be electronegative [2]. Therefore, a 4-coordinate phosphorus can exist as a 3-coordinate phosphorus atom with the lone pair of electrons in use or as a 5-coordinate phosphorus atom holding a negative charge (Figure 1-5).



δ 0 ppm δ+191.8 ppm δ-243.4 ppm

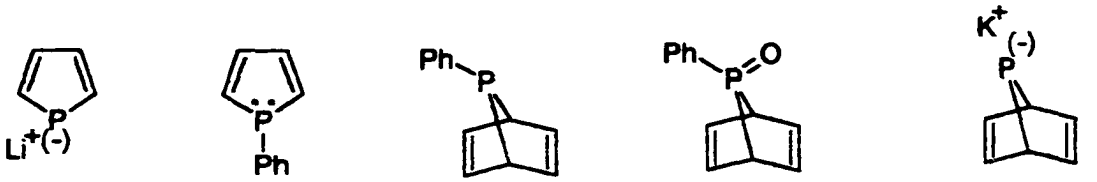
Figure 1-5. Examples of two types of P(IV) species and their ^{31}P NMR chemical shifts [2].

However, five and six coordinate phosphorus atoms in the +5 oxidation state use the d orbitals for their sigma-bond system [2]. The ^{31}P NMR chemical shifts of 5-coordinate phosphorus compounds are affected by the electronegativity of their ligands and steric changes resulting from the distortion of the pentagonal-bipyramidal structure [2]. Shifts are usually found in the range of δ -20 to δ -300 ppm [2]. Phosphorus compounds with a coordination number of 6 have ^{31}P NMR chemical shifts that span a 320 ppm range [2].

Other factors that can influence a ^{31}P NMR chemical shift to a smaller extent are incorporation of phosphorus into a ring, incorporation of phosphorus into a system as a ring bridge, and the nature of the ligands attached to the phosphorus atom. Because of the considerable strain involved in phosphorus-bridged and bicyclic systems, ^{31}P shifts for these types of compounds are usually found at unusually low fields while bulky substituents attached to a phosphorus atom cause upfield shifts. Electron withdrawing groups give rise to a shift downfield and electron donating groups produce an upfield shift.

Therefore, by increasing the electronegativity of atoms attached to the ^{31}P atom, the tendency is increased for a large downfield shift to be observed in the NMR spectrum.

To illustrate the effect of all of the above mentioned factors that can influence ^{31}P NMR chemical shifts, consider the following unsaturated structures (Figure 1-6). A



δ +75 ppm [2] δ +7.8 ppm δ +60-80 ppm (predicted) δ +90-105 ppm [5] δ +1085 ppm [1] (predicted)

Figure 1-6. Phosphorus molecules and the effect of stereochemistry on ^{31}P NMR chemical shift.

phosphole anion is a cyclic, two-coordinate species with a ^{31}P NMR chemical shift of δ +75 ppm. Increasing the coordination of this cyclic species to the three-coordinate 1-phenylphosphole produces a ^{31}P NMR chemical shift of δ +7.8 ppm. If this cyclic, 3-coordinate ^{31}P system becomes a bridged species, a bicyclic compound, the lone pair of electrons will interact with the C=C double bonds, if they are present, increasing the deshielding effect [1] causing the NMR peak to shift downfield to between δ +60 and δ +80 ppm. Upon the attachment of an electronegative group (oxygen) to the phosphorus atom, a ^{31}P NMR chemical shift is expected around δ +80 to δ +90 ppm. Upon cleavage of both the oxygen and phenyl group, a bicyclic, two-coordinate, bridging, anionic phosphorus would remain; this compound has a predicted ^{31}P NMR chemical shift of δ +1085 ppm [1].

The purpose of this research was to synthesize a derivative of a bicyclic, bridging, two-coordinate phosphorus compound and measure its ^{31}P NMR chemical shift. Studies by Chesnut [1] at Duke University in North Carolina predicted a ^{31}P NMR chemical shift

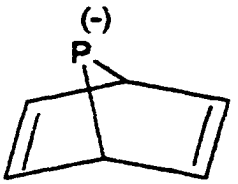


Figure 1-7. The proposed bicyclic, bridging, two-coordinate 7-phosphanorbornadiene compound.

of δ +1085 ppm (Figure 1-7). It was proposed that the steric nature of this bridged system brings the lone pairs of electrons on the bridging phosphorus atom into close contact with the adjacent ring atoms, and specifically the double bonds of the system [1]. This interaction reduces the HOMO- LUMO energy gap by causing π orbitals to lie at higher orbital energies and π* orbitals to lie at lower orbital energies [1]. This causes a large deshielding effect which contributes to the large chemical shift.

This shift is quite important in that it is the largest predicted ^{31}P NMR chemical shift for a conventional organophosphorus molecule [1]. This shift is significantly larger, almost double that of the largest measured ^{31}P NMR chemical shift of δ +668 ppm for the compound 2,4,6-tBuCHP=AsCH(trimethylsilyl) [1].

This prediction can be explained studying 7-phosphanorbornane (7-PNBane), -bornene (7-PNBene), and -bornadiene (7-PNBdiene) (Figure 1-8) [1].

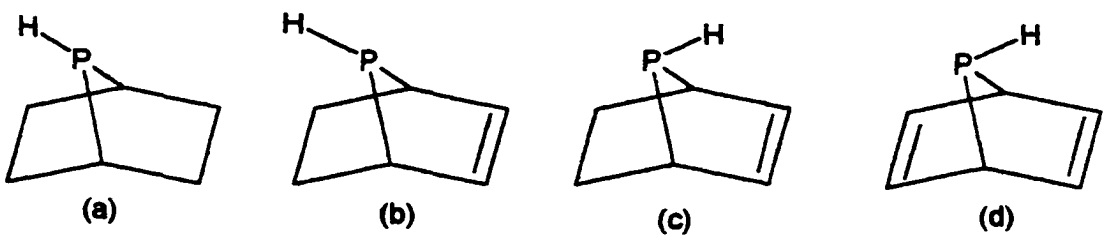


Figure 1-8. Structures for 7-PNBane (a), 7-PNBene (b and c), and 7-PNBdiene (d).

In (a), the lone pair of electrons occupies the highest occupied molecular orbital [1]. When a double bond is added to the system in an anti (b) configuration, the HOMO and

LUMO split into separate π and π^* components which raises the energy of the HOMO, thus decreasing the energy gap between the two orbitals [1].

The addition of another double bond effectively lowers the LUMO because of the presence of another set of π and π^* states [1]. The resultant HOMO-LUMO energy gap becomes reduced further, which enhances the interaction between these orbitals. By studying the neutral, cationic and anionic derivatives of this 7-phosphanorbornadiene structure, a large deshielding effect becomes evident. If species (d) were to be protonated to produce the cationic derivative, a complete removal of the deshielding effect occurs [1]. By deprotonating species (b) and (c) to the corresponding anion derivative, Hartree- Fock calculations predict an extremely large chemical shift relative to the previous two species. Theoretical results [1] also show that the anionic diene exhibits a significantly displaced ^{31}P NMR chemical shift from the anionic 7-PNBene and anionic 7-PNBane. However, the ^{31}P NMR chemical shift of this anionic 7-PNBdiene structure has yet to be determined experimentally. A large deshielding effect is predicted through ab initio Hartree-Fock calculations. These calculations are normally carried out with respect to a bare nucleus when an *absolute* displacement is theoretically determined [3]. This chemical shielding (σ) either produces shifts of positive values (diamagnetic, upfield) or shifts of negative values (paramagnetic, downfield) [3] depending on how the shifts appear relative to a standard shift. Normally, shifts are measured relative to some standard such as the H_3PO_4 mentioned previously. However, the use of absolute shifts eliminates systematic errors that may be hidden by a relative comparison [3]. Calculations of these experimentally unknown structures allow one to predict, confirm, and understand structures otherwise in doubt at a basic level [3]. *Ab initio* calculations of

large molecular systems give bond lengths and angles that are comparable to experimental results to within 0.01-0.03Å and 2-3 degrees, respectively [3]. However, for oxygen and nitrogen containing compounds, a significant difference is observed between theoretical calculations and experimental results of chemical shifts [3]. Hartree-Fock calculations have been used for the great majority of NMR shielding calculations.

If the calculation had not predicted this large chemical shift, a more reasonable ^{31}P NMR chemical shift would have been anticipated. By comparing a 3-coordinate heterocyclic diene and its corresponding norbornadiene (Figure 1-9), a noticeable difference is observed in the ^{31}P NMR chemical shifts. 1-Phenylphosphole has a ^{31}P

δ +7.8 ppm δ +60-80 ppm

Figure 1-9. Structures for a three-coordinate cyclic and bicyclic phosphorus compound.

NMR chemical shift of δ +7.8 ppm. Upon building a norbornadiene with this molecule to produce a bicyclic, bridging, 3-coordinate phosphorus compound (7-phosphanorbornadiene), a chemical shift is expected between δ +60 to δ +80 ppm. A two-coordinate heterocyclic diene in the form of a phosphole anion has a ^{31}P NMR chemical shift of

δ +75 ppm δ +1085 ppm

Figure 1-10. Anionic two-coordinate cyclic and bicyclic phosphorus species.

δ +75 ppm, further downfield of 1-phenylphosphole (Figure 1-10). When this anion is placed into the norbornadiene complex, it would seem to scale the ^{31}P NMR chemical shift to be between δ +600 to δ +800 ppm, but further deshielding of the phosphorus nucleus takes place due to the interaction of two lone pairs of electrons with both double bonds causing the further downfield shift. A reasonable predicted ^{31}P NMR chemical shift would agree with the one calculated by Chesnut [1].

This type of structure will be synthesized in the form of a 2,3-benzo-1,4,5,6-tetra-phenyl-7-phosphabicyclo[2.2.1]hept-5-ene anion (Figure 1-11). This form of the

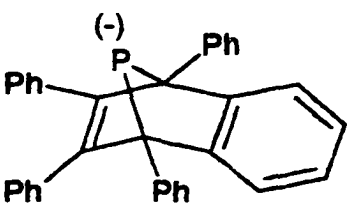


Figure 1-11. The 7-phosphanorbornadiene compound that will be synthesized and measured for its ^{31}P NMR chemical shift.

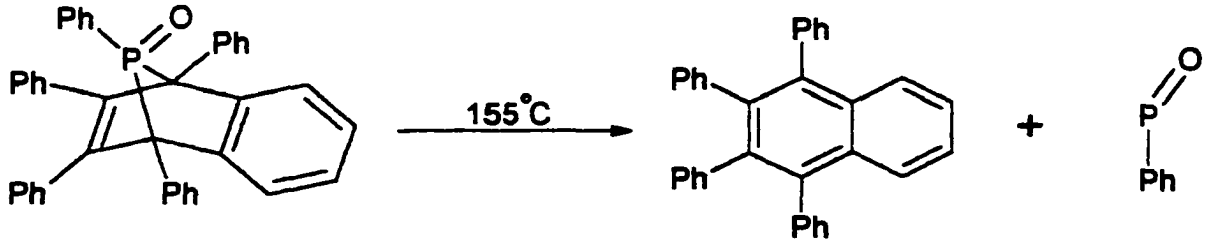
7-phosphanorbornadiene system is chosen since a viable route for its preparation is available.

The proposed synthesis of this compound involves six steps. In the first step a diphenylacetylene dimer will be formed using lithium ribbon under an oxygen-free environment [6,7]. This product mixture will be transferred directly into the next step where it reacts with dichlorophenylphosphine to produce a heterocycle in the form of pentaphenylphosphole. This heterocycle will become oxidized to form pentaphenyl-phosphole-oxide in the third step of the reaction scheme. The fourth reaction step involves the generation of benzyne which will be involved in a Diels-Alder reaction with pentaphenylphosphole-oxide [8]. This produces a 7-phosphanorbornadiene-oxide (7-PNBDO) compound. This product will, then, be reduced using trichlorosilane and

pyridine to yield a 7-phosphanorbornadiene (7-PNBD) derivative in the fifth step of this reaction scheme [9]. Finally, elemental potassium will be used to cleave off a phenyl ligand attached to the bridging phosphorus atom to produce a 7-phosphanorbornadiene anion derivative.

The fifth step of this proposed reaction scheme will involve the reduction of 7-phosphanorbornadiene-oxide to a 7-phosphanorbornadiene derivative. This reduction may impose some problems, so many routes were chosen to ensure the success of the oxygen cleavage. It is important to issue a note of caution concerning these 7-phospha-norbornadiene derivatives. The 7-phosphanorbornadiene-oxide (Scheme 1-1) is stable at room temperature but at elevated temperatures (155°C) we run the risk of debridging this compound. The bridged portion may cleave completely to leave 1,2,3,4-tetraphenyl-

Scheme 1-1. Debridging of 7-PNBdiene derivatives at high temperature.

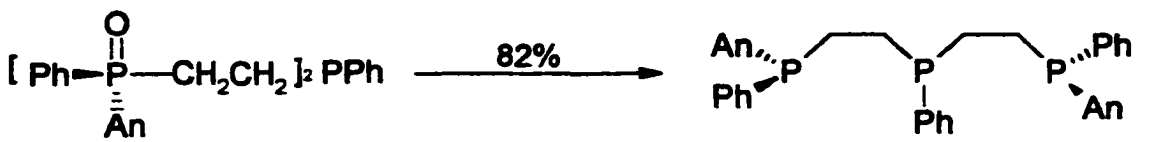


naphthalene and a polymer, poly(phenylphosphinidene-oxide) [10] to relieve the ring strain around the phosphorus atom. Debridging may occur at a lower temperature (<155°C) for the reduced 7-phosphanorbornadiene. Lower temperatures will be used in subsequent reactions to reduce the risk of debridging these species.

A procedure by Quin et al. [9] uses a mixture of trichlorosilane and pyridine in a benzene solvent to reduce various bridged, bicyclic phosphorus-oxide complexes. However, this reaction has not been performed on a 7-phosphanorbornadiene compound,

but only on 7- phosphanorbornene compounds. Hopefully this 7-phosphanorbornadiene-oxide can be reduced successfully without the collapse of the molecule.

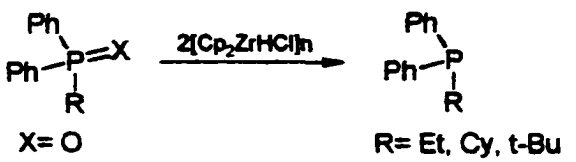
If Quin's method [9] is not successful there are a number of different methods available to reduce the oxide. Johnson and Imamoto [11] reported another procedure that allows a tetra-coordinated phosphine-oxide to be reduced to a phosphine in the



Scheme 1-2. Johnson and Imamoto [18] reduced a phosphine-oxide with $HSiCl_3$/ c- $C_6H_{11}NEt_2$ in acetonitrile.

presence of a $HSiCl_3$/ c- $C_6H_{11}NEt_2$ combination in acetonitrile (Scheme 1-2). Again, this procedure has not been applied to a bridged, bicyclic phosphorus molecule, so it may cause debridging of the 7-PNBDO molecule, or no reaction at all. Phenylsilane may also be substituted for $HSiCl_3$ as it may behave differently.

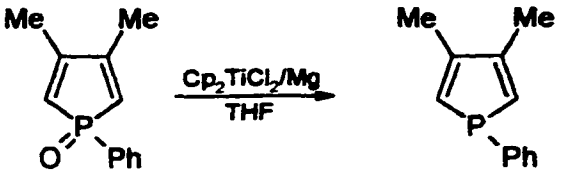
Zablocka *et al.* [12] report the use of the Schwartz reagent, Zirconocene Chloride Hydride (ZCH), in reducing functionalized tetracoordinated pentavalent phosphine-oxide



Scheme 1-3. Zablocka *et al.* [12] reduced a phosphine-oxide using the Schwartz reagent.

to phosphines (Scheme 1-3). Since our research involves a tetracoordinate phosphorus compound, this procedure may work successfully, although the yield is dramatically reduced with ZCH if bulky substituents are coordinated to the phosphorus atom.

Another possible metallocene used by Mathey and Maillet [13] where a phosphole-oxide is reduced involves titanocene dichloride and Mg in refluxing THF (Scheme 1-4). This reagent reduces a bicyclic phosphole oxide but it is unknown if a bridging, bicyclic



Scheme 1-4. Mathey and Maillet [13] reduced a phosphole-oxide via Cp_2TiCl_2/Mg in THF.

phosphorus oxide will show the same result. Problems may occur in refluxing THF because of the potential heat sensitivity of 7-PNBDO.

$Cp_2TiC_2H_4$ is also known to possess the ability to reduce phosphole-oxides. A procedure by Kool *et al.* [14] allowed a partial synthesis of this reagent by reacting titanocene dichloride with trimethylphosphine in the presence of magnesium (Scheme 1-5).

$$Cp_2TiCl_2 + Mg + 2PMe_3 \longrightarrow [Cp_2Ti(PMe_3)_2] + MgCl_2$$

$$Cp_2Ti(PMe_3)_2 + C_2H_4 \longrightarrow Cp_2TiC_2H_4 + 2PMe_3$$

Scheme 1-5. Reaction for the synthesis of $Cp_2TiC_2H_4$, a reagent that reduces phosphole-oxides.

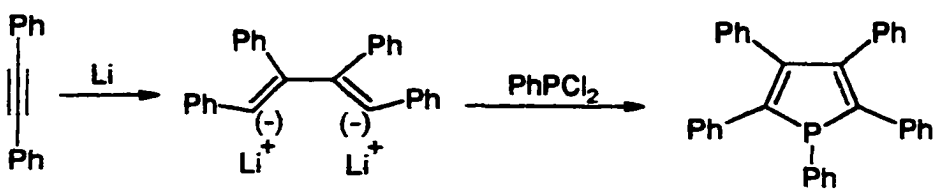
This titanocene trimethylphosphine compound undergoes a ligand exchange with ethylene as

outlined by Alt *et al.* [15]. Ethylene titanocene is extremely air sensitive, so it must be reacted in situ with 7-PNBDO. Also, other ligands such as PPh_3 and $P(n\text{-}Bu)_3$ can be substituted for PMe_3 in order to accelerate ligand exchange.

RESULTS AND DISCUSSION:

A number of the reagents and products used in this research were air-sensitive. Therefore, to reduce the risk of contamination, we have taken appropriate measures, such as flame drying our equipment and working under Nitrogen and Argon atmospheres to maximize oxygen- and moisture-free conditions.

The first goal of this research was to prepare a heterocyclopentadiene in the form of pentaphenylphosphole (**1**). This was done by first creating an organolithium reagent through the dimerization of diphenylacetylene in the presence of elemental lithium ribbon [6,7]. This reaction forms a radical anion via an electron transfer from the lithium to the diphenylacetylene followed by dimerization [6] to form 1,4-dilithio-1,2,3,4-tetraphenylbutadiene. The resulting product mixture was then transferred under an Ar atmosphere into the next reaction where this deep red dilithio compound acts as a nucleophile towards the substituted phosphorus dihalide, $PhPCl_2$, producing **1** (Scheme 1-6).

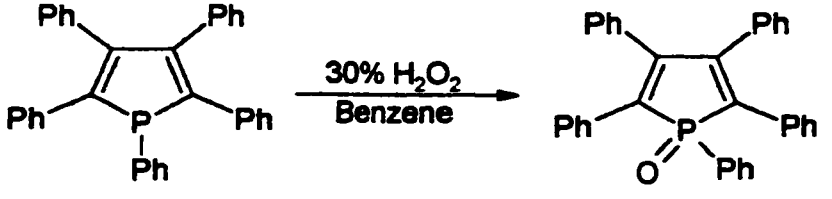


1

Scheme 1-6. A two step synthesis of pentaphenylphosphole.

The dilithio-compound immediately reacted with dichlorophenylphosphine, the dark red colour dissipated and, eventually, a yellow solid, **1**, precipitated. The best yield obtained for this reaction step was 60.7%. This pentaphenylphosphole (PPP) product had a melting point of 256 °C and a ^{31}P NMR chemical shift of δ +16 ppm (App. 1-1) [6,16].

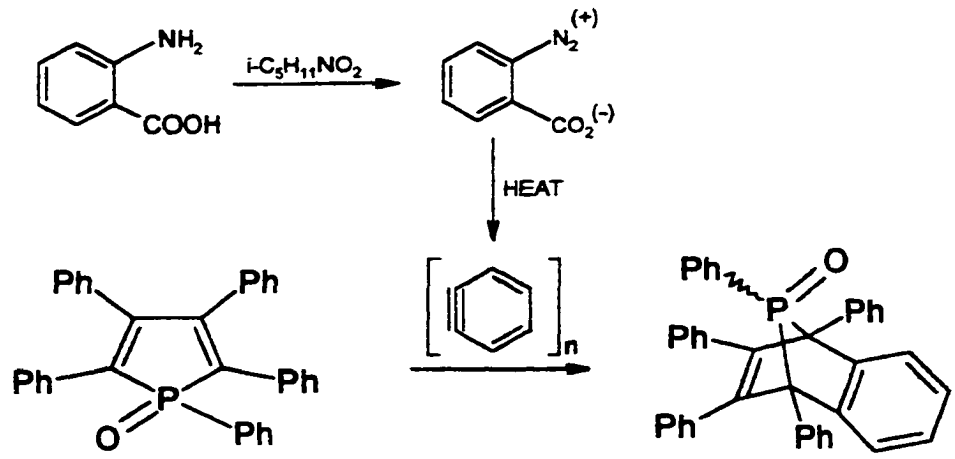
To synthesize the 7-phosphanorbornadiene structure, a Diels-Alder reaction was completed with this 5-membered ring. Unfortunately, 3-coordinate phospholes cannot undergo 1,4-cycloaddition reactions because the lone pair of electrons on the phosphorus atom retain their nucleophilic character and will react with the α,β-unsaturated system [8]. Also, the phosphole ring has quasi-aromatic character. However, in the oxidized 4-coordinate state, the phosphole system lacks the pair of electrons on the phosphorus atom which can act as a nucleophile or participate in cyclic delocalization, so it can behave as an activated diene [8]. Following a repeated synthesis of PPP, the combined products were oxidized with 30% H_2O_2 [16] in dry benzene to yield the 4-coordinate species pentaphenylphosphole-oxide (PPPO, **2**) (Scheme 1-7). This reaction step gave a 68% yield with the product having a melting point of 267 °C and ^{31}P NMR chemical shift of δ +44 ppm (App. 1-2) [8,16]. Normally phosphole-1-oxides spontaneously dimerize [8], but by preparing a compound with 5-phenyl substituents (PPPO) dimerization does not occur due to steric hindrance.



1 **2**

Scheme 1-7. The oxidation of pentaphenylphosphole to pentaphenylphosphole-oxide.

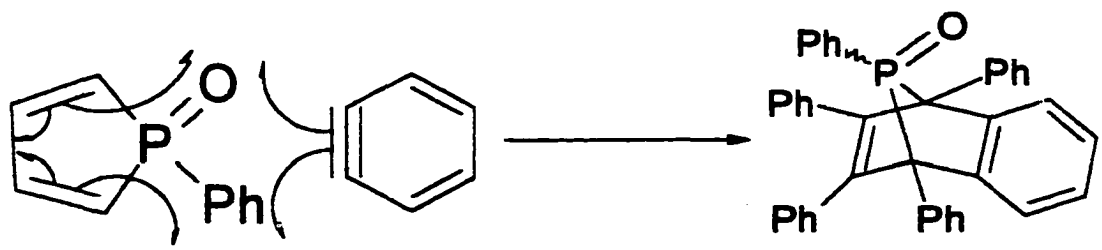
Pentaphenylphosphole-oxide was then reacted with benzyne to give a Diels-Alder reaction product (Scheme 1-8). The benzyne was generated through the diazotization of anthranilic acid using iso-amylnitrite [17]. Benzyne is a short lived intermediate which is immediately trapped by PPPO to give the Diels-Alder reaction.



2 3

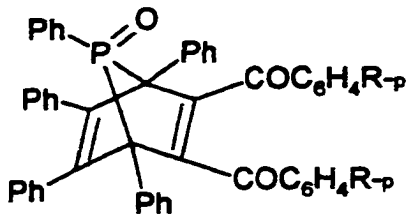
Scheme 1-8. The Diels-Alder reaction involving the in situ generation of benzyne to form only one stereoisomer of 7-PNBDO.

What is conveniently written as the benzyne triple bond is an extremely reactive biradical due to reduced overlap of the π orbitals in the plane of the ring [18]. It does not have true triple bond character as in, for example, acetylene. Because of this modified triple bond, this reaction is not a true Diels-Alder reaction (Scheme 1-9) but a Diels-Alder reaction involving radical



Scheme 1-9. The Diels-Alder reaction between PPPO and benzyne involving radical addition.

addition. The Diels-Alder product (**3**) was attained in 58.5% yield with respect to PPPO (**2**) and had a melting point of 164-166 °C and a ^{31}P NMR chemical shift of δ +95 ppm (App. 1-3). As only a single resonance was observed in the NMR spectrum, it appears that one only stereo-isomer is formed during this reaction. The ^{31}P NMR literature on 7-PNBDO is quite scarce so absolute values cannot be compared, however, similar compounds (**4**) have been synthesized and have ^{31}P NMR chemical shifts within the same range of δ +90 to δ +105 ppm (Figure 1-12) [5].



R = H δ+103.2ppm

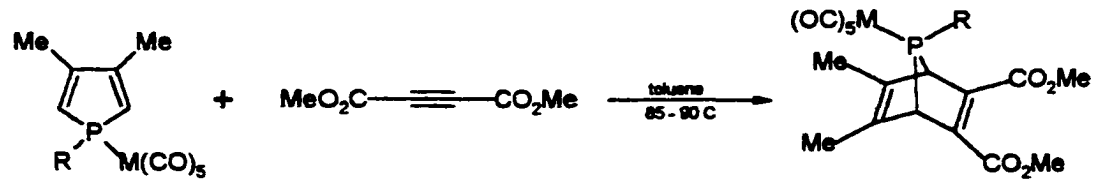
R = Cl δ+102.8ppm

R = CH_3 δ+104.1ppm

4

Figure 1-12. A 7-PNBDO derivative synthesized by Matsumoto and Hashimoto [5].

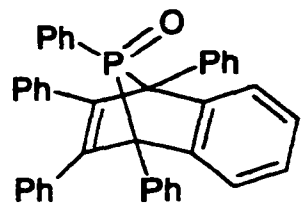
One of the mysteries of compound 3 was the structure and orientation of the oxygen and phenyl ligands attached to the bridging phosphorus atom. Specifically,it was not known which of the ligands was positioned above the aromatic ring of the norbornadiene structure. Marinetti *et al.* [19] proposed that a diene would attack exclusively on the less hindered side (Scheme 1-10) of the phosphole, which in this case would position the oxygen ligand above the fused



Scheme 1-10. The attack of the acetylene takes place on the less hindered side (the side with the R group) of the phosphole.

benzene ring. The chemical shifts for the ^{13}C NMR spectrum were predicted for both possible structures of 3 using the program Advanced Chemistry Development©/C NMR version 1.0. However, the peak positions predicted for both ^{13}C NMR spectra were identical within the accepted theoretical error of ±20 ppm (App. 1-4, 1-5). The ^{13}C NMR spectrum of 3 (App. 1-6) was measured and the observed chemical shifts were similar to the predicted ones for both possible stereoisomers. Next, the 7-PNBDO compound was crystallized and using single crystal x-ray diffraction [20] the structure was determined (App. 1-7) (Table 1-2, 1-3). As a result of the crystallization being performed in benzene, benzene was incorporated into the crystal lattice.

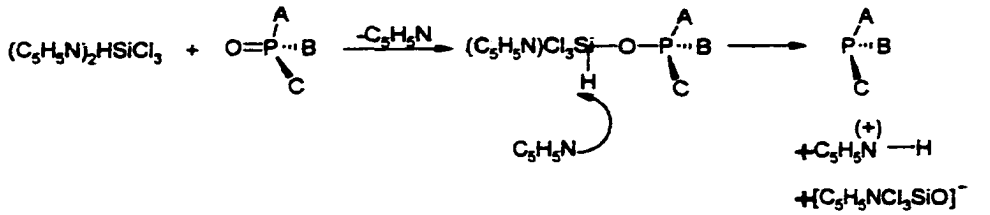
The crystal structure contains two molecules of 7-PNBDO with a separate benzene ring intercalated between them. In addition, microanalysis of these crystals gave a composition of 86.72 ± 0.10% carbon and 5.92 ± 0.08% hydrogen which agrees with the detected two 7-PNBDO molecules and one benzene ring. In summary, 7-PNBDO is formed as a single stereoisomer with its oxygen ligand positioned above the aromatic portion of the molecule (**5**) (Figure 1-13) [20].



5

Figure 1-13. The stereoisomer of 7-PNBDO produced in a modified Diels-Alder reaction.

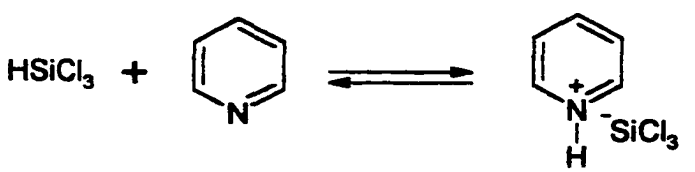
For the next reaction step, the reduction of the oxide, Quin *et al.* [9] have shown that trichlorosilane in the presence of pyridine cleaves an oxygen ligand from a PPPO-type compound. They [9] proposed a 4-centre mechanism for this reaction (Scheme 1-11) in which pyridine forms a complex with the $HSiCl_3$ that cleaves the oxygen ligand and thus gives retention of configuration at the phosphorus centre [9]. When this reduction was carried out using only $HSiCl_3$ on a phosphine-oxide as part of a strained, bridging structure, a small amount of inversion occurred [9]. However, when pyridine was present during this reaction, mixed results produced molecules with both retained and inverted configurations [9]. Therefore, the stereochemistry of the products is difficult to predict for these types of $HSiCl_3$ reductions. This type of reaction has also been performed for a 7-phosphanorbornene-oxide system, where upon $HSiCl_3$ reduction, the phosphorus bridge collapsed except when the reaction was in the presence of pyridine. Here, cleavage of oxygen from the phosphorus atom was successful and the bridge remained intact.



Scheme 1-11. Proposed mechanism for $HSiCl_3$/pyridine reduction of a phosphine-oxide [9].

Therefore, cleavage of oxygen from our compound should produce 2,3-benzo-1,4,5,6,7-penta-phenyl-7-phosphabicyclo[2.2.1]hept-5-ene.

Upon addition of trichlorosilane to a warm solution of benzene, 7-PNBDO and pyridine, a white precipitate clouded the mixture. As $HSiCl_3$ became immersed in an environment of pyridine, it is believed a pyridinium-trichlorosilyl salt is formed in equilibrium with the two reactants (Scheme 1-12). It is not known which side is favoured in this equilibrium. However,



Scheme 1-12. The interaction between $HSiCl_3$ and pyridine to form a pyridinium-trichlorosilyl salt precipitate.

because of the precipitate, acquisition of a ^{31}P NMR spectrum was extremely difficult from this crude mixture. After attempting to work up the product a ^{31}P NMR chemical shift was discovered at δ -49 ppm (Table 1-1) (App. 1-8) which is evidence of a poly(phenylphosphin-idene oxide) that is formed when the norbornadiene structure debridges [9,10]. After repeating this reaction at a lower temperature it was discovered that a small amount of 7-PNBDO had reacted and produced a product with a ^{31}P NMR chemical shift of δ +54 ppm (App. 1-9). This shift is close to the expected range (δ +60 to δ +80 ppm) for 7-PNBD but again only one

Table 1-1. Reaction Conditions for the Reductions of 7-PNBDO.

REACTION WITH 7-PNBDO	SOLVENT	CONDITIONS	RESULTS
5 molar equiv of $HSiCl_3$ 15 molar equiv pyridine [9]	Benzene	·reflux 2 hrs, cool in ice-water bath, hydrolyze with NaOH	·cmpd debridged ·^{31}P shift of δ-49$_{ppm}$
		·same conditions at lower temp (30°C)	·2 peaks at δ+95 and δ+54$_{ppm}$
5 molar equiv $PhSiH_3$ 15 molar equiv Pyridine [9]	Benzene	·heat 2½hrs at 40°C ·cool in ice-water bath with NaOH	·peaks at δ+95 and +41$_{ppm}$
		·heat 24hrs at 40°C	·peaks at δ+95, +41 and +16$_{ppm}$
5 molar equiv of $HSiCl_3$ ($PhSiH_3$) 15 molar equiv 4-dimethylaminopyridine [9]	Benzene	·stir 3hrs at r.t., cool in ice-water bath with NaOH	·no reaction
		·stir at r.t for 72 hrs	·no reaction
		·stir at 40°C for 24hrs	·no reaction
$HSiCl_3$ Cyclohexyldiethylamine [18]	CH_3CN	·Ar atm., 30°C for 3hrs. ·stir with NaOH and extract with benzene	·peak at δ+18$_{ppm}$
		·3hrs at 43°C	·peak at δ+18$_{ppm}$
$PhSiH_3$ Cyclohexyldiethylamine [18]		·3hrs at 30°C	·peaks at δ+96 and +42$_{ppm}$
2 molar equiv Cp_2ZrHCl [17]	THF	·N_2 atm., 40°C for 4 hrs, then room temp. for 48hrs	·no reaction
Cp_2TiCl_2 Mg [19]	THF	·N_2 atm, 35°C for 24hrs	·no reaction
	CH_2Cl_2	·N_2 atm, 35°C for 24hrs	·no reaction
	Benzene	·N_2 atm, 35°C for 24hrs	·peaks at δ+95, +44, +21$_{ppm}$
		·N_2 atm, 85°C for 2½hrs	·peaks at δ+95,+44,+21$_{ppm}$
		·N_2 atm, 85°C for 24hrs	·peaks at δ+44$_{ppm}$

REACTION WITH 7-PNBDO	SOLVENT	CONDITIONS	RESULTS
Cp_2TiCl_2 VinylMgBr	THF	·N_2 atm,-78°C for 1hr, then add 7-PNBDO at 0°C for 1hr	·no reaction
	Toluene	·same	·no reaction
		·increased temp. From 0°C to ~40°C to ~50°C	·no reaction

*Attempts to synthesize [20,21] $Cp_2TiC_2H_4$ via $Cp_2Ti(PPh_3)_2$, $Cp_2Ti[P(n\text{-}Bu_3)]_2$, and $Cp_2Ti(PMe_3)_2$ were unsuccessful.

BF_3•diethyletherate	Benzene	·N_2 atm., r.t. for 5hrs	·no reaction
	THF	·N_2 atm., 0°C for 5hrs	·no reaction
		·N_2 atm, r.t. for 24hrs	·no reaction
$HSiCl_3$ [1]	Toluene	·same	·one peak at δ+162$_{ppm}$
	THF	·same	·one peak at δ+16$_{ppm}$
	Benzene	·stir at room temp. under N_2 atm. from 2-24hrs	·^{31}P peaks at δ-122, +18,+34,+42 ,+43,+45,+58, +59,+162$_{ppm}$
		·stir at room temp. under N_2 atm. for 3hrs	·same peaks as above plus δ+94$_{ppm}$
		·stir in ice bath under N_2 atm. for 3 hrs	·same peaks as above plus δ+94 and +75$_{ppm}$

stereoisomer had been formed. A previous paper by Quin and Bernhardt [19] suggested that one of the carbon-carbon bonds could have broken to form an iso-phosphindoline-oxide derivative. Unfortunately, this product could not be isolated and the reaction could not be repeated so after several attempts this result remained inconclusive. In a separate experiment, another reducing agent (phenylsilane, $PhSiH_3$) was used in an attempt to deoxygenate compound **3**. After 2½ hours, ^{31}P NMR analysis showed unreacted 7-PNBDO and an unexplained peak (δ +41 ppm). After 24 hours there was still little success as ^{31}P NMR analysis revealed peaks at δ +95, +41, and +16 ppm (App. 1-10). The peak positioned at δ +16 ppm had a chemical shift equivalent to that of compound **1** (App. 1-1). It is possible that a small amount of unreacted pentaphenyl-phosphole may have been carried through the reaction sequence.

Next, it was thought that the trouble with this reaction may be a result of the base which is used . A milder base in the form of 4-dimethylaminopyridine (DMAP) was substituted for pyridine in the previous procedure [9]. After 3 hours at room temperature no reaction had occurred. This reaction was subsequently attempted at room temperature for 72 hours with an excess of DMAP but ^{31}P NMR analysis showed only unreacted 7-PNBDO. The temperature was increased to 45 °C for 24 hours still with no success. Another procedure [11] was found which utilized $HSiCl_3$ and cyclohexyldiethylamine (CHDA). When an attempt to reduce 7-PNBDO with $HSiCl_3$/CHDA was first carried out at 30 °C for 3 hours ^{31}P NMR studies determined a peak at δ +18 ppm (App. 1-11). This was indicative of PPP. The temperature of this reaction was increased to 43 °C for 3 hours and the same peak resulted. Through ^{13}C NMR spectroscopy and analytical Gas Chromatography/Mass Spectrometry (GC/MS) analysis of this product, it was confirmed that PPP had been produced. Next, $PhSiH_3$ was substituted for $HSiCl_3$ in this reaction. After 3 hours at 30 °C, ^{31}P NMR studies found peaks at δ +96 and +42 ppm

(App. 1-13). Again, using ^{13}C NMR and GC/MS analysis the data indicated unreacted 7-PNBDO and PPPO.

As stated previously, procedures have been reported in which the reduction of phosphole-oxides used metallocenes. One such reaction [12] involved using bis(cyclopentadienyl)-zirconiumchloride-hydride. A reduction of 7-PNBDO was attempted under light and air sensitive conditions using the zirconium reagent. The 7-PNBDO and Cp_2ZrClH were mixed in a 1:2 ratio. After stirring at 40 °C for 4 hours followed by cooling to room temperature for two days it was evident that only unreacted 7-PNBDO was present in this reaction mixture. This reaction was extended to 72 hours but with no success.

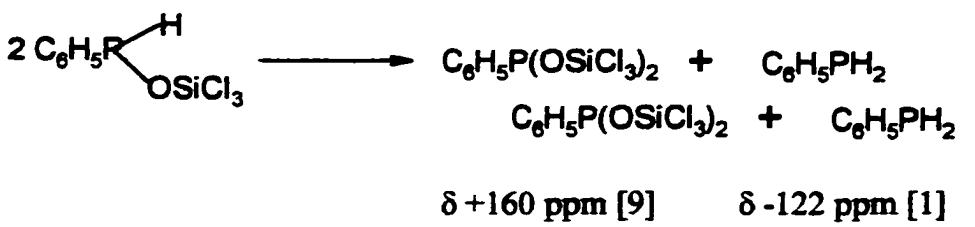
Titanocenedichloride was used in the presence of magnesium in THF and CH_2Cl_2 [13]. Varying the temperature of these reactions was unsuccessful in reducing 7-PNBDO. However, when the solvent was changed to benzene the results became interesting. As the temperature was increased from room temperature to 85 °C, the unreacted 7-PNBDO did not debridge as it usually did above 50 °C. ^{31}P NMR revealed a gradual retro Diels-Alder reaction taking place as the δ +95 ppm peak decreased in intensity and the δ +44 ppm peak (indicative of PPPO) increased in intensity over time until it was the only peak that remained (App. 1-14 and 1-15). Also the small peak at δ +21 ppm that appeared and then disappeared has yet to be assigned.

Another metallocene, bis(ethylene)titanocene had been shown [from 12, 13] to have the ability to cleave an oxygen ligand off of a bridging phosphorus atom. A reaction using this reagent was carried out in THF. ^{31}P NMR studies of the solution at 0 °C revealed unreacted 7-PNBDO and upon warming the mixture to room temperature the result was the same. Upon changing the solvent to toluene and measuring ^{31}P NMR at various temperatures from 0 °C to >50 °C, the 7-PNBDO remained unreacted. No debridging was evident (>50 °C) as had been

previously seen. Another method [14,15] attempted was to prepare the bis(ethylene)titanocene through titanocenedichloride, magnesium, and either PPh_3, $P(n\text{-}Bu)_3$, or PMe_3. Comparing ^{31}P NMR chemical shifts of the products formed with the reactants used, $Cp_2TiC_2H_4$ was not synthesized.

A reaction with borontrifluoride:diethyletherate was also attempted, in the hope that the oxophilicity of boron would result in the reduction of the 7-PNBDO. After 5 hours ^{31}P NMR studies showed no reaction had taken place.

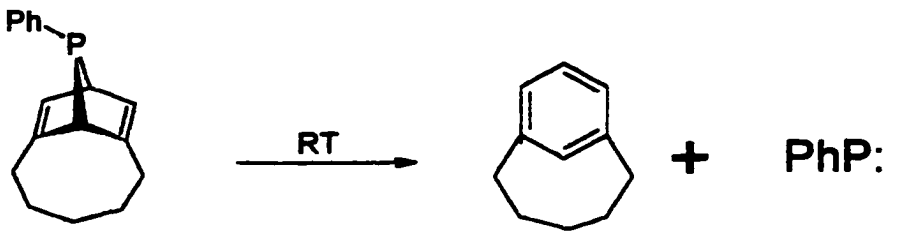
One reaction that did have promise was reducing 7-PNBDO directly with an excess of $HSiCl_3$ in benzene [9]. ^{31}P NMR analysis of the product revealed a variety of peaks (App. 1-16). The peak at δ +160 ppm is explained by Quin *et al.*[9] as the result of a disproportionation of the



Scheme 1-13. A retro McCormack reaction involving the collapse of the phosphorus bridge and a direct interaction with $HSiCl_3$.

phosphorus bridge due to an interaction with the trichlorosilane (a retro-McCormack reaction) (Scheme 1-13). The two peaks located at δ +57 and +58 ppm are close to the expected range (δ +60 to +80 ppm) for both isomers of 7- phosphanorbornadiene. The peak at δ +17 ppm is representative of PPP probably from some kind of retro Diels-Alder reaction and subsequent phosphole-oxide reduction. The peaks at δ +34, +42, +43, and +45 ppm have not been identified. There was extreme difficulty in purifying the targeted product as it appeared to be reactive to air. The peak at δ -41 ppm could possibly be Ph_2PH [2], which has the same ^{31}P NMR chemical shift, caused by debridging of the compound and further reaction. Van Eis *et al.*

[21] report having synthesized a stable 7-PNBD system with a ^{31}P NMR shift of δ+ 112 ppm. This chemical shift is significantly larger than the experimental shifts of δ +58 and +57 ppm acquired in the range for similar structures for 7-PNBenes [21]. Van Eis *et al.* [21] conduct their reaction at room temperature. Once their 7-PNBD is formed, it slowly fragments into a

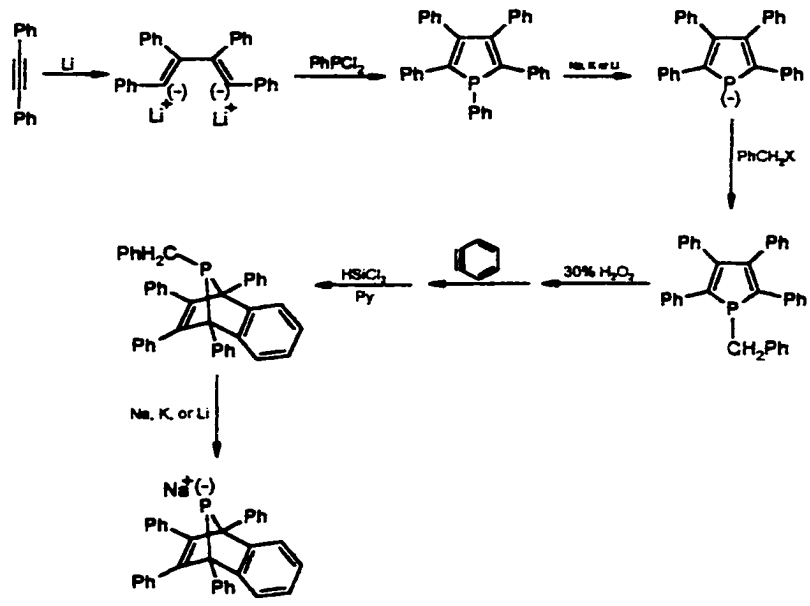


Scheme 1-14. Disproportionation of 7-PNBD at room temperature.

debridged product (half-life of one day) (Scheme 1-14) [21]. They were unable to characterize fully this unstable molecule. The low temperature half-life of the presumed 7-PNBD structure prompted a reappraisal of the 7-PNBDO/$HSiCl_3$/pyridine experiment that was performed in an ice bath. Besides slowing down the reaction, the ^{31}P NMR spectrum revealed a new peak at δ +75 ppm (App. 1-17) which was not observed when the reaction was attempted above room temperature. However, we were unable to reproduce this finding when the experiment was repeated.

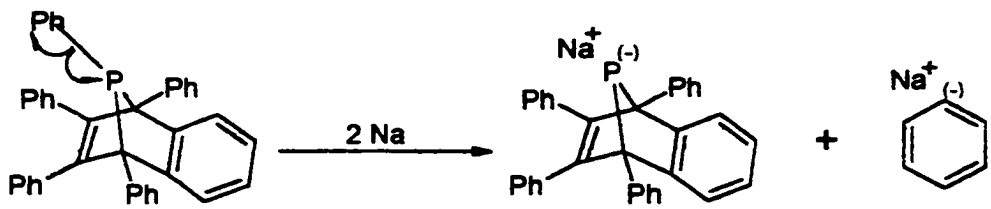
After exhausting many reduction techniques, it is still not known whether the oxygen ligand has been cleaved off of the bridging phosphorus atom. Evidence to support the formation of the 7-PNBD structure is not significant enough to draw any conclusion. Once the products producing the ^{31}P NMR shifts of δ +58 and +59 ppm were synthesized, a piece of elemental sodium was dropped into the crude product mixture and ^{31}P NMR analysis monitored a region further downfield (δ +800 to +1200 ppm) of the spectrum. The proposed radical cleavage is shown in Scheme 1-15. No peaks were discovered in this region. Elemental lithium and

potassium were also used and produced similar results. Interference hindered the detection of any small peaks. Appendix 18 shows the peaks at δ +57 and +58 ppm still remain after this



Scheme 1-16. Proposed cleavage of phenyl ligand to form the 7-PNBD anion.

attempted reduction. Had the 7- PNBD structure been confirmed and the phenyl group cleavage not worked, a better leaving ligand in the form of -CH_2Ph could have been used as shown in a proposed alternative synthesis [22] for the 7-PNBD anion (Scheme 1-16).



Scheme 1-15. Alternate reaction scheme for the synthesis of 7-PNBD with a better leaving ligand on the phosphorus atom.

Chesnut's [1] predicted ^{31}P NMR chemical shift of δ +1085 ppm for a 7-PNBD anion still remains elusive for the time being. More research involving stable 7-PNBDs is required in order to fully characterize and interpret the NMR spectral data. Once strong evidence of a stable

7-PNBD exists more effort can be focussed on the synthesis of a 7-PNBD anion and measuring its experimental ^{31}P NMR chemical shift. This is an area of research needing further exploration.

EXPERIMENTAL:

Phosphorus nuclear magnetic resonance (^{31}P NMR) spectra were measured in 5mm probes on two types of NMR spectrometers. The first four experimental sections of this research were measured at an operating frequency of 80 MHz on a Brüker AC-E 200 spectrometer. The last two experimental sections were measured at 200 MHz on a UNITY Inova 500 NMR spectrometer from Varian. The solvent used for PPP and PPPO was $CDCl_3$ and for the remaining products was C_6D_6. Chemical shifts are reported in parts per million (ppm) from an internal standard of 85% phosphoric acid (H_3PO_4). ^{13}C NMR were also recorded in $CDCl_3$ with $CDCl_3$ as the internal standard (δ +77.0 ppm).

All synthetic experiments were run under a positive pressure of nitrogen or argon gas in flasks that were oven dried. Air and moisture sensitive reagents were transferred via syringe and introduced to the reaction flasks through rubber septa. Benzene was rendered anhydrous by stirring over $CaCl_2$ for four hours followed by storage over 4Å molecular sieves. All other solvents were used as received. Excess solvents were removed *in vacuo* at pressures obtained by a water aspirator drawing on a Buchi rotary evaporator. All compounds were sealed and stored in the refrigerator at a temperature of 4 °C.

Gas chromatography-mass spectrometry (GCMS) was used to assure the identity of the products. GCMS was performed on a Hewlett Packard 5890 Series II gas chromatograph also using a DB-5HT column and helium as the carrier gas. The linear velocity in this system was 35 cm/s. The gas chromatograph was attached to a VG Micromass AutoSpec mass spectrometer which measured the mass spectra of the samples between 52 and 510 mass units with a

resolution of 3000 at 70 e.V. ionizing energy. Mass spectral data are presented in the following form: parent ion (relative intensity), *m/e* of significant fragments (relative intensity).

Melting points were measured using a 60W Gallenkamp® (110/120V) Melting Point Apparatus and are reported as uncorrected values.

Crystallographic data was obtained on a P4 Siemans diffractometer equipped with a Siemans SMART 1K CCD Area Detector [14] at McMaster University in Hamilton, Ontario. The data was processed using the programs SAINT and SADABS and the structure solved using Siemans SHELXTL program library.

PREPARATION OF PENTAPHENYLPHOSPHOLE [6,7]:

This reaction was done in two steps with the synthesis of the dilithio-compound being followed by preparation of the pentaphenylphosphole. Diphenylacetylene (4.0 g, 22.4 mmol) and diethylether (100 mL) were placed in a 250 mL, flame-dried round bottom flask under an argon atmosphere. Lithium ribbon (2.2 cm, 22.4 mmol) was added to the solution. The reaction was stirred for 24 hours at room temperature. The resulting deep red solution contained 1,4-dilithio-1,2,3,4-tetraphenylbutadiene.

For the next part of this procedure, dichlorophenylphosphine (1.52 g, 11.2 mmol) and anhydrous diethylether (60 mL) were placed in another 250 mL, flame-dried round bottom flask under an argon atmosphere. The dilithio solution was transferred into the phosphine solution via a hypodermic syringe (after the transfer was completed, any unreacted lithium was destroyed in the first flask by the addition of ethanol). The resulting yellow solution was stirred for 20 minutes following which the solvent was removed under vacuum. The product was

recrystallized from a minimal amount of dichloromethane(20%)-methanol(80%) solution and filtered to yield yellow, needle-like crystals of pentaphenylphosphole {60.7% yield, ^{31}P NMR (C_6D_6) δ +16 ppm, m.p. 256°C, mol.wt.calc. 464.5; mol.wt.(mass spec.) 464.1}.

PREPARATION OF PENTAPHENYLPHOSPHOLE-OXIDE [16]:

Pentaphenylphosphole (5.0 g) and dry benzene (50 mL) were placed in a 250 mL round bottom flask and the solution was brought to reflux. Excess 30% H_2O_2 (10 mL) was added dropwise to this solution. The solution was allowed to boil for 5 minutes, then cooled to room temperature where PPPO precipitated as crystals slightly darker than PPP {68% yield, ^{31}P NMR (C_6D_6) δ +44 ppm, m.p. 267 °C (lit.[16] m.p. 284-285 °C), mol.wt.calc. 480.5; mol.wt.(mass spec.) 480.1}.

PREPARATION OF 7-PHOSPHANORBORNADIENE-OXIDE [8]:

Iso-amylnitrite (1.38 mL, 10.3 mmol) and dichloromethane (10 mL) were placed in a 25 mL three-necked round bottom flask attached with a condenser, a thermometer, and a 30 mL addition funnel. The solution was heated to reflux (40° to 45 °C) for a few minutes. A mixture of anthranilic acid (1.29 g, 9.4 mmol), PPPO (3.0 g, 6.2 mmol), and reagent grade acetone (4 mL) was added to the solution over a 5 minute period. The reaction was allowed to reflux for 5 hours (35° to 40 °C) and then cooled to room temperature overnight. The solvents were removed under vacuum. Anhydrous diethylether (9.4 mL) and saturated $NaHCO_3$ (6.2 mL) were mixed with the remaining oil. After considerable CO_2 evolution, the two layers were separated in a 60 mL separatory funnel where the $NaHCO_3$ layer on the bottom was discarded. The ether layer

was washed four times with saturated $NaHCO_3$ (1.8 mL), twice with saturated NaCl solution, and dried over anhydrous Na_2SO_4. Once the orange-yellow precipitate had formed, the solution was transferred to a round bottom flask and the solvents were removed under vacuum to produce 2,3-benzo-1,4,5,6,7-pentaphenyl-7-phosphabicyclo[2.2.1]hept-5-ene-oxide {58.5% yield, ^{31}P NMR (C_6D_6) δ +95 ppm, m.p. 164° - 166 °C}. This product crystallized as a dimer with an intercalating molecule of benzene {Found: C, 86.72; H, 5.92. $C_{86}H_{66}O_2P_2$ calculated C, 86.70; H, 5.42}.

7-PNBDO was recrystallized for x-ray analysis by dissolving in a minimal amount of benzene in a 10 mL test tube. N-pentane (30 mL) was poured into a 500 mL erlenmeyer flask. The unsealed test tube was gently placed into the flask and the flask sealed. After a couple of days at room temperature the pentane had slowly diffused into the 7-PNBDO-benzene solution causing crystals of 7-PNBDO to form on the inner surface of the test tube. The solvent was removed by pipette and the crystals dried.

PREPARATION OF 7-PHOSPHANORBORNADIENE [9]:

7-PNBDO (0.050 g, 0.090 mmol) and dry benzene (2 mL) were placed in a 10 mL flame-dried round bottom flask under a nitrogen atmosphere. Trichlorosilane (0.15 mL) was added to the solution via a 1 mL syringe and it was allowed to stir at room temperature for 3 hours. The crude product was run on a ^{31}P NMR analysis to detect possible 7-PNBD {^{31}P NMR (C_6D_6) δ +57.5 and 58.5 ppm}.

PREPARATION OF THE 7-PHOSPHANORBORNADIENE ANION:

The crude 7-PNBD product was placed inside a 5 mm NMR tube. A small piece of sodium metal was added to the NMR tube and a ^{31}P NMR analysis was run to detect the 7-phospha-norbornadiene anion as it was generated *in situ*.

REFERENCES

1. Chesnut, D.B. *"Ab Initio NMR Chemical Shieldings in the Neutral and Charged 7-Phosphabicyclo[2.2.1]-heptane, -heptene, and -heptadiene Systems; the Largest Predicted Downfield Shift for a Conventional Organophosphorus Molecule"*; P.M. Gross Chemical Laboratory: Duke University, Durham North Carolina, personal communication.

2. Verkade, J.G.; Quin, L.D. *"Phosphorus Thirty-one NMR Spectroscopy: Methods in Stereochemical Analysis, vol.8"*; VCH Publishers, Inc.: Deerfield Beach, Florida, 1987.

3. Quin, L.D.; Verkade, J.G. *"^{31}P NMR Spectral Properties in Compound Characterization and Structural Analysis"*; VGH Publishers Inc.: USA, 1994.

4. Emsley, J.; Hall, D. *"The Chemistry of Phosphorus"*; John Wiley and Sons: New York, 1976.

5. Matsumoto, K.; Hashimoto, S. *Heterocycles* **1990**, *30*, 201.

6. Leavitt, F.C.; Manuel, T.A.; Johnson, F.; Matternas, L.U.; Lehman, D.S. *J. Am. Chem. Soc.* **1960**, *82*, 5099.

7. Leavitt, F.C.; Manuel, T.A.; Johnson, F. *J. Am. Chem. Soc.* **1959**, *81*, 3163.

8. Quin, L.D. *"The Heterocyclic Chemistry of Phosphorus"*; John Wiley and Sons: New York, 1976.

9. Quin, L.D.; Caster, K.C.; Kisalus, J.C.; Mesch, K.A. *J. Am. Chem. Soc.* **1984**, *106*, 7021.

10. Stille, J.K.; Eichelberger, J.L.; Higgins, J.; Freeburger, M.E. *J. Am. Chem. Soc.* **1972**, *94*, 4761.

11. Johnson, C.R.; Imamoto, T. *J. Org. Chem.* **1987**, *52*, 2170.

12. Zablocka, M.; Delest, B.; Igau, A.; Skowronska, A.; Majoral, J.P. *Tetrahedron Lett.* **1997**, *38:34*, 5997.

13. Mathey, F.; Maillet, R. *Tetrahedron Lett.* **1980**, *21*, 2525.

14. Kool, L.B.; Rausch, M.D.; Alt, H.G.; Herberhold, M.; Thewalt, U.; Wolf, B. *Angew. Chem., Int. Ed. Engl.* **1985**, *24:5*, 394.

15. Alt, H.G.; Chwind, K.H.; Rausch, M.D.; Thewalt, U. *J. Organometallic Chem.* **1988**, *349*, C7.

16. Campbell, I.G.M.; Cookson, R.C.; Hocking, M.B.; Hughes, A.N. *Synthesis and Chemistry of Phospholes*, **1965**,2184.

17. Mich, T.F.; Nienhouse, E.J.; Farina, T.E.; Tufariello, J.J. *J. Am. Chem. Soc.* **1968**, *45*, 272.

18. Streitwieser, A. Jr.; Heathcock, C.H. *"An Introduction to Organic Chemistry"*; Macmillan Publishing Co., Inc.: New York, 1976.

19. Marinetti, A.; Mathey, F.; Fischer, F.; Mitschler, A. *J. Chem. Soc., Chem. Commun.* **1982**, *667*.

20. Gottardo, G.; Fratpietro, S.; Hughes, A.N.; Stradiotto, M. *Heteroatom Chemistry* **2000**, *11:3*, 182.

21. van Eis, M.J.; Zappey, H.; De Kanter, F.J.J.; De Wolf, W.H.; Lammertsma, K.; Bickelhaupt, F. *J. Am. Chem. Soc.* **2000**, *122*, 3386.

22. Braye, E.H.; Caplier, I.; Saussez, R. *Tetrahedron* **1971**, *27*, 5523.

CHAPTER 2:

AMINO ACID RACEMIZATION

INTRODUCTION:

Deoxyribonucleic Acid (DNA) is an important factor necessary for life. All living cells contain DNA of one form or another. As an organism reproduces or replicates, it passes on copies of its DNA to its progenies. Generation after generation, that same DNA is subjected to mutations that alter its genetic make up. By studying the genetic code of such cells one can discover similarities between species, the sex of a species, relationships between species, diseases or abnormalities specific to a certain species, and the basic evolution of one species into another. DNA provides the ability to trace one's ancestry by comparing specific regions of the genetic code.

Ancient DNA (aDNA) provides the same information, except it must be handled extremely carefully to ensure the purity of the sample. From the time ancient remains are discovered, through its extraction out of the earth, to the acquisition of their aDNA, modern contamination is a big problem. Because aDNA, if intact, is found in minute quantities, it is easily overpowered by the introduction of a small amount of modern DNA into the sample. The quality of preserved aDNA is also dependent on the type of environment to which it has been subjected. If viable aDNA can be extracted without contamination, it can be replicated and amplified in order that it can be analyzed. This is quite a long and tedious process. Sometimes remains are so poorly preserved that no viable aDNA exists in the sample. It is possible to determine if viable DNA is present in ancient remains before a lengthy aDNA extraction process takes place. Amino acids provide good indicators of how well DNA has been preserved in an ancient sample.

It has long been thought that amino acids in ancient samples undergo racemization over time. That is, amino acids are converted from one stereoisomer to another. However, evidence

suggests [1,2] that amino acid racemization occurs according to the environment to which they are exposed. The chemical change that converts amino acids in proteins from one stereoisomer to another was discovered to take place at about the same rate as the degradation of DNA [1]. By determining the extent of the reaction of the amino acids it is possible to determine if the DNA is viable. It also is possible to determine how much of the DNA present can be extracted.

A molecule is said to be chiral if its mirror-image cannot be superimposed on itself. A chiral compound and its mirror-image are known as enantiomers (stereoisomers) [3]. The two molecules have the same molecular weight and the same properties such as melting point, boiling point, density, and solubility in various solvents. Amino acids naturally occur in mirror-image pairs (Laevorotary- and Dextrorotary-forms), but it is the L-form which is used in protein biosynthesis. Once a protein is exposed to conditions such as weak acids or bases, a slow chemical transformation results in the conversion of L-forms to D-forms of the amino acids in the protein [1]. Service [1] explains the predominance of amino acids of the L-form in samples yielding intact DNA and D-forms of amino acids being present in samples where DNA could not be isolated due to degradation. Poinar *et al.* [2] focus on Aspartic Acid (Asp) and noted that if the D/L-Asp ratio is higher than 0.08, no retrievable DNA can be obtained from samples. If the D/L-ratio is lower than 0.08, viable DNA can be readily obtained. They [2] focus on aspartic acid since it undergoes a faster racemization than any other amino acid. For instance, alanine and leucine have a slower rate of racemization. Therefore, if the D/L-ratio for Asp that is lower than that for Alanine (Ala) or Leucine (Leu), contamination by more modern amino acids has occurred [2].

The separation of amino acid enantiomers is made possible through many techniques. Chiral high performance liquid chromatography (HPLC) can be used to separate enantiomers. Amino

acids are derivatized and enantiomeric separation occurs by utilizing a chiral mobile complex on a C_{18} HPLC column [4]. Zhao and Bada [4] derivatized amino acids with o-phthaldialdehyde (OPA) and a chiral thiol, N-acetyl-L-cysteine (NAC) to form diastereomeric complexes that were easily separated by HPLC. In this method a reverse-phase C_{18} column was used in conjuction with a fluorescence detector [4].

Chiral capillary electrophoresis (CE) can also be used to separate enantiomers. Tran *et al.* [5] outlined a procedure employed to determine the extent of amino acid racemization using capillary electrophoresis. Because enantiomers have identical charge to volume ratios, normal solution CE cannot be used [6]. However, if a neutral, chiral, complexing agent (cyclodextrin) is added to the run buffer and selectively interacts with the enantiomers, the charge to volume ratios will differ and separation can be achieved [6]. Chiral capillary electrophoresis (CE) separation is based on cyclodextrin (Figure 2-1). Cyclodextrins (CDs) have many chiral recognition centres which contain a neutral charge and have a small UV absorbance above

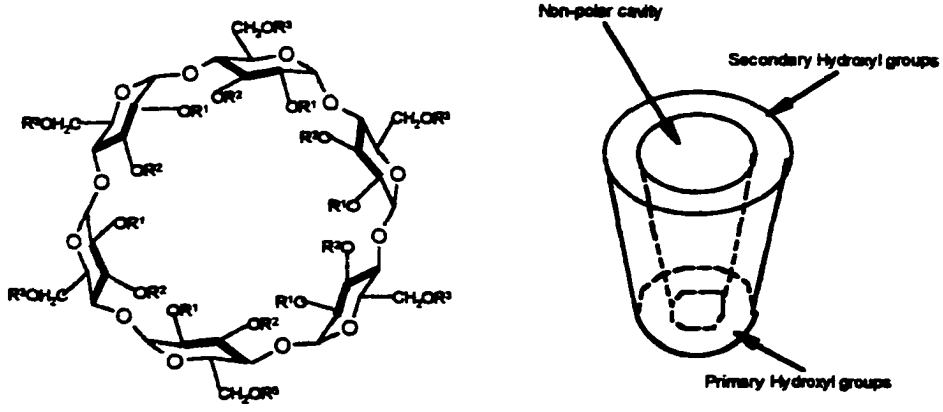


Figure 2-1. The molecular structure of cyclodextrin including the outer surface of the larger rim and its three-dimensional body [7].

200 nm. Each CD unit consists of a non-polar, hydrophobic cavity featuring secondary hydroxyl groups on its larger rim for its enantio-selectivity and primary hydroxyl groups on its smaller rim [7].

The resolution of peaks in chiral CE separations is dependent on cyclodextrin type, pH of the run buffer and cyclodextrin concentration. Cyclodextrins may vary in type due to the number of glucose units present in the ring (Figure 2-1). CDs can also be derivatized to achieve different solubilities and selectivities for the separation process [7]. The concentration of CD may influence the migration time of amino acids. Increasing the concentration increases the probability of complexation with the enantiomer as well as increasing buffer viscosity. This will also affect the peak resolution. For this research, the best peak resolution will be determined by using α-, β-, and γ-CD with a sodium borate buffer, pH 8.5.

The P/ACE™ System MDQ separates the components of a sample inside a fused-silica capillary tube [8]. The sample is usually injected under pressure. A high voltage gradient causes the samples to migrate differently through the capillary [8]. Some factors affecting the migration time are the size, shape and charge of the particles, the electrolyte concentration, pH of the solution, diameter of the capillary used, the length of the capillary and any column pre-treatment [8]. CE has the ability to separate peptides, proteins, nucleic acids, and pharmaceutical drug compounds. It offers the advantage of very low sample consumption due to the actual injection volume being 5 to 50 nL [8]. CE is a fast form of electrophoresis.

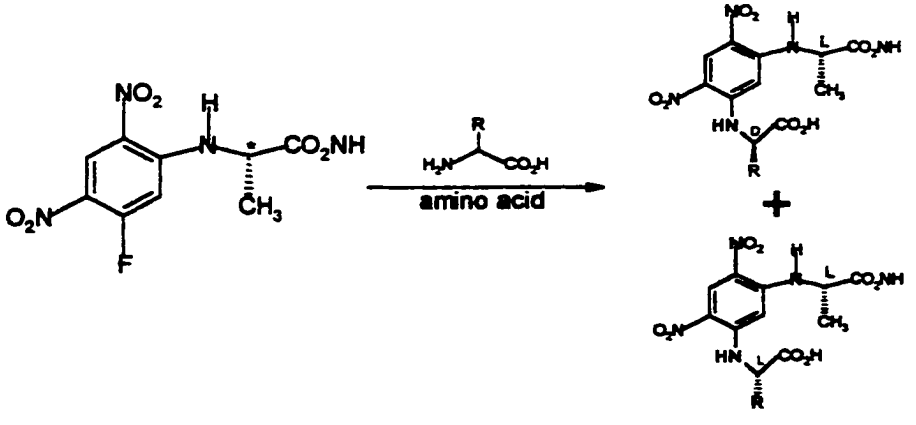
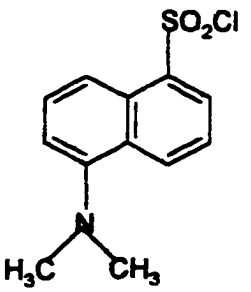


Figure 2-2. The derivatization of L-Marfey's Reagent with amino acids.

Samples of amino acids can be derivatized with L-Marfey's Reagent {Nα-(2,4-dinitro-5-Fluorophenyl)-L-alaninamide} (Figure 2-2) which fluoresces under UV light (App.2-1) so as to be detected. Each amino acid should possess a different migration time during its detection. Should the L-Marfey's Reagent be unsuccessful, another derivatizing agent in the form of dansyl-chloride (Figure 2-3) will be used for the UV detection (App.2-2) of the amino acids.



Dansyl-Chloride

Figure 2-3. The molecular structure of dansyl-chloride.

Should problems with the chiral CE result, HPLC analysis will be used. Because we do not have a chiral HPLC column, the derivatizing agent of choice is L-Marfey's Reagent. When the chiral L-Marfey's Reagent is combined with both the L- and D-form of enantiomeric amino acids, diastereomers are produced (Figure 2-2). These molecules are unique so the HPLC should be able to separate each of the diastereomers. However, if achiral Dansyl-chloride is used as the derivatizing agent, the enantiomers will remain as enantiomers which cannot be separated using regular (non-chiral) HPLC techniques. Also, a phosphate buffer pH 3.3 will be employed instead of the sodium borate buffer (pH 8.5). A buffer with a pH lower than 7 must be used because high pH leads to cleavage of silica from the reverse phase column.

RESULTS AND DISCUSSION:

Overall, this research resulted in an insufficient amount of data to show that this process of detecting amino acid racemization is a viable one. Besides the use of P/ACE™ System MDQ capillary electrophoresis, other techniques were attempted but with few positive results obtained.

The P/ACE™ System MDQ capillary electrophoresis system was well maintained. The capillary itself was cleaned thoroughly with 0.1 N HCl, 0.1 N NaOH, and E-pure water before its daily use as well as between runs (see methods 1-4). The performance of the Beckman P/ACE™ System MDQ was initially tested with a Beckman® test kit consisting of Beckman® Capillary Performance Test Mixture A, Beckman® Capillary Performance Run Buffer A and Beckman® Capillary Regenerator Solution. These reagents were applied using method 2 (see experimental). Results were consistent with those provided by Beckman® so this system appeared to be functioning normally. Optimal peak resolution was obtained through the use of HS-γ-CD in the run buffer. Initial starting conditions involved four recommended methods for use with P/ACE™ System MDQ capillary electrophoresis (methods 1-4).

The maximum voltage of this machine was 30 KV and maximum current was 300 μA. The derivatized amino acids were run at a voltage of 15 KV increasing the voltage to 20 KV in separate runs to determine the migration of the derivatized amino acid peaks. Some problems resulted when a run at a set voltage of 15 KV would only reach 5 KV because the maximum current of the P/ACE™ system was reached before the desired voltage was achieved. This is believed to have been caused by contamination of the run buffer, contamination of the derivatized amino acid sample, or from a cracked or plugged capillary. In some instances

electrical discharges were seen on the electrodes when the current became unstable. Because the submerged ends of the capillary tube could become contaminated during a run which, in turn, may contaminate other solutions in the run, problems of cross-contaminating sample vials from the capillary tube were minimized by dipping the tube ends in E-pure H_2O between moving from vial to vial. Another source of contamination was due to the rubber septa on the vials reacting with the vial contents after a period of time. This reaction produced a slight colour change in the solutions. To minimize this contamination, the solutions were stored in 15 x 45 mm disposable glass vials with a screw cap. Solutions that became coloured were immediately discarded. It is possible that the desired voltage was not achieved due to a neutral capillary, however, the capillary was charged for the recommended [6] period of time before each use. Perhaps the capillary charge was not maintained. The coolant temperature may have affected the results as well, although the optimal temperature of 22 °C [5,9,7] was employed.

As peaks were produced using P/ACE™ System MDQ capillary electrophoresis, the same migration peak appeared for each separate derivatized amino acid sample run in sequence. This same migration peak remained constant between samples but the migration time varied each day the same sequence was run. The derivatized amino acid reference samples should have different migration times. Because L-Marfey's reagent was used, the derivatized L-amino acids should have a lower migration time than the derivatized D-amino acids [5]. Even in a racemic mixture, it appeared that the derivatized D- and L-amino acids would not separate. The concentration of each derivatized amino acid in the run buffer was increased successively each run by varying the injection time (3 to 10 seconds) and increasing the injection pressure (0.3 psi to 3 psi). When the concentration of the derivatized amino acid in the run buffer became too high, the current reached its maximum and the P/ACE™ system shorted out. Another

derivatizing agent, dansyl-chloride, was used in place of L-Marfey's reagent under the same conditions but similar data was obtained.

Since positive results could not be obtained for standard, derivatized amino acids, it was unlikely positive data would be obtained for derivatized amino acids from a modern bone sample. Protein samples were gathered using bone shavings from a modern cow jaw. The glassware used for this experiment was cleaned with reagent grade acetone, rinsed with distilled H_2O and oven-dried to reduce the introduction of impurities into the solutions. The amino acid extract was filtered using a 0.2 μm centrifuge filter, derivatized with L-Marfey's reagent, and run on the P/ACE™ CE system but with minimal success. Each run using the same concentration under the same conditions resulted in different migration peaks each time. The concentration of the derivatized amino acids in the run buffer was varied (injection time and pressure) in sequential runs as well as the voltage (15 to 20 KV) but results still remained unrepeatable. These variations were applied to all four recommended methods (methods 1-4). It is possible that the extraction procedure used to acquire amino acids from bone was not efficient.

The failure of the P/ACE™ System MDQ to detect reproducible peaks resulted in attempts to use other analytical techniques, for example, analytical gas chromatography (GC). Select derivatized amino acids (L-Marfey's reagent) were dissolved in ethyl acetate at a concentration of 2 mg/mL. A 1 μL injection of this solution was run for 35 min. via conditions stated in method 5. L- and D-Thr runs did not produce any migration peaks on the GC, even after increasing the injection volume of derivatized amino acid solution to 2 μL. A more concentrated solution (~5 mg/mL) (Table 2-1) was also used but with the same result. Since solutions of L-Marfey's Reagent did not result in observable peaks when run for 35 min, longer run times (60 min., method 6) were employed for L-Thr, L- and D-Phe at high concentrations

(Table 2-1) but migration peaks failed to appear. The solvent peaks were apparent at the start of the run and some contamination peaks were detected coming off of the column but the GC was unable to detect the derivatized amino acids. It is possible that the derivatized amino acid peaks were migrating at the same speed as the solvent peaks so as to be mixed with them during the analysis. The amino acids may have been sticking to the column which is why a longer run time was employed. Other derivatizing agents were also used such as dansyl-chloride but offered similar results to L-Marfey's reagent. Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was used

Table 2-1. Results obtained on the GC.

Sample	Run Time (min)	Injection (μm)	Concentration (~mg/mL)	Peak(s) Detected
L-Thr	35	1	1	-
D-Thr	35	1	1	-
L-/D-Thr	35	1	1	-
L-Thr	35	3	1	-
L-Thr	35	1	5.8	-
L-Thr	35	1	1	-
D-Thr	35	1	1	-
L-/D-Thr	35	2	1	-
L-Marfey's Reagent	35	1	2	-
L-Thr	60	1	5	-
L-Phe	60	1	5	-
D-Phe	60	1	5	-

as another derivatizing agent but, due to the insolubility of the amino acids in most solvents except water, and the fact that BSTFA will not derivatize amino acids in water, the reaction was quite difficult and could not be attempted. Thus, analyzing derivatized amino acids using analytical gas chromatography was also a non- viable process.

In a final effort to produce some usable results HPLC analysis was attempted. A procedure employed by Clapp *et al.* [10] was used. Although the HPLC detected peaks for each of the

derivatized amino acids, each different derivatized amino acid had the same migration time. It appeared as though L-Marfey's Reagent had the same effect on all of the amino acids, showing no separation between D- and L- pairs, or even between different amino acids. This inability to separate the derivatized amino acids is still not quite understood. A chiral column was not available and the reverse-phase column used could not differentiate between the chiral diastereomers. This column did not treat the diastereomers as unique so the detector saw them as the same molecule. Results may have been different had a chiral column been available.

Studying the extent of amino acid racemization is a process that contains many variables. An abundance of factors seemed to influence the resulting data. The compatibility of the chemistry of this research with the analytical machinery may have played a large part in the production of few positive results. Since results could not be repeated, data acquired was questionable. This research exhausted the time allotted for this project and more time must be spent in determining a viable process to study amino acid racemization.

RECOMMENDATIONS:

There were many variables in this research that contributed to few positive results. Should this research be continued in the future improvements should be made. All solutions involved in P/ACE™ System MDQ capillary electrophoresis should be filtered. This may prevent the electric current from reaching its maximum before the desired voltage is attained. The pH of the run buffer may have to be altered to a pH between 2.5 and 8.5 for separation of derivatized amino acids. Derivatization of amino acids from bone should occur using powdered bone instead of bone shavings. The powdered bone has a greater surface area for a more efficient extraction of amino acids. When detecting derivatized amino acids using Gas Chromatography a longer run time (greater than 60 minutes) or different conditions (other than method 5,6) should be employed. A lower temperature may be required. Using HPLC to detect chiral, derivatized amino acids requires the use of a chiral column. This column would treat the diastereomers as unique so the detector would distinguish between diastereomers and detect different peaks.

EXPERIMENTAL:

All capillary electrophoresis experiments were performed on a BECKMAN P/ACE™ System MDQ capillary electrophoresis system. The system housed a capillary 0.67 cm x 0.50 μm I.D. The run buffer used for each amino acid was made up of boric acid (H_3BO_3) titrated with NaOH to make up a buffer of pH 8.5. This solution was stored in the fridge at 4 °C. All water used in this research was filtered as E-pure water prepared at Lakehead University using the Barnstead® E-Pure-1 Phase Macro Pure Filter System (Model D4631).

All the amino acids and derivatizing agents were purchased from Aldrich. Initially, amino acid derivatizations with L-Marfey's reagent and Dansyl-chloride were carried out in small round bottom flasks and the reactions were heated using a heating mantle to a temperature of 55-60 °C. Subsequently, the amino acids were derivatized in 15×45 mm vials with heating in a VWR® Scientific Heatblock at 58 °C. All derivatizations were carried out in a non-sterile lab environment. The lab bench was covered in large sheets of Whatman™ chromatography paper and latex gloves were used to handle the reagents. Contamination was not an issue as modern amino acid references were used.

The capillary electrophoresis system was controlled by an IBM Corporation™ IBM personal computer with a Pentium II processor, 32.0 megabytes of ram, and Windows™ '95. The Beckman P/ACE™ System MDQ Capillary Electrophoresis ©1996 software version 1.6 was used.

Ultraviolet absorption data in ethanol was acquired on a Perkin-Elmer Lambda 11 UV/Vis spectrometer and the wavelengths of maximum absorption were reported in nanometers.

Analytical gas chromatography (GLC) was performed on a Hewlett Packard 5890 equipped with a flame ionization detector (FID) using a 30 m by 0.35 mm DB-5HT column of (5% phenyl) methylpolysiloxane. The carrier gas used was helium with a flow rate of 2.0 mL/min. Ethyl-acetate was used as the solvent.

PREPARATION OF 0.1 M SODIUM BORATE BUFFER:

Boric Acid (1.2366 g, 0.02 mol) was dissolved in E-pure water (100 mL) to make up a solution of 0.2 M H_3BO_3 in a 250 mL Erlenmeyer flask. The solution was titrated with NaOH solution (5.4 mL, 1.0 M) to a pH of 8.51.

DERIVATIZATION OF AMINO ACIDS [1]:

Amino Acid solutions (L/D-Ala, L/D-Asn, L/D-Phe, L/D-Thr; 0.05 M) were prepared by dissolving the amino acids in E-pure water. L-Marfey's reagent (Nα-(2,4-dinitro-5-fluorophenyl)-L-alaninamide, 0.01 g) was dissolved in reagent grade acetone (1 mL) to produce a 1% solution.

L-Marfey's reagent (140 µL, 1% solution), amino acid (100 µL, 0.05 M) and $NaHCO_3$ (40 µL, 1.0 M) were initially stirred in a 25 mL round bottom flask at 55°- 62 °C in a heating mantle for 15 minutes. Later this experiment utilized 15 x 45 mm vials with screw caps and a more reliable heating source in the form of a heating block (58 °C). After cooling to room temperature, HCl (40 µL, 1.0 M) was added to neutralize the solution. Sodium borate buffer (680 µL, 0.1 M) was used to dilute the product for analysis.

Dansyl-chloride (1% in acetone) was also substituted for L-Marfey's reagent as a UV positive indicator.

ACQUIRING AMINO ACID SAMPLES FROM MODERN BONE [App.2-3]:

A modern bone sample (cow jaw) was acquired and a square inch of its exterior cleaned with course sandpaper. Several large pieces of fresh aluminum foil were placed underneath the bone. An ⅛ inch small sterile drill bit was attached to a Dremel® [770 Type 1, 2 speed, 7.2 V, 7500 rpm] and the cleaned portion of the bone drilled to remove the bone material. About 200 mg of bone shavings were collected on the aluminum foil.

Next, a filter-funnel apparatus was set up so as to suspend a 20 M Hirsch® funnel (capped at its bottom with a rubber stopper) inside a 250 mL beaker. A custom cut piece of filter paper was fitted into the funnel and the funnel filled with 10 mL of 0.25 N HCl and 200 mg of modern bone shavings. The funnel was covered in tinfoil and the entire apparatus placed into a Ziploc™ freezer bag, sealed and stored in a fridge at 4 °C for 24 hours.

The acid was filtered off by removing the rubber stopper, placing the Hirsch® funnel into a 250 mL Erlenmeyer vacuum flask, and attaching a vacuum pump. The walls of the funnel were washed down with 0.25 N HCl, then, another 10 mL of 0.25 N HCl was added. The rubber stopper was replaced and the apparatus stored in the fridge for 24 hours as per the above conditions.

The acid was filtered off as per the above protocol. Ten millilitres of 0.01 N HCl was added to the funnel which was covered with a medium porous material and placed in a dry oven at 58 °C for 24 hours. This solution was collected by placing the bottom of the funnel inside a 10 mL test tube contained within a vacuum flask apparatus and repeating the filtering protocol.

The test tube contents were transferred to a Greiner® 10000 MWCO centrtifuge tube with filter. The solution was centrifuged at 5000 rpm for 15-20 minutes until the collagen became

viscous with a slight amber colour. The centrifuge tube was inverted and spun for 1 minute at 1000 rpm. Then, the product was derivatized as a mixture of amino acids using L-Marfey's Reagent.

RUN (SEPARATION) BUFFER:

The separation buffer prepared for each run of derivatized amino acid was prepared by mixing 20% HS-(α, β or γ)-CD aqueous solution (supplied by Beckman®), E-pure water and sodium borate buffer pH 8.5 in a 1:1:2 ratio respectively. γ-CD seemed to yield slightly better results (better peak resolution).

P/ACE™ SYSTEM MDQ Capillary Electrophoresis:

HCl (0.1 M), Separation buffer (5% HS-CD), E-pure H_2O and capillary regenerator solution (0.1 M NaOH) were each placed in 2 mL glass vials, capped with a rubber septum, placed into the *buffer* tray and put into the CE system. The dilute, derivatized amino acids (~5 mM) were placed in PCR sample vials, capped with rubber septa, placed into the *sample* tray and put into the CE system. Four different recommended methods for running CE were used to optimize peak results.

method 1 [5]: T = 22 °C, UV = 214 nm

EVENT	VALUE	DURATION	SUMMARY
capillary run			
Rinse - pressure	25.0 psi	1.0 min	run buffer, fwd
Inject - pressure	0.4 psi	7.0 sec	sample
Wait			H_2O dip - clean capillary ends
Separate - voltage	20.0 KV	30.0 min	run buffer, reverse polarity
Rinse - pressure	20.0 psi	2.0 min	0.1 M HCl, fwd
Rinse - pressure	20.0 psi	2.0 min	0.1 M HCl, fwd
Rinse - pressure	20.0 psi	1.0 min	H_2O, fwd
Rinse - pressure	20.0 psi	2.0 min	run buffer, fwd

method 2 [9]: T = 22 °C, UV = 214 nm

EVENT	VALUE	DURATION	SUMMARY
capillary equilibration			
Rinse - pressure	25.0 psi	1.0 min	capillary conditioning sol'n, fwd
Rinse - pressure	25.0 psi	1.0 min	H_2O, fwd
Rinse - pressure	25.0 psi	3.0 min	separation buffer, fwd
capillary run			
Rinse - pressure	20.0 psi	1.0 min	run buffer, fwd
Inject - pressure	0.3 psi	4.0 sec	sample
Wait			H_2O dip - clean capillary ends
Separate - voltage	15 KV	5.0 min	run buffer - reverse polarity
Wait			H_2O dip - clean capillary ends
Rinse - pressure	20.0 psi	2.0 min	H_2O

method 3 [6]: T = 20 °C, UV = 214 nm

EVENT	VALUE	DURATION	SUMMARY
capillary equilibration			
Rinse - pressure	20.0 psi	0.5 min	0.1 N HCl, fwd
Rinse - pressure	20.0 psi	2.0 min	H_2O, fwd
Rinse - pressure	20.0 psi	2.0 min	run buffer, fwd
Wait		1.5 min	
Rinse - pressure	20.0 psi	2.0 min	run buffer, fwd
Wait		1.5 min	
Rinse - pressure	20.0 psi	2.0 min	run buffer, fwd
Wait		1.5 min	
Rinse - pressure	20.0 psi	2.0 min	run buffer, fwd
Separate - voltage	3 KV	10.0 min	(100V/cm applied electric field)
capillary run			
Inject - pressure	0.5 psi	3.0 sec	sample
Separate - pressure	15 KV	15.0 min	reverse polarity
Rinse - pressure	20.0 psi	0.5 min	0.1 M HCl, fwd
Rinse - pressure	20.0 psi	2.0 min	H_2O, fwd
Rinse - pressure	20.0 psi	2.0 min	run buffer, fwd

method 4 [7]: T = 22 °C, UV = 214 nm

EVENT	VALUE	DURATION	SUMMARY
capillary equilibration			
Rinse - pressure	20.0 psi	0.5 min	0.1 N HCl, fwd
Rinse - pressure	20.0 psi	2.0 min	H_2O, fwd
Rinse - pressure	20.0 psi	5.0 min	run buffer, fwd

EVENT	VALUE	DURATION	SUMMARY
capillary run			
Rinse - pressure	20.0 psi	1.0 min	0.1 N HCl, fwd
Inject - pressure	0.5 psi	7.0 sec	sample
Separation - voltage	15 KV	15.0 min	run buffer, reverse polarity

ANALYTICAL GAS CHROMATOGRAPHY:

Amino acids (L/D-Thr) were dissolved in ethylacetate (2 mg/mL). A 1 μL injection was used on the GC and the samples were run under method 5 conditions. Longer run times were employed for L/D-Phe and L-Thr with 1 μL injections run under method 6 conditions.

method 5: Conditions used for 35 min. run time on the GC.

Initial temperature	80 °C
Initial time	5 min
Initial rate	10°/min
Final temperature	280 °C
Final time	10 min

method 6: Conditions used for 60 min.run time on the GC.

Initial temperature	80 °C
Initial time	5 min
Initial rate	10°/min
Final temperature	280 °C
Final time	25 min

HIGH PRESSURE LIQUID CHROMATOGRAPHY [10]:

HPLC was run on a Beckman System Gold Programmable Solvent Module 126 with an ASEA Brown Boveri SE120 UV detector. A C_{18} reverse phase column was used to separate the derivatized amino acids stored in phosphate buffer pH 3.3. The phosphate buffer consisted of a solution of ammonium dihydrogen orthophosphate (5.7515 g/L, 50 mM) titrated to a pH of 3.3 with H_3PO_4 (~49.4 mL, 0.05 M) to produce the appropriate buffer solution. UV detection of the

derivatized amino acids occurred at 344 nm. Mobile phase A consisted of a 10:40:50 mixture of acetonitrile, methanol and pH 4.2 acetate buffer (3 mL glacial acetic acid, 1 mL triethylamine, 900 mL H_2O, pH adjusted using NaOH solution). The elution program went as follows.

3 min. Elution of 100% A
9 min. Linear gradient from 100% A to 100% B
11 min. Elution of 100% B
A flow rate of 1.0 mL/min.

REFERENCES

1. Service, R.F. *Science* **1996**, *272*, 810.

2. Poinar, H.N.; Höss, M.; Bada, J.L.; Pääbo, S. *Science* **1996**, *272*, 864.

3. Kotz, J.C.; Purcell, K.F. *"Chemistry and Chemical Reactivity"*; Saunders College Publishing, 1991, 1148.

4. Zhao, M.; Bada, J.L. *Journal of Chromatography* **1995**, *A690*, 55.

5. Tran, A.D.; Blanc, T.; Leopold, E.J. *Journal of Chromatography* **1990**, *516*, 241.

6. eCAP Chiral Methods Development Kit *"Lab VII. CHIRAL SEPARATIONS: MDQ Separation Conditions"*.

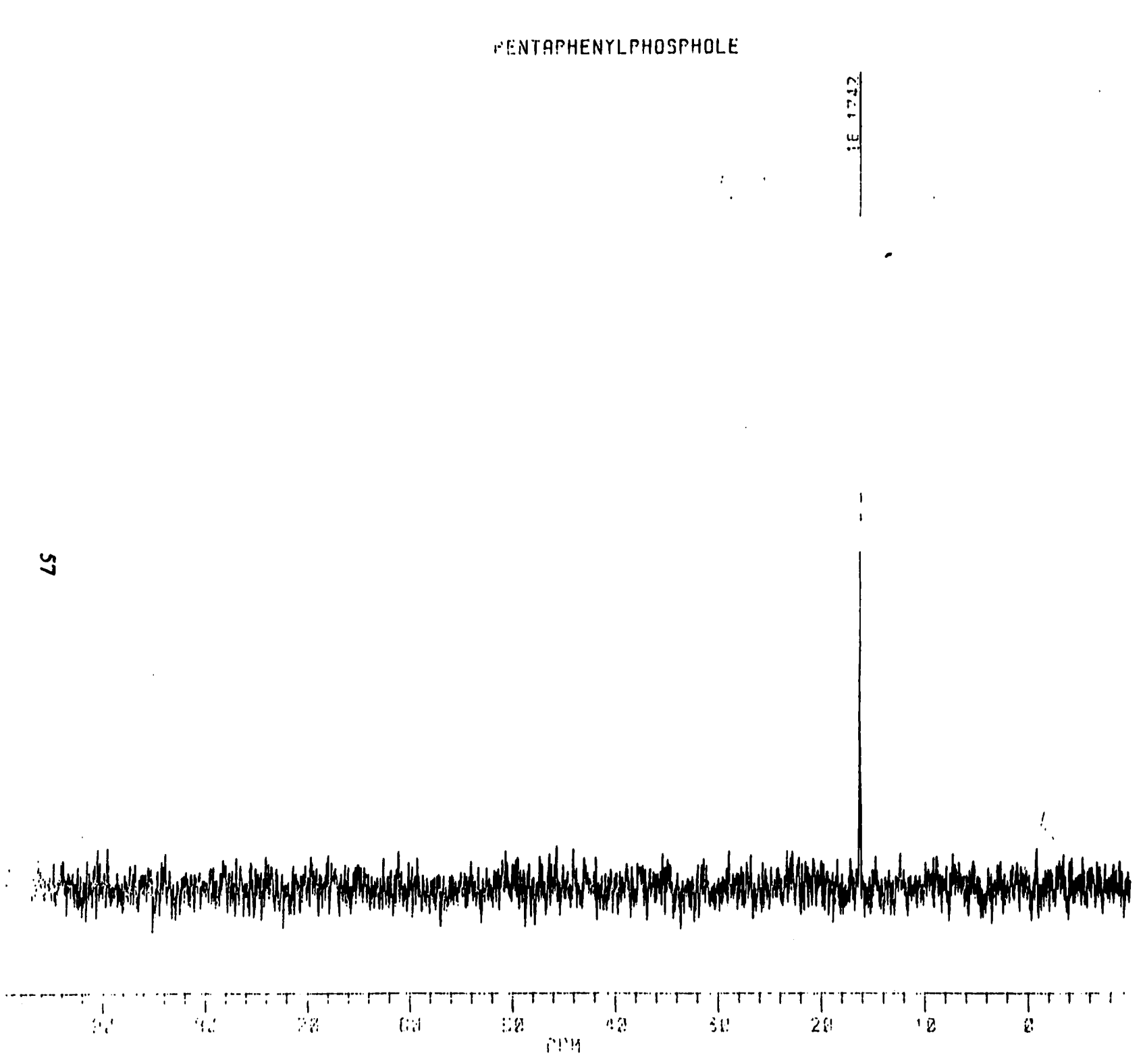
7. Beckman® *"P/ACE™ System MDQ Chiral Methods Development Kit"*; Beckman Instructions 725827-AB, Beckman Instruments Inc., 1997.

8. Beckman® *"P/ACE System MDQ User's Guide"*; Beckman Instruments, Inc., Biotechnology Development Center, Fullerton, CA, 1996.

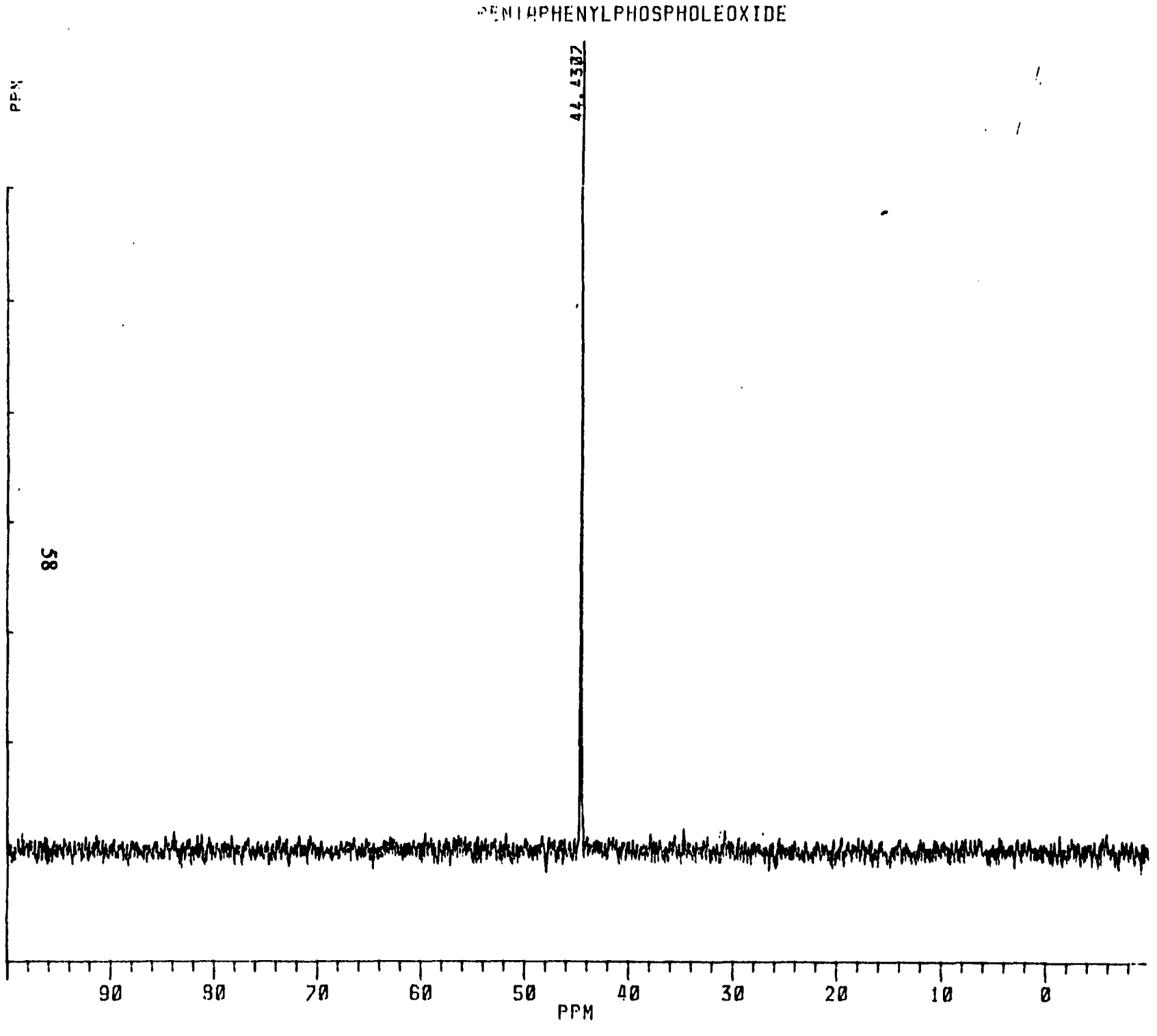
9. Beckman® *"Highly Sulfated Cyclodextrins for P/ACE™ Capillary Electrophoresis System"*; Beckman Instructions 725869-AB.

10. Clapp, C.H.; Swan, J.S.; Poechmann, J.L. *Journal of Chemical Education* **1990**, *69:40*, A122.

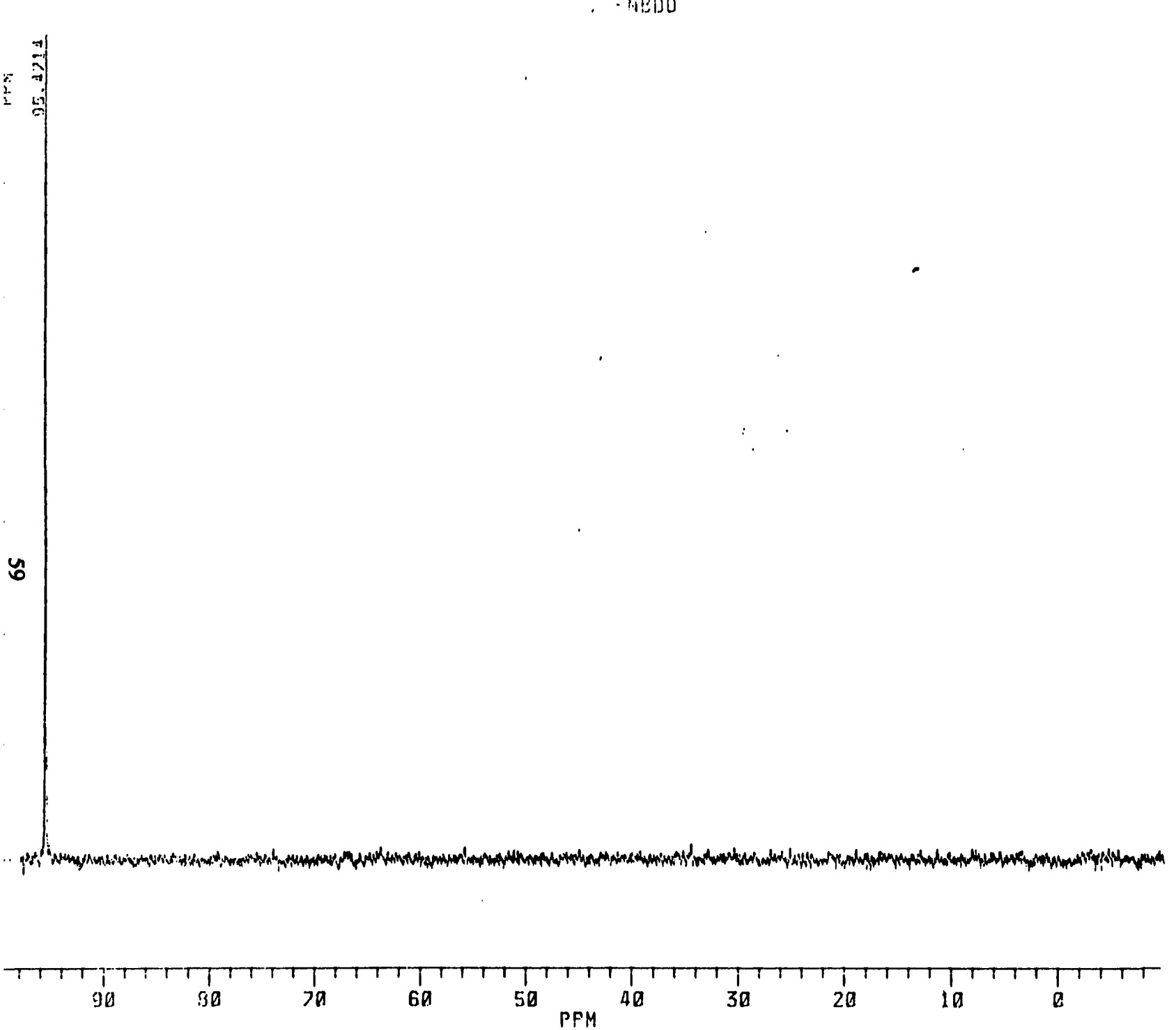
APPENDICES



Appendix 1-1. ^{31}P NMR spectra of pentaphenylphosphole in $CDCl_3$.



Appendix 1-2. ^{31}P NMR spectra of pentaphenylphosphole-oxide in $CDCl_3$.



BRUKER

SWP8K30.S01
DATE 30-11-98

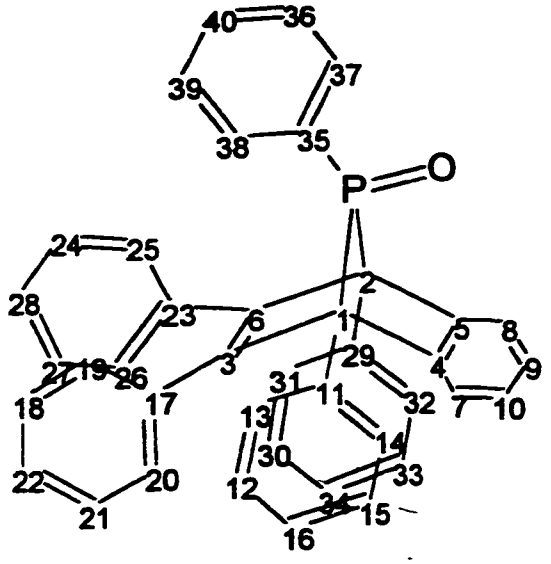
SF 81.015
SY 81.0140000
O1 10000.000
SI 32768
TD 32768
SW 31250.000
HZ/PT 1.907

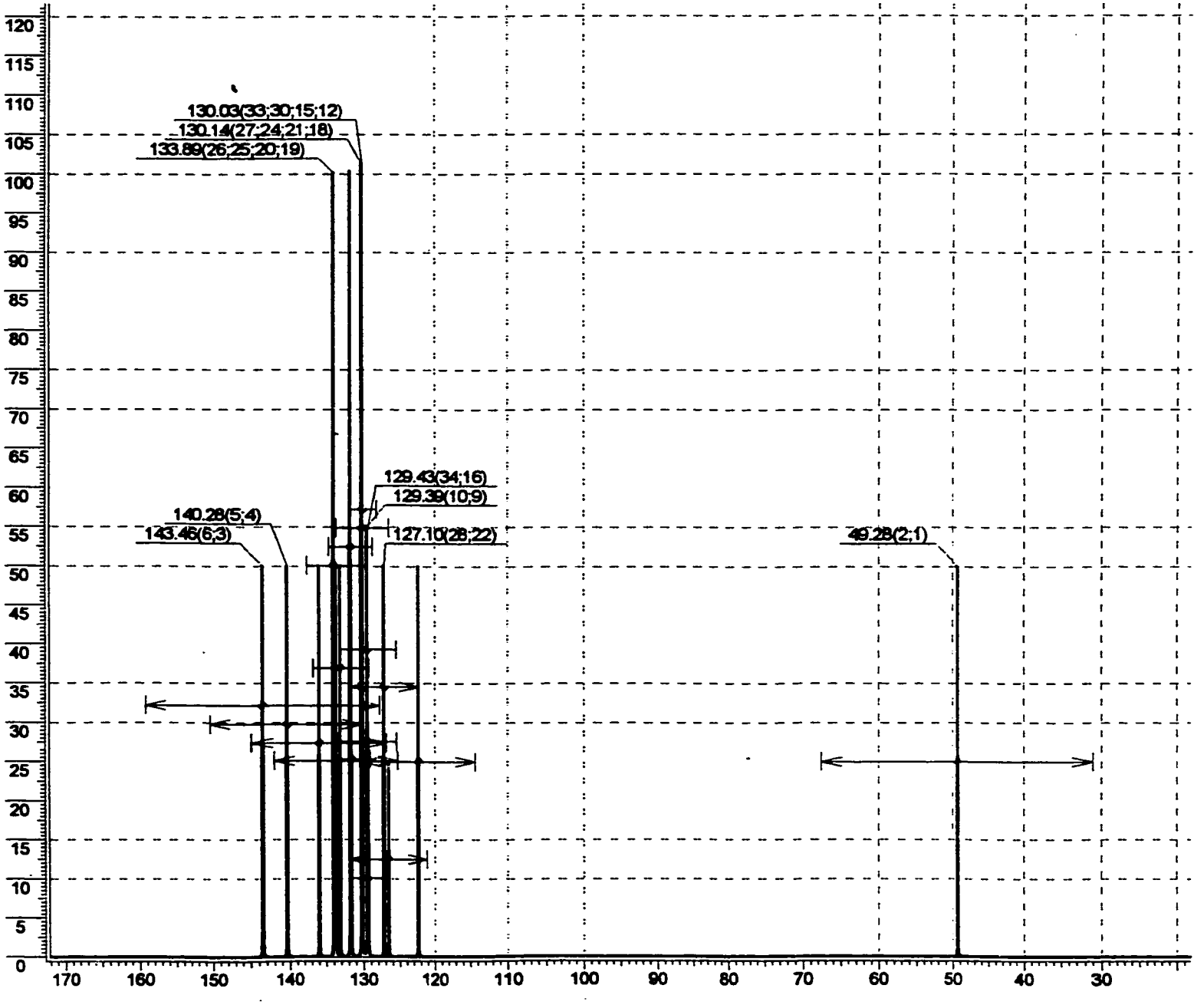
PW 4.0
RD 2.000
AQ .524
RG 4
NS 64
TE 297

FW 39100
O2 3200.000
DP 12L PO

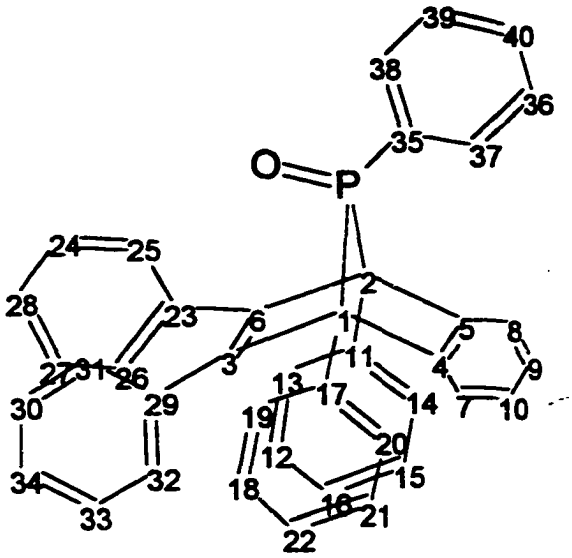
LB 3.000
GB .100
CX 22.00
CY 12.00
F1 100.011P
F2 -9.982P
HZ/CM 405.051
PPM/CM 5.000
SR 1054.53

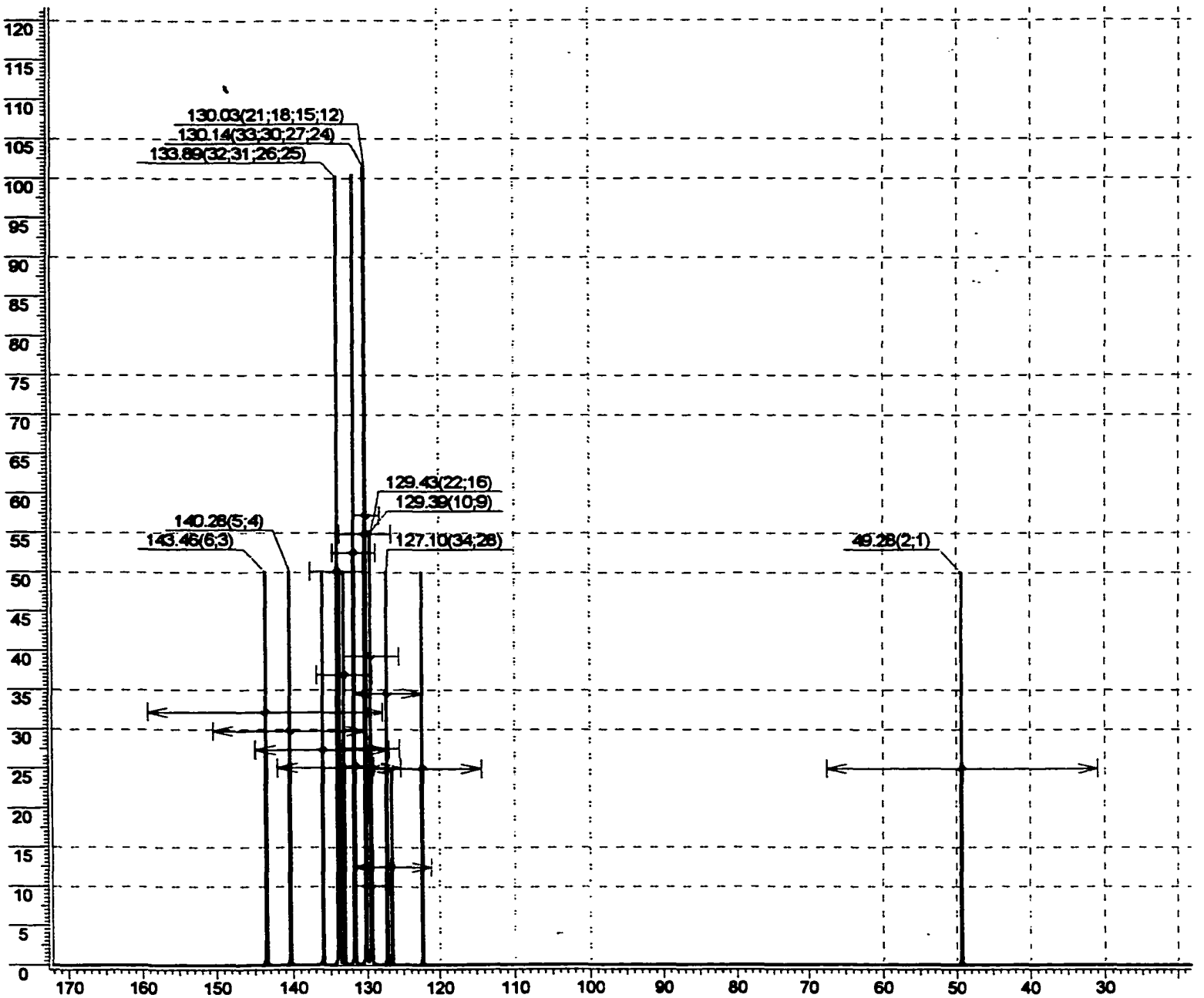
Appendix 1-3. ^{31}P NMR spectra of 2,3-benzo-1,4,5,6,7-pentaphenyl-7-phosphabicyclo[2.2.1]hept-5-ene-oxide (7-PNBDO) in C_6D_6.



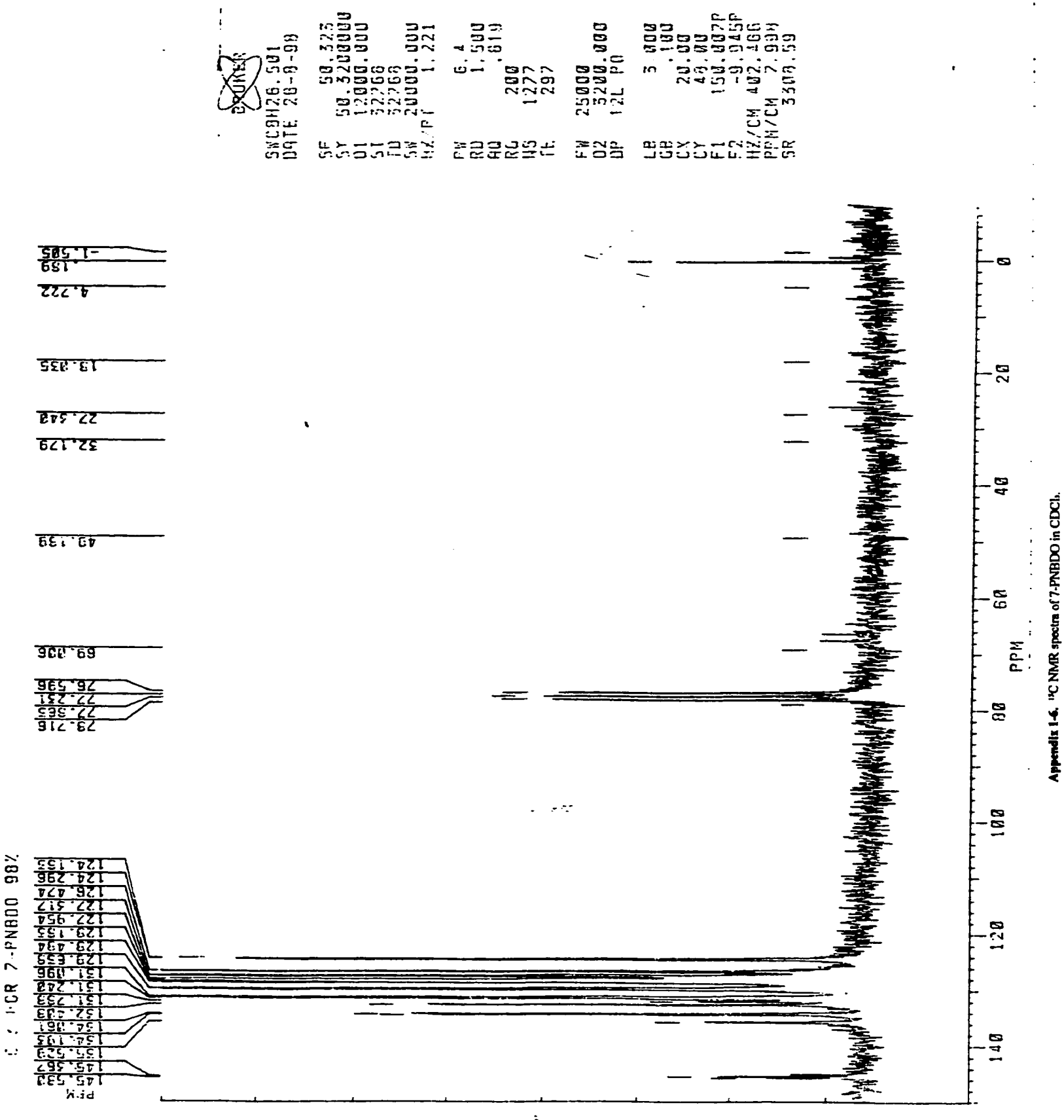


Appendix 1-4. Predicted ^{13}C NMR spectra for one possible stereoisomer of 7-PNBDO within a ± 20 ppm error range.

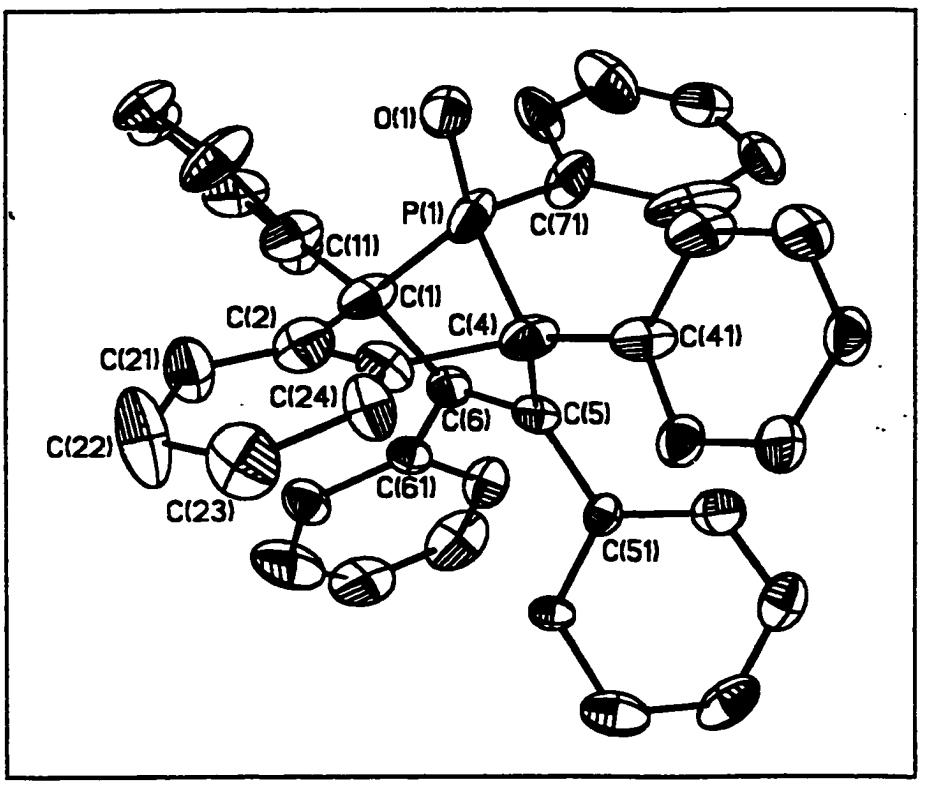




Appendix 1-5. Predicted ^{13}C NMR spectra for the other possible stereoisomer of 7-PNBDO within a ± 20 ppm error range.



Appendix I-6. ^{13}C NMR spectra of 7-PNBDO in $CDCl_3$.



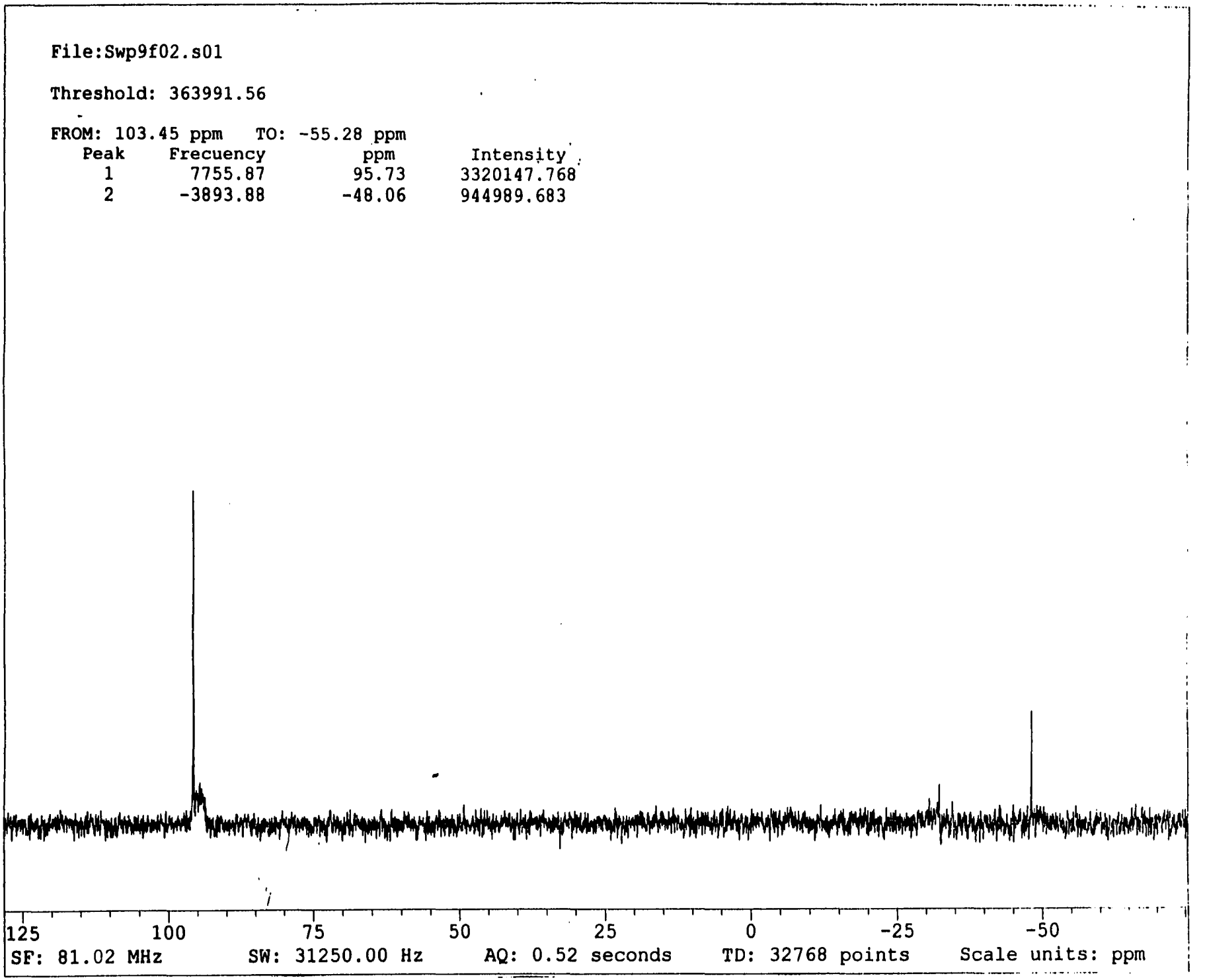
Appendix 1-7. Crystal structure of 7-phosphanorbornadiene-oxide (5).

TABLE 1-2. Crystallographic Collection and Refinement Parameters, for 1·C_6H_6.

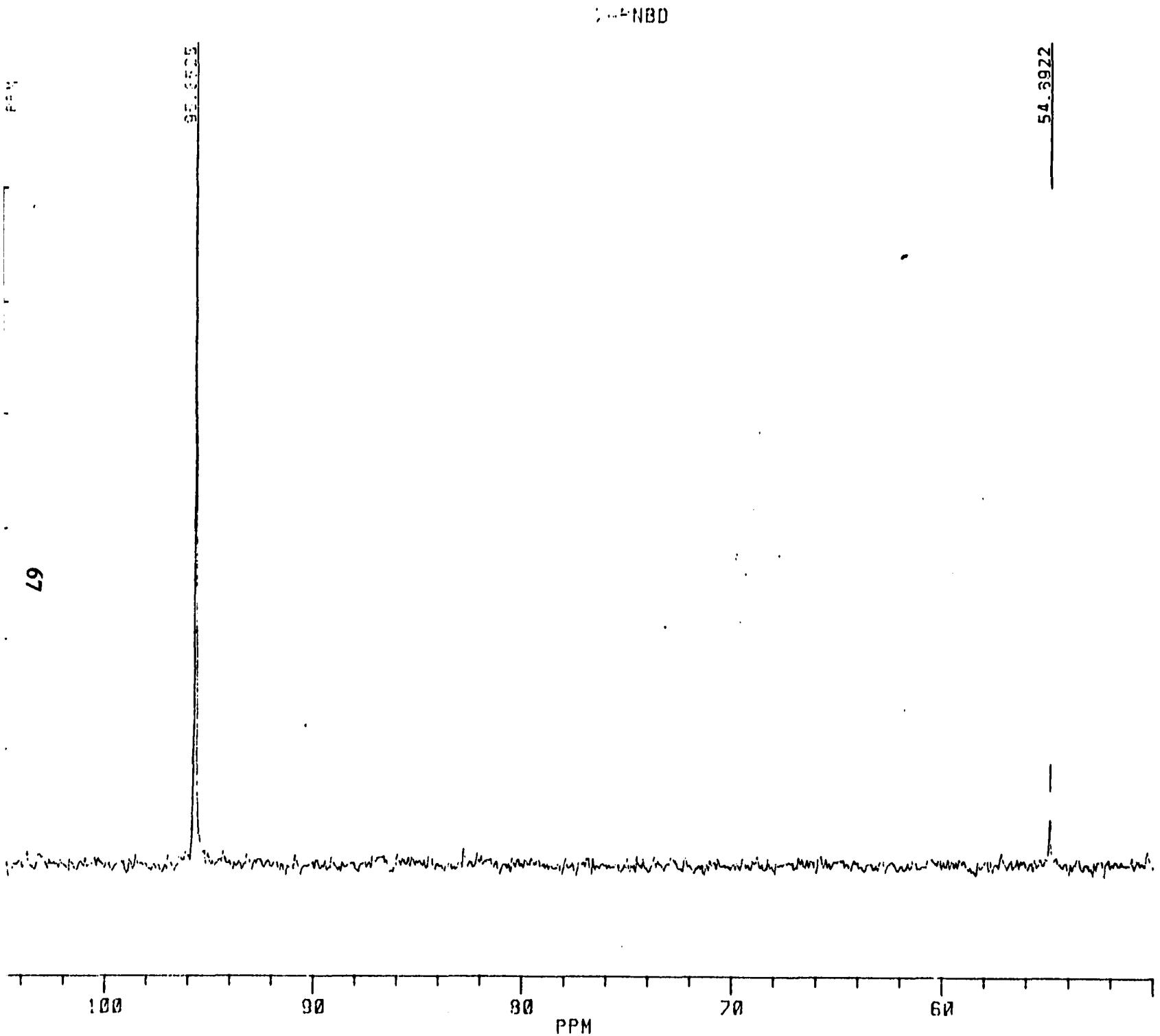
Empirical formula	$C_{46}H_{35}OP$
Molecular Weight	1269.42
Description	yellow prism
Size, mm^3	0.34 x 0.30 x 0.25
Temperature, K	299(2)
Crystal system	monoclinic
Space group	*P*n
a, Å	17.544(1)
b, Å	10.2166(8)
c, Å	19.524(1)
α, deg.	90
β, deg.	90.709(3)
γ, deg.	90
Volume, $Å^3$	3499.3(4)
Z	2
Calcd Density, g/cm3	1.205
Scan Mode	ω-scans
F(000)	1336
θ-range, deg.	1.57 to 22.50
Index ranges	$-21 \le h \le 21$
	$-12 \le k \le 12$
	$-23 \le l \le 24$
No. Refl. Collected	20716
No. Indep. Refl.	8231
Rint	0.0678
Data / restr. / param.	8220 / 8 / 812
GOF on F^2 (all)	0.825
Final R ($I>2\sigma(I)$)*	R1 = 0.0714;
	wR2 = 0.1882
R indices (all data)*	R1 = 0.1541;
	wR2 = 0.2469
Trans., (max., min.)	0.9734, 0.7752
Largest diff. Peak, e/$Å^3$	0.490
Largest diff. Hole, e/$Å^3$	-0.256

TABLE 1-3. Selected bond lengths [Å] and angles [deg.] for both of the independent molecules of 1-C_6H_6.

P(1)-O(1)	1.440(6)	C(101)-P(2)-C(104)	86.1(4)
P(1)-C(4)	1.75(1)	O(2)-P(2)-C(171)	110.9(4)
P(1)-C(1)	1.78(1)	C(101)-P(2)-C(171)	108.5(5)
P(1)-C(71)	1.95(1)	C(104)-P(2)-C(171)	105.4(5)
P(2)-O(2)	1.496(6)	C(11)-C(1)-C(6)	123.5(9)
P(2)-C(101)	1.82(1)	C(11)-C(1)-C(2)	117.3(11)
P(2)-C(104)	1.87(1)	C(6)-C(1)-C(2)	104.3(8)
P(2)-C(171)	1.87(1)	C(11)-C(1)-P(1)	116.7(7)
C(1)-C(2)	1.66(2)	C(6)-C(1)-P(1)	97.7(7)
C(2)-C(3)	1.28(2)	C(2)-C(1)-P(1)	91.2(8)
C(3)-C(4)	1.73(2)	C(41)-C(4)-C(5)	114.7(8)
C(4)-C(5)	1.61(1)	C(41)-C(4)-C(3)	121.4(9)
C(5)-C(6)	1.35(1)	C(5)-C(4)-C(3)	104.6(7)
C(1)-C(6)	1.55(1)	C(41)-C(4)-P(1)	123.2(7)
C(101)-C(102)	1.57(2)	C(5)-C(4)-P(1)	99.5(6)
C(102)-C(103)	1.359(14)	C(3)-C(4)-P(1)	88.7(6)
C(103)-C(104)	1.57(2)	C(111)-C(101)-C(102)	119.3(8)
C(104)-C(105)	1.54(1)	C(111)-C(101)-C(106)	116.3(8)
C(105)-C(106)	1.33(1)	C(102)-C(101)-C(106)	108.5(8)
C(101)-C(106)	1.60(1)	C(111)-C(101)-P(2)	119.8(7)
O(1)-P(1)-C(4)	121.4(5)	C(102)-C(101)-P(2)	91.9(6)
O(1)-P(1)-C(1)	125.1(4)	C(106)-C(101)-P(2)	96.5(6)
C(4)-P(1)-C(1)	91.4(5)	C(141)-C(104)-C(105)	123.5(8)
O(1)-P(1)-C(71)	108.3(6)	C(141)-C(104)-C(103)	119.7(9)
C(4)-P(1)-C(71)	108.2(6)	C(105)-C(104)-C(103)	106.8(7)
C(1)-P(1)-C(71)	99.5(6)	C(141)-C(104)-P(2)	110.8(6)
O(2)-P(2)-C(101)	119.9(5)	C(105)-C(104)-P(2)	94.1(6)
O(2)-P(2)-C(104)	123.2(4)	C(103)-C(104)-P(2)	95.0(6)



Appendix 1-8. ^{31}P NMR spectra using Mestrec® reveals a slight debridging of the 7-PNBDO molecule in benzene.



BRUKER

SWP9913.512
DATE 13-1-99

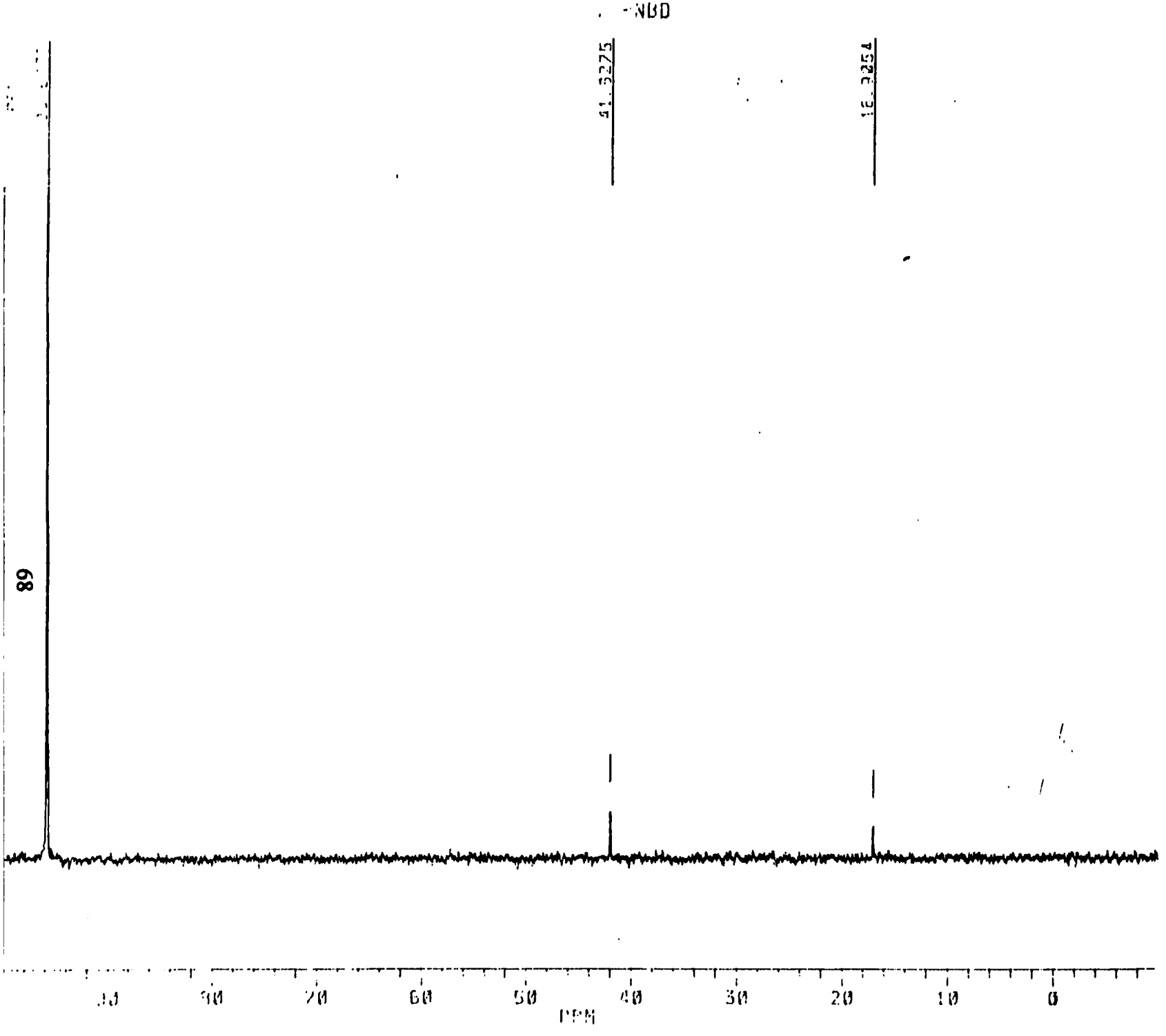
SF 91.015
SY 91.0140000
O1 10000.000
SI 32768
TD 32768
SW 100000.000
HZ/PT 6.104

PW 4.0
RD 2.000
AQ .164
RG 4
NS 64
TE 297

FW 250000
O2 3200.000
DP 12L PD

LB 3.000
GB .100
CX 22.00
CY 12.00
F1 104.993P
F2 49.996P
HZ/CM 202.526
PPM/CM 2.500
SR 1054.53

Appendix 1-9. ^{31}P NMR spectra showing a small peak at δ+54$_{ppm}$ via a trichlorosilane/pyridine reduction in benzene at low temperature.



BRUKER

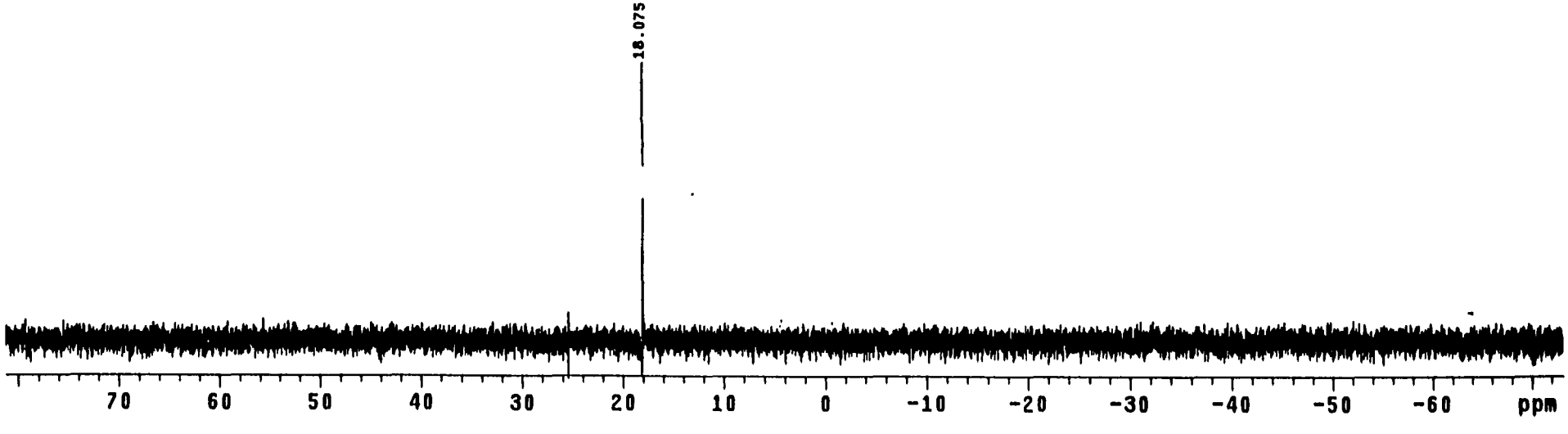
```
SWP31.001
DATE 9-7-99

SF      81.015
SY   81.0140000
O1   10000.000
SI   32768
TD   32768
SW   31250.000
HZ/PT    1.907

PW       4.0
RD       2.000
AQ        .524
RG     400
NS     208
TE     297

FW   39100
O2    3200.000
DP   12L PO

LB       3.000
GB        .100
CX      22.00
CY      12.00
F1     100.011P
F2      -9.982P
HZ/CM 405.051
PPM/CM  5.000
SR    1054.53
```

Appendix 1-10. ^{31}P NMR spectra of a phenylsilane/pyridine reduction on 7-PNBDO in benzene.

CHDA/HSIC13/7-PNBDO

exp1 s2pul

SAMPLE		SPECIAL	
date	Jul 12 2000	temp	22.0
solvent	C6D6	gain	not used
file	exp	spin	not used
ACQUISITION		hst	0.008
sw	68787.6	pw90	9.000
at	1.600	alfa	10.000
np	220120	FLAGS	
fb	38000	il	n
bs	64	in	n
ss	2	dp	y
d1	2.000	hs	ny
nt	512	PROCESSING	
ct	512	lb	1.00
TRANSMITTER		fn	not used
tn	P31	DISPLAY	
sfrq	202.294	sp	-14808.1
tof	11137.3	wp	31239.4
tpwr	59	rf1	28325.2
pw	3.500	rfp	0
DECOUPLER		rp	303.2
dn	H1	lp	-495.3
dof	0	PLOT	
dm	yyy	wc	250
dmm	w	sc	0
dpwr	41	vs	486
dmf	11696	th	13
		at	1
		cdc	
		ph	

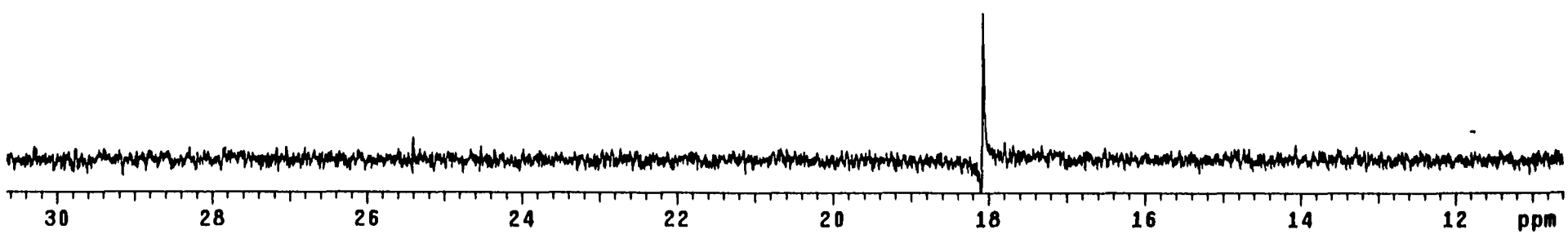


Appendix 1-11. ^{31}P NMR spectra of a reduction of 7-PNBDO using trichlorosilane and cyclohexyldiethylamine in acetonitrile at 30°C.

CHDA/HSIC13/7-PNBDO at 43C

exp1 s2pul

SAMPLE		SPECIAL	
date	Jul 14 2000	temp	22.0
solvent	C6D6	gain	not used
file	exp	spin	not used
ACQUISITION		hst	0.008
sw	66778.0	pw90	9.000
at	1.600	alfa	10.000
np	213690	FLAGS	
fb	37000	il	n
bs	64	in	n
ss	2	dp	y
d1	2.000	hs	ny
nt	516	PROCESSING	
ct	516	lb	1.00
TRANSMITTER		fn	not used
tn	P31	DISPLAY	
sfrq	202.295	sp	2141.8
tof	12148.7	wp	4059.0
tpwr	59	rf1	26308.9
pw	3.500	rfp	0
DECOUPLER		rp	291.5
dn	H1	lp	-480.8
dof	0	PLOT	
dm	yyy	wc	250
dmm	w	sc	0
dpwr	41	vs	376
dmf	11696	th	8
		al cdc ph	

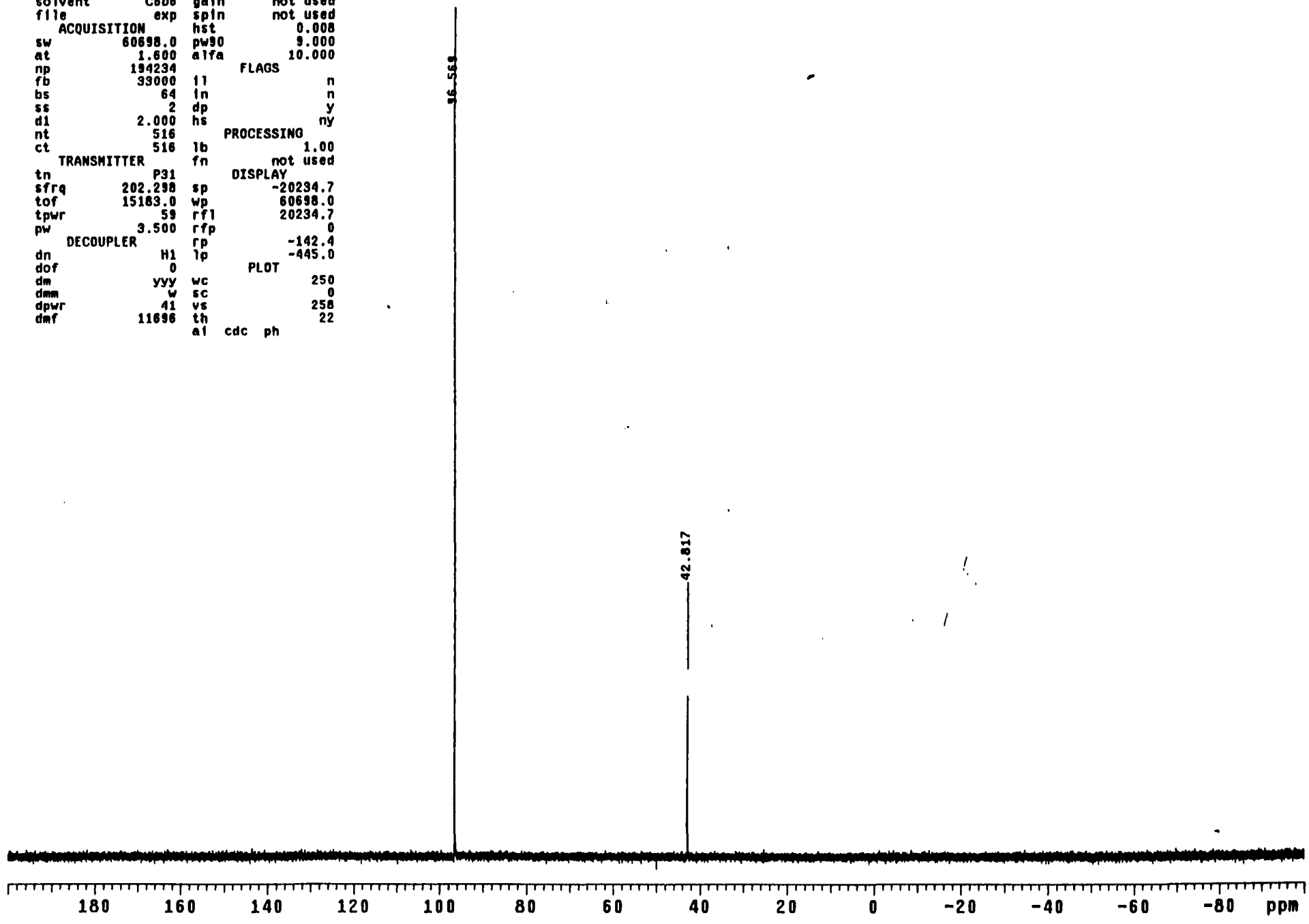


Appendix 1-12. ^{31}P NMR spectra of a reduction of 7-PNBDO using trichlorosilane and cyclohexyldiethylamine in acetonitrile at 43°C.

7-PNBDO/CHDA/PhSiH3
yellow ppt.

exp1 s2pul

SAMPLE		SPECIAL	
date	Jul 11 2000	temp	29.0
solvent	C6D6	gain	not used
file	exp	spin	not used
ACQUISITION		hst	0.008
sw	60698.0	pw90	9.000
at	1.600	alfa	10.000
np	194234	FLAGS	
fb	33000	il	n
bs	64	in	n
ss	2	dp	y
d1	2.000	hs	ny
nt	516	PROCESSING	
ct	516	lb	1.00
TRANSMITTER		fn	not used
tn	P31	DISPLAY	
sfrq	202.298	sp	-20234.7
tof	15183.0	wp	60698.0
tpwr	59	rfl	20234.7
pw	3.500	rfp	0
DECOUPLER		rp	-142.4
dn	H1	lp	-445.0
dof	0	PLOT	
dm	yyy	wc	250
dmm	w	sc	0
dpwr	41	vs	258
dmf	11696	th	22
		ai cdc ph	



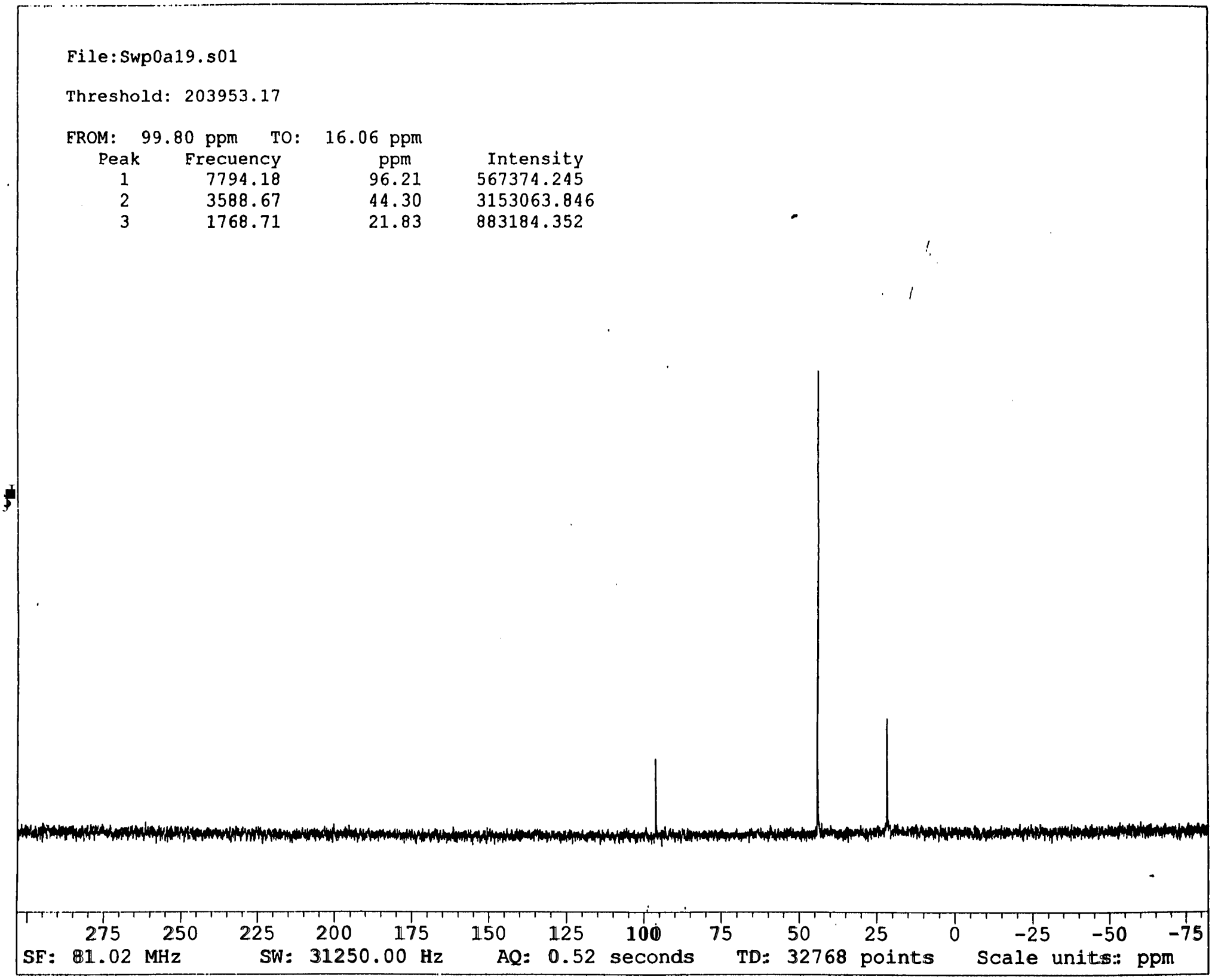
Appendix 1-13. ^{31}P NMR spectra of a reduction of 7-PNBDO using phenylsilane and cyclohexyldiethylamine in acetonitrile.

File:Swp0a19.s01

Threshold: 203953.17

FROM: 99.80 ppm TO: 16.06 ppm

Peak	Frecuency	ppm	Intensity
1	7794.18	96.21	567374.245
2	3588.67	44.30	3153063.846
3	1768.71	21.83	883184.352



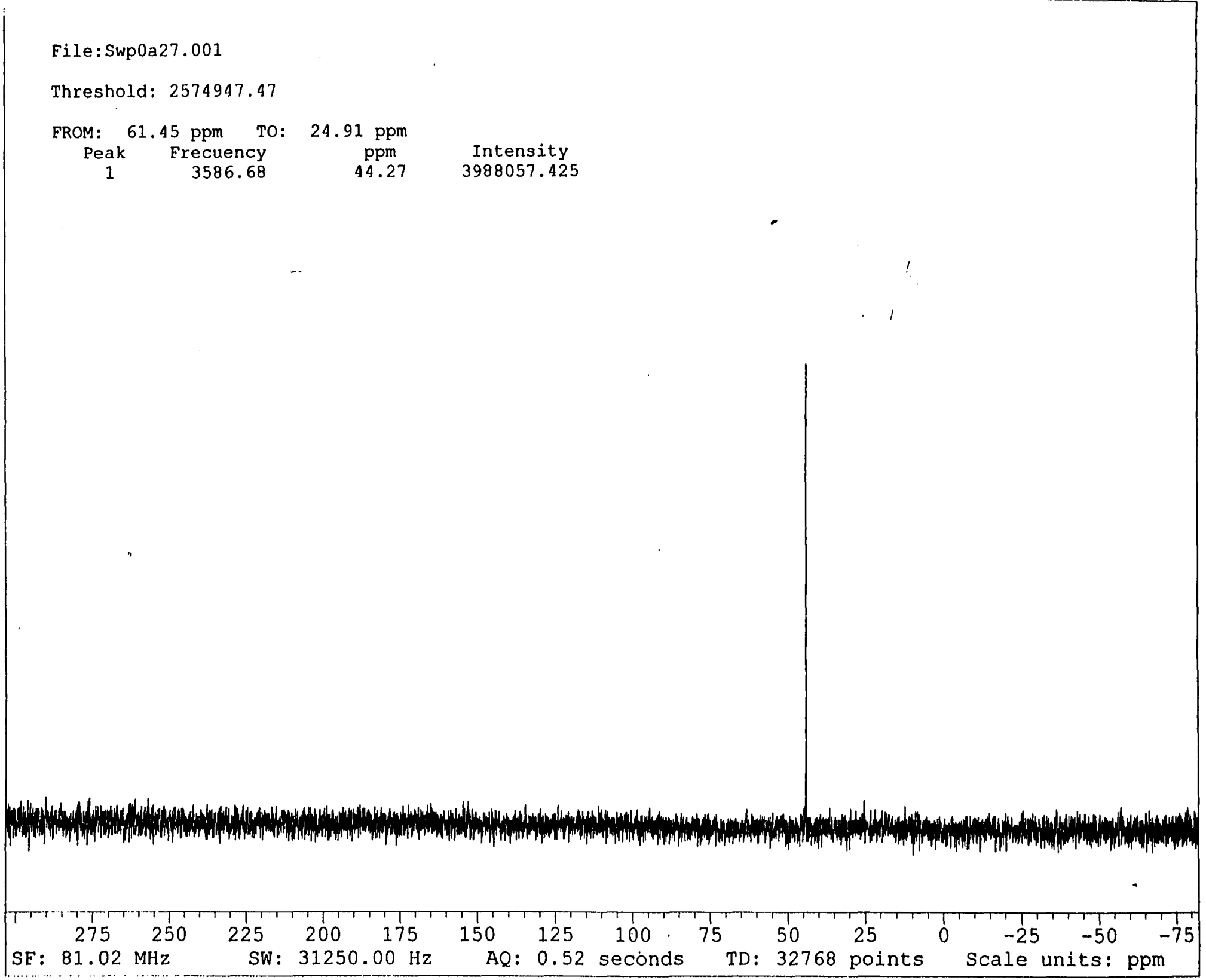
Appendix 1-14. ^{31}P NMR spectra of the products of a reduction of 7-PNBDO using titanocene dichloride/Mg in benzene, initial stages of reaction.

File:Swp0a27.001

Threshold: 2574947.47

FROM: 61.45 ppm TO: 24.91 ppm

Peak	Frecuency	ppm	Intensity
1	3586.68	44.27	3988057.425

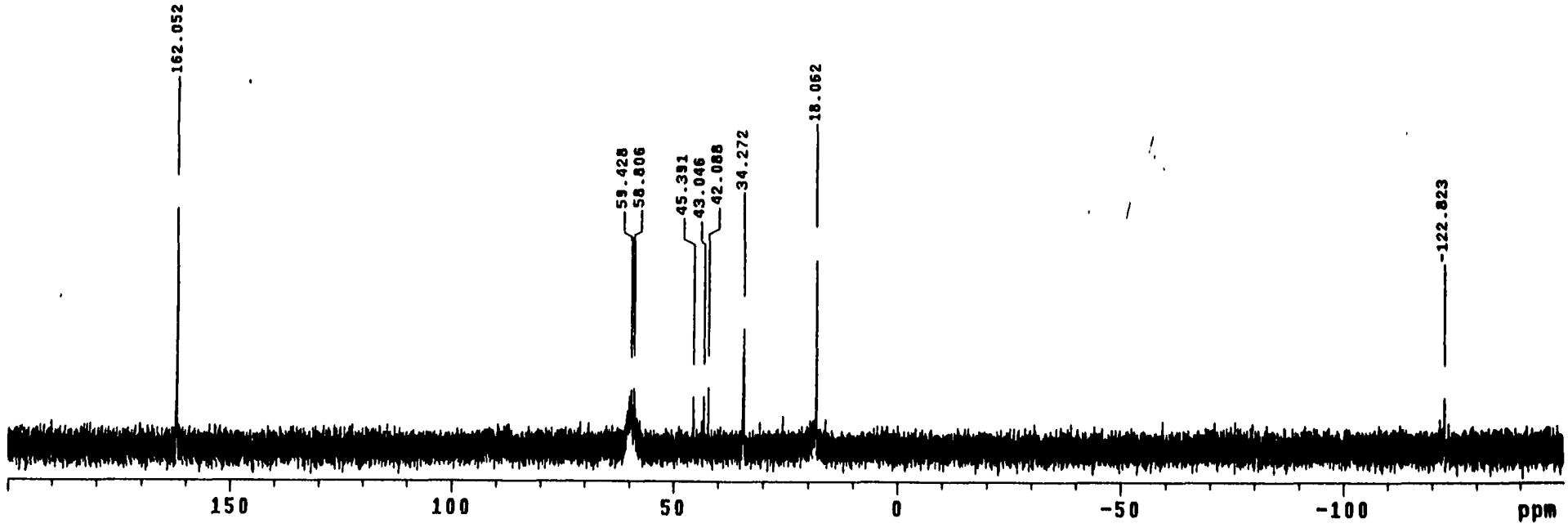


Appendix 1-15. ^{31}P NMR spectra of the products of a reduction of 7-PNBDO using titanocene dichloride/Mg in benzene, final stages of reaction.

Coupled 7-PNBD experiment spectru
m

expl s2pul

SAMPLE		SPECIAL	
date	Jul 12 2000	temp	22.0
solvent	C6D6	gain	not used
file	exp	spin	not used
ACQUISITION		hst	0.008
sw	70796.5	pw90	9.000
at	1.600	alfa	10.000
np	226548	FLAGS	
fb	39000	il	n
bs	64	in	n
ss	2	dp	y
d1	2.000	hs	ny
nt	768	PROCESSING	
ct	768	lb	1.00
TRANSMITTER		fn	not used
tn	P31	DISPLAY	
sfrq	202.293	sp	-30341.0
tof	10125.8	wp	70796.5
tpwr	59	rfl	30341.0
pw	3.500	rfp	0
DECOUPLER		rp	386.4
dn	H1	lp	-549.7
dof	0	PLOT	
dm	ynn	wc	250
dmm	w	sc	0
dpwr	41	vs	642
dmf	11696	th	5
		al	cdc ph

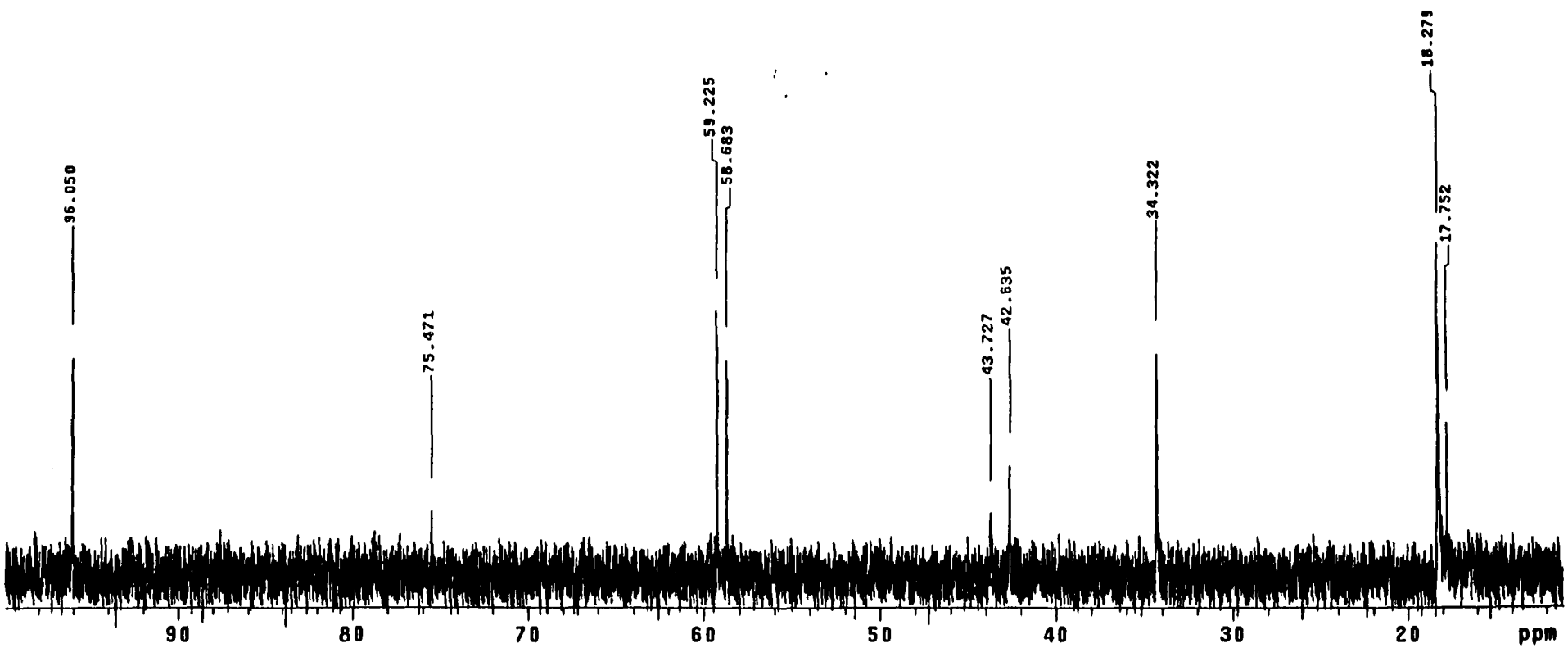


Appendix 1-16. ^{31}P NMR spectra of the reduction of 7-PNBDO with trichlorosilane in benzene.

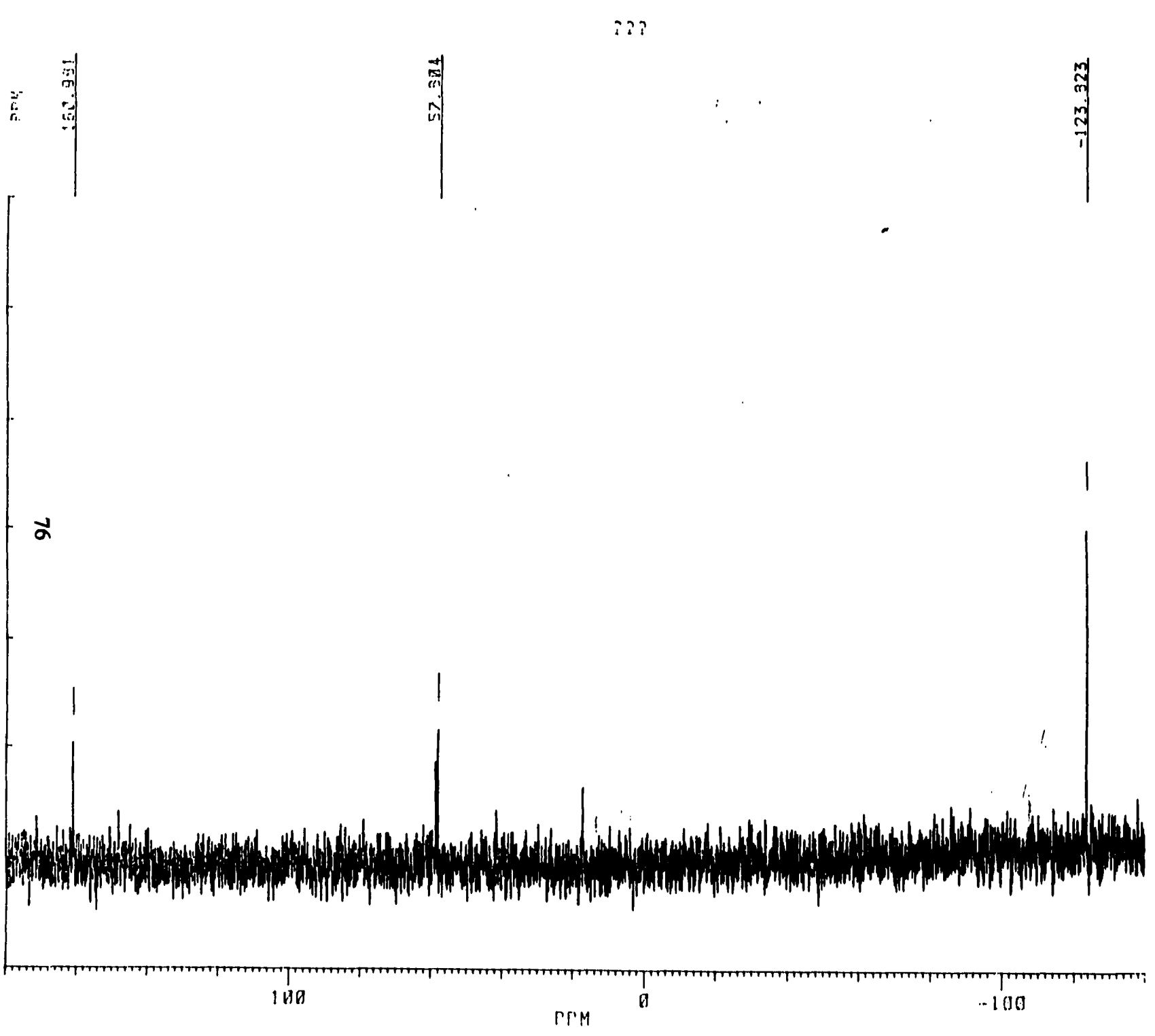
7-PNBDO/HSiCl3/pyridine

exp1 s2pul

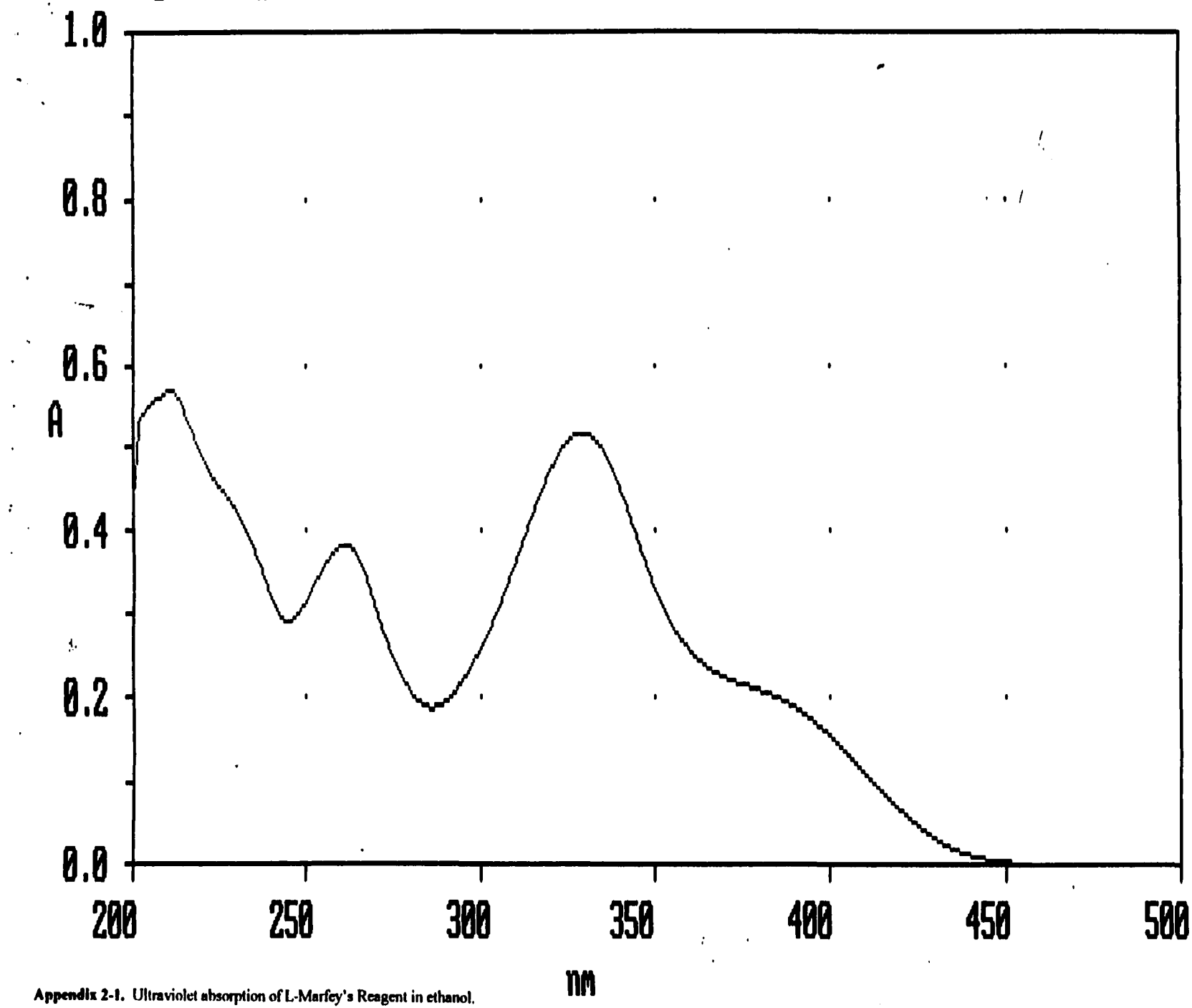
SAMPLE		SPECIAL	
date	Aug 10 2000	temp	5.0
solvent	C6D6	gain	not used
file	exp	spin	not used
ACQUISITION		hst	0.008
sw	76849.2	pw90	9.000
at	1.600	alfa	10.000
np	245918	FLAGS	
fb	42000	il	n
bs	64	in	n
ss	2	dp	y
d1	2.000	hs	ny
nt	1200	PROCESSING	
ct	1200	lb	1.00
TRANSMITTER		fn	not used
tn	P31	DISPLAY	
sfrq	202.290	sp	2224.3
tof	7091.5	wp	17982.3
tpwr	59	rfl	36401.7
pw	3.500	rfp	0
DECOUPLER		rp	-105.2
dn	H1	lp	-374.3
dof	0	PLOT	
dm	yyy	wc	250
dmm	w	sc	0
dpwr	41	vs	1597
dmf	11696	th	8
		al cdc ph	



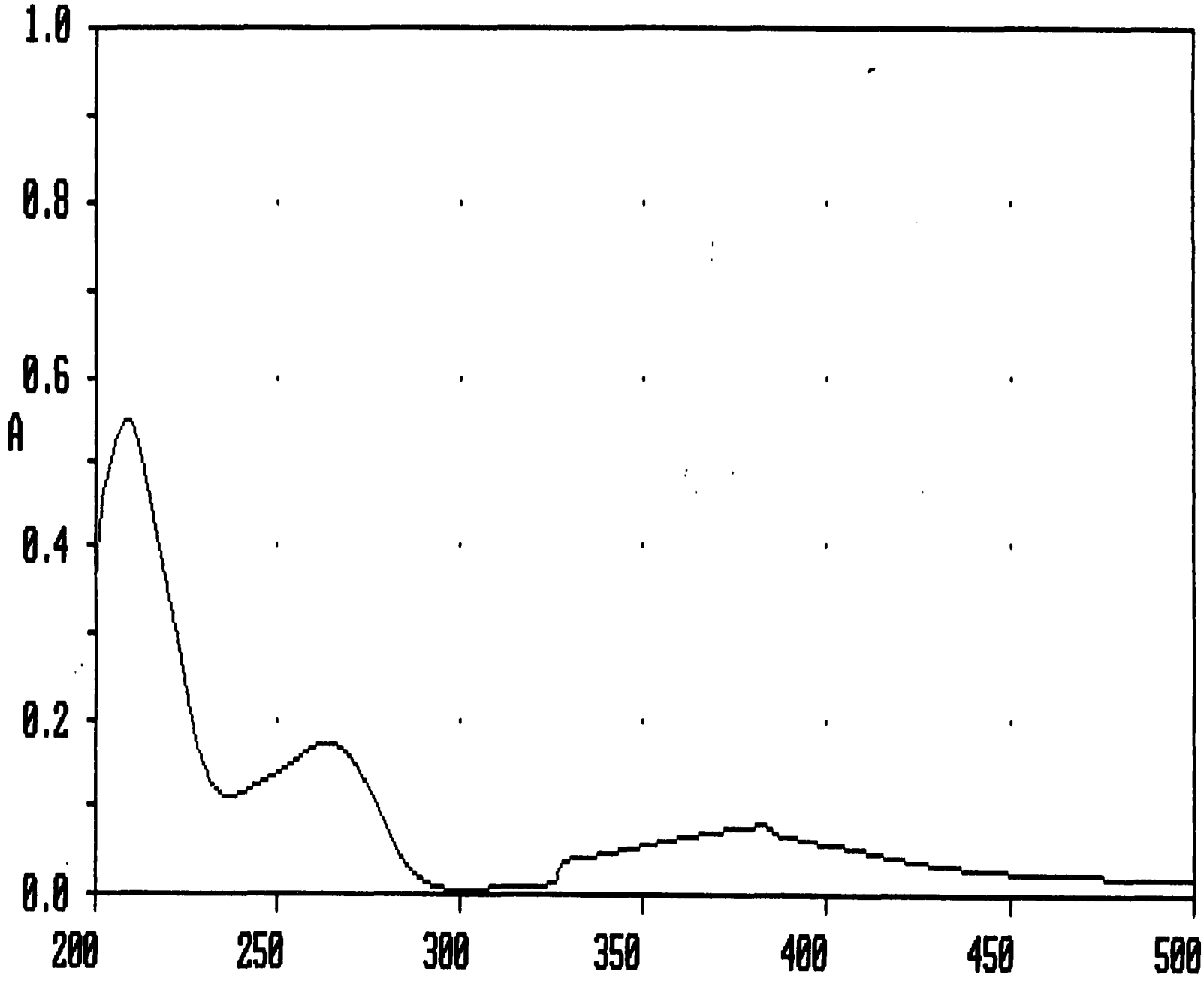
Appendix 1-17. ^{31}P NMR spectra of crude product from 7-PNBDO/HSiCl$_3$/pyridine reaction in ice bath.



Appendix 1-18. ^{31}P NMR spectrum of 7-PNBD reaction with Na metal.



Appendix 2-1. Ultraviolet absorption of L-Marfey's Reagent in ethanol.



Appendix 2-2. Ultraviolet absorption of Dansyl-chloride in ethanol.

AMS Sample Preparation

Bone Sample Preparation

|Collagen|summary|Ninhydrin|summary|Notes|

The dating of bone material can be broken down into several distinct steps.

1. Mechanical cleaning and powdering of the bone
2. Extraction of collagen from the bone
3. Reduction of collagen to amino acids
4. Ninhydrin reaction of amino acids
5. Graphitization

The bone must first be evaluated as to the condition of preservation. Bone that is well preserved will yield collagen that can directly be dated. If the bone material is not well preserved then the further ninhydrin treatment may be required.

If the bone is well preserved then the following steps should be taken.

First, clean a section of the bone approximately one inch square. Use a sterile ¼ inch drill bit tip to remove some of the bone material. It is easiest to use a slow rotation and slight pressure to slowly drill into the bone. Place several pieces of tinfoil beneath the bone. The top piece should be of manageable size. Place the clean area of the bone over the tinfoil and begin to drill into the bone. Shavings from the bone should collect on the tinfoil. If possible collect around 200mg. This will give enough collagen to run several samples. At this point it is easy to tell the condition of the bone. If the bone drills very easily and requires hardly any pressure at all, and the shavings have the consistency of chalk, slate, or powdered rock; then the bone is probably not well preserved. On the other hand, if the shavings come of much like fine wood shavings then the bone probably is well preserved. Three bone samples from the biology department can be used for comparison. They are well preserved.

To extract the collagen a filter funnel and acid procedure must be used. The filter funnel has six parts. The funnel itself, the stopcock, O-ring, washer, paper seal, and the nut. To assemble the funnel first lay out all the pieces.

Use a pair of tweezers to place the filter paper in place. It is usually easier to push the paper down on top of the ceramic filter in a horizontal manner. That is, the filter should be held horizontally while the paper is positioned. Work against the edge of the paper that is furthest from the ceramic filter, otherwise the paper will not completely cover the ceramic filter. Once the paper filter is in place, stretch the paper seal slightly and slide it inside the funnel. The flat side should be upwards. Place the stopcock through the funnel with it in the closed position. The O-ring goes on next, then the washer, and finally the nut. Gently pour the bone shavings into the center of the funnel. The bone is now ready for treatment. Use 0.25N HCl for the first two treatments. Fill the funnel just over the writing that says 1 0 ml. Bone material that is well preserved will float to the top of the acid, much like pumice will. Cover the funnel with a small piece of tinfoil and place in the refrigerator. If only one or two samples are being run then use a large beaker to hold them upright. If more than two samples are being run then the steel plate with drilled holes can be used to hold the funnels. Be sure that the

stopcock is tightly close. After a 24 hour period the acid needs to be drained and discarded. Use the 125ml vacuum flasks for this part. Wet a drilled rubber stopper with distilled water and press the' funnel down into it. Make sure that the funnel extends all the way through the stopper. Press the stopper and funnel onto the top of the vacuum flask and connect the vacuum hose to the side port. With the inline valve closed turn on the pump. Open the inline valve and the stopcock and pump until all the acid has been drained into the flask. Close the stopcock and inline valve. Take care that acid coming into the flask does get into the side port of the flask. After all the acid has been drained, disconnect the pump and remove the funnel from the stopper. Well preserved bone should start taking on the appearance of pulp. Bone that is not well preserved will look more like chips. Repeat this process one more time. Use the new HCl to wash the inside walls of the funnel as some bone material may have collected there. The third time use 0.01N HCl for the treatment. Instead of using tinfoil to cover the funnel use a stopper that has been punctured with a needle. Place the funnel inside the dry oven at 58° Celsius. After 24 hours the acid is ready to be collected.

Place a 13mm fisher culture tube within the 125ml vacuum flask. Position the tube so that its base rests on the perimeter of the flask. Again wet a drilled stopper with distilled water and push the funnel through. The needle punctured stopper can be removed. Push the funnel through only far enough so that the bevel of the funnel is showing. Stopper the flask with the funnel and stopper. Position the flask so that the culture to slants to the right. Note the slant of the bevel on the funnel, position it so that it slants to the left. Slowly work the funnel farther through the stopper until the tip of the funnel almost touches the culture tube. Again with the inline valve in the pumping system closed, connect the hose to the side port and turn the pump on. Open the inline valve for a second or so, then open the stopcock. It is best to take time during this step and miter the flow. If the flow is too fast then it might froth and flow over the edges of the tube. Once all the acid has been collected close the valves and turn off the pump. Very carefully remove the funnel from the stopper and flask. It may be easier to pry the whole stopper and funnel system loose. It is very easy to break the culture tube at this point. Remove the culture tube and cap. The collagen solution is now ready for concentration.

The centrifuge filters used to concentrate the collagen have four parts. The filter with sample reservoir, the filtrate cup, retentate cup, and a cap. Locate the filter and place the large filtrate cup over the filter end. That will leave the reservoir open. Fill the reservoir with the collagen solution using a glass pipette. Do not fill the reservoir any closer to the top than ¼ inch. Place the filter and filtrate cup into one of the centrifuge slots. Place an appropriately filled used filter opposite the new sample. Close the door and spin at 5000 rpm for 15-20 minutes. The reservoir can now be refilled as needed. Empty the filtrate cup as needed so that the system does not become full or unbalanced. As the sample becomes concentrated it will become more viscous. If the sample does not thicken then bone preservation may have been poor. The sample may begin to take on an amber color also. Never let the sample spin down to less than 1/8 inch depth at the filter. A decision to stop spinning may have to be made if it appears as though all the sample may pass through the filter. Once spinning is complete, place the small retentate cup over the reservoir, empty the filtrate cup and place it over the retentate cup. Turn the whole system over.

Place the system in the centrifuge for 1 minute at 1000 rpm. The collagen is now ready for lyophilizing.