THE SYNTHESIS AND EVALUATION OF 1-METHYL-3-PYRROLINES AND 1-METHYLPYRROLES AS SUBSTRATES AND INHIBITORS OF MONOAMINE OXIDASE B

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Thesis submitted in fulfillment of the requirements
for the degree Philosophiae Doctor in Pharmaceutical Chemistry,
at the North-West University, Potchefstroom Campus, South Africa

Promoter: Dr. J.P. Petzer

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Potchefstroom

Dedicated to my husband, Akinwumi, our children, and to my parents, for their love and support throughout the study

"But they that wait upon the Lord shall renew their strength; they shall mount up with wings like eagles; they shall run, and not be weary; and they shall walk, and not faint" – Isaiah 40:31

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PREFACE

The experimental work conducted and discussed in this thesis was carried out in the School of Pharmacy and the Experimental Animal Facility of the North-West University, Potchefstroom Campus, South Africa. André Joubert, Johan Jordaan and Louis Fourie of the SASOL Centre for Chemistry, North-West University, Potchefstroom Campus, South Africa recorded the NMR and MS spectra.

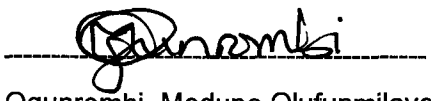
The thesis is presented in an article format and each paper is an individual entity. The research conducted represents original work undertaken by the author, and has not been previously submitted for degree purposes to any other University. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of this thesis. Permission of the co-authors of the papers used in the study has been included. The guides to authors for each paper have also been included.

DECLARATION

This thesis is submitted in fulfillment of the requirements for the degree of the Philosophiae Doctor in Pharmaceutical Chemistry, at the School of Pharmacy, North-West University.

I, Modupe Olufunmilayo Ogunrombi, hereby declare that the dissertation with the title: THE SYNTHESIS AND EVALUATION OF 1-METHYL-3-PYRROLINES AND 1-METHYLPYRROLES AS SUBSTRATES AND INHIBITORS OF MONOAMINE OXIDASE B is my own work and has not been submitted at any other University either in whole or in part.

Signed at Potchefstroom on the 19th September, 2007



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ACRONYMS AND ABBREVIATIONS

AD	Alzheimers' disease
ATP	Adenosine triphosphate
CNS	Central nervous system
DNA	Deoxyribonucleic acid
E	Enzyme
ES	Enzyme-substrate complex
FAD	Flavin adenine dinucleotide
H_2O_2	Hydrogen peroxide
K_{cat}	The turnover number
K_d	Binding constant
K_i	Enzyme-inhibitor dissociation constant
K_m	The Michaelis constant
LBs	Lewy bodies
L-DOPA	Levodopa
MAO	Monoamine oxidase
MAO A	Monoamine oxidase A
MAO B	Monoamine oxidase B
MPP^+	1-Methyl-4-phenylpyridinium
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
PD	Parkinson's disease
PEA	β-Phenylethylamine
ROS	Reactive oxygen species
S	Substrate
SAR	Structure-activity relationship
SET	Single electron transfer
V	Reaction rate
V_{max}	Maximum velocity
λ_{max}	Maximum light absorption

ABSTRACT

Very little is known about why and how the Parkinson's disease (PD) neurodegenerative process begins and progresses. In the course of developments for treatment of PD, the discovery of the inhibition of monoamine oxidase (MAO B) was a conceptual breakthrough, and has now been firmly established. MAO B has also been implicated in the neurodegenerative processes resulting from exposure to xenobiotic amines. For example, MAO B catalyzes the first step of the bioactivation of the parkinsonian inducing pro-neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Additional insight into the mechanism of catalysis of MAO B and the mechanism of neurotoxicity by MPTP is therefore very valuable in the pursuit of the treatment of PD.

The neurotoxic properties of MPTP actually depend on its metabolic activation in a reaction catalyzed by the centrally located MAO B. This reaction leads to the permanently charged 1-methyl-4-phenylpyridinium species MPP^+, a 4-electron oxidation product of MPTP and a potent mitochondrial toxin. The corresponding 5-membered analogue, 1-methyl-3-phenyl-3-pyrroline, is also a selective MAO B substrate. Unlike MPTP, the MAO B-catalyzed oxidation of 1-methyl-3-phenyl-3-pyrroline is a 2-electron process that leads to the neutral 1-methyl-3-phenylpyrrole. MPP^+ is thought to exert its toxic effects only after accumulating in the mitochondria, a process driven by the transmembrane electrochemical gradient. Since this energy-dependent accumulation of MPP^+ relies upon its permanent charge, 1-methyl-3-phenyl-3-pyrrolines and their pyrrolyl oxidation products should not be neurotoxic. We have tested this hypothesis by examining the neurotoxic potential of 1-methyl-3-phenyl-3-pyrroline and 1-methyl-3-(4-chlorophenyl)-3-pyrroline in the C57BL/6 mouse model. The validity of our hypothesis was confirmed when these pyrrolines did not deplete striatal dopamine while analogous treatment with MPTP resulted in 65-73% depletion. Kinetic studies revealed that both 1-methyl-3-phenyl-3-pyrroline and its pyrrolyl oxidation product were present in the brain in relatively high concentrations. Unlike MPP^+, however, 1-methyl-3-phenylpyrrole was cleared from the brain quickly. These results suggest that the brain MAO B-catalyzed oxidation of xenobiotic amines is not, in itself, sufficient to account for the neurodegenerative properties of a compound like MPTP. The rapid clearance of 1-methyl-3-phenylpyrroles from the brain may contribute to their lack of neurotoxicity.

As part of the ongoing investigation into the substrate properties of various 1-methyl-3-phenyl-3-pyrrolinyl derivatives, it was shown in this present study that their respective MAO B catalyzed oxidation products act as reversible competitive inhibitors of the enzyme. We

therefore attempted to determine the effect that specific structural modifications of 1-methyl-3-phenylpyrrole would have on MAO B inhibition potency. Thirteen 1-methyl-3-phenylpyrrolyl derivatives were synthesized and their enzyme–inhibitor dissociation constants (K_i values) for reversible interaction with MAO B determined. In an attempt to quantify the relationship between MAO B inhibitory activity and the physicochemical properties of the substituents, a Hansch-type structure-activity relationship (SAR) study was carried out by multiple linear regression analysis. The most potent inhibitor among the oxidation products considered was 1-methyl-3-(4-trifluoromethylphenyl)pyrrole with an enzyme–inhibitor dissociation constant (K_i value) of 1.30 μM. The least potent inhibitor was found to be 1-methyl-3-phenylpyrrole with a K_i value of 118 μM.

Keywords: monoamine oxidase B; MPTP; 1-methyl-3-phenyl-3-pyrroline; 1-methyl-3-phenyl-3-pyrrole; neurotoxicity; dopamine; striata; reversible inhibitors; competitive inhibition; structure-activity relationship.

OPSOMMING

Weinig is bekend oor die oorsake van die neurodegenerasie wat by Parkinson se siekte (PD) voorkom en wat aanleiding gee tot die verdere verloop daarvan. Die inhibisie van monoamienoksidase B (MAO B) as behandeling vir PD was 'n betekenisvolle deurbraak en is 'n konsep wat tans allerweë erkenning geniet. Dit is aangetoon dat MAO B betrokke is by die neurodegeneratiewe prosesse wat volg ná blootstelling aan xenobiotika. Byvoorbeeld, MAO B kataliseer die eerste stap in die bioaktivering van die proneurotoksien, 1-metiel-4-feniel-1,2,3,6-tetrahidropiridien (MPTP) wat Parkinson-tipe simptome veroorsaak. Verbeterde insig aangaande die meganisme van MAO B-katalise en die meganisme van MPTP se neurotoksisiteit is gevolglik uiters waardevol in die ondersoek na die behandeling van PD.

MPTP se neurotoksiese eienskappe berus in der waarheid op die metaboliese aktivering daarvan as gevolg van die katalise deur sentraalgeleë MAO B. Hierdie reaksie lei tot die permanentgelaaide, 1-metiel-4-piridinium-spesie, MPP^+, 'n 4-elektronoksidasie-produk van MPTP en potente mitochondriale toksien. Die ooreenstemmende 5-lid analoog, 1-metiel-3-feniel-3-pirrolien, is ook 'n selektiewe MAO B-substraat. In teenstelling met MPTP is die MAO B-gekataliseerde oksidasie van 1-metiel-3-feniel-3-pirrolien 'n 2-elektronreaksie waardeur die neutrale 1-metiel-3-feniel-3-pirrool gevorm word. Dit word aanvaar dat MPP^+ se toksiese effekte na vore kom nadat dit in die mitochondria ophoop as gevolg van 'n transmembraangedrewe potensiaalgradiënt. Aangesien hierdie energiegedrewe ophoping van MPP^+ afhanklik is van die permanente positiewe lading daarvan, kan bespiegel word dat die 1-metiel-3-feniel-3-pirroliene en hul pirrolieloksidasieprodukte nie toksies sal wees nie. Ons het hierdie hipotese getoets deur die neurotoksiese potensiaal van 1-metiel-3-feniel-3-pirrolien en 1-metiel-3-(4-chlorofeniel)-3-pirrolien in die C57BL/6-muismodel te ondersoek. Die geldigheid van ons hipotese is bevestig toe gevind is dat hierdie pirroliene nie die striatale dopamien uitgeput het nie in teenstelling MPTP-behandeling wat 'n 65-73% dopamienverlaging veroorsaak het. Kinetiese studies het getoon dat beide 1-metiel-3-feniel-3-pirrolien en sy pirrolieloksidasieproduk in hoë konsentrasies in die brein teenwoordig was, maar in teenstelling met MPP^+ is hierdie verbindings vinnig uit die brein opgeruim. Hierdie bevindings dui daarop dat die MAO B-oksidasie van xenobiotiese amiene nie alleen verantwoordelik is vir die neurodegeneratiewe eienskappe van verbindings soos MPTP nie. Die vinnige uitskeiding van die 1-metiel-3-feniel-3-pirrole uit die brein dra waarskynlik daartoe by dat hulle nie neurotoksies is nie.

In hierdie studie, wat deel uitmaak van 'n omvattende ondersoek van die substraateienskappe van die 1-metiel-3-feniel-3-pirrolinielderivate, is aangetoon dat hul MAO B-gekataliseerde oksidasieprodukte omkeerbare kompetitiewe remmers van die ensiem is. Ons het derhalwe gepoog om vas te stel wat die invloed van spesifieke strukturele veranderings aan 1-metiel-3-feniel-3-pirrool sou hê op die vermoë van dié verbinding om MAO B te inhibeer. Dertien 1-metiel-3-feniel-3-pirrolielderivate is gesintetiseer en hul dissosiasiekonstantes vir ensieminhibisie (K_i-waardes) vir omkeerbare interaksie met MAO B is bepaal. 'n Hansch-tipe stuktuuraktiwiteitstudie (SAR), met meervoudige lineêre regressie is uitgevoer om die verwantskap tussen die MAO B-inhibisievermoë en die fisieschemiese eienskappe van die substituente te bepaal. Die mees potente inhibeerder van die oksidasieprodukte wat ondersoek is was 1-metiel-3-(4-trifluorometielfeniel)pirrool met 'n dissosiasiekonstante vir ensieminhibisie (K_i-waarde) van 1.30 µM. Die inhibeerder met die laagste aktiwiteit was 1-metiel-3-fenielpirrool met 'n K_i-waarde van 118 µM.

Sleutelwoorde: monoamienoksidase B, MPTP, 1-metiel-3-feniel-3-pirrolien, 1-metiel-3-feniel-3-pirrool, neurotoksisiteit, dopamien, striatum, omkeerbare inhibeerders, kompetitiewe inhibisie, struktuuraktiwiteitsverwantskap.

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My Jesus, I adore you for being my **shepherd** and choosing to love me. Your praise shall continually be in my mouth.

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TO WHOM IT MAY CONCERN

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10th September, 2007

Dear Sir / Madam

CO-AUTHORSHIP ON RESEARCH PAPERS

The undersigned, as co-authors of the research articles listed below, hereby give permission to Mrs Modupe Ogunrombi to submit the papers as part of the degree Ph.D. in Pharmaceutical Chemistry at the North-West University, Potchefstroom Campus.

I. Neurotoxicity studies with the monoamine oxidase B substrate 1-methyl-3-phenyl-3-pyrroline.

II. Structure-activity relationships in the inhibition of monoamine oxidase B by 1-methyl-3-phenylpyrroles

Yours sincerely,

J. P. Petzer

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G. Terre'Blanche

S.F. Malan

CHAPTER 1.

INTRODUCTION

1.1 NEURODEGENERATIVE DISEASES

Neuronal loss is an integral part in the normal development of a functional integrated nervous system, and 50%, or more, of all neurons die before adulthood (Oppenheim, 1991). Thus, during development, an initial excess of neurons is produced, and competition within the neuronal population leads to survival of only those neurons that are functionally, temporally, and spatially correct (Cowan *et al.*, 1984). The neurons that do not survive the competition die because of an intrinsic cell suicide program, termed apoptosis, which describes the process of cells disappearing in a non-inflammatory manner (Kerr *et al.*, 1972). Loss of neurons via apoptosis during development is beneficial, but apoptosis that occurs in the mature brain, as may be occurring in neurodegenerative illnesses, is harmful (Holbrook *et al.,* 1996).

Oxygen plays a critical role in cellular respiration as it acts as the final electron acceptor of the electron transport chain, thus driving ATP (adenosine triphosphate) synthesis. Reduction of oxygen, however, occurs in all aerobic organisms and results in the formation of reactive oxygen species (ROS). These species (which include superoxide, hydrogen peroxide and hydroxyl radicals) are directly responsible for the detrimental effects of oxidative stress (Barnham *et al.,* 2004). To counteract the effects of ROS *in vivo*, there are a number of antioxidant mechanisms in place within the cell. These defense mechanisms include superoxide dismutase, catalase, ascorbic acid, and glutathione, amongst others. Oxidative stress and the associated damage to cellular lipids, proteins and deoxyribonucleic acid (DNA) results when these compensatory mechanisms fail to deal with the increasing load of ROS. Interestingly, elevated levels of ROS, which contribute to a decline in cellular function, have been reported to coincide with a number of human pathologies including cancer, cardiovascular disease and neurological disorders (Holbrook *et al.,* 1996).

Neurons may be particularly susceptible to inappropriate activation of apoptosis because their metabolic rates are high and ROS are produced as a normal part of metabolism.

Increased levels of oxidative stress in the brain may be critical for the initiation and progress of neurodegeneration (Youdim & Bakhle, 2006) and neurodegenerative diseases may mimic an accelerated aging process (Holbrook *et al.*, 1996). The number of neurodegenerative disorders is on the increase as average lifespan increases. Under normal conditions, many neurons remain viable and function throughout the lifetime of an individual. However, many people will not complete their lives without excessive death of one or more populations of neurons. The death of hippocampal and cortical neurons is responsible for the symptoms of Alzheimers' disease (AD) while the death of midbrain dopaminergic neurons underlies Parkinson's disease (PD) (Holbrook *et al.*, 1996).

1.2 PARKINSON'S DISEASE

Parkinson's disease (PD) is a common neurodegenerative disease that appears essentially as a sporadic condition. It is currently regarded as the most common neurodegenerative disorder of the aging brain after the Alzheimer's dementia and affects approximately 1% of the population older than 60 years. There is a worldwide increase in the disease prevalence due to the increasing age of human populations. PD etiology remains mysterious, whereas its pathogenesis may be understood as a multifactorial cascade of deleterious factors (Fahn & Przedborski, 2000). The contribution of genetic factors to the pathogenesis of PD is increasingly being recognized. Many researchers now believe that PD results from a combination of genetic susceptibility and exposure to one or more environmental factors that trigger the disease (Jenner & Olanow, 2006). To confirm the genetic implication, especially in the much more common sporadic, or idiopathic PD, a point mutation, which is sufficient to cause a rare autosomal dominant form of the disorder, has been identified in the alpha-synuclein gene on chromosome 4. A defect of complex I of the mitochondrial respiratory chain was also confirmed at the biochemical level (Beal, 1992; Haas, *et al.*, 1995; Jenner & Olanow, 1998). The pathogenesis of Parkinson's disease has recently been linked to oxidative-mediated events including increased monoamine oxidase (MAO) activity and ROS generation. Specifically, age-related increases in brain monoamine oxidase B (MAO B) levels have been proposed to contribute to the neuropathology associated with PD and may explain the increased prevalence of the disease in aged individuals (Soong *et al.*, 1992).

Clinically, PD is characterized by tremor at rest, slowness of voluntary movements, rigidity, and postural instability (Fahn & Przedborski, 2000). The principal biochemical abnormality in PD is the profound deficit in brain dopamine level, primarily, but not exclusively,

attributed to the loss of neurons of the nigrostriatal dopaminergic pathway (Dauer & Przedborski, 2003). This pathway consists of dopaminergic neurons whose cells bodies are located in the substantia nigra pars compacta and whose axons and nerve terminals project to the striatum (Dauer & Przedborski, 2003). A definitive neuropathological diagnosis of PD, however, requires loss of dopaminergic neurons in the substantia nigra and related brain stem nuclei, gliosis, and the presence of intraneuronal proteinaceous inclusions called Lewy bodies (LBs) in the few remaining substantia nigra dopaminergic neurons (Dauer & Przedborski, 2003).

At present, there is no cure for Parkinson's disease and medications or surgery only provide relief from the symptoms, but have not been found to alleviate the underlying dopaminergic neuron degeneration. Thus, PD patients often experience great frustration within a few years of an initially dramatic improvement as the disease inexorably progresses. Another major limitation of current PD medications is their sometimes disabling side effects. For example, the treatment of PD is mainly based on dopamine replacement therapy, which usually is achieved by administration of the dopamine precursor, levodopa (L-DOPA) in combination with a peripheral aromatic L-amino acid decarboxylase inhibitor such as carbidopa or benserazide (Jankovic & Marsden, 1993). Long-term L-DOPA therapy, however, leads to loss of drug efficacy and the onset of unwanted motor complications called dyskinesias (Marsden *et al.*, 1982), which can be as disabling as the parkinsonian symptons themselves. As of yet, the occurrence of L-DOPA-induced motor complications remains a major impediment to the proper management of PD patients. Dopamine agonist drugs are also effective in treating the early symptoms of PD but provoke identical dyskinetic movements as L-DOPA, although with lower incidence (Tolosa & Marin, 1997).

Because these treatment strategies for PD lack selectivity and lead to severe side effects, several studies are currently underway to develop drugs that can delay or even halt the progression of the disease. Alternative therapeutic strategies to treat PD that target non-dopamine receptors with reduced side effect profiles have been under development.

A mechanism-based inactivator of MAO B, (*R*)-deprenyl is being used in combination with L-DOPA as dopamine replacement therapy in PD (Rabey *et al.*, 2000). (*R*)-Deprenyl has also been reported to exert a neuroprotective effect by blocking apoptotic cell death (Tatton & Greenwood, 1991; Tatton, 1993) and may be clinically useful in postponing the emergence of symptoms that require the initiation of L-DOPA therapy in PD patients (LeWitt, 2004). Return of enzyme activity following treatment with inactivators such as (*R*)-

deprenyl requires *de novo* synthesis of the MAO B protein. Aside from the safety considerations associated with irreversible inhibitors, (*R*)-deprenyl is metabolized to (*R*)-methamphetamine, a compound with vasopressor properties (Riederer *et al.,* 2004). For these reasons, studies are still underway to develop safer treatment strategies for PD. Interest in selective inhibitors of monoamine oxidase B has therefore increased in the last years due to their therapeutic potential in aging related neurodegenerative diseases such as PD (Foley *et al.*, 2000; Nicotra *et al.*, 2004). In the next section, various aspects of the enzyme MAO B will be considered with special reference to its role in the treatment of PD.

1.3 MONOAMINE OXIDASE B

Monoamine oxidase B is one of two flavin-dependent isozymes (the other being MAO A) that function in the oxidative deamination of neurotransmitters and exogenous arylalkylamines (Binda *et al.*, 2001). Both isoforms of MAO are approximately 60-kDa and are flavin adenine dinucleotide (FAD)-containing enzymes, bound to the mitochondrial outer membrane through a C-terminal transmembrane polypeptide segment (Mitoma & Ito, 1992). MAO isozymes play a major role in regulating the concentrations of several bioactive amines by performing the most important degradative pathway, the oxidative deamination of the amines (Weyler *et al.,* 1990). The two isozymes are functionally distinct owing to their different affinity for the various substrates. MAO A preferentially carries out the degradation of bulkier endogenous amine neurotransmitters such as serotonin (**1**) and adrenaline (**2**), while MAO B displays greater affinity for small exogenous amines like β-phenylethylamine (**3**) (PEA) (Fowler & Tipton, 1984) (Figure 1) and benzylamine. Dopamine (**4**) is considered a substrate for both MAO forms (Garrick & Murphy, 1980).

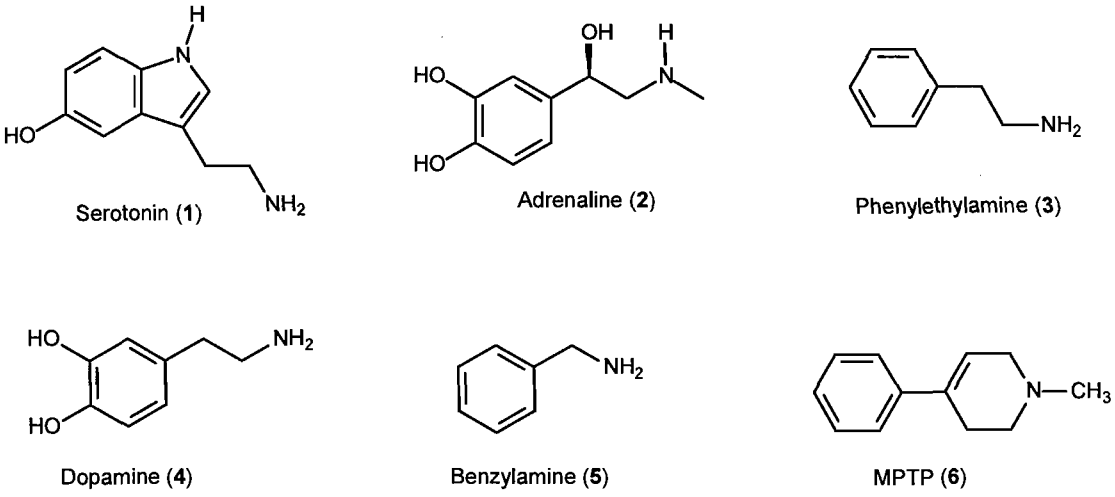


***Figure 1**: Examples of human MAO substrates.*

MAO A and B are encoded by different genes and are expressed in a tissue-specific manner. They are also differently distributed in mammalian brain, with for instance, greater MAO B activity in the basal ganglia (Collins *et al.*, 1970). MAO A is composed of 527 amino acids while MAO B is composed of 520 amino acids. Human MAO A and MAO B share approximately 70% sequence identity, thus the distinct, as well as overlapping specificities in their oxidative deamination of neurotransmitters and dietary amines. Binda *et al.* (2001) showed that as they differ in their substrate, so do they differ in inhibitor specificities. Their crystal structures are dimeric (Figure 2).

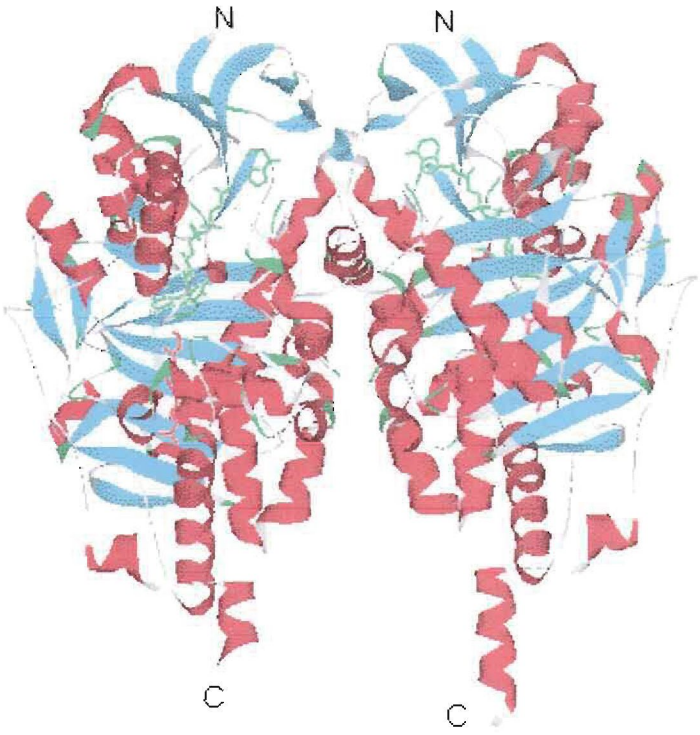


Figure 2: *The crystal structure of human recombinant MAO B (Binda et al., 2001).*

Mechanism-based inactivators of MAO B such as (*R*)-deprenyl bind covalently to the flavin N5 atom on the *re* side of the flavin in the substrate binding site. The substrate binding site is formed by a flat cavity with a volume of 420 $Å^3$. This cavity is lined by a number of aromatic and aliphatic amino acids providing the highly hydrophobic environment (Walker & Edmondson, 1994).

Adjacent to the substrate cavity is a separate, smaller hydrophobic cavity (volume of 290 $Å^3$) lined by residues Phe 103, Pro 104, Trp 119, Leu 164, Leu 167, Phe 168, Leu 171, Ile

199, Ile 316 and Tyr 326. This second cavity is situated between the active site and the protein surface. The side chains of residues Tyr 326, Ile 199, Leu 171 and Phe 168 separate the two cavities (Binda *et al.*, 2001). These observations suggest a mechanism for admission of the substrate into the active site that involves traversing the smaller cavity (termed the 'entrance cavity'). After the substrate reaches the 'entrance cavity', a transient movement of the side chain of Ile 199, one of the four residues separating the entrance from the substrate cavity must occur to allow its diffusion into the active site.

While small molecule inhibitors of MAO B such as isatin binds within the substrate cavity of the enzyme, larger inhibitors such as 1,4-diphenyl-2-butene binds to both the entrance and substrate cavities (Figure 3). Analogous MAO B-specific inhibitors that bind in a manner traversing both cavities include *trans,trans*-farnesol and possibly also (*E*)-8-(3-chlorostyryl)caffeine (Binda *et al.*, 2006). For this dual binding mode to be possible, the side chain of Ile 199 must be rotated into an "open" position that allows the entrance and substrate cavities to fuse. When small molecules are bound to the substrate cavity, the Ile 199 side chain is rotated into the normal closed position, and the two cavities are separated from each other (Figure 4) (Binda *et al.*, 2006).

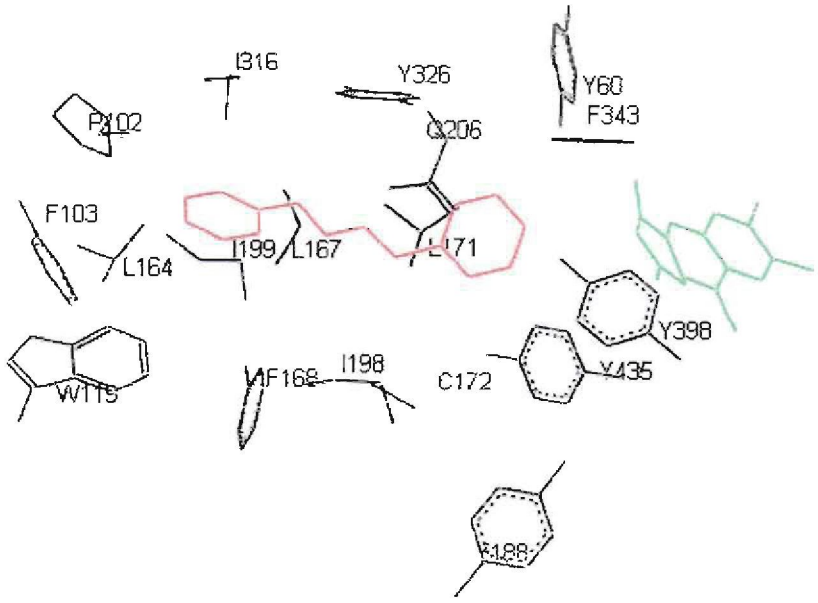


Figure 3: *The active site of human recombinant MAO B with 1,4-diphenyl-2-butene bound (red). The side chain of Ile 199 is rotated into the "open" position allowing for the substrate and entrance cavities to fuse (Binda et al., 2006). The FAD cofactor is shown in green.*

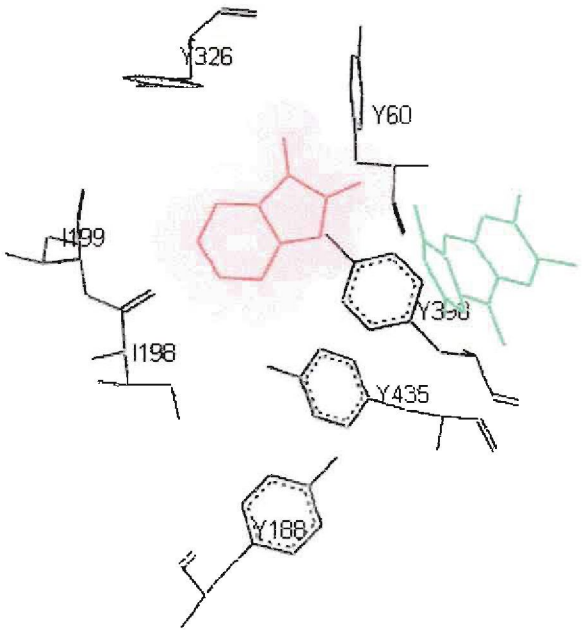


Figure 4: *The active site of human recombinant MAO B with isatin (red) bound to the substrate cavity. Here, the side chain of Ile 199 is rotated into the "closed" position and the substrate and entrance cavities are separated (Hubalek et al., 2005). The FAD cofactor is shown in green.*

Modeling of the binding of a substrate in the active site has been carried out in order to analyze the mechanistic implications of the MAO B three-dimensional structure (Binda *et al.*, 2001). These modeling studies revealed that the benzylamine (**5**) carbon atom undergoing oxidation binds in a highly conserved position in front of the flavin N5 and C4a atoms. The benzylamine methylene carbon was positioned 3.6 Å from flavin N5. The orientation of the aromatic ring was restricted by the flat shape of the cavity. As a result, the amine binds between the phenolic side chains of Tyr 398 and Tyr 435. These residues, together with the flavin, form an aromatic caged environment that is responsible for recognition of the amine functional group. No interaction of the substrate nitrogen atom with any anionic residues was detected, which agrees with the known preference of MAO to bind the deprotonated substrate (Miller & Edmondson, 1999).

The volume of benzylamine substrate is 160 $Å^3$, which is smaller than the volume of the active site cavity (420 $Å^3$). These modeling experiments highlight that the portion of the cavity on the rear side, with respect to the flavin ring, is left unoccupied by the substrate

(Binda *et al.*, 2001). This implies that the cavity may allow an aromatic ring to bind at many positions, further or closer to the flavin. This feature explains the broad substrate and inhibitor specificities of MAO B.

1.4 THE ROLE OF MAO B IN PARKINSON'S DISEASE

Activity measurements of the two MAO isoforms, MAO A and MAO B, in postmortem brain have shown an age-related increase in MAO B but a constant activity of the isozyme A (Novaroli *et al.*, 2006). Moreover, studies have demonstrated that MAO B activity stays unchanged until the 60th year of life and then increases nonlinearly (Delumeau *et al.*, 1994; Strolin & Dostert, 1989). This results in an increased level of dopamine metabolism and production of higher levels of ROS via hydrogen peroxide (H_2O_2) formation (Figure 5). Oxidative deamination of biogenic amines including dopamine and β-phenylethylamine (PEA) by MAO B produces H_2O_2 as a by-product, which is thought to play a role in the etiology of neurodegenerative diseases such as Parkinson's disease. This may be via increased oxidative stress and/or mitochondrial dysfunction (Kumar *et al.*, 2003). Because MAO B is predominantly located in the glial cells (Shih *et al.*, 1999; Mellick *et al.*, 1999), the increase of this enzyme with age may also be attributed to glial cell proliferation associated with neuronal loss (Barnham *et al.*, 2004; Youdim *et al.*, 2004). The increase in MAO B is not merely due to increased glial cell numbers but appears to also involve an increase in enzymatic activity in individual cells. H_2O_2 produced as a consequence of substrate oxidation by MAO B within glia has a high membrane permeability and can diffuse into nearby mid-brain dopaminergic neurons leading to the production of toxic ROS (Halliwell, 1992).

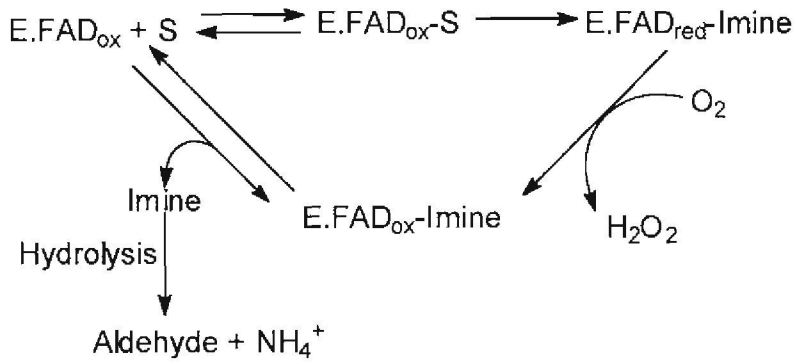


Figure 5: *Scheme for the overall oxidative deamination reaction catalyzed by MAOs. Oxidation of the amine substrates leads to the reduction of FAD. The prosthetic group is reoxidized by molecular oxygen to generate hydrogen peroxide. The imine product is hydrolyzed in a nonenzymatic process.*

The substantia nigra, the brain area affected in PD, contains high numbers of MAO B-positive astrocytes which are themselves protected from the MAO B catalyzed production of H_2O_2 by high levels of glutathione and glutathione peroxidase. H_2O_2, produced within substantia nigra glial cells by MAO B, may be either reduced to H_2O by the glutathione system or diffuse into nearby vulnerable dopaminergic neurons where it may elicit toxic effects (Cohen, 1990) like neuronal degeneration by interacting with free iron to form highly reactive hydroxyl radicals (Youdim *et al.*, 2004). MAO B-catalyzed increased ROS production may contribute to an observed age-related increase in the incidence of mitochondrial damage in the brain, particularly in the substantia nigra (Soong *et al.*, 1992). Interestingly, complex I has been found to be one of the mitochondrial enzymes most affected by oxidative stress (Lenaz *et al.*, 1997) and reductions in the activity of complex I are associated with PD (Beal, 1992; Haas, *et al.*, 1995; Jenner & Olanow, 1998). Elevated MAO B levels, therefore, induce apoptosis in neuronal cells, which may be as a result of the enhanced levels of ROS (Soong *et al.*, 1992).

1.5 THE NEUROTOXIN MPTP

MAO B is implicated in neurodegenerative processes resulting from exposure to xenobiotic amines. The enzyme has been identified as the principal enzyme responsible for the metabolic activation of the proneurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP (**6**)] in the brains of mammals including humans. The MPTP-induced losses of nigrostriatal neurons (Jackson-Lewis *et al.*, 1995) in humans produce a syndrome that is neurochemically, behaviourally and pathologically similar to that observed in patients diagnosed with PD. Ricaurte *et al.* (1986) and Betarbet *et al.* (2000) also reported that selective inhibition of complex I via systemic administration of either MPTP or rotenone show patterns of morphological damage similar to the Parkinsonian brain. The reports that MPTP, a contaminant in the illicit meperidine substitute "new heroin", causes acute Parkinsonian symptoms, dopamine depletion and nigrostriatal degeneration in man, monkey and some other susceptible species seemed to offer an animal model for PD (Cohen *et al.*, 1984). Although both forms of MAO oxidize MPTP sufficiently rapidly *in vitro* to give rise to toxic concentrations of MPP^+ (**8**) (Figure 6), *in vivo*, only the B type plays a role, as judged by the complete prevention of the toxicity of MPTP by (*R*)-deprenyl and pargyline (Cohen *et al.*, 1984). This is so because product inhibition of MAO A rapidly halts its action, while MAO B is much less sensitive to enzyme inactivation by $MPDP^+$ (**7**) and MPP^+ (Singer *et al.*, 1986). A single acute MPTP insult can set in motion a self-sustained cascade of cellular and molecular events with long-lasting deleterious effects (Bové *et al.*, 2005).

The first step of the bioactivation of MPTP is catalyzed by MAO B (Chiba *et al.*, 1984) (Figure 6). MPTP is not the actual toxin but a protoxin. The toxic effects of MPTP are mediated by the pyridinium species MPP^+, a mitochondrial toxin (Nicklas *et al.*, 1985; Ramsay *et al.*, 1991). MPTP, a lipophilic molecule, readily crosses the blood-brain barrier (Irwin & Langston, 1985) and MPP^+ is formed in the glial cells via the MAO B catalyzed oxidation of the parent tetrahydropyridinyl protoxin which generates the unstable dihydropyridinium intermediate $MPDP^+$. A second 2-electron oxidation yields MPP^+ (Figure 6).

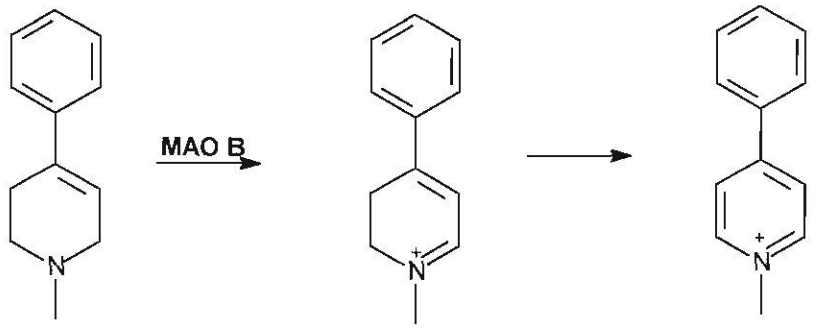


***Figure 6**: The MAO-catalyzed oxidation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.*

The dopamine transporter protein mediates the uptake of MPP^+ into the dopaminergic terminal, where it concentrates in the mitochondrial matrix, inhibiting complex 1 of the electron transport chain and depleting adenosine triphosphate (Ramsay *et al.*, 1991). This leads to neuronal cell death and causes the buildup of ROS that contribute further to nigrostriatal dopaminergic cell destruction, which is ultimately responsible for the severe Parkinsonian syndrome that follows administration of MPTP.

MPTP has selective abilities to effect neuronal death in dopaminergic cells, apparently through a high-affinity uptake process in nerve terminals normally used to reuptake dopamine after it has been released into the synaptic cleft (Calne & Langston, 1983). Such effects lead to gross depletion of dopaminergic neurons which has severe implications on cortical control of complex movements. The direction of complex movement is based from the substantia nigra to the putamen and caudate nucleus which then relay signals to the rest of the brain. This pathway is controlled via dopaminergic neurons, which MPTP selectively destroys, resulting over time in parkinsonism. Evidence that the MAO B catalyzed oxidation of MPTP is an essential step in the expression of MPTP's neurotoxicity

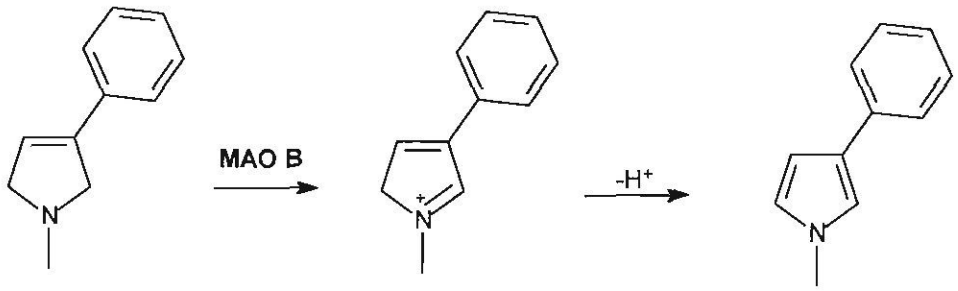
is based on the neuroprotective properties of the selective mechanism-based MAO B inactivator (*R*)-deprenyl (Fuller & Hemrick-Luecke, 1984). Exogenous or endogenous toxins similar to MPTP or rotenone may act in concert with age-related elevations in brain MAO B levels to elicit the disease.

Experimental animals treated with MPTP have become useful models for studying neurodegenerative processes. An enormous body of work regarding the elucidation of the mechanisms of dopaminergic neuron death and the development of experimental neuroprotective therapies has been achieved, thanks to the use of the MPTP mouse model of PD.

Since MAO B has become an attractive drug target for the development of antiparkinsonian agents, research to examine the MAO B substrate and inhibitor properties of various compounds are of interest.

Various cyclic tertiary amines like 1,4-disubstituted-1,2,3,6-tetrahydropyridines and other analogues have been reported to display good MAO B substrate properties. The corresponding piperidinyl and pyrrolidinyl analogues of MPTP are not substrates (Hall *et al.,* 1992), suggesting that the allylamine functionality is important for the catalytic process. Consistent with this view, the MAO B catalyzed oxidation of MPTP occurs regiospecifically at the C-6 allylic position (Ottoboni *et al.,* 1989). Generally, tertiary arylakylamines are very poor substrates of monoamine oxidase, though notable exceptions are MPTP and its analogues (Williams & Lawson, 1998). It seems likely that the presence of the β,γ double bond in the hetero ring of these compounds, an allylic structure, facilitates attack by the enzyme on the methylene group adjacent to the unsaturated centre.

1-Methyl-3-phenyl-3-pyrroline (**9**), a cyclic tertiary arylakylamine, is a structural analogue of MPTP possessing the allylamine functionality and also is one of the reported selective substrates of MAO B (Wang *et al.,* 1998). Unlike MPTP, 1-methyl-3-phenyl-3-pyrroline is not oxidized to permanently charged end products, but to neutral 1-methyl-3-phenylpyrrole (Figure 7).



1-Methyl-3-phenyl-3-pyrroline (**9**) 1-Methyl-3-phenylpyrrole (**10**)

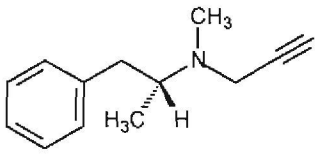
Figure 7: *The MAO B catalyzed oxidation of 1-methyl-3-phenyl-3-pyrroline to 1-methyl-3-phenylpyrrole.*

1.6 MAO B INHIBITORS

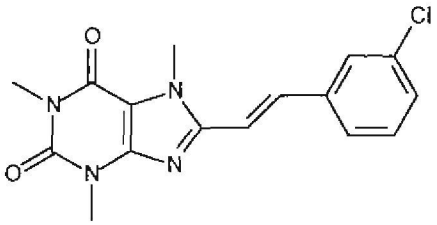
There has been evidence that MAO B inhibitors improve the quality of life in the elderly (Knoll, 1993), suggesting that MAO B inhibitors may antagonize the evolution or progress of neurodegenerative disorders. Specific inhibitors of MAO B constitute a novel and expanding pharmacological class. MAO B inhibition in the brain primarily reduces the catabolism of dopamine and β-phenylethylamine and has therefore found its greatest application in the therapy of neurodegenerative disorders including PD. Inhibition of dopamine oxidation primarily results in the stoichiometric reduction of hydrogen peroxide production which is thought to play a significant role in the etiology of neurodegenerative diseases such as PD.

Interest in MAO B inhibition is mostly stimulated by the desire to elevate the depleted DA concentrations in the striata of PD patients. The beneficial effects of (*R*)-deprenyl (**11**), a mechanism-based inactivator of MAO B, is dependent on the inhibition of the MAO B catalyzed oxidation of dopamine in the CNS, consequently conserving the depleted supply of dopamine in patients diagnosed with early PD (Rabey *et al.*, 2000). Because of the safety considerations associated with irreversible MAO-B inhibitors, such as the requirement of *de novo* synthesis of the MAO B protein for enzyme activity to return, there are at present several studies underway to develop reversible, competitive inhibitors that may offer a safer alternative to the treatment of neurodegenerative disease. Treatments with (*R*)-deprenyl also have the limitation of it being metabolized to (*R*)-methamphetamine, a compound with vasopressor properties (Riederer *et al.*, 2004). Studies have shown that (*E*)-8-(3-chlorostyryl)caffeine (CSC) (**12**), an A_{2A} adenosine receptor antagonist, is also a potent and selective inhibitor of mouse brain MAO B (K_i = 100 nM) but not MAO A (Chen *et al.*, 2002). Khalil *et al.* (2006) reported that *trans,trans*-farnesol (**13**), a component of

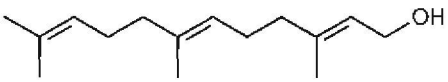
tobacco smoke, is a potent, reversible inhibitor selective for MAO B. Another study has established that 1,4-diphenyl-2-butene (**14**) (K_i = 35 μM), a contaminant of polystyrene bridges, used for MAO B crystallization, and 1,4-diphenyl-1,3-butadiene (K_i = 7 μM) are potent, competitive MAO B-specific reversible inhibitors (Binda *et al.*, 2003; Hubalek *et al.*, 2003).



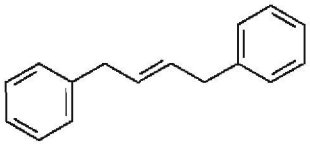
(*R*)-deprenyl (**11**)



(*E*)-8-(3-chlorostyryl)caffeine (**12**)



trans,trans-farnesol (**13**)



1,4-diphenyl-2-butene (**14**)

Figure 8: *The structures of selected inhibitors of MAO B.*

It is therefore clear that the development of specific, reversible MAO B inhibitors are being persistently studied and could eventually lead to clinically useful neuroprotective agents.

1.7 KINETICS OF ENZYME-CATALYZED REACTIONS

1.7.1 Enzyme-catalyzed reactions

Enzymes have localized catalytic sites. The substrate (S) binds at the active site to form an enzyme-substrate complex (ES). Subsequent steps transform the bound substrate into product and regenerate the free enzyme (E) (Figure 9).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

Figure 9*: Enzyme catalyzed reaction. K_1, K_{-1} and K_2 are the rate constants for the individual steps.*

At low concentrations of substrate [S], the enzyme exists in an equilibrium between both the free form E and the enzyme-substrate complex ES. Since the rate of the reaction (V) depends on the concentration of ES, it is sensitive to small changes in [S]. However, at high [S], the enzyme is entirely saturated with substrate and exists only in the ES form. The maximum velocity (V_{max}) is obtained when the entire enzyme is in the form of the enzyme-substrate complex. K_m, the Michaelis constant, is the substrate concentration at which the rate of the reaction velocity is half maximal ($V_{max}/2$) and it is determined experimentally by plotting the graph of reaction rate (V) versus concentration of substrate [S] (Figure 10). The Michaelis-Menten equation expresses the behaviour of various enzymes with different substrate concentrations (Equation 1). The Michaelis-Menten equation for a reaction in the absence of an inhibitor is:

$$V = \frac{V_{\max}[S]}{K_m + [S]}$$

Equation 1: *The reaction velocity, V, as a function of the substrate concentration [S] for an enzyme-catalyzed reaction.*

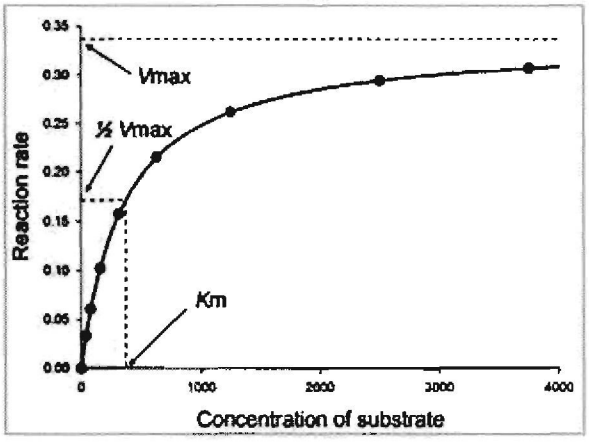


Figure 10: *Saturation curve for an enzyme showing the relation between the concentration of substrate and rate.*

The K_m value may estimate, with certain assumptions, a binding constant (K_d) for the enzyme-substrate complex, that is, if ES is in equilibrium with the free enzyme E and substrate S, K_m is equal to the binding constant for the complex ES. Low values of K_m indicate that the ES complex is held together very tightly and rarely dissociates without the

substrate first reacting to form the product, thus the substrate has high affinity for the enzyme (Rodwell, 1993).

The turnover number, k_{cat}, is the maximum number of molecules of substrate converted to product per active site per unit time and is V_{max} divided by the total enzyme concentration. k_{cat}/K_m, the specificity constant, provides a measure of how rapidly an enzyme can work at low substrate concentration, that is, how efficiently an enzyme converts a substrate into product. It is useful for comparing the relative abilities of different compounds to serve as substrates for the same enzyme. The bigger this number, the better the substrate (Gaál & Hermecz, 1993).

1.7.2 The measurement of the kinetic parameters

Kinetic parameters are determined by measuring the initial reaction velocity as a function of the substrate concentration. The usual procedure for measuring the rate of an enzymatic reaction is to mix enzyme with substrate and observe the formation of product or disappearance of substrate as soon as possible after mixing, that is, when the substrate concentration is still close to its initial value and the product concentration is small. The measurements usually are repeated over a range of substrate concentrations to map out how the initial rate depends on concentration. The relationship between V and [S] is non-linear, resulting in the non-linearity of the plot of V versus [S]. Although the plot is initially linear at low [S], it bends over to saturate at high [S]. This saturation curve does not permit exact measurement of K_m and V_{max}, thus the development of the linearizations of the Michaelis-Menten equation, such as the Lineweaver-Burk plot, also known as the double reciprocal plot. The Lineweaver-Burk plot permits a linear fit to the empirical data using standard regression analysis. This powerful approach has been used successfully for decades to derive the values of K_m and V_{max} in enzymatic reactions from a plot of $1/V$ versus 1/[S]. As shown in figure 11, this is a linear form of the Michaelis-Menten equation and produces a straight line with the equation y = mx + c with a y-intercept equal to $1/V_{max}$ and an x-intercept of the graph representing $-1/K_m$ (Equation 2) (Segel, 1993).

$$\frac{1}{V} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

Equation 2: *The equation for a straight line, y = mx + c with a y-intercept equivalent to $1/V_{max}$ and an x-intercept of the graph representing $-1/K_m$.*

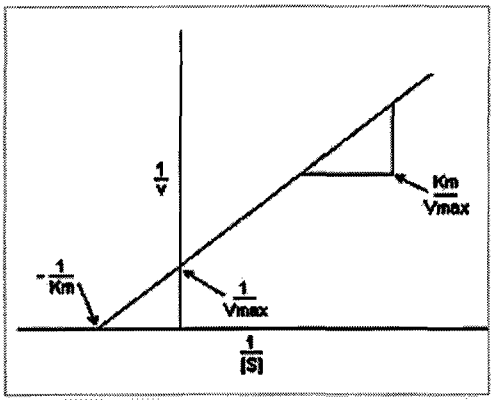


Figure 11: *Lineweaver-Burk or double-reciprocal plot of kinetic data, showing the significance of the axis intercepts and gradient.*

Spectrophotometric techniques are commonly used in such experiments to measure the concentration of a substrate or product continuously as a function of time.

1.7.3 Enzyme inhibition

Enzyme inhibitors are molecules that reduce or abolish enzyme activity. These are either reversible (that is, removal of the inhibitor restores enzyme activity) or irreversible (that is, the inhibitor permanently inactivates the enzyme).

1.7.3.1 Reversible inhibitors

Reversible enzyme inhibitors involve no covalent interactions. In the presence of a reversible inhibitor I, the kinetics of the enzyme can be competitive, non-competitive or mixed, according to their effect on K_m and V_{max}. These different effects result from the inhibitor binding to the enzyme E, to the enzyme-substrate complex ES or to both respectively (Figure 12).

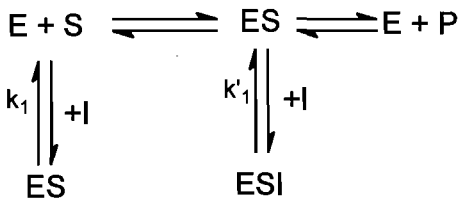


Figure 12: *Kinetic scheme for reversible enzyme inhibition.*

The particular mechanism by which an inhibitor act can be discerned by studying the enzyme kinetics as a function of the inhibitor concentration. A competitive inhibitor acts by decreasing the number of free enzyme molecules available to bind substrate, that is, to form ES, and thus, eventually to form the product (Figure 12). Consequently, a sufficiently high concentration of substrate can eliminate the effect of a competitive inhibitor. Competitive inhibition is represented graphically by the Lineweaver-Burke plot (Figure 13).

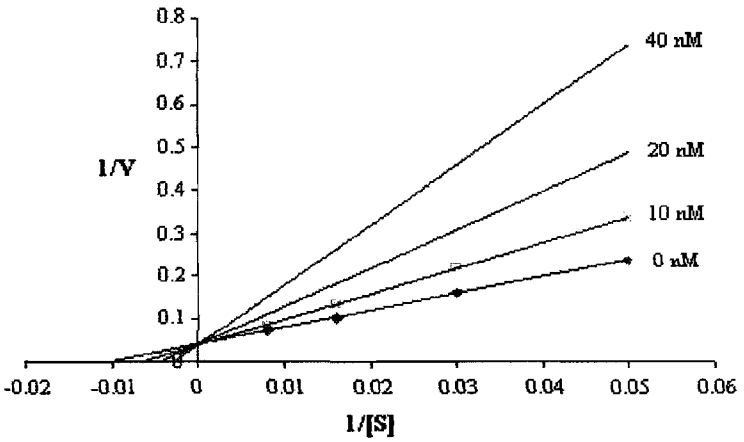


Figure 13: *The double reciprocal plot in the presence of different preset concentrations of a competitive inhibitor.*

The addition of a competitive inhibitor to an enzyme-substrate reaction increases the slope of the straight line while the y-axis intercept remains unchanged. The intercept on the x-axis increases and becomes less negative. Therefore, a competitive inhibitor raises the apparent K_m value of a substrate while V_{max} remains unchanged. Non-competitive inhibitors decrease the value of V_{max} for a substrate, effectively inactivating portions of the enzyme for the substrate (Mason & Lai, 2000). The form of the Michaelis-Menten equation describing the competitive inhibitor-substrate-enzyme relationship for many enzymes is illustrated in equation 3:

$$V = \frac{V_{\max} \times \frac{[S]}{K_m}}{1 + \frac{[S]}{K_m} + \frac{[I]}{K_i}}$$

Equation 3: *Michaelis-Menten equation describing the competitive inhibitor-substrate-enzyme relationship.*

The inverse of this equation expresses the double reciprocal plot in the presence of a competitive inhibitor as described in equation 4:

$$\frac{1}{V} = \frac{K_m}{V_{\max}}\left(1+\frac{(I)}{K_i}\right)\times\frac{1}{[S]}+\frac{1}{V_{\max}}$$

Equation 4: *Michaelis-Menten equation describing the competitive inhibitor-substrate-enzyme relationship.*

The K_i value of a competitive inhibitor is used to describe the affinity of the inhibitor for the active site of the enzyme. In a series of competitive inhibitors, those with the lowest K_i values will cause the highest level of inhibition at a fixed concentration of inhibitor [I]. The K_i value for an inhibitor can be determined from the secondary plot in which the slope of each reciprocal plot is graphed versus the corresponding inhibitor concentration (Figure 14). The x-axis value is equal to $-K_i$. In the presence of a concentration of inhibitor [I] that is approximately equal to K_i the substrate concentration has to double to maintain the same original velocity as in the absence of the inhibitor (Kakkar *et al.*, 1999). Generally it is understood that if plasma or tissue concentrations of a competitive inhibitor are larger than K_i, the inhibition will be physiologically significant. On the contrary, if the plasma or tissue concentrations are lower than K_i, a physiological significant effect is unlikely (Kakkar *et al.*, 1999).

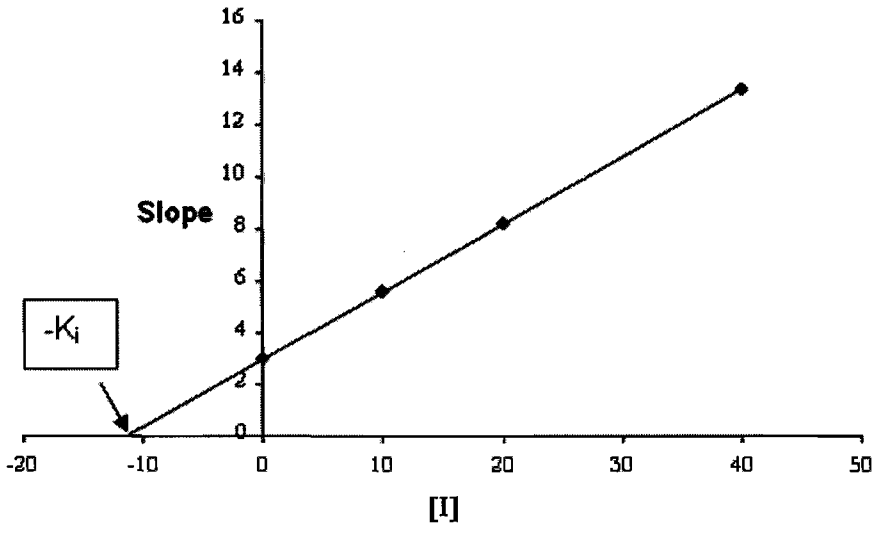


Figure 14: *Secondary plot of the slopes from the double reciprocal plot versus inhibitor concentration.*

1.7.3.2 Irreversible inhibitors

Irreversible inhibitors (inactivators) are compounds that produce irreversible inhibition of the enzyme. They often provide information on the active site by forming covalently linked complexes that can be characterized.

1.7.3.3 Mechanism-based inactivators

A mechanism-based inactivator is an inactive compound whose structure resembles that of either the substrate or product of the target enzyme and which undergoes a catalytic transformation by the enzyme to a species that, prior to release from the active site, inactivates the enzyme. A mechanism-based inactivator requires a step to convert the compound to the inactivating species. This step, which generally is responsible for the observed time dependence of the enzyme inactivation, usually is irreversible. An example of a mechanism-based inactivator of MAO B is (*R*)-deprenyl which first forms a non-covalent complex as an initial, reversible step. Inactivator-enzyme interaction leads to a reduction of the enzyme-bound flavin-adenine dinucleotide (FAD), and concomitant oxidation of the inhibitor. This oxidized inhibitor then reacts with FAD at the N-5-position in a covalent manner, to form a deactivated MAO B-deprenyl combination.

CHAPTER 2.

OBJECTIVE AND SCOPE OF THIS STUDY

In chapter 1, it has been indicated that experimental animals treated with MPTP (**6**) (Figure 6) have become useful models for studying neurodegenerative processes and that in addition to being an important drug target, MAO B is also of interest because of its role as the catalyst that mediates the bioactivation of the pro-neurotoxin MPTP (Chiba *et al.,* 1985). The molecular mechanism by which MPTP selectively damages nigrostriatal neurons and induces a parkinsonian syndrome in mammals, including humans, has thus been the subject of extensive research (Heikkila *et al.,* 1984a; Heikkila *et al.,* 1984b; Nicklas *et al.,* 1985; Langston, 2002). The MAO B catalyzed α-carbon oxidation of the parent compound, MPTP, to yield the corresponding dihydropyridinium species $MPDP^+$ (**7**), is critical to its mode of action. This metabolic intermediate undergoes a second two-electron oxidation to generate the pyridinium metabolite MPP^+ (**8**), the ultimate neurotoxin (Figure 6) (Chiba *et al.,* 1984; Chiba *et al.,* 1985; Ramsay *et al.,* 1991; Markey *et al.,* 1984).

Since this seminal discovery, several tetrahydropyridinyl analogues of MPTP have been prepared and found to be both MAO A and B substrates with differing selectivity among the various compounds (Kalgutkar *et al.,* 1994). Until 1998, 1,4-disubstituted-1,2,3,6-tetrahydropyridines were the only reported cyclic tertiary amines with MAO A and/or B substrate properties. However, Wang *et al.* (1998) showed that 1-methyl-3-phenyl-3-pyrroline was an excellent MAO B substrate. 1-Methyl-3-phenyl-3-pyrroline (V_{max}/K_m = 2054 $min^{-1}mM^{-1}$) is a better and more selective MAO B substrate than MPTP (V_{max}/K_m = 1431 $min^{-1}mM^{-1}$). Semi-empirical calculations suggested that the energy involved in the initial single electron oxidation of the 3-pyrroline analogue to its allylic radical is less (by about 6 kcal/mol) than the corresponding one electron oxidation of MPTP. The pyrrolines are of considerable interest since they are not expected to be metabolized to neurotoxic end-products and therefore may be safe alternatives to tetrahydropyridinyl analogues for mechanistic studies of enzyme function (Figure 7). The allylic amine functionality of 1-methyl-3-phenyl-3-pyrroline is important for the catalytic process since the related piperidinyl and pyrrolidinyl analogues are stable in the presence of MAO B. 1-Methyl-3-phenyl-3-pyrroline (**9**) is oxidized in the presence of MAO B to the isopyrrolinium species which undergoes deprotonation to form the corresponding pyrrole derivative (**10**).

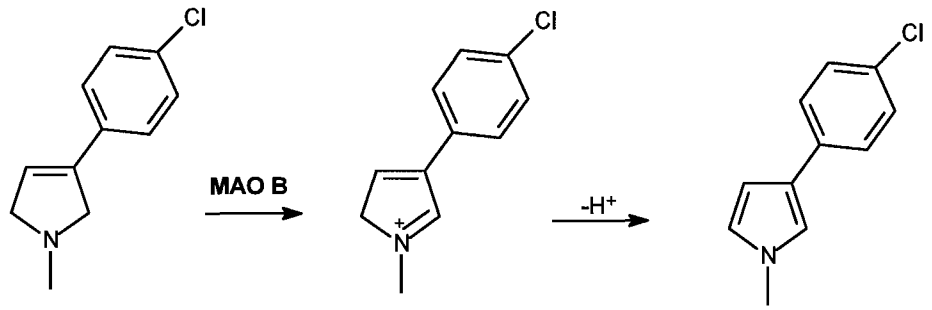
The first objective of this study was to prepare 1-methyl-3-phenyl-3-pyrroline (**9**) and 1-methyl-3-(4-chlorophenyl)-3-pyrroline (**15**). These were to be evaluated *in vivo* as neurotoxic agents and compared to the well-known neurotoxin MPTP. Examining the chemical structures of the MAO-catalyzed oxidation products of 1-methyl-3-phenyl-3-pyrroline, it was postulated that this class of compounds would not mimic the neurotoxic effect observed with MPTP (Wang *et al.,* 1998). It had been demonstrated that the charged MPP^+-like isopyrrolinium intermediate resulting from the two electron oxidation of tertiary 3-pyrrolines undergoes deprotonation, presumably at C-5, to yield the more stable uncharged aromatic pyrrole moiety (Wang *et al.,* 1998). It is generally assumed that the requirement for a positive nitrogenous charge is critical to MPP^+'s mode of toxicity and tertiary 3-pyrrolines offer a unique opportunity to test this hypothesis. Furthermore, demonstrating that this class of MAO substrates are not neurotoxic offers an alternative to MPTP which may encourage more investigators to examine the biological activities of cyclic tertiary amines.

It was also mentioned in chapter 1 that the inhibitors of MAO represent a useful tool for the treatment of neurological and psychiatric diseases. In particular, reversible MAO A inhibitors are used as antidepressant and antianxiety drugs (Volz & Gleiter, 1998), while reversible and selective inhibitors of MAO-B are still under investigation for the treatment and prevention of PD (Van den Berg *et al.,* 2007). The monoamine oxidase B enzyme is thus an interesting target for new drugs to treat Parkinson's disease. The therapeutic role of MAO B inhibitors in Parkinson's disease is particularly of interest because the MAO B isoform appears to be predominantly responsible for dopamine metabolism in the basal ganglia (Collins *et al.,* 1970; Youdim *et al.,* 1972), thus, inhibition of this enzyme in the brain may conserve the depleted supply of dopamine.

The mechanism-based inactivator of MAO B, (*R*)-deprenyl (**11**) (Figure 8), is frequently used in combination with L-DOPA as dopamine replacement therapy in PD (Rabey *et al.*, 2000). From a safety point of view, reversible inhibitors may be therapeutically more desirable than inactivators since MAO B activity can be regained more quickly following withdrawal of the reversible inhibitors. In contrast, return of enzyme activity following treatment with inactivators such as (*R*)-deprenyl, can only be regained via *de novo* synthesis of the MAO B protein which may require several weeks. Aside from the safety considerations associated with irreversible inhibitors, (*R*)-deprenyl is also metabolized to (*R*)-methamphetamine, a compound with vasopressor properties. For these reasons, several studies are currently underway to develop safer reversible and selective inhibitors of MAO B as an alternative to (*R*)-deprenyl (Gnerre *et al.,* 2000). In contrast to (*R*)-

deprenyl, these inhibitors are required to be reversible while retaining selectivity towards MAO B.

A literature survey reveals that reversible inhibition of MAO B by 1-methyl-3-phenylpyrrole (**10**) and its 4-chlorophenyl (**16**) analogue (Figure 15) has previously been demonstrated (Williams & Lawson, 1999). Incidentally, while investigating the substrate properties of various 1-methyl-3-phenyl-3-pyrrolinyl derivatives in this study, it was discovered that their respective MAO B catalyzed oxidation products also act as reversible competitive inhibitors of the enzyme.



1-Methyl-3-(4-chlorophenyl)-3-pyrroline (**15**) 1-Methyl-3-(4-chlorophenyl)pyrrole (**16**)

Figure 15: *The MAO B catalyzed oxidation of 1-methyl-3-(4-chlorophenyl)-3-pyrroline to 1-methyl-3-(4-chlorophenyl)pyrrole.*

Thus, the development of a pharmacophore model for the reversible MAO B inhibition by studying the stereoelectronic properties of some 1-methyl-3-phenylpyrrole (**10**) (Figure 7) analogues was another objective of this study. The pharmacophore model would be applied in the rational design and synthesis of novel, potent reversible and selective inhibitors of MAO B. As part of this second objective, 1-methyl-3-phenylpyrrole (**10**) analogues were synthesized and evaluated as competitive inhibitors of MAO B. The resulting information was used in the rational design of potent reversible inhibitors of MAO B, which are considered to be safer than inactivators such as (*R*)-deprenyl.

With the recent determination of the X-ray crystal structure of MAO B (Binda *et al.,* 2001), inhibitors displaying high potency were docked within the active site of the enzyme. Results from these studies afforded information about the spatial location and the main interactions required for reversible MAO B inhibition by the 1-methyl-3-phenylpyrrole (**10**) class of compounds and further assisted in the rational design of reversible inhibitors that have enhanced potency.

CHAPTER 3.

NEUROTOXICITY STUDIES WITH THE MONOAMINE OXIDASE B SUBSTRATE 1-METHYL-3-PHENYL-3-PYRROLINE

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Neurotoxicity studies with the monoamine oxidase B substrate 1-methyl-3-phenyl-3-pyrroline

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Abstract

The neurotoxic properties of the parkinsonian inducing agent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) are dependent on its metabolic activation in a reaction catalyzed by centrally located monoamine oxidase B (MAO-B). This reaction ultimately leads to the permanently charged 1-methyl-4-phenylpyridinium species MPP^+, a 4-electron oxidation product of MPTP and a potent mitochondrial toxin. The corresponding 5-membered analogue, 1-methyl-3-phenyl-3-pyrroline, is also a selective MAO-B substrate. Unlike MPTP, the MAO-B-catalyzed oxidation of 1-methyl-3-phenyl-3-pyrroline is a 2-electron process that leads to the neutral 1-methyl-3-phenylpyrrole. MPP^+ is thought to exert its toxic effects only after accumulating in the mitochondria, a process driven by the transmembrane electrochemical gradient. Since this energy-dependent accumulation of MPP^+ relies upon its permanent charge, 1-methyl-3-phenyl-3-pyrrolines and their pyrrolyl oxidation products should not be neurotoxic. We have tested this hypothesis by examining the neurotoxic potential of 1-methyl-3-phenyl-3-pyrroline and 1-methyl-3-(4-chlorophenyl)-3-pyrroline in the C57BL/6 mouse model. These pyrrolines did not deplete striatal dopamine while analogous treatment with MPTP resulted in 65–73% depletion. Kinetic studies revealed that both 1-methyl-3-phenyl-3-pyrroline and its pyrrolyl oxidation product were present in the brain in relatively high concentrations. Unlike MPP^+, however, 1-methyl-3-phenylpyrrole was cleared from the brain quickly. These results suggest that the brain MAO-B-catalyzed oxidation of xenobiotic amines is not, in itself, sufficient to account for the neurodegenerative properties of a compound like MPTP. The rapid clearance of 1-methyl-3-phenylpyrroles from the brain may contribute to their lack of neurotoxicity.

Keywords: Monoamine oxidase B; Neurotoxicity; MPTP; 1-Methyl-3-phenyl-3-pyrroline; 1-Methyl-3-phenylpyrrole; Dopamine; Striata

Introduction

The flavoenzyme monoamine oxidase B (MAO-B) has been identified as the principal catalyst responsible for the metabolic activation of the proneurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP (**1**)] in the brains of mammals including humans (Fig. 1) (Chiba et al., 1984; Heikkila et al., 1984b). The molecular mechanisms by which MPTP selectively damages nigrostriatal neurons and induces a parkinsonian syndrome has been the subject of extensive research (Heikkila et al., 1984a; Nicklas et al., 1985; Smeyne and Jackson-Lewis, 2005). Critical to its mode of action is the MAO-B-catalyzed ring α-carbon 2-electron oxidation of the parent compound to yield the corresponding 1-methyl-4-phenyl-2,3-dihydropyridinium species $MPDP^+$ ($\mathbf{2H^+}$). This metabolic intermediate, presumably via the corresponding free base **2**, undergoes a second 2-electron oxidation to generate the 1-methyl-4-phenylpyridinium metabolite MPP^+ ($\mathbf{3^+}$), the ultimate neurotoxin (Chiba et al., 1984; Ramsay et al., 1991; Markey et al., 1984). This process appears to take place mainly in MAO-B rich glial cells (Takada et al., 1990). MPP^+ is thought to accumulate via the plasma membrane dopamine transporter (DAT) (Chiba et al., 1985; Javitch and Snyder, 1984) in nigrostriatal nerve terminals where it localizes within the inner mitochondrial membrane (Sayre et al., 1990). Inhibition of complex I of the mitochondrial respiratory chain by MPP^+ then leads to downstream events such as ATP depletion and

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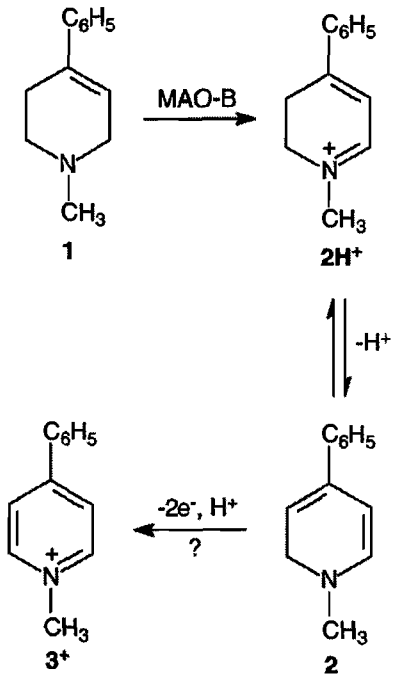

Fig. 1. The MAO-B-catalyzed oxidation of MPTP (**1**) to the corresponding 2,3-dihydropyridinium product $MPDP^+$ ($\mathbf{2H^+}$) and the pyridinium species MPP^+ ($\mathbf{3^+}$).

oxidative stress which eventually result in degeneration of nigrostriatal dopaminergic neurons (Singer et al., 1988). Experimental animals treated with MPTP have become useful models for studying neurodegenerative processes. In a frequently used protocol striatal dopamine concentrations of C57BL/6 mice are measured 7 days after systemic injection (multiple or single doses) of MPTP (Schmidt and Ferger, 2001). The depletion of striatal dopamine is indicative of the permanent loss of nigrostriatal dopaminergic cell bodies in the substantia nigra.

Over the years various tetrahydropyridinyl analogues of MPTP have been found to be MAO-B substrates (Kalgutkar and Castagnoli, 1992). One compound of particular interest is 1-methyl-3-phenyl-3-pyrroline (**4a**), a 5-membered ring analogue that has been shown to be a good and selective substrate of MAO-B (Wang et al., 1998). Unlike MPTP, the MAO-B-generated final metabolite of **4a** is the neutral 1-methyl-3-phenylpyrrole (**5a**). This overall 2-electron oxidation most likely arises via $\mathbf{6H^+}$, the short-lived conjugate acid of the pyrrolyl product **5a** (Fig. 2).

MPP^+ is thought to exert its toxic actions only after accumulating in the inner mitochondrial membrane of the nigrostriatal nerve terminals, a process driven by the transmembrane electrochemical gradient (Sayre et al., 1990). This energy-dependant accumulation of MPP^+ relies upon its permanent charge. Intrastriatal microdialysis studies have established that only permanently charged MPP^+ analogues exhibit dopaminergic neurotoxic properties (Rollema et al., 1990). Interestingly, the neutral *N*-desmethylated form of MPP^+, 4-phenylpyridine, is a much more potent inhibitor of complex I of the isolated respiratory chain than is MPP^+ but since it is not concentrated inside the mitochondria, this compound is not a physiological inhibitor of mitochondrial respiration. Accordingly, chronic exposure of mice to 4-phenylpyridine and 4-phenyl-1,2,3,6-tetrahydropyridine was found not to cause any reduction in striatal dopamine (Perry et al., 1987).

Based on these observations, 1-methyl-3-phenyl-3-pyrrolines and their MAO-B-catalyzed pyrrolyl oxidation products should not be neurotoxic. In this study we have tested this hypothesis by measuring ex vivo striatal dopamine concentrations in C57BL/6 mice 7 days after administration of 1-methyl-3-phenyl-3-pyrroline (**4a**) and 1-methyl-3-(4-chlorophenyl)-3-pyrroline (**4b**). Compound **4b** is also reported to be a MAO-B substrate (Williams and Lawson, 1998). The findings of this study are discussed with reference to the brain concentrations attained by both 1-methyl-3-phenyl-3-pyrroline and its pyrrolyl oxidation product compared to the brain concentrations attained by MPTP, $MPDP^+$ and MPP^+ following systemic treatment of MPTP.

Materials and methods

Caution: MPTP (**1**) is a known nigrostriatal neurotoxin and should be handled using disposable gloves and protective eyewear. Procedures for the safe handling of MPTP have been described previously (Pitts et al., 1986).

Chemicals and instrumentation

All starting materials not described elsewhere were obtained from Sigma-Aldrich and were used without purification. MPTP·HCl, dopamine·HCl and isoprenaline·HCl were purchased from Sigma-Aldrich. Petroleum ether used in this study had a distillation range of 40–60 °C. Melting points (mp) were determined with a Gallenkamp melting point apparatus and all melting points are uncorrected. Proton and carbon NMR spectra were recorded on a Varian Gemini 300 spectrometer. Proton (^1H) spectra were recorded at a frequency of 300 MHz and carbon

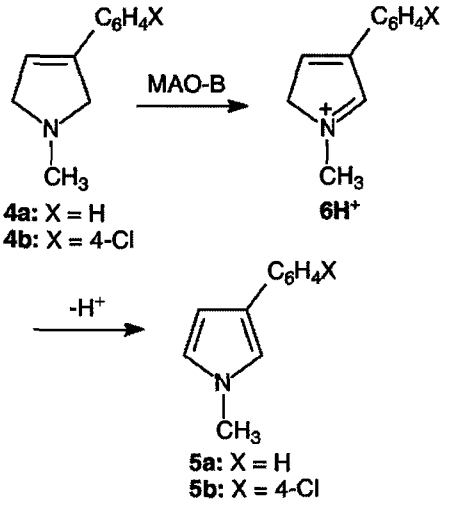

Fig. 2. The MAO-B-catalyzed oxidation of 1-methyl-3-phenyl-3-pyrrolines (**4**) to 1-methyl-3-phenylpyrroles (**5**).

(^{13}C) spectra at 75 MHz. Chemical shifts are reported in parts per million (δ) downfield from the signal of tetramethylsilane added to CD_3OD. Spin multiplicities are given as s (singlet), d (doublet), dd (doublet of doublets), t (triplet) or m (multiplet) and the coupling constants (J) are given in hertz (Hz). Fast atom bombardment mass spectra (FAB-MS) and high resolution mass spectra (HRMS) were obtained on a VG 7070E mass spectrometer. HPLC analyses were performed with an Agilent 1100 HPLC system equipped with an Agilent 1100 series variable wavelength detector. Thin layer chromatography (TLC) was carried out using neutral alumina 60 (Merck) with UV_{254} fluorescent indicator. UV/vis spectral measurements were recorded on a Milton-Roy Spectronic 1201 spectrophotometer.

*Synthesis of 3-phenyl-3-pyrroline hydrochloride (**7**·HCl)*

3-Phenyl-3-pyrroline hydrochloride (**7**·HCl) was prepared from 3-phenyl-3-pyrrolidinol according the method described by the literature (Lee et al., 2002) in a yield of 45%: mp 189–191 °C (ethanol), lit. mp 184 °C (Lee et al., 2002).

*Synthesis of 1-methyl-3-phenyl-3-pyrroline oxalate (**4a**·$C_2H_2O_4$)*

The hydrochloric acid salt of 3-phenyl-3-pyrroline (**7**·HCl) was converted to the corresponding free base with sodium carbonate (2 M). The thick white precipitate that formed was extracted into chloroform and the extract was dried over anhydrous magnesium sulfate. After removal of the chloroform phase, the residue appeared as a light yellow oil which solidified upon cooling to room temperature. The residue (0.55 mmol) was cooled to 0 °C while 98% formic acid (3.3 mmol) was added slowly followed by 40% formaldehyde (3.3 mmol) (Pine and Sanchez, 1971). The reaction mixture was stirred at 80 °C for 3 h and the reaction progress was monitored by TLC on neutral alumina (100% ethyl acetate). During this time, bubbling in the reaction was observed and the reaction turned dark red. Water (5 ml) was added to the reaction followed by an aqueous solution of 5 ml sodium carbonate (2 M). The resulting white precipitate was extracted to diethyl ether and the extract was dried over anhydrous magnesium sulfate. Upon removal of the solvent a light yellow oily residue remained which was converted into its oxalic acid salt by the addition of diethyl ether saturated with oxalic acid. The product was recrystallized from methanol at −20 °C and light yellow colored crystals were obtained in a yield of 30%: mp 152–154 °C. ^1H NMR (300 MHz, CD_3OD): δ 7.49–7.35 (m, 5H), 6.28 (d, 1H, J=1.76 Hz), 4.56 (s, 2H), 4.34 (s, 2H), 3.09 (s, 3H). ^{13}C NMR (75 MHz, CD_3OD): δ 166.71, 139.28, 132.78, 130.18, 129.95, 127.03, 18.83, 63.59, 62.53, 42.95. FAB-MS *m/z*: 160 (MH^+). HRMS calcd. 160.11263, found 160.11260 (MH^+).

*Synthesis of 1-methyl-3-(4-chlorophenyl)-3-pyrroline oxalate (**4b**·$C_2H_2O_4$)*

Compound **4b** was synthesized from 1-methyl-3-(4-chlorophenyl)pyrrole (**5b**) according to the procedure reported in the literature (Williams and Lawson, 1998, 1999) with the following modifications. The oily product obtained from the diethyl ether extract solidified at 0 °C. This solid was placed in a vacuum oven at 40 °C for 24 h. The material was converted into its oxalic acid salt in diethyl ether. Following recrystallization from methanol a colorless solid was obtained in 26% yield: mp 150–152 °C. ^1H NMR (300 MHz, CD_3OD): δ 7.48–7.34 (m, 4H), 6.32 (t, 1H. J=2.19 Hz), 4.55 (d, 2H, J=1.65 Hz), 4.35 (d, 2H, J=1.51 Hz), 3.09 (s, 3H). ^{13}C NMR (75 MHz, CD_3OD): δ 166.63, 137.14, 135.91, 131.50, 130.06, 128.60, 119.85, 63.60, 62.38, 42.97. FAB-MS *m/z*: 194 (MH^+). HRMS calcd. 194.07365, found 194.07371 (MH^+).

*Synthesis of 1,1-dimethyl-3-phenyl-3-pyrrolinium iodide (**8**·I)*

Compound **8**·I served as internal standard for the HPLC analysis of **4a**. The hydrochloric acid salt of 3-phenyl-3-pyrroline (**7**·HCl) was converted to the corresponding free base as described above for the synthesis of **4a**. This free base (1.43 mmol) in 10 ml tetrahydrofurane (THF) was treated with 3 equivalents of iodomethane (4.3 mmol). The reaction mixture was stirred for 1 h at room temperature at which time another 10 ml THF was added followed by an additional 3 equivalents of iodomethane. The suspension was heated under reflux for 30 min, cooled to 0 °C and filtered to collect the light yellow precipitate in a yield of 88%. This product was recrystallized from methanol to give the pure product in a yield of 29%: mp 224–226 °C. ^1H NMR (300 MHz, CD_3OD): δ 7.52–7.38 (m, 5H), 6.36 (m, 1H), 4.82 (dd, 2H, J=2.06, 2.03 Hz), 4.58 (dd, 2H, J=2.20, 2.09 Hz), 3.43 (s, 6H). ^{13}C NMR (75 MHz, CD_3OD): δ 137.97, 132.43, 130.47, 130.03, 127.11, 118.27, 74.11 (t, J=3.4–3.2 Hz), 73.03 (t, J=3.8–3.7 Hz), 54.61 (t, J=4.0–3.9 Hz). FAB-MS *m/z*: 174 (M^+), 175 (MH^+). HRMS calcd. 175.13610, found 175.13610 (MH^+).

*Synthesis of 2-aryl-3-(dimethylamino)allylidene(dimethyl)ammonium perchlorates (**9a–c**)*

Compounds **9a–c** were prepared in relatively high yields from the corresponding phenylacetic acid derivatives, DMF and phosphoryl chloride according to the method described in the literature (Jutz et al., 1969). Compound **9c** served as starting material for the preparation of the HPLC internal standard **5c** (see below). The melting points of compounds were as follows: **9a** mp 203–205 °C (from ethanol), lit. mp 193–194 °C (Jutz et al., 1969); **9b** mp 149–151 °C (from ethanol), lit. mp 142–144 °C (Jutz et al., 1969); **9c** mp 134–136 °C (from ethanol), lit. mp 130–131 °C (Jutz et al., 1969).

*Synthesis of 1-methyl-3-phenylpyrroles (**5a–c**)*

1-Methyl-3-phenylpyrroles (**5a–c**) were synthesized from compounds **9a–c** according to the method described in the literature (Gallagher et al., 1990). The crude products obtained were dissolved in a minimal amount of ethyl acetate and purified on a short column of neutral alumina (Fluka 507C) with 100% petroleum ether (**5a**, **b**) or petroleum ether/ethyl acetate, 90:10 (**5c**) as mobile phase. The fractions containing the product were

recrystallized from an appropriate solvent as indicated below. Compound **5c** served as internal standard for the HPLC analysis of **5a**. The melting points were as follows: **5a** mp 44–46 °C (from petroleum ether), lit. mp 46–47 °C (Gallagher et al., 1990); **5b** mp 112–114 °C (from methanol), lit. mp 117.5–119.5 °C (Gallagher et al., 1990); **5c** mp 120–122 °C (from methanol), lit. mp 126–128 °C (Gallagher et al., 1990).

Steady-state MAO-B activity measurements with **4a** *and* **4b** *as substrates*

Mitochondria were isolated from the tissues of beef liver, baboon liver, mouse liver and mouse brain as described in the literature (Salach and Weyler, 1987) and stored at −70 °C. The mitochondrial isolates were suspended in sodium phosphate buffer (100 mM, pH 7.4, containing 50% glycerol, w/v) and the protein concentrations were determined by the method of Bradford (1976). Since beef liver, baboon liver and mouse liver mitochondria are devoid of MAO-A activity, inactivation of MAO-A was unnecessary where the steady-state kinetic parameters of the oxidation of 1-methyl-3-phenyl-3-pyrrolines by MAO-B were measured (Inoue et al., 1999). The mitochondrial fraction obtained from mouse brain tissue is reported to contain 15% MAO-A activity (Inoue et al., 1999) but since the 1-methyl-3-phenyl-3-pyrrolines (**4a** and **4b**) examined in this study are reported to be MAO-B selective substrates (Wang et al., 1998) inactivation of MAO-A was again not deemed necessary. In order to estimate the K_m and V_{max} values for the oxidation of the 1-methyl-3-phenyl-3-pyrrolines by MAO-B, initial rates were measured at eight substrate concentrations spanning at least two orders of magnitude (6.25–2000 μM). The reactions were carried out in a final volume of 500 μl (in 100 mM sodium phosphate buffer, pH 7.4) and the enzyme concentration used was 0.15 mg mitochondrial protein/ml. All reactions were incubated at 37 °C for 10 min. For this time period the MAO-B-catalyzed production of the 1-methyl-3-phenylpyrrole products was found to be linear. The reactions were terminated by the addition of 70% perchloric acid (10 μl) and the samples were centrifuged at 16,000 *g* for 10 min. The supernatant fractions were removed and the concentrations of the MAO-B-generated 1-methyl-3-phenylpyrrolyl products were measured by HPLC analysis with UV detection (see Chemicals and instrumentation). A Phenomenex Luna C18 column (4.60×250 mm, 5 μm) was used and the mobile phase consisted of 25% distilled water [containing 0.6% (v/v) glacial acetic acid and 1% (v/v) triethylamine] and 75% acetonitrile at a flow rate of 1 ml/min. A volume of 50 μl of the supernatant was injected into the HPLC system and the elution of 1-methyl-3-phenylpyrrole (**5a**) or 1-methyl-3-(4-chlorophenyl)pyrrole (**5b**) was monitored at wavelengths of 270 and 280 nm, respectively. Quantitative estimations of **5a** and **5b** were made by means of linear calibration curves ranging from 1.5 to 25 μM and the initial rates were expressed as nmol of product formed per mg mitochondrial protein per min. The steady-state kinetic data (initial rates as a function of substrate concentration) were fitted to the Michaelis–Menten equation using the nonlinear least-squares fitting routine incorporated into the SigmaPlot software package (Systat Software Inc.). This determination was carried out in triplicate and the K_m and V_{max} values were expressed as means±standard error of the mean (S.E.M.).

Steady-state MAO-B activity measurements with MPTP as substrate

In order to estimate K_m and V_{max} values for the oxidation of MPTP by MAO-B, the same incubation procedures were followed as that described for the 1-methyl-3-phenyl-3-pyrroline substrates with the exception that the incubation time chosen was 12.5 min. The rates of oxidation of MPTP by MAO-B present in the mitochondrial fractions of beef liver, baboon liver, mouse liver and mouse brain were found to remain linear over this time period (results not shown). The concentrations of $MPDP^+$ in the incubations were measured spectrophotometrically at a wavelength of 343 nm (ε=16,000 M^{-1}) (Castagnoli et al., 1997). During the time needed to complete a typical experiment it was found, by HPLC analysis of incubations of 30–90 μM of MPTP with mouse brain mitochondria, that the concentrations of MPP^+ present in the incubations ranged from approximately 4.5% to 10.5% of the corresponding $MPDP^+$ concentrations. These HPLC analyses were carried out as described previously (Petzer et al., 2003).

Animal studies

Animal trials were conducted with retired breeder male C57BL/6 mice (30–35 g, 9–11 months of age) which were housed 5 animals per cage in a temperature (21±0.5 °C) and humidity (50±5% relative humidity) controlled room on a 12 h light–12 h dark cycle with free access to food and water. The animals were provided by the Laboratory animal center of the Potchefstroom campus and protocols for all animal experiments were reviewed and approved by the Research Ethics Committee of the North-West University. All injections were intraperitoneal (i.p.) in a volume of 0.2 ml per 30 g mouse. Sterile saline was used as vehicle for all of the test compounds. Mice were sacrificed by rapid cervical dislocation.

Striatal dopamine measurements

The concentrations of dopamine in dissected mouse striata were determined as described previously, (Harvey et al., 2006) and were expressed as means±S.E.M.

Brain MPTP, $MPDP^+$ and MPP^+ measurements

Immediately after sacrifice, the mouse brains were removed, placed in microcentrifuge tubes and frozen in liquid nitrogen. A portion (~30 mg) of the brain tissue of each mouse was weighed and the concentrations of MPTP, $MPDP^+$ and MPP^+ were determined via HPLC analysis as described previously (Castagnoli et al., 1997). The only modification made to the published procedure was that the internal standard used was changed to 1-methyl-4-(1-methylpyrrol-2-yl)pyridinium iodide (MMP^+) at a final concentration of 1.33 μM. MPTP, $MPDP^+$

and MPP^+ concentrations were expressed as pmol/mg wet weight of tissue (mean±S.E.M.).

Brain measurements of **4a** *and* **5a**

Four groups (n=5/group) of mice were treated with 238 μmol/kg 1-methyl-3-phenyl-3-pyrroline oxalate (**4a**·$C_2H_2O_4$) (see Animal studies above) and sacrificed at 10, 20, 40 and 60 min post-treatment, respectively. Their brains were removed and placed in microcentrifuge tubes. The brain tissues were frozen in liquid nitrogen until further sample preparation. For the measurement of 1-methyl-3-phenylpyrrole (**5a**) concentrations, a portion (~30 mg) of the brain tissue of each mouse was weighed and 10 μl/mg of the homogenizing solution (an aqueous solution of 50% acetonitrile, 0.305 M perchloric acid and 12.5 μM of compound **5c** as internal standard) was added. Following sonication (2×14 s, 14 μm) the samples were placed on ice for 60 min, centrifuged at 16,000 *g* for 15 min and 50 μl of the resulting supernatants were analyzed for 1-methyl-3-phenylpyrrole content by reverse phase HPLC equipped with a UV detection and a Phenomenex Luna C18 analytical column (see Chemicals and instrumentation). The mobile phase consisted of 35% aqueous phase (0.6% glacial acetic acid and 1% triethylamine in distilled water, pH 4.7) and 65% acetonitrile; the solvent was delivered at a flow rate of 1 ml/min. The elutions of **5a** (4.93 min) and internal standard **5c** (4.41 min) were monitored at a wavelength of 270 nm. A linear standard curve was constructed using four calibration standards (3.125–25 μM) prepared in the homogenizing buffer. In order to measure brain concentrations of 1-methyl-3-phenyl-3-pyrroline (**4a**), a portion of brain tissue (~30 mg) of each mouse was weighed and 10 μl/mg of the homogenizing solution (an aqueous solution of 10% acetonitrile, 0.305 M perchloric acid and 12.5 μM of compound **8**·I as internal standard) was added. Following sonication (2×14 s, 14 μm) the samples were placed on ice for 60 min, centrifuged at 16,000 *g* for 15 min and 50 μl of the resulting supernatants were analyzed for 1-methyl-3-phenyl-3-pyrroline content by reverse phase HPLC as described for 1-methyl-3-phenylpyrrole above. The mobile phase consisted of 90% aqueous phase (0.6% glacial acetic acid and 1% triethylamine in distilled water, pH 4.7) and 10% acetonitrile and solvent was delivered at a flow rate of 1 ml/min. The elutions of **4a** (9.66 min) and internal standard **8** (7.88 min) were monitored at a wavelength of 250 nm. A linear standard curve was constructed using four calibration standards (3.125–25 μM) prepared in the homogenizing buffer. The concentrations of **4a** and **5a** were expressed as pmol/mg wet weight of tissue (mean±S.E.M.).

Statistical analysis

Striatal dopamine levels were analyzed using an one-way analysis of variance (ANOVA), followed by multiple comparisons using the Dunnett's *t*-test to compare the experimental groups to the control group. The Statistica software package (StatSoft Inc.) was used for all data analysis and data are expressed as the mean±S.E.M.

Results

Chemistry

1-Methyl-3-phenyl-3-pyrroline (**4a**) was prepared from 3-phenyl-3-pyrroline (7) by the formic acid–formaldehyde methylation procedure (Eschweiler–Clarke reaction) described in the literature (Pine and Sanchez, 1971) (Fig. 3). As expected this method of methylation yielded exclusively the tertiary amine with no trace of the *N,N*-dimethylated pyrrolinium species (**8**) as judged by TLC. In contrast, treatment of 3-phenyl-3-pyrroline with one equivalent of iodomethane yielded a mixture of the tertiary and quaternary amines. Treatment with an excess of iodomethane (6 equiv.) yielded exclusively the pyrrolinium iodide salt (**8**·I) which readily separated from the reaction solvent (THF). The starting material 3-phenyl-3-pyrroline (7) was prepared from glycine ethyl ester and ethyl chloroformate in a synthetic route involving six steps (Wu et al., 1962; Kuhn and Osswald, 1956; Lee et al., 2002) with an overall yield of less than 3%. For this reason the preparation of 1-methyl-3-(4-chlorophenyl)-3-pyrroline (**4b**) was achieved via an alternative route (Fig. 4) (Williams and Lawson, 1998). The key starting material was 2-(4-chlorophenyl)-3-(dimethylamino)allylidene(dimethyl)ammonium perchlorate (**9b**) which was prepared in high yield from 4-chlorophenylacetic acid and DMF (Jutz et al., 1969). Cyclization of **9b** was achieved by treatment with sodium methoxide in anhydrous pyridine to yield 1-methyl-3-(4-chlorophenyl)pyrrole (**5b**) in fair yield (52.8%) (Gallagher et al., 1990). The preparations of 1-methyl-3-phenylpyrrole (**5a**) and 1-methyl-3-(4-methoxyphenyl)pyrrole (**5c**) were achieved in a similar manner starting from phenylacetic acid and 4-methoxyphenylacetic acid, respectively. The 1-methyl-3-(4-chlorophenyl)pyrrole intermediate was converted to the corresponding 1-methyl-3-(4-chlorophenyl)-3-pyrroline (**4b**) by partial reduction with zinc and hydrochloric acid (Williams and Lawson, 1998; Andrews and McElvain, 1929) in an overall yield of 31.9%. In our experience 3-pyrrolines prepared in this manner are often contaminated with what we believe is the corresponding pyrrolidinyl derivatives (Hudson and Robertson, 1967) in amounts ranging from approximately 10–25% as judged by ^1H NMR. We found that in selected instances (for example in the preparation of **4b**, the pyrrolidinyl contaminant may be removed from the 3-pyrrolinyl free base under reduced pressure with slight heating (see

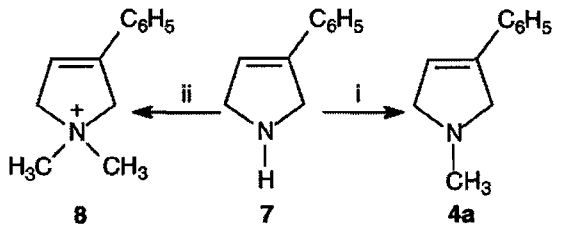

Fig. 3. Synthetic pathways to 1-methyl-3-phenyl-3-pyrroline (**4a**) and 1,1-dimethyl-3-phenyl-3-pyrrolinium iodide (**8**·I). Key: (i) formic acid, formaldehyde, 80 °C; (ii) CH_3I (6 equiv.), K_2CO_3, THF, rt.

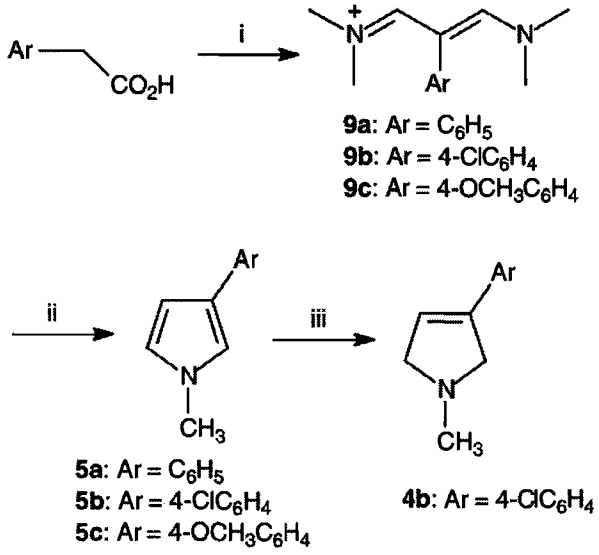

Fig. 4. Synthetic pathway to 1-methyl-3-phenylpyrroles (**5a–c**) and 1-methyl-3-(4-chlorophenyl)-3-pyrroline (**4b**). Key: (i) DMF, $POCl_3$, 80 °C; (ii) $NaOCH_3$, pyridine, reflux; (iii) Zn, HCl, 60 °C.

Materials and methods). Alternatively the impurity can also be removed by fractional crystallization of the oxalate salts.

General enzymology

Both 1-methyl-3-phenyl-3-pyrroline (**4a**) and 1-methyl-3-(4-chlorophenyl)-3-pyrroline (**4b**) have previously been shown to be substrates for MAO-B (Wang et al., 1998; Williams and Lawson, 1998). The 1-methyl-3-phenyl analogue is reported to have a K_m value of 193 μM and a V_{max} value of 397 min^{-1} for oxidation by beef liver MAO-B (Wang et al., 1998) while the K_m and V_{max} values for the oxidation by rat liver MAO-B are reported to be 79 μM and 8.1 nmol/min mg, respectively (Williams and Lawson, 1998). The K_m and V_{max} values for the oxidation of 1-methyl-3-(4-chlorophenyl)-3-pyrroline by rat liver MAO-B are reported to be 67 μM and 10.3 nmol/min mg, respectively (Williams and Lawson, 1998). In the present study we have confirmed both **4a** and **4b** to be good substrates of beef liver, baboon liver, mouse liver and mouse brain MAO-B, and also compared their substrate properties with that of MPTP. In order to compare the substrate properties of **4a**, **4b** and MPTP, it was necessary to determine the steady-state kinetic parameters (K_m and V_{max} values) for all three substrates in a single species since the MAO-B substrate properties often differs between species and even between different tissues in the same species (Inoue et al., 1999). Since the V_{max} values are dependent upon the MAO-B concentration in the mitochondrial preparation used in the assays, and the enzyme concentrations differ from preparation to preparation, cited values could not be used for direct comparison of the substrates. All the substrates were therefore re-examined. In order to measure the steady-state kinetic parameters we chose to measure the extent of oxidation of **4a** and **4b** by HPLC-UV analysis. The concentrations of the MAO-B-catalyzed pyrrole products **5a** and **5b** could be readily measured at wavelengths of 270 and 280 nm, respectively. HPLC-UV was chosen as the analytical technique since background interference in the near-UV wavelength range by the mitochondrial fractions used here as enzyme source was too high to measure the pyrrolyl product concentrations accurately by spectrophotometry. The incubation time of the enzyme-catalyzed reactions were chosen to be 10 min since the oxidation of both substrates (**4a** and **4b**) was found to be linear (results not shown) for at least 12 min at a substrate concentration of 50 μM. Since the mitochondrial fraction obtained from mouse brain tissue is reported to contain 15% MAO-A activity (Inoue et al., 1999) we measured the contribution of MAO-A towards the oxidation of 1-methyl-3-phenyl-3-pyrroline (**4a**) by the mitochondria obtained from this source. The MAO-B isoform was inactivated by pre-incubating the mitochondria with the MAO-B selective inactivator (*R*)-deprenyl (Inoue et al., 1999). Following incubation of **4a** (250 μM) with the MAO-B inactivated mitochondria only trace amounts (0.28±0.03 μM) of the pyrrolyl oxidation product were detected (results not shown). This was less than 5% of the product concentration (8.58±0.31 μM) detected in experiments with mitochondria not previously inactivated. In contrast, when the MAO-A isoform was inactivated by pre-incubating the mitochondria with the MAO-A selective inactivator clorgyline (Inoue et al., 1999) relatively larger amounts (7.18±0.43 μM) of the pyrrolyl product were detected which was approximately 84% of the product concentration detected in experiments with mitochondria not previously inactivated. These results document that MAO-A does not contribute significantly to the oxidation of **4a** by mouse brain mitochondrial isolates and that the steady-state kinetic parameters measured for **4a** are representative of its oxidation by the MAO-B isoform. Since mitochondria obtained from beef liver, baboon liver and mouse liver tissues are devoid of MAO-A activity (Inoue et al., 1999), the oxidation of **4a** and **4b** by these enzyme sources can be exclusively attributed to the action of the MAO-B isoform.

MAO-B substrate properties of **4a** *and* **4b**

As illustrated by example in Fig. 5 the steady-state oxidation of the substrates by MAO-B followed Michaelis–Menten behavior. The K_m and V_{max} values obtained for the oxidation of the two pyrroline substrates and MPTP by beef liver, baboon liver, mouse liver and mouse brain MAO-B are summarized in Table 1. Except for the kinetic data generated with mouse liver mitochondria no significantly large interspecies differences of the K_m values were apparent. The K_m values of **4a**, **4b** and MPTP were significantly larger with mouse liver mitochondria than with the other mitochondrial sources examined here. For example, a K_m value of 461±26.9 μM was observed for the oxidation of **4a** by mouse liver MAO-B while the corresponding value with mouse brain mitochondria was found to be 125±7.04 μM. Similarly, for the oxidation of MPTP, a K_m value of 797±11.5 μM was recorded with mouse liver MAO-B compared to a value of 52.1±3.89 μM observed with mitochondria from mouse brain. These observations are in agreement with the literature which reports a K_m value for the

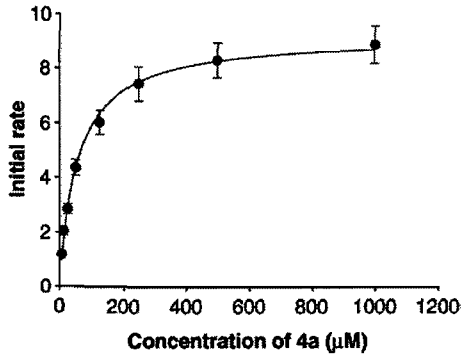

Fig. 5. Determination of the K_m and V_{max} values for the oxidation of **4a** by baboon liver MAO-B. The concentration of **5a** produced was measured by HPLC analysis following a 10 min incubation with 0.15 mg/ml baboon liver mitochondria at 37 °C. The rate data were fitted to the Michaelis–Menten equation using a nonlinear least-squares fitting routine. All measurements were conducted in triplicate and the concentration of **4a** in the incubations ranged from 6.25 to 1000 μM. The initial rates are expressed as nmol/min mg protein of **5a** formed.

oxidation of MPTP by mouse liver MAO-B of 520 μM while a K_m value of 96.8 μM was measured with mouse brain mitochondria (Inoue et al., 1999). The factors contributing to these tissue dependent differences in MAO-B activity remain to be identified. Also of note, the V_{max} values for the oxidation of **4a** and **4b** by mouse brain mitochondrial MAO-B were significantly lower than the corresponding values obtained with beef, baboon and mouse liver mitochondria as enzyme sources. Assuming that there are no large interspecies differences of the turnover numbers (k_{cat}) for the oxidation of **4a** and **4b** by MAO-B, this difference in V_{max} is possibly due to the lower density of MAO-B in brain mitochondrial fractions compared to the liver. The trend has been previously observed in various species where consistently lower V_{max} values have been measured with brain mitochondria as MAO-B source compared to the liver (Inoue et al., 1999). For example baboon liver mitochondria oxidize MPTP with a V_{max} value of 6.3 nmol/min mg protein while with brain mitochondria a V_{max} value of 2.4 nmol/min mg protein was observed. These

Table 1
Steady-state kinetic parameters for the oxidation of **4a**, **4b** and MPTP by MAO-B present in the mitochondrial fractions of beef liver, baboon liver, mouse brain and mouse liver tissue

		K_m (μM)	V^a_{max}	V_{max}/K^b_m
Beef liver	**4a**	56.4±2.82; (193)[c]	9.0±0.5	0.16
	4b	44.8±5.97	9.1±0.8	0.20
	MPTP	138±2.90; (191)[c]	3.1±0.04	0.023
Baboon liver	**4a**	54.6±3.21	9.2±0.7	0.17
	4b	46.1±10.6	4.7±0.4	0.10
	MPTP	173±6.93 (87.5)[d]	6.7±0.1	0.039
Mouse brain	**4a**	125±7.04	0.8±0.02	0.007
	4b	21.0±3.54	0.4±0.02	0.019
	MPTP	52.1±3.89; (40)[e]; (96.8)[d]	0.9±0.1	0.017
Mouse liver	**4a**	461±26.9	5.9±0.2	0.013
	4b	63.5±6.57	2.2±0.3	0.034
	MPTP	797±11.5; (520)[d]	10.6±0.1	0.013

The values are expressed in [a]nmol/min mg mitochondria and [b](min mg protein)$^{-1}$. [c]Wang et al. (1998). [d]Inoue et al. (1999). [e]Castagnoli et al. (1997).

two compounds (**4a** and **4b**) were approximately equally good substrates for MAO-B as judged by the similarity of their V_{max}/K_m values. These V_{max}/K_m values were found to be consistently higher than for MPTP which was confirmed to be also a relatively good substrate of MAO-B. These results are in accordance with literature reports that MPTP and 1-methyl-3-phenyl-3-pyrroline act as good substrates of beef liver (Wang et al., 1998) as well as rat liver (Williams and Lawson, 1998) MAO-B. We have previously shown that the well-known MAO-B substrate benzylamine is oxidized by baboon liver MAO-B with K_m and V_{max} values of 616±23 μM and 63± 2.2 nmol/min mg, respectively (Vlok et al., 2006). This yields a V_{max}/K_m value of 0.10 (min mg protein)$^{-1}$ which compares with the corresponding values obtained for the pyrroline substrates **4a** and **4b** [0.17 and 0.10 (min mg protein)$^{-1}$]. Therefore, consistent with expectation, it can be concluded that **4a** and **4b** are good substrates of MAO-B isolated from a variety of species and therefore these compounds should be oxidized efficiently in vivo to the corresponding pyrrolyl products (**5a** and **5b**).

Neurotoxicity studies

It is well documented that a single intraperitoneal injection of MPTP (95–238 μmol/kg) causes depletion of striatal dopamine in aged C57BL/6 mice while still being sub-lethal (Di Monte et al., 1997; Castagnoli et al., 2001). This depletion measured 7–10 days following treatment is frequently used as marker for the nigrostriatal degeneration resulting from the neurotoxic action of MPP^+. In this study we investigated whether similar

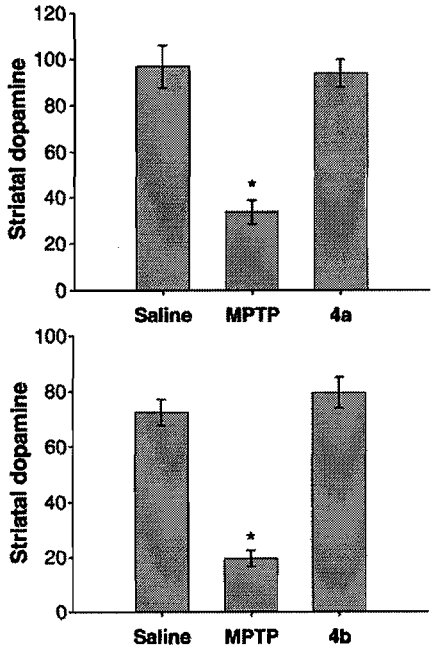

Fig. 6. Striatal dopamine levels of C57BL/6 male mice (n=10 mice/group) treated i.p. with saline, MPTP·HCl (167 μmol/kg), **4a**·$C_2H_2O_4$ (238 μmol/kg) (top) or **4b**·$C_2H_2O_4$ (238 μmol/kg) (bottom). Dopamine levels were measured 7 days after treatment and are expressed as pmol/mg tissue. *Significantly different (p<0.01) from the saline treated group.

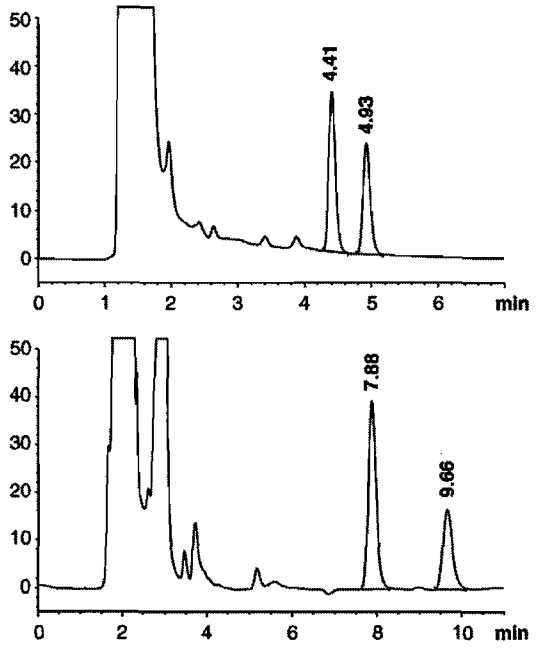

Fig. 7. HPLC-UV tracings showing the presence of 1-methyl-3-phenylpyrrole (**5a**) (top; 4.93 min; λ=270 nm) and 1-methyl-3-phenyl-3-pyrroline (**4a**) (bottom; 9.66 min; λ=250 nm) in the brain tissue of mice sacrificed 10 min following treatment with 238 μmol/kg 1-methyl-3-phenyl-3-pyrroline oxalate (**4a**·$C_2H_2O_4$). The internal standards used were 1-methyl-3-(4-methoxyphenyl) pyrrole (**5c**) (top; 4.41 min) and 1,1-dimethyl-3-phenyl-3-pyrrolinium iodide (**8**·I) (bottom; 7.88 min).

treatment of mice with **4a** and **4b** also results in a loss of dopamine in the striatum and, by association, nigrostriatal injury. Two studies were carried out each containing a control group (n=10 mice) which received saline (0.20 ml/30 g mouse) and a MPTP group (n=10 mice) which received a relatively lower dose of MPTP·HCl (167 μmol/kg). The third group (n=10 mice) received **4a**·$C_2H_2O_4$ or **4b**·$C_2H_2O_4$ at a relatively high dose of 238 μmol/kg. The animals were sacrificed 7 days later, the striata were dissected and the dopamine concentrations were determined by HPLC-ECD analysis as described in the Materials and methods. The results (Fig. 6) show that neither **4a** nor **4b** induced depletion of dopamine with the measured dopamine concentrations at 97% (Fig. 6, top) and 110% (Fig. 6, bottom) of the control values, respectively. In contrast, MPTP treatment significantly (p<0.001) reduced the dopamine levels to 35% (Fig. 6, top) and 27% (Fig. 6, bottom) of the control values.

In vivo kinetic studies

The finding that high doses of **4a** and **4b** do not mimic the characteristic striatal dopamine depletion effect of MPTP is in accordance with the idea that the permanent positive charge of MPP^+ is a key structural feature contributing to its neurotoxic action. While the lack of neurotoxicity by **4a** and **4b** may be explained by this hypothesis, it is also possible that the 1-methyl-3-phenyl-3-pyrrolines are not metabolized as efficiently as MPTP by MAO-B in vivo. This could result in relatively

Table 2
Brain concentrations of **4a** and **5a** in C57BL/6 mice (n=5 mice/group) treated with 238 μmol/kg of **4a**·$C_2H_2O_4$ and sacrificed at the indicated times (min)

Time	Brain concentrations (pmol/mg of wet tissue)	
	4a	**5a**
10	65.5±11.2	53.0±9.89
20	34.1±2.71	37.0±5.29
40	6.92±0.86	6.39±1.60
60	2.20±0.40	3.11±2.54

lower brain concentrations of the pyrrolyl products compared to a concentration of MPP^+ that leads to neuronal injury. Another explanation for the lack of neurotoxicity of **4a** and **4b** is that the bioavailability of 1-methyl-3-phenyl-3-pyrrolines to the brain may be lower than that of MPTP or that the clearance of 1-methyl-3-phenyl-3-pyrrolines and/or their pyrrolyl oxidation products from the brain may be faster than MPTP or MPP^+. To test these theories we injected mice (n=5 mice/group) with 238 μmol/kg of **4a**·$C_2H_2O_4$ and measured the brain concentrations of **4a** and its MAO-B-catalyzed oxidation product **5a** at various time points (10, 20, 40 and 60 min) following treatment (Fig. 7). In a second experiment, mice (n=6 mice/group) similarly received 167 μmol/kg MPTP·HCl and the concentrations of MPTP, $MPDP^+$ and MPP^+ in the brain tissue were measured at 10 min and 90 min following treatment. It is reported that MPTP concentrations reach a maximum at 10 min following i.p. injection while MPP^+ concentrations reach a maximum at approximately 90 min following MPTP injection (Castagnoli et al., 1997). Results in Tables 2 and 3 show that, like MPTP, **4a** reaches maximum brain concentrations relatively early with a measured concentration of 65.5±11.2 pmol/mg tissue at 10 min following treatment. This concentration is similar to the MPTP concentration in the brain of 80.17± 4.99 pmol/mg at 10 min following treatment with a neurotoxic dose of MPTP. The MAO-B-generated pyrrole product **5a** was also found to reach maximal brain concentrations relatively early with a measured concentration of 53.0±9.89 pmol/mg tissue at 10 min following treatment with **4a**. This relatively high concentration of **5a** is in contrast to the MPP^+ concentration in the brain of only 11.17±2.15 pmol/mg tissue at 10 min following MPTP treatment. The pyrrolyl product **5a**, however, was found to clear relatively quickly from the brain; a concentration of only 3.11±2.54 pmol/mg tissue was detected at 60 min post-treatment. This is again in contrast to MPP^+ which was found to be present in the brain in a relatively high concentration of 77.67±4.87 pmol/mg tissue even at 90 min

Table 3
Brain concentrations of MPTP, $MPDP^+$ and MPP^+ in C57BL/6 mice (n=6 mice/group) treated with 167 μmol/kg of MPTP·HCl and sacrificed at the indicated times (min)

Time	Brain concentrations (pmol/mg of wet tissue)		
	MPTP	$MPDP^+$	MPP^+
10	80.17±4.99	25.67±1.23	11.17±2.15
90	4.44±1.60	4.60±1.37	77.67±4.87[a]

[a] MPP^+ concentrations reach a maximum at ~90 min following MPTP injection (Castagnoli et al., 1997).

post-MPTP treatment. The literature reports that MPP^+ concentrations remain relatively high even at a time point of 240 min post-treatment (Di Monte et al., 1997; Castagnoli et al., 1997).

Discussion

In this investigation the nigrostriatal neurotoxic potentials of the 1-methyl-3-phenyl-3-pyrrolines **4a** and **4b** were compared with that of MPTP. It is generally accepted that the neurotoxicity of MPTP is a consequence of its MAO-B-catalyzed metabolism in the brain that ultimately yields the mitochondrial toxin MPP^+ (Chiba et al., 1984; Heikkila et al., 1984b). Evidence suggests that the mitochondrial toxicity and subsequent neurotoxic action of MPP^+ relies on it being permanently charged (Sayre et al., 1990; Rollema et al., 1990). Since the pyrrolyl oxidation products of 1-methyl-3-phenyl-3-pyrrolines are neutral species, **4a** and **4b** are not expected to be neurotoxic.

Here both **4a** and **4b** were shown to act as good substrates for beef liver, baboon liver, mouse liver and mouse brain MAO-B. Judging by the steady-state kinetic parameters, the in vitro MAO-B-catalyzed oxidation of **4a** and **4b** was at least as efficient as that of MPTP and, in some instances, even superior. The results of the neurotoxicity studies, however, show that even at high doses, **4a** and **4b** do not result in depletion of striatal dopamine as observed with MPTP. We conclude, therefore, that these pyrrolines, and probably also other 1-methyl-3-phenyl-3-pyrrolines, are not MPTP-type dopaminergic neurotoxins.

From the in vivo kinetic studies we conclude that **4a** is sufficiently bioavailable to reach brain concentrations similar to those of MPTP following a neurotoxic dose. The pyrrolyl product **5a** also reaches the brain in relative high concentrations which are comparable to the maximum concentration measured for MPP^+. The concentrations measured for **5a** in the brain are probably representative of **5a** generated centrally by the action of MAO-B on **4a** as well as **5a** partitioning from the periphery where it is produced by peripherally located MAO-B and possibly by the cytochrome P450 isozymes. Unlike **5a**, MPP^+ carries a permanent positive charge and is not expected to cross the blood–brain barrier (BBB). Therefore MPP^+ concentrations measured in the brain are representative of centrally generated MPP^+ only. The slow clearance of MPP^+ from the brain may possibly be explained by its low BBB permeability. In contrast pyrrole **5a** is cleared from the brain relatively quickly since it probably crosses the BBB freely. The relatively fast clearance from the brain may be another factor that contributes to the observed lack of neurotoxicity of the 1-methyl-3-phenylpyrroles **5a** and **5b**.

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CHAPTER 4.

STRUCTURE-ACTIVITY RELATIONSHIPS IN THE INHIBITION OF MONOAMINE OXIDASE B BY 1-METHYL-3-PHENYLPYRROLES

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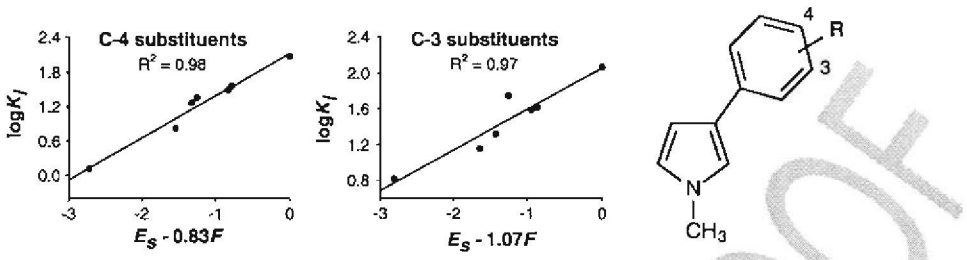

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Structure–activity relationships in the inhibition of monoamine oxidase B by 1-methyl-3-phenylpyrroles

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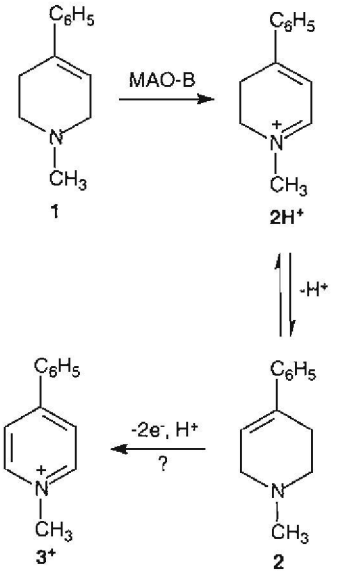
Abstract—1-Methyl-3-phenyl-3-pyrrolines are structural analogues of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and like MPTP are selective substrates of monoamine oxidase B (MAO-B). As part of an ongoing investigation into the substrate properties of various 1-methyl-3-phenyl-3-pyrrolinyl derivatives, it is shown in the present study that their respective MAO-B catalyzed oxidation products act as reversible competitive inhibitors of the enzyme. The most potent inhibitor among the oxidation products considered was 1-methyl-3-(4-trifluoromethylphenyl)pyrrole with an enzyme-inhibitor dissociation constant (K_i value) of 1.30 μM. The least potent inhibitor was found to be 1-methyl-3-phenylpyrrole with a K_i value of 118 μM. The results of an SAR study established that the potency of MAO-B inhibition by the 1-methyl-3-phenylpyrrolyl derivatives examined here is dependent on the Taft steric parameter (E_s) and Swain–Lupton electronic constant (F) of the substituents attached to C-4 of the phenyl ring. Electron-withdrawing substituents with a large degree of steric bulkiness appear to enhance inhibition potency. Potency was also found to vary with the substituents at C-3, again with E_s and F being the principal substituent descriptors.

1. Introduction

The first step in the metabolic activation of the parkinsonian inducing proneurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP, (**1**)], is catalyzed by the flavoenzyme, monoamine oxidase B (MAO-B) (Scheme 1),[1,2] to yield the ring α-carbon 2-electron oxidation product, the corresponding 1-methyl-4-phenyl-2,3-dihydropyridinium species $MPDP^+$ ($\mathbf{2H^+}$). This metabolic intermediate, presumably via the corresponding conjugate base **2**, undergoes a second 2-electron oxidation to generate the 1-methyl-4-phenylpyridinium metabolite MPP^+ ($\mathbf{3^+}$), the ultimate neurotoxin.[3,4] Literature reports various structural analogues of MPTP that have been found to act as good substrates of MAO-B[5,6] as well as of MAO-A.[7] Among these is 1-methyl-3-phenyl-3-pyrroline (**4**) (Scheme 2) which, like MPTP, is a cyclic tertiary allylamine exhibiting selectivity for the MAO-B isoform.[8] The MAO-B catalyzed ring α-carbon



Scheme 1. The MAO-B catalyzed oxidation of MPTP (**1**) to yield the corresponding dihydropyridinium product $MPDP^+$ ($\mathbf{2H^+}$) which spontaneously undergoes a second 2-electron oxidation to generate MPP^+ ($\mathbf{3^+}$).

Keywords: Monoamine oxidase B; Reversible inhibitors; Competitive inhibition; 1-Methyl-3-phenylpyrrole; Structure–activity relationship.
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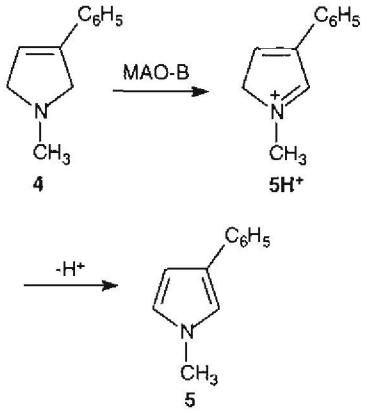


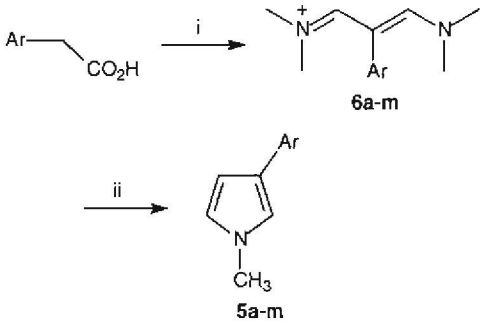
Scheme 2. The MAO-B catalyzed oxidation of 1-methyl-3-phenyl-3-pyrrolinyl derivatives (4) to yield the corresponding 1-methyl-3-phenylpyrrolyl products (5). This oxidation most likely arises via $5H^+$, the short-lived conjugate acid of the pyrrolyl product 5.

2-electron oxidation of **4** yields 1-methyl-3-phenylpyrrole (**5**) as the final product. This oxidation most likely arises via $5H^+$, the short-lived conjugate acid of the pyrrolyl product **5**.[9] As part of an ongoing investigation into the substrate properties of various 1-methyl-3-phenyl-3-pyrrolinyl derivatives, it is shown in the present study that their respective MAO-B catalyzed oxidation products act as reversible competitive inhibitors of the enzyme. A literature survey reveals that reversible inhibition of MAO-B by 1-methyl-3-phenylpyrrole (**5a**) and its 4-chlorophenyl analogue (**5b**) has previously been demonstrated.[10] In an attempt to determine the effect that specific structural modifications of 1-methyl-3-phenylpyrrole will have on MAO-B inhibition potency, we have synthesized 13 1-methyl-3-phenylpyrrolyl derivatives (**5a–m**) and determined their enzyme-inhibitor dissociation constants (K_i values) for reversible interaction with MAO-B. As part of the present SAR analysis, the 1-methyl-3-phenylpyrrolyl derivatives investigated differed only in the substituents on C-3 and C-4 of the phenyl ring. Since inhibitors of MAO-B are currently in use and being investigated for the treatment of neurodegenerative disorders,[11,12] the results of this study may aid in the identification and design of new reversible inhibitors.

2. Results

2.1. Chemistry

The 1-methyl-3-phenylpyrrolyl derivatives (**5a–m**) examined in this study were prepared in relatively good yields (26.6–72.7%) according to a previously reported procedure (Scheme 3).[13] The key starting materials were the 2-aryl-3-(dimethylamino)allylidene(dimethyl)ammonium perchlorates (**6a–m**) which were prepared in very high yields from the appropriately substituted phenylacetic acid derivatives and DMF.[14] Cyclization of **6**, to yield the target pyrrolyl derivatives (**5a–m**), was achieved by treatment with sodium methoxide in anhydrous pyridine. Following purification by column chromatography or by recrystallization from a suitable solvent, the structures and purity of the compounds were verified by mass spectrometry, ^{1}H NMR, and ^{13}C NMR. For those compounds previously reported, the physical data and melting points obtained were compared to the corresponding literature values as cited in Section 4.



Scheme 3. Synthetic pathway to 1-methyl-3-phenylpyrroles (**5a–m**). Reagents and conditions: (i) DMF, $POCl_3$, 80 °C; (ii) $NaOCH_3$, pyridine, reflux.

2.2. Enzymology and inhibition studies

In the present study we have determined the enzyme-inhibitor dissociation constants (K_i values) for reversible interaction of MAO-B with members of a synthetic series of 1-methyl-3-phenylpyrrolyl derivatives (**5a–m**). MAO-B activity measurements were based on the ring α-carbon oxidation of 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (MMTP) to yield the corresponding dihydropyridinium species ($MMDP^+$).[15] The concentrations of $MMDP^+$ produced by this enzymatic reaction were measured spectrophotometrically since $MMDP^+$ absorbs light maximally at 420 nm. At this wavelength neither the enzyme substrate nor the test inhibitors absorb light. Because of these favorable chromophoric characteristics and the in vitro chemical stability of $MMDP^+$, this assay is frequently used to evaluate the potencies of potential inhibitors of MAO-B.[16,17] The mitochondrial fraction obtained from baboon liver tissue was employed as enzyme source since it exhibits a high degree of MAO-B catalytic activity and is devoid of MAO-A activity.[15] Therefore, even though MMTP is a MAO-A/B mixed substrate, its oxidation by baboon liver mitochondria can be attributed exclusively to the action of the MAO-B isoform. The interaction of reversible inhibitors with MAO-B obtained from baboon liver tissue appears to be similar to the interaction with the human form of the enzyme since inhibitors are approximately equipotent with both enzyme sources.[17]

The MAO-B inhibitory properties of **5a–m** first were investigated in order to determine whether the test inhibitors act as time-dependent inactivators or reversible inhibitors of the enzyme. For this study **5e** was selected as a representative test inhibitor. When baboon liver mitochondrial fractions were preincubated with **5e** (5.20 μM) for periods of 0, 15, 30, and 60 min, the rate of MAO-B catalyzed oxidation of MMTP (90 μM) to $MMDP^+$ remained unchanged (results not shown).[18]

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From this result it can be concluded that **5e** interacts reversibly with the active site of MAO-B. The reversibility of enzyme inhibition by **5a–m** was also apparent from the Lineweaver–Burk plots constructed from the kinetic data (see below).

All of the 1-methyl-3-phenylpyrrolyl derivatives (**5a–m**) evaluated were found to be inhibitors of MAO-B. As demonstrated with 1-methyl-3-(3-chlorophenyl)pyrrole (**5h**) (Fig. 1), classical Lineweaver–Burk plots for competitive inhibition were obtained for all of the test inhibitors. The enzyme-inhibitor dissociation constants (K_i values) for the inhibition of MAO-B by **5a–m** are presented in Table 1. The data lead to the conclusion that substitution on the phenyl ring leads to inhibitors with enhanced inhibition potencies since **5a** was found to be the weakest inhibitor. The most potent inhibitor of the series was 1-methyl-3-(4-trifluoromethylphenyl)pyrrole (**5e**) with a K_i value of 1.30 μM. The second most potent inhibitor of the series was 1-methyl-3-(3-trifluoromethylphenyl)pyrrole (**5k**) with a K_i value of 6.55 μM. Substitution at C-4 or C-3 of the phenyl ring with the electronegative CF_3 functional group therefore appears to be the best method of enhancing the binding affinity of 1-methyl-3-phenylpyrrolyl derivatives for the active site of MAO-B. Interestingly, no inhibition of beef liver MAO-B was observed by **5e**, even at a concentration of 200 μM (results not shown). Similarly, no inhibition of beef liver MAO-B was observed by any of the other pyrrolyl derivatives examined here. Further inspection of Table 1 reveals that substitution with bromine at C-4 (**5c**) and C-3 (**5i**) also results in pyrrolyl derivatives with relatively high inhibition potencies. Since substitution at C-4 (**5d**) and C-3 (**5j**) of the phenyl ring with fluorine resulted in relatively weaker inhibitors compared to the bromine analogues **5c** and **5i** and chlorine analogues **5b** and **5h**, it does not appear that potent inhibition is linked exclusively to the presence of electronegative substituents in the phenyl ring.

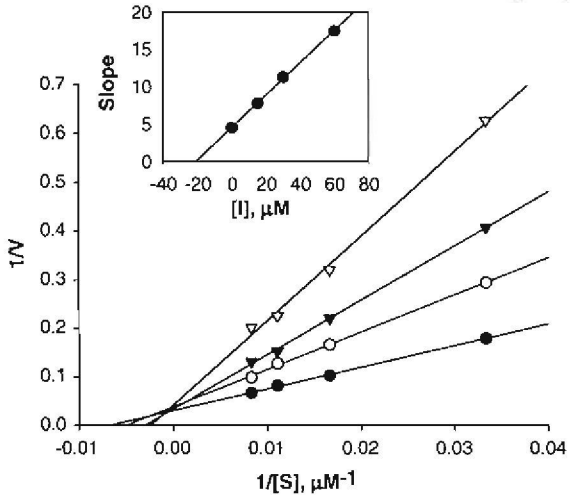
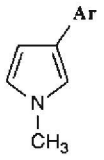


Figure 1. Lineweaver–Burk plots of the oxidation of MMTP by baboon liver MAO-B in the absence (filled circles) and presence of various concentrations of **5h** (open circles, 15 μM; filled triangles, 30 μM; and open triangles, 60 μM). The concentration of the baboon liver mitochondrial isolate was 0.15 mg/mL and the rates are expressed as nmol/min mg protein of $MMDP^+$ formed. The inset is the replot of the slopes versus the inhibitor concentration.

Table 1. The K_i values for the inhibition of MAO-B by 1-methyl-3-phenylpyrrolyl derivatives (**5a–m**)[19]



Compound	Ar	K_i value (μM)[a]	E_s[b]	F[b]
5a	C_6H_5	118	0.00	0.00
5b	4-ClC_6H_4	18.4	−0.97	0.42
5c	4-BrC_6H_4	6.65	−1.16	0.45
5d	4-FC_6H_4	31.0	−0.46	0.45
5e	4-$CF_3C_6H_4$	1.30	−2.40	0.38
5f	4-$CH_3C_6H_4$	22.5	−1.24	0.01
5g	4-$OCH_3C_6H_4$	36.5	−0.55	0.29
5h	3-ClC_6H_4	20.9	−0.97	0.42
5i	3-BrC_6H_4	14.3	−1.16	0.45
5j	3-FC_6H_4	38.9	−0.46	0.45
5k	3-$CF_3C_6H_4$	6.55	−2.40	0.38
5l	3-$CH_3C_6H_4$	56.0	−1.24	0.01
5m	3-$OCH_3C_6H_4$	41.7	−0.55	0.29

The values of the selected physicochemical parameters used in the SAR studies are also listed.
[a] The enzyme source used was baboon liver mitochondrial MAO-B.
[b] Values obtained from Ref. 19.

2.3. Quantitative structure–activity relationships (QSAR)

In an attempt to quantify the relationship between MAO-B inhibitory activity and the physicochemical properties of the substituents, a Hansch-type SAR study was carried out by multiple linear regression analysis. Five parameters were used to describe each substituent. The Taft steric parameter (E_s)[19] and the Van der Waals volume (V_w)[20] were used as descriptors of bulkiness while the lipophilicities of the substituents were described by the Hansch constant (π).[19] The classical Hammett (σ_m or σ_p) and Swain–Lupton (F) constants served as electronic parameters.[19] All the physicochemical values of the substituents were obtained from standard compilations.[19,20] The analogues were divided into two groups—those bearing substituents at C-4 of the phenyl ring (**5b–g**) and those with substituents at C-3 of the phenyl ring (**5h–m**). The unsubstituted 1-methyl-3-phenylpyrrole (**5a**) was considered a member of both groups. Results of the statistical analysis for the two groups are shown in Tables 2 and 3, respectively.

For analogues substituted at C-4 of the phenyl ring (Table 2), the Taft steric parameter (E_s) was the only substituent descriptor that showed a meaningful correlation with the logarithm of the K_i values (expressed in micromolar). Regression analysis of $\log K_i$ with E_s exhibited a relatively good correlation with a R^2 value of 0.91. The statistical F value for the correlation was found to be 51.7, which is higher than the F_{max} value (25.32)[21] for

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Table 2. Correlations of the MAO-B inhibition constants ($\log K_i$) of the 1-methyl-3-phenylpyrrolyl derivatives (**5a–g**) with steric, electronic, and hydrophobic descriptors of the substituents at C-4 of the phenyl ring[a]

Parameter	Slope	y-Intercept	R^2	F[b]	Significance[c]
σ_p	−1.76 ± 0.64	1.40 ± 0.17	0.60	7.54	0.041
F	−1.67 ± 1.18	1.72 ± 0.40	0.29	2.00	0.22
V_w	−1.17 ± 1.05	1.01 ± 0.31	0.20	1.23	0.32
π	−1.30 ± 0.38	1.82 ± 0.22	0.70	11.6	0.019
E_s	0.77 ± 0.11	1.99 ± 0.13	0.91	51.7	0.0008
$E_s + F$	0.71 ± 0.07	2.16 ± 0.09	0.98	79.7	0.0004
	−0.83 ± 0.26				0.032

[a] The logarithm of the K_i values expressed in micromolar was used in the linear regression analysis.
[b] Higher F values indicate a better fit and a regression equation with an F value higher than the critical F value may be judged as significant. Critical F values may be calculated as described recently.[21]
[c] The significance is the fractional probability that the coefficient of the added variable is zero.

Table 3. Correlations of the MAO-B inhibition constants ($\log K_i$) of the 1-methyl-3-phenylpyrrolyl derivatives (**5a** and **h–m**) with steric, electronic, and hydrophobic descriptors of the substituents at C-3 of the phenyl ring[a]

Parameter	Slope	y-Intercept	R^2	F[b]	Significance[c]
σ_m	−1.69 ± 0.49	1.86 ±0.14	0.71	12.0	0.018
F	−1.52 ± 0.63	1.91 ± 0.22	0.54	5.75	0.062
V_w	−0.47 ± 0.29	1.91 ± 0.30	0.34	2.52	0.17
π	−0.84 ± 0.27	1.85 ± 0.16	0.65	9.34	0.028
E_s	0.46 ± 0.12	1.92 ± 0.15	0.73	13.6	0.014
$E_s + F$	0.38 ± 0.05	2.14 ± 0.06	0.97	72.2	0.0013
	−1.07 ± 0.18				0.0039

[a] The logarithm of the K_i values expressed in micromolar were used in the linear regression analysis.
[b] Higher F values indicate a better fit and a regression equation with an F value higher than the critical F value may be judged as significant. Critical F values may be calculated as described recently.[21]
[c] The significance is the fractional probability that the coefficient of the added variable is zero.

95% significance (a higher F value indicates a better fit). All other single-parameter fits with the $\log K_i$ values exhibited poorer statistical correlations. Correlations could be improved by the inclusion of an additional substituent parameter in the regression analysis. A two-parameter fit with E_s and the Swain–Lupton constant (F) yielded a model with a R^2 value of 0.98 and a statistical F value of 79.7 ($F_{max} = 30.18$). For this correlation, the probabilities that E_s and F are zero are 0.04% and 3.2%, respectively. Therefore, the best mathematical description of binding affinity ($\log K_i$) of the C-4 substituted 1-methyl-3-phenylpyrroles (**5a–g**) to MAO-B is:

$$\log K_i = 0.71(\pm 0.07)E_s - 0.83(\pm 0.26)F + 2.16(\pm 0.09)$$
$$(R^2 = 0.98 \text{ and } F = 79.7) \quad (1)$$

Since bulky substituents have increasingly negative Taft steric parameter (E_s) values, the positive correlation observed with E_s (0.71 ± 0.07) indicates that the MAO-B inhibition potency ($\log K_i$) may be enhanced by substitution at C-4 of the phenyl ring with a bulky substituent (Fig. 2). The negative correlation between $\log K_i$ and F (−0.83 ± 0.26) indicates that the potency ($\log K_i$) by which 1-methyl-3-phenylpyrroles inhibit MAO-B may be enhanced by substitution with electron-withdrawing C-4 functional groups. Although not statistically significant, there also appears to be a moderate correlation ($R^2 = 0.70$) between inhibitor binding affinity ($\log K_i$) and the lipophilicity (π) of the C-4 substituents. Since more lipophilic substituents have increasingly positive Hansch constant (π) values, the negative correlation (−1.30 ± 0.38) between π and the $\log K_i$ values indicates that enhancement of the lipophilicity of the C-4 substituents may lead to better inhibition potency. The observed linear correlations of $\log K_i$ with both E_s and π are to be expected since, for the set of substituents examined here, there exists a moderate correlation ($R^2 = 0.68$) between these two substituent descriptors. No significant correlations were observed for any other combination of two substituent descriptors.

For analogues substituted at C-3 of the phenyl ring (Table 3), there appeared to be no single substituent descriptor that showed a meaningful correlation with

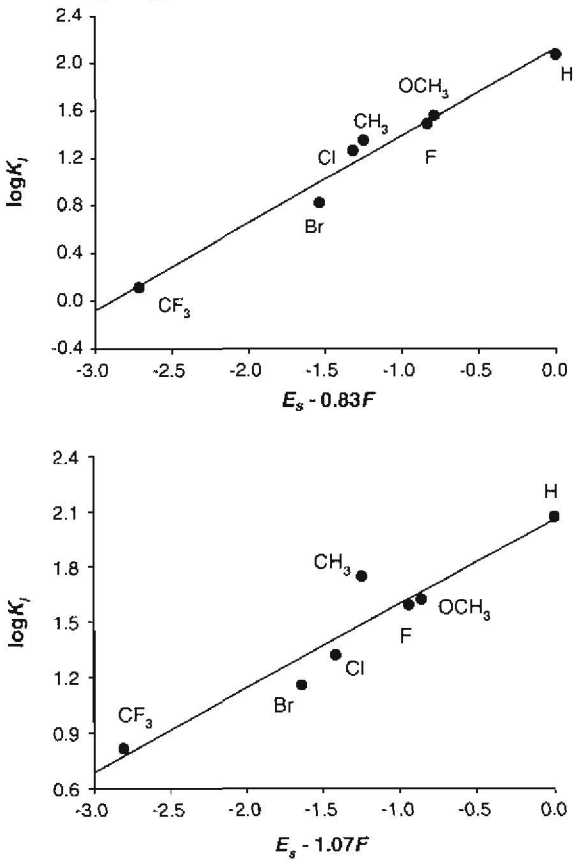

Figure 2. Correlations of the $\log K_i$ values for the inhibition of MAO-B by **5a–m** with the Taft steric parameter (E_s) of the substituents at C-4 (top) and C-3 (bottom) of the phenyl ring. The E_s values were adjusted by the contribution of the Swain–Lupton F constant as indicated on the x-axis titles. The linear regression lines are graphical representations of Eqs. 1 and 2 and the correlation coefficients are 0.98 and 0.97, respectively.

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the $\log K_i$ values. A two-parameter fit with E_s and the Swain–Lupton constant (F), however, yielded a model with a R^2 value of 0.97 and a statistical F value of 72.2 ($F_{max} = 30.18$). For this correlation, the probabilities that E_s and F are zero are 0.13% and 0.39%, respectively. Therefore, the best mathematical description of binding affinity ($\log K_i$) of the C-3 substituted 1-methyl-3-phenylpyrroles (**5a** and **h–m**) to MAO-B is:

$$\log K_i = 0.38(\pm 0.05)E_s - 1.07(\pm 0.18)F + 2.14(\pm 0.06)$$
$$(R^2 = 0.97 \text{ and } F = 72.2) \quad (2)$$

As observed with the analogues substituted at the C-4 position, the positive sign of the E_s parameter coefficient (0.38 ± 0.05) and the negative sign of the F parameter coefficient (-1.07 ± 0.18) suggest that the potency ($\log K_i$) of MAO-B inhibition may be enhanced by substitution with sterically bulky C-3 functional groups that are electron-withdrawing (Fig. 2).

2.4. Modeling studies

In an attempt to better understand the outcomes of the SAR study and to gain additional insight into the binding modes of the inhibitors, molecular docking of all the 1-methyl-3-phenylpyrroles (**5a–m**) within the active site of human MAO-B was performed. Among the crystallographic structures of MAO-B deposited in the Brookhaven Protein Data Bank, the structure with *trans,trans*-farnesol bound to the enzyme (2BK3.pdb)[22] was selected for the docking studies. The choice of this complex was based on the high resolution of the crystallographic structure and the observation that *trans,trans*-farnesol spans both the entrance and substrate cavities of the enzyme active site. As a result, the side chain of Ile-199, which acts as a 'gate' separating the two cavities, is rotated out of its normal conformation to allow for the fusion of the two cavities and the accommodation of larger structures.[23] In order to evaluate the accuracy of the docking procedure, the co-crystallized ligand was redocked within the active site using the LigandFit application of the molecular docking software, Discovery Studio 1.7.[24] The ligand to be docked was first constructed within DS Visualizer Pro and then prepared for the docking simulations using the Prepare Ligands application of Discovery Studio. Following the docking procedure, the docked ligand conformations were further refined using in situ ligand minimization with the Smart Minimizer algorithm. Even though *trans,trans*-farnesol has a relatively high degree of flexibility, the three best-ranked docking solutions obtained exhibited relatively small RMSD from the co-crystallized ligand (1.26 ± 0.18 Å). Within the best-ranked docking solutions, *trans,trans*-farnesol, however, also occupied reversed binding orientations which constituted approximately 40% of the 10 best docking solutions. Therefore, although the orientations of the best-ranked docking solutions obtained with LigandFit closely approximate that of the co-crystallized ligand, reversed binding poses can be expected to be among the docking solutions. Even with this limitation, LigandFit appears to be an effective molecular docking protocol for examining the interactions of the inhibitors with the active site of MAO-B.

The best-ranked docking solutions obtained for all compounds examined (**5a–m**) indicate that the inhibitors traverse both the entrance and substrate cavities of the enzyme, with the pyrrolyl ring extending beyond the boundary between the entrance and substrate cavities while the phenyl ring binds within the substrate cavity. This orientation is probably favored in order to prevent unfavorable interactions between the N–CH_3 and the large polar regions of the substrate cavity and/or to maximize hydrophobic interactions of the N–CH_3 in the entrance cavity.[25] As shown by example with the most potent inhibitor of the series, **5e** (4-CF_3), the phenyl ring is located in the substrate cavity (Fig. 3) while the pyrrolyl nucleus extends into the entrance cavity where the N–CH_3 is stabilized by the hydrophobic environment defined by Phe-103, Trp-119, Leu-164, Leu-167, Phe-168, Ile-316.[25] The best-ranked docking solutions of inhibitors **5b** (4-Cl), **5c** (4-Br), and **5f** (4-CH_3) are virtually superimposed on the binding orientation of **5e**. Inhibitor **5a** (H) and **5d** (4-F) (Fig. 4) also adopt

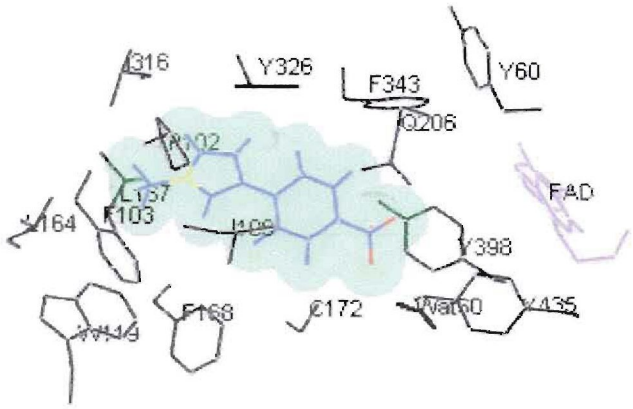

Figure 3. Schematic representation of the most stable complex between **5e** and MAO-B. The inhibitor is displayed in blue, the flavin in purple, and the residues of the enzymatic clefts in black.

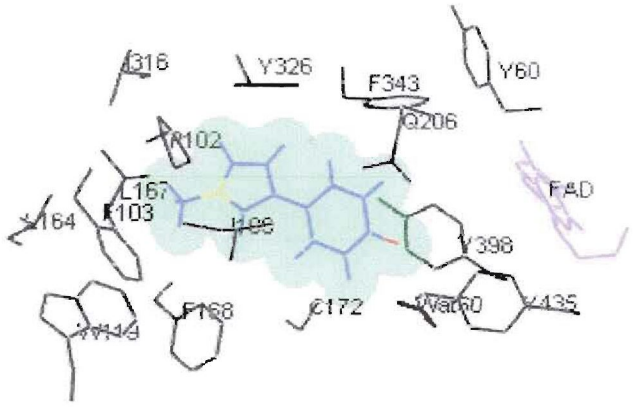

Figure 4. Schematic representation of the most stable complex between **5d** and MAO-B. The inhibitor is displayed in blue, the flavin in purple, and the residues of the enzymatic clefts in black.

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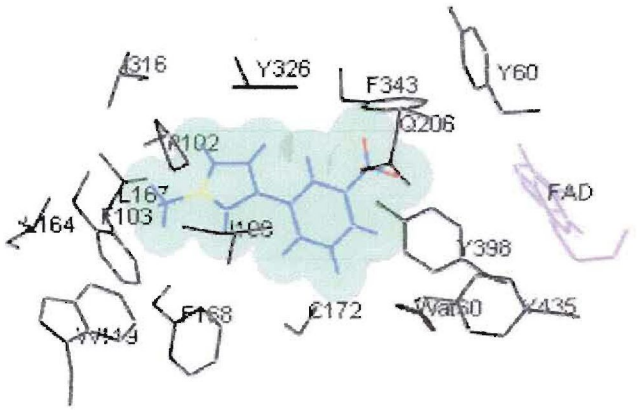


Figure 5. Schematic representation of the most stable complex between **5k** and MAO-B. The inhibitor is displayed in blue, the flavin in purple, and the residues of the enzymatic clefts in black.

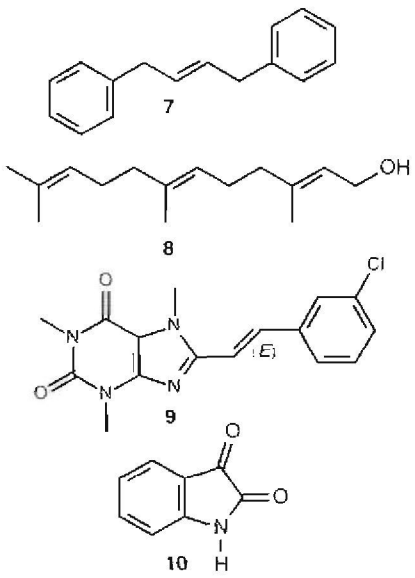
similar binding modes, but the N–CH_3 protrudes to a lesser degree into the entrance cavity and hence is stabilized to a lesser extent (than **5e**) by the hydrophobic environment of the entrance cavity.

The 1-methyl-3-phenylpyrroles substituted at C-3 of the phenyl ring (**5h–m**) adopt similar binding orientations to the C-4 substituted analogues (Fig. 5). The principal difference is that the C-3 substituents are directed toward the small hydrophobic region defined by the apolar residues, Tyr-60, Phe-343, and Tyr-398, while the C-4 substituents are directed in the general direction of the aromatic cage defined by Tyr-398 and Tyr-435.[25] This binding mode presumably maximizes favorable hydrophobic contacts between the C-3 substituent and the enzyme while minimizing unfavorable interactions with the polar environment of the substrate cavity. As expected, the reverse binding modes are also represented among the 10 best-ranked docking solutions of the inhibitors examined, with a frequency of approximately 30–40%. The best solutions were, however, always orientated with the pyrrolyl ring directed toward the entrance cavity.

3. Discussion

This investigation shows that substitution of 1-methyl-3-phenylpyrroles on the phenyl ring has a considerable effect on the potency of MAO-B inhibition displayed by these compounds. For example, substitution at C-4 with a trifluoromethyl functional group (**5e**) increases the inhibition potency by approximately 90-fold compared to the unsubstituted 1-methyl-3-phenylpyrrole (**5a**). The SAR analysis indicates that the inhibition activities correlate with the Taft steric parameter and the Swain–Lupton constant of the substituents at both C-4 and C-3 of the phenyl ring. Inhibitor binding to the enzyme is favored by an increase in both the steric bulk and the electronegativity of the *para* and *meta* substituents.

Interestingly 1-methyl-3-phenylpyrroles do not seem to be inhibitors of beef liver MAO-B since the most potent inhibitor examined here, **5e**, does not inhibit beef liver MAO-B, even at a concentration of 200 μM which is well above its K_i value (1.30 μM) for the inhibition of baboon liver MAO-B. Beef liver MAO-B was also not inhibited by any of the other pyrrolyl derivatives examined here. Other compounds (Scheme 4), 1,4-diphenyl-2-butene (**7**),[23] *trans,trans*-farnesol (**8**)[26], and (*E*)-8-(3-chlorostyryl)caffeine (**9**),[27] have also been reported to inhibit baboon liver and recombinant human MAO-B competitively while having no effect on beef MAO-B. The crystal structures of human recombinant MAO-B in complex with **7** and **8** have shown that these reversible inhibitors exhibit a dual binding mode that involves traversing both the entrance and substrate cavities of the enzyme.[23] The 'gate' separating the two cavities is the side chain of Ile-199 which is rotated out of its normal conformation to allow for the fusion of the two cavities in order to accommodate these larger inhibitors.[23] In contrast to **7** and **8**, the small molecule inhibitor isatin (**10**) has been shown to inhibit competitively all known MAO-B isoforms, including the beef isoform, with similar potencies.[22] Crystal structures of human recombinant MAO-B in complex with isatin[23] have shown that isatin binds within the substrate cavity, leaving the entrance cavity unoccupied. In this instance the side chain of Ile-199 exhibits the normal or 'closed' rotamer conformation. Ile-199 is conserved in all known MAO-B sequences with the exception of beef MAO-B where it is replaced with Phe. The increased size of the Phe aromatic ring relative to Ile is suggested to prevent its occupation of the 'open' rotamer conformation.[22] The only available space for the aromatic ring to occupy is in the entrance cavity of the enzyme which prevents the binding of inhibitors that must traverse both cavities.[22] The observation that **5a–m** does not inhibit beef liver MAO-B suggests that binding of 1-methyl-3-phenylpyrroles to the active site of MAO-B is dependent upon the rotation of Ile-199 out of its normal conformation. This implies that 1-methyl-3-phenylpyrroles also exhibit a



Scheme 4. The structures of the reversible MAO-B inhibitors 1,4-diphenyl-2-butene (**7**), *trans,trans*-farnesol (**8**), (*E*)-8-(3-chlorostyryl)caffeine (**9**), and isatin (**10**).

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dual binding mode that involves interactions with both the entrance and substrate cavities.

While the exact binding orientation of 1-methyl-3-phenylpyrroles in the active site of MAO-B is unknown, molecular docking studies support the argument that the inhibitors occupy both cavities. The most stable protein-inhibitor models generated indicate that the inhibitors may bind to MAO-B with the pyrrolyl moiety protruding into the entrance cavity while the phenyl ring is located within the substrate cavity (Figs. 3–5). For this binding mode to be possible, the side chain of Ile-199 must be rotated into the 'open' conformation. The pyrrolyl N–CH_3 appears to be stabilized by the hydrophobic environment of the entrance cavity and as a result, the structural features of the inhibitor that allow for a higher degree of projection of the N–CH_3 into the entrance cavity, may enhance inhibition potency. One such feature may be enhanced steric bulk of the phenyl substituents. In accordance with this idea, the SAR analysis showed that the inhibition activity correlated with the Taft steric parameter of the substituents at both C-4 and C-3.

The apparent contribution of the Swain–Lupton constant (F) of the phenyl substituents toward the correlations of the inhibition potency with E_s and F (Eqs. 1 and 2) is not well understood. Electron-withdrawing substituents at C-3 and C-4 enhance MAO-B inhibition potency of 1-methyl-3-phenylpyrroles. This is also apparent from the moderate correlations of 0.60 (Table 2) and 0.71 (Table 3) recorded between the $\log K_i$ values and the Hammett electronic parameters (σ_p or σ_m). A possible explanation may be that electron-withdrawing functionalities promote planarity between the phenyl and pyrrolyl rings. The literature supports the idea that planar, heterocyclic compounds frequently act as competitive inhibitors of MAO-B.[28,29] The shift to longer wavelengths of maximal light absorption (λ_{max}) upon substitution of the phenyl ring by electron-withdrawing functional groups (CF_3, Br, and Cl) may support this hypothesis. As shown in Table 4, the more potent inhibitors examined in this study (CF_3, Br, and Cl) exhibited λ_{max} values of 276–286 nm while the relatively less potent inhibitors (H, F, CH_3, and OCH_3) were found to have λ_{max} values of 264–272 nm.

Table 4. The wavelengths of maximal light absorption (λ_{max}) of 1-methyl-3-phenylpyrrolyl derivatives (**5a–m**)

Compound	λ_{max} (nm)[a]	ε (M^{-1})
5a	268	13,880
5b	276	18,260
5c	279	18,660
5d	264	12,360
5e	286	15,060
5f	269	15,460
5g	268	17,280
5h	276	13,700
5i	277	13,880
5j	272	13,420
5k	277	13,640
5l	270	13,660
5m	269	12,220

The extinction coefficients (ε) at these wavelengths are also listed.
[a] All UV/V is spectral measurements were conducted in isopropanol.

In conclusion, even though none of the 1-methyl-3-phenylpyrroles examined here were found to be exceptionally potent inhibitors of MAO-B, this study reveals the general structural features that are important for MAO-B inhibition as well as the modifications that can be made in order to enhance inhibition potency. Features important for inhibition are coplanarity of the aromatic rings and bulky electronegative substituents at the *para* and *meta* positions of the phenyl ring. Structural modifications that may enhance inhibition potency are the enhancement of the distance between the pyrrolyl and phenyl rings (e.g., with an ethylene linker) and the inclusion of additional lipophilic substituents on the pyrrolyl ring (e.g., CH_3 at position 2 and/or 5). Both these modifications will have the effect of promoting hydrophobic burial of the pyrrolyl ring in the entrance cavity of MAO-B.

4. Experimental

Caution. MMTP is a structural analogue of the nigrostriatal neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and should be handled using disposable gloves and protective eyewear. Procedures for the safe handling of MPTP have been described previously.[30]

4.1. Chemicals and instrumentation

All starting materials not described elsewhere were obtained from Sigma–Aldrich and were used without purification. The oxalate salt of MMTP was prepared as described previously.[7] Petroleum ether used in this study was of a distillation range of 40–60 °C. Proton and carbon NMR spectra were recorded on a Varian Gemini 300 spectrometer. Proton (^1H) spectra were recorded in $CDCl_3$ and d_6-DMSO at a frequency of 300 MHz and carbon (^{13}C) spectra at 75 MHz. Chemical shifts are reported in parts per million (δ) downfield from the signal of tetramethylsilane added to the deuterated solvent. Spin multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets) or m (multiplet) and the coupling constants (J) are given in hertz (Hz). Direct insertion electron impact ionization (EIMS), high resolution (HRMS), and fast atom bombardment (FAB-MS) mass spectra were obtained on a VG 7070E mass spectrometer. Melting points (mp) were determined on a Gallenkamp melting point apparatus and are uncorrected. UV–Vis spectra were recorded on a Milton-Roy Spectronic 1201 spectrophotometer. Thin layer chromatography (TLC) was carried out with neutral aluminium oxide 60 (Merck) containing UV_{254} fluorescent indicator.

4.2. Synthesis of 2-aryl-3-(dimethylamino)allylidene (dimethyl)ammonium perchlorates (6a–m)

Synthetic intermediates **6a–m** were prepared in relatively high yields from the corresponding phenylacetic acid, DMF, and phosphoryl chloride according the method described in the literature.[14] The melting points of the compounds reported previously were as follows: **6a** mp

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203–205 °C (from ethanol), lit. mp 193–194 °C[14]; **6b** mp 149–151 °C (from ethanol), lit. mp 142–144 °C[14]; **6c** mp 162–164 °C (from ethanol), lit. mp 152–154 °C[14]; **6f** mp 165–166 °C (from ethanol), lit. mp 150–152 °C[31]; **6g** mp 134–136 °C (from ethanol), lit. mp 130–131 °C[14]; **6h** mp 186–188 °C (from ethanol), lit. mp 180 °C[32]; **6k** mp 114–116 °C (from ethanol), lit. mp 141.5–143 °C[33]; **6l** mp 160–162 °C (from ethanol), lit. mp 164–166 °C.[33] The characterizations of compounds that are previously unreported are summarized below.

2-(4-Fluorophenyl)-3-(dimethylamino)allylidene(dimethyl) ammonium perchlorate (**6d**) was synthesized from 4-fluorophenylacetic acid in a yield of 67.8%: mp 144–146 °C (from ethanol); ^{1}H NMR (d_6-DMSO) δ 2.44 (s, 6H), 3.24 (s, 6H), 7.23–7.37 (m, 4H), 7.69 (s, 2H); ^{13}C NMR (d_6-DMSO) δ 39.30, 48.53, 103.82, 115.25 (d), 128.73 (d), 134.12 (d), 160.47, 163.02, 163.73; FAB-MS *m/z* 221 (M^+).

2-(4-Trifluoromethylphenyl)-3-(dimethylamino)allylidene(dimethyl)ammonium perchlorate (**6e**) was synthesized from 4-(trifluoromethyl)phenylacetic acid in a yield of 72.9%: mp 151–154 °C (from ethanol); ^{1}H NMR (d_6-DMSO) δ 2.42 (s, 6H), 3.26 (s, 6H), 7.56 (d, 2H, J = 7.8 Hz), 7.75–7.79 (m, 4H); ^{13}C NMR (d_6-DMSO) δ 48.59, 103.24, 122.16, 124.96 (q), 125.77, 128.99 (q), 132.95, 137.45, 162.83; FAB-MS *m/z* 271 (M^+).

2-(3-Bromophenyl)-3-(dimethylamino)allylidene(dimethyl) ammonium perchlorate (**6i**) was synthesized from 3-bromophenylacetic acid in a yield of 80.4%: mp 177–178 °C (from ethanol); ^{1}H NMR (d_6-DMSO) δ 2.46 (s, 6H), 3.24 (s, 6H), 7.31–7.41 (m, 2H), 7.57 (t, 1H, J = 1.6 Hz), 7.62–7.65 (m, 1H), 7.69 (s, 2H); ^{13}C NMR (d_6-DMSO) δ 48.55, 103.32, 121.53, 130.21, 131.24, 131.57, 134.44, 135.15, 162.75; FAB-MS *m/z* 281, 283 (M^+).

2-(3-Fluorophenyl)-3-(dimethylamino)allylidene(dimethyl) ammonium perchlorate (**6j**) was synthesized from 3-fluorophenylacetic acid in a yield of 78.1%: mp 158–161 °C (from ethanol); ^{1}H NMR (d_6-DMSO) δ 2.46 (s, 6H), 3.24 (s, 6H), 7.14–7.22 (m, 2H), 7.24–7.31 (m, 1H), 7.43–7.51 (m, 1H), 7.70 (s, 2H); ^{13}C NMR (d_6-DMSO) δ 39.33, 48.54, 103.53, 115.65 (d), 118.97 (d), 128.56 (d), 130.25 (d), 134.98 (d), 159.90, 162.76, 163.17; FAB-MS *m/z* 222 (MH^+).

2-(3-Methoxyphenyl)-3-(dimethylamino)allylidene(dimethyl) ammonium perchlorate (**6m**) was synthesized from 3-methoxyphenylacetic acid in a yield of 75.8%: mp 148–150 °C (from ethanol); ^{1}H NMR (d_6-DMSO) δ 2.47 (s, 6H), 3.23 (s, 6H), 3.77 (s, 3H), 6.85–6.87 (m, 2H), 6.97–7.01 (m, 1H), 7.31–7.36 (m, 1H), 7.67 (s, 2H); ^{13}C NMR (d_6-DMSO) δ 48.46, 55.16, 104.86, 114.47, 117.40, 124.48, 129.41, 133.79, 158.94, 162.73; FAB-MS *m/z* 233 (M^+).

4.3. Synthesis of 1-methyl-3-phenylpyrroles (5a–m)

The 1-methyl-3-phenylpyrrolyl derivatives (**5a–m**) were synthesized from the corresponding 2-aryl-3-(dimethylamino)allylidene(dimethyl)ammonium perchlorates (**6a–m**) according to a modification of the method described in the literature.[13] Sodium wire (143 mmol) was reacted with methanol (48 mL) and the resulting solution was added under an atmosphere of argon to a solution of **6** (64.8 mmol) in 262 mL dry pyridine (distilled over CaH_2 and stored over 4 Å molecular sieves). The reaction was heated under reflux for 24 h. The pyridine was removed via vacuum distillation to obtain a yellow pasty residue to which 100 mL distilled water was added. The resulting suspension was extracted with ethylacetate (3× 100 mL) and the combined organic phases were dried over anhydrous magnesium sulfate (10 g). After the solvent was removed under reduced pressure an oily residue was obtained. The crude product was dissolved in a minimal amount of ethylacetate and purified on a short column (35 × 80 mm) by neutral aluminium oxide chromatography (Fluka 507C) with 100% petroleum ether (**5a–b, f,** and **k–l**) or petroleum ether/ethylacetate, 90:10 (**5g** and **m**), as mobile phase. The fractions containing the product were in most cases recrystallized from an appropriate solvent as cited below. For the synthesis of **5c–e** and **5h–j**, the following modifications were made: following the addition of the 100 mL distilled water to the yellow pasty residue obtained from the vacuum distillation of the pyridine solvent, a suspension was obtained which was stirred for 60 min at room temperature and filtered. A yellow solid residue was obtained which was recrystallized from an appropriate solvent as cited below. All reactions were monitored using neutral aluminium oxide TLC (mobile phase of 100% petroleum ether). The TLC plates were visualized with UV light (254 nm) or by staining with iodine. The melting points of compounds reported previously were as follows: **5a** mp 44–46 °C (from petroleum ether), lit. mp 46–47 °C[13]; **5b** mp 112–114 °C (from methanol), lit. mp 117.5–119.5 °C[13]; **5c** mp 129–130 °C (from methanol), lit. mp 132–133 °C[13]; **5f** mp 49–51 °C (from methanol), lit. mp 55–56 °C[34]; **5g** mp 120–122 °C (from methanol), lit. mp 126–128 °C[13]; **5j** mp 73–75 °C (from methanol), lit. mp 94–96 °C.[13] The characterizations of compounds that were previously unreported are summarized below.

1-Methyl-3-(4-fluorophenyl)pyrrole (**5d**) was synthesized from **6d** in a yield of 31.5%: mp 98–100 °C (from methanol); ^{1}H NMR ($CDCl_3$) δ 3.67 (s, 3H), 6.37 (dd, 1H, J = 1.8, 2.7 Hz), 6.61 (t, 1H, J = 2.5 Hz), 6.83 (t, 1H, J = 2.1 Hz), 7.00 (m, 2H), 7.42 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 36.27, 106.26, 115.29 (d), 118.26, 122.78, 124.17, 126.32 (d), 132.13, 132.17; EIMS *m/z* 175 ($M^{\cdot+}$); HRMS calcd 175.0797, found 175.0811.

1-Methyl-3-(4-trifluoromethylphenyl)pyrrole (**5e**) was synthesized from **6e** in a yield of 41.1%: mp 161–162 °C (from methanol); ^{1}H NMR ($CDCl_3$) δ 3.68 (s, 3H), 6.45 (dd, 1H, J = 1.8, 2.8 Hz), 6.34 (t, 1H, J = 2.5 Hz), 6.96 (t, 1H, J = 2.0 Hz), 7.00 (s, 4H); ^{13}C NMR ($CDCl_3$) δ 36.37, 106.53, 119.39, 123.19, 123.63, 124.75, 125.52 (q), 139.53; EIMS *m/z* 225 ($M^{\cdot+}$); HRMS calcd 225.0765, found 225.0757.

1-Methyl-3-(3-chlorophenyl)pyrrole (**5h**) was synthesized from **6h** in a yield of 33.6%: mp 77–80 °C (from

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methanol); ^{1}H NMR ($CDCl_3$) δ 3.66 (s, 3H), 6.41 (t, 1H, J = 2.3 Hz), 6.62 (t, 1H, J = 2.5 Hz), 6.90 (t, 1H, J = 2.0 Hz), 7.09–7.13 (m, 1H), 7.20–7.26 (m, 1H), 7.34–7.38 (m, 1H), 7.48 (t, 1H, J = 1.9 Hz); ^{13}C NMR ($CDCl_3$) δ 36.30, 106.33, 118.93, 122.95, 123.66, 124.87, 125.05, 129.74, 134.39, 137.87; EIMS *m/z* 191 ($M^{\cdot+}$); HRMS calcd 191.0502, found 191.0490.

1-Methyl-3-(3-bromophenyl)pyrrole (**5i**) was synthesized from **6i** in a yield of 36.9%: mp 82–84 °C (from methanol); ^{1}H NMR ($CDCl_3$) δ 3.65 (s, 3H), 6.39 (dd, 1H, J = 1.8, 2.7 Hz), 6.60 (t, 1H, J = 2.5 Hz), 6.87 (t, 1H, J = 2.0 Hz), 7.15 (m, 1H), 7.22–7.26 (m, 1H), 7.36–7.40 (m, 1H), 7.61 (t, 1H, J = 1.9 Hz); ^{13}C NMR ($CDCl_3$) δ 36.32, 106.34, 118.93, 122.75, 122.96, 123.40, 123.55, 127.80, 127.96, 130.03, 138.18; EIMS *m/z* 235, 237 ($M^{\cdot+}$); HRMS calcd 234.9997, found 235.0003.

1-Methyl-3-(3-trifluoromethylphenyl)pyrrole (**5k**) was synthesized from **6k** in a yield of 51.9%: mp 41–44 °C; ^{1}H NMR ($CDCl_3$) δ 3.68 (s, 3H), 6.45 (dd, 1H, J = 1.8, 2.7 Hz), 6.64 (t, 1H, J = 2.4 Hz), 6.95 (t, 1H, J = 2.0 Hz), 7.39 (m, 2H), 7.62–7.66 (m, 1H), 7.71 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 36.34, 106.37, 119.04, 121.49 (q), 121.69 (q), 122.61, 123.12, 123.69, 126.22, 127.99, 128.92, 136.79; EIMS *m/z* 225 ($M^{\cdot+}$); HRMS calcd 225.0765, found 225.0764.

1-Methyl-3-(3-methylphenyl)pyrrole (**5l**) was synthesized from **6l** in a yield of 72.7%: mp 39–42 °C; ^{1}H NMR ($CDCl_3$) δ 2.38 (s, 3H), 3.68 (s, 3H), 6.45 (dd, 1H, J = 1.8, 2.7 Hz), 6.62 (t, 1H, J = 2.5 Hz), 6.90 (t, 1H, J = 2.0 Hz), 6.97–7.01 (m, 1H), 7.20–7.25 (m, 1H), 7.30–7.35 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 21.50, 36.24, 106.35, 118.51, 122.12, 122.58, 125.11, 125.79, 126.02, 128.43, 135.88, 137.96; EIMS *m/z* 171 ($M^{\cdot+}$); HRMS calcd 171.1048, found 171.1049.

1-Methyl-3-(3-methoxyphenyl)pyrrole (**5m**) was synthesized from **6m** in a yield of 62.0%: mp 67–70 °C; ^{1}H NMR ($CDCl_3$) δ 3.67 (s, 3H), 3.84 (s, 3H), 6.45 (dd, 1H, J = 1.8, 2.7 Hz), 6.62 (t, 1H, J = 2.5 Hz), 6.71–6.75 (m, 1H), 6.91 (t, 1H, J = 2.0 Hz), 7.06 (dd, 1H, J = 1.6, 2.5 Hz), 7.10–7.13 (m, 1H), 7.23–7.08 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 36.23, 55.12, 106.40, 110.59, 110.73, 117.66, 118.71, 122.63, 12.86, 129.46, 137.42, 158.89; EIMS *m/z* 187 ($M^{\cdot+}$); HRMS calcd 187.0997, found 187.1004.

4.4. MAO-B inhibition studies

Mitochondria were isolated from baboon and beef liver tissue as described by Salach and Weyler[35] and stored at −70 °C in 300 µL aliquots. Following addition of an equal volume of sodium phosphate buffer (100 mM, pH 7.4) containing glycerol (50%, w/v) to the aliquots, the protein concentration was determined by the method of Bradford using bovine serum albumin as reference standard.[36] Since the mitochondrial fraction obtained from baboon and beef liver tissue is reported to be devoid of MAO-A activity,[15] inactivation of this isoform was unnecessary. The MAO-A and -B mixed substrate MMTP (K_m = 60.9 µM for baboon liver MAO-B)[15] served as substrate for the inhibition studies. Incubations were carried out in sodium phosphate buffer (100 mM, pH 7.4) and contained MMTP (30–120 µM), the mitochondrial isolate (0.15 mg protein/mL), and various concentrations of the test inhibitors. The final volume of the incubations was 500 µL. The stock solutions of the inhibitors were prepared in DMSO and were added to the incubation mixtures to yield a final DMSO concentration of 4% (v/v). DMSO concentrations higher than 4% are reported to inhibit MAO-B.[29] Following incubation at 37 °C for 15 min, the enzyme reactions were terminated by the addition of 10 µL perchloric acid (70%) and the samples were centrifuged at 16,000*g* for 10 min. The MAO-B catalyzed production of $MMDP^+$ is reported to be linear for the first 15 min of incubation under these conditions.[15] The supernatant fractions were removed and the concentrations of the MAO-B generated product, $MMDP^+$, were measured spectrophotometrically at a wavelength of 420 nm (ε = 25,000 M^{-1}).[15] The initial rates of oxidation at four different substrate concentrations (30–120 µM) in the absence and presence of three different concentrations of the inhibitors were calculated and Lineweaver–Burk plots were constructed. The slopes of the Lineweaver–Burk plots were plotted versus the inhibitor concentration and the K_i value was determined from the x-axis intercept (intercept = $-K_i$). Linear regression analysis was performed using the SigmaPlot software package (Systat Software Inc.). Each K_i value reported here is representative of a single determination where the correlation coefficient (R^2 value) of the replot of the slopes versus the inhibitor concentrations was at least 0.98.

4.5. Time-dependent inhibition studies

In order to determine whether the test inhibitor **5e** acts as a time-dependent inactivator or reversible inhibitor of MAO-B, baboon liver mitochondrial fractions (0.3 mg of protein/mL) were preincubated with **5e** (5.20 µM) for periods of 0, 15, 30, and 60 min at 37 °C.[15] The solvent for this incubation was 100 mM sodium phosphate buffer (pH 7.4). The MAO-A/B mixed substrate, MMTP, at final concentration of 90 µM, was then incubated at 37 °C for 15 min with 0.15 mg protein/mL of the preincubated mitochondria. The final volumes of these incubations were 500 µL and the final concentration of the test inhibitor 2.60 µM. Following termination of the reactions by the addition of 10 µL of perchloric acid (70%), the concentrations of $MMDP^+$ were measured as outlined above. These experiments were carried out in triplicate.

4.6. SAR studies

The values of the substituent descriptors σ_p, σ_m, F, π, and E_s were obtained from Hansch and Leo[19] while those for the Van der Waals volume (V_w) were obtained from compilations by Van de Waterbeemb and Testa.[20] Linear regression analysis of the log K_i values as a function of the substituent descriptor values was carried out with Statistica software package (StatSoft Inc.). In order to estimate the significance of the regression equations, the F statistic was employed. An F value higher than

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the critical F value was judged to be significant. The critical F value (F_{max}) for 95% significance for models constructed from seven $\log K_i$ values (Tables 2 and 3) and which contain one parameter (out of a possible five: V_w, E_s, π, $\sigma_{p/m}$, and F) was calculated to be 25.32, while the F_{max} value for models containing two parameters was calculated to be 30.18.[21]

4.7. Molecular docking studies

All the computational studies were carried out in the Windows-based Discovery Studio 1.7 modeling and simulation environment.[24] The ligands to be docked were constructed within DS Visualizer Pro and then prepared for the docking simulations using the Prepare Ligands application of Discovery Studio. The crystallographic structure of *trans,trans*-farnesol in complex with human MAO-B (2BK3.pdb)[22] was retrieved from the Brookhaven Protein Data Bank (www.rcsb.org/pdb) and the co-crystallized inhibitor was manually deleted. Following typing of the receptor model with the CHARMm forcefield, the binding site was identified by the LigandFit flood-filling algorithm. Automated docking was then carried out with the LigandFit application of Discovery Studio. This docking protocol employed total ligand flexibility whereby the final ligand conformations were determined by the Monte Carlo conformation search method set to a variable number of trial runs. The docked ligands were further refined using in situ ligand minimization with the Smart Minimizer algorithm. All the application modules within Discovery Studio were set to their default values and 10 docking solutions were allowed for each ligand.

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CHAPTER 5.

CONCLUSION

It is generally accepted that the neurotoxicity of MPTP is due to its oxidation in the brain by MAO B which ultimately yields the mitochondrial toxin MPP^+ (Chiba *et al.,* 1984; Heikkila *et al.,* 1984b). In this study, we have compared the potential of 1-methyl-3-phenyl-3-pyrroline and 1-methyl-3-(4-chlorophenyl)-3-pyrroline to act as dopaminergic neurotoxins with that of MPTP. Both compounds have been shown previously to be substrates for MAO B (Wang *et al.,* 1998; Williams & Lawson, 1998) and their oxidation products are neutral species.

1-Methyl-3-phenyl-3-pyrroline and 1-methyl-3-(4-chlorophenyl)-3-pyrroline were shown in the present work to also act as good substrates for beef liver, baboon liver, mouse liver and mouse brain MAO B. It was established in mouse mitochondrial isolates, that MAO A does not significantly contribute to the oxidation of 1-methyl-3-phenyl-3-pyrroline and that the steady-state kinetic parameters measured for 1-methyl-3-phenyl-3-pyrroline are representative of its oxidation by the MAO B isoform. The oxidation of 1-methyl-3-phenyl-3-pyrroline and 1-methyl-3-(4-chlorophenyl)-3-pyrroline by the mitochondrial isolates obtained from beef liver, baboon liver and mouse liver tissues can be exclusively attributed to the action of the MAO B isoform since these tissues are devoid of MAO A activity (Inoue *et al.,* 1999).

This study also showed that 1-methyl-3-phenyl-3-pyrroline is sufficiently bioavailable to reach brain concentrations similar to the MPTP concentrations obtained after a neurotoxic dose of MPTP. The pyrrole product also reaches the brain in relatively high concentrations, which are comparable to the maximum concentration measured for MPP^+. 1-Methyl-3-phenyl-3-pyrroline and 1-methyl-3-(4-chlorophenyl)-3-pyrroline did not, however, mimic the striatal dopamine depletion effect of MPTP and thus, do not act as dopaminergic neurotoxins. These findings are in accordance with the hypothesis that the permanent positive charge of MPP^+ is responsible for its neurotoxic action (Sayre *et al.,* 1990) since 1-methyl-3-phenylpyrrole and 1-methyl-3-(4-chlorophenyl)pyrrole are neutral species. It is speculated that MPP^+ exerts its toxic actions only after accumulating in the inner mitochondrial membrane, a process driven by the transmembrane electrochemical gradient (Sayre *et al.,* 1990). 1-Methyl-3-phenylpyrroles are not predicted to accumulate in the inner mitochondrial membrane and are thus not expected to show any mitochondrial-based cytotoxicity. Inhibition of complex I of the mitochondrial respiratory chain and the resulting depletion of

striatal dopamine by MPP^+ eventually results in degeneration of nigrostriatal dopaminergic neurons. The neutral N-desmethylated form of MPP^+, 4-phenylpyridine, is a much more potent inhibitor of complex I of the isolated respiratory chain than MPP^+, but is not concentrated inside the mitochondria and therefore is not a physiological inhibitor of mitochondrial respiration. In contrast, MPP^+ is a much weaker intrinsic inhibitor of complex I of the electron transport chain, but potent inhibition by MPP^+ occurs by virtue of the fact that MPP^+ undergoes an energy-dependent concentration across the mitochondrial membrane (Sayre *et al.,* 1990), leading to an accumulation of MPP^+ as a result of its permanently charged nature.

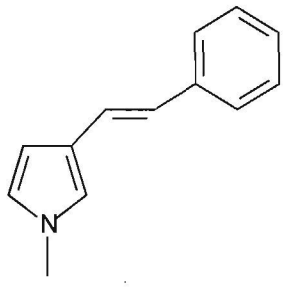
Another goal of this research was to study the MAO B inhibition properties of a series of 1-methyl-3-phenylpyrrole derivatives. This is part of an ongoing investigation into the substrate properties of various 1-methyl-3-phenyl-3-pyrrolines, and it is shown in this study that the 1-methyl-3-phenylpyrrolyl oxidation products reversibly inhibit MAO B. Since inhibitors of MAO B are currently in use, and still being investigated for the treatment of neurodegenerative disorders, the results of this study should contribute to the challenge of identification and design of new reversible inhibitors.

It was found in this study that the most potent inhibitor among the oxidation products considered was 1-methyl-3-(4-trifluoromethylphenyl)pyrrole with an enzyme–inhibitor dissociation constant (K_i value) of 1.30 μM. The least potent inhibitor was found to be 1-methyl-3-phenylpyrrole with a K_i value of 118 μM.

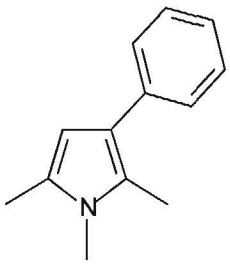
Although the 1-methyl-3-phenylpyrroles examined were found not to be exceptionally potent inhibitors of MAO B, the general features that are necessary for MAO B inhibition as well as the modifications that can be made in order to enhance inhibition potency are revealed. Substitution on the phenyl ring of these compounds has a significant effect on the potency of MAO B inhibition displayed. The SAR analysis indicates that inhibitor binding to the enzyme correlates with an increase in the steric bulk, the electronegativity of the *para* and *meta* substituents, the coplanarity of the aromatic rings and hydrogen bond accepting capability at C-4 substituents of the phenyl ring. Molecular docking studies revealed that the "gate" separating the two cavities of the enzyme, Ile 199, plays a major role. Beef liver MAO B are not inhibited by 1-methyl-3-phenylpyrroles and this is believed to be due to the fact that Ile 199 is conserved in all known MAO B sequences with the exception of beef MAO B where it is replaced with Phe 199.

Structurally, increasing the distance between the pyrrolyl and phenyl ring while retaining planarity could enhance the inhibitory properties of these compounds. For example, preparing compounds with substitution of styryl moiety at C-3 of the pyrrole ring (**17**) instead of the phenyl ring is recommended. 1-Methyl-3-styrylpyrroles are expected to interact more effectively with both entrance and substrate cavities of MAO B than the corresponding 1-methyl-3-phenylpyrroles. Docking studies have shown that part of the entrance cavity is left unoccupied by the phenylpyrroles. Styrylpyrroles may occupy this space leading to enhanced binding affinity.

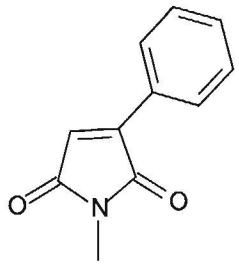
Including additional lipophilic and bulky substituents on the pyrrolyl ring is suggested as modification that may improve inhibition potency because this will lead to enhanced hydrophobic burial of the pyrrolyl ring in the entrance cavity. An example of such a compound is 1,2,5-trimethyl-3-phenylpyrrole (**18**).



1-Methyl-3-styrylpyrrole (**17**)



1,2,5-Trimethyl-3-phenylpyrrole (**18**)



N-methyl-2-phenylmaleimide (**19**)

Figure 16: *Structures of some compounds recommended for future studies.*

Since 1-methyl-3-phenylpyrroles bind to the active site of MAO B only via hydrophobic interactions, modifying these structures to include hydrogen bond acceptors may improve binding affinity. An example of such a modified structure is N-methyl-2-phenylmaleimide (**19**).

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ANNEXURE

Spectral Data

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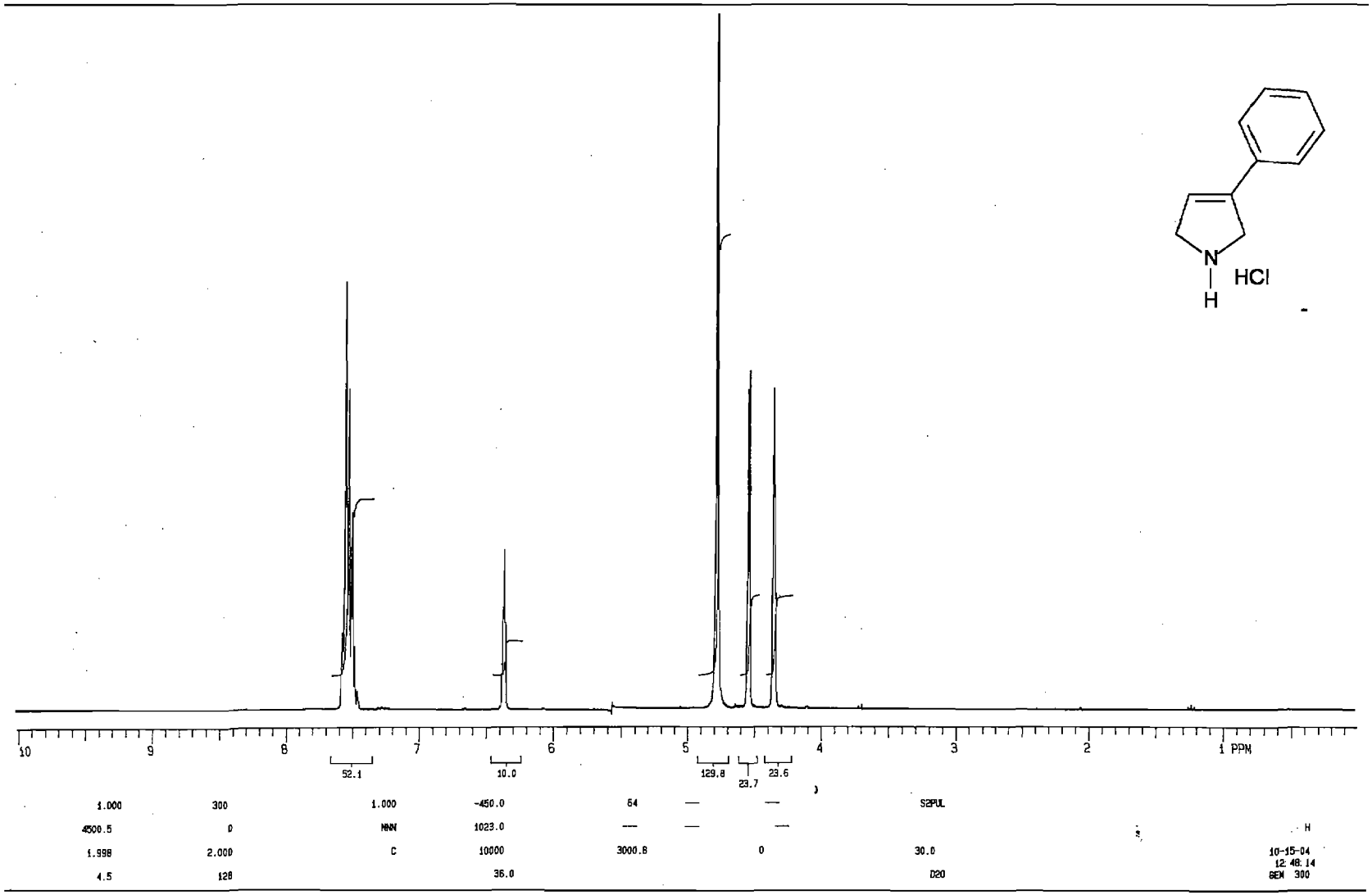
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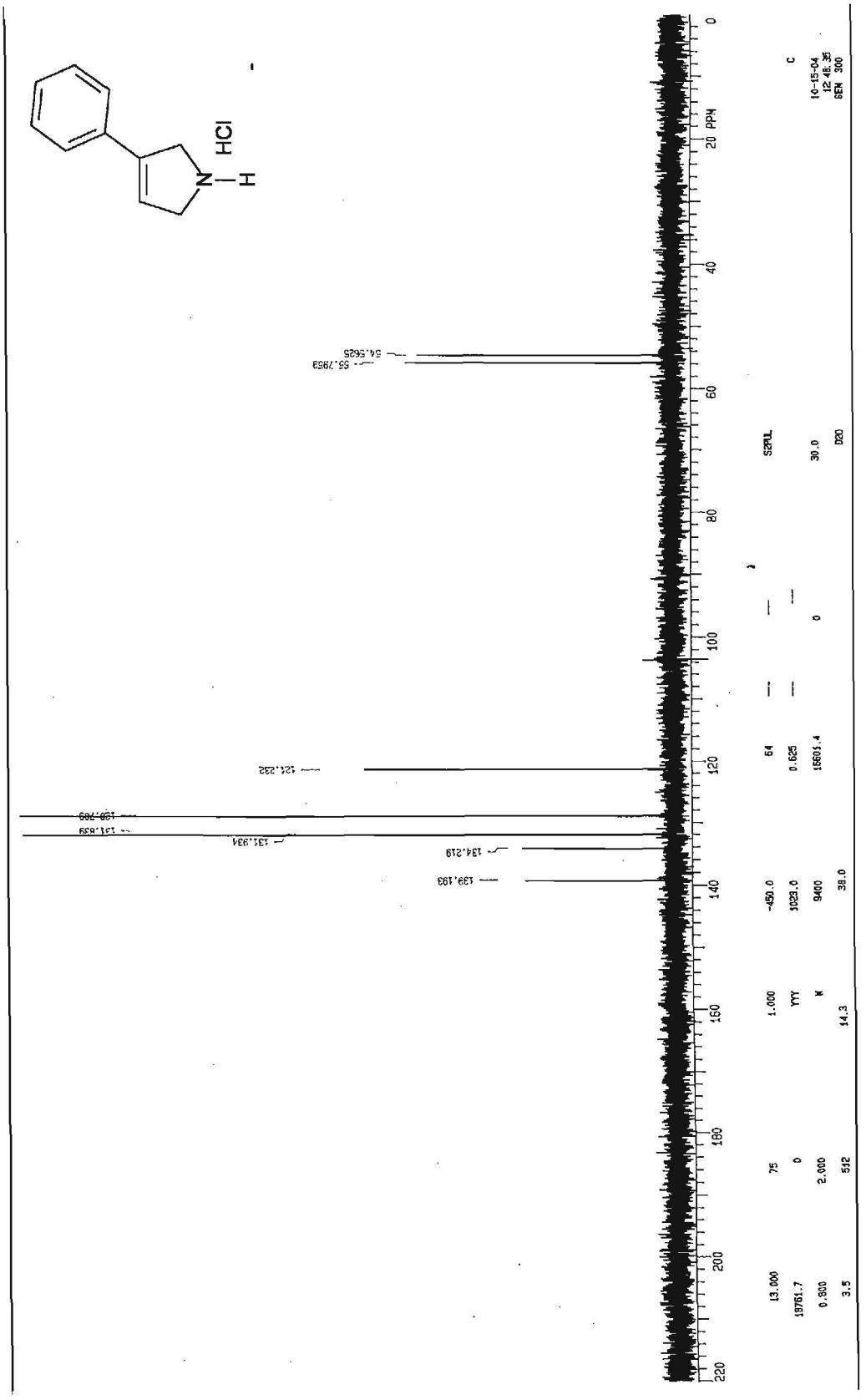
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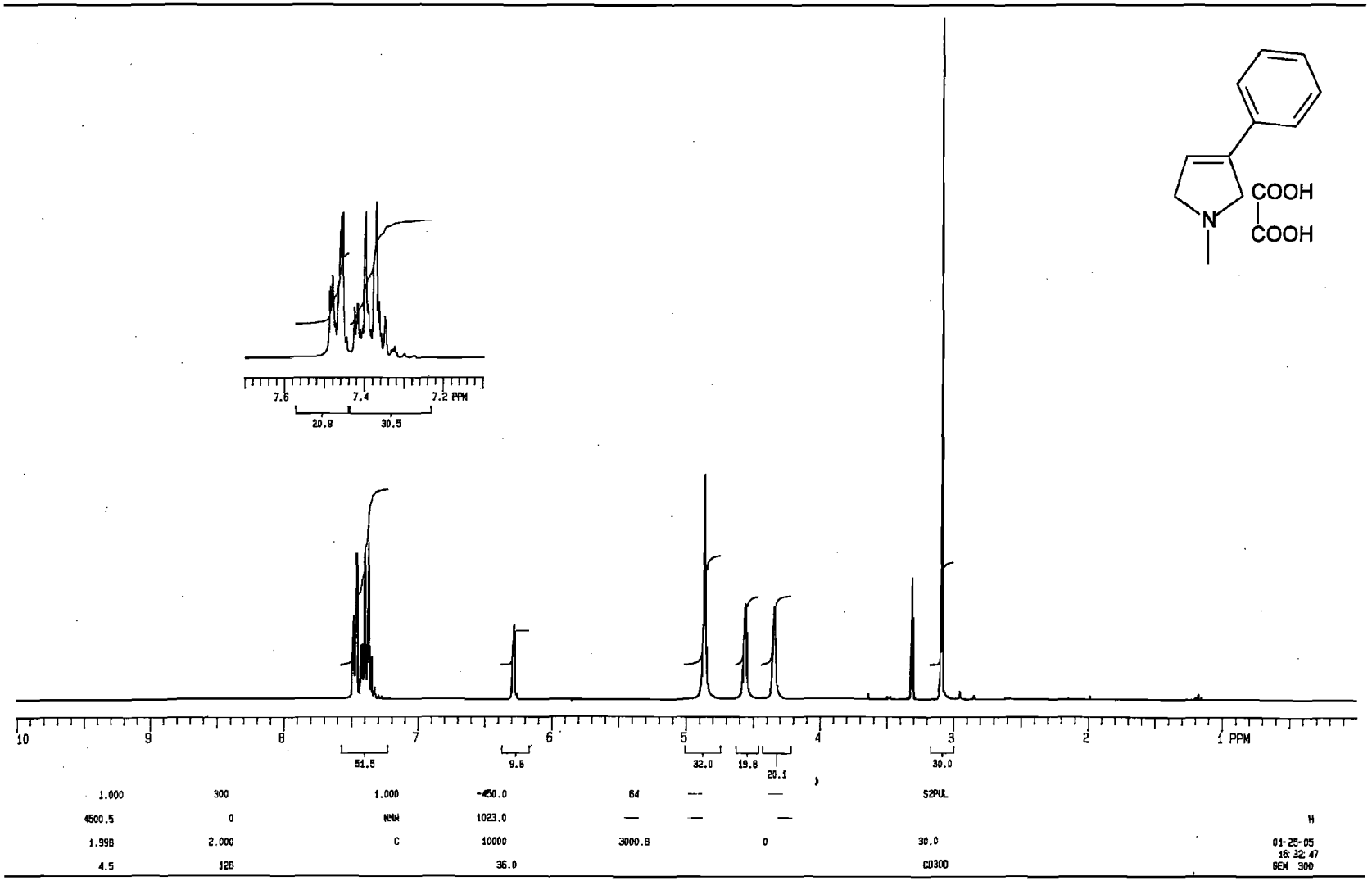
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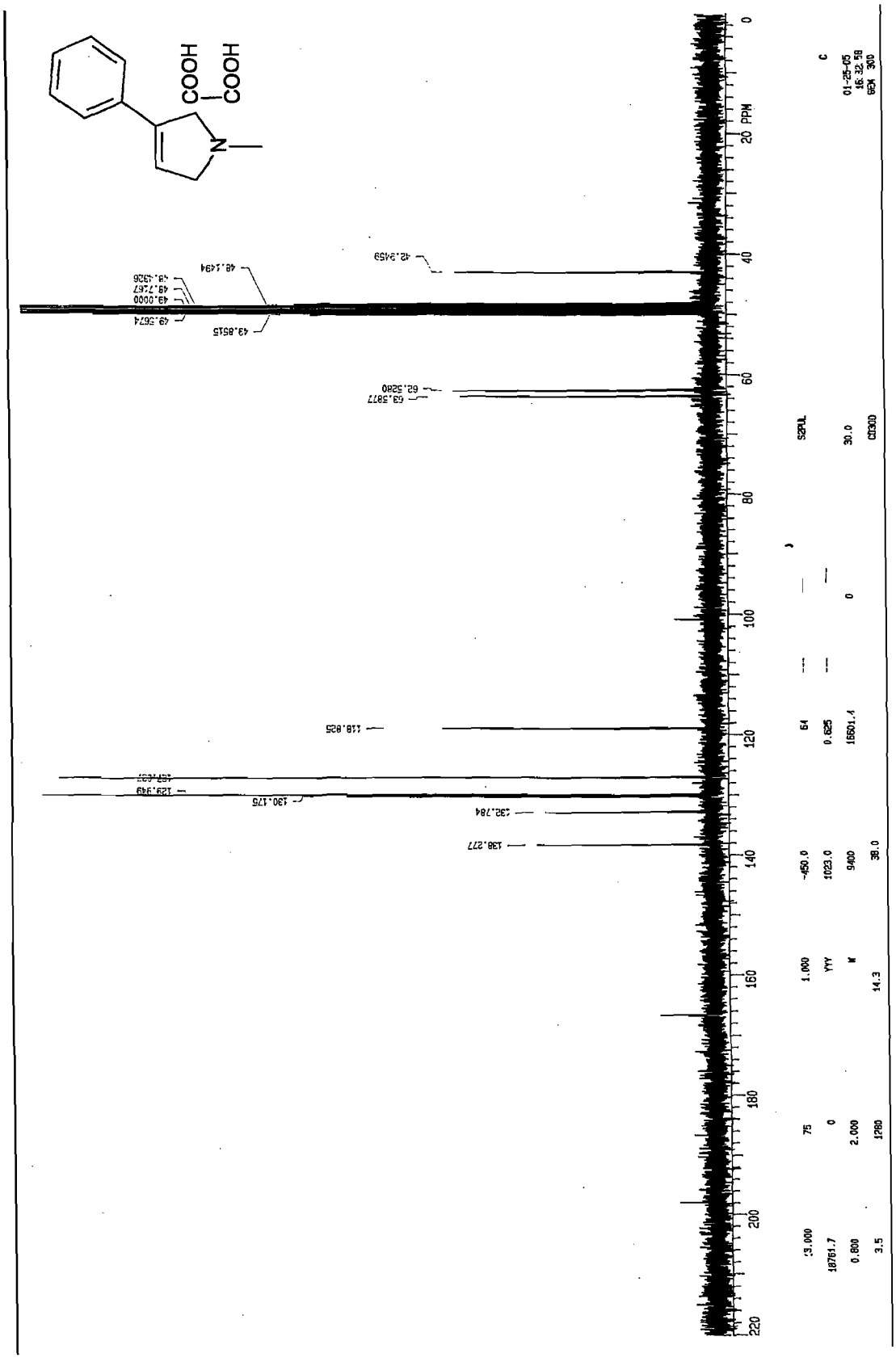
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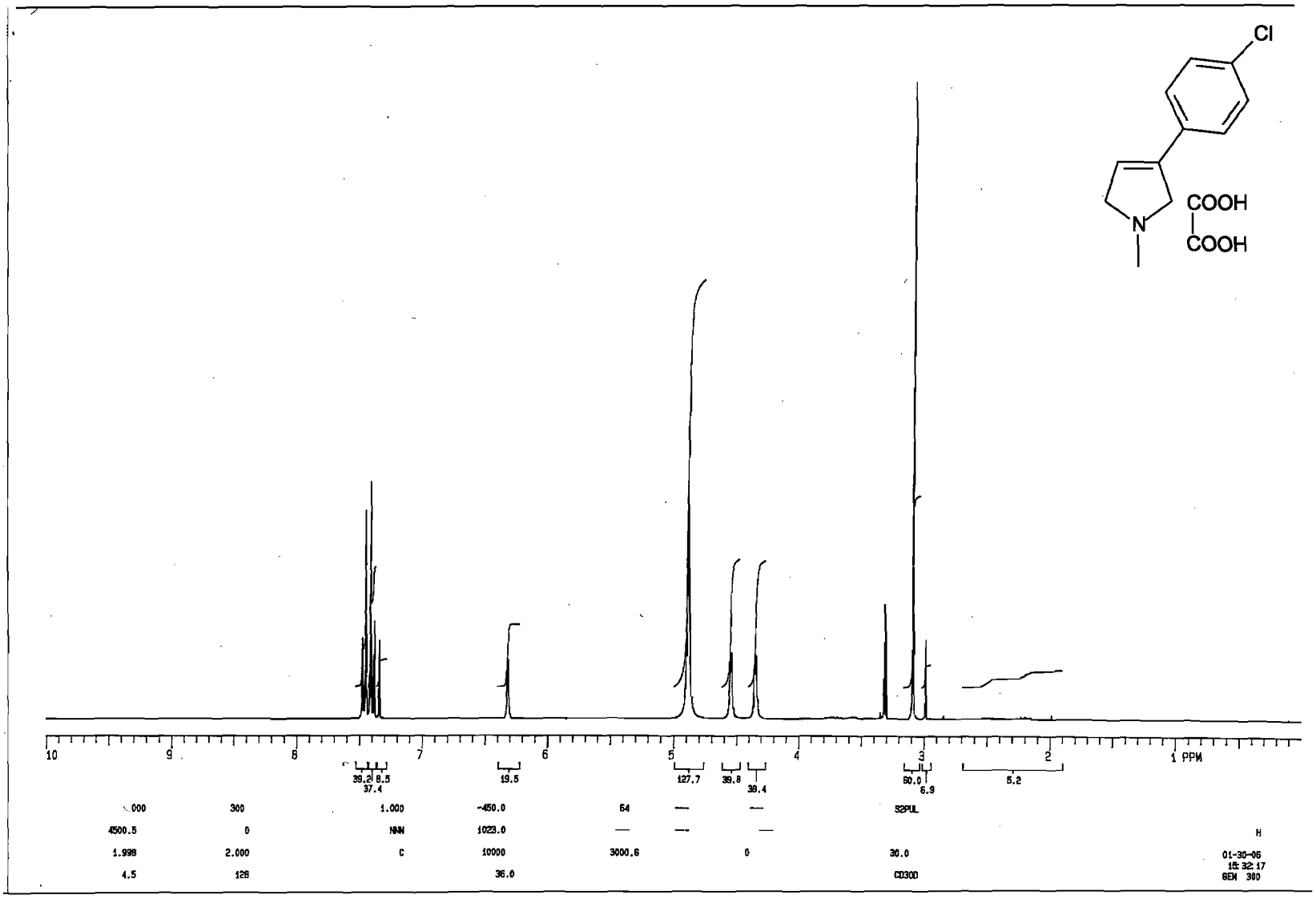
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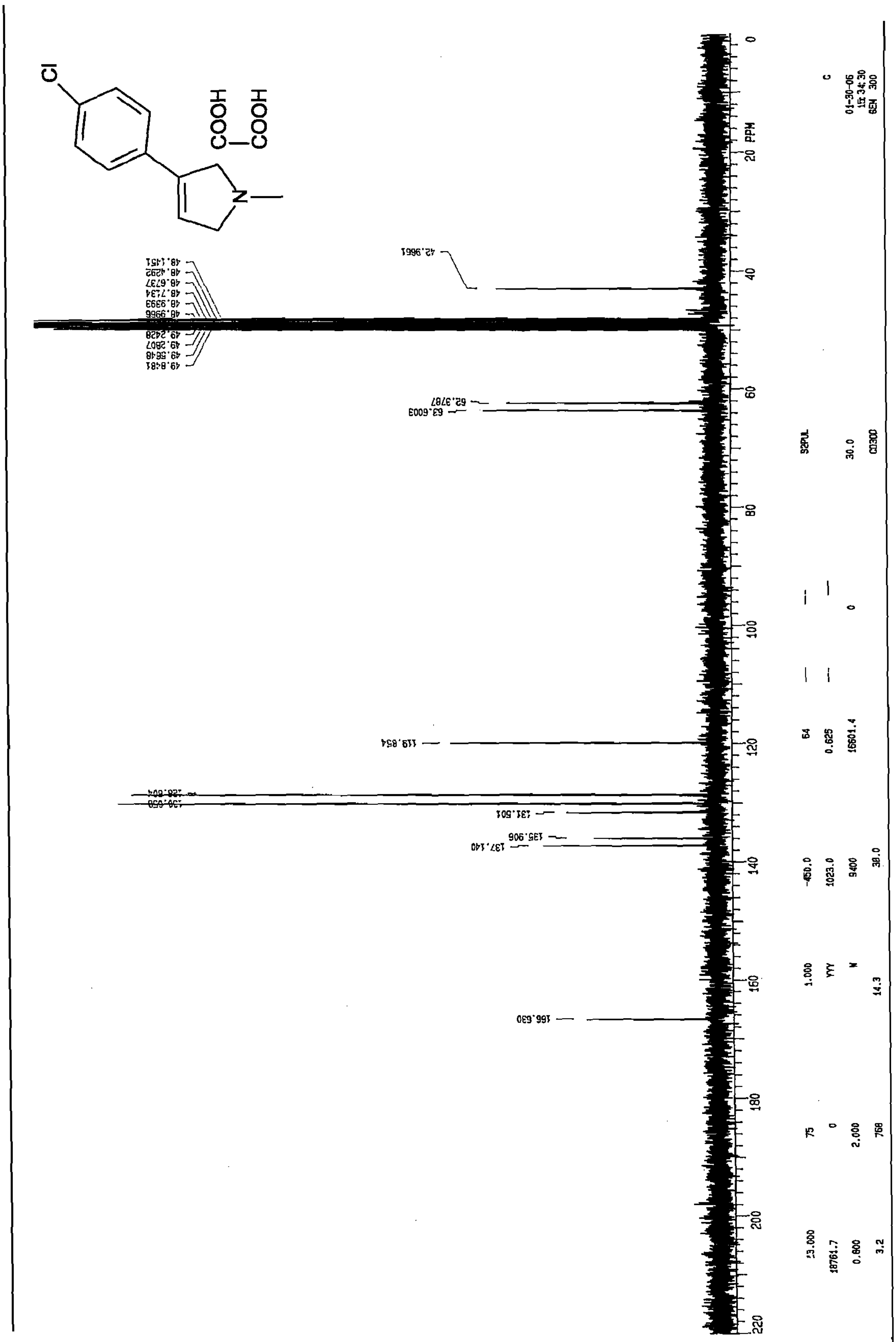
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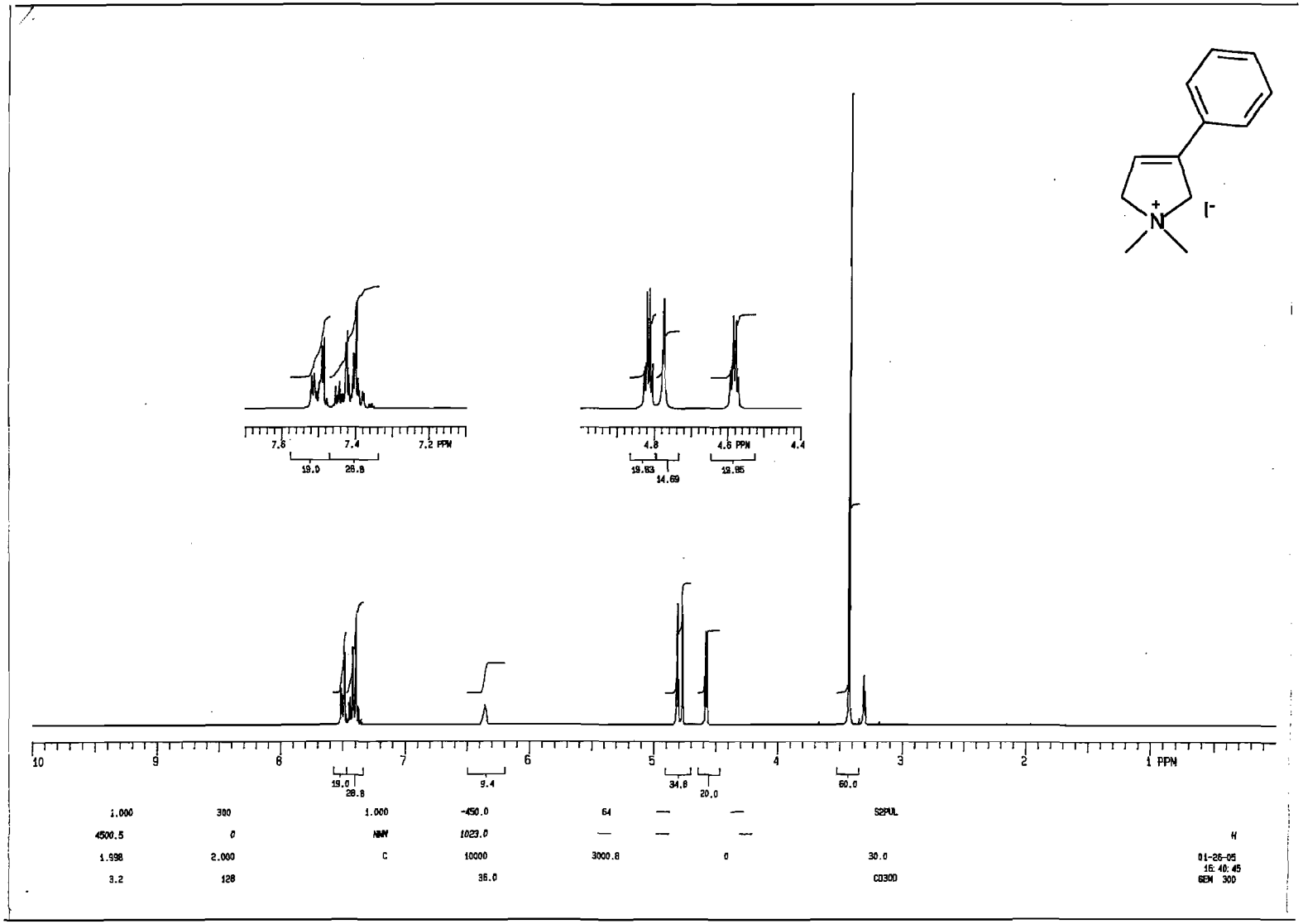
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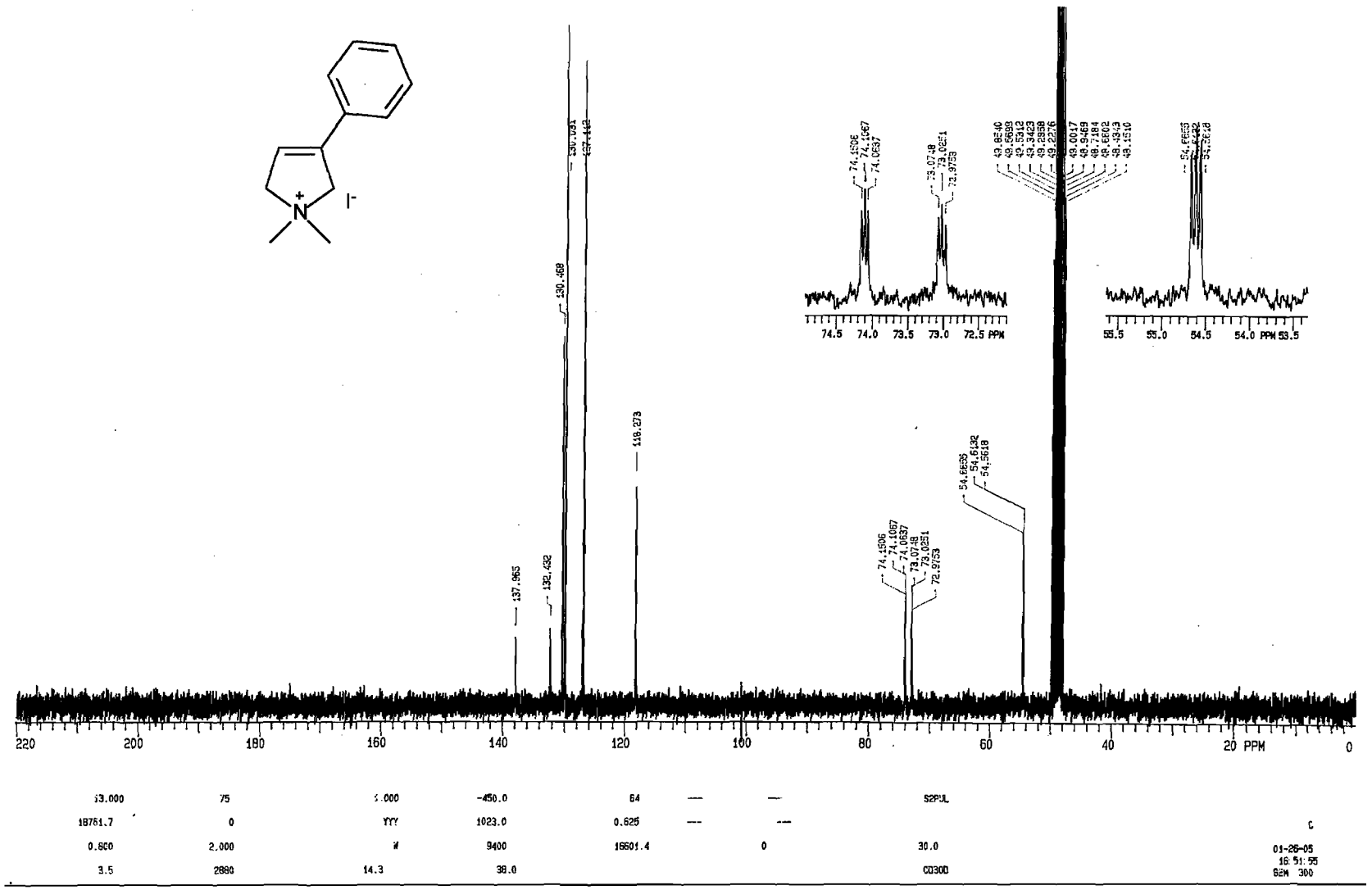
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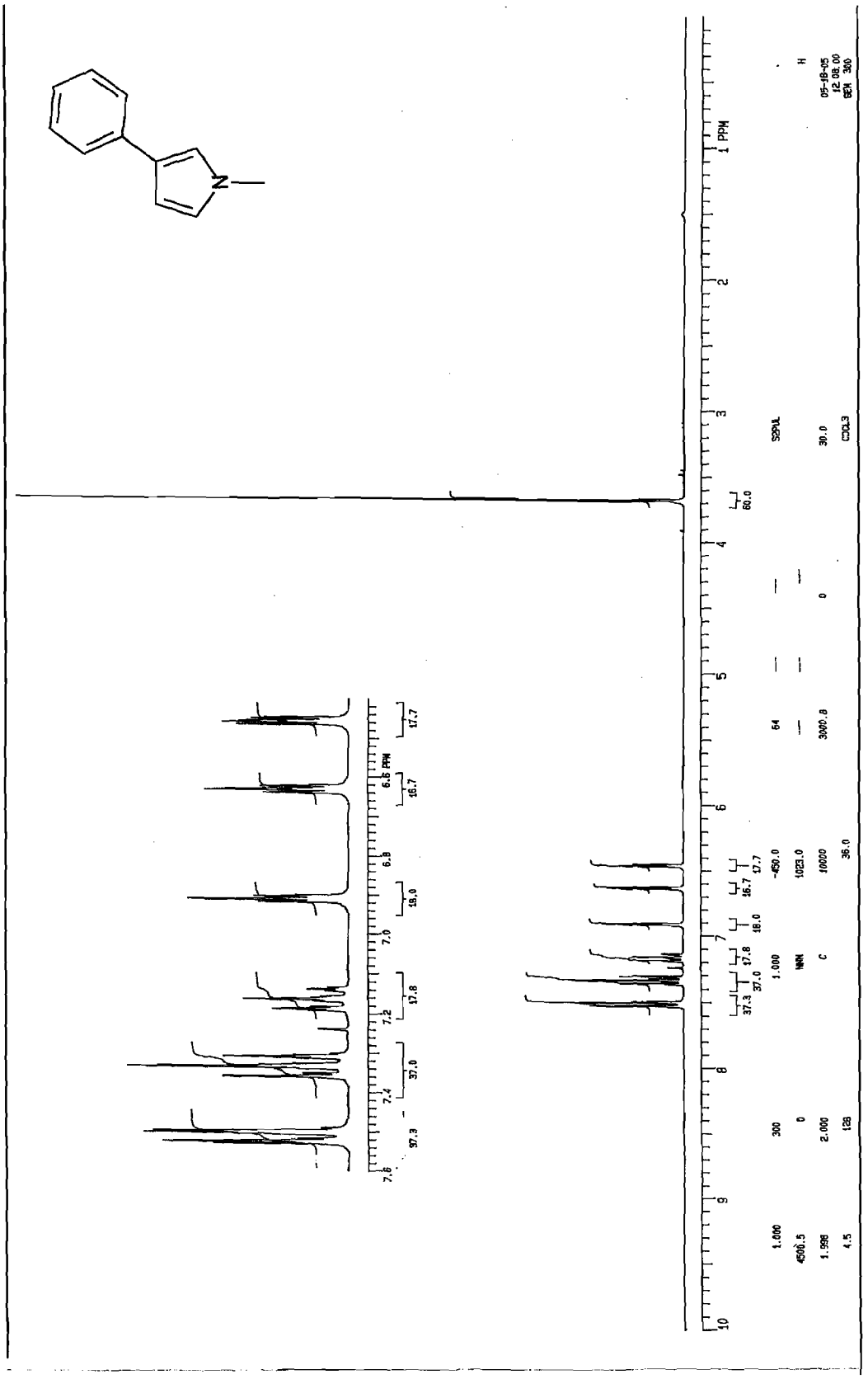

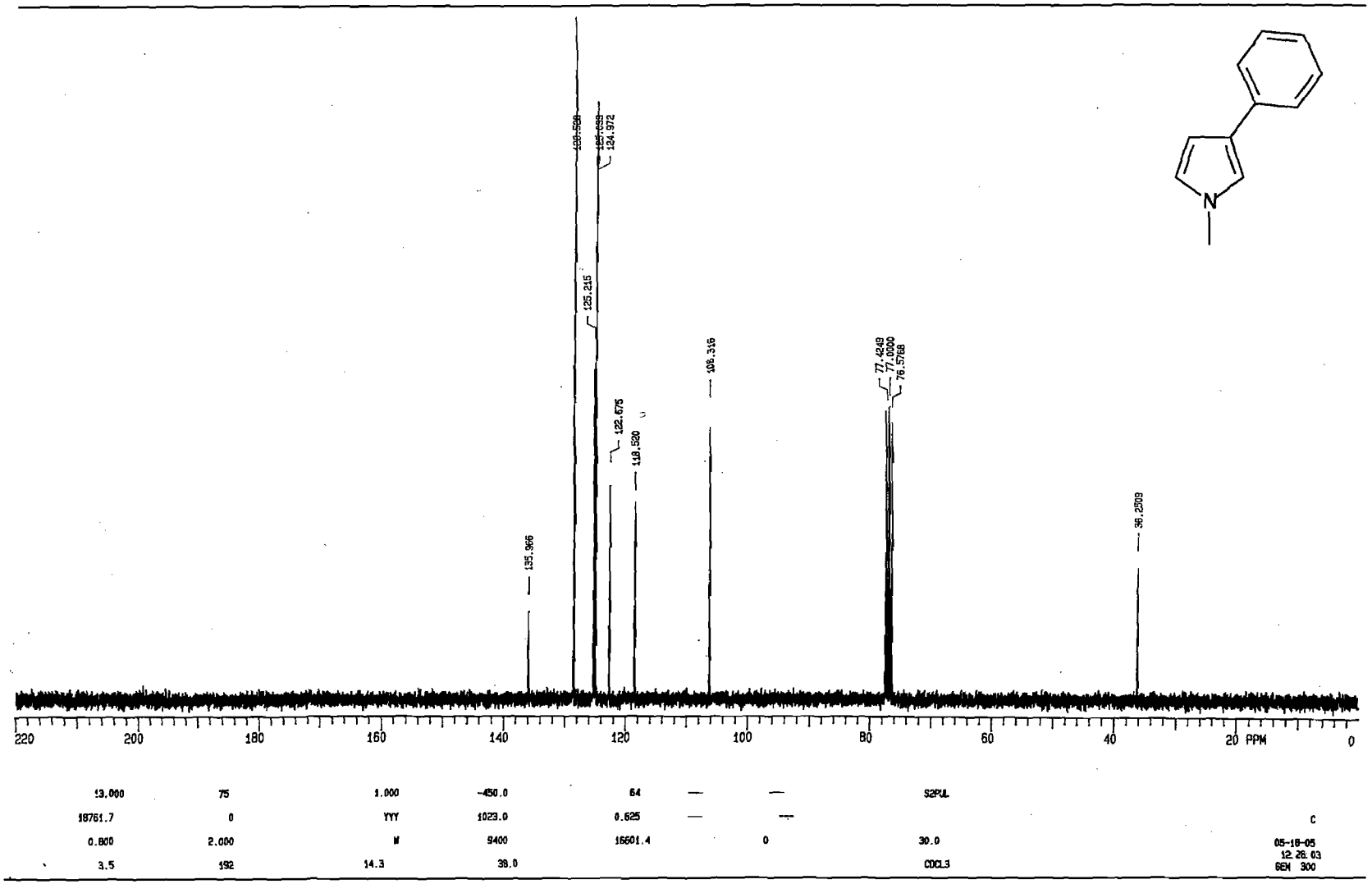
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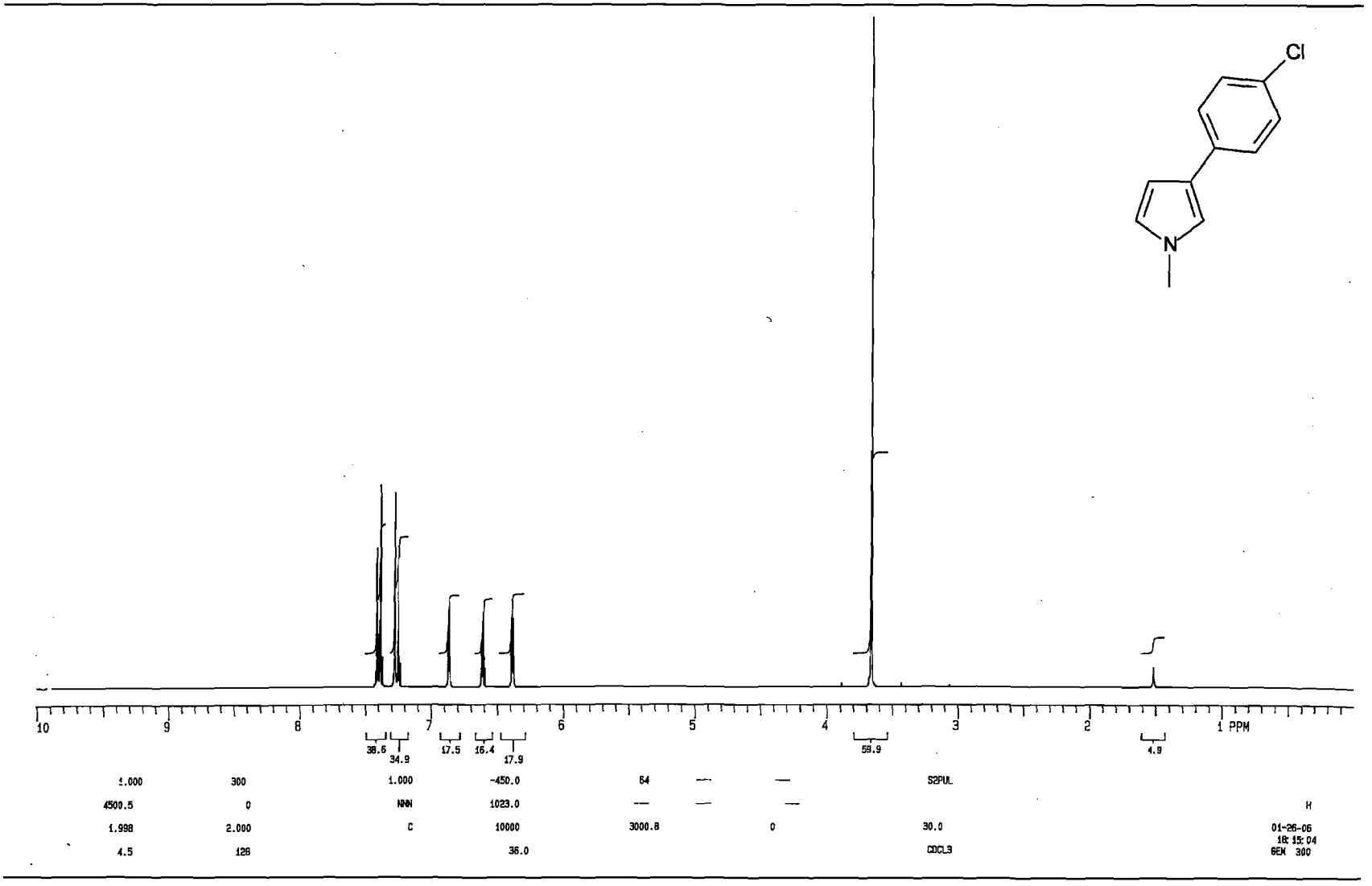
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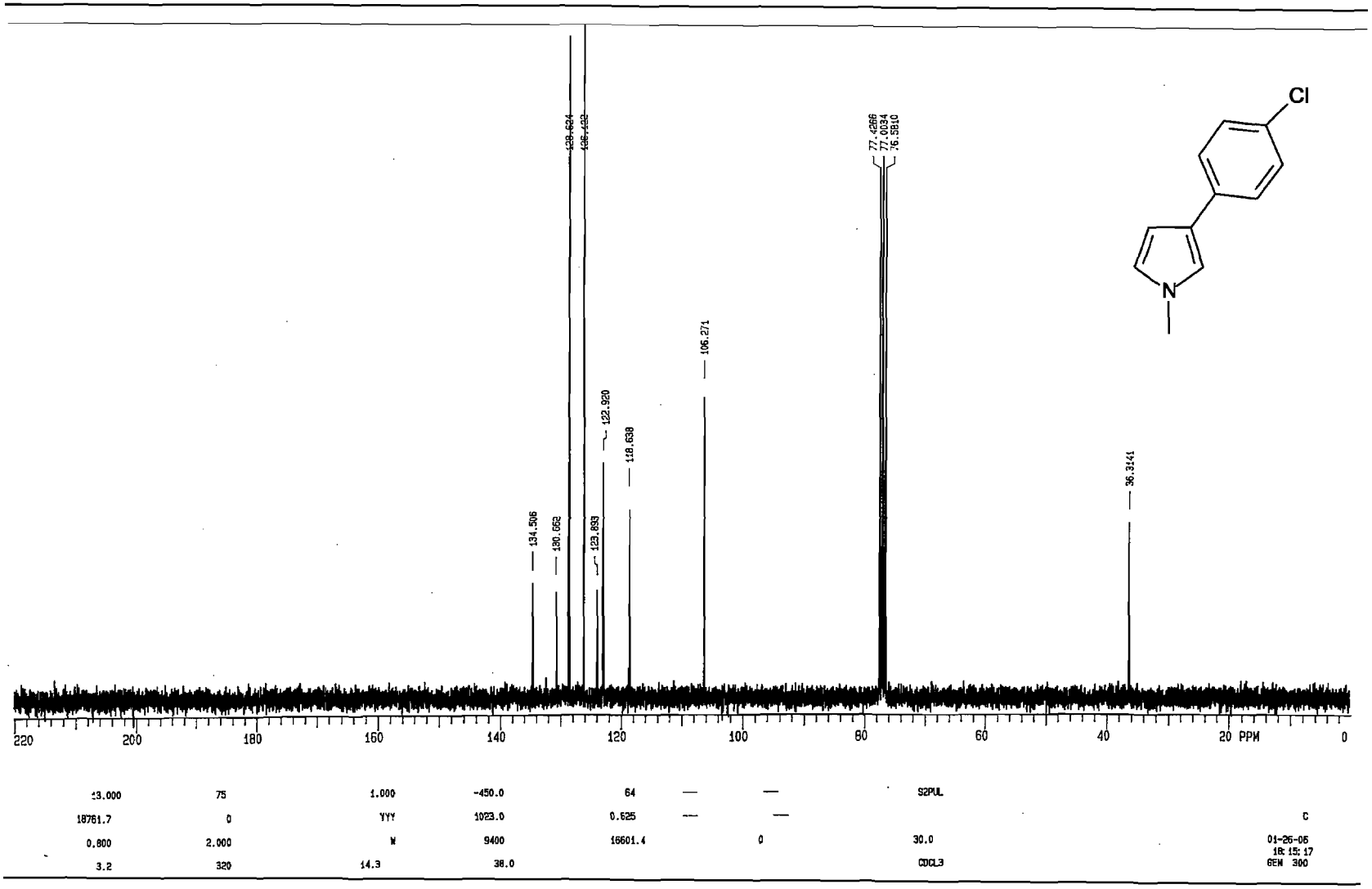
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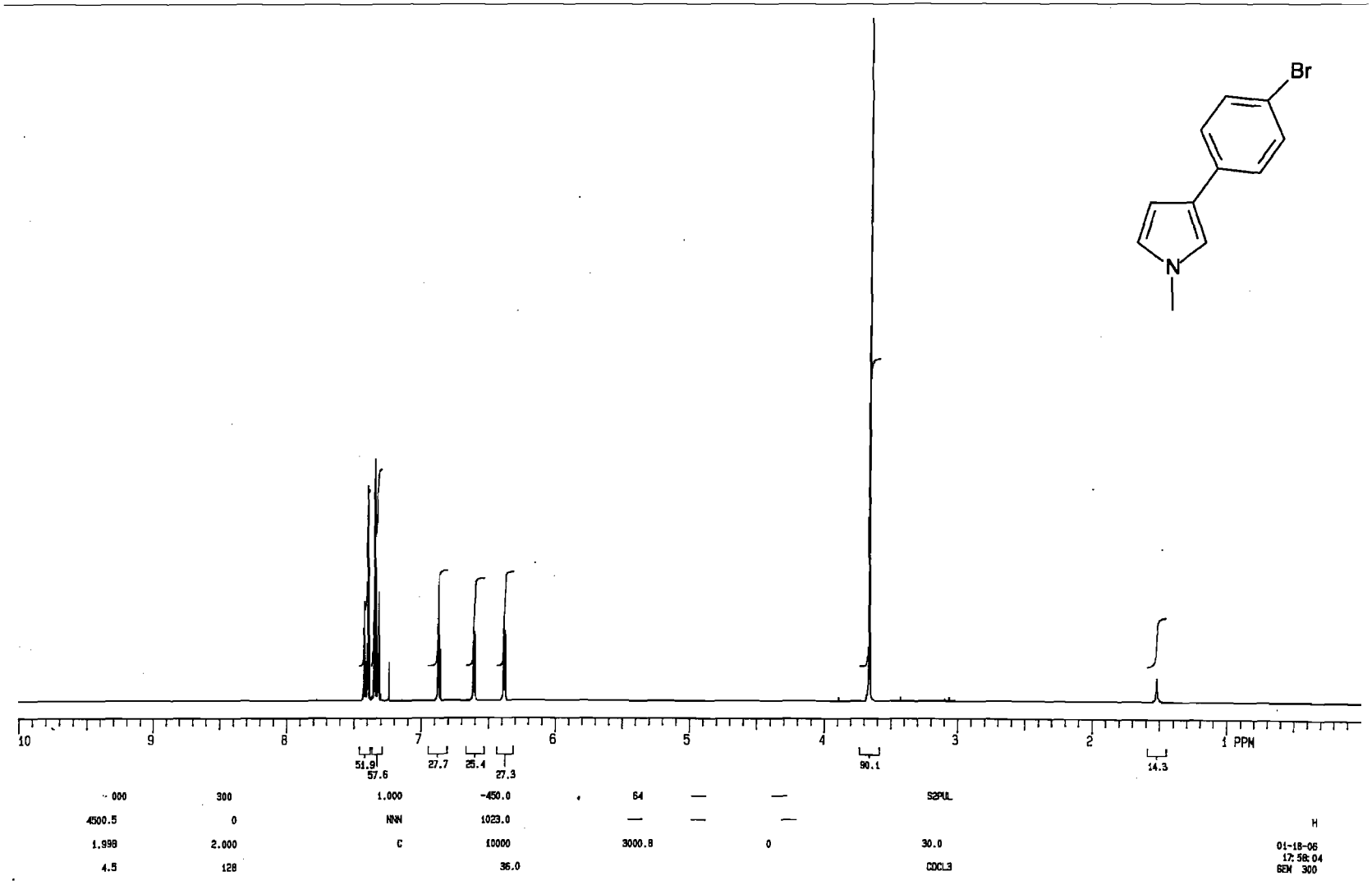
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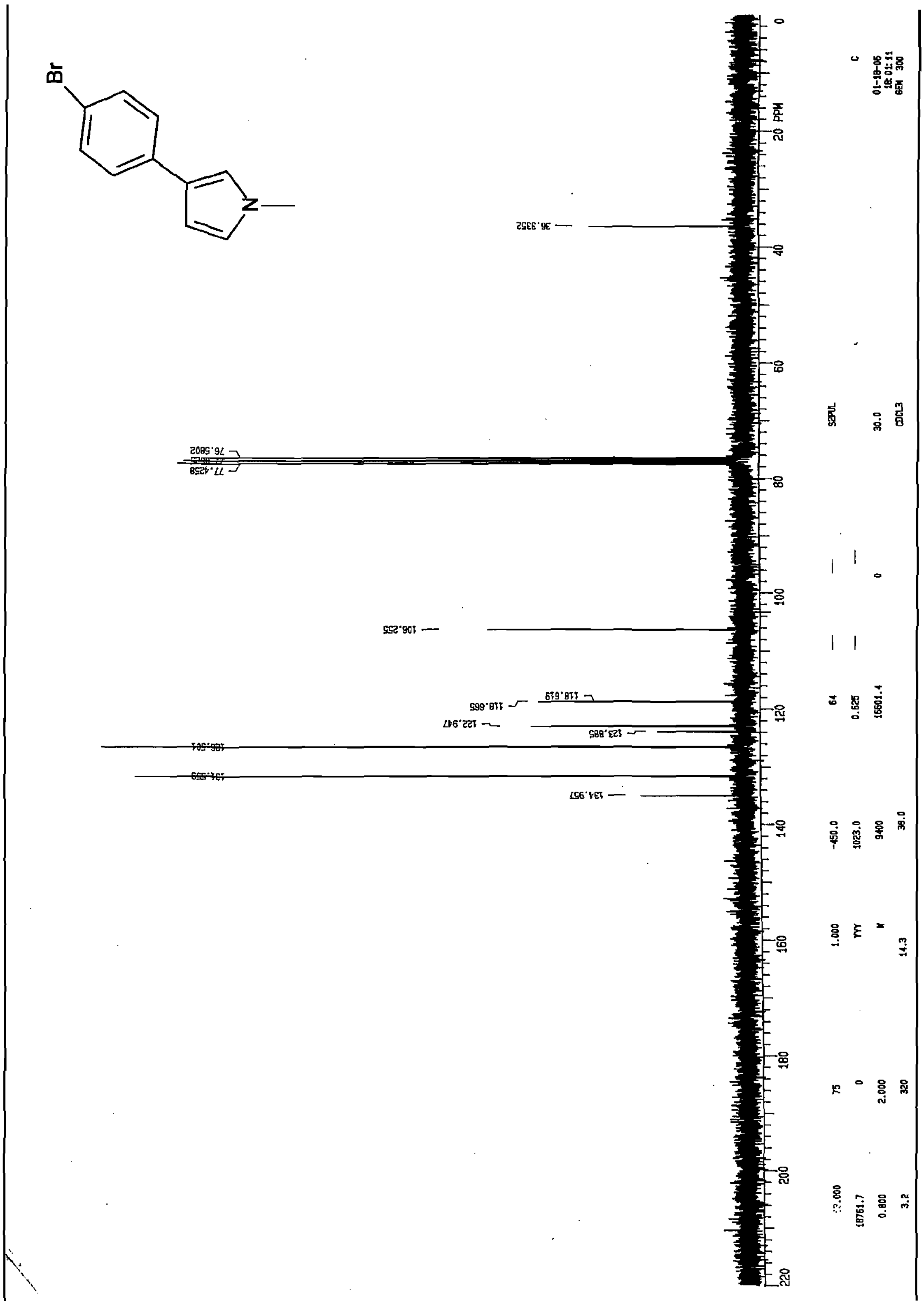
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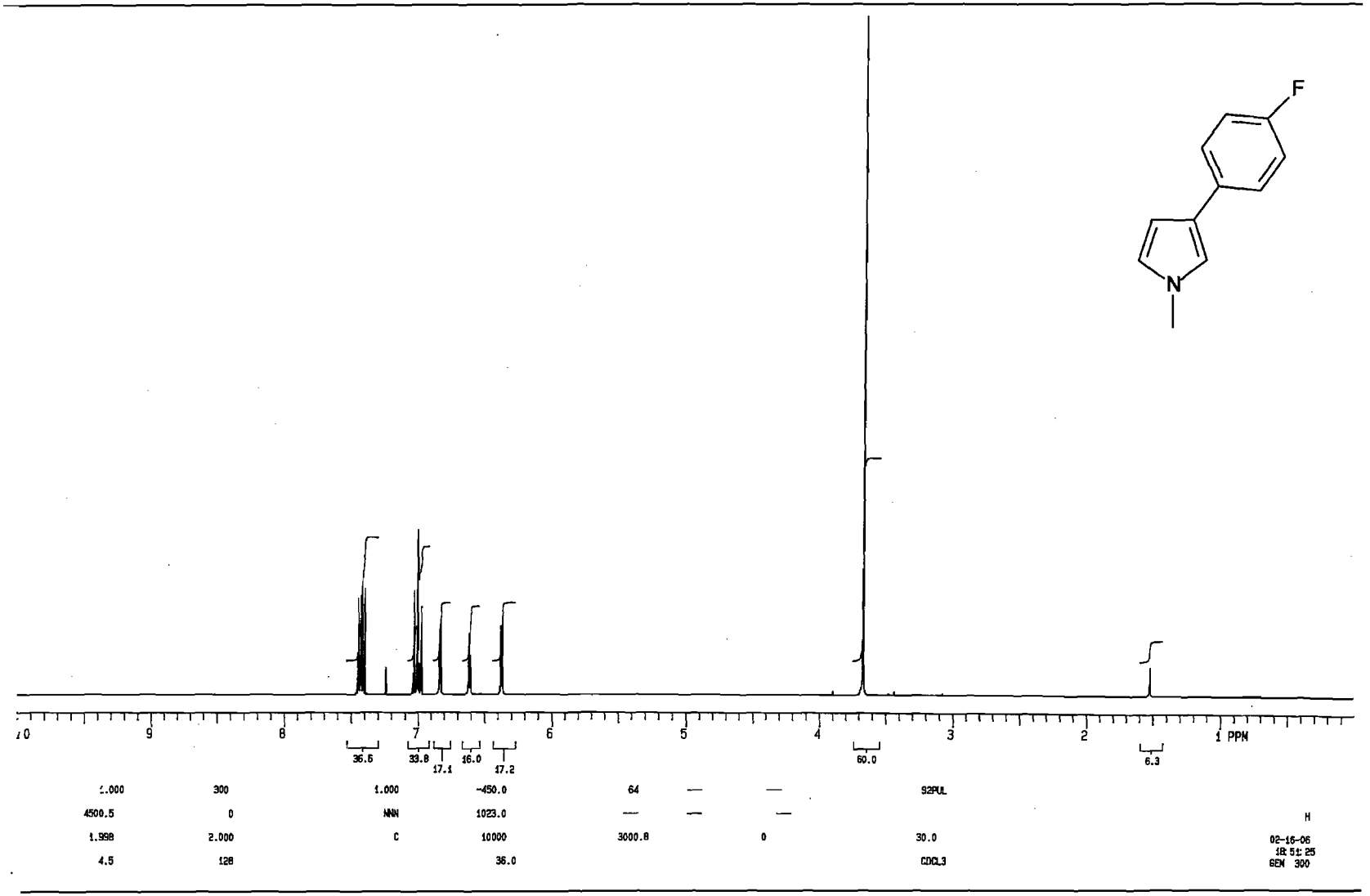
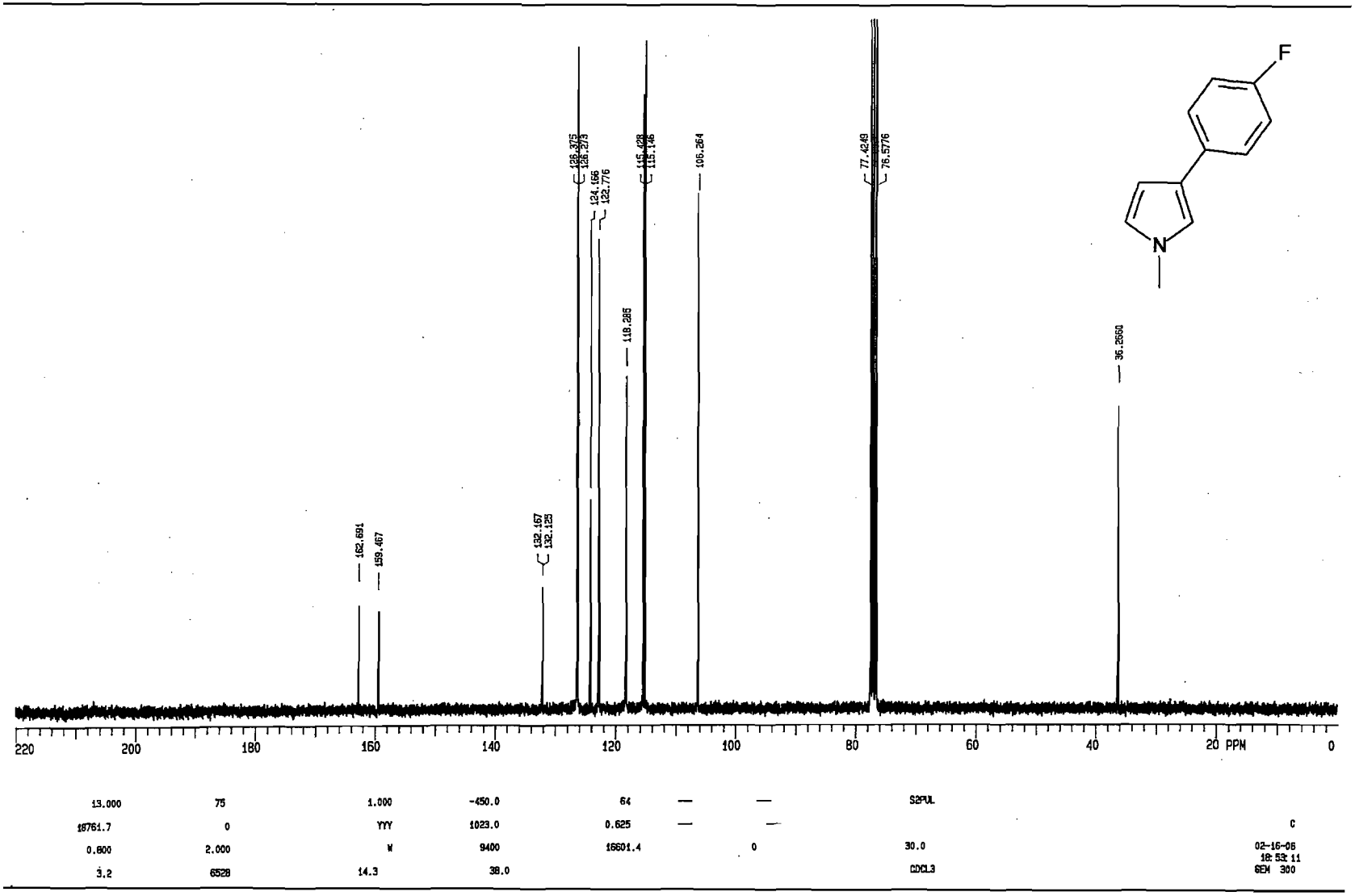
PPM
CDCL3


F
N
10
9
8
7
6
5
4
3
2
1 PPM
36.5
33.8
17.1
16.0
17.2
60.0
6.3
1.000 300 1.000 -450.0 64 — — S2PUL
4500.5 0 NNN 1023.0 — — — H
1.998 2.000 C 10000 3000.8 0 30.0 02-16-06
4.5 128 36.0 CDCL3 18:51:25
GEN 300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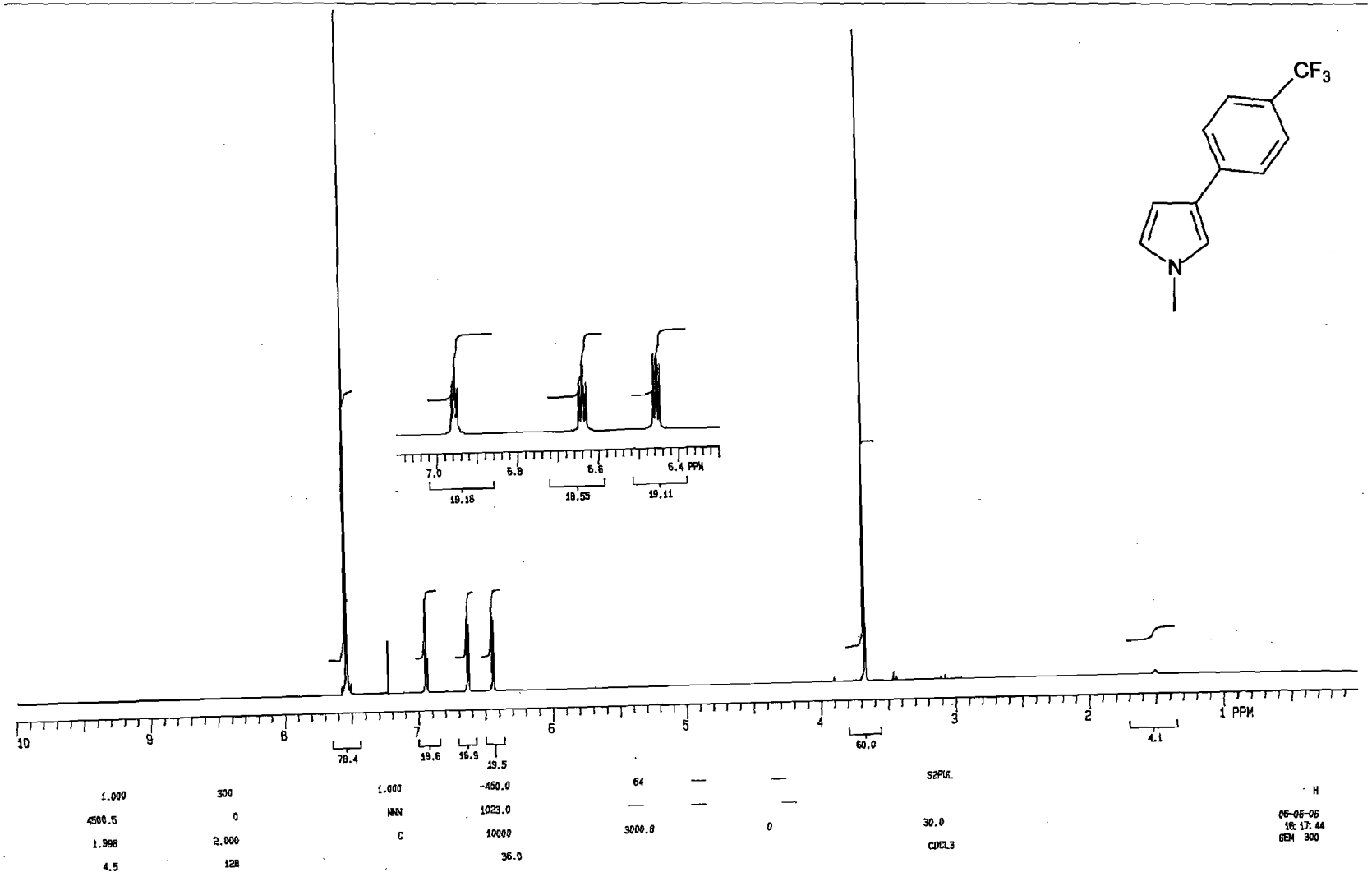
128.375
128.273
124.166
122.776
118.285
115.428
115.146
105.264
77.4249
76.5776
36.2550
220
200
180
160
140
120
100
80
60
40
20 PPM
0
13.000 75 1.000 -450.0 64 S2PUL
16761.7 0 YYY 1023.0 0.625 C
0.600 2.000 W 9400 16601.4 0 30.0 02-16-06
3.2 6528 14.3 38.0 CDCL3 16:53:11
GEM 300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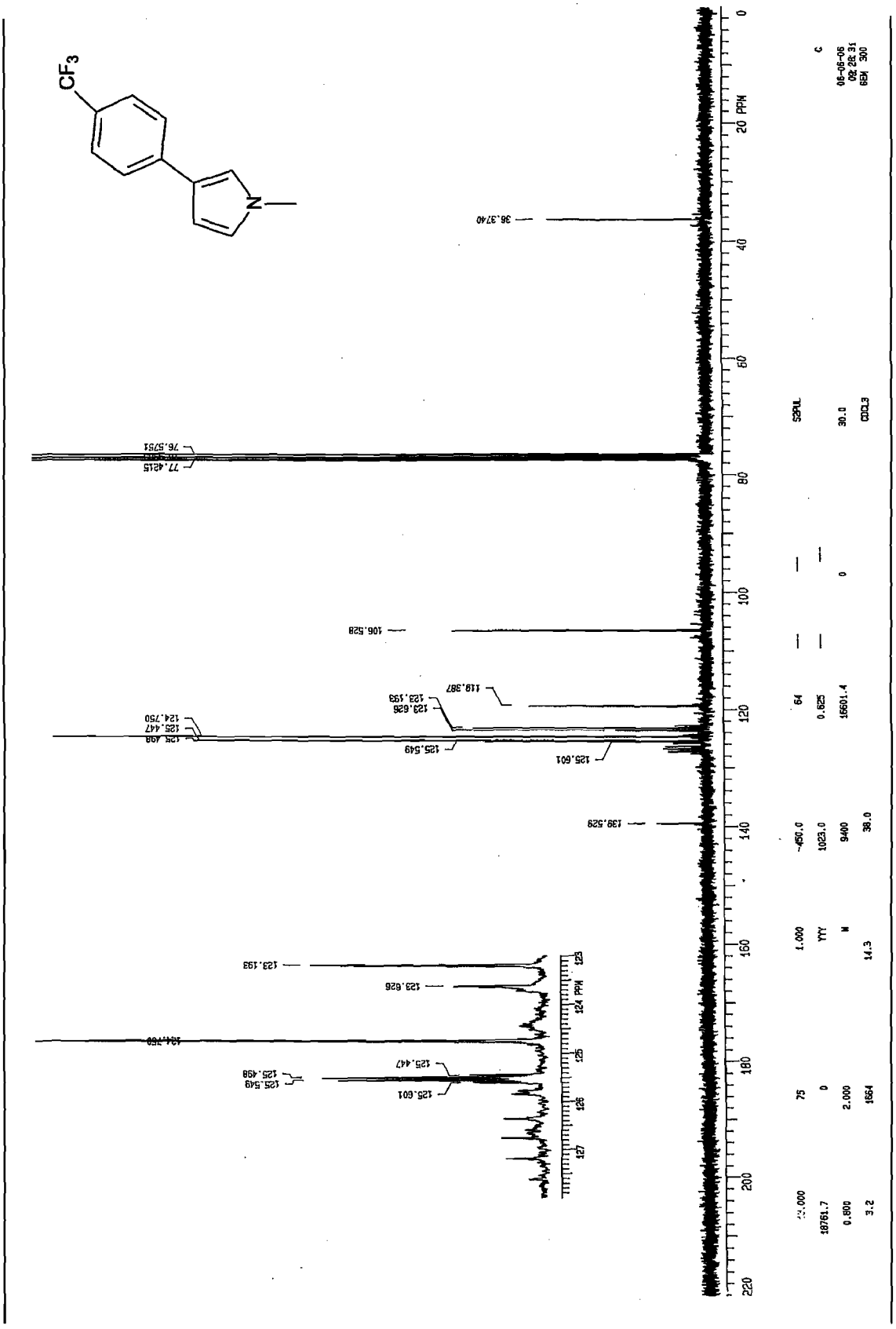
6.8
6.6
6.4 PPM
19.16
18.55
19.11
10
9
8
7
6
5
4
3
2
1 PPM
78.4
19.6
19.9
19.5
60.0
4.1
1.000
4500.5
1.998
4.5
300
0
2.000
128
1.000
NNN
C
-450.0
1023.0
10000
36.0
64
3000.8
0
S2PUL
30.0
CDCL3
H
06-06-06
16:17:44
GEM 300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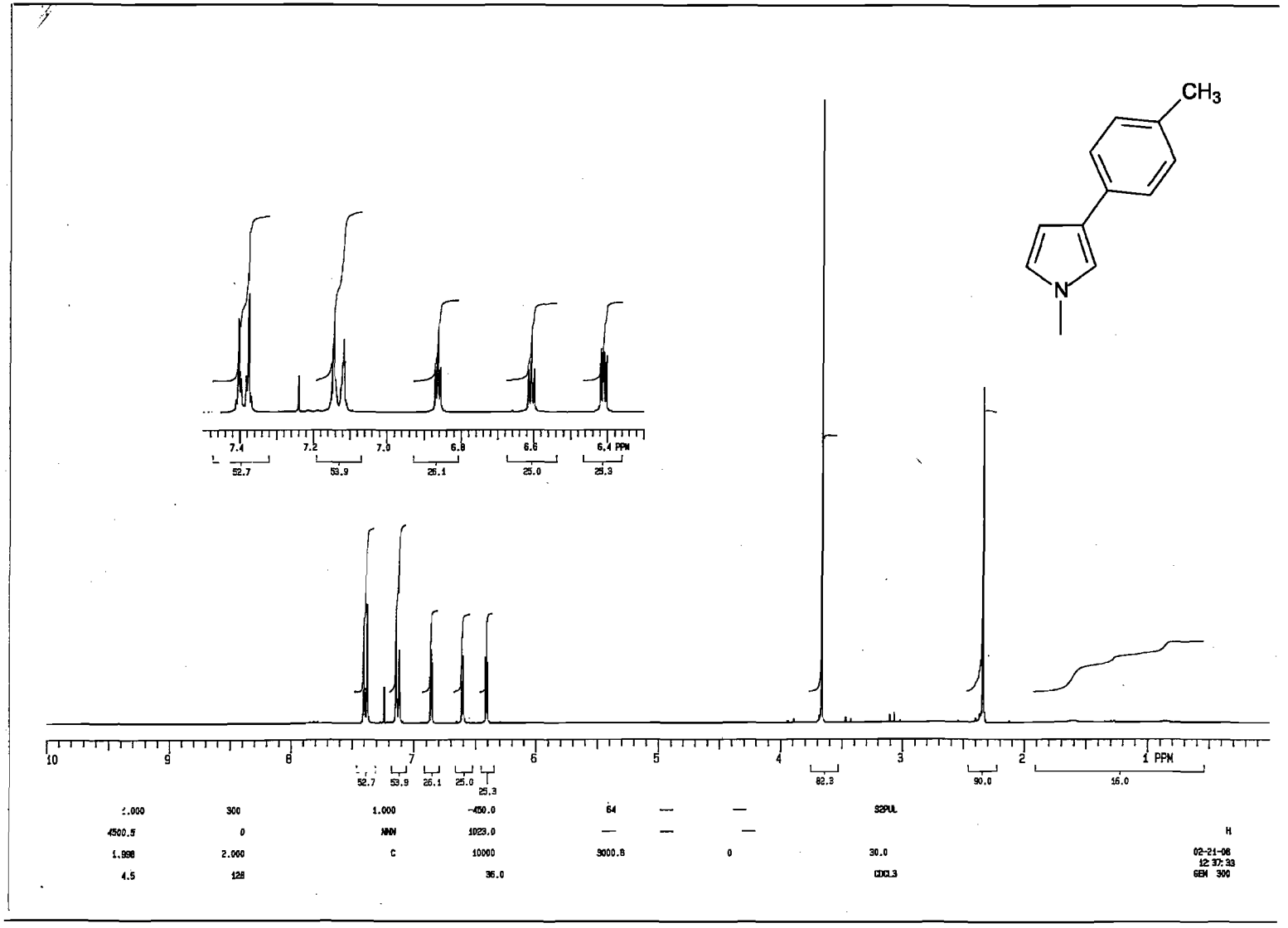
38.3740
76.5751
77.4215
106.528
119.387
123.139
123.626
124.750
125.447
125.498
125.549
125.601
138.529
123.193
123.826
124.750
125.447
125.498
125.549
125.601
20 PPM
124 PPM
SPUL
CDCL3
06-06-06
02:28:31
BBI 300


CH3
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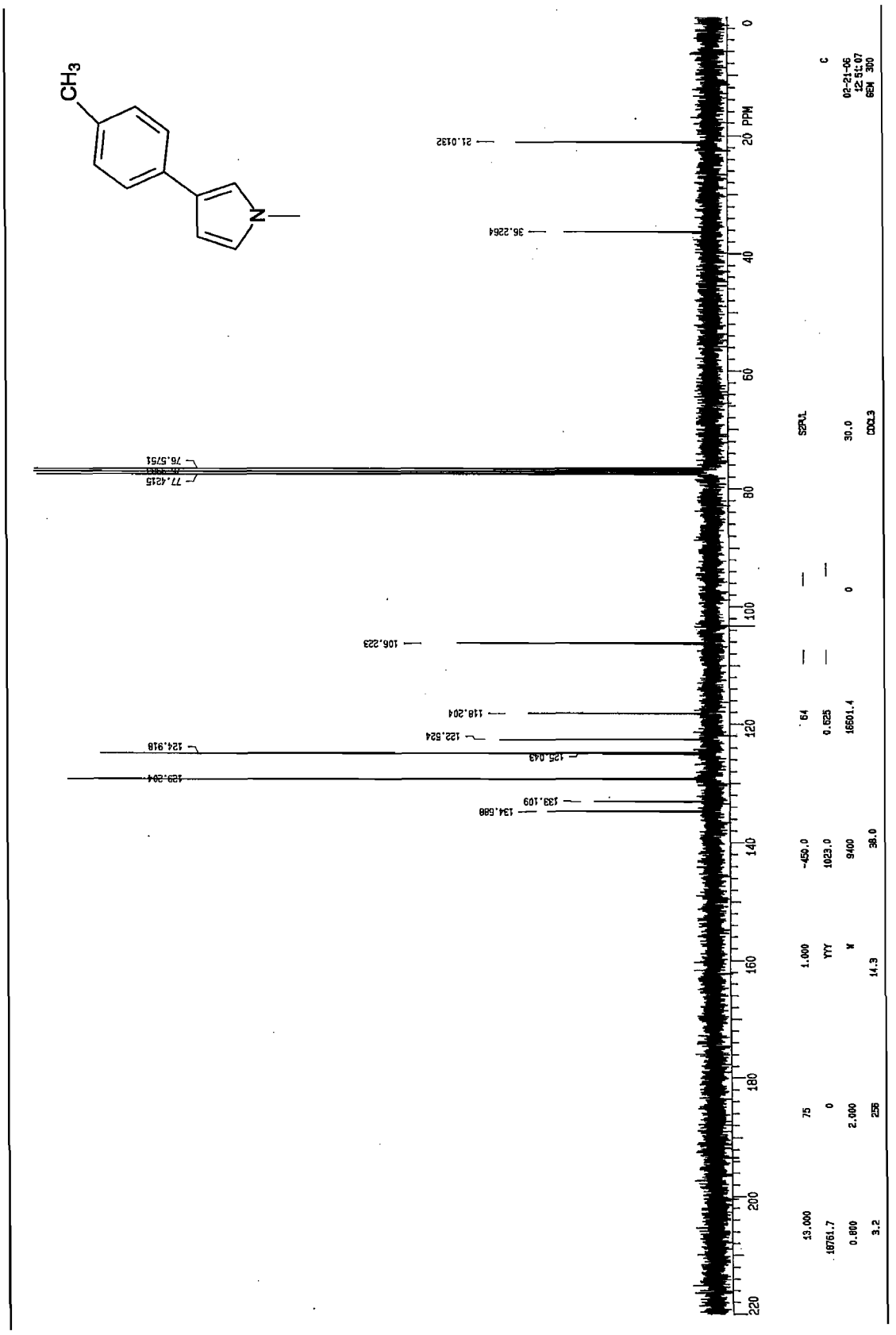
7.4
7.2
7.0
6.8
6.6
6.4 PPM
52.7
53.9
26.1
25.0
25.3
10
9
8
7
6
5
4
3
2
1 PPM
82.3
90.0
16.0
1.000 300 1.000 -450.0 64 — — 32PUL
4500.5 0 NNN 1023.0 — — — H
1.998 2.000 C 10000 9000.8 0 30.0 02-21-06
12:37:33
4.5 128 36.0 CDCL3 GEM 300


CH3
N
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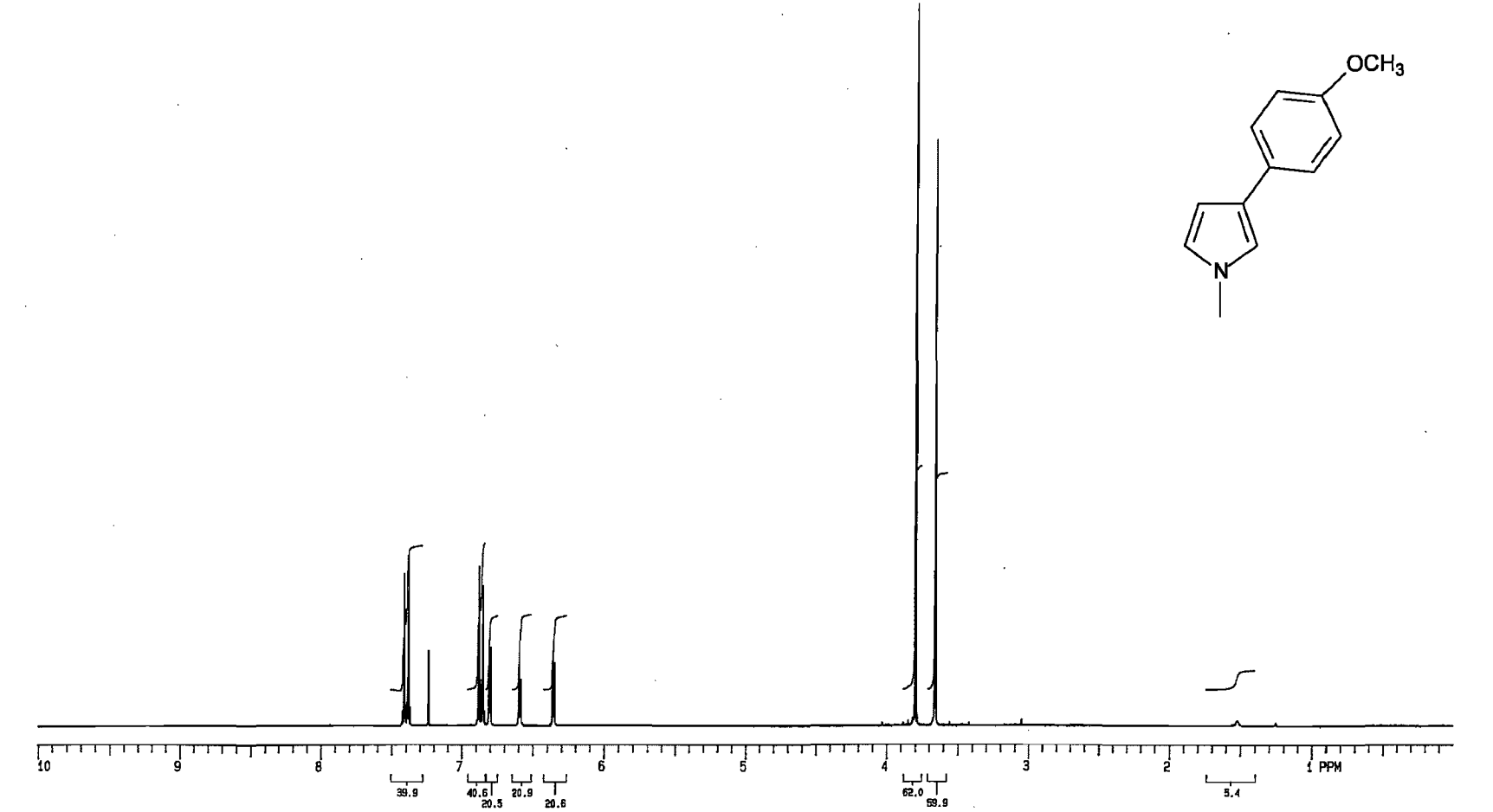
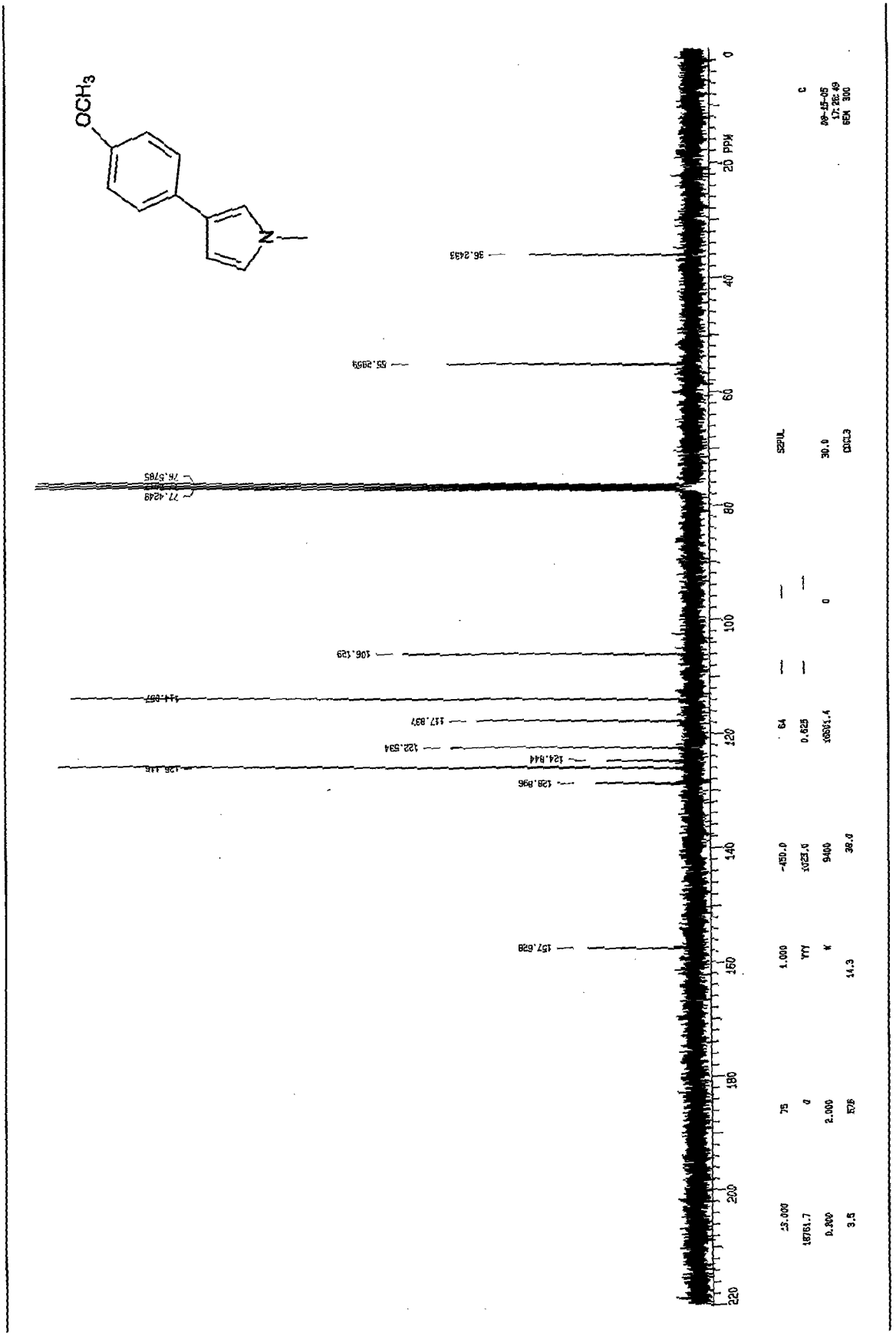
36.2264
76.5751
77.4215
106.223
118.204
122.824
124.918
125.043
129.204
133.109
134.688
0 PPM
20
40
60
80
100
120
140
160
180
200
220
C
02-21-06
12:51:07
SGP1
30.0
CDCl3
0
64
0.625
16601.4
-450.0
1023.0
9400
38.0
1.000
YYY
W
14.3
75
0
2.000
256
13.000
16761.7
0.800
3.2



1.000	300	1.000	-450.0	84	—	—	S2PUL	
4500.5	0	NNN	1023.0	—	—	—		H
1.998	2.000	C	10000	3000.6	0		90.0	08-15-05 17:27:06
4.5	128		36.0				CDCL3	GEN 300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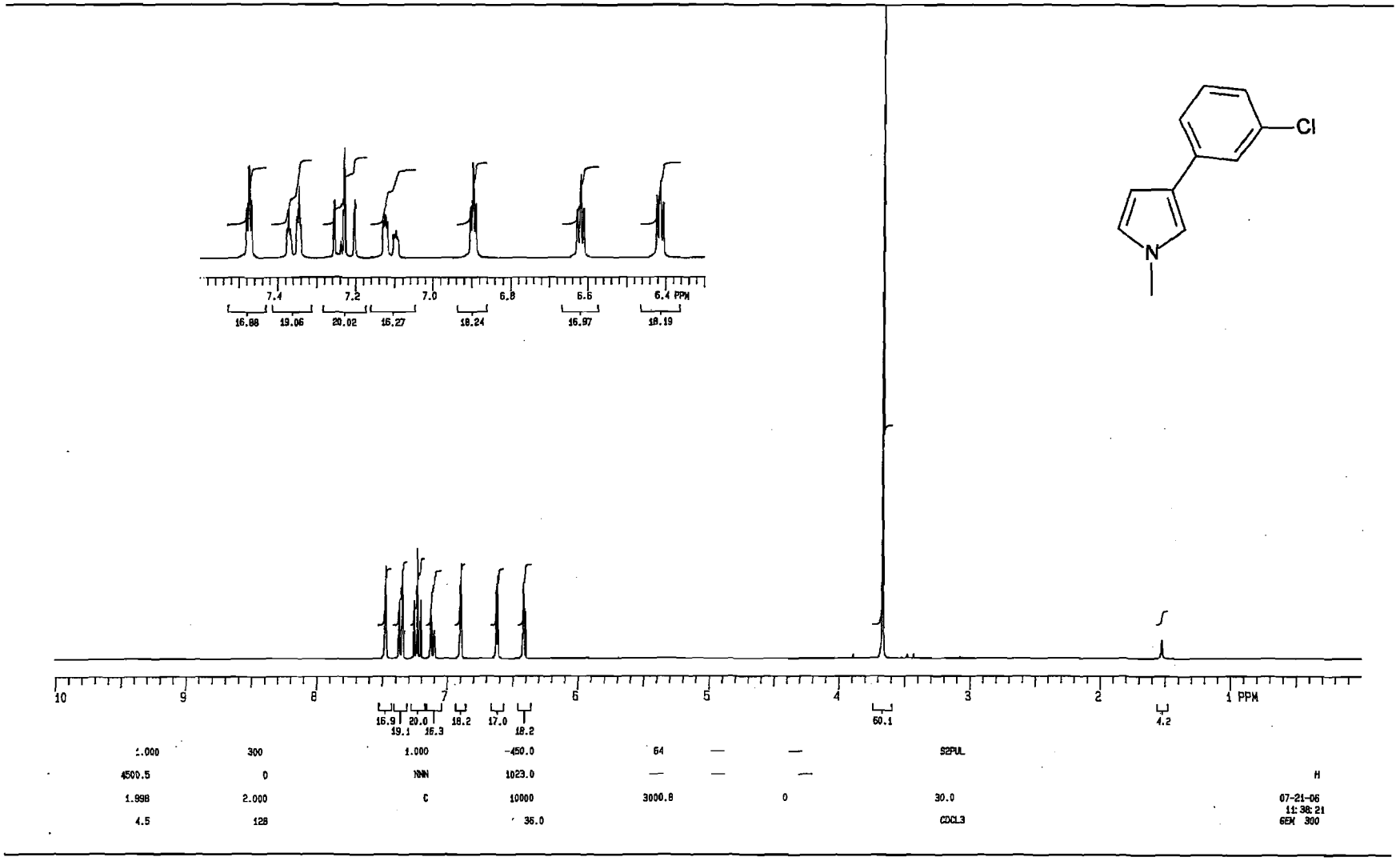
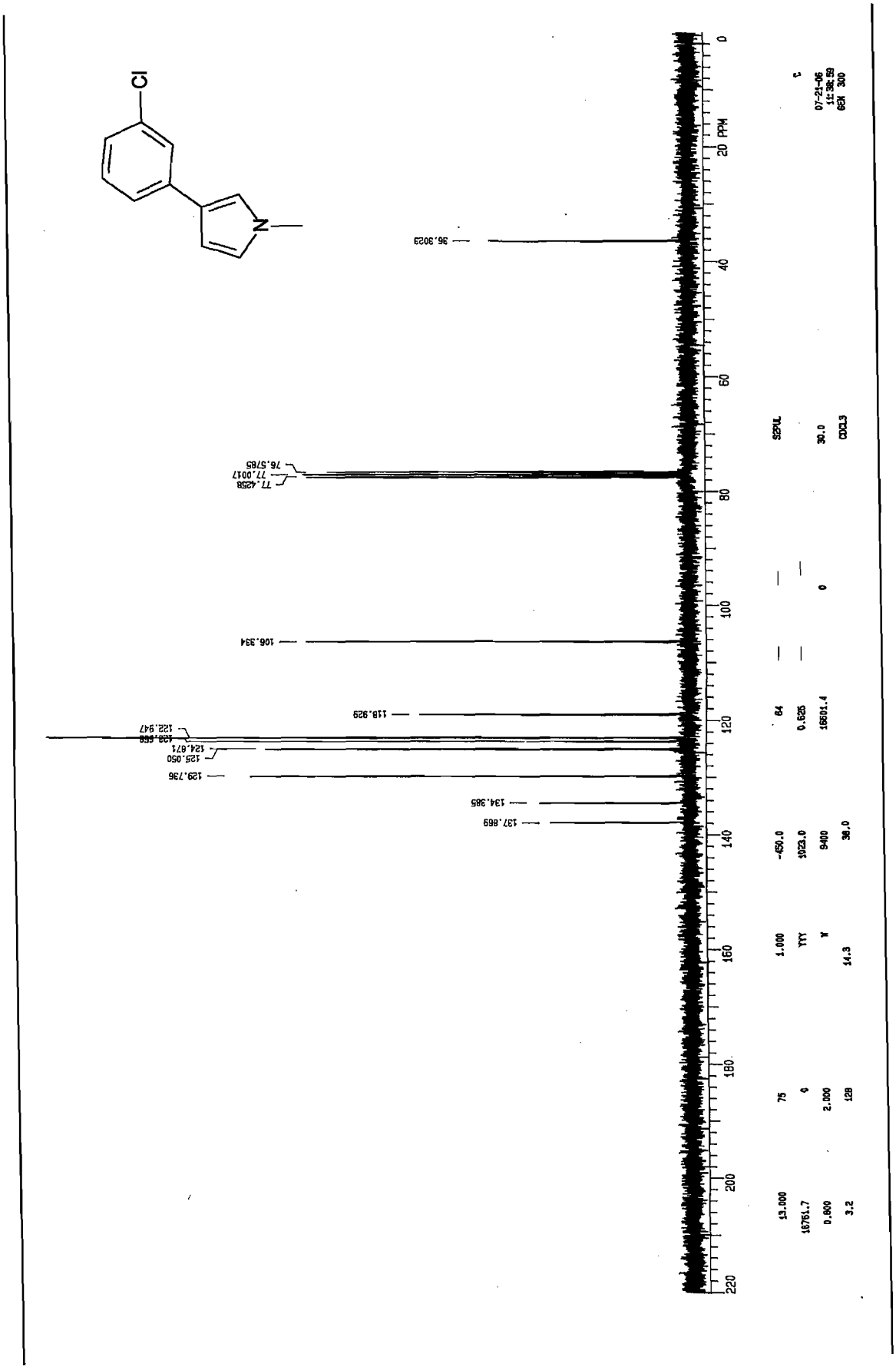
55.2959
77.4248
76.5785
106.129
114.857
117.837
122.534
124.844
128.896
157.628
0
20
40
60
80
100
120
140
160
180
200
220
PPM


Cl
N
7.4
7.2
7.0
6.8
6.6
6.4 PPM
16.88
19.06
20.02
15.27
18.24
16.97
18.19
10
9
8
7
6
5
4
3
2
1 PPM
16.9
19.1
20.0
16.3
18.2
17.0
18.2
60.1
4.2
1.000
300
1.000
-450.0
64
SZPUL
4500.5
0
NNN
1023.0
H
1.998
2.000
C
10000
3000.8
0
30.0
07-21-06
11:36:21
4.5
128
36.0
CDCL3
GEM 300


Cl
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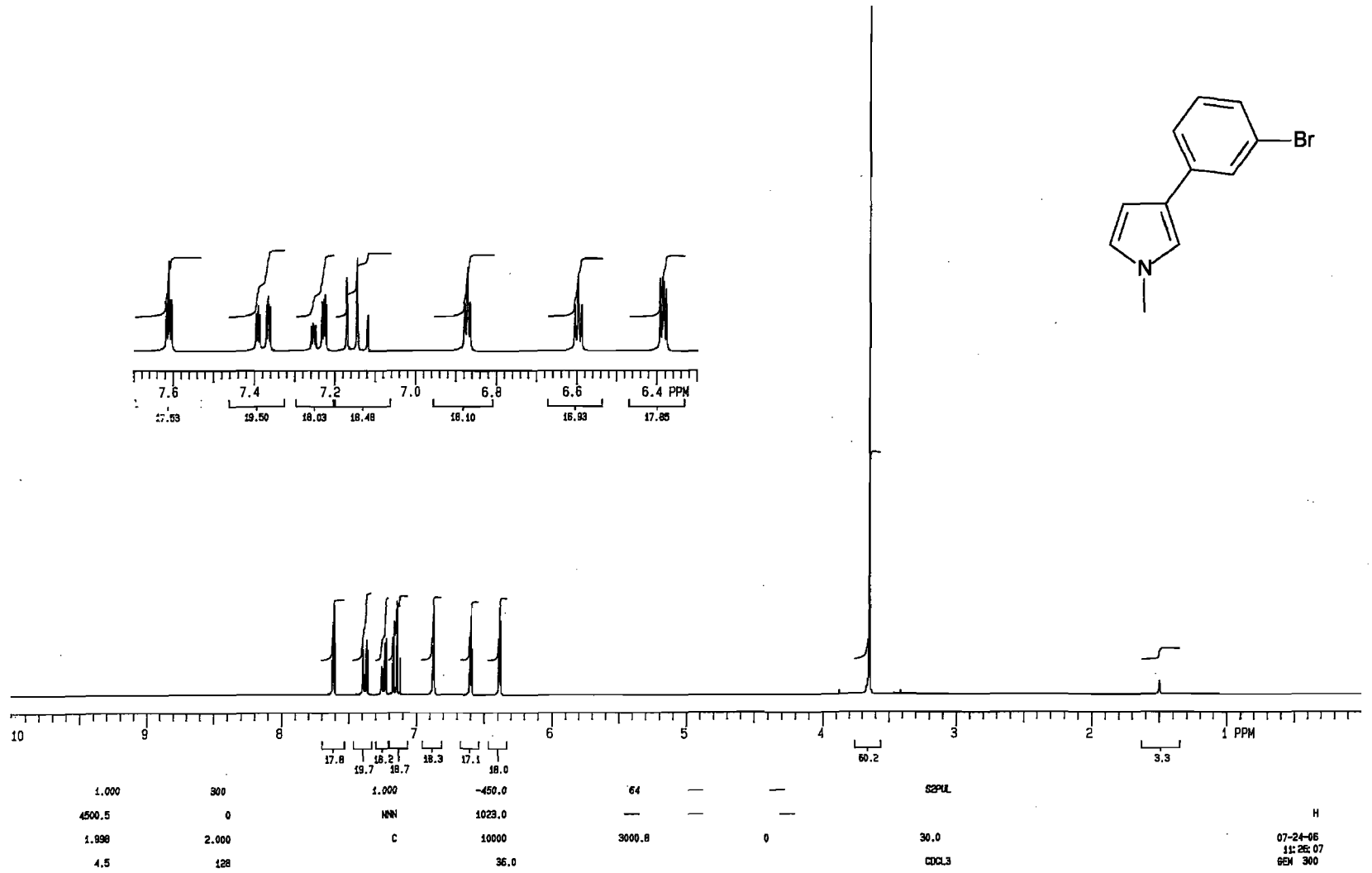
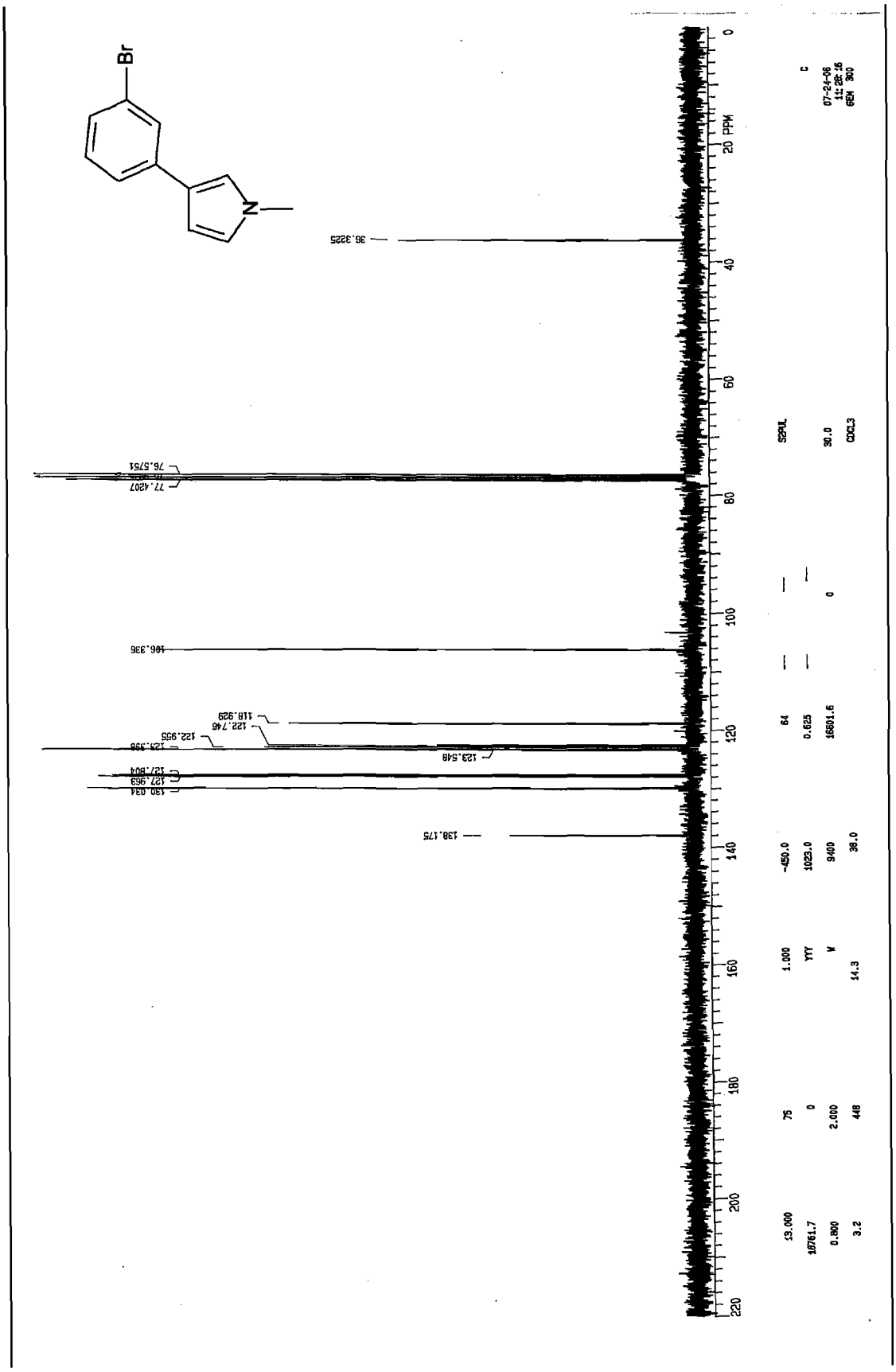
76.5785
77.0017
77.4258
106.334
118.929
122.947
123.668
124.871
125.050
129.736
134.385
137.869
0
20 PPM
40
60
80
100
120
140
160
180
200
220
SERIAL
30.0
CDCL3
07-24-06
11:38:59
DGN 300
64
0.625
16601.4
-450.0
1023.0
9400
30.0
1.000
YYY
N
14.3
13.000
75
0
2.000
128
18751.7
0.800
3.2


Br
N
7.6
7.4
7.2
7.0
6.8
6.6
6.4 PPM
17.53
19.50
18.03
18.48
18.10
16.93
17.85
10
9
8
7
6
5
4
3
2
1 PPM
17.8
19.7
18.2
18.7
18.3
17.1
18.0
60.2
3.3
1.000
300
1.000
-450.0
64
S2PUL
4500.5
0
NNN
1023.0
H
1.998
2.000
C
10000
3000.8
0
30.0
07-24-06
11:25:07
4.5
128
36.0
CDCL3
GEN 300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76.5751
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106.336
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122.746
122.955
123.548
127.804
127.963
130.034
138.175
0
20 PPM
40
60
80
100
120
140
160
180
200
220
C
07-24-06
11:26:16
6EM 300
SEQUL
30.0
CDCL3
64
0.625
16601.6
0
-450.0
1023.0
9409
38.0
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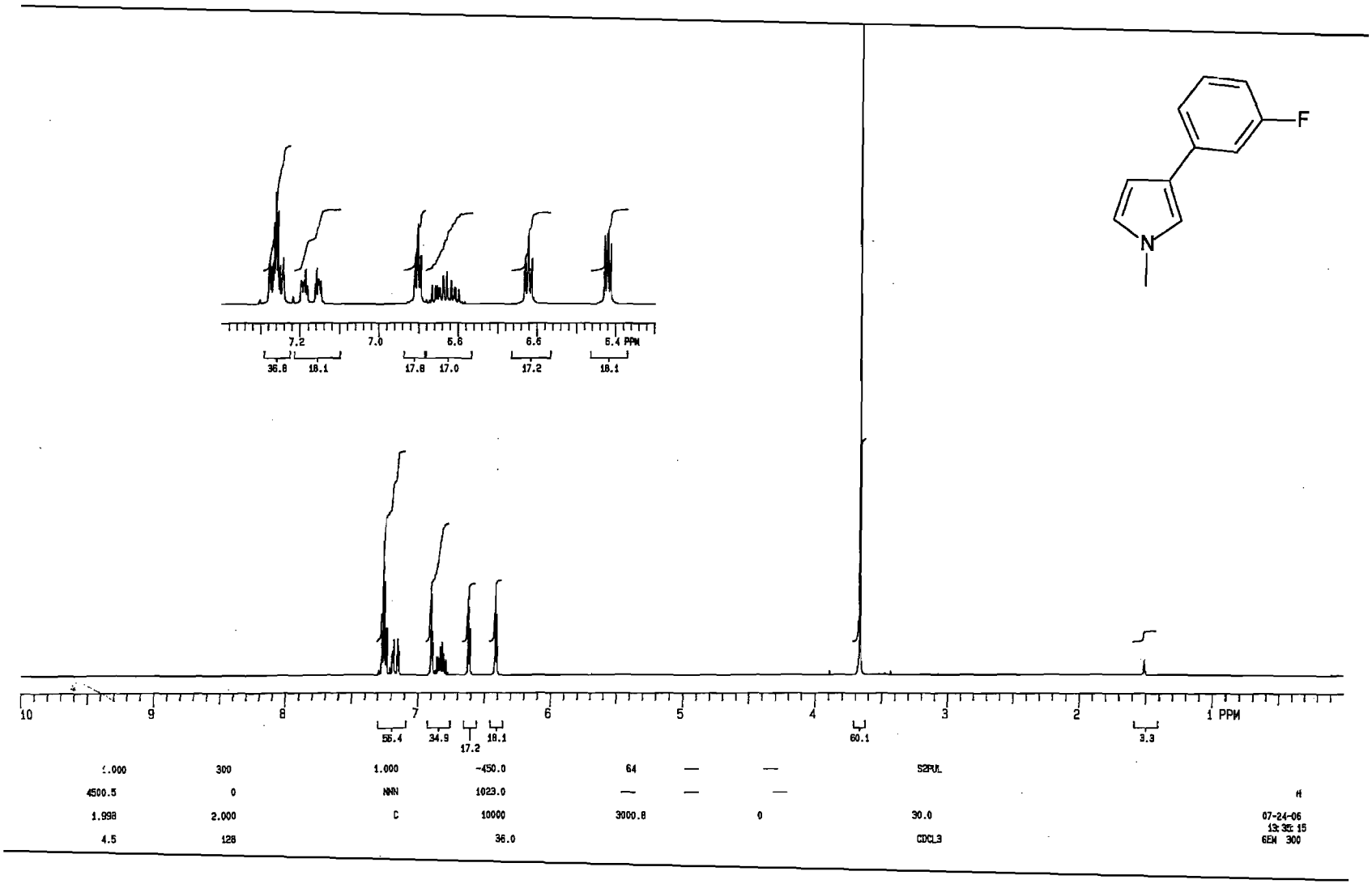
YYY
W
14.3
13.000
16761.7
0.800
3.2
75
0
2.000
448


F
N
7.2
7.0
6.8
6.6
6.4 PPM
36.8
18.1
17.8
17.0
17.2
18.1
10
9
8
7
6
5
4
3
2
1 PPM
55.4
34.9
17.2
18.1
60.1
3.9
1.000 300 1.000 -450.0 64 S2PUL
4500.5 0 NNN 1023.0 H
1.998 2.000 C 10000 3000.8 0 30.0
07-24-06
13:35:15
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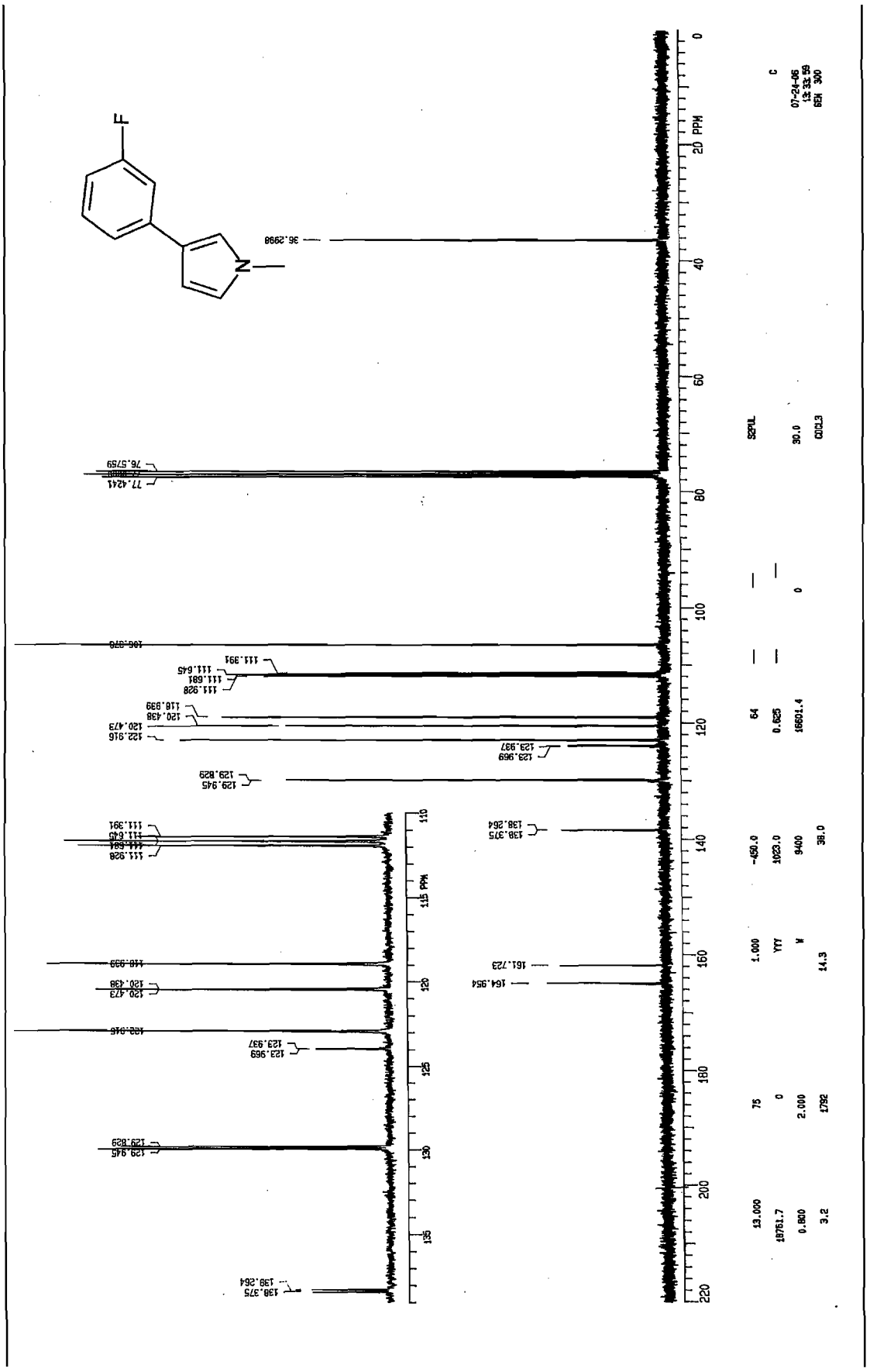
6EM 300


F
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111.645
111.681
111.928
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120.473
122.816
123.969
123.937
129.829
129.945
138.264
138.375
161.723
164.954
0
20 PPM
40
60
80
100
120
140
160
180
200
220
110
115 PPM
120
125
130
135
SCAL
64
0.625
16601.4
-450.0
1023.0
9400
38.0
1.000
YYY
W
14.3
75
0
2.000
1792
13.000
18761.7
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3.2
C
07-24-06
13:33:59
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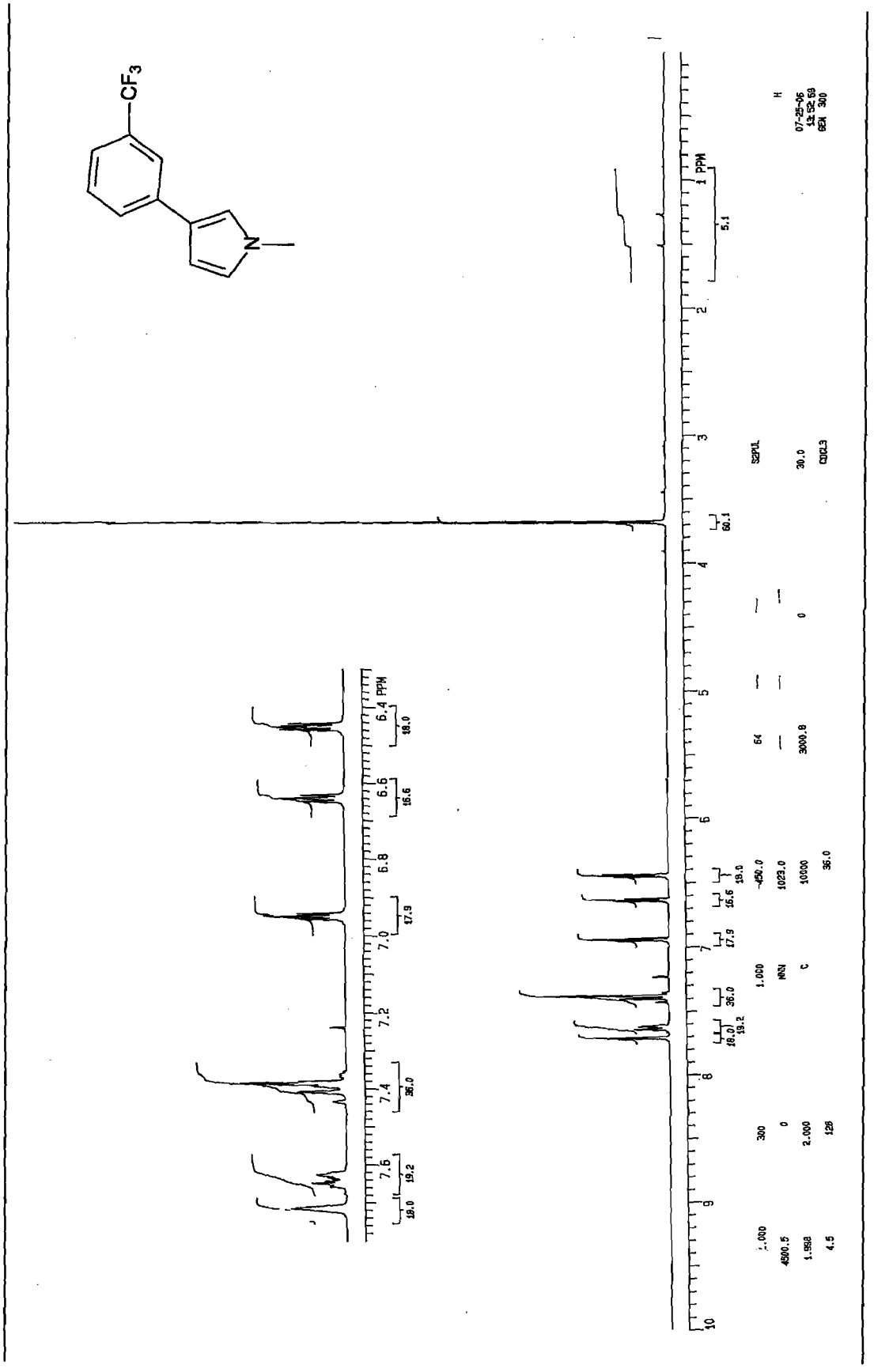
CDCL3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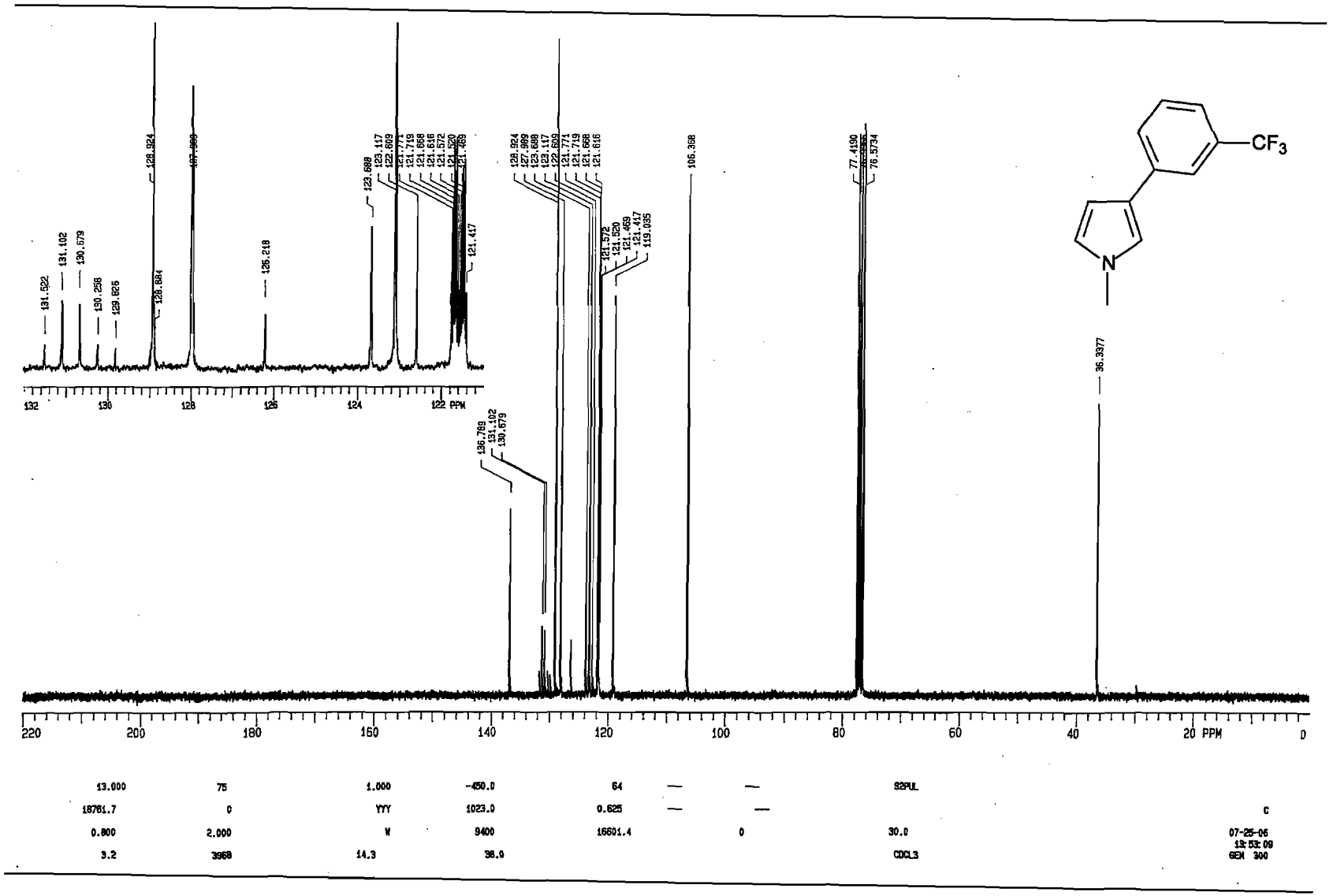
7.4
7.2
7.0
6.8
6.6
6.4 PPM
1 PPM
CDCL3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128.684
127.999
126.218
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123.117
122.609
121.771
121.719
121.688
121.616
121.572
121.520
121.469
121.417
119.035
136.789
106.368
77.4190
76.5734
36.3377
132
130
128
126
124
122 PPM
220
200
180
160
140
120
100
80
60
40
20 PPM
0
13.000 75 1.000 -450.0 64 — — S2PUL
18761.7 0 YYY 1023.0 0.625 — — C
0.800 2.000 W 9400 16601.4 0 30.0 07-25-06
13:53:09
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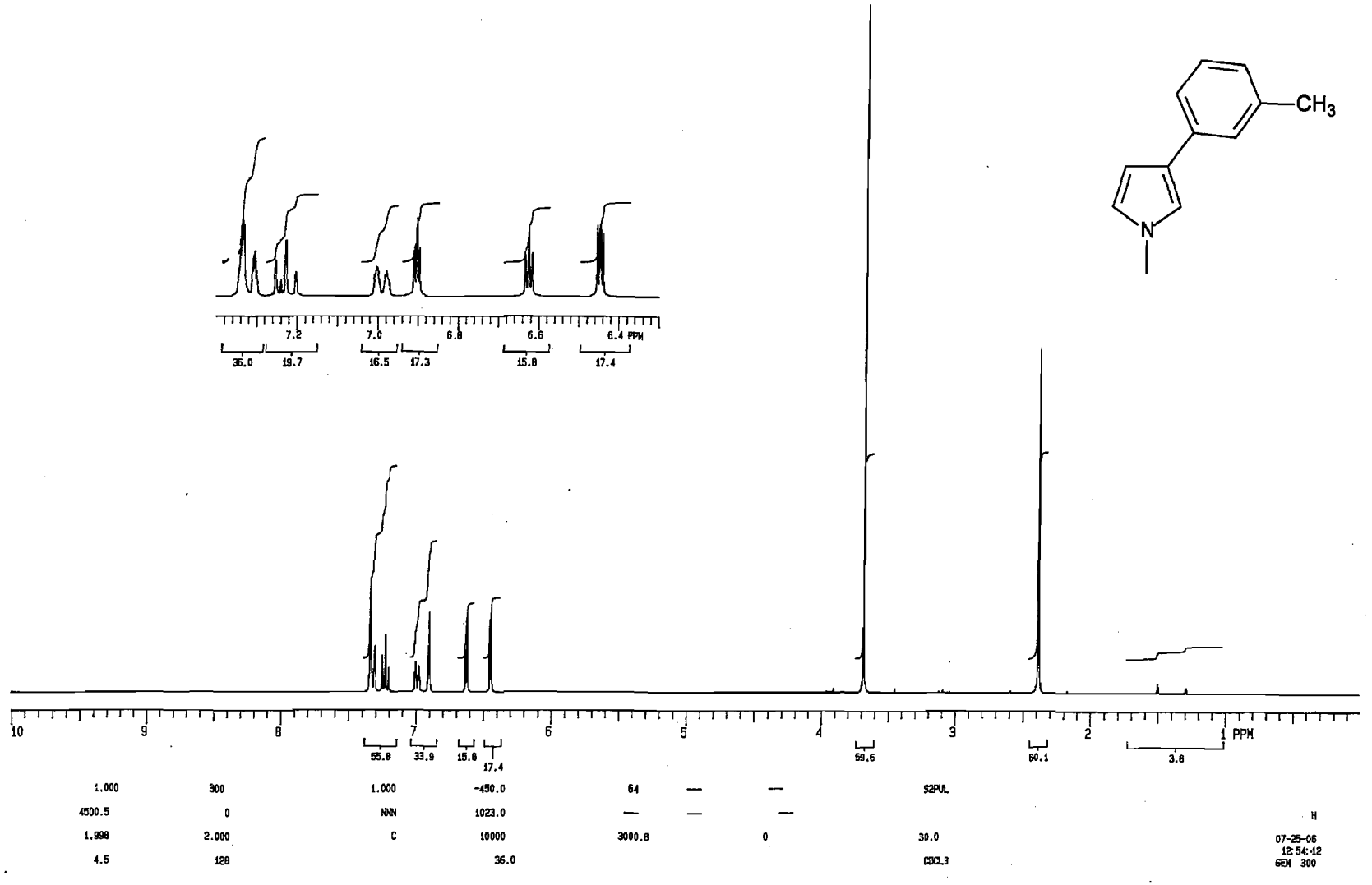
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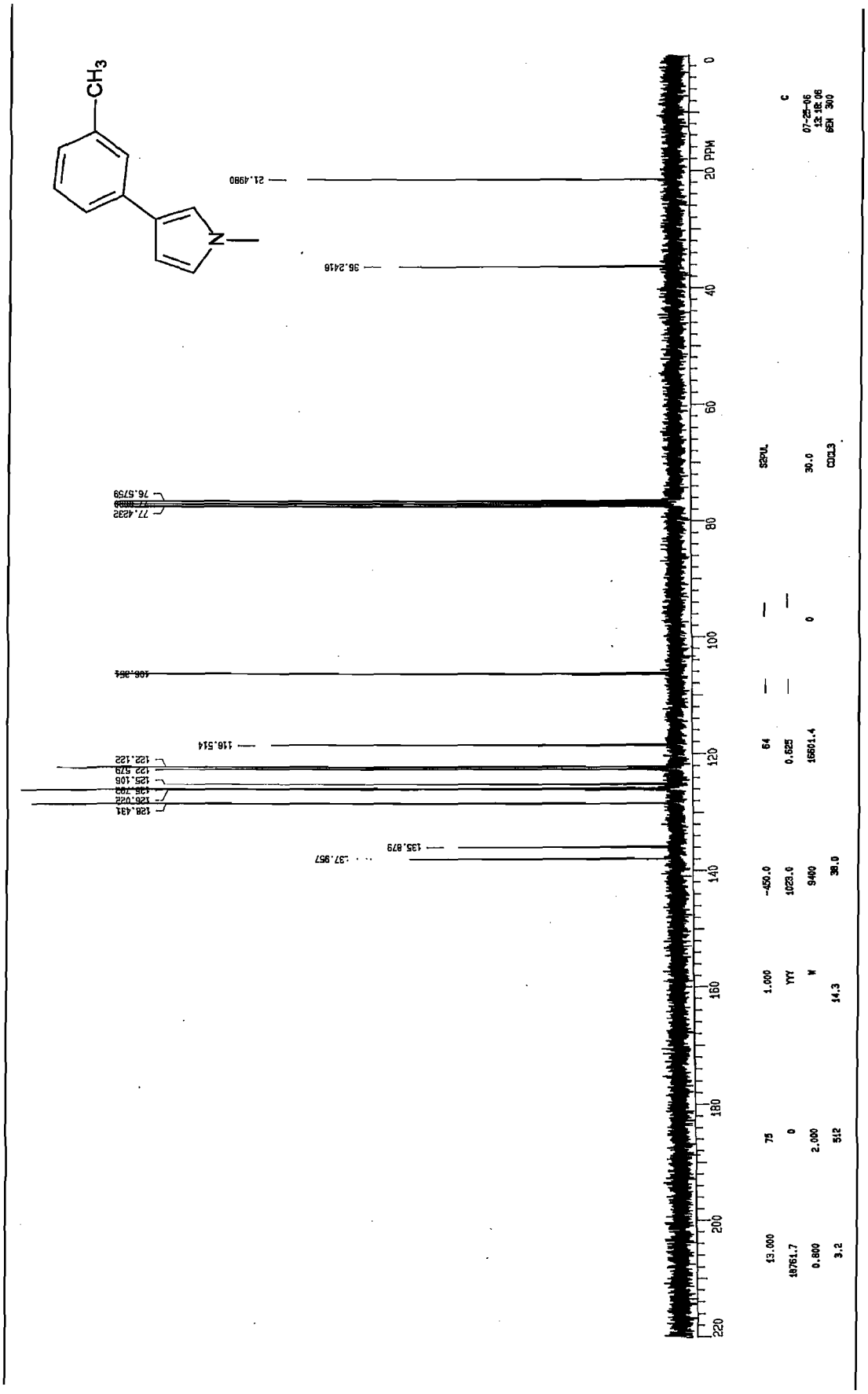
6.8
6.6
6.4 PPM
36.0
19.7
16.5
17.3
15.8
17.4
10
9
8
7
6
5
4
3
2
1 PPM
55.8
33.9
15.8
17.4
59.6
60.1
3.8
1.000 300 1.000 -450.0 64 — — S2PUL
4500.5 0 NNN 1023.0 — — —
1.998 2.000 C 10000 3000.6 0 30.0
4.5 128 36.0 CDCL3
H
07-25-06
12:54:12
GEM 300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77.4232
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122.579
125.108
126.799
126.922
128.434
135.879
137.957
0
20 PPM
40
60
80
100
120
140
160
180
200
220
C
07-25-06
12:18:06
6EH 300
SZPUL
30.0
COCL3
64
0.625
16601.4
0
-450.0
1023.0
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YYY
M
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512
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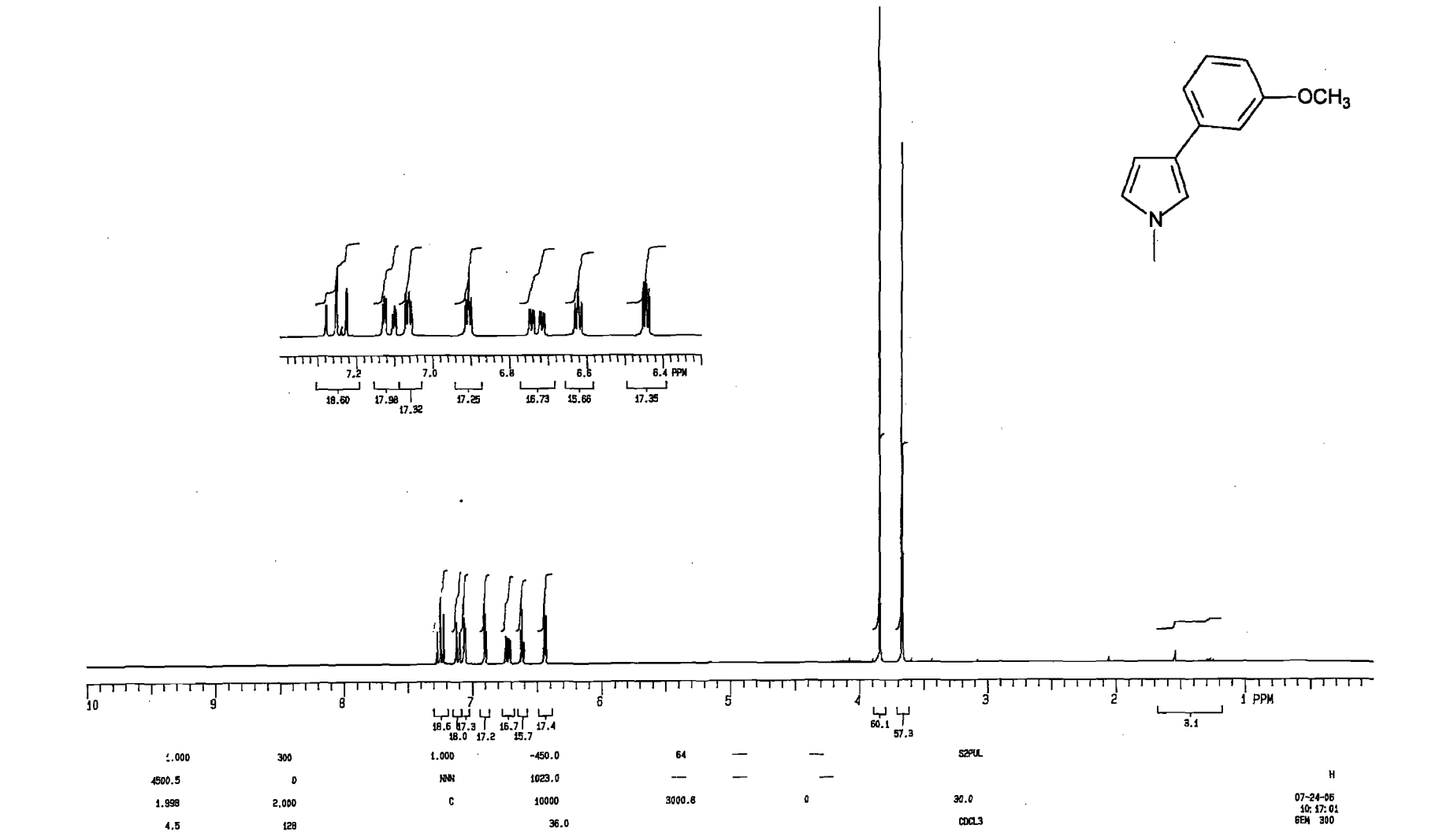
3.2


OCH3
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7.0
6.8
6.6
6.4 PPM
18.60
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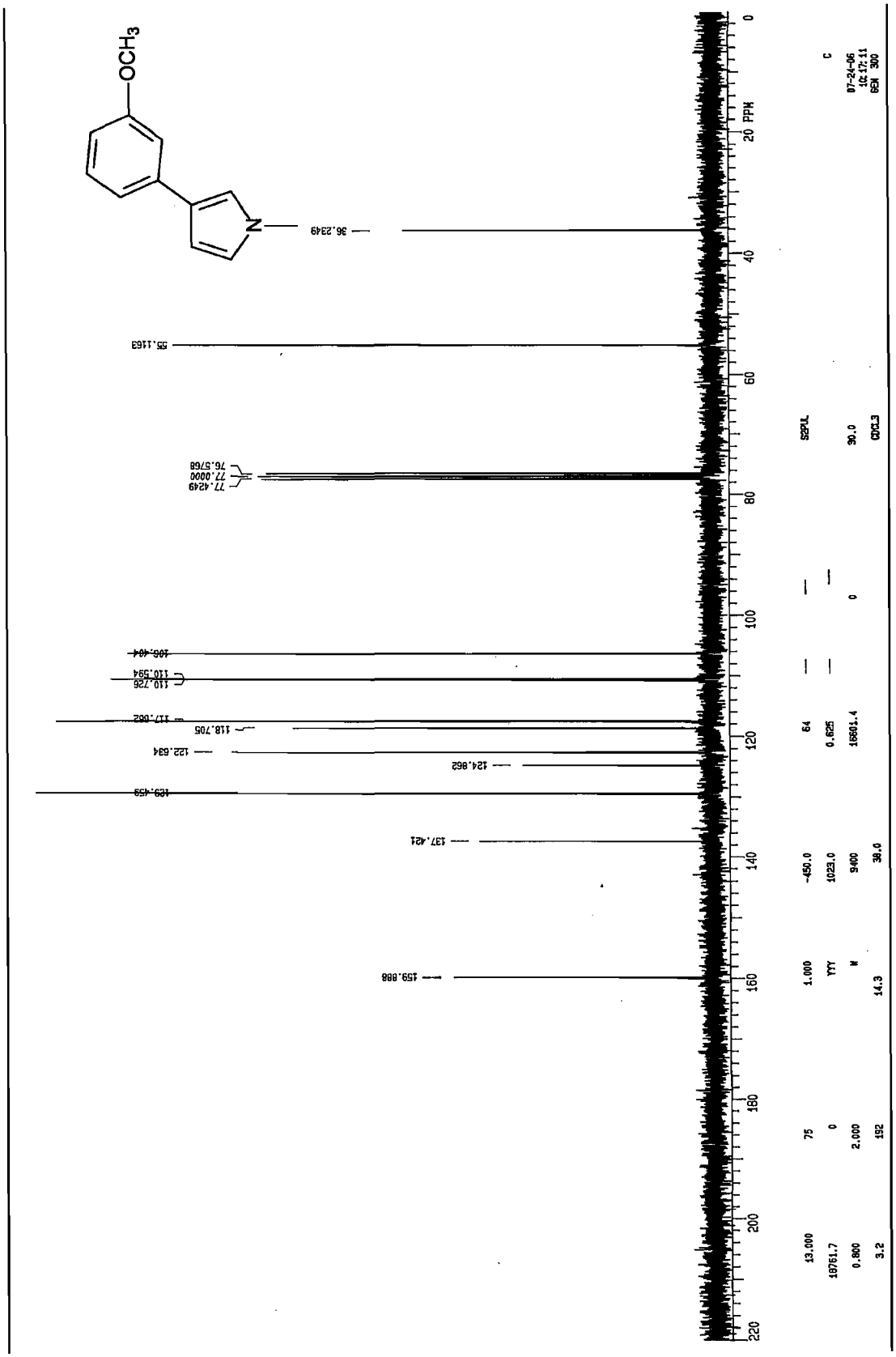
17.32
17.25
16.73
15.66
17.35
10
9
8
7
6
5
4
3
2
1 PPM
18.6
18.0
17.3
17.2
15.7
15.7
17.4
60.1
57.3
3.1
1.000
300
1.000
-450.0
64
S2PUL
4500.5
0
NNN
1023.0
H
1.998
2.000
C
10000
3000.6
0
30.0
07-24-96
10:17:01
4.5
128
36.0
CDCL3
GEN 300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110.594
118.705
122.634
124.862
129.459
137.421
159.888
0
20 PPM
40
60
80
100
120
140
160
180
200
220
C
07-24-96
10:17:11
BSM 300
SGPUL
CDCL3
30.0
0
64
0.625
16601.4
-450.0
1023.0
9400
30.0
1.000
YYY
W
14.3
75
0
2.000
192
13.000
19761.7
0.800
3.2