N-Hydroxyaminopyrenes

The Synthesis and Characterization of Some N-Hydroxyaminopyrenes

By

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TITLE: **THE SYNTHESIS AND CHARACTERIZATION OF SOME N-HYDROXY-AMINOPYRENES**

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Abstract

Nitrated polycyclic aromatic hydrocarbons (nitro-PAH) are environmental contaminants that have been identified in extracts of particulates obtained from automobile exhaust, diesel exhaust, and power plant emissions. One of the most abundant nitro-PAH, 1-nitropyrene, has been found to be a powerful bacterial mutagen and a carcinogen in animal tests. In a bacterial strain (Salmonella typhimurium TA98) 1-nitropyrene undergoes reduction and concomitant covalent binding to DNA, presumably via the N-hydroxy-1-aminopyrene. This labile compound was prepared by the ascorbic acid reduction of 1-nitrosopyrene and was characterized by high performance liquid chromatography (HPLC), combined liquid chromatography-mass spectrometry (LC-MS), ultraviolet-visible (UV-VIS), fluorescence and nuclear magnetic resonance (NMR) spectroscopy (^{1}H and ^{15}N). In addition the pH stability and some reactions of this compound were studied. The 1,6- and 1,8-hydroxylaminonitropyrenes derived from the very potent mutagens 1,6- and 1,8 dinitropyrene were also synthesized and characterized by high performance liquid chromatography, ultraviolet-visible and nuclear magnetic resonance spectroscopy (^{1}H and ^{15}N).

Acknowledgements

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List of Abbreviations

1-AAP	N-Acetyl-1-Aminopyrene
1-AP	1-Aminopyrene
1-HAP	N-Hydroxy-1-Aminopyrene
1-NOP	1-Nitrosopyrene
1-NP	1-Nitropyrene
2-AAF	N-Acetyl-2-Aminofluorene
2-AF	2-Aminofluorene
2-HAAF	N-Acetyl-N-Hydroxy-2-Aminofluorene
2-HAF	N-Hydroxy-2-Aminofluorene
2D-COSY	Two Dimensional Correlated Spectroscopy
AANP	N-Acetyl-Amino-Nitropyrene
Ac	Acetyl
Ac_2O	Acetic Anhydride
ACN	Acetonitrile
amino-PAH	Amino Polycyclic Aromatic Hydrocarbons
ANP	Amino-Nitropyrene
DAP	Diaminopyrene
DEPT	Distortionless Enhancement by Polarization Transfer
dG	deoxyguanosine
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid

DNP	Dinitropyrene
EHO	Epoxy Hydrolase
EtOAc	Ethyl Acetate
HAAF	N-acetyl-N-hydroxy-aminofluorene
HAANP	N-acetyl-N-hydroxy-aminonitropyrene
HANP	N-Hydroxy-Amino-Nitropyrene
HAP	N-Hydroxy-Amino-Pyrene(s)
HPLC	High Performance Liquid Chromatography
INEPT	Insensitive Nuclei Enhancement by Polarization Transfer
k′	$(t_R-t_o)/t_o$ (also known as capacity factor or partition ratio)
LC-MS	Combined Liquid Chromatography-Mass Spectrometry
m-CPBA	m-chloroperoxybenzoic acid
MeOH	Methanol
MFO	Mixed Function Oxidases
MS	Mass Spectrometry
MTBDMSTFA	N-Methyl-N-tert-butyl-dimethylsilyl-trifluoracetamide
nitro-PAH	Nitrated Polycyclic Aromatic Hydrocarbons
NMR	Nuclear Magnetic Resonance Spectroscopy
NP	Nitropyrene(s)
NONP	Nitro-Nitrosopyrene
PAH	Polycyclic Aromatic Hydrocarbons
ppm	parts per million

Prep-LC	Preparative Liquid Chromatography
RIC	Reconstructed Ion Current
RP-HPLC	Reverse-phase HPLC
Rs	Resolution $=((t_{R2}-t_{R1}) / 0.5(w_1 + w_2))$
SEFT	Spin Echo Fourier Transform
TBDMS	tert-Butyldimethylsilyl
TIC	Total Ion Current
t_R	Retention Time
UV-VIS	Ultraviolet-Visible Spectroscopy

1. INTRODUCTION

1.1 Chemicals and Cancer

Exposure to chemicals, either directly or from the environment has been implicated in some research as being responsible for a significant fraction of human cancers [1-5]. One of the best documented examples of a direct link between chemicals and certain human cancers is the high rate of lung cancer in smokers [3]. A partial list of chemicals considered to be carcinogenic to humans includes alkylating agents, aliphatic nitrosamines, a number of aromatic amines, certain polycyclic aromatic hydrocarbons, aflatoxins, vinyl chloride and certain metals [2,3,5,6].

One of the key steps in carcinogenesis (the formation of a cancerous cell) is thought to be the covalent modification of cellular deoxyribonucleic acid (DNA) by the carcinogenic chemical or their metabolites [7,8,9,10]. Although the mechanism of a chemical's reaction with DNA may vary, it is believed that electrophilic species attack at nucleophilic atoms of the heterocyclic DNA bases to form a modified base (referred to as a "mutation") [9,10,11]. Not surprisingly then, most of the chemicals listed above are electrophiles or are capable of being converted into electrophiles by metabolic reactions in cells [12,13].

1.2 Ames Test for Chemical Mutagens

Animal tests and human epidemiological studies have been the methods traditionally used for the identification of chemical carcinogens [3,14,15]. However, animal tests are expensive and require from 6 months to 3 years to complete. Animal studies also suffer from sensitivity problems for weak carcinogens, and furthermore, may be unable to identify the carcinogens in complex mixtures such as environmental samples. From human epidemiological studies it is often difficult to relate cause and effect for exposure to chemical mixtures [14-16].

The Ames microbial mutagenesis bioassay was introduced in 1975 as a test for potential chemical carcinogens [17-19]. It is based on the assumption that chemicals which cause DNA mutations in bacteria are also likely to be carcinogenic in animals. This test is quick (approximately 48 hours), inexpensive compared to any animal test and is useful for screening complex mixtures such as environmental samples [14,15]. The Ames test utilizes specially developed mutant strains of Salmonella typhimurium bacteria [17-19]. Normal cells can synthesize histidine, an amino acid essential for cell growth. These mutant strains of Salmonella cannot synthesize histidine due to a defect in the gene responsible for histidine biosynthesis. Thus, in normal histidine-free growth medium, the Salmonella tester cells will not grow. However, a few cells do undergo spontaneous mutations (Figure 1a) and "revert" to being able to synthesize histidine; this results in the

appearance of a background of a small number of colonies after 48 hours.

Some chemicals lead to a substantially increased rate of mutation above this background level. The number of cell colonies observed above background after forty-eight hours is a function of a chemical mutagen's potency and concentration. A dose-response curve can be obtained for a chemical by determining the number of mutations induced at several different concentrations. The linear portion of the dose-response curve is used to determine a chemical's mutagenicity expressed as the number of revertants per microgram (or nanogram) or revertants per nanomole of the compound [15].

Chemicals which exhibit mutagenicity in the Salmonella tester cells upon direct application to the Salmonella are referred to as **direct-acting mutagens** (Figure 1b) while those compounds that require the addition of an exogenous oxidative metabolising system are said to be **indirect-acting mutagens** (Figure 1c). To provide an approximation of the oxidative metabolism available in human livers, a crude enzyme preparation obtained from homogenized rat liver cells, known as S-9 mix, may be added to the growth medium for the Ames test. The term "direct-acting mutagen" as used in the Ames test may cause some confusion since chemicals that react directly with DNA as well as chemicals being activated by

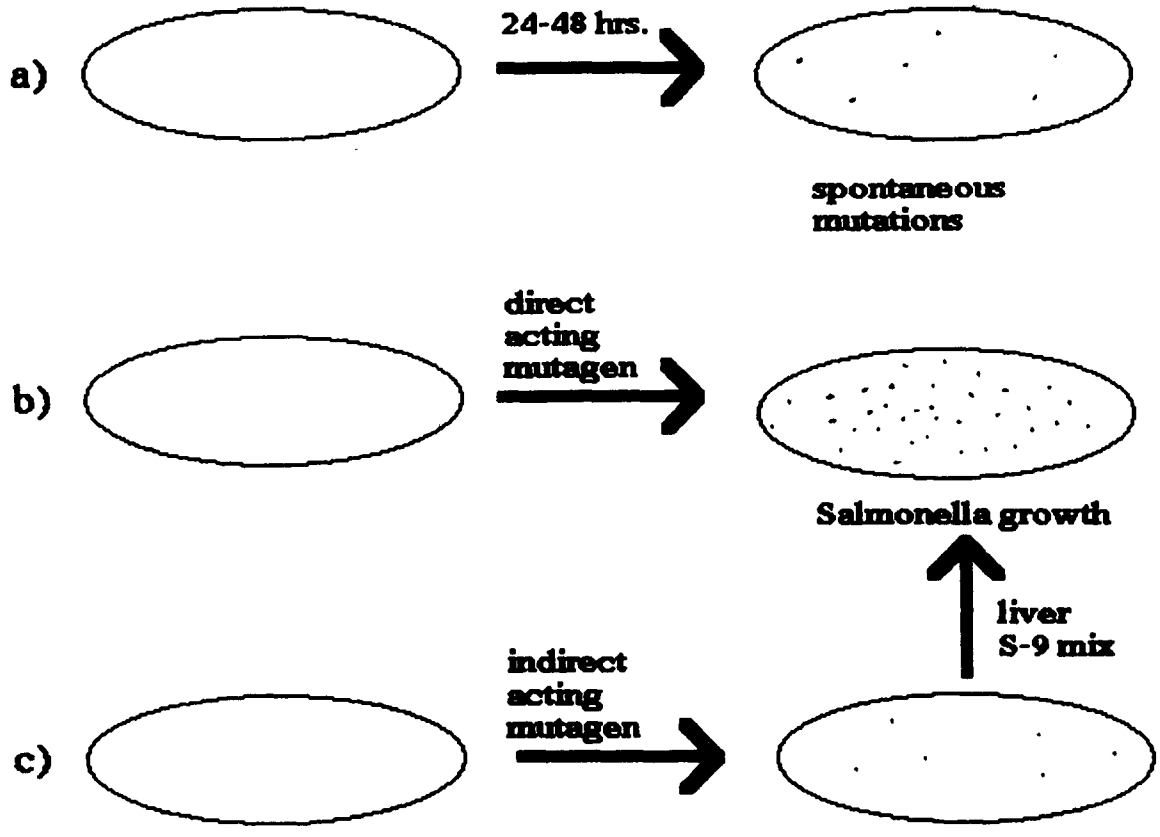


Figure 1: A Representation of the Ames mutagenicity bioassay using Salmonella; a) background mutation levels, b) direct acting mutagens added to system result in colony growth above background levels, c) indirect acting mutagens require activation by exogenous enzymes (S-9 mix) to exhibit mutagenicity.

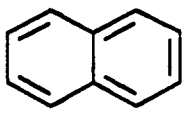
enzymes other than those present in liver S-9 mix are referred to as direct-acting mutagens.

Well over 3000 chemicals have been examined using the Ames test including over 300 chemicals which had been tested for carcinogenicity in animal tests [20]. Over 85% of the positive chemical carcinogens were identified as mutagens [20,21]. This high degree of correlation suggests that the Ames test is a reasonable predictor for many (but not all) chemical carcinogens [21]. As such, the Ames test is recognized as useful but by no means an exclusive screening test for chemicals which may be carcinogenic [21,22].

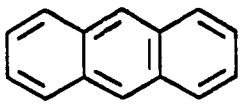
1.3 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) are a class of compounds which consist of two or more fused aromatic rings [23]; examples of these include napthalene 1, anthracene 2, pyrene 3, and benzo(a)pyrene 4 (Figure 2). These compounds are the products of incomplete combustion of organic materials and have been isolated from a wide range of sources including urban airborne particulates [23-25], diesel and automobile exhaust [23,24], coal-fired power plant emissions [26,27], wood stove emissions [27], and tobacco and marijuana smoke [28]. Many of the PAH are demonstrated animal carcinogens [23,25,26,27,29,30,31].

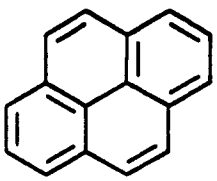
Benzo(a)pyrene (B(a)P) 4 has been studied extensively [30-33] because of its widespread presence in environmental



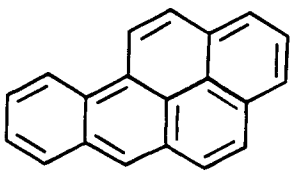
Napthalene 4



Anthracene 2



Pyrene 3



Benzo(a)pyrene 4

Figure 2: Structures of some representative PAH; napthalene 1, anthracene 2, pyrene 3 and benzo(a)pyrene 4.

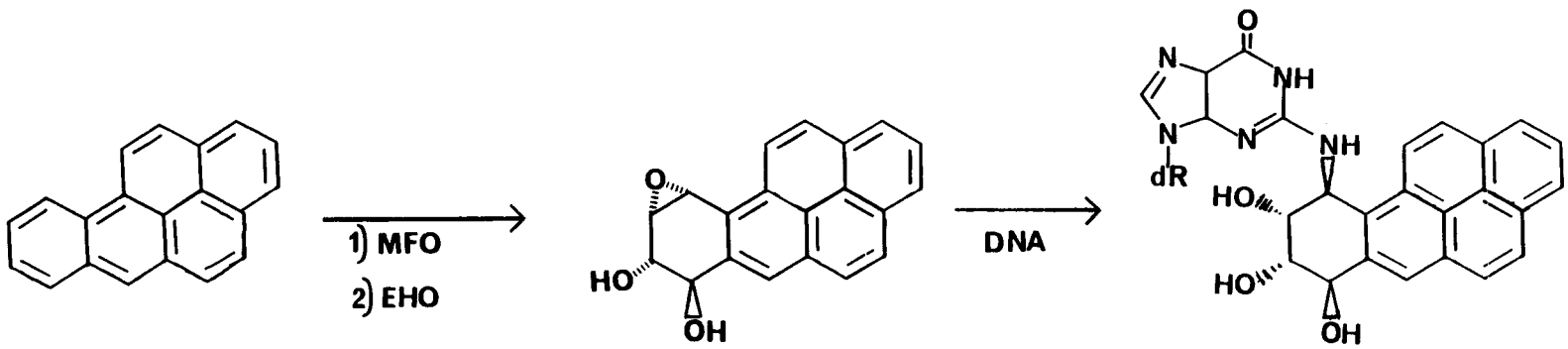
samples and its potency as a carcinogen. In the Ames test B(a)P is a very potent **indirect** mutagen [29,31].

The metabolic pathway established for B(a)P in mammals is shown in Figure 3 [30]. The principal site of reaction for B(a)P with DNA has been shown to be the nucleophilic exocyclic nitrogen atom in the guanine moiety [30-33]. A more detailed discussion on the occurence and metabolism of PAH is beyond the scope of this thesis but is available from other sources[23-33].

1.4 Nitrated Polycyclic Aromatic Hydrocarbons

1.4.1 General

Recent research has shown that the nitrated polycyclic aromatic hydrocarbons (nitro-PAH) are major contributors to the **direct-acting** mutagenicity observed in many environmental samples [34-42]. A number of the nitro-PAH have been identified in extracts from a variety of environmental sources [34-39]. Diesel engine exhausts are a particularly rich source [42-47]. Other sources include gasoline engine exhaust particulates [46], fossil fuelled power plant particulate emissions [42,45], ambient air particulates [48], wood stove emissions [41], cigarette smoke condensates and the carbon black toner formerly used in photocopiers [36]. The environmental sources of nitro-PAH, sampling methods and analytical methodologies for their detection have been reviewed in detail by White [42].



4 5 6

Figure 3: Metabolic pathway for formation of the DNA Adduct 6 from BaP in mammalian livers. A key step is the formation of a specifically configured epoxy diol 5 .

The structures of some typical nitro-PAH are shown in Figure 4. These include; 1-nitronapthalene (1-NN) 7 ,1-nitrofluorene (1-NF) 8, 1-nitropyrene (1-NP) 9, 3-nitrofluoranthene (3-NFA) 10, 6-nitrobenzo(a)pyrene 11 (6-NB(a)P)and 3-nitroperylene (3-NPer) 12.

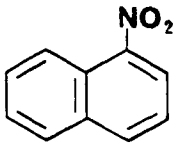
1.4.2 Mutagenicity of Nitro-PAH

The mutagenicity and carcinogenicity of nitro-PAH has been reviewed by Rosenkranz and Mermelstein [40,41]. The majority of nitro-PAH that have been tested in the Ames test are direct-acting mutagens [40]. Of particular significance was the observation that some nitrated pyrenes are the most potent direct-acting bacterial mutagens ever tested [40]. However some nitro-PAH (such as 3-NPer and 6-NB(a)P) exhibit indirect acting mutagenicity [40,41] while others (5-nitroacenapthene (5-NAN)) exhibit both direct- and indirect-acting mutagenicity. The Ames test results obtained for some nitro-PAH are shown in Table 1. All of the nitro-PAH tested thus far except 1-NN have exhibited some carcinogenicity, albeit not at the levels suggested by their high bacterial mutagenicities [40,41].

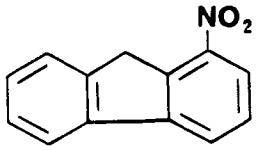
1.4.3 Metabolism of Nitro-PAH

1.4.3.1 Bacteria

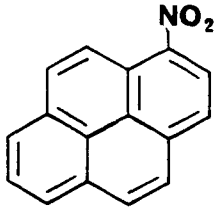
The majority of bacterial studies on nitro-PAH have been performed using the Salmonella typhimurium system [40,41]. In



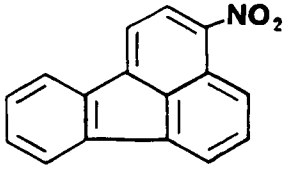
7
1-nitronapthalene



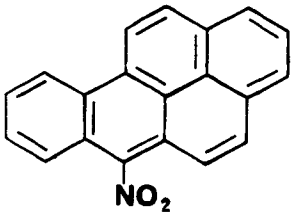
8
1-nitrofluorene



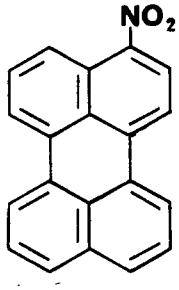
9
1-nitropyrene



10
3-nitrofluoranthene



11
6-nitrobenzo(a)pyrene



12
3-nitroperylene

Figure 4: Structures of some typical Nitro-PAH found in environmental samples; 1-nitronapthalene, 1-nitrofluorene, 1-nitropyrene, 3-nitrofluoranthene, 6-nitrobenzo(a)pyrene and 3-nitroperylene.

Table 1: Mutagenicity of some nitro-PAH determined by the Ames assay using Salmonella typhimurium TA98
(from Rosenkranz and Mermelstein [40,41])

Nitro-PAH	Revertants per nanomole -S9 Mix	+S9 Mix
1-nitronapthalene	n.d.	0.05
2-nitronapthalene	0.01	0.2
1,3-dinitronapthalene	n.d.	0.89
1,5-dinitronapthalene	n.d.	3.3
1,3,6,8-tetranitonapthalene	0.6	0.2
2-nitroanthracene	892	*
9-nitroanthracene	0.8	13.9
2,7-dinitrofluorene	34	471
1-nitropyrene	453	35
1-nitrosopyrene	2130	*
2-nitropyrene	2225	*
1,3-dinitropyrene	144760	4900
1,6-dinitropyrene	183570	37850
1,8-dinitropyrene	254000	75550
1,3,6-trinitropyrene	40700	28330
1,3,6,8-tetranitropyrene	15600	5200
6-nitrobenz(a)pyrene	n.d.	466
3-nitroperylene	1784	<30

notes:
n.d =not detected
*= not available

this system most nitro-PAH behave as direct-acting mutagens. It has been demonstrated that the nitro-PAH do not react directly with the bacterial DNA. Therefore, it is believed that enzymes present in the Salmonella system are converting the nitro-PAH to the biologically active intermediates.

In bacteria, reduction of the nitro group is the major enzymic activation pathway. The enzymes responsible for this enzymic reduction are called nitroreductases. A general pathway for the metabolism of nitro-PAH in bacteria has been proposed (Figure 5) [40,41].

In addition to reduction, some nitro-PAH, for example 3-NPer and 6 NB(a)P, are activated by the exogenous mammalian liver enzymes in S-9 mix to give ring oxidized metabolites [40,41].

1.4.3.2 Mammalian Studies

The mutagenic and genotoxic responses exhibited for the nitro-PAH in mammalian cells can differ from those in bacteria [40,41]. These observations can be accounted for by differences in the transformation to biologically active molecules by the two systems.

Three major metabolic pathways have been recognized for nitro-PAH in mammalian cells [40,41];

i) reduction of the nitro group (Figure 5).

ii) ring oxidation followed by nitro group reduction (a combination of Figures 3 and 5).

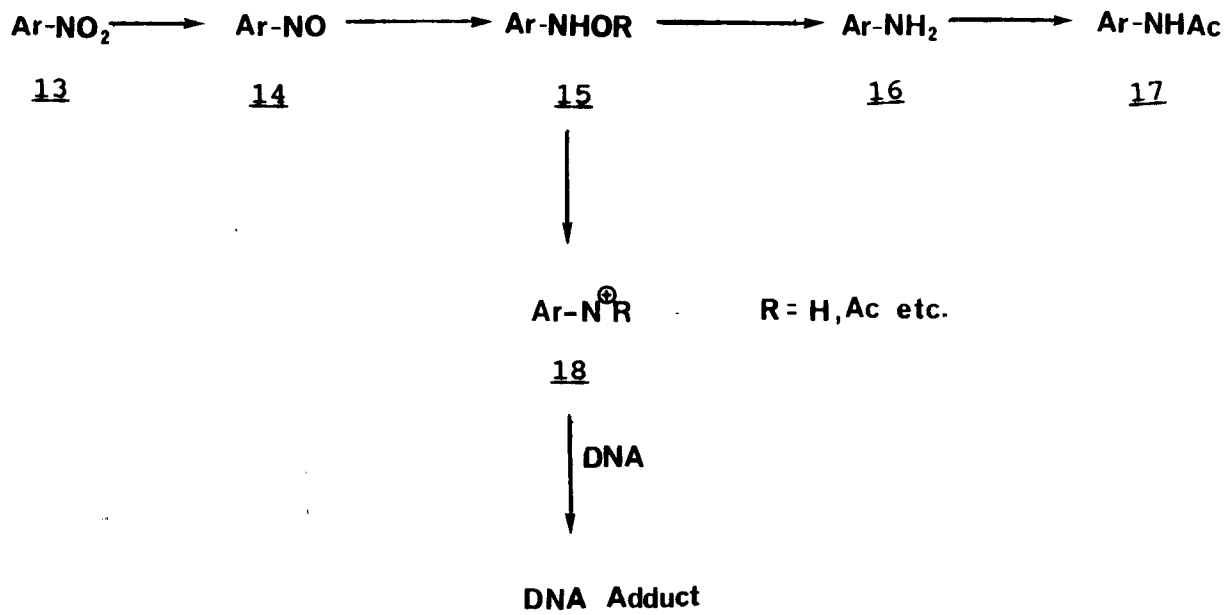


Figure 5: Proposed general metabolic pathway for nitro-PAH in bacterial systems [40,41].

iii) ring oxidation without nitro group reduction, in a manner similar to Figure 3.

1.4.4 Arylhydroxylamines as the Proximate Mutagens in Nitro Group Reductions

For most of the nitro-PAH the experimental evidence points to an arylhydroxylamine 15 (R=H) (N-hydroxy arylamine, hydroxylamino-PAH, N-hydroxy-amino-PAH) or the ester of 15 (R= acetyl etc.) as the reactive proximate mutagen [49,50]. The evidence for this includes the following :

i) in strains of bacteria which are deficient in nitroreductases most nitro-PAH exhibit diminished mutagenicity. In the same bacterial strains, the corresponding arylhydroxylamines display full mutagenicity.

ii) the nitro and nitroso derivatives of many nitro- PAH show substantially higher mutagenicities under anaerobic conditions (under aerobic conditions the arylhydroxylamine may be quickly reoxidized to the nitro or nitroso compound).

iii) none of the nitro, nitroso, amino or N- acetylamino-PAH react directly with DNA whereas the arylhydroxylamines do.

iv) the DNA adducts formed from the nitro-PAH are very similar to those formed from the amino-PAH, suggesting the involvement of a common intermediate for the different classes of compounds [40,41,49].

v) The mutagenicity of arylhydroxylamines and their derivatives has been established from research conducted into the metabolism of carcinogenic aromatic amines [40,49,50].

1.4.5 Chemistry of Arylhydroxlamines

Evidence has been presented above which indicates that arylhydroxylamines derived from nitro-PAH are the actual compounds responsible for the mutagenicity exhibited by nitro-PAH. In view of this, it is appropriate at this stage to review the chemistry of arylhydroxylamines. This section contains a brief review of the synthesis and reactions of arylhydroxylamines. No attempt has been made to cover the synthesis or reactions of their alkylhydroxylamine counterparts. A more extensive coverage of the chemistry of arylhydroxylamines and alkylhydroxylamines may be found in the reviews of Roberts and Patai [51,52]. The biochemistry of arylhydroxylamines has been reviewed in detail by Weisburger and Weisburger [49].

1.4.5.1 Synthesis

In arylhydroxylamines, the nitrogen atom is at an oxidation level intermediate between that of nitroso and amino groups (see Figure 6). Consequently, preparative methods

usually involve;

1) the **reduction** of nitro or nitroso compounds [51-59], or

2) the **oxidation** of amino compounds [51,52].

Biochemically, arylhydroxylamines are formed in a similar manner by **enzymic reduction** (i.e. nitroreductases) or **enzymic oxidation** (i.e. amineoxidases) of appropriate precursors [49,50].

By far the most common and satisfactory method of preparing arylhydroxylamines is <u>via</u> the reduction of the nitrogen atom in a compound at a higher oxidation level [50,51]. A number of reductive methods have been utilized including; metal reductions [54], catalytic hydrogenation [55,56], Grignard reagents [52], sodium- or ammonium-hydrogen sulfide [51,52], ascorbic acid [58], and electrochemical reduction [59]. The yields from these reductions are variable.

The most commonly used oxidative method for preparing arylhydroxylamines is the treatment of a primary aromatic amine with a reagent that donates an oxygen to the amine group (e.g. m-chloroperoxybenzoic acid). However, under the reaction conditions used, the arylhydroxylamines are often further oxidized [51,52].

The synthetic methods which have been utilized to synthesize arylhydroxylamines are outlined in <u>Figure 7</u>.

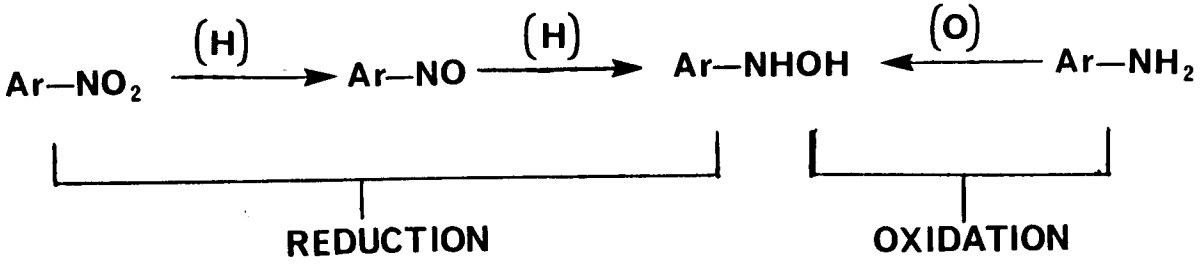


Figure 6: General synthetic strategy employed for the preparation of arylhydroxylamines.

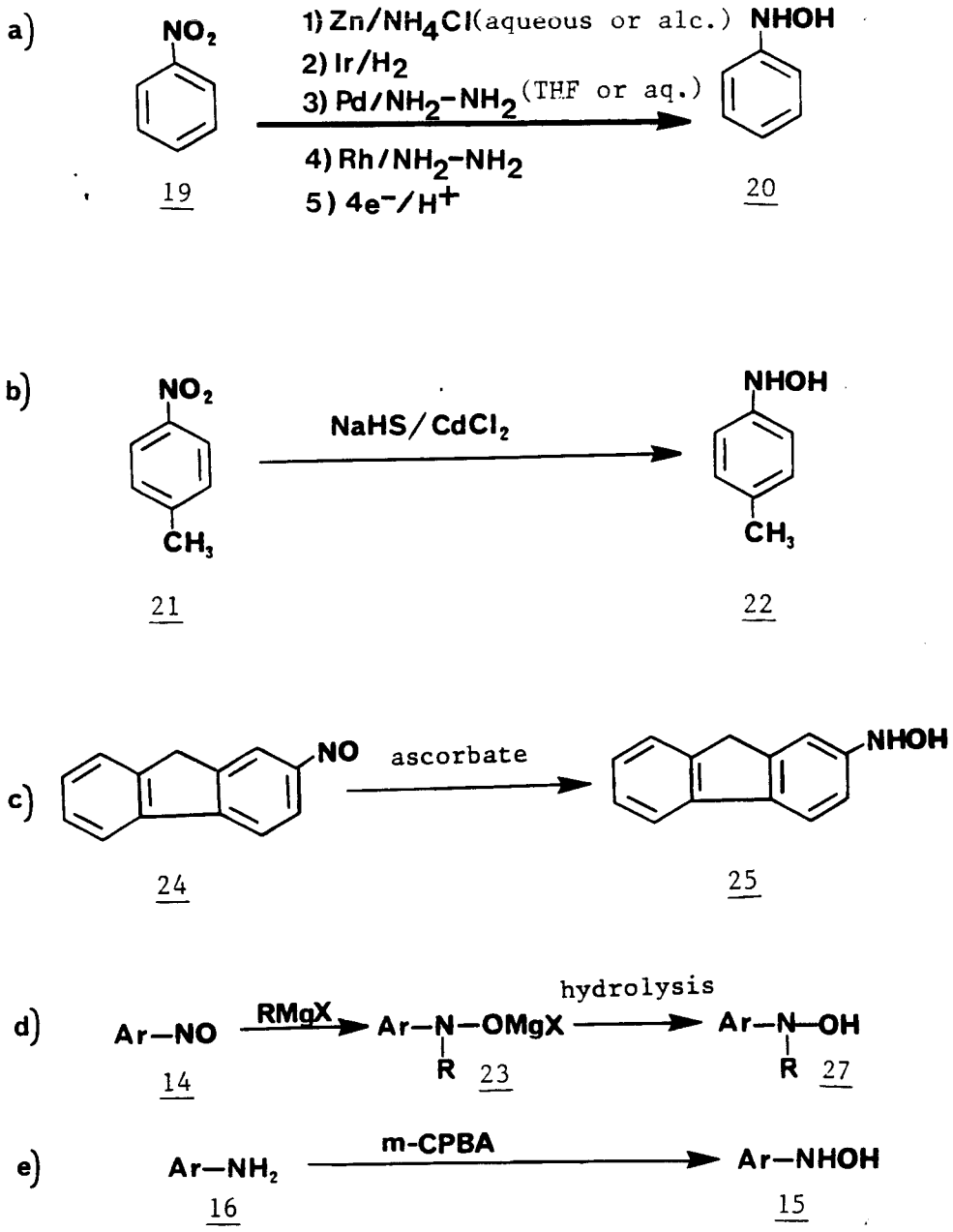


Figure 7: Summary of the synthetic methods which have been used for preparation of arylhydroxylamines, 7(a)- 7(d)= reductions, 7(e)=oxidation.

1.4.5.2 Reactions of Arylhydroxylamines

Arylhydroxylamines readily undergo oxidation, reduction, disproportionation, condensation and rearrangement reactions. They may also react with certain derivatizing reagents [51] (see Figure 8).

Under aerobic conditions, arylhydrxoylamines readily undergo oxidation to give the corresponding nitro and/or nitroso compounds [51]. Arylhydroxylamines may be oxidized to the nitroso compound by mild oxidizing agents such as ferric ammonium sulfate or potassium ferricyanide [51]. In the presence of reducing agents arylhydroxylamines are easily reduced to the corresponding aromatic amine [49,51]. In addition to being easily oxidized or reduced, arylhydroxylamines can undergo a disproportionation reaction whereby two arylhydroxylamine molecules react to give an amine and a nitroso compound [51,56] (this reaction is also called an autoxidation). The amino and nitroso compounds formed may then undergo a condensation reaction with each other to give an azo compound, while an arylhydroxylamine can react readily with a nitroso compound to give an azoxy compound [51]. In protic solvents and solutions containing traces of acid, arylhydroxylamines may undergo the Bamberger rearrangement to ortho- and para- hydroxy,amino-compounds [59,60].

Arylhydroxylamines may react with a variety of esterifying [61] and silylating reagents [61,62] to give

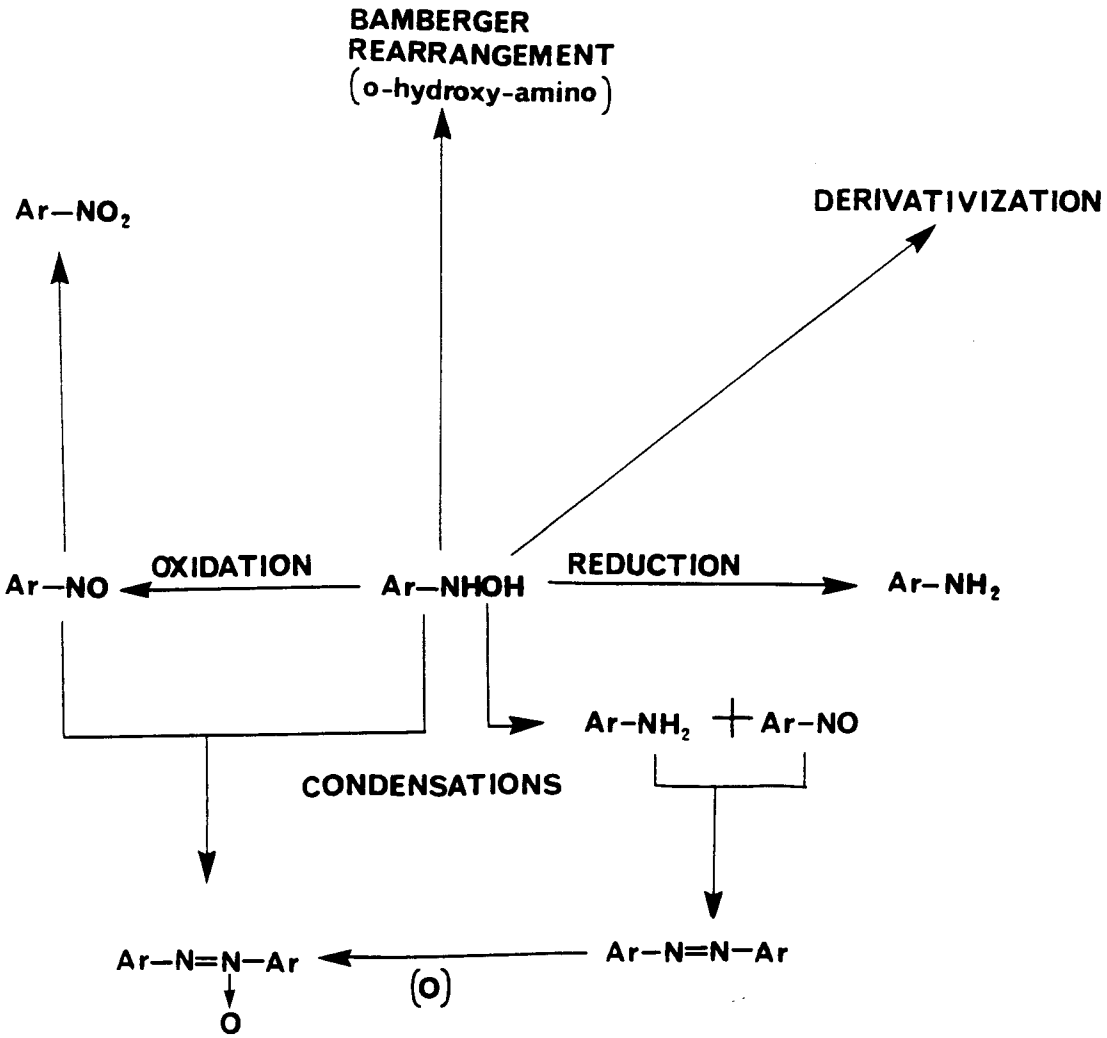


Figure 8: Summary of the reactions of arylhydroxylamines

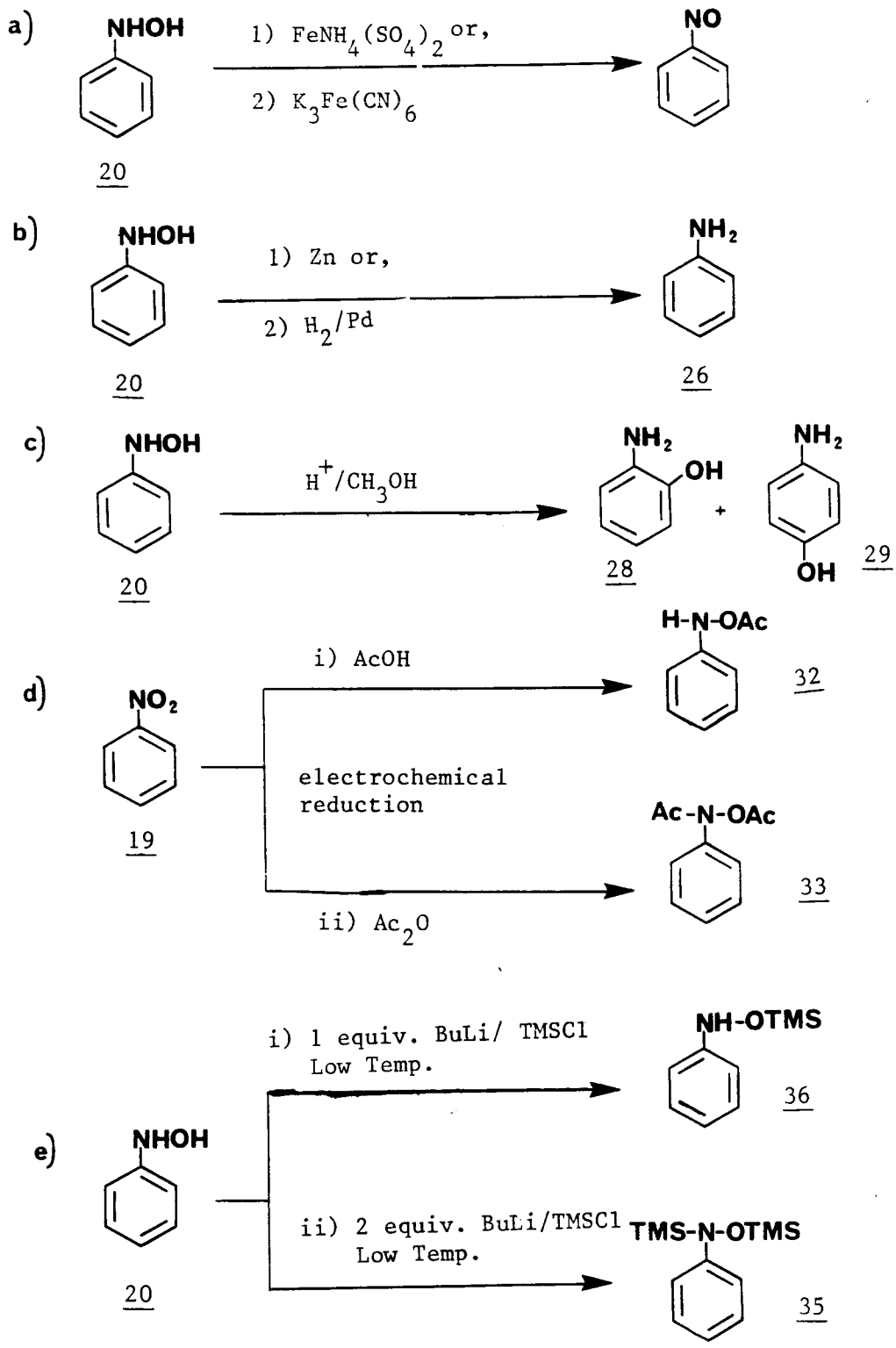


Figure 9: Examples of reactions of which have been observed for arylhydroxylamines

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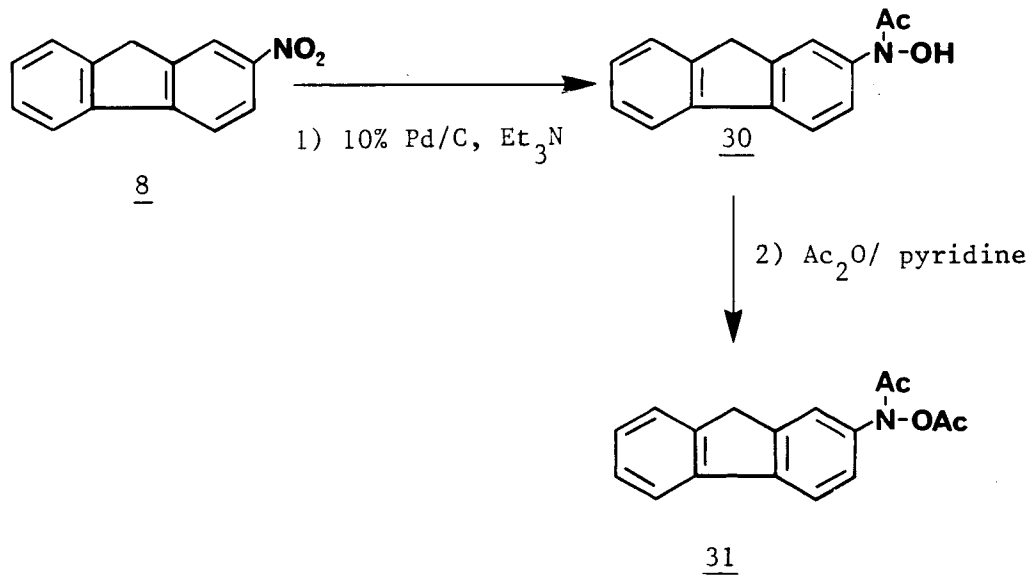
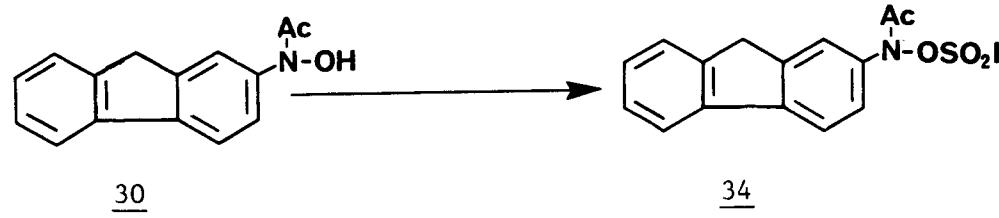


8 30 31

g)



30 34

h) $2\,(Ar-NHOH) \longrightarrow Ar-NO + Ar-NH_2 + H_2O$

Figure 9 (continued)

derivatives which are often even more reactive than the parent arylhydroxylamine [51].

The N-acetyl and/or O-acetyl derivatives of arylhydroxylamines have been prepared by several methods, for example;

i) electrochemical reduction of nitrobenzene 19 in the presence of an acetylating or alkylating agent afforded the N-acetyl- and N,O-bis acetyl or alkyl compound (Figure 9d).

ii) controlled catalytic reduction of 2-nitrofluorene 8 in the presence of acetic anhydride followed by treatment with acetic anhydride in pyridine [61] (Figure 9f).

The sulfate esters of many arylhydroxylamines are very reactive compounds. The sulfate ester 34 was formed by the reaction of N-acetyl-N-hydroxy-2-aminofluorene 30 with sulfuric acid in dimethylformamide (DMF) using dicyclohexylcarbodiimide (DCC) as a dehydrating agent [65].

Silylation is a common method for the protection of functional groups in organic synthesis. However, the silylation of arylhydroxylamines was found to require special conditions. Under these special conditions (low temperature, treatment with one or two equivalents of butyllithium (BuLi) the mono- and bis-trimethylsilyl derivatives 35 and 36 were formed after adding trimethylsilyl chloride to PhNHOH 20 [66].

The reactions outlined above are summarized in Figure 9.

Biochemically, hydroxylamines undergo a variety of reactions which are similar to those above. The biochemical reactions of arylhydroxylamines have been reviewed in greater detail elsewhere [49,67].

1.4.5.3 Nature of the Ultimate Mutagen

The exact mechanism of the reaction between arylhydroxylamines and DNA is currently not fully understood. Many researchers have suggested that the ultimate mutagen involved may be similar to an electron-deficient nitrenium ion 18 derived from 15 (see Figure 5) [12,68-71].

A number of theoretical and chemical studies have indicated the involvement of an electrophilic "nitrenium ion-like" species, however, it is not certain if a discrete, reactive intermediate or a transition state species is involved. For example, the mechanism proposed to account for the formation of the DNA adduct 37 from the sulfate ester of 30 has been postulated by some researchers to involve the formation of a nitrenium ion (Figure 10) [13]. However, no direct evidence was presented for the existence of a nitrenium ion under physiological conditions.

1.4.6 Structure-Activity Relationships

Many studies have been undertaken in an attempt to ascertain if a structure-activity relationship exists for nitro-PAH [40,41,72,73].

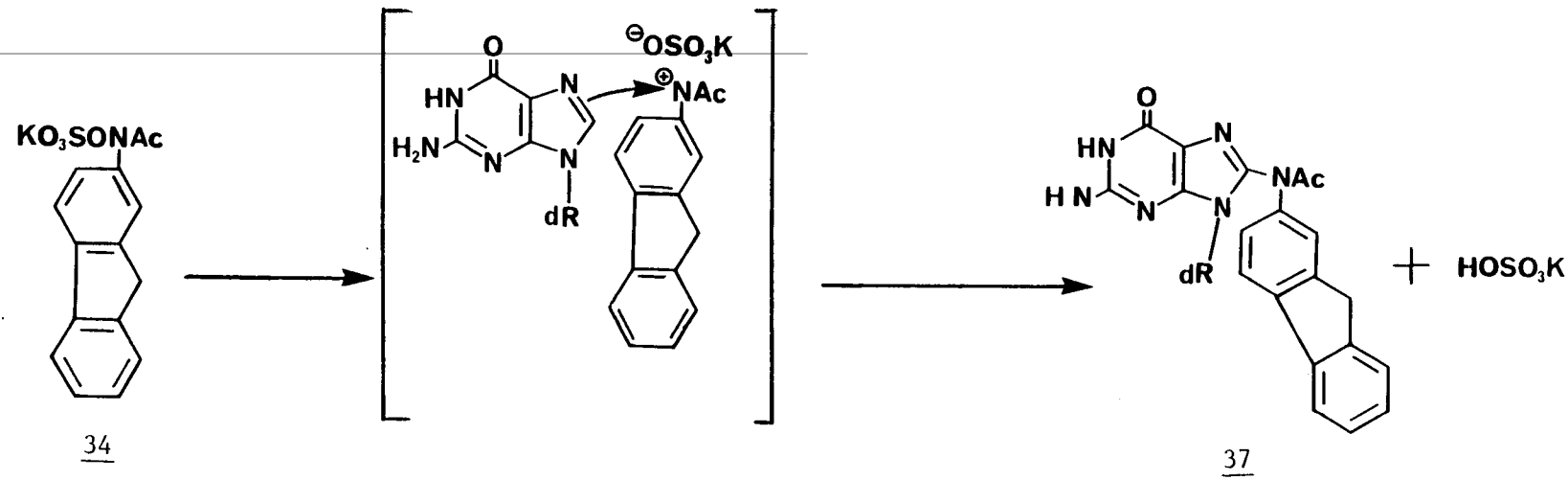


Figure 10: Mechanism proposed by some researchers [13] for the formation of the DNA 37 adduct from the sulfate ester 34 derived from 2-HAAF.

In general, the bacterial mutagenicity of the nitro-PAH has been found to be dependent upon the following factors:

- the number of fused aromatic rings
- the position of the nitro group
- the extent of nitration
- the conformation of the nitro group
- the energy difference between the lowest unoccupied molecular orbital (LUMO) and highest occupied molecular orbital (HOMO).

1.5 The Nitropyrenes

Amongst the most abundant of the nitro-PAH found in environmental samples is 1-nitropyrene(1-NP) 9 [40,41,74-77]. The dinitropyrenes, 1,3-dinitropyrene (1,3-DNP) 38, 1,6-dinitropyrene (1,6-DNP) 39 and 1,8-dinitropyrene (1,8-DNP) 40 are present to a much smaller extent than 1-NP but these compounds are extremely powerful mutagens [40,41,74-79] (see Table 1). These compounds have also been shown to be mammalian carcinogens [79-83].

Previous work by others in this laboratory has shown that when 1-NP is incubated anaerobically in Salmonella typhimurium TA98, it undergoes metabolic reduction resulting in the formation of 1-aminopyrene (1-AP) 43, N-acetyl-1-aminopyrene (1-AAP) 44 and a covalent DNA adduct [83,84]. Subsequently, Howard et al successfully determined the structure of the major adduct as the C-8 guanine derivative 45 [77,85]. The proposed metabolic pathway for 1-NP in Salmonella is shown in Figure 11 [86].

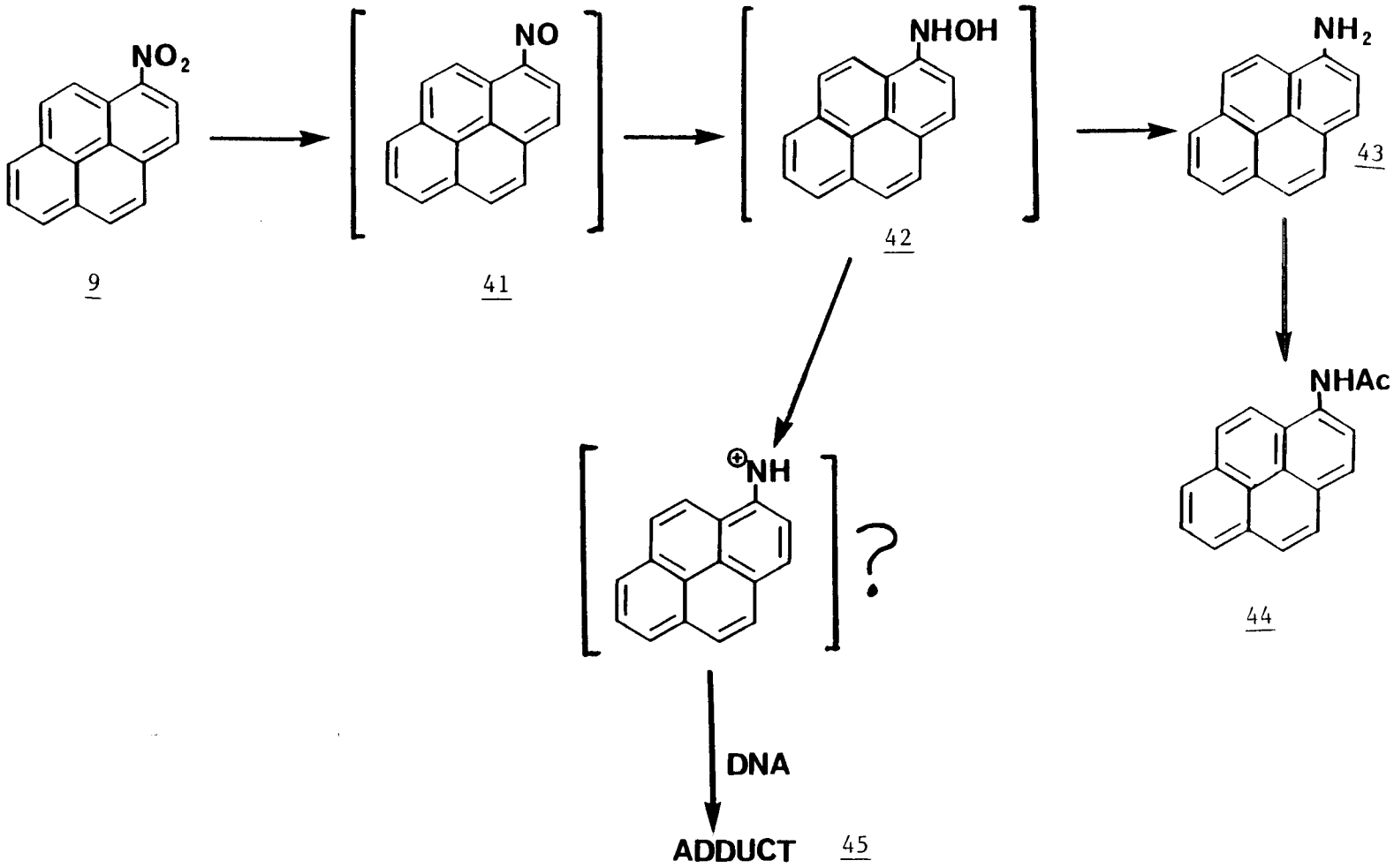
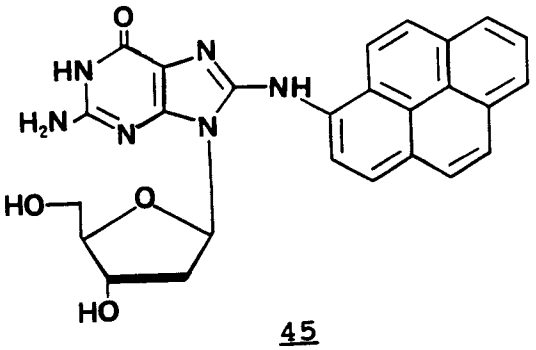


Figure 11:Proposed Metabolic Pathway for 1-NP in Salmonella typhimurium TA98.



45

DNA ADDUCT of 1-NP

Supporting evidence for this metabolic pathway is provided by the observation that when 1-nitrosopyrene (1-NOP) 41 was chemically reduced with ascorbic acid and then reacted with calf thymus DNA *in vitro* a DNA adduct was formed. This DNA adduct was shown to be identical to the *in vivo* adduct 45 by high performance liquid chromatography (HPLC) [77].

During these series of experiments it was found that none of 1-NP, 1-NOP, 1-AP or 1-AAP react directly with DNA [77,83,85,86]. In addition, in the Ames assay 1-AP is an indirect-acting mutagen and requires oxidative activation of the nitrogen atom to become mutagenic [83]. These results indicated that the proximate mutagen formed from the metabolic reduction of 1-NP had the nitrogen atom at the oxidation level of a hydroxylamine. For 1-NP this would correspond to the N-hydroxy-1-aminopyrene (1-HAP) 42.

Initial studies [78,80,87] on the metabolism of 1,8-DNP in *Salmonella typhimurium* TA98 showed a pattern of metabolic reduction similar to that observed for 1-NP; thus, in *Salmonella* 1,8-DNP was found to undergo reduction and covalent binding to DNA. When 1-Nitro-8-nitrosopyrene (1,8-NONP) 46 was chemically reduced with ascorbic acid and reacted with calf thymus DNA *in vitro*, a DNA adduct was isolated [79]. The major DNA adduct isolated from the *in vivo* and *in vitro* experiments was identified as N-(deoxyguanosin-8-yl)-1-amino-8-nitropyrene 47 (Figure 12) [79]. This adduct was originally thought to be

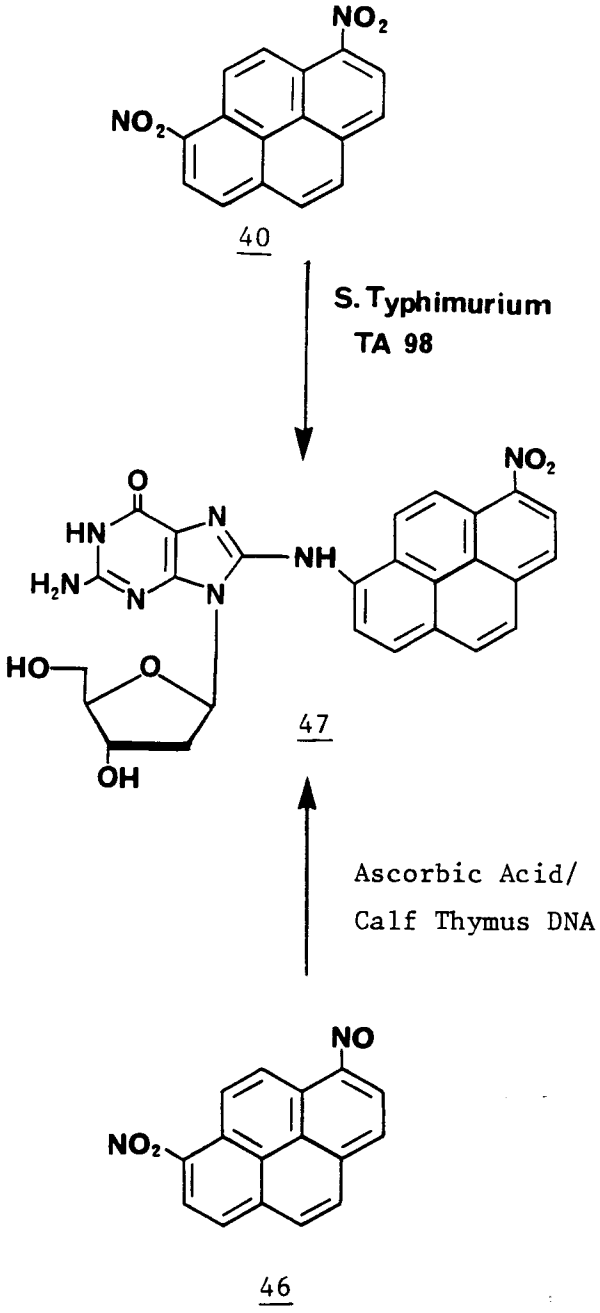
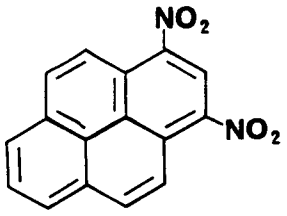
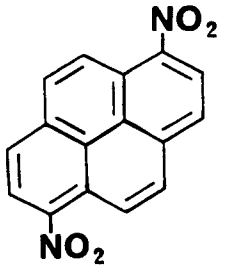


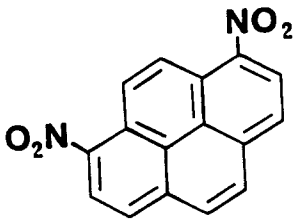
Figure 12: Identity of the DNA adduct (47) formed from in vivo and in vitro experiments with 1,8-DNP.



1,3-DNP 38



1,6-DNP 39



1,8-DNP 40

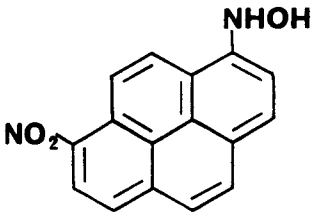
formed by the reaction of N-hydroxy-1-amino-8-nitropyrene 48 (1,8-HANP) with DNA. However, subsequent investigations [87,88,89] have shown that the metabolic pathway differs somewhat from that of 1-NP and that both enzymic **reduction** and **acetylation** are required for mutagenicity to be expressed [78,41,87-90]. Thus, for 1,8-DNP the proximate mutagen is considered to be the O-acetylated compound 49 (1,8-OAc-HANP) derived from the 1,8-HANP. Similar results have since been obtained for the 1,3- and 1,6-DNP compounds[90,91].

1.6 Research Objectives

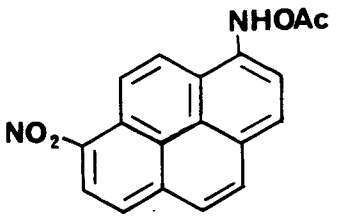
The results of Howard et al [77,85] suggested that the proximate mutagen derived from 1-NP was N-hydroxy-1-aminopyrene (1-HAP) 42 . However, the only evidence presented by the authors for this compound's existence was a high performance liquid chromatography (HPLC) chromatogram of a chemically reduced 1-nitrosopyrene (1-NOP) solution; no further characterization was performed on this compound. Research in this thesis was undertaken to provide more substantial evidence for the existence of 1-HAP.

The specific goals of the project were as follows:

1. To prepare and characterize the N-hydroxy-1-aminopyrene.
2. To study the stability of the N-hydroxy-1-aminopyrene in various solvents and at different pH values.



1,8-HANP 48



1,8-HAANP 49

3. To study the reaction of N-hydroxy-1-aminopyrene directly with nucleosides in order to ascertain the importance of the DNA double helix structure to adduct formation.

4. To prepare a stable derivative of N-hydroxy-1-aminopyrene in order to provide a more readily available source of the compound for chemical and biological testing.

5. To acquire and interpret chemical and spectroscopic data for the mono- and dinitropyrenes and related compounds.

Ultimately this study was an attempt to correlate the chemistry of these proposed metabolites with their observed biological behaviour.

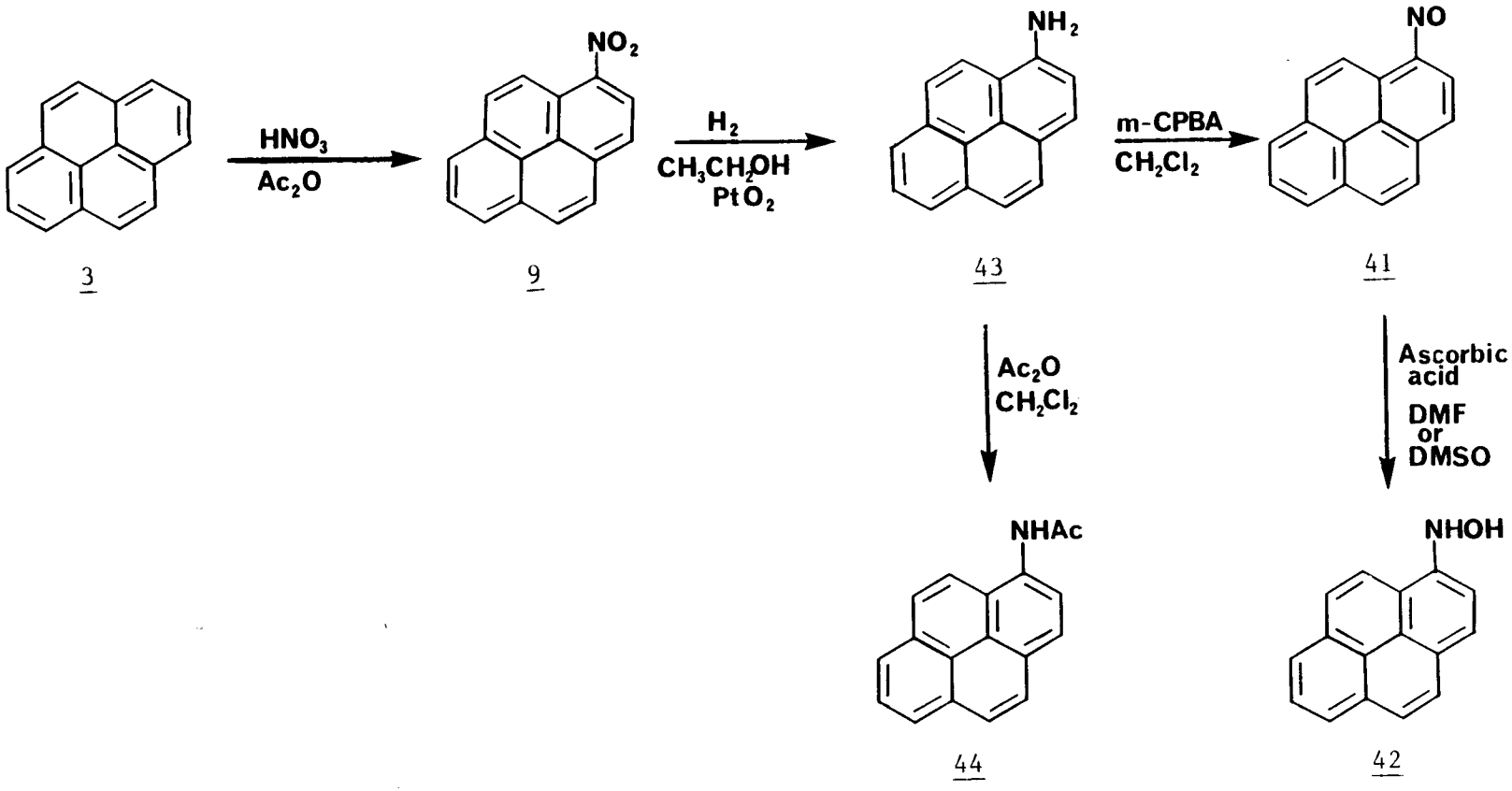
2 RESULTS AND DISCUSSION

At McMaster University a multi-disciplinary approach to the study of nitro-PAH was established involving collaboration between members of the Chemistry and Biochemistry Departments. The synthesis and characterization of compounds used for mutagenicity studies was supervised by Dr. B.E. McCarry (Chemistry Department). The analytical methodologies used were developed under the supervision of Dr. M.A. Quilliam (Chemistry Department). Mutagenicity studies were performed by the research group of Dr. D.R. McCalla (Biochemistry Department).

2.1 SYNTHESES

2.1.1 Synthesis of 1-Nitropyrene and Related Compounds

The synthetic scheme that was followed for the preparation of 1-NP and its identified metabolites is shown in Figure 13. The nitration of pyrene 3, was carried out according to the method of Bavin and Dewar [92,93] and afforded pure 1-NP 9. Hydrogenation of 9 over Adam's catalyst (platinum (IV) oxide) or Pd/C in the manner of Messier et al [83] provided a quantitative yield of 1-AP 43. N-Acetyl-1-aminopyrene (1-AAP) 44 was prepared in > 99% yield by the acetylation of 1-AP with acetic anhydride in dichloromethane (CH_2Cl_2). The oxidation of 1-AP with m-chloroperoxybenzoic acid (m-CPBA) resulted in complete



3 9 43 41 44 42

Figure 13: Synthetic scheme followed for preparation of mono-substituted N-Pyrene Compounds.

oxidation and typically gave a ratio 9:1, 1-NOP(41):1-NP(9) by Reverse Phase High Performance Liquid Chromatography (RP-HPLC) analysis [85]. The 1-NOP was purified before further use by preparative liquid chromatography (prep-LC) on a reverse phase (RP) "Lobar" column. Reverse phase HPLC analysis indicated that the purity of the 1-NOP was now >99.5 %.

Ascorbic acid, a mild reducing agent is known to reduce some nitro- and nitrosoarenes to their N-hydroxy derivatives [58]. Howard et al reported that when a solution of 1-NOP in DMF was treated with ascorbic acid a new peak with a retention time (t_R) shorter than that of 1-AP was observed in the RP-HPLC chromatogram [77,85]. This compound was tentatively identified by them as 1-HAP (42). However, they were unable to obtain a pure sample of 42 for further characterization.

When this experiment was repeated in our laboratory, a similar result was observed by RP-HPLC. Upon the addition of ascorbic acid, the 1-NOP solution immediately decolourized and began to fluoresce blue-green under long wave ultraviolet (UV) light. The ultraviolet-visible (UV-VIS) spectrum of the 1-NOP solution (λ_{max} = 316,468 nm) changed dramatically upon the addition of ascorbic acid to give a spectrum similar in appearance to 1-AP (λ_{max} = 369 nm) but with a λ_{max} = 361 nm. The fluorescence spectrum of the chemically reduced solution had an emission maximum of 482 nm

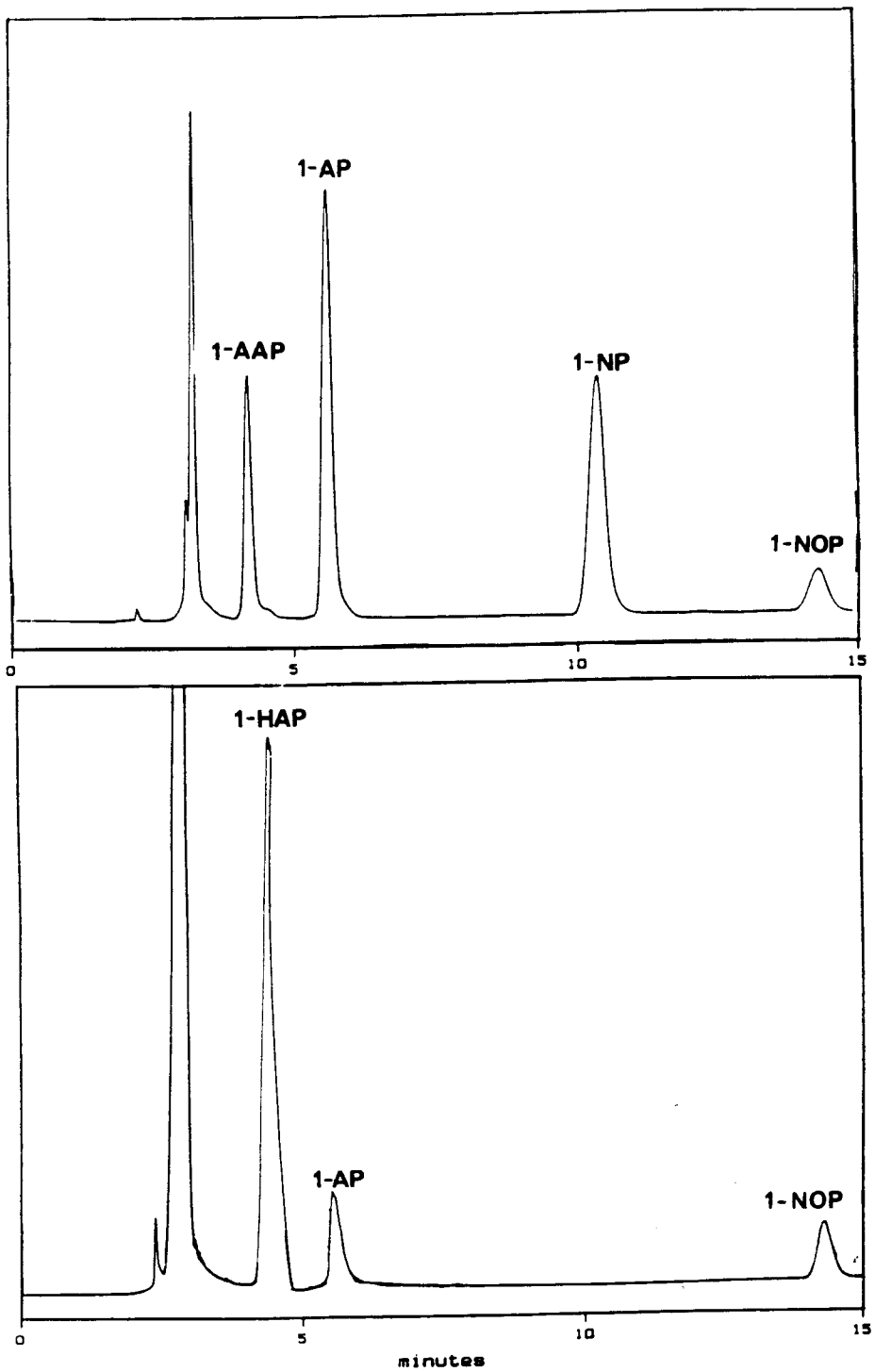


Figure 14: RP-HPLC chromatograms for analyses of a) N-pyrene standards and b) chemically reduced 1-NOP solution. Conditions; 70 % ACN/H_2O, 2 ML/min., 10 um C_{18} column, 4 mmX 25 cm.

(excitation λ = 361 nm) compared to 1-AP which had an emission maximum of 432 nm (excitation λ = 369 nm). Under these same conditions 1-NOP was not fluorescent. Analysis of the reduction solution by RP-HPLC showed 1-AP, 1-NOP and a new, more polar compound (that was suspected to be 1-HAP), typically present in a ratio of 5:10:85 (Figure 14). The use of a HP 1090 liquid chromatograph equipped with a diode array detector (DAD) with a low volume cell allowed the UV-VIS spectra of peaks to be obtained directly as they eluted from the HPLC column. The polar peak (k'=0.97) that had been tentatively assigned to 1-HAP had a UV-VIS spectrum similar to 1-AP) but with λ_{max} at a shorter wavelength (Figure 15).

The peak with k'= 0.97 was collected and its fluorescence spectrum quickly obtained. The emission maximum was found to be at 467 nm ($\lambda_{excitation}$=351 nm) compared to 1-AP which had an emission maximum at 431 nm under similiar conditions ($\lambda_{excitation}$=359 nm) (Figure 16). Identical reaction results were obtained with dimethyl sulfoxide (DMSO) as the solvent. However in acetonitrile (ACN) or methanol (MeOH) complete reduction to 1-AP occurred rapidly, no trace of 1-HAP was observed by HPLC. When the peak with k'=0.97 was collected and reanalysed by RP-HPLC two peaks with t_R's identical to 1-AP and 1-NOP were observed, no peak with k'=0.97 was observed.

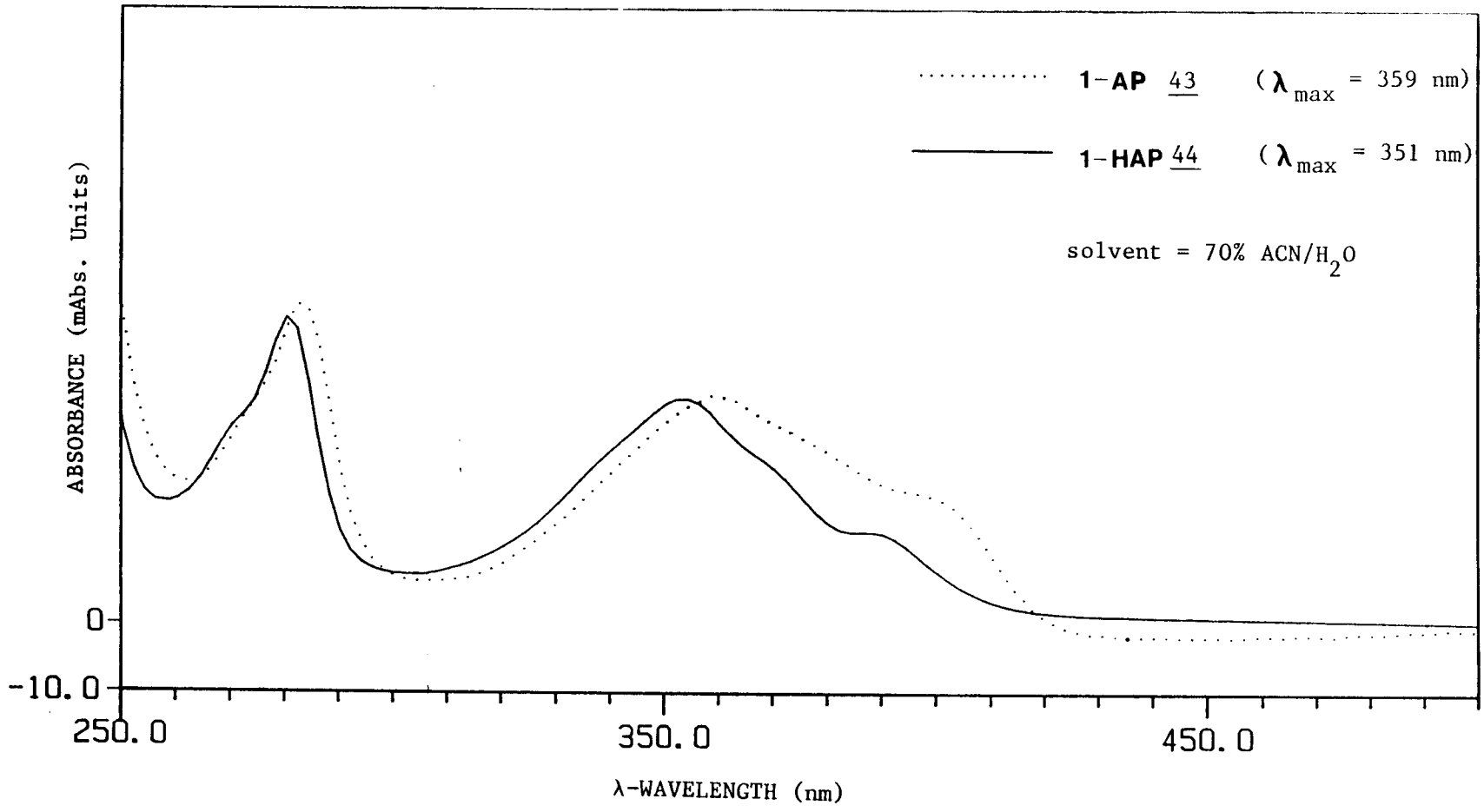


Figure 15: UV-VIS Spectra obtained from RP-HPLC analysis of a chemically reduced 1-NOP solution;a) 1-AP peak and b) peak at k'=0.97 (1-HAP?).

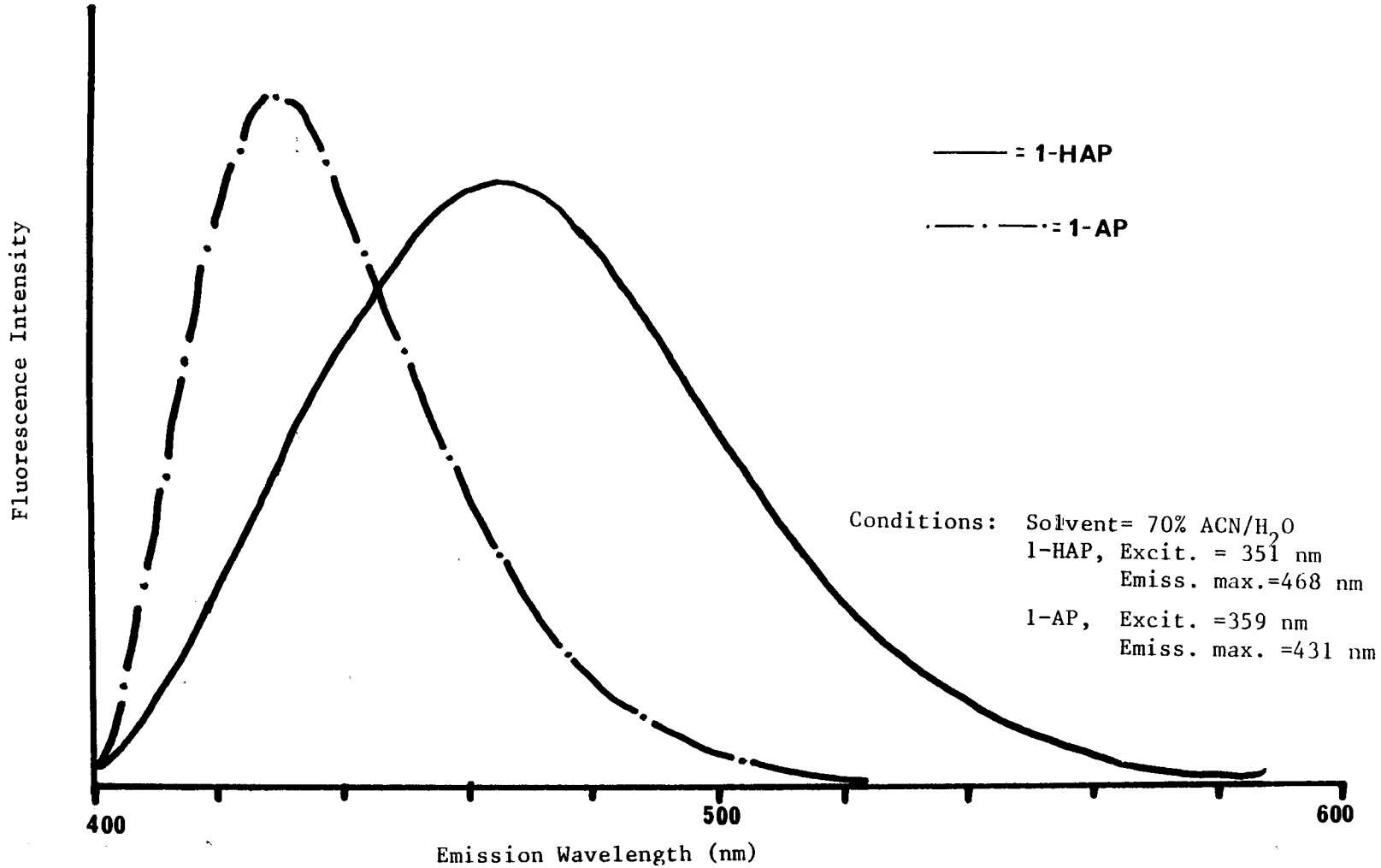


Figure 16: Fluorescence Spectra obtained on 1-AP and 1-HAP peaks from the RP-HPLC analysis (Figure 15b) of a chemically reduced 1-NOP solution.

The chromatographic and spectroscopic data for the compounds shown in Figure 13 are summarized in Table 2 below.

The above results indicated that a new compound was formed in the ascorbic acid reduction of 1-NOP; this compound had an excitation spectrum that was similar to 1-AP, however, the emission spectrum of this compound was significantly different from 1-AP. A similar pattern of spectroscopic properties has been observed for 2-AF 50 and 2-HAF 25 [94].

Since some of the compounds encountered in this research were found to be thermally unstable, RP-HPLC was used for all analyses.

2.1.2 Ascorbic acid Reduction of 1-NOP

The effect of varying the quantity of ascorbic acid used for the reduction of 1-NOP was examined. It was found that if less than two equivalents of ascorbic acid were used, the reduction was not complete and some 1-NOP remained unreduced. However, if two or more equivalents of ascorbic acid were used, the reduction was essentially complete. The reaction appeared to be essentially complete within 10 seconds as monitored by long-wave UV fluorescence. This result suggested that the two electron reduction of 1-NOP to 1-HAP requires two, one-electron transfers. When an ascorbic acid-reduced solution of 1-NOP was studied by electron spin resonance (ESR), no sign of a radical intermediate was observed.

Table 2: Chromatographic and Spectrocopic Data for 1-Nitropyrene and Related Compounds

Compound	Solvent System	k'	λ_{max} (nm)	fluorescence emission λ, (excitation λ)
1-NP	DMF	**	***	not fluorescent
9	70%ACN/H_2O	3.0	290, 378, 404	" "
1-NOP	DMF	**	305, 464	not fluorescent
41	70%ACN/H_2O	5.74	316, 468	" "
1-HAP	DMF	**	361	482, (361)
42	70%ACN/H_2O	0.97	352	467,(352)
1-AP	DMF	**	369	434, (369)
43	70% ACN/H_2O	1.64	359	431, (359)
1-AAP	DMF	**	**	****
44	70% ACN/H_2O	1.05	342	****

Notes: $k'=(t_r-t_o)/t_o$
all data for RP-HPLC was obtained on column D
**= not applicable
***= not measured

2.1.3 Synthesis of Dinitropyrenes and Derivatives

Although the dinitropyrenes (DNP) form a smaller percentage of nitro-PAH emissions they are much more mutagenic. Work at McMaster University [87,89,90] and elsewhere [78] has shown that the mutagenic pathway of 1,8-DNP is different from 1-NP and involves both reduction of the nitro group and O-acetylation of the resulting hydroxylamine. Since many of the DNP metabolites had not been fully characterized, a portion of the work undertaken in this research project involved the synthesis and purification of samples of DNP isomers and related compounds for characterization by UV-VIS, MS etc. The synthetic scheme followed for the preparation of the dinitropyrenes and their derivatives is shown in Figure 17 [78,79].

The nitration of 1-NP with 50 mole equivalents of HNO_3 yielded a mixture of the isomeric 1,3-,1,6- and 1,8-dinitropyrenes (DNP's) (38, 39 and 40). A mixture of the isomeric amino,nitropyrenes (ANP's) 1,3-(51), 1,6-(52) and 1,8-(53) were synthesized by one of two routes;

i) the Zinin polysulfide [95] reduction of a 1,3; 1,6; 1,8-DNP mixture or

ii) basic hydrolysis of a mixture of 1,6- and 1,8- N-acetylamino-nitropyrenes (AANP) 54 and 55.

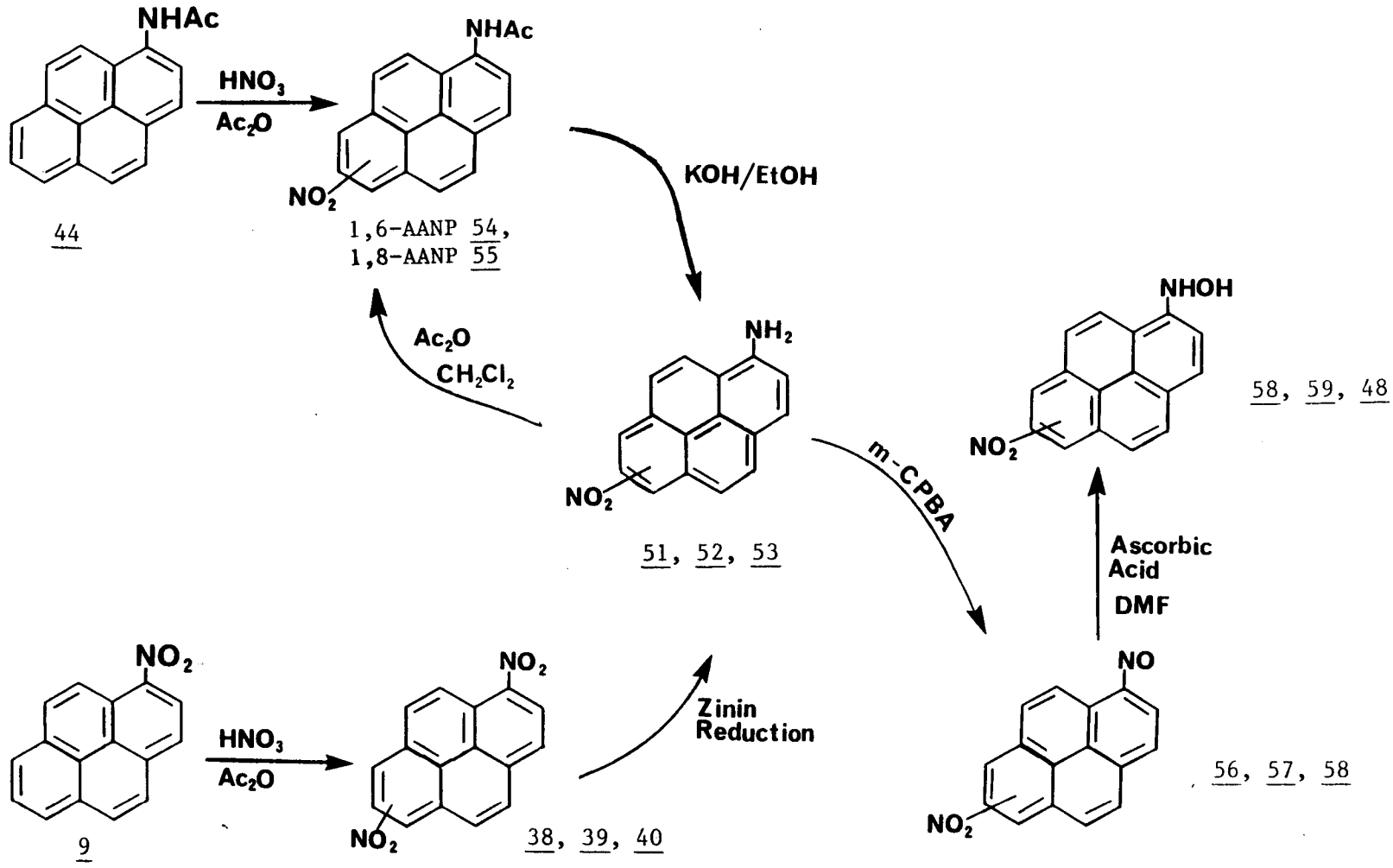


Figure 17: Synthetic scheme followed for the preparation of dinitropyrenes (DNP's) and related compounds.

The isomeric ANP were then separated by column chromatography using activity II alumina as the stationary phase. The activity grade of the alumina proved to be very important for optimized separation of the ANP isomers. Oxidation of an isomerically pure ANP afforded a mixture of the nitro,nitrosopyrene (NONP) and dinitropyrene (DNP). The NONP was purified before further use, in a manner analogous to that used for the 1-NOP, by prep-LC on a "Lobar" column. The reduction of a solution of NONP in DMF or DMSO required a minimum of 5 equivalents of ascorbic acid to cause an immediate change in the colour of the solution. Analysis of a 1,6-NONP 57 reduction solution by HPLC showed a new compound to be present that eluted before the 1,6-ANP. The UV-VIS spectrum for this peak was different from the 1,6-ANP 52. This new compound was tentatively identified as the N-hydroxy-1-amino-6-nitropyrene 59 (1,6-HANP). None of the solutions containing compounds related to DNP were fluorescent, most probably due to quenching by the nitro group. The UV-VIS and RP-HPLC data for these compounds are summarized in Table 3.

Table 3: Chromatographic and Spectroscopic Data for 1,6- and 1,8- substitued N-Pyrenes

Compound	Solvent System	k'	λ_{max} (nm)
1,6-DNP	70% ACN/H_2O	2.47	287, 381, 412
39			
1,6-NNOP	DMF	**	398, 446
57	70%ACN/H_2O	4.13	234, 297, 397, 445
1,6-HANP	DMF	**	480
59	70% ACN/H_2O	0.92	305, 462
1,6-AANP	70% ACN/H_2O	1.14	241, 294, 413
54			
1,6-ANP	DMF	**	316, 512
52	70% ACN/H_2O	1.88	254, 315, 489
1,8-DNP	DMF	**	302, 440
40	70% ACN/H_2O	2.55	300, 440
1, 8-NNOP	DMF	**	302,444
46	70% ACN/H_2O	4.58	291, 398
1,8-HANP	DMF	**	386, 486
48	70% ACN/H_2O	0.89	382, 465
1,8-ANP	DMF	**	520
53	70% ACN/H_2O	1.55	305, 487

Notes:

1. $k' = (t_r - t_o)/ t_o$
2. all data for RP-HPLC was obtained on column E
3. none of the di-substituted compounds were found to be fluorescent
4. ** = not applicable

2.2 Combined Liquid Chromatography-Mass Spectrometry Studies

2.2.1 LC-MS of a 1-NP Standards Mixture

When the peak with k'=0.97 from HPLC analysis of a 1-NOP reduction (tentatively identified as 1-HAP) was collected, solvent removed *in vacuo* and then analysed by probe mass spectrometry (probe MS) only 1-NOP (M^+=231 amu) and 1-AP (M^+=217 amu) were found to be present. No evidence for the existence of 1-HAP with an expected molecular ion of m/z=233 was observed. Repeated attempts to obtain a mass spectrum by performing probe MS on the collected peak failed; in every case only 1-AP and 1-NOP were observed. The 1-NOP and 1-AP may have been formed from a disproportionation of 1-HAP, a reaction has been observed for other arylhydroxylamines [51,96].

The technique of combined liquid chromatography-mass spectrometry (LC-MS) is a fairly recent development which has attracted increasing research attention. This technique involves the on-line combination of HPLC with mass spectrometry in a manner similar to gas chromatography-mass spectrometry (GC-MS). LC-MS is useful for the analysis of compounds that are too labile for GC analysis but which are still volatile enough for MS analysis. At McMaster University a VG moving belt interface was used to transport the column eluant into the MS source (see Figure 18) [48,97].

A 1 uL aliquot of a test solution containing 1-AAP, 1-AP, 1-NOP and 1-NOP was analysed by LC-MS to establish the optimum instrumental conditions. The reconstructed ion current chromatogram (RIC) for m/z=217, an ion common for all the compounds in the test solution, is shown in Figure 19. The "ghosting" seen after the major peaks in Figure 19 was due to incomplete vaporization of compounds from the moving belt interface. The amount of "ghosting" was reduced in subsequent analyses by increasing the amount of heating on the belt.

The mass spectra obtained for the indvidual compounds in the test solution by probe MS and LC-MS analyses are summarized in Table 4. Both 1-NOP and 1-NP were reduced to a much larger extent in the LC-MS system than they were in probe MS. Factors affecting the reduction of nitro-PAH in the source of mass spectrometers have been discussed previously by Quilliam et al [97].

2.2.2 LC-MS of Reduced 1-NOP Solutions

Once the instrumental conditions had been optimized for the mono-N-pyrene standards a solution of 1-NOP in DMF was reduced with ascorbic acid and an aliquot of the solution was analysed by LC-MS. Both ^{14}N and ^{15}N labelled 1-NOP solutions were analysed. The use of isotopes aided the interpretation of mass spectral fragmentation patterns. The molecular ion for the ^{14}N-1-HAP is expected to be m/z=233 and for the ^{15}N labelled compound m/z=234. The reconstructed ion current

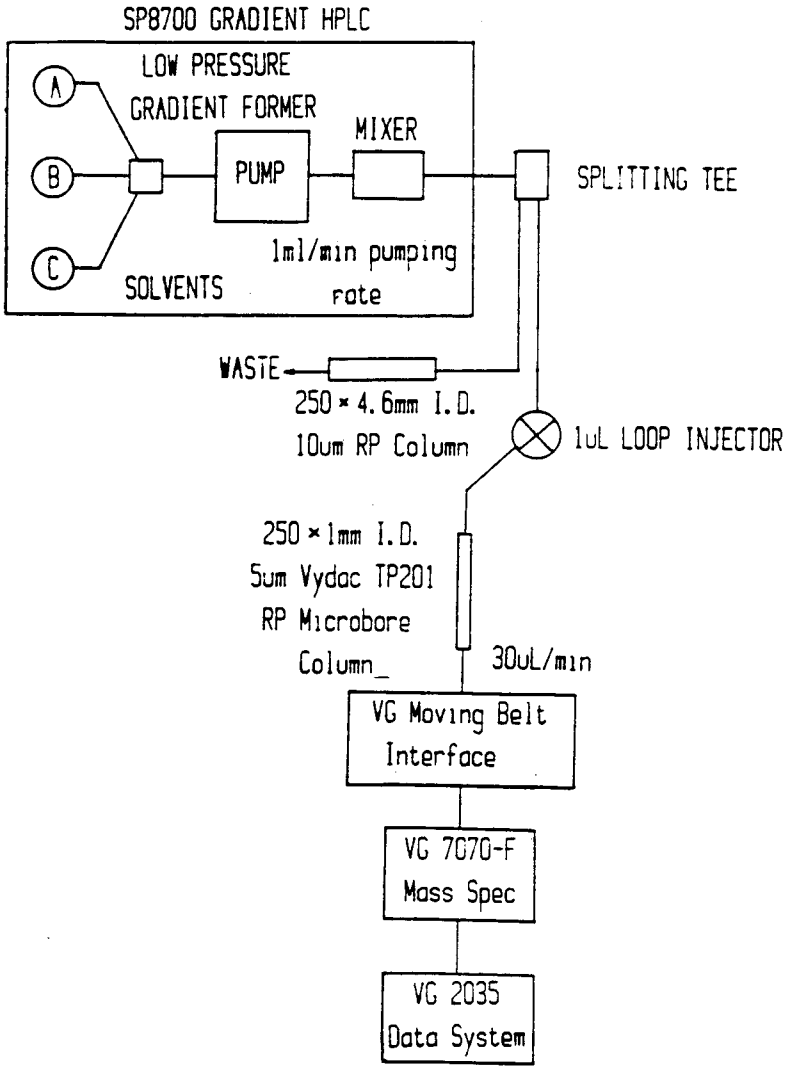


Figure 18: Schematic diagram of the instrumentation used for LC-MS analyses of nitro-PAH.

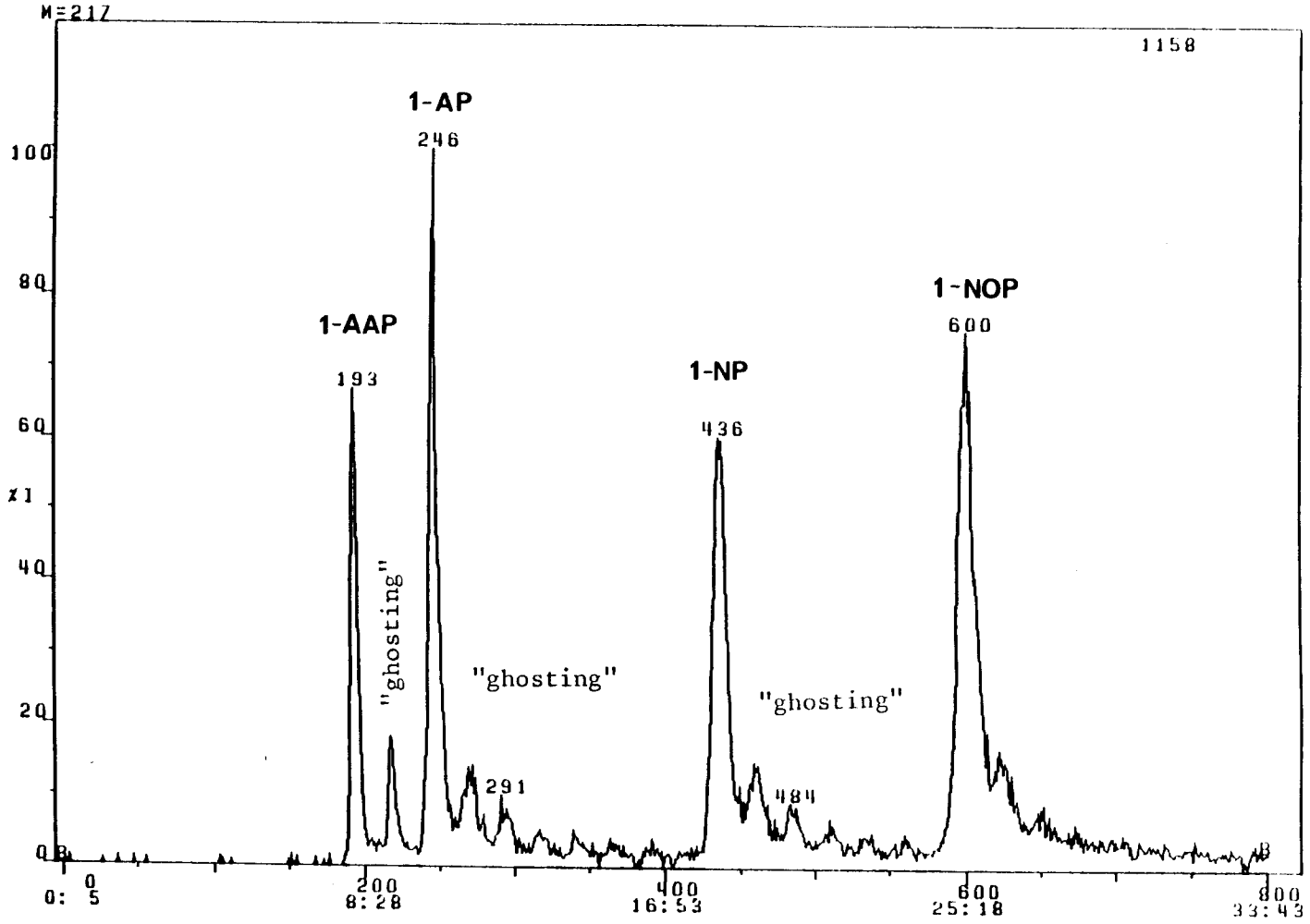


Figure 19: TIC chromatogram obtained from the LC-MS analysis of a mono-N-Pyrene standards mixture.

chromatogram (RIC) for m/z=234 obtained from the LC-MS analysis of a reduced [^{15}N]-1-NOP solution is shown in Figure 20; an identical RIC chromatogram for m/z=233 was obtained from analysis of the [^{14}N] compound. The RIC for the ion m/z=233 in the ^{14}N-1-NOP reduction showed a peak at scan number 144 while the ^{15}N-labelled compound had a peak at scan number 145.

The mass spectra observed for the ^{14}N and ^{15}N compounds are shown in Figure 21a and Figure 21b. In both Figures 21a and 21b the molecular ions correspond to that expected for the 1-HAP compound. The mass spectral data for these two compounds are summarized in Table 5. The fragmentation patterns observed were identical for both the ^{14}N and ^{15}N compounds; in both cases the major fragment lost from the molecular ion is 29 amu, indicating that the nitrogen atom was still incorporated in the molecule after the first fragmentation. The next major fragment ion (m/z=176), was common for both the ^{14}N and ^{15}N compounds, indicating that the nitrogen atom was no longer incorporated in the molecule.

The mass spectral fragmentation pattern has been used previously in the literature for differentiating between the N-OH and C-OH isomers of hydroxylated aromatic amines (Table 6) [94,98]. N-hydroxylated amines show a characteristic loss of 32 amu as the major fragmentation [94,98]. Ortho- and para-hydroxyamines show a different fragmentation pattern, characterized by a loss of 29 amu (corresponding to -COH) as

Table 4: Mass Spectral Data
Obtained for Some Mono-N-Pyrene Compounds
By Probe-MS[1] and LC-MS[2]

Compound	Probe -MS [3]	LC-MS [3]
1-nitropyrene 9	247(86) 217 (43) 201 (100) 189 (42)	247 (35) 218 (19) 217 (100) 216 (16) 201 (43)
1-nitrosopyrene 41	231 (50) 217 (30) 201 (100)	231 (4) 218 (17) 217 (100) 216 (17) 189 (30)
1-aminopyrene 43	217 (100) 189 (20)	218 (19) 217 (100) 216 (16) 189 (23)
N-acetyl- 1-aminopyrene 44	259 (47) 217 (100) 216 (25) 189 (37)	259 (45) 218 (22) 217 (100) 216 (30) 189 (33)

Notes:

1. Probe-MS = probe mass spectrometry with electron impact ionization (EI).

2. LC-MS = combined liquid chromatography-mass spectrometry with EI.

3. MS data is provided as m/z value (relative intensity).

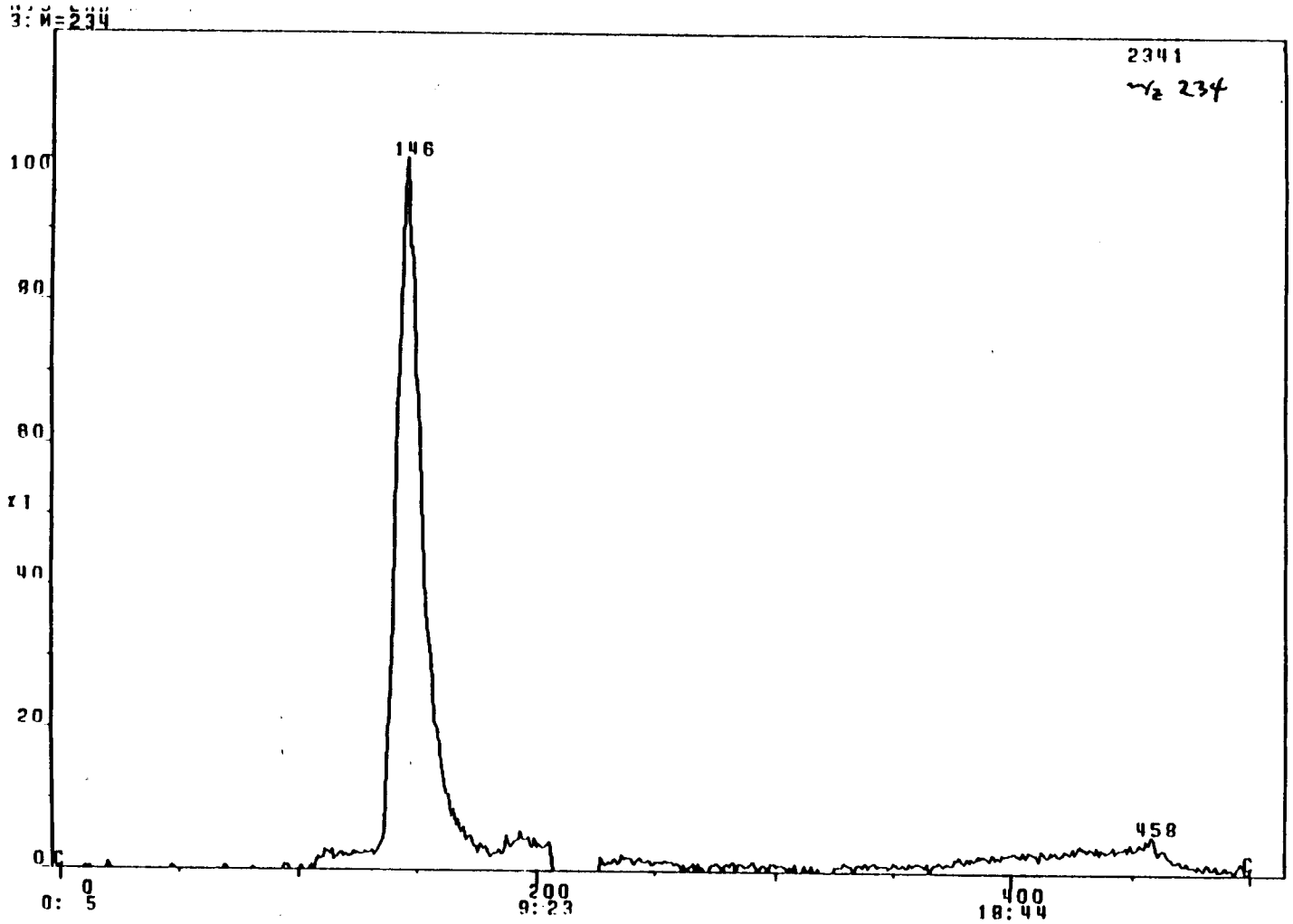


Figure 20: RIC chromatogram (m/z=234) for LC-MS analysis of a reduced ^{15}N-1-NOP solution.

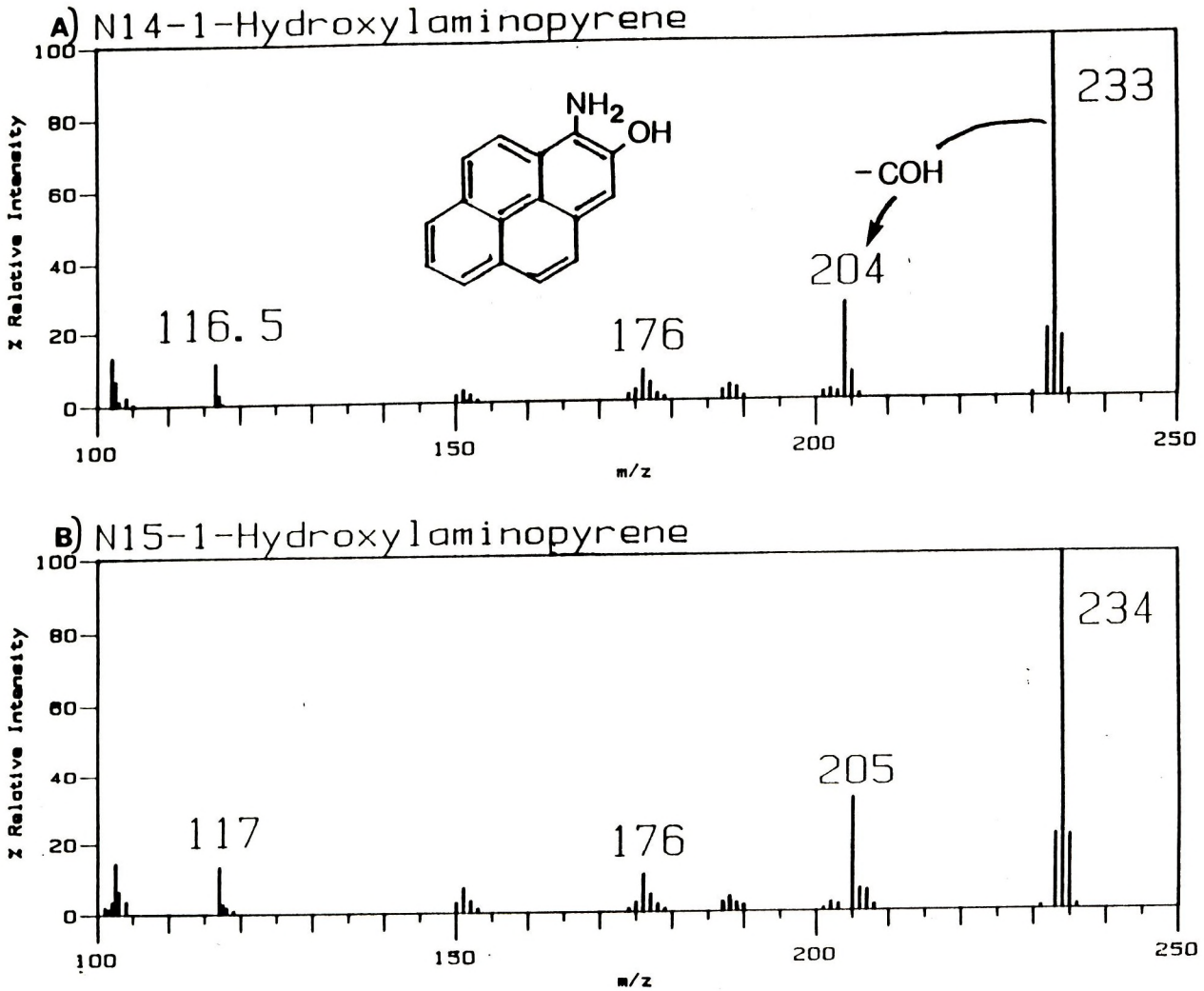


Figure 21: Mass spectra obtained for scan no. 145 in the LC-MS anayses of a chemically reduced 1-NOP in DMF solution

a) ^{14}N labelled compound
b) ^{15}N labelled compound

Table 5: Mass Spectral Data Obtained by LC-MS Analysis of DMF Solutions of Reduced (^{14}N or ^{15}N -) 1-Nitrosopyrene

^{14}N			^{15}N		
m/z	rel. intensity		m/z	rel. intensity	
233	100	(M^+)	234	100	(M^+)
232	21	(M-1)	233	23	(M-1)
204	34	(M-29)	205	36	(M-29)
176	10		176	10	

Table 6: Mass Spectral Data for Some Aromatic Hydroxylamines versus o-hydroxy-Aromatic Amines

N-Hydroxy-1-Aminobenzene[1]

m/z	rel. intensity	fragment
109	35	M^+
107	38	M-2
93	100	M-16
92	52	M-17
65	90	M-44

2-Hydroxy-1-Aminobenzene[2]

m/z	rel. intensity	fragment
109	100	M^+
108	10	M-1
91	5	M-18
80	40	M-29

N-Hydroxy-2-Aminofluorene[1]

m/z	rel intensity	fragment
197	2	M^+
195	25	M-2
181	100	M-16
180	62	M-17
165	70	M-32

3-Hydroxy-2-aminofluorene[3]

m/z	rel. intensity	fragment
197	100	M^+
168	33	M-29
167	31	M-30
152	60	M-45

Notes:data obtained from the following references;

1. Coutts and Mukhertje[98]
2. Hites, R.A.[99]
3. Iorio, M.A. et al[94]

the major fragment [98,99]. The loss of a -COH fragment in the mass spectrometry of phenolic compounds is well documented.A comparison of the mass spectral data in Table 5 with that in Table 6 indicated that the compounds observed by LC-MS were not in fact the 1-HAP, but rather the 2-hydroxy-1-aminopyrene 60 [99]. This compound was presumably formed from the Bamberger rearrangement of 1-HAP (Figure 22).

In an attempt to test this hypothesis, a solution of reduced 1-NOP was reacted under conditions known to result in the Bamberger rearrangement of other arylhydroxylamines [94]. HPLC analysis after reaction showed a major peak (approx. 90% by area) with a t_R identical to 1-AP and a minor peak (approx. 10% by area) that eluted prior to 1-AP. The minor peak was collected and analysed by probe MS. The use of temperature programming on the probe tip allowed a plot of temperature vs. ion current to be obtained for several ions that were indicative of the compound of interest (Figure 23). The ion current vs. probe temperature plot for m/z=204 and 233 was suggestive that the rearranged product 56 was present. Thus, the ion current for m/z=204 & 233 is a maximum when the ion current for m/z=247,231 and 217 are relatively low. All attempts to prepare a pure sample of 60 were unsuccessful.

2.2.3 LC-MS of a Reduced 1-Nitro-8-Nitrosopyrene Solution

A similar set of LC-MS experiments was performed on 1,8-NONP. A solution of 1,8-NNOP in DMF (prepared by J. Fulton), was reduced with ascorbic acid and analysed by LC/MS under the

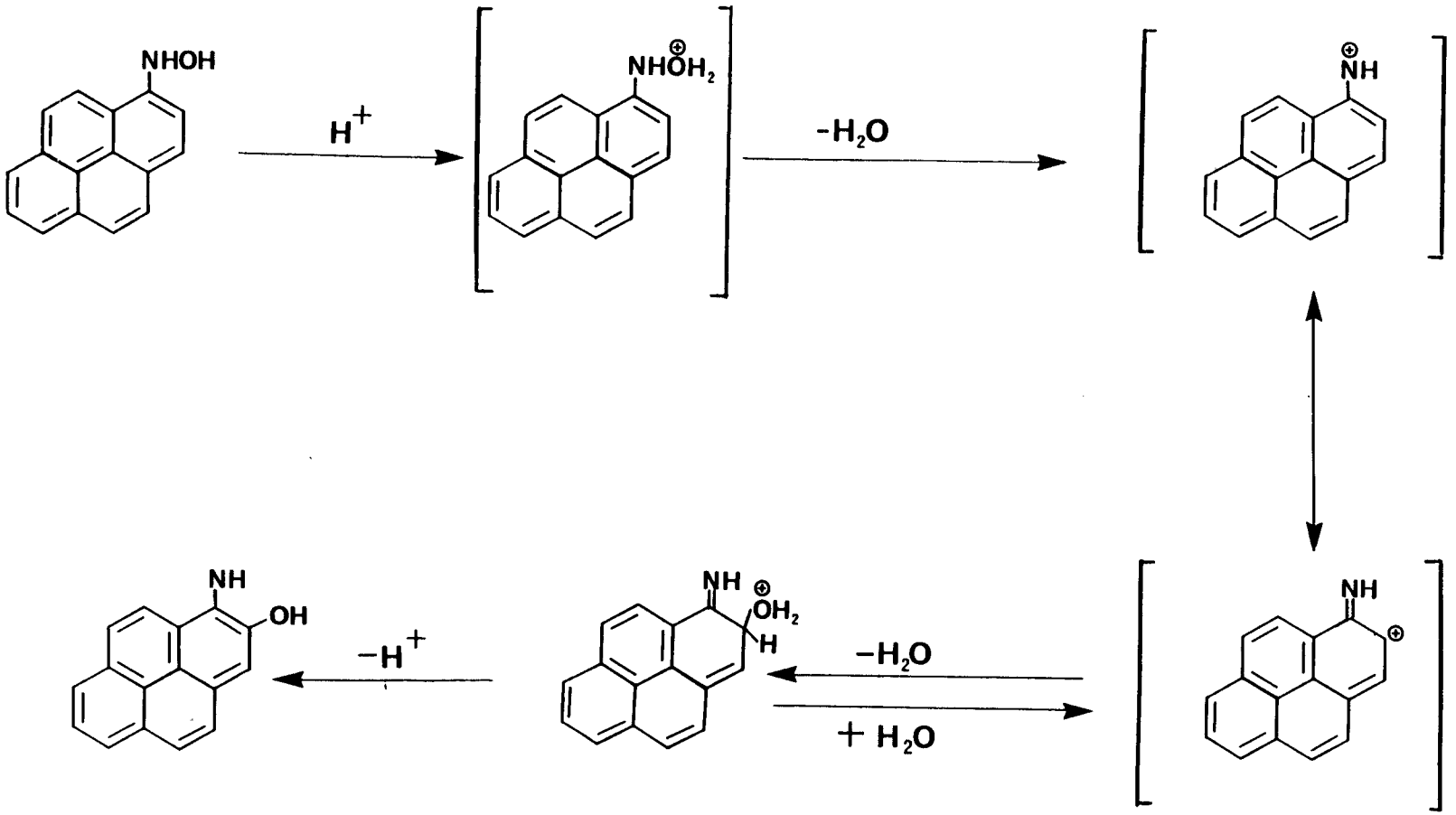


Figure 22: Proposed Mechanism for the Bamberger Rearrangement of 1-HAP.

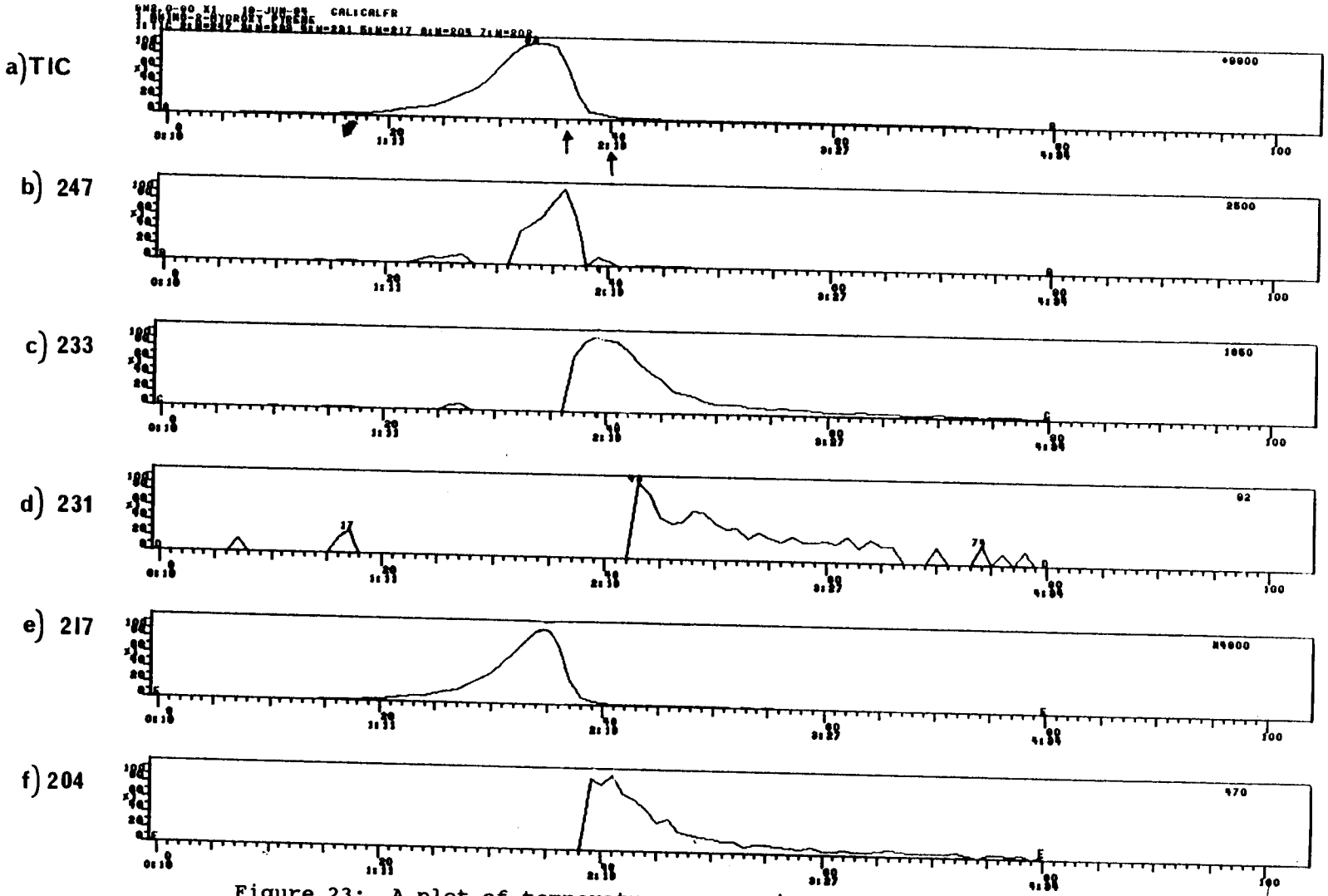


Figure 23: A plot of temperature versus ion current for m/z= 247,233,231,217 and 204 obtained from the probe MS analysis of residues in the RP-HPLC peak from the attempted Bamberger rearrangement of 1-HAP.

same conditions as Section 2.2.2. The TIC for this analysis is shown in Figure 24. The TIC showed peaks at scan numbers 339, 383,400,470 and 508. The peaks at scan numbers 400, 470 and 508 correspond in t_R and mass spectra to 1,8-ANP, 1,8-DNP and 1,8-NNOP. The mass spectrum or the peak at scan number 339 (not shown) was similar but not identical to that of 1,8-ANP. The retention time of this peak however, is significantly different from that for 1,8-ANP. On the basis of this data, it appears that the N-hydroxy-1-amino-8-nitropyrene was being formed but underwent reduction in the mass spectrometer. A similar type of behaviour in mass spectrometer systems has been observed with other arylhydroxylamines, notably 2-HAAF [94]. In contrast to the results observed for 1-HAP, no evidence was observed for rearrangement of 1,8-HANP in the LC-MS system.

2.3. ^{15}N-NMR Studies

2.3.1 ^{15}N-NMR of 1-Nitropyrene and Related Compounds

The LC-MS results presented above indicated that the structure of the compound eluting at scan numbers 144-145 was the 2-OH-1-AP 60, presumably formed from the Bamberger rearrangement of 1-HAP [94]. However, no information was provided by these experiments as to whether the rearrangement was occurring in the DMF solution or somewhere in the LC or LC-MS systems.

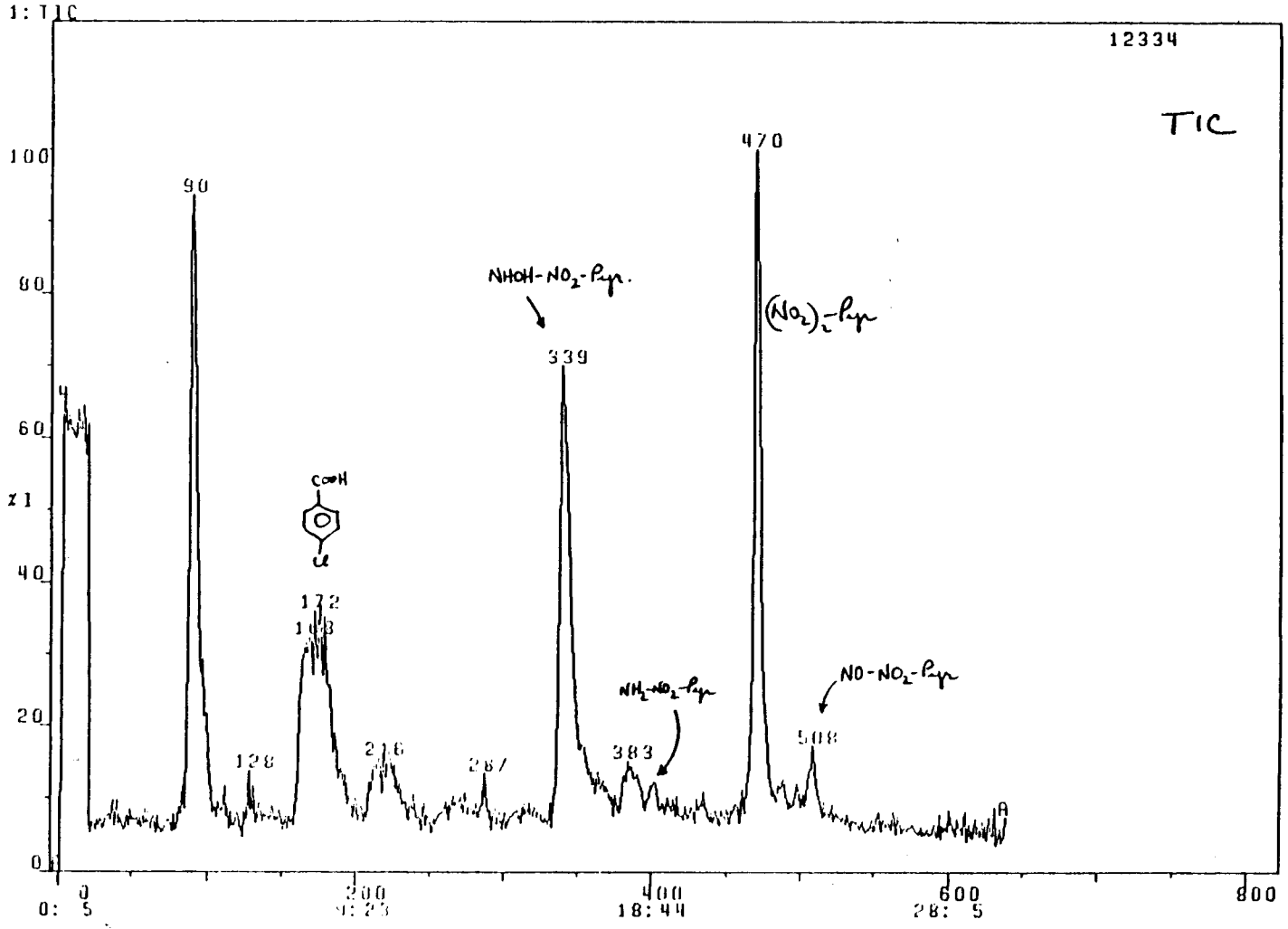


Figure 24: TIC chromatogram for LC-MS analysis of a reduced 1,8-NONP solution.

obtain more information on the chemistry of the reduction of 1-NOP in DMF.

In theory carbon-13 nuclear magnetic resonance (^{13}C NMR) could be used to gain information on the structure of the compounds formed by the ascorbic acid reduction of 1-NOP in DMF and DMSO. However, the low solubility of the N-pyrene compounds being studied and long relaxation times (T_1) observed for the carbon atoms of these compounds in ^{13}C NMR made the use of ^{13}C NMR inconvenient. To illustrate the problem, a ^{13}C NMR spectrum of 10 mg 1-NP in deuterated-DMSO (DMSO-d_6) still showed no C-1 signal after 15 hours of signal acquisition. As a result of these issues the use of ^{13}C NMR was not pursued futher.

Nitrogen-15 NMR is well suited for differentiating between the structures of 1-HAP and 2-OH-1-AP on account of the wide chemical shift range for the nitrogen atom and the ability to differentiate via special pulse sequences such as spin echo Fourier transform (SEFT) between an N-H and a NH_2 group [100]. The use of 99% ^{15}N-labelled compounds plus special pulse sequences (INEPT and DEPT) means that sensitivity is not the problem with ^{15}N NMR that it is with ^{13}C NMR in this application. The chemical shift of the nitrogen atom in ^{15}N NMR varies widely depending on the oxidation state of the nitrogen atom. The range of chemical shifts for a series of N-benzene derivatives is shown in Table 7 [100]. No literature reference was found for ^{15}N chemical shifts of an

oxides are 70 to 100 ppm deshielded compared to the corresponding amine [100]. Based on the data in Table 7 the chemical shift for 1-HAP would be expected to be 50-70 ppm deshielded from the 2-OH-1-AP. In addition, the use of spin-echo fourier transform (SEFT) techniques might allow the two possible structures to be differentiated on the basis of the number of protons attached to the nitrogen atom [101,102].

The ^{15}N NMR spectrum of a solution of 1-NOP in DMF showed a singlet at 893 ppm (Figure 25a). When the 1-NOP was reduced with ascorbic acid the peak at 893 ppm disappeared and a new signal appeared at 137 ppm (Figure 25b). The SEFT pulse sequence showed that this signal was due to a nitrogen atom with only one proton directly attached to it. Under the same acquisition parameters 1-AP had a signal at 60 ppm which showed two attached protons by SEFT [89]. The ^{15}N NMR results for 1-NP and related compounds are summarized in Table 8.

The conclusions from the ^{15}N NMR results presented above are:

- 1-HAP was the ascorbic acid reduction product of 1-NOP.
- in DMF at least, there is no evidence for any rearrangement to a hydroxyamine.

Therefore it apppears that the rearrangement of 1-HAP to 2-OH-1-AP observed by LC-MS is probably occurring in the LC-MS system.

Table 7: ^{15}N NMR data for some N-benzene compounds[1]

Compound	Solvent	δ[2] (ppm)	$^1J_{N-H}$ (Hz)	$^3J_{N-H}$ (Hz)
1-nitrobenzene	neat	370.3	**	-1.94
1-nitrosobenzene	neat	913	**	**
N-hydroxy-1-aminobenzene	DMSO-d_6	**	79.6	**
1-aminobenzene	DMSO-d_6	59.7	82.8	-1.5
N-acetyl-1-aminobenzene	DMSO-d_6	133.2	**	**

Notes:
1. data from Levy and Lichter [100]
2. reference peak not specified
3 ** = no data available

Table 8: 30 MHz ^{15}N NMR Data Obtained for Some Mono-N-Pyrene Compounds

Compound	Solvent	δ (ppm)	$^{1}J_{N-H}$ (Hz)	$^{3}J_{N-H}$ (Hz)
1-nitropyrene 9	DMF	370	**	-1.3
1-nitrosopyrene 41	DMF	893.4	**	***
N-hydroxy-1-aminopyrene 42	DMF	137.35	82.8	***
1-aminopyrene 43	DMF	60.5	85.3	-2.0
N-acetyl-1-aminopyrene 44	DMF	127	***	***
	DMSO-d_6	128.2	84.9	***

Notes:

1. ** = not applicable
2. *** = not availble
3. -chemical shift for all peaks is versus the DMF reference peak at 104.1 ppm.

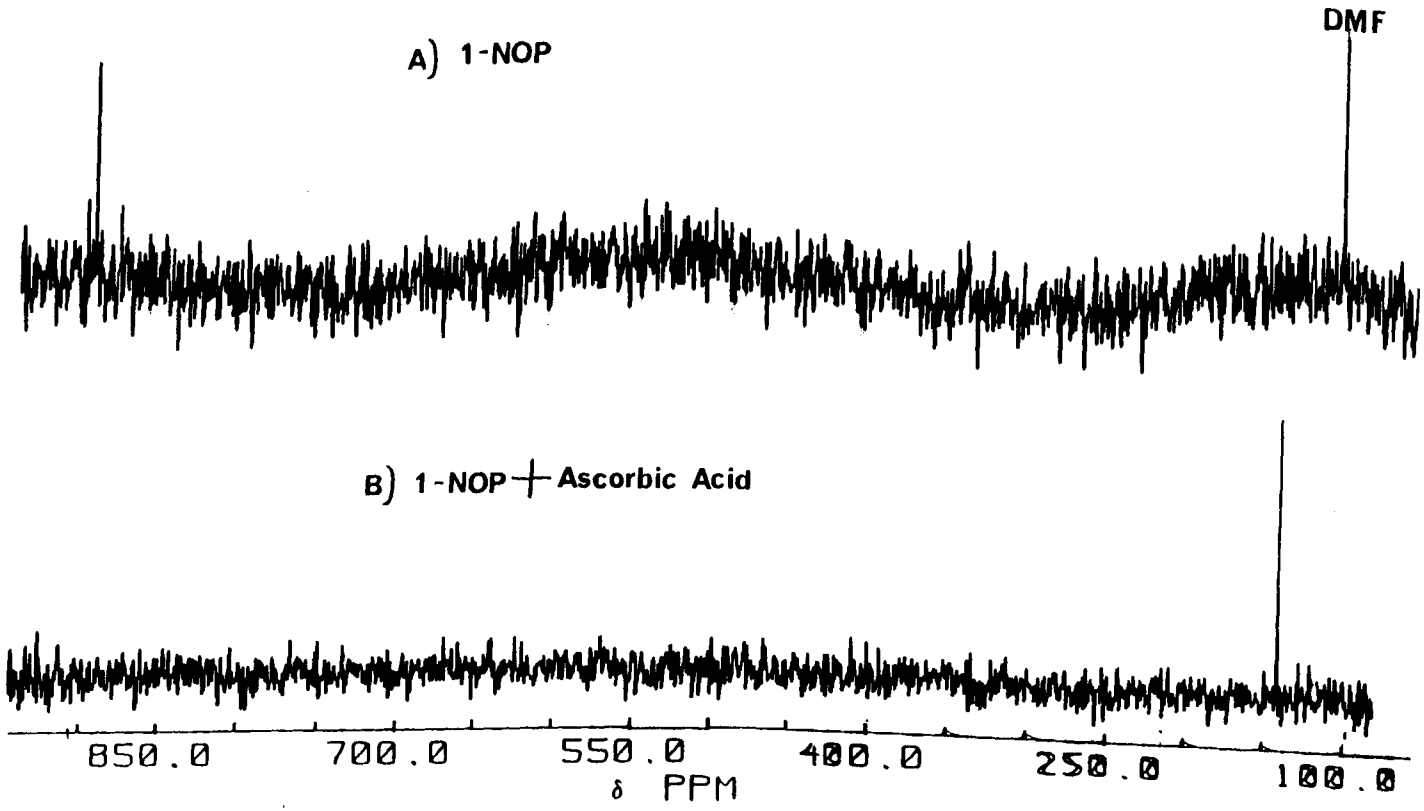


Figure 25: 30MHz ^{15}N NMR Spectra of;
a) 1-NOP and,
b) chemically reduced 1-NOP.
The solvent is DMF.

2.3.2 ^{15}N NMR of Dinitropyrenes and Related Compounds

In a series of parallel experiments to those for mono-N-pyrenes the ^{15}N NMR spectra of a number of 1,6- and 1,8-substituted N-pyrenes compounds were acquired. The results obtained were analogous to those observed for the ^{15}N NMR experiments for the mono-N-pyrene compounds and are tabulated in Table 9 .

The reduction of a NONP results in the disappearance of one nitrogen resonance and the appearance of a new signal that has a chemical shift approximately 70 ppm deshielded from the corresponding ANP. These results indicated that the compound being formed in the reduction of the NONP in DMF was the arylhydroxylamine.

2.4 ^{1}H NMR STUDIES

2.4.1 ^{1}H NMR of 1-NP and Derivatives

Thus far the evidence presented for the formation of 1-HAP in the reaction of 1-NOP has been from HPLC, UV-VIS, Fluorescence, LC-MS and ^{15}N NMR analyses. The acquisition and interpretation of proton (^{1}H) NMR also has the ability to provide unique structural information [101,102].

High field ^{1}H NMR was used for the study of nitro-PAH compounds for the following reasons;

i) the greater sensitivity required due to the small sample sizes that were available.

Table 9: 30 MHz ^{15}N NMR Data for Some Dinitropyrene Derivatives

Compound	Solvent	δ^1N-H (ppm)	δNO_2 (ppm)
1,6-&1,8-AANP (54, 55)	DMSO-d_6	129.71, 129.41	***
1,3-; 1,6- ;1,8- ANP 52; 53 ; 54	DMF	68.35, 68.73	370.7, 375.1, 375.4
1,6; 1,8-HANP 59; 48	DMF	139.6	***

Notes:

1. -measured against DMF external standard peak at 104 ppm
2. *** = not available

The sample size used was governed by,

a) the very low solubility of the compounds being studied.

b) the toxicity of these compounds necessitated as small a sample size as practical.

c) the maximum concentration which could be used before some compounds (notably the DNP's and NNOP's) underwent dimerization or ring stacking.

ii) the high field strength resulted in better separation of the resonances of the aromatic protons on the pyrene moiety.

The reduction of 1-NOP in DMSO was previously shown to give identical experimental results by HPLC analysis to the reduction in DMF solution. Therefore for the ^1H NMR experiments, DMSO-d_6 was used as the solvent. A solution of 1-NOP in DMSO-d_6 (typically 4-10 mM) was prepared and then reduced with an excess of ascorbic acid. The solution immediately decolourized and began to fluoresce blue-green under long-wave UV light. The addition of ascorbate to the solution resulted in the disappearance of the 1-NOP ^1H NMR spectrum (Figure 26a) and the appearance of a new spectrum (Figure 26b) similar in appearance but not identical to that of 1-AP (Figure 26c). The two broad peaks at 9.5 and 8.6 ppm in Figure 26b were tentatively assigned to the N-H and O-H protons although it was not clear which signal was due to which type of proton. The complicated multiplet in the region from 7.8 to 8.3 ppm was due to the aromatic protons and the

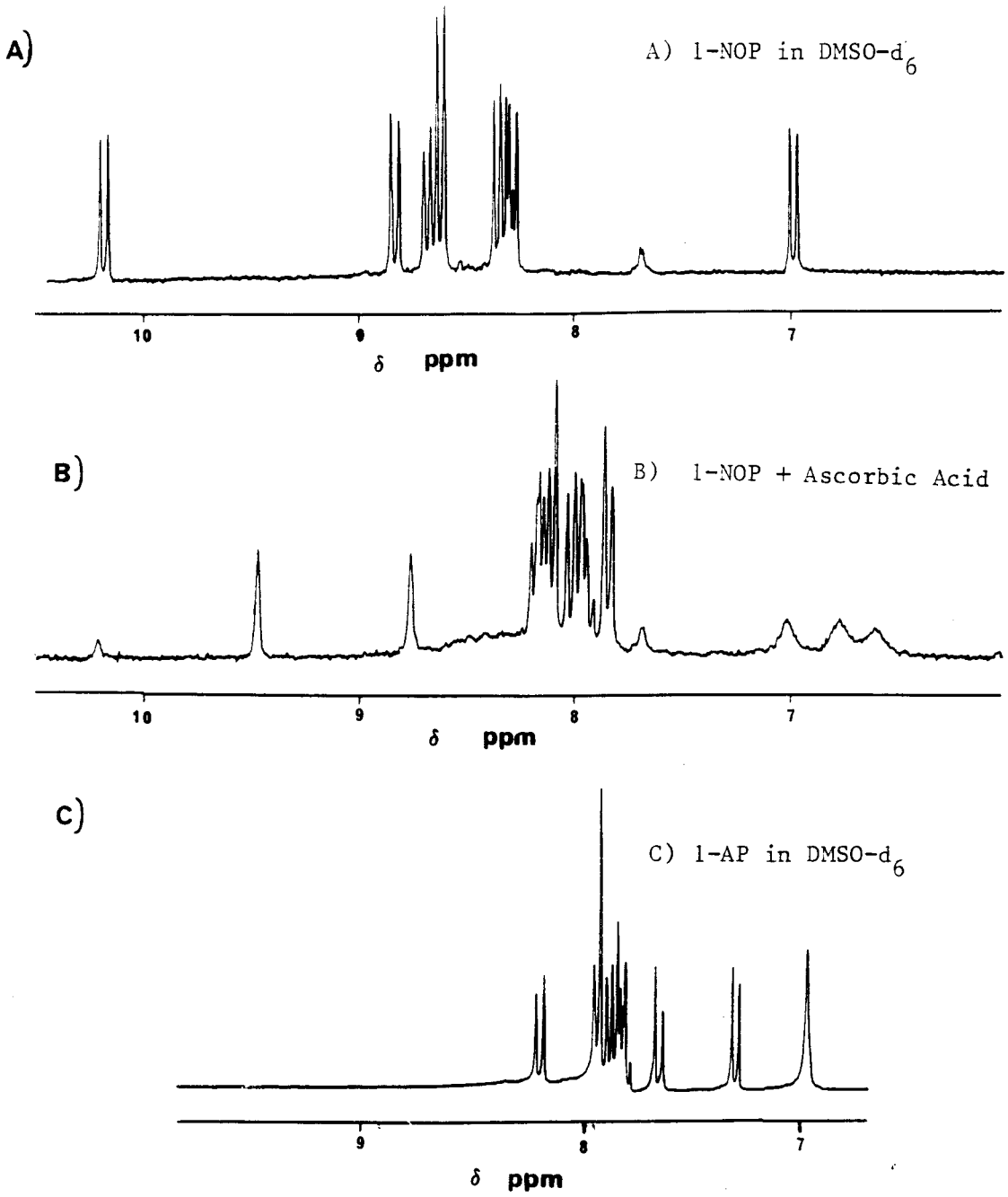


Figure 26: 250 MHz ^{1}H NMR Spectra of: a) 1-NOP, b) 1-NOP + ascorbic acid and c) 1-AP. Solvent= DMSO-d_6.

pattern was too complex to be interpreted by visual inspection.

Since the aromatic region of the ^{1}H NMR spectrum of the reduced 1-NOP solution was so complex it was decided to use the 2D-COSY [101-103] technique to aid in the interpretation. The 2D-COSY contour plot obtained for the region 7.8 to 8.3 ppm of a reduced 1-NOP solution is shown in Figure 27. The use of a contour plot allowed the proton resonances for the individual protons of the pyrene moiety to be more easily assigned. The proton resonances at 9.5 and 8.6 ppm were shown to be coupled to each other but not to any other protons. The ^{1}H NMR data for the compounds derived from 1-NP are summarized in Table 10. All ^{1}H NMR spectra for the nitro-PAH were interpreted with the aid of 2D-COSY techniques and by comparison to the spectra of other related compounds (e.g. 1-NP, 1-NOP, 1-AP and 1-AAP) obtained under the same conditions.

The 2D-COSY technique, while allowing the protons of the aromatic ring of 1-HAP to be assigned, was unable to unequivocally differentiate between O-H and N-H resonances. To resolve this question, a sample of ^{15}N-1-NOP was reduced under identical conditions and the ^{1}H NMR spectrum was analysed (^{1}H NMR spectrum not shown). The peak at 9.3 ppm was now split into a doublet with an ^{15}N-H coupling constant ($^{1}J_{N-H}$) of 82 Hz. When a 2D- J-Resolved experiment (not shown) was performed on this same solution all of the proton-proton couplings were removed and only heteronuclear couplings remained. The 9.3

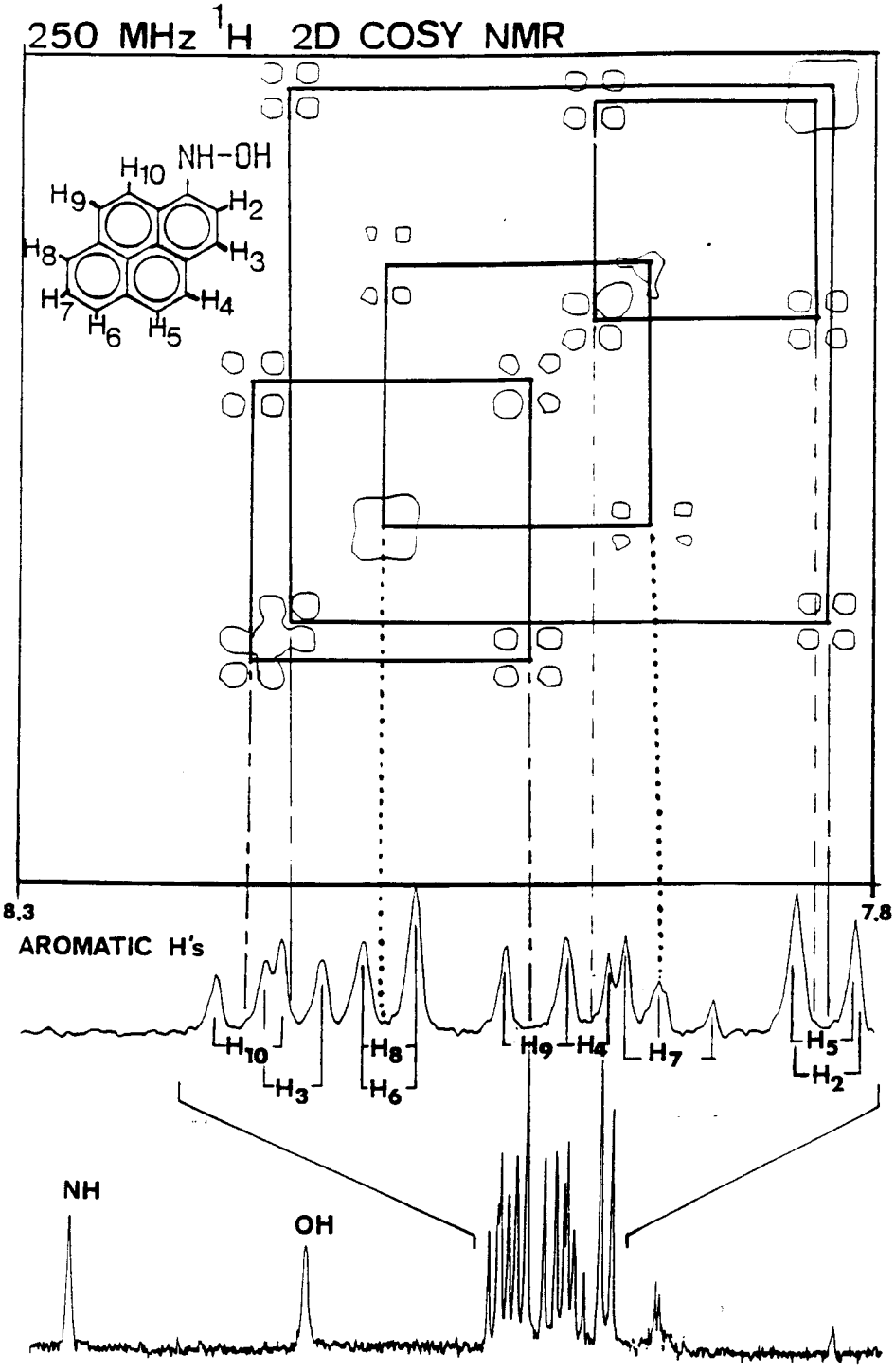


Figure 27 :Contour plot of 250 MHz ^{1}H NMR 2D-COSY spectrum of 1-HAP.

Table 10: ^{1}H-NMR data for 1-nitropyrene and Related Compounds

Compound		Ring Protons H2[a]	H3[a]	H4[b]	H5[b]	H6	H7	H8	H9[c]	H10[c]	OTHER
1-NP	δ	8.75	8.45	8.35	8.47	8.53	8.27	8.55	8.53	8.74	
9	$^3J_{H-H}$	8.47	8.47	8.98	7.98	7.71	7.71	7.71	9.35	9.35	
1-NOP	δ	6.93	8.21	8.29	8.57	8.58	8.27	8.63	8.77	10.11	
41	$^3J_{H-H}$	8.66	8.59	8.5	8.79	7.56	7.6	7.55	9.16	9.08	
1-HAP	δ	7.84	8.15	7.97	7.84	8.09	7.94	8.09	8.01	8.17	9.3 (NH), 8.8 (OH)
42	$^3J_{H-H}$	8.18	8.18	9.13	9.13	7.98	7.75	7.52	9.26	9.26	
1-AP	δ	7.34	7.95	7.70	7.86	7.98	7.85	7.97	7.89	8.24	7.0 (NH)
43	$^3J_{H-H}$	8.3	8.3	8.84	8.84	7.47	7.47	7.47	9.3	9.3	
1-AAP	δ	8.34	8.14	-	8.34	8.11	-	-	8.27	8.38	10.31(NH), 2.27(CH_3)
44	$^3J_{H-H}$	7.97	7.97	-	9.1	8.09	-	-	9.40	9.40	

Notes

a,b,c assignments may be reversed

δ values in ppm versus reference peak of DMSO-d_6 at 2.49

- = unable to determine unequivocally

ppm peak was still showing an 82 Hz coupling. This indicates that the ^{15}N atom is coupled through one bond to the proton at 9.3 ppm. Therefore it was concluded that the proton resonance at 9.3 ppm is due to the N-H and the signal at 8.8 ppm is due to the O-H proton.

2.4.2 ^{1}H NMR Spectroscopy of Dinitropyrenes and Related Compounds

Although the ^{1}H NMR spectra of the 1,6- and 1,8-DNP had been reported [103], there was no information available on the ^{1}H NMR spectra of their associated compounds such the ANP, AANP and HANP. The ^{1}H NMR spectra of these derivatives of the 1,6- and 1,8-DNPs were obtained as solutions in DMSO-d_6. The concentration of solute was determined by the maximum concentration before excessive line broadening was observed. The ^{1}H NMR data for the 1,6- compounds are summarized in Table 11. The data for the 1,8- compounds are shown in Table 12.

The addition of excess ascorbic acid to a solution of the NONP resulted in immediate colour change from orange-brown to deep red-purple. The disappearance of the NONP spectrum was parallelled by the appearance of a new spectrum that was not due to the ANP. Interpretation of ^{1}H NMR spectra was aided by comparison to the NMR spectra of other related compounds.

2.5 REACTIONS OF N-HYDROXY-1-AMINOPYRENE

2.5.1 General Reactions

The reactions that were observed for 1-HAP in this project are summarized in Figure 28. The reactivity of 1-HAP

Table 11: ^{1}H NMR Data for 1,6-DNP and Derivatives

Compound		Ring Protons H2	H3	H4[a]	H5[a]	H7	H8	H9[b]	H10[b]	Others
1,6-DNP	δ	8.86	8.69	8.66	8.84	8.86	8.69	8.66	8.84	
39	$^3J_{H-H}$	8.50	8.34	9.52	9.47	8.50	8.34	9.52	9.41	
1,6-NNOP	δ	8.93	8.82	8.91	10.39	7.01	8.47	8.96	8.61	
57	$^3J_{H-H}$	8.50	8.50	9.26	9.26	8.57	8.57	9.42	9.42	
1,6-HANP	δ	8.19	8.38	8.34	8.53	8.65	7.93	8.48	8.16	N-H = 10.05 O-H = 9.08
59	$^3J_{H-H}$	8.68	8.68	9.42	9.42	8.61	8.61	9.16	9.16	
1,6-ANP	δ	7.46	8.18	8.05	8.60	8.61	8.04	8.25	8.51	N-H = 7.09
52	$^3J_{H-H}$	8.47	8.47	9.07	9.07	8.58	8.58	9.38	9.38	
1,6-AANP	δ	**	8.50	8.37	8.68	8.74	**	8.475	8.61	N-H = 10.53 CH_3 = 2.25
54	$^3J_{H-H}$	**	7.90	9.36	9.36	8.65	**	9.36	9.36	

Notes:

1. all values (chemical shift) are in ppm versus the DMSO -d_6 reference peak at 2.49 ppm
2. coupling ($^3J_{H-H}$) in Hz
3. a,b = assignments may be reversed
4. ** = unable to dertermine

Table 12: ^{1}H NMR Data for 1,8-DNP and Derivatives

Compound		Ring Protons H2	H3	H4	H5	H6	H7	H9	H10	Other
1,8-DNP	δ	8.79	8.69	8.59	8.59	8.69	8.79	8.95	8.95	
40	$^3J_{H-H}$	8.58	8.31	**	**	8.31	8.58	**	**	
1,8-NNOP	δ	8.92	8..71	8.73	##	8.51	7.04	9.24	10.49	
46	$^3J_{H-H}$	8.03	8.53	8.89	##	8.50	8.50	9.64	9.64	
1,8-HANP	δ	7.92	8.40	7.99	8.27	8.49	8.66	8.71	8.56	N-H = 10.08 O-H = 9.08
48	$^3J_{H-H}$	8.59	8.59	8.88	8.69	8.69	8.69	9.74	9.74	
1,8-ANP	δ	7.46	8.21	7.87	8.17	8.05	8.60	8.67	8.67	N-H = 7.09
53	$^3J_{H-H}$	8.49	8.49	8.63	8.63	8.75	8.75	**	**	
1,8-=AANP	δ	8.46	8.50	8.27	8.41	**	8.73	8.76	8.66	N-H = 10.54 CH_3 = ##
55	$^3J_{H-H}$	8.24	8.24	8.99	8.89	**	8.45	9.67	9.67	

Notes: 1. all values (chemical shifts) are in ppm versus the DMSO -d_6 reference peak at 2.49 ppm

2. coupling ($^3J_{H-H}$) in Hz
3. a,b = assignments may be reversed
4. ** = unable to determine
5. ## = not available

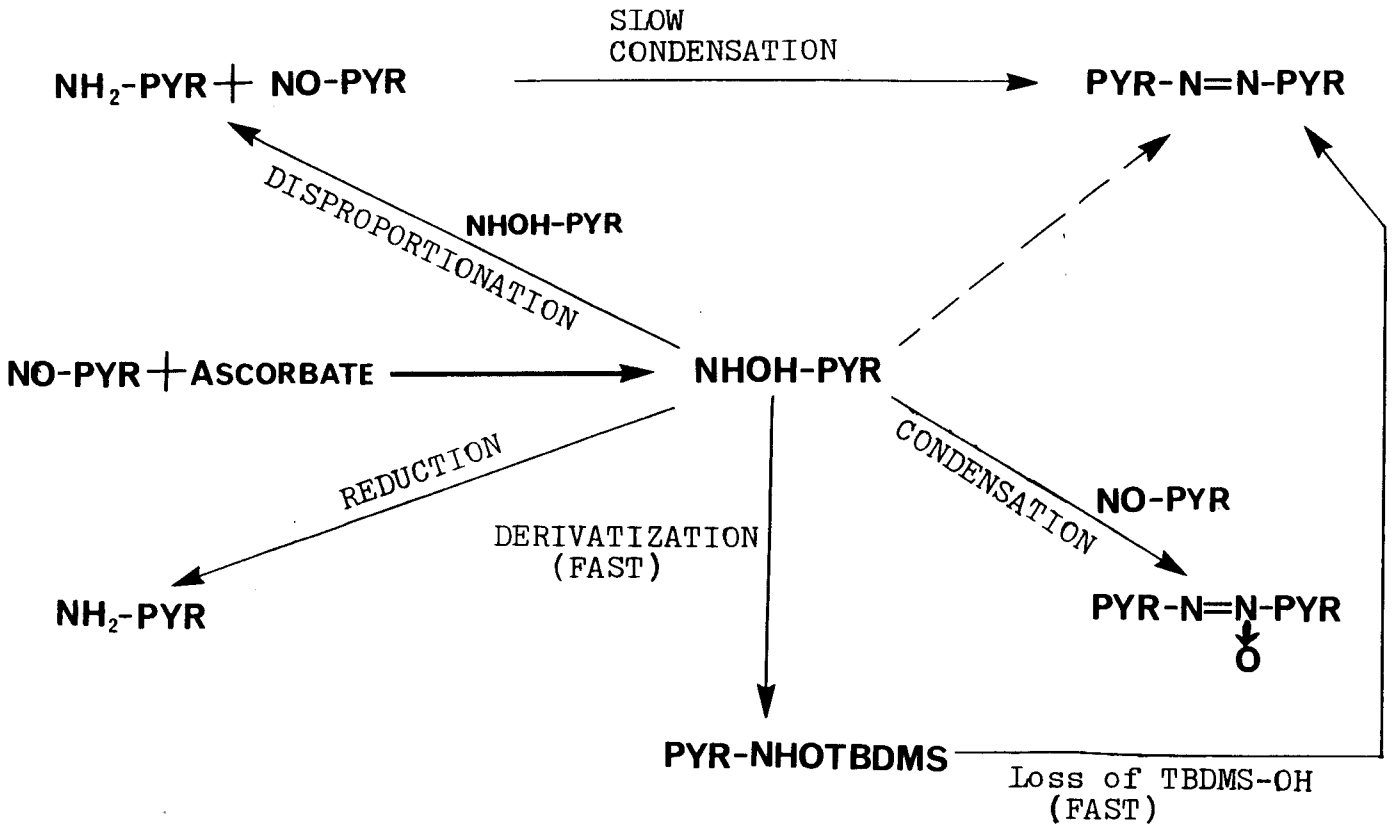


Figure 28: Summary of reactions observed for 1-HAP during this research.

was found to be dependent on the solvent system used. On the RP-HPLC columns used initially (columns A, B, C) the 1-HAP was found to demonstrate poor chromatographic performance due to peak tailing, possibly due to decomposition of the compound on the columns. The use of a buffered (pH 7) mobile phase did not improve the chromatographic performance. The quality of the RP-HPLC chromatograms improved dramatically when a Vydac TP 201 C_{18} column was used. HPLC analyses on the Vydac column indicated a yield of approximately 5% 1-AP and 10%-1-NOP. This contrasts with the ^{1}H and ^{15}N NMR results which show a yield of > 95% for 1-HAP. It was postulated that the 1-HAP decomposition may have been catalysed by any free silanol groups on the HPLC columns. The 1-HAP also appeared to be susceptible to disproportionation in the solvent systems used for HPLC analysis. This statement is supported by the observation that when the 1-HAP peak was collected as it eluted from the HPLC system and reinjected onto the same column no 1-HAP peak was observed. However, two peaks with retention times identical to 1-AP and 1-NOP were observed. Again, the use of buffered mobile phase made no difference to the result. This observation probably explains why it was not possible to obtain a pure sample of 1-HAP for probe-MS analysis.

2.5.2 Reaction of 1-HAP in DMF

The 1-HAP was found to be relatively stable in DMF and DMSO solutions, at room temperature in air the half life ($t_{1/2}$)

was found to be > 24 hours. The decomposition of 1-HAP in DMF was studied by RP-HPLC with UV-VIS diode array detection (DAD). After 96 hours at room temperature the major products observed (based on peak areas at = 254 nm) were:

1-HAP	approximately 0.2%
1-AP	60%
1-NP	4.6%
1-NOP	1.2 %

and two unidentified peaks,

10.8 minutes	17.4 %
13.7 minutes	18.6 %

The peak at 10.8 minutes had a $_{max}$ 395 nm and the peak at 13.7 minutes peak had a $_{max}$ of 416 nm. The two unidentified peaks were collected separately as they eluted from the HPLC system, the solvent was removed in vacuo and the orange-brown residues were analysed by probe MS. Both samples had identical mass spectra showing a molecular ion at m/z=430 and the major fragment ion at m/z=201. Comparison of the t_R, UV-VIS and MS results to that of authentic N,N'-azopyrene standards [91] (prepared by B. E. McCarry via the acid catalysed condensation of 1-AP and 1-NOP) indicated that these two unidentified compounds were the Z- and E- isomers of azopyrene, 61 and 62. The azopyrenes are probably formed in DMF from a condensation reaction of 1-AP and 1-NOP, compounds which in turn are being formed from the disproportionation of 1-HAP.

Both of these reactions have been observed for other arylhydroxylamines [51,104].

In contrast to the results of Howard et al [77,85] and others, no azoxypyrene 63 with a molecular ion of m/z= 446 was observed in the reduced 1-NOP solution. It is possible that azopyrenes were in fact formed under the conditions used by Howard et al and then underwent air oxidation to the azoxypyrene prior to MS analysis. This is supported by the observation of other researchers that the azopyrenes are susceptible to air oxidation [105].

2.5.3 pH Stability of N-Hydroxy-1-aminopyrene

The extent of covalent bonding of 1-NP to DNA was found to be pH dependent, the maxima covalent incorporation occurred in range of pH 5.6 to pH 6 [77]. However, it was not known if this maxima for DNA bonding of 1-NP correlated with the pH stability of 1-HAP since no stability data had been reported for this compound. Therefore, the pH stability and reactivity of 1-HAP was studied at various pH values using RP-HPLC.

Initial studies focussed on finding the optimum ratio of DMF solution: pH 7 buffer required to best mimic aqueous conditions (Table 13). A ratio of 1:19 was decided upon as a good compromise. At lower ratios the system is not truly aqueous as evidenced by the much longer $t_{1/2}$ times. At ratios greater than 1:19 there were problems with the

Table 13: Half life ($t_{1/2}$)[1] of N-hydroxy-1-aminopyrene (1-HAP) in different ratios[2] of DMF to pH 7 buffer[3]

DMF: pH 7 Buffer Ratio	$t_{1/2}$ (seconds)
1 : 3	600
1 : 9	210
1 : 19	90

notes:

1. $t_{1/2}$ = time after mixing where concentration of 1-HAP is 1/2 the original concentration
2. volume:volume, mixing with agitation
(see Chapter 3 (Experimental) for more experimental details)
3. phosphate buffer system

compounds' solubility, and the method detection limit that was obtainable. The $t_{1/2}$ values for 1-HAP as a function of the buffer pH are tabulated in Table 14. The data from Table 14 is plotted in Figure 29. The maximum stability of 1-HAP ($t_{1/2}$= 180 seconds) occurs at a pH of approximately 6. This is in the same general pH region as the maximum for covalent bonding of 1-NP to DNA. The $t_{1/2}$ value of 180 **seconds** in pH 6 buffer contrasts to the value of > 24 **hours** in DMF. Obviously then, the 1-HAP is much more stable in polar aprotic organic solvents.

The RP-HPLC analyses for the pH stability tests showed that the reactions occurring were pH dependent.

1) In the range of pH=2.25-6 the major decomposition product observed for 1-HAP was 1-AP. The lower the pH the shorter the half-life of 1-HAP. The major reaction in this pH region was considered to be an acid catalysed reduction of 1-HAP to 1-AP, possibly due to the excess ascorbic acid present.

2) In the range of pH 6-10 the reactions of 1-HAP are different than in the range of 2.25-6 and apparently much more complex. The major reaction observed was a rapid oxidation of 1-HAP to 1-NOP.

Kadlubar et al [106] have reported a similar pH stability results for 2-HAF and 1-HAF. For these compounds it was found that no oxidation occurs at pH 5 whereas rapid oxidation to the nitroso compound was occurring at pH 7.

Table 14: $t_{1/2}$[1] and log $t_{1/2}$ values for N-hydroxy-1-aminopyrene (1-HAP) at different pH values[2].

pH[3]	$t_{1/2}$ (seconds)		log $t_{1/2}$
2.25	2 (+/- 1)		0.30
3	7 (+/- 2)		0.85
4	35 (+/- 1)		1.54
5	65 (+/- 10)	1.81	
6	180 (+/- 30)	2.26	
7	90 (+/-20)		1.95
8	50 (+/-15)		1.70
9.2	30 (undetermined)		1.48
10	5 (+/-3)		0.70

Notes

1. $t_{1/2}$ = time at which 1-HAP concentration is 1/2 the starting concentration
2. ratio of DMF: buffer = 1:19
3. pH of buffer being used
4. concentration of 1-HAP in DMF is approximately 15 mM

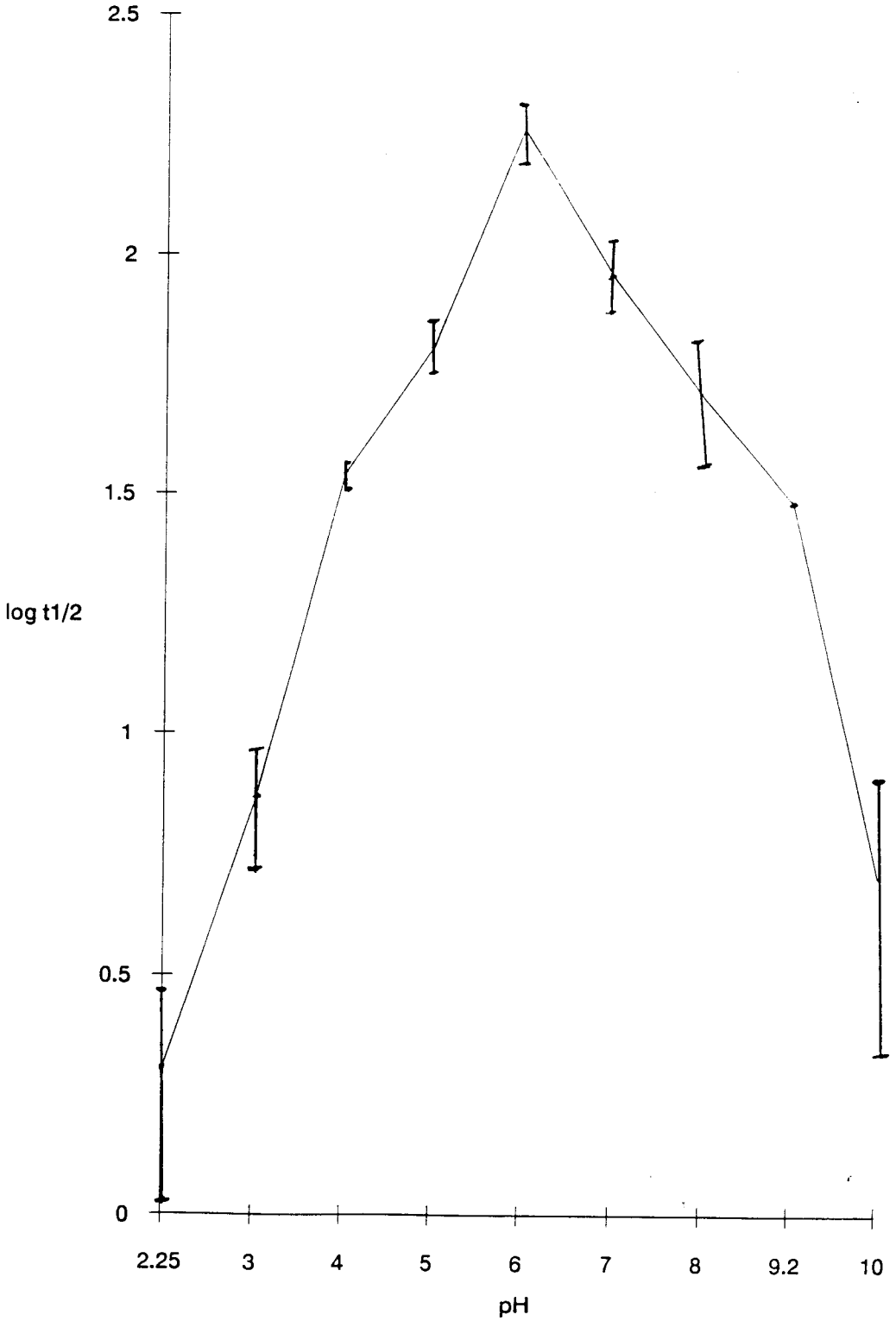


Figure 29: Plot of log $t_{1/2}$ versus pH for 1-HAP. (data from Table 14).

The half-life of 2-HAF in an ethanolic solution was 134 minutes. Under similar conditions (at pH>6) 1-HAP was found to react essentially instantaneously to give 1-NOP .

The maximum stability of 1-HAP at pH 6 corresponds to the pH for the maximum covalent bonding of 1-NP to DNA. The increased binding of 1-NP may be explained in terms of the greater stability of the proximate mutagen (1-HAP) allowing a greater length of time for reaction with DNA to occur.

The pH stability of 1,8-HANP was examined in a similar manner by Dr. J. Fulton [106]. The maximum $t_{1/2}$ for this compound was approximately 100 hours at a 1:3 ratio DMF solution: pH 5.5 buffer. This contrasts to a $t_{1/2}$ of 10 minutes for 1-HAP under the same conditions. The extra stability of 1,8-HANP compared to 1-HAP is probably due to the electron withdrawing effect of the nitro group in 1,8-HANP.

2.5.4 Reaction of 1-HAP with 2'-Deoxyguanosine

From the *in vivo* and *in vitro* studies of 1-NP it is known that the primary site of reaction is with guanine rather than the other heterocyclic bases. The reaction of 1-HAP with a nucleoside fragment containing guanine (2'-deoxyguanosine (dG)) was also studied in this project.

The purpose for this study was two-fold;

i) to examine the importance of the DNA double helix in the formation of the DNA adduct. A reduction in amount of binding to dG may indicate that chemical interactions between the DNA double helix and 1-NP are important, and

ii) as an attempt to find an alternative, simple route for the preparation a DNA adduct for further study.

Solutions of 1-HAP and 2'-deoxyguanosine (dG) were reacted together under conditions similar to those used by researchers to study the reactions of other arylhydroxylamines with dG [108,109]. Aliquots of the reaction solutions were analysed by RP-HPLC with UV and fluorescence detection using a solvent system known to separate DNP nucleoside adducts [90]. No reaction between 1-HAP and dG was detected in any of the solutions. Fluorescence detection is approximately 100 times more sensitive than UV detection for these compounds [107]. Therefore, even trace quantities of a deoxyguanosine adduct were expected to be observed. These results suggested that the DNA double helix structure is important and could assist in DNA adduct formation in vivo and in vitro through steric interactions such as hydrogen bonding or "kinking" perhaps in a manner similar to that which Kadlubar et al found to be operative for 2-HAF [107].

2.5.5 Derivatization

One of the goals of this project was the preparation of suitable derivatives of 1-HAP with enhanced stability which could provide a more direct route to 1-HAP than that currently being used for chemical and biological testing. The derivatization methods considered included; sulfation, acetylation, silylation and electrochemical reduction .

The sulfation of 1-HAP was not attempted in this work since this reaction had resulted in the formation of a derivative that was actually **less stable** than the parent arylhydroxylamine when used for other compounds.

2.5.5.1 Acetylation

Acetylation of other arylhydroxylamines has been reviewed above (Section 1.4.5.2).

The acetylation of 1-HAP was attempted by two methods;

i) Acetic anhydride (Ac_2O), was added to a solution of 1-HAP in DMF in a manner similar to that used by Lotlikar et al [108] for the acetylation of 2-HAF. The 1-HAP was found to decompose rapidly, HPLC analysis showed only 1-AP and 1-NOP with no evidence for an acetylation having taken place. It is probable that the presence of even small quantities of acetic acid resulted in the decomposition of 1-HAP.

ii) The acetylation of 1° and 2° amines and alcohols with N-Acetyl imidazole has been reported [109,110]. The primary advantage of this reagent is that acidic compounds are not liberated as reaction by products. The demonstrated lability of 1-HAP made this an important factor to consider. When N-acetyl imidazole was reacted with 1-HAP in DMF no reaction was detected by RP-HPLC. A possible reason for the lack of reaction may be the relative unreactivity of N-acetylimidazole [110].

2.5.5.2 Silylation

The trimethylsilyl (TMS) group is well known as a protecting group in organic synthesis and analytical chemistry for amines ,hydroxyl and terminal alkynyl groups [111]. Tsui et al [66] have reported the synthesis of the mono-O-TMS and N,O-bis-TMS derivatives of phenylhydroxylamines. However, the TMS group is quite susceptible to hydrolysis in protic media. Much better results have been obtained using tert-butyldimethylsilyl (TBDMS) as the protecting group [112,113]. The TBDMS ethers are approximately 10^4 times more stable than the corresponding TMS ethers and are normally stable in aqueous or alcoholic base [112]. An additional advantage of using silyl compounds as protecting groups is that they may be selectively removed by treatment with fluoride ion (usually as tetrabutylammonium fluoride).

It was felt that the preparation of a TBDMS derivative of 1-HAP should, in theory at least, be possible. This derivative, if prepared could provide a potentially readily available source of 1-HAP upon treatment with TBAF. Normal silylating reagents give acidic byproducts, due to the demonstrated lability of 1-HAP this was undesirable. Mawhinney and Madson reported the development of a new silylating reagent, N-methyl-N-(tert-butyldimethylsilyl) trifluroacetamide (MTBDMSTFA) (64) that it was thought might prove suitable for use on 1-HAP [112]. This reagent gives neutral, easily removed byproducts and

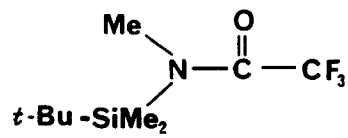
quickly silylates most amine and hydroxyl groups (typical reaction times 5-20 minutes) [112].

When a solution of 1-HAP in DMF was treated with the recommended amount of MTBDMSTFA (approx. 50-fold molar excess) a rapid colour change occurred. The colour remained constant after 5 minutes at room temperature. When the solution was analysed by RP-HPLC after 10 minutes only small quantities of 1-AP, 1-NOP, silylated ascorbic acid and a single major unidentified product were observed. No trace of 1-HAP was observed. The major unidentified chromatographic peak was collected, the solvent removed in vacuo and the residue analysed by UV-VIS spectroscopy and probe MS. Comparison of its HPLC retention time, UV-VIS and mass spectrum with standards indicated that this compound was an azopyrene. No N-silylated products were detected from the attempted silylation of 1-HAP.

Under identical conditions as for 1-HAP, 1-AP was silylated to some extent by MTBDMSTFA (approximately 15% yield by peak area after 14 hours). The reaction was found to give a better yield at elevated temperature (approximately 40% yield at 60°C). Analysis of the solutions by RP-HPLC showed a single new peak, this peak was fluorescent under long wave UV light. The new peak was collected and analysed by UV-VIS and probe MS. The UV-VIS spectrum was similar to 1-AP. The probe MS had a molecular ion (M^+) of m/z=331. A major

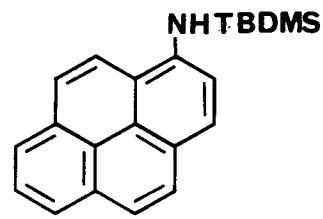
fragment ion of m/z= 57, which is characteristic of TBDMS derivatives[113], was observed in the mass spectrum. This would correspond in structure to the compound N-TBDMS 1-AP 64. A search of the Chemical Abstracts data base revealed that this compound had not been reported previously. When the new peak was collected and reanalysed by RP-HPLC both 1-AP and the original peak were observed. The conclusion here is that the mono-TBDMS derivative (65) of 1-AP was formed from the silylation reaction and that this N-TBDMS derivative was somewhat unstable. The 1-AP is N-silylated by MTBDMSTFA much more slowly than the reaction is occurring with 1-HAP.

The interpretation of the results from these two experiments is that 1-HAP was being O-silylated very rapidly and the O-TBDMS compound formed was then undergoing a further, rapid reaction to form azopyrene. A possible mechanism involves silylation followed by rapid deoxysilylation to form a nitrenopyrene and TBDMS silanol, two nitrenopyrenes may then couple to form azopyrene[114-116] . However, it is very unlikely that a nitrenopyrene exists as a free, discrete intermediate under the experimental conditions in sufficient quantities to couple, it is more probable that a single nitrenopyrene attacks another molecule of O-silylated 1-HAP to start a chain reaction decomposition as presented below in Figure 30 [117].



t-Bu-$SiMe_2$= TBDMS

MTBDMSTFA 64



N-TBDMS-1-AP 65

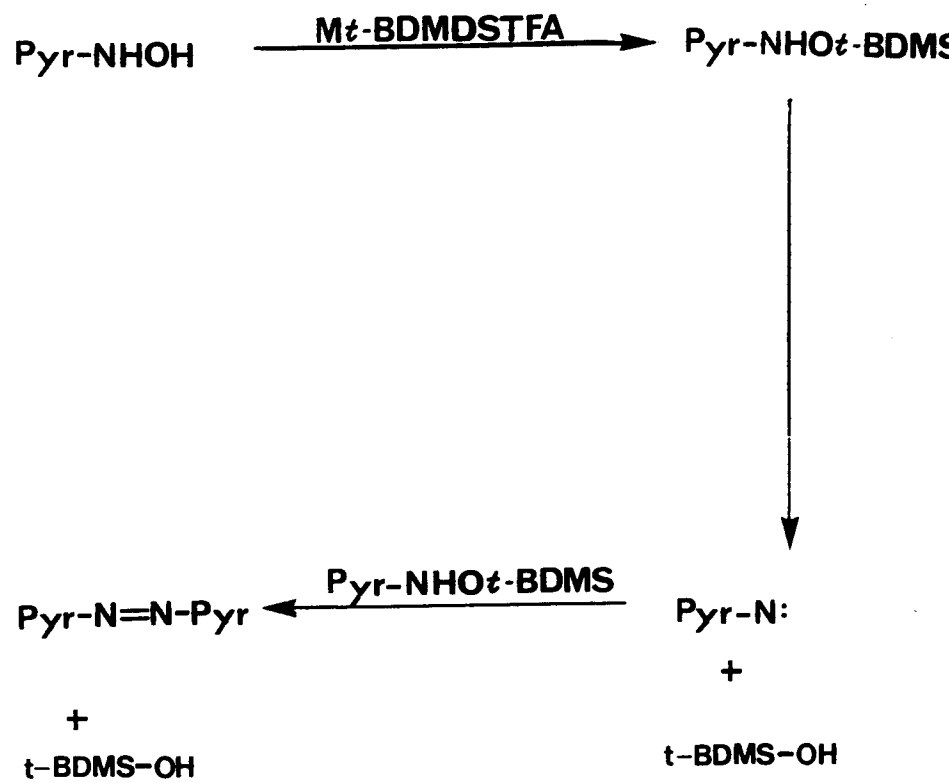


Figure 30: Proposed mechanism for the formation of azopyrenes during the attempted silylation of 1-HAP.

Most of the derivatization methods outlined in Section 1.4.5.2 had failed or were not suitable for 1-HAP. Although Tsui et al [62] were able to prepare the O-TMS and N,O-bis-TMS derivatives of phenylhydroxylamine the conditions required (BuLi) and solvent used (Et_2O) were not practical for 1-HAP.

As a result some alternative methods for the preparation of 1-HAP derivatives were examined.

2.5.5.3 Treatment of 1-Nitrosopyrene with Methyllithium

The addition of Grignard reagents to some aromatic nitroso compounds has been reported to provide the N-substituted hydroxylamines [52]. It was decided to try this method in an attempt to provide an alternative route to a derivative of 1-HAP.

A solution of 1-NOP in dry Et_2O was treated with excess methyllithium, an immediate colour change occurred and the solution became fluorescent under long wave UV light. The UV-Vis spectrum of 1-NOP (λ_{max}= 393,433, 454 nm) disappeared and a new spectrum with λ_{max} at 350 and 477 nm appeared. This new spectrum was different from that of 1-AP (λ_{max}. =362 nm). The fluorescence emission spectrum ($\lambda_{excitation}$=290 nm) had emission maxima at 430 and 530 nm compared to 1-AP which had an emission maximum at 422 nm. In addition, the excitation wavelength maximum for each of the emission maxima were different; the 430 nm emission has an excitation max. at 346 nm while the 530 nm emission has an excitation max. at 477 nm. These two emission maxima could therefore indicate the

existence of two different compounds. The identity of these compounds is not certain. It is suspected that one of the species may be a nitroso radical anion [118]. Support for this proposed identity comes from the fact that nitroso radical anions have been reported to be formed from the addition of Grignard reagents to nitroso compounds [52,118]. Although this was an interesting experimental result, time constraints did not permit further work to be carried out on this reaction.

2.7 Conclusion

The results reported above have shown that:

1) The compound formed in the ascorbic acid reduction of a solution of 1-nitrosopyrene in dimethylformamide or dimethylsulfoxide is N-hydroxy-1-aminopyrene. This compound has been characterized by a combination of RP-HPLC, UV-VIS, fluorescence, ^{1}H and ^{15}N NMR spectroscopy.

2) The technique of combined liquid chromatography-mass sprectrometry allowed the mass spectra of the reduction solution to be obtained after all attempts with probe-MS had failed. The product observed, 2-hydroxy-1-aminopyrene 60, was most likely formed from 1-HAP *via* a Bamberger rearrangement reaction. The results of the ^{1}H and ^{15}N NMR studies on the reduction of 1-NOP in DMF indicated the rearrangement was taking place in the LC-MS system.

3. Although 1-HAP was stable in DMF with a $t_{1/2}$ of >24 hours, it was found to be extremely labile in aqueous media and polar organic solvents. A variety of reactions were observed for 1-HAP in aqueous media including, reduction, oxidation, disproportionation, condensation and rearrangement (Figure 30). The exact reaction observed was dependent on the pH and solvent.

4. The 1-HAP is probably the proximate mutagen derived from 1-NP that reacts *in vivo* with the nucleophilic sites on DNA . The demonstrated mutagenicity of 1-NP may be due to its lability ($t_{1/2}$ approximately 70 sec.) at physiological pH.

5. The reaction of 1-HAP with deoxyguanosine in DMF or pH 6 buffer did not result in the formation of a nucleoside adduct. The importance of the DNA double helix in adduct formation remains to be ascertained for this compound.

6. Sulfation, acetylation and silylation were not suitable methods for preparing a derivative of 1-HAP. Despite all attempts, no stable derivative of 1-HAP was formed.

7. The ascorbic acid reduction of the 1,6-NNOP and 1,8-NNOP in DMF followed a similar pattern of reaction. The HANP's were characterized by HPLC, UV-VIS, ^{1}H and ^{15}N NMR spectroscopy. Additional characterization of 1,8-HANP was performed by LC-MS.

8. Reverse-phase HPLC columns should not be considered completely inert. The Vydac C_{18} RP-HPLC columns in which the silanol groups are more effectively capped proved to be a better column for analysing the 1-NOP reduction solutions. Obviously then, the HPLC column to be used should be chosen with some attention to the chemistry of the compounds which are being studied.

3. EXPERIMENTAL

3.1 Instrumental Conditions

3.1.1 High Performance Liquid Chromatography

All high performance liquid chromatography (HPLC) analyses were performed in an isocratic mode on one of the following instruments:

(i) Beckman Model 110A

(ii) Spectra Physics Model SP8700

(iii) Hewlett Packard Model HP-1090A

Reverse-phase HPLC (RP-HPLC) analyses were carried out using one of the following columns:

(A) Merck RP-18, 5 μm, 4.6 mm x 150 mm

(B) Whatman ODS-2, 10 μm, 4.6 mm x 250 mm

(C) Altex ODS-3, 5 μm, 4.6 x250mm

(D) Vydac TP201 C_{18}, 10 μm, 4.6 x 250 mm

(E) Vydac TP201 C_{18}, 5 μm, 4.6 mm x 250 mm

(F) Vydac TP201 C_{18}, 5μm, 1 mm X 250 mm

(G) Whatman ODS-3, 3μm, 1 mm X 250 mm

Unless otherwise stated the solvent conditions were 70% acetonitrile/water (70% ACN/H_2O) at a flow rate of 2.0 mL/min. The eluant from the column was monitored at 254 nm with a Beckman model 153 UV detector for instruments (i) and (ii). The HP1090A was equipped with an ultraviolet-visible diode array detector. Fluorescence detection was performed using a

Kratos FS 950 Fluoromat instrument with filters set at 365 nm (max.) for excitation and at 418 nm (min.) for emission.

HPLC grade solvents were obtained from Caledon Laboratories, Georgetown, Ontario. HPLC grade water was obtained as needed from a Millipore ultrapurification system. All other solvents used were A.C.S. reagent grade obtained from BDH Chemicals, Toronto, Ontario. Other chemicals were obtained from Aldrich Chemical Corporation, Milwaukee, Wisconsin.

3.1.2 Ultraviolet-Visible Spectroscopy

Ultraviolet-visible (UV-VIS) spectrophotometric measurements were performed on Hewlett Packard HP8451A or Perkin Elmer Lambda 9 instruments. The UV-VIS spectrum of eluting peaks in HPLC analysis were recorded on a Hewlett Packard 1090A liquid chromatograph equipped with a diode-array detector. The extinction coefficient (ϵ) at 254 nm was determined for HPLC measurements. Spectral data are given as $_{max}$followed by (ϵ). Fluorescence spectrum measurements were performed on a Perkin Elmer LS-5 instrument.

3.1.3 Mass Spectrometry

All mass spectrometry (MS) was carried out on a VG 7070F mass spectrometer equipped with a VG 2035 data system. Samples were introduced via either,

(a) a solid probe inlet or

(b) a moving belt interface for combined liquid

chromatography-mass spectrometry (LC-MS).

Electron impact (EI) ionization was used in both cases with an electron energy of 70 eV.

(a) For samples introduced *via* the solid probe inlet system, the sample was taken up in a volatile solvent (typically CH_2Cl_2) and approximately 20 μL of the solution was transferred into a melting point capillary tube. The solvent was removed *in vacuo* and the tube was then flame sealed and submitted for analysis. The sample was evaporated into the source by heating the probe tip linearly to 200°C.

(b) Combined LC-MS was performed by using a VG moving belt interface with a Kapton belt. Vydac C_{18}, 5 μm μbore or Whatman ODS-3, 10 μm μbore columns were used with a mobile phase of 70% ACN/H_2O and a flow rate of 30 μL/min. A 1 μL aliquot of the solution was injected onto the HPLC column. The entire effluent from the μbore column was run onto the belt and an infrared evaporator was used to remove the solvent prior to the vacuum lock system.

This instrumentation has previously been described in more detail by D'Agostino [48] and Quilliam [97].

Mass spectra are reported as m/z value followed by (relative intensity in brackets), (M^+ = molecular ion).

3.1.4 Nuclear Magnetic Resonance Spectroscopy

Proton magnetic resonance (^{1}H NMR) spectra were obtained on Bruker WM-250 or WM-400 instruments operated in the Fourier Transform (FT) mode with a deuterium lock signal using 5mm thin walled NMR tubes. Typical spectral parameters are given in Table 15 (below). Chemical shifts are reported in δ values (ppm) *followed by multiplicity (s = singlet, d = doublet, t =* triplet, m = multiplet), assignment and coupling constant in Hz, (all in brackets). Peak assignment for compounds were determined with the aid of two dimensional correlated spectroscopy (2D-COSY). Unless otherwise stated deuterated dimethylsulfoxide (DMSO-d_6) was used as the solvent and the DMSO-d_6 multiplet at 2.49 ppm was used as the reference peak for ^{1}H NMR analyses. Deuterated solvents for ^{1}H NMR were obtained from M.S. & D. isotopes, Montreal, Quebec.

Nitrogen-15 magnetic resonance (^{15}N-NMR) spectra were obtained on a Bruker AM-300 instrument with 10 mm NMR tubes using DEPT or INEPT techniques. The chemical shifts are reported in δ values (ppm). Unless otherwise stated dimethyl formamide (DMF) was used as the solvent with the DMF peak at 104 ppm used as the reference peak. DMF was used as an external standard (with δ= 104 ppm) for ^{15}N spectra obtained in DMSO.

Table 15: Instrumental Parameters for ^1H NMR

	WM400	WM250
Spectral Width (SW)	6024	4000
Offset Frequency (OF)	8000	5500
Pulse Width (PW)	4.0	3.0
Relaxation Delay (RD)	1.0	1.0
Resolution (Hz/pt)	0.5 to 0.7	0.5 to 0.7

3.1.5 Polarography

Polarography was performed on a Metrohm 626 Polarecord instrument with a dropping mercury working electode, platinum auxiliary electrode and a Ag/AgCl/LiCl (saturated in absolute EtOH) reference electrode. Reagent grade dimethyl formamide (DMF) was pre-dried over a 4 A Linde molecular sieves. Anhydrous tetraethylammonium perchlorate was used as an electrolyte. A micro sample vessel was used allowing a sample size of 1 mg. Samples were deoxygenated with oxygen-free N_2 gas for a minimum of 15 minutes before determining their reduction potentials.

3.2 Synthesis

3.2.1.1 1-Nitropyrene 9

1-Nitropyrene 9 was prepared according to Bavin and Dewar [92]. To a solution of pyrene 3 (Aldrich, 1 g, 0.00495 moles) in 50 mL acetic anhydride was added the nitrating reagent, prepared by the cautious addition of 400 μL of fuming HNO_3(90% W/V) to 10 mL ice-cooled acetic anhydride. After 8 hours at room temperature, the reaction was quenched by the slow

addition of 100 mL H_2O while cooling the solution in an ice bath. (Caution! Solution may overheat and "erupt" if cooling is not used). The resulting yellow precipitate was collected by filtration on a medium scintered glass filter funnel, washed two times with H_2O and then dried in a vacuum dessicator, to yield 1-nitropyrene (1.2 g, 0.00486 moles, 98.1%). RP-HPLC analysis showed this product contained no pyrene.

HPLC Data

	t_R(minutes)	k′
Column A	3.9	7.7
Column B	11.4	9.2
Column C	5	3.6
Column D	4.46	3.3
Column E	4.97	3.0

Polarography

$E_{1/2}$ = -0.85 V vs. saturated calomel electrode(S.C.E)

Spectral Data

UV-VIS MeOH: 232(50,000), 284(13,860), 372, 396, (ϵ_{254}=16,000)

70% ACN/H_2O: 290,378,404

^{1}H NMR

8.58 (d, H2, 8.47)
8.74 (d, H10, 9.35)
8.55 (d, H8, 7.71)
8.53 (d, H9, 9.35)
8.53 (d, H6, 7.71)[a]
8.47 (d, H5, 8.98)[b]
8.45 (d, H3, 8.47)
8.35 (d, H4, 8.98)[b]
8.27 (t, H7, 7.71)
a,b - assignments that may be revesed

MS 247(M^+,60), 217(100), 201(75)

(relative intensities variable)

3.2.1.2 [^{15}N]-1-Nitropyrene

[^{15}N]-1-Nitropyrene was prepared in a similar manner to 1-nitropyrene with some modifications.

Pyrene (1.49 g, 0.00737 moles) was dissolved in acetic anhydride (60 mL). A nitrating solution was prepared by dissolving 660 μL [^{15}N]-HNO_3 (99% atom %, M.S. & D Isotopes, Montreal) in 10 mL acetic anhydride. The nitrating solution was added to the pyrene solution and the combined solutions were stirred for 1.5 hours. The reaction solution was then quenched by adding 250 mL H_2O to the solution while cooling in an ice bath and stirred for a further 1 hour and the yellow precipitate was collected by filtration. The solid was analysed by HPLC and found to contain 1-nitropyrene and pyrene in a 3:2 ratio. (Note: the [^{15}N]-HNO_3 had been titrated with NaOH and found to be 14 M. However, the yield indicates the [^{15}N]-HNO_3 was only 7.5 M. It is suspected that the HNO_3 may be diluted in other acids). These compounds were separated by column chromatography. A silica gel column (33 cm x 20 cm i.d.) was prepared with hexane as the solvent. The [^{15}N]-1-nitropyrene/pyrene mixture (1.44 g) was adsorbed onto 3.55 g silica gel and slurried onto the top of the column. The eluting solvents, the fraction volumes and the percentages of 1-nitropyrene are given in Table 16 below.

Table 16

fraction #	elution volume (mL)	volume collected (mL)	solvent	%1-NP (by HPLC)
1	0-250	250	hexane	-
2	250-300	100	hexane	-
3	300-400	100	hexane	-
4	400-500	100	hexane	-
5	500-600	100	hexane	-
6	600-750	150	hexane	-
7	750-900	200	hexane	-
8	950-1150	200	hexane	-
9	1150-1400	250	hexane/1% acetone	0.3%
10	1400-1900	500	hexane/2% acetone	25%
11	1000-2150	250	hexane/5% acetone	97%
12	2150-2400	250	hexane/10% acetone	96%
13	2400-2650	250	hexane/20% acetone	99%

Fractions 11 and 12 were combined and the solvent was removed in vacuo to yield a yellow solid which contained 5% pyrene. This product was used for further synthesis of [^{15}N] labelled compound. Fraction 13 was used for a pure [^{15}N]-1-nitropyrene sample.

^{15}N NMR δ = 370

3.2.2.1 1-Aminopyrene **43**

1-Nitropyrene 9 was reduced to 1-aminopyrene 43 using the procedure of Messier et al [81]. A suspension of 1-nitropyrene (220 mg, 0.8907 mmoles) in 220 mL methanol (MeOH) was hydrogenated at room temperature and atmospheric pressure over Adams catalyst (Pt(IV)oxide,25 mg). After 2 hours the solution was a clear green colour and fluoresced blue under a long wave UV light. The solution was filtered through Celite and the methanol evaporated in vacuo providing 1-aminopyrene

43 as a slight green solid (192 mg, 0.885 mmoles, 99% yield). This solid fluoresced blue under long wave UV light. RP-HPLC analysis of this solid showed a single fluorescent peak.

HPLC Data

	t_R(minutes)	k′
Column A	1.95	3.3
Column B	5.7	3.8
Column C	2.65	1.48
Column D	2.47	1.40
Column E	2.64	1.64

Spectral Data

UV-VIS MeOH: 240(28,800), 283(23,600), 354,

(ϵ_{254} = 13,600)

DMF: 369, 386(sh), 400(sh)

Et_2O: 241, 284, 362, 401

70% ACN/H_2O: 243, 283, 259, 359

sh = shoulder

Fluorescence

Solvent	Excitation (nm)	Emission max.(nm)
DMF	369	434
ACN	360	441
70% ACN/H_2O	359	431
Ether	362	422

^{1}H NMR

8.24 (d, H10, 9.3)
7.98 (d, H6, 7.47)
7.97 (d, H8, 7.47)
7.95 (d, H3, 8.3)
7.89 (d, H9, 9.3)
7.86 (d, H5[a], 8.84)
7.85 (t, H7, 7.47)
7.70 (d, H4[b], 8.84)
7.34 (d, H2, 8.3)
6.35 (s, NH)

a,b - assignments may be reversed.

MS = 217(M+,100), 189(27)

3.2.2.2 [^{15}N]-1-Aminopyrene

A suspension of [^{15}N]-1-nitropyrene/pyrene (95% 1-nitropyrene) (784 mg, 3.03 mmoles) in 250 mL EtOH was hydrogenated at atmospheric pressure over Adams' catalyst. After 1 hour, analysis HPLC showed no 1-nitropyrene remaining with 1-aminopyrene and pyrene in a ratio of 95:5. The solvent was removed in vacuo yielding 690 mg of light green solid (95% [^{15}N[-1-aminopyrene, 3.02 mmoles, 99.6% yield). For ^{15}N NMR this mixture was not purified. However, for ^{1}H NMR the mixture was purified by prep LC on a Merck RP-8 Lobar column (70% ACN/H_2O, 8 mL/min).

^{15}N NMR δ=60.53

$^1J_{N-H}$= 85.3, $^3J_{N-H}$ = 2.

3.2.2.3 Hydrogenation of 1-Nitropyrene Under More Concentrated Conditions

To a 250 mL round bottom flask containing a stirbar were added 700 mg (2.83 mmoles) 1-nitropyrene 9, 25 mg Adam's Catalyst and 100 mL MeOH. Hydrogenation at atmospheric

pressure for 2 hours afforded a red precipitate and a clear green solution that fluoresced blue under long wave UV light. The red solid was collected by filtration and analysed by mass spectrometry and HPLC/UV-VIS. Mass spectrometry showed fragments at m/z=430 and 201. Analysis by HPLC on column D showed 2 peaks eluting at 10.8 minutes (λ_{max} = 270, 416) and 13.5 minutes. Comparison of these UV-VIS spectra to those of authentic azopyrene samples prepared by B.E. McCarry [91] indicated that the two peaks were due to azopyrenes 61 and 62 (most probably the cis and trans isomers).

3.2.3.1 N-Acetyl-1-aminopyrene **44**

The procedure of Messier et al [83] was employed. To a stirred solution of 100 mg 1-aminopyrene 43 (0.4608 mmoles) in 15 mL dichloromethane (CH_2Cl_2) was added 150 μL acetic anhydride (2.04 mmoles, 4 equivalents). After 1 hour the white precipitate formed was collected by filtration on a coarse fritted filter funnel. HPLC analysis showed a single peak that was not 1-aminopyrene.

HPLC Data

	t_r (minutes)	k′
Column A	1.13	1.5
Column B	4.5	3
Column C	1.87	0.75
Column D	1.98	0.98
Column E	2.05	1.05

Spectral Data

UV-VIS CH_2Cl_2: 242, 276, 340, 380

MeOH: 240, 274, 336, (ϵ_{254}=9,490)

70% ACN/H_2O: 275, 342

^{1}H NMR

10.31	(s, N-H)
8.38	(d, H10, 9.40)
8.34	(d, H2, 7.97)
8.34	(d, H5, 9.10)
8.27	(d, H9, 9.40)
8.14	(d, H3, 7.97)
8.11	(d, H6, 8.09)
2.27	(s, CH_3)

Note: H8, H7, H4 are not assigned

MS = 259(M^+, 45), 217(100), 216(30), 189(33)

3.2.3.2 [^{15}N]-N-Acetyl-1-aminopyrene

A solution of [^{15}N]-1-aminopyrene (95% 1-aminopyrene by weight) (630 mg, 2.76 mmoles) was acetylated with 610 μL acetic anhydride. After 1 hour the [^{15}N]-N-acetyl-1-aminopyrene was afforded as a white precipitate that was collected by filtration (yield = 700 mg, 98.3%).

Analysis of the solid by HPLC showed it to be 95% N-acetyl-1-aminopyrene and 5% pyrene. This mixture was used as is for ^{15}N NMR studies. For ^{1}H NMR the mixture was purified by prep LC on a Merck RP-8 Lobar column (70% ACN/H_2O, 8 mL/min).

^{15}N NMR DMF δ= 127.0

DMSO-d_6 δ= 128.2

$^1J_{N-H}$= 84.9

3.2.4.1 1-Nitrosopyrene **41**

The procedure used was adapted from that of Howard et al [83], m-Chloroperbenzoic acid (m-CPBA) (Sigma, 86% w/w, 210 mg, 1.04 mmoles) in 20 mL of CH_2Cl_2 was added to a stirred solution of 1-aminopyrene 43 (112 mg, 0.516 mmoles) in CH_2Cl_2 under a N_2 atmosphere at 0°C. After 2 hours a 20 μL aliquot analysed by HPLC showed a mixture of 1-nitrosopyrene 41 and 1-nitropyrene 9 in a ratio of 10:1 and some remaining 1-aminopyrene. The reaction was allowed to continue for a further 30 minutes and the solvent was then removed in vacuo. The residue was redissolved in CH_2Cl_2 and passed through a silica gel column (2 cm x 25 cm) to remove 1-aminopyrene and any remaining m-CPBA. A 20 μL aliquot of the eluant was analysed by HPLC and showed a 9:1 ratio of 1-nitrosopyrene to 1-nitropyrene. These were separated by preparative liquid chromatography (prep LC) on a Merck RP-8, Lobar Column (size B, 60% ACN/H_2O, 8 mL/min, resolution (Rs)=1.0). The 1-nitrosopyrene peak, which eluted at 90 minutes was collected and the solvent was evaporated in vacuo. HPLC analysis of the resulting orange brown solid showed it to be pure 1-nitrosopyrene. 1-Nitrosopyrene was found to be susceptible to air oxidation (forming 1-nitropyrene) and was always purified by prep LC prior to use.

HPLC Data

	t_r (minutes)	k′
Column A	5.03	10.17
Column B	15.25	12.8
Column C	6.47	5.06
Column D	5.90	4.8
Column E	6.74	5.74

Polarography

$E_{1/2}$ = -0.63 V (vs. S.C.E.)

Spectral Data

UV-VIS λ_{max} MeOH: 230, 256, 310, 396, 438, 460

DMF: 316, 468

Et_2O: 256, 298, 308, 390, 430, 450

70% ACN/H_2O: 259, 280, 305, 396, 464

ϵ values (MeOH): 234=48371, 254=6900, 302=140, 313=16752, 396=10017, 458=15846

^{1}H NMR

10.11	(d, H10, 9.08)
8.78	(d, H9, 9.16)
8.64	(d, H8, 7.55)
8.58	(d, H6, 7.57)
8.57	(d, H5, 8.79)
8.29	(d, H4, 8.84)
8.27	(t, H7, 7.6)
8.21	(d, H3, 8.59)
6.93	(d, H2, 8.66)

MS = 231 (M^+,41), 217(27), 201(100).

3.2.4.2 [^{15}N]-1-Nitrosopyrene

Purified [^{15}N]-1-aminopyrene (99.5%) was oxidized in CH_2Cl_2 with 2 equivalents of m-CPBA. After 1 hour HPLC analysis showed a 10:1 ratio of 1-nitrosopyrene to 1-nitropyrene. The solution was run through a short silica gel column and the solvent was removed *in vacuo*. HPLC analysis of the red brown solution showed a 8:1 ratio of 1-nitrosopyrene to 1-nitropyrene. For ^{15}N NMR these compounds were not separated. However, for ^{1}H NMR these compounds were separated by prep LC on a Merck RP-8 Lobar column (60% ACN/H_2O, 8 mL/min).

^{15}N NMR δ=893.4

$^{3}J_{N-H}$=1.3

3.2.5.1 N-Hydroxy-1-aminopyrene **42**

N-Hydroxy-1-aminopyrene 42 was synthesized according to Howard *et al* [85]. A 20 mM solution of pure 1-nitrosopyrene was prepared by dissolving 1.4 mg 1-nitrosopyrene 41 (0.0061 mmoles) in 300 μL DMF. To this solution was added 100 μL (2.5 equivalents) of a 150 mM ascorbic acid in DMF solution. The solution immediately decolorized and was found to fluoresce green-blue under long wave light (1-nitrosopyrene is not fluorescent). The UV-VIS spectrum had also changed from a $_{max}$ = 316, 468 nm for 1-nitrosopyrene to a $_{max}$ = 361 nm. HPLC analysis of a 20 μL aliquot on column E showed 3 peaks with retention times of 1.97, 2.64 and 6.74 minutes, corresponding to N-hydroxy-1-aminopyrene, 1-aminopyrene and 1-nitropyrene.

These compounds were typically found in ratios of 85:5:10 (based on peak areas). The use of dried DMF or an argon atmosphere did not improve the yield of N-hydroxy-1-aminopyrene. The reduction of a 1-nitrosopyrene in DMSO solution gave identical results by UV-VIS, fluorescence and HPLC analysis. When solutions of 1-nitrosopyrene in ACN or MeOH were treated with ascorbic acid only 1-aminopyrene was formed; no trace of N-hydroxy-1-aminopyrene was seen by HPLC analysis. Attempts to purify N-hydroxy-1-aminopyrene (for MS etc) by peak collection and solvent evaporation were unsuccessful. In each instance 1-aminopyrene, 1-nitrosopyrene and in some cases azopyrenes were formed in varying quantities. Reduction of a 4 mM 1-nitrosopyrene in DMSO-d_6 solution with ascorbic acid gave a solution that fluoresced green-blue under long wave UV light. Analysis of this solution by ^{1}H NMR showed only N-hydroxy-1-aminopyrene to be present. The N-hydroxy-1-aminopyrene was found to decompose on HPLC columns A and B as evidenced by peak tailing and variability in peak areas

HPLC Data

	t_R(minutes)	k′
Column A	1.05	1.33
Column B	2.93	1.6
Column C	2.2	1.06
Column D	1.95	0.9
Column E	1.97	0.97

Spectral Data

UV-VIS DMF: 361

70% ACN/H_2O: 241, 280, 352, (ϵ_{254} = 9,600)

Fluorescence

DMF: Ex= 361 Em. max.= 482

70% ACN/H_2O: Ex= 352 Em. max = 467

^{1}H NMR

9.47 (s, N-H)
8.77 (s, O-H)
8.17 (d, H10, 9.26)
8.15 (d,H3, 8.18)
8.09 (d, H8, 7.52)[a]
8.09 (d, H6, 7.98)
8.01 (d, H9, 9.26)
7.98 (d, H4, 9.13)[b]
7.94 (d, H7, 7.75)
7.84 (d, H5, 9.13[b]
7.84 (d, H2, 8.18)

a,b - assignments may be reversed.

3.2.5.2 [^{15}N]-N-Hydroxy-1-aminopyrene

[^{15}N]-1-Nitrosopyrene in DMF or DMSO (5 mM solutions) was reduced with 2-3 equivalents ascorbic acid. The solution immediately decolorized and fluoresced green-blue under long wave UV light. For ^{1}H NMR the [^{15}N]-1-nitrosopyrene was purified by prep LC.

^{15}N NMR DMF δ=137.4

$^1J_{N-H}$=82.8

3.2.6. 1,6-Dinitropyrene 39

Purified 1,6-dinitropyrene 39 was obtained from LC Services Corp., Woburn, Mass.

HPLC Data

	t^R(minutes)	k′
Column C	5.88	4.88
Column C (80% ACN/H_2O)	3.76	2.76
Column E (1 mL/min)	6.94	2.47

Spectral Data

UV-VIS 70% ACN/H_2O: 287, 381, 412

^{1}H NMR
8.86 (d,H2 and H7, 8.50)
8.84 (d, H5 and H10, 9.42)
8.69 (d, H3 and H8, 8.36)
8.66 (d, H4 and H9, 9.52)

3.2.7 1,8-Dinitropyrene **40**

Pure 1,8-dinitropyrene 40 was obtained from LC Services Corp., Woburn, Mass., U.S.A.

HPLC Data

	t_r(minutes)	k′
Column C	5.883	4.6
Column C(80% ACN/H_2O)	3.76	2.58
Column D	4.45	3.45
Column E(1 mL/min.)	7.09	2.55

Spectral Data

UV-VIS DMF: 302, 440

70% ACN/H_2O: 300, 440

^{1}H NMR
8.95 (s, H9, H10)
8.79 (d, H7, H2 8.58)
8.69 (d, H6, H3, 8.31)
8.59 (s, H4, H5)

3.2.8 1-Amino-6-nitropyrene **52**

1-Amino-6-nitropyrene 52 was synthesized and purified by N. Altintas.

HPLC Data

	t_R(minutes)	k′
Column C	3.38	2.38
Column E (1 mL/min)	5.77	1.88

Spectral Data

UV-VIS 70% ACN/H_2O: 254, 315, 489

ACN: 318, 398, 482

DMF: 316, 512

^{1}H NMR (DMSO-d_6)

8.61 (d, H7, 8.58)
8.60 (d, H5, 9.07)
8.51 (d, H10, 9.38)
8.25 (d, H9, 9.38)
8.18 (d, H3, 8.47)[a]
8.05 (d, H4, 9.07)
8.04 (d, H8, 8.58)
7.46 (d, H2, 8.47)[a]
7.09 (s, N-H)

(MeOH-d_4)

8.58 (d, H7, 8.58)
8.58 (d, H5, 9.2)
8.45 (d, H10, 9.24)
8.18 (d, H9, 9.24)
8.13 (d, H3, 8.36)
8.01 (d, H8, 8.58)
8.00 (d, H4, 9.20)
7.49 (d, H2, 8.36)

MS: 262 (M^+,95), 232(53), 216(100)

3.2.9 1-Amino-8-nitropyrene **53**

1-Amino-8-nitropyrene 53 was prepared and purified by N. Altintas.

HPLC Data

	t_R(minutes)	k′
Column C	3.73	2.73
Column D	3.08	2.08
Column E (1 mL/min.)	5.09	1.55

Spectral Data

UV-VIS MeOH: 302, 386, 440

DMF: 520

ACN: 302, 386, 486

70% ACN/H_2O: 305, 487

^{1}H NMR

8.67 (s, H9, H10)
8.60 (d, H7, 8.75)
8.20 (d, H3, 8.49)
8.17 (d, H5, 8.62)
8.05 (d, H6, 8.75)
7.87 (d, H4, 8.62)
7.46 (d, H2, 8.49)
7.09 (s, NH_2)

MS = 262(M^+,100), 232(68), 216(89)

3.2.10 N-Acetyl-1-amino-6-nitropyrene **54**

Acetylation of 1-amino-6-nitropyrene 52 in a manner similar to that for 1-aminopyrene 43 afforded N-acetyl-1-amino-6-nitropyrene 54. Specifically, to 19.4 mg (0.074 mmoles) of 1-amino-6-nitropyrene 52 in 15 ml $CHCl_2$ was added 160 μL (1.05 mmoles, 14 equivalents) of acetic anhydride. After 3 hours a yellow precipitate formed which was collected by filtration. HPLC analysis of the solid showed it to be pure N-acetyl-1-amino-6-nitropyrene 54.

HPLC Data

	t_R(minutes)	k′
Column C	2.3	1.3
Column E (1 mL/min)	4.27	1.14

Spectral Data

UV-VIS 70% ACN/H_2O: 241, 294, 413

MeOH: 288, 404

ACN: 302, 415

^{1}H NMR

10.53 (s, N-H)
8.73 (d, H7, 8.65)
8.67 (d, H5, 9.36)
8.60 (d, H10, 9.36)
8.50 (d, H3, 1.90)
8.48 (d, H9, 9.36)
8.37 (d, H4, 9.35)
2.25 (s, CH_3)

Note - H2 and H8 are not assigned.

MS: 304(M^+,100) 262(83), 216(69)

3.2.11 N-Acetyl-1-amino-8-nitropyrene **55**

This compound was prepared in an identical manner to N-Acetyl-1-amino-6-nitropyrene 54 using 1-amino-8-nitropyrene 53 and the same ratio of reagents.

HPLC Data

	t_R(minutes)	k′
Column C	2.26	1.26

Spectral Data

UV-VIS MeOH: 379, 413

^{1}H NMR 10.54 (s, N-H)
8.76 (d, H9, 9.67)
8.73 (d, H7, 8.45)
8.66 (d, H10, 9.67)
8.50 (d, H3, 8.24)[a]
8.46 (d, H2, 8.24)[a]
8.51 (d,H5)[b]
8.27 (d, H4)
a,b - assignments may be reversed.
CH_3,H6 - not assigned

M.S.: 304(M^+,100), 262(96), 232(42), 216(80)

3.2.12 1-Nitro-6-nitrosopyrene **57**

m-CPBA (Sigma, 85% w/w, 24 mg, 0.118 mmoles) was added to a stirred solution of 1-amino-6-nitropyrene 52 (14 mg, 0.0534 mmoles) in 10 mL CH_2Cl_2 under N_2. After 2 hours the solvent was evaporated in vacuo and the residue was taken up in a minimum volume of CH_2Cl_2 and was passed through a short silica gel column (15 cm x 2 cm i.d.). The CH_2Cl_2 was removed in vacuo and the residue (16 mg) was dissolved in 7 mL DMSO. Analysis of a 20 μL aliquot by RP-HPLC showed a 3:2 ratio of 1-nitro-6-nitrosopyrene 57 to 1,6-dinitropyrene 39. These were separated by prep LC on a Merck RP-8 Lobar column 1 60% ACN (H_2O, 8 mL/min). The collected 1-nitro-6-nitrosopyrene peak (t_R = 15.3 minutes) was found to be pure by RP-HPLC analysis. The maximum practical concentration for ^{1}H NMR (to avoid excessive line broadening due to ring stacking) was found to be 4 mM in DMSO-d_6.

HPLC Data

	t_R(minutes)	k′
Column C (80% ACN/H_2O)	5.3	4.3
Column E (1 mL/min)	10.25	4.13

Spectral Data

UV-VIS 70% ACN/H_2O: 234, 297, 397, 445

DMF: 398, 446

^{1}H NMR

10.39 (d, H5, 9.26)
8.95 (d, H9, 9.42)
8.93 (d, H2, 8.50)
8.91 (d, H4, 9.26
8.86 (d, H3, 8.50)
8.61 (d, H10, 9.42)
8.47 (d, H8, 8.57)
7.00 (d, H7, 8.57)

MS: 276(M^+,67), 262(29), 246(39), 232(46), 216(51), 200(100)

3.2.13 1-Nitro-8-nitrosopyrene **46**

This compound was prepared by oxidation of 1-amino-8-nitropyrene 53. m-CPBA (Sigma, 85% w/w, 18 mg, 0.08865 mmoles, 2.03 equiv.) was added to a solution of 1-amino-8-nitropyrene (11.4 mg, 0.0435 mmoles) in 4 mL CH_2Cl_2. After 2 hours the solution was passed through a short silica gel column (15 cm x 2 cm). The CH_2Cl_2 was removed *in vacuo* and the residue was taken up in DMSO. Analysis of a 20 μL aliquot by HPLC showed an 8:1 ratio of 1-nitro-8-nitrosopyrene 46 to 1,8-dinitropyrene 39. These compounds were separated by prep LC on a Merck RP-3 Lobar column (size B,70% ACN/H_2O, 8 mL/min). The 1,8-dinitropyrene had a t_R=46 minutes and the 1-nitro-8-nitrosopyrene eluted at 60 minutes. Analysis of the collected

1-nitro-8-nitrosopyrene peak by HPLC showed it to be >99% 1-nitro-8-nitrosopyrene. The maximum practical concentration for NMR analysis in DMSO-d_6 was found to be 4 mM.

HPLC Data

	t_R(minutes)	k′
Column C (80% ACN/H_2O)	3.76	2.58
Column D	6.78	5.78
Column E (1 mL/min)	11.15	4.58

Spectral Data

UV-VIS DMF: 302, 440

70% ACN/H_2O: 291, 398

^{1}H NMR
10.49 (d, H9, 9.64)
9.24 (d, H10, 9.64)
8.92 (d, H2, 8.53)
8.77 (d, H3, 8.53)
8.75 (d, H4, 8.90)
8.51 (d, H6, 8.50)
7.04 (d,H5, 8.50)
H7- not assigned

MS: 276(M^+,68), 246(53), 216(33), 200(100).

3.2.14 N-Hydroxy-1-amino-6-nitropyrene **59**

A 7 mM solution of purified 1-nitro-6-nitrosopyrene 57 (1.5 mg,0.0054 mmoles) in DMF was reduced with 90 μL (4 equivalents) of a 210 mM ascorbic acid in DMF solution. The solution immediately turned a deep red violet colour. Analysis by HPLC of this solution showed 2 peaks at t_R=3.9 and 5.8 minutes corresponding to N-hydroxy-1-amino-6-nitropyrene 59 and 1-amino-6-nitropyrene 52 in a ratio of 9:1. If less than 3.5 equivalents of ascorbate were used the N-hydroxy-1-aminopyrene underwent rapid reoxidation to 1-nitro-6-

nitrosopyrene. Reduction of a 4 mM solution of 1-nitro-6-nitrosopyrene in DMSO-d_6 with 4 equivalents of ascorbic acid showed only N-hydroxy-1-amino-6-nitropyrene by ^{1}H NMR.

HPLC Data

	t_R(minutes)	k′
Column E (1mL/min.)	3.90	.915

Spectral Data

UV-VIS 70% ACN/$H_2$0: 305, 462

DMF: 480

^{1}H NMR

10.04 (s, N-H)
9.08 (s, O-H)
8.64 (d, H7, 8.61)
8.53 (d, H5, 9.42)
8.48 (d, H9, 9.16)
8.38 (d, H3, 8.68)
8.34 (d, H4, 9.42)
8.19 (d, H2, 8.68)
8.16 (d, H10, 9.16)
7.93 (d, H8, 9.61)

3.2.15 N-Hydroxy-1-amino-8-nitropyrene **48**

A 4 mM solution of 1-nitro-8-nitrosopyrene 46 was prepared by dissolving 1.75 mg in 1.6 mL DMF. A 100 μL aliquot of this solution was removed and reduced with 10 μL (3.75 equivalents) of a 150 mM solution of ascorbic acid in DMF. The solution immediately underwent a colour change from orange-brown to deep red-violet colour. Analysis of the solution by HPLC showed 3 peaks due to N-hydroxy-1-amino-8-nitropyrene 48, 1-amino-8-nitropyrene 53 and 1-nitro-8-nitrosopyrene 46 in a ratio of 96:2.5:1.5. When a 4 mM solution of 1-nitro-8-nitrosopyrene in DMSO-d_6 was reduced

under identical conditions only N-hydroxy-1-amino-8-nitropyrene 48 was detected by ^{1}H NMR.

HPLC Data

	t_R(minutes)	k′
Column D	2.23	1.23
Column E (1 mL/min)	3.77	0.89

Spectral Data

UV-VIS DMF: 386, 486

70% ACN/H_2O: 298, 382, 465

^{1}H NMR

10.08 (s, N-H)
9.09 (s, O-H)
8.71 (d, H9, 9.74)
8.66 (d, H7, 8.70)
8.56 (d, H10, 9.74)
8.40 (d, H3, 8.59)
8.27 (d, H5, 8.88)
8.19 (d, H6, 8.69)
8.00 (d, H4, 8.88)
7.92 (d, H2, 8.58)

3.2.16 [^{15}N]-Dinitropyrene

A suspension of [^{15}N]-1-nitropyrene (55.7 mg, 0.2246 mmoles) in 11 mL acetic anhydride was nitrated at room temperature. The nitrating solution was prepared by diluting 1.10 mL (100 equivalents) fuming [^{14}N] HNO_3 to 10 mL with acetic anhydride. The nitrating solution was added slowly to the nitropyrene and the reaction was left at room temperature for 1 hour. The reaction was then quenched by slowly adding 30 mL of 0.1% H_2SO_4 with ice cooling. The precipitate was collected by filtration. HPLC analysis of the solid showed three peaks which had identical retention times to authentic

standards of the isomeric 1,3:1,6 and 1,8-dinitropyrenes.

3.2.17 1,6- and 1,8-[^{15}N]-N-Acetylaminonitropyrenes

A [^{15}N]-N-Acetyl-1-nitropyrene (600 mg, 2.3 mmoles) suspension in acetic anhydride (23 mL) was nitrated with [^{14}N]-HNO_3 at room temperature. The nitrating solution was prepared by dissolving 1.84 mL (17 equivalents) fuming HNO_3 in 2 mL acetic anhydride. After 3 hours the reaction was quenched by adding 50 mL H_2O with cooling. The yellow-green precipitate was collected by filtration. HPLC analysis of the solid showed a mixture of 1,6 and 1,8-N-acetylamino-nitropyrenes (N-acetyl-1-aminonitropyrenes).

[^{15}N]-NMR DMSO-d_6 δ= 129.5, 129.7 (mixture of isomers)

3.2.18 [^{15}N]-1-Amino-6-nitropyrene and [^{15}N]-1-Amino-8-nitropyrene

The isomeric aminonitropyrenes were prepared by two different methods.

(a) A mixture of the 1,6 and 1,8-[^{15}N]-N-acetylamino-nitropyrenes (619 mg, 2.03 mmoles) were hydrolyzed in 60 mL ethanolic KOH (0.5 M) at 60°C. After 9 hours the solution was transferred to a separatory funnel, diluted with 100 mL H_2O and then extracted with 8 x 100 mL CH_2Cl_2. The CH_2Cl_2 solution was dried with anhydrous Na_2SO_4 and the solvent was removed *in vacuo*. The solid was analysed by HPLC and found to contain the 1,6- and 1,8-aminonitropyrenes as well as a small quantity of unhydrolysed N-acetylaminonitropyrenes. The isomeric aminonitropyrenes were separated on a neutral alumina column

(activity II) with CH_2Cl_2. The activity II alumina was prepared by adding 5 g H_2O to 145 g alumina. After heat evolution had ceased the alumina was slurried into the glass column with hexane/0.5% acetone as the solvent. The aminonitroyrene mixture was dissolved in acetone, adsorbed onto 7.5 g alumina and slurried with hexane: CH_2Cl_2, 1:1, onto the alumina column. Elution of the aminonitropyrenes was begun with hexane/CH_2Cl_2 and then changed to straight CH_2Cl_2 after 50 mL. The fractions collected and elution volume are given in Table 17 below. Fractions 2-4, 5-7, 8-11 and 13-16 were combined. Analysis of these fractions by HPLC showed the area ratio of aminonitropyrenes to be as shown in Table 18 (below).

Table 17

Fraction #	Volume collected (mL)	Solvent
1	0-75	hexane/CH_2Cl_2
2	75-115	CH_2Cl_2
3	115-145	"
4	145-175	"
5	175-205	"
6	205-235	"
7	235-265	"
8	265-295	"
9	295-325	"
10	325-365	"
11	365-405	"
12	405-505	"
13	505-580	"
14	580-630	CH_2Cl_2/MeOH
15	630-680	"
16	680-705	"

Table 18

Fractions	1,6-ANP	1,8-ANP
2-4	4	1
5-7	1	1.6
8-11	1	6.9

The chemical shifts of the ^{15}N atom in the 1,6 and 1,8 compound were found to be identical.

^{15}N NMR DMF: δ=68.75

DMSO-d_6: δ=73.11

(b) A second more direct route to the aminonitropyrenes used the Zinin polysulfide reduction [95]. A basic solution (10 mL) of sodium polysulfide (Na_2S, sulfur, H_2O; 1:4:10) was added to a stirred solution of the [^{15}N]-dinitropyrenes (59.9, 0.2031 mmoles) in 200 mL EtOH/CH_2Cl_2 (1:1). Reduction to the aminonitropyrenes was effected in 30 minutes. Excess sulfide was precipitated by washing 2x with 100 mL 1 mM cadmium chloride. The [^{15}N]-aminonitropyrenes were extracted into CH_2Cl_2 and the solvent was removed in vacuo. Analysis of the solid by HPLC showed a mixture of 1,3, 1,6 and 1,8-aminonitropyrenes in a 1:2:2.33 ratio.

^{15}N NMR NH_2 group, DMF: δ=68.8

DMSO-d_6: δ=73.1

NO_2 group DMF: 374.2, 375.1, 375.4

3.2.19 1,6-and 1,8-[^{15}N]-Nitronitrosopyrenes

A solution of 1,6- and 1,8-[^{15}N]-aminonitropyrenes in CH_2Cl_2 was oxidized with 2 equivalents m-CPBA at room temperature. The solution was passed through a short silica gel column to remove any aminonitropyrene and m-CPBA. The solvent was evaporated in vacuo. Analysis of the orange-brown solid by HPLC showed a 1:6 ratio of dinitropyrenes to nitronitrosopyrenes.

3.2.20 1,6- and 1,8-[^{15}N]-N-Hydroxylaminonitropyrene

A 4 mM solution of a [^{15}N]-Nitro,nitrosopyrene mixture (1,6 and 1,8) was reduced with 4 equivalents ascorbate. The solution immediately changed to a deep red-violet colour.

^{15}N NMR δ=139.6

3.3 Combined Liquid Chromatography-Mass Spectrometry Studies

3.3.1 Combined LC-MS of a Mononitropyrene Standards Mixture

A standard mixture containing approximately equimolar amounts of N-acetyl-1-aminopyrene 44, 1-aminopyrene 43, 1-nitropyrene 9 and 1-nitrosopyrene 41 in ACN was prepared. The solution was filtered through a 0.5 μm filter. An aliquot of the solution was analysed by LC-MS on a Vydac C_{18} μbore column to establish the experimental conditions. The total ion current chromatogram (TIC) showed peaks at scan numbers 193, 246, 438 and 601.

3.3.2 LC-MS of a Reduced 1-Nitrosopyrene Solution

A 5 mM solution of 1-nitrosopyrene (100 μL) was reduced with 3 equivalents of ascorbate. The solution was checked for

green fluorescence under long wave UV light. The solution was then filtered through a 0.5 μm filter. An aliquot of the reaction mixture was analysed by LC/MS using a Vydac C_{18} μbore column (F). The TIC showed peaks at scan numbers 161, 242, 431 and 580. The peak at scan No. 161 had the following mass spectrum: 233(M^+,100), 232(32), 204(36).

3.3.3 Combined LC-MS of N-Hydroxy-1-aminopyrene Solutions

3.3.3.1 [^{14}N]-N-Hydroxy-1-aminopyrene

An 18.7 mM solution of [^{14}N]-1-nitrosopyrene in DMF was reduced with 2.5 molar equivalents of ascorbate. HPLC analysis (column B) of the resulting solution showed reduction to N-hydroxy-1-aminopyrene had taken place. The solution was then filtered through a 0.5 μm filter. A 1 μL aliquot of the solution was analysed by LC-MS on the Whatmen μbore column(G). The peak observed at 144 scans had the following mass spectrum: 233(M^+,100), 232(19), 204(29).

3.3.3.2 [^{15}N]-N-Hydroxy-1-aminopyrene

A 16 mM solution of [^{15}N]-1-nitrosoyrene in DMF was reduced with 2.2 molar equivalents of ascorbate. The resulting solution was analysed by HPLC (column B), which showed N-hydroxy-1-aminopyrene to be present. The solution was filtered through a 0.5 μm filter and then analysed by LC-MS on column G. The TIC had peaks at scan numbers 145, 220 and 458. The peak at 145 scans had the following mass spectrum: 234(M^+,100), 233(19), 205(31).

3.4 Reactions of N-Hydroxy-1-aminopyrene

3.4.1 Bamberger Rearrangement of N-Hydroxy-1-aminopyrene

An 8.65 mM solution of 1-nitrosopyrene (500 μL, 0.00433 mmoles) in DMF was reduced with 5 molar equivalents of ascorbic acid. The resulting solution was fluorescent under long wave UV light. Analysis of an aliquot of the solution by HPLC (column A) showed 3 peaks with t_R=1.2, 1.88 and 5.1 minutes in a ratio of 8:4:1. To this solution was added 1.5 mL of 0.1 N HCl. The acid was then neutralized with 300 μL of 1.0 N NaOH and the solution was extracted with 5 mL ethyl acetate (EtOAc). HPLC analysis of the EtOAc layer showed 2 peaks with t_R=1.41 and 1.88 minutes with areas in a ratio of 1:13. The EtOAc was removed in vacuo. A sample of the residue was submitted for probe MS. analysis: 233(25), 232(8), 217(100), 204(7), 189(31).

Since the probe temperature was raised linearly from ambient to 250°C over 5 minutes a plot of m/z versus scan number (which is directly related to probe temperature) can be made. A plot of m/z = 233 and 204 versus temperature had a maximum at scan number 39. A plot of m/z = 217 had a maximum at scan number 35 and was zero at scan number 39 (Figure 23).

3.4.2 pH Stability of N-Hydroxy-1-aminopyrene

The pH stability of N-hydroxy-1-aminopyrene (as measured by its half-life) in buffered solutions of pH 2.25-10 was determined by RP-HPLC measurements. Chromatography data was acquired on an Apple II+ compatible computer equipped with a

12 bit analog to digital converter and an autoranging amplifier (Interactive Microwave, State College, PA) interfaced and programmed by Dr. M.A. Quilliam (Chemistry Department, McMaster University)

The $t_{1/2}$ values in these experiments were determined in the following manner:

i) typically an approximately 15 mM solution of 1-nitrosopyrene in DMF, containing anthracene (column E, t_R=3.75 minutes) as an internal standard was reduced with 2-3 equivalents ascorbic acid, providing the 1-HAP. An aliquot of the 1-HAP solution was removed and diluted in an appropriate volume of the 200 mM buffer in a Reacti-vial (Wheaton Scientific). The vial was capped and quickly agitated on a vortex mixer to ensure complete mixing. The resulting solution was analysed after 10 seconds. This procedure was then repeated for an analysis at 60 seconds. Based on the results of these two determinations, the experiments were repeated for times greater or less than 60 seconds. The half-life of N-hydroxy-1-aminopyrene in the buffer was time at which the ratio of peak areas for 1-HAP to the anthracene was one half the ratio of peak areas in DMF before adding to the buffer. In DMF the $t_{1/2}$ of 1-HAP was found to be approximately 24 hours. The dilution factor of 1-HAP/DMF in buffer required to mimic an aqueous system was determined by examining the $t_{1/2}$ value in several different ratios DMF: pH 7 buffer (Table 13). As a result of this, a DMF:buffer ratio of 1:19 was decided

upon and was used to determine the $t_{1/2}$ values in Table 14.

3.4.3 Reactivity of N-Hydroxy-1-aminopyrene with Deoxyguanosine

3.4.3.1 N-Hydroxy-1-aminopyrene and Deoxyguanosine in DMF

A 20 mM solution of 1-nitrosopyrene in DMF (600 μL, 0.012 mmoles) with anthracene as an internal standard, was reduced with ascorbate. The resulting solution fluoresced green-blue under long wave UV light. To this solution was added 600 μL (0.012 mmoles) of a 20 mM solution of deoxyguanosine (dG) (Sigma Chemicals) in DMF. The solution was incubated for 40 hours at 37°C. Aliquots of the solution were analysed by HPLC (column E, 50% ACN/H_2O, 2 mL/min) with simultaneous fluorescence and UV detection. Under these conditions no nucleoside adducts were detected.

3.4.3.2 N-Hydroxy-1-aminopyrene and dG in pH 6 Buffer I

A 20 mM solution of 1-nitrosopyrene in DMF (500 μL, 0.01 mmoles) containing anthracene as an internal standard, was reduced with ascorbate. To this solution was added 500 μL (0.0005 mmoles) of a 10 mM dG in pH 6 buffer solution. The solution was incubated at 37°C for 40 hours. When a 20 μL aliquot of the solution was analysed by HPLC (column E, 50% ACN/H_2O, 2 mL/min) with UV and fluorescence detection, no nucleoside adducts were detected.

3.4.3.3 N-Hydroxy-1-aminopyrene and dG in pH 6 Buffer II

A 20 mM solution of 1-nitrosopyrene in DMF (500 μL, 0.01 mmoles) with anthracene as an internal standard, was reduced with ascorbic acid. A 50 μL aliquot (0.001 mmoles) of this solution was added to 500 μL (0.005 mmoles) of a 10 mM solution of dG in pH 6 buffer. The resulting solution was incubated for 40 hours at 37°C. An aliquot was analysed by HPLC (column E, 50% ACN/H_2O, 2 mL/min) with fluorescence and UV detection. No nucleoside adducts were detected.

3.5 Derivatization Reactions

3.5.1 Attempted Acetylation of N-Hydroxy-1-aminopyrene

A 25 mM solution of 1-nitrosopyrene (200 μL, 0.005 mmoles) was reduced with ascorbic acid. The solution immediately began to fluoresce green-blue under long wave UV light. N-Acetylimidazole (9 g, 0.0818 mmoles, 16 molar equivalents) was added to the solution. No immediate change in the colour of the solution was observed. After 8 hours HPLC analyses (column E, mobile phase = 70% ACN/H_2O and 50% ACN/H_2O) indicated that no acetylation had taken place.

3.5.2 Silylation Reactions

All silylations were carried out according to Mawhinney and Madson [112]. The derivatizations were performed in teflon capped ReactiVials (Pierce Chemical Co.) under a dry N_2 atmosphere with DMF as the solvent and N-Methyl-N-(tertbutyldimethylsilyl)trifluoroacetamide (MTDMSTFA) 64 as the silylating reagent.

3.5.2.1 Silylation of N-Hydroxy-1-aminopyrene

A 25 mM solution of 1-nitrosopyrene (50 μL, 0.00125 mmoles) was reduced with ascorbic acid. The solution immediately began to fluoresce green-blue under long wave UV light. MTBDMSTFA 64 (50 μL, 0.20 mmoles, 30 equivalents based on the number of silylable functional groups) was added to the solution. The resulting solution immediately turned an orange-red colour. After 5 minutes no further colour change was observed. A 20 μL aliquot of the solution was analysed by HPLC (column E, 100 % ACN) ten minutes after MTBSTFA was added. The chromatogram showed a peak at 13.7 minutes which was not present in the blanks from silylation of ascorbic acid. When an aliquot of the same solution was analysed again by HPLC, but with a mobile phase of 100% ACN, the chromatogram showed peaks which eluted at approximately 10 minutes. The two peaks were collected separately and analysed by UV-VIS and probe MS. These analyses showed the two peaks to be due to the same compound. (m/z = 430, $_{max}$ = 272, 318, 334, 405). An authentic sample of azopyrene was analysed by HPLC under the same conditions and showed a broad peak at 10-12 minutes.

3.5.2.2 Room Temperature Silylation of 1-Aminopyrene

MTBDMSTFA 64 (40 μL, 0.185 mmoles) was added to 100 μL (0.0025 mmoles) of a 25 mM solution of 1-aminopyrene in DMF. Analysis by RP-HPLC showed no immediate reaction. After 8 hours at room temperature, a 20 μL aliquot was analysed by RP-HPLC (column E,). A new peak with t_R=19.9 minutes was

observed on the chromatogram. The area of this peak was 10% of the 1-aminopyrene peak (t_R=2.64 min). After 24 hours the 19.9 minute peak had grown in area to approximately 28% of the 1-AP peak. The 19.9 minute peak was collected and analysed by M.S. The mass spectrum had m/z = 331, 274, 217, 189, 57. This compound was identified as N-(tertbutyldimethylsily)-1-aminopyrene 65.

3.5.2.3 Silylation of 1-Aminopyrene at 60°C

MTBDMSTFA (40 μL, 0.185 mmoles) was added to 100 μL of 25 mM solution of 1-aminopyrene in DMF. The reaction was left for 8 hours at 60°C. HPLC analyses (column E) at this time showed a new peak in the chromatogram with t_R=21.5 minutes. The area of the new peak was equal to 80% of the 1-aminopyrene peak (t_R=2.64 min) area. The peak which eluted at 21.5 minutes was collected and analysed by UV-VIS, probe M.S. and HPLC.

The compound eluting at 21.5 minutes was not stable under the RP-HPLC conditions used. Thus, when an aliquot of the collected peak was reinjected onto the HPLC, peaks eluted at 2.64 min. and 21.5 min.

Spectral Data

UV-VIS $_{max}$ ACN: 228, 241, 286, 363, 401 nm

Fluorescence

(ACN) excitation = 363 nm; emission max = 425 nm

M.S.: 331(26), 274(50), 217(100), 216(32), 189(69)

3.5.3 Addition of Methyllithium to 1-Nitrosopyrene in Ether

1-Nitrosopyrene (0.05 mg, 0.02 mmoles) was dissolved in 10 mL anhydrous diethyl ether (Et_2O). A 4 mL aliquot of this solution was transferred into a quartz fluorescence cell. A 1.4 M solution of methyl lithium in Et_2O (Aldrich, 200 μL, 0.28 mmoles) was added to the contents of the fluorescence cell. The resulting solution immediately turned pink and became fluorescent under the long wave UV light. The $_{max}$ of the reaction mixture were 350 and 472 nm compared to 1-nitrosopyrene which has $_{max}$=257, 299, 311, 393, 433 and 454 nm in the same solvent. For an excitation wavelength of 290 nm the emission maxima were 430 and 533 nm. The 430 nm emission maximum has excitation maxima at 283 and 346 nm. The 530 nm emission maximum had excitation maxima at 297 and 477 nm. The reaction was terminated by adding 3 drops of 0.1 N HCl to the cell. Immediately after the addition of HCl a UV spectrum of the solution showed $_{max}$ of 278 and 348 nm compared to 1-aminopyrene which has a $_{max}$ at 284, 362 and 401 nm in the same solvent. The fluorescence spectrum of the reaction solution with excitation at 348 nm showed an emission maximum at 430 nm. 1-Aminopyrene in the same solvent with excitation 280 nm had an emission maximum at 422 nm. The Et_2O was evaporated under a stream of N_2. A sample of the residue was submitted for M.S. analysis: m/z (relative intensity), 231(100), 217(87), 216(48), 189(44). The remaining residue when redissolved in MeOH had $_{max}$ = 277, 313 and 350 nm. 1-

Aminopyrene had $_{max}$ of 240, 283 and 350 nm in the same solvent.

4. FUTURE WORK

The majority of the research objectives set out in Section 1.6 were attempted. A number of areas which require further study were indicated by these experimental results.

4.1 Synthesis

Part of the research work presented above involved the synthesis of a number of compounds derived from the mono and dinitropyrenes. Synthetic methods were developed for many of the compounds of interest, however some of the synthetic methods were quite laborious (involving time consuming chromatographic separations) and/or provided low yields. Further work is recommended on the development of more efficient alternative synthetic methods for these compounds, especially derivatives of the dinitropyrenes and isomers of mononitropyrene (eg. 3-nitropyrene).

4.1.1 Hydroxylaminopyrenes

The hydroxylaminopyrenes,1-HAP, 1,6-HANP and 1,8-HANP were successfully prepared in DMF or DMSO by the reduction of the corresponding nitroso compound. These compounds proved to be unstable in many other common organic solvents. In addition, the maximum yield obtained was limited by the solubility of the nitroso compound.

The electrochemical reduction of the nitro or nitroso pyrene in the manner of Wagenknecht [64] offers promise for the preparation of high yields of the free aromatic hydroxylamine under many different conditions. The use of constant

reduction potential should prevent over-reduction to the amine from occurring. Performing the reductions in an atmosphere of N_2 or Ar should prevent reoxidation from occurring. At present there is no experimental apparatus available at McMaster to perform electro-organic synthesis so the proper equipment must be obtained.

It has been noted that pronounced isomer effects were observed for the nitropyrenes [40,41]. Characterization was performed on the hydroxylamine of 1-NP. However, the 3-NP exhibits even greater bacterial mutagenicity than 1-NP. It would be of some interest to prepare and study the hydroxylamines derived from 3-nitropyrene, 2-nitropyrene as well as other nitro-PAH (for example 3-nitroperylene).

4.1.2 Derivatization Reactions

4.1.2.1 Silylation

Unfortunately, all attempts to prepare a stable derivative of 1-HAP were unsuccessful. The results from the room temperature silylation of 1-HAP in DMF with MTBDMSTFA provided some evidence for O-silylation occurring, however, this compound was apparently very unstable (forming azopyrenes) under the experimental conditions and was not isolated. Despite this the silylation of 1-HAP is still promising and further work is recommended. The use of other silylating reagents and conditions should be studied. Tsui *et al* have reported the formation of the O-TMS and N,O-bis-TMS-derivatives of phenylhydroxylamine under special conditions.

Further work could be performed on the characterization of the silylated 1-AP derivative reported above.

The HANPs are apparently more stable than the 1-HAP and the silylation of these compounds may prove easier.

4.1.2.2 Electrochemical Derivatization

A method which is considered very promising for the preparation of a stable derivative of 1-HAP or the HANP is the constant potential electrochemical reduction of the nitropyrene in the presence of a trapping agent such as alkyl halides, acyl halides, silyl halides or other neutral silylating reagents such as MTBDMSTFA. The same methodology as section 4.1.1 could be used. This method could, in principle, allow the acetyl derivative of 1,8-HANP, the postulated proximate mutagen, to be prepared. Once prepared, these compounds could be tested for biological activity.

4.1.2.3 Reaction of Methyllithium with 1-NOP

The experimental results for the addition of methylithium to 1-NOP were interesting and this reaction could be studied further. The effect of solvents and performing the reaction in the presence of trapping agents are areas which should be examined.

4.1.3 Bamberger Rearrangement of 1-HAP

The 1-HAP was found to undergo the Bamberger rearrangement in the LC-MS system and its apparent formation in very low yield was reported above. This reaction could be studied in more detail to prepare a pure sample of the

rearrangement product, 2-hydroxy-1-aminopyrene. This compound is of interest and should be thoroughly characterized (HPLC-UV-VIS, LC-MS, ^{1}H and ^{15}N NMR) and undergo biological testing to see if it differs in mutagenicity from 1-HAP. There was no evidence for the HANP undergoing the Bamberger rearrangement but it is recommended that this reaction be attempted for the HANP.

4.1.4 Reaction of Hydroxlaminopyrenes with DNA

The mechanism of the hydroxylaminopyrenes with DNA is not well understood. Other workers in this research group are currently studying these interactions. The major site of DNA adduct formation has been found to be the guanine moiety with much smaller quantities of other adducts. The importance of the DNA double helix structure in adduct formation has not been ascertained. The reaction of 1-HAP, the HANP and their derivatives with DNA should continue to be studied. The use of poly G DNA of various lengths may allow the mechanisms operating to be deciphered and give a better yield of adducts than present methods.

4.1.5 ^{15}N Labelled Compounds

The preparation of the ^{15}N analogues of all the N-pyrenes studied was carried out as part of this research. These compounds should be used for further chemical and biological testing. The reaction of labelled 1-HAP, 1,6-HANP and 1,8-HANP with DNA would be interesting to study. The ^{15}N compound could also be used in the silylations, electrochemical

reductions, probe MS, LC-MS, ^{15}N and ^{1}H NMR studies to aid in the interpretation of the experimental results.

4.2 Analytical Methodologies

In this project HPLC, LC-MS, UV-VIS, fluorescence and ^{15}N and ^{1}H NMR spectroscopy were used to characterize the compounds being studied. The use of LC-MS in future work should allow the characterization of even unstable compounds including DNA adducts in small quantities. The availability of new LC-MS interfaces, such as Thermospray (Vestec Corp.) and Particle Beam (Hewlett-Packard) should have a positive impact on LC-MS analysis. The wider availability of computerized high field ^{1}H NMR provides for greater sensitivity and simplicity in NMR analysis. There is currently very little information on the ^{15}N NMR of nitro-PAH in the literature. The ^{15}N NMR of the N-pyrenes and other PAH (fluorene, napthalene etc.) should be studied to provide results for comparison to those reported above. Finally it is recommended that the use of fourier transform infrared (FT-IR) should be examined to determine its applicability to the analysis of the nitro-PAH by HPLC-FT-IR etc..

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