SYNTHESIS OF A SERIES OF 4-ANILINO-7-NITROBENZOFURAZANS FOR THE PHOTOCHEMICAL PRODUCTION OF SINGLET OXYGEN

A Thesis

by

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ABSTRACT

SYNTHESIS OF A SERIES OF 4-ANILINO-7-NITROBENZOFURAZANS
FOR THE PHOTOCHEMICAL PRODUCTION OF SINGLET OXYGEN. (August 1985)

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A series of substituted 4-anilino-7-nitrobenzofurazans were synthesized, characterized, and tested for their capability to generate singlet oxygen in liquid solution. The compounds were tested for singlet oxygen production by monitoring the disappearance of the detector molecules, 2,5-dimethylfuran and 1,3-diphenylisobenzofuran, from irradiated reaction solutions. This was the initial step in classifying this series of compounds as prospective photosensitizers for use in the selective inactivation or destruction of biomolecules in vivo. Two compounds in the series, 4-(4´-nitro)-anilino-7-nitrobenzofurazan and 4-(3´-nitro)-anilino-7-nitrobenzofurazan, were found to be efficient producers of singlet oxygen in both detection systems. The related compound, 4-benzylamino-7-nitrobenzofurazan, was observed to generate singlet oxygen when excited by radiation filtered to remove wavelengths below 500 nm.

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CHAPTER I

INTRODUCTION

During the past two decades singlet oxygen, the first excited state of molecular oxygen, has received widespread attention as an important chemical entity in both *in vitro* and *in vivo* systems. It has been invoked as the effective agent in such phenomena as chemiluminescence, photodynamic action, photocarcinogenicity, and decomposition of O_2-rich compounds.[1] Singlet oxygen has been shown to be highly reactive and toxic to living things.[2,3]

Singlet oxygen is now known to be involved in many dye sensitized photooxidations of organic systems. The observed fluorescence quenching of many organic molecules by molecular oxygen has been established to involve a singlet oxygen mechanism in a large number of cases. The excited singlet state oxygen molecule acts as a highly reactive oxidizing agent toward a wide variety of compounds. Sensitized photooxidation reactions are now believed to be the major pathways by which singlet oxygen exhibits toxic effects on biological and biochemical systems.

The destructive capability toward living tissues inherent in dye sensitized photooxidations has been used by a number of workers in the successful treatment of tumors in humans and laboratory animals.[4,5] An organic sensitizer that is capable of generating the highly reactive singlet state of molecular oxygen while binding

selectively to proteins could offer promise as an effective treatment of cancer or other diseases caused by malfunctions at an enzymatic level.

Substituted nitrobenzofurazans have been used extensively as fluorescent probes of enzymes and other biological molecules. Additionally, this class of compounds has been demonstrated, with in vivo experiments, to be effectively cytotoxic toward a wide range of tumors.[6] The purpose of the present research is to synthesize a series of substituted 4-anilino-7-nitrobenzofurazans to determine whether the compounds are capable of efficient generation of singlet oxygen in liquid solution. This would be the initial step in classifying this series of compounds as prospective photosensitizers for use in the selective inactivation or destruction of biomolecules in vivo.

A. Sensitized Photooxidation

Molecular oxygen has long been observed to quench the fluorescence of many organic compounds with diffusion-controlled rate constants, as illustrated in Scheme 1.

$$\text{Sens} + \text{photon} \rightarrow \text{Sens}^*$$

$$\text{Sens}^* + O_2 \rightarrow \text{Sens} + O_2^*$$

Scheme 1.

A very efficient reaction occurs in the presence of suitable acceptors, sensitizers, and oxygen. This process, outlined in Scheme 2, is termed sensitized photooxidation. The following observations can be made of the mechanism: 1) the interaction of

excited state sensitizer and oxygen is involved and acceptors generally do not quench the sensitizer; 2) a kinetically recognizable intermediate is formed quantitatively at O_2 concentrations above 10^{-5} M; 3) the sensitizer is returned to the ground state and can undergo hundreds of cycles; 4) the intermediate either reacts with the acceptor to yield the product or decays. Good acceptors can trap the intermediate even at low concentrations. The intermediate is a very selective electrophile which reacts with dienes, olefins, and polycyclic aromatic hydrocarbons to give endoperoxides or hydroperoxides.[7]

$$\text{Sens}^* + O_2 \rightarrow \text{Intermediate} \begin{array}{l} \nearrow \text{Acceptor-}O_2 \\ \searrow O_2 \end{array}$$

Scheme 2.

Historically, two possibilities for the intermediate have been postulated: 1) excited state oxygen produced by energy transfer from sensitizer to oxygen; and 2) a sensitizer-oxygen complex.[7] Experimentally, the two mechanisms are kinetically equivalent, but several factors point toward the formation of an excited state of molecular oxygen as the intermediate.[2]

The possibility that excited state oxygen was the reactive intermediate in dye sensitized photooxidation reactions was first proposed by Kautsky in the 1930s.[8] Kautsky demonstrated that photooxidation could proceed even if sensitizer and acceptor were physically separated and concluded that the oxidation must have involved the formation of some reactive species capable of

diffusion in the gas phase. Since oxidation was not observed in the absence of oxygen, Kautsky postulated that an excited state of molecular oxygen was the oxidizing agent. Even so, Kautsky's proposal was never accepted in his lifetime. Most workers preferred the sensitizer-oxygen complex mechanism.[9]

The discovery that singlet oxygen was produced in high yield in the chemiluminescent reaction between NaOCl and H_2O_2 reopened interest in singlet oxygen as the reactive intermediate in photooxidation. In 1963 Khan and Kasha studied the chemiluminescence spectroscopically and observed two emission bands at 633.4 nm (15788 cm^{-1}) and 703.2 nm (14221 cm^{-1}). Since the spacing between these bands (1567 cm^{-1}) matched the spacing between ground state vibrational levels of molecular oxygen (1556 cm^{-1}), they correctly identified the emission as resulting from electronically excited oxygen molecules, but incorrectly assigned the 633.4 nm band to a hydrated O_2 molecule with solvent shifted singlet to ground state emission.[10,11] Arnold, Ogryzlo, and Witzke later correctly assigned that emission to a simultaneous transition involving the collision of a pair of singlet oxygen molecules to produce a singlet photon.[12]

In 1964 Foote and Wexler showed that the reaction products of dienes and olefins and singlet oxygen generated from the hypochlorite-H_2O_2 reaction were identical to products formed by dye sensitized photooxidation. Foote compared product distributions and stereoselectivity, relative reactivities of acceptors, and the ratio of decay rate to reaction rate of the intermediate with

more than 30 different olefins in order to arrive at this conclusion.[13] Corey and Taylor demonstrated that singlet oxygen produced from radiofrequency electrodeless discharge reacted with substituted anthracenes and olefins to yield the same products as sensitized photooxidation.[14] On the basis of this evidence, it was generally concluded that singlet molecular oxygen was the reactive intermediate in sensitized photooxidation reactions.

B. Singlet Oxygen

In order to gain an understanding of the excited state of the oxygen molecule, it is necessary to consider the electronic structure of the ground state. The molecular orbital energy level diagram for the oxygen molecule is shown in Figure 1. Molecular oxygen is unusual in that, as a molecule with an even number of electrons, it possesses a paramagnetic ground state, indicating the presence of electrons with unpaired spins. This observation has proved difficult to explain on the basis of valence electronic formulas, but is made understandable with molecular orbital theory. The highest energy occupied orbitals are a pair of $2p\pi^*$ doubly degenerate antibonding orbitals which hold one electron each.[15]

In the singlet state, the spins of the two electrons are paired, while in the triplet state the spins are parallel. The terms singlet, doublet, triplet, etc., arise from the definition of multiplicity: the value of $2S + 1$ where S is the total spin of the electrons in the molecule. Orbital angular momentum coupling rules can be used to show that singlet and triplet electronic states are possible for the oxygen molecule. With an even electron

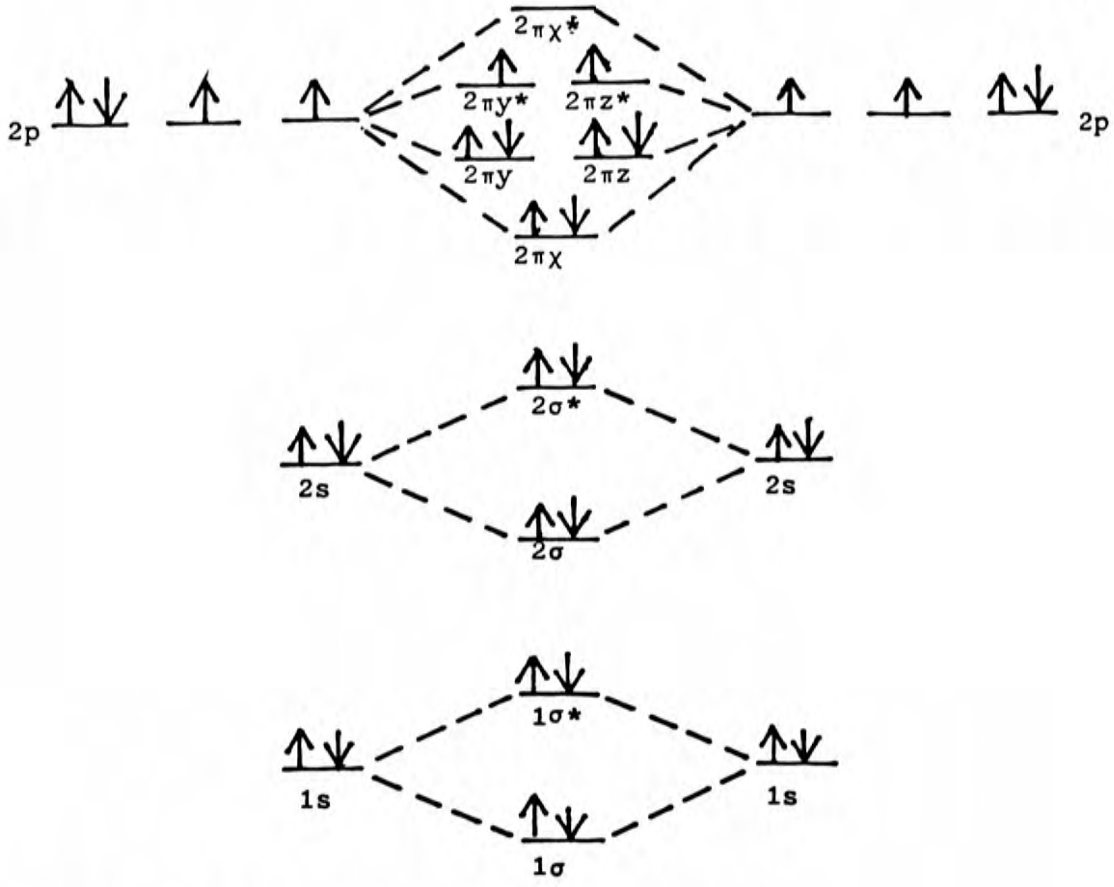

Figure 1. Molecular orbital energy diagram for O_2

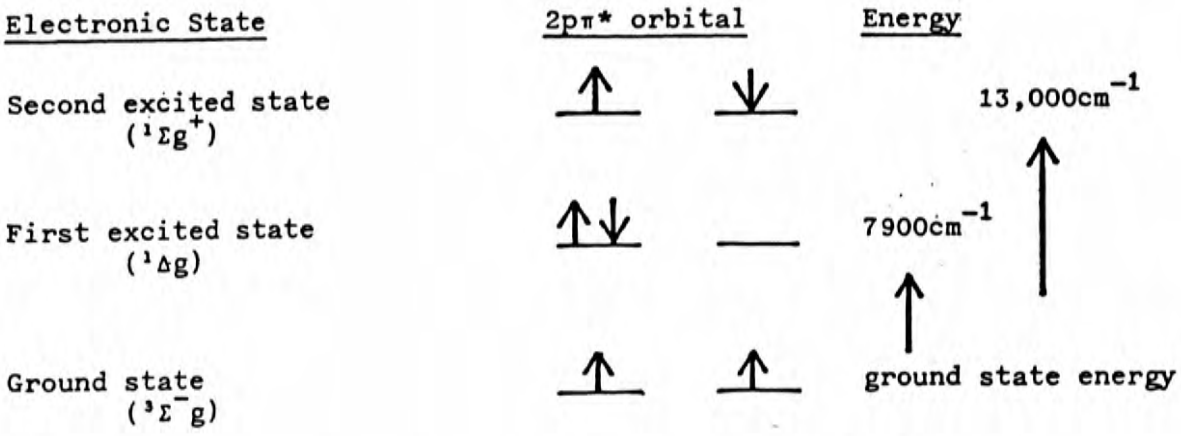

Figure 2. Electronic configurations of excited state O_2

system in which all electron spins are paired, S = 0 and 2S + 1 = 1. The molecule is said to be in a singlet state. In such a system with two electrons of unpaired spins, a triplet state is said to exist since S = 1 and 2S + 1 = 3.[15]

The three lowest energy states of molecular oxygen are shown in Figure 2. Ground state oxygen is designated $^{3}\Sigma g^{-}$ and the two lowest energy excited states are symbolized as $^{1}\Delta g$ and $^{1}\Sigma g^{+}$.[16] Diatomic molecular state term symbols are analogous to atomic state term symbols. The symbols Σ, π, Δ etc., designate electronic states in molecules just as S, P, D, etc., denote electronic configurations in atoms. The left superscript describes the multiplicity of the molecule. The excited states are singlet due to the fact that S = 0 with all electrons paired whether in the same or separate orbitals. The + or - superscript for Σ states indicates whether the electronic wave function changes sign with respect to reflection through the bond axis. The molecular orbitals of homonuclear diatomic molecules based on p atomic orbitals also possess a center of symmetry with respect to inversion. If this operation leaves the function unchanged, the subscript g is applied. Otherwise, the orbital is labelled u, (from the German gerade and ungerade).[17] A detailed analysis of the electronic structure of oxygen is available.[18]

Electronic transitions to both excited states can be observed in the upper atmosphere. Measurements show radiative lifetimes of 45 minutes for $^{1}\Delta g$ state and between 7 to 12 seconds for $^{1}\Sigma g^{+}$ state. Lifetimes are considerably shortened by intermolecular collisions.

In condensed phases the lifetimes are very short: $^1\Sigma g^+ \simeq 10^{-9}$ and $^1\Delta g \simeq 10^{-3}$ seconds.[18] Since the quenching of the $^1\Sigma g^+$ state by the solvent is known to be much faster than reaction with the acceptor, the $^1\Delta g$ singlet state is generally considered to be the reactive intermediate in sensitized photooxidations.[2]

C. Generation of Singlet Oxygen

Generation of singlet oxygen has been achieved experimentally by three important methods: peroxide decomposition, high-frequency discharge, and energy transfer from excited state sensitizer. Both $^1\Delta g$ and $^1\Sigma g^+$ states are produced by the reaction shown in Equation 1 with $^1\Delta g$ production approaching 80%.[19]

$$OCl^- + HO_2^- \text{ ---- } O_2 + Cl^- + OH^- \qquad \text{Equation (1)}$$

Electrical discharges have been used since the early 1900s for producing metastable species in the gas phase.[1]

Sensitizers produce singlet oxygen by energy transfer from an excited electronic state of the sensitizer. This state can be produced by the absorption of ultraviolet or visible light, continuous or pulsed, or by electron beams. The latter excite solvent molecules which rapidly transfer their energy to sensitizer molecules.[20]

The electronic states which may be occupied by sensitizers are illustrated in Figure 3. Direct excitation to a triplet state is spin forbidden and therefore occurs only rarely. The term internal conversion refers to intramolecular interactions by which a molecule passes to a lower energy electronic state without the emission of radiation. External conversion describes processes by

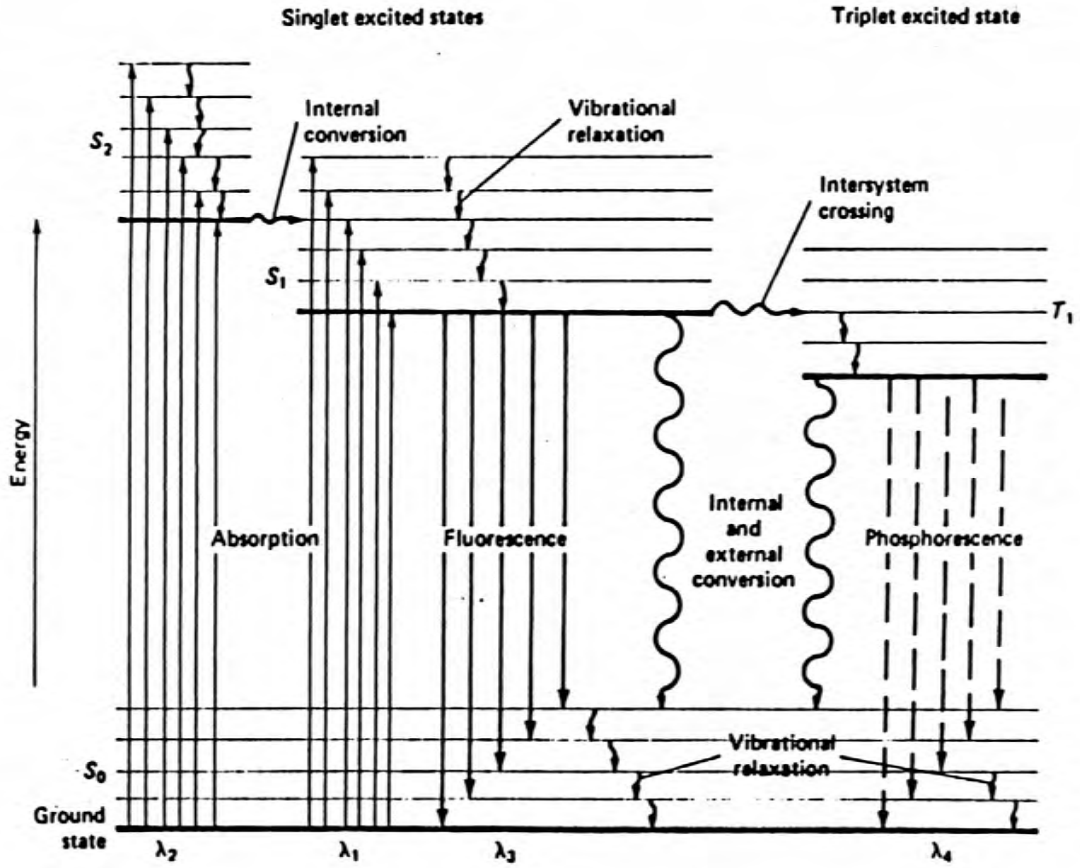

Figure 3. Electronic energy levels available to excited state molecules

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which deactivation of the excited electronic state proceeds through energy transfer to the solvent or other solutes without the emission of radiation. With intersystem crossing the spin of an excited electron is reversed resulting in a change in multiplicity. Intersystem crossing from the singlet state, S1, to the triplet state, T1, is also spin forbidden, but is the major pathway for formation of the T1 state. As with internal conversion, the probability of intersystem crossing is enhanced by close overlap between the vibrational levels of the two electronic states involved in the transition. Vibrational relaxation within a given level is a thermal process but deexcitation from S1 or T1 to the ground state may be accompanied by the emission of light; termed fluorescence and phosphorescence respectively.[21]

The mechanism by which molecular oxygen quenches the fluorescence of many organic molecules has been the subject of thorough investigation in recent years. The interaction of an excited singlet state sensitizer molecule with a ground state oxygen molecule produces a triplet state collision complex. The collision complex is not likely to decay to ground state sensitizer and ground state oxygen due to the large amount of electronic energy to be lost by the complex and the small Franck-Condon factor.[22] It is more probable that the spin-allowed dissociation of the complex to triplet sensitizer and singlet oxygen competes with deactivation of the complex to give triplet sensitizer and ground state oxygen.[23]

Studies have shown that quenching of excited singlet state sensitizer leads to formation of triplet sensitizer and ground state oxygen in most cases.[24] Other experimental results have indicated that the singlet state of some highly fluorescent organic molecules may sensitize singlet oxygen formation by the pathway illustrated in Scheme 3.[25]

$$^1\text{Sens}^* + O_2(^3\Sigma g^-) \rightarrow {}^3\text{Sens}^* + O_2(^1\Delta g) \quad (1)$$

$$^3\text{Sens}^* + O_2(^3\Sigma g^-) \rightarrow {}^1\text{Sens} + O_2(^1\Delta g) \quad (2)$$

Scheme 3.

The majority of energy transfer reactions, resulting in the production of singlet oxygen, take place from the triplet excited state of the sensitizer. The collision complex of excited triplet state sensitizer with ground state oxygen can be either singlet, triplet, or quintet in multiplicity which dictates a one in nine probability of singlet oxygen formation.[2] The singlet complex alone can produce singlet oxygen directly; the triplet complex dissociates to ground state products while the quintet produces triplet sensitizer and ground state oxygen. The observed quenching rate constant of one-ninth of diffusion control or lower has led to the conclusion that the major decay route occurs from the singlet complex; the products of which are ground state sensitizer and $O_2(^1\Delta g)$.[26]

Gijzeman, Kaufman, and Porter found that the most efficient sensitizers possessed triplet energies between 10000 and 15000 cm^{-1}. Studies have shown that the rate of quenching by molecular oxygen

of aromatic molecules with triplet energies greater than 15000 cm^{-1} does not show diffusion control and that the rate is solvent dependent. Two different mechanisms are considered important in explaining this phenomenon: electron exchange and charge transfer interactions.[26]

A great deal of work has been done on photosensitized electron exchange reactions during the past decade.[27,28] Foote and coworkers have suggested the mechanism outlined in Scheme 4 for the electron exchange photooxidations sensitized by electron deficient sensitizers and electron rich substrates, M, in oxygen-aerated polar solvents.[28]

$$\text{Sens} + h\nu \rightarrow {}^1\text{Sens}^* + \text{M} \rightarrow ({}^3\text{Sens}\cdot^- + \text{M}\cdot^+) +$$
$${}^3\text{O}_2 \rightarrow \text{Sens} + \text{M}\cdot^+ + \text{O}_2\cdot^- \rightarrow \text{MO}_2$$

Scheme 4.

The electron exchange mechanism produces a radical cation-superoxide ion pair.[29-33]

Quenching of triplet state sensitizers by molecular oxygen has been postulated to proceed via a charge-transfer mechanism, as illustrated in Scheme 5.[34]

$${}^3\text{Sens}^* + {}^3\text{O}_2 \rightarrow {}^1(\text{Sens}^*\ldots\text{O}_2) \rightarrow {}^1(\text{Sens}^+\text{O}_2^-) \rightarrow {}^1(\text{Sens}\ldots\text{O}_2^*) \rightarrow {}^1\text{Sens} + {}^1\text{O}_2^*$$
$${}^3\text{Sens}^* + {}^3\text{O}_2 \rightarrow {}^3(\text{Sens}\ldots\text{O}_2)^* \rightarrow {}^3(\text{Sens}^+\text{O}_2^-) \rightarrow {}^1\text{Sens} + {}^3\text{O}_2$$

Scheme 5.

The intermediate charge-transfer complex has been found to possess an energy greater than that of the singlet ground state

sensitizer and less than that of the excited triplet state sensitizer.[1] The CT mechanism explains oxygen quenching by processes of intersystem crossing between singlet and triplet C-T complexes with the added possibility of the decay of the triplet complex to ground state products through a CT state. The assumption is that intersystem crossing from the singlet to triplet state of the encounter complex is a slower process than dissociation of the triplet complex into products.[35]

The relative contribution of these two mechanisms is solvent dependent, with charge-transfer becoming more important in polar solvents.[26] The existence of electron exchange and charge transfer mechanisms demonstrates that dye-sensitized photooxidations cannot be blindly assigned to singlet oxygen mechanisms.

D. Decomposition of Singlet Oxygen

Singlet oxygen can undergo deexcitation to the ground state by emission of light and by physical or chemical quenching in solution. Equation 2 represents the biomolecular collision of singlet oxygen molecules in the gas phase leading to emission.[17]

$$2\,{}^1O_2 \rightarrow 2O_2 + \text{photon (633 nm)} \qquad \text{Equation (2)}$$

Singlet oxygen can be quenched in solution by the four different routes outlined in Scheme 6.

(1) $O_2(^1\Delta g) + S \rightarrow {}^3O_2 + S$

(2) $O_2(^1\Delta g) + {}^3O_2 \rightarrow 2\,{}^3O_2$

(3) $O_2(^1\Delta g) + M \rightarrow {}^3O_2 + M$

(4) $O_2(^1\Delta g) + M \rightarrow MO_2$

Scheme 6.

Pathways (1), (2), and (3) result from physical quenching by solvent, S, ground state oxygen, and substrate, M, respectively. Pathway 4 represents the chemical reaction of $O_2(^1\Delta g)$ with the substrate.[36]

Studies in recent years have shown that the singlet oxygen lifetime is solvent dependent.[37] Investigation of the nonradiative relaxation of singlet oxygen in solution has shown conclusively that quenching by pathway (1) involves a specific interaction with the hydrogen atoms of the solvent. The solvent acts as an energy acceptor rather than a donor. The determining factor in the physical quenching of $^1O_2$ by pathway (1) has been shown to be related to the energy of the highest frequency vibrational mode of the solvent.[38]

The lifetimes of singlet oxygen in various solvents has been determined experimentally. Decay measurements reveal singlet oxygen lifetimes ranging from 2 μsec in water and 7 μsec in methanol to 30 μsec in acetonitrile and 60 μsec in chloroform.[39]

A significant isotope effect is observed for the lifetime of singlet oxygen in deuteriated solvents. C- and O- deuteriation gererally increases the singlet oxygen lifetime. Of particular

importance is the observation that the lifetime in D_2O is ten times larger than that in H_2O, providing a powerful means of identification of singlet oxygen in photobiological processes.[37]

The physical quenching of $O_2(^1\Delta g)$ by ground state oxygen has also been studied.[2,40] Amines and β-carotene are known to be effective substrate quenchers of $O_2(^1\Delta g)$ by pathway (3).[41-43] The differentiation between physical and chemical quenching is generally achieved in experiments that compare the extent of the removal, if any, of the quencher to that of a substance known to react exclusively via a chemical pathway with singlet oxygen.[1]

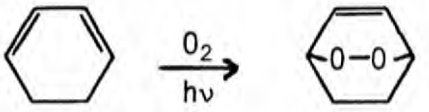
E. Detection of Singlet Oxygen

Detection of singlet oxygen in the gas phase is made comparatively easy by the use of spectroscopic techniques.[2] In condensed phases the detection is confined chiefly to the use of chemical tests; however, the detection of singlet oxygen by direct spectroscopic observation of its emission in solution has recently been reported.[44-46] The emission in solution results in infrared bands at around 1270 and 1590 nm which corresponds to the $^1O_2$ ($^1\Delta g$ ($v = 0$) → $^3\Sigma g^-$ ($v = 0$)) and the $^1O_2$ ($^1\Delta g$ ($v = 0$) → $^3\Sigma g^-$ ($v = 1$)) transitions respectively. The first observation of the 1270 nm emission in solution was made by Krasnovsky[47] and then by Khan and Kasha.[48] Detection of the much weaker 1590 nm emission was reported by Khan[49] and Salokhiddinov.[50]

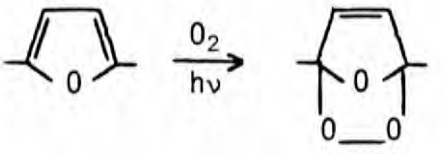
The most widely used detection method of singlet oxygen in solution is the acceptor method. Three generally accepted types of addition reactions of singlet oxygen with unsaturated organic

molecules are known: 1) 1,4 transannular cycloaddition of singlet oxygen to cis-dienes or aromatic hydrocarbons to form cyclic peroxides; 2) 1,2 cycloaddition of singlet oxygen with some olefins to form an unstable dioxetane intermediate which may cleave to yield carbonyl fragments; 3) the reaction of singlet oxygen with olefins to form allylic hydroperoxides.[27]

In reactions with acceptors that are cis-dienes or aromatic hydrocarbons, singlet oxygen behaves as a good dienophile in a manner similar to Diels-Alder reactions. The oxygenation of 1,3-cyclohexadiene and 2,5-dimethylfuran belong to this category.



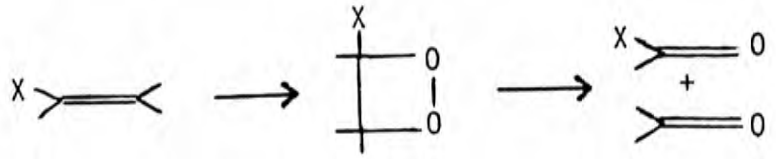
Equation (3)



Equation (4)

The concerted character and low activation energy for these addition reactions have been proven experimentally. The addition of singlet oxygen to aromatic hydrocarbons appears to be a uniquely specific reaction and therefore a very useful diagnostic test for detection of singlet oxygen.[2]

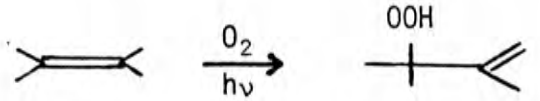
Electron-rich olefins, generally those containing an N, O, or S atom attached to the double bond, react with singlet oxygen to cleave the double bond via a dioxetane intermediate according to Equation 5.[1]



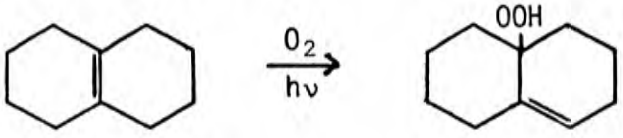
Equation (5)

The existence of dioxetane intermediates in singlet oxygen reactions was definitely established with the discovery of synthetically produced stable dioxetanes and the isolation of a stable dioxetane from the sensitized photooxidation of cis-diethoxyethylene.[51] Several dioxetanes have now been isolated.[52,53] The 1,2-cycloaddition of $^1O_2$ to vinyl ethers, vinyl sulfides, enamines, and tetraalkyl-substituted olefins yields 1,2-dioxetanes.[54] Direct observation of a dioxetane from the singlet oxygen photooxidation of a thioketeneacetal has recently been reported.[55]

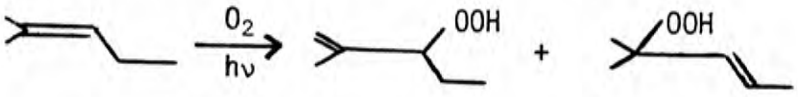
An extensively investigated type of singlet oxygen addition is the "ene" reaction with mono olefins to form allylic hydroperoxides.



Equation (6)



Equation (7)



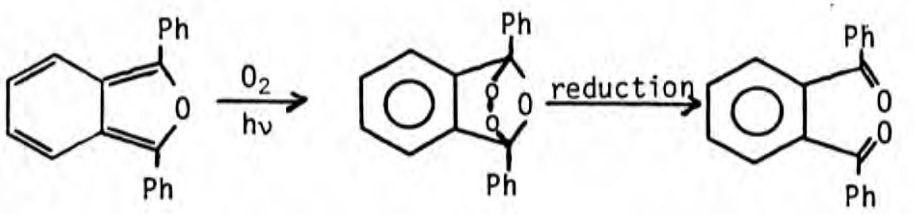
Equation (8)

The oxygen always enters on the same side of the molecule from which the hydrogen leaves and the double bond always shifts.[2]

General requirements for the efficient chemical detection of singlet oxygen are that the reaction between the detector molecule and singlet oxygen should be rapid, completely specific, free of side reactions, and easily tested for products. All of these requirements are usually not met. Of the chemical methods described, only the formation of transannular peroxides from aromatic hydrocarbons is specific for singlet oxygen.[2]

The acceptor 2,5-dimethylfuran (DMF) is very reactive toward singlet molecular oxygen with relatively little physical quenching being involved.[22,25] Gollnick showed that the quantum yield of triplet formation of Rose Bengal in methanol is 0.76 which equals the quantum yield of $^1O_2$ formation and the quantum yield disappearance of DMF, indicating that there is no physical quenching with this acceptor.[56] Wu and Trozzola confirmed the quantum yield of the rubrene-sensitized photooxidation of DMF to equal the yield of singlet oxygen produced.[25]

The reaction of 1,3-diphenylisobenzofuran (DPBF) has also been shown to be a predominantly chemical quencher of singlet oxygen.[20,57]



Equation (9)

Merkel and Kearns showed that the amount of DPBF removed in a laser flash experiment equalled both the amount of singlet oxygen removed and the amount of triplet dye formed.[24] As with DMF, actinometry offers excellent confirmation of chemical quenching rate constants.[36,58] Gorman and associates measured the rate of loss of DPBF over a range of DPBF concentrations using a number of different sensitizers and found all of the rate constants for bleaching of DPBF to lie on a single line. If the reactive intermediate were not the same in each experiment; sensitizer-oxygen complex for example, the sensitizer independence of the rate constant would be unexpected.[20]

In addition to being sensitive diagnostic tests for singlet oxygen, the oxidations of DPBF and DMF can be easily detected by monitoring spectrally the decrease in absorbance at 415 nm and 220 nm respectively.[25,56,59,60]

F. Biological Role of Singlet Oxygen

Oxidative processes are of utmost importance in biological systems. The massive amount of attention focused recently on singlet oxygen along with the realization that it possesses a lifetime sufficient to allow it to react chemically, has prompted many biologists and biochemists, in studies of phototoxicity and photodynamic action, to rationalize their data in terms of singlet oxygen mechanisms. An enormous amount of literature has been produced concerning the biological and biochemical significance of singlet oxygen. Many excellent reviews are available.[3,61]

Singlet oxygen oxidation mechanisms fall under two major categories:

1) Photodynamic Effects - the oxidative destruction of cells and tissues in the presence of light, a sensitizer (extrinsic or intrinsic), and molecular oxygen, and;

2) Metabolic oxidations which occur naturally without radiant excitation.

The requirements for photodynamic action are the same as those for singlet oxygen production: the presence of sensitizers, light, and oxygen. As pointed out previously, these criteria alone are not sufficient to insure a singlet oxygen intermediate (electron transfer, charge transfer, etc.). Oxidations which do not involve $^1O_2$ are termed Type I reactions while those which use a $^1O_2$ intermediate are Type II. Type II behavior is established by 1) enhancement of the process in a D_2O-based system and 2) restriction or prevention of the process in the presence of known $^1O_2$ quenchers; azide ions, 9,10-anthracene-dipropionic acid, diazabicyclooctane, etc. In all of the studies done to date, in which singlet oxygen has been labelled as a participant in lethal or sub-lethal damage, the conclusion has been inferred from indirect evidence: through the presence of oxidation products known to be produced in singlet oxygen reactions, from the effects of D_2O and quenchers, and by the identification of specific products from added substrates.[1] So far, the best substrate for identifying singlet oxygen in biological systems appears to be cholesterol, which has been used to differentiate between singlet oxygen reactions and radical reactions.[3]

Photodynamic action was first described by Raab who found that light was necessary for the death of paramecia placed in a solution of eosin dye.[62] Biochemical photooxidations, from molecules in aqueous solutions to the damage and death of multicellular animals, have since been studied extensively. Spikes offers a comprehensive review of most published work on a variety of systems, both experimental and naturally occurring.[63] A few pertinent examples will be presented here.

A large number of studies have concentrated on the effects of photodynamic action on proteins, amino acids, and nucleic acids. Clear evidence exists for the participation of $^1O_2$ in the photooxidation of some amino acids, particularly histidine, methionine, tyrosine, and tryptophan.[1,64] In addition, the deactivation of the enzymes alcohol dehydrogenase and trypsin, in a photochemical process, is enhanced in D_2O and quenched by NaN_3.[65] Singlet oxygen has been invoked in the photodynamic oxidation of tryptophan residues in several enzymes (lysozyme, papain).[66,67] The yeast Saccharomyces cervisiae exhibits dormancy and genetic changes upon being photosensitized by xanthene, thiazene, and acridine dyes. The effects are enhanced in D_2O and quenched by azide.[68,69] In 1970 Khan and Kasha described a mechanism by which $^1O_2$ could react with cellular DNA to initiate a carcinogenic process involving polycyclic hydrocarbons.[22] The photodynamic inactivation of enzymes and other proteins is thought to involve the oxidation by $^1O_2$ of the side chains of amino acids.[70]

A thorough review of the intervention of singlet oxygen in metabolic events not involving radiant excitation is given by Krinsky.[3] It has been proposed that $^1O_2$ may be the microbicidal agent present in leukocytes. Singlet oxygen formation has been suspected due to the release of superoxide ion and H_2O_2 during phagocytic action and also due to the inability of leukocytes to kill bacteria containing the very effective $^1O_2$ quencher β-carotene.[3]

G. Photosensitized Oxidations in the Treatment of Tumors

The earliest attempt to use the effect of photodynamic action on tissues for the treatment of human tumors was described by Tappenier and Jesionek in 1903.[71] Favorable results were reported using eosin as the photosensitizing dye. The ability of porphyrins to localize in tumor tissue was first recognized in the 1940s.[72] Diamond et al. reported the successful use of crude hematoporphyrin activated by white light in causing the regression of rat tumors in 1972.[73] At about the same time, Dougherty and coworkers observed similar results with a fluorescein dye. The fluorescein dye was injected into subcutaneous tumors in laboratory mice followed by periodic irradiation over a period of days. Subsequent measurement of tumor size revealed dramatic, though not complete, remission of the tumors in all cases. In addition, the fluorescein sensitizer was observed to bind preferentially to the tumor tissue so that healthy tissue was left undamaged by the induced photodynamic action.[74]

Dougherty and coworkers later found hematoporphyrin derivative (HPD) activated by red light to be very effective in the experimental

treatment of tumor systems.[75] Singlet oxygen was later proven to be the cytotoxic agent.[76] Tomson et al. described in 1975 the use of acridine orange in the destruction of mouse epithelial tumor.[77]

Intensive investigation into the use of porphyrins, most notably HPD, in the photoradiation therapy (PRT) of neoplastic tissues has taken place in recent years.[4] A wide variety of histological types of tumors have been successfully treated both in laboratory animals and in human subjects. The success of the technique is due to the tumor localization capability of HPD and to its ability to produce singlet oxygen when irradiated by red light. PRT has proven to be a useful method for treating certain carcinomas which have not responded to chemotherapy, immunotherapy, or ionizing radiation. Relatively little damage is incurred by the normal tissue surrounding the malignancy.[5]

The general method of PRT treatment involves at least a 3 day interval between drug injection and light exposure (by fiber optics) in order to allow for selective uptake of HPD by tumor cells.[5] Research has shown that while HPD is quickly taken up by normal cells (within 2 hours), it is also rapidly lost; although a small amount does remain.[78] The increased concentration or prolonged retention of HPD in tumor tissues is incompletely understood but is believed to result from some biochemical properties unique to malignant cells (protein binding, membrane changes, aberrant biotransformation) or cancer tissue (increased blood supply, avascular zones, absent lymphatics).[79] In addition, the mechanism of cell killing is not known.[4] Both the degree of

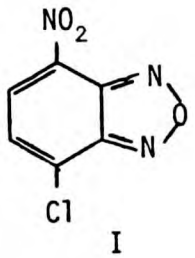
uptake and the strength of photoactivated toxicity have been observed to be a function of the hydrophobicity of the HPD components.[80]

The cellular uptake of HPD is monitored experimentally by the intensity of fluorescence emission by the tissue. The fact that fluorescence is found in tumor cells, after the uptake of HPD, has allowed its use in the successful diagnosis of the early stages of some types of cancer wherein no abnormalities were found by other methods of detection.[81]

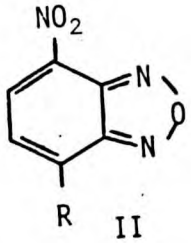
H. Biological Effect of Substituted Nitrobenzofurazans

Substituted nitrobenzofurazans have been found to be effective fluorescent probes and low inhibitors of enzymes and other biomolecules. In addition, this class of compounds has been demonstrated to be effectively cytotoxic toward a wide range of tumors.[6]

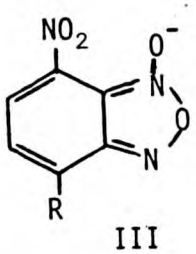
Ghosh reported in 1968 the experimental method for the nucleophilic displacement of the chloro group in 4-chloro-7-nitrobenzofurazan (NBD-Cl) (I) and its N-oxide by a variety of amines and anilines to yield substituted nitrobenzofurazans (II) and benzofuroxans (III).[82]



I



II



III

Ghosh and Whitehouse first noticed in 1968 that several 4-amino derivatives of NBD-Cl were highly fluorescent at low dilutions, and further established that the strong fluorescence of the glycine derivative arose from the substituted amine portion of the molecule. Furthermore, the 4-substituted-7-aminobenzofurazan derivative was about 100-fold less fluorescent than the corresponding 7-nitro derivative. The nitro group exhibited unusual behavior by intensifying the fluorescence and also by shifting both the absorption and emission frequencies.[83]

In addition, Ghosh and Whitehouse observed the 4-benzylamino derivative (BBD) to be about 100-fold more fluorescent than the 4-anilino derivative. It was shown that, in general, substituents on the amino group which restrict its conjugation with the NBD nucleus, such as acetyl or phenyl, cause a drastic loss of fluorescence. The fluorescence of the NBD-amines was best observed in solvents of low polarity and was found to be excited by visible light.[83]

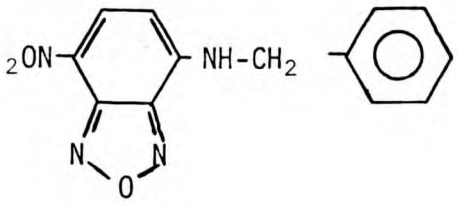
Substituted nitrobenzofurazans have been used extensively as fluorescent tags in various systems. NBD-Cl has been used effectively as an inhibitor or a fluorescent probe with the following enzymes: actin,[84] adenylate kinase,[85] aldolase,[86] ATPase (lys residue),[87] glutamate dehydrogenase,[88] glyceraldehyde-3-P dehydrogenase,[89] lysozyme,[90] papain,[91] peptidase A,[92] phosphorylase a and b (-SH groups),[93,94] and RNA polymerase (cys residue).[95] NBD-Cl has been demonstrated to be fluorogenic molecule with a variety of substances: amines,[96] amino acids,[97]

nitrosamines,[98] morphines,[99] amphetamines,[100] imidazole derivatives,[101] cholesterol,[102] thiophenols,[103] and cysteine, N-acetyl.[104] Trace amounts of carbonyl compounds have been successfully detected with the fluorescence labelling reagent 4-hydrazino-7-nitrobenzofurazan.[105] NBD-n-acylcholines have been demonstrated to behave as fluorescent analogs of acetylcholine.[106]

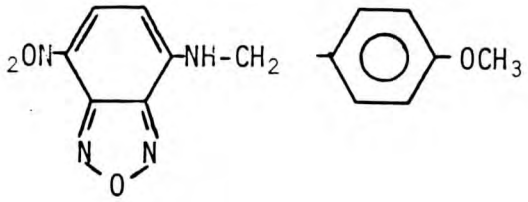
Substituted nitrobenzofurazans and their N-oxides (benzofuroxans) are known to be very effective inhibitors of nucleic acid and protein synthesis in a number of cell lines.[107] At low concentrations, NBD-Cl will break DNA strands and at higher concentrations will inhibit thymidine phosphorylation.[108]

This class of compounds has further been demonstrated, with in vivo experiments on a wide range of transplantable tumors in mice, to offer promise as prospective anti-cancer agents. In addition, the lipophilicity of these compounds is high enough to achieve intracellular concentrations sufficient to influence biosynthetic activities.[6] Further studies, however, have shown that some of the more promising cytostatic candidates are also mutagenic.[109] It has been deduced that the mutagenic properties resulted from the presence of the nitro group and N-oxide functions. A variety of substituted benzofurazans have since been synthesized in an effort to reduce the mutagenicity while retaining the cytotoxicity.[6]

The compound 4-benzylamino-7-nitrobenzofurazan (BBD) (IV) and its p-methoxy analog (V)



IV



V

have been demonstrated to bind specifically to the hydrophobic sites in proteins.[110] BBD is a highly fluorescent molecule which may bind to proteins at sites governed by the para substituent on the benzylamino group of the molecule. A sensitizer which could generate singlet oxygen while binding selectively to proteins could hold promise for selectively denaturing proteins in vivo.[111]

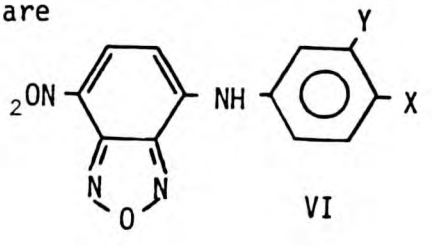
Previous research into the capability of BBD to generate singlet oxygen in solution showed that singlet oxygen production by BBD is either nonexistent or inefficient in comparison to a direct photochemical cycloaddition reaction between BBD and the acceptor molecule. The acceptor molecule employed in these studies was 1,3-cyclohexadiene (CHD). Although actual reaction products were not analyzed, a number of experimental factors led to the supposition of cycloaddition production formation. Both CHD and BBD were removed from the reaction solution. (In sensitized photooxidations, the sensitizer is continuously regenerated.) In addition, the BBD-CHD reaction was photochemical in nature, but did not require the presence of oxygen in order to proceed. An analogous reaction was observed to occur in a BBD-furan system, but not in a BBD-cyclohexene system; suggesting that conjugation

in the acceptor molecule is required for the photochemical reaction to take place. It was concluded that the photochemical reaction occurring was most likely a (4s + 4s) cycloaddition.[111]

Efficiency of singlet oxygen production for a given sensitizer is known to depend on both the triplet energy of the molecule and the lifetime of the molecule in the triplet state. The extremely high fluorescence of BBD may indicate that intersystem crossing in this molecule is of low occurrence. In addition, the triplet energy of the BBD molecule may be too great for proper overlap with the triplet ground state of molecular oxygen.

Fluorescence is generally expected in aromatic molecules or those containing multiple-conjugated bonds with a high degree of resonance stability. Substituent effects are dramatic in fluorescent molecules. Substituents, usually electron-donating groups, that tend to delocalize the pi-electrons can often enhance fluorescence, while electron-withdrawing groups generally decrease or quench fluorescence completely.[112] Alteration of the BBD molecule toward minimizing delocalization of the pi-electrons could possibly enhance intersystem crossing and/or lower the triplet energy of the molecule. The efficiency of singlet oxygen production by the sensitizer might thereby be improved.

4-anilino-7-nitrobenzofurazan and its p-substituted analogs (VI) are



VI

X = OCH_3, $NHCOCH_3$, CH_3, H, Cl, NO_2

Y = NO_2

reasonable candidates for synthesis and subsequent testing for singlet oxygen production. It is postulated that the absence of the methylene group present in BBD could decrease the pi-electron delocalization of the molecule and lower the triplet energy. As previously stated, Ghosh and Whitehouse observed the fluorescence of BBD to be about one hundred times stronger than that of 4-anilino-7-nitrobenzofurazan.[83] Possible substituent effects on both the fluorescence of and singlet oxygen production by the molecules could be observed.

In summary, the question of singlet oxygen production by substituted nitrobenzofurazans may be of practical as well as theoretical importance. The purpose of the present research was to synthesize and test for singlet oxygen production a series of substituted 4-anilino-7-nitrobenzofurazans in an effort to designate this series of compounds as potential candidates for the selective inactivation or destruction of biomolecules in vivo. The compounds were synthesized in accordance with the nucleophilic displacement method reported by Ghosh.[82] The acceptor molecules DMF and DPBF, diagnostic reactants with singlet oxygen, were used to detect singlet oxygen generation by the synthesized nitrobenzofurazans.

CHAPTER II

EXPERIMENTAL

A. General

Melting points were taken on a Fischer-Johns melting point apparatus and are uncorrected. Proton nuclear magnetic resonance spectra were obtained using a Varian EM360A NMR spectrometer. Chemical shifts are presented in parts per million from $(CH_3)_4Si$ reference. A Beckman DB-G grating spectrophotometer was used for recording ultraviolet-visible spectra. Fluorescence spectra were taken on a Baird Nova spectrofluorimeter. The Perkin-Elmer 1330 infrared spectrophotometer was employed to obtain IR spectra. Elemental analyses were performed by Galbraith Laboratories, Inc. of Knoxville, Tennessee.

B. Materials

Nitrogen and oxygen were used as obtained from National Welders Supply Company. Ethyl acetate was dried over $MgSO_4$ and stored over molecular sieve. Chloroform was singly distilled. HPLC grade acetonitrile was used as received from Fisher Scientific. NBD chloride (mp 96-97°C), 1,3-diphenylisobenzofuran (mp 127-129°C), and Rose Bengal (mp > 300°C) were used as received from Aldrich Chemical Company. The 4-benzylamino-7-nitrobenzofurazan (mp 207-208°C) was used as received from Pierce Chemical Company. Dimethylfuran was passed through activated alumina.

The compounds p-anisidine, p-toluidine, and p-chloroaniline were recrystallized from petroleum ether: toluene mixtures to yield white crystals of mp 56-57°C, 42-43°C, and 71-71.5°C respectively. The p-nitroaniline (mp 147-148°C) and m-nitroaniline (mp 112-114°C) were used as received from Eastman and Aldrich Chemical Company, respectively. Aniline was distilled over zinc dust at reduced pressure.

Acetanilide (mp 111-112°C) was used as received from Fisher Scientific. Palladium black was used as received from Aldrich Chemical Company. Cyclohexene was distilled over elemental sodium and stored in the dark.

Thin layer chromatography was performed using Eastman 1318 silica gel chromagram sheets with fluorescent indicator. Reagent grade solvents were used for elution. Spots were observed under both short and long wavelength ultraviolet light.

C. Synthesis of p-Aminoacetanilide

p-Nitroacetanilide was reduced to p-aminoacetanilide by hydrogen transfer from cyclohexene in the presence of a palladium catalyst by the method of Braude, Linstead, and Woolridge.[113] Light pink crystals of p-aminoacetanilide with a m.p. of 161.5-163°C were recovered. ^{1}H NMR ($DMSOd_6$) 9.42 (s, 2 H), 6.84 (m, 4 H) 4.70 (s, 2 H), 1.95 (s, 3 H).

D. Synthesis of Substituted 4-Anilino-7-nitrobenzofurazans

4-(4´-acetamido)-anilino-7-nitrobenzofurazan

A solution of 0.75 g (2.4 mmol) of p-aminoacetanilide and 0.75 g (3.8 mmol) of NBD chloride was refluxed in 100 mL of

anhydrous ethyl acetate for 24 hours. The product solution was washed well with water to remove excess reactants. The organic layer was evaporated to dryness. The resulting residue was recrystallized in 95% ethanol to which a small amount of ethyl acetate had been added. The hot solution was covered and cooled at room temperature until crystal formation occurred. Dark purple crystals of m.p. d285°C were collected. Rf ($CHCl_3$) 0.03, (50:50 vol% benzene:ethyl acetate) 0.08; ir (KBr pellet) 3359,3225,1675, 1614,1574,1531,1492,1406,1355,1301,1259,1177,1034,999,836,594,524 cm^{-1}; ^1H NMR ($DMSOd_6$) 11.00 (s, 1 H), 10.09 (s, 1 H), 8.42 (d, 1 H), 7.53 (m, 4 H), 6.60 (d, 1 H), 2.08 (s, 3 H). Anal. Calcd for $C_{14}H_{11}N_5O_4$: C, 53.68; H, 3.54; N, 22.36. Found: C, 53.11; H, 3.57; N. 22.22.

4-(4´-methoxy)-anilino-7-nitrobenzofurazan

The p-methoxy analog was prepared by allowing p-anisidine to react with NBD-Cl as outlined above and purified in an identical manner to that described for the p-$NHCOCH_3$ analog. Dark green crystals of mp 227-228°C (lit. 223-224°C)[82] resulted. Rf ($CHCl_3$) 0.53, (50:50 vol% benzene:ethyl acetate) 0.58; ir (KBr pellet) 3365,3084,1609,1565,1501,1440,1401,1291,1249,1174,1092,1029,994, 903,843,596,537,496 cm^{-1}; ^1H NMR ($DMSOd_6$) 11.20 (s, 1 H), 8.51 (d, 1 H), 7.28 (m, 4 H), 6.55 (d, 1 H), 3.84 (s, 3 H).

4-(4´-methyl)-7-nitrobenzofurazan

The experimental method for preparation and purification of the p-methyl analog was followed exactly as detailed above using p-toluidine as the aniline compound. Dark red crystals of m.p.

191-193°C were recovered. Rf ($CHCl_3$) 0.58, (50:50 vol% benzene: ethyl acetate) 0.58; ir (KBr pellet) 3343,3090,1569,1495,1437, 1372,1303,1173,1094,1030,994,901,820,597 cm^{-1}; ^1H NMR ($DMSOd_6$) 11.00 (s, 1 H), 8.53 (d, 1 H), 7.35 (s, 4 H), 6.66 (d, 1 H), 2.36 (s, 3 H).

4-anilino-7-nitrobenzofurazan

This compound was prepared as previously described using 0.75 mL (8.2 mmol) of aniline. The purified product yielded red-orange crystals of m.p. 151-152°C (lit. 150-151°C).[82] Rf ($CHCl_3$) 0.55, (50:50 vol% benzene:ethyl acetate) 0.57; ir (KBr pellet) 3290,3067,2925,1564,1494,1446,1403,1310,1167,1097,1035,996,903, 824,781,738,690,587,511 cm^{-1}; ^1H NMR ($DMSOd_6$) 11.10 (s, 1 H), 8.53 (d, 1 H), 7.50 (m, 4 H), 6.72 (d, 1 H).

4-(4´-chloro)-anilino-7-nitrobenzofurazan

The p-chloro analog was prepared and purified as described above using 0.75 g of p-chloro aniline and NBD-Cl. Dark red crystals of m.p. 174-175°C (lit. 168-169°C)[82] resulted. Rf ($CHCl_3$) 0.45, (50:50 vol% benzene:ethyl acetate) 0.51, ir (KBr pellet) 3354,3089,1560,1493,1440,1303,1172,1090,1028,1001,838, 739,599,497 cm^{-1}; ^1H NMR ($DMSOd_6$) 11.20 (s, 1 H), 8.59 (d, 1 H), 7.60 (m, 4 H), 6.80 (d, 1 H).

4-(4´-nitro)-anilino-7-nitrobenzofurazan

A solution containing 1.50 g (5.0 mmol) of p-nitroaniline and 1.50 g (7.6 mmol) NBD chloride was refluxed in 125 mL anhydrous ethyl acetate for one week. The product was purified as previously described yielding orange crystals of m.p. 236-238°C. Rf ($CHCl_3$)

0.07, (50:50 vol% benzene:ethyl acetate) 0.06; ir (KBr pellet) 3286,3171,3100,2924,1617,1570,1519,1449,1333,1249,1106,1035,996, 843,739,603,497 cm^{-1}; ^1H NMR ($DMSOd_6$) 11.30 (s, 1 H), 8.59 (d, 1 H), 8.0 (m, 4 H), 7.13 (d, 1 H). Anal. Calcd for $C_{12}H_7N_5O_5$: C, 47.85; H, 2.34; N, 23.25. Found: C, 47.53; H, 2.31; N,22.43.

4-(3´-nitro)-anilino-7-nitrobenzofurazan

The m-nitro analog was prepared and purified using m-nitro aniline and NBD-Cl in a manner strictly analogous to that outlined for the p-nitro analog. The orange product crystals gave a m.p. of 241-243°C. Rf ($CHCl_3$) 0.11, (50:50 vol% benzene:ethyl acetate) 0.09; ir (KBr pellet) 3339,3099,2926,1599,1565,1531,1506,1317, 1264,1095,1038,997,841,811,733,528 cm^{-1}; ^1H NMR ($DMSOd_6$) 11.40 (s, 1 H), 8.49 (d, 1 H), 7.97 (m, 4 H), 6.91 (d, 1 H).

E. Photooxidation Experiments

For the DMF detection system a simple photochemical reaction vessel was devised as illustrated in Figure 4. The vessel was made of Pyrex which filtered out unwanted ultraviolet radiation. The volume of the reaction chamber was 10 mL with a 1.5 cm pathlength. A 500 watt tungsten lamp kept at a distance of 15 cm was used to irradiate the samples.

Using DMF as the acceptor, the absorbance of DMF was monitored on the Bausch & Lomb Spectronic 21 spectrophotometer at 220 nm with acetonitrile being used as the solvent. In a typical experiment, 10 mL of the DMF (8.5×10^{-5} M)-sensitizer (1.0×10^{-5} to 4.0×10^{-5} M) solution mixture was placed in the photolysis cell. Oxygen, presaturated with the solvent, was passed through the

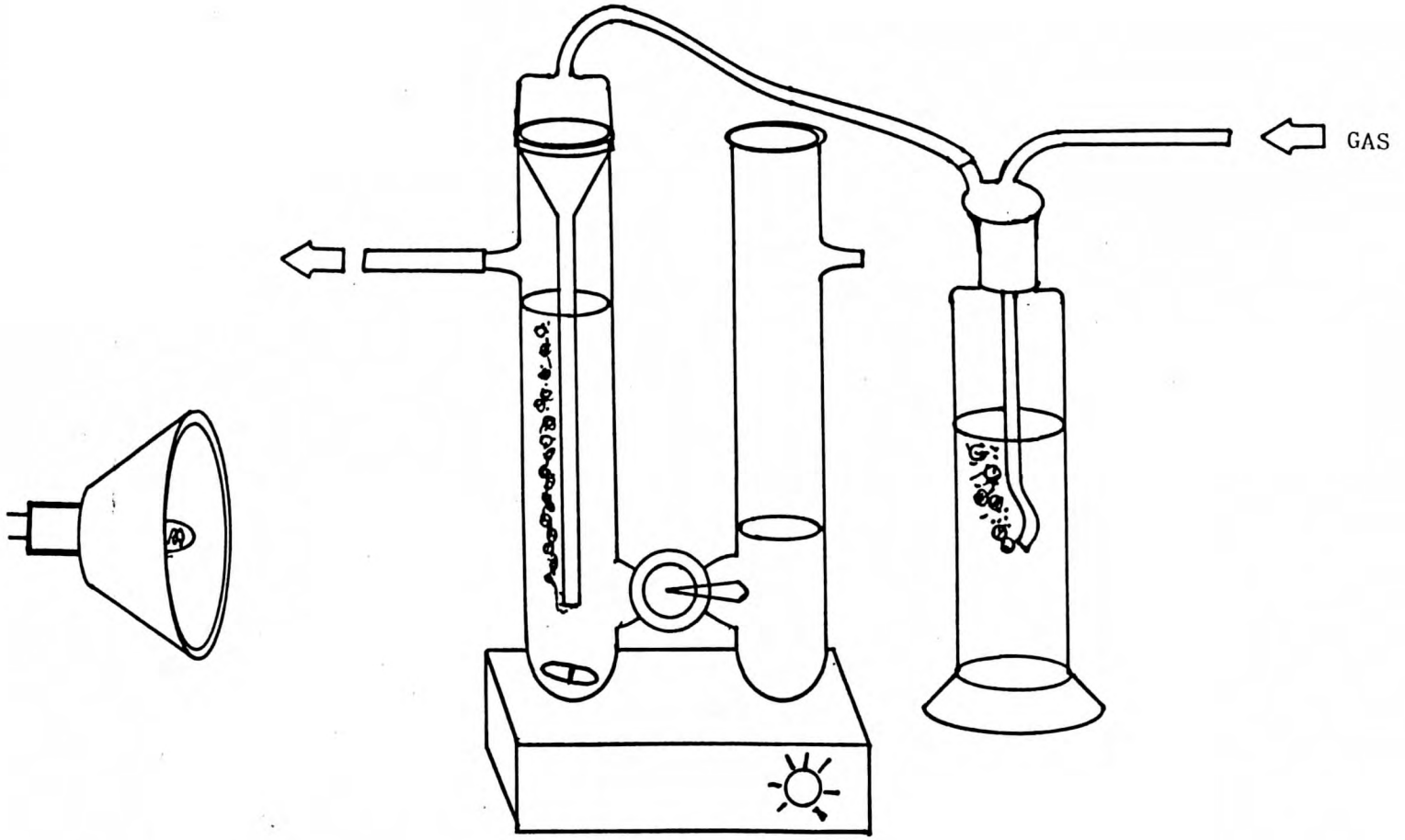


Figure 4. DMF Detection System

well-stirred solution. After 15 minutes, an aliquot was removed for measurement and the remaining solution was irradiated. Aliquots were removed and measured after 20 minutes irradiation and 50 minutes irradiation.

In the control experiments, a DMF solution without the presence of sensitizer was checked for a decrease in absorbance, under the standard conditions described above, using oxygen, nitrogen, and no aerating gas while irradiating the solution. An oxygen aerated DMF solution was tested for a decrease in absorbance with no irradiation. DMF and sensitizer test solutions were run using nitrogen as the aerating gas with irradiation and using oxygen as the aerating gas without irradiation.

Figure 5 illustrates the photochemical apparatus for the DPBF detection system. The reaction volume was 100 mL with a 4.5 cm pathlength. The lamp was kept at a distance of 20 cm from the center of the reaction vessel. The light was filtered to block radiation of wavelengths lower than 500 nm. Solutions were withdrawn by opening the reaction vessel.

In a typical experiment using the DPBF detection system, 100 mL of sensitizer (2.0×10^{-5} M to 4.0×10^{-5} M) in chloroform was bubbled with O_2 for 15 minutes. An absorption spectrum of the sensitizer solution was made and then 1 mL of 2.5×10^{-4} M DPBF was introduced to the solution, yielding a final concentration of 2.5×10^{-5} M, with O_2 bubbling continued for an additional 15 minutes. An aliquot was then withdrawn for measurement and the solution was irradiated. Samples were subsequently removed

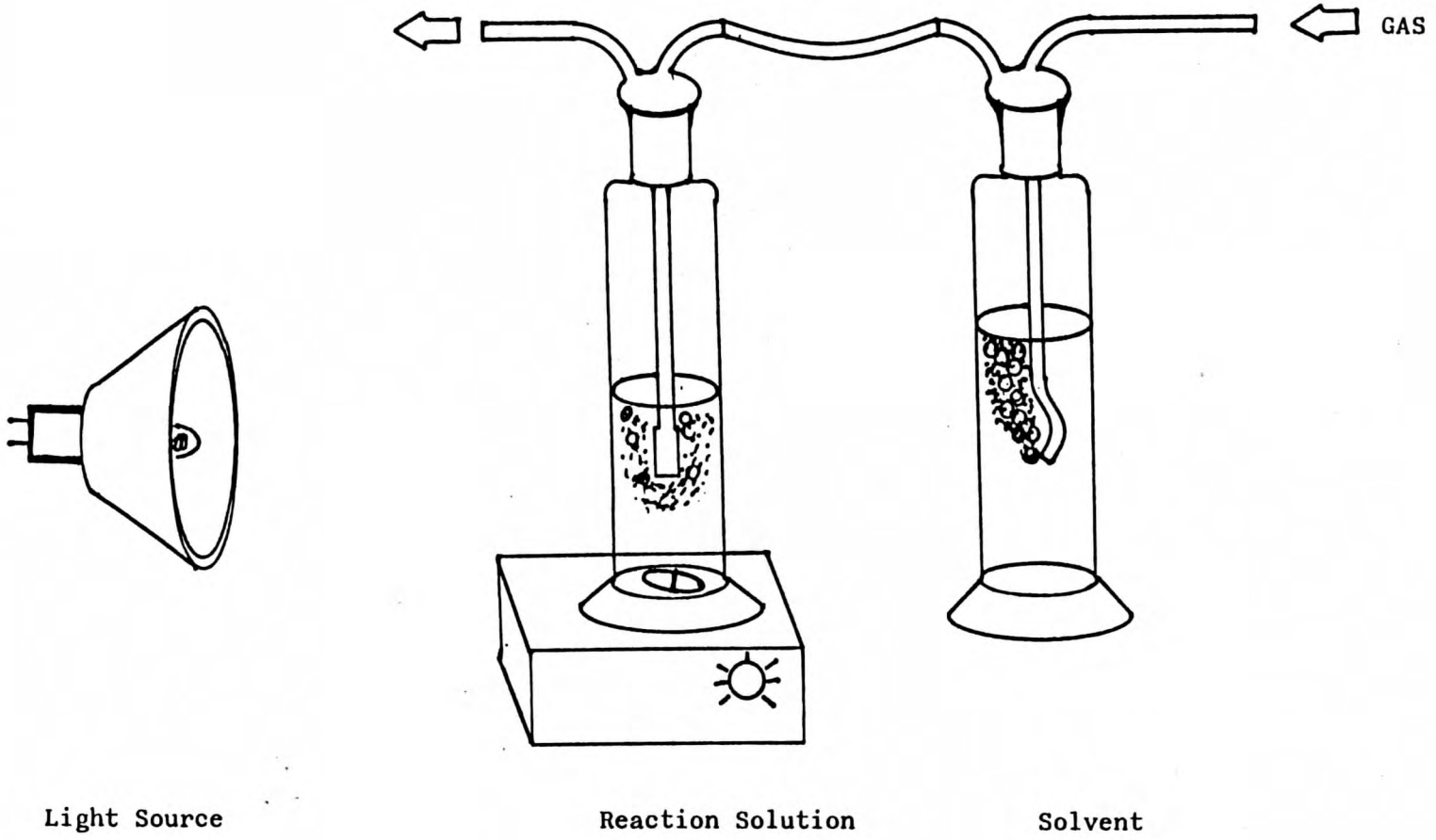


Figure 5. DPBF Detection System

and measured every 30 minutes for a period of 3 hours. The bleaching of DPBF was monitored on the Beckman DB-G scanning spectrophotometer. In the experiments involving N_2 gas, the absorbance measurements were made only at the beginning and the end of the irradiation period in order to avoid exposure of the reaction solution to oxygen in the air.

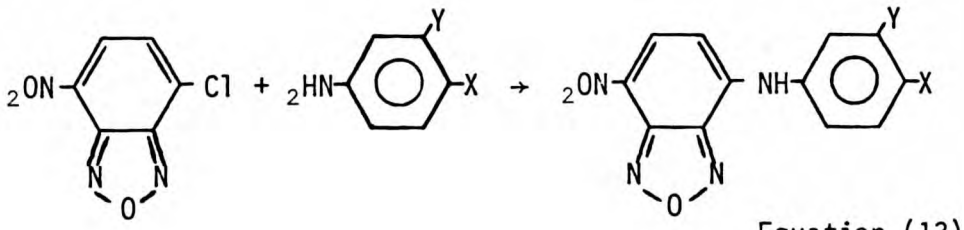
DPBF is known to undergo self-induced photooxidation.[114] By filtering out radiant energy below 500 nm, the self-induced bleaching was greatly reduced, although not completely eradicated. DPBF was found not to undergo self-induced photooxidation in the absence of oxygen. The experiments were carried out in a darkened room in order to reduce the effect of incident light.

CHAPTER III

RESULTS

A. Synthesis of Substituted 4-Anilino-7-nitrobenzofurazans

The substituted 4-anilino-7-nitrobenzofurazans were prepared by a nucleophilic substitution reaction of NBD chloride with aniline and substituted anilines, as outlined in Equation 13.[82]



Equation (13)

Y = H, NO_2

X = $NHCOCH_3$, OCH_3, CH_3, H, Cl, NO_2

B. Spectral Data

The IR spectra for the compounds are presented in the Appendix, Figures 18 through 24. The synthesized nitrobenzofurazans provide very complex IR spectral information with numerous peaks, particularly in the region of 1600-1000 cm^{-1}. The molecules possess no symmetry elements whatsoever; rendering any attempt at assignment of peaks questionable at best. The very similar appearance of the spectra, however, does lend support to the claim of having synthesized an analogous series of compounds.

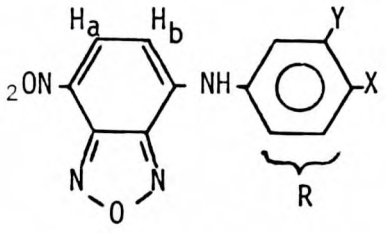
The NMR spectra recorded for the series, Figures 25 through 33, provide excellent confirmation of the structures of the products. The spectra were integrated and the peaks identified in full support of the structures predicted for the compounds.

The chemical shifts experienced by protons in NMR spectra arise from secondary magnetic fields produced by the circulation of electrons surrounding the nucleus. The secondary field opposes the primary field, thus the nucleus is said to be shielded from the full influence of the applied field. An increase in the external field is then required to produce nuclear resonance.

Shielding would be expected to decrease with increasing electronegativity of groups adjacent to the nucleus in question.[115] Examination of Table I reveals a definite pattern in the chemical shifts of the protons, H_a and H_b, attached to the benzofurazan portion of the substituted-7-nitrobenzofurazans, as it relates to the electron withdrawing capability of the anilino portion of the molecule. As expected, resonance effects of electron withdrawal through the benzene ring of the substituted aniline produce an increased deshielding effect and a larger chemical shift as one proceeds through the series, from the compounds containing typically electron donating substituents to those containing typically electron withdrawing substituents.

The uv-visible absorption maxima and the fluorescence emission data of the synthesized compounds are presented in tabular form in Table II. Although the relative degree of fluorescence between the compounds was not quantified through

Table I. Chemical Shifts of Protons H_a and H_b and Aromatic Protons of R in the NMR Spectra of Substituted 7-Nitrobenzofurazans



X	Chemical Shift[c] H_a	H_b	Aromatic Protons on R
$-OCH_3$	8.51	6.57	7.28
$-NHCOCH_3$	8.42	6.60	7.53
$-CH_3$	8.53	6.66	7.35
-H	8.53	6.72	7.50
-Cl	8.59	6.80	7.60
$-NO_2$	8.59	7.13	8.00
$-NO_2$[d]	8.49	6.91	7.97

[c] ppm from TMS

[d] located at position Y

Table II. Visible Absorption and Fluorescence Emission Spectral Data

Compounds[a]	Absorption[b] (λ_{max}, nm)	Fluorescence[b] (λ_{max}, nm)	
		Excitation	Emission
p-OCH_3	465	469	490
p-$NHCOCH_3$	417	404	425
p-CH_3	465	-	-
p-H	460	468	492
p-Cl	455	469	493
p-NO_2	453	460	525
m-NO_2	445	459	523
BBD	463[c]		526[c]

[a] 4-anilino-7-nitrobenzofurazan analogs

[b] $CHCl_3$

[c] dimethylformamide

the acquisition of quantum yields, a qualitative judgment can be made on the basis of the amount of fluorescence visible to the human eye. Of the compounds synthesized, only the p-NO_2 and m-NO_2 analogs could be seen to fluoresce upon exposure to long wavelength ultraviolet radiation.

C. DMF Detection System

The intended purpose of the DMF detection system was that it serve primarily as a screening procedure to identify, for further testing, those compounds which offered evidence of generating singlet oxygen. As the oxidation product of DMF and singlet oxygen was formed, the decrease in concentration of DMF was monitored at 220 nm.[116] The rates of reaction for the various experiments are represented graphically in Figures 6, 7, 8, and 9 with plots of absorbance versus time. The first data point of each curve represents the initial absorbance of the reactants in each test solution. After a short period of aeration, the second data point represents the time at which irradiation of the sample was begun.

Experiments using nitrogen as the aerating gas were run to confirm that the presence of oxygen was necessary for reaction to take place. Nonirradiated reaction mixtures were tested in order to ascertain if the reactions were, in fact, photochemical in nature. As a comparative control, solutions of DMF, without the presence of sensitizer, were tested for bleaching in both oxygen and nitrogen gas (Figure 6a). A slight, steady decrease in absorbance was observed probably due to the fact that the gas

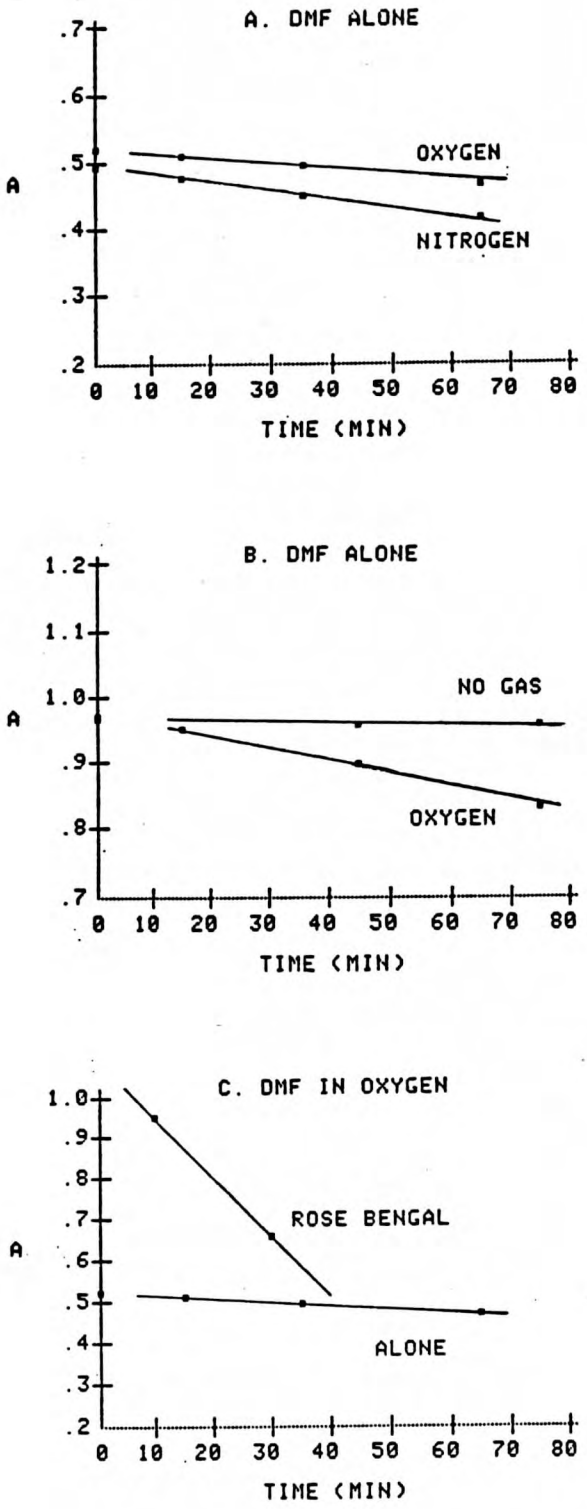


Figure 6. Absorbance at 220 nm as a function of time for DMF test solutions with irradiation beginning after 15 minutes aeration in oxygen or nitrogen gas.

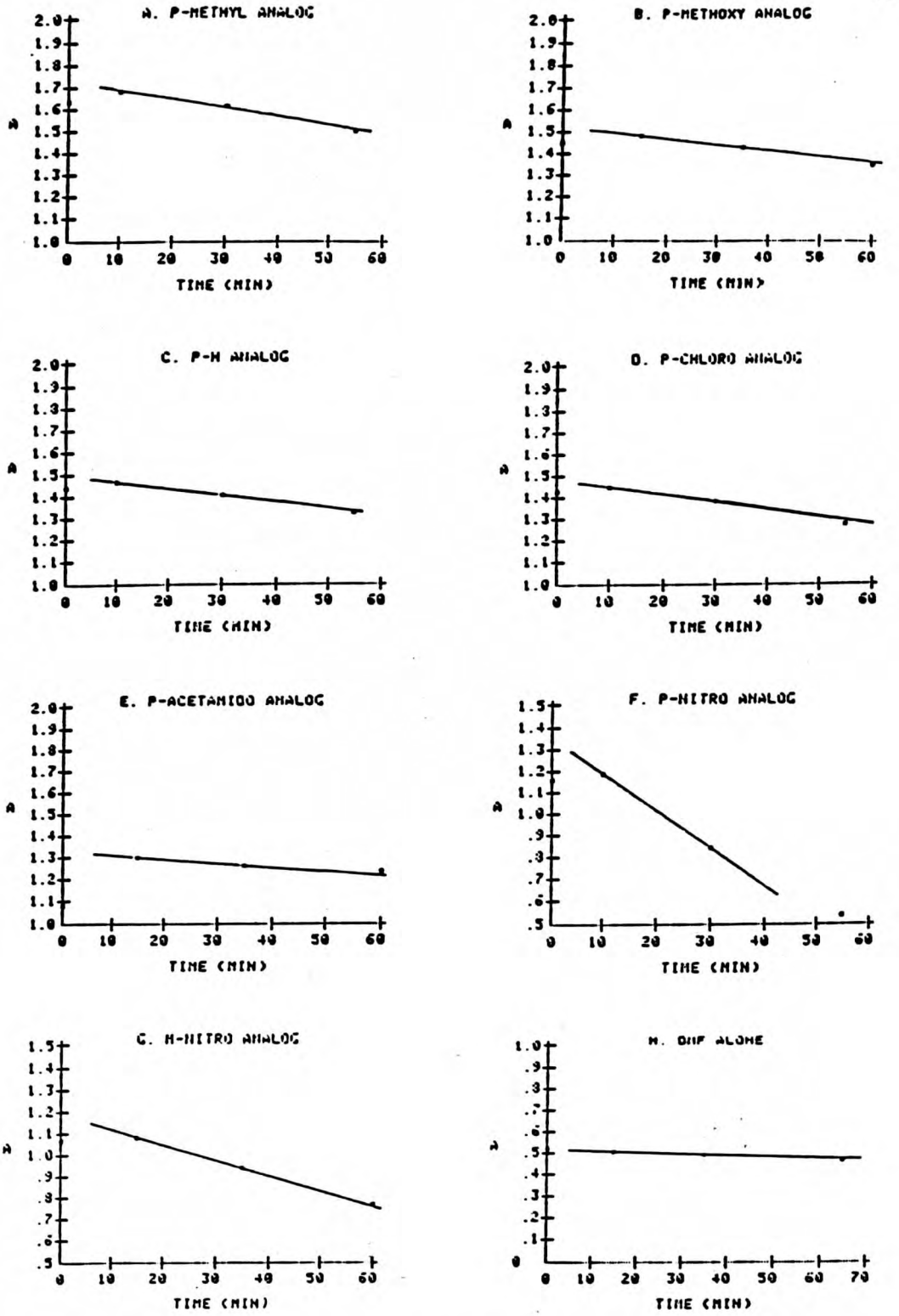


Figure 7. Absorbance at 220 nm as a function of time for DMF (1.5×10^{-4} M) and 4-anilino-7-nitrobenzofurazans (2.5×10^{-5} M to 5.0×10^{-5} M) test solutions with irradiation beginning after 15 minutes oxygen aeration.

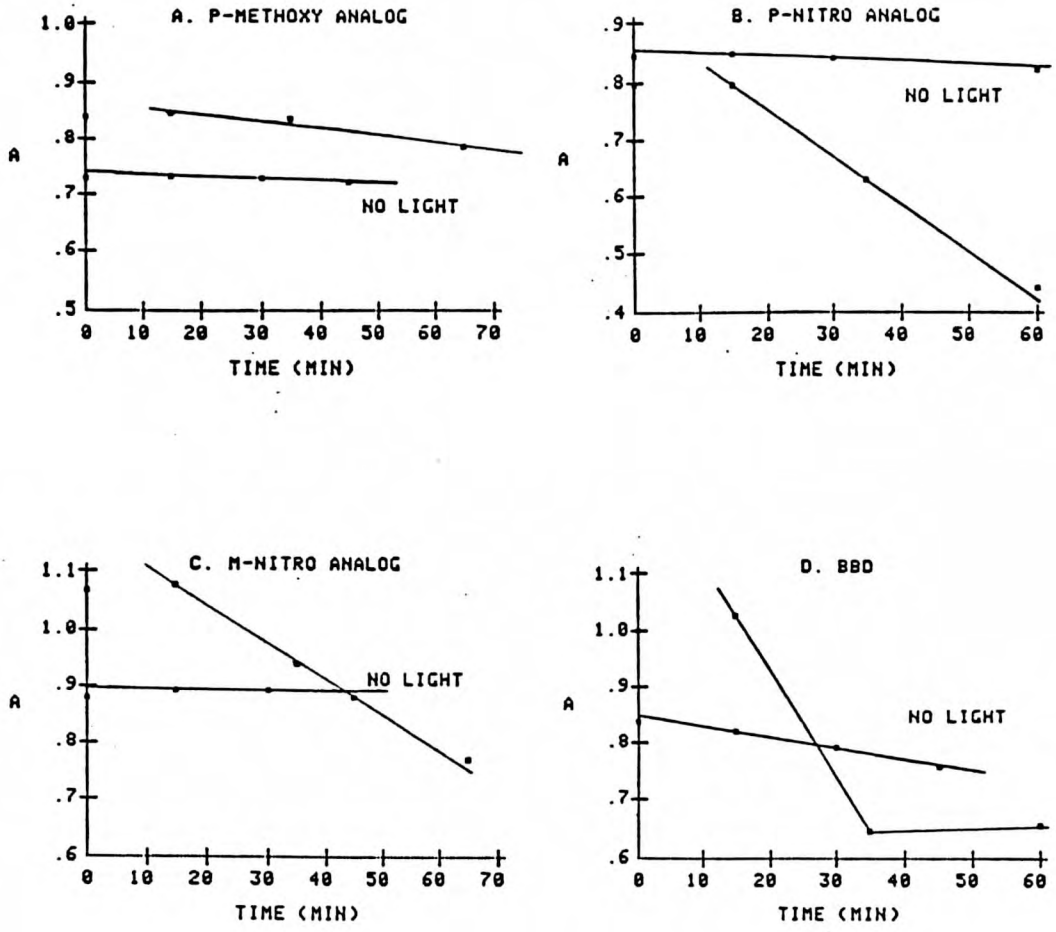


Figure 8. Absorbance at 220 nm as a function of time for DMF (8.5×10^{-5} M) and sensitizer (2.0×10^{-5} M to 4.0×10^{-5} M) oxygen aerated test solutions, both with and without irradiation.

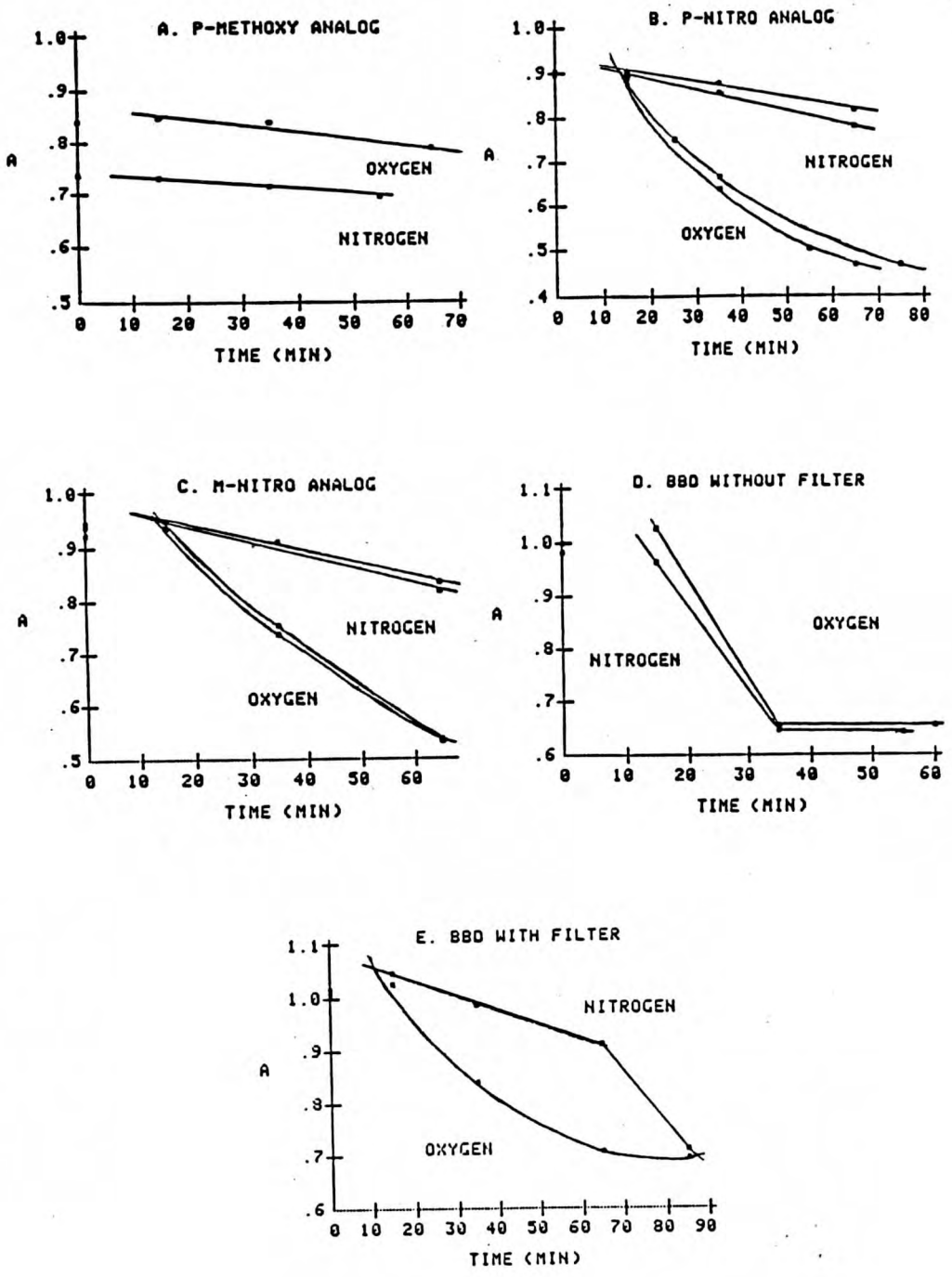


Figure 9. Absorbance at 220 nm as a function of time for DMF (8.5×10^{-5} M) and sensitizer (2.0×10^{-5} M to 4.0×10^{-5} M) test solutions with irradiation beginning after 15 minutes aeration in oxygen or nitrogen gas.

was not presaturated with DMF (Figure 6b). As Figure 6c reveals, the addition to a DMF solution of the known singlet oxygen producer, Rose Bengal, caused a significant decrease in absorbance to occur in comparison to DMF solutions containing no sensitizer.

The plots presented in Figure 7 make clear that upon being tested for singlet oxygen production, only two of the substituted 4-anilino-7-nitrobenzofurazans synthesized offered evidence for efficient generation of singlet oxygen; the p-NO_2 analog and the m-NO_2 analog (Figure 7f,g). The reaction rates for the p-OCH_3, p-$NHCOCH_3$, p-CH_3, p-H, and p-Cl analogs are not significantly different from that of the control slopes of DMF alone in O_2 and N_2 gas (Figure 7a,b,c,d,e,h). As a typical representative of the latter group of compounds, the p-OCH_3 analog was further tested in the absence of oxygen. As can be seen from Figure 9a, the decrease in absorbance of DMF in both cases was very similar to that of the control experiment using DMF alone.

By contrast, as Figure 7f,g illustrates, both the p-NO_2 and m-NO_2 analogs appear to generate singlet oxygen efficiently. The decrease in absorbance of DMF, using p-NO_2 and m-NO_2 analogs as sensitizers, is comparable to that achieved by using Rose Bengal (Figure 6c) as sensitizer. The presence of both light and oxygen are necessary for an efficient reaction to take place, as indicated in Figures 8 and 9. Figure 9c,d also illustrates the reproducibility of the results for the p-NO_2 and the m-NO_2 analogs using either oxygen or nitrogen as the aerating gas.

An efficient reaction also occurs in the presence of BBD as sensitizer. The reaction can be defined as photochemical since irradiation is a requirement for removal of DMF from solution (Figure 8d), but the reaction is not dependent upon the presence of oxygen. As Figure 9d illustrates, the disappearance of DMF is equally dramatic in nitrogen gas as in oxygen gas. The obvious conjecture is that, as previously found,[111] BBD is reacting directly with the acceptor molecule.

D. DPBF Detection System

With the possibility of singlet oxygen production having been established for the p-NO_2 and m-NO_2 analogs, the next step was to corroborate the results using another acceptor molecule. The DPBF detection system was employed for this purpose. The disappearance of DPBF from the reaction solution was followed by observing the decrease in absorbance at 415 nm as the singlet oxygen photooxidation product was formed.

Irradiation of oxygen-aerated DPBF alone causes rapid bleaching of the fluorescent blue-yellow color to yield the singlet oxygen product, 1,2-dibenzoylbenzene.[114] The self-induced photooxidation can be greatly reduced by filtering the excitation light striking the sample.[25] In the present experiments, the use of a filter which blocked radiant energy below 500 nm achieved the minimum amount of bleaching shown in Figures 10 and 12a. The presence of oxygen is a requirement for the self-induced bleaching of DPBF as demonstrated by the extremely slight decrease in absorbance shown in Figures 11 and 12a with nitrogen being used as the

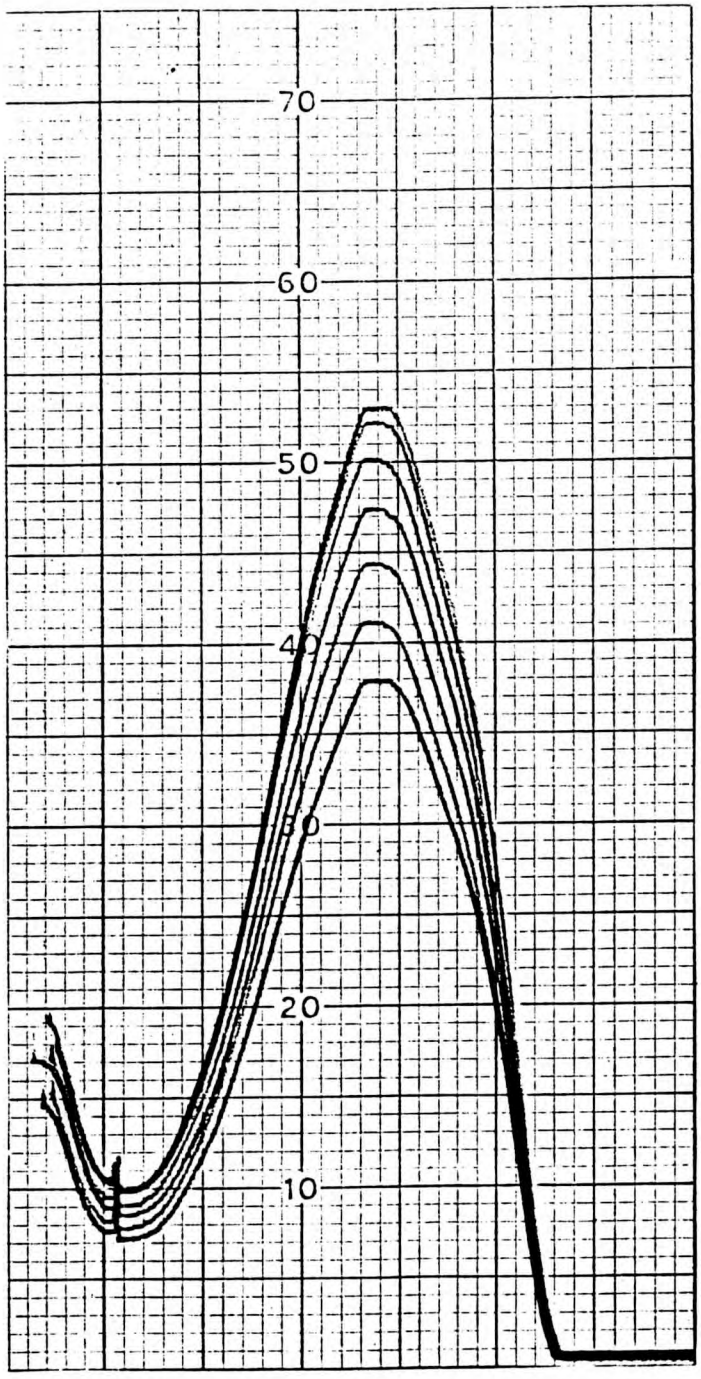


Figure 10. Visible absorption spectra of DPBF in oxygen at 30 minute time intervals.

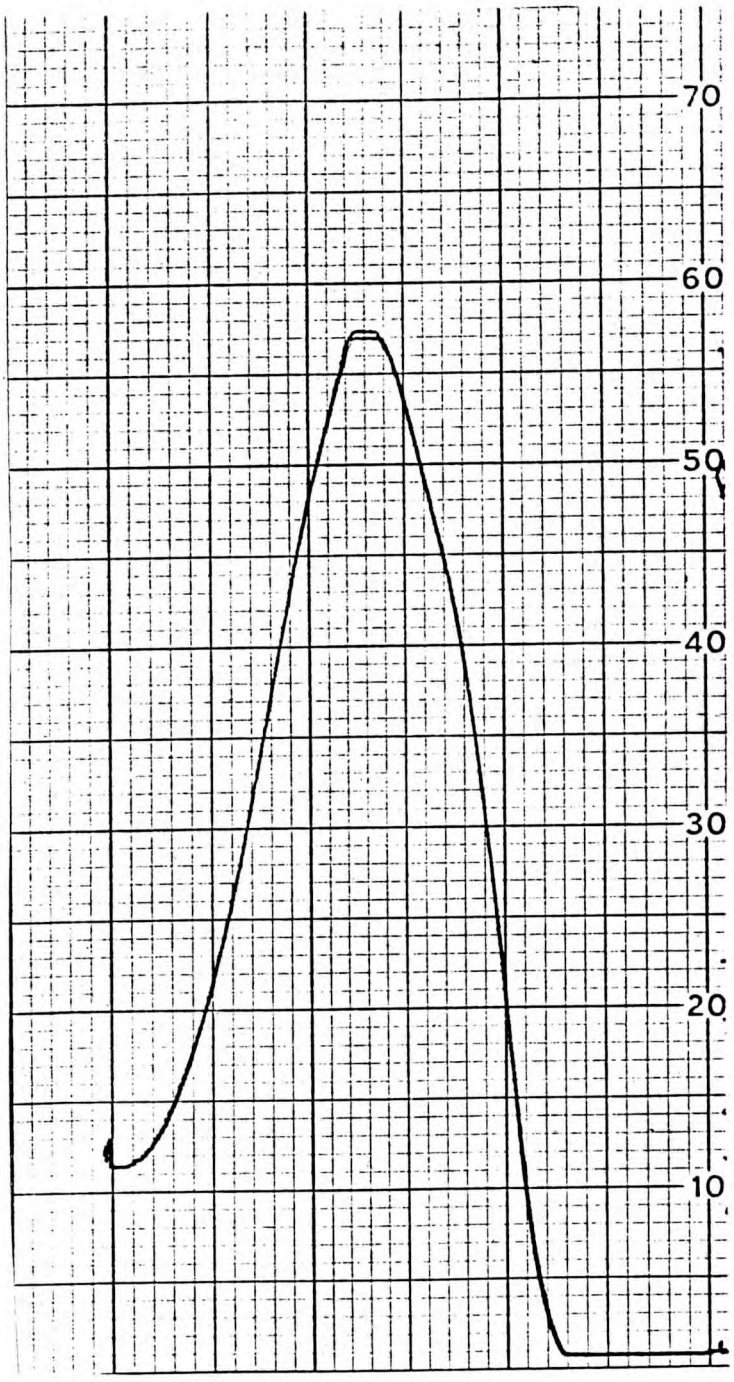


Figure 11. Visible absorption spectra of DPBF in nitrogen at a 3 hour time interval.

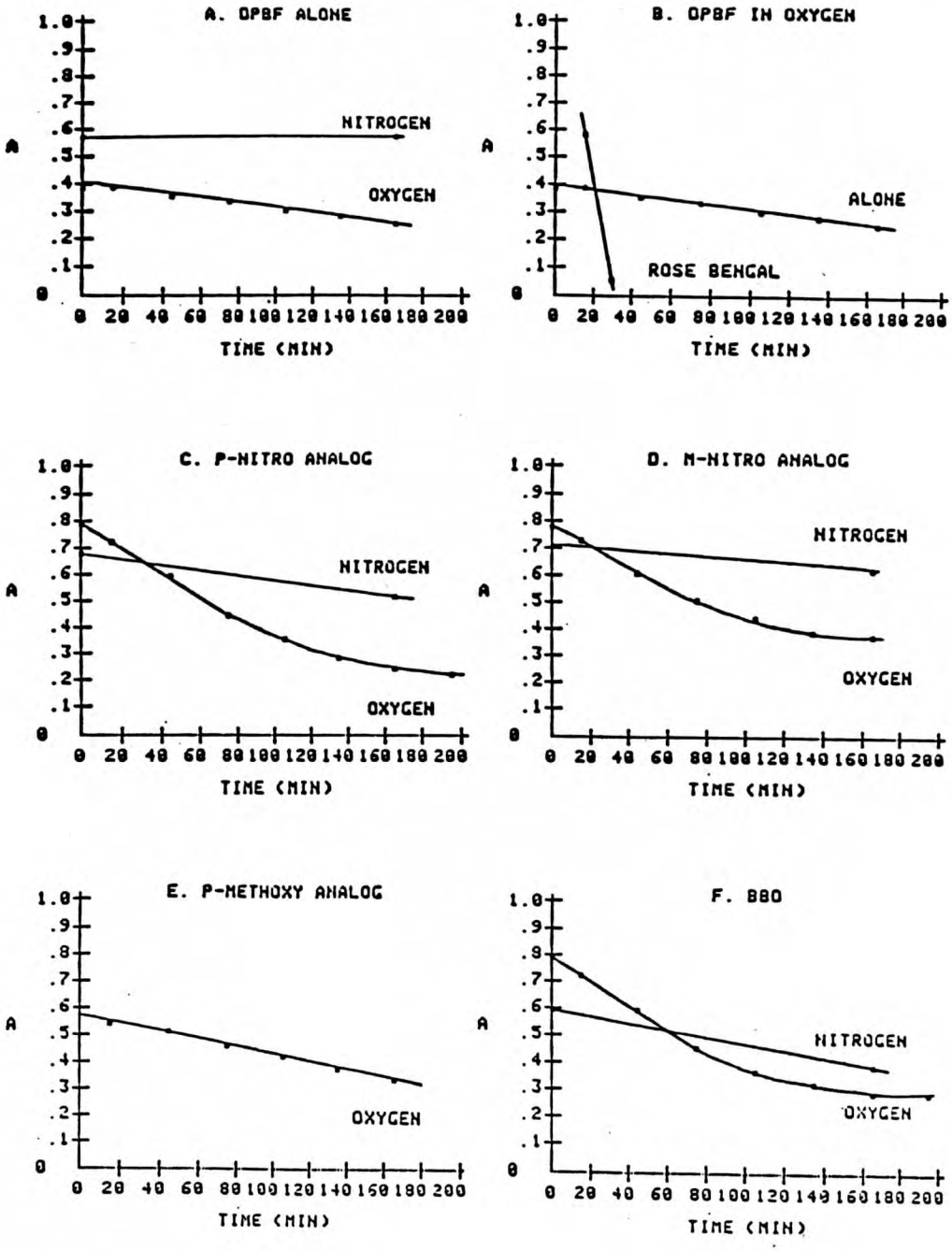


Figure 12. Absorbance at 415 nm as a function of time for DPBF test solutions.

aerating gas. Figure 13 demonstrates the effectiveness of the DPBF system using the known $^1O_2$ producer, Rose Bengal.

Referring to Table II, the absorption maxima of all of the compounds can be seen to overlap to a greater or lesser extent with that of DPBF. The overlap is not so great, however, as to interfere with the observed bleaching of DPBF at 415 nm. By recording the absorption spectrum of the sensitizer solution prior to introducing a one milliliter volume of DPBF, which did not significantly alter the sensitizer concentration, it was possible to follow the decrease in absorbance of the DPBF down to the original absorbance of the sensitizer solution. As can be noted by referring to Figure 14, the decrease in absorbance was halted at the original sensitizer absorbance. As expected according to Beer's Law, Figure 15 illustrates that at 415 nm the absorbances of DPBF and sensitizer are additive.

Using the DPBF detection system, a series of control experiments were run for each compound tested. Reactions were run in the absence of acceptor to confirm that the sensitizer molecules alone were not photochemically reactive. Experiments were done in the absence of light to confirm that the reactions are photochemical. Solutions were tested in nitrogen gas to confirm that the photooxidation product of DPBF is being formed in the reaction. None of the compounds tested showed signs of self-bleaching and, in all cases, the presence of light was found necessary for reaction to take place (Figures 34 through 42 in the Appendix).

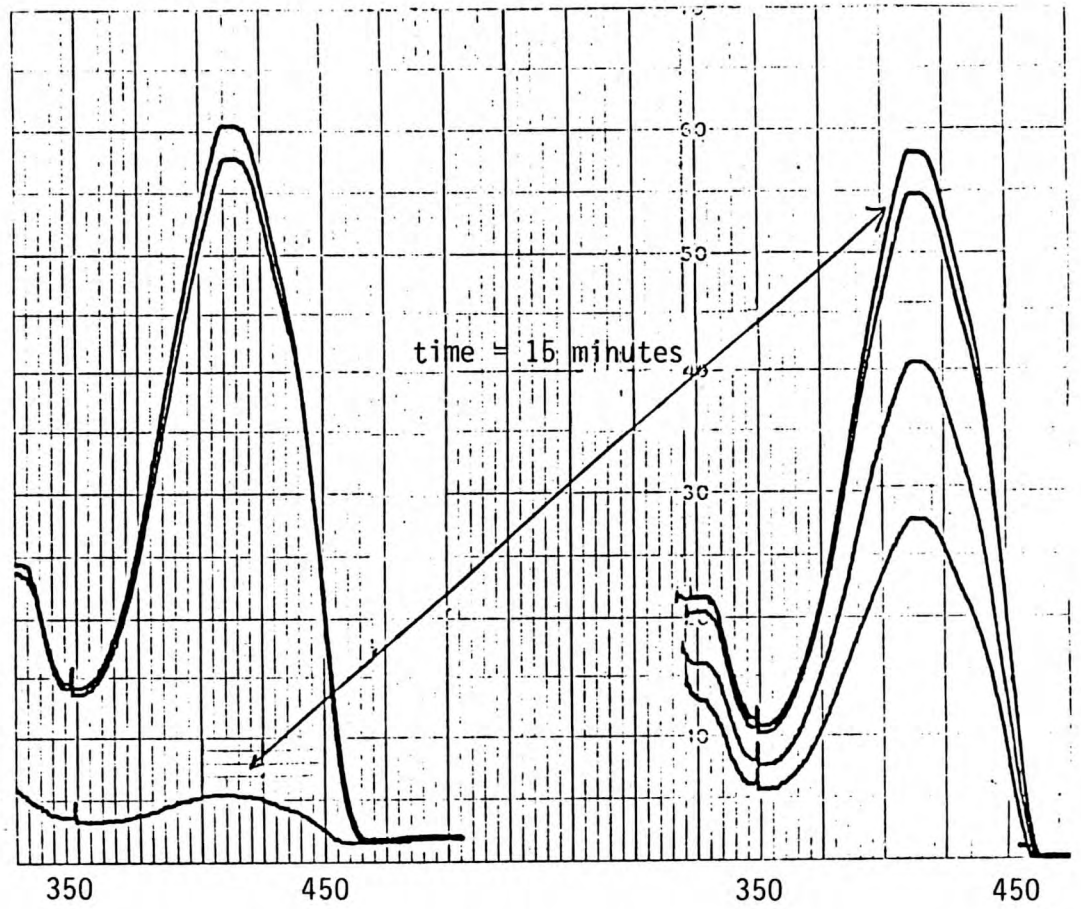


Figure 13. Visible absorption spectra of DPBF and Rose Bengal versus DPBF alone after a 15 minute irradiation time interval.

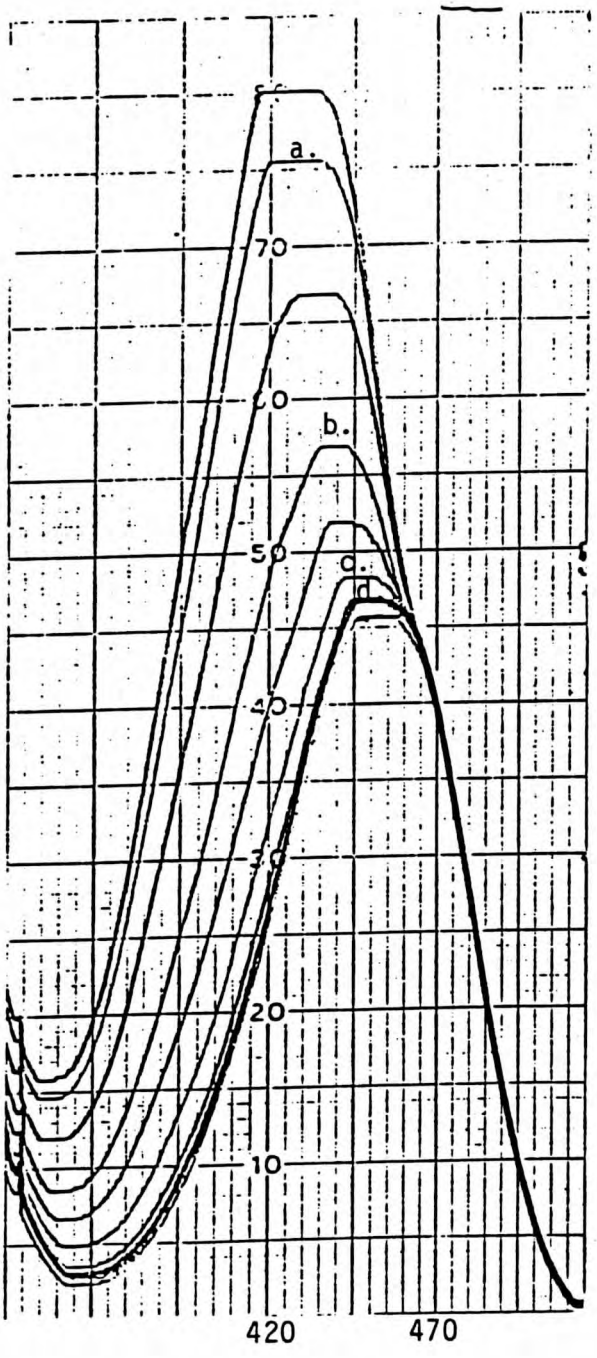


Figure 14. Visible absorption spectra of DPBF and the p-NO_2 analog in oxygen at time intervals; a. 0.25 hrs., b. 1.25 hrs., c. 2.25 hrs., d. 2.75 to 6.25 hrs.

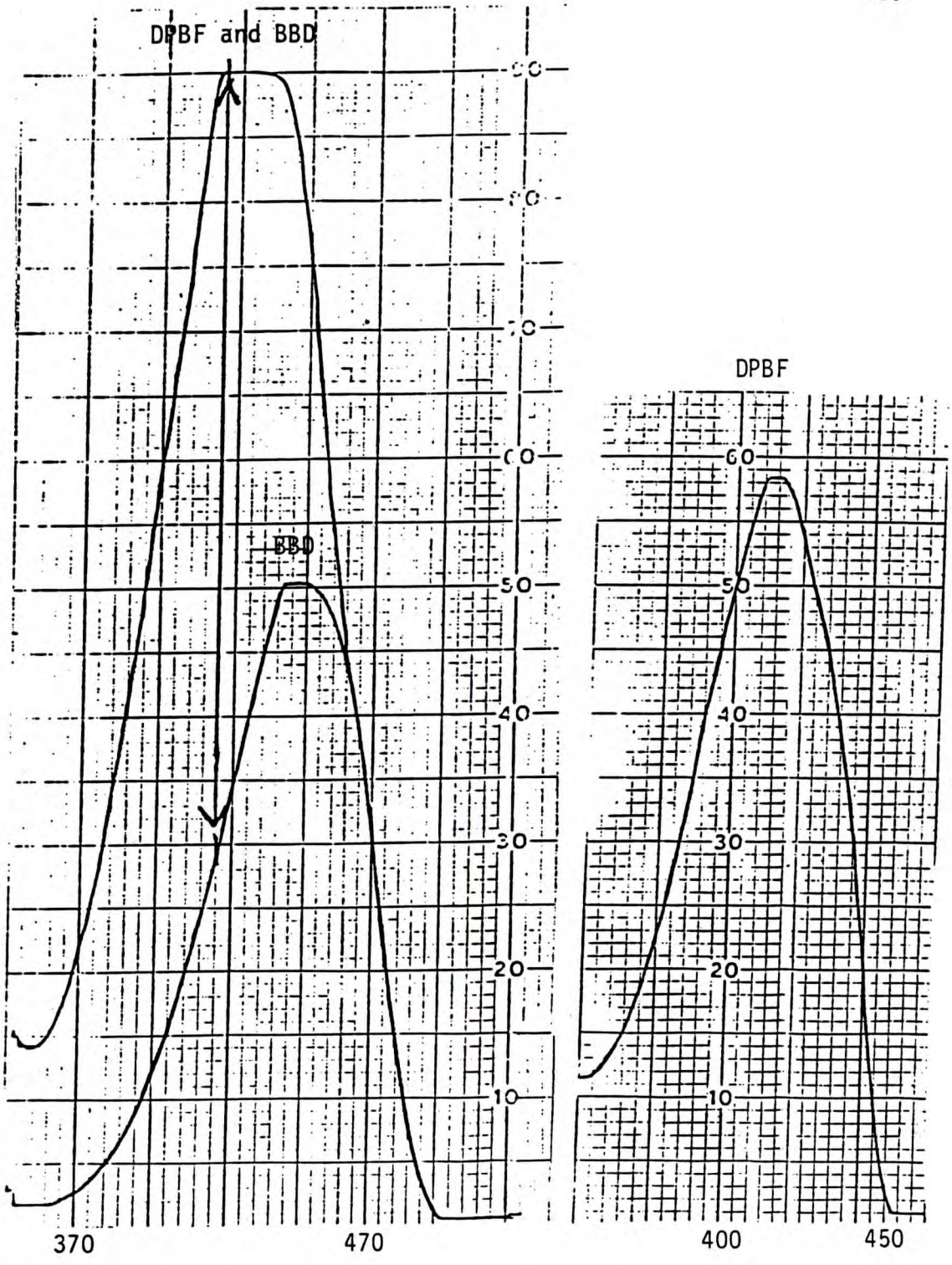


Figure 15. Visible absorption spectra of DPBF and BBD versus DPBF alone.

The results of the DPBF experiments are presented in Figure 12. Using the p-OCH_3 analog as a representative example of the group of compounds which did not generate singlet oxygen, Figure 12e lends support to the results obtained with the DMF detection system. In addition, the p-NO_2 and m-NO_2 analogs appear to generate singlet oxygen in the DPBF system as well as in the DMF system (Figure 12c,d). The difference to be noted is that, while DPBF is known to be the most sensitive singlet oxygen acceptor, the rate of decrease in absorbance as a function of time in the DPBF system is about one order of magnitude less than that in the DMF system. It can be conjectured that, while reducing the amount of self-induced photooxidation of DPBF, filtering of radiation below 500 nm also removes a significant amount of the excitation energy necessary for the sensitizer molecule to reach its triplet state (Table II).

The absorption maximum of Rose Bengal is located at 558 nm (MeOH).[117] As a result, filtering of excitation energy below 500 nm does not affect the rate at which Rose Bengal produces singlet oxygen. Figure 12b shows that the rate of DPBF bleaching in the presence of Rose Bengal is comparable to that of DMF disappearance in the presence of Rose Bengal.

An additional consequence of filtering the light source within the DPBF system was that BBD was observed to give evidence of generating singlet oxygen (Figure 12f). As with the p-NO_2 and m-NO_2 analogs, a significantly different rate of bleaching occurs in the absence of oxygen. By comparison, the rate of

decrease in absorbance of DMF in the presence of BBD was essentially the same in nitrogen as in oxygen (Figure 9d). The overall behavior of BBD in the DPBF detection system closely parallels that of the p-NO_2 and m-NO_2 analogs.

As shown in Figure 16, over an extended period of time the decrease in absorbance of a DPBF-BBD mixture stabilized at the original BBD concentration level, but was then observed to continue at a much reduced rate. The decrease in absorbance of a DPBF and p-NO_2 analog reaction solution, however, halted permanently at the level of the original p-NO_2 analog concentration curve (Figure 14).

In order to ascertain whether the BBD-acceptor photochemical cycloaddition was being suppressed by filtering the light source, the DMF experiment was rerun with BBD while filtering the light source. As Figure 9e shows, screening light below 500 nm allowed BBD to produce singlet oxygen while inhibiting cycloaddition to the acceptor molecule. In fact, the rate of disappearance of DMF in the presence of BBD is now essentially the same as the rate in the presence of the p-NO_2 and m-NO_2 analogs. After 65 minutes irradiation, the filter was removed from the light source. Upon removal of the filter, the cycloaddition reaction appeared to resume until all DPBF had been reacted, as is dramatically illustrated in the plot for nitrogen aeration in Figure 9e.

A more rigorous treatment of the DPBF absorbance could indicate the intervention of singlet oxygen in the reaction mechanism. Singlet oxygen produced by energy transfer from triplet sensitizer can decay via two major pathways, as shown in Scheme 7.

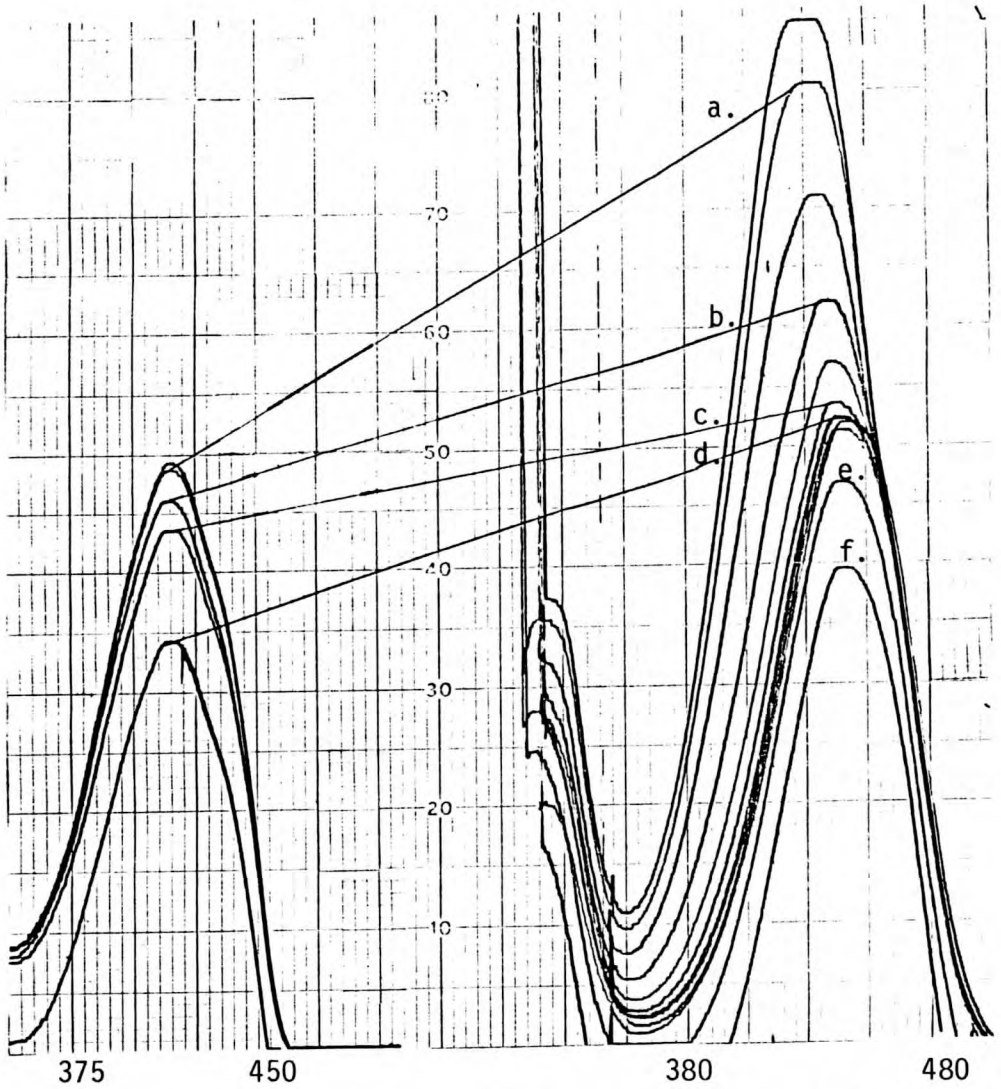


Figure 16. Visible absorption spectra of DPBF and BBD versus DPBF alone in oxygen at time intervals; a. 0.25 hrs., b. 1.25 hrs., c. 2.25 hrs., d. 3.25 hrs., e. 7.5 hrs., f. 9.75 hrs.

$$O_2(^1\Delta g) \xrightarrow{k_d} O_2(^3\Sigma g^-) \qquad (1)$$

$$O_2(^1\Delta g) + Q \xrightarrow{k_r \text{ or } k_q} \text{loss of } O_2(^1\Delta g) \qquad (2)$$

Scheme 7.

Pathway (1) involves the decay of singlet state oxygen to the ground state molecule through interaction with either another oxygen molecule or a solvent molecule. Pathway (2) is the result of physical or chemical quenching of $^1O_2$ by substrate.

When the initial concentration of singlet oxygen is small compared with the initial concentration of DPBF and quencher, Q, the rate expression for the loss of DPBF is

$$\frac{d(-[DPBF])}{dt} = k_r [DPBF] [O_2]^*_o \exp(-\{k_d + k_r [DPBF] + k_q [Q]\}t)$$

Equation (14)

where Q may be either a chemical or physical quencher and $O_2{}_o$ is the initial concentration of singlet oxygen. It can be shown that a plot of $\log(A_T - A_\infty)$ versus time will be linear with a slope $k' = k_d + k_r$ DPBF $+ k_q$ Q where A_T is the absorbance of DPBF at time t, A_∞ is the asymptotic value, k_d is the rate constant for $^1O_2$ decay, k_r is the rate constant for chemical reaction of $^1O_2$ with DPBF, and k_q is the rate constant for quenching of $^1O_2$ by quencher Q.[20,60]

Figure 17 presents plots of $\log(A_T - A_\infty)$ versus time for the p-OCH_3 analog, the p-NO_2 analog, the m-NO_2, and BBD. No attempt at constant temperature control, which would have allowed a

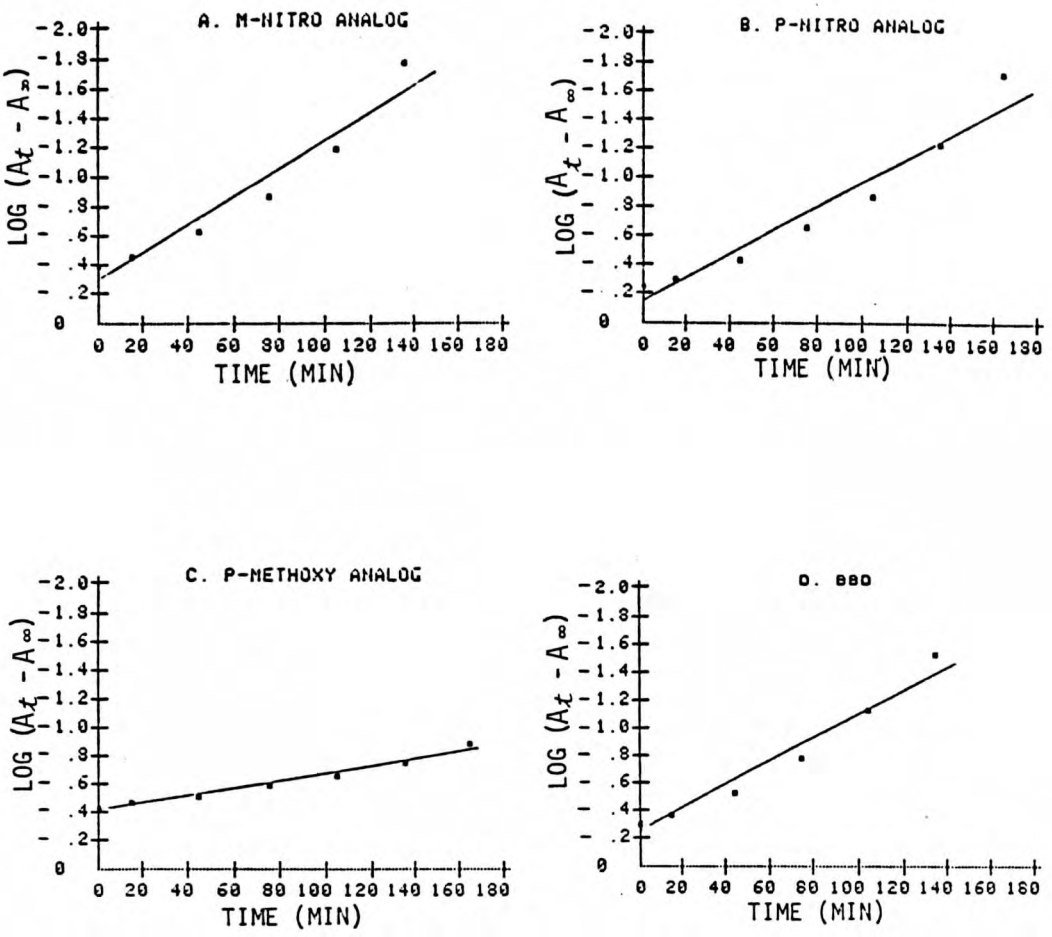


Figure 17. The log function of absorbance at time t minus the asymptotic value as a function of time for DPBF and sensitizer oxygen aerated test solutions.

reliable study of the kinetics of the reactions, was made. In addition, the data plotted is uncorrected for the self-induced bleaching of DPBF. The treatment is used here not to evaluate k', but only to determine if the rate of disappearance of DPBF is consistent with a singlet oxygen mechanism. The results of a linear regression analysis are presented in Table III. While absolute linearity was not approached in the plots, the correlation coefficients are sufficiently high to suggest the participation of singlet oxygen in the reaction mechanisms.

Table III. Linear Regression Analysis of $Log(A_T - A_\infty)$ versus Time for DPBF and Sensitizer Test Solutions

Sensitizer	Correlation Coefficient	Slope
p-OCH_3[a]	-0.987	-2.6×10^{-3}
m-NO_2[a]	-0.968	-9.6×10^{-3}
p-NO_2[a]	-0.972	-8.5×10^{-3}
BBD	-0.982	-8.9×10^{-3}

[a]4-anilino-7-nitrobenzofurazan analogs

CHAPTER IV

DISCUSSION

The characterization, presented in earlier sections, of the substituted 4-anilino-7-nitrobenzofurazans yields firm support to the assertion of having synthesized the desired products. Literature melting points for previously synthesized compounds agree closely with those obtained in the present study.[82] The chemical shifts observed in the NMR spectra behave in a manner which could be predicted according to the proposed structures of the products.

Of the substituted 4-anilino-7-nitrobenzofurazans synthesized, only the p-NO_2 and m-NO_2 analogs offered clear evidence of having generated singlet oxygen. Experimental evidence proved that the presence of light and oxygen was required for reaction to take place with the acceptor molecules DMF and DPBF. No reaction was observed using nitrogen as the aerating gas in either of the detection systems. In addition, only the p-NO_2 and m-NO_2 analogs were found to possess significant fluorescent character. This parallel in behavior is not coincidental. It has long been observed that fluorescent molecules serve as good candidates for fulfilling the role of sensitizer in sensitized photooxidations.[7]

Concerning the fluorescence spectra, an interesting observation to be made is that removal of the intervening methylene

group from the highly fluorescent BBD molecule produced the mildly fluorescent molecule, 4-anilino-7-nitrobenzofurazan. If the absence of the insulating methylene group could so drastically reduce the fluorescence of the p-H analog, it was initially expected that the addition of electron-donating substituent groups to the benzene ring might restore some degree of fluorescent activity to the molecule. Unexpectedly, the strongly electron-withdrawing nitro group was found to enhance fluorescence. While the link between fluorescence and singlet oxygen production was upheld in the present experiments, the analogs predicted to best fulfill the role of sensitizer were located at the opposite end of the electron-donating spectrum from that expected.

Ghosh and Whitehouse have noted the unusual behavior of the nitro group located on the benzofurazan portion of 4-amino derivatives of NBD-Cl in enhancing fluorescence. Studies have established that the amino portion of these molecules was responsible for the fluorescence.[83] In the present studies, the nitro group appears to be capable of enhancing fluorescence while being located on the anilino portion of the substituted 4-anilino-7-nitrobenzofurazans. It is conjectured that the anilino portion of the molecule is responsible for the fluorescence of these compounds, with the nitro substituent being able to attract electron density into the benzene ring sufficiently to allow for stronger fluorescence.

Both the lifetime and the energy of the sensitizer triplet state determine the efficiency of singlet oxygen production. The

most efficient sensitizers have been found to possess triplet energies of between 10000 and 15000 cm^{-1}.[26] The equipment needed to obtain the triplet energies of the synthesized benzofurazans was not available in the present study, however, the acquirement of this information is of obvious importance as a focus for further study.

Although the phosphorescence spectra of the compounds were not obtained, the fluorescence spectra were recorded. While fluorescence spectra do not provide the triplet energies of the molecules, the relative ordering of the energies of the emission maxima could be instructive in this regard. The triplet energy is almost certainly lower, but could not be higher, than the energy of the fluorescent emission maximum.

With respect to increasing energy, the ordering of the emission maxima of the substituted 4-anilino-7-nitrobenzofurazans is _p_-NO_2, _m_-NO_2 > _p_-Cl, _p_-H, _p_-OCH_3 > _p_-$NHCOCH_3$ (Table II). Assuming that the triplet energies of the molecules would follow a similar pattern, the _p_-NO_2 and _m_-NO_2 analogs should possess lower energy triplet states than the remaining compounds. With the assumption that the triplet energies of the molecules are lower than the fluorescence emission energies, referral to Table II shows that the triplet energies of the _p_-NO_2 and _m_-NO_2 (49000 cm^{-1}) analogs are approaching the desired range for efficient singlet oxygen production. It is postulated that the structural effects responsible for the increased fluorescent activity and the increased singlet oxygen generation by the _p_-NO_2 and _m_-NO_2 analogs are also

responsible for the lowering of the triplet energies. The reduction in the energy of the triplet state of the sensitizers could be directly implicated as a primary factor involved in the increased efficiency of singlet oxygen generation.

Further experimentation is needed to determine the precise triplet energies of the synthesized compounds and the effect of structural differences on the triplet energies and on singlet oxygen production. Multisubstituent effects could be studied by placing more than one nitro group on the ring and/or by placing different substituent groups on the ring.

The effects of self-quenching could offer an explanation for the inability of the majority of the synthesized compounds to generate singlet oxygen. Amines have long been recognized as efficient quenchers of singlet oxygen.[42,43] Ogryzlo and Tang reported in 1970 that the rate of quenching of singlet oxygen by a series of amines was related to the ionization potentials of the amines in the gas phase. Ogryzlo suggested a quenching mechanism taking place via a charge-transfer process.[42] Later work showed a definite correlation to exist between the mechanism for the quenching of singlet oxygen and the mechanism for the quenching of the fluorescence of aromatic compounds by amines, lending further support to the proposed charge-transfer intermediate.[118,119]

The primary process is thought to involve the formation of a complex between the electron-donating quencher, Q, and the electron-deficient oxygen, as represented by Scheme 8.

$$Q + O_2(^1\Delta g) \rightarrow (Q\ldots O_2)^1 \rightarrow (Q\ldots O_2)^3 \begin{cases} \nearrow Q + O_2(^3\Sigma g^-) \\ \searrow QO_2 \end{cases}$$

Scheme 8.

The triplet complex may result in either chemical or physical quenching of the oxygen molecule. The formation of an oxidation product requires the presence of an abstractable hydrogen α to the nitrogen.[1]

Chemical quenching of singlet oxygen by the substituted 4-anilino-7-nitrobenzofurazans is not likely, as these compounds do not possess a hydrogen atom α to the anilino nitrogen. The occurrence of physical quenching is, however, a possibility to be considered. The ionization energy of the -NH- group would be expected to decrease with increasing electron withdrawing capability of other substituents on the benzene ring. Consequently, physical quenching of singlet oxygen would be minimized by the presence of a nitro group on the ring.

As was observed in previous studies,[111] the BBD molecule was found to undergo a direct cycloaddition reaction with the acceptor molecule, in this case DMF. With filtering of the light source to allow only wavelengths greater than 500 nm to strike the sample BBD was observed to produce singlet oxygen in both the DMF and DPBF detection systems. If a competition exists between formation of the cycloaddition product and generation of singlet oxygen, the excitation energy striking the sensitizer molecule

is obviously important in determining the outcome of the competitive process. It is conjectured that a lower excitation energy is required for formation of the BBD triplet state than is required for formation of the excited state necessary for the photochemical (4s + 4s) cycloaddition reaction. Consequently, prevention of interaction between higher frequency excitation energy and the BBD sensitizer allowed singlet oxygen generation to supercede cycloaddition product formation.

A promising experimental technique for future research is the use of a tunable laser as the excitation source. It is conceivable that intense, monochromatic light of the proper energy could favor the efficient production of singlet oxygen. Especially in the case of the sensitizer BBD, the use of a laser could greatly benefit the study of singlet oxygen production by inhibiting unwanted side reactions.

Further research aimed at isolating and identifying the photooxidation products of the DPBF and DMF experiments could help to verify the participation of singlet oxygen in these systems. Similarly, the known $^1O_2$ quenchers, -carotene,[41] azide ion,[1] and amines[42,43] could be used to test for the intermediacy of singlet oxygen by adding varying amounts of the quencher to sensitizer-acceptor reaction mixtures and noting the observed effect on the rate of disappearance of DPBF and DMF from the solution.

An additional area for further study is the actual testing of the effects of the sensitizers on biological systems. The capability of the p-NO_2 and m-NO_2 substituted 4-anilino-7-

nitrobenzofurazans to selectively bind to tumor tissue would be of primary interest. These effects could be studied both with and without the experimental conditions required for photodynamic action.

To summarize, a series of substituted 4-anilino-7-nitrobenzofurazans were synthesized, characterized, and tested for singlet oxygen production. Two compounds in the series, the p-NO_2 and m-NO_2 analogs, were observed to generate singlet oxygen efficiently using the detector molecules DMF and DPBF. The compound BBD was observed to produce singlet oxygen effectively only when the light source was filtered to block radiation below 500 nm. These compounds offer promise as potential candidates for the selective inactivation or destruction of biomolecules *in vivo*.

REFERENCES

(1) Gorman, A. A.; Rodgers, M. A. J. Chem. Soc. Rev. 1981, 10, 205.

(2) Kearns, D. R. Chem. Rev. 1971, 71, 395.

(3) Krinsky, N. I. In "Singlet Oxygen"; Wasserman, H. H.; Murray, R. W., Ed.; Academic Press: New York, 1979; Chapter 12.

(4) "Porphyrin Photosensitization"; Kessel, D.; Dougherty, T. J., Ed. In "Advances in Experimental Medicine and Biology", Vol. 160; Plenum Press: New York and London, 1983.

(5) Dougherty, T. J.; Kaufman, J. E. et al. Cancer Research 1978, 38, 2628-2635.

(6) Burkhardt, D.; Ghosh, P. et al. Chem.-Biol. Interactions 1982, 42, 195.

(7) Foote, C. S. Science 1968, 162, 963.

(8) Kautsky, H. Trans. Farad. Soc. 1939, 35, 216.

(9) Weiss, J. Trans. Farad. Soc. 1939, 35, 224.

(10) Khan, A. U.; Kasha, M. J. Chem. Phys. 1963, 39, 2105; 1964, 40, 605.

(11) Khan, A. U.; Kasha, M. Nature 1964, 204, 241.

(12) Arnold, S. J.; Ogryzlo, E. A.; Witzke, H. J. Chem. Phys. 1964, 40, 1769.

(13) Foote, C. S.; Wexler, S. J. Am. Chem. Soc. 1964, 86, 3879.

(14) Corey, E. J.; Taylor, W. C. J. Am. Chem. Soc. 1964, 86, 3881.

(15) Barrow, G. M. "Physical Chemistry", 4th ed.; McGraw-Hill Book Co.: New York, 1979.

(16) Foote, C. S. Accounts Chem. Res. 1968, 1, 104.

(17) Schreiner, R.; Testen, M. E. et al. In "Chemical Demonstrations"; Shakhashiri, B. Z., Ed.; The University of Wisconsin Press: Madison, 1983, Vol. 1, Chapter 2.1.

(18) Kasha, M.; Brabham, D. E. In "Singlet Oxygen"; Wasserman, H. H.; Murray, R. W., Ed.; Academic Press: New York, 1979; Chapter 1.

(19) Murray, R. W.; In "Singlet Oxygen"; Wasserman, H. H.; Murray, R. W., Ed.; Academic Press: New York, 1979; Chapter 3.

(20) Gorman, A. A.; Lovering, G.; Rodgers, M. A. J. J. Am. Chem. Soc. 1978, 100, 4527.

(21) Skoog, D. A.; West, D. M., "Principles of Instrumental Analysis", 2nd ed.; Holt, Rinehart and Winston: Philadelphia, 1980, Chapter 10.

(22) Khan, A. U.; Kasha, M. Ann. N. Y. Acad. Sci. 1970, 171, 5.

(23) Parmenter, C. S.; Rau, J. D. J. Chem. Phys. 1969, 51, 2242.

(24) Stevens, B.; Algar, B. E. J. Phys. Chem. 1968, 72, 3468.

(25) Wu, K. C.; Trozzolo, M. J. Phys. Chem. 1979, 83, 2823; 1979, 83, 3180.

(26) Gijzeman, O. L. J.; Kaufman, F.; Porter, G. J. Chem. Soc., Faraday Trans. 2, 1973, 69, 708; 1973, 69, 721.

(27) Khan, A. U. J. Phys. Chem. 1976, 80, 2219.

(28) Eriksen, J.; Foote, C. S. J. Am. Chem. Soc. 1980, 102, 6083.

(29) Saito, I.; Matsura, T. J. Am. Chem. Soc. 1981, 103, 188.

(30) Foote, C. S.; Steichen, D. S. J. Am. Chem. Soc. 1981, 103, 1855.

(31) Maurette, M. T.; Oliveros, E. et al. Helv. Chim. Acta, Fasc. 2, 1983, 66, 722.

(32) Manring, L. E.; Foote, C. S. J. Phys. Chem. 1982, 86, 1257.

(33) Spada, L. T.; Foote, C. S. J. Am. Chem. Soc. 1980, 102, 391.

(34) Garner, A.; Wilkinson, F. Chem. Phys. Lett. 1977, 45, 432.

(35) Algar, B. E.; Stevens, B. J. Phys. Chem. 1970, 74, 3029.

(36) Evans, D. F.; Tucker, J. N. J. Chem. Soc., Faraday Trans. 2, 1976, 72, 1661.

(37) Ogilby, P. R.; Foote, C. S. J. Am. Chem. Soc. 1983, 105, 3423.

(38) Hurst, J. R.; Schuster, G. B. J. Am. Chem. Soc. 1983, 105, 5756.

(39) Merkel, P. B.; Kearns, D. R. J. Am. Chem. Soc. 1972, 94, 7244.

(40) Matheson, I. B. C.; Lee, J. et al. J. Am. Chem. Soc. 1974, 96, 3343.

(41) Foote, C. S.; Penny, R. W. J. Am. Chem. Soc. 1968, 90, 6233; 1970, 92, 5216.

(42) Ogryzlo, E. A.; Tang, C. W. J. Am. Chem. Soc. 1970, 92, 5034.

(43) Matheson, I. B. C.; Lee, J. J. Am. Chem. Soc. 1971, 94, 3310.

(44) Ogilby, P. R.; Foote, C. S. J. Am. Chem. Soc. 1982, 104, 2069.

(45) Parker, J. G.; Stanbro, W. D. J. Am. Chem. Soc. 1982, 104, 2067.

(46) Rodgers, M. A. J.; Snowden, P. T. J. Am. Chem. Soc. 1982, 104, 5541.

(47) Krasnovsky, A. A., Jr. Photochem. Photobiol. 1979, 29, 29.

(48) Khan, A. U.; Kasha, M. Proc. Natl. Acad. Sci. USA 1979, 76, 6047.

(49) Khan, A. U. Chem. Phys. Lett. 1980, 72, 112.

(50) Salokhiddinov, K. I.; Dzhagarov, B. M. et al. Chem. Phys. Lett. 1980, 76, 85.

(51) Bartlett, P. D.; Mendenhall, G. D.; Schaap, A. P. Ann. N. Y. Acad. Sci. 1970, 171, 79.

(52) Mazur, S.; Foote, C. S. J. Am. Chem. Soc. 1970, 92, 3225.

(53) Zaklika, K. A.; Schaap, A. P. et al. J. Am. Chem. Soc. 1978, 100, 318; 1978, 100, 4916.

(54) Schaap, A. P.; Burns, P. A.; Zaklika, K. A. J. Am. Chem. Soc. 1977, 99, 1270.

(55) Geller, G. G.; Foote, C. S.; Pechman, D. B. Tetrahedron Lett. 1983, 24, 1983.

(56) Gollnick, K.; Schenck, G. O. Pure Appl. Chem. 1964, 9, 507.

(57) Merkel, P. B.; Kearns, D. R. J. Am. Chem. Soc. 1975, 97, 462.

(58) Foote, C. S.; Ching, T. Y. J. Am. Chem. Soc. 1975, 97, 6209.

(59) Stevens, B.; Perez, S. R.; Ors, J. A. J. Am. Chem. Soc. 1974, 96, 6846.

(60) Paquette, L. A.; Kretschmer, G. J. Am. Chem. Soc. 1979, 101, 4655.

(61) "Singlet Oxygen", Wasserman, H. H.; Murray, R. W., Ed.; Academic Press: New York, 1979; Chapters 11 and 12.

(62) Raab, O. Z. Biol. 1900, 39, 524.

(63) Spikes, J. D. In "The Science of Photobiology", Smith, K. C., Ed.; Plenum Press: New York, 1977; Chapter 4.

(64) Grossweiner, L. I.; Kepka, A. G. Photochem. Photobiol. 1972, 16, 305.

(65) Nilsson, R.; Kearns, D. R. Photochem. Photobiol. 1973, 17, 65.

(66) Kepka, A. G.; Grossweiner, L. I. Photochem. Photobiol. 1973, 18, 49.

(67) Jori, G.; Folin, G. et al. Photochem. Photobiol. 1974, 19, 419.

(68) Ito, T.; Kobayashi, K. Photochem. Photobiol. 1977, 26, 581.

(69) Ito, T. Photochem. Photobiol. 1977, 25, 47.

(70) Spikes, J. D.; Straight, R. Ann. Rev. Phys. Chem. 1967, 18, 409.

(71) Tappeiner, H; Jesionek, A. Münchener Med. Wochenschr. 1903, 50, 2042.

(72) Auler, H.; Banzer, G. Z. Krebforsch. 1942, 53, 65.

(73) Diamond, I.; Granelli, S. et al. Lancet, 1972, 2, 1175.

(74) Dougherty, T. J. J. Natl. Cancer Inst. 1974, 52, 1333.

(75) Dougherty, T. J.; Grindey, G. B. et al. J. Natl. Cancer Inst. 1975, 55, 115.

(76) Weiskaupt, K.; Comer, C. J.; Dougherty, T. J. Cancer Research 1976, 36, 2326.

(77) Tomson, S. H.; Emmet, E. A.; Fox, S. H. Cancer Research 1974, 34, 3124.

(78) Berns, M. W., Wile, A. et al. In Ref. 4, 139.

(79) Parrish, J. A. In Ref. 4, 91.

(80) Kessel, D.; Chou, T. H. In Ref. 4, 115.

(81) Vincent, R. G.; Dougherty, T. J. et al. In Ref. 4, 41.

(82) Ghosh, P. B. J. Chem. Soc. (B), 1968, 334.

(83) Ghosh, P. B.; Whitehouse, M. W. Biochem. J. 1968, 108, 155.

(84) Houk, T. W.; Ovnic, M.; Karipides, S. J. Biol. Chem. 1983, 258, 5419.

(85) Price, N. C.; Cohn, M.; Schirmer, R. H. J. Biol. Chem. 1975, 250, 644.

(86) Wong, P. Diss. Abstr. Int. B. 1976, 37, 1676; Chem. Abstr. 1977, 86, 1706x.

(87) Sutton, R.; Ferguson, S. J. Biochem. Soc. Trans. 1984, 12, 467.

(88) Kapoor, M.; Parfett, C. L. Arch. Biochem. Biophys. 1977, 184, 518.

(89) Price, N. C.; Radda, G. K. Biochim. Biophys. Acta 1974, 371, 102.

(90) Aboderin, A. A.; Boedefeld, E. Biochim. Biophys. Acta 1976, 420, 177.

(91) Brocklehurst, K.; Salih, E.; Lodwig, T. S. Biochem. J. 1984, 220, 609.

(92) Baines, B. S.; Brocklehurst, K. Biochem. J. 1982, 205, 205.

(93) Dwek, R. A.; Radda, G. K. et al. Eur. J. Biochem. 1972, 29, 509.

(94) Bennick, A.; Campbell, I. D. et al. Nature (London) New Biol. 1971, 234, 140; Chem. Abstr. 1972, 76, 56030w.

(95) Bratcher, S. C.; Kronman, M. J. Biochem. Biophys. Res. Commun. 1977, 79, 203.

(96) Klimisch, H. J.; Stadler, L. J. Chromatogr. 1974, 90, 141.

(97) Fager, R. S.; Kutina, C. B.; Abrahamson, E. W. Anal. Biochem. 1973, 53, 290.

(98) Klimisch, H. J.; Stadler, L. J. Chromatogr. 1974, 90, 223.

(99) Lo Greco, P.; Cruciatti, A.; Pagani, T. Friuli Med. 1973, 28, 475; Chem. Abstr. 1975, 83, 53110f.

(100) Hopen, T. J.; Briner, R. C. et al. J. Forensic Sci. 1976, 21, 842.

(101) Moree-Testa, P.; Saint-Jalm, Y.; Testa, A. J. Chromatogr. 1984, 290, 263

(102) Rando, R. R.; Bangerter, F. W.; Alecio, M. R. Biochim. Biophys. Acta. 1982, 684, 12.

(103) Amorese, A.; Del Rosso, M. D. et al. Chim. Ind. (Milan) 1976, 58, 452; Chem. Abstr. 1977, 86, 105336t.

(104) Miki, M.; Iio, T. Biochim. Biophys. Acta. 1984, 790, 201.

(105) Gübitz, G.; Wintersteiger, R.; Frei, R. W. J. Liq. Chromatogr. 1984, 7, 839.

(106) Meyers, H. W.; Jürss, R. et al. Eur. J. Biochem. 1983, 137, 399.

(107) Ghosh, P. B.; Whitehouse, M. W. et al. J. Med. Chem. 1968, 11, 305; 1968, 12, 505; 1972, 15, 255.

(108) Kessel, D.; Belton, J. G. Cancer Res. 1975, 35, 3735.

(109) Macphee, D. G.; Robert, G. P. et al. Chem.-Biol. Interact. 1977, 19, 77.

(110) Kenner, R. A.; Aboderin, A. A. Biochem. 1971, 10, 4433.

(111) Crouch, R. L. M. A. Thesis, Drake University, Desk Moines, Iowa, 1978.

(112) Willard, H. H.; Merritt, L. L.; Dean, J. A.; Settle, F. A. "Instrumental Methods of Analysis", 6th ed.; D. Van Nostrand Co.: New York, 1981, Chapter 4.1.

(113) Braude, E. A.; Linstead, R. P.; Woolridge, K. R. H. J. Chem. Soc. 1954, 3586.

(114) Hovey, M. C. J. Am. Chem. Soc. 1982, 104, 4196.

(115) Skoog, D. A.; West, D. M., "Principles of Instrumental Analysis", 2nd ed.; Holt, Rinehart and Winston: Philadelphia, 1980, Chapter 14.

(116) McLachlan, D.; Arnason, T.; Lam, J. Photochem. Photobiol. 1984, 39, 177.

(117) Lamberts, J. J. M.; Schumacher, D. R.; Neckers, D. C. J. Am. Chem. Soc. 1984, 106, 5879.

(118) Davidson, R. S.; Tretheway, K. R. J. Am. Chem. Soc. 1976, 98, 4008.

(119) Young, R. H.; Martin, R. L. J. Am. Chem. Soc. 1972, 94, 5183.

Appendix

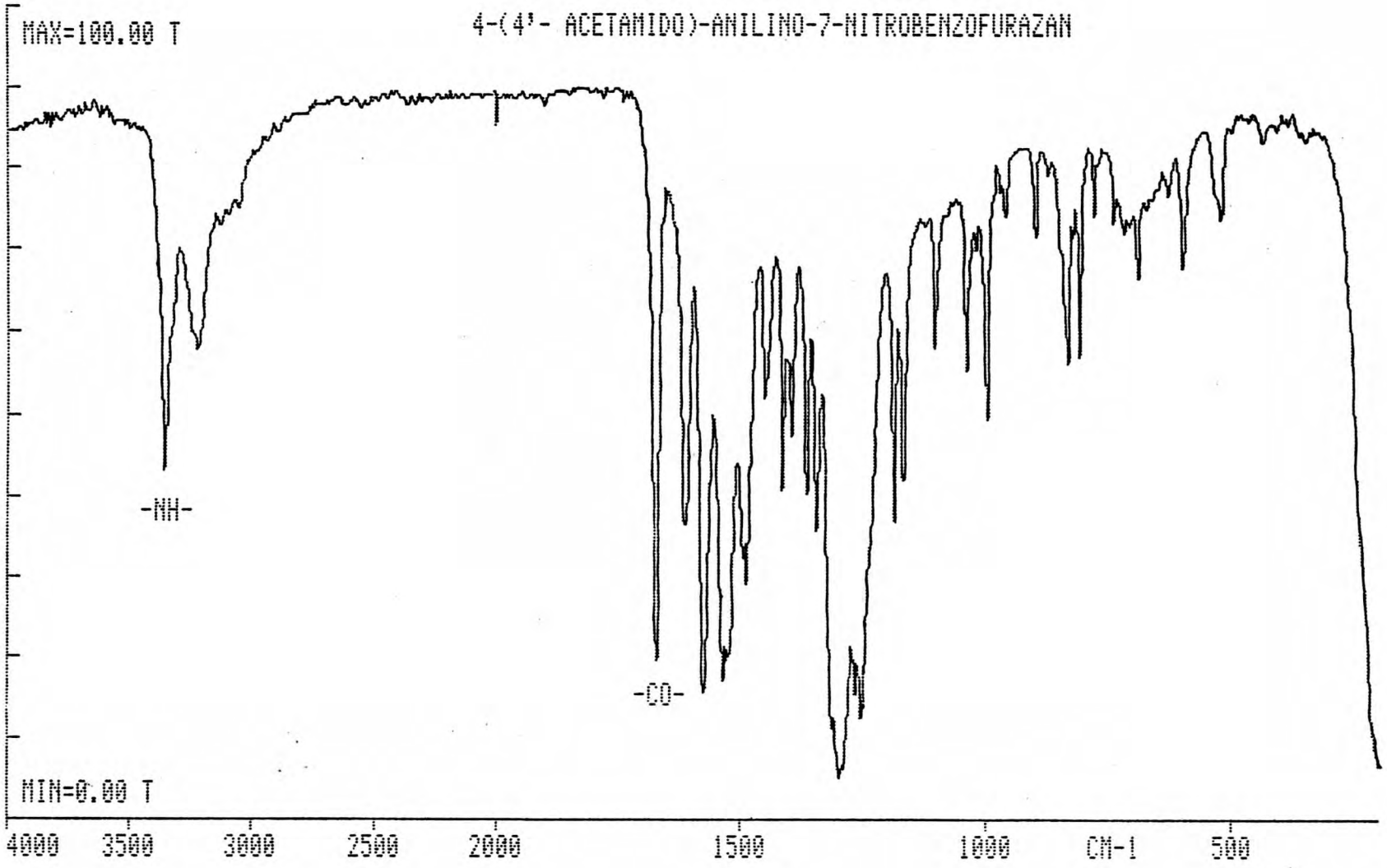


Figure 18. Infrared absorption spectrum of the p-$NHCOCH_3$ analog.

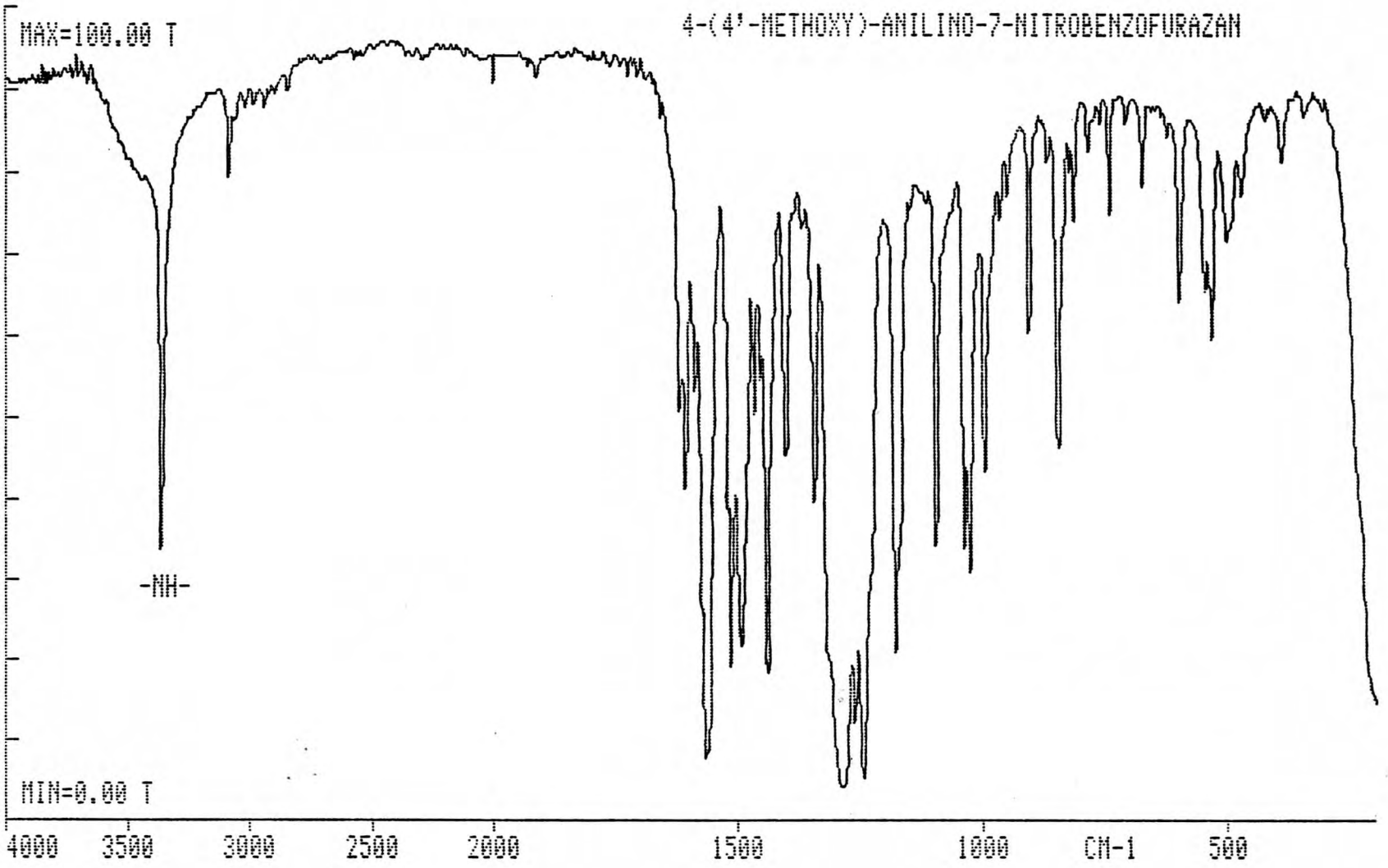


Figure 19. Infrared absorption spectrum of the p-OCH_3 analog.

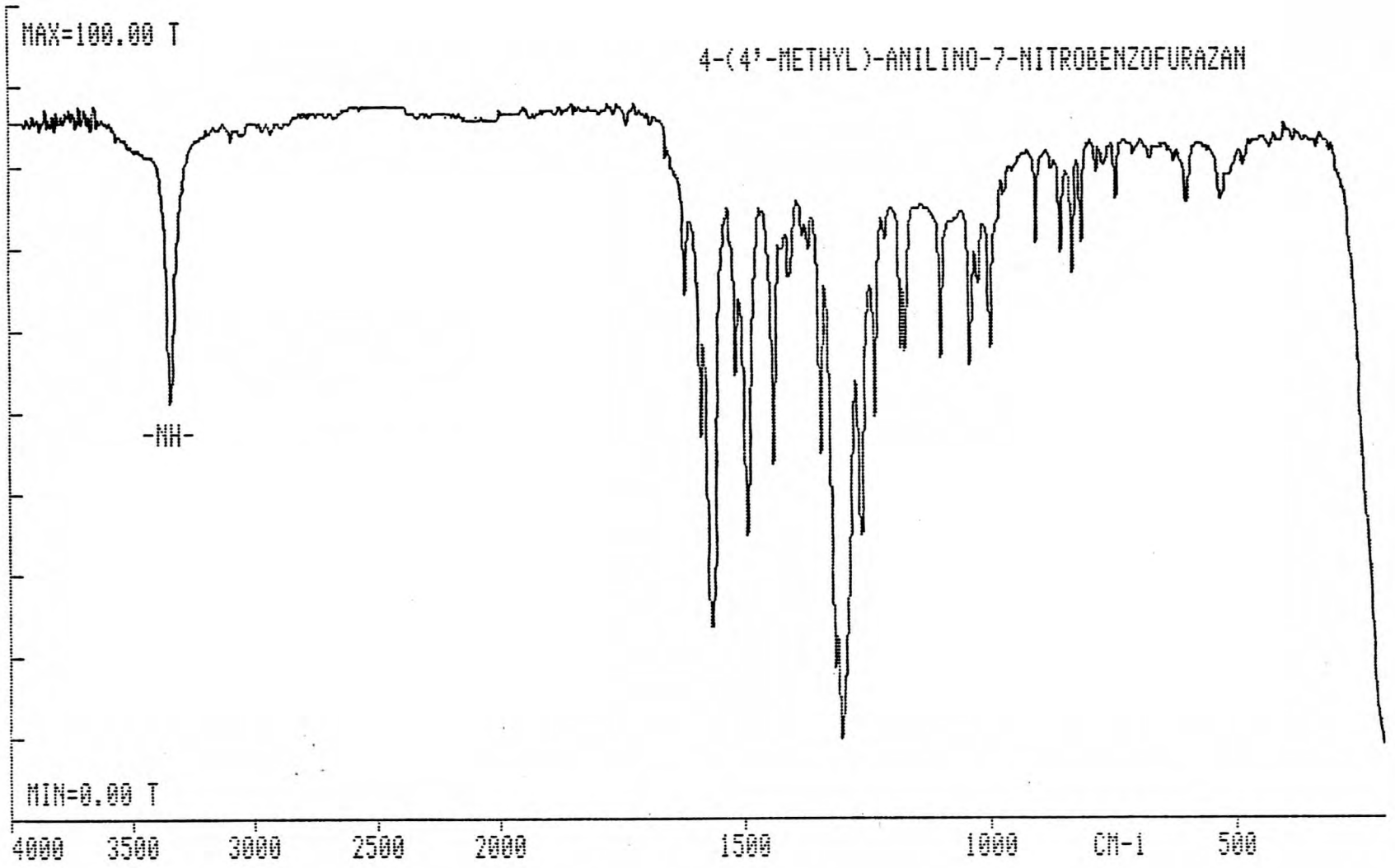


Figure 20. Infrared absorption spectrum of the p-CH_3 analog.

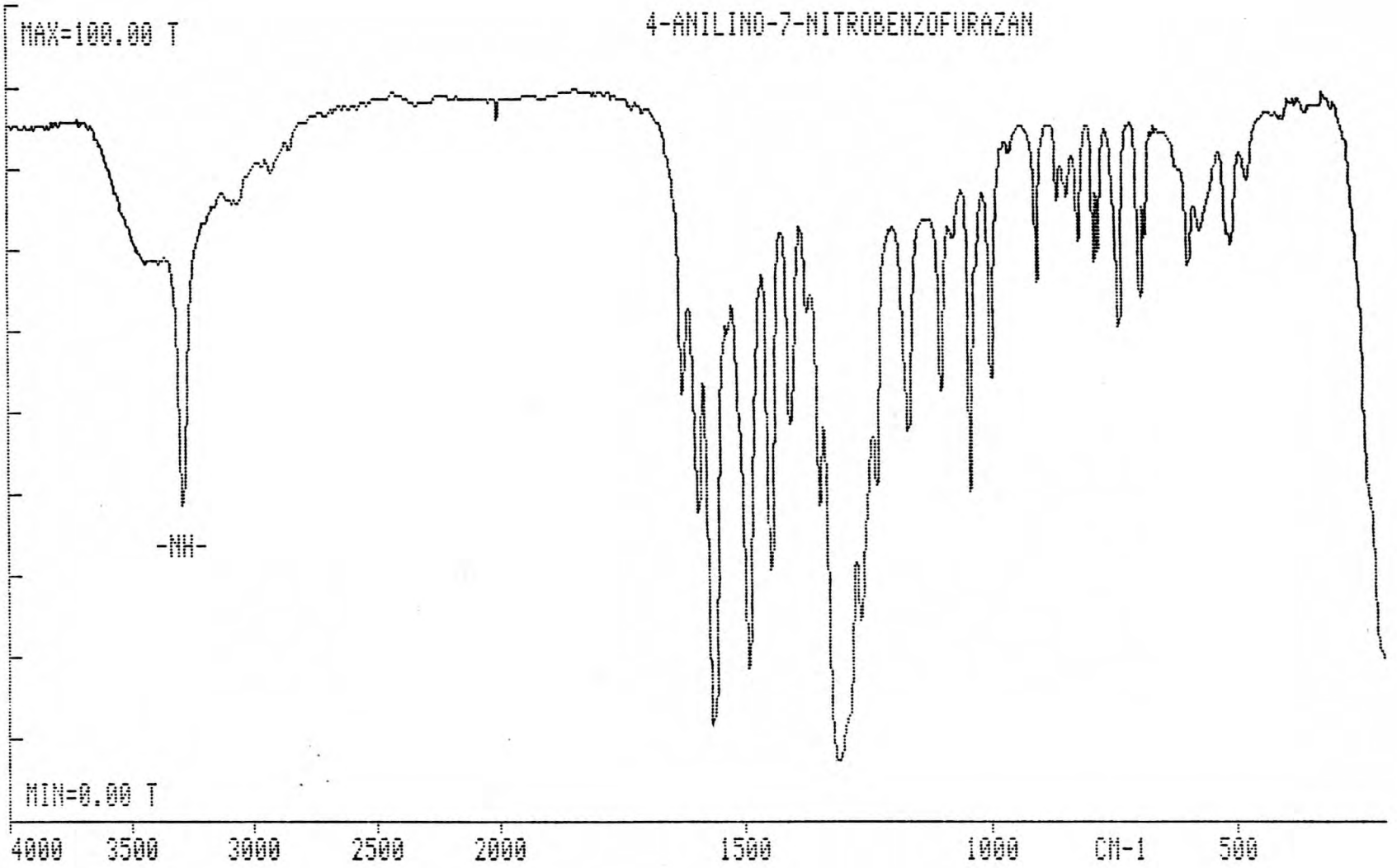


Figure 21. Infrared absorption spectrum of the p-H analog.

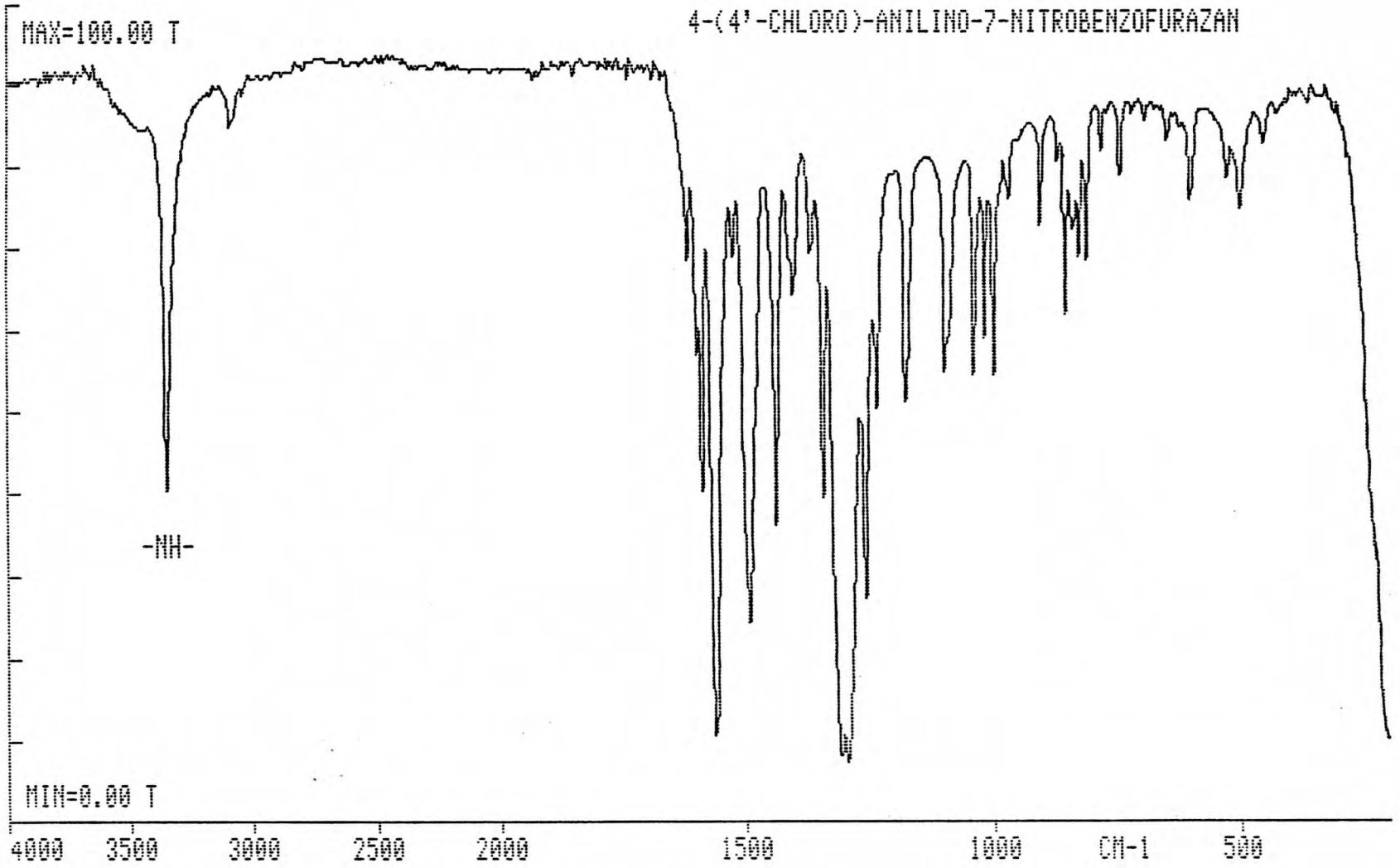


Figure 22. Infrared absorption spectrum of the p-Cl analog.

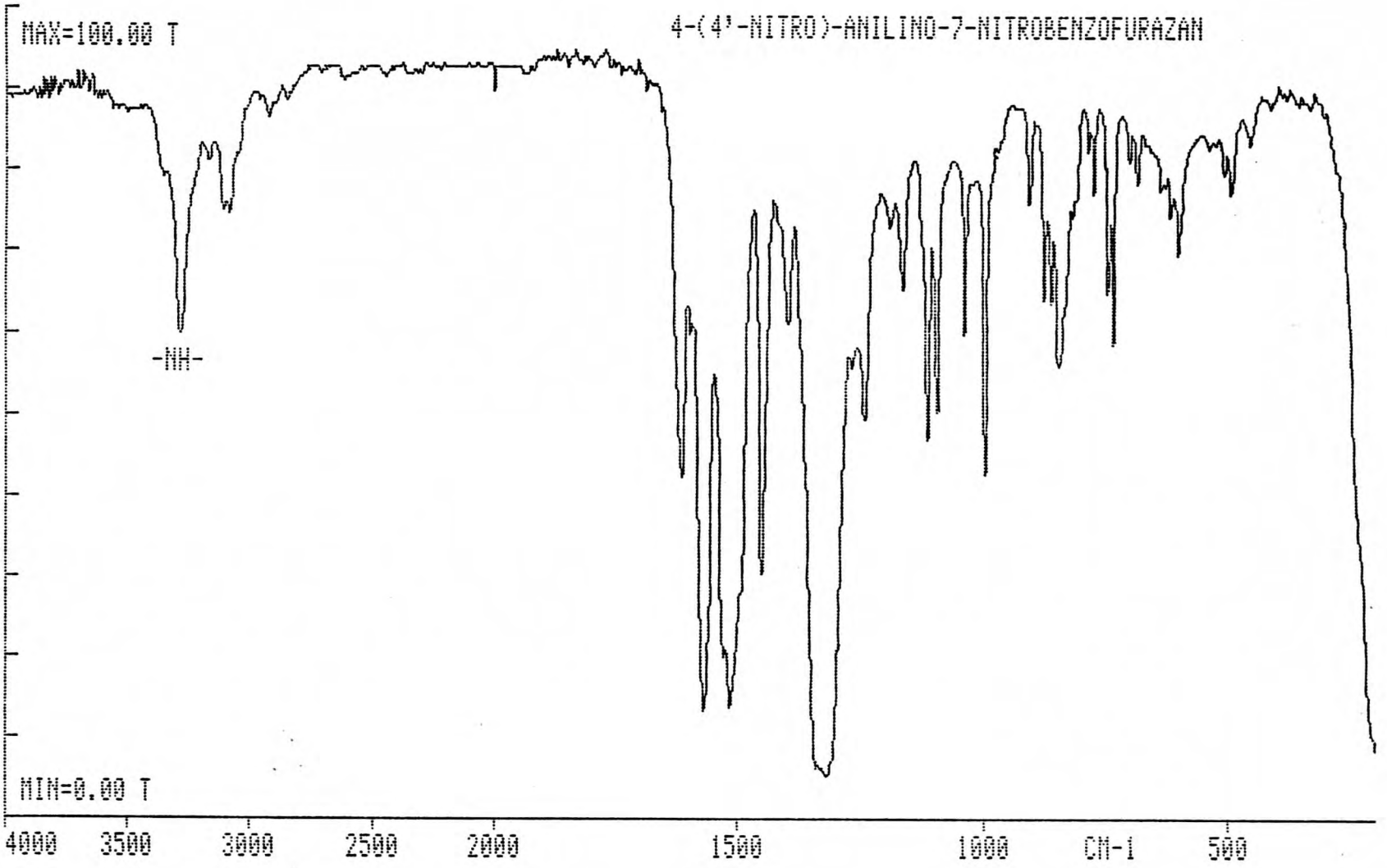


Figure 23. Infrared absorption spectrum of the p-NO_2 analog.

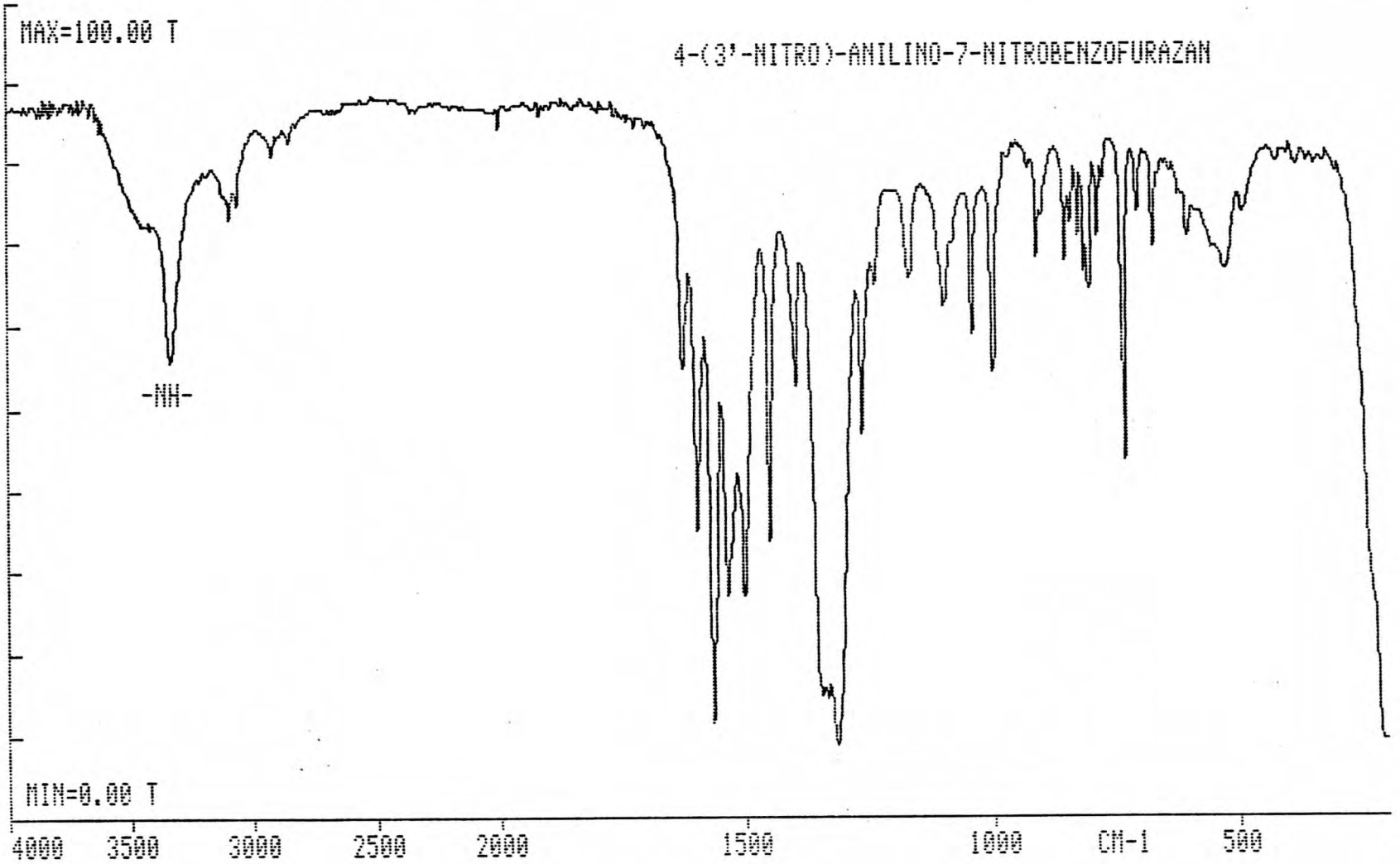


Figure 24. Infrared absorption spectrum of the m-NO_2 analog.

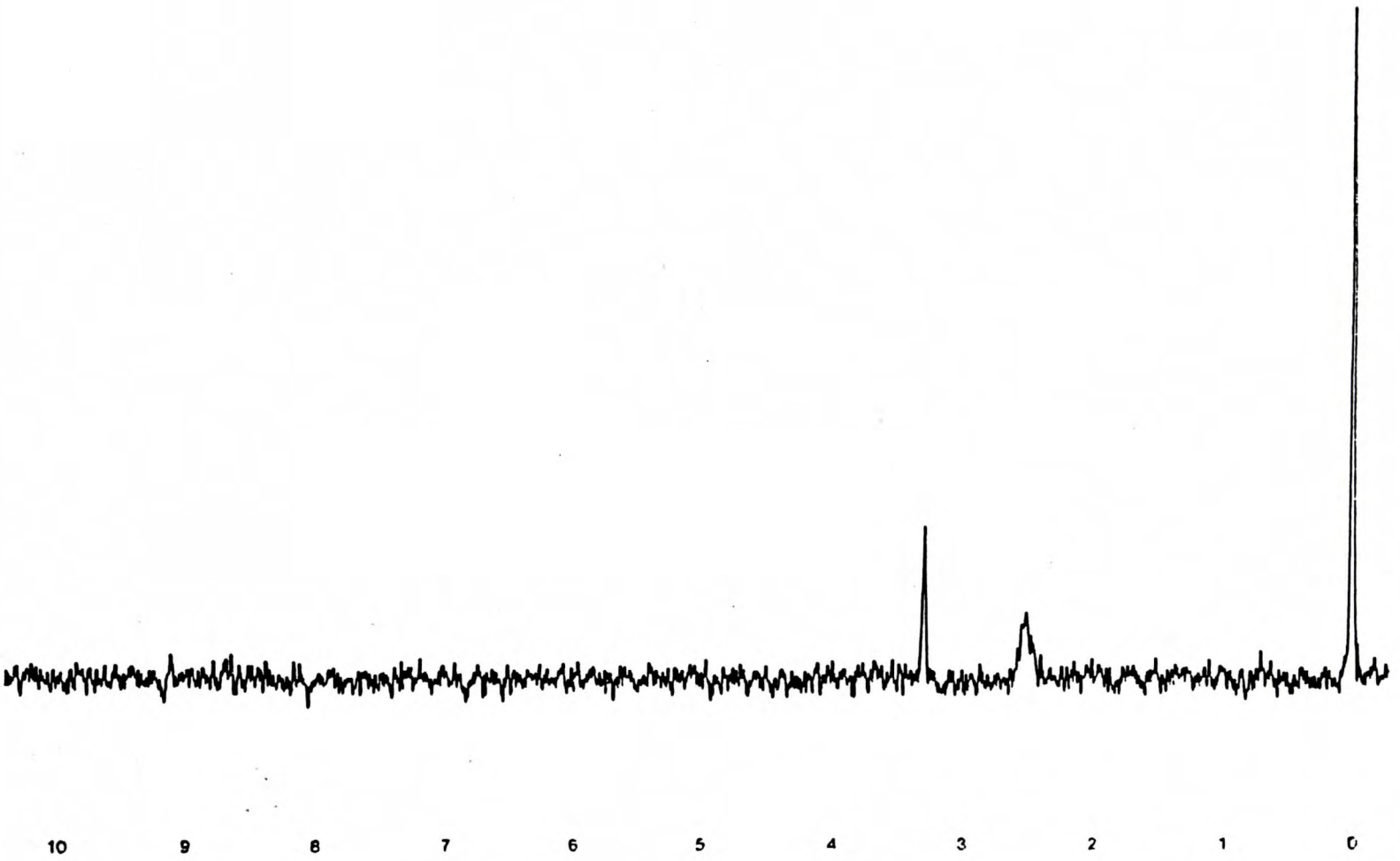


Figure 25. NMR spectrum of Dimethyl Sulfoxide-d_6.

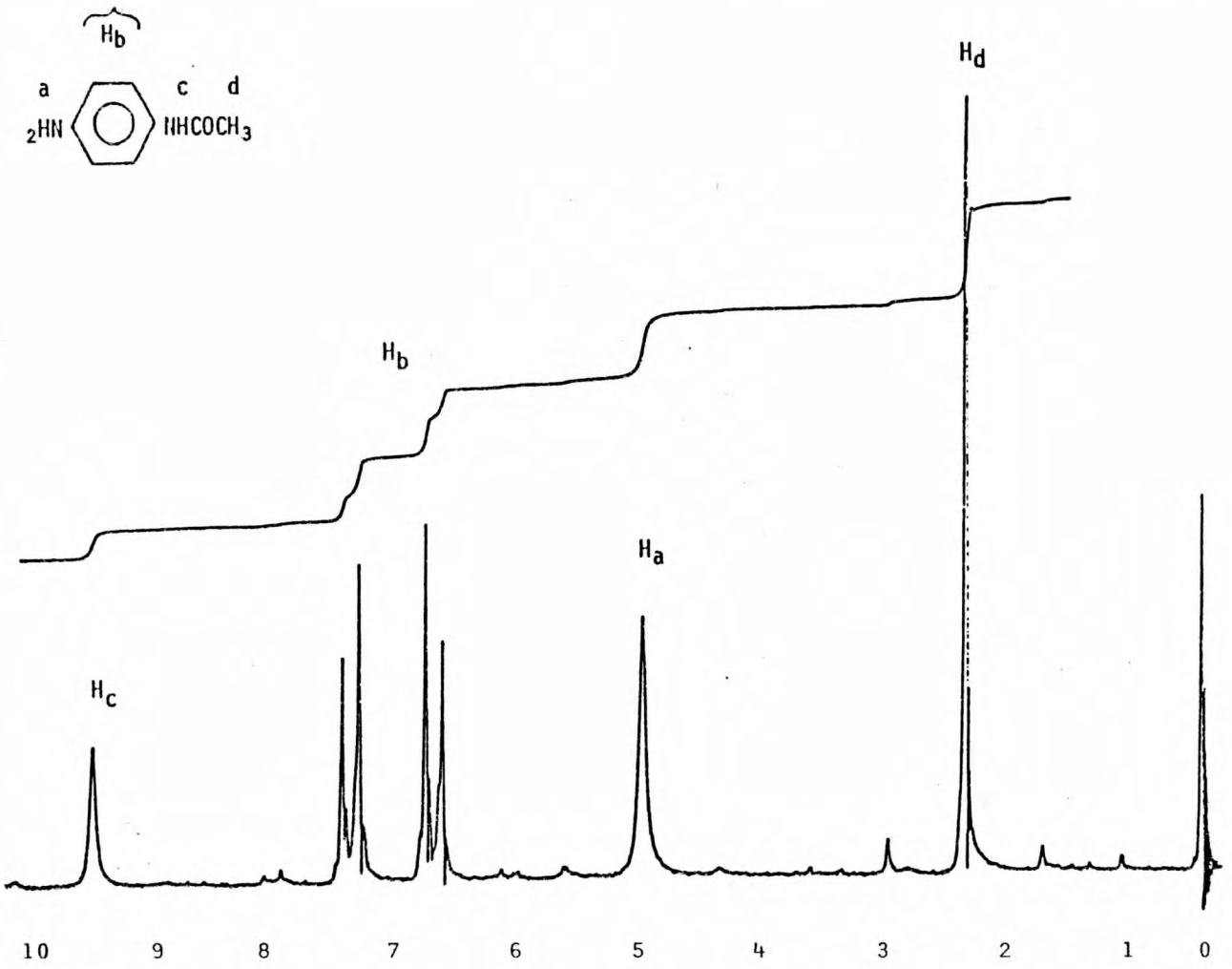


Figure 26. NMR spectrum of p-aminoacetanilide in DMSO-d_6.

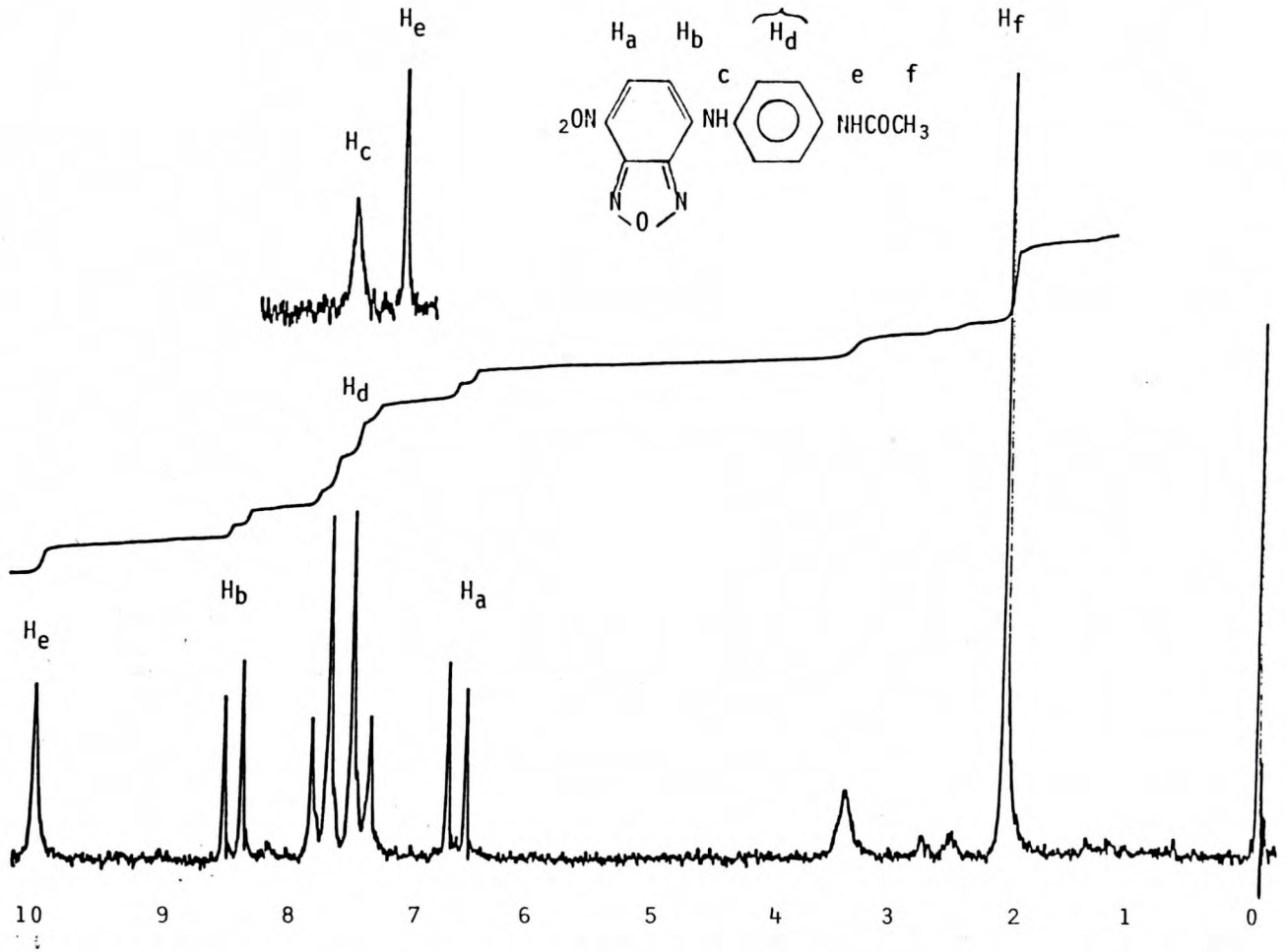


Figure 27. NMR spectrum of 4-(4'-acetamido)-anilino-7-nitrobenzofurazan in DMSO-d_6.

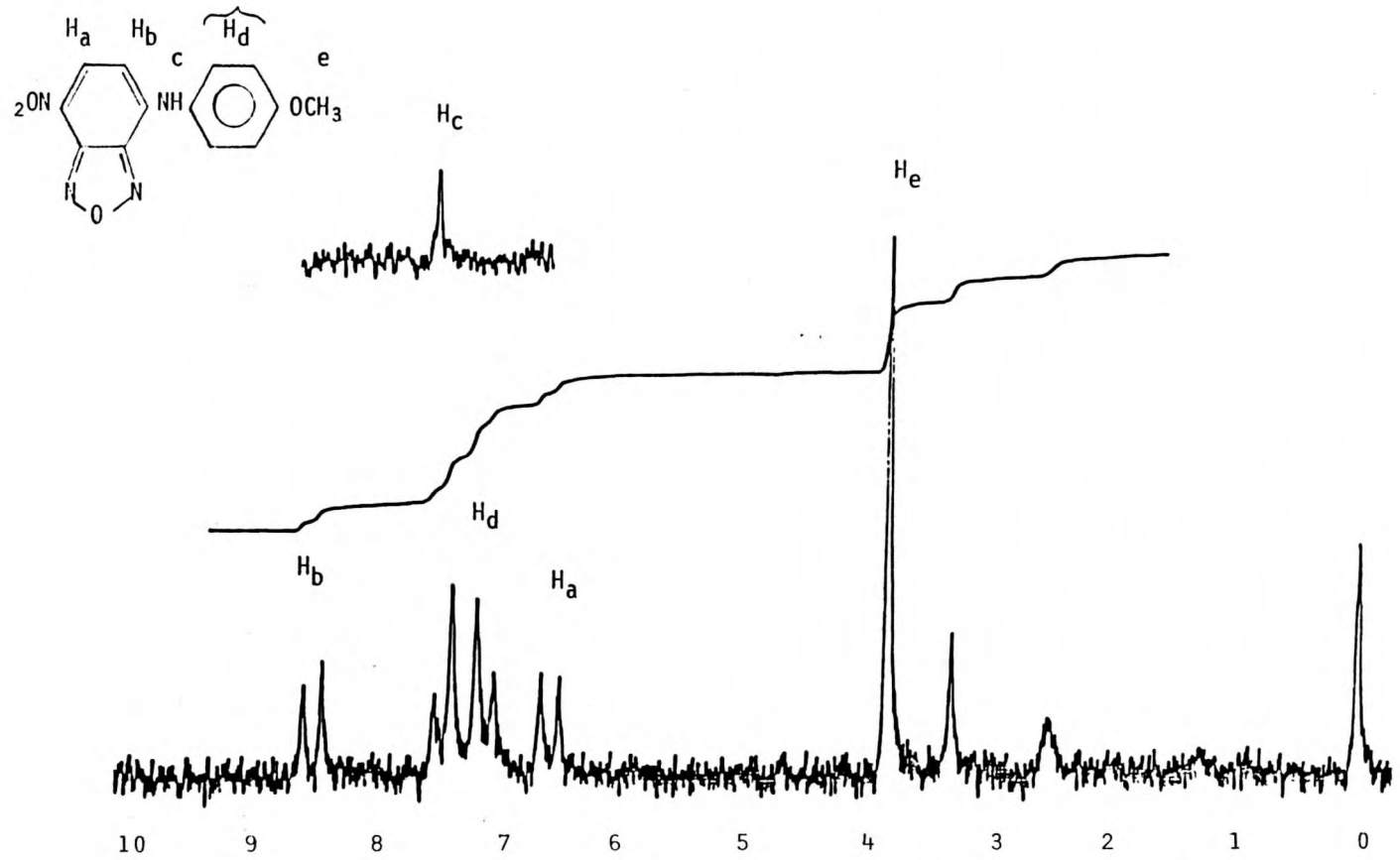


Figure 28. NMR spectrum of 4-(4'-methoxy)-anilino-7-nitrobenzofurazan in DMSO-d_6.

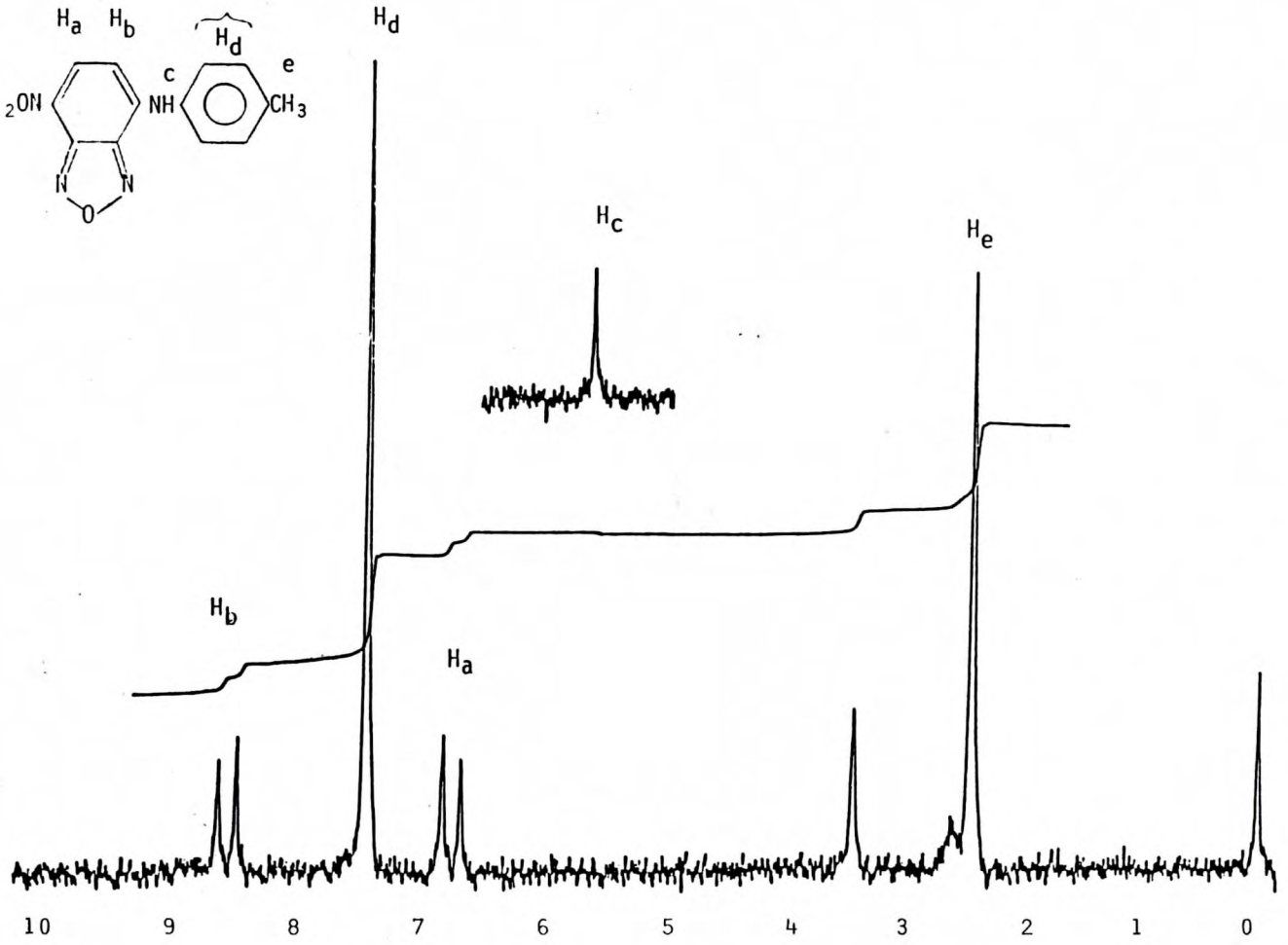


Figure 29. NMR spectrum of 4-(4'-methyl)-anilino-7-nitrobenzofurazan in DMSO-d_6.

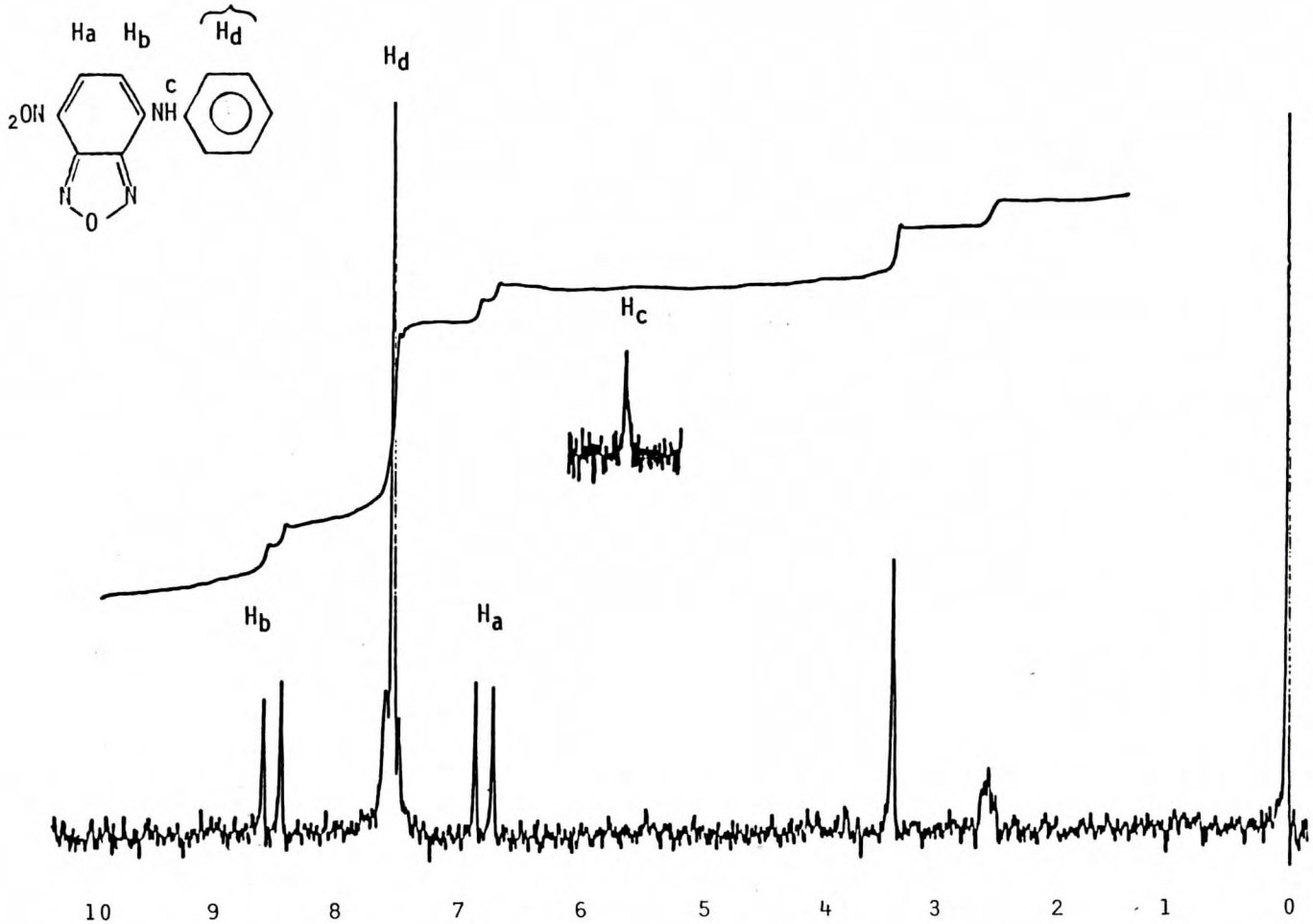


Figure 30. NMR spectrum of 4-anilino-7-nitrobenzofurazan in DMSO-d_6.

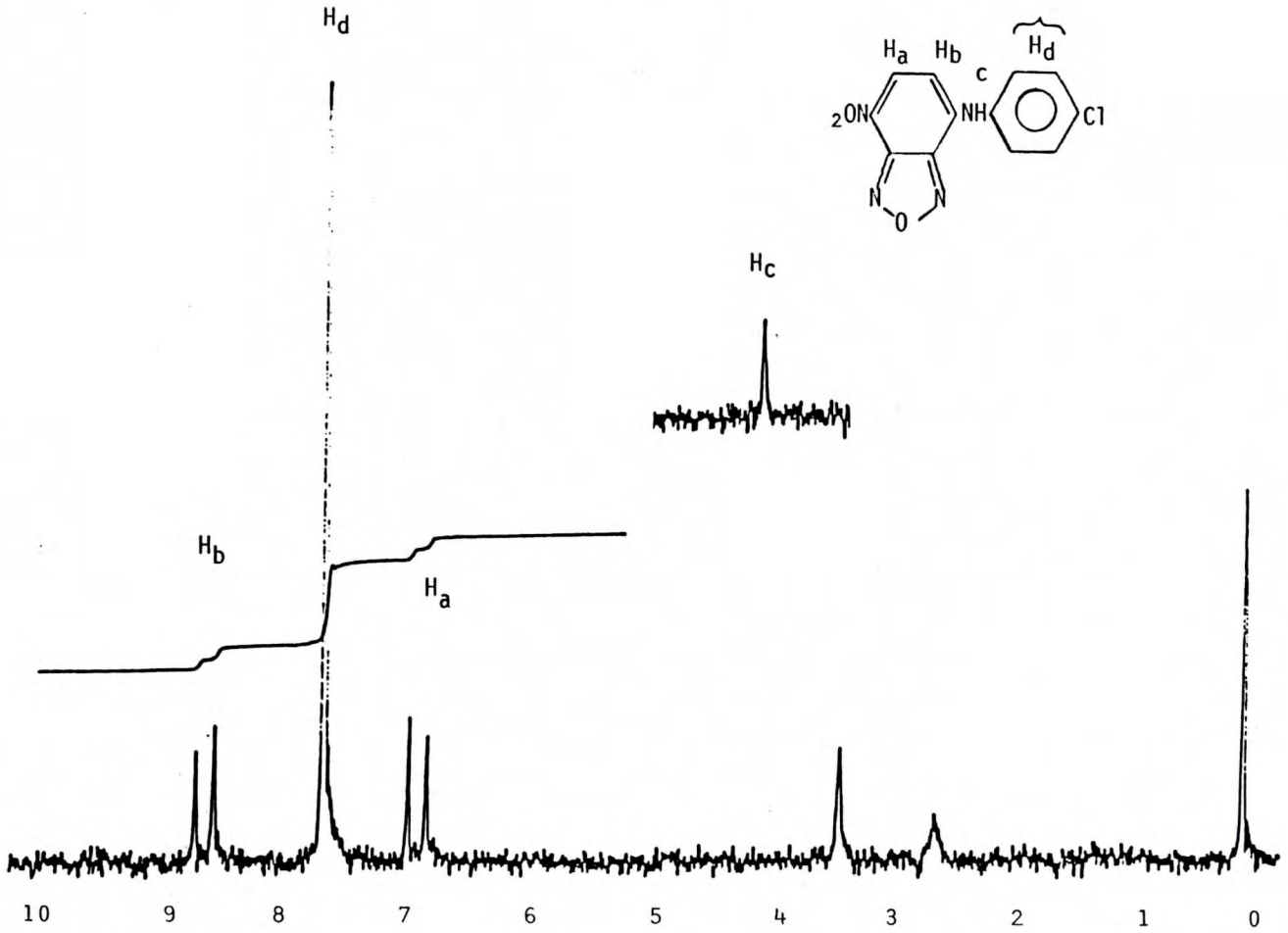


Figure 31. NMR spectrum of 4-(4'-chloro)-anilino-7-nitrobenzofurazan in DMSO-d_6.

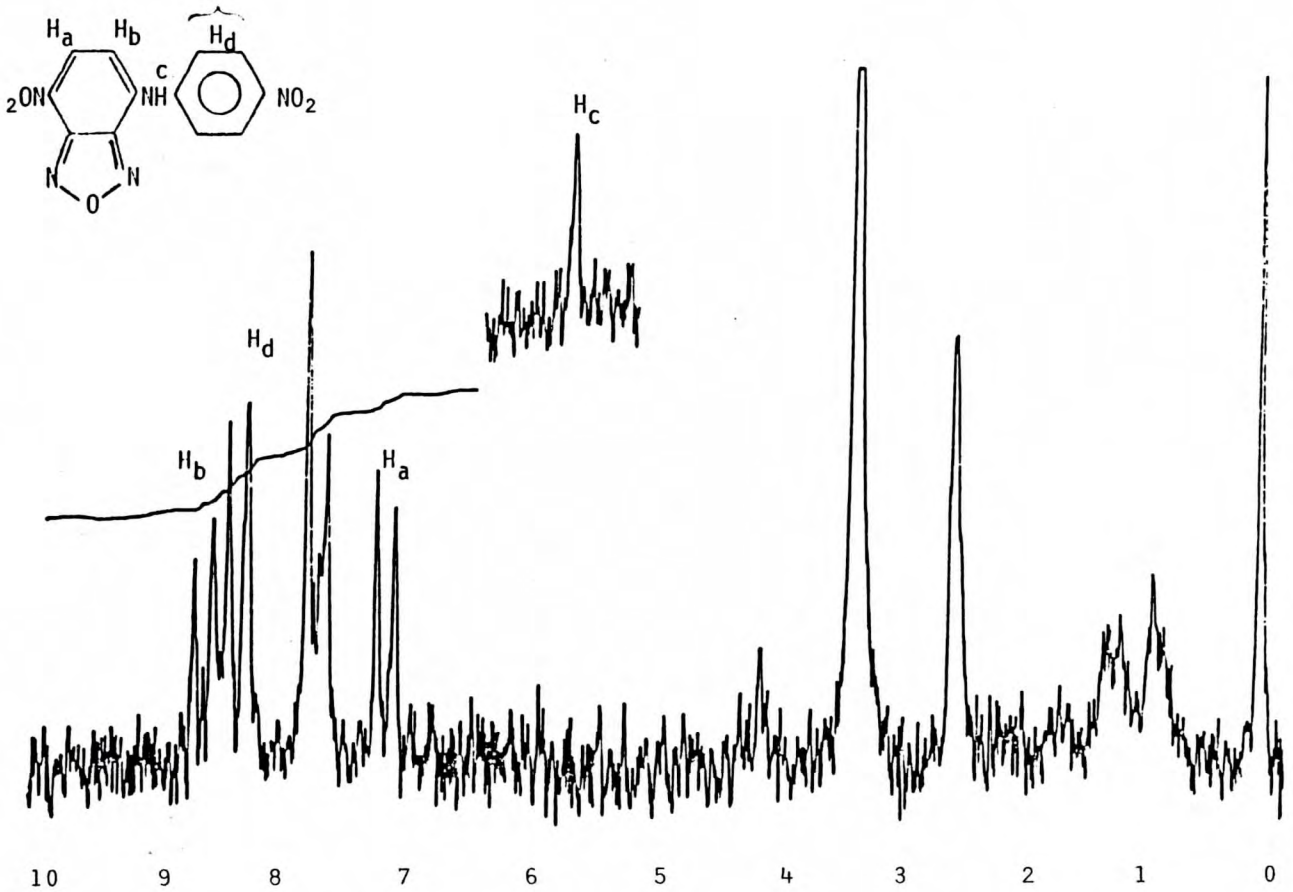


Figure 32. NMR spectrum of 4-(4'nitro)-anilino-7-nitrobenzofurazan in DMSO-d_6.

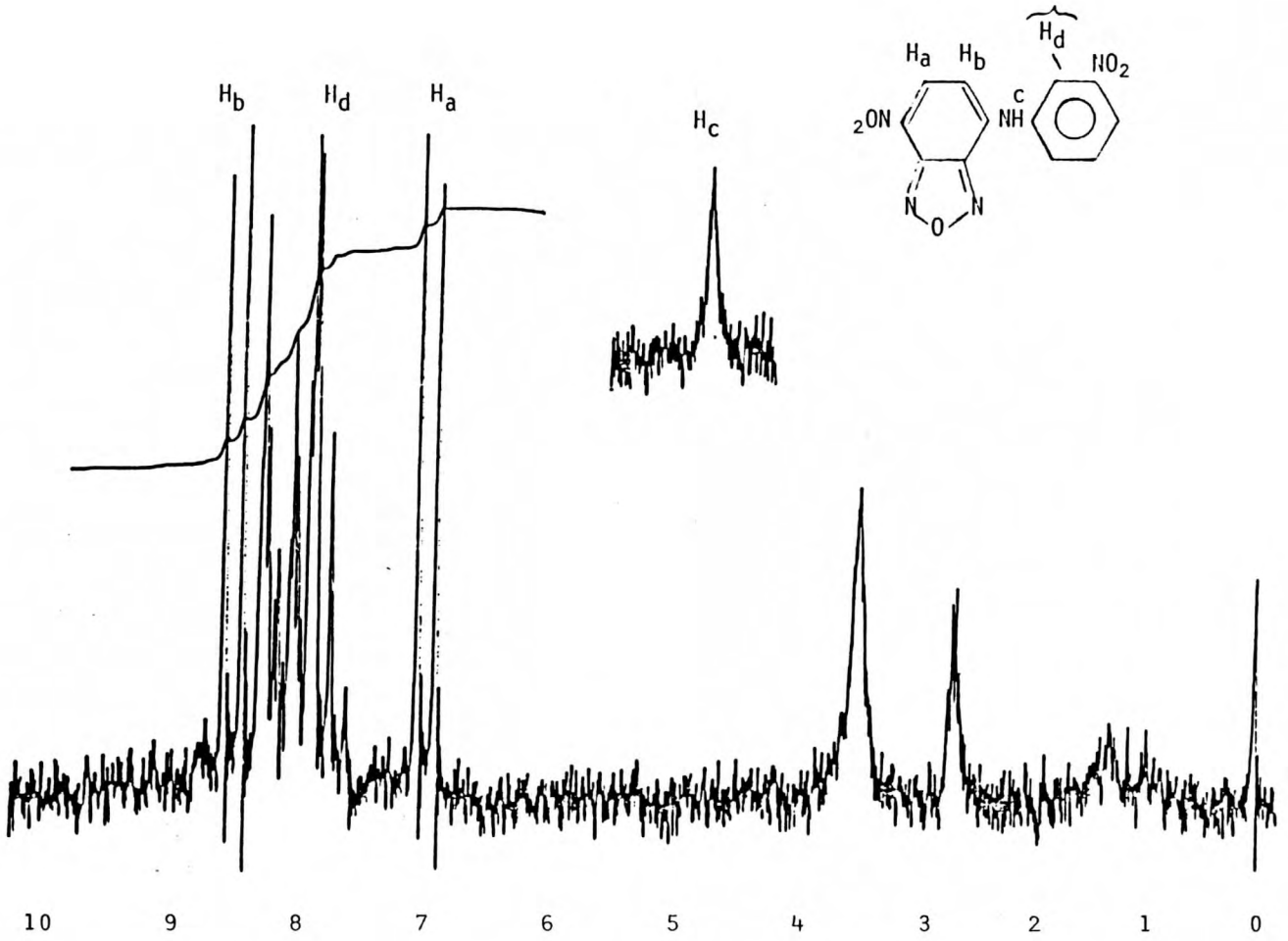


Figure 33. NMR spectrum of 4-(3'-nitro)-anilino-7-nitrobenzofurazan in DMSO-d_6.

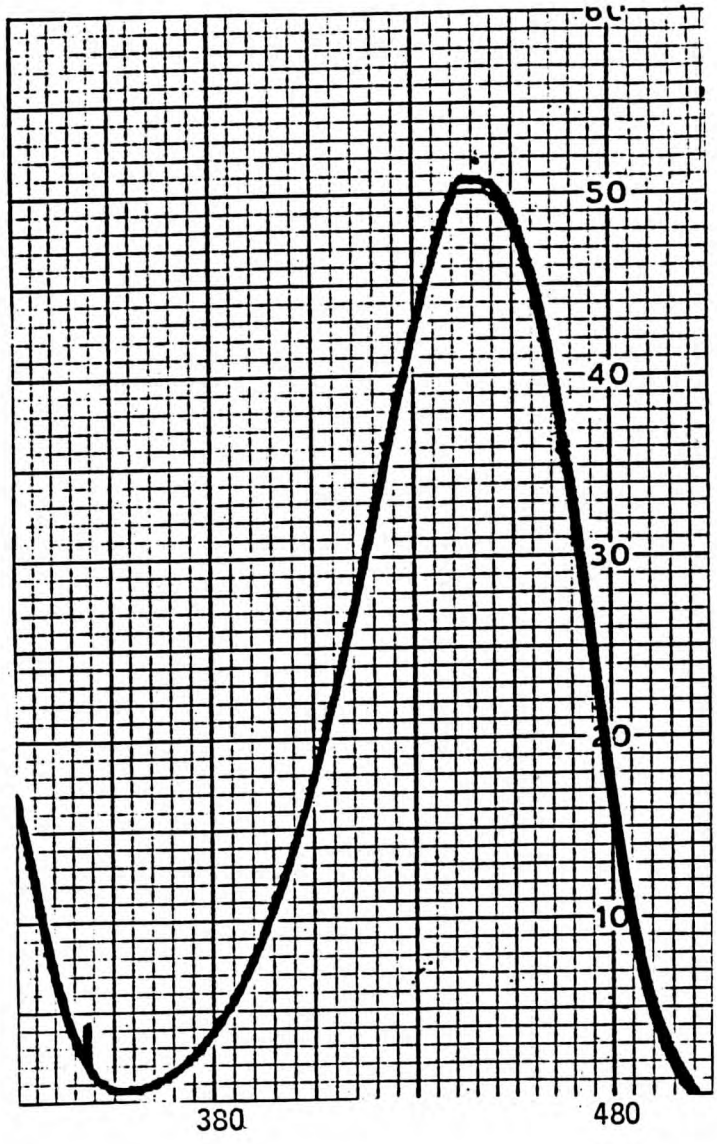


Figure 34. Visible absorption spectra of BBD alone at 30 minute time intervals over a period of 2.5 hours.

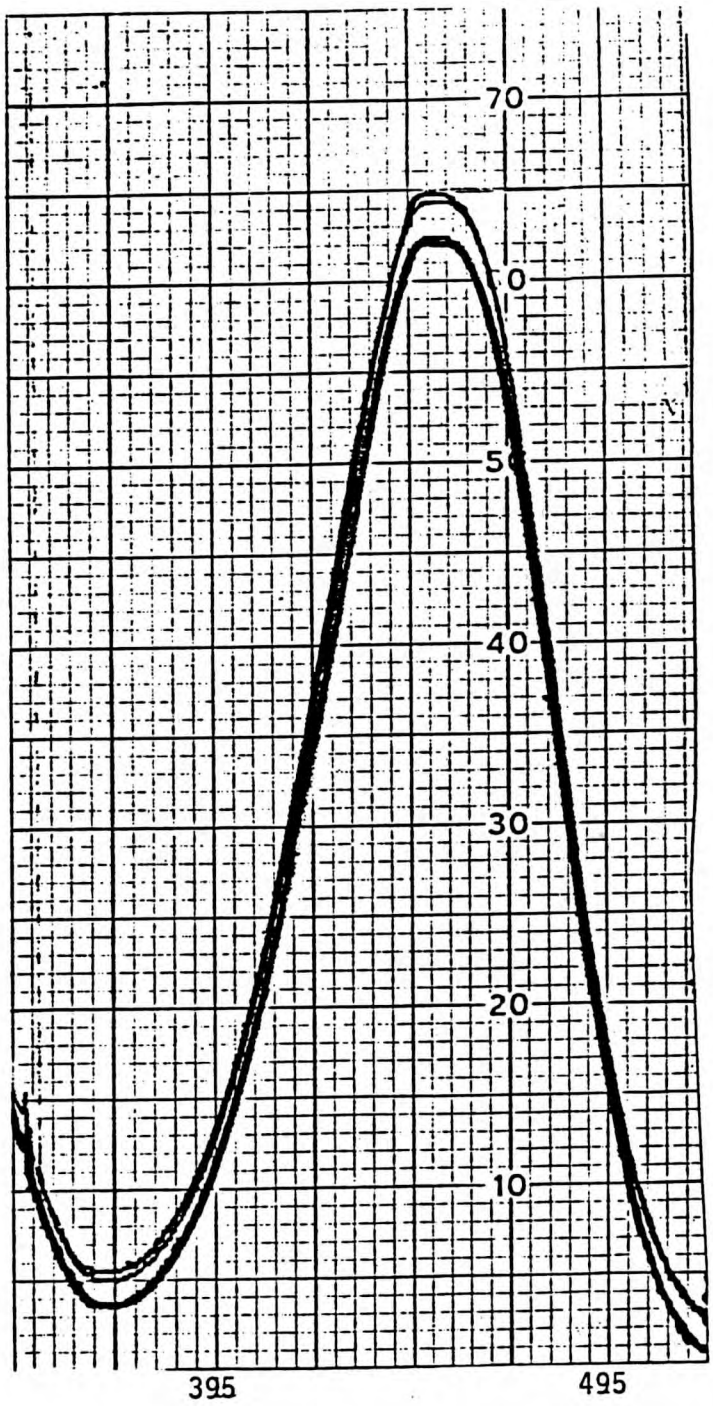


Figure 35. Visible absorption spectra of the p-NO_2 analog at 30 minute time intervals over a period of 2.5 hours.

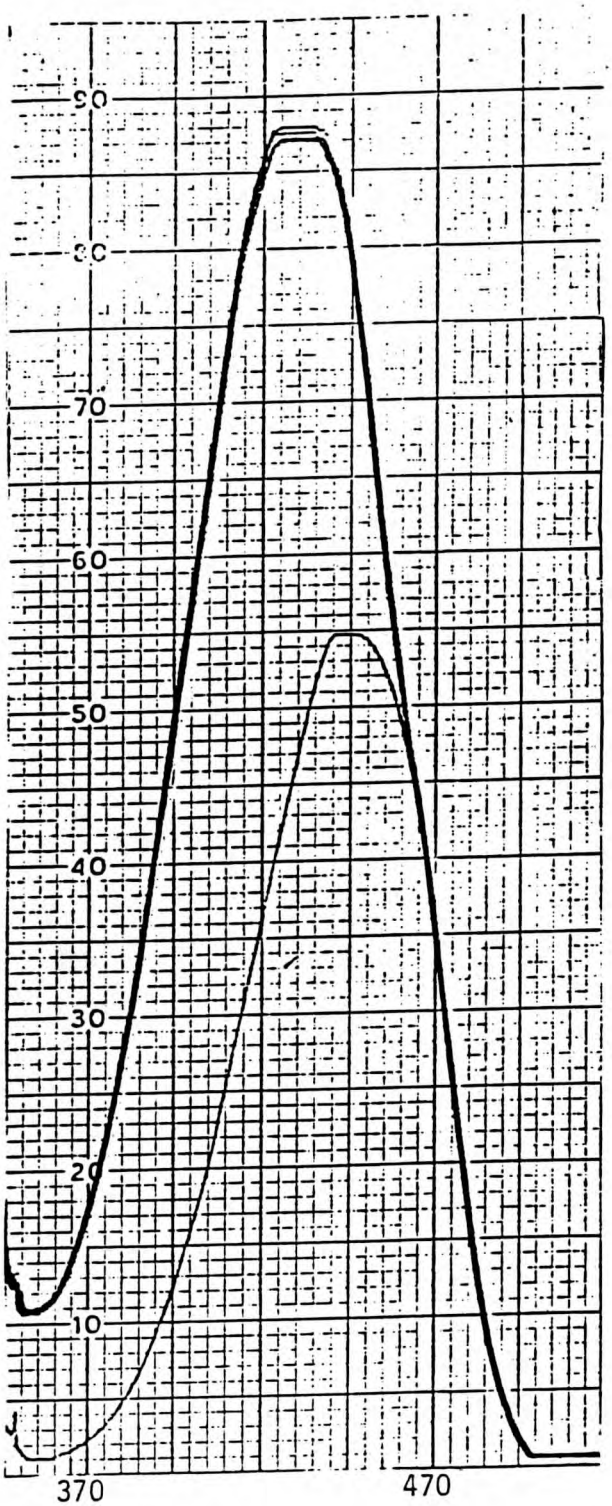


Figure 36. Visible absorption spectra of DPBF and BBD in oxygen with no irradiation at 30 minute time intervals for 2.5 hours.

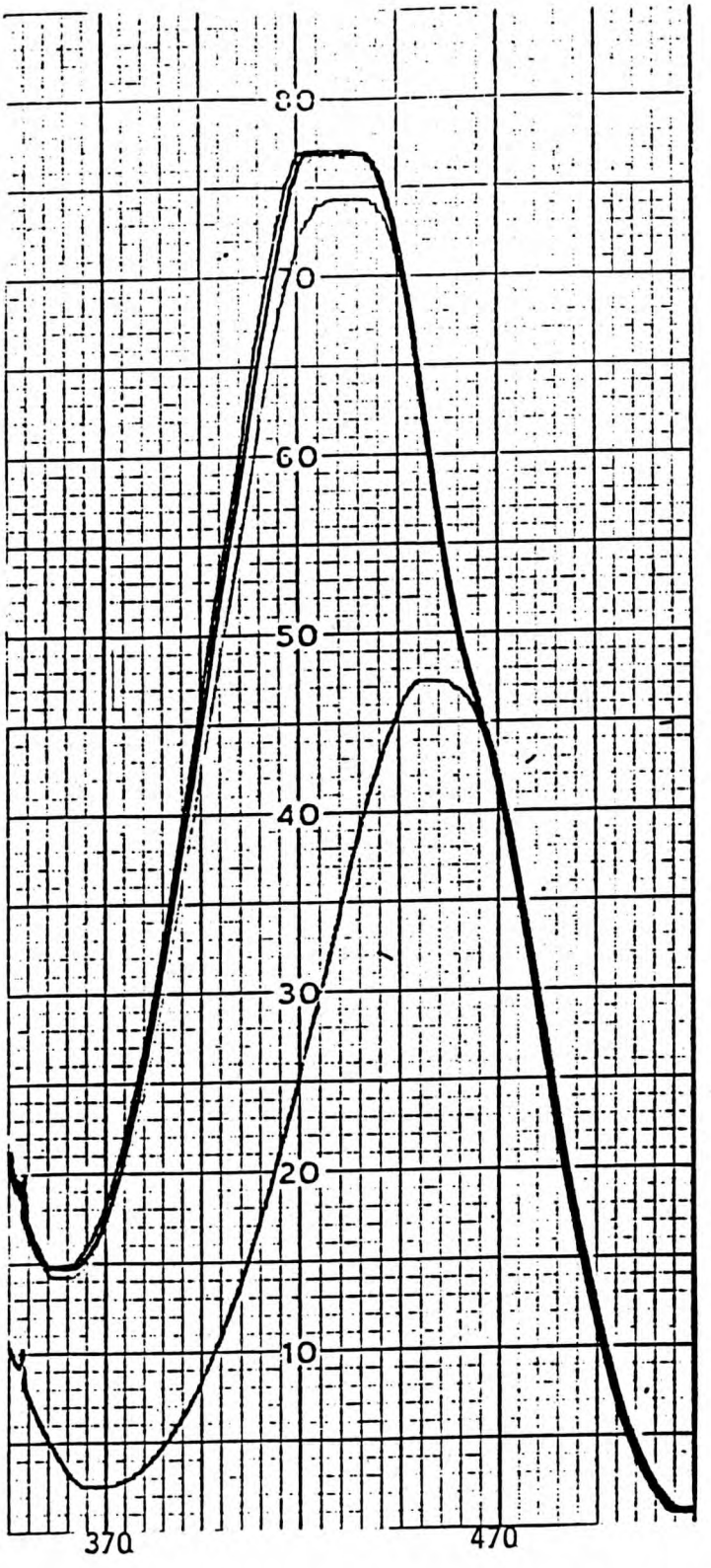


Figure 37. Visible absorption spectra of DPBF and the p-NO_2 analog in oxygen with no irradiation at 30 minute time intervals for 2.5 hours.

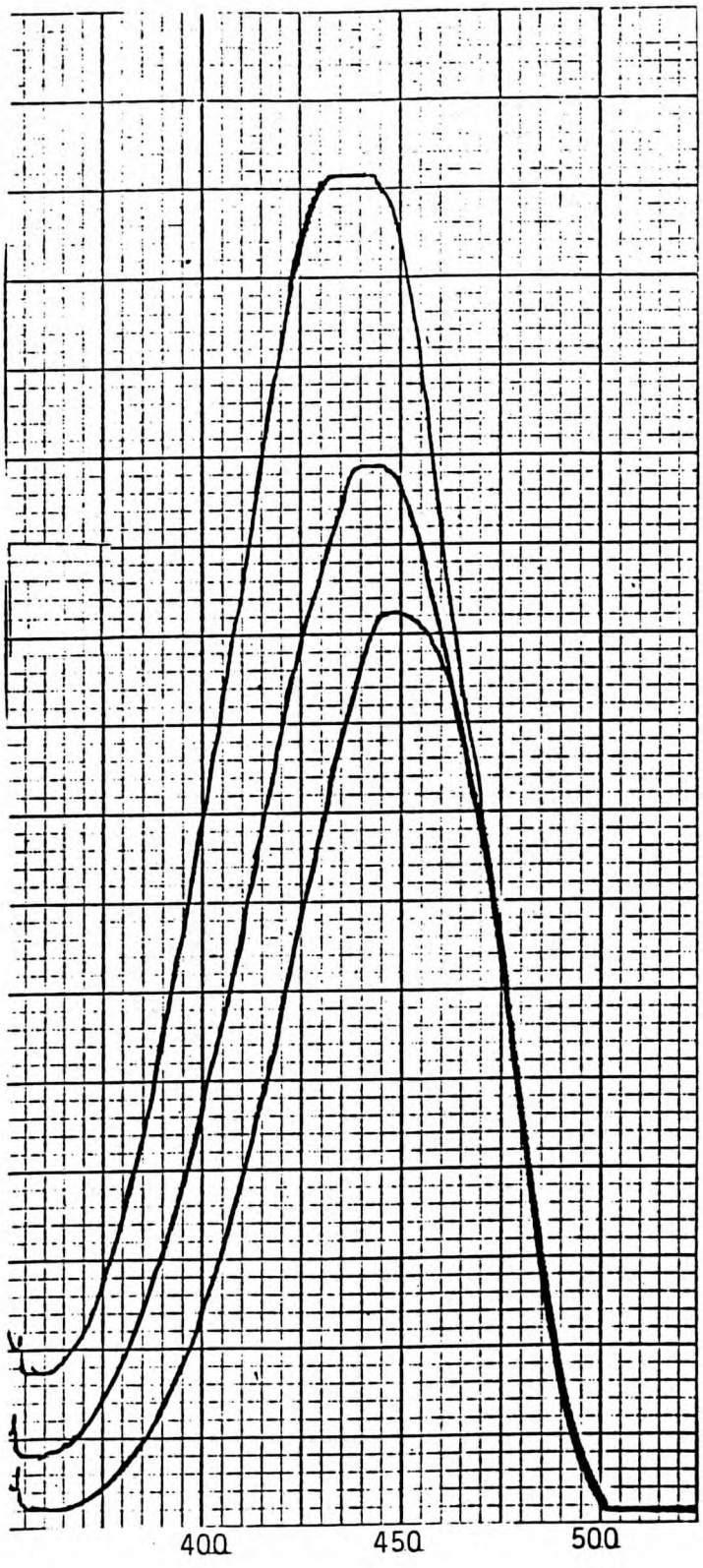


Figure 38. Visible absorption spectra of DPBF and BBD in nitrogen gas at a 3 hour irradiation time interval.

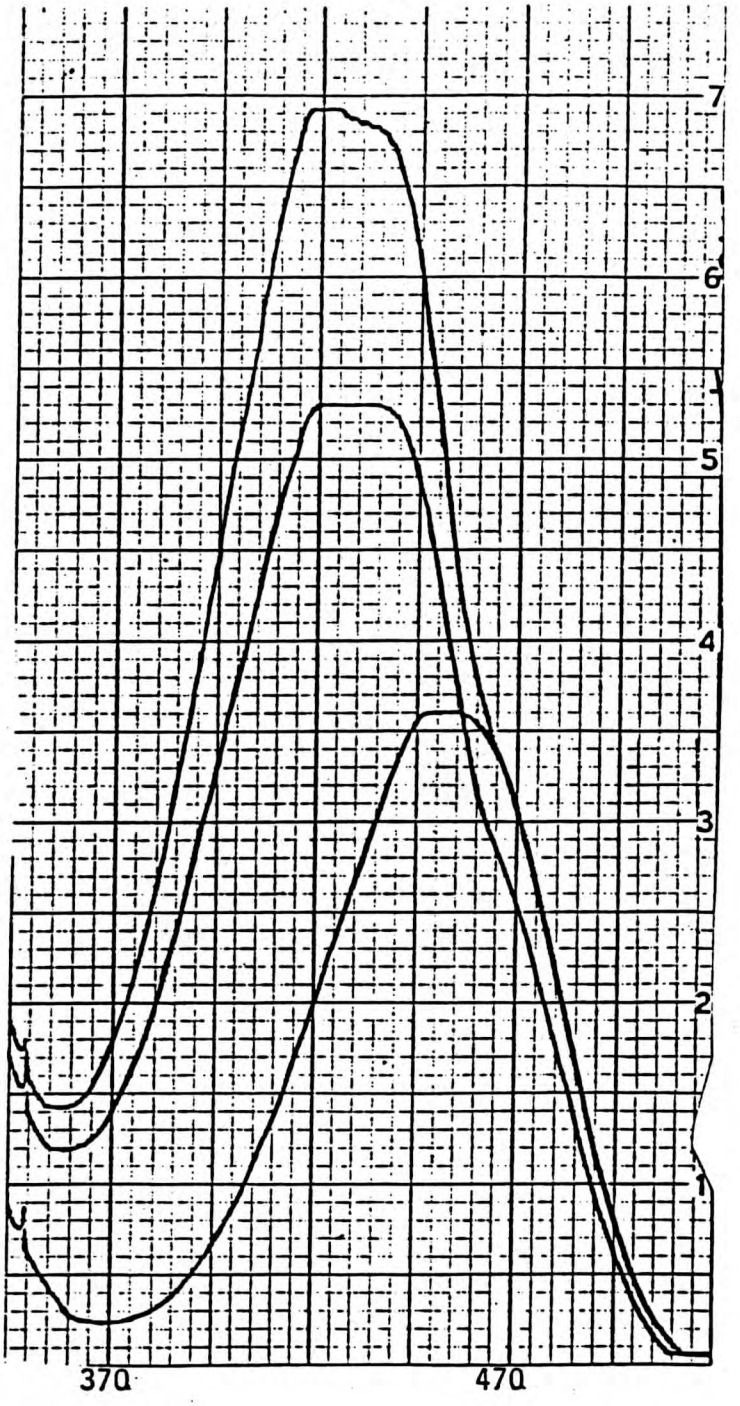


Figure 39. Visible absorption spectra of DPBF and the p-NO_2 analog in nitrogen gas at a 3 hour irradiation time interval.

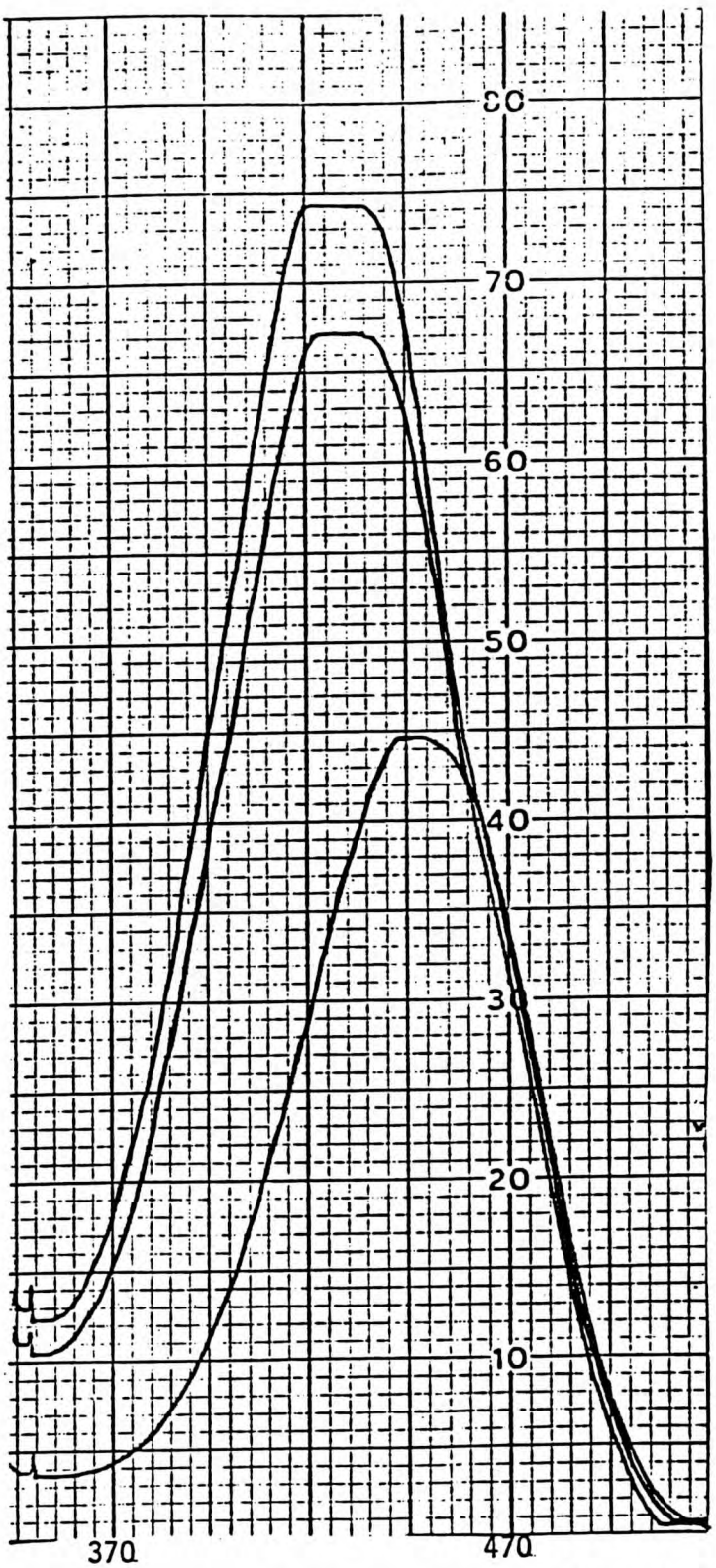


Figure 40. Visible absorption spectra of DPBF and the m-NO_2 analog in nitrogen gas at a 3 hour irradiation time interval.

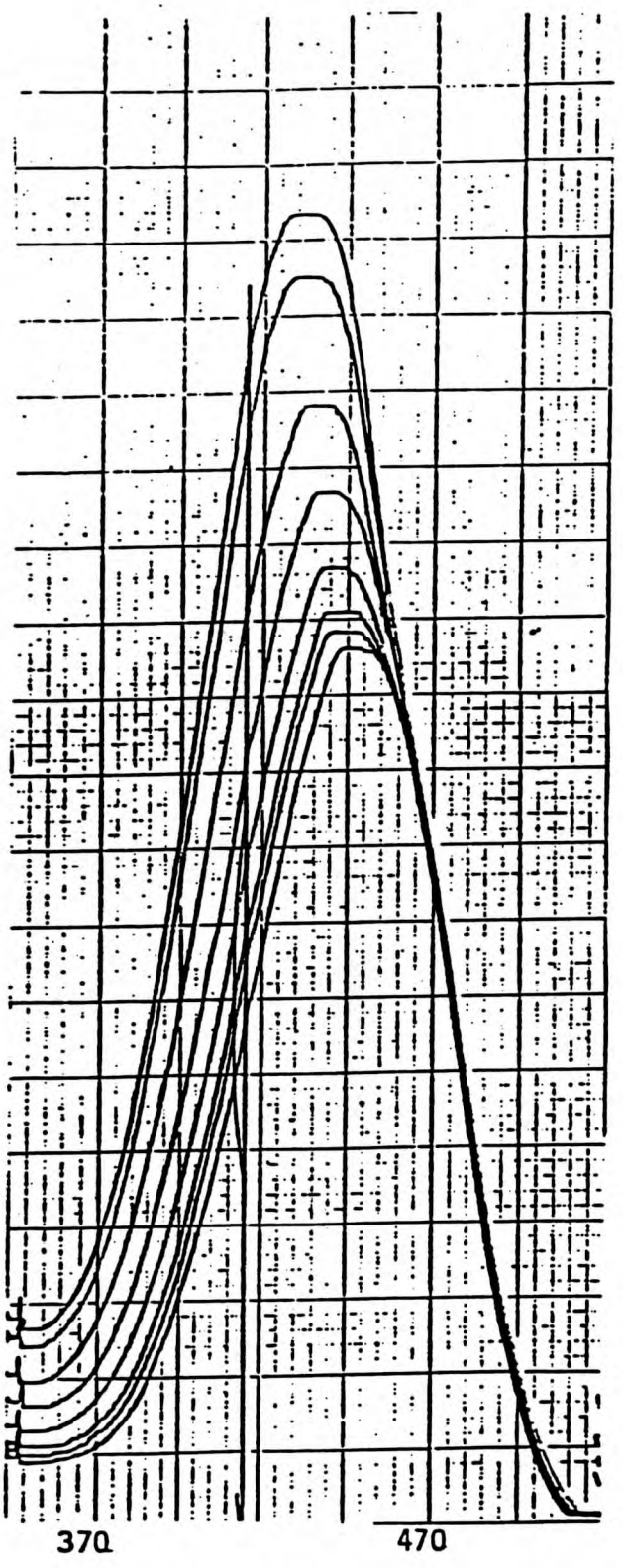


Figure 41. Visible absorption spectra of DPBF and the m-NO_2 analog in oxygen gas at 30 minute irradiation time intervals.

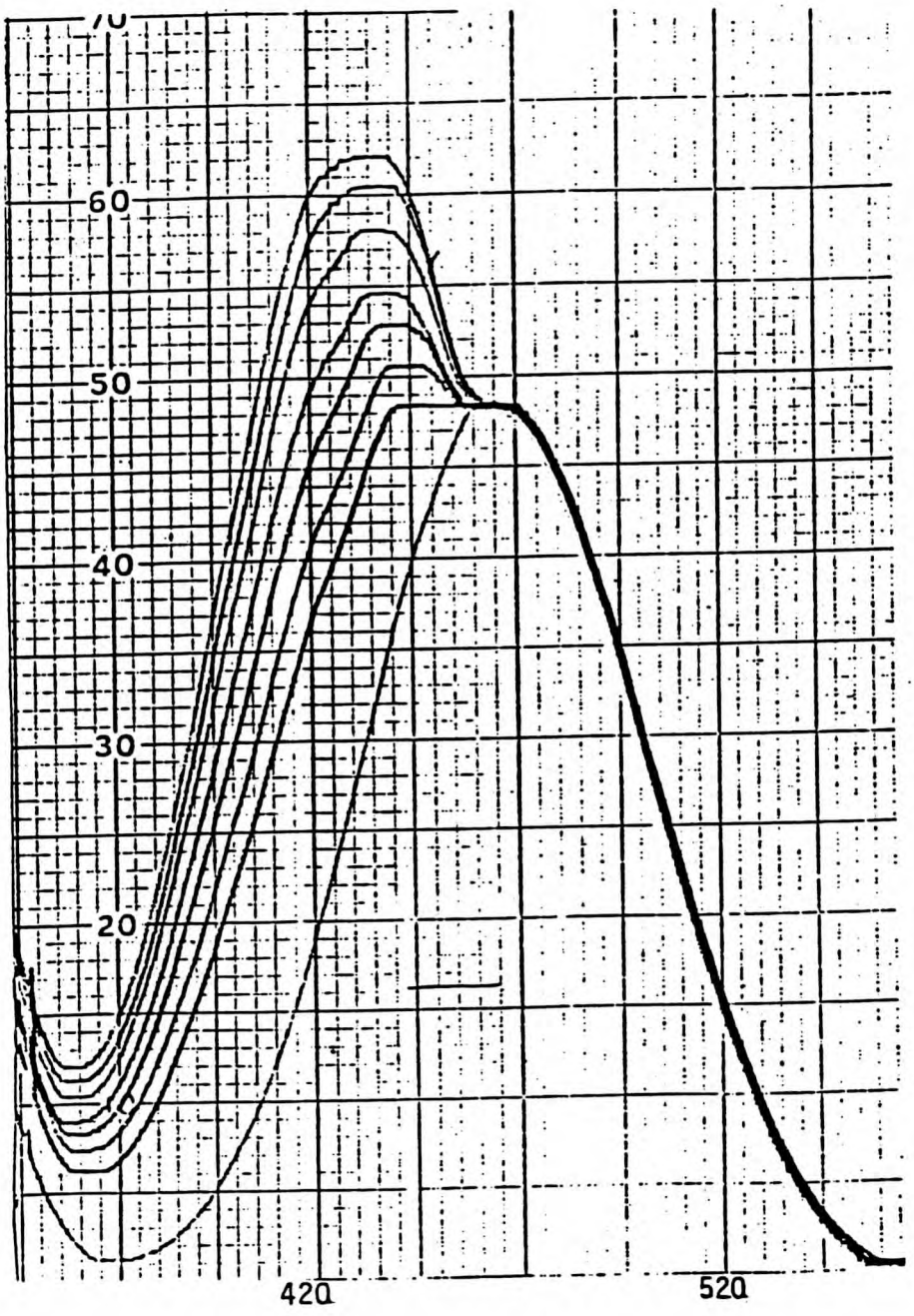


Figure 42. Visible absorption spectra of DPBF and the p-OCH_3 analog in oxygen gas at 30 minute irradiation time intervals.

VITA

Catherine Joines Mader was born in Sparta, North Carolina, on October 2, 1952. After attending Alleghany High School, she received a B.A. in Art from Duke University in 1975. After a several year interval during which she worked, travelled, and reproduced, Ms. Mader entered Appalachian State University in June, 1980. Graduate studies in Chemistry were begun in August, 1982 after acceptance of a teaching assistantship. Master's thesis research was conducted under the direction of Dr. Lawrence Brown with degree requirements being completed in August, 1985.

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