STRUCTURE-ACTIVITY STUDIES OF BIOREDUCTIVE ANTI-CANCER AGENTS

BY

CURTIS OLESCHUK

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Pharmacology and Therapeutics
University of Manitoba
Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION PAGE

STRUCTURE-ACTIVITY STUDIES OF BIOREDUCTIVE ANTI-CANCER AGENTS

BY

CURTIS OLESCHUK

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Abstract

Bioreductive agents are an important class of anti-cancer drugs. The prototypical agent for this class of compounds is Mitomycin C, MMC. MMC is an agent used in the treatment of breast, gastrointestinal, non-small cell lung, and head and neck tumors. These agents are pro-drugs that require intracellular reduction by enzymes, such as NADPH:cytochrome P-450 reductase and DT-diaphorase in order to display maximal activity. The level of DT-diaphorase activity is higher in many solid tumors in comparison to normal tissue. Bioreductive agents that are activated by DT-diaphorase may be used to target tumors high in DT-diaphorase activity. Additionally bioreductive agents are generally more toxic under hypoxic conditions, which may result from more efficient activation by NADPH:cytochrome P-450 reductase. Agents that are activated by NADPH:cytochrome P-450 reductase may be effective for the treatment of hypoxic regions of tumors when used in combination with radiation.

The role of functional groups in modulating bioreductive drug activation by either DT-diaphorase or NADPH:cytochrome P-450 reductase was studied. 2-(Di(chloroethyl)amino)-1,4-benzoquinone, BM was used as a model bioreductive. Three analogs of BM were studied: 5-methoxy-2-(di(chloroethyl)amino)-1,4-benzoquinone (MBM); 5-chloro-2-(di(chloroethyl)amino)-1,4-benzoquinone (CBM); 6-phenyl-2(di(chloroethyl)amino)-1,4-benzoquinone (PBM). MBM was chosen to illustrate the effect of an electron releasing group, CBM was chosen to illustrate the effect of an electron withdrawing group, while PBM was chosen to illustrate the effect of a sterically bulky group.

The cytotoxicities of the BM analogs were measured in H661 cells, a human non-small cell lung carcinoma cell line and in SK-MEL-28, a human melanoma cell line, in the presence and absence, of dicoumarol, an inhibitor of DT-diaphorase. These studies have illustrated that BM and CBM may be inactivated, while MBM may be activated by DT-diaphorase. Addtionally, PBM may not be dependent on DT-diaphorase activity. H661 cells were found to have five times higher NADPH:cytochrome P-450 reductase activity than SK-MEL-28. Using dicoumarol to inhibit DT-diaphorase activity, a comparison of the cytotoxicities of the BM analogs in H661 versus SK-MEL-28 cells illustrated that PBM may be activated by, while CBM may not be dependent on the activity of NADPH:cytochrome P-450 reductase.

The role of a methoxy group in reversing the effect of DT-diaphorase on the cytotoxic activity of BM and MBM was studied. MBM had a less negative first and second redox potential, and was reduced more slowly by DT-diaphorase, in comparison to BM. Addtionally, MBM may, while BM may not be able to re-oxidize. Two theories are suggested to explain the overall effect of each of these differences in reversing the effect of DT-diaphorase on the cytotoxic activity of MBM and BM.

Acknowledgments

My supervisor, Asher Begleiter, for providing the opportunity to carry out a Master's degree and providing a stimulating environment to learn and mature as a scientist. Marsha Leith for her patience and relentless support while I developed my skills in the laboratory. Asher, Marsha, Geoff, Denis, Kristine, Sabine, James, Cheryl, Kerry and many others at the Institute of Cell Biology for their friendship. Countless conversations and interactions with each of these people made my work environment more relaxed and enjoyable.

I extend my sincere appreciation to the Mantioba Health and Research Council for their studentship. I thank Dr. Fiona Parkinson, Department of Pharmacology and Therapeutics, and Dr. David McKinnon, Department of Chemistry for their time and insightful opinions during the review of this thesis. I also thank Dr. David McKinnon for providing laboratory space to carry out synthetic chemistry and for his support during this period.

I would especially like to acknowledge and thank my family. Jerry and Linda Oleschuk (Mom and Dad) for their love. Your thoughfulness and openness provided the opportunity to pursue my interests. You have instilled in me a continued interest in life and the strength to endure many of the challenges it has to offer. David and Dorothy Dalenger (Grandpa and Grandma) for their love and providing the opportunity to carry out my university studies. Rick Oleschuk (Big brother) for his love and guidance through each step of my life.

Last and certainly not least I would like to thank each of my good friends. Jason

Goetz (a.k.a. Jay Richards) for our countless bizarre conversations, which allowed me to explore my thoughts. Chris Chornick, Tricia Chronick, Ryan Ragula, Chantal Olinkin and Kerry Goralski for their peer support

Table of Contents

List of Figures

List of Tables

Abbreviations

ABM	3-acetyl-2-(di(chloroethyl)amino)-1,4-benzoquinone
AZQ	2,5-diaziridine-3,6-bis(carboethoxyamino)-1,4-benzoquinone
BM	2-(di(chloroethyl)amino)-1,4-benzoquinoe
BSA	bovine serum albumin
CB 1954	5-aziridin-1-yl-2,4-dinitrobenzamide
CBM	5-chloro-2-(di(chloroethyl)amino)-1,4-benzoquinone
CDNB	1-chloro-3,4-dinitrobenzene
D3T	1,2-dithiole-3-thione
DMEM/F12 (1:1)	Dulbecco's modified Eagle medium: nutrient powder F-12 (Ham) (1:1)
DMSO	dimethyl sulfoxide
DT-diaphorase	NAD(P)H:(quinone acceptor) oxidoreductase
EO9	3-hydroxymethyl-5-aziridinyl-1-methyl-2(1H-indole-4,7-dione)prop-β-en-α-ol
FAD	flavin adenine dinucleotide
GST	glutathione *S*-transferase
HPLC	high performance liquid chromatography
LB media	Luria broth media
MBM	5-methoxy-2-(di(chloroethyl)amino)-1,4-benzoquinone
MMC	mitomycin C

MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NADH	β-nicotinamide adenine dinucleotide (reduced form)
NADPH	β-nicotinamide adenine dinucleotide phosphate (reduced form)
nmol MTT min^{-1} mg $protein^{-1}$	nanomole of MTT reduced per minute per milligram protein
PBM	6-phenyl-2-(di(chloroethyl)amino)-1,4-benzoquinone
PBS	phosphate buffered saline
TBM	6-*t*-butyl-2-(di(chloroethyl)amino)-1,4-benzoquinone

Introduction

Bioreductive Agents

Overview

Bioreductive agents are a relatively new class of anti-cancer chemotherapeutic pro-drugs that are activated via intracellular enzymatic reduction (Moore, 1977). The prototypical agent is Mitomycin C, MMC. MMC is an anti-tumor antibiotic, which first gained attention as an anti-neoplastic agent in the 1960's (Crooke and Bradner, 1976). Initial clinical trials in Japan showed an overall response rate of 37%, as reviewed by Frank and Osterberg (1960). Since this study, clinical use of MMC as a single agent and in combination therapy has continued (Hortobagyi, 1993; Coia, 1993; Folman, 1993; Schnall and Macdonald, 1993). MMC is widely used in the treatment of breast (Hortobagyi, 1993), gastrointestinal (Schnall and Macdonald, 1993), non-small-cell lung (Folman, 1993) and head and neck tumors (Coia, 1993). In general, studies using MMC as a single agent have produced an overall response rate of 15-30% (Crooke and Bradner, 1976), while combination with other agents has generally produced higher response rates (Hortobagyi, 1993; Walters *et al.*, 1992; Coia, 1993; Folman, 1993; Schnall and Macdonald, 1993). The response rate is limited by the dosage of MMC which can be used. Higher doses of MMC can result in the development of leukopenia and thrombocytopenia (Crooke and Bradner, 1976). Leukopenia and thrombocytopenia are a result of bone marrow suppression (Crooke and Bradner, 1976). The overall response rate and bone marrow toxicity increases with

increasing dosage of MMC (Crooke and Bradner, 1976).

Bioreductive anti-tumor agents can be activated by a number of intracellular enzymes and the mechanisms involved have been extensively studied (Rockwell *et al.*, 1993; Ross *et al.*, 1993). These agents can be activated by one-electron reducing enzymes such as NADPH:cytochrome P-450 reductase (Pan *et al.*, 1984; Keyes *et al.* 1984), xanthine oxidase (Pan *et al.*, 1984; Pritsos *et al.*, 1986) and NADH:cytochrome b_5 reductase (Hodnick *et al.*, 1993), and by two-electron reducing enzymes such as NAD(P)H:(quinone acceptor) oxidoreductase (DT-diaphorase) (Siegal *et al.*, 1992) and xanthine dehydrogenase (Gustafson *et al.*, 1992).

The structures of presently developed bioreductive agents are based on one of three principle reductive elements: a quinone, a nitro group and an N-oxide (Figure I-1). Reduction of each of these reductive elements by one- and two-electron reducing enzymes can directly contribute to the cytotoxicity of a bioreductive anti-tumor agent.

Reduction of a quinone, nitro group, or N-oxide reductive element by a one-electron reducing enzymes generates the corresponding free radical states: semiquinone, nitro radical anion, or nitroxide radical, respectively. Because of their thermodynamic potential, the free radical states of each of the reductive elements are considered extremely reactive and may undergo one of several reactions (Trush *et al.*, 1982). They can add across double bonds, or abstract hydrogen from other molecules (Trush *et al.*, 1982). Each of these reactions may serve to degrade cellular components, such as proteins, lipids and DNA, and is thus considered cytotoxic.

The free radical states of the reductive elements may oxidize to the original fully-

oxidized state by activating molecular oxygen to a univalent superoxide anion (Kappus, 1986). The superoxide anion can rapidly dismutate to form hydrogen peroxide (Kappus, 1986). A metal catalyzed reaction of superoxide anions and hydrogen peroxide can produce extremely reactive hydroxyl radicals (Kappus, 1986). Of the oxygen free radicals produced, the hydroxyl radicals are the most reactive (Kappus, 1986). Similar to the free radical state of each of the bioreductive agents, the hydroxyl radicals may degrade cellular components, such as proteins, fatty acids and nucleic acids. Hydroxyl radicals are integral to radiation-induced cellular damage (Trush *et al.*, 1982).

The free radical state of each of the reductive elements may bind covalently to the tripeptide γ glutamyl cysteinyl glycine, glutathione, (Kappus, 1986). The conjugate may be transported out of the cell, and this serves as a detoxification mechanism (Kappus, 1986).

Finally, the free radical state of each of the reductive elements may be further reduced by a one-electron reducing enzyme to a two-electron reduced state. Depending on the redox potential of the two-electron reduced state, it may or may not undergo a re-oxidation reaction and produce hydroxyl radicals similar to the semi-reduced species. The two-electron reduced state can conjugate with cellular nucleophiles, such as glucuronic acid or sulfate (Prestera *et al.*, 1993(b)). The conjugation products are more readily excreted from the cell, thus this conjugation reaction is also considered a detoxificatio:. mechanism (Prestera *et al.*, 1993(a)). In total, reduction of each of a quinone, nitro, pyridine N-oxide, or pyrimidine N-oxide reductive group by a one-electron reducing enzyme can contribute to the cytotoxicity of a bioreductive agent by direct interaction with cellular components, and/or activation of molecular oxygen. The relative importance of each of these modes of cytotoxicity to the

overall cytotoxicity of a bioreductive agent may be dependent on the bioreductive agent and the level of oxygen (Elwell *et al.*, 1997; Cone *et al.*, 1976).

Reduction of the quinone, nitro, pyridine N-oxide, or pyrimidine N-oxide reductive element by two-electron reducing enzymes will produce a hydroquinone, amino, or fully reduced nitrogen group, respectively. Depending on their stability, each of these reduced species may re-oxidize and produce hydroxyl free radicals, similar to re-oxidation of the free radical state, or react with cellular nucleophiles, such as glucuronic acid or sulfate (Prestera *et al.*, 1993(b)). Thus, reduction of the reductive element by a two-electron reducing enzyme may also directly contribute to the cytotoxicity of a bioreductive agent or to detoxification.

Cytotoxicity directly resulting from reduction of the reductive element is the most important mechanism of action for some bioreductive anti-tumor agents, such as tirapazamine (Elwell *et al.*, 1997) and streptonigrin (Cone *et al.*, 1976) (see Figure I-2). It may be less important for other bioreductive agents such as MMC (Rockwell *et al.*, 1993), 3-hydroxymethyl-5-aziridinyl-1-methyl-2(1H-indole-4,7-dione)prop-β-en-α-ol,EO9,(Bailey *et al.*, 1992), 2,5-diaziridine-3,6-bis(carboethoxyamino)-1,4-benzoquinone, AZQ, (Siegal *et al.*, 1992) and 2-(di(chloroethyl)amino)-1,4-benzoquinone, BM, (Begleiter and Blair, 1984), which are also alkylating agents. Reduction of the reductive element of a bioreductive alkylating agent by one- and two-electron reducing enzymes can indirectly contribute to cytotoxicity by activating the alkylating groups. Reduction of the quinone reductive element of MMC results in the loss of a methoxy and a carboxy-amino leaving group (see Figure I-3). Loss of these leaving groups from the MMC molecule activates two alkylating sites and can result in alkylation of cellular components, such as DNA and proteins. Alkylation of DNA

by both alkylating sites on a single MMC molecule can result in the formation of an inter strand cross-link (Lown *et al.*, 1976; Pan *et al.*, 1986; Pan *et al.*, 1984; Tomasz *et al.*, 1986; Tomasz *et al.*, 1987; Ross *et al.* 1993) (see Figure I-3). Reduction of the quinone reductive element of AZQ can activate aziridinyl groups. The aziridinyl groups can alkylate DNA and proteins, and cross-link DNA (Bailey *et al.*, 1992) (see Figure I-4). Reduction of the quinone reductive group of BM can activate the mustard alkylating group. The mustard group can also alkylate DNA and proteins, and cross-link DNA (Begleiter and Leith, 1990) (see Figure I-5).

Major Activating Enzymes for Bioreductive Agents

DT-diaphorase

DT-diaphorase was first described by Ernster *et al.*, (1958) and partially purified in 1960 (Ernster *et al.*, 1960). DT-diaphorase is primarily located in the cytosol with 5-10% being membrane bound to mitochondria, microsomes and Golgi apparatus (Riley and Workman, 1992). The enzyme, which requires reduced β-nicotinamide adenine dinucleotide (NADH) or reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor for enzymatic activity, has two identical subunits with individual molecular weights of 32 kDa, and contains two molecules of flavin adenine dinucleotide (FAD) (Riley and Workman, 1992). The FAD molecule remains bound during the catalytic activity, and may be integral to the reductive process of DT-diaphorase (Li *et al.*, 1995). The three-dimensional structure of rat liver DT-diaphorase was determined with 2.1 Å resolution by Li *et al.*,

(1995). Their model for the three-dimensional structure localized the binding sites for FAD and NAD(P)H in the dimeric protein. Additionally, their model suggested a direct hydride transfer from NAD(P)H to FAD and from $FADH_2$ to the quinone substrate (which occupies the site vacated by NAD(P)H). This provides a rationale for NAD(P)H and the substrate for DT-diaphorase occupying the same active site (a "ping-pong" mechanism for reduction). Many bioreductive agents are better substrates for rat than human DT-diaphorase (Siegal *et al.*, 1990; Beall *et al.*, 1994). A site-directed mutagenesis study by Chen *et al.*, (1997) found that the catalytic differences between human and rat DT-diaphorase may be due to a difference in a single amino acid at position 104 where human DT-diaphorase has a glycine, while rat DT-diaphorase has a tyrosine. Additionally, this study showed a difference in a single amino acid residue in the binding site of NAD(P)H between rat and human DT-diaphorase. Human DT-diaphorase has an alanine, while rat DT-diaphorase has a threonine at position 130. This structural information may be very important for designing suitable drugs and approaches for human cancer chemotherapy mediated by DT-diaphorase.

NADPH:cytochrome P-450 reductase

NADPH:cytochrome P-450 reductase is a membrane bound protein that is an integral member of the monooxygenase system (Goldstein and Faletto, 1990). This system is important for oxidative metabolism of both endogenous compounds, including fatty acids, steroids and prostaglandins, and exogenous compounds ranging from therapeutic drugs and enviromental toxins to carcinogens. In the monooxygenase system, NADPH:cytochrome P-450 reductase is responsible for redox cycling of cytochrome P-450 (Goldstein and Falleto,

1990). NADPH:cytochrome P-450 reductase is one of only two known mammalian enzymes containing both flavin mononucleotide, FMN, and FAD prosthetic groups (Wang *et al.*, 1997). NADPH is the electron source for reduction reactions by NADPH:cytochrome P-450 reductase. NADPH:cytochrome P-450 reductase accepts a pair of electrons from NADPH as a hydride ion with FAD and FMN being the entry and exit port, respectively, and tranfers these electrons one at a time to cytochrome P-450 or a bioreductive agent (Wang *et al.*, 1997). The redox potentials of each flavin half-reaction have been determined (Iyanagi *et al.*, 1974; Vermillion and Coon, 1978). The enzyme cycles between one- and three- electron reduced levels (or two- and four- electron), with the one-electron reduced semiquinone of the FMN being the highest oxidation state during catalytic turnover (Masters *et al.*, 1965; Backes and Backers, 1988). The amino acid sequence of NADPH:cytochrome P-450 reductase is highly homologous amongst species ranging from yeast to trout to humans (Shen *et al.*, 1993). NAD(P)H:cytochrome P-450 reductase has two functional domains, a hydrophilic C-terminal catalytic domain and a hydrophobic N-terminal domain that serves as an anchor to the endoplasmic reticulum (Kasper, 1971). The N-terminus can be removed by proteolytic cleavage, and the C-terminal is still capable of passing electrons to bioreductive agents, but not to cytochrome P-450 (Wang *et al.*, 1997). The C-terminal catalytic domain can be further segmented into two structural domains. The N-terminal domain is likely to bind FMN and the C-terminal domain is likely to bind FAD and NADPH (Porter and Kasper, 1986; Smith *et al.*, 1994; Narayanasami *et al.*, 1995). The three dimensional structure of rat liver NADPH:cytochrome P-450 reductase has been determined to 2.6 Å resolution by Wang *et al.* (1997). Their model indicated that the FAD and FMN

rings are juxtaposed and the closest distance between them is approximately 4 Å. Additionally, their model indicated a bowl-shaped surface near the FMN-binding site, which is likely to be the binding site of cytochrome P-450 and bioreductive agents. Similar to the structural studies with DT-diaphorase, structural studies of NADPH:cytochrome P-450 reductase may provide very important information for designing suitable drugs and approaches for human cancer chemotherapy mediated by NADPH:cytochrome P-450 reductase.

Enzyme-Directed Approach to Anti-Cancer Drug Development

Current interest in bioreductive agents is due to their potential use in an enzyme directed approach to anti-tumor chemotherapy. The bioreductive anti-tumor agents are activated by one- or two-electron reducing enzymes and may be selectively toxic to tumor cells if the tumor cells have higher levels of activating enzyme(s), or a different microenvironment compared with normal cells (Workman *et al.*, 1993).

It has been shown that DT-diaphorase level and activity is elevated, relative to normal tissue, in primary solid tumors from breast, colon, liver, lung (Schlager and Powis, 1990; Smitskamp-Wilms *et al.*, 1995) and head and neck (Smitskamp-Wilms *et al.*, 1995). The greatest difference was noted for a lung adenoma carcinoma, which was 123-fold higher than normal lung (Schlager and Powis, 1990). In addition to an elevation of DT-diaphorase in established tumors, enzyme levels may be elevated in pre-neoplastic modules (Riley *et al.*, 1992(b)).

The potential for exploiting the increased level and activity of DT-diaphorase in some

tumors can be recognized with EO9. EO9 is a good substrate for, and is activated by DT-diaphorase (Collard *et al.*, 1995). The sensitivity of tumor cell lines to EO9 correlates with the level of DT-diaphorase (Fitzsimmons *et al.*, 1996; Butler *et al.*, 1996; Robertson *et al.*, 1992; Collard *et al.*, 1995; Smitskamp-Wilms *et al.*, 1995). Unfortunately, EO9 has been found to have minimal anti-tumor activity in Phase II clinical trials (Dirix *et al.*, 1996). The poor anti-tumor activity of EO9 may be attributed to the very short plasma half-life of this drug (McLeod *et al.* 1996; Schellen *et al.*, 1996; Workman *et al.*, 1992). Thus, the limitations of EO9 may not be a general phenomena to all bioreductive agents, and the enzyme-directed approach may be applied to anti-tumor chemotherapy using other novel bioreductive agents.

The cytotoxicities of MMC, porfiromycin and tirapazamine are dependent on the extent of oxygenation of the tumor cell. In general, MMC, porfiromycin and tirpazamine are more cytotoxic to cells under hypoxia than aerobic conditions (Workman *et al.*, 1993). The increased cytotoxicity under hypoxia may be the result of more efficient activation by NADPH:cytochrome P-450 reductase (Workman *et al.*, 1993).

Bioreductive agents may be effective anti-tumor treatments for hypoxic regions of a tumor. The presence of regions of low oxygen tension in a variety of tumors is well established and this hypoxia can predispose the failure of some treatments with radiotherapy (Patterson *et al.*, 1995). It has been postulated that bioreductive agents may be useful for treatment of solid tumors when used in combination with radiation therapy and/or other cytotoxins (Workman *et al.*, 1993). Tirapazamine is one of the leading compounds for this approach (Workman *et al.*, 1993). It exhibits a 15 to 200 fold greater toxicity for hypoxic

cells as compared to oxygenated cells (Zeman *et al.*, 1986) and is activated by NADPH:cytochrome P-450 reductase. (Beidermann *et al.*, 1991; Fitzsimmons *et al.*, 1994). The co-administration of tirapazamine with other cytotoxins, notably cisplatin (Holden *et al.*, 1992; Dorie *et al.*, 1993) and also during radiotherapy (Brown *et al.*, 1990) produces selective *in vivo* enhancement of tumor kill. Tirapazamine is currently being studied in Phase II/III clinical trials in combination with conventional cytotoxins (Senan *et al.*, 1997).

It is proposed that the use of bioreductive agents in the treatment of tumors with elevated DT-diaphorase activity may require the use of a bioreductive agent that is very dependent on DT-diaphorase activity, and this approach may be increasingly effective if the agent is specifically activated by DT-diaphorase. Secondly, it is proposed that the use of bioreductive agents in the treatment of hypoxic regions of solid tumors may be more effective using a bioreductive agent that is specifically activated by NADPH:cytochrome P-450 reductase.

The above proposals can be rationalized by identifying the targets of the bioreductive agents for both enzyme-directed approaches. For the use of bioreductive agents to treat tumors high in DT-diaphorase, they must be activated by DT-diaphorase. Activation by NADPH:cytochrome P-450 reductase is not required, and may produce unwanted toxicity. For the use of bioreductive agents to treat hypoxic regions of solid tumors, the agent must be activated by NADPH:cytochrome P-450 reductase. In this case, activation by DT-diaphorase is not required and may likewise produce unwanted toxicity.

The above proposals may also be rationalized from the clinical exerience with MMC. The dosage of MMC used was generally 50-100 mg total, which limited anti-neoplastic

activity (Crooke and Bradner, 1976). In general, the dosage of MMC was limited by the development of myeolopoeisis and thrombopeoisis resulting from bone marrow toxicity (Crooke and Bradner, 1976). Bone marrow is composed of hematopoietic progenitor cells and stromal cells (Ganousis *et al.*, 1992). Stromal cells regulate growth and differentiation of both myeloid and lymphoid progenitors, and are critical in the regulation of hemopoiesis (Lichtman, 1981; Dorshkind, 1990). There are at least four types of stromal cells: adipocyte, endothelial, fibroblast and macrophage. Fibroblasts and macrophage are known to be important in the regulation of hemopoiesis (Dorshkind, 1990). Fibroblasts are believed to have significant levels of DT-diaphorase activity (Thomas *et al.*, 1990). The level of DT-diaphorase activity in fibroblasts is 100-fold less than the activity in some types of tumors, but it is similar to the level in other types of tumors (Fitzsimmons *et al.*, 1996). The level of NADPH:cytochrome P-450 reductase activity in stromal fibroblasts is uncertain. It is postulated that bone marrow toxicity may be the result of the lethal effect of MMC on bone marrow fibroblasts, which may be the result of activation of MMC by DT-diaphorase in these cells. Depletion of the fibroblast population may disrupt hemopoiesis. This theory suggests that anti-tumor activity of all bioreductive agents that are activated by DT-diaphorase may be complicated by bone marrow toxicity. However, the bone marrow toxicity associated with MMC was dose dependent (Crooke and Bradner, 1976), and MMC is a poor substrate for DT-diaphorase (Beall *et al.*, 1995). These together suggest that it may be possible to minimize the bone marrow toxicity associated with treatment of tumors with elevated DT-diaphorase by using an agent that is a better substrate for DT-diaphorase than MMC. EO9 is an example of a better substrate for DT-diaphorase (Beall *et al.*, 1995). It

illustrated no bone marrow toxicity in clinicals trials. Lack of bone marrow toxicity may be due to rapid clearance of EO9 (McLeod *et al.*, 1996; Schellen *et al.*, 1996; Workman *et al.*, 1992).

Bone marrow toxicity may reduce the effectiveness of bioreductive anti-tumor agents in treating hypoxic regions of solid tumors, if the bioreductive agent is a good substrate for and is activated by DT-diaphorase. For such an agent, the dosage required to effectively treat the hypoxic fractions of solid tumors may correspond with a dosage that is toxic to bone marrow. Use of an agent specifically activated by NADPH:cytochrome P-450 reductase to treat hypoxic regions of solid tumors may minimize bone marrow toxicity, since activation by DT-diaphorase is minimized. An example of such an agent is tirapazamine. It is activated by NADPH:cytochrome P-450 reductase (Riley *et al.*, 1992; Walton and Workman, 1990; Wang *et al.*, 1993) and inactivated by DT-diaphorase (Riley and Workman, 1992; Riley and Workman, 1992; Walton and Workman, 1990). In Phase I clinical trials it has shown no bone marrow toxicity (Senan *et al.*, 1997).

Specificity for Activation by DT-Diaphorase or NADPH:Cytochrome P-450 Reductase

It is postulated that specificity for activation of a bioreductive agent by DT-diaphorase or NADPH:cytochrome P-450 reductase may be generated by strategic placement of functional groups on the reductive element of the bioreductive agent. A functional group can potentially generate specificity for activation by altering one of four properties of a bioreductive agent. It may alter affinity for the active site and/or overall rate of reduction of the bioreductive agent by DT-diaphorase or NADPH:cytochrome P-450 reductase, the

stability of either the free radical or two-electron reduced states, the efficiency of the cytotoxic mechanism of the bioreductive agent, or the rate of conjugation of the free radical state with the detoxifying peptide, glutathione, or the two-electron reduced state with detoxifying nucleophiles, such as glucuronic acid and sulfate.

Effect on affinity for active site and/or overall rate of reduction

A functional group may increase or decrease the affinity of a bioreductive agent for DT-diaphorase and/or NADPH:cytochrome P-450 reductase. To a limited extent, an increase in the affinity of a bioreductive agent for DT-diaphorase and/or NADPH:cytochrome P-450 reductase may result in an increase in the rate of reduction of the bioreductive agent by DT-diaphorase and/or NADPH:cytochrome P-450 reductase, respectively. An increase in the rate of reduction of a bioreductive agent by DT-diaphorase or NADPH:cytochrome P-450 reductase may contribute to an increase in cytotoxicity. As an example, Ross *et al.* (1995) studied a series of bioreductive agents and found that increasing affinity for the active site of DT-diaphorase corresponded to an increased toxicity towards a cell line high in DT-diaphorase. Depending on the effect of the functional group on the affinity of the bioreductive agent for DT-diaphorase or NADPH:cytochrome P-450 reductase, specificity for activation by DT-diaphorase or NADPH:cytochrome P-450 reductase may result.

A functional group may increase or decrease the rate at which the bioreductive agent is reduced by DT-diaphorase and/or NADPH:cytochrome P-450 reductase independent of an effect on the affinity for the active site of these enzymes. This may result from an effect of the functional group on rate of the reduction reaction of a bioreductive agent by DT-

diaphorase and/or NADPH:cytochrome P-450 reductase. This would be evident as an increase or decrease in the overall rate of reduction of the bioreductive agent by DT-diaphorase and/or NADPH:cytochrome reductase. An increase in overall rate of reduction of a bioreductive agent by DT-diaphorase and/or NADPH:cytochrome P-450 reductase may result in an increase in cytotoxicity. Depending on the effect of the functional group on the overall rate of reduction of the bioreductive agent for DT-diaphorase or NADPH:cytochrome P-450 reductase, specificity for activation by DT-diaphorase or NADPH:cytochrome P-450 reductase may be generated.

Effect on stability of the free radical and two-electron reduced states

A functional group may affect the stability of the free-radical and two-electron reduced states of a bioreductive agent. As mentioned previously, the free-radical and two-electron reduced states of a bioreductive agent may re-oxidize to the fully oxidized and free radical state, respectively, by activating molecular oxygen. An increase or decrease in the stability of the semi- and two-electron reduced state decreases and increases the rate of re-oxidation of a bioreductive agent, respectively. A change in the rate of re-oxidation of a bioreductive agent may affect the importance of DT-diaphorase or NADPH:cytochrome P-450 reductase for the cytotoxicity of a bioreductive agent.

The production of hydroxyl free radicals may increase with increased rate of re-oxidation and decreased stability of the free radical state. Increased production of hydroxyl free radicals may translate to an increase in the cytotoxicity of the agent. Thus, a decrease in the stability of the free radical state may increase cytotoxicity resulting from reduction to

the free radical state, and increase the importance of NADPH:cytochrome P-450 reductase for the cytotoxicity of the agent. This may generate specificity for activation by NADPH:cytochrome P-450 reductase, if the two-electron reduced state of the agent is quite stable and does not readily re-oxidize.

The extent of alkylation of an alkylating group on a bioreductive agent that is active only in the free radical and/or two-electron-reduced states may decrease with increasing rate of re-oxidation of each of these states. If a functional group increases the stability of the semi-reduced state of a bioreductive alkylating agent, it may reduce re-oxidation and increase the extent of alkylation of the alkylating cytotoxic group. Thus, an increase in stability of the semi-reduced state may increase the importance of NADPH:cytochrome P-450 reductase for the cytotoxicity of the agent. Additionally, an increase in the stability of the two-electron-reduced state may reduce re-oxidation of the two-electron-reduced state and increase the extent of alkylation of an alkylating cytotoxic group. Thus, an increase in the stability of the fully-reduced state of a bioreductive alkylating agent may increase the importance of DT-diaphorase for the cytotoxicity of the agent. Depending on the effect of a functional group on stability of the free radical and two-electron-reduced state of the bioreductive alkylating agent and the rate of alkylation of the free radical and two-electron reduced species, specificity for activation by DT-diaphorase or NADPH:cytochrome P-450 reductase may be generated.

Direct effect on efficiency of the cytotoxic mechanism

Irrespective of an effect that a functional group may have on the stability of the semi- and/or two-electron-reduced states, or the affinity and/or overall rate of reduction by DT-diaphorase and/or NADPH:cytochrome P-450 reductase, the functional group may directly affect the efficiency of the cytotoxic mechanism of a bioreductive agent. The functional group may increase or decrease the rate of alkylation by the alkylating group bound to the reductive element in the free radical and/or two-electron-reduced state of the bioreductive agent. An increase or decrease in alkylation may result in an increase or decrease in cytotoxicity, respectively. Depending on the effect of the functional group on the alkylating activity of the bioreductive agent in the free radical or two-electron-reduced state, specificity for activation by NADPH:cytochrome P-450 reductase or DT-diaphorase may be generated.

Effect on rate of conjugation

As noted earlier, the free radical state of a bioreductive agent may conjugate with glutathione, and the two- electron reduced state of a bioreductive agent may conjugate with glucuronic acid or sulfate. Each of these conjugation reactions may enhance the excretion of the bioreductive agent out of the cell, and can be considered cellular detoxification mechanisms.

A functional group may increase or decrease the rate of conjugation of the free radical state of a bioreductive agent with glutathione, or the two-electron-reduced state with glucuronic acid and/or the two-electron reduced state with sulfate. An increase in the rate of conjugation of the free radical state with glutathione may reduce the concentration of the

free-radical state of the bioreductive agent in a cell, which may reduce the cytotoxic activity of the bioreductive agent following activation by NADPH:cytochrome P-450 reductase. Alternatively, an increase in the rate of conjugation of the two-electron reduced state with either glucuronic acid or sulfate may reduce the concentration of the two electron reduced state of the bioreductive agent in a cell, which may reduce the cytotoxic activity of the bioreductive agent following activation by DT-diaphorase. Thus, depending on the effect of the functional group on conjugation of the free radical state with glutathione and conjugation of the two-electron reduced state with glucuronic acid or sulfate, specificity for activation by activation by NADPH:cytochrome P-450 reductase or DT-diaphorase may be generated.

Structure-Activity Studies

The development of a bioreductive agent that is specifically activated by DT-diaphorase or NADPH:cytochrome P-450 reductase by strategically placing functional groups at various positions on the reductive element requires an understanding of the effect of several functional groups on specificity for reduction by DT-diaphorase or NADPH:cytochrome P-450 reductase. Additionally, it requires a detailed understanding of the effect of the same functional groups on each of the stability of the semi- and two-electron reduced states, the affinity for and overall rate of reduction by DT-diaphorase and NADPH:cytochrome P-450 reductase, the efficiency of the cytotoxic mechanism and the rate of conjugation of the free radical and fully-reduced state. Information from these two areas may be combined to design novel bioreductive agents that will be useful for the enzyme

directed approach to chemotherapy.

There are two fundamental properties to a functional group. The first fundamental property of a functional group is its electronic effect. A functional group may increase or decrease the electron density around atoms to which it is bound. A functional group that increases the electron density around atoms to which it is bound is referred to as electron releasing. Alternatively, a functional group that decreases the electron density around atoms to which it is bound is referred to as electron withdrawing. Electron withdrawing and releasing properties of a functional group can be broken down further. Addition to or removal of electrons from an adjacent atom may be through a π-type bond or a σ-bond. Addition or removal of electrons through a π-bond is referred to as electron release or withdrawl through resonance, respectively. Addition or removal of electrons through a σ-bond is referred to as inductive electron release or withdrawl, respectively.

The second fundamental property of a functional group is steric bulk. This refers to the volume that the functional group occupies. The volume a functional group occupies is dependent on the type of atoms that compose the group and the electron density surrounding each of these atoms.

Functional groups with one of the properties of electron releasing, electron withdrawing, or steric bulk are desirable for study. This type of functional group will illustrate a specific property of a functional group, which may assist in developing generalizations for properties of functional groups. Examples of good functional groups for study are as follows. A methoxy group is a strong electron releasing group with little steric bulk, an acetyl group is a strong electron withdrawing group with little steric bulk, while a

t-butyl group is a weak electron releasing group with steric bulk.

2-(Di(chloroethyl)amino)-1,4-benzoquinone (BM) was chosen to study the role of functional groups on specificity for activation by DT-diaphorase or NADPH:cytochrome P-450 reductase. BM is a model bioreductive agent, which was previously studied in our laboratory (Begleiter, 1983; Begleiter and Leith, 1990). BM and several BM analogs have been synthesized (Makarov *et al.*, 1967; Ross, 1964). BM contains a quinone reductive element similar to MMC, porfiromycin, EO9, AZQ and streptonigrin. BM is a proven bioreductive agent. It has been shown to be 30,000 times more cytotoxic than 2-(di(hydroxyethyl)amino)-1,4-benzoquinone and 600 times more cytotoxic than N,N-di(chloroethyl)aniline in a L5178Y mouse lymphoma cell line (Begleiter and Leith, 1990; Begleiter, 1983). Additionally, BM is believed to be activated by NADPH:cytochrome P-450 reductase and may be inactivated by DT-diaphorase (Begleiter and Leith, 1990). BM has been shown to produce DNA strand breaks, which may be the result of the production of hydroxyl radicals (Begleiter and Leith, 1990; Begleiter and Blair, 1984). Finally, BM also produces DNA cross-links (Begleiter and Leith, 1990; Begleiter and Blair, 1984). Both mechanisms appear to contribute to the cytotoxic activity of BM.

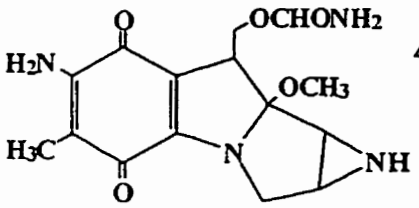
Outline of Research

i. Synthesis of several BM analogs each with a single functional group that is electron withdrawing, electron releasing or sterically bulky.

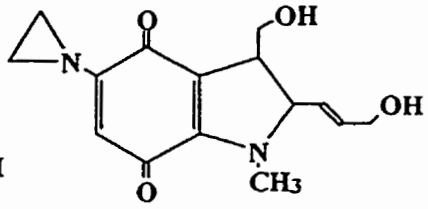
ii. Screen for a dependence of the cytotoxicity of the BM analogs on DT-diaphorase activity and/or NADPH:cytochrome P-450 reductase activity to identify effects of three functional groups.

iii. Study the underlying mechanism for the effect of each functional group on the dependence of the cytotoxicity of a BM analog on DT-diaphorase or NADPH:cytochrome P-450 reductase.

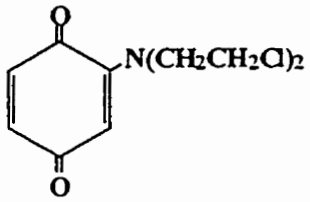
Quinone Reductive Element:



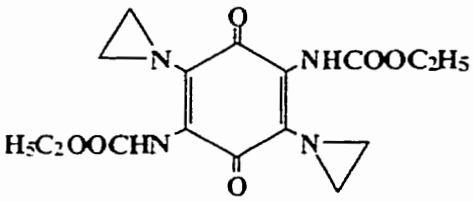
Mitomycin C



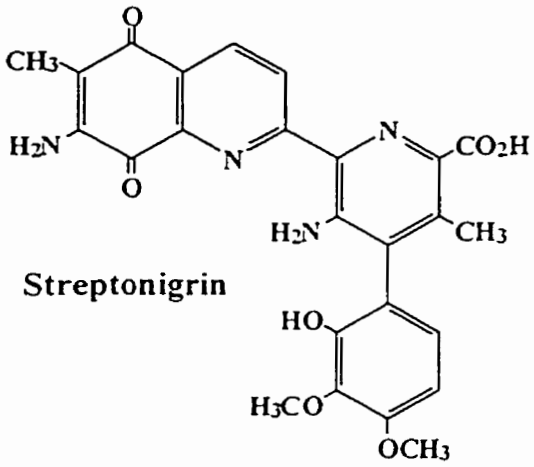
EO9



Benzoquinone Mustard

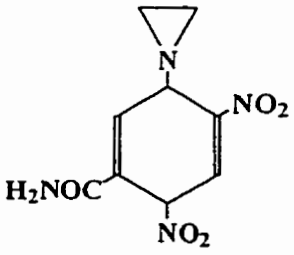


AZQ



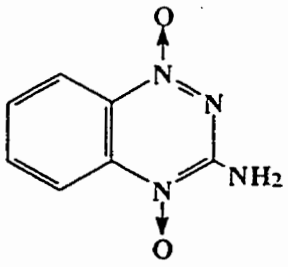
Streptonigrin

Nitro Reductive Element:



CB 1954

N-Oxide Reductive Element:



Tirapazamine

Figure I-1: Examples of bioreductive agents containing one of the three types of reductive elements.

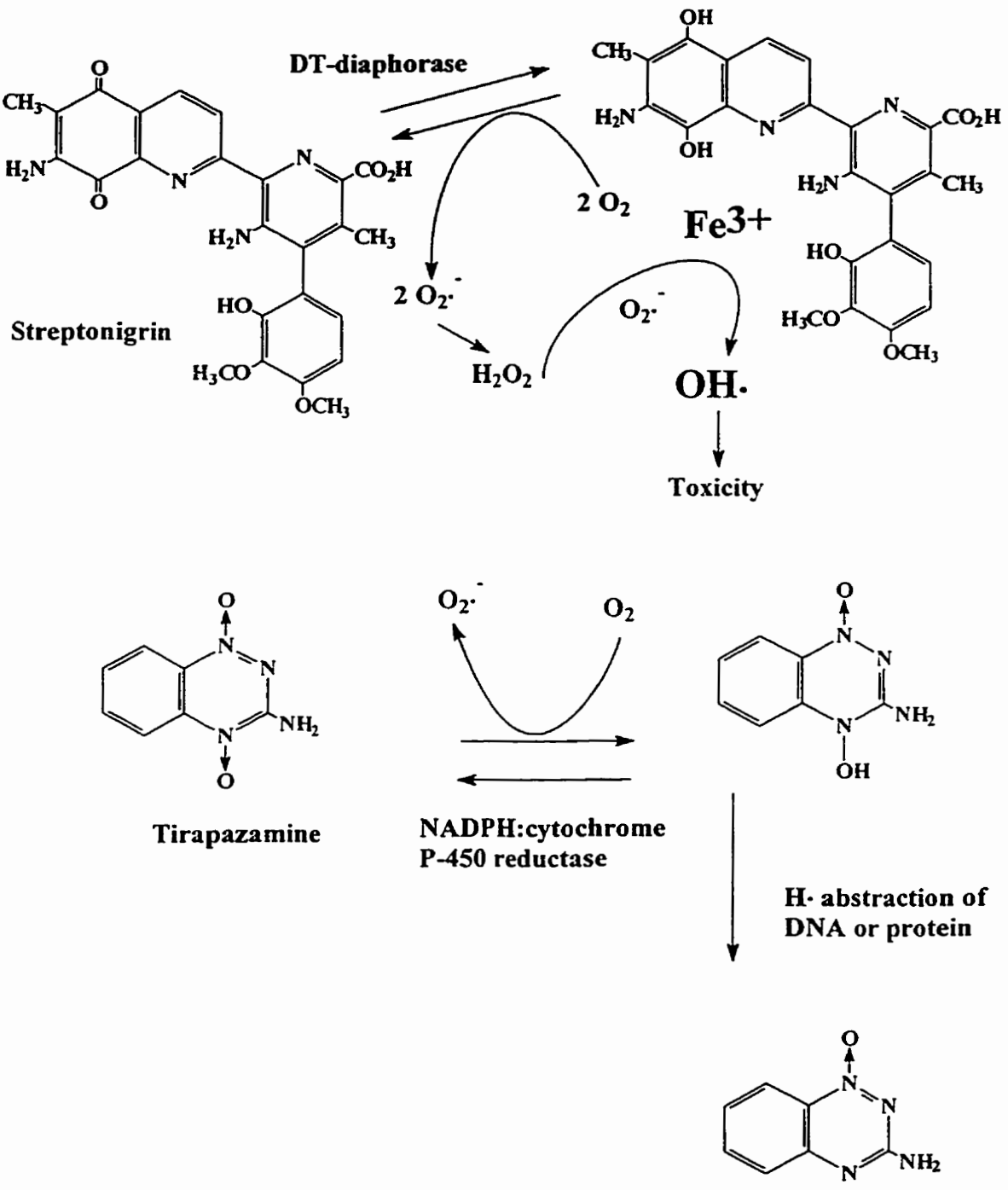


Figure I-2: Model for Streptonigrin and Tirapazamine activation and toxicity.

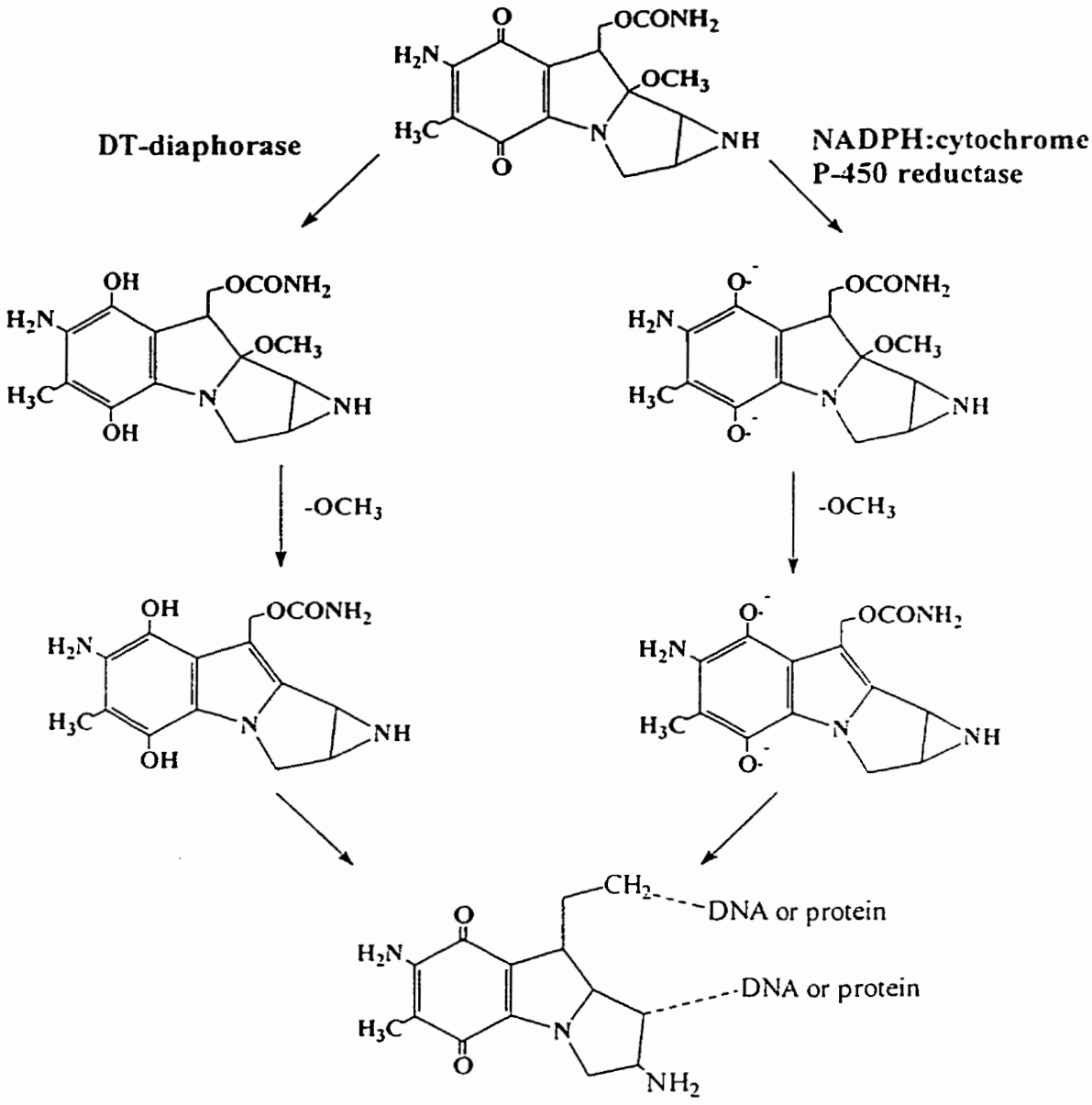


Figure I-3: Model for activation of MMC by DT-diaphorase and NADPH:cytochrome P-450 reductase

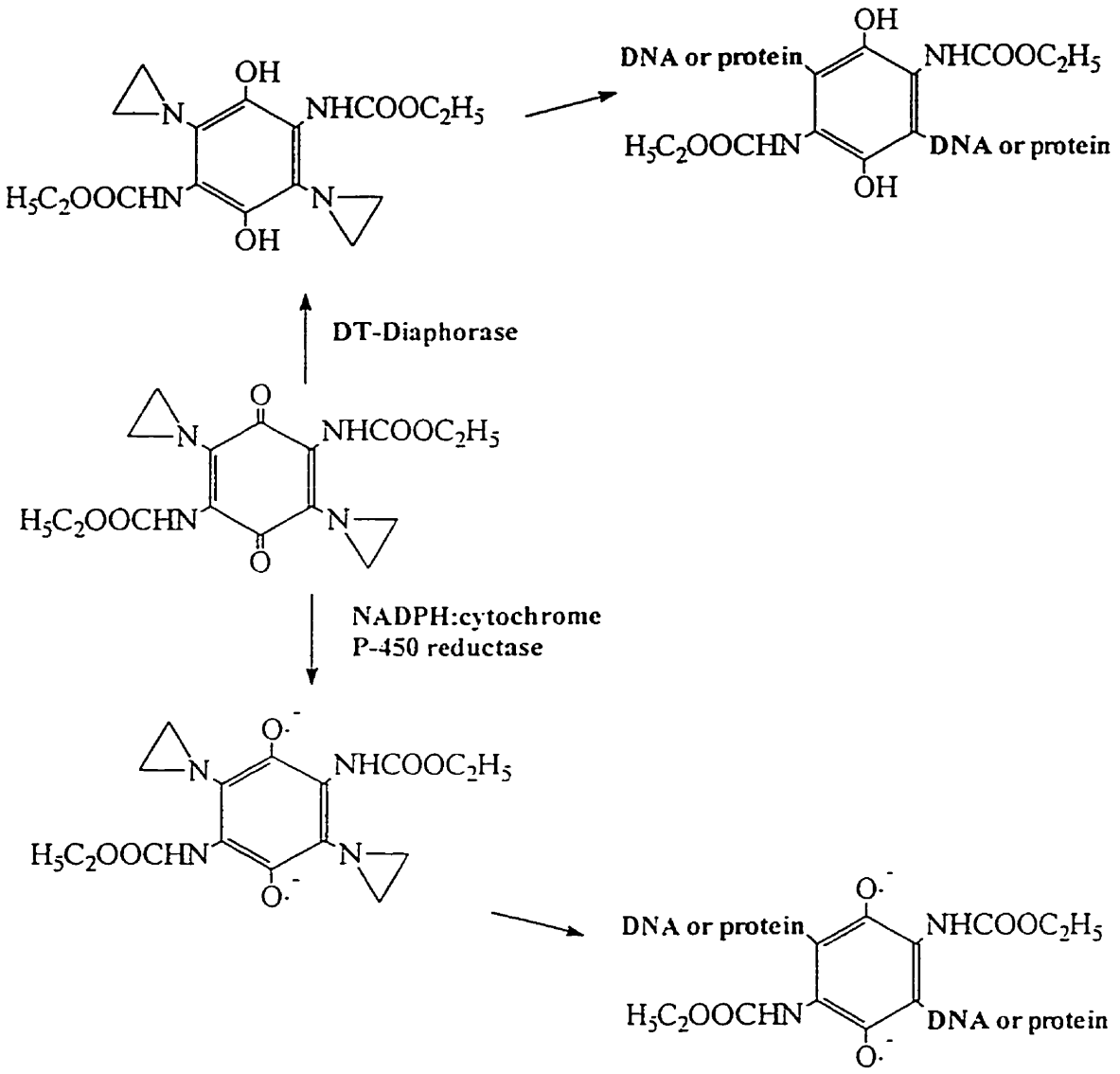


Figure I-4: Model for activation and toxicity of AZQ.

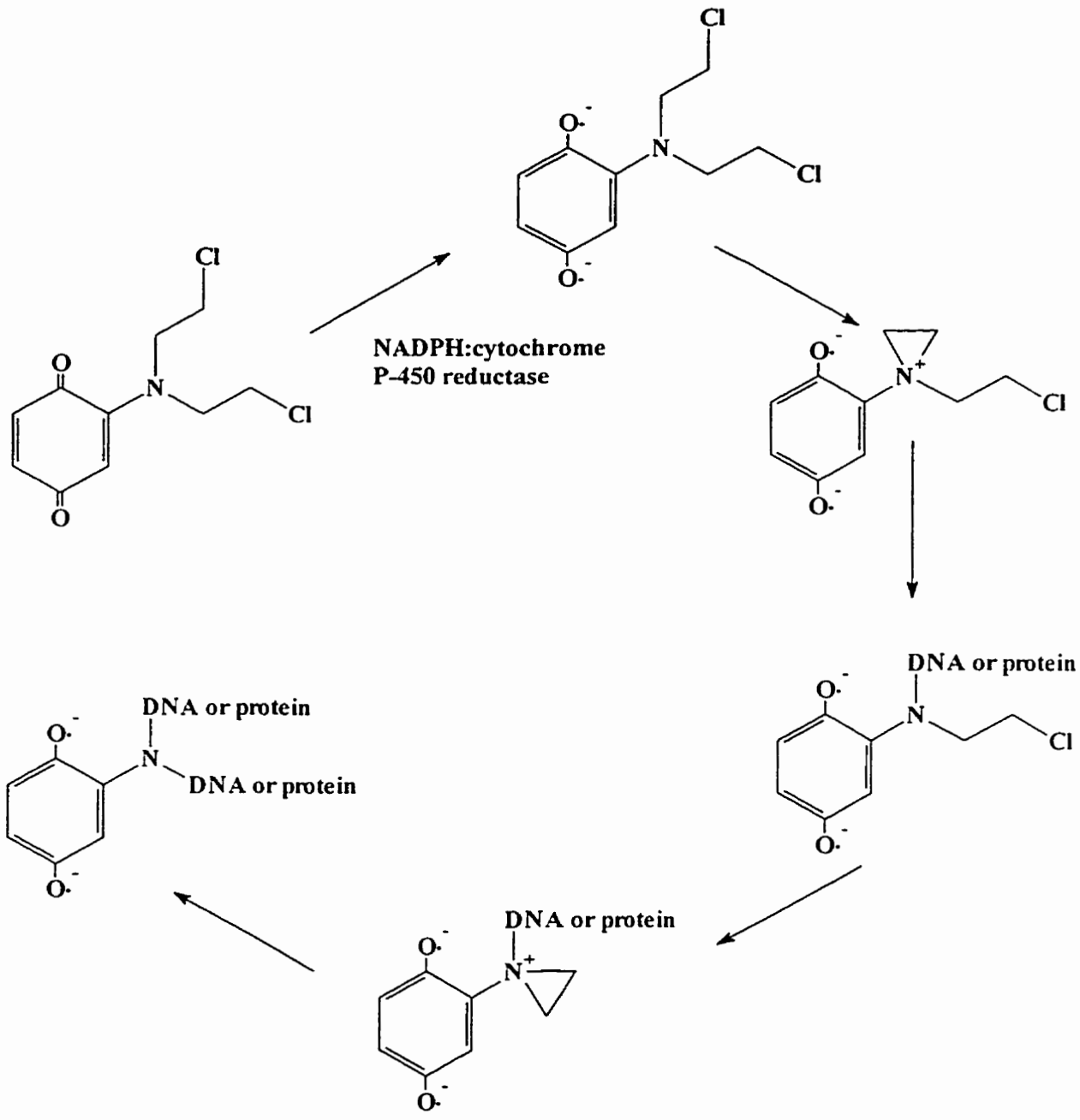


Figure I-5: Model for activation and toxicity of benzoquinone mustard.

Materials and Methods

Materials

All media and fetal bovine serum were obtained from Gibco BRL (Grand Island, NY, USA). All reagents for the DT-diaphorase assay, NADH, FAD, dicoumarol, di(chloroethyl)amine hydrochloride, potassium chloride, Tris HCl, potassium carbonate, vanillin, silver oxide, 1,4-benzoquinone, acetic anhydride, chromic acid, *t*-butyl-hydroquinone, 2-phenyl-1,4-benzoquinone, 2-chloro-1,4-benzoquinone and DEAE Sephadex A-25 anion exchange column were from Sigma (St. Louis, MO, USA). Dichloromethane, methanol, hexanes, 95% ethanol, ethyl acetate, silica gel (1000 mesh), glacial acetic acid, sucrose, benzene, anhydrous ether, H_20_2 solution and potassium fluoride were from Mallinkrodt and Baker Inc. (Phillipsburg, NJ, USA). Dimethyl sulfoxide (DMSO) and dimethyl formamide (DMF) were from BDH Laboratory Supplies (Darset, England). Protein molecular weight standards, Silver stain kit™, Affi-Gel Blue™ (100-200 mesh) affinity column and SDS PAGE reagents were from Biorad (Mississauga, ON, Canada). Cupric acetate, Sephadex G-100 size exclusion column material were from Fisher Scientific (Nepean, ON, Canada). λ Hind III fragments, D_6 acetone, $CDCl_3$ and [α-^{32}P]dATP were from Amersham Life Science (Oakville, ON, Canada). NCI-H661 human non-small cell lung carcinoma cells were obtained from American Type Culture Collection. SK-MEL-28 human malignant melanoma cells were obtained from American Type Culture Collection. HT-29 human colon adenocarcinoma cells were obtained from Dr. J.B. Johnston, Manitoba

Cancer Treatment and Research Foundation, Winnipeg, MB, Canada. EO9 was kindly supplied by Dr. H.R. Hendriks, New Drug Development Office, European Organization for Research and Treatment of Cancer, Amsterdam, The Netherlands.

Synthetic Chemistry

Synthesis of 2-(di(chloroethyl)amino)-1,4-benzoquinone, BM

BM was synthesized using the method developed by Makarova *et al.* (1973). Briefly, di(chloroethyl)amine hydrochloride (0.25 grams, 1.4 mmoles), and potassium fluoride (0.2 grams, 3.2 mmoles) were added to a suspension of 1,4-benzoquinone (0.25 grams, 2.3 mmoles) in 5 mL of H_2O. The resulting reaction mixture was stirred at room temperature for 2 hours. A purple precipitate was collected by filtration, dried and re-crystallized from hexanes to give 0.102 grams (0.41 mmoles) of 2-(di(chloroethyl)amino)-1,4-benzoquinone, BM. The structure was confirmed and purity assessed by ^{1}H nmr analysis in D_6 acetone (Figure M-1): $\delta_{3'and4'}$: 4.29 ppm (dd), $J_{1'3'}$(6.0 Hz)$J_{3'3'}$(2.3 Hz);$\delta_{1'and2'}$: 3.83 ppm (dd), $J_{1'3'}$(2.3)$J_{1'3'}$(6 Hz); δ_3: at 6.15 ppm (d), J_{35}(2.3 Hz); δ_5: 7.03 ppm (dd), J_{35}(2.3 Hz)J_{56}(10 Hz); δ_6: 7.06 ppm (d), J_{56}(10 Hz). Melting point of product: 112-113°C; literature melting point of BM: 112-113°C (Makarova *et al.*, 1973).

Synthesis of 2-methoxy-1,4-dihydroxybenzene

2-Methoxy-1,4-dihydroxybenzene was synthesized using the method developed by Corral (1957). Vanillin (7.6 grams, 45 mmoles) was dissolved into 25 mL of H_2O containing

sodium hydroxide (2 grams, 50 mmoles). The resulting solution was cooled on ice and treated with 17 mL of 10% H_2O_2, added drop-wise over a period of 5 minutes. The reaction was stirred at room temperature for one day. Next the mixture was extracted 3 times with an equal volume of diethyl ether. The extracts were combined, dried with magnesium sulfate and rotary evaporated *in vacuo* to dryness to give a dark brown oil. The dark brown oil was distilled *in vacuo* (5 mm Hg pressure), which produced a clear brown oil that crystallized to a cream paste. The cream paste was re-crystallized using dichloromethane to give 2-methoxy-1,4-dihydroxybenzene (3.5 grams, 25 mmoles). The product was confirmed and purity assessed by ^1H nmr analysis in $CDCl_3$ (Figure M-2): δ_1: 3.84 ppm (s); δ_5: 6.30 ppm (dd), J_{35}(2.8 Hz)J_{56}(8.5 Hz); δ_3: 6.44 ppm (d), J_{53}(2.7 Hz); δ_6: 6.75 ppm (d), J_{56}(8.5 Hz). Melting point of product: 80-85°C; literature melting point of 2-methoxy-1,4-dihydroxybenzene: 82-86°C (Corral, 1957).

Synthesis of 2-methoxy-1,4-benzoquinone

2-Methoxy-1,4-benzoquinone was synthesized using a modified method of Corral (1957). 2-Methoxy-1,4-dihydroxybenzene (3.37 grams, 24 mmoles) was added to an emulsion composed of 50 mL of dichloromethane, 50 mL of 60% glacial acetic acid and chromic acid (2.4 grams, 24 mmoles). The suspension was stirred vigorously for 30 minutes. The dichloromethane layer was removed and the aqueous layer extracted twice with equal volumes of dichloromethane. The dichloromethane extracts were pooled with the original dichloromethane layer, neutralized with sodium bicarbonate, dried with magnesium sulfate and rotary evaporated *in vacuo* to dryness to give 2-methoxy-1,4-benzoquinone (2.4 grams,

17 mmoles). The structure was confirmed and purity assessed by ^{1}H nmr analysis in $CDCl_3$ (Figure M-3): $\delta_{1'}$: 3.83 ppm (s); δ_3: 5.94 ppm (s); $\delta_{5\text{-}6}$: 6.71 ppm (unresolved). Melting point of product: 145-146°C; literature melting point of 2-methoxy-1,4-benzoquinone: 145-146°C (Corral, 1957)

Synthesis of 5-methoxy-2-(di(chloroethyl)amino)-1,4-benzoquinone, MBM

MBM was synthesized using a combination of the method described by Crosby *et al.* (1956) for oxidative amination of 1,4-benzoquinones and Makarova *et al.* (1973) for synthesis of BM. Di(chloroethyl)amine hydrochloride (0.32 grams, 1.8 mmoles), potassium fluoride (0.17 grams, 2.9 mmoles) and cupric acetate (0.36 grams, 1.9 mmoles) were added to a solution of 3-methoxy-1,4-benzoquinone (0.25 gram, 1.8 mmoles) in 5 mL of 95% ethanol. The reaction mixture was stirred at room temperature for 3 days with minimum light exposure. The mixture was filtered and the filtrate was washed 4 times with 20 mL of ethyl acetate. The extracts were pooled with the original supernatant liquid. The pooled solution was washed with an equal volume of 0.1 M HCl to remove any remaining cupric acetate. The ethyl acetate layer was separated, dried with magnesium sulfate and rotary evaporated *in vacuo* to dryness to give a red solid. The red solid was re-crystallized using 95% ethanol to give MBM (0.117 grams, 0.42 mmoles). The structure was confirmed and purity assessed by ^{1}H nmr analysis in $CDCl_3$ (Figure M-4): $\delta_{3'\text{ and }4'}$: 3.72 ppm (dd), $J_{3'1'}$(6.7 Hz)J_{ac}(unresolved); $\delta_{5'}$: 3.82 ppm (s); $\delta_{1'\text{ and }2'}$:3.87 ppm (dd), $J_{1'3'}$(6.3 Hz); δ_3: 5.62 ppm (s); δ_6: 5.72 ppm (s). Melting point of product: decomposed at 183°C; literature melting point of MBM: decomposed at 184-185°C (Ross, 1964).

Synthesis of 6-phenyl-2-(di(chloroethyl)amino)-1,4-benzoquinone, PBM

PBM was synthesized using a combination of the method described by Crosby *et al.* (1956) for oxidative amination of 1,4-benzoquinones and Makarova *et al.* (1973) for synthesis of BM. Di(chloroethyl)amine hydrochloride (2.56 grams, 14.4 mmoles), potassium fluoride (1.33 grams, 22 mmoles) and cupric acetate (2.76 grams, 15 mmoles) were added to a solution of 2-phenyl-1,4-benzoquinone (2.56 grams, 14 mmoles) in 20 mL of 95% ethanol. The resulting mixture was stirred for 3 days in the absence of light. The reaction mixture was applied to a 10 cm x 2 cm silica gel column to remove the cupric acetate. The impure mixture of starting material and addition product, phenylbenzoquinone mustard, isomers was eluted with 50% ethyl acetate in hexanes. Fractions containing the impure mixture were collected, dried with magnesium sulfate and rotary evaporated *in vacuo* to dryness to yield a reddish oil. ^{1}H nmr analysis indicated a 7:1 molar ratio of phenylbenzoquinone mustard, to 2-phenyl-1,4-benzoquinone. Additionally, it indicated the presence of the ortho, meta and para isomers of phenylbenzoquinone mustard in a ratio of approximately 1:2:1, respectively. The meta isomer was purified from the impure mixture, first, by flash chromatography using a 100 mL silica gel column (1000 mesh) with 10% ethyl acetate in hexanes as the mobile phase. Those fractions containing m-phenylbenzoquinone mustard, determined by TLC, were collected and rotary evaporated to dryness *in vacuo*. The resulting semi-solid material was re-crystallized using methanol to give PBM (110 mg, 0.33 mmoles). The structure was confirmed and purity assessed by ^{1}H nmr analysis in D_6 acetone (Figure M-5): $\sigma_{1'-4'}$: 3.93 ppm (unresolved complex of two dd); δ_3: 5.75 ppm (d), J_{35}(2.5 Hz); δ_5: 6.66 ppm (d), J_{35}(2.4 Hz); $\delta_{7'-9'}$:7.44 ppm (m); $\delta_{6' \text{ and } 10'}$: 7.52 ppm (m).

Synthesis of 2-*t*-butyl-1,4-benzoquinone

2-*t*-Butyl-1,4-benzoquinone was synthesized using the method for oxidation of 1,4-dihydroxybenzene developed by Corral (1957). 2-*t*-Butyl-1,4-dihydroxybenzene (7.4 grams, 44 mmoles) was added to a suspension composed of 50 mL of dichloromethane and 50 mL of 60% glacial acetic acid, which contained anhydrous chromic acid (4.4 grams, 44 mmoles). The suspension was stirred vigorously for 30 minutes. The dichloromethane layer was removed, and the aqueous layer extracted with an equal volume of dichloromethane, twice. The dichloromethane extracts were pooled, neutralized with sodium bicarbonate, dried with magnesium sulfate and rotary evaporated to dryness to give 2-*t*-butyl-1,4-benzoquinone (5 grams, 30 mmoles). The product was confirmed and purity assessed by ^{1}H nmr analysis in $CDCl_3$ (Figure M-6): $\delta_{2'\text{-}4'}$: 1.28 ppm (s); δ_3: 6.59 ppm (unresolved); $\delta_{5\text{-}6}$: 6.67 ppm (unresolved).

Synthesis of 6-*t*-butyl-2-(di(chloroethyl)-amino)-1,4-benzoquinone, TBM

TBM was synthesized using a combination of the method described by Crosby *et al.* (1956) for oxidative amination of 1,4-benzoquinones and Makarova *et al.* (1973) for synthesis of BM. Di(chloroethyl)amine hydrochloride (1.5 grams, 8.4 mmoles), potassium fluoride (0.8 grams, 13.8 mmoles) and cupric acetate (1.7 grams, 9.3 mmoles) were added to a solution of 2-*t*-butyl-1,4-benzoquinone (1.4 grams, 8.3 mmoles) in 25 mL of 95% ethanol. The resulting reaction mixture was stirred at room temperature for 3 days with minimum light exposure. The mixture was filtered and the precipitate washed 4 times with 20 mL of ethyl acetate. The extracts were pooled with the original supernatant and washed

with an equal volume of 0.1 M HCl to remove any remaining cupric acetate. The ethyl acetate layer was separated, dried with magnesium sulfate and rotary evaporated *in vacuo* to dryness to give a red/brown oil. ^{1}H nmr analysis indicated the presence of 2-*t*-butyl-1,4-benzoquinone and the addition product, *t*-butyl-1,4-benzoquinone mustard, in a ratio of approximately 1:1. In an attempt to purify *t*-butyl-1,4-benzoquinone mustard , 0.5 grams of the red/brown oil was applied to a 100 mL silica gel column. Fractions were eluted using 10% ethyl acetate in hexanes. Fractions were analyzed by TLC. Fractions containing only one band were combined. ^{1}H nmr analysis indicated the presence of the meta isomer as the major component. Flash chromatography using a 100 mL silica gel column and 10% ethyl acetate in hexanes was carried out a second time. In this case, 0.09 grams of 6-*t*-butyl-2-((dichloroethyl)amino)-1,4 benzoquinone was obtained in 90% purity, as analyzed by ^{1}H nmr. Flash chromatography using a 50 mL silica gel column and 10% ethyl acetate in hexanes was carried out a third time to yield 0.013 grams (0.004 mmoles) of 6-*t*-butyl-2-((dichloroethyl)amino)-1,4-benzoquinone. The structure was confirmed and purity assessed by ^{1}H nmr analysis in $CDCl_3$ (Figure M-7): $\delta_{6'\text{-}8'}$: 1.27 ppm (s);$\delta_{3'\text{-}4'}$: 3.72 ppm (dd), $J_{3'1'}$(5.8 Hz)$J_{3'1}$(4 Hz); $\delta_{1'\text{-}2'}$: 3.75 ppm (dd), $J_{1'3}$(5.7 Hz)$J_{1'3}$(4 Hz); δ_3: 5.60 ppm (d), J_{35}(2.5 Hz); δ_5: 6.44 ppm (d), J_{35}(2.5 Hz).

Synthesis of 5-chloro-2-(di(chloroethyl)amino)1,4-benzoquinone, CBM

CBM was synthesized using the method described by Makarova *et al.* (1973). Di(chloroethyl)amine hydrochloride (0.32 grams, 1.8 mmoles), potassium fluoride (0.17 grams, 2.9 mmoles) and cupric acetate (0.36 grams, 1.9 mmoles) were added to a solution

of 2-chloro-1,4-benzoquinone (0.25 grams, 1.8 mmoles) in 5 mL of 95% ethanol. The reaction mixture was stirred at room temperature for 3 days with minimum light exposure. The mixture was filtered and the filtrate was washed 4 times with 20 mL of ethyl acetate. Extractions were pooled with the original supernatant liquid. The pooled solution was washed with an equal volume of 0.1 M HCl to remove any remaining cupric acetate. The ethyl acetate layer was separated, dried with magnesium sulfate and rotary evaporated to dryness *in vacuo*. The resulting purple solid was re-crystallized using methanol to give CBM (0.09 grams, 0.32 mmoles). The structure was confirmed and purity assessed by ^{1}H nmr analysis in D_6 acetone (Figure M-8): $\delta_{1'-2'}$: 3.87 ppm (m); $\delta_{3'-4'}$: 3.96 ppm (m); δ_3: 5.85 ppm (s); δ_6: 5.94 ppm (s). Melting point of product: 165-166°C; literature melting point of CBM: 165-166°C (Ross, 1964).

Synthesis of 2-acetyl-1,4-benzoquinone

2-Acetyl-1,4-benzoquinone was synthesized using the method described by Kurosawa (1960). 2-Acetyl-1,4-hydroquinone (1 gram, 6.5 mmoles) and freshly prepared anhydrous silver oxide (2 grams , 8.6 mmoles) were added to a flame dried flask containing anhydrous K_2CO_3 (0.65 grams, 6.58 mmoles). A volume of 20 mL of benzene was added, and the resulting suspension was shaken for 10 minutes and filtered. The filtrate was suspended in another 10 mL of benzene and filtered. The supernatant liquids from both filtrations were collected, and rotary evaporated *in vacuo* to dryness to give 0.549 grams of a red/orange semi-solid product. The semi-solid product was suspected to contain 2-acetyl-1,4-benzoquinone in addition to 2-acetyl-1,4-dihydroxybenzene, which was confirmed by ^{1}H

nmr analysis in D_6 acetone (Figure M-9): $\delta_{1'}$; 2.49 ppm (s), δ_3: 6.88 ppm (s); δ_5: 6.88 ppm (d), J_{65}(2.3 Hz); δ_3: 6.90 ppm, J_{65}(2.6 Hz). This product mixture readily decomposed upon standing in air.

Synthesis of 3-acetyl-2-(di(chloroethyl)amino)-1,4-benzoquinone, ABM

The impure oil containing 2-acetyl-1,4-benzoquinone was used in an attempt to synthesize 3-acetyl-2-(di(chloroethyl)amino)-1,4-benzoquinone, since attempts to purify 2-acetyl-1,4-benzoquinone were unsuccessful. The rationale for this approach is that the mustard derivative of acetyl-benzoquinone may be stable enough to allow further purification. Therefore, di(chloroethyl)amine hydrochloride (0.653 grams, 3.7 mmoles), anhydrous K_2CO_3 (1 gram, 7.2 mmoles) and anhydrous silver oxide (1 gram, 4.3 mmoles) were added to a solution of 0.54 grams of the red/orange oil dissolved in 10 mL of anhydrous ether. After 4 hours of stirring with minimum light exposure, the reaction mixture was filtered. The filtrate was washed with 10 mL of anhydrous ether. Supernatant liquid was collected and rotary evaporated *in vacuo* to dryness to yield a red-brown oil. ^{1}H nmr analysis in D_6 acetone indicated the presence of ABM amongst various impurities (Figure M-10): $\delta_{6'}$: 2.45 ppm (s); $\delta_{1'\text{-}4'}$: 3.67 ppm (m); δ_5: 6.67 ppm (d), J_{56}(10 Hz); δ_6: 6.73 ppm (d), J_{56}(10 Hz). The impure mixture readily decomposed after work-up was completed

Analysis of Cytotoxicity of BM Analogs

Preparation of solution of BM analogs

All analogs were prepared fresh on the day of the experiment in dimethyl formamide, DMF. Exposure to light was minimized at all stages due to photochemical decomposition for each of the analogs. All dilutions were carried out in DMF, with the exception of the final dilution into growth media. In all cases, a final concentration of 1% DMF in growth media was used.

Cytotoxicity assay

H661 cells were grown in RPMI 1640 plus 10 % fetal bovine serum. Cells were seeded in Falcon™ 6-well plates at 2.5 x 10^6/well one day prior to the experiment, or 1.5 x 10^6/well two days prior to achieve a final cell number of 3 x 10^6/well. Cells were incubated with, or without, 100 μM dicoumarol in 3.0 mL of fresh RPMI plus 10% fetal bovine serum for 20 minutes at 37°C in 5% CO_2. Media was removed, and cells were treated with, or without, 100 μM dicoumarol and various concentrations of a BM analog in 3 mL RPMI plus 10% fetal bovine serum for 60 minutes at 37°C in 5% CO_2. Cells were washed with 2 mL of citrate saline (130 mM KCl and 15 mM Na citrate), treated with 1 mL of 0.05 % trypsin in citrate saline for 10 minutes at 37°C in 5% CO_2 and suspended in 3 mL RPMI 1640 plus 10% fetal bovine serum. Cells were diluted in RPMI 1640 plus 10% fetal bovine serum, seeded at 1600, 1200, 1000, 800, 400 and 200 cells/well in Falcon™ 96-well plates in triplicate or quadruplicate and incubated at 37°C in 5% CO_2 for 7 days. The surviving cell

fraction was determined by MTT assay, as described by Johnston *et al.* (1994).

SK-MEL-28 cells were grown in DMEM plus 10% fetal bovine serum. Cells were seeded in Falcon™ 6-well plates at 2.5 x 10^6/well one day prior to the experiment, or 1.5 x 10^6/well two days prior to achieve a final cell number of 3 x 10^6/well. Cells were incubated in 3 mL of fresh DMEM plus 10% fetal bovine serum with, or without, 100 μM dicoumarol for 20 minutes at 37°C in 5% CO_2. Media was removed and cells were treated with, or without, 100 μM dicoumarol and various concentrations of the BM analogs in 3 mL DMEM plus 10% fetal bovine serum for 60 minutes at 37°C in 5% CO_2. Cells were washed with 2 mL of citrate saline, treated with 1 mL of 0.05 % trypsin in citrate saline for 20 minutes at 37°C in 5% CO_2 and suspended in 3 mL of DMEM plus 10% fetal bovine serum. Cells were diluted in DMEM plus 10% fetal bovine serum, seeded at 1000, 750, 500, 250 and 125 cells/well in Falcon™ 96-well plates in triplicate or quadruplicate and incubated at 37°C in 5% CO_2 for 9 days. The surviving cell fraction was determined by MTT assay, as described by Johnston *et al.* (1994).

NADPH:cytochrome P-450 reductase activity

NADPH:cytochrome P-450 reductase activity in H661 and SK-MEL-28 was determined in supernatant solutions from cell sonicates by a spectrophotometric assay using cytochrome c as the artificial electron acceptor (Strobel and Digman, 1978)

Purification of DT-Diaphorase

Purification

Human DT-diaphorase was purified from HT-29 human colon carcinoma cells using a modified method of Sharkis *et al.* (1989). HT-29 cells were grown to confluence on 180 Nunc™ 135 mm plates in RPMI 1640 plus 10% fetal bovine serum to give approximately 1 x 10^9 cells. For each plate, cells were washed twice with 12 mL of PBS (150 mM sodium chloride, 5 mM potassium phosphate, pH 7.4) scraped into 5 mL of PBS and spun at 480 xg for 10 minutes to give a 30 mL pellet. The pellet was suspended in 30 mL of 0.25 M sucrose. The suspension was sonicated 3 times for 10 seconds each. The resulting emulsion was ultracentrifuged at 10,000 x g for 90 minutes at 4°C. The supernatant liquid was collected and applied to a 10 mL Affi-Gel Blue™, 100-200 mesh, affinity column that was pre-equilibrated with 0.25 M sucrose in 50 mM Tris HCl (pH 7.2), Buffer A. Using a flow rate of 0.1 mL/min., the column was washed at 4°C with at least 300 mL of Buffer A. The column was washed at 4°C with Buffer A plus 0.5 M KCl to remove proteins non-specifically bound. The wash with Buffer A plus 0.5 M KCl was continued until the absorbance at 280 nm of the eluant was less than 0.02. The column material was removed and suspended in 20 mL of Buffer A containing 0.5 M KCl, 10 mM NADH and 5 mM FAD. The suspension was mixed for 1.5 hours at 4°C and centrifuged at 480 xg for 10 minutes at 4°C. The supernatant was removed, and the pellet resuspended in 20 mL of Buffer A containing 0.5 M KCl, 10 mM NADH and 5 mM FAD. The resulting suspension was mixed for 1.5 hours at 4°C. The suspension was eluted through a mesh filter to remove all Affi-Gel

Blue™ resin. The resulting solution was pooled with supernatant from the first wash with Buffer A containing 0.5 M KCl, 10 mM NADH and 5 mM FAD, and ultrafiltered at 4°C to a final volume of 2 mL using a Centriprep-10™ ultrafilter. At 4°C, the concentrate was applied to an 80 mL Sephadex™ G-100 size-exclusion column pre-equilibrated with 50 mM Tris HCl (pH 7.2). One mL fractions, 60 in total, were eluted using 50 mM Tris HCl (pH 7.2). The fractions were monitored by measuring the absorbance at 280 nM. The DT-diaphorase activity of 10 μL of each fraction was measured using the method described by Prochaska and Santamaria (1988). The elution profile obtained is illustrated in Figure M-11. Fractions 17-20 and 21-27 were pooled and concentrated to 400 μL by ultra-filtration at 4°C using a centriprep-10™ ultra-filter. At 4°C, the concentrates were applied to a 1 mL DEAE Sephadex A-25 anion exchange chromatography column pre-equilibrated with 50 mM Tris HCl (pH 8.9). The column was treated with 50 mM Tris HCl (pH 8.9), and 400 μL fractions were collected. DT-diaphorase activity was measured for 10 μL of each fraction using the method described by Prochaska and Santamaria (1988). Fractions containing DT-diaphorase activity were pooled, and buffered to pH 7-8 by adding an appropriate volume of 1.0 M Tris HCl (pH 4.0). The solution was then ultra-filtered to a final volume of 1 mL, and frozen at -80°C. Purity was assessed using 1, 2, 4 and 8 μg of protein, and SDS PAGE. The gel was stained using Silver Stain™.

DT-diaphorase activity

DT-diaphorase activity for purified DT-diaphorase was determined using menadione as the electron acceptor, as described by Begleiter et al. (1997).

Gel electrophoresis

Protein samples were treated with SDS reducing sample buffer, boiled for 5 minutes and loaded on a 160 x 200 x 1 mm 12% SDS Page gel. A 10 mA current was applied to the gel over 16 hours. Gels were stained using Coomassie Blue R-250 and/or Silver stain™.

Analysis of Rate of Aerobic and Anaerobic Reduction of MBM and BM by DT-diaphorase

Preparation of solutions of BM and MBM

BM and MBM were prepared fresh on the day of the experiment in DMF. Exposure to light was minimized at all stages due to photochemical decomposition for each of the analogs. All dilutions were carried out in DMF, with the exception of the final dilution into 50 mM Tris HCl (pH 7.4). In all cases, a final concentration of 1% DMF in 25 mM Tris HCl (pH 7.4) was used.

Aerobic reduction

Reactions were carried out in 25 mM Tris HCl (pH 7.4) at 25°C using 0.1 μg/mL of DT-diaphorase purified from HT-29 (see Purification of DT-diaphorase), freshly prepared 100 μM NADH, 0.5 μM FAD and 50 μM BM or MBM in a volume of 1 mL. DT-diaphorase was prepared fresh on the day of the experiment from -80°C frozen stock, and activated with 0.01% Tween 20 prior to addition to the reaction mixture.

Aliquots of the reaction mixture were removed at various time points, and immediately frozen at -80°C for HPLC analysis. Samples were kept frozen, prior to analysis, for no longer than 5 days.

Hypoxic reduction

A sealed 1.5 mL Eppendorf™ tube containing reaction buffer (1 mL of 50 mM Tris HCl, pH 7.4) was perforated twice with a needle to create an inlet and outlet hole. A 1.5 inch stainless steel needle was placed through the inlet into the reaction buffer. Using this needle, nitrogen gas with 5% CO_2 was purged into the reaction buffer for at least 1.5 hours at 25°C. DT-diaphorase , prepared using the same method as described for aerobic analysis, was added to the reaction buffer through the outlet hole to give a final concentration of 0.1 μg/mL of DT-diaphorase. Freshly prepared NADH and FAD were added to the reaction buffer through the outlet hole to give a final concentration of 100 μM and 0.5 μM, respectively. Nitrogen gas with 5% CO_2 was bubbled into the reaction buffer for an additional 10 minutes. BM or MBM was added through the outlet hole to the reaction buffer to give a final concentration of 50 μM. Nitrogen gas with 5% CO_2 was bubbled into the reaction vessel for the entire time course.

Aliquots of the reaction mixture were removed through the outlet hole at various time points, and were immediately frozen at -80°C for HPLC analysis. Samples were kept frozen, prior to analysis, for no longer than 5 days.

HPLC analysis of NADH

The rate of reduction of BM and MBM was determined by measuring the rate of consumption of NADH. The rate of consumption of NADH was quantified by HPLC, as described by Gibson *et al.* (1992). Samples were quickly thawed from -80°C storage, and 15 µL of sample was applied to a Phenomenex™ Prodigy 5 µm ODS 150 x 4.6 mm analytical column using a WISP™ autoinjection system and a Waters™ programmable multi-solvent delivery system. The following linear gradient program was used to separate NADH: Solvent A: 10 mM potassium phosphate (pH 6.0), Solvent B: HPLC grade methanol

Time (min.)	Percent A	Percent B
0	100	0
5	95	5
15	20	80
25	20	80

NADH levels were quantified by measuring the absorbency at 340 nM using a Waters™ programmable multi-wavelength detector, and a Hewlett Packard™ integrator. A typical chromatogram is illustrated in Figure M-12. The area under the NADH peak was shown to be linearly related to the concentration of NADH in a range of 0 to 100 µM, as illustrated in Figure M-13.

Redox Potentials

Preparation of solutions of BM analogs

BM analogs were prepared fresh on the day of the experiment in DMF. Exposure to

light was minimized at all stages due to photochemical decomposition for each of the analogs. All dilutions were carried out in DMF, with the exception of the final dilution into 50 mM potassium phosphate buffer (pH 7.4). In all cases, a final concentration of 1% DMF in 50 mM potassium phosphate buffer (pH 7.4) was used.

Cyclic voltammetry

The redox potentials of 500 μM of each BM analog in 50 mM phosphate (pH 7.4) was determined by cyclic voltammetry using a glassy-carbon electrode. Samples were degassed for 5 minutes using nitrogen prior to analysis, and scanned at 50 mV/s over a potential range of +0.30 V to -1.30 V. Redox potentials were measured by Dr. Joseph Wang, Department of Chemistry and Biochemistry, New Mexico State University, New Mexico, USA.

Analysis of Cross-Linking Activity of BM and MBM

Preparation of pBR322 plasmid DNA

E. coli were transformed with pBR322 plasmid purchased from Sigma™ using the Invitrogen™ kit, and grown on an LB agar plate containing 50 μg/mL of ampicillin for 16 hours. Multiple colonies formed, and the plate was stored at 4 °C for one week. A single colony on the plate was used to innoculate 500 mL of Luria Broth ,LB, media containing 50 μg/mL of ampicillin. *E. coli* were grown to saturation by incubating at 37 °C for 12 hours. LB media was centrifuged at 1480 xg for 15 minutes to pellet the *E. coli*. The supernatant

liquid was removed and the pellet suspended in 100 mL of ice cold STE buffer (0.1 M NaCl, 0.01 M Tris HCl, 2 mM EDTA, pH 8.0) and the resulting suspension centrifuged at 1480 xg for 15 minutes. The supernatant liquid was removed and the pellet suspended in 18 mL of solution I (0.05 M glucose, 25 mM Tris HCl, 10 mM EDTA, pH 8.0) followed by the addition of 12 mL of 10 mg/mL lysozyme. The resulting solution was mixed well and left to stand for 10 minutes on ice. A volume of 40 mL of solution II (0.2 M NaOH and 1% SDS) was added and the resulting solution left to stand for 10 minutes on ice. A volume of 20 mL of ice-cold solution III (3 M potassium acetate and 11.5% (v/v) acetic acid) was added and the centrifuge tube inverted several times which produced a flocculent white precipitate. The tube was kept on ice for 10 minutes and centrifuged at 1940 xg for 25 minutes. The supernatant liquid was removed and filtered through 5 layers of cheese cloth into a fresh centrifuge tube. A volume of 50 mL of 2-propanol was added to the filtered supernatant liquid and left to stand at room temperature for 15 minutes. The resulting suspension was centrifuged at 3020 xg for 15 minutes. The supernatant liquid was removed, and the pellet was washed with 50 mL of 70% ethanol. The ethanol solution was aspirated, and the pellet left to dry at room temperature for 10 minutes. The pellet was dissolved in 3 mL of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0), and 0.5 mL of the resulting solution was transferred to each of 6 sterile 1.5 mL Eppendorf™ tubes. A volume of 0.5 mL of ice-cold 5M LiCl was added to each tube, and tubes were centrifuged at 8160 xg for 10 minutes at 4°C. The supernatant liquid from all tubes was transferred equally to 12 fresh sterile 1.5 mL Eppendorf™ tubes. A volume of 0.5 mL 2-propanol was added to each tube, and tubes were left to stand for 10 minutes at room temperature. Tubes were centrifuged at 8160 xg for 10

minutes at room temperature. For each tube, liquid was decanted, and the pellet was washed with 70% ethanol. The ethanol was removed and the pellet left to dry at room temperature for 5 minutes. For each tube, the pellet was dissolved in 500 μL of TE buffer followed by the addition of 500 μL of 6 M NaCl containing 13% (w/v) polyethylene glycol (PEG8000). Contents of each tube were mixed well, and each tube was centrifuged at 11750 xg for 5 minutes at 4 °C. The supernatant liquid was removed and pellets from each tube were dissolved into a total volume of 3 mL of TE buffer. A volume of 0.5 mL of the resulting solution was transferred to each of 6 fresh sterile 1.5 mL Eppendorf™ tubes, and 500 μL of 1:1 phenol/chloroform (equilibrated with TE buffer) added. Contents of each tube was vortexed, and centrifuged at 13800 xg for 3 minutes. For each tube, the upper aqueous layer was removed and transferred to a fresh sterile 1.5 mL Eppendorf™ tube. To each tube, 500 μL of chloroform was added. Tubes were vortexed and centrifuged at 13800 xg for 3 minutes. For each tube, the upper aqueous layer was removed and transferred to a fresh sterile 1.5 mL Eppendorf™ tube. To each tube, 670 μL of ethanol and 23 μL of 3.0 M ammonium acetate (pH 5.2) was added. Tubes were left to stand at -80°C for 15 minutes and centrifuged at 13800 xg for 15 minutes. For each tube, the supernatant liquid was aspirated, and the pellet was washed with 500 μL of 70% ethanol. For each tube, the ethanol wash was aspirated. The pellets from all 6 tubes were dissolved into a total volume of 250 μL of TE buffer. A volume of 20 μL of 10 mg/mL of RNAase was added, and the resulting solution was incubated a 37°C for 1 hour. A volume of 1 μL of the solution was used to determine the purity of the pBR322 DNA preparation by agarose gel electrophoresis. A volume of 9 μL of sterile de-ionized water and 1 μL of Loading buffer (50% (v/v) glycerol, 1mM EDTA

(pH 8.0), 4.6 mg/mL xylene cyanol and 4.6 mg/mL bromophenol blue) was added to the 1 μL of DNA solution, and the resulting solution was loaded onto a 1% TBE agarose gel containing 1 μg/mL ethidium bromide. Additionally, λ *Hind* III fragments from Gibco™ were loaded onto the gel as size markers. The gel was immersed in TBE buffer (89 mM Tris HCl, 89 mM boric acid, 10 mM EDTA, pH 8.3) and a voltage of 80 V was applied to the gel for 2 hours. DNA bands were detected using a transilluminator. For the 1 μL solution of DNA from the maxi preparation, a band at approximately 4.3 kbp was detected corresponding to the length of pBR322 plasmid DNA. Additionally, two other bands corresponding to supercoiled and supercoiled-nicked DNA were detected. Using a comparison of the intensity of DNA band at 4.3 kbp with the intensity of the λ *Hind* III fragments, the concentration of pBR322 plasmid was approximated to be 200 ng/μL.

_Eco_R1 cut of pBR322 plasmid DNA

The pBR322 plasmid DNA prepared, as described above, was cut with *Eco*R1. A volume of 30 μL of *Eco*R1 (5000 units/μL) and 30 μL of 10 times BRL buffer was added to 250 μL of 200 ng/μL pBR322 plasmid DNA. The reaction mixture was incubated at 44 °C for one hour. The reaction mixture was cooled to room temperature, 20 μL of 3.0 M ammonium acetate (pH 5.0) and 660 μL of ethanol were added. The solution was mixed, left to stand at -80 °C for 15 minutes and centrifuged at 13800 xg for 15 minutes at 4°C. The supernatant liquid was aspirated, and the pellet was dissolved in 250 μL of TE buffer.

Gel purification of *Eco*R1 cut pBR322 plasmid DNA

A volume of 20 μL of Loading buffer was added to 200 μL of TE buffer containing approximately 200 ng/μL of *Eco*R1 cut pBR322 plasmid DNA. A volume of 50 μL of the resulting mixture was added to each of 4 wells of a 1% TBE agarose gel. Additionally, λ *Hind* III fragments from Gibco™ were loaded onto the gel as size markers. The gel was submerged in TBE and a voltage of 80 V was applied to the gel for 2 hours. DNA bands were detected using a transilluminator. For each of the 4 lanes used, an intense band of DNA was detected at 3.7 kbp which corresponds to the length of pBR322 plasmid DNA. A slit was cut just in front of this band of DNA, and a 1.5 cm x 0.5 cm strip of NA45 DEAE cellulose membrane inserted into the slit. A voltage of 80 mV was applied to the gel for 20 minutes and membranes were removed. Each membrane was cut into several small segments and distributed equally into 4 Eppendorf™ tubes each containing 200 μL of 1 M NaCl. Tubes were incubated at 68 °C for 16 hours. A volume of 440 μL of ethanol was added to each tube and left at -80 °C for 16 hours. Tubes were centrifuged at 13800 xg for 15 minutes. For each tube, supernatant liquid was aspirated and pellets were dried at room temperature for 10 minutes. A volume of 20 μL of sterile deionized water was added to each tube, and solutions from each tube were combined.

Agarose gel electrophoresis was used to determine the concentration and purity of 1 μL of the 20 μL solution of the *Eco*R1 cut pBR322 plasmid DNA. A volume of 10 μL of sterile deionized water and 1 μL of Loading buffer was added to 1 μL of the purified *Eco*R1 cut pBR322 plasmid DNA and the resulting solution loaded onto a 1% TBE agarose gel. Additionally, λ *Hind* III fragments from Gibco™ were loaded onto the gel as size markers.

The gel was submerged in TE buffer and a voltage of 80 V applied for 2 hours. DNA bands were detected using a transilluminator. A single DNA band at approximately 4.3 kbp was detected for the sample from the purified *Eco*R1 cut pBR322 plasmid DNA. Using a comparison of the intensity of the band at 4.3 kbp with the intensity of the λ *Hind* III fragments, the concentration of the DNA was approximated at 90 ng/μL.

3' end-labeling of EcoR1 cut pBR322 plamid DNA

The 3' end of *Eco*R1 cut pBR322 plasmid DNA was radio-labeled with [α-^{32}P]dATP using Kelnow fragment. A reaction mixture consisting of 0.25 mM dTTP, 50 μCi of [α-^{32}P]dATP, 1 U of Klenow Fragment, 400 ng of purified *Eco*R1 cut pBR322 plasmid DNA, 0.01 M $MgCl_2$, 1 mM DTT and 0.05 mg/mL BSA in 20 μL of 0.05 M Tris HCl (pH 7.5) was incubated at 37°C for 15 minutes. Another 1 U of Klenow Fragment was added to the reaction mixture and the mixture was incubated at 37°C for 10 minutes. Unincorporated DNA was removed using a 3 mL NICK™ Column from Pharmacia Biotech™ (a pre-packed Sephadex G-50 DNA grade column). Labeled *Eco*R1 cut pBR322 plasmid DNA was eluted with 400 μL of TE Buffer.

Agarose gel analysis of cross-linking activity

The cross-linking activity of BM and MBM was studied using the agarose gel assay developed by Hartley *et al.* (1991). In detail, 10 ng of [α^{32}P]dATP labeled pBR322 plasmid DNA was treated with one of 0.5, 1, 5 or 10 μM of MBM or BM in the presence of 0.01 μg purified DT-diaphorase, 100 μM NADH and 5 μM FAD under aerobic conditions at 25°C

for 2 hours in 100 μL of 25 mM Tris HCl/1 mM EDTA (pH 7.4) in silicon coated Eppendorf ™ tubes. For each concentration of BM or MBM reactions were stopped and DNA was precipitated at room temperature by the addition of 100 μL of 2-propanol (or 220 μL of ethanol) and 10 μL of 3.0 M sodium acetate (pH 5.0) and centrifuged at 13800 xg for 20 minutes. Supernatant liquid was removed and 100 μL of 70% ethanol added. The ethanol solution was removed. A volume of 5 μL of sterilized and de-ionized water was added to each tube and tubes were vortexed for 20 minutes. A volume of 5 μL of Strand Separating Buffer (30% DMSO in 1 mM EDTA (pH 8.0) with 0.04% xylene cyanol and 0.04% bromophenol blue) was added, and tubes were vortexed for 20 minutes. A volume of 20 μL of sterilized and de-ionized water was added, and tubes were vortexed for 20 minutes. Tubes were placed in boiling water for 2 minutes, and were rapidly cooled on ice. Samples were loaded onto a 1% TBE agarose gel. Single-stranded and double-stranded controls were generated as follows. [α^{32}P]dATP labeled pBR322 plasmid DNA (10 ng) in a volume of 10 μL of TE buffer was treated with 10 μL of Strand Separating Buffer in a silicon coated Eppendorf tube. The tube was placed in boiling water for 2 minutes, quickly cooled on ice, and loaded onto the agarose gel. This served as the single stranded control. Another 10 ng of [α^{32}P]dATP labeled pBR322 plasmid DNA in a volume of 10 μL of TE buffer was treated with 10 μL of 0.3 M sucrose (0.04% xylene cyanol and 0.04% bromophenol blue), and directly loaded onto the agarose gel. This served as the double stranded control. The agarose gel was submerged in TBE buffer and a voltage of 40 mV applied for 16 hours. The agarose gel was dried at 80 °C for 2 hours, and exposed to a phosphor-imaging plate for 16 hours. The phospho-image was scanned for signals using a phosphorimager, and was viewed using

Molecular Dynamic Phosphorimaging software, version 3.3.

Controls for the cross-linking reaction of BM and MBM were prepared as follows. 10 ng [α^{32}P]dATP labeled pBR322 plasmid DNA (10 ng) was treated with one of each of the following: (a) 0.1 μg/mL of purified DT-diaphorase, 100 μM NADH, 5 μM FAD (b) 50 μM BM (c) 50 μM MBM (d) 0.1 μg/mL purified DT-diaphorase, 50 μM BM (e) 0.1 μg/mL purified DT-diaphorase, 50 μM MBM (f) 50 μM BM, 100 μM NADH, 5 μM FAD (g) 50 μM MBM, 100 μM NADH, 5 μM FAD. All controls were incubated at 25 °C for 4 hours in 100 μL of 25 mM Tris HCl/1 mM EDTA (pH 7.4) in silicon coated Eppendorf™ tubes. DNA was treated and loaded, and the agarose gel was treated using the same protocol described above for preparation and analysis of DNA treated with reduced BM or MBM.

A modified procedure of Hartley *et al.* (1991) was developed. [α^{32}P]dATP labeled pBR322 plasmid DNA (10 ng) was treated with one of 25 or 50 μM BM or MBM in the presence of 0.01μg of purified DT-diaphorase, 100 μM NADH and 5 μM FAD in 100 μL of 25 mM Tris HCl/1 mM EDTA (pH 7.4) in silicon treated Eppendorf ™ tubes for 2 hours. A volume of 10 μL of Loading buffer (1:1 glycerol:1 mM EDTA (pH 8.0), 0.04% bromophenol blue and 0.04% xylene cyanol) was added to each tubes. Tubes were place in boiling water for 2 minutes, cooled quickly on ice, and loaded onto a 1% TBE agarose gel. The agarose gel was submerged in TBE buffer, and a voltage of 40 V applied for 16 hours. The gel was dried at 80 °C for 2 hours, and exposed to a phosphor-image plate over night. The phosphor-image was scanned for signals using a phosphorimager, and was viewed using Molecular Dynamic Phosphorimaging software, version 3.3.

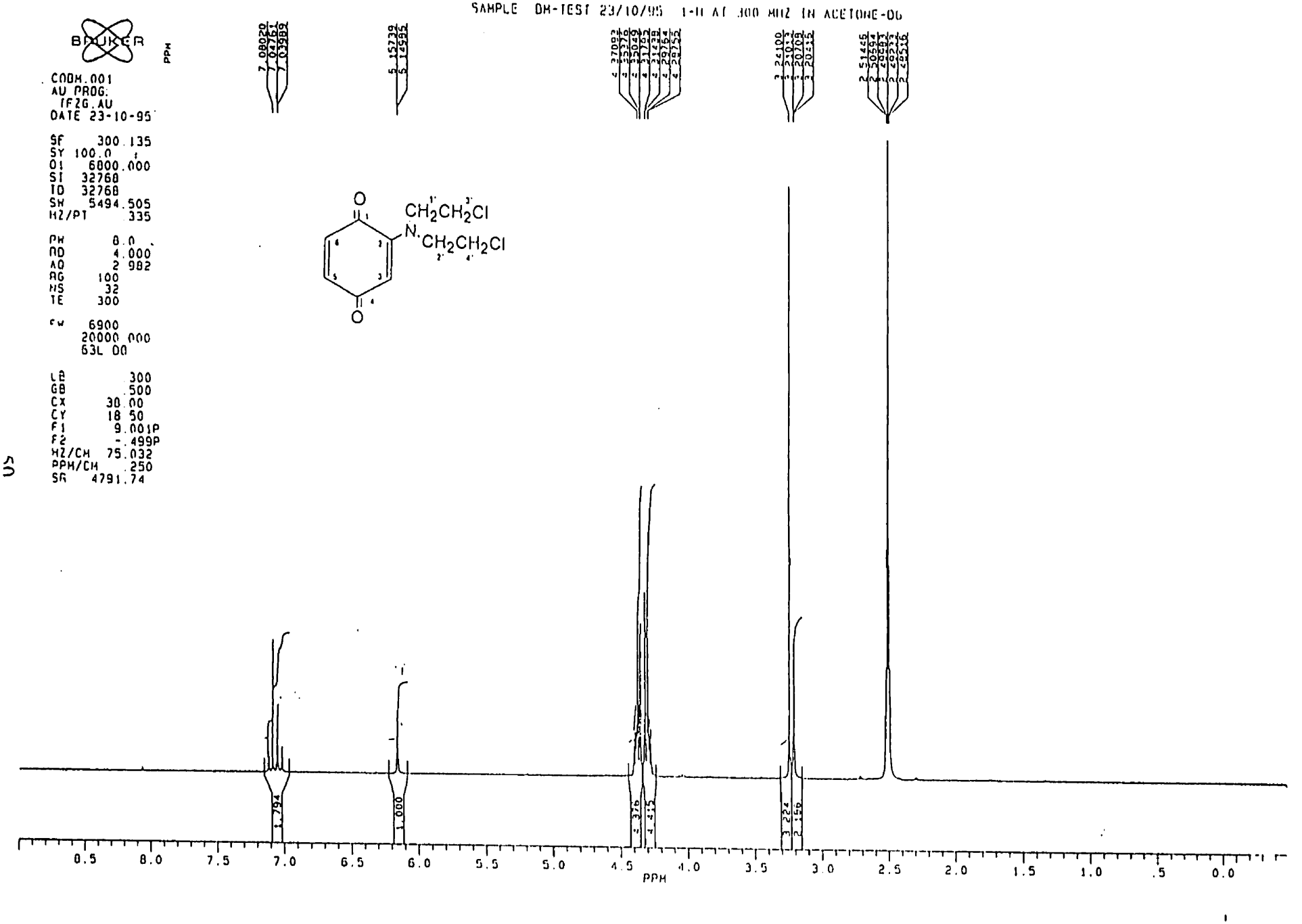


Figure M-1(a): ^{1}H nmr spectrum of BM in D_6 acetone.

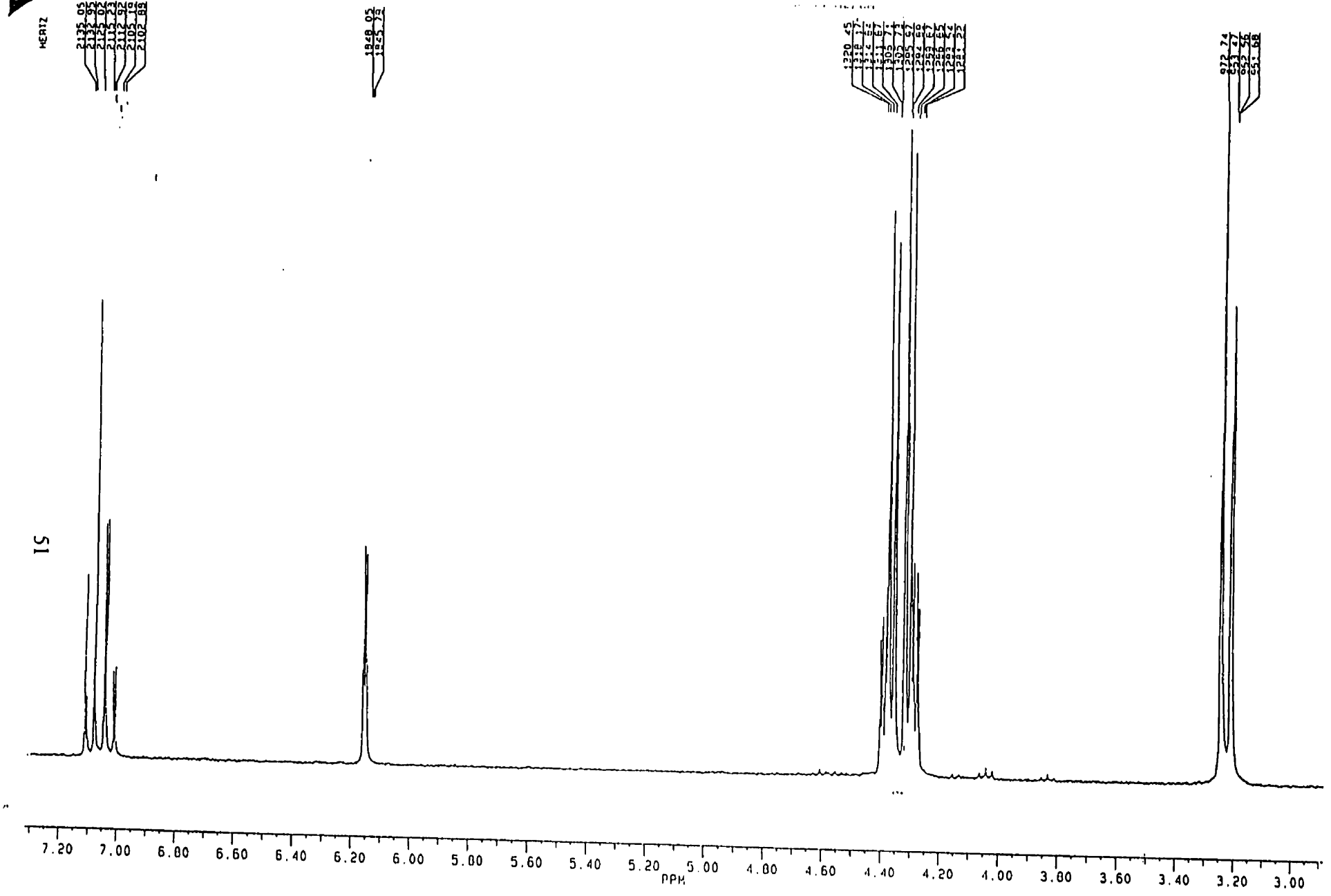
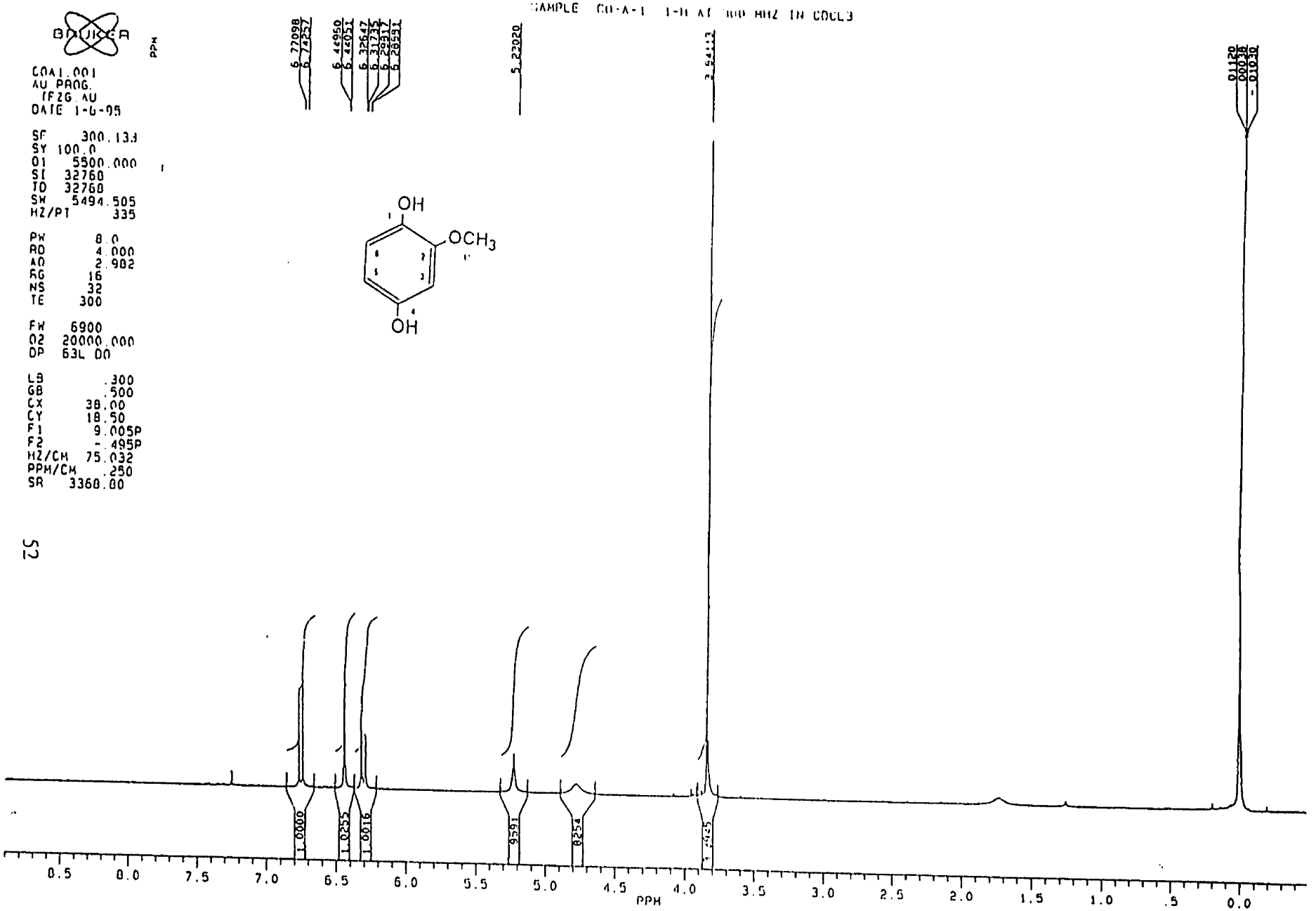


Figure M-1(b): ^{1}H nmr spectrum of BM in D_6 acetone. Expansion of region 3.00 ppm to 7.20 ppm.



52

Figure M-2(a): ^{1}H nmr spectrum of 2-methoxy-1,4-di-hydroxybenzene in $CDCl_3$.

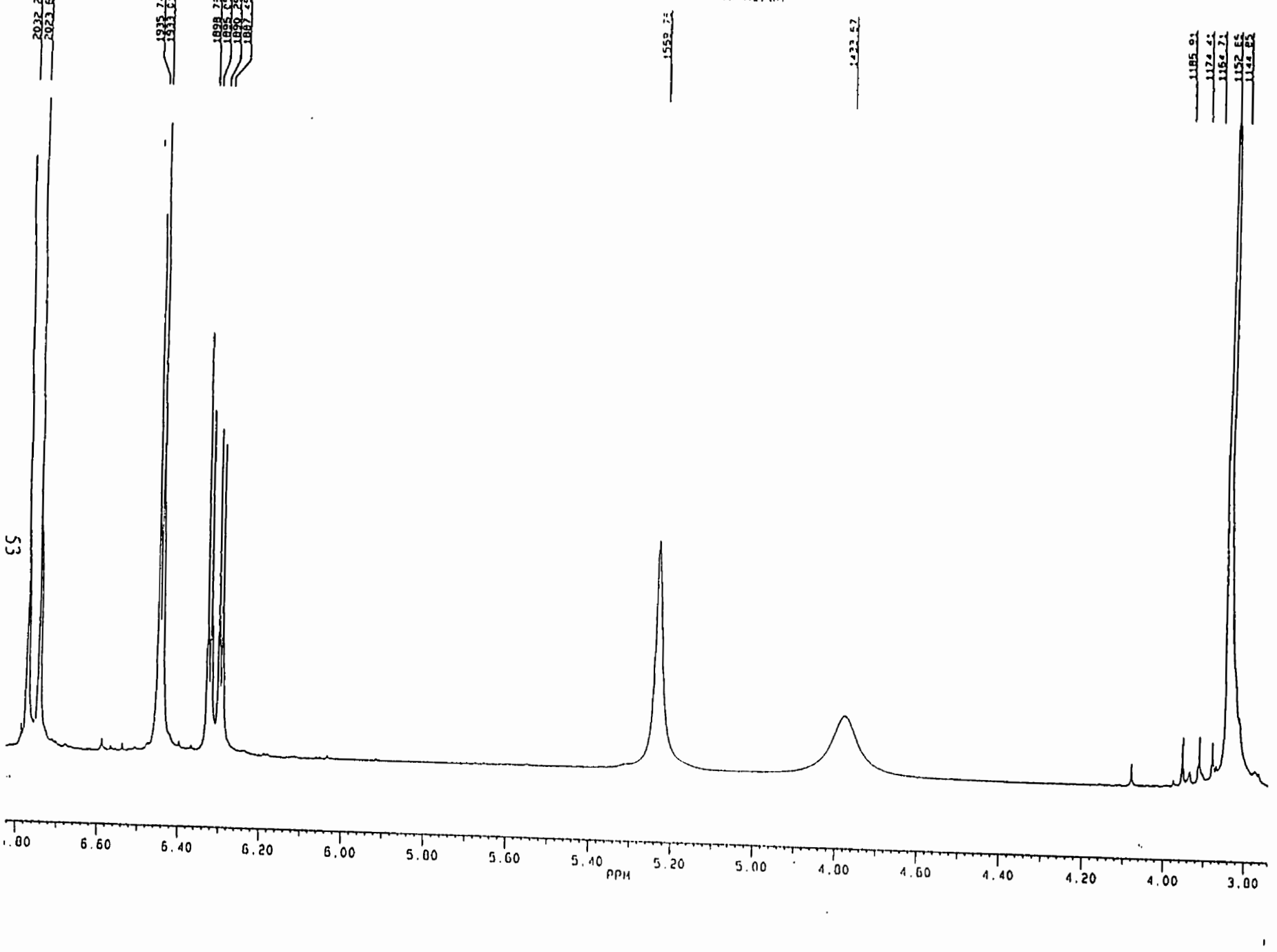


Figure M-2(b): ^{1}H nmr spectrum of 2-methoxy-1,4-di-hydroxybenzene in $CDCl_3$.
Expansion of region 3.80 ppm to 6.60 ppm.

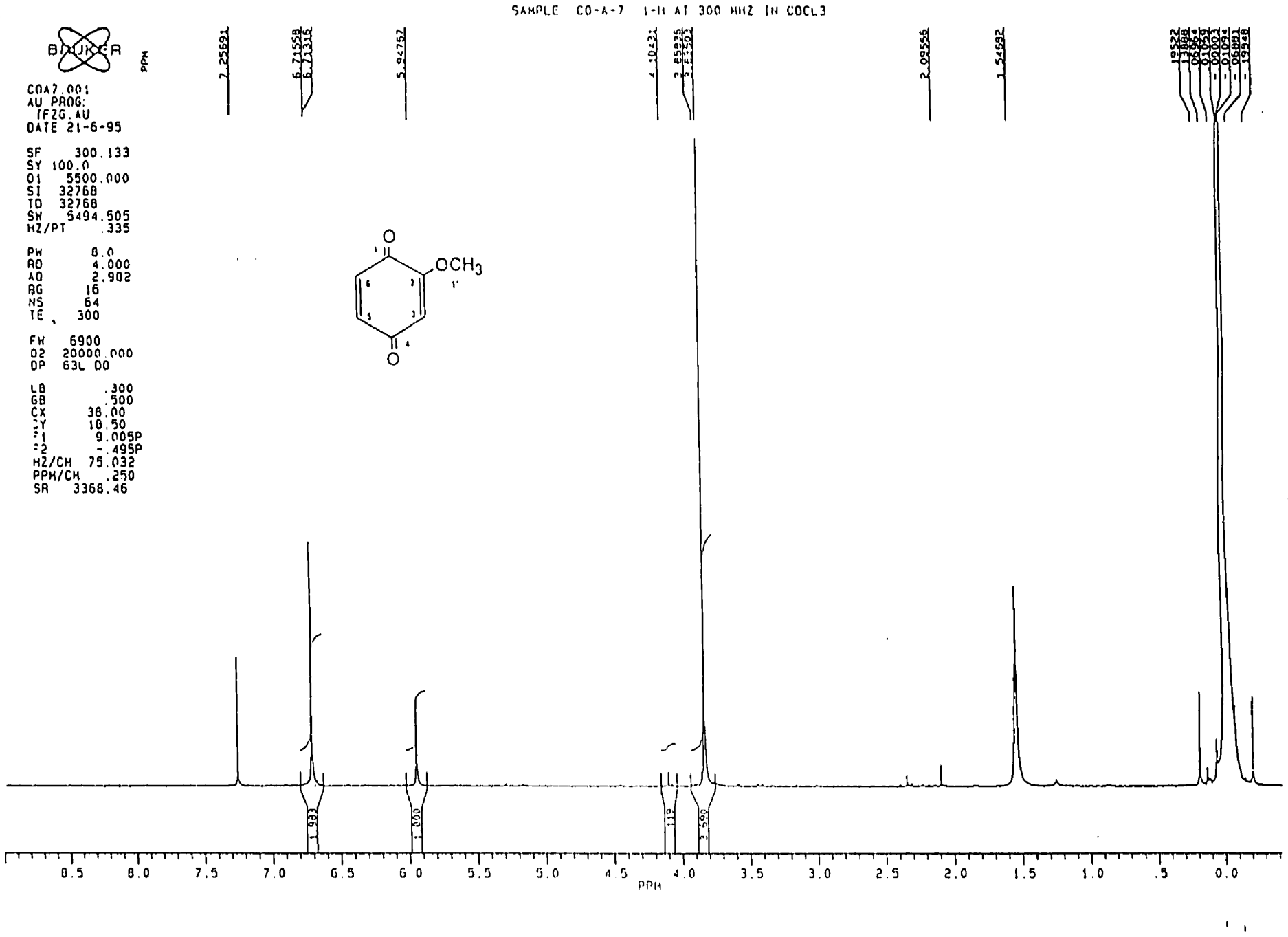


Figure M-3: ^{1}H nmr spectrum of 2-methoxy-1,4-benzoquinone in $CDCl_3$.

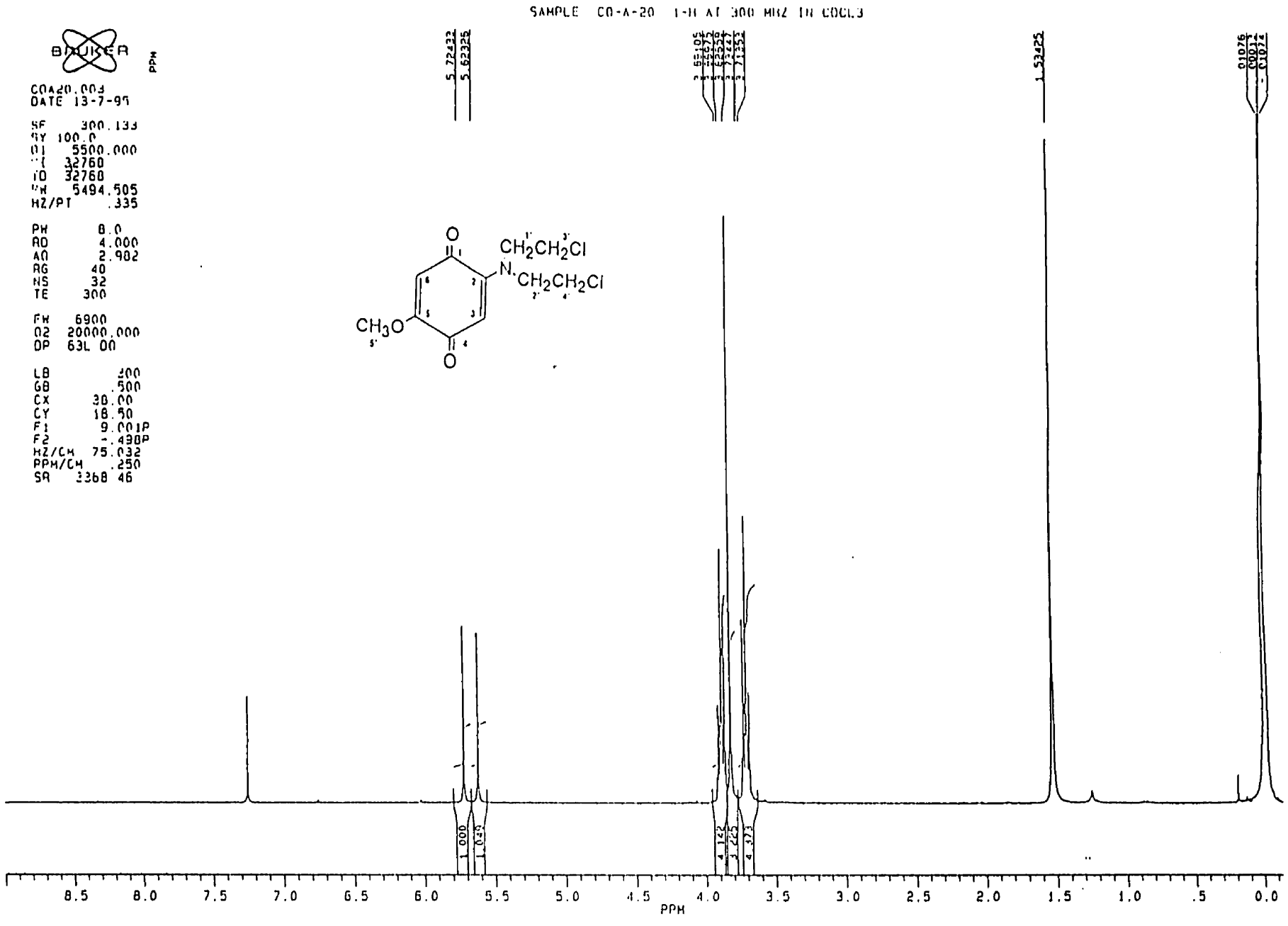


Figure M-4(a): ^{1}H nmr spectrum of MBM in $CDCl_3$.

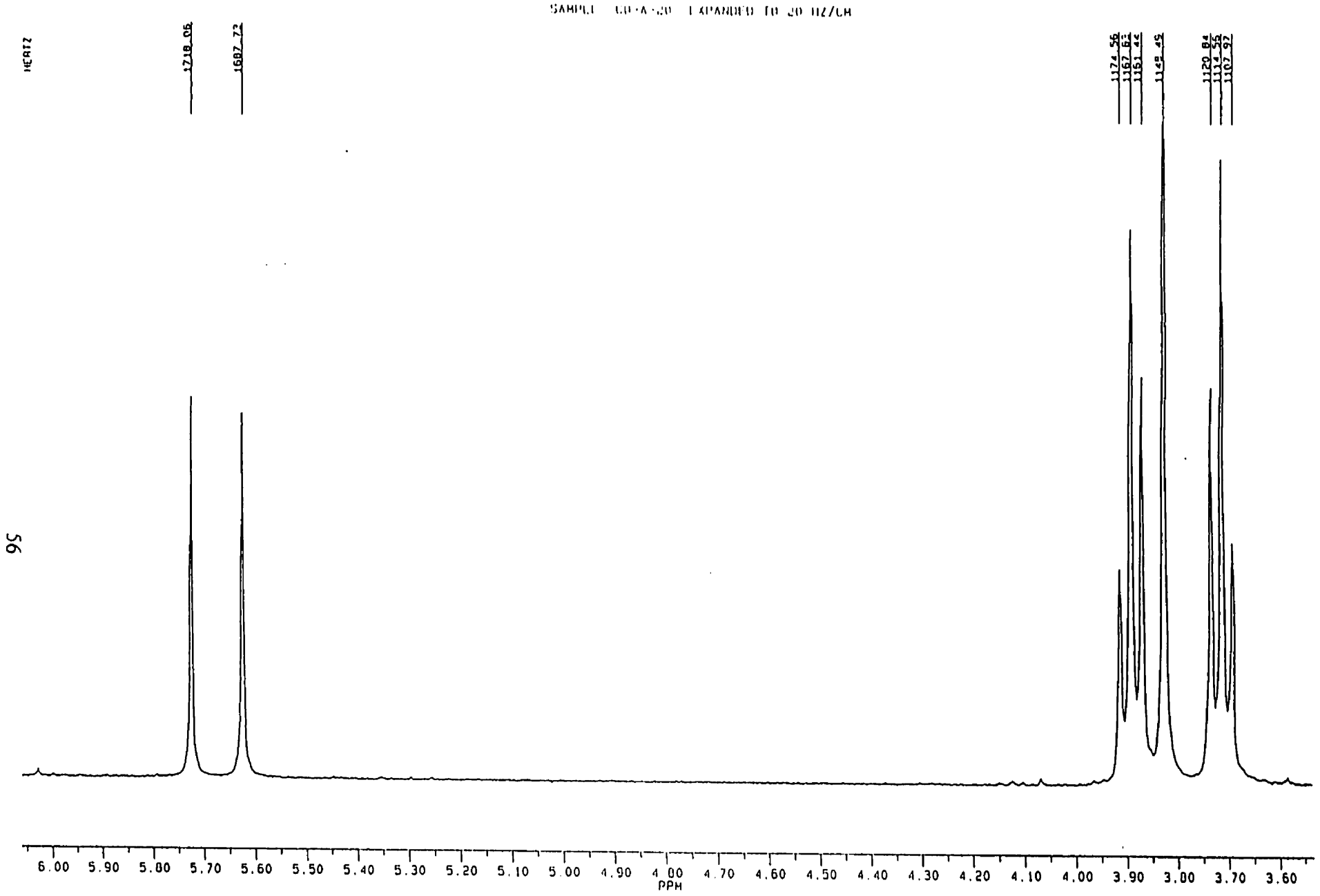


Figure M-4(b): ^1H nmr spectrum of MBM in $CDCl_3$. Expansion of region 3.60 ppm to 6.00 ppm.

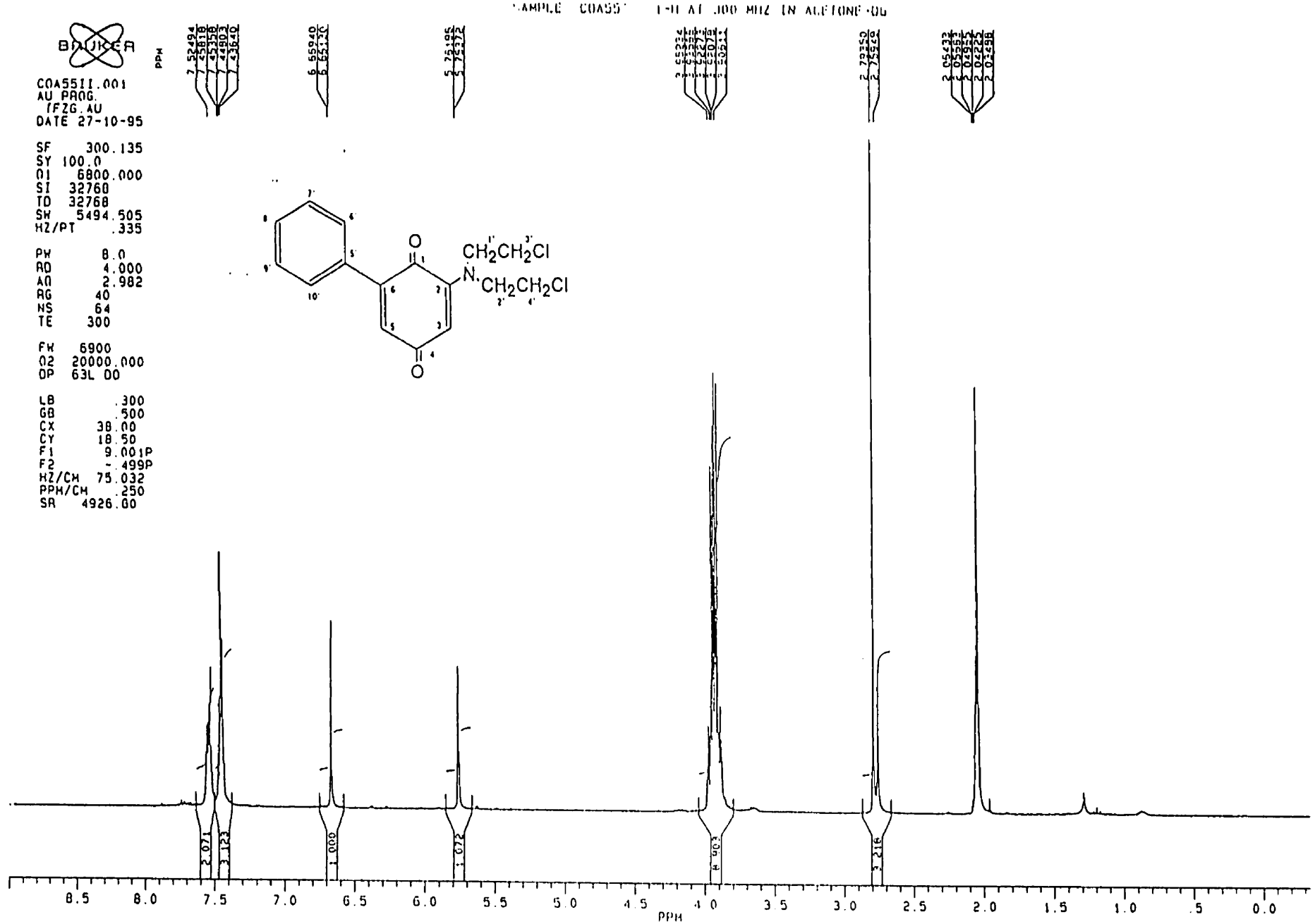


Figure M-5(a): ^1H nmr spectrum of PBM in D_6 acetone.

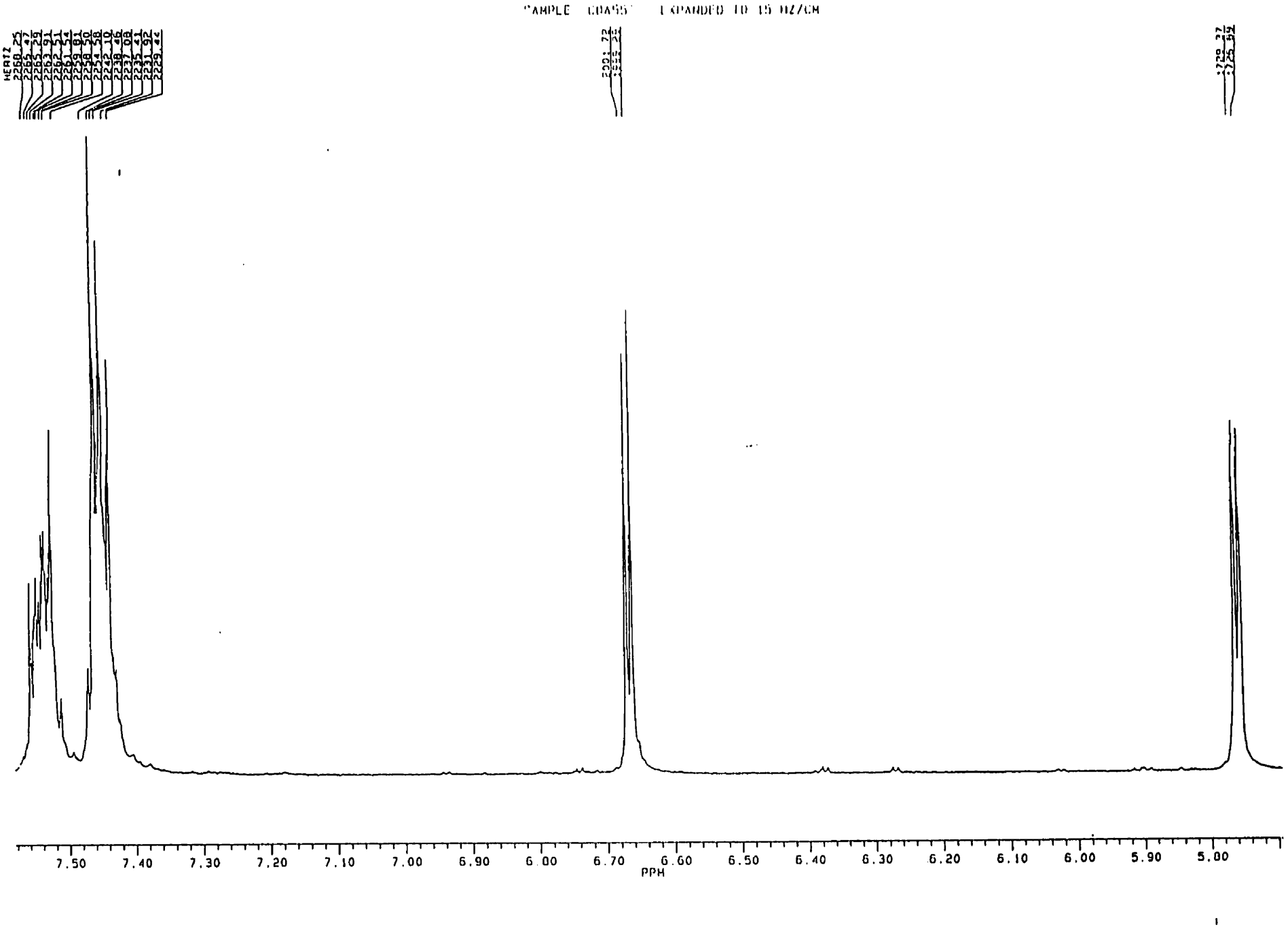


Figure M-5(b): ^1H nmr spectrum of PBM in D_6 acetone. Expansion of region 5.70 ppm to 7.60 ppm.

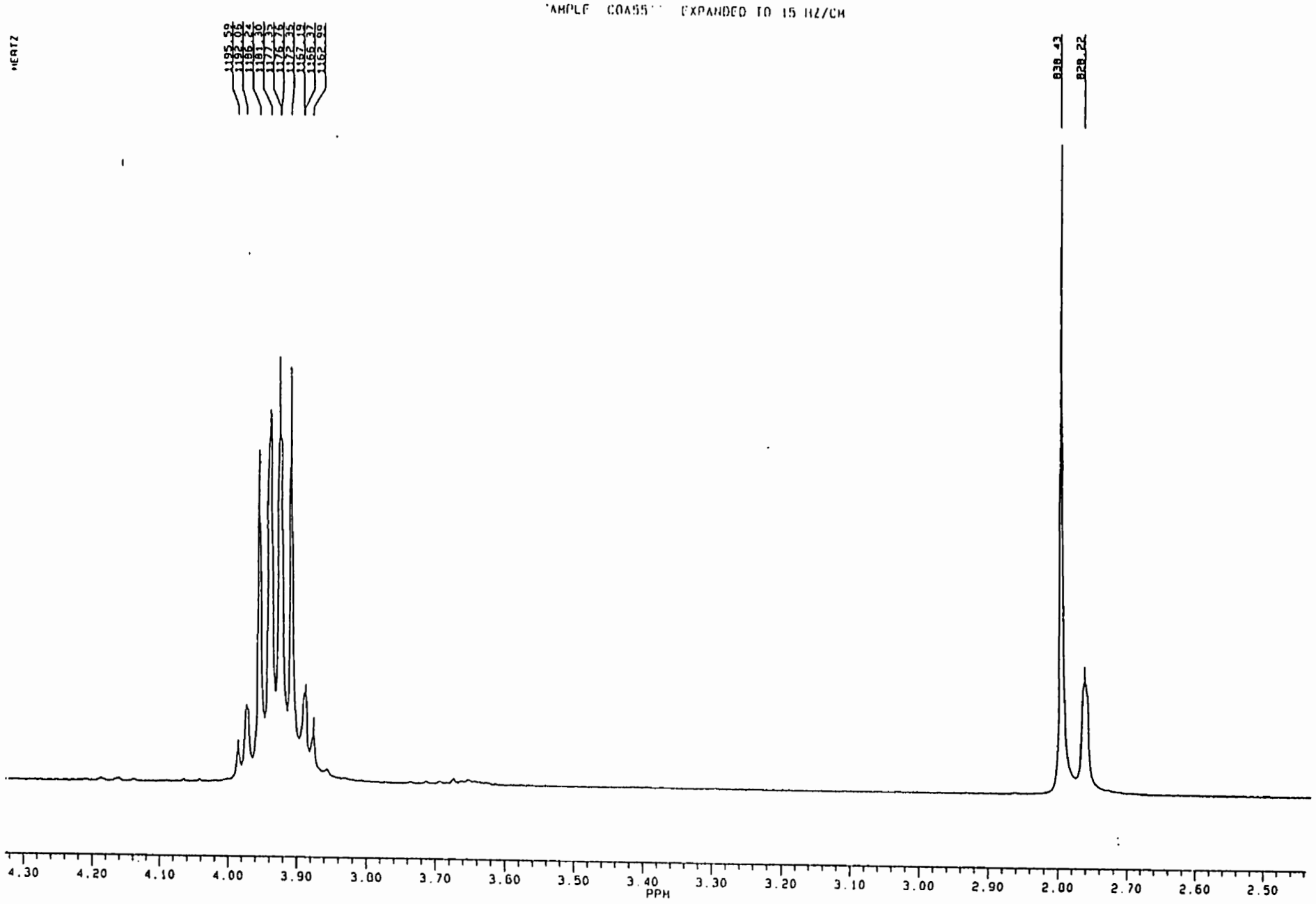


Figure M-5(c): ^{1}H nmr spectrum of PBM in D_6 acetone. Expansion of region 2.50 ppm to 4.30 ppm.

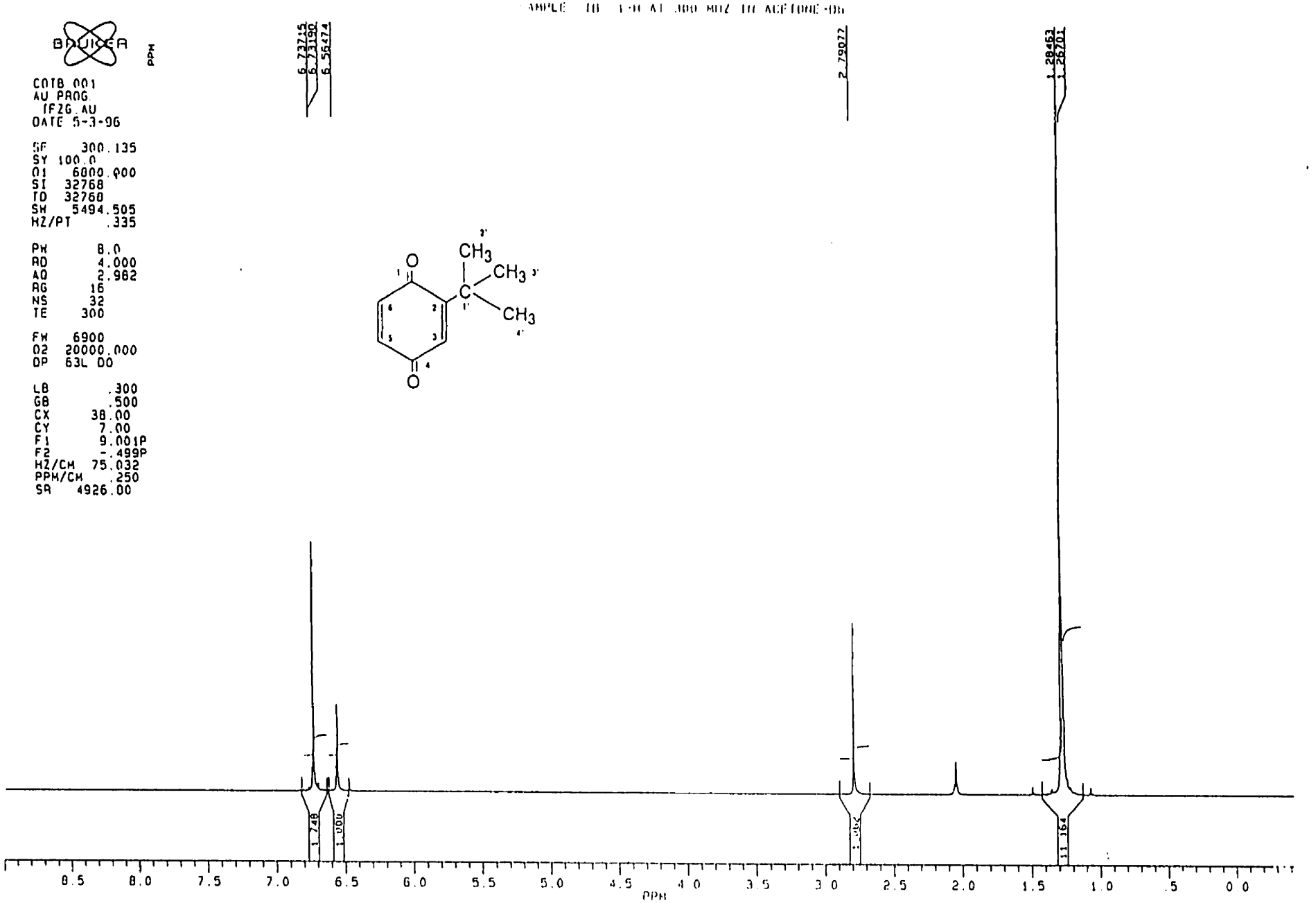


Figure M-6(a): ^{1}H nmr spectrum of 2-*t*-butyl-1,4-benzoquinone in D_6 acetone.

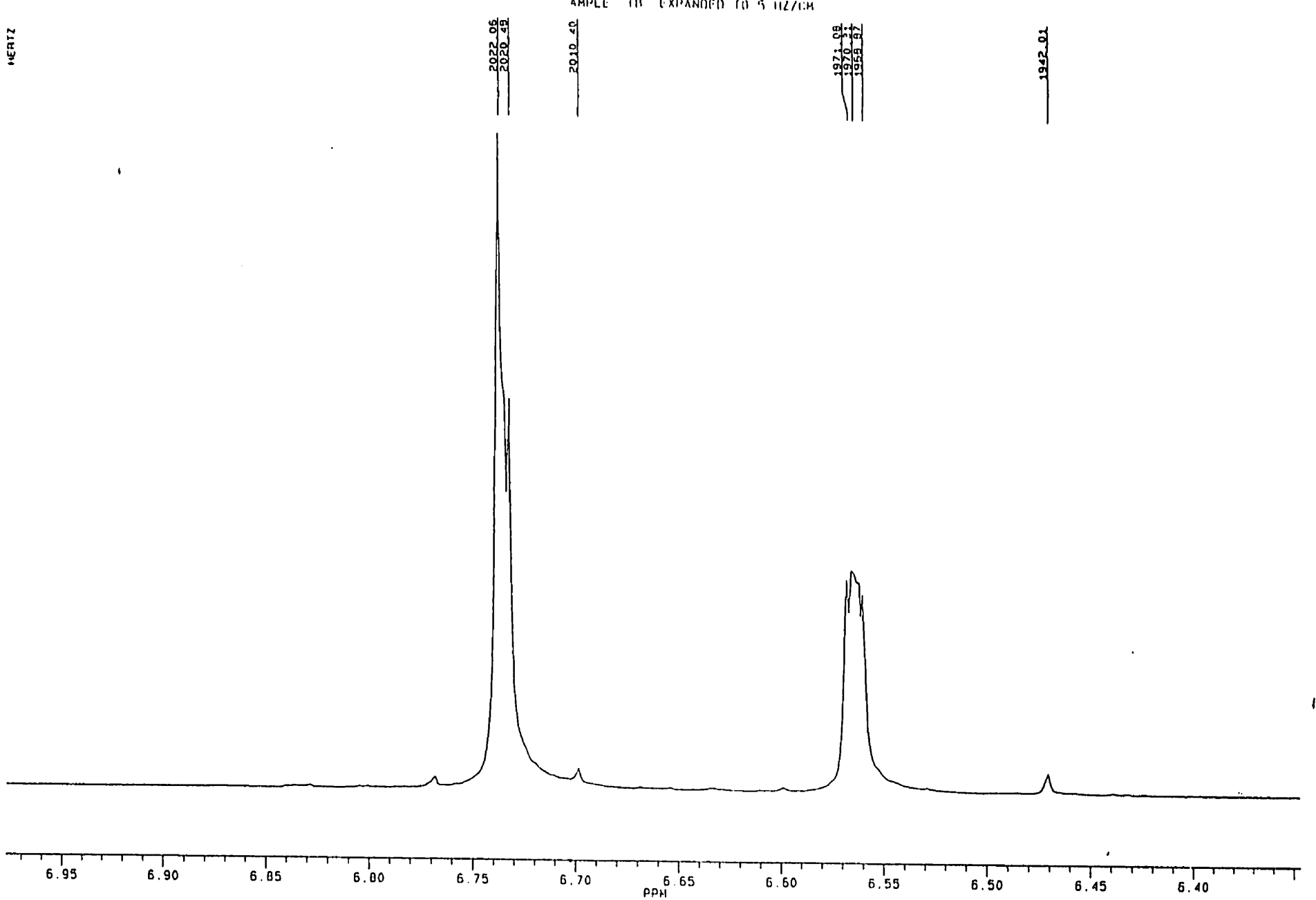


Figure M-6(b): ^{1}H nmr spectrum of 2-*t*-butyl-1,4-benzoquinone in D_6 acetone. Expansion of region 6.40 ppm to 6.95 ppm.

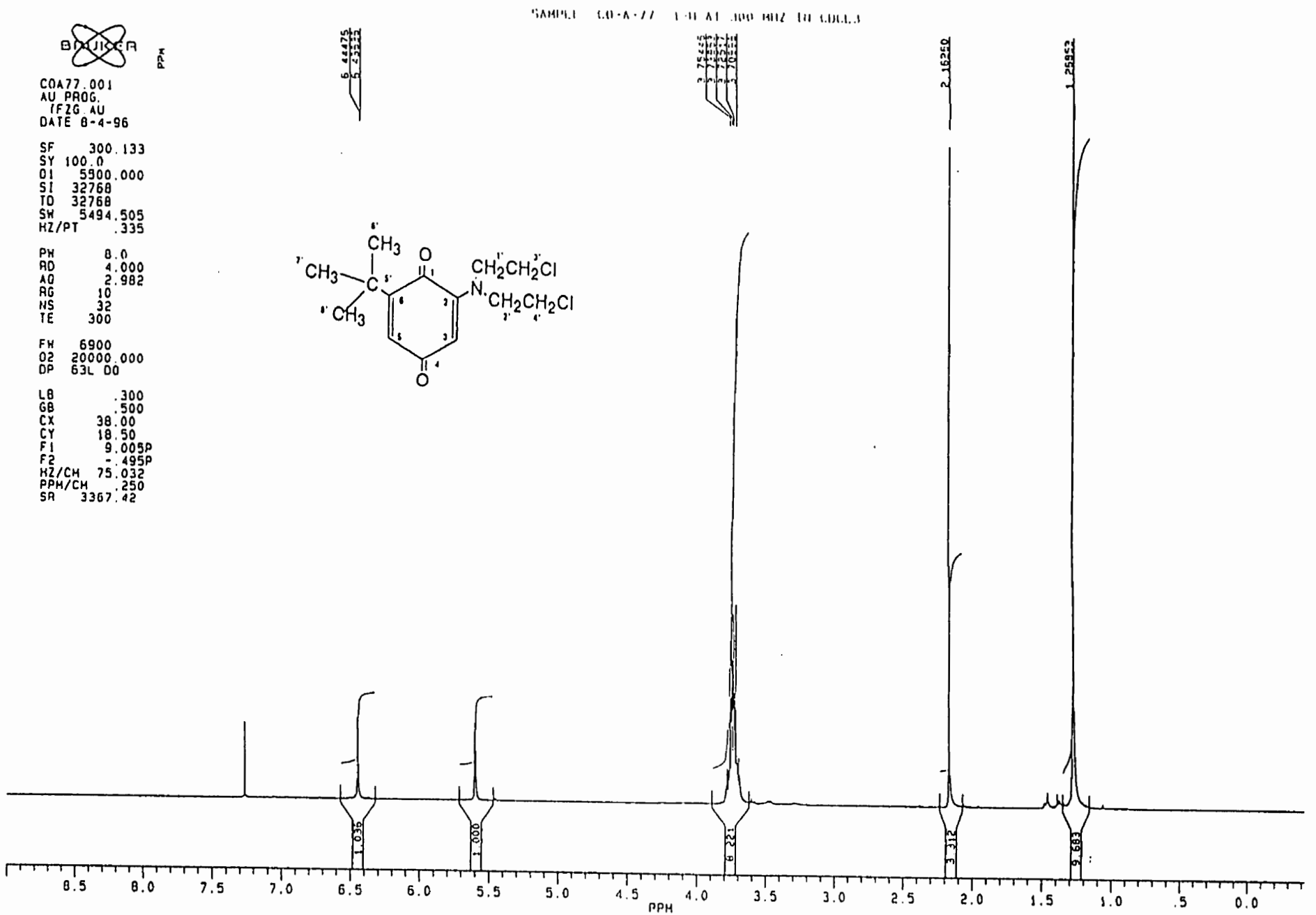


Figure M-7(a): ^1H nmr spectrum of TBM in $CDCl_3$.

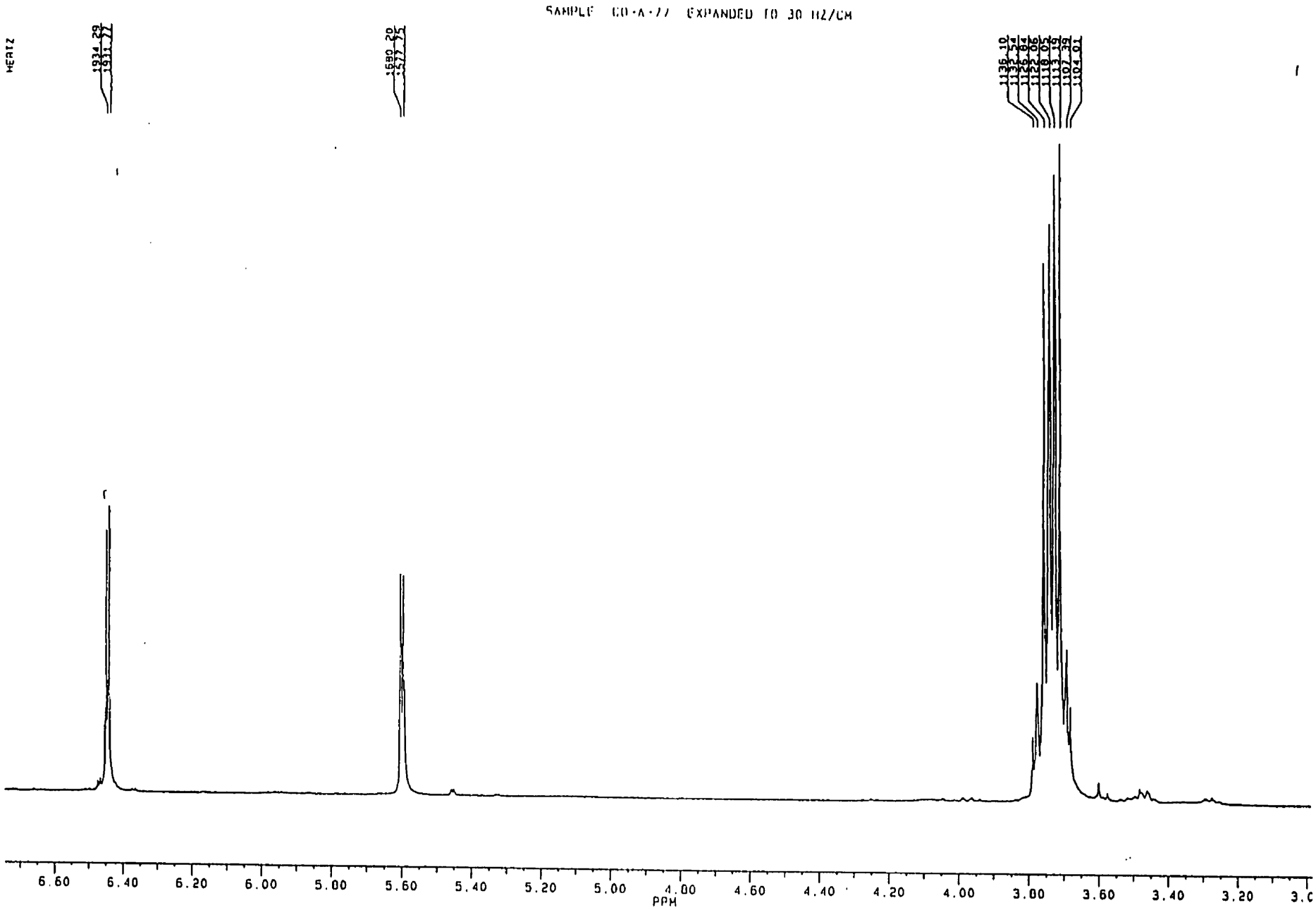


Figure M-7(b): ^{1}H nmr spectrum of TBM in $CDCl_3$. Expansion of region 3.00 ppm to 6.60 ppm.

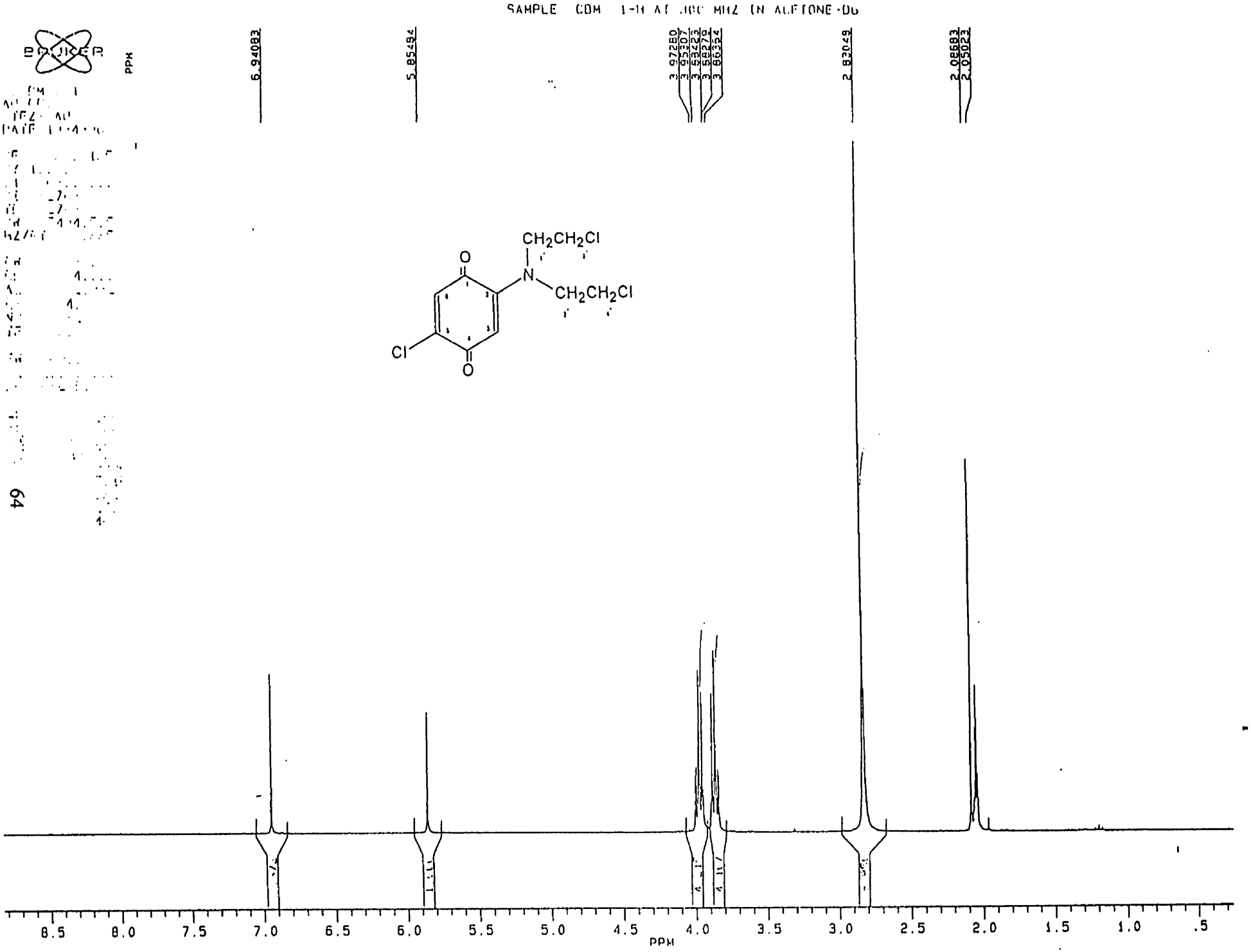


Figure M-8(a): ^{1}H nmr spectrum of CBM in D_6 acetone.

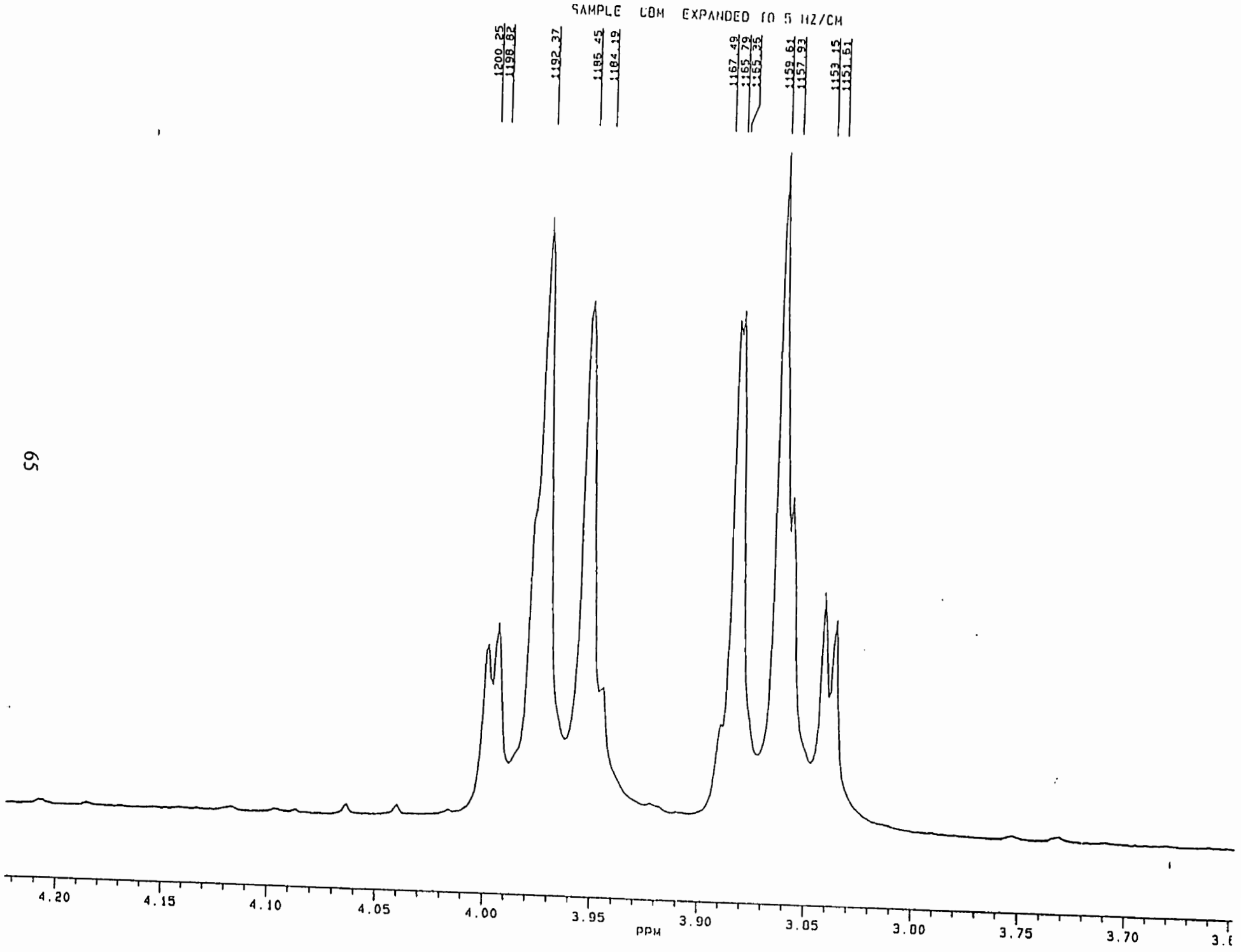


Figure M-8(b): ^1H nmr spectrum of CBM in D_6 acetone. Expansion of region 3.65 ppm to 4.20 ppm.

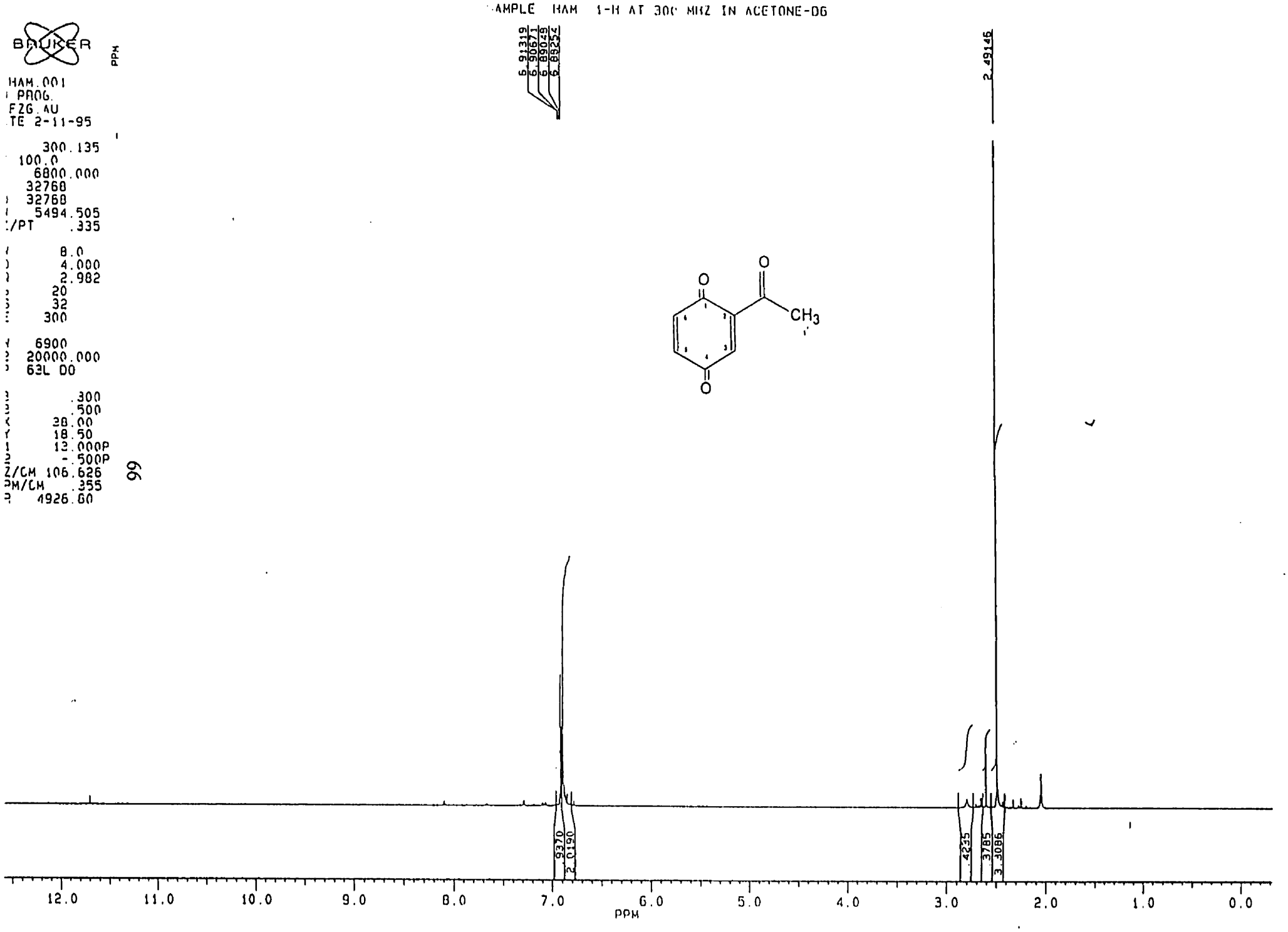


Figure M-9(a): ^{1}H nmr spectrum of 2-acetyl-1,4-benzoquinone in D, acetone.

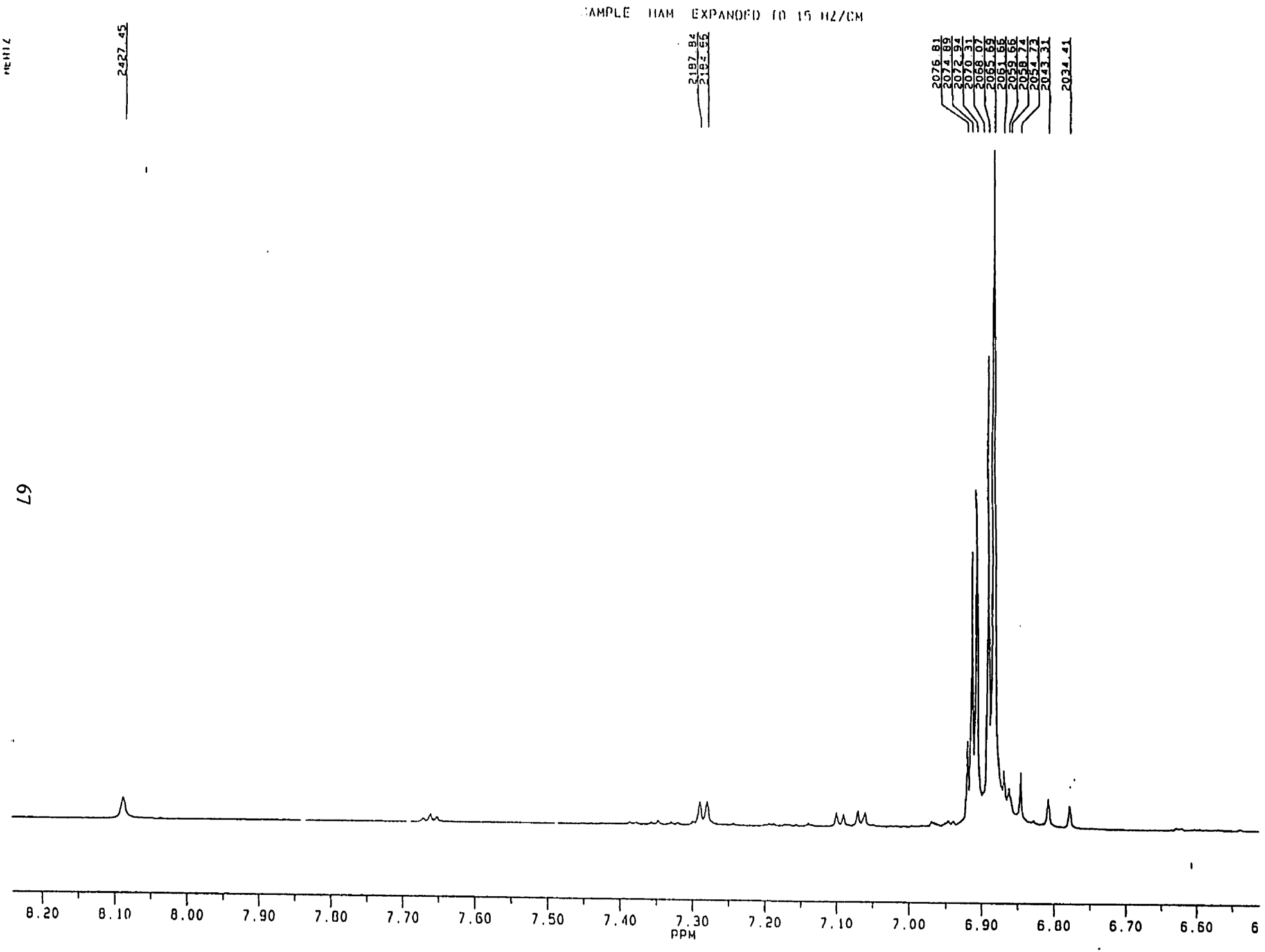


Figure M-9(b): ^{1}H nmr spectrum of 2-acetyl-1,4-benzoquinone in D_6 acetone. Expansion of region 6.40 to 8.20 ppm.

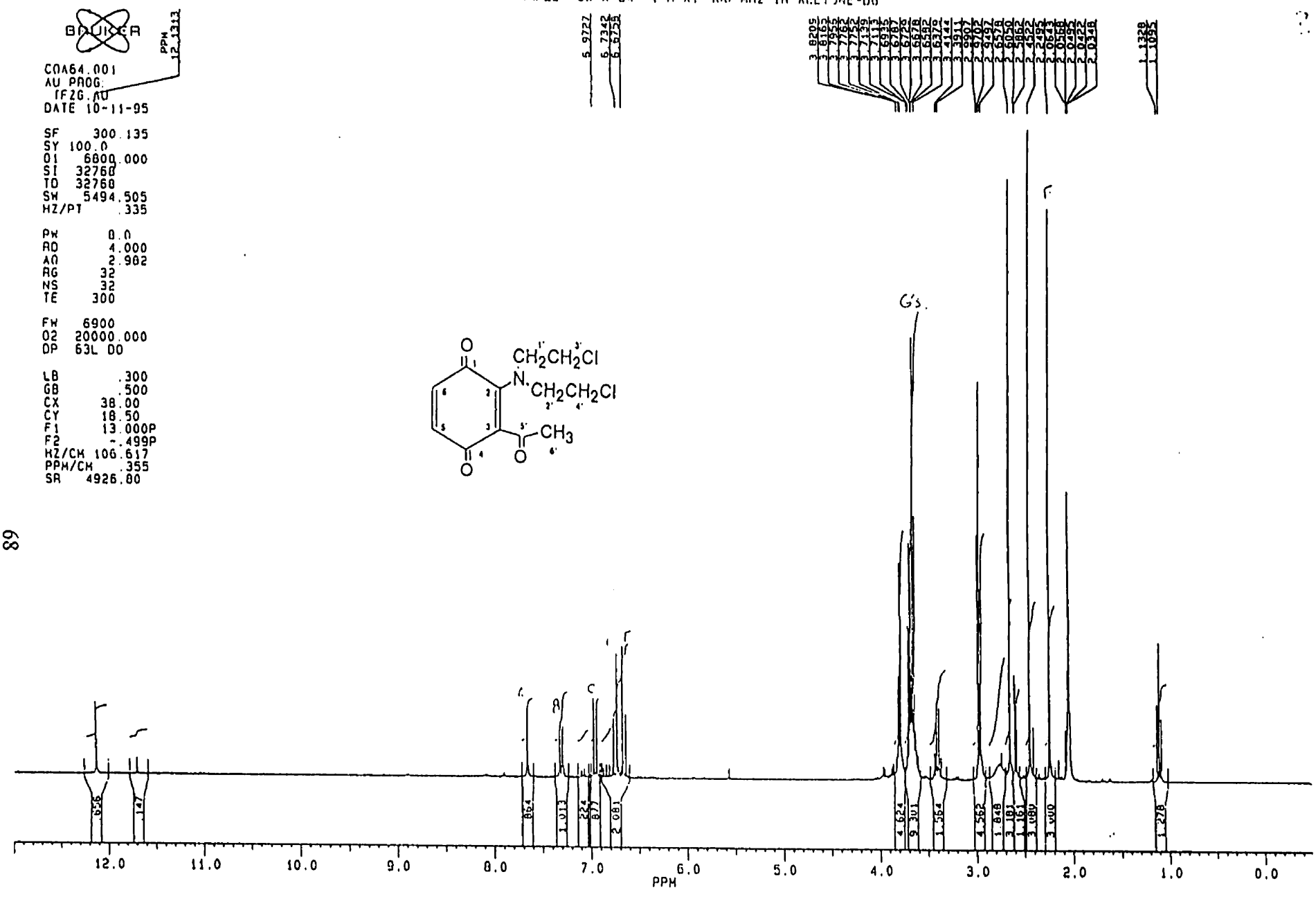


Figure M-10(a): ^{1}H nmr spectrum of ABM reaction mixture in D_6 acetone.

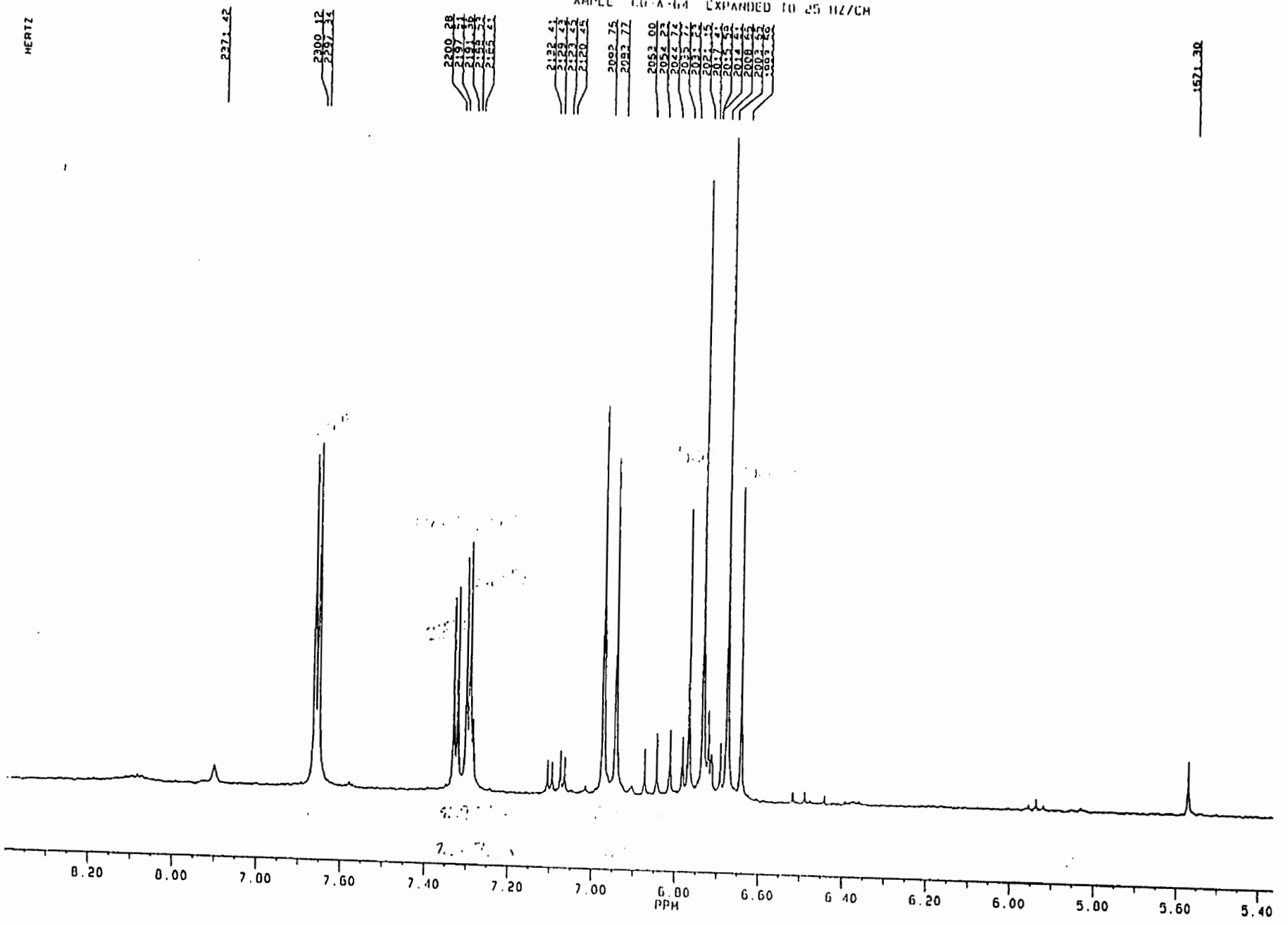


Figure M-10(b): ^{1}H nmr spectrum of ABM reaction mixture in D_6 acetone. Expansion of region 5.40 ppm to 8.20 ppm.

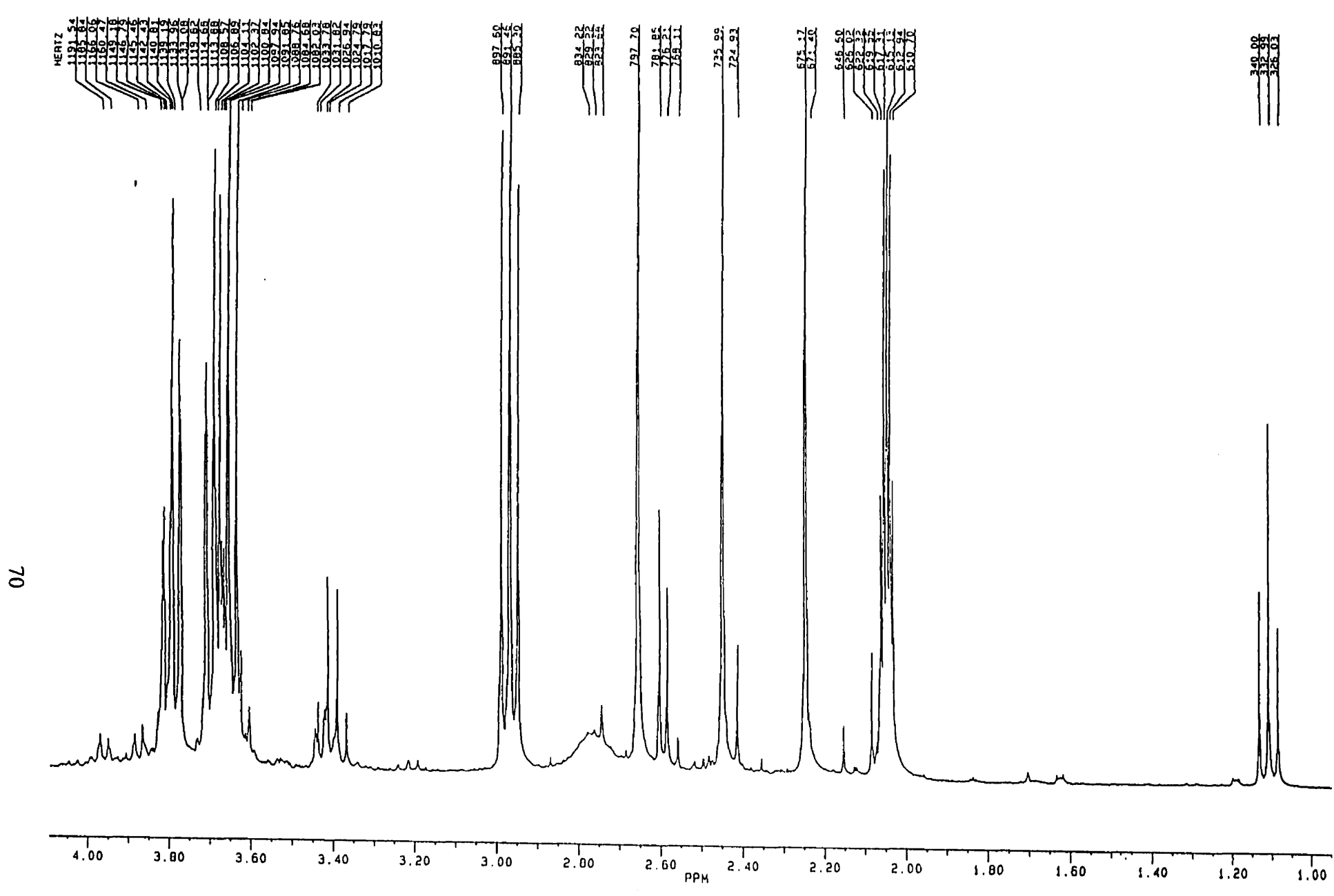


Figure M-10(c): ^{1}H nmr spectrum of ABM reaction mixture in D_6 acetone. Expansion of region 1.00 ppm to 4.00 ppm.

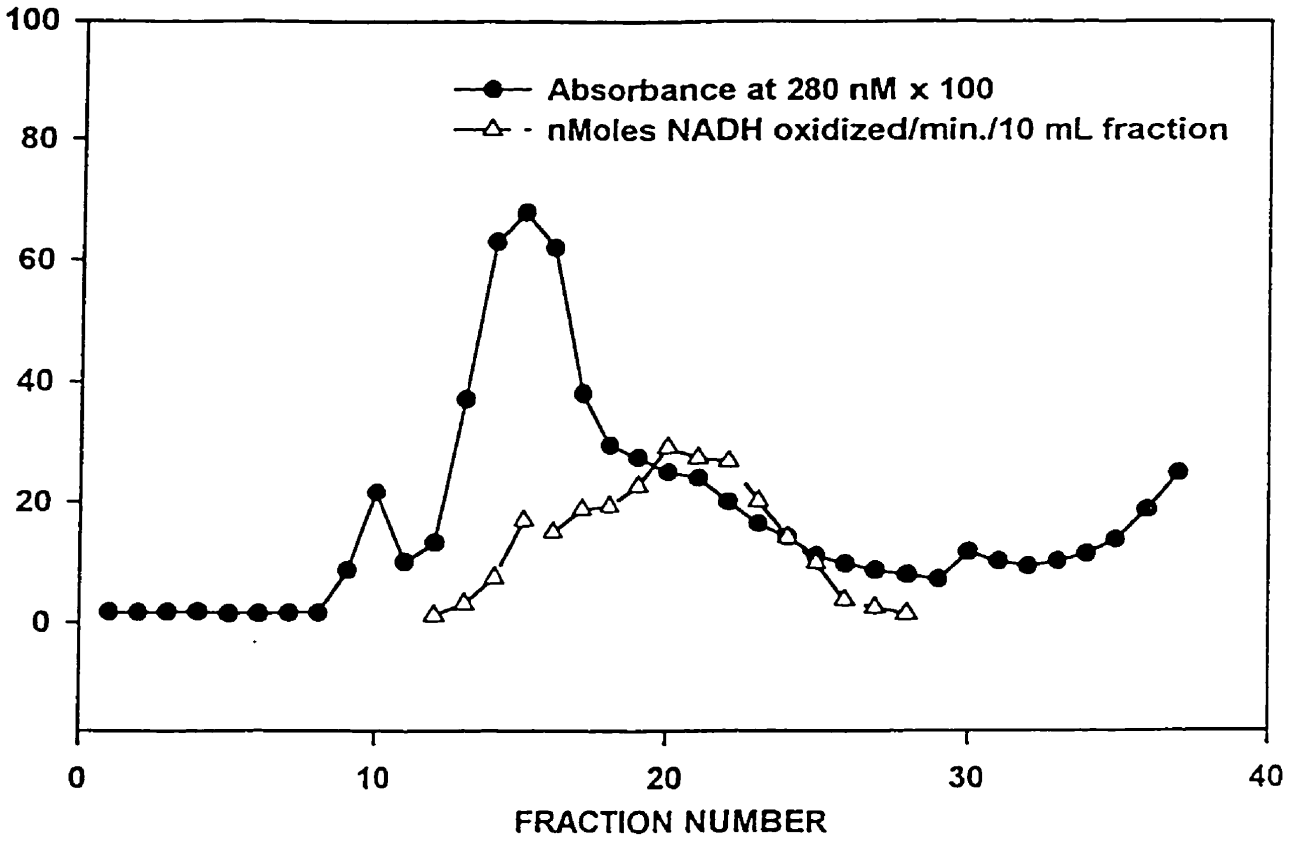


Figure M-11: Elution profile for Sephadex G-100 size exclusion column. An impure mixture of various nucleotide binding proteins were applied to an 80 mL DEAE Sephadex G-100 column. Fractions were eluted using 50 mM Tris HCl (pH 7.4). Fractions were assayed for protein content by measuring absorbency at 280 nm, and DT-diaphorase activity using the method described by Prochaska and Santamaria (1988).

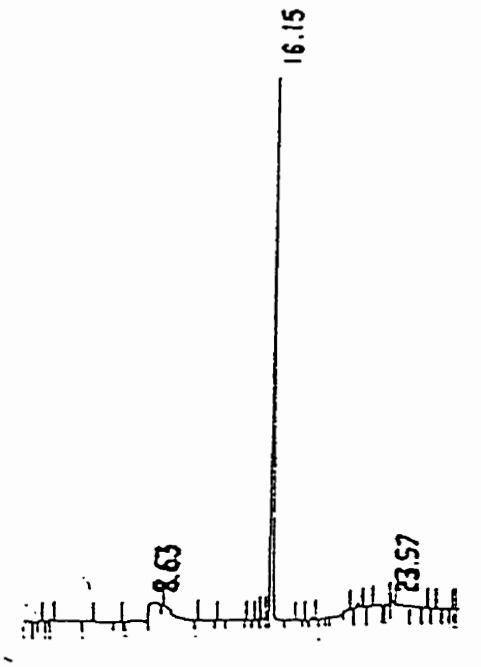


Figure M-12: Chromatogram of 100 μM of NADH in 25 mM Tris HCl (pH 7.4).

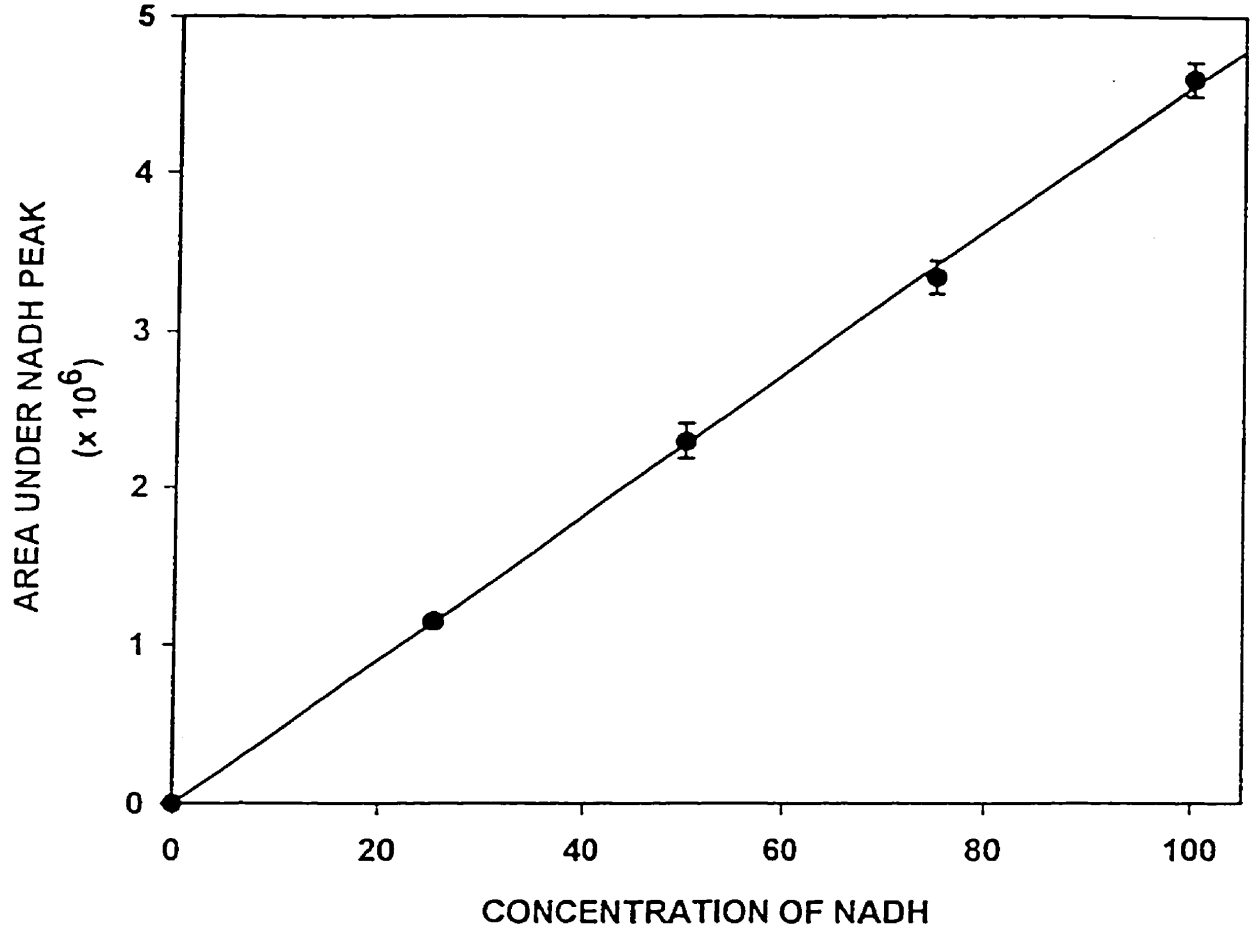


Figure M-13: Calibration curve for HPLC analysis of NADH concentration. NADH was prepared at each of 25, 50, 75 and 100 μM in 50 mM Tris HCl (pH 7.4). Each concentration of NADH was analyzed by HPLC, as described in materials and methods to give the corresponding area under the NADH peak. Points, means of 3 determinations; bars; standard error.

Results

Synthesis of the BM Analogs

Analogs of 2-(di(chloroethyl)amino)-1,4-benzoquinone, BM were chosen to study the effect of functional groups on a quinone ring of a bioreductive agent. Initially, three analogs were chosen, each to illustrate a specific property of a functional group. They were: 5-methoxy-2-(di(chloroethyl)amino)-1,4-benzoquinone, MBM, 6-*t*-butyl-2-(di(chloroethyl)amino)-1,4-benzoquinone, TBM and 3-acetyl-2-(di(chloroethyl)amino)-1,4-benzoquinone, ABM. MBM was chosen to illustrate the effect of a methoxy group, an electron releasing group, TBM was chosen to illustrate the effect of a *t*-butyl group, a steric bulky group, and ABM was chosen to illustrate the effect of an acetyl group, an electron withdrawing group, on the specificity for activation by DT-diaphorase or NADPH:cytochrome P-450 reductase. BM was used as a control in these studies.

Reaction conditions for preparation of each of the BM analogs chosen for study are summarized in Figure R-1. BM was synthesized using the method described by Makarov et al. (1967). MBM was successfully and reproducibly synthesized in sufficient quantity for further study by using a combination of the method described by Crosby et al. (1956) for oxidative amination of 1,4-benzoquinones and Makarova et al. (1973) for synthesis of BM. This method involved use of cupric acetate as an oxidant and the use of 95% ethanol in place of water as the solvent. Preparation of TBM by using similar reaction conditions as used for synthesis of MBM yielded approximately a 1:1:2 mixture of 3-*t*-butyl-2-

(di(chloroethyl)amino)-1,4-benzoquinone, 5-*t*-butyl-2-(di(chloroethyl)amino)-1,4-benzoquinone and 6-*t*-butyl-2-(di(chloroethyl)amino)-1,4-benzoquinone, respectively. 6-*t*-Butyl-2-(di(chloroethyl)amino)-1,4-benzoquinone, TBM (0.013 grams) was purified from the isomeric mixture using silica gel flash-chromatography repeated three times. This quantity of TBM was insufficient for further analysis, and subsequent attempts to prepare this compound were unsuccessful.

As an alternate compound, 6-phenyl-2-(di(chloroethyl)amino)-1,4-benzoquinone, PBM, was chosen for study. PBM contains a phenyl group substituted meta to the di(chloroethyl)amino group on the quinone ring, and may illustrate the effect of a steric bulky group on specificity for activation by DT-diaphorase or NADPH:cytochrome P-450 reductase. Preparation of PBM using similar reaction conditions as used for the synthesis of MBM yielded approximately a 1:1:2 mixture 3-phenyl-2-(di(chloroethyl)amino)-1,4-benzoquinone, 5-phenyl-2-(di(chloroethyl)amino)-1,4-benzoquinone and 6-phenyl-2-(di(chloroethyl)amino)-1,4-benzoquinone (PBM), respectively. PBM was purified from the isomeric mixture using silica gel flash-chromatography twice followed by re-crystallization in 95% ethanol. This method was reproducible and allowed the preparation of PBM in sufficient quantity for further analysis.

Attempts to synthesize ABM using the reaction conditions used for the synthesis of MBM were unsuccessful. Using completely anhydrous conditions and silver oxide in place of cupric acetate, ABM was prepared as an impure mixture. However, ABM readily decomposed and could not be purified for further study. As an alternate compound, 5-chloro-2-(di(chloroethyl)amino)-1,4-benzoquinone, CBM, was chosen for study. CBM

contains a chloro group substituted para to the di(chloroethyl)amino group on the quinone ring, and may illustrate the effect of an electron withdrawing group on specificity for activation by DT-diaphorase or NADPH:cytochrome P-450 reductase. CBM was prepared using a method developed by Makarova et al. (1967). This method afforded CBM in sufficient quantity for further study.

Studies into the Effect of Functional Groups on Cytotoxicity

Dependence of cytotoxicity of BM analogs on DT-diaphorase activity in H661 cells

H661, a human non-small cell lung cancer cell line, was chosen to study the dependence of cytotoxicity of the BM analogs on DT-diaphorase activity. H661 cells have a moderate level of DT-diaphorase activity, 112.7±12.4 nmole/min/mg protein (Begleiter et al., 1997). The cytotoxicities of the BM analogs at 2 μM were studied in H661 cells in the presence, and absence, of 100 μM dicoumarol, an inhibitor of DT-diaphorase. This concentration of dicoumarol completely inhibited the cytotoxicity of EO9 (data not shown). The results are illustrated in Figure R-2. The surviving cell fractions, ratios of the number of viable to non-viable cells, for the BM analogs in the absence of dicoumarol were: 0.23 ± 0.03 for BM, 0.50 ± 0.09 for PBM, 0.40 ± 0.06 for CBM and 0.09 ± 0.01 for MBM. The surviving cell fractions for the BM analogs in the presence of dicoumarol were: 0.13 ± 0.03 for BM, 0.31 ± 0.05 for PBM, 0.30 ± 0.06 for CBM and 0.20 ± 0.01 for MBM. Using $p < 0.05$ as the cut off point for statistical significance, the cytotoxicities of BM and MBM were dependent on the presence of dicoumarol, while the cytotoxicities of CBM and PBM were

not significantly altered by the presence of dicoumarol. Additionally, the cytotoxicity of MBM decreased in the presence of dicoumarol, while the cytotoxicity of BM increased. Thus, in H661 cells, BM and MBM may be dependent on and CBM and PBM may be independent of DT-diaphorase activity. Additionally, BM may be activated by and MBM may be inactivated by DT-diaphorase.

The dependence of cytotoxicity of MBM and BM on the DT-diaphorase activity illustrated at 2 μM BM and MBM in the presence, and absence, of dicoumarol was validated by obtaining a full dose-response curve for the cytotoxicity of each of BM and MBM in the presence, and absence, of dicoumarol. The results are illustrated in Figures R-3 and R-4. The D_{10} , the concentration of drug require to kill 90 % of a tumor cell population, for each of BM and MBM in the presence and, absence of, dicoumarol was determined. In the absence of dicoumarol, MBM and BM had D_{10} of 0.10 ± 0.09 μM and 3.4 ● 0.3 μM, respectively. In comparison, in the presence of dicoumarol MBM and BM had D_{10} of 0.38 ± 0.01 μM and 2.4 ± 0.2 μM. Thus, the results from the full concentration-response data were in agreement with the single concentration data.

Dependence of cytotoxicity of BM analogs on DT-diaphorase activity in SK-MEL-28 cells

The cytotoxicities of BM, MBM, CBM and PBM were studied in SK-MEL-28 cells. SK-MEL-28 is a human melanoma cell line with a DT-diaphorase activity of 586.7±19.6 nmole/min/mg protein (Begleiter et al. 1997). The cytotoxicities of BM analogs at 2 μM were studied in SK-MEL-28 cells in the presence, and absence, of 100 μM dicoumarol. The

results obtained are illustrated in Figure R-5. The surviving cell fractions for the BM analogs in the absence of dicoumarol were: 0.5 ± 0.2 for BM, 0.7 ± 0.2 for PBM, 0.68 ± 0.09 for CBM and 0.065 ± 0.006 for MBM. The surviving cell fractions for the BM analogs in the presence of dicoumarol were: 0.24 ± 0.08 for BM, 0.7 ± 0.1 for PBM, 0.28 ± 0.08 for CBM and 0.29 ± 0.09 for MBM. Using $p < 0.05$ as the cut-off point for statistical significance, the cytotoxicities of MBM, CBM and BM were dependent on the presence of dicoumarol, while the cytotoxicity of PBM was independent of the presence of dicoumarol. Additionally, the cytotoxicities of BM and CBM increased, while the cytotoxicity of MBM decreased in the presence of dicoumarol. Thus, in SK-MEL-28 cells, BM, MBM and CBM may be dependent on and PBM may be independent of DT-diaphorase activity. Additionally, BM and CBM may be inactivated by and MBM may be activated by DT-diaphorase.

Dependence of cytotoxicity of BM analogs on NADPH:cytochrome P-450 reductase activity

The dependence of the cytotoxicities of BM analogs on NADPH:cytochrome P-450 reductase activity were studied using H661 cells and SK-MEL-28. The level of NADPH:cytochrome P-450 reductase activity in H661 cells is 17.2 nmoles/min/mg protein and is 5.83 nmoles/min/mg protein in SK-MEL-28 cells. The cytotoxicities of the BM analogs at 2 μM in the presence of 100 μM dicoumarol in SK-MEL-28 and H661 cells were compared. Dicoumarol was used to minimize complications associated with the difference in DT-diaphorase levels in H661 and SK-MEL-28 cells . The comparison is illustrated in Figure R-6. In the H661 the surviving cell fractions for the BM analogs in the presence of

dicoumarol were: 0.13 ● 0.04 for BM, 0.31 ± 0.05 for PBM, 0.30 ± 0.06 for CBM and 0.20 ± 0.01 for MBM In the SK-MEL-28 the surviving cell fractions for the BM analogs in the presence of dicoumarol were: 0.24 ± 0.08 for BM, 0.7 ± 0.3 for PBM, 0.28 ± 0.08 for CBM and 0.29 ± 0.08 for MBM. Using $p < 0.05$ as the cut off point for statistical significance, PBM had a greater cytotoxicity in H661 than SK-MEL-28 cells while the cytotoxicities of BM, CBM and MBM were similar in SK-MEL-28 and H661 cells. Thus, PBM may be dependent on and BM, CBM and MBM may be independent of NADPH:cytochrome P-450 reductase activity. Additionally, PBM may be activated by NADPH:cytochrome P-450 reductase.

Purification of DT-Diaphorase

DT-diaphorase was purified from HT-29, a human colon cancer cell line. Purification of DT-diaphorase from HT-29 cells was initially carried out, as described by Sharkis et al. (1989). Using this procedure, DT-diaphorase was purified to approximately 70%, as determined by SDS PAGE of 13 μg of protein stained using Coomasie Blue™ (see Figure R-7). This was considered inadequate, and DT-diaphorase was further purified using a DEAE Sephadex column at a pH of 8.0, as suggested by Dr. Su Shu Pan, University of Maryland Cancer Center, Baltimore, MD, USA (private communication). Using this procedure, DT-diaphorase was purified to greater than 98% purity with a dicoumarol inhibitable specific activity of 38,000 nmole MTT reduced/min/mg protein. This corresponded to a 76-fold increase in specific activity. Purity was estimated by SDS PAGE

of 1, 2, 4 and 8 μg of protein stained using Silver stain™ (see Figure R-8).

Redox Potentials of BM Analogs

The redox potentials of BM analogs were determined using cyclic voltammetry. The resulting voltammograms are illustrated in Figures R-9 to R-13. Each voltammogram represents a scan for the current generated by applying various voltages to a solution of the BM analog. Peaks in the voltammogram represent points of maximal current output, which correspond to a redox potential of an analyte in the solution. Thus, peaks correspond to first and second redox potentials of BM analogs.

The first and second redox potentials for each of the BM analogs is summarized in Table 1. CBM, PBM and TBM had similar first redox potentials. The first redox potential of MBM was less than BM, which was less than CBM, PBM and TBM. BM, CBM, PBM and TBM had similar second redox potentials, while MBM had a much less negative second redox potential. The first and second redox potentials are assumed to be the redox potentials of the semiquinone and hydroquinone states, respectively.

Reduction of MBM and BM by DT-Diaphorase

The rate at which DT-diaphorase reduces BM and MBM was studied by measuring the rate of consumption of NADH. NADH is a co-factor for the reduction of substrates by DT-diaphorase. The rate of consumption of NADH was quantified by HPLC, as described

by Gibson et al. (1992).

The level of consumption of NADH was determined at 0, 1, 3, 5, 10 and 20 minutes for BM, and 0, 5, 10, 20, 30, 50, 60 and 120 minutes for MBM. Reactions were carried out under aerobic and hypoxic conditions with a 2:1 ratio of NADH to BM or MBM.

The results obtained for reduction of MBM are illustrated in Figure R-14. The consumption of NADH under aerobic and hypoxic conditions was completed at approximately 60 minutes. At 60 minutes, two equivalents of NADH was consumed under aerobic conditions, while one equivalent of NADH was consumed under hypoxic conditions. Thus, reduction of MBM under aerobic conditions was twice as fast as under hypoxic conditions, and MBM may be able to re-oxidize and continue to be reduced by DT-diaphorase.

The results obtained for reduction of BM are illustrated in Figure R-15. NADH is consumed at a similar rate under aerobic and hypoxic conditions, and the consumption of NADH was completed at approximately 5 minutes under aerobic and hypoxic conditions. Additionally, in contrast to MBM, approximately one equivalent was consumed under both aerobic and hypoxic conditions. Thus, BM was reduced by DT-diaphorase at a similar rate under aerobic and hypoxic conditions, which was faster than the rate at which MBM was reduced by DT-diaphorase. BM does not appear to re-oxidize and continue to be reduced by DT-diaphorase.

Analysis of Cross-Linking Activity of BM and MBM

Various controls were studied to illustrate the effect of the different components of the reaction mixture on the DNA. DNA was treated for 4 hours with each of (a) DT-diaphorase, 100 μM NADH and 5 μM (b) 50 μM BM (c) 50 μM MBM (d) 50 μM BM in the presence of DT-diaphorase (e) 50 μM MBM in the presence of DT-diaphorase (f) 50 μM BM, 100 μM NADH and 5 μM FAD (g) 50 μM MBM, 100 μM NADH and 5 μM FAD. Cross-linking activity was analyzed using an agarose gel method developed by Hartley et al. (1991). The resulting agarose gel is illustrated in Figure R-16. Denatured and non-denatured DNA corresponds to single- and double-stranded DNA, respectively. For each sample, single- and double-stranded DNA correspond to non-cross-linked and cross-linked DNA, respectively. Single stranded bands were present and no double stranded bands were present for all controls.

[α-^{32}P]dATP labelled pBR322 plasmid DNA (10 ng) was treated with one of 0.5, 1, 5 or 10 μM of MBM or BM in the presence of DT-diaphorase, 100 μM NADH and 5 μM FAD under aerobic conditions at 25°C for 2 hours in 25 mM TrisHCl/1 mM EDTA (pH 7.4), and cross-linking activity was analyzed using an agarose gel method developed by Hartley et al. (1991). The resulting gel is illustrated in Figure R-17. Denatured and non-denatured DNA corresponds to single- and double-stranded DNA, respectively. For each sample, single- and double-stranded DNA corresponds to non-cross-linked and cross-linked DNA, respectively. For each sample loaded single- and/or double-stranded DNA bands were not consistently present. Additionally, no dose response of the cross-linking activity was

observed. DNA was present in the location of the loading well for each sample.

A modified procedure of Hartley et al. (1991) was developed in an attempt to eliminate the the inconsistencies associated with the method developed by Hartley et al. (1991). [α-^{32}P]dATP labelled pBR322 plasmid DNA (10 ng) was treated with 25 or 50 μM BM or MBM in the presence of DT-diaphorase, 100 μM NADH and 5 μM FAD for 2 hours. Loading buffer was added to each sample, samples were heat-denatured and directly loaded onto a 1% non-denaturing TBE agarose gel (see Experimental for details). The resulting gel is illustrated in Figure R-18. Results were similar to those obtained using ethanol precipitation.

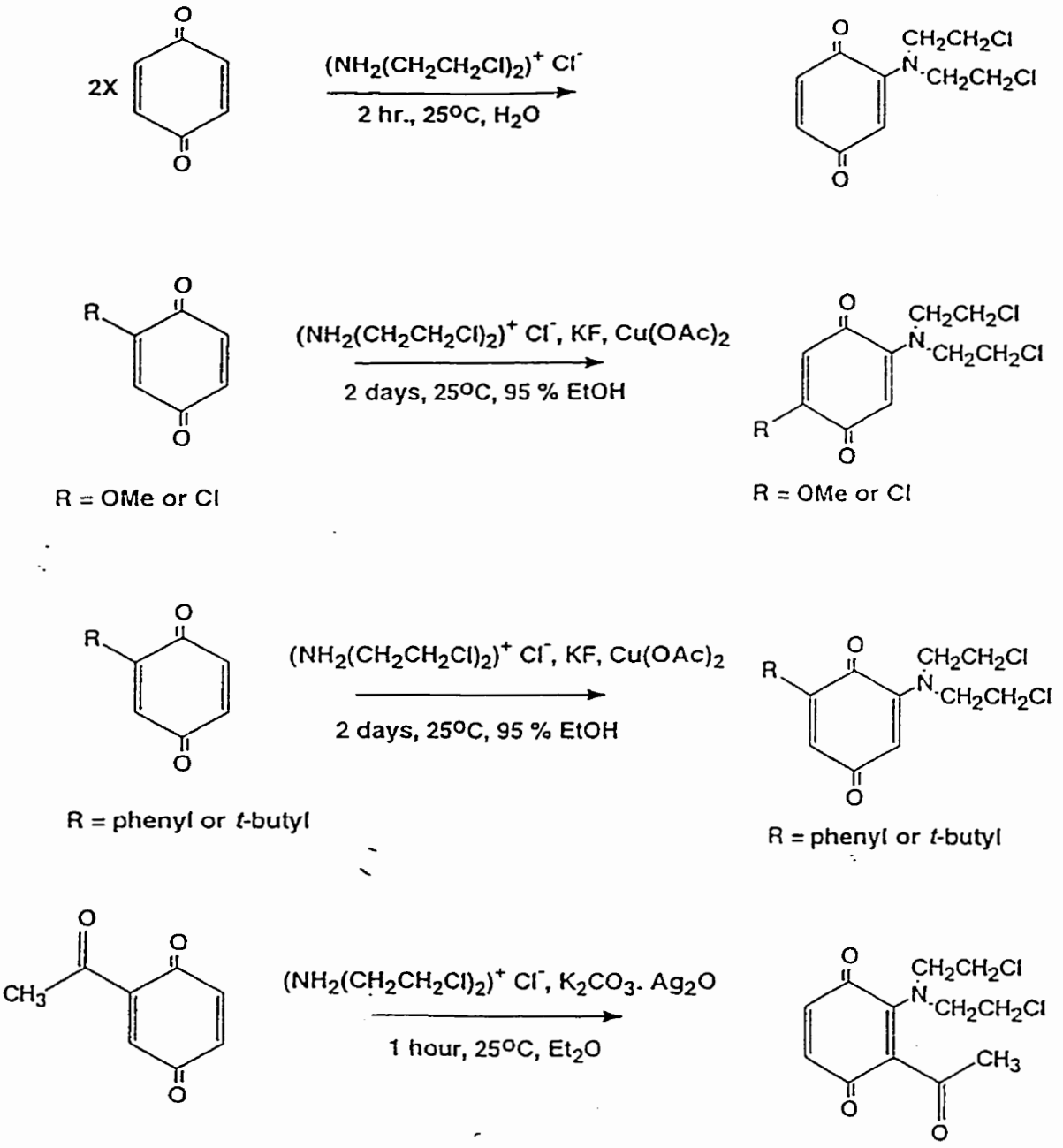


FIGURE R-1: Reaction conditions for the synthesis of the BM analogs.

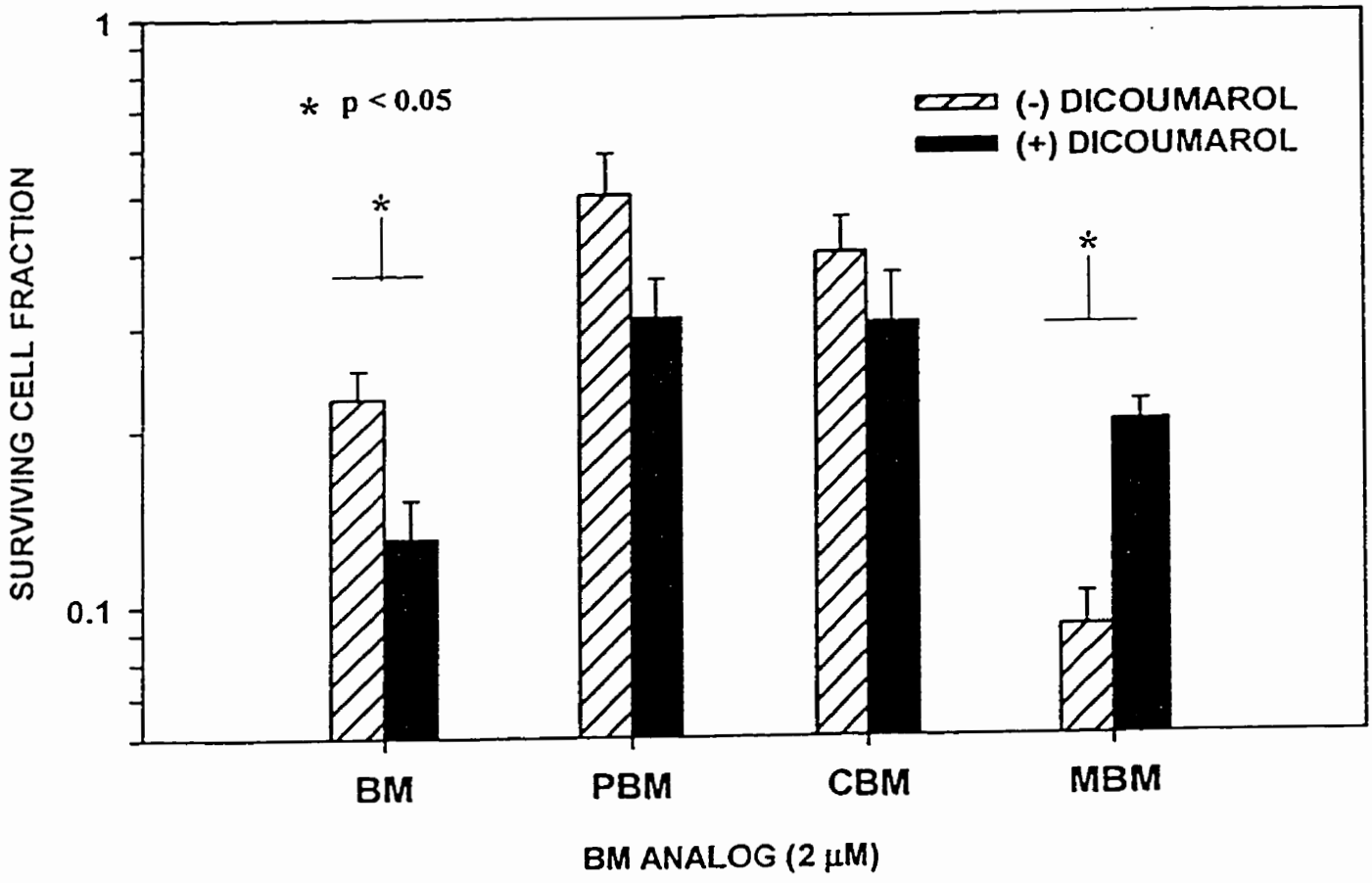


Figure R-2: Cytotoxicity of the BM analogs in H661 human non-small cell lung line in the presence and absence of dicoumarol. Cells were incubated with, or without, 100 μM dicoumarol at 37°C in 5% CO_2 for 20 minutes. Media was removed and cells were incubated with 2 μM of BM analog and with, or without, 100 μM dicoumarol at 37°C and 5% CO_2 for 1 hour. Cells were washed with citrate saline, trypsinized, diluted and incubated at 37°C and 5% CO_2 for 7 days. The surviving cell fraction was determined by MTT assay as described by Johnston et al. (1994). Points, mean of 4 determinations; bars, standard error; *, $p < 0.05$.

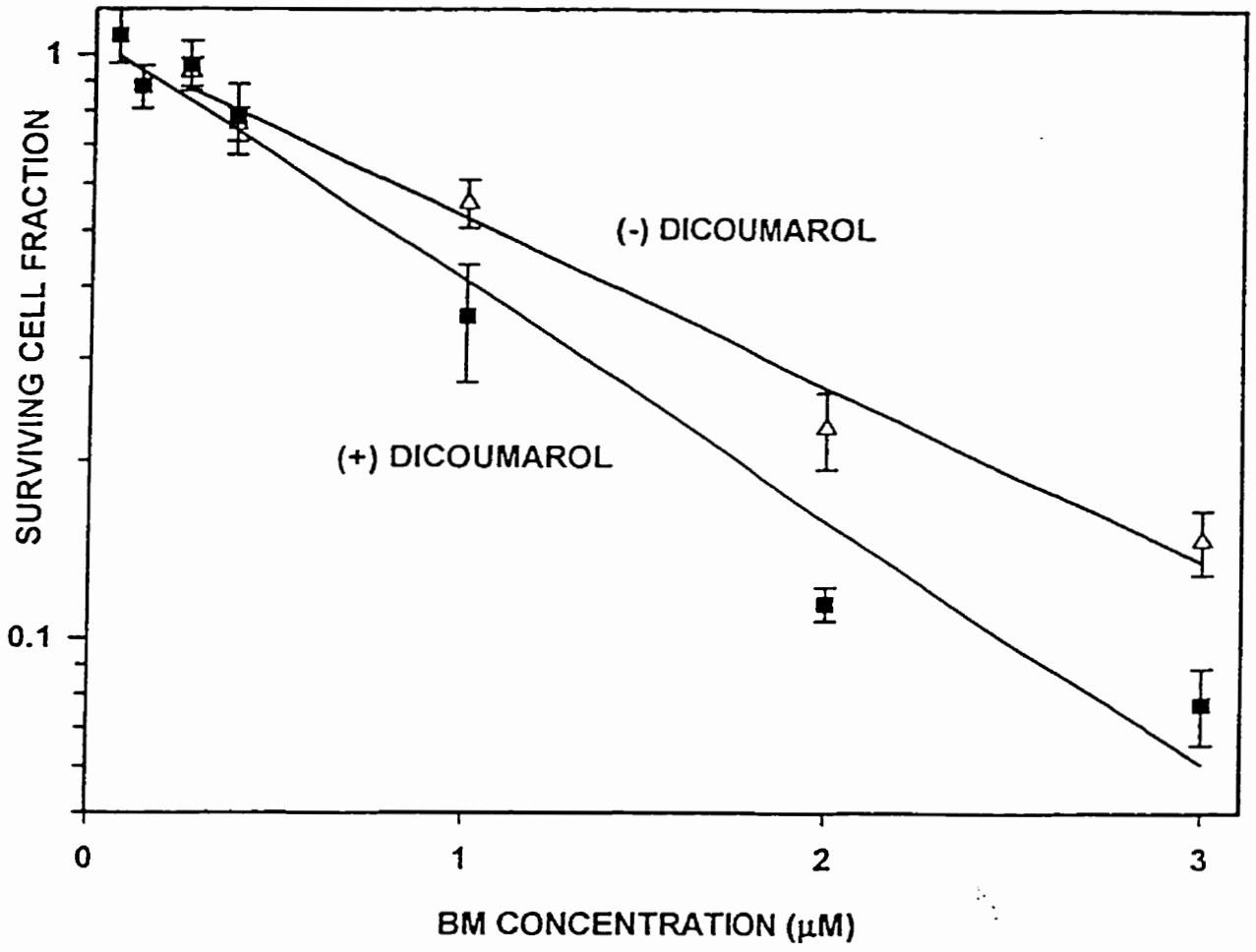


Figure R-3: Cytotoxicity of BM in H661 human non-small cell lung line in the presence and absence of dicoumarol. Cells were incubated with, or without, 100 μM dicoumarol at 37°C and 5% CO_2 for 20 minutes. Media was removed and cells were incubated with various concentrations of BM and with, or without, 100 μM dicoumarol at 37°C and 5% CO_2 for 1 hour. Cells were washed with citrate saline, trysinized, diluted and incubated at 37°C and 5% CO_2 for 7 days. The surviving cell fraction was determined by MTT assay as described by Johnston et al. (1994). Points, mean of 11-15 determinations; bars, standard error; lines, regression lines.

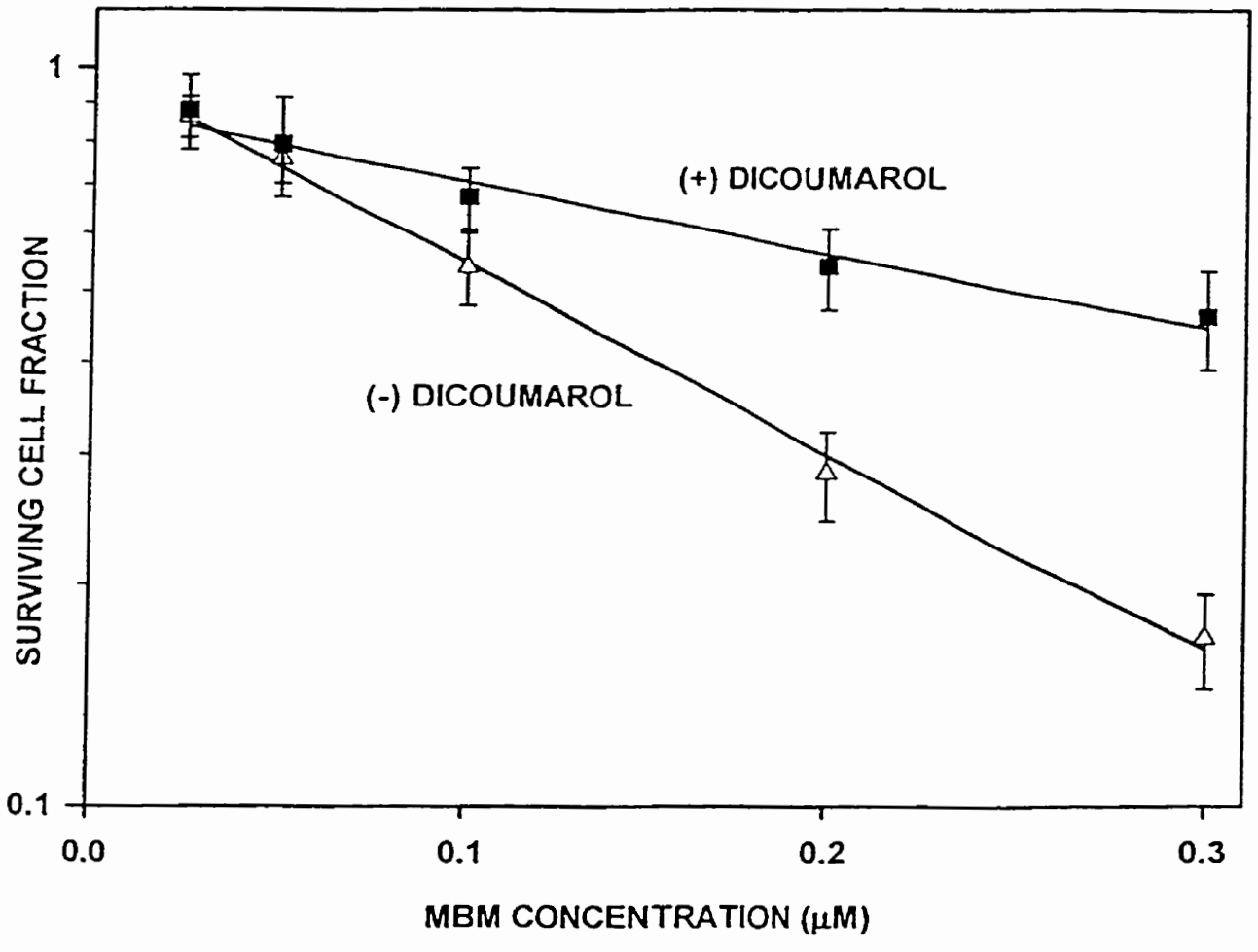


Figure R-4: Cytotoxicity of MBM in H661 human non-small cell lung line in the presence and absence of dicoumarol. Cells were incubated with, or without, 100 μM dicoumarol at 37°C and 5% CO_2 for 20 minutes. Media was removed and cells were incubated with various concentrations of MBM and with, or without, 100 μM dicoumarol at 37°C and 5% CO_2 for 1 hour. Cells were washed with citrate saline, trysinized, diluted and incubated at 37°C and 5% CO_2 for 7 days. The surviving cell fraction was determined by MTT assay as described by Johnston et al. (1994). Points, mean of 11-15 determinations; bars, standard error; lines, regression lines.

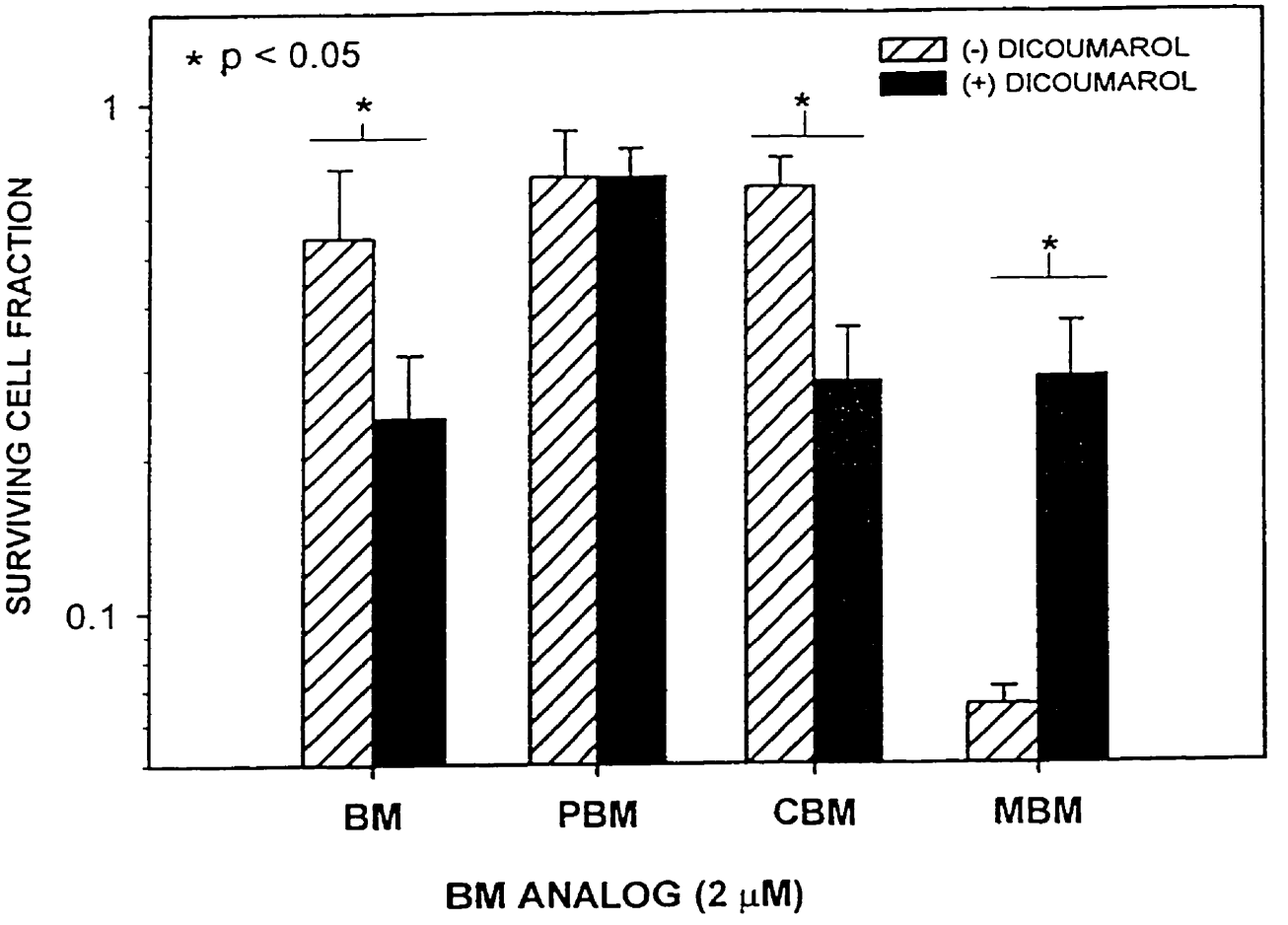


Figure R-5: Cytotoxicity of the BM analogs in SK-MEL-28 human melanoma cell line in the presence and absence of dicoumarol. Cells were incubated with, or without, dicoumarol at 37°C and 5% CO_2 for 20 minutes. Media was removed and cells were incubated with 2 μM of BM analog and with, or without, 100 μM dicoumarol at 37 °C and 5% CO_2 for 1 hour. Cells were washed with citrate saline, trypsinized, diluted and incubated at 37°C and 5% CO_2 for 9 days. The surviving cell fraction was determined by MTT assay as described by Johnston et al. (1994). Points, mean of 4 determinations; bars, standard error; *, $p<0.05$.

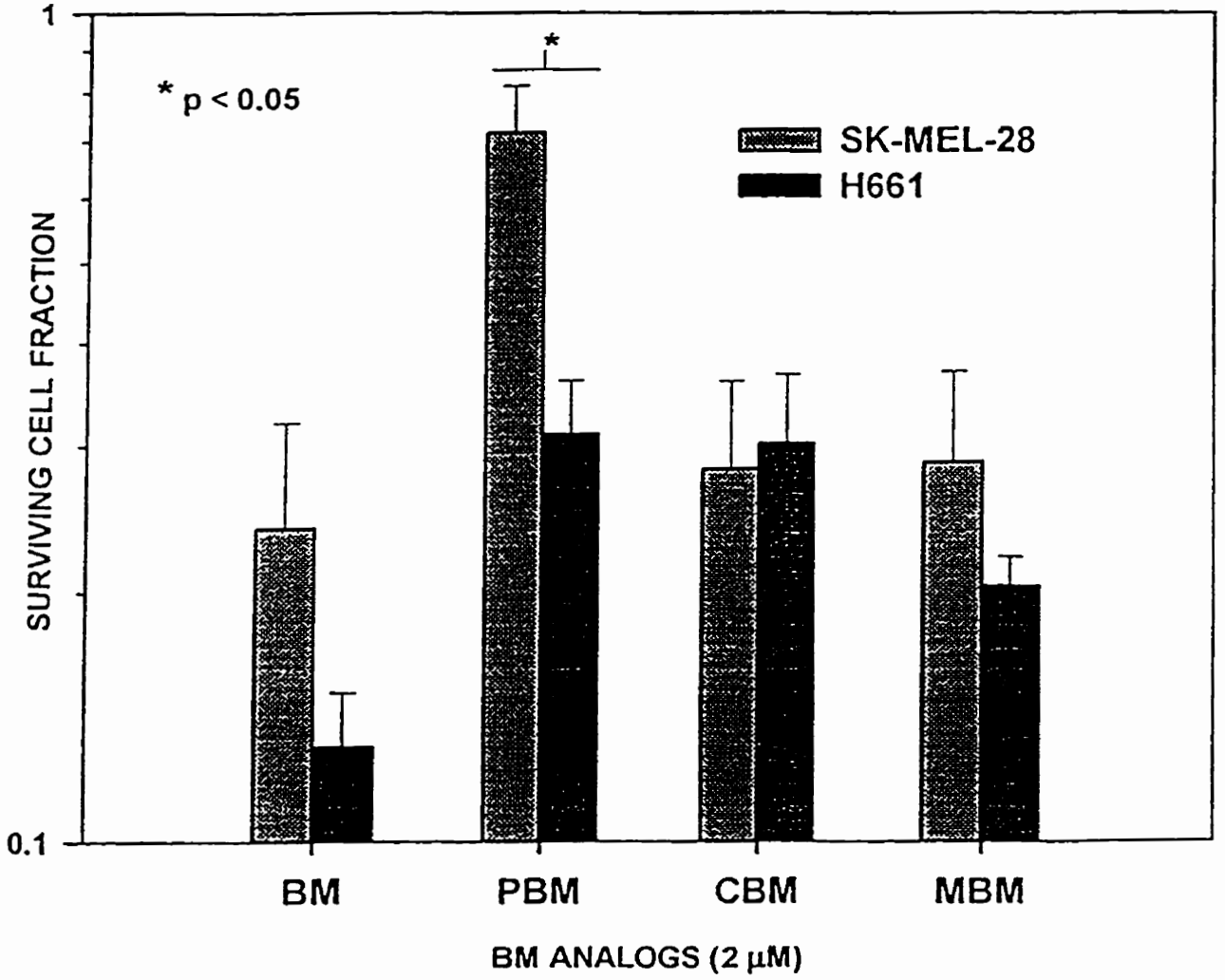


Figure R-6: Comparison of the cytotoxicity of the BM analogs in H661 human non-small cell lung line and in SK-MEL-28 human melanoma cell line in the presence of dicoumarol. Cells were incubated with 100 μM dicoumarol at 37°C in 5% CO_2 for 20 minutes. Media was removed and cells were incubated with 2 μM BM analog and with 100 μM dicoumarol at 37°C in 5% CO_2 for 1 hour. Cells were washed with citrate saline, trypsinized, diluted and incubated at 37°C in 5% CO_2 for 7 days for H661 and 9 days for SK-MEL-28. The surviving cell fraction was determined by MTT assay as described by Johnston et al. (1994). Points , mean of 4 determinations; bars, standard error; *, $p<0.05$.

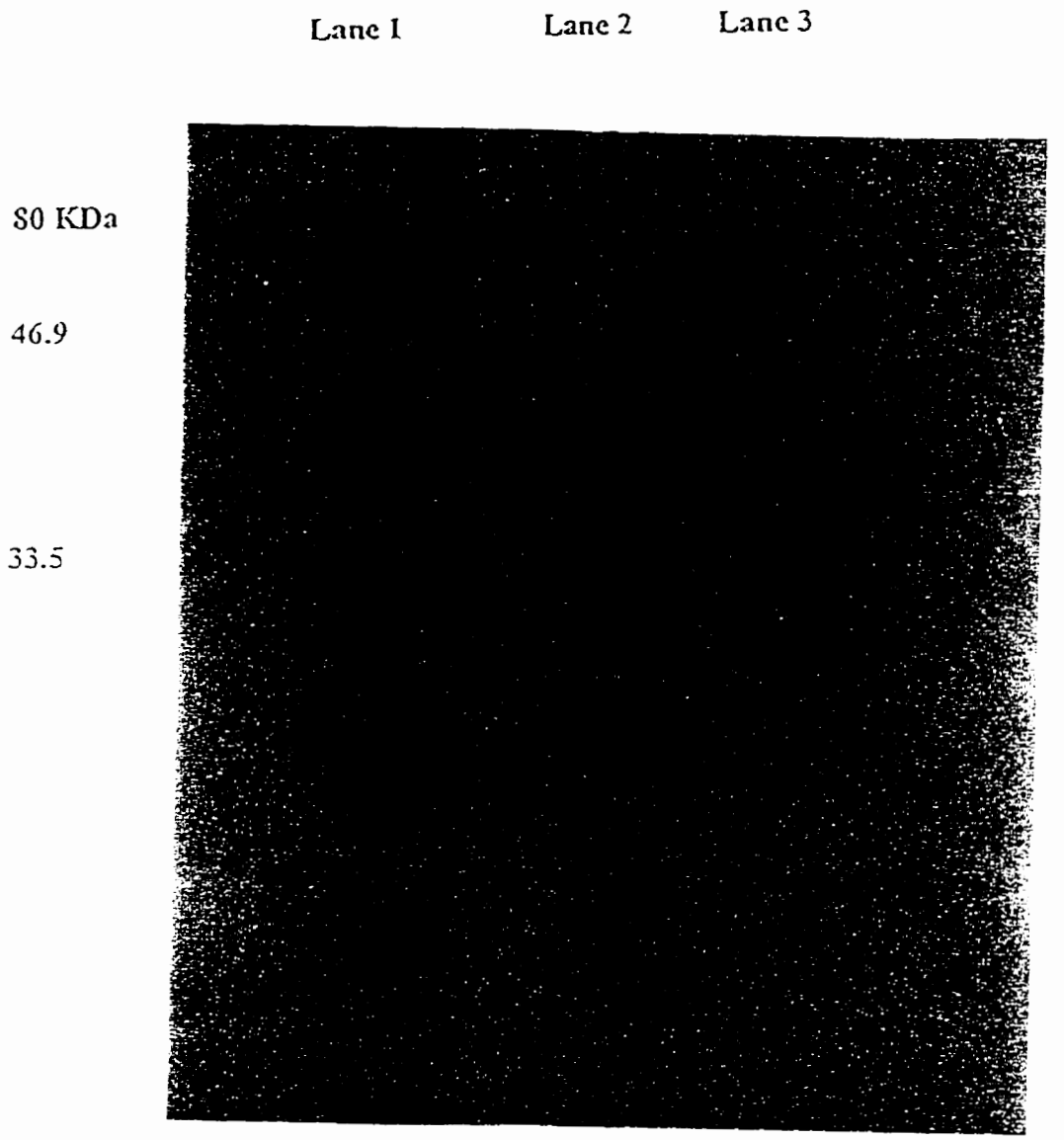


Figure R-7: SDS PAGE gel of human DT-diaphorase purified using the method described by Sharkis et al. (1989). Lane 1: Biorad protein standards: 80, 46.9 and 33.5 kDa (in descending order); Lane 2: 13 μg of protein from concentrate of fractions 17-20 from Sephadex G-100 size exclusion column. Lane 3: 13 μg of protein from concentrate of fractions 21-27 from Sephadex G-100 size exclusion column. A current of 10 mA was applied to the gel overnight and the gel was stained using Silver stain™.

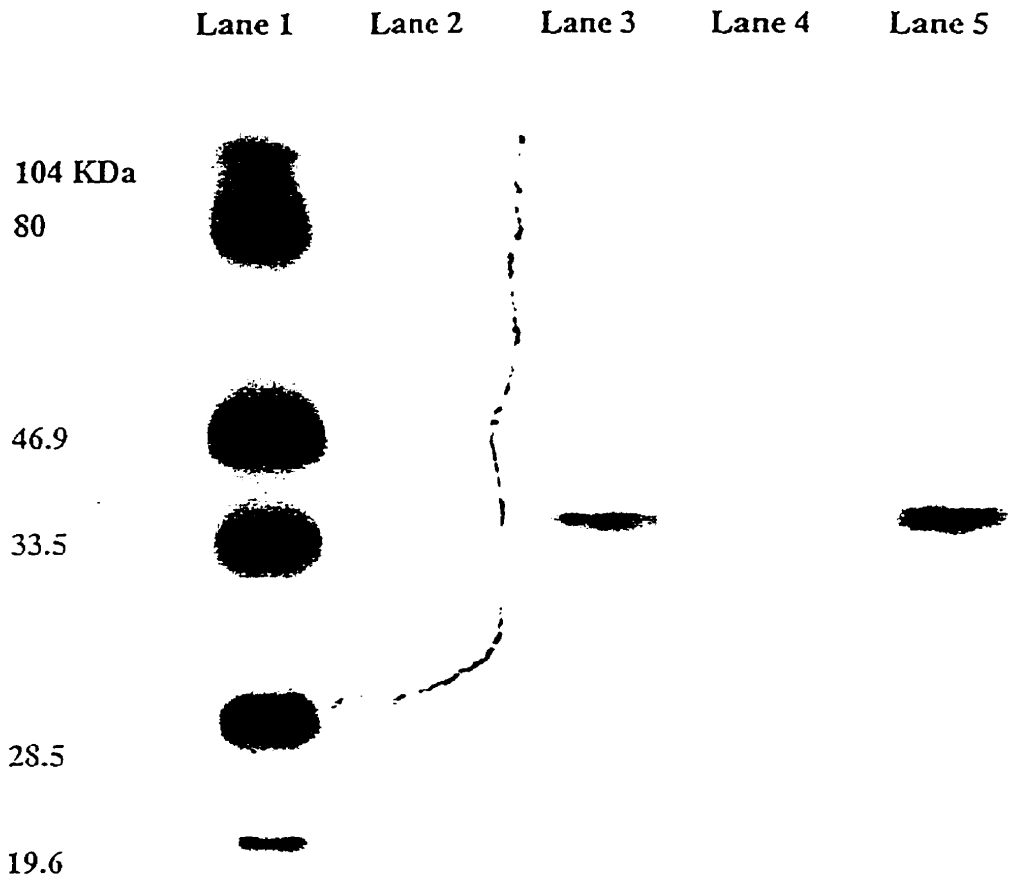


Figure R-8: SDS PAGE of human DT-diaphorase purified using the modified procedure. Lane 1: Biorad protein standards: 140, 80, 46.9, 33.5, 28.5 and 19.6 kDa (in descending order); Lane 2: 1 μg of protein; Lane 3: 4 μg of protein; Lane 4: 2 μg of protein; Lane 5: 8 μg of protein. A current of 10 mA was applied to the gel overnight and the gel was stained using Silver stain™.

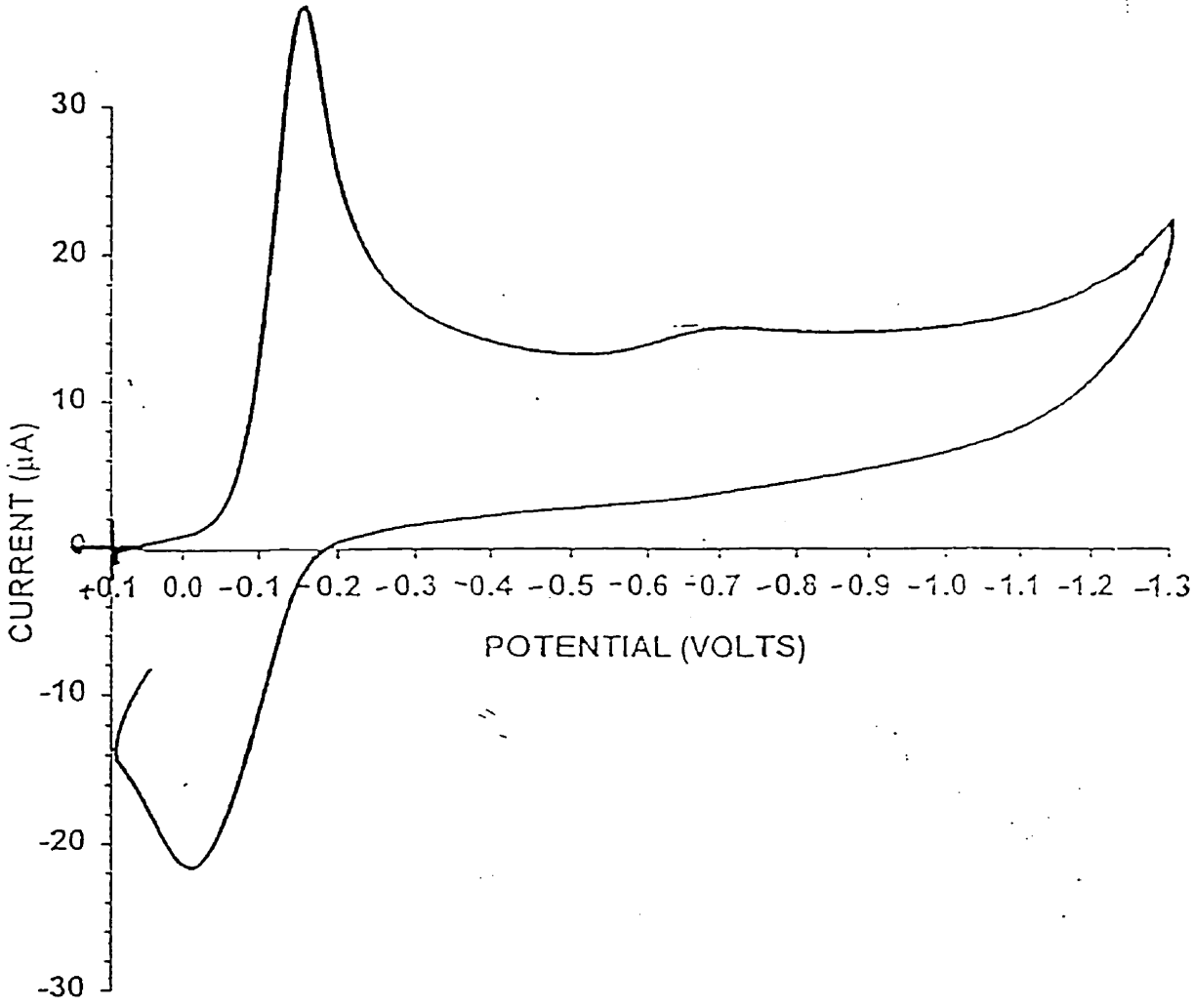


Figure R-9: Cyclic voltammogram of BM. The redox potential of 500 μM BM in 50 mM potassium phosphate (pH 7.4) was determined using a glassy-carbon electrode. Samples were degassed for 5 minutes using nitrogen prior to analysis, and scanned at 50 mV/s over a potential range of +0.1 V to -1.30 V.

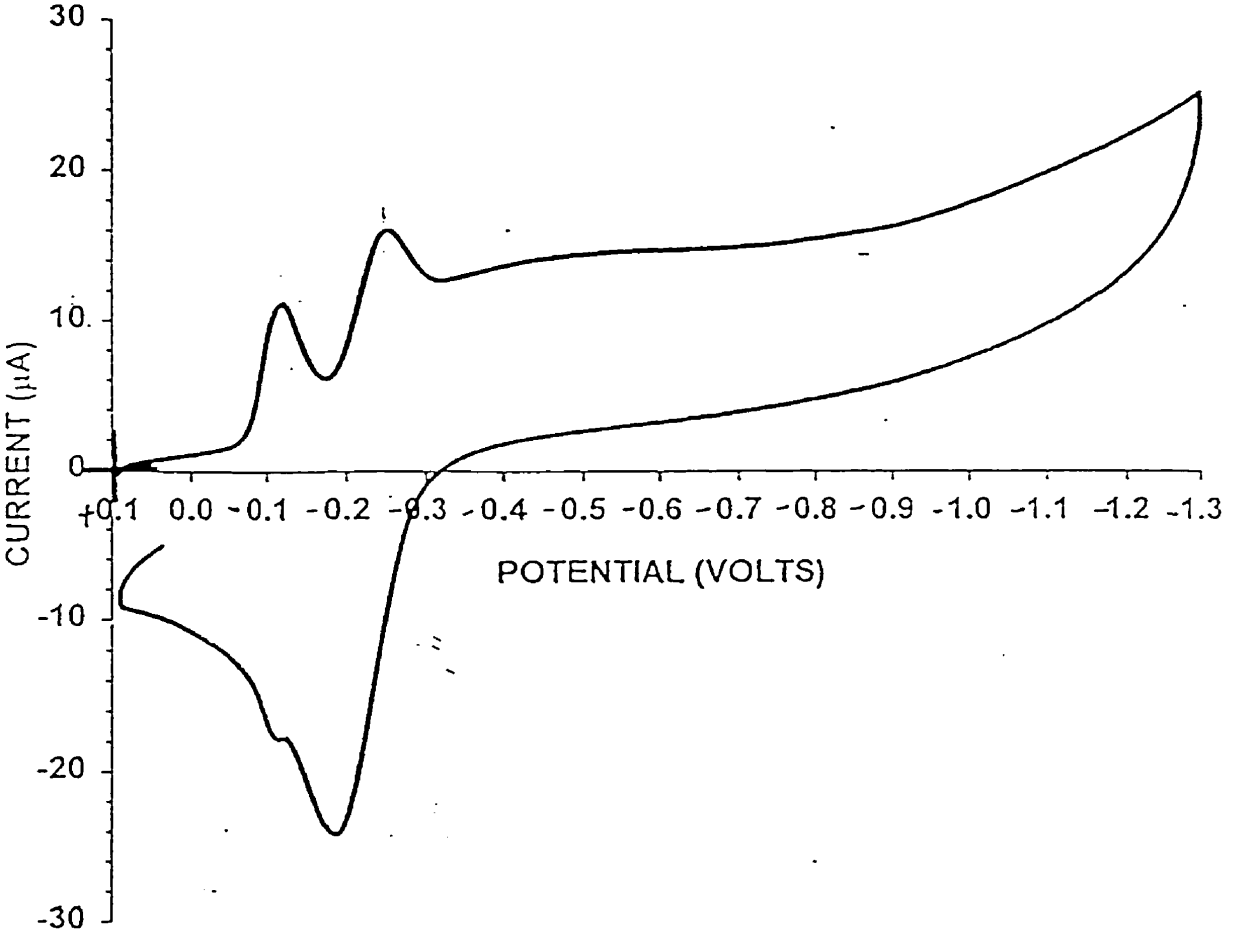


Figure R-10: Cyclic voltammogram of MBM. The redox potential of 500 μM MBM in 50 mM potassium phosphate (pH 7.4) was determined using a glassy-carbon electrode. Samples were degassed for 5 minutes using nitrogen prior to analysis, and scanned at 50 mV/s over a potential range of +0.1 V to -1.30 V.

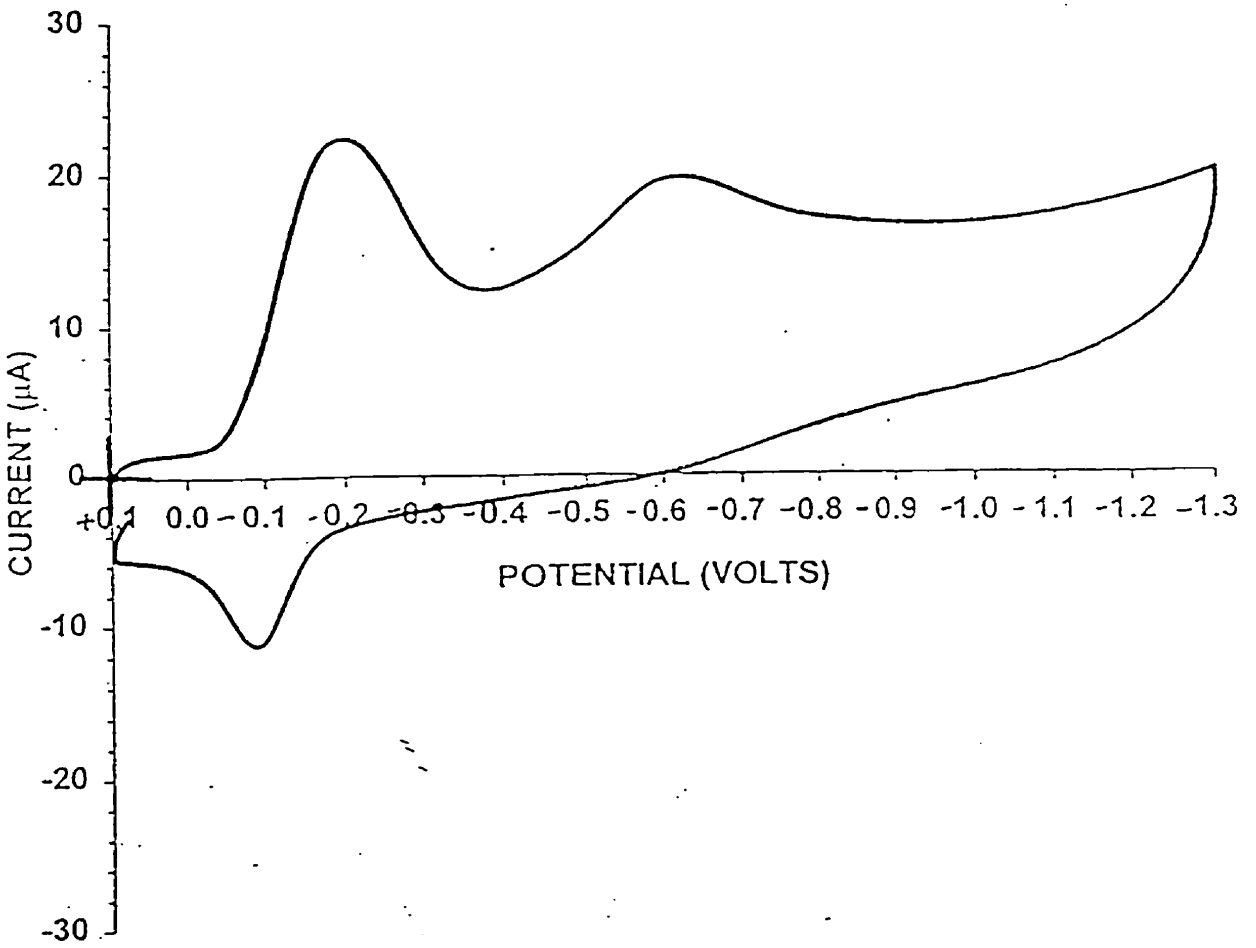


Figure R-11: Cyclic voltammogram of CBM. The redox potential of 500 μM CBM in 50 mM potassium phosphate (pH 7.4) was determined using a glassy-carbon electrode. Samples were degassed for 5 minutes using nitrogen prior to analysis, and scanned at 50 mV/s over a potential range of +0.1 V to -1.30 V.

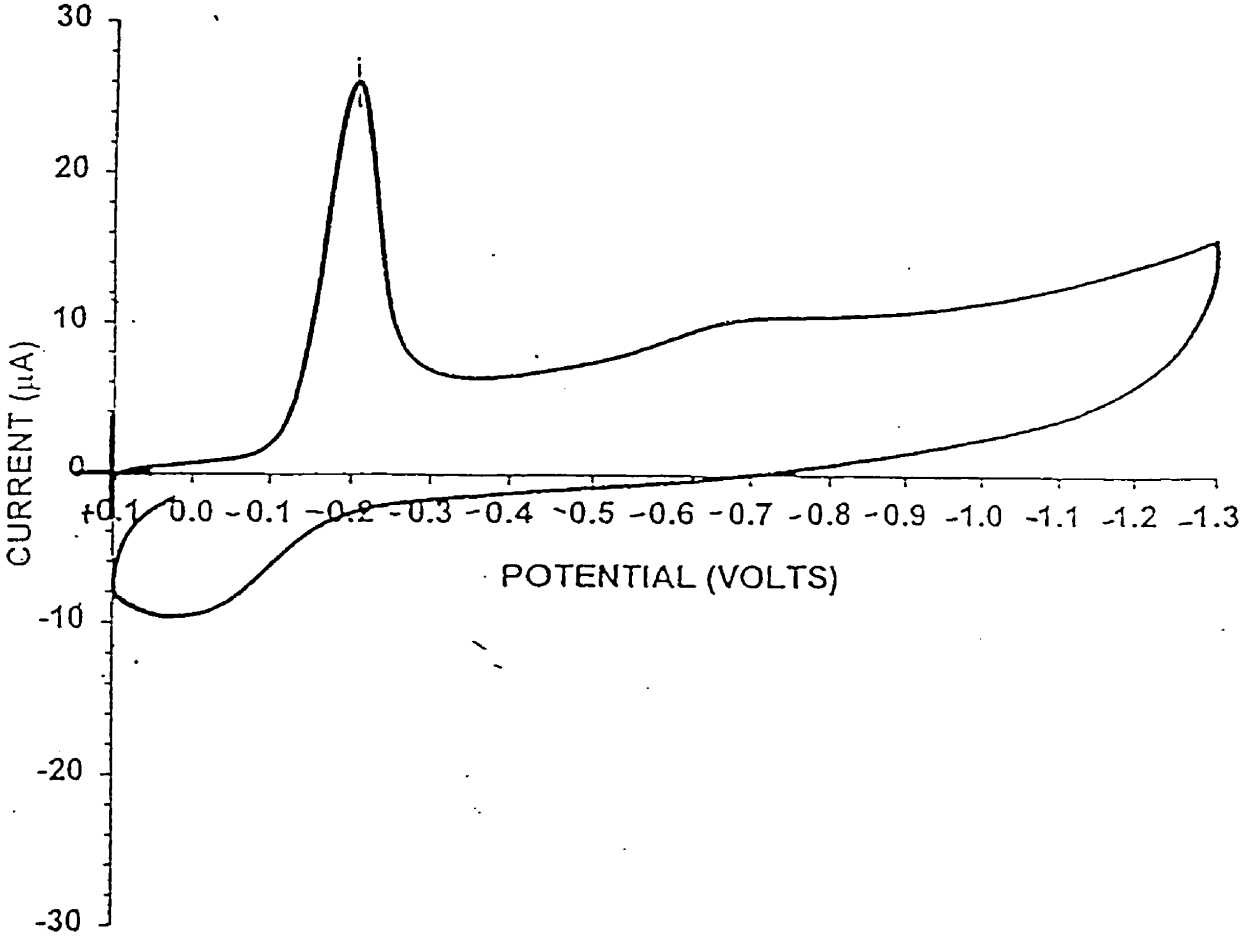


Figure R-12: Cyclic voltammogram of PBM. The redox potential of 500 μM PBM in 50 mM potassium phosphate (pH 7.4) was determined using a glassy-carbon electrode. Samples were degassed for 5 minutes using nitrogen prior to analysis, and scanned at 50 mV/s over a potential range of +0.1 V to -1.30 V.

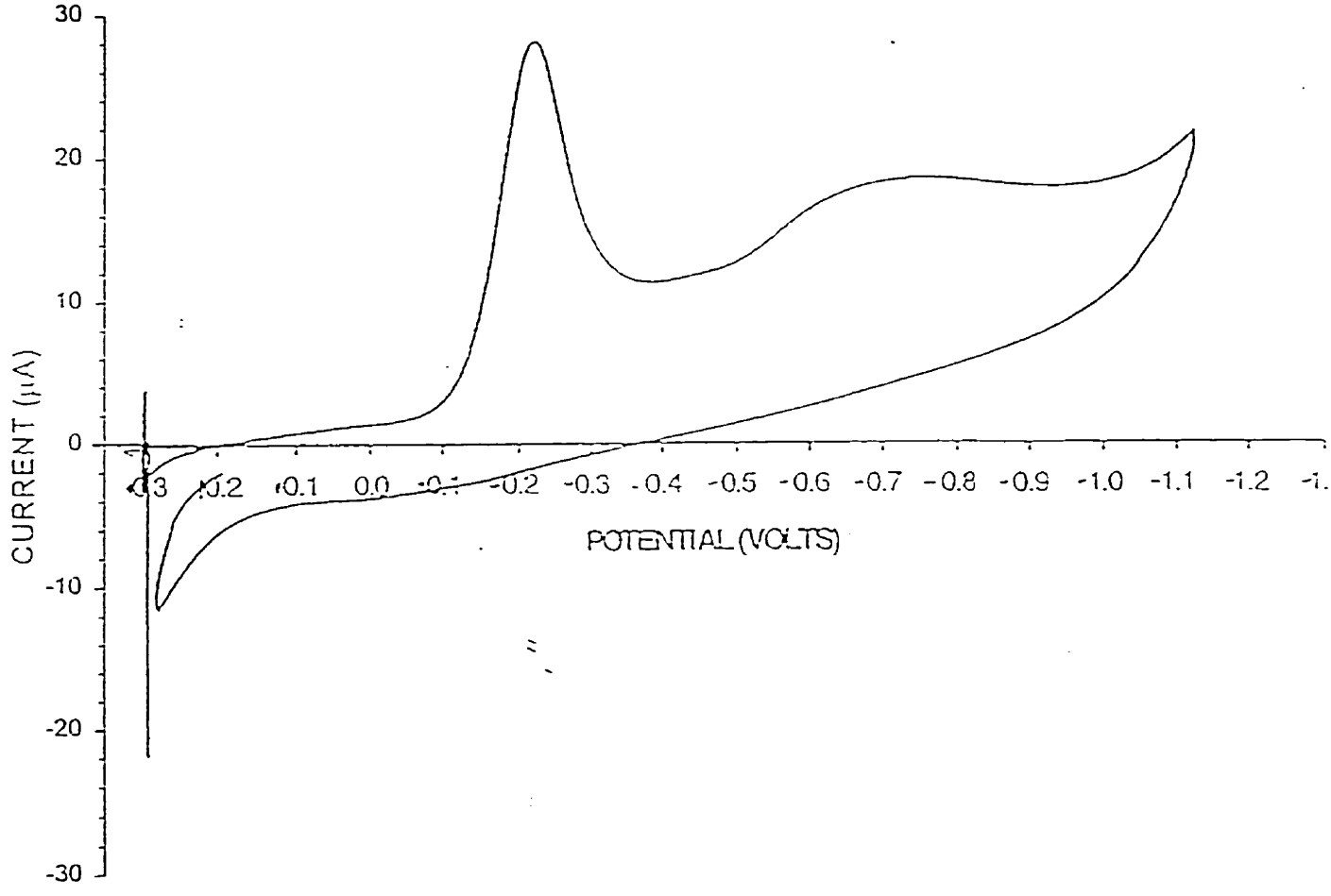


Figure R-13: Cyclic voltammogram of TBM. The redox potential of 500 μM TBM in 50 mM potassium phosphate (pH 7.4) was determined using a glassy-carbon electrode. Samples were degassed for 5 minutes using nitrogen prior to analysis, and scanned at 50 mV/s over a potential range of +0.3 V to -1.30 V.

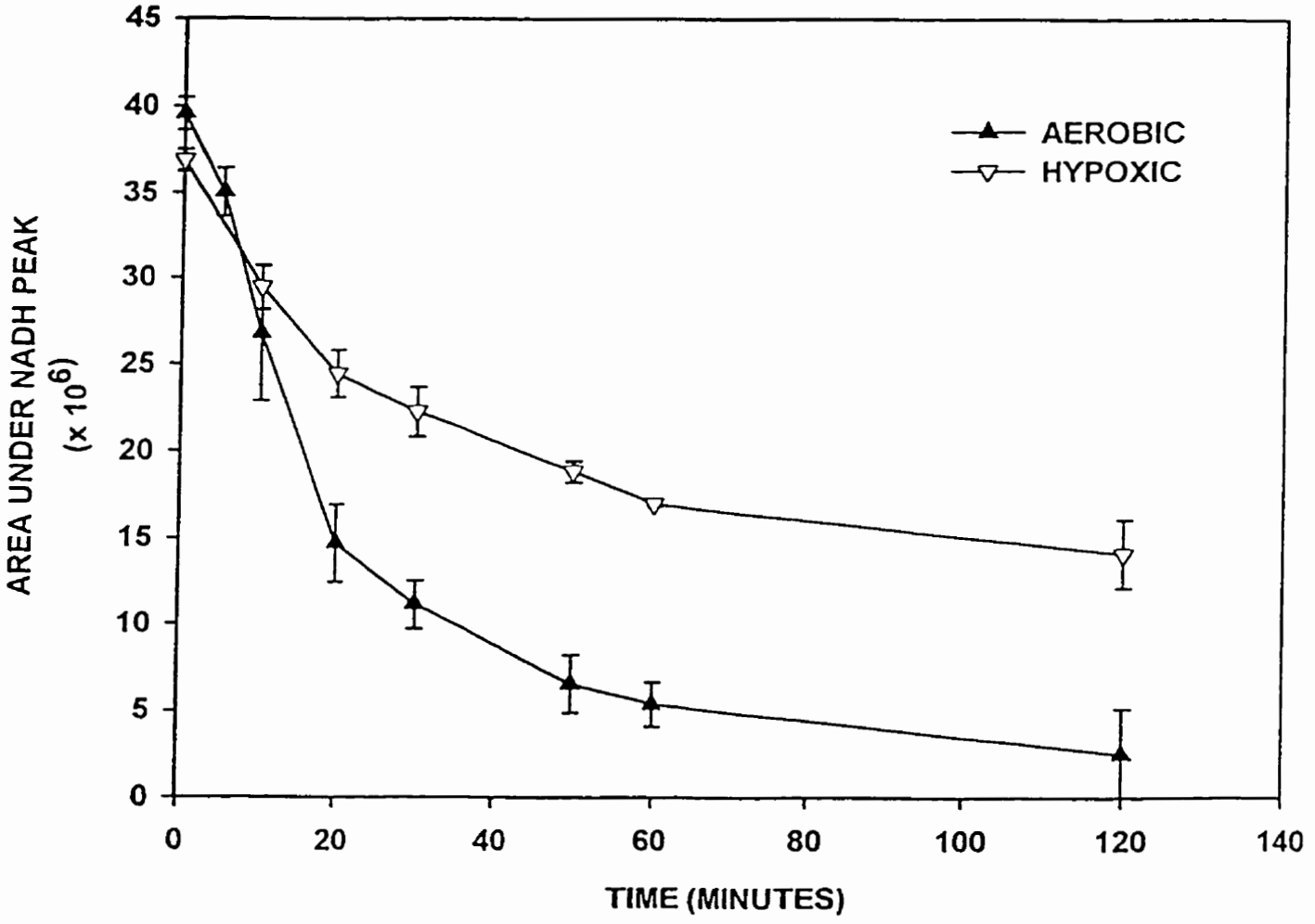


Figure R-14: Time course for aerobic and hypoxic reduction of MBM by human DT-diaphorase purified from HT-29 colon cancer cells. Aerobic and hypoxic reactions were carried out in 50 mM Tris HCl (pH 7.4) at 25°C using 0.1 μg of DT-diaphorase purified from HT-29, 100 μM NADH, 0.5 μM FAD and 50 μM MBM. Consumption of NADH was quantified using HPLC, see materials and methods. Points, mean of 3 determinations; bars; standard error.

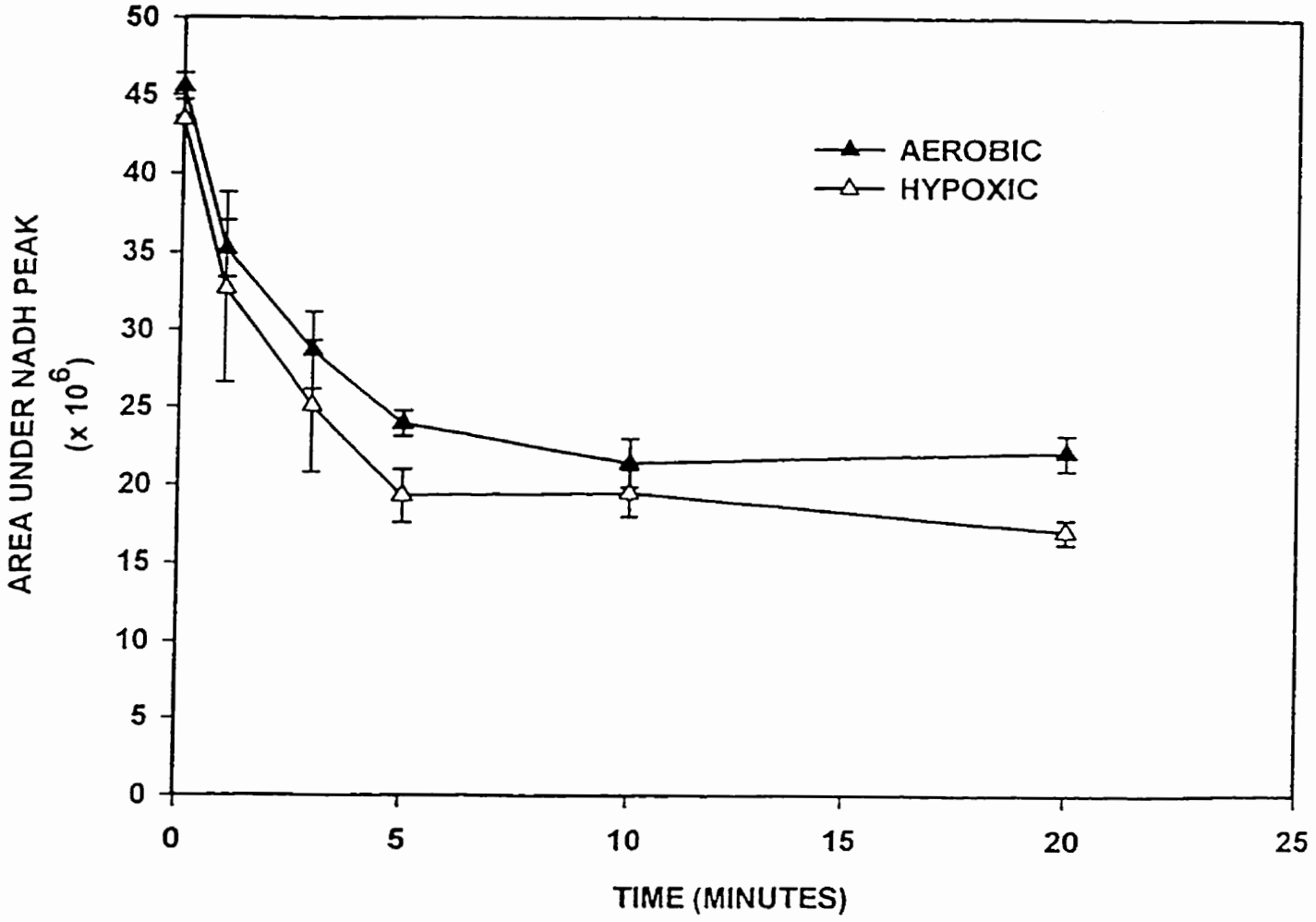


Figure R-15: Time course for aerobic and hypoxic reduction of BM by human DT-diaphorase purified from HT-29 colon cancer cells. Aerobic and hypoxic reactions were carried out in 50 mM Tris HCl (pH 7.4) at 25°C using 0.1 μg of DT-diaphorase purified from HT-29, 100 μM NADH, 0.5 μM FAD and 50 μM BM. Consumption of NADH was quantified using HPLC, see materials and methods. Points, mean of 3 determinations; bars; standard error.

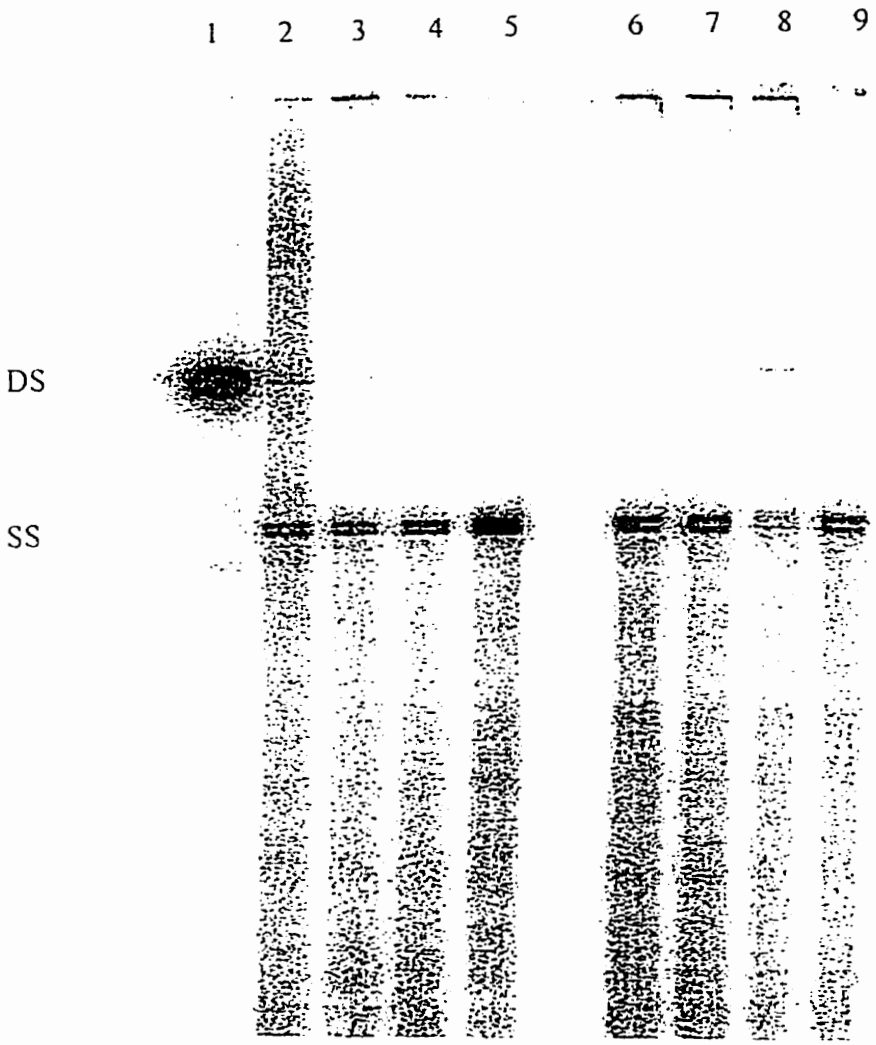


Figure R-16: Cross-linking activity of controls. Various controls were analyzed for cross-linking activity using the agarose gel method developed by Hartley et al. (1991). Lane 1: non-denatured pBR322 plasmid DNA; Lane 2: denatured pBR322 plasmid DNA; Lane 3: pBR322 plasmid DNA treated with 0.1 μg/100 μL purified human DT-diaphorase, 100 μM NADH and 0.5 μM FAD for 3 hours; Lane 4: pBR322 plasmid DNA treated with 50 μM BM for 3 hours; Lane 5: pBR322 plasmid DNA treated with 50 μM MBM for 3 hours; Lane 6: pBR322 plasmid DNA treated with 0.1 μg/100 μL purified DT-diaphorase and 50 μM BM for 3 hours; Lane 7: pBR322 plasmid DNA treated with 0.1 μg/100 μL purified human DT-diaphorase and 50 μM MBM for 3 hours; Lane 8: pBR322 plasmid DNA treated with 50 μM BM, 100 μM NADH and 0.5 μM FAD for 3 hours; Lane 9: pBR322 plasmid DNA treated with 50 μM MBM, 100 μM NADH and 0.5 μM FAD for 3 hours.

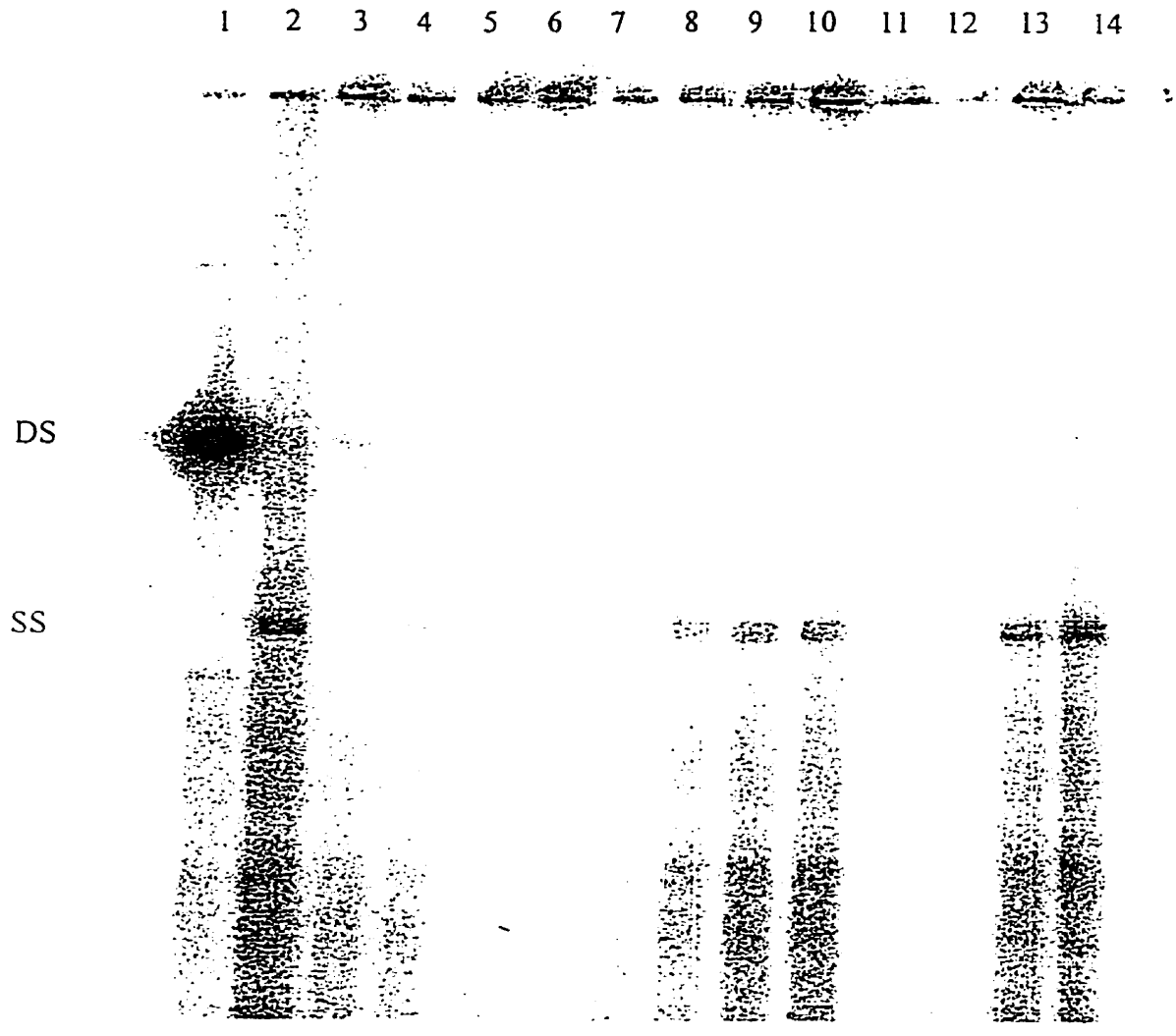


Figure R-17: Cross-linking activity of BM and MBM. The cross-linking activity of BM and MBM was studied using the method developed by Hartley et al. (1991). Lane 1: non-denatured pBR322 plasmid DNA; Lane 2: denatured pBR322 plasmid DNA; Lanes 3-13: pBR322 plasmid DNA treated for 2 hours with 0.1 μg/100 μL purified human DT-diaphorase, 100 μM NADH and 0.5 μM FAD in addition to: Lane 3: 1 μM BM; Lane 4: 5 μM BM; Lane 5: 10 μM BM; Lane 6: 0.5 μM MBM; Lane 7: 1.0 μM MBM; Lane 8: 5 μM MBM; Lane 9: 10 μM BM; Lane 10: 10 μM MBM; Lane 11: 25 μM BM; Lane 12: 10 μM MBM; Lane 13: 25 μM MBM. Lane 14: pBR322 treated with 0.1 μg/100 μL purified human DT-diaphorase for 2 hours.

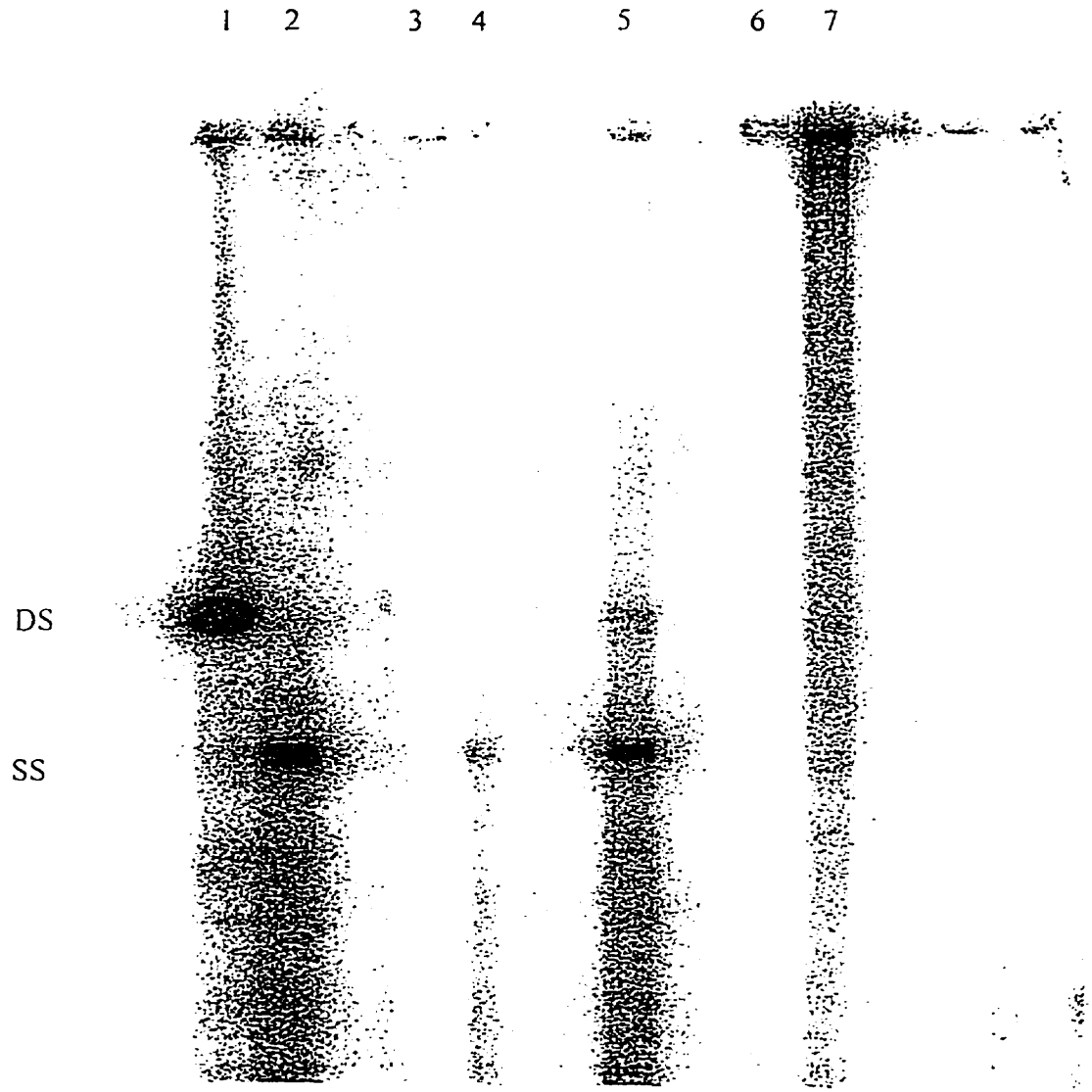


Figure R-18: Cross-linking activity of BM and MBM. The cross-linking activity of BM and MBM was studied using a modified method of the method developed by Hartley et al. (1991). Lane 1: non-denatured pBR322 plasmid DNA; Lane 2: denatured pBR322 plasmid DNA; Lanes 3-7: pBR322 plasmid DNA treated for 2 hours with 0.1 μg/100 μL purified human DT-diaphorase, 100 μM NADH and 0.5 μM FAD in addition to: Lane 3: 10 μM BM; Lane 4: 25 μM BM; Lane 5: no drug was added; Lane 6: 10 μM MBM; Lane 7: 25 μM MBM.

ANALOG	FIRST REDOX POTENTIAL (VOLTS)	SECOND REDOX POTENTIAL (VOLTS)
BM	-0.165	-0.670
MBM	-0.120	-0.240
PBM	-0.200	-0.680
CBM	-0.200	-0.600
TBM	-0.200	-0.650

TABLE 1: Summary of first and second redox potentials for BM analogs. The redox potentials of 500 μM of each of the BM analogs in 50 mM potassium phosphate (pH 7.4) was determined by cyclic voltammetry using a glassy-carbon electrode. Samples were degassed for 5 minutes using nitrogen prior to analysis, and scanned at 50 mV/s over a potential range +0.3V to -0.130 V.

Discussion

Synthesis

Analogs of 2-(di(chloroethyl)amino)-1,4-benzoquinone, BM, were chosen to study the effect of functional groups on specificity for activation of a quinone bioreductive agent by DT-diaphorase or NADPH:cytochrome P-450 reductase. Analogs were chosen to attempt to illustrate the effect of a specific class of functional groups. The analogs initially chosen were: 5-methoxy-2-(di(chloroethyl)amino)-1,4-benzoquinone, MBM, 6-*t*-butyl-2-(di(chloroethyl)amino)-1,4-benzoquinone, TBM and 3-acetyl-2-(di(chloroethyl)amino)-1,4-benzoquinone, ABM. MBM would illustrate the effect of an electron releasing group. TBM would illustrate the effect of a sterically bulky group. ABM would illustrate the effect of an electron withdrawing group.

MBM was successfully prepared in sufficient quantity for further study using a combination of the method described by Crosby et al. (1956) for oxidative amination of 1,4-benzoquinones and Makarova et al. (1973) for synthesis of BM by a Michael-type addition. Preparation of TBM, using a method, similar to that used for the preparation of MBM, produced a mixture of *t*-butyl-2-(di(chloroethyl)amino)-1,4-benzoquinone isomers. TBM could not be purified in sufficient quantity for biological study. The lack of success may be attributed to a reaction of TBM with the silica gel column material. During flash chromatography of the impure isomeric mixture containing TBM, a reaction of the contents of the mixture with the silica gel was noted. This reaction may have reduced the purification

efficiency of the column, which may have reduced the extent to which TBM was purified.

Preparation of ABM, using the method developed for the preparation of MBM was unsuccessful. It was possible that synthesis of ABM using this method was limited by high reactivity of 2-acetyl-1,4-benzoquinone. 2-Acetyl-1,4-benzoquinone may have reacted with various nucleophiles in the reaction mixture, which may complicate and prevent the formation of ABM. Reaction conditions were developed to maximize the reaction of 2-acetyl-1,4-benzoquinone with di(chloroethyl)amine, and minimize the reaction of 2-acetyl-1,4-benzoquinone with other nucleophiles. These reaction conditions involved the use of anhydrous ether, anhydrous potassium carbonate as a drying agent and anhydrous silver oxide as an oxidant. Using these reaction conditions, ABM was prepared amongst other impurities. ABM could not be further purified, and readily decomposed on standing in air.

Since it was not possible to obtain sufficient TBM or ABM for biological studies, alternate compounds, CBM and PBM, were prepared. CBM would illustrate the effect of a mild electron withdrawing group, and PBM would illustrate the effect of a steric bulky group, and may have neutral electronic properties.

CBM was prepared in sufficient quantity for further study using a method developed by Makarova et al. (1973). PBM was prepared in sufficient quantity for further study using a combination of the method described by Crosby et al. (1956) for oxidative amination of 1,4-benzoquinones and Makarova et al. (1973) for synthesis of BM.

Studies into the Effect of Functional Groups on Cytotoxicity

Dependence of cytotoxicity of BM analogs on DT-diaphorase activity in H661 cells

The cytotoxicity of BM, CBM, PBM and MBM were studied in the H661 cell line in the presence and absence of dicoumarol. H661 is a human non-small cell lung carcinoma line shown to possess moderate DT-diaphorase activity, 112.7±12.4 nmoles/min/mg protein (Begleiter et al., 1997). Using dicoumarol, an inhibitor of DT-diaphorase, the cytotoxicity of the agents in the H661 cell line can be studied in the presence and absence of DT-diaphorase activity. Thus, the dependence of the cytotoxicity of the analogs on DT-diaphorase can be studied. Analogs whose cytotoxicity is decreased in the presence of dicoumarol may be activated by DT-diaphorase, while analogs whose cytotoxicity is increased in the presence of dicoumarol may be inactivated by DT-diaphorase. Finally, analogs whose cytotoxicity is not affected by dicoumarol may not be dependent on DT-diaphorase activity.

Initially, the cytotoxicity was studied at 2 μM of BM analog, in the presence, and absence, of dicoumarol (Figure R-2). This provided a preliminary screen for the dependence of cytotoxicity of the BM analogs on DT-diaphorase. The cytotoxicity of BM increased and the cytotoxicity of MBM decreased in the presence of dicoumarol, while the cytotoxicities of CBM and PBM were unaffected by the presence of dicoumarol. Therefore, MBM may be activated by DT-diaphorase, while BM may be inactivated by this enzyme. Additionally, the cytotoxic activity of CBM and PBM may be independent of DT-diaphorase activity.

The dependence of the cytotoxicities of MBM and BM on DT-diaphorase determined

at 2 μM was validated by obtaining full concentration-response curves for each of BM and MBM in H661 cells in the presence, and absence, of dicoumarol (Figures R-3 and R-4). In the absence of dicoumarol, MBM and BM had a D_{10} of 0.10±0.09 μM and 3.4±0.3 μM, respectively. In comparison, in the presence of dicoumarol MBM and BM had a D_{10} of 0.38±0.01 μM and 2.4±0.2 μM, respectively. Thus, the results from the full-concentration response data were in agreement with the single concentration data.

Dependence of cytotoxicity of BM analogs on DT-diaphorase activity in SK-MEL-28 cells

The cytotoxicities of BM, MBM, CBM and PBM were studied at 2 μM in SK-MEL-28 cells in the presence, and absence, of dicoumarol (Figure R-5). SK-MEL-28 is a human melanoma cell line with moderately high DT-diaphorase activity, 586.7±19.6 nmoles/min/mg protein (Begleiter et al., 1997). As with the H661 cells, a comparison of the cytotoxic activity of the BM analogs in SK-MEL-28 cells in the presence, and absence, of dicoumarol may illustrate the dependence of the cytotoxicity of the BM analogs on DT-diaphorase activity and would confirm the results obtained using the H661 cell line.

The cytotoxicities of BM and CBM increased in the presence of dicoumarol, and decreased for MBM. In contrast, the cytotoxicity of PBM was unaffected in the presence of dicoumarol. Therefore, CBM and BM may be inactivated by DT-diaphorase, while MBM may be activated by this enzyme. Additionally, the cytotoxicity of PBM may be independent of DT-diaphorase activity. These results are in agreement with results from the study in the H661 cells for BM, MBM and PBM, but are in disagreement for CBM. The difference

observed for CBM may be due to a difference in the level of DT-diaphorase activity between SK-MEL-28 versus H661 cells. The level of DT-diaphorase is five times higher in SK-MEL-28 than in H661 cells. Thus, a larger change in the cytotoxicity of CBM in the presence, and absence, of diocumarol would be expected using SK-MEL-28 compared with H661, which would be in agreement with the observed results.

It is important to note that MBM retained significant cytotoxic activity in H661 and SK-MEL-28 cells in the presence of dicoumarol. This indicates that MBM may have cytotoxic activity in its unreduced form and/or it may be activated by another reductive enzyme, such as NADPH:cytochrome P-450 reductase. Additionally, the cytotoxic activity of BM was significant in H661 and SK-MEL-28 cells in the absence of dicoumarol. This suggests that, similar to MBM, BM may have cytotoxic activity in its unreduced form and/or it may be activated by other reductive enzymes, such as NADPH:cytochrome P-450 reductase. Finally, the D_{10} of BM and MBM in the H661 cells were significantly different in the the presence of dicoumarol. If we assume that BM and MBM have minimal differences in the cytotoxic activity of their respective unreduced forms, the difference between the cytotoxicity of MBM and BM in the presence of dicoumarol indicates that there may exist differences in the contribution of other reductive enzymes, such as NADPH:cytochrome P-450 reductase, to the activation of BM and MBM.

Dependence of cytotoxicity of BM analogs on cytochrome P-450 reductase activity

The dependence of the cytotoxicity of the BM analogs on NADPH:cytochrome P-450 reductase activity was studied by comparing the cytotoxicity of the BM analogs in SK-MEL-

28 and H661 cells in the presence of dicoumarol (Figure R-6). H661 cells have 3.0 fold higher NADPH:cytochrome P-450 reductase activity than SK-MEL-28 cells. Thus, a comparison of the cytotoxicity of the BM analogs in SK-MEL-28 and H661 cells may illustrate a dependence of the cytotoxicity of the BM analogs on NADPH:cytochrome P-450 reductase. However, SK-MEL-28 have approximately 5-fold higher DT-diaphorase activity than H661 cells. Dicoumarol was used to inhibit DT-diaphorase activity in the cell lines so that it did not interfere with the study of the effect of NADPH:cytochrome P-450 reductase.

Using $p < 0.05$ as the limit for statistical significance, the cytotoxicity of PBM was significantly greater in H661 than SK-MEL-28, while the cytotoxicity of BM, CBM and MBM was not significantly different in SK-MEL-28 and H661 cells. Therefore, the cytotoxicity of PBM may be dependent on NADPH:cytochrome P-450 reductase activity, while BM, CBM and MBM may be independent of NADPH:cytochrome P-450 reductase activity. These results suggest that PBM may be activated by NADPH:cytochrome P450 reductase. It is noted that the cytotoxicities of BM and MBM were 1.8-fold and 1.5-fold greater in H661 and SK-MEL-28 cells, respectively. However, these differences were not statistically significant, although the differences for BM approached statistical significance for ($p < 0.1$).

Summary

In summary, the cytotoxic studies in the H661 and SK-MEL-28 cells in the presence ,and absence, of dicoumarol illustrated that DT-diaphorase may activate MBM , may inactivate BM and CBM, but may have no effect on the cytotoxicity of PBM. Additionally,

the comparison of the cytotoxicity of the BM analogs in H661 versus SK-MEL-28 cells in the presence of dicoumarol illustrated that cytochrome P-450 reductase may activate PBM, but may have no effect on the cytotoxicity of CBM.

Several notes of caution are suggested in the interpretation of the data presented. Firstly, the illustration of dependence of cytotoxicity on DT-diaphorase activity was done at a single concentration in H661 cells for CBM and PBM, and at a single concentration in SK-MEL-28 cells for BM, MBM, CBM and PBM. Additionally, the comparison of the cytotoxicity of BM analogs in H661 cells in the presence of dicoumarol versus the cytotoxicity of BM analogs in SK-MEL-28 cells in the presence of dicoumarol was done using single concentration data. Single concentration data can be misleading, and it is possible that subtle differences not seen at a single concentration may be uncovered with a complete concentration-response study.

A second note of caution is suggested for the concentration of 100 μM dicoumarol used for all studies. This concentration was chosen by studying the effect of various concentrations of dicoumarol on the cytotoxicity of 1 μM EO9 in H661 cells. A concentration of 100 μM was found to completely inhibit the cytotoxicity of 1 μM EO9. On this basis, 100 μM was considered a good concentration to illustrate a maximum difference in the cytotoxicity of the BM analogs in the presence, and absence, of dicoumarol. It is possible that a concentration of 100 μM dicoumarol affects enzymes other than DT-diaphorase and may cause mitochondrial uncoupling (Preusch et al. 1991) which may affect the cytotoxicity of the BM analogs.

A final note of caution is suggested in the interpretation of the comparison of the

cytotoxicity of BM analogs in H661 and SK-MEL-28 cells in the presence of dicoumarol. It is unclear whether the DT-diaphorase activity in H661 and SK-MEL-28 cells was completely inhibited. Residual DT-diaphorase activity may complicate the differences in cytotoxicity of the BM analogs between H661 and SK-MEL-28 cells. Additionally, there may exist other subtleties between these two cell lines that could generate the differences observed. A difference in the activity of DNA-repair enzymes, or detoxification enzymes, such as glutathione S-transferase, may exist between H661 and SK-MEL-28 cells. A difference in the activity of DNA-repair enzymes and glutathione transferase activity may complicate the difference in cytotoxicity of bioreductive agents, such as the BM analogs. Also, a difference in the uptake of the BM analogs into SK-MEL-28 and H661 cells may exist.

Role of a Methoxy Group in Reversing the Effect of DT-Diaphorase for Cytotoxicity of BM versus MBM

The role of a methoxy group in reversing the effect of DT-diaphorase on the cytotoxicity of BM versus MBM, such that BM is inactivated by DT-diaphorase and MBM is activated by DT-diaphorase, was investigated. A methoxy group may affect the dependence of the cytotoxicity of MBM on DT-diaphorase activity by altering the stability of the semi-quinone and/or hydroquinone states of MBM, the rate or ease of reduction of MBM by DT-diaphorase, and/or the cross-linking activity of the mustard group.

Stability of semi-quinone and hydroquinone states of the BM analogs

The stability of the semi-quinone and hydroquinone state of the BM analogs can be predicted by the redox potentials of the semi-quinone and hydroquinone states, respectively. The redox potential of the semi-quinone state and the hydroquinone state is related to the free energy difference between the quinone and semi-quinone states and the quinone and hydroquinone states, respectively, as described by the Nernst equation (Driebergen et al., 1986). The more negative the redox potential of the semi-quinone and hydroquinone state, the less stable the semi-quinone and hydroquinone state, in comparison to the quinone state.

CBM, PBM and TBM had similar first redox potentials (Table 1). The first redox potential of MBM was less than BM, which was less than CBM, PBM and TBM. BM, CBM, PBM and TBM had similar second redox potentials, while MBM had a much less negative second redox potential. The first and second redox potentials are assumed to be the redox potentials of the semi-quinone and hydroquinone states, respectively. The first redox potentials of the BM analogs would predict that the semi-quinone state of MBM is more stable than BM, which is more stable than the semi-quinone state of CBM and PBM. As discussed in the introduction, an increase in the stability of the semi-quinone state of MBM may reduce free radical damage produced by re-oxidation, and increase the cross-linking activity of the semi-quinone of MBM, in comparison to BM. The second redox potential of the BM analogs would predict that the hydroquinone of MBM is more stable than BM, CBM, PBM, and TBM. The effect seen with MBM is similar to a study of the effect of a methoxy group on the redox potential of AZQ (Driebergen et al., 1986). Similar

to the change observed for the semi-quinone states of the MBM and BM, an increase in the stability of the hydroquinone state of MBM may reduce the free radical damage produced by re-oxidation, and increase the cross-linking activity of the hydroquinone of MBM, in comparison to BM. Depending on the contribution of free radical damage versus cross-linking activity to the cytotoxicity of MBM and BM, this may decrease or increase the contribution of DT-diaphorase to the cytotoxicity of MBM, in comparison to BM. Cross-linking activity is the major mechanism of cytotoxicity of BM (Begleiter and Blair, 1984; Begleiter and Leith, 1990). If there is a similar contribution of cross-linking activity to the cytotoxicity of MBM, an increase in the stability of the semi-quinone and/or the hydroquinone state of MBM, in comparison to BM, it would predict that MBM may be more cytotoxic than to BM. This is in agreement with the cytotoxicity results of BM and MBM in H661 cells in the presence, and absence, of dicoumarol.

Reduction of MBM and BM by DT-diaphorase

The rate at which DT-diaphorase reduces BM and MBM was studied by measuring the rate of consumption of NADH. NADH is a co-factor for the reduction of substrates by DT-diaphorase. It donates two electrons for the reduction reaction, and one molecule of NADH is consumed for every molecule of substrate reduced.

For MBM, NADH was consumed more readily under aerobic than hypoxic conditions, and the consumption of NADH under aerobic and hypoxic conditons was completed at 60 minutes (Figure R-14). However, approximately two equivalents of NADH were consumed under aerobic conditions, while approximately one equivalent was consumed

under hypoxic conditions. These results suggest that MBM is reduced more rapidly under aerobic than hypoxic conditions, and that it can re-oxidize and continue to be reduced by DT-diaphorase.

For BM, NADH was consumed at a similar rate under aerobic and hypoxic conditions, and the consumption of NADH was complete at 5 minutes. Additionally, in contrast to MBM, approximately one equivalent was consumed under both aerobic and anaerobic conditions. These results suggest that BM is unable to re-oxidize and continue to be reduced by DT-diaphorase, and that BM is reduced by DT-diaphorase more readily than MBM.

The greater rate of reduction of BM than MBM by DT-diaphorase may result in the reverse effect of DT-diaphorase on the cytotoxicity of BM versus MBM. An optimal rate of production of the hydroquinone by DT-diaphorase may be required to offset competing inactivating reactions of the hydroquinone that would prevent the hydroquinone from carrying out its potentially cytotoxic reactions. The increased rate of formation of the hydroquinone of BM may potentiate the hydrolysis of mustard groups. This may reduce alkylation and cross- linking activity. Alkylation and cross-linking activity may be important for the cytotoxicity of BM (Begleiter et al., 1990). The exact effect that an increased rate of reduction would have on the inactivating reactions, such as hydrolysis, is uncertain.

Redox potentials of MBM and BM predicted that the hydroquinone of MBM would be more stable than that of BM, and BM would redox cycle more readily than MBM, which contradicts the observation that MBM is able, while BM may not be able, to re-oxidize and continue to be reduced by DT-dipahorase. Two interpretations of these contrasting results

are suggested.

(i) The difference between the redox potentials of MBM and BM may be insignificant, and the hydroquinone of BM and MBM may be considered to have similar thermodynamic stability. The re-oxidation reaction of the hydroquinone to the quinone requires an interaction with, and activation of molecular oxygen. The methoxy group may increase the interaction and activation of oxygen with the hydroquinone. This may increase the rate of re-oxidation of MBM, which may explain why MBM is able, while BM may not be able, to re-oxidize and continue to be reduced by DT-diaphorase. The increased formation of reactive oxygen species, like hydroxyl radicals, may contribute to the increased cytotoxic activity of MBM following reduction by DT-diaphorase.

(ii) The hydroquinone of BM and MBM may be considered thermodynamically unstable. The hydroquinone of both BM and MBM may potentially be able to re-oxidize readily. However, an inactivating mechanism may occur for the hydroquinone of BM. Two potential reactions of the hydroquinone of BM are illustrated in Figure D-1. The first reaction, reaction 1, involves the nitrogen group reacting with one of the mustard "arms" to form an aziridinyl group. This aziridinyl group can then alkylate DNA. The second mustard "arm" may similarly form an aziridinyl ring and alkylate DNA thus forming a cross-link. This pathway would contribute to the cytotoxicity of the agent.

Alternatively, as a second reaction, reaction 2, the mustard "arm" may internally cyclize by reacting with the hydroxyl group of the hydroquinone. This cyclized product would not be able to undergo re-oxidation and participate in redox cycling reactions. Alkylation of DNA by the ether group would be unlikely. However, the second mustard

"arm" would be able to form an aziridinyl ring, and alkylate DNA. Thus, the cyclized product would be able to alkylate DNA, but would be unable to cross-link DNA and would have a greatly reduced cytotoxicity. Thus, the cyclization reaction may be considered an inactivating pathway.

For BM, it is possible that internal cyclization, reaction 2, occurs more readily than the formation of the aziridinyl ring, reaction 1 (Figure D-1). This would mean that reduction of BM by DT-diaphorase would lead to inactivation of BM, which would be in agreement with the cytotoxic studies in H661 and SK-MEL-28 cells.

In contrast, it is possible that for MBM the formation of the aziridinyl ring, reaction 1, occurs more readily than the internal cyclization reaction, reaction 2, (Figure D-2). This may be the result of an increase in the electron density around the nitrogen due to donation of electron density from the methoxy group to the nitrogen, inductively and through resonance. The increased electron density around the nitrogen may increase the rate the formation of the aziridinyl ring, reaction 1, so that it occurs more readily than internal cyclization, reaction 2. Thus, the aziridinyl ring would form preferentially, and would be able to alkylate and cross-link DNA. Additionally, the hydroquinone would be able to re-oxidize. Thus, reduced MBM would be able to alkylate and cross-link DNA, and participate in redox cycling reactions. In this case, reduction of MBM by DT-diaphorase would lead to activation of MBM, which would be in agreement with the cytotoxic studies in H661 cells.

Cross-linking activity of BM and MBM

The above explanation for the reverse effect of DT-diaphorase on the cytotoxicity of

BM and MBM is without evidence. To support this theory, analysis of the cross-linking activity of BM and MBM, reduced by DT-diaphorase was attempted using the agarose gel method developed by Hartley et al. (1991). Using this method, [α-^{32}PdATP] labeled and linearlized pBR322 plasmid DNA is incubated with BM and MBM, reduced by DT-diaphorase. The DNA is precipitated, dissolved in DNA-strand separating buffer and heat de-natured. DNA that is cross-linked will remain double-stranded, and DNA that is non-cross-linked will become single-stranded. The single- and double-stranded DNA, corresponding to non-cross-linked and cross-linked DNA, is separated using a non-denaturing TBE agarose gel, and quantified using autoradiography. A ratio of single-stranded to double-stranded DNA will give an estimate of the cross-linking efficiency of BM and MBM reduced by DT-diaphorase.

Attempts to use the method developed by Hartley et al. (1991) were unsuccessful. The DNA could not be consistently loaded onto the gels due to difficulties in solubilizing the DNA for loading. Several attempts were made to overcome the solubility problem. Firstly, the concentration of DMSO in the Strand-Separating Buffer was varied, and other solvents, such as acetone and DMF, were used to attempt to dissolve the DNA. A concentration of 8.5 % DMSO in 1 mM EDTA was found to partly dissolve the DNA treated with various concentration of BM or MBM, reduced by DT-diaphorase. However, the DNA was not consistently present on the agarose gel.

A second attempt to overcome the solubility problem involved the use of a wide concentration range of BM and MBM (0.5 to 50 μM). It was postulated that the DNA may be excessively cross-linked, which may affect its solubility properties. Unfortunately, DNA

treated with 0.5 and 50 μM BM or MBM was similarly insoluble. Additionally, the DNA was not consistently loaded onto the agarose gel and a concentration-response was not observed. In a third attempt, the incubation period was varied between 2 and 4 hours. Again the DNA was not consistently loaded. In a final attempt, the DNA purification step, using ethanol, was eliminated and the reaction mixture was directly loaded onto the agarose gel. Once again, the DNA was not consistently loaded, and precipitation of DNA in the loading wells of the agarose gel was observed.

Summary

In summary, the study of the cytotoxicity of the BM analogs in SK-MEL-28 and H661 cells each in the presense, and absence, of dicoumarol has illustrated that CBM and BM may be inactivated, while MBM may be activated by DT-diaphorase. Additionally, it has illustrated that PBM may not be dependent on DT-diaphorase. A comparison of the cytotoxicity of BM analogs in SK-MEL-28 versus H661 in the presence of dicoumarol has illustrated that PBM may be activated by, while CBM may not be dependent on NADPH:cytochrome P-450 reductase. Studies into the underlying mechanism contributing to the effect of a methoxy group in reversing the effect of DT-diaphorase on the activity of BM and MBM have showed that, in comparison to BM, MBM is more stable in its free-radical and two-electron reduced state; MBM is more slowly reduced by DT-diaphorase, and the hydroquinone of MBM is able to re-oxidize. Two theories are suggested to explain these differences between BM and MBM and their contribution to the observed effect of the

methoxy group in generating the reversed effect of DT-diaphorase on the activity of BM and MBM. Theory 1: The methoxy group may increase the interaction and activation of oxygen with the hydroquinone and the formation of reactive hydroxyl radicals. Theory 2: The methoxy group may reduce the formation of an inactive cyclized product and increase the formation of cross-links with DNA.

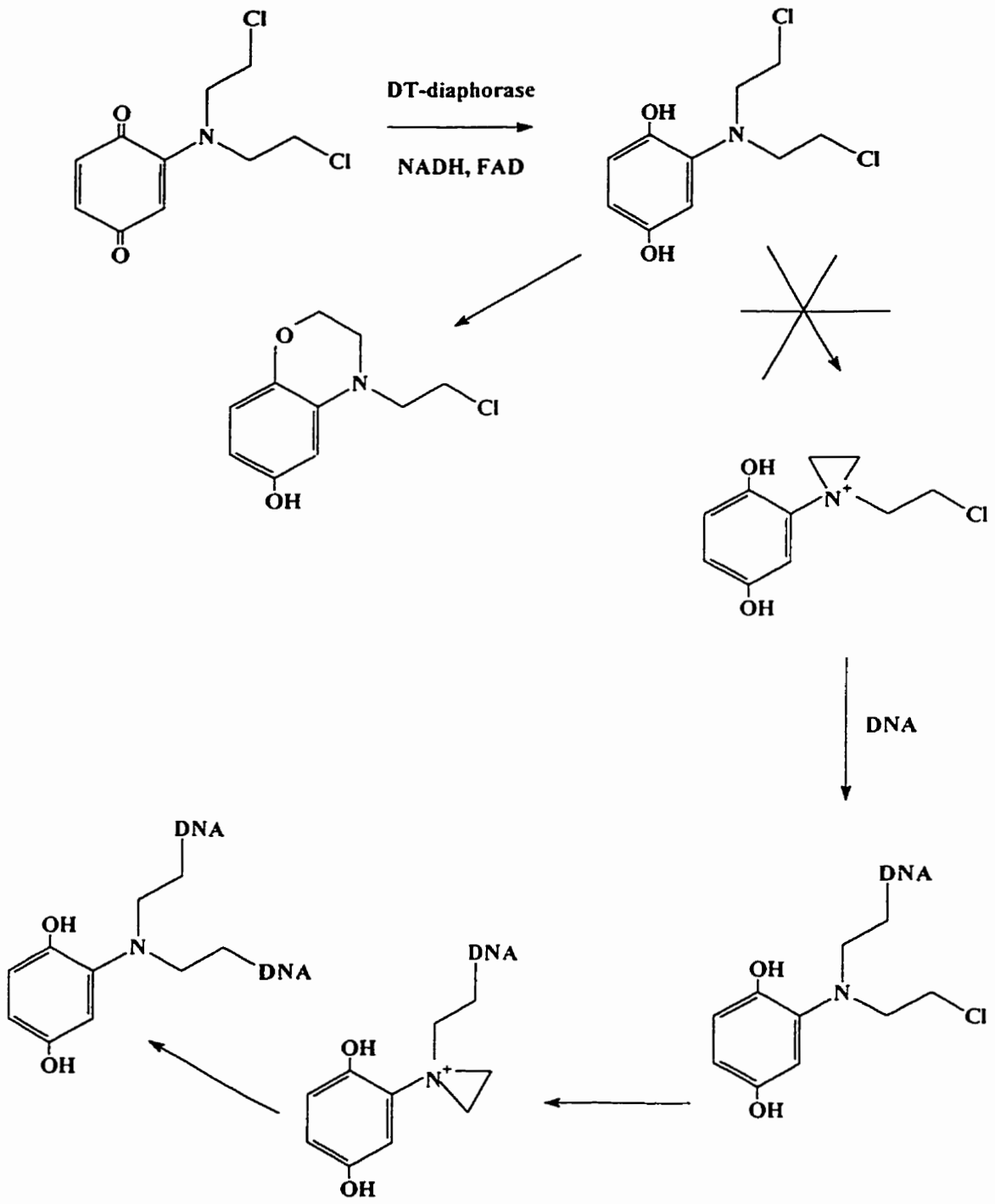


Figure D-1: Postulated mechanism for inactivation of BM by DT-diaphorase.

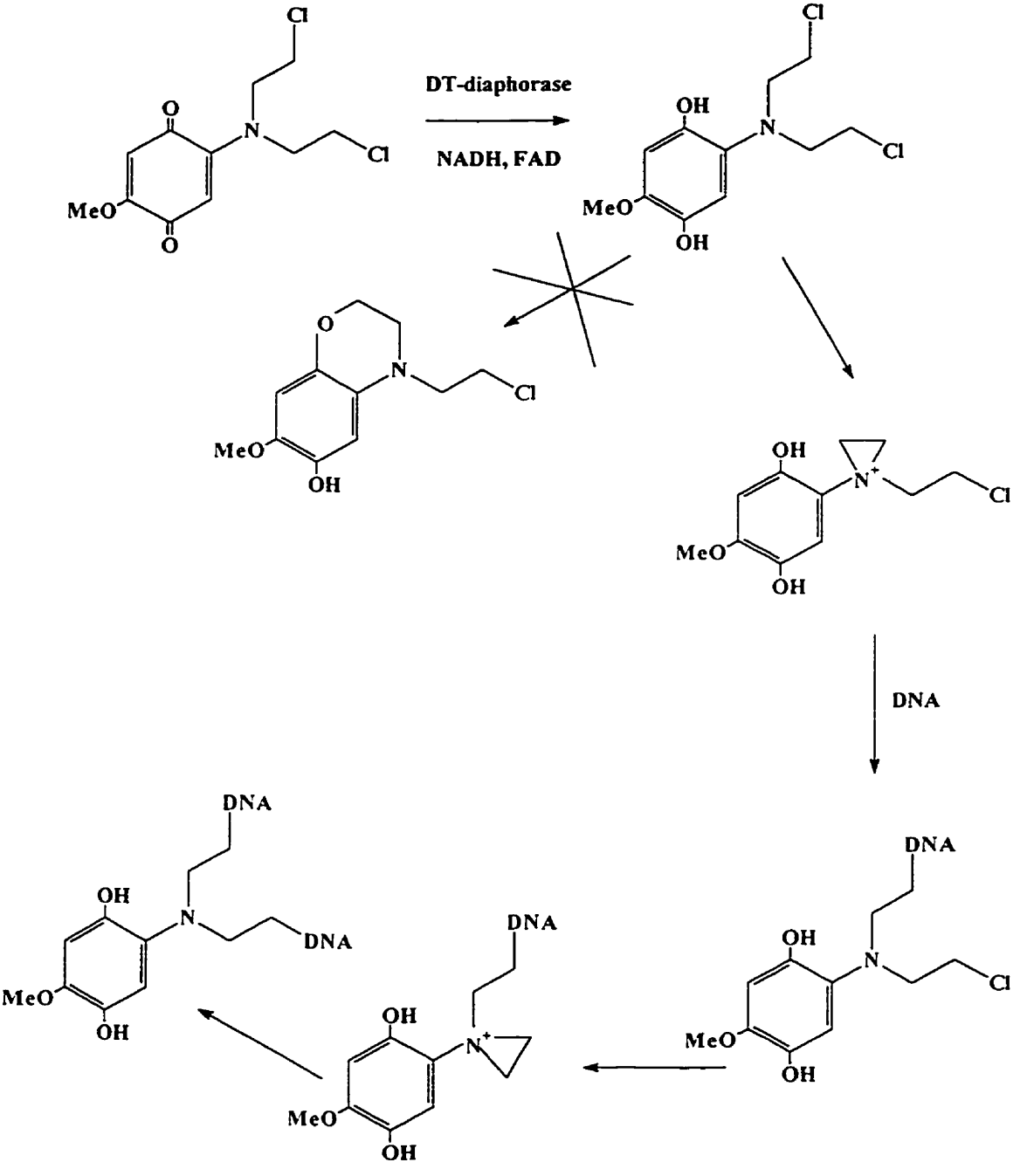


Figure D-2: Postulated mechanism for activation of MBM by DT-diaphorase.

Future Studies

Synthesis and Purification of TBM

PBM was chosen as a substitute for TBM to illustrate the effect of a sterically bulky group. However, a phenyl group has two planar faces, and has less steric bulk than a *t*-butyl group. Additionally, a *t*-butyl group is more electronically inert than a phenyl group. Thus, TBM is an important analog for study to more specifically illustrate the effect of a steric bulky group.

The synthesis of TBM has been worked out, but the purification of the meta isomer from the reaction mixture is difficult and the yield is poor. The current method of purification involves the use of silica gel flash chromatography 3 times. It was observed on each occasion that the TBM reacted with the column, and became irreversibly bound. This complicated the purification process. A better approach may be to use an ODS reverse phase column. ODS column material is silica gel that has been derivatized with ODS. This would prevent the reaction of TBM with the silica gel, which may allow the use of flash chromatography, once. This would simplify the purification process and may increase the yield several fold.

Synthesis of a BM Analog with an Electron Withdrawing Group

ABM was too unstable to be purified for study. As a substitute, CBM was prepared. The chloro group of CBM is a strong inductively withdrawing group, but can donate electrons through resonance (McMurray, 1992). Thus, a chloro group on a quinone ring may be an electron withdrawing group, while a chloro group on a hydroquinone ring may be an electron donating and resonance stabilizing group. Therefore, the electronic properties of a chloro group may be dependent on the redox state of CBM. This is unfavorable for proper analysis of the effect of an electron withdrawing group. Another BM analog is needed that would be an electron withdrawing group in both the quinone and hydroquinone states in order to complete the structure-activity study. Two possible electron withdrawing groups are a carboxyl group and a nitrogen oxide group. These groups are considered strongly electron withdrawing. A carboxyl group would be the preferred choice, because the nitrogen oxide group may itself be reduced by DT-diaphorase.

Synthesis of a Second BM Analog with an Electron Releasing Group

MBM proved to be a very interesting analog. It demonstrated that a methoxy group was able to reverse the role of DT-diaphorase, such that MBM was activated by DT-diaphorase. In order to establish whether this effect is specific to a methoxy group, or can be applied to all electron releasing groups, a BM analog with a different electron releasing group is needed for study . Other electron releasing groups that might be used are an ethoxy,

a butoxy or an amino group.

Studies of the Cytotoxicity of BM Analogs

A complete dose-response study of the cytotoxicity of CBM and PBM in H661 cells in the presence, and absence, of dicoumarol is required. This would validate the results from the single dose study.

The comparison of the cytotoxicity of BM analogs in SK-MEL-28 and H661 cells in the presence of dicoumarol suggested that PBM was dependent on cytohrome P450 reductase levels, but CBM was not. There appeared to be a small dependence for BM and MBM that was not statistically significant. It is possible that a larger difference in the cytochrome P-450 reductase levels would illustrate a dependence of the cytotoxicity of BM and MBM on cytochrome P-450 reductase levels. Thus, it may be better to study the cytotoxicity of BM analogs in MDA-MB-A231 cells that have, or have not, been transfected with cytochrome P-450 reductase. Wild-type MDA-MB-231 is a human breast cancer cell line which has very low cytochrome P-450 reductase activity. Patterson *et al.* (1997) transfected MDA-MB-231 cells with human cytochrome P-450 reductase to produce a cell line that had a 53-fold increase in the cytochrome P-450 reductase activity. A comparison of the cytotoxicity of BM analogs in these two cell lines may amplify the small differences that were seen in the comparison of the cytotoxicity of BM analogs in SK-MEL-28 and H661 cells.

Rate of Reduction of CBM and PBM by DT-Diaphorase

The study of the cytotoxicity of PBM in H661 and SK-MEL-28 cells in the presense, and absence, of dicoumarol has suggested that PBM may not be a substrate for DT-diaphorase. Additionally, the study of the cytotoxicity of CBM in H661 cells in the presence, and absence, of dicoumarol suggested that CBM may not be a substrate for DT-diaphorase. In comparison, the cytotoxicity of CBM in SK-MEL-28 cells in the presence, and absence, of dicoumarol suggested that CBM may be inactivated by DT-diaphorase. Thus, it should be determined whether CBM and PBM are substrates of DT-diaphorase. This could be done by directly studying reduction of PBM and CBM by DT-diaphorase using a method similar to the study of the rate of reduction of MBM and BM by DT-diaphorase.

Rate of Reduction of BM Analogs by Cytochrome P-450 Reductase

The comparison of the cytotoxicity of BM analogs in H661 cells and SK-MEL-28 cells in the presence of dicoumarol suggested that PBM is a substrate of cytochrome P-450 reductase, while BM, CBM and MBM may not be substrates for cytochrome P-450 reductase. Each of these results could be confirmed by a direct study of reduction of BM analogs by cytochrome P-450 reductase.

Analysis of the rate of reduction of BM analogs by cytochrome P-450 reductase would require the measurement of each of the rate of consumption of the BM analog, rate of consumption of NADPH and rate of formation of the hydroquinone of the analog. The

rate of reduction of the BM analog would provide information on the rate at which the BM analog is reduced by cytochrome P-450 reductase to the semiquinone state. Additionally, the rate of reduction of NADPH and rate of formation of the hydroquinone of the BM analog could be compared with the rate of consumption of the BM analog to provide information on the rate of reduction of the semiquinone of the BM analog by cytochrome P-450 reductase to the hydroquinone.

Complete Kinetic Analysis of Reduction of MBM and BM by DT-Diaphorase

A time course analysis of the reduction of MBM and BM by DT-diaphorase has revealed that BM is reduced more readily than MBM. It remains to be determined whether this difference is a result of a difference in the overall rate at which MBM and BM are reduced by DT-diaphorase, or a difference in the affinity of BM and MBM for the active site of DT-diaphorase.

Differences in the overall rate of reduction of MBM and BM by DT-diaphorase could be determined by measuring V_{max}, the maximal initial velocity, of the reduction of both MBM and BM by DT-diaphorase. The greater the V_{max}, the greater the overall rate of reduction.

Difference in the affinity of MBM and BM for the active site of DT-diaphorase could be illustrated by determining the affinity constant, K_M, of BM and MBM for reduction by DT-diaphorase. K_M is the concentration of BM or MBM that produces one-half the maximal initial velocity of reduction of BM or MBM, respectively. The greater the K_m, the lower the

affinity of BM or MBM for the active site of DT-diaphorase.

DT-diaphorase is a ping-pong type enzyme, and the rate of reduction of MBM or BM is dependent on the concentration of MBM or BM, respectively, and the concentration of NAD(P)H. The rate equation for a ping-pong enzymatic reaction is as follows:

$$1/v_o = K_M^A/V_{max}[A] + K_M^B/V_{max}[B] + 1/V_{max}$$ Voet *et al.* (1990).

Where: v_o is the initial rate of the reaction

K_M^A is the affinity constant for substrate A

K_M^B is the affinity constant for substrate B

V_{max} is the maximal velocity of the reaction

Based on this rate equation, V_{max} and K_M for reduction of BM and MBM by DT-diaphorase may be determined by measuring the initial rate of reduction of MBM and BM at various concentrations of BM and MBM for various concentrations of NADH.

Cross- Linking Activity

It has been illustrated that the product of reduction of MBM by DT-diaphorase is able to re-oxidize and continue to be reduced by DT-diaphorase, while the product of reduction of BM by DT-diaphorase is unable to re-oxidize and continue to be reduced by DT-diaphorase. This difference may be due to the ability of the mustard moiety on the product

of reduction of MBM by DT-diaphorase to form aziridinyl rings, versus the inability of the mustard moiety on the product of reduction of BM by DT-diaphorase to form aziridinyl rings (see results and discussion). A difference in the ability of the mustard moiety of reduced BM versus MBM to form aziridinyl rings suggests a difference in the dependence of the cross-linking activity of the mustard moiety on redox state. It would be expected that MBM reduced by DT-diaphorase would form cross- links more efficiently, than BM reduced by DT-diaphorase.

The agarose gel method for analysis of cross-linking activity, developed by Hartley *et al.* (1991), could not be applied to study the cross-linking activity of MBM or BM. The lack of success may be due to an inherent problem with pBR322 DNA. An alternative may be to use Lambda phage DNA. Cross-linking activity of MMC and EO9 reduced by DT-diaphorase using Lambda phage has been studied previously using the the ethidium bromide fluorescence method (Maliepaard *et al.*, 1995).

DNA Strand Break Activity

The illustration that the product of reduction of MBM by DT-diaphorase is able to re-oxidize and continue to be reduced by DT-diaphorase, while the product of reduction of BM by DT-diaphorase is unable to re-oxidize and continue to be reduced by DT-diaphorase additionally suggests that MBM is able to participate in redox cycling reactions to a much larger extent than BM. It would be expected that MBM would be able to create a much larger number of DNA strand breaks than BM.

The DNA strand break produced by the reduction of BM and MBM by DT-diaphorase could be evaluated using a method developed by Chen *et al.* (1995). pBR322 DNA is incubated with BM or MBM in the presence, and absence, of DT-diaphorase. Strand breaks will relax the DNA, and convert it from supercoiled to a more relaxed form. Supercoiled and relaxed pBR322 are separated using agarose gel electrophoresis, and each can be quantified. The extent of strand break activity can be quantified by comparing the amount of supercoiled to relaxed pBR322. The amount of strand breaks in pBR322 treated with BM and MBM in the presence, and absence, of DT-diaphorase could be compared to illustrate differences in DNA strand break activity of BM and MBM reduced and not reduced by DT-diaphorase.

Isolation of the Product of Reduction of BM and MBM by DT-Diaphorase

The products of reduction of BM and MBM by DT-diaphorase could be determined by an ^{1}H nmr analysis of the reduction reaction. The formation of and fate of the respective hydroquinone of BM and MBM could be determined by following the appearance and disappearance of specific proton peaks on the ^{1}H nmr spectrum of the reduction reaction. The formation of the hydroquinone would be expected to produce a significant shift in the resonance, or chemical shift, of each of the hydrogens on the quinone ring. The formation of the aziridinyl ring versus the formation of the cyclized product (see Figures D-1 and D-2) would be expected to produce significantly different shifts in the resonance of the hydrogens on the chloro ethyl groups of the mustard moiety.

Completion of the above studies would provide a solid understanding of the role an electron releasing, electron withdrawing and sterically bulky group in generating specificity for reduction of a benzoquinone mustard bioreductive agent by DT-diaphorase or NADPH:cytochrome P-450 reductase. The information generated from this work may be useful in the design of future bioreductive agents that are specifically activated by DT-diaphorase or NADPH:cytochrome P-450 reductase. Bioreductive agents could be developed containing one or several functional groups, and functional groups would be selected based on the results of the above studies.

References

Amin GC and Shah NM (1943) 2,5-Dihydroxyacetophenone. *Organic Synthesis*, **2**: 42-43.

Backers WL and Recker Backes CE (1988) The Effect of NADPH Concentration on the Reduction of Cytochrome P-450 LM2. *Journal of Biological Chemistry*, **263**: 47-253.

Bailey SM, Suggett N, Walton MI and Workman P (1992) Structure-Activity Relationships for DT-Diaphorase Reduction of Hypoxic Cell Directed Agents: Indoloquinones and Diaziridinyl Benzoquinones. *Internation Journal of Radiation Oncology*, **22**: 649-653.

Beall HD, Lui Y, Seigal D, Bolton EM, Gibson NW and Ross D (1996) Role of NAD(P)H:Quinone Oxidoreductase (DT-Diaphorase) in Cytotoxicity and Induction of DNA Damage by Streptonigrin. *Biochemical Pharmacology*, **51**: 645-652.

Beall HD, Murphy AM, Seigal D, Hargreaves RHJ, Butler J and Ross D (1995) Nicotinamide Adenine Dinucleotide (Phosphate): Quinone Oxidoreductase (DT-Diaphorase) as a Target for Bioreductive Antitumor Quinones: Quinone Cytotoxicity and Selectivity in Human Lung and Breast Cancer Cell Lines. *Molecular Pharmacology*, **48**: 499-504.

Begleiter A, Leith MK, Wang X and Doherty GP (1998) Induction of DT-Diaphorase by 1,2-Dithiole-3-Thiones in Human Tumour and Normal Cells and Effect on Antitumour Activity of Bioreductive Agents. *British Journal of Cancer*, in press.

Begleiter A, Leith MK and Curphey TJ (1996) Induction of DT-Diaphorase by 1,2-Dithiole-3-Thione and Increase of Antitumour Activity of Bioreductive Agents. *British Journal of Cancer*, **74**: S9-S14.

Begleiter A and Leith MK (1993) Role of NAD(P)H:(Quinone Acceptor) Oxidoreductase (DT-Diaphorase) in Activation of Mitomycin C under Acidic Conditions. *Molecular Pharmacology*, **44**: 210-215.

Begleiter A, Robotman E and Leith MK (1992) Role of NAD(P)H:(Quinone Acceptor) Oxidoreductase (DT-Diaphorase) in Activation of Mitomycin C under Hypoxia. *Molecular Pharmacology*, **41**: 677-682.

Begleiter A and Leith MK (1990) Activity of Quinone Alkylating Agents in Quinone-Resistant Cells. *Cancer Research*, **50**: 2872-2876.

Begleiter A, Robotham E, Lacey G and Leith MK (1989) Increased Sensitivity of Quinone Resistant Cells to Mitomycin C. *Cancer Letters*, **45**: 173-176.

Begleiter A, Leith MK, McClarty G, Beenken S, Goldenberg GJ and Wright JA (1985) Characterization of L5178 Murine Lymphoblasts Resistant to Quinone Antitumor Agents. *Cancer Research*, **48**: 1727-1735.

Begleiter A and Blair GW (1984) Quinone-Induced DNA Damage and its Relationship to Antitumor Activity in L5178Y Lymphoblasts. *Cancer Research*, **44**: 78-82.

Begleiter (1983) Cyotocidal Action of the Quinone Group and Its Relationship to Antitumor Activity. *Cancer Research*, **43**: 481-484.

Belcourt MF, Hodnick WF, Rockwell S and Sartorelli AC (1996a) Differential Toxicity of Mitomycin C and Porfiromycin to Aerobic and Hypoxic Chinese Hamster Ovary Cells Overexpressing Human NADPH:Cytochrome P-450 Reductase. *Biochemical Pharmacology* , **93**: 456-460.

Belcourt MF, Hodnick WF, Rockwell S and Sartorelli AC (1996b) Bioactivation of Mitomycin Antibiotics and Hypoxic Chinese Hampster Ovary Cells Overexpressing DT-Diaphorase. *Biochemical Pharmacology*, **51**: 1669-1678.

Benson AM, Hunkeler MJ and Talalay P (1980) Increase of NAD(P)H:Quinone Reductase by Dietary Antioxidants: Possible Role in Protection Against Carcinogenesis and Toxicity. *Biochemistry*, **9**: 5216-5220.

Biedermann KA, Wang J, Graham RP and Brown JM (1991) SR 4233 Cytotoxicity and Metabolism in DNA Repair-competent and Repair-Deficient Cell Cultures. *British Journal of Cancer*, **63**: 358-362.

Bligh HFJ, Bartoszek A, Robson CN, Hickson ID, Kasper CB, Beggs JD and Wolf CR (1990) Activation of Mitomycin C by NADPH:Cytochrome P-450 Reductase. *Cancer Research*, **50**: 7789-7792.

Brown JM and Lemmon MJ (1990) Potentiation by the Hypoxic Cytotoxin SR 4233 of Cell Killing Produced by Fractional Irradiation of Mouse Tumors. *Radiation Oncology*, **50**: 7745-7749.

Butler J, Spanswick VJ and Cummings J (1996) The Autoxidation of the Reduced Forms of EO9. *Free Radical Research*, **29**: 141-148.

Chen S, Knox R, Wu K, Deng PSK, Zhou D, Bianchet MA and Amzel IM (1997) Molecular Basis of the Catalytic Differences among DT-Diaphorase of Human, Rat and Mouse. *The Journal of Biological Chemistry*, **272**: 1437-1439.

Chen W, Blazek ER and Rosenberg I (1995) The Relaxation of Supercoiled DNA Molecules as a Biophysical Dosimeter for Ionizing Radiations: A Feasibility Study. *Medical Physics*, **22**: 1369-1375.

Coia LR (1993) The Use of Mitomycin in Esophageal Cancer. *Oncology Research*, **50:** Suppl 1: 53-62.

Colinas RJ, Hunt DH, Walsh AC and Lawrence DA (1996) Hydroquinone Resistance in a Murine Myeloblastic Leukemia Cell Line. *Biochemical Pharmacology*, **52**: 945-956.

Collard J, Matthew AM, Double JA and Bibby MC (1995) EO9: Relationship Between DT-Diaphorase Levels and Response *in Vitro* and *in Vivo*. *British Journal of Cancer*, **71**: 1199-1203.

Cone R, Hasan SK, Lown JW and Morgan AR (1976) The Mechanism of the Degradation of DNA by Streptonigrin. *Canadian Journal of Biochemistry*, **54**: 219-223.

Corral C (1957) Reaction of 4-Vinylindan and other Styrenes with Substituted p-Benzoquinones. *Rev. Real Acad. cienc. exact. fis. y. mat. Madrid*, **51**: 10-138.

Costantini C, d'Ischia M and Prota G (1994) Persulfate/Copper (II): A Mild and Efficient Oxidising System for the Conversion of Dihydroxybenzenes to Quinones. *Synthesis*, **Short papers**: 1399-1400.

Crooke ST and Bradner WT (1976) Mitomycin C: A Review. *Cancer Treatment Reviews*, **2**: 121-139.

Crosby AH and Lutz RE (1956) A Study of an Oxidative-Amination Method for the Synthesis of Aminoquinones. *Journal of Medicinal Chemistry*, **90**: 1233-1235.

Dirix LY, Tonnesen F, Cassidy J, Epelbaum R, ten Bokkel Huinink WW, Pavlidis N, Sorio R, Gamucci T, Wolff I, Te Velde A, Lan J and Verweij J (1996) EO9 Phase II Study in Advanced Breast, Gastric, Pancreatic and Colorectal Carcinoma by the EORTC Early Clinical Studies Group. *European Journal of Cancer*, **32A**: 2019-2022.

Djuric Z, Corbett TH, Valeriote FA, Heilbrun LK and Baker LH (1995) Detoxification Ability and Toxicity of Quinones in Mouse and Human Tumor Cell Lines Used for Anticancer Drug Screening. *Cancer Chemotherapy and Pharmacology*, **36**: 20-26.

Doostdar H, Demoz A, Burke MD, Melvin WT and Grant MH (1990) Variation in Drug-Metabolizing Enzyme Activities During the Growth of Human Hep G2 Hepatoma Cells. *Xenobiotica*, **4**: 435-441.

Dorie MJ and Brown JM (1993) Tumor-Specific, Schedule Dependent Interaction Between Tirapazamine (SR 4233) and Cisplatin. *Cancer Research*, **53**: 4633-4636.

Doroshow J, Akman S and Forrest G (1992) Effect of DT-Diaphorase Overexpression on Mitomycin C Toxicity for Chinese Hampster Ovary Cells. *Proceeding of the American Association for Cancer Research*, **33**: 507.

Doroshow JH (1986) Role of Hydrogen Peroxide and Hydroxyl Radical Formation in the Killing of Ehrlich Tumor Cells by Anticancer Quinones. *Proceedings of the National Academy of Sciences. USA*, **83**: 4514-4518.

Dorshkind K (1990) Regulation of Hemopoiesis by Bone Marrow Stromal Cells and Their Products. *Annual Reviews of Immunology*, **8**: 111-137.

Dreher D and Junod AF (1996) Role of Oxygen Free Radicals in Cancer Development. *European Journal of Cancer*, **32A**: 30-38.

Driebergen RJ, Holthuis JJM, Hulshoff A, Postma-kelder SJ, Verboom W, Reinhoudt DN and Lelieveld P (1986) Electrochemistry of Potential Bioreductive Alkylating Quinones: Its Use in the Development of New Aziridinylquinones. *Anticancer Research*, **6**: 605-620.

Dulhanty AM and Whitmore GF (1991) Chinese Hampster Ovary Cell Lines Resistant to Mitomycin C under Aerobic but not Hypoxic Conditions are Deficient in DT-Diaphorase. *Cancer Research*, **51**: 1860-1865.

Elwell JH, Sum BG, Ewans JW and Brown JM (1997) Adaptation of Human Tumor Cells to Tirapazamine under Areobic Conditions. *Biochemical Pharmacology*, **54**: 249-257.

Ernster L, Ljunggren M and Danielson L (1960) Purification and some Properties of a Highly Dicoumarol-Sensitive Liver Diaphorase. *Biochemical and Biophysical Research Communications*, **2**: 88.

Ernster L and Navazio F (1958) Soluble Diaphorase in Animal Tissues. *Acta Chemica Scandia*, **12**: 595-602.

Fitzsimmons SA, Workman P, Grever M, Paull K, Camalier R and Lewis AD (1996) Reductase Enzyme Expression Across the National Cancer Institute Tumor Cell Line Panel: Correlation with Sensitivity to Mitomycin C and EO9. *Journal of the National Cancer Institute*, **88**: 259-269.

Fitzsimmons SA, Lewis AD, Riley RJ and Workman P (1994) Reduction of 3-Amino-1,2,4-Benzotriazine-1,4-Di-N-Oxide (Tirapazamine, WIN 59075 SR4233) to a DNA-Damaging Species: A Direct Role of NADPH:Cytochrome P-450 Oxidoreductase. *Carcinogenesis*, **45**: 1503-1510.

Folman RS (1993) Experience with Mitomycin in the Treatment of Non-Small Cell Lung Cancer. *Oncology*, **50 Suppl**: 24-30.

Frank W and Osterberg AE (1960) Mitomycin C (NSC 26980)- An Evaluation of the Japanese Reports. *Cancer Chemotheraphy Reports*, **9**: 114-119.

Ganousis LG, Goon D, Zyglewska T, Wu KK and Ross D (1992) Cell-Specific Metabolism in Mouse Bone Marrow Stroma: Studies of Activation and Detoxification of Benzene Metabolites. *Biochemical Pharmacology*, **42**: 1118-1125.

Goldstein J and Falleto MB (1993) Advances in Mechanisms of Activation and Deactivation of Environmental Chemicals. *Environmental Health Perspectives*, **100**: 169-176.

Gustafson DL and Beall HD, Bolton EM, Ross D and Waldren CA (1996) Expression of Human NAD(P)H:Quinone Oxidoreductase (DT-Diaphorase) in Chinese Hampster Ovary Cells: Effect on the Toxicity of Antitumor Quinones. *Molecular Pharmacology*, **50**: 728-735.

Gustafson DL, Pristos C (1992) Bioactivation of Mitomycin C by Xanthine Dehydrogenase from EMT6 Mouse Mammary Carcinoma Tumors. *Journal of the National Cancer Institute*, **84**: 1180-1185.

Hartley JA Berardini MD and Souhami RL (1991) An Gel Method for the Determination of DNA Interstrand Crosslinking Applicable to the Measurement of the Rate of Total and "Second-Arm" Crosslink Reactions. *Anals of Biochemistry*, **193**: 131-134.

Hoban PR, Walton MI, Robson CN, Godden J, Stratford I, Workman P, Harris AL and

Hickson ID (1990) Decreased NADPH:Cytochrome P-450 Reductase Activity and Impaired Drug Activation in Mammalian Cell Line Resistant to Mitomycin C under Aerobic but not Hypoxic Conditions. *Cancer Research*, **50**: 4692-4697.

Hodnick WF and Sartorelli AC (1993) Reductive Activation of Mitomycin C by NADH:Cytochrome b_5 Reductase. *Cancer Research*, **53**: 4907-4912.

Holden SA, Ticher BA, Ara G, Herman TS and Coleman CN (1992) Enhancement of Alkylating Agent Activity by SR-4233 in the FsaIIC Murine Fibrosarcoma. *Journal of the National Cancer Institute*, **84**: 187-193.

Hortorbagyi GN (1993) Mitomycin C: Its Evolving Role in the Treatment of Breast Cancer. *Oncology*, **50 Suppl**: 1-8.

Iyanagi T, Makino N and Mason HS (1974) Redox Properties of the Reduced Nicotinamide Adenine Dicnucleotide Phosphate-Cytochrome P-450 and Reduced Nicotinamide Adenine Dinucleotide-Cytochrome b_5 Reductase. *Biochemistry*, **13**: 1701-1710.

Johnston JB, Verberg L, Shore T, Williams M, Israels LG and Begleiter A (1994) Combination Therapy with Nucleoside Analogs and Alkylating Agents. *Leukemia*, **8**, Suppl. 1: S140-S143.

Kappus H (1986) Overview of Enzyme Systems Involved in Bio-Reduction of Drugs and Redox Cycling. *Biochemical Pharmacology*, **35**: 1-6.

Kasper CB (1971) Biochemical Distinctions between the Nuclear and Microsomal Membranes from Rat Hepatocytes: The Effect of Phenobarbitol Administration. *The Journal of Biological Chemistry*, **246**: 577-581.

Kennedy KA, McGurl JD, Leondaridis L and Alabaster O (1985) pH-Dependence on Mitomycin C-Induced Cross-Linking Activity in EMT6 Tumor Cells. *Cancer Research*, **45**: 3541-3547.

Kennedy KA, Seigfried JM, Sartorelli AC and Tritton TR (1983) Effects of Anthracyclines on Oxygenated and Hypoxic Tumor Cells. *Cancer Research*, **43**: 54-59.

Keyes SR, Rockwell S and Sartorelli AC (1989) Modification of the Metabolism and Cytotoxicity of Bioreductive Alkylating Agents by Dicoumarol in Aerobic and Hypoxic Murine Tumor Cells. *Cancer Research*, **49**: 3310-3313.

Keyes SR, Rockwell S and Sartorelli AC (1985) Enhancement of Mitomycin C Cytotoxicity to Hypoxic Tumor Cells by Dicoumarol *in Vivo* and *in Vitro*. *Cancer Research*, **45**: 213-216.

Keyes SR, Fracasso PM, Heimbrook DC, Rockwell S, Sliger SG and Sartorelli AC (1984) Role of NADPH:Cytochrome c Reductase and DT-Diaphorase in the Biotransformation of Mitomycin C. *Cancer Research*, **44**: 5638-5643.

Kurosawa E (1961) Quinones. I. The Synthesis of Acetyl-p-Benzoquinone Derivatives and 2-Acetylnaphthoquinone. *Journal of the Chemical Society of Japan*, **34**: 300-304.

Li R, Bianchet MA, Talalay P and Amzel LM (1995) The Three Dimensional Structure of NAD(P)H Quinone Reductase, a Flavoprotein Involved in Cancer Chemoprevention and Chemotherapy: Mechanism of the Two Electron Reduction. *Proceedings of the National Academy of ScienceUSA*, **92**: 8846-8850.

Lichtman MA (1981) The Ultrastructure of the Hemopoietic Environment of the Marrow: A Review. *Experiments in Hematology*, **9**: 391-410.

Lin AJ, Cosby LA, Shansky CW and Sartorelli AC (1972) Potential Bioreductive Alkylating Agents. 1. Benzoquinone Derivatives. *Journal of Medicinal Chemistry*, **15**: 1247-1251.

Lind C, Cadenas E, Hochstein P and Ernster L (1990) DT-Diaphorase: Purification, Properties, and Function. *Methods in Enzymology*, **186**: 287-301.

Lown JW, Begleiter A, Johnson D and Morgan AR (1976) Studies Related to Antitumor Antibiotics. V. Reaction of Mitomycin C with DNA Examined by Ethidium Fluorescence Assay. *Canadian Journal of Biochemistry*, **54**: 110-119.

Makarova AN and Berlin AY (1967) Di-(2-chloroethyl)-Amino-p-Benzoquinones and Their Derivatives. *Zhural Obshchei Khimii*, **37**: 637-642.

Maliepaard M, Wolfs A, Groot SE, de Mol NJ and Janssen LH (1995) Indoloquinone EO9: DNA Interstrand Cross-Linking upon Reduction by DT-Diaphorase or Xanthine Oxidase. *British Journal of Cancer*, **71**: 836-839.

Malkinson AM, Seigal D, Forrest GL, Gadzar AF, Oie HK, Chan DC, Bunn PA, Mabry M, Dykes DJ, Harrison SD and Ross D (1992) Elevated DT-Diaphorase Activity and Messanger RNA Content in Human Non-Small Cell Lung Carcinoma: Relationship to the Response of Lung Tumor Xenografts to Mitomycin C. *Cancer Research*, **52**: 4752-4757.

Marshall RS, Patterson MC and Rauth AM (1991) DT-Diaphorase Activity and Mitomycin C Sensitivity in Non-Transformed Cell Strains Derived from Members of a Cancer Prone Family. *Carcinogenesis*, **12**: 1175-1180.

Marshall RS, Patterson MC and Rauth AM (1989) Deficient Activation by a Human Cell

Strain Leads to Mitomycin Resistance under Aerobic but not Hypoxic Conditions. *British Journal of Cancer*, **59**: 341-346.

Masters BSS, Bilimoria MH and Kamin MH (1965) The Mechanism of 1- and 2- Electron Transfers Catalyzed by Reduced Triphosphopyridine Nucleotide-Cytochrome c Reductase. *Journal of Biological Chemistry*, **240**: 4081-4088.

McLeod HL, Graham MA, Aamdal S, Setanoians A, Groot Y and Lund B (1996) Phase I Pharmacokinetics and Limited Sampling Strategies for the Bioreductive Alkylating Drug EO9. *European Journal of Cancer*, **9**: 1518-1522.

McMurray (1992) *Organic Chemistry* eds. Pantzis HC and Mialousis N (Brooks/Cole Publishing Company) 584-585.

Moore HW (1977) Bioactivation as a Model for Drug Design Bioreductive Alkylation. *Science*, **5**: 527-532.

Narayanasami R, Horowitz PM and Masters BSS (1995) Flavin-Binding Protein Structural Integrity Studies on NADPH-Cytochrome P450 Reductase are Consistent with the Presence of Distinct Domains. *Archives of Biochemistry and Biophysics*, **316**: 267-274.

O'Dwyer PJ, Perez RP, Yao KS, Godwin AK and Hamilton TC (1996) Increased DT-Diaphorase Expression and Cross-Resistance to Mitomycin C in a Series of Cisplatin-Resistant Human Ovarian Cancer Cell Lines. *Biochemical Pharmacology*, **52**: 21-27.

O'Dwyer PJ, Yao KS, Ford P, Godwin AK and Clayton M (1994) Effects of Hypoxia on Detoxicating Enzyme Activity and Expression in HT29 Colon Adenocarcinoma Cells. *Cancer Research*, **54**: 3082-3087.

Pan S, Akman SA, Forrest GL, Hipsher C and Johnson R (1992) The Role of NADPH:Quinone Oxidoreductase in Mitomycin C- and Porfiromycin-resistant HCT 116 Human Colon-cancer Cells. *Cancer Chemotherapy and Pharmacology*, **31**: 23-31.

Pan S, Iracki T and Bachur NR (1986) DNA Alkylation by Enzyme-Activated Mitomycin C. *Molecular Pharmacology*, **29**: 622-628.

Pan S, Andrews PA, Glover CJ and Bachur NR (1984) Reductive Activation of Mitomycin C and Mytomycin Metabolites Catalyzed by NADPH:Cytochrome P-450 Reductase and Xanthine Oxidase. *Journal of Biological Chemistry*, **259**: 959-966.

Patterson AV, Barham HM, Chinje EC, Adams GE, Harris AL and Stratford IJ (1995)

Importance of P450 Reductase Activity in Determining Sensitivity of Breast Tumour Cells to the Bioreductive Drug, Tirapazamine (SR 4233). *British Journal of Cancer*, **72**: 1144-1150.

Patterson AV, Roberstson N, Houlbrook S, Stephens MA, Adams GE, Harris AL, Stratford J and Carmichael J (1994) The Role of DT-Diaphorase in Determining the Sensitivity of Human Tumor Cells to Tirapazamine (SR 4233). *International Journal of Radiation Oncology Biology and Physics*, **2**: 369-372.

Plumb JA, Gerristsen M, Milroy R, Thomson P and Workman P (1994) Relative Importance of DT-Diaphorase and Hypoxia in the Bioactivation of EO9 by Human Lung Tumor Cell Lines. *International Journal of Radiation Oncology Biology and Physics*, **2**: 295-299.

Plumb JA and Workman P (1994) Unsually Marked Hypoxic Sensitization to Indoloquinone EO9 and Mitomycin C in a Human Colon-Tumor Cell Line that Lacks DT-Diaphorase Activity. *International Journal of Cancer*, **56**: 134-139.

Porter TD and Kasper CB (1986) Complete Amino Acid Sequence of NADPH:Cytochrome P450 Reductase from Porcine Hepatic Microsomes. *Biochemistry*, **12**: 2297-2308.

Pospisil J, Lisa E and Buben I (1970) Anitoxidants and Stabilizers-XXII: Comparison of the

Antioxidative Activities of Dihydric Phenols and Their Oxidation Products. *European Polymer Journal*, **6**: 1347-1358.

Powis G, Gasdaska PY, Gallegos A, Sherrill K and Goodman D (1995) Over-Expression of DT-Diaphorase in Transfected NIH 3T3 Cells does not Lead to Increased Anticancer Quinone Drug Sensitivity: A Questionable Role for the Enzyme as a Target for Bioreductively Activated Anticancer Drugs. *Anticancer Research*, **15**: 1141-1146.

Prestera T, Zhang Y, Spencer SR, Wilczak CA and Talalay P (1993a) The Electrophile Counterattack Response: Protection Against Neoplasia and Toxicity. *Advances in Enzyme Regulation*, **33**: 281-296.

Prestera T, Holtaclaw WD, Zhang Y and Talalay P (1993b) Chemical and Molecular Regulation of Enzymes that Detoxify Carcinogens. *Proceedings of the National Academy of Sciences, USA*, **90**: 2965-2969.

Prichard WW (1943) Hydroquinone Diacetate (Quinol Diacetate). *Organic Synthesis*, **2**: 68-69.

Pristos C (1996) Re: Reductase Enzyme Expression Across the National Cancer Institute Tumor Cell Line Panel: Correlation With Sensitivity to Mitomycin C and EO9. *Journal of the National Cancer Institute, Correspondence*, **88**: 1161-1162.

Pristos C and Sartorelli AC (1986) Generation of Reactive Oxygen Radicals through Bioactivation of Mitomycin Antibiotics. *Cancer Research*, **46**: 3528-2532.

Prochaska HJ and Santamaria AB (1988) Direct Measurement of NAD(P)H: Quinone Reductase from Cells Cultured in Microtiter Well: A Screening Assay for Anticarcinogenic Enzyme Inducers. *Analytical Biochemistry*, **169**: 328-336.

Pruesch PC, Seigal D, Gibson NW and Ross D (1991) A Note on the Inhibition of DT-Diaphorase by Dicoumarol. *Free Radical Biology and Medicine*, **11**: 77-80.

Rauth AM, Marshall RS and Kuehl BL (1993) Cellular Approaches to Bioreductive Drug Mechanisms. *Cancer and Metastasis Reviews*, **12**: 153-164.

Rauth AM, Mohindra JK and Tannock IF (1983) Activity of Mitomycin C for Aerobic and Hypoxic Cells *in Vitro* and *in Vivo*. *Cancer Research*, **43**: 4154-4158.

Riley RJ and Workman P (1992a) Enzymology of the Reduction of the Potent Benzotriazine-Di-N-Oxide Hypoxic Cell Cytotoxin SR4233 by NAD(P)H:(Quinone Acceptor) Oxidoreductase Purified from Walker 256 Rat Tumor Cells. *Biochemical Pharmacology*, **43**: 167-174.

Riley RJ and Workman P (1992b) DT-Diaphorase and Cancer Chemotherapy. *Biochemical*

Pharmacology, **43**: 1657-1665.

Robertson N, Stratford IJ, Houlbrook S, Carmichael J and Adams GE (1992) The Sensitivity of Human Tumor Cells to Quinone Bioreductive Drugs: What Role for DT-Diaphorase? *Biochemical Pharmacology*, **44**: 409-412.

Rockwell S, Sartorelli AC, Tomasz M and Kennedy KA (1993) Cellular Pharmacology of Quinone Bioreducitve Alkylating Agents. *Cancer and Metastasis Reviews*, **12**: 165-176.

Rockwell S, Keyes SR and Sartorelli AC (1988) Modulation of the Cytotoxicity of Mitomycin C to EMT6 Mouse Mammary Tumor Cells by Dicoumarol *in Vitro*. *Cancer Research*, **48**: 5471-5474.

Ross D, Beall H, Traver RD, Siegal D, Phillips RM and Gibson NW (1994) Bioactivation of Quinones by DT-Diaphorase, Molecular, Biochemical and Chemical Studies. *Oncology Research*, **6**: 493-500.

Ross D, Siegal D, Beall H, Prakash AS, Mulcahy RT and Gibson NW (1993) DT-Diaphorase in Activation and Detoxification of Quinones: Bioreductive Activation of Mitomycin C. *Cancer and Metastasis Reviews*, **12**: 83-101.

Ross WCJ (1964) Aryl-2-Halogenoalkylarylamines-XXI. The Design of Agents to Exploit Differences in Cellular Oxidation-Reduction Potentials. *Biochemical Pharmacology*, **13**: 969-982.

Sawamura AO, Aoyama T, Tamakoshi K, Mizuno K, Suganuma N, Kikkawa F and Tomoda Y (1996) Transfection of Human Cytochrome P-450 Reductase cDNA and Its Effect on the Sensitivity of Toxins. *Oncology*, **53**:406-411.

Schellen JHM, Planting AST, van Acker BAC, Loos WJ, de Boer-Dennert M, van der Burg MEL, Koier I, Krediet RT and Stoter G an Verweij J (1996) Phase I and Pharmacologic Study of the Novel Indoloquinone Bioreductive Alkylating Cytotoxic Drug EO9. *Journal of the National Cancer Institute*, **86**: 906-912.

Schlager JJ and Powis G (1990) Cytosolic NAD(P)H: Quinone Oxidoreductase (NQO1) Gene in Tumor Cells Compared to Normal Cells of the Same Origin. *Biochemical Pharmacology*, **42**: 1021-1027.

Schnall S and Macdonald JS (1993) Mitomycin Therapy in Gastric Cancer. *Oncology*, **50 Suppl 1**: 70-77.

Senan S, Rampling R, Graham MA, Wilson P, Robin H, Eckardt Jr. N, Lawson N, McDonald A, von Roemeling R, Workman P and Kaye SB (1997) Phase I and

Parmacokinetic Study of Tirapazamine (SR 4233) Administered Every Three Weeks. *Clinical Cancer Research*, **3**: 31-38.

Shen AL and Kasper CB (1993) *in Handbook of Experimental Pharmacology* eds. Schenkman JB and Greim H (Springer, New York) 35-39.

Siegal D, Beall H, Senekeowitsch C, Kasai M, Arai H, Gibson NW and Ross D (1992) Bioreductive Activation of Mitomycin C by DT-Diaphorase. *Biochemistry* **31**: 7879-7883.

Siegel D, Gibson NW, Preusch PC and Ross D (1990) Metabolism of Mitomycin C by DT-Diaphorase: Role in Mitomycin C-Induced DNA Damage and Cytotoxicity in Human Colon Carcinoma Cells. *Cancer Research*, **50**: 7483-7489.

Smith GC, Tew DG and Wolf CR (1994) Dissection of NADPH-Cytochrome P-450 Oxidoreductase into Distinct Functional Domains. *Proceedings of the National Academy of Science USA*, **91**: 8710-8714.

Smitskamp-Wilms E, Hendriks HR and Peters GJ (1996) Development, Pharmacology, Role of DT-Diaphorase and Prospects of the Indoloquinone EO9. *Generic Pharmacology*, **27**: 421-429.

Smitskamp-Wilms E, Giaccone G, Pinedo HM, van der Laan BFAM and Peters GJ (1995) DT-Diaphorase Activity in Normal and Neoplastic Human Tissues; an Indicator for Sensitivity to Bioreductive Agents. *British Journal of Cancer*, **72**: 917-921.

Spanswick VJ, Cummings J and Smyth JF (1996) Enzymology of Mitomycin C Metabolic Activation in Tumour Tissue. *Biochemical Pharmacology*, **51**: 1623-1630.

Strobel HW and Digman JD (1978) Purification and Properties of NADPH:Cytochrome P-450 reductase. *Methods in Enzymology*, **52**: 797-802.

Szmigiero L, Erickson LC, Ewig RA and Kohn KW (1984) DNA Strand Scission and Cross-Linking by Diaziridinylbenzoquinone (Diaziquone) in Human Cells and Relation to Cell Killing. *Cancer Research*, **44**: 4447-4452.

Teicher BA, Lazo SL and Sartorelli AC (1981) Classification of Antineoplastic Agents by Their Selective Toxicities toward Oxygenated and Hypoxic Tumor Cells. *Cancer Research*, **43**: 73-81.

Thomas DJ and Sadler A, Sunrahmanyam VV, Seigal D, Reasor MJ, Wierda D and Ross D (1990) Bone Marrow Stromal Cell Bioactivation and Detoxification of the Benzene Metabolite Hydroquinone: Comparison of Macrophages and Fibroblastoid Cells. *Molecular Pharmacology*, **37**: 255-262.

Tomasz M, Chowdary D, Lipman R, Shimotakahara S, Viero D, Walker V and Verdine GL (1986) Reaction of DNA with Chemically or Enzymatically Activated Mitomycin C: Isolation and Structure of the Major Covalent Adduct. *Biochemistry*, **83**: 6702-6706.

Tomasz M, Lipman R, Chowdary D, Pawlak L, Verdine GL and Nakanishi K (1987) Isolation and Structure of a Covalent Cross-Link Adduct between Mytomycin C and DNA. *Science (Washingston DC)*, **235**: 1204-1208.

Trush MA. Mimnaugh EG and Gram TE (1982) Activation of Pharmacological Agents to Reactive Intermediates. *Biochemical Pharmacology*, **33**: 3335-3346.

Twerdok LE, Rembish SJ and Trush MA (1992) Induction of Quinone Reductase and Glutathione in Bone Marrow Cells by 1,2-Dithiole-3-Thione: Effect on Hydroquinone-Induced Cytotoxicity. *Toxicology and Applied Pharmacology*, **112**: 273-281.

Vermillion JL and Coon MJ (1978) Indentification of the High and Low Potential Flavins of Liver Microsomal NADPH Cytochrome P-450 Reductase. *Journal of Biological Chemistry*, **253**: 8812-8819.

Voet D and Voet JG (1990) *Biochemistry* ed. Stiefel J (John Wiley and Son), 347.

Walters RS, Frye D, Budzar AU, Holmes FA and Hortobagyi N (1992) A Randomized Trial of Two Dosage Schedules of Mitomycin C in Advanced Breast Carcinoma. *Cancer*, **69**: 476-481.

Walton MI, Bibby MC, Double JA, Plumb JA and Workman P (1992) DT-Diaphorase Activity Correlates with Sensitivity to the Indoloquinones EO9 in Mouse and Human Colon Carcinomas. *European Journal of Cancer*, **28A**: 1597-1600.

Walton MI and Workman P (1990) Enzymology of the Reductive Bioactivation of SR4233: A Novel Benzotriazine-Di-N-Oxide Hypoxic Cell Cytotoxin. *Biochemical Pharmacology*, **39**: 1735-1742.

Wang J, Biederman KA, Wolf CR and Brown JM (1993) Metabolism of the Bioreductive Cytotoxin SR 4233 by Tumor Cells: Enzymatic Studies. *British Journal of Cancer*, **67**: 321-325.

Wang M, Roberts DL, Paschke R, Shea TM, Masters BS, Kim JP (1997) Three-Dimensional Structure of NADPH-Cytochrome P450 Reductase: Prototype for FMN- and FAD-Containing Enzymes. *Proceedings of the National Academy of Sciences USA*, **94**: 8411-8416.

Workman P and Stratford IJ (1993) The Experimental Development of Bioreductive Drugs

and Their Role in Cancer Therapy. *Cancer and Metastasis Reviews*, **12**: 73-82.

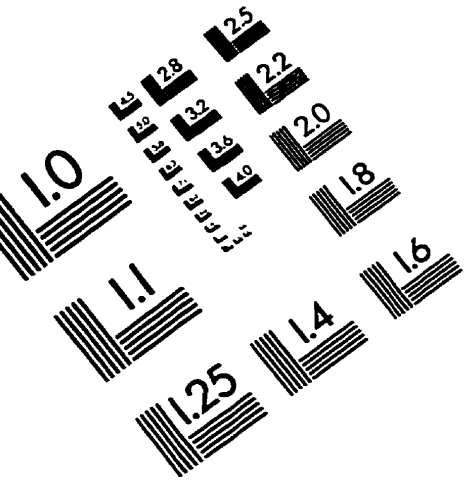
Workman P, Binger M and Kooistra KL (1992) Pharmacokinetics, Distribution and Metabolism of the Novel Bioreductive Alkylating Indoloquinones EO9 in Rodents. *International Journal of Radiation Oncology Biology and Physics*, **22**: 713-716.

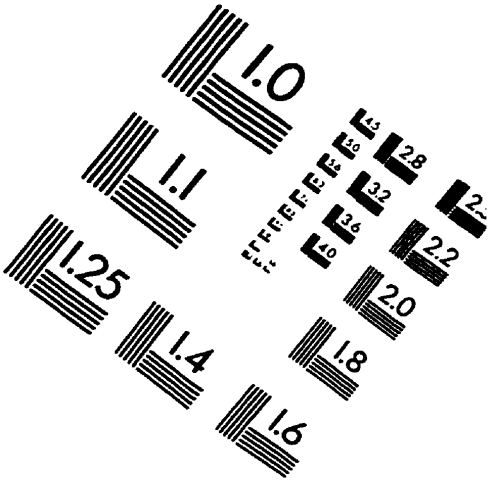
Workman P, Walton MI, Powis G and Schlager JJ (1989) DT-Diaphorase: Questionable Role in Mitomycin C Resistance, but a Target for Novel Bioreductive Drugs? *British Journal of Cancer*, **60**: 800-802.

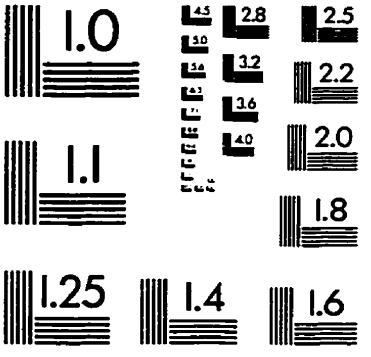
Yoshida T and Tsuda H (1995) Gene Targeting of DT-Diaphorase in Mouse Embryonic Stem Cells: Establishment of Null Mutant and its Mitomycin C Resistance. *Biochemical and Biophysical Resrearch Communications*, **214**: 701-708.

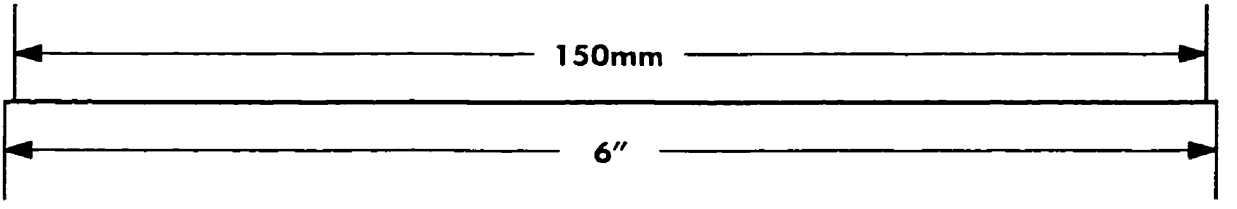
Zeman, EM, Brown JM, Lemmon MJ, Hirst VK and Lee WW (1986) SR 4233: A New Bioreductive Agent with High Selective Toxicity for Mammalian Cells. *International Journal of Radiation Oncology Biology and Physics*, **12**: 1239-1242.

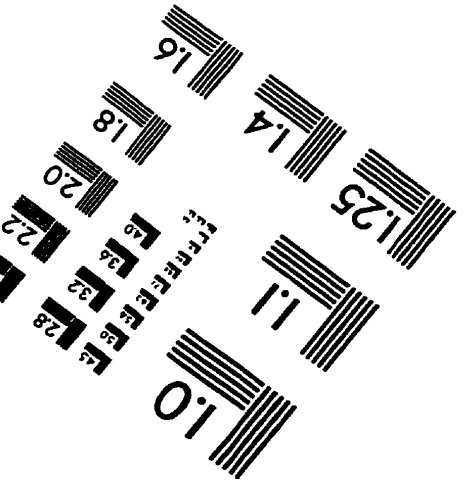
IMAGE EVALUATION
TEST TARGET (QA-3)

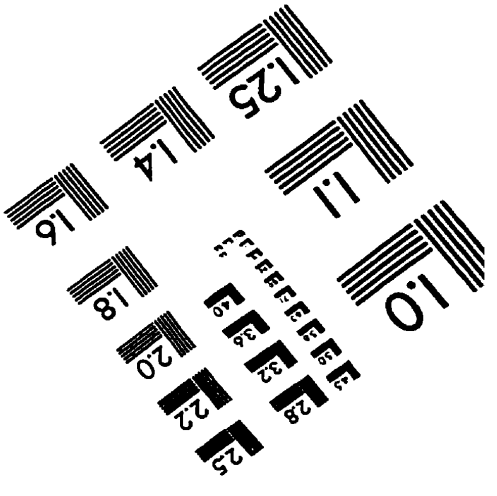












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989