SYNTHESIS AND BIOLOGICAL EVALUATION OF NOVEL DRUG-MONOCLONAL ANTI-CARCINOEMBRYONIC ANTIGEN ANTIBODY IMMUNOCONUGATES

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BY

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Abstract

The objective of this project was to produce novel drug-monoclonal antibody immunoconjugates for site specific drug delivery. The model in these studies consisted of a target, the tumor associated carcinoembryonic antigen (CEA) expressed by a wide range of human solid tumors, and conjugates of doxorubicin (Adriamycin®) (Dox), a potent antibiotic chemotherapeutic drug, covalently linked to a monoclonal anti-CEA antibody by different heterobifunctional cross-linking reagents.

The structure of cross-linking reagents were fully characterized by chemical spectroscopy, infrared (IR), ^{1}H and ^{13}C nuclear magnetic resonance (NMR), and mass spectra (MS). The spacer-Dox derivatives were also characterized by ^{1}H NMR before coupling to thiol groups of thiolated monoclonal anti-CEA antibody. The immunoreactivity of immunoconjugates was evaluated by enzyme linked immunosorbent assays (ELISAs) which showed that all immunoconjugates retained specific binding to CEA. The efficacy of immunoconjugates was determined in MTT (3-4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide) cell survival assays. The Dox 3'-N-amide linked immunoconjugates are not very active *in vitro* with CEA expressing cell lines. However, the Dox C-13 hydrazone linked immunoconjugates are active *in vitro*.

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Glossary of Abbreviations

Ab	Antibody
ABTS	2,2-Azino'-di(3-ethyl benzthiazoline sulphonic acid
Ac	Acetyl
Ac_2O	Acetic anhydride
AcO^-Na^+	Sodium acetate
ADEPT	Antibody directed enzyme prodrug therapy
ADM	Adriamycin
AFP	Alphafetoprotein
AMT	Antibody mediated targeting
Anti-CEA	Monoclonal anti-carcinoembryonic antigen antibody
Ar	Aromatic
^iB	Isobutyl
^tBu	Tert-butyl
CBL	Chlorambucil
CEA	Carcinoembryonic antigen
$ClCO_2{}^iBu$	Isobutyl chloroformate
DMF	N,N-Dimethylformamide
DMSO	Dimethylsulfoxide
Dau	Daunomycin/Daunorubicin
Dox	Doxorubicin
EI(s)	Antibody-enzyme immunoconjugate(s)
ELISA	Enzyme linked immunosorbent assay
Fab	Fragment of antigen binding site
HAMA	Human anti-mouse antibody response

HARA	Human anti-ricin antibody
HCG	Human chorionic gonadotrophin
HETCORR	^{1}H-^{13}C Correlation spectrum
HMD	1,1,1,3,3,3-Hexamethyldisilazane
IC(s)	Immunoconjugate(s)
IR	Infrared [spectroscopy]
IT(s)	Immunotoxin(s)
KOH	Potassium hydroxide
MA	Maleic anhydride
MAb	Monoclonal Antibody
Me	Methyl
MeOH	Methanol
mp	Melting point
MS	Mass spectrometry [spectroscopy]
MTX	Methotrexate
NMR	Nuclear magnetic resonance [spectroscopy]
PBS	Phosphate buffered saline
RAID	Radioimmunodetection
RAIT	Radioimmunotherapy
RAM-HRP	Rabbit anti-mouse Horseradish peroxidase immunoglobulins
RC(s)	Radioimmunoconjugate(s)
RT	Room temperature
$SOCl_2$	Thionyl chloride
SPDP	N-succinimidyl 3-(2-pyridyldithio) propionate

TAA	Tumor associated antigen
TEA	Triethylamine
TFA	Trifluoroacetatic acid
TLC	Thin layer chromatography
TMS	Trimethylsilyl
TMSCl	Chlorotrimethylsilane
TSA	Tumor specific antigen
UV	Ultraviolet [spectroscopy]
VDS	Vindesine

CHAPTER I : INTRODUCTION

I 1.0 CURRENT RESEARCH IN DELIVERY OF ANTICANCER DRUGS

A cancer represents a population of cells within the host body escaping from normal control mechanisms which will continue to grow until the death of the host, unless appropriate treatment is applied. The tumor may be controlled by surgical removal, radiation killing or chemical treatment. Surgery and radiotherapy are only successful in localized cancers while chemotherapy is a major therapeutic approach for the treatment of both localized and metastasized cancers. Cancer chemotherapy has had remarkable success for certain tumors, e.g., childhood leukemia, choriocarcinoma, and testicular carcinoma (DeVita & Hellman, 1982), but it has been relatively ineffective for many of the more common tumors, e.g., lung cancer and colon cancer. All cytotoxic drugs used in cancer therapy share two properties: an ability to inhibit the process of cell division and an inability to distinguish between normal and malignant cells. The effectiveness against these tumors may be increased if drugs are more specific and selective to tumor cells. Two approaches have been used to produce antitumor agents for improving therapy such as design of analogues by chemical modification of the parent drug (Cassady & Douros, 1980) and genetic engineering of antibiotic producing microorganisms (Hutchinson et al, 1989). However, only a few drugs scanned from many hundreds have reached clinical usefulness because of nonspecific toxicity and poor

therapeutic index. In recent cancer chemotherapy research, significant efforts have been directed towards the improvement of anticancer drug delivery. These include pharmacokinetic and pharmacodynamic considerations, therapeutic implications and mechanisms of drug resistance. These approaches appear to be a challenging field in cancer research.

The pharmacokinetic aspect is the study of the rate of change of drug concentration *in vivo* related to drug dose, frequency, and route of administration (Levin, 1986). The pharmacodynamic aspect is the study of the time relationship of pharmacological response elicited by drugs or relationship of drug concentration at a receptor site to biological response (Powis, 1985). Both aspects can be applied to design the dosage of anticancer drugs for achieving the desired and optimal therapeutic effects.

The development of multiple drug resistance (MDR) in tumors has become a challenging problem in cancer chemotherapy. The studies of MDR focus on the mechanisms at the level of membranes, cytoplasms, and DNA repair and gene amplification (Hayes & Wolf, 1990; Burt, Fojo & Thorgeirsson, 1990; Nielsen & Skovsgaard, 1992). The problems of MDR can be overcome by several approaches: 1) drug modification such as increasing lipophilicity (Meyer, Stone & Ullman, 1979); 2) utilization of monoclonal antibodies (MAbs) or monoclonal antibody-drug conjugate against unique glycoproteins of

resistant tumor cells (Pearson et al, 1991; Ohkawa et al, 1992); 3) using calcium channel blockers (Helson, 1984; Cano-Gauci & Riordan, 1987); and 4) decreasing the cytoplasmic level of glutathione (Ozols et al, 1987).

The general approaches for delivery of anticancer drugs in achieving therapeutic improvement are: 1) liposome delivery systems (Weinstein & Leserman, 1984); 2) microparticulate drug delivery systems (Kerr, 1987); 3) macromolecular drug conjugate systems (Maeda & Matsumura, 1989; Seymour, 1992); 4) polymeric controlled-release systems (Laurencin & Langer, 1987; Yokoyama et al, 1992); 5) blood-perfluorochemical exchanges (Hiraga et al, 1987); 6) monoclonal antibody-mediated targeting (Ford & Casson, 1986; Pietersz & Mckenzie, 1992); 7) implantable pumps and reservoirs for regional perfusion (Freeman & Mayhew, 1986); and 8) prodrugs for site-specific delivery by targeted enzymes (Senter et al, 1993).

The approach which represents one of the more significant developments is the use of monoclonal antibodies (MAbs) to concentrate drugs to a tumor. During the past decade, there has been a rapid growth of drug targeting using MAbs as a result of the consolidation of different scientific disciplines, cell biology, recombinant technology and chemistry. Within the area of cell biology, hybridoma technology allows unlimited production of MAb. After immunization of a mouse with human tumor tissue containing

specific tumor associated antigen, the immune system of the mouse processes the antigen and begins to make antibodies. The B cells obtained from excising the spleen are fused with malignant myeloma cells and the fused hybridoma cells are selected from specific medium such as HAT (hypoxanthine /aminopterin/thymidine). The MAbs derived from the hybridoma cells are screened against malignant and normal tissue. The improvement of screening and testing for the immunoreactivity of derived MAbs has accelerated the growth of the drug-targeting discipline. Recombinant DNA technology has allowed the construction of chimeric monoclonal antibodies for drug conjugation which reduce their immunogenicity in humans (Beidler et al, 1988; Fell et al, 1989). Chemistry has accelerated the evolution of the drug-targeting. The importance of chemistry is the design and synthesis of bioconjugates in which the linkages of drugs are compatible to MAbs. These three disciplines create a new dimension that facilitates the advance with monoclonal antibody drug targeting in the treatment of human cancer.

I 2.0 ANTIBODY MEDIATED TARGETING (AMT)

I 2.1 Concepts and Components in Targeted Chemotherapy

The major obstacle in cancer chemotherapy is the lack of selectivity of therapeutic agents for the malignant cells and the toxic side effects to normal cells in therapeutic dosage (Ford & Casson, 1986). Targeted chemotherapy offers an approach to increase drug delivery to the tumor site. This approach was originated from proposals of Paul Ehrlich in the early years of this century. He postulated 'magic bullets' for ligand substrates in drug targeting (Ehrlich, 1906). The basic concept is to link the therapeutic agents to a site-specific carrier directed against tumor cells. Thus, the components of targeted chemotherapy consists of 1) target, 2) carrier, and 3) toxic agent.

II 2.2 Targets

II 2.2 (a) Antigen Specificity

The explicit difference between tumor cells and normal cells is a prerequisite of selectivity for drug targeting. The search for tumor specificity was begun by Enrlich in the early 1900's. The claims for the "tumor specific markers" remained controversial until the demonstrations of tumor specific antigens (TSAs) induced by chemical carcinogens in syngeneic mice were established (Klein el at, 1960; Schreiber et al, 1988). The characterization of oncogenic products

(Harris, 1990) strongly suggests the existent of distinct molecular differences between tumor and normal cells. However, the presence of TSAs in human tumors remains unproven with the exception of the idiotype marker on certain B and T cell lymphomas and leukemias (Old, 1981; Stevenson, George & Glennie, 1990).

The best potential targets for site-specific drug delivery are the tumor associated antigens (TAAs) available in greater content on a tumor cell surface than on normal cells. An ideal tumor marker should possess these several characteristics 1) it should be produced by the tumor cells and be easily detectable in body fluids; 2) it should not be present in healthy cells or benign diseases; 3) it should be present in concentration high enough for screening and detecting early cancer levels; 4) it should directly reflect the bulk of tumor in terms of the quantity of marker; 5) it should be detectable without clinical evidence of a tumor; and 6) it should correlate with the efficacy of anti-cancer therapy (Bates & Longo, 1987). However, no marker meets all of the above criteria.

A wide range of tumor associated antigens were identified and evaluated as tumor markers in cancer diagnosis and management (Table 1) (Bates & Longo, 1987). Many MAbs have been made to antigens strongly expressed by human tumors. Most of these antigens are oncofetal antigens. In solid

tumors, three TAAs, carcinoembryonic antigen (CEA), alphafetoprotein (AFP) and human chorionic gonadotrophin (HCG), are well characterized. Among the above TAAs, CEA is the most well-studied tumor marker. Extensive research has been in progress to dissect CEA at both the cellular and molecular levels (Shively & Beatty, 1985).

Table 1 : Classification of Tumor Markers

Oncofetal antigens	Carcinoembryonic antigen Alphafetoprotein
Ectopic hormones	Adrenocorticotropic hormone Antidiuretic hormone Parathormone and Calcitonin
Placental proteins	Human chorionic gonadotrophin Human placental lactogen Regan isoenzyme (of alkaline phosphatase)
Enzyme	Prostatic acid phosphatase Lactic dehydrogenase

Adapted from Bates & Longo, 1987

I 2.2 (b) Choice of Target Antigen

While considering an antigen as a target for therapy, both the degree of antigen specificity and the number of antigenic molecules per tumor cell are important. The importance of antigen specificity is obvious because it allows the conjugates to anchor on the antigen-positive tumor cells selectively and accurately while sparing antigen-negative

normal cells. However, an entire targeting system would be useless if the expression of the target antigen at the cell surface would not permit the binding of the number of drug-antibody conjugate molecules needed to achieve the therapeutic response.

Most drugs must enter tumor cells to exert an effect; thus, the degree to which a drug-antibody conjugate is taken up by the cells is also important. The uptake is generally facilitated by endocytosis and the degree of endocytosis may vary between different antigens. Conjugates that are internalized may be more effective for delivery of drugs intracellularly.

I 2.2 (c) Characteristics of Targets

Tumor cell heterogeneity is a problem in both diagnosis and treatment of cancer, and this heterogeneity shows a large variety of characteristics (Foulds, 1954; Hart & Fidler, 1981; Fidler & Poste, 1985; Schnipper, 1986). These include sensitivity to chemotherapeutic drugs and expression of tumor antigens (Yeh, Hellström & Hellström, 1981; Albino et al, 1981). The therapeutic problem of drug sensitivity can be approached by MAbs conjugated with multiple drugs or a "cocktail" of conjugates (Oldham, 1991). The antigenic modulation, mutational loss of TAA and shedding of antigens into the body fluids impede the efficacy of using antibody for

therapy. The phenomenon of antigenic modulation in which antibody-coated cells redistribute and then clear the antibody-antigen complexes provides an effective way of escape from immune attack (Gordon & Stevenson, 1981, Gordon et al, 1984). The mutational loss of surface antigens may lead to escape from attack by antibodies or conjugates (Meeker et al, 1985; Glennie et al, 1987). The shedding of antigens into the body fluids may lead to formation of antibody/antigen complexes which are rapidly removed by the reticuloendothelial system (RES).

I 2.3 Carriers

Site specific delivery of chemotherapeutic agents is now receiving more attention in effective cancer chemotherapy. The aim is to develop the carriers that will show a high degree of selectivity in bringing the therapeutic agents into the target sites. An ideal carrier should possess the following properties: 1) the carrier delivers the toxic agent selectively to the target sites; 2) the linkage between the carrier and the toxic agents retains the activities of both agents; and 3) the carrier has reasonable stability for delivery purpose (Ford & Casson, 1986).

I 2.3 (a) Types of Carriers

A wide range of different drug carrier systems, polymers,

macromolecules, nanoparticles, liposomes and monoclonal antibodies are under investigation for the above properties. The most outstanding carriers for targeted drug delivery are antibodies and liposomes.

The use of liposomes as a carrier system improves the therapeutic efficacy of a wide range of drugs (Weinsteain & Leserman, 1984), especially for lipophilic antitumor drugs. Because of their particulate nature, liposome entrapped agents appear to be promising for the treatment of tumors of the reticuloendothelial system (RES) by intravenous administration and tumors of body cavities by intracavitary administration (Perez-Soler, 1989). The liposomal stability and clearance are controlled by the composition of the vesicles themselves (Allen & Chonn, 1987). Thus, liposome composition can determine the amount of drugs delivered to tumors and the uptake of liposomes by tumors (Gabizon & Papahadjopoulos, 1988). However, the problems of using liposomes are nonspecific uptake by the phagocytic cells in the RES, poor permeability out of the blood stream, degradation, toxicity and antigenecity (Gregoriadis, 1990). Immunoliposomes using an antibody as a targeting ligand and a lipid vesicle as a carrier for anticancer agents enhance the site-specific delivery to target site (Wright & Huang, 1989).

The specificity and monoclonality of MAbs offer the best potential carrier system to achieve major advances in targeted

drug delivery.

I 2.3 (b) Antibodies as Carriers

The special immunological properties that enable selected antibodies (Abs) to localize and accumulate in tumor sites favor the application of MAbs as drug-targeting agents. The concept of using MAbs as carriers of therapeutic agents is simple, straightforward and attractive. However, the investigation of antibody mediated targeting (AMT) did not gain attention until the early 1970s. The exploration was hindered by the following impediments: 1) apprehension regarding administration of large quantities of foreign Abs to patients; 2) development of an immune response when these Abs were repeatedly administered; 3) heterogeneity of Abs; 4) poorly defined targets; and 5) lack of relevant preclinical models for *in vivo* testing (Ford & Casson, 1986). Some of these obstacles have been partially or completely reduced.

The discovery of MAbs in 1975 (Köhler & Milstein, 1975) led to the homogeneous unlimited supply of monoclonal antibodies with defined specificities and also opened the avenue for antibody-mediated targeting (AMT). For the production of MAbs, a mouse is usually immunized with the antigen of interest, e.g., human cancer tissue. The mouse is the system of choice because hybridoma technology was developed within the context of mouse genetics (Köhler, 1986).

By these techniques, Abs harvested represent a number of different classes and isotypes. Moreover, IgG antibodies are preferable to IgM because they may concentrate better at the target due to their smaller size. There are two ways to obtain an IgG-producing hybridoma when starting with IgM-making hybridoma: 1) perform "class-switching" (Thammana & Scharff, 1983), and 2) obtain a new hybridoma with the specific antibody isotype when the antigen is already defined (Brown et al, 1980; Brodin et al, 1985).

I 2.4 Monoclonal Antibodies for Cancer Diagnosis and Treatment

MAbs reacting with human TAAs have been used for tumor detection and therapy. The uses of MAbs in the diagnosis of cancer are as follows: 1) immunohistopathological diagnosis; 2) serum probes for tumor markers; and 3) radioimmunodetection (RAID) (Teh et al, 1985).

MAbs can be used either alone or as carriers of toxic agents in cancer treatment (Houghton & Scheinberg, 1986; Byers & Baldwin, 1988; Dillman, 1989; Ford et al, 1990; Pietersz & Mckenzie, 1992). The therapeutic application of MAbs include: 1) mediate destruction of target tumor cells by complement (complement-mediated cytotoxicity); 2) mediate killing of target tumor cells by immune effector cells (antibody-dependent cellular cytotoxicity); 3) direct biological effects on target cells, eg, block cell proliferation; and 4) exert

cytotoxic/cytostatic effect when conjugated to anticancer agents (Houghton & Scheinberg, 1986). The novel approach involving the anti-idiotypic therapy for leukemias and lymphomas (Stevenson, George & Glennie, 1990) is also under consideration. Abs alone are generally not very efficient in the treatment of cancer, but act as carriers when conjugated to toxic agents, giving an appropriate targeting system.

I 2.5 Immunoconjugates for Cancer Diagnosis and Treatment

MAbs which recognize TAAs offer the greatest potential as carriers for site specific targeting. The toxic agents conjugated to MAbs include radioisotopes, toxins, enzymes and anti-cancer drugs (Pietersz & Mckenzie, 1992).

I 2.5 (a) Radioimmunoconjugates (RCs)

Radioimmunoconjugates (RCs), antibodies labelled with radionuclides, in addition to diagnosis and therapy in cancer, have potential in quantifying and studying the pharmacokinetics of MAbs. However, the problems of low localization of radioactivity in tumor, high localization of radioactivity in nontarget organs such as liver, and human antimouse antibody response (HAMA) limit the usefulness of RCs. Thus, recent work has focused on methods for the preparation of RCs to increase their efficacy. Different linking or chelating agents for the attachment of

radioisotopes to MAbs or antibody fragments have been prepared and the feasibility and effectiveness of these conjugates have been widely explored (Garg, Garg & Zalutsky, 1991; Arano et al; 1991; Hadley et al, 1991; Linder et al, 1991b; Griffiths et al, 1992).

The use of radioiodinated antibodies for tumor localization was pioneered by Pressman and Goldenberg (Pressmann & Keighley, 1948; Goldenberg et al, 1978). The methods and reagents for labelling MAbs with radiohalogens have been recently reviewed (Wilbur, 1992). The clinical radioimmunodetection (RAID) trials has been recently summarised and reviewed by Larson (Larson, 1990 & 1991). The radioiodinated antibodies (^{131}I) have been widely used for the diagnosis of a range of tumors; however, ^{131}I is not an ideal radiolabel due to high energy β-emission. The high photon energy results in poor detection efficiency, reduced sensitivity and resolution (Bradwell et al, 1985). Other radionuclides, indium-111 (^{111}In) (Brandt & Johnson, 1992; Subramanian et al, 1992) and technetium-99m (^{99m}Tc) (Linder et al, 1991a), have ideal half lives and photon energies, but they are expensive.

The use of RCs as therapeutic agents offers an attractive approach in cancer therapy, radioimmunotherapy (RAIT). The use of radioisotopes in RAIT has a number of advantages: 1) the small size of isotopes does not greatly alter the

conformation of MAb; 2) the bombardment property of isotopes to the surrounding cells provides effective treatment with heterogeneous tumors in which the tumor cells are killed without the binding of RCs; 3) the conjugates acting over long distance affect the tumor cells in the interior of the tumor. These advantages encourage the clinical studies with RCs, especially ^{131}I (Moseley et al, 1990; Goldenberg et al, 1991; Camera et al, 1991; Meredith et al, 1992). Other isotopes, palladium-109 (Fawwaz et al, 1984), bismuth-212 (Kozak et al, 1986), yttrium-90 (Vaughan, Keeling & Yankuba, 1985; Vriesendorp et al, 1991; Rosenblum et al, 1991) and astatine-211 (Harrison & Royle, 1984; Brown, 1986), are under investigation. The use of interleukin (IL-1) following radioimmunotherapy has been shown to increase survival of mice (Blumenthal et al, 1988) and may be used in the future. Another possibility for RAIT involves the use of boron-10 (^{10}B) in boron neutron capture therapy (Barth, Soloway & Fairchild, 1990; Varadarajan & Hawthorne, 1991).

I 2.5 (b) Immunotoxins (ITs)

Toxins are usually of plant or animal origin and more potent than chemotherapeutic drugs. Low concentrations of toxins are sufficient for cell killing (Yamaizumi et al, 1978; Eiklid, Olsnes & Pihl, 1980). Most toxins are peptides that inhibit the elongation step of protein synthesis. Conjugation

of toxins to MAb, immunotoxins (ITs), represents a potential anticancer agent and a new approach to cancer therapy (Lord, Spooner & Roberts, 1989). Thus, several toxins, ricin, abrin, gelonin, pseudomonas exotoxin A and diphtheria toxin, have been conjugated to MAbs for evaluation. The efficacy of ITs *in vitro* and *in vivo* have been reviewed (Vitetta et al, 1987; Byers & Baldwin, 1988; Hertler & Frankel, 1989). The construction of ITs requires the removal of the cell-binding moiety (B-chain) by chemical cleavage or modification and then the coupling of toxic moiety (A-chain) to MAb via a reducible disulfide bond with the use of a heterobifunctional cross-linker such as N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP). From genetic engineering techniques, the recombinant toxins have been cloned with ligands to form the chimeric toxins for receptor-targeted therapy (Murphy et al, 1986; Kreitman et al, 1992a). By recombinant technology, it is possible to design chimeric toxins with two recognition domains by placing two ligands into different locations of the same toxin to increase efficacy (Kreitman et al, 1992b). Another novel approach is the construction of ITs with a photocleavable linker and their cytotoxicity can be controlled by an external signal such as UV light at the desired site and at the desired time (Goldmacher et al, 1992).

Clinical trials have been performed using ITs for the treatment of chronic lymphocytic leukemia (CLL) (Hertler et

al, 1989), cutaneous T-cell lymphoma (LeMaistre et al, 1991), B cell leukemia (BCL) (Vitetta et al, 1991; Grossbard et al, 1992), metastatic malignant melanoma (Spitler et al, 1987), metastatic breast carcinoma (Gould et al, 1989; Weiner et al, 1989) and graft-versus-host disease (GVHD) (Kernan et al, 1988).

The use of ITs *in vivo* may be hindered by several problems. A number of avenues have been explored to improve their efficacy. The disulfide bond linkage used to prepare ITs is susceptible to reduction *in vivo* and the amount of toxin delivered to target cells will be greatly reduced. Thus, the efficiency is low. New cross-linking agents with a hindered disulfide bond that is more resistant to reduction have been developed (Thorpe et al, 1987; Greenfield, Bloch & Moreland, 1990; Cumber et al, 1992). The failure of ITs to permeate solid tumors limits their potential in therapy. This problem may be solved by reducing the size of IT with the use of fragments of antibody (Fab') (Vitetta et al, 1991). The antibody and toxic moieties of IT are antigenic and provoke immune responses, HAMA and HARA (human anti-ricin antibodies). The problem of HARA may be improved by using a range of different toxin molecules. The use of a chimeric antibody can reduce the immunogenicity of ITs (Siegall et al, 1992). Much future work will be done to improve the efficacy of ITs for targeted therapy.

I 2.5 (c) Antibody-Enzyme Immunoconjugates (EIs)

A recent novel idea is a site specific antibody directed enzyme prodrug therapy (ADEPT) (Bagshawe, 1993). Antibody-enzyme conjugates (EIs) can be targeted to the tumor site in which nontoxic drug precursors (prodrugs) are activated to toxic drugs by targeted enzymes (Bagshawe, 1989; Senter, 1990; Senter et al, 1993). A number of agents have been used *in vitro* and *in vivo* studies (Wallace & Senter, 1991; Jungheim, Shepherd & Meyer, 1992; Svensson et al, 1992; Wang et al, 1992; Vrudhula et al, 1993). The clinical trial from Bagshawe et al. shows the therapeutic effects from this idea, but the problems are HAMA as well as an anti-enzyme antibody (Bagshawe et al, 1991). Recently, a fusion protein consisting of the humanized Ab and the human enzyme may solve the above problem and allow ADEPT *in vivo* (Bosslet et al, 1992).

I 2.5 (d) Antibody-Drug Immunoconjugates (ICs)

Anti-neoplastic drugs as toxic agents for coupling to MAbs is now attracting more interest as the drugs are widely used for therapy and have defined mechanisms of action and pharmacokinetic properties. It would be rational to perform the conjugate Phase I trials with drugs whose antitumor activity and toxicity are known. Then, the toxicity from the conjugate can be compared with the known toxicity of an equal dosage of the nonconjugated drug. Subsequent Phase II trials

can then compare the therapeutic effect of IC with an equal toxic dose of the free drug. The cytotoxic drugs that have been used for conjugation include methotrexate, vindesine, neocarzinostatin, cis-platinum, chlorambucil, 5-fluorouridine, melphalan, daunomycin and adriamycin.

The first report of targeted chemotherapy came out in 1958; Mathé demonstrated the prolongation of survival of mice with methotrexate (MTX) using an antiserum against the mouse leukemia L1210 raised in hamsters (Mathé et al, 1958). The result was due to the synergistic effect of Ab and the drug in noncovalent linkage. For a targeted approach, drugs should be covalently linked to the antibodies (Newman et al, 1977). A lot of preclinical and clinical studies have been reported with different ICs. Clinical trials using human studies were pioneered by Ghose's group, using chlorambucil (CBL) bound to xenogenic antimelanoma globulin (Ghose et al, 1975). One recent report establishes the usefulness of ICs in clinical trials and describes the follow-up results in a large number of colorectal cancer patients treated with chimeric Fab MAb A7-neocarzinostatin (NCS) conjugates (Takahashi et al, 1993). Preclinical studies of vindesine (VDS) linked to MAbs have demonstrated efficacy, both *in vitro* and *in vivo*, of AMT which correlated with target antigen expression (Rowland et al, 1985; Ford et al, 1987b). Other preclinical studies using MAb and derivatives of the vinca alkaloid, desacetylvinblastine

hydrazide, have also demonstrated significant increases in survival times of the animals (Apelgren et al, 1990 & 1993; Starling et al, 1992). Other studies including anthracyclines, Dox (Dillman et al, 1986; Sinkule, Rosen & Radosevich, 1991) and daunorubicin (Dau) (Dillman et al, 1988; Stastry & Das, 1993) and MTX (Kralovec et al, 1989) also showed promising results.

The major problem for the production of ICs is the retention of the activity of both the drug and the antibody. Most conventional conjugation procedures such as glutaraldehyde and carbodiimide result in loss of drug and/or antibody activity. When drugs are conjugated to antibodies, certain chemical groups of the drug can not be modified due to their importance in cytotoxicity to target cells. Thus, the structure-activity relationship of the drugs should be emphasized. Most drugs are hydrophobic and change the solubility of antibody after conjugation, leading to aggregation. Moreover, the linkage of drug to antibody should be stable in the plasma so that the drug is not released prematurely. The synthesis of these conjugate requires extensive chemical manipulations. The cytotoxic effects of the conjugates may depend on drug release or internalization. Thus, more studies will be directed toward the chemical linkage in the preparation of ICs.

I 3.0 RATIONALE FOR THE USE OF A CEA MODEL

Before the evaluation of the role of ICs in chemoimmunotargeting, it was crucial to choose the appropriate target to study. A target should be present on the major solid tumors for which improvement in chemotherapy was urgently needed. The MAbs to the target should be available and suitable for targeting. The target could be easily assayed in the laboratory and the structure well elucidated. CEA is the most widely studied and utilized human tumor marker (Shively & Beatty, 1985) and, in our opinion, is the most suitable target for detailed studies.

I 3.1 Molecular Structure, Evolution and Functional Significance

CEA was first described by Gold and Freedman as a high molecular weight glycoprotein (~180,000) associated with colonic tumors and fetal colon (Gold & Freedman, 1965). The antigenic structure of CEA has been well characterized by using over 52 MAbs (Hammarstrom et al, 1989). The CEA gene family, molecular structure, evolution and functional significance have been extensively reviewed (Rogers, 1983; Shively & Beatty, 1985; Thompson & Zimmermann, 1988; Thompson, Grunert & Zimmermann, 1991).

The function of CEA as an intracellular adhesion molecule was suggested by Benchimol's group (Benchimol et al, 1989).

Thus, CEA has joined the immunoglobulin (Ig) superfamily, involving the basic cell surface recognition events (Williams, 1987).

I 3.2 Clinical Relevance

CEA is the best known TAA which often exists in elevated levels in cancer patients and is associated with most of the common solid tumors that have the highest mortality: >65% of colorectal, >50% of lung, >60% of pancreas, >30% of ovary and breast cancers (Bates & Longo, 1987). CEA was not only identified in the circulation of cancer patients, but also in patients with benign disease, healthy controls and even in normal tissues. However, CEA is still a useful marker for cancer and its level is a valuable parameter in assessing cancer therapy.

I 3.3 CEA as a Model for Antibody Mediated Targeting

CEA is established as a promising target in both antibody mediated diagnosis and treatment. The potential of polyclonal antibodies (Pab) anti-CEA-VDS IC was demonstrated with retention of drug and Ab activities as well as Ab specificity (Johnson et al, 1981; Rowland et al, 1982). Then, an ^{131}I labelled anti-CEA conjugate was used to localize the tumor in patients with advanced metastatic adenocarcinomas (Ford et al, 1983). With the improvement of RAID technique, ^{123}I, ^{99m}Tc and

^{111}In labelled MAb or Fabs have been used to detect CEA expressing tumors (Beatty et al, 1986; Goldenberg et al, 1990).

One of the anti-CEA MAbs, 11-285-14, has been well characterized immunocytochemically (Hockey et al, 1984; Ford, Gallant & Ali, 1985), and confirmed to be reactive only with CEA and non-reactive with normal cross-reacting antigens (NCA) (Price, 1988). The efficacy and specificity of 11-285-14-VDS ICs have been shown *in vitro* and correlated with CEA density in different cancer cell lines (Ford et al, 1987b). These conjugates have also demonstrated their efficacy *in vivo* with xenografts in a nude mouse model (Casson et al, 1987). Further studies of 11-285-14 in AMT with 11-285-14-Dox conjugates have indicated specificity for the CEA target *in vitro* (Richardson et al, 1989).

The rationale for CEA model:

1) The common solid tumors such as colonic, lung, stomach and breast cancers, show high expression of CEA and these are the tumors in which improved chemotherapy is required.
2) CEA is the most well studied, characterized and elucidated TAA.
3) The anti-CEA MAb, 11-285-14, is reactive with CEA expressing cell lines and nonreactive to NCA (Price, 1988).

4) 11-285-14 has been demonstrated to be bound and internalized by CEA expressing cells *in vitro* (Tsaltas, Ford & Gallant, 1992).

5) The CEA model in AMT has been developed from 11-285-14-VDS *in vitro* and *in vivo* as well as 11-285-14-Dox *in vitro* (Ford et al, 1987b; Richardson et al, 1989)

I 4.0 RATIONALE FOR THE USE OF DOXORUBICIN

There is a vast selection of cancer drugs in clinical use and the anthracyclines have the widest spectrum of antitumor activity. Thus, they have aroused considerable interest for cancer treatment. This class of compounds is exemplified by doxorubicin (Dox), an antineoplastic antibiotic, isolated from a culture of Streptomyces peucetius var. caesius and introduced into clinical use in 1976. Dox (Adriamycin®) has the widest range of antitumor activity, ranging from leukemias to a broad spectrum of solid tumors such as breast, lung, stomach and colonic cancers (Blum & Carter, 1974) and may be the most utilized antitumor drug worldwide (Weiss et al, 1986).

I 4.1 Structure and Mechanism of Action

The structural elucidation of Dox shows a characteristic four-ring structure, anthraquinone chromophore, linked to daunosamine via a glycosidic bond and the presence of a ketone

and a hydroxyl at the 13 and 14 position respectively (Figure 1) (Arcamone, Franceschi & Penco, 1969). Dox is chemically named (9S,7S)-7-[(3'-amino-2',3',6'-trideoxy-α-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-9-(hydroxyacetyl)-4-methoxy-5,12-naphthacenedione.

Dox exhibits three principal toxic effects on cells. The first is the interference with DNA metabolism (Zunion, Gambetta & Di Marco, 1975; Liu, 1989). The second is interference with electron transport and cellular respiration (Marcillat, Zhang & Davies, 1989; Demant, 1991). The last one is with lipid peroxidation and disruption of the cellular membrane (Myers et al, 1977; Gianni et al, 1988); thus, Dox may be toxic to cells even without entering the cells (Tritton & Yee, 1982).

I 4.2 Potential in Targeting

Although Dox is very useful in chemotherapy, its efficacy is impeded by toxicities, including myelosuppression, mucositis and alopecia. The greatest concern is the unique cumulative cardiac injury which is the major obstacle to the use of Dox in cancer treatment.

The rationale for producing MAb-Dox immunoconjugate includes:

1) The potential of increasing selectivity to cancer cells and decreasing toxicity to normal cells, thereby

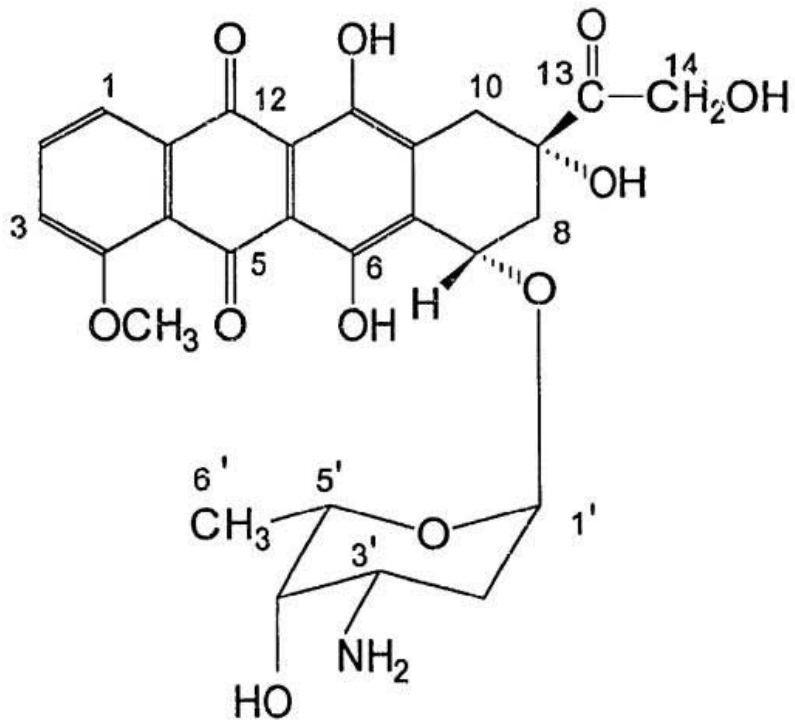


Figure 1. Chemical Structure of Doxorubicin

increasing therapeutic index.

2) Other more potent derivatives of Dox such as cyanomorpholinodoxorubicin, 2 to 1000 times more potent than Dox (Beckman et al, 1988), are not being used in cancer treatment because of their non-specific toxicities, but can be conjugated to MAbs for targeting and may retain anti-tumor activity with lower non-specific toxicity.

I 5.0 METHODS OF CONJUGATION

Bioconjugation chemistry is a descriptive term for the joining of two different molecular functions by chemical or biological means (Meares, 1990). The science and goal of conjugation involve extensive chemical experimentation and product characterization prior to performance of the biological experiments. The multiple steps of the chemical process in preparing and characterizing the bioconjugates provides reliable results and methodology even without encouraging biological results.

The process of joining two molecular components by a covalent bond can be achieved through the use of cross-linking reagents. The components may be proteins, peptides, drugs or nucleic acids. The reagents and methods for protein modification have been reviewed (Means & Feeney, 1990; Lundblad, 1991). The methodology of Ab modification and

chemical linkage of cytotoxic drugs to MAbs will be discussed in sections 5.1 and 5.2 respectively.

I 5.1 Chemical Modification of Antibodies for Conjugation

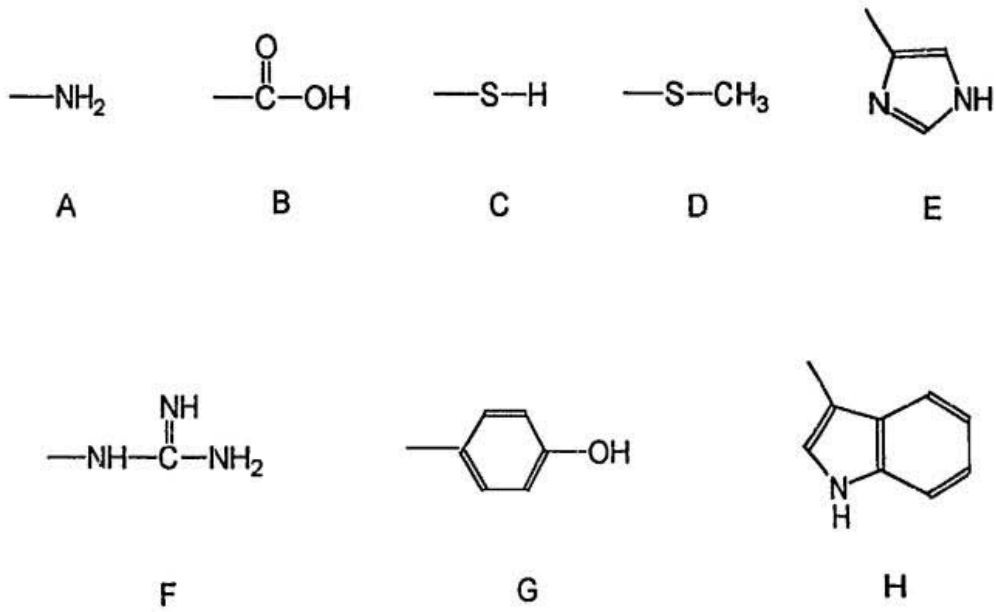
Methods for the chemical modification of Ab continue to expand and develop. The tremendous growth of chemical methods and researchs involve not only fundamental biochemical structure and function, but also pharmacological or medical diagnostic applications.

Conjugation of Abs to other components such as enzymes, toxins, radiolabels or cytotoxic agents depend on the reactivities of the amino acid side chains and the specificities of cross-linkers used. There are twenty amino acids with side chains of different chemical reactivity, but only a few of the amino acid side chains are reactive for modification (Figure 2) (Wong, 1991). The reactivity of Abs that can be chemically modified are also determined by their amino acid composition and the sequence location of the individual amino acids. Lysine residues are usually the most abundant amino acids in proteins and they can be found on the surface of antibody molecules. Thus, the nucleophilic ϵ-amino group of lysine residue will be the common target for chemical modification.

Special reactive groups can be introduced into Abs by chemical modification of the existing functional groups by: 1)

using cross-linking reagents; 2) creating aldehyde groups by carbohydrate oxidation with sodium periodate; and 3) reduction of disulfide bonds by dithiothreitol to expose free thiol groups. The addition of new functionalities serve many purposes including: 1) the incorporation of spacer arms from the protein surface can reduce the steric hindrance caused by other amino acid side chains and decrease the effect of local environment; 2) inactive units can be activated to functional groups for further reaction; 3) the conversion of one functional group into another can either change its specificity or reactivity. Thiolation of Abs plays an important role in conjugation; the thiol is a potent nucleophile in comparison to other groups (Figure 3) (Edwards & Pearson, 1962). Several approaches of a free thiol or protected thiol have been developed for thiolation (Figure 4).

The choice of the cross-linking reagent is critical to the success of conjugation. The reactive groups in cross-linking reagents can be identical or different providing diversity of reagents to bring about covalent bonding between any chemical species. The cross-linking reagents capable of reacting with the side chains of the amino acids of Abs for conjugation may be classified into homobifunctional, heterobifunctional, and zero-length cross-linkers. Over three hundred cross-linkers have been synthesized. The diversity of these linkers is created by researchers for various



Adapted from Wong, 1991

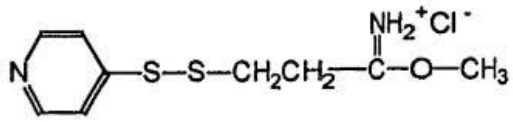
Figure 2. Reactive groups of amino acid side chains. Functional groups A to F are the six most reactive entities. G and H are less reactive.

$$RS^- > ArS^- > RNH^- > RO^- > HO^- > ArO^- > RNH_2 > H_2O$$

Adapted from Wong, 1991

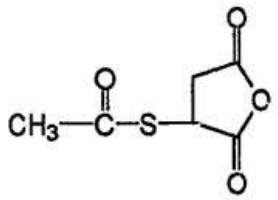
Figure 3. Nucleophilicity of different nucleophiles. Thiolate is the most potent nucleophile.

A.



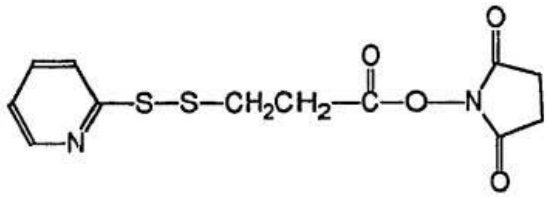
Methyl 3-(4-pyridyldithio)propionimidate hydrochloride

E.



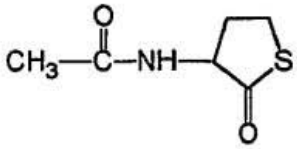
S-acetylmercaptosuccinic anhydride

B.



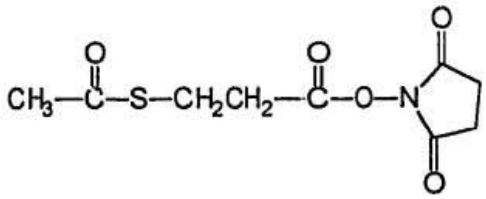
Succinimidyl 3-(2-pyridyldithio)propionate

F.



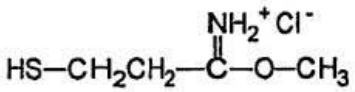
N-Acetylhomocysteine thiolactone

C.



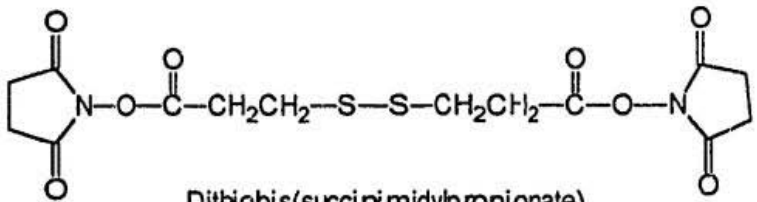
Succinimidyl acetylthiopropionate

G.



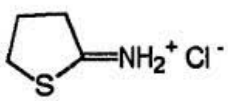
Methyl 3-mercaptopropionimidate

D.



Dithiobis(succinimidylpropionate)

H.



2-Iminothiolane

Figure 4. Common reagents used for thiolation. The reagents A to E contain protected thiol groups which can be activated to generate free thiols after protein thiolation. Free thiol groups can be added to proteins directly with reagents F to H.

applications.

I 5.2 The Linkage of Cytotoxic Drugs to Monoclonal Antibodies

The purpose of linking cytotoxic agents to MAbs is to direct an agent selectively against antigen-bearing cells. A huge amount of literature dealing with the problems and methods of cytotoxic agents conjugated to MAbs has been accumulated, extensively reviewed and discussed (Ghose, Blair & Kulkarni, 1983; Blair & Ghose, 1983; Ghose & Blair, 1987; Pietersz, 1990).

For AMT, an ideal conjugation method should 1) not interfere with either the agent or antibody activity; 2) allow high incorporation of the drug; 3) avoid formation of homopolymers of antibody or agent; 4) avoid aggregation of the conjugate; and 5) be technically straightforward and reproducible (Ghose, Blair & Kulkarni, 1983). Three strategies have been adopted: 1) direct covalent attachment of the drug to Ab, e.g. carbodiimides; 2) using a linker between the drug and Ab, e.g. glutaraldehyde; and 3) attachment of the drug to a high-molecular weight carrier molecule and then conjugating to Ab, e.g. dextran and albumin.

Anthracycline antibiotics play an important role in cancer treatment and represent one of the most investigated classes of antitumor agents. The development of anthracycline-MAb conjugates has been reviewed and summarised

by Hermentin and Seiler (Hermentin & Seiler, 1988). The functional groups suitable for conjugation in Dau and Dox include the daunosamine and the aglycon side chain (Figures 5-8).

The amino group-containing drugs, doxorubicin (Dox) and daunomycin (Dau), can be directly linked to the free carboxyl groups of MAbs in the form of an amide bond by using water soluble carbodiimide, EDCI (1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide) (Hurwitz et al, 1975). Another approach is the use of small spacer molecules as a cross-linker, glutaraldehyde, to couple Dau or Dox to the amino groups of lysine in MAbs (Hurwitz et al, 1975). Both reactions are easy to perform and appear to be the most attractive approaches. The major problem using these reagents is polymerization. It is also difficult to reproduce these couplings. Thus, the procedure for conjugation and the use of cross-linking reagents are stressed.

The chemical linker, which is the heart of the conjugate, determines the activity of the drug either as an integral part of the conjugate or as a releasable component of the conjugate. The choice of a releasable linkage is essential for Dox ICs because the full activity of Dox is required in the free drug form. The development of hydrazone linkers has been shown to allow release of the unmodified drug under mild acidic conditions (Greenfield et al, 1990). A number of

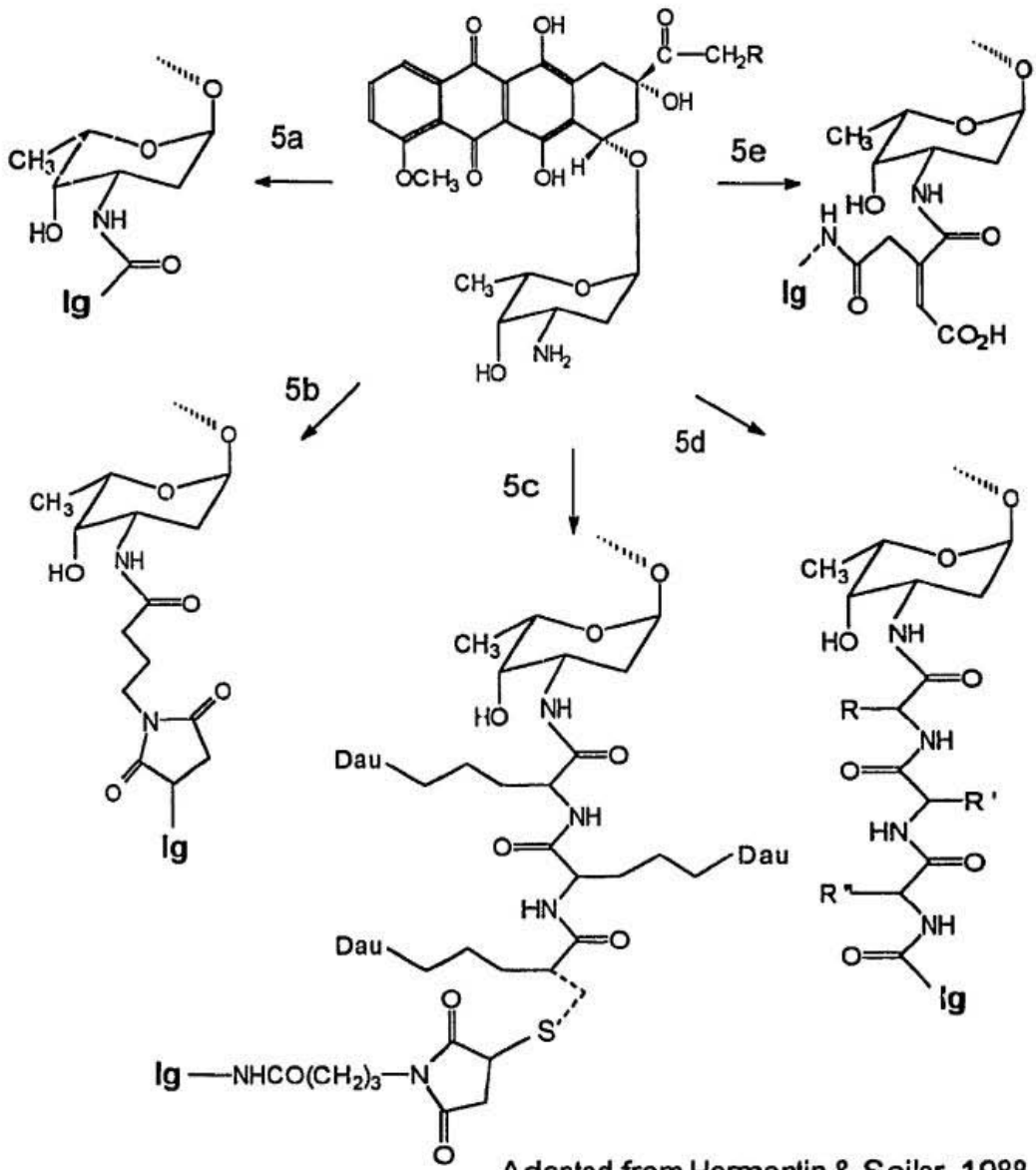
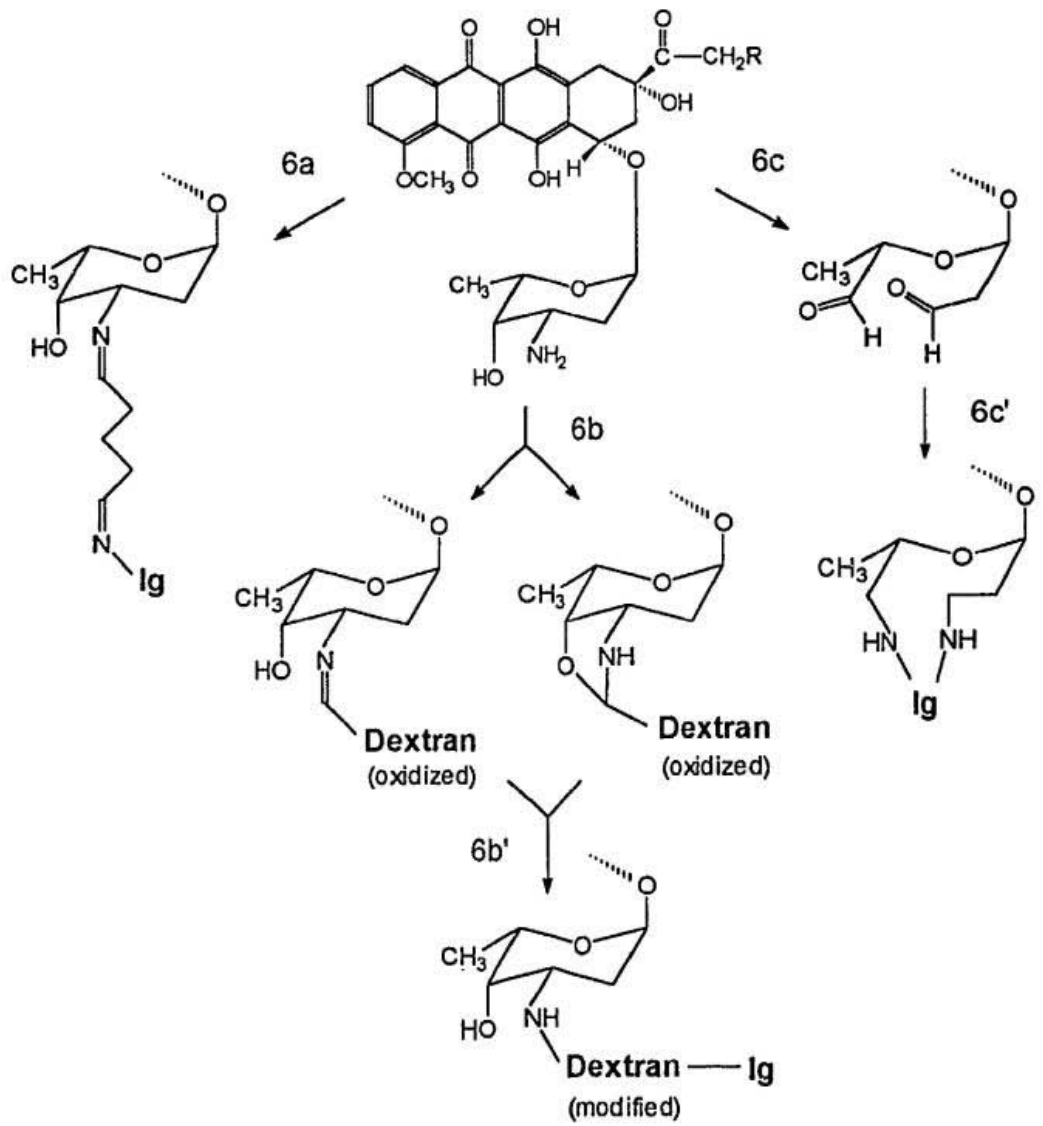


Figure 5. Conjugation via N-acylation of the daunosamine unit

R = H : daunorubicin ; OH : doxorubicin

5a : EDCI / Ig (Hurwitz et al, 1975)
5b : GMBS / reduced Ig (Fujiwara, Yasuno & Kitagawa, 1981)
5c : poly-L-glutamic acid / Ig-maleimide (Kato et al, 1984)
5d : oligopeptide spacers / EDCI / Ig (Trouet et al, 1982)
5e : cis-aconitic anhydride / EDCI / Ig (Shen & Ryser, 1981)

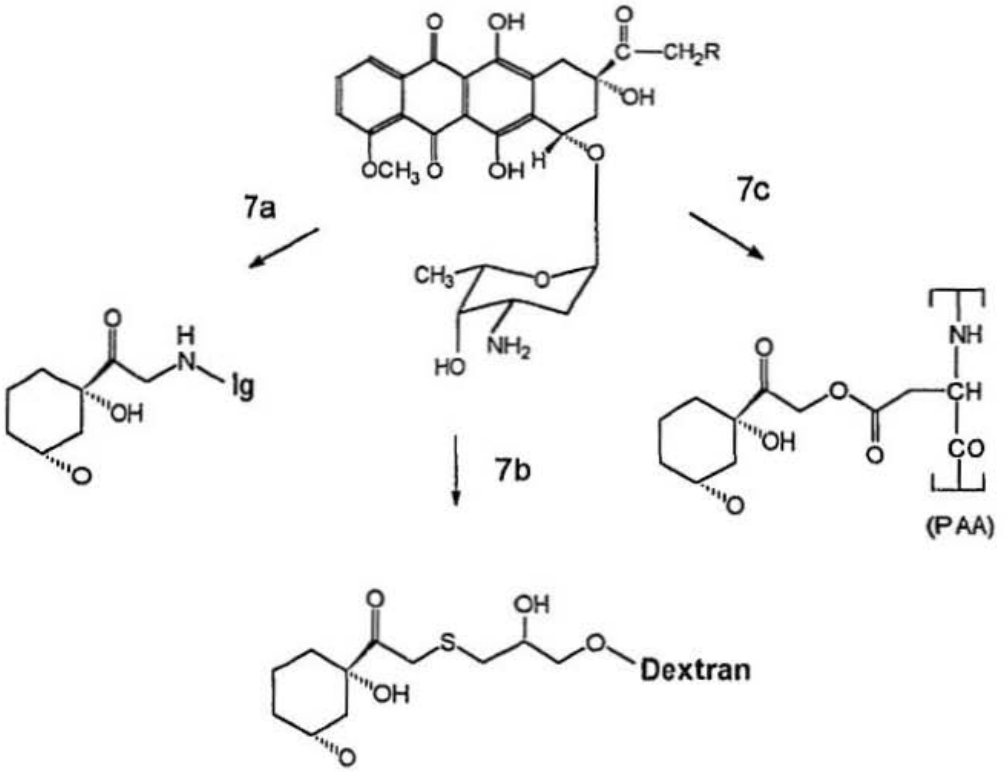


Adapted from Hermentin & Seiler, 1988

Figure 6. Conjugation via N-alkylation of the daunosamine unit

R = H : daunorubicin ; OH : doxorubicin

6a : glutaraldehyde / Ig (Hurwitz et al, 1975)
6b : $NaIO_4$ - oxidized dextran ; 6b' : $NaBH_4$ / Ig (Bernstein et al, 1978)
6c : $NaIO_4$; 6c' : $NaBH_4$ / Ig (Hurwitz et al, 1975)



Adapted from Hermentin & Seiler, 1988

Figure 7. Conjugation via the aglycon-side chain at C-14

R = Br

7a : Amino groups of Ig (Gallego et al, 1984)
7b : 3-mercapto-2-hydroxypropyldextran (Hurwitz et al, 1980)
7c : Carboxyl groups of PAA (Zunino et al, 1982)

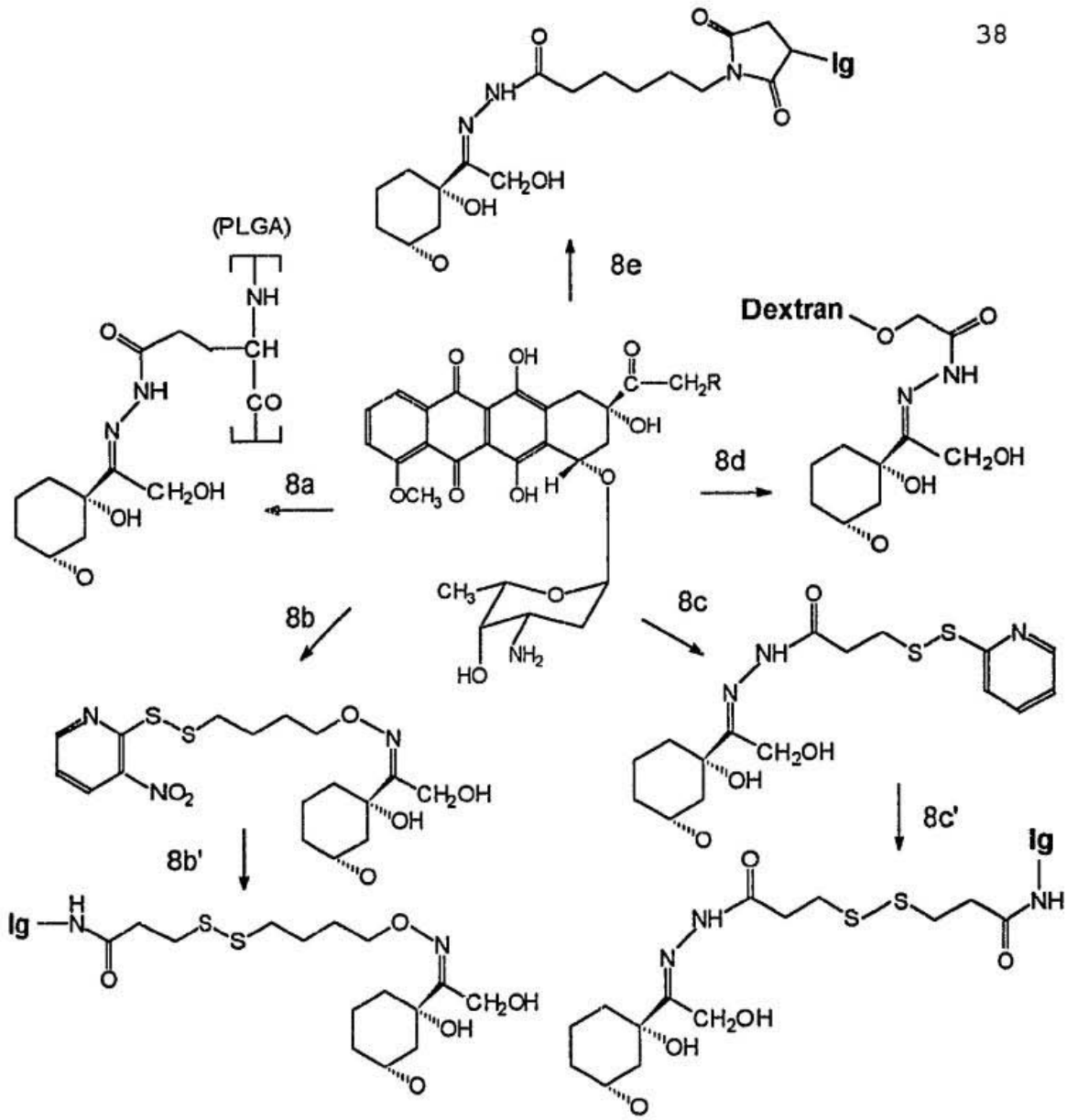


Figure 8. Conjugation via the aglycon-side chain at C-13

R = H : daunorubicin ; OH : doxorubicin

8a : Polyglutamylhydrazide (Hurwitz et al, 1980)
8b : 1-Aminooxy-4-[(3-nitropyridin-2-yl)dithio]butane-HCl
8b' : DTT / N-succinimidyl-3-(pyridyldithio)propionate (SPDP)-Ig (Braslawsky et al, 1991)
8c : SPDP ; 8c' : DTT / SPDP-Ig (Greenfield et al, 1990)
8d : Carboxymethyldextran hydrazide (Hurwitz et al, 1980)
8e : N-Maleoyl-6-aminocaproylhydrazide-HCl (Willner et al, 1993)

different linkers have been developed for this purpose. Heterobifunctional cross-linking reagents are particularly useful for conjugation. The different reactivities of the reactive groups permit the coupling procedure in a stepwise manner, allowing purification and characterization of intermediates before the final conjugation.

I 6.0 EFFICACY OF ANTIBODY-DRUG IMMUNOCONJUGATES (ICs)

Conjugation of cytotoxic drugs to monoclonal antibodies for use in cancer therapy is gaining more attention because many ICs have shown anti-tumor activity in both *in vitro* and *in vivo* models (Ford & Casson, 1986). *In vitro* ICs are generally less effective than the free drug, but it is not necessarily correlated to *in vivo* potential as shown for VDS ICs (Rowland et al, 1985; Casson et al, 1987) and Dox IC (Dillman et al, 1986). Greater efficacy *in vitro* and *in vivo* compared to the free drug has been reported with CBL ICs (Smyth, Pietersz & Classon et al, 1986). The major problems with immunoconjugates have been their low potency, poor access and the HAMA response. It is very important to focus on the factors affecting and improving efficacy.

An improved retention of therapeutic agents on the surface of target cells or the enhanced internalization of such agents into target cells will be important. The ways to attain these purposes include: 1) enhancing antibody affinity

for TAA, 2) increasing Ab uptake into tumor cells; and 3) decreasing desorption from tumor sites. The vasoconstrictive agents that can change the tumor-to-normal tissue perfusion ratio enchance the access of ICs to tumor (Symth, Pietersz & McKenzie, 1987). By using tumor necrosis factor, the antitumor effect and tumor localization of ICs can be enhanced (Symth, Pietersz & McKenzie, 1988). The use of aryl carbohydrate adducts of a radioiodinated Ab that increases intracellular retention (Ali et al, 1990) may be a hint to design some agents for increasing cell surface retention and internalization of therapeutic agents. The attachment of analogs of the peptide "glutamic acid-alanine-leucine-alanine" (GALA) to Fab of NR-ML-05 and NR-LU-10 MAbs has been shown to enhance the retention and internalization of these Fab fragments by tumor cells (Anderson et al, 1993). These approaches may be useful to increase the efficacy of AMT.

The loss of immunoreactivity of MAbs after the high loading of drugs limits IC efficacy. One approach to this problem is to utilize macromolecules such as dextran and albumin (Tsukada, Kato & Umemoto, 1984; Garnett & Baldwin, 1986; Shih & Goldenberg et al, 1991, Heindel et al, 1991) for high levels of drug substitution and then to link the drug conjugates to MAbs. The major advantage of these ICs is the potential for high substitution of drugs per MAb without significant loss of immunoreactivity.

Most drugs must enter tumor cells to exert an effect. Thus, the degree of IC taken up is critical for cytotoxicity at the tumor site. The evidence of Ab internalization after specific binding has been demonstrated (Matzku et al, 1988, Tsaltas, Ford & Gallant, 1992). There has been experimental evidence that some drug-Ab ICs are endocytosed (Hermentin et al, 1990). Once intracellular, the ideal IC can exert toxic effects even with the drug remaining covalently bound to MAb; however, drugs such as anthracyclines in the form of IC may be inactive unless they are cleaved from MAb as free drug. The findings of the acid-sensitive hydrazone linker in Dox ICs suggests that the release of free Dox from MAb in acidic intracellular compartments would be the important step in the action of Dox ICs (Greenfield et al, 1990; Braslawsky et al, 1990 & 1991; Trail et al, 1992 & 1993). Deconjugation via a chemical or enzymatic process in the intracellular compartment, lysosome, to release the free drugs will become the major approach to design the anthracycline ICs for improving therapeutic efficacy. Another avenue to increase the efficacy of ICs is the linking of more potent antitumor agents to MAbs for targeting. One of the Dox derivatives, 3'-deamino-3'-(4-morpholinyl)doxorubicin (MRA), has been conjugated to MAb LM609 for *in vitro* testing (Mueller, Wrasidlo & Reisfeld, 1990). The use of idarubicin-MAb conjugates has shown the complete eradication of smaller

tumors in a human xenograft model (Pietersz et al, 1988). The high specific cytotoxicity of maytansinoid conjugates toward tumor cell lines and low systemic toxicity in mice may open more effective treatments of human cancer (Chari et al, 1992)

Clinical studies have been performed to study the feasibility of AMT in cancer therapy. In these studies there was no toxicity of conjugates administrated to patients, but the results were not really encouraging. Moreover, Dox immunoconjugates showed toxicity in clinical trials. The toxicity was likely due to the Dox non-covalently attached to antibody and the drug was released from the conjugate to cause the toxic effect in patients (Oldham et al, 1988). The major impediment of AMT for the treatment of cancer is the HAMA response (Khazaeli et al, 1988; Tjandra et al, 1990; Petersen et al, 1991). Genetic engineering techniques are being evaluated as a way of overcoming the problem of the HAMA response by producing chimeric, partially human or totally human antibodies (LoBuglio et al, 1989; Gorman et al, 1991; Persson et al, 1991; Zebedee et al, 1992; Gram et al, 1992).

I 7.0 OBJECTIVES OF THESE STUDIES

Targeted chemotherapy using MAb offers a potential avenue to improve site-specific drug delivery to the tumor site. The objectives of this project were: 1) to develop the chemistry of heterobifunctional spacers for linking to drug; 2) to develop the conjugation conditions for linking drug-spacer derivatives to monoclonal antibody; and 3) to test the efficacy of drug-monoclonal antibody immunoconjugates *in vitro*.

CHAPTER II : SYNTHESIS OF HETEROBIFUNCTIONAL SPACERS AND DOXORUBICIN DERIVATIVES

II 1.0 INTRODUCTION

The development of monoclonal antibody-drug (doxorubicin) conjugates (immunoconjugates) has emerged during the past decade. Because of the conventional problems in unwanted cross-linkage and polymerization of the antibody and drugs during conjugation, the new methods of chemical attachment of doxorubicin to MAb is stressed. Heterobifunctional cross-linking reagents are increasingly used for their regiospecificity in coupling drug to MAb for immunotargeted chemotherapy. The modification of daunorubicin (Dau) in 3'-amino group with p-(isothiocyanato)benzoyl chloride and p-(isothiocyanato)phenypropionyl chloride results in 3'-N-amide analogues and the immunoconjugates from these Dau analogues have shown selective toxicity for human ovarian cancer cells (Sweet et al, 1989; Rosik & Sweet, 1990). The maleimidobenzoyl spacers have proved to be useful in the preparation of anthracycline-MAb conjugates (Hermentin et al, 1990). The immunoconjugate containing the doxorubicin (Dox) derivative, maleimidocaproyl doxorubicin hydrazone, were demonstrated to have antigen-specific cytotoxicity *in vitro* and to produce complete tumor regression *in vivo* (Trail et al, 1993; Willner et al, 1993). Therefore, we designed and synthesized a series of new maleimidobenzoyl cross-linking agents which enable the conjugation of 3'-amino group and 13-keto position of Dox to thiolated MAb, resulting in stable

conjugates for targeted chemotherapy.

II 2.0 HETEROBIFUNCTIONAL CROSS-LINKING REAGENT SYNTHESIS

The first spacer maleimidobenzoic acid (MBA) (**3**) was conveniently synthesized from 4-aminobenzoic acid (**1**), requiring only two synthetic steps as shown in Scheme 1. Treatment of **1** with 1.15 molar equivalent of maleic anhydride in acetone/methanol, provided intermediate 4-carboxy-maleimidobenzoic acid (CMBA) (**2**) in good yield (95%). Cyclodehydration of **2** with acetic anhydride as the reagent and solvent, with sodium acetate, followed by hydrolysis, gave the product **3**, also in good yield (78%). Both **2** and **3** were obtained as crude products and detected as a single spot on TLC. From ^{1}H NMR spectroscopy, **2** and **3** were pure and the purity was confirmed by microanalysis.

Two new maleimidobenzoyl spacers, N-(4-maleimidobenzoyl)-6-aminocaproic acid (MBCA) (**4**) and N-(4-maleimidobenzoyl)-11-aminoundecanoic acid (MBUA) (**5**) were prepared directly by coupling of **3** to commercially available amino acids, 6-aminocaproic acid and 11-aminoundecanoic acid respectively, utilizing a procedure by Eirkofer and Ritter with 1,1,1,3,3,3,-hexamethyldisilazane (HMD) as the silylating agent and solvent (Birkofer & Ritter, 1965). Scheme 2 provided an outline of the synthetic steps leading to **4** and **5** starting from **3** and 6-aminocaproic acid or 11-aminoundecanoic

acid.

Trimethylsilylated 6-aminocaproic acid which was moisture sensitive was prepared under inert condition. 6-Aminocaproic acid was refluxed under nitrogen with 1.1 molar equivalent of HMD and two drops of concentrated sulfuric acid, followed by the addition of triethylamine (TEA) and chlorotrimethylsilane (TMSCl) in benzene and stirring overnight at room temperature. Activation of **3** by 1.1 equivalent of isobutyl chloroformate with 1.1 equivalent TEA as hydrogen chloride acceptor at 0°C, followed by coupling to silylated 6-aminocaproic acid (2.0 equivalent), followed by mild hydrolysis, gave the product **4** and starting material detected on TLC. After work-up and flash chromatography, **4** was obtained in 49% yield. The synthesis of **5** was similar to **4** except 1.6 molar equivalent HMD was used. This synthesis gave 46% yield. Analysis of the ^{1}H NMR spectra of both **4** and **5** showed a sharp singlet of the maleimido group at 7.2 ppm, integrating for two protons.

Other two new maleimidobenzoyl spacers, 4-maleimidobenzohydrazide trifluoroacetate salt (MBH) (**15**) and N-(4-maleimidobenzoyl)-6-aminocaprohydrazide trifluoroacetate salt (MBCH) (**16**), were prepared directly from MBA (**3**) and MBCA (**4**) respectively by converting the carboxylic acid group to protected hydrazide and then following the treatment of trifluoroacetic acid, using a procedure modified from Willner et al. (Willner et al, 1993). Scheme 5 provided an outline of

the synthetic steps leading **15** and **16** starting from **3** and **4** respectively.

Activation of **3** by 1.1 equivalent of isobutyl chloroformate with 1.1 equivalent TEA as hydrogen chloride acceptor at 0°C, followed by coupling to *tert*-butyl carbazate (1.0 equivalent) at room temperature gave the crude protected hydrazide, 4-maleimidobenozic acid (*tert*-butyloxycarbonyl) hydrazide (**13**). After work-up and flash chromatography, **13** was obtained in 60% yield. The synthesis of N-(4-maleimidobenzoyl)-6-aminocaproic acid (*tert*-butyloxycarbonyl) hydrazide (**14**) was the same as the procedure above with the starting material **4**. This synthesis gave 72% yield. The protected hydrazide **13** and **14** were converted to hydrazide trifluoroacetate salts, **15** and **16** respectively, in ice-cold trifluoroacetic acid (TFA). Analysis of the ^{1}H NMR spectra of both **15** and **16** showed a sharp singlet of the maleimido group at 7.2 ppm, integrating for two protons.

II 2.1 Doxorubicin-Spacer Coupling Reactions

The heterobifunctional spacers, **3**, **4** and **5**, were used to couple doxorubicin at 3'-NH_2 position to form the 3'-amide bonding. The doxorubicin containing the free-base were obtained by treating doxorubicin hydrochloride (Dox·HCl) with 5% sodium bicarbonate. Selective amide formation of 3'-NH_2 with the various activated maleimidoyl spacers (**3,4,5**) (Scheme

3) was achieved under mild reaction conditions. The desired coupling products, 3'-N-acyl doxorubicin derivatives (**7**,**8**,**9**), were purified by preparative thin layer chromatography and obtained in 41-51 % yield.

The coupling reaction of maleimidobenzoyl spacers, **15** and **16**, to 13-keto position of doxorubicin were achieved by mixing the spacers and Dox·HCl with a catalyzed amount of trifluoroacetic acid (TFA) (Scheme 6) at RT for 1 day. The products, 13-acyl hydrazone doxorubicin, were precipitated out by the addition of acetonitrile and obtained in 85-95 % yield. Desired product can be further purified by TLC.

II 3.0 DISCUSSION

The maleimido derivatives, MBA, MBCA and MBUA, were designed to cross-link the amine group of Dox to thiol groups of antibody. MBA was synthesized using an established literature method and was obtained in an easy way without any purification. Before using 1,1,1,3,3,3-hexamethyldisilazine, several synthetic approaches were unsuccessful in preparing MBCA and MBUA. The major problem was the insolubility of the amino acids in ordinary organic solvents such as dichloromethane and THF in performing the coupling reaction. We tried a polar solvent, hexamethylphosphoramide (HMPA), but the coupling reaction was still unsuccessful. Thus, another synthetic approach outlined in Scheme 4 was adopted. However,

the maleimide was unstable in alkaline condition and was converted to maleamic acid before the hydrolysis of the ester bonding. The conversion was rapid and only a single spot was detected on TLC. We believe that the driving force of this reaction is the release of ring tension upon opening. The product was identified as **12** from ^1H NMR.

The most prominent feature of the organosilicon compounds is the excellent solubility in organic solvents. Substances that are insoluble in organic solvents because of the presence of $-NH_2$, $-CONH_2$, $-OH$, $-CO_2H$ groups, etc. become readily soluble in these solvents with the replacement of their protons by the trimethylsilyl group. 1,1,1,3,3,3-Hexamethyldisilazane is a suitable reagent for direct trimethylsilylation of amino acids which can not be silylated with trimethylchlorosilane, because of their zwitterionic character. The trimethylsilyl amino acids are soluble in ordinary organic solvents; thus, reactions can be carried out in a homogeneous organic phase. In these organic syntheses, the activated carboxylic acid group can be regioselectively coupled to the silylated amino group of amino acids without the formation of the anhydride bonding with the carboxylic acid group. After the coupling reaction, the free carboxylic acid group can be easily recovered by hydrolysis of silyl ester with water. The major problem of this methodology is that the reactions do not proceed completely, some starting material (**3**) is present even

by using an excess of silylated amino acids. This problem is most likely due to the very unstable nature of the silylated amino acid towards hydrolysis, during the various reaction manipulation steps. We believe that if these reaction were performed completely in a glove box, high yield of the desired products would be obtained.

The maleimido spacers, MBH and MBCH, were designed to couple the 13-keto group of Dox to thiolated antibody. These spacers, **15** and **16**, were synthesized from **3** and **4** respectively via the protected hydrazides, **13** and **14** (Scheme 5). The maleimide can be condensed with either *tert*-butyl carbazate or hydrazine, losing the typical maleimide vinyl proton resonance (Heindel et al, 1991). This suggests the competitive Michael addition of the carbazate and hydrazine to maleimide have occured. Moreover, at equimolar concentrations of activated spacers, **3** or **4**, and *tert*-butyl carbazate reacted at room temperature to give stable protected hydrazide. Maleimide was stable in acidic condition; thus, N-protection with *tert*-butoxycarbonyl group was adopted. The *tert*-butyl protective group was removed in excess TFA, giving stable trifluoroacetate salt.

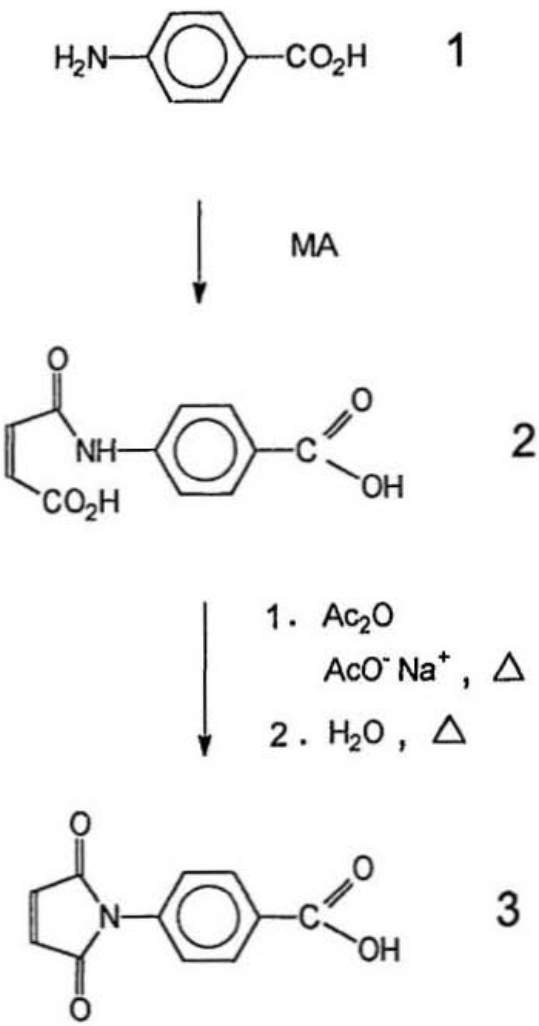
These heterobifunctional spacers consist of two reactive functional groups, maleimide with carboxylic acid or hydrazide, separated by phenyl, N-benzoylpentyl or N-benzoyldecyl spacer. The N-benzoylpentyl and N-benzoyldecyl

spacer arms should limit the possibility of steric hindrance between Dox and MAb. The maleimide and carboxylic acid functional groups were chosen because they show the desired reactivities with amines and thiols respectively. Moreover, these groups are chemically compatible, yielding stable cross-linking reagents. The maleimide and free hydrazide are not compatible to each other. Thus, the hydrazide is complexed with TFA to form the stable salt and to prevent the self condensation reaction.

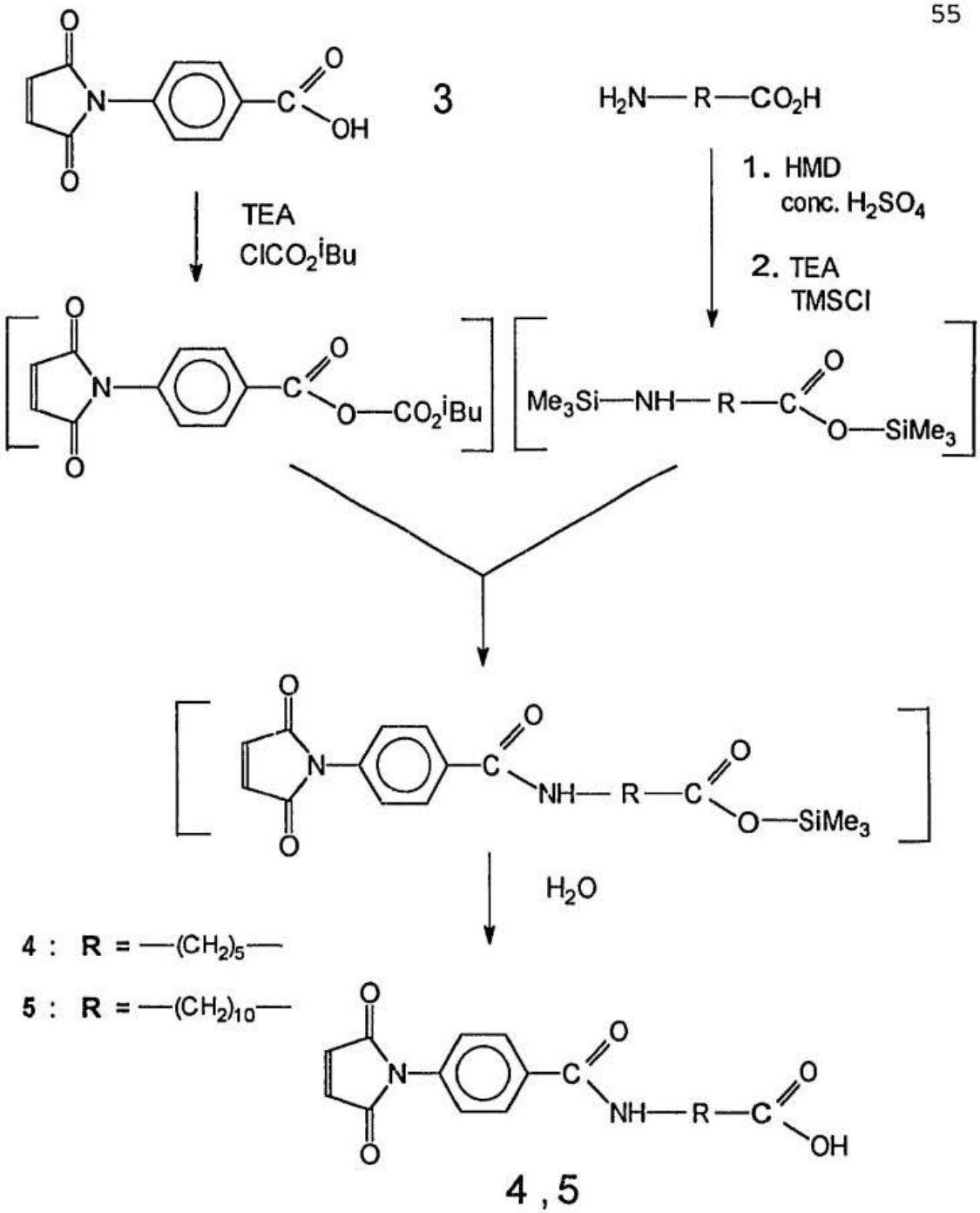
All maleimido derivative spacers, **7**, **8**, **9**, **15** and **16** are easy to prepare and stable during handling. The spacers, **7**, **8** and **9**, are very useful to couple drugs containing amino groups to proteins containing thiols and the resulting amide and thioether bonding provide stable linkage of the conjugate. Various reagents such as N-hydroxysuccimide or thionyl chloride can be used to activate the carboxylic acid group of the linker. However, acid chloride is too reactive and could react with the -OH group in Dox. The N-hydroxysuccimide derivative is less reactive and more selective towards the NH_2 group in Dox; however, the reaction rate will be slow and this may lead to the conjugation reaction between the amino group of Dox and the maleimide moiety of spacer. Thus, isobutyl chloroformate is the preferred reagent to activate the spacers. The reaction time, less than 30 minutes, is enough for the coupling reaction. The spacers are regioselectively

introduced to Dox in an straightforward manner and in reasonable yield.

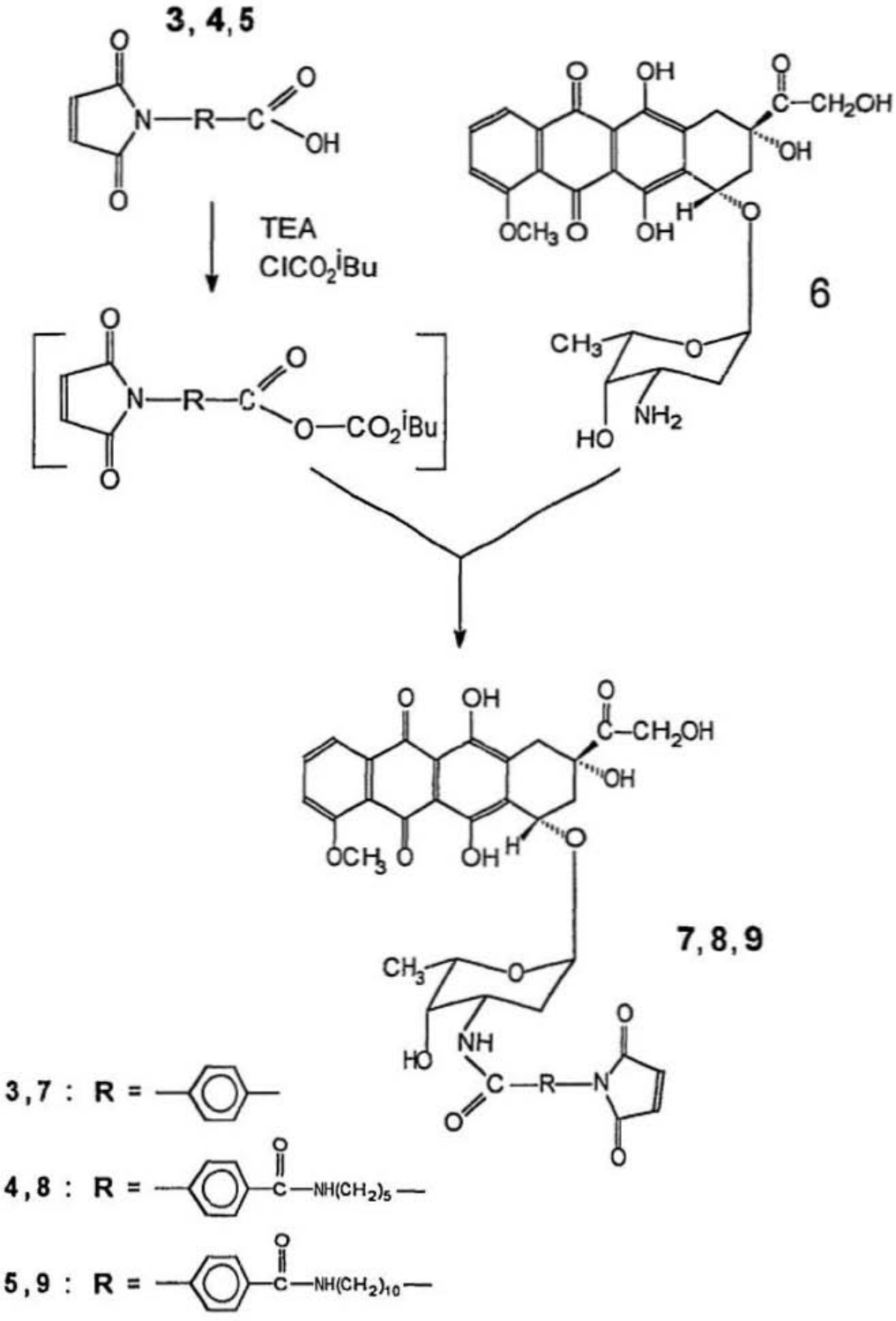
The hydrazide spacers, **15** and **16**, are not very reactive to the keto group of Dox. The addition of catalytic amounts of TFA accelerates the condensation reaction without acid-catalyzed deglycosidation of Dox. The isolation of C-13 hydrazone derivatives of Dox was simple and involved precipitation by the addition of acetonitrile.



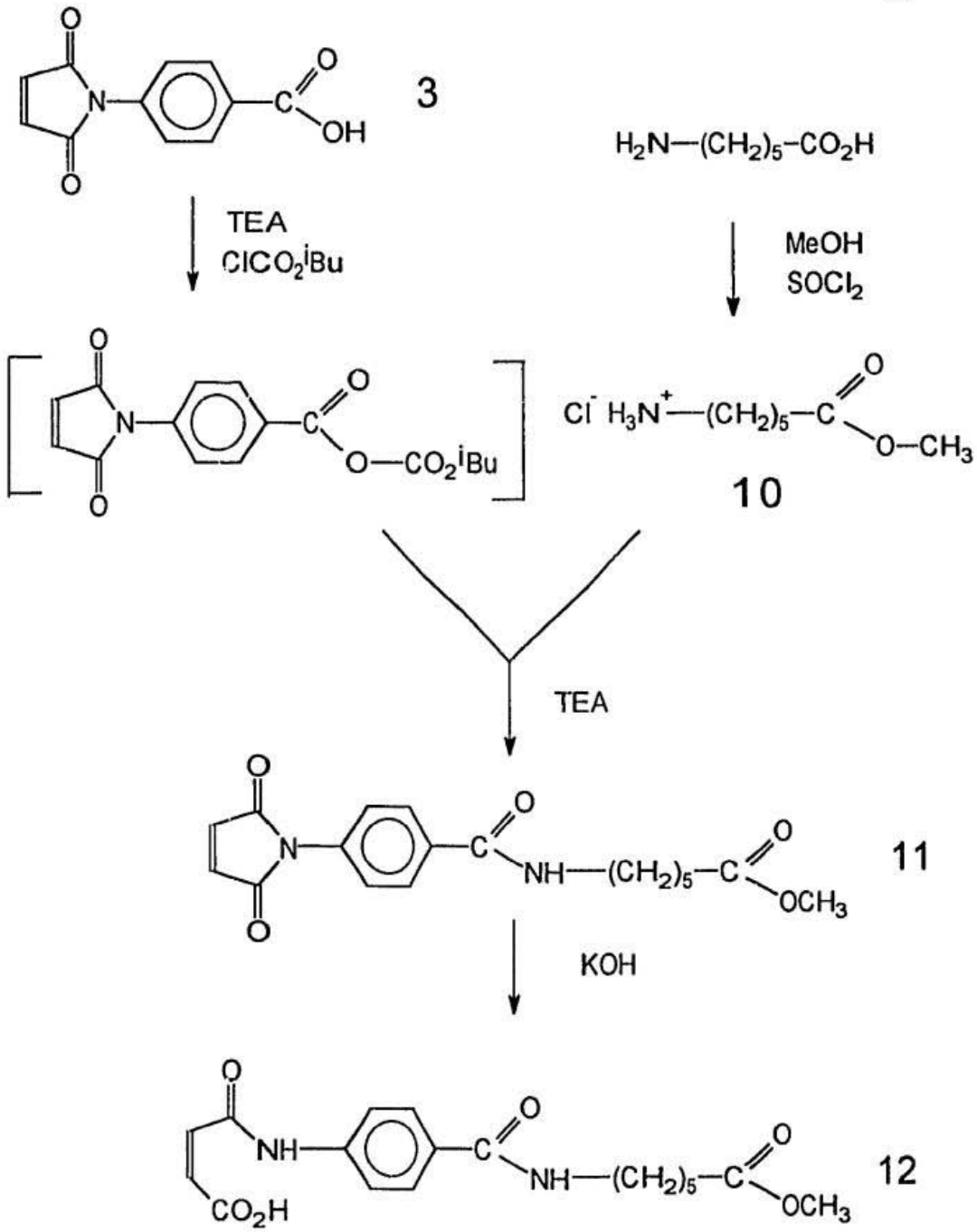
Scheme 1. Synthesis of 4 -carboxylmaleimidobenzoic acid (**2**) and 4-maleimidobenzoic acid (**3**)



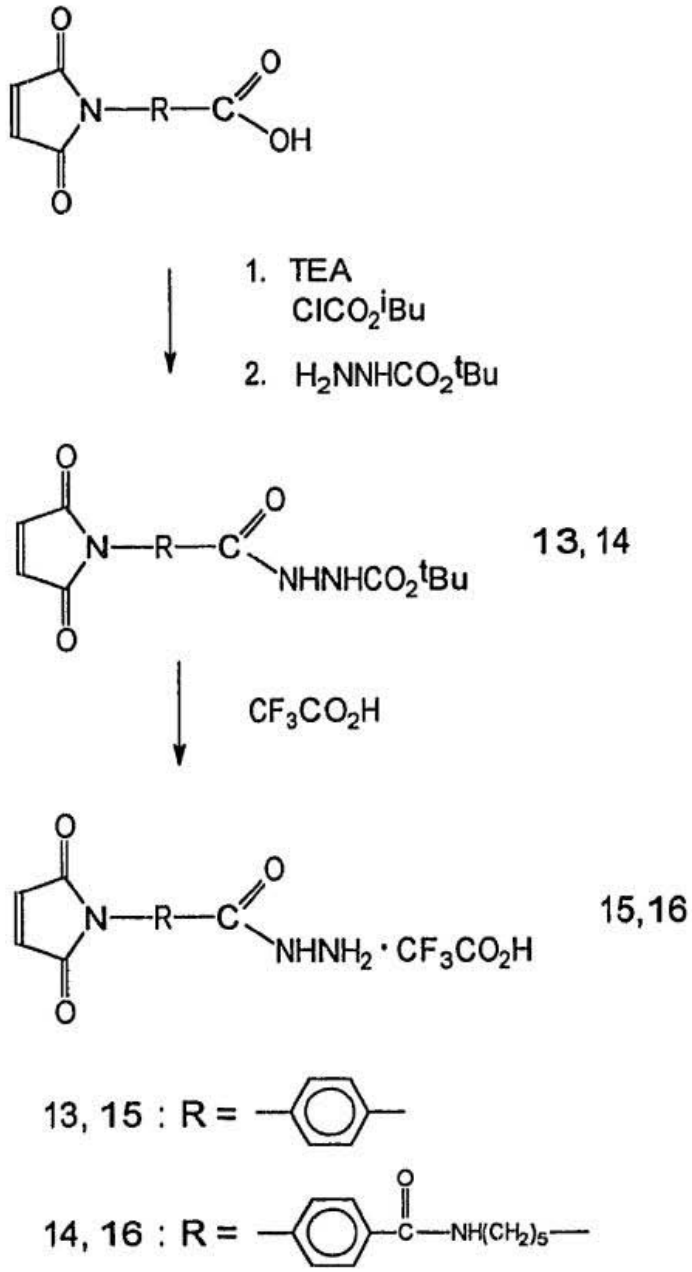
Scheme 2. Synthesis of N-(4-maleimidobenzoyl)-6-aminocaproic acid (**4**) and N-(4-maleimidobenzoyl)-11-aminoundecanoic acid (**5**)



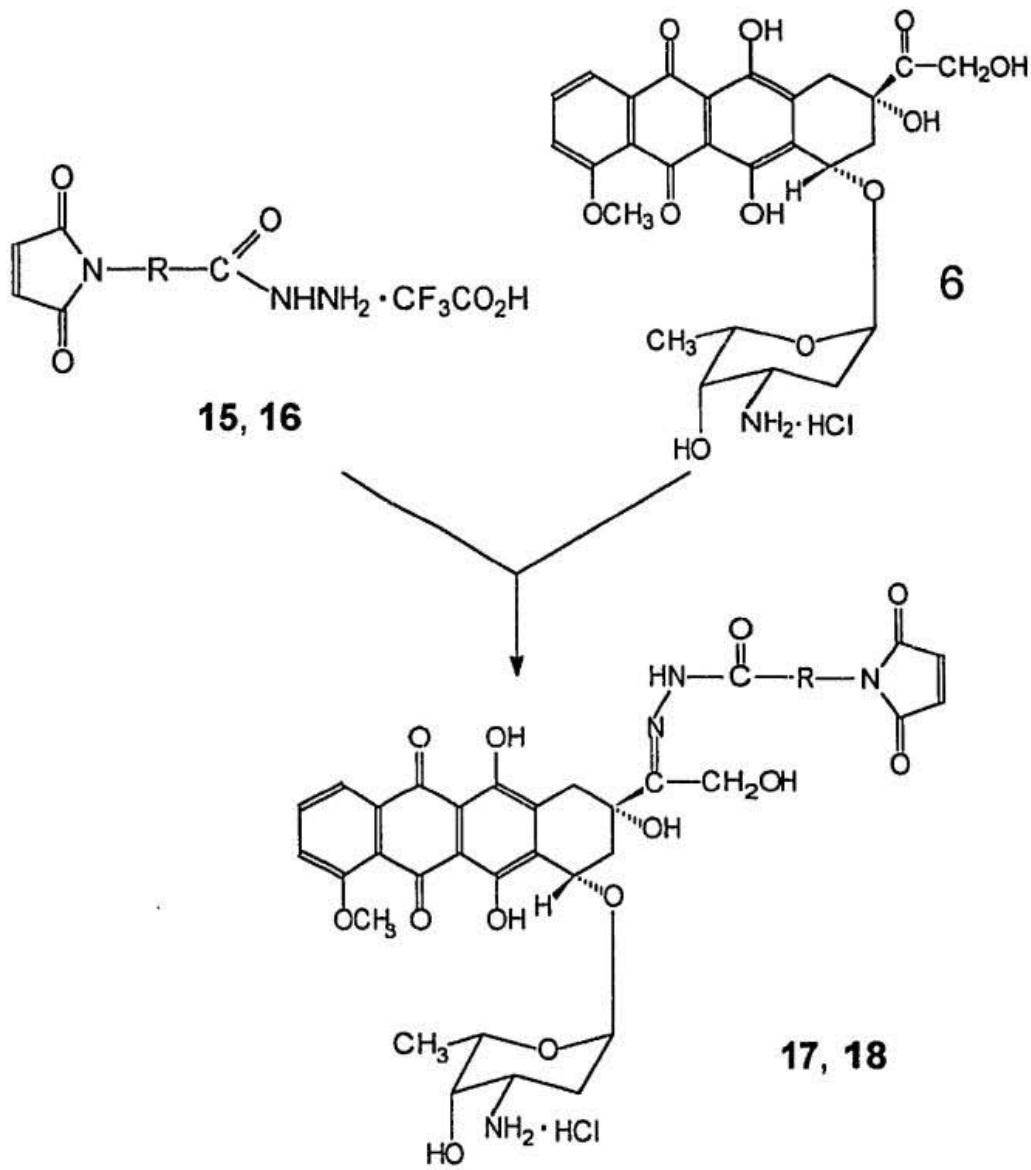
Scheme 3. Synthesis of doxorubicin 3'-N-amide derivatives (**7,8,9**)



Scheme 4. Base-hydrolysis of maleimidobenzoyl ester



Scheme 5. Synthesis of 4-maleimidobenzohydrazide trifluoroacetate salt (**15**) and N-(4-maleimidobenzoyl)-6-aminocaprohydrazide trifluoroacetate salt (**16**)



Scheme 6. Synthesis of doxorubicin C-13 hydrazone derivatives **(17,18)**

II 4.0 EXPERIMENTAL

Materials. Anhydrous reactions were performed under an inert atmosphere of nitrogen. Unless otherwise noted, starting material, reactant and solvents were obtained commercially from Aldrich and were used as such or purified and/or dried by standard means (Perrin & Armarego, 1988). Organic solvents were dried over magnesium sulphate ($MgSO_4$), evaporated on a rotatory evaporator and under reduced pressure. All reactions were monitored by thin-layer chromatography (TLC). The plates were visualized by UV fluorescence. Commercial TLC plates were Sigma T6145 (polyester silica gel 60 Å (0.25mm). Flash chromatography was performed according to the method of Still and coworkers (Still et al.) on Merck grade 60 silica gel, 230-400 mesh. Melting points (mp) were recorded on an Electrothermal 9100 apparatus and are uncorrected. The infrared spectra (IR) were taken on a Nicolet model 205 FT-IR spectrophotometer. Mass spectra assays (MS, m/e) were obtained using a VG Micomass 7070 HS instrument with an ionization energy of 70ev. Elemental analyses were conducted by Microanalysis Laboratories Limited, Markham, Ontario. Nuclear magnetic resonance (NMR) spectra were obtained in deuterated dimethylformamide, dimethyl sulfoxide, acetone or chloroform on a General Electric GE 300-NB (300 MHz) instrument: chemical shifts were measured relative to internal standards:

tetramethylsilane (TMS, δ 0.0 ppm) for ^{1}H and ^{13}C NMR. Multiplicities are described by the following abbreviations: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and so on. The NMR assignments were aided by ^{1}H-^{13}C correlation (HETCORR) 2-D spectra.

4-Carboxylmaleimidobenzoic acid (CMBA) (2)

4-Aminobenzoic acid (**1**) (20 g, 146 mmol) suspended in anhydrous acetone (120 ml) was solubilized by the addition of distilled methanol (20 ml). Then, maleic anhydride (16.49 g, 168 mmol) in anhydrous acetone (40 ml) was added dropwise and stirred at room temperature for 1h. The yellow precipitate was filtered, washed with 100 ml acetone, and vacuum-dried to yield 32.70g (95%) of **2** : mp 242-243°C; IR (KBr) 3500-2500 (CO_2H), 3325(NH), 1700 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 12.84 (bs, 2H, Ar, CO_2H), 10.61 (s, 1H, NH), 7.91 and 7.74 (two d, 4H, J=8.7 Hz, para-substituted phenyl group), 6.50 and 6.33 (two d, 2H, J=12 Hz, maleimido-H); ^{13}C NMR (DMSO-d_6) δ 166.88 (2), 163.63, 142.73, 131.64, 130.19 (2), 125.52, 118.69 (2); MS, m/e: 235 (M^+) Anal. Calcd for $C_{11}H_9N_1O_5$: C, 56.17; H, 3.86; N, 5.96;. Found: C, 56.22; H, 3.92; N, 6.03.

4-Maleimidobenzoic acid (MBA) (3)

The yellow powder of **2** (20g, 85 mmol) was treated with acetic anhydride (40 ml) and anhydrous sodium acetate (3.4g,

41.50 mmol) at 50°C for 2h until a clear solution was formed. The solution was evaporated to dryness and stirred with water (500 ml) at 70°C for 2 h for complete hydrolysis. The white precipitate was filtered off and vacuum-dried to yield 14.45g (78%) of **3** : mp 238-239°C; IR (KBr) 3350-2600 (CO_2H), 1799 and 1715 (C=O) cm-1; ^1H NMR (DMSO-d_6) δ 13.1 (s, 1H, CO_2H), 8.05 and 7.51 (two d, 4H, J=8.5 Hz, para-substituted phenyl group) 7.23 (s, 2H, maleimido-H); ^{13}C NMR (DMSO-d_6) δ 169.52 (2), 166.65, 135.50, 134.86 (2), 129.89 (2), 129.50, 126.11 (2); MS, m/e: 217 (M^+) Anal. Calcd for $C_{11}H_7N_1O_4$: C, 60.83; H, 3.25; N,6.45. Found: C, 60.97; H, 3.36; N, 6.31.

N-(4-Maleimidobenzoyl)-6-aminocaproic acid (MBCA)(4)

Compound **4** was prepared directly from **3** by coupling to 6-aminocaproic acid. A suspension of 6-aminocaproic acid (3.00 g, 22.87 mmol) in 1,1,1,3,3,3-hexamethyldisilazane (5.31 ml, 25.16 mmol), containing 2 drops of concentrated sulfuric acid, was heated at reflux (120-130°C) under nitrogen until complete dissolution. Afterwards, the reaction mixture was boiled for a further 30 min. After cooling, benzene (30 ml), TEA (1.14 ml, 7.62 mmol), and TMSCl (0.97 ml, 7.62 mmol) were added and the mixture was stirred overnight at room temperature to yield the trimethylsilyl ester of N-trimethylsilyl-6-aminocaproic acid. The powder of **3** (2.48 g, 11.44 mmol) was suspended in 40 ml dichloromethane and cooled to 0°C before the addition of

TEA (1.75 ml, 12.56 mmol) and isobutyl chloroformate (1.63 ml, 12.56 mmol). The mixture was stirred for 1h at 0°C and then added to a solution of silylated intermediate in 25 ml dichloromethane. Stirring was continued for 4 h at room temperature. The reaction mixture was diluted with ethyl acetate (200 ml), washed with 0.1N HCl (2 X 100 ml), saturated NaCl (2 X 100 ml), then dried ($MgSO_4$) and evaporated to give crude maleimide. The product was purified by flash chromatography, using two mixtures of solvent (0.1:1:3 acetic acid/acetone/hexane then 0.1:2:3 acetic acid/acetone/hexane) to yield 1.68 g (49%) of **4**: mp 156-157°C; IR (KBr) 3500-2500 (CO_2H), 3466 and 3318 (NH), 3086 (Ar), 2931 and 2868 (alkane) and 1701 (C=O) cm^{-1}; ^1H NMR (DMF-d_7) δ 8.55 (t, 1H, J=5.2 Hz, NH), 8.07 and 7.53 (two d, 4H, J=8.5 Hz, para-substituted phenyl group), 8.03 (s, 1H, CO_2H), 7.24 (s, 2H, maleimido-H), 3.39 (q apparent, 2H, J=6.8 Hz, CH_2N), 2.30 (t, 2H, J=7.3 Hz, CH_2CO), 1.62 (m, 4H, CH_2 x 2), 1.43 (m, 2H, CH_2); ^{13}C NMR (DMF-d_7) δ 175.15, 170.42 (2), 166.31, 135.46 (2), 134.98, 134.76, 128.40 (2), 126.78 (2), 40.08, 34.34, 29.81 (superimposed with the solvent peak), 27.04, 25.29; MS, m/e: 330 (M$^+$). Anal. Calcd for $C_{17}H_{18}N_2O_5$: C, 61.81; H, 5.49; N, 8.48. Found: C, 61.69; H, 5.39; N, 8.33.

N-(4-Maleimidobenzoyl)-11-aminoundecanoic acid (MBUA) (5)

A suspension of 11-aminoundecanoic acid (3.00 g, 14.90

mmol) in 1,1,1,3,3,3-hexamethyldisilazane (5.03 ml, 23.84 mmol), containing 2 drops of concentrated sulfuric acid, was heated at reflux (120-130°C) under nitrogen until complete dissolution. Afterwards, the reaction mixture was boiled for a further 30 min. After cooling, benzene (20 ml), TEA (0.69 ml, 4.94 mmol), and TMSCl (0.63 ml, 4.97 mmol) were added and the mixture was stirred overnight at room temperature to yield the trimethylsilyl ester of N-trimethylsilyl-11-aminoundecanoic acid. The powder of **3** (1.62 g, 7.46 mmol) was suspended in 30 ml dichloromethane and cooled to 0°C before the addition of TEA (1.14 ml, 8.18 mmol) and isobutyl chloroformate (1.06 ml, 8.20 mmol). The mixture was stirred for 1h at 0°C and then added to a solution of silylated intermediate in 25 ml dichloromethane. Stirring was continued for 4h at room temperature. The reaction mixture was diluted with ethyl acetate (200 ml), washed with 0.1N HCl (2 X 100 ml), saturated NaCl (2 X 100 ml), then dried ($MgSO_4$) and evaporated to give crude maleimide. The crude product adsorbed in silica gel (7 g) was layered on a silica gel column and was purified by flash chromatography with solvent 0.1:1:3 acetic acid/acetone/hexane then 0.1:2:3 acetic acid/acetone/hexane to yield 1.38 g (46%) of **5**: mp 162-164°C; IR (KBr) 3500-2500 (CO_2H), 3473 and 3318 (NH), 3079 (Ar), 2945 and 2868 (alkane) and 1715 (C=O) cm^{-1}; ^1H NMR (DMF-d_7) δ 8.55 (t, 1H, J=5.4 Hz, NH), 8.07 and 7.53 (two d, 4H, J=8.5 Hz,

para-substituted phenyl group), 8.03 (s, 1H, CO_2H), 7.23 (s, 2H, maleimido-H), 3.39 (q apparent, 2H, J=6.9 Hz, CH_2N), 2.28 (t, 2H, J=7.3 Hz, CH_2CO), 1.34 (m, 4H, CH_2 x 2), 1.4-1.25 (bs, 12H, CH_2 x 6); ^{13}C NMR (DMF-d_7) δ 175.13, 170.41 (2), 166.30, 135.44 (2), 134.94, 134.79, 128.38 (2), 126.76 (2), 40.11, 34.36, 27.50, 25.54, six peaks hidden by solvent peak; MS, m/e: 400 (M^+). Anal. Calcd for $C_{22}H_{28}N_2O_5$: C, 65.98; H, 7.05; N, 7.00. Found: C, 65.90; H, 7.01; N, 6.92.

Synthesis of 3'-N-amide derivatives of Doxorubicin (7,8,9)

Doxorubicin free base (**6**) (from 5 ml of 2mg/ml of Dox·HCl salt in 0.9% saline, 0.017nmol) was extracted with chloroform from 1 ml 5% $NaHCO_3$ until the aqueous layer was free of the strong orange colour. The extracts were dried over Na_2SO_4, filtered, and evaporated, giving 9.37 mg, assuming 100% recovery.

Synthesis of 3'-N-(4-maleimidobenzoyl)doxorubicin (7)

MBA **(3)** (0.2 g, 0.92 mmol) was suspended in 4 ml dichloromethane and cooled down to 0°C. TEA (144 µl, 1.03 mmol) and isobutyl chloroformate (132 µl, 1.02 mmol) were added and stirred at 0°C for 1 h. The activated MBA (150 µl, 0.032 mmol) was added to the Dox-NH_2 (9.37 mg, 0.017 mmol) in CH_2Cl_2:MeOH (5 ml, 95:5) and stirred at room temperature for 0.5 h. The product was purified by TLC, using CH_2Cl_2/MeOH

(9:1) and then scraped out. The silica gel containing the desired product was washed with CH_2Cl_2/MeOH (20 ml, 9:1) and filtered. After evaporating the solvent from the filtrate, the residue was dissolved in 2 ml hot dichloromethane, cooled down to room temperature and then filtered through cotton. Ethyl ether was added drop by drop to the filtrate for precipitation. After the removal of the solvent, the red precipitate was dried under vacuum to give 5.23 mg (41%) of **7**: ^{1}H NMR ($CDCl_3$) δ 14.00 (s, 1H, OH-11), 13.26 (s, 1H, OH-6), 8.05 (d, 1H, $J_{1,2}$=7.2 Hz, H-1), 7.82 and 7.44 (two d, 4H, J=8.5 Hz, para-substituted phenyl group), 7.79 (t, 1H, $J_{1,2}=J_{2,3}$=8.0 Hz, H-2), 7.39 (d, 1H, $J_{2,3}$=8.4 Hz, H-3), 6.87 (s, 2H, maleimido-H), 6.52 (d, 1H, J=8.4 Hz, NH-3'), 5.55 (d, 1H, $J_{1',2'}$=3.5 Hz, H-1'), 5.32 (m, 1H, H-7), 4.79 (d, 2H, J=4.6 Hz, CH_2-14), 4.59 (s, 1H, HO-4'), 4.35 (m, 1H, H-3'), 4.24 (q, 1H, $J_{4',5'}$=6.7 Hz, H-5'), 4.07 (s, 3H, OCH_3), 3.28 (d, 1H, $J_{10\alpha,10\beta}$=18.5 Hz, H-10β), 3.03 (d, 1H, $J_{10\alpha,10\beta}$=18.5 Hz, H-10α), 2.37 (d, 1H, $J_{8\alpha,8\beta}$=15 Hz, H-8β), 2.19(dd, 1H, $J_{7,8\alpha}$=3.8 Hz, $J_{8\alpha,8\beta}$=15 Hz, H-8α) 1.32 (d, 3H, $J_{4',5'}$= 6.56 Hz, CH_3-6').

Synthesis of 3'-N-[N-(4-maleimidobenzoyl)-6-aminocaproyl] doxorubicin (8)

MBCA (**4**) (0.1 g, 0.30 mmol) was suspended in 4 ml dichloromethane and cooled down to 0°C. TEA (47 μl, 0.34 mmol) and isobutyl chloroformate (43 μl, 0.33 mmol) were added

and stirred at 0°C for 1 h. The activated MBCA (348 μl, 0.026 mmol) was added to the Dox-NH_2 (9.37 mg, 0.017 mmol) in 5 ml CH_2Cl_2:MeOH (95:5) and stirred at room temperature for 0.5 h. The product was purified by TLC, using CH_2Cl_2/MeOH (9:1) and then scraped out. The silica gel containing the desired product was washed with CH_2Cl_2/MeOH (20 ml, 9:1) and filtered. After evaporating the solvent from the filtrate, the residue was dissolved in 2 ml hot dichloromethane, cooled down to room temperature and then filtered through cotton. Ethyl ether was added dropwise to the filtrate for precipitation. After the removal of the solvent, the red precipitate was dried under vacuum to give 7.57 mg (51%) of **8**; ^{1}H NMR ($CDCl_3$) δ 13.96 (s, 1H, OH-11), 13.25 (s, 1H, OH-6), 8.03 (d, 1H, $J_{1,2}$=7.6 Hz, H-1), 7.85 and 7.45 (two d, 4H, J=8.6 Hz, para-substituted phenyl group), 7.78 (t, 1H, $J_{1,2}$=$J_{2,3}$=8.1 Hz, H-2), 7.38 (d, 1H, $J_{2,3}$=8.2 Hz, H-3), 6.88 (s, 2H, maleimido-H), 6.46 (t, 1H, J=5.8 Hz, NH), 5.92 (d, 1H, J=8.6 Hz, NH-3'), 5.48 (s, 1H, H-1'), 5.27 (m, 1H, H-7), 4.75 (d, 2H, J=4.8 Hz, CH_2-14), 4.60 (s, 1H, HO-4'), 4.13 (m, 2H, H-3', H-5'), 4.07 (s, 3H, OCH_3), 3.45 (m, 2H, CH_2N), 3.27 (d, 1H, $J_{10\alpha,10\beta}$=18.0 Hz, H-10β), 3.02 (d, 1H, $J_{10\alpha,10\beta}$=18.0 Hz, H-10α), 2.35 (d, 1H, J=14.7 Hz, H-8β), 2.17(m, 3H, H-8α, CH_2CO), 1.24 (d, 3H, $J_{5',6'}$=6.5 Hz, CH_3-6').

Synthesis of 3'-N-[N-(4-maleimidobenzoyl)-11-aminoundecanoyl doxorubicin (9)

MBUA (**5**) (0.2 g, 0.50 mmol) was suspended in 4 ml dichloromethane and cooled down to 0°C. TEA (77 μl, 0.55 mmol) and isobutyl chloroformate (72 μl, 0.55 mmol) were added and stirred at 0°C for 1 h. The activated MBCA (200 μl, 0.027 mmol) was added to the Dox-NH_2 (9.37 mg, 0.017 mmol) in 5 ml CH_2Cl_2:MeOH (95:5) and stirred at room temperature for 0.5 h. The product was purified by TLC, using CH_2Cl_2/MeOH (9:1) and then scraped out. The silica gel containing the desired product was washed with CH_2Cl_2/MeOH (20 ml, 9:1) and filtered. After evaporating the solvent from the filtrate, the residue was dissolved in 2 ml hot dichloromethane, cooled down to room temperature and then filtered through cotton. Ethyl ether was added drop by drop to the filtrate for precipitation. After the removal of the solvent, the red precipitate was dried under vacuum to give 7.13 mg (45%) of **9**: ^{1}H NMR ($CDCl_3$) δ 13.98 (s, 1H, OH-11), 13.25 (s, 1H, OH-6), 8.04 (d, 1H, $J_{1,2}$=7.7 Hz, H-1), 7.85 and 7.63 (two d, 4H, J=8.5 Hz, para-substituted phenyl group), 7.78 (t, 1H, $J_{1,2}=J_{2,3}$=8.0 Hz, H-2), 7.39 (d, 1H, $J_{2,1}$=8.6 Hz, H-3), 6.88 (s, 2H, maleimido-H), 6.21 (t, 1H, J=5.2 Hz, NH), 5.86 (d, 1H, J=8.6 Hz, NH-3'), 5.50 (d, 1H, $J_{1',2'}$=3.7 Hz, H-1'), 5.29 (m, 1H, H-7), 4.76 (d, 2H, J=5.21 Hz, CH_2-14), 4.56 (s, 1H, HO-4'), 4.15 (m, 2H, H-3', H-5'), 4.07 (s, 3H, OCH_3), 3.45 (m, 2H, CH_2N), 3.28 (d, 1H, $J_{1\alpha,1\beta}$=18.6 Hz,

H-10β), 3.03 (d, 1H, $J_{10\alpha,10\beta}$=18.6 Hz, H-10α), 1.27 (m, 15H, CH_3-6', CH_2 x 6).

6-Aminocaproic acid methyl ester hydrochloride (10)

To a stirred mixture of 6-aminocaproic acid (5.02 g, 38.3 mmol) in absolute methanol (60 ml) at 0°C, thionyl chloride (8.4 ml, 115 mmol) was added dropwise. The ice bath was removed, and the mixture was heated at reflux for 4 h and concentrated *in vacuo*. The product was crystallized from ether-methanol to yield 6.64 g (95%) of **10**: mp 113-115°C; IR (KBr) 3445 (NH), 2951 and 2670 (alkane) and 1732 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.13 (bs, 3H, $NH_3^+Cl^-$), 3.59 (s, 3H, OCH_3), 2.31 (t, 2H, J=7.6 Hz, CH_2N), 2.31 (t, 2H, J=7.3 Hz, CH_2CO), 1.54(m, 4H, CH_2 x 2), 1.31 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ 173.23, 51.28, 38.44, 33.02, 26.55, 25.32, 23.90; MS, m/e: 181 (M^+).

N-(4-Maleimidobenzoyl)-6-aminocaproic acid methyl ester (11)

Compounds **11** was prepared directly from **3** by coupling to 6-aminocaproic acid methyl ester (**10**). A suspension of **3** (1 g, 4.60 mmol) in 30 ml dichloromethane was cooled to 0°C before the addition of TEA (0.64 ml, 4.59 mmol) and isobutyl chloroformate (0.6 ml, 4.63 mmol). The mixture was stirred for 1h at 0°C and then added to a solution of **10** (0.86 g, 4.73 mmol) in 20 ml dichloromethane, containing TEA (0.66 ml, 4.73

mmol). The reaction mixture was stirred for 2h at room temperature. The mixture was washed with H_2O (30 ml), 0.1N HCl (30 ml), 5% $NaHCO_3$ (2 x 30 ml), H_2O (30 ml), then dried ($MgSO_4$) and evaporated to give crude maleimide. The product was purified by flash chromatography with solvent first 1:3 and then 1:2 acetone/hexane to yield 0.76 g (48%) of **11**: mp 83-85°C; IR (KBr) 3335 (NH), 3084 (Ar), 2957 and 2865 (alkane) and 1738, 1726 and 1709 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.54 (t, H, J=5.6 Hz, NH), 7.97 and 7.47 (two d, 4H, J=8.5 Hz, para-substituted phenyl group), 7.21 (s, 2H, maleimido-H), 3.59 (s, 3H, OCH_3), 3.29 (q apparent, 2H, J=6.7 Hz, CH_2N), 2.32 (t, 2H, J=7.4 Hz, CH_2CO), 1.57 (m, 4H, CH_2 x 2), 1.34 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ 173.35, 169.68 (2), 165.49, 134.80 (2), 133.87, 133.71, 127.75 (2), 126.09 (2), 51.19, 39.11, 33.25, 28.78, 25.97, 24.24; MS, m/e: 344 (M^+).

N-(4-Carboxylmaleimidobenzoyl)-6-aminocaproic acid methyl ester (12)

1.4 ml 1N KOH was made up to 5 ml solution with H_2O and then added to compound **11** (0.3 g, 0.87 mmol) in 5 ml THF. The reaction mixture was stirred at 0°C for 30 min. The solution was neutralized with 0.1 N HCl (20 ml) and extracted with ethyl acetate (2 x 50 ml), and then dried ($MgSO_4$) and evaporated to yield 0.27 g (85%) of **12**: mp 173-175°C; IR (KBr) 3400-2400 (CO_2H), 3378 and 3287 (NH), 3044 (Ar), 2957 and 2878

(alkane), 1721, 1703 and 1690 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ 10.55 (s, 1H, CO_2H), 8.36 (t, 1H, J=5.6 Hz, NH), 7.82 and 7.69 (two d, 4H, J=8.7 Hz, para-substituted phenyl group), 6.48 and 6.34 (two d, 2H, J=12 Hz, maleimido-H), 3.58 (s, 3H, OCH_3), 3.23 (q apparent, 2H, J=6.7 Hz, CH_2N), 2.31 (t, 2H, J=7.4 Hz, CH_2CO), 1.54 (m, 4H, CH_2 x 2), 1.33 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ 173.37, 167.01, 165.47, 163.43, 141.06, 131.41, 130.45, 129.66, 128.04 (2), 118.57 (2), 51.22, one carbon hidden by solvent peak, 33.23, 28.87, 25.60, 24.22; MS, m/e: 264 (M^+-98), 120 (M^+-242).

4-Maleimidobenzoic acid (*tert*-butyloxycarbonyl) hydrazide (13)

Compound **13** was prepared directly from **3** by coupling to *tert*-butyl carbazate. The powder of **3** (2.68 g, 12.3 mmol) was suspended in 60 ml dichloromethane and cooled to 0°C before the addition of TEA (1.89 ml, 13.6 mmol) and isobutyl chloroformate (1.76 ml, 13.6 mmol). The mixture was stirred for 1 h at 0°C and then *tert*-butyl carbazate (1.62 g, 12.3 mmol) in 10 ml dichloromethane was added dropwise. Stirring was continued for 2 h at room temperature. The reaction mixture was diluted with ethyl acetate (150 ml) and washed with saturated $NaHCO_3$ (2 x 50 ml), 0.1 N HCl (2 x 50 ml), saturated NaCl (2 x 50 ml), H_2O (50 ml), then dried ($MgSO_4$) and evaporated to give crude protected hydrazide. The product was purified by flash chromatography with two solvent mixtures:

first 1:2 and then 2:3 acetone/hexane to yield 2.44 g (60%) of **13**: mp 182-184°C; IR (KBr) 3545 and 3329 (NH), 3093 (Ar), 2991 (alkane) and 1710 (C=O) cm^{-1}; ^1H NMR (Acetone-d_6) **δ** 9.60 (s, 1H, NH), 8.02 and 7.53 (two d, 4H, J=8.6 Hz, para-substituted phenyl group), 7.07 (s, 2H, maleimido-H), 2.99 (s, 1H, NH), 1.45 (s, 9H, 3 x CH_3); ^{13}C NMR (Acetone-d_6) **δ** 169.06 (2), 165.63, 155.47, 134.79, 134.34 (2), 131.38, 127.68 (2), 125.59 (2), 79.50, 27.30 (3); MS, m/e: 331 (M^+). Anal. Calcd for $C_{16}H_{17}N_3O_5$: C, 57.97; H, 5.17; N, 12.69. Found: C, 58.06; H, 5.14; N, 12.73.

4-Maleimidobenzohydrazide trifluoroacetate salt (15)

The powder of **13** (500 mg, 1.51 mmol) was dissolved in ice-cold trifluoroacetic acid (5 ml) and stirred for 30 min in an ice-bath. The trifluoroacetic acid was removed under vacuum at RT to give compound **15** quantitively: mp 142-144°C; IR (KBr) 3500-2500 (CO_2H), 3277 (NH), 3111 (Ar), 2901 and 2720 (alkane) and 1710 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) **δ** 11.62 (bs, 1H, $NHNH_3^+CF_3O_2^-$), 8.63 (bs, 3H, NH_3^+), 8.01 and 7.56 (two d, 4H, J=8.5 Hz, para-substituted phenyl group), 7.24 (s, 2H, maleimido-H); ^{13}C NMR (DMSO-d_6) **δ** 169.60 (2), 165.43, 135.47, 134.97 (2), 129.38, 128.38 (2), 126.46 (2); MS, m/e: 200 (M^+-145). Anal. Calcd for $C_{13}H_{18}N_3O_5F_3$: C, 44.17; H, 5.14; N, 11.89. Found: C, 44.16; H, 5.06; N, 11.93.

N-(4-Maleimidobenzoyl)-6-aminocaproic acid (*tert*-butyloxy-carbonyl) hydrazide (14)

Compound **14** was prepared directly from **4** by coupling to *tert*-butyl carbazate. The powder of **4** (1.05 g, 3.18 mmol) was suspended in 30 ml dichloromethane and cooled to 0°C before the addition of TEA (0.49 ml, 3.52 mmol) and isobutyl chloroformate (0.45 ml, 3.44 mmol). The mixture was stirred for 1h at 0°C and then *tert*-butyl carbazate (0.42 g, 3.18 mmol) in 10 ml dichloromethane was added dropwise. Stirring was continued for 2 h at room temperature. The reaction mixture was diluted with ethyl acetate (100 ml) and washed with saturated $NaHCO_3$ (2 x 50 ml), 0.1 N HCl (2 x 50 ml), saturated NaCl (2 x 50 ml), H_2O (50 ml), then dried ($MgSO_4$) and evaporated to give crude protected hydrazide. The product was purified by flash chromatography with two solvent mixtures: first 2:3 and then 1:1 acetone/hexane to yield 1.01 g (72%) of **14**: mp 137 - 139°C; IR (KBr) 3310 (NH), 3082 (Ar), 2978 and 2934 (alkane) and 1707 (C=O) cm^{-1}; ^1H NMR (Acetone-d_6) δ 8.80 (s, 1H, NH), 8.00 and 7.48 (two d, 4H, J=8.7 Hz, para-substituted phenyl group), 7.73 (t, 1H, J=4.7 Hz, $N\underline{H}CH_2$), 7.07 (s, 2H, maleimido-H), 3.41 (q apparent, 2H, J=6.9 Hz, CH_2N), 2.88 (s, 1H, NH), 2.21 (t, 2H, J=7.3 Hz, CH_2CO), 1.64 (m, 4H, 2 x CH_2), 1.44 (m, 2H, CH_2, superimposed on ^{t}Bu group), 1.41 (s, 9H, 3 x CH_3); ^{13}C NMR (Acetone-d_6) δ 172.74, 170.22 (2), 166.47, 156.45, 135.39 (2), 135.19, 134.94, 128.45 (2), 126.65

(2), 80.17, 40.24, 34.15, 29.91 (superimposed on acetone peak), 28.36 (3), 27.08, 25.64; MS, m/e: 200 (M^+-244). Anal. Calcd for $C_{22}H_{28}N_4O_6$: C, 59.43; H, 6.35; N, 12.61. Found: C, 59.48; H, 6.31; N, 12.58.

N-(4-Maleimidobenzoyl)-6-aminocaprohydrazide trifluoroacetate salt (MBCH) (16)

Compound **14** (500 mg, 1.12 mmol) was dissolved in ice-cold trifluoroacetic acid (5 ml) and stirred for 30 min in an ice-bath. The trifluoroacetic acid was removed under vacuum at RT to give compound **16** quantitatively as oily yellowish gum: IR (KBr) 3500-2500 (CO_2H), 3107 (Ar), 2944 (alkane) and 1717 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ 10.91 (bs, 1H, $N\underline{H}NH_3^+CF_3CO_2^-$), 9.4 (bs, 3H, NH_3^+), 8.57 (t, 1H, J=5.5 Hz, $CH_2N\underline{H}$), 7.95 and 7.45 (two d, 4H, J=8.5 Hz, para-substituted phenyl group), 7.22 (s, 2H, maleimido-H), 3.28 (q apparent, 2H, J=6.6 Hz, CH_2N), 2.25 (t, 2H, J=7.3 Hz, CH_2CO), 1.57 (m, 4H, 2 x CH_2), 1.34 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ 171.79, 169.75 (2), 165.51, 134.86 (2), 133.95, 133.78, 127.82 (2), 126.22 (2), 40.22 (superimposed by DMSO), 32.73, 28.84, 26.01, 24.49; MS, m/e: 69 (M^+-389). Anal. Calcd for $C_{19}H_{21}N_4O_6F_3$: C, 49.77; H, 4.62; N, 12.23. Found: C, 49.80; H, 4.57; N, 12.27.

Synthesis of 13-[(4-maleimidobenzoyl)hydrazono]doxorubicin (MBH-Dox) (17)

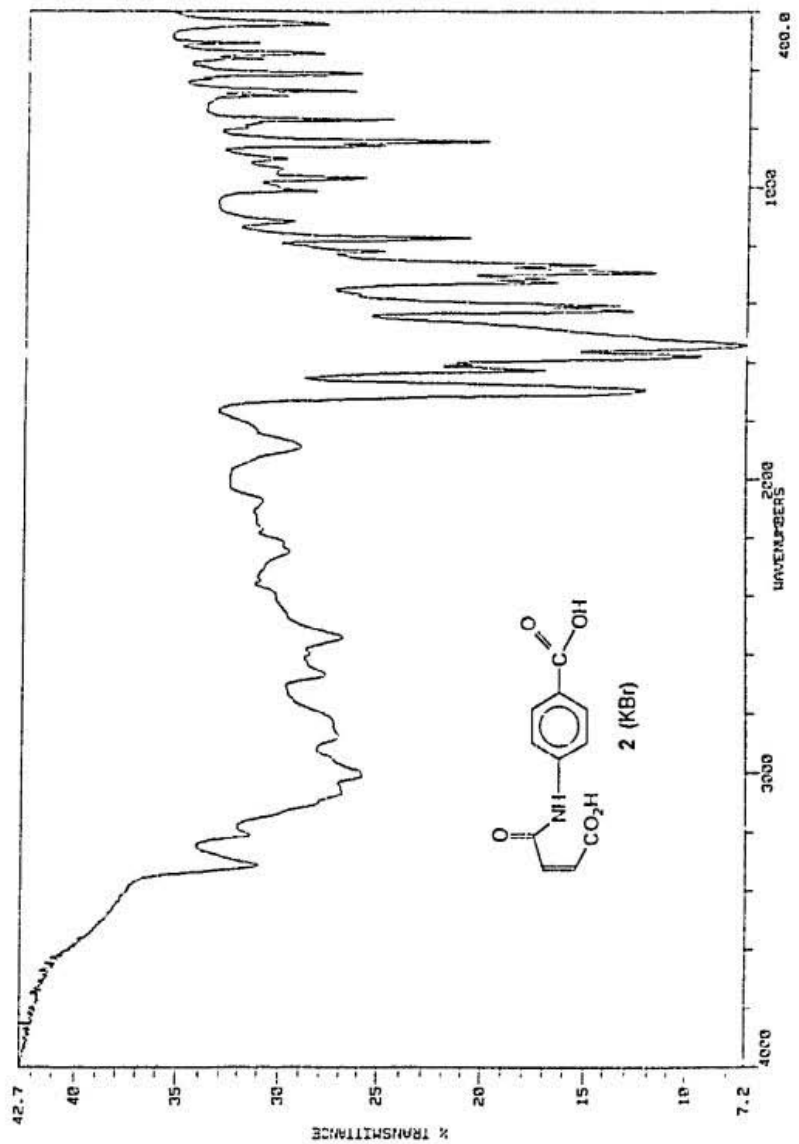
Dox·HCl (20.58 mg, 0.036 mmol) and 4-maleimidobenzohydrazide trifluoroacetate salt (**15**) (41.28 mg, 0.120 mmol) were dissolved in 5 ml methanol with the addition of 3 μl TFA. The mixture, protected from light, was stirred at RT for 1 day. The methanolic solution was concentrated to 1 ml under nitrogen at RT. Acetonitrile (4 ml) was added for precipitation and the suspension was allowed to stand at 4°C for 1 day. The red precipitate was isolated by centrifugation and then dried under vacuum to give crude product, 20.2 mg (72%). The remaining solution was concentrated down to 0.5 ml and then acetonitrile (4 ml) was added for precipitation. The crude product was isolated by the same procedure as above and gave 6.5 mg (23%). The crude product (6.5 mg) was further purified by TLC, using CHC_3/MeOH (85:15) and then scraped out. The silica gel containing the desired product was washed with $CHCl_3$/MeOH (30 ml, 85:15) and filtered. After evaporating the solvent from the filtrate, the residue was dissolved in 2 ml MeOH and then transfered to a test tube. The methanolic solution was concentrated down to 0.5 ml and acetonitrile (3 ml) was added. Precipitation was induced by drying the solution to 1 ml under nitrogen. The suspension was allowed to stand at 4°C for 1 days. The red precipitate was isolated by centrifugation and dried under vacuum to give

4.8 mg (75%) of **17**: ^{1}H NMR (DMSO-d_6) **δ** 14.07 (s, 1H, OH-11), 13.35 (bs, 1H, OH-6), 11.86 (s, 1H, CONHN), 7.93 - 7.64 (m, 9H, NH, NH_3^+, H-1, H-2, H-3 phenylene: H-2, H-6), 7.50 (d, 2H, J=8.4 Hz, phenylene: H-3, H-5), 7.22 (s, 2H, maleimido-H), 5.49 (d, 1H, J= 5.6 Hz, H-1'), 5.31 (bs, 1H, H-7), 5.12 (bs, 1H, HO-14), 4.63 (s, 2H, CH_2-14), 4.56 (d, 1H, J=5.74 Hz, H-5'), 3.98 (s, 3H, OCH_3), 3.55 (bs, 1H, H-4'), 1.18 (d, 3H, $J_{5',6'}$=5.85 Hz, CH_3-6').

Synthesis of 13-[N-(4-maleimidobenzoyl)-6-aminocaproyl hydrazono]doxorubicin (MBCH-Dox) (18)

Dox·HCl (20.34 mg, 0.035 mmol) and N-(4-maleimidobenzoyl)-6-aminocaprohydrazide trifluoroacetate salt (**16**) (53.65 mg, 0.117 mmol, assuming 100% yield obtained from 52 mg of **16**) were dissolved in 5 ml methanol with the addition of 3 μl TFA. The mixture, protected from light, was stirred at RT for 24 h. The methanolic solution was concentrated to 0.5 ml under vacuum at RT. Acetonitrile (4 ml) was added for precipitation and the suspension was allowed to stand at 4°C for 24 h. The red precipitate was isolated by centrifugation and dried under vacuum to give 28.2 mg (90%) of **18**; ^{1}H NMR (DMSO-d_6) **δ** 14.10 (s, 1H, OH-11), 13.32 (s, 1H, OH-6), 10.34 (s, 1H, CONHN), 8.46 (t, 1H, J=5.3 Hz, NH), 7.90 (m, 7H, NH_3^+, H-1, H-2, phenylene: H-2, H-6), 7.60 (dd, 1H, J=4.3 and 2.6 Hz, H-3), 7.42 (d, 2H, J=8.4 Hz, phenylene: H-3, H-5), 7.21

(s, 2H, maleimido-H), 5.49 (bs, 1H, H-1'), 5.31 (bs, 1H, H-7), 4.95 (t, 1H, J=6.4 Hz, HO-14), 4.42 (s, 2H, CH_2-14), 4.04 (q, 1H, J=6.7 Hz, H-5'), 3.95 (s, 3H, OCH_3), 1.18 (d, 3H, $J_{5',6'}$=6.4 Hz, CH_3-6').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C
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8
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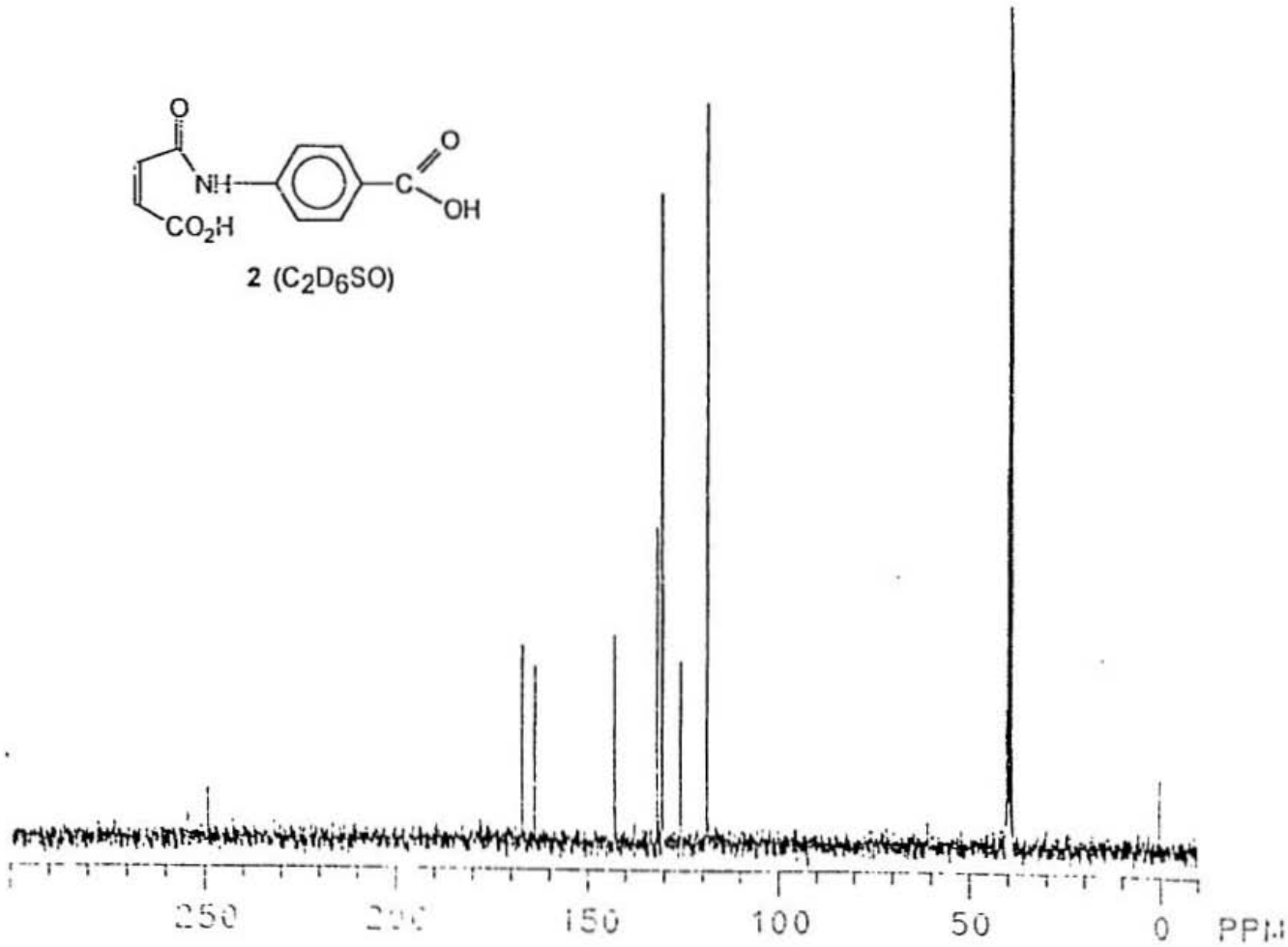
PPM



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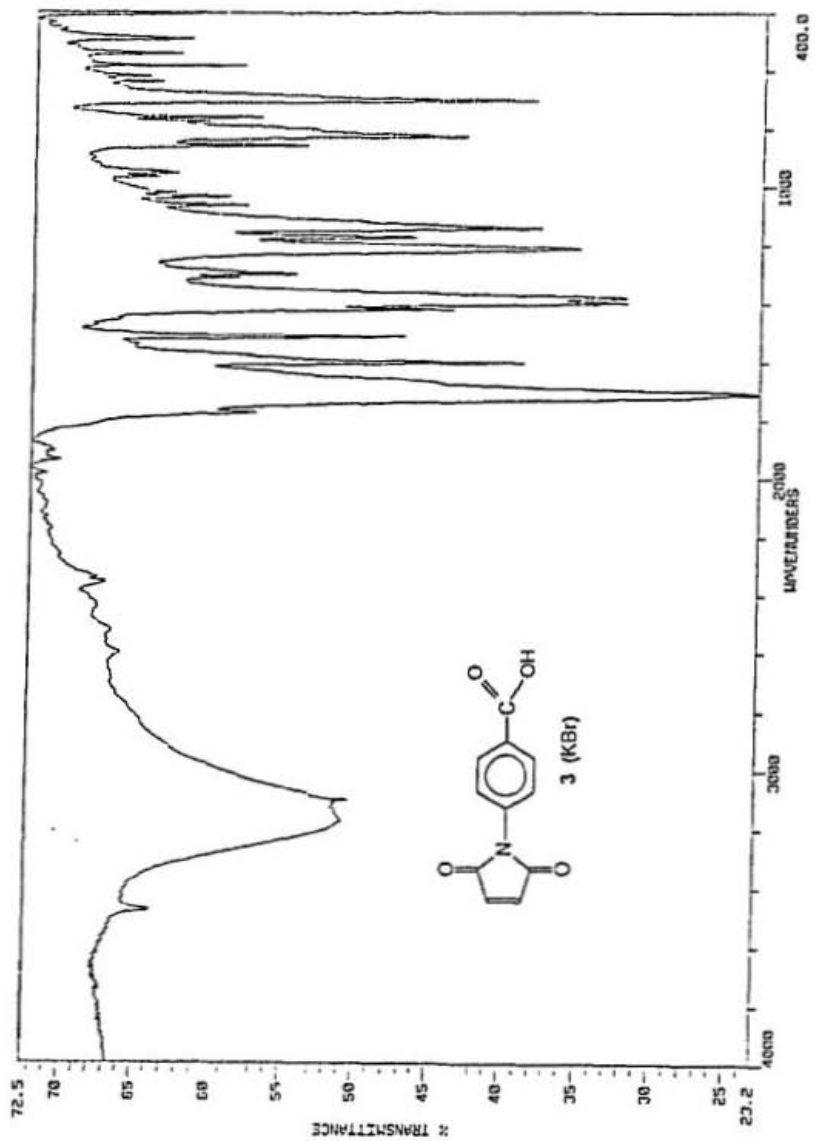
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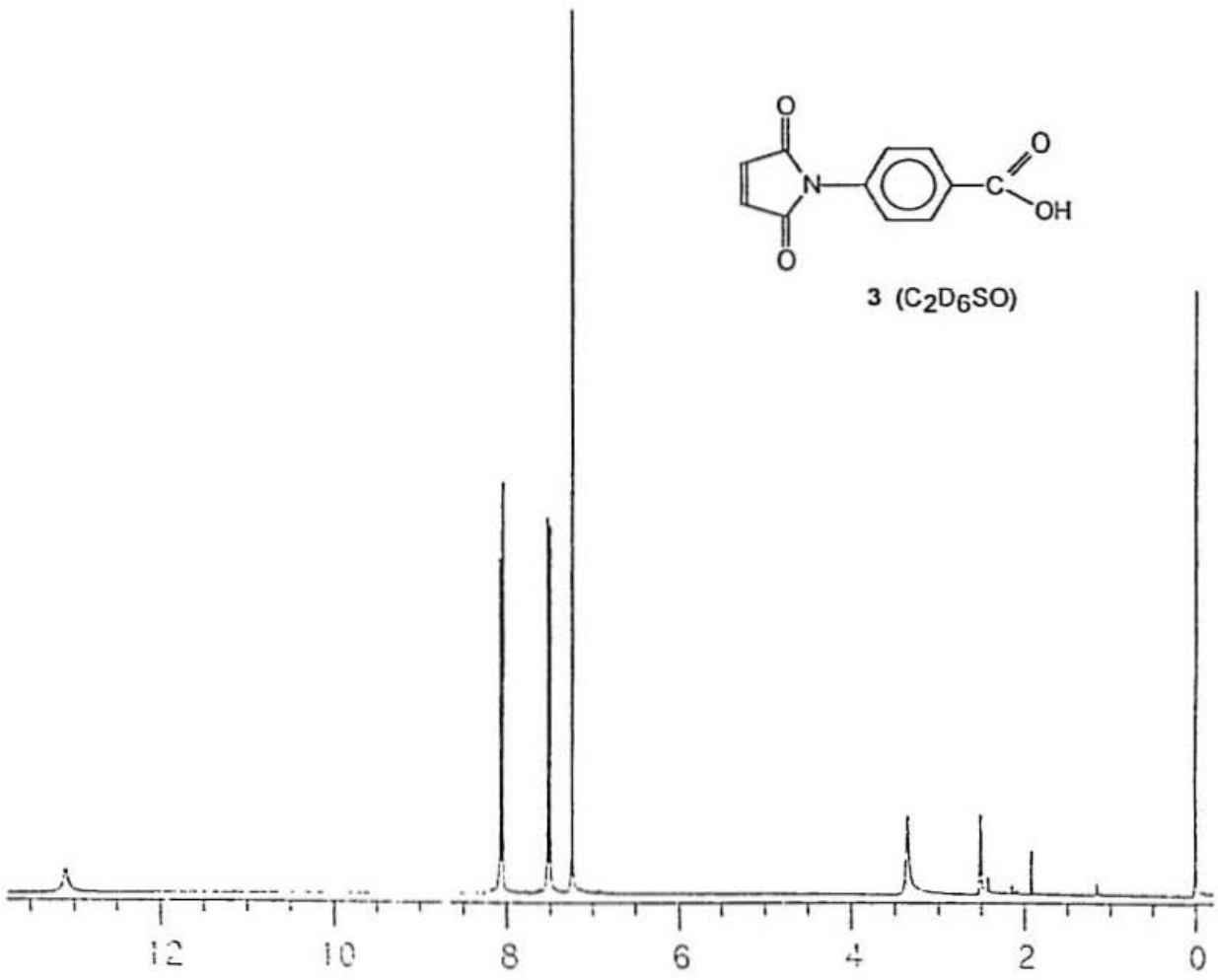

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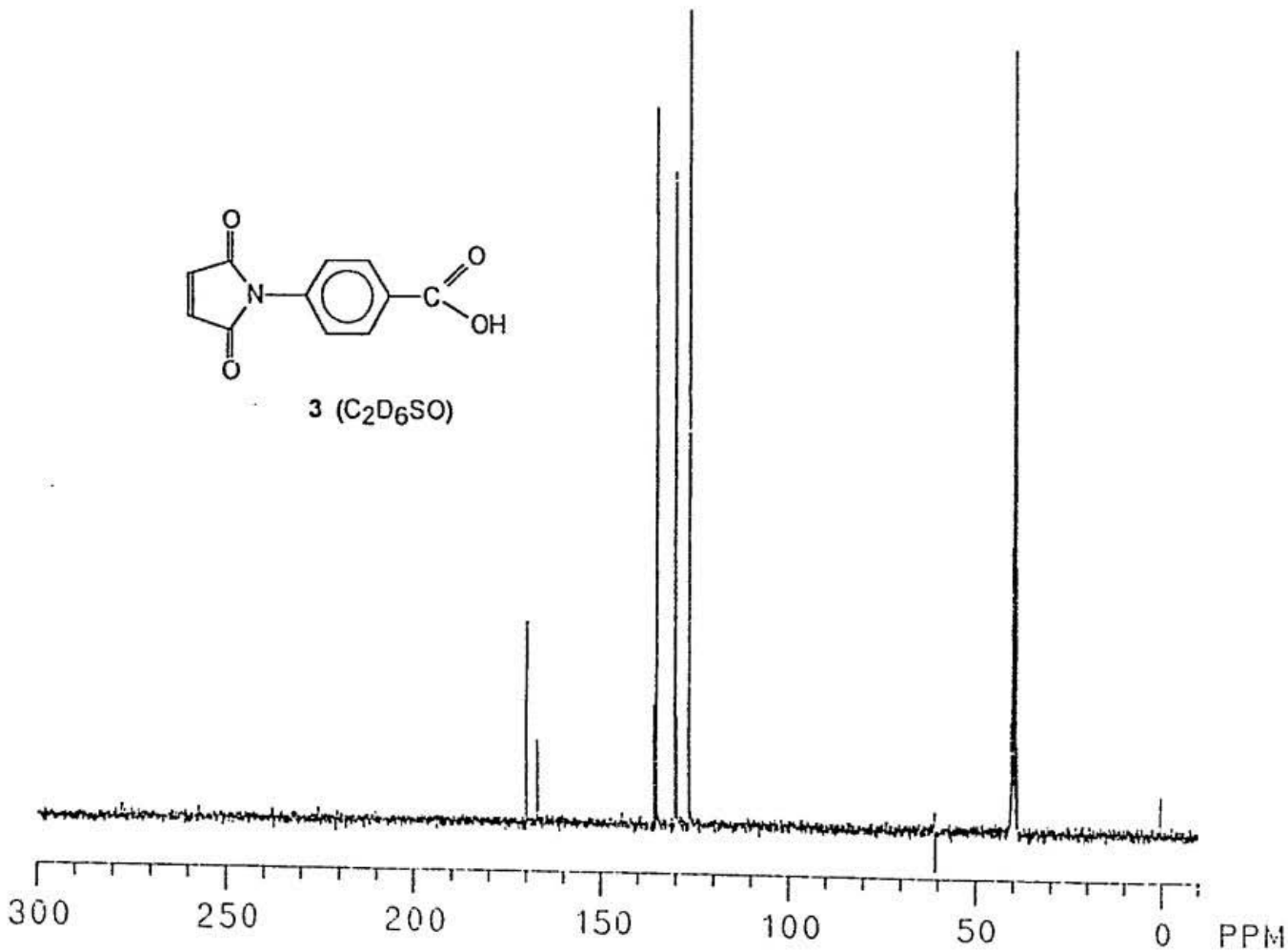

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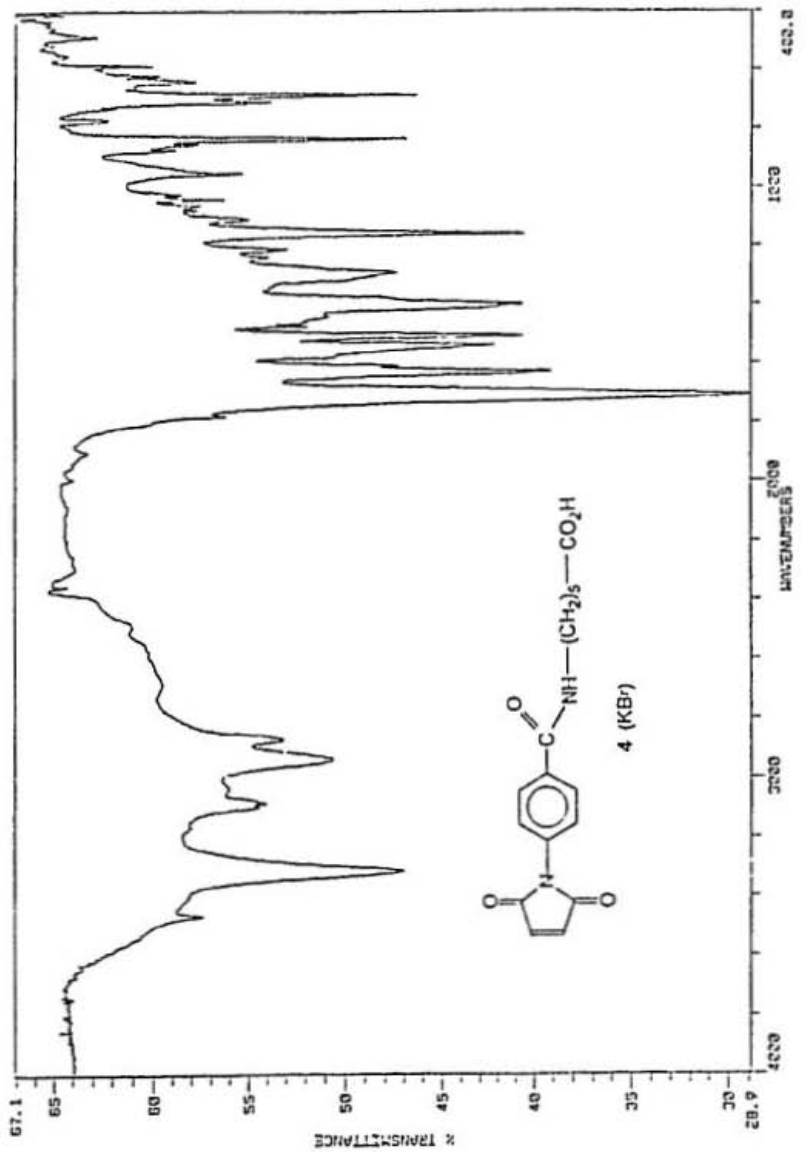
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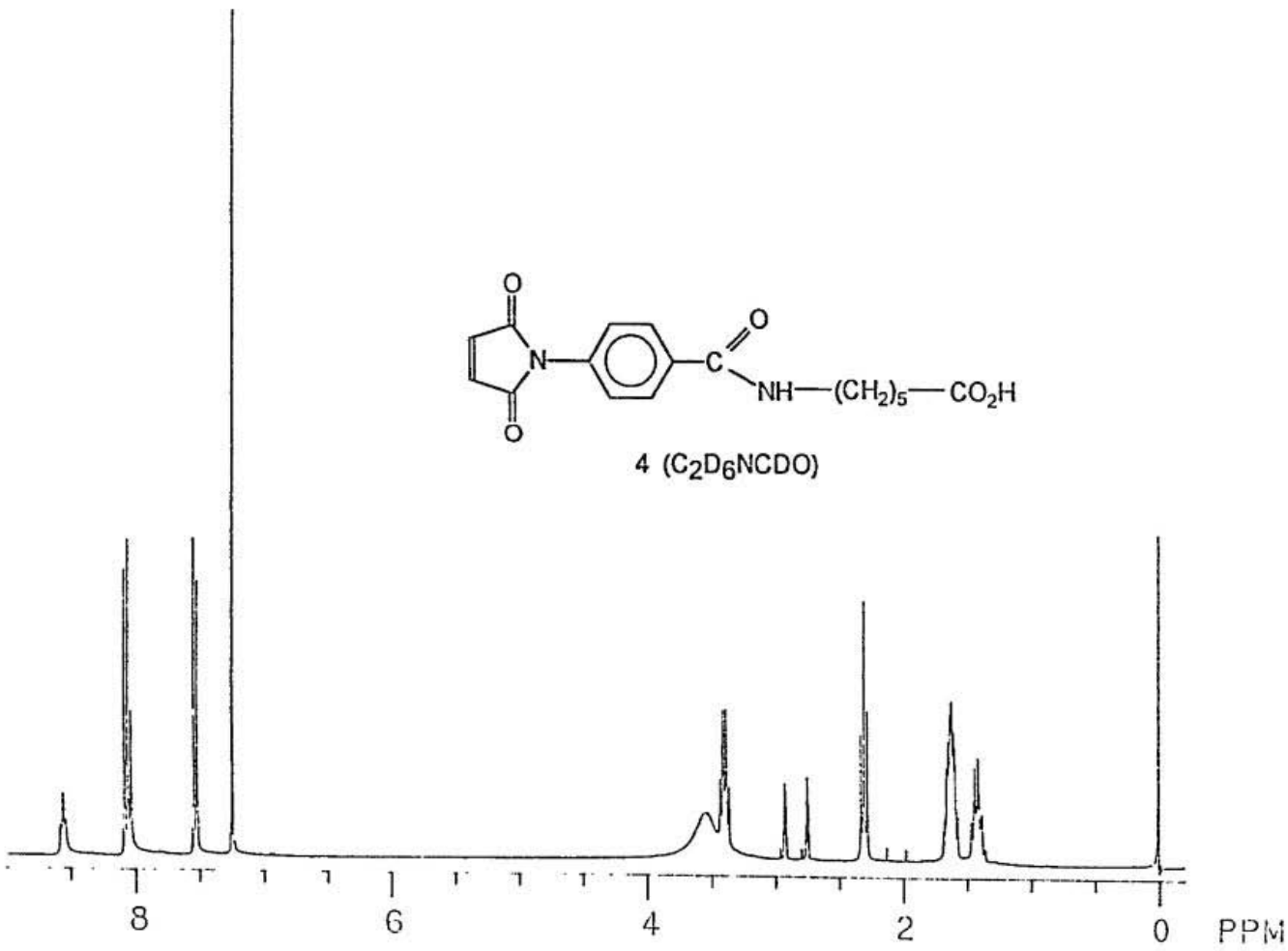
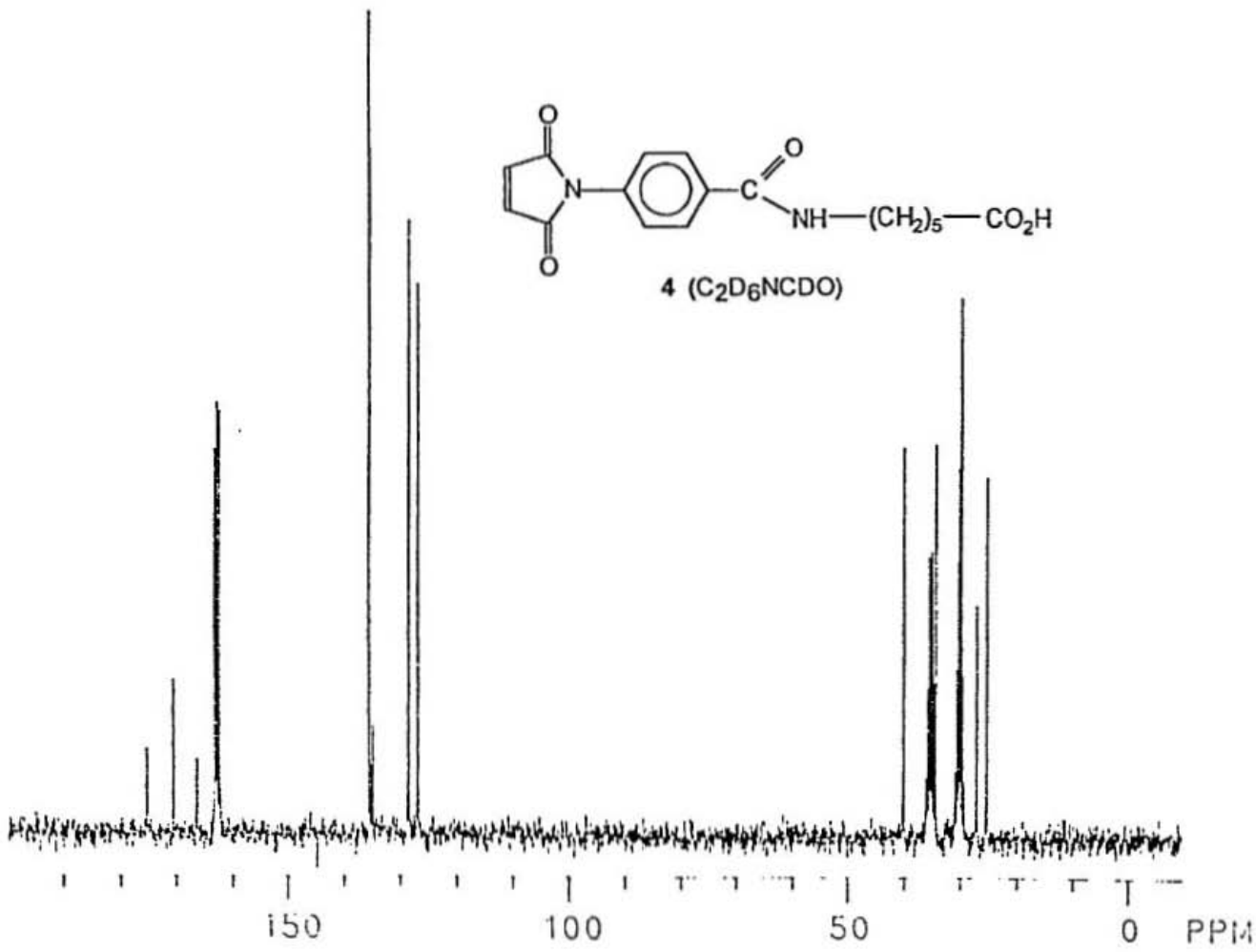


4 (C_2D_6NCDO)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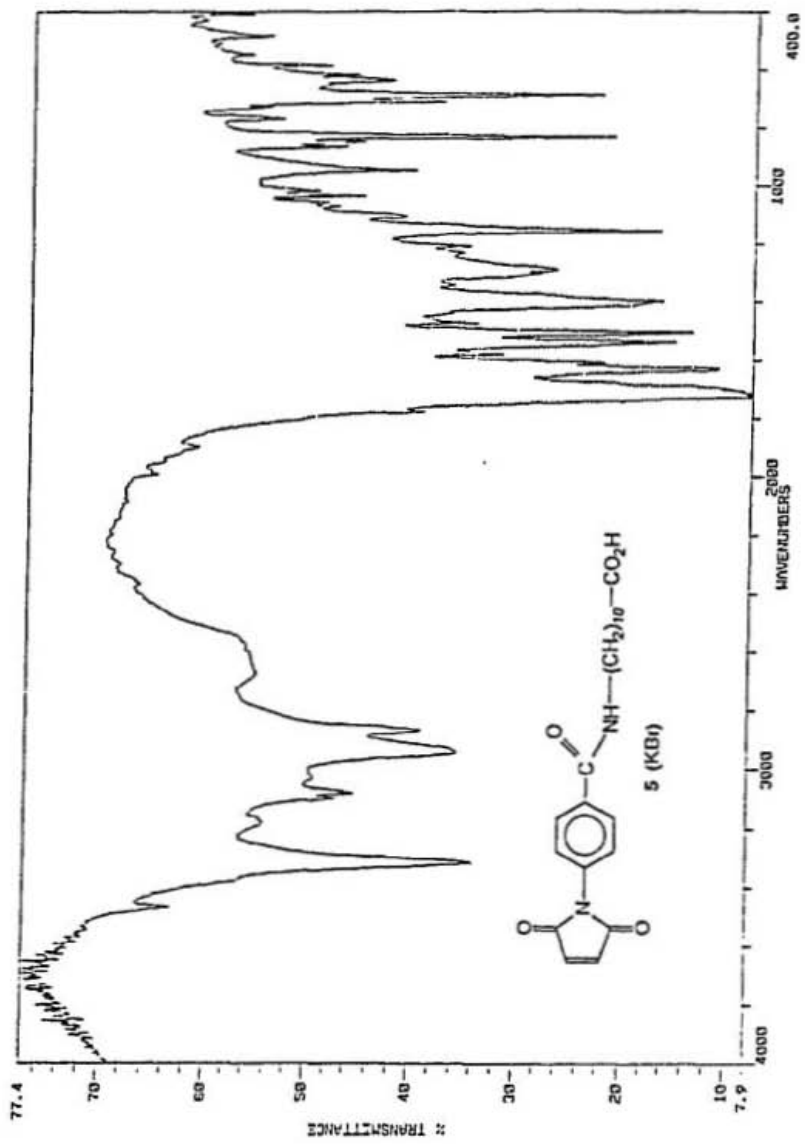
50
0 PPM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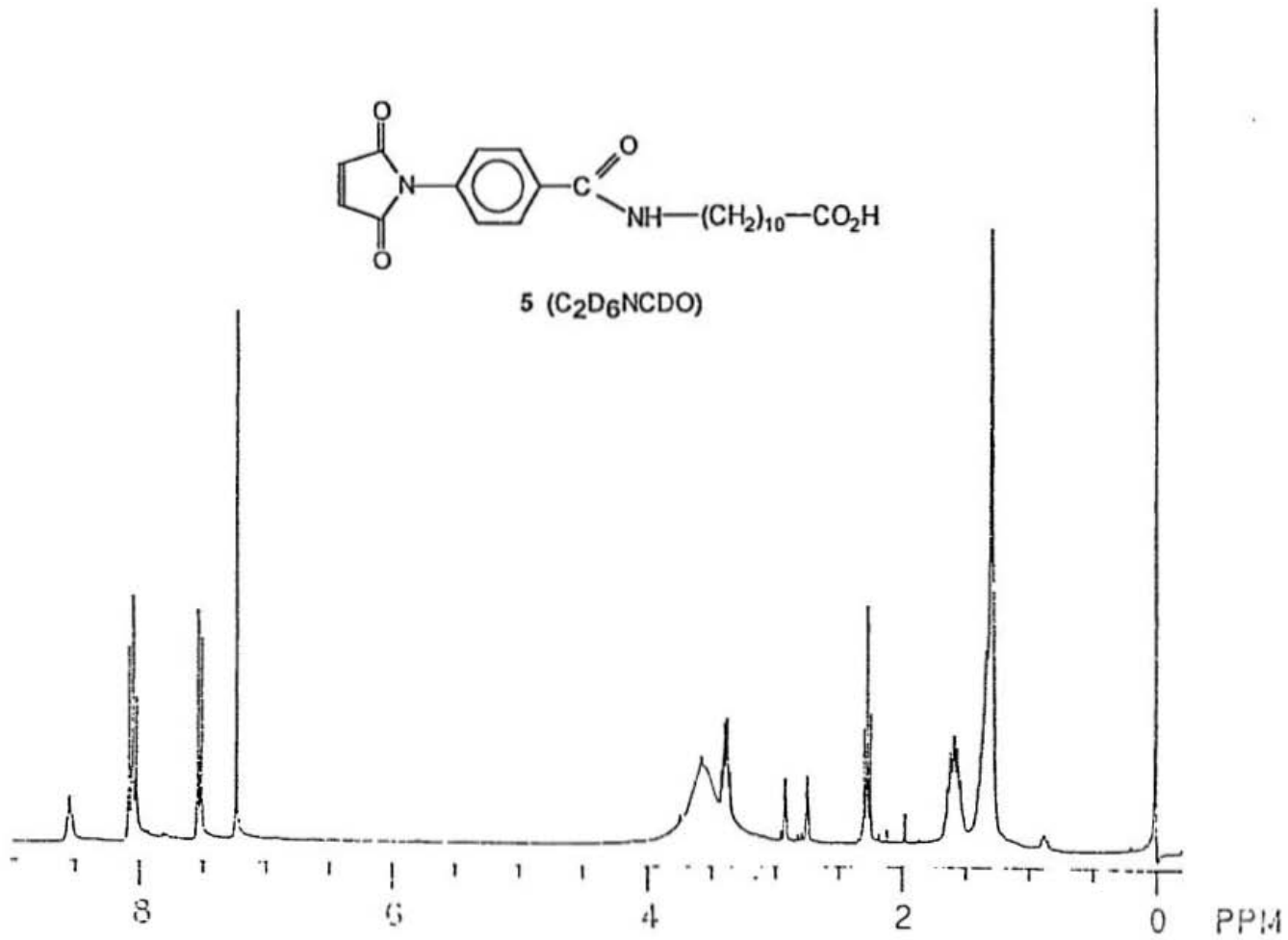
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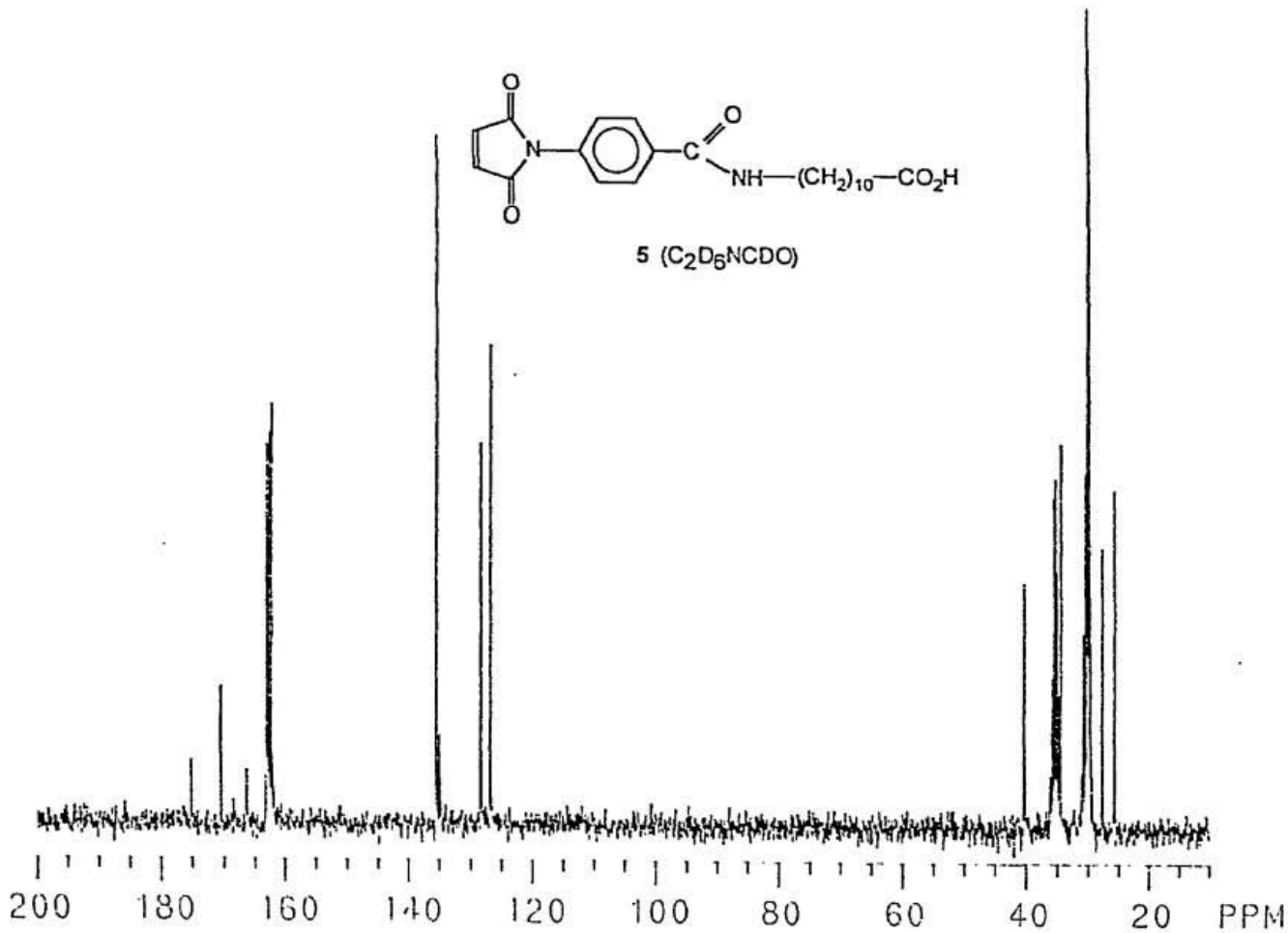
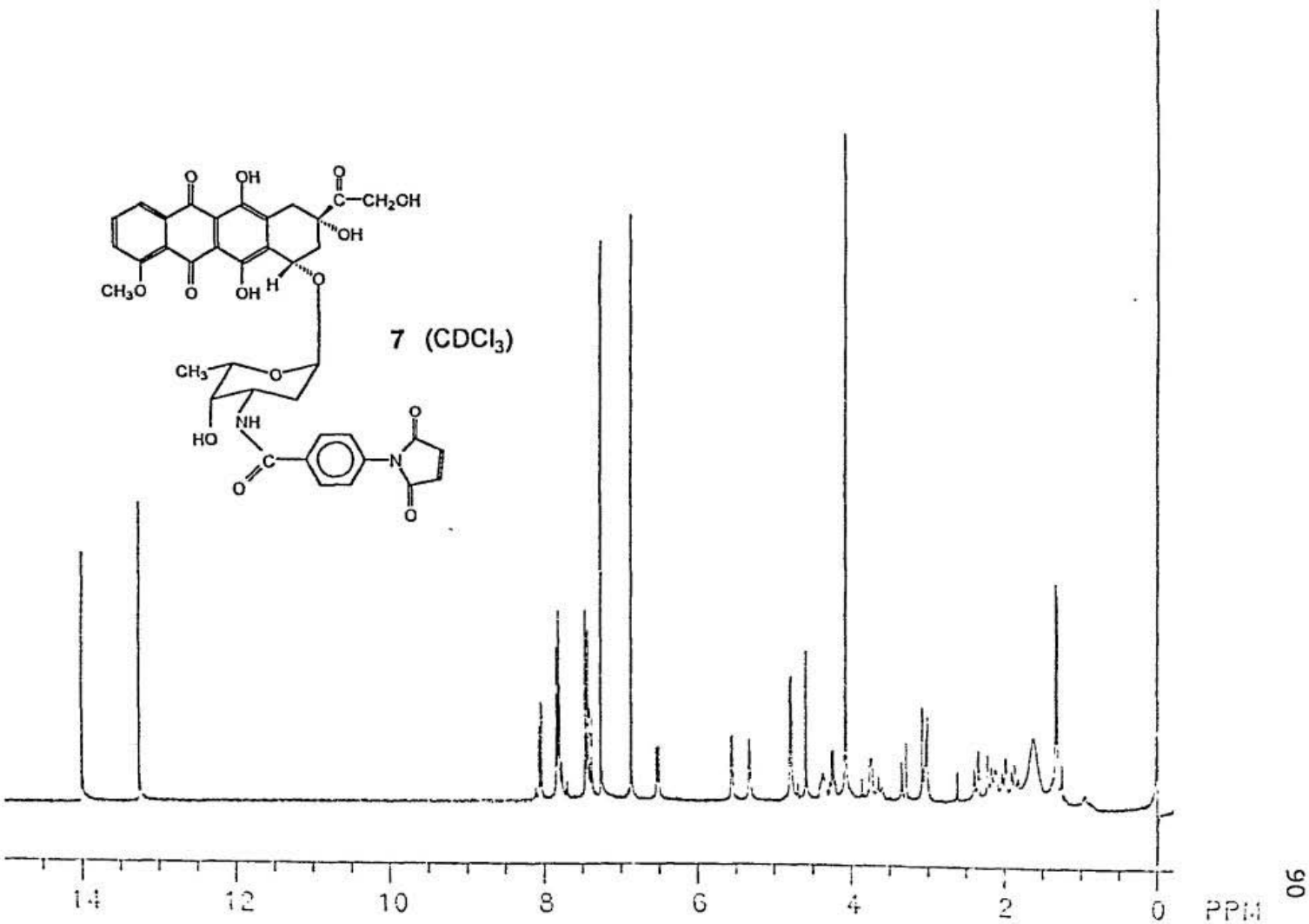


5 (C_2D_6NCDO)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4
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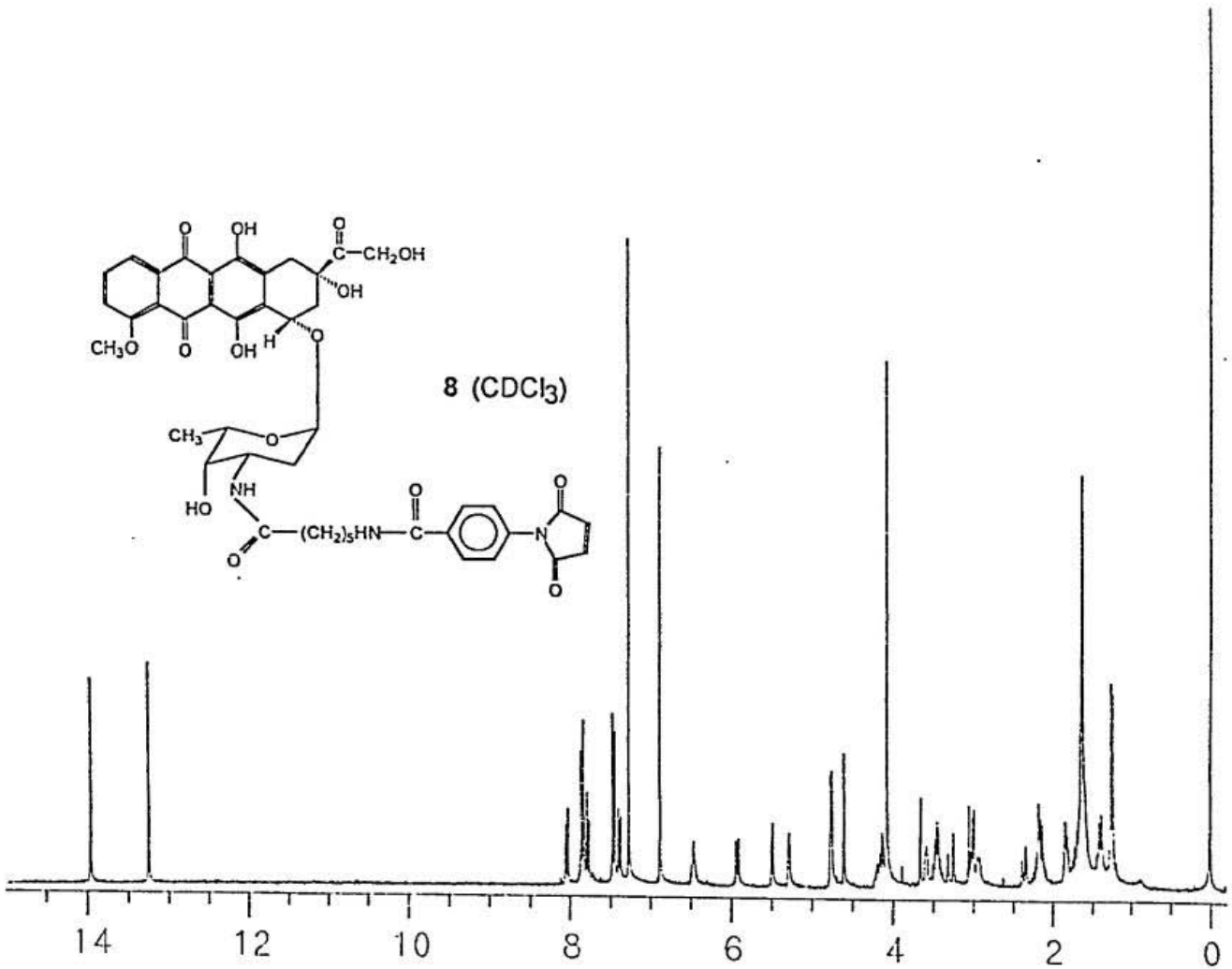
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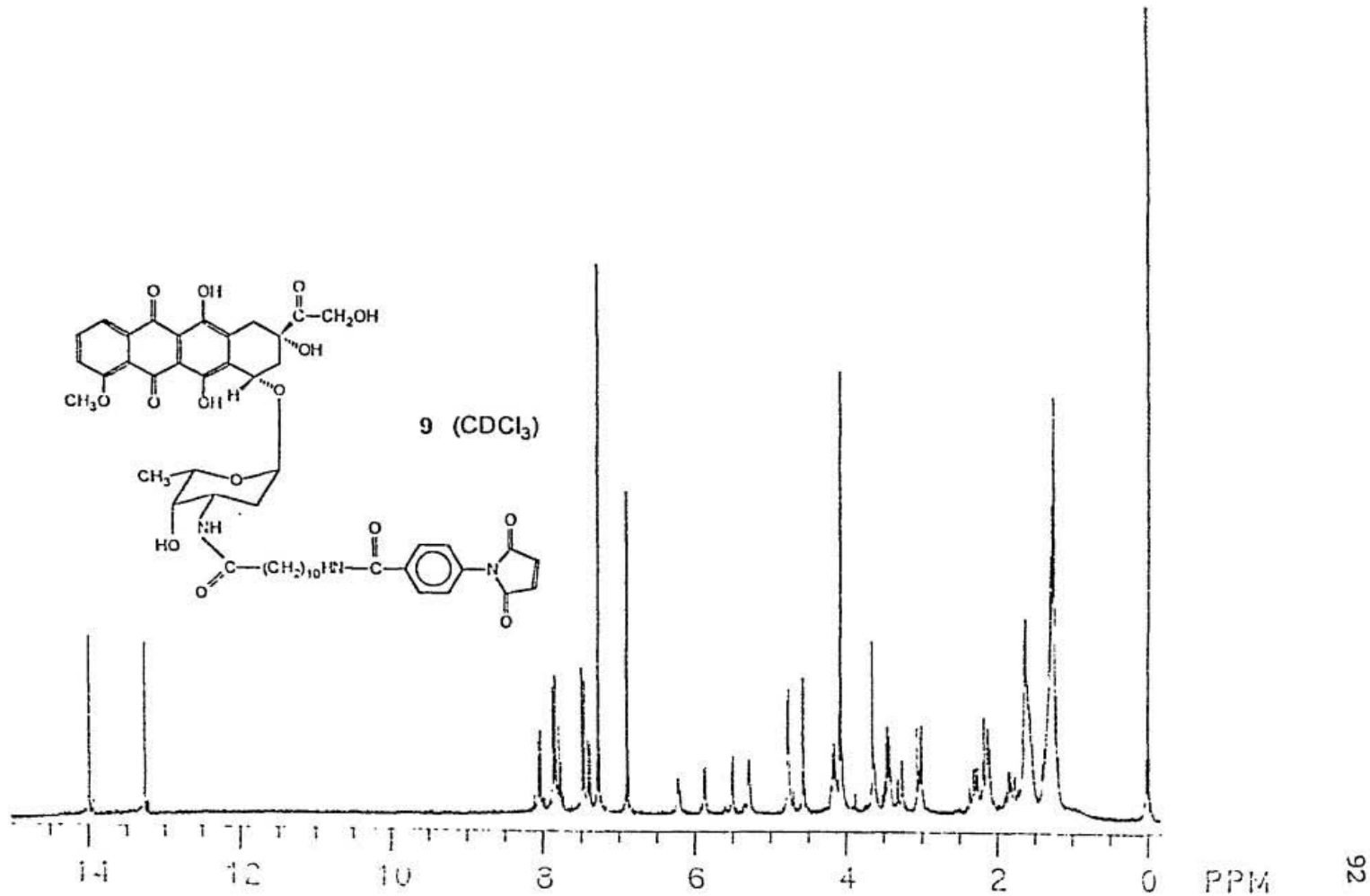
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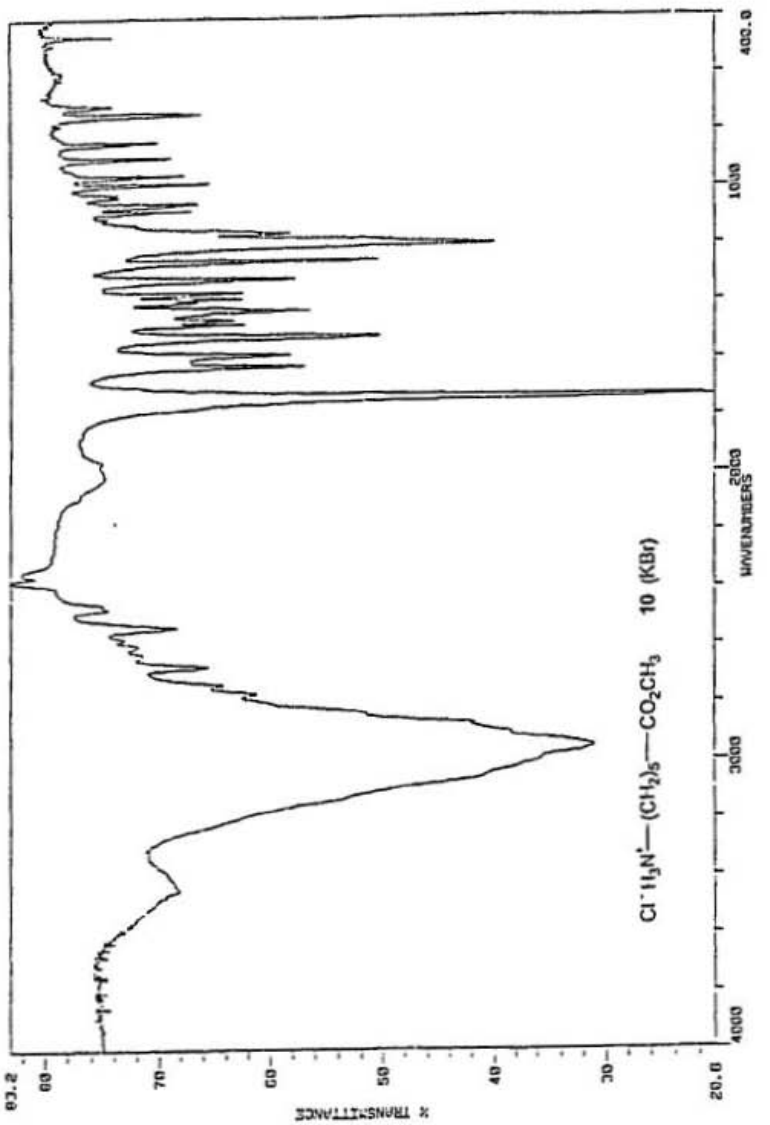
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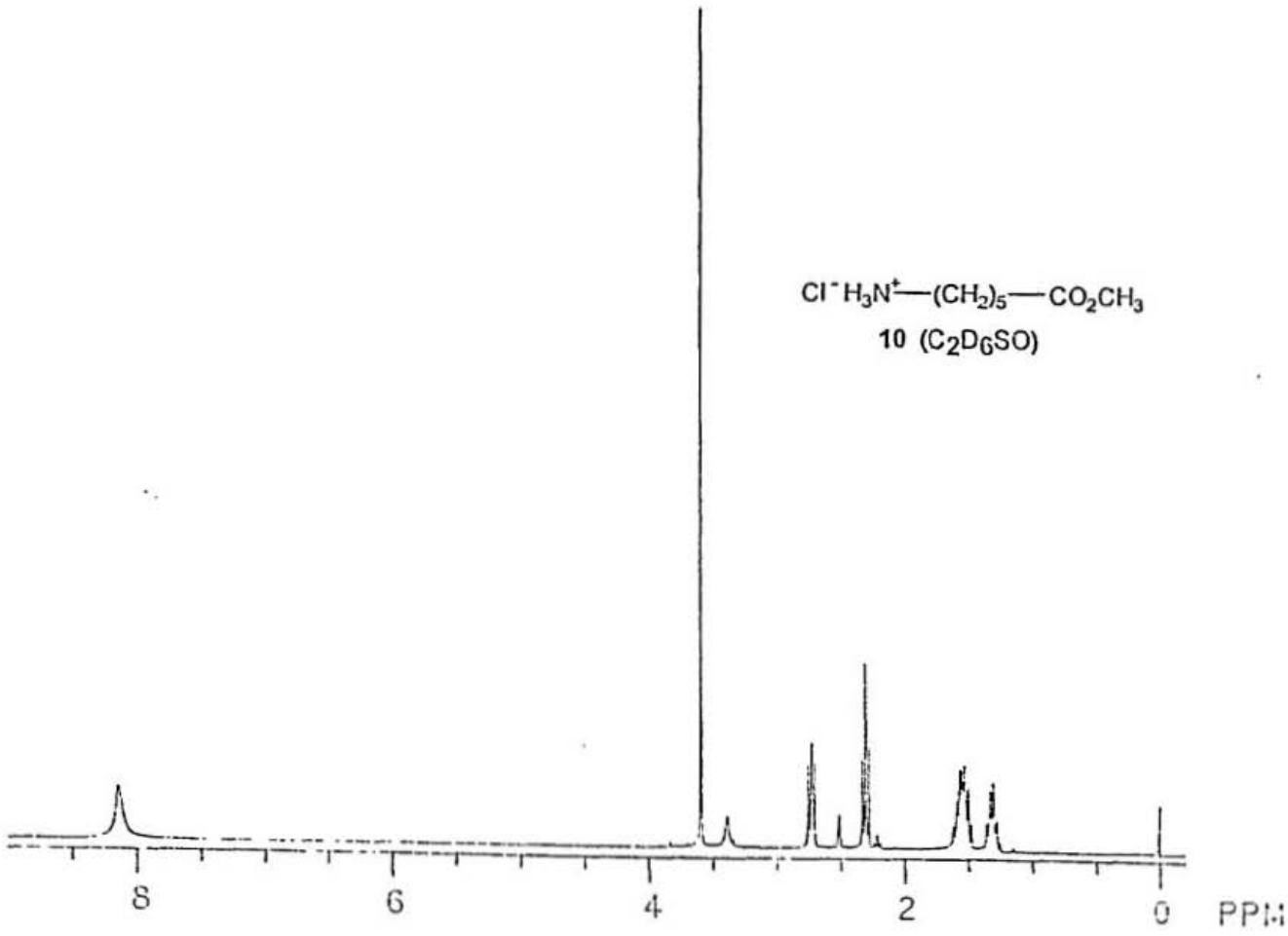
Cl⁻H₃N⁺—(CH₂)₅—CO₂CH₃ 10 (KBr)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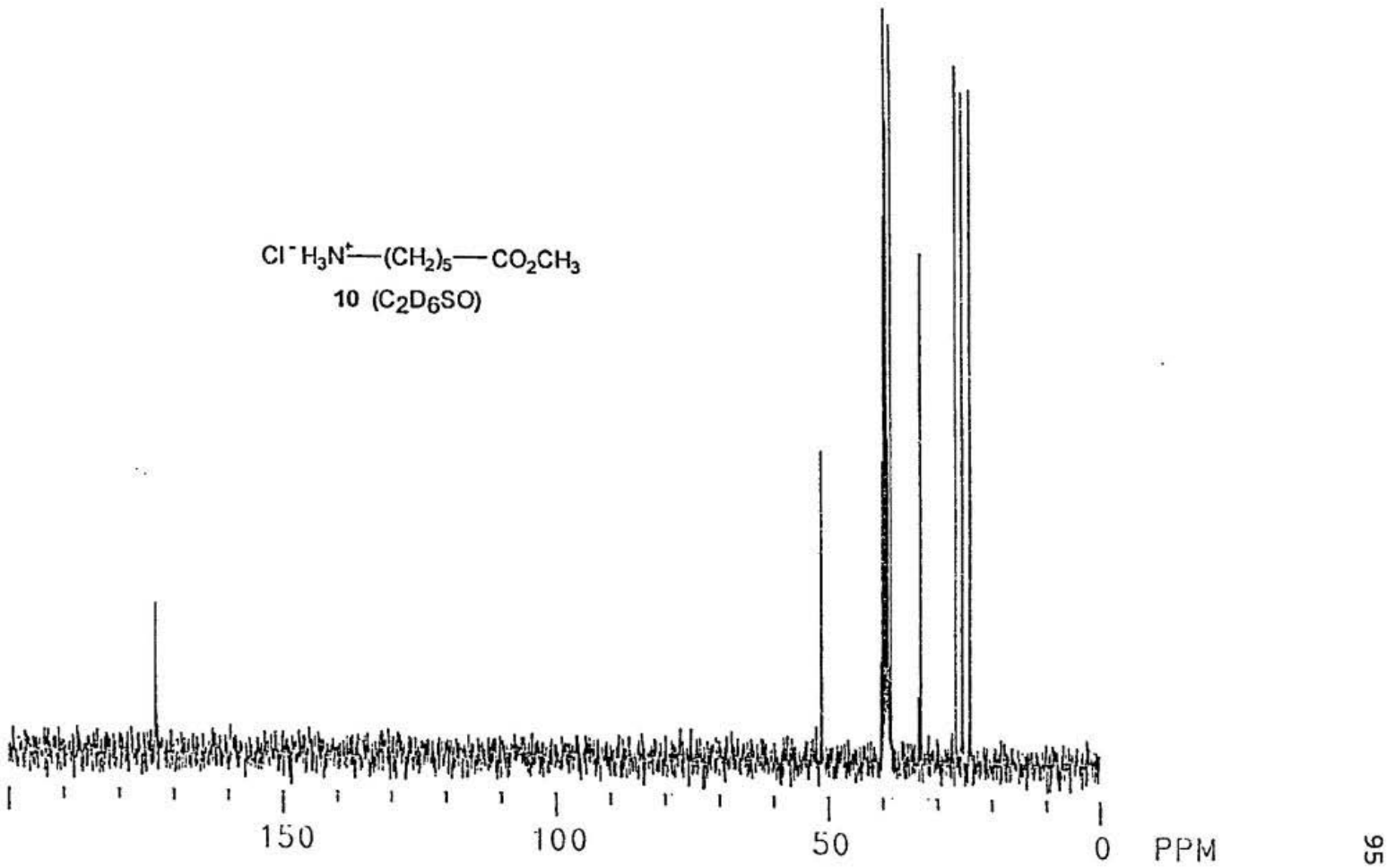
8
6
4
2
0
PPM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100
50
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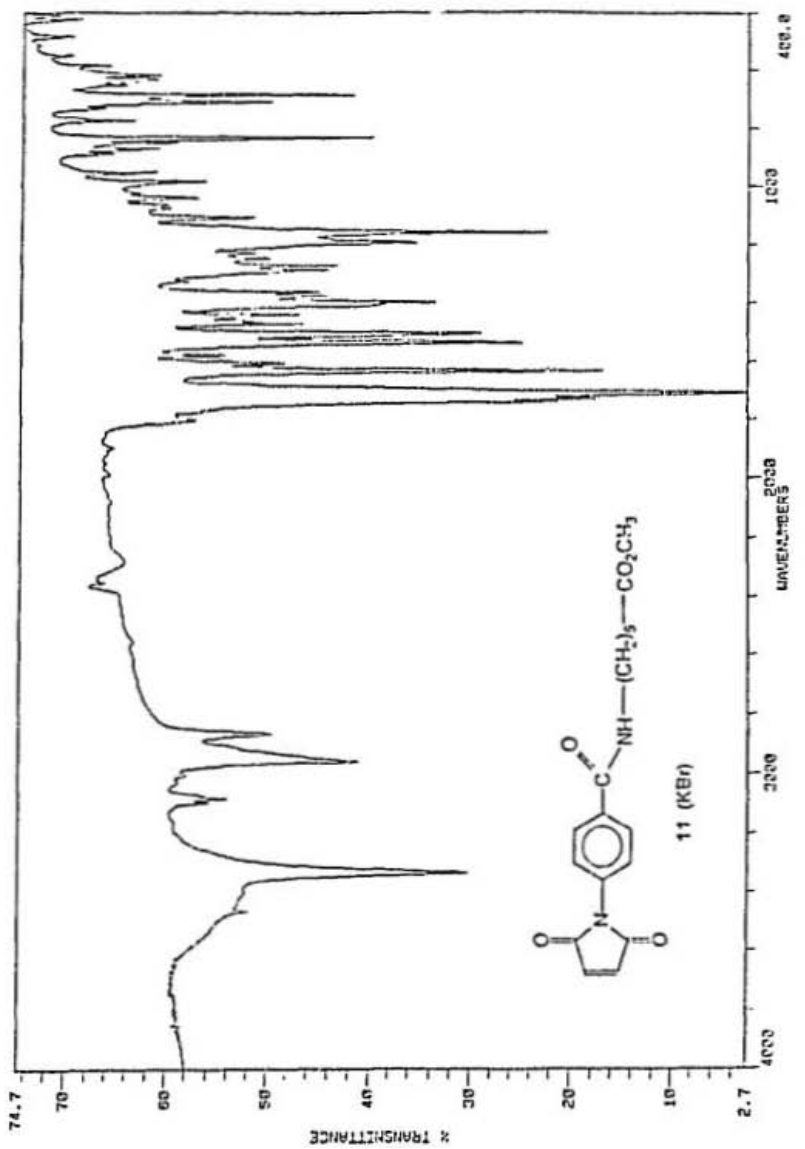
PPM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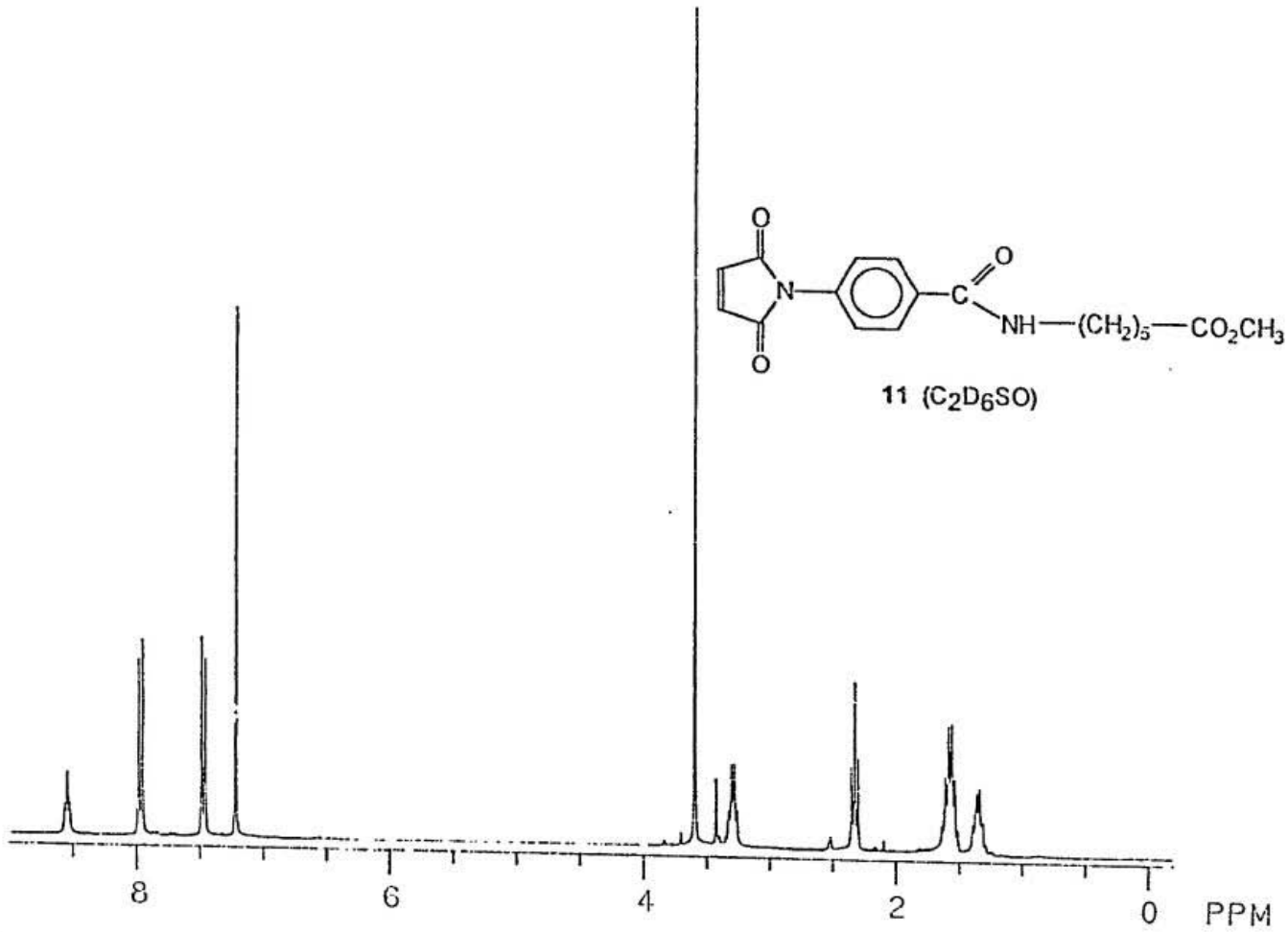
NH—(CH2)5—CO2CH3
N
O
O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4
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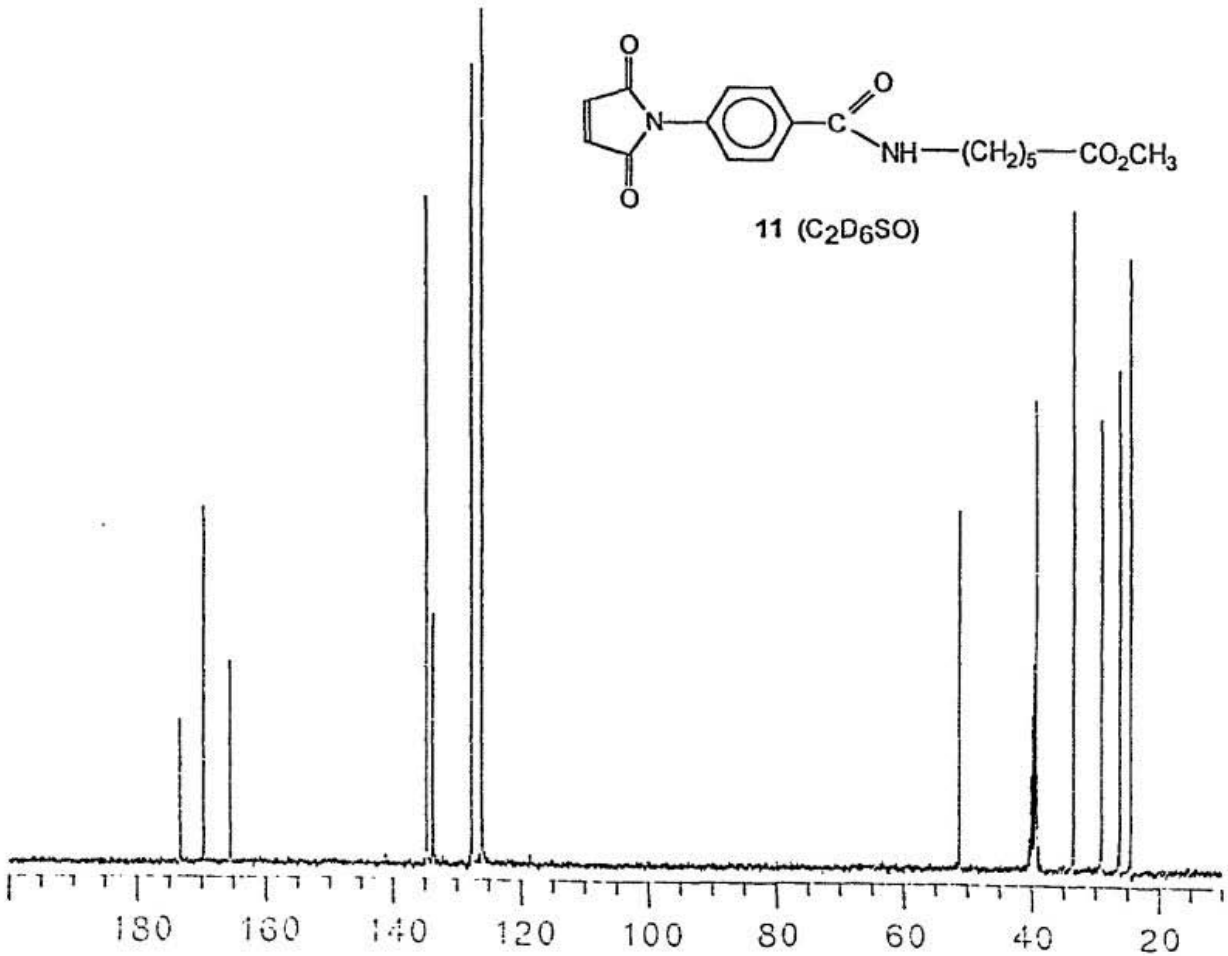
PPM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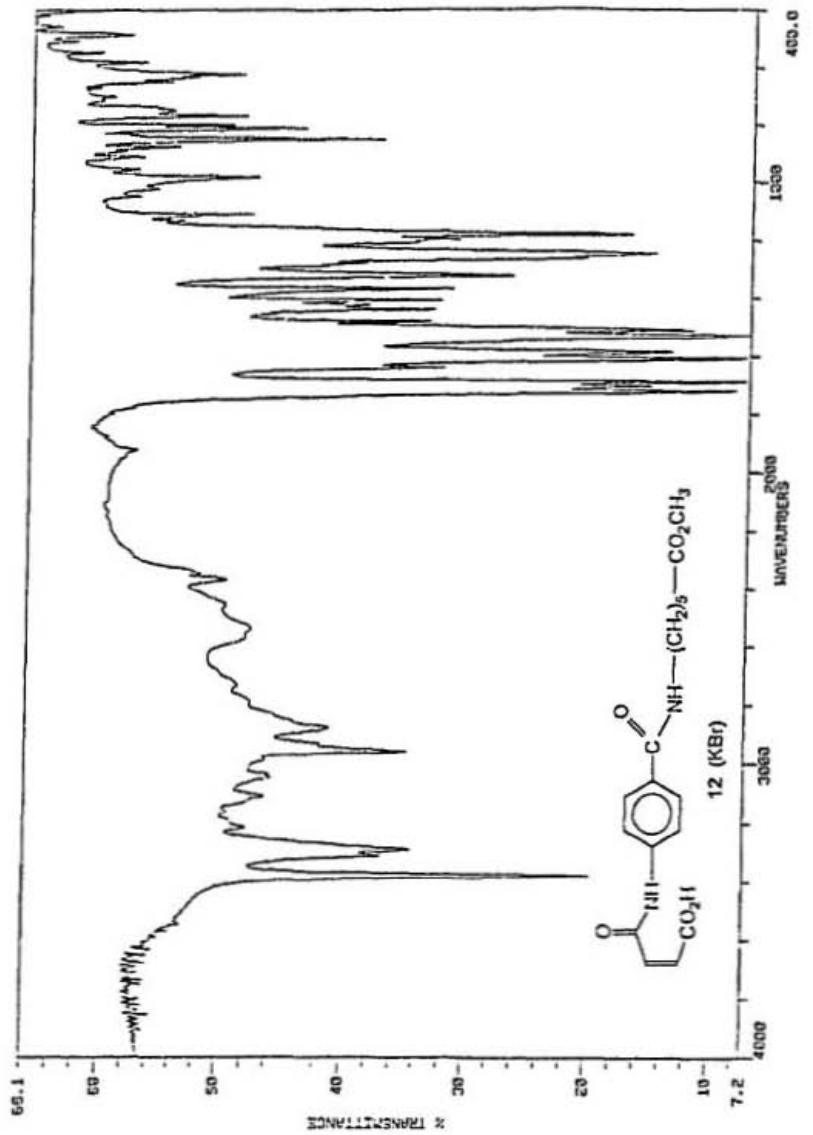
O
N
C
O
NH—(CH2)5—CO2CH3
O
11 (C2D6SO)
180
160
140
120
100
80
60
40
20
PPM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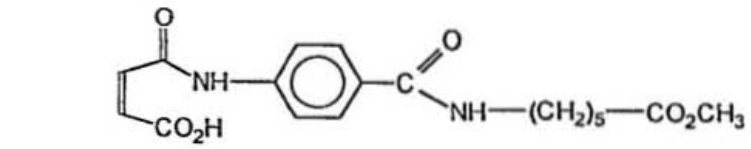
3000
2000
1500
400.0
66.1
7.2
12 (KBr)



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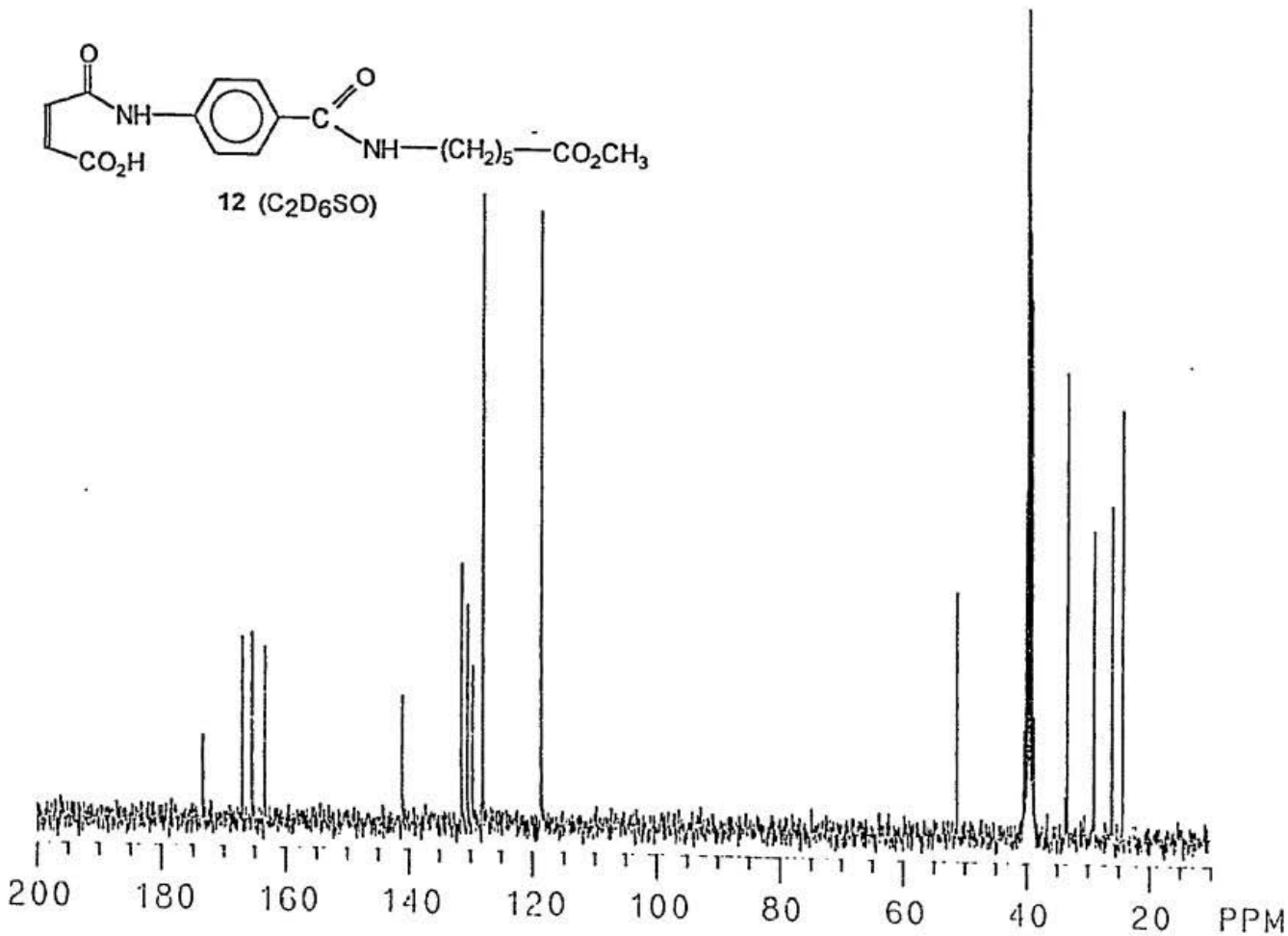
O
NH
CO2H
C
O
NH—(CH2)5—CO2CH3
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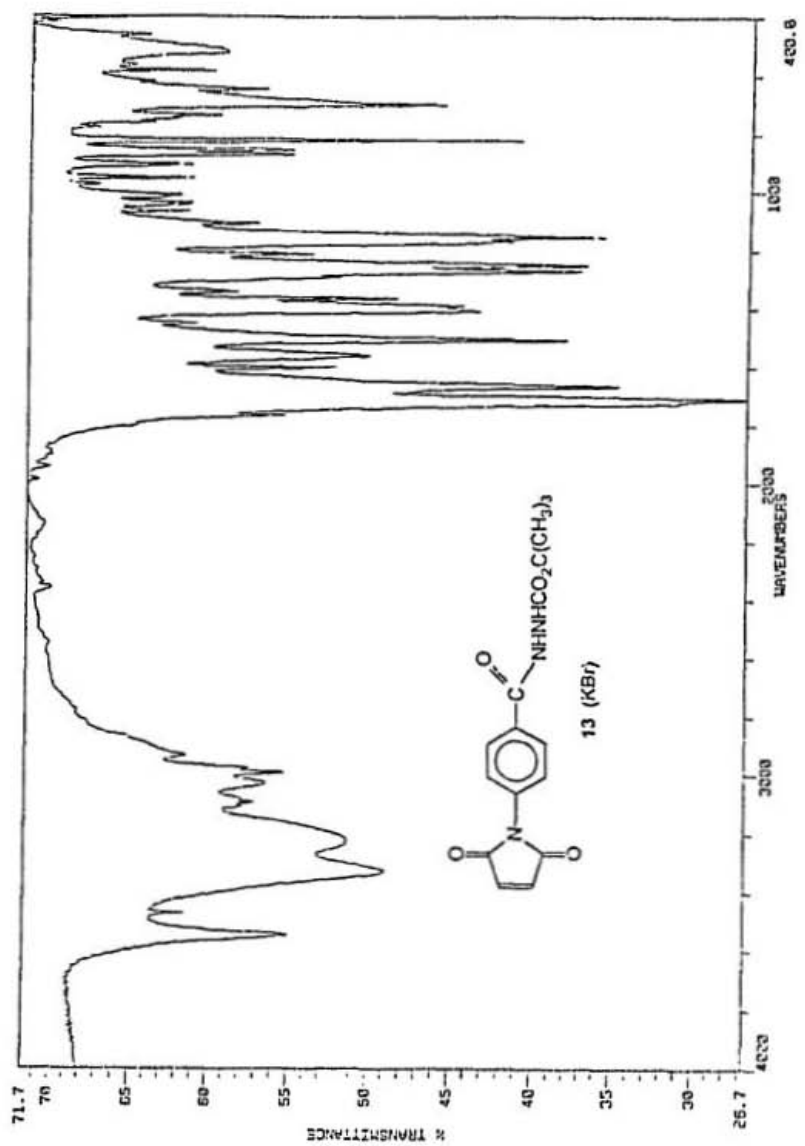
180
160
140
120
100
80
60
40
20
PPM

12 (C_2D_6SO)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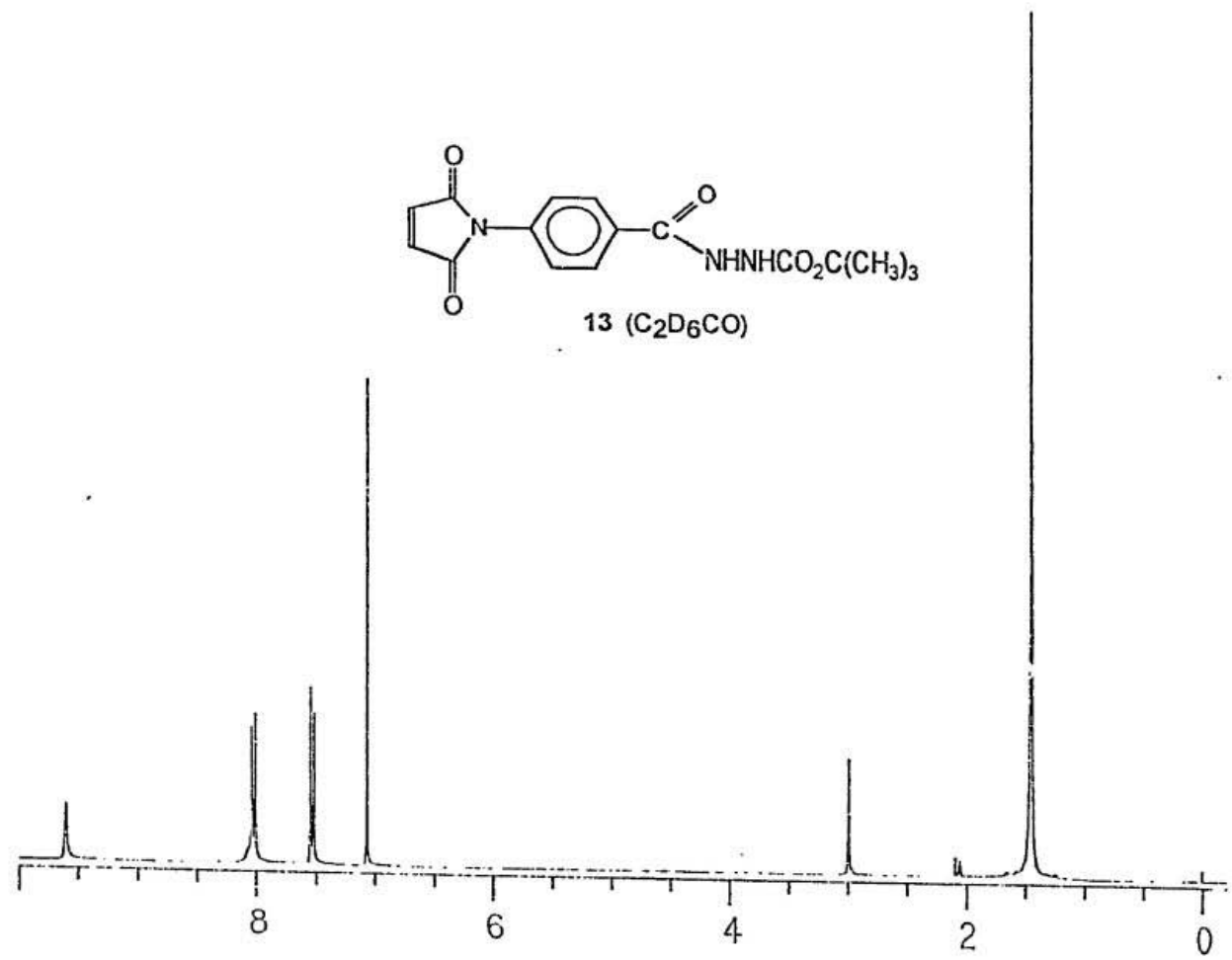

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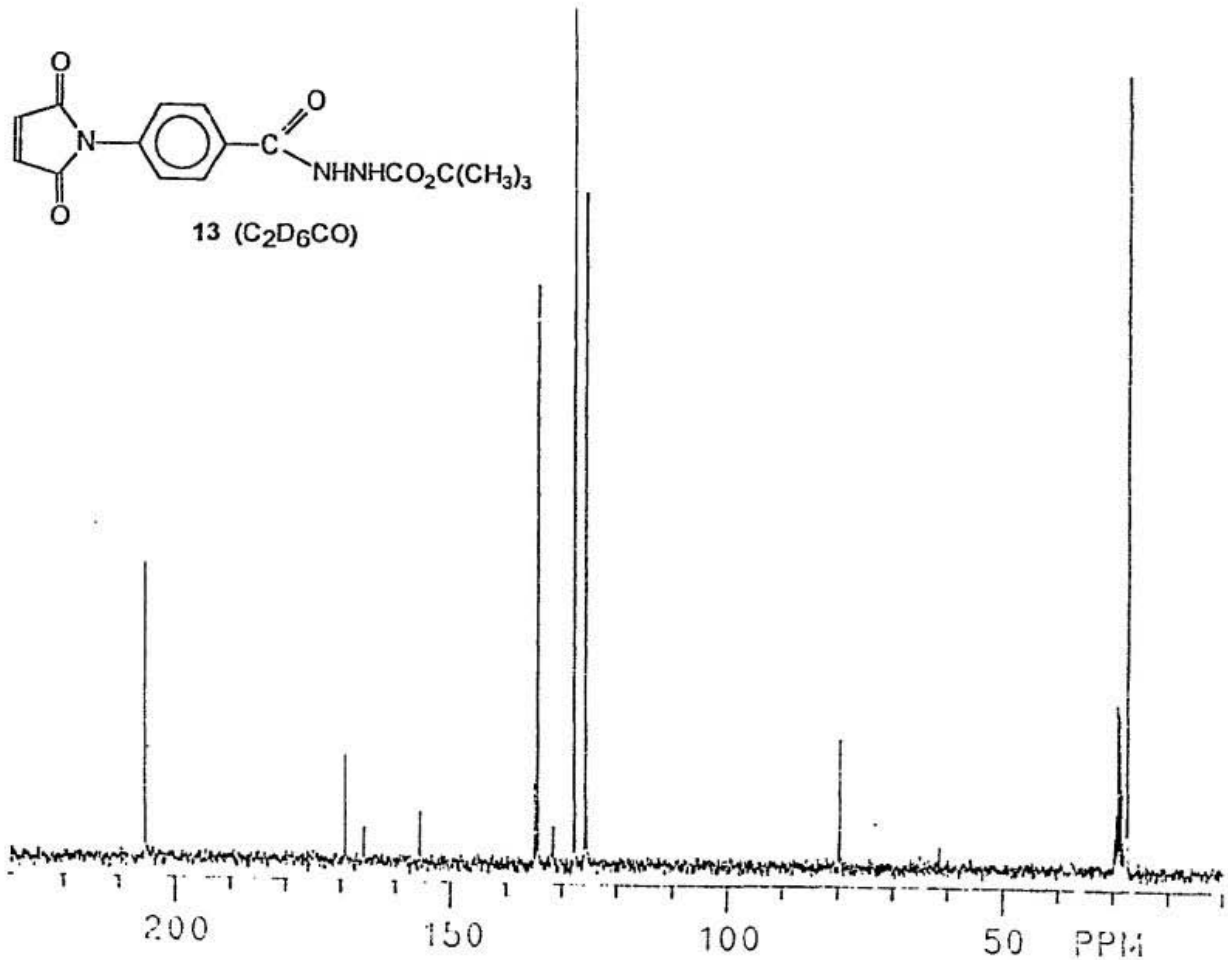

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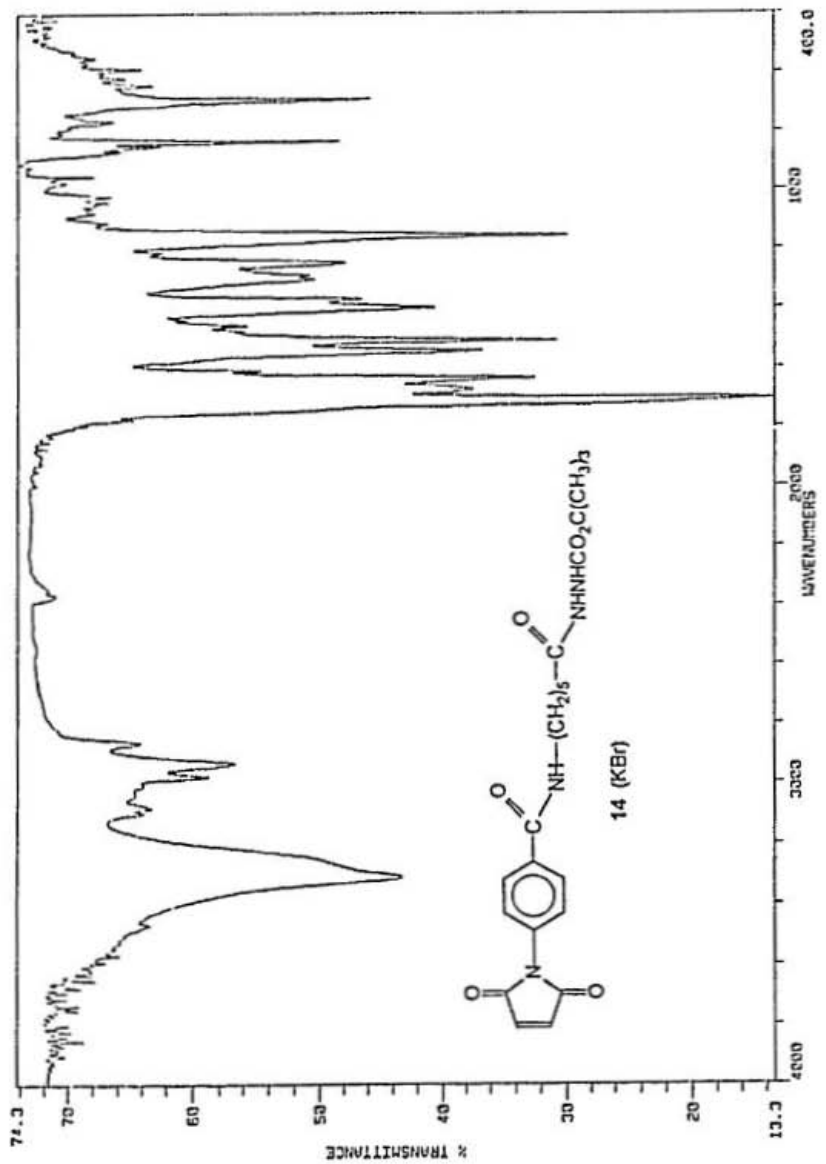
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70
60
50
40
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20
13.3
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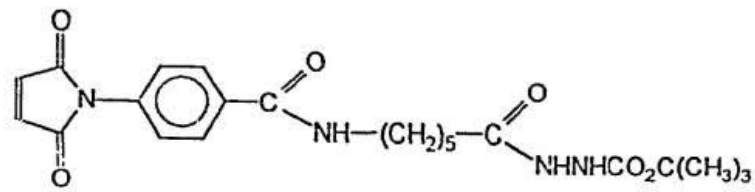
NHNHCO2C(CH3)3
O
N
O
O



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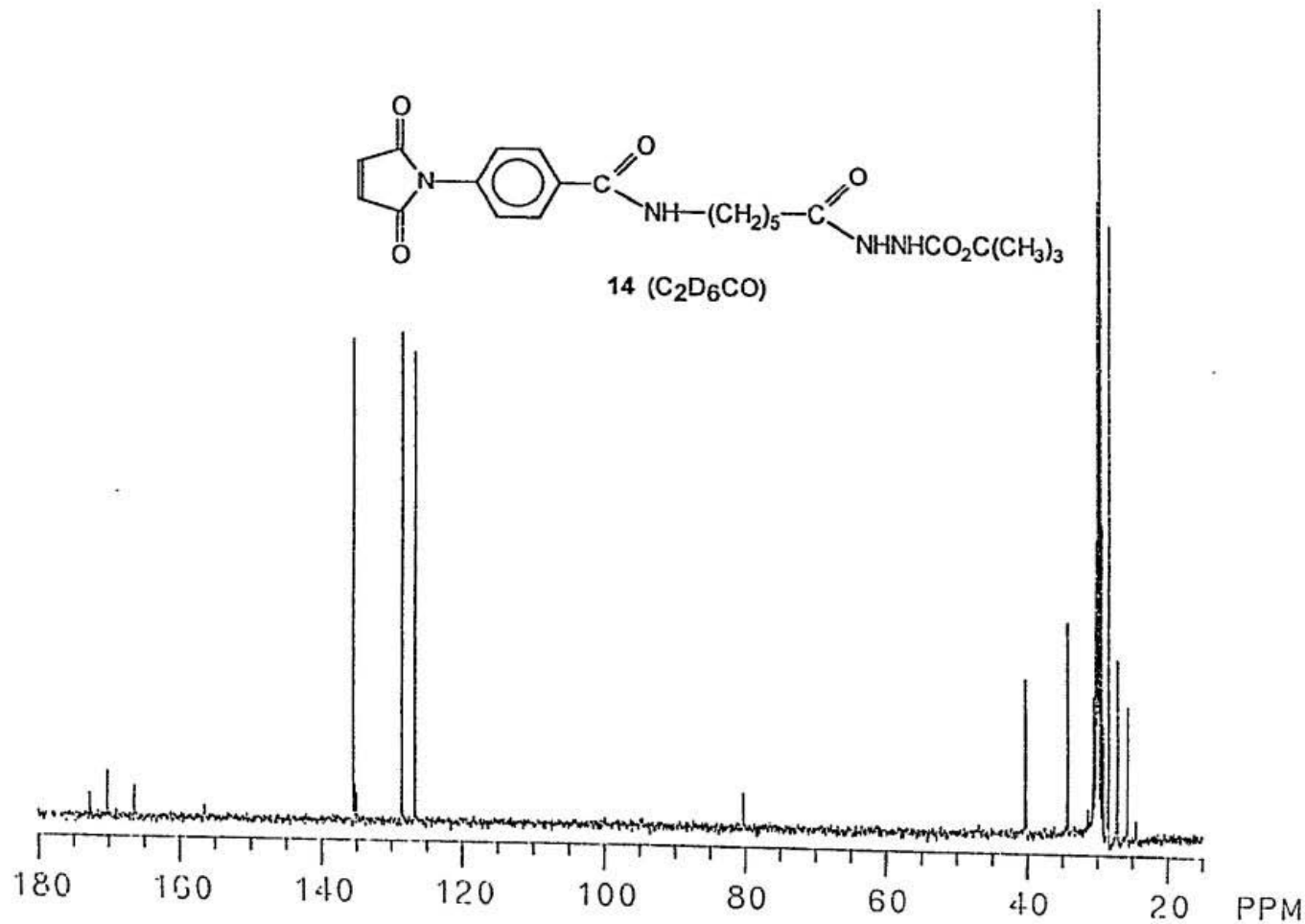
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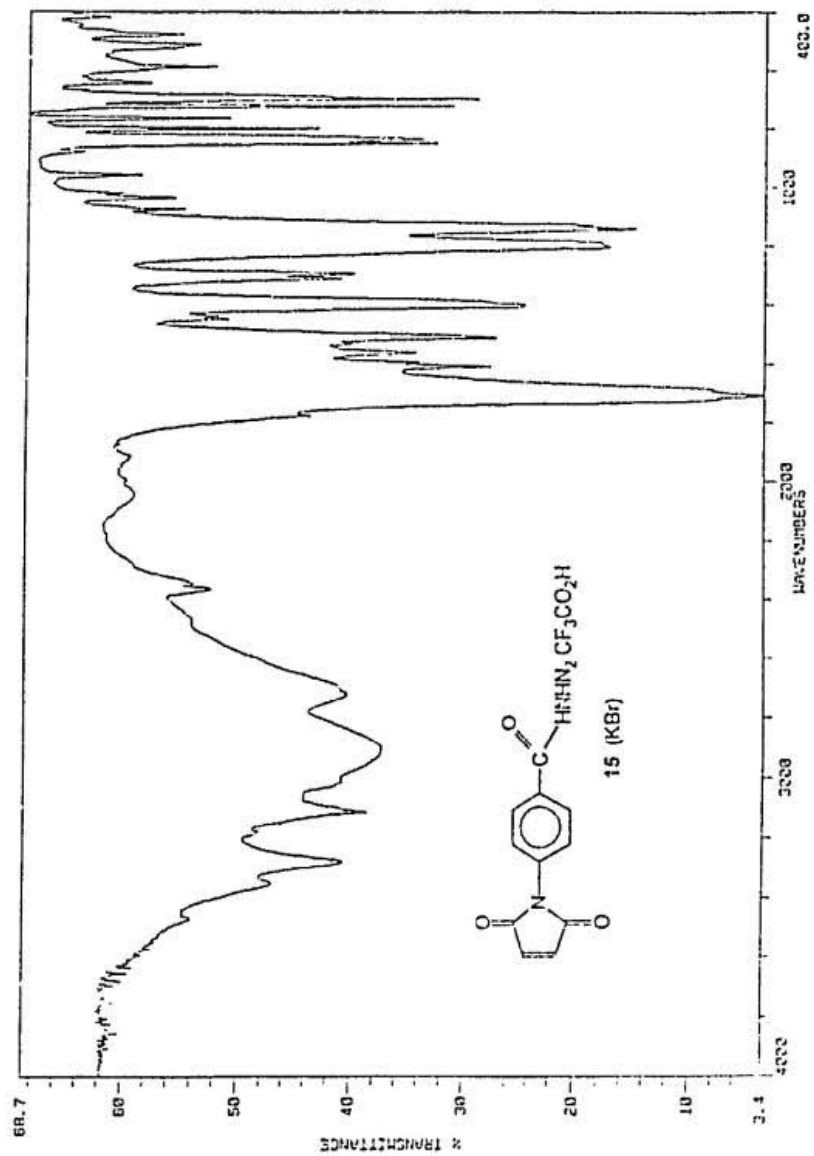
N
O
C
O
NH—(CH2)5—C
O
NHNHCO2C(CH3)3
180
160
140
120
100
80
60
40
20
PPM

14 (C_2D_6CO)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WAVENUMBERS
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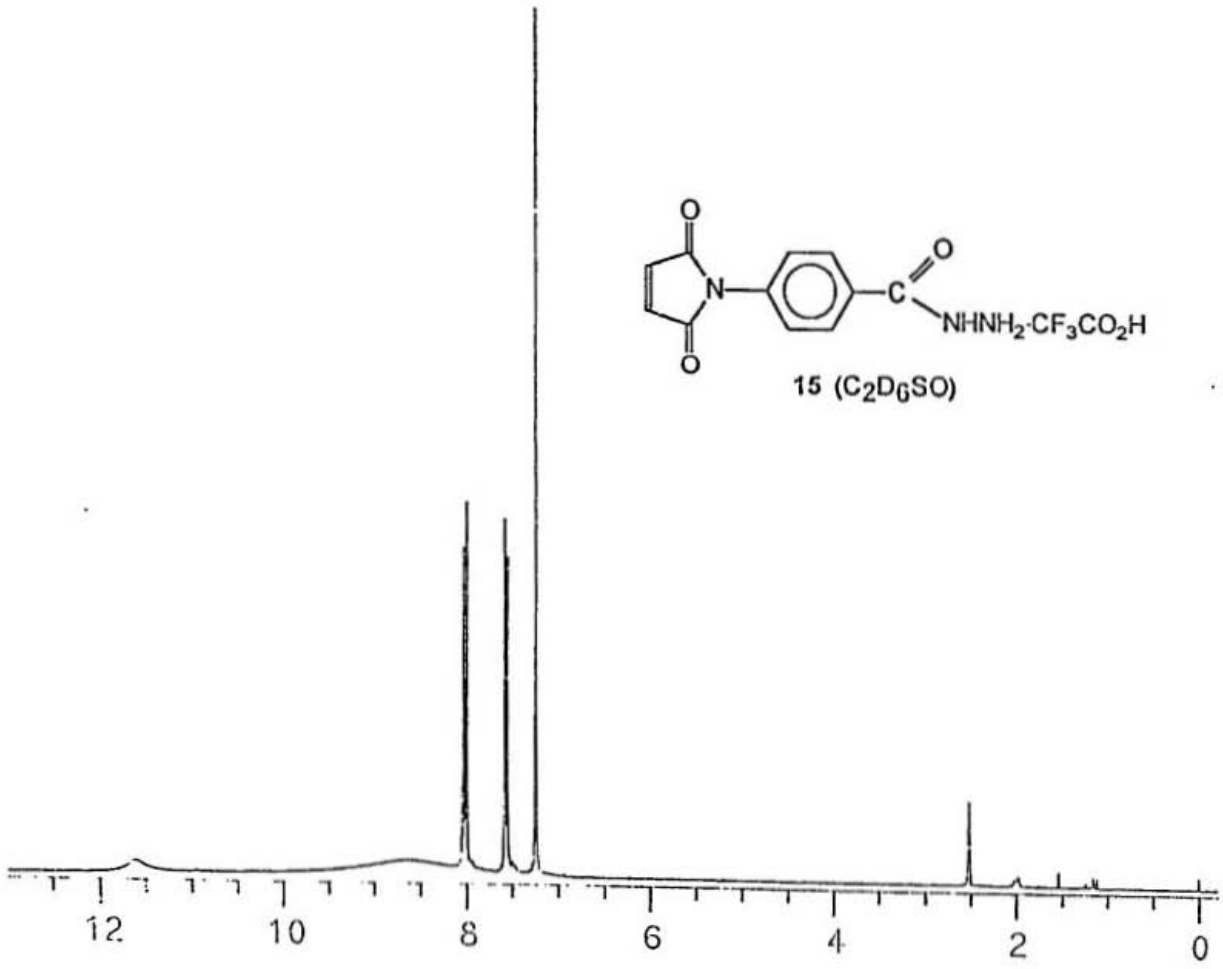
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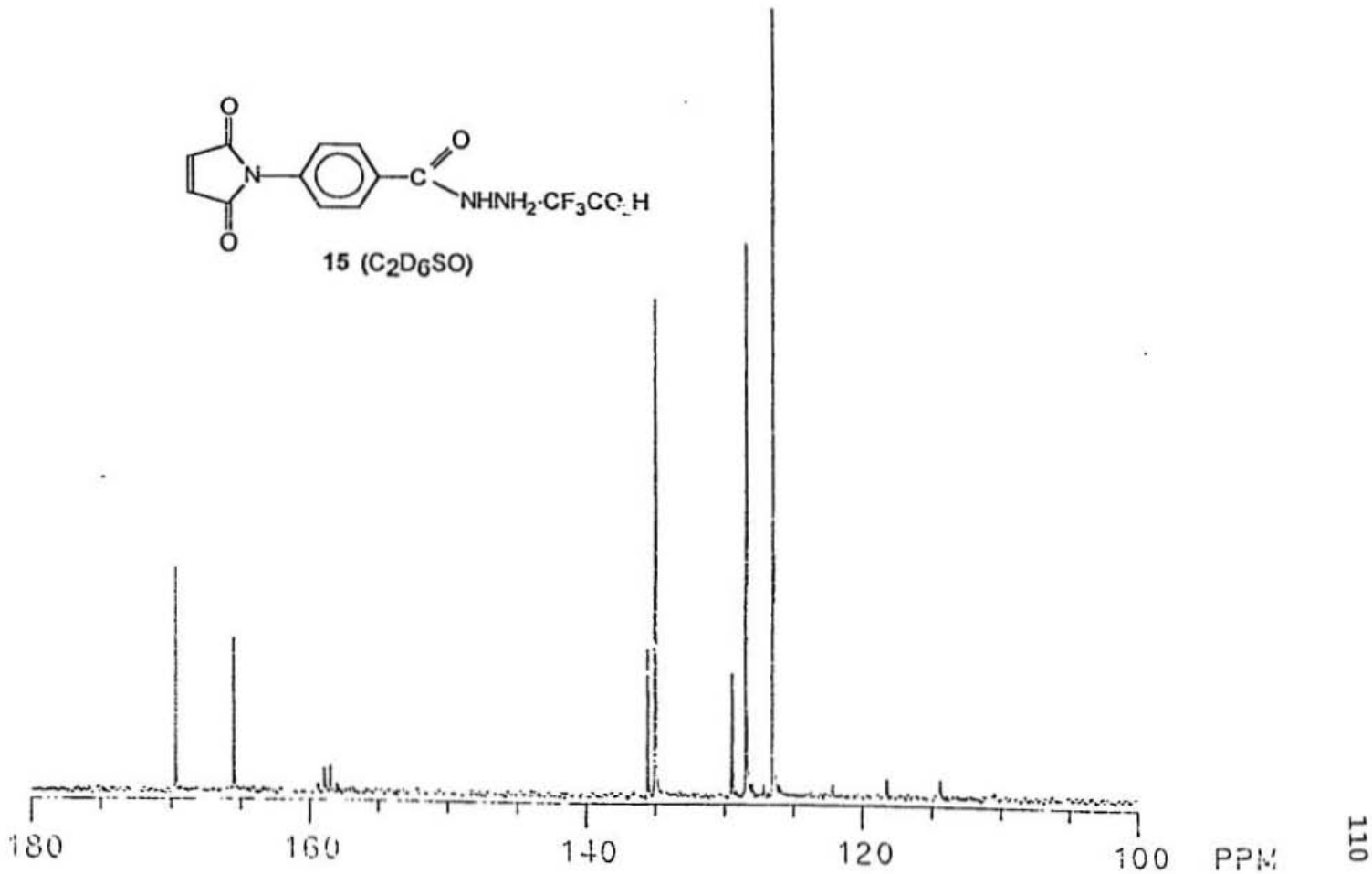
15 (C2D6SO)
12
10
8
6
4
2
0
PPM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C
O
NHNH2·CF3CO2H
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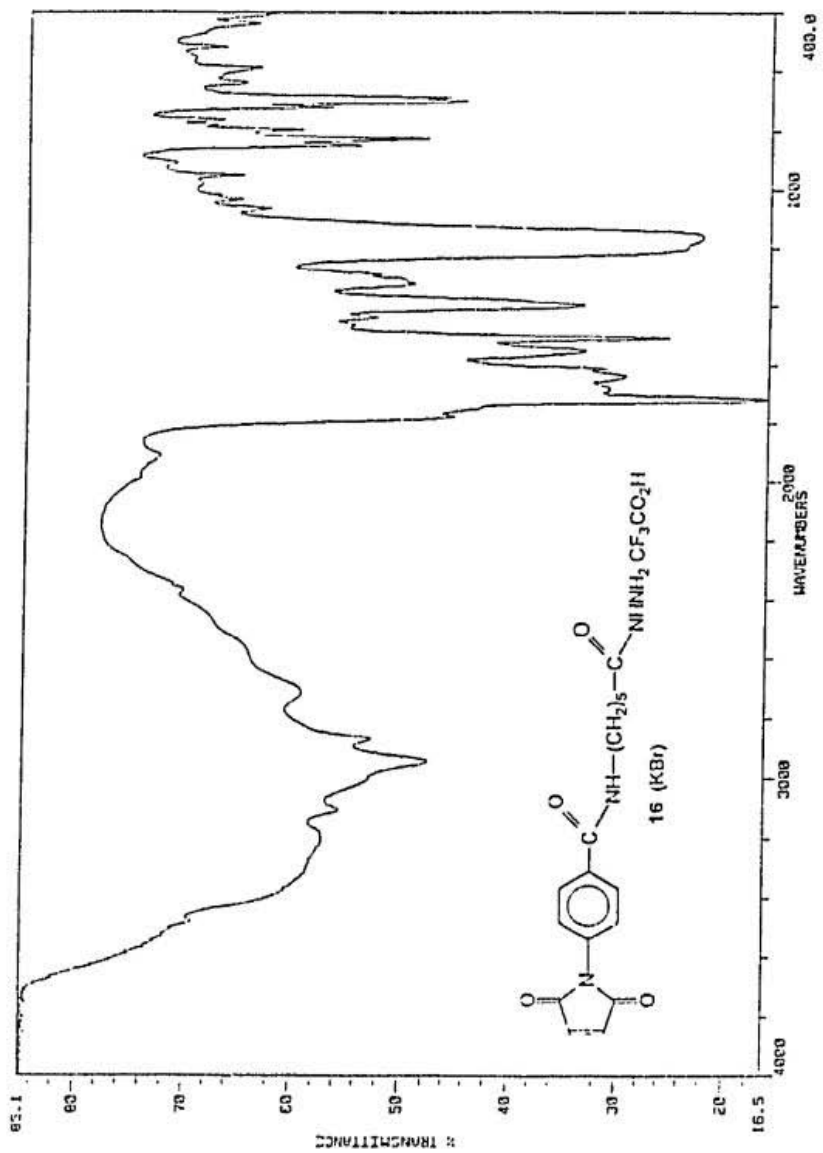
180
160
140
120
100
PPM


% TRANSMITTANCE
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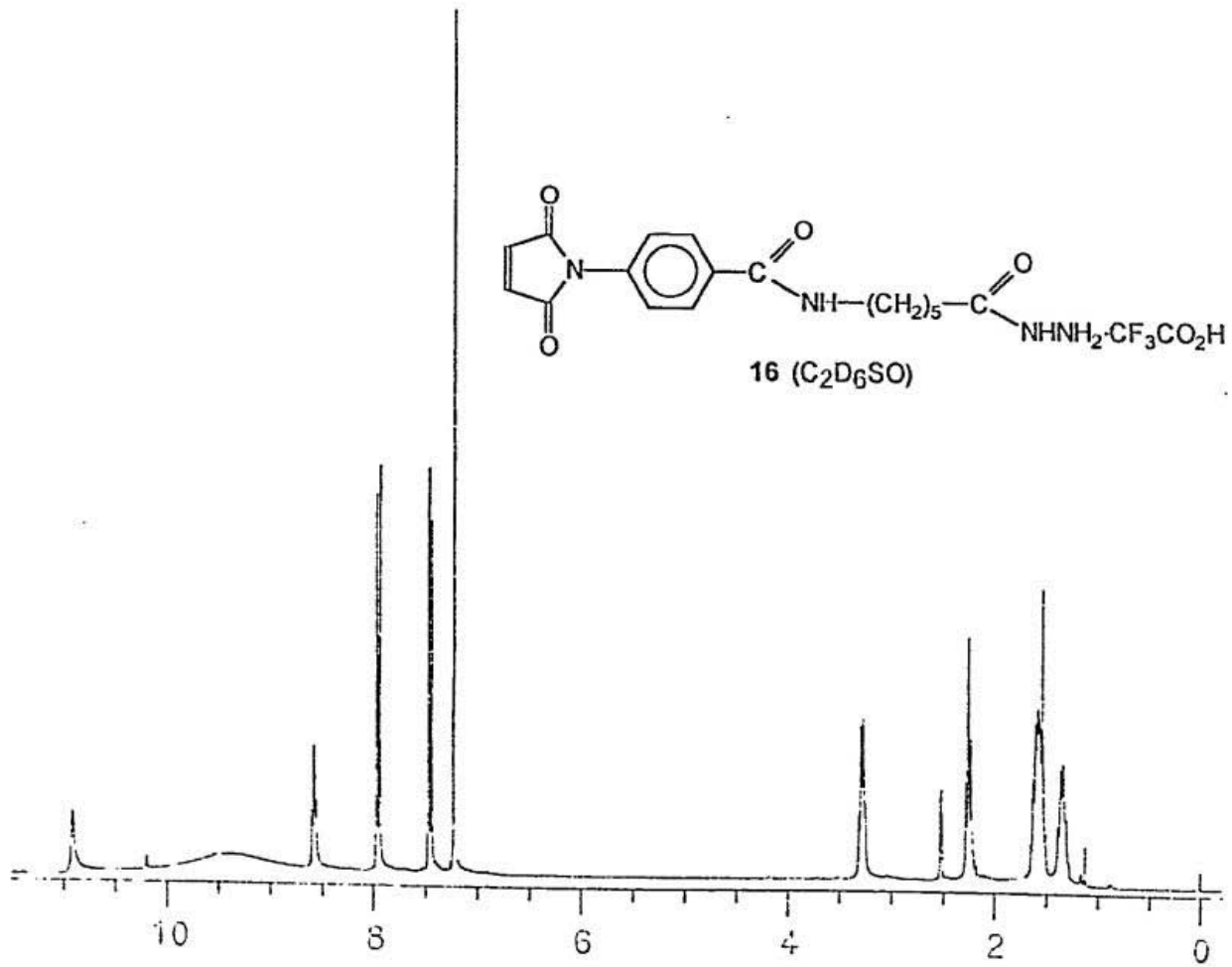
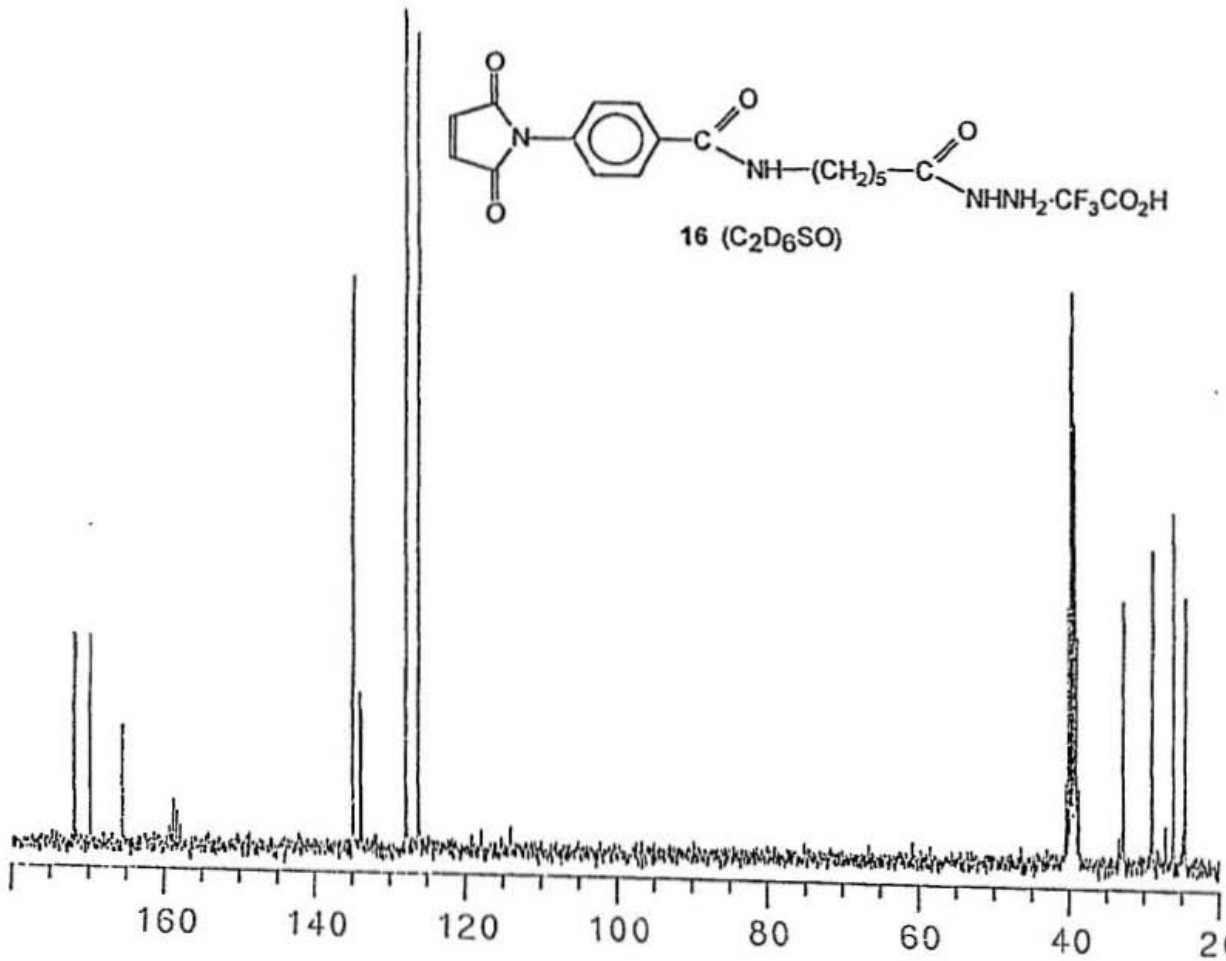
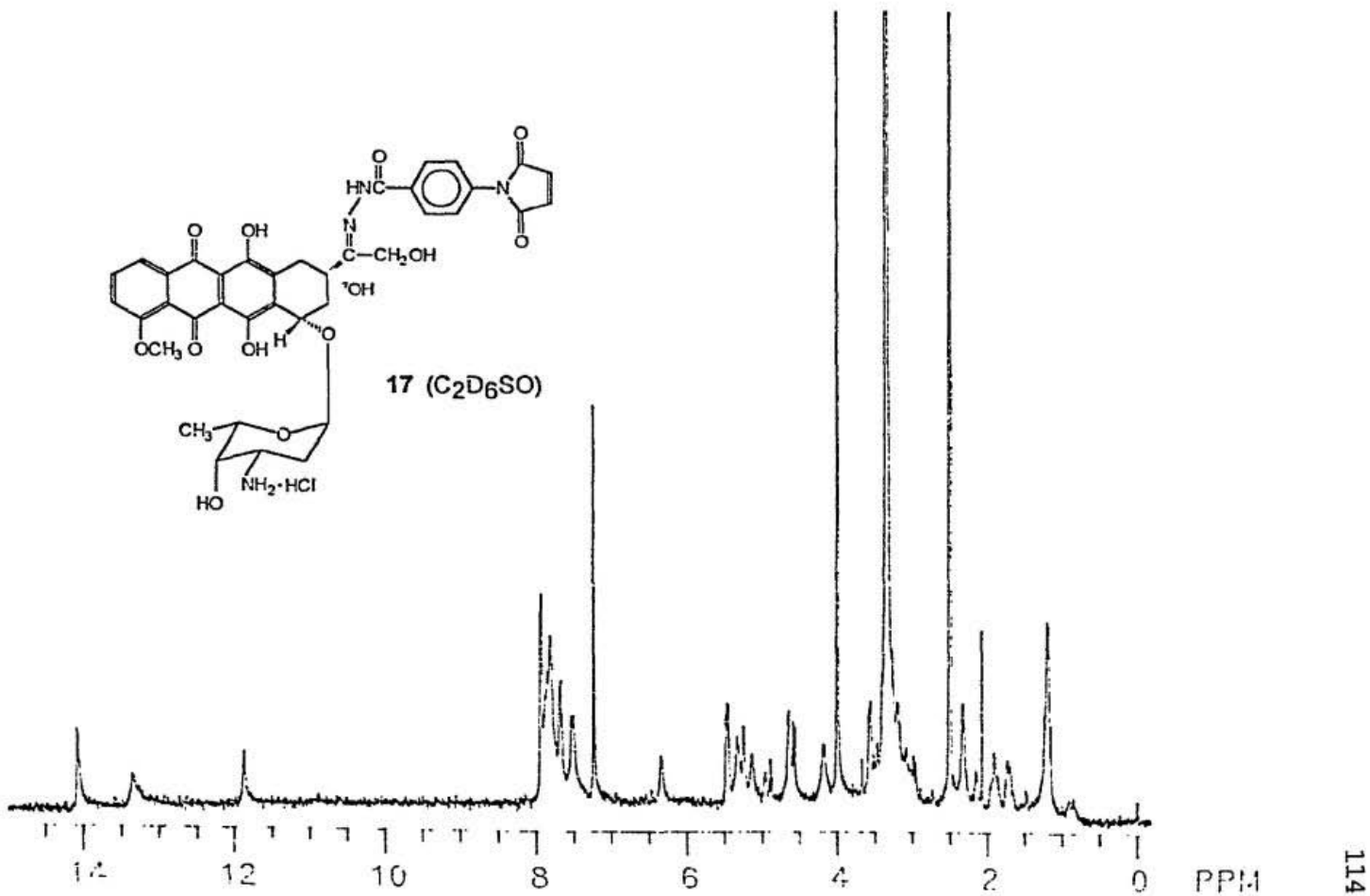
70
80
95.1
16 (KBr)



16 (C_2D_6SO)



16 (C_2D_6SO)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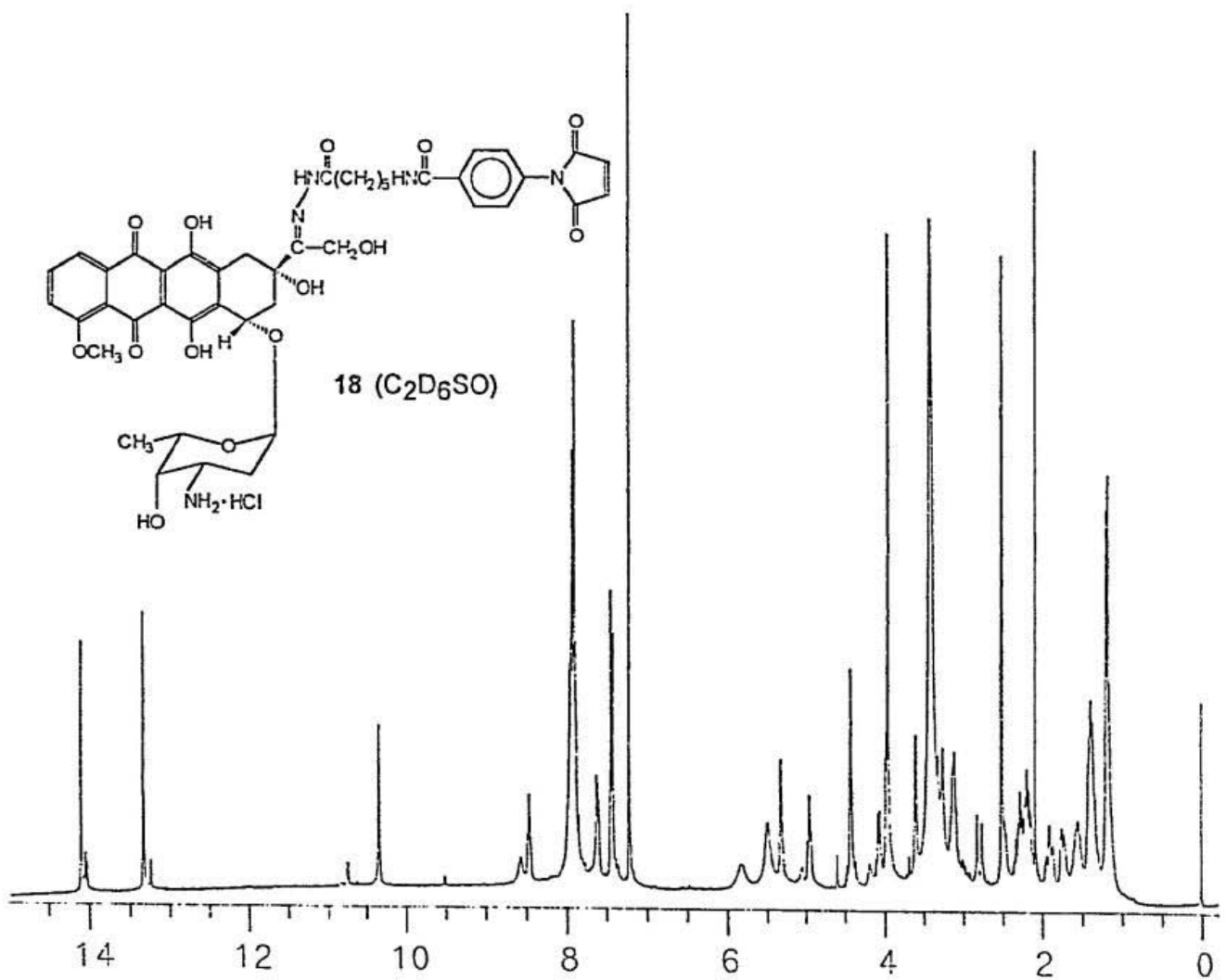
14
12
10
8
6
4
2
0
PPM


18 (C_2D_6SO)
14
12
10
8
6
4
2
0
PPM

CHAPTER III : SYNTHESIS AND BIOLOGICAL EVALUATION OF IMMUNOCONJUGATES

III 1.0 INTRODUCTION

The heterobifunctional spacers, MBA (**3**), MBCA (**4**), MBUA (**5**), MBH (**15**) and MBCH (**16**), were synthesized and coupled to Dox as described in chapter II. The stability and reactivity of the new spacers were determined spectrophotometrically to optimize the conjugation conditions. Monoclonal anti-CEA antibodies (11-285-14) were thiolated by 2-iminothiolane (2-IT) and then allowed to react with maleimide groups of Dox derivatives, **7**, **8**, **9**, **15** and **16** at pH 7.0, giving conjugates, **19**, **20**, **21**, **22** and **23** respectively. The binding activity of 11-285-14 to CEA after thiolation and conjugation was assessed by enzyme-linked immunosorbent assay (ELISA). The Dox-maleimido conjugates were analyzed and characterized by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The efficacy of these conjugates was determined *in vitro* with human tumor cell lines using a colorimetric assay with 3-4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide (MTT).

III 2.0 MATERIALS & METHODS

III 2.1 pH Optimization of Maleimide Reactions

Crossing-linking reagents containing maleimide have been widely used for conjugation reactions. The efficiency of conjugation has been shown to be pH dependent (Knight, 1979). To develop the conjugation conditions, the rate at which the

maleimido derivatives hydrolyze to maleamic acids and the rate at which the maleimido derivatives react with DL-cysteine were characterized at different pHs by spectrophotometer.

Materials

1. Phosphate Buffer (0.1 M, pH 8.0, 1 mM EDTA)

   Monobasic sodium phosphate-H_2O (BDH, Toronto, Ont) 0.73 g

   Anhydrous dibasic sodium phosphate (BDH) 13.44 g

   EDTA (BDH) 0.29 g

   Distilled water 1 L

   Phosphate Buffer (0.1 M, pH 7.0, 1 mM EDTA)

   Monobasic sodium phosphate-H_2O 5.38 g

   Anhydrous dibasic sodium phosphate 8.66 g

   EDTA 0.29 g

   Distilled water 1 L

   Phosphate Buffer (0.1 M, pH 6.5, 1 mM EDTA)

   Monobasic sodium phosphate-H_2O 9.45 g

   Anhydrous dibasic sodium phosphate 4.47 g

   EDTA 0.29 g

   Distilled water 1 L

2. MBA, MBCA and MBUA
3. DL-Cysteine (Aldrich Chem. Co., Milw., WI)
4. N,N-Dimethylformamide (DMF) (Mallinckrodt Inc., Pointe-Claire, Quebec)
5. Spectrophotometer (Beckman DU®-70) (Beckman Instruments

Inc., Fullerton, CA) connected to printer (Panasonic KX-P1091*i*)

Methods

III 2.1.1 Model Base-catalyzed Hydrolysis of Maleimide

Modification of methods from Knight (1979)

1. 1 mM maleimido derivatives (MBA, MBCA and MBUA) in DMF were prepared from 10 mM stock solution.
   10.5 mg MBA/4.835 ml DMF (10 mM)
   30.7 mg MBCA/9.290 ml DMF (10 mM)
   41.3 mg MBUA/10.313 ml DMF (10 mM)
2. 300 μl of 1 mM maleimide (MBA, MBCA or MBUA) in DMF was incubated with 2.7 ml of 0.1 M phosphate buffer (PB), pH 6.5, 7.0 and 8.0, containing 1 mM EDTA, for 1.5 h at room temperature (RT).
3. Hydrolysis of maleimide to maleamic acid was followed spectrophotometrically from 240 to 300 nm.

III 2.1.2 Model Conjugation of DL-Cysteine to Maleimide

1. 300 μl of 1 mM maleimide (MBA, MBCA or MBUA) in DMF was incubated with 2.7 ml of 0.5 mM DL-cysteine in 0.1 M phosphate buffer, pH 6.5 and 7.0, containing 1 mM EDTA, for 1.5 h at RT.
2. The condensation reaction between thiol and maleimide was followed spectrophotometrically between 240 and 300 nm.

III 2.2 Generation of Anti-CEA Monoclonal Antibodies

The mouse hybridoma (11-285-14) which secretes anti-CEA monoclonal antibody was already established in the Oncology Research Laboratory. 11-285-14 is an IgG_1 MAb and has been extensively characterized and evaluated for *in vitro* and *in vivo* targeting (Casson et al, 1985) and was supplied to me after preliminary purification of ascitic fluid on a protein-A affinity column (Ford et al, 1987a).

III 2.3 Thiolation of Anti-CEA Monoclonal Antibodies

There are several approaches by which a free thiol can be linked to the amino group of antibody. 2-Iminothiolane was chosen because it is quite water soluble, it reacts rapidly with amino groups at pH 7 or a little above, it does not require an activation step to release the thiol group, and it preserves the cationic charges of the modified amino groups. The content of thiol was measured by 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) (Riddles, Blakeley & Zerner, 1979).

Materials

1. Phosphate Buffer (0.1 M, pH 8.0, 1 mM EDTA)

   Phosphate Buffer (0.1 M, pH 7.27, 1 mM EDTA)

   Monobasic sodium phosphate-H_2O 3.45 g

   Anhydrous dibasic sodium phosphate 10.65 g

   EDTA 0.29 g

   Distilled water 1 L

2. 2-Iminothiolane-HCl (2-IT) (4.4 mM) (Aldrich Chem. Co., Milw., WI)

   3.17 mg 2-IT in 5.231 ml PB (0.1 M, pH 8.0, 1 mM EDTA)

3. 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) (1 mM) (Sigma, St. Louis, MO)

   3.96 mg DTNB in 10 ml PB (0.1 M, pH 7.0, 1 mM EDTA)

4. Anti-CEA MAb (11-285-14)

   1.32 mg/ml in PB (0.1 M, pH 8.0, 1 mM EDTA)

7. Sephadex G-25 (Pharmacia Fine Chemicals, Upsala, Sweden)
8. Gel filtration column (Pharmacia Fine Chemicals)
9. Dual Path Monitor UV-2 (Pharmacia Fine Chemicals)
10. Spectrophotometer (Beckman DU-60) and printer (Epson LX-810)

Method

Modification of the method of King, Li & Kochoumian (1978).

1. 0.5 ml of 1.32 mg/ml 11-285-14 in PB (0.1 M, pH 8.0, 1 mM EDTA) was treated with 2-IT from 0 to 400 µl and the same PB was added to make up the final volume to 0.54 ml.
2. The mixtures containing 11-285-14 and 2-IT were gently shaken at RT for 2 h.
3. Then, all mixtures were kept frozen or at 4°C until the gel filtration column was ready.
4. The thiolated MAbs were freed of excess 2-IT by passage

through a Sephadex G-25 column equilibrated with PB (0.1 M, pH 7.27, 1 mM EDTA) and collected as a single fraction monitored by UV-absorption. This was set up at RT, but the PB was kept in an ice-bath and flushed with nitrogen.

5. The protein content was assessed spectrophotometrically at 280 nm using a molar extinction coefficient of 214600 M^{-1} cm^{-1}.
6. 100 μl DTNB was added to 900 μl thiolated 11-285-14. The reaction was incubated for 30 min at RT. The thiol content was assessed spectrophotometrically at 412 nm using a molar extinction coefficient of 14150 M^{-1} cm^{-1}.
7. The molar ratio of thiols/MAb was calculated from the following : OD_{412}/OD_{280}
8. A linear regression curve of SH/MAb (abscissa) with 2-IT/MAb (ordinate) was constructed.

III 2.4 Production of Dox-anti-CEA Conjugates

Dox-maleimido derivatives were used for conjugation to anti-CEA MAbs that were thiolated with 2-IT. The conjugation reaction was performed under mild conditions at pH 7.0 at RT.

Materials

1. Phosphate Buffered Saline (PBS) (0.1 M, pH 7.0, 0.9% NaCl, 1 mM EDTA)

   Monobasic sodium phosphate-H_2O 5.38 g

   Anhydrous dibasic sodium phosphate 8.66 g

Sodium Chloride (BDH) 9 g

EDTA 0.29 g

Distilled water 1 L

Phosphate Buffer (0.1 M, pH 7.27, 1 mM EDTA)

Phosphate Buffered Saline (PBS) (0.15 M, pH 7.2) prepared from PBS tablets (Oxoid, Unipath Ltd., England), dissolved water as per manufacturer's instructions.

2. 2-IT (0.1 M)
3. DTNB (1 mM)
4. DMF
5. Anti-CEA MAb (11-285-14)

   1.44 - 1.70 mg/ml in PB (0.1 M, pH 8.0, 1 mM EDTA)
6. Doxorubicin-maleimido derivatives (**7**, **8**, **9**, **17** and **18**)
7. Sephadex G-25 (Pharmacia Fine Chemicals)
8. Gel filtration column (1.6 x 30 cm) (Pharmacia Fine Chemicals)
9. Dual Path Monitor UV-2 (Pharmacia Fine Chemicals)
10. Dialysis tubing (molecular weight cutoff 12000-14000) (Spectrum Medical Industries Inc., Houston, Texas)
11. Amicon ultrafiltration cell fitted with an Amicon PM10 ultrafilter (molecular weight cutoff 10000) (Amicon Corp., Lexington, Mass)
12. Millex GV filter (0.22 μm) (low protein binding) (Millipore Products Division, Bedford, MA)
13. Spectrophotometer (Beckman DU®-60) and printer (Epson LX-

810)

Methods

III 2.4.1 Thiolation of Anti-CEA MAbs

1. 15 - 20 ml of 1.48 or 1.70 mg/ml 11-285-14 in PB (0.1 M, pH 8.0, 1 mM EDTA) were treated with 0.1 M 2-IT from 158 - 197 µl to make the molar ratio of 11-285-14 to 2-IT 1:100.
2. The mixture containing 11-285-14 and 2-IT was gently shaken at RT for 2 hrs.
3. Then, the mixture was kept frozen or at 4°C until the gel filtration column was ready.
4. 3 ml of thiolated MAbs was freed of excess 2-IT by passage through a 1.6 x 30 cm Sephadex G-25 column equilibrated with PBS (0.1 M, pH 7.0, 0.9% NaCl, 1 mM EDTA) and collected as a single fraction monitored by UV-absorption. This was set up at RT, but the PBS was kept in an ice-bath and flushed with nitrogen. The same procedure was repeated for the remaining MAbs-2-IT mixture and the fractions collected were combined together.
5. The thiolated 11-285-14 was concentrated down to 14 - 15 ml by ultrafiltration in an Amicon ultrafiltration cell fitted with an Amicon PM10 ultrafilter (molecular weight cutoff 10000) (Amicon Corp.).
6. 20 - 30 µl DTNB was added to 100 µl 11-285-14 and PB (0.1

M, pH 7.27, 1 mM EDTA) was added to make a final volume of 1 ml. The reaction was incubated for 30 min at RT.

7. The ratio of thiol/11-285-14 was determined as described before.

III 2.4.2 Molar Extinction Coefficient of Dox Derivatives

1. 8.75 - 10 μl of 10 mM Dox 3'-N-amide derivatives (1.71 mg MBA-Dox in 230 μl DMF, 1.61 mg MBCA-Dox in 188 μl DMF and 2.06 mg MBUA-Dox in 222 μl DMF), 10 μl of 13.04 mM Dox-MBH (11.07 mg in 1.070 ml DMF) and 20 μl of 6.24 mM Dox-MBCH (7.20 mg in 1.273 ml DMF) were diluted to 1000 μl by DMF.
2. 500 μl of the above solutions were taken out and further diluted to 1000 μl with DMF. The double dilution procedure was repeated to get five different concentrations of each Dox derivative.
3. The absorbance of each Dox derivative at different concentrations was assessed spectrophotometrically at 280 and 495 nm.
4. The molar extinction coefficient was obtained from a linear regression curve which was constructed from absorbance (ordinate) against Dox derivative concentrations (abscissa).

III 2.4.3 Maleimide Conjugation: Method 1

1. 50 μl of MBA-Dox (1.25 mg in 302 μl DMF, 5.57 mM), MBCA-Dox (3.13 mg in 661 μl DMF, 5.53 mM) or MBUA-Dox (2.04 mg

in 435 μl DMF, 4.80 mM) was diluted to 500 μl with DMF.

2. 500 μl of the above Dox derivative solution was added within 10 min to 2.5 ml of 11-285-14 (1.44 or 1.67 mg/ml containing 6.26, 6.55 or 7.14 thiols/MAb in PBS, pH 7.0, 1mM EDTA, 0.9% NaCl) at RT. Vigorous stirring was continued only for about 5 s after each addition of Dox derivative. The reaction mixture was incubated in the dark for an additional 1 h 20 min period and stirred (every 10 min).
3. The conjugate was then separated from unconjugated Dox derivative by passage through a 1.6 x 30 cm Sephadex G-25 column equilibrated with PBS (0.15 M, pH 7.2 prepared from PBS tablets) and collected as a single fraction monitored by UV-absorption from a UV monitor.
4. Conjugate fractions were concentrated down by ultrafiltration in an Amicon ultrafiltration cell fitted with an Amicon PM10 ultrafilter (molecular weight cutoff 10000).
5. The conjugate solution was transferred to dialysis tubing and dialyzed 2 x 8 h in PBS, pH 7.4 (1L) (prepared from tablets).
6. The conjugates were sterilized through Millex GV filters (0.22 μm) (low protein binding) (Millipore, Bedford, MA)
7. The absorbances of conjugates were determined at 280 nm and 495 nm to estimate protein and drug concentrations

respectively.

III 2.4.4 Maleimide Conjugation: Method 2

1. 25 μl of MBH-Dox (11.07 mg in 1.07 ml DMF, 13.04 mM) diluted to 50 μl with DMF or 50 μl MBCH-Dox (7.20 mg in 1.273 ml DMF, 6.24 mM) was added in 10 μl aliquots every 5 min to 3.0 ml of 11-285-14 (1.56 or 1.62 mg/ml containing 6.55 or 6.59 thiols/MAb in PBS, pH 7.0, 1mM EDTA, 0.9% NaCl) at RT. Vigorous stirring was continued only for about 5 s after each addition of Dox derivative. The reaction mixture was incubated in the dark for an additional 1 h 10 min period and occasionally stirred (every 10 min).
2. The rest of the procedure was the same as section III 2.4.2 (c), steps 3 to 7.

III 2.5 Enzyme Linked Immunosorbent Assay (ELISA)

An anti-CEA semiautomated ELISA using micro cuvettes (Woodhouse, Ford & Newman, 1982; Ford et al, 1987a) was modified to 96 well microtitre plates (Reddy and Ford, 1993). The microtitre plates are a convenient and rapid method for testing large numbers of samples.

Materials

1. Microtitre ELISA plates (ICN Biomedicals Inc., Horsham, PA)
2. Nichiryo digital multichannel pipette and disposable tips

(Fisher Scientific Co., Toronto, Ont)

3. Buffer for ELISA

Carbonate Bicarbonate Buffer (0.1 M, pH 9.2)

Sodium Carbonate (Fisher) 1.59 g

Sodium Bicarbonate (Sigma) 2.93 g

Distilled water 1 L

1% BSA Carbonate Buffer (pH 9.2)

BSA (Sigma) 1g

Carbonate Buffer (0.1 M, pH 9.2) 100 ml

Citrate Phosphate Buffer (0.1 M, pH 4.0)

Citric Acid (BDH) 9.06 g

Dibasic sodium Phosphate 8.16 g

Distilled water 1 L

1% BSA-PBS-Tween Diluent

BSA 1 g

PBS (pH 7.2) 100 ml

Tween 20 (BDH) 100 μl

Saline Tween

Sodium Chloride (0.15M) 8.76 g

Tween (0.1%) 1 ml

Distilled water 1 L

4. 2,2'-azino-di-(3-ethyl-benzthiazoline sulphonic acid) (ABTS) (Sigma, St.Louis, MO). Stock: 27.8 mg/ml in distilled water and stored as 100 μl aliquots at -20°C

100 μl stock

12.5 ml citrate phosphate buffer

1 μl hydrogen peroxide (30%; BDH)

5. Peroxidase conjugated rabbit anti-mouse immunoglobulins (Dako, Denmark)
6. Bio-Tek EL310 EIA plate reader (Mandel Scientific, Rockwood, Ont)
7. CEA (supplied by Oncology Research Laboratory) purified as published (Ford et al, 1987a) in carbonate bicarbonate buffer (0.1M, pH 9.2)
8. 11-285-14 anti-CEA monoclonal antibody (positive control)
9. 1% BSA-PBS-Tween (negative control)

Method

1. ELISA plates were coated with 100 μl per well of 5 μg ml^{-1} CEA in carbonate buffer (pH 9.2) and incubated for 3 hours at 37°C followed by storage in a humidified box at 4°C overnight.
2. The coating solution was pipetted out and stored at -20°C for later use.
3. The plate was washed six times with NaCl-Tween solution using a wash bottle, ensuring that each well was filled.
4. 200 μl of 1% BSA in carbonate buffer was added to each well, as the blocking solution.
5. The plate was then incubated at 37°C for 1 hour.
6. The plate was washed as before (step #3).
7. The test supernatants were prepared at appropriate

dilutions in 1%BSA-PBS-Tween and added, 100 µl per well. The controls used for anti-CEA assays were: positive control, 11-285-14 MAb 2.5 µg/ml; negative control, 1% BSA-PBS-Tween instead of test supernatants. The test was performed in quadruplicate for each dilution.

8. The plate was then incubated at 37°C for 3 hours.
9. This was followed by x 6 washes as before (step #3).
10. Rabbit anti-mouse immunoglobulines - HRP conjugate (RAM) 1:1000 dilution in 1% BSA PBS-Tween was added, 100 µl per well.
11. The plate was then incubated at 37°C for 3 hours.
12. The plate was washed x 6 as before (step #3).
13. Freshly prepared ABTS substrate, 100 µl was added per well and the plate was read after 1 hour at RT at 405 nm single wavelength, by a BioTek EL 310 EIA plate reader (Mandel Scientific, Rockwood, Ontario)

III 2.6 ELISA for Thiolated Anti-CEA Antibody

Materials

1. The materials outlined above, section III 2.5.1
2. Thiolated 11-285-14 in PB (0.1 M, pH 7.0, 1 mM EDTA).

Method

The assay outlined above, section III 2.5.2, was used to test antibody activity of thiolated 11-285-14 (3.35 - 13.50 SH/MAb), with 11-285-14 as a control. Concentrations tested

were 1 x 10^{-5} - 10000 ng/ml and tests were performed in quadruplicate. Standard curves of absorbance (abscissa) with antibody concentration (ordinate) were constructed.

III 2.7 ELISA for Testing Conjugates

Materials

1. The materials outlined above, section III 2.5.1
2. Dox-11-285-14 conjugates in PBS (pH 7.4, PBS tablet).

Method

The assay outlined above, section III 2.5.2, was used to test antibody activity of doxorubicin conjugated 11-285-14, with 11-285-14 as a control. Concentrations tested were 1 x 10^{-5} - 10000 ng/ml and tests were performed in quadruplicate. Standard curves of absorbance (abscissa) with antibody concentration (ordinate) were constructed.

III 2.8 Human Tumor Cell Culture

Human tumor cell lines utilized in this study were already established in culture in the Oncology Research Laboratory (Ford et al, 1987b). They were obtained initially from the American Type Culture Collection, Maryland, USA.

III 2.9 Microcytostasis Assay

Chemosensitivity of cell lines to doxorubicin, doxorubicin derivatives or the monoclonal antibody-doxorubicin

conjugates was assessed with a colorimetric assay that uses the ability of viable cells to reduce a soluble tetrazolium salt, 3-4,5-dimethylthiazol-2,5-diphenyl-tetrazolium bromide (MTT), into an insoluble formazan precipitate (Denizot & Lang, 1986) with modifications (Ford, Richardson & Tsaltas, 1989). The colorimetric assay has the advantages of being safer, less costly and simpler than the radiometric assays.

Materials

1. Human Tumor Cell Line used, with source (obtained from the American Type Culture Collection, Maryland, USA)

Cell line	Source
LS174T	Adenocarcinoma, colon
COLO320DM	Adenocarcinoma, colon

2. Media required for cell Culture, including supplements (all materials supplied by Flow Labs.)

Cell line	Medium	Supplements
LS174T	RPMI-1640 (500 ml)	50 ml fetal calf serum (8.8%) 6 ml glutamine 12 ml penicillin-streptomycin
COLO320DM	Minimum essential medium (500 ml)	50 ml fetal calf serum (8.8%) 6 ml glutamine 6 ml non-essential amino acids 12 ml penicillin-streptomycin

3. Cell suspension 10^5 cells/ml in appropriate medium following trypsinization

4. Microtitre plates, 96 wells (Flow Lab. Inc., McLean, Virginia)
5. Medium-sterile
6. MTT (Sigma) in PBS (stock: 5mg/ml)
7. DMSO (Sigma)
8. Bio-Tek EL310 EIA plate reader (Mandel Scientific)
9. Doxorubicin (Adria Laboratories Inc., Columbus, Ohio)
   Specific doxorubicin concentrations varied in different batches
   Stock solution of Dox in 0.9% NaCl
10. Doxorubicin maleimido derivatives
    Specific doxorubicin derivative concentrations varied in different batches
    Stock solution of Dox 3'-N-amide derivatives in MeOH
    Stock solution of Dox C-13 hydrazone derivatives in 0.9% NaCl
11. 11-285-14 Doxorubicin conjugate
    Specific doxorubicin concentrations and immunoglobulin concentrations varied in different batches

Method

Essentially as for Ford, Richardson & Tsaltas (1989).

1. Under sterile conditions, 100 μl of the cell suspension was dispensed into wells of the microtitre plate, such that each well contained 10^4 cells.
2. The plate was incubated for 24 hrs at 37°C in a

humidified atmosphere of 5% carbon dioxide.

3. Under sterile conditions, medium was aspirated by multiple channel pipette from the wells and doubling dilutions of drug, drug derivatives (Dox 3'-N-amide derivatives added in 5% MeOH) or conjugate were added in a volume of 100 μl to each well. The test was performed in quadruplicate for each dilution, with appropriate control wells that received 100 μl of medium only. 5% MeOH of medium in doubling dilutions were performed in quadruplicate as control in comparision to the Dox 3'-N-amide derivative testing. The plate was incubated for 24 hr at 37°C as before.
4. The medium was aspirated in a similar manner to previously and each well washed 3 times with sterile PBS (200μl) at RT. 100 μl of medium was then added to each well and a 24 hrs recovery period followed.
5. Following removal of the medium by aspiration under sterile conditions after recovery, 100 μl 1:10 dilution of MTT stock was added to each well. A 4 hr incubation period in the humidified incubator at 37°C followed.
6. The medium was aspirated from each well. 100 μl DMSO was added to each well and the plate was agitated on a plate shaker for 20 min.
7. The plate was read spectrophotometrically at 570 and 630 nm.

8. Results from the plate reader were expressed as follows:

Percentage Cell Survival at Each Dilution

$$= \frac{\text{Mean Absorbance at Each Dilution}}{\text{Mean Control Absorbance}} \times 100$$

9. A dose response curve of percentage cell survival (ordinate) against drug concentration (abscissa) was constructed.

III 2.10 SDS-Polyacrylamide Gel Electrophoresis

Gel electrophoresis was performed on a 10% running gel and 5% stacking gel.

Materials

1. Tris-Cl (0.15M, pH 8.8)

   Tris base (Sigma) 18.15 g

   adjust to pH 8.8 with 1M HCl

   final volume to 100 ml with distilled water

   Tris-Cl (0.5M, pH 6.8)

   Tris base 3.0 g

   adjust to pH 6.8 with 1M HCl

   final volume to 50 ml with distilled water

2. Acrylamide : Bisacrylamide (30%T, 2.67%C)

   Acrylamide (Bio-Rad, Richmond, Ca) 29.2 g

   N,N'-Methylenebisacrylamide (Bio-Rad) 0.8 g

   Distilled water 100 ml

   Filter and store at 4°C in the dark (30 days max.)

3. 10% Sodium dodecyl sulphate (SDS) (BDH)
4. 10% ammonium persulphate (Bio-Rad)
5. N,N,N',N'-Tetramethylethylenediamine (TEMED) (Sigma)
6. Sample buffer

   Tris-Cl (0.5M, pH 6.8) 1.0 ml

   Glycerol (Sigma) 0.8 ml

   10% SDS 1.6 ml

   0.05% Bromophenol Blue 0.2 ml

   final volume to 8.0 ml with distilled water
7. Electrode Buffer (pH 8.3)

   Tris base 12 g

   Glycine (Sigma) 57.6 g

   SDS 4 g

   adjust to pH 8.3 with 1M HCl

   final volume to 4 L with distilled water
8. High molecular weight protein standards (Bio-Rad)

Method

1. For preparation of separating gel, 13.35 ml of acrylamide :bis-acrylamide (30% T, 2.67% C), 10 ml Tris-Cl (1.5M, pH 8.8), 0.4 ml 10% SDS and 13.5 ml distilled water were mixed and deaerated under vacuum for 15 min. 0.1 ml 10% ammonium persulphate and 0.02 ml TEMED were added to the mixture, gently shaken and immediately poured into a vertical slab gel with 1.5 mm spacer. Distilled water was layered on top of the gel to prevent evaporation.

The separating gel was allowed to polymerize overnight.

2. For preparation of stacking gel, 1.3 ml of acrylamide :bis-acrylamide (30% T, 2.67% C), 2.5 ml Tris-Cl (0.5M, pH 6.8), 0.1 ml 10% SDS and 4.1 ml distilled water were mixed and deaerated under vacuum for 15 min. 0.1 ml 10% ammonium persulphate and 0.01 ml TEMED were added to the mixture immediately before pouring over the separating gel. A plastic comb was carefully inserted into the stacking gel to form the sample wells.
3. The sample to be analyzed was added to sample buffer making 6 - 12 μg of antibody/50 μl of buffer, 12 μg of conjugate/50 μl of buffer and 4.5 μl of protein standard/ 50 μl of buffer before applying to the sample wells. Electrophoresis was carried out at a voltage of 150 V and constant current of 25 mA for 16 h at RT. The molecular weight of the examined samples was determined from the protein standard.

III 2.11 Coomassie Blue Gel Staining

Materials

1. Fixing solution

   Glycerol 250 ml

   Methanol 200 ml

   Acetic acid 50 ml

2. Staining solution (0.25% Coomassie blue)

Coomassie brilliant blue R 250 (Merck, Darmstadt) 1.25 g

Methanol 125 ml

Acetic acid 50ml

Distilled water 325 ml

3. Destaining solution

Destain #1 and #2 (40% methanol)

Methanol 200 ml

Acetic acid 50 ml

Distilled water 250 ml

Destain #3 (30% methanol)

Methanol 150 ml

Acetic acid 50 ml

Distilled water 300 ml

Destain #4 (10% methanol)

Methanol 50 ml

Acetic acid 50 ml

Distilled water 400 ml

Method

The gel was soaked in fixing solution for 4 hrs and then in staining solution overnight. The gel was washed in destaining solution, 2 hrs in destain #1 and #2, 6 hrs in destain #3 and overnight in destain #4, with gentle shaking.

III 3.0 RESULTS

III 3.1 pH Optimization of Maleimide Reactions

The pH dependence of the coupling reactions between maleimide and thiol were investigated spectrophotometrically by addition of MBA (**3**), MBCA (**4**) and MBUA (**5**) to cysteine thiol at different pHs. The base-catalyzed hydrolysis of maleimide to maleamic acid was also investigated by incubation of MBA, MBCA and MBUA at different pHs. The coupling and hydrolysis reactions are summarized in Figure 9.

Figures 10, 13 and 16 show the absorption spectra recorded during coupling reactions. Tables 2 to 4 and Figures 12A, 15A and 18A illustrate results for the coupling reactions.

For the linkers, MBA, MBCA and MBUA, the rate of coupling, cysteine-to-maleimide condensation, is slower at pH 6.5 and rapid at pH 7.0 (Figures 12A, 15A and 18A). Both MBA and MBUA show higher chromophoric changes at pH 7.0 than at pH 6.5 (Figures 12A and 18A), indicating that more maleimide reacts with cysteine thiol at pH 7.0 than at pH 6.5. MBCA shows virtually the same chromophoric changes at pH 7.0 and 6.5 (Figure 15A).

Figures 11, 14 and 17 show the absorption spectra recorded during hydrolysis reactions. Tables 2 to 4 and Figures 12B, 15B and 18B illustrate results for base-catalyzed hydrolysis reactions.

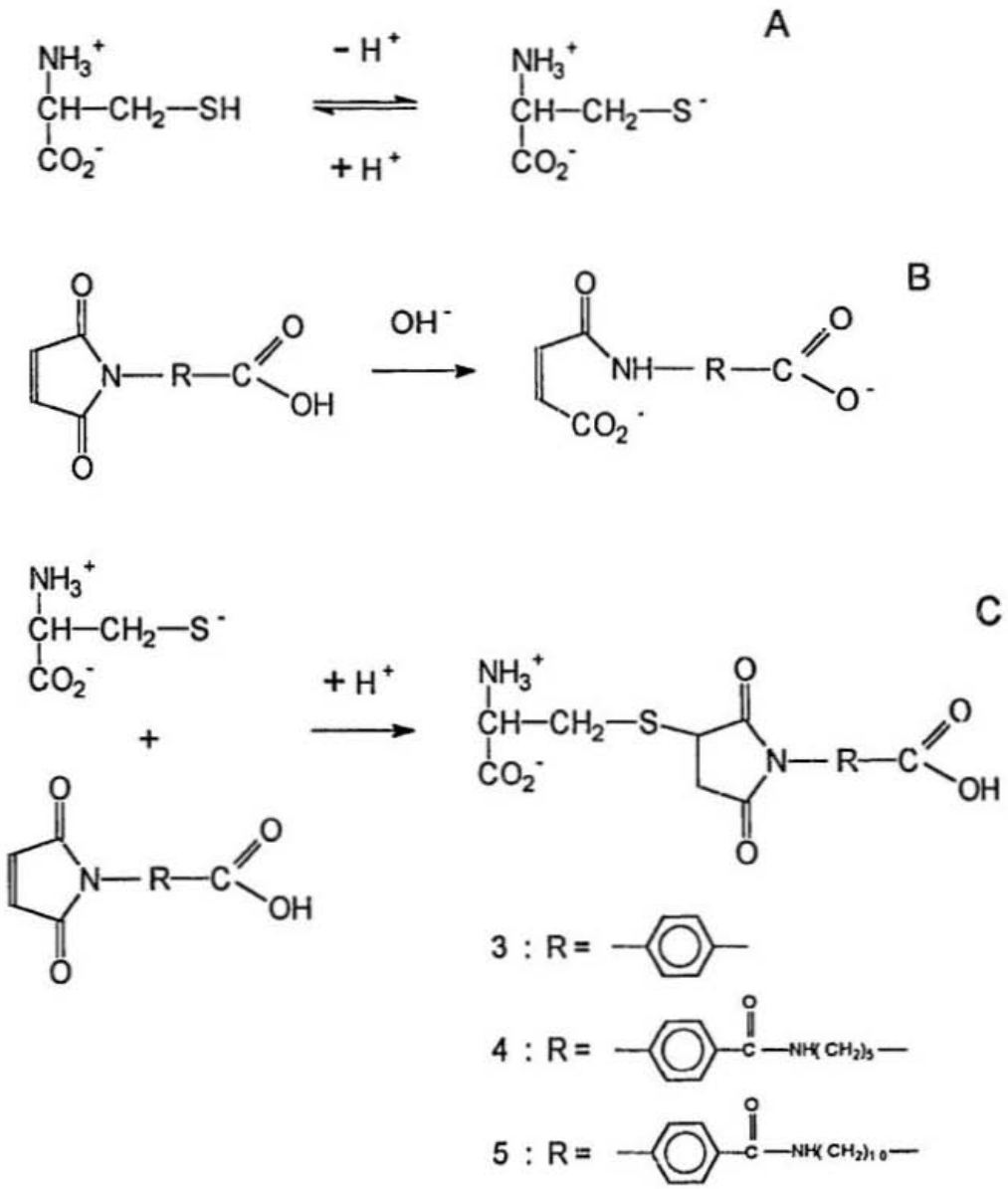


Figure 9. **pH dependence of the coupling reaction between maleimide and thiol.** The existance of cysteine anion in alkaline pH (A). The alkaline hydrolysis of maleimide moieties to maleamic acids (B). The addition reaction of the cysteine anion and the maleimide moieties (C).

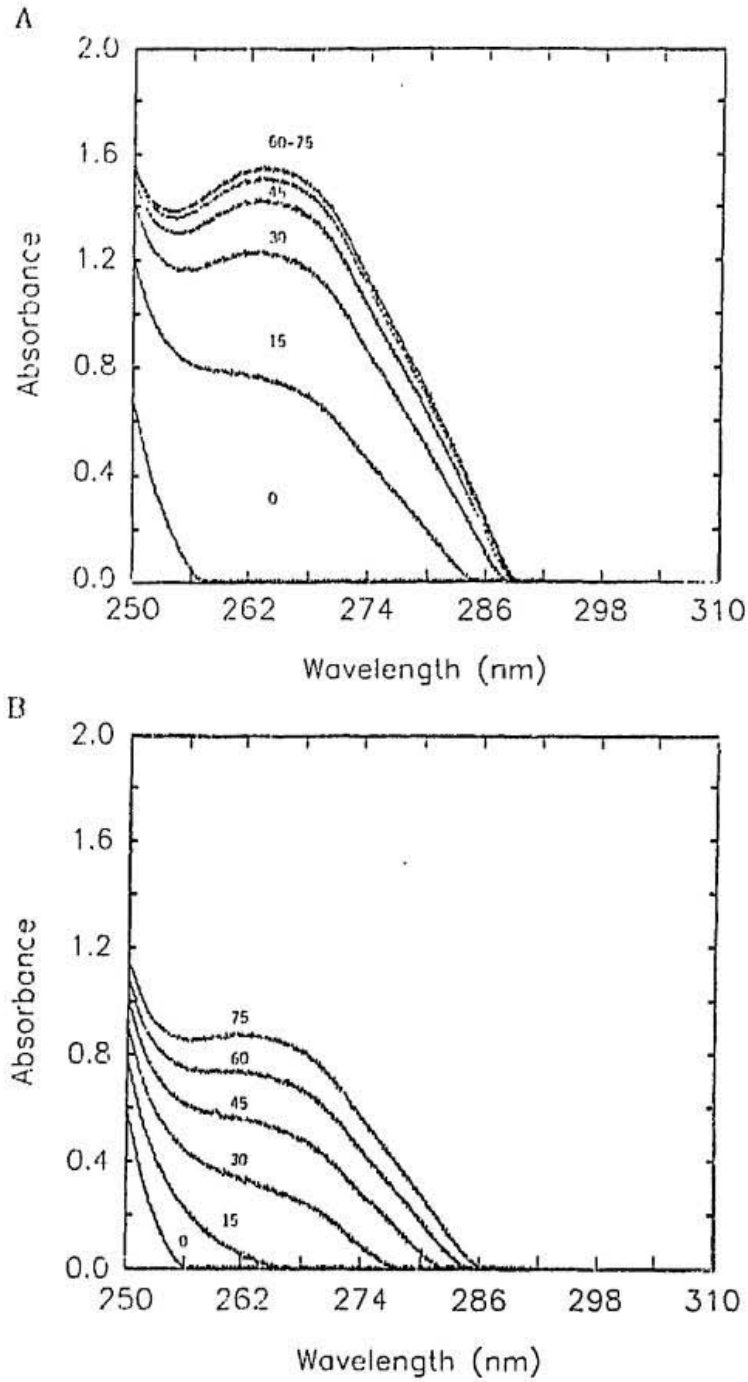


Figure 10. **Chromophoric change of MBA reaction with free thiol.** Absorption spectra were repetitively scanned and recorded at 15 min. intervals for 1¼ h during cysteine-to-MBA condensation. MBA (100 μM) was incubated with excess cysteine (450 μM) in 0.1 M phosphate buffer at pH 7.0 (Panel A), pH 6.5 (Panel B). The number beside each line is the incubation time in minutes.

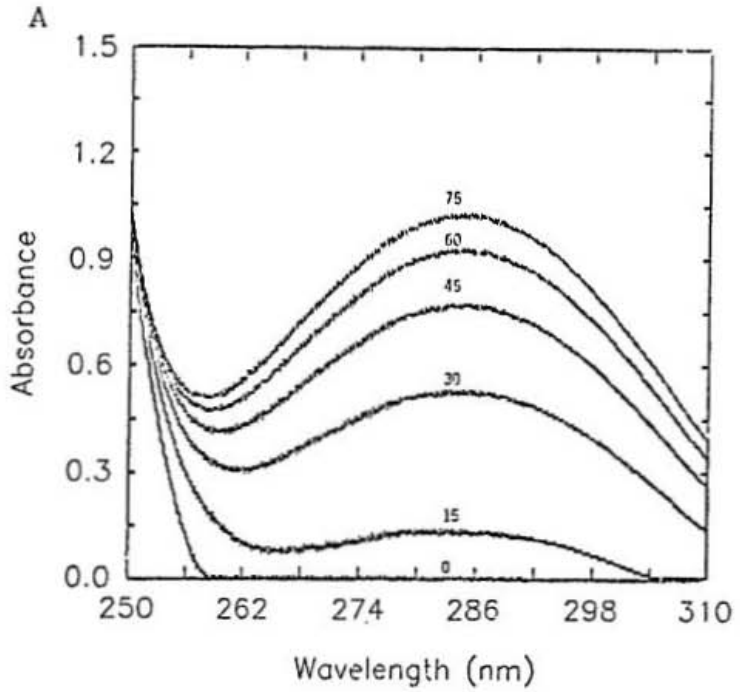
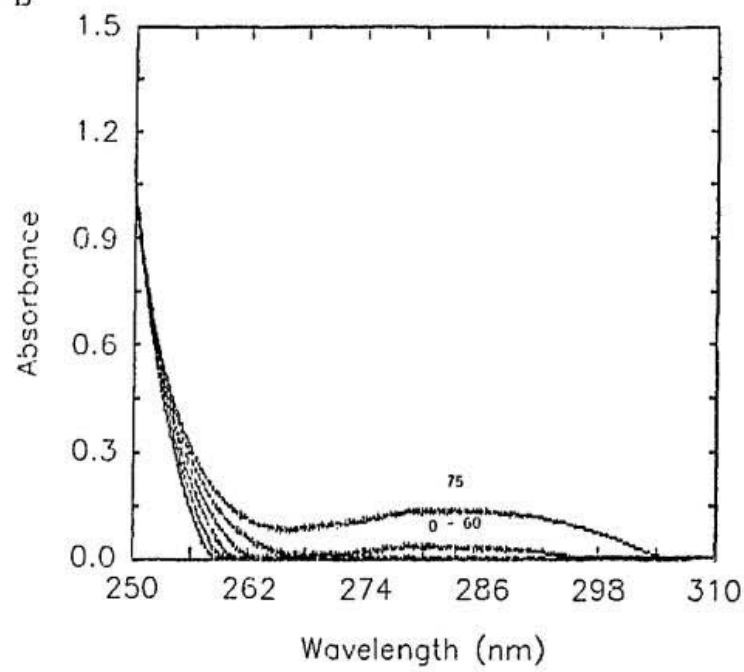


Figure 11. **Chromophoric change of the hydrolysis of MBA.** Absorption spectra were repetitively scanned and recorded at 15 min. intervals for 1¼ h during the hydrolysis of MBA. MBA (100 μM) was incubated in 0.1 M phosphate buffer at pH 8.0 (Panel A), pH 7.0 (Panel B) and pH 6.5 (Panel C). The number beside each line is the incubation time in minutes. (Continued on next page).

B


1.5
1.2
0.9
0.6
0.3
0.0
Absorbance
75
0 - 60
250
262
274
286
298
310
Wavelength (nm)

C

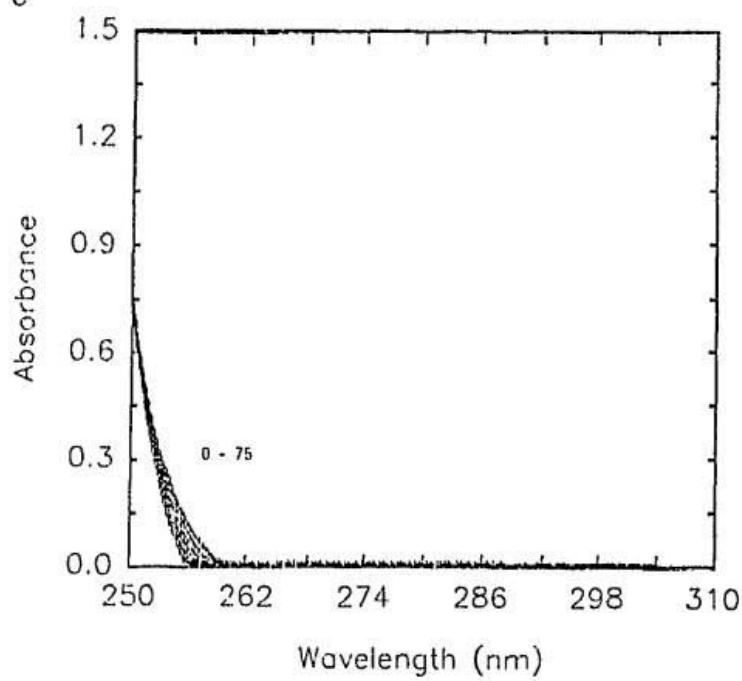

1.5
1.2
0.9
0.6
0.3
0.0
Absorbance
0 - 75
250
262
274
286
298
310
Wavelength (nm)

Table 2. **Absorbance change of MBA at different pHs when undergoing base-catalyzed hydrolysis and conjugation to cysteine.**

Time (min)	Absorbance at 285.5 nm due to base-catalyzed hydrolysis of MBA at pH 6.5	pH 7.0	pH 8.0	Absorbance at 260 nm due to conjugation of cysteine to MBA at pH 6.5	pH 7.0
0	0	0	0	0	0.0254
5	0	0	0	0	0.4601
10	0	0	0.1845	0.0425	0.7671
15	0	0	0.3460	0.1382	0.9891
20	0	0	0.4780	0.2374	1.1528
25	0	0	0.5888	0.3290	1.2738
30	0	0	0.6797	0.4110	1.3635
40	0	0.0175	0.8185	0.5568	1.4800
50	0	0.0808	0.9154	0.6831	1.5475
60	0	0.1402	0.9825	0.7887	1.5845
75	0	0.2247	1.0474	0.9168	1.6170
90	0.0104	0.2988	1.0860	1.0215	1.6332

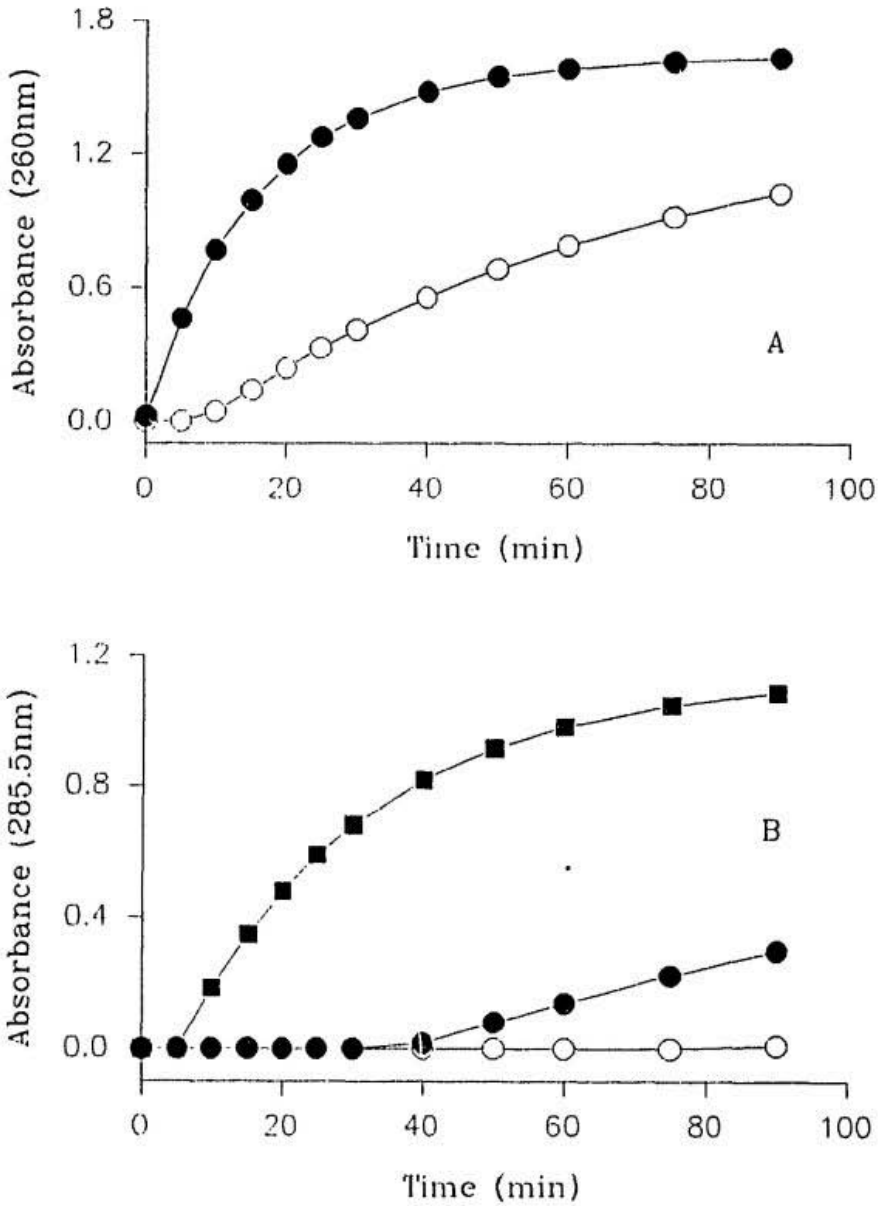


Figure 12. **Optimal condition of coupling reaction between MBA and thiol.** MBA (100 μM) was reacted with cysteine (450 μM) in 0.1 M phosphate buffer at pH 7.0(●) and 6.5 (O) (Panel A). MBA (100 μM) underwent hydrolysis in 0.1M phosphate buffer at pH 8.0 (■), 7.0 (●) and 6.5 (O).

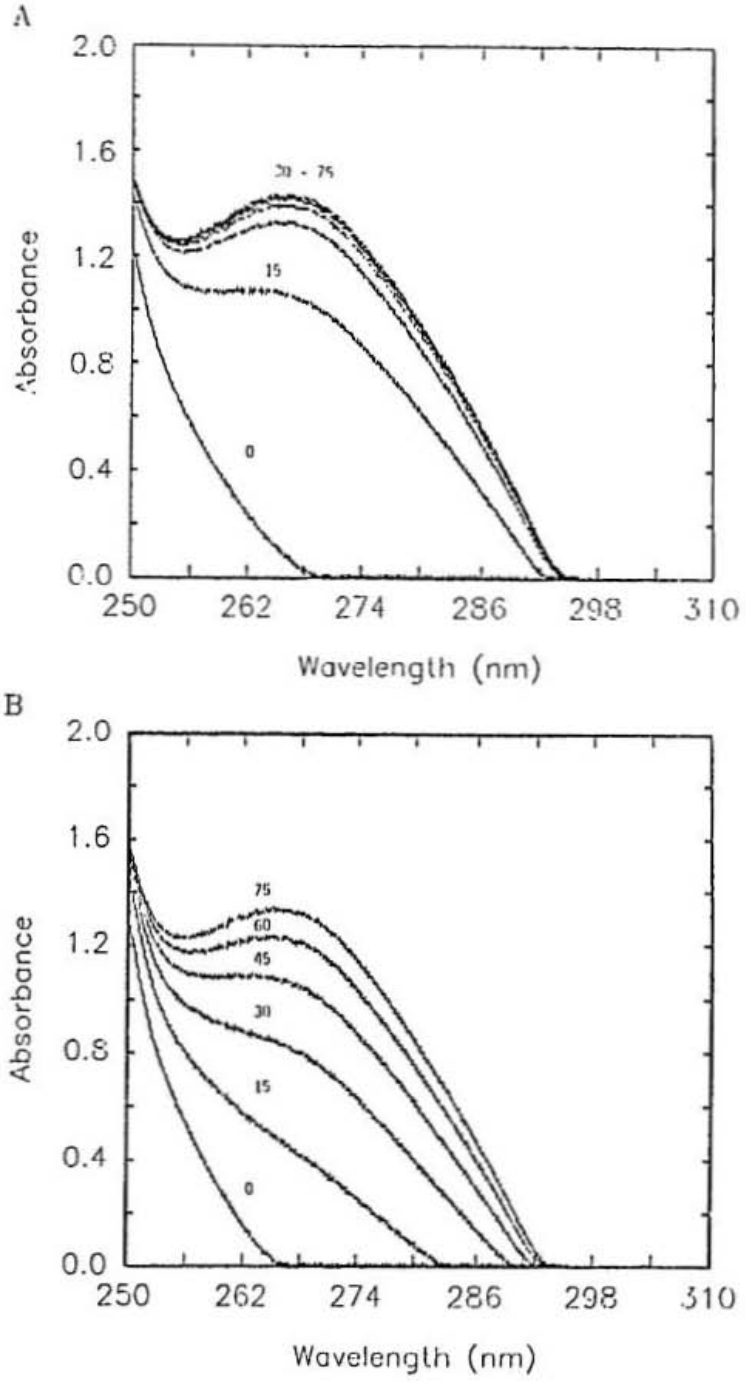


Figure 13. **Chromophoric change of MBCA reaction with free thiol.** Absorption spectra were repetitively scanned and recorded at 15 min. intervals for 1¼ h during cysteine-to-MBCA condensation. MBCA (100 μM) was incubated with excess cysteine (450 μM) in 0.1 M phosphate buffer at pH 7.0 (Panel A), pH 6.5 (Panel B). The number beside each line is the incubation time in minutes.

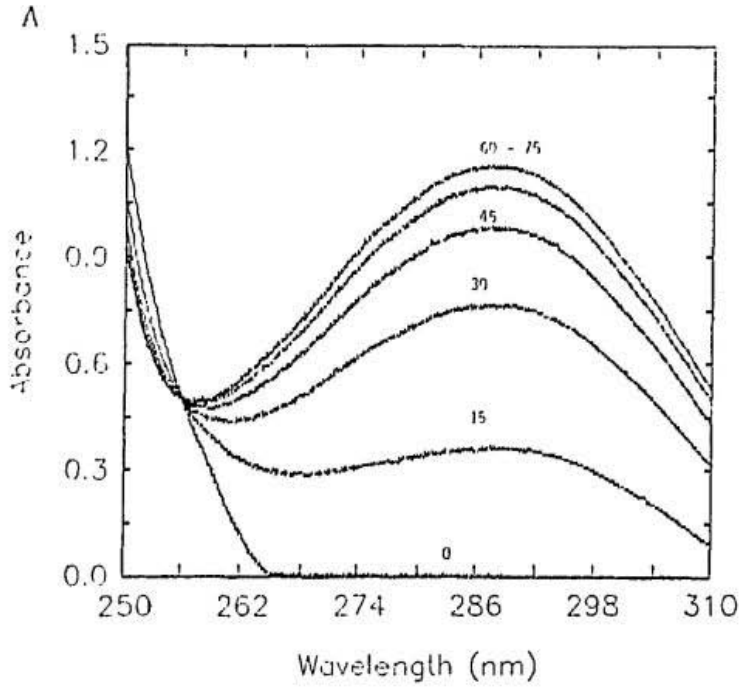
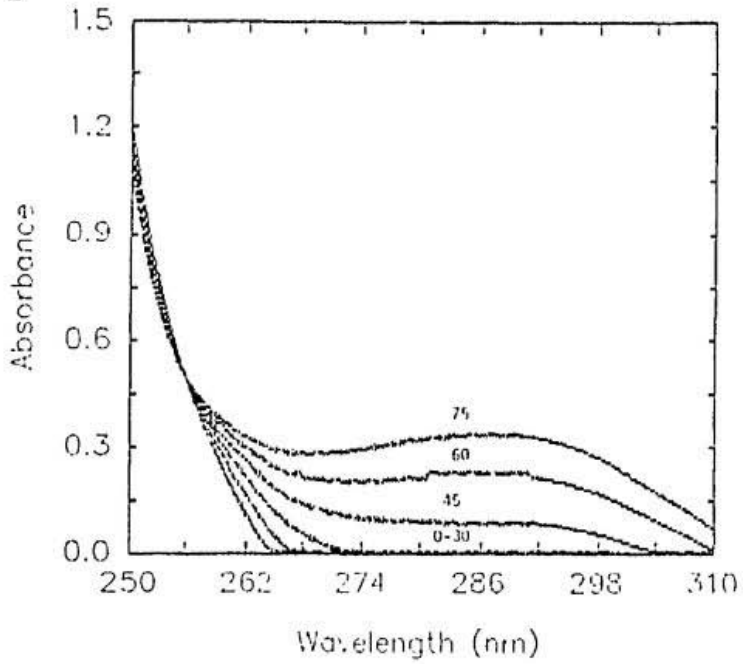


Figure 14. **Chromophoric change of the hydrolysis of MBCA.** Absorption spectra were repetitively scanned and recorded at 15 min. intervals for 1¼ h during the hydrolysis of MBCA. MBCA (100 μM) was incubated in 0.1 M phosphate buffer at pH 8.0 (Panel A), pH 7.0 (Panel B) and pH 6.5 (Panel C). The number beside each line is the incubation time in minutes. (Continued on next page).

B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1.2
0.9
0.6
0.3
0.0
Absorbance
75
60
45
0-30
250
262
274
286
298
310
Wavelength (nm)

C

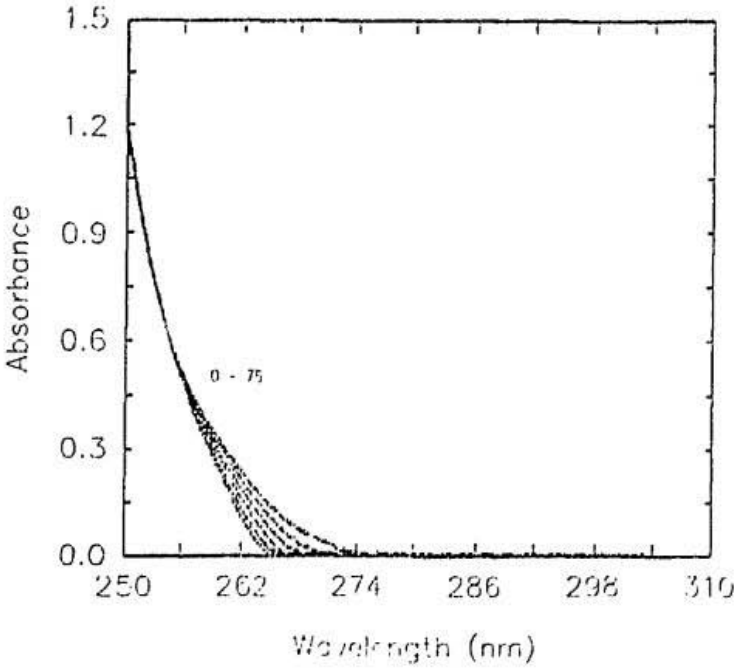

1.5
1.2
0.9
0.6
0.3
0.0
Absorbance
0 - 75
250
262
274
286
298
310
Wavelength (nm)

Table 3. **Absorbance change of MBCA at different pHs when undergoing base-catalyzed hydrolysis and conjugation to cysteine.**

Time	Absorbance at 285.5 nm due to base-catalyzed hydrolysis of MBCA at			Absorbance at 260 nm due to conjugation of cysteine to MBCA at	
(min)	pH 6.5	pH 7.0	pH 8.0	pH 6.5	pH 7.0
0	0	0	0	0.1587	0.1333
5	0	0	0	0.2857	0.5138
10	0	0	0.2812	0.4507	0.7635
15	0	0	0.4930	0.5811	0.9365
20	0	0	0.6630	0.6911	1.0317
25	0	0	0.7982	0.7860	1.1265
30	0	0	0.9061	0.8694	1.1778
40	0	0.0658	1.0647	0.9960	1.2332
50	0	0.1635	1.1621	1.0874	1.2610
60	0	0.2550	1.2272	1.1538	1.2745
75	0	0.3777	1.2828	1.2215	1.2832
90	0	0.4877	1.3130	1.2634	1.2910

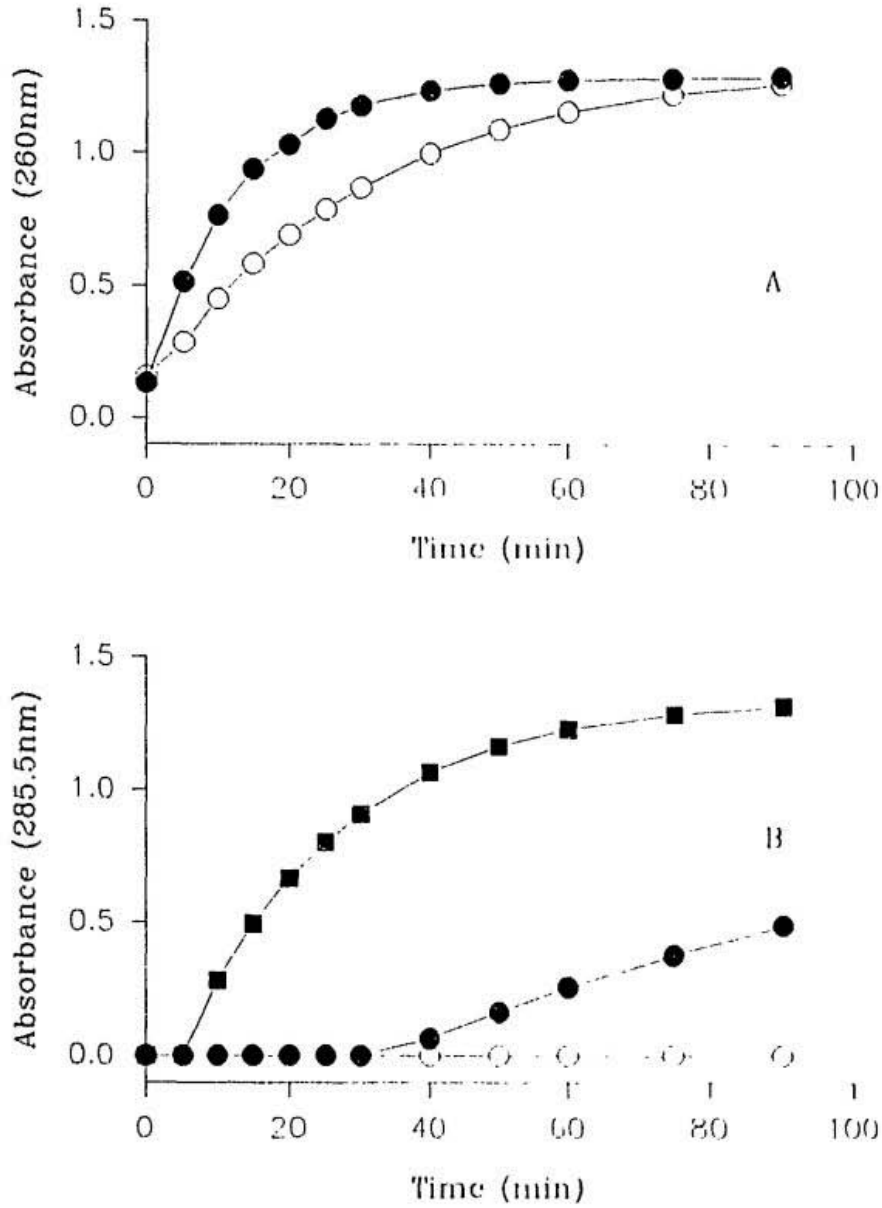


Figure 15. **Optimal condition of coupling reaction between MBCA and thiol.** MBCA (100 μM) was reacted with cysteine (450 μM) in 0.1 M phosphate buffer at pH 7.0(●) and 6.5 (○) (Panel A). MBCA (100 μM) underwent hydrolysis in 0.1 M phosphate buffer at pH 8.0 (■), 7.0 (●) and 6.5 (○).

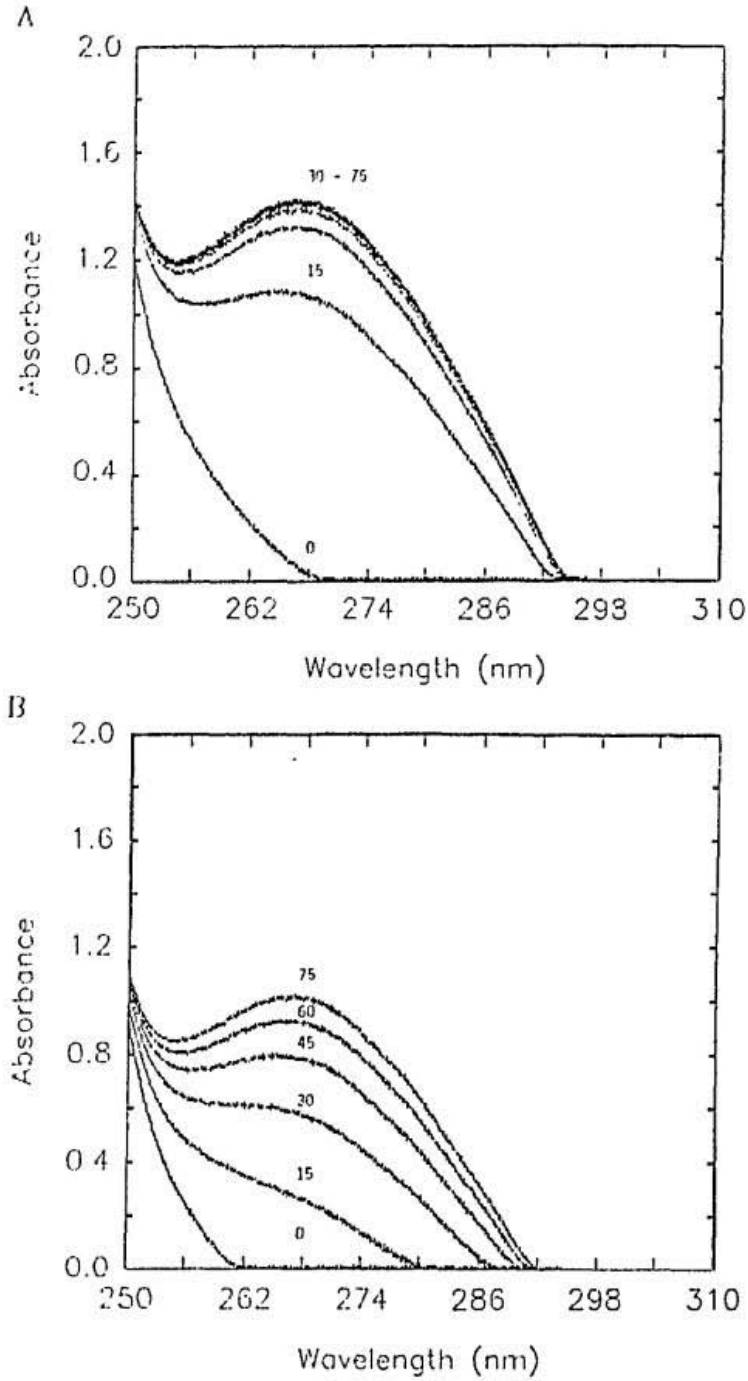


Figure 16. **Chromophoric change of MBUA reaction with free thiol.** Absorption spectra were repetitively scanned and recorded at 15 min. intervals for 1¼ h during cysteine-to-MBUA condensation. MBUA (100 μM) was incubated with excess cysteine (450 μM) in 0.1 M phosphate buffer at pH 7.0 (Panel A), pH 6.5 (Panel B). The number beside each line is the incubation time in minutes.

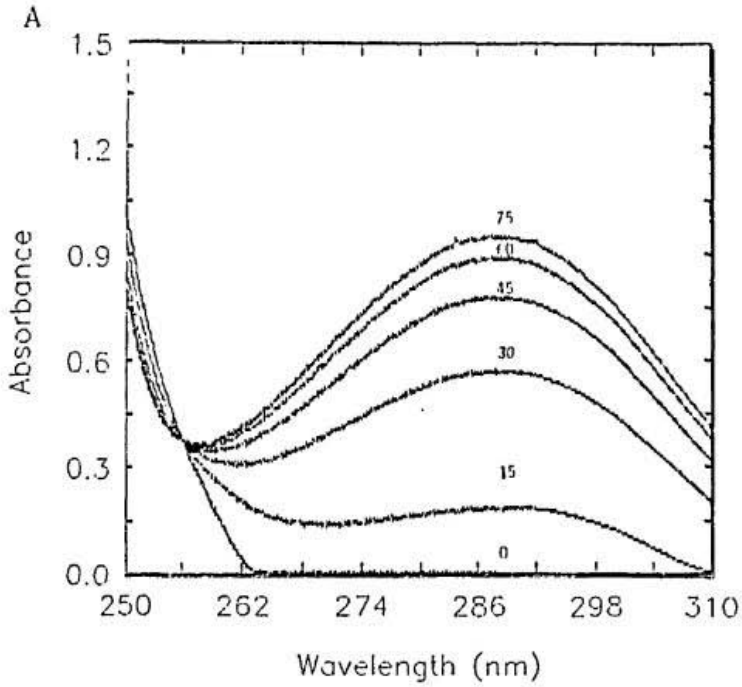
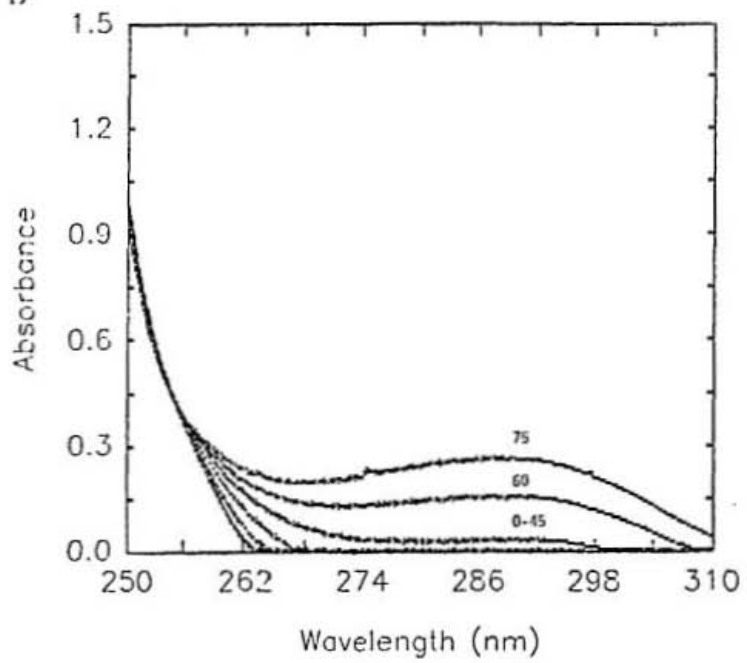


Figure 17. **Chromophoric change of the hydrolysis of MBUA.** Absorption spectra were repetitively scanned and recorded at 15 min. intervals for 1¼ h during the hydrolysis of MBUA. MBUA (100 μM) was incubated in 0.1 M phosphate buffer at pH 8.0 (Panel A), pH 7.0 (Panel B) and pH 6.5 (Panel C). The number beside each line is the incubation time in minutes. (Continued on next page).

B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Absorbance
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262
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286
298
310
Wavelength (nm)

C

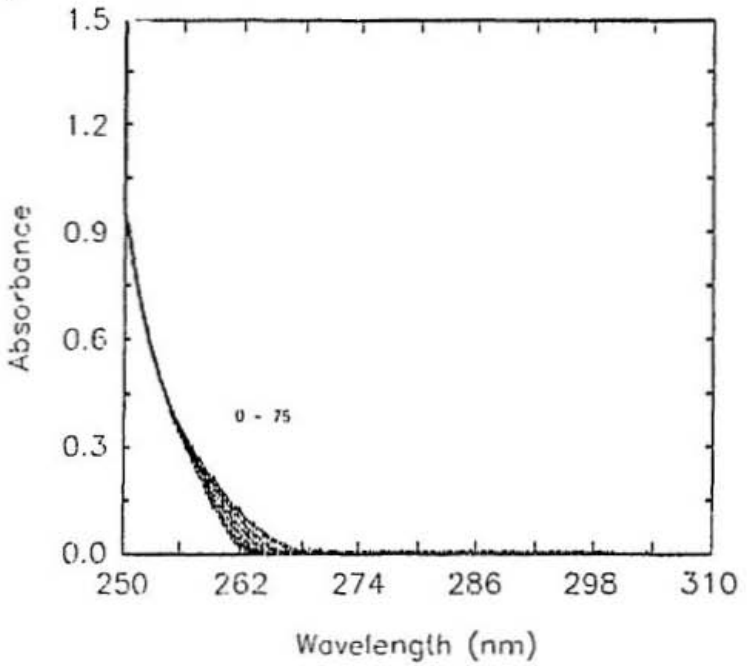
1.5
1.2
0.9
0.6
0.3
0.0
Absorbance
0 - 75
250
262
274
286
298
310
Wavelength (nm)

Table 4. **Absorbance change of MBUA at different pHs when undergoing base-catalyzed hydrolysis and conjugation to cysteine.**

Time	Absorbance at 285 nm due to base-catalyzed hydrolysis of MBUA at			Absorbance at 260 nm due to conjugation of cysteine to MBUA at	
(min)	pH 6.5	pH 7.0	pH 8.0	pH 6.5	pH 7.0
0	0	0	0	0.1718	0.3082
5	0.0062	0	0	0.3271	0.9868
10	0.0064	0	0.1984	0.4610	1.3121
15	0.0067	0	0.4398	0.5761	1.4857
20	0.0071	0	0.6082	0.6818	1.5812
25	0.0077	0	0.7270	0.7742	1.6322
30	0.0077	0	0.8121	0.8567	1.6664
40	0.0081	0	0.9187	0.9970	1.6992
50	0.0090	0.0041	0.9760	1.1042	1.7084
60	0.0092	0.0910	1.0067	1.1894	1.7111
75	0.0098	0.2141	1.0308	1.2808	1.7132
90	0.0107	0.3158	1.0441	1.3490	1.7161

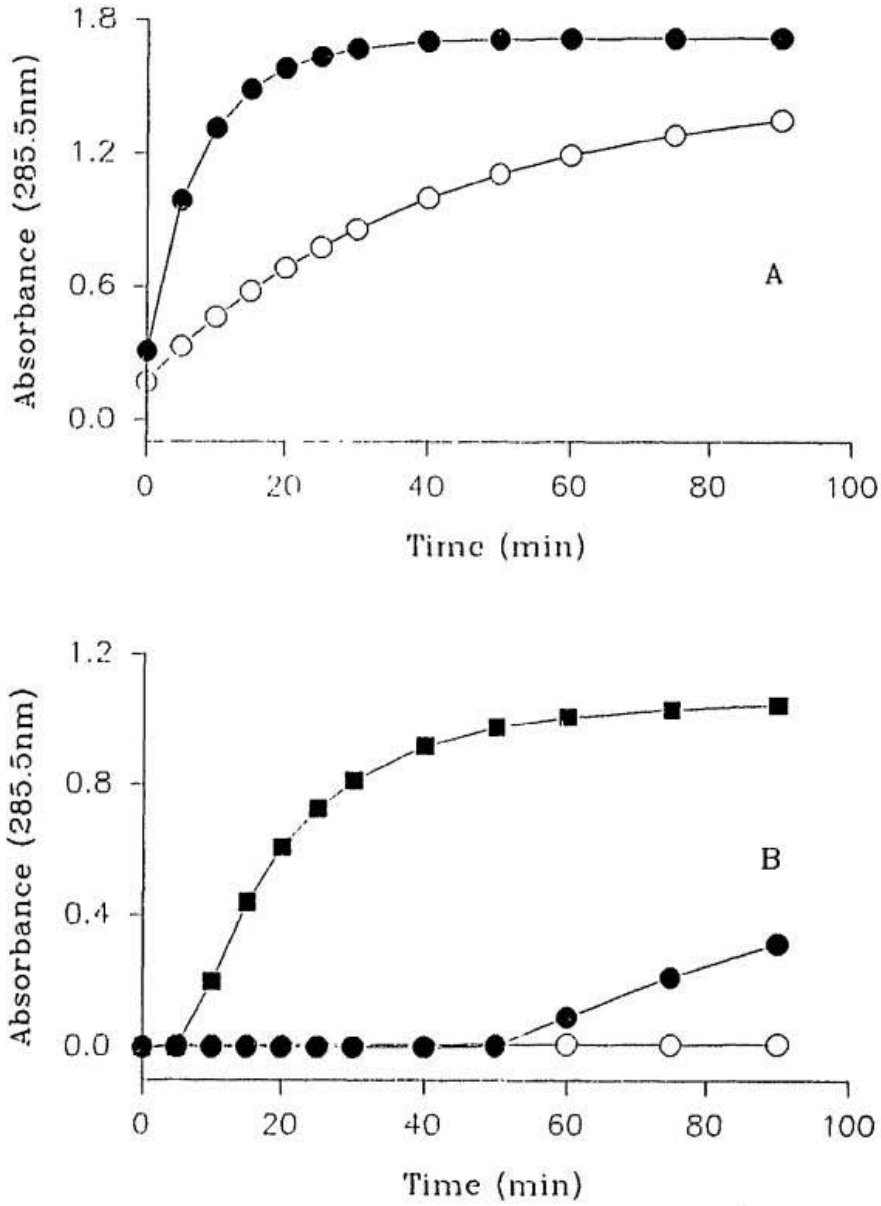

Figure 18. **Optimal condition of coupling reaction between MBUA and thiol.** MBUA (100 μM) was reacted with cysteine (450 μM) in 0.1 M phosphate buffer at pH 7.0(●) and 6.5 (○) (Panel A). MBUA (100 μM) underwent hydrolysis in 0.1 M phosphate buffer at pH 8.0 (■), 7.0 (●) and 6.5 (○).

The rate of chromophoric change is rapid at pH 8.0 and slower at pH 7.0 (Figures 12B, 15B and 18B), indicating that the maleimide is unstable and undergoes hydrolysis at alkaline pH and even at neutral pH. However, at pH 6.5, there is no chromophoric change, indicating that maleimide is stable in slightly acidic conditions.

III 3.2 Thiolation of 11-285-14

11-285-14 was thiolated by 2-iminothiolane (2-IT) (Figure 19). The number of thiol groups introduced on the MAb is highly correlated to the amount of 2-IT used (R-sq = 99.68%, $P < 0.0001$) (Figure 20).

The thiolation is a random process. The thiol groups may be added to the amino group of lysine residues anywhere in the antibody molecule, including in the antigen binding site. To confirm the binding activity of thiolated 11-285-14 to CEA, an antibody ELISA was performed. Figure 21 compares standard curves obtained for the thiolated 11-285-14 containing 3.35 - 13.5 SH/MAb and non-thiolated 11-285-14, confirming CEA binding had been retained although there is some reduction in binding seen after thiolation. The absorbance 0.5 unit and antibody concentration 12.27 ng/ml from the control curve was chosen as a reference point and all the points from other lines under the reference point were plotted out as a linear regression curve. The binding activity decreases as the

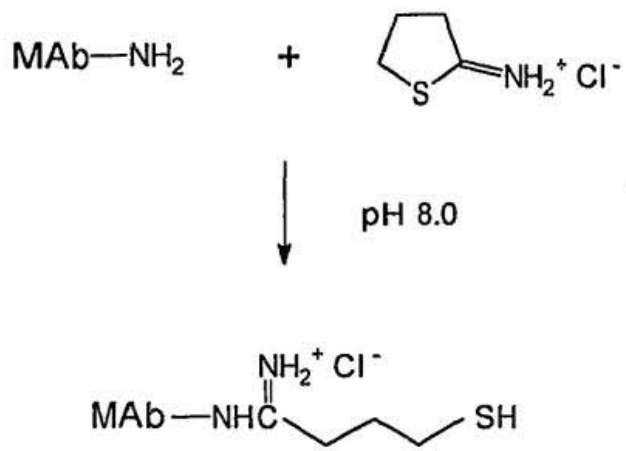

Figure 19. Thiolated MAb prepared by treatment with 2-Iminothiolane.

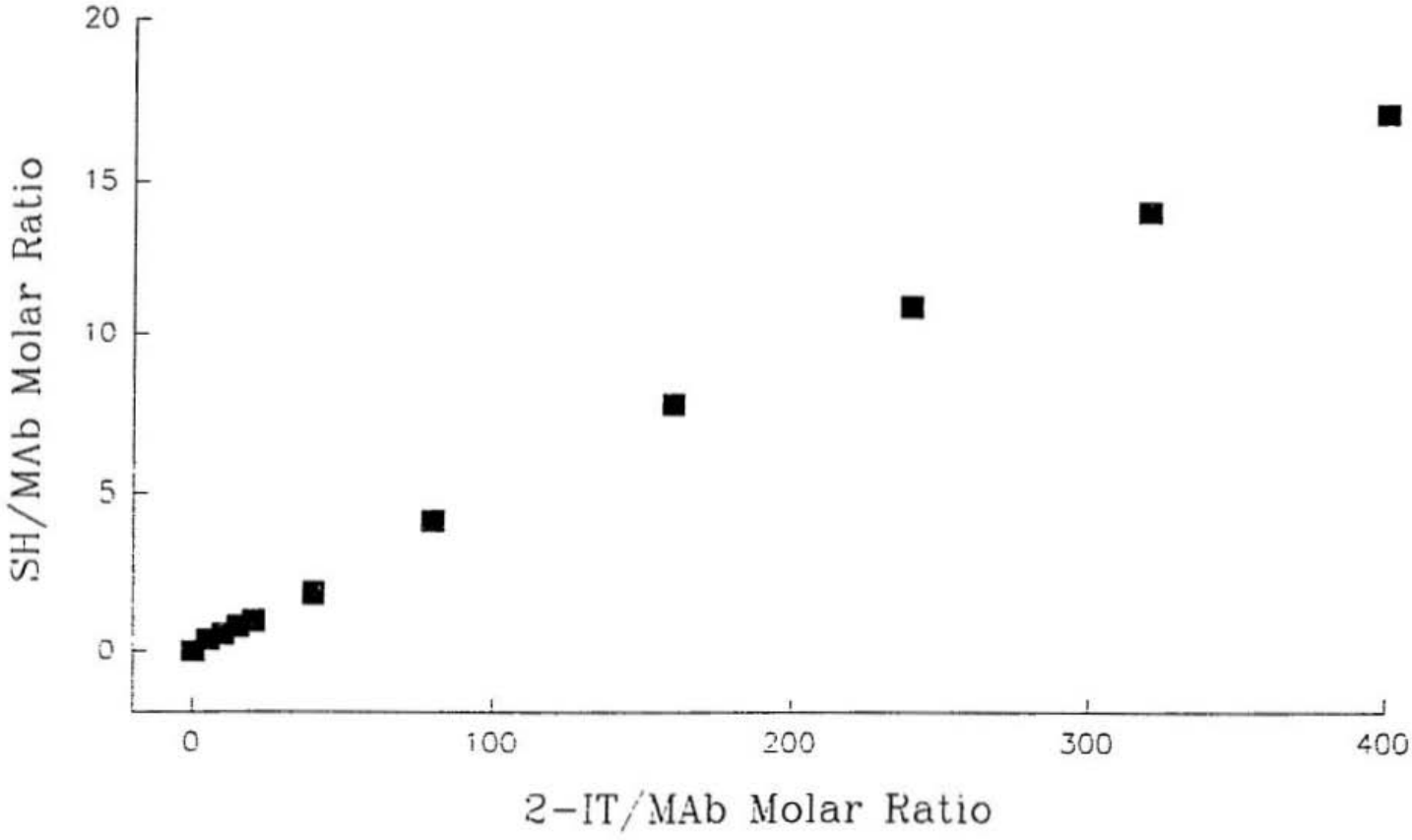

Figure 20. **Scattergram comparing the 2-IT:MAb molar ratio with the final SH:MAb molar ratio achieved after thiolation with 2-IT.**

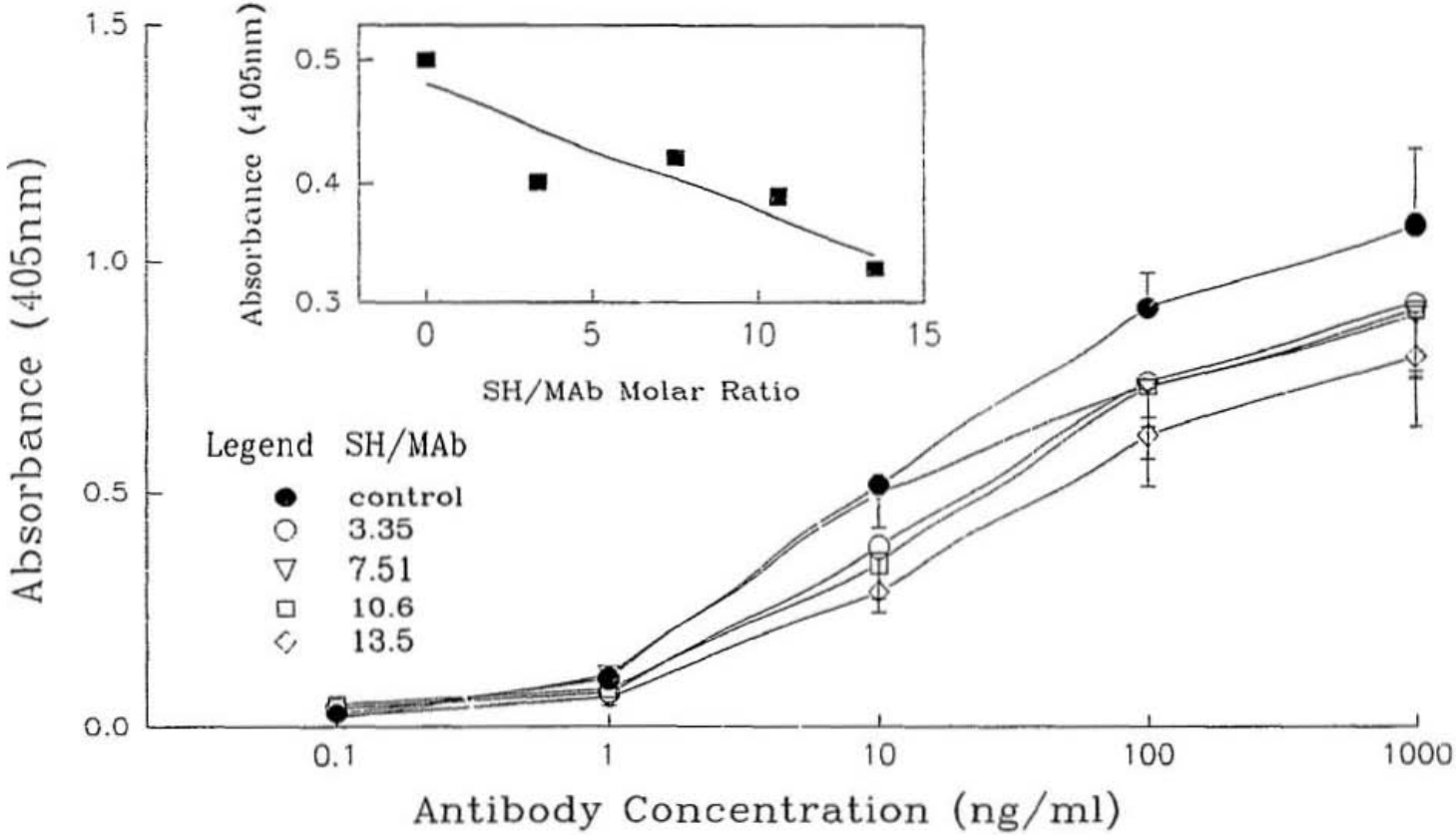

Figure 21. **Standard curves comparing CEA binding of thiolated 11-285-14 with parent 11-285-14 by ELISA (Mean ± S.D.).** Inset: linear regression curve of CEA binding of thiolated and non-thiolated 11-285-14 at antibody concentration 12.27 ng/ml.

substitution of thiol groups increases (R-sq = 78.74%, P =0.0446) (Figure 21, Inset).

III 3.3 Synthesis of Doxorubicin 11-285-14 Conjugates

The molar extinction coefficients of Dox maleimido derivatives are summarized in Table 5. These values were used for calculating the drug concentration and conjugation ratio.

Synthesis of the immunoconjugates was accomplished by thiolating 11-285-14 with 2-IT (Figure 19) and reacting the thiolated 11-285-14 with the Dox maleimido derivatives (Figures 22 and 23). The addition of 11-285-14 containing free thiols to Dox 3'-N-amide or Dox C-13 hydrazone derivatives led to the formation of thioether and amide bonds or hydrazone bonds between Dox and 11-285-14.

With the 2-IT/MAb molar ratio, 100, 6.62 ± 0.32 (S.D.) thiol groups were attached to MAbs with high protein yield (84-85%) (data not shown). 1.68 - 2.58 molecules of drug/molecule of MAb (Table 6) were achieved when 10 equivalents of Dox maleimido derivatives were added to thiolated 11-285-14. The final protein yields following conjugation of drug to MAb were 32 - 60% (Table 7). The total percentage of drug recovery was 3.01 to 7.05 (Table 8). This result showed that the whole process of drug incorporation to MAbs was not efficient and only a low drug recovery was obtained.

Table 5. **Molar extinction coefficient of Dox derivatives 7, 8, 9, 17 and 18.**

		Molar Extinction Coefficient			
compound	λ_{max} (nm)	ϵ	ϵ_{280}	ϵ_{495}	Solvent
7	267	16853	12508	12380	DMF
8	267	16248	11413	13236	DMF
9	267	16017	11754	13061	DMF
17	268	26320	18510	12531	DMF
18	268	16028	12315	13051	DMF
IgG	280	214600			PBS

Dox 3'-N-amide derivatives (7, 8 and 9)

Dox C-13 hydrazone derivatives (17 and 18)

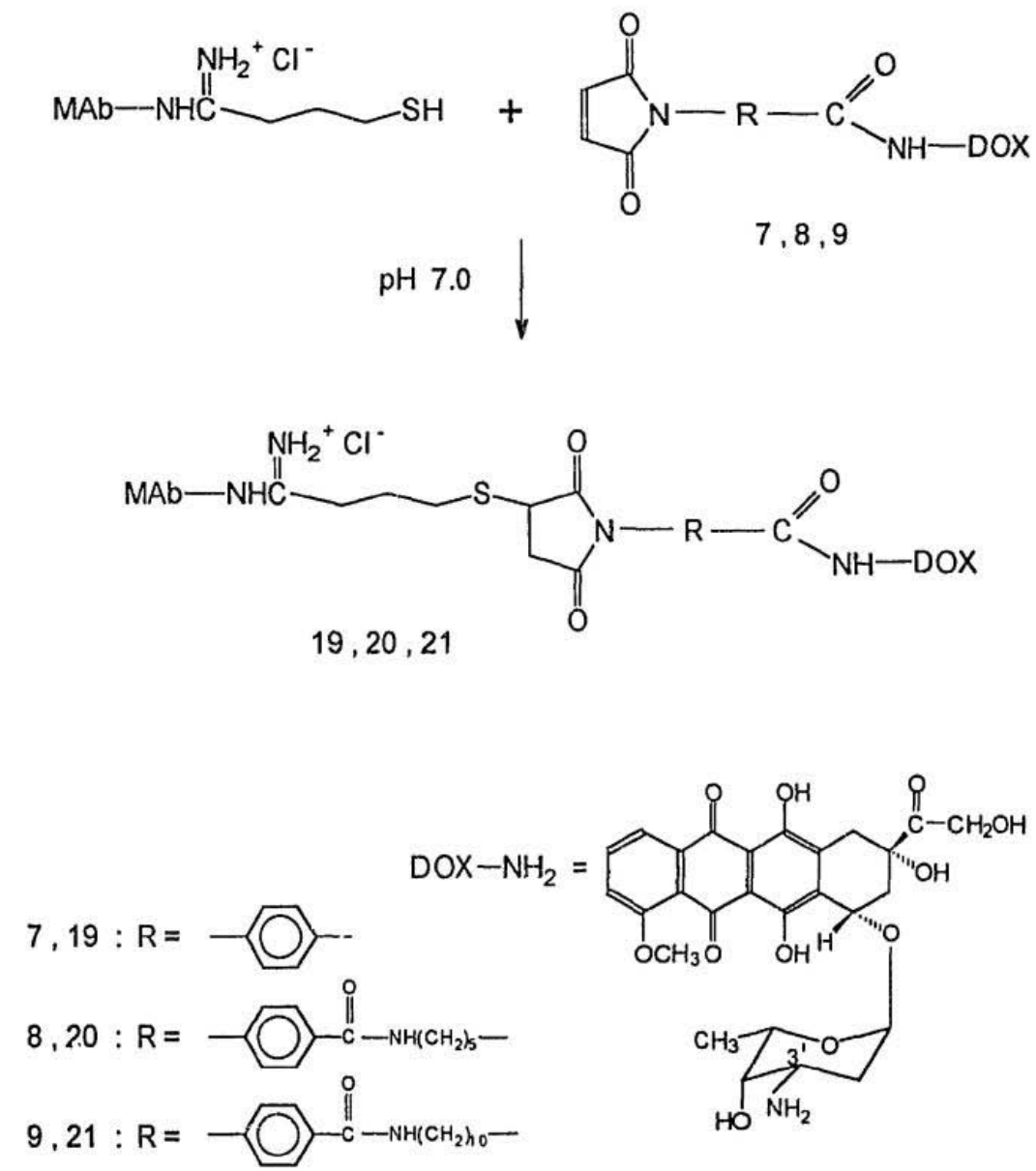

Figure 22. **Synthesis of Dox immunoconjugates by reaction of 2-IT thiolated MAbs with Dox 3'-N-amide derivatives.** Condensation of the Dox derivatives with thiolated MAb leads to formation of thioether bonds with MAb and N-acyl bonds at the 3'-NH_2 position of Dox.

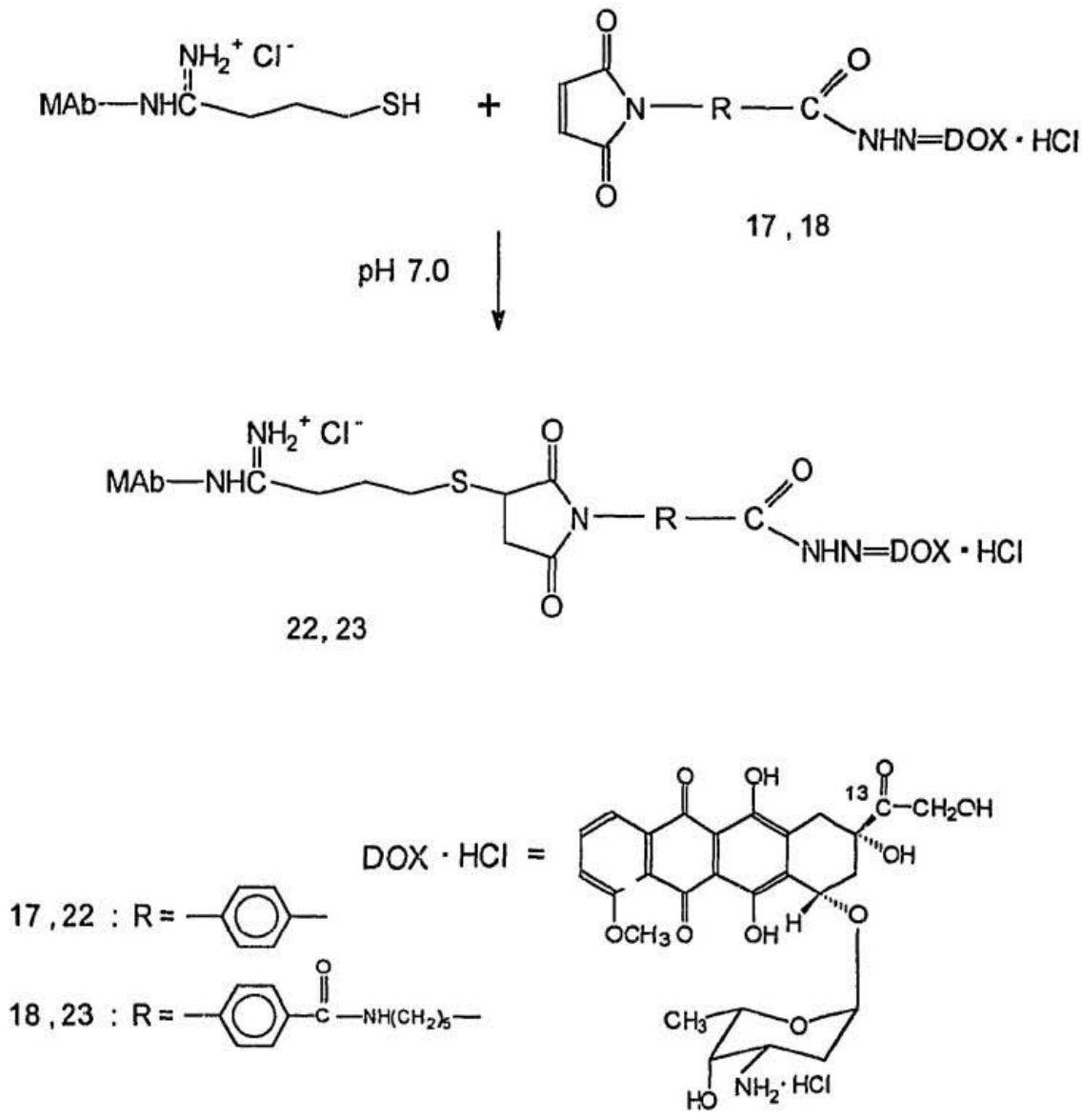

Figure 23. **Synthesis of Dox immunoconjugates by reaction of 2-IT thiolated MAbs with Dox C-13 hydrazone derivatives.** Condensation of the Dox derivatives with thiolated MAb leads to formation of thioether bonds with MAb and C-13 hydrazone bonds at the C-13 position of Dox.

Table 6. **Spectrophotometric evaluation of the Dox-11-285-14 Conjugates**

Conjugate number	absorbance of the conjugate 280nm	495nm	absorbance of the drug content 280nm	protein content 280nm	drug (μM)	antibody (μM)	conjugation ratio drug/antibody
19	1.82	0.24	0.24	1.58	19.01	7.38	2.58
20	2.10	0.24	0.21	1.89	18.33	8.79	2.09
21	1.13	0.11	0.10	1.04	8.11	4.84	1.68
22	1.41	0.15	0.23	1.18	12.32	5.50	2.24
23	1.16	0.14	0.13	1.02	10.82	0.71	2.27

Table 7. **Protein recovery after conjugation reaction**

Conjugate number	SH/MAb	protein used for coupling (mg)	protein recovered after coupling (mg)	% yield
19	7.14	23.38	10.51	45
20	6.55	23.24	13.84	60
21	6.26	18.00	5.81	32
22	6.55	14.58	5.36	37
23	6.59	14.04	4.65	33

Table 8. **Drug recovery after coupling and conjugation reaction**

Conjugate number	percentage recovery of drug after coupling to linker	conjugation reaction	total percentage recovery
19	41	11.61	4.76
20	51	12.54	6.40
21	56	5.38	3.01
22	85	8.29	7.05
23	90	7.49	6.74

III 3.4 *In Vitro* Efficacy

Figures 24 and 25 show the binding activity for immunoconjugates. Dox-MBA and Dox-MBCA conjugates retained most of their binding activity compared to Dox-MBUA conjugate. Dox-MBH and Dox-MBCH conjugates lost some of their binding activity compared to parent 11-285-14.

Dox-MBA, Dox-MBCA, Dox-MBUA, Dox-MBH and Dox-MBCH derivatives and their immmunoconjugates were evaluated *in vitro* for cytotoxicity using the MTT assay. The cell lines, LS174T, which is sensitive to Dox and has high expression of CEA, and COLO320DM, which is also sensitive to Dox and has low expression of CEA, (Ford et al, 1987a; Richardson et al, 1989) were used for these assays. The mean absorbances with the standard deviation for the assays are presented in Tables 9 to 14. The dose-response curves are presented in Figures 26 to 31. Percentage survival of cells is shown along the ordinate and drug, drug derivatives and immunoconjugates, using a logarithmic scale, along the abscissa.

All Dox 3'-N-amide derivatives do not show significant cytotoxic effect on the LS174T cell line when compared to Dox (Figure 26). The Dox-MBA derivative showed the highest cytotoxicity and caused 50% cell survival at ~20 μM while Dox-MBCA caused 50% cell survival at ~40 μM. Dox-MBUA demonstrated no appreciable cytotoxicity and only showed 30% inhibition of cell growth at 40 μM. However, the Dox

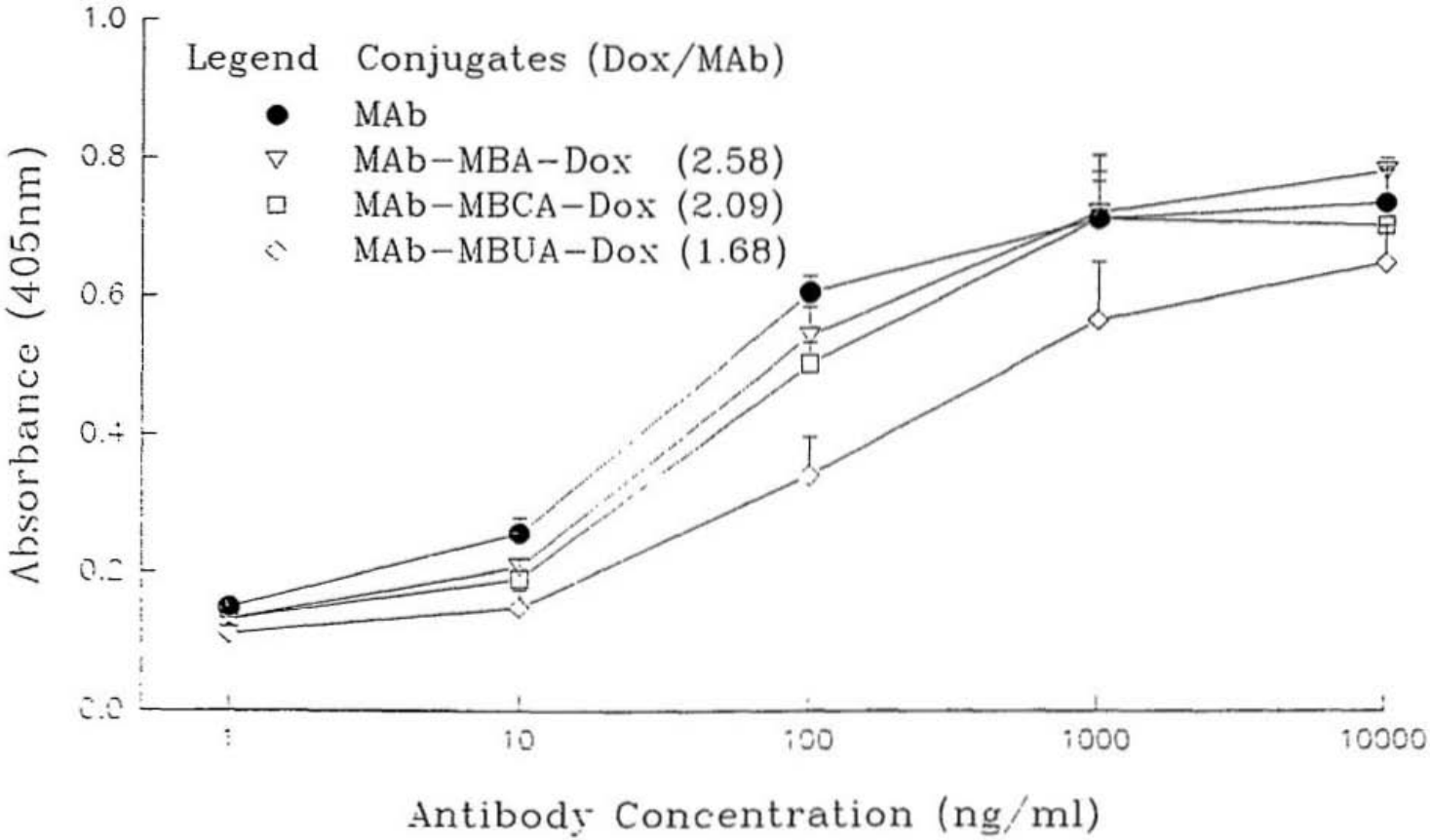

Figure 24. **Standard curves comparing CEA binding of Dox 3'-N-amide immunoconjugates with parent 11-285-14 by ELISA (Mean ± S.D.).**

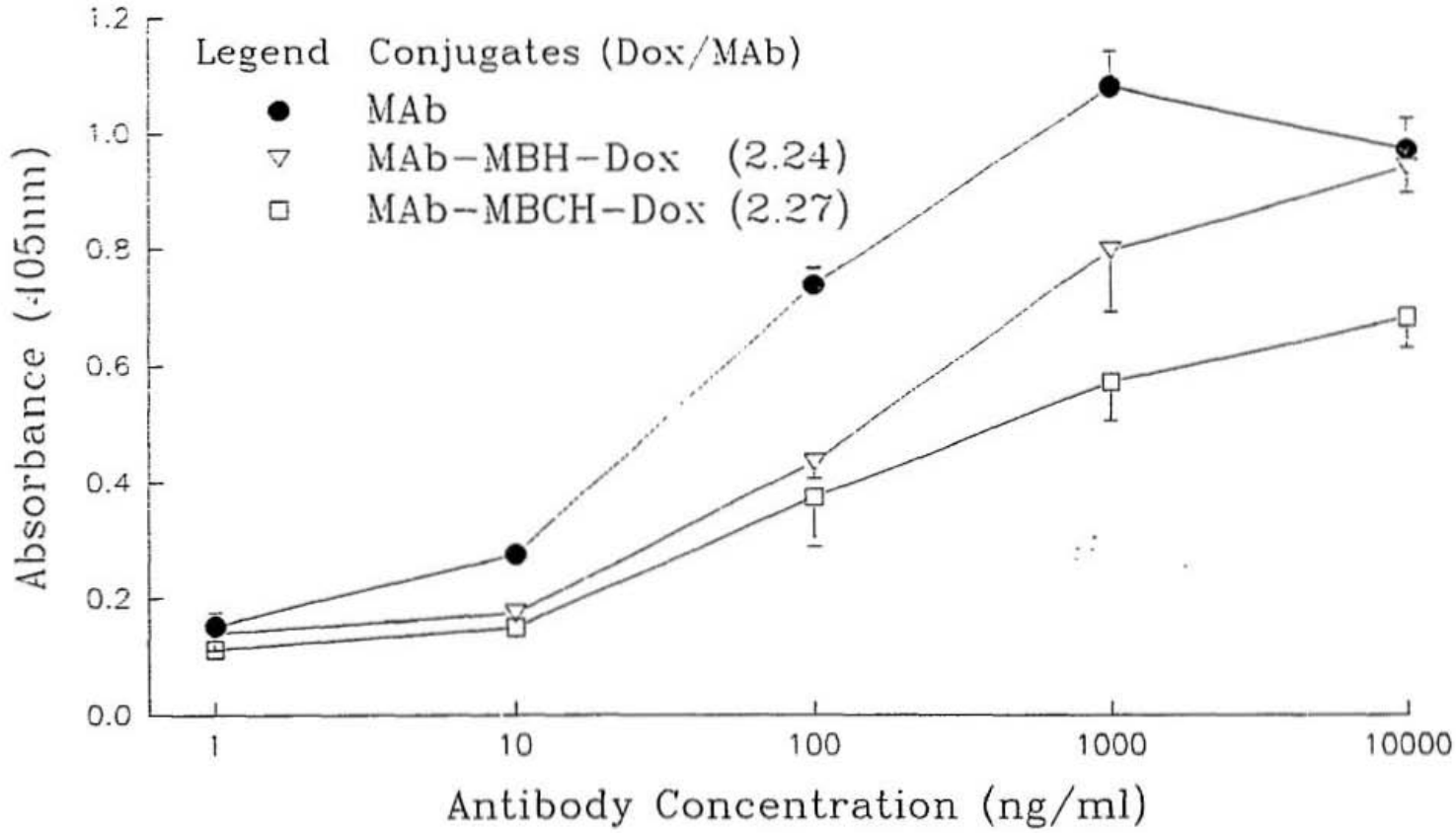

Figure 25. **Standard curves comparing CEA binding of Dox C-13 hydrazone immunoconjugates with parent 11-285-14 by ELISA (Mean ± S.D.).**

Table 9. **Absorbance obtained from MTT assay following 24 hour exposure of LS174T cells to Dox and Dox 3'-N-amide derivatives (Standard Deviation, S.D.)**

	Doxorubicin		Doxorubicin Derivatives					
Doxorubicin	Mean	Percentage	Mean Absorbance			Percentage of Survival		
Concentration	Absorbance	of Survival	MBA	MBCA	MBUA	MBA	MBCA	MBUA
(μM)	(S.D.)	(S.D.)		(S.D.)			(S.D.)	
40	0.059	5.75	0.673	0.461	0.509	42.92	49.60	69.87
	(0.006)	(0.54)	(0.083)	(0.088)	(0.029)	(5.31)	(9.50)	(4.03)
20	0.066	6.49	0.855	1.054	0.707	47.49	65.79	76.67
	(0.032)	(0.31)	(0.068)	(0.140)	(0.143)	(3.77)	(8.74)	(15.48)
10	0.068	6.68	1.347	1.366	0.896	65.52	71.71	92.63
	(0.006)	(0.61)	(0.309)	(0.221)	(0.043)	(15.04)	(11.59)	(4.47)
5.0	0.088	8.63	1.641	1.408	1.015	87.96	85.95	105.29
	(0.008)	(0.83)	(0.193)	(0.141)	(0.072)	(10.32)	(8.63)	(7.51)
2.5	0.193	18.95	1.506	1.553	1.151	95.60	88.31	97.82
	(0.015)	(1.44)	(0.383)	(0.121)	(0.071)	(24.30)	(6.90)	(6.07)

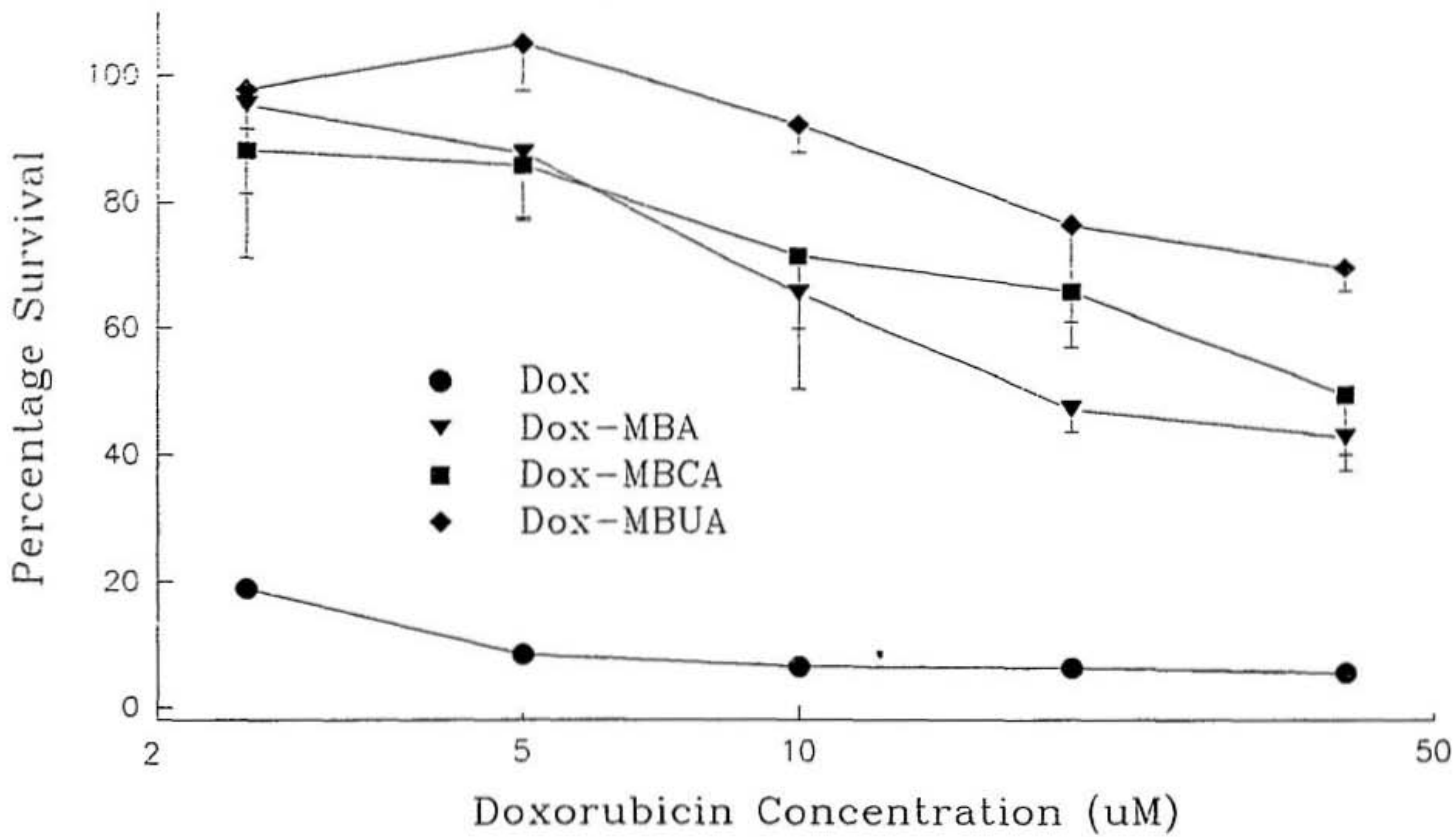

Figure 26. **Dose-Response curves for Dox and Dox 3'-N-amide derivatives on cell line LS174T (mean ± S.D.).**

Table 10. **Absorbance obtained from MTT assay following 24 hour exposure of LS174T cells to Dox and Dox C-13 hydrazone derivatives (Standard Deviation, S.D.)**

	Doxorubicin		Doxorubicin Derivatives			
Doxorubicin Concentration (μM)	Mean Absorbance (S.D.)	Percentage of Survival (S.D.)	Mean Absorbance (S.D.) MBH	Mean Absorbance (S.D.) MBCH	Percentage of Survival (S.D.) MBH	Percentage of Survival (S.D.) MBCH
40	0.059	5.75	0.061	0.039	5.95	5.82
	(0.006)	(0.54)	(0.008)	(0.005)	(0.74)	(0.82)
20	0.066	6.49	0.091	0.045	8.97	6.75
	(0.032)	(0.31)	(0.015)	(0.007)	(1.49)	(1.05)
10	0.068	6.68	0.102	0.061	10.05	9.14
	(0.006)	(0.61)	(0.011)	(0.013)	(1.03)	(1.99)
5.0	0.088	8.63	0.177	0.079	17.37	11.79
	(0.008)	(0.83)	(0.010)	(0.003)	(0.97)	(0.47)
2.5	0.193	18.95	0.308	0.113	30.25	16.83
	(0.015)	(1.44)	(0.036)	(0.003)	(3.55)	(0.37)

1.25	0.354	34.79	0.587	0.194	57.70	28.88
	(0.054)	(5.27)	(0.072)	(0.025)	(7.10)	(3.71)
0.625	0.546	53.64	0.706	0.300	69.39	44.70
	(0.053)	(5.24)	(0.053)	(0.014)	(5.17)	(2.08)
0.313	0.680	66.81	0.832	0.344	81.80	51.31
	(0.104)	(10.18)	(0.155)	(0.036)	(15.24)	(5.39)
0.156	0.899	88.34	0.999	0.419	98.19	62.57
	(0.101)	(9.92)	(0.060)	(0.063)	(5.87)	(9.46)

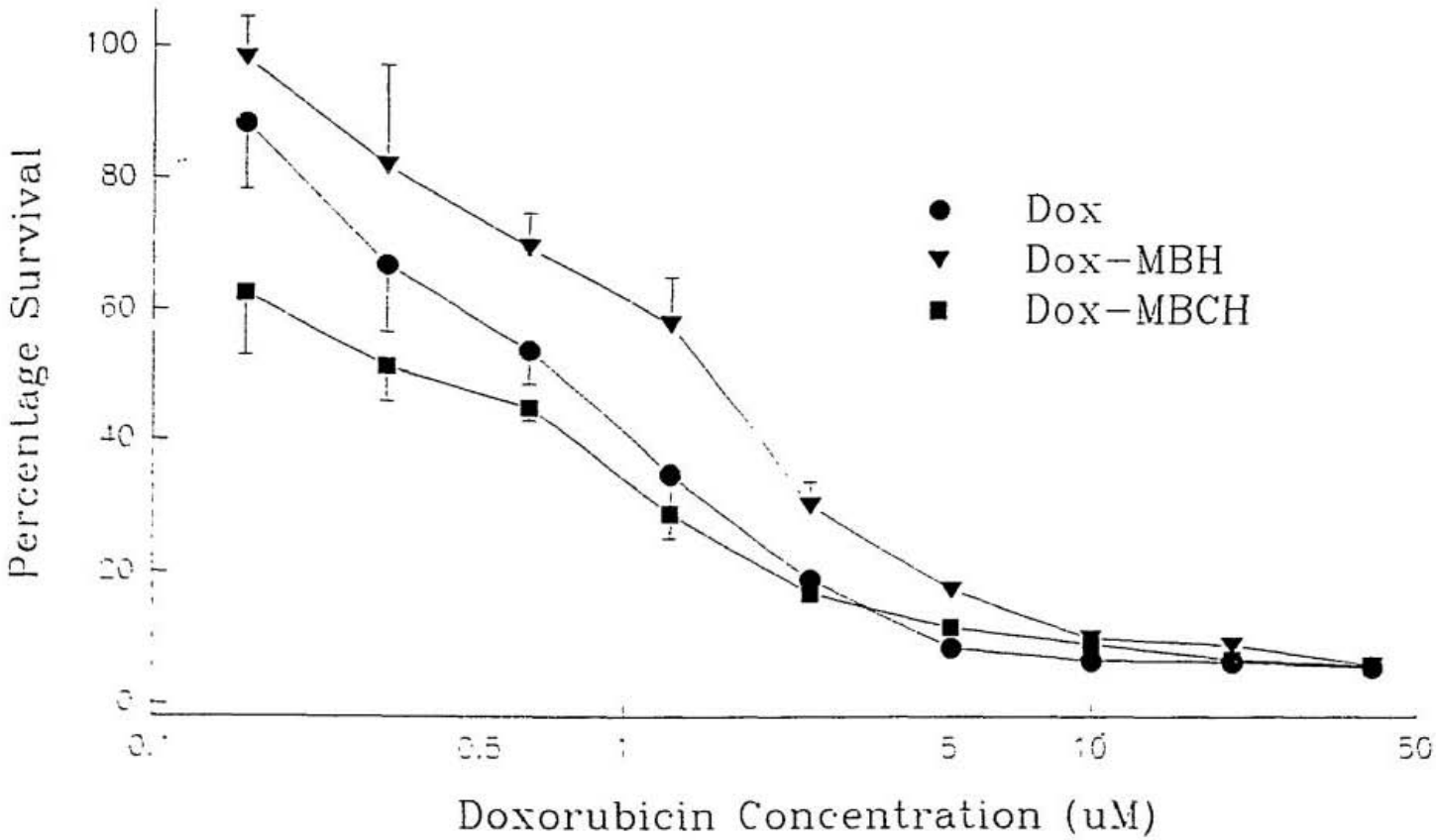

Figure 27. **Dose-Response curves for Dox and Dox C-13 hydrazone derivatives on cell line LS174T (mean ± S.D.).**

Table 11. **Absorbance obtained from MTT assay following 24 hour exposure of COLO320DM cells to Dox and Dox C-13 hydrazone derivatives (Standard Deviation, S.D.)**

	Doxorubicin		Doxorubicin Derivatives			
Doxorubicin Concentration (μM)	Mean Absorbance (S.D.)	Percentage of Survival (S.D.)	Mean Absorbance MBH (S.D.)	Mean Absorbance MBCH (S.D.)	Percentage of Survival MBH (S.D.)	Percentage of Survival MBCH (S.D.)
40	0.025	13.46	0.028	0.040	15.09	18.51
	(0.003)	(1.36)	(0.006)	(0.002)	(3.06)	(0.69)
20	0.034	18.35	0.035	0.041	18.76	18.74
	(0.002)	(0.82)	(0.001)	(0.003)	(0.70)	(1.15)
10	0.033	17.67	0.039	0.038	20.94	17.36
	(0.003)	(1.69)	(0.003)	(0.002)	(1.63)	(0.69)
5.0	0.075	40.78	0.076	0.063	41.46	28.85
	(0.022)	(12.15)	(0.004)	(0.011)	(2.10)	(4.97)
2.5	0.112	60.90	0.110	0.123	59.82	56.44
	(0.027)	(14.64)	(0.022)	(0 025)	(11.70)	(11.36)
1.25	0.165	89.60	0.149	0.177	81.02	81.38
	(0.046)	(25.10)	(0.011)	(0.005)	(6.07)	(2.31)

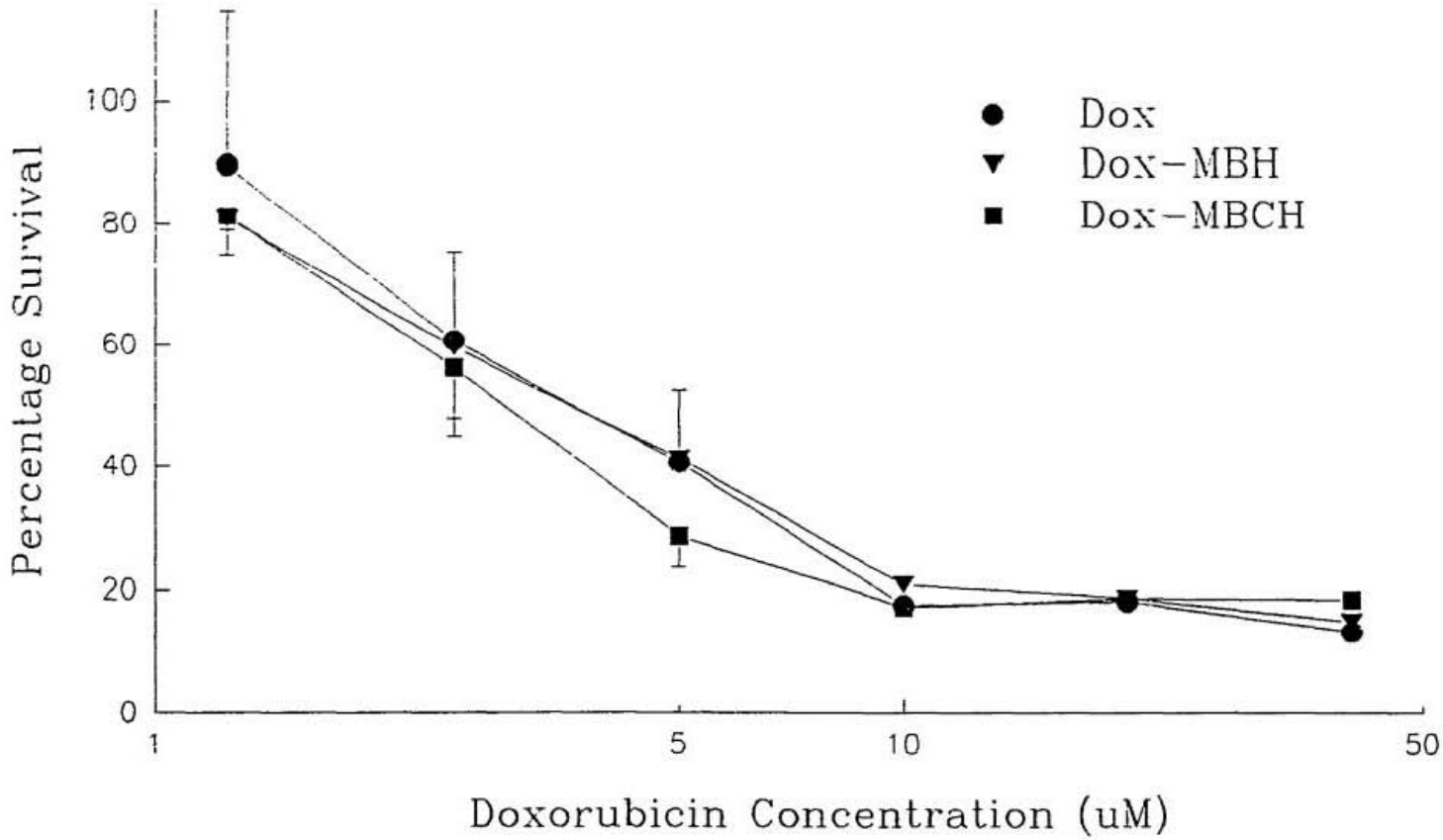

Figure 28. **Dose-Response curves for Dox and Dox C-13 hydrazone derivatives on cell line COLO320DM (mean ± S.D.).**

Table 12. **Absorbance obtained from MTT assay following 24 hour exposure of LS174T cells to Dox and Dox 3′-N-amide immunoconjugates (standrad deviation, S.D.)**

Doxorubicin Concentration (μM)	Doxorubicin Mean Absorbance (S.D.)	Doxorubicin Percentage of Survival (S.D.)	Conjugate Mean Absorbance (S.D.)			Conjugate Percentage of Survival (S.D.)		
			MBA	MBCA	MBUA	MBA	MBCA	MBUA
10	0.037	5.75	0.621	0.472	---*	96.17	73.33	---*
	(0.004)	(0.55)	(0.067)	(0.064)	---*	(10.30)	(9.92)	---*
5.0	0.023	3.57	0.584	0.603	0.619	90.33	93.59	77.50
	(0.003)	(0.49)	(0.062)	(0.072)	(0.115)	(9.57)	(11.20)	(14.43)
2.5	0.041	6.41	0.631	0.506	0.717	97.72	78.61	89.74
	(0.012)	(1.89)	(0.054)	(0.039)	(0.093)	(8.29)	(6.03)	(11.62)
1.25	0.056	8.73	0.704	0.549	0.760	109.02	85.25	95.15
	(0.011)	(1.65)	(0.060)	(0.070)	(0.095)	(9.33)	(10.92)	(11.82)
0.625	0.221	34.28	0.716	0.598	0.734	110.87	92.78	91.90
	(0.050)	(7.68)	(0.058)	(0.040)	(0.060)	(8.90)	(6.18)	(7.51)

* insufficient concentration in *in vitro* testing

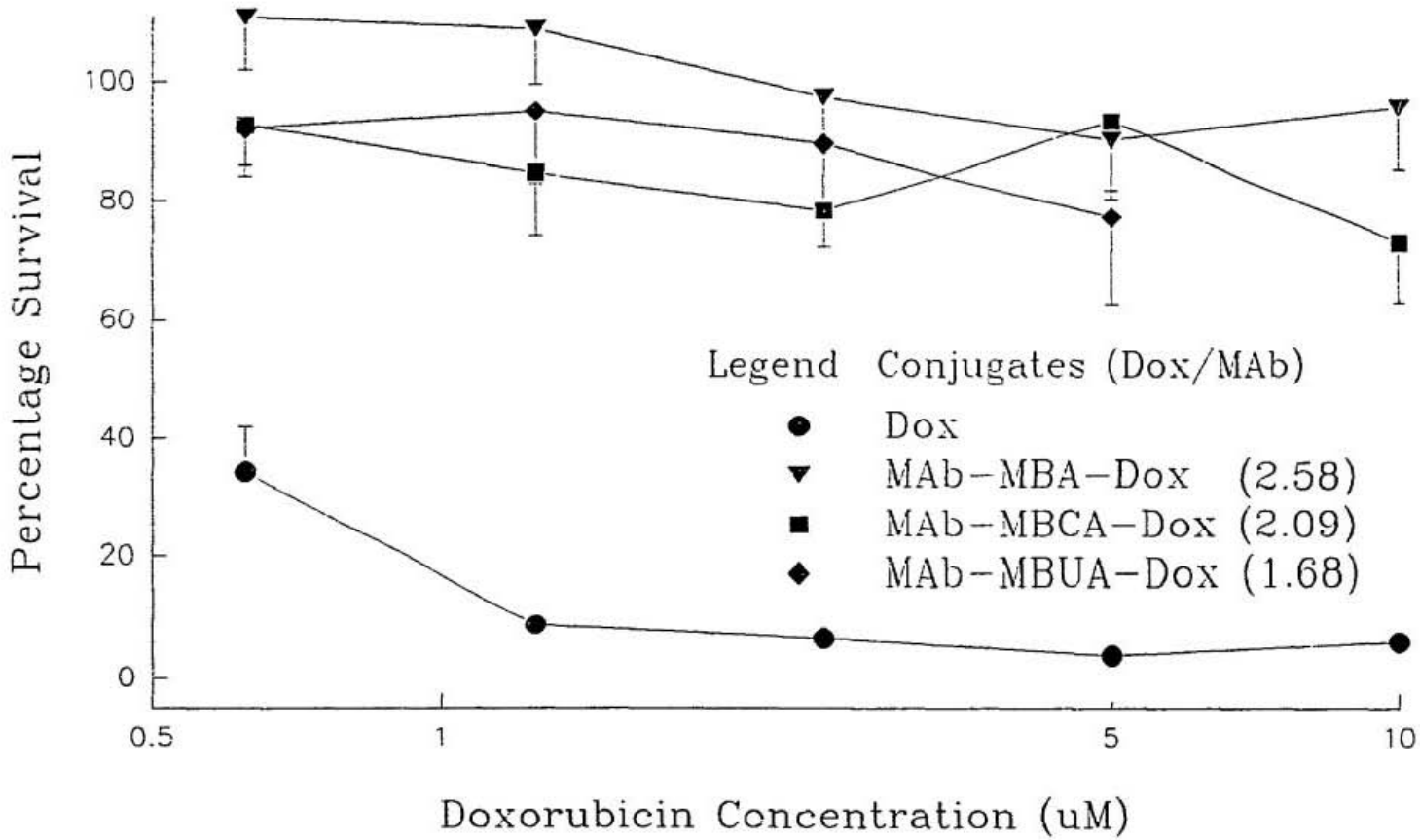

Figure 29. **Dose-Response curves for Dox and Dox 3'-N-amide immunoconjugates on cell line LS174T (mean ± S.D.).**

Table 13. **Absorbance obtained from MTT assay following 24 hour exposure of LS174T cells to Dox and Dox C-13 hydrazone immunoconjugates (Standard Deviation, S.D.)**

	Doxorubicin		Doxorubicin Derivatives			
Doxorubicin	Mean	Percentage	Mean Absorbance		Percentage of Survival	
Concentration	Absorbance	of Survival	MBH	MBCH	MBH	MBCH
(μM)	(S.D.)	(S.D.)	(S.D.)		(S.D.)	
8.0	0.058	8.62	0.517	0.428	52.48	46.00
	(0.003)	(0.49)	(0.010)	(0.030)	(0.97)	(3.17)
4.0	0.079	11.72	0.726	0.589	73.65	63.29
	(0.010)	(1.45)	(0.090)	(0.062)	(9.09)	(6.69)
2.0	0.120	17.84	0.682	0.696	69.18	74.87
	(0.013)	(1.90)	(0.073)	(0.039)	(7.38)	(4.18)
1.0	0.226	33.77	0.878	0.780	89.08	83.85
	(0.003)	(0.46)	(0.086)	(0.064)	(8.68)	(6.87)
0.5	0.350	52.28	0.915	0.852	92.91	91.57
	(0.022)	(3.21)	(0.083)	(0.085)	(8.39)	(9.16)

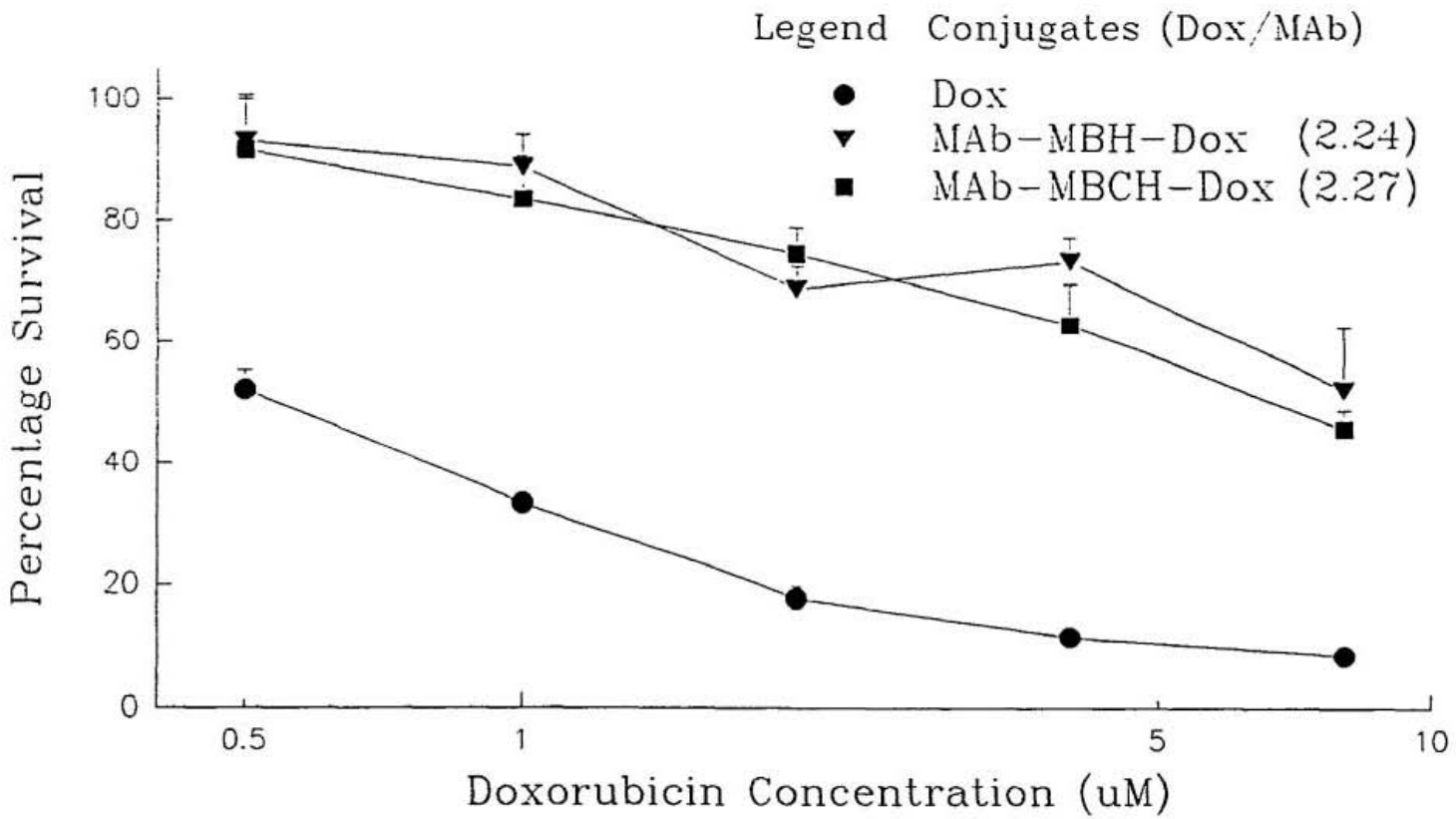

Figure 30. **Dose-Response curves for Dox and Dox C-13 hydrazone immunoconjugates on cell line LS174T (mean ± S.D.).**

Table 14. **Absorbance obtained from MTT assay following 24 hour exposure of COLO320DM cells to Dox and Dox C-13 hydrazone immunoconjugates (Standard Deviation, S.D.)**

	Doxorubicin		Doxorubicin Derivatives			
Doxorubicin Concentration (μM)	Mean Absorbance (S.D.)	Percentage of Survival (S.D.)	Mean Absorbance (S.D.) MBH	Mean Absorbance (S.D.) MBCH	Percentage of Survival (S.D.) MBH	Percentage of Survival (S.D.) MBCH
8.0	0.040	13.91	0.170	0.270	99.40	102.77
	(0.005)	(1.84)	(0.052)	(0.057)	(30.20)	(21.67)
4.0	0.070	24.56	0.179	0.319	104.36	121.60
	(0.017)	(5.90)	(0.046)	(0.050)	(26.81)	(19.14)
2.0	0.138	48.13	0.163	0.309	95.03	117.69
	(0.029)	(10.16)	(0.027)	(0.037)	(15.95)	(13.99)
1.0	0.170	59.48	0.151	0.237	88.03	90.51
	(0.033)	(11.63)	(0.030)	(0.052)	(17.33)	(19.96)
0.5	0.177	61.97	0.165	0.276	96.41	105.39
	(0.021)	(7.39)	(0.025)	(0.065)	(14.58)	(24.95)

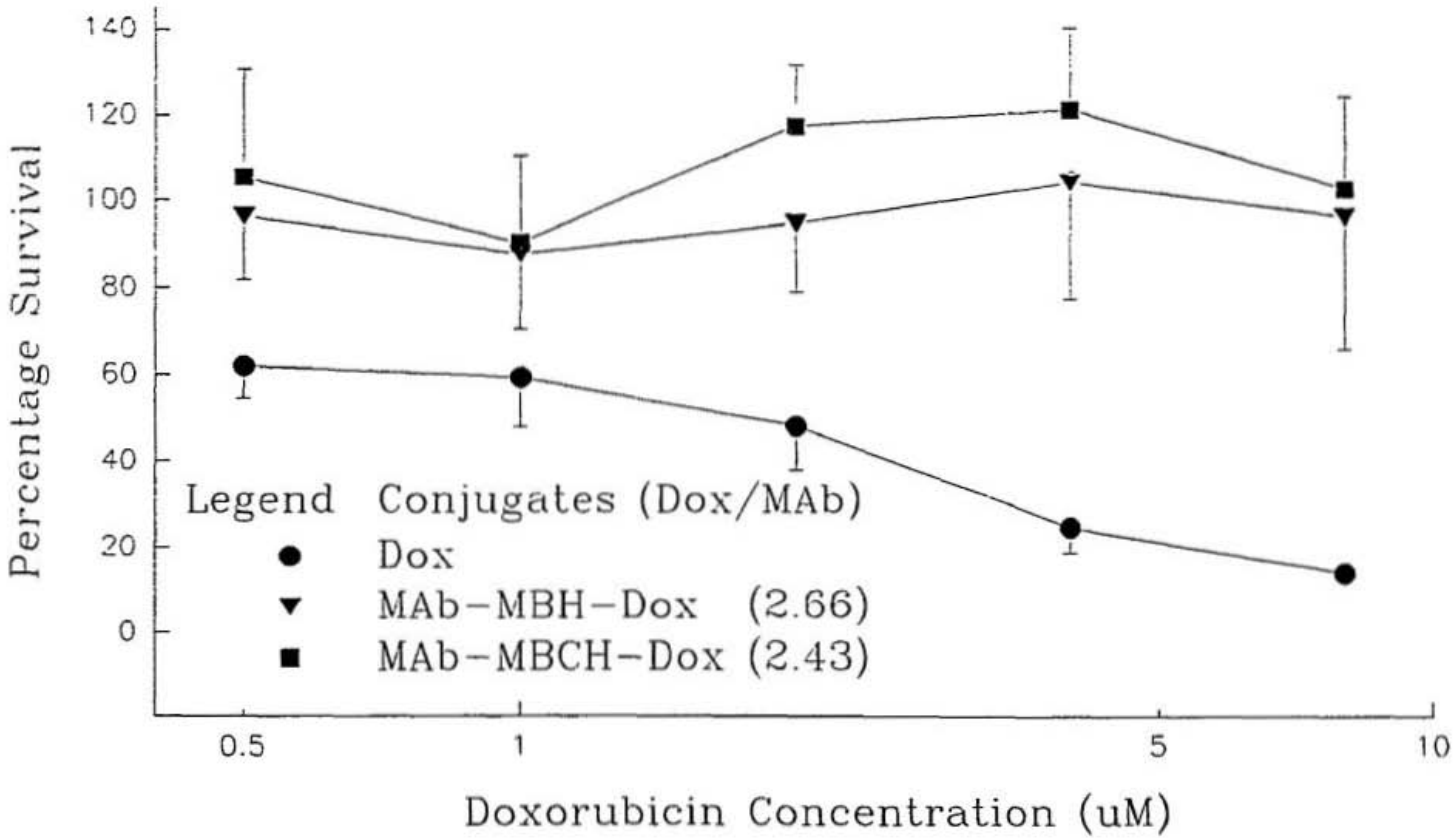

Figure 31. **Dose-Response curves for Dox and Dox C-13 hydrazone immunoconjugates on cell line COLO320DM (mean ± S.D.).**

hydrazone derivatives showed similar cytotoxic effects when compared to Dox on both LS174T and COLO320DM cell lines (Figures 27 and 28). The LS174T cell line showed higher sensitivity to Dox and Dox hydrazone derivatives than the COLO320DM cell line.

Immunoconjugates synthesized from Dox 3'-N-amide derivatives were not sufficiently cytotoxic to effect a 50% inhibition of growth of LS174T cell line (Figure 29). These immunoconjugates exhibited no cytotoxicity even at 10 μM. However, the immunoconjugates prepared from Dox C-13 hydrazone derivatives were sufficiently cytotoxic to achieve 50% inhibition of LS174T cell growth at ~8 μM (Figure 30) and had no appreciable cytotoxic effects on the COLO320DM cell line (Figure 31).

III 3.5 SDS-PAGE Analysis of Antibody and Immunoconjugates

The results of SDS-PAGE analysis of Dox 3'-N-amide and Dox C-13 hydrazone immunoconjugates are shown in Figures 32 and 33 respectively. In both figures, the parent 11-285-14 in the gel (Lane 2) shows one major band which corresponds to the antibody monomer at the position 150,000 KDa and one minor band which presumably corresponds to an impurity. For the gel with Dox 3'-N-amide immunoconjugates (Figure 32), lanes 3 to 5 showing thiolated 11-285-14 with 7.14, 6.55 and 6.26 SH/MAb respectively contain at least two other minor bands of lower

molecular mass in comparison to parent 11-285-14. These bands may be antibody subunits. Lanes 7, 8 and 9, containing immunoconjugates 19, 20 and 21 respectively, show a series of faint bands corresponding in positions to higher molecular masses than antibody monomer. These bands indicate that covalent cross-linking between antibody had occurred. For the gel containing Dox C-13 hydrazone immunoconjugates (Figure 33), lanes 3 and 4 show thiolated 11-285-14 with 6.55 and 6.59 SH/MAb respectively; the faint bands at higher molecular mass in comparsion to parent 11-285-14 (Lane 2) indicate that covalent cross-linking between thiolated antibody had occured. The Dox-MBH and Dox-MBCH immunoconjugates (22 and 23) on lanes 6 and 7 showed a similar pattern to thiolated antibody.

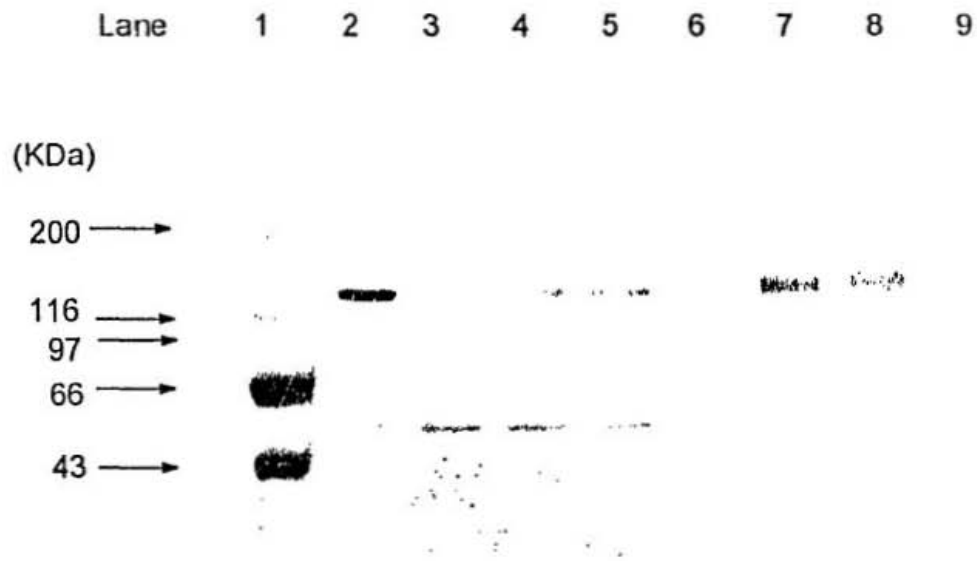

Figure 32. **SDS-PAGE of thiolated 11-285-14 and Dox 3'-N-amide immunoconjugates showing extra PAGE bands, and control, parent 11-285-14.** Lanes are: lane 1, high molecular weight protein standard; lane 2, parent 11-285-14; lanes 3, 4 and 5, thiolated 11-285-14 containing 7.14, 6.55 and 6.26 SH/antibody respectively; lanes 7, 8 and 9, containing immunoconjugates 19, 20 and 21 respectively.

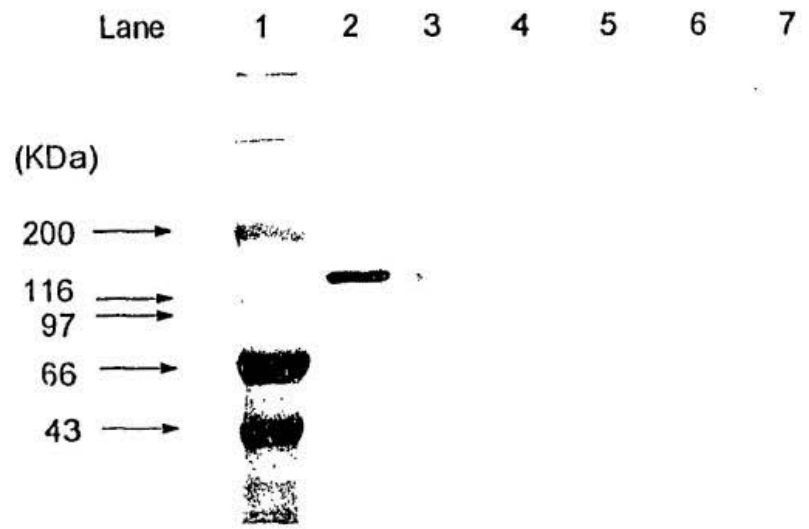

Figure 33. **SDS-PAGE of thiolated 11-285-14 and Dox C-13 hydrazone immunoconjugates showing extra PAGE bands, and control, parent 11-285-14.** Lanes are: lane 1, high molecular weight protein standard; lane 2, parent 11-285-14; lanes 3 and 4, thiolated 11-285-14 containing 6.55 and 6.59 SH/antibody respectively; lanes 6 and 7, containing immunoconjugates 22 and 23 respectively.

III 4.0 DISCUSSION

III 4.1 Introduction

Antibody mediated targeting (AMT) is an attractive approach in chemotherapy. The selectivity of MAb to TAA may attain site-specific drug delivery. However, the major problem in AMT is how to produce drug-MAb conjugates by a reliable method. The conventional methods using EDCI and glutaraldehyde caused a serious side reaction in polymerization of antibodies and drugs. Recently, efforts to investigate the usefulness of AMT have led to the development of a variety of methods and reagents for protein-drug covalent cross-linking. The use of heterobifunctional cross-linking reagents permits the conjugation reaction in a stepwise manner and permits the additional characterization of conjugate. The principle aim of the studies in this thesis was to produce novel cross-linking reagents, to link them to Dox, to characterize them and then to undertake preliminary biological evaluation of them.

Prior to assessing the efficacy of targeted chemotherapy using monoclonal antibody drug immunoconjugates, it is essential to evaluate the various components, target, carrier and toxic agent, of the targeting system. An advantage in the use of drugs as cytotoxic agents is that considerable information has been gained on their use in the treatment of cancer. Dox is one of the most commonly used anticancer

agents in chemotherapy. It is effective for a wide range of cancer, but its use is limited by non-specific toxicity to normal tissues. Thus, Dox is very useful as a toxic agent for AMT. The characterisation of the target, human tumor cell lines expressing the tumor associated antigen CEA, and the carrier, the monoclonal anti-CEA antibody 11-285-14, were well established in the Oncology Research Laboratory. The potential of targeted chemotherapy using vindesine 11-285-14 conjugates had previously been demonstrated *in vitro* and *in vivo* (Casson et al, 1987; Ford et al, 1987b). Preliminary results with Dox-11-285-14 conjugates had also shown efficacy *in vitro* (Richardson et al, 1989). Thus, carrying this work a step further, we investigated the efficacy of Dox-11-285-14 conjugates using novel heterobifunctional spacers.

IV 4.2 pH Optimization of Maleimide Reactions

The heterobifunctional cross-linking reagents containing maleimide are widely used in drug-protein conjugation. The coupling condition of thiol to maleimide is pH dependent. At higher pH, the deprotonated thiol is a strong nucleophilic species for Michael addition to the double bond of maleimide. However, maleimide is susceptible to base-catalyzed hydrolysis in aqueous solution and the product maleamic acid will not react with the cysteine thiol (Gregory, 1955). To understand the extent of the conjugation of MAb to maleimido doxorubicin

derivatives, we characterized the stability and reactivity of the spacers (**3**, **4** and **5**) at several pHs using a spectrophotometric method. These experimental results may be useful to optimize the conjugation reaction between drug and antibody.

The spacers (**3**, **4** and **5**) absorb ultraviolet light in a spectrum characteristic of phenylmaleimide chromophore. Their absorbance maxima are 246 - 249 nm. When maleimides were added to an excess of cysteine at pH 7.0 and 6.5, coupling occured cleanly with the smooth conversion of the major phenylmaleimide absorption into a new chromophore centered at 262 - 266 nm (Figures 10, 13 and 16). As expected in a reaction in which the deprotonated thiol is a strong nucleophilic species, the rate of coupling, cysteine-to-spacer condensation, was slower at pH 6.5 and more rapid at pH 7.0 (Table 2, 3 and 4, and Figure 12A, 15A and 18A). At pH 7.0, more deprotonated thiols exist for Michael addition. Neither MBA nor MBUA showed the same chromophoric change at pH 7.0 and 6.5 within a 1.5 h incubation time. It may suggest that less maleimide reacts with cysteine thiol at pH 6.5.

We next investigated the rate of hydrolysis of the spacers at several pH values. The spectra, Figure 11, 14 and 17, clearly indicate the chromophore of the newly formed maleamic acid, 284 - 286 nm, during hydrolysis at different pHs and the results are summmarized in Tables 2, 3 and 4, and

Figures 12B, 15B and 18B. At pH 6.5 an aqueous solution of maleimide did not show appreciable absorption in the range 270 - 310 nm and no absorbance developed within 1.5h incubation time. The maleimide was stable at pH 6.5. However, after incubation at pH 8.0, strong absorbance in the region 270 - 310 nm was obtained. The chromophoric change was rapid at pH 8.0 and slower at pH 7.0 (Figures 12B, 15B and 18B). These observations suggested that alkaline hydrolysis of maleimide was rapid at pH 8.0 and slow at pH 7.0.

The conjugation reaction between the maleimide of Dox derivatives and the thiol groups of MAb proceeds more slowly than the model reaction of the spacers and cysteine because of the steric hindrance to reaction, local ionization in protein or dilution factor. The rate of hydrolysis of maleimide can limit the extent of conjugation of MAb to maleimido-Dox derivatives and pH 6.5 will be the ideal conjugation condition. However, based on the experimental results, less conjugation occured in MBA and MBUA when the chromophoric changes are compared (Figures 12A and 18A). After a certain degree of thiolation of MAb optimal conjugation may not be attained at pH 6.5. Thus, pH 7.0 was chosen to be the optimal condition for the maleimide-thiol coupling reaction.

III 4.3 Thiolation of 11-285-14

Chemical cross-linking of biological components such as

drug-protein conjugation usually involves chemical modification of proteins. Chemical conjugation of proteins to drugs depend on the reactivities of the constituents of proteins and the specificities of the cross-linking reagents. Amino groups of lysines are generally the most abundant functional groups on the surface of protein molecules that are susceptible for conversion to other functionalities. The conversion of amino to sulfhydryl groups becomes the most attractive approach for conjugation and the most potent nucleophile, thiol, increase the reactivity of protein for conjugation. Several reagents have been employed to introduce thiol groups into proteins and 2-iminothiolane (2-IT) is the most commonly used one. It is quite water soluble and reacts rapidly with amino groups at pH 7.0 or above. After the addition of thiol moieties, the cationic charges of the modified amino groups can be preserved. Moreover, the thiols show high reactivity to various maleimidyl groups.

11-285-14 was subjected to different degrees of thiolation with different amounts of 2-IT (Figure 20). The number of thiol groups was determined by DTNB at pH 7.27. DTNB is not a stable reagent. Dissolved in phosphate buffer (pH 7.0) and kept in the dark at -20°C, this is stable indefinitely. The number of thiol groups incorporated into the MAb is highly correlated to the amount of 2-IT used (R-sq = 99.68%, $P < 0.0001$) (Figure 20). The thiolated MAbs were

separated on a Sephadex G-25 column rather than dialysis because serious protein precipitation occured during dialysis.

The generation of reactive thiol groups on the MAb is easily controlled by using 2-IT, but the addition of thiols is a random process. There is no preferred group or subset of lysines that react faster than the others with 2-IT (Figure 20). There may be a wide spread of individual MAb molecules containing thiol groups higher and lower than the average. The binding activity of thiolated 11-285-14 to CEA may not be uniform from molecule to molecule.

Before using the thiolated 11-285-14 for conjugation reactions, binding studies were performed to confirm the binding of antibody to CEA by ELISA. The binding activity of 11-285-14 decreases as the number of thiol groups on antibody increases (Figure 21), presumably because of the thiolation on the active-site lysines. PAGE (Figures 32 and 33) showed the major bands of MAb at MW 150,000 from non-thiolated 11-285-14 and thiolated 11-285-14. The parent 11-285-14 used had been partly purified by protein A affinity chromatography. However, one contaminant around 190,000 was observed. From lane 3 - 5 in figure 32, the absence of clear bands higher than 150,000 (other than the 190,000 band) indicates that there is no covalent cross-linking between antibody. The extra bands at MW lower than 150,000 may be due to the breakdown of antibody and they are not in the parent antibody

(Lane 2). In figure 33, the faint bands at molecular weight higher than 150,000 in lanes 3 and 4 indicate the covalent cross-linking between thiolated antibody. However, use of 2-IT is a quite reliable method for thiolation without serious protein-protein cross-linking before protein-drug conjugation. Probably, serious cross-linking between antibodies does not occur because of low protein concentration (1.48 - 1.70 mg/ml) and low thiol substitution (6.26 - 7.14 SH/MAb) in our experiments.

III 4.4 Production of Drug Monoclonal Antibody Conjugates

Dox-11-285-14 conjugate showed an unresolved spectrum (λ_{max} 487 nm) relative to Dox derivatives (Figure 34). It may be the hypochroism (ϵ ~ 8000 M^{-1} cm^{-1}) which is characteristic of stacking aggregation of the anthracycline chromophore (Menozzi et al, 1984). The unresolved spectrum may be due the microenvironment of antibody which affects the chromophore of Dox conjugated to antibody. The molar extinction coefficient used in our experiment to estimate the drug bound to antibody is between 12380 and 13236 M^{-1} cm^{-1} (Table 5). The drug to protein ratio may be underestimated with these values.

The conjugation of drugs to antibodies is chemically difficult, especially for the highly hydrophobic drugs conjugated to hydrophilic antibodies. A number of methods have been extensively reviewed and result in 4 - 10 molecules

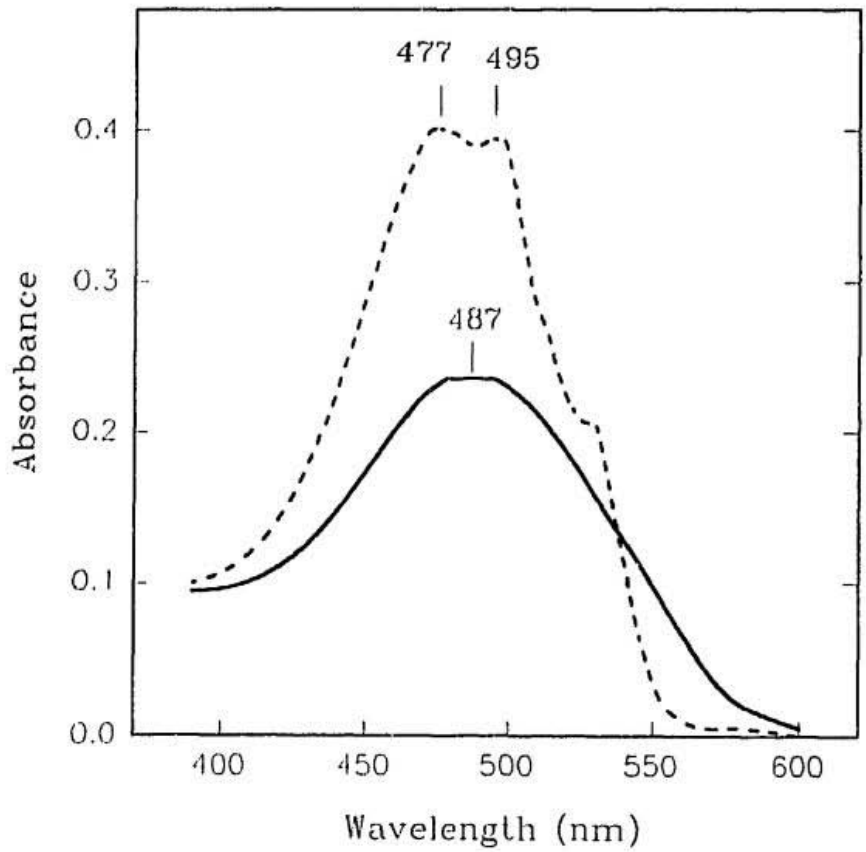

Figure 34. **Light absorption spectra of MBA-Dox derivative (---) and MBA-Dox conjugate (——)**

of drug per molecule of antibody (Pietersz, 1990). During the conjugation reaction, there is loss of antibody activity, up to 10-fold loss of drug activity and an inevitable protein precipitation. Antibody-daunorubicin conjugates prepared with succinyl linkages were prone to aggregation at 3 - 4 molecules of drug per molecule of antibody (Aboud et al, 1989). The conjugates prepared from IgG and Dox derivatives containing a reactive N-hydroxysuccinimide ester moiety (Dox-DSP) showed an extensive protein precipitation with an average of substitution of 1 to 2 Dox molecules per molecule of IgG (Demant, Jensen & Sehested, 1991).

The incorporation of spacer moieties may improve the coupling efficiency. The longer spacer arm should have increased the efficiency of the coupling reaction. At the beginning of the synthesis of our immunoconjugates, the problem of protein precipitation was very serious even in the presence of a 10-fold excess of drug. The gel filtration column retained most of the protein. After the improvement of the method, the drug was added in aliquots instead of one batch. All conjugation solutions were turbid and only Dox-MBA and Dox-MBCA conjugates appeared to have no aggregation during the conjugation reaction. The control containing Dox derivatives with non-thiolated antibodies or Dox with thiolated antibody remained as clear reddish solutions without any sign of protein precipitation. Only Dox-MBUA showed very

high non-covalent binding to non-thiolated 11-285-14, giving a conjugate containing 3.60 molecules of drug per molecule of antibody and high protein recovery. It is clear that the behavior of thiolated antibody or non-thiolated antibody with Dox-maleimido derivatives is different.

Hydrophobic protein-drug interactions and protein structure may affect the properties of the conjugation products and be responsible for the protein aggregation. Demant et al. (1991) suggest that protein-anthracycline complex formation is cooperative and involves two steps: initial self-association of anthracycline into aggregated structures, subsequent binding of protein at the aggregate surface and the formation of a structural resemblance to lipoproteins with a hydrophobic core stabilized by hydrophilic coat. It is possible that Dox-maleimido derivatives tend to form lipoprotein like structures and become precipitated in aqueous solutions. Willner et al. (1993) suggest that the tendency of protein precipitation during conjugation resides in the antibody and not the linkers because it persists with thiolation by either N-succinimidyl-3-(2-pyridinyldithio)propionate (SPDP) or 2-IT. Protein yields of 50 - 80% and 20 - 50% were obtained for SPDP-thiolated and 2-IT-thiolated MAbs respectively (Greenfield et al, 1990). Thus, the loss of positive charge when lysine amino groups react with SPDP but not with 2-IT is not responsible for the

protein precipitation. The problem of precipitation can be overcome when thiolation of lysines is abandoned. The dithiothreitol (DIT) reduction of MAb may be a more promising method (Willner et al, 1993). Final protein yields of 32 - 60% were obtained in our conjugation reactions (Table 7). A yield as high as 60% is reasonable in comparsion to the yields obtained by other investigators. The loss of protein may in part be due to the Amicon filters used for ultrafiltration and Millex GV filters used for sterilization. These filters retained some of the conjugates.

SDS-PAGE analysis of Dox-11-285-14 conjugates (**19**, **20**, **21**, **22** and **23**) showed extra bands of molecular weight (MW) higher than 150,000 (MW of mouse IgG_1). It suggests that covalent cross-linking between immunoconjugates occured. All immunoconjugates, except Dox-MBUA conjugate, showed a most intensely stained band at MW 150,000, indicating that most of the immunoconjugate remained as a monomer.

III 4.5 *In Vitro* Efficacy

All Dox 3'-N-amide derivatives were less toxic than the unconjugated drug against the CEA positive cell line, LS174T; Dox-MBUA derivatives failed to reach 50% inhibition of cell growth at 40 μM concentration. However, the cytotoxicity of the Dox C-13 hydrazone derivatives was of similar potency to Dox on both the CEA positive cell line, LS174T, and the CEA

negative cell line, COLO320DM; LS174T is more sensitive to Dox in comparsion to COLO320DM. The binding of Dox to DNA by intercalation is one of the most acceptable theories for its cytotoxicity. Structure-activity studies with anthracyclines have shown that the sugar residue is an important contributor to cytotoxicity. Dox analogues bearing N-acyl substituents are less potent than those bearing a free amino group (Yamamoto, Acton & Henry, 1972; Gabbay et al, 1976). However, recent X-ray crystallographic studies of anthracycline-oligodeoxynucleotide complexes indicates that the amino group does not interact with DNA (Frederick & Wang et al, 1990).

It has been suggested that anthracycline immunoconjugates do not show significant cytotoxicity if the drugs are not deconjugated from antibody at target site (Hermentin & Seiler, 1988). The attachment of Dox to antibody with acid-sensitive spacers and the release of Dox from immunoconjugates after internalization inside the lysosome compartment enable the drug to retain pharmacological activity. The tritylated 3'-N-[p-aminobenzoyl]-amide-daunorubicin was unstable in acetic acid and detritylation at pH 4 caused the release of daunorubicin. According to these authors, the aromatic ring promotes the hydrolysis of the 3'-N-amide linkage (Rosik & Sweet, 1990). Previous work in this laboratory has shown that the anti-CEA monoclonal antibody 11-285-14 is internalized by LS174T cells, but is not internalized by COLO320DM (Tsaltas,

Ford & Gallant, 1992). The internalization favors acidic cleavage of the drug from the immunoconjugate if, of course, it is hydrolysable. However, with a similar compound, Dox-MBA derivative and immunoconjugate, did not show any *in vitro* activity. This may be due to maleimide preventing the hydrolysis of aromatic amide bonding between MBA and Dox. The Dox(hydrazone-linked) immunoconjugates were more potent than Dox 3'-N-amide immunoconjugates but not as potent as their starting material, Dox C-13 hydrazone derivatives.

The results from ELISA showed the retention of binding activity of immunoconjugates (Figures 24 and 25). Because of the low drug to protein ratios (1.68 - 2.58 molecule of drug/molecule of MAb), all preparations will contain non-conjugated antibody. Inevitably, it is anticipated that the non-conjugated antibody will be responsible for some of the reactivity of immunoconjugates in ELISA. Thus, it is hard to prove the selectivity and binding activity of these immunoconjugates. However, it is quite clear that within MAb-MBA-Dox and MAb-MBCA-Dox conjugates there were little or no MAb inactivation whereas with the MAb-MBUA-Dox, MAb-MBH-Dox and MAb-MBCH-Dox conjugates there were a reduction in CEA binding. Although the binding activity has been apparently retained, the very poor cytotoxicity of Dox 3'-N-amide immunoconjugates is probably due to a reduced intracellular release of the active drug from the conjugates.

Because of the difficulty and serious protein precipitation during conjugation reaction in high drug substitution, Dox-11-285-14 conjugate preparations were obtained in low drug substitution. As a result, insufficient drug substitution did not permit significant cytotoxic effects to be observed and prevented a conclusion being reached on the efficacy of the immunoconjugates compared to the unconjugated Dox. The final purified and concentrated preparations only permitted cytotoxicity testing over a limited range. However, even if the drug substitution of Dox 3'-N-amide immunoconjugates was increased, we anticipate that cytotoxicity would probably still be modest.

In vitro testing results from Dox 3'-N-amide derivatives and immunoconjugates were matched with each other; no activity was obtained from any drug derivative and its conjugate. If the drug derivatives do not exhibit any cytotoxicity, one might expect that the immunoconjuagtes should not show any activity when tested *in vitro*. However, there have been a few reports in the literature where drug derivatives have been inactive *in vitro* but after linkage to antibody they have shown increased cytotoxicity of the immunoconjugates compared to unconjugated drug (Garnett et al, 1986, Smyth et al, 1986) and this is why we proceeded with the production of immunoconjugates. Also, a recent report by Henn et al 1993 indicated that, in general, 5-fluro-2'-deoxyuridine

derivatives had no activity *in vitro*. However, one immunoconjugate was non-specifically cytotoxic to both antigen positive and negative cells. These observations therefore cannot be explained by the increased uptake of the immunoconjugates by the target cells and suggest that the conjugation procedure has resulted in loss of selectivity of the carrier antibody. This is not the same as the results we obtained with the Dox 3'-N-amide immunoconjugates where there was no activity with antigen positive cells.

The cytotoxicity of the Dox(hydrazone-linked) immunoconjugates was less than the coressponding Dox hydrazone derivatives but showed significant specificity after conjugation of antibody. The dose-response curves show a gradual reduction in the percentage of surviving cells as the immunoconjugate concentration increases when the high CEA expression cell line, LS174T, is evaluated (Figure 30). Selectivity is confirmed by the absence of conjugate effect on the low CEA expressing cell line, COLO320DM, with the same concentration range (Figure 31). Although the Dox(hydrazone-linked) immunoconjugates show efficacy in *in vitro* testing, these immunoconjugates were just sufficiently cytotoxic to achieve 50% inhibition of LS174T at 8 μM. The efficacy of immunoconjugates depends on the density of antigen expressed on the target cells and the quantity of drug delivered per antibody molecule. Thus, immunoconjugates prepared with a

higher drug substitution should be more potent than low drug substitution because more drug is delivered per antibody molecule bound to target cell. Because of the protein aggregation and preciptation, drug substitutions, 2.24 - 2.27, obtained in Dox(hydrazone-linked) immunoconjugates is probably too low for good antitumor activity. These assays require considerably higher drug concentration or higher drug substitution which were not possible here because of the protein precipitation.

The use of heterobifunctional spacers with hydrazide and maleimide to cross-link Dox and antibody will be the current approach to prepare Dox immunoconjugate for antibody mediated targeting. Immunoconjugates (BR96-Dox) prepared from conjugating BR96 to (6-maleimidocaproyl) hydrazone of Dox were evaluated in *in vitro* and in human xenograft models (Trail et al, 1993; Willner et al, 1993). Treatment of established human tumors in athymic rats with BR96-Dox conjugates produced complete tumor regressions and long term cures. BR96 is a chimeric (mouse-human) MAb which binds to a TAA that is closely related to Lewis Y (Le^y) expressed on the surface of human carcinoma cells of the breast, colon, lung, and ovary. However, Le^y also exits in a wide range of normal cells such as blood cells. The first criterion of AMT is antigen specificity in which the antigens must be strongly expressed in tumors, allowing the conjugates to target the antigen-

positive tumor cells and sparing antigen-negative normal cells. CEA is strongly expressed by most carcinomas of the colon, stomach and breast. Moreover, CEA is present in much smaller amounts on normal tissues and is relatively specific for tumor. Thus, Dox-anti-CEA conjugate is a more suitable model for detailed studies in AMT. In the future, treatment of human cancer with immunoconjugates may provide the additional therapeutic option hoped for.

III 5.0 IMMUNOCHEMOTHERAPY : PROSPECTS AND FUTURE STUDIES

The ultimate aim of any new therapeutic approach is to apply it in the clinic. The discovery of hybridoma technology opened the new generation of immunochemotherapy using monoclonal antibodies as carriers of therapeutic agents. Before proceeding through Phase I, II and III Clinical Trials, it is important to characterize the immunoconjugates and then assess their efficacy and toxicity using a relevant pre-clinical model.

The use of CEA as a target has potential clinical applications in both diagnosis and therapy. The broad clinical evaluation and the well-characterized response and toxicity profiles of chemotherapeutic agents make them attractive for linkage to antibodies. Drug immunoconjugates have been shown to retain both *in vitro* and *in vivo* cytotoxicity in many investigations.

Several fundamental questions are outstanding and extensive investigation is required before the initiation of clinical trials. The most common problem is the difficulty in effectively binding chemotherapeutic agents to antibodies. The improvement of conjugation procedures is esential to optimize the yield of immunoconjugates. The determination of the nature, covalent or non-covalent, and the chemical properties, releasable or non-releasable, of the linkage between the drug and antibody provide the information about

the stability of immunoconjugates. The fate of the conjugate following systemic administration is unclear.

The work presented in this thesis provide some valuable information for future studies: 1) the synthesis of heterobifunctional spacers is well characterized; 2) the production of immunoconjugates can be performed in a stepwise manner, permitting additional characterization; 3) immunoconjugates prepared from Dox 3'-N-amide derivatives are not active in *in vitro* testing; and 4) Dox(hydrazone-linked) immunoconjugates are active in *in vitro* and appear to provide a more promising approach.

Further investigation of the correct spacer groups for effective release of drug and the understanding of the exact mechanism of release of the drug at target site appear to be essential for development of doxorubicin conjugates. The improvement of conjugation condition and purification of immunoconjugate are essential to prevent protein aggregation and precipitation. These will hopefully contribute to the development of better conjugates for antibody mediated targeting.

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