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Synthesis and characterization of site-specific porphyrin-antibody conjugates

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SYNTHESIS AND CHARACTERIZATION OF SITE-SPECIFIC PORPHYRIN-ANTIBODY CONJUGATES

by

SHANGDE CAI

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THE CITY UNIVERSITY OF NEW YORK

Abstract

Synthesis and Characterization of Site-Specific Porphyrin-Antibody Conjugates

by

Shangde Cai

Advisor: Prof. David K. Lavallee

The porphyrin-antibody conjugation strategy reported in this work has potential for use of nuclear medicine due to the robust chelation of radionuclides by porphyrins and the inherent specificity of the antibody-antigen interaction.

A method for regiospecific covalent modification of antibodies at the oligosaccharide moieties of the hinge region of the antibodies was developed and the characterization of the covalent porphyrin-antibody conjugates by using sodium dodecylsulfate polyacrylamide gel electrophoresis(SDS-PAGE) is described.

An asymmetrical bifunctional porphyrin, 5-(4-aminophenyl)-10,15,20,-tris(4-sulfonatophenyl) porphine (H_2AS_3P) and its *N*-p-nitro-benzylated derivative(*N*-bzHAS_3P) were synthesized. The former had been used for testing the possibility of attachment of the aniline group of H_2AS_3P to the aldehyde group produced by mild oxidation of oligosaccharide moieties in antibodies. The conjugation was

verified by SDS-PAGE slab gel which revealed coloration due to the porphyrin at the same position as the antibody's heavy chain band, demonstrating that the porphyrin covalently binds to the heavy chains of antibodies.

To be useful in rapid metallation of the porphyrin-antibody conjugates, the porphyrin attached to antibodies must be *N*-substituted. To synthesize an *N*-substituted porphyrin, it was necessary to protect the aniline functionality during the *N*-alkylation reaction. The phthalimide group proved stable enough to resist the high temperature required for alkylation of a pyrroleninic nitrogen atom of the porphyrin. The phthalimide moiety was then deprotected to recover the amino group which is necessary for antibody conjugation.

Acknowledgements

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I owe tremendous thanks to my wife, Yuzhen Hong, for her spiritual and financial supports of this work.

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Chapter 1

Introduction

Targeting of radioactivity to tumors in the human body using specific antitumor antibodies dates from the early studies and concepts of Paul Ehrlich (1), who described antibodies as "magic bullets", and the pioneering work of David Pressman, who, using antibodies labeled with isotopes of iodine (2), initially demonstrated that antibodies could target tumors in living systems. The discovery of monoclonal antibodies by Kohler and Milstein (3) in 1975 gave new emphasis to the use of antibodies as radiopharmaceuticals.

The antibodies from immune heterosera that arise from the natural immunization of animals are a mixture of many different types of antibodies which vary in important antibody properties such as subclass, specificity and affinity. These antibodies are called "polyclonal", because they arise from the simultaneous immunization of many different lymphocytes when the animal is exposed to an antigen. In contrast, monoclonal antibodies are homogeneous in these properties. The basic principle is that once the animal is making polyclonal antibody against the antigen, plasma B cells are obtained by excising the spleen, harvesting the B cells, and fusing them with malignant myeloma cells (4,5). Thousands of distinct hybridomas may be produced in a single fusion from B lymphocytes and myeloma

cells. These individual hybrid cells can be separated one from another and will divide and grow indefinitely in proper culture medium *in vitro*. From a single hybridoma, an entire colony of identical cells will be formed. Each cell can produce a single type of antibody of specificity for the antigen to which the parent lymphocyte was immunized. The cells in the colony are "monoclonal" as they all are from a single progenitor cell. The antibody produced by this group of cells is called "monoclonal antibody." Because of ease of production and greater homogeneity, since 1975 many monoclonal antibodies have been produced against tumor-associated antigens from most of the common human tumors, which has led to the use of radiolabeled antibodies in clinical trial in cancer. Most of these studies have been concerned with tumor detection, although a growing number are concerned with tumor therapy (6-17). During the past two decades, the clinical utility of radioimmunodetection has been well established for many common human tumors, such as colorectal, melanoma, breast and ovarian. But the practicality of this technique was not universally accepted (18) and the most challenging problem was the low uptake of radiolabeled antibody by tumor.

Radioimmunoconjugates are usually made of three major components: radionuclides, antibodies and/or metal chelators. To make improvements in use of radioimmunoconjugates for *in vivo* diagnosis and therapy of human cancers, researchers are mostly concerned about three aspects, 1) choice of

radionuclides, 2) stability of the radioligand and 3) covalent modification of the antibody.

The choice of radionuclide usually begins with an examination of the nuclear properties of the atom. If the radionuclide is to be used for diagnosis, the match between the gamma-ray energy and the imaging device is of prime concern. For therapeutic radionuclides, the absorbed radiation dose is the most important characteristic. Using beta ray emitters as potential therapeutic radionuclides has often been reported with encouraging results, such as ^{67}Cu (0.192 MeV β_1^-, 0.161 MeV β_2^-, 0.132 MeV β_3^-; imaging gamma ray 0.184 MeV, $t_{1/2}$ = 61.9 h) (19-24), ^{90}Y (2.228 MeV β^-, $t_{1/2}$ = 64.1 h) (25-27) and ^{131}I (0.183 β^-, 0.364 MeV γ, $t_{1/2}$ = 192 h) (28-31). ^{67}Cu and ^{131}I can be used for both therapy and diagnosis. Alpha-emitting radionuclides have also been suggested as radiolabels for therapeutic applications of antibodies, such as ^{211}At ($t_{1/2}$ = 7 h) (32).

Among the radionuclides most often suggested for diagnostic imaging, ^{123}I (0.159 MeV γ, $t_{1/2}$ = 13 h) and ^{99m}Tc (0.140 γ, $t_{1/2}$ = 6 h) have the best decay characteristics, in comparison with the others: ^{67}Ga (0.185 MeV γ, $t_{1/2}$ = 78 h), ^{111}In (0.172 MeV γ_1, 0.247 MeV γ_2, $t_{1/2}$ = 67.4 h), and ^{131}I. However, ^{123}I is a cyclotron product, which is much less available than the convenient generator product ^{99m}Tc. Technetium-99m became popular not only because of its ideal nuclear properties for imaging with the Anger gamma camera, but because of its ready availability from the ^{99}Mo generator

system (33). Since the nuclear properties suitable for imaging and the quality of commercial supply available, ^{111}In is also of wide application in modern radioimmunodetection technology (7,8,34-41)

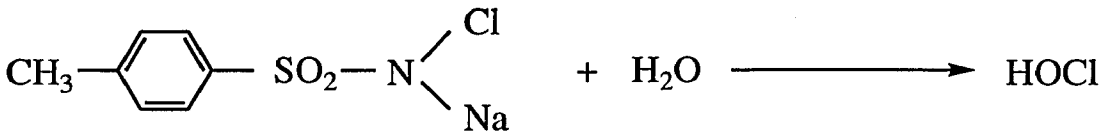
The sensitivity and specificity of tumor detection using conventional gamma camera imaging generally has not been sufficient for clinical use. Although using better imaging devices is one method to improve radioimmunoimaging, for example, the use of single-photon emission computed tomography (SPECT) with ^{123}I-labeled Mabs (monoclonal antibodies) (42,43), positron emission tomography (PET) is might further improvement in radioimmunoimaging. PET has more accurate quantitative capabilities than SPECT, which could be of great value in determining tumor and normal tissue dosimetry prior to therapy. A number of proteins have been labeled with positron emitters, such as ^{11}C (44,45), ^{68}Ga (46,47), ^{18}F (48,49) ^{64}Cu (21,50). While recent applications of fluorine-18 in PET to measure regional cerebral glucose metabolism use ^{18}F labeled deoxyglucose ([^{18}F]FDG) (51), labeling antibodies with ^{18}F has been considered due to nearly 100% of ^{18}F decays is positron emission (52-57). For example, Zalutsky and his coworkers reported that the Mab fragment of Mel-14, an antibody reactive with gliomas and other tumors, was labeled by reaction with *N*-succinimidyl-4-[^{18}F]fluoro-benzoate ([^{18}F]SFB) (58). The tumor-to-normal tissue ratio for ^{18}F-labeled Mel-14 fragment was 40:1 for brain, suggesting

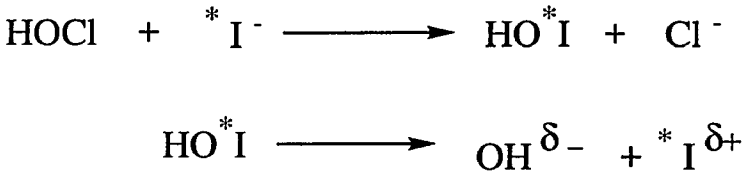
that it may be feasible to use ^{18}F-labeled antibody fragments for imaging tumors by PET.

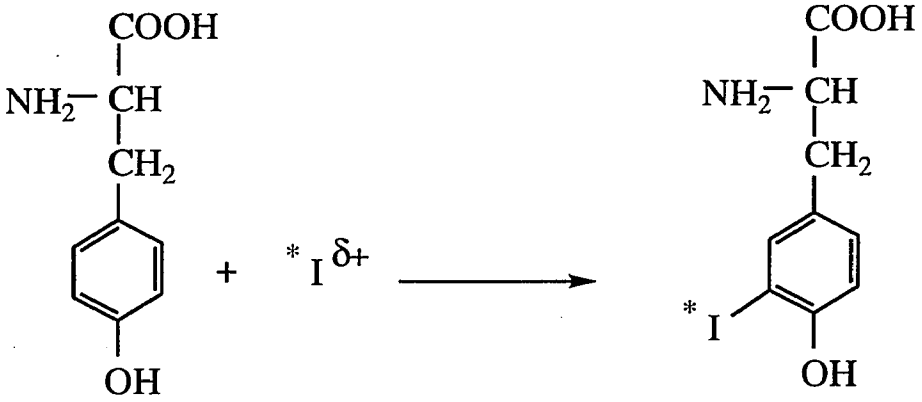
Covalent labeling with radiohalogens, especially iodine, is a conventional scheme of introducing radioactive isotopes into antibodies. In this procedure, the chemical modification and radiolabeling occur in the same step, but the choice of isotope is limited. As mentioned at the beginning of the chapter, the first labeling of antibodies with radionuclides was performed with ^{131}I (2). It is desirable to replace iodinated radiopharmaceuticals used in nuclear medicine by ones using bifunctional chelate complexes with radioactive metals such as ^{99m}Tc and ^{111}In. The reason is basically the chemical instability of ^{131}I labeled compounds compared to those labeled with ^{99m}Tc and other radionuclides.

Most radioiodination methods involve the use of oxidizing agents, for example, Chloramine-T (*N*-chloro-4-methylbenzenesulfonamide sodium salt), to convert stable I^- ion to quasi-stable I^+ before electrophilic substitution in aromatic amino acid residues of antibodies such as tyrosine residues (Figure 1-1). These methods may not be useful in nuclear medicine when the amino acid residue most easily iodinated is located within the antigen binding site, because iodination could result in loss of immunoreactivity. Besides, the oxidizing agents used in the scheme are capable of

Figure 1-1. Chloramine-T iodination of the tyrosine of the antibody.


CH3
SO2
N
Cl
Na
+ H2O
HOCl


HOCl + *I- → HO*I + Cl-
HO*I → OH δ- + *I δ+


COOH
NH2
CH
CH2
OH
+ *I δ+
*I

denaturing antibodies by breaking peptide bonds (59). The halogenation of the antibody can also be achieved by conjugating a prehalogenated molecule to the antibody as mentioned above (57). The halogenated *N*-hydroxysuccinimide ester conjugates with proteins by forming amide bonds between the amino groups of lysine residues of the antibody and the carbonyl groups, which have been activated by the *N*-hydroxysuccinimide. But one of the major disadvantages of this method is the susceptibility of the *N*-hydroxysuccinimide ester group to hydrolysis during conjugation with the antibody. Water competes with the amino groups of the lysine residues and results in a lowering of yield. Additionally, as discussed by Gansow (35), none of the iodine isotopes are optimal for either radioimaging or radiotherapy: ^{123}I has a too short half life; the x-ray from ^{125}I is too weak; ^{131}I is not only undesirable for imaging due to its long half life and cytotoxic beta radiation but also modestly successful in therapy because of the low dose from this weak β-emitter.

The method of labeling antibodies with radioactive metals using bifunctional chelating agents added flexibility in terms of the chemical reactions involved, the choice of radiiosotope, and the time of addition of the radionuclide (60,61). The development of a variety of bifunctional chelating agents, which form stable antibody conjugates and bind metal ions very tightly, as well as the availability of isotopes with convenient half-lives and useful radiation for imaging and therapy, has made this approach to

radioimmunoimaging and radioimmunotherapy a practical technique. An advantage of this technique is that the chemistry and the addition of radionuclide are two separate steps. Antibodies with chelates covalently attached may be prepared, purified, characterized, and stored in nonradioactive form. Just before the modified antibodies are used for actual imaging and therapy purpose, the radioactive metal ion may be added in a simple, rapid step. In this way a higher specific activity is obtained than when radiolabeling and utilization are more widely separated in time. Furthermore, handling of the modified antibody is more convenient, safe, and economical.

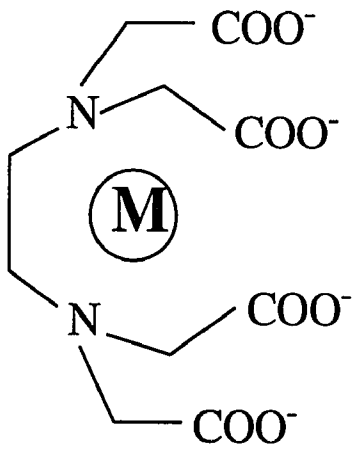
The relative merit of chelation labels vs. halogen labels is continuing subject of controversy in the antibody radiopharmaceutical literature. An optimal balance between each of several criteria is required in choosing an appropriate label for an antibody. First of all, the labeling procedure should not cause the radiolabeled antibody to lose its immunoreactivity. Secondly, the radionuclide should be stably bound to the antibody and remain at the specific site in *vivo*. Finally, the nuclide should have suitable nuclear properties either for radioimmunoimaging or for radioimmunotherapy. Therefore, the bifunctional chelating agents to be used for antibody radiolabeling should have functional groups reactive with the antibody to form a covalent bond which will be stable in *vivo*. And the metal complex with the desired radionuclide should be stable *in*

vivo under physiological conditions to prevent non-target cells from radiation damage. They also should be nontoxic and have no effect on the immunoreactivity of the antibody.

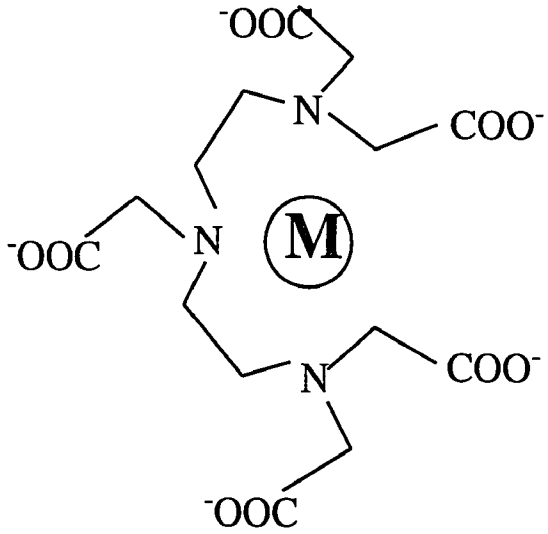
Commonly used nuclide chelators are such amide compounds as ethylenediamine-tetraacetic acid (EDTA), diethylenetriaminopentaacetic acid (DTPA) and 1,4,7,10-tetraazacyclododecane-*N,N',N",N'"*-tetraacetic acid (DOTA) (Figure 1-2) with a variety of functional groups. The antibody-metal conjugates prepared using these chelating agents are generally quite stable. For example, *p*-benzenediazonium-EDTA (62) was one of the first compounds supposed to be coupled with tyrosine and histidine. The high pH required, however, caused loss of immunoreactivity. A carboxymethoxybenzyl-EDTA chelator was prepared to link the lysine moiety at pH 5 using the carbodiimide method (37). *p*-Isothiocyanatobenzyl-DTPA conjugated to antibodies also at the amino group of lysine residues was reported to be stable with ^{111}In *in vivo* (63). The low amount of radioactivity in the liver, from the results of *in-vivo*-distribution test, was an indicator of the stability of the ^{111}In complex. Furthermore, chelated metal ions from metabolized antibodies are rapidly excreted via the kidney, in contrast to the fate of iodine, which concentrated in the thyroid.

A particularly attractive feature of this type of labeling is the versatility of the chemistry involved in the actual antibody modification. Since a variety of functional group may be incorporated into chelating compounds, it is

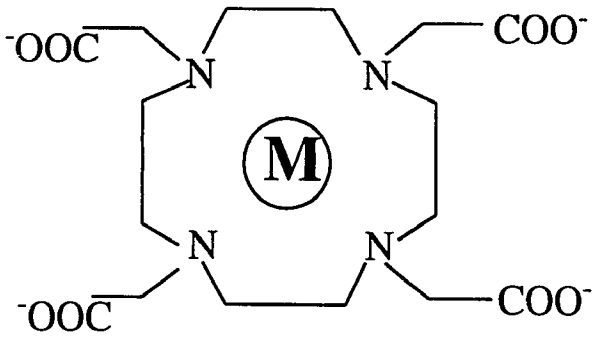
Figure 1-2. Schematic chelate structures of ethylenediaminotetraacetic acid (EDTA), diethylenetriaminopentaacetic acid (DTPA) and 1,4,7,10-tetraazacyclododecane-*N*,*N*',*N*",*N*'"-tetraacetic acid (DOTA).



EDTA



DTPA

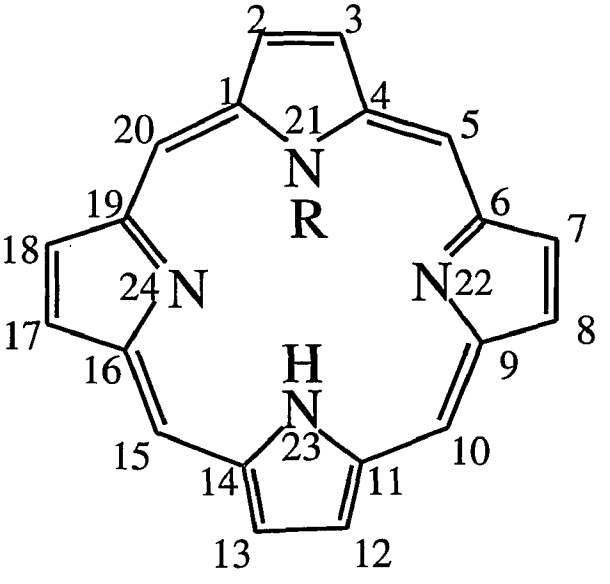


DOTA

possible to experiment with various reaction types and choose the one that gives the highest specific radioactivity with the least loss of immunoreactivity. For example, copper-67 complexes of noncyclic EDTA and DTPA derivatives were reported to decompose very rapidly in serum. Meares *et al.* synthesized a series of bifunctional chelators based on macrocyclic ligands such as DOTA and TETA, 1,4,8,11-tetraazacyclotetradecane-*N,N',N", N"'*-tetraacetic acid. These macrocyclic species exhibit remarkable kinetic inertness with copper (64-68).

The reason Meares *et al.* chose copper is because transition metals such as copper possess excellent nuclear, electronic, and chemical properties for medical applications (69-72). Copper is a biologically essential metal. Since any essential metal which escapes its normal metabolic pathway can be very toxic to the organism, complexes of such metals serve as effective cytotoxic agents. We have concentrated on another class of macrocyclic chelator, the porphyrins (Figure 1-3), for chelating agents of such transition metallic radionuclides as copper and cobalt in use primarily for gamma and PET imaging. Naturally occurring porphyrins are a variety of metal complexes such as iron porphyrins presented in hemoglobin and myoglobin, magnesium porphyrins found in the chlorophylls and bacteriochlorophylls. Copper porphyrins are found in the feather pigments of some birds (73). Thus at least some porphyrins are biologically compatible. Synthetic porphyrins are not readily metabolized, unlike chelates with

Figure 1-3. The tetrapyrrocyclic ring structure of an *N*-substituted porphyrin with the IUPAC recommended numbering system.


2
3
1
4
20
21
5
N
R
19
6
7
18
24 N
N 22
17
8
16
H
N
9
15
23
10
14
11
13
12

carboxylic groups and amide bonds. Copper porphyrins are reported to be extremely stable. For example, Mercer-Smith *et al.* demonstrated that less than a 1% loss of copper from the ^{67}CuTCPP (5,10.15.20-tetrakis(4-carboxyphenyl)porphinato-[^{67}Cu]copper(II)) occurs during the incubation with the challengers, such as EDTA and DTPA, under simulated physiological conditions, for 12 days (21).

The porphyrins used for chelating Cu(II) are *N*-substituted bifunctional porphyrins, e.g., the *N*-*p*-nitrobenzyl-5,10.15.20-tetrakis(4-carboxyphenyl)porphyrin. When the copper is complexed, because of the tendency to form very stable planar complexes, the *N*-substituent is cleaved and the copper porphyrin complex attains a planar conformation which has lower energy. Non-transition metals which are not capable of cleaving the *N*-substituent to form planar macrocycle complexes, therefore, do not form stable complexes. The choice of *N*-substituent is important because the rate of cleavage by copper(II) varies by several orders of magnitude for different *N*-substituents, the most rapidly cleaved *N*-substituents are those which readily form carbocations (133), such as *N*-allyl and *N*-benzyl groups. The *N*-benzyl porphyrin-antibody conjugates can be radiolabeled with $^{67}CuCl_2$ under mild reaction conditions (82).

Another key issue in the use of chelates for metallic radioisotopes is the need for covalent modification of antibodies by conjugation to chelating agents. In general, such modification can cause more or less a decrease in the

immunoreactivity (74). Rodwell *et al.* demonstrated that the carbohydrate moieties on antibodies could be used to attach various substances at specific positions away from the antibody-antigen combining site (41). This site-specific modification was shown to preserve the immunoreactivity even with relatively high loadings of a substance. Standard techniques involving lysine modification to attach chelating agents onto antibodies resulted in severe loss of immunoreactivity when the conjugates contain more than one chelators per antibody (41,74,75).

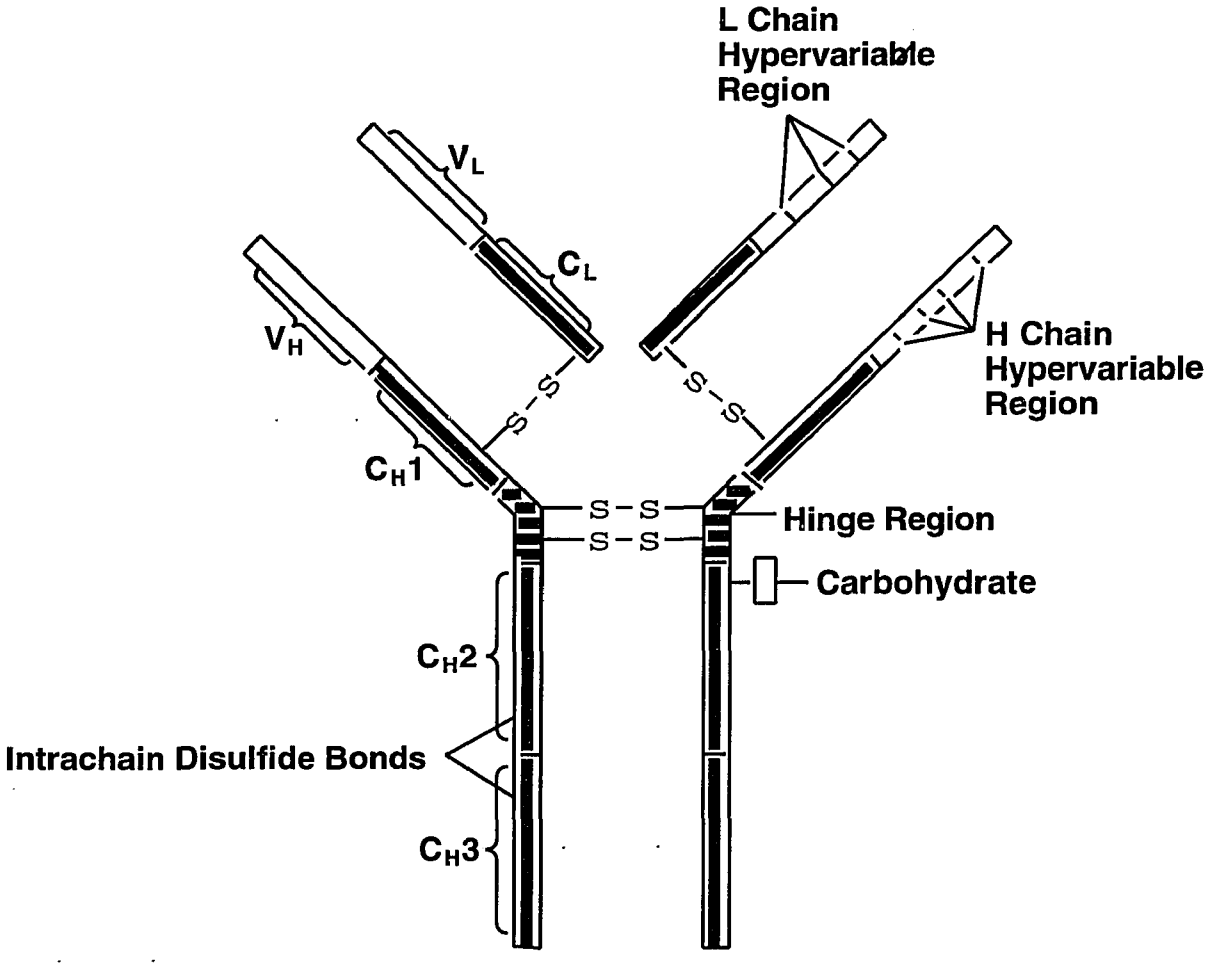
The analysis of localization and biodistribution of radiolabeled antibodies after the injection of the antibody with ^{111}In-DTPA complexes into xenograft-bearing mice and normal mice as control was reported by Rodwell's group (76). The images show that the radiolabeled antibody localizes well to the xenograft and that this localization is immunospecific, in comparison with the images obtained from a normal, non-tumor-bearing mouse and one injected with an irrelevant ^{111}In-labeled antibody conjugate. There was no selective localization of radionuclides seen in both of the control images. In non-tumor-bearing animals, the tissue-to-blood ratios (radiation counts per minute per gram of organ divided by cpm/g blood) are all less than 1, indicating there is no selective localization to any normal organ . The high tumor-to-blood ratios of 19.5:1 shows that the site-specifically modified antibody conjugate localizes well to

this xenograft. Accumulation in other organs is very low with tumor-to-blood ratios just above 1.

Site-specific modification refers to covalent chemical modifications at a specific site on an antibody molecule, in contrast to the general methods, which result in modification of a certain class of sites on antibodies, such as lysine, tyrosine, or aspartic or glutamic acid side chains. The "class-specific" modification can lead to decreased immunoreactivity. This can be illustrated by the schematic structure of immunoglobulin G molecules which are among the simplest type of immunoglobulins.

The immunoglobulin G (IgG) has two longer polypeptides (heavy chains), each of about 50 kDa, and two shorter ones (light chains), each of about 25 kDa. There are disulphide bonds between the light chains and heavy chains as well as between the heavy chains. The disulfide bonds are only found in the constant region of the light chains and heavy chains, not the hypervariable region, where there are antigen-binding sites. Carbohydrate moieties are located only in the constant regions of heavy chains, near the hinge of Y shaped IgG macromolecules (Figure 1-4) (77-80). The most accessible sites for drug attachment on antibodies are the ε-amino groups of the lysine residues and the carbohydrates. The latter groups can be oxidized by periodates to generate aldehyde functionalities from the vicinal diols (81). The amino groups

Figure 1-4. A representation of an IgG class of antibody. V_L--variable region of light chains; V_H--variable region of heavy chains; C_L--constant region of light chains; C_H--constant region of heavy chains.

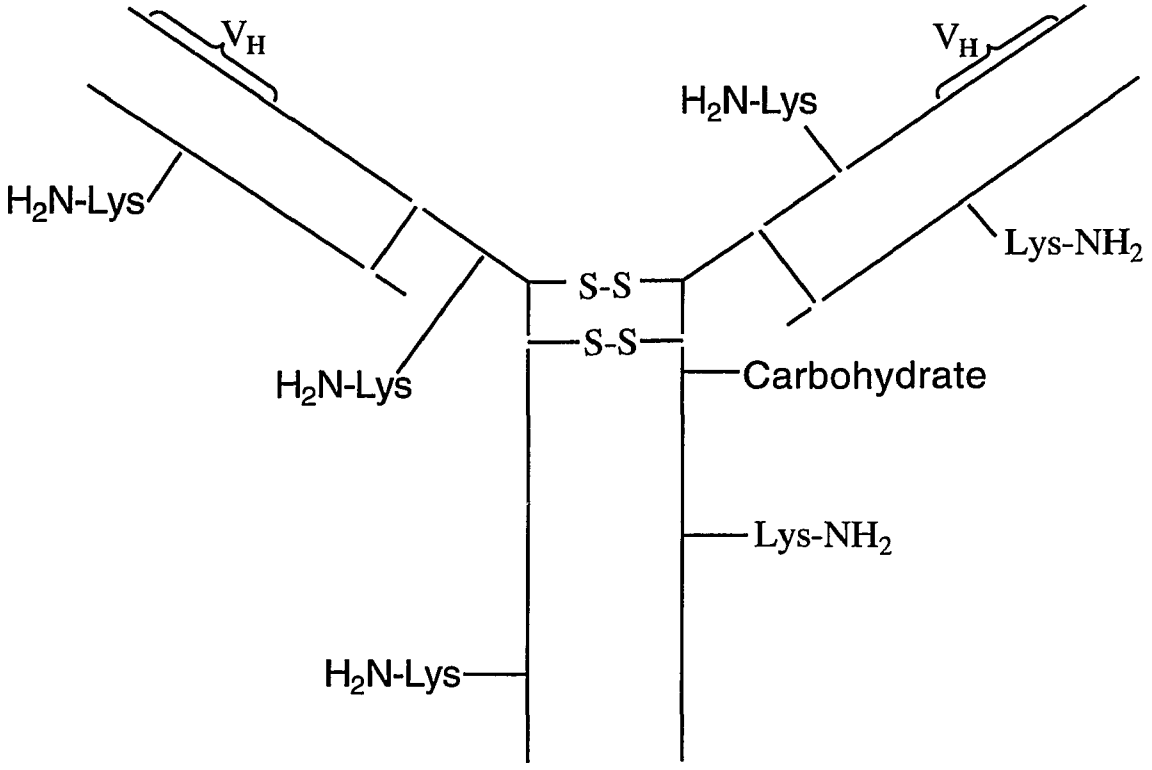

L Chain
Hypervariable
Region
V_L
C_L
V_H
H Chain
Hypervariable
Region
S-S
S-S
S-S
C_H1
S-S
Hinge Region
Carbohydrate
C_H2
Intrachain Disulfide Bonds
C_H3

of the lysine side chains are randomly located all over the antibody molecule, but the carbohydrates are regiospecific and remote from the antigen-binding sites (Figure 1-5) (80).

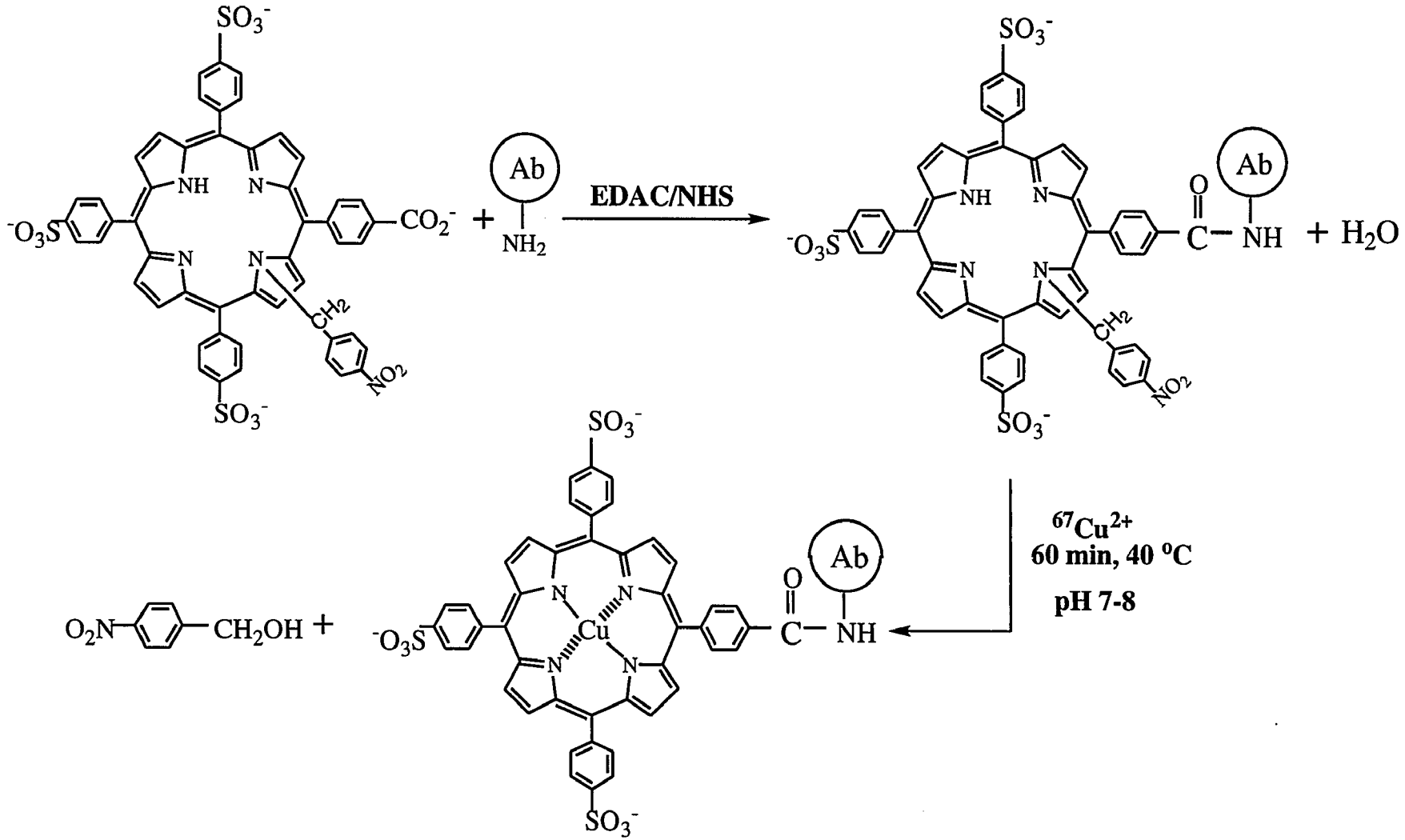
The previous work in our laboratory, in collaboration with the researchers at the University of Utah, Michigan Molecular Institute and Los Alamos National Laboratory, involving coupling of a carboxylate functionality in a porphyrin with lysine residues in an antibody. The porphyrin used was *N*-nitrobenzyl-5-(4-carboxyphenyl)-10,15,20-tris(4-sulfonatophenyl)porphyrin (*N*-bzHCS$_3$P). Activated by *N*-hydroxysuccinimide (NHS) in the presence of ethyldimethylaminopropylcarbodiimide (EDAC), the carboxylate group of the porphyrin coupled to the amino group of lysine side chains of antibodies (Scheme 1-1), while the three sulfonate groups ensure the water solubility of the porphyrin. The antibody-porphyrin conjugate was metallated by loss of the *N*-nitrobenzyl group under very mild conditions (82).

Since the amide linkages between the porphyrin and the antibody might be located in the hypervariable regions near the antigen-binding sites, affecting the immunoreactivity of the antibody, site-specific labeling has been thought to be preferable. A porphyrin and a porphyrin-like macrocycle have previously been coupled at the oligosaccharide site of an antibody, but, in both cases, large linking moieties terminating in aliphatic amines were employed. One of our collaborators first attached the carboxyl peripheral group of

Figure 1-5. Antibody sites of covalent attachment. The amino groups of lysine chains are randomly all over the antibody molecule, but the carbohydrate moiety is regiospecific and remote from the variable domains.


V_H
V_H
H_2N-Lys
H_2N-Lys
H_2N-Lys
Lys-NH_2
S-S
S-S
Carbohydrate
Lys-NH_2
H_2N-Lys

Scheme 1-1. The conjugation of *N*-bzCS_3P to antibodies via the amino group of lysine side chains.


SO3-
NH
N
-O3S
CO2-
Ab
NH2
EDAC/NHS
C
NH
+ H2O
CH2
NO2
O2N
CH2OH +
Cu
67Cu2+
60 min, 40 °C
pH 7-8

N-bzCS_3P to a polyamine starbust dendrimer and then attached an amine functionality on the dendrimer (83) to the oligosaccharide site using the periodate method (84) (Scheme 1-2). In the other case, Oseroff et al. coupled a chlorin via its peripheral carboxyl group to a modified dextran polymer and then used an amine moiety on the modified polymer for attachment to the antibody in a similar manner (85).

To link a macrocyclic chelator to the carbohydrate portion of an antibody, it is necessary to have the appropriate functionality such as a hydrazine, hydrazide or amine group, as well as a variety of those which derived from these groups (63,86-95). The carbohydrate region is not as sterically accessible as the amino groups of lysine side chains due to the relatively large size of antibodies and porphyrins, so a "bridge" between the porphyrin and the antibody seems to be desired.

With the above considerations, my project was to synthesize a porphyrin such as that shown in Figure 1-6. Since the aniline was thought to be much less nucleophilic than the aliphatic amine. it was felt that at least one methylene group would be required between the reactive group (R) and the peripheral phenyl group. The traditional way to make such asymmetric porphyrins was by a crossed-Rothemund condensation (96-102), that is, three parts of benzaldehyde and one part of corresponding *p*-substituted one, i.e. *p*-hydrazinobenzaldhyde, condensed with four equivalents of pyrrole. It was, however, very difficult to make such

Scheme 1-2. The *N*-nitrobenzyl-5-(4-carboxyphenyl)-10,15,20-tris(4-sulfonatophenyl)porphyrin (*N*-bzHCS$_3$P) attached to the oligosaccharide site of the antibody through a polyamine starbust dendrimer.

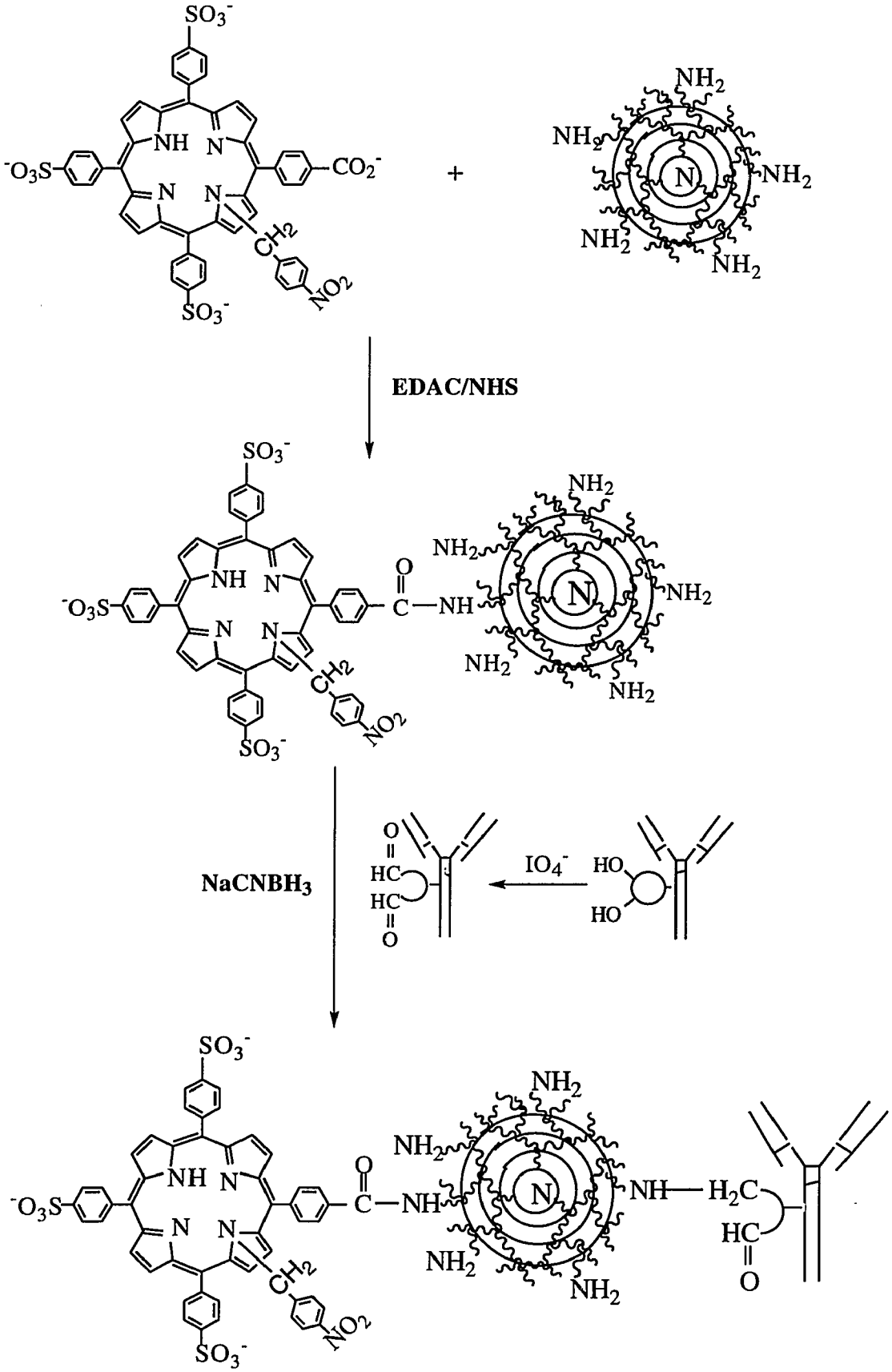
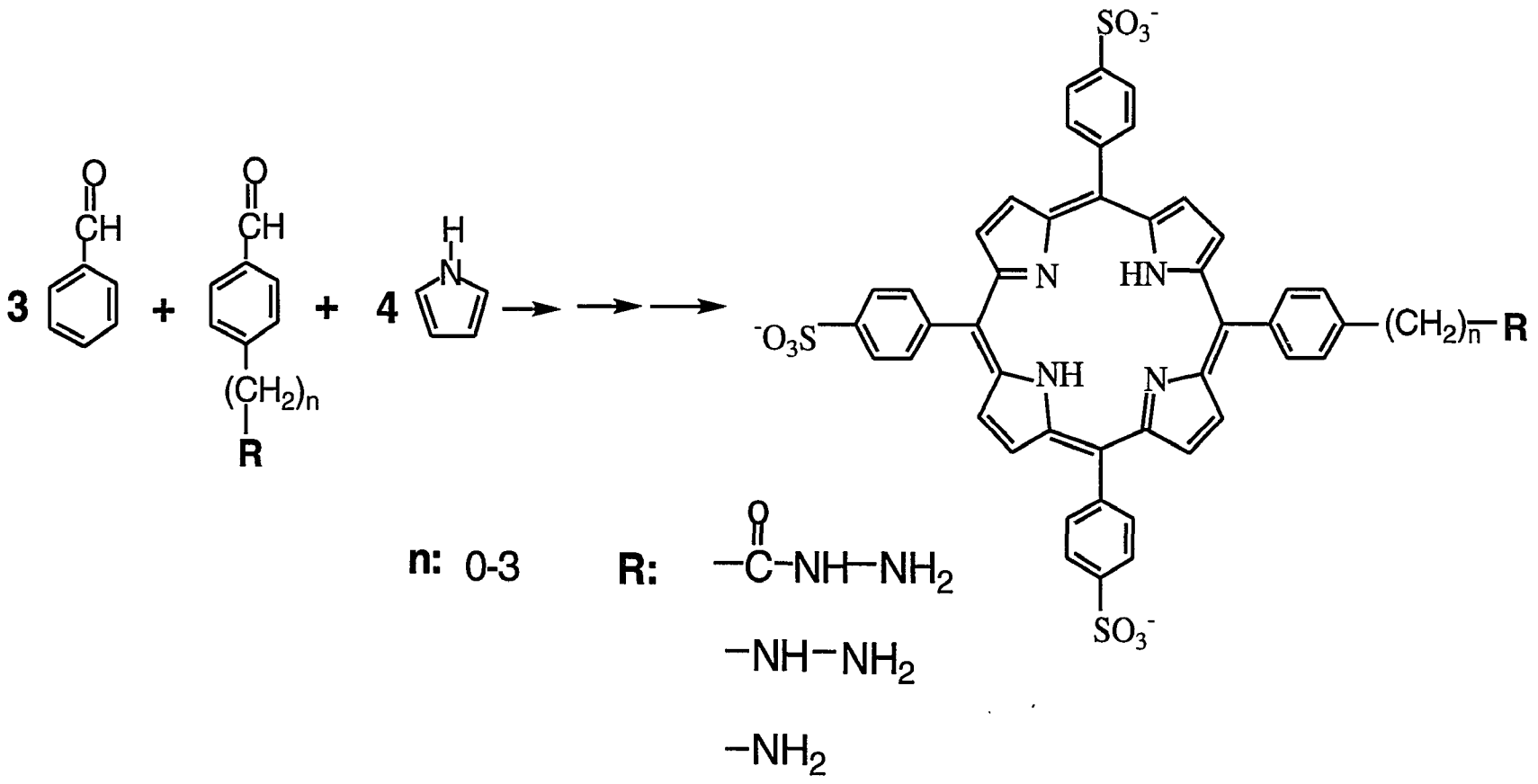

EDAC/NHS
NaCNBH3
IO4-

Figure 1-6. The crossed-Rothemund condensation to make asymmetrical bifunctional porphyrins with reactive groups (R) was not practicable. The number of methylene groups between the phenyl group and the reactive group (R) would rather be more than zero.

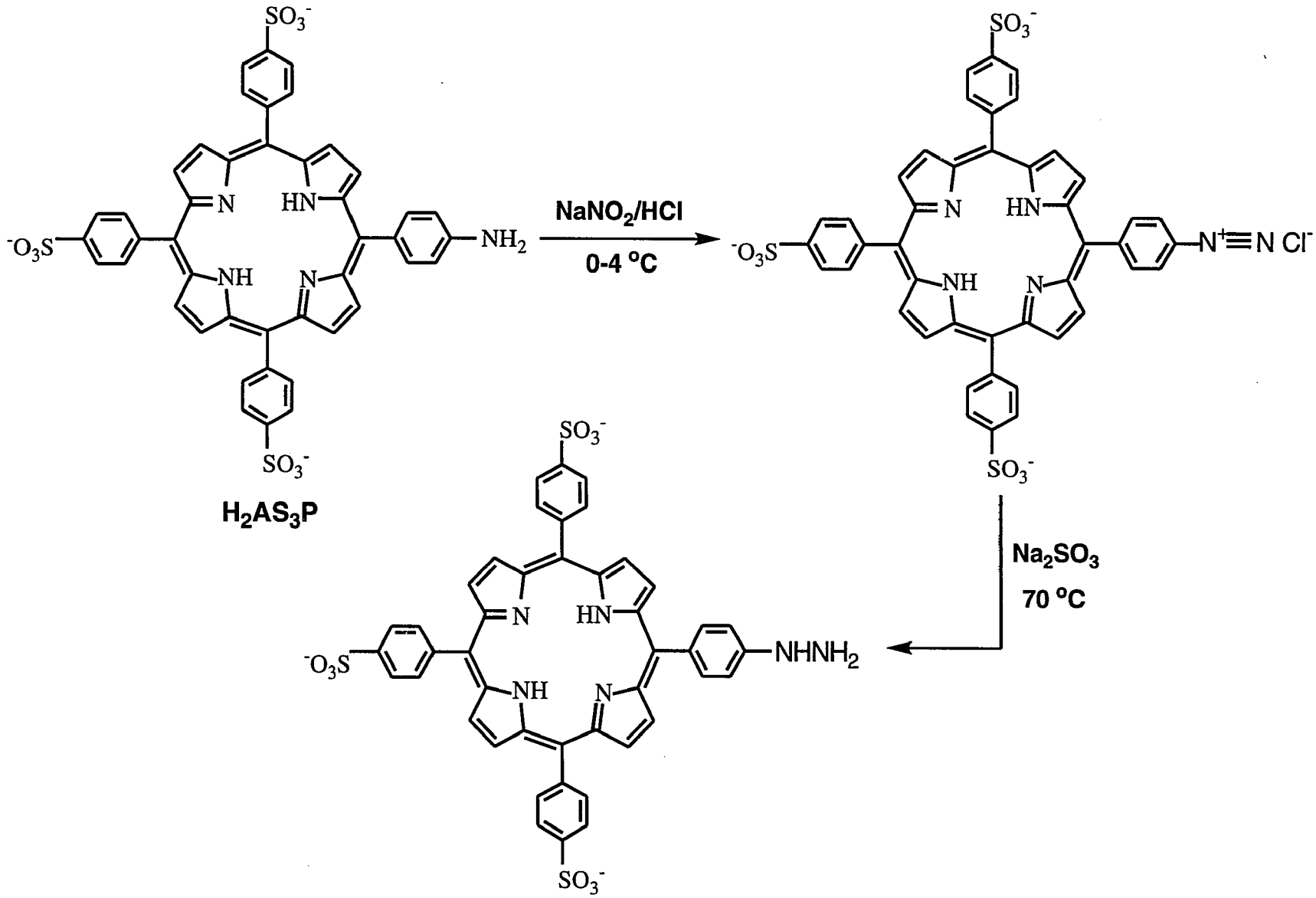

3
+
+
4
n: 0-3
R:
SO3-
-O3S
N
HN
NH
N
(CH2)n
R
SO3-
-NH-NH2
-NH2

starting materials or to protect those reactive groups which would be used for coupling with antibodies during the condensation of the aldehydes with pyrrole. While we sought a method to convert one of the peripheral phenyl groups of the tetrakis-5,10,15,20-phenyl porphyrin (TPP) to the nucleophilic reactive group, Kruper *et al.*(103) published their procedure of mono-nitration of TPP followed by the reduction to an aniline group.

At first we synthesized the asymmetrical bifunctional porphyrin, 5-(4-aminophenyl)-10,15,20-tris(4-sulfonatophenyl) porphyrin (H_2AS_3P) by using the Kruper's method and tried to convert the aniline group to phenyl hydrazine either before sulfonation or after (Figure 1-7). The attempt failed mainly due to the difficulties in purification.

We then focused attention on the aniline group itself and the project was planned in two steps. The first was to evaluate coupling with aniline NH_2. After a method for coupling the aniline group directly with antibodies through the carbohydrates without a linking molecule was developed, then the second step, synthesizing a *N*-substituted derivative of H_2AS_3P (*N*-bzHAS_3P) and attaching it to the antibody, was undertaken. The antibody conjugates with *N*-substituted porphyrins would become the precursor for radiolabeling with metallic radionuclides because only the *N*-substituted porphyrin could complex radioisotope ions readily under biological conditions (82,104).

Figure 1-7. Attempt to convert the aniline group of the 5-(4-aminophenyl)-10,15,20-tris(4-sulfonatophenyl)porphyrin to the phenyl hydrazine failed mainly due to the difficulties in purification of the intermediate and the product.


SO3-
-O3S
N
HN
NH
N
NH2
NaNO2/HCl
0-4 °C
N+≡N Cl-
H2AS3P
Na2SO3
70 °C
NHNH2

In this study, a commercially available polyclonal rabbit immunoglobulin G (RIgG) was employed and the porphyrin-antibody conjugates were characterized by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for both 5-(4-aminophenyl)-10,15,20-tris(4-sulfonatophenyl)porphyrin and *N*-*p*-nitrobenzyl-5-(4-aminophenyl)-10,15,20-tris(4-sulfonatophenyl)porphyrin. SDS-PAGE was used to monitor the conjugation reaction.

Chapter 2

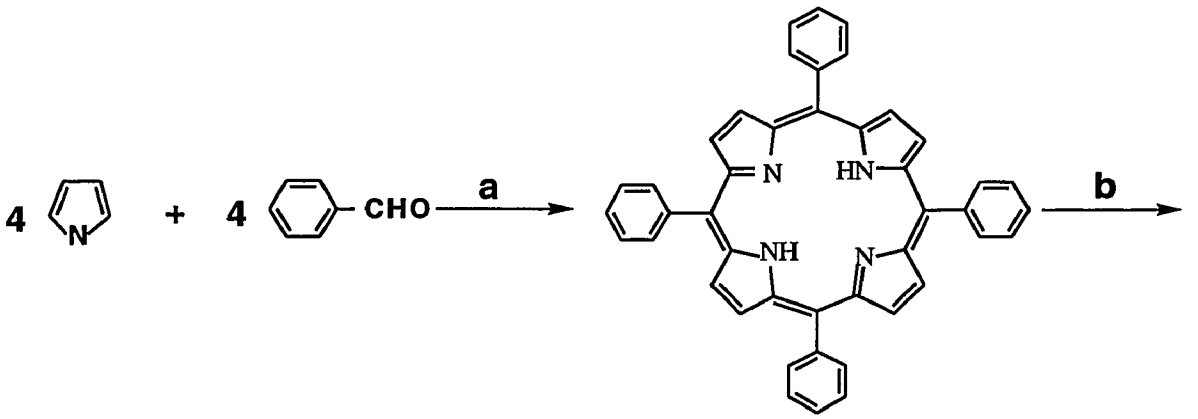
Synthesis and Characterization of 5-(4-Aminophenyl)-10,15,20-Tris(4-Sulfonatophenyl)Porphyrin and Its Conjugate with Immunoglobulin G

2.1 Introduction:

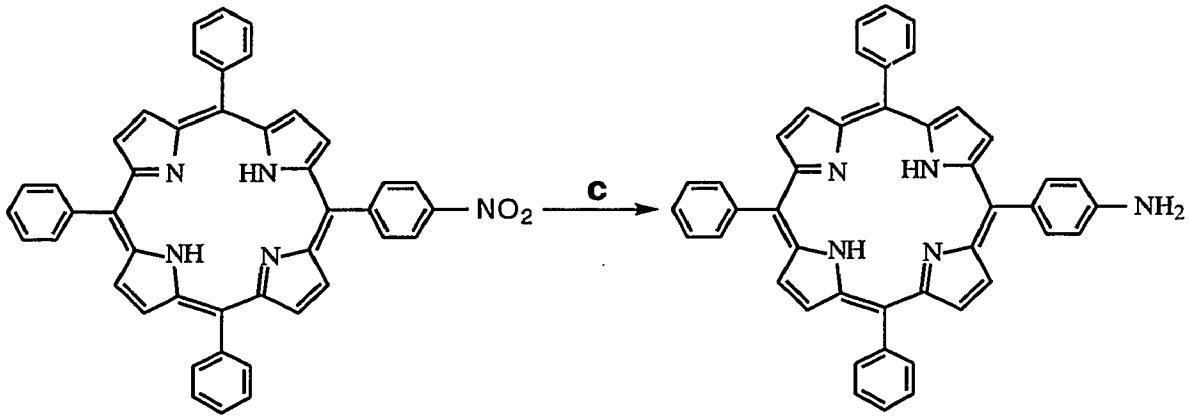
In the first part of project the (aminophenyl)trisulfonatophenylporphyrin (H_2AS_3P) was synthesized and regiospecifically incorporated into immunoglobulin G (IgG) molecules. This work demonstrates that a relatively rigid and sterically hindered synthetic porphyrin can be attached directly to the antibody and in addition, that an aniline functionality is sufficiently reactive for this application.

Instead of the crossed-Rothemund condensation, using the Kruper's procedure, a symmetrical porphyrin, meso-substituted tetraphenylporphyrin (TPP) was mono-nitrated at the para position of one of the peripheral phenyl rings with fuming nitric acid added at a very slow flow rate. The nitro group was then reduced to amine group by stannous chloride. The other three phenyl rings were finally mono-sulfonated at the para positions (Scheme 2-1). The advantages of the Kruper's method used are (a) the starting material, TPP, is readily available (98,100-101,105-107), either commercially at low expense or via a one-step synthesis under uncomplicated conditions; (b) there is no need to protect and deprotect the

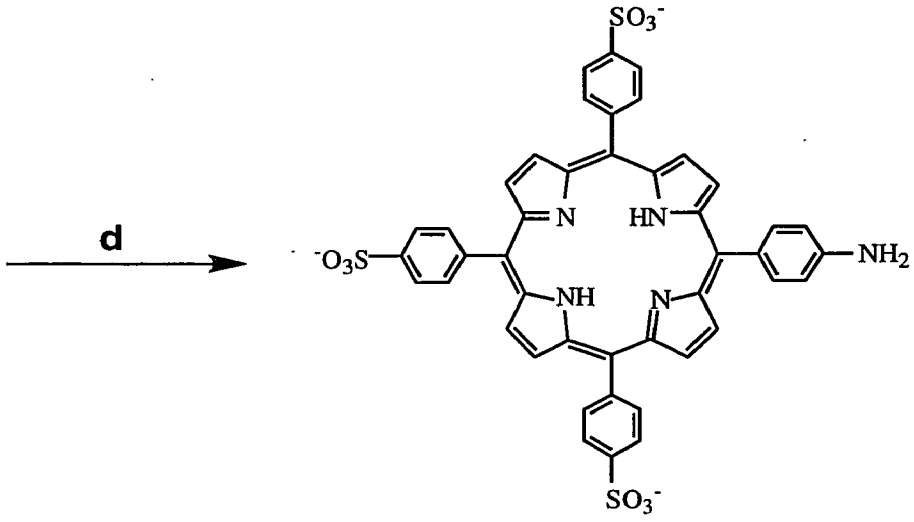
Scheme 2-1. Synthesis of the 5-(4-aminophenyl)-10,15,20-tris(4-sulfonatophenyl)porphyrin (H_2AS_3P). Reaction conditions: (a) propionic acid, reflux; (b) fuming HNO_3, $CHCl_3$, 0-4 °C; (c) $SnCl_2$, concentrated HCl, reflux; (d) concentrated H_2SO_4, 70 °C.



H_2TPP (1)



(2) (3)



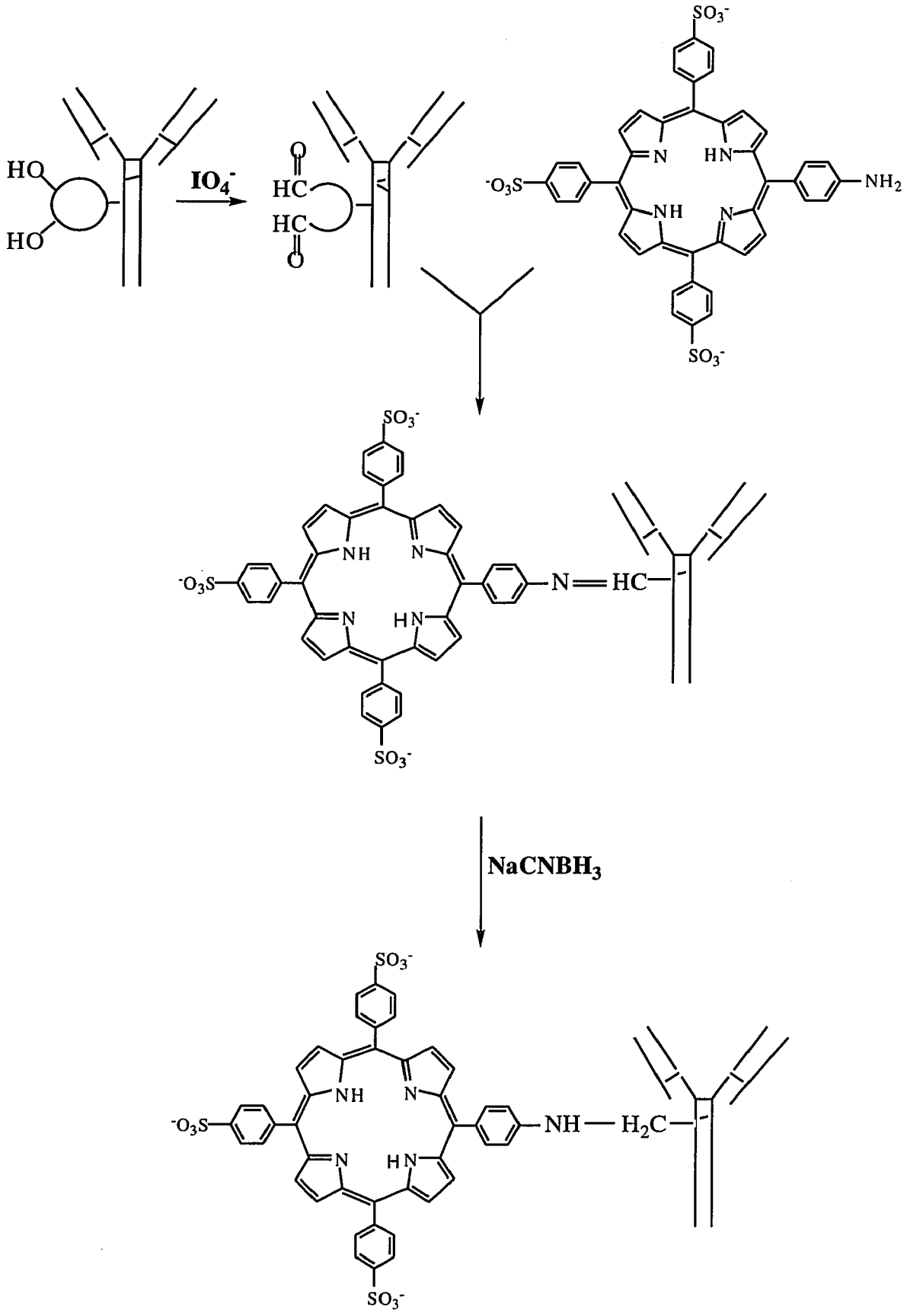
H_2AS_3P (4)

reactive functionality; (c) the asymmetric mono-reactive functionality enables the coupling with antibodies without any crossliking among them (19); (d) the sulfonate groups ensure the solubility in the aqueous media, which is demanded by the reaction with antibodies.

Having such a bifunctional chelator in hand, it is then appropriate to explore the regioselective modification of an antibody. Since the chemical procedure used for conjugation with the immunoglobulin molecule must be mild and also specific for a certain site, the location of the oligosaccharides in the constant region of the immunoglobulin molecule was thought to be favorable for incorporating the nuclide chelates (108-110). The structure of the carbohydrate substrate has been elucidated for several immunoglobulins (111-113). Mild treatment of cis-glycols of the sugars with sodium periodate gives di-aldehydes, which are suitable for Schiff base formation with amines. We have investigated optimal conditions for such treatment of a rabbit immunoglobulin G(RIgG) with the periodate and the subsequent reaction of the oxidized antibody with the amino group of H_2AS_3P (Scheme 2-2).

The porphyrin-antibody conjugate has a much greater molecular weight than the free porphyrin. However, unreacted porphyrins cannot be separated completely from the conjugates by using conventional gel filtration, the method used in biological experiments to purify macromolecules (114-120). Unfortunately, porphyrins strongly adhere to proteins non-

Scheme 2-2. The conjugation reaction of H_2AS_3P and IgG.


HO
HO
IO4-
HC
HC
O
O
SO3-
-O3S
N
HN
NH
N
NH2
SO3-
SO3-
NH
N
-O3S
N
HN
N
HC
SO3-
NaCNBH3
NH
N
-O3S
N
HN
NH
H2C
SO3-

covalently, that is, they are "sticky". Therefore, gel filtration using Sephadex G-25 is not capable of completely separating free porphyrin from a porphyrin-antibody non-covalent combination, as seen from the behavior of control samples containing a mixture of porphyrin and antibody that was not coupled. When such samples are eluted from a Sephadex G-25 column, a yellow band, lighter in intensity than the covalent conjugates, is eluted.

Considering the fact that any charged ion or group will migrate when placed in an electric field and that the electric force would be stronger than the London dispersion force which is the main cause of the porphyrin sticking, polyacrylamide gel electrophoresis was chosen to completely purify and then characterize the conjugates. In polyacrylamide gel electrophoresis (PAGE), the separation is dependent on both charge density (the ratio of charge to mass) and size of proteins. The gel is a porous media in which the pore size is of the same magnitude as the size of the polypeptide molecules. Therefore, a molecular sieving effect occurs in addition to separation due to migration in the electric field (121). Thus two peptides of different sizes but identical charge densities could be separated by PAGE since the molecular sieving effect would cause difference in the migration rates of the molecules.

Antibodies can be dissociated into light and heavy chains by dissociating agents. PAGE can be used to determine the location of the porphyrin on either light or heavy chain

because the size difference between the heavy chains and the light chains of antibodies is large enough. The antibody conjugates with porphyrins were denatured by heating at 95 ^{0}C in the presence of excess dissociating agent, sodium dodecyl sulphate (SDS), and a thiol reagent (to cleave disulphide bonds), 2-mercapto-ethanol. Under these conditions, most of heavy chains and light chains were dissociated and bound to SDS in a certain ratio (1.4 g of SDS per gram of polypeptide) (121). The SDS-polypeptide complexes have almost identical charge densities due to the predominant negative charge provided by SDS. Thus they migrate in polyacrylamide gels according to polypeptide sizes (122-123).By referring to the position of polypeptides of known molecular mass electrophoresed on the same slab gel, the heavy chain and light chain bands could be identified.

An electrophoretic experiment using stained and unstained antibodies was used to determine the incorporation position by the porphyrin. All the protein bands on the slab gel could be stained by an organic dye, while the unstained porphyrin-polypeptide conjugate band was visualized by the coloration of the porphyrin. Two groups of the same set of samples were loaded on a slab gel. After electrophoresis, the gel was cut in half, each having one group of samples. One was then stained, the other not. By aligning the two parts of the gel and comparing the band positions, it could be determined whether or not the porphyrin couples to the antibody and if it binds to the heavy chains or the light

chains. If the porphyrin did not attach to the antibody covalently, there would not be any color of porphyrin at either the position of the heavy chain band or that of the light chain band on the unstained gel. The unconjugated pophyrin band would be seen at the very low molecular weight region which is much farther from the light chain band due to its much smaller molecular weight. In addition, if the attachment is not regiospecifical at the sugar moieties, the porphyrin's color would appear at both heavy chain and light chain regions. Only the location of the porphyrin at the heavy chain position can demonstrate regiospecific conjugation since the covalent bonding between the porphyrin and the antibody should occur at the oligosaccharide region, which is only found in the heavy chains of antibodies. The porphyrin and antibodies fixed in the electrophoresis gel can be detected by visible and ultraviolet absorption. However, the staining and unstaining methodology we used is no doubt the simpler qualitative determination of location of the coupled pophyrin.

The coupling yield were determined by recovering the conjugate sample from the electrophoresis gel using electroelution. It was then scanned at 415 nm for porphyrin absorbance and at 280 nm for protein absorbance. Since the unreacted, sticky porphyrin was removed completely after SDS-PAGE, the recovered conjugate solution was very pure. Therefore, the ratio of the two absorbances reflected the ratio of the numbers of porphyrins and antibodies as long as

their extinction coefficients were known. This is a simple, effective and accurate method to determine the conjugation yield.

2.2 Materials and Instruments:

The polyclonal rabbit immunoglobulin G (RIgG) was purchased from Sigma Chemical Co. (St. Louis, MO.) and used without further purification. Gel electrophoresis reagents were obtained from Bio-Rad (Richmond, CA.). The gel filtration resin Sephadex G-25 was purchased from Pharmacia Fine Chemicals (Piscataway, NJ.) A peristaltic pump, also from Pharmacia, was used to control the flow rates of the gel filtration. The "Mighty Small" SE200 slab gel electrophoresis unit was purchased from Hoefer Scientific Instruments (San Francisco, CA.). The electroelution of slab gel was carried out on Little Blue Tank electroelution system from ISCO (Lincoln, NE.). A DU-8 UV-Visible Spectrophotometer from Beckman Instruments, Inc. (Irvine, CA.) and GE QE-300 NMR spectrometer were used for compound identification and determination of number of porphyrin per antibody. Elemental analysis data was obtained from Galbraith Laboratories, Inc. (Knoxville, TN).

All other chemical reagents, dialysis membrane, and organic solvents were purchased from Sigma, Aldrich Chemical Co., or Fisher Scientific.

2.3 Results and Discussion:

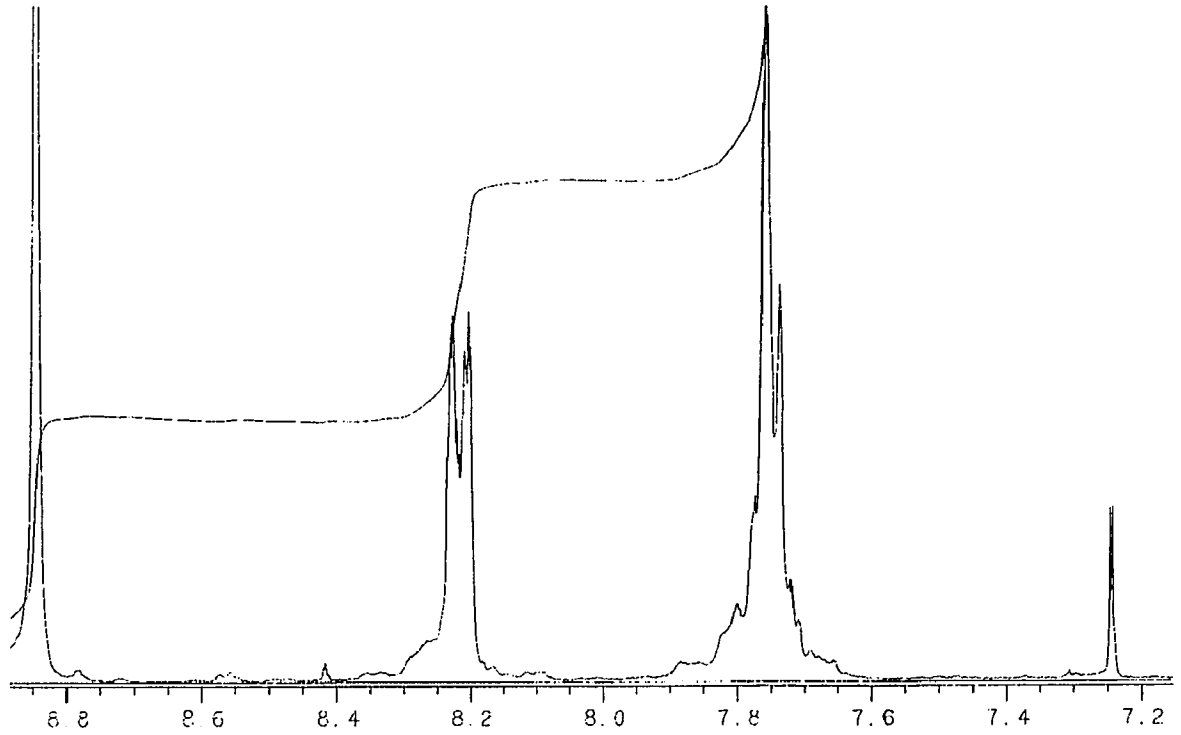
2.3.1 Synthesis of the Porphyrin

2.3.1.1 Synthesis of Meso-substituted Tetraphenylporphyrin (H_2TPP)

The Adler-Longo method (98) was used in this synthesis. Although this compound is commercial available and the reaction yield is low, it is worthwhile to synthesize H_2TPP in the lab because of the low expense of the starting materials. Two procedures were investigated. The pyrrole was freshly distilled in both cases. In one case (procedure A) all of benzaldehyde was put in refluxed propionic acid first, then added pyrrole slowly into the mixture. In the other (procedure B) the pyrrole and benzaldehyde were mixed first, which was then slowly added to the refluxed propionic acid. The yields in the both procedures were about the same (5-6%) and there are no differences between their ^1H NMR spectra in $CDCCl_3$ (Figure. 2-1), from which it can be seen that the H_2TPP is a symmetrical compound: a singlet at 8.85 ppm indicates eight β-pyrrolenine hydrogen atoms of porphyrin ring, a multiplet 8 H at 8.22 ppm is contributed by the ortho hydrogens of four peripheral phenyls, another multiplet 12 H at 7.75 ppm represents the meta and para hydrogens of the phenyls. Yet the latter procedure seemed more reasonable in terms of lab technique and that the yield was a little bit higher by 0.5%.

2.3.1.2 Direct Nitration of Tetraphenylporphyrin

Figure 2-1. The 300 MHz ^1H NMR spectra of the solution of meso-substituted tetraphenylporphyrin (H_2TPP) (substance **1** in Scheme 2-1) in $CDCl_3$. The upper one is for the product from procedure A, the lower from procedure B. There is no difference between them. (The peak located at 7.25 ppm is contributed by trace of $CHCl_3$ in solvent).


8.8
8.6
8.4
8.2
8.0
7.8
7.6
7.4
7.2

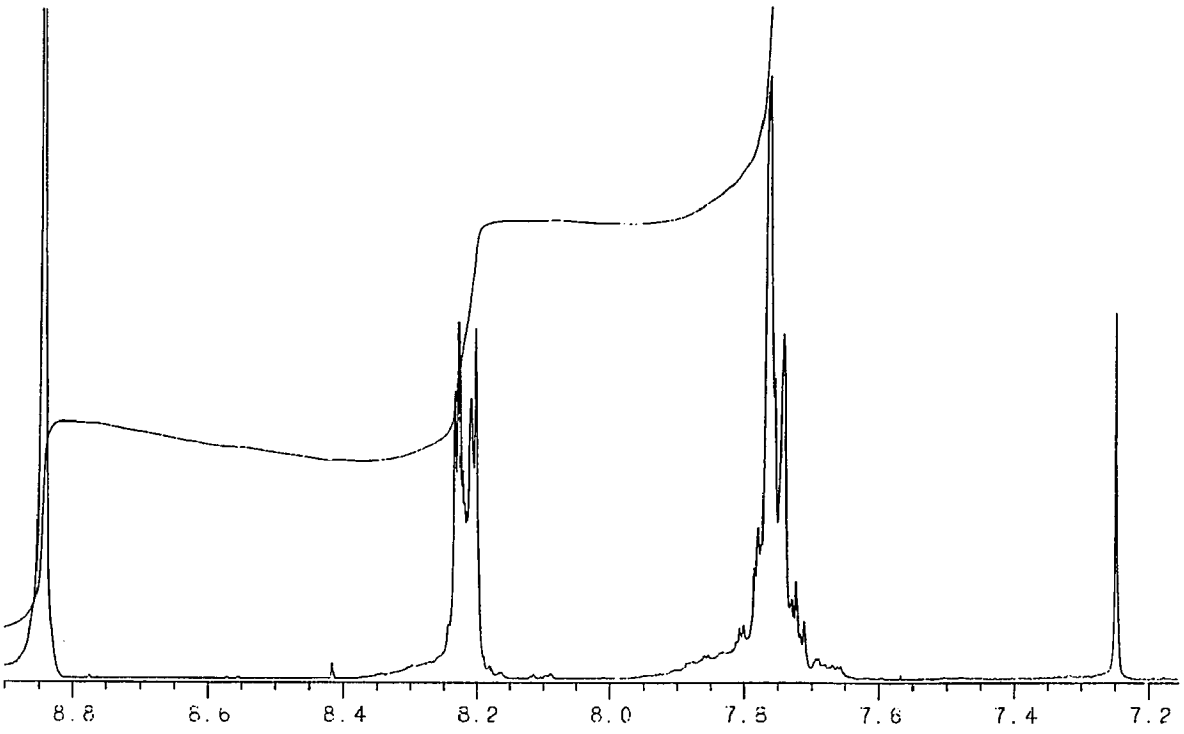
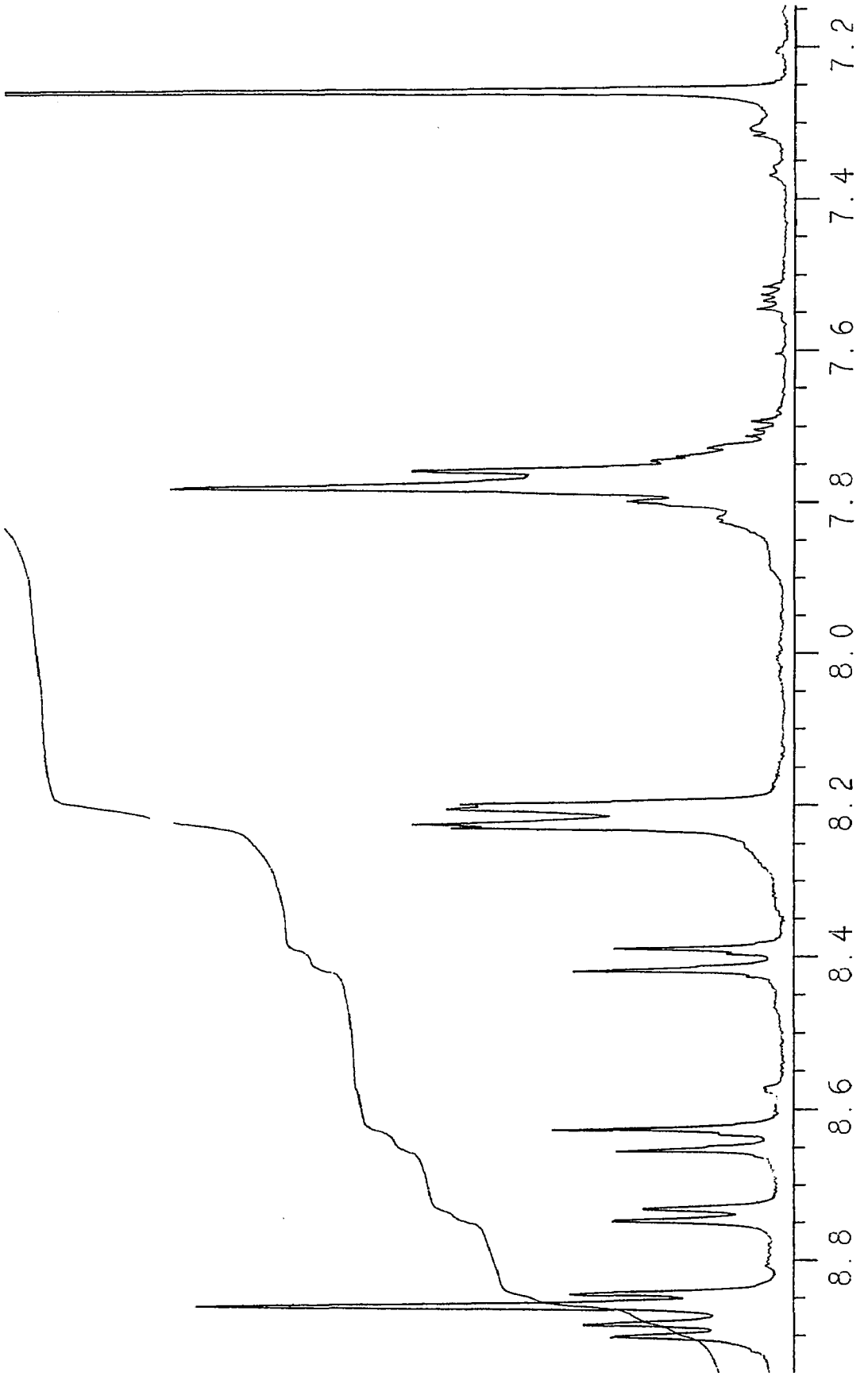

8.8
8.6
8.4
8.2
8.0
7.8
7.6
7.4
7.2

Figure 2-2. The 300 MHz ^{1}H NMR spectrum of a solution of 5-(4-nitrophenyl)-10,15,20-triphenylporphyrin (substance **2** in Scheme 2-1) in $CDCl_3$. (The peak located at 7.26 ppm is contributed by trace of $CHCl_3$ in solvent).


7.2
7.4
7.6
7.8
8.0
8.2
8.4
8.6
8.8

With excess fuming nitric acid (density 1.45 g mL^{-1}), nitration of phenyl groups of tetraphenylporphyrin (H_2TPP) at the para position occurred stepwise. Figure 2-2. shows the ^1H NMR spectrum of the mononitroporphyrin in which the 8 H β-pyrrolenine hydrogen peak is no longer singlet due to loss of symmetry of the porphyrin. It became three peaks of different chemical shifts: one singlet at 8.86 ppm from the four hydrogens far away from the nitrophenyl ring, one doublet at 8.88 ppm from the two hydrogens close to the nitrophenyl group and the other doublet at 8.73 ppm from the two in the middle. Another pair of two-proton doublets at 8.39 ppm and 8.63 ppm from the nitrophenyl ring indicates the para position was substituted. The original eight-proton ortho hydrogen multiplet and twelve-proton meta/para hydrogen multiplet are now assigned for six ortho hydrogens and nine meta/para hydrogens of the three non-substituted phenyl rings.

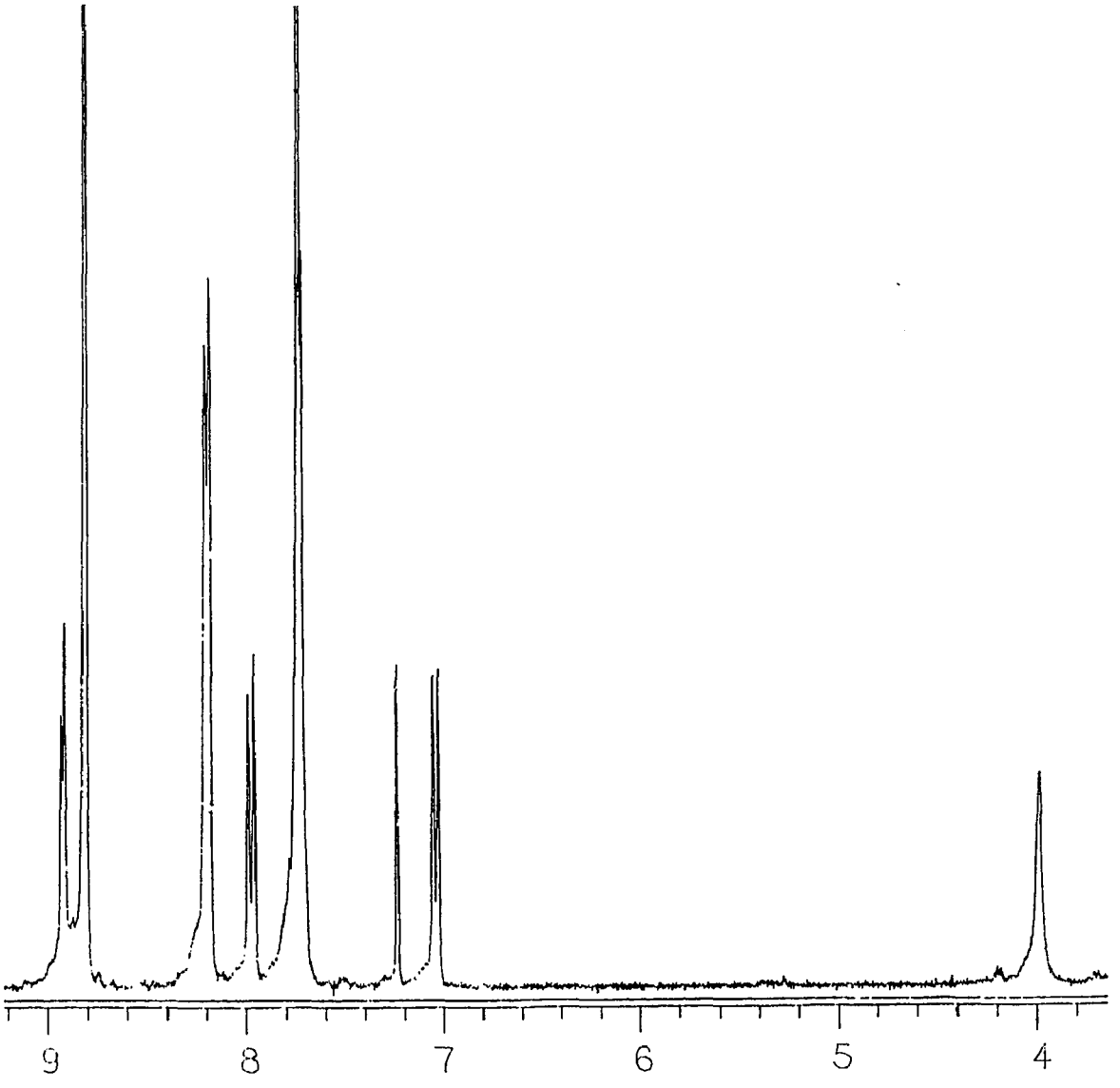
The rate of nitration and the selectivity for mononitroporphyrin were dependent upon the concentration of the starting material, H_2TPP, and the amount of excess HNO_3. When the solvent volume was less, the conversion from H_2TPP to mononitroporphyrin was faster, but a greater amount of the two dinitroporphyrin isomers was produced. If the solvent volume is too large, however, the complete conversion of H_2TPP to mononitroporphyrin was very slow. Under the same conditions, dinitroporphyrins, as well as trinitroporphyrin, was formed before the H_2TPP was completely converted to the

mononitroporphyrin. At the same time, macrocycle degradation increased due to exposure to the concentrated acid. Monitored at intervals by TLC, the reaction was controlled to such an extent that maximum amount of mononitroporphyrin was produced with minimal retention of starting materials, multinitroporphyrins, and degradation residues left. The highest yield of mononitroporphyrin obtained from the reactions was 57% and the lowest was 26%. Optional reaction conditions are given in the experimental section of this chapter. A modification of the solvent system used by Kruper and coworkers to isolate the desired product from column chromatography is also described in the experimental section.

2.3.1.3 Reduction of 5-(4-Nitrophenyl)-10,15,20-triphenyl-porphyrin

The reduction of (nitrophenyl)triphenylporphyrin was carried out according to a modification of Kruper's procedure (Section 2.4.1.3). The nitroporphyrin was stirred in refluxing hydrochloric acid for one hour before adding stannous chloride. This modification improved the dissolution of the starting material in a concentrated aqueous acid. The (aminophenyl)triphenylporphyrin was obtained in 80% yield from the improved procedure. The ^{1}H NMR spectrum of the aminoporphyrin (Figure.2-3) revealed the difference of electronic inductive and conjugate effects between the nitro group and the amino group. First, the three β-pyrrolenine hydrogen peaks (8.88, 8.86, and 8.73 ppm in Figure 2-2)

Figure 2-3. The 300 MHz ^{1}H NMR spectrum of a solution of 5-(4-aminophenyl)-10,15,20-triphenylporphyrin (substance **3** in Scheme 2-1) in $CDCl_3$. (The peak located at 7.26 ppm is contributed by trace of $CHCl_3$ in solvent).

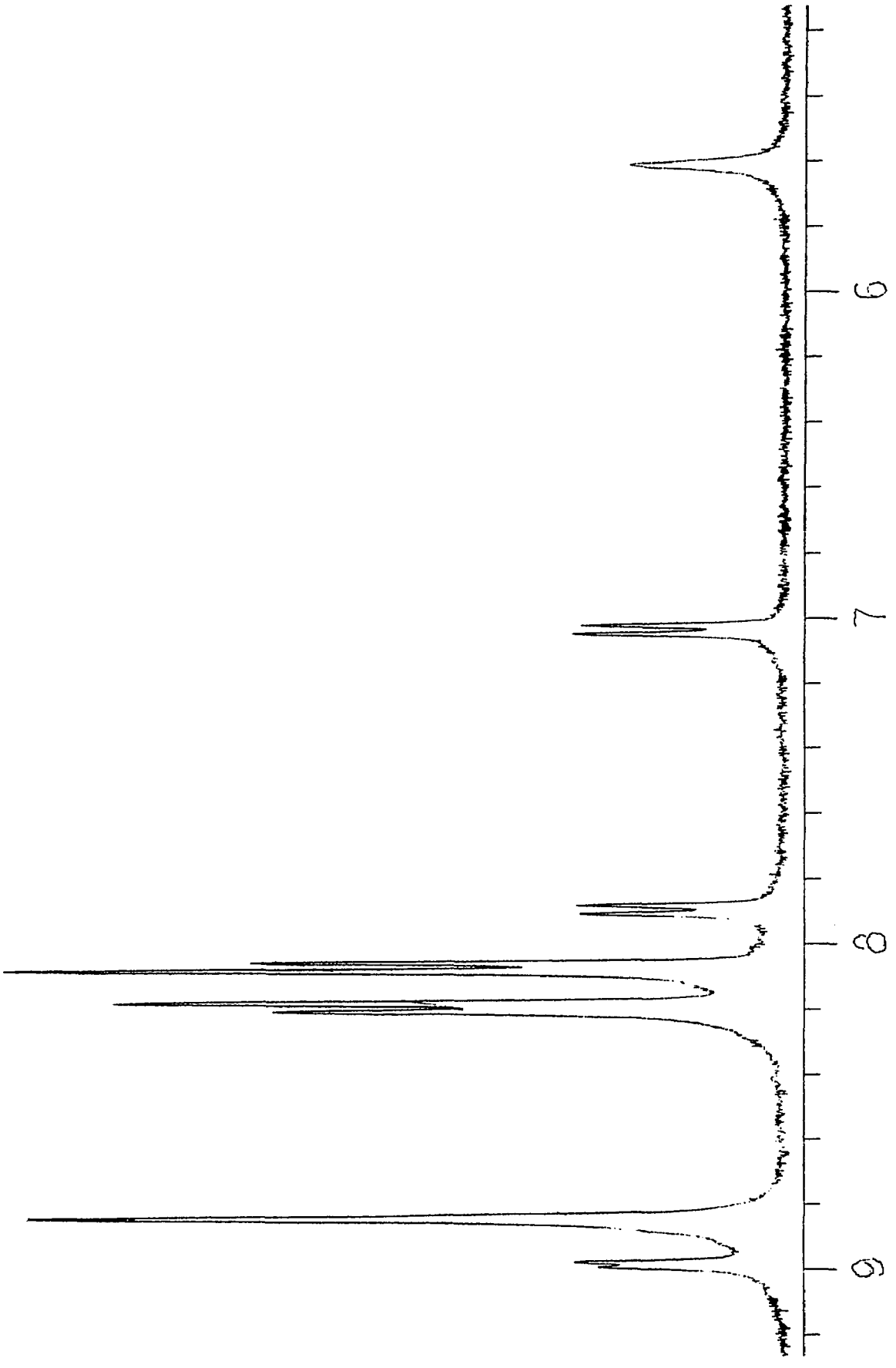

9
8
7
6
5
4

merged to two: one two-proton doublet from two β-pyrrolenine hydrogens close to the aminophenyl ring and one six-proton singlet from other six β-pyrrolenine hydrogens (8.93 and 8.83 ppm in Figure 2-3). The doublet pair (7.97 and 7.0 ppm) from ortho/meta hydrogens of the aminophenyl ring moving upper field (from 8.63 and 8.39 ppm in Figure 2-2) was the second change. Finally, the broad singlet at 3.95 ppm demonstrates the formation of amino group.

2.3.1.4 Sulfonation of 5-(4-Aminophenyl)-10,15,20-triphenyl-porphyrin

Comparing the NMR spectrum of 5-(4-aminophenyl)-10,15, 20-tris(4-sulfonatophenyl)porphyrin (H_2AS_3P) (Figure 2-4) with that of (aminophenyl)triphenylporphyrin (Figure 2-3), it can be seen that the peak at 8.21 ppm from six ortho hydrogens of the three phenyl groups and the peak at 7.74 ppm from nine meta and para hydrogens in the Figure 2-3 became the pair of doublets at 8.07 ppm and 8.19 ppm shown in the Figure 2-4: one from six ortho hydrogens and one from six meta hydrogens, demonstrating the sulfonation of the all three phenyl groups at the para position. The H_2AS_3P was isolated as the potassium salt rather than as the sodium or ammonium salts which result from Kruper's method. It was found that the potassium salt is more soluble in water than the sodium salt. The ammonium salt is not useful for our project because it would be hydrolyzed and would compete with the aniline group in the reaction with antibodies. Another alternation, in the purification

Figure 2-4. The 300 MHz ^1H NMR spectrum of a solution of 5-(4-aminophenyl)-10,15,20-tris(4-sulfonatophenyl)porphyrin (H_2AS_3P) (substance **4** in Scheme 2-1) in DMSO-d_6.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6

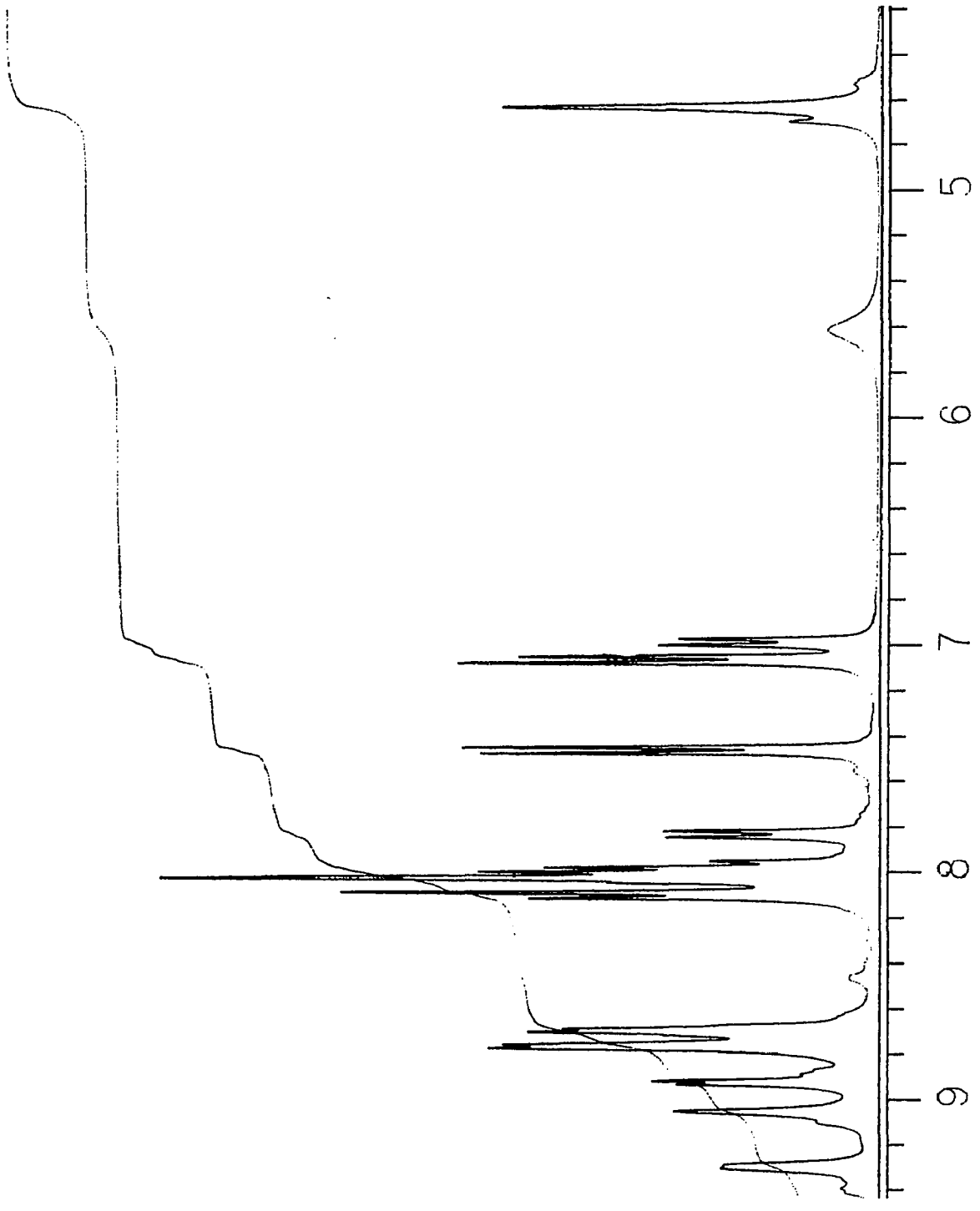
procedure was also made. Since sulfonated dark green suspension was so fine that it would pass through a glass fritted funnel, the suspension could not be washed in the fritted funnel. With centrifuging in intervals, the suspension was washed thoroughly with deionized water

2.3.2 Determination of the Apparent Molecular Weight and the Extiction Coefficient of 5-(4-Aminophenyl)-10,15,20-tris(4-sulfonatophenyl)porphyrin (H_2AS_3P)

In order to determine the extinction coefficient of the product in the aqueous solution, the apparent molecular weight was determined by the ^1H NMR spectral method using tetrakis(4-*N*-methylpyridyl)porphyrin tetra-*p*-tosylate salt (TMPyP-4), a well defined compound(F.W. 1364), as the standard. Samples of TMPyP-4 and H_2AS_3P were dissolved in DMSO-d_6 and the ^1H NMR spectrum of the mixture obtained. The molar ratio of TMPyP-4 to the H_2AS_3P is equal to the ratio of the integral value per TMPyP-4 proton to that of H_2AS_3P. The number of moles of the standard sample is known, so the apparent molecular weight of H_2AS_3P can be determined. Its extinction coefficient was obtained from the absorption spectrum and the apparent molecular weight.

The apparent molecular mass of H_2AS_3P is 1400 based on the molar ratio obtained from the ^1H NMR spectrum of the mixture of TMPyP-4 and the H_2AS_3P (Figure 2-5) and the mass of H_2AS_3P used to prepare the sample.

Figure 2-5. The 300 MHz ^{1}H NMR spectrum of a solution of the mixture of TMPyP-4 and the H_2AS_3P in DMSO-d_6. The integral value per hydrogen for TMPyP-4 is 0.23, that of H_2AS_3P 0.53. So the molar ratio of TMPyP-4 to H_2AS_3P is 1:2.3.


5
6
7
8
9

2.3.3 Preparation and Characterization of the Porphyrin-Antibody Conjugate

Every preparation of the conjugate of the porphyrin H_2AS_3P with the rabbit immunoglobulin G (RIgG) should be followed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) to check the extent of the reaction, because, as mentioned above, unreacted porphyrin sticks to antibodies and can only be separated by the SDS-PAGE. The conditions required for SDS-PAGE in this particular use must be optimized. Neither of the two relative investigations could be first accomplished with the other as a reference. Numerous trials were required for the optimization and development of the SDS-PAGE separation method for these conjugates.

The proper pH and temperature are very important to the conjugation reaction. First, oxidation of IgG with sodium periodate must be carried out in a solution at a pH that does not affect antibody activity. A lower pH is appropriate for the oxidation of IgG to avoid polymerization through inter-molecular Schiff base formation, as pointed out by Murayama, *et al.* (124). Polymerization was evident at the top of the gel, showing that these molecular weights are too high for them to migrate through the gel. There was also a problem with the gel itself because of the acrylamide concentration needed to be completely adjusted, too. The pH condition was so critical for optimal oxidation of IgG that the pH of the

commercial antibody solutions proved problematic. Solid antibody preparatives were employed to avoid the difficulty.

Determining the temperature for optimal oxidation was not so complicated as the pH, but the optimal temperature was a function of the reaction time. Various combinations have been published: 4 hr at 4°C or 30 min at room temperature, by Murayama *et al.*(97); 8 h at room temperature, by Willan *et al.*(82); 1 h at 4°C, by Rodwell *et al.*(22); and etc.. Reactions requiring long period at room temperature were not effective in our trials. To prevent antibodies from losing immunoreactivity, the combination of a low temperature and long period of time, i.e. 8 h at 4°C, was preferable. Subsequent reaction of the oxidized IgG with amine, through Schiff base formation, occurred in good yield at either 4°C or room temperature, overnight or for 8 h. The appropriate pH of the buffer is determined by the reactivity of the amine. For the non-*N*-substituted porphyrin H_2AS_3P, the reaction was run at pH 6.0, which is similar to the most common published conditions for this type of reaction.

The SDS-PAGE technique was improved concurrently. To get good resolution of sample components, a discontinuous buffer system has been used in SDS-PAGE. In this technique a slab gel made of two parts of different composition and pH is used. The sample is loaded at the top of a large-pore stacking gel placed on top of a small-pore resolving, or running, gel (125). The optimal acrylamide concentrations of both stacking gel and running gel were determined by many

trials so that the proteins in sample solutions would stack tightly in sharp bands before migrating into the running gel, and be resolved in well separated regions of the running gel. Otherwise, the proteins would either stay in the stacking gel, or the resolution in the running gel was poor.

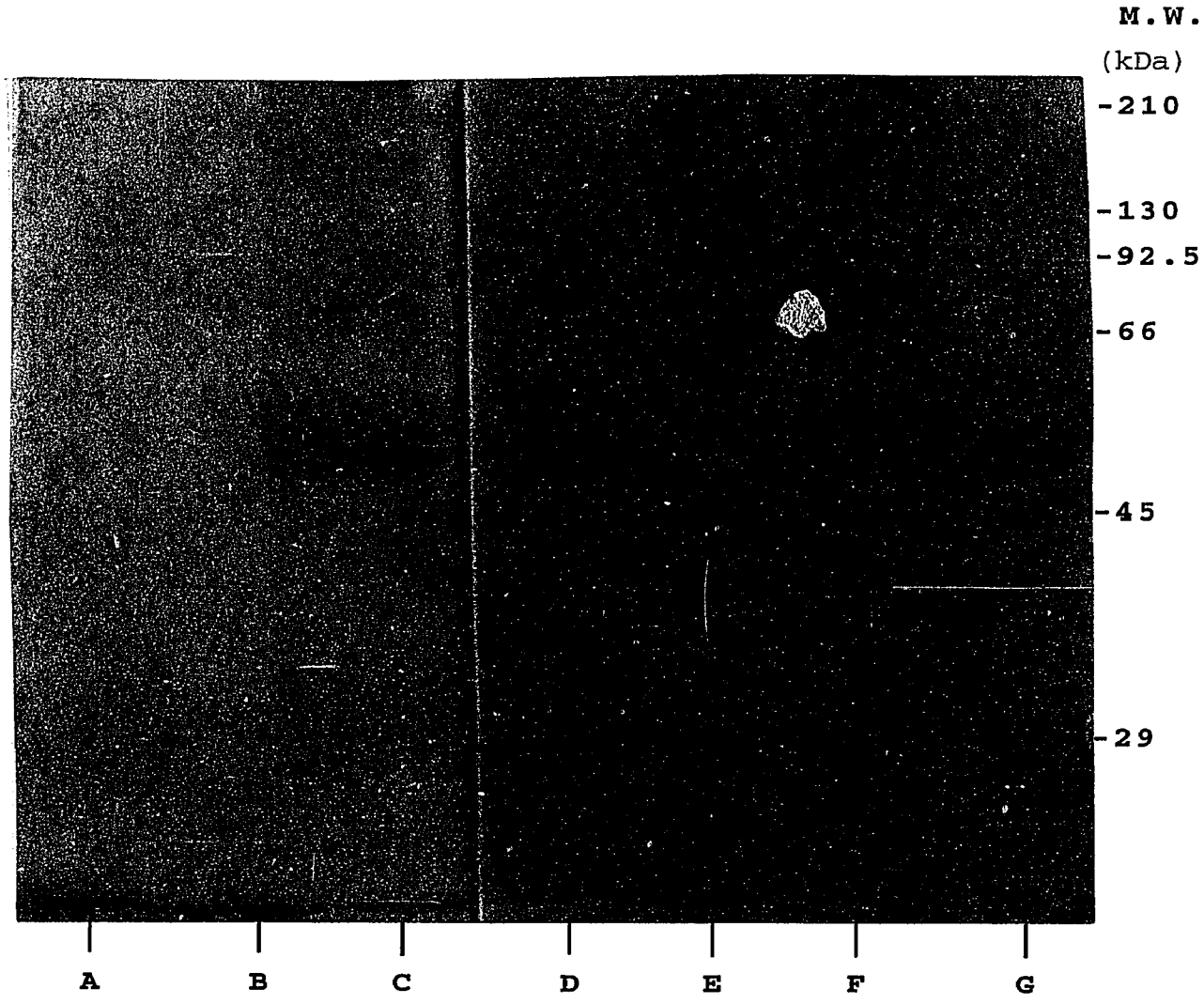
Another condition important for good gel resolution is the denaturing of conjugate samples. In almost all studies of electrophoresis, protein samples are dissociated into their individual subunits. The most common dissociating agent used is sodium dodecyl sulphate (SDS). A thiol reagent is used to cleave the disulphide bonds between the heavy chains and the light chains of antibodies.

The result of SDS-PAGE (Figure 2-6) demonstrates the site-specific attachment of antibodies with porphyrins. The antibody RIgG, when reduced with 2-mercaptoethanol, dissociated into individual polypeptides: heavy chains, on which the oligosaccharide is attached, and light chains, of molecular mass 50-70 kDa and 22-23 kDa, respectively, as seen on the stained gel (D) (F) (G). The standard is in lane (E).

There is difference in color between the heavy chain band of the conjugate (F) and the other bands due to heavy chains. All bands but the one assigned to the conjugate are blue due to the protein stain. The conjugate band, however, is greenish due to the mix of blue and yellow, the color of the porphyrin H_2AS_3P.

Additional evidence for the site-specific attachment of porphyrin is shown on the unstained gel. The yellow spot in

Figure 2-6. SDS-PAGE slab gel. (A)-(C) unstained. (D)-(G) stained. (A) free porphyrin, (B) control, (C) conjugate, (D) antibody RIgG, (E) standard, (F) conjugate, (G) control.


M.W.
(kDa)
-210
-130
-92.5
-66
-45
-29
A
B
C
D
E
F
G

lane (C) appears at the same height as that of heavy chains on the stained gel and there are no yellow at the positions of the light chains.

Since there are no aldehydes generated from the oxidized oligosaccharide in the control sample (B) or the free porphyrin sample (A), the porphyrin could not bind to the antibody. In these samples, the porphyrin migrated to the bottom of gel. The porphyrin-antibody non-covalent combination which could not be separated by gel filtration was separated by electrophoresis, as noted in lanes (B) and (G). Unstained lane (B) only shows the separated porphyrin, stained lane (G) can show the separated antibody.

The SDS-PAGE results indicate that the yield for conjugation is high but not quantitative. There is a very faint yellow coloration at the bottom of the gel in the covalent conjugate sample lane (C) after electrophoresis, which again indicates that Sephadex separation of unconjugated porphyrin is incomplete. Hydrolysis of the covalent linkage between the porphyrin and the oligosaccharide moiety was ruled out as the cause for this coloration because the extent of coloration does not differ if samples are electrophoresed within 1 or 2 hours of conjugation or if they are allowed to remain at room temperature for 24 hours before electrophoresis. SDS-PAGE is, therefore, required for analytical separation.

2.3.4 Determination of the Number of Porphyrins Coupled to the Antibody

The ratio of the visible absorption of porphyrin to the UV absorption of the heavy chain of the antibody obtained from a spectrum (Figure 2-7) of the porphyrin-antibody (heavy chain) conjugate solution after electroelution, is 1:1.5. Using the extinction coefficients for H_2AS_3P at 414 nm, 3.3×10^5 M^{-1} cm^{-1} (see calculation in Section 2.4.4), and for the heavy chain of the antibody at 278 nm, 1.0×10^6 M^{-1} cm^{-1}, the molar ratio of heavy chain to porphyrin was calculated as 1:2. Therefore, on average, there are 4 porphyrins covalently attached to each antibody. It is likely that there is a distribution of conjugates with different porphyrin to antibody ratios, averaging about 4:1. Isoelectric focusing (126) may be used to determine the relative amounts of each conjugates in such mixtures.

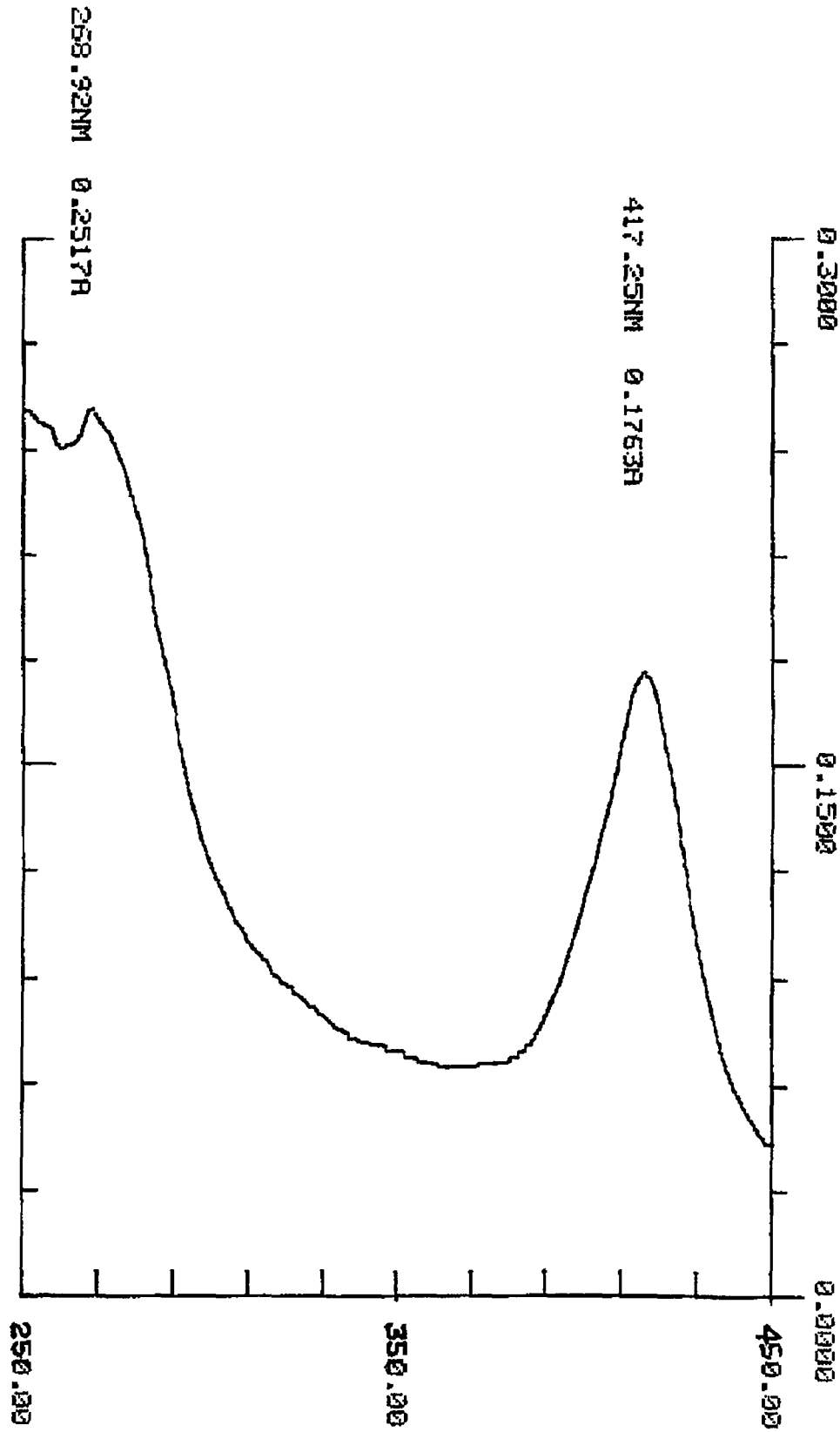
2.4 Experimental Section:

2.4.1 Synthesis of 5-(4-Aminophenyl)-10,15,20-Tris(4-Sulfonatophenyl) Porphine

2.4.1.1 5,10,15,20-Tetraphenylporphyrin (H_2TPP) (**1**)

Procedure A: Benzaldehyde (13.3 mL, 0.133 mol) was dissolved in 500 mL of propionic acid in a 1000 mL two neck round bottom flask, and the solution was then heated to refluxing. Freshly distilled pyrrole (9.3 mL, 0.133 mol) was slowly added into the stirred solution of benzaldehyde from an additional funnel over half an hour. After continued

Figure 2-7. The UV-visible spectrum of a porphyrin-antibody (heavy chain) conjugate solution after electroelution from a SDS-PAGE slab gel.


258.42NM 0.2517A
417.25NM 0.1763A
0.3000
0.1500
0.0000
250.00
350.00
450.00

refluxing for another 30 min with magnetic stirring, the reaction was cooled and left at room temperature overnight under a drying tube. The mixture was cooled at 0-4 °C in a ice bath for two hours and then filtered through a sintered-glass funnel. The residue was thoroughly washed with three 200 mL portions of cold methanol and then three 200 mL portions of 60 °C hot water. The precipitate was vacuum dried at about 100 °C to give 3.83 g crude H_2TPP.

Procedure B: Benzaldehyde and pyrrole, the same amount of both as in the procedure A, mixed in an additional funnel were added into 500 mL of refluxing propionic acid dropwise. The reaction was refluxed for another 30 min and cooled, and allowed to stand overnight at room temperature The same procedure of purification as above was taken to give 4.1 g of crude product.

The same ^1H NMR ($CDCl_3$) spectra for product obtained by both procedures was obtained: δ 8.85 (s, 8 H, β-pyrrole), 8.22 (m, 8 H, ortho phenyl), 7.75 (m, 12 H, meta/para phenyl). Elemental analysis was not obtained for H_2TPP since it is a well known compound.

2.4.1.2 5-(4-Nitrophenyl)-10,15,20-triphenylporphyrin (**2**)

Tetraphenylporphyrin (4.0 g, 6.5 mmol) was dissolved in 600 mL of distilled chloroform under nitrogen at room temperature. The solution was put in a ice bath after H_2TPP completely dissolved. Fuming nitric acid (5 mL, 115 mmol, density 1.45 g mL^{-1}) was added from a pressure-equalizing

addition funnel to the solution at 0-4 °C. The addition lasted two hours. The reaction was monitored with TLC by using hexane-methylene chloride (1:1 in volume) on silica plates. The product (R_f = 0.47) started to appear 90 min after completion of adding the nitric acid. The starting material (R_f = 0.71) was almost completely converted and the dinitrated porphyrin (R_f = 0.26) started to appear. The reaction mixture was washed with six 300 mL portions of distilled water and dried over magnesium sulfate for two hours. After filtration, the filtrate was concentrated to 50 mL and was loaded onto a silica column (15 in.× 3 in. Fisher 60-200 mesh) and eluted with hexane-methylene chloride (50/50 in volume). The second band (mononitropoprphyrin) was collected and the solvent was removed using a rotary evaporator. The product was vacuum dried to give 5-(4-Nitrophenyl)-10,15,20-triphenylporphyrin in 57% yield.

^{1}H NMR($CDCl_3$) δ: 8.88 (d, 2 H. J = 5.1 Hz, β-pyrrole), 8.86 (s, 4 H, β-pyrrole), 8.73 (d, 2 H, J = 4.7 Hz, β-pyrrole), 8.63 (d, 2 H, J = 8.6 Hz, nitrophenyl), 8.39 (d, 2 H, J = 8.7 Hz, nitrophenyl), 8.20 (m, 6 H, ortho phenyl), 7.77 (m, 9 H, meta/para phenyls), -2.80 (s, 2 H, pyrrole NH). Anal. Calcd. for $C_{44}H_{29}N_5O_2 \cdot C_6H_{14}$: C, 80.46; H, 5.76; N, 9.39; O, 4.29. Found: C, 80.07; H, 5.54; N, 9.39; O, 4.12.

2.4.1.3 5-(4-Aminophenyl)-10,15,20-triphenylporphyrin(**3**)

To (nitrophenyl)triphenylporphyrin (2.25 g, 3.41 mmol) was added 72 mL of concentrated hydrochloric acid in a 250-mL

three neck round bottom flask under nitrogen. The mixture was refluxed for 1 h to dissolve the porphyrin. Stannous chloride (anhydrous) (2.0 g, 10.5 mmol) was then added, and the reaction was refluxed for another 1 h. The TLC by using a 1:1 mixture of hexane and methylene chloride on silica plates showed complete conversion of starting material (R_f = 0.68) to product (R_f = 0.22). The reaction was cooled and poured into 300 mL of cold distilled water, and the pH of the solution was brought to 8.0 with concentrated ammonium hydroxide. The aqueous solution was then extracted with six 300 mL portions of methylene chloride. The organic phases were combined and dried over magnesium sulfate. After filtration, the solution was concentrated to 80 mL and applied to a silica column with a 1:1 mixture of hexane and methylene chloride as the first eluent to remove a small amount of unreacted porphyrin. The second and main band was then eluted with methylene chloride, leaving degradation products on top of the column, to give (aminophenyl)triphenylporphyrin in 80% yield.

^{1}H NMR($CDCl_3$) δ: 8.93 (d, 2 H, J = 4.7 Hz, β-pyrrole), 8.83 (s, 6 H, β-pyrrole), 8.21 (m, 6 H, ortho phenyl), 7.97 (d, 2 H, J = 8.6 Hz, aminophenyl), 7.74 (m, 9 H, meta/para phenyls), 7.0 (d, 2 H, J = 8.3 Hz, aminophenyl), 3.95 (s, 2 H, amino), -2.70 (s, 2 H, pyrrole NH). Anal. Calcd. for $C_{44}H_{31}N_5$: C, 83.91; H, 4.96; N, 11.12%. Found: C, 84.05; H, 4.96; N, 11.02.

2.4.1.4 5-(4-Aminophenyl)-10,15,20-tris(4-sulfonatophenyl)-porphyrin, Tripotassium Salt (**4**)

(Aminophenyl)triphenylporphyrin (1.65 g, 2.6 mmol) was dissolved in 72 mL of concentrated sulfuric acid. The solution was heated at 70 °C for 2 days and then stirred at room temperature for 3 days under nitrogen atmosphere. The dark green suspension was poured into 500 mL of distilled water and left overnight. The supernatant was decanted and the precipitate washed with two 300 mL portions of distilled water with centrifuging in the interval. An aqueous potassium hydroxide solution (2 mL, 2 N) was added to the slurry and the porphyrin dissolved. The aqueous porphyrin solution was dialyzed against distilled water by using a M.W.500 cutoff membrane to give purified tripotassium salt of (aminophenyl)-tri(*p*-sulfonatophenyl)porphyrin in 80% yield.

^{1}H NMR (DMSO-d_6) δ: 8.98 (m, 2 H, β-pyrrole), 8.84 (s, 6 H, β-pyrrole), 8.19 (d, 6 H, J = 7.6 Hz, sulfonatophenyl), 8.07 (d, 6 H, J = 7.7 Hz, sulfonatophenyl), 7.89 (d, 2 H, J = 8.1 Hz, aminophenyl), 7.03 (d, 2 H, J = 7.9 Hz, aminophenyl), 5.61 (s, 2 H, amino), -2.85 (s, 2 H, pyrrole NH). Anal.Calcd. for $C_{44}H_{28}N_5O_9S_3K_3 \cdot 9H_2O$: C,46.09; H, 4.04; N, 6.11; S, 8.39; O, 25.12. Found: C, 44.88; H, 4.37; N, 5.64; S, 8.75; O, 25.69.

2.4.2 Synthesis of Porphyrin-Antibody Conjugates

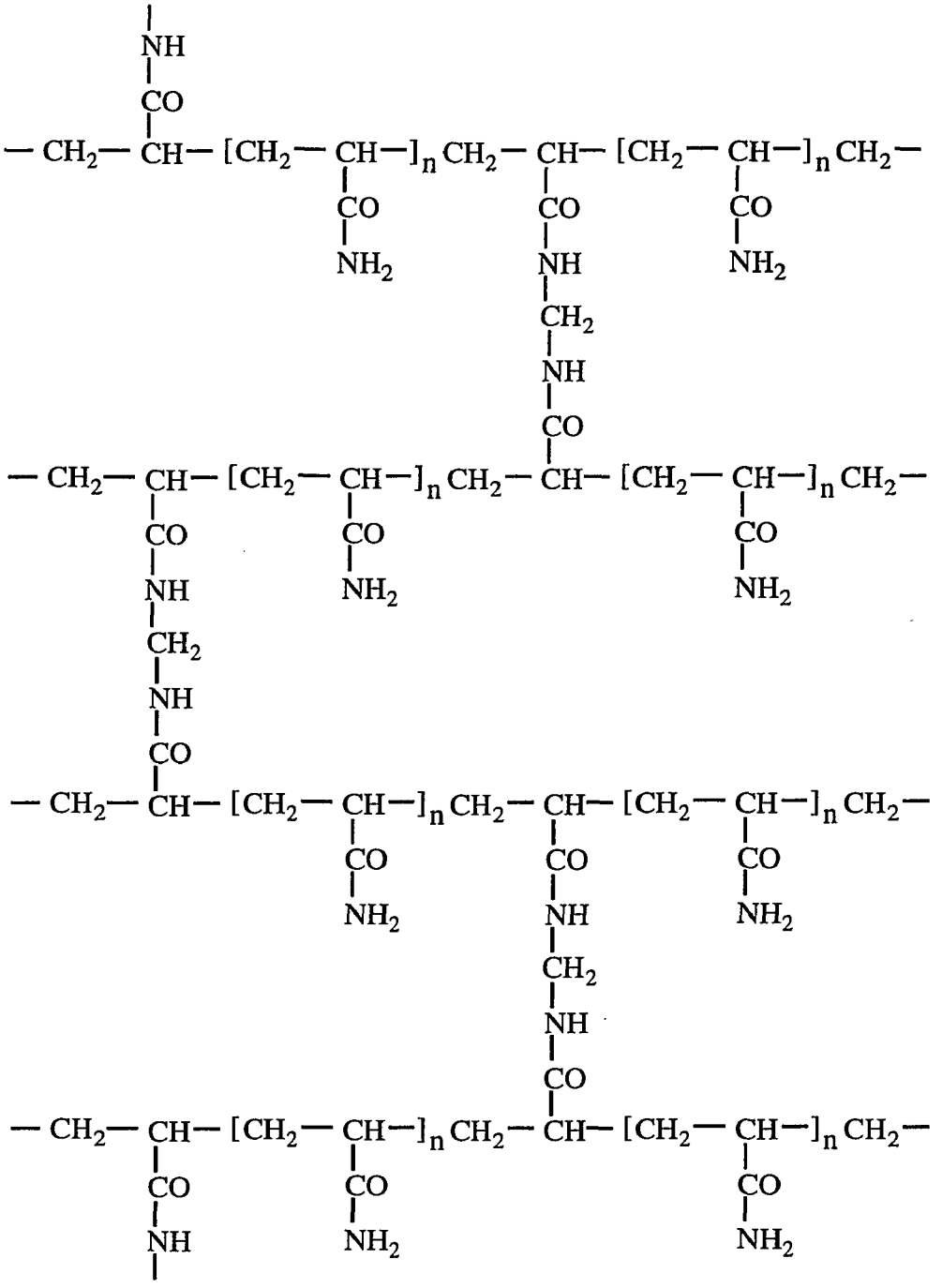
Rabbit immunoglobulin G (IgG) (20 mg, Sigma) was dissolved in 0.9 mL of acetate buffer (0.02 M, pH 4.0) at 0-4 °C with stirring. Sodium periodate solution (0.1 mL, 40 mg in

1 mL of the acetate buffer) was added to the antibody solution and the reaction was stirred in the dark at 0-4 °C for 6 h. The oxidized antibody was purified by passing it through a Sephadex G-25 column (8 cm × 1 cm) equilibrated with phosphate-buffered saline (0.02 M, 0.15 M NaCl, pH 6.0) at 0-4°C. Fractions containing antibodies (detected by UV absorption at 280 nm) were collected to give a total volume of 1.5 mL. This solution was combined with about a 100-fold molar excess of the porphyrin H_2AS_3P solution (0.5 mL, 0.2 g in 5 mL of the phosphate buffer of pH 6.0) and stirred at room temperature for 1 h. Sodium cyanoborohydride solution (0.01 mL, 80 mg in 1 mL of distilled water) was then added, and the reaction kept in the dark at room temperature overnight with stirring. The porphyrin-antibody conjugates were purified from the large excess of free porphyrin on a Sephadex G-25 column (8 cm × 1 cm) with a phosphate buffer at pH 6.0 as a eluent. The yellow conjugate band eluted in 35-45 min with a peak volume of about 2.5 mL at a very slow flow rate (≤0.05 mL/min) controlled by a peristaltic pump. The conjugate solution was stored at -20 °C.

2.4.3 Gel Electrophoresis

Procedures used in this section are mostly those of Hames (121). Polyacrylamide gel was prepared by polymerization of acrylamide monomer into long chains, which, meanwhile, were crosslinked with *N,N'*-methylene bisacrylamide (usually abbreviated to bisacrylamide) to build up a covalent

Figure 2-8. The structure of polyacrylamide gel. Acrylamide monomers are polymerized into long chains which, meanwhile, are crosslinked by *N,N'*-methylene bisacrylamide



meshwork (Figure 2-8). Otherwise, the polymerization without crosslinker results only in a viscous solution. The polymerization of acrylamide was initiated by ammonium persulphate and catalysed by *N,N,N',N'*-tetramethyl-ethylenediamine (TEMED). The rate of polymerization increased when either the TEMED or ammonium persulphate concentration was increased. The pore size of the gel decreased when the total concentration of monomer (acrylamide plus bisacrylamide) was increased. The total concentration of monomer was represented by %T in grams per 100 mL (i.e. w/v), and the percentage (w/w) of crosslinker in the total monomer is represented by %C. When C = 5%, the pore size would reach a minimum (127), but above about T = 15% , the %C required for minimum pore size should increase with the value of %T (128). The ratio of acrylamide to bisacrylamide commonly used was 37.5:1 (2.6%C). A 3%T stacking gel and 10%T resolving gel with the Laemmli SDS-discontinuous buffer system (125) proved to be suitable in the electrophoresis of porphyrin-antibody conjugates.

2.4.3.1 Preparation of Stock Solutions

- *Acrylamide-bisacrylamide* (30:0.8), was prepared by dissolving 30 g of premixed acrylamide/bisacrylamide (37.5:1) (Bio-Rad) in 73 mL of distilled, deionized water with stirring for 30 min. The solution was stored at 4 °C in a dark bottle.

- *TEMED*, used as supplied and stored at 4 °C in a dark bottle.
- *Ammonium persulphate* (10%, w/v), 1.0 g of ammonium persulphate was dissolved in 10 mL of deionized water. This solution should not last for more than 1-2 months.
- *SDS* (10%, w/v), prepared by dissolving 10 g of SDS in distilled water to 100 mL with low heating and stirring until all the SDS dissolved. The solution was stored at room temperature.
- *50% glycerol*, 5.0 mL of glycerol was mixed with 5.0 mL of distilled water.
- *Stacking gel buffer stock*: 1.0 M Tris-HCl (pH 6.8); 12.1 g of Tris was dissolved in 60 mL of distilled water, adjusted to pH 6.8 by adding HCl, and brought to 100 mL final volume with distilled water.
- *Resolving gel buffer stock*: 2.0 M Tris-HCl (pH 8.8); 24.2 g of Tris was dissolved in 80 mL of distilled water, adjusted to pH 8.8 with HCl, and brought to 100 mL final volume with water.
- *Reservoir buffer stock*: 72.05 g of glycine, 15.12 g of Tris, and 5.0 g of SDS were dissolved in and made to 500 mL with distilled water.
- *Sample buffer stock*: 0.625 mL of 1.0 M Tris-HCl (pH 6.8), 0.2 g of SDS, 1.0 mL of glycerol, and 2 mg of Bromophenol Blue (used as a tracking dye) were dissolved in and made to 10 mL with distilled water.

- *2% agarose*, 2 g of agarose was dissolved in 100 mL of distilled water with heating and stirring. It gelled when cooled to room temperature.
- *Stain solution*: 2 g of Coomassie Blue R-250 was dissolved in 400 mL of methanol, brought to 925 mL with distilled water, and 75 mL of glacial acetic acid added. The solution was filtered through a Whatman No. 1 filter paper to remove any insoluble material.
- *Destain solution*: 30% methanol, 7.5% acetic acid; 300 mL of methanol was mixed with 625 mL of distilled water, and 75 mL of glacial acetic acid was then added to bring the final volume to 1000 mL.

2.4.3.2 Preparation of Gel Mixture

The 10%T resolving gel mixture was made of as follows:

a. Acrylamide-bisacrylamide (30:0.8), 2.5 mL
b. 2.0 M Tris-HCl (pH 8.8), 1.875 mL
c. Distilled, deionized water, 3.025 mL
d. 10% SDS, 0.075 mL
e. 10% Ammonium persulfate, 0.025 mL
f. TEMED, 0.005 mL

The 3%T stacking gel mixture was as follows:

a. Acrylamide-bisacrylamide (30:0.8), 0.320 mL
b. 1.0 M Tris-HCl (pH 6.8), 0.502 mL
c. Distilled, deionized water, 1.010 mL
d. 10% Ammonium persulfate, 0.010 mL
e. TEMED, 0.002 mL

In both cases, the last two items, ammonium persulfate and TEMED, were mixed with others just before casting.

2.4.3.3 Preparation of Slab Gels

By using the "Mighty Small" slab gel electrophoresis unit SE 200, a miniature vertical slab gel of polyacrylamide was cast in a sandwich formed by a glass plate, spacers (1.0 mm thick), and a notched alumina plate. The 8 × 10 cm sandwich was clamped against an upper buffer reservoir pod; the notched alumina plate forms one side of the completed upper buffer reservoir. The bottom of the sandwich was sealed by 2% agarose which was melted at 60 °C, run as a line on a warmed glass plate along the bottom of the sandwich, drawn up into the sandwich by capillary effect, and cooled to form a gel plug. The resolving gel mixture (10%T) was poured to within 2 cm of the notch of the alumina plate after the agarose had solidified. Water saturated n-butanol (1 mL) was carefully overlaid, and it floated on the top of the gel, making the edge smooth and horizontal. When the resolving gel was polymerized after 40-50 min, the butanol was removed. The gel was rinsed several times with distilled water, and drained with a piece of tissue. The stacking gel mixture (3%T) was then layered on top of resolving gel using a Pasteur pipette, and a comb was inserted to form sample wells. After the stacking gel polymerized, the upper buffer reservoir-gel sandwich assembly was placed in the lower buffer reservoir with springs to hold it erect. The reservoir

buffer stock (Tris-glycine pH 8.3, 12 mL) was diluted by 10 times with distilled water and was poured into upper and lower buffer reservoirs, each holding about 60 mL. The level of buffer in upper buffer reservoir should be above the notch of the alumina plate after the comb was gently removed, and the level of buffer in lower reservoir should be higher than the bottom of gel in the sandwich, meaning the sample wells in stacking gel are full of reservoir buffer and the resolving gel connected the lower buffer through the agarose gel plug. When two electrodes in the upper and lower reservoirs were connected to a power supply the electric current circulated through the stacking gel and resolving gel. At this point, the gel is ready for loading samples and running electrophoresis.

2.4.3.4 Preparation of Samples

Procedures to prepare SDS-PAGE sample stock solutions which were to be combined with sample buffer and 2-mercaptoethanol and run electrophoresis on a slab gel under the same conditions were as follows:

a. Free Porphyrin:

5-(4-Aminophenyl)-10,15,20-tris(4-sulfonato-phenyl)-porphyrin (H_2AS_3P) (2 mg) was dissolved in 1 mL of distilled water.

b. Control Sample:

Rabbit immunoglobulin G (RIgG) (5 mg) was dissolved in 1 mL of the phosphate-buffered saline (0.02 M, 0.15 M NaCl, pH

6.0), combined with 0.025 mL of the porphyrin H_2AS_3P solution (0.2 g in 5 mL of the phosphate buffer of pH 6.0), and stirred in the dark at room temperature overnight. The mixture of H_2AS_3P and RIgG was then passed through a Sephadex G-25 column (8 cm × 1 cm) at a very slow flow rate (≤0.05 mL/min), and a yellow band eluted with a peak volume of about 1.5 mL, which was to be used as the control sample in SDS-PAGE.

c. Porphyrin-antibody Conjugate:

The porphyrin-antibody conjugate solution (2.5 mL) purified by Sephadex G-25 (see Section 2.4.2) was concentrated by lyophilization to 1 mL.

d. RIgG:

Antibody RIgG (1 mg) was dissolved in 1 mL of phosphate buffer solution (0.01 M, pH 7.2).

e. Standard:

High molecular mass standard mixture (3 mg, Sigma) included myosin (212 kD), β-galactosidase (130 kD), phosphorylase a (92.5 kD), bovine serum albumin (68 kD), egg albumin (45 kD), and carbonic anhydrase (29 kD) was dissolved in 1 mL of phosphate buffer solution (0.01 M, pH 7.2).

With 1.0-mm-thick gel and 10-well comb, the appropriate sample volume loaded on the gel was found to be 20 μL. Samples were prepared according to the recipe in Table and treated at 95 °C for 2 min. They were cooled to 0-4 °C and ready for being loaded onto the slab gel.

Table Recipe for sample preparation using the SDS-discontinuous buffer system

	Sample	Sample buffer	2-mercpto-ethanol	H_2O
(A) Free H_2AS_3P	2 µL	2 µL		16 µL
(B) Control	12 µL	2 µL	2 µL	4 µL
(C) Conjugate	10 µL	2 µL	2 µL	6 µL
(D) RIgG	5 µL	2 µL	2 µL	11 µL
(E) Standard	2 µL	2 µL	2 µL	14 µL
(F) Conjugate	5 µL	2 µL	2 µL	11 µL
(G) Control	6 µL	2 µL	2 µL	10 µL

2.4.3.5 Sample Loading and Electrophoresis

Samples were loaded in the order of (A) through (G) on the gel from left to right, leaving two wells blank between (C) and (D). Each sample was underlayered in a well by using a fine-tipped syringe. The glycerol increased the density of the sample so that, when applied to the gel, the sample remained as a well-defined overlay and did not undergo convective mixing with the reservoir buffer in the wells. The electrophoresis unit was then connected to a power supply with the anode (+) connected to the lower reservoir and the cathode (-) connected to the upper reservoir, and electrophoresis was carried out at 18 mA constant current for about two hours. The power supply was turned off when the tracking dye reached the bottom of the gel (below the surface of the lower buffer).

2.4.3.6 Gel Staining and Destaining

Before it were peeled from the alumina plate, the gel was cut in half between (C) and (D). The right half of gel containing samples (D), (E), (F), (G) was put in a tray and stained with the acidic Coomassie Blue R-250 solution for 30 min at room temperature. The left of gel containing samples (A), (B), (C) was put in another tray and immersed with the destaining solution. The stained gel was rinsed thoroughly with water and then left in destain solution for at least 48 h at room temperature to remove the excess stain. The longer the destaining, the clearer the gel background obtained.

These two pieces of gel were placed on filter-paper and dried by using a gel drier (Bio-Rad). The result of the gel electrophoresis was explained in the Section 2.3.3.

2.4.4. Determination of the Apparent Molecular Weight and the Extiction Coefficient of 5-(4-Aminophenyl)-10,15,20-tris(4-sulfonatophenyl)porphyrin (H_2AS_3P)

Samples of tetrakis(4-*N*-methylpyridyl)porphine tetra-*p*-tosylate salt (TMPyP-4) (12.4 mg, F.W. 1363.63) and 5-(4-aminophenyl)-10,15,20-tris(4-sulfonatophenyl)porphine tripotassium salt (H_2AS_3P) (29.4mg) were mixed and dissolved in DMSO-d_6. The ^1H NMR spectrum of the above solution was taken. The molar ratio of TMPyP-4 to H_2AS_3P, obtained from the ^1H NMR spectrum (Figure 2-4), is 1:2.3. The number of moles of the standard sample is known, so the apparent molecular weight of H_2AS_3P was calculated as 1,395. The visible absorption of the 4.3 mM H_2AS_3P aqueous solution at the wavelengths 414 nm is 1.4, therefore, the extinction coefficient at 414 nm of the porphyrin H_2AS_3P was figured out as 3.3×10^5 M^{-1} cm^{-1}. Similarly, from the UV absorption at 278 nm for the antibody whole molecule, the extinction coefficient is 2.0×10^6 M^{-1} cm^{-1}. Since two moles of heavy chain polypeptides can be yielded from one mole of antibody molecule by SDS-PAGE, the extinction coefficient of the heavy chain was thought to be the half of that of antibody, say, 1.0×10^6 M^{-1} cm^{-1}. The ratio of the visible absorption of porphyrin to the UV absorption of heavy chain of the antibody, obtained from the

UV-visible spectrum of the porphyrin-antibody (heavy chain) conjugate solution which was gained by electroelution, is 1:1.5. Using their extinction coefficients, the molar ratio of heavy chain to porphyrin was calculated as 1:2. We concluded that there are average 4 porphyrins covalently attaching to an antibody.

2.4.5. Electroelution and Determination of the Number of Porphyrins Coupled to the Antibody

Using the "Little Blue Tank" electroelution system (ISCO), the yellow porphyrin-antibody conjugate band on a unstained slab gel was excised and put in an electroelution trap composed of two cells, each with a dialysis membrane at the bottom allowing passage of electrolytes or ions with molecular weights smaller than 5,000. The trap was placed on the boundary wall between two buffer reservoirs, with the two cells down to the reservoirs respectively at the opposite sides of the boundary. The gel was put in the cell of the trap in the cathod (-) buffer reservoir. The electric current was conducted through the the reservoir buffer (0.1M sodium phosphate, pH 6.0) which filled up the trap and cover both the cells. The conjugate molecules were electroeluted from the gel, migrated towards the other cell of the trap which was immersed in the anode (+) buffer reservoir, and retained in the 200-μL concentration well by the dialysis membrane. Run at constant current of 10 mA per trap (more than one traps could be placed in the "little blue tank" for

electroelution at one time), the electroelution time was about 2 h. Buffer solution was taken out of the trap with care to avoid convective mixing with the sample solution, which was then pipeted out to be used for UV-visible spectroscopy. The number of coordinated porphyrins per antibody was determined by the relative absorbances of the Soret peak of the porphyrin near 416 nm and that of the peak of the protein near 280 nm.

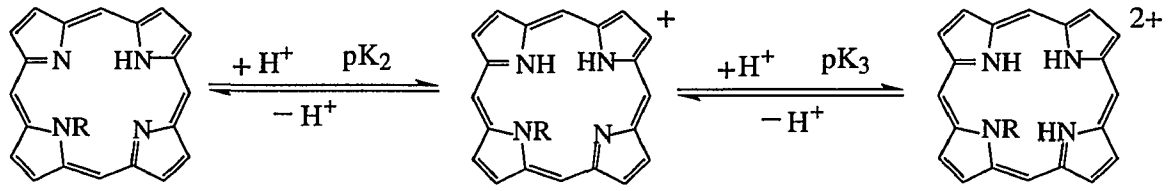
Chapter 3

Synthesis and Characterization of Novel *N*-*p*-nitrobenzyl-5-(4-Aminophenyl)-10,15,20-Tris(4-Sulfonatophenyl)Porphyrin and Its Conjugate with Immunoglobulin G

3.1 Introduction:

Porphyrins substituted at a pyrroleninic nitrogen atom with a variety of substituents including benzyl groups, phenyls, alkyl and acyl groups have been studied in our laboratory for a number of years (104). These porphyrins exhibit different chemical properties from non-*N*-substituted porphyrins mainly due to the slanted *N*-substituted pyrrole ring which distorts the planarity usually observed for non-*N*-substituted porpyrins (129-131). Lavallee and coworkers investigated in detail the two most important consequences of the distortion of the porphyrin ring. First, it increases basicity of the pyrroleninic nitrogen atom, especially the pK value for the free base-monocation equilibrium (Figure 3-1) (104). Secondly, the *N*-substituted porphyrins complex with metal ions as much as 10^5 times faster than the corresponding non-*N*-substituted porphyrins and the *N*-substituent of these complexes can be readily displaced by a nucleophile to produce non-*N*-substituted metalloporphyrins. (Figure 3-2) (104). Under proper conditions, for example, using the *N*-benzyl substituent, the two step reaction of metalation of

Figure 3-1. Successive protonations of *N*-substituted (upper) and non-*N*-substituted (lower) porphyrins. The pK_2 is much greater than the pK_3 for non-*N*-substituted porphyrins.


N
HN
NR
N
+ H+
pK2
− H+
NH
HN
NR
N
+
+H+
pK3
− H+
NH
HN
NR
HN
2+

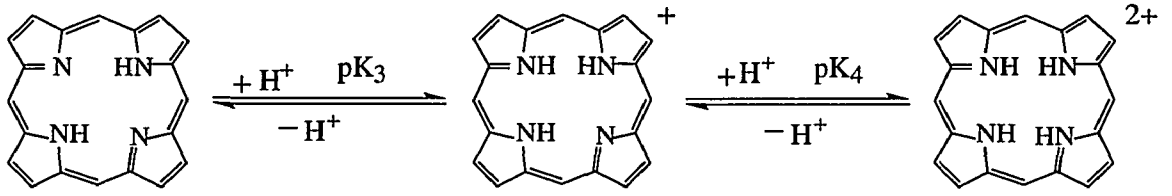
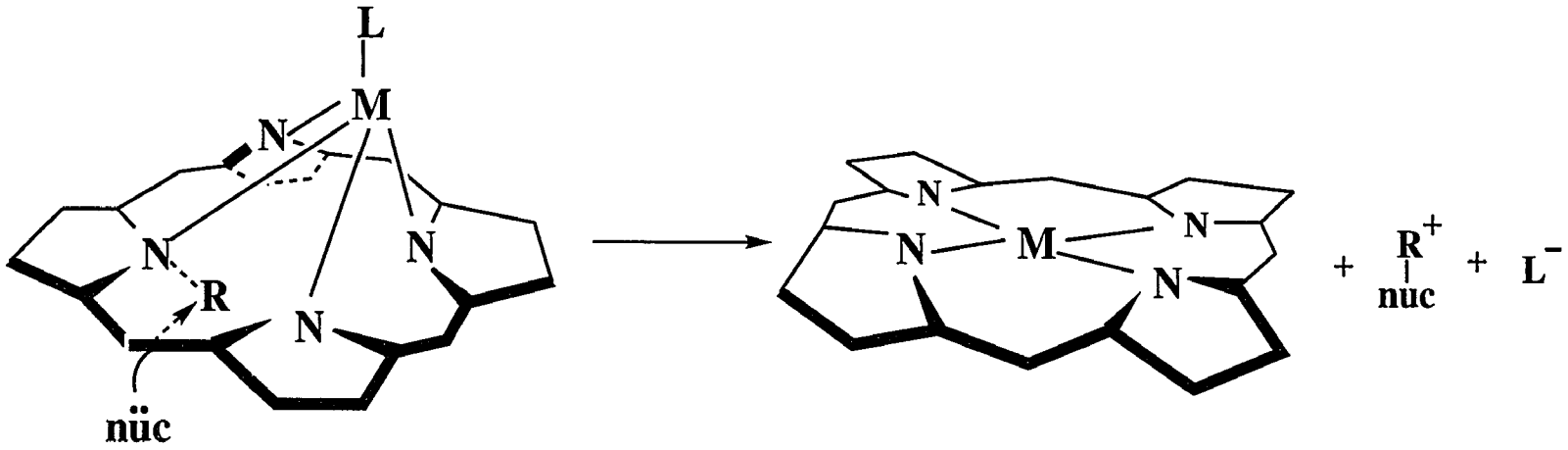

N
HN
NH
N
+ H+
pK3
− H+
NH
HN
NH
N
+
+H+
pK4
− H+
NH
HN
NH
HN
2+

Figure 3-2. Nucleophilic displacement of an *N*-substituent of the metal complex of *N*-substituted porphyrins.


L
M
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R
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nüc
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N
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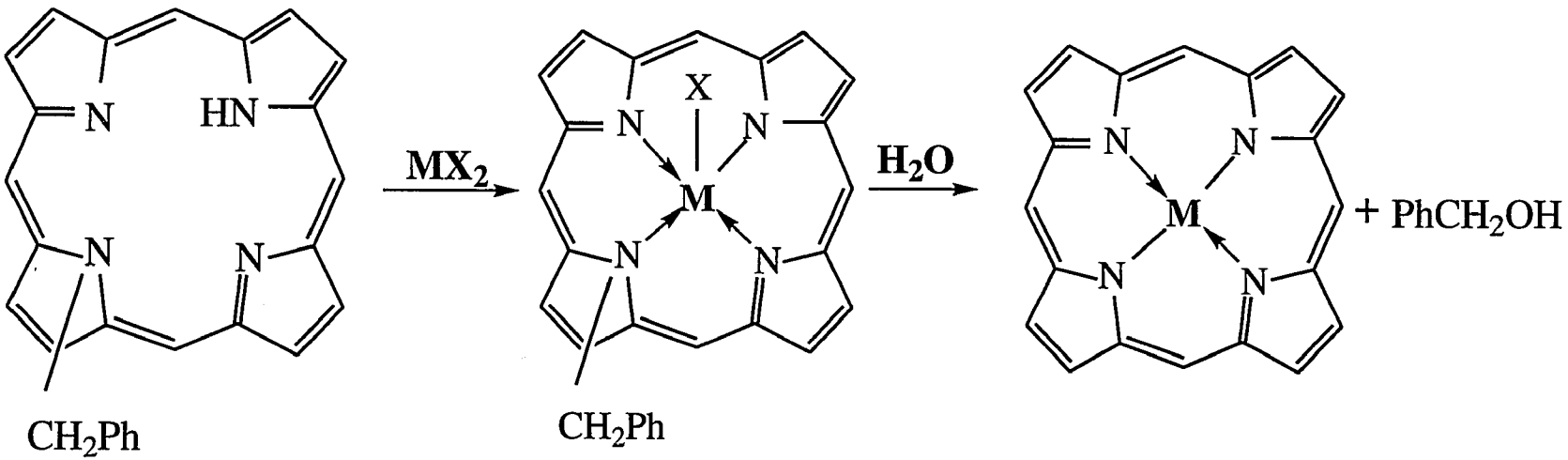
N
+
R+
nuc
+
L−

the *N*-substituted porphyrin and removal of the substituent can occur in aqueous solvents at neutral pH at 40 °C (where proteins are stable). In contrast, direct formation of a complex between the metal ion and the corresponding non-*N*-substituted porphyrin normally requires refluxing for 30 min to 24-48 h. Rapid metalation of *N*-substituted porphyrin has been applied to radiolabeling porphyrin-antibody conjugates for medical diagnosis by our collaboraters (84, 132) (Scheme 1-1).

Lavallee and coworkers demonstrated that the rate determining step in the reaction of *N*-benzylporphyrins with metal ions such as Co(II), Cu(II), Ni(II) and Pd(II) to give the corresponding non-*N*-substituted metalloporphyrins (Scheme 3-1) is debenzylation (82). The study of the effect of the *N*-substituent on the nucleophilic displacement of the *N*-substituent showed that the *p*-nitrobenzyl group is the most reactive among those groups mentioned at the beginning of this section (133). It has been also observed that the *N*-*p*-nitrobenzyl group is stable during the sulfonation of periphery phenyls of the porphyrin. All the above features of the chemistry of *N*-substituted porphyrins, as well as the achievement in the regiospecific conjugation of antibodies with the 5-(4-aminophenyl)-10,15,20-tris(4-sulfonatophenyl)-porphyrin (H_2AS_3P) described in the Chapter 2, make the *N*-*p*-nitrobenzyl-5-(4-aminophenyl)-10,15,20-tris(4-sulfonatophenyl)porphyrin (*N*-bzHAS_3P) an ideal chelator candidate for radioimmunoconjugates. Herein I report that 1) the synthesis

Scheme 3-1. The metal complexation of *N*-benzylporphyrins. The rate limiting step is debenzylation. **M**: Co(II), Cu(II), Ni(II) and Pd(II).


N
HN
N
N
CH2Ph
MX2
X
N
N
M
N
N
CH2Ph
H2O
N
N
M
N
N
+ PhCH2OH

of the *N*-bzHAS$_3$P by using *p*-nitrobenzyldiphenylsulfonium tetrafluoroborate carried out in a glass tube with Teflon™ stopper under high pressure; 2) the protection and deprotection of aniline functionality of the porphyrin during the synthesis of the *N*-bzHAS$_3$P and 3) the attempted synthesis and characterization of the regiospecific covalent conjugate of *N*-bzHAS$_3$P and immunoglobulin G.

3.2 Materials and Instruments:

In addition to those described in the Section 2.2, all other chemical reagents and organic solvents were purchased from Sigma, Aldrich, or Fisher Scientific. The glass tube with Teflon™ stopper were obtained from Fisher. Affi-Gel 10 affinity chromatographic support which is used to verify the amine functionality is obtained from Bio-Rad.

3.3 Results and Discussion:

3.3.1 N-p-Nitrobenzylation of the Porphyrin

Electrophilic substitution of a pyrroleninic nitrogen atom of the porphyrin by a p-nitrobenzyl group, according to Lavallee's procedure (82), could have been provided by reaction in dichloromethane, either at room temperature overnight or at 105-120 °C under high pressure in a sealed vessel for 1-2 h, with *p*-nitrobenzyldiphenylsulfonium tetrafluoroborate. The *N*-substitution reaction could be monitored by visible spectroscopy since the *N*-substituent changes the energy of the absorption of light significantly

(134-135) (Figure 3-3 and Figure 3-4) and normally the basic aqueous solution of non-*N*-substituted porphyrins is reddish brown, that of *N*-substituted porphyrins dark green. The reaction at room temperature overnight did not provide a significant yield in this case. However, quite good product yield was provided by the high pressure reaction.

The *p*-nitrobenzyldiphenylsulfonium tetrafluoroborate was prepared according to the method of Badet and Julia (136) using diphenylsulfide and p-nitrobenzyl alcohol with methanesulfonic acid as a catalyst under flowing argon (Scheme 3-2). The reaction yield was below 20%, however, the starting materials are relatively inexpensive. The white crystalline product had the melting point of 158 °C and good stability when stored in a freezer.

3.3.2 Protection of the Amino Functionality of the Porphyrin

In the total synthesis of *N*-*p*-nitrobenzyl-5-(4-aminophenyl)-10,15,20-tris(4-sulfonatophenyl)porphyrin, the most important strategy was the protection of the reactive amino group before the electrophilic *N*-substitution attack by benzyl cation and the recovery of the amino functionality to allow the porphyrin to be conjugated with an antibody after the *N*-substitution reaction. The protecting group must have sufficient stability to resist the harsh conditions needed for the *N*-substitution reaction. It should also have sufficient reactivity to be cleaved to recover the amino group.

Figure 3-3. The visible spectrum of 5-(4-aminophenyl)-10,15,20-tris(4-sulfonatophenyl)porphyrin (H_2AS_3P) at pH 12, H_2O, room temperature.

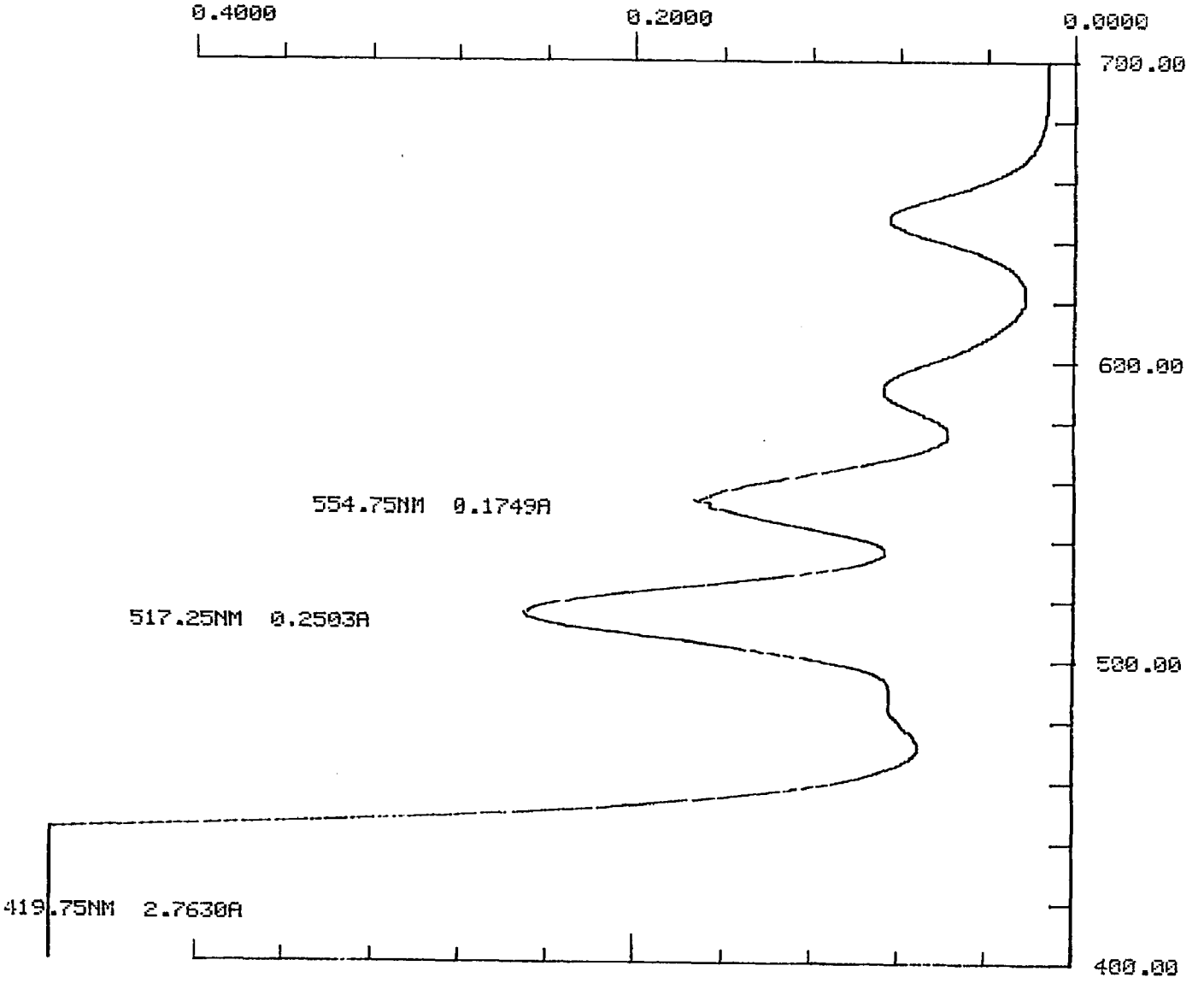
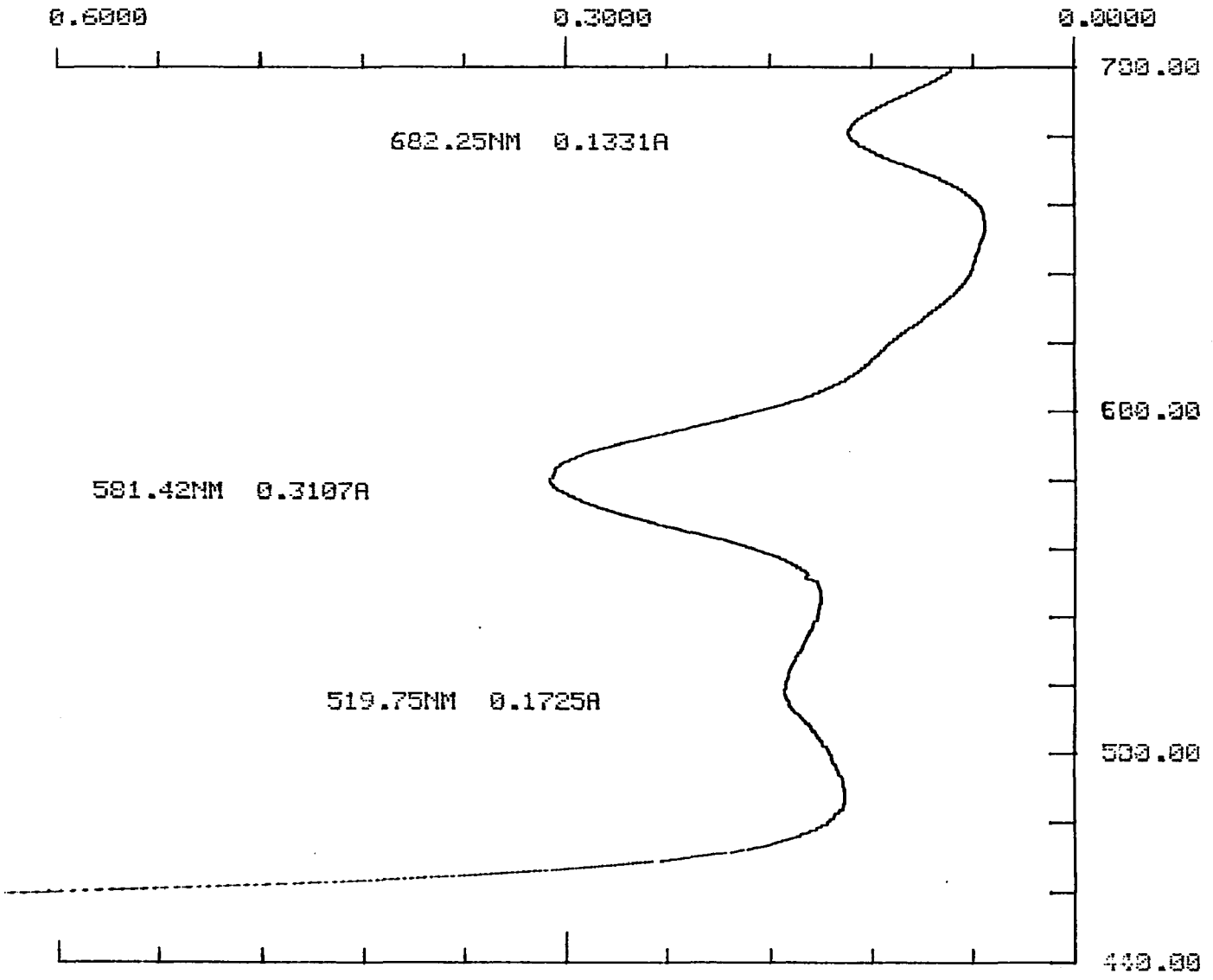
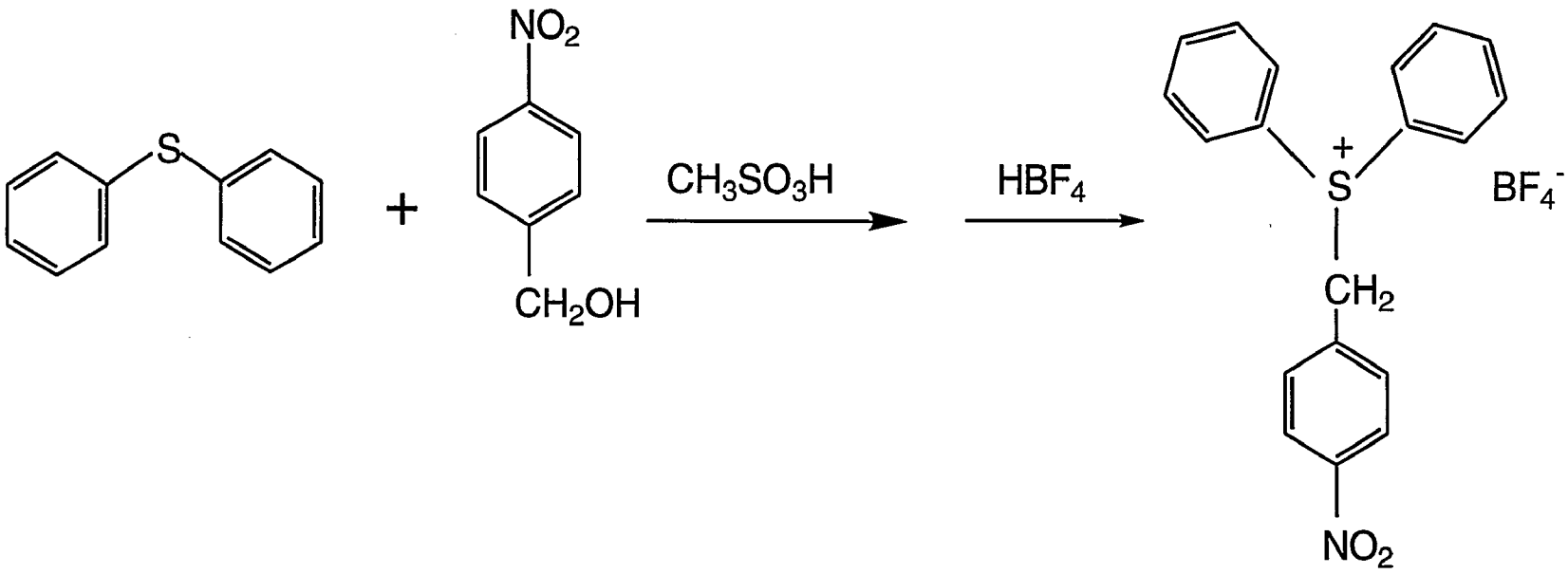

0.4000
0.2000
0.0000
700.00
600.00
554.75NM 0.1749A
517.25NM 0.2503A
500.00
419.75NM 2.7630A
400.00

Figure 3-4. The visible spectrum of *N*-*p*-nitrobenzyl-5-(4-aminophenyl)-10,15,20-tris(4-sulfonatophenyl)porphyrin (*N*-bzHAS_3P) at pH 12, H_2O, room temperature.


0.6000
0.3000
0.0000
700.00
682.25NM 0.1331A
600.00
581.42NM 0.3107A
519.75NM 0.1725A
500.00
440.00

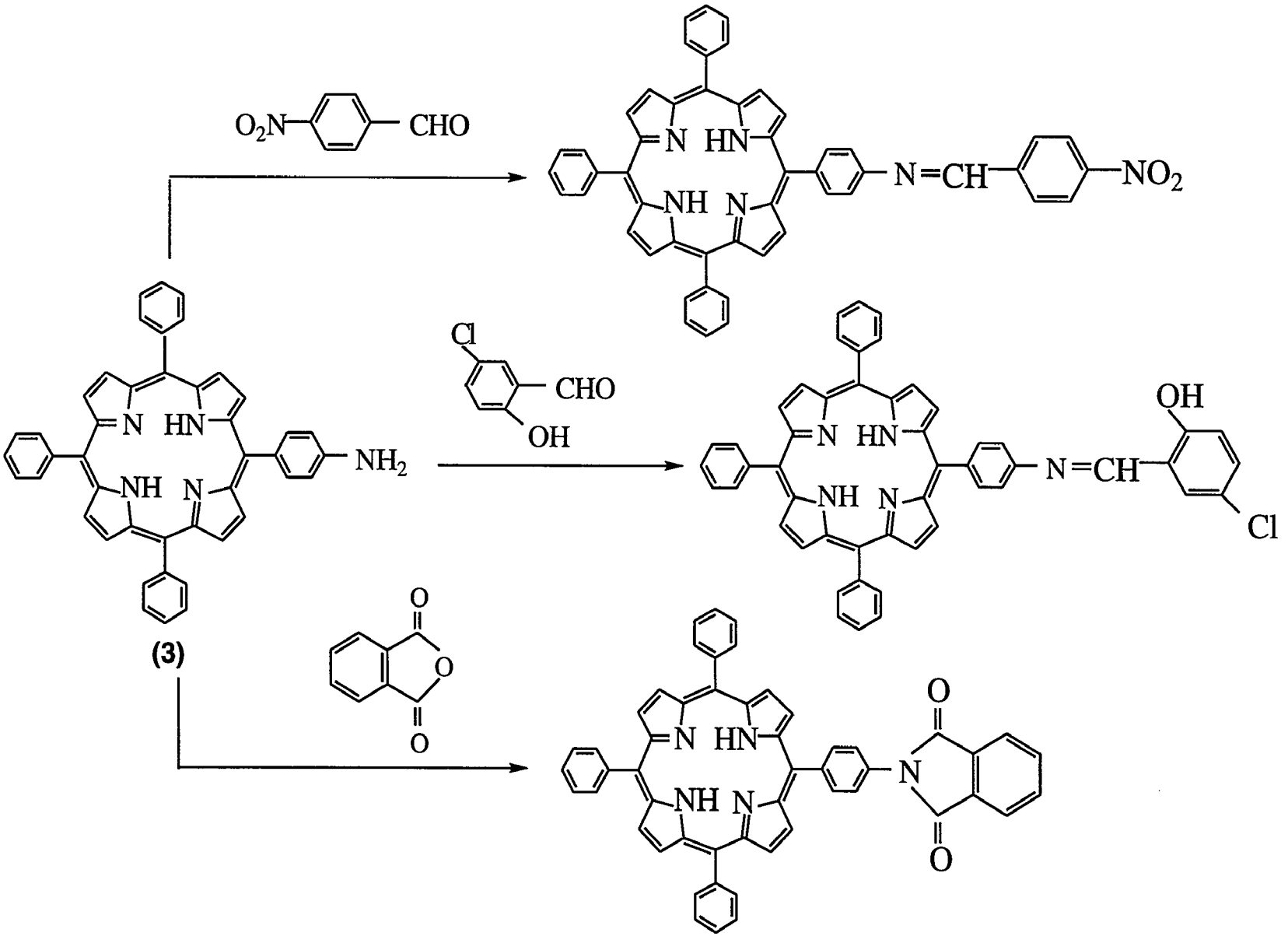
Scheme 3-2. The preparation of p-nitrobenzyldiphenylsulfonium tetrafluoroborate.


S
+
NO2
CH2OH
CH3SO3H
HBF4
+
S
BF4-
CH2
NO2

Three commercially available, inexpensive reagents were tried for use in protecting the amino group of the porphyrin: 1) *p*-nitrobenzaldehyde; 2) 5-chlorosalicylaldehyde and 3) phthalic anhydride. The former two reacted with amine to form Schiff bases: *N*-*p*-nitrobenzylidene and *N*-5-chlorosalicylidene groups, the latter one forming an *N*-phthaloyl group.(137-139). These protection methods are illustrated schematically in Figure 3-5. The 5-(4-aminophenyl)-10,15,20-triphenyl-porphyrin (compound **3** in the scheme 2-1) was protected and then reacted with the diphenyl-p-nitrobenzylsulfonium salt. After the pyrroleninic nitrogen had been substituted by p-nitrobenzyl group, the protecting group was cleaved and the *N*-benzyl-(aminophenyl)-triphenylporphyrin sulfonated to form the *N*-benzyl-(aminophenyl)tri(*p*-sulfonatophenyl)-porphyrin (*N*-bzHAS$_3$P). It is important to note that di-*N*-substitution is possible on the both two pyrroleninic nitrogens and it is also possible for the protecting groups to be cleaved during the electrophilic substitution reaction, exposing the amino group to benzylation. The difficulty of cleavage of the protecting group is another problem which could lead to loss of the reactive amino group.

The following sections describe protection of the amino group of the porphyrin with phthalic anhydride as well as unsuccessful attempts with p-nitrobenzaldehyde and 5-chlorosalicylaldehyde.

Figure 3-5. The protection for the amino group of (aminophenyl)triphenylporphyrin. The top: formation of *N*-*p*-nitrobenzylideneamine; the middle: formation of *N*-5-chlorosalicylideneamine; the bottom: formation of phthalimide.


O2N
CHO
N HN
NH N
N=CH
NO2
Cl
CHO
OH
NH2
(3)
OH
Cl
O
O
O
O
O

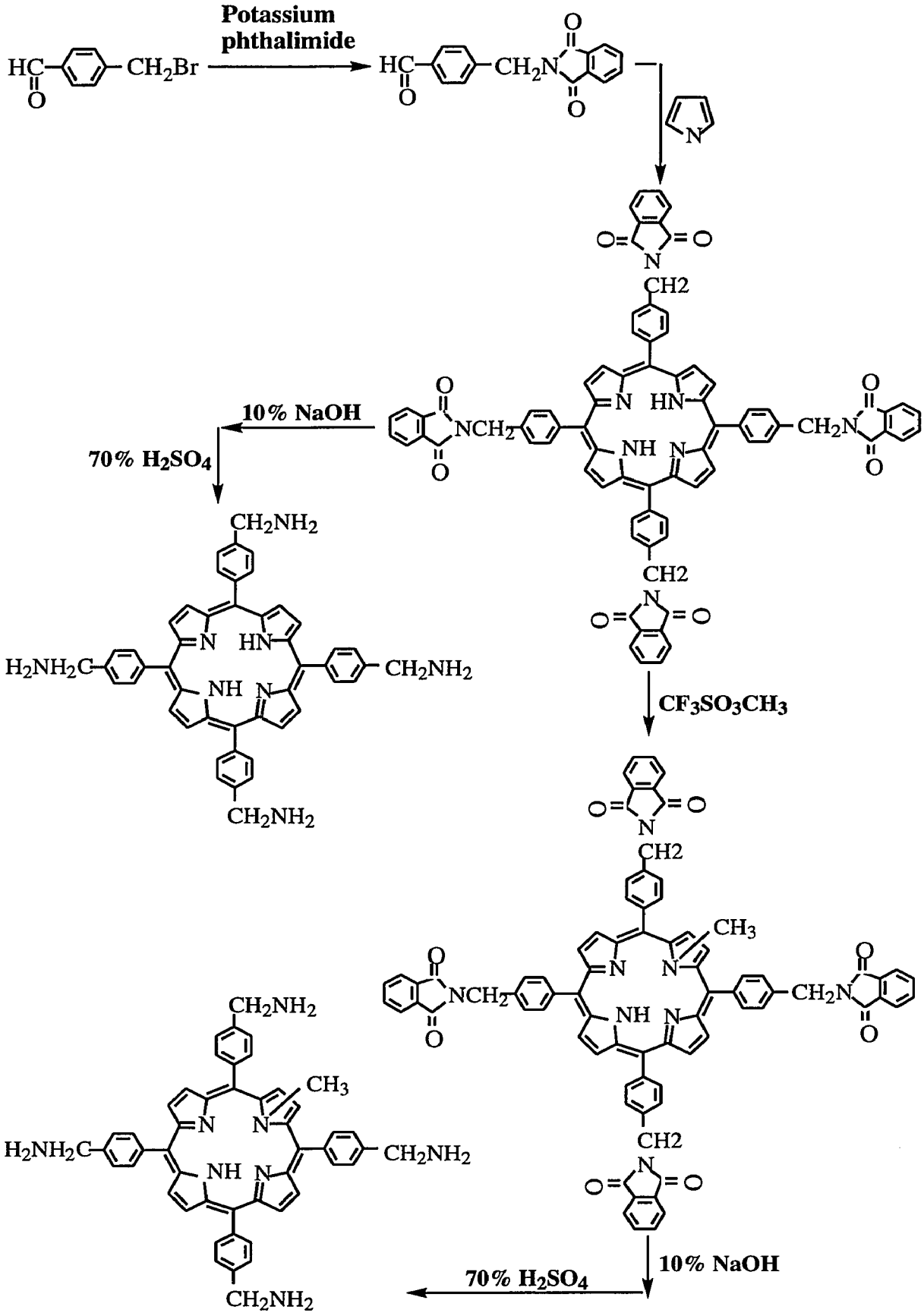
3.3.2.1 Protection by Phthalic Anhydride

Phthalimide was used by Xu previously in our laboratory as a protecting group in her synthesis of tetrakis(*p*-aminomethylphenyl)porphyrins (TAMPP) and *N*-methyl-tetrakis(*p*-aminomethylphenyl)porphyrins (Scheme 3-3)(140). This method yielded benzylamine in good yield by cleavage of the imide bonds in a two-step hydrolysis process: the solution of 10% sodium hydroxide first converted the imide functionality into an amide group; then the 70% sulfuric acid converted the amide to the amine.

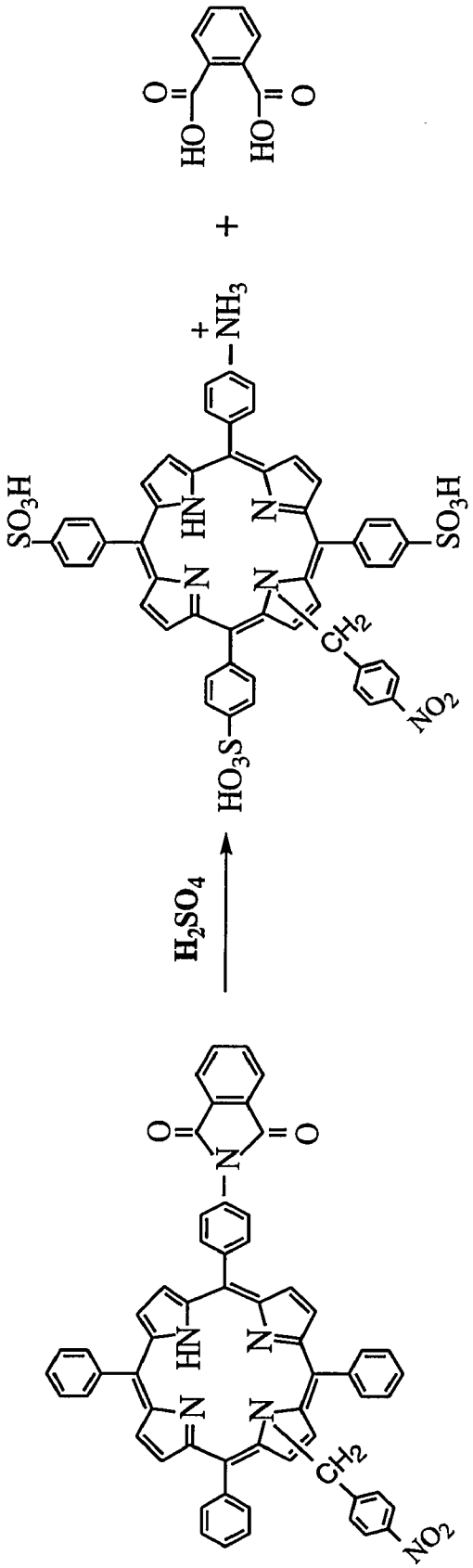
According to literature precedents, the phthalimido groups can be removed by hydrazine (139,141-142), acidic media (141-143), or alkaline (143). Since the porphyrin should be sulfonated by using sulfuric acid after deprotection, we designed a one-step reaction to cleave the phthalimido group as well as to fulfill the sulfonation (Scheme 3-4). The design seemed straightforward, but the result of reaction was uncertain because the ^{1}H NMR spectrum of the sulfonated porphyrin was poorly resolved. Reactions of the porphyrin with oxidized antibodies were, therefore tried to see if the porphyrins could incorporate antibodies by their amino functionality. But SDS-PAGE slab gels showed no sign of covalent conjugation between the porphyrin and the heavy chain of the antibody.

Protection of the aniline group by phthalimide was continued only after the two other commonly used protecting groups, *p*-nitrobenzylidene and chlorosalicylidene, had been

Scheme 3-3. The synthesis of tetrakis(*p*-aminomethylphenyl)-porphyrins (TAMPP) and *N*-methyl-tetrakis(p-aminomethylphenyl)porphyrins.


Potassium phthalimide
10% NaOH
70% H2SO4
CF3SO3CH3
70% H2SO4
10% NaOH

Scheme 3-4. The one-step reaction to cleave the phthalimido group and to fulfill the sulfonation did not succeed.


O
HO
HO
O
+
+NH3
SO3H
SO3H
HN
N
N
N
CH2
NO2
HO3S
H2SO4
O
O
N
HN
N
N
N
CH2
NO2

employed. As I discussed subsequently, these protecting groups proved to be unstable. Although it might be expected that sodium hydroxide would cleave the substituent on the pyrroleninic nitrogen of the porphyrin, in fact, the compound proved to be stable. After treatment with sodium hydroxide, one of the imide bonds of the phthalimide was cleaved and became a carboxylate, increasing the polarity of the compound so greatly that the R_f of the sample changed from 0.82 to zero on a silica TLC plate. The visible spectrum of the carboxylate indicated that it was still a *N(pyrroleninic nitrogen)*-substituted porphyrin (similar to the Figure 3-4). This result encouraged us to explore the second step of hydrolysis with an acid since the p-nitrobenzyl group substituted on the pyrroleninic nitrogen showed great stability during the hydrolysis of the *N*-nitrobenzalidene and the *N*-chlorosalicylidene and even sulfonation with concentrated sulfuric acid. The TLC showed the carboxylate (R_f = 0) was converted to a less polar compound (R_f = 0.42) after the second hydrolysis by sulfuric acid and the ^{1}H NMR spectrum indicated the amino group was really recovered by eventual cleavage of the phthalimide.

Figure 3-6 showed the ^{1}H NMR spectrum of the (*p*-phthalimidophenyl)triphenylporphyrin. The two quadruple peaks at 7.88 and 7.67 ppm indicated the 4 H from the phthalic phenyl group.

Figure 3-7 showed the ^{1}H NMR spectrum of the *N*-nitrobenzyl(*p*-phthalimidophenyl)triphenylporphyrin. The peaks

for the eight β-pyrrolenine hydrogens at 8.5-8.9 ppm region became more complicated due to two *N*-substituted isomers. The peaks for the 4 H from the phthalic phenyl group were still there.

Figure 3-8 showed the ^1H NMR spectrum of the *N*-nitrobenzyl(aminophenyl)triphenylporphyrin. The peaks for the 4 H from the phthalic phenyl group disappeared and there was a broad peak near 4 ppm, which disappeared after the $CDCl_3$ solution of sample was treated with a drop of D_2O and shaken thoroughly (Figure 3-9), indicating that this peak was from the recovered amino hydrogen which would easily exchange with the deuterium of the D_2O (144).

3.3.2.2 Attempted Protection by *p*-Nitrobenzaldehyde

(Aminophenyl)triphenylporphyrin was converted to (*N*-*p*-nitrobenzylideneaminophenyl)triphenylporphyrin by refluxing (aminophenyl)triphenylporphyrin with a ten-fold excess of *p*-nitrobenzaldehyde overnight. The conversion was verified by TLC (see experimental section). The extra benzaldehyde was eliminated, using column chromatography, after the *N*(pyrroleninic nitrogen)-substitution reaction of the protected porphyrin, because the product is of larger polarity difference from the benzaldehyde than (*N*-*p*-nitrobenzylideneaminophenyl)triphenylporphyrin is, which allows the separation by the column chromatography. However, multiple products were obtained. The green product separated as several spots on a TLC. In order to cleave the protecting

Figure 3-6. The ^{1}H NMR spectrum of a solution of the (*p*-phthalimidophenyl)triphenylporphyrin in $CDCl_3$.

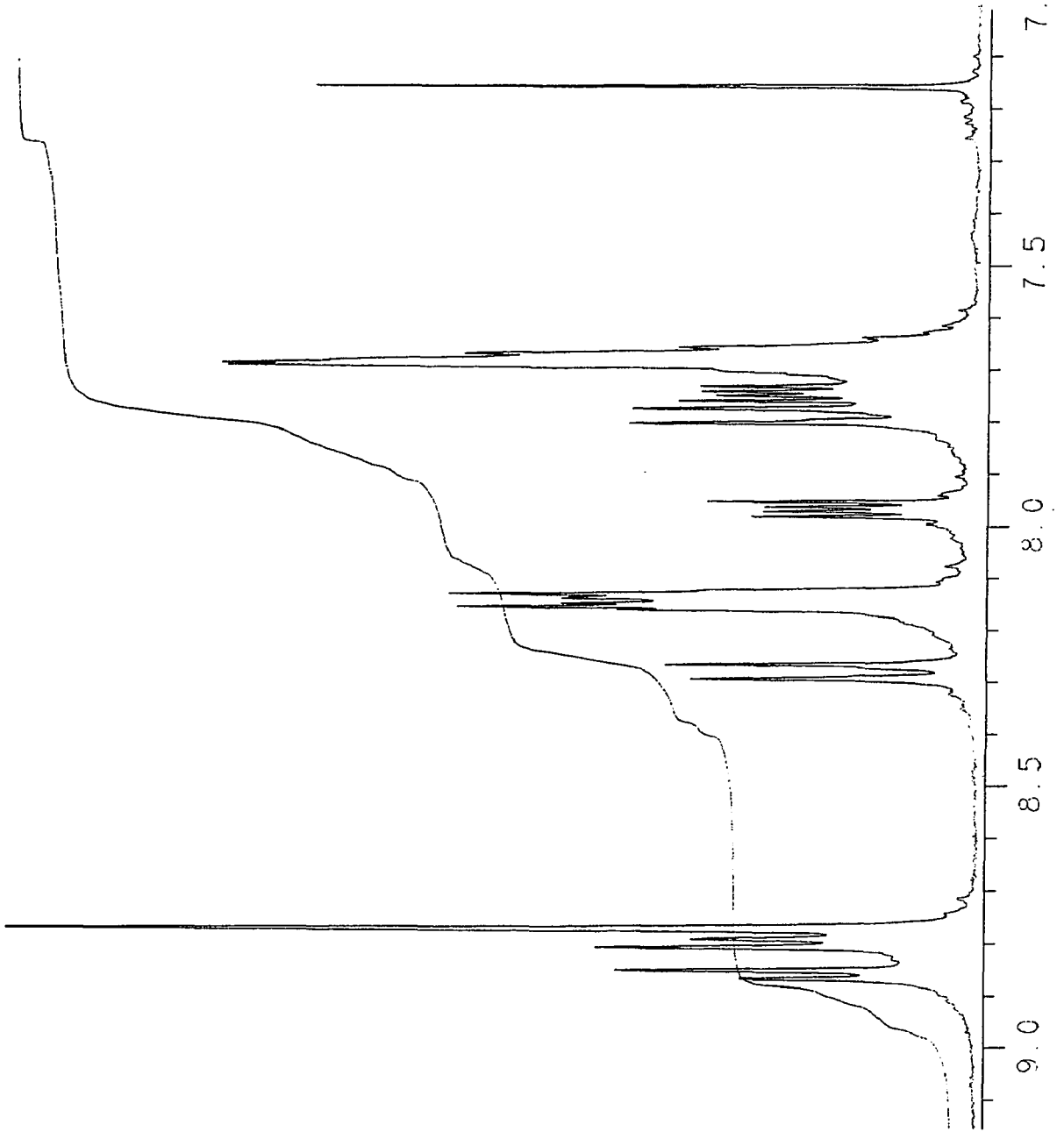

7.
7.5
8.0
8.5
9.0

Figure 3-7. The ^1H NMR spectrum of a solution of the *N*-nitrobenzyl-(*p*-phthalimidophenyl)triphenylporphyrin in $CDCl_3$. (It is likely that all possible N-substituted isomers are present in equal amount.)

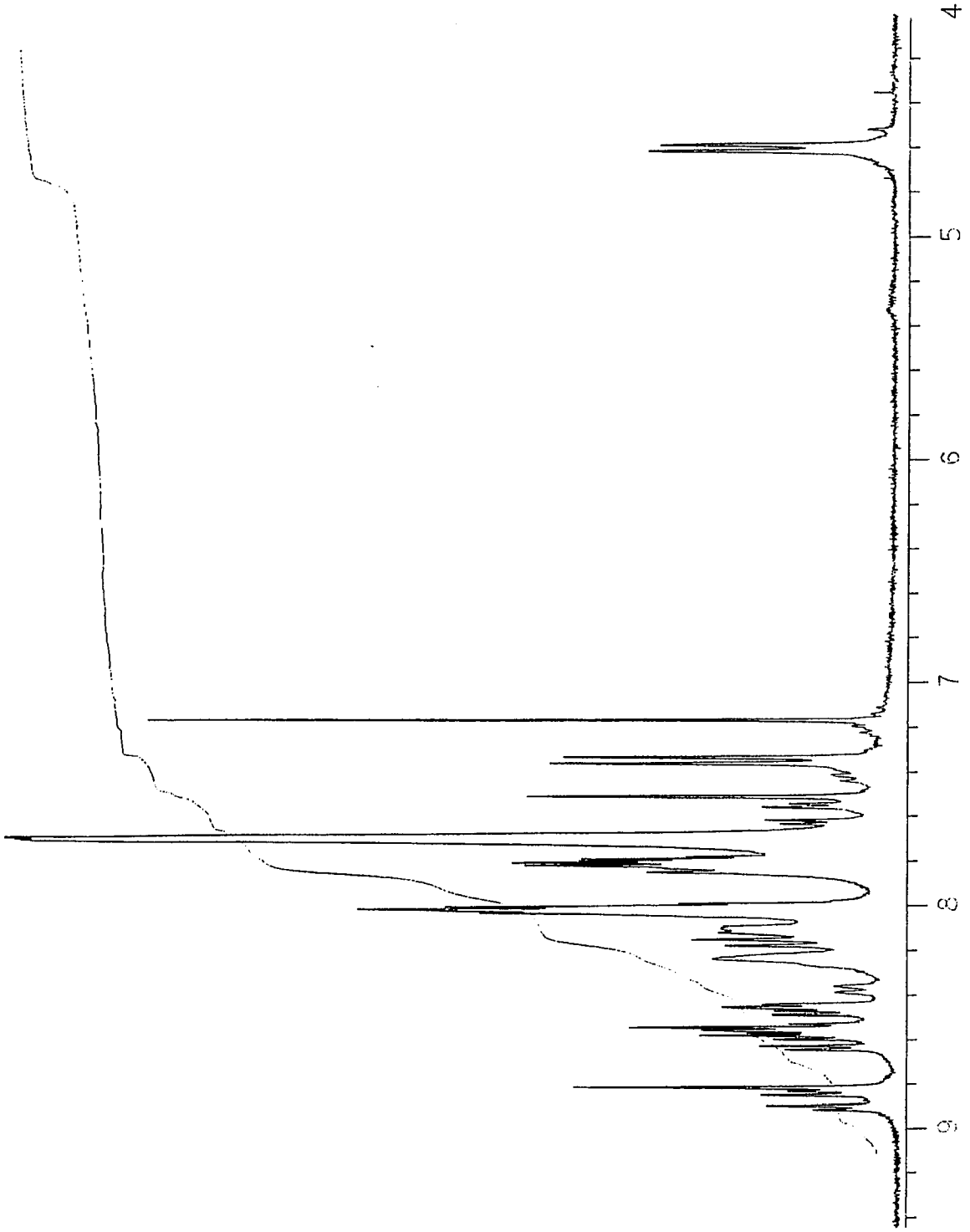

4
5
6
7
8
9

Figure 3-8. The ^1H NMR spectrum of a solution of the *N-p*-nitrobenzyl-(aminophenyl)triphenylporphyrin in $CDCl_3$. (It is likely that all possible N-substituted isomers are present in equal amount.)

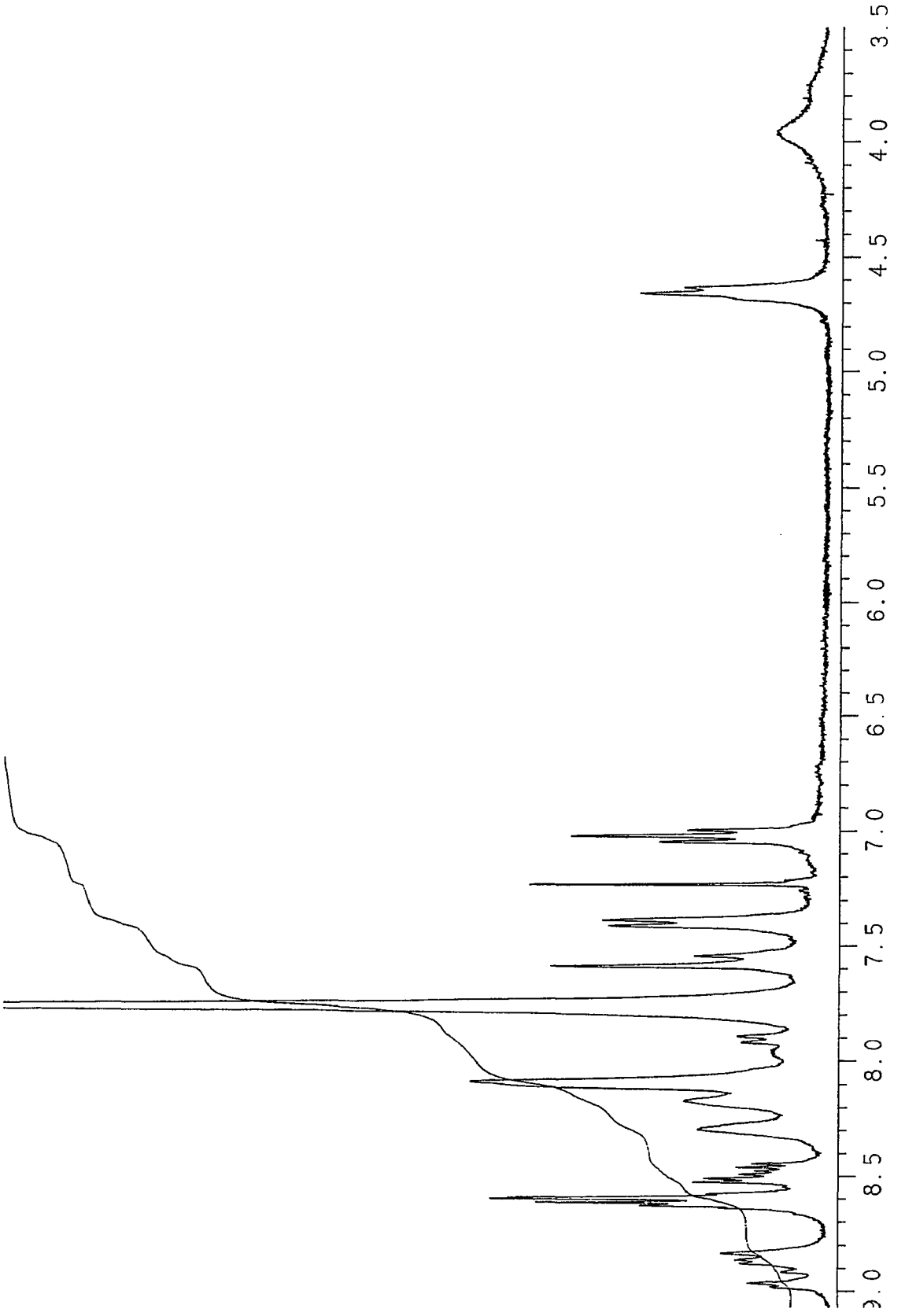
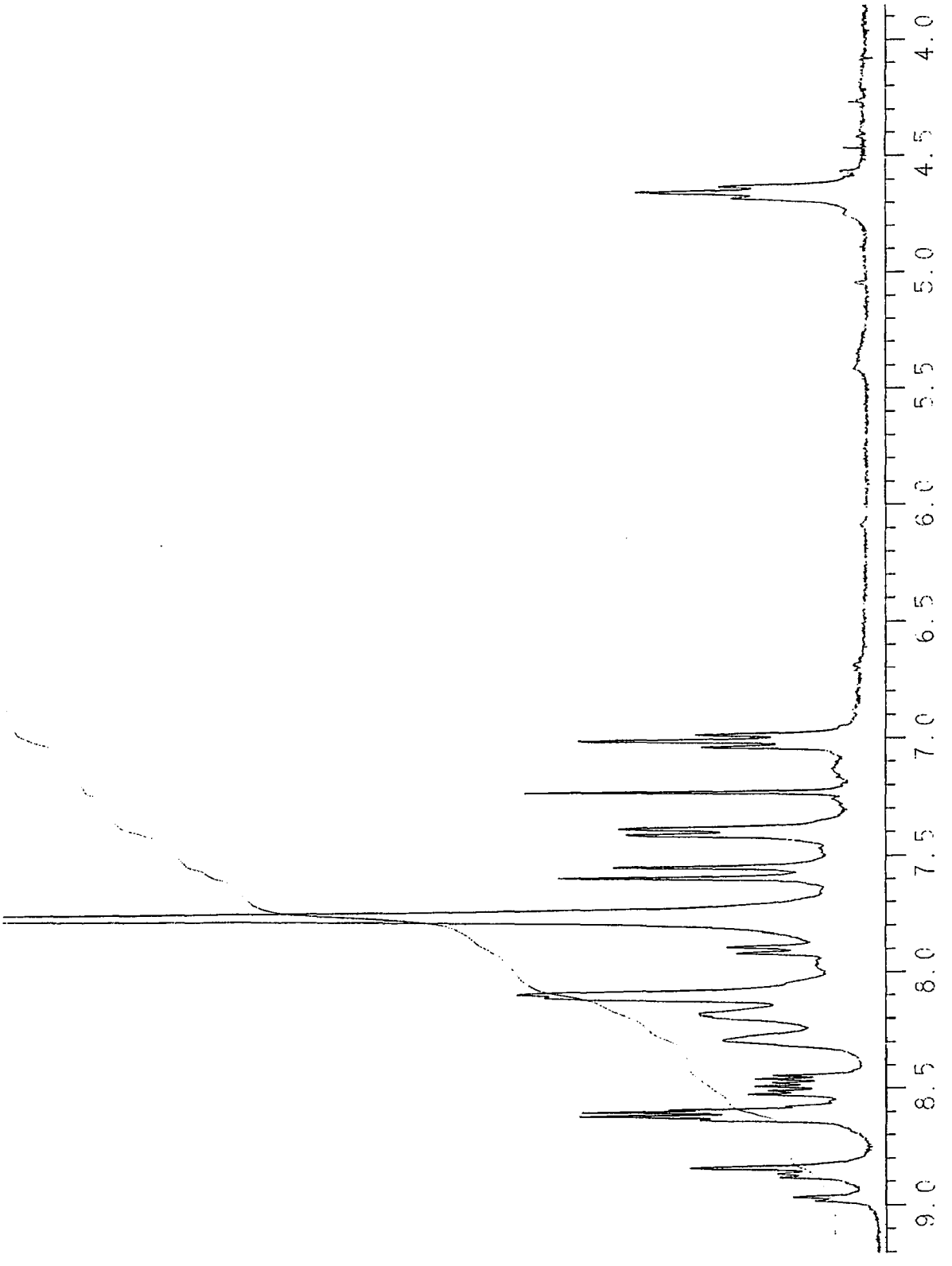

3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0

Figure 3-9. The ^1H NMR spectrum of a solution of the *N-p*-nitrobenzyl-(aminophenyl)triphenylporphyrin in $CDCl_3$ mixed with D_2O. The broad peak near 4 ppm disappeared (as compared to the Figure 3-8). (It is likely that all possible N-substituted isomers are present in equal amount.)


9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0

group the mixture was treated with several kinds of organic or inorganic acids, including *p*-toluenesulfonic acid, acetic acid and hydrochloric acid, in different concentrations, but the were no significant changes shown on TLC plate in comparison to the untreated sample. The acid-treated mixture was then chromatographed through a silica column with a series of solvents in the order of increasing polarity to separate all components of the mixture. None of these separated bands showed any peak with amino characteristics in the ^{1}H NMR spectrum. After being sulfonated, none of these fractions covalently bound to oxidized antibodies, which was shown by SDS-PAGE. Some other (*N*-*p*-nitrobenzylideneamino-phenyl)triphenylporphyrin was prepared and purified by a silica column with another solution (see experimental section). The protected porphyrin was characterized by ^{1}H NMR spectroscopy and, unexpectedly, it was found that this porphyrin was just like the starting material, (aminophenyl)triphenylporphyrin. Checking again with the TLC, it was seen that most of protected porphyrin was converted back. It was realized that the (*N*-*p*-nitrobenzylidene-aminophenyl)triphenylporphyrin was not stable, especially when the extra benzaldehyde was removed and the product was exposed to the air for a long period of time. The product was temporarily stable when the purification step was skipped right before reacting with the diphenyl-*p*-nitrobenzylsulfonium salt, but it expected to be cleaved during the high temperature reaction such that recovered

amino groups were also substituted by the nitrobenzyl groups like the pyrroleninic nitrpgen atoms were. That is also the reason why there were different kinds of *N*-substituted products shown on the TLC silica plate and they could not be recovered amino groups by treatment with various acids which were normally used to cleave the C=N bond of *N*-benzalidenes or other kinds of Schiff base.

3.3.2.3 Attempted Protection by 5-Chlorosalicylaldehyde

N-salicylidene amines are expected to be more stable. than corresponding benzaldehyde analogs, due to strong intramolecular hydrogen bonding between the salicylidene nitrogen and the phenolic proton. For protection of amino groups, 5-chlorosalicylaldehyde was, therefore, used in a reaction with (aminophenyl)triphenylporphyrin in methylene chloride with acetic acid as a catalyst. The green *N*-substitution product was also a mixture, similar to the case of the porphyrin protected by the *N*-nitrobenzalidene mentioned above. The green mixture was treated with hydrochloric acid in order to hydrolyze the protecting group. ^{1}H NMR spectra were obtained for every component of the hydrolyzed mixture. One of the spectra showed a broad peak near 4 ppm indicating the sample was hydrolyzed and the amino group recovered (compare Figure 3-10 and Figure 3-11).

This *N*-*p*-nitrobenzyl-(aminophenyl)triphenylporphyrin was then sulfonated at the para positions of the three phenyl groups and the *N*-*p*-nitrobenzyl-(aminophenyl)tri(*p*-sulfona-

tophenyl)porphyrin reacted with the oxidized antibodies. The SDS-PAGE showed covalent linkage between the porphyrin and the antibody, that is, there was a yellow band at the position of the heavy chains of IgG molecules on a unstained slab gel (145). It was clear, however, that the *N*-5-chlorosalicylidene was not stable during the *N*-subatitution reaction. Only a small fraction of the protecting groups remained to yield *N*-*p*-nitrobenzyl-(*N*-5-chlorosalicylideneaminophenyl)triphenylporphyrin which was then hydrolyzed to recover the amino group. A better protecting group for the amino function, therefore, was still needed.

3.3.3 Synthesis of N-p-Nitrobenzyl-5-(4-aminophenyl)-10,15,20-tris(4-sulfonatophenyl)porphyrin (N-bzHAS$_3$P)

After the trial of protection by phthalimide proved to be successful the synthesis of *N*-*p*-nitrobenzyl-5-(4-aminophenyl)-10,15,20-tris(4-sulfonatophenyl)porphyrin (*N*-bzHAS$_3$P) was designed as illustrated schematically in Scheme 3-5. Reaction of (aminophenyl)triphenylporphyrin (compound **4** in the Scheme 2-1) and phthalic anhydride was tried at first by refluxing in 1,4-dioxane (bp 108 °C). The product was a very polar material which had zero mobility on a silica TLC plate using methylene chloride as a developing solvent and it only dissolved in *N*,*N*-dimethylformamide (DMF) or dimethyl sulfoxide (DMSO). It was not stable and some of it decomposed to (aminophenyl)triphenylporphyrin after a week. The down field peak at about 12 ppm in its ^{1}H NMR spectrum showed it to

Figure 3-10. The ^{1}H NMR spectrum of a solution of (*N*-5-chlorosalicylideneaminophenyl)triphenylporphyrin in $CDCl_3$.

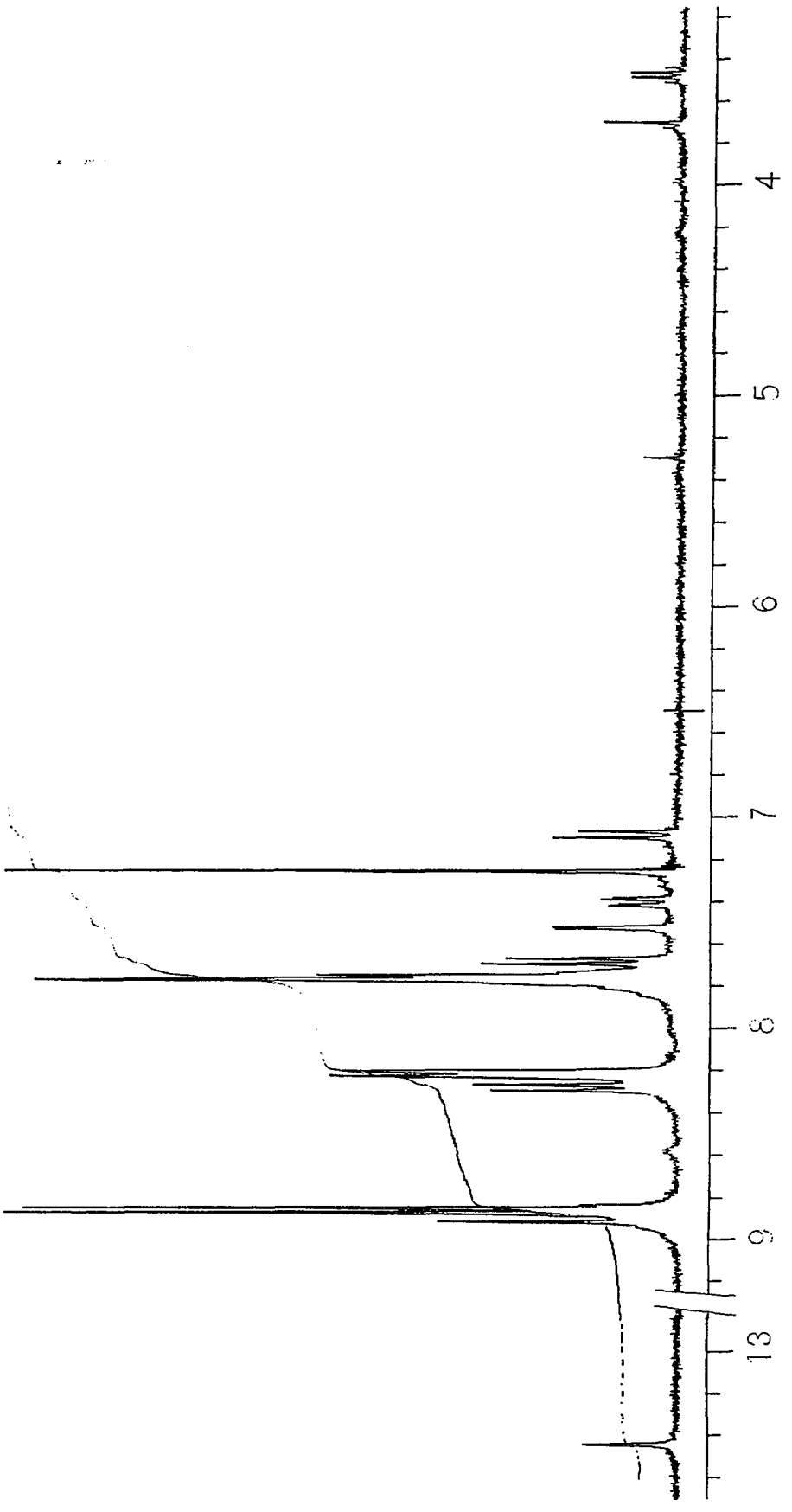
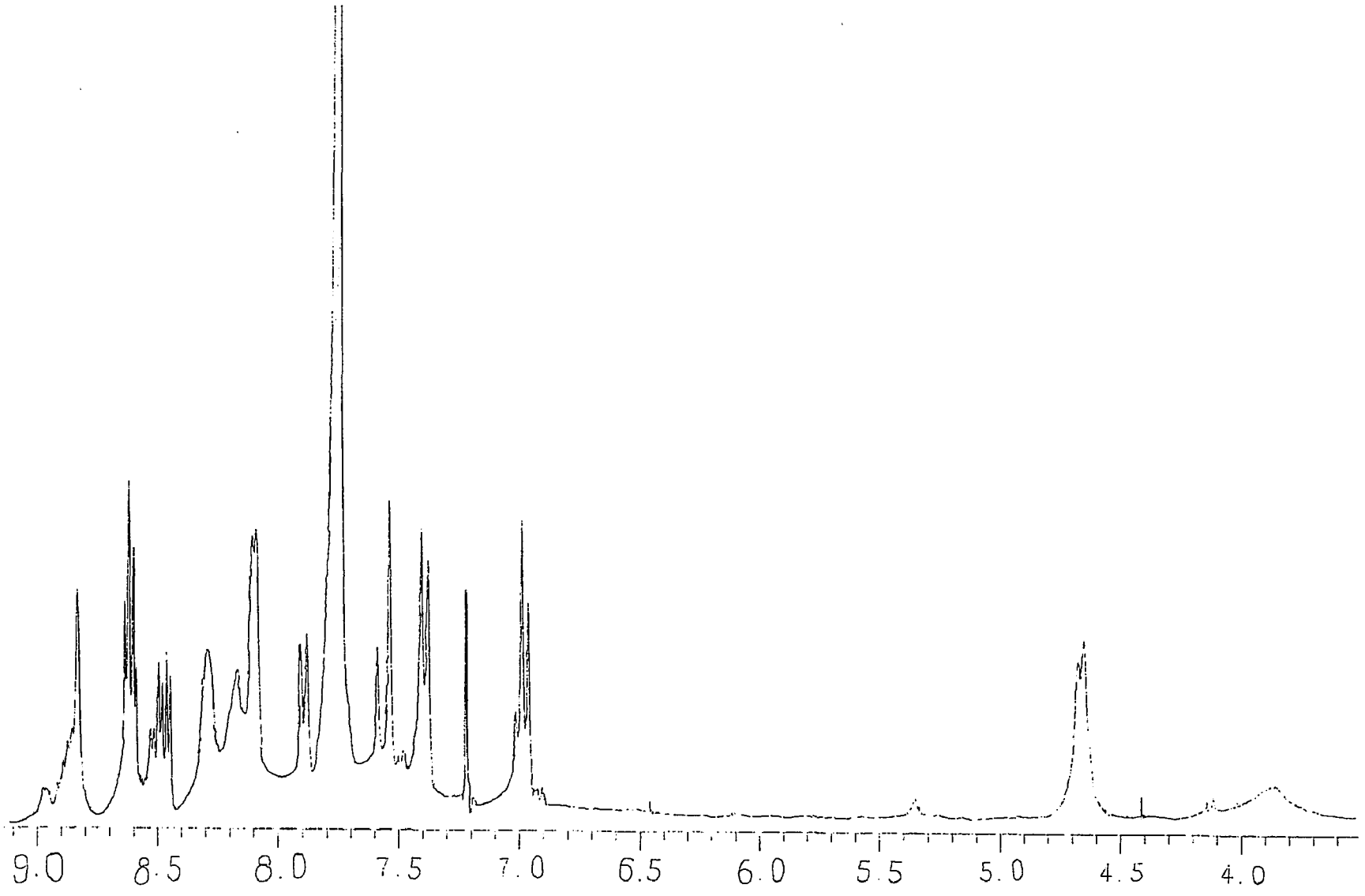
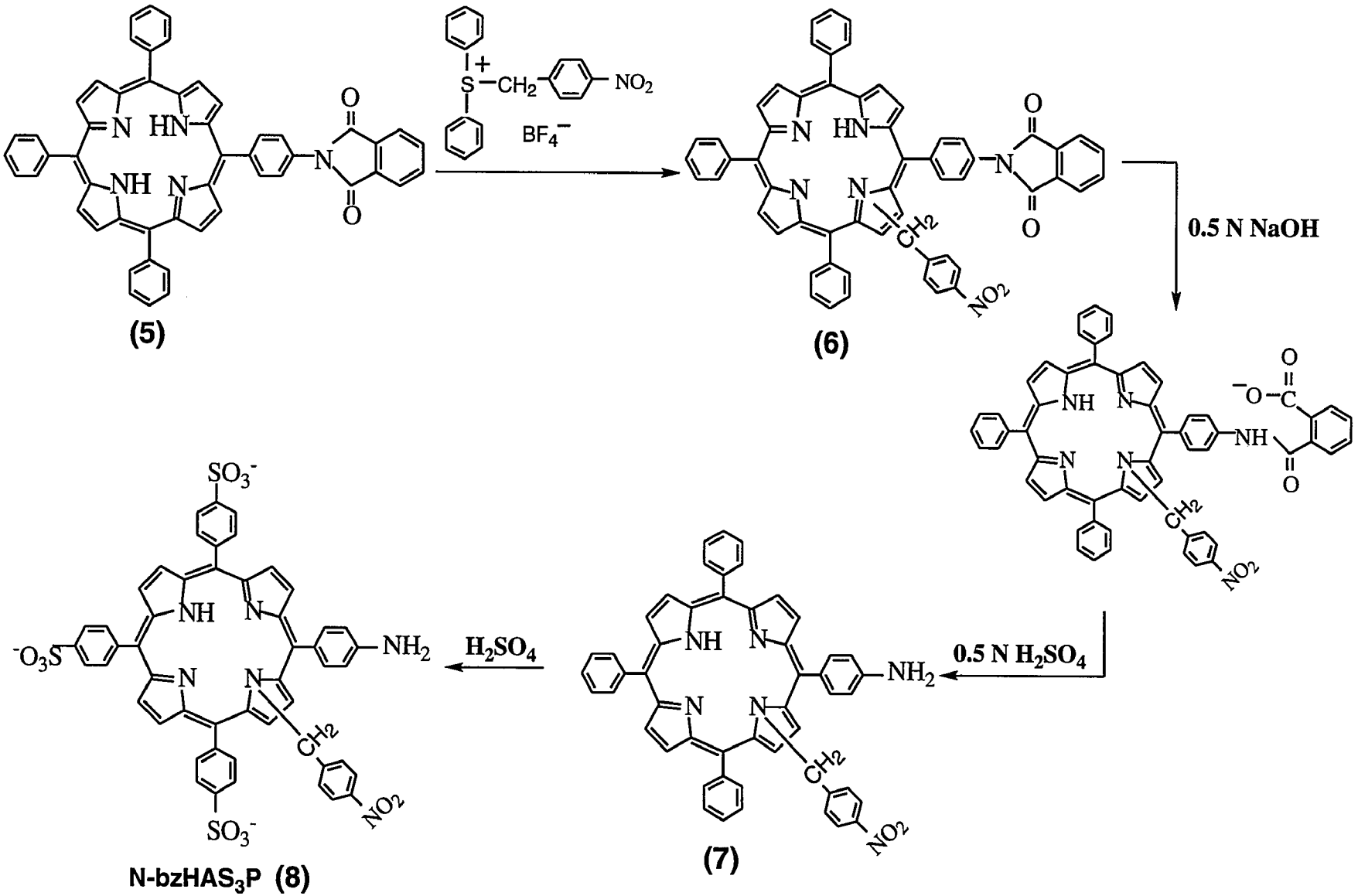

13
9
8
7
6
5
4

Figure 3-11. The ^1H NMR spectrum of a solution of hydrolyzed *N*-*p*-nitrobenzyl-(*N*-5-chlorosalicylideneaminophenyl)triphenylporphyrin in $CDCl_3$. (It is likely that all possible N-substituted isomers are present in equal amount.)


9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0

Scheme 3-5 The design for synthesis of *N*-*p*-nitrobenzyl-5-(4-aminophenyl)-10,15,20-tris(4-sulfonatophenyl)porphyrin (*N*-bzHAS_3P).


(5)
(6)
0.5 N NaOH
0.5 N H_2SO_4
H_2SO_4
(7)
N-bzHAS$_3$P (8)

be a o-carboxy benzamide rather than a phthalimide. The (*p*-phthalimidomethylphenyl)triphenylporphyrin (**5**) was only formed when (aminophenyl)triphenylporphyrin and a large excess of phthalic anhydride were fused at high temperature (146). It was actually less polar and had greater mobility than unreacted porphyrin on the silica TLC with methylene chloride. It also dissolved in low polarity solvents such as methylene chloride. Purification of product was attempted by washing with basic aqueous solution, but one of the imide bonds appeared to be broken (as seen a non-mobile spot on a TLC plate) The protected porphyrin was then purified by washing with 1,4-dioxane to eliminate most of extra phthalic anhydride from the reaction mixture and further by silica column chromatography with methylene chloride to elute the product, leaving the other little free phthalic anhydride in the column.

The *N*-substitution reaction was carried out in the way described in the Section 3.3.1. The yield of mono-*N*-substituted product (**6**) after purification by using alumina column chromatography was 70%. The alumina column chromatography needed less polar solvent than that used in the silica column to elute the *N*-substituted porphyrin and leaving less porphyrin on the gel.

Sulfonation of (**7**) was carried out in a similar way to the procedure by Tsutsui (147). Satisfactory ^{1}H NMR spectra could not be obtained. All peaks in the aromatic region became broad and could not be assigned definitely. There was

not a peak clearly indicating the presence of amino group because a large water peak just located near 4-5 ppm where the amino peak used be.

3.3.4 Attempted Synthesis and Characterization of the N-Substituted Porphyrin-Antibody Conjugate

The synthesis and characterization of the *N*-substituted porphyrin-antibody conjugate was tried in the same way as done with the non-*N*-substituted porphyrin, 5-(4-aminophenyl)-10,15,20-tris(4-sulfonatophenyl)porphyrin (H_2AS_3P), and the antibody, rabbit immunoglobulin G. That is, antibodies are oxidized by periodate salts to produce aldehyde functionalities, followed by conjugation with amine functionalities of porphyrins. Since the *N*-*p*-nitrobenzyl-5-(4-aminophenyl)-10,15,20-tris(4-sulfonatophenyl)porphyrin (*N*-bzHAS_3P) has different basicity from the H_2AS_3P, the optimal pH for Schiff base formation between the amino groups of the porphyrin and the aldehyde groups of the oxidized antibody should be different from what is needed for H_2AS_3P. To determine appropriate reaction conditions, the conjugation of *N*-bzHAS_3P and the antibody was carried out in a series of media of pH values 5, 6, 7, 8.5, and 10. Unexpectedly, the *N*-bzHAS_3P did not covalently link to the antibodies under any of these conditions.

Because the amine group is not really apparent in the NMR spectrum of the porphyrin *N*-bzHAS_3P, another technique was required to verify its presence. An *N*-hydroxysuccinimidyl

terminal agarose gel is used to bind molecules with accessible primary amines. The non-N-substituted analog to *N*-bzHAS$_3$P, H$_2$AS$_3$P, really bound to this chromatographic support, whereas an analog porphyrin without the amine functionality, *N*-*p*-nitrobenzyl-5,10,15,20-tetra(4-sulfonatophenyl)porphyrin (*N*-bzTSPP), did not bind covalently. After these control experiments were performed, the presence of the amine group on *N*-bzHAS$_3$P was verified since this porphyrin bound strongly to the *N*-hydroxysuccinimidyl terminal agarose gel (148).

3.4 Experimental Section:

3.4.1 Synthesis of p-Nitrobenzyldiphenylsulfonium Tetrafluoroborate

p-Nitrobenzyl alcohol (23 g, 0.15 mole) was dissolved in 20 mL of methanesulfonic acid (0.3 mole) under argon flow and stirred at room temperature. Diphenylsulfide (17 mL, 0.1 mole) was added drop by drop into the solution cooled to 5 °C with an ice bath. After addition of diphenylsulfide, the argon flow was stopped and the reaction mixture was brought to room temperature and stirred for 3 h. The mixture was washed with three 100 mL portions of 4:1 diethyl ether/hexane solution. The residue was cooled in an ice bath and 20 mL of water was added slowly. Aqueous solution of 46% tetrafluoroboric acid was added drop by drop into the stirred cooled mixture, which was then allowed to come to room temperature and stirred for 30 min. The product was extracted

with three 100 mL portions of methylene chloride. The organic solutions were combined and about 30 mL of saturated solution of sodium bicarbonate was added to make the solution slightly basic. After being dried with magnesium sulfate, the solution was filtered, and reduced in volume by a rotary evaporator. After adding about 50 mL of methanol, 400 mL of diethyl ether was added to cause precipitation. The white crystals were filtered, washed with diethyl ether several times and air dried. Yield 14%, m.p. 158-160 °C.

3.4.2 Synthesis of 5-(4-Phthalimidophenyl)-10,15, 20-triphenylporphyrin (**5**) *from 5-(4-Aminophenyl)-10,15,20-triphenylporphyrin* (**4**)

Phthalic anhydride (0.67 g, 4.5 mmol) was ground in a mortar and then mixed with 5-(4-aminophenyl)-10,15,20-triphenylporphyrin (**4**) (0.28 g, 0.45 mmol). The mixture was heated molten and dried in a round bottom flask. Some of the anhydride recrystallized on the wall of the flask. The residue was dissolved in 200-mL methylene chloride, followed by addition of 100-mL 1,4-dioxane. The solution was washed with six 300 mL portions of water. Most of the extra phthalic anhydride dissolved in the dioxane and was removed in the water. After washing with water the volume of organic solution was reduced to about 200 mL and some of phthalic anhydride was detected under UV light (long wavelength). Further purification by silica column chromatography with methylene chloride to elute the product and leave the other

free phthalic anhydride on the column, gave the 5-(4-phthalimidomethylphenyl)-10,15,20-triphenylporphyrin (**5**) in 90% yield. A later experiment showed that the extra phthalic anhydride could be thoroughly removed just by washing with 1,4-dioxane-water.

^{1}H NMR ($CDCl_3$) δ: 8.93 (d, 2 H, J = 5 Hz, β-pyrrole), 8.87 (d, 2 H, J = 5 Hz β-pyrrole), 8.85 (s, 4 H, β-pyrrole), 8.36 (d, 2 H, J = 8 Hz, phthalimidophenyl), 8.22 (m, 6 H, ortho phenyl), 8.03 (dd, 2 H, J_1 = 5 Hz, J_2 = 3 Hz, phthalic phenyl), 7.87 (d, 2 H, J = 8 Hz, phthalimidophenyl), 7.81 (dd, 2 H, J_1 = 5 Hz, J_2 = 3 Hz, phthalic phenyl), 7.77 (m, 9 H, meta/para phenyls), -2.75 (s, 2 H, pyrrole NH). Anal. Calcd. for $C_{52}H_{31}N_5O_2 \cdot 1/2\ CH_2Cl_2 \cdot 1/2\ H_2O$: C, 77.7; H, 4.33; N, 8.63; O, 4.93%. Found: C, 78.78; H, 4.73; N, 8.45; O, 4.87%.

3.4.3 Synthesis of N-p-Nitrobenzyl-5-(4-phthalimidophenyl)-10,15,20-triphenylporphyrin (**6**) *from 5-(4-Phthalimidophenyl)-10,15,20-triphenylporphyrin* (**5**)

The protected porphyrin (5) (0.43 mg, 0.5 mmol) was mixed with *p*-nitrobenzyldiphenylsulfonium tetrafluoroborate (0.86 mg, 2 mmol) and dissolved in 250 mL of methylene chloride in a high-pressure glass tube sealed by a Teflon™ stopper (114). The solution was stirred and heated in an oil bath. The temperature of the oil bath was raised to 120 °C and the solution was stirred at that temperature for 1.5 h. After the vessel had cooled to room temperature, it was placed into an ice bath for a half hour to ensure that the

pressure inside fell to atmosphere pressure. The aluminum seal was carefully unscrewed. The dark green solution was neutralized by adding 1 M aqueous ammonia until the porphyrin solution turned dark brown and was then washed with five 200 mL portions of water. After being reduced in volume, the organic solution was applied to an alumina column (18 in. × 2 in., Fisher, 80-200 mesh) equilibrated with the solution of 30% hexane in methylene chloride. The unreacted porphyrin and remaining sulfonium salt were eluted as the first band (pink) with the solution of 30% hexane in methylene chloride. Elution was continued with the same solution until no sulfonium salt was eluted. Methylene chloride was then used to elute the desired compound as the second band (green on the column, eluted as a purple solution). After crystallization from methylene chloride and acetonitrile (1:1 in volume), the yield was 70%.

^{1}H NMR ($CDCl_3$) δ: 8.91 (d, 1 H, β-pyrrole), 8.85 (m, 2 H, β-pyrrole), 8.63 (d, 1 H, β-pyrrole), 8.55 (m, 2 H, β-pyrrole), 8.47 (m, 2 H, ortho phenyl), 8.22 (s, 1 H, ortho phenyl), 8.16 (d, 2 H, J = 8.4 Hz, phthalimidophenyl), 8.12 (m, 1 H, ortho phenyl), 8.02 (m, 4 H: 2 ortho phenyl; 2 phthalic phenyl), 7.83 (d, 2 H, J = 8.8 Hz, phthalimidophenyl), 7.80 (m, 2 H, phthalic phenyl), 7.71 (m, 9 H, meta/para phenyls), 7.51 (m, 2 H, β-pyrrole), 7.34 (d, 2 H, J = 8.6 Hz, *N*-benzyl ring), 4.59 (d, 2H, J = 8 Hz, *N*-benzyl ring), -3.49 (d, 2 H, *N*-benzyl methylene). Anal.

Calcd. for $C_{59}H_{38}N_6O_4$: C, 79.0; H, 4.27; N, 9.38; O, 7.14%. Found: C, 78.31; H, 4.95; N, 8.78; O, 7.18%.

3.4.4 Synthesis of N-p-Nitrobenzyl-5-(4-aminophenyl)-10.15.20-triphenylporphyrin (**7**) *from N-p-Nitrobenzyl-5-(4-phthalimidophenyl)-10,15,20-triphenylporphyrin* (**6**)

N-substituted protected porphyrin (**6**) (150 mg) was dissolved in 90 mL of 1,4-dioxane, followed by addition of 6N NaOH (10 mL) and water to bring the final concentration of NaOH to 0.5 N. The solution was refluxed for 20 min. After it was cooled and neutralized with acetic acid, the mixture was extracted with 150 mL of methylene chloride. The organic solution was washed with three 200 mL portions of water and evaporated with a rotary evaporator until all solvent was removed. The crude carboxy-benzamide-*N*-substituted-porphyrin residue (Scheme 3-5) was directly used in the second hydrolysis step. It was dissolved in 100 mL of 1,4-dioxane, followed by addition of 5 mL of concentrated sulfuric acid and water to bring the final concentration of H_2SO_4 to 0.5 M. The solution was refluxed for 2 h. After the solution was cooled and neutralized with aqueous ammonia, the porphyrin was extracted with 150 mL of methylene chloride and then washed with water. The organic phase was reduced in volume and applied to an alumina column equilibrated with methylene chloride. After the unhydrolyzed porphyrin was eluted with methylene chloride, a solution of 1% acetone in methylene chloride was used to elute the desired band. Fractions

containing only the hydrolyzed porphyrin band were combined, giving *N-p*-nitrobenzyl-5-(4-aminophenyl)-10.15.20-triphenyl-porphyrin (**7**) in 60% yield.

^{1}H NMR ($CDCl_3$) δ: 8.98 (d, 1 H, β-pyrrole), 8.83 (m, 2 H, β-pyrrole), 8.62 (m, 3 H, β-pyrrole), 8.51 (m, 2 H, ortho phenyl), 8.25 (m, 1 H, ortho phenyl), 8.18 (m, 1 H, ortho phenyl), 8.08 (m, 2 H, ortho phenyl), 7.91 (d, 2 H, aminophenyl) 7.75 (m, 9 H, meta/para phenyls), 7.56 (m, 2 H, β-pyrrole), 7.40 (d, 2 H, aminophenyl), 7.06 (t, 2 H, *N*-benzyl ring), 4.65 (t, 2 H, *N*-benzyl ring), 4.03 (s, 2 H, amino), -3.45 (m, 2 H, *N*-benzyl methylene). Anal. Calcd. for $C_{51}H_{36}N_6O_2 \cdot H_2O$: C, 78.24; H, 4.86; N, 10.73; O, 6.13%. Found: C, 78.72; H, 5.56; N, 9.63; O, 5.45%.

3.4.5 Synthesis of N-p-Nitrobenzyl-5-(4-aminophenyl)-10,15,20-tris(4-sulfonatophenyl)porphyrin, Trisodium Salt (N-bzHAS$_3$P) (**8**) *from N-p-Nitrobenzyl-5-(4-aminophenyl)-10.15.20-triphenylporphyrin* (**7**)

The hydrolyzed *N*-substituted porphyrin (100 mg, 0.13 mmol) was mixed with 8 mL of concentrated sulfuric acid.and heated on a steam bath overnight. After cooling to room temperature, the dark green solution was poured into 250 mL of water. The sulfonated porphyrin precipitated at room temperature and the supernatant was carefully poured off. The precipitates were washed with two 100 mL portions of distilled water with centrifuging in between. Aqueous sodium hydroxide (1 mL, 1 N) was added to the slurry and the

porphyrin totally dissolved. The aqueous porphyrin solution was dialyzed against distilled water by using M.W. 500 cutoff membrane to give purified trisodium salt of (aminophenyl)-tri(*p*-sulfonatophenyl)porphyrin in 75% yield.

Anal. Calcd. for $C_{51}H_{33}N_6S_3O_{11}Na_3 \cdot 16H_2O \cdot 3NaOH$: C, 41.33; H, 4.56; N, 5.68; O, 32.45; S, 6.50; Na, 9.33%. Found: C, 38.21; H, 5.06; N, 4.20; O, 32.36; S, 6.54; Na, 8.22%.

3.4.6 Attempted Synthesis of N-p-nitrobenzyl-(N-p-nitrobenzylideneaminophenyl)triphenylporphyrin

5-(4-aminophenyl)-10,15,20-tri-phenylporphyrin (**4**) (300 mg, 0.48 mmol) and p-nitrobenzaldehyde (725 mg, 4.8 mmol) dissolved in 25 mL of methylene chloride and refluxed overnight. With the TLC of silica plates in methylene chloride it was seen that the starting material (R_f = 0.22) was converted to the product (R_f = 0.47) almost quantitatively and the R_f of p-nitrobenzaldehyde was the same as that of the product. Because of the difficulty of separating the product from the extra *p*-nitrobenzaldehyde and the large difference of polarity between *N*-substituted porphyrins and non-*N*-substituted one, the protected (aminophenyl)triphenyl-porphyrin was not purified immediately. The mixture was reacted directly with the diphenyl-p-nitrobenzylsulfonium salt (430 mg, 0.96 mmol) in methylene chloride at 120 °C in the sealed glass tube with a Teflon stopper for 1.5 h. The solution was cooled and neutralized with 15 mL of 2 M aqueous ammonium hydroxide. The mixture was washed with three 200 mL

portions of water. The green spot of product migrated very little on a TLC plate under the same conditions as above. Therefore, the *p*-nitrobenzaldehyde remaining from the preceding reaction, as well as unreacted protected and unprotected porphyrin from the previous reaction, were now easily separated from the *N*-substituted product by chromatography on a silica column. The green product was a mixture of *N*-substituted porphyrins which separated as several spots on a silica TLC using a solution of 2% of acetone in methylene chloride as developing solvent.

After being dried in the air, the *N*-substituted-(*p*-nitrobenzylideneaminophenyl)triphenylporphyrin was added *p*-toluenesulfonic acid (200 mg, 0.8 mmol) dissolved in 25 mL of acetone and 1 mL of water. The solution refluxed overnight. An aliquot of solution was neutralized with trimethylamine and run a TLC under the same condition as above, but no significant changes were shown on TLC plate in comparison to the unhydrolyzed sample. The solution was added 0.02 mL of concentrated hydrochloric acid and continued to reflux for 4 h, but there was still no change on TLC.

The acid-treated mixture was neutralized with ammonium hydroxide, washed with water and then chromatographed through a silica column with a series of solvents in the order of increasing polarity. The first green band was eluted with solution of 1% of acetone in methylene chloride, the last green band came out with 6% acetone in methylene chloride. The green residue, composed of macrocycle degradation

products, was eluted with 15% of acetone in methylene chloride. ^{1}H NMR spectra were made for every fraction of the mixture and none of these showed any peak with amino group characteristics.

(*N-p*-nitrobenzylideneaminophenyl)triphenylporphyrin was prepared again in the same way as above and was purified by a silica column with solution of 70% of hexane in methylene chloride. In this solvent system the mobility of *p*-nitrobenzaldehyde was greater than that of the product. After all extra benzaldehyde was eluted out, which could be detected by a UV lamp (long wavelength), the desired porphyrin band came out slowly with the same developing system. Reduced volume by a rotary evaporator, the collected porphyrin solution was dried in a beaker open to the air over a weekend. Further dried in a vacuum oven, the porphyrin was run by ^{1}H NMR spectroscopy and, unexpectedly, it was found that this porphyrin was just like the starting material, i.e. (aminophenyl)triphenylporphyrin. Checking again with the TLC, it was seen that most of the protected porphyrin was converted back.

3.4.7 Attempted Synthesis of N-p-Nitrobenzyl-(N-5-chlorosalicylideneaminophenyl)triphenylporphyrin

5-(4-aminophenyl)-10,15,20-tri-phenylporphyrin (**4**) (300 mg, 0.48 mmol) and 5-chlorosalicylaldehyde (750 mg, 4.8 mmol) dissolved in 50 mL of methylene chloride, and 0.3 mL of glatial acetic acid was added. The solution refluxed

overnight. The TLC of silica plates with 1:1 mixture of methylene chloride and hexanes showed that starting material (R_f = 0.16) was converted to product (R_f = 0.71) almost quantitatively. Without acetic acid, refluxing overnight could not make complete conversion. After neutralization by an aqueous solution of ammonium hydroxide, washing with water and extraction by chloroform, the protected porphyrin was chromatographed by a silica column. With the solution of 30% of methylene chloride in hexane, the extra 5-chloro-salicylaldehyde was eluted and the first band, i.e. the protected porphyrin, eluted by using the solution of a 1:1 mixture of methylene chloride and hexane. A significant amount of the second and also the last band eluted with methylene chloride was unexpectedly found by TLC to be the starting porphyrin. These should not have been so much because the TLC before the column chromatography showed clearly that conversion was complete. The *N*-salicylidene must have decomposed and the (aminophenyl)triphenylporphyrin reformed. The decomposition is attributed to the washing of the reaction mixture with basic aqueous solution to purify the product.

The porphyrin protected by *N*-5-chlorobenzalidene was then reacted with p-nitrobenzyldiphenylsulfonium salt under the same condition as the porphyrin protected by the *N*-nitrobenzalidene. The green *N*-substitution product was also a mixture, seen as about five spots on the TLC plate, similar to the case of the porphyrin protected by the *N*-

nitrobenzalidene mentioned above. The green mixture was treated with hydrochloric acid in order to hydrolyze the protecting group, *N*-5-chlorobenzalidene. A similar procedure to that described in the last section was using to separate the mixture, though more tedious. ^{1}H NMR spectroscopy was run for every component of the mixture and one of the spectra showed a broad peak near 4 ppm of chemical shift indicating the sample was hydrolyzed and the amino group recovered.

3.4.8 Verification of Primary Amine Groups by Using an Affinity Gel

Affi-Gel 10 (20 mL) was put in a glass fritted funnel. the supernatant solvent was drained and the gel was washed with five bed volumes of cold isopropanol. A porphyrin solution (10 mg in 2 mL of DMSO) was added to the gel and a uniform suspension was made by agitating. The mixture was then washed with three 50 mL portions of 1 M ZnCl water solution and the supernatants were drained. With porphyrins possessing amine functionality, the gel retained the color of the corresponding porphyrin, meaning the porphyrin bound to the gel and could not be washed off from the gel by the ZnCl salt solution. With the analog porphyrin without amine groups, the gel turned to its original color after washing, meaning the porphyrin did not bind to the gel covalently.

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