STRUCTURE OF THE ACTIVE COMPONENT OF HEMATOPORPHYRIN DERIVATIVE (HPD) AS A TUMOR-LOCALIZING REAGENT

By

Shinji Takamura

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Chemistry

1985

ABSTRACT

STRUCTURE OF THE ACTIVE COMPONENT OF HEMATOPORPHYRIN DERIVATIVE (HPD) AS A TUMOR-LOCALIZING REAGENT

By

Shinji Takamura

A model compound of hematoporphyrin was synthesized to characterize the structure of the active tumor-localizing component of hematoporphyrin derivative (HPD) which has been used for detection and phototherapy of malignant tissues. This active component of HPD was identified as a diporphyrin joined by an ester linkage. The effect of solvent on the structural conformation of this diporphyrin ester has also been examined by spectroscopic methods.

TO MY FAMILY

ACKNOWLEDGEMENTS

The author expresses his appreciation to Dr. Chi K. Chang for his guidance, patience, and support throughout the course of this work.

Appreciation is also extended to the members of Dr. Chang's research group for their helpful discussions and encouragement, and to Dr. Brian Musselman for providing useful references and his successive cooperation through this project.

The financial support of Michigan State University is gratefully acknowledged.

And finally, the author wishes to thank his wife, Hiromi, for her encouragement, patience and understanding.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

INTRODUCTION

Historical Perspective

The phototoxic effect ("photodynamic action") of administered porphyrin in mammals appears to have been first observed by Hausmann[1] in mice and by Meyer-Betz[2] in man over 70 years ago. The effect was known in certain porphyrias long before this, but the cause was not known. The localization of administered porphyrins in tumor tissue was recognized[3] in the 1940's, and this behavior was explored further in the 1950's in work that led to the development of the hematoporphyrin derivative (HPD) preparation.[4,5]

It was not until 1972 that these two ideas concerning the biological activity of porphyrins (that is, photodegradation of tissue and localization in tumors) came together successfully when Diamond and his colleagues[6] demonstrated that crude hematoporphyrin, when activated by white light, could produce regression in transplanted glioma tumors in rats. In 1975, this result was confirmed and extended by Berenbaum[7] and by Dougherty.[8]

Since then, clinical application, called "photoradiation therapy" (PRT) or "photodynamic therapy

(PDT), has been developed for eradication of malignant tumors in humans and animals utilizing HPD as a photosensitizing drug. Clinical successes have stimulated interest in fundamental research into porphyrin photosensitization phenomena.

Clinical Procedures

The selective concentration of porphyrins at tumor sites, following HPD injection, has previously been utilized only for the tumor-imaging purpose.[9-12] The introduction of light completes the tumor destruction procedure. The therapy begins with the administration of a porphyrin photosensitizer to the subject (usually intravenous injection) prior to illumination of the tumor. Preferential accumulation of the pigment at neoplastic loci occurs between 24-96 hours, at which time exposure of the activated tumor to red light, delivered via fiber optics, results in photodestruction of the cell.[13,14]

Photoradiation therapy is particularly valuable in treating lung cancer in older persons who frequently have poor pulmonary or cardiac function which precludes other therapies such as radiotherapy and chemotherapy due to side effects.[15] The combination of HPD phototherapy and surgery for treatment of brain tumors[16] provides an example of the potential usefulness of PRT in combination with another therapeutic modality.

Porphyrin photoradiation therapy has also been successful in eradicating a group of spontaneous tumors in pet cats and dogs.[17] These animals had received no other therapy, and their tumors had not become widely disseminated. The analogous use of photoradiation therapy in man as an early mode of tumor therapy may result in an increased incidence of successes.[18]

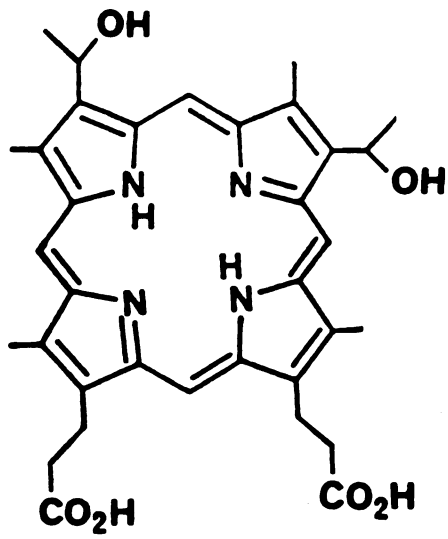
Porphyrins can also photosensitize a variety of microbial cell types.[19] PRT might, therefore, be useful for eradication of persistent superficial infections resistant to conventional antibiotic therapy.

HPD Localization

Chemical studies have shown that photoradiation therapy of many tumors can be carried out with minimal damage to adjacent normal tissues. This selective effect is not, however, a universal phenomenon. In the case of the eye, porphyrin accumulation by normal tissue[20] was observed. The precision targeting of irradiation, therefore, will be needed if PRT is to be used for therapy of ocular tumors. Other instances of accumulation of HPD components by "normal" tissues may be found. Porphyrin components of HPD also accumulate in another normal tissue: the skin. Clinical reports cited above mention the transient photosensitization of skin which follows HPD administration. This result was not unexpected. Using a labeled HPD in the

mouse, Gomer and Dougherty[21] had reported substantial accumulation of the label in the skin of these animals.

Hematoporphyrin Derivative (HPD) - Its Chemical Nature



1

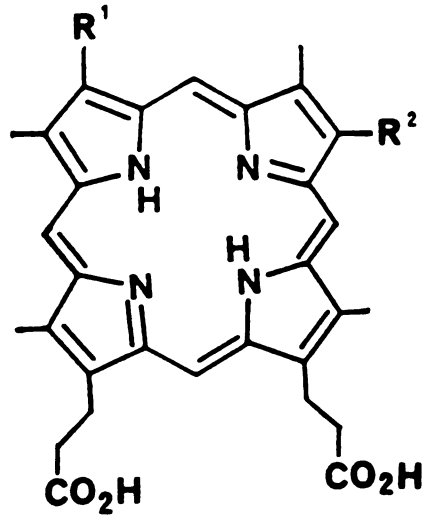
Hematoporphyrin IX 1 and especially the related product known as "Hematoporphyrin Derivative" (HPD) have been the most used compounds in tumor diagnosis and phototherapy. HPD was first prepared by Lipson et. al.[5] in 1960.

Hematoporphyrin 1 dihydrochloride was treated with 5% sulfuric acid in acetic acid to give a solid material. They referred to this as "hematoporphyrin derivative", and this name is retained here. In biological studies, it was treated with base to overcome the solubility problem into aqueous media prior to injection. This treatment is now known to cause chemical changes in the solid derivative, and hence, the material prepared for injection is called real

"HPD", and the original HPD is changed to HPA (acetylated hematoporphyrin) due to the major components of this.

$$\text{I} \xrightarrow[1\text{ h, }20^{\circ}\text{C}]{5\%\ H_2SO_4\ \text{in HOAc}} \text{HPA} \xrightarrow[1\text{ h, }20^{\circ}\text{C}]{0.1\text{N NaOH}} \text{HPD}$$

HPA is now known to be a complex mixture (Figure 1). It was separated and characterized by P. S. Clezy et. al.[22] in the form of the corresponding dimethyl ester derivatives and found to be a mixture of both acetylation and dehydration products. Components of HPA and HPD were also analyzed conveniently as the free acids by high-pressure liquid chromatography (HPLC) employing a reverse-phase system[23,24] and identified by comparison with authentic porphyrin-dicarboxylic acids.



1	$R^1 = R^2 = CH(OH)CH_3$
2a	$R^1 = CH(OH)CH_3$; $R^2 = CH(OAc)CH_3$
2b	$R^1 = CH(OAc)CH_3$; $R^2 = CH(OH)CH_3$
3a	$R^1 = CH = CH_2$; $R^2 = CH(OH)CH_3$
3b	$R^1 = CH(OH)CH_3$; $R^2 = CH = CH_2$
4	$R^1 = R^2 = CH(OAc)CH_3$
5a	$R^1 = CH = CH_2$; $R^2 = CH(OAc)CH_3$
5b	$R^1 = CH(OAc)CH_3$; $R^2 = CH = CH_2$
6	$R^1 = R^2 = CH = CH_2$

Figure 1. Components of HPA

The composition of the HPA mixture is somewhat variable, but the main components are 0,0'-diacetylhematoporphyrin **4** and O-acetylhematoporphyrin **2a,b** with smaller amounts of the 8(3)-(1-acetoxyethyl)-3(8)-vinyldeuteroporphyrin isomers **5a,b** and the corresponding alcohols **3a,b** as shown in Figure 2.

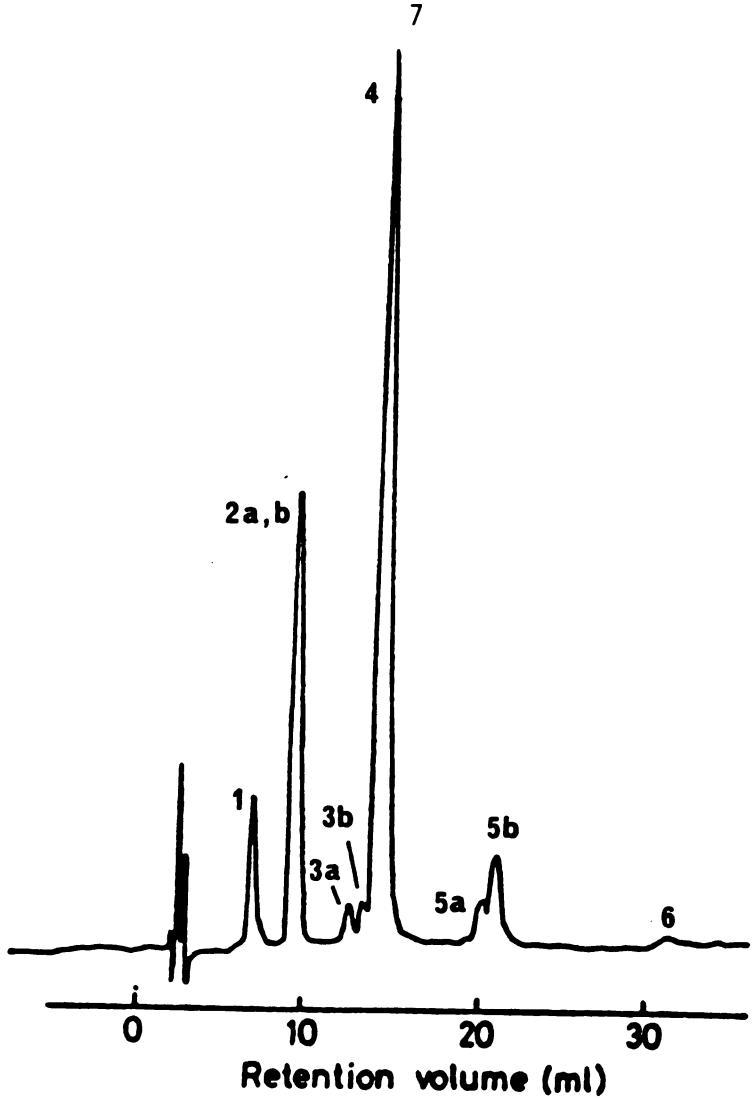


FIGURE 2. Separation of components of haematoporphyrin derivative by analytical h.p.l.c. using the DuPont Zorbax™ ODS column eluted with aqueous tetrahydrofuran–acetate buffer.

On the other hand, no acetylhematoporphyrin is found in alkali-treated HPD. This is expected because, evidently, hydrolysis and elimination reactions occur in alkaline solution. According to the HPLC analysis, the alkali treatment leads to hematoporphyrin 1, the hydroxyethyl vinyldeuteroporphyrin isomers 3a,b, and protoporphyrin 6.

However, these products (1, 3a, 3b, 6) are inactive in the biological assay. Only the acetylated porphyrins (i.e.,

the mono- and diacetates of hematoporphyrin 2 and 4, which are major components of HPD) and acetoxylethylvinyldeuteroporphyrin 5 are active.[25]

It was postulated that another type of substance must be formed in the alkali treatment. The active component here is a porphyrin, possibly a dimer or oligomer, which is retained on the column during the normal separation by HPLC. This postulation is supported by the following observations:

(i) The crude material obtained from the spent column is active without further alkali treatment.

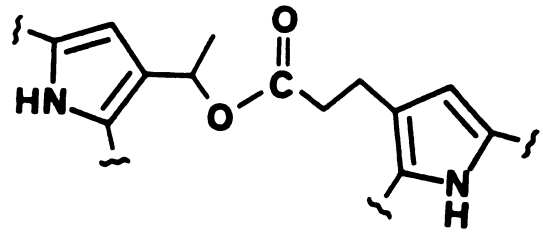
(ii) The activity develops over 30 minutes when HPA or the mono- or diacetates of hematoporphyrin are treated with alkali.

(iii) Gel-filtration, size-exclusion chromatography, is also useful to isolate this active component from the other porphyrins.[26]

Recently, fast atom bombardment (FAB) mass spectrometry (MS) has been utilized for the analysis of HPD[27,28] and confirmed the presence of mono-, di-, and triporphyrins.

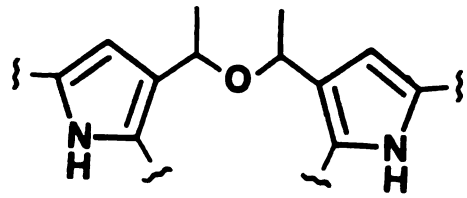
Three different dimer structures were suggested based on condensation of hemaporphyrin acetates.[25,29]

1. ESTER



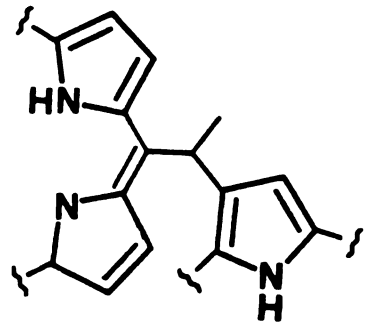
Hydrolyzed fairly readily. Elimination possible.
Dimeric structure possible with four such bonds!

2. **ETHER**



More difficult to hydrolize. Elimination possible (to give vinyl and hydroxyethyl groups).

3. **CARBON-CARBON BOND**



Derived from electrophilic substitution of benzylic-type carbonium ion at meso position. Likely to be quite stable.

Tentative characterization of this dimeric compound as a dihematoporphyrin ether had been proposed.[27] The assignment of the ether structure to the porphyrin was based largely on the stability of the bond connecting the two porphyrin moieties in 1N NaOH. However, no decisive spectroscopic evidence to characterize the dimeric structure has been obtained yet.

Purpose of This Work

The mechanism of pharmacological activity and the biochemical basis for the higher affinity of HPD for tumor

cells are still unknown, partly owing to the incomplete characterization of the active HPD components. As most of the HPD has been characterized as ineffective monoporphyrins, the use of large doses of HPD has been required for successful therapy, and this causes the increase in skin photosensitization.

It is, therefore, obviously of considerable interest to separate the active component of HPD and establish its chemical identity. The major difficulty associated with separation and analysis of HPD arises from the fact that the hematoporphyrin has two hydroxyl and two propionic acid groups positioned unsymmetrically on the porphyrin ring. Statistically, there are 3 ether structural isomers or 4 esters possible between two hematoporphyrin molcules, and each consists of diastereotopic isomers. In reality, either set of compounds is likely to be present during the HPD preparation and, no doubt, this situation has made the isolation and characterization for a specific structure a nightmare.

With the goal of answering the question concerning the structure of the active component of HPD, model studies have been proposed to simplify the situation. The evidence demonstrating that the active components of HPD are dimeric and trimeric hematoporphyrins linked via an ester bond is presented in this work.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

I. Synthesis of 17-(2-carboxyethyl)-8,12-diethyl-3-(1-hydroxyethyl)-2,7,13,18-tetramethyl-21H,23H-porphine 7

A hematoporphyrin model compound 7, which contains the necessary functional groups for coupling but has no possibility of forming isomeric products, was designed to simplify the problem of characterization of HPD (Figure 3).

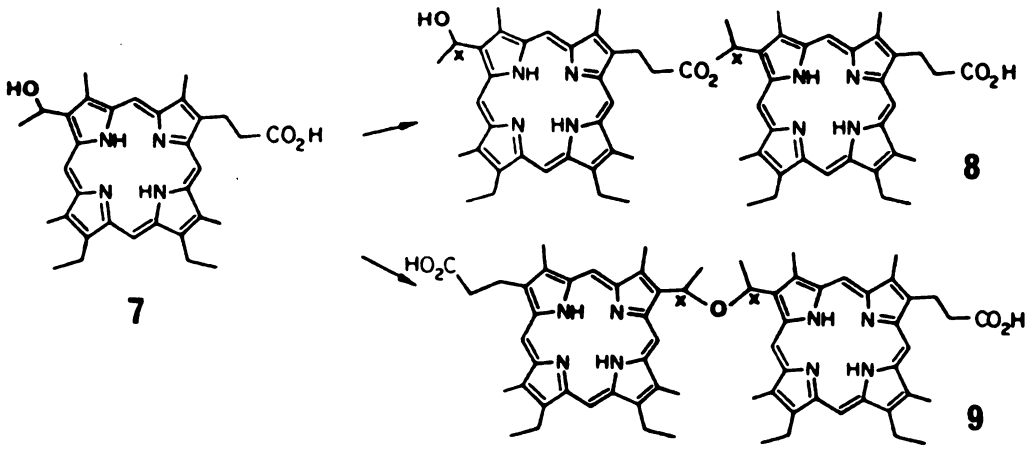


Figure 3. Two proposed structures for the dimeric porphyrin

The synthesis of porphyrin 7 is shown in Figures 4 and 5. Diborane generated from ethereal boron trifluoride and sodium borohydride was employed to reduce 4-ethoxycarbonylmethyl pyrrole 10 to 2'-hydroxyethyl pyrrole 11. Successful results were obtained by using excess diborane.[31] Replacement of the hydroxyl group with chlorine

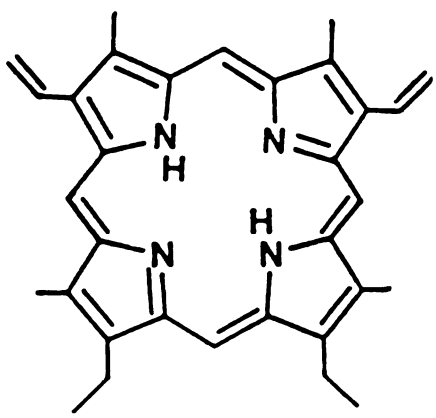
was accomplished with thionyl chloride in benzene at reflux, but the product contained a small amount of impurity, possibly from air oxidation. The higher yield without any impurity was obtained when the reaction was carried out under inert gas. The benzyl group of pyrrole 12 was removed quantitatively by catalytic hydrogenolysis with 10% palladium on carbon to give pyrrole-2-carboxylic acid 13.

The procedure of Clezy, et. al.[31], was used to convert t-butyl pyrrole-2-carboxylate 14 to 2-formyl pyrrole 15. Trifluoroacetic acid hydrolyzes the t-butyl ester and decarboxylates the resultant acid. Then the α-free pyrrole is formylated by orthoformate. This pyrrole turns greenish when left in the air. 5,5'-Dimethylpyrromethene 16 was obtained by condensation of 2-formyl pyrrole 15 and α-free pyrrole resulting from decarboxylation of pyrrole-2-carboxylic acid 13.

In the synthesis of porphyrin 18 via dipyrromethenes, the symmetric 5,5'-dibromodipyrromethene 17 is selected as it is simple to prepare. 5,5'-Dimethyldipyrromethene 16 was condensed with 5,5'-dibromodipyrromethene 17 in anhydrous formic acid in the presence of one equivalent of bromine[32] to give porphyrin 18 in 40% yield.

2'-Chloroethylporphyrin 18 was readily converted in high yield into the corresponding vinylporphyrin 19 by treatment with sodium hydroxide in refluxing aqueous pyridine as reported by Clezy, et. al.[34] In addition to

dehydrochlorination, the hydrolysis of the ring ester was accomplished conveniently. Thin-layer chromatography (TLC) analysis indicated the presence of a minor product moved rapidly on the plate. ^{1}H-NMR analysis of this fraction gave a highly symmetric spectrum and showed vinyl protons, ring methyl and ring ethyl protons, and indicated the absence of the propionic acid side chain, suggesting diethyl-tetramethyl-divinylporphyrin **21** shown below. This product was a very minor product formed during the porphyrin condensation and can be separated easily at this stage by column chromatography.



21

Treatment of vinyl porphyrin **18** with saturated hydrogen bromide-acetic acid produces an HBr adduct in the manner consistent with Markovnikov's rule. The adduct was then immediately hydrolyzed with water and neutralized with aqueous sodium hydroxide to give the desired model hematoporphyrin, 1'-hydroxyethyporphyrin **7**.

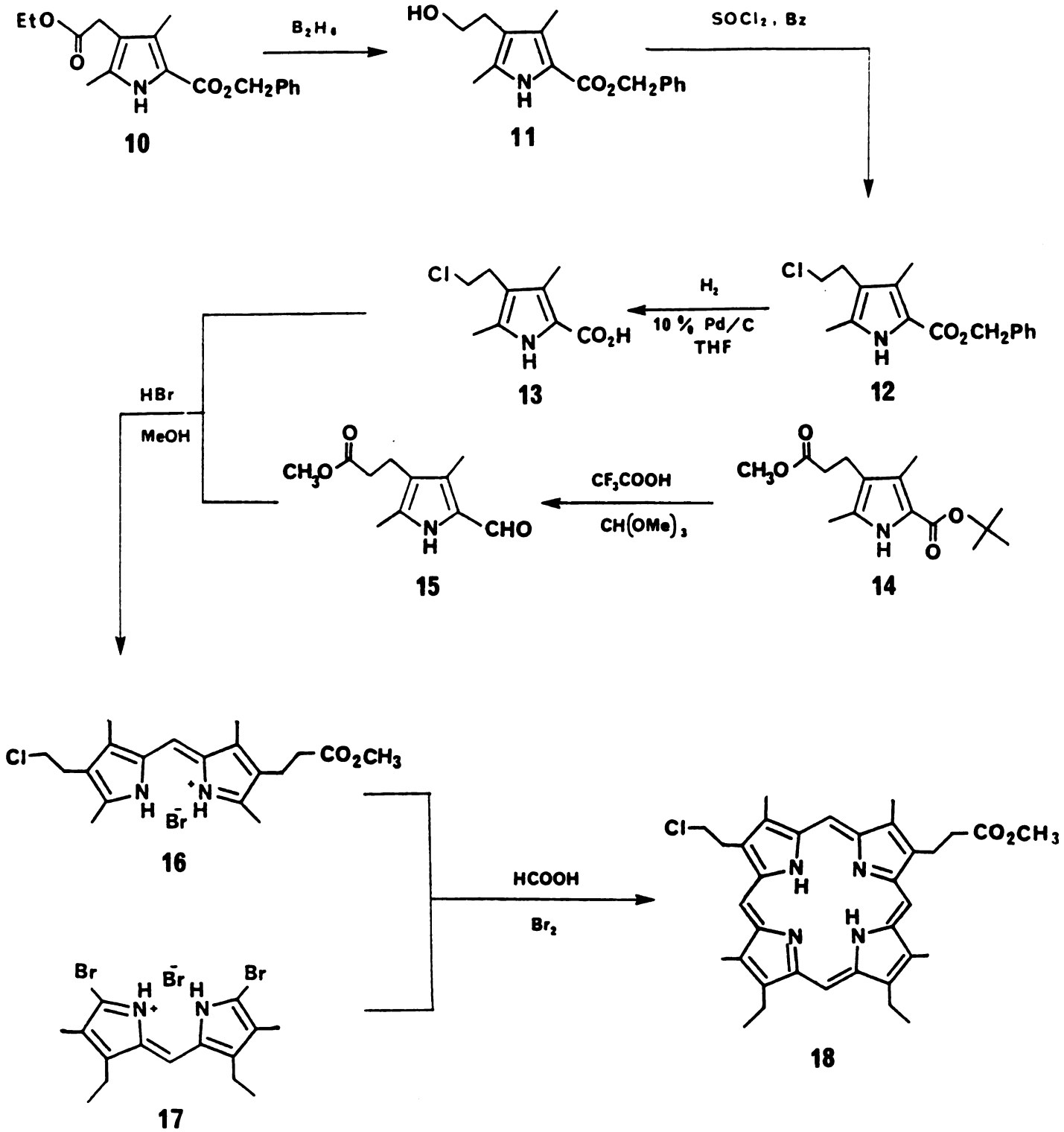


Figure 4. Synthesis of porphyrin via dipyrromethenes

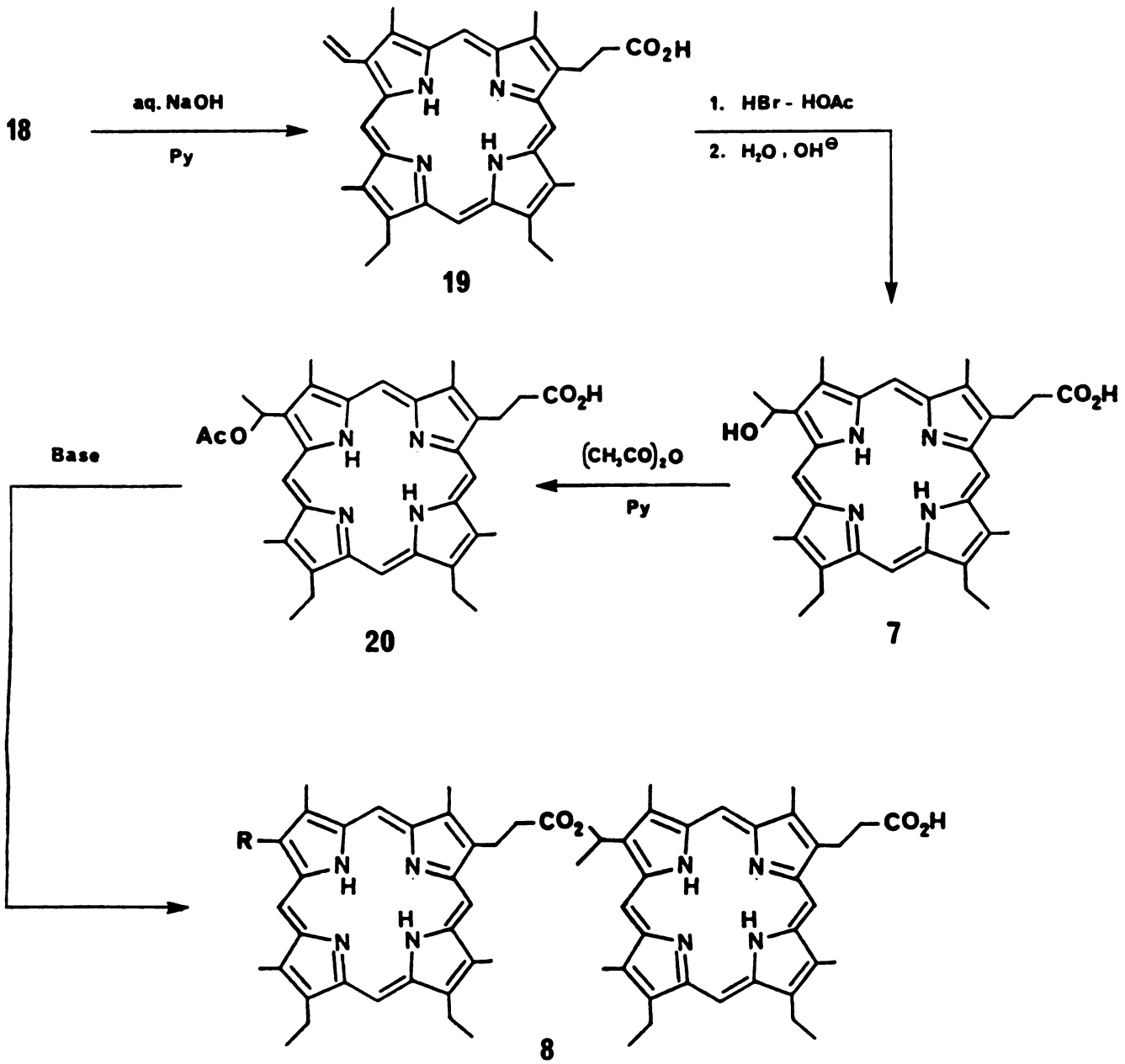


Figure 5. Modification of the ring substituents to a model compound and formation of diporphyrins.

II. Isolation and Characterization of Dimeric Components of Model HPD.

As mentioned previously, it has been reported that the main components of HPA are di- and monoacetylhematoporphyrins[22,23] and the acetylated porphyrins only become biologically active when treated with alkali.[25] These findings suggest that the use of pure acetylated porphyrin, rather than the H_2SO_4-acetic acid treated mixture (HPA) for alkali treatment, should bring promising results.

1'-Hydroxyethylporphyrin **7** was acetylated effectively with acetic anhydride in dry pyridine to give 1'-acetoxyethylporphyrin **20**, which was then treated with 0.1N sodium hydroxide solution. In basic conditions, both hydrolysis and elimination reactions could be expected. Careful comparison of the retention times of the reaction products with those of authentic samples in high pressure liquid chromatography (HPLC) and thin-layer chromatography (TLC) indicated the presence of 1'-hydroxyethylporphyrin **7** as a hydrolysis product, vinylporphyrin **19** as an elimination product and 1'-acetoxyethylporphyrin **20** as an unreacted starting material. Besides these three fractions, another rapid-moving brownish fraction was detected on the TLC plate, which was later shown to be dimeric components.

Analysis of this product mixture by fast atom bombardment mass spectroscopy (FAB-MS) resulted in the

generation of relatively intense ion species at m/z 1059, 1041 and 1023 (Figure 6). The mass of two 1'-hydroxyethylporphyrin 7 molecules is 1076; covalent bonding of these molecules via an ether or ester bond results in the loss of water to give a mass of 1058; protonation[28] of this porphyrin dimer yields an ion mass of 1059 which corresponds to the m/z 1059 component identified in the mass spectrum. Ions at m/z 1041 and 1023 may result from successive loss of water molecules from the molecular ion species. These water losses may have occurred either during FAB-MS analysis or prior to analysis during the HPD synthesis procedure. A second, less intense ion species is present in the spectrum at m/z 1081; this ion arises due to the presence of sodium salt in the sample carried over from the buffer solvent. In this case, the sodium adduct (Na^+) rather than the protonated species is produced under FAB ionization conditions. Though FAB-MS analysis confirmed the presence of dimeric components in the product mixture, it can not distinguish between the two possible oligomer linkages, ether or ester, as proposed for such a dimer.[29]

The reaction was repeated in various organic solvents; tetrahydrofuran, methanol, dimethylsulfoxide and acetone, and monitored by TLC. No significant difference in the product mixture was observed with these solvents. The rapid-moving dimer fraction increased gradually to a certain extent and then stopped. No further increase of this fraction was seen even with longer reaction time. The

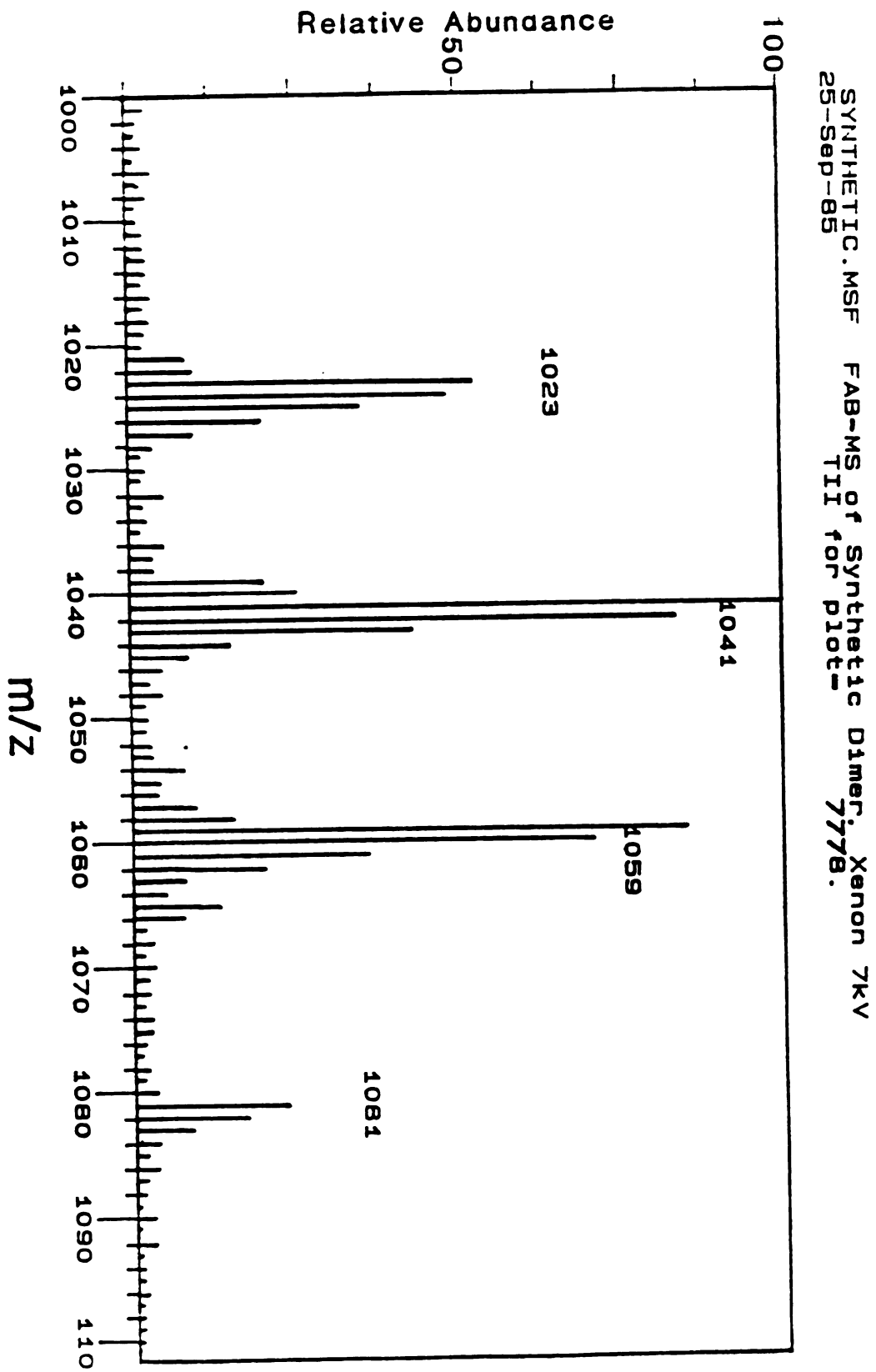


Figure 6. Fast atom bombardment mass spectrum (FAB-MS) of the product mixture

reaction in pyridine, however, did not give any dimer or starting material **20** but the hydrolysis and elimination products **7** and **19**. This seems to indicate that the starting material is hydrolyzed too fast to form a dimer; or even if the dimer is formed, the dimer is linked by an ester bond and hydrolyzed immediately under the reaction conditions. The higher reaction temperature (ca. 60°C) facilitated the elimination reaction to give more vinylporphyrin **19**. Using sodium bicarbonate as base repressed the hydrolysis reaction but the formation of the dimer also slowed down.

The ether structure was proposed by Dougherty[27], based on the finding that the tumor-localizing component of HPD was hydrolyzed in aqueous acid but not in aqueous base. An easy method to distinguish between the two possible linkages, ether or ester, may be by ^{1}H-NMR spectroscopy. Characteristic difference between these two is expected in the chemical shift of the methyne proton (marked X) in Figure 3. **8** would give two different peaks, while **9** would give only one. Previous ^{1}H-NMR spectra of the tumor-localizing component of HPD were too messy to prove the structure due to the difficulty in isolation of a single compound among a few possible isomers.

Since this model compound forms no dimeric isomers, the isolation should be much easier. Characteristic to the dimer fraction is a quenched fluorescence; a spot of the dimer on TLC appears as a brownish dark area, consistent with the result obtained for the fractionated-active

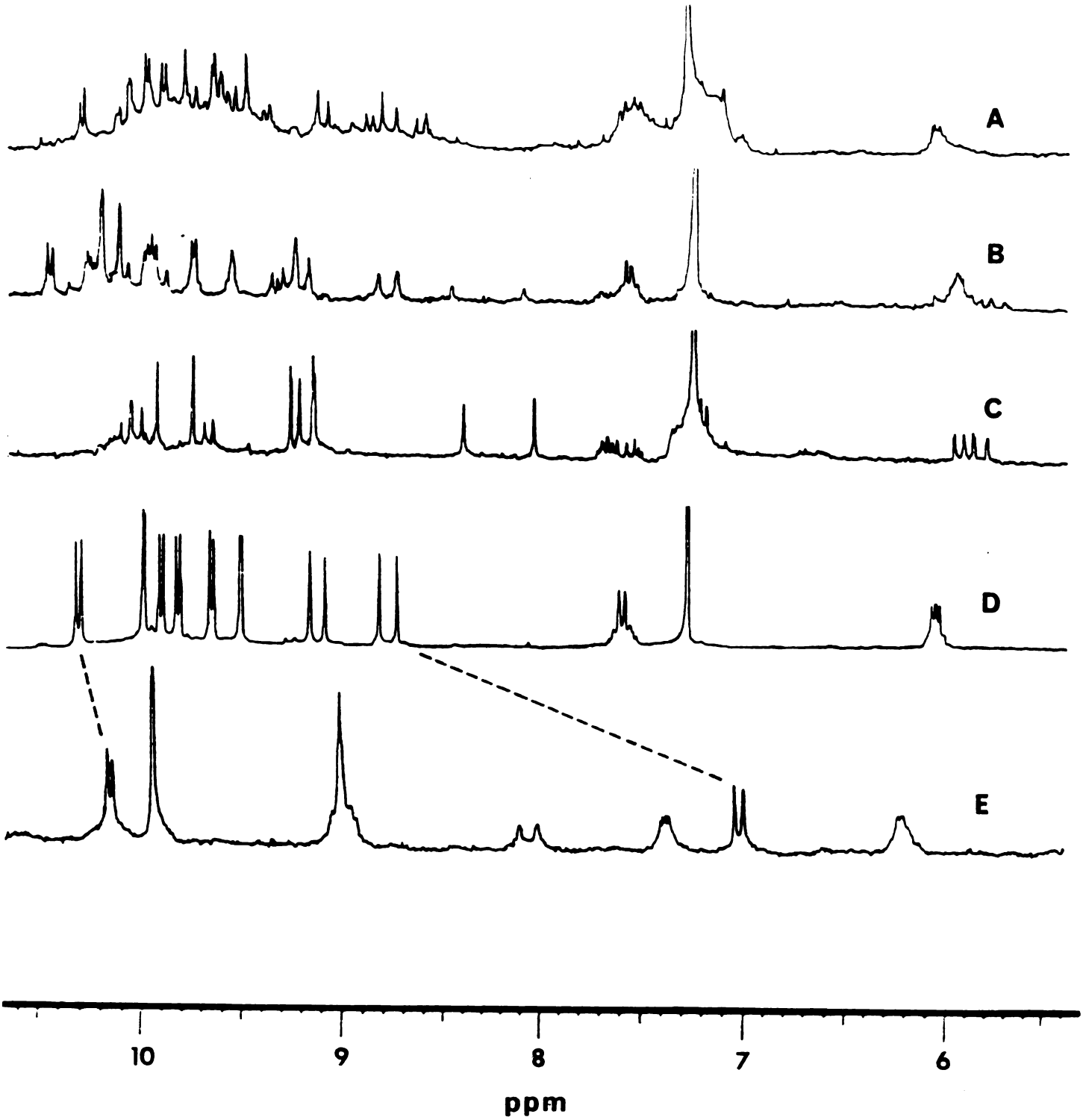


Figure 7. ^{1}H-NMR spectra of chromatographed fractions

A. Fractionated diporphyrin-free acid
B. Fractionated diporphyrin methyl ester
C. Vinyldiporphyrin methyl ester
D. 1'-Methoxyethyldiporphyrin methyl ester in $CDCl_3$
E. 1'-Methoxyethyldiporphyrin methyl ester in CD_3CN

component of HPD.[26] Separation of the product mixture was attempted by column chromatography, followed by thin-layer chromatography over silica gel using 5-10% methanol in methylene chloride as eluent. The isolated rapid-moving dimer fraction, however, failed to give a clean spectrum (Figure 7A) as HPLC analysis indicated that this fraction was not pure enough. Further purification of this sample on silica gel did not give improved NMR spectra. The difficulty in separation appeared to arise from the polar propionic acid side-chain which causes streaking badly on silica gel.

The separation process became much easier when the product mixture was esterified with diazomethane and chromatographed. This time the fraction of dimeric component moved more slowly than the methyl ester of vinylporphyrin **19** as well as 1'-acetoxyethylporphyrin **20**. This may be because all components became less polar after esterification of the acid side-chain, and the larger diporphyrin now has more interaction with silica gel than those monomers. Without esterification, the dimer fraction moves faster than any other monomers because monomeric porphyrins with a free propionic acid side-chain have a higher polarity than the dimeric porphyrins with one extra unit of a non-polar macrocyclic porphyrin ring.

All monomer fractions isolated were identified by direct comparison (TLC, ^{1}H-NMR) with authentic materials. ^{1}H-NMR spectrum of the isolated dimer fraction indicated two

different peaks in almost equal ratio at 6.06 and 7.58 ppm (Figure 7B). The investigation of this dimeric component by TLC, ^{1}H-NMR and FAB-MS indicated that more than one type of dimer was formed. Further separation gave two compounds whose spectra are shown in Figures 7C and 7D. Figure 7C shows vinyl protons at 5.84 and 5.96 as doublet-doublet. Figure 7D shows two different types of methyne protons clearly at 6.05 ppm (quartet) and 7.59 ppm (quartet) in equal ratio. These results rule out the possibility of an ether linkage for the diporphyrin and prove that the diporphyrins are linked by an ester group.

Mechanistically, the formation of an ester linkage is not only understandable but expected. Under the alkaline conditions employed in the preparation of HPD, the nucleophilic substitution reaction, illustrated in Figure 8, is very similar to the mechanism identified in classical experiments of Philips[33] for establishing the optical activity associated with S_N2 reactions (Figure 8).

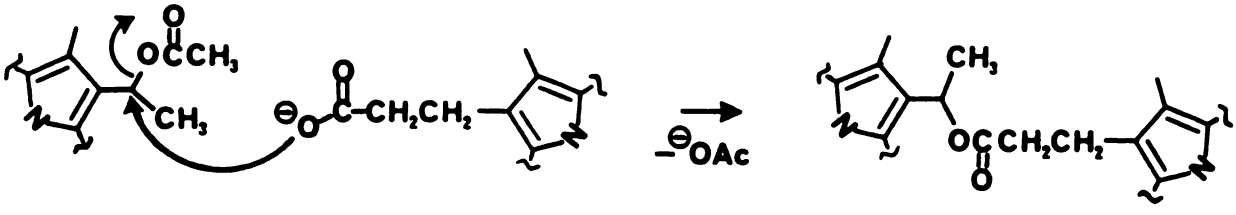


Figure 8. Possible mechanism in HPD preparation

Another observation consistent with this mechanism was made during the acetylation of the model hematoporphyrin 7 using 10% acetic acid anhydride in pyridine. In addition to 1'-acetoxyethylporphyrin **20**, a small amount of the

diporphyrin was formed due to the generation of the carboxylate anion as a nucleophile between the acid side-chain and pyridine.

FAB-MS analysis of the isolated diporphyrin shows clean and intense ion species at m/z 1087 though the protonated methyl ester of the dimer **8b** has the mass of 1073. The difference of 14 mass units is due to the formation of a methyl ether **8d**. The reason for this is that the originally produced diporphyrin **8c** is labile and undergoes solvolytic changes relatively readily when it is developed on the silica gel TLC plate using methanol-methylene chloride as a solvent. Methanolysis, however, does not occur without silica gel which facilitates the solvolysis catalytically. A similar result has been reported by Clezy, et. al.[22], except that they obtained ethyl ether derivatives due to the ethanol present as a stabilizer in chloroform. Therefore, chromatography with diporphyrin esters, particularly in the presence of alcohol, should be avoided.

^{1}H-NMR studies of the model diporphyrin compound yielded the following results. When dissolved in the non-polar solvent $CDCl_3$, each of eight meso protons in the dimer appeared as a doublet so that a total of sixteen prominent resonance signals appeared in the spectrum with chemical shifts between 8.7 to 10.3 ppm (Figure 7D). In contrast, the ^{1}H-NMR spectrum of the monomer contains, at maximum, four signals in the range between 9.7 and 10.3 ppm. This

difference may arise from interactions between the two-ring systems in the diporphyrin molecule which create a different and stable chemical environment for each meso hydrogen. The good resolution of these signals and the large chemical shifts apparent in the spectrum are indicative of a well-defined structure.

Analysis of the model compound in a more polar solvent was completed in order to examine the effect of different solvents on the conformation of the dimer. The ^{1}H-NMR spectrum taken in acetonitrile-d^{3} is shown in Figure 7E. The appearance of meso hydrogen signals from 6.9 to 10.2 ppm represents a doubling of the range of chemical shifts apparent in the non-polar solvent. This indicates that the meso hydrogens interact with each other to a greater extent in the polar solvent and, therefore, suggests that a more rigid, tightly bound dimer conformation exists in the polar environment.

The addition of a small amount of trifluoroacetic acid to the sample gave no sharp signals but broad ones. This technique is well known to break aggregation of certain porphyrins by use of charge repulsion between molecules via protonation of the porphyrin nitrogens. This result indicates that close association between the porphyrin rings of the dimer is disrupted upon protonation.

Absorption studies of the model compound in polar and non-polar solvents support the finding that a dimer is present in a form which allows for close proximity between

the two porphyrin rings. The absorption spectra of the dimer and the monomer are shown in Figure 9. The spectra in methanol show a significant blue-shift of the Soret peak (21nm) and a small red-shift in the visible bands for the diporphyrin, consistent with the spectral properties of previously synthesized co-facial diporphyrins.[35,36] In methylene chloride, the Soret blue-shift appears to be much less (< 5nm with reference to 7). These observations suggest that, while the two porphyrin rings of the dimer are closely associated in most solvents, their interaction in a polar solvent is tighter and more extensive than that observed in a non-polar solvent.

The results presented in this thesis demonstrate that the principle tumor-localizing component of HPD is porphyrin oligomers interconnected with ester bonds.[37] The ester linkage, containing four carbons and one oxygen between the two porphyrin rings, provides sufficient length for the co-facial interaction of the two rings. The degree of the interaction depends on the polarity of the solvent (i.e., the more polar solvent leads to the stronger interaction of the rings).

These properties of dimeric porphyrins could provide a possible explanation for HPD localization in tumor tissue. The binding and transport of HPD components by low-density lipoprotein (LDL) has been previously documented.[38] We postulate that the dimer conformation present in LDL is similar to that in the non-polar solvent. After the LDL

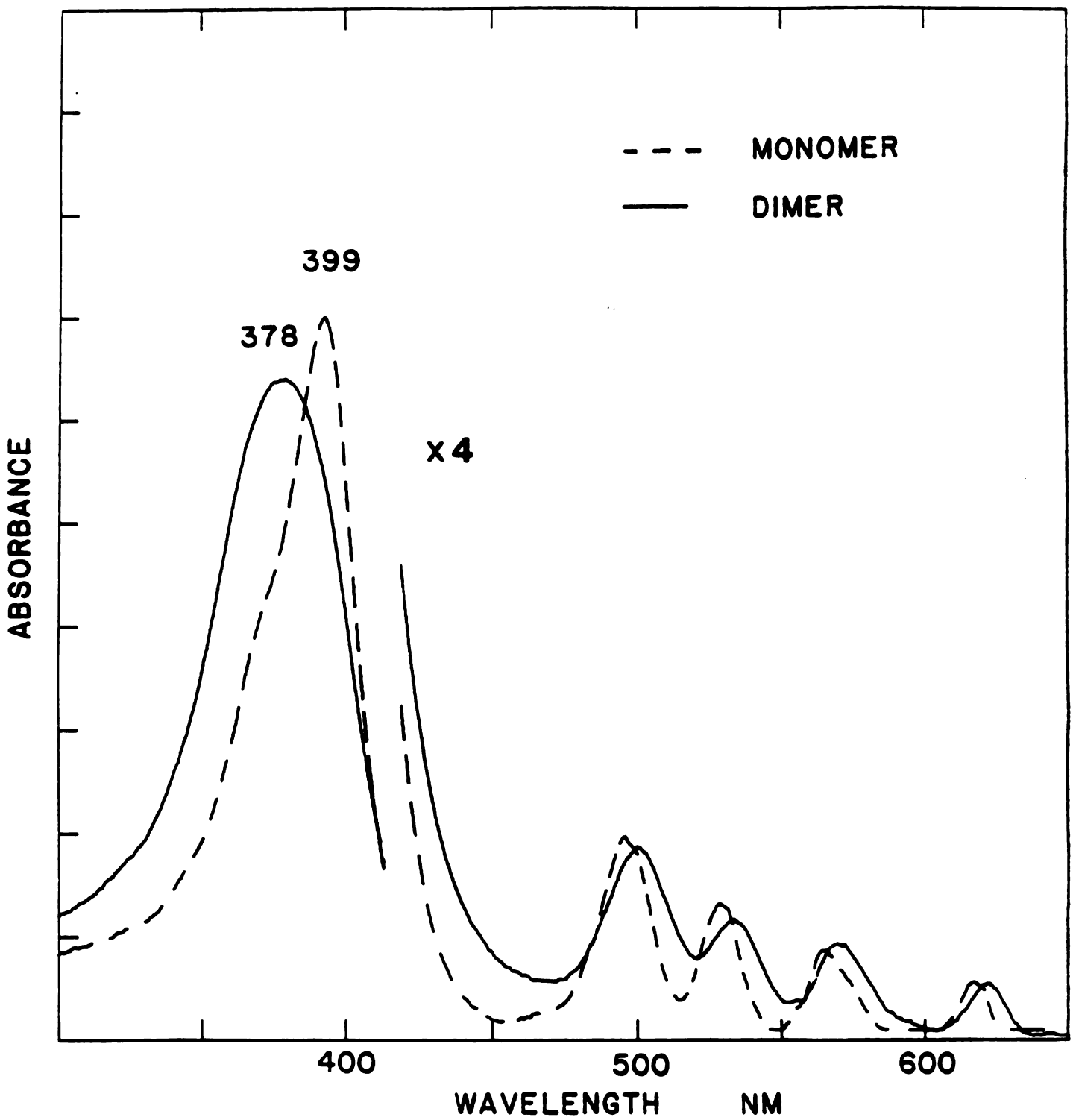


Figure 9. Visible absorption spectra of the diporphyrin 8d methyl ester (solid line) versus the starting material 7 (broken line) in methanol

enters the cell, the dimer is released into the cytosol, where the polar environment directs a change in the dimer conformation to the more tightly bound dimer observed in polar solvent. The dimer structure is selectively retained in the cell while monomers diffuse out of the cell through the membrane. It is not known at present whether the dimer may become protonated or metallated resulting in further conformational changes which could be important in controlling the rate of hydrolysis and the lifetime of the porphyrin in the cell. However, with the understanding of the chemical structure of the active component of the tumor-localizing agent, we are now able to define approaches which may answer important questions regarding the mechanism of localization and photodynamic activity of HPD.

EXPERIMENTAL

EXPERIMENTAL

GENERAL

NMR spectra were obtained on a Varian T-60 or on a Bruker WM-250 instrument with tetramethylsilane (TMS) as internal standard. Mass spectra were done on a Finnigan 4021 with an INCOS data system. UV-visible spectra were taken on a Varian Cary 219 machine with 1cm quartz cells. Melting points are uncorrected. Fast-atom bombardment (FAB) mass spectrometry (MS) analysis were completed on a Vacuum Generators ZAB-2F double-focusing mass spectrometer equipped with an Ion Tech FAB Gun. Solvents for FAB matrix were made up of thioglycerol, dithiothreitol, and dithioerythretol (2:1:1), addition of 0.1M trifluoroacetic acid to the matrix facilitated the ionization of the porphyrins during FAB analysis.[29]

A HEMATOPORPHYRIN MODEL COMPOUND

Benzyl 4-(2-hydroxyethyl)-3,5-dimethylpyrrole-2-carboxylate 11

Boron trifluoride-ether complex (120 ml) was added dropwise to sodium borohydride (25g, 0.66 mol) in bis-(2-methoxy-ethyl)ether (50 ml), and the diborane generated was

passed in a slow stream of argon through a solution of benzyl 4-ethoxycarbonylmethyl-3,5-dimethylpyrrole-2-carboxylate (20g, 0.063 mol) in tetrahydrofuran (100 ml) for 45 minutes. Methanol was then carefully added until effervescence ceased. The solvents were removed under reduced pressure, and the hydroxyethylpyrrole was recrystallized from methylene chloride-hexane as needles: yield 13.8g (79.6%); m.p. 115-116°C (lit.[39] m.p. 120-121°C); NMR ($CDCl_3$) δ 1.58 (br s, 1H), 2.18 (s, 3H), 2.28 (s, 3H), 2.62 (t, 2H), 3.63 (t, 2H), 5.29 (s, 2H), 7.36 (s, 5H), 9.0 (br s, NH); mass spectrum, m/e (rel. inten.) 91 (100), 134 (15), 242 (19), 273 (9, molecular ion).

Benzyl 4-(2-chloroethyl)-3,5-dimethylpyrrole-2-carboxylate 12

Benzyl 4-(2-hydroxyethyl)-3,5-dimethylpyrrole-2-carboxylate (10.0g, 0.037 mol) was dissolved in benzene (200 ml) and anhydrous potassium carbonate (20g, 0.145 mol) was added, followed by thionyl chloride (6 ml, 0.082 mol). The mixture was refluxed under argon for 3 hours. The solution was filtered and evaporated to dryness under reduced pressure, and the residue was chromatographed (silica gel-methylene chloride) and recrystallized from methylene chloride-hexane: yield 10.0g (93.6%); m.p. 119-120°C (lit.[30] m.p. 120-121°C); NMR ($CDCl_3$) δ 2.20 (s, 3H), 2.28 (s, 3H), 2.81 (t, 2H), 3.52 (t, 2H), 5.31 (s, 2H), 7.40 (s, 5H), 8.97

(br s, NH); mass spectrum, m/e (rel. inten.) 91 (100), 242 (11), 291 (6, molecular ion), 293 (2).

4-(2-Chloroethyl)-3,5-dimethylpyrrole-2-carboxylic acid 13

Benzyl 4-(2-chloroethyl)-3,5-dimethylpyrrole-2-carboxylate (4.0g, 0.014 mol) and 10% palladium-charcoal (0.7g) were stirred under hydrogen (1 atm, room temperature) in tetrahydrofuran (125 ml). The reaction was stopped when hydrogen uptake ceased. The mixture was filtered and evaporated to dryness under reduced pressure: yield 2.73g (98.7%); m.p. 112-114°C; NMR ($CDCl_3$) δ 2.23 (s, 3H), 2.30 (s, 3H), 2.83 (t, 2H), 3.51 (t, 2H), 8.77 (br s, NH); mass spectrum, m/e (rel. inten.) 134 (100), 152 (50), 201 (13, molecular ion), 203 (4).

4-(2-Methoxycarbonylethyl)-2-formyl-3,5-dimethyl pyrrole 15

Finely ground 2-t-butoxycarbonyl-4-(2-methoxycarbonyl-ethyl)-3,5-dimethylpyrrole (2.0g, 0.007 mol) was added in portions (0.2g) to stirred trifluoroacetic acid (10 ml, 0.130 mol) and the solution was warmed up to 40°C. After 5 minutes, trimethyl orthoformate (3 ml, 0.017 mol) was added in one portion and stirring was continued at 40°C for a further 5 minutes. Water was then slowly added and the dark oil was extracted with ether. The ether layer was washed with aqueous ammonia until the aqueous phase became pH~7, and then washed with water twice. The organic layer was

evaporated to dryness under reduced pressure. The crude product was purified by chromatography on silica gel to give the formylpyrrole as pale yellow needles: yield 0.6g (40.3%); m.p. 121-124°C (lit.[40] 128-129°C); NMR ($CDCl_3$) δ 2.27 (s, 6H), 2.50 (t, 2H), 2.62 (t, 2H), 3.67 (s, 3H), 9.44 (s, 1H), 9.61 (br s, NH); mass spectrum, m/e (rel. inten.) 44 (100), 69 (16), 136 (7), 209 (2, molecular ion).

4-(2-Chloroethyl)-4'-(2-methoxycarbonylethyl)-3,3',5,5'-tetramethyl-2,2'-dipyrromethene hydrobromide 16

4-(2-Chloroethyl)-3,5-dimethylpyrrole-2-carboxylic acid (5.3g, 0.026 mol) and 4-(2-methoxycarbonylethyl)-2-formyl-3,5-dimethylpyrrole (5.2g, 0.025 mol) were suspended in methanol (10 ml). This mixture was treated immediately with 48% hydrobromic acid (6 ml). The orange solid was formed after heating on a steam bath. The solid was triturated in ether, collected by filtration and dried *in vacuo*: yield 9.0g (84.1%).

3-(2-Chloroethyl)-8,12-diethyl-17-(2-methoxycarbonylethyl)-2,7,13,18-tetramethyl-21H,23H-porphine 18

4-(2-Chloroethyl)-4'-(2-methoxycarbonylethyl)-3,3',5,5'-tetramethyl-2,2'-dipyrromethene hydrobromide (9.0g, 0.0209 mol) and 5,5'-dibromo-3,3'-diethyl-4,4'-dimethyl-2,2'-dipyrromethene hydrobromide (10.5g, 0.0226 mol) were suspended in anhydrous formic acid (85 ml) and treated with bromine (1.2 ml). The mixture was heated in an

oil bath (ca. 80°C) for 2.5 hours. The solvent was then allowed to boil off over a period of 30 minutes with further heating in the bath up to 130°C during which time the porphyrin was formed. To the residue was added methanol (300 ml) and concentrated sulfuric acid (25 ml), followed after 10 minutes by trimethyl orthoformate (100 ml). After standing overnight, protected from moisture, the reaction mixture was evaporated to dryness. The crude product was chromatographed over silica gel using methylene chloride. A dark non-fluorescent forerun was discarded. The porphyrin-containing fractions were diluted with methanol (500 ml) and evaporated under reduced pressure until the product crystallized as sparkling purple blades: yield 3.84g (39.5%); NMR ($CDCl_3$) δ 4.07 (s, 2H, NH), 1.85 (t, 6H, CH_3), 3.18 (t, 2H, CH_2), 3.33 (s, 3H, CH_3), 3.45 (s, 3H, CH_3), 3.52 (s, 3H, CH_3), 3.56 (s, 3H, CH_3), 3.67 (s, 3H, CH_3), 4.01 (m, 4H, CH_2), 4.24 (m, 4H, CH_2), 9.70 (s, 1H, meso), 9.75 (s, 1H, meso), 9.88 (s, 1H, meso), 9.99 (s, 1H, meso).

<u>17-(2-Carboxyethyl)-8,12-diethyl-2,7,13,18-tetramethyl-3-vinyl-21H,23H-porphine 19</u>

A solution of 3-(2-chloroethyl)-8,12-diethyl-17-(2-methoxycarbonylethyl)-2,7,13,18-tetramethyl-21H,23H-porphine (0.200g, 0.351×10^{-3} mol) in pyridine (90 ml) was refluxed under argon for a few minutes and then sodium hydroxide (0.6g, 0.015 mol) in water (20 ml) was added and heating continued for 1.5 hours. At the end of the reaction,

aqueous acetic acid (6M) was added to neutralize excess sodium hydroxide, followed by water (200 ml). The mixture was concentrated under reduced pressure and the precipitate was collected and dried *in vacuo* overnight. The crude product was purified by chromatography on silica gel using methylene chloride-methanol as eluent: yield 0.180g (98.7%); NMR ($CDCl_3$) δ 1.88 (t, 6H, CH_3), 3.30 (t, 2H, CH_2), 3.60 (3s, 12H, ring-CH_3), 4.05 (q, 4H, CH_2), 4.37 (t, 2H, CH_2), 6.15 (dd, 1H, vinyl-H), 6.32 (dd, 1H, vinyl-H), 8.25 (dd, 1H, vinyl-H), 10.00 (s, 2H, meso), 10.05 (s, 1H, meso), 10.14 (s, 1H, meso); λ_{max} (CH_2Cl_2) nm 402 (Soret), 502, 538, 571, 625. The NMR spectrum is shown in Figure 10.

<u>17-(2-Carboxyethyl)-8,12-diethyl-3-(1-hydroxyethyl)-2,7,13,18-tetramethyl-21H,23H-porphine 7</u>

17-(2-Carboxyethyl)-8,12-diethyl-2,7,13,18-tetramethyl-3-vinyl-21H,23H-porphine (0.200g, 0.835 x 10^{-3} mol) was stirred with the saturated hydrogen bromide-acetic acid solution (4 ml) in subdued light for 24 hours at room temperature (ca. 20°C). The mixture was poured into excess water (15 ml), and after allowing 5-10 minutes for hydrolysis of the HBr-adduct, it was neutralized with aqueous NaOH. The precipitate was collected and washed with water. The product was dried *in vacuo* overnight: yield 0.202g (97.6%); NMR ($CDCl_3$) δ 1.86 (t, 6H, CH_3), 2.12 (d, 3H, CH_3), 3.26 (t, 2H, CH_2), 3.52 (m, 12H, ring-CH_3), 4.07 (m, 4H, CH_2), 4.30 (t, 2H, CH_2), 6.26 (q, 1H, CH), 9.94 (s,

1H, meso), 9.98 (s, 1H, meso), 10.04 (s, 1H, meso), 10.26 (s, 1H, meso); λ_{max} (CH_2Cl_2) nm 401 (Soret), 499, 534, 568, 621. The NMR spectrum is shown in Figure 11.

3-(1-Acetoxyethyl)-17-(2-carboxyethyl)-8,13-diethyl-2,7,13,18-tetramethyl-21H,23H-porphine 20

17-(2-Carboxyethyl)-8,12-diethyl-3-(1-hydroxyethyl)-2,7,13,18-tetramethyl-21H,23H-porphine (0.100g, 0.186 x 10^{-3} mol) in pyridine-acetic anhydride (9:1, 3 ml) was stirred in subdued light under argon for 4 hours at room temperature (ca. 20°C). The mixture was frozen in an acetone-CO_2 bath and treated with glacial acetic acid (4 ml). The solid was allowed to warm up in an ice bath while being constantly agitated with a spatula. Ice-cold water (30 ml) was added in portions with continued stirring, and the precipitated porphyrin was collected and washed with ice-cold water. The crude product was purified by chromatography on silica gel, using methylene chloride-methanol as eluent: yield 0.097g (90.0%); NMR ($CDCl_3$) δ 1.84 (m, 6H, CH_3), 2.30 (s, 3H, acetyl-CH_3), 2.23 (d, 3H, CH_3), 3.28 (t, 2H, CH_2), 3.56 (s, 3H, ring-CH_3), 3.61 (s, 3H, ring-CH_3), 3.66 (s, 3H, ring-CH_3), 3.74 (s, 3H, ring-CH_3), 4.05 (m, 4H, CH_2), 4.36 (t, 2H, CH_2), 7.55 (q, 1H, CH), 10.02 (s, 1H, meso), 10.03 (s, 1H, meso), 10.09 (s, 1H, meso), 10.40 (s, 1H, meso); λ_{max} (CH_2Cl_2) nm 400 (Soret), 499, 535, 568, 621. The NMR spectrum is shown in Figure 12.

Preparation of diazomathane from p-toluenesulfonylmethyl-nitrosoamide (Diazald)

2-(2-Ethoxyethoxy)-ethanol (6.0 ml) and ether (3.5 ml) were added to a solution of potassium hydroxide (1.0g, 0.0178 mol) in water (1.8 ml). The solution was placed in a 50 ml three-necked (one was stoppered) distilling flask fitted with a dropping funnel and an efficient condenser in an oil bath at 70°C. As the distillation of the ether started, a solution of p-toluenesulfonylmethylnitrosoamide (3.5g, 0.0164 mol) in ether (30 ml) was added through the dropping funnel slowly. During the distillation, the soltuion was stirred efficiently, and the yellow ethereal diazomethane was distilled out. When the dropping funnel was empty, another portion of ether (10 ml) was added slowly, and distillation was continued until the distilling ether was colorless.

MODEL HEMATOPORPHYRIN DERIVATIVE

(I) Preparation

Method 1

3-(1-Acetoxyethyl)-17-(2-carboxyethyl)-8,13-diethyl-2,7,13,18-tetramethyl-21H,23H-porphine (0.020g, 0.034 x 10^{-3} mol) was dissolved in tetrahydrofuran (0.5 ml) and 0.1 N NaOH solution (0.5 ml) was added. The mixture was stirred in subdued light at room temperature (ca. 20°C). The reaction was monitored by TLC (silica gel) using 5-10%

methanol in methylene chloride as eluent and stopped after no more increase of the rapid-moving fraction on TLC was recognized. It generally took 2-6 hours. The solution was brought to pH 5 by dropwise addition of dilute hydrochloric acid and shaken with tetrahydrofuran (20 ml) and saturated sodium chloride solution (20 ml). Ethyl acetate (30 ml) was added, and the mixture was shaken. The organic layer was washed with water twice, separated and taken to dryness.

Method 2

Acetylated porphyrin (0.020g, 0.034×10^{-3} mol) was dissolved in DMSO (0.5 ml) and 0.1 N $NaHCO_3$ solution (0.5 ml) was added. The reaction was carried out as described above. After acidification, excess ice-cold water was added (ca. 5 ml) until the porphyrin was precipitated. (A small amount of sodium chloride was added when the precipitation did not occur.) The solid was collected by filtration, washed with water and dried *in vacuo*. The reaction mixture gave four different spots on TLC.

(II) Fractionation of Porphyrin-free Acid

The product prepared in the preceding section was chromatographed on silica gel using 5% (v/v) methanol in methylene chloride as eluent. From this column, the slowest fraction (C) was isolated from the earlier ones (A and B). Fraction C was identified as hydroxyethyl porphyrin **7**. The early fractions (A and B) were separated on a plate of silica gel. Fraction B was found to be a mixture of vinyl-

and acetoxyethyl porphyrins, **19** and **20**. ^{1}H-NMR spectrum of fraction A was not clear enough to determine whether the component was a dimer or a mixture of different porphyrins.

(III) Fractionation of Porphyrin Methyl Ester

The product mixture (50 mg) was dissolved in methylene chloride (2.0 ml) and treated with excess ethereal diazomethane freshly prepared from p-toluenesulfonylmethyl-nitrosamide (Diazald). The mixture was stirred in subdued light at room temperature. The solvents were evaporated gradually in a fume cupboard. The residue was dried *in vacuo*. TLC analysis of this residue indicated five major fractions.

The mixture was treated with column chromatography on silica gel using methylene chloride to give, first, two fractions and, later, the fractions were eluted using 5% (v/v) acetone in methylene chloride. Further separation was carried out for these later fractions with a TLC plate using 2% (v/v) acetone in methylene chloride.

Fraction 1 - This was obtained as the first fraction by elution with methylene chloride in the first separation and identified as vinyl porphyrin methyl ester.

Fraction 2 - This was obtained as the second fraction by elution with methylene chloride in the first separation and identified as acetoxyethyl porphyrin methyl ester.

Fraction 3 - This fraction was obtained as the first major band from the second separation using the plate

(silica gel) and identified as vinyldiporphyrin methyl ester. ^{1}H-NMR ($CDCl_3$) characteristic peaks δ 5.74 (1H, dd); δ 5.96 (1H, dd), 7.65 (1H, m), 8.06 (1H, s), 8.42 (1H, s), 9.17 (2H, d), 9.22 (1H, s), 9.27 (1H, s), 9.79 (1H, s), 9.97 (1H, s); FAB-mass spectrum m/z (rel. inten.) 1055 (100), 1056 (76), 1057 (16).

Fraction 4 - This fraction was obtained as the second major band from the second separation using the place and identified as methoxyethyl-diporphyrin methyl ester. ^{1}H-NMR ($CDCl_3$) characteristic peaks δ 6.06 (1H, q), 7.60 (1H, q), 8.78 (1H, d), 9.14 (1H, d), 9.52 (1H, d), 9.67 (1H, d), 9.83 (1H, d), 9.91 (1H, d), 10.00 (1H, d), 10.33 (1H, d); FAB-mass spectrum m/z (rel. inten.) 1087 (100), 1088 (86), 1089 (44). The NMR spectrum is shown in Figure 13.

Fraction 5 - This fraction was obtained as the last major band from the plate separation and identified as hydroxyethylporphyrin methyl ester.

APPENDIX

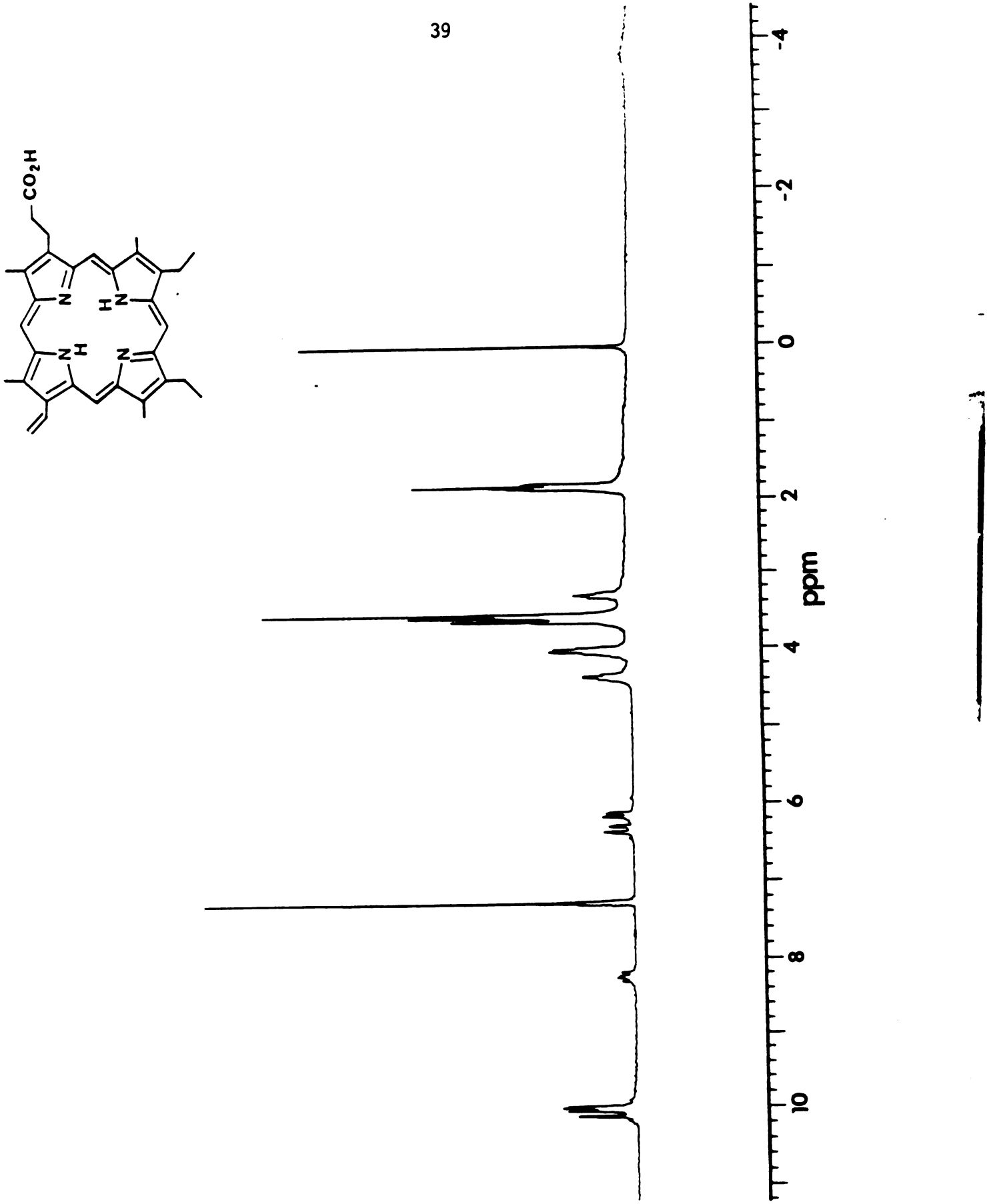


Figure 10. 250 MHz ^{1}H-NMR spectrum of vinylporphyrin 19

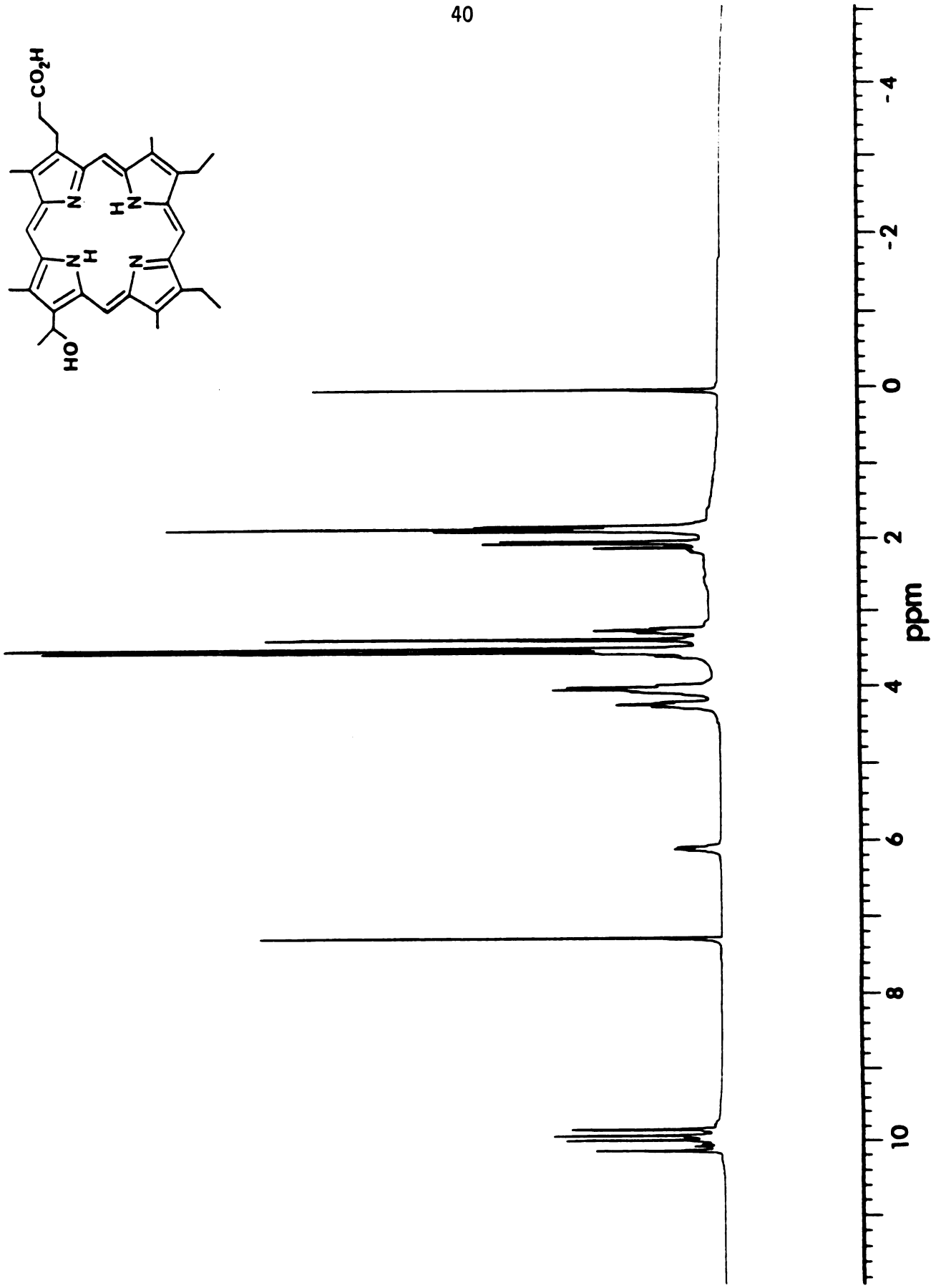
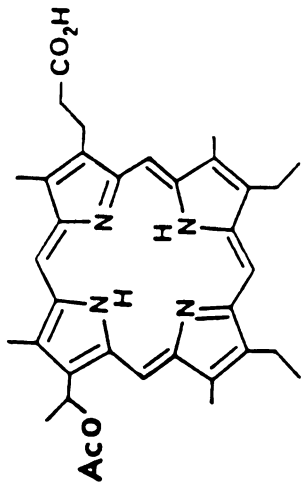


Figure 11. 250 MHz ^{1}H-NMR spectrum of a hematoporphyrin model compound 7



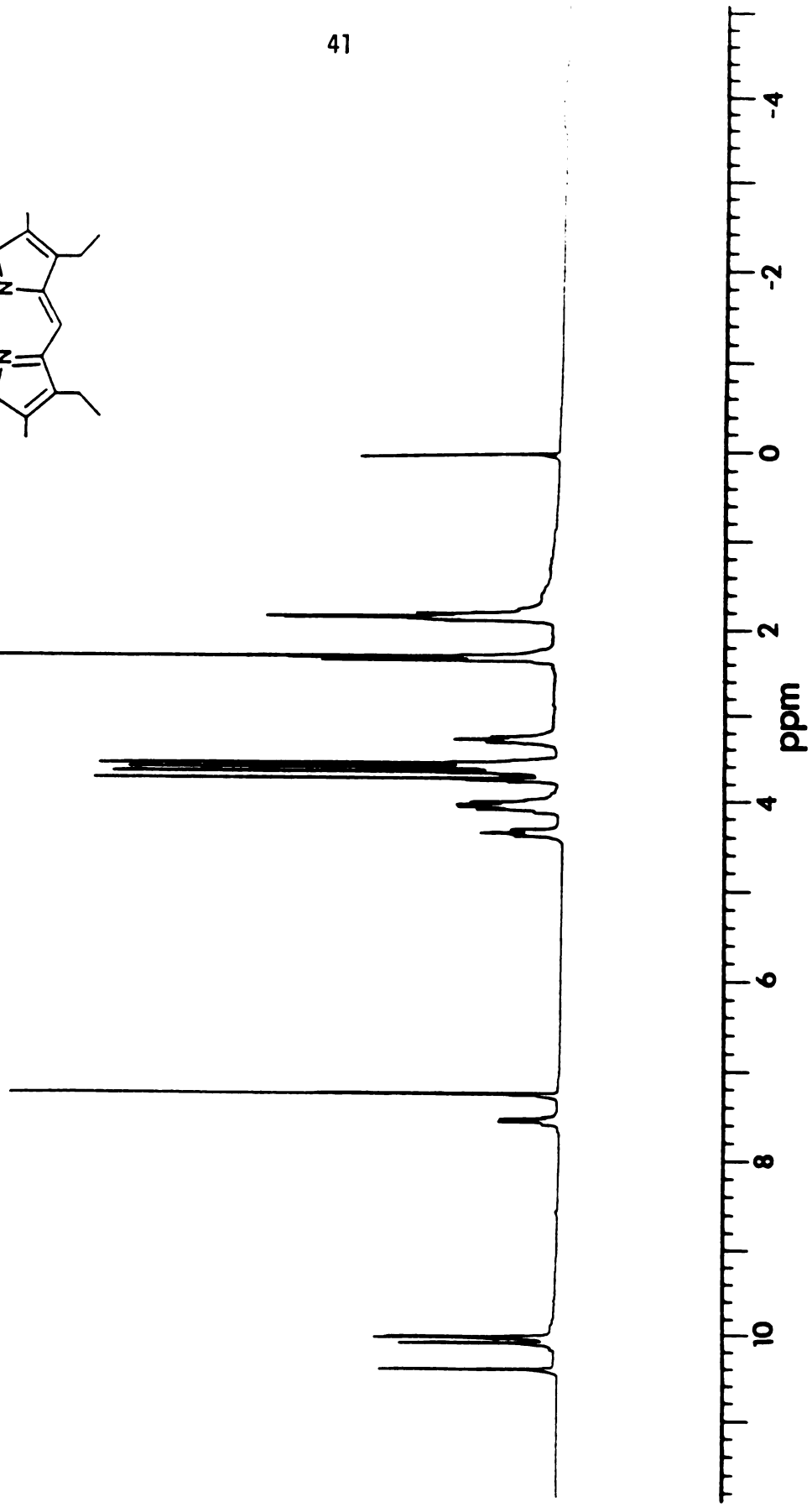


Figure 12. 250 MHz ^{1}H-NMR spectrum of 1'-acetoxyethylporphyrin 20

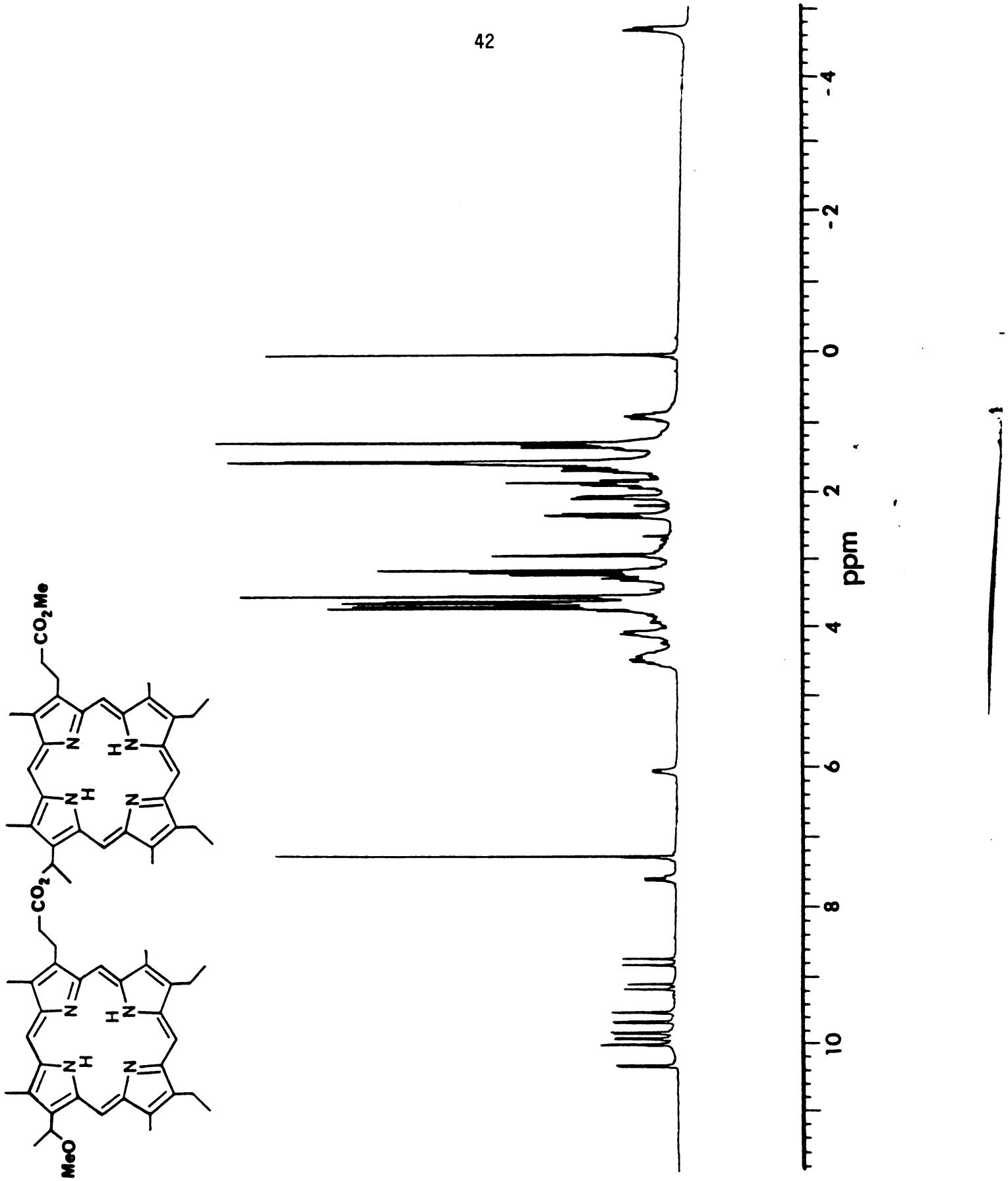


Figure 13. 250 MHz ^{1}H-NMR spectrum of 1'-methoxyethyldiporphyrin 8d methyl ester

LIST OF REFERENCES

LIST OF REFERENCES

1. Hausmann, W. *Biochem. Z.* **1911**, *30*, 276.

2. Meyer-Betz, F. *Deutsch. Arch. Klin. Med.* **1913**, *112*, 476.

3. Auler, H.; Banzer, G. *Z. Krebforsch.* **1942**, *53*, 65; Figge, F. H. J.; Weiland, G. S.; Manganiello, L. O. *Proc. Soc. Exp. Biol. Med.* **1948**, *64*, 640.

4. Schwartz, S.; Absolon, K.; Vermund, H. *Univ. Minnesota Med. Bull.* **1955**, *27*, 7.

5. Lipson, R. L.; Baldes, E. J. *Arch. Dermatol.* **1960**, *82*, 508; Lipson, R. L.; Baldes, E. J.; Olsen, A. M. *J. Natl. Cancer Inst.* **1961**, *26*, 1.

6. Diamond, I.; McDonagh, A. F.; Wilson, C. B.; Granelli, S. G.; Nielsen, S.; Jaenicke, R. *Lancet.* **1972**, *ii*, 1175.

7. Kelly, J. F.; Snell, M. E.; Berenbaum, M. C. *Brit. J. Cancer* **1975**, *31*, 237.

8. Dougherty, T. J.; Grindey, G. B.; Fiel, R.; Weishaupt, K. R.; Boyle, D. G. *J. Natl. Cancer Inst.* **1975**, *55*, 115.

9. Benson, R. C.; Farrow, G. M.; Kinsey, J. H.; Cortese, D. A.; Zinke, H.; Utz, D. C. *Mayo Clin. Proc.* **1982**, *57*, 548.

10. Hayata, Y.; Kato, H.; Konaka, C.; Ono, J.; Matsushima, Y.; Yoneyama, K.; Nishimiya, K. *Chest.* **1982**, *82*, 10; *Recent Results Cancer Res.* **1982**, *82*, 121; *Cancer* **1983**, *51*, 50.

11. King, E. G.; Doiron, D. R.; Man, G.; Profio, A. E.; Huth, G. *Recent Results Cancer Res.* **1982**, *82*, 90; *Cancer* **1982**, *49*, 777.

12. Profio, A. E.; Carvlin, M. J.; Sarnaik, J.; Wudl, L. R. in "Porphyrins in Tumor Phototherapy" (Edited by Andreoni, A. and Cubbedu, R.), pp 321-337, Plenum Press, New York (1984).

13. Dougherty, T.; Kaufman, J.; Goldfarb, A.; Weishaupt, K.; Boyle, D.; Mittleman, A. *Cancer Res.* **1978**, *38*, 2628.

14. Gomer, C. J.; Smith, D. M. *Photochem. Photobiol.* **1980**, *32*, 341.

15. Kato, H., et. al. in "Porphyrin Photosensitization" (Edited by Kessel, D. and Dougherty, T. J.), pp 23-39, Plenum Press, New York, 1983.

16. McCulloch, G. A. J.; Forbes, I. J.; See, K. L.; Cowled, P. A.; Jacka, F. J.; Ward, A. D. in "Porphyrin Localization and Treatment of Tumors" (Edited by Doiron, D. R. and Gomer, C. J.), pp 709-717, Alan R. Liss, New York, 1984.

17. Dougherty, T. J.; Thoma, R. E.; Boyle, D. G.; Weishaupt, K. R. *Cancer Res.* **1981**, *41*, 401.

18. Kennedy, J. in Reference 15, pp 53-62.

19. Bertolini, G.; Dall'Aqua, M.; Vazzoler, M.; Salvato, B.; Jori, G. *Med. Biol. Environ.* **1982**, *10*, 239-242.

20. Gomer, C. J.; Doiron, D. R.; Jester, J. V.; Szirth, B. C.; Murphree, A. L. *Cancer Res.* **1983**, *43*, 721-727.

21. Gomer, C. J.; Dougherty, T. J. *Cancer Res.* **1979**, *39*, 146-151.

22. Clezy, P. S.; Hai, T. T.; Henderson, R. W.; van Thuc, L. *Aust. J. Chem.* **1980**, *33*, 585-597.

23. Bonnett, R.; Ridge, R. J.; Scourides, P. A.; Berenbaum, M. C. *J. Chem. Soc. Perkin Trans. I* **1981**, 3135-3140.

24. Moan, J.; Sandberg, S.; Christensen, T.; Elander, S. in Reference 15, pp 165-179.

25. Berenbaum, M. C.; Bonnett, R.; Scourides, P. A. *Br. J. Cancer* **1982**, *45*, 571-581.

26. Dougherty, T. J.; Boyle, D. G.; Weishaupt, K. R.; Henderson, B. A.; Potter, W. R.; Bellnier, D. A.; Wityk, K. E. in Reference 15, pp 3-13.

27. Dougherty, T. J.; Potter, W. R.; Weishaupt, K. R. in Reference 12, pp 23-35; and in Reference 16, pp 301-314.

28. Musselman, B. D.; Watson, J. T.; Chang, C. K. *Org. Mass. Spect.*, in press.

29. Bonnett, R.; Berenbaum, M. C. in Reference 15, pp 241-250.

30. Games, D. E.; Jackson, A. H.; O'Hanlon, P. J. *J. Chem. Soc. Perkin I* **1976**, 2501.

31. Clezy, P. S.; Fookes, C. J. R.; Liepa, A. J. *Aust. J. Chem.* **1972**, *25*, 1979.

32. Paine, J. B., III; Chang, C. K.; Dolphin, D. *Heterocycles* **1977**, *7*, 813.

33. Philips, H. *J. Chem. Soc.* **1923**, *123*, 44.

34. Clezy, P. S.; Fookes, C. J. R. *Aust. J. Chem.* **1977**, *30*, 217.

35. Chang, C. K. *ACS Adv. Chem. Ser.* **1979**, *173*, 162.

36. Chang, C. K.; Abdalmuhdi, I. *Angew. Chem. Int. Ed. Engl.* **1984**, *23*, 164.

37. Chang, C. K.; Takamura, S.; Musselman, B.; Kessel, D., presented at International Conference on Excited States of Porphyrins, November 17-19, 1985, Little Rock, Arkansas, *ACS Adv. Chem. Ser.*, in press.

38. Reyftman, J.; Morliere, P.; Goldstein, S.; Santus, R.; Dubertret, L.; Legrange, D. *Photochem. Photobiol.* **1984**, *40*, 721.

39. Carr, R. P.; Jackson, A. H.; Kenner, G. W.; Sach, G. S. *J. Chem. Soc. (C)* **1971**, 487.

40. Davies, J. L. *J. Chem. Soc. (C)* **1968**, 1392.