

Uptake of Protoporphyrin and Violet Light Photodestruction of *Propionibacterium acnes*

Thor B. Melø

Institute of Physics, AVH, University of Trondheim, 7055 Dragvoll, Norway

Z. Naturforsch. **42c**, 123–128 (1987); received June 18/September 15, 1986

Propionibacterium acnes, Protoporphyrin, Photoinactivation, Photosensitization, Protoporphyrin Uptake

The uptake of protoporphyrin IX by *Propionibacterium acnes* in suspension has been studied by fluorescence spectroscopy. Protoporphyrin, after it was injected into a cell suspension, was firstly bound to receptors on the cell surface and in this state protoporphyrin was non-fluorescent. Subsequently, probably as a result of lateral diffusion in the cell wall, these protoporphyrin-receptor complexes formed dimers. The final step in the overall uptake process of protoporphyrin by the cells from the surroundings consisted in a jump of such dimers from waterlike to lipidlike compartments in the cell membrane where protoporphyrin became fluorescent. The lipidlike compartments in the cells had a limited binding capacity of protoporphyrin.

The fraction of surviving cells versus light dose has also been studied for varying amounts of protoporphyrin added to the cell suspensions. The survival curves were exponentially decaying with the irradiation time and there was a direct proportionality between the inverse slope of the survival curves and the intensity of protoporphyrin fluorescence from the lipidlike compartments. The relevance of these results to the therapy of Acne vulgaris is also discussed.

Introduction

When the human skin is illuminated by blue light a punctate pink fluorescence may be observed due to the presence of *Propionibacterium acnes* (*P. acnes*) colonies in the pores. These bacteria produce and accumulate porphyrins which are fluorescent [1]. A high density of *P. acnes* in the cutaneous follicular orifices may, under certain circumstances, elicit a disease referred to as Acne vulgaris.

Porphyrins, present in these bacteria in a high concentration compared to other cells, are known to be photodynamic sensitizers [2]. In similar systems, for instance erythrocytes with an increased level of protoporphyrin (PP), the action of light may be lethal [2]. *P. acnes* will also be photoinactivated due to the presence of endogenously produced porphyrins [3]. The aim of this paper is to investigate if porphyrins added from outside will have a photodynamic action on *P. acnes*. The mechanisms for the uptake of porphyrins added to *P. acnes* in suspension, in order to enhance the effect of light, will also be investigated. This study is related to investigations within the field

of cancer phototherapy, where the uptake of hematoporphyrin by human cells, in order to sensitize the cells to light, has been studied [4].

Materials and Methods

Bacteria and growing conditions

Serotype I (CN 6278) of *P. acnes* was grown on agar plates made from phosphate buffered Eagles medium (pH = 6.7). During growth the plates were kept in a semianaerobic atmosphere (about 2% O₂) at 37 °C in darkness. A Gas Pak system (BBL Gas Pak) was used to produce and maintain a semi-anaerobic atmosphere during growth. The bacteria were harvested at the age of 4 days and suspended in phosphate buffered saline (PBS) with a pH = 6.8.

Chemicals

The chemicals used were either purchased from Sigma Chemical Company or Porphyrin Products. A stock solution of protoporphyrin IX was prepared in the following way: 6 mg of PP was dissolved into a mixture of 5 ml ethanol and 5 ml 0.01 M NaOH. Before each experiment when PP was added to the cell suspensions, 1 ml of this stock solution was diluted in 100 ml PBS (pH = 6.8). Referred to PP in monomeric form, the concentration of this solution was 10⁻⁵ M. A defined volume of this diluted solution was

Abbreviations: PP, protoporphyrin IX; CTAB, N-Cetyl-N,N,N-trimethyl-ammonium bromid; *P. acnes*, *Propionibacterium acnes*.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/87/0100–0123 \$ 01.30/0



Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung-Keine Bearbeitung 3.0 Deutschland Lizenz.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung“) beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

This work has been digitalized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition “no derivative works”). This is to allow reuse in the area of future scientific usage.

transferred to the suspensions by mean of a micropipette.

Photodestruction of cells

One hour after the addition of a defined amount of PP to the cell suspensions aliquots of 100 μ l were taken from the cell suspensions and diluted in PBS by a factor of 10^5 .

These diluted cell suspensions were irradiated by a 900 W photoradiator (Applied Photophysics, Model 5350) equipped with a Xenon lamp. The wavelength of the exciting beam was 400 nm, the bandwidth of the light 10 nm and the irradiance was $65 \text{ W} \cdot \text{cm}^{-2}$. 20 μ l of these irradiated cell suspensions was spread out on Bactoagar in Petri dishes. The degree of photoinactivation of the cells was found by counting the number of colonies formed after incubation of the illuminated plates for 7 days at 37°C in darkness. The number of colonies on notirradiated plates was ranging between 400 and 900 depending upon the cell density in the initial suspensions.

The fraction of PP not bound to the cells was determined in the following way: Equal amounts of PP were added to a cell suspension and its blank. The suspension was centrifuged and the detergent N-Cetyl-N,N,N-trimethyl-ammoniumbromid (CTAB) (0.04% b.w.) was added to both supernatant and blank. The fluorescence was then measured from both supernatant and blank and the intensity ratio gives the fraction of unbound PP.

Instrumentation

The fluorescence measurements were done by a standard rightangled fluorimeter, which is described elsewhere [5]. In order to avoid scattered light to reach the detection system a band pass filter (BP 40, type Oriel) was placed in the exciting beam and a long pass filter (LP 54, type Oriel) on the emission side of the fluorimeter. Absorption and turbidity measurements were done by a PYE-UNICAM SP8-200 UV/visible spectrophotometer. The irradiances were measured by a thermopile detector, type S15, Sensors Inc.

Results

Protoporphyrin uptake by *P. acnes*

After an injection of a defined amount of PP into a cell suspension the fluorescence intensities at both

618 nm, indicative of PP in water phase, and at 634 nm, reflecting the amount of PP in a hydrophobic phase, were measured as a function of time. The fluorescence yield of PP in water is low and the emission maximum is at 620 nm. In hydrophobic media the fluorescence yield is much higher than in water and the emission peak is at 634 nm. The fluorescence intensity from PP which enters the aqueous phase surrounding the cells reached rather quickly an equilibrium value, while the fluorescence from PP in lipidlike compartments of the cells much more slowly reached its equilibrium value (see Fig. 1). This observation indicates that more than one step is involved in the overall uptake process of PP from the surroundings into the cells.

In Fig. 2A the fraction of unbound PP (PP in the water phase) is plotted versus the amount of PP

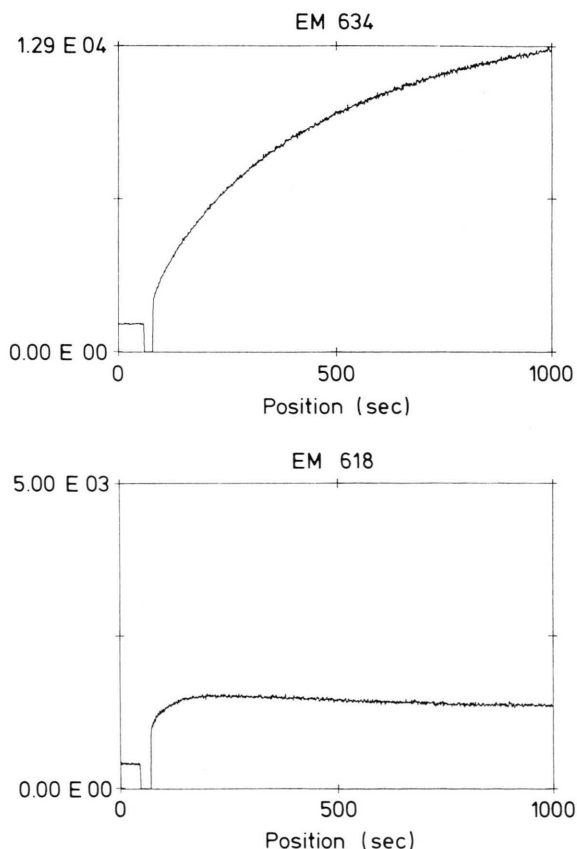


Fig. 1. The time dependence of the fluorescence intensities at 634 nm, where the lipidlike emission has a maximum, and at 618 nm, indicative of protoporphyrin in water, after addition of protoporphyrin to a cell suspension.

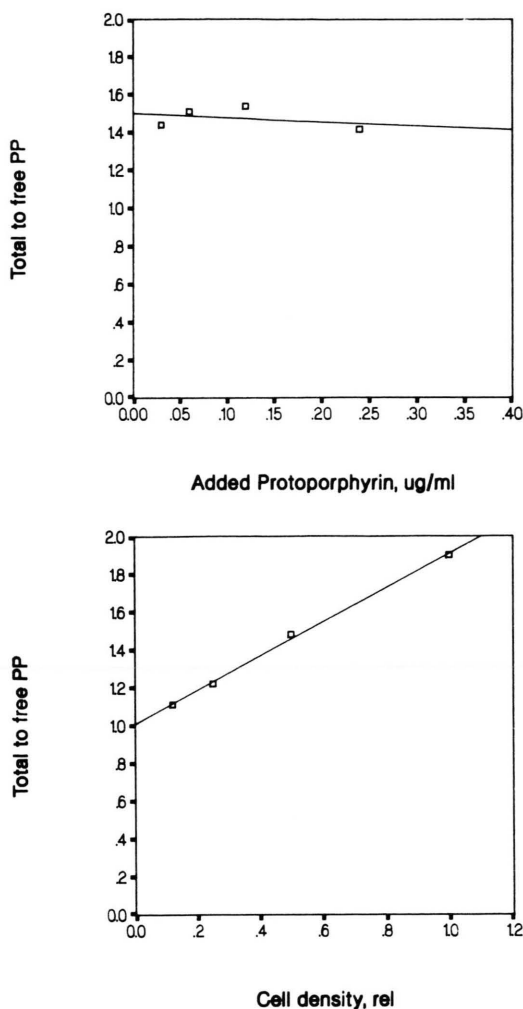


Fig. 2. The ratio between amount of protoporphyrin added to a cell suspension and free amount (not bound to cells *versus*): A) Total porphyrin added to cell suspension of constant cell density. B) Cell density for a fixed total protoporphyrin addition.

added to cell suspensions of identical cell densities. The ratio of unbound to total PP added is constant for all PP additions. The fraction of non-bound PP was also determined as a function of cell density (Fig. 2B), and an inverse relationship was found.

In Fig. 3A the final fluorescence intensities (the equilibrium value) from PP bound to lipidlike compartments of the cells are plotted versus the total amount of PP added to suspensions of identical cell densities. This relation is sigmoidal and the leveling off at high PP amounts indicates that the lipidlike

compartments of the cells have a limited binding capacity of PP.

In Fig. 3B the inverse values of the fluorescence intensity from PP in lipid phase and cell density are plotted and a linear relation is seen in the high concentration range.

In Fig. 4A the initial time dependence of the 634 nm fluorescence intensity is shown for suspensions of identical cell densities after injection of a controlled amount of PP in each case. Above a certain PP concentration the initial fluorescence rise is

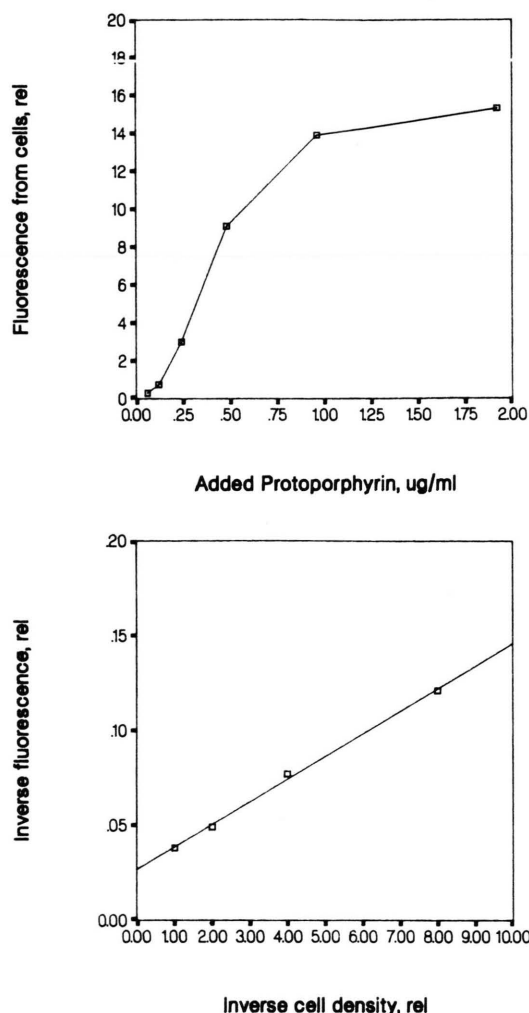


Fig. 3. A) The final fluorescence intensities at 634 nm from cell suspensions of equal densities versus amount of added protoporphyrin. B) Inverse fluorescence at 634 nm from cell suspensions *versus* inverse cell density for equal protoporphyrin additions.

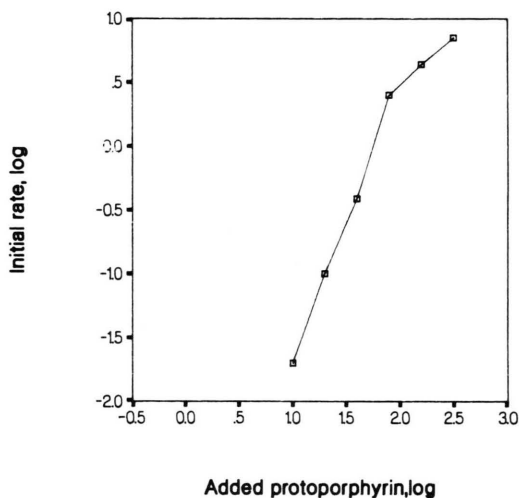
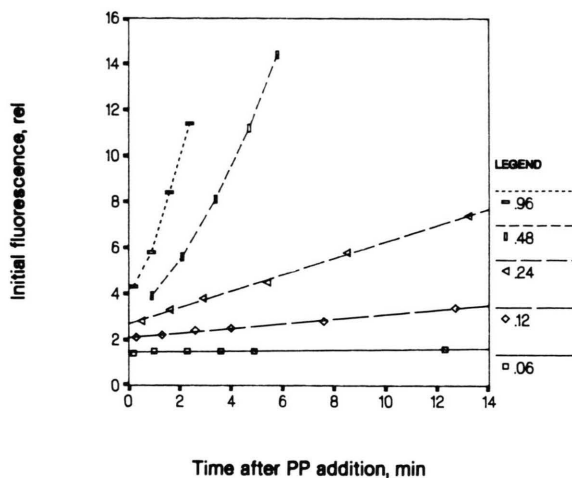


Fig. 4. A) The initial fluorescence increase from cell suspensions of equal densities after additions of increasing amounts of protoporphyrin. B) The initial rate of fluorescence increase *versus* concentration of added protoporphyrin in a logarithmic plot.

more than linear with time and below this critical concentration the initial fluorescence is in proportion to the square of added PP concentration (see Fig. 4B).

Photodestruction of *P. acnes*

P. acnes cells were illuminated by 400 nm light and in Fig. 5A the fraction of surviving cells is plotted *versus* exposure time. The survival curves are exponential with time which indicates that the lethal action is obtained by one hit. A hit means here a lethal

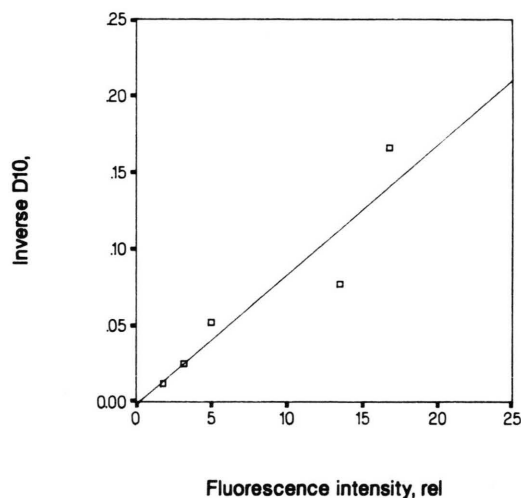
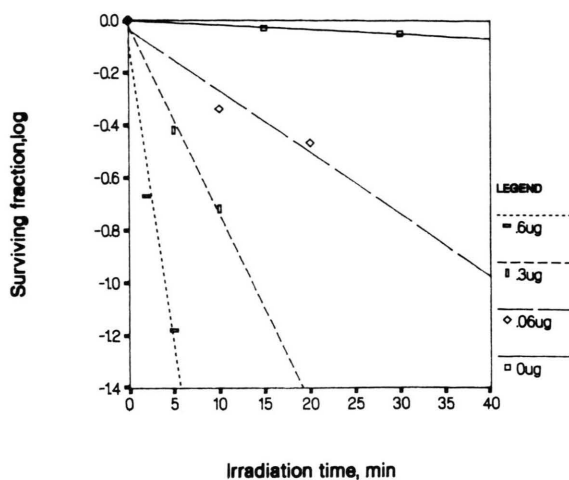


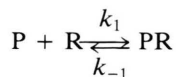
Fig. 5. A) The fraction of surviving cells versus irradiation time for various additions of protoporphyrin. B) The relation between inverse irradiation dose giving 10% survival and relative fluorescence from lipidlike environments in the cells.

photooxydation of a particular site in the cell. The slope of the survival curves in semilogarithmic plots is in proportion to the amount of PP in the lipid phase, measured as fluorescence intensity at 634 nm from the cells (see Fig. 5B).

Discussion

The degree of protoporphyrin binding to *P. acnes*, in an intermediate state where PP is non-fluorescent, did both depend upon the concentration of added PP

and cell density (see Fig. 2). This indicates that this adhesion of PP to the cells is a bimolecular process:



where R is some receptor on the cell surface and P a protoporphyrin molecule. At equilibrium the reaction rates in both directions are equal:

$$k_1 \cdot N_f \cdot (N_r - N_b) = k_{-1} \cdot N_b$$

where N_f and N_b are the numbers of free and bound PP molecules. N_r is the number of protoporphyrin receptors and $(N_r - N_b)$ is the number of available receptors. A rearrangement of this expression gives the equation of Scatchard:

$$N_b/N_f = K \cdot (N_r - N_b) \quad K = k_1/k_{-1}$$

showing that a linear relation exist between the ratio of bound to unbound PP and bound PP. Realizing that the total amount of added PP, N_0 , either may be bound or unbound, one has that $N_b + N_f = N_0$. By means of this relation N_b , which is difficult to measure, can be eliminated from the Scatchard equation. One then has, when $N_r \gg N_b$:

$$N_f/N_0 = 1/(1 + 1/(K \cdot N_r)).$$

This relation is indeed in accordance with the experimental results. It predicts a constant ratio between free to total PP and a linear relation between the inverse of free PP and the number of receptors, which is in proportion to the cell density (see Fig. 2).

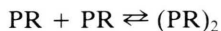
Subsequent to this adhesion reaction the PP entities will slowly become fluorescent reflecting some further rearrangement reaction. The sigmoidal relation between the final fluorescence intensity from the lipid compartment and the total concentration as well as the initial superlinear fluorescence increase with time are in line with a cooperative binding of PP to the lipidlike compartments in the cell. Based on the square dependence of the initial fluorescence increase to total concentration a particular kind of cooperative uptake of bound non-fluorescent PP in waterlike environments (probably the cell wall) to lipidlike compartments will be proposed where two steps are involved:

1) An encounter between two protoporphyrin-receptor complexes PR. This step will include lateral diffusion in the cell wall and a kind of dimer formation between two such complexes on the encounter.

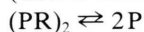
2) A jump of the dimerization product from hydro-

philic to hydrophobic environments. PP in this hydrophobic phase is fluorescent.

The equations for these processes may be written:



(lateral diffusion and dimerization)



(jump from water to lipid phase).

Both kinetic and equilibrium data are explained by this model. At small PP additions the number of dimers at equilibrium in the cell membrane is $N_d = K_d \cdot N_b^2$, where K_d is the equilibrium constant for the monomer-dimer reaction introduced above and N_b again the number of bound PP molecules which was determined by the bimolecular reaction between PP and receptors. This will explain the first part of the sigmoidal curve describing the relation between the final fluorescence intensity and the amount of added PP. The final part of this curve is explained by the second equation when in addition it is assumed that the lipid compartment of the cells has a limited binding capacity. The Hill equation is then valid and the relation between the fluorescence intensity from the lipid compartment and the binding capacity, which is proportional to the cell density, obey this relation (see Fig. 3B).

The initial reaction rate for the dimerization is:

$$d(PR)_2/dt = k_1^d \cdot N_b^2,$$

k_1^d is forward reaction constant

and the initial rate for the second reaction will be:

$$d(2P)/dt = k_2 \cdot (PR)_2,$$

k_2 is the probability for jump

which explains that, when the reaction rates are comparable, the number of PP molecules in the lipid phase, which is monitored by fluorescence, will be:

$$2P = k_1^d \cdot k_2 \cdot N_b^2 \cdot t^2/2,$$

or that the initial fluorescence increase is quadratic with time. When the first reaction is much faster than the second reaction, which is the case when the cell surface density of bound PP is relatively large, the initial fluorescence increase is linearly related to time simply because the first reaction is completed before the next reaction has started. When the first reaction is much slower than the second reaction the initial rate of fluorescence increase will also be linear with time. In this case, however, the initial reaction rate is related to the square of bound PP entities, which is seen in Fig. 4B.

There is a direct proportionality between the slope of the survival curves, measured as the time necessary to obtain 10% survival (D_{10}) and the amount of PP in lipidlike compartments of cells, measured as the fluorescence intensity at 634 nm. This indicates that it is the PP which is localized in these regions which is responsible for cell death.

Similar results were obtained by Kjeldstad *et al.* [6] when hematoporphyrin derivative (Hpd) was added to *P. acnes* in suspension. The degree of cell inactivation was also in this case in proportion to the fraction of Hpd which was fluorescent.

The uptake and localization of porphyrins in cells is also of great interest in cancer therapy [4]. The time scale for protoporphyrin uptake in *P. acnes* is the same as for Hpd in cancer cells. This indicates that a common uptake mechanism may exist in the two cases and that therefore *P. acnes* may act as a model system in the study of porphyrin uptake by cells.

P. acnes, the natural inhabitant of the skin, play a role in the pathophysiology of the disease Acne vulgaris. The frequency of Acne vulgaris is correlated to a high density of *P. acnes* in the pilosebaceous follicles. The growing conditions for *P. acnes* in the pores are quite comparable to those in an ordinary laboratory chemostat. In the pore there is a constant production of a nutrient, the sebum, and there is a con-

stant flow of matter, due to excretion, out of the pore. Antibiotics are mainly used in the treatment of Acne vulgaris, in order to reduce the density of the bacteria in the pores, and the density of *P. acnes* in the follicles can be monitored by fluorescence measurements from the skin under Woods light [7]. In this paper, as well as in other ones [3, 8], it has been shown that *P. acnes* are inactivated by violet light, which is also present at natural light conditions. A reduced density of *P. acnes* may therefore also be obtained by additional violet light illumination. In this paper it is shown that the sensitivity of the cells to light will depend upon the concentration of protoporphyrin in the *P. acnes*. The production of protoporphyrin, which is to shown in a subsequent paper, will both upon the oxygen concentration around the cells as well as the pH in the surrounding medium. In line with these facts, it can be suggested that, if the pH value in some pores are aberrated, the number of *P. acnes* inhabiting the pore might increase due to a reduced light sensitivity. Work are also in progress where the porphyrin content in each pore is determined by microfluorometry.

Acknowledgements

I kindly thank Prof. A. Johnsson for his encouragement during this work and his reading and revising of the manuscript.

- [1] E. Cornelius and G. D. Ludwig, *J. Invest. Dermatol.* **49**, 368–370 (1967).
- [2] F. R. Hopf and D. G. Whitten in: *The Porphyrins*, Vol. II (Edited by D. Dolphin), p. 161–166, Academic Press 1978.
- [3] B. Kjeldstad, *Z. Naturforsch.* **39c**, 300–302 (1983).
- [4] T. Christensen and J. Moan, *Cancer Lett.* **9**, 105–110 (1980).
- [5] B. Klockare, T. B. Melø, and A. Johnsson, *Physiol. Plant.* **46**, 101–108 (1979).
- [6] B. Kjeldstad, T. Christensen, and A. Johnsson, in: *Methods of Porphyrin photosensitization* (Edited by D. Kessel), p. 155–159, Plenum Publishing Corp. 1985.
- [7] K. J. McGinley, G. F. Webster, and J. J. Leyden, *Brit. J. Dermatol.* **102**, 437–441 (1980).
- [8] B. Kjeldstad and A. Johnsson, *Photochem. Photobiol.* **43**, 67–70 (1986).