5,7,12,14-Tetramethyldimethoxybenzo[*b*,*i*][1,4,8,11]tetraazacyclodecine, A New Tumoricidal Pseudo-porphyrin

Leszek Czuchajowski* and Matthew Morra

Department of Chemistry and Department of
Plant, Soil and Entomological Sciences, University of Idaho
Moscow, ID 83843

Eugeniusz Kubaszewski and Tadeusz Malinski*

Department of Chemistry, Oakland University,
Rochester, MI 48309

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The title compound, a mixture of two isomers differing in the position of a methoxy substituent in one benzene ring, was obtained in a Ni-templated synthesis directly as a water soluble dihydrochloride of the free base. The cyclic voltametry study indicated that in a neutral solution the reduction and oxidation are irreversible one electron processes, the latter leading to cation radical undergoing polymerization, a process followed by deposition of a film on the electrode. In 1,2-dichloroethane the cation radical is oxidized to a dication, both species being much less stable than those originating from *meso*-tetraphenylporphyrin. The title compoud at 2.5 x 10^{-5} *M* in Tris buffer showed a 50% inhibition of the growth of malignant melanoma cells as compared to a 44% inhibition shown by a water soluble *meso*-monomethoxy-tris(*N*-methylpyridinium)porphyrin. The exposure to light for 30 minutes at 2.5 times smaller concentration increased the inhibition caused by the pseudo-porphyrin from 9% to 49%.

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Introduction.

In the last decade a large amount of research has focused upon tetraazaannulene macrocyclic ligands [1-4]. These ligands are particularly well suited models for naturally occuring systems because they provide a stable N4 environment whose coordination closely resembles that of porphyrins and corrin rings found in biological systems. It also resembles the nitrogen-coordinated amino- and transition metal complexes. One of the simplest representatives of these ligands, 5,7,12,14-tetramethyldibenzo-[*b*,*i*][1,4,8,11]tetraazacyclodecine, has already proved to be an ideal synthetic candidate for studying porphyrin-like structure-function correlations [5]. There were many successful efforts to achieve periferal substitution on its framework, the appended substituents providing points of attachment for further structural modifications. As the consequence, these substituents might create the possibility for synthesis of even more complex compounds serving as new biologically important models. The substituents in question represent alkyls, (fluoro)acetyl, (fluoro)benzoyl or benzooxazolyl located at the 6, 13 centers [6] and the chloro, bromo, nitro or ester substituents attached to the benzene rings [7].

In the course of synthesizing analogs of our new porphyrinyl-nucleoside systems [8,9] in which a porphyrin is replaced by a pseudo-porphyrin, we became interested in the particular use of dibenzo[*b*,*i*]tetraazaannulene substituted in each benzene ring by a methoxy group. This compound attracted our attention because of its synthesis, electrochemical behavior and tumoricidal activity against malignant melanoma cells.

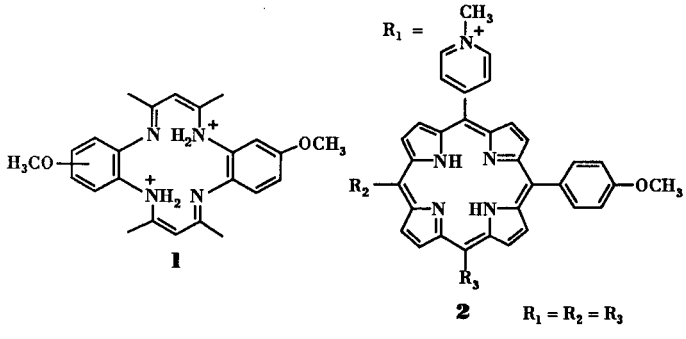
In recent research on tumoricidal effects, porphyrin-based structures have shown promise. This is mainly related to their role in photodynamic therapy in which porphyrins act as photosensitizers [10]. Also, there is growing interest in the interactions of water soluble porphyrins with DNA. The interaction and/or intermolecular interactions which are involved [11] opened a new area of interest in the bioapplicability of (metallo)porphyrins. To the authors' best knowledge, pseudo-porphyrins of the tetraazaannulene-type have never been used in research related to their interaction with normal or malignant melanoma cells, one of the reasons being their lack of solubility in water.

Results and Discussion.

Tetraazaannulene systems are usually prepared in template synthesis reactions, with nickel as a templating center [12]. This implies the necessity of demetallation if a free base tetraazaannulene (a "pseudo-porphyrin") is the primary goal of the synthesis or if another metal is required in the core. However, removal of nickel is a lengthy and rather delicate process. We have found (see Experimental) that the dimethoxy derivative in question can be formed directly as a free base in spite of using a nickel salt as a templating coreagent. It was obtained as the dihydrochloride which showed excellent solubility in water due to the presence of the ammonium centers.

The examination of the electrochemical behavior of the pseudo-porphyrin in question, **1**, was based on cyclic voltammetry and spectroelectrochemistry. Typical cyclic voltammograms obtained for **1** in aqueous solutions at different *p*H values are shown in Figure 2 in which also the peak potentials are given. At neutral *p*H one reduction

(peak I) and one oxidation (peak II) are observed at -1.21 and 0.86 V, respectively. Both peaks are due to irreversible one-electron process. In 0.1 *M* hydrochloric acid both peaks are shifted to more positive potentials, 10 mV for the reduction and 100 mV for the oxidation. This indicates that the presence of protons facilitates reduction and inhibits oxidation. In basic solution (0.1 *M* sodium hydroxide) the reduction peak is split into two peaks observed at -0.44 (peak I') and -0.98 (peak I). Both peaks are due to one electron transfer process which is followed by the formation of an anion radical. The ratio of the current for peaks I/I' increases with decreasing *p*H, but in all cases the sum of the currents is equal. This indicates the existance of an equilibrium, presumably between the protonated and deprotonated forms of **1**.



1 **2** $R_1 = R_2 = R_3$

Figure 1. 5,7,12,14-Tetramethyldimethoxybenzo[*b*,*i*][1,4,8,11]tetraazacyclodecine•2HCl (a pseudo-porphyrin), **1**, and meso-tris(*N*-methyl-4-pyridinium)-*p*-methoxyphenylporphyrin, **2**.

A controlled potential electrolysis was performed in order to verify the equilibrium between the peaks I and I', the potential applied being by 50 mV more negative than that of the peak I. The coulometric data obtained indicate that a total of 1.05 ± 0.07 faraday is transformed in the processes I' and I. Thus, it seems likely that the split peaks observed for the reduction of **1** in 0.1 *M* sodium hydroxide can be ascribed to the reduction of double deprotonated species that is easily reduced at -0.44 V and to the reduction of the singly deprotonated form reduced at -0.98 V. Both reduction processes involve the production of a radical. No oxidation of **1** is observed in a basic solution.

One can expect that the one electron transfer oxidation of **1** in aqueous neutral and acidic solution should lead to the formation of a cation radical which undergoes dimerization and/or polymerization. This was found for tetraazaannulene complexes in nonaqueous media [7a,13]. Polymerization of pseudo-porphyrin **1** followed by the deposition of a film an indium-tin oxide transparent electrode was indeed observed by the continuous scan cyclic voltammetry in the range 0.0-1.0 V. The obtained film is characterized by the uv-vis bands at 290 and 520 nm. In 1,2-dichloroethane solution the pseudo-porphyrin **1** is oxidized also in one electron transfer reaction to a cation radical (at 1.15 V). However, contrary to the aqueous solution, this cation radical can be further oxidized to a dication at 1.45 V (Figure 2). It is characteristic that the potentials of oxidation of **1** in the solution of 1,2-dichloroethane are very similar to those observed for meso-tetraphenylporphyrin [14]. The cation radical and dication originating from the latter porphyrin are, however, much more stable than those obtained from **1**. No oxidation was observed in the dimethyl sulfoxide solution. The reduction of pseudo-porphyrin **1** in nonaqueous (Figure 3) as well as in aqueous solvents (Figure 2) involves one-electron transfer and the production of an unstable anion radical. It is very characteristic that the oxidation of **1** to cation radical in aqueous solution is by 200-300 mV easier than the analogous oxidation of porphyrins and phthalocyanins.

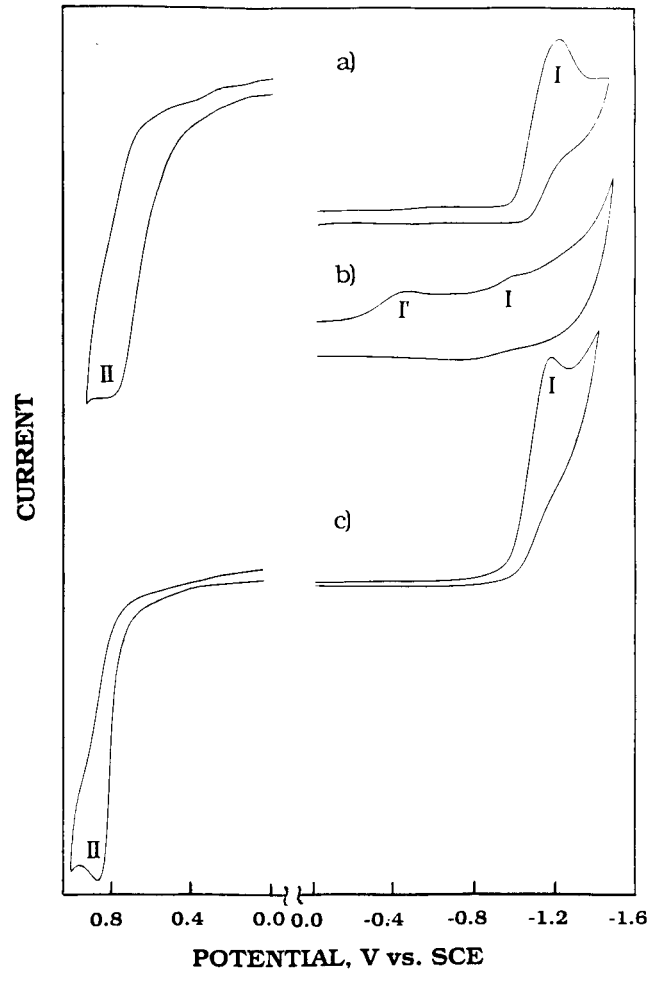


Figure 2. Cyclic voltammograms of **1** in aqueous solutions; (a) 0.1 *M* $LiClO_4$; (b) 0.1 *M* NaOH; (c) 0.1 *M* HCl. Voltammetric peak potentials (V vs SCE) for **1**, Ox-Ep and Red-Ep: (a) 0.86, -1.21, respectively; (b) —, -0.44 and -0.98; (c) 0.96, -1.20.

The thin-layer uv-vis spectroelectrochemistry shows the spectral changes taking place during the processes of oxidation and reduction, Figures 4-6. The electronic absorption spectra were taken before and during the processes of controlled potential reduction and oxidation, respectively in the 200-800 nm and 275-875 nm regions. The original spectrum of **1** in aqueous solution at *p*H 7 has the high intensity bands at 267, 280, 330 (ill defined band) nm and a very broad band at 530 nm. During the reduction the high intensity band at 280 nm decreases while the band at 530 nm increases and shifts to 505 nm. An isobestic point is observed at 328 nm. During the oxidation process more distinctive changes of the spectrum are observed. The band at 280 nm is changed similarly to that observed for the reduction, however, the band at 330 nm increases its intensity and shifts bathochromically to 350 nm, while the broad peak at 530 nm disappears. The electronic absorption spectrum of **1** in 0.1 *M* sodium hydroxide solution is characterized by the bands at 265 and 315 nm (Figure 5). During the reduction the band at 315 decreases its intensity while a new band at 390 nm appears and grows. A clear isobestic point at 358 nm is observed. During the reduction carried on in 0.1 *M* hydrochloric acid the band at 280 nm decreases and the band at 331 nm grows (Figure 6a). Also the decrease of the 490 nm band is observed and its bathochromic shift to 575 nm. During the oxidation process one observes the decrease of the intensity of the 280 nm band and the increase and the hypsochromic shift of the bands at 331 and 490 nm (Figure 6b).

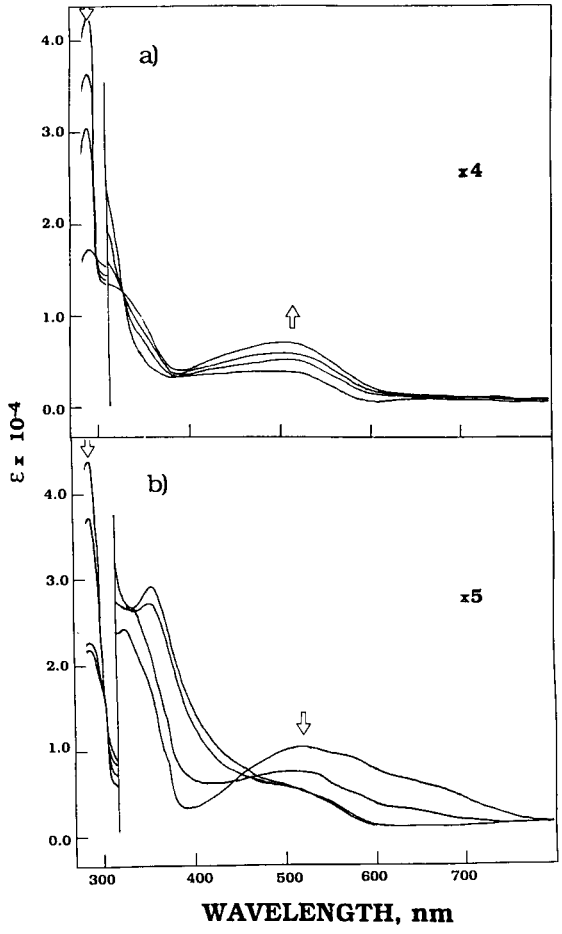


Figure 4. Time resolved uv-vis spectra obtained during (a) reduction; (b) oxidation of **1** from aqueous 0.1 *M* $LiClO_4$.

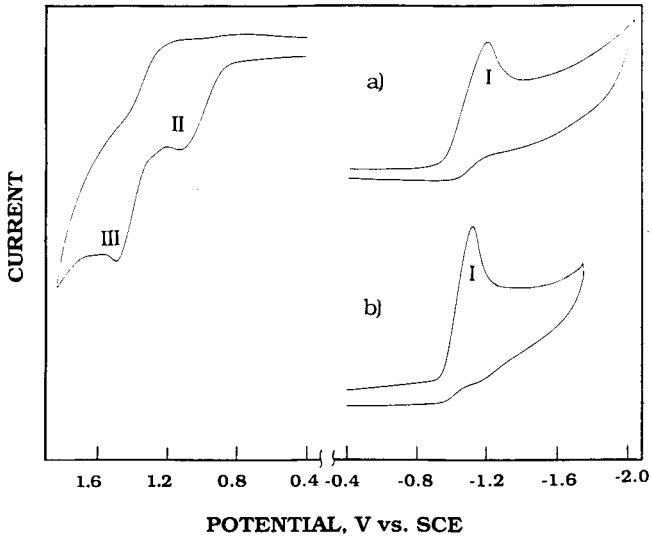


Figure 3. Cyclic voltammograms of **1** in nonaqueous solutions; (a) 0.1 *M* in 1,2-dicholorethane; (b) 0.1 *M* in dimethyl sulfoxide. Voltammetric peak potentials (V vs SCE), Ox-Ep snd Red-Ep: (a) 1.15, 1.45 and -.17, respectively; (b) —, -1.13.

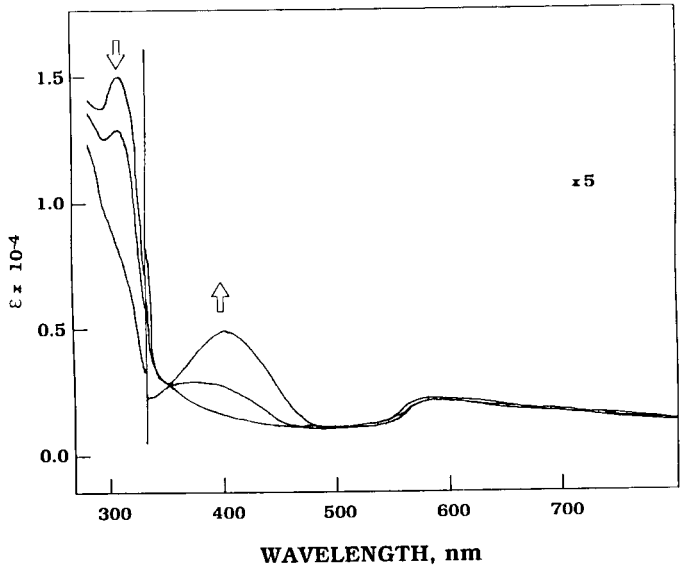


Figure 5. Time resolved uv-vis spectra obtained during reduction of **1** in aqueous 0.1 *M* NaOH.

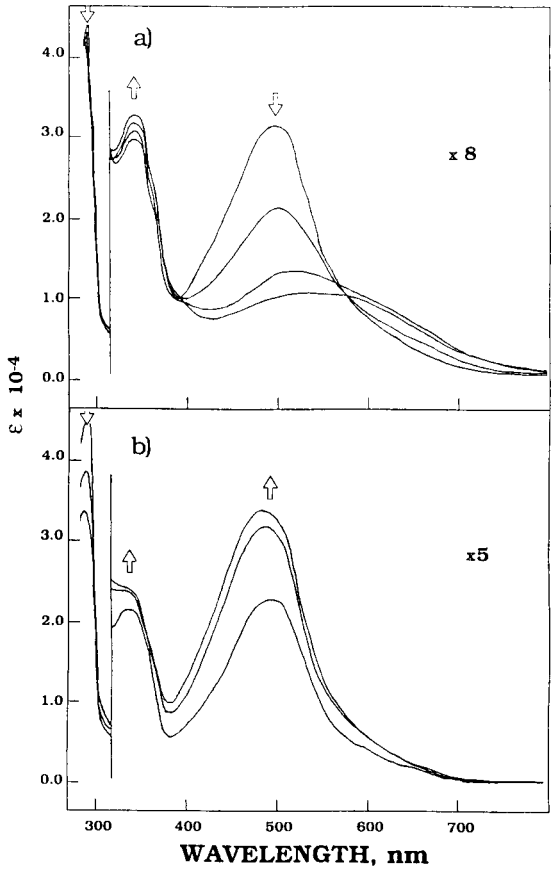


Figure 6. Time resolved uv-vis spectra obtained during (a) reduction; (b) oxidation of **1** from aqueous 0.1 *M* HCl.

In connection with the facts that in aqueous solution both the oxidation and the reduction of the pseudo-porphyrin **1** occur and that this compound shows a relatively rich spectrum in the uv-vis region, it is likely that photo-oxidation and/or photoreduction can take place. Our preliminary measurements have shown that indeed some significant changes were observed in the uv-vis spectra of the neutral, basic and acidic solutions of **1** after exposure to the visible light; after 4 hours the band at 266 nm characteristic for the neutral solution totally disappeared and the band at 430 nm shifted hypsochromically to 460 nm. Such a behavior is consistent with the described spectroelectrochemical characteristics of the oxidation-reduction of the pseudo-porphyrin.

Contrary to a rather simple synthesis of the water soluble pseudo-porphyrin **1**, the synthesis of water soluble porphyrins is usually associated with some additional synthetic steps as compared to water insoluble porphyrins. These steps most ofter involve the introduction of *N*-methyl-4-pyridinium meso-substituent(s). Since some of our water soluble porphyrinyl-nucleosides containing the latter substituent showed strong tumoricidal effects against malignant melanoma cells [15], we were encouraged to check activity of the new pseudo-porphyrin species **1** of tetraazaannulene-type. It contained the methoxy substituent which was also present in the meso- (*p*-methoxyphenyl)-tris(*N*-methyl-4-pyridinium)porphyrin, **2**, applied as a representative of water soluble porphyrins. At a concentration of 2.5 x 10^{-5} *M* in Tris buffer (*p*H 7.2) the synthesized pseudo-porphyrin **1** showed a malignant cell death rate 50% after 3 days of action, an activity which surpassed that of the real porphyrin system **2** characterized by a 44% death rate. The tumoricidal activity in question showed a strong dependence on the concentration in Tris buffer and was strongly influenced by exposure to light. At a concentration of **1** at 10^{-5} *M*, inhibition of malignant melanoma cell growth was only 9%, however, exposure to light for 30 minutes increased the inhibition to 49% (see Table 1).

The effect of **1** on the growth of malignant cells equalled that of our porphyrinatocobalt(II)-thymidine which appeared at a 2.5 times smaller concentration [16]. At present is is difficult to make any suggestions concerning the reasons for the observed behavior of the pseudo-porphyrin in question. One can expect, that further accumulation of data concerning both the tetraazaannulene-type pseudo-porphyrins and the properly related porphyrins will eventually allow useful generalizations.

EXPERIMENTAL

Synthesis.

Materials and Methods.

The following commercially available chemicals from Aldrich were used in the synthesis of **1** without further purification: 4-methoxy-1,2-phenylenediamine dihydrochloride, 2,4-pentanedione, nickel diacetate tetrahydrate, anhydrous methanol, chloroform and acetone. The chemicals used in the synthesis of **2** were

Table 1

Effect of Pseudo-porphyrin **1** and Porphyrin **2** (solutions in Tris buffer) on the Growth of M21-HPM Malignant Melanoma Cells; Number of Cells as Percent of Control and Standard Deviations

	Psedo-porphyrin **1**		Porphyrin **2**	
Treatment /concentration	% of Control	Standard deviation	% of Control	Standard deviation
1 μg/ml ($10^{-6}M$)	98.5	±1.1		
10 μg/ml ($10^{-5}M$) no light	89.2	±1.7		
30 min light	49.2	±1.8		
25 μg/ml ($2.5 \times 10^{-5}M$)	49.5	±1.1	66.0	±3.1

the same as described in [9]. Fast atom bombardment mass spectrometry was performed on a V6 Micromass 70/70 mass spectrometer with a 11/250 data system, 3-nitrobenzyl alcohol applied as a matrix. The ^1H nmr spectra were recorded on a Bruker IBM AF 300 MHz Fourier transform spectrometer. Electronic absorption spectra were recorded on a Perkin-Elmer Lamda 4C uv-vis spectrometer model C 688-0002.

5,7,12,14-Tetramethyldimethoxybenzo[*b*,*i*][1,4,8,11]tetraazacyclodecine Dihydrochloride **1**.

Nickel diacetate tetrahydrate (1.743 g, 0.007 mole), 4-methoxy-1,2-phenylenediamine dihydrochloride (3.0 g, 0.014 mole) and 2,4-pentanedione (1.4 g, 0.014 mole) were added to anhydrous methanol (250 ml) and refluxed under nitrogen for 48 hours. The mixture was evaporated to about 80 ml under reduced pressure and left standing for 48 hours in a refrigerator. The precipitated dark violet crystals were filtered and washed with acetone-chloroform 4:1; yield 52%. From the concentrated filtrate another 19% of product was obtained; ms: fab $(M+1)^+$ 479 m/z and 440 m/z, the latter corresponding to mono-hydrochloride; ^1H nmr (deuteriodimethyl sulfoxide): ppm 10.30 (br s, 2H, $\overset{+}{N}H_2$), 9.61 (br s, 2H, $\overset{+}{N}H_2$), 6.58 (d, 8.7 Hz, 2H ar), 6.41 (m, 2H ar), 6.23 (d, 2.2 Hz, 2H ar), 4.08 (s, 2H, C[6,13]-H), 3.64 (s, 6H, OCH_3), 1.79 (s, 6H, CH_3), 1.75 (s, 6H, OCH_3); uv-vis (water): nm 267, 278, 327, 504 (broad); (water, 0.1 *M* lithium perchlorate): 267, 280, 330, 530; (water, 0.1 *M* sodium hydroxide): 265, 305; (water 0.1 *M* hydrochloric acid): 266, 280, 331, 490; (1,2-dichloroethane, 0.1 *M* tetrabutylammonium perchlorate): 270, 280, 340, 546; (dimethyl sulfoxide, 0.1 *M* tetrabutylammonium perchlorate): 261, 430.

Anal. Calcd. for $C_{24}H_{30}Cl_2N_4O_2$: C, 60.67; H, 6.28; Cl, 14.64; N, 11.71; O, 6.69. Found: C, 60.52; H, 6.31; Cl, 14.70; N, 11.52.

Meso-5,10,15-tris(*N*-methyl-4-pyridinium)-20-(*p*-methoxyphenyl)-porphyrin **2**.

This reference water soluble porphyrin was obtained as already described [9]. First, the meso-tris(4-pyridyl)-*p*-methoxyphenylporphyrin was obtained by cross condensation of 4-pyridinecarboxaldehyde and *p*-anisaldehyde with pyrrole in boiling propionic acid, the proper product separated by column chromatography on a silica gel column, chloroform/methanol 30:1 applied as an eluent. Then the *N*-methylation was achieved by the methyl iodide-nitromethane mixture; the iodine tri-*N*-methylpyridinium salt was finally converted to a chloride salt.

Electrochemical and Spectroelectrochemical Methods.

Cyclic voltammetric and differential pulse voltammetric measurements were made with IBM EC 225 voltammetric analyzer. An omnigraphic Houston 1000 X-Y recorder was used to record the current voltage output for sweep rates between 0.002 and 0.30 v/s. Current voltage curves were collected on digital storage oscilloscope with an X-Y recorder attached. Coulometry was performed with a PAR Model 273 potentiostat. A conventional three electrode system was used. This consisted of a platinum working electrode (diameter 2 mm), a platinum wire counterelectrode and saturated SCE as the reference electrode. The uv-vis spectra were obtained with a Tracor Northern multichannel analyzer. The system was composed of a Tracor Northern 6050 spectrometer containing a crossed Czerny-Turner Spectrograph and a Tracor Northern 1710 multichannel analyzer. Spectra were recorded by a double-array detector. Spectroelectrochemistry was performed in thin-layer cell followed the design of Lin *et al* [17] with calculated path length of 0.5 mm and platinum mesh as a working electrode. The spectrum was taken over the region of 275-875 nm. Glass plates covered with indium-tin oxide and electrochemically deposited films were used to obtain uv-vis spectra of the polymeric pseudo-porphyrin. Indium-tin oxide coated glass was washed successively with acetone and methanol in an ultrasonic bath five minutes, rinsed with distilled water, and used as a working electrode. After the polymerization, the film on indium-tin oxide was rinsed with distilled water. The applied solvents were as follows: 1,2-dichloroethane (Fisher Scientific Company), hplc grade, twice distilled from phosphorus pentoxide; dimethyl sulfoxide (Eastman), analytical grade, distilled over calcium hydride prior to use; the supporting electrolyte, tetrabutylammonium perchlorate (Eastman), twice recrystallized from ethanol, dried and stored in a vacuum at 45°. All solvents were deareated with nitrogen each time prior before the working solution of a reagent was prepared.

The Effect on the Proliferation of Human M21-HPB Malignant Melanoma Cells [15,16].

Cells were obtained from Hybritech Inc., and were cultured in RPMI 1640 medium. Culture medium was supplemented with 10% fetal bovine serum, 50 μM HEPES, 50 μg/ml gentamicin and 10 μg/ml amphotericin B. Culture medium was purchased from Irvine Scientific, fetal bovine serum from HyClone Laboratories and other supplements from Sigma Chemical Company. Cells were grown in T-25 flasks (Nunc, Naperville, IL) and maintained at 37° in a humidified atmosphere with 5% carbon dioxide. Initially, 1 x 10^6 cells were plated in flasks and incubated for 24 hours allowing cells to attach to the substratum. The culture medium was then supplemented with 0, 1, 10 or 25 μg/ml of **1** or **2**, the Tris buffer of *p*H 7.2 at 37° serving as the solvent. The cells were allowed to grow for three more days and were then harvested with 0.25% trypsin in PBS for counting. Viable cells were counted in an Elzone automated particle counter Model 380PC, Particle Data Inc. The control samples contained the solution in Tris buffer of sodium chloride providing the same concentration of chloride anion as that in samples **1** and **2**. The results are reported as mean values of triplicates and the standard deviations. In some experiments the T-25 flasks containing the cells were exposed to four floodlights for 30 minutes.

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