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STRUCTURE/ACTIVITY RELATIONSHIPS AMONG PHOTOSENSITIZERS RELATED TO PHEOPHORBIDES AND BACTERIOPHEOPHORBIDES

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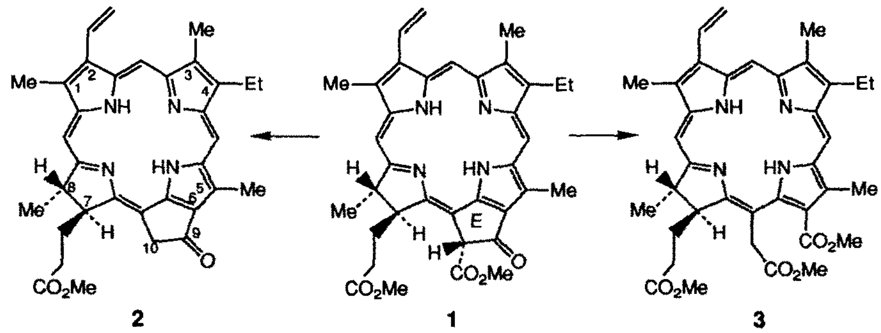
Abstract. A series of long wavelength absorbing photosensitizers related to pheophorbide-a, pyropheophorbide-a, bacteriopheophorbide-a and chlorin-e_6 were synthesized and evaluated for their comparative *in vivo* tumorcidal activity and skin phototoxicity vis-a-vis Photofrin®. Structure/function relationships are discussed. All new photosensitizers were found to have high production of $^1O_2$ and reduced skin phototoxicity compared to Photofrin®.

In recent years there has been great interest in synthesis of long wavelength photosensitizers for use in photodynamic therapy (PDT). The resulting deeper tissue penetration should allow larger tumors to be treated by comparison with Photofrin®. Another desirable objective, compared with Photofrin®, is synthesis of sensitizers with reduced residual skin phototoxicity. A large number of dyes such as phthalocyanines, naphthalocyanines, purpurins, bacteriochlorins, aspartyl acid derivatives of chlorin-e_6, benzoporphyrin derivatives, and sapphyrins have been reported as potential photosensitizers in the last several years.[1] However, the mode of action and structural requirement(s) for an effective photosensitizer are not clear and predictable. In our initial efforts to elucidate the structure of Photofrin®, we synthesized a series of porphyrin dimers and trimers with ester, ether, and carbon-carbon linkages.[2-6] From *in vivo* results with these compounds we, and various other groups, observed that changing the substituents at peripheral positions of the porphyrin nucleus results in a remarkable difference in photosensitizing activity.[7-10]



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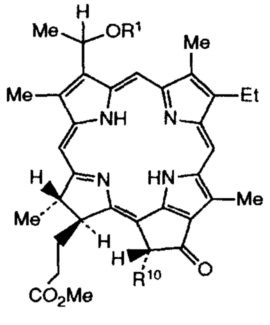
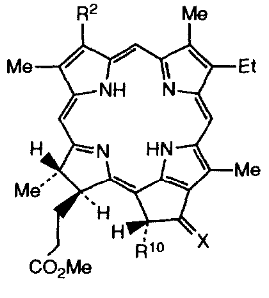
Among long-wavelength absorbing photosensitizers, pheophorbide-a, which is a degradation product of chlorophyll-a, has received increasing attention due to its high production of $^1O_2$ and great abundance in Nature.[11,12] In the methyl pheophorbide series, we have previously shown that photosensitizing efficiency increased with a decrease in polarity of the pheophorbide.[13] We now report the synthesis and comparative *in vivo* photosensitizing activity of a series of photosensitizers related to chlorin-e_6, pheophorbides, and bacteriopheophorbides.

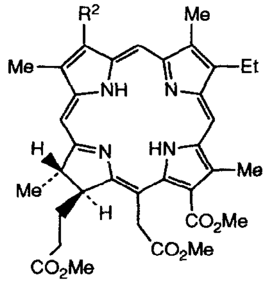
The main objective of our study was to understand the structure/activity relationships among photosensitizers in a particular series, and compare these results with those from other well-investigated photosensitizers. This study, we hope will help us to understand the generic structural requirements for an effective photosensitizers. For our studies, methyl pheophorbide-a **1**, methyl pyropheophorbide-a, **2** (in which the methoxycarbonyl attached to isocyclic ring E is replaced by H) and chlorin-e_6 trimethyl ester, **3**, obtained using standard methodology by opening the isocyclic ring in **1**, were used as the starting materials. Methyl pheophorbide-a **1** was isolated from *Spirulina maxima* alga by following our earlier method.[14] It was converted into methyl pyropheophorbide-a **2** in quantitative yield by refluxing in collidine. Basic hydrolysis of **1** produced chlorin-e_6 trimethyl ester **3** in 80% yield.[15] In order to compare the effect of the length of alkyl side chain upon biological activity in the methyl pyropheophorbide series, a number of alkyl ether derivatives **5**, **6**, and **7** were synthesized. For example, for the preparation of methyl 2-(1-methoxyethyl)-2-devinylpyropheophorbide-a **5**, methyl pyropheophorbide-a **2** was first reacted with 30% HBr/acetic acid. The intermediate 2-(1-bromoethyl) derivative which resulted was not isolated, but was immediately reacted with methanol to afford the desired methyl ether product in 75% yield after the column chromatography. Other alkyl derivatives were prepared by following the same approach but using different alcohols in the last step. The *in vivo* results have shown that among all the alkyl ether derivatives, methyl 2-(1-hexyloxyethyl)pyropheophorbide-a **7** (λ_{max} 660 nm; Figure 1) was superior.



4 R^1 = n-hexyl; R^{10} = CO_2Me
5 R^1 = Me; R^{10} = H
6 R^1 = n-butyl; R^{10} = H
7 R^1 = n-hexyl; R^{10} = H

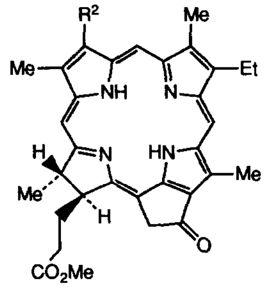


8 R^2 = $CH{=}CH_2$; R^{10} = CO_2Me; X = S
9 R^2 = $CH{=}CH_2$; R^{10} = H; X = S
10 R^2 = CHO; R^{10} = CO_2Me; X = O
11 R^2 = CHO; R^{10} = H; X = O

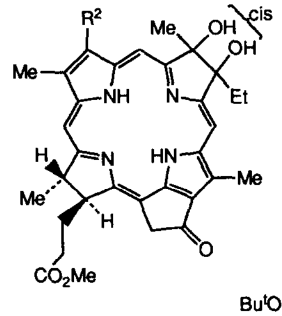


12 R^2 = CH(O-hexyl)Me
13 R^2 = CHO

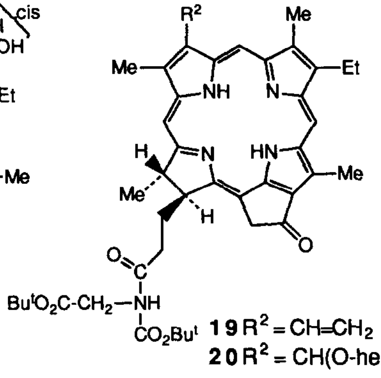
In order to compare the effect of secondary (1-alkoxyethyl) versus primary (alkoxymethyl) ether derivatives, methyl 2-(hexyloxymethyl)-pyropheophorbide-a **14** was prepared from the corresponding methyl 2-(1-hydroxymethyl) pyropheophorbide **15**, which in turn was prepared in almost quantitative yield by $NaBH_4$ reduction of the 2-formyl derivative **11**. Replacement of vinyl with formyl group in methyl pheophorbide-a (to give **10**), methyl pyropheophorbide-a (to give **11**), and chlorin-e_6 (to give **13**) gave a red shift in the optical spectrum of 26 nm producing a strong long wavelength absorption at 690 nm. These compounds were prepared in >85% yield by reacting the corresponding pheophorbide **1** and **2** with OsO_4 and $NaIO_4$. Sulfur analogues of biologically active compounds often show enhanced biological activity, compared with the oxygen analogue. Thus, by following the method of Cava et al.[16] thione derivatives **8** and **9** were obtained in 47% yield by reacting the corresponding pheophorbide with the Lawesson's reagent; both products had a strong long wavelength absorption at 702 nm (Figure 1). Other investigators[17,18] have independently used this methodology in preparing thione porphyrins and bacteriochlorins.



14 $R^2 = CH_2O$-hexyl
15 $R^2 = CH_2OH$
16 $R^2 = Et$



17 $R^2 = Et$
18 $R^2 = CH(O$-hexyl$)Me$



19 $R^2 = CH{=}CH_2$
20 $R^2 = CH(O$-hexyl$)Me$

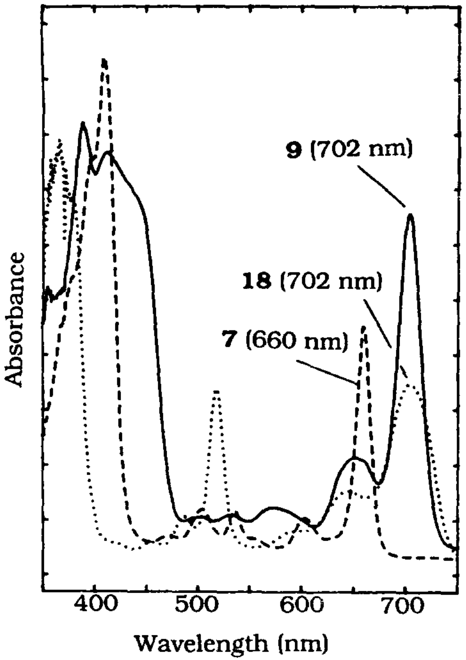


Figure 1: Optical Spectra, in CH_2Cl_2, of pheophorbides **7** and **9**, and bacteriopheophorbide **18**.

Due to their long wavelength absorption properties, bacteriochlorins have been proposed as potential useful candidates for use in photodynamic therapy.[1] Some bacteriochlorophylls and bacteriochlorins have been shown to photosensitize cells *in vitro* and have exhibited photodynamic activity *in vivo*.[19,20] To understand the effect of the hexyl ether moiety on the biological activity of this class of compounds, dihydroxy-pyrobacteriopheophorbides **17** and **18** were synthesized. For the synthesis of **17**, methyl mesopyropheophorbide-a **16**, obtained by hydrogenation of **2**, was reacted with OsO_4 in pyridine. Subsequent treatment with H_2S gas cleaved the intermediate osmium complex and a mixture of diastereoisomers with *cis*-vicinal hydroxyl groups (either up or down relative to the (7-S),(8-S) absolute stereochemistry in ring D) was isolated in 80% yield. Reaction of **7** with OsO_4, under similar reaction conditions, gave bacteriochlorin **18** (λ_{max} 702 nm; Figure 1) in excellent yield. In the chlorin series, the monoaspartyl derivative of chlorin-e_6 (MACE) has been reported to be an effective photosensitizer *in vitro* compared with Photofrin®. *In vivo* studies have shown that MACE is an effective tumor photosensitizer with rapid clearance properties if light treatments were performed 3 to 4 h after drug administration. Neither tumor cure nor normal tissue damage could be induced when the drug was administered (up to 50 mg/kg) 24 h prior to light treatment.[21] To compare the biological activity of such derivatives in the pyropheophorbide series, t-butyl aspartyl derivatives **19** and **20** (with or without hexyl ether groups) were synthesized. For the preparation of these compounds the carbodiimide derivative obtained from the corresponding carboxylic acid was reacted with di-t-butyl aspartyl ester and the desired photosensitizers were isolated in 65 and 70% yield, respectively.

Biological Activity; A Comparative Study:

The *in vivo* photosensitizing ability (30+ day tumor response and skin photosensitivity) of all the new photosensitizers was compared with that of Photofrin® in the SMT-F tumor growing subcutaneously in DBA/2 mice, a method previously described by Dougherty et al.[22] and by Gomer and Ferriao.[21] Table 1 presents comparative *in vivo* antitumor activities of selected photosensitizers studied here; data for Photofrin® are also included. Photosensitizers were injected at various doses and were treated either 24 h or 3 h post injection.

Table 1: Comparative *in vivo* Antitumor Activity of Selected Photosensitizers†

Compound	Dose (mg/kg)	Absorbance λmax	Time (h) betw. injection and light treatment	% Tumor Response (days)#		
				1-2	7	30
Photofrin®	4.2	630	24	100	80	20
	5.0	630	24	100	95	44
Chlorin-e$_6$ trimethyl ester hexyl ether (**12**)	1.0*	660	24	100	75	0
	0.3	660	24	100	0	0
Methyl pheophorbide-a hexyl ether (**4**)	1.0*	660	24	100	100	50
	0.3	660	24	100	90	0
	0.3	660	3	100	100	20
Methyl pyropheophorbide-a hexyl ether (**7**)	0.3	660	24	100	100	50
	0.25	660	24	100	85	35
Methyl pyropheophorbide-a thione (**9**)	5.0	702	24	0	0	0
	5.0	702	3	100	100	80
Methyl 2-formylpheophorbide-a (**10**)	5.0	690	24	80	60	0
Methyl 2-formylpyropheophorbide-a (**11**)	2.5	690	24	0	0	0
	2.5	690	3	67	67	0
2-Formylchlorin-e$_6$ trimethyl ester (**13**)	5.0	690	24	0	0	0
Methyl 2-(hexyloxymethyl)-pyropheophorbide-a (**14**)	0.3	660	24	100	33	0
Di-t-butyl aspartyl pyropheophorbide-a hexyl ether (**20**)	1.0	660	24	100	100	0
	0.3	660	24	100	67	0
Methyl dihydroxymesobacteriopheophorbide-a (**17**)	0.3	702	3	0	0	0
Methyl dihydroxybacteriopheophorbide-a hexyl ether (**18**)	0.3	702	3	0	0	0

† 4-6 mm diameter tumors exposed to 75 MW/cm^2 for 30 min to deliver 135 J/cm^2 from a tunable dye laser tuned to the maximum red absorption peak. #Non-palpable tumors. *At higher doses there was 100% mortality after light exposure.[13]

The results can be summarized as follows: in the pyropheophorbide series it was observed that by increasing the length of the alkyl ether carbon chain, there was an increase in photosensitizing activity. We have previously reported[13] similar observation in a series of alkyl ether derivatives of methyl pheophorbide-a **1** and chlorin-e_6 trimethyl ester **3**. However, the hexyl ether derivative of methyl pyropheophorbide **7** was found to be somewhat superior to the other analogues and showed promising activity even at a dose of 0.3 mg/kg [treated after 24 h (100% tumor response at day 7, 50% tumor response at day 30)]. The hexyl ether derivative obtained from methyl 2-(hydroxymethyl)-pyropheophorbide-a **14** showed better photosensitizing activity than methyl pyropheophorbide-a **2**, was less effective than **7**. Similarly, the thione and formyl pyropheophorbides **9** and **11** were found to be more active than their methyl pheophorbide-a analogues **8** and **10**; for example, thione derivative **9** at a dose of 5.0 mg/kg when treated after 3 h showed 80% tumor response at day 30, but at the same dose the oxo-derivative **2**, was inactive. Similarly, formyl derivative **11**, at a dose of 2.5 mg/kg (treated after 3 h) showed 67% tumor necrosis at day 7. The aspartyl derivative **20** did show some improvement in photosensitizing ability compared with **2**, but was found to be less active than **7**. As shown in Table 1, in the bacteriochlorin series, the dihydroxymesobacteriopyropheophorbide-a **17** and its hexyl ether derivative **18** were found to be inactive at a dose of 0.3 mg/kg when tumors were exposed to light 3 h post i.p. injection. Thus, they appear to be less effective than 2-(1-hexyloxy)ethylpyropheorbide-a **7**, but further studies at variable doses and time intervals are in progress.

From these preliminary *in vivo* results, it can be seen that the five membered isocyclic ring appears to play an important role in photosensitizing ability of the compounds. Removal of the ester group at position 10 (ring E) of methyl pheophorbide-a slightly increases *in vivo* photosensitizing efficacy. These results were consistent in a series of photosensitizers with variable substituents. All these compounds showed high production of $^1O_2$ as measured following the literature procedure.[23] To date, only photosensitizers **4** and **7** have been tested for skin phototoxicity and these showed more rapid attenuation of normal tissue photosensitivity with time after administration vis-a-vis Photofrin®. From Table 1 it can be clearly seen that among all of the photosensitizers reported herein, compound **7** showed the highest photosensitizing ability with only minor skin photosensitivity.

Detailed biological studies with these photosensitizers will be reported elsewhere. All the new photosensitizers were characterized by proton NMR, spectrophotometry, elemental analysis, and/or high resolution mass spectroscopy.

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