Photophysical properties of 5,10,15,20-tetrakis(*m*-hydroxyphenyl)-porphyrin (m-THPP), 5,10,15,20-tetrakis(*m*-hydroxyphenyl)chlorin (m-THPC) and 5,10,15,20-tetrakis(*m*-hydroxyphenyl)bacteriochlorin (m-THPBC): a comparative study

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For the tumour photosensitisers m-THPP, m-THPC, and m-THPBC in methanol the following photophysical properties have been measured: absorption and fluorescence spectra, E_S, Stokes shift, Φ_f, Φ_T, τ_T, dioxygen quenching rates, and Φ_Δ for air-saturated and oxygen-saturated solutions. The properties of the first excited triplet state are quite uniform across this series. The quantum yields of singlet oxygen formation are high, being 0.43–0.46 in air-saturated methanol, and 0.59–0.62 in oxygen-saturated methanol. For a constant substitution pattern and a standard given light dose, tumour photonecrosis *in vivo* appears to parallel the product of drug dose and the molar extinction at the irradiation wavelength in the red region of the visible spectrum.

There has been increasing interest in recent years in the potential applications of photosensitizing molecules and visible light in the treatment of cancer.[1,2] This treatment, which results in the destruction of the cancer tissue, depends on the presence of molecular oxygen, and hence this is an example of the photodynamic effect. The treatment is commonly referred to as photodynamic therapy (PDT). In mechanistic terms the process appears to depend primarily on singlet oxygen production, leading to a Type II photoreaction, thus:

$$P(S_0) \xrightarrow{h\nu} P(S_1) \xrightarrow{\text{isc}} P(T_1)$$

$$P(T_1) + {}^3O_2 \longrightarrow P(S_0) + {}^1O_2$$

$$\text{Biomolecules } (S_0) + {}^1O_2 \longrightarrow \text{products (resulting in membrane damage and cell death)}$$

(where P = photosensitizer, S_0 = ground state singlet, S_1 = first excited singlet state, T_1 = first excited triplet state, ${}^3O_2$ = ground state triplet oxygen, and ${}^1O_2 = {}^1\Delta_g$ singlet oxygen), so that the quantum yield of singlet oxygen formation is a significant parameter. Radical processes (Type I photoreactions) occur concomitantly, but appear generally to play a less important role in cellular damage.[3]

PDT received a considerable stimulus in 1993 when regulatory approval was given (originally in Canada) for the use of Photofrin, a commercial preparation of haematoporphyrin derivative, in the treatment of certain cancers. However haematoporphyrin derivative is a complex mixture,[4] and hence there has been worldwide activity in the search for more effective, more selective photosensitizers which are single substances. Because of the transmission characteristics of human tissue (red > blue), photosensitizers with strong absorption in the red have been sought.[2]

Amongst these "second generation" photosensitizers, a series of compounds closely related to 5,10,15,20-tetrakis(*m*-hydroxyphenyl)porphyrin (m-THPP) **1** have been particularly promising. This series comprises the parent porphyrin **1**; the corresponding dihydroporphyrin or chlorin **2** [5,10,15,20-tetrakis(*m*-hydroxyphenyl)chlorin, m-THPC]; and the corresponding 7,8,17,18-tetrahydroporphyrin or bacteriochlorin **3** [5,10,15,20-tetrakis(*m*-hydroxyphenyl)bacteriochlorin, m-THPBC]. In experimental animal assays the tumour photonecrotic activity increases in the sequence **1** < **2** < **3**.[5] *In vivo* comparisons of m-THPC (**2**) with other photosensitisers (m-THPP, p-THPP, p-THPC, Photofrin) for both tumour photonecrotic activity and tumour selectivity showed that m-THPC **2** was the most promising compound of those examined.[6] The first clinical results[7] for m-THPC appeared in 1991. The drug is now at an advanced stage of clinical trial.[8]

The photophysical properties of the porphyrin, m-THPP **1** were described earlier.[9] In this paper we report photophysical data for the related reduced compounds m-THPC **2** and m-THPBC **3**, and make comparisons between the properties of the three oxidation levels.

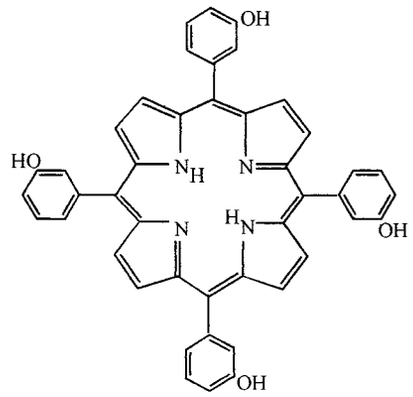
Experimental

Compounds **1**, **2** and **3** were prepared as described.[5] Compound **3** was a gift from Scotia QuantaNova plc (Guildford) and contained about 5% of the chlorin **2**. Since these compounds (and especially **3**) are subject to photobleaching, spectroscopic measurements were carried out on fresh solutions, which were handled in minimal ambient light.

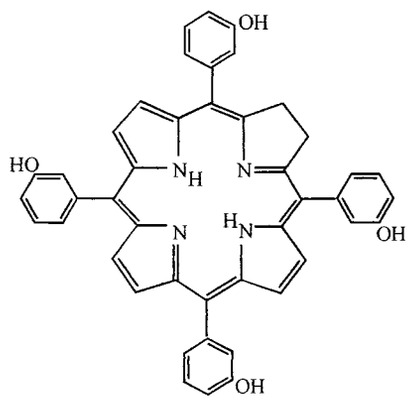
Methylene blue (British Drug Houses Ltd), haematoporphyrin (Sigma-Aldrich), and β-carotene (Hoffmann la Roche) were used as received. Anthracene (Aldrich) was recrystallized from ethanol before use. Methanol (spectroscopic grade, British Drug Houses Ltd), monodeuteriated methanol, CH_3OD (Cambridge Isotope Laboratories) and cyclohexane (spectroscopic grade, Rathburn Chemicals Ltd) were all used as received.

Instrumentation

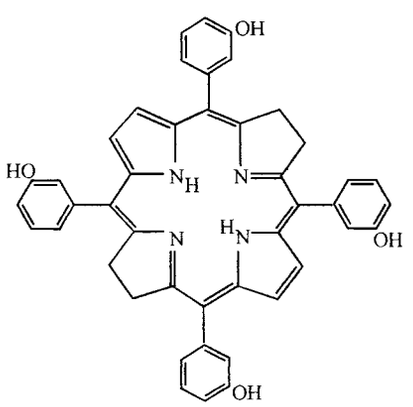
Kinetic absorption measurements were made using the third harmonic (355 nm) of a Spectron Q-switched Nd-YAG laser as described previously.[10] Time resolved singlet oxygen luminescence was detected *via* the (0,0) phosphorescence band



1 m-THPP



2 m-THPC



3 m-THPBC

$$O_2(^1\Delta_g) \longrightarrow O_2(^3\Sigma_g^-) + h\nu(1270\ nm)$$

centred at 1270 nm using a Judson germanium diode (G-050, active diameter = 0.5 cm) coupled to a Judson preamplifier.

Absorption and fluorescence measurements were recorded using a Perkin Elmer Lambda 2 UV–VIS spectrometer and a Perkin Elmer LS50 luminescence spectrometer, respectively.

Methods

The fluorescence quantum yield was determined using haematoporphyrin ($\Phi_f = 0.09$)[11] as the standard, and an excitation wavelength of 500 nm.

Triplet state absorption coefficients were obtained using the energy transfer method for m-THPBC and the complete conversion method for m-THPP and m-THPC.[12] In the energy transfer method β-carotene was used as the reference standard, for which $\Delta\varepsilon_T^{515} = 187\,000$ dm^3 mol^{-1} cm^{-1}.[13] In the complete conversion method the reference was anthracene in cyclohexane, for which $\Delta\varepsilon_T^{422} = 64\,700$ dm^3 mol^{-1} cm^{-1} and $\Phi_T = 0.71$.[14,15]

For the determination of the triplet state quantum yields (Φ_T) the comparative method was used, again using anthracene in cyclohexane as reference. Deaerated solutions of the sensitizers were optically matched at the laser excitation wavelength.

The rate constants (k_q) for oxygen quenching of the triplet states were determined in methanol by kinetic absorption measurements on various O_2–N_2 mixtures. Five single shot kinetic absorption traces were signal-averaged for each measurement and good single exponential fits were obtained for all sensitizers. The oxygen concentration in air-saturated methanol was taken to be 2.1×10^{-3} mol dm^{-3}.[16]

For singlet oxygen quantum yield determinations, air-equilibrated solutions of the sensitizers were optically matched at the laser excitation wavelength, along with that of the reference standard for which the singlet oxygen quantum yield had previously been determined. Solutions were prepared in 1 × 1 cm quartz cells with absorbances of 0.5 at 355 nm. Time resolved luminescence at 1270 nm was recorded following laser excitation. At each laser intensity the recorded luminescence trace was obtained by signal averaging ten single shots. The averaged traces were fitted with a single exponential which was extrapolated to $t = 0$. Plots of I_o (the extrapolated signal intensity at $t = 0$) *versus* laser intensity were found to be linear up to a laser intensity of 4 mJ pulse^{-1}. Within this laser intensity range eight data points were obtained for each plot. Since the gradient of the I_o *versus* laser intensity plots are proportional to Φ_Δ, the values of Φ_Δ may be obtained by comparison with the gradient obtained for the reference standard. The standard employed was haematoporphyrin in monodeuteriated methanol, where $\Phi_\Delta = 0.53$ and 0.64 for air-saturated and oxygen-saturated solutions respectively.[17]

Results and discussion

The measurements on the three compounds considered here have all been made with methanol as solvent at concentrations where aggregation effects are not important. For example, the spectrum of m-THPC in methanol accurately follows Beer's Law over the concentration range 4.6×10^{-6} to 7.34×10^{-4} M with the band width at half height for the Soret band remaining constant (1870 cm^{-1}) over this range.[18] The results of our present study are summarized in Table 1. It is worth pointing out that this is the only series where, for the *same substitution pattern*, biological assays *in vivo* are available at all three oxidation levels. To this is now added an extensive set of photophysical parameters available at the same three levels.

Ground state absorption spectra

Band I shifts to lower energy as expected with the reduction of the porphyrin to the dihydro and 7,8,17,18-tetrahydro derivatives. The porphyrin→chlorin change is relatively small (644→650 nm) whereas reduction of the chlorin to bacteriochlorin is accompanied by a substantial shift (650→735 nm). At the same time a marked hyperchromic effect, which is believed to be important for the clinical application, is observed along the series.

Fluorescence spectra

The fluorescence spectra of **1** and **2** show two bands (0–0, 0–1) to lower energy of Band I in absorption as is commonly observed for compounds in these series. The bacteriochlorin behaves differently. It shows only one band (746 nm) to lower energy of Band I in absorption, and two weaker bands (612, 653 nm) at *higher* energies. A likely explanation for this is that the 653 nm emission is due to the small percentage of the chlorin **2** which is always present in the bacteriochlorin **3**. This view is supported by the observation that when the excitation wavelength is 680 nm, then only one emission band, that at 746 nm, is observed. However this explanation does not explain the 612 nm emission, and the matter is being examined further.

Table 1 Some photophysical properties of m-THPP **1**, m-THPC **2**, and m-THPBC **3** in methanol

		m-THPP **1**	m-THPC **2**	m-THPBC **3**
1.	λ_{max} Band I/nm	644	650	735
	ε_{max}/M^{-1} cm^{-1}	3400	29600	91000
2.	λ_{max} fluoroescence/nm	649, 715	653, 720	612, 653, 746
	for excitation at λ/nm	415	415	500
3.	Stokes shift/cm^{-1}	117	71	198
4.	E_s [(1 + 2)/2]/kJ mol^{-1}	185	183.5	161.5
5.	Φ_f	0.12	0.089	0.11
6.	Φ_T	0.69	0.89	0.83
7.	τ_T/s	1.2×10^{-4}	0.50×10^{-4}	0.53×10^{-4}
8.	λ_{max} $T_1 \rightarrow T_2$/nm	440	445	400
	ε_{max} $T_1 \rightarrow T_2$/M^{-1} cm^{-1}	40400	19300	41900
9.	O_2 quenching rate constant k_q/M^{-1} s^{-1}	1.9×10^9	1.8×10^9	2.5×10^9
10.	Φ_Δ, air-saturated	0.46	0.43	0.43
11.	Φ_Δ, oxygen-saturated	0.59	0.59	0.62

For all three compounds Φ_f values are low and rather similar to one another.

Energies of excited states

The Stokes shifts are similar to one another for all three compounds (*ca.* 100 cm^{-1}). The singlet energies are taken as the means of λ_{max} in absorption (Band I) and emission. The values for the porphyrin and the chlorin are similar [but porphyrin (185 kJ mol^{-1}) is greater than chlorin (183.5 kJ mol^{-1})], with the value for the bacteriochlorin being much lower (161.5 kJ mol^{-1}).

The phosphorescence of m-THPP in methanol at 80 K has been reported[9] to be very weak ($\Phi_p \sim 10^{-5}$), but gave an E_T value of 137 (±0.05) kJ mol^{-1} and a singlet–triplet energy gap of 48 kJ mol^{-1}. It has not been possible to observe phosphorescence from the chlorin **2** or the bacteriochlorin **3** under similar conditions, but it is known[19] that the singlet–triplet energy gap in the related zinc(II) *meso*-tetraphenylchlorin is 43 kJ mol^{-1}. If a similar value applies to **2** and **3**, then the estimated E_T values would be 140.5 kJ mol^{-1} for the chlorin **2** and 119 kJ mol^{-1} for the bacteriochlorin **3**, both values being appreciably above the E_S value for singlet oxygen (94 kJ mol^{-1}).

Triplet properties

The triplet properties of the three oxidation levels are similar. The triplet absorption maximum occurs at 440–445 for **1** and **2**, and at 400 for **3**. The triplet quantum yields are high and fall in the range 0.69–0.89, with triplet lifetimes of *ca.* 10^{-4} s. The rates of quenching of the triplet by dioxygen are *ca.* 2×10^9 M^{-1} s^{-1}, while the quantum yields of singlet oxygen formation, Φ_Δ, are 0.43–0.46 for air-saturated methanol and 0.59–0.62 for oxygen-saturated methanol. The higher values in oxygen-saturated solution reflect the effects of oxygen-enhanced intersystem crossing.[20]

Evidently on the basis of triplet properties all three of the compounds under consideration appear to be potentially valuable photosensitizers for PDT. *In vivo* assay[5,21] indicates however that destruction of *ca.* 5 mm of tumour tissue at a constant light dose (10 J cm^{-2}) at Band I requires a photosensitizer dose of 6.25 μmol kg^{-1} of the porphyrin **1**, 0.75 μmol kg^{-1} of the chlorin **2**, and 0.39 μmol kg^{-1} of the bacteriochlorin **3**.

This leads us to the conclusion that in this series there is an approximate relationship between biological activity and molecular extinction at the wavelength of irradiation. This is illustrated in Fig. 1 which shows a plot of depth of tumour necrosis for a single animal model under standard conditions[5,6] against the product of photosensitizer dose (μmol kg^{-1}) and molecular extinction at the Band I maximum (the wavelength at which the tumour is irradiated). Two features emerge from this plot. Firstly, because a tumour damage depth of ~0.1 mm (four points in Fig. 1) cannot be considered to be a positive result,

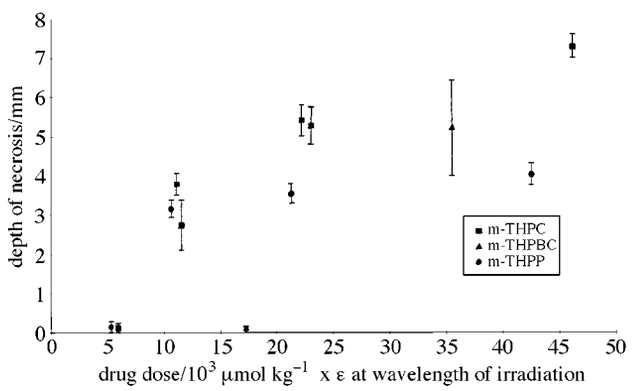


Fig. 1 Plot of depth of tumour photonecrosis against the product of drug dose and molecular extinction at Band I for m-THPP **1** (●), m-THPC **2** (■) and m-THPBC **3** (△). Biological data from references 5 and 6. The threshold for activity of m-THPP and m-THPC is seen to fall between 5000 and 10000 on the horizontal axis: the threshold for m-THPBC is less certain because of insufficient data points.

there appears to be a threshold to the biological damage. Secondly, above this threshold, and at a constant light dose (10 J cm^{-2}), tumour damage is roughly proportional to the product of photosensitizer dose and Band I ε_{max} throughout the series, although there is only one observation in the active part of the plot for the bacteriochlorin **3**. Presumably this sort of relationship can only arise when (i) triplet properties are fairly similar, as demonstrated here, and (ii) other physical properties (solubility, partition coefficient) are similar, so that the cellular distributions are more or less the same. The latter similarity is considered to arise with these substances because they have the same substitution pattern. As a consequence, the solubility and partition characteristics are regarded as being governed principally by the four phenolic groups at the periphery of each molecule.

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