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Carotenohematoporphyrins as Tumor-Imaging Dyes. Synthesis and *In Vitro* Photophysical Characterization*

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ABSTRACT

Multichromophoric dyes for use in tumor imaging have been synthesized and photophysically characterized. Structurally, these dyes are dyads and triads that consist of one or two carotenoid polyenes covalently attached to hematoporphyrin (HP) or hematoporphyrin dimethyl ester (HPDME) moieties *via* ester linkages. The ground-state absorption of each compound shows that the electronic interaction between the chromophores is small. The fluorescence quantum yield for the dyad monocaroteno-HPDME is 0.033 and the dicaroteno-HPDME triads have yields between 0.016 and 0.007, all of which are reduced with respect to the parent compound HPDME (0.09). Global analysis of the transient fluorescence decays of the dyads and triads requires two exponential components (~5–6 ns and ~1–2 ns) to fit the data, while a single exponential component with a lifetime of 9.3 ns describes the decay data of the parent HPDME. Possible mechanisms for the observed porphyrin fluorescence quenching by the nearby carotenoid are discussed. Nanosecond transient absorption reveals a carotene triplet with maximum absorption at 560 nm and a 5.0 μs lifetime. No transient was detected at 450 nm, indicating rapid (≤10 ns) triplet energy transfer from the hematoporphyrin to the carotenoid moieties in fluid as well as in rigid media. The yield of triplet energy transfer from the porphyrin to the carotenoid moiety is unity. Singlet oxygen, $O_2(^1\Delta_g)$, studies support the transient absorption data, as none of these compounds is capable of sensitizing $O_2(^1\Delta_g)$. Liposome vesicles were used to study the photophysical characteristics of the dyes in phospholipid membranes. Singlet oxygen was not sensitized by the dyads and triads in liposomes. Transient absorption measurements suggest that the triads are substantially aggregated within the phospholipid bilayer, whereas aggregation in the dyads is less severe.

INTRODUCTION

Many studies have chronicled the development of new drugs for photodynamic therapy (PDT) of tumors, as well as for the early diagnosis of malignancy, which are based on the fluorescence emission typical of chromophores such as porphyrins (1–3). A significant problem frequently encountered with employing PDT-active drugs in diagnosis is that selective uptake by the neoplastic tissue is not high, leading to the presence of these drugs in other tissues such as skin (4,5). The accumulation of photoactive drugs in skin results in photosensitization, even in ambient room light. Singlet oxygen is thought to be responsible primarily for the skin sensitization effect.

In an effort to combat this problem, carotenoporphyrin derivatives have been synthesized as prototypes of imaging agents for the early detection of malignancy (6). It has been shown that the phototoxicity is eliminated when the porphyrin moieties bear covalently attached carotenoids. Close proximity of the carotenoid moiety to the porphyrin and effective electronic coupling between the chromophores promotes extremely rapid energy transfer from the porphyrin triplet state to the carotenoid, yielding the carotenoid triplet state (7). The quenching of the lifetime of the porphyrin triplet state to <10 ns renders it kinetically incapable of $O_2(^1\Delta_g)$ sensitization.

The carotenoporphyrins of previous studies showed preferential localization in tumors (6). The specific accumulation of these compounds in tumor tissue is thought to be a consequence of the lipophilic character of both the porphyrin and the carotenoid moieties, coupled with their transport mechanisms by lipoproteins within the plasma and the profusion of lipoprotein receptors on malignant cells (8,9).

In the present work, we have extended the studies to carotenohematoporphyrins. Hematoporphyrins have been shown to be useful especially as PDT agents and their tumor-localizing and -sensitizing properties have been extensively studied in many laboratories (1). The investigation of the effect of the carotenoid moiety on the delivery of the hematoporphyrin fluorophore to tumors is one of the consid-

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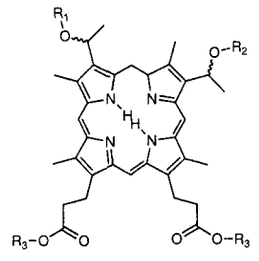
‡ *Abbreviations:* HP, hematoporphyrin; HPDME, hematoporphyrin dimethyl ester, PDT, photodynamic therapy; DMAP, 4-dimethylaminopyridine; THF, tetrahydrofuran; TLC, thin-layer chromatography.



1 R_1, R_2 = -H, R_3 = $-CH_3$

2 R_1, R_2 = -COPh, R_3 = $-CH_3$

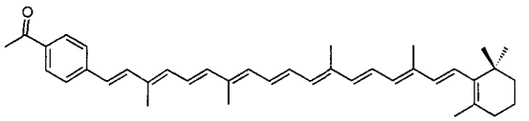
3 R_1 = -H, R_2 = Car_1, R_3 = $-CH_3$

4 R_1, R_2 = Car_1, R_3 = $-CH_3$

5 R_1, R_2 = Car_2, R_3 = $-CH_3$

6 R_1, R_2 = Car_2, R_3 = -H

Car_1 =



Car_2 =

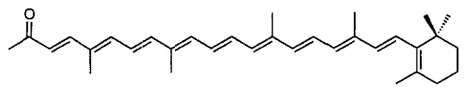


Figure 1. Carotenohematoporphyrins and hematoporphyrin models: (**1**) HPDME; (**2**) 2,4-dibenzoylhematoporphyrin dimethyl ester; (**3**) monocarotenohematoporphyrin dimethyl ester; (**4**) dicarotenohematoporphyrin dimethyl ester; (**5**) dicarotenohematoporphyrin dimethyl ester (no internal phenyl groups); (**6**) dicarotenohematoporphyrin (no internal phenyl groups).

erations that motivated the preparation of the dyads and triads of this study.

MATERIALS AND METHODS

Synthesis

Hematoporphyrin dimethyl ester (HPDME) (**1**) was prepared from hematoporphyrin (HP) (Porphyrin Products Inc.) using standard procedures. The triads and dyads (see Fig. 1) were characterized by ^1H NMR and mass spectra. The ^1H NMR spectra were recorded on a Varian Unity spectrometer at 500 MHz. The samples were dissolved in deuteriochloroform with tetramethylsilane as an internal standard, unless otherwise indicated. Mass spectra were obtained with a matrix-assisted time-of-flight spectrometer (Vestec Laser Tec Research Instrument; matrix used: sulfur). Measured mass/charge ratios (*m/z*) and ^1H NMR data are listed for each compound.

Dibenzoylhematoporphyrin dimethyl ester **2**. A portion of **1** (100 mg, 0.16 mmol) 25 mL of dichloromethane, 0.5 mL of pyridine and 3 mg of 4-dimethylaminopyridine (DMAP) were mixed in a 100 mL flask. The mixture was stirred under an atmosphere of N_2 until all the solid had dissolved. Benzoyl chloride (37 μL, 0.32 mmol) was added to the mixture and allowed to react for 24 h. Because not all of the starting material or the monobenzoylated products were consumed after that time, an extra amount of a benzoyl chloride (37 μL) was added and the reaction was allowed to proceed for another 48 h. The reaction mixture was washed first with an aqueous solution of citric acid and then with brine. The solvents were evaporated from the organic layer under vacuum and the residue was chromatographed on silica gel (dichloromethane/2% acetone) to give 120 mg (90% yield) of **2**. ^1H-NMR δ(ppm): −3.71 (2H, brs, -NH), 2.44–2.48 (6H, m, 2 -OCHCH_3, 4 -OCHCH_3), 3.27–3.30 (4H, d, t, J = 12.6 Hz, J = 5.5 Hz, $\overline{CH_2CH_2CO_2CH_3}$), 3.62 (6H, s, β CH_3 × 2), 3.65 (6H, s, CO_2CH_3), 3.81 (3H, s, β CH_3), 3.84 (3H, s × 2, β CH_3), 4.40 (4H, d, t, J = 12.6 Hz, J = 5.5 Hz, $CH_2CH_2CO_2CH_3$), 7.43–8.34 (12H, m, Ar, 2 -OCHCH_3, 4 -OCHCH_3), 10.07 (1H, s, *meso* H), 10.12 (1H, s, *meso* H), 10.53 (1H, s, *meso* H), 10.60 (1H, s, *meso* H); MS *m/z* 808 (M + 1)$^+$; UV/visible (CH_2Cl_2) 273, 400, 480, 500, 534, 570, 624 nm.

Monocarotenohematoporphyrin dimethyl ester **3** *and dicarotenohematoporphyrin dimethyl ester* **4**. To a 100 mL round-bottomed flask equipped with a stirring bar and a nitrogen line was added 0.17 g (0.32 mmol) of 7′-apo-7′-(4-carboxyphenyl)-β-carotene (10) and 30 mL of dry benzene. To the resulting suspension was added 0.15 mL (2.0 mmol) of pyridine followed by 58 μL (0.8 mmol) of thionyl chloride. The solid carotenoid acid rapidly reacted to form a dark red solution characteristic of the carotenoid acid chloride. The mixture was stirred for 30 min and after this time the solvent was removed under vacuum. A portion of benzene (30 mL) was added and the solvent evaporated a second time to dryness. The carotenoid acid chloride was redisolved in dichloromethane (20 mL) and 0.15 mL of pyridine. To a 250 mL round-bottomed flask equipped with a stirring bar and a nitrogen line were added 0.1 g (0.16 mmol) of **1**, 30 mL of dichloromethane and a catalytic amount of DMAP. Once all the solids had dissolved, the carotenoid acid chloride solution was added *via* a transfer tube. The reaction was allowed to proceed for 3 days and its progress was followed by thin-layer chromatography (TLC). The reaction mixture was diluted with dichloromethane and extracted first with aqueous sodium bicarbonate and then with brine. The organic layer was dried over Na_2SO_4 and filtered, and the filtrate was concentrated under vacuum. The residual pyridine was removed by redissolving the solid in toluene (200 mL) and evaporating the solvent under vacuum. Flash chromatography of the crude product (toluene/5–15% ethyl acetate) resulted in two fractions. Each fraction was further purified by flash chromatography on silica gel. The less polar fraction was eluted with dichloromethane/1% acetone as solvent, yielding 60 mg (23%) of triad **4** as a mixture of stereoisomers. ^1H NMR (500 MHz, $CDCl_3$), δ (ppm): −3.67 (2H, brs, -NH), 1.03–104 (12H, s, Car 16-CH_3, Car 17-CH_3), 1.46–1.48 (4H, m, Car 2-CH_2), 1.60–1.64 (4H, m, Car 3-CH_2), 1.72 (6H, s, Car 18-CH_3), 1.97–2.03 (28 H, m, Car 4-CH_2, Car 19-CH_3, Car 20-CH_3, Car 19′-CH_3, Car 20′-CH_3), 2.46–2.50 (6H, m, 2 -OCHCH_3, 4 -OCHCH_3), 3.30 (4H, q, J = 8.5 Hz, $CH_2CH_2CO_2CH_3$), 3.65 (6H, s, CO_2CH_3), 3.69–3.79 (6H, s, β CH_3 × 2), 3.82 (3H, s, β CH_3), 3.80 (3H, s, β CH_3), 4.43 (4H, q, J = 8.5 Hz, $CH_2CH_2CO_2CH_3$), 6.11–7.25 (28H, m, Car=CH-), 7.50–7.52 (4H, m, Car 1′-Ar, Car 5′-Ar), 7.75–7.77 (2H, m, 2 -OCHCH_3, 4 -OCHCH_3), 8.27–8.30 (4H, m, Car 2′-Ar, Car 4′-Ar), 10.09 (1H, s, *meso* H), 10.14 (1H, s, *meso* H), 10.56 (1/2H, s, *meso* H), 10.57 (1/2H, s, *meso* H), 10.64 (1H, s, *meso* H); MS *m/z* 1509 (M + 1)$^+$; UV/visible (CH_2Cl_2) 285, 402, 480, 500 sh., 624 nm. MS: *m/z* 1661 (M + 1)$^+$. The more polar faction was chromatographed using dichloromethane/5–8% acetone as solvent, resulting in 70 mg (38%) of dyad **3** as a mixture of stereo and regioisomers. ^1H NMR (500 MHz, $CDCl_3$), δ (ppm): −3.75 (2H, brs, -NH), 1.03–1.05 (6H, s, Car 16-CH_3, Car 17-CH_3), 1.46–1.52 (2H, m, Car 2-CH_2), 1.61–1.64 (2H, m, Car 3-CH_2), 1.72 (3H, s, Car 18-CH_3), 1.97–2.03 (14 H, m, Car 4-CH_2, Car 19-CH_3, Car 20-CH_3, Car 19′-CH_3, Car 20′-CH_3), 2.22 (3/4H, d, J = 6.5 Hz 2 -OCHCH_3 or 4 -OCHCH_3), 2.23 (3/4H, d, J = 5.5 Hz 2 -OCHCH_3 or 4 -OCHCH_3), 2.26 (3/4H, d, J = 6.5 Hz 2 -OCHCH_3 or 4 -OCHCH_3), 2.28 (3/4H, d, J = 6.6 Hz 2 -OCHCH_3 or 4 -OCHCH_3), 2.47–2.49 (3H, m, 2 -OCHCH_3 or 4 -OCHCH_3), 3.26–3.32 (4H, m, $CH_2CH_2CO_2CH_3$), 3.60–3.82 (18H, m, CO_2CH_3, β CH_3 × 4), 4.36–4.42 (4H, m, $CH_2CH_2CO_2CH_3$), 6.11–6.99 (15H, m, Car=CH-, 2 -OCHCH_3 or 4 -OCHCH_3), 7.50 (2H, d, J = 6.0 Hz, Car 1′-Ar, Car 5′-Ar), 7.75 (1H, q, J = 7.0 Hz 2 Car-OCHCH_3 or 4 Car-OCHCH_3), 8.26–8.30 (2H, m, Car 2′-Ar, Car 4′-Ar), 10.05 (1H, s, *meso* Hs), 10.12 (1H, s, *meso* H), 10.44 (1/4H, s, *meso* H), 10.46 (1/4H, s, *meso* H), 10.51 (1/4H, s, *meso* H), 10.53 (1/4H, s, *meso* H), 10.55 (1/2H, s, *meso* H), 10.58 (1/2H, s, *meso* H); MS: *m/z* 1144 (M+1)$^+$; UV/visible (CH_2Cl_2) 314, 400, 484, 568, 624 nm.

Dicarotenohematoporphyrin dimethyl ester **5**. To a 100 mL round-bottomed flask equipped with a stirring bar and a three-way adapter was added 183 mg (0.399 mmol) of 6′-apo-β-caroten-6′-oic acid (10), 30 mL of dichloromethane and 46 μL (0.415 mmol) of *N*-methylmorpholine. The mixture was stirred vigorously for several minutes under an N_2 atmosphere and then 73 mg (0.415 mmol) of 2-chloro-4,6-dimethoxy-1,3,5-triazine was added. The solution was stirred at room temperature under N_2 for 4 h. The TLC indicated formation of an orange product of intermediate polarity. At this point, **1** (0.100 g, 0.160 mmol) was added to the reaction mixture followed by 46 μL (0.415 mmol) of *N*-methylmorpholine and 51 mg (0.418 mmol) of DMAP. The mixture was stirred at room temperature under N_2 for 24 h. The crude reaction mixture was washed

with a dilute aqueous solution of citric acid, then with aqueous sodium bicarbonate. The organic layer was dried over sodium sulfate and filtered, and the filtrate was concentrated. Column chromatography with dichloromethane/1–3% acetone yielded 155 mg (64%) of dicarotenohematoporphyrin dimethyl ester **5** as a mixture of stereoisomers. ^{1}H-NMR δ (ppm): −3.69 (2H, brs, -NH), 1.03 (12H, s, Car 16-CH_3, Car 17-CH_3), 1.45–1.48 (4H, m, Car 2-CH_2), 1.57–1.68 (4H, m, Car 3-CH_2), 1.71 (6H, s, Car 18-CH_3), 1.95–2.04 (28 H, m, Car 4-CH_2, Car 19-CH_3, Car 20-CH_3, Car 19′-CH_3, Car 20′-CH_3), 2.37–2.41 (6H, m, 2 -OCHCH_3, 4 -OCHCH_3), 3.27–3.33 (4H, m, $CH_2CH_2CO_2CH_3$), 3.65 (6H, s, CO_2CH_3), 3.67 (3H, s, β CH_3), 3.69 (3H, s, β CH_3), 3.79 (3H, s, β CH_3), 3.84 (3H, s, β CH_3), 4.42 (4H, m, $CH_2CH_2CO_2CH_3$), 6.10–6.72 (26H, m, Car=CH-), 7.57–7.67 (4H, m, Car=CH-, 2 -OCHCH_3, 4 -OCHCH_3), 10.08 (1H, s, *meso* H), 10.13 (1H, s, *meso* H), 10.47 (1/2H, s, *meso* H), 10.48 (1/2H, s, *meso* H), 10.56 (1H, s, *meso* H); MS *m/z* 1509 $(M + 1)^+$; UV/visible (CH_2Cl_2) 285, 402, 480, 500 sh., 624 nm.

Dicarotenohematoporphyrin diacid **6**. To a 50 mL round-bottomed flask was added 20 mg (0.013 mmol) of dicarotenohematoporphyrin dimethyl ester **5** and 20 mL of tetrahydrofuran (THF) freshly distilled from $LiAlH_4$. Once the solid had dissolved, 0.4 mL of 10% of aqueous KOH was added. The solution was stirred at room temperature under an atmosphere of N_2 for 20 h. After this time TLC indicated that a negligible amount of starting material remained. The reaction mixture was poured into water and the pH was adjusted to 6–7 with citric acid. The dicarotenoporphyrin was then extracted with several fractions of chloroform/10–15% methanol. The combined fractions were washed with water and the solvent was evaporated to give 20 mg (quantitative yield) of **6**. ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$), δ (ppm): 1.04 (12H, s, Car 16-CH_3, Car 17-CH_3), 1.49 (4H, m, Car 2-CH_2), 1.62 (4H, m, Car 3-CH_2), 1.72 (6H, s, Car 18-CH_3), 1.95–2.03 (28 H, m, Car 4-CH_2, Car 19-CH_3, Car 20-CH_3, Car 19′-CH_3, Car 20′-CH_3), 3.20–3.32 (4H, m, $CH_2CH_2CO_2H$), 2.39–2.38 (6H, m, 2 -OCHCH_3, 4 -OCHCH_3), 3.63 (3H, s, β CH_3), 3.69 (3H, s, β CH_3), 3.79 (3H, s, β CH_3), 3.85 (3H, s, β CH_3), 4.35–4.47 (4H, m, $CH_2CH_2CO_2H$), 6.14–6.74 (26H, m, Car=CH-), 7.59–7.64 (4H, m, Car=CH-, 2 -OCHCH_3, 4 -OCHCH_3), 10.13 (1H, s, *meso* H), 10.20 (1H, s, *meso* H), 10.48 (1H, s, *meso* H), 10.56 (1H, s, *meso* H); MS: *m/z* 1481 $(M + 1)^+$; UV/visible (CH_2Cl_2) 286, 402, 476, 494 sh., 624 nm.

Spectroscopic studies

Steady-state fluorescence and fluorescence excitation spectra were measured using a SPEX Fluorolog-2 fluorometer. Transient fluorescence decays were measured using a time-correlated single-photon counting system. The excitation beam was generated by a frequency-doubled, mode-locked Coherent Antares Nd:Yag laser coupled to a synchronously pumped, cavity-dumped dye laser. Rhodamine 6G dye was used and 580 or 590 nm output was selected. Detection was performed by a Hamamatsu microchannel plate photomultiplier. The instrumental response function was ~35 ps. Multiexponential fits and global data analysis were performed using custom-designed software (11).

Nanosecond transient absorption measurements were performed using a previously described spectrometer (12). The excitation beam was generated by an OPOTEK OPO pumped by the third harmonic (355 nm) of a Continuum Surelite Nd:YAG laser. The laser pulse width was ~5 ns.

Time-resolved $O_2(^1\Delta_g)$ luminescence measurements were made using the laser system of the transient absorption apparatus. The excitation wavelength varied, and the data were acquired at a 5 Hz repetition rate. The luminescence was detected by a cryogenic germanium diode detector (North Coast Instruments model EO-817PP) through an interference filter with the transmission maximum at 1270 nm. The analog signal from the diode was amplified, digitized with a LeCroy transient recorder and analyzed using a computer.

Liposome preparation

Liposomes were prepared using a standard reverse-phase evaporation technique. Synthetic dioleoyl phosphatidylcholine and L-α-phosphatidylserine (brain, sodium salt, Avanti Polar Lipids) in dichloromethane were mixed in a 3:2 ratio (5 mg total combined weight) and dried under a stream of N_2. The HP derivatives were dissolved in 1 mL of chloroform and were added to the flask containing the lipids. To this mixture 0.75 mL of diethyl ether and 1.0 mL of KCl (50 m*M*) were added. This solution was sonicated for 5 min in a bath sonicator and then the volatile solvents were evaporated under vacuum (rotatory evaporator) for a total of 30 min. The resulting liposome solution was passed through a Sephadex G-100 column to remove any micelles or aggregated forms of HP derivatives and/or lipids.

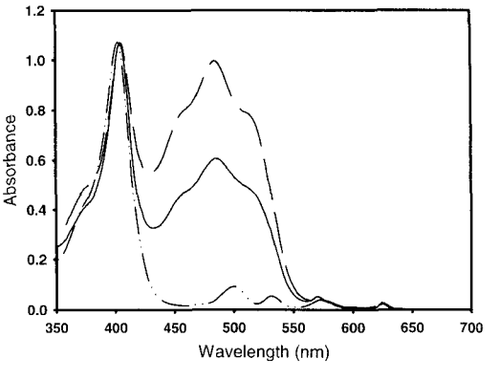


Figure 2. Absorption spectra of **1** (— · · —), **3** (—) and **4** (— — —). All spectra were taken in toluene. The spectra have been normalized at the peak of the Soret band (~403 nm).

RESULTS

Synthesis

Triads **4** and **5** and dyad **3** were prepared by covalently attaching **1** through an ester linkage to a carotenoid acid. The esterification reaction was carried out with **1** and either the corresponding carotenoid acid chloride or the coupling reagent 2-chloro-4,6-dimethoxy-1,3,5-triazine and the carotenoid acid. Triad **6** was obtained by controlled basic hydrolysis of **5**. The commercially available HP from which **1** was prepared is obtained by hydration of hemin and consists of a mixture of stereoisomers. Thus, the dyad and triads of this study are also isomeric mixtures. The diastereomeric relationship of some of the isomers in these mixtures is demonstrated by their ^{1}H NMR spectra (*e.g.* see *meso* protons of **4** and **5**). The mixture in the case of **3** includes regioisomers as well as stereoisomers.

Absorption spectra

Figure 2 shows the absorption spectra of **1**, **3** and **4**. The amplitudes have been normalized at the Soret band maximum (403 nm). The spectrum of **1** has bands at 403, 500, 533, 570 and 625 nm that are characteristic of free base porphyrins. Compounds **3** and **4** also exhibit the carotene absorption with a maximum at 482 nm. The HP Q_y bands located at 568 and 623 nm are visible in **3** and **4**, while the Q_x bands are obscured by the strong carotenoid bands. The carotenoid absorption maximum of **5** is shifted to 475 nm due to the absence of the phenyl group in the carotenoid moiety, but the spectrum is otherwise similar to that of **4**.

The multichromophoric species **3–6** have absorption spectra that are essentially a linear combination of those of the model porphyrin and model carotenoid(s). This is an indication that the electronic interaction between the chromophores is small.

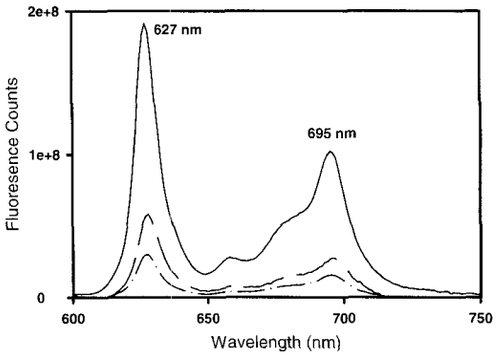


Figure 3. Fluorescence spectra of compound **1** (—), **3** (— — —) and **4** (— · —), with excitation at 580 nm. All compounds were dissolved in toluene and were not purged of oxygen.

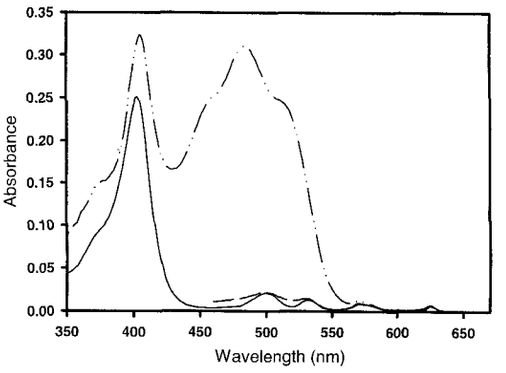


Figure 4. The corrected excitation spectrum of **4** (— — —), along with the ground-state absorption spectra of **1** (—) and **4** (— · · —). The solvent was toluene and all traces were normalized in the region of 625 nm where the carotenoid absorbance is negligible.

Steady-state fluorescence

The steady-state fluorescence spectra of model compound **1**, dyad **3** and triad **4** with excitation at 580 nm are shown in Fig. 3. These spectra were taken at equal absorbance at the excitation wavelength and show the quenching of fluorescence in dyad **3** and triad **4** with respect to **1**. Steady-state fluorescence quantum yields were measured using free-base tetraphenylporphyrin (Φ_f = 0.11) as a standard (13). The fluorescence quantum yield for **1** is calculated to be 0.09, which is consistent with published values (14). The fluorescence quantum yield of dyad **3** is 0.033 and triads **4** and **5** have quantum yields of 0.016 and 0.007, respectively. The magnitude of fluorescence quenching is consistent with previous studies of free base porphyrin–carotene dyads (7).

Figure 4 presents a corrected excitation spectrum of **4** that has been normalized to the absorption spectrum of **1** in the 625 nm region, where the carotenoid does not have appreciable absorption. It is readily apparent that there is no detectable singlet–singlet energy transfer from the carotene to the porphyrin, because the excitation spectrum has comparable intensity to the underlying HP absorption spectrum. The lack of singlet energy transfer from the carotenoid to the porphyrin is characteristic of all the carotenohematoporphyrins of this study.

Fluorescence lifetimes

The fluorescence quenching was further investigated by time-resolved fluorescence studies using the single-photon counting method. Solutions (~1 × 10^{-5} *M*) of dyad **3** and triads **4** and **5** were excited with 9 ps laser pulses at 590 nm, and the fluorescence was measured as a function of time at nine wavelengths in the 600–710 nm region. The decays were analyzed globally as a sum of exponential components. The lifetimes and amplitudes at the peak of fluorescence are shown for each compound in a series of solvents in Table 1. As an example, Fig. 5 shows a global fit of the fluorescence decay of **5** in hexane (χ^2 = 1.08). The two significant decay components have lifetimes of 4.4 ns and 1.6 ns. A third component with a lifetime of 0.001 ns, corresponding to solvent Raman scattering, was required in all the fits and is not included in Table 1 or Fig. 5.

As shown in Table 1, two significant components were present in each of the fluorescence decays of **3**, **4** and **5**. Typically, previously studied carotenoporphyrins exhibit fluorescence decays that can be fitted with a single fluorescence lifetime. In order to investigate a possible explanation for the double exponential decay observed for **3**, **4** and **5**, a kinetic model (15,16) was used in which an equilibrium between the excited singlet state of the porphyrin (C–^{1}P) and a possible porphyrin–carotene charge-separated state ($C^{\bullet+}$–$P^{\bullet-}$) was established. Because the energy of $C^{\bullet+}$–$P^{\bullet-}$ and therefore the equilibrium constant for the electron transfer process should be sensitive to solvent polarity, fluorescence decays were measured in solvents with different dielectric constants. Acetonitrile and cyclohexane or *n*-hexane were used as polar and nonpolar solvents, respectively, representing a change in dielectric constant of ~20-fold. The forward (k_{cs}) and reverse (k_{-cs}) electron transfer rate constants and the rate constant for charge recombination to the ground state (k_{cr}) were determined. From k_{cs} and k_{-cs} the thermodynamic driving force for photoinduced electron transfer (ΔG°_{cs}) between C–^{1}P and $C^{\bullet+}$–$P^{\bullet-}$ was calculated. In the case of **3** and **4**, k_{cs} doubled when going from nonpolar to polar solvent. However, k_{-cs} was found to increase for **4** and decrease for **3** for the same change of solvents. From these results a small increase in driving force, ~40 mV, was estimated when going from nonpolar to polar solvent in the case of **3**, while the opposite effect (~40 mV less driving force) was estimated when going from nonpolar to polar solvent in the case of **4**.

Nanosecond transient absorption spectroscopy

Compound **1** exhibits a transient absorption with a maximum at 440 nm, which corresponds to the porphyrin triplet state. The lifetime of this transient is 65 μs in a toluene solution that has been purged of oxygen by argon, and the triplet quantum yield (Φ_T) is 0.72 (14). In the case of **3**, **4** and **5**, the porphyrin triplet state is not observed. Within 10 ns, a new transient with a maximum absorbance at 560 nm is observed. This is the carotenoid triplet species. Thus, the porphyrin triplet is quenched within the time resolution of the spectrometer by triplet energy transfer to the carotenoid. Figure 6 shows the decay of the carotenoid triplet absorption of **3** and **4** detected at 560 nm following excitation at 590 nm. The quantum yield of triplet energy transfer is unity and

Table 1. Transient fluorescence data for **3**, **4** and **5** (τ_1, τ_2) and χ^2 for each global fit*

Compound	Solvent	ϵ	τ_1 (ns)	A_1	τ_2 (ns)	A_2	χ^2
3	Cyclohexane	2.0	6.3	0.81.	1.3	0.19	1.04
	Toluene	2.4	6.1	0.63	2.6	0.37	1.04
	Methylene chloride	9.1	4.8	0.56	1.8	0.44	1.08
	Acetonitrile	37.5	4.9	0.60	1.8	0.40	1.05
4	Decalin	2.0	5.9	0.47	2.3	0.53	1.06
	n-Hexane	1.9	5.1	0.32	2.8	0.68	1.08
	Toluene	2.4	4.6	0.35	2.2	0.65	1.07
	Methylene chloride	9.1	3.7	0.25	1.6	0.75	1.06
	Acetonitrile	37.5	4.8	0.24	1.6	0.76	1.08
5	*n*-Hexane	1.9	4.4	0.83	1.6	0.18	1.08
	Toluene	2.4	4.3	0.15	1.2	0.85	1.11

*The solvent is indicated for each set of data. All solutions were $\sim 1 \times 10^{-5}$ *M*. ϵ is the dielectric constant of the solvents.

the carotene triplet has a lifetime of 5 μs. The lifetime and transient absorption maximum are both characteristic of the carotenoid triplet (7). The lifetime of this signal is significantly quenched upon saturation of the solution with oxygen.

Triplet energy transfer studies have also been carried out in polystyrene films and in methyl-THF glass at 77 K to determine the extent of triplet energy transfer in solid media. In methyl-THF glass at 77 K, the triplet state of the porphyrin in **1** has a lifetime of ~2.5 ms with an absorbance maximum at 440 nm. This is characteristic of the porphyrin triplet and consistent with previous studies of other porphyrins in glasses (17). No porphyrin triplet signal could be detected in methyl-THF at 77 K in the polychromophoric compounds **3** and **4** and the decay of the carotenoid triplet signal (λ_{max} = 570 nm) was detected. The carotenoid triplet signal of compounds **3** and **4** have lifetimes of 9 μs and 13 μs, respectively. Thus, triplet energy transfer from the porphyrin to the carotenoid is rapid and efficient even in these solid matrices. The rise time of the carotenoid triplet signal in each medium is faster than the resolution of the spectrometer.

The porphyrin triplet state of compound **1** has a lifetime of ~260 μs and an absorption maximum at 450 nm in a polystyrene film in an argon-purged cell. Under similar conditions, compound **3** exhibits a signal with a lifetime of 4.0 μs and a maximum at 560 nm, and the equivalent signal in **4** has a lifetime of 3.5 μs and a maximum at 570 nm. Thus, triplet energy transfer from the porphyrin to the carotenoid occurs in polystyrene films as well. Figure 6 shows the decay of the carotenoid triplet state of **4** in the film.

Singlet oxygen sensitization

Because of the rapid transfer of triplet energy from the porphyrin to the carotenoid, it was expected that $O_2(^1\Delta_g)$ sensitization would not occur in the carotenohematoporphyrins. Figure 7 presents the decay of $O_2(^1\Delta_g)$ emission at 1270 nm after irradiation with a laser pulse of ~5 ns at 625 nm of samples of **1** and **5** in sealed cells saturated with O_2. Compound **1** has a quantum yield of $O_2(^1\Delta_g)$ sensitization of 0.5 (18) and the $O_2(^1\Delta_g)$ generated has a lifetime of 25 μs in toluene, in agreement with previously reported value (19). Compounds **3**, **4** and **5** do not generate any detectable $O_2(^1\Delta_g)$ under these conditions.

Photophysics in liposomes

In order to model the photophysics of these imaging dyes in tissues, they were inserted into lipid vesicle membranes and studied spectroscopically.

The Soret absorption bands of all the compounds in li-

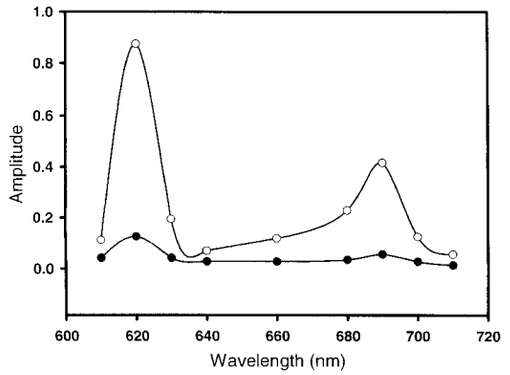


Figure 5. Decay-associated fluorescence emission spectrum obtained by exciting a $\sim 1 \times 10^{-5}$ *M* solution of triad **5** in hexane with 590 nm, ~9 ps laser pulses. Global analysis of the data at the indicated wavelengths gave two decay components with time constants of 4.4 (○) and 1.6 (●) ns.

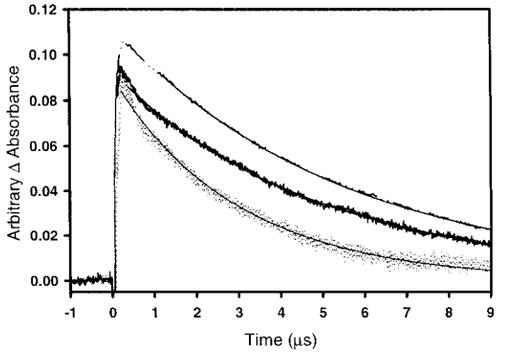


Figure 6. Carotenoid triplet decay for **3** (— · · —) and **4** (—) in argon-purged toluene; sample concentrations were $\sim 10^{-5}$ *M*; single exponential fit (—); lifetime 5 μs. Carotenoid triplet decay for **4** in a polystyrene film (· · · · · ·) exposed to air; single exponential fit (—); lifetime 3.5 μs. Excitation was at 430 nm and detection was at 570 nm.

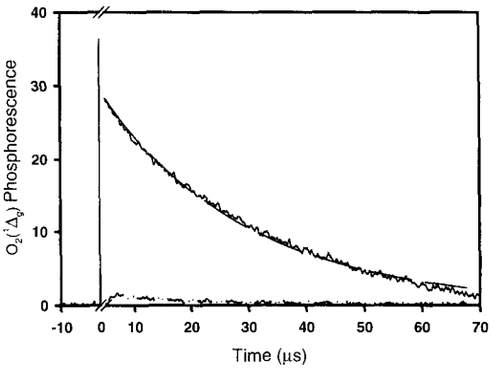


Figure 7. $O_2(^1\Delta_g)$ phosphorescence decay for solutions of **1** (—) and **5** (— · · —) at 1270 nm following a ~5 ns 625 nm laser pulse. Samples were dissolved in toluene, enclosed in a cuvette and saturated with oxygen.

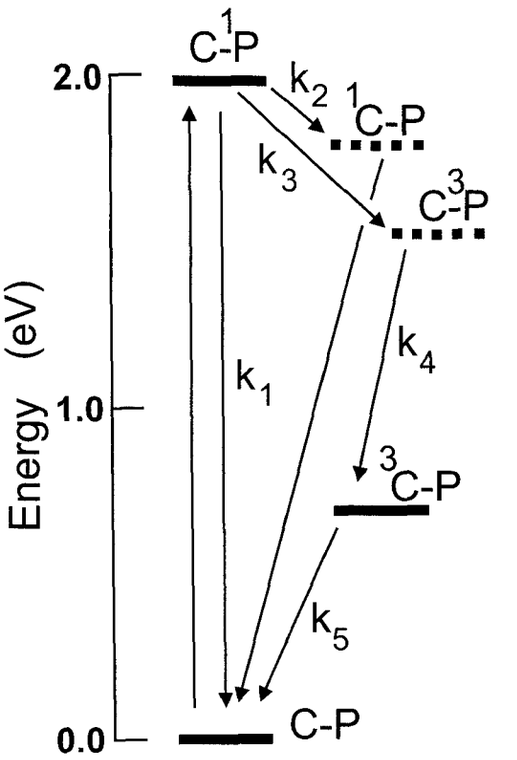
posomes are broadened. The characteristic carotene absorption is visible with some loss of definition. Line broadening and loss of definition of the spectra of these dyads and triads are a sign of aggregation. Triplet–triplet energy transfer studies and fluorescence measurements were designed to investigate further the aggregation phenomenon of these dyes in liposomes.

Liposome samples of **3**, **4** and **5** were prepared, purged with argon and excited at 430 nm, and the transient triplet absorption signal of each was measured. Compound **3** has a detectable triplet decay signal with a 6 μs lifetime and a maximum of absorbance at 560 nm, which is typical of the carotenoid triplet species. Quantitative measurements of triplet quantum yields were hindered by the variable turbidity of the liposome solution in the 430 nm region, which makes the fraction of incident light absorbed by the chromophore difficult to determine accurately.

Compounds **4** and **5** do not have any detectable triplet signal in the 550–600 nm or 440–470 nm regions when inserted into liposomes. These samples do have a transient absorption from ~700 nm to 950 nm with a lifetime of 50 ns. This signal does not correspond to any known transient absorption of carotenoids. The transient absorption of the carotenoid cation radical for a carotenoid of this length occurs at considerably longer wavelengths (7). Moreover, the addition of the reducing agent sodium dithionite, which is known to quench the carotenoid cation radical, does not quench the observed signal.

To investigate further the nature of this signal, compound **5** was allowed to form aggregates in methanol and transient absorption measurements were carried out. A solution of **5** in a mixture of methanol/dichloromethane (3:1) was placed under a stream of argon and the dichloromethane was partially evaporated, whereupon the solution turned cloudy. With this suspension a transient signal with absorption between 700 and 950 nm was observed with a lifetime of 50 ns. Addition of dichloromethane restored a clear solution and the transient absorption disappeared. Therefore, it appears that **4** and **5** are indeed aggregated within the liposomal membrane and that the observed signal corresponds to an excited state of an aggregate.

Compounds **3**, **4** and **5** all retain some of their fluorescence after insertion into lipid vesicles. The steady-state



Scheme 1. High energy states of dyads and triads and their decay pathways. The dashed lines indicate estimated energy levels.

maxima are located at the same wavelengths as are found in the solution studies (627 and 695 nm). However, the major component of the transient fluorescence is quenched to ~100–150 ps for all three dyes. A sample of **5** in an aggregated form in methanol exhibited a multicomponent fluorescence decay with a principal component of 300 ps.

Samples of **3** and **5** in liposomes were used to determine if $O_2(^1\Delta_g)$ could be generated by the compounds when aggregated in liposomes. Following irradiation with a ~5 ns laser pulse at 625 nm, no 1270 nm emission was detected for either compound. Although the 5 μs lifetime of $O_2(^1\Delta_g)$ in aqueous solution is near the limit of the time resolution of the flash spectrometer, it is possible to detect $O_2(^1\Delta_g)$ on this time scale and to make qualitative estimations. Compound **5** was allowed to aggregate in methanol and was tested for $O_2(^1\Delta_g)$ sensitization. The lifetime of $O_2(^1\Delta_g)$ in methanol is 10 μs, which is readily detectable on our system. However, no $O_2(^1\Delta_g)$ was detected in this aggregate, which is in agreement with the data for the triads in liposomes.

DISCUSSION

Scheme 1 shows the pathways of relaxation of the excited states of dyad **3** and triads **4** –**6**. The lowest excited singlet state of the porphyrin moiety ($C-^1P$) can be prepared by direct excitation of this chromophore. The fluorescence excitation spectrum shown in Fig. 4 does not show significant intensity over the absorption of the carotenoid moiety (maximum at ~480 nm). Thus, singlet energy transfer from the carotenoid to the porphyrin does not occur to a measurable extent in any of these systems.

The fluorescence of the dyad and triads **3–6** is considerably quenched in comparison to that of reference porphyrins **1** or **2**. Although the mechanism of quenching of the fluorescence of porphyrins by carotenoids has been actively investigated, questions about the details of this process still remain (20). If the relevant carotenoid S_1 level is lower in energy than that of the porphyrin S_1 state, energy transfer from the porphyrin to the carotenoid could occur. Until recently this mechanism was considered energetically improbable because the S_1 state of an 11 double-bond carotenoid was thought to be above the S_1 state of most porphyrins. However, recent estimations locate the S_1 state of β-carotene and related carotenoids at ~700 nm (20,21). The S_1 state of the HP of this study, taken as midway between the maximum of the redmost absorption band and the maximum of the fluorescence band, is at 625 nm. Thus, from the standpoint of energetics singlet–singlet energy transfer from the HP to the carotenoid is a possible mechanism for the quenching of the porphyrin fluorescence observed in **3–6**.

Quenching of the porphyrin fluorescence by electron transfer from the carotenoid to the porphyrin S_1 state is another possible mechanism. The resulting charge-transfer state $C^{\bullet+}P^{\bullet-}$ would have a characteristic transient absorption spectrum but could be difficult to detect due to its extremely short lifetime. In a related bichromophoric system, in which a carotenoid was linked to a porphyrin modified to be an unusually good electron acceptor, such a transient species has been reported and electron transfer established as the quenching mechanism (22). Also, in related systems the fluorescence quenching of the porphyrin was significantly greater in polar solvents such as acetonitrile than in less polar solvents such as toluene (22,23). This observation can be interpreted as an indication of the formation of charge-separated states such as $C^{\bullet+}$–$P^{\bullet-}$ from C–P^1.

In the present study, two lifetimes were necessary to fit the transient fluorescence data, but neither lifetime showed a marked dependence on solvent polarity. A kinetic model in which the two-exponential decay results from equilibrium between C–P^1 and $C^{\bullet+}$–$P^{\bullet-}$ (15,16) was used to analyze the data and to extract k_{cs}, k_{-cs} and k_{cr}. While k_{cs} doubled when going from a nonpolar to a polar solvent in both **3** and **4**, the effect is not significant when the 20-fold difference in dielectric constants of the solvent is taken into account. Moreover, even though ΔG^{o}_{cs} becomes more negative (by ~40 mV) when going from a nonpolar to a polar solvent in the case of **3**, it shows exactly the opposite trend in the case of **4**. Because this model yields unreasonable results, it does not seem likely that there is an equilibrium between C–^{1}P and a charge-separated state. It is also unlikely that the two components are due to aggregation of the compound in solution. The addition of 10% methanol to a methylene chloride solution of the compounds, which usually disrupts aggregates, had little effect on the amplitude and lifetime of the two decay components. Also, it is unlikely that aggregation of the compounds would be similar across a broad range of solvents. The most likely possibility for the origin of the two lifetime components is the presence of two diastereomeric or conformationally distinct forms of the compounds.

The triplet state of the porphyrin (^{3}P) generated by pathway 3 in Scheme 1 is readily evident in the case of porphyrins **1** and **2**. However, ^{3}P is not observed in any of the carotenoporphyrins, because pathway 4 leads rapidly to the formation of ^{3}C–P. Efficient triplet–triplet energy transfer from the porphyrin to the carotenoid was also observed in solid matrixes such as polystyrene films or in methyl-THF at 77 K. This was a rather unexpected result in view of previous studies (17,24) and the following considerations. Triplet–triplet energy transfer primarily occurs by an electron-exchange mechanism that requires orbital overlap of the donor and the acceptor. When spacers such as methylene groups have been interposed between the porphyrin and the carotenoid of previously studied dyads, rapid energy transfer has been observed in solution at room temperature. However, in a rigid plastic matrix at room temperature, or in an organic glass at 77 K, the energy transfer process ceased and only the transient absorption of ^{3}P at 450 nm could be detected. An explanation for the different behaviors of these dyads in rigid media and in fluid solutions is that the most stable conformation of the molecule in solution has the carotenoid extended out, away from the porphyrin and insulated from it by the linkage bonds. In rigid media the extended conformation with poor orbital overlap predominates and triplet–triplet energy transfer is not observed. In fluid solution, on the other hand, relatively unrestricted intramolecular motions around the flexible linkage between the chromophores bring the two π-electron systems into momentary contact, allowing the triplet–triplet energy transfer to take place.

Triplet–triplet energy transfer in rigid media, as observed with the carotenoporphyrins of the present study, requires electronic coupling between the carotenoid and the porphyrin through orbital overlap that is not a function of intramolecular motions. Thus, either the methyne carbon and ether oxygen that separate the chromophores are not acting as effective insulators or a static through-space contact between the π systems is possible. The latter possibility was investigated by molecular mechanics calculations that show that the most stable conformations of **3–6** have the carbonyl ester at ~2.8–2.9 Å from the nearest pyrrole carbon. Because the carbonyl function is in conjugation with the carotenoid π-system, a through-space orbital overlap of the carotenoid and the porphyrin π-systems is provided by this Van der Waals contact. Therefore, fast triplet–triplet energy transfer can occur in both fluid solution and rigid media.

In studies of other carotenoporphyrin dyads, a through-space-electronic coupling has been observed and the conformational proximity of both chromophores has been demonstrated by the presence of large upfield chemical shifts in the ^{1}H NMR spectra of the carotenoid protons (25). The ^{1}H NMR spectra of **3–6** do not exhibit unusual chemical shifts of the carotenoid protons. This observation agrees with the molecular mechanics studies that show that the lowest-energy conformations are L shaped with the phenyl carotenoid protons at a distance of ~5 Å from the nearest pyrrole carbon.

Both dynamic and thermodynamic parameters of the carotenoporphyrin triplet states combine to prevent the sensitization of $O_2(^1\Delta_g)$. The kinetic limitation arises from the extremely rapid intramolecular triplet–triplet energy transfer to yield the triplet carotenoid, which is much faster than the diffusion-limited energy transfer from the HP triplet to O_2

to yield $O_2(^1\Delta_g)$. The resulting triplet carotenoid species is significantly below $O_2(^1\Delta_g)$ in energy and therefore the energy transfer process between ^3C–P and O_2 to generate $O_2(^1\Delta_g)$ is thermodynamically forbidden (26).

CONCLUSIONS

The combined photophysical properties of the carotenohematoporphyrins of this study clearly demonstrate their potential as imaging agents of neoplastic tissue. The dyads and triads are significantly fluorescent in all of the solvents tested as well as in liposomes, which are models of the cellular environment. The molecular architecture of the linkage between the porphyrin and the carotenoid allows a though-space orbital overlap of the π-systems with the concomitant result of rapid triplet–triplet energy transfer from the porphyrin to the carotenoid moiety, which is independent of the temperature and fluidity of the media. The carotenoid triplet species is the lowest electronically excited state of the carotenohematoporphyrins; it decays harmlessly to the ground state. Singlet oxygen is not formed, suggesting that these compounds will not be phototoxic in animal tissue.

Specific accumulation of related carotenoporphyrins in tumor tissue has been observed. Pharmacokinetic studies of some of the carotenohematoporphyrins described in the present study in parallel with the extensively tested HP should answer the question of the role of the carotenoid in the specific delivery of these drugs to neoplastic tissue.

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