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Carboxylate modified benzylidene cyclopentanone dyes for one- and two-photon excited photodynamic therapy

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ABSTRACT

Benzylidene cyclopentanone dyes have high photosensitization activity and large two-photon absorption (TPA) cross-section in near infrared region. To explore their application potentials in two-photon excited photodynamic therapy (TPE-PDT), a series of carboxylate modified benzylidene cyclopentanone dyes were synthesized by varying number of carboxylate groups. Their linear and nonlinear photophysical properties, reactive oxygen yields and *in vitro* PDT activities were investigated systematically. The results showed that the water solubility of these dyes was significantly enhanced after introducing carboxylate groups. All dyes exhibited large TPA cross-section around 1000–1400 GM at 840 nm. In addition, they could generate both superoxide anion radical and singlet oxygen species under illumination. However, only the dye **Y1** modified by one carboxylate group presented strong PDT activity to human rectal cancer 1116 cells, which indicated that moderate water–lipid amphipathy was a very important factor for PDT dyes. Finally, **Y1** was proved to have large potential in TPE-PDT by *in vitro* experiments.

1. Introduction

Two-photon excited photodynamic therapy (TPE-PDT) is a novel phototherapy technology [1–3], which attracts much attention in recent years due to its advantages of deep biological tissue penetration and highly selective targeting damage. Compared to conventional PDT [4–6], in which photosensitizing drugs generate cytotoxic reactive oxygen species (ROS), such as superoxide anion radical ($O_2^{-\bullet}$) or singlet oxygen ($^1O_2$), through absorbing one photon under visible illumination, TPE-PDT achieves photocytotoxicity through absorbing two photons of long wavelength simultaneously. Therefore a large two-photon absorption cross-section (σ) is an important prerequisite for TPE-PDT photosensitizers.

Conventional clinical PDT drugs were used in early TPE-PDT studies. However, their σ values were too small to be suitable clinically, for example, the σ value of Photofrin was only 7.4 GM [3] at 850 nm and the σ value of Verteprofin was 51 GM [7] at 900 nm ($1\,GM = 10^{-50}\,cm^4\,s\,molecule^{-1}\,photon^{-1}$). In the last decade, a great number of compounds with large σ values were reported. However, only a few of them could be used as photosensitizer directly for TPE-PDT [8]. The main reason is most of reported compounds are hydrophobic, having no biocompatibility. In fact, water–lipid amphiphilic photosensitizers are more photodynamically active than hydrophobic or hydrophilic ones because hydrophility is necessary when photosensitizers transport in the aqueous biological fluids (such as blood, intercellular substance or cytoplasm) and lipophility is required when photosensitizers diffuse into phospholipid membranes or cellular organs. Thus a combination with hydrophilic and lipophilic properties is important for a PDT photosensitizer [9,10].

Recently various strategies have been employed to increase biocompatibility of photosensitizers with large two-photon absorption (TPA) cross-section [11–16]. Among them, modifications of hydrophobic photosensitizers with water-soluble polyethylene glycol or carboxylate groups have been proven to be efficient. However, till now, most of these studies have been focused on porphyrin derivatives [17]. The reason for this preference should be due to the successful application of hematoporphyrin derivatives as clinical PDT drugs in last decades together with natural porphyins frequently occurring in living matter. Less attention has been paid on other photosensitizers. Usually, the specificity of a photosensitizer on cancer tissues is considered a prerequisite for PDT application. Till now, most of PDT studies show that the selective tumor uptake of a sensitizer is probably not mainly due to the special properties of the sensitizer, but rather to differences in the physiologies between tumors and normal tissues [18,19]. The special retention of a sensitizer in the malignant tissue is a consequence of different kinetics of the sensitizer removal from malignant and healthy tissues. The removal from healthy tissue is faster. The concentration

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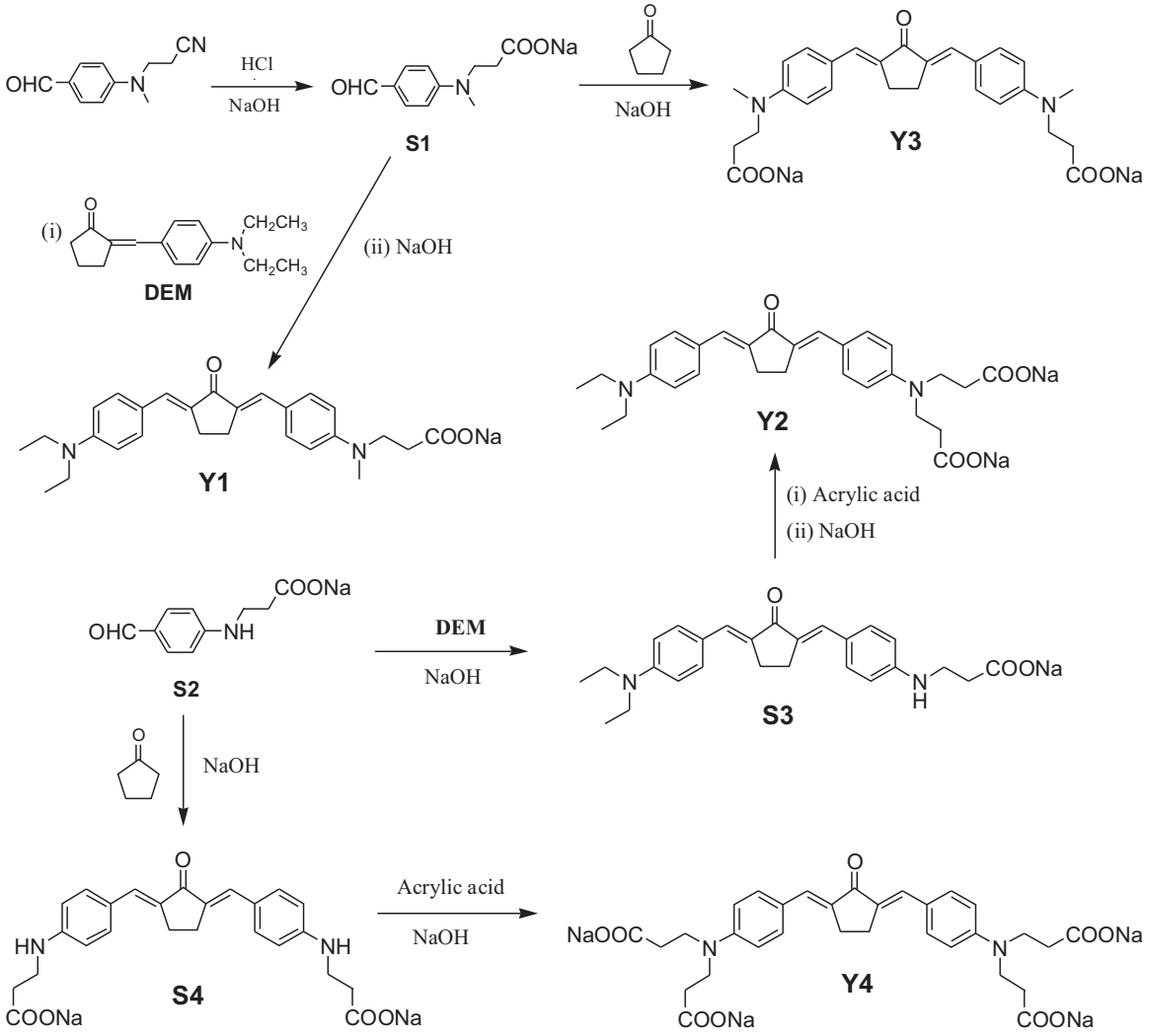

Scheme 1.

difference depends on the nature of the sensitizer. This result brings more vitality for exploring new photosensitizers. In our previous work, benzylidene cyclopentanone dyes were found having large σ values (400–3300 GM around 800 nm, which are comparatively high based on their simple molecular structures) and high photosensitizing efficiencies in two-photon polymerization [20–22], indicating they could have potential in TPE-PDT. However, these dyes were all hydrophobic. In this work, four carboxylate modified benzylidene cyclopentanone dyes **Y1**–**Y4** were synthesized (Scheme 1). Their solubility, photophysical properties and PDT activities were investigated systematically.

2. Experimental

2.1. Materials

5,5-Dimethyl-1-pyrroline-N-oxide (DMPO), 2,2,6,6-tetramethyl-4-piperidone (TEMP) and 9,10-dimethylanthracene (DMA) were purchased from Sigma–Aldrich Chemical Company. Cyclopentanone was purchased from Tianjin Jinke Institute of Fine Chemical. 3-((4-Formylphenyl)-(methyl)-amino)-propanenitrile was obtained from Wenzhou Longsheng Chemical Co. Ltd. Dulbecco's modified Eagle's medium (DMEM, low glucose, containing 100 unit/mL penicillin, 100 mg/mL streptomycin) and phosphate buffered saline (PBS, pH = 7.4) were purchased from Beijing Solarbio Science & Technology Co. Ltd. Cell counting Kit-8 (CCK-8) was from Beyotime Institute of Biotechnology. Fetal bovine serum (FBS) was obtained from Hangzhou Sijiqing Co. Ltd. Other A.R. grade reagents were all from Beijing Beihua Co. Ltd., and used after purification by common methods. Sodium 3-(4-formylphenylamino)-propanoate (**S2**) and 2-(4-(diethylamino)-benzylidene)-cyclopentanone (**DEA**) were prepared according to literature [23,24]. Human rectal cancer 1116 cells (HRC-1116 cells, from Department of Laser Medicine, Chinese PLA General Hospital) were grown in culture medium of DMEM supplemented with 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells were suspended at a concentration of 1×10^5 cells/mL.

2.1.1. Sodium 3-[(4-formylphenyl) (methyl)-amino]-propanoate (*S1*)

Sodium hydroxide (140 mmol, 5.6 g) and 3-((4-formylphenyl)-(methyl)-amino)-propanenitrile (47.8 mmol, 9 g) were dissolved in 200 ml water. The reaction mixture was refluxed for 4 h, then cooled and filtrated. A dilute HCl solution was added dropwise into the filtrate under stirring. Precipitate was collected, washed with water (3×150 mL), and neutralized with a dilute NaOH solution. The neutralized solution was dried under vacuum to afford 10.5 g of yellow solid, yield 96%. ^{1}H NMR (400 MHz, D_2O) δ (ppm): 2.44 (t, J = 7.16 Hz, 2H), 3.03 (s, 3H), 3.71 (t, J = 7.36 Hz, 2H), 6.81 (d, J = 9.04 Hz, 2H), 7.72 (d, J = 9.08 Hz, 2H), 9.43(s, 1H).

*2.1.2. 2-[4-[(2-Sodiumcarboxylate-ethyl)(methyl)-amino]-benzyliden]-5-[4-(diethylamino)-benzyliden]-cyclopentanone (**Y1**)*

DEA (11.2 mmol, 2.72 g) and **S1** (10 mmol, 2.28 g) were mixed in methanol (70 mL) with 0.09 g NaOH as catalyst. The reaction mixture was refluxed under stirring for 24 h. After cooling, the solvent was removed by rotary evaporation. The residue was dissolved in water and neutralized with a dilute HCl solution. Precipitate was collected, washed with water (3 × 50 mL), and further purified by column chromatography on silica gel ($V_{CHCl_3} : V_{MeOH} = 100 : 3$). The eluted product was neutralized with dilute NaOH solution, and dried under vacuum to afford red solid **Y1** (1.73 g, yield 40%). ^{1}H NMR (400 MHz, DMSO-d6) δ (ppm): 1.12 (t, J = 7.04 Hz, 6H), 2.07 (t, J = 7.36 Hz, 2H), 2.95 (s, 3H), 2.99 (s, 4H), 3.4 (q, J = 7 Hz, 4H), 3.54 (t, J = 7.12 Hz, 2H), 6.74 (d, J = 8.92 Hz, 4H), 7.27 (s, 2H), 7.48 (dd, J_1 = 6.52 Hz, J_2 = 2.52 Hz, 4H). HRMS-ESI: m/z Calcd. for $C_{27}H_{33}N_2O_3$ $[M-Na^+ + 2H^+]^+$: 433.2491, found: 433.2477.

*2.1.3. 2-[4-[Bis-(2-sodiumcarboxylate-ethyl)-amino]-benzyliden]-5-[4-(diethylamino)-benzyliden]-cyclopentanone (**Y2**)*

DEA (5.7 mmol, 1.4 g) and **S2** (5 mmol, 1.07 g) were dissolved in ethanol (32 mL) and heated to reflux under nitrogen. 0.05 g NaOH was added as catalyst. After 6 h of reflux, the reaction mixture was cooled and filtrated. The solid was collected and washed with ethanol (3 × 30 mL). Intermediate **S3** was obtained with a yield of 86%. ^{1}H NMR (400 MHz, D_2O) δ (ppm): 0.93 (t, J = 7.04 Hz, 6H), 2.36 (t, J = 7.12 Hz, 2H), 2.43 (s, 4H), 3.03 (q, J = 6.96 Hz, 4H), 3.22 (t, J = 7.04 Hz, 2H), 6.39 (d, J = 8.32 Hz, 2H), 6.56 (d, J = 8.36 Hz, 2H), 7.09 (d, J = 8.6 Hz, 2H), 7.17 (t, J = 8.24 Hz, 4H).

S3 (4.3 mmol, 1.9 g) was dissolved in hydrochloric acid (1 mol/L, 7 mL). 34 mL acrylic acid was added. The reaction mixture was stirred at 80 °C for 8.5 h under nitrogen, then cooled and filtrated. The filtrate was added into water (200 mL) and stirred at 5 °C for 3 h. Precipitate was collected, washed with water (30 mL). The product was neutralized with dilute NaOH solution, dried to afford 0.72 g of dark red solid (yield 32%). ^{1}H NMR (400 MHz, D_2O) δ (ppm): 1.08 (t, J = 7.12 Hz, 6H), 2.47 (t, J = 7.64 Hz, 4H), 2.57 (s, 4H), 3.22 (q, J = 6.96 Hz, 4H), 3.59(t, J = 7.72 Hz, 4H), 6.21 (d, J = 8.92 Hz, 2H), 6.77 (d, J = 8.88 Hz, 2H), 7.17 (s, 2H), 7.32 (d, J = 8.96 Hz, 2H), 7.41 (d, J = 8.88 Hz, 2H). HRMS-ESI: m/z Calcd. for $C_{29}H_{35}N_2O_5$ $[M-2Na^+ + 3H^+]^+$: 491.2546, found: 491.2546.

*2.1.4. 2,5-Bis-[4-[(2-sodiumcarboxylate-ethyl)(methyl)-amino]-benzylidene]-cyclopentanone (**Y3**)*

S1 (40 mmol, 9.172 g) and cyclopentanone (20 mmol, 1.68 g) were mixed in water (80 mL). 0.3 g NaOH was added as catalyst. After 6 h of reflux, the reaction mixture was dispersed into ethanol (600 mL) and stirred at 5 °C for 3 h. Precipitate was collected, washed with ethanol (50 mL), and dried to afford 2.7 g of dark red solid (yield 27%). ^{1}H NMR (400 MHz, D_2O) δ (ppm): 2.31 (t, J = 7.52 Hz, 4H), 2.43 (s, 4H), 2.73 (s, 6H), 3.45 (t, J = 7.68 Hz, 4H), 6.57 (d, J = 8.84 Hz, 4H), 7.09 (s, 2H), 7.25 (d, J = 8.8 Hz, 4H). HRMS-ESI: m/z Calcd. for $C_{27}H_{31}N_2O_5$ $[M-2Na^+ + 3H^+]^+$: 463.2233, found: 463.2225.

*2.1.5. 2, 5-Bis-[4-[bis-(2-sodiumcarboxylate-ethyl)-amino]-benzylidene]-cyclopentanone (**Y4**)*

S4 and **Y4** were synthesized by similar procedure described above. **S4** (yield 40.6%). ^{1}H NMR (400 MHz, D_2O) δ (ppm): 7.27 (d, J = 8.4 Hz, 4H), 7.12 (s, 2H), 6.57 (d, J = 8.4 Hz, 4H), 3.18 (t, J = 7.0 Hz, 4H), 2.50 (s, 4H), 2.38 (t, J = 7.0 Hz, 4H). HRMS-ESI: m/z Calcd. for $[M-2Na+3H]^+$: 435.1925, found: 435.1915. **Y4** (yield 78.4%). ^{1}H NMR (400 MHz, D_2O) δ (ppm): 7.46 (d, J = 8.8 Hz, 4H), 7.18 (s, 2H), 6.74 (d, J = 8.8 Hz, 4H), 3.54 (t, J = 7.4 Hz, 8H), 2.76 (s, 4H), 2.43 (t, J = 7.4 Hz, 8H); HRMS-ESI: m/z Calcd. for $C_{31}H_{35}N_2O_9$ $[M-4Na+5H]^+$: 579.2347, found: 579.2337.

2.2. Method

UV–Vis absorption spectra were measured on a Hitachi U-3900 spectrophotometer with a concentration of 10^{-5} mol/L. One-photon fluorescence spectra were performed on a Hitachi F-4500 fluorescence spectrophotometer with a concentration of 10^{-5} mol/L. ^{1}H NMR spectra were obtained on Bruker DPX400 spectrometer. Mass spectra were carried out on Bruker Apex IV FTMS. Fluorescence quantum yields (Φ_f) were measured in dilute solutions using Rhodamine 6G in ethanol as a standard (Φ_f = 0.95) [25].

Electron paramagnetic resonance (EPR) spectra were monitored on Bruker ESP-300E. DMPO and TEMP were employed as trapping agents for superoxide anion radical and singlet oxygen, respectively. Samples were dissolved in oxygen-saturated dimethyl sulfoxide (DMSO) with a concentration of 10^{-4} mol/L for **Y1**–**Y3** and 2×10^{-5} mol/L for **Y4**. After the addition of spin-trapping reagent, the oxygen-saturated solution was irradiated with a 532 nm laser. Quantum yield of singlet oxygen (Φ_Δ) was measured by monitoring the time-resolved laser photolysis experiment [26]. A 532 nm diode laser was adopted as light source. 9,10-Dimethylanthracene (DMA) was used as trapping agent for singlet oxygen, and Rose Bengal was used as a standard (Φ_Δ = 0.76 in methanol, 0.162 in DMSO, 1.0 in octanol) [27].

TPA cross-section (σ) values of four compounds in methanol solution (2×10^{-4} mol/L) were determined using the two-photon excited fluorescence (TPEF) technique with femtosecond laser pulses. The excitation light source used was a mode-locked Tsunami Ti:sapphire laser (720–880 nm, 80 MHz, <130 fs). Rhodamine B in methanol solution (10^{-4} mol/L) was used as Ref. [28].

Lipid–water partition coefficient (PC) which showed relative solubility in octanol versus PBS was obtained by the stir-flask method [29]. Photosensitizers were diluted to a concentration of 20 μmol/L in 5 mL PBS. 5 mL n-octanol was added. The mixture of the two solvents were stirred gently for 24 h, then centrifuged at 4000 rpm for 10 min to separate the phases. The lipid–water PC values were calculated by the ratio of photosensitizer concentration in n-octanol to that in PBS solution.

Before *in vitro* experiments, **Y1**–**Y4** were dissolved in PBS to make a series of concentration. In dark cytotoxicity test, flat-bottom 96-well plates were divided into two groups: experimental group (E) and reference group (R). Experimental group was seeded with 100 μL HRC-1116 cell suspension per well, while reference group was filled with 100 μL culture medium. One well was kept as blank in each group. After 24 h incubation, 10 μL PBS was added into blank wells (B). The rest of experimental wells and reference wells (X) were injected with 10 μL PBS solutions of dyes with proper concentration to make the final concentrations of dyes are 2, 5, 10, 25, 50 μg/mL.

Cell viability was measured using CCK-8 assay [30]. After 4 h incubation with dyes, 10 μL CCK-8 was added into each experimental well and 10 μL PBS was added into each reference well. Cells were continuously incubated for another 2 h. The absorbance of each well at 450 nm was determined using a microplate reader. Cell viability was calculated by the following equation:

$$\text{Cell viability}\,(\%) = \left(\frac{A_{EX} - A_{RX}}{A_{EB} - A_{RB}}\right) \times 100\%$$

Here A stands for absorbance; subscription E and R represents experimental and reference group; X is well with a given concentration; B is blank well.

In photocytotoxicity test (one-photon PDT activity experiment), the same operation is performed except that the final concentrations of dyes are 0.25, 0.5, 1, 2, 5 μg/mL. Moreover, after 4 h of

incubation with dyes, cells were irradiated with a 532 nm diode laser (20 mW/cm^2) for 20 min. Then they were continuously cultured in dark for another 24 h before cell viability was monitored using CCK-8 assay.

In two-photon induced cytotoxicity experiment, HRC-1116 cells were incubated with 2 μg/mL dye solution for 4 h, followed by irradiation under a mode-locked femtosecond Ti:sapphire laser (Tsunami Ti:sapphire, 800 nm, 80 MHz, 80 fs) through an objective (10×, NA = 0.4, Olympus). The sample was fixed on a xyz-step motorized stage controlled by a computer. Before illumination, the focused laser point was adjusted to the middle of a targeted cell. The illumination intensity was 16 mW. Blank group was observed under the same condition in the absence of photosensitizer.

3. Results and discussion

3.1. Synthetic strategy

Scheme 1 provides the synthesis route of target dyes **Y1**–**Y4**. Sodium carboxylate groups were introduced to the aryl moieties of a 2,5-bis[4-(diarylamino)-benzylidene]-cyclopentanone chromophore to improve its hydrophility by base-catalyzed condensation of carboxylate modified aromatic aldehydes **S1**–**S2** with cyclopentanone or **DEA**. **S1** was synthesized by hydrolysis reaction of the nitrile group in 3-((4-formylphenyl)-(methyl)-amino)-propanenitrile with very high yield (96%). **S2** was obtained by addition reaction of 4-aminobenzaldehyde with acrylic acid [23]. Due to the strong electron-withdrawing ability of aldehyde group at the para-position of amino group in 4-aminobenzaldehyde, the Michael addition of 4-aminobenzaldehyde with acrylic acid gave only the nomo carboxylate adduct **S2**. When the condensation reaction of **S2** with cyclopentanone or **DEA** finished, the nucleophilicity of nitrogen in **S3** and **S4** increased. Thus, the further Michael addition reaction of **S3** or **S4** with acrylic acid provided target compound **Y2** and **Y4**.

3.2. Linear photophysical properties

The UV–Vis absorption spectra as well as emission spectra of dye **Y1** in THF, DMSO, methanol and PBS are shown in Fig. 1. The spectra of other three dyes **Y2**–**Y4** are quite similar to that of **Y1**. Due to the small difference of the electron donating abilities of their terminal substitutes, only small variations in the absorption and emission maxima of four dyes can be found. The linear photophysical properties of four dyes are all listed in Table 1. **Y4** is not soluble in THF, so no data is available. Moreover, the molar extinction coefficients of **Y2**–**Y3** in THF are also unavailable because their solubility in THF is very poor. The red-shift of the maximum absorption band and the enlarged Stokes shift ($\Delta\nu$) with increasing solvent polarity (from THF to DMSO) confirm the absorption is due to a π–π^* transition and the emission is mainly from an intermolecular charge transfer state [31,32]. From DMSO (a polar aprotic solvent) to methanol (a less polar but protic solvent compared to DMSO), a further red-shift of the absorption band and a large increase of Stokes shift should be due to the hydrogen bond interaction between dyes and methanol. In addition, the sharp decreases of singlet oxygen and fluorescence quantum yields in methanol compared to data in DMSO are also relative to the hydrogen bond interaction. The further decrease of fluorescence quantum yield in PBS versus in methanol is in agreement with solvent-polarity-induced nonradiative decay [33]. However, the solution viscosity shows an opposite effect. Octanol which possesses relative high viscosity sharply reduces radiationless transition [34], leading to exceptional high singlet oxygen and fluorescence quantum yields of **Y1**. It is a pity that other three dyes cannot be dissolved in octanol, no singlet oxygen and fluorescence quantum yields could be obtained for these dyes in this solvent. Because octanol is a lipid-like solvent, the action of dyes in cells could be more similar to that of dyes in octanol compared to other solvents.

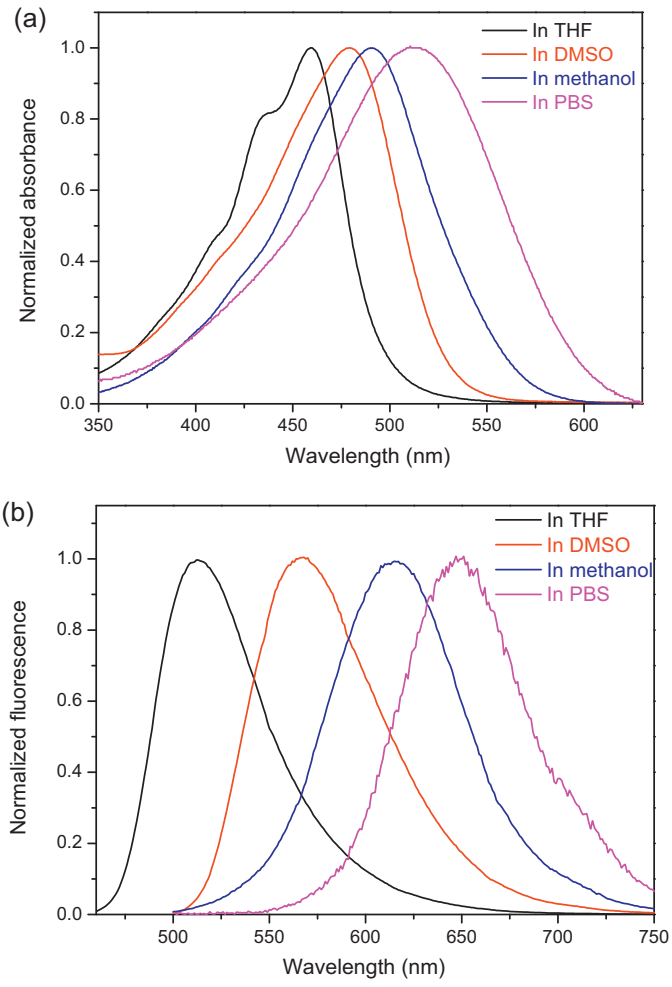

Fig. 1. Normalized absorption and emission spectra of **Y1** in different media.

3.3. Photosensitization activities

PDT is based on the dye-sensitized photooxidation of biological matter in the target tissue. There is much evidence to suggest that reactive oxygen species (ROS) are the major damaging species in PDT [19]. As typical ROS, superoxide anion radical ($O_2^{-\bullet}$) and singlet oxygen ($^1O_2$) are generated through Type I and Type II mechanisms [35,36] respectively. Both of them are based on excited triplet state of photosensitizer. The generation of ROS can be monitored by EPR spectra [37]. A typical EPR signal (inset in Fig. 2a) of DMPO–$O_2^{-\bullet}$ adduct with hyperfine couple constants (α_N = 12.7 G, α_β^H = 10.3 G, α_γ^H = 1.3 G) was trapped in DMSO solutions of four dyes plus DMPO as $O_2^{-\bullet}$ trapping reagent under irradiation with a 532 nm laser. Using TEMP as $^1O_2$ trapping reagent, the EPR spectrum (inset in Fig. 2b) of the oxidation product TEMPO with triplet peaks in equal intensity (α_N = 15.9 G) was also observed in DMSO solutions of four dyes under identical irradiation.

The time dependent of EPR signal intensity are illustrated in Fig. 2. Due to the poor solubility of **Y4** in DMSO, its concentration (2×10^{-5} mol/L) is only one fifth of the concentration (10^{-4} mol/L) of other three dyes, leading to weak EPR signal intensity. For **Y1**–**Y3**, considering their different extinction coefficient at 532 nm

Table 1
Linear and nonlinear photophysical properties of **Y1**–**Y4** in different media.

	Solvent	λ_{max}^{abs} (nm)	λ_{max}^{fl} (nm)	ε_{max} (10^4 M^{-1} cm^{-1})	$\Delta\nu$ (cm^{-1})	Φ_f	Φ_Δ	σ_{max} (GM)
Y1	THF	460	513	6.22	2246			
	DMSO	479	565	7.48	3178	0.184	0.067	
	Octanol	487	575	7.42	3142	0.358	0.201	
	Methanol	491	614	6.23	4080	0.024	0.016	1026
	PBS	512	646	3.71	4051	0.002		
Y2	THF	461	511		2123			
	DMSO	479	565	8.99	3178	0.187	0.056	
	Methanol	492	617	7.41	4118	0.032	0.021	1401
	PBS	516	645	6.21	3876	0.003		
Y3	THF	454	511		2457			
	DMSO	473	561	6.26	3316	0.154	0.058	
	Methanol	488	613	5.28	4179	0.024	0.019	1053
	PBS	507	644	4.78	4196	0.002		
Y4	DMSO	473	558	6.94	3221	0.146	0.053	
	Methanol	489	613	4.89	4137	0.050	0.042	1167
	PBS	513	644	5.27	3965	0.005		

λ_{max}^{abs} is one-photon absorption band maximum; ε_{max} is molar extinction coefficient at λ_{max}^{abs}; λ_{max}^{fl} is fluorescence band maximum; Φ_f is fluorescence quantum yield; Φ_Δ is singlet oxygen quantum yield; σ_{max} is the maximum TPA cross section obtained within 720–870 nm.

(**Y2** > **Y1** > **Y3**), their photosensitizing efficiency are very similar. The results of EPR spectra demonstrate that all four dyes can effectively generate ROS through both Type I and Type II mechanisms. Although the $^1O_2$ quantum yields (Φ_Δ) of four dyes (listed in Table 1) are low in polar solvent such as DMSO and methanol, the Φ_Δ of **Y1** in lipid-like octanol as 0.201 is acceptable. Considering target points of PDT are normally lipoidic cellular membrane or cellular organs (such as mitochondria and lysosome), the Φ_Δ in octanol is more useful.

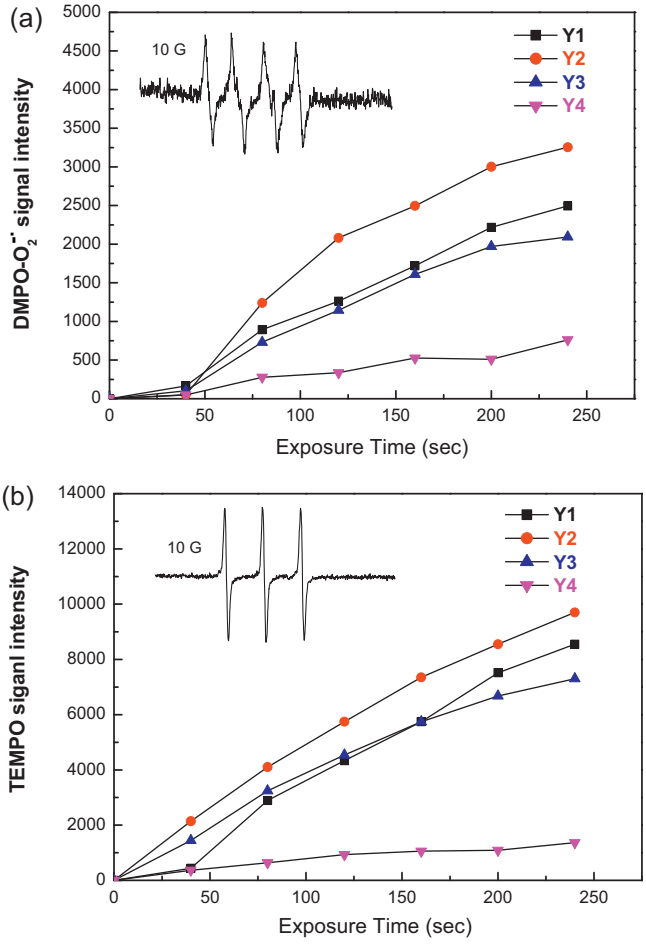

Fig. 2. EPR signal intensity of DMPO–$O_2^{-\bullet}$ adduct (a) and TEMPO (b) as a function of irradiation time (irradiated at 532 nm) for oxygen-saturated DMSO solution of dyes ($(1 \times 10^{-4}$ mol/L for **Y1**–**Y3** and 2×10^{-5} mol/L for **Y4**). Inset: the EPR spectra of DMPO–$O_2^{-\bullet}$ (a) and TEMPO (b).

3.4. Two-photon absorption cross section

The two-photon excitation spectra of **Y1**–**Y4** in methanol are showed in Fig. 3. Compared to their linear absorption peaks around 490 nm, the energy band of TPA is coincide in energy with the vibronic shoulder of the 1PA. This suggests that it is most probably vibronically enhanced **S0** → **S1** transition. Within the whole measured range (720–870 nm), the maximum σ (σ_{max}) of **Y1**–**Y4** are 1026 GM, 1401 GM, 1053 GM and 1167 GM, respectively. These data are quite comparable to that of 2,5-bis-[4-(diethylamino)-benzylidene]-cyclopentanone (1032 GM in N,N-dimethylformamide), indicating that the TPA property of benzylidene cyclopentanone core has little influenced after modification with terminal hydrophilic group. Among these four dyes, **Y2** shows the largest σ value due to the stronger electron-donating capability of ethyl than that of methyl or $-CH_2CH_2COONa$ group. Considering their high TPA cross sections, these four dyes are expected to have great application prospect on TPE-PDT.

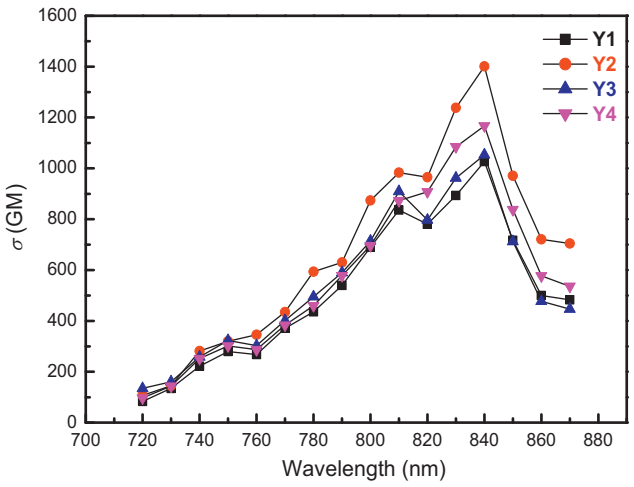

Fig. 3. Two-photon excitation spectra of **Y1**–**Y4** in methanol.

Table 2
Solubility in PBS and octanol–water partition coefficient (PC) of **Y1**–**Y4**.

Compound	Solubility in PBS (mg/mL)	log PC
Y1	0.8	2.92
Y2	>5	0.25
Y3	>5	−0.65
Y4	>5	−0.79

3.5. Evaluation of amphiphilicity

It is clinically necessary that photodynamic drug should be water–lipid amphiphilic in order to pass both hydrophilic and lipophilic barriers in biological system. Normally, it is evaluated by octanol–water partition coefficient (PC) [38]. In this work, different number of carboxylate group was introduced to balance the hydrophobicity of benzylidene cyclopentanone core. The results showed that the water-solubility of dyes could be sharply improved as increasing number of carboxylate group (Table 2). The solubility of **Y1** in PBS is 0.8 mg/mL, while that of **Y2**–**Y4** is over 5 mg/mL. However, the polarity of carboxylate is so strong that the modified target dye will lost its lipophilicity when more than one carboxylate is linked. **Y2**–**Y4** are almost insoluble in apolar solvents, whereas **Y1** with log PC of 2.92 still keeps good solubility in organic phase, behaving as a water–lipid amphiphilic molecule.

3.6. One-photon PDT effect

A wide range of dye's concentration was investigated in both dark cytotoxicity and photocytotoxicity tests. Dark cytotoxicity test was determined in darkness to exclude the influence of photodynamic effect. Therefore, cell viability in dark cytotoxicity test could be used to assess the toxicity of a dye indirectly. Photocytotoxicity test was carried out under irradiation of a 532 nm laser, so cell viability could evaluate the PDT effect of a dye. The results show (Fig. 4) that cell viability decreases with increasing dye's concentration. **Y2**–**Y4** are safely in dark even at concentration of 50 μg/mL (cell viability over 70%), while evident dark cytotoxicity of **Y1** appears at concentration of 25 μg/mL. The phototoxicity of **Y2**–**Y4** is limited, while **Y1** achieves very clear phototoxicity at concentration of

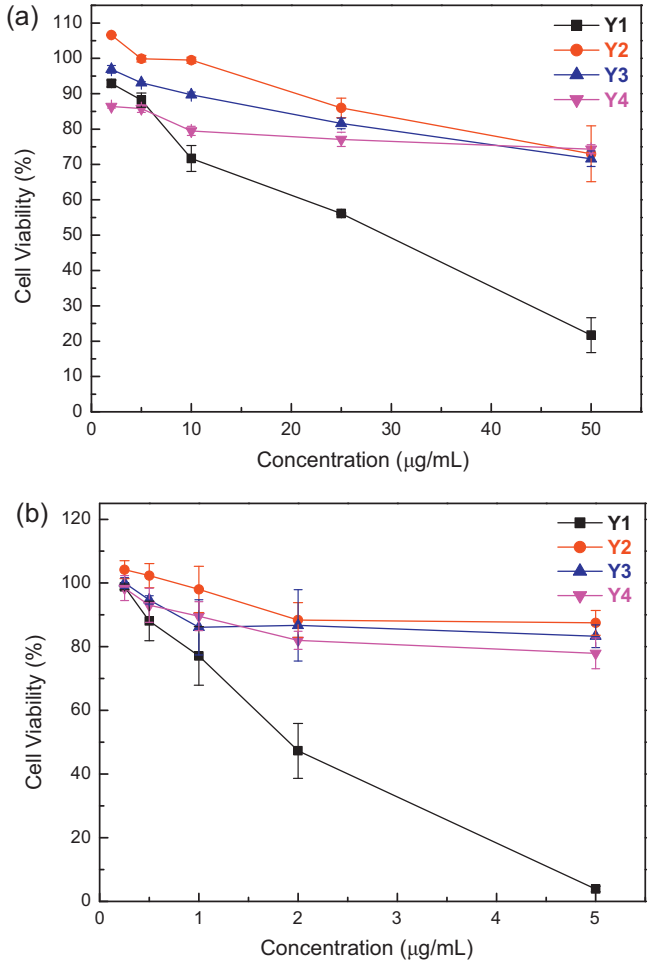

Fig. 4. Dark cytotoxicity (a) and photocytotoxicity (b) of **Y1**–**Y4** toward HEC-1116 cells using CCK-8 assay. The error bars denote standard deviation from three replicates. Dyes were dissolved in PBS before injected into culture medium.

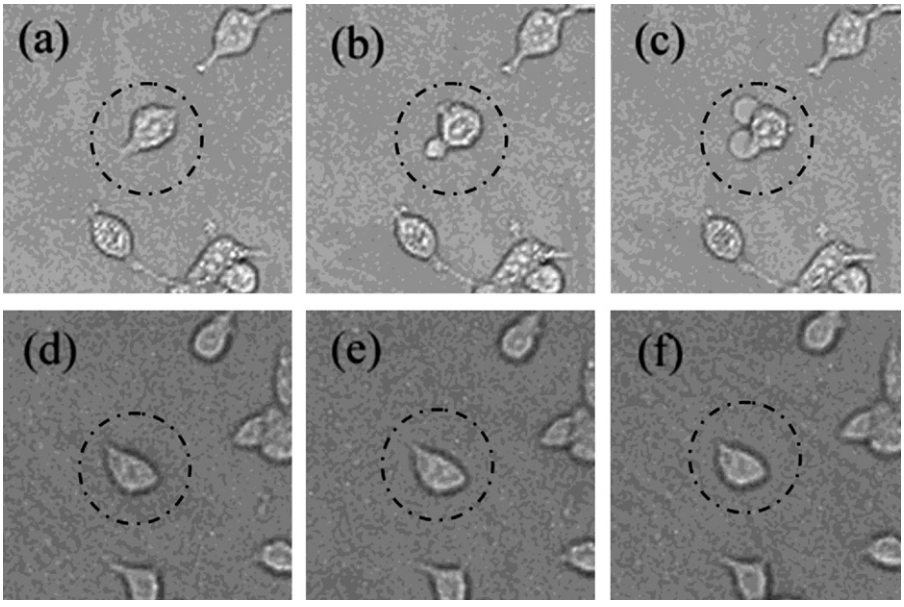

Fig. 5. Experimental group (a–c): Photographs of HRC-1116 cells incubated with 2 mg/mL PBS solution of **Y1** before irradiation (a); after 1 min irradiation (b); 10 min after irradiation (c). Blank group (d–f): Photographs of HRC-1116 cells before irradiation (d); after 10 min irradiation (e); 10 min after irradiation (f).

2 μg/mL. At concentration of 5 μg/mL, **Y1** presents only 3.9% cell viability under irradiation.

The strong PDT activity of **Y1** should be due to its proper water–lipid amphiphilicity because it is the only amphiphilic dye among four dyes. For their lack of lipophilicity, **Y2**–**Y4** cannot be efficiently absorbed by tumor cells. The result confirms the great importance of amphiphilicity for PDT dyes. Though the dynamic range of **Y1** is limited (2.5–25 μg/mL), it shows remarkable PDT efficiency compare to other three dyes. If the photosensitizer's dose is properly chosen (for example at 5 μg/mL), **Y1** is still a potential candidate for PDT.

3.7. Two-photon induced cytotoxic effect

A single cell in the circular region (Fig. 5) was exposed to a focused 800 nm laser beam. A targeted cell incubated with **Y1** in experimental group was irradiated for 1 min. The cellular edema formed soon after treatment (Fig. 5b) and became more visible 10 min later (Fig. 5c), indicating a clear photo-damage of a tumor cell. In addition, cells out of laser beam exhibited no change during the entire process. In blank group, a targeted cell which was treated without photosensitizers showed no significant change after 10 min irradiation (Fig. 5e and f). It was confirmed that both laser and photosensitizer were indispensable to exert a cytotoxic effect on tumor cells. The results prove that the **Y1** has great potential in TPE-PDT.

4. Conclusion

Through introducing carboxylate group, four water soluble benzylidene cyclopentanone dyes **Y1**–**Y4** were synthesized. Among them, only the dye **Y1** modified by one carboxylate group presented proper water–lipid amphopathy, other three dyes had good water solubility but lost lipophilicity. The results of EPR spectra demonstrated that all four dyes could effectively generate ROS through both Type I and Type II mechanisms. Their maximum σ were all above 1000 GM at 840 nm. However, only **Y1** showed strong one- and two-photon excited PDT activity to human rectal cancer 1116 cells. It was proven that introduction of carboxylate group was a successful way to improve biocompatability of benzylidene cyclopentanone dye but the modified degree must be controlled within a proper range to keep its water–lipid amphipathy, which was a very important factor to maintain the PDT activity of dyes.

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