ISSN 0006-2979, Biochemistry (Moscow), 2020, Vol. 85, No. 7, pp. 773-780. © Pleiades Publishing, Ltd., 2020.
Published in Russian in Biokhimiya, 2020, Vol. 85, No. 7, pp. 907-915.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM20-107, June 19, 2020.

Phytofluene as a Highly Efficient UVA Photosensitizer of Singlet Oxygen Generation

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Received April 29, 2020
Revised May 26, 2020
Accepted June 4, 2020

Abstract—Phytoene and phytofluene – uncolored C_{40} carotenoids with short chain of conjugated double bonds (3 and 5, respectively) – are known to be universal precursors in biosynthesis of colored carotenoids in photosynthesizing organisms. It is commonly recognized that C_{40} carotenoids are photoprotectors of cells and tissues. We have shown that phytofluene is an exception to this rule. By measuring photosensitized phosphorescence of singlet oxygen ($^1O_2$) we found out that phytofluene was very effective photosensitizer of $^1O_2$ formation in aerated solutions under UVA irradiation (quantum yield of 85 ± 5%), whereas phytoene was almost inactive in this process. It was demonstrated that both carotenoids quench singlet oxygen in the dark. The obtained quenching rate constants [$(4 \pm 1) \times 10^6$ $M^{-1}\cdot s^{-1}$ for phytoene and $(2 \pm 0.5) \times 10^7$ $M^{-1}\cdot s^{-1}$ for phytofluene] were smaller than the rate constant of the diffusion-controlled reactions by 3-4 orders of magnitude. Thus, both carotenoids displayed rather weak protector properties. Moreover, phytofluene due to its high photosensitizing activity might be considered as a promoter of cell photodamage and a promising UVA photosensitizer for medical purposes.

DOI: 10.1134/S0006297920070056

Keywords: phytofluene, phytoene, singlet oxygen, photosensitization, UVA

INTRODUCTION

Colored carotenoids with nine and more conjugated double bonds (CDBs) synthesized by green plants and photosynthetic bacteria are recognized as efficient protectors of living tissues against photooxidative stress. It has been suggested that protection is based on capabilities of carotenoids to efficiently deactivate the triplet states of chlorophylls and bacteriochlorophylls, quench singlet oxygen ($^1O_2$), and scavenge free radicals ([1, 2] and refs therein). On the contrary, visual pigment – retinal, which is polyene with short CDB chain (6 CDBs), is an efficient photosensitizer of $^1O_2$ formation in non-polar environment ([2, 3] and refs therein). This process is thought to cause photodynamic damage of retina and various eye diseases ([4] and refs therein). The C_{40} carotenoids with short CDB chains (3-7 CDBs) (phytoene, phytofluene, ζ-carotene) are known to be formed in photosynthetic bacteria, green plants, and algae as universal precursors in biosynthesis of colored carotenes and xanthophylls that have a long chain of CDBs [1]. Based on these facts, it was suggested that polyenes with short CDB chains might cause photodynamic damage of photosynthetic tissues [5]. In addition, it was recently found that the membranes of purple bacteria photosensitize oxygenation of bacteriochlorophyll B-850 and $^1O_2$ production under blue-green light, which is predominantly absorbed by carotenoids ([6-10] and refs therein). On the other hand, phytoene and phytofluene are present in commonly consumed foods and are among the predominant carotenoids in human plasma and tissues. Moreover, they were shown to

Abbreviations: CDB(s), conjugated double bond(s); $^1O_2$, singlet oxygen; UV, ultra violet; Φ_Δ, the quantum yield of $^1O_2$ generation.

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react with free radicals and therefore, were used as nutrients providing protection of skin and other tissues against oxidative stress and UV light ([11, 12] and refs therein). Nevertheless, to the best of our knowledge, systematic analysis of $^1O_2$ generation by C_{40} carotenoids – precursors in β-carotene biosynthesis – has never been carried out, although flash photolysis studies of the triplet states of certain compounds of this group have been reported [13, 14]. Recently, we observed in the preliminary test measurements that phytofluene was an exceptionally strong photosensitizer of $^1O_2$ formation, which exceeds by this ability all-trans retinal. This observation stimulated us to detailed investigation of $^1O_2$ generation by this carotenoid and its biosynthetic precursor phytoene using measurements of photosensitized phosphorescence of $^1O_2$ [15]. The obtained results are represented below.

MATERIALS AND METHODS

All-trans phytoene and phytofluene were obtained from cells of two sulfur photosynthetic bacteria *Ectothiorhodospira haloalkaliphila* (ATCC 51935^T strain) and *Allochromatium vinosum* (MSU^T strain). According to earlier nomenclature, this bacterium was named *Allochromatium minutissimum*. The cell culture of *E. haloalkaliphila* was obtained from Professor J. F. Imhoff (Helmholtz Center for Ocean Research, Germany), and the *A. vinosum* culture was provided by the Department of Microbiology, Lomonosov Moscow State University). Bacterial cells were grown anaerobically in the presence of 53 and 71 μM of diphenylamine (DPA) ("Reachim", Russia) under 90 W/m^2 white light on the Pfennig's and Larsen's media. The collected cells were disrupted by sonication using a UZG13-0.1-22 ultrasound generator ("Ultrasound technology", Russia). Intact cells and fragments of cell walls were removed by centrifugation in a K24 centrifuge ("Janetzki", Germany) for 10 min at 4500 rpm and 10,000 rpm for *E. haloalkaliphila* and *A. vinosum*, respectively [16-18]. Carotenoids were extracted from bacterial membranes using a mixture of acetone, methanol and petroleum ether and followed by purification using chromatography techniques. More details on the carotenoid isolation procedure were presented in [18].

Singlet oxygen was detected using measurement of its own infrared phosphorescence at 1270 nm, which arose due to the energy transfer from the triplet state of the photosensitizer molecules to oxygen followed by population of the excited singlet ($^1\Delta_g$) state of oxygen molecules. Measurements were carried out using a laser/LED spectrometer recently assembled at the A. N. Bach Institute of Biochemistry, Russian Academy of Sciences [19]. The spectrometer allowed phosphorescence detection upon excitation by continuous LEDs with the emission maxima at 281, 350, and 371.5 nm (AQMEPAN, China) and 399.5 nm (Polironic, Russia), and pulsed LED with the emission maximum at 405 nm (Alkom Medica, Russia). LED radiation was focused onto a 5 mm spot on the surface of a quartz cuvette with the test solution. Intensity of the excitation light was controlled by a ThorLabs PM-100D power meter with a S120VC sensor head (ThornLabs, USA). Phosphorescence of $^1O_2$ was recorded at a 90° angle by a cooled FEU-112 photomultiplier (Ekran Optical systems, Russia) with the S-1 spectral response through the cut-off filters that transmit IR light at $\lambda \geq 1000$ nm and one of three interchangeable interference filters with transmission maxima at 1230, 1270, and 1310 nm and half-width of 10 nm. The steady-state phosphorescence intensity was measured with a digital millivoltmeter (Econix-Expert, Russia).

For kinetic measurements, samples were irradiated with pulses of blue LED at 405 nm. Pulse duration and pulse repetition rate varied from 1 to 10 μs, and from 5 to 100 Hz, respectively [19]. The photomultiplier signal passed through a preamplifier and was directed to a USB board operating in the time-resolved photon-counting mode (Parsec, Russia). The time constant of the recording system was about 10 ns. The counting board, which was launched by an additional pulse of the control unit synchronously with the LED pulse, divided the time interval between flashes into 512 or 1024 channels. Kinetic curves were obtained by accumulating photomultiplier pulses in each channel. The quantum yields of $^1O_2$ generation by carotenoids were measured using an organic photosensitizer phenalenone (perinaphthenone, 1H_phenalen-1-one) (Merk, EU) as a reference, which had a wide absorption band with maximum at 350 nm and considerable absorbance at 405 nm. The quantum yield of $^1O_2$ photogeneration by phenalenone is known to be close to 1 (0.95 ± 0.05) [20, 21]. The experiments were performed using specially purified hexafluorobenzene (C_6F_6) and perflorohexane as solvents (Piminvest, Russia).

RESULTS

Figure 1 illustrates chemical structures of the carotenoids under study. It is seen that phytoene has 3 CDBs and phytofluene – 5 CDBs.

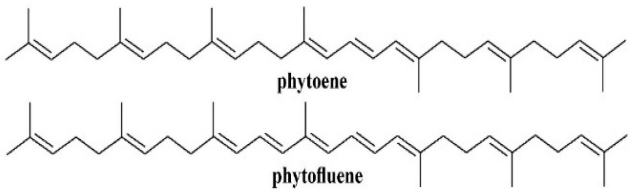

Fig. 1. Structural formulae of the studied carotenoids.

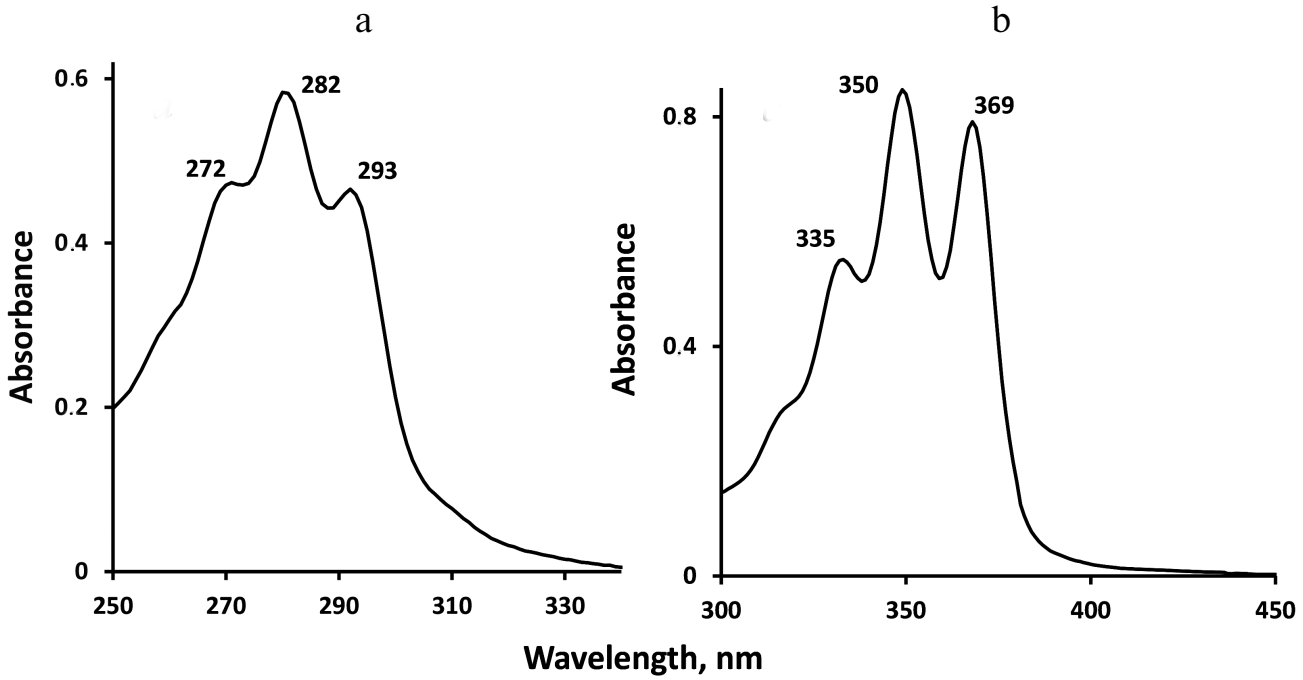

Fig. 2. Absorption spectra of phytoene in perfluorohexane (a) and phytofluene in hexafluorobenzene (b) (with absorbance of the solvents subtracted) in the 1 cm quartz cuvettes.

Absorption spectra of carotenoids are shown in Fig. 2. It is seen that the absorption maxima of phytoene are in the UV-B region between 250 and 310 nm, therefore for spectroscopic measurements phytoene was dissolved in perfluorohexane, which transmits light in this wavelength range. Absorption maxima of phytofluene are in the UV-A region between 320 and 400 nm. However, phytofluene is poorly soluble in perfluorohexane. Therefore, phytofluene was dissolved in hexafluorobenzene, which was transparent in the UVA region but did not transmit the UV-B light. That is why different solvents were used for spectroscopic analyses of the carotenoids. There were also other reasons of using hexafluorobenzene and perfluorohexane. Molecules of these solvents have no hydrogen atoms and, hence, weakly quench $^1O_2$. Therefore, the lifetimes (τ_Δ) and intensities of $^1O_2$ phosphorescence are several hundred times greater than in the organic solvents containing H-atoms, where the $^1O_2$ lifetimes usually vary in the range 10-300 μs ([19] and refs therein). The use of less expensive hydrogen-free solvents containing chlorine (CCl_4 and freon) appeared to be impossible for our studies because under irradiation in the chlorine-containing solvents rapid photodestruction of carotenoids occurred due to an efficient photochemical reaction. In fluoro-containing solvents such reaction was not observed. The following molar absorption coefficients (in $M^{-1}\cdot cm^{-1}$) were used for calculation of the pigment concentrations: phytoene, 50,000 (at 282 nm); phytofluene, 73,000 (at 350 nm) [22], and phenalenone 97,000 (at 351 nm) [20, 21].

Decays of photosensitized $^1O_2$ phosphorescence in perfluorohexane and hexafluorobenzene after excitation by short pulses of the violet LED (405 nm) are presented in Fig. 3. Phenalenone was employed as a photosensitizer. The decays were exponential with the lifetimes of 16 ms in hexafluorobenzene and 30 ms in perfluorohexane.

Both phytoene and phytofluene practically do not absorb light at the wavelength of pulsed LED (405 nm), therefore for excitation of these carotenoids cw LED radiation at 281 and 371 nm was employed. Under irradiation by these LEDs, phelanenone solutions emitted rather strong phosphorescence of $^1O_2$ with a maximum at 1270 nm in both solvents.

Figure 4 illustrates relative quantum yields of $^1O_2$ phosphorescence in solutions of phytoene and phenalenone. Phytoene was studied in perfluorohexane under excitation at 281 nm (3.5 mW). Very weak luminescence was observed at 1270 nm under these conditions in phytoene solutions. Phenalenone absorbance at 281 nm was low, therefore phenalenone was irradiated by the LED at 371 nm to obtain reliable results. Absorbance of both compounds was adjusted to 0.13 at the excitation wavelengths that corresponded to the concentrations of 2.6 μM for phytoene and 1.7 μM for phenalenone. After normalization to the number of incident photons and subtraction of the background luminescence signal emit-

ted by the solvents without photosensitizers, we established that under the applied conditions the intensity of the $^1O_2$ phosphorescence in the phytoene solutions was ≈100 times lower than in the phenalenone solution.

It is known that phytoene and phytofluene are able to moderate quenching of $^1O_2$, although accurate measurements of the rates of this process have not been reported [23]. Quenching activity of phenalenone is known to be very low ([19] and refs therein). In order to find out how quenching of $^1O_2$ by phytoene affects the relative yield of $^1O_2$ phosphorescence in phytoene solutions, we studied quantitatively the rate of $^1O_2$ quenching by phytoene. This was performed based on measuring the decay rates of the phenalenone-photosensitized $^1O_2$ phosphorescence upon addition of phytoene. Hexafluorobenzene was employed as a solvent, because both phytoene and phenalenone were reasonably soluble in hexafluorobenzene, whereas both carotenoids were poorly soluble in perfluorohexane. Figure 4b shows that quenching obeys the Stern–Volmer equation:

$$k_{obs} = k_o + k_q[Car], \quad (1)$$

where k_{obs} is the observed rate constant of phosphorescence decay (in s^{-1}) ($k_{obs} = 1/\tau_\Delta$, where τ_Δ is the $^1O_2$ lifetime) after LED pulses in solutions containing both photosensitizer and *Car*; k_0 is the rate constant of the phosphorescence decay in phenalenone solutions without *Car*; k_q is the bimolecular rate constant of $^1O_2$ quenching by Car; [*Car*] is the molar concentration of the carotenoid.

It follows from Fig. 4b that $k_q = (4 \pm 1) \times 10^6$ $M^{-1}\cdot s^{-1}$ for phytoene. This value agrees with the data reported by Mathews–Roth et al. indicating that $k_q < 10^7$ $M^{-1}\cdot s^{-1}$ [23]. Hence, phytoene is a rather poor quencher of $^1O_2$. It follows from the Stern–Volmer plot presented in Fig. 4b that phytoene at the concentration of 2.6 μM increases the decay rate of $^1O_2$ phosphorescence in hexafluorobenzene by a factor of 1.16 ($k_q[Car]/k_o$) = 0.16). By rewriting Eq. (1) using the steady-state phosphorescence intensities, the following equation is obtained:

$$I_o/I_q = 1 + (k_q/k_o)[Car], \quad (2)$$

where I_o and I_q are the steady-state intensities of phenalenone-photosensitized $^1O_2$ phosphorescence in the absence and in the presence of a phytoene, respectively. It can be estimated from this equation that in hexafluorobenzene, quenching by phytoene causes 16% reduction of the steady-state intensity of $^1O_2$ phosphorescence compared to the solution of phenalenone alone.

Taking into account that the $^1O_2$ lifetime in perfluorohexane is two-fold longer (or k_o is two-fold lower) than in hexafluorobenzene (Fig. 4), and k_q are equal in both solvents, one can calculate using Eq. (2) that quenching of $^1O_2$ by phytoene (2.6 μM) in perfluorohexane should

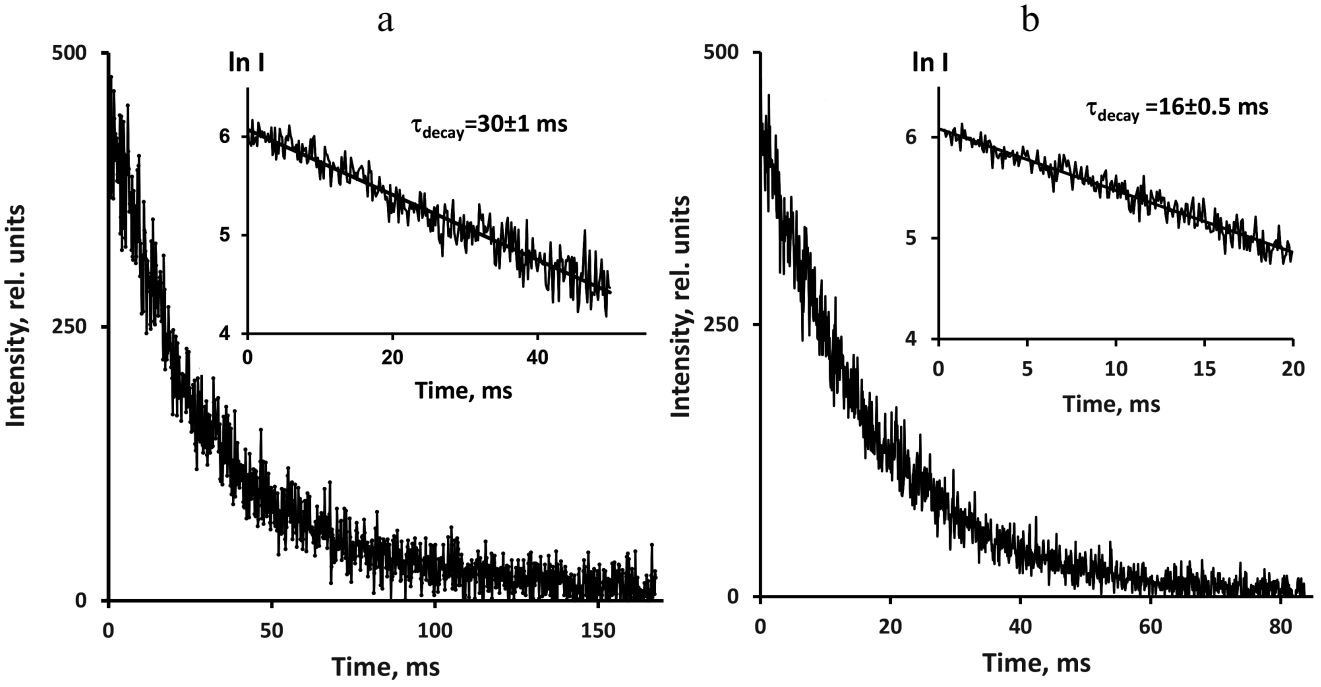

Fig. 3. Decays of $^1O_2$ phosphorescence in solutions of phenalenone (~3 μM) in perfuorohexane (a) and hexafluorobenzene (b) after irradiation with 10 μs LED pulses (405 nm) in the presence of air. The decays were obtained using a pulse repetition rate of 5 and 10 Hz after 30- and 15-min signal accumulation. Average excitation power was 30 and 60 μW, respectively. The insets show semilogarithmic plots of the decay curves.

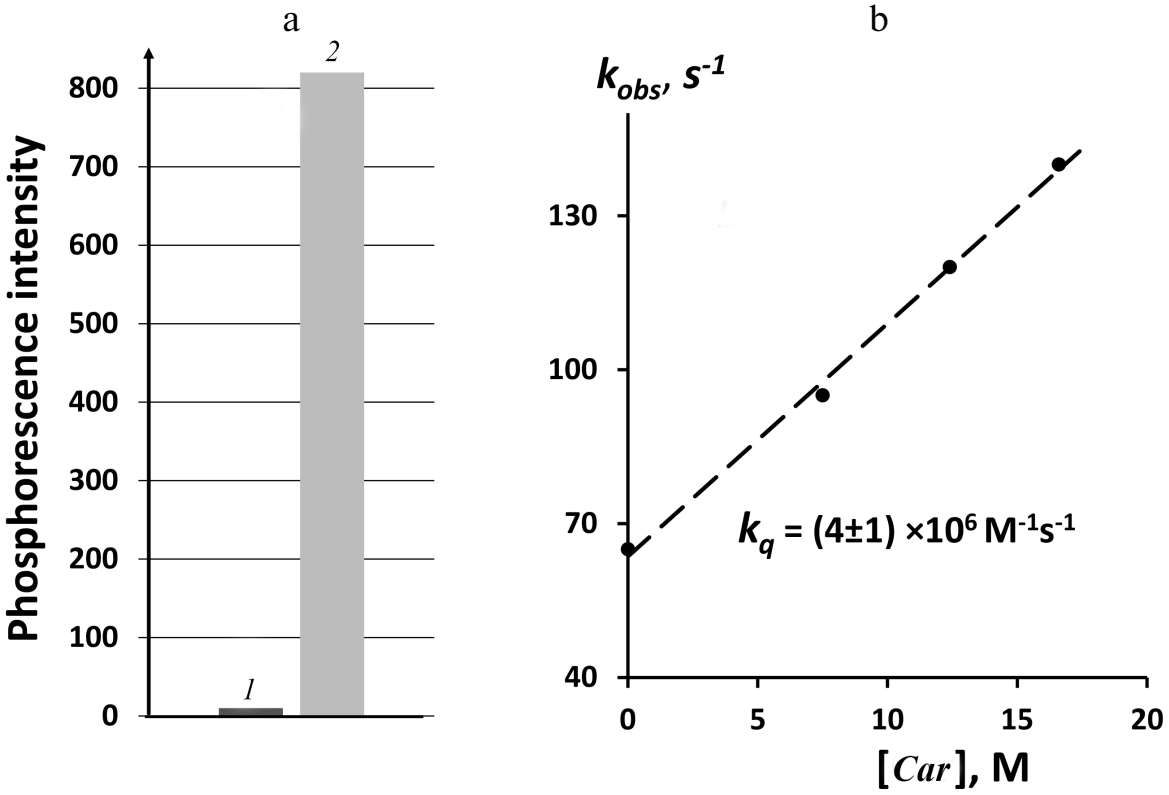

Fig. 4. Generation and quenching of $^1O_2$ by phytoene. a) Relative intensities of photosensitized phosphorescence of $^1O_2$ in solutions of phytoene (*1*) and phenalenone (*2*) in perfluorohexane with equal absorbance (0.13) at the excitation wavelengths (281 and 371 nm, respectively); b) quenching of phenalenone-photosensitized $^1O_2$ phosphorescence in hexafluorobenzene by addition of phytoene, excitation wavelength was 405 nm.

reduce the phosphorescence intensity by a factor of 1.3 ($(k_q[Car]/k_o) \approx 0.3$). Hence, $^1O_2$ quenching by phytoene cannot explain low intensity of $^1O_2$ phosphorescence in the phytoene solution (Fig. 4). It should be concluded that this effect is due to the very low quantum yield of $^1O_2$ generation (Φ_Δ) by phytoene. Based on these results, we can argue that the phytoene Φ_Δ is less than 2%. This value agrees with the flash photolysis data of Bensansson et al., who reported that the yield of phytoene triplet state is very low, being less than 0.002 [14]. Studies of phytoene solutions are currently underway to generate more detailed data.

Phytofluene was studied in hexafluorobenzene under excitation by both cw LED at 371 nm (2-3 mW) and pulsed LED at 405 nm. After irradiation of phytofluene solution by the cw LED a rather strong IR phosphorescence with the spectral maximum at 1270 nm was observed (Fig. 5). After addition of 50% acetone, which led to a sharp decrease in the $^1O_2$ lifetime, the photosensitized phosphorescence weakened by approximately 500-fold (Fig. 5). Similar effect was observed in the phenalenone solutions in hexafluorobenzene [19]. The action spectrum of this phosphorescence evaluated using three LEDs with fixed wavelengths 350, 371, and 399 nm (not shown) corresponded to the absorption spectrum of phytofluene.

Due to low absorbance of phytofluene at the wavelength of our pulsed LED (405 nm), it was difficult to perform accurate time-resolved measurement of $^1O_2$ phosphorescence. Nevertheless, weak signal of the $^1O_2$ phosphorescence was detected under the pulsed irradiation of rather concentrated (6.2 μM) phytofluene solution with absorbance of ~0.01 at 405 nm (Fig. 6). The decay time was about 5 ms, being about three-fold shorter than in the dilute solution of phenalenone alone (Fig. 3). This fact indicated that phytofluene quenched $^1O_2$ at the used concentration. In order to verify this observation, a solution was prepared, which contained phytofluene of the same concentration and phenalenone with absorbance of 0.1 at 405 nm. Strong phosphorescence at 1270 nm was observed in this solution. Its lifetime was found to coincide with the phosphorescence lifetime in the solution of pure phytofluene (Fig. 6). Moreover, the initial intensity of phosphorescence in phenalenone solution was about 10 times stronger than in the solution of phytofluene. Hence, relative phosphorescence intensities of both solutions were proportional to the values of their absorbance at the wavelength of excitation. This fact suggests that the

quantum yields of $^1O_2$ generation by phytofluene and phenalenone were approximately the same.

Further experiments were directed to more detailed investigation of phytofluene ability to generate and quench $^1O_2$. Figure 6b shows the Stern–Volmer plot [Eq. (1)] for quenching of $^1O_2$ phosphorescence photosensitized by phenalenone (2 μM) upon addition of phytofluene. It follows from this plot that the rate con-

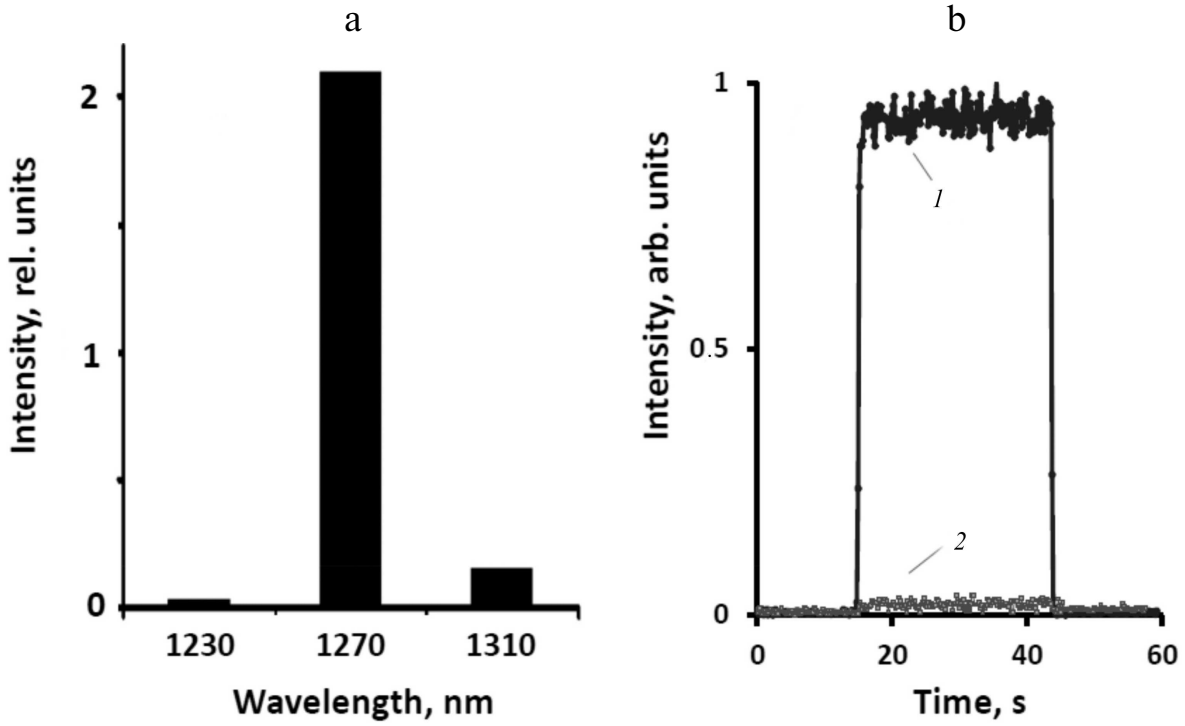

Fig. 5. Parameters of $^1O_2$ phosphorescence photosensitized by phytofluene in hexafluorobenzene. a) Phosphorescence spectrum; b) intensity of phosphorescence before (*1*) and after (*2*) addition of 50% acetone. Phosphorescence was excited by cw LED at 371 nm (≈3.5 mW).

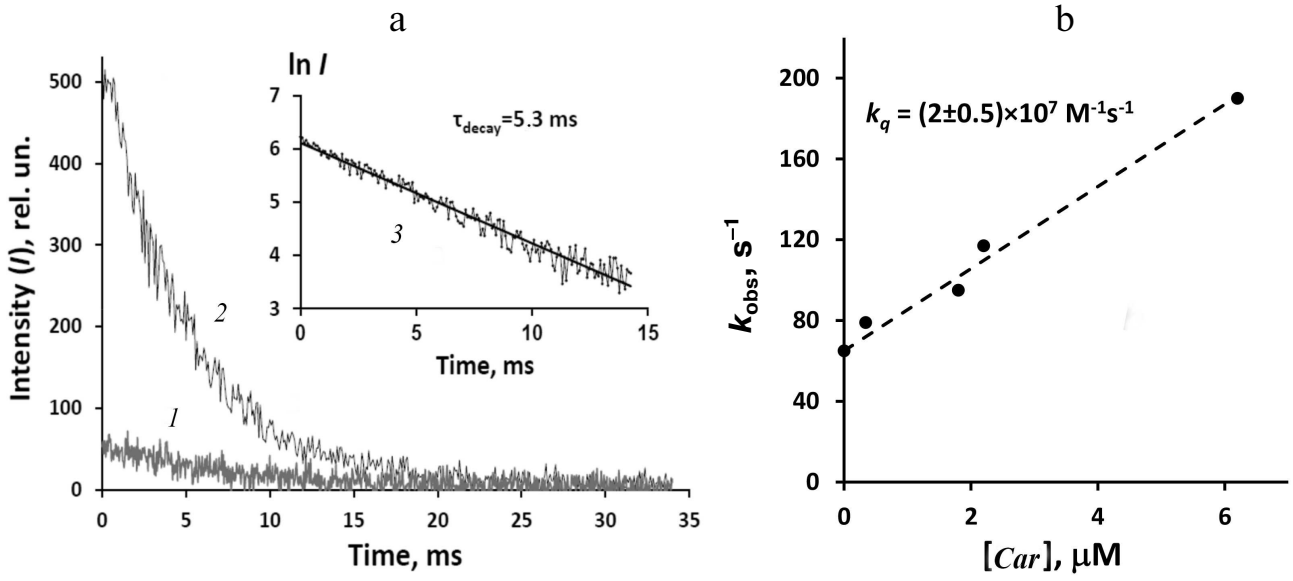

Fig. 6. Generation and quenching of $^1O_2$ phosphorescence by phytofluene in hexafluorobenzene. a) Decays of phosphorescence after irradiation with LED pulses (10 μs, 405 nm) in solution of phytofluene (6.2 μM) (*1*) and in solution of phenalenone with added 6.2 μM phytofluene (*2*) (pulse repetition rate 30 Hz; averaging time – 15 min); semilogarithmic plot of the curve *2* (*3*). b) Stern–Volmer plot for phytofluene quenching of $^1O_2$ phosphorescence photosensitized by phenalenone (2 μM). See additional explanations in the text.

Quantum yields of $^1O_2$ generation and rate constants of $^1O_2$ quenching by carotenoids studied in the present work

Carotenoid	CDB	Φ_Δ	k_q, $M^{-1}\cdot s^{-1}$
Phytoene*	3	<0.02	$(4 \pm 1) \pm 10^6$
Phytofluene	5	0.85 ± 0.05	$(2 \pm 0.5) \pm 10^7$

* Φ_Δ for phytoene was obtained in hexafluorohexane, other data – in hexafluorobenzene.

stant of $^1O_2$ quenching by phytofluene is $(2.0 \pm 0.5) \times 10^7$ $M^{-1}\cdot cm^{-1}$. This value agrees with the estimate of $k_q < 10^8$ $M^{-1}\cdot s^{-1}$ reported by Mathews–Roth et al. [23].

To determine the quantum yield of $^1O_2$ generation (Φ_Δ) by phytofluene, the intensities of $^1O_2$ phosphorescence were compared in the solutions of phytofluene and phenalenone in hexafluorobenzene of equal absorbance (0.15) at 371 nm. This absorbance corresponded to the concentrations of phytofluene and phenalenone of about 2 and 1.4 μM, respectively. Under these conditions, the $^1O_2$ phosphorescence intensity in the phytofluene solution was about 60% of that in the phenalenone solution. Reduction of $^1O_2$ phosphorescence in the phytofluene solution is partially due to the quenching of $^1O_2$ by phytofluene (Fig. 6b). Using Eq. (2) and obtained k_q for phytofluene, one can calculate that 2 μM phytofluene causes 1.5-fold reduction in the phosphorescence intensity compared to the solution of phenalenone alone. Taking into account this quenching and the value of Φ_Δ for phenalenone equal to 0.95 [20, 21], we arrive to the following value of Φ_Δ for phytofluene: $\Phi_\Delta = 0.95 \times 0.6 \times 1.5 = 0.85$ with estimated error of ±5%. Table summarizes the data obtained.

DISCUSSION

Our data demonstrate that phytoene and phytofluene, which are universal precursors in biosynthesis of colored carotenoids with the long CDB chains in plant and bacteria, differ greatly in their ability to photosensitize and quench $^1O_2$. Phytoene was much less efficient in both these processes. Phytofluene is more active as a $^1O_2$ quencher than phytoene but still the quenching activities of both carotenoids are much (by 2-3 orders of magnitude) lower than those of lycopene or β-carotene [2, 5, 15]. At the same time, phytofluene shows very high photosensitizing activity. To the best of our knowledge this property of phytofluene was observed in the present paper for the first time. The data suggest that upon photoexcitation, phytofluene efficiently populate rather long-lived triplet state, which is capable to efficiently transfer its energy to oxygen. We could not find any information on the quantum yield and lifetime of phytofluene triplets in the literature. However, energy of the triplet state (E_T) can be estimated from the linear dependence between E_T^{-1} and number of the CDBs reported by Bensasson et al. [14]. Using this linear function, we obtained that E_T = 1.45 ev for phytofluene, hence it is higher than the energy of the $^1\Delta_g$ state of oxygen (1 ev). Therefore, generation of $^1O_2$ by triplet phytofluene is energetically allowed.

In addition, it is known that phytofluene emits rather strong fluorescence at 500 nm with the quantum yield of 0.05 ± 0.01 at room temperature [24, 25]. This is unusual for C_{40} carotenoids, whose fluorescence yield is normally less than 10^{-3} [24, 25]. Hence, by photophysical properties phytofluene resembles more chlorophyll and porphyrins than C_{40} polyenes. The data suggest, that phytofluene should be an efficient promoter of photochemical processes. In our opinion this fact changes views on the biological roles of this compound. In particular, it may be involved in biological effects of UV-A light [26], photodamage of the photosynthetic apparatus and skin tissues. Moreover, this compound can be considered as a potential UV-A photosensitizer in cosmetology and medicine. However considering high photosensitizing activity of phytofluene, the use of this compound for protection of skin from ultraviolet radiation seems questionable.

Acknowledgements. The authors are grateful to companies "Polironik Ltd." (Moscow) and "Alkom Medika Ltd." (St. Petersburg) for technical assistance and to "Pimimvest" (Moscow) for purification of the solvents.

Funding. This study was financially supported in part by the Russian Foundation for Basic Research (projects Nos. 18-04-00684-a and 19-04-00331-a), and by the State Assignments of the Federal Research Centre for Biotechnology, Russian Academy of Sciences, Moscow, and by the Federal Research Centre "Pushchino Scientific Center for Biological Research", Russian Academy of Sciences, Pushchino, Russia.

Ethics declarations. The authors declare that they have no conflicts of interest. This article does not contain any studies involving human participants or animals performed by any of the authors.

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